Online Data Supplement

Acceleration of crossbridge kinetics by protein kinase A phosphorylation of cardiac myosin binding protein C modulates cardiac function

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ONLINE DATA SUPPLEMENT FOR EXPANDED MATERIAL AND METHODS

Myofibril Preparation

This protocol produces intact myofibrils that can be used for ATPase assay, kinase reactions, immunofluorescence, gel electrophoresis, and western blotting. The base rigor buffer consisted of KCl 60 mmol/L, $MgCl_2$ 2 mmol/L, and 3-(N-Morpholino) propanesulfonic acid 4-Morpholinepropanesulfonic acid (MOPS) 20 mmol/L at pH=7.4. One can substitute 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) 20 mmol/L pH=7.4 for MOPS. Different steps of the preparation procedure and different follow-on experiments dictated adding other components to the base rigor buffer. All work was done at 4^oC. Steps were as follows. (1) Excise heart and place immediately in rigor buffer with 10 mmol/L 2,3-butanededione monoxime (BDM) followed with gentle compressions and rinse to remove all blood. Krebs Hensleit solution (NaCl 119 mmol/L, glucose 12 mmol/L, KCl 4.6 mmol/L, $NaHCO_3$ 2.5 mmol/L, KH_2PO_4 1.2 mmol/L, $MgCl_2$ 1.2 mmol/L, $CaCl_2$ 1.8 mmol/L) after bubbling in 95%O2-5%CO2 for 30 minutes or relaxing solution (KCl 100 mmol/L, HEPES 20 mmol/L, ethylene glycol tetra acetic acid (EGTA) 2 mmol/L, adenosine triphosphate (ATP) 4 mmol/L, pH=7) was used for this step. The subsequent steps needed to be free of ATP so that crossbridges remain in rigor to maintain sarcomeric integrity. (2) Place washed hearts in 5 ml of rigor buffer with protease inhibitor cocktail (Sigma P8340 at volume:volume ratio of 1:100; P8340 consisted of [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] = AEBSF, aprotinin, bestatin, E-64, leupeptin, and pepstatin-A), EGTA 1 mmol/L , and phosphatase inhibitor cocktail (cocktail

consisted of okadaic acid 0.1 μmol/L, and Sigma P2850 at volume:volume ratio of 1:100; P2850 consisted of cantharidin, bromotetramisole, and microcystin). Alternatively, okadiac acid at 5 μmol/L can be used. The advantage of okdaic acid is its water solubility and lack of interference with kinase reactions. The phosphatase inhibition cocktail for inhibition of protein phosphatase 1A (PP-1A), protein phosphatase 2A (PP-2A), and protein phosphatase 2B (PP-2B) consisted of okadiac acid 0.5 μmol/L and cyclosporine-A 1 μmol/L. This combination provides ease of use and reduced exposure to okadiac acid. (3) Polytron 20 seconds 3-times to mechanically break the heart to myocyte sized fragments. (4) Place the polytroned solution in a Dounce homogenizer (glass pestle/tube-bulb) and pump for 35-times to further shear tissue. (5) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 10 minutes. (6) Remove supernatant. (7) Re-suspend pellet in 5ml of rigor buffer with 1% Triton-X100 and 1 mmol/L EGTA + protease inhibitor cocktail; all re-suspensions were at about 1:40 for pellet volume to solution volume ratio which is about 5 ml in most cases, (8) Place re-suspension in Dounce homogenizer and pump 5 times. (9) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 5 minutes. (10) Remove supernatant; EGTA was used to chelate intracellular calcium; however, EGTA needs to be removed for protein concentration analysis. (11) Re-suspend pellet in rigor buffer with 1% Triton-X100 + protease inhibitors and incubate for 20 minutes with gentle agitation twice during the extraction time. (12) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 5 minutes. (13) Remove supernatant. (14) Re-suspend in rigor buffer. (15) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml Falcon tube at 2500 RPM (1500g) for 5 minutes. (16) Remove supernatant, (17) Re-suspend in rigor buffer with 1 μg/μl of bovine serum albumin (BSA); this is needed to prevent clumping of myofibrils. (18) Centrifuge in Beckman J2-HC with JS4.3 rotor in 15ml

Falcon tube at 2500 RPM (1500g) for 5 minutes. (19) Repeat steps 16-18. (20) Add rigor buffer with 1 μg/μl BSA to pellet at about (1:1) ratio for maximum concentration; and measure final volume. This usually yields a final concentration of 6-8 μg/μl. (21) Take 3-samples of 10-μl volume and dilute to 50-μl for Pierce BCA protein assay (23225) and use the average of 3 to estimate concentration. (22) As necessary, place the final preparation in 50% glycerol for long term storage. The freshly prepared myofibrils will retain near full ATPase activity for about 24 hours when stored at 4^{o}C.

Western Blots

Western blots were used to determine total expression of transgenes. Sets of four different amounts of total myofibril homogenate from each mouse were loaded onto polyacrylamide gels. Western blots were done using a rabbit polyclonal anti-cMyBP-C antibody[1] at a dilution of 1:10,000. The slopes of plots of anti-cMyBP-C antibody intensity *versus* total protein load for each cMyBP-C KO mouse with insertion of a transgene was divided by the slope for a normal WT mouse on the same blot to determine the percent expression of mutant (t3SA) or WT (tWT) cMyBP-C. Mouse monoclonal anti-myc (Upstate® 05-419 clone 9E10) antibody at 1:250 dilution was used to verify expression of the transgene.

Exogenous Protein Kinase A Phosphorylation

Exogenous PKA treatment was done to phosphorylate cMyBP-C and cTnI in myofibrils and mechanical preparations. The lyophilized catalytic subunit of PKA was purchased from Sigma (P2645) and kept frozen until use. PKA was thawed on ice on the day of the experiment, subsequently equilibrated to room temperature for 12 minutes, and then re-suspended. All stock solutions and solutions containing myofibrils were equilibrated to room temperature before mixing with PKA. Deviation from the warming and equilibration sequence greatly reduced PKA activity. The protocol for phosphorylating myofibrils involved bathing 100 μg of freshly prepared myofibrils in 50 μl of solution containing PKA catalytic subunit, KCl 60 mmol/L, $MgCl_2$ 10 mmol/L, and ATP 2 mmol/L at 30^{o}C for 60 minutes. The ratio of enzyme to total myofibrillar protein (weight/weight) was 0.05 (1 unit of PKA/μl according to Sigma specifications for activity). The reaction was stopped with the addition of 4X SDS sample buffer (Tris-HCl 0.25 mol/L pH 8.8, dithiothreitol 0.32 mol/L, sodium dodecyl sulfate 12% weight/V, glycerol 40% V/V, bromophenol blue 0.08% weight/V) which solubilized the proteins. There were no detectable differences in levels of phosphorylation when the reaction was run at 30^{o}C or 22^{o}C. See stretch activation portion for exogenous *in vitro* PKA phosphorylation of the mechanical preparation.

Pro-Q Diamond Phosphoprotein and Sypro-Ruby Staining

Pro-Q Diamond phosphoprotein stain was used for detection and semi-quantative estimation of amount of phosphorylated proteins. Sypro-Ruby was used for checking for protein loading on all proteins. Myofibrils were prepared with above protocol with protein concentration estimated

by using Pierce BCA assay. Myofibrils were loaded at total protein amounts of 4 μg, 8 μg, and 12 μg into different lanes of 10% Bio-Rad Criterion polyacrylamide gels. Electrophoresis was done at 175V for 62 minutes. The following steps stained the gel for phosphorylated protein: (1) Remove gel from cassette and wash with de-ionized (ddH_2O) water twice. (2) Rock gel in fixative solution (50% methanol, 10% glacial acetic acid) on an orbital shaker for 3-sessions of 30 minutes each with complete solution exchange between each session. (3) Wash gel with ddH_2O 10-times followed by 6 sessions of 5-minute ddH_2O wash on orbital shaker. (4) From this point onward, care was taken to eliminate potential of photobleaching. All staining and washing were done in a container completely opaque to light. Furthermore, all solution changes took place in a darkened environment. (5) Stain gel with 75 ml of Pro-Q Diamond (Molecular Probes:P-33300) on orbital shaker for 90 minutes with rotating the container by 90^o at 45 minute mark. (6) Wash with ddH_2O 2-times. (7) Complete 4-sessions of 25 minutes de-staining with Pro-Q Diamond De-staining solution (Molecular Probes: P-33311) on orbital shaker with complete solution exchange for all sessions; change to new stain-free container after 2^{nd} de-stain session. (8) Complete 4 sessions of 5-minute ddH_2O wash on orbital shaker. (9) Molecular Imager F/X (Bio-Rad) with laser excitation at 532 nm and long pass filter at 555 nm was used to capture Pro-Q Diamond fluorescence image. (10) Post-stain gel with 60 ml of Sypro Ruby (Molecular Probes: S12000) over night. (11) De-stain with Sypro-Ruby de-stain solution (10% methanol, 7% glacial acetic acid) for 4 sessions of 30 minutes each with complete solution exchange for each session. (12) Wash with ddH_2O with 6 sessions of 5 minutes each. (13) UVP EC3 imaging system at UV source with 302 nm excitation filter and long pass 560 nm emission filter was used to capture Sypro-Ruby fluorescence image. (14) As necessary, secondary post-stain with Coomassie blue to provide readily visualized gel.

Phosphorylation Gel Analysis

We developed a method that minimized staining variability, optical to digital capture variability, and loading of protein of interest variability to compare phosphorylation levels of a specific protein across multiple mouse lines and conditions. Bio-Rad Laser-Pix software was used to convert images of stained gels to quantitative values for analysis. Each band of interest has its own background band. Total count intensity of band was calculated as

[Equation 1]

$$Total\ Intensity = Band\ Area\ X\ (Mean\ Intensity - Mean\ Background\ Intensity)$$

Value of 0 intensity for 0 protein load was inserted as another data point. Linear regression was done on the bands of interest from lanes with different total myofibril protein loading to calculate the slope of total count/protein load for each mouse and experiment condition. Differences in slopes reflect differences in properties that are a fraction of total amount of protein (e.g. fraction of phosphorylated cMyBP-C, fraction of cMyBP-C versus total protein …etc.). This method eliminates the possibility that total myofibril protein load differences would be interpreted as differences in fractional quantities e.g. fraction of cTnI that is phosphorylated. Calculated P-slope is the total ProQ count versus total myofibril protein load. The calculated L-slope is the total Sypro-Ruby count versus total myofibril protein load. The regression r^2 values nearly always exceeded 0.97 for slopes calculated from Pro-Q Diamond and Sypro-Ruby staining. P-slope can be described by the following.

[Equation 2]

$$PSlope\left(\frac{ProQ}{total\ myofibril\ protein\ loaded}\right) = CFF\left(\frac{ProQFluorescence}{Protein(\text{experiment})PO4}\right) \times PPF\left(\frac{Protein(\text{experiment})PO4}{Protein(\text{experiment})}\right) \times EPF\left(\frac{Protein(\text{experiment})}{total\ myofibril\ protein\ loaded}\right)$$

CFF is the captured fluorescence factor, PPF is the phosphorylated experimental protein fraction, and EPF is experimental protein fraction from the estimated total myofibril protein. CFF is a function of dye binding efficiency, fluorescence characteristics of the dye, excitation intensity, and collections electro-optics (e.g. photomultiplier gain for BioRad system, optical resolution, and charged couple device (CCD) density per pixel for UVP system). The ratio of the experiment P-slope to the WT baseline P-slope within each gel provided a value that should be free of differences in CFF between gels as the effects of CFF would be cancelled. The following set of equations describes the cancelling process. First, using equation [2], one can write the ratio of P-slopes as:

[Equation 3]

$$\frac{PSlope_{Experiment}}{PSlope_{WTBaseline}} = \frac{CFF_{Experiment} \times PPF_{Experiment} \times EPF_{Experiment}}{CFF_{WTBaseline} \times PPF_{WTBaseline} \times EPF_{WTBaseline}}$$

Since CFF within the same gel should be the same, then, $CFF_{WTbaseline}$ cancels $CFF_{Experiment}$ to yield equation 4.

[Equation 4]

$$\frac{PSlope_{Experiment}}{PSlope_{WTBaseline}} = \frac{PPF_{Experiment} \times EPF_{Experiment}}{PPF_{WTBaseline} \times EPF_{WTBaseline}}$$

Similarly, the ratio of L-Slopes of Sypro-Ruby cancels the CFFs to allow estimation of the ratio of L-slopes as:

[Equation 5]

$$\frac{LSlope_{Experiment}}{LSlope_{WTBaseline}} = \frac{EPF_{Experiment}}{EPF_{WTBaseline}}$$

Thus, equation 4 divided by equation 5 (i.e. P-slope ratio / L-slope ratio) will cancel the EPF of a specific protein to yield the ratio of phosphorylated protein of interest for an experiment to the phosphorylated protein of interest in the WT baseline.

[Equation 6]

$$\frac{\left[\frac{PSlope_{Experiment}}{PSlope_{WTBaseline}}\right]}{\left[\frac{LSlope_{Experiment}}{LSlope_{WTBaseline}}\right]} = \frac{\frac{PPF_{Exp} \times EPF_{Experiment}}{PPF_{WTBaseline} \times EPF_{WTBaseline}}}{\frac{EPF_{Experiment}}{EPF_{WTBaseline}}} = \frac{PPF_{Experiment}}{PPF_{WTBaseline}}$$

In summary, ratio of P-slopes provides independence from variance of fluorescence properties between gels, and combined ratio provides independence from variance of fraction of protein of interest as a part of total myofibril protein between sample preparations. This analysis method works well provided that a stable WT baseline exists. The summary equation from all the above now reduces to the following.

[Equation 7]

$$PL\left(\frac{experiment}{WT_{baseline}}\right) = \frac{[ProQ\_slope(experiment)/ProQ\_(WT_{baseline})]}{[Sypro\_slope(experiment)/Sypro\_slope(WT_{baseline})]}$$

A protocol of ensuring stable WT baseline phosphorylation without using pharmacological intervention was used. The protocol involves: (1) moving the mice from central housing to experiment location the night before the procedure, (2) providing a quiet environment for the over-night stay, and (3) anesthetizing each specific mouse free from sight of surgery and minimizing the scent of blood (ie. surgery done under the hood and changing gloves between each mouse.)

Dual Wavelengths Immunofluorescence

Dual wavelengths immunofluorescence of Anti-cMyBP-C and Anti-cMyc was used to demonstrate incorporation of transgenic MyBP-C. Myofibril suspension at a concentration of 2-4 μg/μl was prepared. Unless otherwise noted, all buffer was rigor buffer with 1 μg/μl of BSA. All rocking was done on an orbital shaker. A 50 μl aliquot of myofibril preparation was dropped onto a #1.5 cover slip. A 90 sec wait allowed adherence of myofibrils to the cover slip. Dipping

the cover slip in the rigor buffer washed away the unattached myofibrils. Placing the cover slip with myofibril side down onto 50 μl of 3% goat serum in a Petri dish for 15 minutes blocked non-specific binding sites. Buffer was added until the cover slip floated up, the cover slip was turned over, and the dish rocked gently for 5 minutes to wash away the goat serum. A mixture of primary antibodies was prepared ahead of time. This mixture with final dilutions included polyclonal rabbit anti-cMyBP-C antibody[1] at 1:250 dilution and monoclonal mouse anti-Myc antibody (Upstate 05-419 clone 9E10) at 1:25 dilution. The concentration of primary anti-body needed to be optimized for each specific antibody; however, increasing concentration to 40 times needed for Western blots provided a good starting point. Placing the cover slip with myofibril side down onto 50 μl of primary antibody mixture in a Petri dish started the primary antibody binding reactions. This was continued for 60 minutes at 4^{o}C without rocking in a covered Petri dish. Fresh buffer was added until the cover slip floated, the cover slip was inverted, and the dish rocked gently for 5 minutes to wash away the primary antibodies. A mixture of secondary antibodies was prepared ahead of time. All handling procedures involving fluorescence secondary antibodies were done in lowest possible light conditions. The secondary antibodies mixture with final dilutions consisted of Molecular Probes goat anti-rabbit AlexaFluor647® at 1:250 dilution and Molecular Probes goat anti-mouse AlexaFluor488® at 1:250 dilution. The cover slip with myofibril side down was placed onto 50 μl of the secondary antibody mixture in a Petri dish. The incubation was continued for 30 minutes at room temperature without rocking in a covered Petri dish that was completely protected from light. Two 5-minute washes as described earlier removed the secondary antibodies. The cover slip with myofibril side down was placed onto 200 μl of 4% para-formaldehyde and incubated for 10 minutes to fix the samples. Dilution with wash buffer and two 5-minutes washes removed the para-formaldehyde. A drop of

Molecular Probes Prolong Gold (P36930) antifade mounting reagent was placed on a microscope slide. The cover slip with myofibril side down was carefully placed onto the drop of antifade mounting reagent without trapping bubbles. The cover slip was immobilized on the slide with clear finger nail polish application to its 4 corners. After 24 hours of curing, the edges of the cover slip were sealed with clear finger nail polish. A Zeiss LSM 5 Pascal microscope system with Plan Apo-chromatic 100X oil immersion objective was used for imaging. The initial locating of myofibrils and focus adjustments were done using the UV light source under visual guidance. Actual imaging was done using laser excitation, optical filters, and photomultiplier. A HeNe laser with 633 nm excitation and a 650 nm long pass filter generated signals to create images for anti-cMyBP-C immunofluorescence. An argon laser with 488 nm excitation and 505-530 nm band pass filter generated signals to create images for anti-cMyc immunofluorescence.

Strech Activation on Skinned Myocardium

Stretch activation was assessed in skinned myocardium[2, 3] to quantify the effects of PKA phosphorylation of regulatory proteins on crossbridge kinetics. The protocol was as follows: Sarcomere length was set to 2.1 μm, an initial activation was done at pCa 4.5 (pCa = $-\log_{10}[Ca^{2+}]$) to establish maximum force, pCa was varied to determine pCa for half-maximal force (i.e. pCa 5.75), and finally a rapid stretch equivalent to 1% of fiber length was applied and held for 5 seconds. PKA phosphorylation was done using 1-unit PKA/μL at 22^{o}C instead of 30^{o}C to prevent potential degradation of skinned myocardium.[2] The PKA phosphorylation solution was washed out by 4-times volume exchange. Phosphate incorporation due to PKA phosphorylation was assessed from similarly treated myofibrils, as described above. The forces

measured were P_0, the pre-stretch baseline; P_1, the force difference between P_0 and the peak force immediately post-stretch; P_2, the force difference between P_0 and the minimum at the end of the post-stretch decay; P_3, the force difference between P_0 and the peak of delayed force development; and P_{df}, the force difference between P_3 and P_2. Rate constants k_{rel} and k_{df} were determined for the P_1 to P_2 force decay and the P_2 to P_3 delayed force development (stretch activation), respectively. Fitting $A(1-\exp(-k_{rel} \cdot t))$ to the force decay from the peak force immediately post stretch (P_1) to the minimum force (P_2) yielded rate constant k_{rel}, where A is amplitude and t is time. Calculating the inverse of the half-time for delayed force development from P_2 yielded rate constant k_{df}. Forces were normalized to P_0 to allow comparisons between experiments.

Echocardiography

Echocardiography was done using a Visualsonics Vevo 770 system with a 30 MHz probe to record intact *in vivo* cardiac structure and function. Left ventricular (LV) chamber dimensions, LV posterior wall (PW) thickness, and fractional shortening (FS) were measured using M-mode in the parasternal short-axis view at the level of the papillary muscle. Ejection fraction (EF) was estimated using Vevo 770 software from the 2-dimensional parasternal long-axis view of the left ventricle in the EKV™ (EKG-based kilohertz visualization) mode. The EKV™ mode reconstructs one averaged cardiac cycle cine with 1000 frames per scan line; thus, the reconstructed cine is averaged from greater than 1000 actual heart beats. Blood flow Doppler and tissue movement Doppler were done in the apical 4-chamber view. Isovolumetric relaxation time (IVRT) was measured from the blood flow Doppler spectra. The ratio of the blood flow Doppler

peak (E) at mitral inflow to the tissue movement Doppler peak (e') at the lateral mitral annulus (E/e')[4] provided another index of diastolic function. With the exception of EF (which is already essentially averaged over ~ 1000 cardiac cycles), all measured parameters were averages from at minimum of 3 cardiac cycles. TH performed and CT read all the echocardiography.

Dobutamine (10 μg/g) was administered by intraperitonial (IP) injection to elicit a maximum β_1 response that was sustained for 15 minutes[5] in order to maximize *in vivo* PKA phosphorylation. Dobutamine was injected IP after the baseline measurements were completed. Post-dobutamine imaging was initiated 8 minutes after IP injection. Phosphoprotein gel staining of contractile proteins from similarly treated mice showed that phosphorylation peaked 10 minutes after injection, followed by a slight decrease in phosphorylation at 15 minutes (data not shown). This post-dobutamine injection time window allowed only EF and FS to be recorded for assessment of dobutamine augmentation.

Mice were maintained in a consistent controlled state. Anesthesia was maintained with continuous inhalation of isoflurane. Body temperature was kept constant at 37^{o}C using a heat lamp and a heating pad with continuous feedback control. Continuous cardiac monitor followed the heart rate. Anesthesia was adjusted to ensure heart rate did not fall below 400 beats per minute (BPM) and mice did not wake-up during the procedure. All mice reported in the study had complete recovery from procedure.

Mouse Age for β-Myosin Heavy Chain Expression

Mice from all four lines of: (1) normal wild type "WT", (2) cMyBP-C knock-out "KO", (3) transgenic expression of non-PKA-phosphorylatable mutant cMyBP-C onto KO background "t3SA", and (4) transgenic expression of WT cMyBP-C onto KO background "tWT" were aged to 90 ± 7 days at the time of heart extraction to ensure age differences did not contribute to differences in relative β myosin heavy chain (βMHC) expression with respect to (αMHC).

Colloidal Coomassie Staining for MHC

Colloidal coomassie blue (CCB) staining on large format (18 X 16 cm) 6% gel[6] was chosen as the initial assessment of βMHC expression due to its stoichiometric binding to protein, sensitivity, and maximum separation. Myofibril preparations were made from hearts of 4 different mice on each line of mice. Stacking gels contained 3%T, 15%C, 10% glycerol, 0.13 mol/L Tris (pH 6.8) and 0.1% SDS with N-N' diallyltartardiamide (DATD) used as the cross-linker. Separating gels contained 6%T, 2.6%C, 10% glycerol, 0.37 mol/L Tris (pH 8.8) and 0.1% SDS with DATD as the cross-linker. %T and %C are calculated as the following.

[Equation 8]

$$\%T = \left(\frac{acrylamide\ (mg) + \ DATD\ (mg)}{volume\ (ml)}\right) \times 100$$

[Equation 9]

$$\%C = \left(\frac{DATD\ (mg)}{acrylamide\ (mg) + \ DATD\ (mg)}\right) \times 100$$

Gel polymerized in room temperature for 1 hour and overnight at 4°C. Electrophoresis was done using a Hoefer SE 600 Vertical Electrophoresis Unit with 0.75 millimeter spacers and a Bio-Rad PowerPac 300 power supply. The running buffer consisted of 25 mmol/L Tris (base), 192 mmol/L glycine and 0.1% SDS, pH 8.3. The gels ran at constant current of 16 mA for 9 hours at 4°C. Coomassie blue staining was done for 16 hours. The composition is as follows: 10% phosphoric acid, 20% methanol, 10% ammonium sulfate, 0.12% G-250 Coomassie.[7] Gel was de-stained in ddH_2O for minimum of 3 hours or until background became clear. Gels were imaged with UVP EC3 system. The relative proportions of α and β MHC isoforms were determined by densitometric analysis of CCB stained gels using LaserPix software.

Dual Wavelengths Fluorescent Western Blot for MHC Isoforms

We adapted a dual wavelengths fluorescence based western blot method to analyze α and β MHC expression to (1) confirm colloidal coomassie findings, (2) reduce the time required from 4 days for colloidal coomassie staining to 1 day for dual wavelength fluorescence western blot, and (3) provide a more easily manipulated final product than large 6% gel for handling and imaging. Aliquots of myofibril preparations from 16 hearts (4 mice from each of 4 lines) were used. Electrophoresis was done on 5% Bio-Rad Criterion gel at 150 volts for 4 hours at 4°C with pre-cooled running buffer. Replacing existing running buffer with pre-cooled running buffer at 4°C

every 80 minutes was necessary to maintain the integrity of gel and to produce sharp bands. Protein bands were transferred to 0.45 μm nitrocellulose membranes under conditions of 55 volts for 90 minutes at 4^{o}C with transfer buffer (20% methanol, 0.1% SDS, 0.3% Tris, 1.4% glycine). PVDF membrane had too much background noise. Using 0.2 μm nitrocellulose membrane captured more protein at the cost of an increase in background noise. Membranes was blocked with Odyssey blocking buffer (Li-Cor 927-4000) for 1-hour at room temperature. Mouse monoclonal anti-total MHC antibody (Chemicon, MAB1552) at 1:100 dilution and rabbit polyclonal anti-βMHC (Sigma/Atlas, HPA00129, affinity isolated) antibody at 1:500 dilution were suspended in Odyssey blocking buffer with 0.1% polyoxyethelene sorbitan monolaurate (Tween 20) and were incubated with the membrane for 1-hour in room temperature with gently orbital shaking. Changing to new container, immediate wash with Tris-buffered saline with Tween "TBST" (NaCl 0.15 mol/L, Tris 0.02 mol/L pH 7.4 , 0.05% Tween 20), and followed by 4 session of 10-minute washes with TBST cleared non-specific binding. Secondary fluorescent antibodies consisted of IRDye800CW goat anti-mouse (Li-Cor, 926-32210, cross-adsorbed, 778 nm excitation/795 nm emission) at 1:10,000 dilution and Alexa Fluor 680 goat anti-rabbit (Molecular Probes, A21109, highly cross-adsorbed, 679 nm excitation/702 nm emission) at 1:10,000 dilution. From this point onward, all steps were done in minimal lighting to prevent photo-bleaching. A mixture of secondary antibodies was incubated with the membrane for 50 minutes in room temperature with gentle orbital shaking and completely shielded from all light. All washes were done with TBST. Changing to a new container, an immediate wash followed by one 5 minute wash, 2 times of 30 minutes, and then 3 times of 5 minutes greatly reduced background noise. An Odyssey infrared imaging system (Li-Cor) provided the double wavelength excitation and captured the double wavelength emission image. Anti-total

MHC/IRDye800CW fluorescence reported the amount of total MHC. Anti-βMHC/Alexa Fluor 680 fluorescence reported the amount of βMHC. Quantification was done using the Odyssey software. A dried membrane can be imaged repeatedly over multiple days without significant changes.

Determination of Titin Phosphorylation

Cardiac titin phosphorylation of in all 4-lines were done using the previously mentioned methods of myofibril preparation and Pro-Q Diamond phosphoprotein staining with followed by Sypro-Ruby total protein staining. The following protocol was used to produce minimally phosphorylated myofibrils for use as a comparative standard across all gels: lightly anesthetize WT mouse with isoflurane, injection metoprolol (specific β_1 adrenergic blocker) IP at 5 μg/g (drug/body weight) dose, allow mouse to recover for 30 minutes, and then re-anesthetize mouse for removal of heart. The following precautions and modification were used to optimize detection of titin phosphorylation: (1) freeze solubilized myofibril suspension in 50% glycerol to -80^{o}C immediately and use within 3 months, (2) add β-mercaptoethanol to a final concentration of 10 mmol/L in top well of Bio-Rad Criterion cassette just before electrophoresis to minimize protein cross-linking during the migration, (3) use 7.5% gel, (4) use myofibrils from metoprolol treated WT heart (WT_{bBlock}) as a comparative baseline so that WT basal phosphorylation could be included in the analysis.

[Equation 10]

$$PL\left(\frac{experiment}{WT_{bBlock}}\right) = \frac{[ProQ\_slope(experiment)/ProQ\_(WT_{bBlock})]}{[Sypro\_slope(experiment)/Sypro\_slope(WT_{bBlock})]}$$

ONLINE DATA SUPPLEMENT FOR EXPANDED RESULTS

βMHC Expression

Colloidal Coomassie Blue (CCB) staining with corroborating dual wavelength western blotting (2WL-WB) showed that t3SA line has increased βMHC expression of at most ~10% of total MHC (Online Figure I and Online Table I) in comparison to WT at 3-months of age but tWT had similar βMHC expression as WT. Both CCB staining and 2WL-WB showed that comparative expression of βMHC across all four lines is KO > t3SA > tWT=WT (Online Figure I and Online Table I). As supported by similar stretch activation kinetics at basal conditions between WT, tWT, and t3SA, this increase in βMHC expression did not affect function.

Differences in age of mice and methodology can explain the small increase in measured % βMHC from our prior reported value of ~13%[8] to the current value of ~19%. Mice in this study are about 4-weeks older than in the prior study. CCB and 2WL-WB have different protein binding characteristics than the silver staining used in the prior study.[8]

Titin Phosphorylation

ProQ-Diamond phosphoprotein staining and subsequent Sypro-Ruby total protein staining to adjust for loading differences showed that basal titin phosphorylation is similar across all 4-lines (WT = KO = t3SA = tWT, see Online Figure II and Online Table I).

Steady State Force/pCa Measurements

Myocardium from all 4-lines showed similar steady state force response to variations in activating calcium at basal conditions and after PKA treatment (see Online Table II). PKA treatment did not change maximum force but did decrease calcium sensitivity across all 4-lines (see Online Table II). Thus the PKA-induced decrease in calcium sensitivity does not involve phosphorylation of cMyBP-C but instead is due to phosphorylation of cTnI.[9]

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ONLINE DATA SUPPLEMENT FIGURE LEGENDS

Online Figure I: Both colloidal coomassie blue (CCB) staining and dual wavelength fluorescence western blot (2WL-WB) showed corroborating % expression of βMHC of KO > t3SA > tWT=WT. 2 μg and 1.25 μg of myofibril from each heart were loaded onto gels for CCB staining and 2WL-WB respectively. (I.A) Gray scaled image showed CCB staining of both αMHC and βMHC. Red showed anti-total MHC fluorescence. Green showed anti-βMHC fluorescence. Yellow showed matched portion of anti-total MHC attributable to βMHC. (I.B) % βMHC expression was calculated from CCB image. Separately, % βMHC expression was also calculated from anti-total MHC fluorescence image using matching yellow band as confirmatory guide for portion of total MHC attributable to βMHC. n=4 hearts for each line, * denotes $p < 0.05$ *vs.* WT, # denotes $p < 0.05$ *vs.* KO. (I.C) alternatively, ratio of anti-βMHC fluorescence *vs.* anti-total MHC fluorescence from was calculated. n=4 hearts for each line, * denotes $p < 0.05$ *vs.* WT, # denotes $p < 0.05$ *vs.* KO.

Online Figure II: All 4-lines showed similar basal titin phosphorylation. (II.A) Example of ProQ phosphoprotein and Sypro-Ruby total protein staining. Titin appeared as the most consistently phosphorylated protein across all mice on ProQ staining. ProQ staining also verified that t3SA and βBlock (metoprolol) treated WT have decreased cMyBP-C phosphorylation. Sypro-Ruby staining showed similar protein loading on all lines. Sypro-Ruby also verified that KO is missing cMyBP-C. (II.B) n=4 hearts for all lines. There is no detectable difference in titin phosphorylation across all lines (t3SA = tWT = WT = KO).

Online Table I: Summary of βMHC expression and Titin Phosphorylation Values

Measurement Means ± SEM	WT, n=4	KO, n=4	t3SA, n=4	tWT, n=4
% βMHC by CCB	0#	18.7 ± 1.2*	8.5 ± 1.8*#	0.8 ± 0.8 #
% βMHC by 2WL-WB	0.9 ± 0.1	19.4 ± 0.7*	9.9 ± 1.3*#	2.4 ± 1.1#
βMHC IInt/total MHC IInt	0.013 ± 0.001	0.109 ± 0.006*	0.038 ± 0.008*#	0.013 ± 0.001#
% Titin phosphorylation *vs.* WT treated with βBlock	70.8 ± 16.5	73.9 ± 8.7	63.4 ± 8.6	61.6 ± 9.5

"*" denotes $p < 0.05$ *vs.* WT. "#" denotes $p < 0.05$ *vs.* KO. Abbreviations are: colloidal coomassie blue (CCB), dual wavelength fluorescence western blot (2WL-WB), Integrated Fluorescence Intensity (IInt). Metoprolol 5μg/g (drug/body weight) IP was used as βBlock at 30 minutes prior to heart extraction.

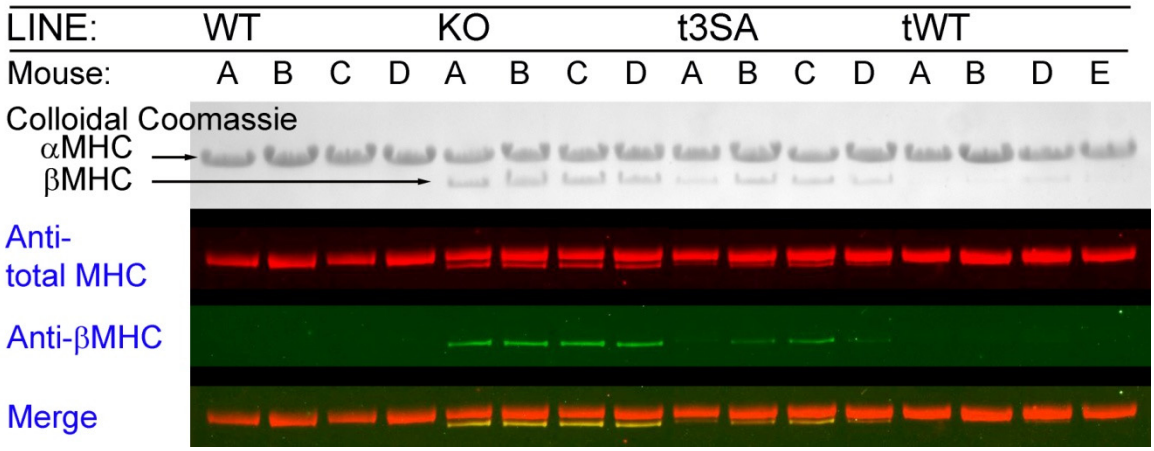
Online Table II: Summary of Steady State Force/pCa Results

	WT, n=8	tWT, n=8	t3SA, n=8	KO, n=8
FMax (mN/mm^2)	19.26 ± 2.06	18.93 ± 1.76	19.25 ± 1.47	20.01 ± 2.07
FMax + PKA (mN/mm^2)	18.54 ± 2.19	18.24 ± 1.87	18.36 ± 1.33	18.73 ± 1.84
Fmin (mN/mm^2)	0.74 ± 0.11	0.71 ± 0.13	0.69 ± 0.11	0.74 ± 0.13
Fmin + PKA(mN/mm^2)	0.65 ± 0.11	0.63 ± 0.10	0.59 ± 0.08	0.63 ± 0.12
n_H	3.80 ± 0.18	3.78 ± 0.23	3.80 ± 0.22	3.76 ± 0.15
n_H+PKA	3.71 ± 0.20	3.86 ± 0.20	3.85 ± 0.19	3.76 ± 0.13
pCa_{50}	5.76±0.02	5.75±0.02	5.76±0.02	5.73±0.02
pCa_{50}+PKA	5.65±0.03*	5.65±0.02*	5.68±0.03*	5.65±0.02*

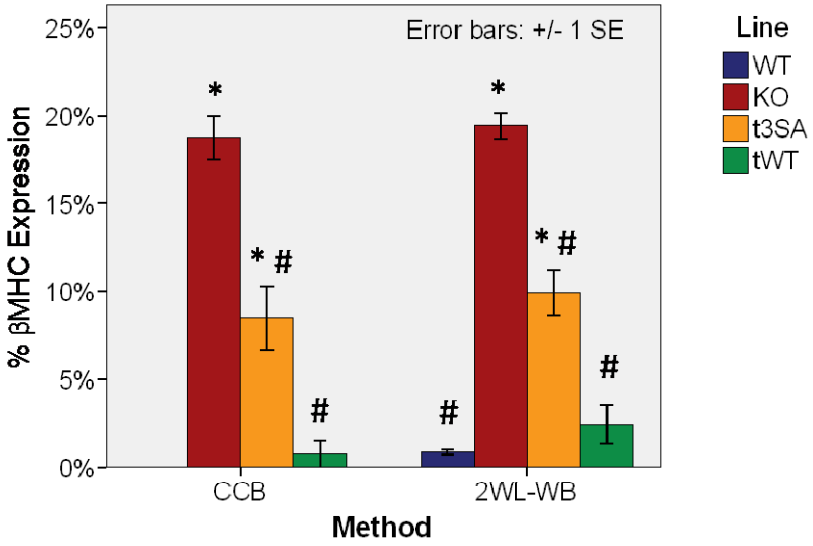
"*" denotes $p < 0.05$ when compared to basal condition without PKA

For force-pCa relationship, normalized force versus calcium data points were fitted to Hill's equation $\frac{[Ca^{2+}]^N}{[K_{50}]^N + [Ca^{2+}]^N}$ where K_{50} is $[Ca^{2+}]$ for 50% force, N is the Hill coefficient, and $pCa_{50}=-\log_{10}[K_{50}]$.

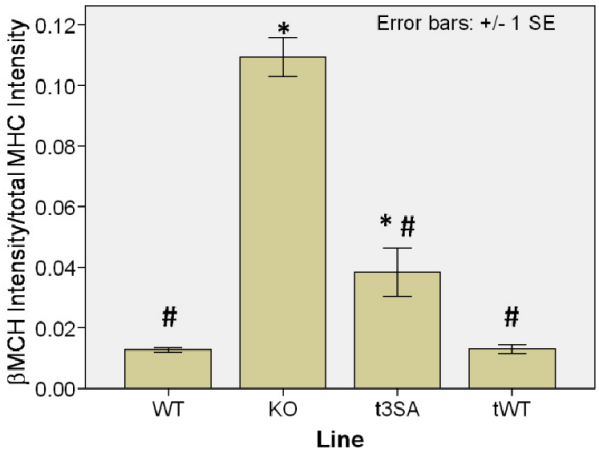
Online Figure I.A



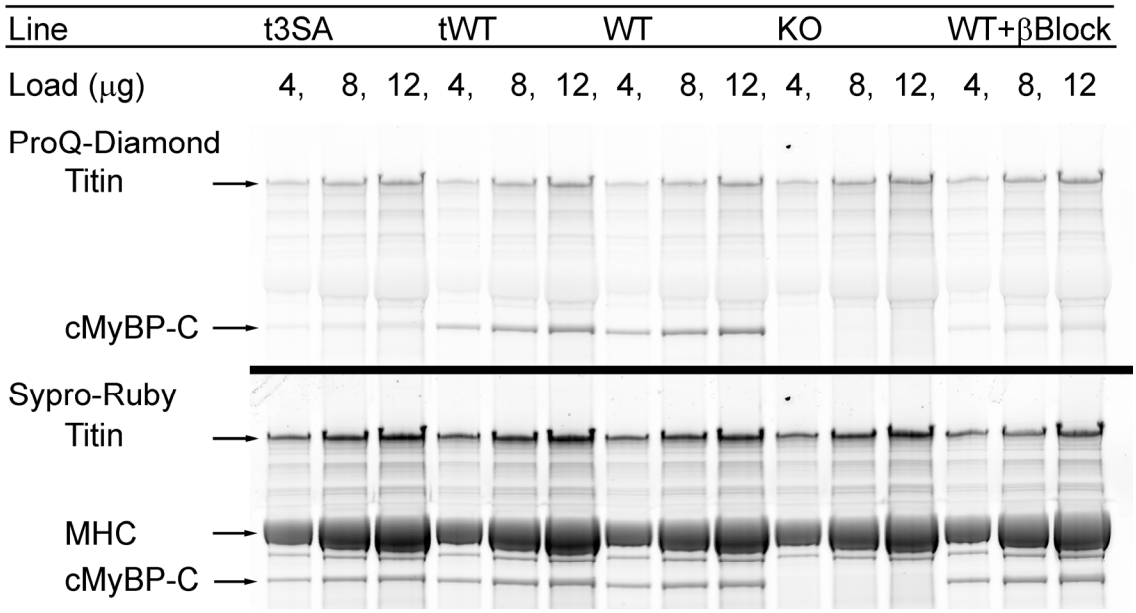
Online Figure I.B



Online Figure I.C



Online Figure II.A



Online Figure II.B