SUPPLEMENTAL MATERIAL

Data S1.
SUPPLEMENTAL METHODS

Animal models. Two transgenic mouse-models of HCM carrying the R92Q and the E163R mutations in *TNNT2* gene with C57BL/6 genetic background were generated in Dr. Tardiff's Lab [1, 2] and employed for this study. Heterozygous progenitors were bred with C57BL/6 females (Charles River) and the genotypes of the offspring determined by PCR on DNA from tail biopsy specimens. Animals used for experiments were 6-8 month old WT and heterozygous R92Q and E163R males weighing 30-45 g. Animals were maintained and bred at the animal facility of the University of Florence, Italy, according with the local ethical committee rules.

Echocardiography. In collaboration with Toscana Life Sciences (Siena, Italy) we performed echocardiographic measurements in WT, R92Q and E163R mice. We used the dedicated instrumentation Visualsonics Vevo 2100. Mice were first anesthetized in an induction chamber with isoflurane (induction, 4%–5%; maintenance, 1%–2.5% in oxygen from a precision vaporizer) and then positioned prone on a heated bed. In each mouse we performed Parasternal Long Axis (PSLAX) and measured the thickness of the interventricular septum (IVS), left ventricle posterior wall (LVPW), left ventricular interior diameter (LVID), Short Axis (SAX), Simpson's LV volume reconstruction, PV Flow, LV Diastolic Function and isovolumic relaxation time (IVRT), isovolumic contraction time (IVCT), no flow time (NFT), aortic ejection time (AET).

Dissection of ventricular trabeculae. Left and right ventricular trabeculae were selected because of their similarity with fascicles in the cardiac wall [3]. Mice were heparinized (5000 UI/ml) and anesthetized by inhaled isoflurane. The heart was rapidly excised, perfused retrogradely via the proximal aorta with a modified Krebs-Henseleit solution (KH solution) and placed in a dissection dish beneath a binocular microscope. KH solution contained (mM): 120 NaCl, 5 KCl, 2 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 0.50 Ca^{2+} and 10 glucose, pH 7.4 equilibrated with 95%O_2 5%CO_2. During retroperfusion and dissection the potassium concentration in the KH solution was raised to 15 mM/L to stop spontaneous beating of the heart. BDM 20 mM/L was also added to the solution to minimize contractures following cutting damages. Right ventricle was opened by cutting along the anterior

interventricular junction. Thin unbranched trabeculae, running between the free wall of the right ventricle and the atrioventricular ring, were dissected by cutting through the atrioventricular ring on one extremity and removing a portion of the right ventricular wall on the other one. Cross-sectional muscle area was calculated with the assumption of an ellipsoid shape.

Mechanical measurements on intact trabeculae. Ventricular trabeculae were mounted between the basket-shaped platinum end of a force transducer (KG7A, Scientific Instruments Heidelberg, Germany) and a motor (Aurora Scientific Inc., Aurora, Canada), both connected to micromanipulators, in a glass-bottomed heated horizontal tissue bath with platinum wires for field-stimulation[4]. Sarcomere length was measured by laser diffraction [3]. A custom-made Labview software was used for motor control, stimulation and force signal recording. Muscles were allowed to stabilize at baseline conditions (30°C, 2 mmol/L [$CaCl_2$], 1 Hz stimulation) for at least 20-30 min and were gradually stretched to optimal initial sarcomere length (2.15±0.03 μm) before starting the experimental protocol. Twitch force and kinetics were studied with the following protocols. (I)Response to Isoproterenol: twitch force was measured at 1 Hz while muscles were exposed to a saturating dose of isoproterenol (1μM); recordings were started 10 minutes after solution change. (II) Force frequency relationship: trabeculae were paced at different stimulation frequencies (0.1 to 8 Hz) to assess changes of steady-state twitch force. (III) Post rest potentiation and premature beats: stimulation pauses of different duration (2 to 60 s) were inserted after the last contraction of a steady series at 3 Hz to evaluate post-rest potentiation of the first stimulated beat after the pause, as well as the occurrence of spontaneous beats during the pause.

Mechanical measurements on skinned trabeculae. Ventricular trabeculae, dissected as described above, were skinned by overnight incubation in relaxing solution (containing 20 mM MOPS, 10 mM EGTA, 1 mM free Mg21, 5 mM MgATP22, 12 mM creatine phosphate, 10 IU/ml creatine kinase, pH adjust to 7.0 with KOH), added with 0.5% Triton X100. Triton was then removed and the skinned preparations were mounted horizontally between a force transducer and a motor by means of T-clips. Force-PCa curves were obtained as previously described[5]. Muscles were activated by transferring

them manually between baths containing different pCa solutions and the pCa-force relationship was determined. Three types of solutions were employed: relaxing solution (pCa 9) with 5 mM EGTA, pre-activating solution with 0.5 mM EGTA and 4.5 mM 1,6-diamino hexane-N,N,N',N'-tetraacetic acid (HDTA), and a maximal activating (pCa 4.5) solution with 5 mM Ca-EGTA. Relaxing and maximal activating solutions were mixed in different proportions to obtain activating solutions with various pCa's. Solutions were applied in the sequence: relaxing, pre-activating, activating, relaxing. All solutions contained: 60 mM BES (N,N-bis[2-hydroxyethyl]-2-aminoethane sulphonic acid); 5.83 mM Na2ATP2, 7.4 mM MgCl. Potassium propionate was added to adjust the final ionic strength to 0.20 M. pH was adjust to 7.1 with KOH at 20°C.

Sarcomere energetics in skinned trabeculae.

The experimental procedures, solutions and equipment used for functional measurements were as described previously [6, 7]. Isometric force and ATPase activity were measured at maximal and submaximal [Ca^{2+}] at 20°C. During the measurement trabeculae were kept inside a small (30 µl) chamber with thin quartz windows.

ATPase activity was measured using an enzyme coupled assay. In this assay the ATP regeneration from ADP and phosphoenol-pyruvate, catalyzed by the enzyme pyruvate kinase, is coupled to the oxidation of NADH to NAD+ and the reduction of pyruvate to lactate, catalyzed by L-lactic dehydrogenase. NADH oxidation was measured photometrically from the absorbance at 340 nm of near-UV light. The absorbance signal was calibrated by adding 0.5 nmol ADP to the solution in the measuring chamber. ATPase activity in the muscle could be derived from the slope of the absorbance signal. The Ca^{2+}-activated ATPase activity was calculated by subtracting the basal ATPase activity (measured in relaxing solution) from the NADH oxidation during contraction and normalized to the volume of the trabeculae. The tension cost (energy cost of tension generation) is expressed as the ratio ATPase activity/tension).

Mechanics of single myofibrils. Thin strips of the mouse ventricular samples were incubated overnight in ice-cold relaxing solution (pCa 8.0, pH 7.0, in mmol l–1: ionic strength 200, EGTA 10,

Tris 10, MgATP 5, freeMg2+ 1)with 1% Triton X-100 added. Triton was then removed and the strips were homogenized in the same relaxing solution to produce myofibril suspensions that were stored at 0–4°C and used for experiments for up to 4 days. Techniques for mechanical measurements in human cardiac myofibrils were as previously described [8, 9]. Briefly, myofibrils were transferred to a temperature controlled chamber filled with relaxing solution (pCa 8.0, 15°C). The selected myofibril was mounted (initial sarcomere length around 2.2 μm) between a cantilever force probe and a glass needle mounted on the shaft of a length control motor. Myofibrils were maximally activated and fully relaxed by rapid solution switching between two continuous streams of solutions (pCa 8.0 and 4.5). All solutions contained a MgATP-regenerating system and a cocktail of protease inhibitors.

ATP hydrolysis measurements from myofibril suspensions.

Steady state ATP hydrolysis of ventricular mouse myofibrils in relaxing solution was measured using Malachite Green (MLG) Phosphate Assay (BioAssay Systems Hayward, CA, USA). Briefly, myofibrils were washed and resuspended with modified relaxing solution which did not contain MgATP, protease inhibitors and CP. The concentration of myosin in the suspension was determined spectrophotometrically as in Herrmann et al. (1994) from the absorbance at 280nm (BioSpectrometer®, Eppendorf Hamburg, DE) in 2% SDS, with E1%1cm = 7 and assuming that myosin had a MW of 5 x 105 D and represented 50% of total protein content (typically 0.2-0.3 mg/ml;[14,15]). Mean heads concentration in reaction solution (250 µl) was set about 0.5 µM. The reaction was started in a constant-temperature bath at 30 °C by adding 50 µl of 5 mM MgATP dissolved in the same experimental solution (final concentration 1mM). The reaction was arrested by acid quench at different times by adding 250 µl of ice-chilled 0.67 M Trichloroacetic acid (TCA). Phosphate assay was performed adding 20 µl of MLG reagent to 80 µl of acid-quenched reaction mixtures and measuring the absorbance at 630 nm after 30 min incubation at room temperature. ATP hydrolysis was expressed as the ratio of [Pi] to [myosin head] (µM/µM).

Isolation of single cardiomyocytes from mouse hearts. Ventricular cardiomyocytes were isolated by enzymatic dissociation as previously described [4]. The animal was heparinized (5,000 U/kg, i.p.) and deeply anesthetized with Isoflurane. The excised heart was immediately bathed in cell isolation solution and perfused retrogradely via the proximal aorta. Cell isolation solution contained 120 NaCl, 1.2 $MgCl_2$, 10 KCl, 1.2 KH_2PO_4, 10 glucose, 10 Hepes and 20 taurine, 5 pyruvate, oxygenated with O_2 (Table 3); pH 7.2 (adjusted with NaOH). The coronary arteries were then perfused with this solution at 37°C for 3-4 min at a constant flow of 3 mL/min. The solution was then switched to a recirculating enzyme solution made of the same buffer with the addition of 0.1 mg/mL Liberase (Liberase TM for mouse, Roche Applied Sciences). After 8-9 min, the ventricles were excised and cut into small pieces in buffer solution supplemented with 1 mg/ml BSA. Gentle stirring facilitated further dissociation of the myocytes. The cell suspension was let to settle and the cell pellet was resuspended in a modified Tyrode buffer (see below) with 0.05 mM $CaCl_2$ supplemented with 1 mg/ml BSA. Calcium concentration was gradually raised to 1.0 mM.

Intracellular Ca^{2+}-transients measurements. Freshly isolated cardiomyocytes were incubated with FluoForte Reagent (ENZO Life Sciences) and plated on a glass bottomed dish with platinum electrodes for electrical field stimulation, as previously described[10]. We measured fluorescence emitted at 505-525 nm with a fast camera (Photometrics Cascade 128+) during fixed excitation at 490 nm with a monochrome LED source, while stimulating the cells at 1Hz, 3Hz and 5Hz using <3ms square pulses. Measurements were performed at steady state by averaging 20-50 subsequent Ca^{2+} transients.

In order to quantify the occurrence of arrhythmic events, cells were stimulated at 5Hz for at least 30 seconds and then stimulation was abruptly stopped for 15s, while recording intracellular Ca^{2+} movements. We quantified the occurrence of Ca^{2+} waves (slow raise kinetics, amplitude 20-50% of a regular transient) and spontaneous Ca^{2+} transients (fast upstroke, amplitude comparable to a regular transient). The protocol was repeated in the presence of ISO.

Drugs. Isoproterenol (ISO) (SIGMA) was stocked in distilled water solution with 1 mg/ml ascorbic acid as an anti-oxidant, and then used at 0.1 μM and 1 μM.

Confocal Microscopy. Isolated cardiomyocytes were incubated for 20 minutes with 5μM of Di-3-ANEPPDHQ (Thermo-Fisher) membrane dye, resuspended in Tyrode buffer, placed atop a glass-bottomed chamber (MaTek) and observed using a 63x oil immersion objective on a Leica TCS5 inverted microscope. The excitation wavelength was 488 nm and the emission filter was set at 504-649 nm. A single image was recorded from each cell by choosing a plane comprising the central portion of the cell. Images were then analyzed offline with ImageJ, using an automatic analysis plugin (TTorg)[11].

Western Blot. Western blot analysis was performed by a standard method [12] on proteins isolated from LV specimens from WT, R92Q and E163R mice, as previously described[13]. The following antibodies were used: phospho-CaMKII at threonine 287, NFAT1c, phosphorylated-NFAT1c, cardiac Troponin I (cTnI) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); CaMKIIδ (Abcam, Cambridge, MA); phospho-PLB at serine 16 (Millipore, Billerica, MA); PLB (Thermo-Fisher, Waltham, MA); SERCA2a (Badrilla, Leeds, UK); phospho-cTnI (Cell Signalling Technology, Danvers, MA). Relative intensity of individual bands from Western blots was quantitated using ImageJ software and normalized to GAPDH. The ratio for control was assigned a value of 1.

Statistical Analysis. Data from cells and muscles are expressed as mean±SEM (Standard Error of Mean) values obtained from a number of independent determinations on different samples: number of samples and mice for each set of measurements is indicated in figure legends. To faithfully compare different sets of measurements, sensitivity analysis was performed for each statistical comparison[14] using SPSS 23.0 (IBM, USA), in order to account for:

1- Non-gaussian distribution (Shapiro-Wilk test).

2- Heteroschedasticity (Levene's test).

The statistical tests used to calculate P-values for each dataset were different depending on the properties of individual datasets and on the number of groups to compare. The specific tests used for each comparison are indicated with abbreviations in the respective figure legends.

1- For sets variables where a single measurement from each mouse is included (e.g. echocardiography data, western blot results), the three different groups (WT, R92Q-KET and R92Q-RAN) were compared using one of the following tests, using SPSS 23.0:

(a) One-Way ANOVA with Tukey correction, for normally-distributed homoscedastic datasets.

(b) Kruskal-Wallis test with Dunn's multiple comparison test, for non-Gaussian-distributed datasets (as identified with the Shapiro-Wilk test)

(c) Welch's ANOVA with Games-Howell test, when there was a significant difference among the variances of the different groups heteroscedastic groups (identified by Levene's test).

2- In some datasets, average data derives from multiple samples (myocytes, trabeculae) from different mice. The intrinsic correlation among different cells or trabeculae from the same mice (within-subject correlation) could influence the outcome of the results, especially when a different number of samples from each mouse is included in the overall means. We estimated within-subject correlation for each variable using multi-level one-way analysis-of-variance (ANOVA). In brief, the variance among the cells/trabeculae from each single subject is compared with the overall variance. When the estimated intra-class correlation exceeds 0.10, it is considered to have a significant effect on the results, requiring multi-level analysis for comparison. In order to account for intra-subject correlation, we used linear mixed models to compare data groups, using the *xtmixed* function of the STATA 12.0 program (StataCorp, USA). When Levene's test reported unequal variances in unpaired comparisons, linear mixed models were adjusted for heteroschedasticity (via independent calculation of residuals in the two groups). The Probability (*P*) values were calculated from linear mixed models. For multiple comparisons, we used the Tukey post-hoc test. $P<0.05$ was considered statistically significant.

Study approval. Animals were bred and used for experiments in accordance with Italian and European regulations for animal handling and care and all experimental protocols were approved by the local

committee for animal welfare. In agreement with current regulations, the protocol was submitted to the Committee for Animal Welfare of the Italian Ministry of Health on February 2014 and approved on March 2014.

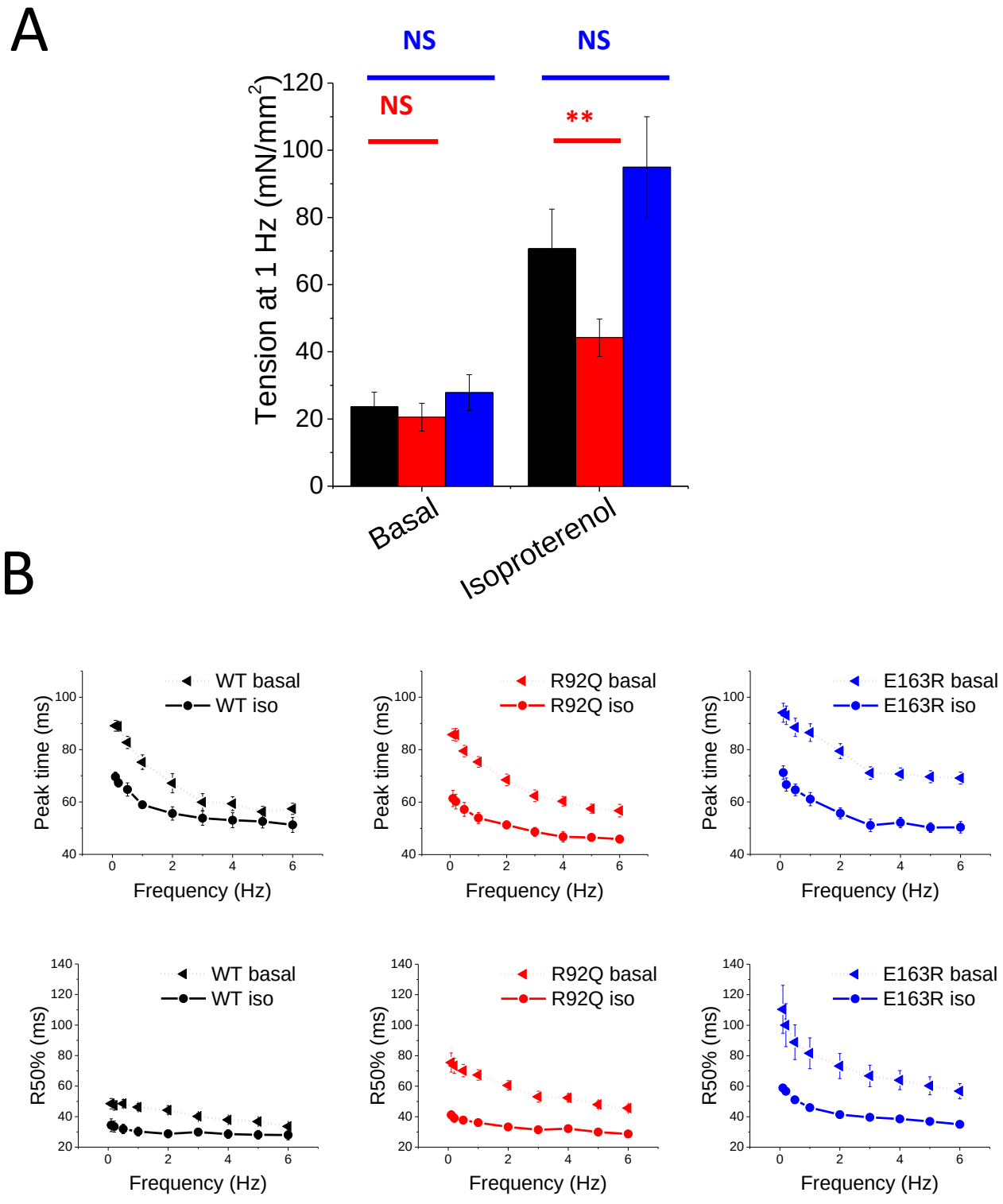

Figure S1. Steady-state contractions with Isoproterenol. (A) Steady-state 1 Hz twitch tension at basal conditions and in the presence of Isoproterenol 10^{-7}M. **(B)** Time to peak and time from peak to 50% relaxation (R50%, right), measured in steady state twitches at different stimulation frequencies at basal conditions and in the presence of Isoproterenol 10^{-7} M.

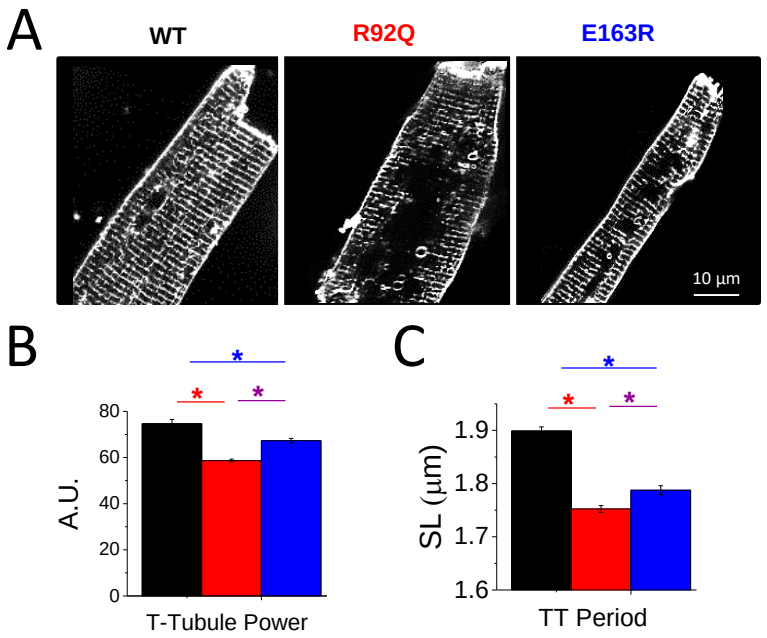

Figure S2. Alterations of t-tubules. (A) Representative confocal images from isolated LV cardiomyocytes stained with di-3-aneppdhq from WT (right), R92Q (middle) and E163R (right) hearts. Horizontal bar equals 10 µm. **(B)** T-tubule Power, as calculated using the TTorg ImageJ plugin, in cardiomyocytes from the three study groups. **(C)** Mean T-tubule period (equivalent to sarcomere length) in resting LV cardiomyocytes from the three cohorts of mice. (**B-C)** Means ± S.E. from 68 WT (4 mice), 142 R92Q (6 mice) and 69 E163R (4 mice) cardiomyocytes. Statistics: One-way ANOVA with Tukey correction.*P<0.05.

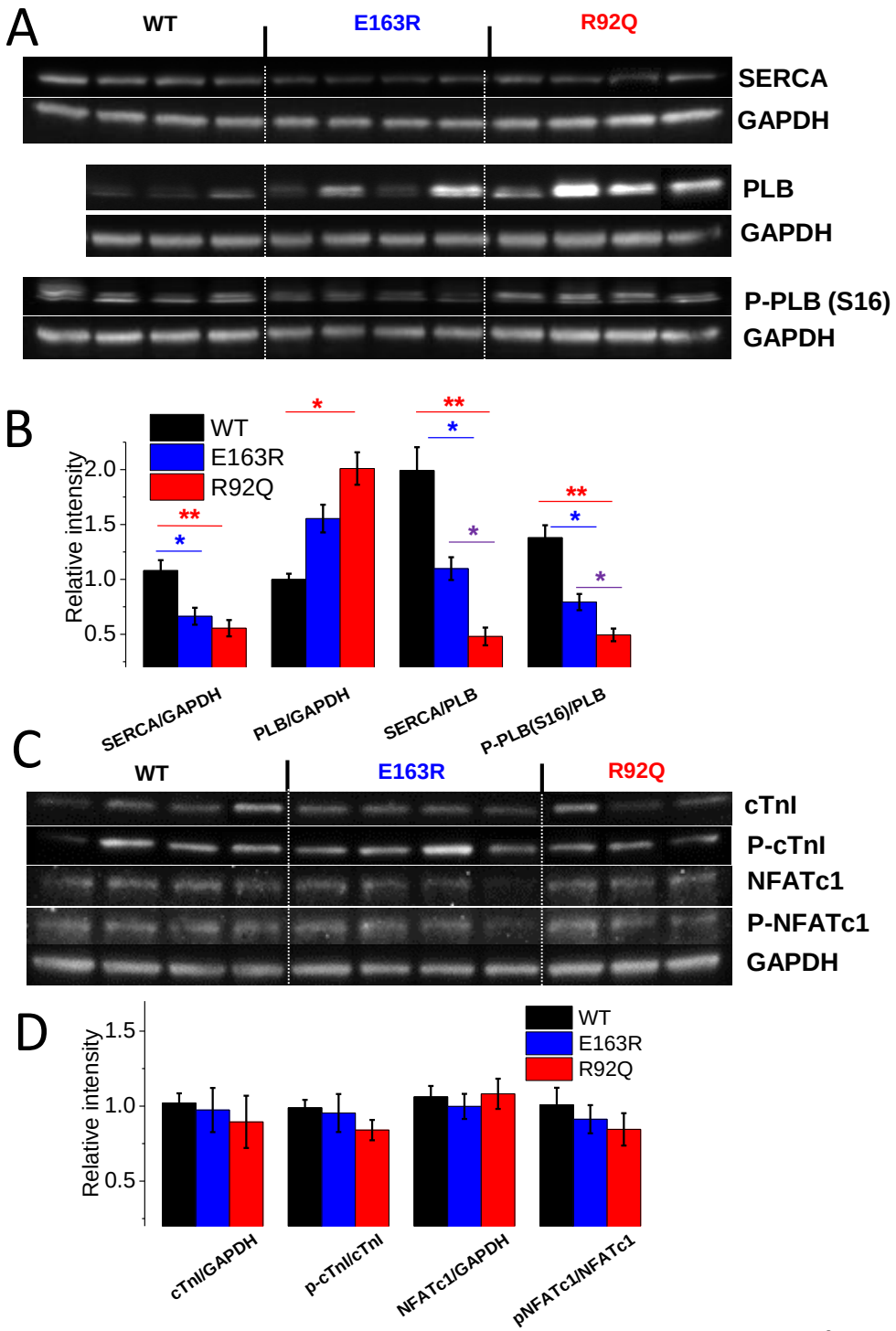

Figure S3. Additional protein studies. (A) Representative Western blots for total SERCA, phospholamban (PLB), Phosphorylated PLB at Serine 16 (P-PLB(S16)) and GAPDH. **(B)** Average relative intensity values of SERCA, PLB, SERCA/PLB and p-PLB(S16)/PLB ratios in WT, R92Q and E163R hearts (8 samples for each groups). **(C)** Representative Western blots for total cardiac troponin-I (cTnI), phosphorylated TnI (P-cTnI), total NFATc1, phosphorylated NFATc1 (P-NFATc1) and GAPDH. **(D)** Average relative intensity values of cTnI, P-cTnI, NFATc1, P-NFATc1 in WT, R92Q and E163R hearts (5 samples for each groups). (**B-D**)The intensity of individual SERCA bands was quantified and normalized to that of GAPDH. Relative intensities of WT were set at 1. Statistics: One-way ANOVA with Tukey correction.*P<0.05. **P<0.01.

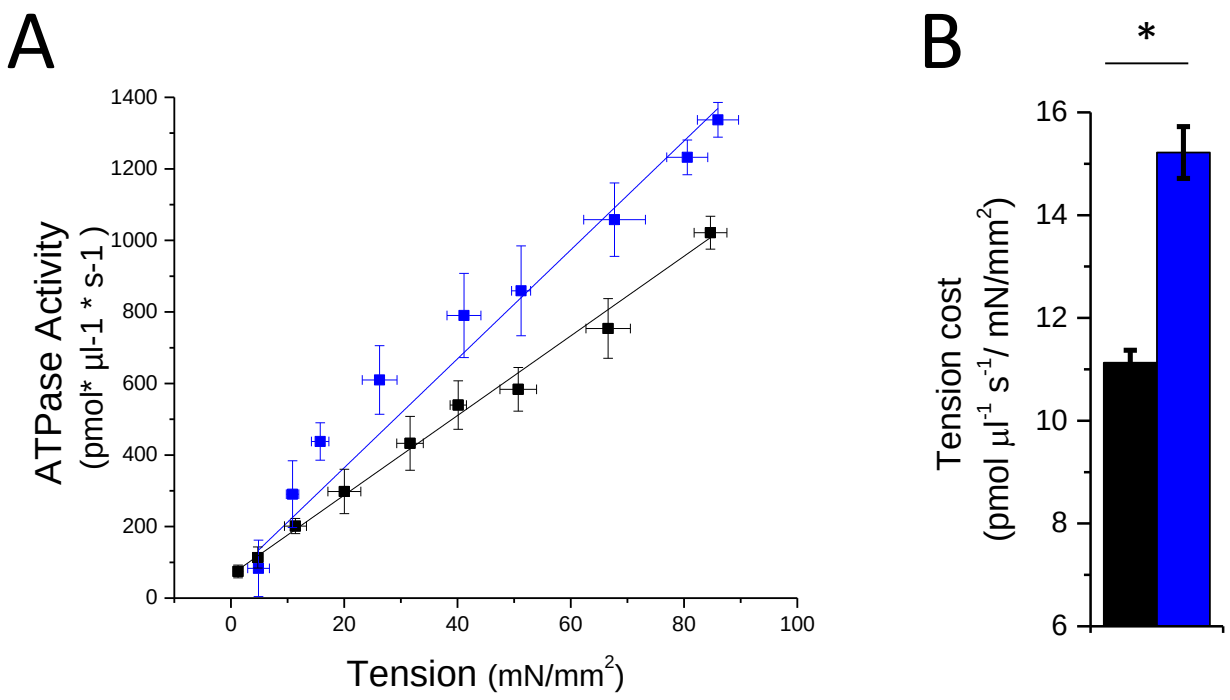

Figure S4. Effect of the E163R mutation on the relationship between steady-state isometric force and ATPase activity. (A) TC was determined from the slope of the relationship between ATPase activity and active tension measured at various pCa. ATPase versus active tension values were obtained from 10 WT and 8 E163R trabeculae. (B) Mean slopes of the force-ATPase relations in the two groups.

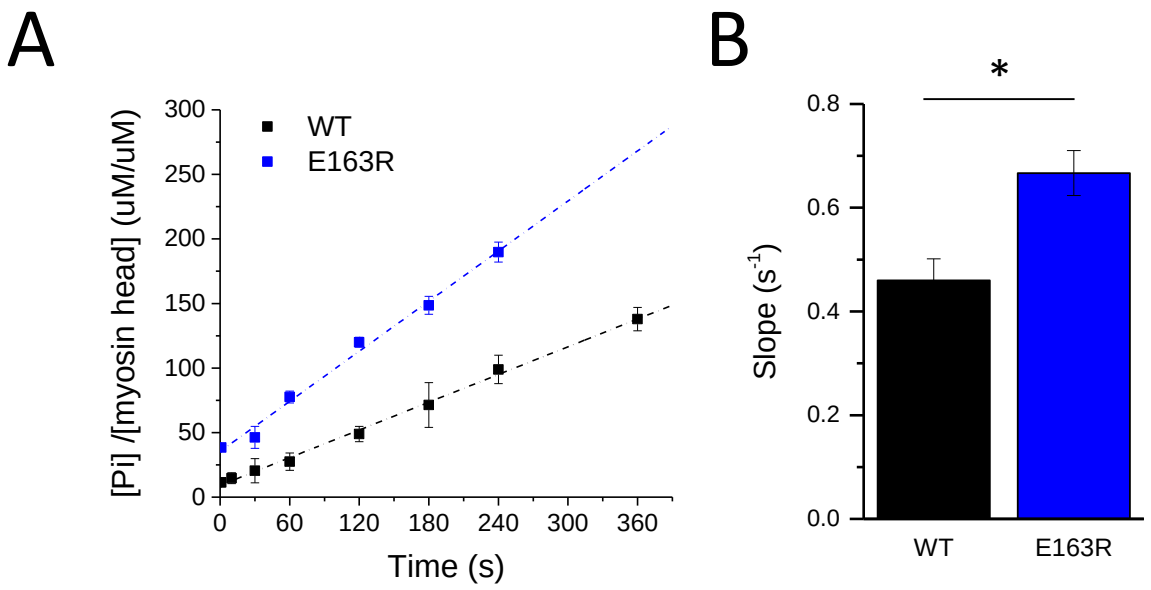

Figure S5. Steady-state ATP hydrolysis from myofibril suspensions in relaxing solution. A) Representative ATPase activity for WT (black square) and E163R (red square) myofibrils measured in relaxing condition using malachite green phosphate assay and normalized by myosin head concentration. Each point is the Mean ± SEM of 2 measurements from the same batch of myofibrils. **B)** Average slope of regression lines (s^{-1}) for WT (N=6 hearts) and E163R (N=2 hearts) myofibrils.

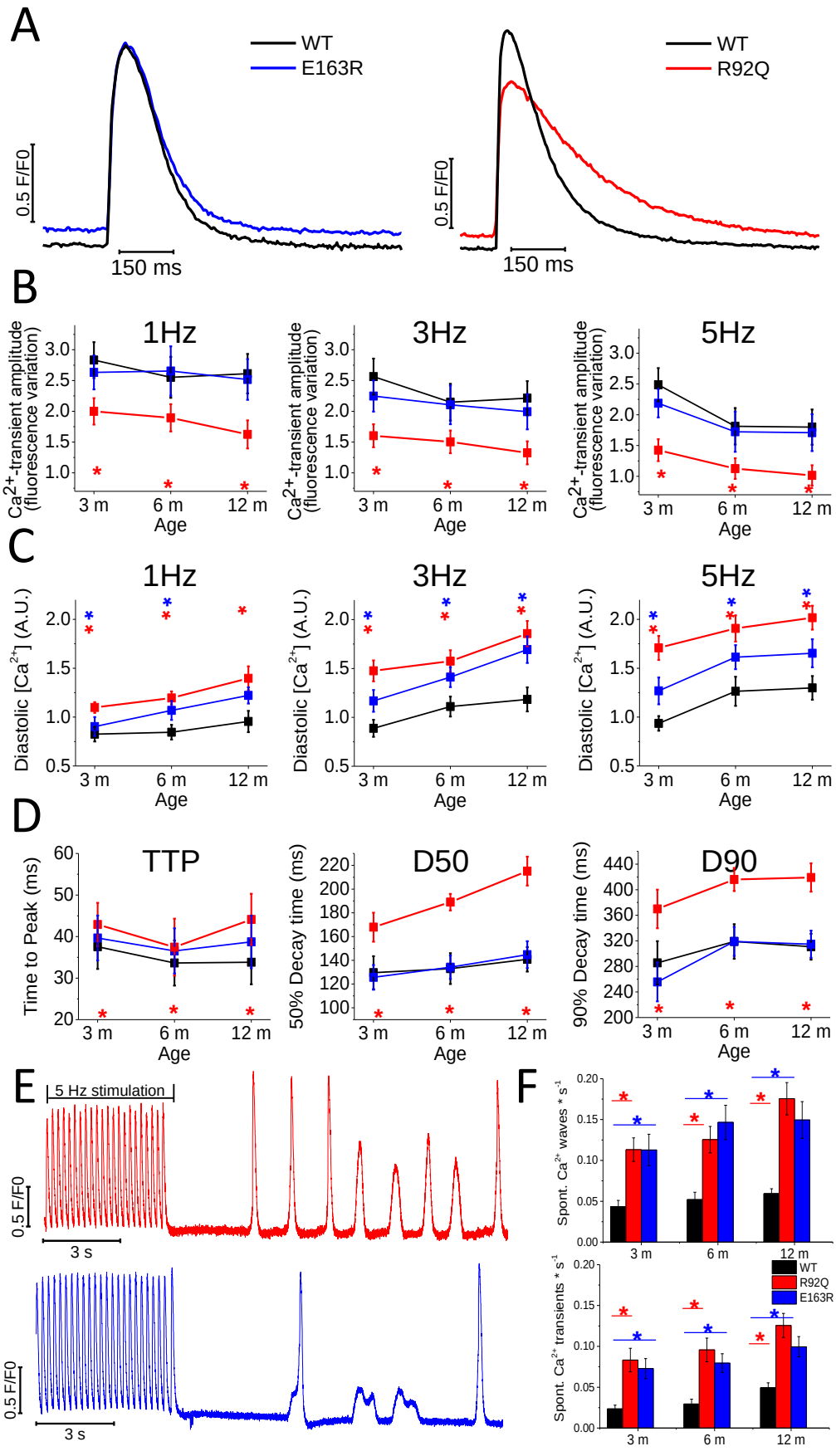

Figure S6. Intracellular Ca^{2+} handling in transgenic mice of different age. A) Representative superimposed Ca-transients elicited at 1 Hz from myocytes of 3 month-old WT and E163R mice (left), as well as WT and R92Q mice (right). **B)** Amplitude of Ca-transients at 1Hz (left), 3Hz (center) and 5Hz (right) in cells from WT, E163R and R92Q mice of differente ages (3 months, 6 months and 12 months). **C)** Diastolic [Ca] during steady-state stimulation at 1Hz (left), 3Hz (center) and 5Hz (right) in cells from WT, E163R and R92Q mice of different ages. **D)** Kinetics of Ca-transients elicited at 1Hz in cells from mice of different ages. Time to peak (TTP) on the left, Time to 50% decay (center) and Time from peak to 90% decay (right). **E)** Representative Ca-fluorescence traces of the protocol used to elicit spontaneous Ca-release events from 3 month-old mice: R92Q above and E163R below. **F)** Frequency of spontaneous Ca-release events during pauses in mice of different age: Ca-waves above and Ca-transients below. **B-D, F)** 3 months: means±SEM from 38 WT (2 mice), 44 E163R (3 mice) and 39 R92Q (2 mice) cells; 6 months: 65 WT (3 mice), 65 E163R (4 mice) and 55 R92Q (3 mice) cells; 12 months: 55 WT (3 mice), 41 E163R (2 mice) and 53 R92Q (3 mice) cells. Statistics: linear mixed models Tukey correction.

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