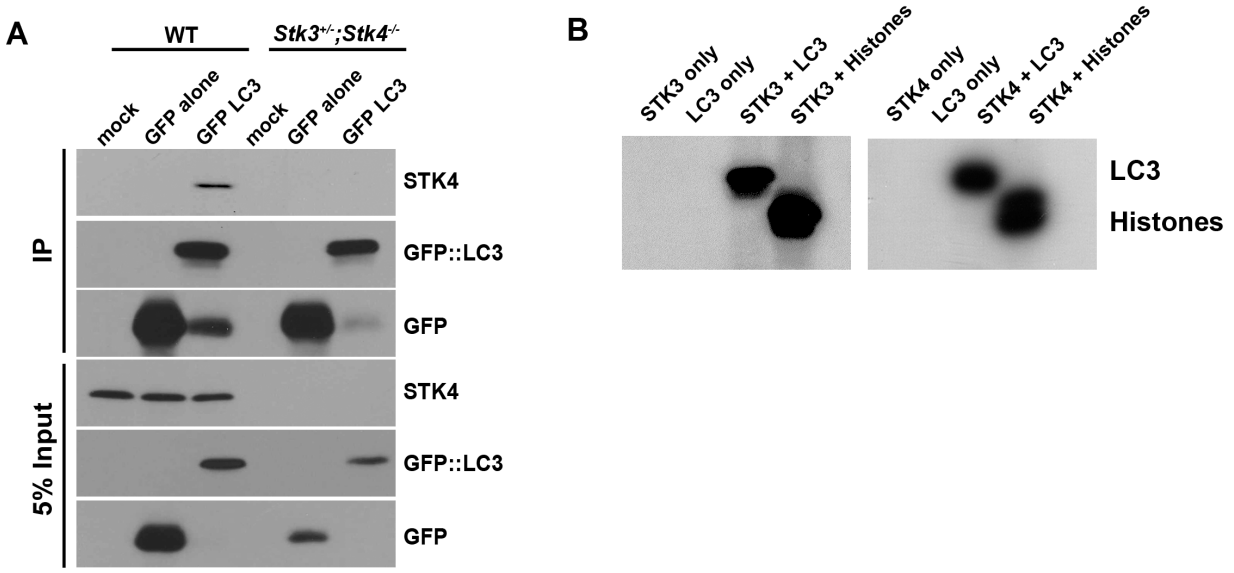

Figure S1 (related to Figure 1). STK4 interacts with and directly phosphorylates LC3

(**A**) *In vivo* interaction between LC3 and STK4 demonstrated by co-immunoprecipitation and western blotting. Wild-type and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were transfected with vectors encoding GFP alone or GFP-tagged LC3. Lysates were immunoprecipitated with GFP-Trap and complexes were resolved by SDS-PAGE (200–300 µg of total protein) followed by immunoblotting with antibodies to STK4, LC3, or GFP. Lysates equivalent to 5% of immunoprecipitate input were analyzed as controls. Data are representative of 3 independent experiments.

(**B**) *In vitro* STK3/STK4 kinase assays. Recombinant STK3 or STK4 kinases were incubated with His-tagged LC3 or with histone H2B as a positive control (Cheung et al. 2003) in the presence of gamma ^{32}P-(C)-ATP. Samples were resolved by SDS-PAGE and subjected to autoradiography. Data are representative of 3 independent experiments.

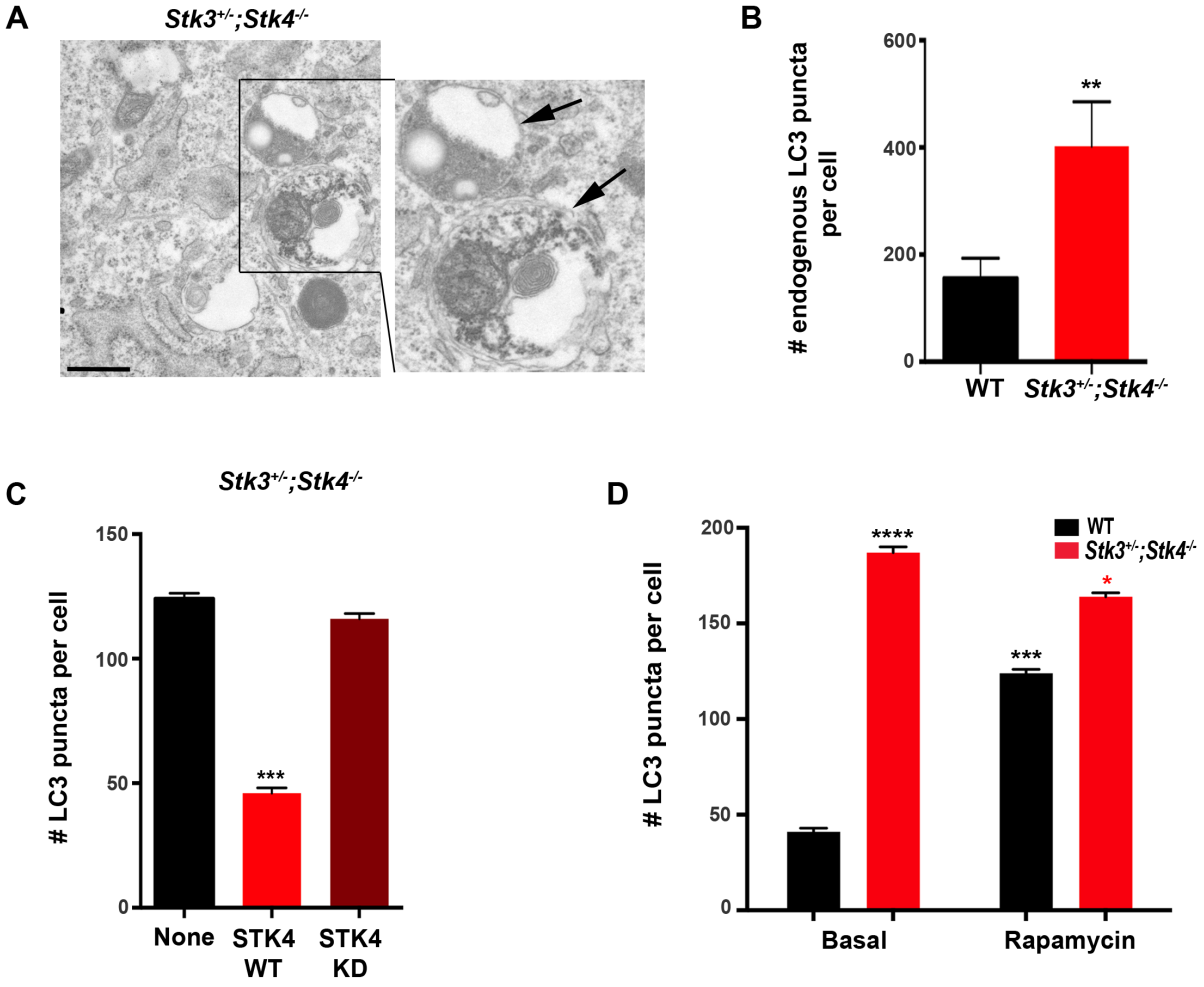
A
Stk3+/-;Stk4-/-
B
endogenous LC3 puncta per cell
600
400
200
0
**
WT
Stk3+/-;Stk4-/-
C
Stk3+/-;Stk4-/-
LC3 puncta per cell
150
100
50
0
***
None
STK4 WT
STK4 KD
D
LC3 puncta per cell
200
150
100
50
0
****
***
*
WT
Stk3+/-;Stk4-/-
Basal
Rapamycin
E
WT
Stk3+/-;Stk4-/-
Starvation +BafA
0hr 1hr 2hr 4hr 0hr 1hr 2hr 4hr
LC3-I
LC3-II
Tubulin
Exp#1 1X 3X 8X 12X 5X 8X 7X 8X
Exp#2 1X 4X 9X 14X 5X 6X 7X 9X
Exp#3 1X 2X 5X 9X 7X 6X 9X 10X

Figure S2 (related to Figures 1 and 2). Autophagy phenotypes observed in *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs

(**A**) Representative electron micrographs of *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs. Enlargement of the boxed area containing double membrane-bound autophagosomes (black arrows) is depicted to the right. Scale bar = 1 μm.

(**B**) Quantification of endogenous LC3 puncta in WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs. Mean ± SEM from at least 15 cells. $^{**}P < 0.01$ by Student's *t*-test. Data are representative of 2 independent experiments.

(**C**) Quantification of basal GFP::LC3 puncta in *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs expressing wild-type STK4 (STK4-WT) or kinase-dead STK4 (STK4-KD, K59R substitution). Cells were transfected with the indicated constructs for 36 h before analysis. Mean ± SEM of 10 cells. $^{***}P < 0.001$ by one-way ANOVA. Data are representative of 3 independent experiments.

(**D**) Quantification of GFP::LC3 puncta in WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs incubated with 200 nM rapamycin for 2 h or left untreated (basal). Mean ± SEM of 10-15 cells. $^{**}P < 0.01$, $^{***}P < 0.001$ by one-way ANOVA. Black and red asterisks indicate comparisons to WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ cells, respectively, under basal conditions. Data are representative of 2 independent experiments.

(**E**) Western blot analysis of WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs exposed to a time course of combined starvation and bafilomycin A (BafA) treatment. Cell lysates (20 μg total protein) were resolved by SDS-PAGE and subjected to western blotting with anti-LC3B antibody. Tubulin was probed as a loading control and the ratios of LC3-II/tubulin were calculated as shown below the representative blot with the 0 h condition in WT cells set to 1X. The blots are representative of 3 independent experiments. Quantification of other experiments are shown below.

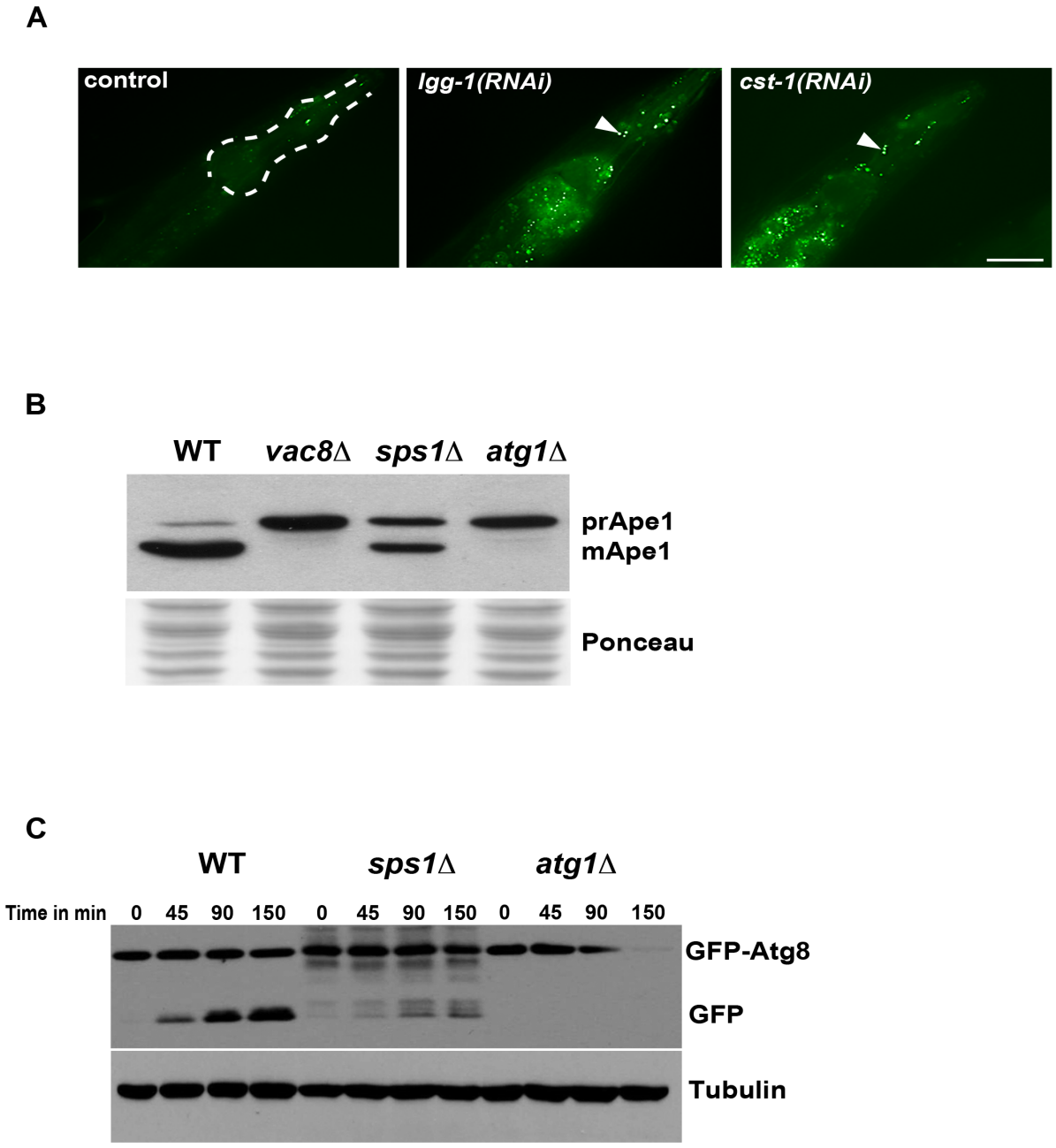

Figure S3 (related to Figure 3). P62 measurement in *C. elegans* and characterization of autophagy defects in deletion mutant of the putative *S. cerevisiae* STK4 ortholog Sps1

(**A**) Representative fluorescence micrographs of *C. elegans* expressing SQST-1/p62::GFP fed from hatching with bacteria expressing control, *lgg-1/Lc3,* or *cst-1/Stk4* dsRNA. Micrographs of the anterior region [pharynx (outlined) and anterior intestine] were taken on day 1 of adulthood. Arrows indicate GFP::p62 foci. Scale bar, 50 μm. For quantification, see Figure 3D.

(**B and C**) BLAST analysis of the *S. cerevisiae* genome (http://inparanoid51.sbc.su.se/cgi-bin/index.cgi and www.yeastgenome.org*)* for proteins with the highest amino acid sequence homology to mammalian STK4 retrieved Sps1 as the top candidate (data not shown). To examine whether loss of *SPS1* in yeast results in autophagy defects similar to that observed for loss of *Stk3/Stk4* in mammalian cells and *cst-1/Stk4* in *C. elegans*, processing of endogenous preApe1 protein through the Cvt pathway (Scott et al. 1996) (**B**) and cleavage of GFP-Atg8 fusion protein via autophagy (Klionsky et al. 2012) (**C**) was assessed.

(**B**) Wild-type (WT) or mutant yeast (BY4742) strains containing deletions in *SPS1*, *VAC8*, or *ATG1* were grown in rich medium and cell extracts were analyzed by western blot with anti-Ape1 antibodies. Total protein staining (Ponceau S) was performed to confirm uniform loading of protein lysates (50 μg). Reduced Ape1 maturation, as indicated by lower levels of the mature form (mApe1) compared with WT, was observed in *sps1*Δ mutants, indicating at least a partial defect in the Cvt pathway. As expected, little or no Ape1 processing was observed in *atg1*Δ or *vac8*Δ mutants (controls). Data are representative of 3 independent experiments.

(**C**) Starvation-induced autophagy phenotypes of WT, *sps1*Δ, and *atg1*Δ mutants transformed with GFP-Atg8 reporter plasmids. Actively growing (mid-log) cultures of each strain were transferred to nitrogen starvation media for up to 150 min. Autophagy-dependent cleavage of the GFP-Atg8 fusion protein was monitored by western blot analysis of cell lysates (50 μg protein) with anti-GFP antibody. Accumulation of cleaved GFP (lower band) following starvation was delayed in the *sps1*Δ mutant compared with WT yeast, indicating an *SPS1*-specific defect(s) in autophagy. Tubulin was probed as a loading control. Data are representative of 2 experiments.

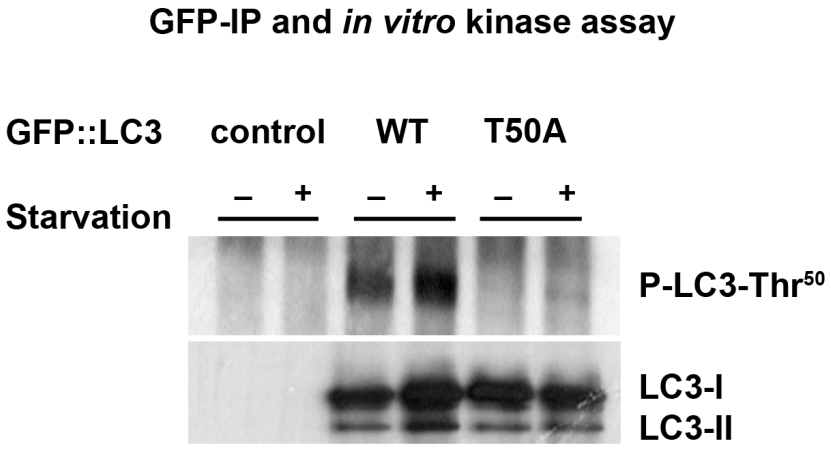

Figure S4 (related to Figure 4). Analysis of P-LC3-Thr^{50} antibody

In vitro STK4 kinase assays. Wild-type MEFs were transfected for 36 h with constructs encoding GFP alone (control), GFP::LC3 (WT), or GFP::LC3-Thr^{50A} mutant (T50A). Cells were left untreated or exposed to starvation for 2 h. Lysates were immunoprecipitated with GFP-Trap beads and the complexes were incubated with purified STK4 and non-radiolabeled ATP (see Methods). Samples were resolved by SDS-PAGE and subjected to western blotting with anti P-LC3B-Thr^{50} antibody (upper panel) or anti-LC3B antibody (lower panel). Data are representative of 4 experiments.

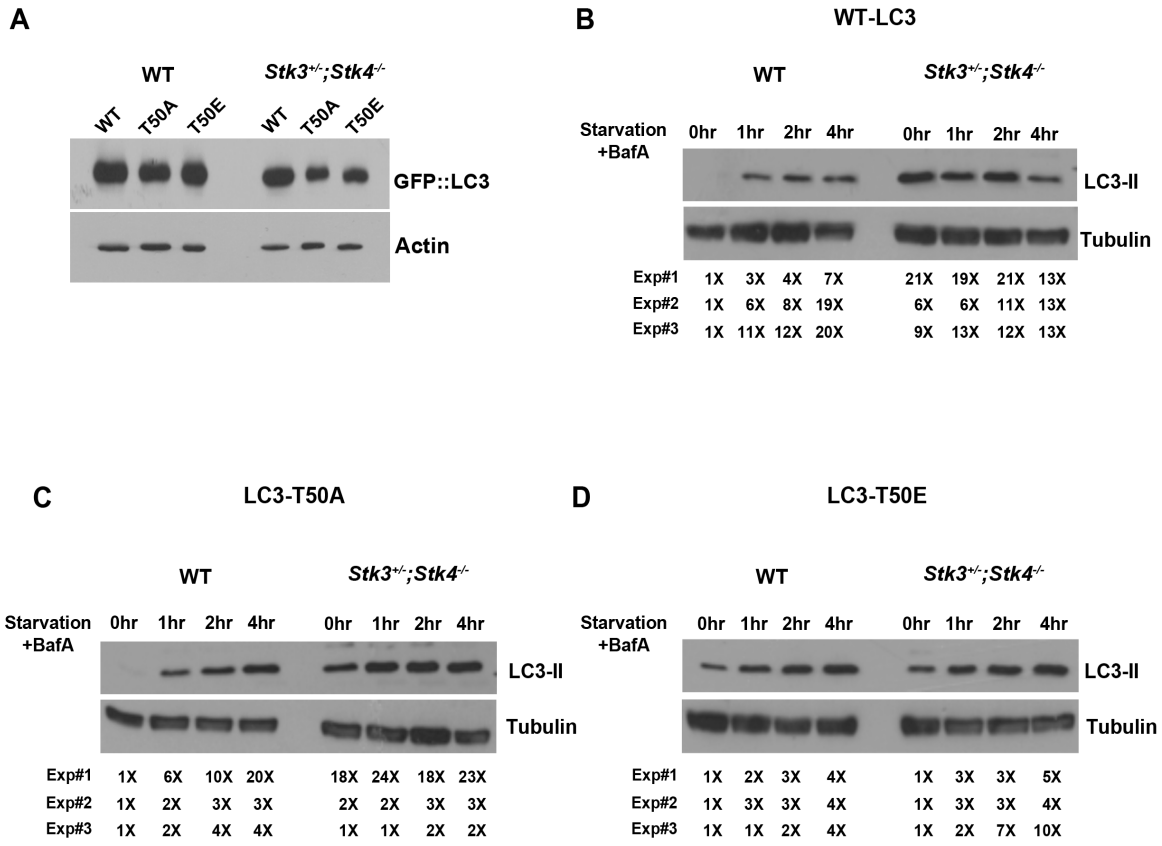

Figure S5 (related to Figure 5). *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs expressing LC3-T50E respond to autophagy stimulus

A) Western blot analysis of wild-type (WT) and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs transiently transfected with wild-type, T50A, or T50E GFP::LC3 constructs. Cell lysates (20 μg total protein) were resolved by SDS-PAGE and subjected to western blotting with anti-GFP antibody. Actin was probed as a loading control. Data are representative of at least 3 independent repeats.

(**B-D)** Western blot analysis of WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs stably expressing GFP-tagged WT-LC3 (**B**), LC3-T50A (**C**) or LC3-T50E (**D**) constructs. Cells were exposed to a combined regimen of starvation and bafilomycin (BafA) treatment over the indicated time course because individual exposure to either starvation or BafA did not result in a robust accumulation of LC3-II in WT cells. Cell lysates (20 μg total protein) were

resolved by SDS-PAGE and subjected to western blotting with anti-LC3B antibody. Tubulin was probed as a loading control and the ratios of LC3-II/tubulin were calculated as shown below the representative blot with the 0 h condition in WT cells set to 1X. The blots are representative of 3 independent experiments. Quantification of other experiments are shown below. Only LC3-II protein is depicted since LC3-I levels were faint at the exposures best suited for LC3-II.

While of interest to this flux analysis, we note that we were unable to consistently measure p62 levels due to varying levels of p62 from lysate to lysate (data not shown).

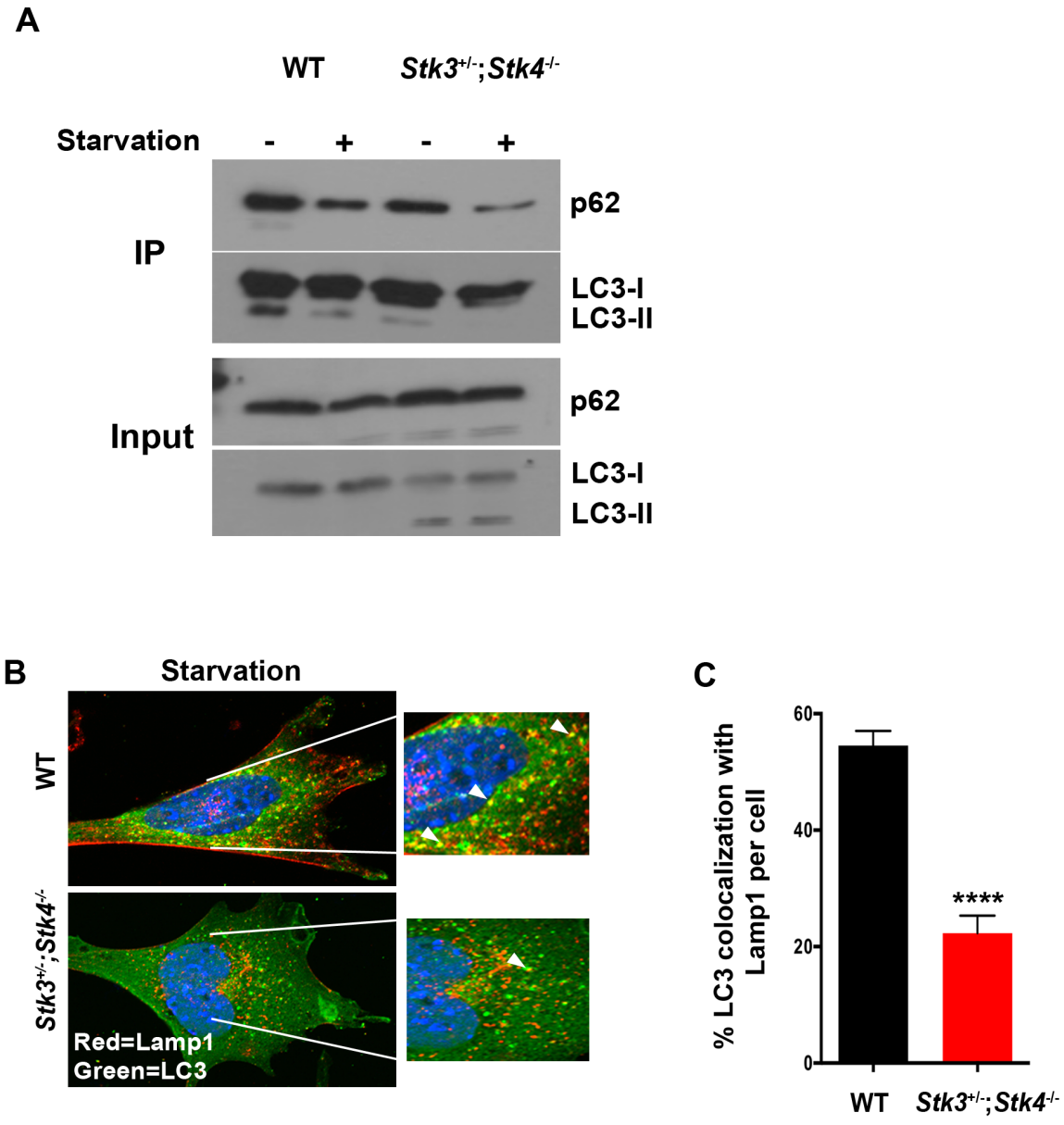
A
WT
Stk3+/-;Stk4-/-
Starvation
-
+
-
+
IP
p62
LC3-I
LC3-II
Input
p62
LC3-I
LC3-II
B
Starvation
WT
Stk3+/-;Stk4-/-
Red=Lamp1
Green=LC3
C
% LC3 colocalization with Lamp1 per cell
60
40
20
0
****
WT
Stk3+/-;Stk4-/-
D
WT
Stk3+/-;Stk4-/-

Figure S6 (related to Figure 6). *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs display a defect in fusion of autophagosomes with lysosomes

(**A**) *In vivo* interaction between LC3 and p62 demonstrated by co-immunoprecipitation and western blotting. Wild-type (WT) and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs were transfected with GFP-tagged WT LC3 for 36 h and either left untreated (-) or exposed to starvation medium for 2 h (+). Lysates were immunoprecipitated with GFP-Trap and complexes were resolved by SDS-PAGE (200–300 µg of protein) followed by immunoblotting with antibodies to p62 or LC3B. Lysates equivalent to 5% of the immunoprecipitate input were analyzed as controls. Data are representative of 3 independent experiments. Starvation condition was included to test for potentially enhanced p62 binding due to activation of autophagy. Interestingly, 2 h starvation resulted in a modest decrease in the interaction between LC3 and p62 in both cell types. Time-course starvation analysis will be necessary to resolve this result.

(**B**) Representative confocal fluorescence micrographs of WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs expressing GFP::LC3 and stained with anti-LAMP1 antibody to visualize colocalization events (arrow heads) between LC3 (green, autophagosomes) and LAMP1 (red, lysosomes). Nuclei are stained blue with DAPI. Insets depict magnification of the boxed area. Arrowheads indicate colocalization events between LC3 and LAMP1. Scale bar = 10 µm.

(**C**) Quantification of colocalization events represented in (B). Data shown represent results of ~ 35-40 cells and Mean ± SEM. ****$p < 0.0001$ by Student's *t*-test. Data are representative of 4 independent experiments.

(**D**) Representative confocal fluorescence micrographs of WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs depicting intracellular cathepsin B activity. Cells were treated with 200 nM rapamycin for 1 h and stained with Magic Red substrate for 30 mins. No background stain was observed in untreated WT or *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ cells (data not shown). Arrowheads indicate accumulated cathepsin B substrate, which fluoresces when cleaved by active cathepsin (see Methods for details). Note that the Magic Red Substrate is known to accumulate in lysosomes and in mitochondria (www.immunochemistry.com). Scale bar = 10 µm. Data are representative of 3 independent repeats.

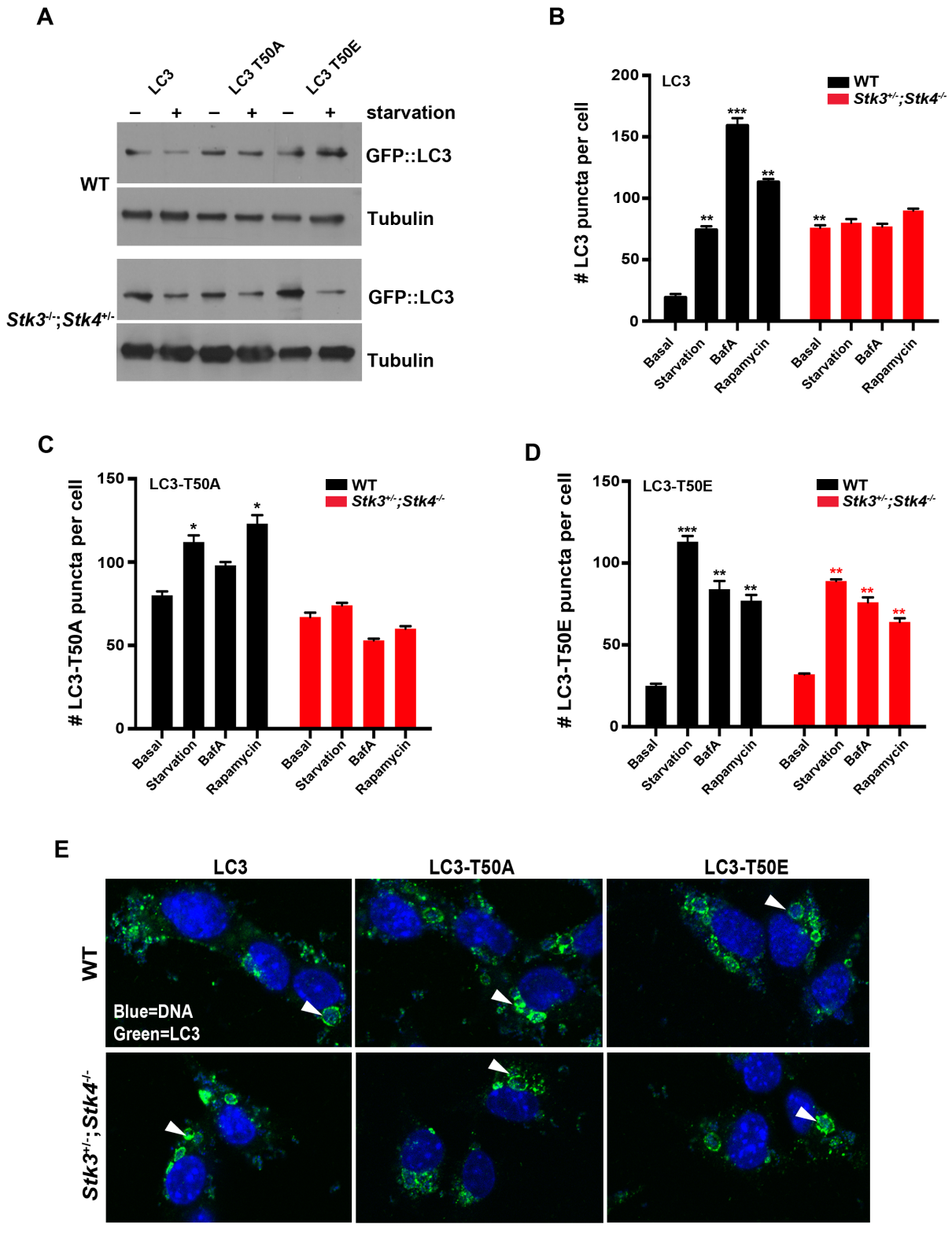
A
LC3
LC3 T50A
LC3 T50E
– + – + – +
starvation
WT
GFP::LC3
Tubulin
Stk3-/-;Stk4+/-
GFP::LC3
Tubulin
B
LC3
WT
Stk3+/-;Stk4-/-
LC3 puncta per cell
200
150
100
50
0
Basal
Starvation
BafA
Rapamycin
C
LC3-T50A
LC3-T50A puncta per cell
D
LC3-T50E
LC3-T50E puncta per cell
E
LC3
LC3-T50A
LC3-T50E
WT
Stk3+/-;Stk4-/-
Blue=DNA
Green=LC3

Figure S7 (related to Figure 7). Characterization of WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs stably expressing LC3 mutant constructs

(**A**) Western blot analysis of GFP-tagged LC3, LC3-T50A, and LC3-T50E mutants in stable transfectants of wild-type (WT, upper two panels) and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ (lower two panels) MEFs. Cell extracts were analyzed by SDS-PAGE and western blotting with anti-GFP antibody. Tubulin was probed as a loading control. Data are representative of 2 independent experiments.

(**B-D**) Quantification of GFP::LC3 puncta in WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs stably expressing GFP-tagged LC3 (**B**), LC3-T50A (**C**), or LC3-T50E (**D**) constructs. Clones were incubated for 2 h in starvation medium or in basal medium in the presence or absence of 200 nM rapamycin or 50 nM BafA. Stable expression of the constructs in both WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs recapitulated the response observed in transient transfectants (compare with Figure 5 in the main text). Mean ± SEM of 10 cells. **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA. Black and red asterisks indicate comparisons to WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ cells, respectively, under basal conditions. Data are representative of 2 independent experiments.

(**E**) Confocal fluorescence micrographs of WT and *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs stably expressing LC3 mutant constructs incubated with GAS for 2 h and then stained for GFP::LC3 (green) and DNA (DAPI, blue). Note that DAPI stains several GAS cells in addition to the single MEF nucleus. Scale bar = 10 μm. Arrowheads indicate GAS surrounded by GFP::LC3-bound autophagosomes. Data are representative of 2 independent experimental repeats.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES (in alphabetic order)

Antibodies

The following primary antibodies diluted in TBST buffer were used: Rabbit anti-LC3 (Cell Signaling Technology 3868S, 1:1000 dilution), mouse anti-GFP (Santa Cruz Biotechnology, SC-9996, 1:1000 dilution), rabbit anti-STK4 (Cell Signaling Technology, 3682S, 1:1000 dilution), rabbit anti-p62 (MBL, PM045Y, 1:1000 dilution), rabbit anti-LAMP1 (Abcam, ab24170), rabbit anti-ATG16L (MBL, PM040), mouse anti-Flag (Sigma, F3165), chicken anti-GFP (Aves Labs Inc, GFP-1020), mouse anti-tubulin (Cell Signaling Technology, 3873P, 1:2000 dilution), and rabbit anti-β-actin (Cell Signaling Technology, 4967, 1:10,000 dilution).

The peptide sequence containing p-Thr^{50} of LC3B - QLPVLDK[pT]KFLVPD, along with the production of a polyclonal antibody to this site was manufactured in rabbits (21st Century Biochemicals). Rabbits were immunized over an 84-day period with production bleeds taken multiple times. Sera were characterized by dot-blot and western analysis against the aforementioned LC3B peptide as well as recombinant full-length LC3B protein. Those bleeds with the highest immuno-reactivity from one of the two rabbits were used to generate the final antibody via affinity purificaition using the peptide immunogen and immunodepleted using the unmodified peptide (QLPVLDKTKFLVPD).

Autophagy assays in mammalian cells

For visualizing GFP::LC3 puncta, cells were transfected with GFP::LC3 construct (as described in main methods) and stained with DAPI (Sigma) for 10 mins, at a final concentration of 1 to 0.1 μg/mL, followed by confocal microscopy (described below). For analysis of endogenous LC3, LAMP1 and ATG16L, the cells were fixed in 4% paraformaldehyde for 15 min, washed 3 times in PBS and blocked for 1 h in 5% donkey serum and 0.3% Triton X-100 in PBS. Following blocking, cells were stained with the corresponding primary antibodies diluted at 1:100 concentration in blocking buffer overnight, with the exception of LAMP1 antibody which required only 2 h of incubation for robust staining. Cells were washed 3 times in PBS and stained with anti-rabbit Alexa

Fluor 594 secondary antibodies (Molecular Probes, 1:1000) for 1 h. For Syntaxin17 analysis, cells were transfected with 0.5 µg FLAG-Syntaxin17 plasmid and stained with anti FLAG antibody followed by anti-mouse Alexa Fluor 594 secondary antibody (Molecular Probes, 1:1000) as described above. Cells were then washed 3 times in PBS and counterstained with DAPI and mounted in Fluoromount-G (Southern Biotech). The figures show representative images from 10–15 random fields (63× objective) for each condition and biological replicate. All confocal microscopy was performed at the Waitt Advanced Biophotonics Center (Salk Institute for Biological Studies) on an LSM 780 spectral confocal microscope mounted on an inverted Axio Observer Z1 frame (Carl Zeiss). Typically, confocal slice thickness was 0.9 µm for both fluorescence channels, with ~10 slices taken to encompass the three-dimensional entirety of the cells in the field of view. Maximum intensity projections of each region were calculated for subsequent quantification and analysis. Quantification was performed as previously described (Doyon et al. 2011) with IMARIS software (Bitplane, Zurich). An appropriate threshold was set and kept constant for the entire analysis. Images (10–15 per experiment) were acquired under the same conditions and the enumerated counts from these images were averaged and designated as a single point for data representation and statistical analysis.

For visualizing colocalization events between LC3 with LAMP1, cells were transfected with GFP::LC3 and RFP::LAMP1, fixed and analyzed by staining with GFP and LAMP1 antibodies as above. Images were acquired in the GFP and RFP channels and merged to compare the two signal patterns, and plot profiles derived from the merged images were quantified manually. Experiments were repeated 2–5 times, as indicated. Statistical analysis of biological repeats was performed by two-tailed Student's *t*-test or one-way ANOVA using GraphPad Prism 5.0 software (GraphPad Software Inc.).

Autophagy assays in *C. elegans* and *S. cerevisiae*

The *cst-1*(*tm1900*) deletion strain (FX1900) was kindly provided by the Mitani Lab (National Bioresource Project for the Nematode, Japan) and the SQST-1/p62::GFP reporter strain [HZ859 (Tian et al. 2010)], was a generous gift from Dr. Hong Zhang

(Institute of Biophysics, Chinese Academy of Sciences). *cst-1*(*tm1900*) was out-crossed three times to the wild-type strain and allotted the strain name MAH208. *C. elegans* strains were maintained and cultured under standard conditions at 20°C using *E. coli* OP50 as a food source. EM analysis was performed as previously described (Lapierre et al. 2011).

For measuring autophagy flux in *C. elegans*, GFP-positive LGG-1 puncta was counted in wild-type [DA2123, (Kang et al. 2007)] and *cst-1(tm1900)* animals (AGD814) in response to bafilomycin A (BafA) or control treatment as follows: BafA (BioViotica) was mixed with Texas Red Dextran, 3000 MW (Molecular Probes) to a final solution of 50 uM + 2.5 ug/ul of Texas Red in 0.2% DMSO and injected into anterior intestinal area of adult Day 1 *C. elegans* as described in Wormbook (http://www.wormbook.org, chapter on Transformation and Microinjection). Animals were allowed to recover on NGM plates with OP50 for two hours. Those that were red and still moving were chosen for confocal microscopy. Adult worms were mounted on a 2% agarose pad + 1% 1.5 M NaN_3 in 3ul of 1.5 M NaN_3. Worms were imaged using a LSM Zeiss 710 scanning confocal. Z stacks were taken at 0.6 μm slices. GFP excitation/emission was limited to 493/523 nm to eliminate background fluorescence due to auto-fluorescence of the worm. At least 10 Day-1 adults were imaged for each condition. Quantification of GFP::LGG-1 puncta in seam cells was done using ImageJ (http://imagej.nih.gov/ij/) and graphed using GraphPad.

For analysis of p62::GFP foci, HZ859 animals were raised on *lgg-1* and *cst-1* dsRNA-expressing bacteria from hatch. On Day 1 of adulthood, 15-20 animals were visualized with a Leica DFC310 FX camera and p62 foci enumerated using Image J software. RNAi clones were from the Julie Ahringer library (Kamath et al. 2003; Rual et al. 2004). Data were analyzed by Student's *t*-test or ANOVA using GraphPad.

Autophagy measurements in yeast were performed as described (Cheong et al. 2005). In brief, yeast were grown in YPD (Yeast Extract-Peptone-Dextrose) liquid medium, and starvation was induced by transferring cultures to synthetic medium lacking a nitrogen source (SD-N medium) for up to 150 min. Cell lysates were precipitated using TCA and analyzed by SDS-PAGE followed by western blotting with anti-Ape1 (ab2717, Abcam) or anti-GFP antibodies.

Cathepsin activity assay

Measurement of Cathepsin activity was performed according to manufacturer's instructions (937, Magic Red™ Cathepsin B Assay Kit, ImmunoChemistry). Briefly, cells were grown on coverslips and treated with 200nM Rapamycin for 2 h to induce autophagy. Cells were incubated in Magic Red substrate for 30 min and cover slips were processed for confocal microscopy as above, without fixation. Rapamycin was chosen instead of starvation as an autophagy stimulus since *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEFs were highly sensitive to the combination of starvation medium and Magic Red substrate.

Expression constructs

Mammalian expression vectors: The following expression plasmids were used in this study: GFP::LC3 (gift of Dr. Shaw), mCherry::GFP::LC3 and RFP::LAMP1 (Gift of Dr. Cuervo), HA-STK4 (Addgene plasmid 12203, gift of Dr. Chernoff) and FLAG-Stx17 (Addgene plasmid 45911, gift of Dr. Mizushima). Point mutations were introduced into the GFP::LC3 and HA-STK4 sequences using a QuikChange II site-directed mutagenesis kit (Agilent Technologies) with the following primers:

(a) For conversion of threonine 50 to alanine (T50A) in GFP::LC3:

Forward: GCTTCCTGTTCTGGATAAAGCAAAGTTCCTTGTACCTGAC,

Reverse: GTCAGGTACAAGGAACTTTGCTTTATCCAGAACAGGAAGC.

(b) For conversion of threonine 50 to glutamate (T50E) in GFP::LC3:

Forward: GCTTCCTGTTCTGGATAAAGAAAAGTTCCTTGTACCTGAC,

Reverse: GTCAGGTACAAGGAACTTTTCTTTATCCAGAACAGGAAGC.

(c) To generate kinase-dead HA-STK4, the critical lysine 59 required for ATP binding was changed to an arginine (Creasy et al. 1996) with the following primers:

Forward: GACCGGCCAGATTGTTGCTATTAGGCAAGTTCCTGT,

Reverse: ACAGGAACTTGCCTAATAGCAACAATCTGGCCGGTC.

Bacterial expression vector: His-tagged LC3 was generated by inserting human LC3 cDNA into the pET28A vector (Clontech) by PCR cloning with the following primers:

Forward: GTCCACGGATCCATGCCGTCGGAGAAGACCTTCAAGC,

Reverse: GGACTCTCGAGTTACACTGACAATTTCATCCCGAACG.

Infection with group A *streptococcus*

Group A *streptococcus* (GAS) strain NZ131 (serotype M49) was grown in Todd-Hewitt broth (THB, Difco) at 37°C to mid-logarithmic phase (OD_{600} = 0.4). GAS were added to a monolayer of MEFs in antibiotic-free medium at a multiplicity of infection of 50:1. The plates were centrifuged at 500 ×*g* for 5 min and incubated for 1 h at 37°C in a 5% CO_2 incubator. The medium was replaced with medium containing 10 μg/ml penicillin and 100 μg/ml gentamicin and the cells were incubated for an additional 1 h at 37°C to eliminate extracellular bacteria. For quantifying bacterial clearance, the treated cells were lysed in 0.025% Triton X-100 followed by trituration. The lysates were serially diluted and plated on Todd-Hewitt agar for enumeration of bacterial CFU.

For confocal microscopy of GAS and LC3, infected MEFs were processed for antibody staining against LC3 and confocal microscopy as described above in "Autophagy assays in mammalian cells".

Mammalian cell lines

Stk3$^{+/-}$:*Stk4*$^{-/-}$ and *Atg7*$^{-/-}$ MEFs and C2C12 myoblasts (gift from Drs. Yingzhi Yang, Randy Johnson, Shu-Ichi Matsuzawa and Hetzer, respectively) were maintained in DMEM (Cellgro) supplemented with 10 or 20% fetal bovine serum (FBS), 1% antibiotics (Life Technologies), 0.5% BME, and 10% non-essential amino acids (Life Technologies). Since *Stk3*$^{+/-}$:*Stk4*$^{-/-}$ MEFs exhibited growth retardation between passages 6-10, they were immortalized with SV40 as described (Tevethia and Ozer 2001). Untransformed MEFs were used in **Figures 1A, 1E, S2A and S2B**, whereas the rest of the *Stk3*$^{+/-}$;*Stk4*$^{-/-}$ MEF data were generated using SV40 transformed. MEFs stably expressing the various LC3 constructs were generated as previously described (Carter et al. 1996). Several clones of each genotype were tested for stable protein expression and maintained under G418 selection.

Mass spectrometry

In-gel digestion of LC3 was performed by standard methods with LysC (Promega). Titanium dioxide (TiO_2)-based phosphopeptide enrichment and reversed-phase HPLC-tandem mass spectrometry (MS/MS) using an LTQ Orbitrap Velos Pro Mass Spectrometer equipped with electron transfer dissociation was performed as described previously (Ma et al. 2013). HPLC-MS/MS data were searched against an international protein index (IPI) human protein database and filtered using Trans-Proteomic Pipeline (Seattle Proteome Center), as described previously (Ma et al. 2013).

Recombinant protein purification and kinase assays

BL21 Rosetta *E. coli* (Novagen) were transformed with His-tagged LC3 bacterial expression vector, grown in LB broth for 16 h, and induced to express recombinant protein by the addition of 0.6 mM IPTG for 3 h. The recombinant protein was purified with a Ni-NTA spin kit (Qiagen) and dialyzed using Slide-a-Lyzer dialysis cassettes (Pierce) into *in vitro* kinase assay buffer. Recombinant active STK3 (S6573) and STK4 (M9697) kinases were purchased from Sigma Inc.

For *in vitro* kinase assays, recombinant STK3 or STK4 (5–10 ng per reaction) was incubated for 30 min at 30°C with 1–5 µg of recombinant His-LC3B or histone H2B (positive control substrate, Sigma) in kinase assay buffer (20 mM Tris, pH 7.5, 10 mM magnesium chloride, 1 mM EGTA, and 1 mM DTT) containing 10 µCi ^{32}P-ATP (Perkin Elmer). The reactions were stopped by boiling in 2X SDS sample buffer and the proteins were resolved by SDS-PAGE. The gels were dried and imaged using standard autoradiography. For kinase assays in which recombinant GFP::LC3 was used as the substrate, GFP::LC3 was immunoprecipitated from MEFs with GFP-Trap beads (Allele Biotech), and the cells were lysed in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 1% NP-40, protease and phosphatase inhibitor cocktails, and 1 mM PMSF. The beads were then washed 3 times with lysis buffer and twice with kinase assay buffer. The resulting enriched GFP::LC3 preparation was used as a substrate for *in vitro* kinase reactions, as described above, except that cold ATP was used. The kinase reaction was stopped by boiling in 2X SDS

sample buffer, and the proteins were resolved by SDS-PAGE and then subjected to western blotting with rabbit P-LC3-Thr^{50} antibody (21st Century Biochemicals).

To generate samples for mass spectrometric analysis, the *in vitro* kinase reactions were performed as described above except radiolabeled ATP was replaced with 1 mM cold ATP, and the reaction was incubated at 30°C for a total of 1 h, with a second addition of kinase after 30 min incubation.

Transmission electron microscopy (TEM)

Cultured cells were processed for TEM using the method of Gilula et al., 1978. Briefly, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, washed briefly in buffer, and post-fixed in 1% osmium tetroxide. Following a further buffer wash, the cells were treated with 0.5% tannic acid and 1% sodium sulfate, washed in buffer, dehydrated in an ethanol series, transitioned to 2-hydroxypropyl methacrylate, and finally embedded in LX112 (Ladd Research). Pieces of the flat embedded resin were glued to a blank block face and 60 nm sections were cut, mounted on copper slot grids coated with parlodion, and stained with uranyl acetate and lead citrate. Sections were examined on a Philips CM100 electron microscope (FEI) at 80kV, and images were collected using a Megaview III CCD camera (Olympus Soft Imaging Solutions GmbH).

Western blotting

Cell lysates were made by incubation in either western lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, protease and phosphatase inhibitor cocktails [Roche Diagnostics], and 1 mM PMSF) or IP-lysis buffer (20 mM Tris, pH 7.5, 150 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 1% NP-40, protease and phosphatase inhibitor cocktails and 1 mM PMSF) for 20 min at 4°C. Lysates were centrifuged and the supernatants were subjected to SDS-PAGE (5–20 µg total protein/sample, as indicated in figure legends) followed by western blot analysis. The blots were blocked in 3% BSA in Tris-buffered saline containing 1% Tween-20 (TBST) and incubated overnight with primary antibodies diluted in 3% BSA/TBST. Blots were washed and incubated with HRP-conjugated anti-mouse (Sigma, 1:10,000 dilution) or anti-rabbit (Sigma, 1:2000 dilution) secondary antibodies for 1 h and washed

again. Finally, blots were developed with Pierce ECL western blotting substrate (Pierce) and visualized by autoradiography on Amersham Hyperfilm (GE Healthcare Biosciences). Quantification was performed by utilizing ImageJ software.

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