SUPPLEMENTAL MATERIAL

MCUb induction protects the heart from post ischemic remodeling

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Online Methods:

Please see the Major Resources Table in the Supplemental Materials.

Animals

All mice used in this study were in the *C57BL/6* genetic background, and equal ratios of both male and female animals were employed. All mice were compared directly to WT controls in the same background, at the same age. Mice were housed and cared for in an AAALAC-accredited animal care facility at Cincinnati Children's Hospital Medical Center and University of California, Davis, which meets or exceeds the requirements of the Office of Laboratory Animal Welfare. Mice were inspected daily by veterinary technicians to ensure healthy conditions. All experimental use of mice was approved by the Institutional Animal Care and Use Committee (IACUC).

Cardiac-specific MCUb overexpressing transgenic mice were generated using the cardiac-specific tetracycline (Tet)-off expression system as described previously.[22] Briefly, a tetracycline transactivator (tTA TG) mouse line with cardiac-specific promoter (α-myosin heavy chain, α-MHC) was crossed with an α-MHC tetracycline-operator containing promoter transgenic line in which the MCUb cDNA from mouse was cloned downstream to allow inducible expression, such that in the absence of tetracycline or doxycycline expression was robust, but with addition of tetracycline or doxycycline expression was extinguished. However, for this study we never shut down the transgenic system, so MCUb expression was constitutive from birth onwards. Transgenic mice were generated in the gene-targeting core at Cincinnati Children's Hospital using the standard practice of injecting the entire agarose gel-purified DNA construct of interest into newly fertilized oocytes in the C57BL/6 genetic background, same as the WT control genetic background.

Mcub-null mice were generated from previously targeted ES cells using the "knockout-first allele" strategy that is targeted between the 1st and 2nd exon with a β-galactosidase/neomycin cDNA cassette that typically disrupts allele expression (*Mcub*$^{tm1a(KOMP)Mbp}$, European Conditional Mouse Mutagenesis program, MGI ID: 1914065). Homozygous targeted mice with this insertion were fully null for MCUb protein expression and were otherwise viable and overtly normal. Mice were also crossed with FLPe mice (the Jackson Laboratory) expressing Flipase to remove the β-galactosidase/neomycin cDNA cassette flanked by FRT sites (through the germline), leaving the allele only with loxP sites flanking exon #2. This provided us with *Mcub*$^{fl/fl}$ mice, which we used for mouse embryonic fibroblast (MEF) generation and cell studies. We also utilized *Mcu*$^{fl/fl}$ mice that were published previously[41] to generate MEF cell lines, and

crossed *Mcu*$^{fl/fl}$ mice with *Mcub*$^{fl/fl}$ mice to generate *Mcu*$^{fl/fl}$*Mcub*$^{fl/fl}$ double floxed mice to produce double targeted MEFs.

Surgery

Ischemia-reperfusion (I/R) injury was performed as previously described.[24,41] Briefly, mice were anesthetized by inhaled 1.5% isoflurane and surgery was conducted in a sterile environment to minimize complications. Mice were closely monitored after surgery and received necessary pain relief treatment (Buprenorphine, 0.03mg/mL, subcutaneous injection) to minimize discomfort. For infarct area measurement, mice were challenged with reversible left coronary artery ligation for 30 minutes or 60 minutes, depending on the experiment, followed by 24 hours of reperfusion. Following reperfusion, the left coronary artery was re-ligated, and Evans blue dye was injected into the left ventricle to demark the non-ischemic tissue. Hearts were excised, sectioned and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) to visualize viable myocardium. Pictures of stained heart sections were obtained by Leica DFC310 FX (Leica Microsystems). Ischemia area and area at risk were quantified by ImageJ software. For cardiac functional analyses post I/R injury, mice undergoing 60 minutes of ischemia were allowed to recover for 2 or 4 wks and then subjected to echocardiographic measurements. For assessment at different timepoints post I/R injury, mice were challenged with 60 minutes of ischemia, and the left ventricle of the hearts were harvested at different time-points post injury (1, 3 or 7days post I/R injury) for either Western blotting analysis or real-time PCR analysis, which will be described in more below.

For myocardial infarction (MI) injury, mice were challenged with permanent irreversible left coronary artery ligation and pain medication was given (Buprenorphine, 0.03mg/mL, subcutaneous injection). Mice were sacrificed and hearts collected for analysis at different time-points post injury (1, 3 or 7days post M/I injury). Where possible, mice were analyzed in a manner blinded to the investigator. Randomization was not necessary because all mice were genetically identical and of similar ages.

Remote Ischemic Pre-Conditioning

Remote ischemic pre-conditioning (RIPC) was performed in mice at 9-12 weeks of age. Mice were anesthetized in a sealed chamber filled with 3% isoflurane. Then mice were moved to a platform with a warming pad for the remote ischemic pre-conditioning procedure while under continued anesthesia (1.75% isoflurane). A silk tie was placed around the femoral artery in the hindlimb of the mouse where it was occluded for 5 min followed by 5 min release as one cycle, for a total of four cycles a day, for 7 consecutive days total.[42,43] A Huntleigh Mini Dopplex (Doppler D900, Huntleigh Healthcare) was used to ensure the artery

was occluded during the procedure. Mice were then sacrificed, and the hearts were removed and processed for RNA, protein, and for purification of mitochondria.

Echocardiography

Echocardiography was conducted on mice anesthetized with 2% isoflurane and placed in the supine position with paws pulled outwards and taped down on a temperature-controlled electrocardiogram board. Because echocardiography is a non-invasive procedure pain relief was not needed. The Vevo 2100 Imaging System and software (VisualSonics, Toronto, ON, Canada) were used to obtain and analyze M-mode recordings at the short-axis midpapillary region. All mice subjected to echocardiography were analyzed in a blinded manner.

Cell culture model

Mouse embryonic fibroblasts (MEFs) were generated and cultured as described previously.[23] Briefly, MEFs were generated by harvesting *Mcu*$^{fl/fl}$, *Mcub*$^{fl/fl}$, or *Mcu*$^{fl/fl}$/*Mcub*$^{fl/fl}$ embryonic day 10.5 embryos. After the removal of the head and internal organs, the remainder of the bodies were passed through a 25-gauge needle and plated on 10 cm^2 tissue culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific) containing 10% bovine growth serum (BGS, Thermo Fisher Scientific), penicillin-streptomycin (Thermo Fisher Scientific), and nonessential amino acids (Invitrogen). After two passages, the MEFs were subjected to simian virus 40 (SV40) large T antigen immortalization by infecting with an SV40 T antigen–expressing lentivirus (Addgene, plasmid 22298, pLenti CMV/TO SV40 small + large T). Once immortalization was achieved, cells were treated with an adenovirus expressing Cre recombinase (AdCre) for gene deletion for 48 hours before harvesting. Control MEFs were genetically identical within an experiment except they lacked AdCre infection. Deletion of the targeted gene was verified with western blotting.

Western blotting

Heart tissue or isolated mitochondria were lysed in RIPA buffer (Thermo Fisher Scientific) on ice containing protease/phosphatase inhibitor cocktail (cOmplete EDTA-free protease inhibitor cocktail, PhosSTOP phosphatase inhibitor cocktail, Roche). Then protein lysates were loaded onto a 12% SDS-PAGE gel and electrophoresed at 100 volts for approximately 2-3 hours. The separated proteins were transferred onto a 0.2 μm nitrocellulose membrane (Bio-Rad Laboratories) at 100 volts for 1 hour on ice. The only exception was when separating EMRE, which used a 15% SDS-PAGE gel at 110 volts for 1 hour followed by transferring at 80 volts for 1 hour. Western blots were performed using different antibodies at 4°C overnight: MCU (1:500, Cell Signaling,

Catalog#14997), MCUb (1:250, custom-made from YenZym Antibodies, LLC), MICU1 (1:500, Cell Signaling, Catalog#12524S), MICU2 (1:500, Bethyl Laboratories, Catalog#A300-BL19212), EMRE (1:500, Bethyl Laboratories, Catalog#A300-BL19208), NCLX (slc24a6, 1:500, Abcam, Catalog#ab83551), OXPHOS cocktail (1:5000, Abcam, Catalog#ab110413), VDAC (1:5000, Abcam, Catalog#ab14734), and GAPDH (1:5000, Fitzgerald, Catalog#10R-G109A). Fluorescence-based secondary antibodies were used against different species of antibodies and were incubated at room temperature for 1 hour (1:10,000, LI-COR Biosciences, Catalog#926-32211; # 925-68070). Membranes were analyzed using Odyssey CLx Imaging System (LI-COR Biosciences).

Real-time qPCR measurement

RNA extraction was performed from the left ventricle of the heart, and all the steps were performed on ice at 4°C. The left ventricle of the heart was homogenized in 1.5 mL Eppendorf tubes containing Trizol™ Reagent (Thermo Fisher Scientific) with a tissue-tearor (Bio Spec Products Inc.). Homogenates were centrifuged at 12,000 g for 10 minutes, and the supernatant was transferred to new 1.5 mL Eppendorf tubes. Chloroform (1:5 volume ratio to supernatant) was added, and samples were vortexed for 15 seconds followed by 3 minutes of incubation at room temperature. Samples were then centrifuged at 12,000 g for 15 minutes. The aqueous phase was transferred to clean 1.5 mL Eppendorf tubes. Isopropanol (2.5:1 volume ratio to chloroform) was added, and samples were mixed and incubated for 8 minutes at room temperature. RNA pellets were obtained by centrifuging at 12,000 g, and then cleaned with 75% ethanol, and then dissolved in RNase-free water. RNA concentration was measured with a Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific), and cDNA synthesis was performed with SuperScript® III First-Strand Synthesis SuperMix (Thermo Fisher Scientific). RT-qPCR was performed with C1000 Touch Thermal Cycler (Bio-Rad Laboratories) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories). RT-qPCR cycling conditions were: 95 °C for 30 seconds, [95 °C for 15 seconds, 60 °C for 30 seconds, followed by fluorescence reads] (40 cycles), 65 °C to 95 °C melting curve with 0.5 °C inclement every 5 seconds, followed by fluorescence reads. The cycle threshold values were normalized to the values obtained from the RPL7 internal loading control. Data were presented as fold change relative to sham controls. Sequences of primers used in RT-PCR are listed as follows: *MCU* 5'-GTGCCCTCTGATGACGTGACGG-3 (forward) 5'-ATGACAAGCTTAAAGTCATG-3' (reverse); *MCUb* 5'-GAAGAGCCAAGTGGAGAGCA-3' (forward) 5'-TTCCGACCGGGCTTCTATTG-3' (reverse); *MICU1* 5'-ACACCCTCAAGTCTGGCTTAT-3' (forward) 5'-TTCCCATCTTTGAAGTGCTTCTT-3' (reverse); *MICU2* 5'-TCGGCGCAGAAAAATTATTTGG-3' (forward), 5'-

GTGTCATGTAATACTCTCCGTCG-3' (reverse); *EMRE* 5'-TCTACACCGTACCGGGCAG-3' (forward) 5'-AGTGTCCCGACATAGAGAAAGG-3' (reverse); *RPL7* 5'-GAAGCTCATCTATGAGAAGGC-3' (forward) 5'-AAGACGAAGGAGCTGCAGAAC-3' (reverse).

Mitochondrial Isolation and analyses

Heart or MEF mitochondria were isolated by differential centrifugation as described previously.[41,23] Briefly, hearts were harvested from mice and minced in MS-EGTA buffer (225 mmol/L mannitol, 75 mmol/L sucrose, 5 mmol/L HEPES and 1 mmol/L EGTA, pH 7.4). The MEFs were grown to confluency on 4 Nunc Bioassay Dishes (Sigma-Aldrich) and then harvested and pelleted in MS-EGTA buffer. The tissue lysates/cells were then suspended in 7 ml of buffer and homogenized with a Potter-Elvehjem tissue homogenizer. The homogenates underwent 600 g centrifugation for 5 minutes, and the supernatants were transferred for second spin at 13,000 g for 10 minutes to generate mitochondrial pellets.

Baseline mitochondrial Ca^{2+} content was measured by a Calcium Detection Kit (Abcam). Briefly, isolated mitochondria were re-suspended in de-ionized H_2O and sonicated for 5 minutes. One hundred micrograms of mitochondria were added for assessment as recommended in the vendor-supplied protocol (Abcam).

For the cardiac mitochondrial Ca^{2+} uptake assay, isolated heart mitochondria were re-suspended in KCl buffer (125 mmol/L KCl, 20 mmol/L HEPES, 2 mmol/L $MgCl_2$, 2 mmol/L KH_2PO_4, and 40 µmol/L EGTA, pH 7.2). Mitochondrial Ca^{2+} uptake was measured using a PTI QuantaMaster™ Spectrofluorometer (HORIBA), with excitation as 506 nm and emission as 533 nm. One milligram of isolated heart mitochondria was added to the KCl buffer containing 1 µmol/L malate, 7 µmol/L pyruvate, 250 nmol/L of Calcium Green 5-N (Thermo Fisher Scientific). Malate and pyruvate were substrates to activate mitochondrial function. Calcium Green 5-N was used as Ca^{2+} indicator in the solution. Twenty micromolar $CaCl_2$ solution in 2 µl was added sequentially (as indicated by arrows in figure graphs) until mitochondria swelled and no longer took up Ca^{2+}. MEF mitochondrial Ca^{2+} uptake assays were conducted as above but with 2 mg of mitochondria isolated from *Mcu*$^{fl/fl}$, *Mcub*$^{fl/fl}$, or *Mcu*$^{fl/fl}$*Mcub*$^{fl/fl}$ MEF cell lines.

Oxygen consumption rate was determined using a Seahorse Extracellular Flux (XF) Analyzer (Agilent Technologies) as described previously.[23] Isolated heart mitochondria (25 µg) were loaded onto XF24 cell culture microplates (Agilent Technologies) in KCl buffer containing 25 mmol/L pyruvate, 10 mmol/L malate

and 5 mmol/L ADP and subjected to the Mito Stress Test Kit (Agilent Technologies) using the standard protocol provided by Agilent Technologies. Pyruvate, malate, and ADP were used as substrates for proper mitochondrial function. Briefly, after basal respiration was measured, the mitochondria were treated sequentially with 2 μmol/L oligomycin, 5 μmol/L carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and 0.5 μmol/L rotenone. Oligomycin, an ATP synthase inhibitor, was used to block ATP-linked respiration. And the remaining oxygen consumption rate was referred to as proton leak. FCCP is a potent uncoupler of oxidative phosphorylation that achieves maximal respiration rate. Rotenone is used to interfere with electronic transport chain in mitochondria by inhibiting Complex I.

Ca^{2+} and mPTP measurement in cardiomyocytes

Adult mice cardiac ventricular myocytes were isolated using previously described methods.[25] Isolated adult myocytes were pretreated with 5 μmol/L thapsigargin, 50 μmol/L cytochalasin D and loaded with 10 μmol/L Rhod2-AM. During and after permeabilization with saponin (50 μg/ml), cells were perfused with incubated with internal solution (in mmol/L: K-aspartate 100, KCl 40, $MgCl_2$ 0.5, EGTA 0.5, HEPES 10, maleic acid 2, glutamic acid 2, pyruvic acid 5, KH_2PO_4 0.5, Mg-ATP 5, pH 7.2).

To measure mitochondrial Ca^{2+} uptake, free cytosolic Ca^{2+} was acutely increased to 2 μM and the Rhod2 fluorescence signal was measured over the next 14 minutes. Ru360, a mitochondrial Ca^{2+} uptake inhibitor, was used as a positive control. To measure basal mitochondrial Ca^{2+}, the myocytes pretreated as above were equilibrated with an internal solution of 100 nmol/L [Ca^{2+}] (mimicking basal cytosolic [Ca^{2+}]). F_{min} and F_{max} were measured at 0 and 4 μmol/L [Ca^{2+}], respectively with internal solution containing 5 μmol/L ionomycin (Ca^{2+} ionophore), 5 μmol/L FCCP and 1 μmol/L oligomycin to dissipate mitochondrial membrane potential. This allows equilibration of [Ca^{2+}] across the inner mitochondrial membrane. To measure Na^+-dependent mitochondrial Ca^{2+} efflux in saponin-permeabilized myocytes after mitochondrial Ca^{2+} loading, the perfusate was switched to a Ca^{2+}-free buffer with 10 mmol/L Na^+ internal solution. Mitochondrial Ca^{2+} decline was fit by a single exponential decay during the first 5 minutes to obtain a time constant (R squared > 0.99).

To access mitochondrial Ca^{2+} efflux, myocytes were pre-exposed to lower ionomycin (1 μM), and mitochondria were loaded with Rhod-2AM and 2 μM $CaCl_2$ (for equal Ca^{2+} loading, control myocytes were treated with 2 μM Ru360). Mitochondria were exposed to 2 μM $CaCl_2$ and then we measured the decline in Rhod-2 fluorescence following a switch to buffer containing no Ca^{2+} and 10 mM Na^+. Here, NCLX mediated Ca^{2+} efflux was assessed by normalizing the Ca^{2+}

decline rate in the presence of Na^+ to that in the absence of Na^+. First 5 minutes of decay was used to calculate NCLX efflux rate via one phase decay fitting.

mPTP opening in permeabilized adult myocytes was measured with 100 nmol/L Tetramethyrhodamine, Methyl Ester (TMRM, λex=543 nm, λem=552-617 nm), a potential-sensitive dye. Permeabilized cells were first washed with standard intracellular relaxation solution containing (in mmol/L) EGTA 2, HEPES 10, K-aspartate 135, Mg-ATP 5, reduced glutathione 10, glucose 10, $MgCl_2$ 0.7, pH7.2 at RT. Then, the solution was switched to Na^+-free internal solution with low Ca^{2+} buffering capacity and 100 nM free Ca^{2+} containing (in mmol/L): EGTA 0.05, $CaCl_2$ 0.0234, HEPES 20, K-aspartate 100, KCl 40, $MgCl_2$ 0.551, maleic acid 2, glutamic acid 2, pyruvic acid 5, KH_2PO_4 0.5, MgATP 5, pH 7.2. Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were monitored to indicate mPTP opening and 10 μmol/L CsA was used as a control to desensitize mPTP opening. The frequency of mPTP opening is counted as the number of mitochondria that lost membrane potential normalized by total number of mitochondrial monitored over 10 minutes. Experiments were performed with confocal microscopy (Nikon, ×40 objective) using 2D frame mode. And images were analyzed by ImageJ software (NIH, https://imagej.nih.gov/ij/).

Ca^{2+} sparks and transient measurements

Intact ventricular myocytes were loaded with Fluo-4 AM dye (5 μmol/L) for 30 minutes, transients and sparks were recorded as previously described.[25,26] Calcium transients were obtained by field stimulation at 1 Hz in normal Tyrode's buffer with 1.8 mmol/L Ca^{2+}. Sarcoplasmic reticulum (SR) Ca^{2+} load was evaluated by the Ca^{2+} transient amplitude upon rapid application of caffeine (10 mmol/L). Images were acquired with confocal microscopy (Nikon, ×40 objective) using line scan mode with excitation at 488 nm, emission at >505 nm. Images were analyzed using ImageJ software and Sparkmaster.[27]

Histology and transmission electron microscopy

Mouse hearts were isolated and fixed in 10% formalin solution overnight. Samples were then processed and embedded in paraffin. Heart were sectioned at 5 μm using a HM 355S Automatic Microtome (Thermo Fisher Scientific). Both hematoxylin and eosin-stained (H&E) and Masson's trichrome staining were performed on these heart sections. Images were obtained using Olympus BX60 microscope at 10X objective. Heart sections were also stained by Sirius Red staining, and images were obtained using Leica DFC310 FX (Leica Microsystems) at 2X objective. Percentage of fibrosis was quantified by ImageJ software. Transmission electron microscopy was performed as previously described.[44,45] Briefly, heart tissues were harvested, fixed in 3.5% glutaraldehyde and 0.15% sucrose in 0.1 M sodium cacodylate, pH 7.4, post-

fixed in 1% OsO_4, dehydrated, and embedded in epoxy resin. Imaging was performed using a Hitachi 7600 electron microscope with magnification at 3500X.

Co-immunoprecipitation (Co-IP)

Heart mitochondria were isolated and digested in IP lysis buffer (20 mmol/L Tris pH 7.4, 1 mmol/L DTT, 0.25% NP-40, 140 mmol/L NaCl, 10 mM KCl, 0.25% Triton X-100, adding protease/phosphatase inhibitor cocktail as mentioned above) at 4°C for 2 hours by gentle rotation. Dynabeads™ protein G (Thermo Fisher Scientific) were used for protein immunoprecipitation. Dynabeads™ protein G was bound to MCU antibody (Cell Signaling) according to the vendor protocol. Briefly, 50 µl of dynabeads were transferred to a 1.5 ml Eppendorf tube. The tube was placed on the DynaMag™-2 Magnet (Thermo Fisher Scientific) and supernatant was removed to get dynabead pellet. Five micrograms of MCU antibody was diluted in 200 µl 1X phosphate-buffered saline (PBS) with 0.1% Tween™ 20, and to the dynabeads pellet. The mixture was incubated at 4°C by gentle rotation for 2 hours to get MCU-bound dynabeads. MCU-bound dynabeads were then placed on the DynaMag™-2 Magnet and were re-suspended in 50 µl of PBS with 0.1% Tween™ 20. Four milligrams of heart mitochondria were mixed with 20 µl of MCU-bound dynabeads, and the mixture was incubated at 4°C overnight by gentle rotation. Samples were then put on the DynaMag™-2 Magnet to get the antigen-antibody-bead complex. Elution buffer (1X PBS with 1% Triton X-100 and loading dye) was added to the pellet. And antigens bound to MCU-bound dynabeads were eluted by boiling the samples at 95°C for 15 minutes. Samples were then used for performing western blots for EMRE.

Statistics

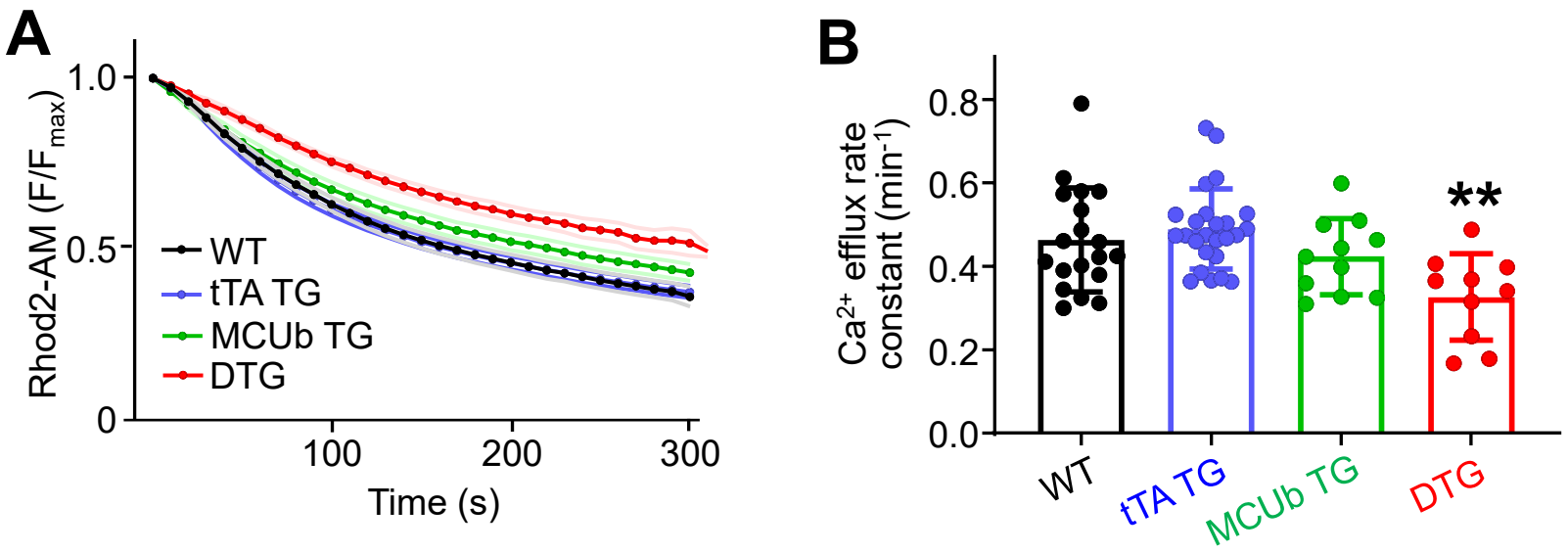
Data were presented as mean ± SEM. Group sizes were determined by experience with similar studies performed within the Molkentin laboratory, and by an a priori power analysis for a two-tailed, two-sample t-test with an α of 0.05 and power of 0.8, in order to detect a 10% difference in signal at the endpoint. Animals were grouped without blinding or randomization because they were genetically identical and of the same age and housed together. However, experiments were blinded were possible when two or more people could be recruited to conduct the front setup of the experiment versus the backend analysis of data (i.e. for all surgical models and echocardiography).

For experiments involving a surgery (I/R and MI) mice that died before the desired endpoint were excluded from analysis because they were obviously missing. No difference was observed in survival rates between groups in each experiment. All surviving animals were included in endpoint measurements and

none were excluded. Moreover, in all surviving mice we performed Grubb's 2-sided outlier test (α0.05) to document extreme outliers. No animals achieved this extreme outlier criteria and hence none were excluded. The Grubb's Outlier Test was performed using GraphPad Prism 7 software. For all other experiments, no samples or animals were excluded from analysis. All statistical analysis was conducted using GraphPad Prism 7 software. The normality of all data was ascertained using the Shapiro-Wilk normality test. For statistical analysis comparing only two groups (Fig 3C-D, 4D-E, 4G-K, 5B, 5G-I, 6B; Online Fig IVB-C), student's t-test was used and a p-value of < 0.05 was considered significant. For statistical analysis comparing multiple groups with one variable (Fig 1A, 2F-G; Online Fig IB, IIA-E), a one-way ANOVA was used to analyze sample variance. If the one-way ANOVA confirmed a significant difference between groups, a post hoc Bonferroni test for multiple comparisons was performed and an adjusted p value of < 0.05 was considered significant. For statistical analysis with two variables (Fig 2H, 3E, 5D-F; Online Fig IVD), a two-way ANOVA was used to determine possible interactions between the variables. If the two-way ANOVA confirmed a significant interaction between variations, a post hoc Bonferroni's multiple comparisons test was performed and an adjusted p value of < 0.05 was considered significant.

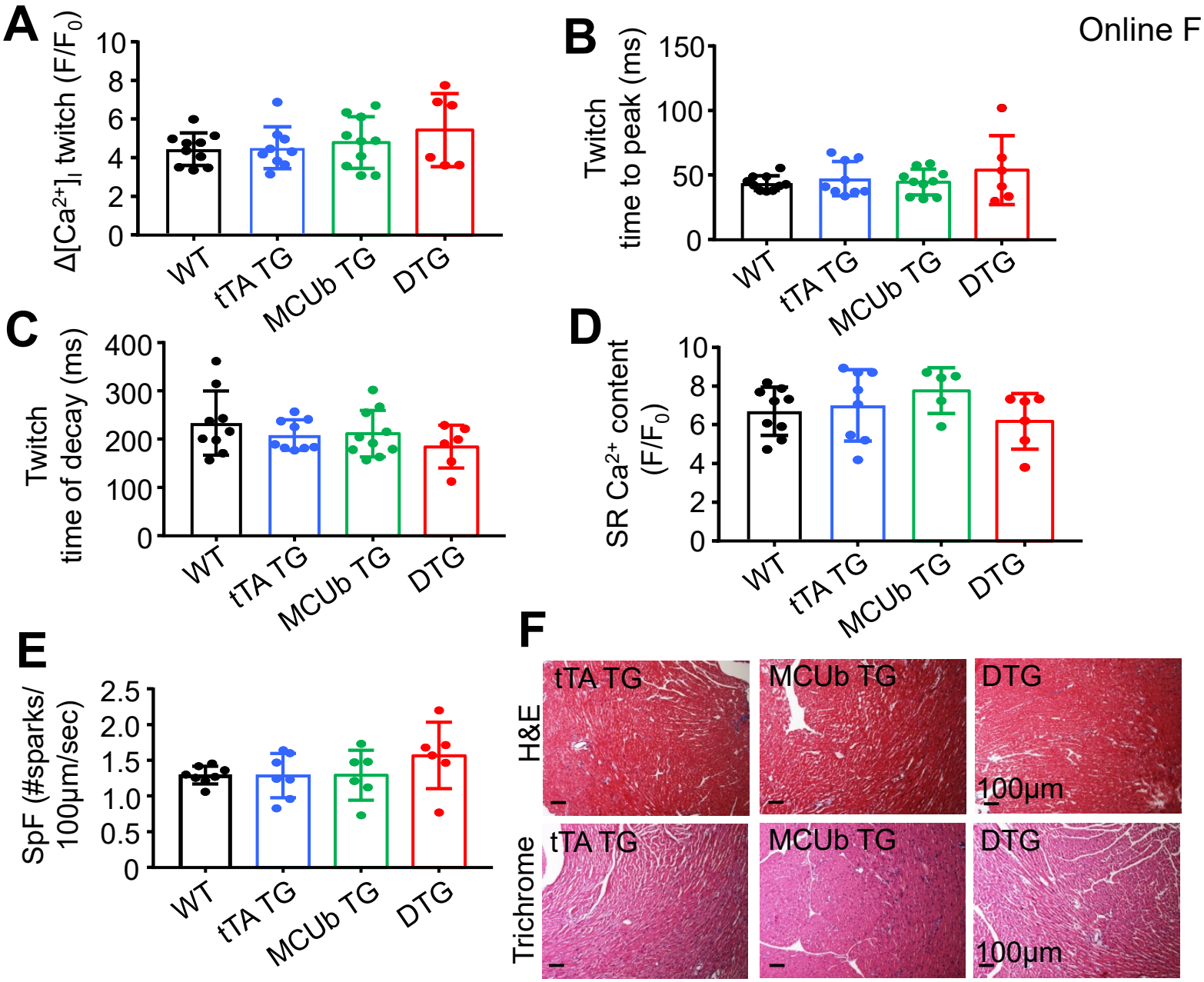
Please see the Major Resources Table in the Supplemental Materials.

Online Figure I



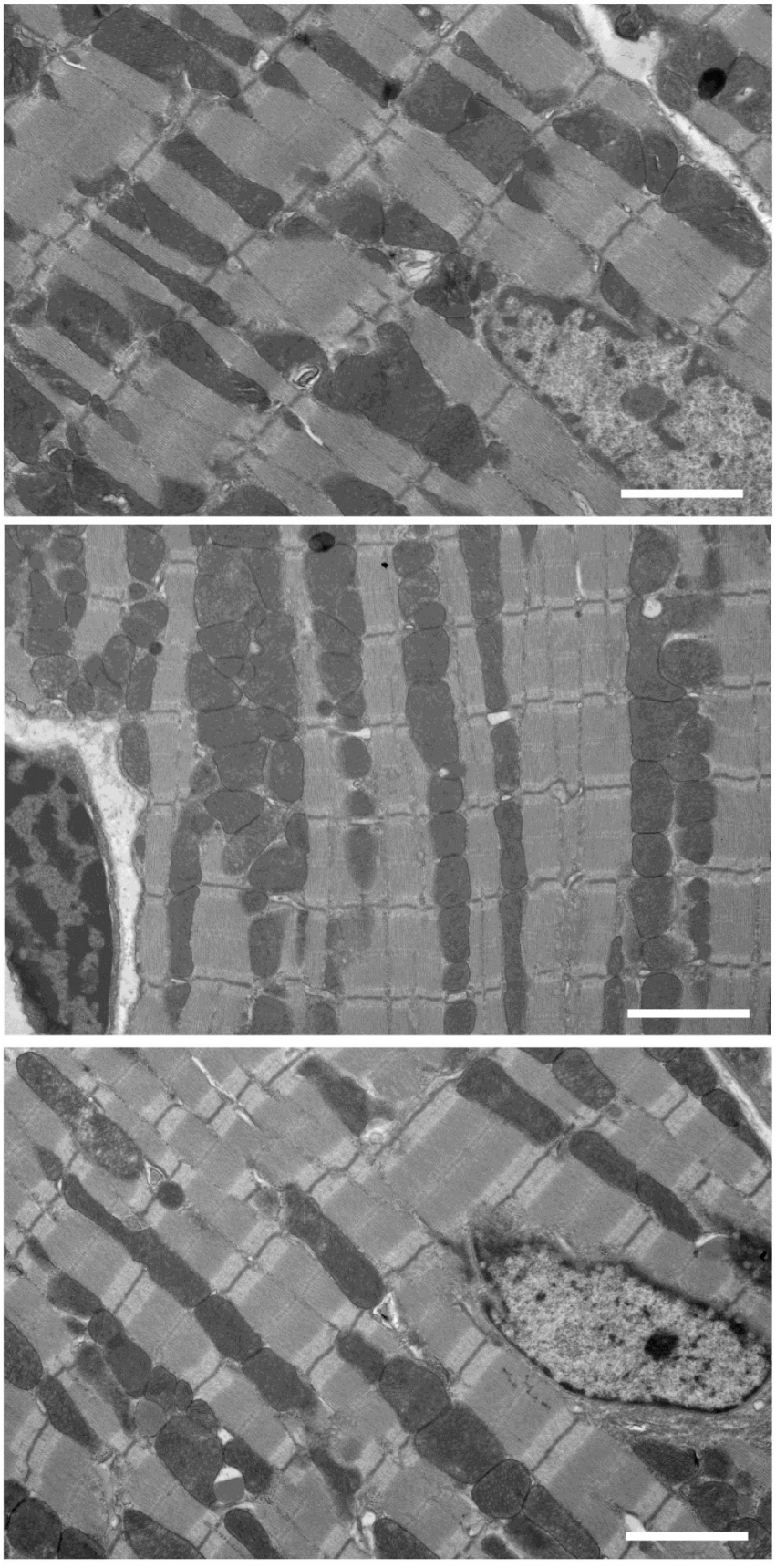
Online Figure I. Mitochondrial Ca^{2+} efflux was reduced in cardiac-specific MCUb overexpressing mice. A) Average fluorescence traces of mitochondrial Ca^{2+} efflux induced by 10 mmol/L Na^{+} in isolated cardiomyocytes from hearts of the indicated groups of mice. B) Average rates of mitochondrial Ca^{2+} efflux derived from traces in panel A. n=19 myocytes from WT mice, n=26 myocytes from control tTA TG mice, n=11 myocytes in MCUb single TG mice, n=10 myocytes in DTG mice. Data presented as mean ± SEM. One-way ANOVA and post hoc Bonferroni test was used for statistical analysis. **p<0.01 vs WT by post hoc Bonferroni test.

Online Figure II

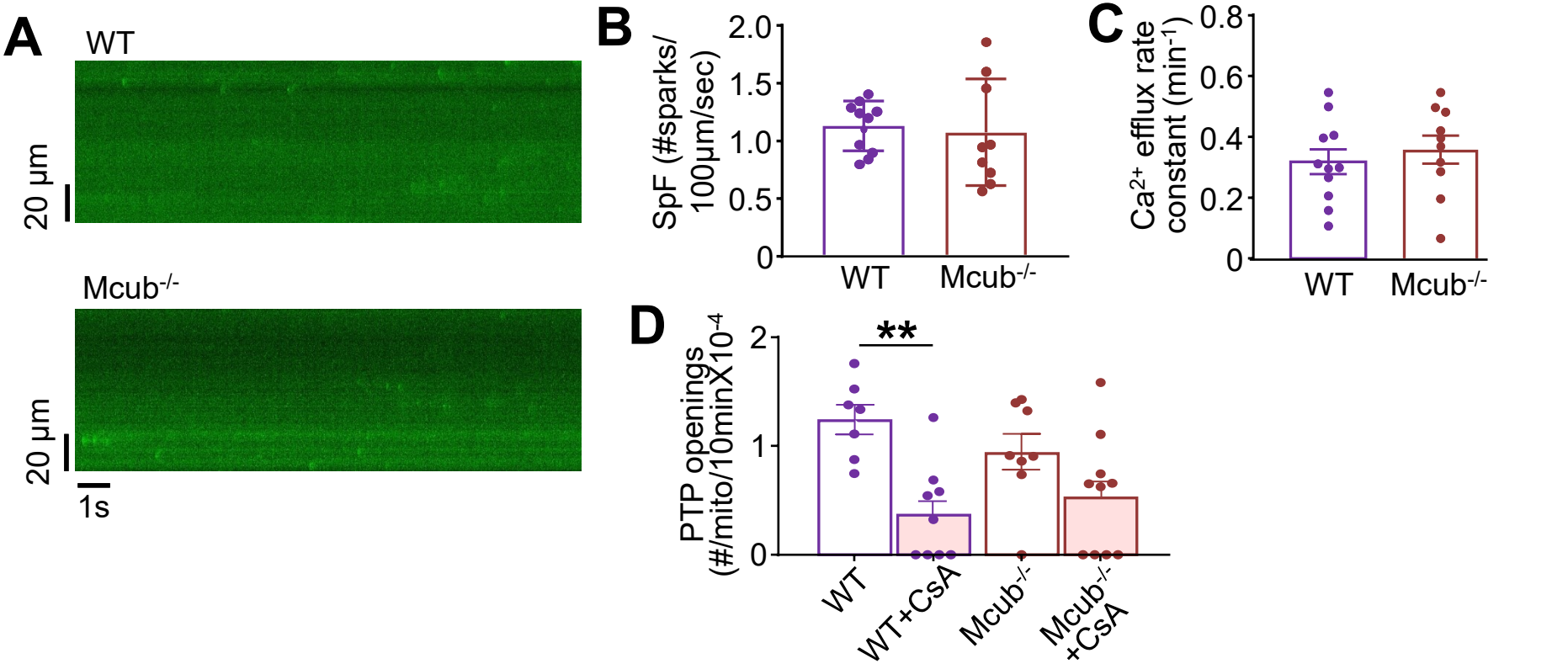


Online Figure II. Cardiomyocyte-specific MCUb overexpression did not change intracellular Ca^{2+} handling or histology. A-C) Intracellular Ca^{2+} transient amplitude, twitch time to peak and twitch decay (tau) and from adult cardiomyocytes from hearts of the indicated groups of mice. n=10 in WT group, n=9 in tTA TG group, n=10 in MCUb TG group, n=6 in DTG group. Data presented as mean ± SEM. One-way ANOVA showed no statistical differences. D) SR Ca^{2+} content measurement from adult cardiomyocytes isolated from hearts of the indicated groups of mice. n=9 in WT group, n=8 in tTA TG group, n=5 in MCUb TG group, n=6 in DTG group. Data presented as mean ± SEM. One-way ANOVA showed no statistical difference between the groups. E) Diastolic Ca^{2+} spark frequencies from adult cardiomyocytes from hearts of the indicated groups. n=10 in WT group, n=9 in tTA TG group, n=10 in MCUb TG group, n=6 in DTG group. Data presented as mean ± SEM. One-way ANOVA showed no statistical differences between the groups. F) Representative hematoxylin and eosin-stained (H&E) or Masson's trichrome-stained histological heart sections from the indicated groups of mice. Scale bars are shown (100 μm).

Online
Figure III

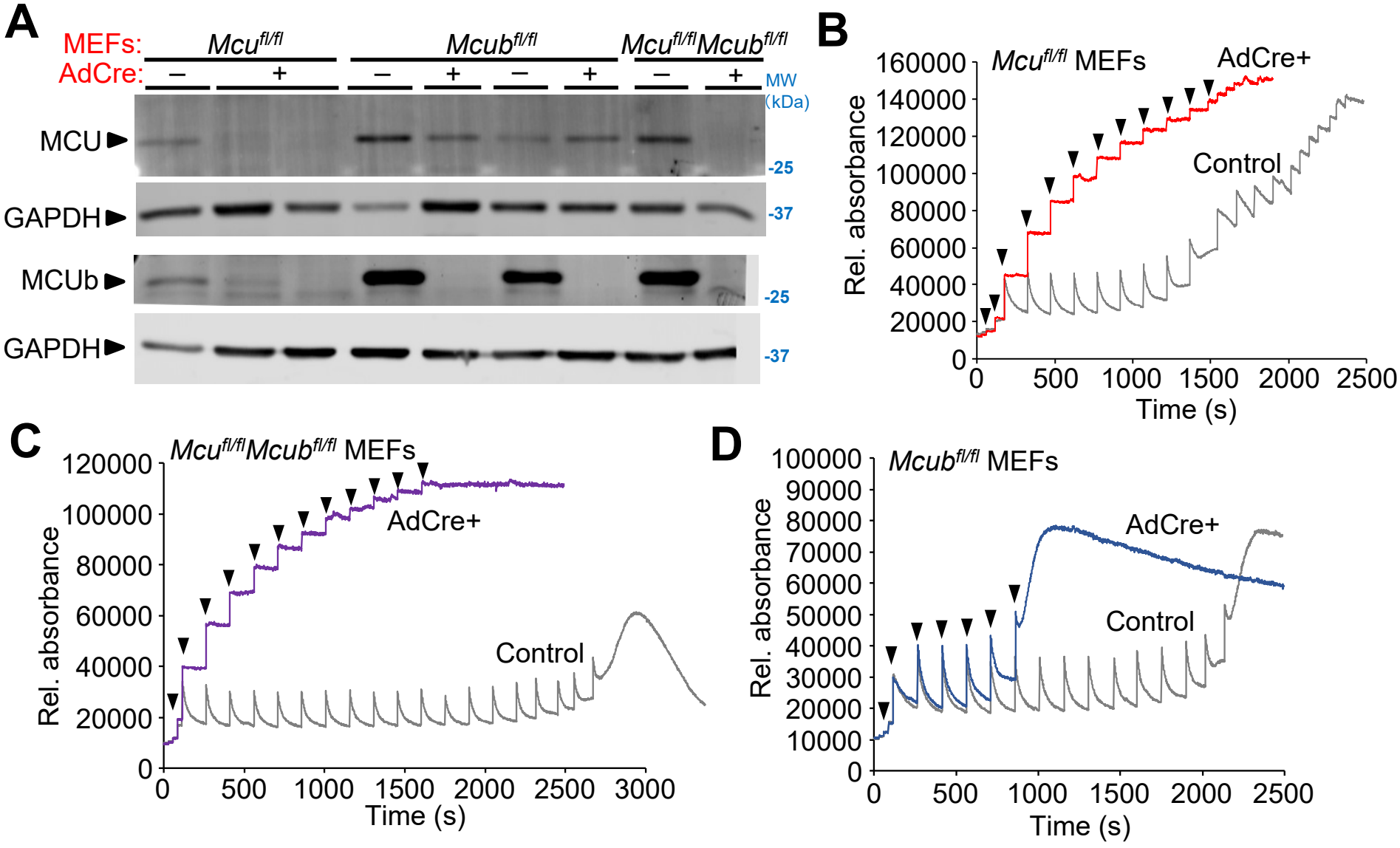


Online Figure III. Mitochondrial ultrastructure shows no pathology when MCUb expression is altered in the heart. Representative images of transmission electron microscopy at 3500X magnification from mouse hearts taken from the indicated groups of mice at 2-3 month of age. Four individual mouse hearts were analyzed in each group. Scale bar represents 2 μm. WT = wildtype; DTG = double transgenic for MCUb overexpression.

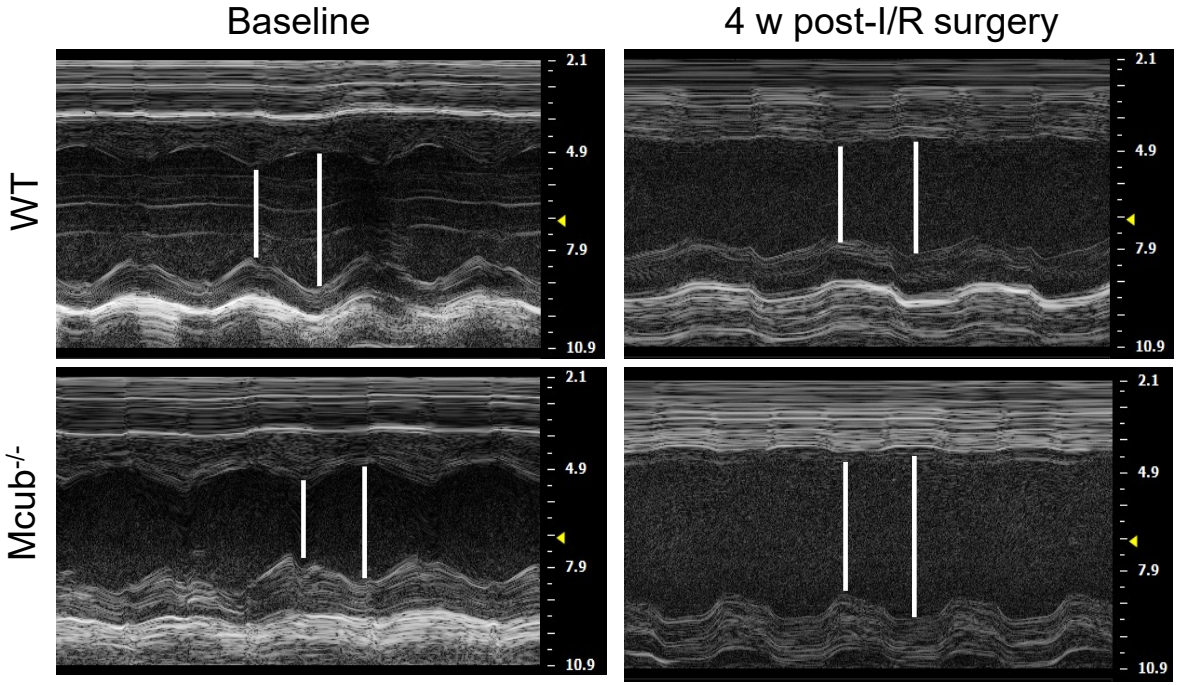


Online Figure IV. Deletion of the *Mcub* gene did not alter Ca^{2+} handling and, mitochondrial Ca^{2+} efflux or mPTP opening frequency. A) Representative 2D line scans of Ca^{2+} spark frequency from adult cardiomyocytes from hearts of the indicated groups of mice. B) Quantification of diastolic Ca^{2+} spark frequency from adult cardiomyocytes from hearts of the indicated groups. n=10 in WT group, n=9 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. C) Quantification of NCLX mitochondrial Ca^{2+} efflux rate with one exponential fit of decay, assessed by Rhod2 fluorescence. n=10 in WT group, n=9 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. D) Quantification of mPTP opening frequency with 100 nM free Ca^{2+} challenge, with tetramethylrhodamine methyl ester (TMRM) used as indicator in adult cardiomyocytes from hearts of mice of the indicated groups. CsA was used as a desensitizing control. n=7 in WT group, n=9 in WT group with CsA treatment, n=8 in *Mcub*$^{-/-}$ group, n=10 in *Mcub*$^{-/-}$ group with CsA treatment. Data presented as mean ± SEM. Two-way ANOVA and post hoc Bonferroni test were used for statistical analysis. A significant interaction was found between treatment and genotype ($p<0.05$). **$P<0.01$ versus WT by post hoc Bonferroni test.

Online Figure V



Online Figure V. *Mcub* deletion in MEFs showed altered mitochondrial Ca^{2+} uptake capacity. A) Western blot of MCU and MCUb on isolated mitochondria from MEFs of the indicated genotypes. MEFs were treated with vehicle or Cre-expressing Adenovirus (AdCre), and cells were harvested for western blot. GAPDH was used as the protein loading control. B–D) Mitochondrial Ca^{2+} uptake assays measured as relative mitochondrial absorbance (swelling) in 2 mg of isolated MEF mitochondria from the indicated genotypes. MEFs were first infected 48 hours prior with AdCre in the indicated genotypes and the control condition was genetically identical MEFs without AdCre. Calcium Green-5N was used as Ca^{2+} indicator. Arrows indicate addition of 20 μM $CaCl_2$ bolus to the assay.



Online Figure VI. Echocardiographic measurement shows greater dilation on *Mcub*$^{-/-}$ mice post-I/R injury. Representative M-mode echocardiographic images from the indicated mice at baseline or 4 weeks post-I/R injury. White lines indicate left ventricle internal diameter, both end-diastolic and end-systolic (longer being diastolic).