Supplemental Methods

1. Animals

The investigation conforms to the guide for the care and use of laboratory animals published by the NIH (Publication No. 85-23, revised 1985). The *Mybpc3*-KI mice were generated by targeted insertion of a G>A transition on the last nucleotide of exon 6 and were maintained on the Black swiss background [1].

2. Adult mouse ventricular myocytes

Adult ventricular myocytes were isolated from 13-week-old Het, KI and WT mice as previously described [2]. Briefly, hearts were excised from heparinized (5,000 U/kg body weight) and anesthetized (ketamine/xylazine) mice, mounted in a Langendorff perfusion apparatus, and retrogradely perfused through the aorta with a Ca^{2+}-free modified Tyrode solution containing 10 mM 2,3-butanedione monoxime (BDM) at 37°C for 3 min. Perfusion was then switched to the same solution containing 75 units/ml Liberase Blendzyme 3 (Roche Diagnostics) for 10 min. The left ventricular tissue was excised, minced and gently pipette-dissociated. Collagenase activity was stopped by the addition of fetal bovine serum (10%). Cell suspension was then sequentially washed in 25 µM, 100 µM, 200 µM and 1 mM Ca^{2+}-Tyrode and resuspended in 1.5 mM Ca^{2+}-Tyrode at room temperature.

3. Assessment of myofilament function in skinned mouse ventricular trabeculae

Mechanical function of skinned mouse cardiac trabeculae was assessed at 18 °C, using equipment and protocols similar to those described in detail previously [3]. In brief, 7-8-week-old WT, Het and KI mice had been treated with the β-blocker propranolol (0.5 g/l in drinking water) for 3 days beforehand in order to reduce the catecholamine-induced phosphorylation of intracellular proteins that can occur during anaesthesia and heart dissection. Hearts were removed and stored in liquid N_2 until the day of use. Hearts were thawed in relaxing solution and thin trabeculae were dissected from the right ventricle (2-6 muscles from each heart). Trabeculae were then chemically permeabilized ("skinned") in skinning solution for 3 h at 4°C and washed twice with fresh relaxing solution. Following washing, skinned trabeculae were stored in relaxing solution containing 50% glycerol at -20°C for up to 3 days, until they were used for the assessment of myofilament function. Myofilament function was assessed at a sarcomere length of 2.2 µm. Skinned steady-state force-*p*Ca relationship was determined experimentally and fitted to the Hill equation to yield maximal Ca^{2+}-activated force (max *F*), the log of [Ca^{2+}] required for 50% of maximal activation (pCa_{50}), and the Hill coefficient (*nH*).

4. Sarcomere shortening and Ca^{2+} transients

Morphological and functional criteria for using a ventricular myocyte for the experiments were: i) rod-shape, ii) no membrane blebs, iii) no hypercontractile zones, iv) no spontaneous contractions, and v) stable contraction amplitude at 0.25 Hz. Sarcomere length (SL) was first assessed under resting conditions (i.e., electrical stimulation stopped for 10 min) in individual myocytes incubated in 1.5 mM Ca^{2+}-Tyrode with or without 10 mM BDM. Then, cells were incubated in Ca^{2+}-Tyrode containing 5 µM Fura-2-AM (Molecular Probes) for 40 min and rinsed twice with the Ca^{2+}-Tyrode solution. Sarcomere shortening and Ca^{2+} transients of intact myocytes were acquired at 240 Hz upon electrical field stimulation (0.25 to 3 Hz pacing frequency, with 4 ms duration, 10 V). Since sarcomere shortening parameters were similar in both Fura-2-loaded and -unloaded cells, results were pooled. Sarcomere shortening and Ca^{2+} transients were acquired using an integrated IonOptix device (IonOptix corporation) as described previously [2]. Ratiometric Fura-2 fluorescence measurements

were performed using optical excitation filters of 380 and 360 nm. Emitted fluorescence (510 nm) was background subtracted. The $[Ca^{2+}]_i$ was calculated from ratiometric measurements according to a modified method from Grynkiewicz and collaborators [4-5]. Determination of the parameters of the Grynkiewicz equation were routinely achieved in myocytes. Since no significant difference was found between the 3 mouse groups, calculated parameters were pooled and the same mean values were used for all $[Ca^{2+}]_i$ calculations (R_{max}=3.84, R_{min}=0.55 and β=5.62). All experiments were conducted at room temperature. Caffeine was used to determine the sarcoplasmic reticulum (SR) Ca^{2+} content and to evaluate the activity of the Na^+/Ca^{2+} exchanger (NCX1) [6-7]. After 60–90 s stimulation at 1 Hz, superfusing solution was rapidly switched to a solution containing 10 mM caffeine for 7–8 s.

5. Echocardiography

Transthoracic echocardiography was performed using the Vevo 2100 System (VisualSonics, Toronto, Canada). Ten-week-old WT, Het and KI mice were anesthetized with isoflurane (1.5-2%) and assured to a warming platform in a supine position. Anesthetic depth was monitored by electrocardiogram and respiration rate. Body temperature was kept at 37°C. Conventional measurements were obtained from B-mode recordings using a MS 400 transducer (18-38 MHz) with a frame rate of 230-400 frames/s. Two-dimensional short axis views were recorded at the mid-papillary level. Parasternal long axis views were recorded at the plane of the aortic valve with a concurrent visualization of the left ventricular (LV) apex. Anterior wall and posterior wall thickness, LV diameter and cavity area were recorded according to standard procedures. Colour M-mode tissue Doppler imaging of the mitral inflow profile was obtained from a modified apical four-chamber view. The transducer was placed over the apex and angulated anteriorly. The direction and the sampling depth were adjusted to obtain the highest velocity and pulsed wave Doppler of the mitral inflow was recorded from the position at which the velocity was maximal. Peak early (E) and atrial (A) mitral inflow velocities were measured [8-9]. Tissue Doppler recordings were recorded at the septal corner of the mitral annulus in the same apical four-chamber view. Peak early (E´) and atrial (A´) velocities were measured. All measurements were taken from three cardiac cycles and averaged. Measurements were obtained by an examiner blinded for the genotype. All images were recorded digitally and off-line analysis was performed using the Vevo 2100-software.

6. Immunoblot analysis

Immunoblot analysis was carried out as previously described [10-11]. Briefly, ventricular tissue or skinned myocytes were lyzed in SDS buffer, proteins were separated by SDS-electrophoresis using 15, 10 and 7.5% polyacrylamide mini-gels (Protean system III, Biorad), and transferred to a nitrocellulose or polyvinylidene difluoride membrane. Each immunoblot was stained with coomassie blue or Ponceau S to assure equal protein loading. Membranes were blocked with 5% dry milk powder (Roth) and 2% BSA (Sigma) in TBS-Tween (Tris buffered saline containing 0.1% Tween-20) for 2 h. Subsequently, primary antibodies were added and incubated for 2 h or overnight in blocking solution. Mouse monoclonal antibodies were directed against □-actinin (Sigma, A7811, 1:1000), β-MHC (Sigma, M8421, 1:4000), NCX1 (Abcam, ab6495, 1:5000), total phospholamban (total-PLB, Badrilla, A010-14, 1:5000), and phosphorylated PLB (Ser16-PLB and Thr17-PLB, Badrilla, both 1:5000). Rabbit polyclonal antibodies were directed against calsequestrin (CSQ, Dianova, PA1-913, 1:2500), sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2; Santa Cruz, N-19, sc-8095, 1:500), phosphorylated cardiac troponin I (pSer23/24-cTnI, Cell Signaling, 1:1000), phosphorylated cMyBP-C (pSer282-cMyBP-C; Enzo Life Sciences; used at 1:2,000-1:5000), total cardiac troponin I (Total-cTnI, Cell Signaling, 1:1000 or Chemicon, 1:30000), total cMyBP-C, kind gift from Mathias Gautel,1:30000). Following several washing steps in TBS-Tween, HRP-

conjugated secondary antibodies (Dianova or Jackson ImmunoResearch Laboratories) were applied for 60 min. Detection was performed with the chemiluminescent substrate SuperSignal® West Dura (Pierce) or enhanced chemiluminescence (GE Healthcare). Specific protein bands were quantified by densitometry using the SynGene software (ChemiGenius; GeneTools, SynGene, UK) or with a calibrated densitometer (GS-800), using Quantity One® 1-D analysis software (Bio-Rad).

7. Statistical analysis

Data are expressed as mean±SEM. Comparisons were performed using Student's t-test, or using one-way or two-way ANOVA followed by Bonferroni's post-tests. A value of $P<0.05$ was considered statistically significant.

8. References

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