Supporting Information

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SI Materials and Methods

Reagents. Antibodies used in the present studies included monoclonal antibodies to prohibitin and calnexin, which were purchased from Santa Cruz Biotechnology. Monoclonal antibodies to MFN1, MFN2, and OPA1 were from Abcam. Donkey anti-Rabbit and donkey anti mouse IgG horseradish peroxidase-conjugated antibodies were purchased from GE Healthcare. Donkey anti-mouse and donkey anti-rabbit FITC-conjugated antibodies were from Santa Cruz Biotechnology. Rabbit anti-VDAC1 monoclonal antibody was from Cell Signaling. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), rotenone, antimycin, oligomycin, and DPI sulfate were purchased from Invitrogen.

Animals Care. Male and female C57BL/6J mice, 4–6 wk of age, were purchased from Jackson Laboratory. All animals were maintained in an environmentally controlled facility with diurnal light cycle and free access to water and either a standard rodent chow (Harlan Teklad 2018) or a high-fat diet from Research Diets (catalog no. D12492). All experiments involving animals were performed in compliance with approved institutional animal care and use protocols according to National Institutes of Health guidelines (1).

Immunofluorescence Confocal Microscopy and Image Analysis. For immunofluorescence staining, the cells were fixed in 4% (vol/vol) paraformaldehyde for 10 min, washed twice with PBS, and then permeabilized with 0.1% Triton X-100 for 10 min. Fixed cells were preincubated for 30 min in PBS containing 5% (wt/vol) BSA at room temperature. Cells were stained with primary antibody (anti-Myc monoclonal antibody; 1:500 dilution) for 3 h at room temperature, followed by incubation with secondary antibody conjugated with FITC (1:1,000 dilution; Santa Cruz Biotechnology). For mitochondrial staining, cells were stained with MitoTracker Red (final concentration, 100 nM) for 5 min in 37 °C incubator, washed with PBS three times, 5 min each wash. The cells were then analyzed under confocal microscopy (Leica TCS SP2 AOBS) equipped with a bipolar temperature controller. Images were processed using Adobe Photoshop 7.0, and quantitative analyses were performed using ImageJ (National Institutes of Health). All experiments were performed at least three times with similar results.

EM Analysis. Cells were fixed in 4% (vol/vol) paraformaldehyde and 5% (vol/vol) glutaraldehyde, stained sequentially in 2% (wt/vol) OsO_4 and 1% uranyl acetate, dehydrated by a series of ethanol washes and embedded in Embed-812 resin for sectioning and analysis. Samples were analyzed with the use of a JEOL 1200EX transmission electron microscope.

mtDNA Mutation Assays. For mtDNA isolation, MEF and C2C12 cells were homogenized and a mitochondrial fraction was isolated as previously described (2). Mitochondria were then lysed in the presence of 0.5% SDS and 0.2 mg/mL proteinase K in 10 mM Tris-HCl, 0.15 M NaCl, and 0.005 M EDTA (3). mtDNA was purified by phenol/chloroform extraction and ethanol precipitation. The random mutation capture assays were performed as previously described (4). Briefly, mtDNA was digested with TaqI for 5h and then diluted in a 96 well format and probed with primers flanking the TaqI restriction site to detect mtDNA genomes that contained a mutation in the TaqI restriction site. A control pair of primers was used to detect the amount of mtDNA genomes that was interrogated. PCR was carried out in 20 μL reactions using the ABI StepOnePlus and 95 °C SYBRGREEN PCR Master Mix. Quantitative PCR amplification was carried out using the following programs: step 1, 95 °C for 10 min; step 2, 95 °C for 15 s; step 3, 60 °C for 1 min; step 4, repeat step 2 and step 3 forty times; and step 5, melt curve from 65 °C to 95 °C.

Mitochondrial Fusion Assay. C2C12 cells stably expressing ACLAT1 or vector control were seeded with 5×10^5 per 6 cm^2 plate and transfected with mitochondria-targeted green fluorescent protein (mtEGFP) or with mitochondria-targeted dsRED2 (mtDsred2), respectively. After 30 h, individual pools of cells, respectively, expressing mtEGFP and mtDsRed2 were mixed and coplated at a 1:1 ratio onto 13-mm round coverslips. Fusion was then induced after 6 h by a 60-s treatment with a 50% (wt/vol) solution of PEG 1500 in PBS (Sigma), followed by extensive washes in DMEM supplemented with 10% (vol/vol) FCS. To inhibit protein synthesis, cycloheximide (20 μg/mL) was added 30 min before fusion and kept in all solutions and cell culture medium used subsequently until cells were fixed for 10 min with ice-cold 4% (vol/vol) formaldehyde in PBS. After three washes with PBS, coverslips were mounted on slides and kept in a dark box at 4 °C overnight.

MEF Preparation. The female mice at embryonic day 12 were euthanized. Embryos were dissected out on a dish with HBSS. Head, limbs, and internal organs were removed. The remaining embryo was minced with a scalpel or razor blade and transferred to a 15-mL tube with 4 mL of collagenase solution (2 mg/mL collagenase IV, 0.7 mg/mL DNase I and 10 mg/mL hyaluronidase); 4 mL of collagenase solution was also used to wash the dish to get all the cells. Tubes were rotated for 30–60 min in a 37 °C incubator until all of the tissue chunks were gone. The digested solution was filtered through 100-μm mesh to 50-mL tubes filled with chilled DMEM (30 mL). The samples were centrifuged at 9,000 × *g* for 5 min, and the cell pellet was washed again with 25 mL of DMEM. Then, cells were then seeded with complete medium.

XF24 Bioenergetic Assay. OCR was measured using the Seahorse XF24 analyzer (Seahorse Bioscience), as described previously (2). After equilibration, the test reagent were preloaded in the reagent delivery chambers of the O_2 sensor cartridge and injected into the wells after the XF respirometry read the basal OCR. OCRs (pmol/min) were obtained. After the baseline measurement, 70 μL of a testing agent prepared in assay medium was then injected into each well to reach the desired final concentration. This was followed by mixing for 2 min to expedite compound exposure to cellular proteins, and OCR measurements were then carried out.

ROS Measurements. Mitochondria were isolated from C2C12 cells stably expressing vector and ALCAT1. The intracellular ROS generated from the isolated mitochondria was analyzed by measuring hydrogen peroxide (H_2O_2) with Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) in 100 μL of reaction mixture, which contained 100 μM Amplex Red, 0.5 U/mL HRP, 0.3% BSA (fatty acid free), 121 mM KCl, 1.9 mM KH_2PO_4, 1.0 mM EGTA, 19.4 mM Tris-HCl, and 50 μM succinate as fuel source. In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometry to produce the red fluorescent oxidation product resorufin, which can be measured at excitation and emission wavelengths of 545 and 590

nm, respectively. The assay was calibrated with known H_2O_2 concentrations. Amplex Red conversion to resorufin was monitored at indicated time points using a Molecular Devices SpectraMaxM5/M5e microplate reader controlled by PC SoftMax Pro-5 software.

TBARS Assay. The lipid peroxidation was analyzed by the thiobarbituric acid reactive substance method according to the instruction from TBARS Assay Kit (Cayman). Cells (5×10^7) were collected in 0.8 mL of PBS and sonicated three times for 5-s intervals at 40 V setting over ice. Then, the mixture was centrifuged at 300 × *g* for 10 s, and the protein concentration was tested with Pierce BCA Protein Assay kit. Supernatant (100 μL) was thoroughly mixed with a stock solution containing 100 μL of SDS, 5.3 mg of thiobarbituric acid, 2 μL of thiobarbituric acid acetic acid, and 2 μL of thiobarbituric acid sodium hydroxide. This mixture was heated at 100 °C for 1 h, and then the reaction was stopped by placing the vials on ice for 10 min. The vials were centrifuged at 1,600 × *g* for 10 min at 4 °C, and the clear supernatant was collected and loaded to wells (200 μL/well) in a 96-well flat bottom plate. Sample absorbance at 532 nm was read on a microplate reader. MDA concentration was calculated from a standard curve and normalized as micromoles of MDA per microgram of protein. This assay was triplicated for each sample.

Statistical Analysis. Statistical comparisons were performed using two-tailed nonpaired *t* tests to determine the difference between the two C2C12 cell lines and between ALCAT1$^{-/-}$ and WT mice. Data are expressed as means ± SEM. *P* less than 0.05 was considered statistically significant.

1. National Institutes of Health (1985) *Guide for the Care and Use of Laboratory Animals*. (National Institutes of Health, Bethesda, MD) publication no. 86–23.
2. Li J, et al. (2010) Cardiolipin remodeling by ALCAT1 links oxidative stress and mitochondrial dysfunction to obesity. *Cell Metab* 12:154–165.
3. Vermulst M, Bielas JH, Loeb LA (2008) Quantification of random mutations in the mitochondrial genome. *Methods* 46:263–268.
4. Chen H, et al. (2010) Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell* 141:280–289.

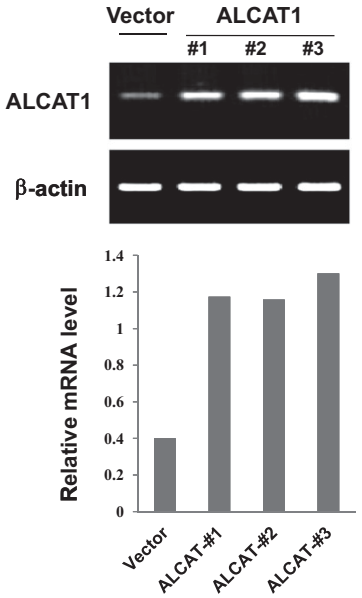

Fig. S1. RT-PCR analysis of ALCAT1 mRNA expression level in C2C12 cells stably expressing ALCAT1. C2C12 cells stably transfected with ALCAT1 expression vector or an empty vector were selected for individual clones. The C2C12 cell lines stably expressing ALCAT1 or vector control were analyzed for ALCAT1 mRNA expression by RT-PCR analysis.

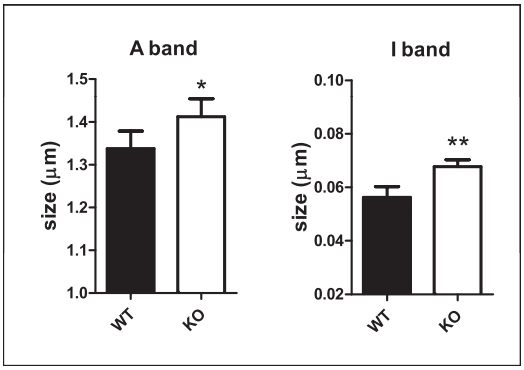

Fig. S2. Quantification of A and I band from isolated skeletal muscle from ALCAT1 KO mice and WT controls. EM analysis were carried out to examine mitochondrial morphology and fiber structure of isolated skeletal muscle from KO and WT mice as shown in Fig. 2 *E* and *F*. Quantification of A and I band, which represent myosin filaments and actin filaments respectively, was carried out by measuring width of individual band from several sections of the EM picture ($n = 5$). *$P < 0.05$, **$P < 0.01$.

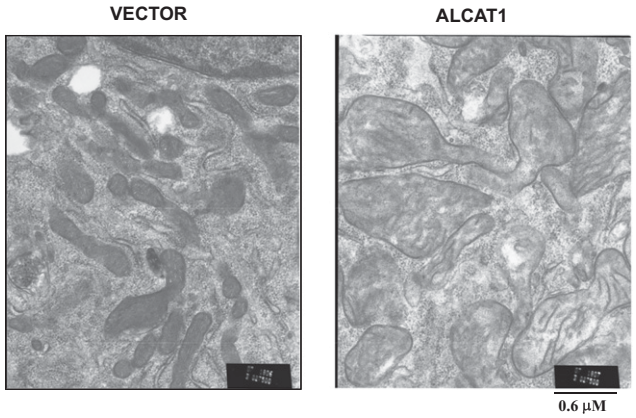

Fig. S3. Overexpression of ALCAT1 in C2C12 cells caused mitochondrial swelling. C2C12 cells stably transfected with ALCAT1 expression vector or an empty vector were selected for individual clones. The C2C12 cell lines stably expressing ALCAT1 or vector control were analyzed for mitochondrial morphology by electron microscopy. ALCAT1 overexpression caused severe mitochondrial swelling, as evidenced by enlarged mitochondria and damaged cristae.

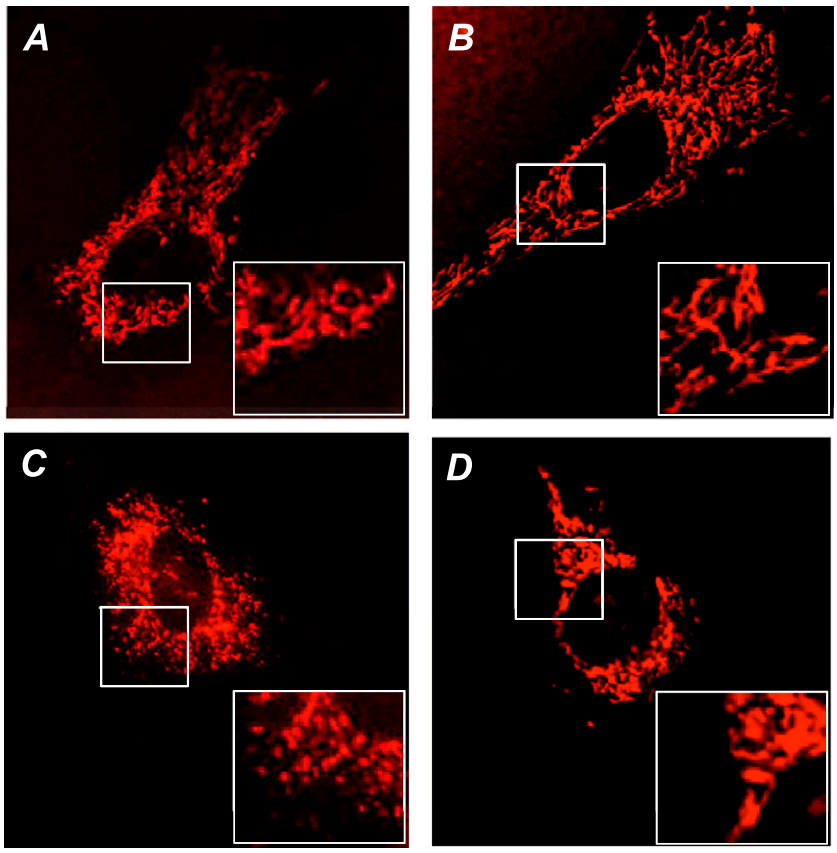

Fig. S4. ALCAT1 deficiency prevented mitochondrial fragmentation in response to oxidative stress in MEFs. Isolated MEFs from ALCAT1 KO mice (*B* and *D*) and the WT control mice (*A* and C) were cultured in the absence (*A* and *B*) or presence (*C* and *D*) of 0.5 mM H_2O_2 for 2 h, followed by analysis of mitochondrial network by staining with MitoTracker Red. In contrast to vector control, ALCAT1 deficiency prevented mitochondrial fragmentation in response to oxidative stress.

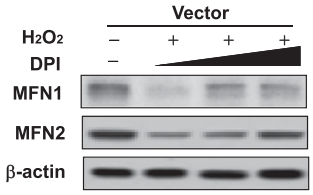

Fig. S5. Antioxidant treatment prevents MFN depletion in C2C12 cells. C2C12 cells stably expressing the vector control were cultured with increasing dose (0, 1, and 5 μM) of DPI for 1 h and then treated with 1.5 mM H_2O_2 for 1 h. The cells were then harvested and analyzed for the expression of MFN1 and MFN2 by Western blot analysis using.

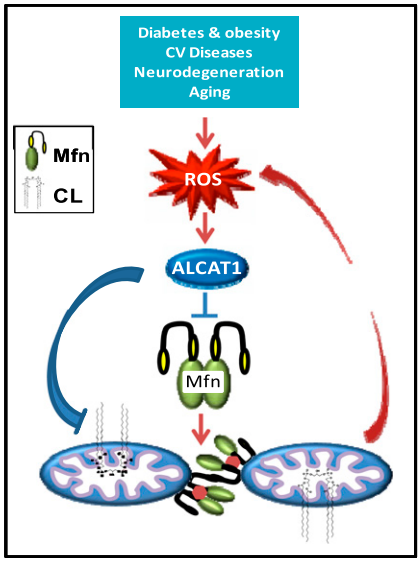

Fig. S6. Schematic model depicting a role of ALCAT1 in mitochondrial fragmentation. Accordingly, ALCAT1 expression is up-regulated by oxidative stress associated with aging and age-related diseases. Cardiolipin remodeling by ALCAT1 produces aberrant cardiolipin species commonly found in age-related diseases, such as enrichment of DHA content in cardiolipin, leading cardiolipin peroxidation, which causes depletion of MFN expression. MFN deficiency causes mitochondrial fragmentation, further exacerbating ROS production and mitochondrial dysfunction.