Supplemental information

Experimental Procedures

Antibodies and reagents. Anti-Vps34 (#4263 for WB), Beclin1 (#3738 for WB), phospho-ACC S79 (#3661), ACC (#3662), phospho-AMPKα T172 (#2531), AMPKα (#2532) and LC3 (#2775 for WB) antibodies were purchased from Cell signaling technology. Anti-UVRAG (M160-3), Atg14L (PD026 for IP), and LC3 (PM036 for immunostaining) antibodies were obtained from MBL. Anti-Vps34 (Echelon, Z-R015) and Beclin1 (Bethyl A302-567A) antibodies were used for immunoprecipitation. Anti- α-tubulin (T6199), vinculin (V9264), Flag (F3165), and Atg14L (A6358 for WB) antibodies were obtained from SIGMA, Anti-Ha, and Myc antibodies were from Covance. Anti-p62 (SQSTM1, GP62-C), Vps34 (38-2100 for WB), and p150 (Vps15, A302-571A) antibodies were purchased from Progen Biotechnik, Zymed, and Bethyl, respectively. Anti-phospho Beclin1 S91/S94 and Vps34 T163/S165 antibodies were generated by immunizing rabbits with the corresponding phosphopeptides. The phosphospecific antibodies were affinity purified (Cell Signaling Technology). Mutagenesis was performed based on Quik-Change mutagenesis (Stratagene).

Cell culture, transfection, and viral infection. HEK293 cells or 293A were cultured in high glucose (25 mM) DMEM (Invitrogen, #11965) medium containing 10% fetal bovine serum (FBS; Invitrogen) and 50 μg/ml penicillin/streptomycin. The MEFs were grown in the high glucose (25 mM) DMEM culture medium (complete medium) supplemented with β-mercaptoethanol (Invitrogen), 1 mM pyruvate (SIGMA), and non-essential amino acid mixture (Invitrogen). Glucose starvation in this study was carried out to switch the culture medium from the complete medium to the same medium except no glucose (Invitrogen, #11966) unless described otherwise. Transfection with polyethylenimine (PEI, SIGMA) was performed as previously reported (Horbinski et al., 2001). To establish Beclin1 and Vps34 knockdown stable MEFs, lentiviral construct (pLKO.1-TRC system, Addgene) containing shRNA targeting mouse

Beclin1 (target sequence, 5′ -CTCAGGAGAGGAGCCATTTAT-3′) and mouse Vps34 (target sequence, 5'-TCACGAGATGTACCTGAACGTGAT-3') was generated, respectively, and co-transfected with viral packaging plasmids (psPAX2 and pMD2.G) into HEK293 cells. The resulting viral supernatant was applied to MEFs and the stable pools were obtained in the presence of 5 μg/ml puromycin (SIGMA). Retrovirus harboring the expression plasmids (pQCXIH) encoding wild-type or mutant Beclin1 (or Vps34), which produce CBP (Calmodulin Binding Peptide)/SBP (Streptavidin Binding Peptide) at N-terminus of the target protein, was introduced into the stable knockdown MEFs. To avoid the effect of shRNA on reconstituted gene expression, the genes encoding Beclin1 or Vps34 were silence-mutated accordingly. Beclin1 (or Vps34) reconstituted MEFs were selected with 0.25 mg/ml hygromycin (Invitrogen) in the complete culture medium.

Western blot and immunoprecipitation. Cells were lysed with Mild Lysis Buffer [MLB, 10 mM Tris, pH7.5, 2 mM EDTA, 100 mM NaCl, 1% NP-40 , 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche)]. These cell lysates were used for western analyses. For immunoprecipitation, the indicated antibody was coupled with protein G-sepharose (GE Healthcare life science) in 1% BSA in TBST [20 mM Tris, pH.8.0, 170 mM NaCl, and 0.05% Tween-20]. The antibody-conjugated beads were added to the cell lysates and incubated at 4ºC for 2 hrs. The resulting beads were washed with MLB five times and used for further analyses.

Cell death assay. Cell viability was determined by cell counting with trypan blue staining (SIGMA, T8154) according to manufacture's instruction. Also, apoptotic cells were examined by the detection of Annexin V/propidium iodide (PI) double-positive cells under flow cytometry (BD Biosciences).

***in vitro* PI(3)P ELISA assay.** Total amount of PI(3)P was measured in a quantitative and competitive ELISA format assay according to the manufacture's manual (Echelon Biosciences). Briefly, after following

a lipid extraction protocol, the lipid fraction obtained from the cells were incubated with a PI(3)P detector protein, then added to a PI(3)P-precoated microplate for competitive binding. A peroxidase-conjugated antibody against PI(3)P detector protein was added and colorimetric detection was carried out to determine PI(3)P detector protein binding on the plate. Cellular PI(3)P quantities were calculated by the standard curve from non-linear fitting of PI(3)P standards.

Protein purification and AMPK kinase assay. The bacterial expression constructs (pGEX-KG) containing the indicated genes were transformed into DH5α or BL21 codon plus (Stratagene). Cells were induced to protein overexpression under 0.1-0.5 mM IPTG at 18°C. Cells were resuspended in PBS containing 0.5% Triton X-100, 5 mM β-mercaptoethanol, 2 mM EDTA, and 1 mM PMSF, followed by ultrasonication. The proteins were purified by a single step using glutathione bead according to manufacture's protocol (GE Health Science). Purified proteins were dialyzed against 20 mM Tris, pH8.0, and 10% glycerol. 0.5 μg of bacterial purified GST-Beclin1 or GST-Vps34 proteins were used for AMPK assay (10 ng of AMPK α/β/γ complex, Cell signaling technology or Genescript) as substrates to determine phosphorylation sites. The phosphorylation reaction was carried out under the condition as previously described (Kim et al., 2001). Phosphorylation of GST-Beclin1 or Vps34 proteins was determined by ^{32}P-autoradiogram and the protein level was normalized by comassie staining of the membrane.

Preparation of Atg14L protein. Full length Atg14L protein was expressed as ZZ-TEV-His-Atg14L fusion protein in Sf9 cells on Baculovirus expression system according to manufacture's instruction (Invitrogen). The cells were harvested by centrifugation and resuspended in hypotonic buffer (20 mM Tris-HCl pH7.5, 5 mM KCL, 1 mM $MgCl_2$, 1 mM DTT, and protease inhibitors), followed by dounce homogenates with pestle (WHEATON) 30 times. Atg14L proteins were purified as previously reported (Fan et al., 2011). The purified protein was eluted by TEV cleavage in the elution buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5 mM DTT, and 20 mM Glycerol).

Lambda phosphatase/AMPK treatment *in vitro*. To determine the effects of phosphorylation on the lipid kinase activity of Vps34 complex, the indicated Vps34 complex was pre-incubated with either AMPK or lambda phosphatase (λ PPase) *in vitro*. The Vps34 complex was immunoprecipitated as indicated in *Figure legend*. The immune-complex was incubated with either 5U of λ PPase (Cell signaling technology) in the phosphatase buffer or 0.5-5 ng of purified AMPK in Kinase Assay buffer [KA, 20 mM HEPES, pH7.4, 1 mM EGTA, 0.4 mM EDTA, 5 mM $MgCl_2$, and 0.05 mM DTT] in the presence of 0.2 mM AMP and 0.1 mM cold ATP at 37C for 15 min. The Vps34 bound beads were extensively washed with MLBS buffer (MLB containing 0.01% SDS) and followed by KA buffer. Vps34 complex was recovered by a centrifugation and used for Vps34 lipid kinase assay.

Immunofluorescence staining. The indicated MEFs were plated on 4-well chamber slides at a density of $5x10^4$ cells, and the following day, the cells were starved with glucose for 3 hrs. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature (RT), and permeabilized with 50 μg/ml digitonin in the blocking buffer (0.1% normal goat serum in PBS) for 10 min. The cells were washed twice with the blocking buffer. After 30 min of blocking with the same buffer, cells were incubated with anti-LC3 antibody (1:1000) for 1 hr at RT. The cells were washed with PBS and then incubated with Alexa Fluor488-conjugated anti-rabbit IgG antibody (Invitrogen, 1:1000) for 1 hr. For co-staining of PI(3)P, cells were incubated again with biotinylated-GST-2xFYVE proteins (1 μg/ml) for 30 min at RT, and then incubated with NL557-conjugated streptavidin (R&D systems-NL999, 1:1000) for 30 min at RT. ProLong Gold antifade reagent with DAPI (Invitrogen) was used for mounting and images were taken using a Zeiss LSM confocal microscope.

Image quantification. For quantification, a set of control and experimental images was processed with same parameters using Photoshop and Image J software. Firstly, images were converted to 8-bit gray

images and set a threshold on Photoshop. Gray images were then converted to 8-bit binary black & white using process > binary > make binary tool of ImageJ software. To determine the total area of marker-positive cells, the binary images were then analyzed for particles (size (pixel^2): 0-infinity, circularity: 0.00-1.00). To quantify the co-localization percentage (%), green color- and red color-channel images were converted to black & white images and overlayed using Photopshop software. Overlaying dots were counted from at least 5 different images. Statistical significance (P-value) was determined by two-sided Wilcoxon rank sum test using Mstat software (Dr. Drinkwater, University of Wisconsin).

Electron microscopy. MEFs were fixed in modified Karnovsky's fixative (1.5% glutaraldehyde, 3% paraformaldehyde and 5% sucrose in 0.1 M cacodylate buffer, pH 7.4) for 8hrs, followed by treatment with 1% osmium tetroxide in 0.1 M cacodylate buffer for additional 1hr. They were stained in 1% uranyl acetate and dehydrated in ethanol. Samples were embedded in epoxy resin, sectioned (60 to 70 nm), and placed on Formvar and carbon-coated copper grids. Grids were stained with uranyl acetate and lead nitrate, and the images were obtained using a JEOL 1200EX II (JEOL, Peabody, MA) transmission electron microscope and photographed on a Gatan digital camera (Gatan, Pleasanton, CA).

Supplemental Figure legends

Fig. S1. AMPK differentially regulates different Vps34 complexes in response to glucose starvation, Related to Figure 1.

(A) Stoichiometry of Vps34 complexes in different cell lines. The Vps34 complexes were immunoprecipitated against Vps34 and Atg14L, respectively, from the indicated cell lines, and then the subunit proteins in each complex were analyzed by western blots. Also, expression level of Vps34 complex subunit proteins in the tested cell lines were examined (Lysates).

(B) Schematic chart for the preparation of different Vps34 complexes. U2OS cell lines harboring inducible Flag-Beclin1 were induced with 200 ng/ml of doxycyclin (Dox) for overnight (Sun et al., 2008). Total-

Beclin1 was immunoprecipitated by Flag antibody and then eluted by adding 3x Flag peptide (denoted as Bec-Total). The purified Bec-Total was used for subsequent immunoprecipitation for Atg14L or UVRAG containing complex (denoted as Atg14L, VIC3 and UVRAG, VIC4), respectively. To obtain Atg14L- and UVRAG-free Beclin1 complex, "Bec-Total" fraction was incubated with the beads conjugated with both Atg14L and UVRAG antibodies. The flowthrough fraction (the resulting supernatant of Atg14L/UVRAG double IP, representing both Atg14L and UVRAG immuno-depleted fraction) was used for Beclin1-IP (denoted as Bec, VIC2). Also, Beclin1-free Vps34 complex was prepared from the Beclin1-depleted cell lysates (Unbound fraction of first Flag-IP, denoted as Vps34, VIC1).

(C) AMPK activation upon glucose starvation. Wild-type MEFs were starved with glucose for 3 hrs and AMPK activation was examined by phosphorylation of AMPKα T172 (P-AMPK T172), of which phosphorylation is essential for activation, and phosphorylation of its direct downstream target, ACC1 (P-ACC S79)

(D) Vps34 protein levels of different complexes used in the Vps34 lipid kinase assay in Fig.1F. Because not all samples could be loaded on a single SDS gel, the ATG14L IP samples were run twice for comparison.

(E) Expression of dominant negative AMPKα blocks the regulation of Vps34 complex by glucose starvation. HA-Vps34, Myc-Beclin1, Flag-Atg14L, and Flag-UVRAG were co-expressed with kinase inactive form of AMPKα (AMPK-DN) in HEK293 cells as indicated. The cells were starved with glucose for 3 hrs and the Vps34 complex was immunoprecipitated by HA (for VIC1), Myc (for VIC2), or Flag (VIC3 or VIC4). The immune-complex was subjected to *in vitro* Vps34 lipid kinase assay. The immunoprecipitation of anti-mouse IgG was included as a negative control (CON). Two different exposures of the autoradiography (L.E. denotes fro longer exposure) are shown in the top two panels. The Vps34 kinase activity was normalized by the Vps34 protein level and shown as relative fold, in which the activity of glucose-rich condition defines to be one (the bottom panel, n=3). Also, the relative activity of the

indicated complex was compared to VIC1 which was defined as one (the second panel from the bottom). Error bars represent S.D.

(F) Co-expression of wild-type AMPKα inhibits non-autophagy Vps34 complexes (VIC1, VIC2) and activates pro-autophagy VPS34 complexes (VIC3, VIC4). HA-Vps34, Myc-Beclin1, Flag-Atg14L, and Flag-UVRAG were transfected into HEK293A cells in the presence of either AMPKα wild-type (W) or kinase inactive (D) constructs as indicated. Vps34 complex was immuno-purified for *in vitro* Vps34 lipid kinase assay and the kinase activity is represented as described in Fig.S1C (Error bars represent S.D., n=3).

(G) Activation of AMPK is sufficient to regulate Vps34 complex. HA-Vps34, Myc-Beclin1, and Flag-Atg14L were transfected into HEK293A cells as indicated. The cells were incubated with glucose-free medium, or treated with 2 mM 2-Deoxyglucose (2-DG), or 5 mM Metformin (Metf) in glucose-rich medium for 3 hrs. Vps34-Beclin1 complex and Vps34-Beclin1-Atg14L complex were prepared by Beclin1-IP (by Myc antibody) and Atg14L-IP (by Flag antibody), respectively. *In vitro* Vps34 lipid kinase reaction was carried out and one representative data from two-independent experiments was shown. Also, cell lysates were probed with phospho-ACC (S79) to examine AMPK activation upon the starvation.

Fig. S2. AMPK directly activates pro-autophagy Vps34 complex via phosphorylation, Related to Figure 2.

(A) Schematic diagram for *in vitro* phosphorylation and dephosphorylation of Vps34 complex by AMPK and lambda phosphatase (λ PPase), respectively. To examine whether AMPK regulates Vps34 complex via phosphorylation, Vps34 immune-complex isolated from glucose-rich culture was incubated with AMPK in the presence of cold ATP. AMPK was removed and the samples were treated with λ PPase *in vitro*. In parallel, to determine whether glucose starvation regulates Vps34 complex by phosphorylation, Vps34 immune-complex was treated with λ PPase. After removing λ PPase, the resulting immune-complex was further treated with AMPK *in vitro* to determine whether AMPK–mediated phosphorylation alone is sufficient to mimic the effect of glucose starvation on Vps34 complexes. The immune-complex

was extensively washed with MLBS after *in vitro* treatments and Vps34 lipid kinase assay was determined.

(B) AMPK directly activates Atg14L containing Vps34 complex. Endogenous Vps34 complex was immunoprecipitated by Atg14L antibodies from wild-type MEFs cultured in glucose rich medium. The immune-complex was treated with increasing amount (5, 15, 30 ng) of AMPK in the presence of cold ATP *in vitro.* After incubation, AMPK was removed and then Vps34 lipid kinase assay was determined. Also, the immune-complex incubated with 30 ng of AMPK without cold ATP was included as a control (w/o ATP).

(C) Activation of the Atg14L-containing (VIC3) and UVRAG-containing (VIC4) Vps34 complex by AMPK or glucose starvation. Wild-type MEFs were incubated with or without glucose for 3 hrs and endogenous VIC3 and VIC4 were immuno-precipitated by Atg14L and UVRAG antibodies, respectively. The immune-complex isolated from un-starved cells was treated with AMPK followed by lambda phosphatase. Also, the immune-complex from the starved cells was treated with lambda phosphatase followed by AMPK as indicated. Vps34 kinase activity was normalized by the Vps34 protein level and shown as relative fold (Error bars represent S.D., n=3).

(D) Atg14L directly enhances Vps34 activity. Ha-Vps34 and Myc-Beclin1 were co-transfected into HEK293 cells and the Vps34-Beclin1 complex was immunoprecipitated by Myc antibody. The immune-complex was incubated with the increasing amount (10, 20, 40 ng) of purified Atg14L protein and washed with MBLS to remove unbound Atg14L from the complex. The resulting immune-complex was subjected to Vps34 lipid kinase assay. The amount of Vps34 and Atg14L protein in the complex was determined by western blot.

(E) Undetectable PI-lipid kinase activity in the purified Atg14L protein. Purified Atg14L activated the Beclin1 complex (VIC2) but itself had no detectable PI-lipid kinase activity even when three times more Atg14L protein was used (lane 5) than the amount used to activate the Beclin1 complex *in vitro* (Lane 2, 3).

(F) C-terminus of Atg14L plays important roles not only to enhance of basal activity of Vps34 complex but also to activate the complex by AMPK. Systematic Atg14L deletion constructs (Flag-Atg14L, see the schematic diagram at bottom) were co-transfected with Vps34, Beclin1, and AMPK into HEK293 cells as indicated. Atg14L containing Vps34 complexes were immunoprecipitated by Flag antibody and the lipid kinase activity was determined. Vps34 activity was normalized by Vps34 protein level in IPs and the relative Vps34 activity was shown as fold (Error bars represent S.D., n=3).

Fig. S3. AMPK directly phosphorylates Vps34 and Beclin1, Related to Figure 3.

(A) *in vitro* AMPK phosphorylation assay against ATG proteins. The indicated autophagy proteins were immunoprecipitated from the transfected HEK293 cells. The purified proteins were used as a substrate for *in vitro* AMPK phosphorylation assay. Phosphorylation was determined by ^{32}P-autoradiogram and the amount of each protein was determined by western blots. The arrows denote the full-length of the recombinant protein of interest.

(B-E) The indicated GST-Beclin1 (or GST-Vps34) full-length (WT) and deletion fragments were purified from *E. coli* and were used as substrates for *in vitro* AMPK assays. **(B-C)** AMPK phosphorylates Beclin1 S91/S94 *in vitro*. Deletion analyses indicated that the major phosphorylation of Beclin1 by AMPK was in the fragment between 86-128. Consistently, internal fragment of Beclin1 (78-148) was phosphorylated by AMPK. Mutation of S91 and S94 to alanines abolished the majority of phosphorylation in the Beclin1 fragment 1-148. **(D-E)** AMPK phosphorylates Vps34 T163/S165 *in vitro*. Major phosphorylation was observed in the fragment between 131-192. Mutation of serine and threonine conserved in vertebrates demonstrated that T163/S165 were AMPK target sites. GST and GST-TSC2F (TSC2 fragment 1300-1367 containing AMPK phosphorylation site at S1345) were used as a negative and a positive control for AMPK reaction, respectively. Phosphorylation was determined by ^{32}P-autoradiograph and the protein levels were examined by Coomassie staining. Note: Overexpression of Vps34 proteins in bacteria is not

successful to obtain the intact protein with the expected molecular weight. There are several degraded fragments. Arrows indicate full-length target protein of interest.

(F) Evaluation of phospho-specific antibodies for Beclin1 S91/S94 and Vps34 T163/S165 phosphorylation. Phosphoantibodies were prepared by immunizing rabbits with synthetic phosphopeptides (Cell Signaling). Recombinant GST-Beclin1 (1-148) or Vps34 (1-192) fragments were purified from bacteria and 500 ng of the indicated recombinant fragments were phosphorylated by AMPK *in vitro*. After reaction, 5 ng of the GST-proteins were used to test specificity of the indicated phospho-antibodies by western blot. Non-phosphorylatable mutants, GST-Beclin1 (1-148) S91/94A and GST-Vps34 (1-192) T163/165A, were used as negative controls.

(G, H) Beclin1 S91/S94 (**G**) and Vps34 T163/S165 (**H**) are phosphorylated by AMPKα co-expression in the transfected cells. Flag-Beclin1 wild-type and the S91/94A were co-transfected with either AMPKα wild-type (W) or kinase-inactive mutant (D) into HEK293 cells. Flag-Beclin1 was immuno-precipitated and phosphorylation of Beclin1 S91/S94 was examined by western blot. Similar experiment was performed for Vps34 phosphorylation against immunoprecipitated Ha-Vps34 proteins from the transfected HEK293 cells.

Fig. S4. Atg14L determines the effects (either activation or inhibition) of AMPK on Vps34 complex, Related to Figure 5.

(A) Atg14L enhances Beclin1 S91/S94 phosphorylation by AMPK. VIC2 (without Atg14L) and VIC3 (with Atg14L) were immuno-purified from HEK293 cells co-transfected with or without AMPKα as indicated. WT and SA denote wild type and S91/94A mutant of Beclin1, respectively. Phosphorylation level of Beclin1 S91/S94 was examined by western blot.

(B) Phosphorylation of Vps34 T163/S165 is repressed by Atg14L. HA-Vps34 and Myc-Beclin1 were co-transfected into HEK293 cells with either vector (Vec) or Flag-Atg14L in the presence or absence of

AMPKα as indicated. Vps34 protein was immuno-precipitated by HA antibody and T163/S165 phosphorylation was determined by western blot.

(C) Beclin1 in VIC3 is a better substrate than in VIC2 for AMPK phosphorylation *in vitro*. Flag-Beclin1 was transfected into HEK293 cells. Myc-Beclin1 was co-transfected with Flag-Atg14L as indicated. Flag-Beclin1 and Myc-Beclin1 were immunoprecipitated from the transfected cells that were cultured in glucose-rich medium. The immune-complexes were subjected to *in vitro* phosphorylation by AMPK for the indicated times. Beclin1 S91/S94 phosphorylation was determined by western blot. Protein levels of Beclin1 and Atg14L in the IP complex were examined by western blots. Endo. and exo. denote the endogenous and ectopically expressed Atg14L, respectively.

Fig. S5. Beclin1 S91/S94 phosphorylation is required for autophagy induction, Related to Figure 6.

(A) Knockdown of either Beclin1 or Vps34 suppresses LC3 modification in response to glucose starvation. MEF cells were stably infected with lentivirus expressing shRNA for Scramble (Scr), Vps34 (Vps34-KD), and Beclin1 (Bec-KD), respectively. The knockdown cells (knockdown efficiency was shown in Fgi.6A, 7A) were starved with glucose for the indicated time. Cell lysates were resolved to examine autophagy induction by LC3 western. Protein was normalized by α-tubulin (α-Tub).

(B) Beclin1 S91/S94 phosphorylation is important for p62 degradation by glucose starvation. The indicated control (Scramble, Scr), Beclin1 knockdown (Beclin1-KD), and Beclin1-reconstituted (WT or S91/S94A mutant, SA) MEFs were incubated in glucose-free medium for 3 hrs and p62 (SQSTM1) protein, an autophagy marker that is degraded during autophagy, was examined. p62 protein level was normalized by vinculin and shown as relative folds (Error bars represent ± S.D., n=3).

(C) Beclin1 S91/S94 phosphorylation is required for glucose-starvation induced LC3 puncta formation (Confocal images for Fig.6C) . The Beclin1 knockdown (KD) and Beclin1-reconstituted (KD/WT and KD/SA) MEFs were starved with glucose for 3 hrs and induction of autophagy and cellular PI(3)P level were examined by LC3 and GST-2xFYVE staining, respectively. Representative confocal images are shown

(scale bar, 10 μm). LC3 and PI(3)P double-positive puncta, marked by the overlap staining of LC3 and GST-2xFYVE, are shown in boxes with higher magnification.

(D) Beclin1-S91/94A mutant is defective in autophagy induction. Beclin1-MEFs were starved with glucose for 3 hrs and the autophagy vacuoles were examined on EM (bar, 1μm). Higher magnification of boxed areas is shown, in which autophagosome/autolysosome-like structures are indicated by arrows (black boxes; bar, 500 nm).

(E) Autophagy-related PI(3)P is increased in response to glucose starvation whereas overall PI(P) level is decreased. MEFs stably expressing GFP-DFCP1 were incubated with or without glucose for 3 hrs. Total PI(3)P level was examined by immunostaining with GST-2xFYVE probe and autophagy-related PI(3)P was determined by the number of GFP-positive puncta under confocal fluorescence microscopy (Top, confocal images). The area of GFP-DFCP1 and the overlap of GST-2xFYVE and GFP-DFCP1staining were quantified (Bottom). Results shown represent mean ± S.D. of 10-15 randomly selected images of the cells.

Fig. S6. Vps34 T163/S165 phosphorylation plays a role in cell protection from glucose starvation, Related to Figure 7.

(A) Vps34 T163/S165 phosphorylation is dispensable for p62 degradation upon glucose starvation. The indicated control (Scramble, Scr), Vps34 knockdown (Vps34-KD), and Vps34-reconstituted (WT or T163/165A mutant, SA) MEFs were incubated in glucose-free medium for 3 hrs. p62 degradation was determined as described in Fig.S5B (Error bars represent **±** S.D., n=3).

(B) Vps34 T161/S163A mutant is functional in autophagy (confocal images for Fig.7C). Similar experiments were performed for Vps34 knockdown and reconstituted MEFs as described in Fig.S5C.

(C) Autophagy analysis by electron microscopy (EM). Similar experiments were performed for Vps34 knockdown and reconstituted MEFs as described in Fig.S5D.

(D) Vps34 T163/S165 phosphorylation is required for cell survival in response to glucose starvation. MEF cells were starved with glucose for the indicated time periods and then cell viability was measured

by cell counting with tryphan blue staining. Cell viability was shown as a % of the viability under glucose-rich condition as 100% (Error bars represent ± S.D., n=4; **, $p<0.01$).

Fig. S7. Supplemental information for Discussion.

(A) *In vitro* Vps34 lipid kinase assays with increasing amount of immunoprecipitated Vps34 complex. Endogenous Beclin1 and Atg14L were immunoprecipitated from MEFs incubated with or without glucose for 3 hrs. The increasing amount of Beclin1 and Atg14L immune complexes were subjected to *in vitro* Vps34 lipid kinase assay. ^{32}P-PI(3)P was resolved by thin-layer-chromatography and the Vps34 protein was determined by western blot.

(B) Metabolic activities of AMPKa-DKO MEFs. AMPKa wild-type (WT) and double knockout (DKO) MEFs were incubated with a fresh culture medium containing high glucose (25 mM) and the glucose usage level was monitored by measuring the glucose level in the culture medium at the indicated time points (Top panel). In parallel, AMPK-WT and DKO MEFs were incubated with glucose-free medium for the indicated time periods and then cellular lactate level was determined by lactate measurement kit according to the manufacturer's instruction (Cayman chemical, Bottom panel). (Error bars represent ± S.D., n=3)

(C) The majority of Vps34 complex in MEFs is non-autophagy complexes (free of both Atg14L and UVRAG). To further confirm that majority of Vps34 complex interacts with neither Atg14L nor UVRAG (Fig.1A) and that it is not an artifact of immunoprecipitation (disrupting the complex formation due to antibody) of Vps34 antibody used in immunoprecipitation, total Vps34 protein was harvested by pull-down method instead of using Vps34 antibody. To this end, Vps34 was pull-down by Streptavidin beads from Vps34-KD/WT MEFs, in which endogenous Vps34 was knock-down and N-terminal SBP (streptavidin binding peptide)-Vps34 was re-introduced to a level similar to endogenous Vps34 in wild-type MEFs (See the expression level at Fig.7A). In parallel, Atg14L and UVRAG were immunoprecipitated. When normalized to similar levels of Beclin1, much less Atg14L or UVRAG was recovered in the SBP-

Vps34 pulldown. These data indicate that the majority of VPS34 is not in complex with either Atg14L or UVRAG, consistent with results of immunoprecipitation with specific antibodies (Fig.1B-D).

(D) Vps34 lipid kinase activity of Vps34-pull down complex was also inhibited by glucose starvation. SBP-Vps34 complex prepared by streptavidin pulldown (panel A) was subjected to lipid kinase assay. At the same time, Vps34-IP complex was prepared for comparison. Vps34 proteins in the pull-down and the IP complexes were examined by western blots. Glucose starvation inhibited lipid kinase activity of both preparations.

(E) *In vitro* Vps34 lipid kinase assay using two different Beclin1 preparations. To test whether the antibody used in IP interferes with Vps34 complex formation and its lipid kinase activity, Beclin1 was prepared by IP or pull-down method from reconstituted MEF cells (See the expression level of Beclin1 in Fig.6A). The cells were incubated with or without glucose for 3 hrs before assay. The Vps34 kinase assay and the complex analysis were performed in the condition normalized Beclin1 protein levels.

(F) Overexpression of Atg14L enhances autophagy in response to glucose starvation. Flag-Atg14L was transfected into HEK293 cells and then the cells were starved with glucose for 3 hrs in the absence or presence of 10 mM NH_4Cl. Autophagy was determined by LC3 western analysis. Protein level was normalized by α-tubulin and total Atg14L protein, including endogenous and ectopically expressed Atg14L, was immunoblotted with Atg14L antibody.

(G) UVRAG can not overcome the loss of Atg14L in glucose starvation-induced autophagy. Atg14L siRNA pool (Dharmacon) was transfected into HEK293 cells, in which Flag-UVRAG was transfected at the following day. After 2 day post-transfection, the cells were starved with glucose for 3 hrs in the presence or absence of 10 mM NH_4Cl. Autophagy was determined by western blot against LC3. Protein level was normalized by α-tubulin and the expression level of Atg14L and UVRAG were also determined.

(H,I) The role of ULK1/2, Vps34, and Beclin1 in autophagy induced by glucose starvation or rapamycin treatment. Wild-type MEFs, ULK1-KO/ULK2-KD (Kim et al., 2011), Vps34-KD, and Beclin1-KD MEFs were

incubated with either glucose-free medium or 50 nM rapamycin for 8 hrs **(H)** and for 3 hrs **(I)**,

respectively. Autophagy induction was determined by LC3-II western blots.

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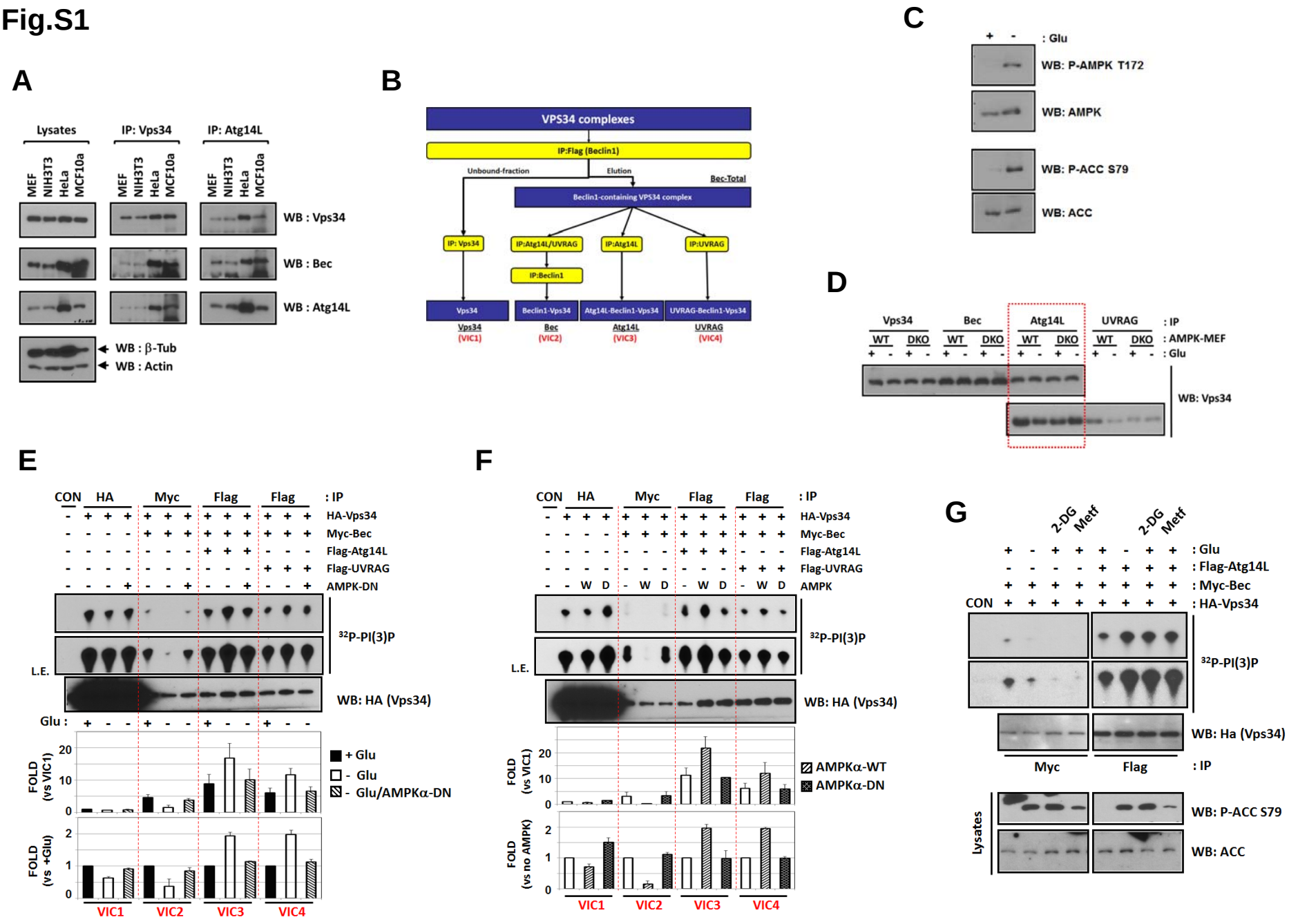
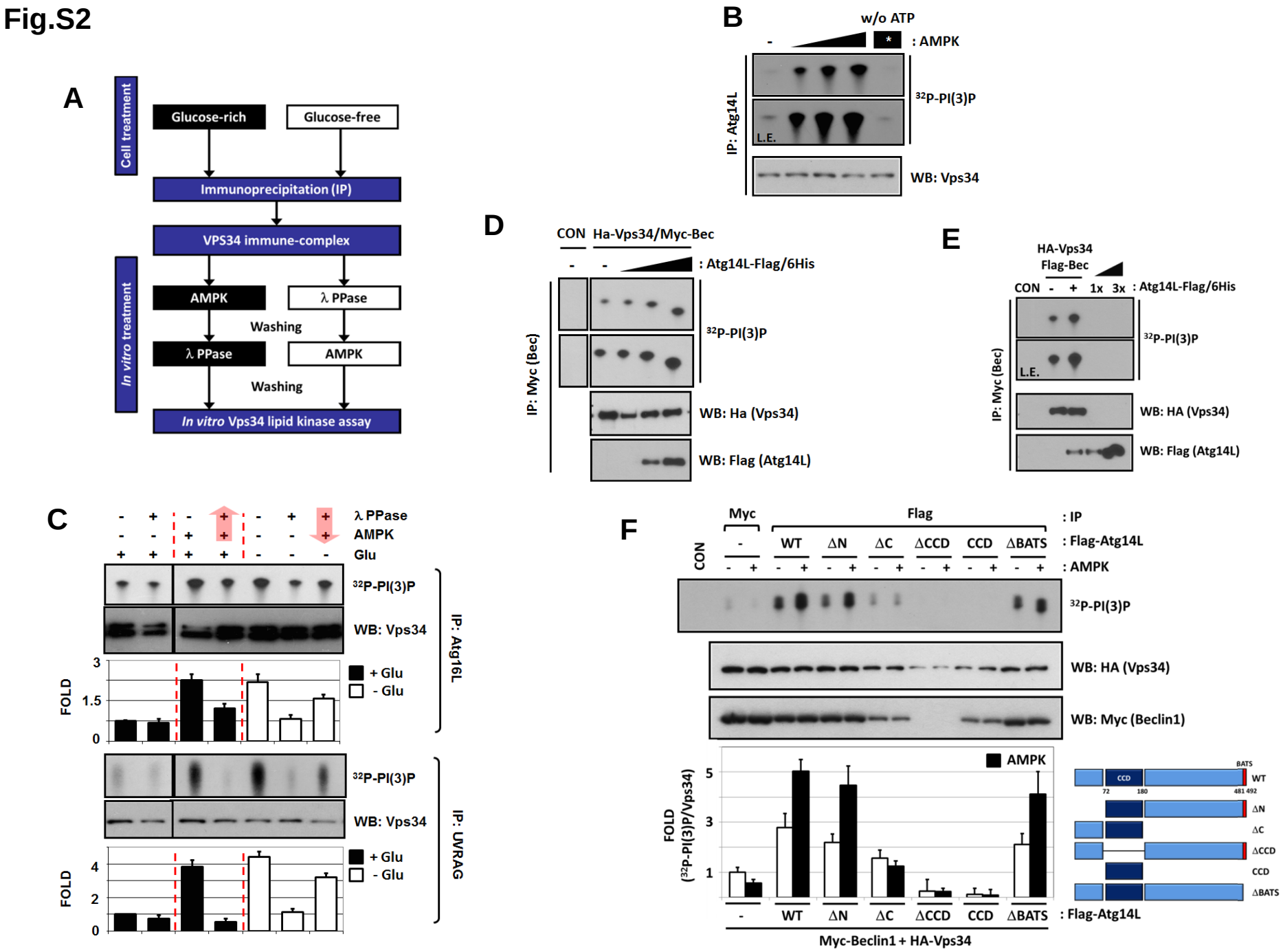
Supplemental Figure1

Fig.S1



Supplemental Figure2



Supplemental Figure3

Fig.S3

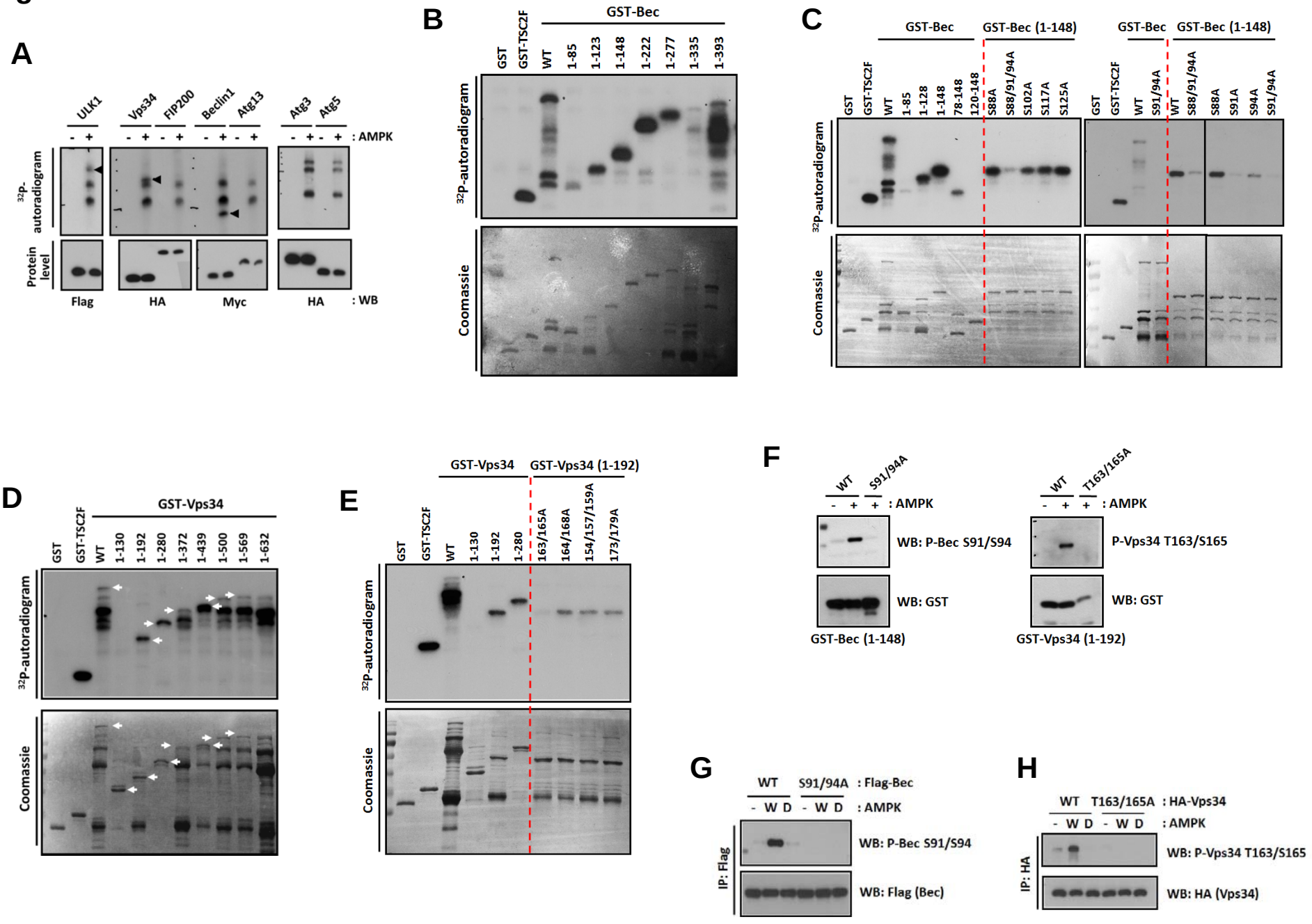
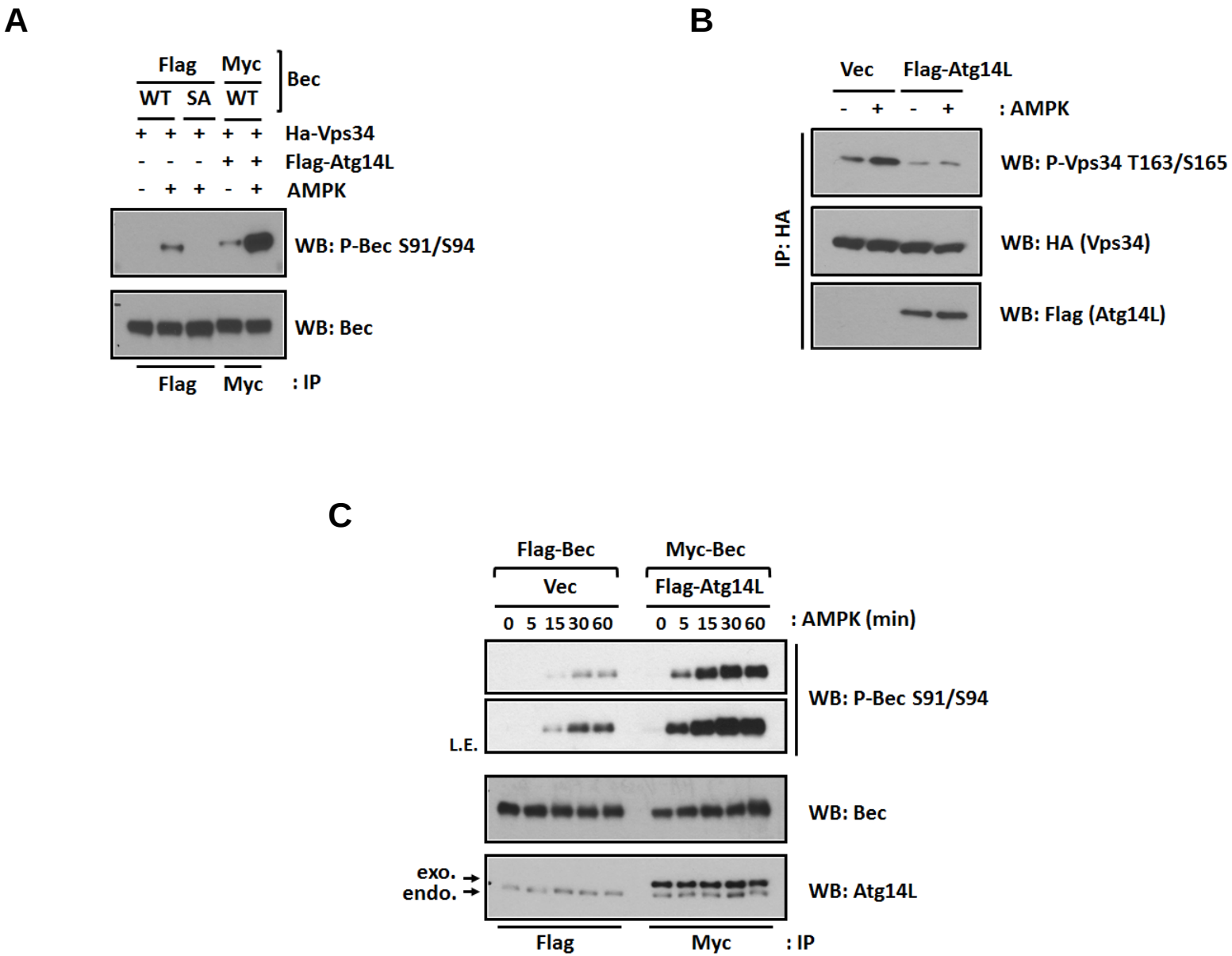
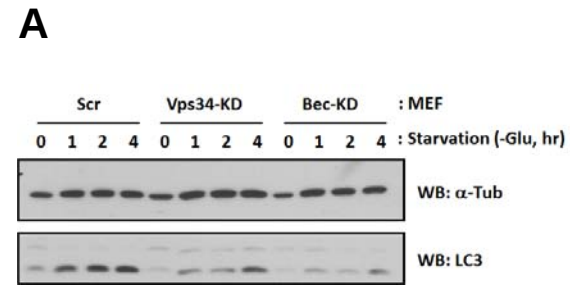


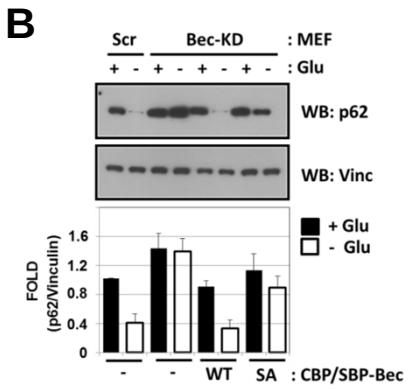
Fig.S4

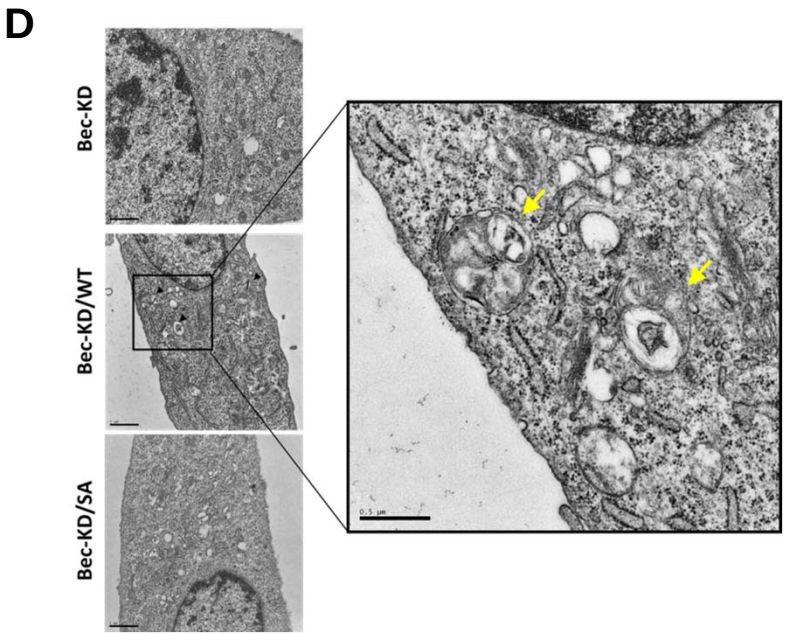

A
Flag
Myc
WT
SA
WT
Bec
+ + + + +
Ha-Vps34
- - - + +
Flag-Atg14L
- + + - +
AMPK
WB: P-Bec S91/S94
WB: Bec
Flag
Myc
: IP
B
Vec
Flag-Atg14L
- + - +
: AMPK
IP: HA
WB: P-Vps34 T163/S165
WB: HA (Vps34)
WB: Flag (Atg14L)
C
Flag-Bec
Myc-Bec
Vec
Flag-Atg14L
0 5 15 30 60
0 5 15 30 60
: AMPK (min)
WB: P-Bec S91/S94
L.E.
WB: Bec
exo.
endo.
WB: Atg14L
Flag
Myc
: IP

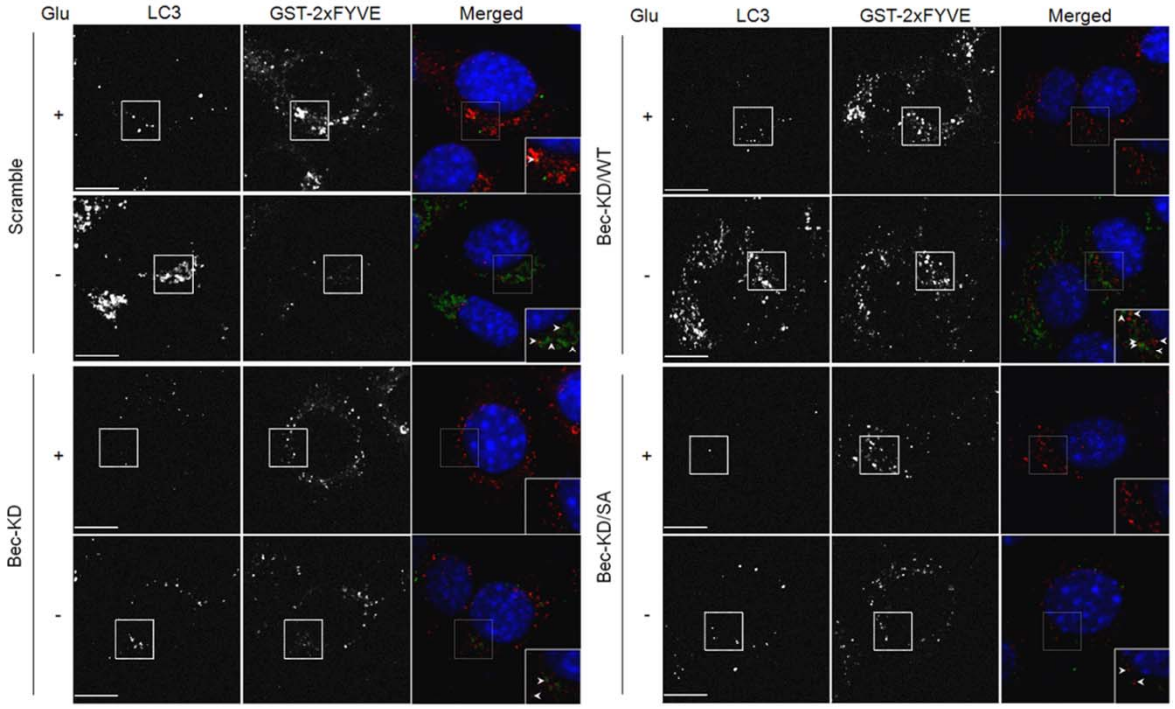
Supplemental Figure5

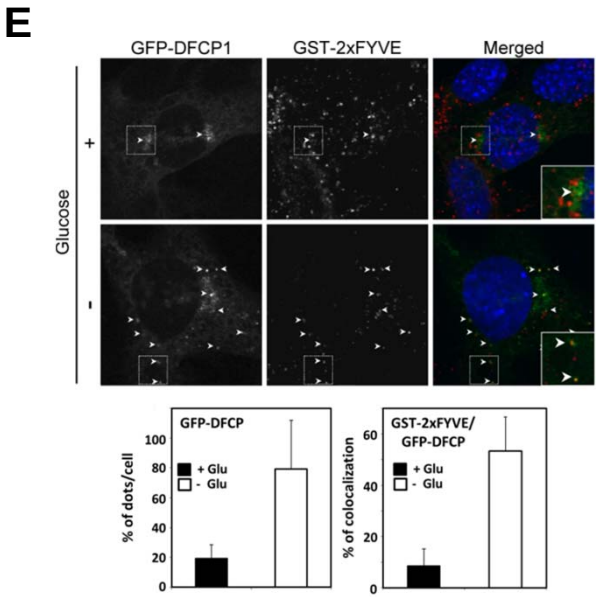
Fig.S5





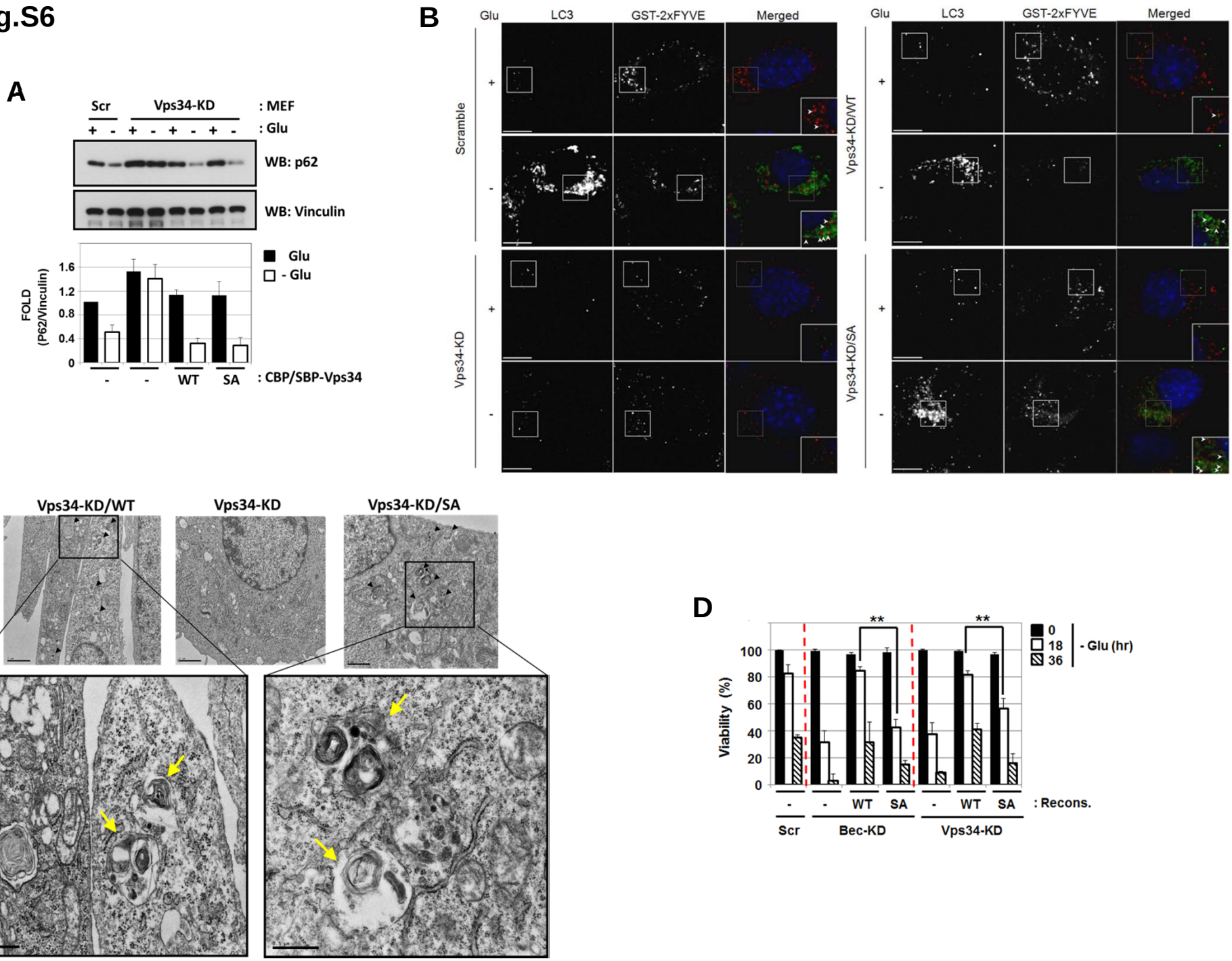






Supplemental Figure6

Fig.S6



Supplemental Figure7