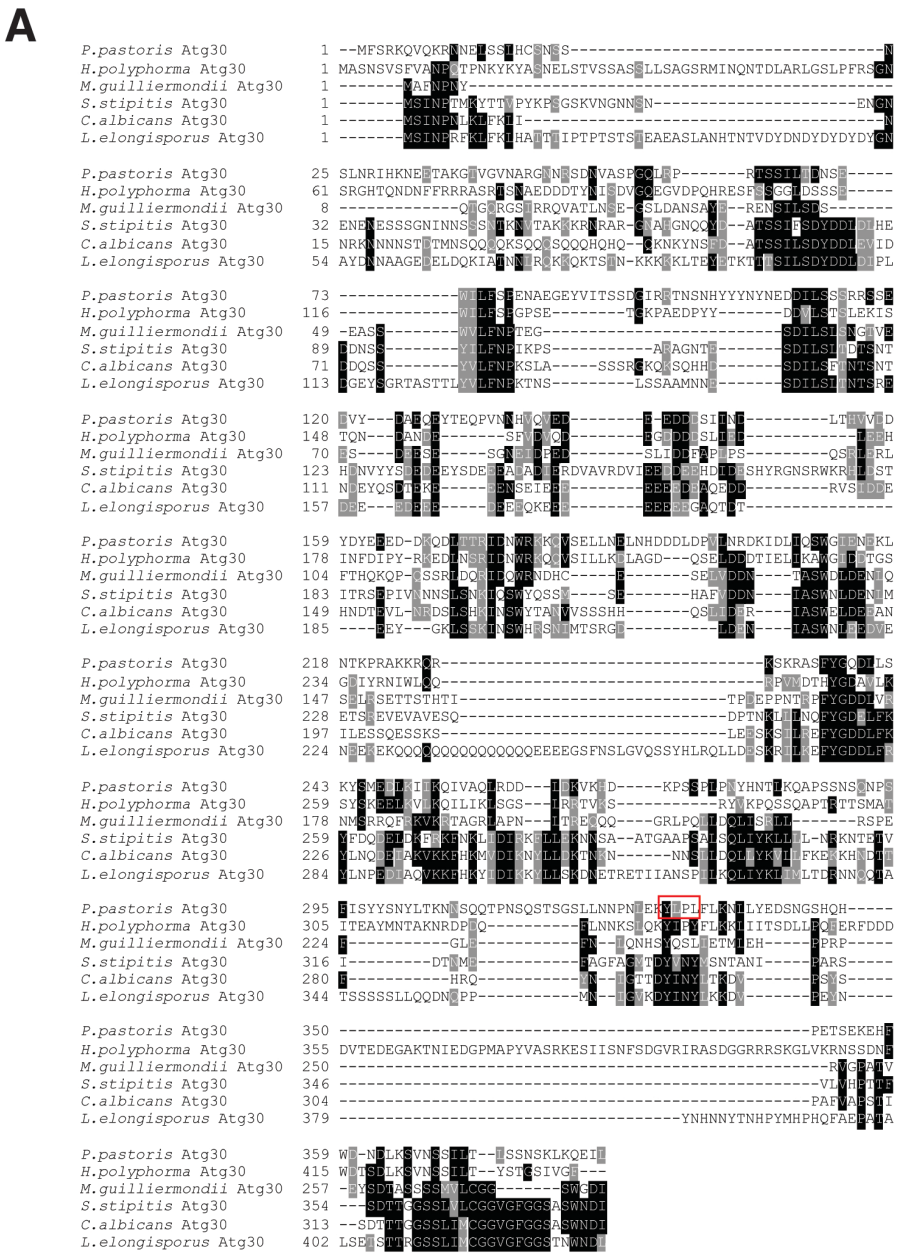


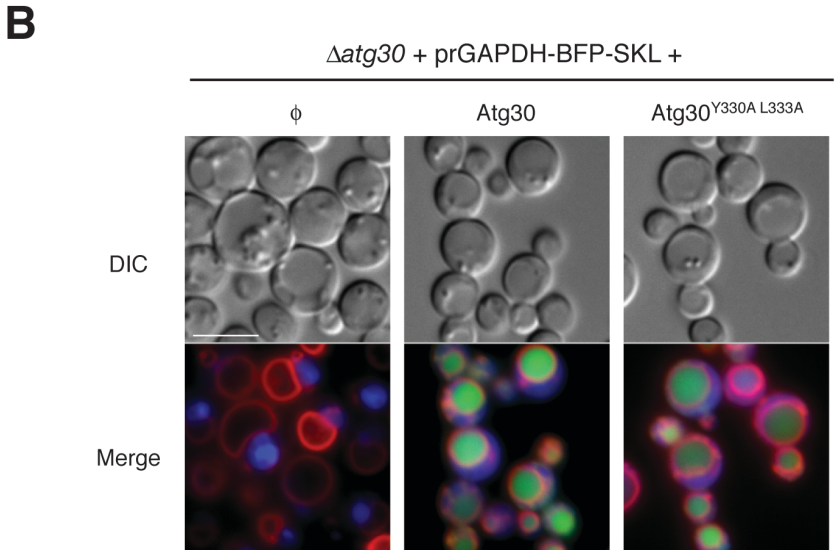
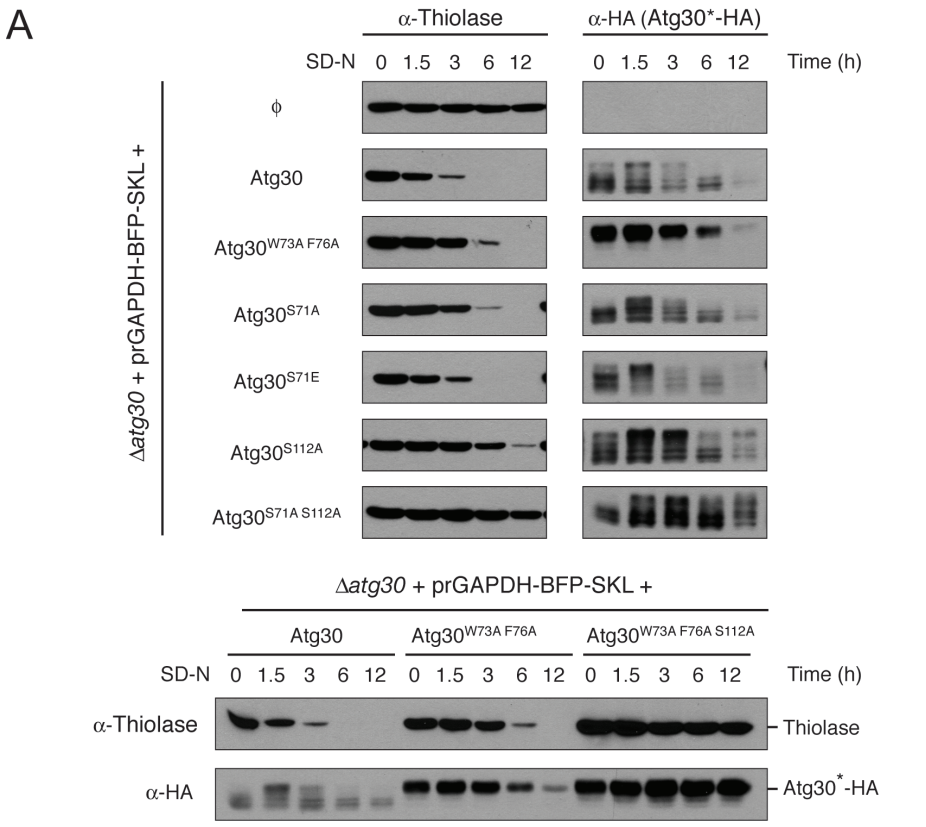
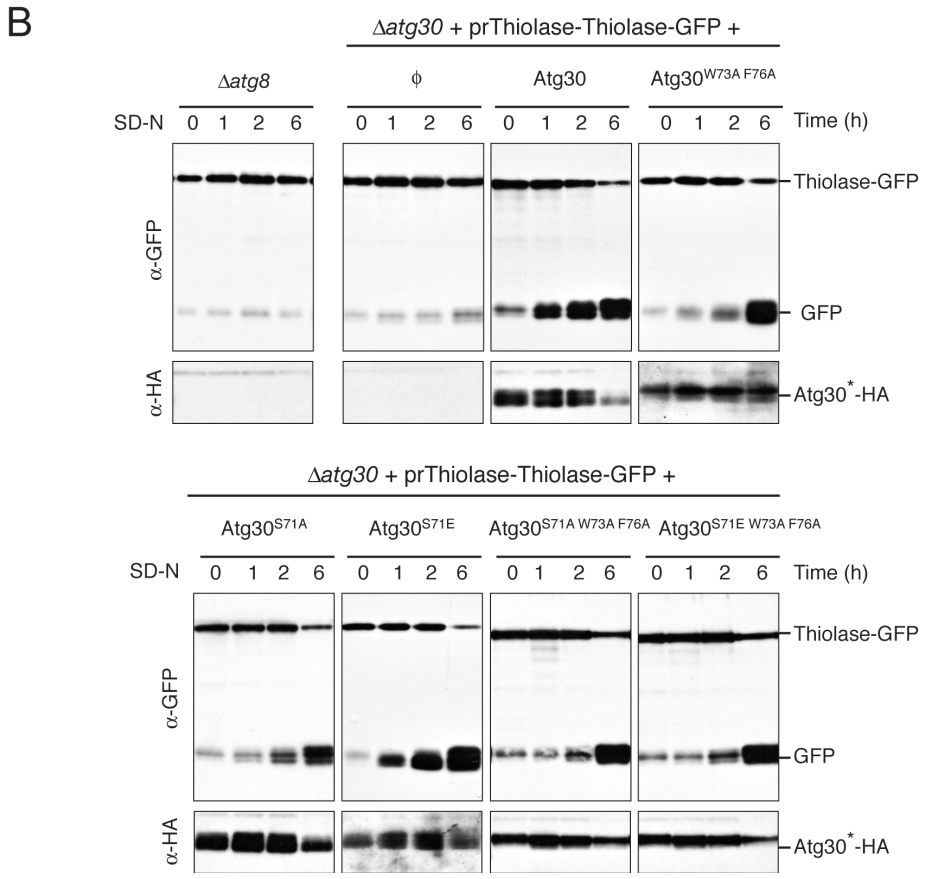


Figure S1. Non-conserved and non-functional AIM in Atg30. (**A**) Multiple sequence alignments of Atg30 homologs (identical residues are indicated with black boxes and similar residues with grey boxes). Sequences, accession numbers, abbreviation of organism names and methodology used for the alignments are in Materials and Methods. Red box indicates a putative AIM in Atg30. (**B**) Pexophagy assay by fluorescence microscopy analysis of methanol-induced peroxisomes labeled with BFP-SKL and Atg30-GFP. ϕ: Δ*atg30*; Atg30: WT; Atg30$^{Y330A\ L333A}$: putative AIM mutant. Vacuoles are labeled with FM4-64. Scale bar: 5 μm.


A
α-Thiolase
α-HA (Atg30*-HA)
SD-N 0 1.5 3 6 12
0 1.5 3 6 12 Time (h)
Δatg30 + prGAPDH-BFP-SKL +
ϕ
Atg30
Atg30W73A F76A
Atg30S71A
Atg30S71E
Atg30S112A
Atg30S71A S112A
Δatg30 + prGAPDH-BFP-SKL +
Atg30
Atg30W73A F76A
Atg30W73A F76A S112A
SD-N 0 1.5 3 6 12 0 1.5 3 6 12 0 1.5 3 6 12 Time (h)
α-Thiolase
Thiolase
α-HA
Atg30*-HA


B
Δatg30 + prThiolase-Thiolase-GFP +
Δatg8
ϕ
Atg30
Atg30W73A F76A
SD-N 0 1 2 6 0 1 2 6 0 1 2 6 0 1 2 6 Time (h)
α-GFP
Thiolase-GFP
GFP
α-HA
Atg30*-HA
Δatg30 + prThiolase-Thiolase-GFP +
Atg30S71A
Atg30S71E
Atg30S71A W73A F76A
Atg30S71E W73A F76A
SD-N 0 1 2 6 0 1 2 6 0 1 2 6 0 1 2 6 Time (h)
α-GFP
Thiolase-GFP
GFP
α-HA
Atg30*-HA

C

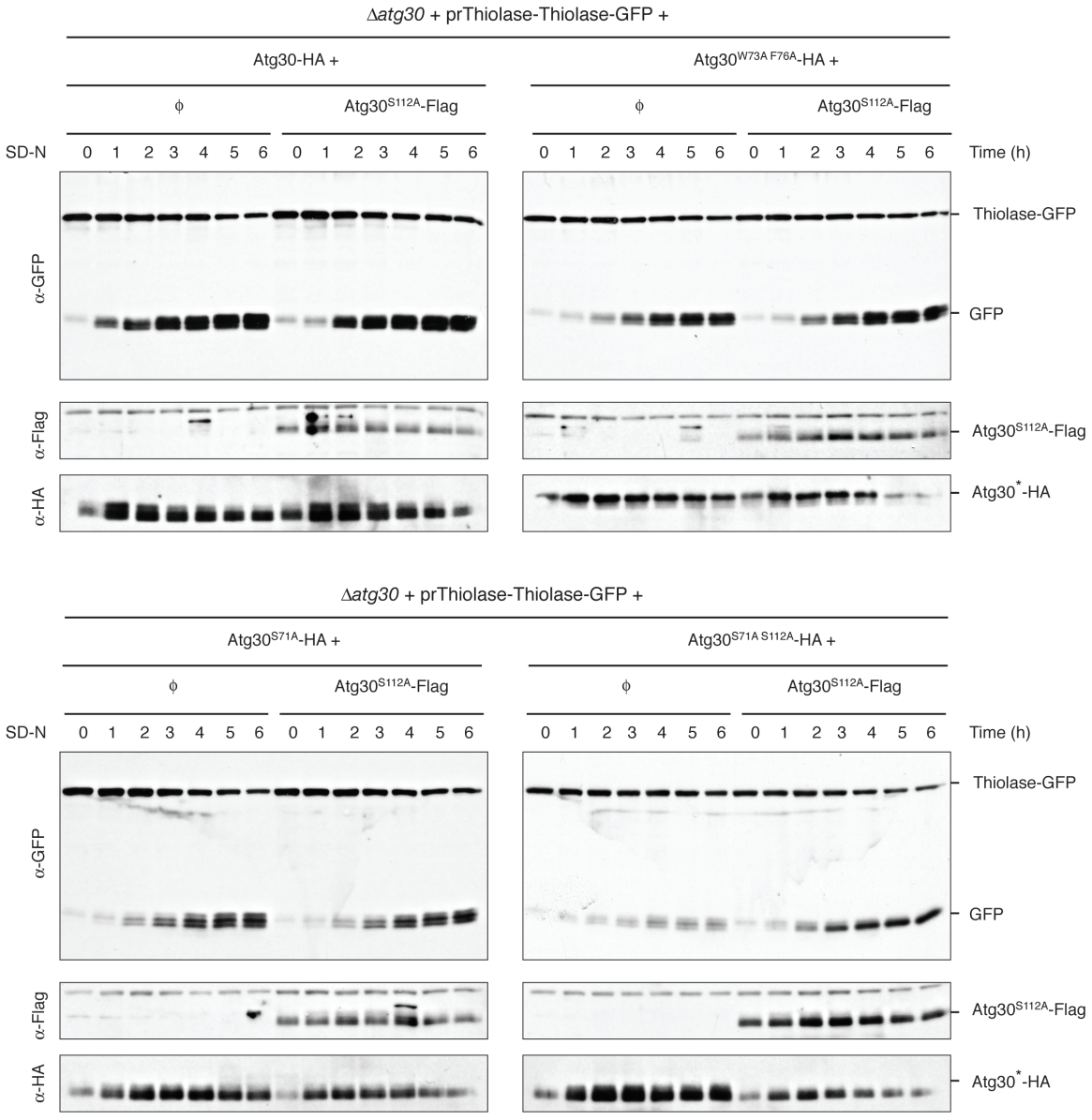
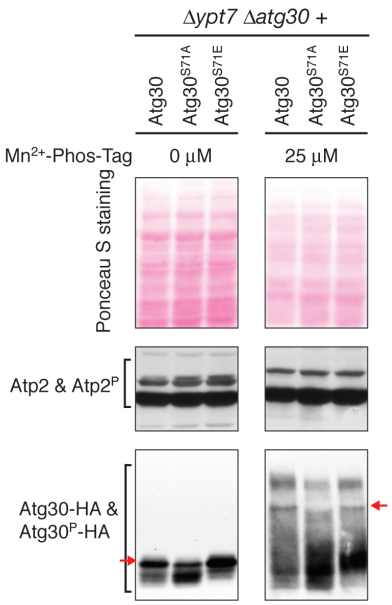


Figure S2. Pexophagy experiments monitored by degradation of thiolase (**A**) or by appearance of free GFP (**B** and **C**). Degradation of oleate-induced peroxisomes was stimulated by shifting cells to SD-N. The presence of the specified proteins was examined by immunoblotting using α-*Sc*thiolase, α-GFP, α-HA and/or α-Flag antibody. (**A**) Pexophagy in Δ*atg30* (ϕ), WT (Atg30) and Atg30 mutants was monitored by thiolase levels. (**B** and **C**) Pexophagy was monitored by appearance of free GFP. *: Atg30 wild-type or mutant forms.

A



B

Rank	Score	SPI (%)	VML	Variable sites	Sequence
1	10.41	60.7	1.3	S71s	(R) T S S I L T/D N s E W/I|L|F/S|P E N R (E) *

C

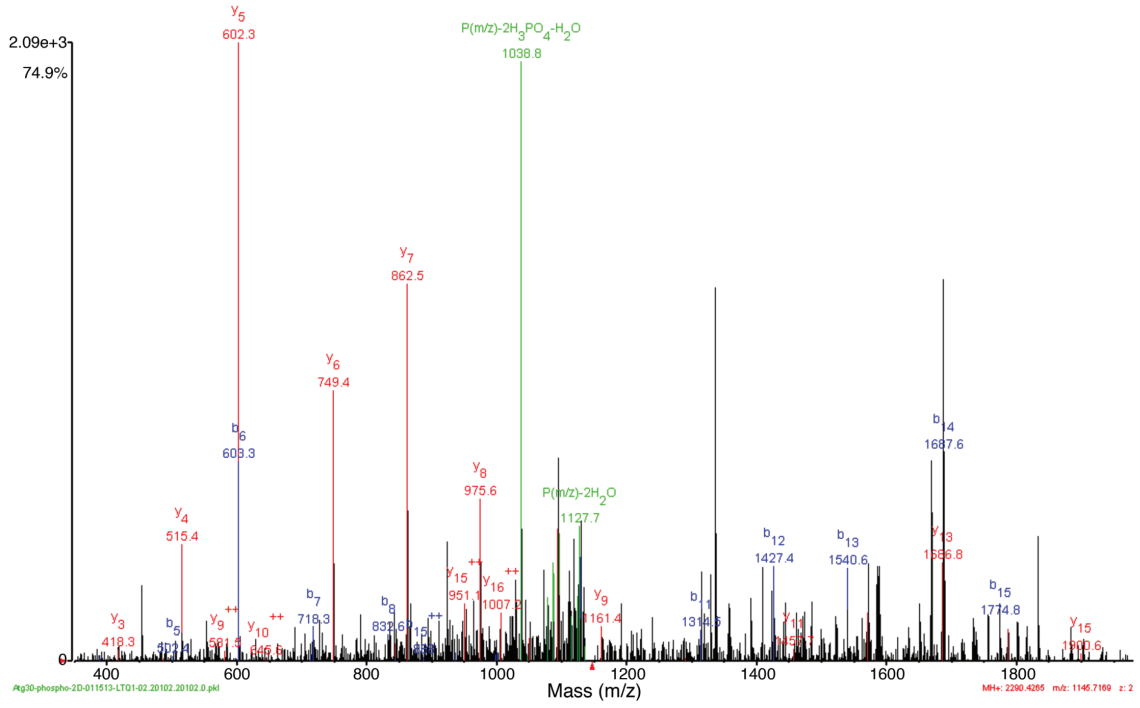


Figure S3. Phosphorylation of S71 in the Atg30 protein. (**A**) Mobility shift detection of Atg30 and Atg30 phosphomutants (Atg30^{S71A} and Atg30^{S71E}) proteins. Phosphorylated

molecules were separated using Phos-tag acrylamide as described in the Methods section of Supplementary information. Cells were grown in oleate medium for 15 h and shifted to SD-N for 30 min. Ponceau S staining and Atp2 were used as a loading control. Red arrow indicates a band absent in $Atg30^{S71A}$. (**B**) Table containing result of Spectrum Mill search result of S71 phospho-peptide. SPI (%): Score Peak Intensity. VML: Variable Modification Localization score [1]. The site is considered localized when VML > 1.1. *: / = y ions; | = both b and y ions. (**C**) MS2 spectra revealing the S71 phospho-peptide.

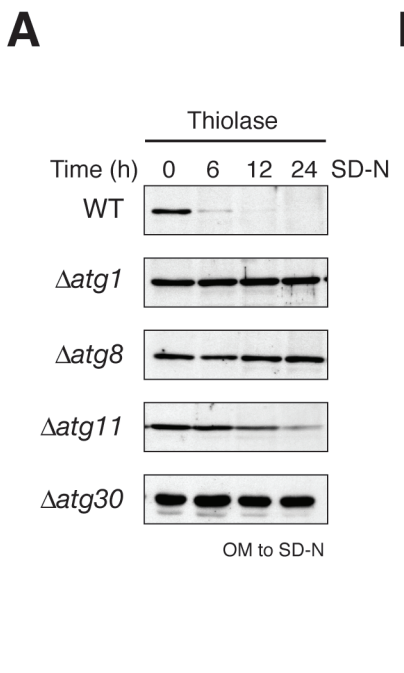


Figure S4. Atg8 and Atg11 are indispensable for pexophagy. (**A**) Pexophagy experiments of oleate-induced peroxisomes shifted to SD-N followed by degradation of thiolase. (**B**) Large phagophore membrane formation in WT, Δ*atg8* and Δ*atg11* cells monitored with GFP-Atg26 during pexophagy upon switch from methanol to SD-N [2].

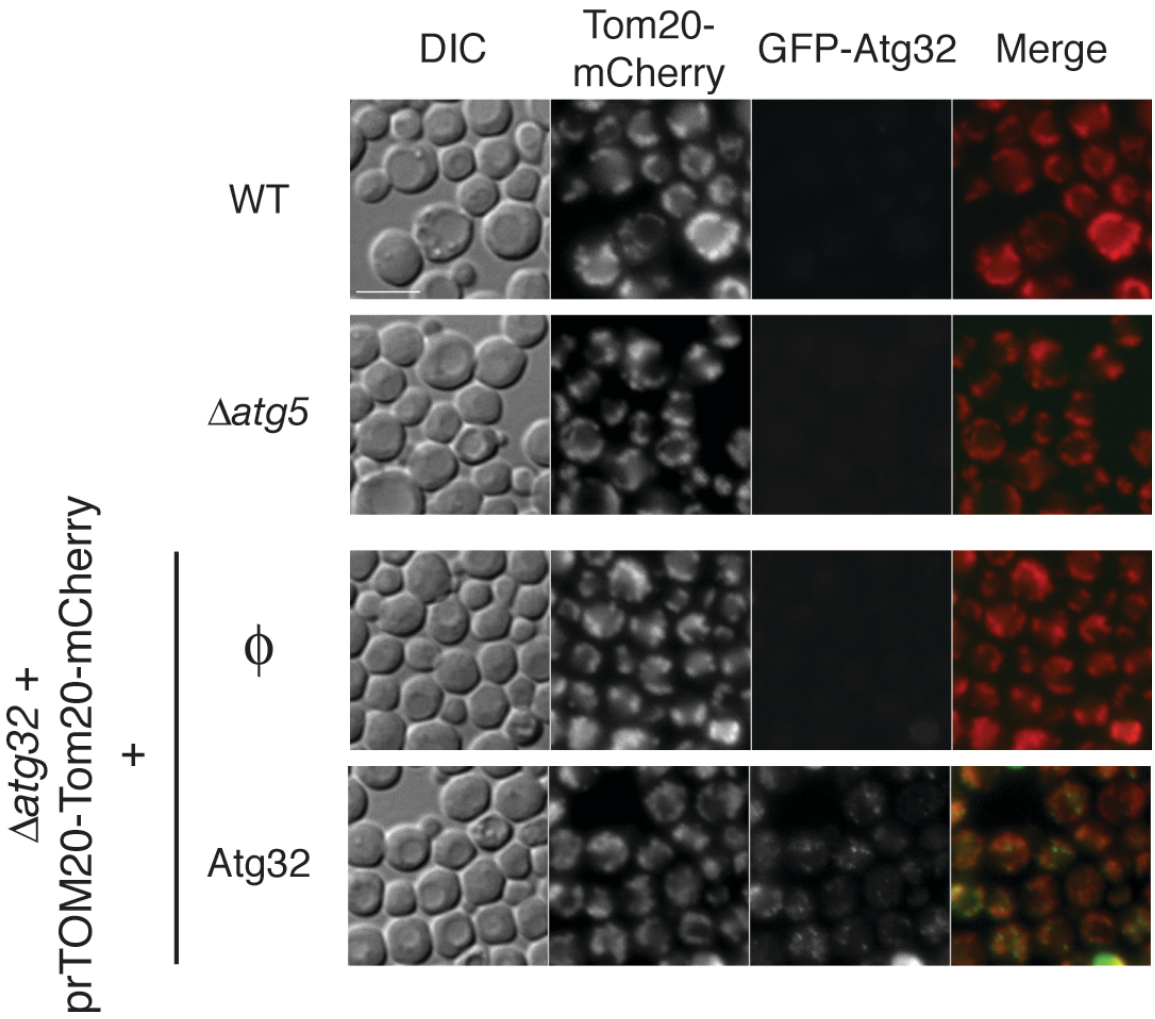
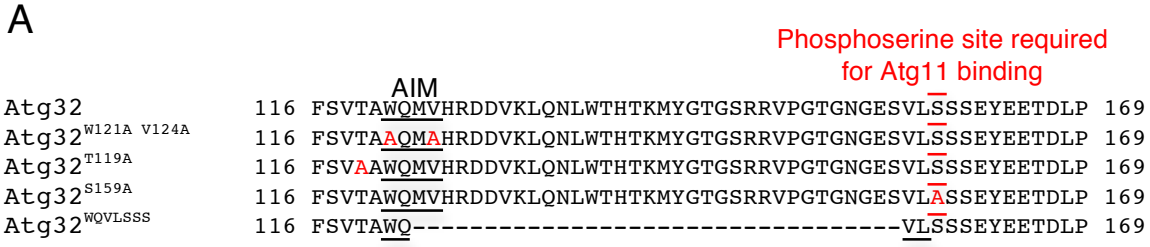


Figure S5. The *P. pastoris* Atg32 localizes on mitochondria. Cells grown in YPL medium for 12 h. The fusion protein, GFP-Atg32, colocalized with Tom20-mCherry at the mitochondria. prTOM represents the promoter of the *TOM20* gene. Scale bar: 5 μm.

A



B

Figure S6. Interactions between *P. pastoris* Atg32 and Atg8. (**A**) Atg32 sequences from aa 116-169 of wild-type (Atg32), the T119A mutant, the AIM mutant ($Atg32^{W121A\ V124A}$), the S159A mutant ($Atg32^{S159A}$) affecting the Atg11 binding site and a deletion of the sequence between the AIM and phosphosite required for Atg11 binding in Atg32 ($Atg32^{WQVLSSS}$). (**B**) Two-hybrid assays between Atg32 wild-type or mutants and Atg8. Phosphomimic T119E was included in $Atg32^{WQVLSSS}$ to detect the interaction with Atg8 because this site on the heterologous protein is not phosphorylated in *S. cerevisiae*.

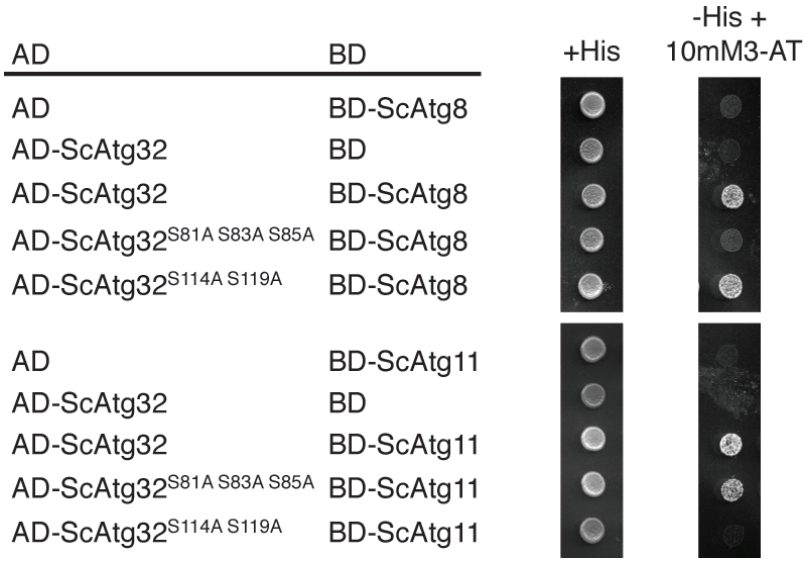


Figure S7. Two-hybrid protein-protein interaction analysis of ScAtg32, ScAtg8 and ScAtg11. The receptor was mutated at Serine(s) upstream of the AIM (ScAtg32$^{S81A\ S83A\ S85A}$) and at the Atg11-binding site (ScAtg32$^{S114A\ S119A}$).

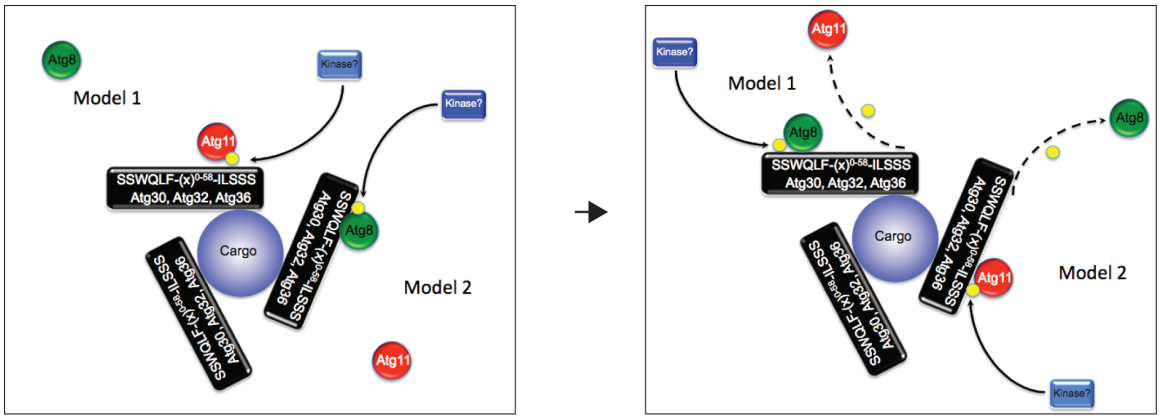


Figure S8. Model for interactions between autophagy receptors and the core autophagic machinery. See text for details.

Tables

Table S1. *Pichia pastoris* strains

Description	Strain	Genotype	Reference
	GS115	*his4*	[3]
WT	PPY12	*his4, arg4*	[4]
Δ*atg1*	R12	GS115 Δ*atg1::Zeocin*	[5]
Δ*atg5*	Sjcf935	PPY12 Δ*atg5::Zeocin*	This study
Δ*atg8*	Sjcf925	PPY12 Δ*atg8::KanMX*	[6]
Δ*atg11*	R8	GS115 Δ*atg11::Zeocin*	[7]
Δ*atg30*	Sjcf959	PPY12 Δ*atg30*::*KanMX*	This study
Δ*atg30*	Sjcf936	PPY12 Δ*atg30::Zeocin*	[6]
Δ*atg32*	Sjcf1715	PPY12 Δ*atg32::KanMX*	This study
Δ*ypt7*	Srrm197	PPY12 Δ*ypt7*::*KanMX*	[8]
Δ*ypt7* Δ*atg30*	Sjcf1736	PPY12 Δ*ypt7*::*KanMX* Δ*atg30::Zeocin*	This study

Table S2. *Saccharomyces cerevisiae* strains

Strain	Description	Genotype	Reference
WT	BY4742 + Thiolase-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, pot1::Thiolase-GFP (HIS5)	[9]
ΔSc*atg1*	Δ*atg1* + Thiolase-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, atg1::KanMX4, pot1:: Thiolase -GFP (HIS5)	[9]
ΔSc*atg36*	Δ*atg36* + Thiolase-GFP	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, atg36::KanMX4, pot1:: Thiolase-GFP (HIS5)	This study

Table S3. *Pichia pastoris* plasmids

Plasmid	Promoter	Fusion protein	Integration locus	Selectable marker
pJCF143	*ATG30*	Atg30-Flag	*ATG30*	Zeocin
pJCF208	*ATG8*	GFP-Atg8	*HIS4*	*HIS4*
pJCF213	*ATG30*	Atg30-GFP	*HIS4*	*HIS4*
pJCF291	*ATG11*	GFP-Atg11	*ARG4*	*ARG4*
pJCF388	*ATG11*	Flag-Atg11	*ARG4*	*ARG4* or Zeocin
pJCF392	*GAPDH*	GFP-Atg26	*HIS4*	*HIS4*
pJCF401	*GAPDH*	BFP-SKL	*HIS4*	*HIS4* or Geneticin
pJCF402	*GAPDH*	BFP-SKL	*ARG4*	*ARG4*
pJCF419	*ATG8*	GFP-Atg8	*HIS4*	*HIS4* or Hygromycin
pJCF523	*TOM20*	Tom20-mCherry	*ARG4*	*ARG4* or Hygromycin
pJCF592	*ATG32*	Atg32-GFP	*HIS4*	*HIS4*
pJCF648	*ATG30*	Atg30-HA	*HIS4*	*HIS4*
pJCF681	*ATG32*	Atg32-HA	*HIS4*	*HIS4*
pJCF688	*TOM20*	Tom20-GFP	*ARG4*	*ARG4* or Hygromycin
pJCF694	*ATG8*	myc-Atg8	*HIS4*	*HIS4* or Hygromycin
pJCF701	*THIOLASE*	Thiolase-GFP	*ARG4*	*ARG4*

Table S4. *Saccharomyces cerevisiae* plasmids

Plasmid	Fusion protein 1	Fusion Protein 2	Background vector	Selectable marker
pJCF589-F115	BD-PpAtg8	none	pGBT9	*TRP1*
pJCF226	AD-PpAtg30	none	pGAD-GH	*LEU2*
pJCF590	AD-PpAtg32	none	pGAD-GH	*LEU2*
pJCF659	BD-ScAtg8	none	pGBT9	*TRP1*
pJCF660	BD-ScAtg11	none	pGBT9	*TRP1*
pJCF652	AD-ScAtg32	none	pGAD-GH	*LEU2*
pJCF653	AD-ScAtg36	none	pGAD-GH	*LEU2*
pJCF800	AD-ScAtg8	none	pGBT9	*TRP1*
pJCF801	BD-ScAtg8	NLS-ScAtg11	pBridge	*TRP1*
pJCF804	BD-ScAtg11	NLS-ScAtg8	pBridge	*TRP1*
pJCF805	HA-ScAtg36	none	pRS415	*LEU2*

Methods

Media used to grow strains include: YPD (2% glucose, 2% bacto-peptone, 1% yeast extract); YNB (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate); nitrogen starvation medium or SD-N (0.17% yeast nitrogen base without amino acids and ammonium sulfate; 2% glucose); oleate medium for *P. pastoris* (YNB; 0.079% complete supplement mixture [CSM], 0.05% yeast extract, 0.02% Tween-40, 0.2% oleate), oleate medium for *S. cerevisiae* (1% oleate, 5% Tween-40, 0.25% yeast extract, 0.5% peptone, and 5 mM phosphate buffer) and methanol medium (YNB; 0.079% CSM, 0.05% yeast extract, 0.5% methanol); YPL (2% lactic acid, 2% bacto-peptone, 1% yeast extract, pH 5.5). All cultures were grown at 30°C.

In silico analysis

Atg30 and ScAtg32 protein sequences were used as queries in Standard Protein BLAST analyses on dataset of the non-redundant (nr) protein sequences, using default parameters and the algorithm PSI-BLAST (Position-Specific Iterated BLAST) at the National Center for Biotechnology Information (NCBI). The organism names, abbreviations and GenBank accession numbers of Atg30 homologs are the following: *Candida boidinii* (*Cb*, BAL15150), *Clavispora lusitaniae* (*Cl*, EEQ38313), *Spathaspora passalidarum* (*Sp*, EGW35335), *Candida tropicalis* (*Ct*, EER31772), *Millerozyma farinosa* (*Mf*, CCE81513), *Meyerozyma guilliermondii* (EDK37477), *Candida parapsilosis* (*Cp*, CCE40725), *Candida dubliniensis* (*Cd*, CAX44784), *Lodderomyces elongisporus* (*Le*, EDK43552), *Scheffersomyces stipitis* (*Ss*, EAZ63411), *Candida albicans* (*Ca*, EEQ42629), *Debaryomyces hansenii* (*Dh*, CAG86787), *Candida tenuis* (*Cte*, EGV64948) and *Hansenula polymorpha* (*Hp*, EFW95627). The Atg32 homologs: *Hansenula polymorpha* (EFW94927), *Pichia pastoris* (*Pp*, CAY71556), *Kluyveromyces lactis* (*Kl*, CAH02615), *Zygosaccharomyces rouxii* (*Zr*, CAR29352), *Ashbya gossypii* (*Ag*, AAS53654), *Candida glabrata* (*Cg*, CAG60013), *Vanderwaltozyma polyspora* (*Vp*, EDO18565), *Tetrapisispora phaffii* (*Tp*, CCE63093), *Lachancea thermotolerans* (*Lt*, CAR23100), *Torulaspora delbrueckii* (*Td*, CCE90351), *Kazachstania africana* (*Ka*, CCF57872), *Eremothecium cymbalariae* (*Ec*, AET39104), *Naumovozyma castellii* (*Nc*, CCC70913), *Scheffersomyces stipitis* (ABN68377), *Spathaspora passalidarum*

(EGW34613), *Candida tropicalis* (EER35239), *Debaryomyces hansenii* (CAG88355), *Millerozyma farinosa* (CCE81282) and *Clavispora lusitaniae* (EEQ41615). Multiple sequence alignments were done using ClustalW2 and manually adjusted if required. The sequence logo was obtained using the combined multiple sequence alignments of all Atg30 and Atg32 homologs in the GenBank database (up to Nov 2011, described the supplementary information) and the default setup of the WebLogo server (http://weblogo.berkeley.edu/logo.cgi).

Mobility shift detection of phosphorylated proteins

One ml of Δ*ypt7* Δ*atg30* cells expressing Atg30, Atg30^{S71A} and Atg30^{S71E} was collected after peroxisome proliferation in oleate medium for 15 h and shifted to nitrogen starvation for 30 min. and TCA precipitated. Samples were resolved in a 10% SDS-PAGE gel with 25 μM Phos-Tag acrylamide (Wako Cat. #300-93523) and 25 μM $MnCl_2$ to improve the separation of phosphoproteins. Phos-Tag acrylamide gels were treated according manufacturer's recommendation and analyzed by Western blot.

Co-immunoprecipitation with and without phosphatase treatement

A) Peroxisomes purification: 300 hundred OD of cells were grown 15-16 h in oleate medium, washed twice with dH_2O and transferred to glucose medium, SD-N at 2 OD/ml for 0.5 h. The pellet weight was used to determine the volume of Zymolase buffer (0.5 M KCl, 5 mM MOPS/KOH buffer [pH 7.2] 10 mM Na_2SO_4; 4 ml/g of cells) and concentration of Zymolase-100T (Nacalai Tesque) to add to cells (0.5 mg/1g cells). Cells were then incubated at 30°C (80 rpm for 45 min) and spheroplasts were centrifuged (2200 x g, 8 min, 4°C) before being resuspended in 1 homogenization buffer (5 mM MES/KOH [pH 5.5], 1 M Sorbitol with 10 mM NaF, 1 mM PMSF, 1 mM protease inhibitors: PIC yeast, Leupeptin and Aprotinin; 2 mL/g of cells) and subjected to 20 strokes in a Dounce homogenizer. The cell homogenate was centrifuged twice at low speed to remove cell debris (500 x g, 10 min, 4°C) and the supernatant spun at high speed (200, 000 x g, 30 min, 4°C). The pellet was retrieved and resuspended in 0.8 mL of IP lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1 mM EDTA, 0.2% Triton X-100, and 10 mM NaF, 1 mM PMSF, 1 mM protease inhibitors: PIC yeast, Leupeptin and

Aprotinin). The addition of 250 μl acid-washed glass beads and vortexing (1 min x 5 times) lysed the peroxisome fraction, which was further solubilized with rotation (30 min, 4°C). The supernatant was recovered after centrifugation (20, 000 x g, 10 min, 4°C) and total protein concentration was measured using the Bradford assay. The supernatant was diluted to 200 μg of protein in 1mL with IP lysis buffer; 10 μl of sample was used for the input lane (Input).

B) Co-immunoprecipitation: 100 μl of EZ-View HA-beads (Sigma) were added to the supernatant containing 200 μg of protein in 1 ml and rotated for 16 hr, 4°C. The samples were either washed four times with IP lysis buffer (5 mL for 10 min) or were further phosphatase treated. HA-Beads after the wash were resuspended in 100 μL of 1% SDS and 1x loading buffer and boiled at 100°C for 10 min to elute, followed by SDS-PAGE and immunoblotting.

C) Lambda Protein Phosphatase (λPP) treatment: the protein complex purified using EZ-View HA-beads from Δ*atg30* cells, with or without Atg30-HA expression, was collected in duplicate and washed once with 5 ml of IP lysis buffer and twice with 500 µl of NEBuffer for PMP (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, pH 7.5). The HA-bead complex was resuspended in 50 µl of NEBuffer for PMP with 1.5 µl of λPP (+; NEB, 400,000 U/ml) and without λPP (-), and incubated at 30°C for 1 h. To stop the reaction and clean the HA-bead complex, two 5 ml short washes with IP lysis buffer, as well as two 5 ml washes for 10 min with IP lysis buffer were used. Finally, samples were eluted with 100 µl of 1% SDS and 1x loading buffer, boiled and subjected to SDS-PAGE and immunoblotting.

Proteins purification and sequencing

A) Proteins purification: HA-tagged Atg30, mutated at position 81 (A81R) to reduce the size of the tryptic peptide encompassing S71 (from 33 to 20 aa), was expressed in Δ*atg30* cells. Cells were grown overnight on oleate medium and transferred for 30 min into SD-N before extraction. Atg30-HA purification was performed from 3000 OD equivalents of yeast cells. The cells pellet was washed in phosphate-buffered saline (pH 7.4) and lysed with 5 mL of glass beads (vortexed 5 times for 2 min at 4°C) in 20 ml IP lysis buffer (50

mM Tris-HCl, pH 7.5, 100mM NaCl, 0.1mM EDTA, 0.2% Triton X-100, 10 mM NaF, 1mM PMSF, 1mM protease inhibitors cocktail: PIC yeast, Leupeptin and Aprotinin). The cells debris was removed by centrifugation at low speed (500 × g, 10 min). The membrane protein solubilization was performed by incubation of the supernatant at 4°C for 1 h with rotation followed by centrifugation (21,000 × g, 10 min). One mL of EZ-View HA-beads (Sigma) was added to the supernatant and incubated overnight. Beads were washed five times with 10 mL of the IP lysis buffer for 5 min and three times with 5 mL of 50 mM HEPES pH 7.2.

B) Mass spectrometry: proteins bound to EZ-View HA-beads were digested with 0.5 µg trypsin at 37°C overnight. Phosphopeptides were enriched by metal oxide affinity capture (CeO2) and analyzed by 2D LC MS/MS using an LTQ tandem mass spectrometer.

An Agilent 1200 HPLC system (Agilent Technologies) delivered a flow rate of 600 nL min^{-1} to a 3-phase capillary chromatography column through a splitter. Using a custom pressure cell, 5 µm Zorbax SB-C18 (Agilent) was packed into fused silica capillary tubing (250 µm ID, 360 µm OD, 30 cm long) to form the first dimension reverse phase column (RP1). A 5 cm long strong cation exchange (SCX) column packed with 5 µm PolySulfoethyl (PolyLC) was connected to RP1 using a zero dead volume 1 µm filter (Upchurch, M548) attached to the exit of the RP1 column. A fused silica capillary (200 µm ID, 360 µm OD, 20 cm long) packed with 5 µm Zorbax SB-C18 (Agilent) was connected to SCX as the analytical column (RP2). The electrospray tip of the fused silica tubing was pulled to a sharp tip using a laser puller (Sutter P-2000). The peptide mixtures were loaded onto the RP1 column using the custom pressure cell. Peptides were first eluted from the RP1 column to the SCX column using a 0 to 80% acetonitrile gradient for 150 min. The peptides were then fractionated by the SCX column using a series of salt gradients (from 5 mM to 1 M ammonium acetate for 20 min), followed by high resolution reverse phase separation using an acetonitrile gradient of 0 to 80% for 120 min.

Spectra were acquired on LTQ-XL linear ion trap tandem mass spectrometers (Thermo Electron Corporation, San Jose, CA) employing automated, data dependent acquisition. The mass spectrometer was operated in positive ion mode with a source temperature of 250 °C. As a final fractionation step, gas phase separation in the ion trap was employed to separate the peptides into 3 mass classes prior to scanning; the full MS scan range was

divided into 3 smaller scan ranges (400–800, 800–1,200, and 1,200–2,000 Da) to improve dynamic range. Each MS scan was followed by 5 MS/MS scans of the most intense ions from the parent MS scan. A dynamic exclusion of 1 min was used to improve the duty cycle.

The raw data were extracted and searched using Spectrum Mill v4.01 (Agilent Technologies). MS/MS spectra with a sequence tag length of 1 or less were considered to be poor spectra and were discarded. The remaining MS/MS spectra were searched against NCBI non-redundant protein database limited to *P. pastoris* taxonomy. The enzyme parameter was limited to full tryptic peptides with a maximum mis-cleavage of 1. All other search parameters were set to SpectrumMill's default settings (carbamidomethylation of cysteines, +/- 2.5 Da for precursor ions, +/- 0.7 Da for fragment ions, and a minimum matched peak intensity of 50%). Ox-Met, n-term pyro-Gln, and phosphorylation on Serine, Threonine, or Tyrosine were defined as variable modifications. A maximum of 2 modifications per peptide was used. Spectra with score of 10 or higher were manually inspected. Phosphorylation sites were localized to a particular amino acid within a peptide using the variable modification localization (VML) score in Agilent's Spectrum Mill software [1].

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