Supplemental Material

Expanded Materials and Methods

Statistical analyses. Please refer to **Online Dataset S1** Excel file for detailed summary of all the statistical tests performed in the study, broken down by individual figures and figure panels. For binary comparisons between genotypes, when $N \geq 6$ animals or hearts, t-test was used for normally distributed data and Mann-Whitney Rank Sum test when normality test failed ($P<0.05$ with Shapiro-Wilk test). When, for binary comparisons, multiple cells were measured from several animals, a linear mixed effects regression model was used to determine statistical significance. The model included fixed effects for genotype and random intercept effects for the individual mice[46]. Where data could not be assumed to be normally distributed from literature nor accurately estimated, due to few animals and/or few cells per animal, the model was constructed on data transformed to an approximately normal distribution (zero mean and unit variance) by the Rankit procedure. Modeling was done in SPSS software (IBM). Low N (animal<6) was generally used for dependent variables obtained from either 1) live individual cells or a cell group defined by the microscope field of view on a coverslip, or from aliquots of cell suspensions or 2) fixed tissue/cells and/or subcellular formations (morphometric analysis, electron microscopy). For these experiments, besides N, n (>>N) is also accounted as the number of individual cells or coverslips (the latter when all/most cells on the field responded similarly to a stimulus). Multiple comparisons for *in vivo* or *ex vivo* organ function studies, where, besides the main genotypes (linker-expressing vs. non-expressing control and/or doxy-linker) sex differences or drug (cyclosporine A) effects were also factored in, 2-Way

ANOVA was used, with repeated measures (RM) for multiple time points or multiple stimuli. Using SigmaStat, assumption checking was automatically performed for the normality of distribution (Shapiro-Wilk test) and for equal variance (passed at $P \geq 0.05$). If/when normality or equal variance failed or $N<6$, the data were rank-transformed using Rankit and the ANOVA was run on the ranks. When ANOVA found a significant interaction ($P<0.05$), p values were adjusted by a post hoc multiple comparison procedure (Holm-Sidak method) ; either 'all pairwise' or 'against control' depending on the data layout (indicated in each figure in gray box). For the echocardiography parameters recorded during high-dose isoproterenol challenge (Figs. 7, S5), a complication was that some of the animals died before the last time point (24h) was recorded. These missing data would have been prohibitive for the 2-Way RM ANOVA. To circumvent the issue, in these experimental datasets, a 2-Way RM ANOVA was performed separately for the 0min-0.5min-30min period, that included practically all animals (1 Doxy female. who died before 30 min, was excluded); then, for the full 0min-24h period, on the smaller cohort that survived 24h. For the latter, only the multiple comparison against or at the 24h time point were accounted.

To compare cellular responses to uninterrupted incremental stimulation (NADH/FAD autofluorescence responses to incremental electric pacing), we modeled the dependence of the NADH/FAD ratios on the pacing frequency using generalized estimating equations (GEE) with a nested, hierarchical covariance structure to account for variations between experimental days, technical replicates and individual cells. This model output is summarized in Table S1. GEE modeling was done using Statsmodels (Python).No study-wide multiple comparison adjustment was applied. The Holm-Sidak correction was used

for tests of multiple hypotheses within the same data set following ANOVA or linear mixed-effect regression models (Fig4C,D).

Experimental randomization, inclusion and exclusion criteria, handling of outliers. **Randomization**. Whenever more than one pair of Ctr vs Lnk animals were used for in vivo experiments (e.g. echocardiography), attention was paid to perform the procedure on the animals by alternating between groups to avoid potential biased variations in the individual waiting time in the cages at the performance site. When males and females were both used, the alternation between groups was done in male-male, female-female pairs. For the experiments with live cells, myocyte batches isolated simultaneously from a pair of Lnk and Ctr hearts were handled as pairs for randomization too (e.g. technical repetitions for fluorescence-based live-cell assays on coverslips or in aliquots of permeabilized cell suspensions were performed by alternating Lnk with Ctr and the different treatment conditions in each repetition cycle. **Inclusion/exclusion**: as described earlier, mouse inclusion was primarily based on age range. Animals displaying distress or appearing sick were excluded. When echocardiography was performed with heart rate recording, animals with <400/min basal heart rate were excluded (concern of relative isoflurane overdose). For VCMs, isolation batches with $\geq$85% viable cell yield were included. For live-cell imaging the cells included in analysis should have maintained their 'healthy rod shape' until the last analyzed data point. See further, experiment-specific incusion/exclusion details at the description of experiments. **Outliers**: For N<10, when each N represented an animal or heart, we were conservative with regard to outliers. Suspected outlier was tested using a box plot, where outlier was defined as <(Q1-1.5*IQR) or >(Q3+1.5*IQR), where Q1 and Q3 are the 25% and 75% quartiles and IQR

is the inter-quartile range (the box size, Q3-Q1). Confirmed outlier was double-checked for potential technical/human error reasons. Technical outliers were either corrected (in case of fixable analytical/calculation error) or removed/excluded. If no technical reason was identified for the large difference, the data point was handled as a potential biological variance. In such cases, P values are shown both via including the outlier and (*excluding the outlier*), as it is shown for CII in Figure S3C. For larger n, such as cells, non-technical outlier was excluded when it was so extreme that grouping the data points in a 6-bin histogram, the outlier rendered the rest of the data to the lower or upper 2 bins (separated from the rest of the data by 2 empty bins).

Materials, chemicals, biologicals, pharmaceuticals. Chemicals and reagents were purchased from Sigma-Aldrich and Thermo Fisher Scientific, unless otherwise specified. Reagents for transmission electron microscopy (TEM), FIB-SEM microscopy and histology preparations were from Electron Microscopy Sciences. Protein assay kits, Western blot (WB) reagents, were from Bio-Rad. Fluorescent Ca^{2+} indicator Fura-2 acetoxymethyl ester was from AAT Bioquest. Primary antibodies were as follows (dilutions when not specified are for WB). Anti-MCU rabbit polyclonal from Cell Signaling Technologies (CST D2Z3B, WB 1:500, IF 1:100), anti-RyR2 mouse monoclonal (MA3–916, WB 1:500, IF 1:50) from Thermo Scientific. Anti-RFP (1:100 for immunogold) from Life Technologies, PGC1α (WB 1:1000 RRID:AB_1640773), Calsequestrin (WB 1:1000, RRID:AB_303865), Calreticulin (WB 1:1000, RRID:AB_2275208), SERCA (WB 1:1000, RRID:AB_325502). Secondary antibodies for IF were Alexa Fluor®647 (A21244, 1:200) and Alexa Fluor®488 (A21200, 1:200) from Thermo Fisher. Secondary antibody for immunogold was Fluoronanogold-AlexaFluor488 and gold enhancer GoldenhanceTM

EM Plus from Nanoprobes. Cell culture media and supplements were from Gibco. Calf serum from HyClone. Secondary antibodies for WB were LiCor IRDye 680RD (926-68022 anti-mouse) or LiCor IRDye 800CW (926-32213 anti-rabbit). Collagenase type 2 was from Worthington-Biochem, laminin from Corning. Seahorse assay kit was from Agilent Technologies. Doxy-diet**:** Modified 5001 diet w/200ppm doxycycline from Animal Specialties and Provisions (Quakertown, PA).

Creating the Linker-Mouse. FVB/N ^{Tg}(Myh6/-tetO-linker) and FVB/N ^{Tg}(Myh6/tTA) mice were each crossed with an Mfn2$^{LoxP/LoxP}$ strain with mixed background, heterozygously expressing cardiac muscle-specific nuclear-directed αMHC-'turbo'Cre[7] (conditional Mfn2KO). Owing to apparent mosaicism, turboCre was bred out; leading to the $^{Tg/Tg}$(Myh6/tetO-linker) x $^{Tg/Tg}$(Myh6/tTA) x Mfn2$^{fl/fl}$ 'linker-mouse' (Figure 1A). To suspend linker expression, doxycycline-supplemented chow ('Doxy-diet') was provided at libitum (estimated ~200 mg/kg/day) for 3-7 weeks.

PCRs and genotyping Ear clippings were digested overnight at 53^{o}C in Tail Lysis Buffer1 (BioWorld) supplemented with 0.4mg/mL proteinase K (Thermo Fisher Scientific). Nucleic Acids are precipitated by adding 1x volume of 100% Isopropanol and centrifuged 16,000*g (10 min). Pellet was washed twice with 90% Ethanol containing 10 mM Tris-HCl and resuspended in TE Buffer (10 mM Tris 0.1 mM EDTA, pH 8.5). Genotyping is performed on 1μL of isolated DNA using Kodak DNA Polymerase Master mix (abmgood) and 0.5μM primers pairs against Linker (5'GAAGCCTAGCCCACACCAGAAATG, 5'AGGACACCTAGTCAGACAAAATATG),

Tetxoff (5'AGCGCATTAGAGCTGCTTAATGAG,

5'GTCGTAATAATGGCGGCATACTAT), or NNT(5'Forward, 5'Reverse).

Primary Mouse Cardiomyocyte Isolation from P14 and older mice

VCM were isolated as described in[30]. Briefly, perfusion buffer was composed of (in mM) NaCl 120, KCl 14.7 KH_2PO_4 0.6, Na_2HPO_4 0.6, $MgSO_4$ 1.2, $NaHCO_3$ 4.6, Taurine 30, BDM 10, Glucose 5.5, Na-HEPES 10. pH 7.0. Myocyte digestion buffer: collagenase type II 2mg/ml in 50 ml perfusion buffer. Myocyte stopping buffer: 10% calf serum, $CaCl_2$ 12.5μM in Perfusion Buffer (50 ml). Myocyte Plating Medium: MEM 48.5 ml supplemented with BSA 1%, 100U/ml Penicilin, 2 mM L-glutamine, 5 mM BDM, ITS - 5 ug/ml insulin, 5 ug/ml transferrin, 5 ng/ml selenium. Mice were injected with 200 units of heparin, IP. After 5 minutes, the mouse was euthanized by cervical dislocation, the chest opened and the heart excised with well-exposed ascending aorta, which was then cannulated for retrograde perfusion. Langendorff perfusion was performed with 37°C perfusate, at 4ml/min flow rate: 3 min with perfusion buffer followed by 2 min digestion buffer; then 15 µl 100 mM $CaCl_2$ was added to the digestion buffer reservoir. When the heart was digested (~10-11 min, checked by palpation), it was transferred to and suspended in digestion buffer (the latter step under a laminar flow culture hood). Once the cells were dissociated, myocyte stopping buffer was added in excess and the calcium gradually reintroduced in three steps using stopping buffer containing (in μM) 100, 400, 900 µl $CaCl_2$ (between each step myocytes were spun down gently @20*g for 3 min). Finally, the cells are resuspended in myocyte plating medium and viable cells counted using a NucleoCounter NC-200 (ChemoMetec A/S, Denmark) and NucleoView

software. Based on experience, a minimum of 85% viable cells (predominantly with 'heathy rod shape') was needed for reliable functional assays (batches with <85% viable cells were deemed unsuccessful and excluded from further use). The cells were then plated to laminin-coated dishes in the desired densities for further analysis/assays (except for the experiments in cell suspension). Plated cells were left to adhere for at least 1h in a sterile CO2 incubator.

Primary Mouse Cardiomyocyte Isolation from P7 mice

P7 mice were sacrificed by decapitation. Hearts were excised and incubated for 30 minutes in the collagenase buffer used for adult VCM isolation[30] at 37°C. Individual hearts were resuspended up and down gently every 5 minutes until complete tissue disaggregation. Then, the cardiomyocytes suspension was centrifuged at 30 g for 3 minutes to pellet down the cells. Finally, the cardiomyocytes were plated onto laminin-coated coverslips (see above).

Heart homogenate for whole ventricle lysates was prepared as described in[30]. Briefly: mice were euthanized by cervical dislocation, and the heart immediately excised, washed, and placed in isolation buffer, containing 225 mM mannitol, 75 mM sucrose, 20 mM HEPES, 0.1 mM EGTA, and 1 g/liter BSA, pH 7.4. After excising the heart, all steps were done on ice, and centrifuge steps at 4 °C. Ventricular muscle tissue was minced, rinsed, and then homogenized with a 5-ml glass-Teflon homogenizer (Wheaton) (20 up-down strokes at 300 rpm). Alternatively, LV Lysate was obtained by dissecting the LV from the excised heart, rinsing it in PBS, and flash freezing the tissue in Liquid N2. The frozen tissue was placed in a chilled mortar containing Cell Signaling Lysis Buffer supplemented

with 0.1% SDS and HALT Protease and Phosphatase inhibitors (Thermo Fisher) and homogenized using the pestle. Isolated Ventricular Cardiomyocytes were pelleted at 100g and resuspended in Cell Signaling Lysis Buffer supplemented with 0.1% SDS and HALT Protease and Phosphatase inhibitors.

Echocardiography and acute high-dose adrenergic stimulation by Isoproterenol.

Echo was performed with a Vevo 2100 high-frequency linear array ultrasound system with MS-550D 40MHz transducer (VisualSonics) in a blinded fashion (the person operating the instrument was not aware of the mouse groups). Mice were anesthetized with 3% isoflurane (90s) then placed in a supine position on a thermostated operating platform. During acquisition 1.5% isoflurane in 100% oxygen was administered via nosecone. Baseline Echo data were collected in B-mode and M-mode in mid-papillary level short-axis orientations. For acute response to adrenergic stimulation (single bolus of isoproterenol, 300 mg/kg i.p.), after baseline, Echo recordings were taken 30s, 30 min, 24h post injection. In some subsets, 60s and 60 min points were also collected. All Echo parameters were calculated by VevoLab 3.2.0 software, which uses Artificial Intelligence-based Auto-LV technology in the functional and anatomical analysis of the left ventricle to avoid inter-operator variability. Body surface area for the calculation of CI (equivalent) was approximated as $BW^{2/3}$.

Blood pressure recordings.

BP was monitored using a BP-2000 system consisting of a control unit, 6 channel mouse platform, six mouse holders, BP-2000 analysis software, cuff elastic endcaps and cuffs.

The mice for blood pressure recording were first acclimatized in calm environment, at least 3 times (3 different days) before a final recording. The mice in the cages were pre-exposed to the red light for 5 min to slightly increase their body temperature for higher blood flow to the tail. After pre-warming of mice, they were placed to the heated platform (37°C) and covered by mouse holder. The tails were pulled out of the holder through a 'tail- hole'; cuffs were placed on the tails, and the tails were taped to the platform to minimize any movement. The data from software were exported to Excel and then further analyzed in SigmaPlot. Only systolic readings were used owing to frequent errors with the diastolic ones.

Cardiac performance assessment via graded maximal exercise on treadmill. The test followed the protocol by Petrosino et al[18]. Acclimated mice were subjected to an incremental speed and incline routine (incline raised from 0^o to 15^o final over 10 min, while the speed raised from 0 m/min to 24 m/min over 15 min then by 1 m/min in each minute until exhaustion, Figure S3D). Animals were acclimated to the 6-lane mouse treadmill (Columbus Instruments, Columbus, OH, USA) in 3 sessions, with 60 hours of rest between each session. Mice were placed on the steady treadmill for 3 minutes and shock grid in the rest area was activated (to 3 Hz). Then, the belt was activated at walking speed (6 m/min) for 5 min followed by progressive revving up to 12 m/min in total duration of 12 min. After the last acclimation session, 1 week of recovery was allowed. The actual test involved both, increments in incline and speed and was run until the point of exhaustion. First, mice were placed on the steady treadmill at 0° incline and the shock grid was activated. After 3 min, belt speeds and incline were increased sequentially as follows (speed, duration, grade): (walking 6 m/min, 2 min, 0°)→(9 m/min, 2 minutes, 5°)→(12

m/min, 2 min, 10°)→(15 m/min, 2 min, 15° (max. incline)). From here, at maximum (15º) incline, the speed was increased further every minute until exhaustion in the following steps: (m/min)→18→21→23→+1/each minute. Exhaustion (endpoint for treadmill) was defined, as the point, when mice (any portion of their body) maintained continuous contact with the shock grid for 5 seconds. The time on belt, and the maximum attained speed were documented.

Cardiac I/R.

The cardiac I/R protocol was adapted from[41]. Briefly, hearts were quickly dissected into ice-cold Krebs buffer, cannulated via the aorta, and perfused through a Langendorff apparatus with Krebs buffer at constant flow perfusion pressure of 80 mmHg connected to data acquisition device Powerlab (ADInstruments) with LabChart software. Left ventricular (LV) pressure was monitored with a water-filled latex balloon inserted into the LV and inflated to obtain an end-diastolic pressure (LVEDP) of 8-10 mmHg. LV developed pressure (LVdevP) was calculated as the difference between LV systolic pressure and LVEDP. After 20 minutes of equilibration, hearts were subjected to 40 minutes of no-flow global ischemia, then 60 minutes of reperfusion with Krebs–Henseleit bicarbonate buffer (KHB) or KHB supplemented with CsA (2 μM). After 60 min of reperfusion, heart slices (prepared placing by hearts inside the heart matrices and cut with blade to 0.5 mm slices) were incubated in 1% triphenyl tetrazolium chloride to outline the area of necrosis. The slices (total – 5 slices) were placed on a black background plate and images were taken. The images of every slice of the hearts were loaded into SAT software (software for the Semi-Automated Analysis). The contrast caused by infarct brightness was adjusted and used for extraction of the infarct area. The software generated the infarct size for each

slice of the heart. The area of each slice was evaluated in Fiji J. The infarct size from each slice was expressed as portion of infarct in the whole heart (infarct size of slice * area of slice / total area of the heart). Finally, the infarct sizes expressed as portion of the heart occupied by infarct, were summarized from all 5 slices.

The records from LVDVP were analyzed in LabChart Reader software. The average from max dP/dt (the first derivative of the pressure waves) was analyzed from following time points– min 1, 3, 5, 7, 10, 15 from basal (stabilization) period; min 1, 3, 5, 10, 15, 20, 30, 40 from ischemia period; and start, min 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60 from reperfusion period.

Histology.

Hearts were fixed either via perfusion (I/R injury) with 2.5% glutaraldehyde/0.15 M Na-cacodylate buffer, pH 7.4 or by immersion (after basal Echo) in 2% paraformaldehyde/2% glutaraldehyde in 0.15 M Na-cacodylate; then embedded in paraffin and sectioned using a microtome at the Department of Pathology Anatomy and Cell Biology histology core facility. Sections were stained with hematoxylin/eosin (H&E) or Masson's trichrome and imaged by an EVOS (M7000, Invitrogen^{TM}) digital microscope. Fibrosis was quantified In FIJI (Image J) by masking the area of tissue (all red area) and fibrotic areas (blue area) in at least 5 different field snapshots from each heart specimen at 20x direct magnification. The fibrotic area is expressed as the percentage (portion) of fibrotic tissue to the total cardiac tissue area in each field.

Immunofluorescence

Immunofluorescence staining followed standard protocols. Briefly, freshly isolated VCMs were fixed with 5% paraformaldehyde/PBS for 15 min. 3% Bovine Serum Albumin and 0.2% Triton X-100 in PBS were used for blocking and permeabilization. Antibodies were diluted in PBS/1% BSA, and 0.2% Triton X-100. To assess nonspecific binding of secondary antibodies, negative controls without primary antibody were used. SlowFade® was used for mounting on microscope slides; or myocytes plated to glass-bottom dishes were directly imaged in PBS, without mounting. Immunofluorescence was imaged using a Zeiss LSM880MP confocal microscope equipped with Airyscan super-resolution (1.7x beyond the diffraction limit) detector module. A Zeiss 63x/ 1.4 NA oil plan-apochromat differential interference contrast objective was used to obtain all images. Image post-processing was done using Zen software from Zeiss and the Canvas X vectographic software (ACD Systems).

NADH/FAD Autofluorescence imaging in Isolated VCMs

The freshly isolated VCMs plated on laminin-coated glass coverslips were mounted to quick-change electrical stimulation chamber equipped with 2 Pt electrodes (RC-47FSLP, Warner Instruments). The chamber was then mounted on the thermostated (@37°C) microscope stage and connected to a custom-built multi-channel perfusion system with manual on/off switches. The field-stimulation buffer contained 150 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 2 mM anhydrous $MgCl_2$, 1 mM glucose, 2.5 mM pyruvate, 5 mM creatine, 5 mM Taurine, 2 mM $CaCl_2$ and 100 nM Iso. Baseline was recorded when AVMs were perfused with perfusion buffer. FS was applied as biphasic electrical pulses of 20-mV amplitude with stepwise increases in frequency using a MyoPacer Stimulator (IonOptix) with frequencies 1-2-5Hz (30s each). After stimulation, perfusion was stopped and the

rest of additions were performed manually (by pipetting): caffeine (10 mM), NaCN (40μM), rotenone (0.25μM) with antimycin A (10μM) and FCCP (5μM) with oligomycin (2.5 μM). Imaging was performed using an Olympus IX70 inverted microscope (Fluo/340, 20x/0.7 water objective) fitted with a Lambda DG-4 ultra-high speed wavelength-switch illuminator (Sutter Instruments) and a 512 × 512 Evolve back-illuminated EMCCD camera (Teledyne Photometrics). Cells were excited alternately using 360/20 nm and 480/10 nm band-pass filters, UVND 0.4, with emission filter 455/50 nm and 535/20 nm. Single-cell records were analyzed with the custom-made software, Spectralyzer, then further with Sigmaplot. As a correction to a bleed-through to the FAD channel, the minimum (fully reduced, upon cyanide/rotenone/antimycin A addition) fluorescence was subtracted. To avoid erroneously high NADH/FAD ratios owing to the subtraction, cells with <20 unit leftover fluorescence were uniformly excluded from the ratio calculation. <17% of the cells were excluded this way.

Fluorescence Imaging of $[Ca^{2+}]_c$ signals in adherent VCMs

The freshly isolated VCMs plated to laminin-coated glass coverslips were loaded with 5 μM fura-2 acetoxymethyl ester dissolved into the plating buffer in the presence of 0.003% Pluronic® F127 (Sigma-Aldrich P-2443) for 20 min at room temperature and washed. Then, coverlips were mounted into quick-change electrical stimulation chamber equipped with 2 Pt electrodes (RC-47FSLP, Warner Instruments). Chamber was connected to the custom-built perfusion system, and mounted to the thermostated (37^{o}C) stage of an Olympus IX81 inverted microscope (Fluo/340, 40x oil immersion objective, NA 1.35) fitted with a custom rapid-switch LED illumination system (T-LED, Sutter Instruments) and a Hamamatsu ORCA 4.0 EMCCD camera (512x512). Fura 2 was excited alternately at 340

nm and 380 nm, and emission >500 nm was captured. Image collection rate was 45 ms/frame. The assay (field-stimulation) buffer contained 150 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 2 mM anhydrous $MgCl_2$, 1 mM glucose, 2.5 mM pyruvate, 5 mM creatine, 5 mM Taurine, 2 mM $CaCl_2$ and 100 nM Iso. After baseline recording, myocytes were electrically stimulated @1 Hz for ~1 min followed by ~30 s rest then @ 5 Hz for ~30 s and ~30 s rest, then again @1 Hz for ~1 min and ~30 s rest. Switching between stimulation frequencies was done manually and timed by a lab timer. After the final rest, saturating caffeine (10 mM) was added directly (pipetting) to the chamber at stopped perfusion. Pacing-associated trains of [Ca2+] spikes were analyzed by reading and averaging the peak (systolic) values and inter-peak minimum (diastolic) values for each cell. For caffeine response analysis, only the cells presenting a [Ca2+]c spike after caffeine addition were included. (To minimize photo-damage owing to the high-frequency UV-exposure, these experimental recordings were kept short and, likely because of the manual mixing, some of the cells did not show a response in the recorded period).

TMRM accumulation kinetics in adult VCMs.

Freshly isolated VCMs from control or linker mice were plated onto laminin-coated coverslips as described above and imaged using a confocal microscope Zeiss LSM 880 while kept in a solution containing NaCl 150mM, KCl 5.4 mM, Hepes 10mM, CaCl2 2mM, Glucose 1mM, Pyruvate 2.5mM, Creatine 5mM, and Taurine 5mM. A bolus of TMRM 25 nM was added to the chamber, and the fluorescence intensity was monitored every 10 s. 5 minutes after TMRM addition, $\Delta\Psi_m$ was dissipated by adding FCCP 5µM to release the accumulated TMRM back to the bulk incubation medium.

Immunogold.

Freshly isolated VCMs were plated to Thermanox plastic coverslips (13 mm diameter), fixed with 5% paraformaldehyde/PBS for 15 min, washed with PBS and quenched with 50 mM glycine in PBS. Cells were permeabilized and blocked by 3% BSA and 0.2% Triton-X in PBS at room temperature gently rocking for 30 min. The blocking buffer was aspirated and cells were incubated with primary antibody for 1 hour at room temperature under gentle rocking; followed by washing 3x with 1% BSA in PBS, secondary blocking with 5% goat serum in PBS for 30 min at room temperature, then incubation with secondary antibody (Fluoronanogold anti-mouse/anti-rabbit Fab' AlexaFluor®488) under gentle rocking for 1 hour at room temperature. Further washes followed with PBS (3x), then 3x with 0.1% Triton X-100/diH_2O. Gold enhancement (GoldEnhance^{TM} EM Plus, Nanoprobes) was done according manufacturer's instruction; colloidal gold particles were developed for 3 min, after which the cells were intensively washed with diH_2O and stored in 1.6% glutaraldehyde/PBS and 0.2% tannic acid at 4°C overnight. The next day, cells were washed with 0.1 M sodium cacodylate and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate for 60 min at dark in 4°C. The cells were then washed with diH_2O, contrasted with 1% aqueous uranylacetate, dehydrated in graded alcohol for 2 min in each 20%, 50%, 70%, 90%, 3×100%; infiltrated and embedded in Durcupan resin and polymerized for 48h. From the Durcupan blocks, pieces containing the cells were cut out and remounted to resin 'dummy blocks' using acrylic glue (Gorilla Glue) so that the Thermanox coverslip remained exposed as sectioning surface. Ultrathin sections were obtained using by ultramicrotome and images were obtained using by TEM (as described in TEM section).

Extracellular flux analysis to determine OCR

Freshly isolated adult myocytes were resuspended in plating medium (MEM, BSA, Penicilin/streptomycin, glutamine, BDM, ITS). Prior to plating, Seahorse V7 24-well tissue culture plates were coated with laminin (20 µg/ml, Invitrogen mouse laminin, 23017). The cardiomyocytes were plated to reach final density ~15-40%. Plated cardiomyocytes were let sit for 10 min then the plating medium was topped to 200 µl final volume followed by additional 30-40 min incubation (37°C, 2.1% CO_2 incubator). Plating medium was then replaced by plating medium without BDM for additional 30 min. Finally, plating medium was replaced with 500 µl seahorse assay medium for glucose/glutamine-fueled oxidative phosphorylation (unbuffered DMEM supplemented with 4 mM glutamine, 25 mM glucose and 0.1 mM pyruvate, pH 7.4). Cell density in the wells was determined using the EVOS system and plate was incubated in a hybridization oven at 37°C for 40 min.

Seahorse XF24 assay. O_2 consumption and extracellular acidification rate (OCR, ECAR) of the plated myocytes was measured using the Seahorse Bioscience XF24 Flux Analyzer. The XF24 automated protocol consisted of 10 min delay following microplate insertion, baseline OCR/ECAR measurements [5 × (2 min mix, 2 min wait, 2 min measure)], followed by injection of port A (60 µl DMEM) then OCR/ECAR measurement [2 × (2 min mix, 2 min wait, 2 min measure)], then injection of port B (60 µl, oligomycin OM – final concentration 4 µg/ml) and OCR/ECAR [2 × (2 min mix, 2 min wait, 2 min measure)], injection of port C (60 µl, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone FCCP – final concentration 1µM) and OCR/ECAR measurement [2 × (2 min mix, 2 min wait, 2 min measure)] and injection of port D (60 µl – antimycin A, final concentration 10 µM) and

OCR/ECAR measurement [2 × (2 min mix, 2 min wait, 2 min measure)]. When the assay finished, the images for cell density were taken.

Transmission electron microscopy

For perfusion fixation, animals were heparinized, then euthanized by cervical dislocation. The heart were cannulated through aorta on a Langendorff apparatus. First, the hearts were perfused by 1Ca Tyrode solution for 5 min (in mmol/l: 135 NaCl, 5.4 KCl, 5 MgCl2, 1 CaCl2, 0.33 NaH_2PO_4, 10 HEPES, pH 7.3), followed by perfusion with Ca^{2+}-free Tyrode solution (in mmol/l: 135 NaCl, 5.4 KCl, 5 $MgCl_2$, 0.02 $CaCl_2$, 0.33 NaH_2PO_4, 10 HEPES, pH 7.3), and finally with 2.5% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH 7.4). Small pieces from left ventricle and papillary muscle were cut (~1 mm^3), overnight postfixed in 4°C in 2% osmium tetroxide partially reduced by 0.8% $K_4Fe(CN)_6$ in 0.15 M Na-cacodylate buffer. Samples were contrasted en bloc with 1% aqueous uranylacetate, dehydrated in graded series of acetone, embedded in Spurr's resin. Longitudinal, ultrathin sections (65–80 nm) were cut from the resin-embedded blocks with a diamond knife (Diatome-US, USA) using a Leica UCT ultramicrotome and caught on copper grid covered with formvar film. Images of longitudinal oriented cardiomyocytes were obtained via an FEI Tecnai 12 TEM fitted with an AMT XR-111 10.5 Mpx CCD camera at 3,200 – 15,000× magnification (80 kV).

TEM morphometric analysis of mitochondria and the Mito-jSR associations was performed using ImageJ/FIJI (NIH). To get the general information about mitochondrial morphology and abundance (the area of mitochondria and perimeter), a mask was drawn over the sarcoplasmic area in cardiomyocyte longitudinal sections. The percent of the

sarcoplasmic area covered by mitochondria was used for determination of mitochondrial density. For morphometric analysis of Mito-jSR associations longitudinally arranged 'beads-on-string' interfibrillar mitochondria were selected[7] and a custom Python plugin (MitoCareTools[43], Figure 4B), tailored for Mito-jSR analysis[44], was used to extract the metrics. The tracing of 3 membranes in TEM images is important for analysis: mitochondrial perimeter, transversal (T) side of mitochondrion and jSR in contact with T-side of mitochondrion. The plugin calculates point-by-point the interface distances radial/perpendicular to the mitochondrial surface; finds the cutoff (<50 nm) distance limits; divides the OMM contact segment (within the cutoff) to distance bins and normalizes bins to the transversal side length (or mitochondrial perimeter depending on how it is set; see Figure 4B). 10 nm distance bins were requested either cumulatively (larger bin includes the smaller ones) or as exclusive, distance-range bins. Mask of mitochondria, the transversal side (line segment) of outer mitochondrial membrane and the interfacing jSR membrane line (in distance < 50 nm) were created. Mean gap distance, length of transversal side of mitochondria, length of jSR and of the interface (mitochondria outer membrane-jSR) values were obtained by the plugin.

FIB SEM

Sample preparation. The isolation and primary fixation of hearts was performed as described for TEM. After primary fixation, the samples were postfixed overnight in 4°C in 2% OsO_4 partially reduced by 0.8% $K_4Fe(CN)_6$ in 0.15 M Na-cacodylate buffer. Postfixation steps were adapted from a serial block face sectioning volume scope specimen prep protocol provided by the ThermoFisher team ('Dresden protocol'). The

heart pieces were incubated in 0.2% tannic acid in water for 10 min at room temperature; rinsed with diH_2O; incubated in 1% OsO_4 in diH_2O for 30 min at room temperature; rinsed with diH_2O; then, incubated in pre-heated solution (60^oC, 30 min to facilitate dissolving) of 1% thiocarbohydrazide in diH_2O for 20 min at room temperature. This was followed by one more round of incubation in 1% OsO_4 in water for 30 min at room temperature, washing (4x) with diH_2O and contrasting with 1% uranylacetate in 25% methanol overnight at 4°C. Next day, the samples were washed with diH_2O, incubated en block with lead aspartate (0.02 M lead nitrate in 0.03 M sodium aspartate, pH 5.5) for 30 min at 60°C, rinsed 2× in diH_2O and dehydrated in graded series of ethanol and finally in 100% acetone. The samples were gradually infiltrated by Acetone/Epon with 2:1 – 1:1 – 1:2, mixing ratios followed by 2× 100% Epon and polymerization in 100% Epon for 24-48 h at 60^oC in a dry oven. Sample quality was checked in thin sections via TEM. Samples were then sent to Thermo Fisher Scientific, where they were mounted to SEM stubs and (sputter)-coated with a thin layer of carbon or iridium film.

FIB-SEM 3D automated data acquisition: The acquisition of a stack of images was defined by 2 consecutive steps; 1- site preparation; 2- automated data acquisition. Both were performed in a Helios G4 UC or Helios 5 UX (Thermo Scientific) FIB-SEM system. Site preparation: The preparation process involved using a Gallium ion beam to mill away areas of the sample around the region of interest, creating ‘trenches’ on each side as well as in the front of what is later defined as the “block face”. This process is necessary to provide the detectors and electron beam access to the region of interest to alternatively mill material away from the block face and image the freshly revealed block face. During this process, a 30 kV FIB was used to deposit a protective platinum layer on the area of

interest, and to create a fiducial to support automation. **Automated acquisition**: 3D automated data acquisition was performed using the Thermo Scientific Auto Slice and View software. During this process, a magnetic immersion lens was used to acquire a SEM image after each slice removed by the FIB. Auto Slice And View software was used to automatically control the FIB-SEM throughout the process. The FIB current used for each slice was 440 pA, and the target slice thickness was 5 nm. An in-column backscattered electron detector was used to acquire the SEM images. The landing energy of the SEM was 2 keV, with a beam current of 100 pA, and a dwell time of 10 µs. Scanning dimensions were adjusted to acquire 5 nm isotropic voxels. To maintain focused SEM image acquisition, Auto Slice And View software automatically applied an auto focus routine at specified intervals. 649+ SEM images were automatically acquired from the area of interest. **Image Segmentation**: The stack of tiff images captured by the FIB-SEM was loaded into the Thermo Scientific Amira software. Prior to segmentation the following steps were applied to the entire stack. Images were aligned to each other using the align-slice module. The stack was then filtered using first a 2D Gaussian filter with a Kernel of 0.8 followed by a sharpening filter (Deblur) using a factor of 1. Labels corresponding to mitochondria and parts of mitochondria that needed to be highlighted were created in the segmentation editor part of Amira. The mitochondria of choice were first selected manually every 20 slices and an interpolation of the selection was used to create a full 3D selection of each mitochondria separately. After, a Threshold approach was applied, only working from the interpolated selection described above. This highlighted the Mitochondria's cristae. Finally, a VolumeRendering technique was used to visualize the segmented mitochondria and a snapshot of their 3D shape and

organization was exported from Amira. For some of the 3D reconstructions and segmentations FIJI Image J plugin was used (Registration/Linear Stack Alignment with SIFT) for stack alignment and the Object Research Systems (ORS) Dragonfly software for the segmentation.

Western Blotting

Protein content was quantified using a BCA protein assay (Thermo Fisher Scientific). Equal quantities of protein for each sample was diluted in Laemmli buffer (60 mM Tris, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, pH 6.8). Samples were heated at 95°C for 10 minutes and resolved with SDS-PAGE. Protein was transferred to nitrocellulose membranes with the trans-blot turbo rapid semi-dry transfer (Biorad). Total protein was stained either with Ponceau S solution (0.1% Ponceau S in 5% Acetic Acid) and imaged or with No-Stain Protein Labeling Reagent according to manufacturer's instructions (Thermo Fisher Scientific). Membranes were subsequently blocked in Everyblot blocking buffer (Biorad). Membranes were probed 4°C overnight or 2hr at RT with primary antibodies diluted in PBS-T (0.05% Tween). Membranes were washed 3 times with PBS-T(0.1%Tween) and probed for 1 hour at RT with secondary antibodies against rabbit(RRID:AB_621848) or Mouse(RRID:AB_10715072) diluted 1:10000 in PBS-T (0.5% Tween). Membranes were imaged using Azure C600 Imaging system 3seconds IR700 Exposure and 3 minutes IR800 Exposure. Band densitometry was performed using Empiria Studio version 2.2(LiCor). **Respiratory Complex detection**. 50µg of cardiomyocyte lysate in Laemmli's Sample Buffer were heated at 37°C for 10 minutes prior to being loaded into

a 10% SDS PAGE gel and run via Western blotting conditions described above. Individual complexes were probed using a pre-mixed cocktail of 5 mouse monoclonal antibodies (RRID:AB2629281) at a dilution of 1:1000 overnight at 4°C. Secondary antibody staining and acquisition were performed via standard Western blotting protocol.

mRNA assays. VCMs or fresh ventricle wall pieces are transferred into and stored in RNA-Later solution (Thermo Fisher Scientific). RNA is isolated from the solution using the Monarch Total RNA miniprep kit (New England Biolabs) according to manufacturer's instruction. Purified RNA is quantified using 260/280 ratio on a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific) and equal amounts are added in triplicate to a 380 well plate with 0.4 μM primers, Luna® Universal One-Step Reaction buffer, and WarmStart RT Enzyme mix (New England Biolabs). qPCR is performed on a QuantStudio5 (Thermo Fisher Scientific) and cycled according to the polymerase manufacturer's instructions. Primer pairs used are as followed: GAPDH(5'CCTGCACCACCAACTGCTTA, 5'AGTGATGGCATGGACTGTGG), RFP(5'CACTACGACGCTGAGGTCAA, 5'GTTGTGGGAGGTGATGTCCA), RMDN3(5'TTCCAGCTGCTGCTCAACAA, 5'GAGCAGCCTCTGCTTCTTCT),and MCU(5'CACCACACTGTGCATTGAGC, 5'GCACCAGAGTGGTCCTCTTC). The inverse of the average ΔCT values for each target gene and time point were compared control vs linker as well as temporally within each group.

Calcium retention capacity assay (Fluorometric Measurements of $[Ca^{2+}]_c$)

Primary adult cardiomyocytes were isolated as described in Primary Mouse Cardiomyocyte Isolation; the final VCM pellet was resuspended in Ca^{2+}-free extracellular storage buffer (120 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 0.2 mM $MgCl_2$, 20 mM HEPES, 0.1mM EGTA, pH7.4), counted and stored on ice in aliquots of 800,000 cells. The aliquots were pelleted and washed into an intracellular medium (ICM,120 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 20 mM HEPES at pH 7.2) supplemented with protease inhibitors (pepstatin, leupeptin, antipain 1 μg/mL each), saponin 20μg/ml, malate/TRIS 2mM, pyruvate/TRIS 2 mM, MghCl2 100 μM, thapsigargin 10 μM, TMRM 1μM in a stirred plastic cuvette (Sarstedt), inside a temperature-controlled (Peltier @37°C) multiwavelength excitation dual-wavelength emission fluorometer (DeltaRAM, Horiba/PTI). $[Ca^{2+}]_o$ was monitored using fura-2 LoAff (1 uM) at excitation 340 nm and 380-nm and emission 500-nm at 20 points/s acquisition rate, while $\Delta\Psi_m$ was simultaneously monitored as the TMRM fluorescence at excitation 540 nm and emission 580 nm. Cells were added 20s after the start of the recording. Parallels were recorded with or without addition of mPTP blocker cyclosporine A (CSA 5 μM) at 120s. In all recordings, at 290s NCLX inhibitor CGP-37157 (20 uM) was added. At 500s 3.75 uM CaCl2 bolus was added (using a Hamilton syringe via an addition port without pausing the data acquisition), repeatedly in every minute, until there was no calcium uptake. Then, a mixture of oligomycin (5 ug.ml) and FCCP (2 μM) was added to dissipate $\Delta\Psi_m$ and release all Ca^{2+} from the mitochondria. Calibration of the Ca^{2+} signals was carried out at the end of the measurements with the sequential addition of 1mM $CaCl_2$ and 10mM EGTA/Tris (pH 8.5); and molar $[Ca^{2+}]$ values were calculated using the Grynkiewicz equation $[Ca^{2+}]$ (nM)=Kd*((R-R_{min})/(R_{max}-R))/(S_{f2}/S_{b2})[45]. VCMs of 2 hearts

were pooled together in each experimental group (4 repetitions). One experiment consisted of 3-7 parallels/genotype in ‘no-CsA experiments’, and 2-4 parallels/genotype in ‘CsA experiments’. The number of added calcium pulses for the individual parallels was averaged for each of the independent experiments.

GEE Regression Results

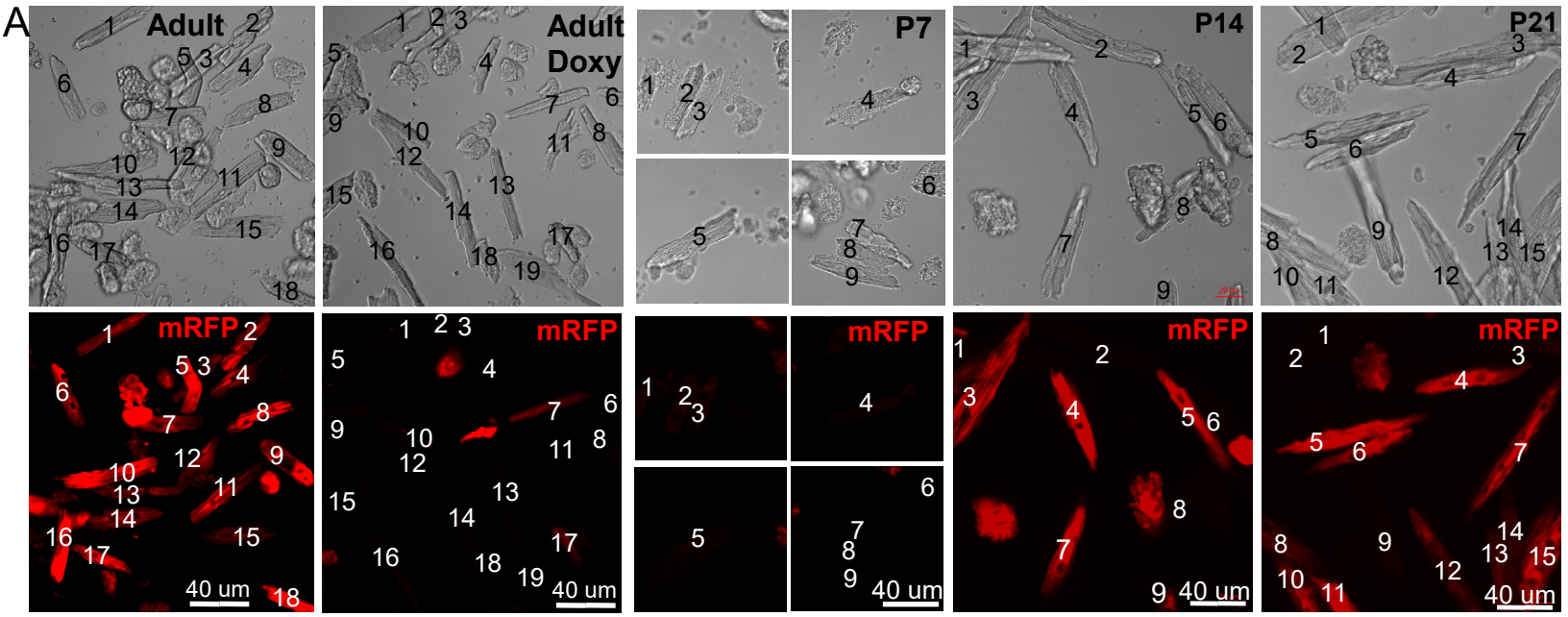
Dep. Variable:	vals	No. Observations:	1372
Model:	GEE	No. clusters:	7
Method:	Generalized	Min. cluster size:	108
	Estimating Equations	Max. cluster size:	284
Family:	Gaussian	Mean cluster size:	196.0
Dependence structure:	Nested	Num. iterations:	8
Date:	Fri, 18 Nov 2022	Scale:	204.536
Covariance type:	robust	Time:	14:51:20

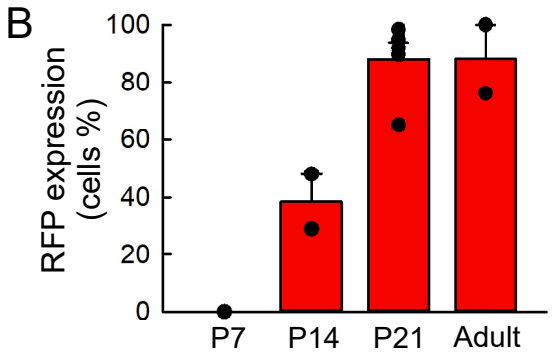
	coef	std err	z	P>|z|	[0.025	0.975]
const	16.2866	2.752	5.918	0.000	10.893	21.680
L	-4.5834	1.778	-2.577	0.010	-8.069	-1.098
stim	-0.6525	0.370	-1.766	0.077	-1.377	0.072
L*stim	0.8011	0.298	2.690	0.007	0.217	1.385

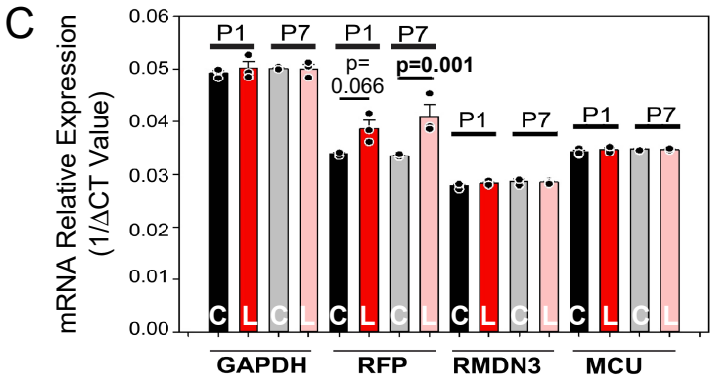
Skew:	6.0896	Kurtosis:	63.9886
Centered skew:	6.7736	Centered kurtosis:	76.0289

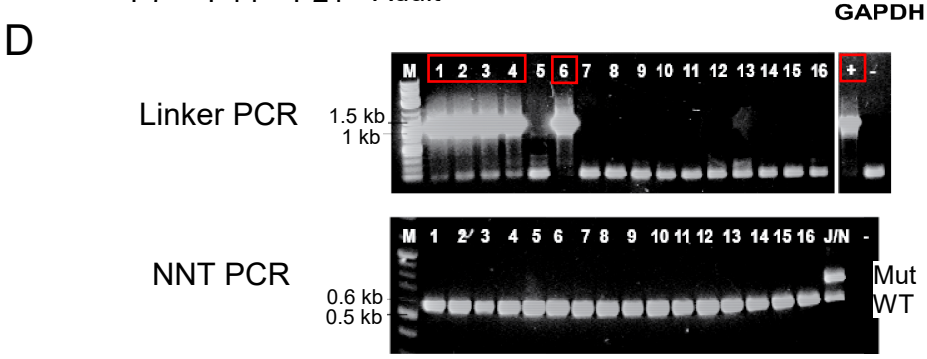
Table S1. GEE Regression results (for Figure 5C). Const is the estimate for the control at baseline; L is the linker baseline relative to control; stim is the effect of pacing in control (per Hz); and L*stim is the interaction term. Interpretation wise: there is a baseline difference, the effect of stimulation in control is marginal, but it is different from the response in Linker.

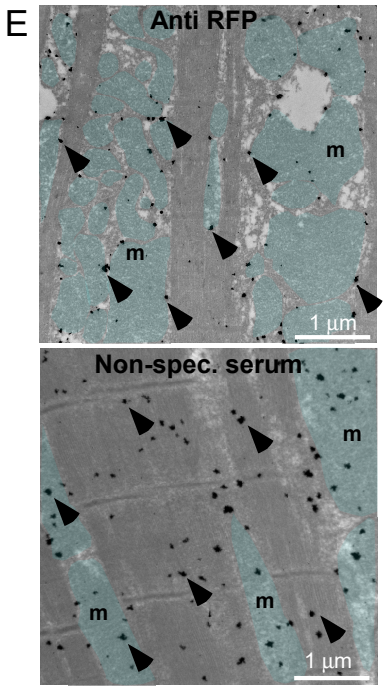
Figure S1


A
Adult
Adult Doxy
P7
P14
P21
mRFP
40 um


B
RFP expression (cells %)
100
80
60
40
20
0
P7
P14
P21
Adult


C
mRNA Relative Expression (1/ΔCT Value)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
P1
P7
p= 0.066
p=0.001
C
L
GAPDH
RFP
RMDN3
MCU


D
Linker PCR
1.5 kb
1 kb
NNT PCR
0.6 kb
0.5 kb
Mut
WT


E
Anti RFP
Non-spec. serum
m
1 µm

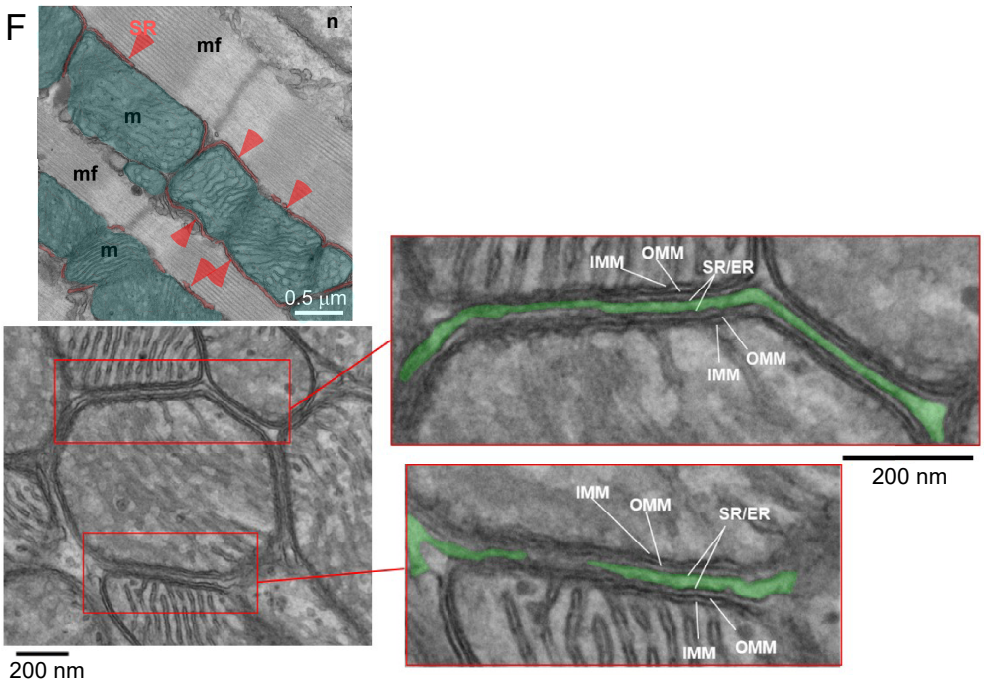
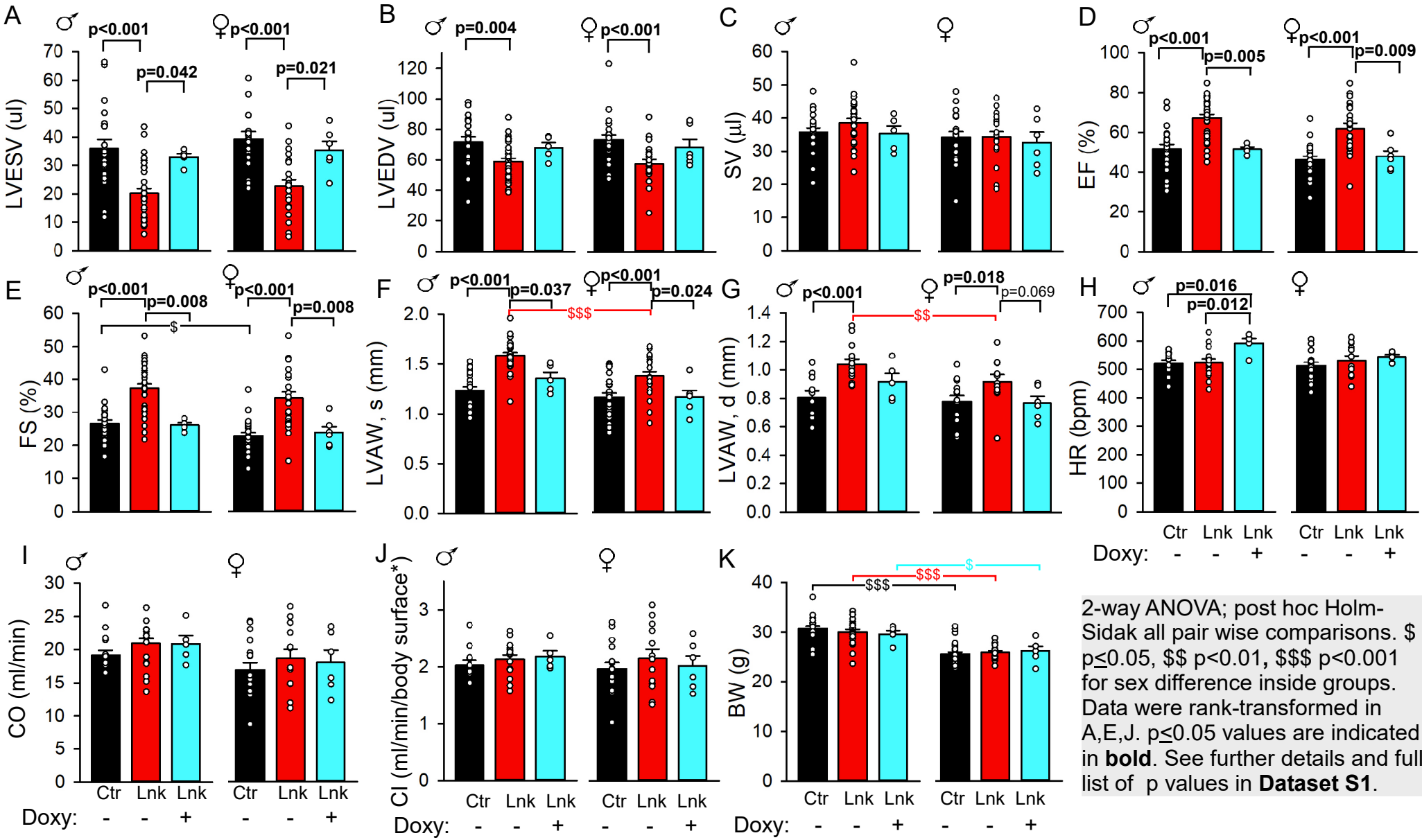

F
SR
mf
n
m
0.5 µm
G
IMM
OMM
SR/ER
200 nm

Figure S1. Onset of linker expression, validation of linker localization, mitochondria and SR/ER interactions in the adult linker myocytes. A. Exemplar mRFP immunofluorescence (lower row) and phase contrast (upper row) images of VCMs from adult linker-mice (Adult) and adult Doxy-linker-mice (Adult Doxy, 4 weeks on Doxy-diet) and VMCs isolated at the indicated age (P7, P14, P21). Matching numbers in the vertical image pairs correspond to the same point on the field where a cell is located. **B**. Occurrence of red-fluorescent VCMs that were isolated from linker mice of the indicated ages. Linker mRFP is detectable from P14 in a fraction of the myocytes while at P21 it is similarly expressed as in adult myocytes. N=3(P7), 2(P14), 5(P21), 2(Adult). **C.** Linker (RFP) mRNA is detectable in P7 but not P1 mice. L, linker; C, control. N=3 hearts each. P calculated by 2-way ANOVA using rank-transformed (by Rankit) data (see also Dataset S1). **D.** PCR amplification of the two linker transgenes (Linker PCR: upper band Myh/6-tetO-linker, lower Myh6/tTA) and the wild type (WT) and mutant (Mut) NNT genes (NNT PCR). Linker mice are lanes 1-4 and 6; control are lanes 5 and 7-16. **E.** TEM Immunogold (pre-embedding) labeling of mRFP (black dots) in linker VCMs. Note that the gold particles predominantly localize around mitochondria; while in the control (non-specific serum w/o primary antibody) only background signal is visible and dots are randomly covering all area of cardiomyocytes. **F.** Representative high-magnification TEM micrograph of interfibrillar mitochondria (cyan hue), tightly surrounded by a network of SR (pink hue, arrows). **G.** Mitochondria-SR interfaces in a mitochondrial cluster shows that the network SR (nSR) infiltrates the large dense mitochondrial clusters; in fact, the linker may promote mitochondrial clustering via connecting multiple mitochondria to the same SR tubule. Zoomed areas show individual membranes of mitochondria (OMM, IMM) and sarco/endoplasmic reticulum (SR/ER).

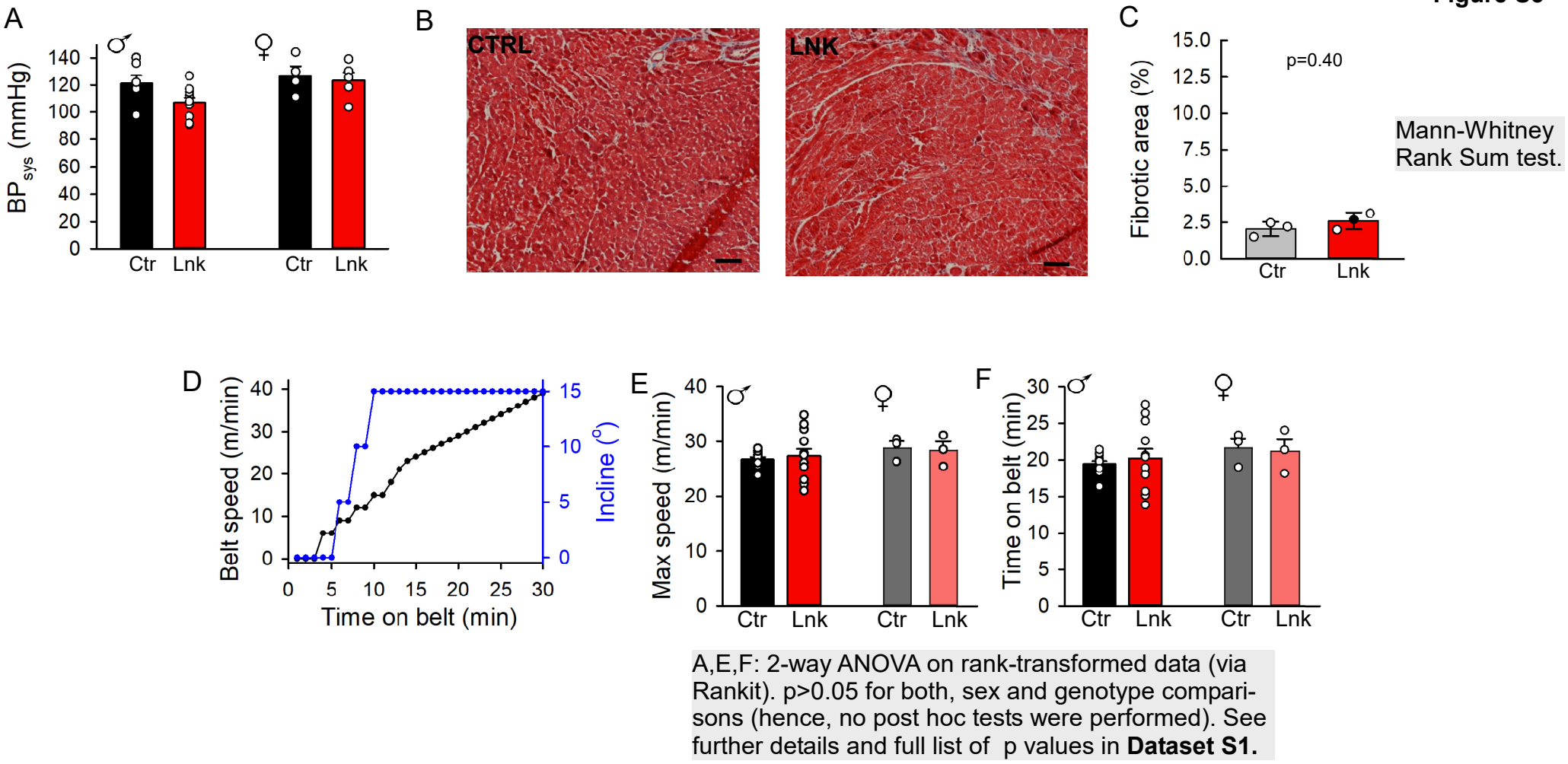
Figure S2



2-way ANOVA; post hoc Holm-Sidak all pair wise comparisons. $ $p \leq 0.05$, $$ $p < 0.01$, $$$ $p < 0.001$ for sex difference inside groups. Data were rank-transformed in A,E,J. $p \leq 0.05$ values are indicated in **bold**. See further details and full list of p values in **Dataset S1**.

Figure S2. Basal cardiac phenotype of the linker-mouse hearts. A-K. Basal echocardiography/ECG parameters from Figure 2 in absolute values. See animal numbers in main Fig.2.

Figure S3



Mann-Whitney Rank Sum test.

A,E,F: 2-way ANOVA on rank-transformed data (via Rankit). $p>0.05$ for both, sex and genotype comparisons (hence, no post hoc tests were performed). See further details and full list of p values in **Dataset S1.**

Figure S3. Cardiovascular phenotypes in the linker-mice. A. Systolic blood pressure (BP) readings of control (Ctr) and linker (Lnk) mice. (Means±S.E., n=8 Ctr male, 10 Lnk male, 4 Ctr female, 8 Lnk male.) **B**. Representative images of Masson's trichrome-stained LV tissue sections. Scale bar: 100 μm. **C**. Bar chart of the fibrotic areas (% of total tissue area) from LV tissue sectionsN= 3 Ctr and 2 Lnk females and 1 Lnk male (black-filled circle). **D-F**. Cardiac performance assessment via graded maximal exercise on treadmill. **D.** Graphic scheme of the protocol. Mice were subjected to a combined incrementing incline/speed regime until exhaustion. Performance parameters collected are the maximum attained speed (**E**) and the corresponding time spent on the treadmill belt (**F**). (Means±S.E., n=12 each, male Ctr and Lnk mice and 3 each, female Ctr and Lnk mice.)

Figure S4

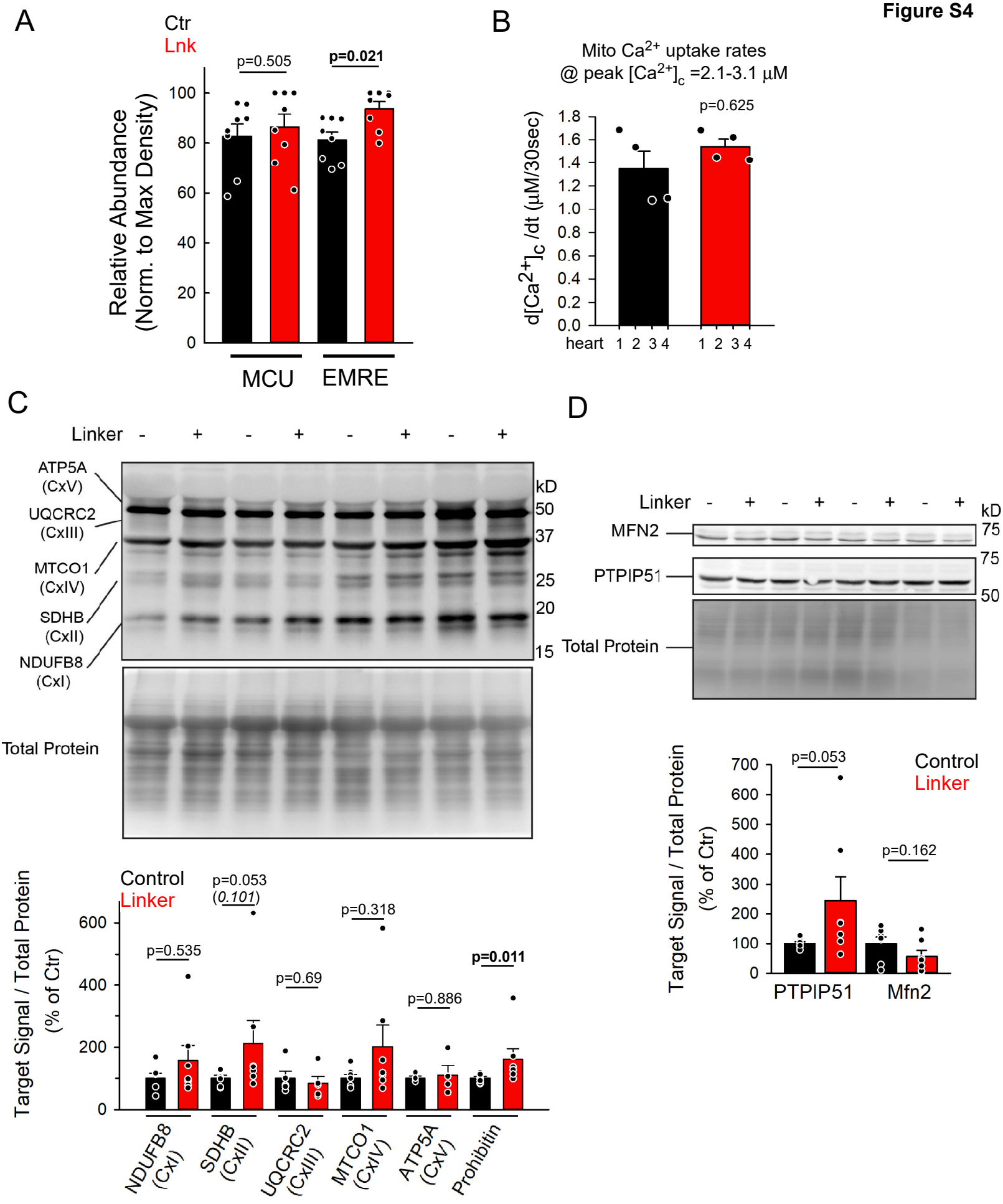

A
Ctr
Lnk
p=0.505
p=0.021
Relative Abundance (Norm. to Max Density)
MCU
EMRE
B
Mito Ca2+ uptake rates
@ peak [Ca2+]c =2.1-3.1 µM
p=0.625
d[Ca2+]c /dt (µM/30sec)
heart 1 2 3 4 1 2 3 4
C
Linker
ATP5A (CxV)
UQCRC2 (CxIII)
MTCO1 (CxIV)
SDHB (CxII)
NDUFB8 (CxI)
kD
Total Protein
Control
Linker
p=0.053 (0.101)
p=0.535
p=0.69
p=0.318
p=0.886
p=0.011
Target Signal / Total Protein (% of Ctr)
NDUFB8 (CxI)
SDHB (CxII)
UQCRC2 (CxIII)
MTCO1 (CxIV)
ATP5A (CxV)
Prohibitin
D
Linker
MFN2
PTPIP51
Total Protein
p=0.053
p=0.162
Control
Linker
Target Signal / Total Protein (% of Ctr)
PTPIP51
Mfn2

Figure S4. Protein abundances of mitochondrial membrane proteins in control and linker heart lysates. A. Relative expression of mitochondrial Ca^{2+} uniporter components MCU and EMRE. Pooled data from LV and VCM lysates. Band densities are normalized to the maximum per blot for the same protein and same type of sample (heart or myocyte lysate) (n=8 CTr and 8 Lnk; 4 VCM lysates and 4 ventricular homogenates). **B**. Mitochondrial Ca^{2+} uptake rates assessed as the $[Ca^{2+}]_c$ decay rate following the addition of Ca^{2+} boluses in suspensions of permeabilized VCMs, as described for Figure S7B. To sample similar segment of the cooperative $[Ca^{2+}]$ activation curve of the channel, only the Ca^{2+} pulses with peak $[Ca^{2+}]$=2.1-3.1 μM range (relevant at the local jSR-mitochondrial Ca^{2+} transfer) were included. To better isolate mitochondrial Ca^{2+} influx, the assays were performed in the presence of CSA 2 μM and CGP37157 20μM (blocked mPTP and NCLX efflux routes). SR Ca^{2+} uptake was blocked by thapsigargin (10μM) N=4 independent experiments (2 technical replicates each). **C**. Relative abundance of mitochondrial respiratory complex subunits. Complex(C) I component NADH:ubiquinone oxidoreductase subunit B8 (NDUF88), CII component succinate dehydrogenase complex iron sulfur subunit B (SDHB), CIII component ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), CIV component mitochondrial cytochrome c oxidase I (MTCO1), and CV component ATP synthase F1 subunit alpha (ATP5A). **D.** Relative abundance of endogenous proteins with mitochondria-ER/SR tethering roles, Ptpip51 and mitofusin-2 (Mfn2). For (**C,D**) band densities are normalized to the total protein in the lane (using Thermo Fisher's Total Protein Reagent), then, in each preparation type (VMC and LV lysates), to the mean of the Ctr. N=7 hearts each (3 VMC and 4 LV lysates). All bars show Means ± SEs. **P values were obtained** either via Mann-Whiney rank sum test (A,C), Wilcoxon Signed Rank test (B) or t-test (D).

Figure S5

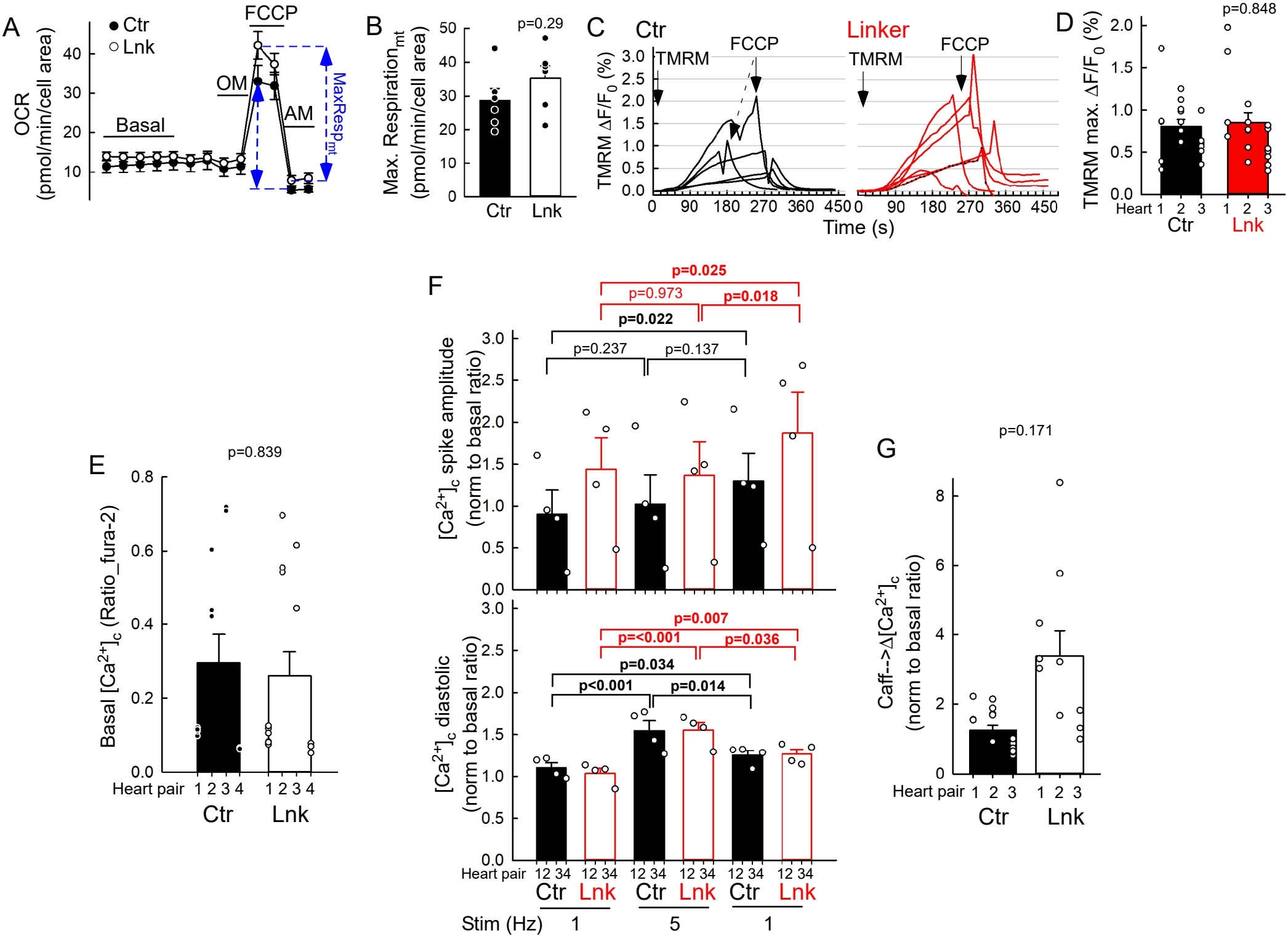
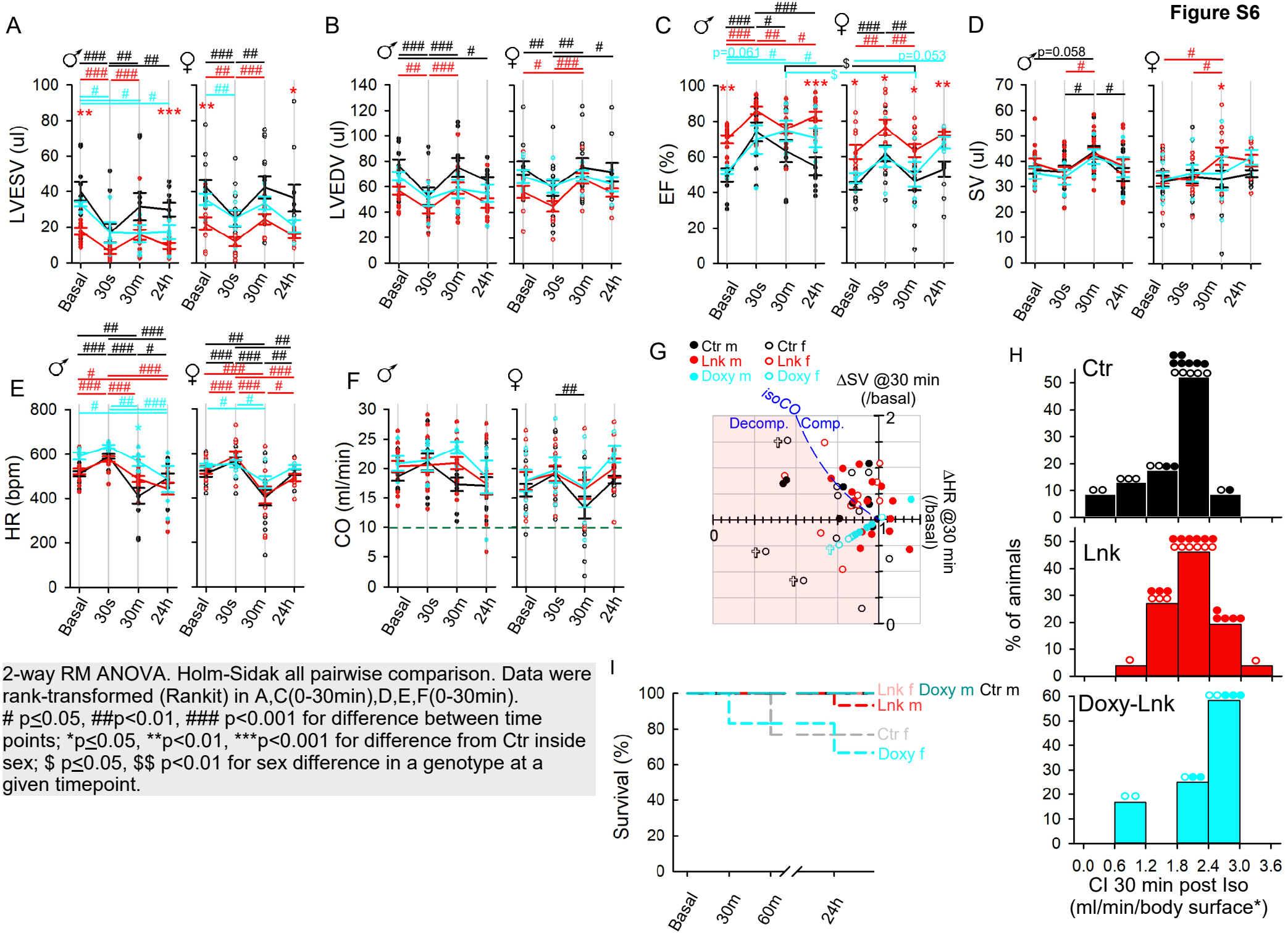

A
Ctr
Lnk
FCCP
OM
AM
Basal
MaxResp$_{mt}$
OCR (pmol/min/cell area)
B
Max. Respiration$_{mt}$ (pmol/min/cell area)
p=0.29
Ctr
Lnk
C
Ctr
Linker
TMRM
FCCP
TMRM ΔF/F$_0$ (%)
Time (s)
D
p=0.848
TMRM max. ΔF/F$_0$ (%)
Heart 1 2 3 1 2 3
Ctr
Lnk
E
p=0.839
Basal [Ca^{2+}]$_c$ (Ratio_fura-2)
Heart pair 1 2 3 4 1 2 3 4
Ctr
Lnk
F
p=0.025
p=0.973
p=0.018
p=0.022
p=0.237
p=0.137
[Ca^{2+}]$_c$ spike amplitude (norm to basal ratio)
p=0.007
p=<0.001
p=0.036
p=0.034
p<0.001
p=0.014
[Ca^{2+}]$_c$ diastolic (norm to basal ratio)
Heart pair 12 34 12 34 12 34 12 34 12 34 12 34
Ctr Lnk Ctr Lnk Ctr Lnk
Stim (Hz) 1 5 1
G
p=0.171
Caff-->Δ[Ca^{2+}]$_c$ (norm to basal ratio)
Heart pair 1 2 3 1 2 3
Ctr
Lnk

Figure S5. Functional cellular phenotyping of the linker-mouse VCMs (Related to Figure 4). A-B. Glucose/glutamine-fueled respiration of VCMs measured by Seahorse XF24. **A**. Oxygen consumption rates (OCR) normalized to cell density measured at basal condition, after adding oligomycin (OM 4 µg/ml, for basal ATP synthase activity and H^+ leak), FCCP (1 µM, for maximum uncoupled respiration) and antimycin A (10 µM, for non-mitochondrial respiration). **B**. Cumulated maximum mitochondrial respiration values. Means±S.E. n=6 VCM preps (3 male, 3 female) each from Ctr and Lnk hearts. P values (all >0.08, Dataset S1) were calculated by 2-way RM ANOVA (A) on rank-transformed data followed by Holm-Sidak all-pairwise post hoc comparison and by paired t-test (B). **C.** Time courses of TMRM accumulation to the mitochondria of individual VCMs recorded using Airyscan imaging. Traces are pooled from all the cells recorded in a single day; fluorescence intensity is normalized to the baseline (at the time of TMRM addition). When fluorescence increase appeared to slow down, FCCP (2 µM) was added to dissiptate $\Delta\Psi_m$. **D**. Cumulated maxima of normalized TMRM intensities from (C). Means±s.e., n=27 Ctr and 19 Lnk VCMs from 3 batches (hearts), 5-13 individual VCM/batch. P calculated by mixed random intercept model (Dataset S1). **E-G (related to Figure 4E-I):** $[Ca^{2+}]_c$ responses in individual VMCs expressed as fura-2 ratio changes normalized to the basal ratio. In vitro calibration to obtain molar values for $[Ca^{2+}]_c$ were unsuccessful (yielded too many out-of-range cells). Since in each experiment the basal Fura2 ratio (**E**) was similar between Ctr and Lnk, but the experiment-to-experiment variations were large, the ratio changes are normalized to the initial (basal) ratio. N and the statistical comparisons are the same as in the corresponding panels of Figure 4E-I. The dot plots overlaid over the bar graphs correspond to individual coverslips (E,F) or cells (G). They are aligned with the corresponding numeric indices (x axis ticks) of experiments. The same number index for Ctr and Lnk indicates an experimental pair (normalization to the Ctr in Figure 4E-I has been performed across these experimental pairs).

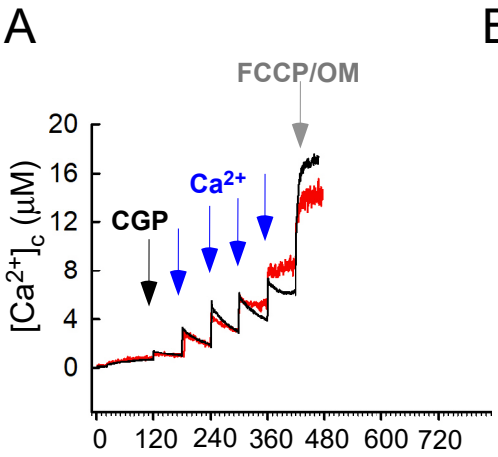
Figure S6

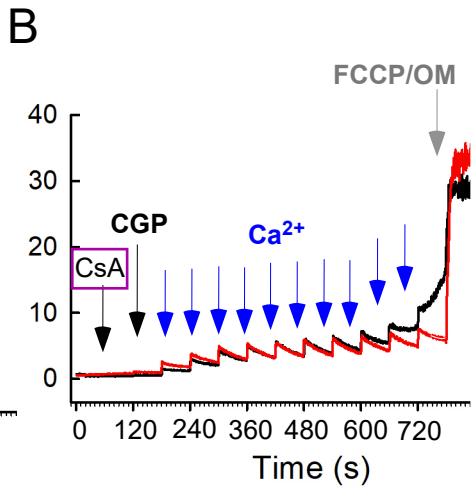


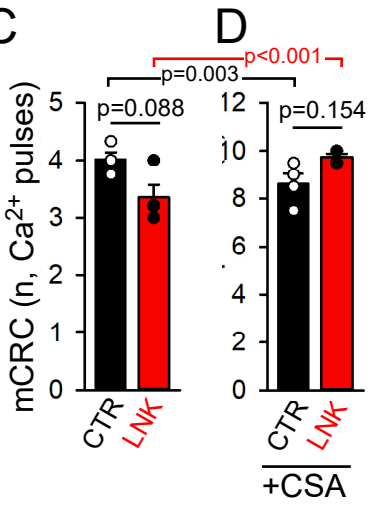
2-way RM ANOVA. Holm-Sidak all pairwise comparison. Data were rank-transformed (Rankit) in A,C(0-30min),D,E,F(0-30min).
p≤0.05, ##p<0.01, ### p<0.001 for difference between time points; *p≤0.05, **p<0.01, ***p<0.001 for difference from Ctr inside sex; $ p≤0.05, $$ p<0.01 for sex difference in a genotype at a given timepoint.

Figure S6. Improved resistance to acute massive β-adrenergic stress in the linker-mice. Main Figure 7 supplemented with the Doxy-linker cohorts (cyan color code). The Doxy-linker female indicated with ✞, died after an additional recording at 60 min (this time point is not shown, since it has been done only for a subset of animals).

Figure S7







2-way ANOVA. Rank-transformed data. Holm-Sidak all pairwise com-parison.

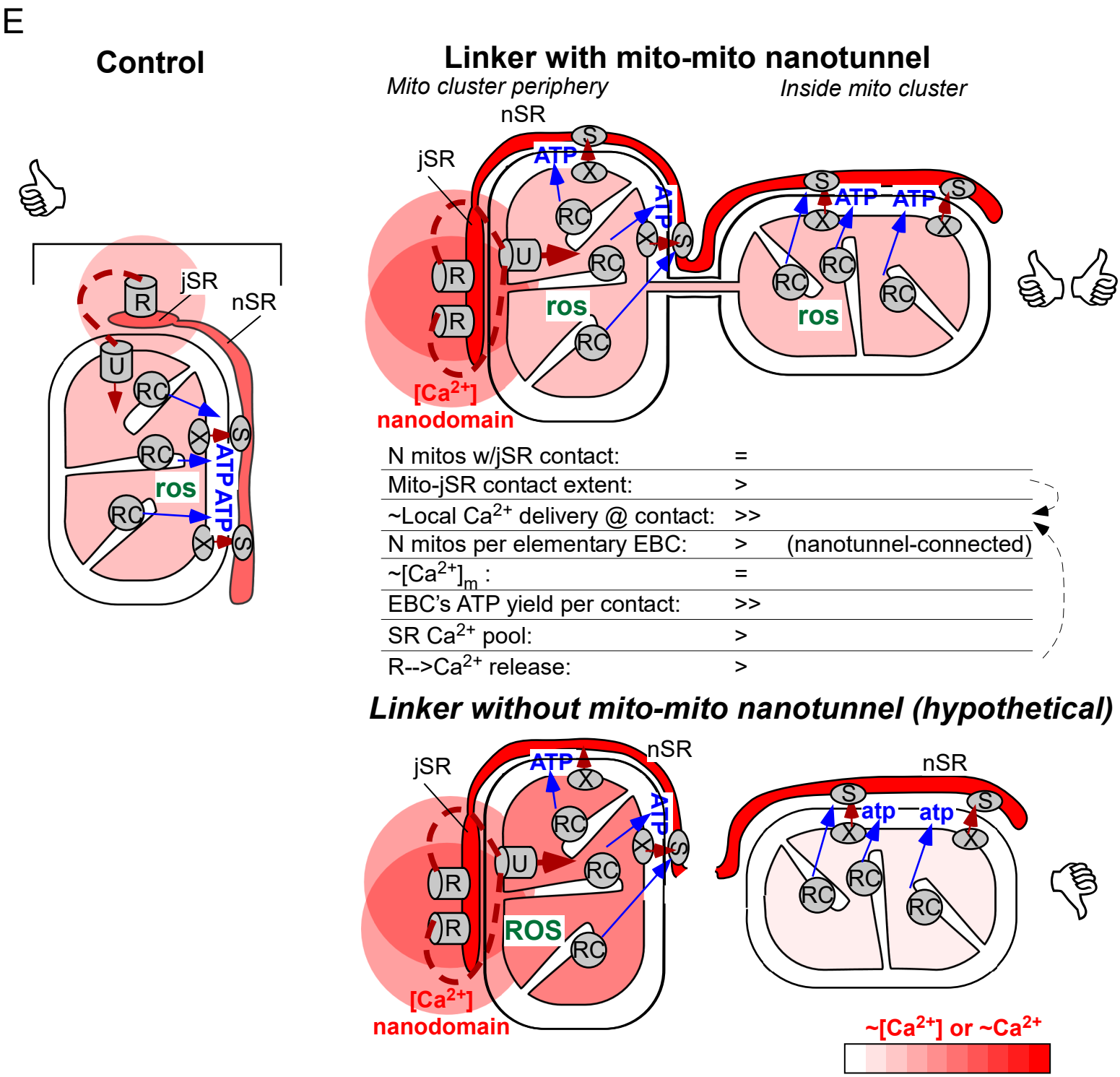


Figure S7. A-D mCRC assays in suspensions of permeabilized VCMs. A-B: representative timecourses of [Ca^{2+}] in the cytosolic compartment ([Ca^{2+}]$_c$) measured using Fura-LoAff. SERCA blocker thapsigargin (10 μM) was present throughout, NCLX blocker CGP37157 (CGP, 20 μM) was added at 120s. Subsequently, Ca^{2+} pulses ($CaCl_2$ 3.75 μM) were repeatedly applied every minute until the cessation or reversal of mitochondrial Ca^{2+} uptake (post-peak downward deflection flattened or turned to upward deflection). Finally, uncoupler (FCCP/OM, FCCP 2 μM/Oligomycin 5μg/ml) was added to fully release the sequestered Ca^{2+}. In (B), CSA 5 μM was added at 60s to inhibit mPTP. **C-D** Cumulated mCRCs expressed as the number of tolerated Ca^{2+} pulses. Means±S.E., N=4 pairs of VCM preps, each prep pooled from 2 hearts; 2-4 technical replicates (time-course recordings) for each group.

E. Proposed model for how inter-mitochondrial 'nanotunneling' allows safe improvement of EBC upon linker-mediated enhancement of individual mitochondria-jSR contacts and local Ca^{2+} transfer. Nanotunneling increases (doubles in this simplification) the mitochondrial mass involved in the elementary EBC event, attributed to a given mitochondrion-jSR contact. This keeps [Ca^{2+}]$_m$ in a 'safe range' despite the enhanced local Ca^{2+} delivery. R, RyR2; RC, respiratory chain; S, SERCA; U, uniporter (MCU channel complex); X, Na^+/Ca^{2+} exchanger (NCLX). Red arrows and dashed lines: Ca^{2+} fluxes. Blue arrows: ATP fluxes (ATP>atp). Estimated EBC-associated ROS increases are also indicated (ROS>ros). Shades of red approximate [Ca^{2+}] or Ca^{2+} content (for SR); concentric 'clouds' around Rs depict RyR2-mediated local Ca^{2+} release (nanodomain). The listed parameters are referenced to control: same as control (=), increased (>); >> indicates an increase involving multiple, already increased upstream factors (dashed arrows).