Supplementary Data

Pex3-anchored Atg36 tags peroxisomes for degradation in *Saccharomyces cerevisiae*

Alison M. Motley, James M. Nuttall and Ewald H. Hettema

Supplementary figures legends **Fig. S1. Analysis of peroxisome biogenesis in *atg36Δ* cells.** (A) WT and *atg36Δ* cells expressing GFP-PTS1 from the *HIS3* promoter were grown under various conditions and multiple images of each condition were captured (sample of images shown). Bar, 5 μm. (B) Peroxisomes were counted and cells were classed according to peroxisome number: class A cells contain 1-4 peroxisomes/cell, class B cells contain 5-10 peroxisomes/cell, and class C cells contain >10 peroxisomes/cell. Peroxisomes in at least 200 cells of each strain were counted per growth condition. (C) WT, *pex3Δ* and *atg36Δ* cells were grown on an oleate-containing plate. (D) WT cells expressing Pex11 fused to monomeric GFP (Pex11-mGFP) were grown in oleate medium for 18 h. (E) Pex11-GFP localises to peroxisomes in cells grown in oleate (18 h) and switched to SD-N medium (3 h).

Fig. S2. Endogenous expression of Atg36-GFP and Atg36-mGFP

Fluorescence images of WT or *pex3Δ* cells expressing genomically tagged Atg36-GFP and plasmid-encoded HcRed-PTS1 expressed from the *HIS3* promoter under various growth conditions. Multiple fluorescent images were acquired in Z-Axis and flattened into a single

image. Bright field image is a single plane. White arrowheads indicate auto-fluorescence in the vacuole of cells expressing only HcRed-PTS1. This auto-fluorescence is detectable under most conditions using these long exposure times, but is less pronounced in logarithmically growing cells. Peroxisomes cluster when Atg36

is tagged in the genome at its C-terminus with GFP or monomeric GFP (mGFP). Bar, 5 μm.

Fig. S3. Comparison of Atg36 expression. (A) *atg36Δ* cells containing untagged *GAL1-ATG36* expressed for 5 h in galactose medium. Peroxisomes are labeled with Pex11-GFP and cluster upon overexpression of *ATG36*. (B) *atg36Δ* cells containing N- or C- tagged Atg36 were grown for 22 h in glucose before switching to galactose medium for the times indicated. Right hand panel shows expression of endogenous Atg36 under the same conditions. (C) Titration of galactose-induced expression of *ATG36-GFP vs*. endogenous expression. Samples from (B) were reloaded. Pgk1 blot used as loading control and ∫λ of total lysate loaded indicated. Galactose- induced expression of *ATG36-GFP* is at least 50 –100 fold higher than endogenous expression. (D) Phosphatase treatment was performed on Atg36-PtA lysates. -, no CIP; +, 20 U CIP. Top

and bottom panels show lysates incubated at 25°C and 37°C, respectively. Cells were grown 22 h in oleate medium and starvation samples were switched to SD-N medium for 3 h. CIP treatment clearly affects the mobility of Atg36 under oleate conditions, indicating that Atg36 is phosphorylated. Under starvation conditions, CIP treatment does not result in a single band of the same mobility as that seen under oleate conditions. This implies that modification of Atg36 under starvation conditions is different to that under oleate conditions. This may reflect a different, CIP-resistant phosphorylation or a different type of modification.

Fig. S4. *pex3-177* is affected specifically in pexophagy.Pex11-GFP pexophagy assay in *pex3Δ* cells transformed with plasmids encoding three *pex3* alleles isolated in the pexophagy screen or the Inp1 non-binding *pex3-1* allele. *atg1Δ* cells are included as a negative control for pexophagy. Cells were grown 18 h in oleate

medium (-) and switched to SD-N medium for 22 h (+). GFP* indicates the relative protease-resistant degradation product indicative of vacuolar breakdown. (B) Quantification of peroxisome distribution in *pex3Δ* cells expressing *PEX3*, *pex3-177*, or *pex3-1* alleles with peroxisome marker GFP-PTS1. At least 100 budding cells containing peroxisomes were counted per strain. Peroxisome segregation is unaffected in *pex3-177* cells. (C) *pex3Δ* cells containing *pex3-177* and Pex11-GFP were mated with *pex19Δ* cells containing HcRed-PTS1 to show that peroxisomes in *pex3-177* cells are functional in import. (D) (top panel) *pex3Δ* cells expressing GFP-PTS1 from the oleate-inducible *CTA1* promoter and *pex3* alleles as indicated were assayed for pexophagy. (central panel) *pex3Δ* cells expressing GFP-PTS1 from the *HIS3* promoter were grown on glucose to log phase. Black arrowheads indicate empty mother cells. (bottom panel) Inp1-GFP expressed on a plasmid from the *INP1* promoter in *pex3Δ* cells containing alleles as indicated were grown to log phase on glucose. Expression of Inp1-GFP causes empty buds as indicated by white arrowheads. Quantification of Inp1-GFP puncta revealed that approx. 15% *pex3-177* mother cells have no Inp1-GFP puncta (all mother cells in *PEX3* cultures contain Inp1-GFP puncta); 45% *pex3-177* mother cells have 1-4 puncta per cell (30% in *PEX3* cells); 40% *pex3- 177* mother cells have have 5 – 10 puncta per cell (60 % in *PEX3* cells), and no *pex3-177* mother cells have 11 or more Inp1-GFP puncta per cell (10 % in *PEX3* cells). Inp1-GFP puncta are completely absent in *pex3-1* cells, reflecting the inability of this allele to bind Inp1. Although the number of Inp1-GFP puncta are reduced in *pex3-177* cells, peroxisome distribution is normal

(Suppl Fig. 4B). The reduced numbers of Inp1-GFP puncta may result from reduced levels of pex3-177 protein (see E). (E) Western blot analysis of Pex3 levels in WT, *pex3-1* and *pex3-177* cells using anti-Pex3.

Fig S5. Mutational analysis of AIM/LIR-like sequences in Atg36.

AIMs are characterised by the presence of an aromatic residue at position 1 and a bulky hydrophobic residue at position 4. Atg36 contains two putative AIMs according to the consensus [W/Y]xx[L/I/V] (Kirkin et al., 2009; Noda et al., 2010). However, the identification recently of the LIR of optineurin broadens the consensus to include an F at position 1 (Johansen and Lamark, 2011; Wild et al., 2011), and according to this consensus, there are a further six AIM/LIR-like sequences in Atg36. We tested the effect of mutations (to AxxA) of all of these putative AIMs for pexophagy activity and binding to peroxisomes. All known *S.cerevisiae* AIMs contain a W at position 1. Atg36 contains one such motif (W282/L285), but mutation of this conserved motif had no consequence for Atg36 function. Of the remaining seven AIM-like motifs, mutation of only one of them, Y191/L194, results in reduced pexophagy. This mutant version of Atg36 is still recruited to peroxisomes, but peroxisomes remain dispersed in the cytosol and are not degraded. Importantly, a single substitution of the aromatic residue to leucine (Y191L (which comprises the residue present at this position in most Atg36 yeast orthologues)) has no effect on Atg36 function in pexophagy.

Besides the aromatic residue at position 1 and the bulky hydrophobic residue at position 4, ionic interactions between negatively charged residues at position X-3, X-2, X-1, X2 and X3 of AIM/LIRs and positively charged residues in Atg8/LC3 contribute to binding (Noda et al., 2010). Mutation of the negatively charged residue at position -2 relative to Y191 (D189A) did

not affect Atg36 function in pexophagy. Frequently these negative charges are replaced by serine or threonine residues and their phosphorylation may regulate binding to Atg8/LC3 as has been shown for optineurin binding to LC3/GABARAP (Wild et al.,

2011). There are no serine/threonine residues flanking position 1. However, there are two serine/threonine residues in the vicinity (S-4 and T+5 relative to position 1 (Y191), but single or double mutations in these residues to either glutamic acid or alanine had no consequence for Atg36 function. These combined observations lead us to conclude that Y191/L194 does not constitute an AIM. Furthermore, the other AIM-like motifs of Atg36 are not essential for Atg36 function.

(A) Sequence alignments of AIM/LIR motifs in autophagy receptors. Aromatic residues are shown in red, negatively charged residues in green, and hydrophobic residues in blue. Positions are shown according to the canonical consensus sequence. The top four proteins are human and the bottom three are *S. cerevisiae* proteins.

(B) Schematic representation of two AIM-like motifs in Atg36 are shown aligned with yeast orthologues. Ag, *Ashbya gossypii;* Zr, *Zygosaccharomyces rouxii;* Vp, *Vanderwaltozyma polyspora;* Lt, *Lachancea thermotolerans*. The remaining LIR-like motifs in Atg36 all contain an F in position 1 and include: F33/L36, F47/L50, F53/V56, F138/L141, F215/I218, F238/L241.

(C) GFP-tagged *atg36* mutants were expressed in *atg36Δ* cells containing Pex11-mRFP and assayed for pexophagy by growing for 18 h on oleate then switching to starvation medium for 22 h. Top panels, WT; central panels, W282A/L285A mutant; bottom panels, Y191A/L194A mutant.

(D) Pexophagy was assayed by Pex11-GFP breakdown after growth of cells for 18 h on oleate (0) then switching for 22 h to starvation medium (left panel). Only Y191A/L194A shows reduced pexophagy. Similarly, when these alleles are tagged with GFP and subjected to the same pexophagy assay (right panel), free GFP (*) that results from Atg36-GFP degradation in the vacuole is strongly reduced in cells expressing Atg36 Y191A/L194A. Size

bar, 5 μm.

(E) Mutations in Atg36 that were tested for their effects on pexophagy. +, no effect; -, reduced pexophagy.

Supplementary Materials and Methods

CIP treatment

Briefly, yeast cells (50 OD) were sonicated in phosphatase buffer (100 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM DTT, 4 mM Pefabloc SC, one complete protease inhibitor cocktail tablet/25 ml) and lysates obtained by centrifugation (13,000 x g, 5 min, 4°C). For calf intestinal alkaline phosphatase treatment (CIP) treatment, lysates corresponding to approximately 5 OD equivalents were treated with 20 U CIP (New England BioLabs) at 25°C for 20 min. CIP was not added to untreated samples. The reaction was stopped by addition of 5% TCA and precipitated protein was resuspended in 1x SDS-PAGE buffer and boiled at 95°C for 5 min.

Table S1 Yeast strains used in this study

Genotype	Reference
BY4741 MATa *his3-1 leu2-0 met15-0 ura3-0*	Euroscarf
BY4742 MATα *his3-1 leu2-0 lys2-0 ura3-0*	Euroscarf
BJ1991 MATα *ura3-251 leu2 trp1 prb1-1122 pep4-3 gal2*	(Jones, 1977)
C13 abys 86 MATα *ura3Δ5 leu2-3 112 his3 pra1-1 prb1-1 prc1-1 cps1-3*	(Heinemeyer et al., 1991)
BY4741 *atg36Δ::kanMX4*	Euroscarf
BY4741 *atg1Δ::kanMX4*	Euroscarf
BY4742 *atg11Δ::kanMX4*	Euroscarf
BY4741 *atg19Δ::kanMX4*	Euroscarf
BY4741 *pex14Δ::kanMX4*	Euroscarf
BY4741 *atg8Δ::kanMX4*	Euroscarf
BY4741 *atg32Δ::kanMX4*	Euroscarf
BY4741 *APE1*-GFP*::HIS5*	This study
BY4741 *atg36Δ::kanMX4 APE1*-GFP*::HIS5*	This study
BY4741 *atg1Δ::kanMX4 APE1*-GFP*::HIS5*	This study
BY4741 *atg19Δ::kanMX4 APE1*-GFP*::HIS5*	This study
BY4741 *OM45*-GFP*::hphMX4*	This study
BY4741 *atg36Δ::kanMX4 OM45*-GFP*::hphMX4*	This study
BY4741 *atg1Δ::kanMX4 OM45*-GFP*::hphMX4*	This study
BY4741 *atg36Δ::kanMX4 pex3Δ::loxP*	This study
BY4741 *pex19Δ::kanMX4*	Euroscarf
BY4741 *pex3Δ::kanMX4*	Euroscarf
BY4742 *ATG36*-PtA*::HIS5*	This study
BY4741 *atg1Δ::kanMX4 ATG36*-PtA*::HIS5*	This study
BY4741 *atg1Δ::kanMX4 atg36Δ::HIS5*	This study
BY4741 *atg1Δ::kanMX4 pex3Δ::loxP*	This study
BY4741 *atg32Δ::kanMX4 pex3Δ::loxP*	This study
BY4741 *atg32Δ::kanMX4 pex3Δ::loxP OM45*-GFP*::hphMX4*	This study
BY4741 *atg32Δ::kanMX4 pex3Δ::loxP OM45*-GFP*::hphMX4 atg36Δ::HIS5*	This study
C13 abys 86 *ATG36*-PtA*::HIS5*	This study
C13 abys 86 *atg1Δ::LEU2 ATG36*-PtA*::HIS5*	This study
C13 abys 86 *atg1Δ::HIS5 MVP1*-PtA*::kanMX4*	This study
BY4742 *ATG36*-GFP*::hphMX4*	This study
BY4741 *atg1Δ::kanMX4 ATG36*-GFP*::hphMX4*	This study
BY4741 *pex3Δ::kanMX4 ATG36*-GFP*::hphMX4*	This study
BY4741 *atg17Δ::kanMX4*	Euroscarf
BY4742 *atg11Δ::kanMX4 atg17Δ::HIS5*	This study
BY4741 *Δpho13::MET15 Δpho8::pho8 60-HIS3*	(Ohashi and Munro, 2010)
BY4741 *atg1Δ::KanMXΔpho13::MET15 pho8Δ::pho8 60-LEU2*	(Ohashi and Munro, 2010)
BY4741 *atg36Δ::URA3 pho13Δ::MET15 pho8Δ::pho8 60-*	This study

HIS3	
BY4742 *ATG36*-mGFP::*HIS5*	This study

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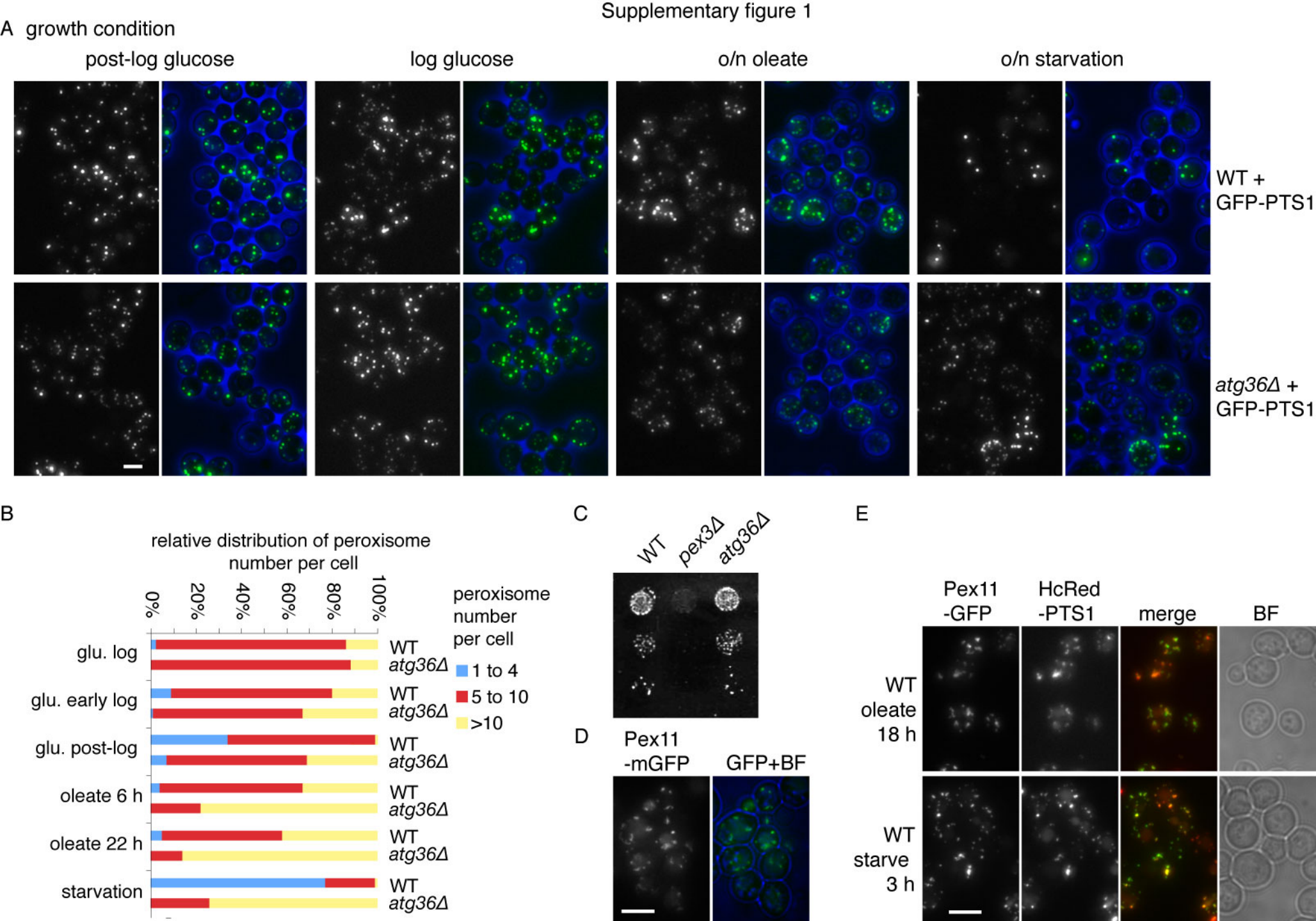
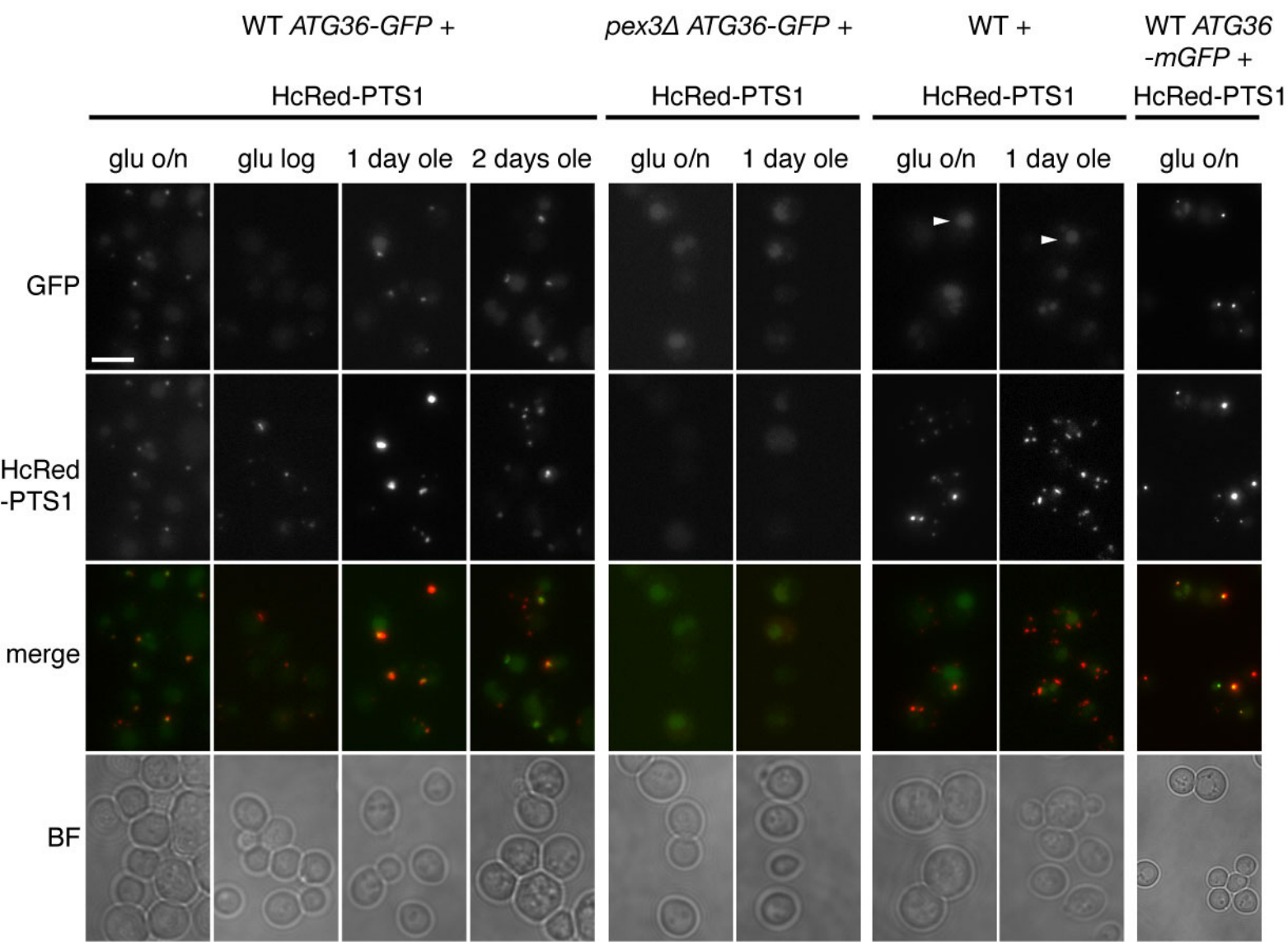
Supplementary figure 1



Supplementary figure 2



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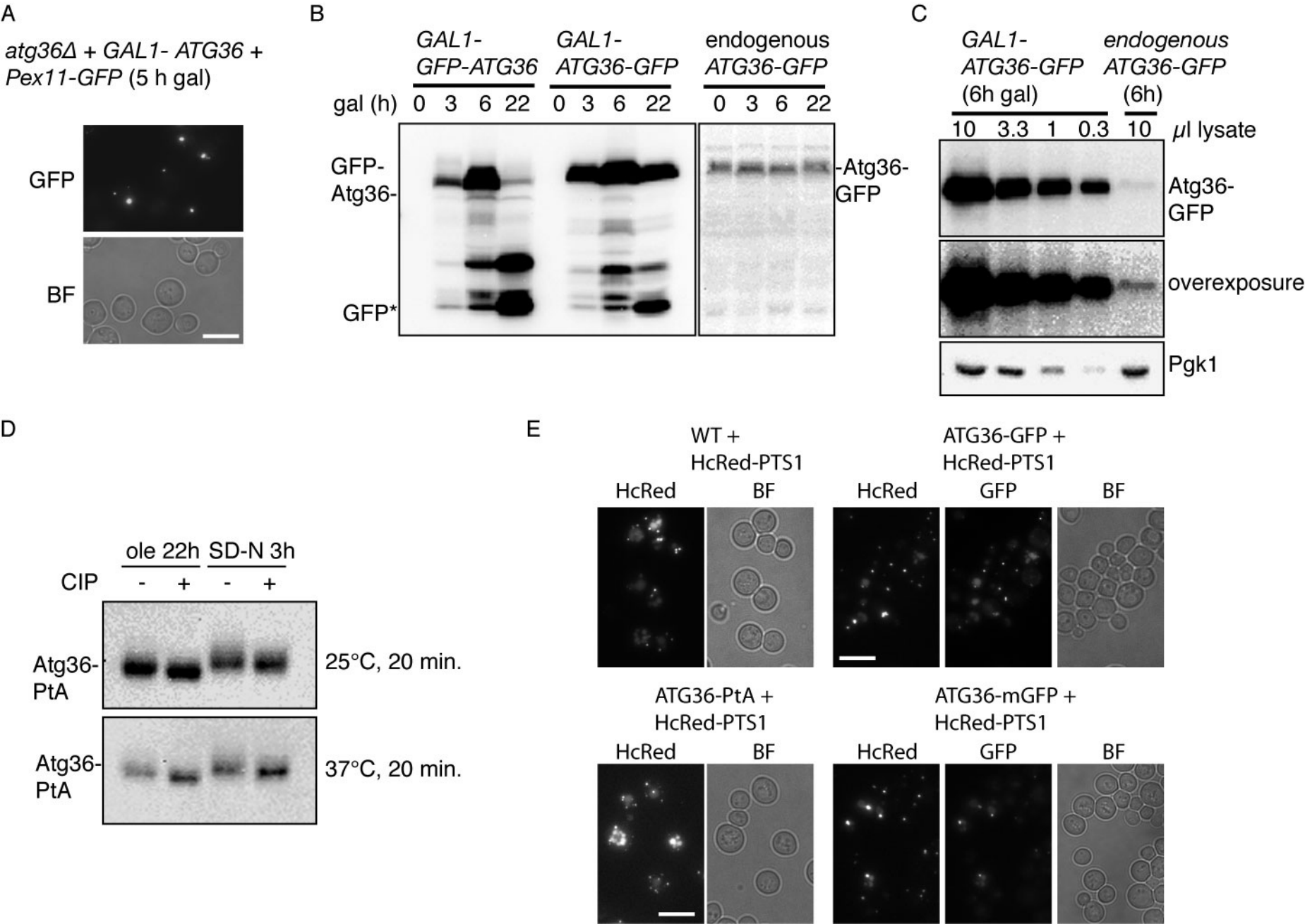
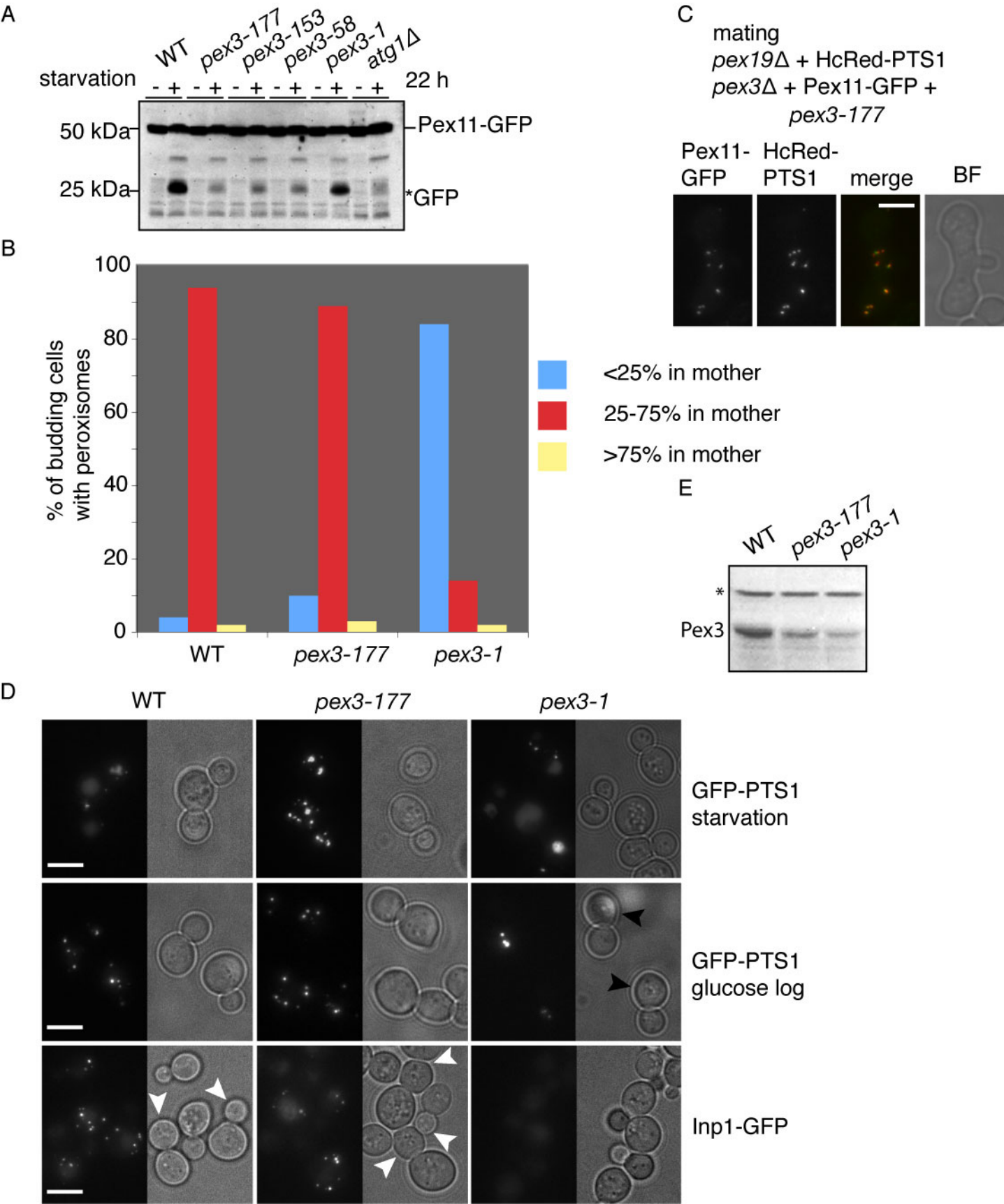


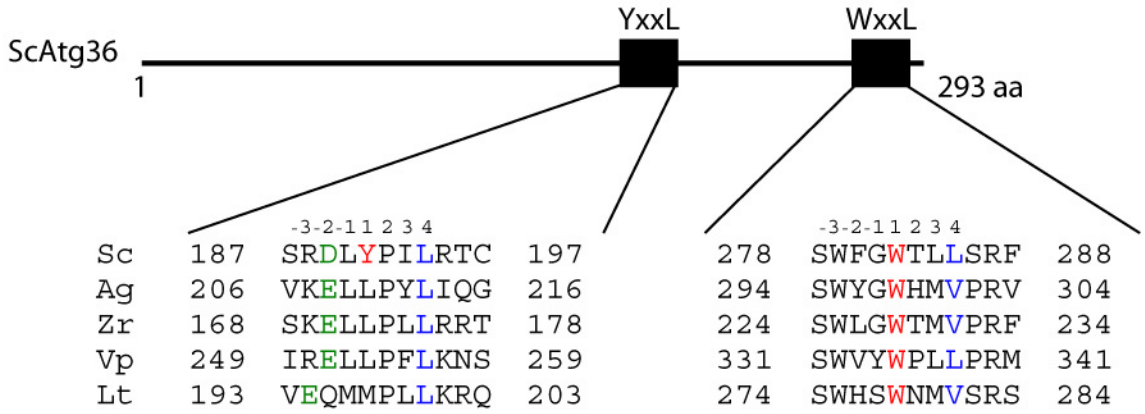
Supplementary figure 4



A

	AIM/LIR
POSITIONS	-3-2-1 1 2 3 4
P62	GDDDWTHLSS
NBR1	SSEDYIIILP
NIX	LNSSWVELPM
OPTINEURIN	SEDSFVEIRM
ScAtg32	ISGSWQAIQP
ScAtg19	KALTWEEL
ScAtg34	RPFTWEEI

B



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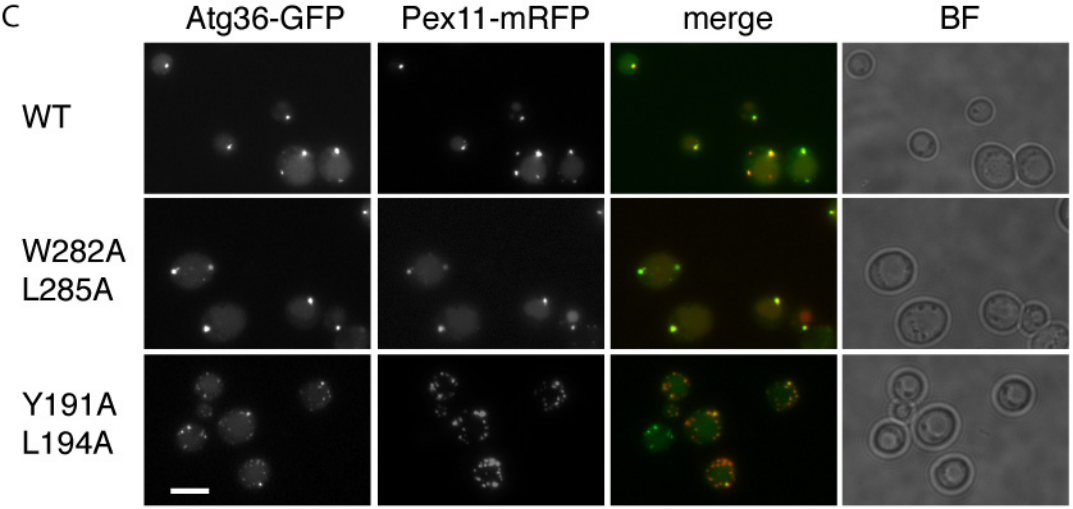

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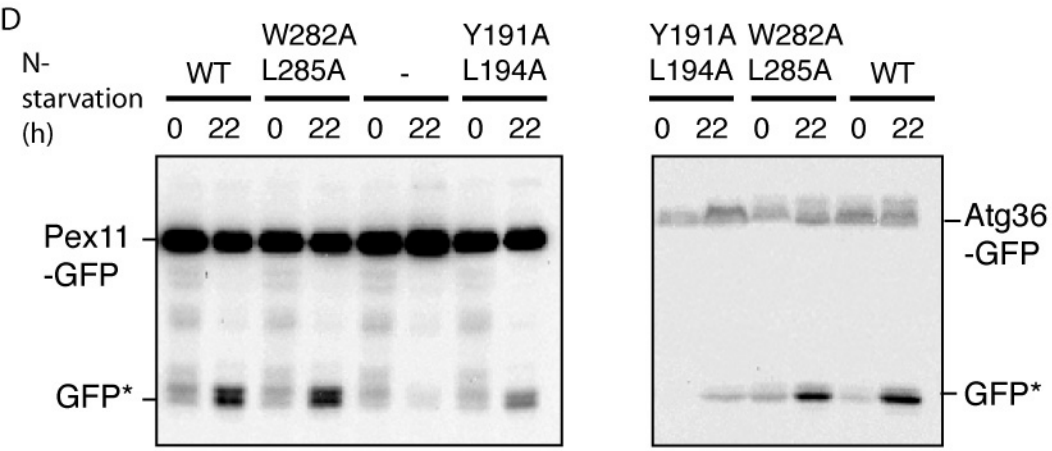

E

Effects of various mutations in Atg36 on pexophagy

Atg36	**Pexophagy**
WT	+
Δ284	+
W282A/L285A	+
Y191A/L194A	-
D189A	+
Y191L	+
S187A	+
S187E	+
T196A	+
T196E	+
S187A/T196A	+
S187E/T196E	+

Supplementary figure 5