Supplemental Experimental Procedures

Animal treatments. Where indicated, mice were starved for 24 or 48 hours with water *ad libitum*. Mice of all genotypes were kept on a standard chow diet (LabDiet #5058) and on a regular 12-hour dark/light cycle, except for the experimental subgroup maintained in a High Fat Diet (Research Diets D12492, 60% kcal% fat) for 16 weeks. Isoprenaline hydrochloride (20mg/kg b.w.; Sigma) was injected intraperitoneally two times within 24 hours and leupeptin (20mg/kg b.w.; Fisher Scientific) was injected intraperitoneally 2 hours before euthanasia and tissue harvesting. Control (Ctr) animals in these studies received saline. Primary hepatocytes were isolated by collagenase perfusion and iso-density percoll centrifugation (Kreamer et al., 1986) by the Marion Bessin Liver Research Center Core Facilities. All genotyping, breeding and treatments in this study were done according to protocol and all animal studies were under an animal study protocol approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

Antibodies. All antibodies were against mouse protein and were diluted at 1:1000 except where specified. The antibodies against the cytosolic tail of rat and mouse LAMP-2A, -B and -C were prepared in our laboratory (Cuervo and Dice, 1996; Zhang and Cuervo, 2008) and the antibodies against total LAMP-2 (clone GL27A) and LAMP-1 (clone 1D4B, 1:2000) were from the Developmental Hybridoma Bank (University of Iowa, Iowa City, Iowa). The antibodies against Actin (1:5000), GAPDH (1:3000), and Cyclophillin A (1:5000) were from Abcam, Cathepsin D (1:500) from Santa Cruz, p62 from Enzo Life Sciences, Mdh1 (1;500), Gpd2, Acadl, Cyp27A1 (1:500) from Novus Biologicals, and LC3, Pyr. Carb, Aldo A, Eno 1, Acsl 1, Akt, pAKT Ser 473, GS, and pGS Ser 641 from Cell Signaling Technology.

Generation of LAMP-2A$^{f/f}$ and liver-specific LAMP-2A knockout mice. A LAMP-2A knockout mouse was generated using a targeting vector that contained a 5kb long homology arm of the *lamp2a gene*, a loxP sequence and an FNFL (Frt-Neo-Frt-Loxp) cassette flanking exon 9A

(508bp: 60885-61392 bp) of the *lamp2* gene and a 2kb short homology arm of the *lamp2* gene. This targeting vector, which also carried the DTA (Diptheria toxin alpha chain) sequence, was electroporated into PTL1 ES cells, selected with G418 and DTA to reduce random integration and then screened for homologous recombinants by PCR and Southern blot analysis. Positive ES clones were injected into C57BL/6 blastocysts to generate chimeric mice. Mice carrying the floxed LAMP-2A allele ($L2A^{f/f}$) were crossed with Albumin-Cre C57BL/6 mice to generate liver-specific LAMP-2A conditional knockout mice (Albumin-Cre-$L2A^{f/f}$, referred throughout this work as L2AKO) (**Fig 1**). All results presented in this study are the mean values of data obtained with mice from two founder lines. Except where indicated, we used 4-6 months old male mice for all studies. Standard biochemical tests, markers of liver damage, functional tests and analysis of weight and metabolic parameters revealed no differences between wild-type mice and Alb-Cre mice, thus we used both interchangeably as control (Ctr). Litter size and sex segregation for Ctr and L2AKO mice were comparable.

Histology and electron microscopy. For immunohistochemistry, deparaffinized and unstained liver sections were processed using standard procedures for epitope retrieval, quenched, and blocked before incubation with the desired primary antibody. We used Image J software (NIH) to quantify the number of particles, size, and cellular area occupied in 15-20 different randomly taken micrographs for each condition after thresholding. Electron microscopy for liver was done after fixation of liver blocks (1 mm^3 in size) with 2% paraformaldehyde and 2% gluteraldehyde in 0.1M sodium cacodylate buffer followed by postfixation staining with 1% osmium tetroxide and 1% uranyl acetate. After dehydratation, resin embedding and ultrathin sectioning samples were viewed on a JEOL 1200EX transmission electron microscope at 80 kV. Morphometric analysis was performed in micrographs using Image J software and classification of autophagic vacuoles was done following the standard criteria (Singh et al., 2009) as follows: autophagosomes were distinguished as double membrane vesicles with content of similar density as the surrounding cytosol and comprised often of recognizable cellular structures;

autophagolysosomes were identified as single or partially double membrane vesicles of content of lower density than the surrounding cytosol and comprised of amorphous content or partially degraded cellular structures. In both cases, the limiting membrane had to be denuded of ribosomal particles.

Metabolic phenotyping. Body weight was measured weekly during the study period. To determine energy expenditure, mice were adapted to individual metabolic chambers for 3 days to acclimate prior to data collection. Metabolic measurements (oxygen consumption, carbon dioxide production, food intake, and locomotor activity) were obtained continuously every 8 minutes using a CLAMS (Columbus Instruments) open-circuit indirect calorimetry system for 8 days in the following sequence: 3 days fed, 2 days starvation, and 3 days refeeding. Mice were weighed and underwent body composition analysis prior to and after their analysis in the metabolic chambers.

Glycemic control and glycogen content. Prior to the glucose tolerance test, mice were fasted for 6 hours and then injected with glucose (1g/kg b.w. i.p. injection). Blood glucose levels were monitored before and 15, 30, 60, and 120 min after injection using blood collected from tail veins and a glucometer (Accu-Chek Compact, Roche). For the insulin sensitivity test, mice were fasted for 4 hours and injected with human insulin (0.75U/kg b.w. i.p. injection). Tail blood was collected before and 15, 30, 60, and 90 minutes after injection and glucose levels were determined as described above. For the pyruvate tolerance test, mice were starved overnight and injected with a bolus of sodium pyruvate (2g/kg b.w. i.p. injection) and tail blood was used to assess glucose levels as described above before and 15, 30, 60, and 90 minutes after injection. For biochemical quantification of hepatic glycogen content (Vaitheesvaran et al., 2010), livers were dissected, weighed, and resuspended in 1ml of 6% perchloric acid. After homogenization, lysates were spun at 1,000g for 20 minutes and the supernatant was combined with 5 volumes of 100% ethanol to precipitate glycogen over 48 hours at 4°C. Pellets were collected, washed in ethanol and dissolved in 0.1M sodium acetate pH 4.5. Amyloglucosidase

was used to hydrolyze glycogen into glucose and glucose levels were determined colorimetrically using the Quantichrom Glucose Assay Kit (Bioassay Systems). Glycogen content was normalized to liver weight.

Quantification of liver and serum lipids. Lipids were extracted from liver homogenates (1mg total protein) with chloroform:methanol (2:1) and the collected organic phase was dried under a stream of nitrogen. For separation and identification of lipid species, dried down lipids were re-dissolved in chloroform and spotted along with lipid standards for various lipid species onto glass silica TLC plates (EMD Biosciences) pre-cleared with chloroform:methanol (1:1) (Gross et al., 2010). Samples were run 1/3 of plate length in chloroform/methanol/acetic acid/water (25:15:4:2), dried and subjected to a second run for the remaining 2/3 plate length in heptane/isopropyl ether/acetic acid (60:40:4). After drying, a third run to 1/3 plate length in ethyl acetate/isooctane/acetic acid (45:15:10) was performed and dried plates were sprayed to saturation with 3% cupric-acetate/8% phosphoric acid in water and allowed to dry overnight at room temperature. Plates were developed by consecutive incubation at 80°C for 3 minutes and at 200°C until all samples were visible. Plates were scanned and band densities were analyzed by Image J software. For analysis of metabolites in serum, blood was collected by retro-orbital bleeding, serum was separated by centrifugation and FFA (HR Series NEFA, Wako Diagnostics), Glycerol (Free Glycerol Reagent, Sigma), TG (Infinity Triglyceride, Thermoscientific), and ketone (β-Hydroxybutyrate LiquiColor® Reagent, Stanbio) levels were determined by colorimetric assays.

Extracellular flux analysis. Using the Seahorse Biosciences extracellular flux analyzer, glycolytic flux (ECAR) and oxygen consumption rates (OCR) were measured in the XF 96-well analyzer for fibroblasts and in the XF 24-well analyzer for primary hepatocytes isolated one day prior to analysis from Ctr and L2AKO mice. MEFs were seeded in the 96-well Seahorse plate at 15,000-30,000 cells per well and either kept in DMEM with 10% FBS or exchanged for serum-depleted media 16h before the experiment. For primary hepatocytes, the 24-well Seahorse plate

was coated with Collagen I and incubated at 37°C for 48h before cell seeding. Hepatocytes were isolated from Ctr or L2AKO livers and resuspended in DMEM with 5% FBS, 1% PSF, and Gentamicin (50µg/ml) and diluted to a final concentration of 300,000 cells/ml. A volume of 100µl (or 30,000 cells) of the cell suspension was added gently to each well, leaving 4 wells blank as controls (with media only). For serum-starvation, cells were washed three times with RPMI media, 1% PSF, and 50µg/ml Gentamicin and incubated overnight at 37°C. Respiratory capacity of isolated mitochondria obtained from Ctr and L2AKO mouse livers was measured following the protocol published by Seahorse Biosciences. After determining protein concentration of the isolated fractions, mitochondria were diluted to a concentration of 5µg/50µl in mitochondria assay solution (MAS) with succinate (10mM). A volume of 50µl of diluted mitochondria was loaded carefully into each well of the Seahorse plate (5µg of mitochondrial protein/well) and the plate was centrifuged for 20 min at 2,000g at 4°C. After the spin, 450µl of MAS with 10mM succinate was added to each well, bringing the final volume up to 500µl and transferred to the XF 24-well analyzer. Oxygen consumption rates of isolated mitochondria were measured to assess the degree of coupling between the electron transport chain (ETC) and oxidative phosphorylation machinery (OXPHOS). State 2 of mitochondrial respiration was measured under basal conditions in the presence of the substrate succinate. State 3 was initiated by addition of ADP (4mM), state 4 with the addition of oligomycin (2.5µg/ml), and state 3µ was induced with FCCP-mediated uncoupling (4µM) to stimulate respiration. The respiratory ratios were calculated as follows: (OCR of state 3)/(OCR of state 4) and (OCR of state 3µ)/(OCR of state 4).

Lipogenic and lipolytic analyses. Lipogenesis was assessed in cultured cells by measuring ^{14}C-glycerol (Perkin Elmer) incorporation into intracellular triglyceride (TG) stores. Briefly, cells were treated with DMEM containing ^{14}C-glycerol (1µCi/ml) and upon collection, cells were washed three times with PBS and lipids were extracted twice with 1ml of isopropanol/hexane (2:3) for 30 min at RT, then dried under a nitrogen stream. Lipids were separated by TLC (Gross

et al., 2010) and radioactive TG was quantified by PhosphorImager analysis and represented as rate of TG production per hour using arbitrary units obtained from ImageJ software after normalizing to protein concentration. To quantify the rates of triglyceride hydrolysis, cells were incubated with 100μM ^{14}C-oleic acid complexed to BSA (7:1 molar ratio) for 18h overnight as previously described (Goldstein et al., 1983). The next morning, cells were washed three times in PBS and chased in the presence of 5μM Triascin C for 1, 3, and 6 hours to inhibit re-esterification and TG biosynthesis (Brasaemle et al., 2000; Parkes et al., 2006). Radioactive TLC plates were scanned and the decline in radiolabeled intracellular TG over time was quantified using ImageJ software. Counts were normalized to total protein from cells extracted in 0.1N NaOH/0.1% SDS. To measure fatty acid β-oxidation rates, cells were labeled with 100μM ^{3}H-palmitate-BSA complex (0.4 μCi/ml) (Goldstein et al., 1983) and after 6 hours, the cell media was collected and centrifuged to remove debris. Subsequently, 100μl of media was extracted with 900μl of 20mM Tris-HCl and 5% (w/v) activated charcoal by rotating for 30 min at RT to absorb ^{3}H-palmitate-BSA and fatty acids. Samples were then centrifuged at 16,000g for 15 min and 200μl of the supernatant fraction (the aqueous phase) was removed to determine fatty acid oxidation by measuring tritiated water ($[^{3}H]_{2}0$) using scintillation counting. Counts were normalized to total protein in cells extracted in 0.1N NaOH/0.1% SDS (Miranda et al., 2011). To measure the rate of hepatic VLDL secretion *in vivo*, mice were fasted overnight to prevent intestinal secretion of chylomicrons. Blood was collected by retro-orbital puncture before and 1, 3, and 5 hours after i.p. injection of Poloxamer-407 (Sigma). Serum was collected and plasma triglyceride was measured as described above.

Zoxazolamine-induced paralysis test. We analyzed the clearance time of zoxazolamine, a muscle relaxant metabolized by the liver, as an index of hepatic function. We gave mice a single i.p. injection of zoxazolamine (150mg/kg) in olive oil, placed them on their backs and recorded the time required to regain the righting reflex after the paralysis induced by this compound (Zhang and Cuervo, 2008).

Quantitative Proteomics and Protein Pathway Analysis. Liver lysosomes active for CMA were isolated from 24 hour-starved Ctr and L2AKO mice treated or not with leupeptin two hours before isolation. Three different sets of lysosomes from three different animals were separately analyzed for purity, integrity, electrophoretic patterning and enrichment in markers of CMA lysosomes by immunoblot. Quantitative proteomics analysis was performed using iTRAQ multiplex (Applied Biomics) in the three animals under the four different conditions: Ctr mice untreated, Ctr mice treated with leupeptin, L2AKO mice untreated and L2AKO mice treated with leupeptin. For each protein hit the average ratio(s) for the protein, the number of peptide ratios that contributed and the geometric standard deviation were determined. Values in the three experimental groups were compared to untreated Ctr and are represented as the average of folds (lysosomes isolated from untreated Ctr mice are given a value of 1). CMA substrate proteins were defined as those for which leupeptin treatment resulted in increase in lysosomal levels >20% and with a reduction in leupeptin response of $\geq$20% in the L2AKO. Validation of the subset of proteins of interest was performed by immunoblotting in lysosomal fractions isolated from independent mice. The protein sets catalogued as CMA substrates from the iTRAQ experiments were analyzed using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems). Each identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base.

Microarray analysis. We extracted mouse liver total RNA using TRIzol (Invitrogen) and purified with RNeasy chromatography (Qiagen) from Ctr and L2AKO mice, fed and 48h starved. cDNA was synthesized and labeled using a Whole Transcript Expression Kit (Ambion) and GeneChip WT Terminal Labeling and Controls Kit (Affymetrix), respectively. Samples were hybridized to Affymetrix mouse 1.0 ST GeneChip arrays (Catalog Number 901171). Data were processed and normalized using the oligo package. Data were normalized using Robust Multiarray Average (RMA) method and a *t*-test was performed. Genes with Benjamini & Hochberg adjusted $p<0.05$ were considered significant and filtered for fold change greater than ±1.1, resulting in

156 upregulated and 90 downregulated genes in Ctr versus KO under fed conditions and 216 upregulated and 35 downregulated during starvation out of the 28944 genes amplified. Raw data and analysis details have been submitted to the NCBI Gene Expression Omnibus database to comply with MIAME guidelines. Gene Ontology terms were performed using GOMiner and IPA. Gene set was filtered to remove genes without entrez or GO annotation, and selects genes with an IQR > 0.5. The full microarray raw data has been deposit in GEO accession number GSE49553.

Quantitative real-time PCR. QT-PCR was done using the TaqMan One-Step RT-PCR Master Mix reagent (Applied Biosystems) with the following primers: 5'AGGTGCTTTCTGTGT CTAGAGCGT3' and 5'AGAATAAGTACTCCTCCCAGAGCTGC3' for LAMP-2A; 5'ATGTGC TGCTGACTCTGACCTCAA3' and 5'TGGAAGCACGAGACTGGCTTGATT3' for LAMP-2B; 5'G GTGCTGGTCTTTCAGGCTTGATT3' and 5'ACCACCCAATCTAAGAGCAGGACT3' for LAMP-2C and 5'TAGTGCCCACATTCAGCATCTCCA3' and 5'TTCCACAGACCCAAACCTGTCACT3' for LAMP-1.

Power analysis and additional statistical analysis. Power analysis was used to determine the number of animals required for each analysis based on the average values of enrichment and recovery for the specific fraction using endogenous markers for each compartment (for the isolated fractions), the previous differences that we have found when manipulating CMA in the in vitro system (for the analysis of CMA components/activity), and our observations on animals subjected to similar diets and data obtained by the Animal Physiology Core in this mouse strain (for metabolic studies). With the calculated sample size and a two-sided Type 1 error rate of 5% it was predicted >80% power to detect effects >1.5 in the parameters analyzed. None of the animals were excluded from the study and randomization was not required as breeding were set to have in all litters Ctr and experimental animals allowing for comparisons among littermates. Blinding was used whenever pooling of animals was not required.

Supplemental References

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