SUPPLEMENTAL MATERIAL

Autophagy is required for sortilin-mediated degradation of apolipoprotein B100

Short title: Autophagy and ApoB100 degradation

Authors; Jaume Amengual PhD[1], Liang Guo PhD[1], Alanna Strong MD/PhD[2], Julio Madrigal-Matute PhD[3], Haizhen Wang PhD[1], Susmita Kaushik PhD[3], Jeffrey L. Brodsky PhD[4], Daniel J. Rader MD[2], Ana Maria Cuervo MD/PhD[3], Edward A. Fisher MD/PhD[1*].

Affiliations: [1]Division of Cardiology, NYU School of Medicine, New York, United States; Department of Medicine, NYU School of Medicine, New York, United States; Marc and Ruti Bell Program in Vascular Biology, NYU School of Medicine, New York, New York, USA. [2]Institute for Translational Medicine and Therapeutics, Cardiovascular Institute, and Institute for Diabetes, Obesity and Metabolism, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania, USA. [3]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, New York, USA. [4]Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA.

Corresponding author: Edward A. Fisher, MD, PhD; edward.fisher @nyumc.org.

Leon H. Charney Professor of Cardiovascular Medicine Director, Marc and Ruti Bell Vascular Biology Program and the Center for the Prevention of Cardiovascular Disease
NYU School of Medicine, Smilow 7
522 First Ave., NY, NY 10016
212-263-6636
212-263-9115 (fax)

DETAILED METHODS

Reagents and Antibodies

EXPRE^{35}S-Protein Labeling Mix was purchased from Perkin Elmer Life Sciences (Waltham, MA). Protein A-Sepharose was obtained from Invitrogen (Life Technologies, Grand Island, NY). All the other chemicals, unless stated otherwise, were the highest grade available and purchased from Sigma (St. Louis, MO).

For immunohistochemical analyses, chicken anti-apoB (Abcam, Cambridge, MA), mouse anti-sortilin (BD Biosciences, San Jose, CA), rabbit anti-sortilin (Abcam), rabbit anti-LC3 (MBL international, PM036, Woburn, MA), rabbit anti-Atg16L (MBL international), rabbit anti-giantin (Abcam), mouse anti-EEA1 (BD Biosciences), rat anti-LAMP1 (Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA), rabbit anti-RAB11 (Abcam), rabbit anti-p62/SQSTM1 (Enzo Life Sciences, Farmingdale, NY), and rabbit anti-STX17 (Sigma) were used as primary antibodies. Secondary antibodies conjugated to Alexa fluorophores were purchased from Molecular Probes (Life Technologies).

For western blot analyses, goat anti-apoB (Calbiochem, Billerica, MA), rabbit anti-ATG5 (Novus Biologicals, Litttleton, CO), mouse anti-GFP (Santa Cruz Biotechnologies, Dallas, TX), mouse anti-sortilin (BD Biosciences), mouse anti-tubulin (Abcam), rabbit anti-ATG7 (Cell Signaling, Danvers, MA), rabbit anti-GADPH (Millipore, Temecula, CA), rabbit anti-p62 (Abcam), rabbit anti-LC3 (MBL international) rat anti-LAMP1 (DSHB), goat anti-Cathepsin D (Santa Cruz Biotechnologies), rabbit anti-ERp72 (Enzo life sciences), and transferrin receptor (ThermoFisher Scientific, Waltham, MA), were used as primary antibodies.

For immunoprecipitation analyses, goat anti-apoB (Calbiochem), mouse anti-Myc tag (Cell Signaling), mouse anti-HA tag (Life Technologies), and rabbit anti-sortilin (Abcam) were used.

Plasmids, Adeno virus (AV) and Adeno-associated Virus (AAV)

pEGFP-C1-hApg5 was a gift from Dr. Noboru Mizushima (Addgene plasmid # 22952), pEGFP-Atg14L was a gift from Dr. Tamotsu Yoshimori (Addgene plasmid # 21635), pcDNA3.1-myc-hSortilin (Wild-type sortilin) was a gift from Dr. Francis S. Lee [1], pcDNA3.1-hPro-Sortilin (an inactive form of sortilin generated by mutation of the furin recognition site) was a gift from Dr. Anders Nykjaer [2], and pcDNA3.1-proBDNF-HA was a gift from Dr. Barbara Hempstead [3].

Adenoviral vectors encoding the reporter gene LacZ or sortilin were used to infect primary hepatocytes at $1x10^8$ infectious units/ml from 24 to 48 h before the experiments. Viruses were kindly provided by Dr. Elena Aikawa [4].

Adeno-associated viral (AAV) vectors encoding cre recombinase, GFP, and sortilin under the control of the liver-specific thyroxine-binding globulin promoter were used. Viruses were provided by the University of Pennsylvania Vector Core. AAVs were injected intraperitoneally with $1x10^{11}$ viral genomes/mouse for each AAV into 12-week old mice. Two weeks later, mice were subjected to the experimental protocols described below.

Cell Culture

Rat hepatoma McArdle RH-7777 (McA) cells obtained from the American Type Culture Collection (ATCC; Manassas, VA) were grown in culture plates coated with type I collagen from calf skin (Sigma Aldrich; St. Louis, MO). Wild-type McA cells stably expressing human apoB100 [5] as well as wild-type sortilin or GFP stably overexpressing cells [6] were cultured in DMEM (Cellgro; Manassas, VA) supplemented with 10% FBS (Gemini; Alachua, FL), 10% horse serum, 1% L-glutamine (Cellgro), and penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37°C, 5% CO_2.

Doxycycline (DOX)-inducible HUH7 cells were grown in DMEM supplemented with 10% FBS, 1% L-glutamine, and penicillin/streptomycin at 37°C, 5% CO_2, as previously described [6]. DOX (700ng/ml) was added for 72 h to induce gene expression before performing the corresponding experiments.

Primary mouse hepatocytes were isolated and cultured from *Apobec1*$^{-/-}$ mice (which express only apoB100 in their livers) as previously described [7]. Lysosome-dependent degradation was inhibited by addition of 30 μM E64D and 100 μM leupeptin or 100 nM vacuolar ATPase [V-ATPase] inhibitor bafilomycin [8]. Autophagosome-lysosome fusion was disrupted by addition of 50 μM vinblastine. Autophagosome formation was inhibited by addition of the class III PtdIns3K inhibitor 3-methyladenine (3-MA, 10 mM) to the media. Proteasome-dependent ER-associated degradation (ERAD) was inhibited with MG-132 added to the media at a final concentration of 25 μM.

Animals and Husbandry

Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 (Apobec1) knockout mice (*Apobec1*$^{-/-}$) on a B6 genetic background were a generous gift from Dr. Janet D. Sparks with permission from Dr. Nicholas Davidson [9]. Autophagy related 5 (*Atg5*) floxed mice B6.129S-Atg5<tm1Myok>, here referred as *Atg5*$^{f/f}$, were obtained from Dr. Steven Burden with permission from Dr. Noboru Mizushima [10]. Mice were maintained on a regular chow diet with *ad libitum* access to food and water at 24°C in a 12:12 h light–dark cycle. Double compound mutants (*Apobec1*$^{-/-}$*Atg5*$^{f/f}$) were generated at the New York

University animal facility following standard breeding procedures. All animal procedures and experiments were approved by the New York University Institutional Animal Use and Care Committee.

Metabolic Labeling Experiments

Steady-State labeling studies: McA hepatocytes were rinsed twice with warm PBS, and preincubated for 60 min in experimental medium (DMEM without methionine/cysteine (Gibco), supplemented with 1% FBS, 1% L-glutamine and Penicillin/Streptomycin). Cells were metabolically labeled with 120 µCi/ml of [^{35}S]-protein labeling mix for 3 h at 37ºC. At the conclusion of the incubation period, conditioned media were collected and centrifuged at 1200 rpm for 3 min in a clinical centrifuge to remove any detached cells. The cells were washed with cold PBS, and lysed with 1 ml of lysis buffer (6.1 mM Na_2HPO_4, 4.5 mM $NaHPO_4$, 88.4 mM NaCl, 36.5 mM LiCl, 1% SDS, 0.01% sodium deoxycholate, 1% triton X-100) for 30 min on ice by gentle shaking. A protease inhibitor cocktail (Roche, Indianapolis, IN) and PMSF were added to conditioned media and cell lysates, which were subsequently used for immunoprecipitation of labeled apoB100.

Pulse-Chase labeling studies: After the indicated treatments, cells were pre-incubated in experimental medium for 60 min and pulse-labeled with 240 µCi/ml of [^{35}S]-protein labeling mix (Perkin Elmer Life Sciences, Waltham, MA) for 15 min. Cells were washed with warm PBS and chased in experimental medium supplemented with an excess of unlabeled methionine (1.5 mg/mL) and cysteine (0.5 mg/mL). Chase time points were 30 min and 2 h in the case of apoB100 and HA-tagged proBDNF, and 30 min, 2 h, and 4 h for Myc-tagged sortilin experiments. At the end of the chase period, conditioned media were collected and centrifuged as above to remove detached cells. Cells were washed twice with warm PBS, and lysed in 1 mL of lysis buffer for 30 min on ice by gentle shaking. Both protease inhibitor cocktail and PMSF were added to conditioned media and cell lysates, which were used for immunoprecipitation of labeled apoB100, HA-tagged proBDNF, Myc-tagged sortilin, and albumin.

Immunoprecipitation and Quantification of [^{35}S]*-labeled Proteins*

To immunoprecipitate labeled apoB or Myc-tagged sortilin, cell lysates or conditioned media were mixed with a 5x NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.4, 1% triton X-100, 0.1% SDS) to reach a final concentration of 1x NET, together with 5 µg of the corresponding antibody and 50 µl of protein A sepharose beads (Life Technologies). The mixture was incubated overnight at 4°C with gentle shaking in the presence of the corresponding antibodies (see above). After incubation, sepharose beads were washed three times with 1x NET buffer and immunoprecipitated proteins were released from the

sepharose beads by addition of sample buffer (0.125 M Tris HCl pH 6.8, 4% SDS, 6 M urea, 1 mM EDTA, 10 mM DTT, 25 mM β-mercaptoethanol), followed by 5 min heating at 95°C.
Samples were resolved with 4-6% urea (apoB100) or 4-10% (Myc-sortilin, proBDNF-HA) SDS-PAGE gels, fixed with a mixture of acetic acid: methanol: water (10:40:50), washed with ddH_2O, and dried. Quantification of labeled proteins was performed by densitometry using the Typhoon Trio laser scanner (GE Healthcare, Pittsburgh, PA). Gel loading was normalized to total labeled protein obtained after precipitation with trichloroacetic acid (TCA).

Crosslinking and Sortilin Co-immunoprecipitation

For co-immunoprecipitation studies, cells were washed with ice-cold PBS and proteins were crosslinked with dithiobis [succinimidyl propionate] (DSP) for 1 h at room temperature. The cells were then thoroughly washed and lysed with RIPA buffer (50 mM Tris HCl pH8, 150 nM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). After protein quantification, endogenous sortilin was immunoprecipitated using an anti-sortilin antibody (Abcam) in the presence of protein A sepharose beads. To disrupt the binding between proteins, cells were incubated with protein loading buffer supplemented with 100 mM of dithiothreitol, followed by 5 min heating at 95°C.

Determination of Triglyceride and ApoB100 Secretion Rates in Mice

After an overnight fasting period, mice were given an intra-peritoneal injection of 200 µCi of [^{35}S] protein labeling mix (Perkin Elmer) combined with 1000 mg/kg pluronic F127 poloxamer-407 (BASF, Florham Park, NJ) to inhibit lipoprotein clearance from plasma [11]. Triglyceride secretion rate was calculated using plasma collected at 0, 1, 2 and 4 h post-injection. Total plasma apoB100 secretion was determined by taking 2 µl of plasma from the 1 h time point and separating the proteins by SDS-PAGE followed by densitometric quantification using the Typhoon Trio laser scanner. Animals were sacrificed at the end of the last time point (4 h), and plasma and livers were harvested. For separation of apoB100 in plasma VLDL, 250 µl of plasma (at time-point 4 h) was ultra-centrifuged (Beckman TL-100) at 100,000 rpm for 3.5 h to a density of <1.006 g/ml. Equal volumes of VLDL samples were subjected to 4% SDS-PAGE, fixed, dried, and subjected to fluorography and densitometry as described above. The relative quantity of both total plasma apoB100 and VLDL-apoB100 were normalized to total plasma albumin secreted. Albumin quantification (loading control) was determined by western blot analysis.

Isolation of Subcellular Fractions

Autophagosomes (APGs), autolysosomes (AUTLs), and lysosomes were isolated from livers of 6 h-fasted mice injected 3 h before liver dissection with vinblastine (0.5 mg/kg) to increase abundance of APGs.

After homogenization and differential centrifugation to separate unbroken cells, nuclei and a large fraction of mitochondria, the 17,000 × g resulting pellet was subjected to centrifugation in a discontinuous metrizamide (AK Scientific, CA), density gradient as described previously [12]. Fractions were recovered from the different metrizamide interfaces (APG in the 20–15%, AUTL in the 20–24% and lysosomes in the 26–24%) and washed by centrifugation in 0.25 M sucrose. Cytosolic fractions were prepared by centrifugation of the 17,000 × g supernatant at 100,000 × g for 1 h. The pellet of this centrifugation was used as the ER–enriched fraction.

Immunoblotting

Proteins were extracted from cell lysates (McA or primary mouse hepatocytes) and tissue lysates (liver) in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40). Total protein amounts were quantified using Bio-Rad DC assay kit (Bio-Rad, Hercules, CA). In general, 20-80 µg of protein homogenate were separated by SDS-PAGE and subsequently electroblotted onto PVDF membranes (Bio-Rad). Membranes were blocked with fat-free milk powder (5% w/v) dissolved in Tris-buffered saline (15 mM NaCl and 10 mM Tris/HCl, pH 7.5) containing 0.01% Tween 100 (TBS-T), washed, and incubated overnight at 4°C with the appropriate primary antibody (see above). Infrared fluorescent-labeled secondary antibodies were prepared at 1:15,000 dilution in TBS-T with 5% fat-free milk powder and incubated for 1 h at room temperature. Goat anti-mouse conjugated to 800CW (Li-Cor Biosciences, Lincoln, NE), donkey anti-rabbit conjugated to 680CW (Li-Cor), donkey anti-goat conjugated to 800CW (Li-Cor), or donkey anti-rat conjugated to 800CW (Li-Cor) were detected using the Odyssey Fc infrared imaging system (Li-Cor). Protein band quantification was performed using ImageJ (NIH, Bethesda, MD).

Immunofluorescence and Confocal Microscopy

McA rat hepatocytes and primary mouse hepatocytes were grown on fibronectin-coated glass coverslips. After the corresponding treatments media were removed and cell monolayers washed with ice-cold PBS. Cells were then fixed with 7.5% of neutral buffered formalin solution containing 0.025% of glutaraldehyde for 10 min at room temperature. For immunodetection in whole liver samples a small liver piece of tissue was fixed with 10% neutral buffered formalin solution (Sigma) for at least 24 h at 4°C. After fixation, tissue samples were immersed in a PBS solution containing 30% sucrose (w/v) until sinking, being further embedded in Optimal Cutting Temperature (OCT) compound (Sakura, Torrance, CA). Thirty-micrometer-thick sections were cut on a Leica cryostat CM1850 (Leica Microsystems, Buffalo Grove, IL), and three to six free-floating sections from each mouse were used (3 to 6 animals per experimental group) for immunohistrochemical analyses.

The specimens were then washed and permeabilized with PBS containing 0.1% Triton (PBS-T) or 0.1% digitonin, and blocked for 1 h with PBS supplemented with 5% bovine serum albumin and 2% goat serum (Sigma). Afterwards, specimens were incubated overnight in blocking buffer containing the corresponding primary antibodies (see above) at a 1:200 concentration. Specimens were washed and incubated for 1 h at room temperature with the corresponding secondary antibodies (see above) at a 1:400 dilution in blocking buffer. Secondary antibodies conjugated to Alexa fluorophores were purchased from Molecular Probes (Life Technologies). DAPI was used to stain nuclei.

For some co-localization experiments, when two primary antibodies were raised in rabbits, one of the primary antibodies was detected using the regular procedure (described above), while the second primary antibody was conjugated with Alexa fluorophore 488 using a commercially available kit (Life Technologies).
Confocal images were acquired with a Leica TCS SP5 II confocal microscope by using a 405 diode laser (excitation 405 nm), a multiline argon laser (excitation 488 nm), and two HeNe lasers (excitation 543 and 633 nm) with a 40× Apochromat, numerical aperture 1.25 – Oil objective and with a 63× Apochromat, numerical aperture 1.40- Oil objective.

In the case of isolated APG, vesicles were incubated with primary antibodies for 10 min at room temperature, followed by incubation with fluorescence-conjugated secondary antibodies for an additional 10 min as previously described [13]. Labeled vesicles were recovered by centrifugation and spotted on a glass slide. Following fixation with 8% formaldehyde in 0.25 M sucrose for 15 min, the slides were visualized with a 100× objective (numerical aperture 1.4) using an Axiovert 2000 fluorescence microscope. The percentage of co-localization was calculated using the JACoP plug-in of ImageJ (NIH) after an appropriate threshold was established.

Electron Microscopy

The Tokuyasu method was used for immuno-electron microscopy [14]. Briefly, cultured cells were fixed with freshly made 2% paraformaldehyde in PBS containing 0.2% glutaraldehyde, pH 7.2-7.4 for 4 h at 4°C. After washing with PBS, the cells were embedded with 10% gelatin, infused with sucrose, and cryosectioned using Leica UC6 cryo-ultramicrotome at 80 nm thickness onto 200 mesh carbon-formvar coated copper grids. For double immunolabeling, the grids were blocked with 1% cold water fish skin gelatin (Sigma) for 5 min, incubated with chicken-anti apoB antibody at room temperature for 2 h, and 1 h of 18 nm gold-conjugated donkey anti-chicken IgG secondary antibody (Jackson ImmunoReasearch Laboratories, Inc., West Grove, PA). After fixing with 1% glutaraldehyde for 10 min, rabbit-anti sortilin antibody was applied, and 5 nm protein A gold (Cell Microscopy Center, University Medical Center

Utrecht, 35584 CX Utrecht, The Netherlands) was used for secondary antibody. The grids were fixed in 1% glutaraldehyde for 5 min, washed with distilled water, contrasted, embedded in a mixture of 3% uranyl acetate and 2% methylcellulose at a ratio of 1 to 9, examined under Philips CM-12 electron microscope (FEI; Eindhoven, The Netherlands) and photographed with a Gatan (4k x2.7k) digital camera (Gatan, Inc., Pleasanton, CA).

siRNA Transfection and Knock-down

For siRNA experiments, cells were transfected with SiGenome siRNA pools (control scrambled siRNAs, rat *Atg7*, *human apoB100*) (Dharmacon, Waltham MA) using DharmaFECT 4 Transfection Reagent in Opti-MEM media (Life Technologies) for a minimum of 48 h according to the manufacturer's protocol. The efficiency of the knockdown was assessed by western blot or RT-PCR using the OneStep qPCR quantitect kit from Qiagen (Hilden, Germany).

RNA Isolation and Quantitative Real Time (QRT)-PCR Analysis

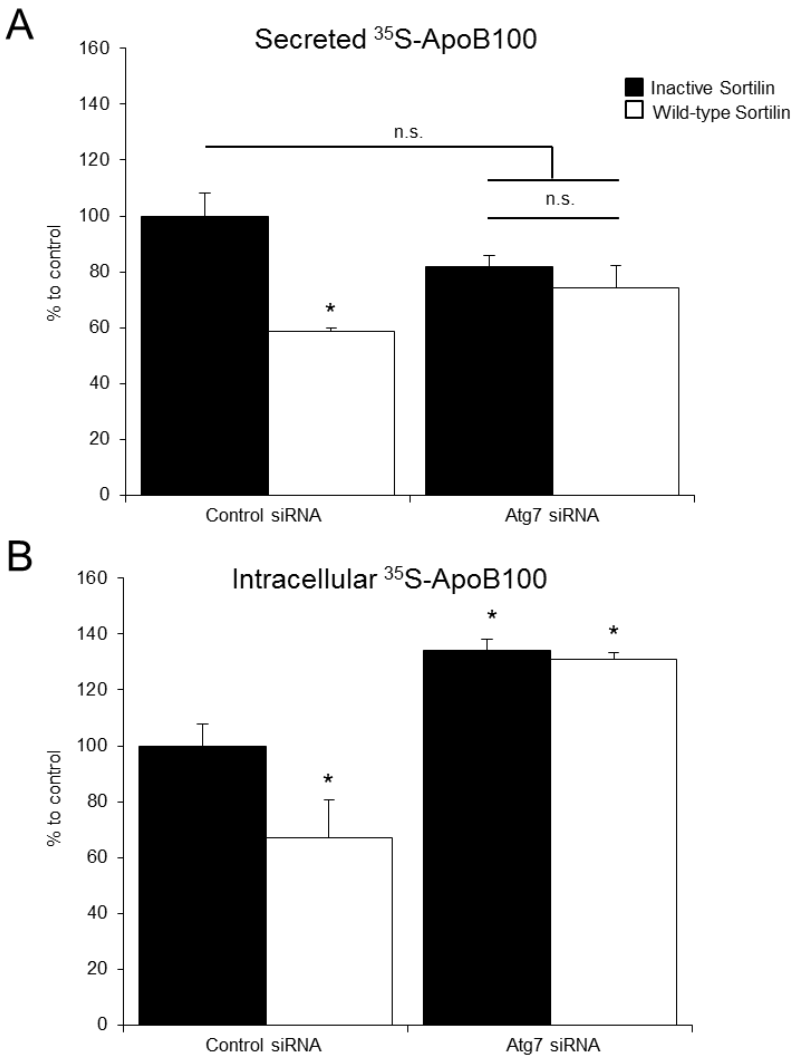
RNA was isolated from animal tissues or cultured cells using TRIzol Reagent following the manufacturer's instructions (Life Technologies). Two µg of total RNA was reverse transcribed to cDNA using the Verso cDNA Synthesis kit (ThermoFisher Scientific). QRT-PCR was carried out with TaqMan chemistry and probes (Applied Biosystems) for human LC3 (Hs01076567_g1), human GAPDH (Hs02758991_g1), mouse ATG5 (Mm01187303_m1), mouse GAPDH (Mm99999915_g1), rat ApoB (Rn01499054_m1), rat ATG7 (Rn01492725_m1), and rat GAPDH (Rn01775763_g1). Gene expression analyses were performed with the ABI Step One Plus QRT-PCR machine (Applied Biosystems).

Statistical Analysis

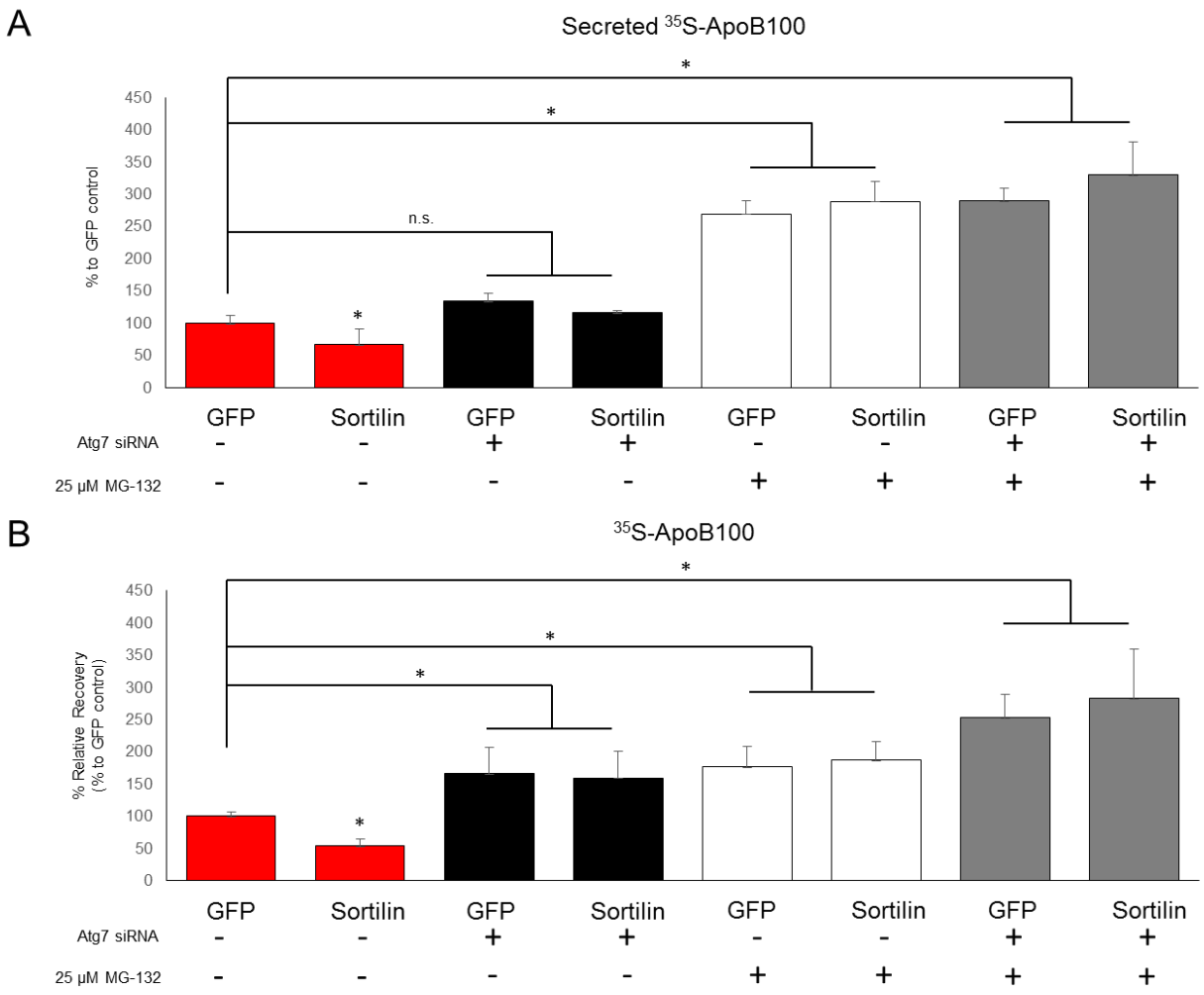
All experiments were conducted in at least triplicate. Data are expressed as the mean ± SEM. Statistical differences were analyzed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Normal distribution of the sample groups were analyzed using the D'Agostino-Pearson omnibus and the Shapiro-Wilk normality tests. Statistical significance was then determined by unpaired two-tailed Student's t-test with a threshold of significance set at $p<0.05$.

BIBLIOGRAPHY

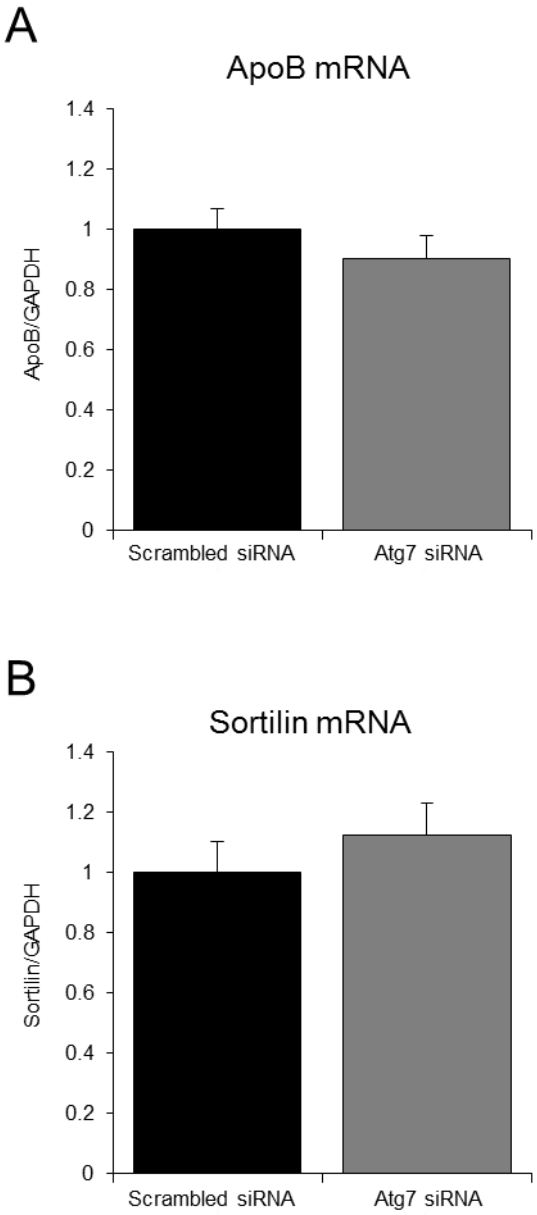
1. Chen ZY, Ieraci A, Teng H, Dall H, Meng CX, Herrera DG, Nykjaer A, Hempstead BL and Lee FS. Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2005;25:6156-66.
2. Munck Petersen C, Nielsen MS, Jacobsen C, Tauris J, Jacobsen L, Gliemann J, Moestrup SK and Madsen P. Propeptide cleavage conditions sortilin/neurotensin receptor-3 for ligand binding. *The EMBO journal*. 1999;18:595-604.
3. Evans SF, Irmady K, Ostrow K, Kim T, Nykjaer A, Saftig P, Blobel C and Hempstead BL. Neuronal brain-derived neurotrophic factor is synthesized in excess, with levels regulated by sortilin-mediated trafficking and lysosomal degradation. *The Journal of biological chemistry*. 2011;286:29556-67.
4. Goettsch C, Hutcheson JD, Aikawa M, Iwata H, Pham T, Nykjaer A, Kjolby M, Rogers M, Michel T, Shibasaki M, Hagita S, Kramann R, Rader DJ, Libby P, Singh SA and Aikawa E. Sortilin mediates vascular calcification via its recruitment into extracellular vesicles. *The Journal of clinical investigation*. 2016;126:1323-36.
5. Yao ZM, Blackhart BD, Linton MF, Taylor SM, Young SG and McCarthy BJ. Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. Evidence that the length of apolipoprotein B has a major effect on the buoyant density of the secreted lipoproteins. *The Journal of biological chemistry*. 1991;266:3300-8.
6. Strong A, Ding Q, Edmondson AC, Millar JS, Sachs KV, Li X, Kumaravel A, Wang MY, Ai D, Guo L, Alexander ET, Nguyen D, Lund-Katz S, Phillips MC, Morales CR, Tall AR, Kathiresan S, Fisher EA, Musunuru K and Rader DJ. Hepatic sortilin regulates both apolipoprotein B secretion and LDL catabolism. *The Journal of clinical investigation*. 2012;122:2807-16.
7. Andreo U, Guo L, Chirieac DV, Tuyama AC, Montenont E, Brodsky JL and Fisher EA. Insulin-stimulated degradation of apolipoprotein B100: roles of class II phosphatidylinositol-3-kinase and autophagy. *PloS one*. 2013;8:e57590.
8. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, Adhihetty PJ, Adler SG, Agam G, Agarwal R, Aghi MK, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy*. 2016;12:1-222.
9. Hirano K, Young SG, Farese RV, Jr., Ng J, Sande E, Warburton C, Powell-Braxton LM and Davidson NO. Targeted disruption of the mouse apobec-1 gene abolishes apolipoprotein B mRNA editing and eliminates apolipoprotein B48. *The Journal of biological chemistry*. 1996;271:9887-90.
10. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T and Mizushima N. The role of autophagy during the early neonatal starvation period. *Nature*. 2004;432:1032-6.
11. Millar JS, Cromley DA, McCoy MG, Rader DJ and Billheimer JT. Determining hepatic triglyceride production in mice: comparison of poloxamer 407 with Triton WR-1339. *Journal of lipid research*. 2005;46:2023-8.
12. Marzella L, Ahlberg J and Glaumann H. Isolation of autophagic vacuoles from rat liver: morphological and biochemical characterization. *The Journal of cell biology*. 1982;93:144-54.
13. Koga H, Kaushik S and Cuervo AM. Altered lipid content inhibits autophagic vesicular fusion. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2010;24:3052-65.
14. Tokuyasu KT. Immunochemistry on ultrathin frozen sections. *The Histochemical journal*. 1980;12:381-403.



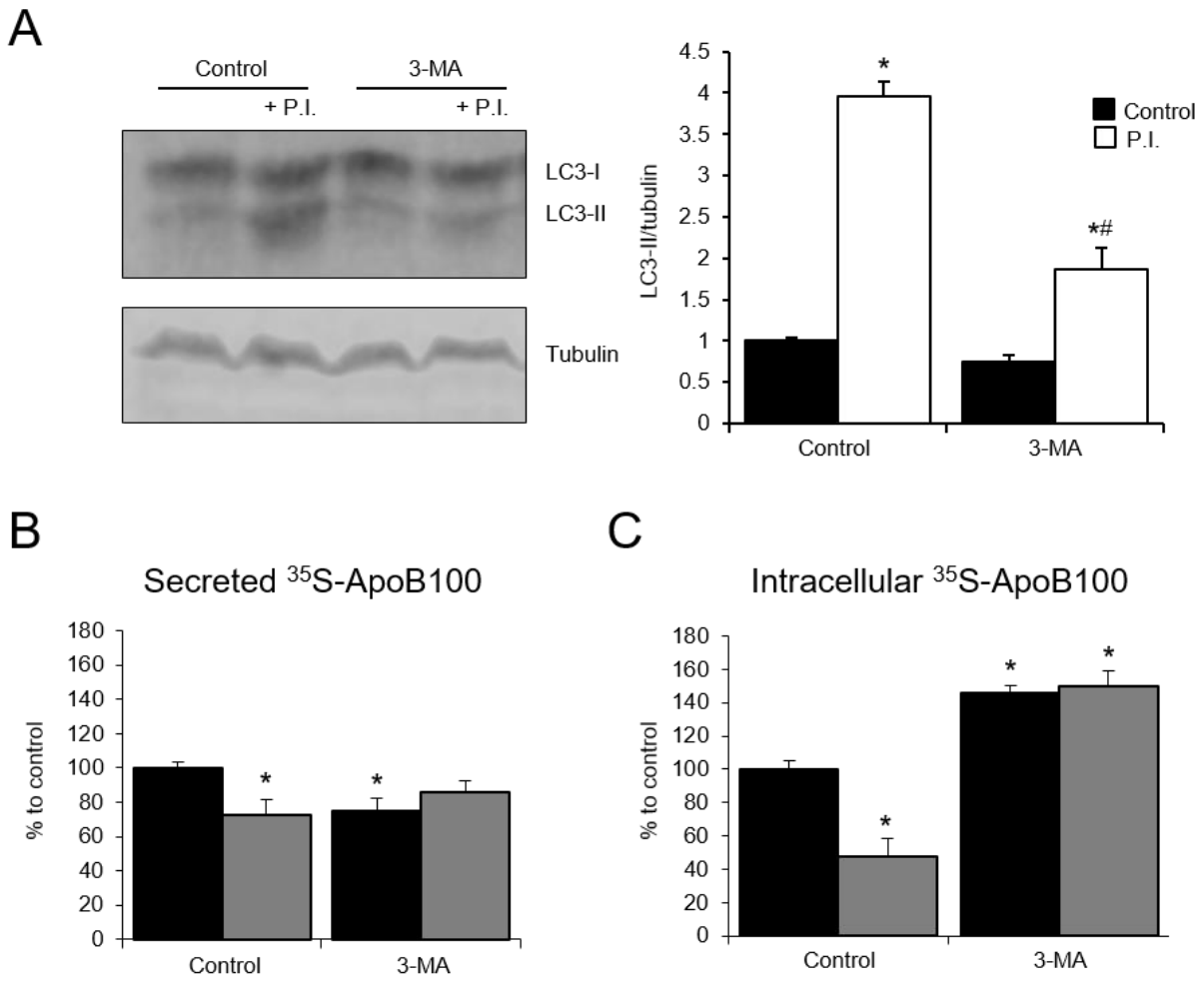
Online Figure I. ***ApoB100 metabolism in vitro after over expression of wild type and inactive sortilin in control and autophagy-deficient rat hepatic cells.*** McA hepatic cells stably expressing human apoB100 were transfected with *Atg7* siRNA or with scrambled siRNA for 24 h. Then, cells were transfected with either a plasmid containing the full-length myc-tagged human sortilin (Wild-type Sortilin, pcDNA3.1-h*SORT1*-myc) or a dominant negative sortilin ("Inactive Sortilin"; pcDNA3.1-hPro-Sortilin) generated by mutation of the furin recognition site, which prevents sortilin binding to apoB100. **(A)** Secreted apoB100 and **(B)** intracellular apoB100 recoveries showing the amount of apoB100 at the end of the chase period. Numerical data represent the means ± SEM. *, $p<0.05$ versus Control siRNA. Not significant differences are indicated with 'n.s.'.



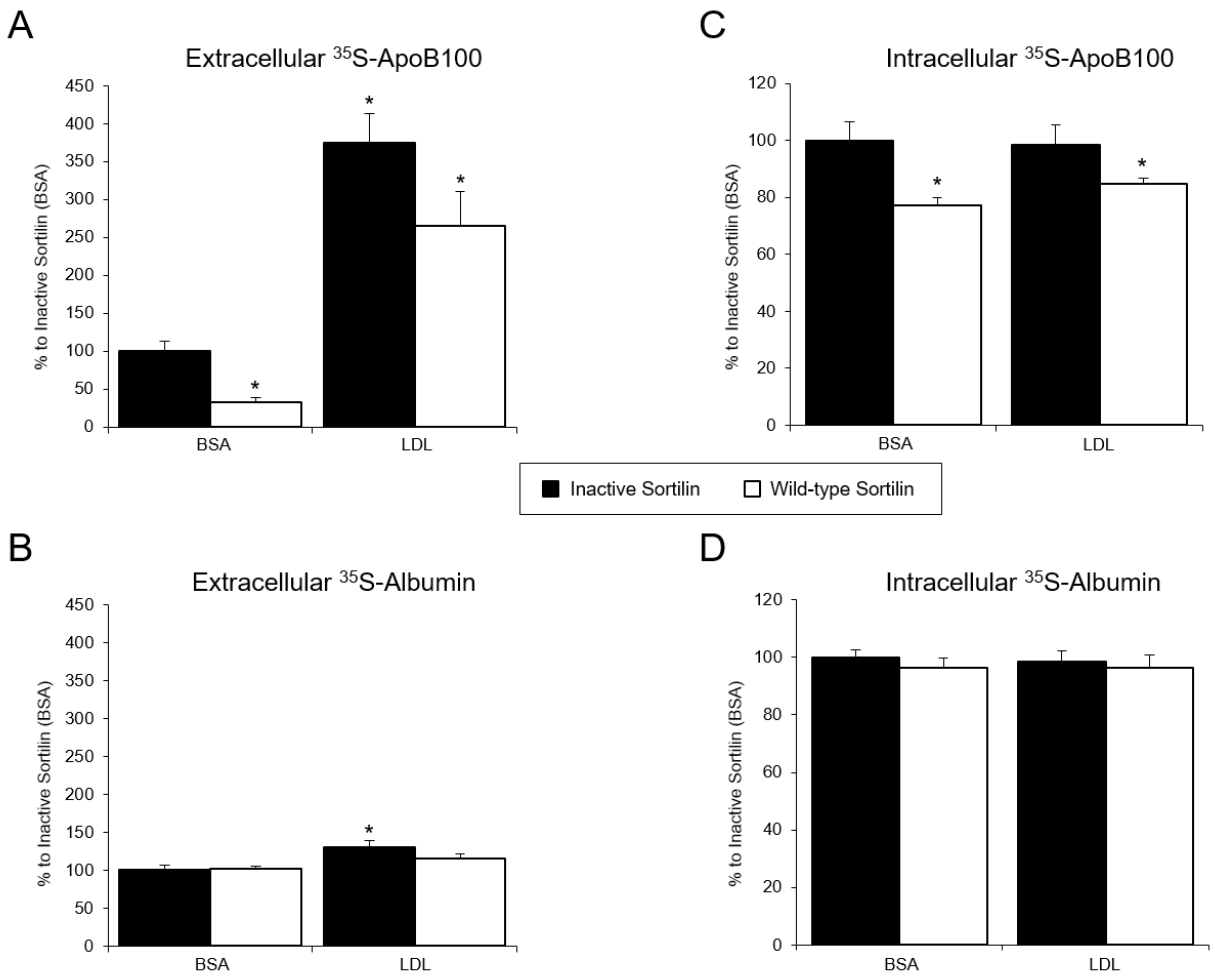
Online Figure II. ApoB100 recovery in McA cells stably over-expressing GFP or sortilin under different conditions. McA rat hepatic cells were cultured in normal growth medium and transfected with Atg7 siRNA or with scrambled siRNA for 48 h. In some cases, cells were incubated with the proteasome inhibitor MG-132 (25 µM) for 2 h prior the pulse-chase labeling with [35S] protein labeling mix (see methods for details). **(A)** Secreted apoB100 and **(B)** total relative recovery at the end of the pulse chase period. Results are expressed as percentage apoB100 recovery relative to GFP cells under normal (Control) conditions. Numerical data represent the means ± SEM. *, $p<0.05$ versus control group (left bar).



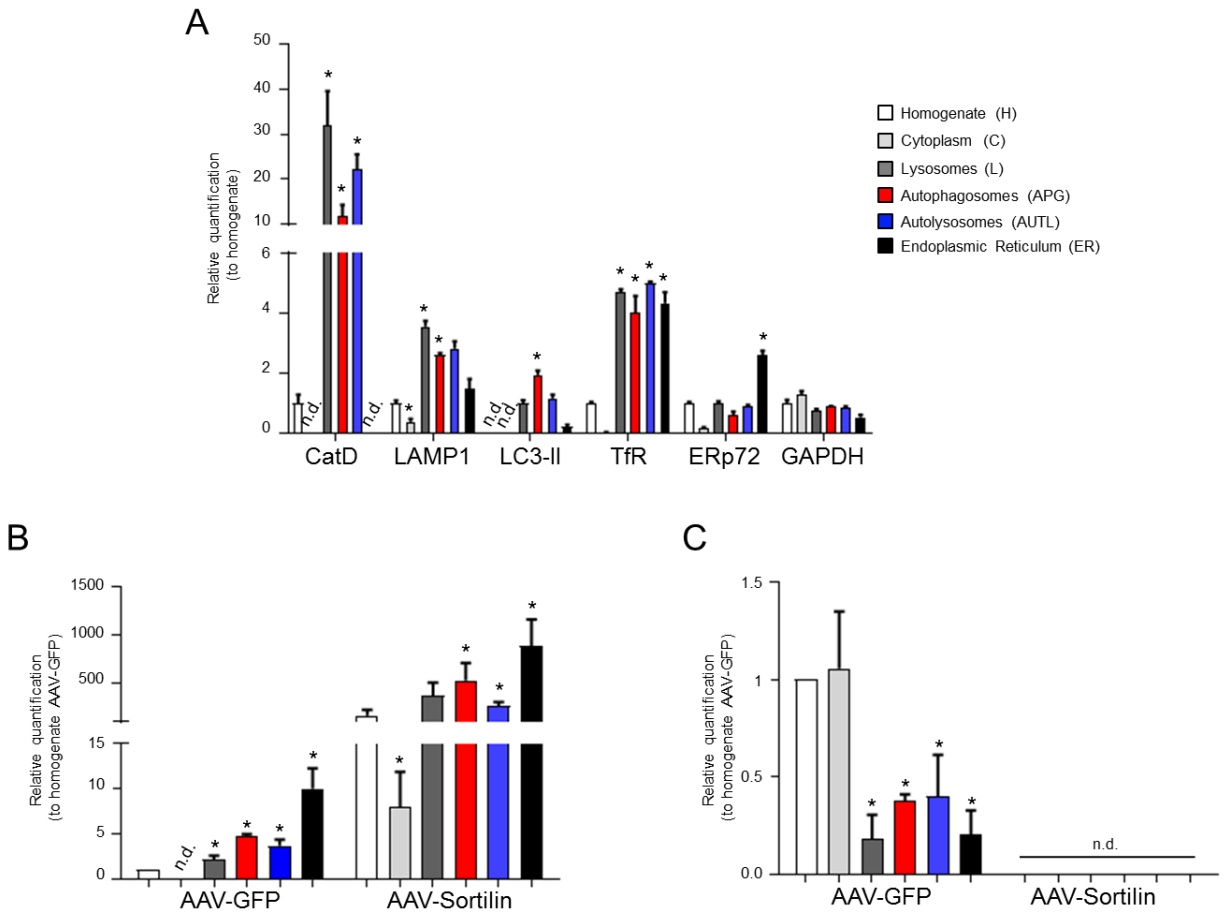
Online Figure III. McA hepatic cells stably expressing human apoB100 were transfected with either scrambled siRNA or Atg7 siRNA to determine changes on apoB and sortilin mRNA expression. Numerical data represent the means ± SEM.



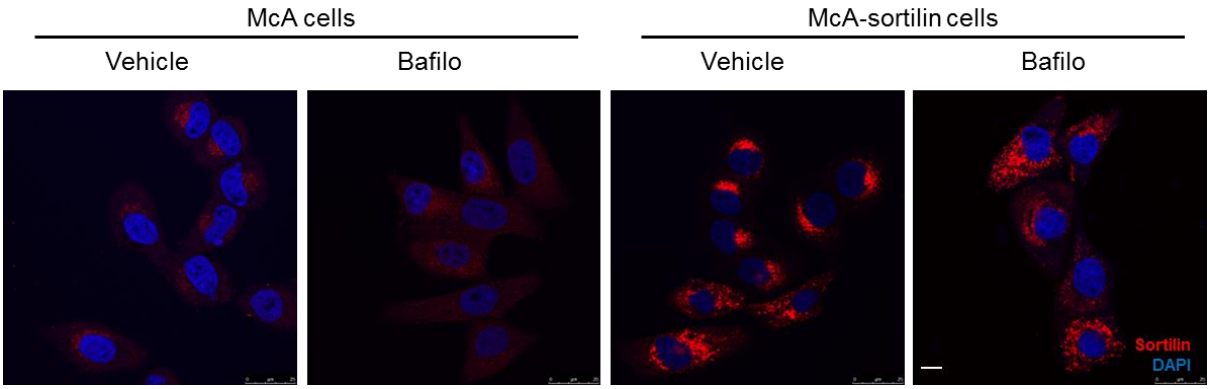
Online Figure IV. *Autophagy inhibitor 3-methyladenine (3-MA) blocks sortilin-mediated apoB100 degradation in McA cells.* McA rat hepatic cells stably expressing GFP or sortilin were cultured in normal growth medium and subjected to a steady state metabolic labeling (see methods for details) **(A)** Representative western blot showing the effect of 3-MA blocking the autophagic flux. Data are displayed as the relative differences in **(B)** secreted apoB100 and **(C)** intracellular apoB100 between cells transfected with GFP (set to 100%) vs. sortilin. Numerical data represent the means ± SEM. *, $p<0.05$ versus McA GFP.



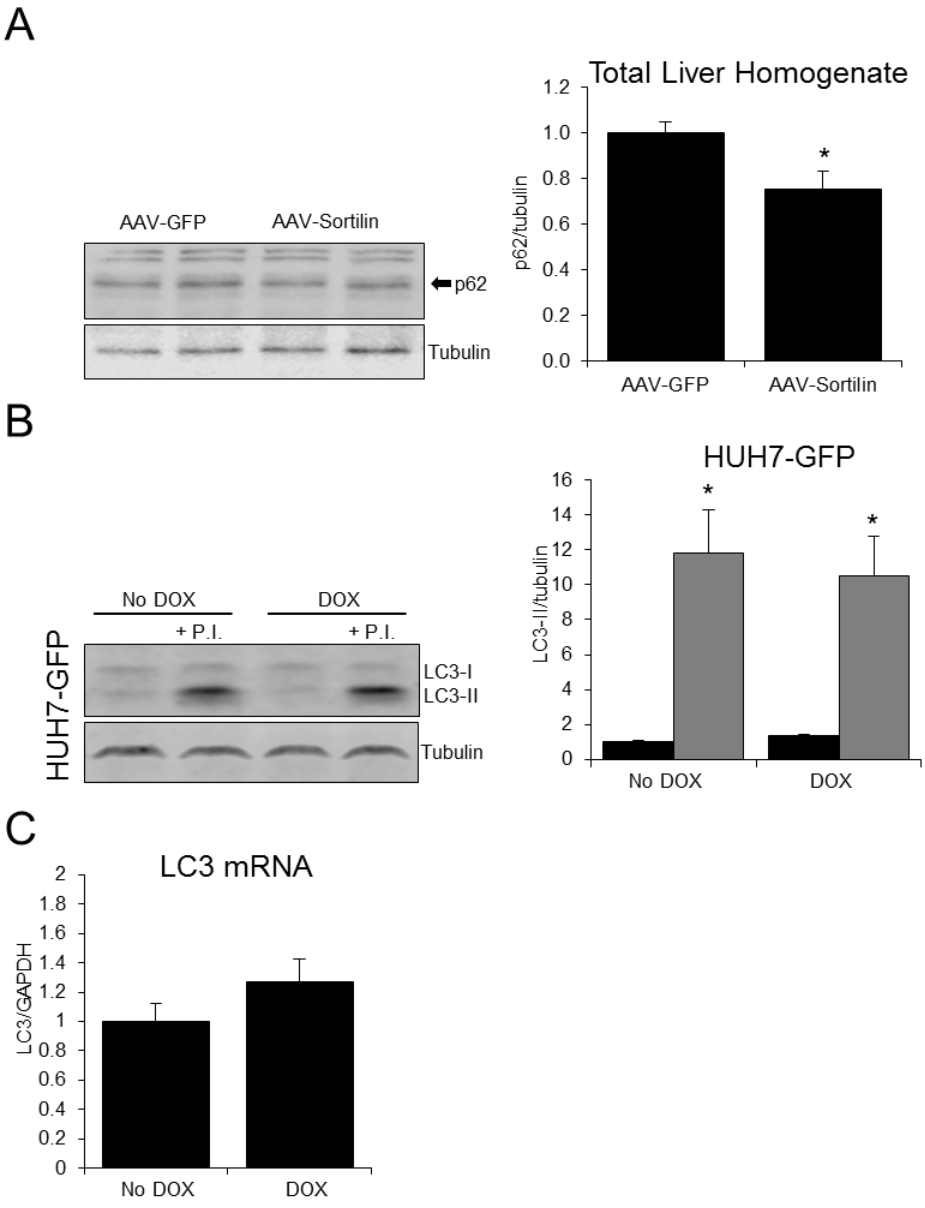
Online Figure V. *Sortilin-mediated intracellular apoB100 degradation is independent of reuptake of newly-secreted apoB100.* McA hepatic cells stably expressing human apoB100 were transfected with either a plasmid containing the full-length myc-tagged human sortilin (Wild-type Sortilin, pcDNA3.1-hSort1-myc) or a dominant negative sortilin ("Inactive Sortilin"; pcDNA3.1-hPro-Sortilin) generated by mutation of the furin recognition site, which prevents sortilin binding to apoB100. When cells reached sub-confluency (80%-90%), they were washed and amino acid-starved for 1 h before being subjected to a steady state metabolic labeling (see methods for details). To competitively prevent the reuptake of newly secreted apoB100 during the metabolic labeling, cells were incubated in the presence of 500 μg/ml of unlabeled LDL or BSA (as a control). **(A)** extracellular apoB100, **(B)** extracellular albumin **(C)** intracellular apoB100, and **(D)** intracellular albumin. Numerical data represent the means ± SEM. *, $p<0.05$ inactive sortilin versus wild-type sortilin, two-tailed Student's t-test.



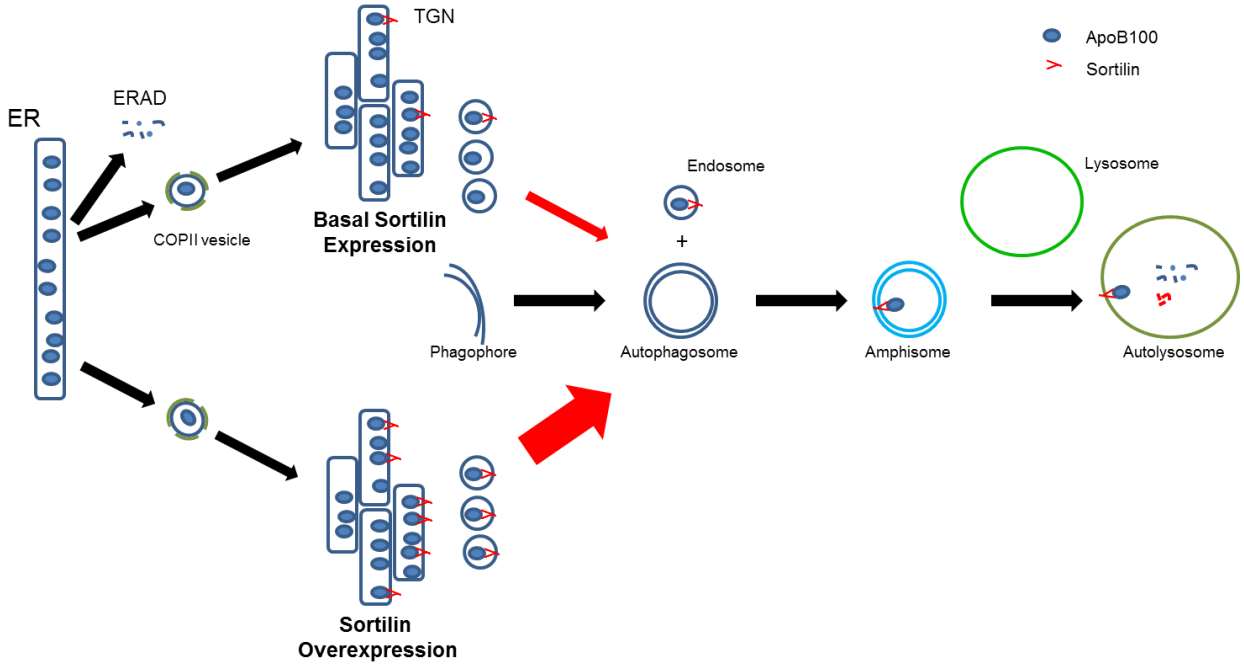
Online Figure VI. *Western blot quantifications from main Figure 5.* Numerical data represent the means ± SEM. *, $p<0.05$ versus total homogenate (H) using two-tailed Student's t-test. (n.d.); not detected.



Online Figure VII. Wild-type McA cells (McA cells) and McA cells overexpressing sortilin (McA-sortilin cells) were exposed to 100 nM of bafilomycin (Bafilo) or vehicle control (DMSO) for 3 hours, fixed and immunostained for sortilin (red). Nuclei were stained with DAPI (blue). White bar, 10 µm.



Online Figure VIII. (A) Age and sex matched *Apobec1*$^{-/-}$ mice (n=6/group) received a single intraperitoneal injection containing adeno-associated vectors (AAV) under control of the liver-specific thyroxine-binding globulin promoter to induce the overexpression of either GFP or sortilin two weeks before tissue harvesting. Immunoblotting for total liver levels of p62 and tubulin are shown. **(C)** Doxycycline (DOX)-inducible HUH7 cells overexpressing GFP (HUH7-GFP) were used to determine autophagic flux. **(C)** LC3 mRNA levels were measured in HUH7-sortilin cells exposed to control medium (no DOX) and DOX-containing medium. P.I.; protease inhibitors. Numerical data represent the means ± SEM. *, $p<0.05$versus control (AAV-GFP Figure B; control medium (no P.I.) Figure D) using two-tailed Student's t-test.



Online Figure IX. *Schematic representation of the two major apoB100 degradatory pathways and the implication of sortilin in the post-ER secretory pathway*. ApoB100 is directed to the trans-Golgi network (TGN) from the endoplasmic reticulum (ER) in COPII vesicles or degraded by the ER-associated degradation (ERAD). Once in the TGN, sortilin binds apoB100 and both are delivered to the autophagosome via an endosome, forming an amphisome. When sortilin is over-expressed, apoB100 will be preferentially directed to the autophagosome (thick red bar) for autophagic degradation. The fusion of the autophagosome and the endosome will form an amphisome, which will further fuse with the lysosome to form an autolysosome, where apoB100 and sortilin are degraded.