SUPPLEMENTAL MATERIAL

Elevated MCU expression by CaMKIIδB limits pathological cardiac remodeling

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Short title: CaMKIIδB upregulates MCU for heart protection

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Expanded Methods

The authors declare that all supporting data are available within the article and the Online Data Supplement. The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. Raw data are available from the corresponding author on reasonable request.

Animals

All animal procedures were performed in accordance with the approved protocol by the Institutional Animal Care and Use Committee (IACUC) of the University of Washington. The experiments included in this study used both male and female mice. Mice and rats were housed under standard conditions provided with standard rodent chow and water ad libitum in a vivarium with a 12 hr light/dark cycle at 22°C. The global MCU KO mice were on mixed C57BL/6 and CD1 background and were from Dr. Toren Finkel (University of Pittsburg).[7] The CaMKIIδ knockout mice were from Dr. Joan Heller Brown (University of California San Diego).[25] The floxed/floxed Stop polyA MCU (F/F) mice were generated by Cyagen on C57BL/6 background (Charles River) through pronuclear injection of the pPB[Exp]-CBh>LSL:{mMCU/3XFLAG vector (VectorBuilder). The F/F mice were crossed with αMHC-MerCreMer transgenic mice (MCM, maintained on C57BL/6 background, Charles River). At ~8 weeks of age, tamoxifen (5 mg/kg/day, intraperitoneal injection for 2 days) was used to induce the overexpression of MCU in the heart (MCU TG). The F/F mice and MCM mice with the same tamoxifen treatments were used as controls for the MCU TG mice. To generate cardiomyocyte specific MCU KO mice (cKO), we crossed the previously reported MCU homozygous “floxed” mice ($MCU^{fl/fl}$)[9] with MCM mice, both are on C57BL/6 background. At ~8 weeks of age, the mice received an intraperitoneal injection of tamoxifen (40 mg/kg) for 5 days, which was sufficient to induce MCU deletion in cardiomyocytes.[9] Sample size was determined by power analysis and based on our previous experiments and literatures.[43, 44]

Heart hypertrophy model in mice

To generate chronic β-AR activation-induced heart hypertrophy model, isoproterenol (ISO, 10 mg/kg/day dissolved in saline) were injected subcutaneously in mice of both genders at the age of 8-10 weeks for up to 4 weeks. Littermates were randomly assigned to ISO or vehicle (saline) injection. Echocardiography was performed in conscious adult mice using a high-resolution ultrasound system (Vevo 2100 or 3100, VisualSonics) with an 18-38 MHz MS-400 transducer. The following parameters were monitored digitally in M-mode: LV dimensions at diastole (LVDd) and systole (LVDs) and anterior and posterior wall thickness at diastole and systole. Fractional shorting (FS) and ejection fraction (EF) were calculated from these parameters. All measurements were averaged over five consecutive cardiac cycles. The echo measurements and calculations were done by an investigator who did not know the experimental setting (genotypes and treatments).[45] Mice with clear signs of heart hypertrophy in ISO group were included in the study.

Histological studies for heart hypertrophy and fibrosis were done by a histology core facility at the University of Washington on the South Lake Union campus. Briefly, hearts were fixed in 4% paraformaldehyde in PBS (pH 7.4), embedded in paraffin, and sectioned at a thickness of 5 μm. Standard hematoxylin and eosin (HE), Picrosirius, or wheat germ agglutinin (Sigma, Cat. No. L4895) staining was performed.

Human heart samples

The human heart samples were collected in a previous study and the study protocol was approved by local ethics committee.[46] The failing heart samples (HF) were obtained from the left ventricular (LV) free wall or apex of the heart during heart transplantation or implantation of a LV assist device. The non-failing heart samples (NF) were obtained from the LV free wall of donor hearts found unsuitable for transplantation for a variety of reasons such as history of blood

transfusion in the emergency room, unacceptable age, or history of resuscitation before death etc. All donors had no history, macroscopic, or laboratory signs of cardiac diseases.

Adult cardiomyocyte isolation, culture, gene transfer, and treatment

The isolation and culture of adult cardiomyocytes from rat or mouse followed our established protocols reported previously.[43] Briefly, Adult Sprague Dawley rat (200-250 g, Harlan) or mouse (wild type, MCU KO or MCU TG) was anesthetized by intraperitoneal injection of pentobarbital and the heart quickly removed. The heart was cannulated via ascending aorta, mounted on a modified Langendorff perfusion system, and perfused with oxygenated Krebs-Henseleit Buffer (KHB) supplemented with collagenase II (Worthington, Cat No. LS004177) and hyaluronidase (Sigma, Cat. No. H3506) at 37°C. Rod shaped adult cardiomyocytes were collected and plated on 6 well plates or glass coverslips coated with 20 μg/mL laminin (Life Technologies, Cat. No. 23017-015). The cells were cultured in serum-free M199 medium (Sigma, Cat. No. M2520) supplemented with 10 mmol/L glutathione, 26.2 mmol/L sodium bicarbonate, 0.02% bovine serum albumin and 50 U/mL penicillin–streptomycin. Adenovirus-mediated gene transfer was conducted 2 hr after cell plating at a multiplicity of infection of 50-100 and the cells were cultured for 48 hr (for mouse cardiomyocytes) or 72 hr (for rat cardiomyocytes). ISO (0.01-10 μmol/L, Sigma, Cat. No. I6504) was added in culture medium up to 24 hr before measurements or sample collection. In some experiments, cyclosporin A (CsA, 100 nmol/L, Sigma, Cat No. 30024), KN93 (0.5 μmol/L, Sigma, Cat. No. 422708), Tetrakis(acetoxymethyl) 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA-am, 100 nmol/L, TCI America, Cat. No. T2845), or Cal XI (1 μmol/L, Sigma, Cat. No. 208743) was added shortly before ISO.

Measurements of intracellular Ca^{2+}, ATP, mPTP opening, and cell death

Confocal imaging was conducted to monitor mitochondrial, cytosolic, or SR Ca^{2+} levels in quiescent cells and Ca^{2+} transients during electrical pacing.[47] Custom-designed chambers were

used to mount the cells on the stage of confocal microscope (Leica TCS SP8 with a 40 x 1.3 NA oil-immersion objective). To determine steady state mitochondrial Ca^{2+} levels, we used mitochondria-targeted genetic Ca^{2+} indicators mt-PeriCam and mt-GCaMP6f in intact cardiomyocytes.[48, 49] To estimate the amount of releasable mitochondrial matrix Ca^{2+}, we monitored FCCP-induced mitochondrial Ca^{2+} depletion in permeabilized cardiomyocytes loaded with rhod-2 salt (Invitrogen, Cat. No. R14220) as previously reported.[9] The normalized changes in rhod-2 fluorescence from the same cell before and after FCCP treatment were used to estimate the relative levels of free matrix Ca^{2+}. We used Twitch2B, a ratiometric Ca^{2+} indicator, to measure diastolic cytosolic Ca^{2+} levels.[50] We used GCaMP6f (in rat cardiomyocytes) or fluo-8 AM (in mouse cardiomyocytes, 10 μmol/L, AAT Bioquest, Cat. No. 21083) to measure electrical pacing (1 Hz)-induced Ca^{2+} transients. To monitor steady state SR Ca^{2+} levels, we used the SR-targeted Ca^{2+} indicator D1ER[51]. We used fluo-8 AM, Na^{+}- and Ca^{2+}-free solutions, and intact cardiomyocytes to determine caffeine (10 mmol/L)-induced SR Ca^{2+} release and SERCA2a-mediated Ca^{2+} reuptake as described previously.[52] For confocal imaging, intact cells were incubated in modified Tyrode's solution (in mmol/L: 138 NaCl, 0.5 KCl, 20 HEPES, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1 $CaCl_2$, 5 Glucose, pH 7.4) at room temperature. mt-PeriCam, mt-GCaMP6f or GCaMP6f was excited at 405 and 488 nm and the emissions collected at >490 nm. Rhod-2 was excited at 552 nm and emission collected at 560-700 nm. Twitch2B was excited at 405 nm and the emissions collected at 485-510 nm and 525-610 nm. D1ER was excited at 405 nm and the emissions collected at 450-505 nm and 515-560 nm. Fluo-8 was excited at 488 nm and the emission collected at >490 nm. For monitoring cytosolic or mitochondrial ATP levels, we used a genetic ATP indicator ATeam.[23] The ATeam was excited at 405 nm and emissions collected at 420-505 and 515-700 nm. Confocal images were taken at 1024 resolution. Digital image analysis used the software of the confocal microscope, ImageJ, and customer designed programs coded in interactive data language.[43]

Laser-induced mPTP opening followed a protocol reported previously.[53] Briefly, tetramethylrhodamine methyl ester (TMRM, 20 nM) was loaded to cells without washing and linescan confocal image (1000 lines) was taken by exciting the cells at 552 nm and emission collected at >560 nm at a scanning speed of 100 ms/line.

Cardiomyocyte death was detected by using trypan blue (MP Biomedicals, Cat. No. 1691049) exclusion assay. At least 20-50 cells were counted in each image and four images analyzed in each sample. To determine apoptosis, caspase 3 activity was measured by using the Caspase-Glo 3/7 Assay kit (Promega, Cat. No. G8090).

Measurement of mitochondrial Ca^{2+} uptake

Mitochondrial Ca^{2+} uptake was measured in freshly isolated mitochondria from the heart and followed a previous published protocol.[54] Briefly, 250 μg mitochondria were added into 1 mL assay buffer (120 mmol/L KCl, 10 mmol/L NaCl, 1 mmol/L KH_2PO_4, 20 mmol/L HEPES-Tris, pH 7.2) with 1 μmol/L rotenone, 5 mmol/L succinate and 800 nmol/L Fura-FF (Cayman Chemical Company, Cat. No. 20451). Mitochondria were gently stirred and the fluorescence signals were monitored by using a spectrofluorometer (QuantaMaster 80 dual emission Spectrofluorometer, Photon Technology International, Inc.) with 340 and 380 nm excitation and 525 nm emission for Fura-FF to calculate the ratiometric changes. Pulses of $CaCl_2$ (25 μmol/L) were added at an interval of 150 s until the detection of massive Ca^{2+} release due to the opening of mPTP.

Measurement of oxygen consumption rate (OCR)

The OCR was measured by using a Seahorse XF24 Extracellular Flux Analyzer. Adult rat cardiomyocytes (1000 cells per well) were plated in the XF24 cell culture microplates in M199 medium. Ad-LacZ, Ad-MCU or Ad-DNMCU were added, cells were cultured for 48 hr, and ISO (100 nM) were added for another 24 hr. For seahorse assay, cells were washed and incubated in XF assay medium supplemented with 10 mM glucose, 1mM pyruvate and 2 mM glutamine

for 30 min at 37°C without CO_2. Oligomycin (2.5 μg/ml), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 3 μM), and antimycin A (1 μM) plus rotenone (1 μM), were injected sequentially into the wells to assess basal respiration, proton leak, and spare (maximal) respiratory capacity as we reported before.[55]

Construction of adenoviral vectors

To make adenoviral vectors for *in vitro* gene delivery in adult cardiomyocytes, we co-transfected the pBHGloxΔE1, 3Cre plasmid (Microbix) with pDC316 shuttle vector (Microbix) containing the gene of interest into HEK293 cells using Lipofectamine 2000 (Invitrogen, Cat. No. 11668). Through homologous recombination, the gene of interest was integrated into the E1-deleted adenoviral genome.[56] The viruses were amplified in HEK293 cells, aliquoted, and stored at -80°C.

We generated adenoviral vectors containing the following genes: N-terminal 3 x Flag tagged MCU (NM_001033259) or dominant negative MCU (DNMCU, D260Q/E263Q),[5, 57] N-terminal 3 x Flag tagged CREB (NP_034082.1) or dominant negative CREB (DNCREB, S133A), N-terminal HA tagged CaMKIIδB (XM_017590614.1) or its mutations (S332E, S332A, or K43A), C-terminal GFP-tagged, cytosol-targeted or nucleus-targeted CaMKII inhibitory peptide (AIP, KKALRRQEAVDAL) (c-AIP or n-AIP),[58, 59] C-terminal RFP-tagged, cytosol-targeted or nucleus-targeted CaMKII inhibitory protein CaMKIIN (c-CaMKIIN or n-CaMKIIN, NM_033259.2),[60-62] C-terminal RFP tagged protein kinase A inhibitory peptide (PKI, MTDVETTYADFIASGRTGRRNAIHD, Genbank: AAA40867),[63] D1ER (Addgene, Cat. No. 36325), GCaMP6f (Addgene, Cat. No. 40755) or mt-GCaMP6f, and Twitch2B (Addgene, Cat. No. 100040). For AIP or CaMKIIN, the nuclear targeting sequence (PKKKKRV) or the cytosolic location sequence (ALQKKLEELELDE) were used, respectively. Correct location of the differentially targeted AIP or CaMKIIN was determined by confocal microscopy with DAPI co-staining of the nucleus. The adenoviruses of HA-CaMKIIδC, HA-DNCaMKIIδC, PeriCam, β-

galactosidase (LacZ), mitochondrial targeted GFP (mGFP), and ATeam were generated and reported previously.[43, 55] The Ad-PP2B/CALNA3 (calcineurin, CaN) was purchased (Vector Biolabs, Cat. No. 1537). Mouse Gsα gene (NP_001297012.1) was amplified by RT-PCR and the constitutively activity Gsα (caGsα, Q227L) was generated by point mutation.[24] Calmodulin (CaM, NP_033920.1) was amplified by RT-PCR and dominant negative CaM (DNCaM) was generated by point mutations in all 4 EF hands: D20A, D56A, D93A, and D129A.[64] The fusion protein MCU-ATeam or DNMCU-ATeam was generated by inserting ATeam gene after the C terminus of MCU or DNMCU and before the stop codon. For point mutations in CaMKIIδB, CREB, MCU, Gsα and CaM genes, we used QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Cat. No. 200521) and the primers were shown in Supplemental Table 1 (underline indicates mutated sequence).

The adenoviruses harboring shRNA for mouse CaMKIIδ (NM_001346636.1) (Ad-shCaMKII) was generated using the following hairpin forming oligonucleotides (hairpin sequence underlined): 5'-gagcaactgattgaagcta<u>ttcaagaga</u>tagcttcaatcagttgctc-3'. Apa I and Hind III site was added at the 5' and 3' ends, respectively. The sense and antisense oligonucleotides were synthesized, annealed, and subcloned into a modified pDC311 vector.[56] Recombinant adenoviruses were generated in HEK293 cells as described above.

Luciferase activity assay

Four different fragments (from -1780, -1221, -1020, or -878 to the start codon) of the promoter region of mouse MCU gene (ID: 215999) were amplified by PCR and cloned into a dual luciferase reporter pNLCol1 vector (Promega, Cat. No. N146A) to drive luciferase expression. Full length MCU promoters containing mutations in the 4 CRE elements (I: -1214 to -1207, II: -1139 to -1132, III: -1034 to -1027, and IV: -900 to -893) were generated by using QuikChange II XL Site-Directed Mutagenesis kit and the primers were shown in Supplemental Table 2 (underline indicates the mutated sequence).

H9C2 cells were plated in 96-well plates and transfected with 0.2 μg of the indicated luciferase vectors per well or control vector using Fugene (Promega, Cat. No. E269A) for 24 hr. Then, the cells were incubated with ISO for another 24 hr. The luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega, Cat. No. E1910).

Recombinant protein preparation and *in vitro* phosphorylation assay

CREB was subcloned into pET30a vector and transfected into *E. coli* strain BL21 (DE3) (New England Biolabs, Cat. No. C2527H). When the cell density reached an OD600 of ~1.0, protein expression was induced by adding 0.5 mmol/L isopropyl 1-thio-β-D-galactopyranoside (Thermo, Cat. No. R0391). The cells were cultured at 4°C for 2 days before collecting. The 6 x His tagged CREB was purified from cell lysate by using Nickle resin (Ni Sepharose 6 Fast Flow, GE Healthcare, Cat. No. 17-5318-06) followed by gradient imidazole elution. The purified recombinant CREB was evaluated by SDS-PAGE and coomassie brilliant blue staining, which revealed a purity of ~90% and a molecular weight of ~42 kD. HA-CaMKIIδB or its mutations (K43A, S332A, or S332E) were expressed in HEK293 cells for 72 hr. Cells were harvested and homogenized in Tris-HCl buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl and 0.5% Triton X100, pH 7.4) via sonication. The anti-HA magnetic beads (Pierce, Cat. No. 88836) were used to concentrate and immobilize HA-CaMKIIδB. Calmodulin was purchased (Millipore, Cat. No. 208690). The immobilized HA-CaMKIIδB beads (5 μL) were incubated with purified CREB (2 μmol/L) in 100 μL reaction buffer containing 20 mmol/L Tris-HCl, 150 mmol/L NaCl, and 25 μmol/L ATP (pH7.4) for 30 min at 30°C in the presence or absence of 1 mmol/L $CaCl_2$ and/or 1 mmol/L calmodulin. After the incubation, 25 μL 5 x loading buffer were added and samples incubated at 70°C for 10 min to stop the reaction. CREB phosphorylation was determined by Western blotting using anti-p-CREB (1:1,000, Cell Signaling, Cat. No. 9198) or anti-CREB (1:2,000, Cell Signaling, Cat. No. 9197) antibody.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed according to the supplier's protocol (Pierce Agarose ChIP kit, Cat. No. 26156). Briefly, adult cardiomyocytes were treated with 1% formaldehyde for 10 min to cross-link proteins and chromatin. The reaction was stopped by adding 0.125 mol/L glycine for 5 min. Cells were washed with cold PBS twice, detached in PBS buffer at 4°C, and centrifuged down. The chromatin was purified by micrococcal nuclease digestion and passing through a column, and analyzed on a 1% agarose gel to determine the shearing efficiency. As a negative control, normal IgG was used instead of CREB antibody (Santa Cruz, Cat. No. sc-168) in immunoprecipitation. For positive control, we detected the binding between mouse GAPDH promoter and RNA Polymerase II by immunoprecipitation using anti-RNA Polymerase II antibody (Thermo Scientific, Cat. No. 1862243). The DNA sequences that bound to CREB or RNA Polymerase II were amplified by PCR and the primers were shown in Supplemental Table 3.

Electrophoretic mobility shift assay (EMSA)

The probes for biotin-labeled CRE IV, biotin-labeled mutant CRE IV (underline indicates the mutated sequence) and unlabeled competitor CRE IV were shown in Supplemental Table 4. The sense and antisense oligonucleotides were synthesized and annealed to form double-stranded DNA probes. CREB/CRE IV complexes were formed by incubating 1 μg of biotin-labeled CRE IV DNA probe, biotin-labeled CRE IV mutant DNA probe (negative competitor), or unlabeled CRE IV DNA fragment (positive competitor) with 1 μg CREB (6 x His tagged CREB was purified from HEK293 cells) in 10 μl buffer (50 mmol/L Tris-HCl and 150 mmol/L NaCl) for 30 min at 22°C. Then, 6.5% non-denaturing polyacrylamide gel (5 x Tris-borate-EDTA (TBE) buffer 1 mL, 30% acrylamide/bis 2.2 mL, 80% glycerol 80 μL, 10% $(NH_4)_2S_2O_8$ 90 μL, tetramethylethylenediamine 10 μL, and ddH_2O 6.62 mL) was prepared freshly and pre-run in pre-chilled 0.5 x TBE buffer for 10 min at 120V on ice. The CREB/CRE IV complexes were

loaded on the gel and run at 100V for 50-60 min. The proteins/probe complex was transferred to Zeta-Probe Blotting Membrane (Bio-rad, Cat. No. 162-0158), the free or bound biotin-CRE IV were immobilized by using a Stratagene UV Crosslinker for 1 min. The membrane was blocked by 5% BSA in TBST for 30 min, incubated with HRP conjugated streptavidin (1:20000, Millipore, Cat. No. 18-152) in 5% BSA in TBST for another 30 min at room temperature, and washed 3 times. Biotin-CRE IV bands were detected by chemiluminescence. The bands corresponding to the CREB/biotin-CRE IV complexes were shifted upwards as compared to the unbound free biotin-CRE IV.

Co-immunoprecipitation

Co-immunoprecipitation followed the same procedure as we reported before.[43] Briefly, adult cardiomyocytes were lysed by 3 freeze-thaw cycles in PBS with 0.1% Triton X-100 and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were incubated with 10 μL of Protein A/G agarose beads (Pierce, Cat. No. 20423) for 2 hr to remove endogenous IgG and centrifuged at 5,000 rpm for 5 min to remove the beads. Protein samples (1 mL, 1.5 mg/mL) were incubated with rabbit anti-CaMKIIδ antibody (1:100, GeneTex, Cat. No. GTX111401), rabbit anti-CREB (1:50, Santa Cruz, Cat. No. sc-186), or 20 μL rabbit IgG-agarose beads (Sigma, Cat. No. A2909) for 12 hr at 4°C. Then, protein A/G agarose beads (20 μl) were added to enrich the primary antibody/protein complex. After 4 hr incubation, the beads were washed with PBS containing 0.1% Triton X-100 for 5 times. Proteins were eluted by using 30 μL of 0.1 mol/L glycine (pH=2.7) and loaded on 12% SDS-PAGE. Western blotting was done by using mouse anti-CREB (1:1000, Cell Signaling, Cat. No. 9104), goat anti-CaMKIIδ (1:1000, Santa Cruz, Cat. No. sc-5392), or rabbit anti-calcineurin (1:2500, Cell Signaling, Cat. No. 2614) antibodies followed by secondary antibodies.

Mass spectrometry analysis

Rat adult cardiomyocytes were infected with adenovirus containing HA-CaMKIIδB for 48 hr. The cells were washed with ice-cold PBS and lysed with buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl, 0.5% Triton X-100, pH=7.4) containing a protease inhibitor (Pierce, Cat. No. A32953). The lysate was incubated with 20 µL anti-HA magnetic beads (Pierce, Cat. No. 88836) for 16 hr to enrich HA-CaMKIIδB. The beads were centrifuged down and incubated with 50 µL de-phosphorylation reaction buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$ and 1 µmol/L calmodulin (Millipore, Cat. No. 208690), pH=7.4 with or without 500U calcineurin (Sigma, Cat. No. C1907) for 2 hr at 37°C. After incubation, the proteins were denatured, separated by SDS-PAGE, and stained by Coomassie brilliant blue. The band corresponding to HA-CaMKIIδB was cut from the gel and subjected to trypsin digestion using an in gel digestion kit (Thermo, Cat. No. 89871).

All samples were analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher) equipped with a nano-Acquity UPLC system (Waters) and in house developed nano spray ionization source. Samples were loaded from the autosampler onto a 100 µm ID Integrafrit trap (NewObjective) packed with Reprosil-Pur C18-AQ 120 Å 5 µm material (Dr. Maisch) to a bed length of 2.5 cm at a flow rate of 2 µL/min. After loading and desalting for 10 min with 0.1% formic acid plus 2% acetonitrile (LCMS grade from Fisher), the trap was brought in-line with a pulled fused-silica capillary tip (75-µm i.d.) packed with 35 cm of Reprosil-Pur C18-AQ 120 Å 5 µm (Dr. Maisch). Peptides were separated using a linear gradient from 5-30% solvent B (LCMS grade 0.1 % formic acid in acetonitrile, Fisher) in 90 min at a flow rate of 300 nL/min. The column temperature was maintained at a constant 50°C during all experiments.

Peptides were detected using a data-dependent (DDA) method. Survey scans of peptide precursors were performed in the orbitrap mass analyzer from 375 to 1575 m/z at 120K resolution (at 200 m/z) with a 7e5 ion count target and a maximum injection time of 50 ms. The instrument was set to run in top speed mode with 3 sec cycle for the survey and the MS/MS scans.

Orbitrap Fusion (Thermo Fischer Scientific) raw files were converted to the mzXML format using the ReAdW converter. Comet (version 2018.01 rev. 3) was used to search the mzXML files against a Rat UniProt sequence database appended with common contaminants and the Rat_Flag-ha-CaMKII_delta_B protein (totaling 31,679 sequence entries). The database search parameters were: 20 ppm precursor tolerance, isotope_offset 3, tryptic digest allowing 2 missed cleavages, 15.9949 variable modification on methionine, 79.966331 variable modification on serine/threonine/tyrosine, 57.021464 static modification on cysteine, and fragment_bin_tol 0.02. An expectation value cutoff of 10e-7 was applied to the Comet search results.

Reverse transcription quantitative PCR (RT-qPCR)

Expression of hypertrophy-related genes and MCU were determined by RT-qPCR using SYBR green probe (Thermo, Cat. No. 4309155). Total RNA were isolated from freshly isolated heart tissues or cultured adult cardiomyocytes by using a Direct-zol RNA MiniPrep Kit (Zymo Research, Cat. No. R2050) and reverse transcribed to cDNA by using Maxima First Strand cDNA Synthesis Kit (Thermo, Cat. No. K1671). The primers used for qPCR measurements are listed in Supplemental Table 5. The RT-qPCR were repeated 4 times for each gene and the results normalized to 18S ribosomal RNA.

CaMKIIδB S332 phosphospecific antibody production

The CaMKIIδB S332 phosphospecific antibody was developed by Sino Biological, Inc. Briefly, antigen phosphopeptide (Ac-CGVKKRKpS^{332}SSSV-NH2) was designed, synthesized, and conjugated to Keyhole limpet hemocyanin (KLH) to immunize rabbits. Ten weeks after the immunization, serum was collected. The titer of antiserum was determined by ELISA (≥1:64000) using phosphopeptide antigen. The antiserum was purified by AG affinity chromatography. The purified antibody was further verified by ELISA using phospho- or non-phosphopeptide.

Western blotting

Whole heart tissues or intact adult cardiomyocytes were lysed in lysis buffer in the presence of a cocktail of proteinase/phosphatase inhibitors (Sigma, Cat. No. MSSAFE) and centrifuged at 12,000 g for 30 min at 4°C. To separate nuclear and cytosolic fractions in cultured cardiomyocytes, we used the NE-PER™ nuclear and cytoplasmic extraction kit (Thermo, Cat. No. 78833). The cells were collected, washed, suspended in hypotonic buffer, and homogenized by gentle sonication. After centrifugation, nuclei pellet and cytoplasmic fraction were separated and nuclei resuspended in a low-salt buffer. To obtain mitochondrial proteins, we used the protocol reported previous for isolating mitochondria from the heart.[54]

The extracted proteins were separated by 12% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Cat. No. IPVH00010), and the membranes blocked and incubated with various primary antibodies at 4°C overnight followed by appropriate secondary antibodies. Immunoblot signals were detected by chemiluminescence (Millipore, Cat. No. WBKLS0100) and the images quantified by ImageJ. Detailed information of the antibodies used are listed in Supplemental Table 6.

Blue-Native PAGE

Purified mitochondria from mouse heart (50 μg) were incubated for 15 min on ice in 4X NativePAGE sample buffer (Invitrogen, Cat. No. BN2003) with 2% digitonin (Invitrogen, Cat. No. BN2006). Samples were centrifuged at 16000 g, for 15 min at 4°C and NativePAGE 5% G-250 Sample Additive (Invitrogen, Cat. No. BN2004) was added to reach the final concentration of 0.2%. The electrophoresis running buffer (Invitrogen, Cat. No. BN2001) was prepared according to the manufacturer's protocol for NativePAGE 4-16% Bis-Tris Gel (Invitrogen, Cat. No. BN1002BOX). Running buffer was cooled to 4°C and electrophoresis was run at 4°C. Protein standard (Invitrogen, NativeMark, Cat. No. LC0725) was used to estimate the molecular weight. The gel was run at 80V for 30 min with Light Blue Cathode Buffer (Invitrogen, Cat. No. BN2002)

and then at 250V for 4 hr. The gel was rinsed with transfer buffer (190 mmol/L glycine, 27 mmol/L Tris, and 10% methanol) containing 2% SDS for 30 min, and the proteins transferred to 0.45 μm PVDF membrane at 0.25 A for 60 min in a mini transfer tank (Bio-Rad). The membrane was incubated in 100% methanol for 2 min and stained with fast Green to reveal molecular weight marker and then rinsed with distilled water for 5 min before blocking and antibody incubation.

Biochemical assays

Biochemical kits were used to measure caspase 3/7 activity (Caspase-Glo 3/7 assay kit, Promega, Cat. No. G8090), calpain activity (Calpain-Glo protease assay kit, Promega, Cat. No. G8501), calcineurin activity (Calcineurin cellular activity assay kit, Millipore, Cat. No. 207007), and ATP content (ATP bioluminescence assay kit CLS II, Roche, Cat. No. 11699695001) according to the manufacturers' protocol.

Statistics

Data are shown as mean ± standard error (SEM). When multiple experiments using different numbers of animals were pooled for statistical analysis, the range of animal numbers was indicated in the figure legend. Statistical analyses used GraphPad Prism 7.02 software. Shapiro-Wilk normality test was performed to determine the data distribution. Non-normally distributed data were analyzed by nonparametric tests: Kruskal-Wallis test followed by Dunn's post hoc analysis for >2 groups or Mann-Whitney test for 2 groups. Normally distributed data were analyzed by using parametric tests: one-way analysis of variance (ANOVA) followed by Tukey test for >2 groups or unpaired Student's t-test for 2 groups. Multiple testing adjustment was included in the Dunn's test and Tukey test through controlling the Type I error for the family of comparisons. Experiments consisting of two categorically independent variables and one dependent variable were analyzed by two-way ANOVA. A P value <0.05 was considered statistically significant.

Supplemental Table 1. List of primers for generating mutations.

Gene	Mutation	Primer sequence
CaMKIIδB	K43A	forward: 5'-ctactggacaagagtatgctgccgcaattatcaacaccaaaaagctttc-3' reverse: 5'-gaaagctttttggtgttgataattgcggcagcatactcttgtccagtag-3'
CaMKIIδB	S332E	forward: 5'-ccatcatctgaacactcgaactctccttccttttctttaccccatcc-3' reverse: 5'-ggatggggtaaagaaaaggaaggagagttcgagtgttcagatgatgg-3'
CaMKIIδB	S332A	forward: 5'-ctgaacactcgaactggccttccttttctttaccc-3' reverse: 5'-gggtaaagaaaaggaaggccagttcgagtgttcag-3'
Gsα	Q227L	forward: 5'-gctcatcgcgcaggccgcccaca-3' reverse: 5'-tgtgggcggcctgcgcgatgagc-3'
CREB	S133A	forward: 5'-gaaatcctttcaaggaggcctgcctacaggaaaattttga-3' reverse: 5'-tcaaaattttcctgtaggcaggcctccttgaaaggatttc-3'
MCU	D260Q	forward: 5'-acgggctccatgatctgccacgagtactccc-3' reverse: 5'-gggagtactcgtggcagatcatggagcccgt-3'
MCU	E263Q	forward: 5'-agtaggtgacgggctgcatgatgtcccac-3' reverse: 5'-gtgggacatcatgcagcccgtcacctact-3'
CaM	D20A	forward: 5'-tgccgtcaccatctttagcgaatagggagaaagct-3' reverse: 5'-agctttctccctattcgctaaagatggtgacggca-3'
CaM	D56A	forward: 5'-tgccattgccatcagcagccacttcgttgatcata-3' reverse: 5'-tatgatcaacgaagtggctgctgatggcaatggca-3'
CaM	D93A	forward: 5'-accattcccatccttggcaaacactcggaaggc-3' reverse: 5'-gccttccgagtgtttgccaaggatgggaatggt-3'
CaM	D129A	forward: 5'-gtccgtcgccatcaatagctgcttctctgatcatt-3' reverse: 5'-aatgatcagagaagcagctattgatggcgacggac-3'

Supplemental Table 2. List of primers for generating the CRE element mutations.

CRE element	Mutation	Primer sequence for mutation
CRE I: tgacgcaa	aaaagcaa	forward: 5'-aaactcttgtgaaaaaaacttaaaagcaaagggtcgcgac-3' reverse: 5'-gtcgcgacccttgctttaaagtttttttcacaagagttt-3'
CRE II: tgaggtca	aaaagtca	forward: 5'-caagagaaagttgagaaaagtcagtccaagccaggcatgat-3' reverse: 5'-atcatgcctggcttggactgactttctcaactttctcttg-3'
CRE III: tgacttaa	aaaattaa	forward: 5'-ctagcctcaggatgtagtaaaaattaatctcaacaaaaatccg-3' reverse: 5'-cggatttttgttgagattaatttttactacatcctgaggctag-3'
CRE IV: tgaggtca	aaaagtca	forward: 5'-ggatggtgaggttggaggtgagttaaaagtcatcctgggct-3' reverse: 5'-agcccaggatgactttttaactcacctccaacctcaccatcc-3'

Supplemental Table 3. List of primers for ChIP assay.

Target	Antibody	Primer sequence
MCU promoter	CREB	CRE IV forward: 5'-aaaagggtaattctacgctgg-3' CRE IV reverse: 5'-ttttgctaagatagcaagtaga-3'
GAPDH promoter	RNA Polymerase II	GAPDH forward: 5'-ctgcagtactgtggggaggt-3' GAPDH reverse: 5'-caaaggcggagttaccagag-3'

Supplemental Table 4. List of probes for EMSA assay.

Probe	5' biotin labeling	Probe sequence
CRE IV	Yes	sense: 5'-biotin-ggttggaggtgagtttgaggtcatcctgggctacatagagcat-3' antisense: 5'-biotin-atgctctatgtagcccaggatgacctcaaactcacctccaacc-3'
Mutant CRE IV	Yes	sense: 5'-biotin-ggttggaggtgagttaaaagtcatcctgggctacatagagcat-3' antisense: 5'-biotin-atgctctatgtagcccaggatgacttttaactcacctccaacc-3'
Competitor CRE IV	No	sense: 5'-ggttggaggtgagtttgaggtcatcctgggctacatagagcat-3' antisense: 5'-atgctctatgtagcccaggatgacctcaaactcacctccaacc-3'

Supplemental Table 5. List of primers for RT-qPCR.

Description	Species	Sequence
ANP	Mouse	forward: 5'-attgacaggattggagcccagagt-3' reverse: 5'-tgacacaccacaagggcttaggat-3'
BNP	Mouse	forward: 5'-gccagtctccagagcaattca-3' reverse: 5'-gggccatttcctccgactt-3'
Col1a2	Mouse	forward: 5'-caagcatgtctggttaggagag-3' reverse: 5'-cgactaggacagagtaggtgaa-3'
MCU	Mouse	forward: 5'-tcgtggagaggttagaggac-3' reverse: 5'-agaatgccaaactgggtgg-3'
18S rRNA	Mouse	forward: 5'-gtaacccgttgaaccccatt-3' reverse: 5'-ccatccaatcggtagtagcg-3'

Supplemental Table 6. List of antibodies for Western Blotting.

Name	Company	Cat. No.	Species	Titer
Actin	Sigma	A2103	Rabbit	1:10000
BIP	Cell Signaling	3177	Rabbit	1:2000
Calcineurin	Cell Signaling	2614	Rabbit	1:2500
CaMKII(p-Thr287)	Signalway	11287	Rabbit	1:2500
CaMKIIδ	GeneTex	GTX111401	Rabbit	1:2000
CaMKIIδ (A-17)	Santa Cruz	sc-5392	Goat	1:1000
CaMKIIδB (p-Ser332)	Custom made	NA	Rabbit	1:100
Caspase3	Cell Signaling	9664	Rabbit	1:500
CREB	Cell Signaling	9197	Rabbit	1:2000
CREB	Santa Cruz	sc-186	Rabbit	1:2000
CREB	Cell Signaling	9104	Mouse	1:1000
CREB (p-Ser133)	Cell Signaling	9198	Rabbit	1:1000
EMRE (SMDT1)	Sigma	HPA032117	Rabbit	1:2000
FLAG	Sigma	F1804	Mouse	1:5000
HA	Biolegend	901514	Mouse	1:5000
Lamin A	Santa Cruz	sc-20680	Rabbit	1:1000
MCU	Cell Signaling	14997	Rabbit	1:2500
MCUb	Sigma	HPA048776	Rabbit	1:2000
MICU1	Sigma	HPA037480	Rabbit	1:1000
PDH	Cell Signaling	3205	Rabbit	1:2000
PDH-α1 (p-Ser293)	Cell Signaling	31866	Rabbit	1:2000
PDI	Cell Signaling	3501	Rabbit	1:2000
PLN (Thr17)	Badrilla	A010-13	Rabbit	1:2000
SERCA2	Cell Signaling	4388	Rabbit	1:5000
SLC24A6 (NCLX)	Sigma	SAB2102181	Rabbit	1:500
VDAC1	Cell Signaling	4661	Rabbit	1:5000
Anti-Goat IgG	Millipore	AP107P	Rabbit	1:10000
Anti-Mouse IgG	Millipore	AP181P	Goat	1:10000
Anti-Rabbit IgG	Millipore	AP187P	Goat	1:10000
Anti-Mouse IgG (Light chain specific)	Jackson ImmunoResearch	115-035-174	Goat	1:10000
Anti-Rabbit IgG (Light chain specific)	Jackson ImmunoResearch	211-032-171	Mouse	1:10000

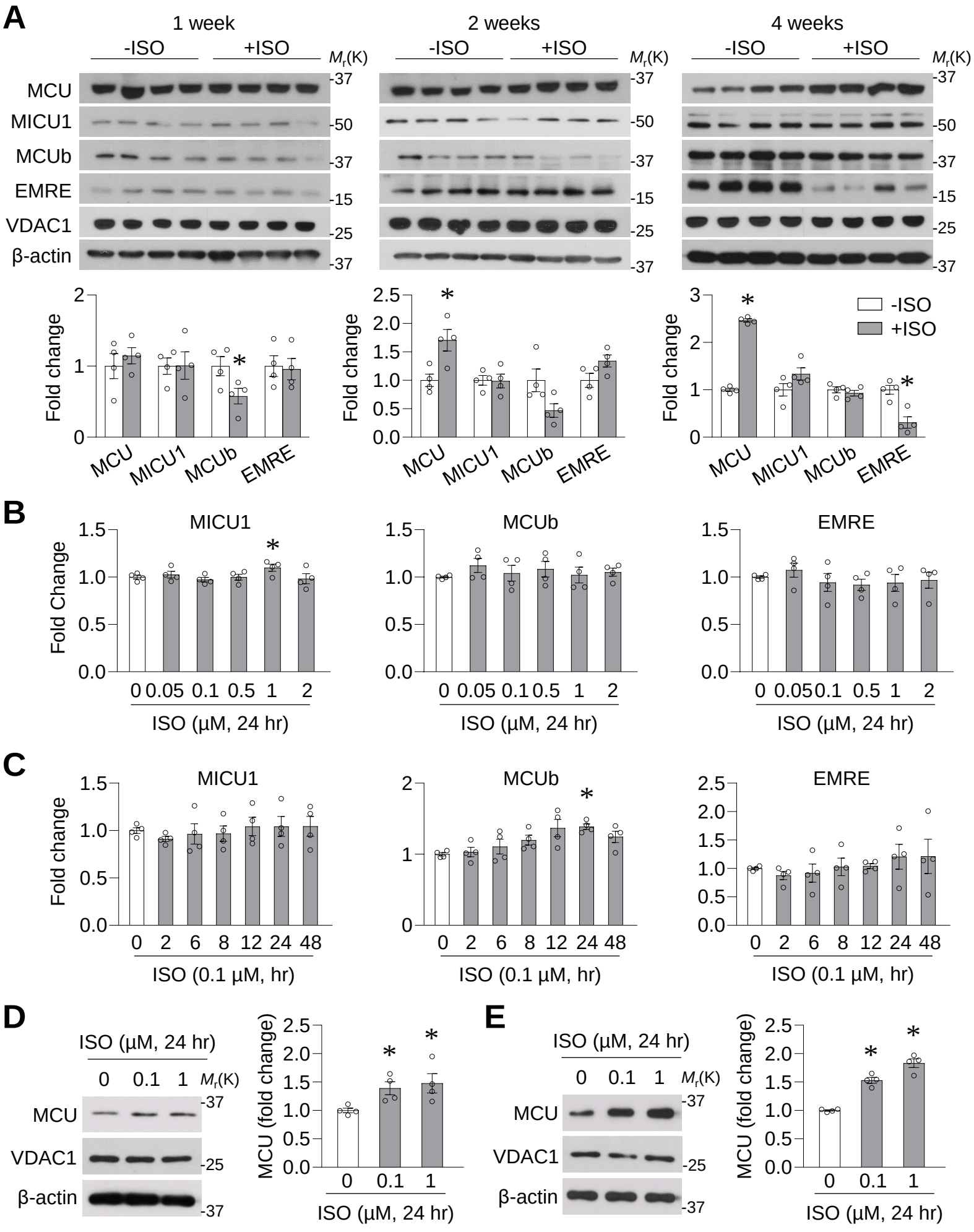

A
1 week
2 weeks
4 weeks
-ISO
+ISO
M_r(K)
MCU
MICU1
MCUb
EMRE
VDAC1
β-actin
-37
-50
-37
-15
-25
-37
Fold change
-ISO
+ISO
B
MICU1
MCUb
EMRE
Fold Change
0 0.05 0.1 0.5 1 2
ISO (μM, 24 hr)
C
MICU1
MCUb
EMRE
Fold change
0 2 6 8 12 24 48
ISO (0.1 μM, hr)
D
ISO (μM, 24 hr)
0 0.1 1
M_r(K)
MCU
VDAC1
β-actin
-37
-25
-37
MCU (fold change)
ISO (μM, 24 hr)
E
ISO (μM, 24 hr)
0 0.1 1
M_r(K)
MCU
VDAC1
β-actin
-37
-25
-37
MCU (fold change)
ISO (μM, 24 hr)

Figure S1. Effects of ISO on MCU and its associated proteins. A, Representative Western blotting images and summarized data showing the levels of MCU and its associated proteins: MICU1, MCUb, and EMRE in whole heart samples after ISO administration for 1 week (*: P=0.0506 vs. –ISO for MCUb), 2 weeks (*: P=0.0018 vs. –ISO for MCU), or 4 weeks (*: P=6.21E-08 or 0.0043 vs. –ISO for MCU or EMRE, respectively). n=4. **B**-**C**, Summarized data showing dose- (**B**) and time-dependent (**C**) changes in MICU1, MCUb, and EMRE protein levels in adult mouse cardiomyocytes after ISO incubation. In **B**, *: P=0.0002 vs. –ISO for MICU. In **C**, *: P=0.0002 vs. –ISO for MCUb. n=4. **D**-**E**, Representative images and summarized data showing dose-dependent effects of ISO on MCU protein levels in adult rat cardiomyocytes (**D**, *: P=0.0182 or 0.03430 vs. –ISO for 0.1 or 1 μM ISO groups, respectively) or H9C2 myoblast cells (**E**, *: P=5.95E-05 or 3.96E-05 vs. –ISO for 0.1 or 1 μM ISO groups, respectively). n=4.

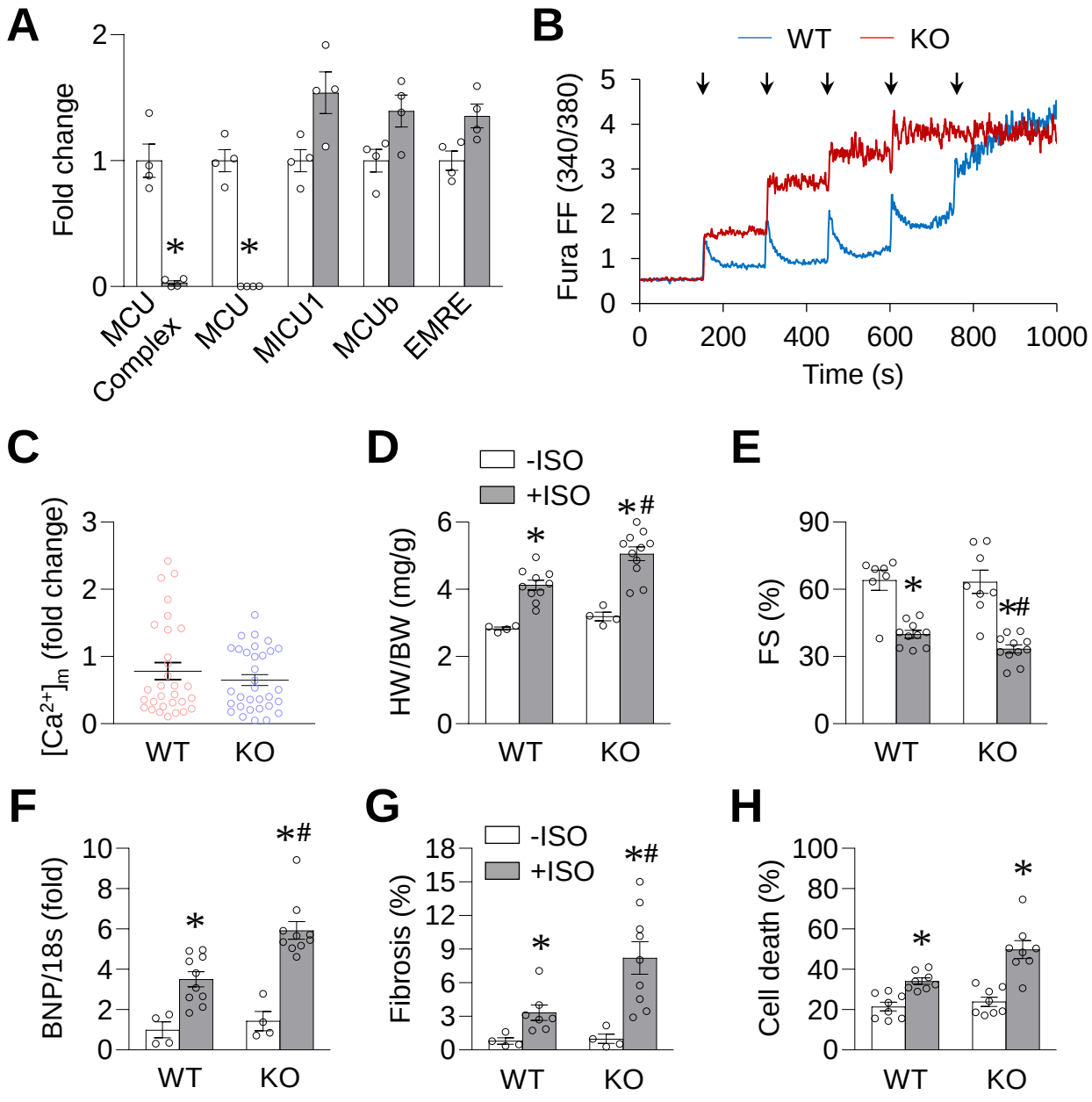

A
Fold change
MCU Complex
MCU
MICU1
MCUb
EMRE
B
WT
KO
Fura FF (340/380)
Time (s)
C
[Ca2+]m (fold change)
WT
KO
D
-ISO
+ISO
HW/BW (mg/g)
E
FS (%)
F
BNP/18s (fold)
G
Fibrosis (%)
H
Cell death (%)

Figure S2. Characterization of the MCU KO mouse model. A, Summarized data showing the levels of MCU complex and MCU and its associated proteins: MICU1, MCUb, and EMRE in MCU KO mice whole heart samples. *: P=0.0003 or 2.68E-05 KO vs. WT for MCU complex or MCU, respectively. n=4. **B**, Representative traces showing Ca^{2+} uptake by freshly isolated mitochondria from the hearts of WT or MCU KO mice. n=4. Arrows indicate the additions of Ca^{2+} (25 μmol/L each time). **C**, Mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$) monitored in freshly isolated and permeabilized adult cardiomyocytes from WT or MCU KO mice. n=30-32 cells from 5 mice in each group. **D**, Heart weight to body weight ratio (HW/BW). *: P=0.0002 WT+ISO vs. WT-ISO, *: P=4.34E-06 KO+ISO vs. KO-ISO, #: P=0.0017 KO+ISO vs. WT+ISO. n=5-6 for -ISO groups and n=10-11 for +ISO groups. **E**, Left ventricular fractional shortening. *: P=6.97E-07 WT+ISO vs. WT-ISO, *: P=4.72E-05 KO+ISO vs. KO-ISO, #: P=0.0308 KO+ISO vs. WT+ISO. n=7-11. **F**, mRNA level of brain natriuretic peptide in mouse heart. *: P=0.0023 WT+ISO vs. WT-ISO, *: P=6.91E-05 KO+ISO vs. KO-ISO, #: P=0.0005 KO+ISO vs. WT+ISO. n=4 for -ISO groups and n=10 for +ISO groups. **G**, Summarized data on Picrosirius red staining for fibrosis in mouse heart. *: P=0.0267 WT+ISO vs. WT-ISO, *: P=0.0082 KO+ISO vs. KO-ISO, #: P=0.0151 KO+ISO vs. WT+ISO. n=4 for -ISO groups and n=7-9 for +ISO groups. **H**, ISO (0.1 μmol/L, 24 hr) induced cell death in adult cardiomyocytes from WT or MCU KO mice. *: P=0.012 WT+ISO vs. WT-ISO, *: P=0.0096 KO+ISO vs. KO-ISO. n=4.

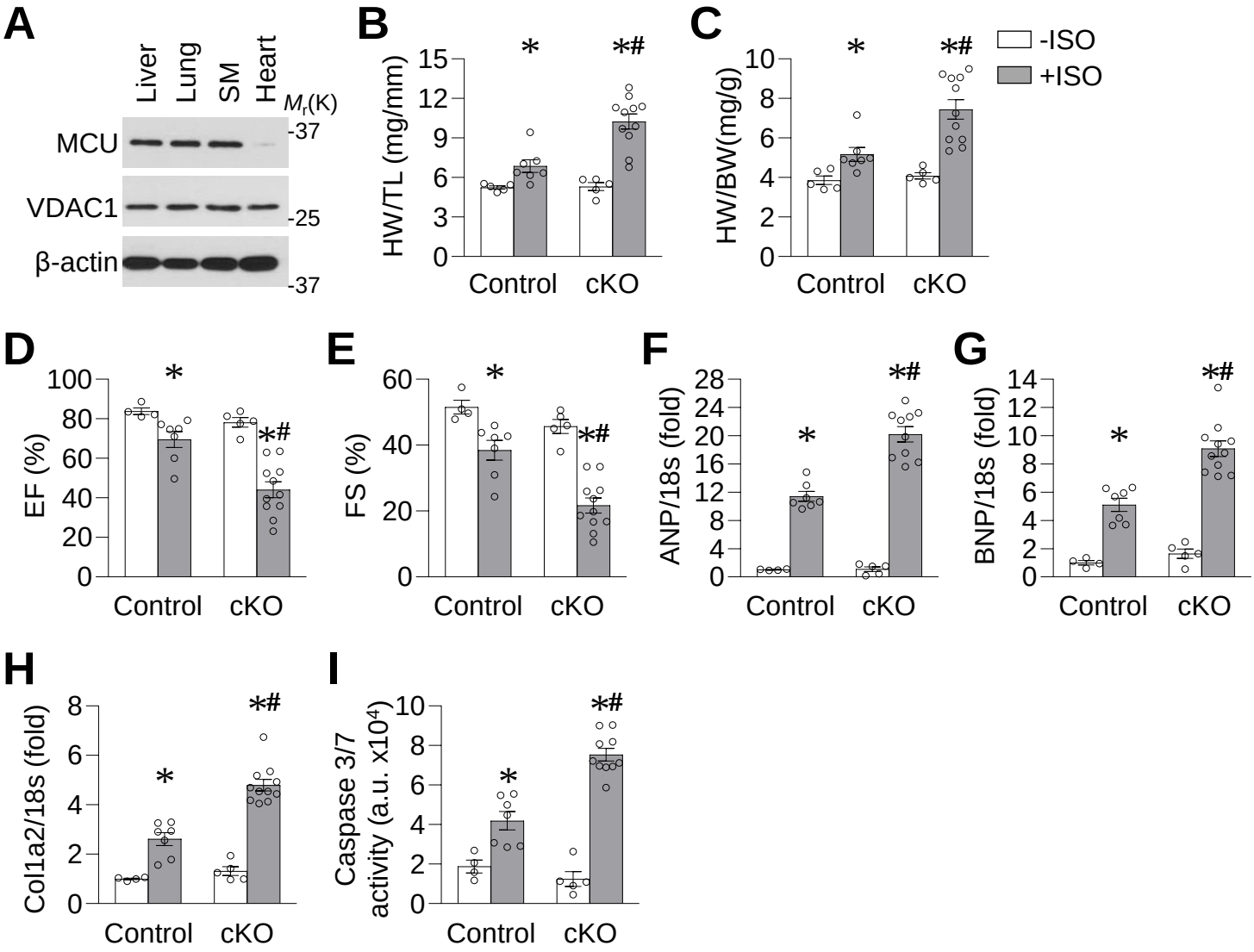

A
Liver
Lung
SM
Heart
Mr(K)
MCU
-37
VDAC1
-25
β-actin
-37
B
HW/TL (mg/mm)
Control
cKO
C
HW/BW(mg/g)
-ISO
+ISO
D
EF (%)
E
FS (%)
F
ANP/18s (fold)
G
BNP/18s (fold)
H
Col1a2/18s (fold)
I
Caspase 3/7 activity (a.u. x10^4)

Figure S3. Inducible, cardiomyocyte-specific MCU KO (cKO) exacerbated cardiac hypertrophy and dysfunction induced by chronic β-AR stimulation. A, Representative Western blotting images showing the deletion of MCU in the heart of cKO mice 4 weeks after tamoxifen induction (40 mg/kg/day for 5 days at the age of 8 weeks). **B**-**C**, Heart weight to tibia length ratio (HW/TL, **B**, *: P=0.0201 Control+ISO vs. Control-ISO, *: P=0.0003 cKO+ISO vs. cKO-ISO, #: P=0.0013 cKO+ISO vs. Control+ISO) and heart weight to body weight ratio (HW/BW, **C**, *: P=0.0147 Control+ISO vs. Control-ISO, *: P=0.0003 cKO+ISO vs. cKO-ISO, #: P=0.0081 cKO+ISO vs. Control+ISO) with (+ISO) or without (-ISO) 4-week ISO administration (10 mg/kg/day). n=5 for -ISO groups and n=7-11 for +ISO groups. Control mice are $MCU^{fl/fl}$ mice with the same tamoxifen treatment. **D**-**E**, Left ventricular ejection fraction (EF, **D**, *: P=0.032 Control+ISO vs. Control-ISO, *: P=6.21E-05 cKO+ISO vs. cKO-ISO, #: P=0.0003 cKO+ISO vs. Control+ISO) and left ventricular fractional shortening (FS, **E**, *: P=0.0014 Control+ISO vs. Control-ISO, *: P=1.4E-05 cKO+ISO vs. cKO-ISO, #: P=0.0002 cKO+ISO vs. Control+ISO) in mouse heart. n=4-5 for -ISO groups and n=7-11 for +ISO groups. **F**-**H**, mRNA levels of atrial natriuretic peptide (ANP, **F**, *: P=1.65E-06 Control+ISO vs. Control-ISO, *: P=1.09E-07 cKO+ISO vs. cKO-ISO, #: P=1.79E-05 cKO+ISO vs. Control+ISO), brain natriuretic peptide (BNP, **G**, *: P=0.0001 Control+ISO vs. Control-ISO, *: P=5.09E-05 cKO+ISO vs. cKO-ISO, #: P=0.0003 cKO+ISO vs. Control+ISO) or collagen type I α2 chain (Col1a2, **H**, *: P=0.0014 Control+ISO vs. Control-ISO, *: P=9.83E-08 cKO+ISO vs. cKO-ISO, #: P=2.77E-06 cKO+ISO vs. Control+ISO) in mouse heart. n=4-5 for -ISO groups and n=7-10 for +ISO groups. **I**, Caspase 3/7 activity in mouse heart. *: P=0.007 in mouse heart. Control+ISO vs. Control-ISO, *: P=1.29E-07 cKO+ISO vs. cKO-ISO, #: P=1.85E-05 cKO+ISO vs. Control+ISO. n=4-5 for -ISO groups and n=7-10 for +ISO groups.

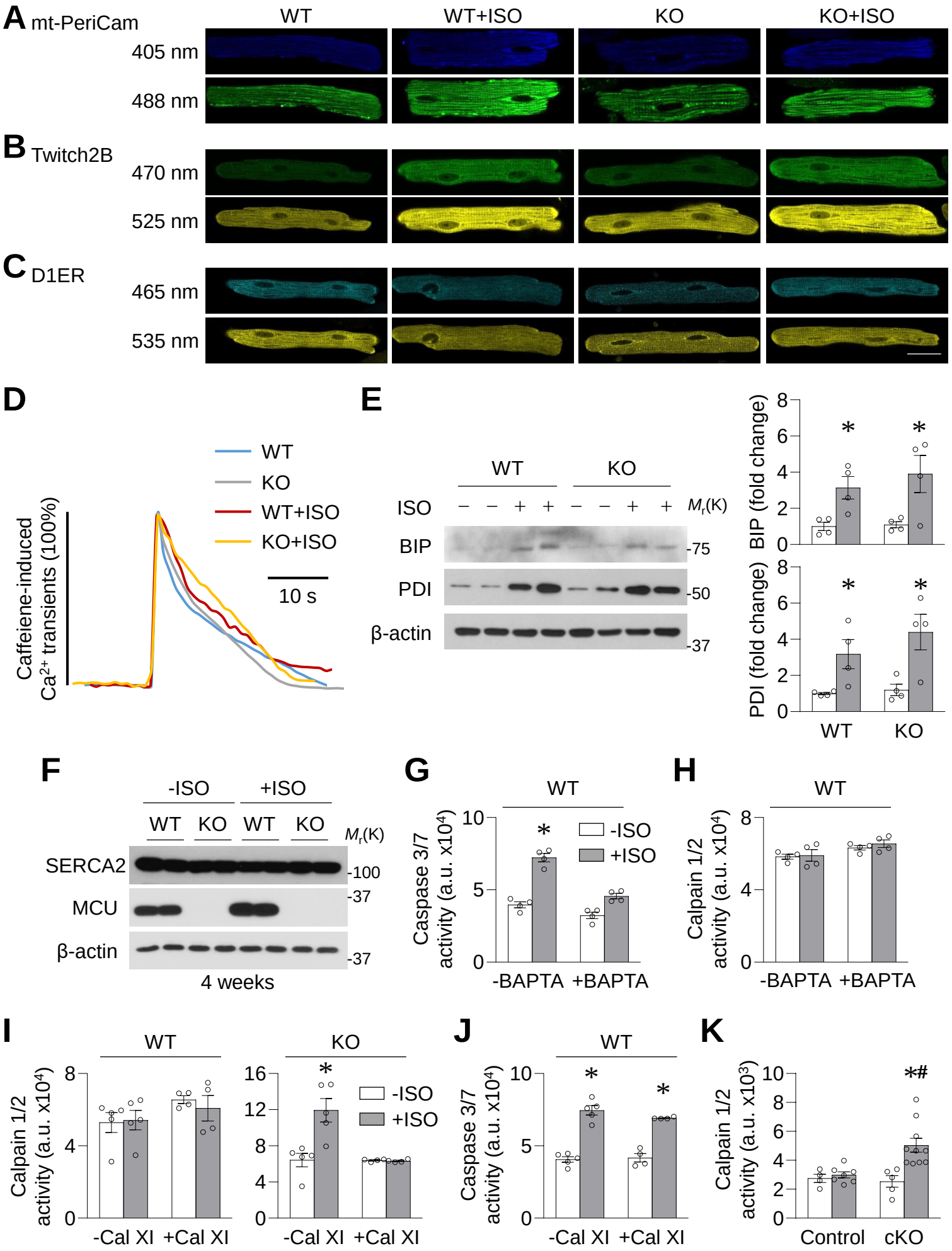

A mt-PeriCam
WT
WT+ISO
KO
KO+ISO
405 nm
488 nm
B Twitch2B
470 nm
525 nm
C D1ER
465 nm
535 nm
D
WT
KO
WT+ISO
KO+ISO
Caffeine-induced Ca2+ transients (100%)
10 s
E
WT
KO
ISO
Mr(K)
BIP
PDI
β-actin
-75
-50
-37
BIP (fold change)
PDI (fold change)
WT
KO
F
-ISO
+ISO
WT
KO
WT
KO
Mr(K)
SERCA2
MCU
β-actin
-100
-37
-37
4 weeks
G
WT
Caspase 3/7 activity (a.u. x10^4)
-ISO
+ISO
-BAPTA
+BAPTA
H
WT
Calpain 1/2 activity (a.u. x10^4)
-BAPTA
+BAPTA
I
WT
KO
Calpain 1/2 activity (a.u. x10^4)
-Cal XI
+Cal XI
-ISO
+ISO
J
WT
Caspase 3/7 activity (a.u. x10^4)
-Cal XI
+Cal XI
K
Calpain 1/2 activity (a.u. x10^3)
Control
cKO

Figure S4. MCU modulated Ca^{2+} homeostasis during chronic ISO incubation. A-C, Representative confocal images of mt-PeriCam (**A**), Twitch2B (**B**) and DIER (**C**). Scale bars = 20 μm. **D**, Representative trace of caffeine-induced Ca^{2+} transients as a measurement of endo/sarcoplasmic reticulum (SR) Ca^{2+} release and reuptake. **E**, endo/sarcoplasmic reticulum stress markers: binding immunoglobulin protein (BIP) and protein disulfide isomerase (PDI) in WT or MCU KO hearts after 4 weeks of ISO administration. For BIP, *: P=0.0187 WT+ISO vs. WT-ISO, *: P=0.036 KO+ISO vs. KO-ISO; for PDI, *: P=0.0351 WT+ISO vs. WT-ISO, *: P=0.0231 KO+ISO vs. KO-ISO. n=4. **F**, Representative Western blotting images showing SERCA2 protein levels in WT or MCU KO hearts after 4 weeks of ISO administration. n=4. **G-H**, Effects of BAPTA-AM (0.1 μmol/L) on ISO (0.1 μmol/L, 24 hr) induced caspase 3/7 activity (**G**, *: P=9.98E-05 +ISO vs. –ISO) or calpain1/2 activity (**H**) in adult cardiomyocytes from WT mice. n=4. **I**, Effects of calpain inhibitor XI (Cal XI, 1 μmol/L) on ISO-induced calpain1/2 activity in WT or MCU KO cardiomyocytes. *: P=0.0019 +ISO vs. –ISO. n=4. **J**, Effects of calpain inhibitor XI (Cal XI, 1 μmol/L) on ISO-induced caspase 3/7 activity in WT cardiomyocytes. *: P=9.98E-05 or 7.91E-05 +ISO vs. –ISO for -Cal XI or +Cal XI groups, respectively. n=4. **K**, Calpain1/2 activity in mouse heart. *: P=0.005 cKO+ISO vs. cKO-ISO, #: P=0.0041 cKO+ISO vs. Control+ISO. n=4-5 for -ISO groups and n=7-10 for +ISO groups.

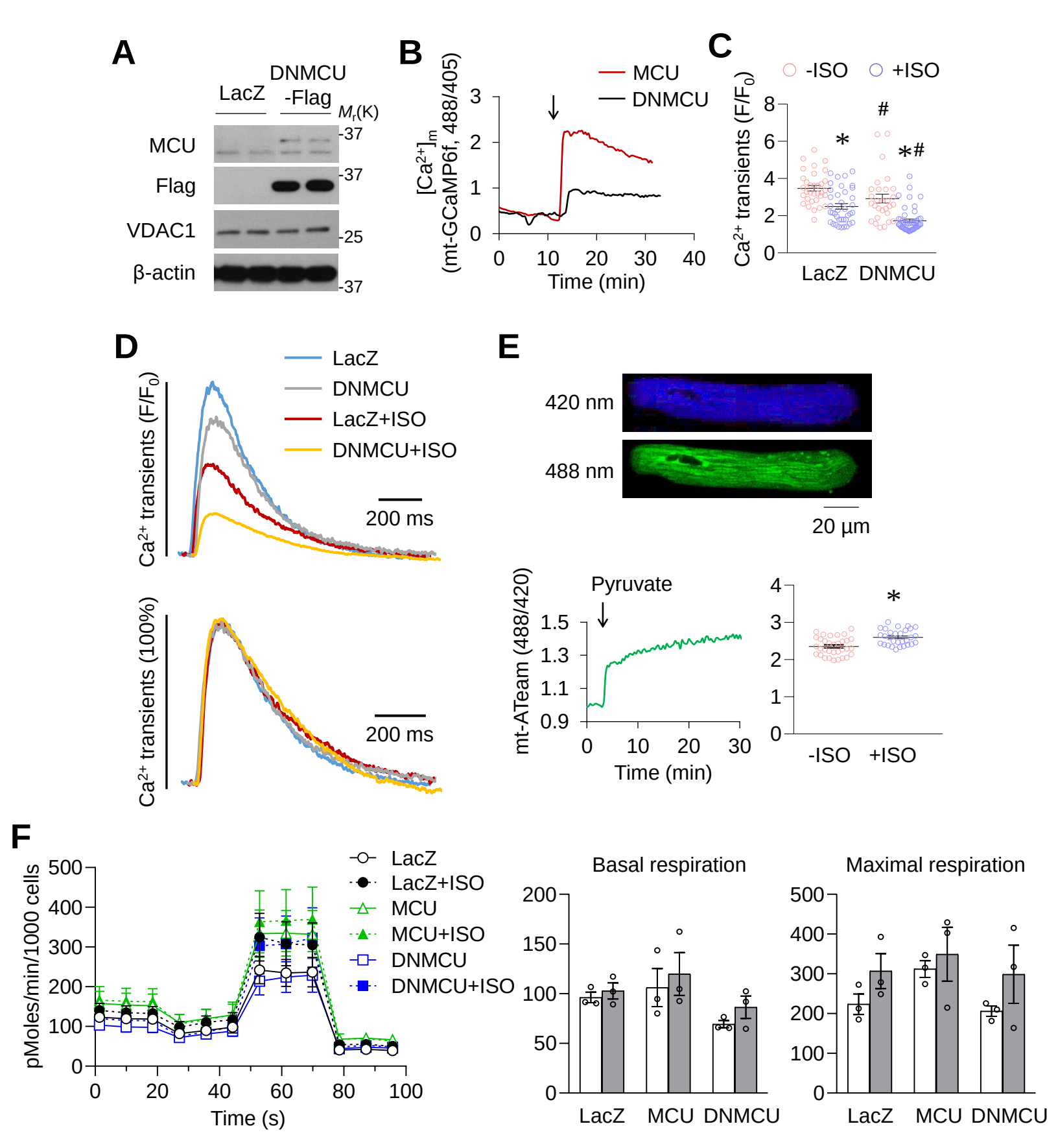

A
LacZ
DNMCU -Flag
M_r(K)
MCU
Flag
VDAC1
β-actin
-37
-37
-25
-37
B
MCU
DNMCU
$[Ca^{2+}]_m$ (mt-GCaMP6f, 488/405)
Time (min)
C
-ISO
+ISO
Ca^{2+} transients (F/F_0)
LacZ
DNMCU
D
LacZ
DNMCU
LacZ+ISO
DNMCU+ISO
Ca^{2+} transients (F/F_0)
200 ms
Ca^{2+} transients (100%)
200 ms
E
420 nm
488 nm
20 μm
Pyruvate
mt-ATeam (488/420)
Time (min)
-ISO
+ISO
F
LacZ
LacZ+ISO
MCU
MCU+ISO
DNMCU
DNMCU+ISO
pMoles/min/1000 cells
Time (s)
Basal respiration
Maximal respiration
LacZ
MCU
DNMCU

Figure S5. DNMCU and mt-Ateam expression in cardiomyocytes. A, Western blotting images showing the expression of Flag-tagged DNMCU in adult rat cardiomyocytes 48 hr after adenovirus-mediated gene transfer. **B**, Representative traces showing mitochondrial Ca^{2+} uptake detected by mt-GCaMP6f in permeabilized adult rat cardiomyocytes overexpressing MCU or DNMCU. Arrow indicates the addition of Ca^{2+} (1 μmol/L). n=3. **C**, Amplitude of pacing-induced cytosolic Ca^{2+} transients (GCaMP6f) in adult rat cardiomyocytes overexpressing dominant negative MCU (DNMCU) and incubated with ISO (0.1 μmol/L, 24 hr). *: P=2E-05 LacZ+ISO vs. LacZ-ISO, #: P=0.0483 DNMCU-ISO vs. LacZ-ISO, *: P=6.2E-07 DNMCU+ISO vs. DNMCU-ISO, #: P=1.663E-05 DNMCU+ISO vs. LacZ+ISO. n=33-60 cells from 5 rats. **D**, Representative traces showing the original (upper) and normalized Ca^{2+} transients in cardiomyocytes under electric pacing (1 Hz). **E**, Representative images (left) showing the expression pattern of mitochondria-targeted ATeam (mt-ATeam), response of mt-ATeam to pyruvate (10 mmol/L, lower left), and mt-ATeam fluorescence after ISO (0.1 μmol/L, 24 hr) incubation (lower right) in adult rat cardiomyocytes. *: P=6.402E-05 +ISO vs. –ISO. n=30-33 cells from 4-5 rats. **F**, Mitochondrial respiration measured by Seahorse XF24 Extracellular Flux Analyzer. The original traces of oxygen consumption rate (OCR) and summarized data of basal and maximal respiration are shown. Adult rat cardiomyocytes overexpressing LacZ, MCU or DNMCU were incubated with ISO (100 nM) for 24 hr. N=3.

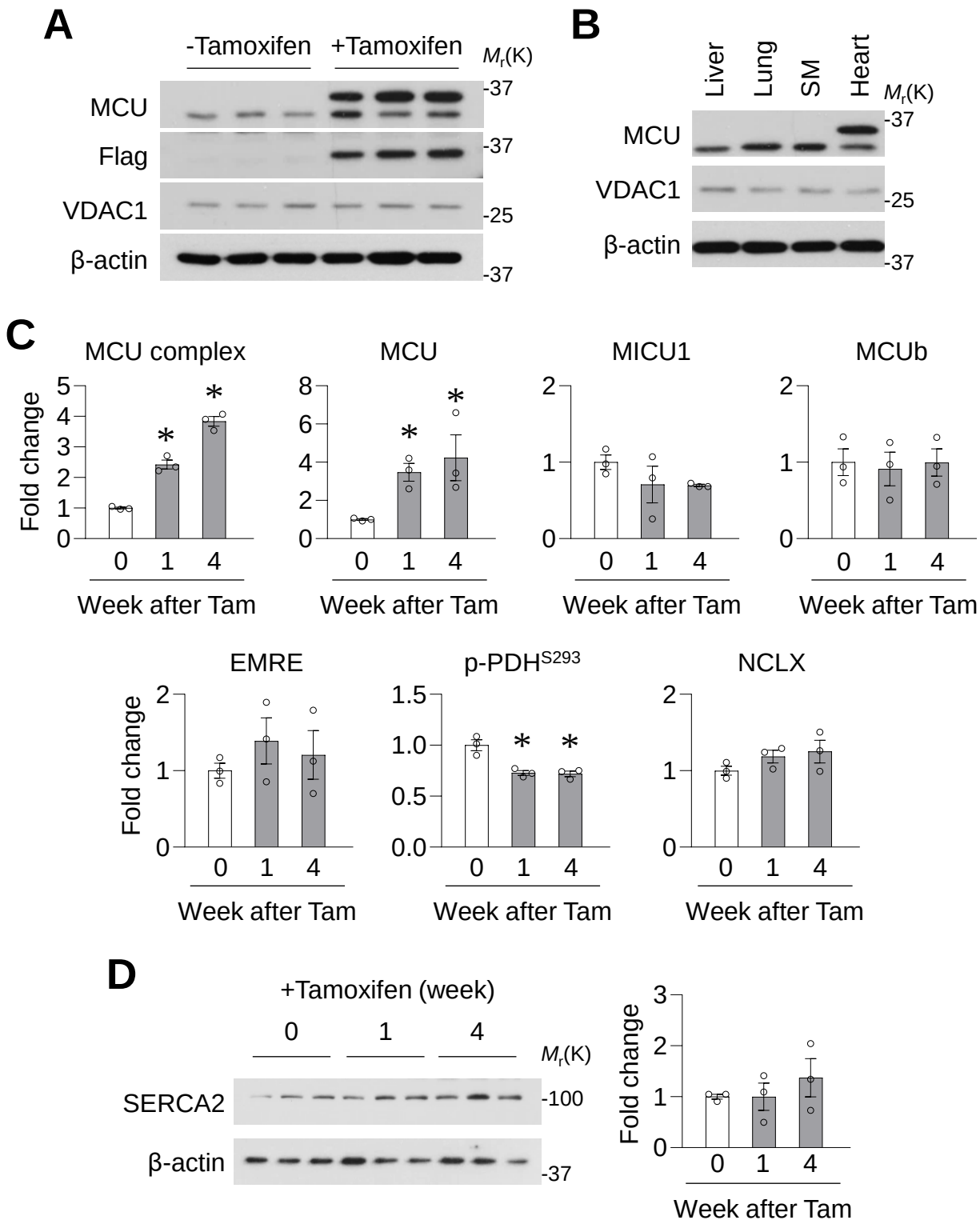

A
-Tamoxifen
+Tamoxifen
Mr(K)
MCU
Flag
VDAC1
β-actin
-37
-37
-25
-37
B
Liver
Lung
SM
Heart
Mr(K)
MCU
VDAC1
β-actin
-37
-25
-37
C
MCU complex
MCU
MICU1
MCUb
Fold change
Week after Tam
EMRE
p-PDHS293
NCLX
D
+Tamoxifen (week)
0
1
4
Mr(K)
SERCA2
β-actin
-100
-37
Fold change
Week after Tam

Figure S6. Characterization of the inducible and heart specific MCU TG mice at baseline. **A**, Representative Western blotting images showing the expression of Flag-tagged MCU in whole heart samples of MCU TG mice (F/F crossed with MCM and after tamoxifen induction). **B**, Representative Western blotting images showing that Flag-tagged MCU was only expressed in the heart after induction. SM: skeletal muscles. **C**, Summarized data showing the levels of MCU complex, MCU, MICU1, MCUb, EMRE, NCLX, and PDH phosphorylation in mitochondria from the hearts of MCU TG mice at 0, 1, or 4 weeks after tamoxifen (Tam) induction. *: P=0.0007 1 or 4 weeks vs. 0 week for MCU complex; *: P=0.0062 or 0.0045 1 or 4 weeks vs. 0 week for MCU; *: P=0.0097 or 0.0095 1 or 4 weeks vs. 0 week for p-PDH^{S293}. n=3. **D**, Western blotting images and summarized data showing SERCA2 levels in whole heart samples of MCU TG mice after tamoxifen induction. n=3.

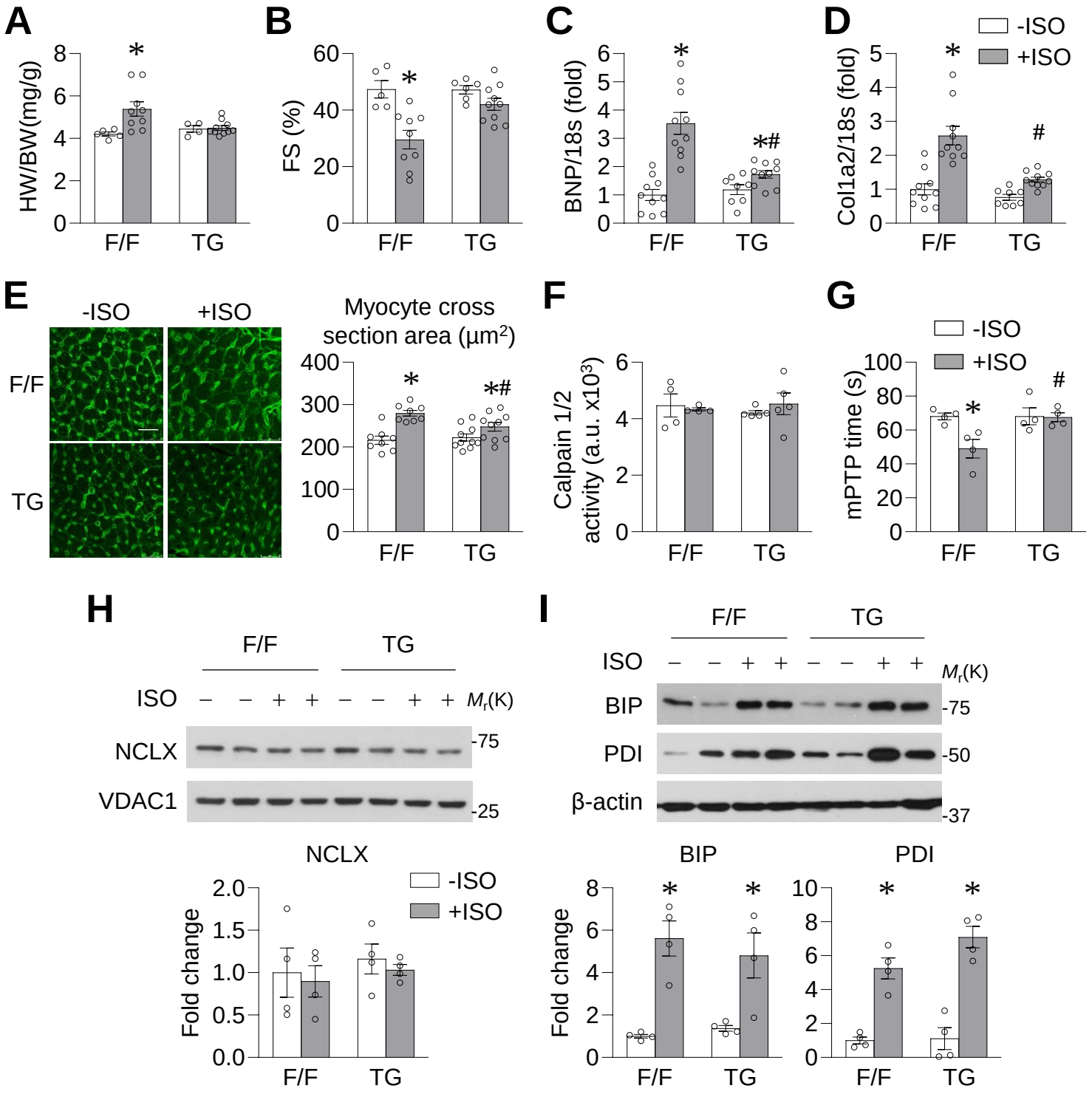

A
HW/BW(mg/g)
F/F
TG
B
FS (%)
C
BNP/18s (fold)
D
-ISO
+ISO
Col1a2/18s (fold)
E
-ISO
+ISO
F/F
TG
Myocyte cross section area (μm²)
F
Calpain 1/2 activity (a.u. x10³)
G
mPTP time (s)
H
ISO
Mr(K)
NCLX
VDAC1
-75
-25
Fold change
I
BIP
PDI
β-actin
-75
-50
-37

Figure S7. MCU TG ameliorated heart dysfunction induced by chronic β-AR activation. **A**, Heart weight to body weight ratio (HW/BW) in MCU floxed/floxed (F/F) or MCU TG (TG) mice after 4-week ISO administration. *: P=0.0199 F/F+ISO vs. F/F-ISO. n=5 for -ISO groups and n=9-10 for +ISO groups. **B**, Left ventricular fractional shortening in mice after 4-week ISO administration. *: P=0.004 F/F+ISO vs. F/F-ISO. n=5-6 for -ISO groups and n=9-10 for +ISO groups. **C**-**D**, mRNA levels of BNP (**C**, *: P=7.9E-05 F/F+ISO vs. F/F-ISO, *: P=0.041 TG+ISO vs. TG-ISO, #: P=0.0004 TG+ISO vs. F/F+ISO) or Col1a2 (**D**, *: P=0.0005 F/F+ISO vs. F/F-ISO, #: P=0.0002 TG+ISO vs. F/F+ISO) in mouse hearts. n=8-10. **E**, Representative images and summarized data of wheat germ agglutinin staining showing the quantification of cardiomyocyte cross-sectional area in control (F/F) or TG mouse hearts with or without ISO administration for 4 weeks. *: P=9.77E-05 F/F+ISO vs. F/F-ISO, *: P=0.0494 TG+ISO vs. TG-ISO, #: P=0.025 TG+ISO vs. F/F+ISO. n=8-10. **F**, Calpain1/2 activity measured in F/F or TG hearts after ISO administration for 4 weeks. n=4-5. **G**, mPTP time in F/F or TG cardiomyocytes after ISO (0.1 μmol/L, 24 hr) incubation. *: P=0.007 F/F+ISO vs. F/F-ISO, #: P=0.0104 TG+ISO vs. F/F+ISO. n=4. **H**, Western blotting images and summarized data showing NCLX level in mitochondria from F/F or TG hearts after ISO administration for 4 weeks. n=4. **I**, Endo/sarcoplasmic reticulum stress markers (BIP and PDI) in F/F or TG hearts after ISO administration for 4 weeks. For BIP, *: P=0.0014 F/F+ISO vs. F/F-ISO, *: P=0.0183 TG+ISO vs. TG-ISO, for PDI, *: P=0.0006 F/F+ISO vs. F/F-ISO, *: P=0.0006 TG+ISO vs. TG-ISO. n=4.

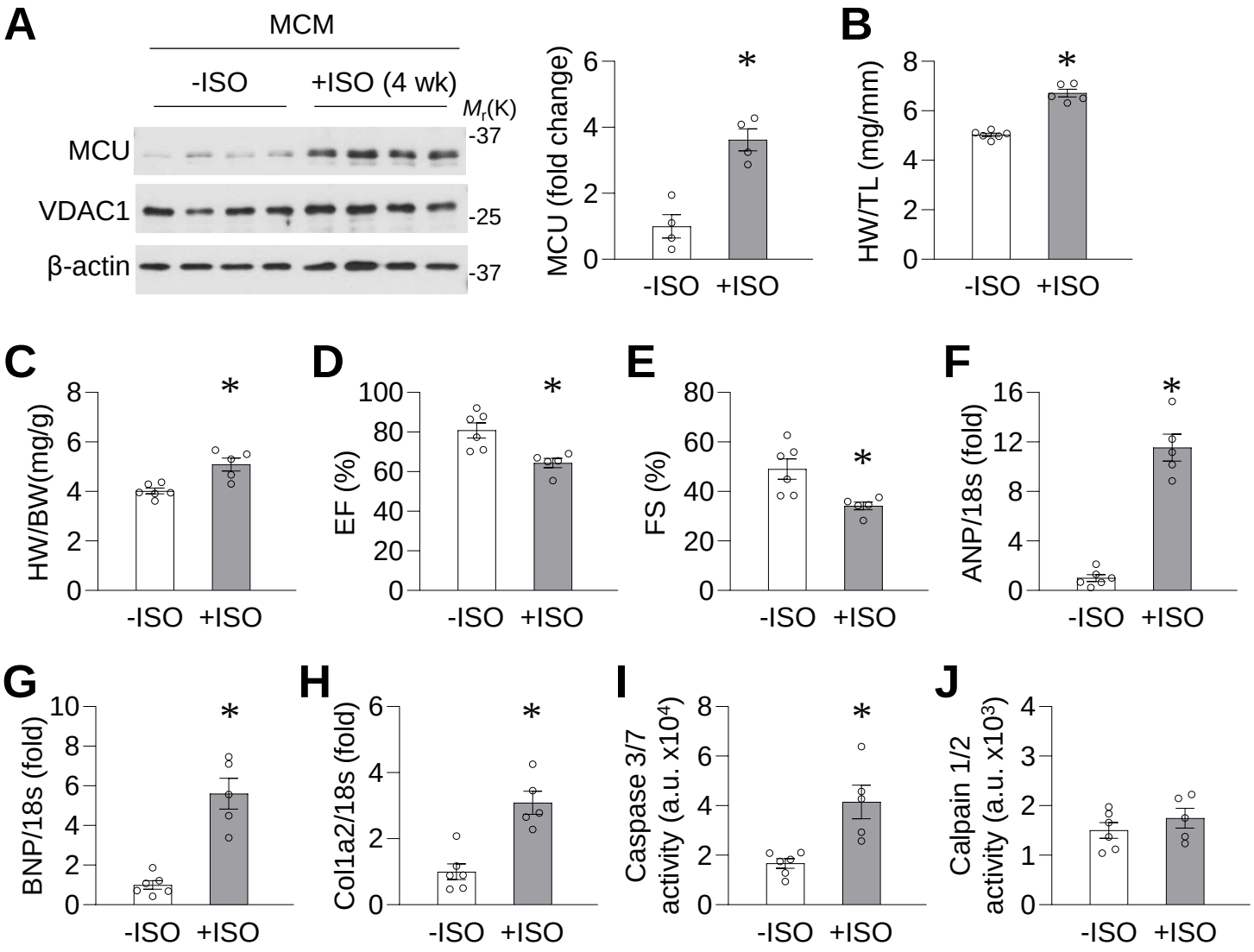

A
MCM
-ISO
+ISO (4 wk)
M_r(K)
MCU
VDAC1
β-actin
-37
-25
-37
MCU (fold change)
B
HW/TL (mg/mm)
C
HW/BW(mg/g)
D
EF (%)
E
FS (%)
F
ANP/18s (fold)
G
BNP/18s (fold)
H
Col1a2/18s (fold)
I
Caspase 3/7 activity (a.u. x10⁴)
J
Calpain 1/2 activity (a.u. x10³)
-ISO
+ISO
*

Figure S8. The α-myosin heavy chain promoter driven tamoxifen-inducible Cre recombinase (MCM) mice exhibited heart hypertrophy and dysfunction during chronic β-AR stimulation. A, Representative Western blotting images and summarized data showing MCU protein level in MCM mice after tamoxifen induction (5 mg/kg/day, intraperitoneal injection for 2 days) and with (+ISO) or without (-ISO) ISO administration (10 mg/kg/day, 4 weeks). *: P=0.0012 +ISO vs. –ISO, n=4. **B**-**C**, Heart weight to tibia length ratio (HW/TL, **B**, *: P=2.35E-06 +ISO vs. –ISO) and heart weight to body weight ratio (HW/BW, **C**, *: P=0.003 +ISO vs. –ISO) of MCM mice. n=6 for -ISO group and n=5 for +ISO group. **D**-**E**, Left ventricular ejection fraction (EF, **D**, *: P=0.0067 +ISO vs. –ISO) and left ventricular fractional shortening (FS, **E**, *: P=0.003 +ISO vs. –ISO) in MCM mice. n=6 for -ISO group and n=5 for +ISO group. **F**-**H**, mRNA levels of atrial natriuretic peptide (ANP, **F**, *: P=0.0122 +ISO vs. –ISO), brain natriuretic peptide (BNP, **G**, *: P=0.0001 +ISO vs. –ISO) and collagen type I α2 chain (Col1a2, **H**, *: P=0.0007 +ISO vs. –ISO) in MCM mice hearts. n=6 for -ISO group and n=5 for +ISO group. **I**-**J**, Caspase 3/7 activity (**I**, *: P=0.004 +ISO vs. –ISO) and Calpain1/2 activity (**J**) in MCM mice hearts. n=6 for -ISO group and n=5 for +ISO group.

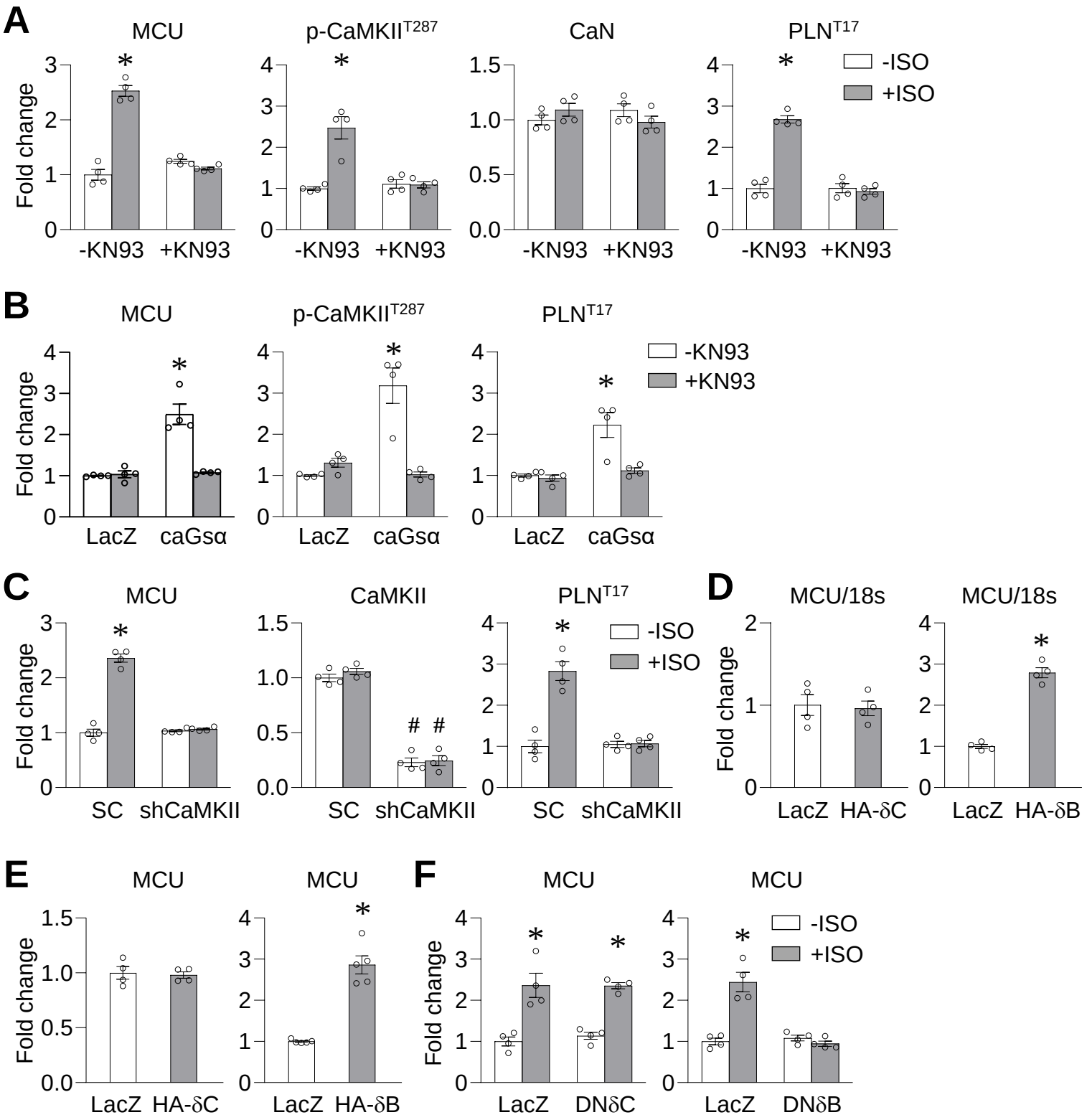

A
MCU
p-CaMKII^{T287}
CaN
PLN^{T17}
Fold change
-ISO
+ISO
-KN93
+KN93
B
MCU
p-CaMKII^{T287}
PLN^{T17}
Fold change
-KN93
+KN93
LacZ
caGsα
C
MCU
CaMKII
PLN^{T17}
Fold change
-ISO
+ISO
SC
shCaMKII
D
MCU/18s
Fold change
LacZ
HA-δC
HA-δB
E
MCU
Fold change
LacZ
HA-δC
HA-δB
F
MCU
Fold change
-ISO
+ISO
LacZ
DNδC
DNδB

Figure S9. CaMKIIδB regulated MCU expression. A, Effects of CaMKII inhibitor KN93 (0.5 μmol/L) on ISO (0.1 μmol/L, 24 hr)-induced MCU upregulation (*: P=3.2E-05 +ISO vs. –ISO), CaMKII phosphorylation at T287 (p-CaMKII^{T287}, *: P=0.0017 +ISO vs. –ISO), calcineurin (CaN) protein levels and phospholamban phosphorylation at T17 (PLN^{T17}, *: P=1.55E-05 +ISO vs. –ISO). n=4. **B**, Effects of KN93 on constitutive active Gsα (caGsα)-induced MCU upregulation (*: P=0.0012 caGsα-KN93 vs. LacZ-KN93), p-CaMKII^{T287} (*: P=0.0025 caGsα-KN93 vs. LacZ-KN93) and PLN^{T17} (*: P=0.0114 caGsα-KN93 vs. LacZ-KN93). n=4. **C**, Effects of CaMKII shRNA (shCaMKII) on ISO-induced MCU upregulation (*: P=9.34E-06 +ISO vs. –ISO), CaMKII protein levels (#: P=5.89E-06 shCaMKII-ISO vs.SC-ISO, #: P=4.66E-06 shCaMKII+ISO vs.SC+ISO) and PLN^{T17} (*: P=0.0006 +ISO vs. –ISO). SC, scrambled control shRNA. n=4. **D-E**, Effects of overexpression of HA-tagged wild type CaMKIIδC (HA-δC) or CaMKIIδB (HA-δB) on MCU mRNA levels (**D**, *: P=1.08E-05 HA-δB vs. LacZ) or protein levels (**E**, *: P=2.95E-05 HA-δB vs. LacZ). n=4. **F**, Effect of overexpression of dominant negative CaMKIIδC (DNδC) or dominant negative CaMKIIδB (DNδB) on ISO-induced MCU upregulation. Left panel, *: P=0.0048 LacZ+ISO vs. LacZ-ISO, *: P=3.87E-05 DNδC+ISO vs. DNδC-ISO. Right panel, *: P=0.0011 LacZ+ISO vs. LacZ-ISO. n=4.

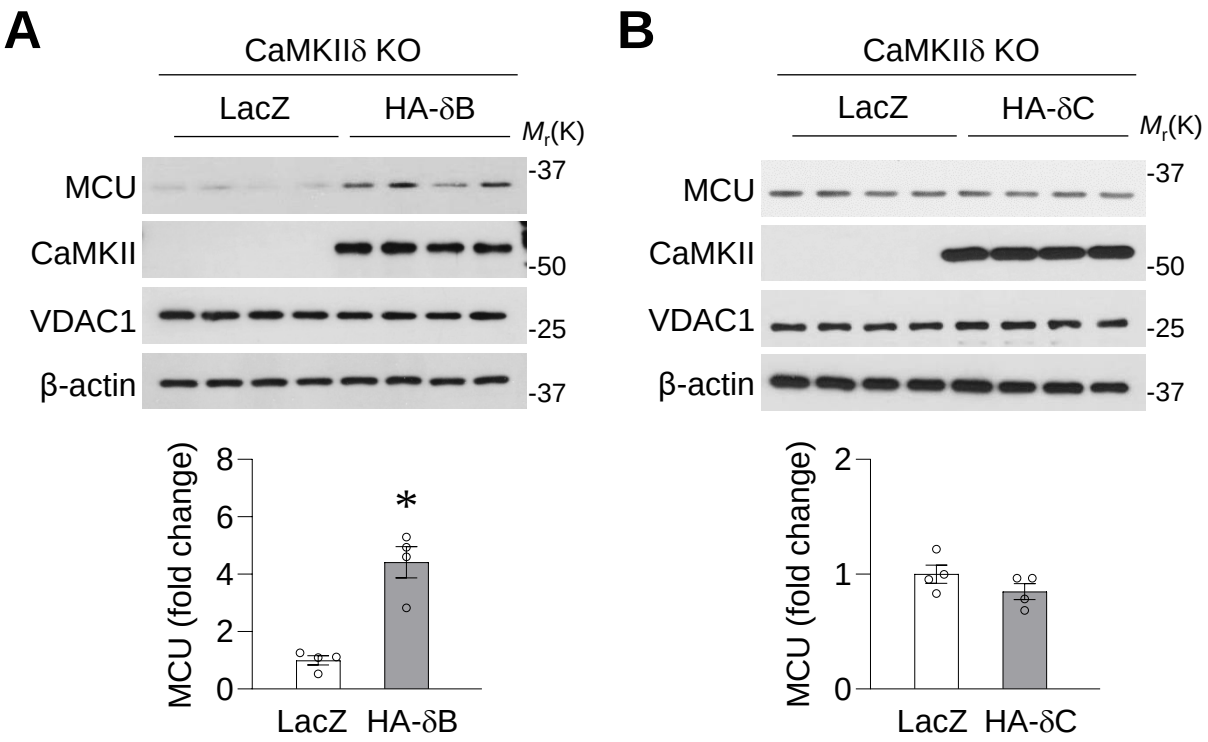

A
CaMKIIδ KO
LacZ
HA-δB
Mr(K)
MCU
CaMKII
VDAC1
β-actin
-37
-50
-25
-37
MCU (fold change)
8
6
4
2
0
*
LacZ
HA-δB
B
CaMKIIδ KO
LacZ
HA-δC
Mr(K)
MCU
CaMKII
VDAC1
β-actin
-37
-50
-25
-37
MCU (fold change)
2
1
0
LacZ
HA-δC

Figure S10. CaMKIIδB overexpression on CaMKIIδ null background upregulated MCU. Western blotting images and summarized data showing the protein levels of MCU in adult cardiomyocytes isolated from CaMKII KO mice and overexpressing CaMKIIδB (**A**) or CaMKIIδC (**B**). *: P=0.001 HA-δB vs. LacZ. n=4.

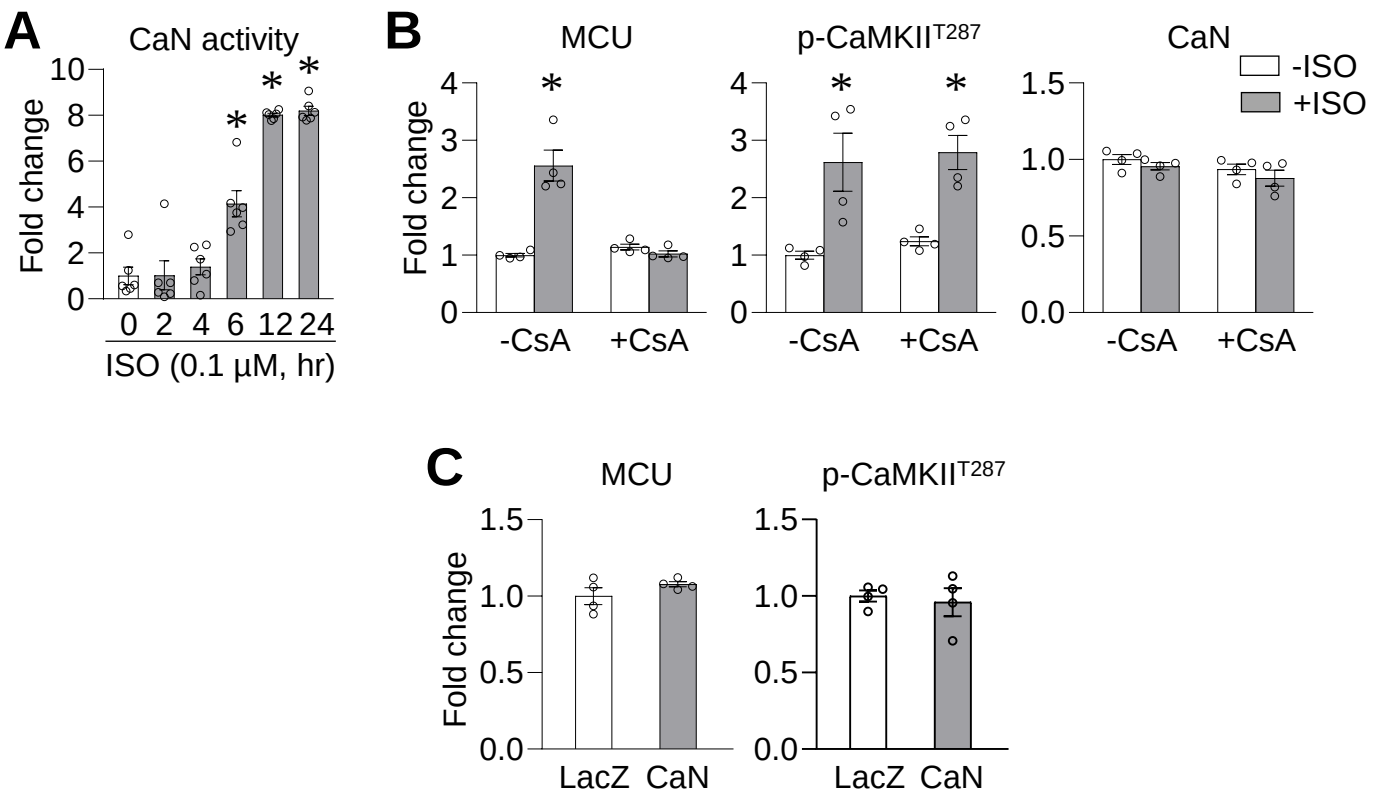

A
CaN activity
Fold change
10
8
6
4
2
0
0 2 4 6 12 24
ISO (0.1 μM, hr)
B
MCU
p-CaMKII T287
CaN
-ISO
+ISO
Fold change
-CsA
+CsA
C
MCU
p-CaMKII T287
Fold change
LacZ CaN

Figure S11. Role of calcineurin in ISO-induced MCU upregulation. A, Calcineurin (CaN) activity at different time points after ISO (0.1 μmol/L) incubation. *: P=0.001, 5.71E-09 or 1.19E-08 6, 12, or 24 hr vs. 0 hr, respectively. n=4. **B**, Effects of CaN inhibitor cyclosporine A (CsA, 1 μmol/L) on ISO-induced MCU upregulation (*: P=0.0012 +ISO vs. –ISO), CaMKII phosphorylation at T287 (*: P=0.019 +ISO vs. –ISO, *: P=0.0024 CsA+ISO vs. CsA-ISO), and CaN protein levels. n=4. **C**, Effects of CaN on MCU protein levels and CaMKII phosphorylation at T287.

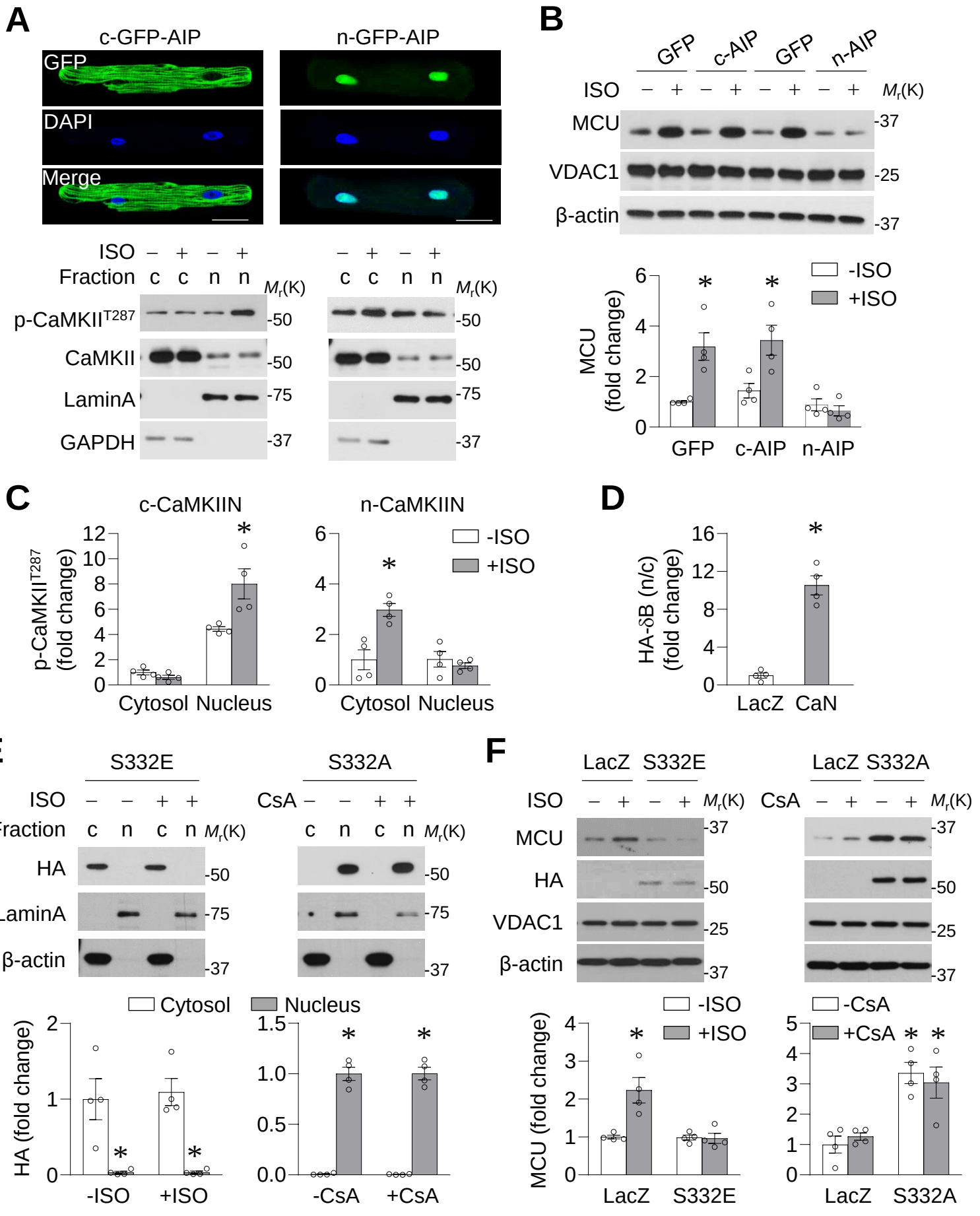

A
c-GFP-AIP
n-GFP-AIP
GFP
DAPI
Merge
ISO
Fraction
p-CaMKII^{T287}
CaMKII
LaminA
GAPDH
M_r(K)
B
GFP
c-AIP
n-AIP
MCU
VDAC1
β-actin
MCU (fold change)
-ISO
+ISO
C
c-CaMKIIN
n-CaMKIIN
p-CaMKII^{T287} (fold change)
Cytosol
Nucleus
D
HA-δB (n/c) (fold change)
LacZ
CaN
E
S332E
S332A
CsA
HA
HA (fold change)
-CsA
+CsA
F
LacZ
S332E
S332A
MCU (fold change)

Figure S12. The phosphorylation status of S332 regulated nuclear translocation of CaMKIIδB and MCU expression. A, Targeting GFP-tagged AIP to cytosol (c-GFP-AIP) or nucleus (n-GFP-AIP) abolished ISO-induced CaMKII phosphorylation at T287 in the corresponding compartments. **B**, n-AIP but not c-AIP blocked ISO-induced MCU upregulation in adult mouse cardiomyocytes. *: P=0.0071 GFP+ISO vs. GFP-ISO, *: P=0.0233 c-AIP+ISO vs. c-AIP-ISO. n=4. **C**, Effects of overexpression of cytosol-targeted and RFP-tagged CaMKIIN (c-CaMKIIN) or nucleus-targeted and RFP-tagged CaMKIIN (n-CaMKIIN) on ISO (0.1 μmol/L, 24 hr)-induced CaMKII phosphorylation at T287 in cytosolic or nuclear fraction. Left panel, *: P=0.0255 +ISO vs. –ISO in nuclear fraction. Right panel, *: P=0.0054 +ISO vs. –ISO in cytosolic fraction. n=4. **D**, Effect of overexpression of calcineurin (CaN) on nuclear vs. cytosolic location of HA-tagged CaMKIIδB (HA-δB). *: P=9.95E-05 CaN vs. LacZ. n=4. **E**, Effects of ISO on intracellular location of CaMKIIδB S332E (Left panel, *: P=0.0016 or 0.0011 Nucleus vs. Cytosol in –ISO or +ISO groups, respectively) or CsA on the location of CaMKIIδB S332A (Right panel, *: P=5.43E-06 or 3.84E-06 Nucleus vs. Cytosol in –CsA or +CsA groups, respectively) in adult mouse cardiomyocytes. n=4. **F**, Effects of S332E or S332A mutations on MCU expression in adult cardiomyocytes. Left panel, *: P=0.0106 +ISO vs. -ISO. Right panel, *: P=0.002 or 0.0149 S332A vs. LacZ or S332A+CsA vs. LacZ+CsA, respectively. n=4.

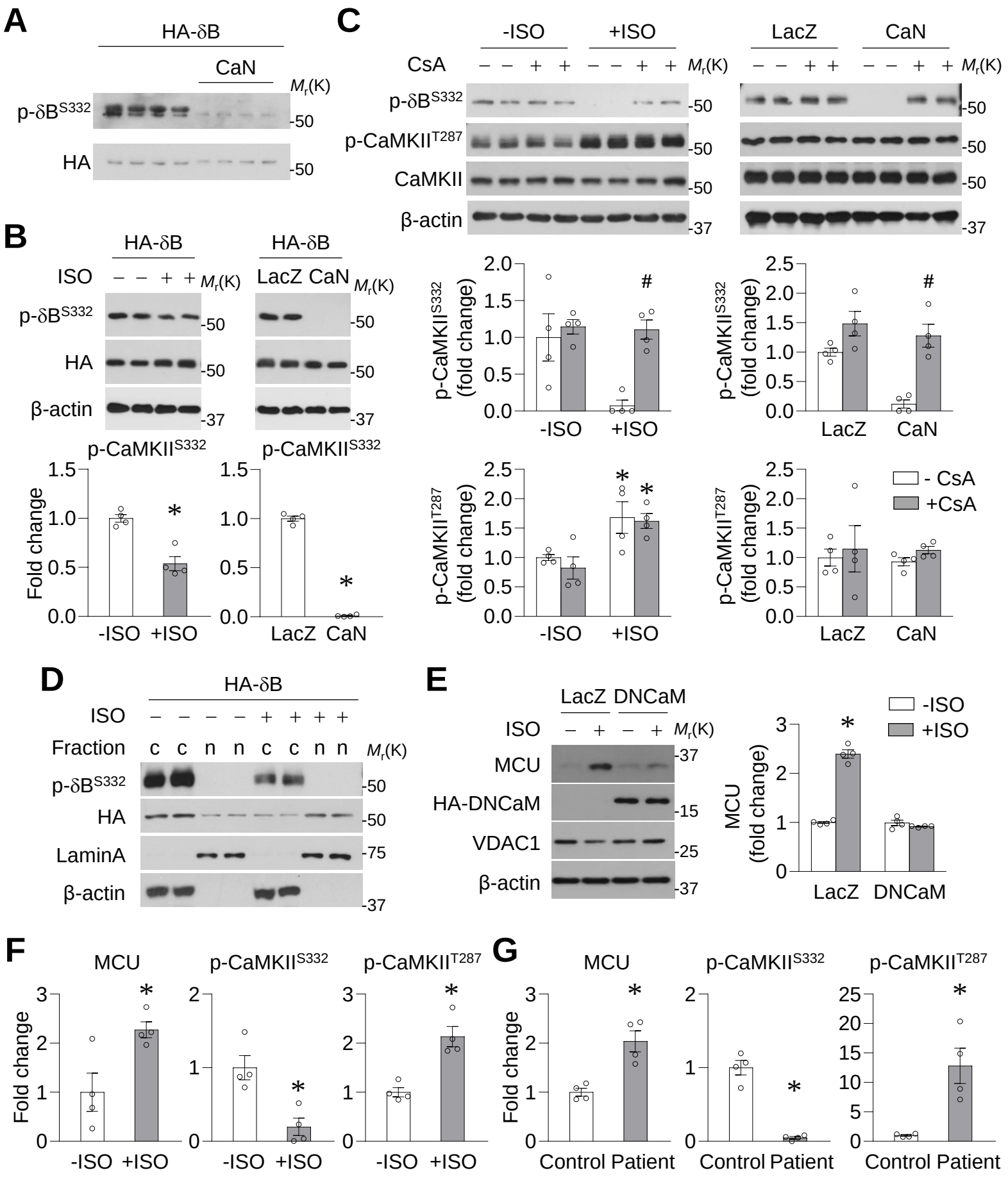
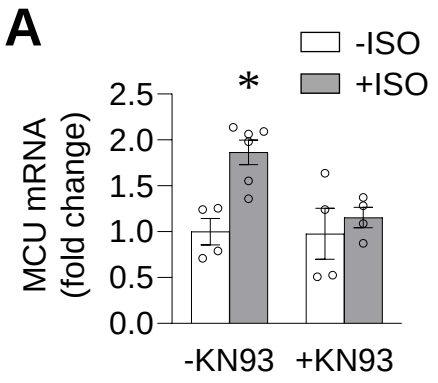
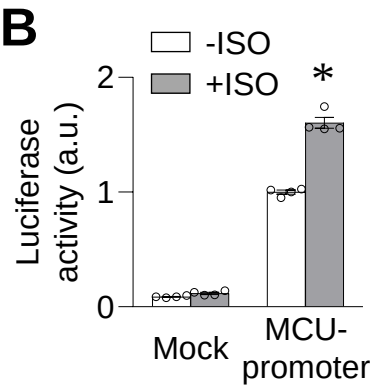

A
HA-δB
CaN
M_r(K)
p-δB^{S332}
-50
HA
-50
C
-ISO
+ISO
LacZ
CaN
CsA
– – + + – – + + M_r(K)
– – + + – – + + M_r(K)
p-δB^{S332}
-50
p-CaMKII^{T287}
-50
CaMKII
-50
β-actin
-37
B
HA-δB
HA-δB
ISO
– – + + M_r(K)
LacZ CaN M_r(K)
p-δB^{S332}
-50
HA
-50
β-actin
-37
p-CaMKII^{S332}
p-CaMKII^{S332}
Fold change
-ISO +ISO
LacZ CaN
p-CaMKII^{S332} (fold change)
-ISO +ISO
LacZ CaN
p-CaMKII^{T287} (fold change)
-ISO +ISO
LacZ CaN
- CsA
+CsA
D
HA-δB
ISO
– – – – + + + +
Fraction c c n n c c n n M_r(K)
p-δB^{S332}
-50
HA
-50
LaminA
-75
β-actin
-37
E
LacZ DNCaM
ISO
– + – + M_r(K)
MCU
-37
HA-DNCaM
-15
VDAC1
-25
β-actin
-37
-ISO
+ISO
MCU (fold change)
LacZ DNCaM
F
MCU
p-CaMKII^{S332}
p-CaMKII^{T287}
Fold change
-ISO +ISO
-ISO +ISO
-ISO +ISO
G
MCU
p-CaMKII^{S332}
p-CaMKII^{T287}
Fold change
Control Patient
Control Patient
Control Patient

Figure S13. CaMKIIδB de-phosphorylation at S332 promoted its nuclear translocation and MCU expression. A, Western blotting images showing that a custom-made phosphospecific antibody detected CaMKIIδB S332 de-phosphorylation by CaN *in vitro* in the presence of calmodulin (CaM) and Ca^{2+}. n=4. **B**, Effects of ISO or CaN on S332 phosphorylation status of HA-δB in adult mouse cardiomyocytes. *: P=0.0013 +ISO vs. –ISO, *: P=2.07E-08 CaN vs. LacZ. n=4. **C**, Effects of CaN inhibitor cyclosporine A (CsA) on ISO- or CaN-induced T287 phosphorylation and S332 de-phosphorylation of endogenous CaMKII in adult mouse cardiomyocytes. Left panels, #: P=0.0005 +ISO+CsA vs. +ISO-CsA, *: P=0.00476 +ISO-CsA vs. –ISO-CsA, *: P=0.013 +ISO+CsA vs. –ISO+CsA. Right panels, #: P=0.0014 CaN+CsA vs. CaN-CsA. n=4. **D**, Effects of ISO-induced S332 de-phosphorylation on the nuclear or cytosolic location of HA-δB in adult mouse cardiomyocytes. n=4. **E**, Effects of overexpressing HA-tagged dominant negative calmodulin (DNCaM) on ISO-induced MCU upregulation. *: P=4.04E-06 LacZ+ISO vs. LacZ-ISO. n=4. **F**-**G**, Summarized data showing protein levels of MCU and CaMKIIδB phosphorylation at T287 or S332 in mouse hearts after 4-week ISO administration (**F**) or in failing heart samples from patients (**G**). For **F**, *: P=0.024, 0.0077 or 0.0023 +ISO vs. –ISO for MCU, p-CaMKII^{S332} or p-CaMKII^{T287}, respectively. For **G,** *: P=0.004, 8.29E-09 or 0.0078 Patient vs. Control for MCU, p-CaMKII^{S332} or p-CaMKII^{T287}, respectively. n=4.





C

CRE	Score	Start	End	Predicted sequence
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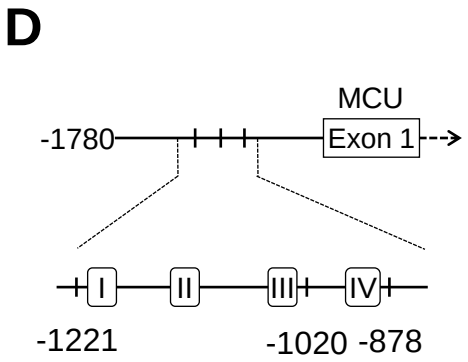
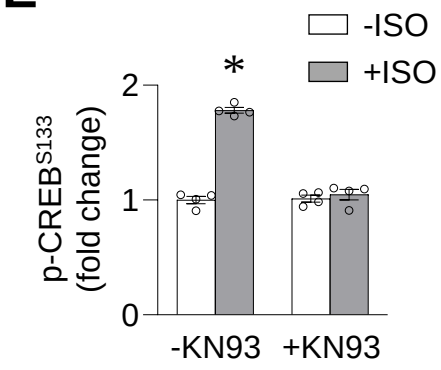
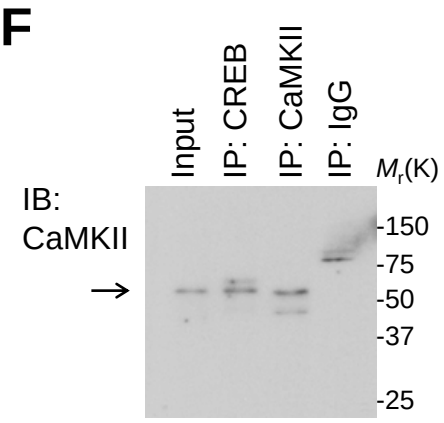
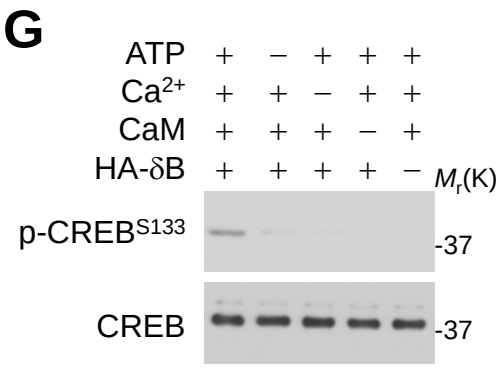
II	9.77	-1139	-1132	TGAGGTCA
III	6.39	-1034	-1027	TGACTTAA
IV	9.77	-900	-893	TGAGGTCA









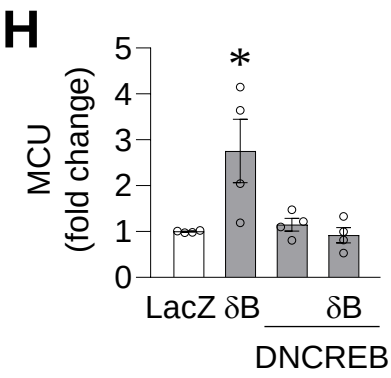


Figure S14. CaMKIIδB regulated MCU gene expression via CREB. A, RT-qPCR analysis of MCU mRNA levels in adult mouse cardiomyocytes after ISO (0.1 μmol/L, 24 hr) incubation with or without KN93 (0.5 μmol/L). *: P=0.0027 +ISO vs. –ISO. n=4. **B**, Luciferase activity assay showing MCU promoter activity in H9C2 cells after ISO incubation. *: P=1.91E-05 +ISO vs. –ISO. n=4. **C**-**D**, The sequences (**C**) and location (**D**) of the 4 CREB binding elements (CRE I-IV) in MCU promoter. **E**, Effects of KN93 on ISO-induced CREB phosphorylation at S133 in adult mouse cardiomyocytes. *: P=1.34E-06 +ISO vs. –ISO. n=4. **F**, Co-IP analysis showing the binding of CaMKII with CREB in adult cardiomyocytes. **G**, *In vitro* phosphorylation assay showing that CaMKIIδB phosphorylated CREB at S133 in the presence of ATP, Ca^{2+}, and calmodulin. In **F**-**G**, similar results were obtained from 4 repeats. **H**, Effects of dominant negative CREB (DNCREB) on HA-CaMKIIδB (δB)-induced MCU upregulation in adult mouse cardiomyocytes. *: P=0.0433 δB vs. LacZ. n=4.