Supplementary Material

Reduction in Myofilament Ca^{2+} Sensitivity Partially Ameliorates the Cardiac Phenotype in Hypertrophic Cardiomyopathy Linked to a TnT-R92Q Mutation

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1. Supplementary Methods

SDS-PAGE and Immunoblotting

Excised heart samples were immediately frozen in liquid nitrogen and stored at -80°C. Heart samples (10-20 mg) were homogenized with a Bead Ruptor 24 Elite as previously described (Batra et al., 2021; Capote et al., 2021). The homogenized sample was split equally for whole homogenate and myofibril preparations. The myofibril preparation was prepared with 1% (v/v) Triton X-100 (Solaro et al., 1971). The pellet was washed with SRB without Triton X-100 and resuspended 1:5 relative to the original tissue weight in industrial-strength buffer (ISB: 8 M urea, 2 M thiourea, 50 mM Tris pH 6.8, 3% v/v SDS, 75 mM DTT, and 0.05% bromophenol blue (Fritz et al., 1989). The whole homogenate preparations were solubilized at 1:5 relative to the original tissue weight in the ISB buffer. Protein concentrations were determined with 660 nM Protein Assay (ThermoFisher, 22660) with IDCR reagent.

Whole homogenate protein samples were loaded (10-25 μg/lane) on 12 or 15% (w/v) total acrylamide SDS-PAGE gels, with 0.5% (w/v) bis-acrylamide as previously described (Fritz et al., 1989). The gels were cast in Bio-Rad's Criterion Cell for most experiments except for myosin heavy chain and regulatory light chain (RLC) separations described below. Myosin heavy chain isoform separation was carried out in 6% (w/v) total acrylamide SDS-PAGE as previously described (Warren and Greaser, 2003) and stained with Coomassie G-250 (Bio-Rad, 1610786). The RLC separations utilized Phos-tag SDS-PAGE as previously described, with minor modifications (Kinoshita et al., 2006), and with 4 μg/lane of myofibril heart sample loaded onto the gel. RLC was separated into multiple bands corresponding to unphosphorylated (U), one (P1), and two (P2) phosphorylation sites, all within the same lane, allowing simple ratio analysis. The Phos-tag gel was 12% (w/v) total acrylamide, 3.3% (w/v) bis-acrylamide, 50 μM Phos-tag, 100 μM MnCl, and poured into 1 mm-thick Bio-Rad mini gel glass plates. The gel was run in a Bio-Rad mini gel apparatus at 20 mA for 75 min at room temperature, and then the proteins were transferred to the immunoblot membrane.

The protein transfers were done as previously described with some modifications (Matsudaira, 1987). The proteins were transferred onto 0.2 μm polyvinylidene difluoride (PVDF) membrane in 10 mM CAPS pH 11.0 without methanol at 20-30 V for 90 min. The transfer of the Phos-tag gels required preincubation with 10 mM CAPS pH 11.0 and 5 mM EDTA for 10 min, repeated once, and then washed once in 10 mM CAPS pH 11.0 buffer before transferring at 30V for 90 min. After the transfer, the membranes were blocked with either 5% (w/v) non-fat dry milk (NFDM) in 50 mM Tris-HCl pH 7.5, 200 mM NaCl with 0.1% (v/v) Tween-20 (TBST) or 2% BSA-TBST. The immunoblots were incubated in primary antibodies overnight at 4°C, washed in TBST, incubated in secondary antibodies at room temperature for 1.5 hrs, and washed in TBST. See Supplemental Table 5 for the specific antibody information. The membranes were developed with ECL (ThermoFisher, 34096 or Bio-Rad, 170-5061), imaged with Chemidoc MP (Bio-Rad), and analyzed with ImageLab (Bio-Rad, v. 6.0.1). The data were statistically analyzed and graphed with GraphPad Prism v 9.3.1 or 10.0.3.

To determine overall phosphorylation levels of myofilament proteins, myofibril heart samples (7 μg/lane) were loaded onto 15% (w/v) total acrylamide SDS-PAGE. The gel was stained with a Pro-Q Diamond stain (Invitrogen, P33301) following the manufacturer's recommendations. The gel was imaged with Bio-Rad's Chemidoc MP imager, after which the gel was stained with Coomassie G-250 (Bio-Rad, 1610786) following the manufacturer's recommendations. The images were analyzed using Bio-Rad's Image Lab V 6.0.1 and Microsoft Excel 360. The data were statistically analyzed and graphed with GraphPad Prism v 9.3.1 or 10.0.3.

Echocardiography

B-mode, M-mode, pulsed-wave Doppler, and tissue Doppler images were obtained as previously described (Alves et al., 2014; Chowdhury et al., 2020) in four groups of animals. Mice were anesthetized with 3-4% isoflurane in an induction chamber, followed by maintenance at 1-3% isoflurane concentrations through a respirator. Body temperature was monitored by a rectal probe and maintained at 37°C. Electrode conduction gel was applied to the distal extremities, which were taped to electrodes. Upper abdominal and anterior chest wall hair was removed and cleaned away before applying acoustic conduction gel. The left atrial diameter was assessed by B-mode and M-mode images acquired in the parasternal long-axis window at the aortic root level. B-mode and M-mode images were used for multiple parasternal short-axis windows (apical, mid-ventricular, and basal), with the mid-ventricular/papillary level singled out for assessment of posterior and anterior wall thickness and ventricular luminal diameter during both systole and diastole to calculate fractional shortening, stroke volume, and cardiac output. The mice were then repositioned to the Trendelenburg position to obtain B-mode and pulse-wave Doppler images of the apical four-chamber window for mitral inflow measurements and tissue Doppler for septal mitral annular velocities. All measurements and calculations were averaged from three consecutive cycles and performed according to the American Society of Echocardiography guidelines. Data analysis was performed with the VevoLab 5.5.1. Analytic Software.

High-quality coronary flow velocity signals were obtained under isoflurane-induced anesthesia, as described above. The coronary vasodilator properties of isoflurane are well known, so we strictly controlled the level of isoflurane input and heart rate to ensure the accuracy of the collected data. Coronary flow measurements were performed on a modified parasternal long-axis view as previously described (Chang et al., 2015). From the low parasternal short-axis view, a search for diastolic color velocity in the anterior interventricular groove, followed by clockwise rotation to achieve alignment of the color jet, was performed. The sample volume position was consistent in all mice during the measurements.

Fibrosis Assessment

The deparaffinized sections were stained for collagen depositions (fibrosis) using the Picro Sirius Stain kit (Abcam, ab) according to the manufacturer's instructions. The Trichrome stain kit was intended for visualization of collagenous connective tissue fibers in tissue sections. Coverslips were mounted with Krystalon toluene-based mounting medium (Harleco, 64969-71). Next, images of whole heart sections were taken by a Zeiss Axio Imager Z2 (Germany) brightfield microscope with a motorized stage for tiling. Tiles (region of scanning) were fused using native Zen stitching. Analysis of fibrosis levels in whole heart scans of apex/apical and midventricular levels was done using ImageJ (NIH ver. 1.53k14) in heart sections. The Trichrome-stained fibrosis images were analyzed by taking the original RGB image color channels and selecting the color channel corresponding to the trichrome stain. The channel was then manually adjusted to the pixel threshold values that best fit the collagenous staining. The area was measured using ImageJ's Measure tool with the Limit to Threshold property enabled. The tissue/background was determined by minimal auto-thresholding of the same channel. The fraction of the collagenous area was calculated by dividing the collagenous area by the tissue area. Localized fibrosis was assessed by 2048 x 2048 square pixel window selection of regions of interest (coronary artery regions – CA, right ventricular insertion – RVI, intraventricular septum- IVS, lateral free wall- LW). Levels of fibrosis were measured as percent collagenous area to tissue area (within the scanned window).

Immunohistochemistry (IHC) and Histology

Mice were anesthetized with 5% isoflurane, and the hearts were excised and placed into cold PBS. The hearts were quickly sliced at the midpapillary level and placed into biopsy cassettes, followed by fixation in 10% neutral buffered formalin (Milipore-Sigma, HT501128), then washed and stored in 70% Ethanol. Next, samples were paraffin-embedded, and non-consecutive transverse sections were cut and applied to microscope slides (Research Histology Core, UIC). The formalin-fixed and paraffin-embedded sections were baked at 60°C and then deparaffinized with 100% xylene (2 x 7 min) followed by rehydration with incremental washes of decreasing aqueous ethanol (100% for 2 x 5 min, 95% for 5 min, 70% for 5 min, and 50% for 5 min) solutions, and washed in distilled water for 20 min and used for staining or continued on for IHC. Next, antigen retrieval was performed using sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 95°C for 40 min.

Sections were then blocked in 5% BSA in PBS Tween 20 (PBST) (0.1% Tween-20) for 1 hour at room temperature. To visualize vessels, sections were incubated in rat monoclonal anti-CD31 antibody (1:10, cat. DIA-310, Dianova) and α-SMA. To detect YAP, rabbit polyclonal anti-YAP (1:100) was used in 1% BSA TBST and incubated overnight at 4°C. Next, after three 5-minute washes with PBST, sections were incubated with secondary antibodies for 2 hours at room temperature. Next, sections were washed three times for 5 min and incubated with DAPI (4',6-diamidino-2-phenylindole) for nuclear counterstaining for 20 min at room temperature. Sections were then washed in TBST and mounted with a mounting medium preserving fluorescent signal (ThermoFisher Scientific, P10144). For a negative primary antibody (NPA) control, we omitted the primary antibodies. All sections were airy-scanned at 16-bit values in the regions of interest with a Zeiss LSM880 confocal microscope (Germany). The camera used for acquisition had a single GaAsP photomultiplier tube (PMT), and the light was filtered by emission filters (EF5) with acoustic-optical tunable filters (AOTF) to adjust the necessary brightness.

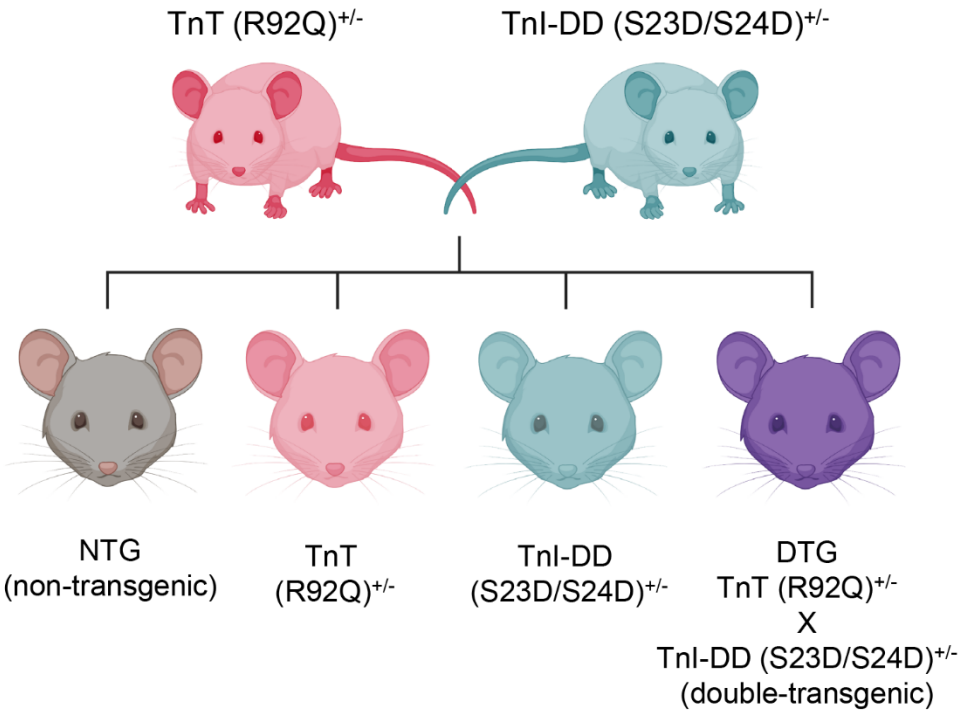
All slides were scanned at 1024 x 1024-pixel size with 16-bit depth values with an objective C-apochromatic 63x/1.2 W Korr FCS M27. Channels with their properties included: (1) Channel 1 (633nm) with gain of 800, and ILP (illumination power) 1.50%; (2) Channel 2 (561 nm) with gain of 800, and ILP (illumination power) 4.00%; (3) Channel 3 (488 nm) with gain of 850, and ILP (illumination power) 4.00%; and (4) Channel 4 (405 nm) with gain of 750, and ILP (illumination power) 1.00%. Images were acquired using ZEISS Black 2.3 SP1 software and analyzed using ZEISS Blue edition 3.2 software.

YAP signal intensity measurement in coronary vessels.

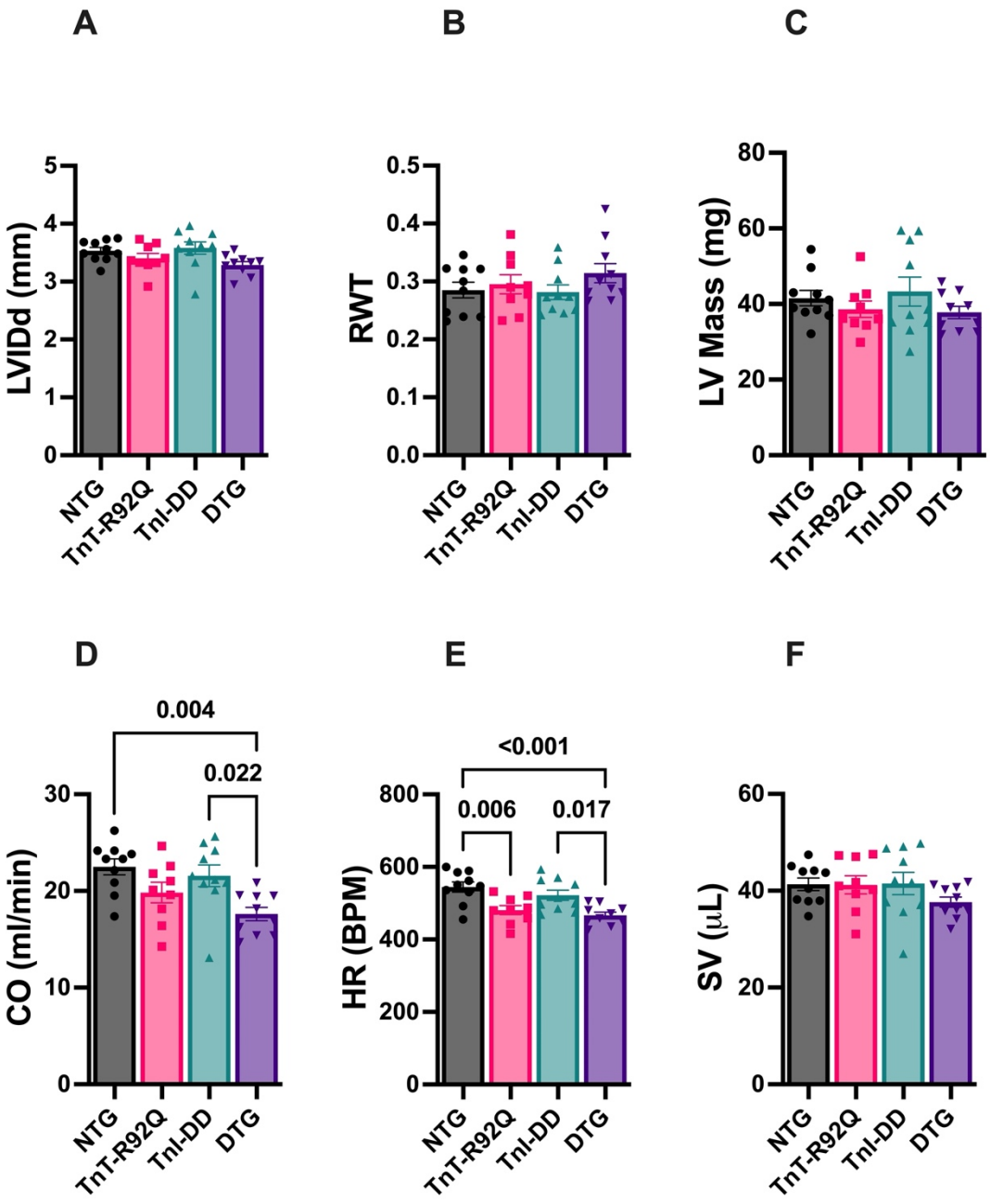
Acquired images were then uploaded to Image J ver. 1.54 for fluorescence intensity measurements. Three non-consecutive slides per animal in each group were analyzed. An area of coronary vessels was outlined, and a signal intensity corresponding to YAP mean fluorescence expressed in pixels was collected. The values were plotted onto graphs, and statistical analysis was performed (see details in Statistical analysis).

2. Supplementary Data

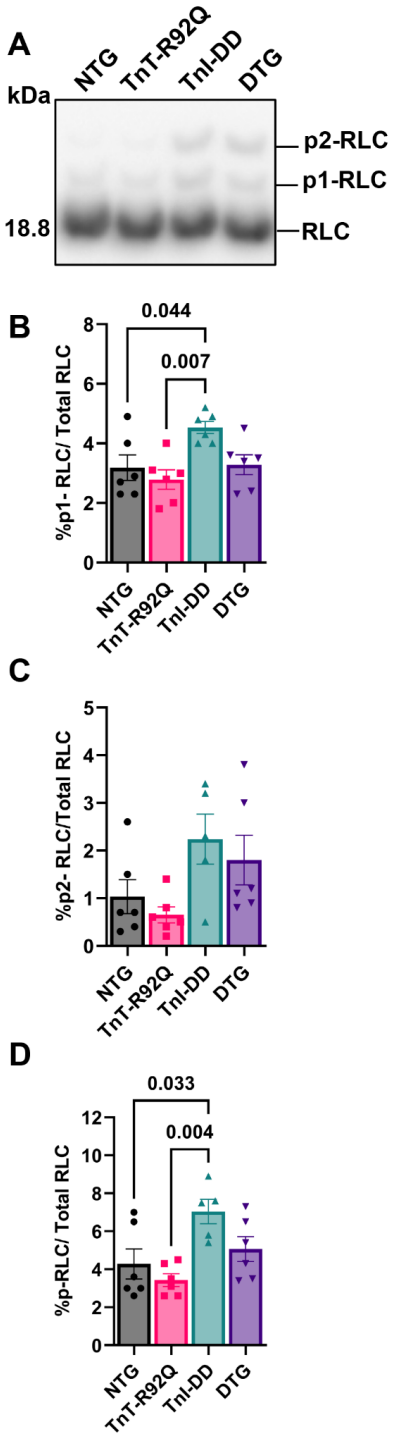
a. Supplementary Figures



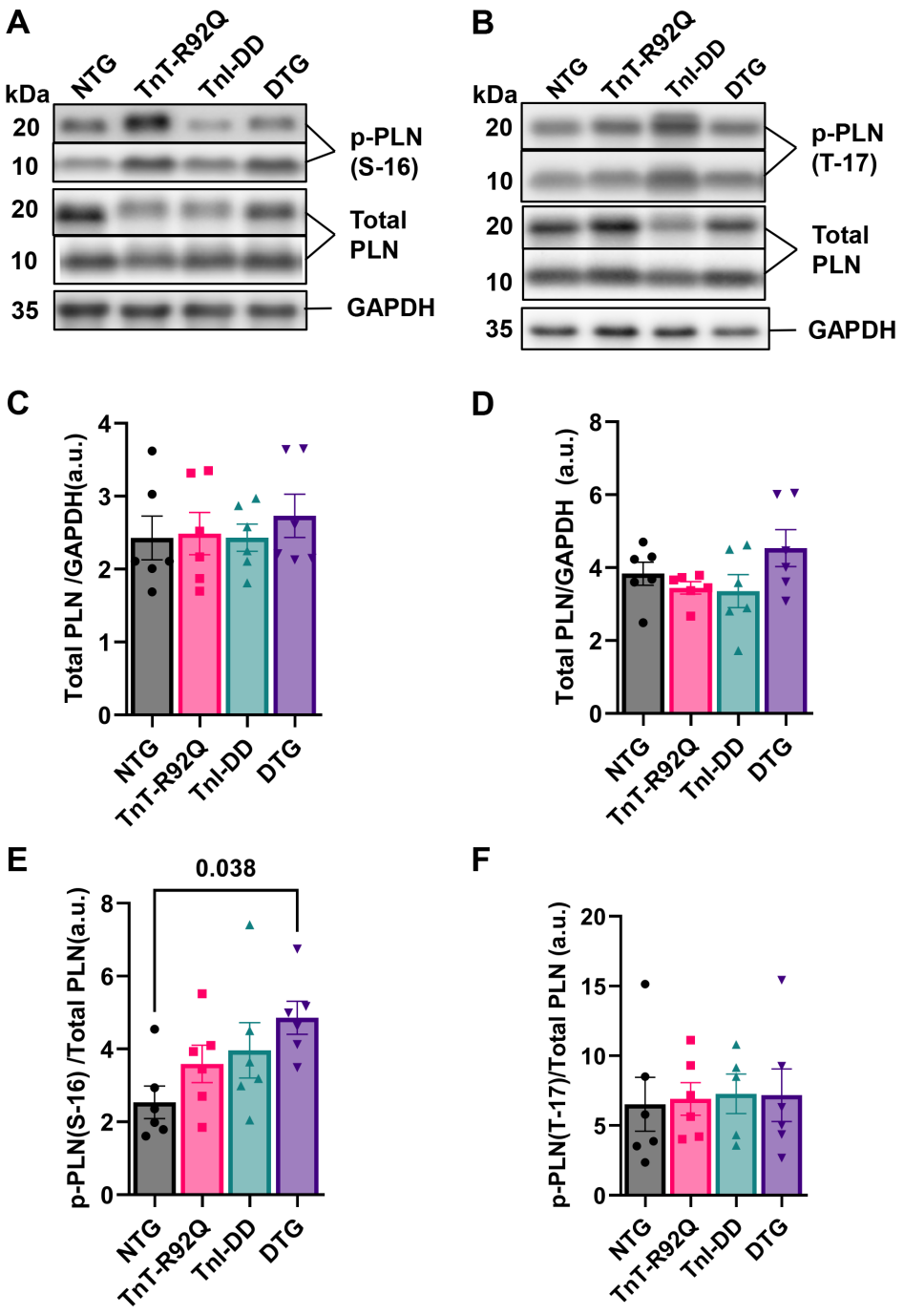
Supplemental Figure 1. The schematic representation of breeding the mice and the generation of experimental groups. NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic.



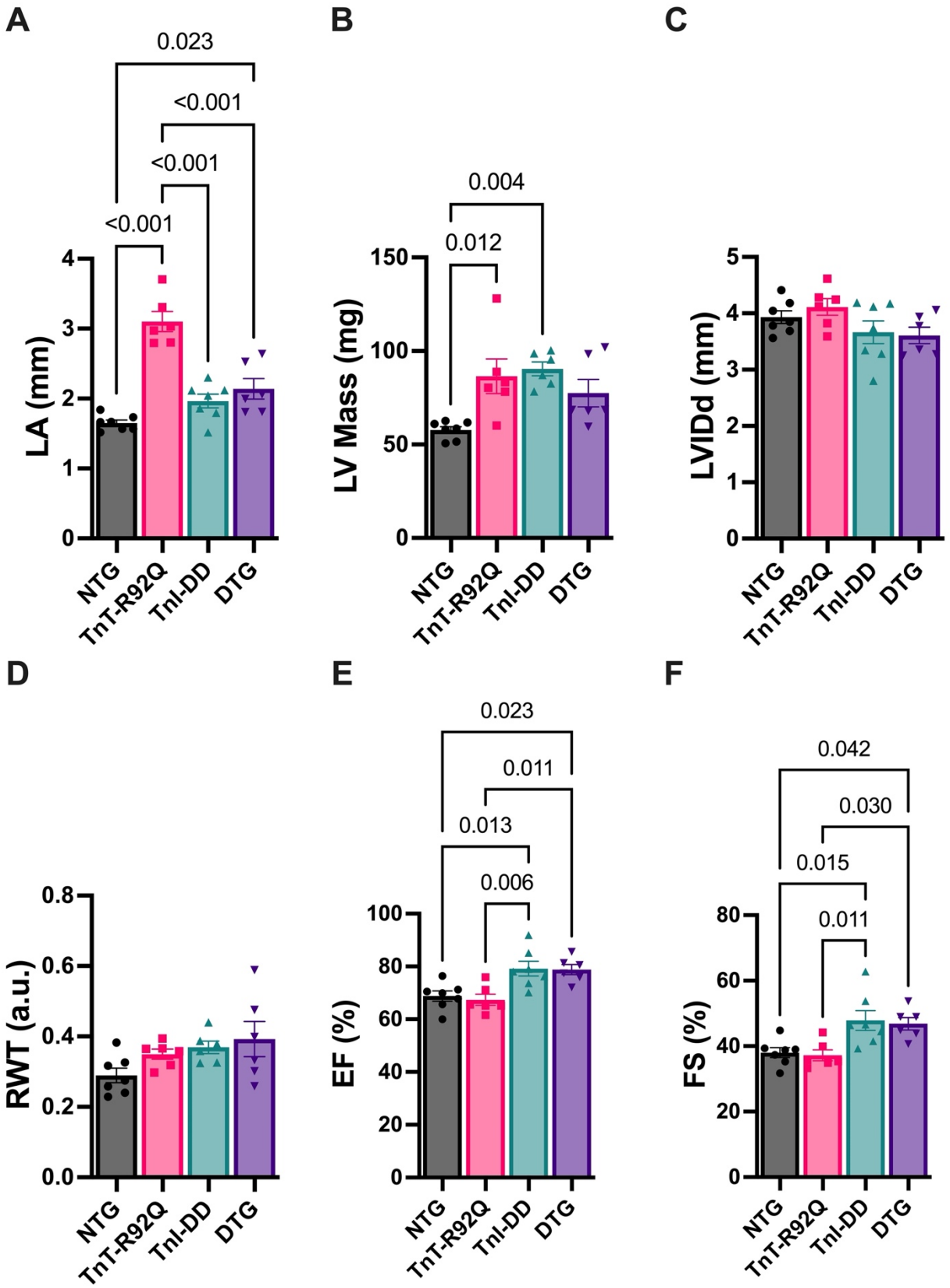
Supplemental Figure 2. Morphological, systolic, and diastolic parameters in NTG, TnT-R92Q, TnI-DD, and DTG hearts at 28 days of age. **(A)** left ventricular internal diastolic diameter (LVIDd, **(B)** relative wall thickness (RWT), **(C)** left ventricular mass calculated based on echocardiography (LV Mass), **(D)** cardiac output (CO), **(E)** heart rate (HR), **(F)** stroke volume (SV). Data are presented as mean ± SEM. n=9-10; Data were analyzed by 1-way ANOVA followed by Tukey's multiple comparisons test. NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic.

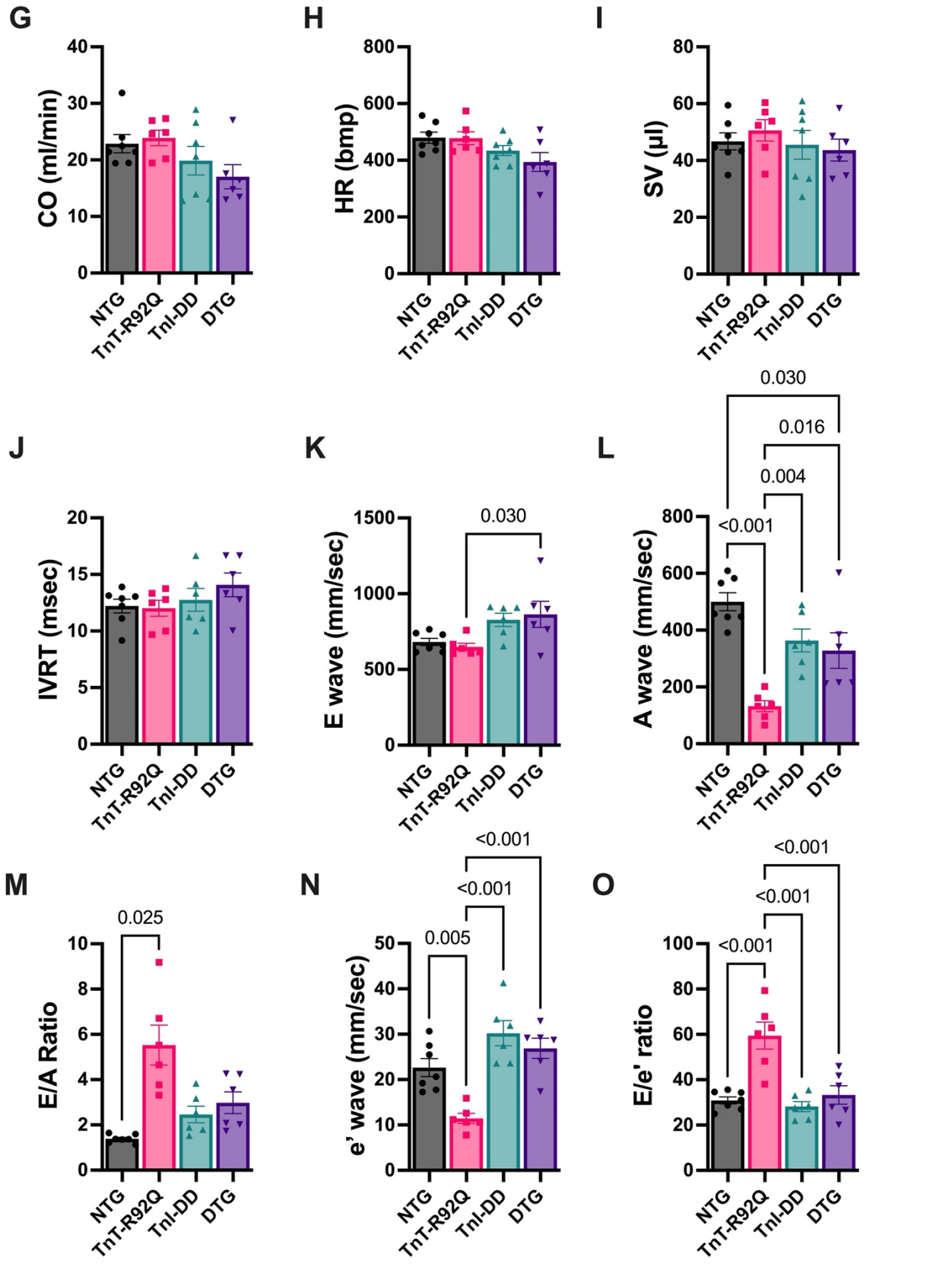


Supplemental Figure 3. **Phosphorylation (p) of the regulatory light chain (RLC) in isolated myofilaments via Western blot PhosTag separation**. **(A)** Representative Western blot PhosTag image of regulatory light chain. **(B)** Quantitation of p1-RLC site compared to total RLC abundance. **(C)** Histogram of p2-RLC site compared to total RLC abundance. **(D)** Histogram of all p-RLC sites compared to total RLC abundance. Data reported as mean ± SEM, n=5-6. Data were analyzed by 1-way ANOVA followed by Tukey's test. NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic.



Supplemental Figure 4. Total phospholamban (PLN) abundance and phosphorylation (p) in whole heart homogenates. **(A)** Representative Western blot images of p-PLN at serine 16, Total PLN, and GAPDH loading control. **(B)** Representative Western blot images of p-PLN at threonine 17, Total PLN, and GAPDH loading control. **(C)** Histogram of total PLN/GAPDH abundance. **(D)** Histogram of total PLN/GAPDH abundance. **(E)** Histogram of p-PLN (S-16)/total PLN abundance. **(F)** Histogram of p-PLN (T17)/total PLN abundance. Data reported as mean ± SEM, n=5-6. Data were analyzed by 1-way ANOVA followed by Tukey's test. NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic.


A
0.023
<0.001
<0.001
<0.001
LA (mm)
0
1
2
3
4
NTG
TnT-R92Q
TnI-DD
DTG
B
0.004
0.012
LV Mass (mg)
0
50
100
150
NTG
TnT-R92Q
TnI-DD
DTG
C
LVIDd (mm)
0
1
2
3
4
5
NTG
TnT-R92Q
TnI-DD
DTG
D
RWT (a.u.)
0.0
0.2
0.4
0.6
0.8
NTG
TnT-R92Q
TnI-DD
DTG
E
0.023
0.011
0.013
0.006
EF (%)
0
20
40
60
80
100
NTG
TnT-R92Q
TnI-DD
DTG
F
0.042
0.030
0.015
0.011
FS (%)
0
20
40
60
80
NTG
TnT-R92Q
TnI-DD
DTG


G
CO (ml/min)
40
30
20
10
0
NTG
TnT-R92Q
TnI-DD
DTG
H
HR (bmp)
800
600
400
200
0
I
SV (µl)
80
60
40
20
0
J
IVRT (msec)
20
15
10
5
0
K
E wave (mm/sec)
1500
1000
500
0
0.030
L
A wave (mm/sec)
800
600
400
200
0
0.030
0.016
0.004
<0.001
M
E/A Ratio
10
8
6
4
2
0
0.025
N
e' wave (mm/sec)
50
40
30
20
10
0
<0.001
<0.001
0.005
O
E/e' ratio
100
80
60
40
20
0
<0.001
<0.001
<0.001

Supplemental Figure 5. Morphological, systolic, and diastolic parameters in NTG, TnT-R92Q, TnI-DD, and DTG hearts at 16 weeks of age. (A) left atrial diameter (LA), **(B)** left ventricular mass calculated based on echocardiography (LV mass), **(C)** left ventricular internal diastolic diameter (LVIDd, **(D)** relative wall thickness (RWT), **(E)** ejection fraction (EF), **(F)** fractional shortening (FS), **(G)** cardiac output (CO), **(H)** heart rate (HR), **(I)** stroke volume (SV), **(J)** isovolumic relaxation time (IVRT), **(K)** peak velocity of early diastolic mitral flow (E) wave, **(L)** peak velocity of late diastolic mitral inflow A wave, **(M)** E/A ratio represents peak velocity of early diastolic mitral flow divided by peak velocity of late diastolic mitral inflow, **(N)** peak velocity of early diastolic mitral annual motion **(O)** E/e' ratio represents peak velocity of early diastolic transmitral flow divided by peak velocity of early diastolic mitral annual motion. Data are presented as mean ± SEM. n=6-7 Data were analyzed by 1-way ANOVA followed by Tukey's multiple comparisons test (panels A-C, E-L, N-O). RWT and E/A ratio data were analyzed using 1-way ANOVA, followed by Dunnett's T3 multiple comparisons test. NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic.

Supplemental Table 1. Morphological, Systolic, and Diastolic Parameters Evaluated by Echocardiography at 28 Days of Age.

Parameter		NTG (n=10)	TnT-R92Q (n=9)	TnI-DD (n=10)	DTG (n=10)
LA (mm)	**Mean**	1.52	2.12	1.53	1.64
	SE	0.058	0.059	0.084	0.054
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			<0.001	
	NTG vs. TnI-DD			>0.999	
	NTG vs. DTG			0.536	
	TnT-R92Q vs. TnI-DD			<0.001	
	TnT-R92Q vs. DTG			<0.001	
	TnI-DD vs. DTG			0.579	
LV mass (mg)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	41.54	38.63	43.30	37.78
	SE	2.040	2.174	3.810	1.617
	ANOVA P=0.404				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.4360	
	NTG vs. TnI-DD			0.6276	
	NTG vs. DTG			0.3021	
	TnT-R92Q vs. TnI-DD			0.2144	
	TnT-R92Q vs. DTG			0.8183	
	TnI-DD vs. DTG			0.1333	
LVIDd (mm)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	3.53	3.40	3.58	3.29
	SE	0.06	0.08	0.11	0.06
	ANOVA P=0.047				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.650	
	NTG vs. TnI-DD			0.972	
	NTG vs. DTG			0.129	
	TnT-R92Q vs. TnI-DD			0.396	
	TnT-R92Q vs. DTG			0.738	
	TnI-DD vs. DTG			0.051	
LVISd (mm)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	1.93	1.68	2.02	1.48
	SE	0.06	0.12	0.09	0.05
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.1868	
	NTG vs. TnI-DD			0.8504	
	NTG vs. DTG			0.0033	
	TnT-R92Q vs. TnI-DD			0.0357	
	TnT-R92Q vs. DTG			0.3528	
	TnI-DD vs. DTG			0.0004	

RWT		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	0.29	0.30	0.28	0.31
	SE	0.014	0.016	0.013	0.017
	ANOVA P=0.401				
	Tukey's multiple comparisons test			Individual P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			0.963 0.998 0.498 0.919 0.805 0.403	

EF (%)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	75.15	83.40	74.15	86.80
	SE	0.618	1.898	1.622	1.686
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			0.005 0.968 <0.001 <0.001 0.421 <0.001	

FS (%)		**NTG** (n=9)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	43.18	52.07	42.50	56.49
	SE	0.556	2.323	1.469	2.680
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			0.019 0.995 <0.001 0.008 0.401 <0.001	

CO (ml/min)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	22.50	19.83	21.57	17.62
	SE	0.819	1.066	1.124	0.682
	ANOVA P=0.004				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			0.212 0.894 0.004 0.569 0.363 0.022	

HR (bpm)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	544.7	480.8	521.9	466.9
	SE	14.16	12.47	14.02	8.48
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.006	
	NTG vs. TnI-DD			0.567	
	NTG vs. DTG			<0.001	
	TnT-R92Q vs. TnI-DD			0.120	
	TnT-R92Q vs. DTG			0.866	
	TnI-DD vs. DTG			0.017	
SV (μl)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	41.35	41.21	41.50	37.66
	SE	1.29	1.86	2.31	1.04
	ANOVA P=0.317				
	Tukey's multiple comparisons test			Individual P Value	
	NTG vs. TnT-R92Q			>0.999	
	NTG vs. TnI-DD			>0.999	
	NTG vs. DTG			0.412	
	TnT-R92Q vs. TnI-DD			>0.999	
	TnT-R92Q vs. DTG			0.471	
	TnI-DD vs. DTG			0.378	
IVRT (msec)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	10.86	12.28	9.70	13.56
	SE	0.377	0.364	0.464	0.320
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.067	
	NTG vs. TnI-DD			0.160	
	NTG vs. DTG			<0.001	
	TnT-R92Q vs. TnI-DD			<0.001	
	TnT-R92Q vs. DTG			0.116	
	TnI-DD vs. DTG			<0.001	
E wave (mm/sec)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	836.9	715.8	963.8	851.0
	SE	28.36	36.30	25.83	31.36
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.042	
	NTG vs. TnI-DD			0.025	
	NTG vs. DTG			0.987	
	TnT-R92Q vs. TnI-DD			<0.001	
	TnT-R92Q vs. DTG			0.019	
	TnI-DD vs. DTG			0.055	

A wave (mm/sec)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	666.1	469.5	552.1	592.0
	SE	27.45	46.25	24.06	26.17
	ANOVA P=0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			<0.001 0.061 0.342 0.274 0.047 0.799	
E/A Ratio		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	1.26	1.61	1.79	1.46
	SE	0.026	0.119	0.127	0.071
	Kruskal-Wallis test P value <0.001				
	Dunn's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			0.040 <0.001 0.343 >0.999 >0.999 0.177	
e' (mm/sec)		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	28.68	16.95	30.84	23.32
	SE	1.593	1.623	1.253	1.608
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			<0.001 0.743 0.074 <0.001 0.030 0.006	
E/e'		**NTG** (n=10)	**TnT-R92Q** (n=9)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	30.06	45.25	31.70	37.54
	SE	2.140	4.287	1.441	1.973
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			0.001 0.968 0.182 0.004 0.178 0.382	

NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic. Data presented as mean ± SEM. n = Sample sizes.

LA = left atrium, LV mass = left ventricle mass, LVIDd = left ventricular internal diameter at diastole, LVISd = left ventricular internal diameter at systole, RWT = relative wall thickness, EF = ejection fraction, FS = fractional shortening, CO = cardiac output, HR = heart rate, SV = stroke volume, IVRT = isovolumic relaxation time, E wave = peak velocity of early diastolic transmitral flow, A wave = peak velocity of late diastolic transmitral flow, e' – peak velocity of early diastolic mitral annular motion.

Supplemental Table 2.

Coronary Flow Parameters Evaluated by Echocardiography at 28 Days of Age.

Parameter		NTG (n=10)	TnT-R92Q (n=8)	TnI-DD (n=10)	DTG (n=10)
Diastolic AT (msec)	**Mean**	17.20	23.37	16.97	22.35
	SE	1.188	0.932	0.642	0.996
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			<0.001	
	NTG vs. TnI-DD			0.998	
	NTG vs. DTG			0.003	
	TnT-R92Q vs. TnI-DD			<0.001	
	TnT-R92Q vs. DTG			0.889	
	TnI-DD vs. DTG			0.002	
Mean Diastolic Velocity (mm/sec)		**NTG** (n=10)	**TnT-R92Q** (n=8)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	380.7	314.6	433.0	352.6
	SE	34.94	9.830	27.62	33.34
	ANOVA P=0.060				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.433	
	NTG vs. TnI-DD			0.583	
	NTG vs. DTG			0.900	
	TnT-R92Q vs. TnI-DD			0.047	
	TnT-R92Q vs. DTG			0.817	
	TnI-DD vs. DTG			0.220	
Peak Diastolic Velocity (mm/sec)		**NTG** (n=10)	**TnT-R92Q** (n=8)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	639.8	534.6	720.3	591.0
	SE	63.71	17.68	44.60	57.71
	ANOVA P=0.102				
	Tukey's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q			0.512	
	NTG vs. TnI-DD			0.674	
	NTG vs. DTG			0.902	
	TnT-R92Q vs. TnI-DD			0.085	
	TnT-R92Q vs. DTG			0.877	
	TnI-DD vs. DTG			0.284	

Mean Systolic Velocity (mm/sec)		**NTG** (n=10)	**TnT-R92Q** (n=8)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	104.5	92.07	127.9	72.82
	SE	7.532	10.95	8.544	7.609
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Individual P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			0.744 0.243 0.056 0.036 0.409 <0.001	
Peak Systolic Velocity (mm/sec)		**NTG** (n=10)	**TnT-R92Q** (n=8)	**TnI-DD** (n=10)	**DTG** (n=10)
	Mean	161.2	131.1	195.2	111.3
	SE	12.41	15.21	13.82	10.58
	Kruskal-Wallis test P<0.001				
	Dunn's multiple comparisons test			Adjusted P Value	
	NTG vs. TnT-R92Q NTG vs. TnI-DD NTG vs. DTG TnT-R92Q vs. TnI-DD TnT-R92Q vs. DTG TnI-DD vs. DTG			>0.999 0.954 0.095 0.078 >0.999 <0.001	

NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic. Data presented as mean ± SEM. n = Sample sizes. AT = acceleration time.

Table 3. Skinned Fiber Bundles Ca^{2+} Force Measurements.

Parameter	Groups			
	NTG	**TnT-R92Q**	**TnI-DD**	**DTG**
n	8	9	8	8
pCa_{50}	5.73 ± 0.021	6.07 ± 0.033	5.69 ± 0.020	5.98 ± 0.009
	ANOVA P<0.01			
	Tukey's multiple comparisons test		Adjusted P Value	
	NTG vs. TnT-R92Q		<0.001	
	NTG vs. TnI-DD		0.018	
	NTG vs. DTG		<0.001	
	TnT-R92Q vs. TnI-DD		<0.001	
	TnT-R92Q vs. DTG		0.048	
	TnI-DD vs. DTG		<0.001	
Hill Coefficient	4.56± 0.218	3.29 ± 0.273	5.39 ± 0.187	3.79 ± 0.254
	Kruskal-Wallis test P<0.001			
	Dunn's multiple comparisons test		Adjusted P Value	
	NTG vs. TnT-R92Q		0.082	
	NTG vs. TnI-DD		0.750	
	NTG vs. DTG		0.583	
	TnT-R92Q vs. TnI-DD		<0.001	
	TnT-R92Q vs. DTG		>0.999	
	TnI-DD vs. DTG		0.006	
Max Tension (mN/mm^2)	32.00 ± 2.564	23.26 ± 2.286	24.96 ± 2.472	28.00 ± 2.564
	Kruskal-Walllis test P=0.175			
	Dunn's multiple comparisons test		Adjusted P Value	
	NTG vs. TnT-R92Q		0.186	
	NTG vs. TnI-DD		0.957	
	NTG vs. DTG		>0.999	
	TnT-R92Q vs. TnI-DD		>0.999	
	TnT-R92Q vs. DTG		>0.999	
	TnI-DD vs. DTG		>0.999	

NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic.

Data are presented as mean ± SE. Data were compared using a 1-way ANOVA test, followed by the Tukey test (pCa_{50} data) or Kruskal-Wallis test, followed by Dunn's test (Hill coefficient and Max Tension data).

Supplemental Table 4. Morphological, Systolic, and Diastolic Parameters Evaluated by Echocardiography at 16 weeks of Age.

Parameter		NTG (n=7)	TnT-R92Q (n=6)	TnI-DD (n=7)	DTG (n=6)
LA (mm)	Mean	1.65	3.10	1.97	2.14
	SE	0.042	0.143	0.098	0.147
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			<0.001	
	NTG vs. TnI-DD			0.186	
	NTG vs. DTG			0.023	
	TnT-R92Q vs. TnI-DD			<0.001	
	TnT-R92Q vs. DTG			<0.001	
	TnI-DD vs. DTG			0.682	
LV mass (mg)		NTG (n=7)	TnT-R92Q (n=6)	TnI-DD (n=6)	DTG (n=6)
	Mean	57.72	86.49	90.42	77.44
	SE	1.832	9.212	3.700	7.338
	ANOVA P=0.003				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.012	
	NTG vs. TnI-DD			0.004	
	NTG vs. DTG			0.115	
	TnT-R92Q vs. TnI-DD			0.968	
	TnT-R92Q vs. DTG			0.725	
	TnI-DD vs. DTG			0.455	
LVIDd (mm)		NTG (n=7)	TnT-R92Q (n=6)	TnI-DD (n=7)	DTG (n=6)
	Mean	3.93	4.11	3.66	3.61
	SE	0.112	0.148	0.203	0.146
	ANOVA P=0.124				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.849	
	NTG vs. TnI-DD			0.607	
	NTG vs. DTG			0.485	
	TnT-R92Q vs. TnI-DD			0.217	
	TnT-R92Q vs. DTG			0.162	
	TnI-DD vs. DTG			0.994	
RWT		NTG (n=7)	TnT-R92Q (n=6)	TnI-DD (n=6)	DTG (n=6)
	Mean	0.290	0.350	0.369	0.393
	SE	0.0207	0.0144	0.0177	0.0500
	Brown-Forsythe ANOVA test P=0.131; Welch's ANOVA test P=0.077				
	Dunnett's T3 multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.184	
	NTG vs. TnI-DD			0.072	
	NTG vs. DTG			0.392	
	TnT-R92Q vs. TnI-DD			0.938	
	TnT-R92Q vs. DTG			0.942	
	TnI-DD vs. DTG			0.997	

EF (%)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=7)	**DTG** (n=6)
	Mean	68.78	67.36	79.18	78.79
	SE	1.934	2.112	2.752	1.925
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.970	
	NTG vs. TnI-DD			0.013	
	NTG vs. DTG			0.023	
	TnT-R92Q vs. TnI-DD			0.006	
	TnT-R92Q vs. DTG			0.011	
	TnI-DD vs. DTG			>0.999	
FS (%)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=7)	**DTG** (n=6)
	Mean	38.03	37.22	47.84	46.83
	SE	1.516	1.667	3.032	1.883
	ANOVA P=0.002				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.994	
	NTG vs. TnI-DD			0.015	
	NTG vs. DTG			0.042	
	TnT-R92Q vs. TnI-DD			0.011	
	TnT-R92Q vs. DTG			0.030	
	TnI-DD vs. DTG			0.988	
CO (μl/min)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=7)	**DTG** (n=6)
	Mean	22.87	23.90	19.87	17.03
	SE	1.614	1.363	2.537	2.121
	ANOVA P=0.107				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.983	
	NTG vs. TnI-DD			0.693	
	NTG vs. DTG			0.198	
	TnT-R92Q vs. TnI-DD			0.501	
	TnT-R92Q vs. DTG			0.121	
	TnI-DD vs. DTG			0.750	
HR (bmp)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=7)	**DTG** (n=6)
	Mean	479.7	478.0	434.0	393.9
	SE	19.47	22.43	17.63	33.35
	ANOVA P=0.054				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			>0.999	
	NTG vs. TnI-DD			0.489	
	NTG vs. DTG			0.072	
	TnT-R92Q vs. TnI-DD			0.553	
	TnT-R92Q vs. DTG			0.096	
	TnI-DD vs. DTG			0.624	

SV (μl)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=7)	**DTG** (n=6)
	Mean	46.71	50.61	45.55	43.66
	SE	2.991	3.762	5.063	3.842
	ANOVA P=0.691				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.903	
	NTG vs. TnI-DD			0.997	
	NTG vs. DTG			0.950	
	TnT-R92Q vs. TnI-DD			0.813	
	TnT-R92Q vs. DTG			0.650	
	TnI-DD vs. DTG			0.987	
IVRT (msec)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=6)	**DTG** (n=6)
	Mean	12.21	12.01	12.75	14.08
	SE	0.6033	0.7081	1.003	1.048
	ANOVA P=0.336				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.998	
	NTG vs. TnI-DD			0.966	
	NTG vs. DTG			0.403	
	TnT-R92Q vs. TnI-DD			0.927	
	TnT-R92Q vs. DTG			0.349	
	TnI-DD vs. DTG			0.701	
E wave (mm/sec)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=6)	**DTG** (n=6)
	Mean	680.8	648.5	827.1	863.6
	SE	23.86	24.06	43.48	85.60
	ANOVA P=0.013				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.964	
	NTG vs. TnI-DD			0.174	
	NTG vs. DTG			0.063	
	TnT-R92Q vs. TnI-DD			0.086	
	TnT-R92Q vs. DTG			0.030	
	TnI-DD vs. DTG			0.955	
A wave (mm/sec)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=6)	**DTG** (n=6)
	Mean	500.2	132.2	363.7	328.1
	SE	31.74	19.48	40.18	62.48
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			<0.001	
	NTG vs. TnI-DD			0.108	
	NTG vs. DTG			0.030	
	TnT-R92Q vs. TnI-DD			0.004	
	TnT-R92Q vs. DTG			0.016	
	TnI-DD vs. DTG			0.930	

E/A Ratio		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=6)	**DTG** (n=6)
	Mean	1.383	5.523	2.459	2.979
	SE	0.0698	0.884	0.3667	0.4777
	Brown-Forsythe ANOVA test P=0.002; Welch's ANOVA test P=0.002				
	Dunnett's T3 multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.025	
	NTG vs. TnI-DD			0.146	
	NTG vs. DTG			0.093	
	TnT-R92Q vs. TnI-DD			0.073	
	TnT-R92Q vs. DTG			0.165	
	TnI-DD vs. DTG			0.935	
e' (mm/sec)		**NTG** (n=7)	**TnT-R92Q** (n=6)	**TnI-DD** (n=6)	**DTG** (n=6)
	Mean	22.64	11.44	30.22	26.89
	SE	1.981	1.100	2.769	2.224
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			0.005	
	NTG vs. TnI-DD			0.074	
	NTG vs. DTG			0.481	
	TnT-R92Q vs. TnI-DD			<0.001	
	TnT-R92Q vs. DTG			<0.001	
	TnI-DD vs. DTG			0.695	
E/e' Ratio		**NTG** (n=7)	**TnT-R92Q** (n=7)	**TnI-DD** (n=6)	**DTG** (n=6)
	Mean	30.89	59.43	28.17	33.27
	SE	1.574	5.967	2.225	4.050
	ANOVA P<0.001				
	Tukey's multiple comparisons test			Adjusted P value	
	NTG vs. TnT-R92Q			<0.001	
	NTG vs. TnI-DD			0.952	
	NTG vs. DTG			0.967	
	TnT-R92Q vs. TnI-DD			<0.001	
	TnT-R92Q vs. DTG			<0.001	
	TnI-DD vs. DTG			0.778	

NTG, non-transgenic; TnT-R92Q - transgenic mice expressing TnT-R92Q, TnI-DD – transgenic mice expressing TnI-S23,24D, DTG - double transgenic. Data presented as mean ± SEM. n = Sample sizes. LA = left atrium, LV mass = left ventricle mass, LVIDd = left ventricular internal diameter at diastole, RWT = relative wall thickness, EF = ejection fraction, FS = fractional shortening, CO = cardiac output, HR = heart rate, SV = stroke volume, IVRT = isovolumic relaxation time, E wave = peak velocity of early diastolic transmitral flow, A wave = peak velocity of late diastolic transmitral flow, e' – peak velocity of early diastolic mitral annular motion.

Supplemental Table 5. Antibodies for Western blot and immunohistochemical staining.

Target Antibodies	Cat. number	Supplier	Dilution
WB			
Rb YAP (WB)	14074S	Cell Signaling Technology	1:1000; 5% NFDM + TBST
Rb Phospho-YAP Ser127	4911	Cell Signaling Technology	1::1000; 2% BSA + TBST
Ms Calsequestrin2	Ag13246	Proteintech	1:10000; 5% NFDM + TBST
Ms GATA4	Sc-25310	Santa Cruz	1:100; 5% NFDM + TBST
Rb phospho-GATA4 Ser105	Ab5245	Abcam	1:2000;5% NFDM + TBST
Rb ERK1/ERK2	9102	Cell Signaling Technology	1:1000; 2% BSA + TBST
Rb Phospho-ERK1/ERK2	76299	Abcam	1:2000; 2% BSA + TBST
Ms PLN	A010-14	Badrilla	1:5000; 5% NFDM + TBST
Rb Phospho-PLN Ser16	07-052	EMD Millipore	1:1000; 5% NFDM + TBST
Rb Phospho-PLN Thr17	A010-13	Badrilla	1:2500; 5% NFDM + TBST
Rb CAMKII	A010-56AP	Badrilla	1:2000; 5% NFDM + TBST
Rb Phospho-CAMKII	PA5-37833	Invitrogen	1:1000; 2% BSA + TBST
Rb SERCA2a	A010-23	Badrilla	1:20000; 5% NFDM + TBST
Ms GAPDH (HRP conjugate)	51332	Cell Signaling Technology	1:1000; 2% BSA + TBST

Rb GAPDH	2118	Cell Signaling Technology	1:1000; 2% BSA + TBST
Ms GAPDH	47724	Santa Cruz	1:200; 2% BSA + TBST
Rb alpha/beta Tubulin	2148	Cell Signaling Technology	1:2000; 2% BSA + TBST
Hs-anti-mouse-HRP 2°antibody	7076S	Cell Signaling Technology	1:20,000; 5% NFDM + TBST
Gt-anti-rabbit-HRP 2°antibody	7074S	Cell Signaling Technology	1:20,000; 5% NFDM + TBST
Ms Troponin I	10R-T123K	Fitzgerald Industries International	1:5000; 2% BSA + TBST
Ms Troponin T	564766	BD Biosciences	1:1000; 2% BSA + TBST
Rb MyBP-C	custom	Gift from Rick Moss	1:10000; 5% NFDM + TBST
Ms RLC	ALX-BC-1150-S-L001	Enzo Life Sciences	1:1000; 5% NFDM + TBST
Rt CD31 (IHC)	DIA310	Dianova	1:10; 1%BSA + TBST
Ms α-SMA (IHC)	AB7817	Abcam	1:100; 1%BSA + TBST
IHC			
Rt CD31	DIA310	Dianova	1:10; 1%BSA + TBST
Rb YAP	14074S	Cell signaling Technology	1:100; 1%BSA + TBST
Ms α-SMA	AB7817	Abcam	1:100; 1%BSA + TBST

Gt-anti-rat Alexa Fluor633	A21094	ThermoFisher Scientific	1:1000; 1%BSA + TBST
Gt-anti-rabbit Alexa Fluor568	A11011	ThermoFisher Scientific	1:1000; 1%BSA + TBST
Chicken anti-mouse Alexa Fluor 488	A21206	ThermoFisher Scientific	1:1000; 1%BSA + TBST

Abbreviations used: Ms, mouse antibody; Rb, rabbit antibody; Hs, horse antibody; Gt, goat antibody: NFDM, non-fat dry milk; TBST, Tris-buffered saline with 0.1% (v/v) Tween-20; HRP, horseradish peroxidase; BSA, bovine serum albumin.

References

Alves, M.L., Dias, F.A.L., Gaffin, R.D., Simon, J.N., Montminy, E.M., Biesiadecki, B.J., et al. (2014). Desensitization of myofilaments to Ca2+ as a therapeutic target for hypertrophic cardiomyopathy with mutations in thin filament proteins. *Circ Cardiovasc Genet* 7(2)**,** 132-143. doi: 10.1161/CIRCGENETICS.113.000324.

Batra, A., Warren, C.M., Ke, Y., McCann, M., Halas, M., Capote, A.E., et al. (2021). Deletion of P21-activated kinase-1 induces age-dependent increased visceral adiposity and cardiac dysfunction in female mice. *Mol Cell Biochem* 476(3)**,** 1337-1349. doi: 10.1007/s11010-020-03993-3.

Capote, A.E., Batra, A., Warren, C.M., Chowdhury, S.A.K., Wolska, B.M., Solaro, R.J., et al. (2021). B-arrestin-2 Signaling Is Important to Preserve Cardiac Function During Aging. *Front Physiol* 12**,** 696852. doi: 10.3389/fphys.2021.696852.

Chang, W.T., Fisch, S., Chen, M., Qiu, Y., Cheng, S., and Liao, R. (2015). Ultrasound based assessment of coronary artery flow and coronary flow reserve using the pressure overload model in mice. *J Vis Exp* (98)**,** e52598. doi: 10.3791/52598.

Chowdhury, S.A.K., Warren, C.M., Simon, J.N., Ryba, D.M., Batra, A., Varga, P., et al. (2020). Modifications of Sarcoplasmic Reticulum Function Prevent Progression of Sarcomere-Linked Hypertrophic Cardiomyopathy Despite a Persistent Increase in Myofilament Calcium Response. *Front Physiol* 11**,** 107. doi: 10.3389/fphys.2020.00107.

Fritz, J.D., Swartz, D.R., and Greaser, M.L. (1989). Factors affecting polyacrylamide gel electrophoresis and electroblotting of high-molecular-weight myofibrillar proteins. *Anal Biochem* 180(2)**,** 205-210.

Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K., and Koike, T. (2006). Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics* 5(4)**,** 749-757. doi: 10.1074/mcp.T500024-MCP200.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 262(21)**,** 10035-10038.

Solaro, R.J., Pang, D.C., and Briggs, F.N. (1971). The purification of cardiac myofibrils with Triton X-100. *Biochim Biophys Acta* 245(1)**,** 259-262. doi: 0005-2728(71)90033-8 [pii].

Warren, C.M., and Greaser, M.L. (2003). Method for cardiac myosin heavy chain separation by sodium dodecyl sulfate gel electrophoresis. *Anal Biochem* 320(1)**,** 149-151. doi: 10.1016/s0003-2697(03)00350-6.