Cardiac Myosin-Binding Protein-C Phosphorylation and Cardiac Function

Sakthivel Sadayappan, James Gulick, Hanna Osinska, Lisa A. Martin, Harvey S. Hahn, Gerald W. Dorn II, Raisa Klevitsky, Christine E. Seidman, Jonathan G. Seidman, Jeffrey Robbins

Abstract—The role of cardiac myosin binding protein-C (cMyBP-C) phosphorylation in cardiac physiology or pathophysiology is unclear. To investigate the status of cMyBP-C phosphorylation in vivo, we determined its phosphorylation state in stressed and unstressed mouse hearts. cMyBP-C phosphorylation is significantly decreased during the development of heart failure or pathologic hypertrophy. We then generated transgenic (TG) mice in which the phosphorylation sites of cMyBP-C were changed to nonphosphorylatable alanines (MyBP-C(AllP)). A TG line showing ≈40% replacement with MyBP-C(AllP) showed no changes in morbidity or mortality but displayed depressed cardiac contractility, altered sarcomeric structure and upregulation of transcripts associated with a hypertrophic response. To explore the effect of complete replacement of endogenous cMyBP-C with MyBP-C(AllP), the mice were bred into the MyBP-C(t/t) background, in which less than 10% of normal levels of a truncated MyBP-C are present. Although MyBP-C(AllP) was incorporated into the sarcomere and expressed at normal levels, the mutant protein could not rescue the MyBP-C(t/t) phenotype. The mice developed significant cardiac hypertrophy with myofibrillar disarray and fibrosis, similar to what was observed in the MyBP-C(t/t) animals. In contrast, when the MyBP-C(t/t) mice were bred to a TG line expressing normal MyBP-C (MyBP-C WT), the MyBP-C(t/t) phenotype was rescued. These data suggest that cMyBP-C phosphorylation is essential for normal cardiac function. (Circ Res. 2005;97:0-0.)

Key Words: mouse □ mouse mutants □ muscle □ muscle contraction □ myocardial contractility

Understanding the structure/function relations for cardiac myosin binding protein-C (cMyBP-C) is clinically relevant, as cMyBP-C mutations are a widely recognized cause of familial hypertrophic cardiomyopathy.1 Various cMyBP-C transgenic (TG) and gene-targeted mouse models have demonstrated the importance of the protein for long-term integrity of sarcomeric structure and for maintaining normal cardiac contractility.2,3 Functional insight can be gained from appreciating the crucial structural differences between cMyBP-C and the skeletal isoform. Only the cardiac isoform contains an extra immunoglobulin domain at the N terminus (C0), an insertion of 28 residues within the C5 domain, and thre3ee phosphorylation sites (Ser273, -282, -302) that are substrates for cAMP-dependent protein kinase A (PKA), Ca2+-calmodulin-activated kinase and protein kinase C.

In vivo, PKA-mediated phosphorylation of cMyBP-C is linked to modulation of cardiac contraction.4 On adrenergic stimulation, PKA phosphorylates Ser273, -282, and -302, whereas protein kinase C phosphorylates only Ser273 and -302.5 These residues, located near the N terminus of the protein, are of particular interest, as this region binds to the S2 segment of the myosin heavy chain (MHC),6,7 which is close to the lever arm domain of myosin. It has been hypothesized that cMyBP-C/MHC interactions are dynamically regulated by the phosphorylation/dephosphorylation of cMyBP-C.8 In vitro experiments showed that after phosphorylation of cMyBP-C by PKA, the thick filaments exhibited a relative loose structure,9 preventing binding to myosin and, thus, changing the maximum Ca2+-activated force (F max). Electron microscopy of isolated thick filaments confirmed that phosphorylation of cMyBP-C initiates crossbridge movement away from the thick-filament backbone, although this interaction may also be determined by the MHC isoform that is present.10 In theory, cMyBP-C phosphorylation could change both filament orientation and contractile mechanics,7,11 although cMyBP-C phosphorylation did not alter the Ca2+ sensitivity of Mg2+-ATPase activity in reconstituted contractile protein systems.12,13 Additionally, phosphorylation of cMyBP-C did not affect the Ca2+ sensitivity of Mg2+-ATPase activity, force-Ca2+ relationship, or sarcomere length dependency of contraction in intact skinned fiber experiments,14,15 suggesting that MyBP-C phosphorylation plays a relatively minor role in regulating contraction. Given the above body of data, the role that cMyBP-C phosphorylation plays in either normal or pathological cardiac function remains unclear.

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From the Department of Pediatrics (S.S., J.G., H.O., R.K., L.A.M., J.R.), Division of Molecular Cardiovascular Biology, Cincinnati Children’s Hospital Medical Center, OH; Department of Medicine (H.S.H., G.W.D), University of Cincinnati, OH; Department of Genetics (C.E.S., J.G.S), Howard Hughes Medical Institute and Harvard Medical School, Boston, Mass; and Cardiovascular Division (C.E.S.), Brigham and Women’s Hospital, Boston, Mass.
Correspondence to Jeffrey Robbins, MLC 7020, 3333 Burnet Ave, Cincinnati, OH 45229-3039. E-mail jeff.robbins@chmc.org
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We wished to determine whether cMyBP-C phosphorylation played an essential role in the maintenance of cardiac function. We first defined the phosphorylation status of cMyBP-C in the normal and diseased heart and showed that significant changes occurred during development of cardiac pathology. We then generated a TG mouse model in which the known phosphorylation sites in cMyBP-C were converted to nonphosphorylatable alanines (MyBP-CAllP) and compared this model to a model with comparable levels of TG expression of normal cMyBP-C (MyBP-CWT). Both TG models were subsequently bred into the homozygous cMyBP-Cx0 background. The data establish that cMyBP-C phosphorylation is essential for normal cardiac function.

Materials and Methods
An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Transgenic and Targeted Lines
The cDNA for mouse cMyBP-C (MyBP-CWT) was obtained by RT-PCR using RNA isolated from the mouse cardiac ventricle. The cMyBP-C phosphorylation sites (Ser273, Ser282, and Ser302), along with 2 adjacent sites that could be potentially phosphorylated (Thr272, Thr281), were converted to alanine and a sequence encoding the myc epitope incorporated as described previously. The cDNA was subcloned into the mouse germline transmission vector, termed MyBP-CAllP, and was bred with mice that expressed less than 10% of normal cMyBP-C to nonphosphorylatable alanines (MyBP-CAllP) and compared this model to a model with comparable levels of TG expression of normal cMyBP-C (MyBP-CWT). Both TG models were subsequently bred into the homozygous cMyBP-Cx0 background. The data establish that cMyBP-C phosphorylation is essential for normal cardiac function.

Molecular and Protein Analyses
For assessment of cMyBP-C transcript size, expression levels, and expression of hypertrophic marker genes, RNA transcript and blot analyses were performed as described. Enriched myofibrillar proteins were isolated using F60 buffer and solubilized in urea buffer. MyBP-CAllP was identified via SDS-PAGE followed by Western blots using anti-myc monoclonal antibodies and anti-cMyBP-C rabbit polyclonal antibodies raised against the CO–C1 domains.

Phosphorylation and Isoelectric Focusing
Myofibrillar proteins were treated with phosphatase and PKA as described. One-dimensional isoelectric focusing (IEF) was performed to identify the phosphorylated forms.

Cardiac Function
In vivo cardiac function was assessed both by noninvasive echocardiography in nonsedated or sedated animals and by invasive catheterization.

Results
Phosphorylation States of cMyBP-C in Normal and Heart Failure Mouse Models
As is the case for a number of other contractile proteins, cMyBP-C can be reversibly phosphorylated at multiple sites in response to altered physiological conditions. However, the in vivo phosphorylation levels of the protein under basal and stressed conditions are not well defined. To understand the level of phosphorylation in the nontransgenic (NTG) animals before we perturbed the cMyBP-C protein complement, cMyBP-C phosphorylation under normal and stressed conditions was determined using IEF followed by Western blots using anti-myc monoclonal antibodies and anti-cMyBP-C rabbit polyclonal antibodies raised against the CO–C1 domains.

Figure 1. Cardiac MyBP-C phosphorylation levels in normal and pathological states, A, One-dimensional IEF (pH 5 to 7) of total myofilament proteins followed by Western blot analysis using cMyBP-C antibody. Proteins were obtained from the following samples: hearts that had undergone sham operation for TAC, 24 hours (lane 1); 24 hours post-TAC (lane 2); 180 days sham (lane 3); 18 days post-TAC (lane 4); a NTG control (lane 5); 8-week-old calcineurin TG mouse (lane 6); 7- to 8-month-old MLP-deficient mouse (lane 7); a β-MHC TG mouse (lane 8); ischemic reperfusion (IR), 24 hours sham (lane 9); 24 hours post-IR (lane 10); 5 days post-IR sham (lane 11); and 5 days post-IR (lane 12). The 4 forms of cMyBP-C based on its phosphorylation status are shown as P0, P1, P2, and P3, which correspond to the de-, mono-, bi-, and triphosphorylated forms, respectively, with isoelectric points of 6.1, 5.9, 5.7, and 5.5. B, MHC isoform shifts. Using the samples from A, the α- and β-MHC isoforms were separated in a 5% glycerol gel. The data showed the expected isoform shifts characteristic of activation of the fetal gene program in the disease models (lanes 4, 6, 7, and 12). C, cMyBP-C phosphorylation in cardiac disease. Samples are as in A, and average values are shown as a percentage of total cMyBP-C (n=3). The quantitative data are shown in tabular form in supplemental Table 1.
dilated heart failure,\(^2^2\) and animals that had been subjected to ischemia reperfusion. Because the degree to which cMyBP-C and its phosphorylated forms regulate cardiac function may depend, in part, on the MHC isoform that is present,\(^2^3\) we also determined cMyBP-C phosphorylation levels in a mouse model in which we had replaced approximately 70% of the normal \(\alpha\)-MHC with \(\beta\)-MHC.\(^2^4\)

Under basal conditions, the mono-, bi-, and triphosphorylated species of cMyBP-C made up >90% of the cMyBP-C population (Figure 1A, lane 5). A \(\beta\)-MHC shift, in of itself, had no effect on the normal phosphorylation pattern failure (Figure 1B, lane 8). In all of the models in which cardiac function was significantly compromised by either surgical or genetic manipulation, total cMyBP-C phosphorylation, particularly the triphosphorylated species, was decreased (Figure 1A and 1C; supplemental Table I). Invariably, in the animals that displayed overt cardiac failure as determined by labored breathing, anasarca, and failure to groom, the triphosphorylated state was reduced or absent (Figure 1, lanes 4, 6, 7, and 12). These data show that increased dephosphorylation of cMyBP-C is associated with contractile dysfunction and heart failure.
To investigate the role of cMyBP-C phosphorylation in relation to cardiac function, we generated a construct (MyBP-CAllP) in which the 3 phosphorylation sites (Ser273, -282, and -302) and 2 neighboring potential alternative phosphorylation sites (Thr272 and -281) were altered to alanine (Figure 2A). Previously, we prepared a TG mouse that expressed myc-tagged, normal cMyBP-C (line 21, MyBP-CWT),2 and mice from NTG (lane 1), MyBP-CWT/(t/t) (lane 3), and MyBP-CAllP/(t/t) (lane 4) were either untreated (Control) or treated with PKA or phosphatase (Phosp). PKA-treated myofilaments show levels of MyBP-CAllP in the MyBP-CWT background that are essentially identical to NTG levels. Lane 2, which corresponds to the protein derived from MyBP-C(t/t) hearts, showed no detectable signal and was omitted. E, Myosin isoform shifts in the samples shown in D. MyBP-CWT expression completely rescued the cardiac phenotype observed in MyBP-C(t/t) hearts in terms of the shift to the fetal hypertrophic pattern, as evidenced by β-MHC expression. In contrast, MyBP-CAllP failed to prevent reversion to the fetal MHC isoform program. M indicates size markers.

**Genetic Modulation of cMyBP-C**

The phosphorylation motif of cMyBP-C is conserved among the human, mouse, chicken, and frog (Figure 2A). To estimate the degree of replacement of the endogenous cMyBP-C with the transgenically-encoded species, we used representative Northern (top 2 panels) and dot-blot (bottom) analyses of cMyBP-C mRNA from the left ventricles of 12-week-old NTG (lane 1), homozygous MyBP-C(t/t) (lane 2), MyBP-CWT/(t/t) (lane 3), and MyBP-CAllP/(t/t) (lane 4) mice hybridized with a cMyBP-C probe. GAPDH was used as a loading control. B, SDS-PAGE analysis shows the absence of cMyBP-C in the homozygous MyBP-C(t/t) hearts, replacement of endogenous cMyBP-C at normal levels in the MyBP-CWT/(t/t) and MyBP-CAllP/(t/t) crosses, and conservation of the other contractile protein levels in these animals. Lanes are numbered as in A. C, Western blot analysis shows the absence of endogenous cMyBP-C in the homozygous MyBP-CWT/(t/t) and MyBP-CAllP/(t/t) hearts in the samples shown in D. MyBP-CWT expression completely rescued the cardiac phenotype observed in MyBP-C(t/t) hearts in terms of the shift to the fetal hypertrophic pattern, as evidenced by β-MHC expression. In contrast, MyBP-CAllP failed to prevent reversion to the fetal MHC isoform program. M indicates size markers.

**Histological and Ultrastructural Consequences of MyBP-CAllP**

The gross histology of 3-month MyBP-CAllP hearts was unremarkable, with no obvious abnormalities, fibrosis, calcification, or disarray compared with NTG controls (Figure 3A). However, transmission electron microscopy revealed subtle ultrastructural changes. Whereas NTG hearts showed typical, well-organized and aligned sarcomeres with regularly distributed mitochondria, sarcoplasmic reticulum, and T-tubules (Figure 3B and 3C), MyBP-CAllP hearts occasionally displayed regions that lacked the regular sarcomere–mitochondria distribution, and some sarcomeres showed altered H-zones and M-lines that were relatively ill defined (Figure 3B, lower panel; Figure 3C, lower panels).

The lack of definition at the central part of the sarcomere is reminiscent of the altered sarcomeres that were observed in mice in which cMyBP-C expression was ablated.2 To confirm that MyBP-CAllP was being incorporated normally into the sarcomere, we performed immunohistochemical analyses with both anti-myc and polyclonal anti–cMyBP-C antibodies. Both MyBP-CAllP and MyBP-CWT proteins were incorporated normally into the sarcomere (Figure 3C).

Despite the subtle ultrastructural changes, no cardiac hypertrophy and/or dilation in the MyBP-CAllP hearts showed 2 species when treated with PKA, the nonphosphorylated MyBP-CAllP (P0), and the phosphorylated endogenous cMyBP-C (P2, P3), allowing us to estimate the degree of replacement of the endogenous cMyBP-C with the transgenically-encoded species. Approximately 40% replacement with the MyBP-CAllP occurred in lane 262, which is equivalent to the level of replacement previously measured in the MyBP-CWT-expressing hearts derived from line 21.
tected. The heart/body weight ratios did not significantly differ between NTG (0.53 ± 0.05, n = 8) and MyBP-CAllP (0.60 ± 0.05, n = 8; P < 0.08) littermates at 3 months. However, we reasoned that the ultrastructural changes reflected a structural deficit that might result in subtle alterations in the transcriptional patterns and, indeed, found that β-MHC and atrial natriuretic factor (ANF) transcript levels, which can serve as sensitive molecular markers for cardiac stress, were significantly increased in MyBP-CAllP hearts when compared with either NTG or MyBP-CWT samples (Figure 3D and 3E).

Depressed Cardiac Function in MyBP-CAllP Mice

On the basis of the altered ultrastructure, we considered that partial replacement of cMyBP-C with a nonphosphorylatable form might affect whole-organ function. Cardiac function was evaluated both noninvasively and by catheterization in the intact animals at 3 months. M-mode echocardiography in nonsedated animals showed that the MyBP-CAllP animals had normal cardiac dimensions and function under baseline conditions (supplemental Table II). As cMyBP-C phosphorylation occurs in response to alterations in intracellular calcium levels (activating calcium/calmodulin-dependent kinase) and β-adrenergic stress (activating PKA), we reasoned that adrenergic stimulation might reveal a deficit in the ability of the MyBP-CAllP animals to respond to dobutamine. Hemodynamic load can be significantly altered via β-adrenergic stimulation, activation of PKA, and the subsequent phosphorylation of phospholamban, cTnl, and MyBP-C. Phosphorylation of cTnl and phospholamban leads to increases in crossbridge cycling and enhanced relaxation, but the role that cMyBP-C plays in these processes, if any, is unclear. Cardiac function was assessed by in vivo catheterization and dobutamine stimulation. The complete data are shown in supplemental Table III. At baseline in this model, we were able to detect significant differences in the basal left ventricular (LV) end-systolic pressure (83.6 ± 1.0), dP/dtmax (6051.1 ± 241.5), and dP/dtmin (−5541.1 ± 151.2) in the MyBP-CAllP hearts, compared with the NTG and MyBP-CWT groups. The MyBP-CAllP hearts had decreased contractile performance (peak dP/dtmax) at maximum dobutamine stimulation, suggesting that a lack of cMyBP-C phosphorylation inhibits maximum contractility. To confirm that these changes were not attributable to compensatory phosphorylation of the other contractile proteins, such as the myosin light chains, the phosphorylation status of these proteins was examined using 2D electrophoresis and found to be unchanged in the MyBP-CAllP mouse hearts (supplemental Figure I).

MyBP-CAllP Fails to Rescue the MyBP-C(WT) Phenotype

The data indicated that partial replacement with a nonphosphorylatable cMyBP-C led to both structural and functional
deficits. If phosphorylatable cMyBP-C serves an essential function in the heart, complete replacement of endogenous cMyBP-C with MyBP-CWT should lead to significant functional deficits or even death. To test this hypothesis, line 262 was bred with MyBP-C(t/t), which should result in a homogenous complement of MyBP-CAllP-, as MyBP-C(t/t) produces only a small amount of protein that is truncated and nonfunctional. The MyBP-CWT line was also bred to MyBP-C(t/t) to confirm that a transgenic strategy could, in fact, rescue the MyBP-C(t/t) phenotype. Northern and dot-blot analysis confirmed robust expression of both MyBP-CWT and MyBP-CAllP- in the mice and confirmed that MyBP-CAllP-“replacement” in the MyBP-C(t/t) background was complete (Figure 4D).

The MyBP-C(t/t) mice have been characterized previously. The homozygous animals are viable but soon after birth display a progressive dilated cardiomyopathy. Myocyte hypertrophy, disarray, fibrosis, and calcification are observed, and these progress as the animals mature. The effectiveness of MyBP-CWT expression in preventing reactivation of a fetal transcription program was underscored by the lack of β-MHC expression, which serves as a sensitive marker for a nascent hypertrophic response (Figure 4E).

TG expression of MyBP-CWT also effectively rescued the overt hypertrophy displayed by the MyBP-C(t/t) hearts. In contrast, equal levels of MyBP-CWT expression did not rescue the MyBP-C(t/t)-induced hypertrophy (Figure 5A). The MyBP-C(t/t) and MyBP-C(t/t) mouse hearts showed markedly enlarged chambers, with significant cardiac hypertrophy and myocyte disarray, as compared with the NTG and MyBP-CWT mouse hearts (Figure 5B). Light microscopic analyses showed pathology typical of cardiac hypertrophy in both the MyBP-CWT and MyBP-CWT mice, whereas the MyBP-CWT-derived sections appeared normal (Figure 5C and 5D). Heart/body weights in the MyBP-CWT and MyBP-CWT mice were significantly elevated, whereas, in contrast, the values derived from the MyBP-CWT mice were essentially normal, confirming the absence of physiological hypertrophy (Figure 5E).

Ultrastuctural analysis showed the expected lack of M-band definition in the MyBP-CWT sarcomeres, as described previously in this model. In contrast, regular A- and I-bands and M-lines in both the MyBP-CWT and MyBP-CWT compared with MyBP-C(t/t) sarcomeres were apparent (Figure 6A). To confirm correct incorporation of MyBP-CAllP- and MyBP-CWT in the MyBP-C(t/t) background, we performed immunohistochemistry with both cMyBP-C and myc antibody. As expected, cMyBP-C was absent in the MyBP-C(t/t) hearts, but each transgenically encoded species showed the expected pattern of incorporation at approximately equal levels (Figure 6B).

The inability of MyBP-CWT to rescue the MyBP-C(t/t) phenotype was confirmed at the functional level. M-mode echocardiography showed the MyBP-CWT and MyBP-C(t/t) mice had increased LV end-diastolic and end-systolic dimensions, as well as reduced fractional shortening, whereas normal shortening fractions were observed in the MyBP-CWT hearts (Figure 7A and the Table). Although both MyBP-C(t/t) and MyBP-CWT mice incorporate normally into the sarcomere, MyBP-CWT expression appears to be able to rescue the MyBP-C(t/t) phenotype, whereas MyBP-C(t/t) cannot. To define this more completely, we looked for activation of the fetal gene program in these mice, as upregulation of ANF, brain natriuretic peptide (BNP), β-MHC, and skeletal α-actin and downregulation of α-MHC, phospholamban (PLN), and the sarcoplasmic reticulum Ca2+ pump (SERCA) often serve as sensitive markers for hypertrophy or cardiac stress. The data illustrate the completeness of the rescue, as no differences, even at the molecular level, were detected between the NTG and MyBP-CWT groups (Figure 7B and 7C). In contrast, ANF, β-MHC, BNP, and skeletal α-actin were upregulated, whereas α-MHC, PLN, and SERCA were significantly downregulated in both the MyBP-CWT and MyBP-CWT groups (Figure 7B and 7C), a pattern consistent with compromised cardiac function.

**Discussion**

Heart failure is associated with diminished β-adrenergic responsiveness, loss of cardiac contractility, abnormalities in Ca2+ handling, and altered contractile protein phosphorylation. Although cMyBP-C is extensively phosphorylat-
Cardiac Function Assessed by M-Mode Echocardiography

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>sd (mm)</th>
<th>LVED (mm)</th>
<th>LVES (mm)</th>
<th>hs (mm)</th>
<th>HR (bpm)</th>
<th>FS (%)</th>
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<tr>
<td>NTG</td>
<td>12(n=6)</td>
<td>0.89±0.16</td>
<td>3.8±0.49</td>
<td>0.78±0.15</td>
<td>2.24±0.29</td>
<td>1.2±0.17</td>
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<tr>
<td>MyBP-CWT:(t/t)</td>
<td>12(n=7)</td>
<td>1.02±0.16#</td>
<td>4.98±0.7#</td>
<td>0.9±0.24</td>
<td>4.23±0.62#</td>
<td>1.16±0.2</td>
</tr>
<tr>
<td>MyBP-CWT:(t/t)</td>
<td>12(n=7)</td>
<td>0.95±0.1#</td>
<td>3.7±0.39</td>
<td>0.91±0.23</td>
<td>2.21±0.24</td>
<td>1.4±0.28</td>
</tr>
<tr>
<td>MyBP-CWT:(t/t)</td>
<td>12(n=8)</td>
<td>1.02±0.13**</td>
<td>4.5±0.51**</td>
<td>0.96±0.16</td>
<td>3.57±0.51**</td>
<td>1.29±0.18**</td>
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Echocardiographic measurements were averaged from at least 3 separate cardiac cycles: septal thickness in diastole (sd), LV end-diastolic thickness (LVED), free wall thickness during diastole (hd), LV end-systolic thickness (LVES), septal thickness in systole (hs), heart rate (HR), and fractional shortening (FS). The MyBP-CWT:(t/t) and MyBP-CWT:(t/t) hearts demonstrated increased LV diastolic and systolic diameter as well as reduced fractional shortening. The statistical significance of differences was determined by unpaired Student t-tests. Data are expressed as means±SE. #P<0.001, #P<0.05: significant difference between MyBP-CWT:(t/t) and NTG. **P<0.001, *P<0.05: significant difference between MyBP-CWT:(t/t) and MyBP-CWT:(t/t). ¶¶P<0.001, ¶P<0.05: significant difference between MyBP-CWT:(t/t) and NTG.
filament, changes their orientation, increases the degree of order of the crossbridges, and decreases crossbridge flexibility.23,31,34,35 Although our data do not address crossbridge mechanics directly, it is clear that the mutant cMyBP-C is present in the sarcomere in a pattern that is indistinguishable from normal protein but appears to lack some critical function necessary for maintaining the overall sarcomere architecture, as manifested by the alterations observed in the sarcomere–mitochondrial spatial relationships. Further studies using these mouse models should provide valuable insight into the mechanical consequences of cMyBP-C phosphorylation.

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References


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From the Division of Molecular Cardiovascular Biology, Cincinnati Children’s Hospital Medical Center (S.S., J.G., H.O., R.K., L.A.M., J.R), Department of Medicine, University of Cincinnati (H.S.H., G.W.D), Cincinnati, OH, Department of Genetics, Howard Hughes Medical Institute and Harvard Medical School (C.E.S., J.G.S) and Cardiovascular Division, Brigham and Women’s Hospital, Boston, MA (C.E.S.).

Running title: Cardiac Myosin Binding Protein-C

Correspondence to Jeffrey Robbins PhD, Division of Molecular Cardiovascular Biology, MLC 7020, 3333 Burnet Ave, Cincinnati, OH 45229-3039 Tel: (513) 636-8098; Fax: (513) 636-5958. E-mail: jeff.robbins@cchmc.org
Materials and Methods

Transgene Construction

The cDNA encoding the endogenous form of mouse cardiac MyBP-C (MyBP-C\textsuperscript{WT}) was obtained by RT-PCR using total RNA isolated from the mouse cardiac ventricle. Full length MyBP-C\textsuperscript{WT} (~3.8kb) was subcloned, completely sequenced in both directions and compared with Genebank cDNA database (Accession number: NM_008653). The known phosphorylation sites (Ser-273, -282 and -302)\textsuperscript{1} and two neighboring potentially alternative phosphorylation sites (Thr-272 and -281) were converted to alanines using standard PCR-based methods and subcloned into the ApoI and SphI restriction sites. This construct was named MyBP-C\textsuperscript{AllP-}. Sequence encoding the human c-myc peptide (EQKLISEEDL) was inserted after the initiation methionine codon to differentiate the transgenic (TG) species from endogenous cMyBP-C.\textsuperscript{2,3} Earlier studies confirmed that introduction of the c-myc epitope was benign.\textsuperscript{3,4} The cDNAs were subcloned into the mouse \(\alpha\)-myosin heavy chain promoter and the construct purified from the plasmid backbone after NotI digestion. The DNAs were used to generate multiple lines of FVB/N TG mice.\textsuperscript{5} Six founders were obtained for this construct, the one chosen for further experimentation, line 262, contained 11 copies of the MyBP-C\textsuperscript{AllP-} transgene and expressed the protein at a level such that approximately 40% of endogenous cMyBP-C was replaced with the TG species.

MyBP-C\textsuperscript{(t/t)} Background.

MyBP-C\textsuperscript{(t/t)} mice,\textsuperscript{6} were back-crossed for more than 6 generations into the FVB/N background. MyBP-C\textsuperscript{AllP-} mice (line 262) were crossed with homozygous MyBP-C\textsuperscript{(t/t)} mice to ensure the absence of endogenous phosphorylatable cMyBP-C. An animal that expresses the normal cMyBP-C isoform,\textsuperscript{3} MyBP-C\textsuperscript{WT}, was also bred into the MyBP-C\textsuperscript{(t/t)} background to serve as a control. Except where noted, 5-6 mice, 12-15 weeks old of
mixed gender were used in each experiment after preliminary experiments showed no gender differences.

**Genotyping**

DNA samples were extracted from ear clips at 10 days and PCR was employed in two different reaction sets for either the cMyBP-C transgene or MyBP-C\(^{(t/t)}\). The forward (5’-TGTCAGCTTCAACAAAGAAGCCAAG-3’) and reverse (5’-CTTCAGGACCTTGAGACACTTTCTTC-3’) oligonucleotide sequences were used for genotyping cMyBP-C transgene. Oligonucleotide sequences for MyBP-C\(^{(t/t)}\) genotyping have been described.\(^6\)

**Mouse Models of Heart Failure**

Four different mouse models of heart failure were used to determine if cMyBP-C phosphorylation changed during the development of cardiac pathology:

1. **TG model.** This mouse (supplied by Dr. Jeffery Molkentin, Cincinnati) over-expresses the active form of calcineurin in the heart, developing cardiac hypertrophy and heart failure that mimics human heart disease.\(^7\) It shows a dramatic increase in heart size with the heart weight/body weight averaging 5-fold greater as compared to control littermates at 8 weeks post-birth.

2. **Gene targeted model.** The striated muscle-specific LIM-only protein MLP (muscle LIM protein) homozygous knockout mice (supplied by Dr. Pico Caroni, Basal, Switzerland) show dilated cardiomyopathy and heart failure.\(^8\)

3. **Pressure overload hypertrophy.** Acute pressure overload is typically used as a means of inducing a pathological profile of cardiac hypertrophic growth and ventricular remodeling. Initiation of pressure overload induced hypertrophy in the first 2 days post-surgery is associated with immediate early gene activation although less is known of the signaling factors or genes that sustain the long-term hypertrophic growth of the
myocardium and its transition to dilated failure. Pressure overload hypertrophy was induced by constriction of the transverse aortic arch (TAC). The arch was visualized through a median sternotomy and 7-0 silk ligature was tied around the aorta (27-gauge constriction) between the right brachiocephalic and left common carotid arteries. Heart weight and body weight ratios were calculated to confirm the presence of cardiac hypertrophy. Total myofibrillar proteins were obtained from both ventricles at either 1 day or 18 days after TAC surgery.

(4) Ischemia-reperfusion. In order to induce myocardial infarction, cardiac ischemia-reperfusion injury was performed in eight-week old FVB/N mice as described previously. The thoracotomy was closed and the mice were revived for a 1 hour ischemic period, after which the knot was released and the heart reperfused for 24 hours or 5 days. Upon completion of the reperfusion period, mice were sacrificed by CO₂ asphyxiation and the hearts quickly removed for analysis of infarction injury with 2% triphenyl tetrazolium chloride. Infarcts were ~40-45% of the left ventricle after 5 days of reperfusion. Total myofibrillar proteins were obtained from both ventricles, and included the area not at risk, area at risk, and infarcted area.

Molecular Analyses

For evaluating cMyBP-C transcript levels, hearts were homogenized in Tri-Reagent (Molecular Research Center) and total RNA extracted according to the manufacturer’s protocol. For assessment of cMyBP-C transcript size, northern blot analyses were performed using 7.5 µg of total RNA, as described. Expression levels were determined by RNA dot blot analysis with γ³²P-labeled cMyBP-C and human growth hormone (hGH) probes using transcript-specific oligonucleotides. GAPDH was used for the loading control. Expression levels of atrial natriuretic factor (ANF), brain natriuretic protein (BNP), α-skeletal actin, α- and β-myosin, phospholamban and SERC2a were
used to detect activation of transcription reflecting cardiac hypertrophic and/or heart failure.

**Protein Analyses**

To identify modifications at the protein level, enriched myofibrillar proteins were isolated using F60 buffer (60 mmol/L KCl, 30 mmol/L imidazole, 7.2 mmol/L MgCl₂, pH 7.0) with protease/phosphatase inhibitors (Cocktail I and II, Sigma) as described,¹¹ and solubilized in urea buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 20 mmol/L spermine, 1 mmol/L PMSF and 20 mmol/L DTT). The presence of MyBPC₅₅ protein was confirmed in SDS-PAGE (4-15% criterion gradient Tris-glycine pre-cast gels, Bio-Rad) followed by western blots using an anti-c-myc monoclonal antibody (clone 9E10, Roche) and an anti-cMyBP-C rabbit polyclonal antibody raised against the C0-C1 domains.¹²,¹³ Myc-tagged MyBP-C⁵⁵ was used as a positive control and for comparing protein levels.

**In Vitro PKA Phosphorylation and Phosphatase Treatment**

Total enriched myofibrils were isolated as described above in the presence of 1% Triton X-100 (Fisher Scientific) and protease/phosphatase inhibitors (Cocktail I and II, Sigma). The PKA phosphorylation assays contained 100 µg of freshly isolated total skinned myofibrils as described previously.¹⁴ Myofibrils were incubated 90 minutes at 37°C in 100 µl of 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 20 mmol/L MgCl₂, 10 µM ATP and 30 U of the PKA catalytic subunit (PKAce kit, Novagen). A PKA inhibitor, PKI 6-22 Amide (Calbiochem), was used for control studies to confirm the specificity of PKA activity. Similarly, 100 µg of freshly isolated total myofibrils were treated with 10 U of alkaline phosphatase (Roche) in 1× dephosphorylation buffer (0.5 mol/L Tris-HCl and 1mmol/L EDTA, pH 8.5). Myofibrils were washed 3 times in F60 and solubilized in 7 mol/L urea buffer for IEF. Protein was determined by the Bradford method (Bio-Rad).
**Measurement of MyBP-C Phosphorylation**

One dimensional isoelectric focusing (IEF) was used to detect the multiple phosphorylated species of a subset of the contractile proteins (de-, mono- bi- and tri-phosphorylated) as described in detail previously$^{12,13}$ with some modifications. IEF slab gels were prepared using empty criterion cassettes (Bio-Rad) consisting of 5% Duracryl (Genomic Solutions), 5% glycerol, 1× of both pH 5-7 and pH 3-10 ampholites (Bio-Rad) were degassed for 1 hour and the three catalysts (riboflavin-5′-phosphate (FMN), ammonium persulfate, and N,N,N,N,-tetramethylethylenediamine (TEMED) added separately. Total myofibrillar protein was loaded in buffer containing 10 mol/L urea, 40% glycerol, 1× of both pH 5-7 and pH 3-10 ampholites, and 2% Triton X-100 with 0.025% bromophenol blue. The cathode buffer consisted of 30 mmol/L NaOH (pH 10.1), and the anode buffer consisted of 15 mmol/L phosphoric acid (pH 2.4) (Bio-Rad). Standard IEF markers (Bio-Rad) were used for calculating isoelectric points (pI). The running conditions were 1 W for 2 hours, 2 W for 30 minutes and 500 V for 2 hours per gel. The transfer took place in 0.7% acetic acid (pH 3), placing the nitrocellulose membrane towards the anode at 200 mA for 10-12 hours at 4°C. After the transfer, an immunoblotting procedure was performed using a polyclonal anti-cMyBP-C antibody as described.$^{2,3}$ Percentage phosphorylation was expressed as cMyBP-C (phosphorylated species)/(P0+P1+P2+P3) x 100%.

**Two-Dimensional Electrophoresis**

Myofilament proteins suspended in 8 mol/L urea buffer were analyzed via two-dimensional analysis to assess the phosphorylation status of selected contractile proteins. Total myofilament proteins, 50-75 µg, were diluted in 185 µl IPG rehydration buffer (Bio-Rad) containing 1× ampholytes (Bio-Rad) and subjected to IEF on IPG strips (11 cm, pH 4-7 or pH 7-10 linear gradient, Bio-Rad) according to the manufacture’s instructions. This was followed by 10-20% gradient SDS-PAGE (Criterion gradient Tris-
glycine pre-cast gels, Bio-Rad). The gels were stained with SYPRO-Ruby (Bio-Rad) for 8-12 hours and scanned on the Typhoon 9400 scanner (Amersham Biosciences) or transferred to nitrocellulose membranes (Bio-Rad) to quantitate the level of myofibrillar protein phosphorylation by western blotting.

**Echocardiography Measurements**

For two-dimensional M-mode echocardiography, mice were anesthetized with 2% isoflurane (Table 1). In some cases, conscious unsedated mice were used (Table 2S, Online Supplement), and hearts visualized with a Hewlett Packard Sonos 5500 instrument and a 15 MHZ transducer as described previously.\(^\text{15}\) Measurements were taken three times per animal from different areas and then averaged for left ventricular (LV) diastolic and systolic dimensions (LVEDD and LVESD) and septal and posterior wall thickness (SWT and PWT), from which fractional shortening (FS) and LV mass was derived. Pulsed wave Doppler was used to measure aortic ejection time (E-TIME) and calculate the velocity of circumferential shortening, \(V_{cf}\) (FS/E-TIME). Diastolic transmitral inflow Doppler indices included the following: peak E wave velocity, peak A wave velocity, E wave deceleration time, and isovolumic relaxation time (IVRT).

**In Vivo Cardiac function and β-adrenergic Responsiveness**

Invasive hemodynamic studies was performed in the intact animal as previously described.\(^\text{16}\) Mice were anesthetized with ketamine-thiobutabarbital and following tracheotomy (PE-90), the right femoral artery and vein were cannulated with polyethylene tubing for measuring systemic arterial pressure and for the infusion of experimental drugs. To assess myocardial performance, closed chest animals were studied with a high fidelity 1.4F Millar micromanometer tipped catheter placed retrograde across the aortic valve into the LV (via right carotid cut-down) for pressure measurement and determining \(dP/dt_{\text{max}}\) and \(dP/dt_{\text{min}}\). Cardiovascular responses to
increasing doses of dobutamine were determined during 3 minute constant infusions (0.1 µl/min/gm body weight), and average values determined during the final 30 seconds of each infusion. Online heart rate, telemetry, LV developed pressure, +dP/dt, and -dP/dt were archived on a Macintosh G4 using Maclab software.

**Histochemistry**

Heart weight/body weight was measured to determine if hypertrophy had occurred. For histopathological examinations, the hearts were removed while still beating from deeply anesthetized mice, drained of blood, and fixed in 10% formalin. The hearts were bisected longitudinally, dehydrated through a graded series of alcohols, and laid open before being paraffin embedded. Step-serial sections (5 µm) were taken from 2-3 hearts per group. Sections were stained with hematoxylin-eosin or Masson’s trichrome and images evaluated with the SPOT software (Diagnostic Instruments, Inc) using an Olympus BX60 microscope. The presence of necrosis, fibrosis, myocyte disarray and calcification were evaluated by an expert who was blinded to genotype.

**Immunohistochemistry**

Localization and integration of cMyBP-C into the sarcomere was determined by confocal microscopy. Hearts were perfused in 4% paraformaldehyde/cardioplegic buffer (50 mmol/L KCl, 5% dextrose in phosphate buffered saline (PBS)), fixed overnight in 4% paraformaldehyde/PBS at 4°C, transferred to 10% sucrose for 3 hours, followed by 30% sucrose for at least overnight. After fixing, the hearts were embedded in OCT, frozen on dry ice and the blocks stored at -80°C until use. Five µm frozen sections were fixed on microscope slides (SuperfrostPlus, Fisher) and stored at -80°C. Before processing for immunostaining, the frozen sections were allowed to dry at room temperature for 15 minutes. The slides were placed in a slide jar and microwaved with antigen retrieval buffer (10 mmol/L citric acid and 0.05% Tween 20, pH 6.0) for 2, 5 and 25 minutes at
power levels 100, 20 and 10, respectively (Kenmore) and then cooled to room
temperature. The slides were washed with PBS (3 times, 5 minutes each), incubated in
blocking solution (1% BSA, 0.1% cold water fish skin gelatin, 0.05% NaN₃ and 0.1%
Tween-20 in PBS) for 1 hour at room temperature and probed with 1:1300 diluted
polyclonal antibody against cMyBP-C (C0-C1) in PBS for overnight at 4°C. The slides
were washed with PBS 3 times for 5 minutes each, incubated with blocking solution for
20 minutes, and incubated with secondary goat anti-rabbit IgG antibody (1:100)
conjugated with Alexa-488 (green fluorescence) in PBS. Finally, the slides were washed
with PBS 3 times for 5 minutes each, and mounted with Vecta Shield. For long-term
storage, immuno-labeled slides were placed in the dark at 4°C. The vector biotin-avidin
blocking and M. O. M. kits (Vector Laboratories, Inc) were used for immunostaining
with the monoclonal anti-myc antibody and with the Alexa-488 conjugated goat anti-
mouse IgG antibody. Specimens were examined using confocal microscopy (Nikon PCM
2000) and software SimplePCI v.4 (C.Imaging Systems) by an investigator blinded to
genotype.

Transmission Electron Microscopy

Structural analyses at the light and electron microscopy levels were performed as
described.²,³ For electron microscopic ultrastructural analysis, mice were anesthetized
with isofluorane and the hearts fixed by perfusion with 3.5% glutaraldehyde in
cardioplegic buffer for 2 minutes, followed by 3.5% glutaraldehyde in 100 mM
cacodylate buffer (pH 7.3) for 2 minutes. The fixatives were gravity fed (600 mm) into
the hearts through the apex and right ventricle. Immediately after the hearts started to fill
with the fixative, the aorta and pulmonary artery were cut to allow the output of liquids.
The hearts were then excised and subsequently separated into six regions: left atrial flap,
right atrial flap, left ventricular free wall, right ventricular free wall, septum, and apex.
Each region was divided into small (1 mm) fragments and fixed in the glutaraldehyde-
cacodylate fixative overnight at 4°C. The tissue fragments were post-fixed on ice in 1% O₃O₄ in cacodylate buffer, dehydrated in acetone, and embedded in a Poly/Bed 812 resin mixture. Thin sections were counterstained with uranyl acetate and lead citrate and examined on a Zeiss 912 transmission electron microscope at an accelerating voltage of 100 kV. Multiple sections were cut from 2-4 mice of mixed gender and >50 fields were observed by a blinded observer.

**Statistical Analysis**

Data are expressed as means±SE. The significance of differences among means was evaluated either using analysis of variance (ANOVA) or Student’s t test. Statistically significant differences were considered as $P<0.05$. 
Figure 1S. Phosphorylation status of myosin essential light chain (ELC1v) and regulatory light chain (RLC2v) in MyBP-C<sup>WT</sup> (WT), MyBP-C<sup>AllP–</sup> (AllP–) and MyBP-C<sup>(t/t)</sup> (t/t) mouse hearts. Two-dimensional electrophoresis (pH 4-7) was performed as described previously<sup>17</sup> to determine if any compensatory changes in the phosphorylation status of ELC1v and RLC2v had occurred in response to the presence of MyBP-C<sup>AllP–</sup> protein. Changes in phosphorylation status of these proteins can sometimes be associated with heart failure.<sup>18</sup> No significant differences could be detected between the NTG, MyBP-C<sup>WT</sup> and MyBP-C<sup>AllP–</sup> samples.
Table 1S. Quantification of cMyBP-C phosphorylation species (n=3)

<table>
<thead>
<tr>
<th>Lanes</th>
<th>Models</th>
<th>%P3</th>
<th>%P2</th>
<th>%P1</th>
<th>%P0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24 hours Sham</td>
<td>23.1 ± 1.1</td>
<td>43.1 ± 5.4</td>
<td>27.1 ± 3.0</td>
<td>6.7 ± 1.3</td>
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<tr>
<td>2</td>
<td>24 hours TAC</td>
<td>11.5 ± 1.0‡</td>
<td>45.2 ± 1.7</td>
<td>30.6 ± 2.4</td>
<td>12.6 ± 0.9‡</td>
</tr>
<tr>
<td>3</td>
<td>18 day Sham</td>
<td>21.7 ± 1.4</td>
<td>43.7 ± 3.3</td>
<td>26.7 ± 1.0</td>
<td>7.9 ± 0.9</td>
</tr>
<tr>
<td>4</td>
<td>18 day TAC</td>
<td>2.2 ± 2.9†</td>
<td>13.0 ± 3.1†</td>
<td>16.6 ± 4.4††</td>
<td>68.1 ± 10.1†</td>
</tr>
<tr>
<td>5</td>
<td>NTG</td>
<td>24.3 ± 1.8</td>
<td>42.9 ± 3.6</td>
<td>25.3 ± 2.3</td>
<td>7.5 ± 1.1</td>
</tr>
<tr>
<td>6</td>
<td>Calcineurin TG</td>
<td>2.0 ± 1.1*</td>
<td>22.9 ± 1.3*</td>
<td>27.0 ± 4.6**</td>
<td>57.2 ± 3.9*</td>
</tr>
<tr>
<td>7</td>
<td>MLP-KO</td>
<td>1.5 ± 0.9*</td>
<td>24.4 ± 1.8*</td>
<td>34.7 ± 3.8**</td>
<td>39.4 ± 4.8*</td>
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<tr>
<td>8</td>
<td>β-MHC TG</td>
<td>27.0 ± 0.8</td>
<td>47.7 ± 1.9</td>
<td>21.7 ± 3.8</td>
<td>3.6 ± 2.7</td>
</tr>
<tr>
<td>9</td>
<td>24 hours Sham</td>
<td>32.6 ± 2.9</td>
<td>45.9 ± 0.5</td>
<td>18.3 ± 5.5</td>
<td>3.2 ± 2.7</td>
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<tr>
<td>10</td>
<td>24 hours IR</td>
<td>34.4 ± 1.8</td>
<td>45.4 ± 0.5</td>
<td>15.9 ± 0.7</td>
<td>4.3 ± 2.3</td>
</tr>
<tr>
<td>11</td>
<td>5 day Sham</td>
<td>23.4 ± 16.1</td>
<td>52.9 ± 9.0</td>
<td>20.4 ± 3.8</td>
<td>3.3 ± 4.7</td>
</tr>
<tr>
<td>12</td>
<td>5 day IR</td>
<td>25.2 ± 3.2</td>
<td>24.9 ± 3.4##</td>
<td>14.3 ± 4.0</td>
<td>34.7 ± 4.3#</td>
</tr>
</tbody>
</table>

One dimensional IEF focusing (pH 5-7) of total myofilament proteins followed by western blot analysis using cMyBP-C antibody as described in detail in the Online Supplemental Materials and Methods. A representative western blot is shown in Figure 1A. The percent of de-, mono, di- and tri-phosphorylated MyBP-C (P0, P1, P2, and P3, respectively) is shown for each model. Signal on the membrane was quantitated using a STORM 860 (GE Healthcare) and ImageQuant v.5.2. Percentage of phosphorylation is expressed as cMyBP-C (Phosphorylated species)/(P0+P1+P2+P3)X100. Values are expressed as means±SE.

‡‡P<0.05 and ‡P<0.001, significant difference vs 24 hours TAC and 24 hours Sham.

††P<0.05 and †P<0.001, significant difference vs 18 day TAC and 18 day Sham

**P<0.05 and *P<0.001, significant difference vs with NTG

##P<0.05 and #P<0.001, significant difference vs 5 day IR and 5 day Sham
Table 2S. Cardiac Function Assessed by M-mode Echocardiography

<table>
<thead>
<tr>
<th>Groups</th>
<th>LVED (mm)</th>
<th>LVES (mm)</th>
<th>SW (mm)</th>
<th>PW (mm)</th>
<th>HR (bpm)</th>
<th>E-TIME</th>
<th>LVM (g/m2)</th>
<th>FS (%)</th>
<th>V&lt;sub&gt;cf&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG</td>
<td>3.1 ± 0.08</td>
<td>1.7 ± 0.1</td>
<td>0.7 ± 0.01</td>
<td>0.7 ± 0.02</td>
<td>693.3 ± 27.2</td>
<td>38.2 ± 1.1</td>
<td>63.4 ± 3.7</td>
<td>47.2 ± 2.2</td>
<td>11.7 ± 0.8</td>
</tr>
<tr>
<td>WT</td>
<td>3.3 ± 0.15</td>
<td>1.8 ± 0.2</td>
<td>0.6 ± 0.02</td>
<td>0.6 ± 0.02</td>
<td>711.6 ± 14.2</td>
<td>40.8 ± 1.6</td>
<td>65.3 ± 5.7</td>
<td>46.0 ± 3.7</td>
<td>10.5 ± 1.2</td>
</tr>
<tr>
<td>AllP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.2 ± 0.19</td>
<td>1.7 ± 0.1</td>
<td>0.7 ± 0.02</td>
<td>0.7 ± 0.02</td>
<td>683.5 ± 13.7</td>
<td>40.9 ± 1.3</td>
<td>66.2 ± 8.1</td>
<td>47.0 ± 2.8</td>
<td>10.9 ± 1.1</td>
</tr>
</tbody>
</table>

Conscious unsedated mice at 3 months were used. Measurement values were averaged from at least 3 separate cardiac cycles: left ventricular (LV) end diastolic thickness (LVED), LV end systolic thickness (LVES), septal wall thickness (ESW), posterior wall thickness (PW), aortic ejection time (E-TIME), LV mass (LVM), fractional shortening (FS), heart rate (HR) and velocity of circumferential shortening, V<sub>cf</sub> (FS/E-TIME). The MyBP-C<sup>AllP</sup>-TG hearts demonstrated normal cardiac function compared to NTG and MyBP-C<sup>WT</sup> TG cohorts (n=6). Data are expressed as means±SE.
Table 3S. Cardiac Function Assessed by In Vivo Catheterization

<table>
<thead>
<tr>
<th>Groups</th>
<th>Heart rate (bpm)</th>
<th>LVP (mm Hg/s)</th>
<th>dP/dt\text{max} (mm Hg/s)</th>
<th>dP/dt\text{min} (mm Hg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Dobutamine</td>
<td>Basal</td>
<td>Dobutamine</td>
</tr>
<tr>
<td>NTG</td>
<td>400 ± 4.7</td>
<td>606 ± 11.3</td>
<td>108.6 ± 3.0</td>
<td>121.8 ± 4.0</td>
</tr>
<tr>
<td>WT</td>
<td>425 ± 4.8</td>
<td>625 ± 6.4</td>
<td>92.1 ± 2.4</td>
<td>99.9 ± 1.0</td>
</tr>
<tr>
<td>AllP\text{-}\text{NTG}</td>
<td>383 ± 5.5#</td>
<td>548 ± 7.2</td>
<td>83.6 ± 1.0**</td>
<td>99.6 ± 1.5</td>
</tr>
</tbody>
</table>

Measurements of LV hemodynamic parameters, including heart rate, peak LV pressure (LVP), LV dP/dt\text{max} (an index of myocardial contractility) and LV dP/dt\text{min} (an index of myocardial relaxation), were determined. The LV dP/dt\text{max} and dP/dt\text{min} in the MyBP-C\text{AllP}\text{-}mice was markedly decreased by 76% and 78%, respectively, compared with to the NTG and MyBP-C\text{WT} mice. LV dP/dt\text{max} and dP/dt\text{min} in MyBP-C\text{AllP}\text{-}TG mice remained substantially lower than in NTG and MyBP-C\text{WT} TG mice. All data are presented as means±SE. **P<0.05 and *P<0.001 vs NTG. #P<0.05 and ##P<0.001 vs WT (n = 6) for all cohorts.
References


15. Hahn HS, Marreez Y, Odley A, Sterbling A, Yussman MG, Hilty KC, Bodi I, Liggett SB, Schwartz A, Dorn GW, 2nd. Protein kinase Ca negatively regulates

