Methods:

Disclosure: The authors declare that all supporting data are available within the article. Constructs used will be made available upon request from the authors.

Animal model: Animal experimentation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Johns Hopkins Animal Care and Use Committee. Hartley guinea pigs (~2 month-old; HillTop Lab Animals) were housed in an animal facility at the Johns Hopkins University. All guinea pigs were randomly assigned to different experimental groups. The heart failure and sudden cardiac death model was previously established using male guinea pigs, therefore only male guinea pigs were used in the present study.

Male animals were anesthetized with 4% isoflurane in a closed box for 4min, and then intubated. Animals were ventilated with oxygen and 2% isoflurane. Ascending aortic constriction (AC) was produced by tying a suture around the ascending aorta using an 18-gauge needle as a spacer, which was then removed. Sham-operation was performed following the same procedure without tying the suture. When animals were breathing spontaneously after the procedure, bupronex (0.05mg/kg) was administered via intramuscular injection for analgesia and animals were observed until full recovery. Isoproterenol was administered daily by i.p. injection at 1 mg/kg for the first week after surgery and at 2 mg/kg for another 3 weeks (ACi). For in vivo functional studies and isolated perfused hearts, the Normal control group was subjected to the sham operation and daily isoproterenol injection but no aortic constriction. For in vitro cellular functional studies, the Normal group did not receive the daily isoproterenol injection. All animals at endpoint (4 weeks after aortic banding surgery) were included for analyses.

Production of Adenoviral vector: Adenoviral vectors were constructed with the Gateway system (Invitrogen, Inc.). A 3xFLAG tag was added to C-terminal of the guinea pig MCU sequence with a Gly-Gly-Ser-Gly-Gly linker. The final vector expressed 2 genes: MCU-3xFLAG driven by an EF1α promotor and a reporter, nuclear-targeted CFP (nCFP), driven by a cytomegalovirus promoter. Transfections, adenovirus amplification, and purification were performed following the protocol of the ViraPower Adenoviral Expression System (Invitrogen, Inc.). Titer of adenovirus of 1-5x10^{11} (pfu/ml) was used for *in vivo* studies.

Immunoblotting: Mitochondria were isolated from hearts as described previously[52], solubilized, and boiled in 1x LDS sample buffer for SDS-PAGE. The protein mixture was separated on a 4-12% NuPAGE gel (1 mm, Invitrogen). Samples were run at room temperature for 35 min at 200 V. Proteins were transferred to nitrocellulose membranes with iBlot (Invitrogen, Inc.), using program 3 for 7 min. Membranes were stained with Direct Blue (Sigma-Aldrich) to evaluate the transfer efficiency. Membranes were blocked for 1 h using Odyssey® blocking buffer (Li-Cor Biosciences) and incubated with primary antibodies (MCU, Cell Signaling Technology #14997; Flag tag, Millipore Sigma, #F3165; VDAC, Abcam, #14734; EMRE, Creative Diagnosis, #DPABAH-09221,) overnight at 4°C. Antibody binding was visualized with an infrared imaging system using IRDye secondary antibodies (800CW Donkey anti-Rabbit IgG, #926-32211 and 680RD Donkey anti-Mouse IgG, #926-68072; Odyssey, Licor Biosciences) and quantification of band intensity was performed using the Odyssey Application Software 3.0.

Fluorescence recordings of mCa^{2+} dynamics and ROS in intact cardiomyocytes: Myocyte isolation and mCa^{2+} and ROS recordings were performed as described previously[53]. In brief, cells with MityCam expression were imaged every 25ms at resting state, followed by 0.1 and 1Hz stimulation in the presence of 100nM ISO and then returned to resting state. MityCam fluorescence was measured with ImageJ

(https://imagej.nih.gov/ij/index.html) and expressed as 1-F/F0. To monitor ROS production, myocytes were loaded with 2μM CM-DCFDA and CM-DCF fluorescence was excited 485nm, with emission measured at 525nm. In the presence of 100nM ISO, fluorescence was recorded for 0.5min in the resting state and then 3min at 4 Hz stimulation.

Mitochondrial Ca^{2+} uptake in permeabilized cardiomyocytes: Myocytes isolated from normal hearts injected with Ad-MCU-flag-nCFP or ACi hearts injected with Ad-MCU-flag-nCFP or Ad-nCFP were placed on coated coverslips, which were then mounted in a perfusion chamber. Cells were then permeabilized with 100 μM digitonin and loaded with 2 μM Rhod-2-AM in perfusion buffer containing (in mM): KCl 137, KH_2PO_4 2, HEPES 20, $MgCl_2$ 2.5, EGTA 0.02, Glutamate 5, malate 5, thapsigargin 0.01, blebbistatin 0.01. To record the fluorescence of Rhod-2, microscopic fields of view were selected to include both viral vector-transduced (nCFP positive) and non-transduced myocytes. The initial linear rapid mCa^{2+} uptake rate was analyzed by pairwise comparisons between vector-transduced and non-transduced cells with MCU transduction as a fixed effect and "dish" nested within "heart" and "heart" as a random effects. After a brief baseline recording in Ca^{2+}-free buffer the perfusate was switched to the same buffer containing 2 μM free Ca^{2+}.

Sarcomere shortening, Ca^{2+} transient, SR Ca^{2+} loading: Ventricular myocytes were loaded with 3 μM Fura2-AM (Invitrogen, Molecular Probes, Carlsbad CA) in a modified Tyrode's solution containing (in mM) NaCl 138, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, NaH_2PO_4 0.33, and Glucose 10 (pH 7.4 with NaOH) for 15min. After rinsing, cells were placed in a perfusion chamber with a flow-through rate of 2 ml/min, and sarcomere length and whole cell Ca^{2+} transients were recorded using an inverted fluorescence microscope (Nikon, TE2000), and IonOptix (Myocam®) software. To measure SR Ca^{2+} loading and RyR leak, myocytes were stimulated at 2hz for 1 min in the presence of ISO and then switched to Na^+ and Ca^{2+} free perfusate with or without 1 mM tetracaine followed by rapid application of 10 mM caffeine.

Assessment of RyR2 disulfide bonds: Hearts were excised and perfused with 10 ml fresh ice-cold cysteine-preservation buffer containing 250 mM HEPES-KOH, pH 7.7; 5mM EDTA, 2mM DTPA, 0.1mM Neocuproine, and 20mM N-ethylmaleimide to block free thiols. The left ventricle was then cut off, immersed in the same buffer, minced into small pieces (1-mm), incubated with shaking for 5min, frozen in liquid N_2, and stored at -80°C. Sarcoplasmic reticulum (SR) vesicles were then prepared, as previously described[54]. Briefly, 300mg frozen tissue was homogenized in 2ml buffer containing 375mM sucrose, 7.5mM HEPES, 3.75 mM EDTA, 1mM Na_3VO_4, 20mM NaF, and protease inhibitor cocktail (Roche) on ice, followed by centrifugation at 3000 g for 15min at 4°C. The supernatant was transferred to a new tube and centrifuged at 20,000 g for 15 min at 4°C. The supernatant was transferred to pre-cooled tubes and ultra-centrifuged at 110,000 g for 1hr at 4°C. The pellet was resuspended with 100 μl RIPA buffer containing protease inhibitor cocktail and stored at -80°C. To label the disulfide bonds, 300μg of SR sample was diluted with PBS to 100 μl and incubated with 10mM tris(2-carboxyethyl)phosphine) (TCEP) at RT for 15 min. The reduced sample was then incubated with 0.1mM Alexa Fluor 680 C2 Maleimide at RT for 1hr to fluorescently label the newly released free thiols. Labelled samples were then incubated with 1 μg RyR2 antibody (ABclonal, A0298) at 4°C overnight, followed by immunoprecipitation using Dynabeads (ThermoFisher Scientific). Samples were eluted from the beads, separated on a 3-8% NuPage protein gel, and wet-transferred to a PVDF membrane. The membrane was washed 3 times with TBST buffer and imaged with the Odyssey Imaging system (Li-Cor Biosciences).

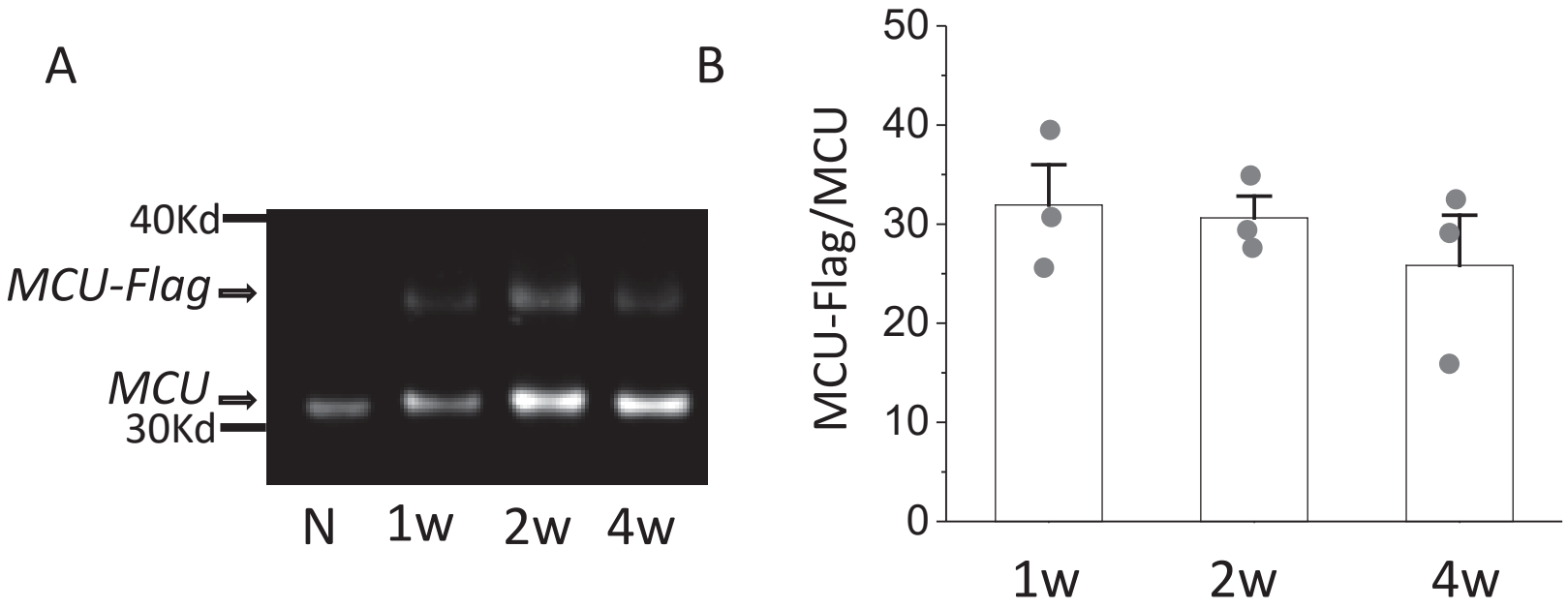
Echocardiography: Transthoracic echocardiography was performed on conscious non-anesthetized guinea pigs by using a Vevo 2100 high-resolution in vivo imaging system with 24 MHz transducer VisualSonics, Toronto, ON, Canada) and analyzed with the Advanced cardiovascular package software (VisualSonics).

Hemodynamics and OCR in Langendorff-perfused hearts: The *ex vivo* measurement of hemodynamics and oxygen consumption rate (OCR) were performed on Langendorff-perfused hearts as previously described.[55] In brief, hearts were mounted on a Langendorff apparatus attached to a PowerLab system (AD Instruments). A buffer-filled latex balloon was inserted into the LV. Outflow buffer was collected from pulmonary artery and oxygen concentration of the outflow was measured with an oxygen probe attached to PowerLab system. HR, LVDP, maximal rates of contraction and relaxation (±dP/dt), and O_2 concentration of the outflow were recorded on a computer. OCR was calculated and normalized to dry heart weight. After a 10min equilibration period, hearts were subjected to a 10min baseline recording, followed by application of 25nM ISO and another 15 minutes of recording. The hemodynamic parameters were determined by taking the average of a period of 1 min at a steady state before and after ISO application.

Histological studies: Hearts were excised and rapidly immersed into ice-cold saline solution. The aorta was then cannulated with a 16G needle, retrogradely perfused with 4% phosphate-buffered paraformaldehyde, and immersion-fixed overnight in the same fixative. Following fixation, the specimens were submitted to a core facility at Johns Hopkins University, the Reference Histology Laboratory, where the specimens were embedded in paraffin and 5 µm sections from the mid-ventricular region were processed and stained with Masson's Trichrome to assess the interstitial fibrosis. The interstitial collagen fraction was determined using computer-assisted image analysis (Image J).

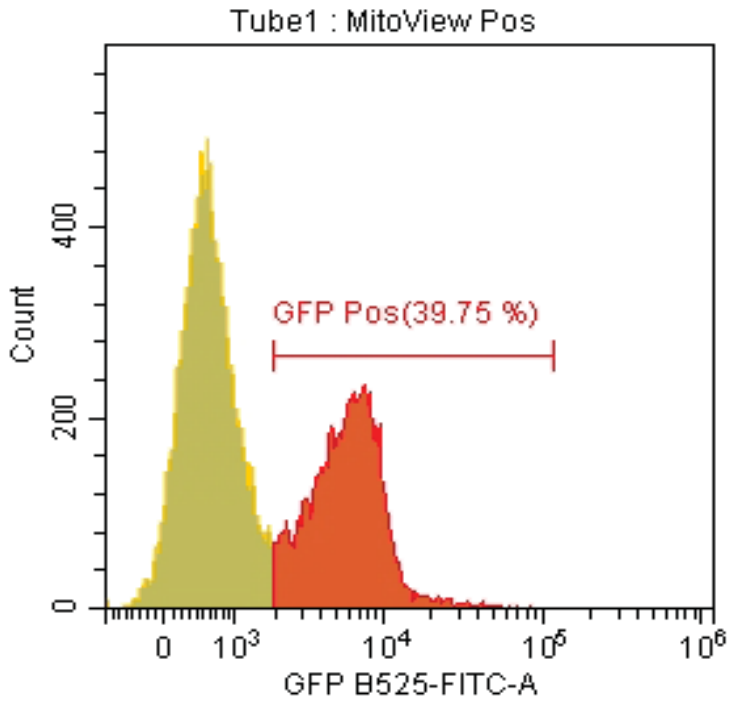
Statistical Analysis: Statistical analyses were performed using JMP Pro (Ver. 14.3, SAS Institute, Inc.). Sample sizes were designed with adequate power according to the literature and our previous studies. Normal distribution of data was determined by the Shapiro-Wilk test. In experiments with small sample size, normality was validated by analysis of the same parameters measured in our previous studies (Online Supplemental Materials). Data are expressed as mean ± SEM. Images or recordings with measurements close to the mean of group were used for representative figures. To account for potential hierarchical clustering effects[56], a nested model with 2-way ANOVA was used to analyze data from isolated cardiomyocytes, with data from individual "Cells" nested within "Animal" (both random effects) and "treatment group" as a fixed effect. Data from ex vivo and in vivo studies were analyzed using 1-way ANOVA. Tukey HSD was used for multiple comparisons following ANOVA; no corrections were made across tests. Sample sizes are provided in the Figure legends. Cells for in vitro studies were isolated from 3-5 hearts.

Online Figure I



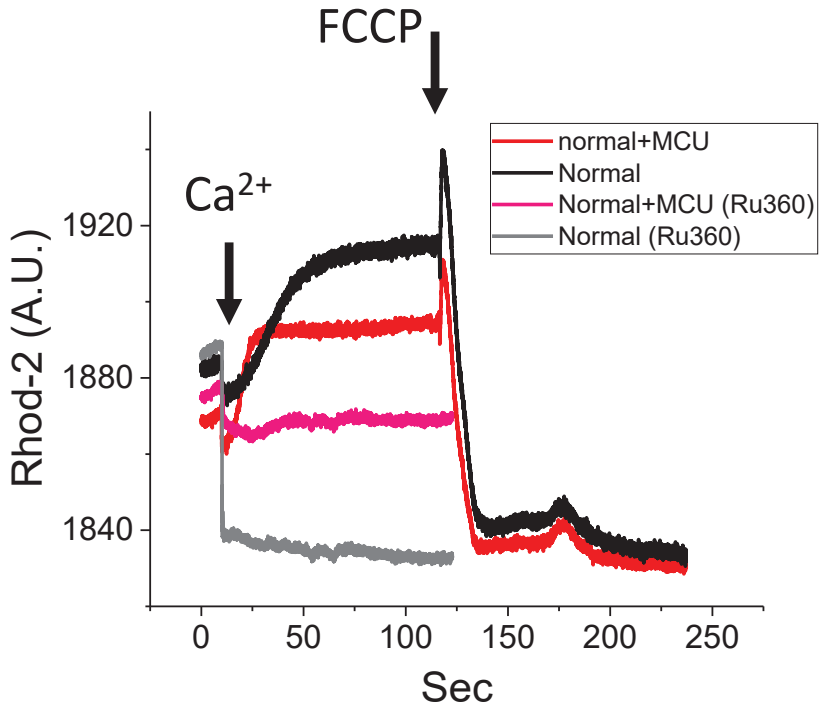
Online Figure I. Adenoviral vector-mediated MCU overexpression at 1 week, 2 weeks, and 4 weeks after injection. A) Representative image of Western blot for MCU showing endogenous MCU and MCU-flag in the protein samples from left ventricle without Ad-MCU-flag injection (N), or at 1w, 2w, and 4w after Ad-MCU-flag injection. B) Average intensity of MCU-flag normalized to endogenous MCU in Ad-MCU-flag injected left ventricles at 1, 2, and 4 week after injection (n=3) showing that the expression level of MCU-flag did not significantly decline after 4 weeks. Data were analyzed with 1-way ANOVA followed with post-hoc Tukey multiple comparisons.

Online Figure II



Online Figure II. Flow cytometry analysis of Adenovirus-mediated MCU gene delivery. Adenovirus expressing MCU-GFP fusion protein was injected into left ventricle of a guinea pig heart. Mitochondria were isolated 1 week later and loaded with MitoView 633. Mitochondria were counted with flow cytometry with high sensitivity detection. In the population of energized mitochondria (MitoView positive), about 40% were GFP positive.

Online Figure III



Online Figure III. Representative raw recording of Rhod-2 fluorescence in permeabilized cardiomyocyte showing Ru360 inhibition of mCa^{2+} uptake and FCCP-induced mCa^{2+} release. Cardiomyocytes isolated from Normal heart with injection of Ad-MCU-flag were permeabilized and loaded with rhod-2-AM in Ca^{2+}-free buffer. Black and grey traces represent recording in non-transduced myocytes and red and pink represent transduced myocytes. After switch to a buffer containing 2μM Ca^{2+}, Rhod-2 fluorescence increased rapidly (black and red). However, the increase was inhibited in cells treated with 1μM Ru360 (pink and grey). After mCa^{2+} reached steady state (black and red), application of 5mM FCCP induced release of mCa^{2+}.

Online Figure IV

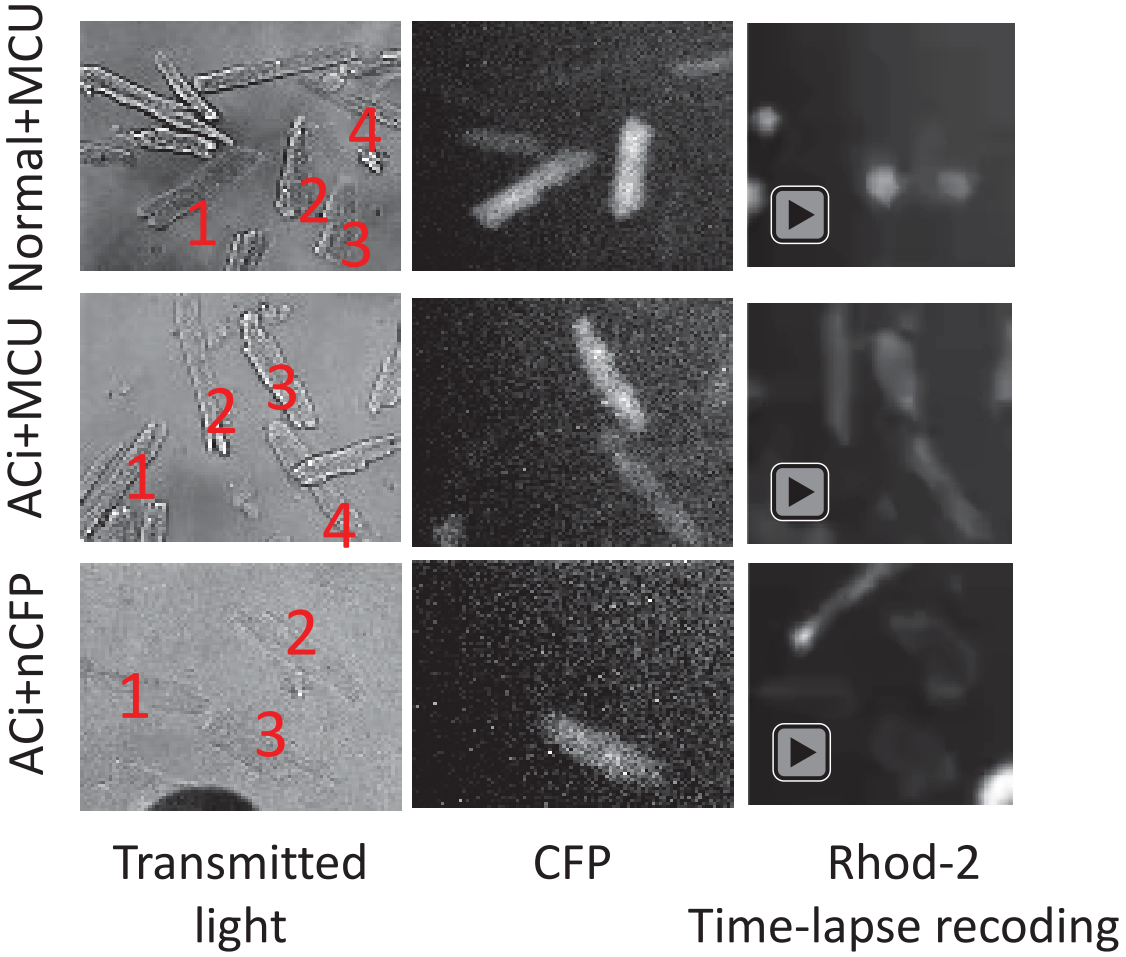
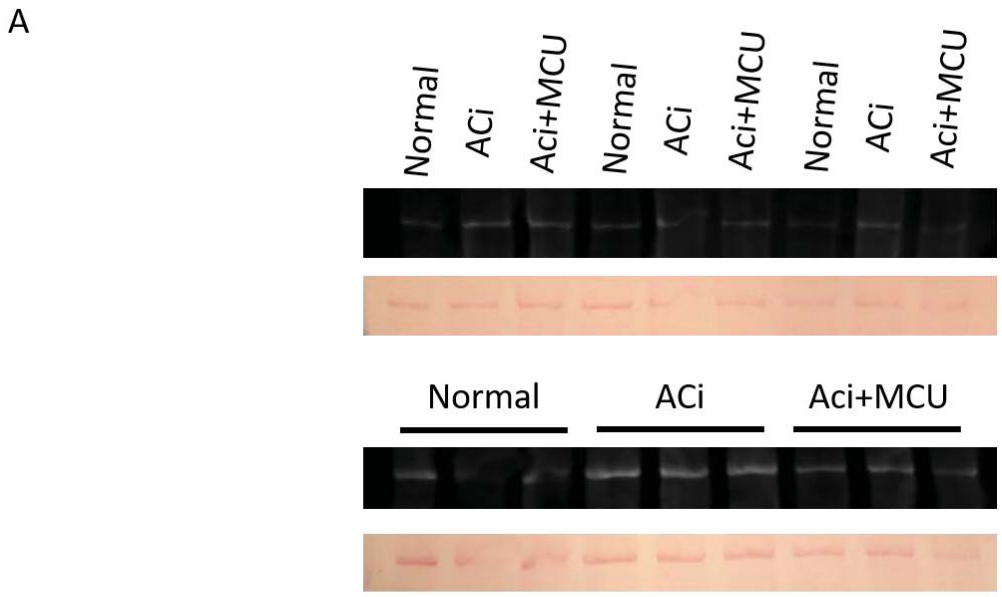


Figure S4. Images of representative recordings in Figure 2A. Cardiomyocytes were isolated from Normal (Normal+MCU) and ACi (ACi+MCU) hearts with injection of Ad-MCU-flag and ACi heart with injection of control virus (ACi+nCFP). In the transmitted light images, cells used for measurement of rhod-2 signal were labelled with numbers and included both transduced (CFP positive) and non-transduced (CFP negative) cells. Representative Rhod-2 fluorescence image sequences for individual cells are shown in embedded videos (right panels). Video sequences were filtered using the FFT Bandpass Filter function in Fiji, all other images are unprocessed.

Online Figure V. **MCU overexpression reversed Redox modification of RyR in ACi heart.**

The normalized fluorescence of RyR2 in the ACi group was about 84% higher than that in Normal hearts indicating more disulfide bond formation in RyR of ACi hearts. MCU overexpression in ACi hearts significantly reduced Alexa 680 labeling of RyR2 to the levels of normal hearts.



Online Figure V. SR vesicles prepared from Normal, ACi, and ACi+MCU hearts were used for disulfide crosslink labelling. After blocking free thiols, disulfide links were reduced and labeled with Alexa 680. Immunoprecipitated RyR2 was separated using 3-8% SDS-PAGE and transferred to a PVDF membrane. A) Images of Alexa 680-labelled RyR2 and Ponceau S stain for normalization in 2 separate experiments. B) The membrane showing Ponceau S staining in the 2 experiments.