SUPPLEMENTAL MATERIAL

Early Sensitization of Myofilaments to Ca^{2+} Prevents Genetically Linked Dilated Cardiomyopathy in Mice

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Short title: Myofilament Sensitization as a Therapy for DCM

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METHODS

Generation of new transgenic (TG) mice.

New TG mouse lines were generated by crossbreeding existing lines of mice, TG mice with mutated tropomyosin (Tm) at position 54 (TmE54K) [1] and TG mice expressing skeletal isoform of troponin I (ssTnI) [2]. All mice used in this work were in the same mixed genetic background. Four groups of mice were used for experiments: 1) NTG (non transgenic), which expresses wild-type Tm and cardiac TnI; 2) ssTnI, which expresses wild-type Tm and ssTnI; 3) Tm54, which expresses Tm54 and cardiac TnI; 4) Tm54/ssTnI (DTG), which expresses Tm54 and ssTnI.

All animal procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Review Board of the University of Illinois at Chicago.

pCa-Force relationship in skinned fiber preparation

Measurements of pCa-force relations were performed as previously described [3, 4] with slight modifications. Mice were anesthetized by intra-peritoneal injection of pentobarbital sodium (50 mg/kg). Hearts were quickly excised and left ventricle (LV) papillary muscles dissected into fiber bundles (4-5 mm long and 150-250 μm in diameter) in cold, high relaxing (HR) buffer (10 mmol/L EGTA, 6.5 mmol/L $MgCl_2$, 42 mmol/L KCl, 6.2 mmol/L ATP, 10 mmol/L creatine phosphate, 100 mmol/L BES, pH 7.0, ionic strength 180 mmol/L) plus protease inhibitors (2.5 mg/L pepstatin A, 1 mg/L leupeptin, and 50 mmol/L PMSF). Fiber bundles were next mounted between a force transducer and a

micro-manipulator using cellulose-acetate glue and skinned in high relaxing buffer containing 1% Triton X-100 for 30 minutes at 22^{o}C. Resting sarcomere length was set at 2.0 μm using laser diffraction and measurements to determine cross sectional area of the fiber were made. After skinning, the fibers were washed in HR solution and were then sequentially bathed in a series of solutions containing increasing Ca^{2+} concentrations (pCa 8 to 4.5). All experiments were performed at 22^{o}C. Isometric tension was recorded on a chart recorder and the pCa-force-relation was fitted to the Hill equation using nonlinear regression analysis with GraphPad Prism 6. From this fitted curve, we derived the pCa_{50} (pCa required to produce 50% of the maximal force obtained), max tension and Hill coefficient.

Echocardiography

Mice were anesthetized with 1% isoflurane in 100% oxygen by face mask. Body temperature was maintained at 36-37^{o}C. Echocardiography was performed using a Vevo 770 High-Resolution In Vivo Imaging System and RMVTM 707B scan head with a center frequency of 30 MHz (VisualSonics, Toronto, ON, Canada) as previously described [5, 6]. Echocardiographic studies were performed in 5 months old mice. M-Mode images of the left ventricle (LV) outflow tract (LVOT) and left atrium (LA) were taken from the left parasternal long axis view. The parasternal short axis view at the level of the papillary muscles was used to measure the LV internal dimension (LVID), inter-ventricular septal wall thickness (SWT) and posterior wall thickness (PWT). Pulsed Doppler was performed with the apical four-chamber view. The mitral inflow was recorded with

the Doppler sample volume at the tip of the mitral valve leaflets, at the center of the mitral valve orifice. In order to measure time intervals, the Doppler sample volume was moved toward the LVOT and both the mitral inflow and LV outflow were obtained in the same recording. Three parameters of the LV diastolic function were evaluated: 1) E/A ratio = maximal velocity of blood flow in the early diastole (E) / maximal velocity of blood flow in the late diastole (A); 2) E wave deceleration time (DT), which was the time from E to the end of the early diastole; and 3) LV isovolumic relaxation time (IVRT), which was the time measured from the aortic valve closure to the mitral valve opening. Additional information about the diastolic function was obtained with tissue Doppler imaging (TDI). Peak myocardial velocities in the early (E_m) and late (A_m) diastole were obtained with the sample volume at the septal side of the mitral annulus in the four chamber view. All measurements and calculations were averaged from 3 consecutive cycles and performed according to the American Society of Echocardiography guidelines [7, 8]. Data analysis was performed with the Vevo 770 Analytic Software.

In situ hemodynamic

In situ pressure-volume measurements were performed as previously described [6, 9, 10]. Mice (14-week old) were anesthetized with an initial IP dose of etomidate (10 mg/kg), morphine (1 mg/kg) and urethane (0.75-1 g/kg). The degree of anesthesia was monitored by toe pinch, and subsequent injections (one-fifth of initial dose) were administered until a reflex withdrawal of the toe was no longer observed. Mice were placed on a thermally controlled table and

maintained at 37°C via rectal monitoring of body temperature. A tracheotomy was performed using a steel intubation cannula (1.2 mm diameter; Hugo Sachs Electronik-Harvard, Germany) inserted into the airway and secured with suture. The right common carotid artery was then isolated, the distal end tied off with 6-0 suture, and the artery cannulated with an ultra-miniature P-V catheter (1.4F PVR1045, Millar Instruments, Houston, TX, USA). The transducer was advanced down the right carotid artery, into the aorta, through the aortic valve and into the LV. LV pressure (LVP) and volume were continuously monitored and digitally recorded on Chart software (v.5.5, AD Instruments). To record P-V loops in different loading conditions, an abdominal access was obtained to allow transitory vena cava occlusion right bellow the diaphragm. A right femoral vein was dissected and cannulated with a stretched PE-10 tubing to infuse 0.9% saline. This tubing was connected to a 250µL glass syringe mounted on a Model 355 micro infusion pump (Sage Instruments, Cambridge, MA).

Assessment of B-MHC abundance

The expression level of βMHC was assessed as previously described [11].

2D_DIGE Gels

Mouse cardiac tissue was flash frozen in liquid nitrogen and stored in a -80ºC freezer. Myofibrils were purified from frozen cardiac tissue as described [12] 2D-DIGE gels were run to determine the post translational modifications of troponin I, troponin T, tropomyosin, regulatory myosin light chain and myosin binding protein C. The first dimension utilized 4.7-5.9 pH 18cm IPG strips (BioRad) to fully separate tropomyosin, regulatory myosin light chain and troponin T. [13]. To

separate MyBP-C in the first dimension we ran 3-11 pH 24cm IPG strips (GE Healthcare) [14]. The troponin I was separated in the first dimension with 7-11 pH 18cm IPG strips (GE Healthcare) [15]. In all strips we loaded from 12.5-25 µg per channel (Cy2, Cy3 and Cy5) with a total of 37.5-75µg per strip. We incorporated an internal standard with all gels to normalize gel to gel variability. The 2nd dimension gel was a 12% SDS-PAGE run in a criterion gel box (BioRad) . The gel images were analyzed with PDQuest v 8.0 advanced (BioRad) and the optical densities were then statistically compared.

Western blots

Apexes from the left ventricles of 5 months old mice were homogenized in urea/thiourea sample buffer (1:20) using a ground-glass homogenizer and protein was prepared as previously described[12]. Protein concentration was determined using the RCDC Protein Assay (BioRad). For all gels 10-20 µg of protein were separated on SDS/polyacrylamide gels (10% for ERK, Akt and GSK3 and 15%) and transferred to either 0.2µm nitrocellulose or 0.2µm PVDF membrane. After transfer, the membranes were blocked for 1 hour at room temperature in blocking buffer containing 5% nonfat dry milk in TBS-T (100 mmol/L Tris/HCl, 0.9% NaCl, 0.1% Tween-20, pH 7.5). For immuno-detection, the membranes were probed with specific primary anti-rabbit antibodies from Cell Signaling, MA, USA : 1) Phospho (T202,Y204 sites of phosphorylation)-p44/42 MAPK (mitogen-activated protein kinase 1) Erk1/2 (extracellular signal-regulated kinase 1 and 2) 1:1000; 2) p44/42 MAPK (Erk1/2) 1:2500; 3) Phospho (Ser21/9) -GSK-3α/β (Glycogen synthase kinase 3 alpha/beta) 1:1000; 4) GSK-

3α/β, 1:2000; 5) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1:50,000; 6) Phospho (S473)-Akt (protein-kinase-B)1:2000; 7) Akt 1:2000. Membranes were also probed with antibodies from Abcam Cambridge, MA, USA: 1) Phospho (S105)-GATA4 (GATA binding factor-4) 1:1000; 2) GATA4 1:1000; and anti-mouse Troponin I from Fitzgerald Acton, MA, USA. The membranes were incubated with the primary antibodies at 4ºC overnight in incubating buffer (TBS-T with 1% BSA), then washed several times in TBS-T and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature (Anti-rabbit IgG HRP, 1:5,000 Promega, Wisconsin, USA or anti-mouse IgG Fab specific from Sigma St Louis, MO, USA). The HRP activity was detected using chemiluminescence reagent (Amershan ECL plus, GE healthcare, Buckinghamshire, UK) and exposure to autoradiograph film or developed using the BioRad Chemidoc XRS+ System (BioRad, Hercules, CA). All densitometry was either conducted using the ImageJ Software (National Health Institute, USA) or Image Lab (BioRad, Hercules, CA). To assess equal loading and total protein expression levels, the membranes were stripped in Restore buffer (Thermo Sci.) and probed with anti-GAPDH. Immunoblots showed modest variations in loading, and consistent total protein abundances as assessed by GAPDH. The phosphorylation ratio was therefore quantified as phosphorylation ratio = (phosphorylated/total *100%) with total = (total/ GAPDH * 100%).

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6. Data in the figures are presented as mean ± SE, n=number of samples. Data in tables are presented as mean ± SE, n=number of samples. Differences among four groups were analyzed by one-way ANOVA followed by post-hoc analysis. The name of the post-hoc test used is specified either in the figure legend or in the text. Differences were considered significant when $P<0.05$.

References

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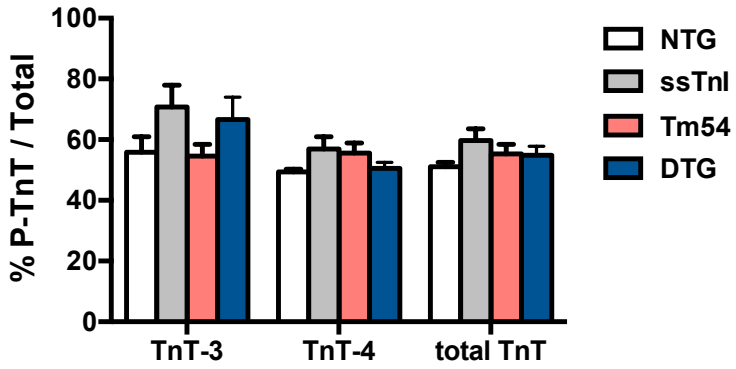
Supplemental Table 1
Diastolic and systolic function evaluated by echocardiography.

	NTG	ssTnI	Tm54	DTG
EF (%)	65.36 ± 2.175	80.13 ± 2.704*†‡	29.22 ± 1.396*	65.13 ± 2.348‡
FS (%)	35.62 ± 1.675	48.75 ± 2.423*†‡	13.79 ± 0.708*	35.62 ± 1.790‡
S_m (mm/s)	26.56 ± 1.104	26.62 ± 1.157†‡	17.37 ± 1.375*	21.86 ± 1.283*‡
HR (beats/min)	496.9 ± 8.93	472.5 ± 18.2	514.4 ± 9.32	434.4 ± 18.7*‡
SV (µL)	46.35 ± 1.910	51.62 ± 2.947‡	37.76 ± 2.382*	52.66 ± 2.048‡
CO (mL/min)	23.12 ± 1.282	24.50 ± 1.906	19.38 ± 1.146	22.93 ± 1.459
E/A	1.54 ± 0.102	1.23 ± 0.075‡	3.25 ± 0.412*	1.57 ± 0.058‡
DT (ms)	26.02 ± 1.584	26.80 ± 1.988	21.16 ± 1.802	30.54 ± 1.700‡
IVRT (ms)	13.85 ± 0.588	16.80 ± 0.703*	15.98 ± 0.576*	17.86 ± 0.934*
E_m (mm/s)	30.65 ± 1.448	24.10 ± 1.526*	25.01 ± 0.558*	24.10 ± 1.526*
A_m (mm/s)	28.20 ±1.758	28.30 ± 1.643†‡	18.85 ± 1.814*	21.25 ± 0.326*
E/E_m	34.23 ± 2.751	33.35 ± 3.641	34.02 ± 1.058	34.84 ± 2.602

Ejection fraction (EF), fractional shortening (FS), peak myocardial velocity (S_m), heart rate (HR), stroke volume (SV), cardiac output (CO), peak velocity of mitral blood inflow in early diastole (E) to peak velocity of mitral blood inflow in late diastole (A) (E/A ratio), E wave deceleration time (DT), isovolumic relaxation time (IVRT), peak myocardial velocity in early diastole (E_m), peak myocardial velocity in late diastole (A_m) and E to E_m ratio (E/E_m). The data are presented as mean ± SE (n=number of animals). Differences among groups were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparisons test. * significantly different vs. NTG; † significantly different vs. DTG; ‡ significantly different vs. Tm54; n=7-8

A.



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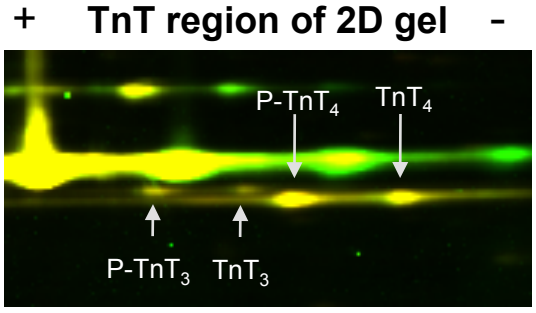
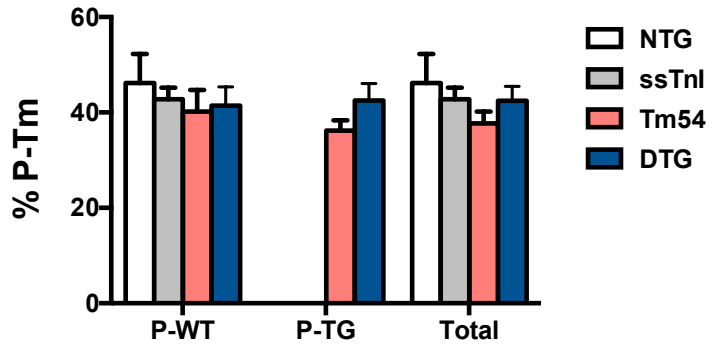


NTG Cy5 (red), Tm54 Cy3 (green)

Supplemental Figure 1.

Analysis of Troponin T (TnT) phosphorylation. **A**. Histogram of the 2D-DIGE TnT quantification. Data shown as mean ± SEM, n=6. **B.** Representative 2D-DIGE region of interest showing a merged image with NTG and Tm54 samples. No significant differences were found in TnT among the samples. TnT-3 = troponin T isoform 3, TnT-4 = troponin T isoform 4, P = site of phosphorylation.

A.



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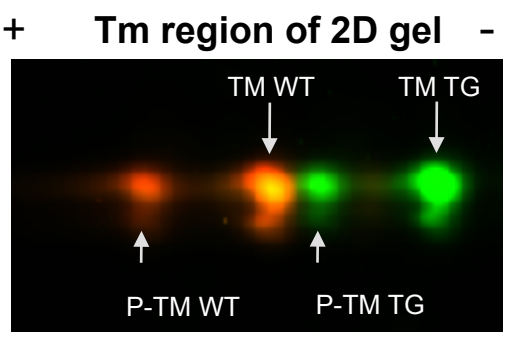
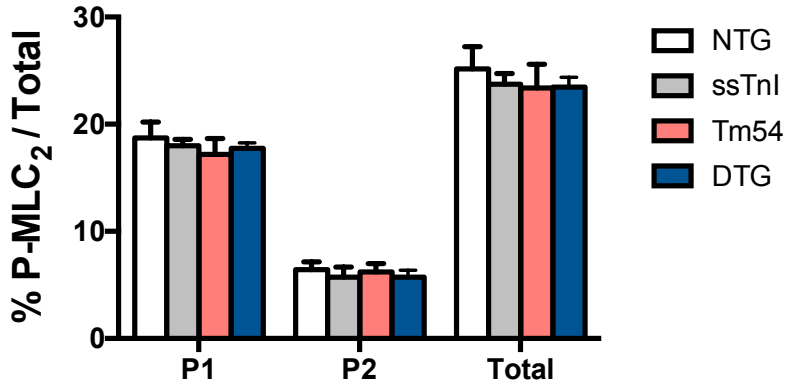


NTG Cy5 (red), Tm54 Cy3 (green)

Supplemental Figure 2

Analysis of tropomyosin (Tm) phosphorylation. **A**. Histogram of the 2D-DIGE tropomyosin quantification. Data shown as mean ± SEM, n=6. **B**. Representative 2D-DIGE region of interest showing a merged image with NTG and Tm54 samples. No significant differences were found in tropomyosin among the samples that contained tropomyosin. Note the NTG and ssTnI did not express any of the Tm54 protein. WT = wild-type tropomyosin, TG = Tm54, P = site of phosphorylation.

A.



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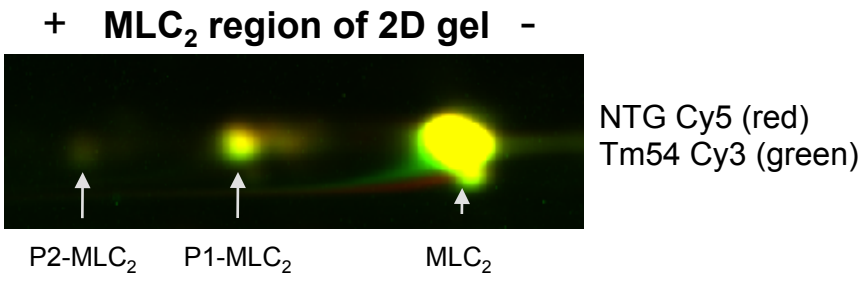
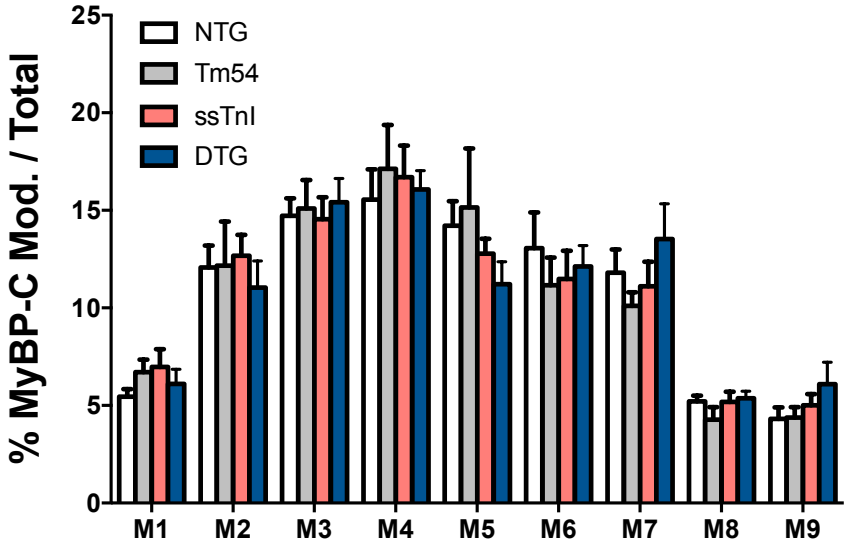


Supplemental Figure 3

Phosphorylation of regulatory myosin light chain (MLC_2). **A**. Histogram of the 2D-DIGE regulatory myosin light chain quantitation. Data represent RLC_2 phosphorylation and are shown as mean ± SEM, n=6. **B**. Representative 2D-DIGE region of interest showing a merged image with NTG and Tm54 samples. No significant differences were found in regulatory myosin light chain among the samples.

A.



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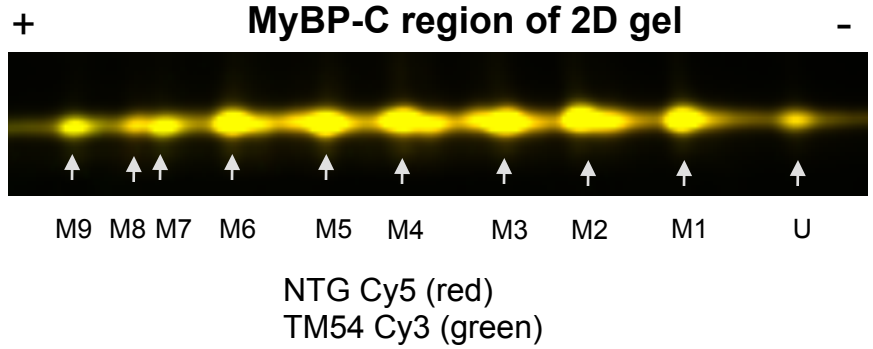

Supplemental Figure 4

Myosin binding protein C (MyBP-C) analysis of post-translational modifications. A. Histogram of the 2D-DIGE myosin binding protein C quantification. Data are presented as modification of M1 to M9 sites and are shown as mean ± SEM, n=6. **B**. Representative 2D-DIGE region of interest showing a merged image with NTG and Tm54 samples. No significant differences were found in myosin binding protein C among the samples.