Enhanced cardiac contractile function with increased sarcomere length (SL) is, in part, mediated by a decrease in the radial distance between myosin heads and actin. The radial disposition of myosin heads relative to actin is modulated by cardiac myosin binding protein-C (cMyBP-C), suggesting that cMyBP-C contributes to the length-dependent activation (LDA) in the myocardium. However, the precise roles of cMyBP-C in modulating cardiac LDA are unclear. To determine the impact of cMyBP-C on LDA, we measured isometric force, myofilament Ca$^{2+}$-sensitivity ($pCa_{50}$) and length-dependent changes in kinetic parameters of cross-bridge (XB) relaxation ($k_{rel}$), and recruitment ($k_{rec}$) due to rapid stretch, as well as the rate of force redevelopment ($k_r$) in response to a large slack-restretch maneuver in skinned ventricular multicellular preparations isolated from the hearts of wild-type (WT) and cMyBP-C knockout (KO) mice, at SLs 1.9 μm or 2.1 μm. Our results show that maximal force was not significantly different between KO and WT preparations but length-dependent increase in $pCa_{50}$ was attenuated in the KO preparations. $pCa_{50}$ was not significantly different between WT and KO preparations at long SL (5.82 ± 0.02 in WT vs. 5.87 ± 0.02 in KO), whereas $pCa_{50}$ was significantly different between WT and KO preparations at short SL (5.71 ± 0.02 in WT vs. 5.80 ± 0.01 in KO; p < 0.05). The $k_r$, measured at half-maximal Ca$^{2+}$-activation, was significantly accelerated at short SL in WT preparations (8.74 ± 0.56 s$^{-1}$ at 1.9 μm vs. 5.71 ± 0.40 s$^{-1}$ at 2.1 μm, p < 0.05). Furthermore, $k_{rel}$ and $k_{df}$ were accelerated by 32% and 50%, respectively at short SL in WT preparations. In contrast, $k_r$ was not altered by changes in SL in KO preparations (8.03 ± 0.54 s$^{-1}$ at 1.9 μm vs. 8.90 ± 0.37 s$^{-1}$ at 2.1 μm). Similarly, KO preparations did not exhibit length-dependent changes in $k_{rel}$ and $k_{df}$. Collectively, our data implicate cMyBP-C as an important regulator of LDA via its impact on dynamic XB behavior due to changes in SL.

**Keywords:** cMyBP-C, length-dependent activation, sarcomere length, myofilament function, cross-bridge kinetics

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**INTRODUCTION**

Length-dependent activation (LDA) is the mechanism by which force production in the heart becomes more sensitive to Ca$^{2+}$ as the sarcomere length (SL) is increased (Allen and Kentish, 1985). Although it is well recognized that LDA underlies the Frank-Starling’s Law of the heart, the cellular and molecular mechanisms that modulate this process are still poorly understood mainly because LDA involves a dynamic and complex interplay between a multitude of thick- and thin-filament-based mechanisms (De Tombe et al., 2010). The thick-filament-based mechanisms involve augmentation of strong crossbridge (XB) formation followed by enhancement in the myofilament Ca$^{2+}$ sensitivity upon a reduction in the myofilament lattice spacing and the radial distance between the thick and thin filaments at long SL (Fuchs and Smith, 2001). The strongly-bound XBs then cooperatively recruit additional near-neighbor XBs into the force-bearing state (Gordon et al., 2000; Regnier et al., 2004). The thin-filament-based mechanisms involve an increased affinity of troponin C (TnC) to Ca$^{2+}$ when the neighboring TnC sites are bound with Ca$^{2+}$ and the increased affinity of TnC to Ca$^{2+}$ is also a result of a positive feedback effect of the strongly-bound XBs (Hannon et al., 1992; Moss et al., 2004; Li et al., 2014). Furthermore, the cooperative effect between neighboring troponin-tropomyosin (Tn-Tm) complexes also impacts the Ca$^{2+}$ binding properties of the thin-filament (Butters et al., 1997; Farman et al., 2010) and thus influence the LDA in cardiac muscle (for details on LDA refer to reviews by Konhilas et al., 2002; Hanft et al., 2008; De Tombe et al., 2010; Campbell, 2011).

Earlier investigations have proposed that LDA in cardiac muscle is influenced by various sarcomeric proteins such as TnC (Gulati et al., 1991), TnI (Konhilas et al., 2003; Tachampa et al., 2007), TnT (Chandra et al., 2006), myosin heavy chain (Korte and McDonald, 2007), essential light chain (Michael et al., 2013), and titin (Fukuda et al., 2003). In addition to the aforementioned sarcomeric proteins, it is also possible that cardiac myosin binding protein-C (cMyBP-C) may be an important modulator of cardiac LDA because cMyBP-C is uniquely positioned in the sarcomere to interact with both the thick- and thin-filaments (Squire...
et al., 2003; Shaffer et al., 2009; Previs et al., 2012; Sadayappan and De Tombe, 2012; Mun et al., 2014), and has been shown to be important in regulating key aspects of dynamic XB behavior (Stelzer et al., 2006a,b; Coulton and Stelzer, 2012), and providing structural rigidity to the myofilament lattice (Palmer et al., 2011).

Importantly, recent evidence from low-angle X-ray diffraction experiments showed that cMyBP-C tethers the myosin XBs closer to the thick-filament backbone and that ablation of cMyBP-C results in the radial displacement of XB towards the thin-filament (Colson et al., 2007). The role of cMyBP-C in LDA is also underscored by the observation that length-dependent increase in myofilament Ca2+ sensitivity was blunted in cardiac preparations from patients with cMyBP-C mutations (Van Dijk et al., 2012; Sequeira et al., 2013). However, the precise roles of cMyBP-C in modulating length-dependent changes in cardiac contractile dynamics are still unknown. Therefore, to determine the impact of cMyBP-C on length-dependent changes in contractile dynamics, we utilized skinned myocardium from a cMyBP-C knock-out (KO) mouse model (Harris et al., 2002), and measured steady-state contractile parameters and we also used stretch activation experiments to measure the kinetic parameters. We measured Ca2+-activated maximal force, myofilament Ca2+ sensitivity (pCa50), rate of force redevelopment (ktr), rate of XB relaxation (krel), and rate of XB recruitment (krec) at short (1.9 μm) and at long (2.1 μm) SLs. Our results show that the length-dependent increase in pCa50 was attenuated in the KO preparations compared to wild-type (WT) preparations. Furthermore, length-dependent changes in dynamic contractile parameters ktr, krel, and krec were blunted in KO preparations compared to WT preparations, indicating that cMyBP-C plays a critical role in the myofilament-mediated response in cardiac LDA.

**MATERIALS AND METHODS**

**ETHICAL APPROVAL AND ANIMAL TREATMENT PROTOCOLS**

This study was performed according to the protocols laid out in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, Revised 1996), and was conducted according to the guidelines of the Institutional Animal Care and Use Committee at Case Western Reserve University. Mice aged 3–6 months, of both sexes, and belonging to SV/129 strain were used according to the guidelines of the Institutional Animal Care and Use Committee at Case Western Reserve University. Mice aged 3–6 months, of both sexes, and belonging to SV/129 strain were used for the experiments. KO mice used in this study were previously generated and well-characterized (Harris et al., 2002). WT mice expressing normal, full-length cMyBP-C in the myocardium were used as controls.

**ESTIMATION OF cMyBP-C CONTENT AND PHOSPHORYLATION STATUS OF SARCOMERIC PROTEINS IN WT AND KO HEART SAMPLES**

Cardiac myofibrils were isolated from frozen mouse ventricles on the day of the experiment (Gresham et al., 2014). A piece of the frozen tissue was thawed in a fresh relaxing solution, homogenized, and the myofibrils were then skinned for 15 min with 1% Triton X-100 (Cheng et al., 2013). Skinned myofibrils were then resuspended in fresh relaxing solution containing protease and phosphatase inhibitors (PhosSTOP and cOmplete ULTRA Tablets; Roche Applied Science, Indianapolis, IN, USA) and stored on ice. To determine the cMyBP-C content and myofilament protein phosphorylation status, ventricular samples were solubilized by adding Laemmli buffer and were heated to 90°C for 5 min. For Western blot analysis, 10 μg of cardiac myofibrils were electrophoretically separated on 4–20% Tris-glycine gels (Lansa Walkersville Inc., Rockland, ME, USA) at 180 V for 60 min. Proteins were transferred to PVDF membranes and incubated overnight with a primary antibody that detects cMyBP-C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described previously (Cheng et al., 2013). For Pro-Q phosphoprotein analysis, 2.5 μg of solubilized cardiac myofibrils were electrophoretically separated at 180 V for 85 min then fixed and stained with Pro-Q diamond phosphoprotein stain (Invitrogen, Carlsbad, CA, USA) to assess the phosphorylation status of sarcomeric proteins. After imaging the Pro-Q stained gels, the gels were counterstained with Coomassie blue to determine if there are any changes in the isoform expression of sarcomeric proteins. Densitometric scanning of the stained gels was done using Image J software (U.S. National Institutes of Health, Bethesda, MD, USA) (Gresham et al., 2014).

**PREPARATION OF SKINNED VENTRICULAR MULTICELLULAR PREPARATIONS AND Ca2+ SOLUTIONS FOR EXPERIMENTS**

Skinned ventricular multicellular preparations were prepared as described previously (Cheng et al., 2013; Gresham et al., 2014). In brief, ventricular tissue was homogenized in a relaxing solution and skinned for 60 min using 1% Triton-X 100. Multicellular preparations with dimensions ~100 μm in width and 400 μm in length were selected for the experiments. The composition of various Ca2+ activation solutions used for the experiments was calculated using a computer program (Fabiato, 1988) and known stability constants (Godt and Lindley, 1982). All solutions contained the following (in mM): 100 N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 15 creatine phosphate, 5 dithiothreitol, 1 free Mg2+, and 4 MgATP. The maximal activating solution (pCa 4.5; pCa = -log [Ca2+]free) also contained 7 EGTA and 7.01 CaCl2; while the relaxing solution (pCa 9.0) contained 7 EGTA and 0.02 CaCl2; and the pre-activating solution contained 0.07 EGTA. The pH of the Ca2+ solutions was set to 7.0 with KOH and the ionic strength was 180 mM. A range of pCa solutions, containing varying amounts of [Ca2+]free, were then prepared by mixing appropriate volumes of pCa 9.0 and 4.5 stock solutions and the experiments were performed at 22°C.

**EXPERIMENTAL APPARATUS FOR THE ESTIMATION OF ISOMETRIC FORCE AND FORCE-pCA RELATIONSHIPS**

Detergent-skinned ventricular preparations were held between a motor arm (312C; Aurora Scientific Inc., Aurora, Ontario, Canada) and a force transducer (403A; Aurora Scientific Inc.) as described previously (Merkulov et al., 2012; Cheng et al., 2013). Changes in the motor position and signals from the force transducer were sampled (16-bit resolution, DAP5216a, Microstar Laboratories; Bellevue, WA) at 2.0 kHz using SL control software (Campbell and Moss, 2003). As previously described (Stelzer et al., 2006a,b,c), the experimental set up was positioned on the stage of an inverted microscope (Olympus; Tokyo, Japan) that was fitted with a 40X objective and a closed-circuit television camera (model WV-BL600; Panasonic, Tokyo, Japan). To illuminate the multicellular preparations, we used the light emanating from...
a halogen lamp and the light was passed through a cut-off filter (transmission >620 nm) before reaching the preparation. Bitmap images of the preparations were captured using an AGP 4X/2X graphics card and its associated software (ATI Technologies) to assess the SL of our preparations during the experiment. For all mechanical measurements, SL of the muscle preparations was set to 1.9 or 2.1 \( \mu m \) in a relaxing solution and submaximal force (\( P \)) developed at each pCa was normalized to maximal force (\( P_o \), at pCa 4.5) i.e., \( P/P_o \) to construct the force-pCa relationships. The force-pCa data were fit using the equation \( P/P_o = \frac{[Ca^{2+}]^{nH}}{[Ca^{2+}]^{nH} + [Ca^{2+}]^{1+H}} \), where \( n_H \) is the Hill coefficient and \( k \) is the pCa required to produce half-maximal activation (i.e., pCa_{50}) (Gresham et al., 2014).

**MEASUREMENT OF THE RATE OF FORCE REDEVELOPMENT (\( k_{fr} \))**

\( k_{fr} \) was measured at 50% of maximal activation in WT and KO muscle preparations to assess the rate of XB transitions from weak- to strong-binding states (Brenner and Eisenberg, 1986; Campbell, 1997). Measurement of \( k_{fr} \) in Ca\(^{2+}\)-activated muscle preparations was performed according to a mechanical slack-restretch protocol described previously (Stelzer et al., 2006b; Chen et al., 2010; Cheng et al., 2013). Skinned muscle preparations were transferred from relaxing (pCa 9.0) to an activating Ca\(^{2+}\) solution (pCa ranging from 6.2 to 4.5), and once the muscle preparations attained steady-state isometric force, they were rapidly slackened by 20% of their original muscle length and were held for 10 ms using a high-speed length control device (Aurora Scientific Inc.). The slackening was followed by the brief period of unloaded shortening which resulted in a rapid decline in force due to detachment of the strongly-bound XBs. The muscle preparation was then rapidly restretched back to its original length and the time course of force redevelopment was measured. \( k_{fr} \) for each slack-restretch maneuver was estimated by linear transformation of the half-time of force decay and force redevelopment.

**STRETCH ACTIVATION EXPERIMENTS TO MEASURE DYNAMIC CONTRACTILE PARAMETERS**

Stretch activation experiments were carried out as previously described (Cheng et al., 2013; Gresham et al., 2014), except that in this study a 2% stretch of initial muscle length perturbation was utilized. Muscle preparations were placed in pCa solutions that produced submaximal force (\(~50\%\) of maximal force), and were allowed to develop a steady-state force. Muscle preparations were then rapidly stretched by 2% of their initial muscle length and were held at the increased length for 5 s before being returned back to their initial muscle length. The characteristic features of the stretch activation response in cardiac muscle have been described previously (Stelzer et al., 2006d; Ford et al., 2010), and the stretch activation parameters measured are presented in Figure 1. In brief, a sudden 2% stretch of muscle length elicits an instantaneous rise in force (P1) in the muscle preparation, which is due to the strain of elastic elements within the strongly-bound XBs (Phase 1). The force then rapidly declines because the strained XBs rapidly detach (Phase 2) and equilibrate into a non-force producing state, with a characteristic rate constant \( k_{rel} \). Following this phase of rapid decline, force development occurs gradually (Phase 3), with a characteristic rate constant \( k_{df} \), which is a result of length-induced recruitment of new XBs into the force-bearing state (Stelzer et al., 2006d; Gresham et al., 2014). Stretch activation amplitudes were normalized to prestretch Ca\(^{2+}\)-activated force and were measured as described previously (Desjardins et al., 2012; Gresham et al., 2014). \( k_{rel} \) and \( k_{df} \) were estimated using a linear transformation of the half-time of force decay and force redevelopment.

**DATA ANALYSIS**

Data were analyzed using Two-Way analysis of variance (ANOVA). One factor in this analysis was cMyBP-C variant (WT or KO), and the second was SL (1.9 or 2.1 \( \mu m \)). Therefore, we used Two-Way ANOVA to test the hypothesis that the effect of the SL on a given contractile parameter depended on the cMyBP-C variant.
EFFECT OF ABLATION OF cMyBP-C ON THE EXPRESSION AND PHOSPHORYLATION LEVELS OF SARCOMERIC PROTEINS

Western blot analysis of WT and KO ventricular samples was done using a primary antibody that detects cMyBP-C protein (Cheng et al., 2013). As predicted, cMyBP-C is present in the WT sample but is completely absent in the KO sample (Figure 2A). SDS gels loaded with ventricular samples from WT and KO hearts were stained with Coomassie blue or Pro-Q Diamond stain to assess the effects of cMyBP-C KO on sarcomeric protein isoform expression and phosphorylation levels (Figures 2B,C, respectively). As reported in our recent studies (Desjardins et al., 2012; Merkulov et al., 2012) the KO hearts exhibited a slight increase (16 ± 3%) in the level of β-myosin heavy chain (MHC) expression (data not shown). Consistent with our previous studies (Desjardins et al., 2012; Merkulov et al., 2012), the expression and phosphorylation levels of other regulatory contractile sarcomeric proteins such as cardiac TnT, cardiac TnI, and regulatory light chain were not different between WT and KO skinned myocardium (Figures 2B,C).

EFFECT OF cMyBP-C ON LENGTH-DEPENDENT CHANGES IN Ca2+-ACTIVATED MAXIMAL FORCE PRODUCTION

To assess the effect of cMyBP-C on length-dependent changes in thin-filament activation, Ca2+-activated maximal force production (at pCa 4.5) was measured at SL's 1.9 and 2.1 μm in WT and KO muscle preparations (values are shown in Table 1). Two-Way ANOVA (see Data analysis under Methods section for details) revealed no significant interaction effect, but revealed a significant main effect (P < 0.005) of SL on Ca2+-activated maximal force production. To probe the determining factor for the significant main effect, subsequent post-hoc tests were carried out. These post-hoc tests using multiple planned pairwise comparisons showed that maximal force production was not significantly different between WT and KO groups at either SL (Table 1). However, maximal force (Fmax) was significantly decreased by ~34% and ~38% at short SL vs. long SL in WT and KO groups, respectively (Table 1). Similar trends were observed regarding the Ca2+-independent forces measured at pCa 9.0 (Fmin) in WT and KO groups (Table 1). Collectively, our results demonstrate that cMyBP-C does not impact the length-dependent changes in Ca2+-activated maximal force and Ca2+-independent force production.

Table 1 | Steady-state mechanical properties of WT and KO ventricular multicellular preparations.

| Group | Fmin (mN/mm²) | Fmax (mN/mm²) | nH | pCa50 |
|-------|--------------|--------------|-----|--------|
| SL 1.9 μm | | | | |
| WT | 0.82 ± 0.12 † | 17.29 ± 1.98 * | 3.41 ± 0.32 * | 5.71 ± 0.02 * |
| KO | 0.90 ± 0.13 * | 14.93 ± 1.56 * | 2.30 ± 0.08 † | 5.80 ± 0.01 * |
| SL 2.1 μm | | | | |
| WT | 2.14 ± 0.28 | 26.32 ± 2.93 | 2.47 ± 0.22 | 5.82 ± 0.02 |
| KO | 1.97 ± 0.25 | 23.98 ± 2.58 | 2.49 ± 0.23 | 5.87 ± 0.02 |

Fmin: Ca2+-independent force measured at pCa 9.0; Fmax: maximal Ca2+-activated force measured at pCa 4.5; nH: Hill coefficient of the force-pCa relationship; pCa50: pCa value required for the generation of half-maximal force. Values are expressed as mean ± s.e.m., from 7 to 17 multicellular preparations and 3 to 5 hearts per each group.

† Significantly different from the corresponding group at SL 2.1 μm; P < 0.05.
‡ Significantly different from the corresponding WT group at SL 1.9 μm, P < 0.05.
diac thin-filaments are more sensitive to Ca$^{2+}$ in the KO group. Collectively, our results demonstrate that the effect of cMyBP-C on length-dependent changes in cooperative mechanisms in the sarcomere.

ANOVA revealed no significant interaction effect, but revealed significant main effects of SL ($P < 0.005$) and cMyBP-C ($P < 0.005$) on pCa$_{50}$. Subsequent post-hoc tests revealed that the main effect of SL was because of the following: pCa$_{50}$ significantly increased upon increasing the SL from 1.9 to 2.1 μm as indicated by a leftward shift in the force-pCa relationships in both WT and KO groups (Figures 3A, B). The SL-dependent increase in pCa$_{50}$ ($\Delta$pCa$_{50}$) was attenuated in KO group (indicated by arrows in B, C). Two-Way ANOVA revealed a significant interaction effect ($P < 0.05$) on n$_H$ suggesting that cMyBP-C influences the effect of SL on n$_H$. Subsequent post-hoc tests revealed that n$_H$ significantly increased at SL 1.9 μm in WT but not in KO group. Determinations were made from 7 to 10 multicellular preparations and 3 to 4 hearts per each group. Values are reported as mean ± s.e.m.

**EFFECT OF cMyBP-C ON LENGTH-DEPENDENT CHANGES IN THE RATE OF FORCE REDEVELOPMENT ($k_{tr}$)**

$k_{tr}$ is a measure of XB transition rate from a weakly- to a strongly-bound XB state (Brenner and Eisenberg, 1986; Campbell, 1997). We have previously shown that ablation of cMyBP-C accelerates submaximal $k_{tr}$ at long SL (Stelzer et al., 2006b)—indicating that KO group exhibited an accelerated rate of XB turnover from weak- to strong-bindings states. We now sought to determine whether such effects are also observed at short SL in the KO group. Therefore, we measured $k_{tr}$ at 1.9 and 2.1 μm to gain insights into the effect of cMyBP-C on length-dependent changes in $k_{tr}$. Two-Way ANOVA revealed a significant interaction effect ($P < 0.005$) on $k_{tr}$ suggesting that cMyBP-C influenced the effect of SL on $k_{tr}$. The cause of the interaction effects was assessed by post-hoc multiple pairwise comparisons which showed that submaximal $k_{tr}$ was accelerated in KO compared to WT group at long SL (Figure 4; Table 2) as reported earlier (Stelzer et al., 2006b). Furthermore,
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Figure 4 | Effect of cMyBP-C on length-dependent changes in the rate of force redevelopment ($k_{df}$). $k_{df}$ was measured at 50% level of activation in WT and KO groups at SLs 2.1 and 1.9 µm using a mechanical slack-restretch protocol (Gresham et al., 2014). Two-Way ANOVA revealed a significant interaction effect ($P < 0.005$) on $k_{df}$ and post-hoc tests showed that $k_{df}$ was significantly accelerated by $\sim 53\%$ at short SL vs. long SL in WT group. However, such a trend was absent in the KO group. Furthermore, $k_{df}$ was significantly accelerated by $\sim 56\%$ in KO vs. WT group at long SL.

Table 2: Dynamic contractile parameters of WT and KO ventricular multicellular preparations.

| Group          | $k_{fs}$ (s$^{-1}$) | $k_{rel}$ (s$^{-1}$) | $k_{df}$ (s$^{-1}$) | P1 |
|----------------|---------------------|----------------------|---------------------|----|
| SL 1.9 µm      |                     |                      |                     |    |
| WT             | 8.74 ± 0.56*        | 45.01 ± 4.06*        | 12.26 ± 1.53*       | 0.535 ± 0.022* |
| KO             | 8.03 ± 0.54         | 36.96 ± 2.94         | 9.64 ± 0.39         | 0.543 ± 0.048  |
| SL 2.1 µm      |                     |                      |                     |    |
| WT             | 5.71 ± 0.40         | 34.21 ± 2.12         | 8.17 ± 0.52         | 0.604 ± 0.016  |
| KO             | 8.90 ± 0.37†        | 45.76 ± 4.01†        | 11.27 ± 0.67†       | 0.528 ± 0.026† |

$k_{fs}$: rate force redevelopment; $k_{rel}$: rate of force decay; $k_{df}$: rate of force redevelopment; $P_1$: the magnitude of peak force attained following a rapid stretch of muscle length in an isometrically-activated muscle preparation. $k_{rel}$ and $k_{df}$ were estimated using a linear transformation of the half time of force decay and force redevelopment. Values are expressed as mean ± s.e.m. from 6 to 13 multicellular preparations and 3 to 4 hearts per each group. $\ast$Significantly different from the corresponding WT group at SL 2.1 µm; $P < 0.05$. $\dagger$Significantly different from the corresponding WT group at SL 2.1 µm; $P < 0.05$.

$k_{rel}$ was significantly accelerated by $\sim 53\%$ at short SL compared to long SL in WT group (Figure 4; Table 2). However, an acceleration of $k_{rel}$ at short SL was absent in KO group (Figure 4) such that differences in $k_{rel}$ between WT and KO groups observed at long SL were no longer apparent at short SL. These results indicate that cMyBP-C mediates the length-dependent changes in XB turnover rate.

Effects of cMyBP-C on Length-Dependent Changes in the Rates of Stretch-Induced XB Relaxation ($k_{rel}$) and XB Recruitment ($k_{df}$)

Our data shows that cMyBP-C affects the length-dependent changes in the XB turnover rate, $k_{tr}$ (Figure 4). Because $k_{tr}$ is proportional to the sum of $f$ (rate of XB attachment) + $g$ (rate of XB detachment) according to a two-state XB model (Brenner, 1988), we sought to determine if the effect of cMyBP-C on length-dependent changes in $k_{tr}$ were due to changes in either the rate of XB detachment or the rate of XB attachment kinetics, or both. We used stretch activation experiments (described in the methods section) to measure $k_{rel}$ and $k_{df}$ which are measures of the rates of XB detachment and XB recruitment, respectively (Cheng et al., 2013; Gresham et al., 2014).

Two-Way ANOVA revealed a significant interaction effect ($P < 0.05$) on $k_{rel}$ suggesting that cMyBP-C influenced the effect of SL on the rate of XB detachment kinetics. The cause of the interaction effect was evident from the post-hoc multiple pairwise comparisons which revealed that $k_{rel}$ was significantly accelerated by $\sim 52\%$ at short SL in WT group but such an acceleration of $k_{rel}$ at short SL was absent in KO group (Figure 5A; Table 2). Furthermore, in agreement with recent studies (Stelzer et al., 2006a; Merkulov et al., 2012), our data shows that $k_{rel}$ was significantly accelerated by $\sim 34\%$ in KO group compared to WT group at long SL (Figure 5A; Table 2).

Two-Way ANOVA revealed a significant interaction effect ($P < 0.005$) on $k_{df}$ suggesting that cMyBP-C influenced the effect of SL on the rate of XB recruitment into the force-bearing state. The cause of the interaction effect was assessed using post-hoc tests which revealed that $k_{df}$ was significantly accelerated by $\sim 50\%$ at short SL in WT but such an acceleration of $k_{df}$ at short SL was absent in KO (Figure 5B; Table 2). Furthermore, in agreement with a previous study (Stelzer et al., 2006a), our data shows that $k_{df}$ was significantly accelerated by $\sim 38\%$ in KO compared to WT at long SL. Thus, our stretch activation data shows that both $k_{rel}$ and $k_{df}$ were accelerated at short SL in WT but such trends were absent in the KO (Figures 5A,B). These findings suggest that the absence of acceleration of $k_{tr}$ at short SL in the KO group (Figure 4) is due to a combined effect of the absence in the accelerations of $k_{rel}$ and $k_{df}$ at short SL in KO group (Figure 5). Collectively, our results suggest that cMyBP-C modulates length-dependent changes in the kinetics of XB detachment and attachment in cardiac muscle.

Effect of cMyBP-C on Length-Dependent Changes in the Magnitude of Stretch-Induced Increase in Muscle Fiber Stiffness ($P_1$)

Our data shows that the XB detachment rate ($k_{rel}$) was accelerated at short SL compared to long SL in WT group (Figure 5A). Also, $k_{rel}$ was accelerated at long SL in KO compared to WT group (Figure 5A). We sought to determine whether such accelerations in $k_{rel}$ could have arisen from a decrease in the muscle fiber stiffness because changes in $k_{rel}$ can be correlated with changes in stiffness of XBs (Stelzer et al., 2006c). We imposed a sudden 2% stretch in muscle length in an isometrically-contracting muscle preparation and measured the magnitude of the elicited instantaneous increase in force ($P_1$ in Figure 1). $P_1$ is a result of a rapid distortion of the elastic regions of the strongly-bound XBs (Stelzer and Moss, 2006; Ford et al., 2010; Cheng et al., 2013) and is an index of the muscle fiber stiffness because both $P_1$ and muscle fiber stiffness are well correlated to the number of parallel and force-producing XBs that are bound to actin prior to the imposed stretch in muscle length (Campbell et al., 2004; Ford et al., 2010; Cheng et al., 2013).
Two-Way ANOVA revealed no significant interaction effect and main effects on P1. Post-hoc tests showed that P1 significantly decreased ($P = 0.036$) at short SL compared to long SL in WT group (Figure 6; Table 2). However, such a decrease in P1 at short SL was absent in KO group. Furthermore, P1 was significantly decreased ($P = 0.032$) at long SL in KO compared to WT (Figure 6; Table 2). These results suggest that a decrease in the muscle fiber stiffness contributed, at least in part, to the acceleration of $k_{rel}$ observed at short SL compared to long SL in WT, and also at long SL in KO compared to long SL in WT (Figure 5A). Decreased XB stiffness could enhance strain-induced rates of XB detachment by increasing XB compliance such that XB's detach rapidly (Stelzer et al., 2006c; Cheng et al., 2013)—indicating that changes in P1 can be correlated with changes in $k_{rel}$. Thus, it is likely that the absence of differences in P1 at long and short SL's in KO group (Figure 6) may have contributed to the lack of differences we observed in $k_{rel}$ at long and short SL's in KO group (Figure 5A). Collectively, our data suggests that cMyBP-C modulates length-dependent changes in the rate of XB detachment via its impact on the muscle fiber stiffness.

**DISCUSSION**

Given the lack of our understanding regarding cMyBP-C’s role in cardiac LDA, we performed a detailed investigation of different aspects of cardiac contractile function both in the presence and absence of cMyBP-C and at SL’s 1.9 and 2.1 μm. Results from our studies demonstrate that length-dependent changes in contractile dynamics are significantly impacted in the absence of cMyBP-C in the cardiac sarcomere. Novel findings from our experiments show an attenuated length-dependent response with respect to steady-state myofilament Ca$^{2+}$ sensitivity of force generation, and profoundly blunted length-dependent XB cycling dynamics in ventricular preparations isolated from hearts lacking cMyBP-C—suggesting that cMyBP-C is a key modulator of cardiac LDA.

**ABLIATION OF cMyBP-C ATTENUATES THE LENGTH-DEPENDENT CHANGES IN MYOFILAMENT Ca$^{2+}$ SENSITIVITY**

An increase in myofilament Ca$^{2+}$ sensitivity ($pCa_{50}$) upon an increase in SL is a hallmark of LDA (Kentish et al., 1986; Dobesh et al., 2002). The effect of cMyBP-C on LDA is important to study because it is known that LDA is depressed in human hearts expressing cMyBP-C mutations (Van Dijk et al., 2012; Sequeira et al., 2013). Our results show that although the absence of cMyBP-C did not affect maximal force production (Table 1), it did attenuate the SL-mediated increase in Ca$^{2+}$ sensitivity ($ΔpCa_{50}$) (Figures 3A–C), a result that agrees with an earlier report (Cazorla et al., 2006). Cazorla et al reported an attenuated $ΔpCa_{50}$ in the KO when compared to the WT myocardium.

| SL 2.1 μm | SL 1.9 μm |
|----------|----------|
| KO       | KO       |
| WT       | WT       |

Figure 6 | Effect of cMyBP-C on length-dependent changes in the magnitude of sudden-stretch induced increase in muscle fiber stiffness (P1). P1 was calculated from the force responses elicited upon a sudden 2% stretch in muscle length imposed on isometrically-contracting ventricular preparations (Stelzer et al., 2006c). Two-Way ANOVA revealed no significant interaction effect and main effects on P1. Post-hoc tests showed that P1 significantly decreased at short SL vs. long SL in WT group but such a trend was absent in KO group. $k_{rel}$ significantly accelerated by ∼34% in KO vs. WT group at long SL (A). Two-Way ANOVA revealed a significant interaction effect ($P < 0.005$) on $k_{rel}$ and post-hoc tests showed that $k_{rel}$ significantly accelerated by ∼50% at short SL vs. long SL in WT group but such a trend was absent in KO group. In addition, $k_{rel}$ significantly accelerated by ∼39% in KO vs. WT group at long SL (B). Determinations were made from 6 to 13 multicellular preparations and 3 to 4 hearts per each group. Values are reported as mean ± s.e.m. *$P < 0.05$; **$P < 0.005$. 

| SL 2.1 μm | SL 1.9 μm |
|----------|----------|
| KO       | KO       |
| WT       | WT       |

Figure 5 | Effect of cMyBP-C on length-dependent changes in the rates of XB detachment ($k_{rel}$) and XB recruitment ($k_{rec}$). Isometrically-activated ventricular preparations were subjected to a sudden 2% stretch in their muscle length and the elicited force responses were used to estimate (A) $k_{rel}$ and (B) $k_{rec}$ in WT and KO groups at SL's 2.1 and 1.9 μm as described in the methods section (Cheng et al., 2013; Gresham et al., 2014). Two-Way ANOVA revealed a significant interaction effect ($P < 0.05$) on $k_{rel}$ and post-hoc tests showed that $k_{rel}$ significantly accelerated by ∼32% at short SL vs. long SL in WT group but such a trend was absent in KO group. $k_{rel}$ significantly accelerated by ∼34% in KO vs. WT group at long SL (A). Two-Way ANOVA revealed a significant interaction effect ($P < 0.005$) on $k_{rel}$ and post-hoc tests showed that $k_{rel}$ significantly accelerated by ∼50% at short SL vs. long SL in WT group but such a trend was absent in KO group. In addition, $k_{rel}$ significantly accelerated by ∼39% in KO vs. WT group at long SL (B). Determinations were made from 6 to 13 multicellular preparations and 3 to 4 hearts per each group. Values are reported as mean ± s.e.m. *$P < 0.05$; **$P < 0.005$. 

| SL 2.1 μm | SL 1.9 μm |
|----------|----------|
| KO       | KO       |
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| SL 2.1 μm | SL 1.9 μm |
|----------|----------|
| KO       | KO       |
| WT       | WT       |

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| SL 2.1 μm | SL 1.9 μm |
|----------|----------|
| KO       | KO       |
| WT       | WT       |

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ABLATION OF cMyBP-C BLUNTS THE LENGTH-DEPENDENT CHANGES IN XB CYCLING KINETICS

As demonstrated previously (Stelzer et al., 2006b), our present data show that \( k_{tr} \) was accelerated in KO compared to WT group at long SL (Figure 4). Because changes in \( k_{tr} \) indicate a shift in the equilibrium in the transitions between the closed to open states (McKillop and Geeves, 1993) of the thin-filament (Campbell, 1997), our data suggests that in the absence of cMyBP-C the thin-filaments are shifted more toward the open state. To understand the impact of cMyBP-C on length-dependent changes in XB transitions/cycling from weak- to strong-binding states, we measured \( k_{tr} \) at short and long SLs. Our data show that \( k_{tr} \) was accelerated at short SL compared to long SL in WT group (Figure 4) indicating the XB cycling is accelerated at short SL. In support of this observation, an earlier study showed that loaded shortening velocity and \( k_{tr} \) were significantly accelerated at short SL compared to long SL in skinned rat cardiac myocytes (Korte and McDonald, 2007). The mechanism for such an increase in \( k_{tr} \) at short SL may arise from the acceleration of XB cycling kinetics such that there are more XBs working against a constant load because of increased XB flexibility which allows XBs to radially extend toward the thin-filament at short SL (Korte and McDonald, 2007). Increased flexibility of XBs at short SL may also arise due to decreased stiffness of titin (Granzier and Irving, 1995), a consequence of which is a decreased force exerted by titin on cMyBP-C which can in turn lead to decreased constraint imposed by cMyBP-C on the myosin XBs (Korte and McDonald, 2007).

Because \( k_{tr} \) encompasses both the rates of XB attachment (\( f \)) and detachment (\( g \)) (Brenner and Eisenberg, 1986), we determined if increased \( k_{tr} \) observed at short SL in WT was due to increase in either \( f \) or \( g \), or both. Using length perturbation experiments (Gresham et al., 2014), we measured the rates of force development (\( k_{df} \) and force decay (\( k_{rel} \)), parameters that are analogous to \( f \) and \( g \). Our data shows that acceleration of \( k_{tr} \) at short SL in WT group was indeed due to a combination of increases in both \( k_{df} \) and \( k_{rel} \) (Figure 5). Our data also shows that both \( k_{df} \) and \( k_{rel} \) did not increase at short SL in KO skinned myocardium such that the values were not significantly different from those at long SL (Figure 5). A recent study (Tanner et al., 2014) showed that XB detachment rates were accelerated in papillary muscle isolated from KO hearts compared to WT hearts but only under a \( \beta \)-MHC background at very long SL (2.2–3.3 \( \mu \)m). Under an \( \alpha \)-MHC background, XB detachment rates displayed a slight non-statistically significant increase in KO papillary muscles compared to WT papillary muscles, in contrast to the larger accelerations in XB detachment we observed in KO multicellular preparations at shorter SL (i.e., 2.1 \( \mu \)m), isolated from hearts expressing predominantly \( \alpha \)-MHC. Taken together, our data shows that an absence of acceleration in \( k_{tr} \) at short SL in KO group was due to the absence of accelerations in both \( k_{df} \) and \( k_{rel} \) at short SL (Figure 5). Thus, our study suggests that the mechanisms influencing length-dependent changes in XB transitions between weak- to strong-binding states are blunted in the absence of cMyBP-C.

ABLATION OF cMyBP-C BLUNTS LENGTH-DEPENDENT CHANGES IN MUSCLE FIBER STIFFNESS AND COOPERATIVE MECHANISMS

To test whether changes in XB detachment (as assessed by \( k_{rel} \), Figure 5) due to cMyBP-C ablation or changes in SL were related to altered XB compliance and muscle fiber stiffness, we estimated the magnitude of the instantaneous increase in force \( P_1 \), a parameter that represents the stretch-induced strain of the strongly-bound XBs and an indicator of XB stiffness (Ford et al., 2010; Cheng et al., 2013). Our measurements showed that \( P_1 \) values were decreased in WT at short SL compared to long SL, and also in KO at long SL when compared to WT at long SL (Figure 6, Table 2). Because \( P_1 \) can be correlated to \( k_{rel} \) (Stelzer et al., 2006c; Cheng et al., 2013), our results are consistent with the idea that decreased muscle fiber stiffness contributed to the observed acceleration in the XB detachment. Significantly, our results demonstrate that muscle fiber stiffness decreased at short SL compared to long SL in WT but not in KO group (Figure 6),...
suggesting that the lack of length-dependent changes in $k_{rel}$ seen in KO group (Figure 5) may have been related to the lack of length-dependent changes in the muscle fiber stiffness (Figure 6), because muscle fiber stiffness in KO group is already significantly lower than WT group at long SL.

To test whether changes in XB recruitment, (as assessed by $k_{df}$, Figure 5) due to cMyBP-C ablation or changes in SL were related to changes in cooperative mechanisms, we estimated the Hill coefficient, $n_H$ from the pCa-tension relationships. Our estimates showed that $n_H$ values were increased in WT at short SL compared to long SL (Figure 3D), a result that is consistent with previous studies (Ford et al., 2012; Gollapudi et al., 2012). This suggests that enhanced cooperative mechanisms may have accelerated the XB recruitment rate at short SL in WT group. This increase in $n_H$ may be a result of enhanced Ca$^{2+}$ binding to Tn, near-neighbor interactions among Tn-Tn, XB-Tn, and XB-XB (Razumova et al., 2000; Campbell et al., 2001). Notably, such an increase in $n_H$ at short SL was absent in KO (Figure 3D) which may have likely contributed to the absence of length-dependent changes in $k_{rel}$ in the KO group (Figure 5B). In the context of the KO model, it is likely that depressed cooperative XB-XB (Razumova et al., 2000; Moss et al., 2004), XB-Tn (Razumova et al., 2000; Chandra et al., 2007) and XB-Ca$^{2+}$/TnC (Li et al., 2014) interactions at short SL may have contributed to the blunting of the increase in $n_H$ with decrease in SL. Therefore, our data shows that length-dependent changes in cooperative mechanisms are depressed when cMyBP-C is absent in the sarcomere.

**CONCLUSIONS**

Our study provides evidence to show that cMyBP-C plays a key role in fine-tuning length-dependent cardiac contractile function via its impact on myofilament responsiveness to Ca$^{2+}$, XB cycling kinetics, and muscle fiber stiffness. Taken together, our findings suggest that impaired LDA may contribute to depressed myocardial contractile function in human patients harboring mutations in cMyBP-C that ultimately cause a significant decrease in the amount of cMyBP-C expression in the sarcomere.

**AUTHOR CONTRIBUTIONS**

Ranganath Mamidi and Julian E. Stelzer contributed to the conception and design of the experiments. Ranganath Mamidi, Kenneth S. Gresham, and Julian E. Stelzer participated in performing the experiments, data acquisition, data analysis, data interpretation, drafting, and revising the manuscript. All authors approved the final version of the manuscript.

**ACKNOWLEDGMENTS**

This work was supported by the National Heart, Lung, and Blood Institute Grant (HL-114770-01). We would like to thank Heather Butler, Department of Ophthalmology/Endocrinology at Case Western Reserve University for help with maintaining our mouse colonies.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.