MATERIALS AND METHODS

Yeast strains and media

Standard protocols were used for yeast manipulations (Kaiser, 1994). Cells were cultured at 30 ℃ in SD+CA medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.5% casamino acids, and 2% glucose) supplemented with appropriate nutrients. Autophagy was induced by transferring the cells to SD (-N) medium (0.17% yeast nitrogen base, without amino acids and ammonium sulfate, and 2% glucose).

BY4741 (*MATa his3D leu2D met15 Dura3D*) and other ATG disruptants used in this study are listed in Supplemental Table 1. BY4741 was purchased from Invitrogen. Media and methods for gene disruption have been described previously (Mark S. Longtine et al, 1998).

Plasmids and other materials

Each full-length *atg* gene with its endogenous promoter and terminator was amplified by PCR and ligated into YEplac181, YCplac111 and pRS316 plasmids with appropriate restriction endonucleases. The ORF region and the downstream 600bp was amplified by PCR and inserted into YEplac181 plasmid after the GAL4 promoter and the N-GST tag sequence.

Purification of GST-tagged proteins

To express each ATG protein for purification, plasmid YEplac181, containing the GAL4 promoter and the ORF of the *atg* gene, was transformed into BY4741. Cells were incubated in up to 2 liters of SD-Leu medium containing 2% raffinose instead of glucose from OD_{600}=0.2 until OD_{600}=0.8-1.2 with rotation in a 30 ℃ incubator. Galactose was added at a final concentration of 2% to induce GST-ATG expression for 2-4 hours. 1 liter of the culture was harvested as non-starved cells. The remaining cells were washed three times with SD (-N) medium containing 2% raffinose and galactose. The cells were then starved for 1 hour in this SD (-N) medium and then harvested and lysed together with the non-starved cells. GST-ATG protein was purified with Glutathione Sepharose TM 4B (GE Healthcare) as previously described.(Lu et al., 2011). Cells were vortexed with glass beads to break them open with phosphatase inhibitor, then centrifuged at high speed. The supernatant was collected and incubated with Glutathione Sepharose for 2 hours. The column containing the lysate was washed slowly by wash buffer with a high NaCl concentration, and then eluted by reduced GSH (Sigma).

Mutagenesis of phosphorylation sites

Site-specific mutagenesis was performed with a simple PCR method. The target phosphorylation sites were mutated to alanine by nucleic acid replacement. Mutagenesis primer sequence was designed by QuikChange Primer Design tool from Agilent Technologies, containing sequences 15bp upstream and downstream of the

single mutation site. The sequence was much longer if two or more near sites were mutated in one primer (Supplemental table 2). YCplac111 or pRS316 plasmid containing *atg* genes with its endogenous promoter and terminator were amplified using mutagenesis primers. Anneal 10min at 55℃ for 16 thermal cycles.

The products were cut by Dpn I (New England Biolabs) and transformed into competent *E.coli*. Mutations were confirmed by sequencing.

Fluorescence microscopy

For fluorescence microscopy, cells were grown to OD_{600}=0.8-1.0 in appropriate selective medium and shifted to SD (-N) medium for various lengths of time as described (Cheong et al., 2005). The cells were observed at room temperature using FV-1000 (Olympus) confocal microscopes. The percentages of cells with vacuolar GFP-Atg8 fluorescent signals were determined by counting 100 cells in three separate experiments.

Mass spectrum analysis

For LC-MS/MS analysis, peptides were separated by a 90 min gradient elution at a flow rate of 0.250 μl/min with a Thermo-Dionex Ultimate 3000 HPLC system, which was directly interfaced with a Thermo LTQ-Orbitrap Velos pro mass spectrometer. The analytical column was a homemade fused silica capillary column (75 μm ID, 150 mm length; Upchurch, Oak Harbor, WA) packed with C-18 resin (300 A, 5 μm; Varian, Lexington, MA). Mobile phase A consisted of 0.1% formic acid, and mobile

phase B consisted of 80% acetonitrile and 0.1% formic acid. An LTQ-Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur 2.2 software. A single full-scan mass spectrum in the Orbitrap (400–1800 m/z, 30,000 resolution) was followed by 20 data-dependent MS/MS scans in an ion trap at 35% normalized collision energy (CID).

MS/MS spectra from each LC-MS/MS run were searched against ATG proteins in the *Saccharomyces cerevisiae* database using the Proteome Discoverer (Version 1.4) search algorithm. The search criteria were as follows: full tryptic specificity was required; two missed cleavages were allowed; carbamidomethylation was set as a fixed modification; oxidation (M) was set as a variable modification; precursor ion mass tolerance was 10 ppm for all MS acquired in the Orbitrap mass analyzer; and fragment ion mass tolerance was 0.8 Da for all MS2 spectra acquired in the LTQ. A high confidence score filter (FDR < 1%) was used to select the "hit" peptides and their corresponding MS/MS spectra were manually inspected.

We also manually verified the intensity of each phospho-peptide and compared the phosphorylation ratio between cells in full medium and starvation medium.

GPS prediction

The GPS (Group-based Prediction System, currently updated to version 3.0) software was constructed with a modified version of the GPS (Group-based Phosphorylation Scoring) algorithm. Different protein kinases (PKs) exhibit distinct recognition specificities and each PK modifies only a limited subset of substrates. It is accepted that short linear motifs (SLMs) around the phosphorylation sites (p-sites) provide

primary specificity. Eukaryotic PKs were classified into a hierarchy with four levels: group, family, subfamily, and single PK. Based on the hypothesis that similar PKs recognize similar SLMs, a predictor GPS algorithm was constructed for each PK and used to directly predict the potential PKs for each non-annotated p-site from the phospho-proteomic studies. iGPS is based on the GPS algorithm. The iGPS (GPS algorithm with the interaction filter, or in vivo GPS) software was used mainly for the prediction of *in vivo* site-specific kinase-substrate relations (ssKSRs). In iGPS, protein-protein interaction (PPI) information was added as the major contextual factor to filter out potential false-positive hits, in order to obtain a more accurate result.

Supplemental Table1

Yeast strains used in this sutdy.

Name	**Genotype**	**Reference**
BY4741	*MATa his3Δ1 leu2Δ met15Δ ura3Δ*	This study
ScLY1	BY4741, *pRS316[GFP-ATG8]*	This study
ScLY2	BY4741, *pRS315[GFP-ATG8]*	This study
ScLY3	BY4741, *YEPlac181 [Gal1-GST-ATG31]*	This study
ScLY4	BY4741, *atg31Δ::kanMX6*	This study
ScLY6	ScLY4, *YEPlac181 [Gal1-GST-ATG31]*	This study
ScLY27	BY4741, *atg1Δ::kanMX6, YEPlac181 [Gal1-GST-ATG1]*	This study
ScLY28	BY4741, *atg3Δ::kanMX6, YEPlac181 [Gal1-GST-ATG3]*	This study

ScLY29	BY4741, *atg4*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG4]*	This study
ScLY30	BY4741, *atg6*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG6]*	This study
ScLY31	BY4741, *atg8*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG8]*	This study
ScLY32	BY4741, *atg15*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG15]*	This study
ScLY33	BY4741, *atg16*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG16]*	This study
ScLY34	BY4741, *atg17*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG17]*	This study
ScLY35	BY4741, *atg18*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG18]*	This study
ScLY36	BY4741, *atg27*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG27]*	This study
ScLY37	BY4741, *atg29*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG29]*	This study
ScLY38	BY4741, *atg31*Δ*::kanMX6, YEPlac181 [Gal1-GST-ATG31]*	This study

Supplemental table 2

Mutagenesis primers

Atg1 primer	
T226A	ACGTCATTAGCAGAAgCTCTTTGTGGCTCACCA
	TGGTGAGCCACAAAGAGcTTCTGCTAATGACGT
Y332A	GAAGACTTGTCTTCTgcTGAATTGGAAGATGA
	TCATCTTCCAATTCAgcAGAAGACAAGTCTTC
S343A	TTACCTGAGTTAGAAgCCAAATCAAAAGGTATT
	AATACCTTTTGATTTGGcTTCTAACTCAGGTAA
S351A	AAAGGTATTGTAGAAgcTAATATGTTCGTTTCT
	AGAAACGAACATATTAgcTTCTACAATACCTTT
S356A	AATATGTTCGTTgCTGAGTATTTATCTAAACAG
	CTGTTTAGATAAATACTCAGcAACGAACATATT
S436A	GTGGTAGAGAAGAAAgCGGTTGAAGTTAATTCA
	TGAATTAACTTCAACCGcTTTCTTCTCTACCAC

S515A	GTGGATAGACGCTTGgCTATATCCTCGCTGAAT
	ATTCAGCGAGGATATAGcCAAGCGTCTATCCAC
T590A	CTGAAATTAGATAATgCTAATATCGTTAGTATT
	AATACTAACGATATTAGcATTATCTAATTTCAG
S621,T622, T623A	TCTCAAATTGTTCCATTAgCAgCAgCATTAAAAGGCATGGCTAAC
	GTTAGCCATGCCTTTTAATGcTGcTGcTAATGGAACAATTTGAGA
S677A	AAGGGTAGAACTTTAgCTGCCACATCTCAGTTG
	CAACTGAGATGTGGCAGcTAAAGTTCTACCCTT
T685A	TCTCAGTTGAGTGCAgCTTTCAATAAACTACCA
	TGGTAGTTTATTGAAAGcTGCACTCAACTGAGA
S769A	AGATTCAAGCATGCTgCTGAGGTAGCTGAAAAT
	ATTTTCAGCTACCTCAGcAGCATGCTTGAATCT
S783A	GAAGAAAAAGGTAGTgCGGAAGAGCCAGTATAT
	ATATACTGGCTCTTCCGcACTACCTTTTTCTTC
Atg3	
Y168, Y169A	ATGGCGCAAGAAAGGgcTgcCGACCTTTATATTGCG
	CGCAATATAAAGGTCGgcAgcCCTTTCTTGCGCCAT
Y172A	AGGTATTACGACCTTgcTATTGCGTACTCGACA
	TGTCGAGTACGCAATAgcAAGGTCGTAATACCT
T177A	TATATTGCGTACTCGgCATCTTATAGGGTCCCT
	AGGGACCCTATAAGATGcCGAGTACGCAATATA
S230A	TCAGTGTTATCTGTTgCCATTCATCCATGTAAG
	CTTACATGGATGAATGGcAACAGATAACACTGA
Atg4	
T483A	GTCCTTGTAGAGAAGGAAgCGGTAGGTATTCAC
	GTGAATACCTACCGcTTCCTTCTCTACAAGGAC
S488A	GTAGGTATTCACgcTCCTATTGATGAAAAA
	TTTTTCATCAATAGGAgcGTGAATACCTAC
Atg6	
S32, T37A	ATTTGCTACTTTCAAATAATgCAATTATCACTGCAgCGAATGAAAATGTCATCAGCAAC
	GTTGCTGATGACATTTTCATTCGcTGCAGTGATAATTGcATTATTTGAAAGTAGCAAAT
S231A	AAAGAAAAGCAGTATgCCCATAATCTTTCGGAA
	TTCCGAAAGATTATGGGcATACTGCTTTTCTTT
Atg15	
T22, T24A	CTAGGATGCATTCTAgCGCTTgCAGTGCTCTGCCTTATT

	AATAAGGCAGAGCACTGcAAGCGcTAGAATGCATCCTAG
Atg16	
S17,T21, S23A	AAAGCAAAGGAGGAAAGAgcCAATCCACAAgCAGATgcCATGGATGATTTGTTAATT
	AATTAACAAATCATCCATGgcATCTGcTTGTGGATTGgcTCTTTCCTCCTTTGCTTT
S50A	TTGTTTCAAGATAATgcTGGCGCCATTGGTGGC
	GCCACCAATGGCGCCAgcATTATCTTGAAACAA
Atg18	
S41A	TTCGGAAAATTTTATgCAGAGGACAGTGGGGGC
	GCCCCCACTGTCCTCTGcATAAAATTTTCCGAA
T56, S57A	GTCGAGATGTTGTTCTCCgCCgCGTTACTAGCCCTCGTTGGG
	CCCAACGAGGGCTAGTAACGcGGcGGAGAACAACATCTCGAC
S140,S142, S146A	CCACGTGGCCTTATGGCTATGgCTCCTgCGGTAGCCAACgcCTATTTAGTGTATCCATCACCACCA
	TGGTGGTGATGGATACACTAAATAGgcGTTGGCTACCGcAGGAGcCATAGCCATAAGGCCACGTGG
S173A	AACAATATCACATTGgCAGTTGGTGGCAACACA
	TGTGTTGCCACCAACTGcCAATGTGATATTGTT
S192A, S195A	GATCAGCAAGATGCTGGCCATgcTGACATTgcCGACTTGGATCAGTATTCGAGC
	GCTCGAATACTGATCCAAGTCGgcAATGTCAgcATGGCCAGCATCTTGCTGATC
S214A	GCGGATCCAACAAGCgcTAACGGCGGTAACAGC
	GCTGTTACCGCCGTTAgcGCTTGTTGGATCCGC
T234A	GTATTCAACTTGGAAgCATTACAGCCAACCATG
	CATGGTTGGCTGTAATGcTTCCAAGTTGAATAC
S349A	TCGCTCGATACCACCgcTATCGATGCGCTGAGT
	ACTCAGCGCATCGATAgcGGTGGTATCGAGCGA
T393A	CGAAGAGCTGCCAGAgCATTGGGTCAGATTTTC
	GAAAATCTGACCCAATGcTCTGGCAGCTCTTCG
Atg27	
T93	AAGGATGCTATCACCgCTCAAATTATAGATTTT
	AAAATCTATAATTTGAGcGGTGATAGCATCCTT
T211	TACGCTCTACTGTTTgCATTGATATACCTGATG
	CATCAGGTATATCAATGcAAACAGTAGAGCGTA
Atg29	

T33A	TTTGAGTGGAATGGAgCAAAGGAGCGACAGCTT
	AAGCTGTCGCTCCTTTGcTCCATTCCACTCAAA
S43A	GCTTTGGACAATGGTAgCAAATTTGAATTATTC
	GAATAATTCAAATTTGcTACCATTGTCCAAAGC
S127A	TAAATGTATCCGCAgcCCCACTGACCACGGAAA
	TTTCCGTGGTCAGTGGGgcTGCGGATACATTTA
S187A	GAAATGGAGTGCGGTgcTTCAGATGACGATTTAT
	ATAAATCGTCATCTGAAgcACCGCACTCCATTTC
Atg31	
S38, S40, T41, S44A	ATCAACCAGCATACAATAATGAAgCAAAGgcCgCGGACGGAgcTGATTATGCAATGTTTCCCACTAACATTAAG
	CTTAATGTTAGTGGGAAACATTGCATAATCAgcTCCGTCCGcGgcCTTTGcTTCATTATTGTATGCTGGTTGAT
S116A	ATGAGCTTTTGTCACATAGGACAAATGCTCTCAGCTTGGAGG
	CCTCCAAGCTGAGAGCATTTGTCCTATGTGACAAAAGCTCAT
S135A	TTATCATCACATGGTGATGATAAAGCTAATGATGAAGAGGAAGAAC
	GTTCTTCCTCTTCATCATTAGCTTTATCATCACCATGTGATGATAA
S143A,S146A	ATCTAATGATGAAGAGGAAGAACTTGCCGTTGACGCTGATAGATTCAGAGTGGACTC
	GAGTCCACTCTGAATCTATCAGCGTCAACGGCAAGTTCTTCCTCTTCATCATTAGAT
S153A	ATAGATTCAGAGTGGACGCGGACATTGAGCTCGAT
	ATCGAGCTCAATGTCCGCGTCCACTCTGAATCTAT
S174A	TCTGTCTCCTTTTCTTCGCGATTTGGCTTTGAATGACTTAATTAAACTCTAC
	GTAGAGTTTAATTAAGTCATTCAAAGCCAAATCGCGAAGAAAAGGAGACAGA
s195A	TGAACAATTACAAATGCTCTCCAATGCCGTATGAAAGCAAAAATGA
	TCATTTTTGCTTTCATACGGCATTGGAGAGCATTTGTAATTGTTCA

Supplemental table 3

Phosphorylation sites identified by mass spectrum in full medium and starvation medium.

Protein name	Phospho-sites in full medium	Phospho-sites in starvation medium
Atg1	T226, Y332, S351, S356, S436, T590, S621, S677, S783	T226, S343, S356, S436, S515, T590, S621, T685, S769, S783.
Atg3	Y168, Y169, Y172, S230	Y168, Y169, Y172, T177, S230
Atg4	T483, S488	T483, S488
Atg6	S32, T37, S231	S32, T37, S231
Atg15	T22, T24	T22, T24
Atg16	S17, T21, S23, S50	S17, T21, S23, S50
Atg18	S41, T56, S57, S140, S142, S146, S192, S195, S214, T234	S140, S142, S146, S173, S195, S214, T234, S349, T393
Atg27	T93, T211	T93, T211
Atg29	T33, S43, S127, S187	T33, S43, S127, S187
Atg31	S38, S40, T41, S44, S116, S135, S143, S146, S153, S174, S195	S38, S40, T41, S44, S116, S135, S143, S146, S153, S174, S195