SUPPLEMENTAL DATA

Autophagy-related Protein 32 Acts as an Autophagic Degron and Directly Initiates Mitophagy

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SUPPLEMENTAL METHODS

Protein Phosphatase Treatment Assays—For protein phosphatase assays, *atg32Δ pep4Δ prb1Δ* cells expressing Atg32–HA were pregrown in SDCA, and transferred to SDGCA. 2.0 OD_{600} units of cells were collected at the 32 h time point, and subjected to alkaline lysis and TCA precipitation. The pellet was resuspended in a reaction buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM DTT, 0.5 mM EDTA, 0.01% Brij-35, 2 mM $MgCl_2$), treated with or without lambda protein phosphatase (λ-PPase) in the presence or absence of PPase inhibitor at 30˚C for 1 h. Samples corresponding to 0.2 OD_{600} units of cells were loaded per lane.

Autophagy Assays—For enzymatic measurement to quantify autophagic activity, cells were subjected to an alkaline phosphatase (ALP) assay as reported previously (1).

SUPPLEMENTAL REFERENCES

1. Noda, T., Matsuura, A., Wada, Y., and Ohsumi, Y. (1995) *Biochem. Biophys. Res. Commun.* **210**, 126-132
2. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) *Yeast* **14**, 115-132
3. Okamoto, K., Kondo-Okamoto, N., and Ohsumi, Y. (2009) *Dev. Cell* **17**, 87-97
4. Noda, N. N., Kumeta, H., Nakatogawa, H., Satoo, K., Adachi, W., Ishii, J., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2008) *Genes Cells* **13**, 1211-1218
5. James, P., Halladay, J., and Craig, E. A. (1996) *Genetics* **144**, 1425-1436

TABLE S1
Yeast strains used in this study

Name	Genotype	Background
KOY76	***MAT*a** *his3Δ1 leuΔ0 met15Δ ura3Δ0*	BY4741 (2)
KOY186	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2*	(3)
KOY545	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-ATG32]	(3)
KOY546	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-ATG32-3HAn]	(3)
KOY556	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316]	
KOY576	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg8::CgHIS3*	
KOY631	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3*	
KOY637	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(AQAA)-3HAn]	
KOY675	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(AAATA)-3HAn]	
KOY717	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3 atg8::hphNT1*	
KOY729	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316]	
KOY731	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-ATG32-GFPn]	
KOY765	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(1-388)-3HAn]	
KOY766	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(1-388)-TA^{mito}-3HAn]	
KOY780	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-ATG32-3HAn]	
KOY781	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg11::CgHIS3* [pRS316-ATG32-3HAn]	
KOY782	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg1::CgHIS3* [pRS316-ATG32-3HAn]	
KOY783	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg2::CgHIS3* [pRS316-ATG32-3HAn]	
KOY785	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg9::CgHIS3* [pRS316-ATG32-3HAn]	
KOY786	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg14::CgHIS3* [pRS316-ATG32-3HAn]	
KOY788	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(1-388)-3HAn]	
KOY789	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(AAA)-3HAn]	
KOY791	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(1-388)-GFPn]	
KOY799	BY4741 *atg32::natNT2 VPH1-mCherry::kanMX6 POT1-GFP::hphNT1*	
KOY876	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-atg32(1-388)-TA^{mito}-3HAn]	
KOY877	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-atg32(1-388)-TM^{pexo}-3HAn]	
KOY879	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg1::CgHIS3* [pRS316]	
KOY880	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg1::CgHIS3* [pRS316-atg32(1-388)-TA^{mito}-3HAn]	
KOY881	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg1::CgHIS3* [pRS316-atg32(1-388)-TM^{pexo}-3HAn]	
KOY887	BY4741 *atg32::natNT2 VPH1-mCherry::kanMX6 POT1-GFP::hphNT1* [pRS316]	
KOY889	BY4741 *atg32::natNT2 VPH1-mCherry::kanMX6 POT1-GFP::hphNT1* [pRS316-atg32(1-388)-TM^{pexo}-3HAn]	
KOY895	BY4741 *atg32::natNT2 VPH1-mCherry::kanMX6 POT1-GFP::hphNT1 atg1::CgHIS3* [pRS316]	
KOY896	BY4741 *atg32::natNT2 VPH1-mCherry::kanMX6 POT1-GFP::hphNT1 atg1::CgHIS3* [pRS316-atg32(1-388)-TM^{pexo}-3HAn]	
KOY936	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(1-388)-TA^{mito}-3HAn]	
KOY937	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3* [pRS316-atg32(1-388)-TA^{mito}-GFPn]	
KOY1091	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3 atg8::hphNT1* [pRS316-ATG8] [pRS315-ATG32]	
KOY1092	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3 atg8::hphNT1* [pRS316-ATG8] [pRS315-atg32(AQAA)]	
KOY1093	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3 atg8::hphNT1* [pRS316-atg8(R62A)] [pRS315-ATG32]	
KOY1094	BY4741 *atg32::natNT2 his3Δ1::GPD^{p}-mtDHFR-mCherry::CgHIS3 atg8::hphNT1* [pRS316-atg8(R62A)] [pRS315-atg32(AQAA)]	
KOY1149	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg8::CgHIS3* [pRS316-ATG8] [pRS315-ATG32-3HAn]	
KOY1152	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg8::CgHIS3* [pRS316-atg8(R62A)] [pRS315-atg32(AQAA)-3HAn]	
KOY1185	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg1::CgHIS3* [pRS315-ATG32-3HAn] [pRS316]	
KOY1186	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg1::CgHIS3* [pRS315-ATG32-3HAn] [pRS316-ATG1]	
KOY1188	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg1::CgHIS3* [pRS315-ATG32-3HAn] [pRS316-atg1(D211)]	
KOY1198	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg8::CgHIS3* [pRS316-ATG8] [pRS315-ATG32]	
KOY1199	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg8::CgHIS3* [pRS316-atg8(R62A)] [pRS315-atg32(AQAA)]	
KOY1203	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2 atg3::CgHIS3* [pRS316-ATG32-3HAn]	
KOY1334	BY4741 *atg19::hphNT1 ATG11-2xGFPhy::kanMX6 his3Δ1::TEF^{p}-mtmCherry::CgHIS3*	
KOY1336	BY4741 *atg19::hphNT1 ATG11-2xGFPhy::kanMX6 his3Δ1::TEF^{p}-mtmCherry::CgHIS3 atg32::zeoNT3*	
KOY1418	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-atg32(AAA)-3HAn]	
KOY1419	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-atg32(AAATA)-3HAn]	
KOY1420	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-atg32(AAA)]	
KOY1421	BY4741 *pep4::kanMX6 prb1::hphNT1 atg32::natNT2* [pRS316-atg32(AAATA)]	

TABLE S2
Plasmids used in this study

Plasmid	Relevant characteristrics		Source or reference
pRS316-ATG32-3HAn	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		(3)
pRS316-atg32(1-388)-3HAn	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-atg32(1-388)-TA^{mito}-3HAn	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*	Atg32(1-388) + Gem1(618-662)	this study
pRS316-atg32(1-388)-TM^{pexo}-3HAn	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*	Atg32(1-388) + Pex15(315-383)	this study
pRS315-atg32(AQAA)	*CEN LEU2* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		(3)
pRS316-atg32(AQAA)	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS315-atg32(AQAA)-3HAn	*CEN LEU2* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-ATG8	*CEN URA3*		(4)
pRS316-atg8 (P52A/R67A)	*CEN URA3*		Nakatogawa[#]
pRS316-ATG32	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-atg32(AAA)	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-atg32(AAATA)	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-atg32(AAA)-3HAn	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-atg32(AAATA)-3HAn	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-atg32(AQAA)-3HAn	*CEN URA3* 580 bp *5′-UTR* & 744 bp *3′-UTR* from *ATG32*		this study
pRS316-ATG1	*CEN URA3*		Kamada[$]
pRS316-atg1(D211A)	*CEN URA3*		Kamada[$]
pGAD-ATG32	*2μ LEU2 GAL4AD*		(3)
pGBD-C1	*2μ TRP1 GAL4BD*		(5)
pGBD-ATG8	*2μ TRP1 GAL4BD*		(4)
pGBD-ATG11	*2μ TRP1 GAL4BD*		Ohsumi Lab[§]
pGAD-atg32(51-529)	*2μ LEU2 GAL4AD*		this study
pGAD-atg32(101-529)	*2μ LEU2 GAL4AD*		this study
pGAD-atg32(151-529)	*2μ LEU2 GAL4AD*		this study
pGAD-atg32(Δ86-122)	*2μ LEU2 GAL4AD*		this study
pGAD-atg32(Δ123-150)	*2μ LEU2 GAL4AD*		this study

[#]H. Nakatogawa (Tokyo Institute of Technology, Yokohama, Japan), unpublished.
[$]Y. Kamada (National Institute for Basic Biology, Okazaki, Japan), unpublished.
[§]Ohsumi lab (Tokyo Institute of Technology, Yokohama, Japan), unpublished.

TABLE S3
Data collection and refinement statistics

	Atg8-Atg32^{AIM} complex
Data collection statistics	
Beamline	SPring8 BL41XU
Space group	*I*23
Cell parameters (Å)	a = 104.84
Resolution range (Å)	50.0 – 3.0 (3.11 – 3.00)[a]
Observed reflections	28775
Unique reflections	3969
Completeness	100.0 (100.0)
R_{merge} (I)	0.069 (0.380)
Refinement statistics	
Resolution range (Å)	50.0 – 3.0
No. of protein atoms	912
No. of sulfate molecules	2
No. of water molecules	5
R / R_{free}	0.226 / 0.268
Rmsd from ideality distance (Å)	0.009
angle (°)	1.42

[a]Values in parentheses refer to the outer shell.

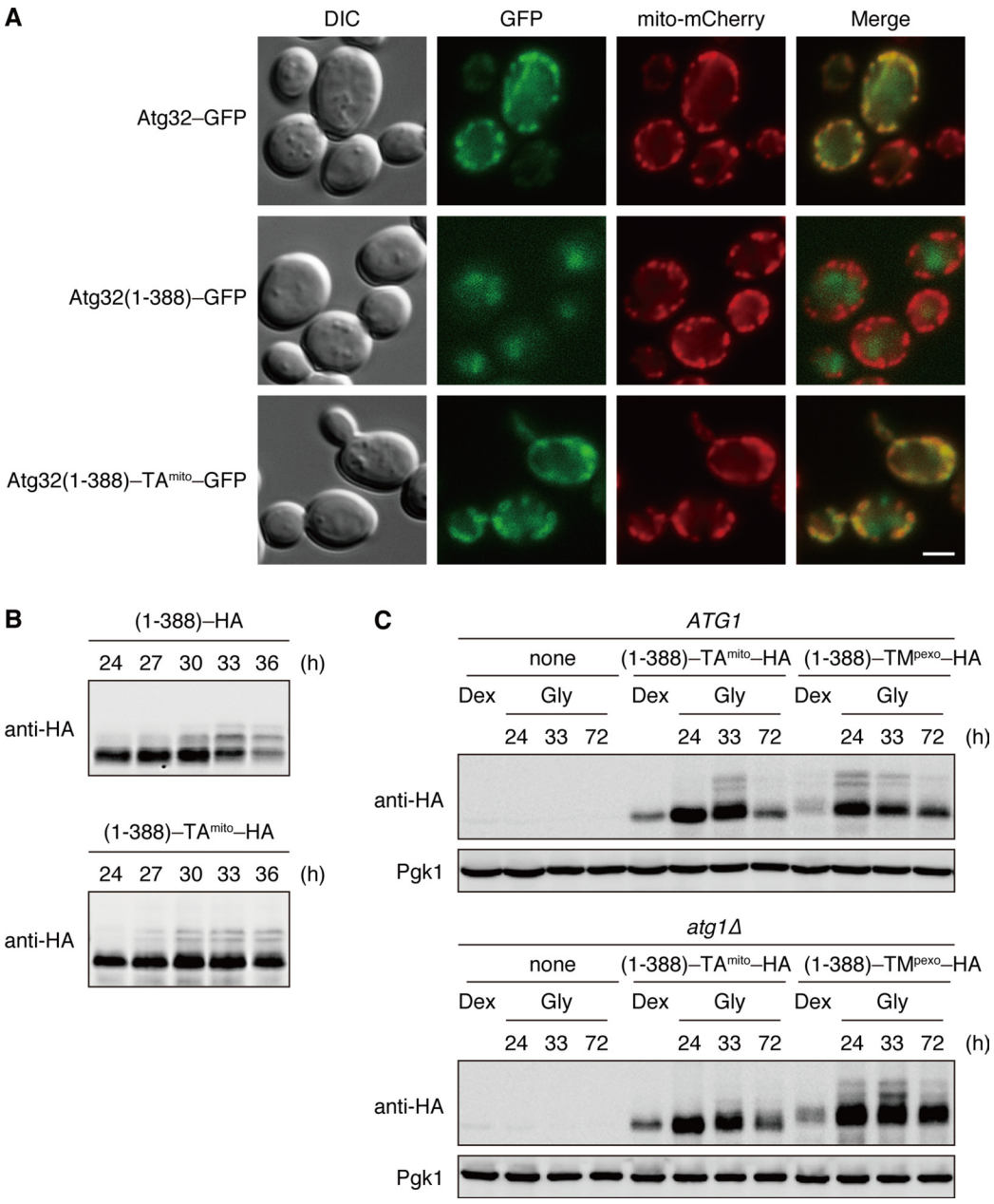

FIGURE S1. **The Atg32 cytosol domain is stably expressed.** *A*, Cells expressing a mitochondrial matrix-localized DHFR–mCherry (mito-mCherry) were transformed with a low-copy plasmid that encodes Atg32–GFP, Atg32(1-388)–GFP, or Atg32(1-388)–TA^{mito}–GFP under the endogenous promoter, grown in glycerol medium for 24 hr, and observed by fluorescence microscopy. All strains are *atg32*-null derivatives. Scale bar, 2 μm. *B*, Expression patterns of the HA-tagged Atg32 variants, (1-388)–HA and (1-388)–TA^{mito}–HA (see Fig. 1*A*), in glycerol-grown cells. All strains are vacuolar protease-deficient, *atg32*-null derivatives. Cells were collected at the indicated time points, and subjected to western blotting. *C*, Cells containing or lacking Atg1 (*ATG1* or *atg1Δ*) were transformed with a low-copy, empty plasmid (none), or the one that encodes (1-388)–TA^{mito}–HA, or (1-388)–TM^{pexo}–HA (see Fig. 1, *B* and *C*). Both Atg32 variants were expressed under the *ATG32* promoter. All strains are vacuolar protease-deficient, *atg32*-null derivatives. Cells were collected at the indicated time points, and subjected to western blotting. Pgk1 was monitored as a loading control.

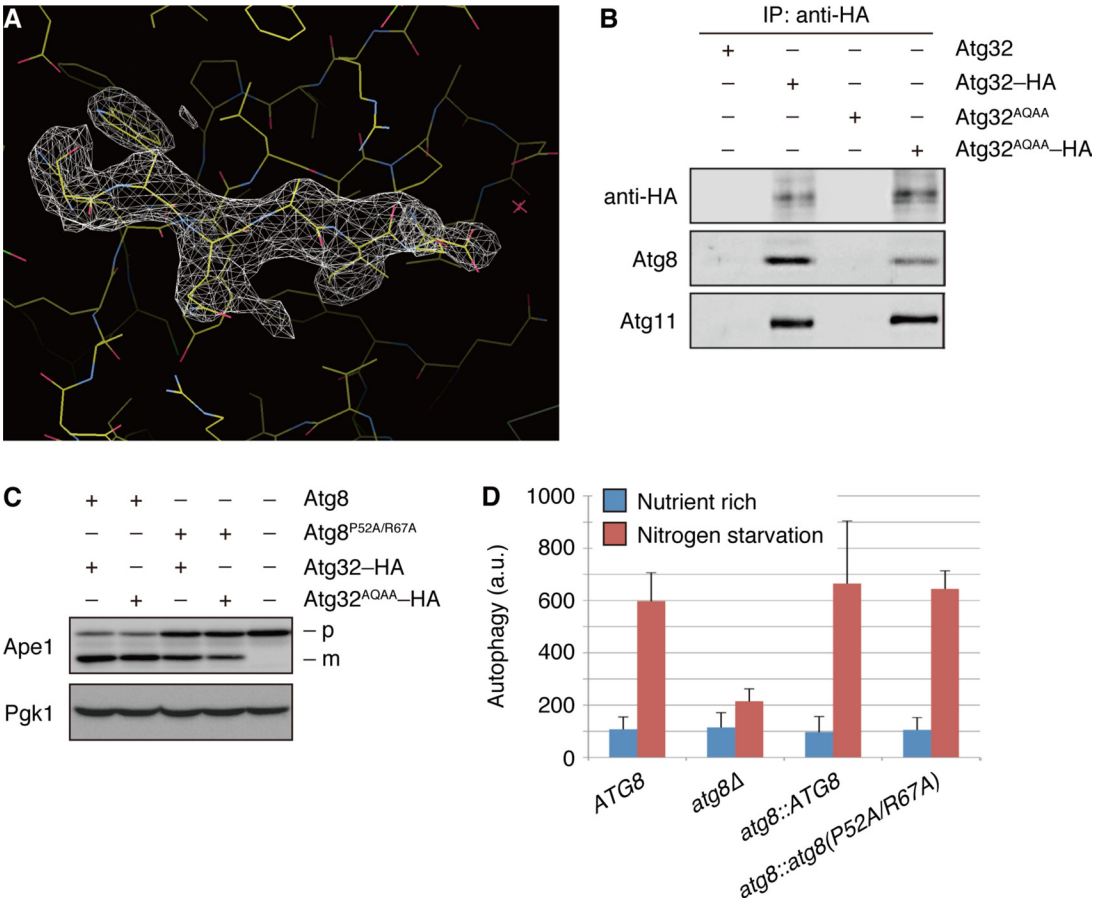

FIGURE S2. **Atg8-Atg32 AIM peptide interaction interface.** *A*, Final annealed *Fo - Fc* omit electron density map for the Atg32(SWQAIQ)$^{85\text{-}90}$ peptide bound toAtg8. The map is contoured at 2.7 σ and is shown with white meshes. The final model of the Atg8-Atg32(SWQAIQ)$^{85\text{-}90}$ peptide complex is shown with stick models, in which C, N and O atoms are colored yellow, blue and red, respectively. The figure was prepared using the program COOT. *B*, Coimmunoprecipitation assays fro cells expressing the indicated variants of Atg8 and Atg32 grown in glycerol medium for 30 hr. All strains are vacuolar protease-deficient, and *atg32*-null derivatives. Mitochondria-enriched fractions were solubilized, and subjected to immunoprecipitation using anti-HA antibody-conjugated agarose beads. The eluted immunoprecipitates (IP) were analyzed by western blotting. *C*, Cells expressing wild-type and the indicated variants of Atg8 and Atg32 were grown in glucose medium to mid-log phase, collected, and subjected to western blotting. All strains are vacuolar protease-deficient, *atg8*- and *atg32*-double null derivatives. Ape1, a cargo in the Cvt pathway, is synthesized in the cytosol as a precursor (p), transported to the vacuole, and processed to be a mature form (m). Pgk1 was monitored as a loading control. *D*, Wild-type (*ATG8*), atg8-null (*atg8Δ*), Atg8 knock-in (*atg8::ATG8*), or Atg8$^{P52A/R67A}$ knock-in (*atg8::atg8(P52A/R67A)*) cells expressing cytosolic Pho8 were grown in YPD (nutrient rich), and incubated further in SD-N (nitrogen starvation). Cells were collected, and subjected to alkaline phosphatase assays. Data represent the averages with bars indicating standard deviations.

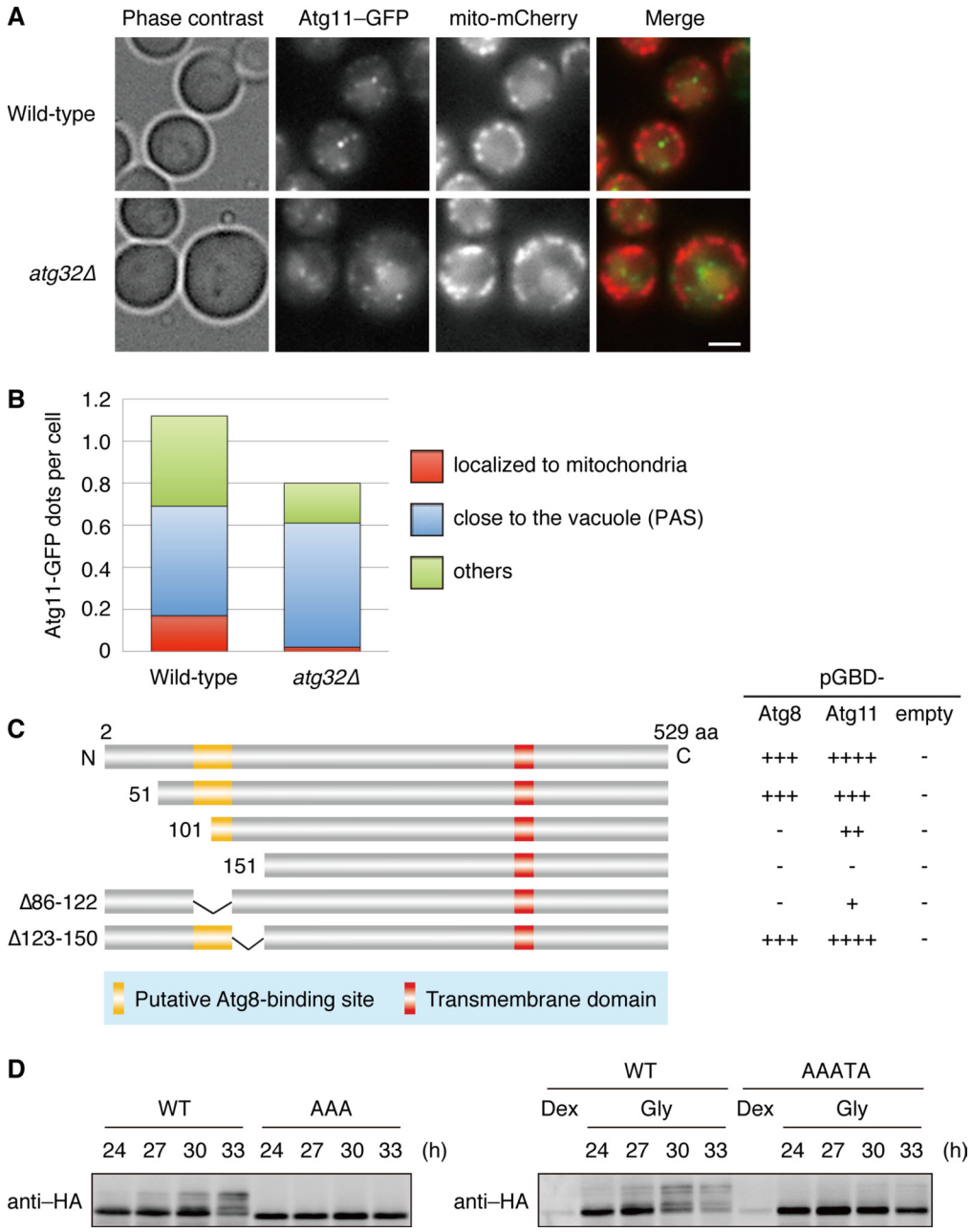

FIGURE S3. **Atg11 targets to mitochondria via Atg32.** *A*, *GFP* was integrated into the chromosomally encoded *ATG11* of wild-type or *atg32Δ* cells expressing a mitochondrial matrix-localized mCherry (mito-mCherry). Cells were grown in glycerol medium for 30 hr, and analyzed by fluorescence microscopy. Scale bar, 2 μm. *B*, At least 200 cells of each strain displaying Atg11–GFP dots observed in *A* were scored, and the localization patterns were classified into the indicated categories. *C*, Cells were transformed with two yeast two-hybrid assay plasmids pGAD-*ATG32* wild-type or -*atg32* mutants (see schematic representations), and pGBD-*ATG8*, -*ATG11*, or -empty constructs, and grown on +His and –His+3AT plates for 4-5 days. *D*, Expression patterns of HA-tagged Atg32 (WT), $Atg32^{AAA}$ (AAA) for $EEE^{120-122}$, or $Atg32^{AAATA}$ (AAATA) for $SSDTS^{115-119}$ in glucose- (Dex) or glycerol-grown (Gly) cells (see Fig. 4*A*). All strains are vacuolar protease-deficient, *atg32*-null derivatives. Cells were collected at the indicated time points, and subjected to western blotting.

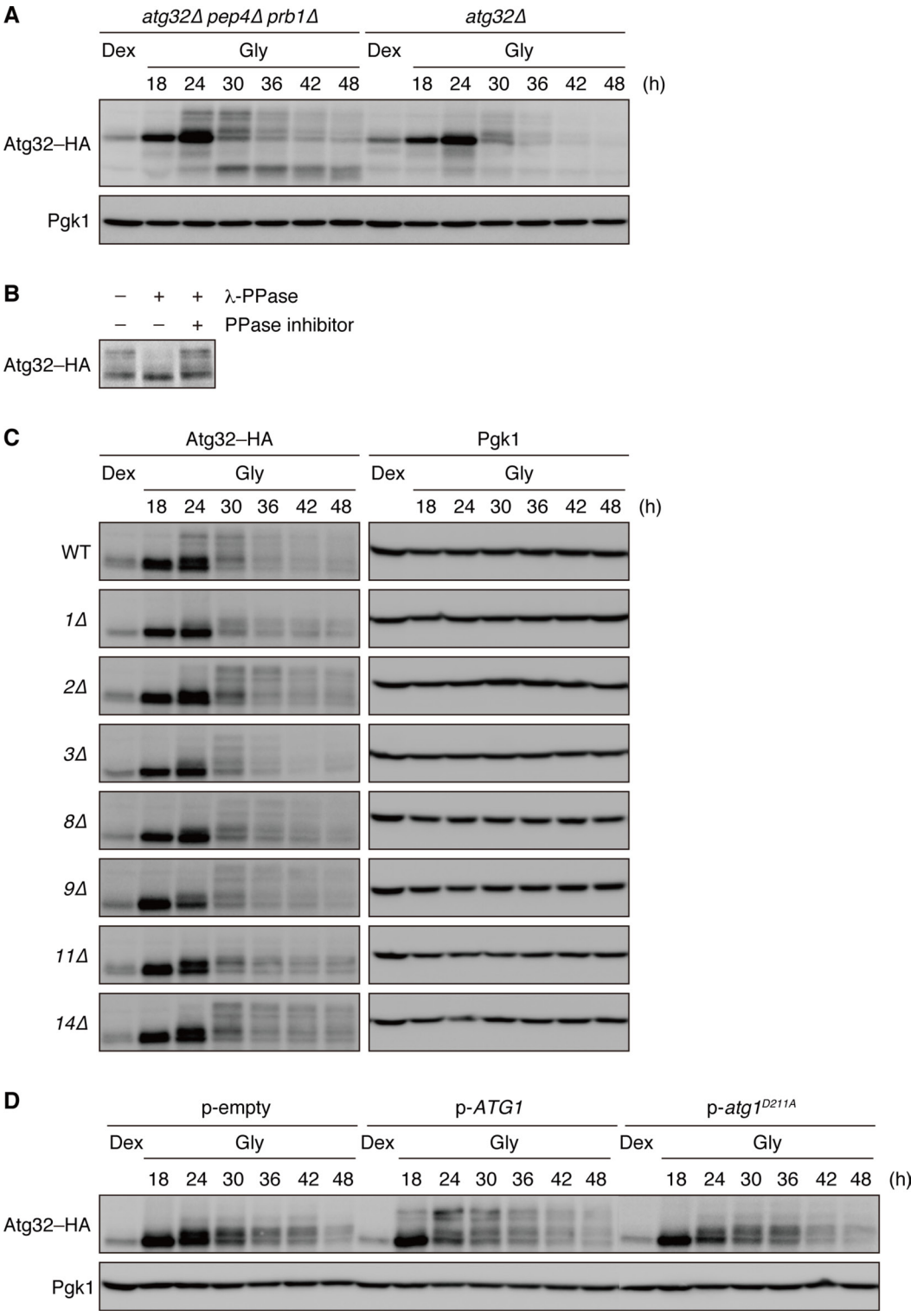

FIGURE S4. **Atg32 is phosphorylated during respiratory growth.** *A*, Expression of Atg32–HA in glucose- (Dex) or glycerol-grown (Gly) cells lacking the endogenous Atg32 that are vacuolar protease-deficient (*atg32Δ pep4Δ prb1Δ*) or -competent (*atg32Δ*). HA-tagged Atg32 was expressed from a low-copy plasmid under the *ATG32* promoter. Cells were collected at the indicated time points, and subjected to western blotting. Pgk1 was monitored as a loading control. *B*, For protein phosphatase

treatment, *atg32Δ pep4Δ prb1Δ* cells expressing Atg32–HA were grown in glycerol medium, collected at the 32 hr time point, and subjected to alkaline lysis and TCA precipitation. The pellet was resuspended in a reaction buffer, treated with or without lambda protein phosphatase (λ-PPase) in the presence or absence of PPase inhibitor. *C*, Expression patterns of Atg32–HA in autophagy-competent (WT) and *atg1~14* null mutant (*1Δ~14Δ*) cells grown in glucose (Dex) or glycerol (Gly) medium. All strains are vacuolar protease-deficient, *atg32*-null derivatives. Cells were collected at the indicated time points, and subjected to western blotting. *D*, Vacuolar protease-deficient cells lacking both Atg32 and Atg1 were transformed with a plasmid that encodes Atg32–HA, and an empty (p-empty) or the one that encodes wild-type Atg1 (p-*ATG1*) or a kinase-dead mutant (p-$atg1^{D211A}$). Expression of these proteins was controlled under the endogenous promoters. Cells were grown in glucose (Dex) or glycerol (Gly) medium, collected at the indicated time points, and subjected to western blotting.

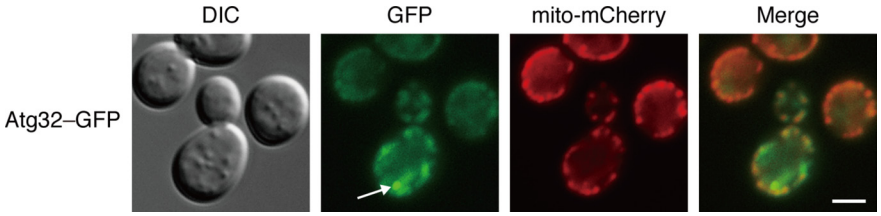

FIGURE S5. **Atg32 exhibits a dot-like localization pattern.** Cells expressing a mitochondrial matrix-localized DHFR–mCherry (mito-mCherry) were transformed with a low-copy plasmid that encodes Atg32–GFP under the endogenous promoter, grown in glycerol medium for 24 hr, and observed by fluorescence microscopy. This strain is an *atg32*-null derivative. Arrow indicates a discrete dot. Scale bar, 2 μm.