PNAS

**Supporting Information for**

# Myosin binding protein H-like regulates myosin binding protein distribution and function in atrial cardiomyocytes

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## Supplemental Methods

**Isolation of mouse atrial cardiomyocytes.** Cardiomyocytes were isolated as previously described (1). Mice were anesthetized with 5% inhaled isoflurane vaporized in 100% oxygen. Sedated mice were administered a 50 U intraperitoneal injection of sodium heparin solution to prevent blood coagulation and were allowed to recover for 20 minutes. Mice were again anesthetized, secured prone, followed by lateral incisions through the ribs and removal of the heart and lungs. The heart was bathed in a 35 mm culture dish with ice cold Tyrode's solution with no calcium (143 mM NaCl, 2.5 mM KCl, 16 mM $MgCl_2$, 11 mM glucose, 25 mM $NaHCO_3$, pH 7.4). A 24-gauge animal feeding needle (Cadence Science) was attached to a 1 mL syringe filled with ice cold Tyrode's solution with no calcium. The needle was inserted into the aorta and tied securely with a 6-0 silk suture. The heart was perfused with this solution at ~1 mL/min, followed by transfer of the cannula to a Langendorff apparatus with a constant pressure set to 80 mmHg. The Langendorff apparatus contained Tyrode's solution with no calcium and a modified Krebs-Henseleit buffer for digestion (0.15% collagenase type 2 (Worthington), 0.1% BDM, 0.1% glucose, 100 U/mL penicillin/streptomycin, 112 mM NaCl, 4.7 mM KCl, 0.6 mM $KH_2PO_4$, 40 μM $CaCl_2$, 0.6 mM $Na_2HPO_4$, 1.2 mM $MgSO_4$, 30 μM phenol red, 21.4 mM $NaHCO_3$, 10 mM HEPES, and 30 mM taurine, 1x insulin-transferrin-selenium (Gibco), pH 7.4) maintained at 37 °C with water-jacketed glassware (Radnoti) and a recirculating water bath. The hearts were perfused with digestion solution for 7 minutes followed by removal of the atria, fine mincing with fine spring scissors, and trituration using a plastic transfer pipette every five minutes for 40 minutes. Atrial tissue was allowed to settle between each trituration and the supernatant was filtered through a 100 μm cell strainer into a stop buffer (digestion buffer with no collagenase, 40 μM $CaCl_2$, and 1% bovine serum albumin). After complete digestion of the tissue, the cells were centrifuged for 1 minute at 100 × G. The pellet was resuspended in stop buffer, centrifuged again for 1 minute at 100 × G, and resuspended in a final buffer (stop buffer with no bovine serum albumin). Ventricular cardiomyocytes were isolated in the same manner except the hearts were digested for 5 minutes followed by serial trituration until the tissue was fully broken apart, approximately 6 minutes. Glass coverslips (10 mm diameter) were coated with 20 μg/mL laminin (23017-015 Gibco) in PBS for one hour. Cardiomyocytes were added in final buffer and allowed to adhere to the coverslips for one hour at 37 °C.

**Immunofluorescence staining for super resolution imaging.** Coverslips with adhered cardiomyocytes were put into the wells of a 24-well plate. The cells were washed with 500 μL of phosphate buffered saline and incubated in 4% paraformaldehyde for 10 minutes at 4 °C. The cells were washed with PBS and then permeabilized in PBS with 0.25% Triton X-100 for 25 minutes at 4 °C. The cells were blocked with a solution of 20% FBS and 0.1% Triton X-100 in PBS for 30 minutes at 4 °C and then incubated with primary antibodies (MyBP-HL, Pocono, custom; cMyBP-C, Santa Cruz E7; Actinin, Sigma A7811) in 2% FBS and 0.1% Triton X-100 overnight at 4 °C. The cells were washed three times in cold PBS containing 0.1% Triton X-100 and incubated in an appropriate secondary antibody. Secondary antibodies conjugated with Alexa-488 (Invitrogen A21202) or Alexa-568 (Invitrogen A10042) were used for super-resolution microcopy. Coverslips were again washed in PBS with 0.1% Triton X-100 three times, with the second wash containing 1 μM Houchest solution (Invitrogen H3570) to visualize nuclei. Cover slips were mounted using ProLong Gold anti-fade reagent (Thermo P10144) onto glass slides.

**Protein isolation.** Atria and ventricles from frozen mouse hearts or cryoarchived human heart samples were homogenized in ice cold F60 buffer (60 mM KCl, 30 mM Imidazole, 2 mM $MgCl_2$) in a Biospec bead homogenizer with 2.3 mm zinc-silica beads for one minute. Sarcomere proteins were fractionated by centrifugation at 12,000 × G for 10 minutes at 4 °C, followed by re-homogenization of the pellet with F60 with 0.1% Triton X-100 and centrifugation at 12,000 × G for 10 minutes at 4 °C for three total washes and then final resuspension in urea buffer (50 mM Tris-HCl, pH 7.5, 4 M urea, 1 M thiourea, 0.4% CHAPS, 20 mM spermine, 20 mM dithiothreitol), as previously described. Protein concentration was determined using a Bradford assay and equal concentration protein homogenates were made using additional urea buffer and a Laemmli 2 × loading dye. Proteins were separated on a 4 – 15% acrylamide SDS gel (BioRad) with tris-glycine SDS running buffer. Proteins were transferred to a PVDF membrane with 0.2 µm pore size via wet transfer apparatus (BioRad) using a tris-glycine buffer with 20% methanol. Proteins were detected with primary antibodies against MyBP-HL (custom), cMyBP-C (Santa Cruz, E7), myosin heavy chain (Developmental Studies Hybridoma Bank, MF20), or cMyBP-C phosphorylation-specific antibodies for S273, S282, and S302 (Sadayappan Lab). Secondary antibodies linked with horseradish peroxidase enzymes (Jackson Immuno Research) and Pico or Femto ECL detection reagents (Thermo) were used to visualize protein abundance using an iBright imaging platform (Thermo). The peptides used for quantification were assumed to have similar ionization efficiencies and indicative of the molar abundance of each protein (2).

**Quantitative mass spectrometry.** Small (1 – 2 mg) pieces of atrial tissue were flash frozen on liquid nitrogen and shipped on dry ice to the University of Vermont. The tissue was thawed, solubilized in RapiGest SF Surfactant (Waters Corporation), reduced, alkylated with iodoacetamide, and digested into peptides using trypsin as described for mouse ventricular tissue (3). The peptides were separated by ultra-high pressure liquid chromatography (LC) on a 1 mm Acquity HSS T3 column (Waters Corporation) and directly infused into a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) through an electrospray ionization source (3). Data were collected in data-dependent MS/MS mode. Peptides were identified by SEQUEST using a mouse proteome database downloaded from UniProt, and LC peak areas of peptides of interest were extracted using Proteome Discoverer 2.2 (Thermo Fisher Scientific). LC peak areas were imported into Microsoft Excel and the relative molar abundances were determined using a label-free quantitative routine (3).

***In silico* modeling of MyBP-HL and MyBP-C localization.** The localization and distribution of MyBP-HL and MyBP-C molecules within atrial sarcomeres was predicted by combining the results from the immunofluorescence imaging and quantitative mass spectrometry using an analytical model as previously described for skeletal muscle MyBP-C isoforms (4). To determine the MyBP-HL and MyBP-C doublet spacing in the structured illumination microscopy images from the *Mybphl* wild-type, heterozygous, and homozygous mice, multiple regions of interest of approximately 5 × 1.5 microns were selected from the images. A custom-written Python script was used to generate multiple intensity profiles across the sarcomeres within each region of interest. The individual intensity profiles were spatially aligned to one another using a cross-correlation based routine in Python. The aligned intensity profiles from each group were compared to images generated *in silico* using an analytical model developed to predict the localization and distribution of MyBP-C molecules in skeletal muscle sarcomere (4). The analytical model renders images with overlapping 2-dimensional Gaussian point spread functions of known spacing along the thick filament, to mimic fluorescence *in situ*. The initial

assumption of the model was that MyBP-HL and MyBP-C molecules can bind to any of 17 myosin helical repeats along each half of the thick filament. Next, the distance between the first MyBP-HL and MyBP-C molecule with respect to the center of the thick filament (M-line) was predicted, and then molecules were distributed within 43 nm myosin repeats relative to this initial position. The model assumed that the total number of MyBP-HL and MyBP-C molecules estimated from the quantitative mass spectrometry data were distributed between these 43 nm repeats on each half of a thick filament and no more than 3 molecules were allowed at any repeat. The fluorescence signal associated with each MyBP-HL and MyBP-C molecule was rendered as a 2-dimensional Gaussian point spread function with a full width at half maximum (FWHM) of 400 nm. The intensity of the effective point spread function was scaled to reflect the number of molecules at each 43 nm repeat. The position of repeats was iteratively adjusted relative to the center of the thick filament (maintaining the 43 nm periodicity) as well as the occupancy (or lack thereof). For each iteration, the intensity of overlapping point spread functions was summed, an image was created, and fitted with a dual Gaussian distribution for comparison to the structured illumination microscopy images.

**Isometric cardiomyocyte force measurements.** These experiments were performed as previously described (5, 6) with changes made for atrial cardiomyocytes. Frozen mouse atria or ventricle samples were incubated in isolation buffer (9.81 mM KOH, 2 mM EGTA, 7.11 mM $MgCl_2$, 10 mM imidazole, 108.01 mM KCl, 5.8 mM ATP 10 mM DTT) with protease inhibitors (ThermoFisher 78437), phosphatase inhibitors (ThermoFisher 78420), and 0.3% Triton X-100 and homogenized (OMNI International GLH 850) for three pulses of 1 second homogenizations. The homogenized tissue was strained through a 70 µm cell strainer and allowed to rest on ice for 20 minutes. The skinned cell suspension was then centrifuged three times at 300 x g for 2 minutes (atria) or 120 x g for 2 minutes (ventricle). The supernatant was removed, and the pellet was washed with fresh isolation buffer without triton after every spin. For atrial preparations care was taken not to disturb the pellet. Cells were then diluted in 200 – 400 µL (atria) or 600 – 1000 µL (ventricle) of isolation solution, depending on quantity and quality of cells, and 40 µL of the cell suspension was pipetted onto a cover slip on a custom microscope stage platform. Activating solution (10 mM $Ca^{2+}$-EGTA, 28.12 mM $K^+$ propanoate, 100 mM BES, 6.2 mM $MgCl_2$, 6.32 mM ATP, 10 mM creatine phosphate, 10 mM DTT, protease inhibitors, pH 7.02) and relaxing solution (10 mM EGTA, 47.58 mM $K^+$ propanoate, 100 mM BES, 6.54 mM $MgCl_2$, 6.24 mM ATP, 10 mM creatine phosphate, 10 mM DTT, protease inhibitors, pH 7.02) were mixed at 90, 85, 80, 75, and 65% activating/relaxing to make a range of solutions with varying calcium concentrations. Individual skinned cardiomyocytes were selected based on their size and appearance, with highly branched or visibly deformed cells excluded. The needle tips of the force transducer (Kronex AE801) and length controller (Thor Labs) were coated in Norland Optical Adhesive 63. The needles were attached to cardiomyocytes and then UV treated to firmly attach the cells. Cells were exposed to varying calcium concentrations via a multi-channel solution switching manifold. Cell force was measured using custom LabView software and cell cross sectional area was determined using calibrated rulers for cell width and depth.

**Single myofibril force measurements.** Myofibrils were isolated from the atria of WT and null mice as described (7-9). Briefly, left atrial tissue was demembranated in rigor solution (50 mM Tris, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.0) with 0.05% Triton X-100 and protease inhibitors (10 µM leupeptin, 5 µM pepstatin, 200 µM phenyl-methylsuphonylfluoride, 10 µM E64, 500 µM $NaN_3$, 2 mM dithioerythritol) overnight at 4

°C overnight. Then, the demembranated tissue was washed in rigor solution and resuspended in bath solution (pCa 9.0; 100 mM $Na_2EGTA$; 1 M potassium propionate; 100 mM $Na_2SO_4$; 1M MOPS; 1M $MgCl_2$; 6.7mM ATP; and 1 mM creatine phosphate; pH 7.0) with protease inhibitors and homogenized for seven seconds two times with a Tissue Tearor (Cole-Parmer).

***Immuno-electron microscopy.*** Immunolabeling of MyBP-HL, cMyBP-C (C5-C7) and I103 titin epitopes was performed on skinned atrial tissue bundles from 2-month-old WT mice. Atria were removed from hearts and skinned in relaxing solution (40 mM BES, 10 mM EGTA, 6.56 mM $MgCl_2$, 5.88 mM Na-ATP, 1.0 DTT, 46.53 mM K-propionate, 15 mM creatine phosphate, pH 7.0) containing 1 % (w/v) Triton X-100 (Ultrapure, Thermo Fisher Scientific) and protease inhibitors (0.4 mM leupeptin, 0.1 mM E64 and 0.25 mM PMSF), overnight at 4 °C in a rotator. Subsequently, the skinned atrial tissue was rinsed in relaxing solution containing inhibitors for 5 h, and bundles of fibers were prepared under a dissecting microscope, sectioning along the surface of the atrial curvature and following the orientation of the parallel fibers. Bundles of fibers were pinned down with aluminum T-clips in a sylgard dish with wells, stretched at ~25 – 30 % from their slack length and processed by the pre-embedding technique previously described (10). Atrial tissue bundles were fixed in 3.7 % paraformaldehyde in 10 mM PBS pH 7.2 for 30 min at 4 °C, and after rinsing in the same buffer, they were incubated with 50 mM glycine in PBS followed by blocking with 0.5 % bovine serum albumin (BSA) in PBS containing protease inhibitors and 0.05 % Tween 20. Single or double labeling of atrial tissue bundles was performed incubating 48 h at 4°C with the rabbit polyclonal antibodies against MyBP-HL (0.231 mg/mL, 1:40), cMyBP-C (C5-C7) (0.87 mg/mL, 1:15) and I103 titin (0.2 mg/mL, 1:25), followed by rinsing in PBS containing protease inhibitors and incubation with the secondary antibodies Alexa Fluor-568 goat anti-rabbit antibody IgG (ab175471. Invitrogen, 1:30) or Fab goat anti-rabbit antibody IgG (AP 132, Millipore, 1:25) as appropriated, 24 h at 4 °C. Controls were used by replacing each primary antibody with PBS and 0.5 % BSA solution containing protease inhibitors as before. Afterward, bundles of atrial tissue were fixed with 3 % glutaraldehyde and processed by transmission electron microscopy. Briefly, bundles of fibers were post-fixed in 1 % $OSO_4$ in the same PBS buffer, dehydrated in an ethanol graded series, infiltrated with propylene oxide, and transferred to a 1:1 mix of propylene oxide:Araldite 502/Embed 812 resin for polymerization 48 h at 60 °C. Ultrathin sections (90 nm) were obtained in a Reichert-Jung ultramicrotome and contrasted with 1 % potassium permanganate and lead citrate. Imaging was performed in a TECNAI Spirit G2 transmission electron microscope (FEI, Hillsboro, OR) with a side-mounted AMT Image Capture Engine 6.02 (4Mpix) digital camera operated at 100 kV. Density profiles from digital images were analyzed using ImageJ v1.49 (NIH, MD, USA) to determine MyBP-HL and I103 titin epitope distances across the A-band. Values were expressed as the mean ± SEM and corrected for shrinkage (caused by TEM procedure) using the A-band known value of 1.6 μm (11).

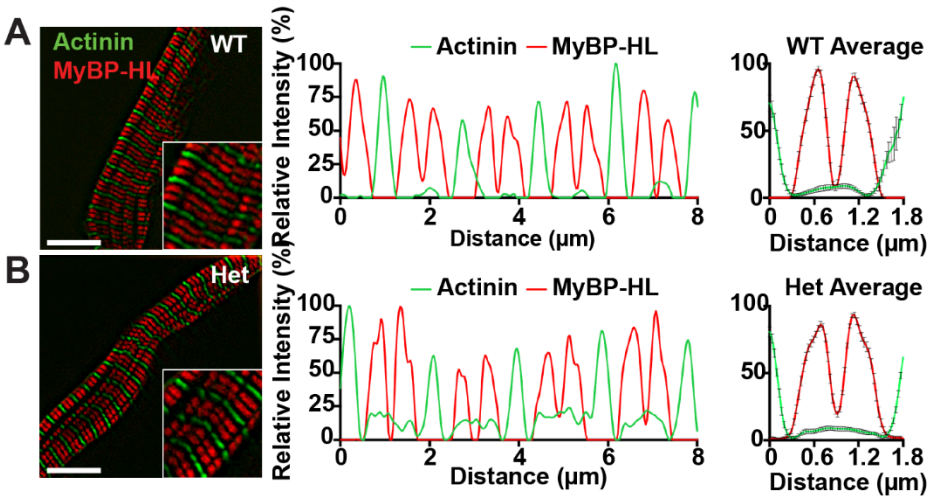


**Fig. S1.** Structured illumination microscopy of wild type (A) and *Mybphl* heterozygous null (B) mouse isolated atrial cardiomyocytes reveals MyBP-HL (red) localizing in an A-band doublet pattern interdigitating with actinin (green) stained Z-disks in wild type and *Mybphl* heterozygous mice. Scale bar = 10 μm.

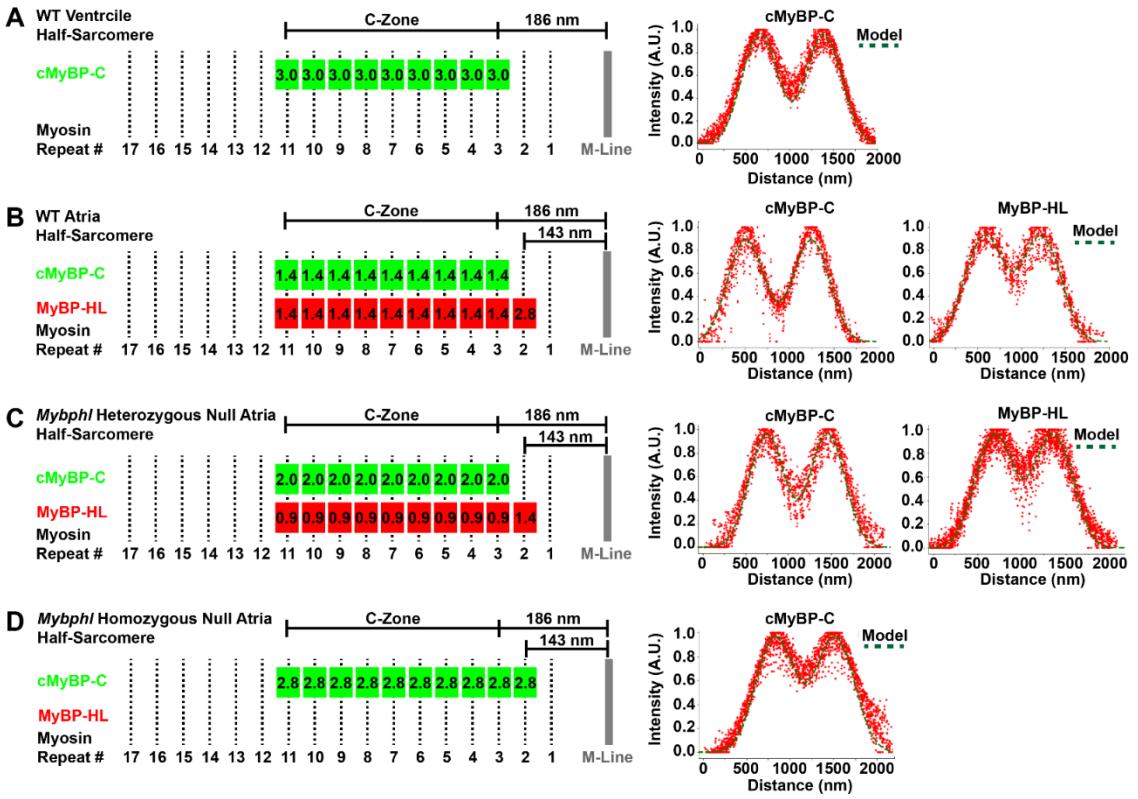


**Fig. S2.** Computational modeling supports cMyBP-C replacing MyBP-HL in the medial repeat in *Mybphl* null sarcomeres. (A) Representative confocal microscopy image of an isolated ventricular cardiomyocyte stained with the cMyBP-C E7 antibody (left). Point-spread function model of the relative cMyBP-C abundance taken from mass spectrometry, with three cMyBP-C molecules per 43 nm myosin repeat, and the known nine cMyBP-C repeats found in ventricular cardiomyocytes (green line), compared to fluorescence intensity traces from confocal images (red dots) (right). Sarcomere schematic showing every 43 nm spaced myosin repeat and the number of cMyBP-C molecules per 43 nm repeat in ventricular cardiomyocytes (bottom). (B) Point spread model of wild type atrial cardiomyocytes with cMyBP-C and MyBP-HL myosin repeat distributions illustrating binding of both proteins to nine myosin repeats, with MyBP-HL binding to one additional medial myosin repeat 143 nm from the M-line (myosin repeat 2). (C) Point spread model of heterozygous *Mybphl* null atrial cardiomyocytes showing cMyBP-C binding closer to the M-line with reduced MyBP-HL. (D) Point spread model in *Mybphl* homozygous null cardiomyocytes shows replacement of the MyBP-HL with 3 cMyBP-C molecules per 43 nm myosin repeat.

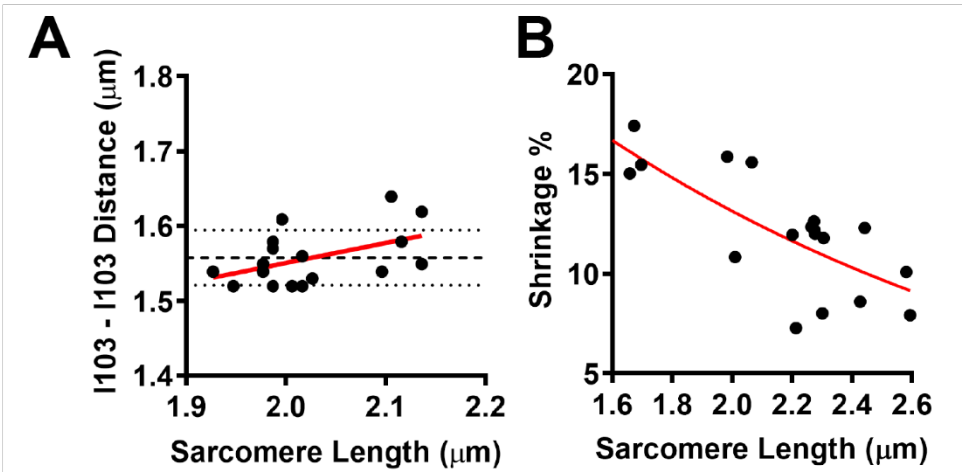


**Fig. S3.** (A) The titin I103 epitope marked the lateral bounds of the A band and was measured as 1558 ± 9 nm. (B) Sample shrinkage was calculated by comparing the known distance of the titin I103 epitopes to the sample at various observed sarcomere lengths.

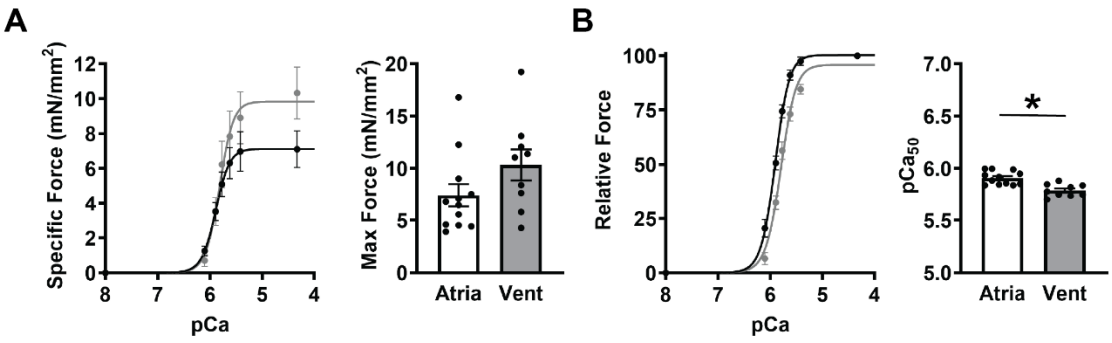


**Fig. S4.** (A) Specific force (force/cross sectional area) of permeabilized atrial and ventricular cardiomyocytes from WT mice. N = atria: 5 mice, 16 cells; ventricle: 4 mice, 12 cells. (B) Relative force traces show a significantly higher calcium sensitivity of force development in wild-type atrial cardiomyocytes compared ventricular cardiomyocytes. N = atria: 5 mice, 16 cells; ventricle: 4 mice, 12 cells * = $P < 0.05$ by Welch's T-test.

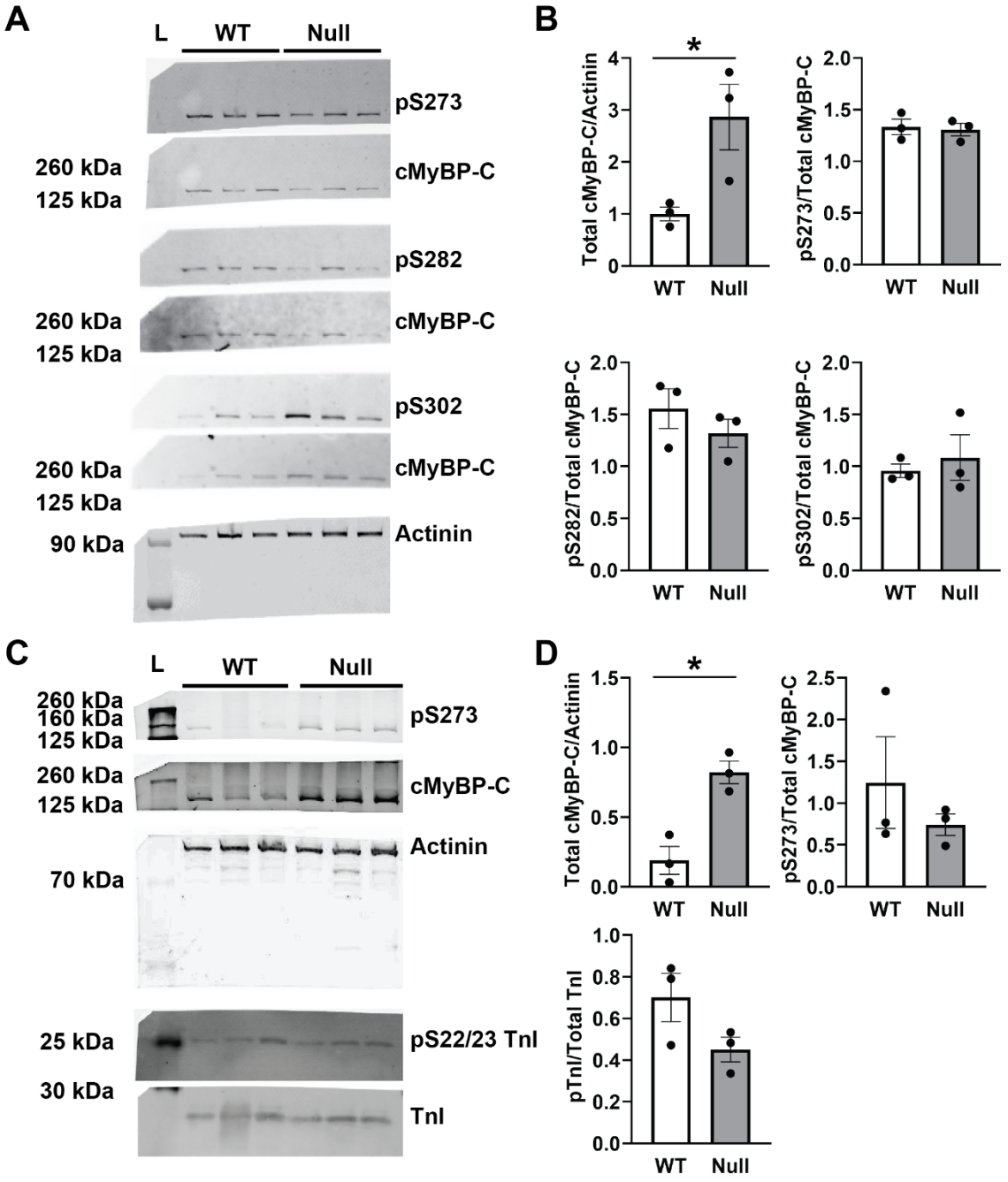


**Fig. S5.** (A) Immunoblots on protein lysates made from the remnants of the myofibril samples used in Figure 4. Three WT and three Null samples had sufficient material to quantify. (B) Quantification of the post-myofibril isolation samples show the expected increase in cMyBP-C levels, but the levels of cMyBP-C phosphorylation normalized to total cMyBP-C levels is not significantly different. (C) Immunoblots of total atrial lysates also assessed phosphorylation levels of cMyBP-C and cTnI. (D) Quantification again shows increased cMyBP-C in Mybphl null atria, with no significant difference in phosphorylation at S273 of cMyBP-C or S22/23 of cTnI. * = $P < 0.05$ by Student's t-test.

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