Supporting Information

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SI Materials and Methods

Generation of Vps34$^{f/f}$ Mice. Vps34 (Pik3c3) gene conditional knockout ES cells in C57BL/6 background were purchased from Sanger Institute, in which LoxP sequences were inserted flanking exon 4. A promotorless cassette encoding β-galactosidase and neomycin resistance flanked by FRT sequences was inserted. Blastocyst injection was performed in University of Alabama at Birmingham transgenic core, followed by breeding to germ line. The β-galactosidase and neomycin resistance cassette was deleted by breeding to FLP mice. Genotyping was carried out by tail clips and PCR to distinguish the floxed and wild-type alleles, and the existence of the cre gene. Genotyping was performed with the following primers: (Pik3C3-5′arm) 5′-CCTGTTTCCTATCCCTGGCATTCC-3′ and (Pik3C3-3′arm) 5′-GGTTTGTGCAACAGAGAGCTAAGC-3′.

Cells and Culture. Primary mouse embryonic fibroblasts (MEFs) were generated from day 13–16 embryos from Vps34$^{+/f}$ matings. Early passage MEFs were immortalized by transfection with SV40 large T-antigen. Immortalized MEFs were infected with adenoviral GFP for Vps34$^{+/+}$ or adenoviral Cre-GFP for Vps34$^{-/-}$ for 24 h. A minimum of 4 d after infection was given to allow sufficient knockout before experiments were performed. Immunofluorescent experiments were performed at a minimum of 6 d after infection to allow for loss of adenoviral GFP expression.

MEFs were cultured in DMEM supplemented with 10% (vol/vol) FBS (HyClone Fetal Clone III), 100 units per mL penicillin, 100 μg/mL streptomycin and 2 mM glutamine. For starvation assays, MEFs were cultured in serum-free media for 6 h, amino acid-free media (Hanks' buffer supplemented with 10% (vol/vol) dialyzed serum, 100 units per mL penicillin, 100 μg/mL streptomycin, 4.5 g/L glucose) for 12 h, or Hanks' buffer (with 10 mM Hepes) for 2 h.

Mouse hepatocyte were isolated from wild-type and Vps34$^{-/-}$ animals. Hepatocytes were isolated by anesthetizing the mice with ketamine/zylazine, and the livers were perfused through the inferior vena cava. Buffers used for the perfusion were heated to 37 °C and aerated with 5% CO_2/95% O_2. The livers were perfused first with ≈40 mL of Krebs Ringer buffer (122 mM NaCl, 5.6 mM KCl, 5.5 mM glucose, 25 mM $NaHCO_3$, and 20 mM Hepes at pH 7.4) containing 10 U per mL heparin, followed by the same volume of Krebs Ringer buffer with 20 mg of collagenase type I (Worthington). The digested livers were removed, and the cells were washed out with DMEM/F12 medium (Sigma) supplemented with 5% FBS, 1 g/L fatty-acid free BSA, and 1% antibiotic/antimycotic. The cells were pelleted by gentle centrifugation and washed with hepatocyte wash buffer (Invitrogen) containing 1 g/L fatty acid-free BSA. The cells were suspended in DMEM supplemented with 5% FBS and antibiotics and plated on collagen-coated dishes. After incubation at 37 °C in 5% CO_2 for 3–4 h, the attached cells were rinsed with PBS and then incubated overnight in Medium 199 (Invitrogen) supplemented with 100 nM dexamethasone, 1 nM insulin, 100 nM 3,3,5-triiodo-L-thyronine, and antibiotics.

Mouse ventricular myocytes were isolated and cultured as described (1).

Cell Transfection and Retroviral Infection. MEFs were transfected by Lipofectamine 2000 (Invitrogen). Retroviral infection was performed as described (2) to establish Vps34$^{f/f}$ MEFs stably expressing GFP-LC3 or GFP-FYVE.

Plasmids. GFP-LC3 constructs were described (3). mCherry-GFP-LC3 was a gift from Terje Johansen (University of Tromso, Tromso, Norway) (4). GFP-FYVE was a gift from Deborah Brown (Stony Brook University, Stony Brook, NY). GFP-DFCP1 is a gift from Nicholas Ktistakis (Babraham Institute, Cambridge, UK) (5).

Reagents and Antibodies. DMEM and Hanks' buffer were purchased from Invitrogen, E64D (used at 10 μg/mL) from EMD, and Pepstatin A (used at 10 μg/mL) from Sigma. The following antibodies were used for Western blotting: Vps34 (1:1,000; Cell Signaling Technology), LC3 (1:2,000, Cell Signaling Technology), p62/SQSTM1 (1:200,000; Abnova), β-tubulin (1:10,000; Sigma), Atg5 (to detect free form, 1:500; Abgent; to detect conjugated form, 1:1,000; Sigma), Atg12 (1:500; Cell Signaling Technology), Lamin B1 (1:1,000; Santa Cruz Biotechnology), Ubiquitin (1:5,000; Covance), S6 (1:1,000; Cell Signaling Technology) and Phospho-S6 Serine 240 (1:1,000; Cell Signaling Technology), 4-EBP1 and phospho-4EBP1 (1:1,000; Cell Signaling Technology), EGFR (1:500; Abcam). The following antibodies were used for immunofluorescence: LC3 (1:300; MBL), Atg12 (1:200; Cell Signaling Technology), LAMP1 (1:50; Developmental Studies Hybridoma Bank), Rab7 (1:100; Cell Signaling Technology), Rab5 (1:100; Santa Cruz Biotechnology). The following reagents were used for the transferrin recycling assay: deferoxamine mesylate (100 μM; Sigma-Aldrich), mouse transferrin (Rockland), mouse transferrin biotin-conjugated (Rockland), and VECTASTAIN ABC-AmP Western Blotting Immunodetection Kit (Vector Laboratories).

Acridine Orange Staining. MEFs (1.5×10^5) were plated on glass-bottomed dishes. After overnight recovery, cells were incubated with 0.3 mL of Acridine Orange Staining solution (Invitrogen) in 0.7 mL of full media for 30 min at 37 °C. Cells were then immediately observed by deconvolution microscope.

Measurement of Long-Lived Protein Degradation. The assay was performed as described (2). Briefly, MEFs (2×10^4) were plated into a 12-well plate. After overnight recovery, cells were labeled with 0.5 μCi/mL ^{14}C-L-valine in L-valine–free medium (Invitrogen). Twenty-four hours after labeling, cells were washed three times with PBS and incubated in complete medium plus 10 mM unlabeled L-valine for 24 h to chase out short-lived proteins. Cells were washed again for three times with PBS and cultured either in complete medium or in serum-free medium, both containing 10 mM unlabeled L-valine. The supernatant was collected and precipitated with ice cold trichloroacetic acid (TCA) at a final concentration of 10% (vol/vol). The TCA-soluble radioactivity was measured by liquid scintillation counting. At the end of the experiments, cells were precipitated with 10% ice cold TCA, washed with 10% TCA, and dissolved in 0.2 M NaOH, and the radioactivity was measured. The degradation of long-lived protein was calculated by the radioactivity in TCA-soluble supernatant normalized against the total ^{14}C-radioactivity present in supernatants and cell pellets.

Vps34 Kinase Activity Assays. Vps34$^{f/f}$ MEFs were infected with vector or Cre virus. Upon reaching confluence, cell layers on 10-cm tissue culture plates were washed with 5 mL of ice-cold PBS and 5 mL of ice-cold wash buffer (20 mM Tris at pH 7.5, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaF, and 10 mM sodium pyrophosphate). The cells were then scraped into lysis buffer (wash buffer containing 100 μM orthovanadate, 1%

Nonidet P-40, 10% (vol/vol) glycerol, and 200 μM phenylmethylsulfonyl fluoride), and the lysates were incubated on ice for 15–20 min with frequent vortexing. The lysates were centrifuged at 18,000 × *g* for 15 min at 4 °C, and protein concentration of the supernatants was determined by Bradford assay (Bio-Rad Laboratories). Equal amounts of supernatant protein were mixed with antibody to Vps34 (Cell Signaling Technology) or Beclin 1 (Santa Cruz Biotechnology) and kept on ice overnight, then the immunocomplexes were pulled down with protein A-agarose. The beads were washed, divided, and used for Vps34 assays and Western blotting as described (2). Briefly, the kinase assays were initiated by the addition of γ-[^{32}P]ATP (PerkinElmer) and L-α-phosphatidylinositol (Sigma-Aldrich). The reaction product was isolated by TLC and visualized by autoradiography.

EGFR Degradation and Transferrin Recycling. For EGFR degradation, cells were plated at 1.5×10^5 cells per well in a 6-well plate. Once adherent, cells were serum-starved overnight. Cells were then stimulated with 100 ng/mL EGF in serum-free medium for the indicated time points. Cells were rinsed with PBS once and lysed in sample buffer [10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.1% bromophenol blue, 5% (vol/vol) β-mercaptoethanol, Tris pH6.8].

For transferring recycling, cells were plated at 1.0×10^5 cells in 3-cm plates. Once adherent, cells were serum starved for 4 h, then stimulated with 50 μg/mL biotinylated-transferrin (Rockland) in serum-free medium for 30 min at 37 °C. Plates were placed on ice and washed twice with ice cold DMEM for 2 min each. Cells were stripped for 1 min in stripping buffer (10 mM acetic acid and 150 mM NaCl at pH 3.5) on ice, then washed twice with ice cold DMEM. Cells were chased with 0.5 mg/mL unlabeled transferrin (Rockland) with the addition of 0.1 M deferoxamine mesylate for the indicated time points. Conditioned medium and cell lysate was collected. After SDS/PAGE and transfer to a nitrocellulose membrane, biotinylated-transferrin was detected by streptavidin detection kit (Vectastain).

mTOR Stimulation. Cells were plated at 2.5×10^5 cells per well in a 6-well plate. Once adherent, cells were starved overnight in serum-free medium. Cells were then incubated in high-salt glucose buffer (10 mM Hepes at pH 7.4, 140 mM NaCl, 4 mM KCl, 2 mM $MgSO_4$, 1 mM KH_2PO_4, and 10 mM glucose) for 2 h and restimulated with 2× MEM amino acids (Sigma) for 30 min. Cell lysates were collected in general lysis buffer (1:100 ProteCEASE-M, 0.2 mM PMSF, 0.1 mM Na_3VO_4, 20 mM β-glycerol phosphate disodium, 10 mM NaF, and 1 mM $Na_4P_2O_7$).

Electron Microscopy. Tissue samples were collected freshly and fixed in 4.0% (wt/vol) paraformaldehyde and 2.5% (vol/vol) EM grade glutaraldehyde in 0.1 M PBS (pH 7.4). Cell samples were fixed in 2.5% EM grade glutaraldehyde in 0.1 M PBS (pH 7.4). After fixation, samples were placed in 2% (wt/vol) osmium tetroxide for tissues and 1% for cells in 0.1 M PBS (pH 7.4), dehydrated in a graded series of ethyl alcohol, and embedded in Epon resin for tissues and Durcupan resin for cells. Ultra-thin sections of 80 nm were cut with a Reichert-Jung UltracutE ultramicrotome and placed on formvar-coated slot copper grids. Sections were counterstained with uranyl acetate and lead citrate and viewed with a FEI Tecnai12 BioTwinG2 electron microscope. Images were acquired with an AMT XR-60 CCD Digital Camera System.

For the immuno-gold EM analysis, cells were serum-starved for 6 h, then fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde in PBS for 1 h at room temperature, and washed for 5 min three times in PBS. Cells were permeabilized and blocked with 0.2% saponin, 10% (wt/vol) BSA, 10% (vol/vol) goat serum, and 0.1% fish gelatin in PBS for 30 min at room temperature. After a brief wash in PBS, cells were incubated with rabbit–anti-GFP antibody (6) in 10% (wt/vol) BSA PBS overnight at 4 °C. Cells were washed for 10 min six times in PBS, stained for 2 h at room temperature with an anti-rabbit secondary antibody conjugated to 1.4-nm gold particle (Nanoprobes), washed for 10 min six times, and refixed for 10 min with 1% glutaraldehyde in PBS. A gold enhancement kit (Nanoprobes) was used to increase the size of the gold particles (3 min of enhancement) to allow visualization by electron microscope. The samples were then processed with standard procedure, as described (2).

Histological Studies. For cryosections, the tissue was embedded directly in O.C.T. compound (Sakura Finetek), stored at −80 °C, and sectioned into 6-μm sections. For paraffin-embedded sections, tissue was fixed in 10% (vol/vol) neutral buffered formalin overnight, dehydrated in gradually increasing concentrations of ethanol, perfused in paraffin at 60 °C overnight, and embedded in paraffin the next day. Tissue was sectioned into 6-μm sections, and paraffin tissue was dewaxed and rehydrated in decreasing concentrations of ethanol solutions. Immunohistochemistry was performed according to standard protocol unless otherwise noted. Briefly, for PAS staining, a Periodic Acid-Schiff kit (Sigma) was used and counterstained with hematoxylin. For Oil Red O staining, cryosections were brought to room temperature, fixed in 10% neutral buffered formalin, washed in running tap water, rinsed with 60% (vol/vol) isopropanol, and stained with Oil Red O solution (Sigma). Sections were then rinsed with 60% isopropanol, washed with distilled water, and mounted in glycerin jelly. Tissue sections were observed and imaged under Zeiss inverted Axiovert 200M microscope.

Immunofluorescence. Cells were plated at $3–5 \times 10^4$ cells per well on gelatin-coated glass coverslips in 24-well plates. After treatment, cells were fixed in 4% (wt/vol) paraformaldehyde (PFA) in PBS for 20 min at room temperature. Cells were washed twice with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were washed three times with PBS and blocked in 10% BSA in PBS for 1 h. Primary antibodies were added in 5% (wt/vol) BSA in PBS plus 0.1% Tween-20 (PBST) overnight at 4 °C. Cells were washed four times with PBS. Fluorophore-conjugated secondary antibodies were added in 5% BSA in PBST for 1 h at room temperature with gentle shaking. Cells were washed three times with PBST, twice with PBS, and then mounted with Immuno-Mount.

Echocardiograms. Echocardiography was performed as described (1). M-mode images were used for ventricular measurements that were obtained from nine cardiac cycles for each animal.

1. Lu Z, et al. (2009) Loss of cardiac phosphoinositide 3-kinase p110 alpha results in contractile dysfunction. *Circulation* 120:318–325.
2. Dou Z, et al. (2010) The class IA phosphatidylinositol 3-kinase p110-beta subunit is a positive regulator of autophagy. *J Cell Biol* 191:827–843.
3. Ullman E, et al. (2008) Autophagy promotes necrosis in apoptosis-deficient cells in response to ER stress. *Cell Death Differ* 15:422–425.
4. Pankiv S, et al. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 282:24131–24145.
5. Axe EL, et al. (2008) Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. *J Cell Biol* 182:685–701.
6. Zhong Y, et al. (2009) Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinase complex. *Nat Cell Biol* 11: 468–476.

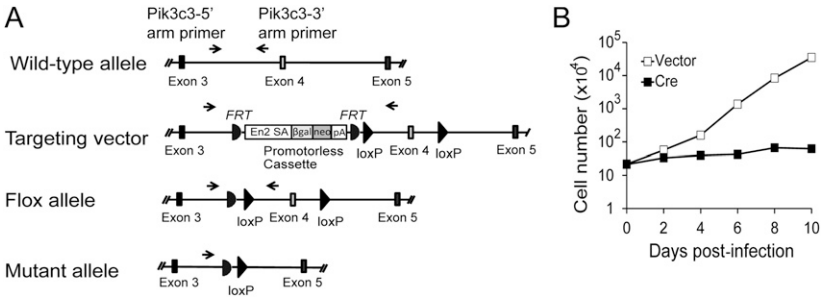

Fig. S1. (*A*) Schematic representation of the Vps34 conditional knockout strategy. LoxP sites flank exon 4 of the Vps34 gene. Expression of the Cre recombinase results in excision of exon 4 and a frame shift causing a deletion of 755 of the 887 amino acids of Vps34. (*B*) Immortalized Vps34$^{f/f}$ MEFs were infected with adenoviral vector control or adenoviral Cre. Cells were counted via hemocytometer on the indicated days, and cell number is plotted. Data shown is an average of three countings ± SEM. Error bars are too narrow to be seen.

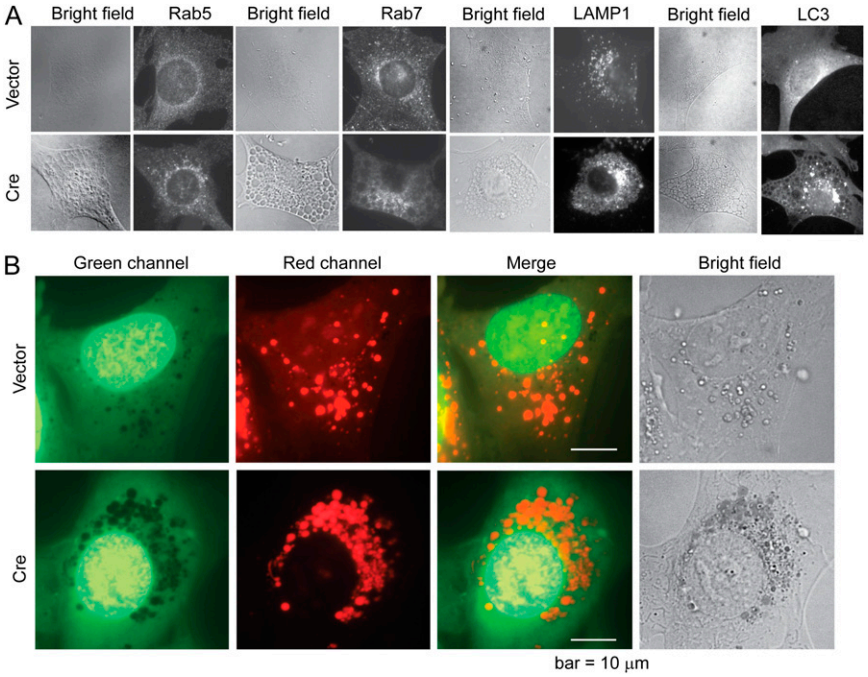

Fig. S2. Characterization of the large-sized vacuoles in Vps34-null cells. (*A*) Vector control or Cre-infected Vps34$^{f/f}$ MEFs were stained with antibodies against the early endosome marker Rab5, late endosome marker Rab7, lysosomal protein LAMP1, and LC3. Fluorescence and phase contrast images were taken. Note that the large-sized vacuoles are decorated at least partially by Rab7 and LAMP1, but not by LC3. (*B*) Cells were incubated with acridine orange (AO) staining solution. Live cells were immediately observed. Whereas neutral AO emits green fluorescence, protonated AO emits red fluorescence, indicative of acidic environment. Note that Vps34-null cells exhibited an accumulation of acidic structures, which colocalized with the large-sized translucent vacuoles (see bright field).

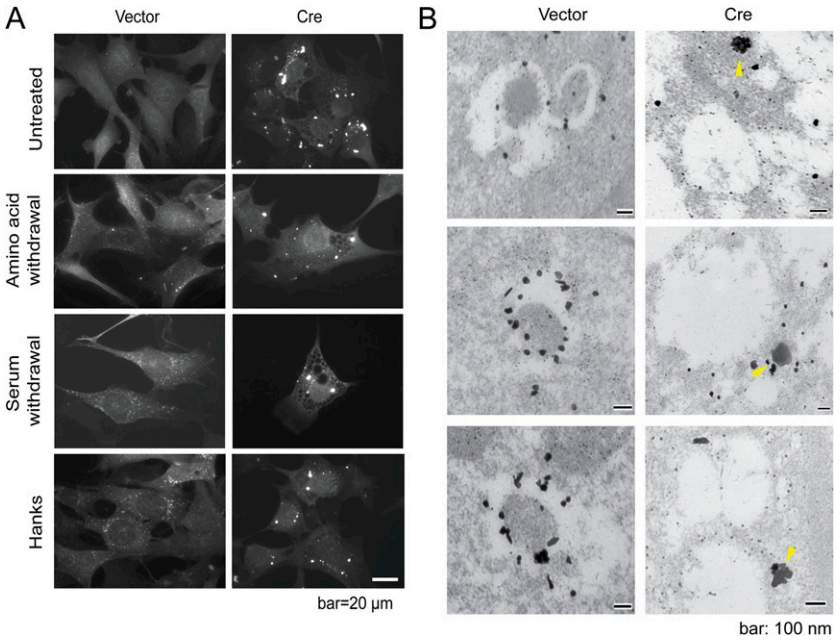

Fig. S3. Nutrient starvation-induced autophagy is blocked in Vps34-null MEFs. (*A*) Vps34$^{f/f}$ MEFs stably expressing GFP-LC3 were infected with vector control or Cre. Seven days after infection, cells were starved in Hanks' buffer for 2 h, serum-free media for 6 h, or amino acid-free media for 12 h. Note that nutrient starvation-induced GFP-LC3 puncta in vector control-infected cells, whereas larger GFP-LC3 aggregates appeared in Vps34-null cells, which did not further increase upon starvation. (*B*) For the immuno-gold EM analysis, vector and Cre-infected GFP-LC3 expressing cells were serum-starved for 6 h and subjected to an anti-GFP antibody, then processed using an anti-rabbit secondary antibody conjugated to 1.4-nm gold particle (Nanoprobes). A gold enhancement kit (Nanoprobes) was used to increase the size of the gold particles (3 min of enhancement) to allow visualization by electron microscope. Note that in vector-infected cells, the gold particles are present on the autophagosome membrane and in the cargo, whereas in Cre-infected cells, the gold particles form aggregates that are not associated with the membrane structures (yellow arrowheads). The smaller black particles in both vector and Cre cells are nonspecific background signal from the gold enhancement processing according to manufacturer's instruction.

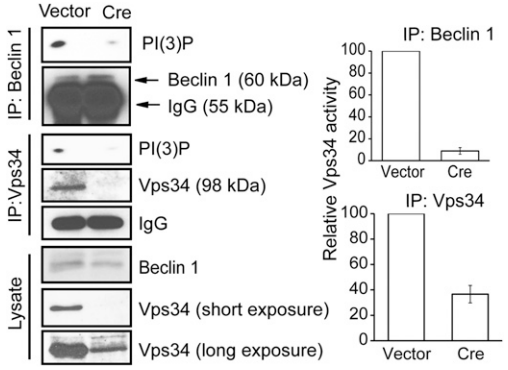

Fig. S4. Decrease of Vps34 or Beclin 1-associated PI(3)P in Vps34-null cells. (*Left*) Lysates of vector or Cre-infected Vps34$^{f/f}$ MEFs were subjected to immunoprecipitation with antibodies against Vps34 or Beclin 1. Western blots of the immunoprecipitates and cell lysates were probed with the indicated antibodies. Vps34 kinase activity was assayed in the immunoprecipitates. Vps34 activity [indicated by the level of ^{32}P-labeled PI(3)P on the autoradiograms] was quantified by densitometry and normalized to that of the vector-infected cells. The average of three independent assays ± SEM is shown at *Right*.

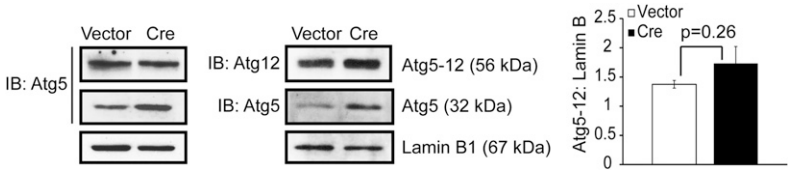

Fig. S5. Atg5-12 conjugation is not affected by Vps34 deletion. Lysate from vector control or Cre-infected cells was collected at 8 d after infection and probed with antibodies to Atg5 or Atg12 to detect conjugated Atg5-Atg12 and free Atg5. The level of conjugated Atg5-Atg12 was normalized to Lamin B1 (n = 4).

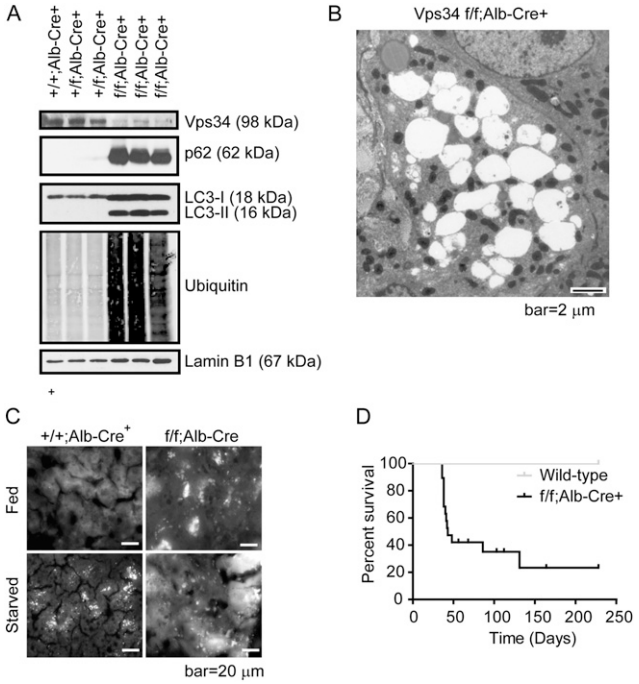

Fig. S6. Characterization of Vps34-null liver. (*A*) Autophagy flux is blocked in the Vps34-null liver. Whole liver lysates were prepared from 6- to 13-wk-old mice with indicated genotypes and were analyzed with indicated antibodies. (*B*) As seen in Vps34-null MEFs (Fig. 1*E*), Vps34-null livers also contain multiple large empty vacuoles representing swollen endosomes. (*C*) GFP-LC3 transgenic mice with indicated liver genotypes were fed or fasted for 24 h. Liver cryosections were observed, and representative images are shown. Note that fasting resulted in increased GFP-LC3 puncta in wild-type liver, whereas Vps34-null liver showed large-size GFP-LC3 aggregates in both fed and fasted conditions. (*D*) Kaplan–Meier survival curve of Alb-Cre;Vps34$^{f/f}$ mice.

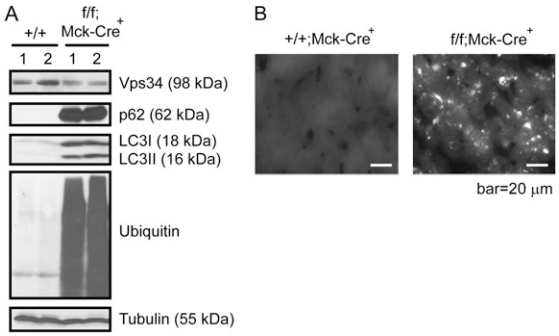

Fig. S7. Autophagy flux is blocked in the Vps34-null heart. (*A*) Whole heart lysates were prepared from 6- to 13-wk-old mice with indicated genotypes and were analyzed with indicated antibodies. (*B*) GFP-LC3 transgenic mice with indicated heart genotypes were subjected to heart cryosection. Representative images are shown. Note the presence of large size GFP-LC3 aggregates in Vps34-null heart.