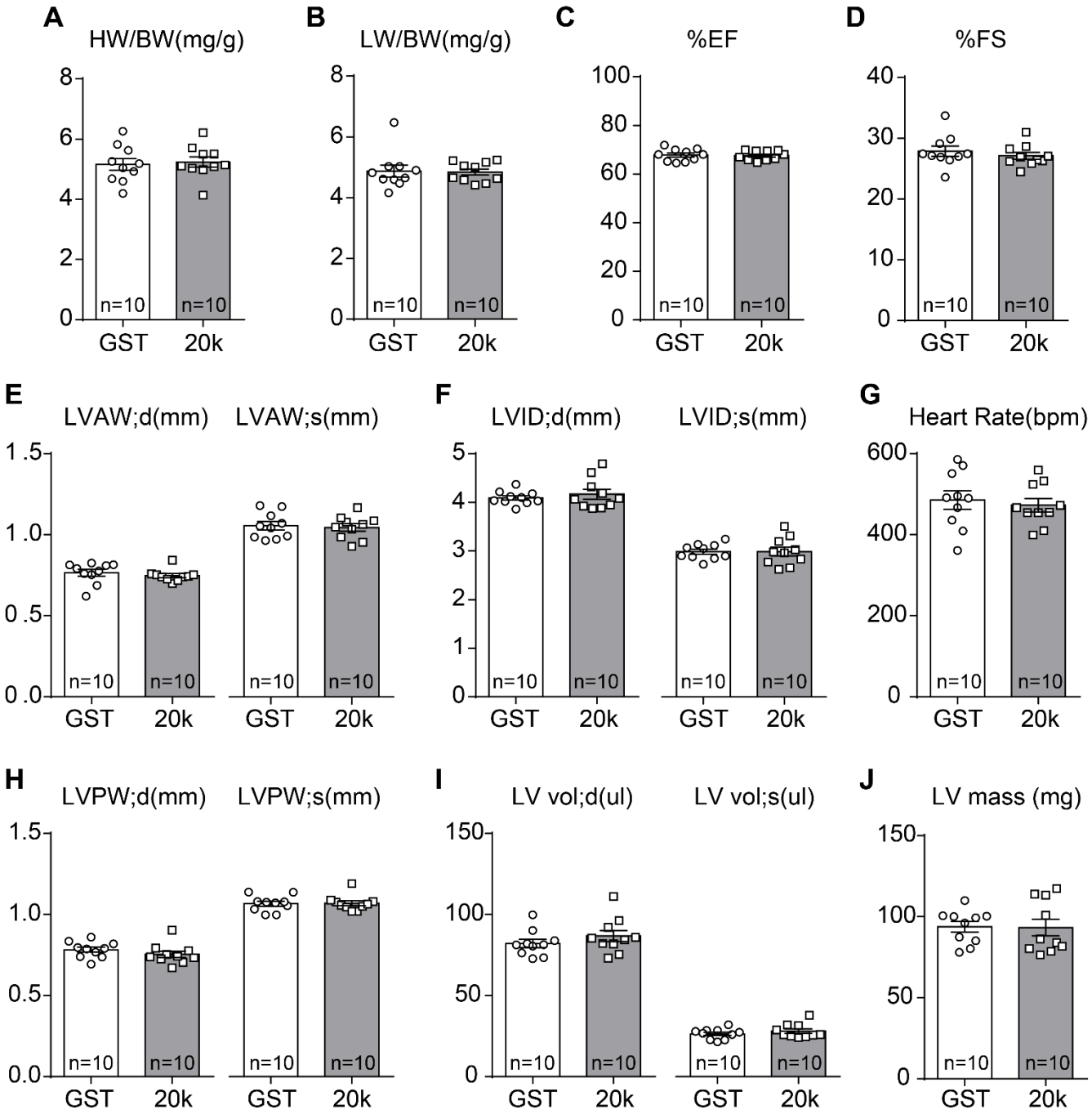
Supplemental Materials



Supplemental Figure 1. Extended GJA1-20k expression does not lead to cardiac pathology. Structural and functional parameters recorded from AAV9-GJA1-20k or AAV9-GST expressing hearts at 4 weeks following AAV9 introduction. **A)** Heart/body weight ratio (mg/g). **B)** Lung/body weight ratio (mg/g). Echocardiography measurements include: **C)** % Ejection fraction. **D)** % Fractional shortening. **E)** Left ventricular anterior wall thickness (mm; diastole and systole). **F)** Left ventricular internal dimension (mm; diastole and systole). **G)** Heart rate (beats per minute). **H)** Left ventricular posterior wall thickness (mm; diastole and systole). **I)** Left ventricular volume (ul; diastole and systole). **J)** Left ventricular mass (mg).

Extended Methods

Isolation of adult mouse ventricular cardiomyocytes.

Ventricular cardiomyocytes were isolated from mice hearts as previously described (1). Adult (12 to 14 weeks old) male C57BL/6 mice (The Jackson Laboratory 000664) were injected with heparin (100 IU, i.p.) 20 to 30 mins before dissection. Mice were anesthetized with isoflurane and cervical dislocation was performed and hearts removed rapidly and immersed in ice-cold calcium-free perfusion buffer containing (in mmol/L) NaCl 120.4, KCl 14.7, KH_2PO_4 0.6, Na_2HPO_4 0.6, 5 $MgSO_4$-$7H_2O$ 1.2, Na-HEPES 10, $NaHCO_3$ 4.6, taurine 30, butanedione monoxime (BDM) 10 and glucose 5.5 (final pH=7). Extraneous tissue was removed, and the aorta cannulated under a dissection scope. Hearts were retrograde perfused through the aorta (2.5ml/min) (Radnoti Langendorff perfusion apparatus) with calcium-free perfusion buffer which was then switched to calcium-free digestion buffer (30ml) (perfusion buffer containing collagenase II [2mg/ml] from Worthington Biochemical). The hearts were then further digested with digestion buffer containing 100 μmol/L $CaCl_2$ (20mL). Hearts were removed from the perfusion apparatus and placed in a Petri dish containing 2 ml digestion buffer and 3 ml of stop buffer (perfusion buffer with 10% FBS and 12.5μM $CaCl_2$). The atria were removed and the ventricles were pulled into 10-12 equally sized pieces. Heart tissue was then gently dispersed into cell suspension using plastic transfer pipettes. The cell suspension was collected in a 15ml falcon tube, brought to 10 ml with stop buffer and centrifuged at 40 x g for 3 min. Damaged myocytes and non-myocytes were removed by a series of washes in 10 ml of stop buffer containing, sequentially, 100, 400, or 900 μmol/L $CaCl_2$. Cardiomyocytes were pelleted by centrifugation at 40 x g for 3 min after each wash. After the final wash, cardiomyocytes were resuspended in cardiomyocyte culture medium (ScienCell), plated onto dishes pre-coated with 10

μg/ml Laminin (BD Biosciences) and maintained for 2 hours in a humidified atmosphere of 5% CO_2 at 37^{o}C to allow them to adhere. The cardiomyocytes were then washed once with culture medium and placed in the 37^{o}C incubator with 5% CO_2.

Immunostaining and confocal imaging

For tissue immunofluorescence, cryosections (10 μm) were fixed in 4% PFA for 20 minutes at room temperature, blocked and permeabilized at room temperature for 1 hour with 10% normal goat serum (NGS) and 0.5% TritonX-100 in PBS. The tissue sections were incubated with primary antibodies diluted in 5% NGS and 0.1% TritonX-100 in PBS (antibody diluent) at 4°C overnight. The primary antibodies used are: mouse anti-Cx43 (N-terminus epitope, 1:200, Acris AM32558SU-N), rabbit anti-Cx43 (C-terminus epitope, 1:1000, Sigma-Aldrich C6219) and chicken anti-GFP (1:500 Abcam ab13970). The following day, the sections were washed (3x5min washes) with PBS then incubated for 1 hour at room temperature with the respective secondary antibodies conjugated to anti-mouse or anti-rabbit Alexa Fluors (1:500, Life technologies). Tissue sections were then washed (3x5min) with PBS and coverslips were mounted using ProLong gold antifade reagent containing DAPI. The tissue sections were imaged using a Nikon Eclipse T*i* imaging system with a ×100/1.49 Apo objective, a spinning disk confocal unit (Yokogowa CSU-X1) with 561 and 647-nm diode-pumped solid-state lasers, and an ORCA-Flash 4.0 Hamamatsu camera (C11440), controlled by NIS Elements software. Maximum intensity projections of 12.5 μm confocal z-stacks was obtained for imaging. Colocalization signal between Cx43-CT and Cx43-NT signal was assessed using JACoP (Just Another Colocalization Plugin) for ImageJ.

Mitochondria purification

The mitochondria purification assay was performed as previously described (2). The mouse heart or isolated cardiomyocytes were manually homogenized in 1.5 ml of isolation Buffer A with BSA (in mM: 230 Mannitol, 70 sucrose, 10 HEPES, 2 EDTA pH 7.2 with KOH and 1 mg/mL fatty acid free BSA) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 1,300x g for 3 min at 4ºC and 10% of the supernatant was removed as input and stored at -80ºC. The remainder of the supernatant was then carefully collected and centrifuged at 10,000x g for 10 min at 4ºC. The resulting pellet which contains crude mitochondria was gently resuspended in 55ul of isolation buffer A with no BSA while the supernatant which contains the F1 fraction was stored at -80ºC. The resuspended crude mitochondria pellet was gently overlaid on 3 mL of 30% (vol/vol) Percoll solution in Buffer B (250 mM sucrose, 10 mM Hepes, 1 mM EDTA-Na2, pH7.4) (0.9 mL Percoll pure + 2.1 mL Buffer B). The mitochondria were ultracentrifuged at 50,000x g for 45 min at 4ºC. After the ultracentrifugation, 2 clear layers of mitochondria were observed, F2 and F3. The F2 and F3 fractions were carefully collected in separate tubes and resuspended in isolation Buffer A (without BSA) and centrifuged at 12,000x g for 5 min at 4ºC. The resulting pellet from each fraction was washed twice with 1 mL of isolation buffer A (without BSA) to remove all percoll and then resuspended in RIPA lysis buffer and stored with Nupage LDS sample buffer at -80ºC.

Molecular Biology

Human *GJA1* cDNAs (Open Biosystems) encoding full-length Cx43 and smaller isoforms were cloned into pDONR/221 to generate Gateway entry clones (ThermoFisher Scientific) as previously described (3). Using these clones, destination vectors (pDEST)

encoding C-terminal V5 or GFP-tagged proteins were subsequently made. All constructs are driven by the cytomegalovirus (CMV) promoter. Internal methionine start sites in GJA1-20k were mutagenized to leucine (QuickChange Lightning Mutagenesis Kit, Agilent) to ensure single isoform expression as described (3).

Cell culture and transfection

Cells were seeded at a concentration of 3x10^6 cells (HEK293T, Life Technologies) on 10 cm culture dishes and the cells were maintained in culture medium (DMEM media, antibiotic free, containing 10% FBS, non-essential amino acids and sodium pyruvate) (ThermoFisher Scientific) at 37ºC in a humidified atmosphere of 5% CO_2 for 16-24 hours. The HEK293T cells were transfected with pDEST-GST-GFP or pDEST-GJA1-20k-GFP (3.5ug plasmid/10 cm dish) using Fugene HD (Promega) according to manufacturer's instructions. 16-24 hours after transfection, the cells were washed 2x with culture medium and transfection efficiency was assessed by examining GFP fluorescence.

Proteinase k protection assay

HEK293T cells (Life Technologies) were transfected with GFP tagged GJA1-20k as described above and mitochondria were isolated using the mitochondria isolation kit for cultured cells with dounce homogenization method according to manufacturer's instructions (ThermoFisher Scientific catalog# 89874)(4). The cells in the dish were scraped with 800 ul of Isolation Reagent A before the douncing step. Following the 700 xg centrifugation step for 10min at 4ºC, the supernatant was then centrifuged at 3000 xg for 15min to obtain a more purified fraction of mitochondria (according to manufacturer's instructions). Following the 3000

xg centrifugation step, the mitochondria pellet was incubated in a 1ug/ml PK solution (ThemroFisher Scientific, catalog# AM2548) diluted in Isolation Reagent C on ice for 30 minutes. The mitochondria were pelleted by centrifugation at 12,000 xg for 5 minutes at 4^{o}C and washed once with Isolation Reagent C. The mitochondria were centrifuged again at 12,000 xg for 5 minutes at 4^{o}C and the pellet was resuspended in 1 X RIPA buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 1% TritonX-100, 1% Soduim Deoxycholate, 1mM NaF, 0.2mM Na_3VO_4 and 2x Halt Protease and Phosphatase Inhibitor Cocktail). Loading buffer (Nupage LDS sample buffer NP0007, ThermoFisher Scientific) was added at 1x final concentration prior to running the western blot.

Super resolution STORM imaging

The crude mitochondria pellet isolated from adult mouse ventricular cardiomyocytes, infected with an adenovirus vector for V5-GJA1-20k, was resuspended in isolation buffer A (in mM: 230 Mannitol, 70 sucrose, 10 HEPES, 2 EDTA pH 7.2 with KOH) and plated onto a laminin pre-coated 35 mm glass bottom dish for 1:30 hours at 4^{o}C. The mitochondria were then fixed with 4% PFA at room temperature for 10 minutes followed by blocking and permeabilization for 10 minutes at RT in 1% NGS with 0.5% TritonX-100 in PBS. The mitochondria were then incubated at 4°C overnight with primary antibodies diluted in 1% NGS with 0.1% TritonX-100 in PBS. Primary antibodies used were: rabbit anti-Tom20 (1:200; Santa Cruz biotechnologies sc11415) and mouse anti-V5 (1:200, Sigma-Aldrich V8012). The following day, the mitochondria were incubated for 1 hour at room temperature with the respective secondary antibodies conjugated to anti-mouse or anti-rabbit Alexa Fluors (1:500, Life technologies) and after 6 washes (5 min each) in PBS the mitochondria were left to air dry

and then stored in the dark at room temperature. At the day of STORM imaging, freshly made oxygen-scavenging buffer system was added to the mitochondria-containing dishes to enable effective photoswitching. Briefly, 10 mmol/L cysteamine (Sigma) was added to GLOX (0.5 mg/mL glucose oxidase, 40 μg/mL catalase, 10% glucose) in 50 mmol/L Tris (pH8.0) buffer with 10 mmol/L NaCl. All images were collected using a Nikon Eclipse Ti microscope with a 100 x objective with 1.49 numerical aperture total internal reflection fluorescent (TIRF) objective and NIS Elements software with STORM module. The STORM images (signals within 500nm Z-depth from the coverslips) were acquired using lasers (488, 561 from a self-contained 4 line laser module with AOTF) and captured by a high speed iXon DU897 Ultra EMCCD camera. Fresh STORM imaging buffer was exchanged every hour to maintain photoswitching properties of the sample (5). The STORM module in Nikon Element software was used to obtain and analyze the images to generate 3-dimensional projections of V5-20K/Tom20 and V5-GFP/Tom20 images at high resolution (XY-resolution, 10-20nm, Z-resolution, 50nm).

Western blotting

Heart tissue samples and mitochondrial fractions were lysed in either RIPA buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 1% TritonX-100, 1% Soduim Deoxycholate, 1mM NaF, 0.2mM Na_3VO_4 and 2x Halt Protease and Phosphatase Inhibitor Cocktail) or in mitochondrial isolation Buffer A with BSA. The protein concentration was quantified using Bio-Rad DC protein assay. Protein lysates and the mitochondrial fractions were mixed with loading buffer (Nupage LDS sample buffer NP0007, ThermoFisher Scientific) at 1x final concentration and then aliquoted and stored at –80°C. Prior to running the western blot, the samples were thawed and incubated at room temperature for 45 minutes then subjected to SDS-PAGE

electrophoresis using NuPAGE Bis-Tris gels (4-12%) with MOPS or MES running buffers (Invitrogen) according to the manufacturer's instructions. Gels were transferred to FluoroTrans PVDF membranes (Pall), which were subsequently fixed by soaking in methanol and air drying before rewetting with methanol and blocking for 1 hour at room temperature with 5% nonfat milk or 5% BSA (for phospho antibodies) in TNT buffer (0.1% Tween20, 150 mM NaCl, 50 mM Tris pH 8.0). Membranes were then incubated overnight with primary antibodies diluted in 5% milk or 5% BSA in TNT. Primary antibodies used were rabbit anti-Actin (1:3000, Sigma-Aldrich A2103), rabbit anti-$CaV_{1.2}$ (1:500, Alomone Labs ACC-003), mouse anti-Cx43 (1:1000, Fred Hutchinson Cancer Research Center CT1), mouse anti–N-cadherin (1:1000, BD Biosciences 610921), mouse anti-Na^+/K^+ ATPase (1:1000, Millipore 05-369), rabbit anti-Tom20 (1:1000, Santa Cruz Biotechnologies sc1145), rabbit anti-PGC-1α (1:1000, Novus Biologicals NBP1-04676), rabbit anti-NRF1 (1:5000, Abcam ab175932), rabbit anti-mtCO2 (1:500, abcam ab198286), rabbit anti-mtTFA (1:1000 Abcam ab138351), mouse anti-CoxIV (1:1000, Abcam ab14744), mouse anti-Gapdh (1:5000, Abcam ab8245), rabbit-anti p-Drp1(Ser616; active form of Drp1) (1:500, Cell Signaling D9A1), chicken anti-Mitofusin1 (1:500, Abcam ab107129), mouse anti-tubulin (1:1000, Sigma-Aldrich T6199). The following day, the membranes were washed 3x (5minute washes) with TNT buffer before incubation for 1 hour at room temperature with the respective secondary antibodies (anti-rabbit or anti-mouse Alexa Flours 1:500, Life technologies) diluted in 5% milk or 5% BSA in TNT. Following incubation with secondary antibody, membranes were washed 5x (5minute washes) with TNT buffer, soaked in methanol, and allowed to air dry. Membranes were then imaged using the Versadoc MP 4000 fluorescent western detection system (Bio-Rad). Quantity One (Bio-Rad) analysis software was used to

quantify individual band intensities. The samples were normalized to Actin or Gapdh and graphs were plotted as fold change and statistical analysis performed using GraphPad Prism 6 software.

AAV9 production and gene delivery

AAV9 vectors containing GST-GFP or GJA1-20k-GFP driven by the CMV promoter were produced in human embryonic kidney 293 cells and were purified by Welgen, Inc. (Worcester, MA). Adult C57BL/6 male mice received 100 microliters of 3×10^{10} vector genomes (vg) of AAV9-GST-GFP or AAV9-GJA1-20k-GFP through retro-orbital injection (6). The AAV9 vectors were delivered 4 weeks prior to cardiomyocyte or heart tissue collection and analysis. Vector encoded GFP expression in heart tissue was confirmed by immunofluorescence detection at 4 weeks post injection.

Transmission Electron microscopy

Left ventricular tissue was isolated from AAV9-GST-GFP or AAV9-GJA1-20k-GFP mice with AAV9 vectors delivered 4 weeks prior to tissue collection. Hearts were perfused with 2% glutaraldehyde and 2% paraformaldehyde in PBS for 10 minutes and left ventricular tissue was post-fixed with 1% osmium tetroxide and incubated in 3% uranyl acetate (7). Samples were then dehydrated in ethanol, treated with propylene oxide, embedded in Spurr resin (Electron Microscopy Services), and sectioned using an ultramicrotome (UCT, Leica). Sections were mounted on EM grids and stained with uranyl acetate and lead citrate and prepped for imaging.

Images were acquired using the JEM1200-EX, JEOL microscope equipped with a digital camera (BioScan 600W, Gatan). Percent of cell area occupied by mitochondria was assessed using imageJ. All electron microscopy work was done by the core facility at the Electron Imaging Center of The California NanoSystems Institute, UCLA.

Assessment of Mitochondrial DNA copy number

Genomic DNA was extracted using NucleoSpin Tissue kit (MACHEREY-NAGEL, catalog# 740952) from left ventricular tissue isolated from AAV9-GJA1-20k and AAV9-GST expressing mice at 4 weeks post AAV9 expression. Mitochondrial copy number was then assessed using Mouse Mitochondrial DNA Copy Number Assay Kit according to manufacturer's instructions (Detroit R&D, catalog# MCN3) and a Real-Time PCR system (Bio-Rad, CFX-Connect) (8). DNA concentration used for Real-Time PCR was 5 ng/ul for each heart sample.

Measurement of mitochondrial membrane potential

Acutely isolated cardiomyocytes from AAV9-GST or AAV9-GJA1-20k expressing hearts were loaded with Mitotracker JC1 (ThermoFisher Scientific) at 5 μg/mL (in perfusion buffer with 1.8mM $CaCl_2$) and incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 for 30 minutes (9). Cells were washed three times (with perfusion buffer containing 1.8mM $CaCl_2$) before live cell imaging. The cardiomyocytes were imaged using a Nikon Eclipse T*i* imaging system with a ×100/1.49 Apo objective, a spinning disk confocal unit (Yokogowa CSU-X1) with 488 and 561-nm diode-pumped solid-state lasers, and an ORCA-Flash 4.0 Hamamatsu camera (C11440), controlled by NIS Elements software. Maximum intensity projections of 12.5 μm

confocal z-stacks was obtained for imaging. The ratio of red to green fluorescence intensity was used as indicator of mitochondrial membrane potential and quantified using imageJ.

Seahorse mitochondrial respiration assay

The mitochondrial oxygen consumption was evaluated using Seahorse XF Cell Mito stress Test kit according to the manufactures' instructions (10). Briefly, 24 well Agilent Seahorse cell culture microplate were coated with 20 ug/uL laminin overnight. Adult cardiomyocytes (1000 cells) isolated from AAV9-GST or AAV9-GJA1-20k hearts were plated in each well and allowed attachment for one hour. The cells were then switched to XF base media supplemented with 5.5 mM glucose, 1 mM pyruvate and 4 mM L-glutamine, and incubate in 0% CO2 for one hour before the test. FCCP (Carbonyl cyanide p-trifluoromethoxyphenylhydrazone) at 0.8 uM was used to elicit maximal respiration. Complex I and III inhibitors (Rotenone and antimycin A) were used at 50 uM to measure non-mitochondrial respiration. ATPase synthase inhibitor oligomycin at 3 uM does not inhibit oxygen consumption, consistent with the state-4 like bioenergetic profile of quiescent adult cardiomyocytes (10).

Superoxide measurement

Acutely isolated cardiomyocytes from AAV9-GST or AAV9-GJA1-20k expressing hearts were loaded with MitoSOX Red (ThermoFisher Scientific) at 5 μg/mL (in perfusion buffer with 1.8mM $CaCl_2$) and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 20 minutes. Cells were washed three times (with perfusion buffer containing 1.8mM $CaCl_2$) before live cell imaging. The cardiomyocytes were imaged using a Nikon Eclipse T*i* imaging system with a ×100/1.49 Apo objective, a spinning disk confocal unit (Yokogowa CSU-X1) with 561-

nm diode-pumped solid-state lasers, and an ORCA-Flash 4.0 Hamamatsu camera (C11440), controlled by NIS Elements software. Maximum intensity projections of 12.5 µm confocal z-stacks was obtained for imaging. The red fluorescence intensity was used as indicator of mitochondrial superoxide production and quantified using imageJ.

Echocardiography

Transthoracic echocardiography imaging was performed on anesthetized animals (4 weeks post AAV9-GJA1-20k or AAV9-GST injection) using a VEVO 3100 ultrasound machine with MX5550D transducer (VisualSonics) (11). LV volume, %EF, %FS and heart rate measurements were determined from the B-mode long axis view. LVID, LVPW, LVAW and LV mass measurements were determined from the M-mode short axis view.

Langendorff-perfused mouse heart preparation, Ischemic Preconditioning and I/R injury.

The Langendorff-perfused mouse hearts were subjected to ischemia/reperfusion injury as previously described (12, 13). Adult male C57BL/6 mice (The Jackson Laboratory 000664) (12 to 14 weeks old) were injected with heparin (100 IU, I.P) 20 to 30 minutes prior to the Langendorff procedure. Mice were anesthetized with isoflurane and cervical dislocation was performed and hearts were removed quickly by a midsternal incision and placed into ice-cold modified pH 7.4 Krebs-Henseleit (K-H) solution of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2.H_2O$ 2.5, $MgCl_2.7H_2O$ 1.2, $NaHCO_3$ 24, KH_2PO_4 1.2, glucose 11, EDTA 0.5. Under a dissecting microscope, the aortic opening was immediately cannulated and tied on a 23-gauge stainless steel blunt needle. The heart was attached to a Langendorff apparatus (Radnoti) and perfused through the aorta at a constant rate of 2.5 ml/min with the K-H buffer.

The K-H solution was constantly gassed with 95% O_2/5% CO_2. Perfusion medium was passed through water-jacketed tubing and cylinders, and the temperature was maintained at 37°C with a temperature-controlled circulating water bath. The hearts were allowed to equilibrate for 20 minutes to achieve a steady state before they were subjected to 30 minutes of global ischemia, followed by 60 minutes of reperfusion. Control hearts were perfused continuously throughout the protocol. During no-flow ischemia, the heart was immersed in warm K-H buffer in order to maintain warmth and moisture.

For the ischemic preconditioning experiments, the isolated mouse hearts were allowed to equilibrate for 20 minutes to achieve a steady state before they subjected to 4 repeated cycles of 3.5 minutes ischemia followed by 5 minutes reperfusion (14). After the 4 cycles of ischemic preconditioning, the hearts were subjected to prolonged I/R of 30 minutes ischemia and 60 minutes reperfusion.

For ECG recordings, electrodes (140155-M, Radnoti) were used to record the electrocardiogram and heart rate throughout the experiment using labchart software (ADInstruments) with Power lab and Bio Amp hardware (ADInstruments). A Millar MIKRO-TIP (SPR-671) pressure catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle from the left atrium to measure left ventricular pressure throughout the experiment using a Bridge Amp (ADInstruments). Left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP) and heart rate were monitored and recorded continuously using PowerLab system (ADInstruments). Hearts were paced at 360 beats per minute using a pacing electrode (140157M, Radnoti) and a stimulus delivered from a stimulator (stimulus

isolator, ADInstruments) (ADInstruments Colorado Springs, CO). After the initial 20 min stabilization, hearts were excluded from further study if they exhibited one or more of the following exclusion criteria: LVEDP higher than 20 mmHg; LVDP less than 50 mmHg; intrinsic heart rate less than 280 bpm or irregular; or aortic regurgitation(13). Hearts were then subjected to 30 min of global ischemia followed by 1 hour of reperfusion. Pacing was initiated at 2 min after reperfusion(13).

Myocardial infarction model

Four weeks post AAV9-GST-GFP or AAV9-GJA1-20k-GFP delivery, mice were given a dose of analgesia (0.1 mg/kg Buprenex and 5 mg/kg Carprofen), injected with an anesthetic cocktail (10 mg/kg Xylazine and 100 mg/kg Ketamine), intubated using the Biolite intubation system (Braintree Scientific), and mechanically ventilated at 0.2 cm3 tidal volume/100 respirations per minute with oxygen and isoflurane inhaled anesthesia (Minivent type 845, Harvard Apparatus). Left anterior descending coronary artery ligation was performed as previously described (15). Complete occlusion of the vessel was verified by visible blanching of the myocardium distal to the tie. After verifying that the mice were breathing spontaneously, they were injected with analgesia (Buprenex) and observed until recovery was complete. No animals died due to surgery. At 72 hours post ligation, mice were euthanized and hearts were collected for infarct size analysis.

Infarct Size analysis

Infarct size analysis was done as previously described (16). Briefly, Triphenyl tetrazolium chloride (TTC) staining was used for postmortem determination of infarct size.

Transverse heart slices 1 millimeter thick were immersed in freshly made 1% TTC solution for 20 minutes at 37 ^{o}C, followed by fixation (10% neutral formalin, Sigma-Aldrich) for 30 minutes before imaging. Viable myocardium was stained red by the conversion of the TTC redox indicator into a precipitate by mitochondrial dehydrogenases. The extent of red staining indicated viable tissue containing active mitochondria, whereas white areas marked nonviable infarcts. Using ImageJ, the area of the infarcted region of each slice was manually traced, measured, and expressed as a percentage of the total slice area. Infarct size was corrected to the weight of each slice as follows: Weight infarct = (WeightSlice 1 x %InfarctSlice 1) + (WeightSlice 2 x %InfarctSlice 2) + (WeightSlice 3 x %InfarctSlice 3) + (WeightSlice 4 x %InfarctSlice 4).

Statistical analysis

All quantitative data were expressed as mean ± SEM and analyzed using Prism 6 software (GraphPad). For comparison between two groups, unpaired two-tail Student's t test or Mann-Whitney U-test was performed. For comparison between three groups, Kruskal-Wallis test with Dunn's multiple comparisons test was performed. The Seahorse data in Figures 7C-7D and the intraventricular pressure data in Figures 10C-10D were analyzed using two-way ANOVA with Sidak's or Holm- Sidak's multiple comparisons test. Shapiro-Wilk normality test was performed for all data. Two-sided p-values are reported and a p-value of less than 0.05 was deemed statistically significant.

Human Tissue Collection

Cold cardioplegia was perfused antegrade prior to cardiectomy, and the explanted heart was placed immediately in ice-cold physiologic solution. Full-thickness samples from the base of the

left ventricle were cleaned rapidly of all epicardial fat and embedded in OCT medium. The embedded tissue was submerged in liquid N2-chilled isopentane to snap-freeze before storage at –80°C.

Study Approval

Study Approval: Mice

C57BL/6 mice were obtained from JAX and were maintained under sterile barrier conditions. All procedures were reviewed and approved by Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Study Approval: Human

De-identified human heart lysates from patients with end stage ischemic cardiomyopathy undergoing transplantation and non-failing controls who died from non-cardiac reasons were obtained from the Cedars-Sinai Heart Institute (CSHI) Biobank, which stores plasma as well as tissue and lysates from heart explants acquired under informed consent using a protocol approved by institutional review board from the Cedars-Sinai Medical Center Office of Research Compliance.

1. Smyth JW, Vogan JM, Buch PJ, Zhang SS, Fong TS, Hong TT, et al. Actin cytoskeleton rest stops regulate anterograde traffic of connexin 43 vesicles to the plasma membrane. *Circulation research.* 2012;110(7):978-89.
2. Singh H, Lu R, Rodriguez PF, Wu Y, Bopassa JC, Stefani E, et al. Visualization and quantification of cardiac mitochondrial protein clusters with STED microscopy. *Mitochondrion.* 2012;12(2):230-6.
3. Smyth JW, and Shaw RM. Autoregulation of connexin43 gap junction formation by internally translated isoforms. *Cell Rep.* 2013;5(3):611-8.
4. Li GB, Zhang HW, Fu RQ, Hu XY, Liu L, Li YN, et al. Mitochondrial fission and mitophagy depend on cofilin-mediated actin depolymerization activity at the mitochondrial fission site. *Oncogene.* 2018;37(11):1485-502.

5. Fu Y, Shaw SA, Naami R, Vuong CL, Basheer WA, Guo X, et al. Isoproterenol Promotes Rapid Ryanodine Receptor Movement to Bridging Integrator 1 (BIN1)-Organized Dyads. *Circulation.* 2016;133(4):388-97.
6. Basheer WA, Xiao S, Epifantseva I, Fu Y, Kleber AG, Hong T, et al. GJA1-20k Arranges Actin to Guide Cx43 Delivery to Cardiac Intercalated Discs. *Circulation research.* 2017;121(9):1069-80.
7. Fu Y, Zhang SS, Xiao S, Basheer WA, Baum R, Epifantseva I, et al. Cx43 Isoform GJA1-20k Promotes Microtubule Dependent Mitochondrial Transport. *Front Physiol.* 2017;8:905.
8. Medeiros DM. Assessing mitochondria biogenesis. *Methods.* 2008;46(4):288-94.
9. Perry SW, Norman JP, Barbieri J, Brown EB, and Gelbard HA. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques.* 2011;50(2):98-115.
10. Readnower RD, Brainard RE, Hill BG, and Jones SP. Standardized bioenergetic profiling of adult mouse cardiomyocytes. *Physiol Genomics.* 2012;44(24):1208-13.
11. Hong T, Yang H, Zhang SS, Cho HC, Kalashnikova M, Sun B, et al. Cardiac BIN1 folds T-tubule membrane, controlling ion flux and limiting arrhythmia. *Nature medicine.* 2014;20(6):624-32.
12. Smyth JW, Hong TT, Gao D, Vogan JM, Jensen BC, Fong TS, et al. Limited forward trafficking of connexin 43 reduces cell-cell coupling in stressed human and mouse myocardium. *J Clin Invest.* 2010;120(1):266-79.
13. Gao D, Zhang L, Dhillon R, Hong TT, Shaw RM, and Zhu J. Dynasore protects mitochondria and improves cardiac lusitropy in Langendorff perfused mouse heart. *PloS one.* 2013;8(4):e60967.
14. Rodriguez-Sinovas A, Sanchez JA, Gonzalez-Loyola A, Barba I, Morente M, Aguilar R, et al. Effects of substitution of Cx43 by Cx32 on myocardial energy metabolism, tolerance to ischaemia and preconditioning protection. *The Journal of physiology.* 2010;588(Pt 7):1139-51.
15. Kolk MV, Meyberg D, Deuse T, Tang-Quan KR, Robbins RC, Reichenspurner H, et al. LAD-ligation: a murine model of myocardial infarction. *Journal of visualized experiments : JoVE.* 2009(32).
16. Redfors B, Shao Y, and Omerovic E. Myocardial infarct size and area at risk assessment in mice. *Exp Clin Cardiol.* 2012;17(4):268-72.