ONLINE APPENDIX

Animal studies

All investigations conformed to the NIH Guide for the Care and Use of Laboratory Animals and are in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of California, San Diego. Type 2 diabetes (T2D) was induced in 12 week old C57BL/6 mice with a high fat diet (HFD, 60% calories from fat) (Envigo) and a single STZ injection of 75mg/kg (1). Control groups were maintained on regular lab chow. The progression of T2D was monitored over the course of 6 months by measuring glucose levels in fasting and glucose challenge conditions, fed-state insulin, glycated hemoglobin, cholesterol, HDL, LDL, and triglyceride levels. Cholesterol, HDL, LDL, and triglyceride levels were measured by the UCSD Mouse Phenotyping Service using standard procedures. Insulin and HbA1c levels were measured in plasma and blood, respectively, using commercially available mouse-specific kits (Crystal Chem). Glucose tolerance test (GTT) was conducted at 1, 3, and 6 months post-STZ/HFD and insulin tolerance test (ITT) was performed 6 months post-STZ/HFD. For GTT, mice were IP injected with 10% glucose (10 µl/g) after overnight fasting, and blood was drawn to measure glucose levels at 0, 30, 60, and 120 minutes after glucose injection. For ITT, 0.5 units/kg of Humulin R insulin was IP injected after 4 hours of fasting, and blood was drawn at 0, 15, 30, 60, 90, and 120 minutes after insulin injection. A time-course metabolic characterization of the T2D model is reported in Supplemental Table 1

Experiments took into account only male mice since female rodents are generally known to display decreased sensitivity to STZ due to estradiol's ability to protect pancreatic β cells from apoptosis induced by oxidative stress (2).

Adenovirus and adeno-associated virus (AAV) vectors

A complete list of primers used to clone the recombinant proteins used in this study is available in Supplemental Table 2. MCUb was cloned from mouse heart and subsequently a

dominant negative MCUb was generated by introducing two point mutations (CTG>CAG and TGT>AGT) resulting in W246R and V251E using the QuikChange Site-Directed Mutangenesis kit (Stratagene). MCUb WT and MCUb$^{W256R/V251E}$ were then cloned into pENTR1A using the In-Fusion HD Cloning Kit (TakaraBio) according to the manufacturer's instructions. Adenoviruses were generated using the ViraPower Adenoviral Gateway expression kit (Invitrogen). MCUb$^{W246R/V251E}$ was also cloned into the pAAV shuttle vector which was used by the University of California, San Diego Vector Development Core to generate MCUb$^{W246R/V251E}$ encoding, liver-detargeted, double-stranded AAV serotype 9.45 particles (3). The sequence of each vector was confirmed by restriction enzyme analysis as well as DNA sequencing at every cloning step. Adenovirus and AAV encoding the mitochondria-targeted, circularly-permuted green fluorescent proteins inverse Pericam (Mitycam) and MCU were already available and their generation has been reported (4).

Isolation and culture of neonatal cardiomyocytes

Mouse neonatal cardiomyocytes (NCM) were isolated as already described (4) using the Worthington Neonatal Cardiomyocyte Isolation System (Worthington Biochemical, Lakewood, NJ) following the manufacturer's instructions. Cells were plated onto gelatin-coated culture dishes in culture medium consisting of 4:1 Dulbecco's modified Eagle's medium (DMEM)-M199, 10% horse serum, 5% fetal bovine serum, and 1% penicillin-streptomycin-amphotericin B (Fungizone). Cells were allowed to adhere and establish for 72 h before changing to basic experimental culture medium (4.5:1 DMEM-M199, 2% fetal bovine serum, and 1% penicillin-streptomycin-amphotericin B) supplemented with glucose at physiological concentration (5.5 mM) for 24 h. Cells were transduced for 48h with Adv-MCU (MOI=20). Adv-MCUb (MOI=100), or Adv-dnMCUb (MOI=20).

Isolation and culture of adult cardiomyocytes

Adult cardiomyocytes were isolated from mouse hearts as described previously (5). Isolated cardiomyocytes were plated in 4.5 g/l glucose DMEM supplemented with blebbistatin (5 µg/ml) on laminin and adenoviral or siRNA/Lipofectamine® RNAiMAX (Invitrogen) pre-coated

surfaces, and cultured for 24 h. Ncor2 silencing was performed using an Ncor2-specific Silencer™ siRNA (Ambion, #4390771). Pharmacological inhibition studies using CAMKII (KN-93, Sigma), PKA (H89, BIOLOG Life Science Institute), and PKG (RP8-PET, BIOLOG Life Science Institute) were initiated 24 h after plating, and following plating medium removal and substitution with fresh DMEM containing 10% FBS, 1% antibiotics, and 100 μM kinases inhibitors.

***In vitro* sgRNA transcription and purification**

A 20 bp sequence targeting the murine MCUb promoter (TGTCGCCGCCCCAGTCGGCG) was identified using the "ATUM gRNA Design Tool" and was inserted into the middle of a 58-bp primer behind a T7 promoter sequence (sequence available in Supplemental Table 2). The custom primer was then used with a reverse template (sequence available in Supplemental Table 2) in a DNA polymerase extension reaction to generate a dsDNA template. The dsDNA template was used with the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) to generate ssRNA ~100 bases in length. The reaction was DNase-treated, and full-length RNA was purified by isolating the correct length after running on a denaturing 1% agarose gel with 1% v/v bleach (6). A negative control sgRNA targeting the first exon of the human MCU gene (CAGGAGCGATCTACCTGCGG) (7) was similarly prepared.

Expression and purification of recombinant 7XHis-dCas9-3×FLAG fusion protein.

pX330-CIB1-dCas9 (Addgene) was used as a template to clone a 7XHis-dCas9-3×FLAG fusion protein into pQTEV. Recombinant 7XHis-dCas9-3XFLAG was expressed and purified from *E. coli* BL21 Rosetta (DE3), as similarly reported by Cividini and colleagues (8). Rosetta cells carrying pQTEV-7XHis-dCas9-3XFLAG were cultured at 37°C until A600 reached ≈0.4. Recombinant expression was induced by incubating the bacterial culture with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (Genesee Scientific, Inc.) at RT overnight. The next day, bacterial cells were harvested and lysed by sonication in 50 mM Tris-HCl (pH 7.4). Cleared lysate was supplemented with 200 mM NaCl and incubated for 2 h with nickel-nitrilotriacetic acid-agarose resin (Qiagen) pre-equilibrated in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl with constant mixing.

Beads were then washed with 50 mM Tris-HCl (pH 7.4), 200 mM NaCl supplemented with 5 mM imidazole until A280 ≈0.1. An imidazole gradient (5–500 mM) was then applied, and fractions where collected. Fractions that exhibited dCas9 expression by SDS-PAGE and Coomassie staining were pooled and loaded onto a G-50 resin column to remove excess imidazole. Eluted fractions were then concentrated using a SpeedVac concentrator SVC200H (Savant) and pooled, and protein concentration was determined for further experiments.

MCUb gene promoter pull-down

The MCUb gene promoter was pulled-down using an approach similar to the one described by Tsui and colleagues (9). Cardiomyocytes were isolated from 6 control, and 6 T2D mouse hearts and fixed for 30 min with 2 mM DSG-PBS and subsequently with 1% PFA-PBS for 15 min at RT. The fixed cardiomyocyte preparations from each group were then pooled together, lysed in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.1% Na-Deoxycholate, 0.5% N-lauryl sarcosine, 1x protease and phosphatase inhibitors (Bio-Rad), and cell lysates were sonicated at ≈13W on ice to fragment chromatin until 0.1 - 1 kb fragment enrichment was achieved (10 cycles of 10 sec on, 30 sec off). Cardiomyocyte lysates were then quickly centrifuged to get rid of residual cell debris, and protein content was quantified according to standard procedures. Equal amounts of lysed cardiomyocyte preparations were then combined with pre-formed sgRNA-dCas9 ribonucleoprotein complexes (15 µg dCas9 were combined with 75 µg sgRNA overnight at 4˚C under continuous rotation in 50 mM Tris-HCl pH7.4 supplemented with 1 mM DTT) and incubated overnight at 4˚C under continuous rotation. The following day 50 µl of anti-FLAG M2 Affinity Agarose Gel (Sigma) which had been pre-equilibrated in lysis buffer was added to the mixtures and samples were kept at 4˚C overnight. FLAG-tagged dCas9, and co-precipitated genomic DNA fragments and proteins, were eluted from the anti-FLAG M2-conjugated agarose beads after washing them 5 times with lysis buffer, and elution was performed with 0.2 mg/ml FLAG peptide. One-third of the total eluted volume was then digested for 2h at 55˚C with 1 µg/ml protease (Takara Bio), and genomic DNA fragments were subsequently isolated using the DNA

Clean and Concentrator kit (Zymo Research) and used for RT-qPCR analysis of MCUb promoter fragments. The remaining two-thirds of the total eluted volume was digested with trypsin and underwent LC-MS/MS analysis.

LC-MS/MS analysis

dCas9 co-precipitated proteins and lysed cardiomyocyte preparations were trypsin-digested and analyzed by ultra-high pressure liquid chromatography (UHPLC) coupled with tandem mass spectroscopy (LC-MS/MS) using nano-spray ionization (10). Phospho-peptides were enriched by passing the digested cardiomyocytes preparations over TiO_2 (High-Select™ TiO_2 Phosphopeptide Enrichment Kit, ThermoFisher) and metal-based resins (IMAC) (High-Select™ Fe-NTA Phosphopeptide Enrichment Kit, ThermoFisher) as described by manufacturer protocol. Those peptides not retained by TiO_2 and IMAC were pooled and analyzed, as follows, and were used to describe the cardiomyocyte proteome. The nanospray ionization experiments were performed using an Orbitrap fusion Lumos hybrid mass spectrometer (Thermo) interfaced with nano-scale reversed-phase UHPLC (Thermo Dionex UltiMate™ 3000 RSLC Nano System) using a 25 cm long, 75 μm ID glass capillary packed with 1.7 μm C18 (130) BEHTM beads (Waters corporation). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of ACN (Acetonitrile) at a flow rate of 375 μl/min for 2 h. The buffers used to create the ACN gradient were: Buffer A (98% H_2O, 2% ACN, 0.1% formic acid) and Buffer B (100% ACN, 0.1% formic acid). Mass spectrometer parameters are as follows: an MS1 survey scan using the orbitrap detector (mass range (m/z): 400-1500 (using quadrupole isolation), 120000 resolution setting, spray voltage of 2200 V, Ion transfer tube temperature of 275 C, AGC target of 400000, and maximum injection time of 50 ms) was followed by data dependent scans top speed for most intense ions, with charge state set to only include +2-5 ions, and 5 second exclusion time, while selecting ions with minimal intensities of 50000 at in which the collision event was carried out in the high energy collision cell (HCD Collision Energy of 30%), and the fragment masses were analyzed in the ion trap mass analyzer (with ion trap scan rate of turbo, first mass

m/z was 100, AGC Target 5000 and maximum injection time of 35ms). Protein identification and label free quantification was carried out using Peaks Studio 8.5 (Bioinformatics solutions Inc.). A complete list of proteins showing significant differences between CTR and T2D (p-values ≤ 0.05) is reported in Supplemental Spreadsheet 1. The proteomics, phospho-proteomics and dCas9 pull-down experiments reported in this paper are deposited into the Proteome Xchange and PRIDE repositories: Project accessions: PXD021282 and PXD021284.

Phosphoproteomics analysis

Phosphopeptides were identified as those retained by the TiO_2 and IMAC resin and displaying at least one phosphate residue (mol mass = +79.97) per peptide. Only phospho-peptides that were detected in every sample for each biological group were considered. The quantification of every phosphopeptide was normalized over the amount of protein detected in LC-MS/MS analysis of peptides that were not retained by the IMAC resin. Every phosphopeptide that was detected multiple times or had the same phosphorylated residue was averaged. Normalized phosphopeptides were averaged between biological groups. Protein ontology analysis was conducted using Metascape (11). An analysis of the most abundant serine-centered-phospho-motifs in cardiomyocytes treated with AAV-dnMCUb was performed using the probability logo generator from the Schwartz Lab (12). A complete list of proteins with phosphopeptides showing ≥ 2-fold differences between T2D+AAV-dnMCUb and T2D+AAV-ctr groups is reported in Supplemental Spreadsheet 1.

Analysis of proteins co-precipitated with dCas9

Identification of subcellular compartmentalization and biological processes of proteins whose peptides were identified by LC-MS/MS analysis was conducted after *in vitro* MCUb pull-down with sgRNA-MCUb/dCas9 from CTR and T2D hearts using uniprot.org (13). Supplemental Spreadsheet 1 lists all proteins that were found to have co-precipitated with dCas9. These proteins are organized by molecular function, specific biological function, and broad functional categories.

RNA-seq

Mouse hearts were lysed in Trizol (Thermo) and RNA was extracted by adding chloroform. RNA was then precipitated from the aqueous phase using isopropyl alcohol, washed with 70% ethanol and resuspended in diethyl pyrocarbonate (DEPC)-treated ddH_2O before RNA quality was assessed with the Agilent TapeStation system by the Institute for Genomic Medicine (IGM) at University of California, San Diego. Strand-specific total RNA-seq libraries from ribosomal RNA-depleted RNA were prepared using the TruSeq Stranded Total RNA Library kit (Illumina) according to the manufacturer-supplied protocol. Completed libraries were sequenced 75 bp on an Illumina NextSeq 500 instrument to a depth of approximately 30 million reads per sample. Sequencing reads were aligned to the GRCm38/mm10 version of the mouse genome using STAR with default parameters (14). Only reads that aligned uniquely to the genome (MAPQ > 10) were kept for downstream analysis. Gene expression levels were calculated by counting reads that overlapped with GENCODE-defined exons in a strand specific manner, using HOMER's command analyzeRepeats.pl (15), allowing reads to be assigned to more than one transcript if the exons overlap. Raw, unnormalized read counts for genes in each sample were then analyzed using DESeq2 to identify significantly regulated genes (15). Functional gene enrichment and pathway analysis was performed using Metascape (11). Supplemental Spreadsheet 1 lists gene names for all genes with transcripts showing significant differences (Adj. p-value ≤ 0.05) between groups. The accession number for the RNA-seq experiment reported in this paper is NCBI GEO: GSE145294.

Chromatin immunoprecipitation of isolated adult cardiomyocytes (ChIP)

Isolated adult cardiomyocytes were fixed, lysed, and sheared as described already in previous sections. Anti-Ncor2, PPARγ, PPARα, or normal anti-rabbit/-mouse IgG control (5 µg each) was added to equal amounts of lysed and sheared pooled cardiomyocyte preparations, and samples were kept under constant rotation at 4ºC overnight. The following day samples were incubated with 20 µl of Pierce™ Protein A/G Magnetic Beads (ThermoFisher) for 2-4 h at 4ºC

under constant rotation, before beads were washed twice with low-salt wash buffer (20 mM Tris HCl pH 8.0, 0.2 mM EDTA, 150 mM NaCl, 0.1% DSD, 1% Triton X-100), twice with high-salt wash buffer (20 mM Tris-HCl pH 8.0, 0.2 mM EDTA, 500 mM NaCl, 0.1% DSD, 1% Triton X-100) and once with LiCl wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% IGEPAL CA630, 1% deoxycholate) and TE buffer. Elution was performed in 200 mM NaCl, 1% SDS, 300 µg/ml Proteinase K (Takara Bio) for 2 h at 55ºC followed by 65ºC overnight for de-crosslinking. Genomic DNA fragments were subsequently isolated using the DNA Clean and Concentrator kit (Zymo Research). qPCR analysis of MCUb promoter fragments was carried out using two sets of primers amplifying ≈150 bp long fragments positioned at promoter positions -150 and -1000. Averaged Ct values for Ncor2/PPARγ/PPARα/IgG were normalized over the Ct values for the input samples (1% lysed and sheared preparations prior withheld prior IP), and Ct values relative to Ncor2, PPARγ, or PPARα IP were compared with Ct values relative to IgG control IP.

Mitochondrial glucose and fatty acid oxidation

Glucose and fatty acid metabolism were measured in isolated working hearts as described previously (5, 17). Briefly, hearts were perfused with a modified Krebs-Henseleit Buffer containing 11 mM glucose and 0.8 mM palmitate bound to 3% bovine serum albumin (fatty acid-free). [5-^{3}H]glucose was used to measure glucose oxidation and [9,10-^{3}H]palmitate was used to measure fatty acid oxidation.

Mitochondrial ATP production

Mitochondrial ATP production was inferred from monitoring the linear increase of D-luciferin bioluminescence when luciferase was supplied with ATP from mitochondria. 50 µg of isolated mitochondria were resuspended in 5 mM Tricine pH 7.8, 5 mM $MgSO_4$, 0.1 mM EDTA, 1 mM DTT, 0.15 mg/ml D-luciferin, and 1 µg/ml recombinant firefly luciferase (Molecular probes), in the presence of 5 mM succinic acid, 5 mM glutamic acid, 5 mM malic acid, and 5 mM ADP. Luminescence was recorded for 10 min on a Biotek Synergy 2 multimode plate reader, taking readings every minute at 590/530 nm. As negative technical controls, the assay was conducted

in the absence of either ADP or mitochondria, or in the presence of 70 µM oligomycin, an ATP synthase inhibitor.

SERCA2a activity assay

Sarcoplasmic reticulum (SR) calcium uptake was assessed similarly as reported by Bidwell and Kranias (18). Mouse hearts or NCM were homogenized in 15 volumes of ice-cold homogenization buffer (25 mM Imidazole, pH 7.0). 0.35 ml homogenates were then combined with 2.8 ml uptake buffer (40 mM Imidazole, pH 7.0; 100 mM KCl; 5 mM K-Oxalate; 10 mM Na-Azide; 4.5 mM $MgCl_2$; 200 mM Ca^{2+}; and $^{45}Ca^{2+}$ (250 cpm/µl) (Perkin Elmer)). Reactions were started by adding 0.35 ml of 30 mM ATP in 25 mM Imidazole pH 7.0. Duplicate samples of 0.5 ml were filtered at defined time intervals through 25 mm diameter, 0.45 µm pore nitrocellulose filters using the Millipore 1225 sampling manifold. Filters were washed twice with uptake buffer and dissolved in 0.5 ml acetone before adding 5 ml of EcoLite(+) Liquid Scintillation Cocktail (MP Biomedicals) and cpm were counted using a Beckman Coulter LS6500 Liquid Scintillation Counter. Values were expressed as nmol Ca uptake/min/mg SR protein.

qPCR

qPCR analysis of endogenous cardiac mRNAs was conducted according to standard procedures. RNA was isolated as described in the RNA-seq method. cDNA synthesis was performed using the SuperScript™ III First-Strand Synthesis System (ThermoFisher) and poly(A)+-selected RNA primed with oligo(dT) was retro-transcribed. An Applied Biosystems QuantStudio 3 Real-Time PCR System, and PowerUp SYBR Green Master Mix (Applied Biosystems) were used. Relative quantification was calculated using the ΔΔCT method with mouse ribosomal 16S rRNA or RPL32 as housekeeping genes. Analysis of genomic DNA in dCas9 pull-down experiments was conducted using the same qPCR equipment and master mix reagent. Relative quantification of the MCUb gene promoter fragment was calculated using the ΔΔCT method, referring to the amount of MCUb gene promoter pulled-down by a non-specific

sgRNA-dCas9 ribonucleoprotein complex (dCas9+human MCU sgRNA). A complete list of primers used in qPCR studies is reported in Supplemental Table 2.

PLA assay in isolated cardiomyocytes

Isolated cardiomyocytes from T2D hearts were transduced with Adv-MCU-FLAG, Adv-MCUb-FLAG, or Adv-dnMCUb-FLAG vectors as described above for 24 h and subsequently a proximity ligation assay was carried out similarly as reported by Fernandez-Sanz and colleagues (19). Cardiomyocytes were fixed (99.6% acetone cooled at −20°C, 5 min), permeabilized (0.025% Triton X-100 in phosphate buffered saline (PBS)), and incubated at 4°C overnight while being simultaneously labeled with anti-FLAG and either anti-MCU, -EMRE, -MICU1, or -MICU2 antibodies in PBS/1% BSA (primary antibody dilutions are reported in Supplemental Table 3). The following day samples were washed 3x (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween20) and then incubated with the corresponding secondary antibodies (Alexa anti-mouse-561 and Alexa anti-rabbit-488) conjugated with the PLA oligonucleotide probes (PLA probe anti-rabbit PLUS, and PLA probe anti-rat MINUS; Olink Bioscience) for 1 h at 37°C. For ligation and amplification reactions, Duolink *in situ* detection kit recommendations were followed (Olink Bioscience). Samples were then stained with Hoechst 33342 nuclear dye (Invitrogen) and coverslips were mounted with ProLong Diamond Antifade Mountant (ThermoFisher Scientific) on slides. Images were captured with a DeltaVision deconvolution microscope system (Applied Precision) using a 60x objective at the University of California, San Diego, School of Medicine Light Microscopy Facility. At least 5 fields/biological sample were imaged, and ≈20 serial optical sections, spaced by 0.2 µm, were acquired. The datasets were deconvolved using SoftWorx software (Applied Precision) on a Silicon Graphics Octane work station.

Co-immunoprecipitations

Cardiomyocytes were isolated from hearts as described above and immediately fixed for 15 min at RT with 4% PFA/PBS. Fixed cells were then washed 2x with PBS and resuspended in RIPA buffer supplemented with 1x protease and phosphatase inhibitor cocktail (Halt,

ThermoFisher Scientific). Samples were briefly sonicated, lysates were cleared by brief high-speed (10,000 rpm) centrifugation, and anti-MCUb immunoprecipitation was performed using Protein A/G Plus-Agarose beads (Santa Cruz) following the manufacturer's recommendations. Immunoblot analysis of MCUb, MCU, MICU1, and MICU2 in the MCUb immunoprecipitated samples was performed as described below in the "Western blot analysis" paragraph.

Western blot analysis

Protein levels were assessed by western blot analysis of 50 – 100 µg as previously described (5,8). A complete list of antibodies and the details of their usage can be found in Supplemental Table 3. Images were acquired with the ChemiDoc MP System (Bio-Rad). Band density was quantified with ImageJ software as previously described (5,8). Pierce Reversible Protein Stain Kit for PVDF Membranes was used as loading control for western blots. PVDF membranes were stained and total loaded protein was evaluated following the manufacturer's instructions.

Langendorff preparations

Heart function was assessed *ex vivo* as previously described by us (20, 21). Pressure development was recorded digitally (1 kHz) by connecting an intraventricular balloon to a 2 F Millar pressure transducer. Hearts were paced at 400 bpm and the resulting pressure waves were analyzed for pressure development. In adrenergic stimulation studies, 1 µmol/l isoproterenol (Sigma) was infused as reported by Grimm and colleagues (22).

Size-exclusion chromatographic analysis

Mitochondria were isolated from 3 mice/group and subsequently pooled together and lysed in RIPA buffer. 2-8 mg of pure cardiac mitochondrial protein lysate were fractionated by gel filtration using a Sephadex G-200 (Sigma) 1.6/60 cm column that was equilibrated with PBS at a flow rate of ≈1 ml/min. 1 ml fractions were collected in PBS and concentrated with a SpeedVac SVC200H concentrator (Savant). 50 µg were then loaded and run on NuPAGE 4–12% Bis-Tris gels (Invitrogen) under reducing conditions and anti-FLAG immunoblot was performed. Prior to

experiments, the gel filtration chromatography was calibrated using gel filtration globular protein standards from BioRad.

Creatine Kinase activity

The muscle creatine kinase activity and the consequent production of phosphocreatine was determined in mouse heart homogenates (25 mM Imidazole, pH 7.0) by following the lactate dehydrogenase (LDH)-dependent oxidation of NADH to NAD+ at 340 nm in the presence of creatine, ATP, phosphoenolpyruvate (PEP), and pyruvate kinase (PK) (23). The assay buffer contained 1 mM NADH, 5 mM ATP, 50 mM creatine, 130 mM KCl, 6 mM $MgCl_2$, 0.4 mM PEP, 15 U/ml LDH, 7 U/ml PK and 15 mM Tris (pH 8.8). Absorbance was monitored on a Biotek® Synergy 2 multimode reader.

Measurement of cardiac cAMP levels and PKA activity

For measurement of cardiac cAMP levels, mice were deeply anesthetized and beating hearts were flash-frozen by clamping the cardiac tissue with liquid nitrogen-cooled clamping tools. Frozen hearts were subsequently pulverized, weighed, resuspended in 10% trichloroacetic acid, homogenized, and centrifuged for 30 mins at 12,000 rpm at 4˚C. The supernatant was then mixed with 5 volumes of dd$H_2$0-saturated diethyl ether and the two phases were allowed to separate prior to diethyl ether removal. This step was repeated 4 more times. cAMP levels were measured upon serial dilutions using a commercially available cAMP ELISA kit (Abcam ab65355) following manufacturer's instructions. Absorbance proportionality between dilutions was used to judge quality of the measurements. Calibration curve-inferred pmol values were normalized per µg of pulverized tissue.

For cardiac PKA activity, sections of frozen heart were Dounce homogenized in ice-cold 10 mM potassium phosphate pH 7.0, 1 mM EDTA, 25 mM β-mercaptoethanol, and protease inhibitor cocktail (Millipore/Sigma #539131). The homogenate was sonicated for 20 sec on low power at 4°C. The lysates were cleared by centrifugation at 21,000 x g for 10 mins at 4°C. Cleared lysates were used for *in vitro* kinase assays; final reaction conditions (40mM HEPES pH 7.4, 10

mM $MgCl_2$, 1 mM theophylline, 10 mM β-glycerolphosphate, 10 µM Na_3VO_4, 1 µM DT-2, 60 µM ATP, 0.6 mCi $^{32}PO_4$-g-ATP, and 0.52 mg/ml Kemptide with or without 10 µM cAMP). Kinase reactions were run for 1.5 min at 30°C and stopped by spotting on P81 phosphocellulose paper. P81 paper was washed four times in two liters of 0.452% o-phosphoric acid. The paper was rinsed once in 95% EtOH, and dried in an 80°C oven. Phosphate incorporation was measured by liquid scintillation counting and expressed as pmol phosphate incorporated per µg protein per minute.

Isolation of Mitochondria

Mitochondria were isolated as previously reported (8).

PDC activity

PDC was measured in mitochondrial lysates obtained from freshly isolated mitochondria using a commercially available activity microplate kit (Abcam, ab109902) and following the manufacturer's instructions.

cCa^{2+} and mCa^{2+} transients

Approximately 1 h after plating (48 hours post-transduction) isolated adult or neonatal cardiac myocytes were loaded with Indo-1 AM (0.02 mg/ml) for 20 min at room temperature in an atmosphere of 5% CO_2/95% air. cCa^{2+} transients were recorded in respiratory buffer with 2 mM $CaCl_2$ containing 25 mM HEPES at room temperature beginning 15 – 20 min after loading with Indo-1 AM, and transients were analyzed as already described (24). mCa^{2+} transients were recorded using Mitycam and analyzed as described (5). Transients were recorded upon electric stimulation at 0.3 Hz.

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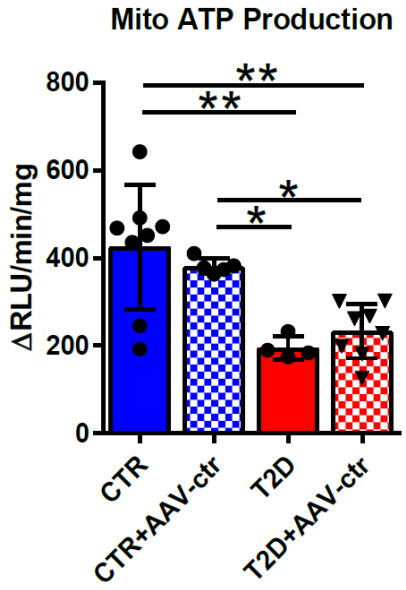
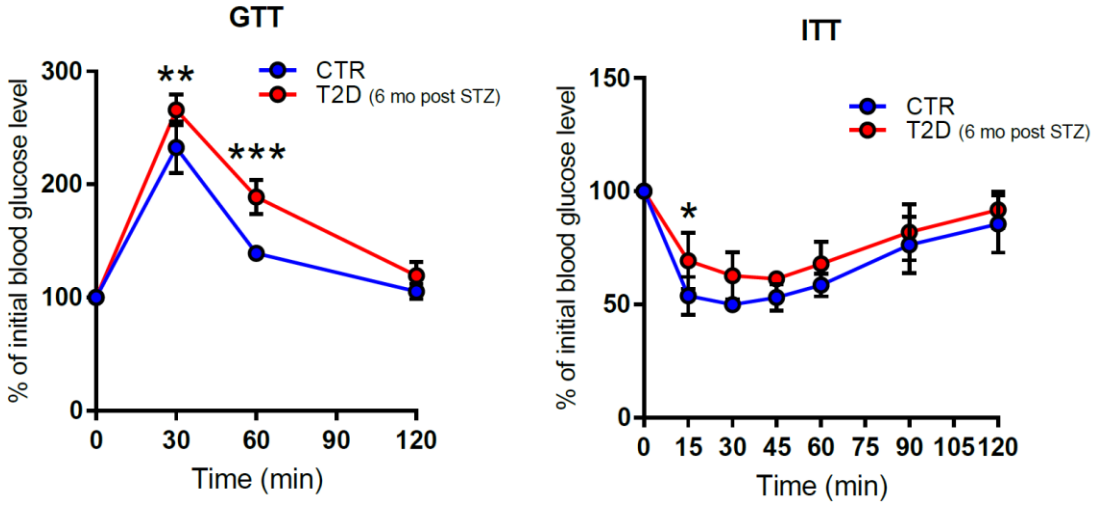
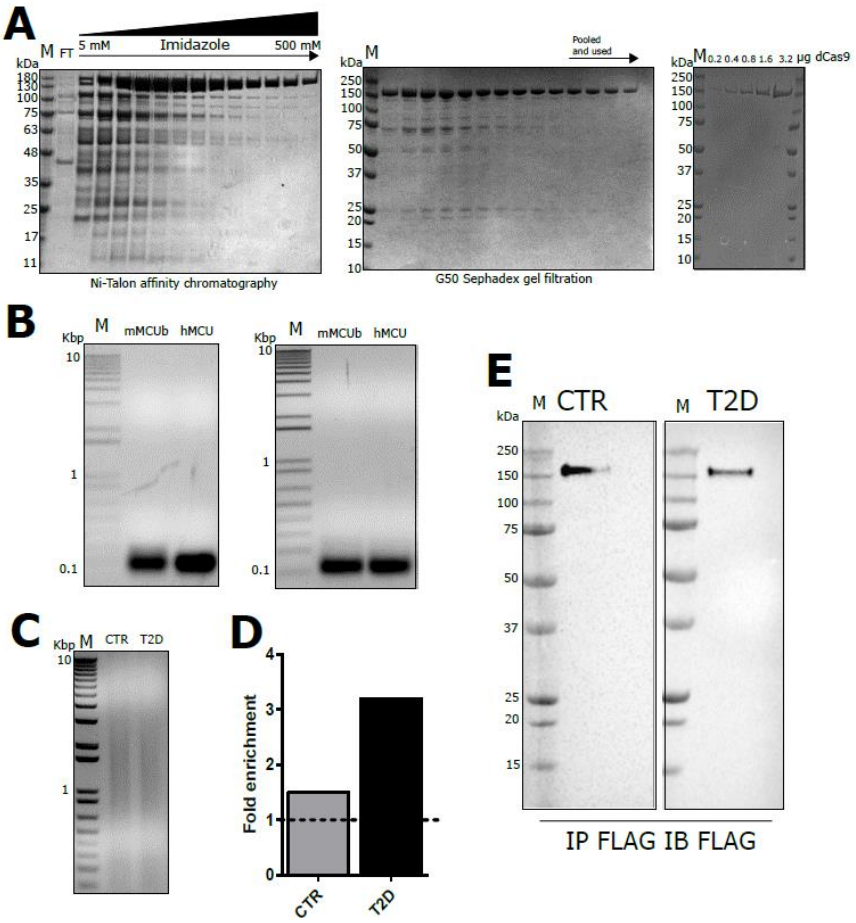
SUPPLEMENTAL FIGURES



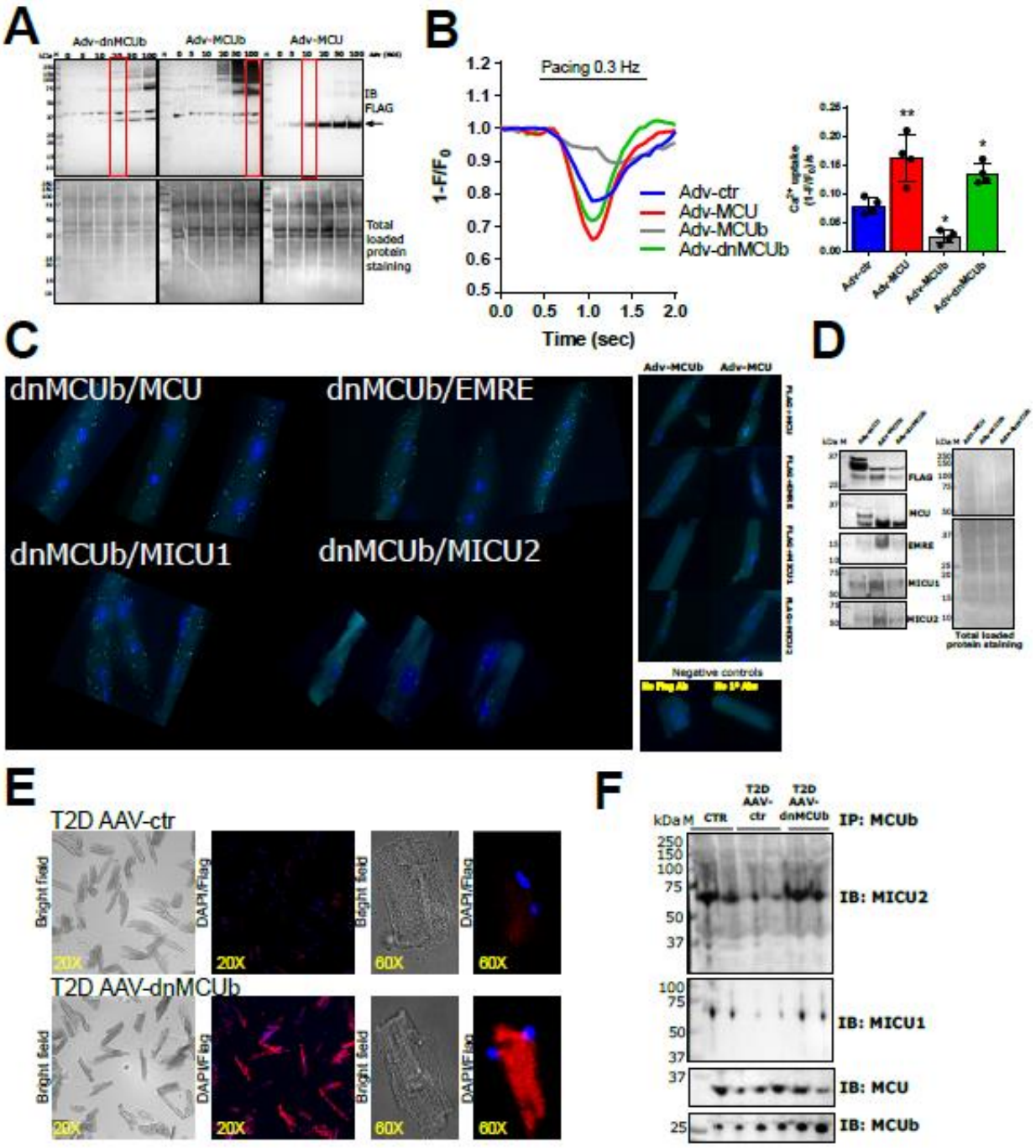
Supplemental Fig. 1 *In vivo* AAV-ctr (control) delivery does not influence mitochondrial energy production. Mitochondrial ATP production rates (CTR, N=8; CTR+AAV-ctr, N=5; T2D, N=4; T2D+AAV-ctr, N=8). Data are presented as mean±SD. One-way ANOVA followed by Dunnett's multiple comparisons test was used for statistical analysis. *Adj.p <0.05, **Adj.p <0.01.



Supplemental Fig. 2 Glucose (GTT) and Insulin (ITT) tolerance tests 6 months post-STZ injection and HFD. Two-way ANOVA followed by Sidak's multiple comparisons test was used for statistical analysis. *Adj.p <0.05, **Adj.p <0.01, ***Adj. p <0.001. GTT data were obtained from N=5 CTR, N=3 T2D; ITT data were obtained from N=9 CTR, N=10 T2D.



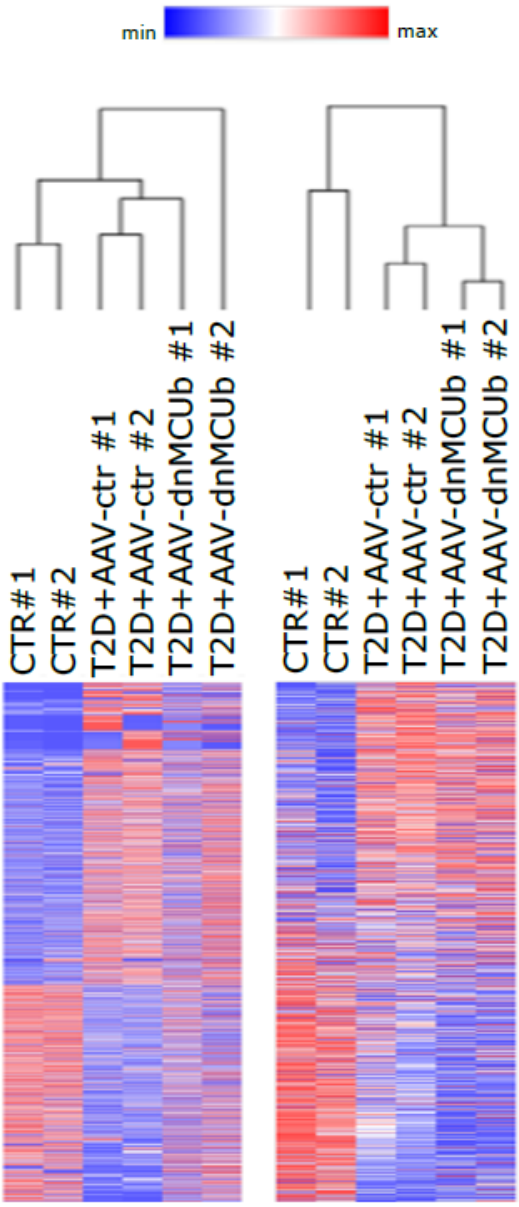
Supplemental Fig. 3 *In vitro* MCUb promoter pull-down. A) Recombinant expression and purification of 7XHis-dCas9-3×FLAG Fusion Protein (dCas9). dCas9 was expressed in *E. coli* BL21 Rosetta, purified with a Ni-Talon affinity resin for His-tagged proteins, eluted with an imidazole gradient (5 – 500 mM) and washed on a Sephadex G-50 column to remove imidazole. The purest fractions were pooled and concentrated. Coomassie blue staining of the Bolt Bis-Tris Plus polyacrylamide gels is reported. **B)** Generation of MCUb promoter-targeting sgRNA *in vitro*. Double-stranded DNA template (left) carrying the T7 promoter and sgRNA specific for the murine MCUb promoter (mMCUb) or human first exon MCU (hMCU) was used with the HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) to generate ssRNAs ~100 bases in length (right), shown on a denaturing 1% agarose gel with 1% v/v bleach. **C)** Chromatin fragmentation obtained from pooled CTR and T2D cardiomyocyte preparations. **D)** qPCR analysis of the presence of the MCUb promoter in dCas9-precipitated samples. Data are presented as fold enrichment of MCUb promoter in sgRNA-mMCUb-dCas9- vs sgRNA-hMCU-dCas9-complex-mediated immunoprecipitates. **E)** Western blot analysis of the presence of the FLAG-tagged dCas9 in FLAG-tagged dCas9 immunoprecipitates.



Supplemental Fig. 4 Development of an AAV-based strategy to introduce a dominant-negative mutant MCUb (dnMCUb) into T2D cardiac mitochondria. (A-B) A dnMCUb adenoviral vector was engineered, tested, and compared with correspondent MCU and MCUb vectors in neonatal cardiac myocyte (NCM) primary cultures. **A)** NCM were transduced at a multiplicity of infection (MOI) per cell ranging from 0 – 100, and western blot analysis for

recombinant FLAG-tagged proteins was conducted. NCM were transduced in subsequent experiments with Adv-dnMCUb at MOI=20, Adv-MCUb at MOI=100, and Adv-MCU at MOI=10. **B)** Representative mCa^{2+} transients measured in NCM. mCa^{2+} transients were assessed in paced-contracting NCM (0.3Hz) using Mitycam which was delivered *in vitro* by Adv-Mitycam 24 h before cells were transduced with Adv-MCU, Adv-MCUb, Adv-dnMCUb, or Adv-ctr (control). Transients were recorded 48 h post-transduction and analysis of mitochondrial Ca^{2+} import rate is reported. mCa^{2+} uptake rates were quantified and are reported in graph as averaged maximal drop of Mitycam fluorescence (F) obtained by electrical stimulation over normalized stable Mitycam fluorescence (F_0=1) at time zero prior to initiation of electrical stimulation, per second [$(1-F/F_0)/s$]. **(C-D)** The ability of dnMCUb to incorporate into the MCUC was investigated using PLA assays in adult cardiomyocytes isolated from T2D hearts and transduced with either FLAG-tagged MCU, MCUb, or dnMCUb. **C)** Representative fluorescence detection of *in situ* interaction between FLAG-tagged dnMCUb (left panel), MCUb or MCU (right panels) and the MCUC complex members MCU, EMRE, MICU1, and MICU2. As negative controls (bottom right) cells were incubated only with either anti-FLAG primary antibody or no primary antibody. **D)** Representative western blots of recombinant FLAG-tagged MCU, MCUb, and dnMCUb as well as endogenous levels of MCU, EMRE, MICU1, and MICU2 in cardiomyocyte preparations from T2D hearts used for PLA assays. Total loaded protein stained on PVDF membranes is shown as loading control. **(E-F)** A dnMCUb adeno-associated viral vector (AAV) was engineered and delivered *in vivo*. Presence of dnMCUb protein expression was investigated. **E)** Anti-FLAG immunofluorescence staining of cardiomyocytes isolated from T2D mice given either AAV-ctr or AAV-dnMCUb. **F)** MCUb was immunoprecipitated from cardiomyocytes isolated from CTR, T2D+AAV-ctr, and T2D+AAV+dnMCUb, and western blot analysis of co-precipitated MCU, MICU1, MICU2, and MCU was conducted. Data in A) and B) are representative of 4 experiments. Data in C) and D) are representative of experiments replicated 3 times. Data in E) and F) are representative of adult cardiac myocyte preparations and immunoprecipitations from 2 mice per group. One-way ANOVA

followed by Dunnett's multiple comparisons test was used for statistical analysis in B).*Adj.p <0.05, **Adj.p <0.01.



Supplemental Fig. 5 *In vivo* dnMCUb delivery does not trigger cardiac remodeling. Heat maps of the transcriptomics (left) and proteomics (right) analyses are presented. Hierarchical clustering was obtained using Spearman rank correlation. Each row represents one gene or protein. A color gradient was introduced to visualize relative levels (blue=low levels, red=high levels). dnMCU transgene expression did not significantly alter either the transcriptome nor the proteome towards CTR levels, as evidenced by hierarchical clustering.

SUPPLEMENTAL TABLES

	1 month		3 months		6 months	
	CTR	**T2D**	**CTR**	**T2D**	**CTR**	**T2D**
Cholesterol (mg/dl)	56.6 ± 11	116.3 ± 12.7**	91.6 ± 13.4	139.3 ± 26.6*	138.4 ± 23.2	202.7 ± 32.9***
HDL Cholesterol (mg/dl)	50 ± 11.3	96 ± 9.5***	79.2 ± 13.9	115 ± 9.2***	94.1 ± 8.3	146.5 ± 19.9***
LDL, Calculated (mg/dl)	1 ± 1	14 ± 4	3 ± 2	14.33 ± 7	8.1 ± 6.4	45 ± 14.4***
Triglyceride (mg/dl)	27.3 ± 2.9	33 ± 3.6	33.5 ± 5.1	50.3 ± 2.1***	37.5 ± 7.1	56.8 ± 6.9***
BW (g)	23.6 ± 1.2	29.3 ± 2.3	27.44 ± 3.3	38.5 ± 4.4***	32.5 ± 1.4	45.5 ± 6.5***
FBG (mg/dl)	103.7 ± 5.9	115.2 ± 10.8	83.4 ± 12.4	141.6 ± 14.7	122 ± 29.2	216.3 ± 82.2**
GTT 1hr (mg/dl)	182.3 ± 24.5	188.7 ± 16.2	136 ± 25.6	249.2 ± 60**	189.3 ± 6.3	319 ± 72.6***
Insulin (ng/ml)	0.64 ± 0.18	1.5 ± 1.2	0.8 ± 0.1	2.2 ± 0.2 *	1.3 ± 0.5	2.9 ± 0.2 **
Hba1c (%)	3.1 ± 0.3	3.4 ± 0.4	3.2 ± 0.3	3.2 ± 0.2	3.2 ± 0.6	3.6 ± 0.4
Hba1c (mmol/mol)	10 ± 4	14 ± 4	12 ± 3	12 ± 2	12 ± 6	16 ± 4

Supplemental Table 1. Time course metabolic characterization of the T2D mouse model. Data were collected 1, 3, and 6 months post-STZ/HFD and are presented as mean ± SD. Two-way ANOVA followed by Sidak's multiple comparisons test was used for statistical analysis.*Adj.p <0.05, **Adj.p <0.01, ***Adj.p <0.001.

Supplemental Table 2: List of primers used in this study.

MCUb Endogenous Cloning From Mouse Heart
MCUb FWD 5'-ATGCCAGGAGCTCTGTCCGG
MCUb REV 5'-CTAGTTCTTCTCGCTGGCTT
Site-Directed Mutagenesis Primers
MCUb W246R/V251E FWD 5'-ggccAGGcttacttggtggGAGtactcctgggatatc
MCUb W246R/V251E REV 5'-gatatcccaggagtaCTCccaccaagtaagCCTggcc
In-Fusion Cloning Primers for pENTR1a-MCUb
FWD 5'-atcacactggcggccgctcgagcATGCCAGGAGCTCTGTCCGG
REV 5'-agaatagggccctctagatgcatCTACTTATCGTCGTCATCCTTGTAATCGTTCTTCTCGCTGGCTTCCT
In-Fusion Cloning Primers for AAV2 Shuttle Vector
MCUb constructs
FWD 5'-tctcgagctcaagct*ccacc*ATGCCAGGAGCTCTGTCCGG
REV 5'-ccgctttacttgtacCTACTTATCGTCGTCATCCTTGTAATCGTTCTTCTCGCTGGCTTCCT
Primers to clone 3XFLAG-DCAS9-6HIS Into pQTEV
FWD 5'-agggatccgggtcgactatggataaaaagtattctattgg
REV 5'-tctatcaacagtcgatcacttgtcatcgtcatccttgtaatcgatgtcatgatctttataatcaccgtcatggtctttgtagtcgtcacccccaagctgtgaca-3'
Primers to Produce sgRNA Directed Towards The Mouse MCUb Gene Promoter
FWD 5'-TTAATACGACTCACTATAG*TGTCGCCGCCCCAGTCGGCG* GTTTTAGAGCTAGAAATAGC-3'
REV 5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'
Primers to Produce sgRNA Directed Towards The Human MCU First Exon
FWD 5'-TTAATACGACTCACTATAG*CAGGAGCGATCTACCTGCGG* GTTTTAGAGCTAGAAATAGC-3'
REV 5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3'
qPCR Primers
MCUb mRNA
FWD 5'-AGTTACCTTCTTCCTGTCGTTTGCG
REV 5'-CAGGGATTCTGTAGCAAGG
MCUb gene promoter (Region#1)
FWD 5'-AAAATACCCAGCCTGGAGCC
REV 5'-TAACGACGCATCCTCATCCG
MCUb gene promoter (Region#2)
FWD 5'-ACTGACAGTCCTTTGGCTCG
REV 5'-GGCAGGAGTTAAGCATTGCG
Ncor2 mRNA
FWD 5'-AAGTTCAGAGCTGGCTTCCA
REV 5'-TGACCAGTTCCTCCCATGTT
16S
FWD 5'-GGCAAACACGAACCCCGCCT
REV 5'-GTCAGGATACCGCGGCCGTT
RPL32
FWD 5'-TCGTCAAAAAGAGGACCAAGAAG
REV 5'-CCGCCAGTTTCGCTTAATTT

Supplemental Table 3: List of primary and secondary antibodies used in this study.

Primary Antibodies	Protein	Manufacturer/Catalog#	Application/Dilution
	Anti-MCU	Sigma HPA016480	WB 1:1000, PLA 1:50
	Anti-MCUb	Sigma HPA048776	WB 1:500, PLA 1:50
	Anti-EMRE	Santa Cruz SC-86337	WB: 1:200, PLA 1:50
	Anti-MICU1	Sigma HPA037480	WB 1:500, PLA 1:50
	Anti-MICU2	Abcam ab101465	WB 1:500, PLA 1:50
	Anti-FLAG M2	Sigma F3165	WB 1:1000, PLA 1:50, IF 1:50
	Anti-pPLN	Thermo Scientific PA5-38317	WB 1:500
	Anti-SERCA2a	Santa Cruz SC-376235	WB 1:1000
	Anti-Ncor2	Abcam Ab5802	WB 1:1000, IP 5 µg
	Anti-PPARα	Millipore MAB3890	IP 5 µg
	Anti-PPARγ	Santa Cruz SC-271392	IP 5 µg
Secondary Antibodies	**Host**	**Manufacturer/Catalog#**	**Application/Dilution**
	Anti-Mouse IgG	Santa Cruz SC-2025	IP 5 µg
	Anti-Rabbit IgG	Cell Signaling 2729	IP 5 µg
	Anti-Mouse IgG-HRP-conjugated	ThermoFisher Scientific 31430	WB 1:5000
	Anti-Rabbit IgG-HRP-conjugated	Cell Signaling 7074	WB 1:5000
	Duolink® In Situ PLA® Probe Anti-Mouse PLUS	Sigma DUO92001	PLA 1:5
	Duolink® In Situ PLA® Probe Anti-Rabbit MINUS	Sigma DUO92005	PLA 1:5