SUPPLEMENTAL MATERIALS

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Supplemental Methods

Generation of mouse model and administration of raloxifene

We made a cardiac-specific RRM deletion mouse model. *The cRbm20$^{\Delta RRM}$* mice had exons 6 and 7 of *Rbm20* flanked with LoxP sites and the mice expressed MerCreMer recombinase protein under transcriptional control of cardiac specific α-myosin heavy chain (MHC6) promoter[1]. Administration of raloxifene, a selective estrogen receptor modulator, led to Cre-mediated excision of exons 6 and 7 of the *Rbm20* gene [2] in cardiomyocytes in a time specific manner. Male, *MCM;cRbm20$^{\Delta RRM}$* mice that were heterozygous for the mutant *Rbm20* allele and for the MerCreMer transgene, on C57BL/6J background, were utilized in this work. Raloxifene hydrochloride (RR09, TSZ Chem) was dissolved in warmed dimethyl sulfoxide (50mg/mL) and was administered via intraperitoneal (i.p.) injection at 40/mg/kg/day once daily for 8 consecutive days. The control animals received i.p. injection of dimethyl sulfoxide (DMSO) at an equivalent volume. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona and the NIH "Using Animals in Intramural Research" guidelines.

TAC/DOCA surgery

Minimally invasive transverse aortic constriction (TAC) was performed on ~ 8 week old male mice as described previously with modification[3]. Briefly, mice were anesthetized using a single injection of ketamine and xylazine (120mg/kg and 12mg/kg, I.P.) and a 5mm horizontal incision was made at the first left intercostal space. The thymus was temporarily retracted to visualize the aortic arch and a 7-0 silk suture was passed under the aorta between the right innominate and left carotid arteries. The suture was ligated around a blunted 27 gauge needle and the needle was quickly removed. The chest wall and skin were closed. Sham animals underwent the same procedure except the suture was not ligated around the aorta. An additional incision was made in the right

flank of the animal and a subcutaneous pocket was created by blunt dissection. A deoxycorticosterone acetate (50mg/pellet, 21 day release) or placebo pellet (Innovative Research of America) was implanted. The skin was closed and the mice were allowed to recover in a ThermoCare warmer.

Mouse Echocardiography

Mice were anesthetized under 1% isoflurane (USP, Phoenix) in oxygen mixture, then placed in dorsal recumbence on a heated platform for echocardiography. Transthoracic echo images was obtained with a Vevo 2100 High Resolution Imaging System (Visual-Sonics, Toronto, Canada) using the model MS550D scan head designed for murine cardiac imaging, and MS250 scan head was used to measure aortic flow velocity at the site of constricted aorta. Care was taken to avoid animal contact and excessive pressure which could induce bradycardia. Body temperature was maintained at 37°C. Imaging was performed at a depth setting of 1 cm. Images were collected and stored as a digital cine loop for off-line calculations. Standard imaging planes, M-mode, Doppler, and functional calculations were obtained according to American Society of Echocardiography guidelines. The parasternal long axis view and mid wall cross sectional view of the left ventricle (LV) were used to guide calculations of percentage fractional shortening, percentage ejection fraction, and ventricular dimensions and volumes. In addition, the left atrial dimension was measured in the long-axis view directly below the aortic valve leaflets. Passive LV filling peak velocity, E (cm/sec), and atrial contraction flow peak velocity, A (cm/sec), were acquired from the images of mitral valve Doppler flow from tilted parasternal long axis views. A sweep speed of 100 mm/sec was used for M-mode and Doppler studies. Considering that heart rate positively correlates with systolic performance, the heart rates of animals during echocardiographic study were maintained in the range of 500 -550 beats/minute for M-mode, 450-500 beats/minute for B-mode and 350 to 450 beats/minute for Doppler studies.

Conscious echo. A separate group of mice were consciously echoed while scruffing the skin at the nape of the neck and a standard short axis (M-mode) cine loop was recorded at the level of the papillary muscles to assess chamber dimensions (LV systolic and diastolic dimensions (LVDs, LVDd)) wall thickness (WT), and cardiac function via fractional shortening (%FS).

In-vivo pressure-volume measurements

An in-vivo pressure-volume analysis was performed in mice using a SciSense Advantage Admittance Derived Volume Measurement System and 1.2F catheters with 4.5 mm electrode spacing (SciSense, London, Ontario, Canada). Mice were anesthetized and ventilated with 1% isoflurane using an SAR-1000 Ventilator (CWE Inc) and body temperature maintained at 37^{o}C using a TC-1000 Temperature Controller (CWE Inc). Anesthetized mice were secured and a bilateral subcostal incision was made. The diaphragm was opened to expose the heart. The catheter was inserted into the LV via apical approach. The IVC was located and occluded during a sigh (pause) in ventilation to acquire load-independent indexes. Data acquisition and analysis was performed in LabScribe2 (iWorx, Dover NH). EDPVR was analyzed using a monoexponential fit ($P = C + Ae^{\beta V}$) with the exponent (β) reported as the stiffness[4].

Cell Isolation

Cells were isolated as described previously[5]. Briefly, mice were heparinized (1,000 U/kg, i.p.) and euthanized by cervical dislocation under isoflurane. The heart was removed and cannulated via the aorta with a blunted 21-gauge needle for retrograde coronary perfusion. The heart was perfused for 4 min with perfusion buffer ([in mmol/L] 90 NaCl, 34.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$, 12 $NaHCO_3$, 10 $KHCO_3$, 10 HEPES, 10 taurine, 5.5 glucose, 5 BDM, 20 Creatine, 5 Adenosine and 5 Inosine, pH 7.4), followed by digestion buffer (perfusion buffer plus 0.05 mg/ml Liberase TM research grade; Roche Applied Science, and 13 μM $CaCl_2$) for 20 min. When the heart was flaccid, digestion was halted and the heart was placed in myocyte stopping buffer

(perfusion buffer plus bovine calf serum 0.08 [BCS]/ml and 8 µM $CaCl_2$) with protease inhibitors ([in mmol/L] 0.4 Leupeptin, 0.1 E64, and 0.5 PMSF (Peptides International,Sigma-Aldrich)). The left ventricle was cut into small pieces, and the rest of the heart was discarded. The small pieces of left ventricle were triturated several times with a transfer pipette and then filtered through a 300µm nylon mesh filter.

Passive stress measurement in skinned cardiomyocytes

Mouse cells, isolated as explained above, were skinned for 7 mins in relaxing solution ([in mmol/L] 40 BES, 10 EGTA, 6.56 $MgCl_2$, 5.88 Na-ATP, 1.0 DTT, 46.35 K-propionate, 15 creatine phosphate, pH 7.0) with protease inhibitors ([in mmol/L] 0.4 leupeptin, 0.1 E64 , and 0.5 PMSF) and 0.3% Triton X-100 (Ultrapure; Thermo Fisher Scientific). Cells were washed extensively with relaxing solution pCa 9 and stored on ice. Skinned myocytes were used for mechanic studies within 48 hours after time of cell isolation. Myocyte suspension was added to a room temperature flow-through chamber mounted on the stage of an inverted microscope (Diaphot 200; Nikon). A skinned myocyte was glued at one end to a force transducer (Model 406A or 403A, Aurora Scientific). The other end was bent with a pulled glass pipette attached to micromanipulator so that the myocyte axis aligned with the microscope optical axis and cross sectional area (CSA) was measured directly. The cross sectional images of skinned cells were analyzed by ImageJ 1.41 software (National Institutes of Health) and were used to convert measured force to stress. Then, the free end of the cell was glued to a servomotor (Model 308B, Aurora Scientific) that imposes controlled stretches. Sarcomere length (SL) was measured with a MyocamS and SarcLen acquisition module (IonWizard 6.2, IonOptix Co, MA) attached to a computer. To correct for ~20% lattice expansion during skinning process[6], CSA of skinned cells were divided by a correction factor of 1.44. Passive stress was measured in relaxing solution pCa 9 with protease inhibitors at room temperature. Cells were stretched from slack length at a speed of 1 base length/sec to SLs of 2.1, 2.2 and 2.3 µm, followed by a 20 sec hold and then a release back to

the original length. Recovery time of at least 7 mins in between stretches was used to prevent memory-effects in subsequent measurements. Data were collected using a custom LabVIEW VI (National Instruments, Austin TX) at a sample rate of 1 kHz. Measured forces were converted to stress (force/unit undeformed CSA). The stress during the 1 base length/sec stretch was plotted against the SL and fitted with a monoexponential curve to derive stress-SL relationships. Passive stiffness was determined from the derivative of the passive stress vs SL relation.

Measurement of Ca^{2+} transient in intact cardiomyocytes

Intact cardiomyocytes were isolated as described above, then Ca^{2+} was reintroduced to cardiomyocyte suspension to a final concentration of 1 mM. Isolated LV cardiomyocytes were incubated with Fura-2 AM 2 μm (F-1225, Life Technologies) in stopping buffer(perfusion buffer plus bovine calf serum 0.08 mg/ml buffer and 1 mM $CaCl_2$) for 10 min at room temperature and resuspended in 1.8 mM Ca^{2+} D-MEM/F-12 (12400, Gibco, Life Technologies). An inverted microscope (IX-70; Olympus) was used with a chamber that had platinum electrodes to electrically stimulate cells and a perfusion line with heater control and suction out to maintain a flow rate of ~2 ml/min. All intact cell experiments were performed at temperature 37°C in D-MEM/F-12 (12400, Gibco, Life Technologies) plus 10 μg/mL insulin (I9278, Sigma-Aldrich). Cells were field-stimulated at 2 Hz frequency by MyoPacer stimulator (IonOptix Co, MA). All images were recorded with X40 objective lens. Data were collected using an IonOptix FSI A/D board and IonWizard 6.2.2.61 software (IonOptix Co, MA) with SarcLen modules to determine SL. Fluorescence was measured ratiometrically with the Ion Optix photometry system (IonOptix Co, MA). Fura-2 was excited alternately at 340 and 380 nm, and emission was recorded at 510 nm. Background fluorescence was subtracted for each excitation wavelength. Ratio of fluorescence intensities excited at 340 nm and 380 nm was used as a relative measurement of cytoplasmic Ca^{2+}, the ratio transient was fitted by monotonic transient analysis software (IonWizard 6.2.2.61). All measurements were carried out at 37°C.

Measurement of passive myocardial stiffness of left ventricular free wall

Mice were placed under isoflurane anesthesia, cervically dislocated, and the ribcage was rapidly removed to access the heart. One ml of HEPES pH 7.4(in mM: NaCL, 133.5; KCl, 5; NaH2PO4, 1.2; MgSO4,1.2; HEPES, 10) solution containing 30μM KCl and 30 mM BDM was injected into the LV through the apex, after which time the heart noticeably relaxed and ceased pumping. The heart was removed and the LV was isolated from the other chambers. The apex and base were removed from the LV leaving a cross sectional slice approximately 2mm thick. The septum and RV attachment regions were discarded and the LV free wall tissue was placed in relaxing solution. Endocardial fibers (apical to base orientation) were dissected and discarded. Once visualized, 400μM mid-myocardial fibers (circumferential orientation) were carefully removed and skinned in fresh relaxing solution pH 7.0 with 1% Triton-X-100 (Pierce, IL, USA) overnight at ~3°C and protease inhibitors (phenylmethylsulfonyl fluoride (PMSF), 0.5 mM; leupeptin, 0.04mM; E64, 0.01mM), then washed for one hour with relaxing solution. Phosphatase inhibitor cocktail 2 (P5726, Sigma-Aldrich) 1 ml was added into 100 ml of relaxing solution. Immediately following the wash with relaxing solution, 150-250 μm (diameter) strips were dissected and aluminum clips were placed on both ends of the preparation. The aluminum clips were attached to a strain gauge force transducer and high-speed length motor and the preparation was submersed in relaxing solution. Thickness and width of the preparation were measured and CSA was calculated assuming an elliptical cross-section. Sarcomere length (SL) was measured on line by laser diffraction. Relaxed fibers were stretched (100%/sec) from their slack length to sarcomere lengths of 2.0, 2.1, 2.2, and 2.3 μm with a 12 minute rest between stretches. To determine the collagen contribution to passive force, thick and thin filaments were extracted from the sarcomere, removing titin's anchors in the sarcomere. Curves were fit to data obtained from identical stretches before and after extraction to determine the total and ECM-based stress respectively. Titin-based

stress was taken to be the difference between these two curves. All data was fit to an exponential growth equation where passive stress is a function of sarcomere length as shown below:

$$\sigma = a_0(e^{(L-a_2)/a_1} - 1)$$

where σ=passive stress L=sarcomere length,a_0, a_1, and a_2 are empirical constants for initial elastic modulus, time constant of the fit, and slack sarcomere length respectively. To determine stiffness the derivative of the stress vs sarcomere length curve was used.

Confocal immunofluorescent imaging for in-vivo cell dimension and sarcomere length (SL)

Mice were placed under isoflurane anesthesia, cervically dislocated, and the ribcage was rapidly removed to access the heart. The hearts were arrested with a Krebs solution containing high KCl (35 mM) and BDM 2,3-butanedione monoxide (20 mM), through LV apical puncture to ensure that hearts were at diastasis during fixation. The hearts were rapidly excised, transversely cut, embedded in Tissue-Tek O.C.T. compound (Ted Pella Inc) and immediately frozen in 2-methylbutane-cooled in liquid nitrogen, then stored at -80 °C. The specimens were sectioned into 20 μm thick sections (Microm HM 550; Thermo Scientific). Tissues were permeabilized with 0.2% Triton X-100 in PBS for 20 min at room temperature, then were blocked with 2% bovine calf serum plus 1% normal donkey serum in PBS and incubated overnight with primary antibodies (rabbit anti-Laminin 1:400 (Sigma #L9393), rabbit anti connexin43 1:400 (Sigma#C6219), and mouse anti alpha-actinin 1:5000 (Sigma #A7811).Then the tissues were washed with PBS for 30 min and incubated with secondary antibodies at room temperature. The secondary antibodies were Alexa Fluor 568 Goat anti-Rabbit (Red, Molecular Probes #A11011) and Alexa Fluor 488 Goat anti-Mouse (Green, Molecular Probes #37977A). Tissues were washed with PBS and quickly washed with distilled water. Coverslips were mounted onto slides with Vectashield mounting media with DAPI (Vector Laboratories Cat# H-1200). Images were captured using a Leica SP5 confocal microscope with 40X oil (numerical aperture 1.25) objective. The tissues were excited at 543, 405 and 488 nm. Cells in mid-myocardial fibers (circumferential orientation) of the LV wall were used

for measurement. An average of 5 fields were observed for each section, and average of 3 sections were observed for 1 heart. The images were analyzed by the ImageJ software. Cellular dimensions were analyzed by the free hand area selection tool and tracing the periphery of the cell. Cell length was determined by ferret diameter and cell width was determined by min ferret diameter. The 1D plot profiles were determined along the cell length to obtain sarcomeric striations from alpha-actinin stained Z discs. The plot profiles were analyzed using fityk1.2.1 (http://fityk.nieto.pl). Well-defined peaks were fitted with Gaussian curves and the SL was calculated from average peak to peak distance.

Exercise testing

Mice were tested using a 6-lane rodent treadmill system (Exer 3/6, Columbus Instruments, Columbus, OH). After acclimation running at low speed (10 m/min for 10 minutes), exercise testing was performed by having mice run at progressively increasing speeds (speed steps of 4 m/min every 3 minutes). Maximal speed and running distance were determined when the mouse left the treadmill and remained on a shock pad for 5 sec. All animals were given water and standard rodent feed ad libitum pre- and post-exercise.

Quantification of protein expression

Flash-frozen LV tissues were prepared as previously described[7-9]. Briefly, the LV tissues were flash frozen in liquid nitrogen and solubilized between glass pestles cooled in liquid nitrogen. Tissues were primed at -20^{o}C for a minimum of 20 min, then suspended in 50% urea buffer ([in mol/L] 8 Urea, 2 Thiourea, 0.05 Tris-HCl, 0.075 Dithiothreitol with 3% SDS and 0.03% Bromophenol blue pH 6.8) and 50% glycerol with protease inhibitors ([in mmol/L] 0.04 E64, 0.16 Leupeptin and 0.2 PMSF) at 60^{o}C for 10 min. Then the samples were centrifuged at 13000 rpm for 5 min, aliquoted and flash frozen in liquid nitrogen and stored at -80^{o}C.

Titin isoform analysis was performed as previously described [9]. Briefly, the solubilized samples were electrophoresed on 1% agarose gels using a vertical SDS-agarose gel system (Hoefer)[10]. Gels were run at 15 mA per gel for 3 h and 20 min, then stained using Coomassie brilliant blue (Acros organics), scanned using a commercial scanner (Epson 800, Epson Corporation, Long Beach CA) and analyzed with One-D scan (Scanalytics Inc, Rockville MD). Each sample was loaded in a range of five volumes and the integrated optical density (IOD) of titin and MHC were determined as a function of loading volume. The slope of the linear relationship between IOD and loading was obtained for each protein to quantify expression ratios.

Expression levels were quantified with western blotting as previously described [11]. Solubilized samples were run on a 0.8% agarose gel in a vertical gel electrophoresis chamber. Gels run at 15 mA per gel for 3 h and 20 min were then transferred onto PVDF membranes (Immobilon-FL, Millipore) using a semi-dry transfer unit (Trans-Blot Cell, Bio-Rad, Hercules CA). Blots were stained with Ponceau S (Sigma) to visualize the total protein transferred. Blots were then probed with primary antibodies (see Table) followed with secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra (Biotium Company, Hayward CA). Blots were scanned using an Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln NE) and the images were analyzed using Li-COR software. Ponceau S scans were analyzed in One-D scan to normalize WB signal to protein loading. A list of primary antibodies used in western blot studies is provided as shown below.

Antibody	Source	Host	Dilution
RBM20	Gift from Dr.Gotthardt, MDC Berlin	Rabbit	1:500
Cypher	Santa Cruz Biotechnology	Mouse	1:200
LDB3	Novus Biologicals	Rabbit	1:800
PLBpT17	Badrilla	Rabbit	1:1500
PLB	Badrilla	Mouse	1:1500
pS282 cMyBP-C	Enzo Life Sciences	Rabbit	1:2500
MyBP-C	Santa Cruz Biotechnology	Mouse	1:500
p53	Millipore	Mouse	1:1000
CaMKIIδ	Badrilla	Rabbit	1:5000
GAPDH	Thermo Pierce	Mouse	1:5000
β-tubulin	Cell Signaling Technologies	Rabbit	1:1000

Picrosirius red (PSR) staining

Histology with Picrosirius red (PSR) staining measured the collagen volume fraction in LV cross-sections. The collagen volume fraction (CVF) was measured on tissues fixed in glutaraldehyde. These fixed hearts were sliced radially into sections, embedded, sectioned, and stained using Picrosirius Red to quantify collagen content. Stained sections were then imaged on a Zeiss microscope (Imager.M1) and analyzed for collagen content using custom Axiovision scripts. Collagen fiber length and width were analyzed by image J software. The free hand line selection tool was used for fiber length measurement. A 1D plot profile was determined across the fiber width. The plot profile was analyzed using fityk1.2.1. Well-defined peaks were fitted with Gaussian curves and fiber widths were calculated as 2 X HWHM (half width at half maximum).

Statistics

Statistical analysis was performed in Graphpad Prism (GraphPad Software, Inc). In all Figures and Tables (except for the below listed exceptions) a one-way ANOVA, without repeated measures, with a Bonferroni post-hoc analysis that calculates *p*-values corrected for multiple comparisons was performed to assess differences among 3 experimental groups (Sham, TAC/DOCA *cRbm20*$^{\Delta RRM}$ -DMSO and TAC/DOCA *cRbm20*$^{\Delta RRM}$-raloxifene). **Figures 4A, 5A, S5A, S5B:** A mono-exponential curve fit was used in cellular and LV myocardial passive stiffness measurements. A mono-exponential fit was selected based on visual inspection of the fit and low obtained residuals. We used nonlinear regression analysis with a least squares fitting method to determine the differences of individual curve fits among the three experimental groups. Curve fits were compared using an Extra sum-of-squares F-test in GraphPad Prism. A non-significant p-value ($p>0.05$) indicates that one curve can fit all data, while a significant p-value ($p\leq0.05$) indicates that each different data requires its own curve fit. **Figure 7:** Two-way ANOVA with repeated measures, with a Bonferroni post-hoc analysis was used for the treadmill running study to compare the differences of 3 experimental groups at 3 time points, the difference at 4 weeks post-surgery was shown as bar graphs in Figure 7B. **Table S3:** Two-way ANOVA without repeated measures, with a Bonferroni post hoc analysis was performed to assess differences in conscious echocardiogram of nonsurgical *MCM; cRbm20*$^{\Delta RRM}$ +/- mice after raloxifene induced Cre recombinase RBM20 inhibition. In both Supplemental Tables S1 and S3 the different time points were not obtained from the same animals. For example measuring titin isoform expression at different times after raloxifine injection requires us to sacrifice the animals and this precludes using the same animal for different time points. Thus we were not able to use a repeated measures test.

Results are shown as mean ± SEM. $p \leq 0.05$ was taken as significant. The numbers of animals or cells are shown in the figure legends in the following order: Sham, TAC/DOCA *cRbm20*$^{\Delta RRM}$-DMSO and TAC/DOCA *cRbm20*$^{\Delta RRM}$-raloxifene.

Supplemental Tables

Titin Isoform Ratio (Isoform/total titin)	Wk0	Wk1	Wk2	Wk3	Wk4	Wk5
N2BAsc (~3.6 MDa) /total titin	0	0.04 ± 0.03	0.09 ±0.02	0.19 ±0.03****	0.20 ±0.02****	0.19 ±0.01***
N2BAsc (~3.5 MDa) /total titin	0	0.04 ±0.01	0.14 ±0.04**	0.26 ±0.02****	0.26 ±0.02****	0.32 ±0.01****
N2BA (~3.3 MDa) /total titin	0.15 ± 0.02	0.14 ± 0.01	0.08 ±0.02	0.08 ±0.02	0.07 ±0.02	0.05 ±0.01*
N2B /total titin	0.69 ± 0.02	0.62 ± 0.06	0.50 ±0.06	0.31 ±0.04****	0.30 ±0.03****	0.28 ±0.01****
Titin degradation/ total titin	0.17 ±0.01	0.19 ± 0.05	0.18 ±0.01	0.16 ±0.02	0.17 ±0.01	0.17 ±0.01
Total titin/MHC6	0.13 ±0.02	0.20 ±0.06	0.16 ±0.02	0.26 ±0.04	0.25 ±0.02	0.25 ±0.03

Table S1. Titin isoform expression in LV myocardium of non-surgical *cRbm20*$^{\Delta RRM}$ mice after raloxifene injection. Expression level of N2BAsc titins increased while the level of N2B titin and wild-type N2BA titin decreased over time. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 vs. Week 0. (n=5,5,6,6,6,4 mice for week 0,1,2,3,4,5 respectively)

Echocardiography At 2 weeks post-surgery	**Sham (n= 20)**	***cRbm20*$^{\Delta RRM}$ - DMSO (n= 29)**	***cRbm20*$^{\Delta RRM}$ - raloxifene (n= 37)**
Heart rate (bpm)	527± 10	479± 9**	464± 7****
LVID;d (mm)	3.79± 0.05	3.83± 0.05	3.92± 0.04
WT;d (mm)	0.85± 0.02	0.96± 0.02**	0.94± 0.02**
LVID;s (mm)	2.74± 0.06	2.78± 0.06	2.83± 0.06
WT;s (mm)	1.13± 0.02	1.28± 0.02***	1.27± 0.02***
LV mass (mg)	88.7±2.2	107.0±3.2***	103.2±2.6**
Eccentricity	4.5±0.1	4.1±0.1*	4.2± 0.1
LV Vol;d (µl)	64.4 ±2.0	66.3± 1.5	68.9± 1.8
LV Vol;s (µl)	26.7 ±1.3	27.1± 1.4	29.5±1.5
SV (µl)	37.7± 1.3	39.1± 1.1	39.4 ±1.1
FS (%)	30.8± 0.9	31.7± 1.3	30.3± 0.9
EF (%)	58.7±1.3	60.0±1.7	57.8±1.3
LA dimension (mm)	2.08±0.03	2.41±0.02****	2.38± 0.03****
MV E (mm/s)	768.1± 28.1	814.3± 27.8	820.2± 30.7
MV A (mm/s)	530.9 ±35.4	457.6± 25.6	455.9± 23.0
E decel time (ms)	24.7± 0.5	21.2±0.5****	21.6±0.5****
MV E/A	1.55± 0.10	2.03± 0.16	2.10± 0.14*
MV e´ (mm/s)	-23.0± 1.3	-18.6± 0.9*	-19.2± 0.9*
MV a´ (mm/s)	-23.8± 1.3	-20.2± 1.2	-19.4± 0.9*
e´/a´	0.99± 0.05	0.97± 0.06	1.05± 0.06
E/e´	-34.9±1.8	-46.3± 2.0**	-45.6± 2.6**

Table S2. Echocardiographic parameters 2 weeks post TAC/DOCA surgery (before expression of N2BAsc titins in raloxifene injected mice). LV, left ventricle; d,diastole; s,systole; LVID, LV internal dimension; WT, wall thickness. Eccentricity indicates LVIDd/WTd; Vol, volume; SV, stroke volume;FS, fractional shortening; EF, ejection fraction; LA, left atrium; MV, mitral valve. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 vs. Sham. No significant difference in any echo parameters between *cRbm20*$^{\Delta RRM}$-DMSO and *cRbm20*$^{\Delta RRM}$-raloxifene groups.

Conscious Echocardiography	Raloxifene			DMSO (vehicle)		
	Baseline	1 week after raloxifene	3 weeks after raloxifene	Baseline	1 weeks after DMSO	3 weeks after DMSO
HR (BPM)	541 ±27	512 ±13	630 ±8*,###	561 ±43	616 ±18##	642 ±20*,###
LVID;d (mm)	2.92 ±0.12	2.73 ±0.08	2.80 ±0.08	3.12 ±0.31	3.12 ±0.04	2.90 ±0.07
WT;d(mm)	1.20 ±0.05	1.17 ±0.03	1.19 ±0.03	1.03 ±0.01	1.08 ±0.02	1.14 ±0.02
LVID;s(mm)	1.79 ±0.17	1.53 ±0.07	1.67 ±0.05	1.89 ±0.14	1.64 ±0.15	1.73 ±0.06
WT;s(mm)	1.63 ±0.08	1.60 ±0.02	1.57 ±0.04	1.39 ±0.03	1.53 ±0.08	1.47 ±0.03
Eccentricity	2.45 ±0.17	2.34 ±0.11	2.36 ±0.05	3.03 ±0.33	2.90 ±0.08#	2.55 ±0.11
EF (%)	81.3 ±4.4	90.3 ±1.5	86.8 ±1.2	79.6 ±0.1	84.1 ±2.84	84.1 ±2.3
SV (µl)	27.9 ±2.1	24.7 ±1.4	25.9 ±2.3	31.0 ±7.2	31.5 ±2.7	28.5 ±2.0

Table S3. Conscious echocardiography of non-surgical *cRbm20*$^{\Delta RRM}$ mice before, at 1 week and at 3 weeks after raloxifene/DMSO administration. Results showed that raloxifene does not cause a reduction in contractility and does not cause changes in chamber geometry. HR, heart rate; LVID, left ventricular internal dimension; WT,wall thickness; d,diastole ;s,systole; Eccentricity indicates LVIDd/WTd; EF, ejection fraction; LV, left ventricle; SV ,stroke volume. *p≤0.05 vs. baseline of raloxifene group, #p≤0.05, ##p≤0.01, ###p≤0.001 vs. at 1week after raloxifene (n=7 and 4 mice for raloxifene and DMSO group.)

Supplemental Figures

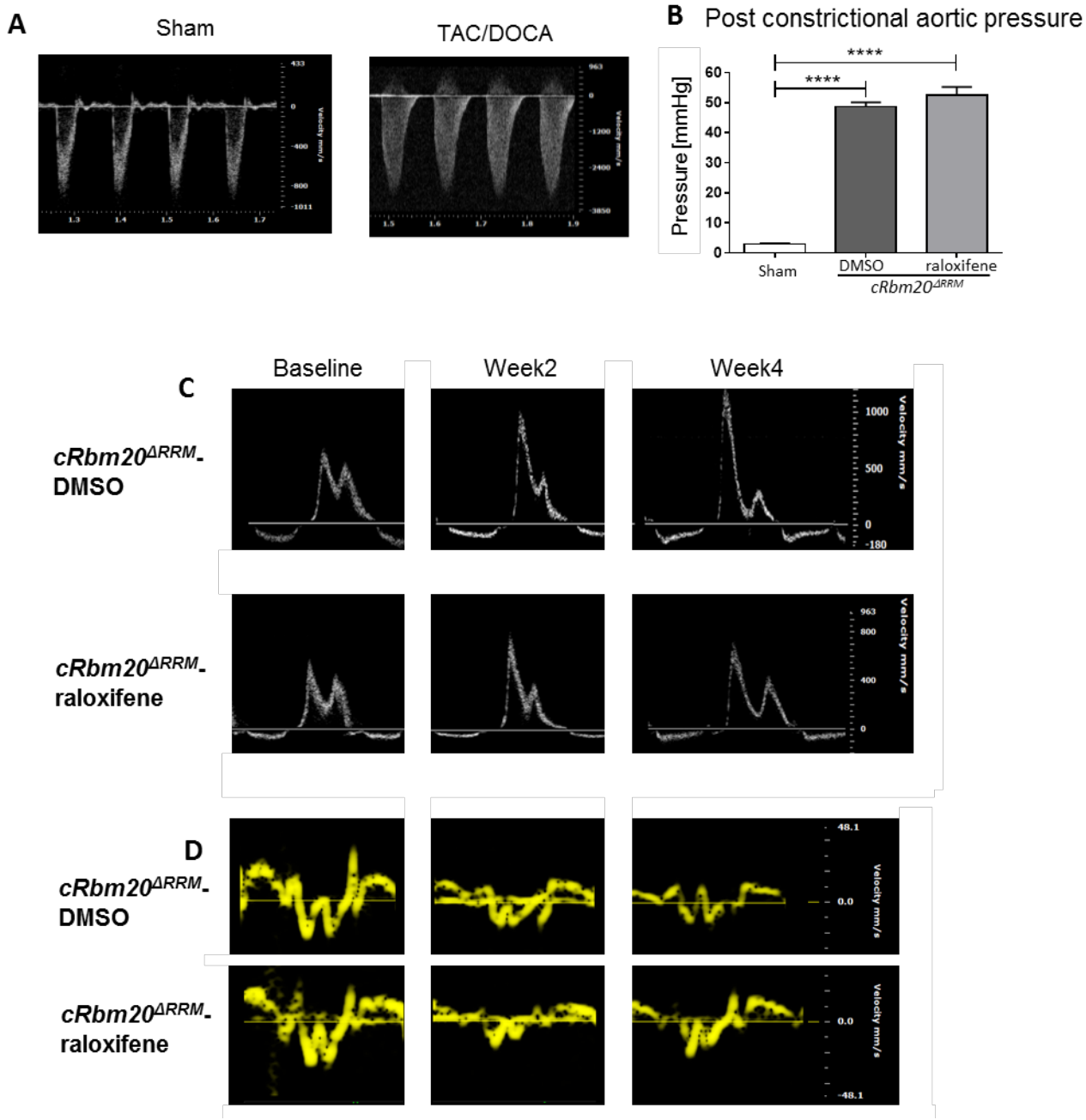

Figure S1A. (A) Pulse wave Doppler echocardiography showed increased post constrictional aortic flow velocity in TAC/DOCA mice (note that the Y axis scales of sham and TAC/DOCA are different). **(B)** Aortic pressure at post constrictional site were equally elevated in both TAC/DOCA groups. ****p≤0.0001. (n=20 sham, 29 *cRbm20*$^{\Delta RRM}$-DMSO, 37 *cRbm20*$^{\Delta RRM}$-raloxifene mice). **(C)** Mitral inflow pattern and **(D)** Mitral annular velocity of *cRbm20*$^{\Delta RRM}$-DMSO (top) and *cRbm20*$^{\Delta RRM}$-raloxifene (bottom) revealed progressive diastolic dysfunction and a restrictive filling pattern in *cRbm20*$^{\Delta RRM}$-DMSO while *cRbm20*$^{\Delta RRM}$-raloxifene showed recovery of diastolic dysfunction.

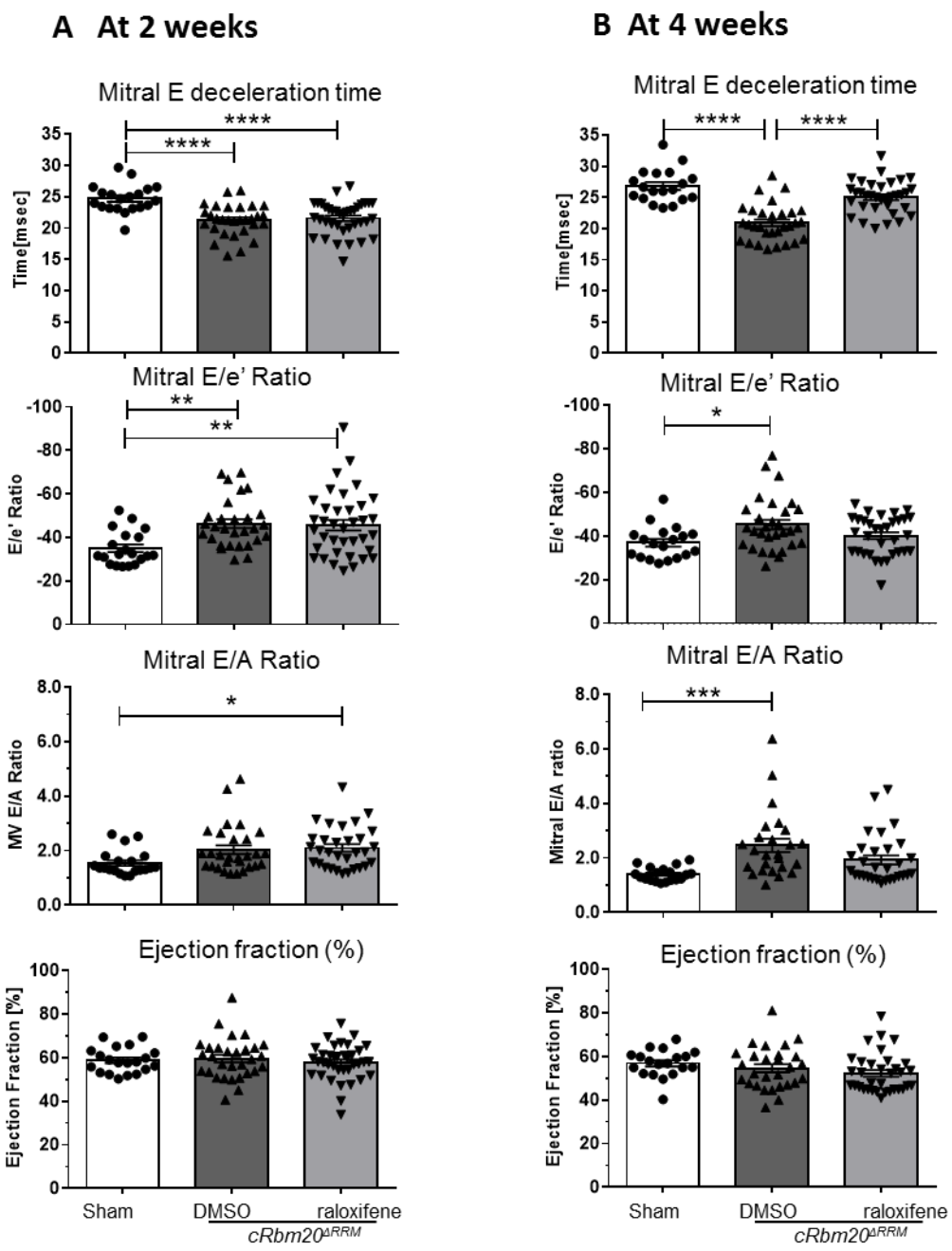

Figure S1B. Echocardiography: diastolic and systolic function 2 weeks (Panel A) and 4 weeks (Panel B) after TAC/DOCA surgery (raw data were shown). *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. (n=20 sham, 29 *cRbm20*$^{\Delta RRM}$-DMSO, 37 *cRbm20*$^{\Delta RRM}$-raloxifene).

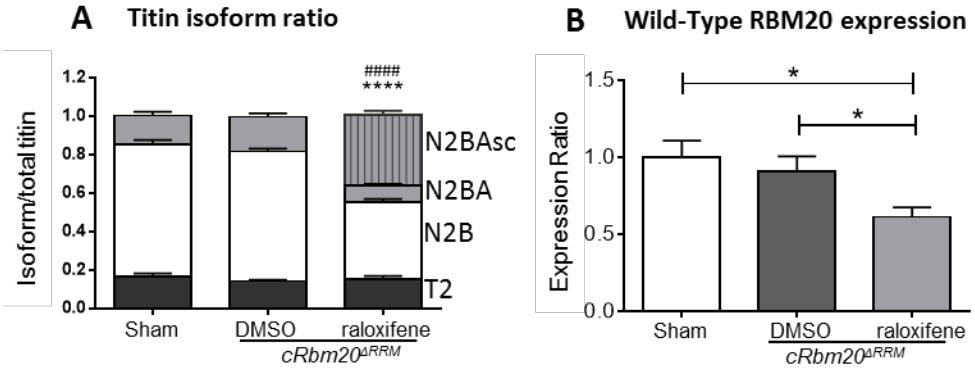

Figure S2. A) SDS-agarose analysis of titin expression and B) Western blot analysis of RBM20 expression in *cRbm20*$^{\Delta RRM}$ mice at 4 weeks post TAC/DOCA surgery. (A) Titin isoform expression in *cRbm20*$^{\Delta RRM}$-DMSO mice was not different from sham animals. *cRbm20*$^{\Delta RRM}$-raloxifene mice expressed super compliant titin N2BAsc (striped area) which accounts for ~45% of total titin. N2BAsc is expressed at the expense of N2B titin (white area) and N2BA titin (grey). ****p≤0.0001 vs. Sham. $^{\#\#\#\#}$p≤0.0001 vs. *cRbm20*$^{\Delta RRM}$-DMSO. **(B)** RBM20 protein is ~40% reduced in *cRbm20*$^{\Delta RRM}$-raloxifene hearts. *p≤0.05. (n=18, 29, 37 mice for titin isoform study, n=9,12,14 mice for RBM20 study).

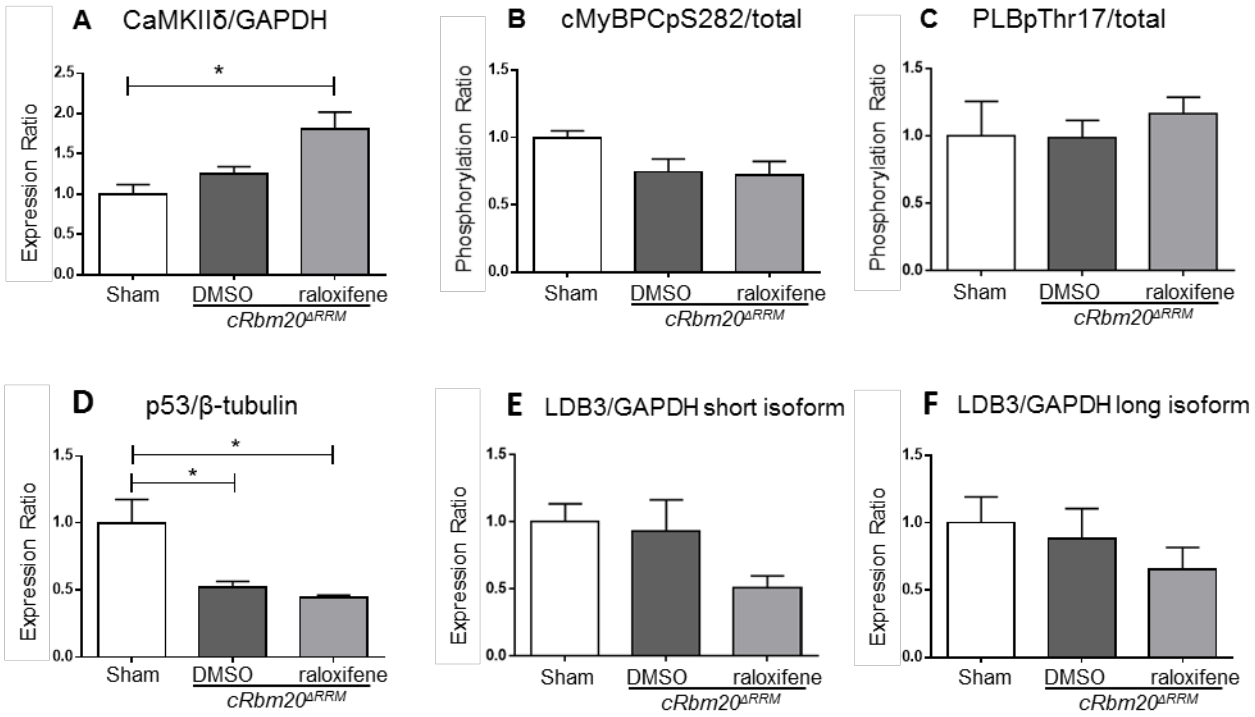

Figure S3. Western blot analysis of non-titin substrates of RBM20. (A) Results showed an increased expression of CaMKIIδ in *cRbm20*$^{\Delta RRM}$-raloxifene mice vs sham, but no difference vs. *cRbm20*$^{\Delta RRM}$-DMSO. Phosphorylation of cMyBPCpSer282 **(B)** and PLBThr17 **(C)** were normal. **(D)** Expression level of p53 was similarly decreased in both TAC/DOCA groups, suggesting a consequence of the TAC/DOCA procedure rather than RBM20 inhibition. For the short **(E)** and long **(F)** cardiac isoforms of LDB3, no differences were found. *p≤0.05. (n= 4 sham, 6 *cRbm20*$^{\Delta RRM}$-DMSO, 8 *cRbm20*$^{\Delta RRM}$ -raloxifene mice)

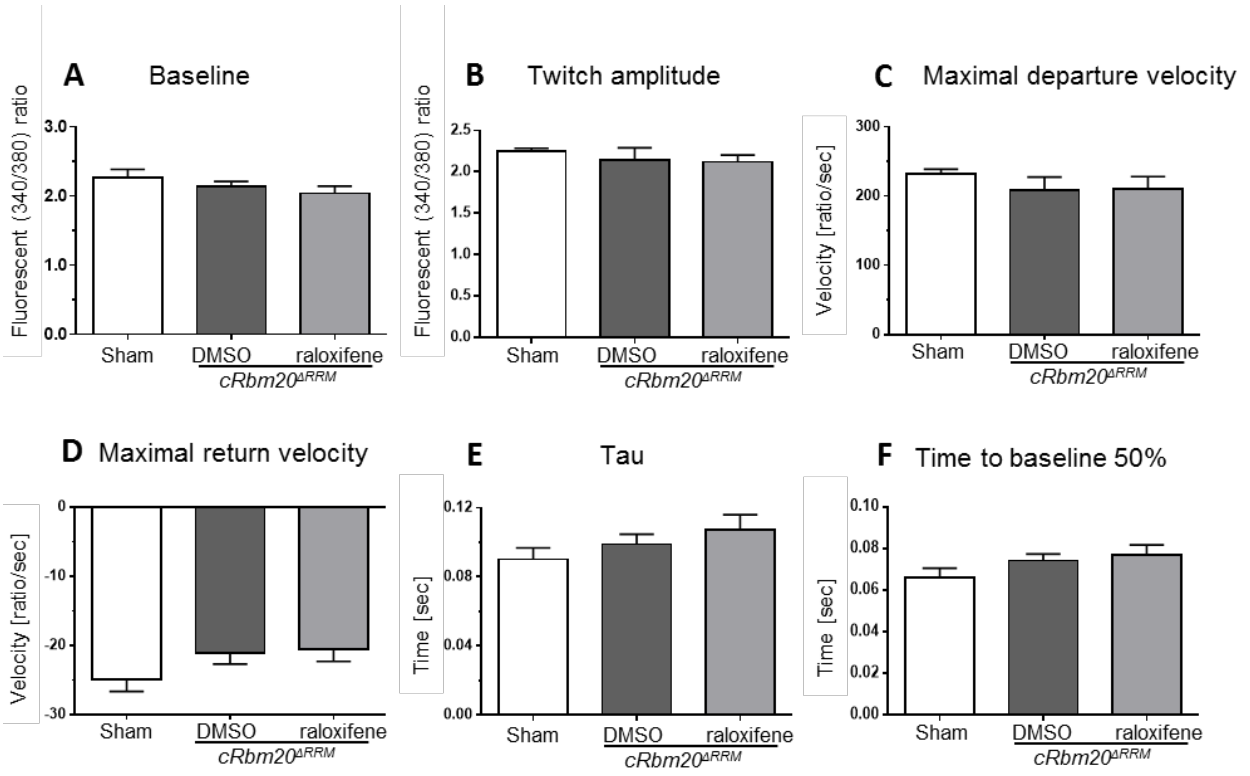

Figure S4. Ca^{2+} handling of isolated cardiomyocytes. Fura-2 340/380 ratio was measured in intact cardiomyocytes stimulated at 2 Hz. Baseline signal **(A)**, twitch amplitude **(B)** ,maximal rising velocity **(C)** as well as Ca^{2+} reuptake parameters; maximal decaying velocity of signal **(D)**, exponential decay time constant (Tau) **(E)** , and time for the transient to return 50% **(F)** were not significantly different among three experimental groups. (n= 148,202,191 cells 6,9,8 mice.)

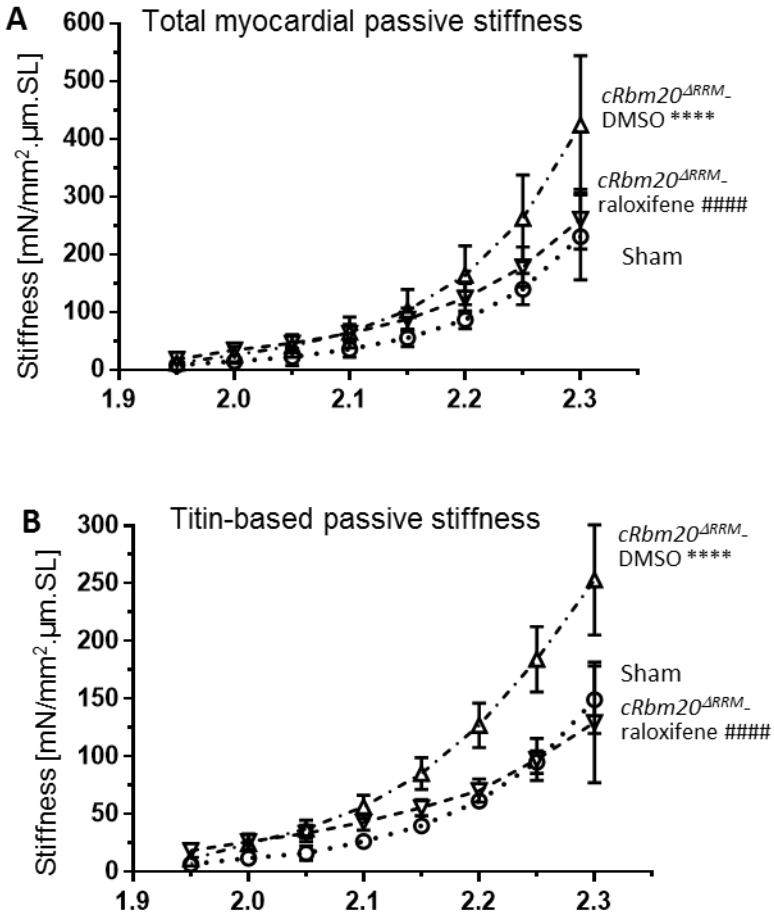

Figure S5. Myocardial passive stiffness of LV free wall. The relation of total myocardial stiffness (A) and (B) titin-based stiffness vs. SL of sham (circle), *cRbm20*$^{\Delta RRM}$-DMSO (upward triangle) and *cRbm20*$^{\Delta RRM}$-raloxifene (inverted triangle) are shown. **(A)** Total stiffness-SL relations was increased in *cRbm20*$^{\Delta RRM}$-DMSO mice but was normalized in the raloxifene group. **(B)** Titin-based stiffness was highest in *cRbm20*$^{\Delta RRM}$-DMSO but was normalized in *cRbm20*$^{\Delta RRM}$-raloxifene mice. (For analysis, see Supplemental Methods.) ****p≤0.0001 vs. Sham, ####p≤0.0001 vs. *cRbm20*$^{\Delta RRM}$-DMSO. (n=4 sham, 6 *cRbm20*$^{\Delta RRM}$-DMSO, 6 *cRbm20*$^{\Delta RRM}$-raloxifene mice)

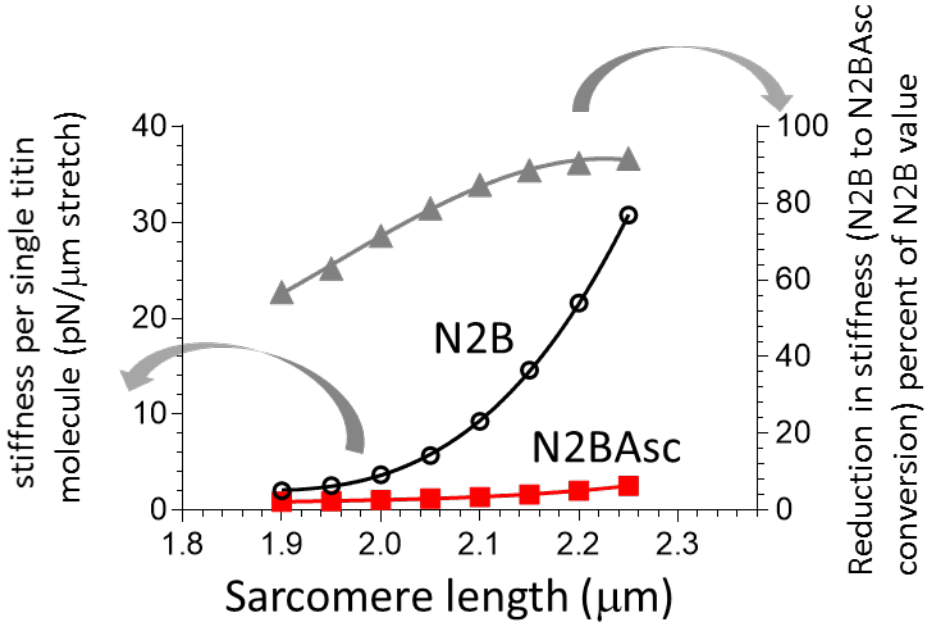

Figure S6. Wormlike chain (WLC) simulations to estimate the effect of converting N2B titin into N2BAsc on passive stiffness. The force-SL relation of a single titin molecules (N2B cardiac titin isoform) was calculated using the wormlike chain (WLC) equation with three serially-linked WLCs, representing the combined proximal and distal tandem Ig segments, the PEVK, and N2B-Us spring elements. The contour length (Lc) of tandem Ig segments was 200 nm (40 Ig domains with an average spacing of 5 nm). The PEVK Lc was 70 nm and the N2B-Us Lc was 180 nm. To account for the inclusion of an extra 34 Ig domains and 300 amino acid PEVK residues the contour length of the tandem Ig segments was increased by 34 x 5 nm (170 nm) and that of the PEVK by 300 x 0.35 nm (105 nm). The persistence lengths (PL) were 12 nm (tandem Ig segments), 1.0 nm (PEVK) and 0.3 nm (N2B-Us). (For experimental evidence that underlies these values and additional details, see Perkins et al 2015[12]. The obtained force-SL relations were converted to stiffness – SL relations by calculating the local change in force.

Supplemental References

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