

Supplemental Material

Supplemental Methods

Experimental models

Taz-KD mice

Generation and characterization of the Taz-KD mouse model has been described elsewhere.¹⁴ Mice were obtained from Jackson Laboratories (B6.Cg-Gt(ROSA)26Sor^{tm37(H1/tetO-RNAi:Taz)Arte/ZkhuJ} (stock No. 014648). This strain expresses shRNA against Taz mRNA in the ROSA26 locus under control of the tetracycline-response element (Tet-On). The genetic background of these mice is C57BL/129S6.

Animals were fed with standard rodent chow enriched with doxycycline (625 mg/kg). For breeding, female mice were fed with this diet one week before and after mating. During mating, doxycycline was withdrawn to avoid male infertility. The genotype of the pups was assessed by RT-PCR. Mice were housed in a pathogen-free facility under 12 h light/darkness cycles and controlled temperature and humidity. Animals were maintained in IVC housing and manipulated in Class IIA laminar flow biosafety cabinets to prevent cross-contamination. Sentinel animals were regularly tested for pathogens using either serology or PCR on tissue, feces, and pelt swabs. Sentinel animals were housed 2 per cage on the bottom shelf of each ventilated rack. Cages were sampled systematically so that every cage was sampled over a 4-week period on any given rack. Sentinel mice were exposed to colony animal bedding for 6-8 consecutive months. Animals were used for experiments at 10, 20, and 50 weeks of age. Since we did not identify gender-specific differences in cardiac function *in vivo* or in mitochondrial function *in vitro*, both male and female mice were used for the study.

Overall study timeline

	10	20	50
	Time (weeks)		
Cine-MR Imaging	X	X	X
Echocardiography	X	X	X
P/V loops/dobutamine	X		
ECG analysis		X	
EC coupling/redox	X	X	X
Isolated mitochondria (respiration, ROS)	X	X	X
Molecular biology	X	X	X
Redox histology		X	X

Since the study was exploratory in nature when commenced, no primary or secondary endpoints were defined. Also, since no effect sizes could be foreseen, in particular for the alterations of excitation-contraction coupling and the interplay with mitochondrial energetics (the primary goal of our study), no reasonable power calculation was possible for animal calculations before the start of experiments. All mice that appeared of normal health were included, but excluded if they showed signs of disease or suffering. For invasive hemodynamics and ECG recordings including treatments with CGP-37157 or vehicle, animals were randomly assigned to the experimental groups in alternating order. Also here, since no indications from the literature exist on the hemodynamic response of Taz-KD mice to dobutamine or a comparable β -adrenergic agonist, no reasonable power calculation was possible before starting the experiments. For all *in vitro* and *in vivo* experiments, the persons who performed the experiments and/or data analyses were blinded to the genotypes and treatment groups. No mice died during the observation period up to 50 weeks.

iPSC-derived cardiac myocytes

The generation of cardiac myocytes from iPSCs (WT-D2) of a control individual and a BTHS patient [TAZ10 (c.590 G>T)] was described previously.^{8, 93} Feeder-free adherent cultures of iPSCs were passaged every 4-5 days by using the non-enzymatic Versene solution. Differentiation of iPSCs into cardiac myocytes was induced by RPMI 1640 medium supplemented with 2 mM L-glutamine and B27 without insulin.⁹⁴ The GSK3 inhibitor CHIR99021 (10 μ M) was added to the culture for 24 hours, the Wnt inhibitor IWP2 (5 μ M) was added at day 3 for 2 days and the culture medium was changed to RPMI+B27+insulin (RPMI 1640 medium supplemented with 2 mM L-glutamine and B27 with insulin, at day 7. At day 20, the cells were replated as monolayers into geltrex-coated culture dishes. Cardiac myocytes were selected by substituting glucose for 4 mM lactate in the culture medium for 6 days between days 20 and 30.⁹⁵

Method details

Mitochondria isolation from mouse heart

For mitochondria isolation, mice were sacrificed by intraperitoneal injection of ketamine hydrochloride (100 mg/kg; Pfizer, Karlsruhe, Germany) and xylazine hydrochloride (10 mg/kg; Bayer Healthcare, Berlin, Germany). The protocol for mitochondria isolation from adult mouse heart was first described by Mela and Seitz.⁹⁶ All media used in the procedure were ice-cold, and all steps were performed on ice or at 4°C. After the animal was sacrificed, the heart was excised and rinsed in a Petri dish filled with isotonic isolation solution containing (in mM): mannitol 225, sucrose 75, HEPES 2, EGTA 1 (pH 7.4). Atrial and non-cardiac tissue was removed, and ventricles were opened to remove blood clots. The heart was then transferred into a 2 mL tube containing 500 μ L isolation solution and minced with scissors. Subsequently, the minced heart was transferred to a 5 mL homogenizer (Teflon pestle) and manually homogenized in 1 mL isolation solution for 14 minutes. The homogenate was centrifuged at low speed (400 g) in a pre-cooled (4°C) centrifuge for 5 minutes to remove cell debris. The supernatant obtained from the first centrifugation was centrifuged at high speed (7,700 g) for 10 minutes, and the pellet - containing mitochondria - was washed in 1 mL mitochondrial suspension solution containing (in mM) mannitol 225, sucrose 75, HEPES 2 (pH 7.4) and centrifuged again twice at high speed (7700 g) for 10 minutes. The pellet obtained from the final centrifugation step was resuspended in 100-150 μ L mitochondrial suspension solution and used for experiments.

Mitochondria isolation from mouse skeletal muscle

The protocol for mitochondria isolation from mouse skeletal muscle was first described by Chappel and Perry.⁹⁷ All media used in the procedure were ice-cold, and all steps were performed on ice or at 4°C. After the animal was sacrificed, hindlimb skeletal muscle was rapidly excised and rinsed in a Petri dish filled with basic medium containing (in mM) KCl 140, HEPES 20, MgCl₂ 5, EGTA 1 (pH 7.0). Connective tissue or adipose tissue is removed and skeletal muscle is placed in a 2 mL tube containing 500 μ L homogenization medium (same composition as basic medium, plus ATP 1 mM and BSA 1%; pH 7.0) containing 1 U of subtilisin A, a serine protease produced by *Bacillus subtilis*, and minced with scissors. The skeletal muscle is then transferred to a 5 mL homogenizer (Teflon pestle) and manually homogenized in 1,5 mL homogenization medium for 14 minutes. The

homogenate is centrifuged at low speed (800 g) in a pre-cooled (4°C) centrifuge for 10 minutes to remove cellular debris. The supernatant obtained from the first centrifugation is centrifuged at high speed (12,000 g) for 10 minutes, and the pellet, containing mitochondria, was resuspended in 200 μ L basic medium.

Mitochondria isolation from mouse brain

For mitochondria isolation from mouse brain, the brain was extracted, rinsed with isolation solution (same as used for mitochondria isolation from mouse heart) and minced with scissors. The tissue was divided in two parts and transferred to a 5 mL homogenizer (Teflon pestle) and manually homogenized in a buffer consisting of 1 ml isolation solution and 0.16 mg/mL bacterial protease type XXIV; using 10 strokes with a loose-fitting glass pestle followed by a tight-fitting pestle. The homogenate was centrifuged at 2000 g for 3 minutes at 4°C in a pre-cooled centrifuge. The pellet was resuspended in 1 mL of isolation solution and bacterial protease mixture and centrifuged again at 2000g for 3 minutes. The supernatant obtained from these two centrifugations were pooled and centrifuged at 12000 g for 8 minutes. The resulting pellet, containing mitochondria, was resuspended in 100 μ L of mitochondrial suspension solution (same as used for mitochondria isolation from mouse heart).

Mitochondrial respiration

Mitochondrial respiration was measured polarographically using a Clark electrode (Hansatech) covered with an ultrathin Teflon membrane (Hansatech). Experiments were performed at 37°C. For each experiment, 400 μ g mitochondria were suspended in 2 mL air-saturated respiration buffer containing (in mM) KCl 134, $MgCl_2$ 2.5, KH_2PO_4 2, EGTA 0.5, HEPES 20 (pH 7.2) inside the oxygraph chamber (State 1). Subsequently, mitochondria were supplied with saturating concentrations of the complex I substrates pyruvate and malate (5 mM each). Under this condition, which we refer to as respiratory State 2, respiration is limited by the low level of ADP, and O_2 consumption is mainly due to H^+ leak across the IMM. Thereafter, oxidative phosphorylation was stimulated by the addition of ADP (final concentration: 1 mM), achieving respiratory State 3. Finally, State 4 was obtained by adding the complex V inhibitor oligomycin (1.25 μ M).

Mitochondrial H_2O_2 emission

H_2O_2 emission from isolated mitochondria was measured with Amplex UltraRed, a sensitive and H_2O_2 -specific indicator that is converted to the fluorescent molecule resorufin (λ_{exc} 535 nm; λ_{em} 595 nm) upon reaction with H_2O_2 . Oxidation of Amplex UltraRed by H_2O_2 is catalyzed by horseradish peroxidase (HRP). The assay was performed at 37°C using a fluorescence plate reader (Infinite M200Pro, Tecan) in a 96-well transparent bottom plate. The experiment was conducted with 200 μ L respiration buffer containing (in mM) KCl 134, $MgCl_2$ 2.5, KH_2PO_4 2, EGTA 0.5, HEPES 20 (pH 7.2) per well containing 165 μ g mitochondria and the following concentrations of assay reagents: Amplex UltraRed 10 μ M, HRP 0.5 U/mL, and SOD 100 U/mL. Respiratory state 1, 2, and 3 were obtained using the same substrates used for mitochondrial respiration measurements; subsequently, the complex III blocker antimycin A (15 μ M) was added as a positive control to induce superoxide formation at the ETC. Results are obtained by averaging at least 3 replicates of the same experimental condition. Background fluorescence in the absence of mitochondria was measured for each condition and subtracted accordingly.

Mitochondrial superoxide formation

Superoxide (O_2^-) formation in isolated mitochondria was monitored by measuring the EPR spectra of the radical formed by the reaction of O_2^- with the redox-activated cyclic hydroxylamine spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; 300 μM) using a Bruker spectrometer (ESP300e) equipped with a standard 4102ST cavity holding the capillary support quartz glass finger. The temperature of the finger was controlled and maintained at 37°C throughout the experiment. The sample containing mitochondria (12 μg per replicate), CMH, as well as different combinations of substrates/inhibitors was filled in a 50 μL glass capillary, which was immediately transferred into the capillary holder. To correct for unspecific radical production, control experiments were performed with single components of the assays and CMH. As described previously,²³ the EPR spectra of the CMH radical were measured with modulation amplitude of 0.1 mT and a microwave power of 20 mW at a time constant of 20.48 ms for 8 times and evaluated with a customized program (Medeia) which measures the peak-to-peak intensity and width of one (or several) lines of a radical visible in a time series. In the absence of line width changes and saturation effects, the intensity information can be translated to radical concentration by calculating the integral of the monitored line and considering the multiplicity of the radical signal. This value is compared to a reference sample of known concentration (100 μM Tempol) recorded under identical conditions for quantitative measures.

Permeability transition pore (PTP) assay

Ca^{2+} retention capacity of isolated mitochondria was determined by exposing mitochondria to repeated Ca^{2+} pulses (final free Ca^{2+} concentration 10 μM each pulse) and simultaneously measuring extramitochondrial Ca^{2+} levels with the fluorescence indicator Calcium Green 5-N (λ_{exc} 503 nm; λ_{em} 535 nm) using a fluorescence plate reader (Infinite M200Pro, Tecan). The assay was performed at 37°C in a 96-well transparent bottom plate. The experiment was conducted in PTP buffer containing (in mM) KCl 120, mannitol 70, sucrose 25, HEPES 20, KH_2PO_4 5, EGTA 0.02. For each replicate, 165 μg mitochondria were dissolved in 200 μL PTP buffer. Mitochondria were incubated with potassium glutamate (5 mM) and sodium malate (2.5 mM) with or without the PTP inhibitor cyclosporine A (2 μM) for 15 minutes. Calcium Green 5-N (1 μM) was added immediately before starting the experiment. Ca^{2+} was added by an automatic injector to each well every 120 s. The amount of Ca^{2+} required to obtain 10 μM free Ca^{2+} for each addition was calculated with Maxchelator (<http://maxchelator.stanford.edu/CaEGTA-TS.htm>). Results were calculated by averaging at least 2 technical replicates of the same experimental condition.

Aconitase activity

Aconitase activity was determined by measuring absorption changes of NAD(P)H every minute at 340 nm using a fluorescence plate reader (Infinite M200Pro, Tecan). The assay was performed in a total volume of 200 μL reaction buffer at 37°C after one freeze/thaw cycle to break mitochondria. For each replicate, 25 μg of mitochondria or cytosol were supplied with (in mM) Tris-HCl 36, cysteine 0.8, MgCl_2 0.4, NADP^+ 0.2, citrate 30 (pH 7.4).

Cardiac myocyte isolation from mouse heart

Isolation of adult ventricular myocytes from mouse heart was performed using a modified Langendorff perfusion system, as described by O'Connell and colleagues (AfCS Research Reports; Vol. 1 No. 5 CM). Mice were anaesthetized with 5% isoflurane, 250 I.U. unfractionated heparin, and

0.33 mg carprofen. When pedal reflexes could not be elicited anymore, the heart was exposed and excised together with the aortic arch, the aorta was cannulated, and retrograde perfusion was started. Before reaching the heart, the perfusate was warmed up to 37°C by flowing through a glass coil immersed in a water bath at 37°C. The composition of the solutions used in the isolation is provided in the [Table I](#).

First, the heart was perfused with solution A for 4 minutes to remove the remaining blood from the coronary vessels; subsequently, the heart was digested with solution B for 5 to 8 minutes. Solution B contains Liberase TH, a mixture of collagenase I, II, and the protease thermolysin, which degrades intercellular junctions. To stop digestion of the tissue, the heart was removed from the Langendorff apparatus, the ventricles were opened and rinsed with a 1:1 to mixture of solutions A and C at room temperature. Intact cardiac myocytes were released by softly rinsing the heart in the solution A/C mixture for 2-3 minutes, after which the solution was transferred to a 15 mL tube. The cell pellet obtained by sedimentation was resuspended in 5 to 10 mL (depending on cell viability and the yield of the isolation) of solution D and transferred to a Petri dish. Finally, cardiac myocytes were gradually re-exposed to the physiological concentration of extracellular Ca^{2+} in 5 steps, each step lasting 4 minutes: 50 μM $\rightarrow$ 100 μM $\rightarrow$ 200 μM $\rightarrow$ 500 μM $\rightarrow$ 1 mM CaCl_2 . Thereafter, the solution was again transferred to a 15 mL tube and cardiac myocytes were obtained by sedimentation for 8-10 minutes. The pellet was resuspended in 10 mL of culture medium (M199 + FCS 5% + penicillin and streptomycin 1% + 10 mM HEPES) and stored at 37°C.

Experiments with intact cardiac myocytes

Experiments with intact cardiac myocytes were performed using a customized IonOptix setup (IonOptix, Milton, MA, USA) coupled to an inverted epifluorescence microscope (Eclipse Ti, Nikon GmbH, Düsseldorf, Germany). All experiments were performed at 37°C. During the experiment, cells were continuously superfused with Normal Tyrode's (NT) solution containing (in mM): NaCl 130, KCl 5, MgCl_2 1, CaCl_2 1, Na-HEPES 10, glucose 10, sodium pyruvate 2 and ascorbic acid 0.3 (pH 7.54).

For experiments shown in [Figures 2A-H, 5D-H, 6A-C, II, and VI](#) adult ventricular myocytes were field-stimulated at 0.5 Hz for 120 s, subsequently superfused with NT solution containing isoproterenol (30 nM), and when the isoproterenol-induced increase in contractility became evident, stimulation frequency was increased to 5 Hz for 180s. For experiments shown in [Figures 5I, J and 7A](#), cardiac myocytes were superfused with NT solution containing the NCLX inhibitor CGP-37157 (10 μM ; [Figure 5I, J](#)), the K_{ATP} channel sensitizer pinacidil (100 μM), the K_{ATP} channel blocker HMR-1098 (30 μM ; [Figure 7A](#)), or vehicle (DMSO) before being subjected to Iso/5 Hz stimulation.

In the experiment shown in [Figures 2I-P](#), cardiac myocytes were field-stimulated at 0.5 Hz for 120 s. Subsequently, field-stimulation was stopped and one pulse of caffeine (10 mM) was applied to induce Ca^{2+} release from the SR. Stimulation at 0.5 Hz was resumed until sarcomere shortening was again comparable to baseline conditions. Thereafter, cardiac myocytes were superfused with NT solution containing isoproterenol (30 nM) until a visible increase in sarcomere shortening could be observed, and stimulation frequency was increased to 5.0 Hz for 120 s. A second caffeine pulse was applied immediately after stopping field-stimulation.

During the experiments described above, sarcomere shortening was measured together with NAD(P)H and FAD autofluorescence ([Figures 5D-J; IIA, B; VI](#)), Indo-1-AM fluorescence to monitor $[\text{Ca}^{2+}]_i$ ([Figures 2A, E-P; IIC-F](#)), tetramethylrhodamine methyl ester (TMRM; 10 nM) to monitor mitochondrial membrane potential $\Delta\Psi_m$ ([Figure 5F](#)) or cellular ROS formation with 5-(6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (DCF; 1 μM ; [Figure 6A-C](#)). Calibration of NAD(P)H and FAD signals was performed by applying carbonyl cyanide-p-

trifluoromethoxyphenylhydrazine (FCCP; 5 μ M) and cyanide (4 mM) to induce complete oxidation and reduction, respectively, of the mitochondrial pyridine nucleotide pools.

Patch clamp experiments

To determine mitochondrial and cytosolic Ca^{2+} levels in the same cardiac myocyte (**Figure 5A-C**), we employed a patch-clamp dual-fluorescence approach that we previously established.²⁵ Briefly, cardiac myocytes were incubated for 2 hours with rhod-2-acetoxymethylester (rhod-2-AM), a Ca^{2+} indicator that segregates primarily into mitochondria, and subsequently cytosolic remnants of rhod-2-AM were removed by dialyzing the cytosol with a pipette solution containing Indo-1 penta- K^+ salt (75 μ M). Indo-1 penta- K^+ salt does not pass membranes and thus reports specifically $[\text{Ca}^{2+}]_c$.²⁵ Additionally, the pipette solution contained (in mM): potassium glutamate 130, KCl 19, MgCl_2 0.5, Na-HEPES 15, Mg-ATP 5 (pH 7.2). Subsequently, cardiac myocytes were voltage-clamped in whole-cell configuration (37°C, pipette resistance 2-4 M Ω) and subjected to the experimental stress protocol described above by depolarizing the cell from -80 to +10 mV for 50 ms at 0.5 or 5 Hz, respectively.

Force measurements in intact cardiac myocytes

To monitor force development in isolated cardiac myocytes (**Figure 7B-E**), we used the IonOptix force transducers as described previously.⁵⁹ In this experiment, cardiac myocytes were superfused with NT solution containing (in mM) NaCl 130, KCl 5, MgCl_2 1, CaCl_2 2, Na-HEPES 10, glucose 10, sodium pyruvate 2, and ascorbic acid 0.3 (pH 7.54). Briefly, cantilever of the force transducer and the piezo motor were covered first with “precoat” for a few seconds and thereafter coated with specially-formulated biocompatible adhesive (MyoTak). Subsequently, the holders were transferred into the chamber, in which cardiac myocytes were placed on a 2% BSA-laminated cover slip. Beating cells with the correct orientation were attached to both cantilevers and then stretched with the piezo motor up to a sarcomere length of 1.9 μ m. The measurements were performed at room temperature. The stimulation frequency was 1 Hz, and after the final sarcomere length was reached, stimulation frequency was kept for 40 s at 1 Hz and then for 60 s at 4 Hz.

Force measurements in membrane-permeabilized cardiac myocytes

The composition of all solutions was calculated based on a computer software as previously described.⁴⁴ The pH of all solutions was adjusted to 7.1 at 15°C by KOH and ionic strength was adjusted to 180 mM with KCl. The relaxing solution contained (in mM) free Mg^{2+} 2, MgATP 1, EGTA 20, BES 10, and phosphocreatine (PCr) 14.5. Maximal Ca^{2+} activating solution consisted of (in mM) 2 free Mg^{2+} , 1 MgATP, 20 EGTA, 10 BES and 32 μ M free Ca^{2+} . Ca^{2+} activating solutions with lower free $[\text{Ca}^{2+}]$ were obtained by mixing of the Ca^{2+} activating and relaxing solutions and assuming an apparent stability constant of the Ca^{2+} -EGTA complex of $10^{6.35}$.⁴⁴

Frozen cardiac tissue samples from 10 w/o *Taz*-KD and WT mice were thawed in relaxing solution and cardiac myocytes were mechanically isolated with a tissue ruptor (Qiagen). Cardiac myocytes were chemically permeabilized by incubation for 5 minutes in relaxing solution containing 0.5% (v/v) Triton-X100 and glued between a force transducer and a servo motor (Aurora Scientific). Isometric force measurements were subsequently performed in single cardiac myocytes at sarcomere length of 2.2 μ m. Average sarcomere lengths were determined by means of a spatial Fourier transformation. Absolute tensions were normalized to cardiac myocyte cross-sectional area and expressed as developed tension (in kN/m²). Passive force (F_{pass}) was determined by shortening the

myocyte in relaxing solution by 30% of its length. Active tension ($\text{Ca}^{2+}\text{-F}_{\text{act}}$) was calculated as $\text{F}_{\text{act}} = \text{F}_{\text{total}} - \text{F}_{\text{pass}}$. In addition, the rate constant of tension redevelopment (k_{TR}) was determined using a slack-restretch test: after steady state force was reached, cardiac myocytes were shortened within 1 ms to 70% of their original length and restretched to the original length after 50 ms. A single exponential was fitted to the force redevelopment to determine the rate constant of force redevelopment: k_{TR} was determined at maximal and submaximal $[\text{Ca}^{2+}]$ to assess cross-bridge cycling kinetics.

Force- Ca^{2+} relations were fit by a non-linear fit procedure to a modified Hill equation using GraphPad Prism 8 as follows:

$$P(\text{Ca}^{2+}) / P_0 = [\text{Ca}^{2+}]^n H / (K^n H + [\text{Ca}^{2+}]^n H)$$

where P is steady-state force. P_0 denotes the steady isometric force at either saturating $[\text{Ca}^{2+}]$, nH describes the steepness of the relationship, and K represents the $[\text{Ca}^{2+}]$ at which force is half-maximal ($0.5 \times P_0$). Myofilament Ca^{2+} affinity is denoted as EC_{50} .

Redox histology

To map the *in vivo* redox status, the *Taz*-KD mouse model was crossbred with a transgenic model expressing the mitochondrial H_2O_2 sensor mito-roGFP2-Orp1⁵³ on a C57BL/6N background. Mice were sacrificed via cervical dislocation. Immediately afterwards, the thorax was opened and cardiac perfusion was performed. Hereby, the circulatory system was perfused with 25 mL N-Ethylmaleimide (NEM, 50 mM, diluted in PBS) at ~5 mL/min. Thereafter, hearts were removed and dissected in 50 mM NEM solution. Fixation was done overnight at room temperature with 4% paraformaldehyde. On the following day, the samples were washed for 15 min in PBS, embedded in Tissue Tek OCT Compound (Fisher Scientific) rapidly frozen using liquid nitrogen-cooled isopentane and cut at 5 μm using a Leica CM 3050 S cryostat (Leica, Germany). For imaging, a filter-based fluorescence microscope (Axio Observer 7, Zeiss) with a 20x air objective was used. The roGFP2-Orp1 fluorescence was measured at 500 to 550 nm with either 405 (BP 430/60, Zeiss filter) or 488 nm (BP 470/40 HE, Zeiss filter) excitation.

The images obtained at 405 and 488 nm excitation were analyzed separately and individual thresholds for signal intensity were set to remove background and overexposed pixels (Fiji imaging software). Fluorescence ratio 405/488 images were calculated by combining the image masks resulting from 405 and 488 nm thresholds and displaying the ratio. The resulting image was visualized using ImageJ lookup table "Fire". Image analysis was performed using a macro coded by the investigator, thus minimizing the risk of bias. The presented change in ratio (Figure 6F) results from normalizing all ratios to the average of the WT ratio.

Gene expression analyses

RNA was prepared from mouse LV tissue using peqGold TriFast (PeqLab) extraction reagent per manufacturer's protocol. For cDNA preparation 2 μg of RNA were digested with DNase (PeqLab) and then reverse-transcribed using the HighCap cDNA RT Kit (Applied Biosystems) according to the manufacturer's protocol. TaqMan PCR was conducted in a StepOne plus thermocycler (Applied Biosystems) using TaqMan GenEx Mastermix (Applied Biosystems). Signals were normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) controls. No template controls were used to monitor for contaminating amplifications. The ΔCt was used for statistical analysis and $2^{-\Delta\Delta\text{Ct}}$ for data presentation. Probes used to amplify the transcripts are listed in the Table II.

SDS-page and Western blot

Western blot analysis was performed either to determine total protein content using complete tissue homogenate or to compare protein content in isolated mitochondria and cytosol (see mitochondrial isolation). Mouse LV myocardium, skeletal muscle, and brain tissue was homogenized in total lysis buffer containing Tris-HCl 100 mM, SDS 4%, and glycine 20%, complete protease inhibitors (Roche), and phosphatase inhibitors (Roche). The homogenate was centrifuged at 16.000 g for 20 minutes and protein concentration was estimated by BCA assay. For each lane, 50 µg of protein was separated on 10 or 12% SDS-PAGE gels and electrophoretically transferred to nitrocellulose membrane (0.2 µm pore size, Schleicher and Schuell). Membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat dry milk for 120 minutes at room temperature and exposed to the primary antibodies (listed in the [Table II](#)) diluted 1:1000. Secondary antibodies (goat anti-mouse 1:2500; rabbit anti-goat 1:5000) were incubated for 60 minutes at room temperature. Proteins were visualized by enhanced chemiluminescence according to the manufacturer's guidelines (Amersham Pharmacia Biotech, Freiburg, Germany). Quantification was performed using the high-resolution chemiluminescence Fusion SL Detection System (PepLab, Erlangen, Germany) and analyzed by FusionCapture Advanced Software (PepLab, Germany). Data are presented as intensity optical density (IOD).

Blue Native-PAGE and Western blot

Mitochondria were isolated from tissues as described above and the protein concentration was estimated by BCA assay. For BN-PAGE analysis, 100 µg (brain and heart) or 200 µg (skeletal muscle) of mitochondria were solubilized in digitonin buffer containing (in mM) Tris-HCl 20, NaCl 50, EDTA 0.1, PMSF 2, glycerol 10%, (w/v) digitonin 1%; pH 7.4, for 15 minutes on ice. After a clarifying spin, the samples were separated on a 4-13% BN-PAGE.⁹⁸ Native protein complexes were transferred on a PVDF membrane (Immobilon-P 0.45 µm pore size, Merck Millipore) followed by immunoblotting with antibodies directed against NDUFB8, UQCRC1, COX5A (all generated by Peter Rehling), and MCUa. In addition, expression levels of the proteins were analyzed by SDS-PAGE and immunoblotting using antibodies specific for OXPHOS proteins and MCUa.

Magnetic resonance imaging

All MRI experiments were performed using a 9.4 Tesla horizontal bore animal scanner (Bruker BioSpin 94/20, Ettlingen, Germany) equipped with Paravision 5.1 operating software, with a linear resonator and a receive only 2 × 2 element phased-array surface coil developed for imaging of the mouse heart. During imaging experiments, animals were anesthetized using a mixture of isoflurane and oxygen (1.0 to 2.5% /99.0 to 97.5%), applied via a nose mask at a flow rate of 1.0 to 2.0 L/min. Mice were positioned prone in a dedicated cradle including the coil, and a water-heated blanket was placed over the body to prevent hypothermia. Core temperature was measured using a rectal sensor. ECG signals were recorded with external electrodes attached to the front paws and a pressure transducer attached to the abdominal wall allowed for monitoring of the respiration rate. For determination of end-diastolic and end-systolic volumes, a multi-slice retrospectively self-gated cine fast low angle shot (FLASH) sequence was employed in short axis orientation covering the entire left ventricle from base to apex (protocol Intragate-FLASH). Sequence settings were set to TR/TE of 5.6/1.5 ms, flip angle 20°, 8 consecutive slices, slice thickness 0.7 mm, 200 averages, field of view 25.6 × 25.6 mm, matrix 256 × 256 (resulting in a resolution in plane of 100 µm × 100 µm). For every slice position 20 cine image frames were reconstructed. With the same sequence, four-chamber cine images were acquired for strain analysis with imaging parameters set to TR/TE 5.6/1.4 ms, flip angle

20°, 1 slice, slice thickness 1.0 mm, 200 averages, field of view 20.0 × 20.0 mm, matrix 200 × 200 (resulting in a resolution in plane of 100 μm × 100 μm). Images were exported from the scanner in DICOM format, and image analysis was performed by manually contouring LV images using the freely available software Segment.⁹⁹ The investigator was blinded to the genotype of the animals during image analysis.

Echocardiography

Echocardiography was performed with the Visualsonics Vevo® 770 High-Resolution *in vivo* Micro-Imaging System (Visualsonics Inc., Scanhead: RMV707B, 15-45 MHz, cardiac mouse). Mice were anesthetized with a mixture of isoflurane (3%) and O₂ (97%) at a flow rate of 1 L/min. The animal was placed in a supine position on a pad with embedded ECG. During the measurement, anesthesia was maintained with a mixture of isoflurane (1-1.5%) and O₂ (98.5-99%) at a flow rate of 1 L/min using a rodent nose mask. Heart rate was maintained at ~500 beats per minute (bpm) with these regimens. Body temperature was monitored via a rectal probe and maintained at 37.0±1.5°C throughout the entire procedure via a heated platform and a heat lamp. Chest hair was removed and a layer of preheated ultrasound gel was applied to the chest.

Two-dimensional (B-mode) images were acquired in parasternal long- and short-axis view with guided one-dimensional (M-mode) recordings at the midventricular level apical of the papillary muscle in both views. Interventricular septum (IVS), left ventricular posterior wall (LVPW), and left ventricular internal diameter (LVID) were measured in parasternal long-axis projection during at least three cardiac cycles and averaged. ECG and respiration gating were used to suppress imaging artifacts due to respiration and cardiac movements.

Hemodynamic and ECG recording in vivo

Closed chest hemodynamic studies of aortic pressure and LV function were performed with a retrogradely inserted 1.4F PV catheter (SPR-839NR) and an MPVS Ultra pressure-volume loop system (Millar Instruments, Houston, TX, USA). Data were collected with a PowerLab 8/35 throughout the complete experiment and analyzed with LabChart 8.1 Pro (ADInstruments, Sydney, Australia). After 30 min of stabilization, calibrated pressure-volume loops were continuously recorded under 1.5% isoflurane anaesthesia; body temperature was kept constant at 37°C. Comprehensive investigation of LV performance started with the recording of systemic blood pressures in the aortic arch. After moving the catheter forward to the left ventricle, blood pressure, pressure/volume loops, and the first derivatives of LV pressure, i.e., +dP/dt and -dP/dt, were recorded. At this stage, CGP-37157 (0.02 mg/kg) or vehicle were administered via the right jugular vein, and potential CGP-37157 effects on cardiac performance were recorded after 15 min incubation time. Afterwards, dobutamine was infused with a starting concentration 10 ng/g*min up to a final concentration of 40 ng/g*min. To determine the end-diastolic pressure/volume relationship (EDPVR) a transient inferior vena cava occlusion was then performed during simultaneous measurement of pressure/volume loops. EDPVR values were determined by fitting the end-diastolic points during vena cava occlusion using a linear regression fit: $P_{ed} = EDPVR * V_{ed} + \text{intercept}$. Based on these linear fits, EDPVR was calculated as the average slope (EDPVR in mmHg/μL). Mice were sacrificed under deep anesthesia, the hearts were weighed, LV tissue was dissected and snap frozen for further investigations.

To record ECGs *in vivo*, mice were anesthetized using 1.5% isoflurane and kept on a warming plate (37°C) throughout the experiment. Lead I and II ECG recordings were captured at a sampling rate of 4,000 points per second (ADInstruments, PowerLab 8/35 equipped with BIOamplifiers) and time

periods and intervals of the ECG recordings were analyzed with the built-in ECG module of LabChart Pro 8.

Myocardial fibrosis

Interstitial fibrosis was assessed by Picro Sirius red staining as described in previous publications.¹⁰⁰ In short, LV tissue samples were fixed using 4% formalin/PBS solution and embedded in paraffin. LV circumferential sections 6 µm thick were cut with a microtome. Subsequently, samples were deparaffinized, hydrated, and incubated in 0.1% Sirius red in a saturated solution of picric acid. Excess Sirius red was removed with 0.01 N HCl. Picro Sirius red staining identifies collagen type I and type III by yellow-red and green birefringence, respectively. Ten fields from the middle third of the left ventricle were photographed at 100x magnification with a Nikon E600 microscope. The quantification displayed in **Figure 1L** corresponds to the percent area of collagen on total myocardial tissue as analyzed with the digital software Lucia G.

8-hydroxy-2'-deoxyguanosine (8-OHdG) staining

Histology slices of LV myocardium were deparaffinized and pre-treated in Coplin jars with 0.05% citraconic anhydride solution (pH 7.4) for 1 hour at 98°C. Slices were then incubated overnight in primary antibody at 4°C and thereafter with the appropriate secondary antibody at 37°C for 40 minutes. To detect hydroxyl radical-induced DNA oxidation, slices were incubated with a polyclonal antibody against 8-OHdG (Abcam) diluted 1:300 in citrate buffer. To identify cardiac myocytes, a monoclonal antibody against α -sarcomeric actin (Sigma-Aldrich) was used. FITC- and TRITC-conjugated anti-goat IgG and anti-mouse IgM (Dianova) were used as secondary antibodies. Wash steps were performed with 4x saline-sodium citrate buffer. Sections were counterstained with DAPI (Calbiochem) and mounted with fluorescent mounting medium (Vectashield, Vector Laboratories). All sections were evaluated using a Nikon E600 epifluorescence microscope (Nikon, Germany) with appropriate filters.

Supplemental Tables

Table I. Solutions for cardiac myocyte isolation

	Solution A	Solution B	Solution C	Solution D
NaCl	113 mM	113 mM	113 mM	113 mM
KCl	4.7 mM	4.7 mM	4.7 mM	4.7 mM
KH ₂ PO ₄	0.6 mM	0.6 mM	0.6 mM	0.6 mM
Na ₂ HPO ₄	0.6 mM	0.6 mM	0.6 mM	0.6 mM
MgSO ₄	1.2 mM	1.2 mM	1.2 mM	1.2 mM
NaHCO ₃	12 mM	12 mM	12 mM	12 mM
KHCO ₃	10 mM	10 mM	10 mM	10 mM
HEPES	10 mM	10 mM	10 mM	10 mM
Phenol red	32 µM	32 µM	32 µM	32 µM
BDM	10 mM	10 mM	10 mM	10 mM
Taurine	30 mM	30 mM	30 mM	30 mM
Glucose	5.5 mM	5.5 mM	5.5 mM	5.5 mM
CaCl ₂		12.5 µM	12.5 µM	12.5 µM
Liberase TH		0.08 mg/mL		
Trypsin		0.14 mg/mL		
FCS			10%	5%

Table II. Materials used in the study

Reagent	Producer	Identifier
Antibodies		
Actin (α-sarcomeric)	Sigma-Aldrich	Cat#A2172
Catalase	Cell Signaling Technology	Cat#14097S
Cytochrome c oxidase subunit 4	Abcam	Cat#ab14744
Cytochrome c oxidase subunit 5	Generated by Peter Rehling	N/A
EMRE (C22orf32)	Santa Cruz Biotechnology	Cat#sc-86337
Glutathione peroxidase 1	Abcam	Cat#ab108427

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Millipore	Cat#MAB374
MICU1	Cell Signaling Technology	Cat#12524S
MICU2	Abcam	Cat#b101465
Mitochondrial H ⁺ /Ca ²⁺ exchanger	Generated by Peter Rehling	N/A
MCUa	Cell Signaling Technology	Cat#14997S
MCUb (Ccadc109b)	Abcam	Cat#ab170715
MCUR1	Cell Signaling Technology	Cat#13706S
NADH dehydrogenase 1 beta subcomplex subunit 8 (NDUFB8)	Generated by Peter Rehling	N/A
Peroxiredoxin 1	Cell Signaling Technology	Cat# 8499
Peroxiredoxin 2	Abcam	Cat# ab50862
Peroxiredoxin 3	Ab Frontier	Cat#LF-PA0030
Phospholamban	Badrilla	A010-14
Phospholamban pSer16	Badrilla	A010-12
Phospholamban pThr17	Badrilla	A010-13
SERCA	Thermo Fisher Scientific	MA3-919
Superoxide dismutase 1	Santa Cruz Biotechnology	Cat#sc-11407
Superoxide dismutase 2	Santa Cruz Biotechnology	Cat#sc-30080
Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 (UQCRC1)	Generated by Peter Rehling	N/A
Total OXPHOS rodent Ab cocktail	Abcam	Cat#ab110413
Voltage-dependent anion-selective channel 3 (VDAC3)	Generated by Peter Rehling	N/A
8-OHdG	Abcam	Cat#ab10802
2 ^{ary} Ab goat anti-mouse	Bio-Rad	Cat#170-6516
2 ^{ary} Ab rabbit anti-goat	Bio Rad	Cat#172-1034
Chemicals, Peptides, and Recombinant Proteins		
Acrylamide/bisacrylamide (30%)	Bio-Rad	Cat#1610158
Adenosine diphosphate (ADP)	Sigma-Aldrich	Cat#01905; CAS 20398-34-9
Amplex™ UltraRed	Thermo Fisher Scientific	Cat#A36006
Adenosine triphosphate (ATP)	Sigma-Aldrich	Cat#A2383; CAS 34369-07-8

B-27™ Supplement, minus insulin	Thermo Fisher Scientific	Cat#A1895601
BES	Sigma-Aldrich	Cat#B9879; CAS 10191-18-1
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A7030
2,3-butanedione monoxime (BDM)	Sigma-Aldrich	Cat#B0753; CAS 57-71-6
Caffeine	Sigma-Aldrich	Cat# C0750
Calcium Green™-5N	Invitrogen	Cat#C3737
Carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP)	Sigma-Aldrich	Cat#C2920; CAS 370-86-5
CGP-37157 (CGP)	Sigma-Aldrich	Cat#C8874; CAS 75450-34-9
CHIR99021	Sigma-Aldrich	Cat#361559
Collagenase type II	Worthington Biochemical	Cat#LS00417
cOmplete™ Protease Inhibitor Cocktail	Roche	Cat#11697498001
Cyanide	Sigma-Aldrich	Cat# 380970
Cyclosporine A	Sigma-Aldrich	Cat#59865-13-3; CAS 59865-13-3
DAPI stain	Calbiochem	Cat#508741; CAS 28718-90-3
2',7'-dichlorodihydrofluorescein diacetate (DCF)	Thermo Fisher Scientific,	Cat#D6883; CAS 4091-99-0
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D8418; CAS 67-68-5
DNAase	VWR PeqLab	Cat#VWRC13-1091
Doxycycline-enriched rodent chow	Ssnif Spezialdiäten GmbH	Cat#A153D70623
2,4-dinitrophenol (DNP)	Sigma-Aldrich	Cat#D198501; CAS D198501
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	CAS 60-00-4
Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)	Sigma-Aldrich	Cat#E8145; CAS 13368-13-3
Foetal calf serum (FCS)	Thermo Fisher Scientific	Cat# 26140079
Heparin	Braun AG	N/A
HighCap cDNA RT Kit	Applied Biosystems	Cat#4368814
Horseradish peroxidase (HRP)	Sigma-Aldrich	CAS 9003-99-0
Hydrogen peroxide (30%)	Merck	Cat#107209
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich	CAS 7365-45-9

1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH)	Enzo Life Sciences	Cat#ALX-430-117
HMR-1098	Sigma-Aldrich	Cat#SML2767; CAS 261717-22-0
Immobilon-P (pore size: 0.45 µm)	Merck Millipore	Cat#
Indo-1 acetoxymethyl ester (Indo-1-AM)	Thermo Fisher Scientific	Cat#I1223
Indo-1 pentapotassium salt	Fisher Scientific	Cat#I1202
Isoflurane	Abbott GmbH & Co. KG	N/A
Isoprenaline/isoproterenol (Iso)	Sigma-Aldrich	Cat#I6504; CAS 5984-95-2
Isopropanol	Merck	Cat#109634; CAS 67-63-0
IWP-2	Sigma-Aldrich	Cat#506072
Ketaset (Ketamine)	Zoetis	N/A
Lactobionic acid	Sigma-Aldrich	Cat#153516; CAS 96-82-2
Laminin	Roche Diagnostics	Cat#11 243 217 001
Liberase, Thermolysin high (TH)	Sigma-Aldrich	Cat#5401135001
MyoTak	IonOptix	N/A
N-Ethylmaleimide (NEM)	Sigma-Aldrich	CAS 128-53-0
Nitrocellulose membrane	Merck	Cat# IPVH00010
Oligomycin A	Sigma-Aldrich	Cat#75351; CAS 579-13-5
PeqGold TriFast™	VWR PeqLab	N/A
Phenol red	Sigma-Aldrich	CAS 143-74-8
Phosphocreatine (PCr)	Sigma-Aldrich	Cat#P7936; CAS 19333-65-4
Pinacidil	Sigma-Aldrich	Cat#P154; CAS 85371-64-8
cOmplete™ Protease Inhibitor Cocktail	Roche	Cat#11697498001
PhosSTOP™ (phosphatase inhibitor)	Sigma-Aldrich	Cat#4906845001
Proteinase, bacterial, type XXIV	Sigma-Aldrich	Cat#P8038; CAS 9014-01-1
Proteinase K	Thermo Fisher Scientific	Cat#EO0491
Rhod-2-acetoxymethylester (rhod-2-AM)	Thermo Fisher	Cat#R1244
RPMI 1640 medium	Thermo Fisher Scientific	Cat#11875101
Sodium dodecylsulfate (SDS)	Carl Roth	Cat#0183; CAS 151-21-3
Subtilisin A	Sigma-Aldrich	Cat#P5380
Superoxide dismutase (SOD)	Sigma-Aldrich	Cat#S9697
TaqMan GenEx Mastermix	Applied Biosystems	Cat#4369016

Taurine	Merck	Cat#808616; CAS 107-35-7
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	Cat#T9281
Tetramethylrhodamine methyl ester (TMRM)	Thermo Fisher Scientific	Cat#T668
Tissue-Tek® O.C.T. Compound	Sakura	Cat#4583
Tris Base	Carl Roth	Cat#5429; CAS 77-86-1
Tris-HCl	Carl Roth	Cat#9090; CAS 1185-53-1
Triton X-100	Thermo Fisher	Cat# 327371000
TWEEN® 20	Sigma-Aldrich	CAS 9005-64-5
VECTASHIELD® Antifade Mounting Medium	Vector Laboratories	N/A
Versene™ solution	Thermo Fisher Scientific	Cat#15040066
Xylazine	Bayer AG	N/A
Experimental Models: Cell Lines		
TAZ10	Generated in Peter Rehling's laboratory	Dudek et al. ⁸
Experimental Models: Organisms/Strains		
<i>Taz</i> -KD [B6.Cg-Gt(ROSA)26 ^{Sortm37(H1/tetO-RNAi:Taz)Arte/ZkhuJ}]	The Jackson Laboratory	Acehan et al. ¹⁴
Mito-roGFP2-Orp1 <i>Taz</i> -KD	This study	Fujikawa et al. ⁵³
Oligonucleotides		
<i>Anp</i>	Applied Biosystems	Mm01255747_g1
<i>Ctgf</i>	Applied Biosystems	Mm01192932_g1
<i>Gapdh</i>	Applied Biosystems	Mm99999915_g1
<i>Mcua</i>	Applied Biosystems	Mm01168773_m1
<i>Taz</i>	Applied Biosystems	Mm00504978_m1
Software and Algorithms		
Prism 6.0	GraphPah	http://www.graphpad.com
Image J	NIH	https://imagej.nih.gov/ij/
IonWizard	IonOptix	N/A
Segment	MedViso	¹⁴

Table III. Body and organ weight.

	10 weeks of age		20 weeks of age		50 weeks of age	
	WT (n=5)	<i>Taz</i> -KD (n=6)	WT (n=5)	<i>Taz</i> -KD (n=7)	WT (n=24)	<i>Taz</i> -KD (n=19)
BW (g)	22.84±1.58	21.44±1.93	24.63±2.03	25.49±1.22	30.61±1.09	25.25±0.99 ^{†‡}
HW (mg)	129.5±5.5	119.5±6.4	175.6±23.2	174.1±10.7	168.1±6.3	147.9±6.4 [†]
HW/BW (mg/g)	5.71±0.17	5.67±0.25	7.04±0.45	6.85±0.31	5.51±0.11	5.94±0.25
Liver weight (mg)	1039±103	1058±130	1032±88	1267±69	1342±52	1214±59
Lung weight (mg)	141.7±5.67	135.2±6.9	155.3±9.3	151.9±10.0	161.1±3.1	150.5±3.8

Abbreviations. BW: body weight; HW: heart weight.

Table IV. Parameters of cardiac function.

	10 weeks of age		20 weeks of age		50 weeks of age	
	WT (n=5)	Taz-KD (n=4)	WT (n=5)	Taz-KD (n=4)	WT (n=16)	Taz-KD (n=12)
CO (mL/min)	16.26±1.14	12.57±1.37	16.45±1.49	11.56±1.50*	16.31±0.80	10.15±1.10 ^{†‡}
CO/BW (mL/min/g)	NA	NA	0.68±0.06	0.44±0.05*	0.53±0.02	0.40±0.03 ^{†‡}
HR (bpm)	433.20±28.66	423.25±13.52	436.60±27.60	410.75±45.66	433.4±12.76	444.5±13.18
LVEF (%)	73.95±3.99	63.83±6.80	69.68±1.78	52.33±3.26*	75.42±1.38	54.61±4.22 ^{†‡}
LVEDV (μL)	51.40±3.20	46.75±3.12	54.20±3.63	54.00±3.08	49.94±2.04	42.5±2.77 [†]
LVESV (μL)	13.80±2.97	17.25±3.86	16.60±1.81	26.00±3.14	12.31±0.89	19.58±2.57 [†]
SV (μL)	37.60±1.50	29.50±2.50	37.60±2.06	28.00±1.00*	37.63±1.64	22.67±1.97
LVEDD (mm)	3.68±0.16	3.90±0.16	3.72±0.18	3.83±0.75	3.65±0.04	3.62±0.11
LVESD (mm)	2.26±0.10	2.67±0.37	2.28±0.06	2.68±0.14	2.05±0.06	2.56±0.06 ^{†‡}
LVPW (mm)	0.68±0.02	0.77±0.12	0.70±0.05	0.73±0.05	0.78±0.03	0.72±0.04
IVS (mm)	1.00±0.03	1.07±0.07	0.96±0.05	1.08±0.09	0.98±0.04	1.06±0.05

Abbreviations. BW: body weight; HR: heart rate; IVS: interventricular septum; LVEDD: left ventricular end-diastolic diameter; LVESD: left ventricular end-systolic diameter; LVEDV: left ventricular end-diastolic volume; LVESV: left ventricular end-systolic volume; LVEF: left ventricular ejection fraction; LVPW: left ventricular posterior wall; NA: not available; SV: stroke volume; Taz-KD: tafazzin-knockdown; WT: wild-type.

Table V. Assessment of force- Ca^{2+} relationship in membrane-permeabilized and mechanically preloaded cardiac myocytes.

	WT (n=18/4)	Taz-KD (n=15/4)
Area (μm^2)	332 $\pm$ 50.5	315 $\pm$ 49.6
F_{max} (kN/m²)	17.2 $\pm$ 1.6	15.2 $\pm$ 1.7
F_{pass} (kN/m²)	2.3 $\pm$ 0.2	2.0 $\pm$ 0.4
nH	2.11 $\pm$ 0.15	1.91 $\pm$ 0.15

Abbreviations. F_{max}: maximal force development; F_{pass}: passive force development; nH: Hill's coefficient for Ca^{2+} cooperative activation. Errors are indicated as standard error of the mean (SEM); n-numbers are indicated as numbers of hearts or animals for **Tables III, IV** and as numbers of cardiac myocytes / animals for **Table V**.

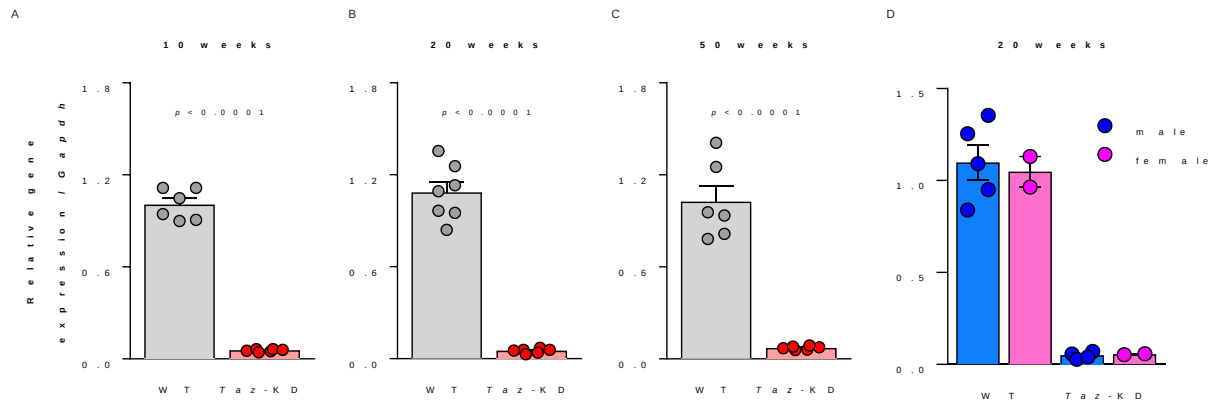
indicates $p < 0.05$ with 2-way ANOVA;

*, **, ***, ****, *****: $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ with Bonferroni post-test;

†, ‡, †‡, ‡‡ = $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ with unpaired Student's t-test.

Supplemental Figures

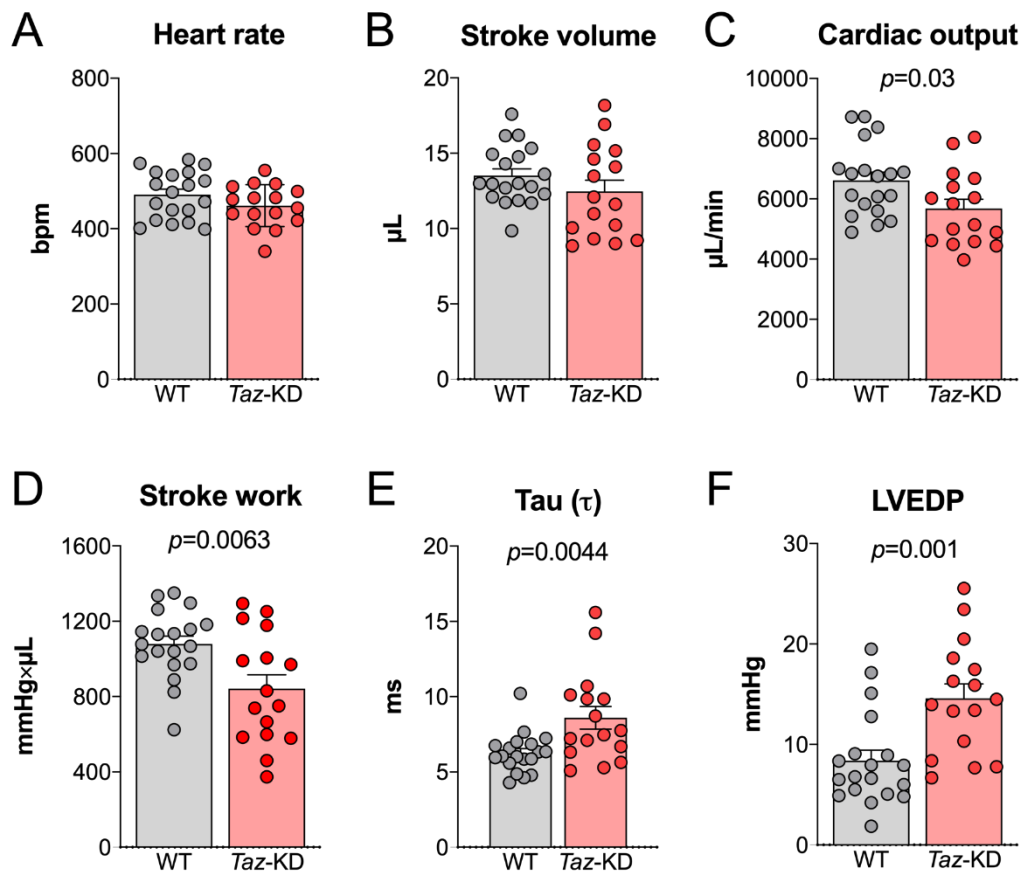
Figure I. *Taz* mRNA levels are suppressed in *Taz*-KD hearts.



Tafazzin mRNA levels in 10 (**A**), 20 (**B, D**), and 50 w/o mice (**C**; n=6 per group, except for 20 w/o WT, n=7). D, Tafazzin mRNA levels in 20 weeks old male and female WT and *Taz*-KD mice. There was no impact of gender on the downregulation of *Taz* in 2-way ANOVA analysis.

Data represent mean $\pm$ SEM (indicated by error bars); n-numbers are indicated as numbers of hearts or animals; statistical significance was determined by unpaired Student's t-test (**A-C**) or 2-way ANOVA (**D**).

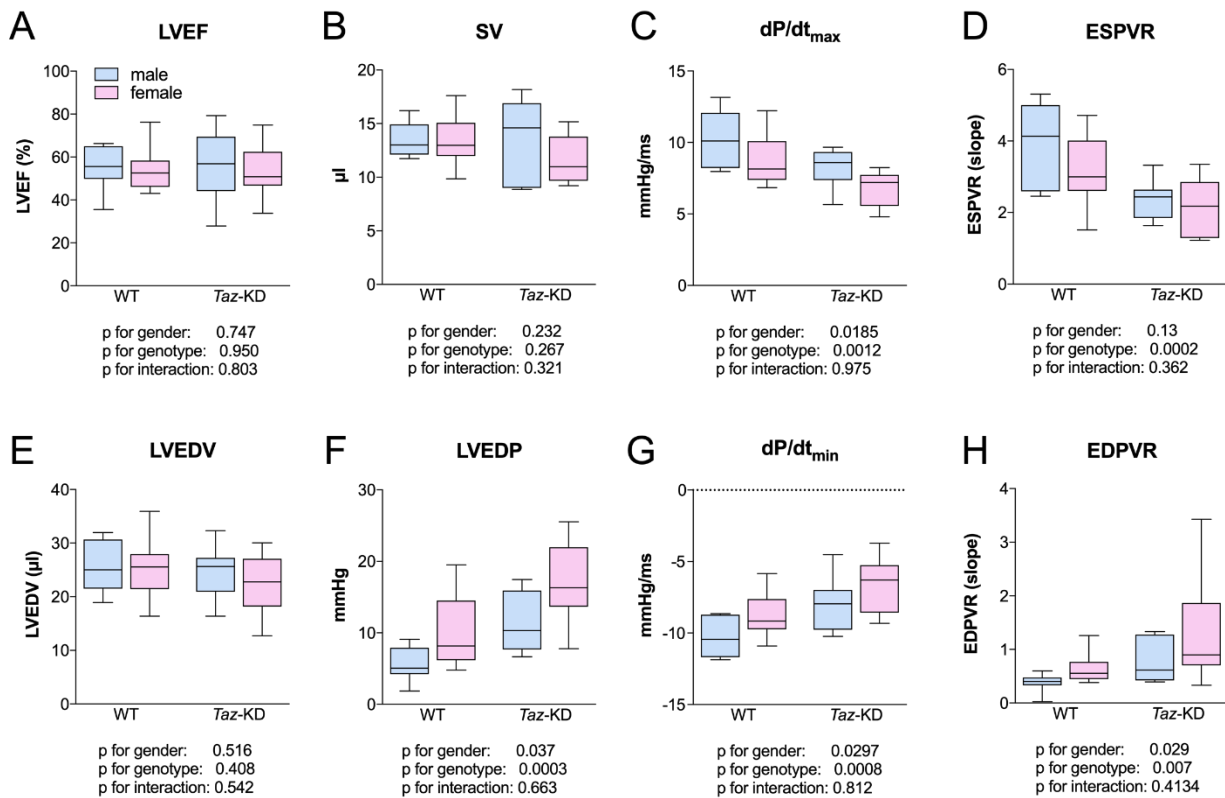
Figure II. Invasive assessment of systolic and diastolic function in *Taz*-KD mice.



Invasive hemodynamic assessment of heart rate (HR; **A**), left ventricular (LV) stroke volume (**B**), cardiac output (**C**), LV stroke work (**D**), the relaxation time constant tau (τ ; **E**) and LV end-diastolic pressure (LVEDP; **F**) in 10 w/o WT and *Taz*-KD mice (WT $n=19$; *Taz*-KD $n=16$).

Data represent mean $\pm$ SEM (indicated by error bars); n-numbers are indicated as numbers of hearts or animals; statistical significance was determined by unpaired Student's t-test.

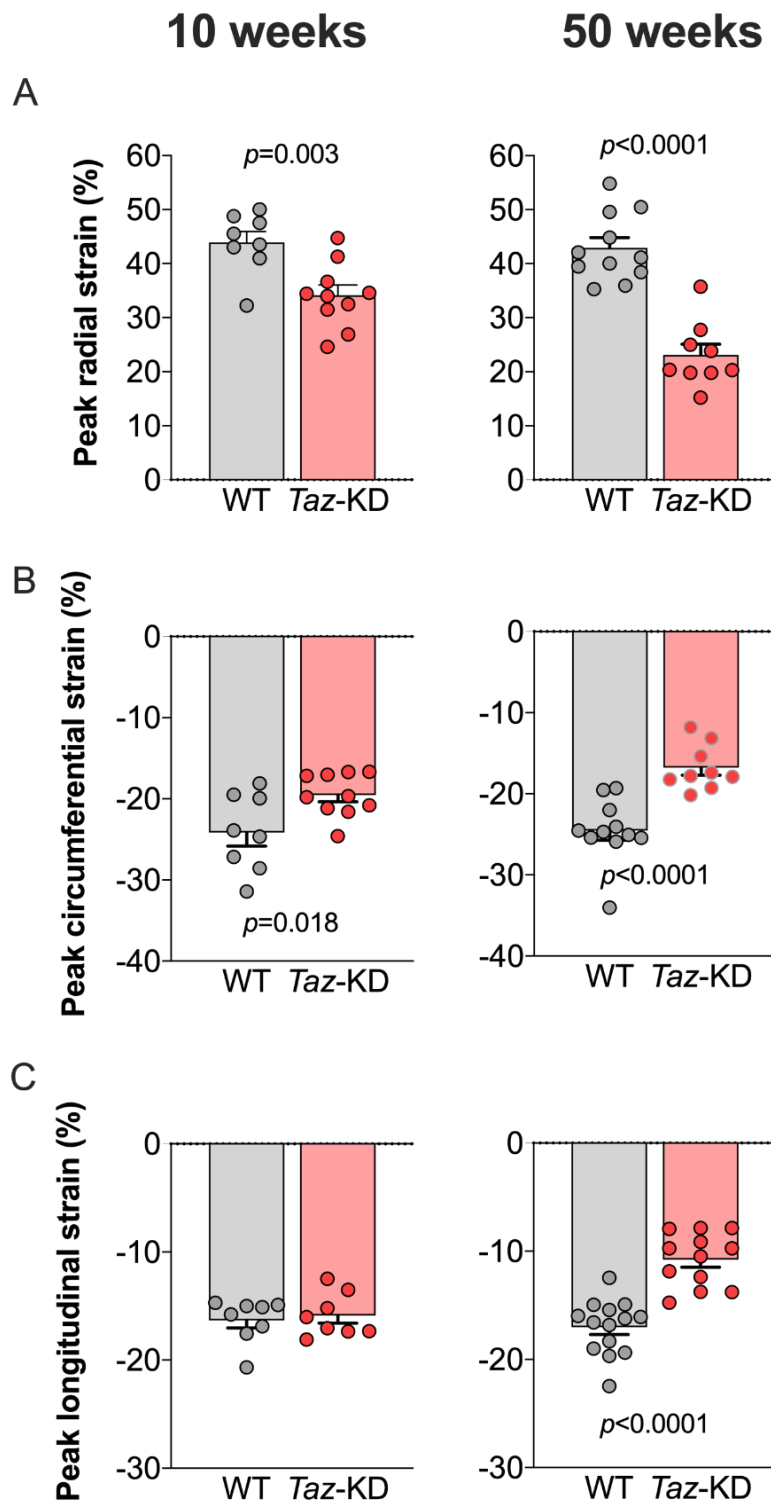
Figure III. Absence of gender-specific differences in *Taz*-KD cardiomyopathy.



Invasive hemodynamic assessment of left ventricular (LV) ejection fraction (LVEF; **A**), stroke volume (SV; **B**), maximal rate of LV pressure rise (dP/dt_{max}; **C**), end-systolic pressure volume relationship (ESPVR; **D**), LV end-diastolic volume (LVEDV; **E**) and end-diastolic pressure (LVEDP; **F**), maximal rate of decay of LV pressure (dP/dt_{min}; **G**) and end-diastolic pressure-volume relationship (EDPVR; **H**) in WT and *Taz*-KD male and female mice, respectively (WT: male n=7, female n=12; *Taz*-KD: male n=7, female n=9).

Data represent mean ± SEM (indicated by error bars); n-numbers are indicated as numbers of hearts or animals; statistical significance was determined by 2-way ANOVA analysis to test the dependence of the respective parameters on gender, genotype, and the interaction between both factors, respectively.

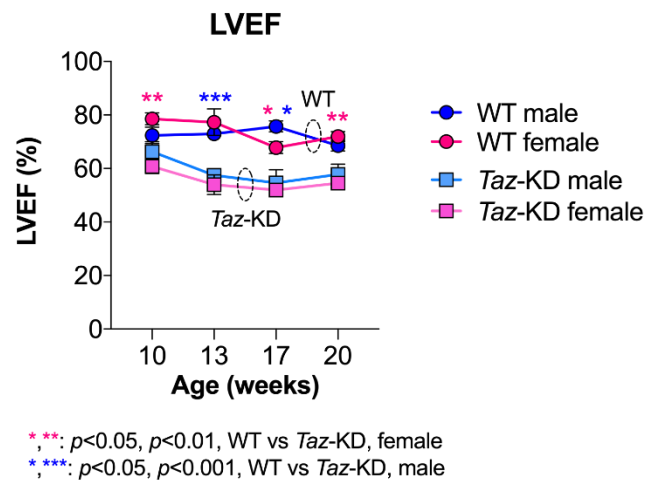
Figure IV. Left ventricular strain is impaired in *Taz*-KD mice at 10 and 50 weeks of age.



Left ventricular peak radial (A), circumferential (B), and longitudinal (C) strain in 10 (left) and 50 w/o (right) mice (10 weeks, WT $n=8$, *Taz*-KD $n=10$; 50 weeks: WT $n=11$, *Taz*-KD $n=9$).

Data represent mean $\pm$ SEM (indicated by error bars); n -numbers are indicated as numbers of hearts or animals; statistical significance was determined by unpaired Student's t -test.

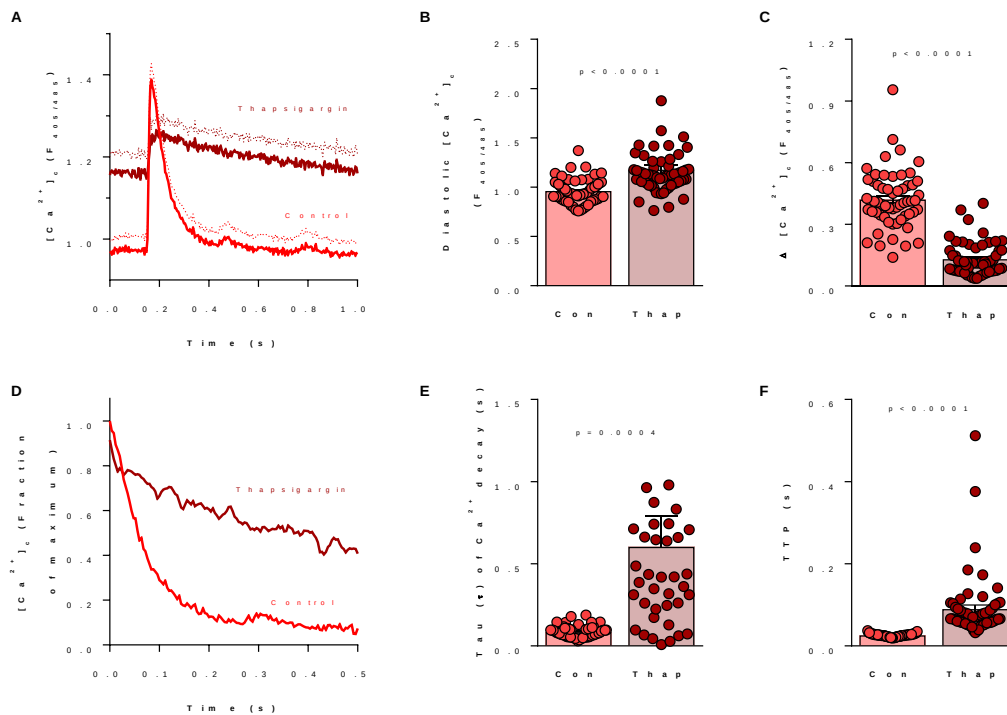
Figure V. Left ventricular ejection fraction in male vs female *Taz*-KD and WT mice.



Assessment of left ventricular ejection fraction by magnetic resonance imaging in male WT (n=7), female WT (n=3), male *Taz*-KD (n=6) and female *Taz*-KD (n=6) at 10, 13, 17 and 20 weeks, respectively.

Data represent mean $\pm$ SEM (indicated by error bars); n-numbers are indicated as numbers of hearts or animals; statistical significance was determined by 2-way ANOVA analysis with Tukey's multi comparison test.

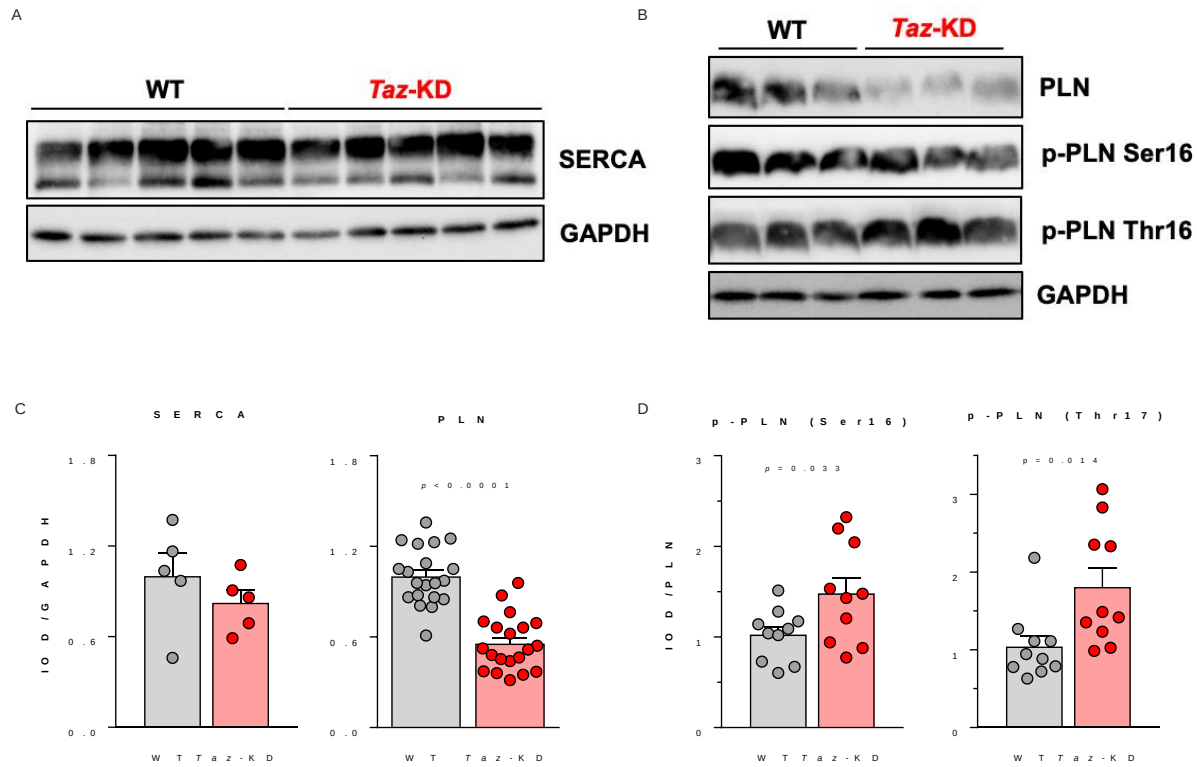
Figure VI. Effects of SERCA inhibition on cytosolic Ca^{2+} transients in cardiac myocytes.



Cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_c$) transients in *Taz*-KD myocytes field-stimulated at 1 Hz before (Control; Con) and after application of the SERCA inhibitor thapsigargin (Thap; 1 μM). **A**, averaged original traces of $[\text{Ca}^{2+}]_c$ transients. **B**, diastolic $[\text{Ca}^{2+}]_c$; **C**, amplitude of $[\text{Ca}^{2+}]_c$ transients; **D**, decay of $[\text{Ca}^{2+}]_c$ normalized to the maximum; **E**, time constant of $[\text{Ca}^{2+}]_c$ decay; **F**, time-to-peak (TTP) of $[\text{Ca}^{2+}]_c$.

Data represent mean $\pm$ SEM (indicated by error bars); statistical significance was determined by paired Student's t-test in n=61, n=58 and n=61 cells for panels **B**, **C** and **F**, respectively; and by unpaired t-test in n=67 (Control) and n=37 (Thapsigargin) cells in panel **E**.

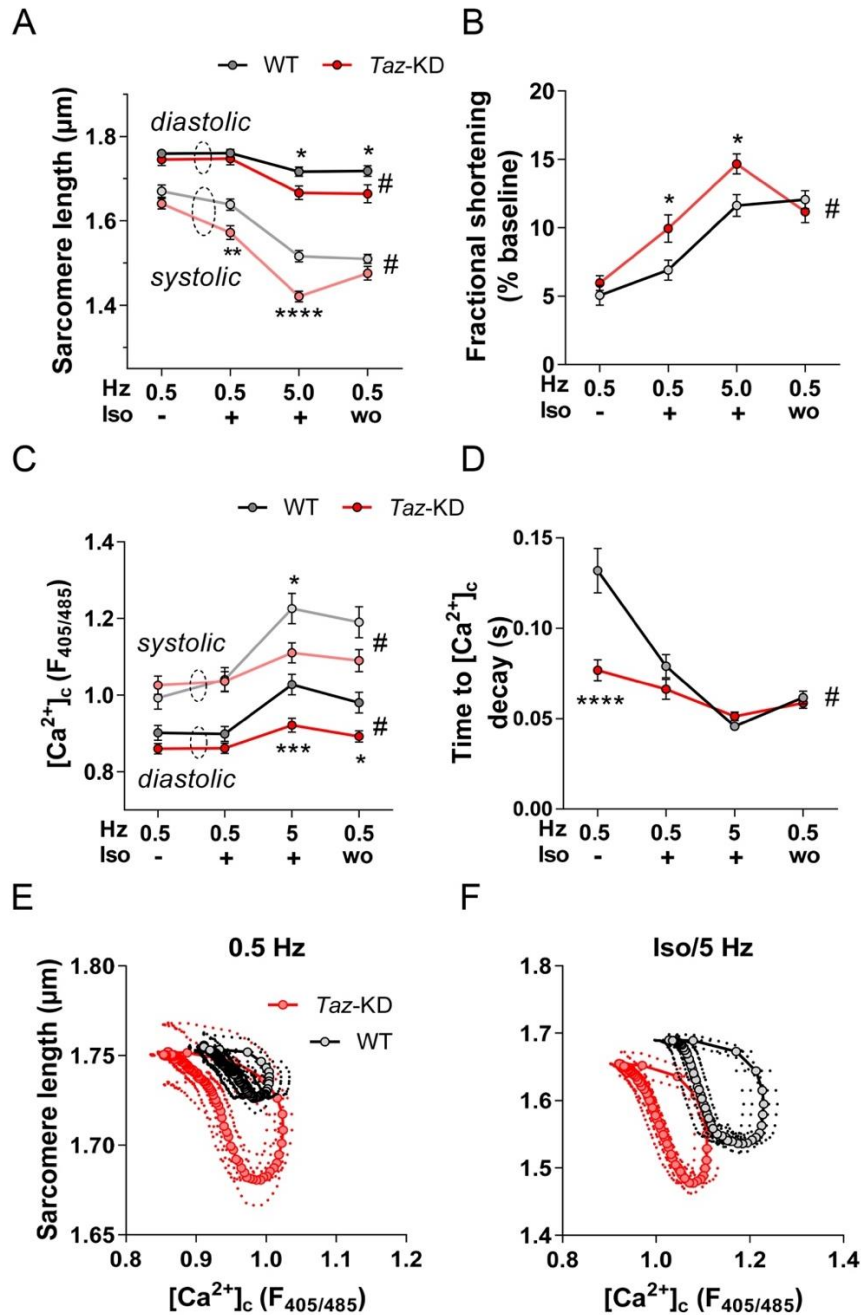
Figure VII. SERCA and phospholamban protein expression and phosphorylation in *Taz*-KD and WT hearts.



Western-blot analysis of SERCA, phospholamban (PLN), and phosphorylation of PLN at Serine 16 (Ser16) and Threonine 17 (Thr17), the PKA- and CaMKII phosphorylation sites, respectively. **A** and **B**, original blots of SERCA; **C**, quantification of total protein of SERCA (n=5 per group) and PLB (n=20 experiments from n=10 hearts per group); **D**, quantification of phosphorylation of PLB at Ser16 and Thr17 related to total protein of PLB (n=10 per group), respectively.

Data represent mean $\pm$ SEM (indicated by error bars); n-numbers are indicated as numbers of hearts or animals; statistical significance was determined by unpaired Student's t-test.

Figure VIII. Enhanced myofilament Ca^{2+} affinity and faster $[\text{Ca}^{2+}]_c$ decay in cardiac myocytes from 50-week-old *Taz*-KD mice.



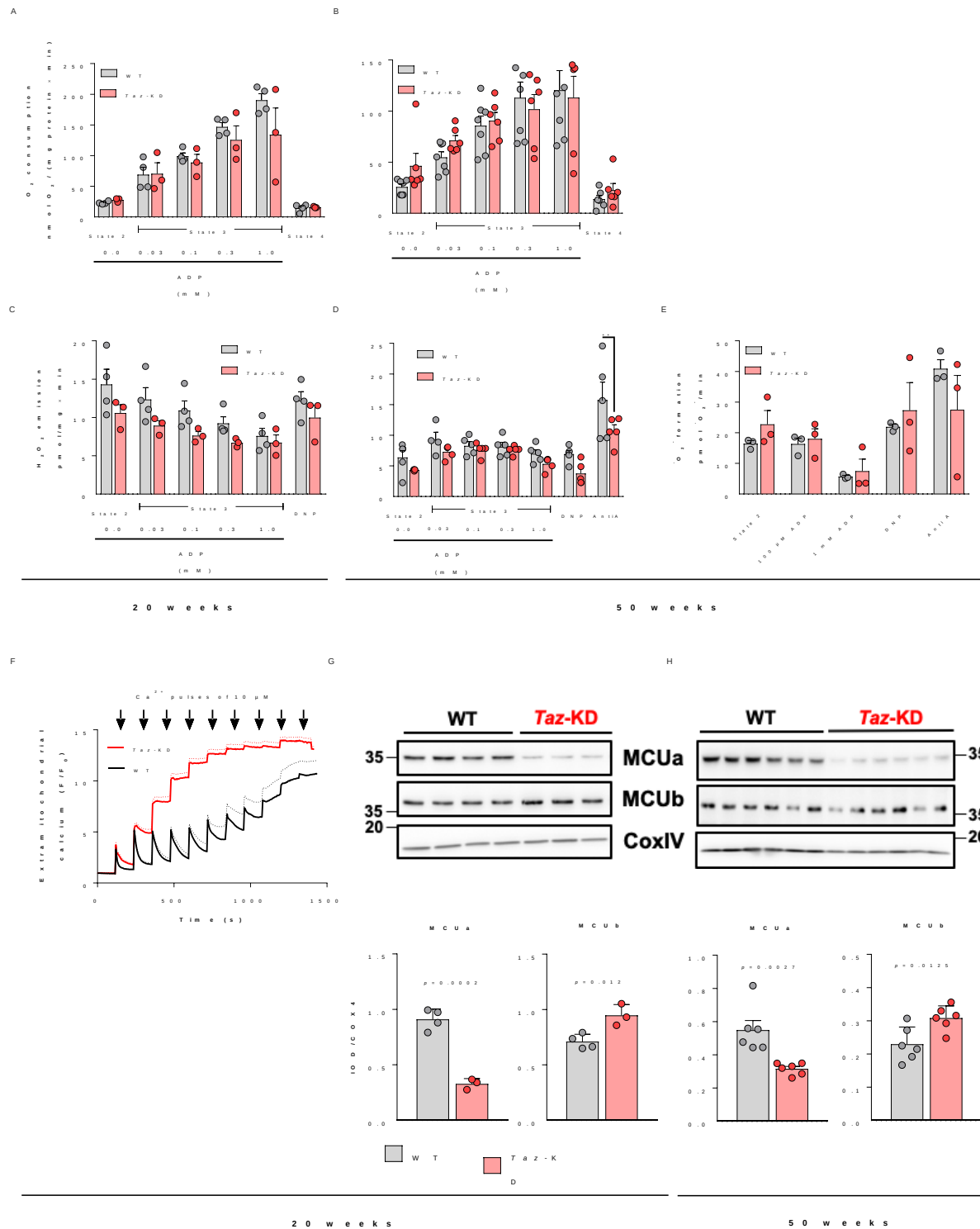
Sarcomere length (A) and fractional shortening (B) of intact cardiac myocytes isolated from 50 w/o animals (WT $n=30/4$, *Taz*-KD $n=26/4$). Cytosolic Ca^{2+} (C, $[\text{Ca}^{2+}]_c$) and time to $[\text{Ca}^{2+}]_c$ decay of intact cardiac myocytes (D) isolated from 50 w/o animals and loaded with Indo-1 (WT $n=21/3$, *Taz*-KD $n=19/3$). $[\text{Ca}^{2+}]_c$ plotted against sarcomere length of cardiac myocytes at 0.5 Hz (E) and Iso/5 Hz stimulation (F). Data extracted from experiments in C and D (WT $n=21/3$, *Taz*-KD $n=19/3$).

Error bars and dotted lines [in (E) and (F)] indicate standard error of the mean (SEM); n-numbers are indicated as numbers of cardiac myocytes / animals.

indicates $p < 0.05$ with 2-way ANOVA;

*, **, ***, ****, *****: $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ with Bonferroni post-test.

Figure IX. Unchanged respiration and ROS emission, but decreased MCU expression and Ca^{2+} uptake in cardiac mitochondria from 20- and 50-week-old *Taz*-KD mice.



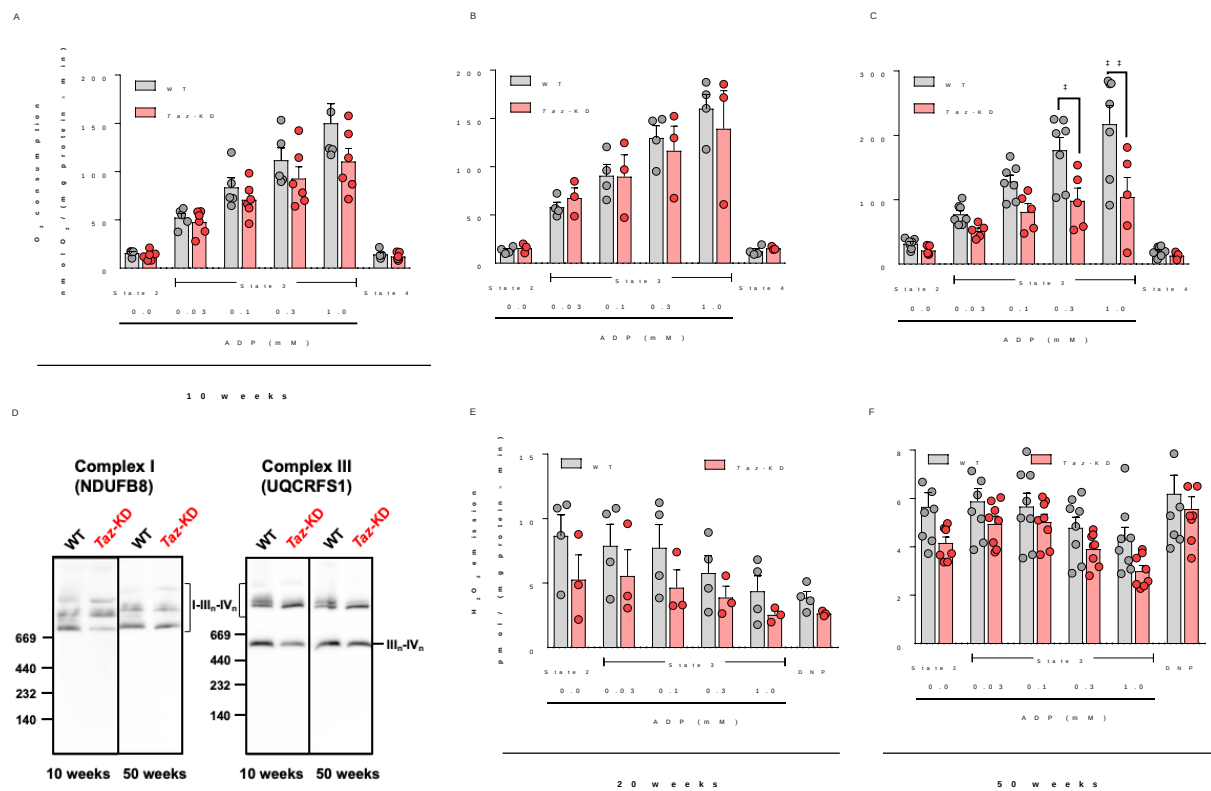
O_2 consumption of cardiac mitochondria from 20 (A) and 50 w/o mice (B; 20 weeks: WT n=4, *Taz*-KD n=3; 50 weeks: WT n=7, *Taz*-KD n=6). H_2O_2 emission from cardiac mitochondria from 20 (C) and 50 w/o mice (D; 20 weeks: WT n=4, *Taz*-KD n=3; 50 weeks: n=5 per group). Superoxide ($\cdot\text{O}_2^-$) formation in cardiac mitochondria from 50 w/o mice (E; n=3 per group). Ca^{2+} retention capacity of cardiac mitochondria from 20 w/o mice (F; WT n=4, *Taz*-KD n=3). Protein levels of MCUa and MCUB

in the heart of 20 (**G**) and 50 w/o mice (**H**; 20 weeks: WT n=4, *Taz*-KD n=3; 50 weeks: n=6 per group).

Error bars and dotted lines [in (**F**)] indicate standard error of the mean (SEM);
n-numbers are indicated as numbers of hearts or animals;

** $p < 0.01$ with Bonferroni post-test.

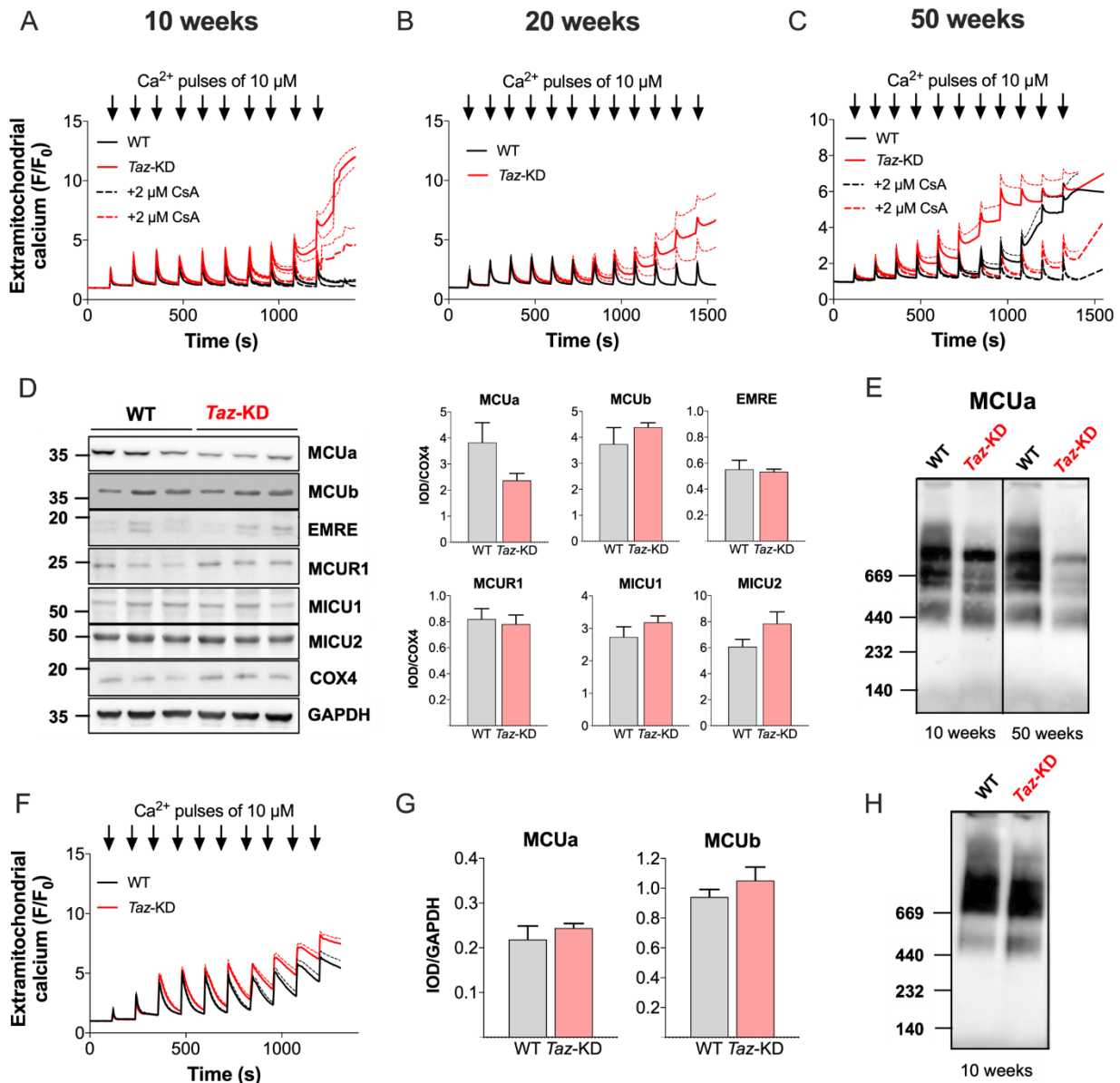
Figure X. Unchanged respiration and H₂O₂ emission in skeletal muscle mitochondria from *Taz*-KD mice.



A, O₂ consumption of skeletal muscle mitochondria from 10 w/o mice (WT n=5, *Taz*-KD n=6). **B**, BN-PAGE analysis of the NADH dehydrogenase 1 β subcomplex subunit 8 (NDUFB8) of complex I and the complex III component Rieske in skeletal muscle mitochondria from 10 and 50 w/o mice. O₂ consumption (**C**) and H₂O₂ emission (**D**) of skeletal muscle mitochondria from 20 w/o mice (WT n=4, *Taz*-KD n=3). O₂ consumption (**E**) and H₂O₂ emission (**F**) of skeletal muscle mitochondria from 50 w/o mice (O₂ consumption: WT n=7, *Taz*-KD n=5; H₂O₂ emission: n=8 per group).

Error bars indicate standard error of the mean (SEM);
n-numbers are indicated as numbers of hearts or animals;
†, ‡ = $p < 0.01$, $p < 0.0001$ with unpaired Student's t-test.

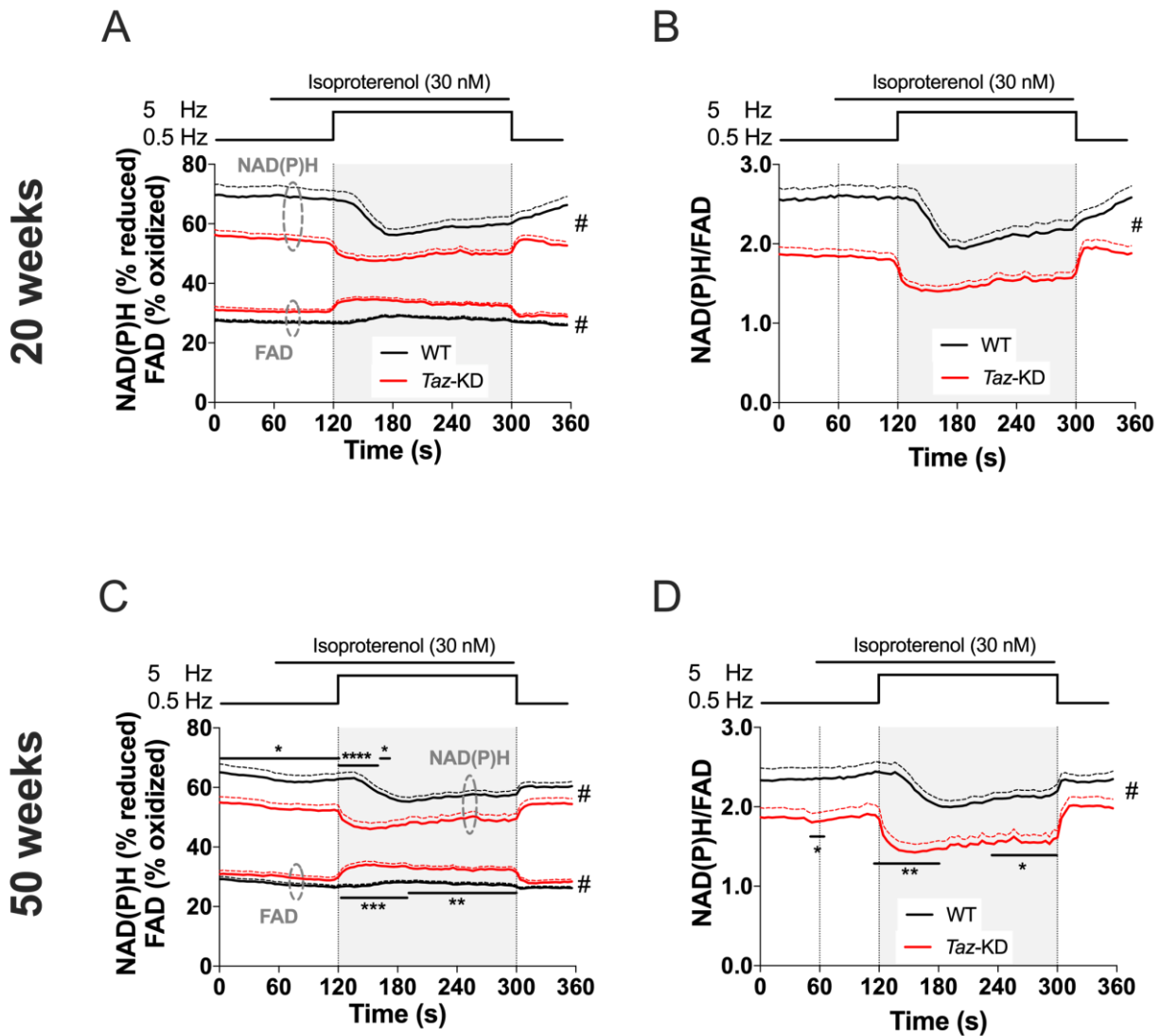
Figure XI. Preserved mitochondrial Ca^{2+} uptake in skeletal muscle and brain mitochondria of *Taz*-KD mice.



Ca^{2+} retention capacity of skeletal muscle mitochondria from 10 (A), 20 (B), and 50 w/o mice (C; 10 weeks: WT n=6, *Taz*-KD n=4; 20 weeks: WT n=4, *Taz*-KD n=3; 50 weeks: n=8 per group). D, protein levels of the MCU complex subunits MCUa, MCUb, EMRE, MCUR1, MICU1, and MICU2 in skeletal muscle of 10 w/o mice (n=6 per group). E, BN-PAGE analysis of MCUa in skeletal muscle of 10 and 50 w/o mice. F, Ca^{2+} retention capacity of brain mitochondria from 10 w/o mice (n=4 per group). G, protein levels of MCUa and MCUb in brain of 10 w/o mice (n=6 per group). H, BN-PAGE analysis of MCUa in brain from 10 w/o mice.

Error bars and dotted lines [in (A) to (C) and (F)] indicate standard error of the mean (SEM); n-numbers are indicated as numbers of hearts or animals.

Figure XII. Oxidation of mitochondrial NAD(P)H/FAD redox state in cardiac myocytes from 20- and 50-week-old *Taz*-KD mice.



Autofluorescence of NAD(P)H and FAD (**A**) and NAD(P)H/FAD ratio (**B**) in intact cardiac myocytes from 20 w/o mice (WT n=12/3, *Taz*-KD n=24/3). Autofluorescence of NAD(P)H and FAD (**C**) and NAD(P)H/FAD ratio (**D**) in intact cardiac myocytes from 50 w/o mice (WT n=30/4, *Taz*-KD n=26/4).

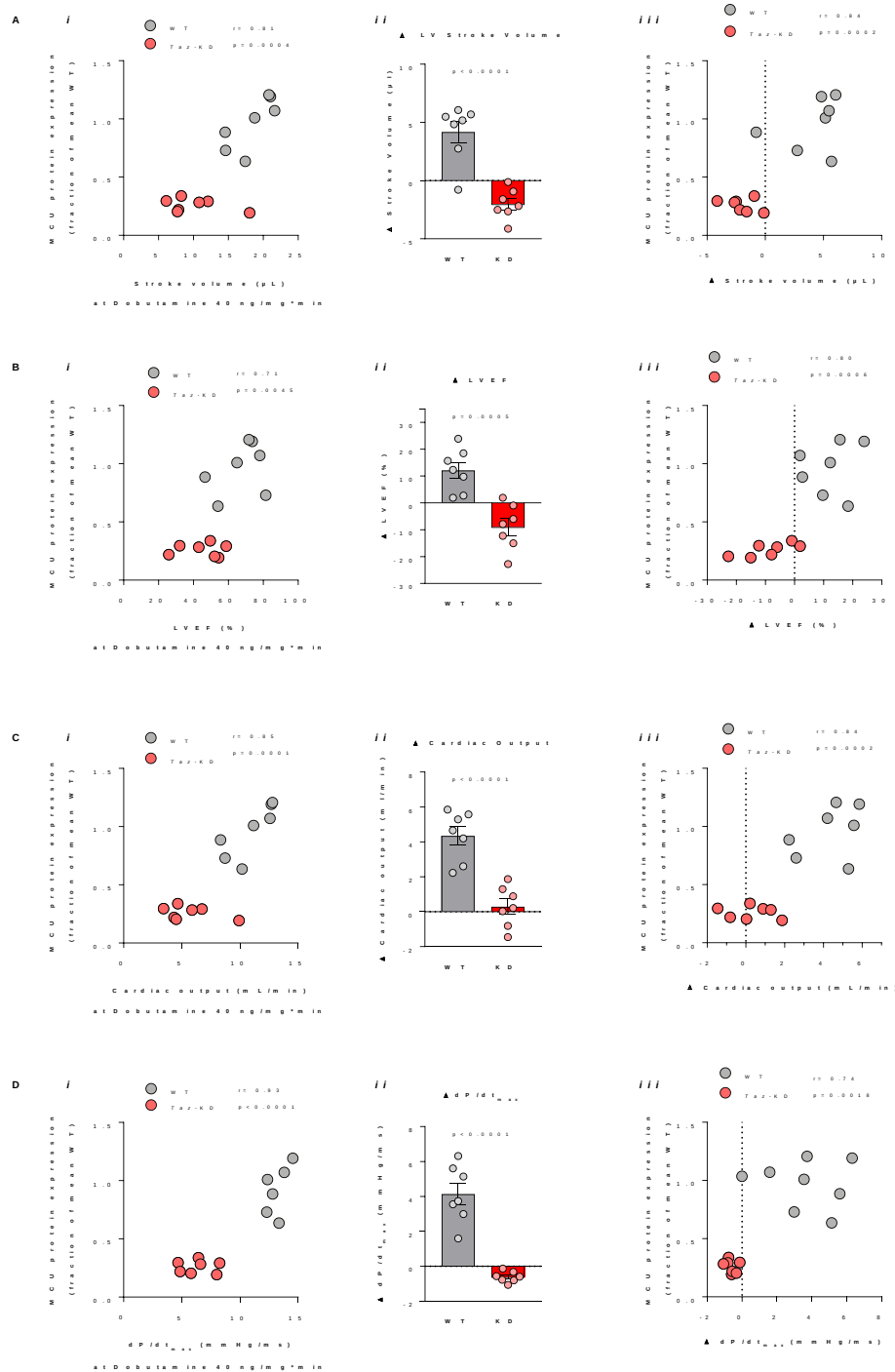
Dotted lines indicate standard error of the mean (SEM);

n-numbers are indicated as numbers of cardiac myocytes / animals;

indicates $p < 0.05$ with 2-way ANOVA;

*, **, ***, ****, *****: $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$ with Bonferroni post-test.

Figure XIII. Cardiac inotropic reserve correlates with MCUa protein levels in *Taz*-KD and WT hearts.



Correlation of left ventricular (LV) stroke volume (**A**), LVEF (**B**), cardiac output (**C**), and $dP/dt_{\max}$ (**D**) at the maximum dose of dobutamine (40 ng/g*min) with MCUa protein expression from the same hearts in WT and *Taz*-KD animals after pretreatment with CGP-37157, respectively (n=7 per group; **all i**). In **ii**, changes (Δ) in the same hemodynamic parameters from baseline to maximum dobutamine are shown and in **iii**, the correlations of these changes with MCUa protein expression, respectively. Error bars indicate standard error of the mean (SEM); n-numbers are indicated as numbers of animals. Comparison between groups in **ii** performed with unpaired t-test.