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Revealing the mechanism of how cardiac myosin-binding protein C N-terminal fragments sensitize thin filaments for myosin binding

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Cardiac muscle contraction is triggered by calcium binding to troponin. The consequent movement of tropomyosin permits myosin binding to actin, generating force. Cardiac myosin-binding protein C (cMyBP-C) plays a modulatory role in this activation process. One potential mechanism for the N-terminal domains of cMyBP-C to achieve this is by binding directly to the actin-thin filament at low calcium levels to enhance the movement of tropomyosin. To determine the molecular mechanisms by which cMyBP-C enhances myosin recruitment to the actin-thin filament, we directly visualized fluorescently labeled cMyBP-C N-terminal fragments and GFP-labeled myosin molecules binding to suspended actin-thin filaments in a fluorescence-based single-molecule microscopy assay. Binding of the C0C3 N-terminal cMyBP-C fragment to the thin filament enhanced myosin association at low calcium levels. However, at high calcium levels, C0C3 bound in clusters, blocking myosin binding. Dynamic imaging of thin filament-bound Cy3-C0C3 molecules demonstrated that these fragments diffuse along the thin filament before statically binding, suggesting a mechanism that involves a weak-binding mode to search for access to the thin filament and a tight-binding mode to sensitize the thin filament to calcium, thus enhancing myosin binding. Although shorter N-terminal fragments (Cy3-C0C1 and Cy3-C0C1f) bound to the thin filaments and displayed modes of motion on the thin filament similar to that of the Cy3-C0C3 fragment, the shorter fragments were unable to sensitize the thin filament. Therefore, the longer N-terminal fragment (C0C3) must possess the requisite domains needed to bind specifically to the thin filament in order for the cMyBP-C N terminus to modulate cardiac contractility.

cardiomyopathy | muscle | contractility | single molecule | regulated thin filaments

Cardiac myosin-binding protein C (cMyBP-C) modulates cardiac contraction at the level of the sarcomere. Mutations in the cMyBP-C gene are a major cause of hypertrophic cardiomyopathy (1), a disease that affects up to 1 in 200 people (2) and is the leading cause of sudden cardiac death in young adults (1, 3). cMyBP-C is composed of 11 subdomains from C0 to C10 (Fig. 1A), 8 Ig-like and 3 Fn(III)-like with a proline-alanine-rich region between C0 and C1, and a linker between C1 and C2 (i.e., M-domain) (4). Each cMyBP-C molecule is tethered through its C terminus (5) to the myosin thick filament (6), while its N-terminal domains are free to contact either the myosin head region or the actin thin filament (7) (Fig. 1A). Specifically, cMyBP-C interactions with myosin have been shown to occur through the S2 region (8–11), the myosin regulatory light chain (RLC) (12) and the S1 head (13), which may serve to stabilize myosin's superrelaxed state (14–16) through interactions with the recently defined myosin mesa region (13, 17). In addition to myosin binding, cMyBP-C interacts with actin via its N-terminal domains (C0–C2) (18–21). These binding-partner interactions are believed to functionally impact cardiac contractility. For example, cMyBP-C knockout mouse cardiomyocytes (22) demonstrate a decrease in calcium sensitivity while N-terminal fragments infused into skinned fibers

(11) result in an increase in calcium sensitivity. Accordingly, fluorescence assays in muscle cells (10) have demonstrated that cMyBP-C N-terminal fragments activate muscle at low calcium levels and inhibit maximal activity at high calcium, suggesting dual roles in modulating contraction in vivo.

Mechanistically, muscle activation occurs by calcium-dependent shifts in the position of tropomyosin on the thin filament (23, 24). Tropomyosin binds axially along the thin filament (Fig. 1A) and shifts azimuthally when calcium binds to the tropomyosin-associated troponin protein complex. Three positions of tropomyosin have been structurally (24) and functionally (25) defined: blocked (sterically preventing myosin binding), closed (myosin can bind weakly), and open (myosin binding is unperturbed). It has been proposed that the cMyBP-C N terminus could sensitize the thin filament to calcium through the displacement of tropomyosin from its blocked to its closed position, to enhance myosin binding (21). The function of the cMyBP-C N-terminal subdomains C0C3, C0C1, and C0C1f (i.e., C0C1 including the first 17 M-domain residues) have been extensively characterized for their ability to sensitize the thin filament to calcium using in vitro motility (18, 26, 27), ATPase assays (19), and electron micrographic studies (18, 21, 28). Our previous studies and that of others show that all three bind to actin, but only the C0C3 fragment can both activate

Significance

Diverse demands on cardiac muscle require the fine-tuning of contraction. Cardiac myosin binding protein-C (cMyBP-C) is involved in this regulation; however, its precise molecular mechanism of action remains uncertain. By imaging the interactions of single myosin and cMyBP-C molecules interacting with suspended thin filaments in vitro we observe cMyBP-C N-terminal fragments assist activation and modulate contraction velocity by affecting myosin binding to the thin filament. Fluorescent imaging of Cy3-labeled cMyBP-C revealed that it diffusively scans the thin filament and then strongly binds to displace tropomyosin and activate at low calcium. At high calcium, cMyBP-C decorates the filament more extensively, reducing myosin binding through competing with binding sites. Understanding the mechanism of MyBP-C action has important implications for heart disease.

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The authors declare no conflict of interest.

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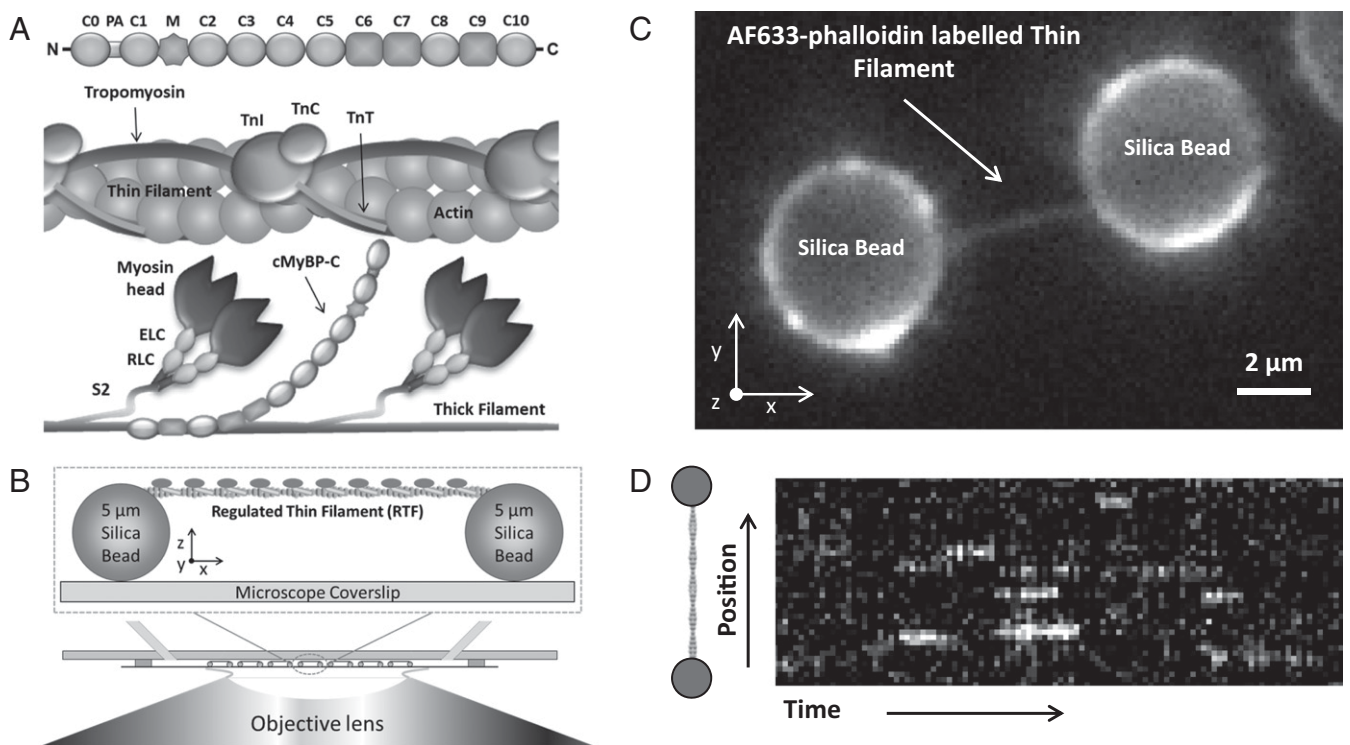


Fig. 1. Thin filaments in vivo and in the single-molecule tightrope assay. (A) The subdomain structure of cMyBP-C and its spatial relationship to the thin filament (actin decorated with tropomyosin and the troponin complex TnI, TnI, and TnC) and myosin-containing thick filament in the sarcomere. (B) The thin-filament tightrope assay. Regulated thin filaments are suspended between surface-adhered beads using a microfluidic device and visualized using a high-N.A. objective lens. (C) A regulated thin filament labeled with AF633-phalloidin can be visualized suspended between two beads. (D) Representative kymograph of interactions between 15 nM S1-GFP and a thin-filament tightrope at high calcium (pCa 4) and 0.1 μM ATP. Each x-axis pixel corresponds to a frame (300 ms), and each pixel on the y axis corresponds to 126.4 nm along the tightrope.

thin filament sliding at low (0.1 μM) calcium and inhibit sliding at high (100 μM) calcium levels. In contrast, the shortest fragment, the murine C0C1, does neither, while the C0C1f with its additional 17 residues of the M-domain can only inhibit thin filament sliding at high calcium (18). These differential functions may allow assignment of specific functional capacities to cMyBP-C's N-terminal domains.

To define the molecular basis of thin-filament activation by cMyBP-C, we used a single-molecule suspended thin filament assay (Fig. 1B), in which we could directly observe the calcium-dependent binding of individual fluorescently labeled myosin-S1 molecules to the thin filament (29). Using all three murine cMyBP-C N-terminal fragments (Fig. 2B), C0C3, C0C1, and C0C1f, we show that cMyBP-C's N terminus (C0C3) can maximally activate the thin filament, permitting myosin-S1 to bind even at low calcium. However, at high calcium with the thin filament maximally activated cMyBP-C can decrease myosin-S1 binding. By imaging fluorescently labeled cMyBP-C N-terminal fragments we observed these molecules individually binding directly to the thin filament. Once bound, the cMyBP-C fragments both diffuse along the thin filament and bind statically, with enhanced static binding observed only for the C0C3 fragment at high calcium levels that maximally activate the thin filament. These fragment binding and motion behaviors on the thin filament provide direct mechanistic insight into how cMyBP-C achieves both calcium sensitization at low calcium and reduced activation at high calcium, and thus modulation of cardiac contractility.

Results

The Effect of cMyBP-C N-Terminal Domains on S1-GFP Myosin Binding to the Thin Filament. We studied how each of the three N-terminal fragments C0C3, C0C1f, and C0C1 (Fig. 2) affects myosin binding

to thin filaments (18–21), by directly measuring individual S1-GFP molecules interacting with thin filaments in their presence. Using 1 μM unlabeled cMyBP-C N-terminal fragments, we determined the level of S1-GFP (15 nM) binding to the thin filament at low and high calcium (0.1 μM and 100 μM , respectively). The fragment concentration was chosen based on past motility studies in which 1 μM C0C3 both sensitized the thin filament to calcium and inhibited thin filament motility at maximally activating calcium concentrations (30). Fig. 2A shows representative kymographs for S1-GFP binding. At low calcium in the absence of C0C3, the kymograph (Fig. 2A, Upper Left) shows very few S1-GFP binding events, consistent with the thin filament's not being fully activated, as previously described (29). At high calcium in the absence of C0C3, the thin filament is activated, as evidenced by significant S1-GFP binding to the thin filament (Fig. 2A, Upper Right). In the presence of C0C3 the situation is reversed. At low calcium, considerable S1-GFP binding is observed (Fig. 2A, Lower Left), whereas at high calcium these interactions are rare (Fig. 2A, Lower Right). To quantify changes in S1-GFP binding, we calculated the mean fluorescence intensity per pixel for each tightrope. Minimally, 10 tightropes were observed under the same experimental condition (Fig. 2B). To compare the effects of a given cMyBP-C fragment on S1-GFP binding, the average fluorescence intensity per pixel was normalized to that at low calcium concentration in the absence of fragments (Fig. 2B). At low calcium, addition of C0C3 significantly increased S1-GFP binding [$57.0 \pm 7.6\%$ (SEM)] vs. no fragment, equivalent to that observed at high calcium in the absence of C0C3 [$55.7 \pm 13.2\%$ (SEM)]. However, addition of C0C3 at high calcium also significantly reduced S1-GFP binding by $38.3 \pm 15.8\%$ (SEM) vs. the absence of C0C3 at high calcium. Interestingly, no significant difference in S1-GFP binding was seen as a result of the introduction of either shorter fragment,

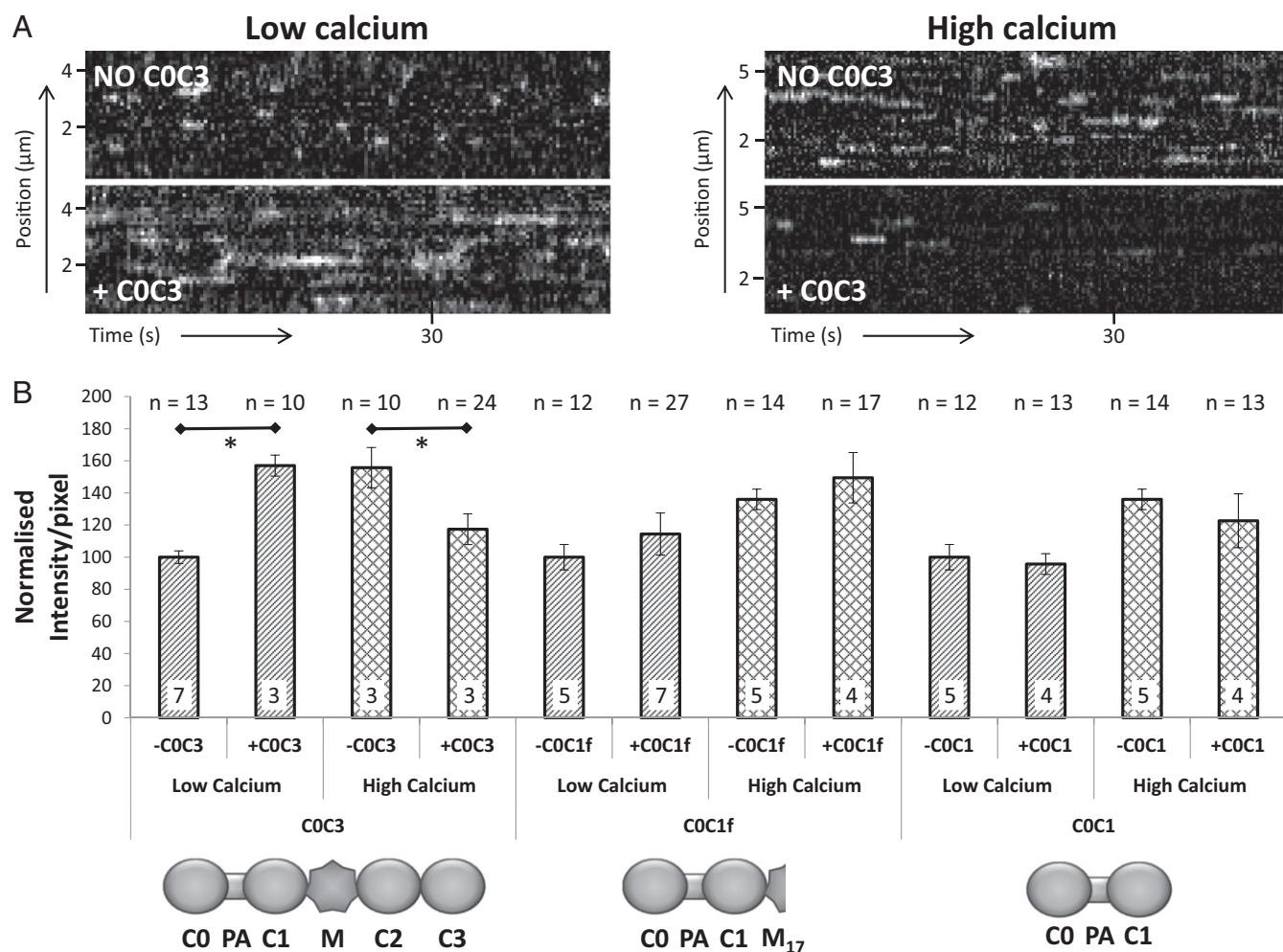


Fig. 2. The calcium-dependent effects of cMyBP-C fragments on S1-GFP binding to regulated thin filaments. (A) Representative kymographs of S1-GFP binding to thin filaments in the absence and presence of COC3, at low and high calcium, collected at 3.3 fps. (B) Bar chart of the average intensity/pixel ratio in the kymographs of S1-GFP binding in the absence and presence of different cMyBP-C N-terminal fragments; the values have been normalized to each respective control experiment (i.e., no fragment present at low $[Ca^{2+}]$). Data were collected using 15 nM S1-GFP with or without 1 μ M of fragment, with n values representing the number of tightropes imaged per condition. The number of experimental repeats (flowcells) is shown at the bottom of the bars. Results showed a significant increase $[57.0 \pm 7.6\%$ (SEM)] in S1-GFP binding in the presence of COC3 at low calcium and reduction $[38.3 \pm 15.8\%$ (SEM)] at high calcium; no significant difference was seen when using the shorter fragment COC1f $[14.4 \pm 15.3\%$ (SEM), $P = 0.42$ at low calcium and $13.4 \pm 16.9\%$ (SEM), $P = 0.42$ at high calcium] and COC1 at low $[-4.3 \pm 10.3\%$ (SEM), $P = 0.70$] and high calcium $[-13.4 \pm 18.0\%$ (SEM), $P = 0.44$]. *Significant at $P < 0.075$.

COC1f or COC1, at both low and high calcium. These results suggest that the shorter fragments are missing critical domains essential for modulating S1-GFP binding to the thin filament in a calcium-dependent manner.

Imaging N-Terminal cMyBP-C Fragments Binding to the Thin Filament.

As described above, only the COC3 fragment affected S1-GFP binding to the thin filament in a calcium-dependent manner. Could the differential effects seen for the COC3 fragment compared with the shorter COC1f and COC1 fragments be related to a difference in their binding to the thin filament? Therefore, we imaged 20 nM Cy3-tagged COC3, COC1f, and COC1 interacting with thin filaments at both low and high calcium. Fig. 3 shows clear binding of all three fragments to the thin filaments using long-exposure (1 s) still images. These images were analyzed to determine the average number of fragment molecules bound per micrometer of thin filament. To ensure a more accurate determination of closely spaced fluorescent fragments that could not be spatially resolved, we used fluorescence spot intensity to estimate the number of molecules per spot. First, we determined the fluorescence intensity for

single Cy3-COC3 molecules from thin filament binding images at low calcium (Fig. 3A, Left), where fluorescent spots were easily discernible. Even in these conditions there was considerable variance in spot intensity, suggesting that such spots were due to closely spaced, neighboring Cy3-COC3 molecules. To extract the fluorescent intensity of a single molecule, we fitted the distribution of intensities at low calcium to multiple Gaussians (29, 31) as detailed in *SI Appendix, Fig. S1*. Using the fluorescence intensity of a single Cy3, we converted each fluorescence spot intensity along a tightrope into the number of bound molecules and used this to calculate the average number of cMyBP-C fragments bound per micrometer of thin filament. For Cy3-COC3 (Fig. 3A and D), there was a significant greater than fivefold increase in binding at high vs. low calcium $[6.27 \pm 1.15$ molecules per μ m (SEM, $n = 2$) vs. 0.84 ± 0.17 molecules per μ m (SEM, $n = 11)$, respectively]. This was not the case for Cy3-COC1f (Fig. 3B and D), where no significant ($P = 0.55$) change in decoration of thin filaments (in molecules per μ m) was observed between low and high calcium $[1.17 \pm 0.21$ (SEM, $n = 9$) vs. 1.56 ± 0.77 (SEM, $n = 5)$ molecules per μ m, respectively]. However, significantly more Cy3-COC1 (Fig. 3C and D)

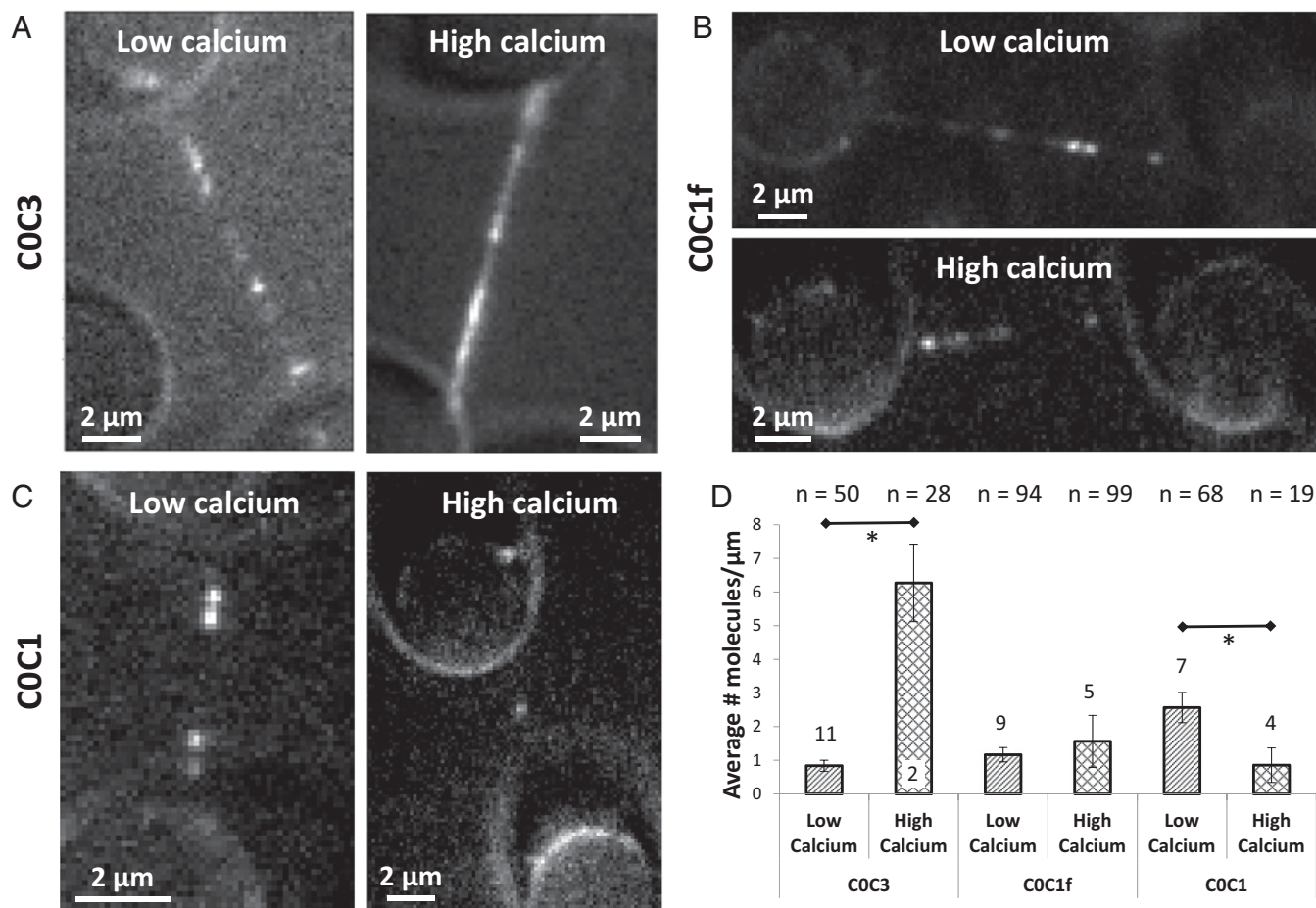


Fig. 3. Fluorescent cMyBP-C N-terminal fragments associate with thin filament tightropes. Representative images of tightropes decorated using 20 nM Cy3-tagged (A) COC3, (B) COC1f, and (C) COC1, in low and high calcium conditions. (D) Bar chart indicating the average number \pm SEM of Cy3-tagged N-terminal cMyBP-C fragments bound per micrometer micrometer of tightrope. n = number of tightropes imaged, above and within the bars are flowcell numbers. *Significant at $P < 0.075$. Further examples of clustered binding are shown in *SI Appendix, Fig. S2*.

bound to thin filaments at low [2.57 ± 0.45 (SEM, $n = 7$) molecules per μm] vs. high calcium [0.85 ± 0.51 (SEM, $n = 4$) molecules per μm]. In summary, only COC3 binding to the thin filament was substantially affected by calcium, resulting in increased binding at high calcium.

At high calcium, the binding of Cy3-COC3 did not occur uniformly across thin filaments; instead, binding occurred in clusters (Fig. 3 and *SI Appendix, Fig. S2*), suggesting the possible existence of coordinated binding. To quantify this, we determined the number of molecules per fluorescent spot for Cy3-COC3, the only fragment that showed increased binding at high calcium. Fig. 4 shows the distribution of number of molecules within each fluorescent spot or cluster on a thin filament for Cy3-COC3 at low and high calcium. At low calcium, the majority of fluorescent spots contain only a single Cy3-COC3; however, at high calcium the distribution is quite broad with its peak at four Cy3-COC3 molecules per spot. To investigate the nature of these distributions we first tried fitting the high-calcium data to a Poisson distribution. The fit as shown in Fig. 4 (dotted line) does not adequately describe the data; however, the Poisson distribution has a peak value of 5.2, which suggests that Cy3-COC3 is binding in clusters. For a more meaningful fit of Cy3-COC3 binding, we used the mechanistic explanation of myosin binding to a calcium-regulated thin filament, which is based upon the accessibility of actin binding sites afforded by motions of tropomyosin across the thin filament (25). We propose that tropomyosin movements from the blocked to the closed

state that occur either transiently or through calcium induction result in the exposure of cMyBP-C binding sites. Thus, upon Cy3-COC3 binding, Cy3-COC3 could stabilize the closed state, and because of the inherent stiffness of tropomyosin (32) this would expose more cMyBP-C binding sites locally on the thin filament. This mechanism is analogous to that of thin-filament activation by rigor myosin binding (33, 34). Therefore, here we utilized the model we described previously to explain the calcium-dependent activation of the thin filament (29) to fit Cy3-COC3 binding data in Fig. 4.

In brief, the model of Cy3-COC3 binding to the thin filament (i.e., actin), which can adopt different states of activation, can be summarized by the following chemical equilibrium:



A_B is actin in the blocked state, A_{Cl} is actin in the closed state and able to bind cMyBP-C (M), and $A_{Cl} \cdot M$ is actin in the closed state able to bind to d molecules of M . The probability of M being attached to the thin filament ($prob_{att}$) is given by the ratio of time spent attached (τ_{on}) vs. the sum of τ_{on} and the time spent detached (τ_{off}):

$$prob_{att} = \frac{\tau_{on}}{\tau_{on} + \tau_{off}},$$

where τ_{on} is a measured parameter (*SI Appendix, Fig. S3*). τ_{off} depends on the proportion of thin filaments in the $A_{Cl} \cdot M$ state

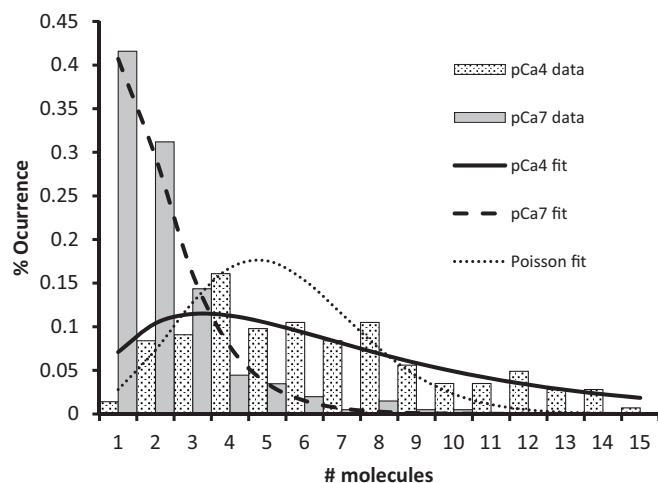


Fig. 4. Histogram of cMyBP-C cluster sizes. The percent occurrence of fluorescent molecules bound to the thin-filament tightropes in clusters was determined based on the fluorescence intensity of a single Cy3-C0C3 (*SI Appendix, Fig. S1*). Plotted at low calcium (solid bars) and high calcium (dotted bars), data are fitted to the relationship shown in the main text. A best-fit Poisson distribution is shown with the dotted curve. The sum square differences of the Poisson vs. chemical kinetic model are 14.8 and 1.92, respectively, for the high-calcium dataset.

($pA_{Cl}M$; see *SI Appendix* for mathematical description) and because Cy3-C0C3 binding results in the exposure of more sites on actin, this allows more (i.e., d) Cy3-C0C3 to bind in an effective cooperative unit. Therefore, τ_{off} is expressed as follows:

$$\tau_{off} = 1 / (d \cdot pA_{Cl}M).$$

As in Desai et al. (29), the probability of the exact number (n) of cMyBP-C molecules binding is $prob_{att}^n \times (1 - prob_{att})^{1-n}$, and this equation was used for fitting (Fig. 4).

Fig. 4 shows the fit to the probability of the exact number of Cy3-C0C3 binding performed by the Microsoft Excel GRG Nonlinear fitting engine to globally fit the high and low calcium conditions simultaneously. The affinity (K_{ca}) of calcium binding to thin filaments was fixed at $0.7 \mu\text{M}$ (pCa_{50} of ~ 6.15) based on previous studies (29, 35). Since the size of the cooperative unit is a property of tropomyosin's stiffness, we also fixed the size of the cooperative unit (d) at 11, as previously determined (29). τ_{on} was measured at high calcium as $5.1 (\pm 3.1 >95\% \text{ CI})$ s (*SI Appendix, Fig. S3*). The only fitted parameter K_2 , the affinity of Cy3-C0C3 for the $A_{Cl}M$ state of the thin filament, was found to be $0.18 (\pm 0.02; >95\% \text{ CI}) \mu\text{M}$.

N-Terminal cMyBP-C Fragments Display Two Modes of Motion on Thin Filaments. Dynamic imaging of cMyBP-C fragments on thin filament tightropes unexpectedly revealed that in addition to N-terminal fragments binding statically to thin filaments [Fig. 5A, horizontal trace (arrowhead)] a proportion of the fragments appeared to diffuse randomly along the thin filament [Fig. 5A, trace with y-axis movement (star)]. Statically bound molecules were defined as not showing any apparent movement greater than one pixel in both dimensions (equivalent to 120 nm and 1 s) during the entire detection time window of >200 s [Fig. 5A, horizontal trace (arrowhead)]. This behavior was calcium-dependent for Cy3-C0C3 molecules; the proportion of kymograph traces showing diffusive Cy3-C0C3 motion decreased significantly ($P < 0.075$) from $19.4 \pm 4.6\%$ (SEM, $n = 11$) at low calcium to $6.0 \pm 1.8\%$ (SEM, $n = 8$) at high calcium (Fig. 5B). By comparison, Cy3-C0C1f showed no significant difference ($P = 0.203$) in the proportion of

diffusive molecules at both low and high calcium, $27.3 \pm 2.2\%$ (SEM, $n = 9$) and $32.3 \pm 3.1\%$ (SEM, $n = 6$), respectively. This was also the case for Cy3-C0C1 $24.5 \pm 6.4\%$ (SEM, $n = 7$) vs. $47.7 \pm 26.2\%$ (SEM, $n = 3$) at low and high calcium, respectively ($P = 0.2542$). We also observed that some of the diffusing molecules would switch to static binding during their interaction with the thin filament (*SI Appendix, Fig. S4*), which was interpreted as being temporarily bound to a specific site in a partially exposed region of the thin filament; nonetheless, these molecules were classified as diffusive.

We calculated the diffusional characteristics summarized in *SI Appendix, Table S1* using mean squared displacement (MSD):

$$MSD = \frac{1}{n} \sum_{i=1}^n (x_i(t) - x_i(0))^2 = 2Dt^\alpha,$$

where D is the diffusion constant of the molecule, t is the analysis time window, $x_i(t)$ is the position at time t , $x_i(0)$ is the starting reference position, and α is a coefficient that denotes the nature of the diffusion, where values close to 1 indicate random diffusion (36). Diffusion constants were transformed to log values before averaging and errors propagated to real numbers to provide the values in *SI Appendix, Table S1*.

Diffusion along a lattice can be described as multiple discrete steps with an average stepping rate. This definition allows us to calculate the average step size of the fragments on the thin filament, provided the stepping rate is known. We can calculate the stepping rate from attached lifetimes obtained from laser trap studies (37) that indicated C0C3, C0C1f, and C0C1 attach with two lifetime populations, <30 ms and >200 ms. Given that the longer events were ~ 60 times less frequently observed, and more likely to represent the a population of the stronger interactions that we report here (*SI Appendix, Fig. S3*), we used the short lifetime to calculate the step size for each fragment at low calcium, according to the equations below (38):

$$\text{stepping rate} = \frac{1}{\tau} = \frac{2D}{l^2}$$

$$\therefore \text{step size} = l = \sqrt{2D\tau},$$

where τ is the attached lifetime, D is the diffusion constant, and l is the average step size. This provides an average step size of 17 ± 3 nm (SD) for Cy3-C0C3, 12 ± 2 nm (SD) for Cy3-C0C1f, and 7 ± 1 nm (SD) for Cy3-C0C1. Due to the method of calculating these values it was not possible to generate a significance test; therefore, these values provide a range of step size from approximately one to three actin monomers.

Discussion

cMyBP-C plays a critical role in modulating cardiac contractility (39, 40), as evidenced by the high prevalence of cMyBP-C mutations in hypertrophic cardiomyopathy (41). One way for cMyBP-C to modulate cardiac contractility is to alter the sensitivity of the thin filament to calcium (10, 18, 27, 39), as reported here. To determine the underlying mechanism by which cMyBP-C enhances thin-filament activation at low calcium levels (18, 26), we directly visualized the impact of cMyBP-C N-terminal fragments on the binding of individual myosin (S1-GFP) molecules along a single suspended actin-thin filament. Specifically, we observed that the C0C3 N-terminal fragment enhanced myosin binding to thin filaments at low calcium (i.e., increased calcium sensitivity) and reduced myosin binding at high calcium. These results are consistent with our previous in vitro motility studies and that of others (18, 26), showing that cMyBP-C's N terminus is sufficient to sensitize the thin filament to calcium and to modulate thin-filament sliding velocities at high calcium. The binding of

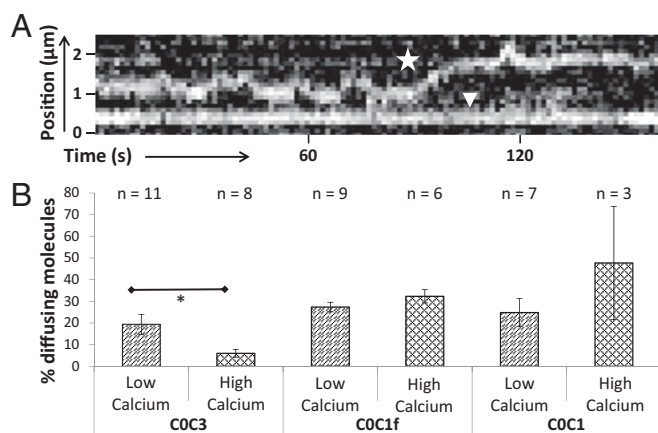


Fig. 5. cMyBP-C fragments diffuse on and statically bind to thin-filament tightropes. (A) An example of a kymograph representing the two types of interaction seen with cMyBP-C (using Cy3-C0C3 as an example). Diffusion on the thin-filament tightrope is indicated by a star and a stationary molecule is indicated with an arrowhead. (B) A bar chart providing the mean fraction \pm SEM of diffusing molecules measured at low and high calcium concentrations. *n* values refer to the number of flowcells. *Significant at $P < 0.075$. Data were collected for 200 s or more using 20 nM of either C0C3, C0C1f, and C0C1 at low calcium; at high calcium, to compensate for decoration levels that enable individual molecules to be distinguished, 5 nM C0C3 and C0C1f or 20 nM C0C1 was used.

Cy3-C0C3 itself to the thin filament is seen to occur in clusters and this binding most likely displaces tropomyosin to a position that facilitates myosin binding (10, 18, 20). Surprisingly, ~20% of total Cy3-C0C3 binding events show diffusive behavior on the thin filament at low calcium, which may reflect the behavior of cMyBP-C's N terminus within the sarcomere as it searches for its binding site on the thin filament.

Specific cMyBP-C N-Terminal Domains Are Necessary for Thin-Filament Activation. Activation of the thin filament at high calcium triggered significant S1-GFP binding, as expected (Fig. 2). Interestingly, the C0C3 N-terminal fragment sensitized the thin filament to calcium so that at low calcium, S1-GFP binding was equivalent to that for a fully calcium-activated thin filament (Fig. 2). The shorter N-terminal fragments (C0C1f and C0C1), with the C2 and C3 domains and a portion or the entire M-domain removed, were still capable of binding to thin filaments (Fig. 3). However, these fragments failed to sensitize the thin filament at low calcium (Fig. 2), measured as no change in S1-GFP binding in their presence. The failure of these shorter, murine cMyBP-C N-terminal fragments to sensitize the thin filament agrees with ATPase (19), *in vitro* motility (18, 26), and fragment-infused fiber data (11), suggesting that murine N-terminal fragments require at least C0-C2 to observe maximal thin filament calcium sensitization compared with the human N-terminal cMyBP-C fragments, where C0C1 and C1 alone are sufficient to sensitize the thin filament to calcium (19, 20, 42, 43). This species difference has been attributed to alternate sequences in the Pro-Ala linker between C0 and C1 as well as C1 itself (44).

The three-state model for thin-filament regulation (25) provides a useful mechanistic context for how C0C3 sensitizes the thin filament to calcium. Specifically, calcium binding to the thin filament permits tropomyosin movement on actin to expose myosin binding sites, shifting tropomyosin from the "blocked" to the "closed" position. As a result of myosin binding, tropomyosin is further displaced to the "open" or "myosin" position (24), therefore propagating the binding of additional myosins (29). By C0C3 binding to the thin filament at low calcium as it transiently enters the closed position, this N-terminal fragment may stabilize tropomyosin in the closed position, allowing myosin binding to

actin, as supported by structural studies of tropomyosin movement caused by C0C2 binding to thin filaments (18). Since our data show a distinct difference between binding at low and high calcium concentrations, this may also suggest that additional binding sites become available once tropomyosin is displaced from the blocked position. The clustered or possible coordinated binding of C0C3 most likely represents binding to newly accessible actin adjacent to the location of a previously bound C0C3; however, intermolecular interactions between C0C3 molecules may also contribute.

Modes of cMyBP-C N-Terminal Interactions with the Thin Filament.

The modular architecture of cMyBP-C provides the potential to structurally compartmentalize its functional capacities. Even though the very N-terminal domains, C0C1 and C0C1f, bind to actin (20, 21, 45, 46) as we have also shown previously (47), only C0C3 possesses the ability to sensitize the thin filament to low calcium while decreasing activation levels at high calcium. Unique to our study is the observation that upon binding of these N-terminal fragments to the thin filament, the fragments either remain stationary or undergo a diffusive-like movement along the thin filament. Specifically, for C0C3, how do these two distinct modes of interaction (i.e., 20% diffusing and 80% stationary) contribute to the thin-filament sensitization at low calcium? Multiple sites of N-terminal fragment interactions with the thin filament have been proposed based on actin cross-linking (48), fragment binding to actin in the laser trap (37), and electron microscopy studies (18, 20, 21, 46). At low calcium, tropomyosin on the thin filament should be in a dynamic equilibrium between the blocked and closed states (49). Presumably, the stationary Cy3-C0C3 molecules are stereospecifically bound to the thin filament through multiple binding sites, which could effectively shift tropomyosin's equilibrium position to being predominantly closed and thus sensitize the thin filament to calcium. At low calcium the Cy3-C0C1 and Cy3-C0C1f have the same distribution of diffusing and stationary molecules, and therefore the stationary fractions are not bound in the same manner as Cy3-C0C3, given the inability of these shorter fragments to sensitize the thin filament for myosin binding. As individual Ig-domains, both C0 and C1 can bind to the thin filament in several different configurations (20, 42), which is not the case for longer C0C2 and C0C3 fragments (21, 50). These structural studies support our proposal that the stationary Cy3-C0C3 molecules are the result of multiple sites of interaction with the thin filament, necessary to achieve calcium sensitization.

For the 20% of the Cy3-C0C3 fragments that diffuse at low calcium, we observed transient switching from diffusion to static binding on thin filaments (*SI Appendix*, Fig. S4). Such behavior would suggest that the diffusing molecules were interacting with regions of the thin filament more stably. However, for those transient static interactions that did not lead to extended binding this may suggest incomplete formation of a binding interface with the thin filament, which would otherwise result in activation and C0C3 recruitment. When diffusing, C0C3 molecules must weakly interact with the thin filament with a diffusional step size that we estimate to be between ~7 and ~17 nm, consistent with one to three actin monomers. This diffusive mode of motion may allow the N terminus of cMyBP-C to maintain contact with and scan the thin filament until such time that specific cMyBP-C binding sites are exposed on actin as tropomyosin undergoes its dynamic equilibrium between blocked and closed positions on the thin filament. Once statically bound to these sites, cMyBP-C may stabilize tropomyosin in the closed position (18, 25). This may also explain why at high calcium where the thin filament is predominantly in the closed state (25), Cy3-C0C3 binding to the thin filament is significantly increased (Fig. 3D) and almost entirely stationary (Fig. 5B) as cMyBP-C binding sites on actin would be readily exposed.

The clustering of C0C3 may compete with myosin for actin binding sites, as proposed in structural studies (18, 20, 21, 46) and cosedimentation competition studies (51), explaining the reduction in myosin binding observed on tightropes at high calcium (Fig. 2). Therefore, competitive inhibition of myosin binding by the N terminus of cMyBP-C may be one contributing factor to the reduced thin-filament velocity observed both in the C-zone of native thick filaments (52) and in the motility assay at high calcium (18, 26). However, reduced cross-bridge numbers in limited locations on the thin filament may not be enough to slow motility. It is possible that cMyBP-C binding to the thin filament may provide a load against which the remaining cross-bridges work, in turn slowing velocity (53, 54). Is *in vivo* clustering of cMyBP-C on the thin filament possible? cMyBP-C is positioned every ~43 nm along the thick filament within the C-zone. Since each thin filament in the muscle lattice is surrounded by three thick filaments, the individual cMyBP-C N termini from these thick filaments could cluster at a common point on the thin filament. In addition, HCM mutations of MyBP-C are known to lead to truncations (55), which could potentially compete with myosin as previously suggested (22). Fluorescence detection of Cy3-C0C3 binding and clustering at low vs. high calcium enabled us to fit the data by a chemical equilibrium model to estimate the affinity of these cMyBP-C fragments for thin filaments as 0.18 μM . This value is ~10-fold tighter than the ~2 μM reported for unregulated actin from ATPase inhibition studies (19) and up to 25-fold tighter than the affinities reported for mouse C0C2 using actin cosedimentation assays (48, 56). Some studies reporting the lower binding affinities (48) used bare actin, whereas thin filaments were used in the present study. Recent findings suggest that tropomyosin may bind cMyBP-C's N terminus (57); therefore, tropomyosin itself could act as a cofactor in the clustered binding of C0C3.

Conclusion

In this paper we addressed the nature of thin-filament activation/inhibition by the N terminus of cMyBP-C. Using a newly developed single-molecule imaging assay, we propose a mechanism by which cMyBP-C interacts with thin filaments using two different binding regimes in relaxing and activating calcium conditions. At low calcium (relaxing conditions), cMyBP-C senses the thin filament for changes in activation state due to positional fluctuations of tropomyosin. Sensing occurs by a weak binding mode in which the N terminus diffuses from actin monomer to monomer. Once specific cMyBP-C binding sites are encountered that allow subdomains C0 through C3 to bind more tightly, cMyBP-C can then effectively shift the tropomyosin equilibrium position from the blocked to the closed state, activating the thin filament. At high calcium (activating conditions), where calcium binding to troponin itself shifts tropomyosin from the blocked to the closed position, cMyBP-C can bind tightly to the thin filament and compete for myosin binding. It is important to note that our studies define the cMyBP-C N-terminal interactions with the thin filament but do not rule out potential interactions with the myosin head region as contributing to the thin filament sensitization. Regardless, our studies provide at least one molecular model for how cMyBP-C carries out its function within the confines of the sarcomere *in vivo*.

Materials and Methods

Protein Purification. Actin and myosin were purified from chicken pectoralis muscle (58), and recombinant human cardiac tropomyosin and troponin

were expressed in *Escherichia coli* and further purified as described previously (59). Murine cMyBP-C fragments were bacterially expressed with a C-terminal His-tag for purification using a nickel affinity column. Myosin-S1 was produced from whole myosin by papain digestion (60) and labeled with EGFP by exchange of the endogenous RLC with recombinantly expressed EGFP-tagged RLC (29); this construct is termed S1-GFP. Using the proteolytically derived single-headed S1 construct, instead of full-length myosin or heavy meromyosin, simplified interpretation and quantification of the data. Thin filaments were fully reconstituted by incubating actin, tropomyosin, and troponin overnight at 4 °C in 4 mM imidazole, pH 7, 2 mM MgCl_2 , 1 mM DTT, and 0.5 mM ATP (61) with a molar ratio actin:tropomyosin:troponin of 2:0.5:0.25. In all experiments, thin filaments were labeled with AF633-phalloidin (A22284; Invitrogen) for imaging in the flow chamber. For experiments involving cMyBP-C N-terminal fragments binding to thin filaments, the N-terminal fragments (i.e., C0C3, C0C1f, and C0C1; see Fig. 2B) were fluorescently labeled with a maleimide-Cy3 (PA23031; GE Healthcare) as in ref. 62, achieving ~30% labeling efficiency. Based on the C0C3 structure, there is only one surface-exposed reactive cysteine (C248) and thus only one Cy3 dye per labeled N-terminal fragment (62).

The Thin-Filament Tightrope Assay. To nullify any potential surface interference and to permit full, 3D accessibility to all myosin and cMyBP-C binding sites on the thin filament, we suspended thin filaments between surface-adhered beads to create tightropes (Fig. 1B) as described in detail previously (29). Briefly, silica beads were functionalized with 340 $\mu\text{g}/\text{mL}$ poly-L-lysine and adhered to a glass coverslip by infusion into a microfluidic flow cell. To generate tightropes, 500-nM thin filaments were passed across the silica beads multiple times back and forth using a syringe pump. Imaging was performed using a custom-built oblique angle fluorescence microscope (38) that excites the sample with a continuous-wave 20-mW, 488-nm DPSS laser (JDSU) focused off-center at the back-focal plane of a 100 \times objective (1.45 N.A.) to achieve obliquely angled illumination. We detected images (Fig. 1C) through an Optosplit III (Cairn Research) projected onto a Hamamatsu OrcaFlash 4.2 camera.

Data Collection and Analysis. All experiments were performed in either high (100 μM , pCa 4) or low (0.1 μM , pCa 7) calcium buffers of 25 mM imidazole, pH 7.4, 4 mM MgCl_2 , 1 mM EGTA, and KCl adjusted to a final ionic strength of 51 mM using MaxChelator (63). Ten or more tightropes were imaged per condition (details of the conditions are provided in *Results*). Videos of S1-GFP binding to thin filaments were acquired at three frames per second (fps) for up to 1 min, while videos of Cy3-labeled cMyBP-C fragments were acquired at 1 fps for up to 5 min. To determine the spatial and temporal dynamics of S1-GFP or Cy3-labeled cMyBP-C fragments interacting with thin filaments, we transformed movies into kymographs where fluorophore position was plotted along the y axis and time on the x axis (Fig. 1D). Analysis of bound fluorophore position was performed using custom-written macros in ImageJ available online (kadlab.mechanicsanddynamics.com). To measure the extent of S1-GFP binding we integrated the fluorescence intensity over the entire kymograph and normalized to the total number of pixels. All statistical *P* values mentioned were calculated using an unpaired *t* test, and *P* values <0.075 were considered significant. All SEM values were calculated using the number of flowcells, and in the case of comparative calculations SEM is a propagated error and therefore no *n* value is given. For data fitted using Microsoft Excel, the >95% CI values were calculated using the method of Kemmer and Keller (64).

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