The C Terminus of Cardiac Troponin I Stabilizes the Ca²⁺-Activated State of Tropomyosin on Actin Filaments

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Rationale: Ca^{2+} control of troponin–tropomyosin position on actin regulates cardiac muscle contraction. The inhibitory subunit of troponin, cardiac troponin (cTn)I is primarily responsible for maintaining a tropomyosin conformation that prevents crossbridge cycling. Despite extensive characterization of cTnI, the precise role of its C-terminal domain (residues 193 to 210) is unclear. Mutations within this region are associated with restrictive cardiomyopathy, and C-terminal deletion of cTnI, in some species, has been associated with myocardial stunning.

Objective: We sought to investigate the effect of a cTnI deletion -removal of 17 amino acids from the C terminus on the structure of troponin-regulated tropomyosin bound to actin.

Methods and Results: A truncated form of human cTnI ($cTnI_{1-192}$) was expressed and reconstituted with Troponin C and Troponin T to form a mutant troponin. Using electron microscopy and 3D image reconstruction, we show that the mutant troponin perturbs the positional equilibrium dynamics of tropomyosin in the presence of Ca^{2+}. Specifically, it biases tropomyosin position toward an "enhanced C- state" that exposes more of the myosin-binding site on actin than found with wild-type troponin.

Conclusions: In addition to its well-established role of promoting the so-called "blocked-state" or "B-state," cTnI participates in proper stabilization of tropomyosin in the "Ca^{2+}-activated state" or "C-state." The last 17 amino acids fulfill this stabilizing role. The data are consistent with a "fly-casting" model in which the mobile C terminus of cTnI ensures proper conformational switching of troponin–tropomyosin. Loss of actin-sensing function within this domain, by pathological proteolysis or cardiomyopathic mutation, may be sufficient to perturb tropomyosin conformation. (*Circ Res*. 2010;106:00-00.)

Key Words: troponin ∎ thin filament ∎ myocardial stunning ∎ cardiomyopathy

Lesions of the myofilament proteins are a common cause of inherited and acquired forms of heart disease.[1,2] Such defects in thin filament protein, cardiac troponin (cTn)I, have been implicated in both hypertrophic and restrictive cardiomyopathy, as well myocardial stunning. For example, mutations within the C-terminal domain of cTnI gene that cause amino acid substitutions R192H, G203S, and K206Q lead to hypertrophic and/or restrictive cardiomyopathy,[3,4] whereas removal of the C-terminal 17 amino acids from cTnI by Ca^{2+}-dependent proteolysis has been implicated in models of myocardial stunning,[5–9] a condition that arises from brief ischemia that substantially depresses contractile function without causing cell death.[2,10] In fact, expression of truncated $cTnI_{1-193}$, at levels <20% relative to endogenous cTnI, is sufficient to substantially compromise systolic and diastolic function[11] in mice. Moreover, C-terminal degradation of cTnI has been observed in patients undergoing coronary artery bypass graft surgery.[12] Given the role of this domain of cTnI in genetic and acquired heart disease, efforts are underway to fully understand its function.

TnI is 1 of the 3 subunits that comprise the troponin complex that regulates muscle contraction by controlling the position of tropomyosin on actin filaments in response to Ca^{2+}.[13,14] Known as the inhibitory subunit of troponin, it prevents myosin binding to actin, in diastole, by maintaining tropomyosin over the outer edge of actin filaments. In systole, Ca^{2+} binds to the Ca^{2+}-receptor subunit of troponin, TnC, which causes a conformational change that promotes its interaction with cTnI and coincident release of TnI inhibitor regions from actin. This causes the average position of tropomyosin to shift across the face of actin and thereby expose myosin-binding sites that are then accessible for myosin to begin crossbridge cycling.[15]

The domains of TnI, which contain multiple binding sites for TnC, TnT, actin, and tropomyosin, have been extensively

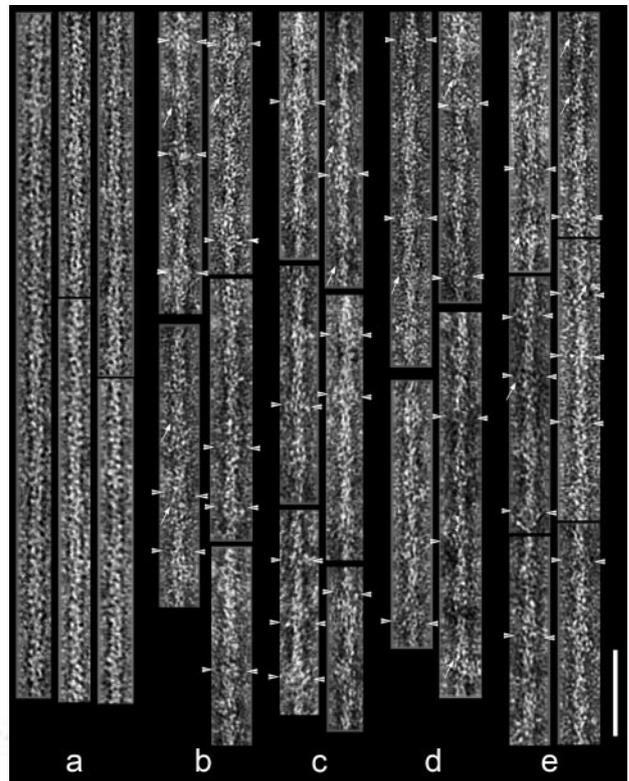| Non-standard Abbreviations and Acronyms	
B-state	blocked state of the thin filament
C-state	Ca^{2+}-induced closed state of the thin filament
cTn	cardiac troponin
$cTnI_{1-192}$	truncated cTnI lacking 17 amino acids at the C terminus
EM	electron microscopy
M-state	myosin-induced fully active open state of the thin filament
PIPES	piperazine-N,N'-bis(ethanesulfonic acid)
Tn	troponin

Figure 1. Electron micrographs of reconstituted thin filaments. a, Bare actin filaments. b, Actin filaments reconstituted with wild-type troponin and tropomyosin in Ca^{2+}-free buffer. c, Actin filaments reconstituted with wild-type troponin and tropomyosin in the presence of Ca^{2+}. d, Actin filaments reconstituted with mutant troponin and tropomyosin in Ca^{2+}-free buffer. e, Actin filaments reconstituted with mutant troponin and tropomyosin in the presence of Ca^{2+}. Arrowheads point to the globular heads of the troponin complex. The white arrows highlight tropomyosin strands. Scale bar=50 nm.

characterized,[16] and their organization has been clarified by the low and high Ca^{2+} crystal structures of cardiac and skeletal muscle troponin.[17,18] However, only an incomplete picture of regulatory switching of tropomyosin on actin can be garnered from the troponin structures, because the C terminus of TnI is unresolved, owing to its high flexibility.[19] Given the pathological significance of lesions with the C terminus of cTnI, we sought to determine how truncation of TnI affects the prime function of troponin, namely, its ability to modulate tropomyosin position on actin filaments. We discuss newly acquired structural data in the context of recent biochemical and biophysical studies of $cTnI_{1-192}$ and a newly proposed model of cTnI function.[20]

Methods

Protein Preparation

F-Actin and bovine cardiac tropomyosin were purified by standard methods.[21] Methods describing the expression, purification, and reconstitution of troponin subunits are described in the Online Data Supplement, available at http://circres.ahajournals.org.

Electron Microscopy

Thin filaments were reconstituted by mixing actin, tropomyosin, and wild-type or mutant troponin in a ratio of 7:2:2 (F-actin: 10 to 20 μmol/L) in both low- and high-calcium buffers (low Ca^{2+}: 5 mmol/L PIPES/5 mmol/L sodium phosphate buffer [pH 7.1], 100 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.2 mmol/L EGTA, 1 mmol/L NaN_3, 1 mmol/L DTT; high-Ca^{2+}: same buffer supplemented with 2 mmol/L $CaCl_2$). Uranyl acetate staining is described in the Online Data Supplement. Electron microscopy was carried out on a Philips CM120 transmission electron microscope using low-dose methods (12 e$^-$/Å), the details of which are described elsewhere.[22–24]

Three-Dimensional Image Reconstruction From Electron Micrographs

Electron micrographs were digitized and analyzed by 2 distinct yet complementary methods of image reconstruction.[22,23] First, data were analyzed by helical reconstruction, a Fourier-space filtering and averaging method, using the Brandeis Helical Package essentially as detailed in.[23] Given the subtle, yet statistically and biologically significant, changes that we observed in thin filament structure, the results were cross-validated by further analysis of micrographs from an independent protein preparation using the real-space single-particle averaging method of Egelman,[25] as described by Pirani et al.[22] (For a comparison of results obtained from both reconstruction methods, see Figure III in the Online Data Supplement at http://circres.ahajournals.org.)

Results

Electron Microscopy and Three-Dimensional Reconstruction of Wild-Type and $cTnI_{1-192}$-Containing Thin Filaments

Thin filaments were formed from F-actin, cardiac tropomyosin and troponin complexes under conditions known to saturate the filaments with regulatory proteins.[22–24] "Wild-type" and mutant troponin complexes, reconstituted from subunits expressed in *Escherichia coli*, were used for comparison. The mutant troponin contained a truncated form of cTnI (TnI_{1-192}) but included otherwise normal troponin subunits, human cTnC and cTnT. Filaments were negatively stained in uranyl acetate and recorded by low-dose electron microscopy.[23] Electron microscopy (EM) of the thin filaments showed characteristic double-helical arrays of actin monomers, tropomyosin strands, and troponin densities repeating with a 40 nm periodicity (Figure 1). EM images of reconstituted filaments prepared from separately expressed and purified proteins were analyzed independently by the first and last authors; the raw images and 3D reconstructions generated from the 2 data sets were indistinguishable from each other and thus combined for analysis here. Filaments were studied by both helical reconstruction of relatively long
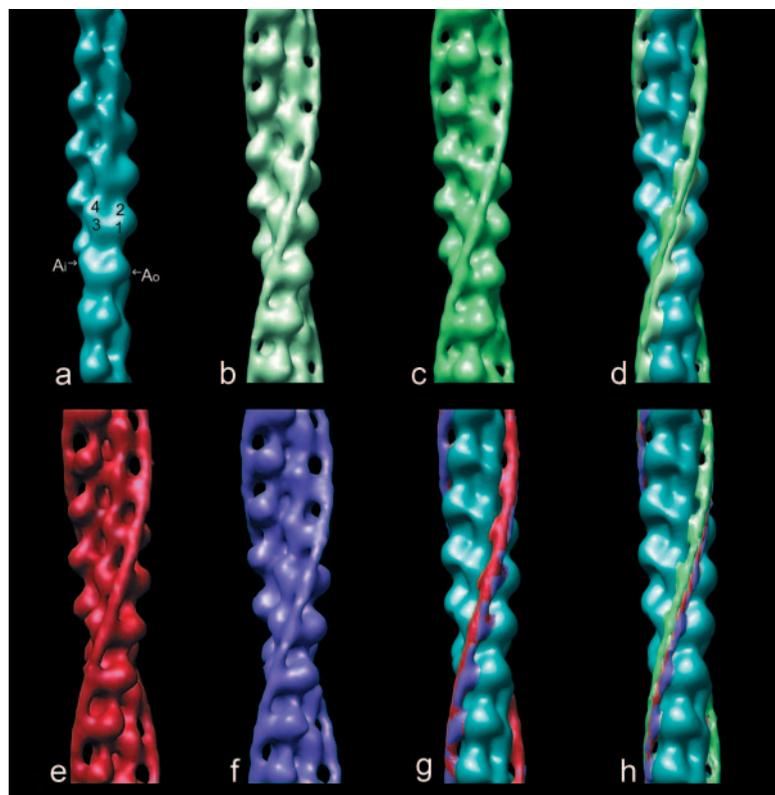
Figure 2. Mutant troponin affects the average position of tropomyosin on actin. Three-dimensional reconstructions of filaments are depicted in longitudinal view. b through d show 3D structures of filaments incubated in Ca^{2+}, whereas e through g depict reconstructions of Ca^{2+}-free filaments. The bare actin filament is depicted in a. Subdomains 1 and 2 comprise the outer domain of actin (A_o), whereas the inner domain (A_i) consists of subdomains 3 and 4. b depicts actin–tropomyosin with wild-type troponin in Ca^{2+}. c depicts of mutant-controlled tropomyosin on actin in Ca^{2+}. In d, the positions of tropomyosin from b and c are superimposed. e and f show the average position of tropomyosin conferred by wild-type and mutant troponin, respectively, in the absence of Ca^{2+}. The results of e and f are superimposed in g. All structures are superimposed in h.

filament stretches ($\approx$200 to 400 nm)[26] and by single-particle methods on filament segments ($\approx$40 nm)[25]; results from the 2 methods were completely consistent and reproducible.

Reconstructions of thin filaments showed actin subunits and densities that were attributable to tropomyosin (Figure 2). The longitudinally continuous tropomyosin strands were well defined in both control filaments containing "wild-type" troponin–tropomyosin and in filaments containing the mutant $cTnI_{1–192}$. Inspection of the reconstructions showed that the mutation did not interfere with the ability of tropomyosin to undergo a Ca^{2+}-induced shift from the outer domain (A_o) to the inner domain (A_i) of actin; thus, the impact of both the wild-type troponin and mutant troponin on directed tropomyosin movement is normal in both sets of filaments. In fact, in high Ca^{2+} conditions, tropomyosin localized further onto A_i in filaments containing mutant cTnI (Figures 2c and 3c) than it did in filaments with the wild-type TnI (Figures 2b and 3b). Thus, whereas the direction of the tropomyosin movement was the same in both samples, the magnitude of the movement was greater in the mutant (superimposed in Figure 2A and 2B [d]). As a consequence, less lingering tropomyosin density touched

A_o at high Ca^{2+} in the mutant than in the wild-type. In contrast, no obvious differences in tropomyosin position on F-actin were found for the low Ca^{2+} data (Figures 2g and 3g).

The Effect of $TnI_{1–192}$ at High Ca^{2+} Is Statistically Significant

Helical projection, ie, projection of densities down the helical axis of F-actin and tropomyosin, provides a means of defining the average position of tropomyosin relative to actin in reconstructions. Comparison of helical projections confirmed that tropomyosin is localized differently in wild-type and mutant thin filaments, but again such a distinction was only detected for the high Ca^{2+}-treated sample. The distinction was subtle but became obvious following difference density analysis that isolated the respective tropomyosin densities from actin. Here, maps of F-actin (no tropomyosin) were simply subtracted from those of thin filaments. The resulting tropomyosin densities then were superimposed on reference maps of bare F-actin and compared (Figure 4e). In the presence of Ca^{2+}, tropomyosin controlled by mutant troponin, containing $cTnI_{1–192}$ was shifted azimuthally by $\approx$9° more than it was by wild-type troponin (Figure 4e).
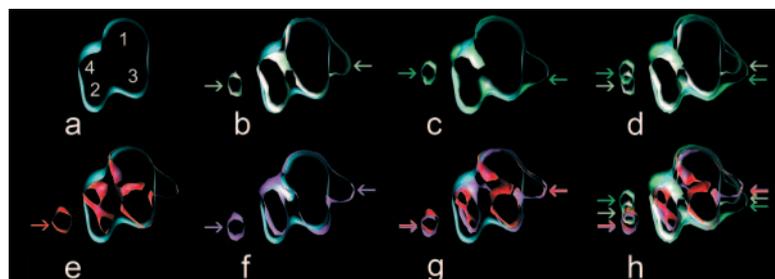

Figure 3. Cross-sections of wild-type– and mutant-controlled thin filaments. Images correspond to those described in Figure 2. b through d, Structures in Ca^{2+}. e through h, Structures in EGTA. b, Wild-type plus Ca^{2+}. c, Mutant plus Ca^{2+}. b and c are superimposed in d. e, Wild-type in EGTA. f, Mutant in EGTA. e and f are superimposed in g. All structures are superimposed in (h).
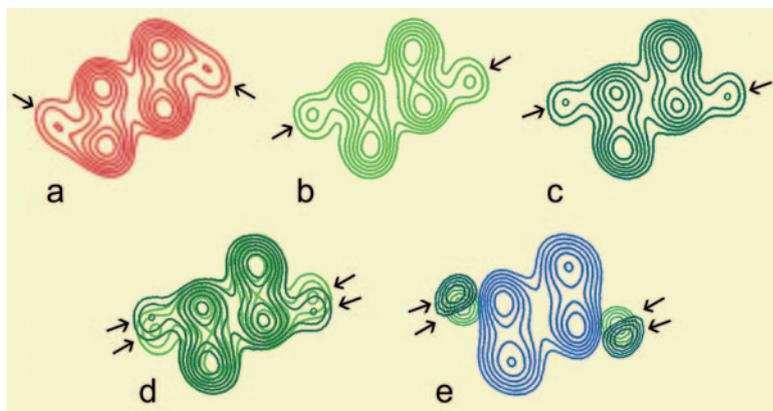
Figure 4. Helical projections illustrate the impact of mutant troponin on the tropomyosin position. a, Actin–tropomyosin–troponin filaments in Ca^{2+}-free solution. Tropomyosin sits on the outer domain of actin. b, In Ca^{2+}, tropomyosin adopts an average position over the inner domain of actin. c, Mutant troponin containing $cTnI_{1-192}$ is also responsive to Ca^{2+} and tropomyosin again adopts and average position over the inner domain of actin. d, Superimposing results from b and c shows that tropomyosin, controlled by mutant troponin, has shifted azimuthally to adopt an average position further from the outer actin domain by $\approx 9°$. e, To better visualize the image densities arising from the tropomyosin strands, the image density of bare actin filaments was subtracted from b and c. The densities of tropomyosin controlled by wild-type (light green) or mutant troponin (dark green) were superimposed over the helical projection of bare actin (blue). The difference in the centroid positions of tropomyosin density were statistically significant at >95%.

Point by point analysis of the maps using Student t test methodology[27,28] showed that this difference in tropomyosin position was statistically significant at 95% confidence levels. (Also see Online Figure IV, which demonstrates further that the distinctions noted are statistically significant.) Because the average position of tropomyosin in the mutant is further from the low Ca^{2+}, blocking state than it is in control filaments, we call it the "enhanced-on state position." Differences in tropomyosin positions for low Ca^{2+} filaments were not obvious or statistically significant.

Tropomyosin Equilibrium Position on Thin Filaments Is Altered by $cTnI_{1-192}$

Tropomyosin is thought to oscillate laterally over a narrow region of the flat surface of actin[22,29]; however, in the presence of troponin, its equilibrium balance becomes more biased toward specific regulatory positions on actin,[24,30,31] namely, those of the low Ca^{2+} B-state or the high Ca^{2+} C-state. The results above suggest that the mutant caused a rebalancing between positional states or possible development of a new equilibrium position for tropomyosin. Cross-correlation tools[32,33] comparing the experimental data to thin filament models with different tropomyosin locations were used to sort and classify short filament segments into positional categories. An analysis of high Ca^{2+} filaments indicated that ≈ 3.5 times more mutant filament data fitted better to the "enhanced C-state" than to the wild-type C-state position, whereas the reverse was true for wild-type data, where more of the data belonged to the C-state category (Table).

Discussion

Control of Tropomyosin Conformation by TnI_{1-192}

The C-terminal half of cTnI harbors 3 well-characterized domains: (1) an actin-binding region that inhibits actomyosin

Table. Distribution of Filament Segments Sorting to Different Regulatory States

Sample	B-State (%)	C-State (%)	Enhanced C-State (%)
Ca^{2+}-treated filaments with wild-type troponin	29	43	28
Ca^{2+}-treated filaments with mutant troponin	26	17	57

ATPase activity (inhibitory peptide; residues 128 to 147); (2) a region that binds to the N-terminal domain of troponin C in the presence of Ca^{2+} (switch peptide; residues 148 to 163); and (3) a second actin-binding site (residues 168 to 188). The function of the remaining C-terminal residues (residues 189 to 210) is largely unknown. In the absence of Ca^{2+}, this highly flexible domain[19] adopts a more defined structure as it binds to actin. Image reconstruction of thin filaments saturated with the C-terminal half of TnI show that the inhibitory region binds to actin at its N terminus (subdomain 1). Residues downstream of the inhibitory peptide span the cleft of the long-pitch helical actin strands, much like the smooth muscle inhibitory protein caldesmon,[34] and drape over subdomains 3 and 4 of the adjacent actin, where they abut tropomyosin and stabilize it in the blocked state (B-state).[31]

The human $cTnI_{1-192}$ construct, like the form that recapitulates the phenotype of myocardial stunning in mice,[11] lacks the last 17 amino acids. Previous biochemical studies[35] showed that $cTnI_{1-192}$, alone, bound both actin and actin-tropomyosin with the same affinity as full length cTnI. Yet when $cTnI_{1-192}$ was reconstituted into troponin, the complex could not fully inhibit ATPase activity in the absence of Ca^{2+}. This suggested that either cTnI could not maintain tropomyosin in a fully competent B-state or that equilibrium dynamics between the B- and C-states of the thin filament of might be altered. As shown in Figures 2g and 3g, mutant troponin caused no statistically discernible difference in the average position of tropomyosin in the presence of EGTA. Thus, residues 193 to 210 of cTnI, downstream of its major actin-binding regions, are not required to generate the B-state position of tropomyosin.

In the presence of Ca^{2+}, the mutant troponin displays higher maximal Ca^{2+}-activated actin–tropomyosin–S1 ATPase than does the wild-type troponin.[35,36] Similar observations of both higher basal and Ca^{2+}-activated ATPase activity[35] were noted in studies of the murine variant of restrictive cardiomyopathy mutant, R193H.[37] Wild-type troponin could confer comparable maximal activity, provided that thin filaments were fully activated by noncycling NEM-S1 heads. The data could be best explained by a shift in the tropomyosin equilibrium from the inactive to the active state[37] or, in the context of a 3-state structural model, a shift that would favor

transition to the myosin-induced state (M-state). Here, incorporation of $cTnI_{1-192}$ into troponin, in the presence of Ca^{2+}, evinces a tropomyosin position shifted further over the inner domain of actin (ie, subdomains 3 and 4) than typically observed with wt troponin in Ca^{2+}. The 9° azimuthal shift of tropomyosin, although subtle, is most discernible in the cross section of the filament (Figure 3d) and the helical projection (Figure 4e). The tropomyosin displacement is approximately half of the width of tropomyosin and just shy of the fully activated M-state observed in the presence of Ca^{2+} and docked myosin-S1 heads.

Note that the average position of tropomyosin is a function of the frequency with which one regulatory configuration or another is adopted, ie, the "enhanced C-state" is not a fixed position on actin but rather is associated with a readjusted distribution of positional states (Table). Tropomyosin regulated by wild-type troponin can also adopt the enhanced C-state, albeit less frequently, ie, in 28% of wild-type filaments versus 57% among mutant filaments. Therefore, the position of tropomyosin defined by mutant troponin is not a new structural state, per se, but rather a perturbation of the natural equilibrium distribution of tropomyosin on actin. This increases the propensity for tropomyosin to be found further over the inner domain of actin that is comprised of subdomains 3 and 4. Hence, actin can more easily bind myosin crossbridges.

The data in Figures 2d, 3d, and 4e are the first to depict alterations of thin filament structure by a pathological lesion of troponin, and they provide insight into how $cTnI_{1-192}$ alters the Ca^{2+} sensitivity of myofilaments. Specifically, we and others have shown that $cTnI_{1-192}$ increases the Ca^{2+} sensitivity of the ATPase reaction,[35,36] and $cTnI_{1-192}$ incorporation into rat trabeculae and human myofibrils increases the Ca^{2+} sensitivity of steady-state isometric tension. To determine the mechanism, Tachampa et al[36] measured the effect of the mutant on Ca^{2+}-affinity for TnC within the troponin complex. Although isolated troponin showed no difference, Ca^{2+} affinity was increased when mutant troponin was bound to thin filaments.[36] However, myofilament Ca^{2+} sensitivity also reflects the degree to which Ca^{2+}-binding would ultimately affect tropomyosin movement. The primary novel finding of this study is that Ca^{2+} binding to mutant troponin shifts the average position of tropomyosin not to the normal C-state but to a state that more closely resembles myosin-induced M-state over subdomain 3 and 4 of actin, thereby exposing more of the myosin binding site on actin. Thus, the integrity of the C terminus of TnI appears to mediate proper equilibrium transitions between the B-, C-, and near–M-states, and its effect can only be observed properly in the context of the entire thin filament.

Intact cTnI is therefore necessary for stabilization of the natural C-state of tropomyosin on actin, in addition to its established role as stabilizer the B-state in the absence of Ca^{2+}. Ca^{2+}-activation of the thin filament is widely held to involve removal of cTnI from actin as the switch peptide of cTnI (residues 148 to 163) binds to the N-terminal domain of TnC. However, simple removal of cTnI inhibition in Ca^{2+}, and release of the B-state, is inconsistent with recent biochemical studies showing that cardiomyopathy mutations within distinct C-terminal domains of cTnI perturb tropomy-

osin equilibrium differently.[37,38] Whereas some mutations, notably within the inhibitory peptide, appear to exhibit B-state defects, others closer to the C terminus are more consistent with defects of the C-state. Moreover, in vitro motility analysis has shown that the sliding velocity of thin filaments regulated by TnI_{G203S} and TnI_{K206Q} was Ca^{2+}-sensitized and ultimately higher in maximum Ca^{2+}, indicative of greater thin filament activation.[39] Therefore, lesions within the last 17 amino acids of cTnI may cause aberrant thin filament activation by destabilizing the C-state of tropomyosin in favor of a conformation that more closely resembles the fully active M-state.

Proposed Mechanism of C-State Stabilization by the C Terminus of TnI

If cTnI is an active participant in C-state stabilization, the salient question is, how. Recently, an innovative "fly-casting" hypothesis has been proposed[20] to describe the manner by which the highly-disordered C-terminal region of cTnI might contribute to muscle regulation. When Ca^{2+} binds to the N-terminal of TnC, and the TnC-binding switch peptide of cTnI binds the hydrophobic pocket of TnC, the TnI inhibitory peptide and second actin-binding domain are removed from actin in the process. However, the fly-casting hypothesis posits that residues that lie C-terminal to the TnI switch peptide (the mobile domain) would continue to participate in long-range sampling, or sensing, of the thin filament via ionic interactions. This would effectively catalyze TnI binding to actin when Ca^{2+} dissociates from TnC.[19,20] If the weak transient ionic interactions between the mobile domain of cTnI and actin destabilize binding of the TnI switch peptide to the N-terminal domain of TnC,[20] then C-terminal deletions of thin filament-bound cTnI would, conversely, confer higher affinity for TnC. Indeed, we noted this previously, as $cTnI_{1-192}$–mediated inhibition of actin–tropomyosin–activated ATPase was more easily reversed by TnC in the presence of Ca^{2+} (see Foster et al[35] and figure 3B therein).

From the perspective of the fly-casting model, $cTnI_{1-192}$ is deficient in 2 ways. It lacks the last 17 amino acids and therefore casts a shorter "fishing line" with which to "sense" actin. It also lacks 4 basic residues, 3 lysine and 1 arginine. Because Lys and Arg are critical determinants of actin-binding affinity in the inhibitory region[40] and second actin-binding sites of TnI,[41] these residues in the C terminus of TnI may also interact weakly/transiently with Asp and Glu residues on the actin filament. These weak TnI–actin interactions could well be sufficient to stabilize tropomyosin in a wild-type C-state, given the correspondingly low local affinity of tropomyosin for F-actin.[42] We suspect that pathophysiological changes to the C terminus of TnI that abrogate its transient ionic interactions with actin, or impinge on its flexibility, may destabilize the C-state and thereby favor movement of troponin–tropomyosin to an enhanced-C-state. Biochemical studies have shown that cardiomyopathies arising from cTnI mutations alter the equilibrium positions of tropomyosin[37,38] and lesions within the C terminus, D190H R192H, G203S, and R206Q likewise exert Ca^{2+}-sensitizing effects on contractility.[38,39] These mutants would also be worth investigating structurally.

Role of the C Terminus of cTnI in Heart Function

Although difficult to extrapolate complex functional sequelae from static structures, the conformation of tropomyosin conferred by the mutant troponin suggests possible mechanisms by which deletion of the C terminus of cTnI might influence heart function. The exposure of myosin-binding sites on actin is a key determinant of the rate of crossbridge attachment (so-called f_{app} in the nomenclature of 2-state crossbridge models).[43] Our results with the mutant troponin would therefore favor a higher rate of crossbridge attachment at any given Ca^{2+} concentration, which is consistent with recent work on stunned rat myocardium.[44] However, work with both models of stunned myocardium, and the mutant troponin in rat trabeculae, shows that force production is substantially compromised[11,36,44,45] and at a higher energetic cost.[36,44] This is indicative of increased crossbridge turnover that stems from an offsetting increase in the rate of crossbridge detachment (g_{app}).[44,45] It is possible that destabilization of tropomyosin from the C-state toward the M-state by the mutant may decrease an energetic barrier to crossbridge detachment. Finally, functional studies of the mutant troponin have shown lower cooperativity of Ca^{2+}-activated force production in rat trabeculae,[36] human cardiomyocytes,[46] and in a transgenic mouse model of myocardial stunning.[11] We submit that stunning characterized by low levels of cTnI truncation could well cause destabilization of tropomyosin as seen here, and thereby dampen propagation of Ca^{2+} activation along the thin filament.

Indeed, previous work in a transgenic mouse model of stunning indicates that systolic and diastolic heart function is compromised when only 9% to 17% of the troponins contain truncated cTnI.[11] Destabilization of tropomyosin position would be expected to have large functional consequences in muscle, even with such low levels of cTnI proteolysis. Given the semirigid nature of tropomyosin, local destabilization would be propagated beyond a single troponin–tropomyosin regulatory unit. In other words, because thin filament regulatory switching is cooperative, an effect at one site, in this case because of mutant cTnI, will have a delocalized effect on neighboring sites even if those sites contain wild-type troponin.

Summary

In conclusion, a key issue regarding thin filament activation in striated muscle is: what are the precise molecular determinants that govern the movement of tropomyosin on actin, namely, what protein interactions influence its equilibrium position in the presence and absence of Ca^{2+}? Increased Ca^{2+} sensitivity observed in multiple studies of $cTnI_{1-192}$ involves both higher affinity of Ca^{2+} for troponin[36] and alteration of tropomyosin conformation on actin. This change in conformation reveals that cTnI actively stabilizes the natural C-state tropomyosin position in the presence of Ca^{2+}. In diastole, residues 128 to 192 of cTnI are sufficient to generate the B-state, whereas determinants within the last 17 amino acids are critical to the C-state in systole. This work informs our understanding of myocardial stunning models characterized by cTnI proteolysis at the C terminus and suggests a framework for the consideration of restrictive and/or hypertrophic cardiomyopathy mutations within the same domain.

Disclosures

None.

Novelty and Significance

Lesions within the C terminus of cardiac troponin (cTn)I have severe consequences for heart function, yet this region has no assigned molecular function that would help refine models of contraction or explain its pathophysiology. Here, we have shown that a critical function of this unstructured domain is to stabilize the Ca^{2+}-activated state of the thin filaments. Removal of the C terminus of TnI perturbs the structure and equilibrium movements of tropomyosin on actin in the presence of Ca^{2+}. This is first documented change in thin filament structure caused by a pathological modification of cTnI. The data suggest an expanded role for cTnI in muscle regulation. It is more than a simple inhibitory protein that promotes muscle relaxation in diastole; it is an active participant in proper myofilament activation in systole. Because defects in the C terminus of cTnI can be propagated along tropomyosin strands, a small degree of proteolysis, as might occur in myocardial stunning, can have a disproportionate effect on heart function.

What Is Known?

- Mutations or proteolysis within the C terminus of cTnI cause heart dysfunction.
- The function of this intrinsically disordered domain is ill defined.
- There is currently no structural framework that helps us understand why mutations in this domain would be harmful.

What New Information Does This Article Contribute?

- The C terminus of cTnI stabilizes the Ca^{2+}-activated state of tropomyosin on actin, likely through transient ionic interactions with actin.
- This tropomyosin-stabilizing function means that cTnI actively participates in proper myofilament activation in systole in addition to its established role of promoting muscle relaxation in diastole.
- Cardiomyopathy mutations within the C terminus of cTnI that affect charge or flexibility may mimic its deletion, altering the natural movements of tropomyosin and, in turn, influencing myofilament crossbridge interactions.

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The C Terminus of Cardiac Troponin I Stabilizes the Ca^{2+}-Activated State of Tropomyosin on Actin Filaments

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Supplement Material

Preparation of Proteins

Actin was prepared from rabbit (NZW) skeletal muscle according to the method of Spudich and Watt[1]. Bovine cardiac tropomyosin was purified according to Tobacman and Adelstein[2]. Human cardiac isoforms of TnT, TnC and TnI and TnI1-192 were cloned in the pet-11d vector and expressed in BL21(DE3) pLysS under carbenicillin/chloramphenicol selection, induced by the addition of 0.5 mM Isopropyl β-D-1-thiogalactopyranoside. *E. coli* pellets were harvested by centrifugation at 4000 rpm in a Beckman JS-4.2 rotor. Pellets were washed with phosphate buffered saline containing 5 mM EDTA. Pellets were stored at -80°C until use. Pellets were thawed and resuspended in a lysis buffer containing 50 mM HEPES, 350 mM KCl, 5 mM EGTA, 1 mM EGTA, 1mM DTT, 1mM PMSF pH 7.0 (approx 200 mL per 2L of original culture). Lysozyme was added to a final concentration of 0.5 mg/mL and allowed to stir at 4°C for 20 min. The solution was subjected to sonication at 20 W, five times for 1 minute, at 1 minute intervals, on ice. It was subsequently centrifuged at 10000xg for 10 min at 4°C. Supernatants were discarded and troponin subunits were purified from inclusion bodies in the pellet. TnI and TnI mutants formed tight, highly insoluble inclusion bodies that were washed by resuspending the pellet in a buffer containing 20 mM Tris-HCl, 1M Urea, 5mM EDTA, 1% (w/v) Triton X-100, 1mM DTT, 1mM PMSF pH 7.0 and subsequently recentrifuging at 10000 x g for 10 min. The same wash step was omitted from preparations of TnT, since it tended to solubilize the protein. Inclusion bodies were resuspended in 50 mM HEPES, 8 mM Urea, 5 mM EDTA, 1mM DTT, pH 8.0 (approx. 50 mL) and allowed to sit on ice for 30 min to ensure full solubilization. Solubilized protein was clarified by centrifugation at 100000xg for 30 min. TnT, TnI and TnC were purified by cation exchange chromatography using CM-cellulose or UnoS FPLC column. The columns were equilibrated in 20 mmol/L Tris-HCl, 6 mol/L urea, 1 mM EDTA, 1 mM DTT pH 8.0. Proteins were eluted with a linear gradient of NaCl. TnC was purified from soluble *E.coli* lysates by Phenyl-Sepharose chromatography as described by Gopalakrishna & Anderson[3].

Reconstitution of the Troponin Complexes

Briefly, the troponin subunits were first dialyzed into buffer containing 5 mmol/L MOPS (or PIPES; pH 7.2), 6 mol/L urea, 1mol/L NaCl, 5mmol/L NaH_2PO_4, 3 mmol/L $MgCl_2$, 0.2 mmol/L EGTA, 1mmol/L dithiothreitol. TnT, TnC, and TnI or TnI$_{1-192}$ were mixed in equimolar ratios and refolded by successive dialysis, first against the same buffer containing 2M urea, 1M KCl, then against buffers, without urea, containing 1 mol/L, 0.5 mol/L and 0.1mol/L NaCl. The purity and stoichiometry of the proteins used to reconstitute the complexes was assessed by SDS-PAGE (see Online Figure I). The biochemical and biophysical properties of reconstituted mutant troponin have been characterized extensively *in vitro* and in muscle fibers[4-6].

Assembly of Thin Filaments and Negative Staining

Thin filaments were reconstituted by mixing actin, tropomyosin and wild-type or mutant troponin in a ratio of 7:2:2 (F-actin: 10-20 μmol/L) in both low- and high-calcium buffers (low Ca^{2+}: 5 mmol/L PIPES/5 mmol/L sodium phosphate buffer (pH 7.1), 100 mmol/L NaCl, 3 mmol/L $MgCl_2$, 0.2 mmol/L EGTA, 1mmol/L NaN_3, 1mmol/L DTT; high-Ca^{2+}: same buffer supplemented with 2 mmol/L $CaCl_2$). Cosedimentation experiments showed that mutant and wild-type troponins bound to actin-tropomyosin with comparable stoichiometry, indicating that C-terminal truncation of TnI did not interfere with proper thin filament assembly (Online Figure II). Samples were diluted 20-fold, quickly applied to carbon-coated grids, and negatively stained with 1%-2% uranyl acetate. The suitability of negative staining for the study of thin filament structure at 20 to 25 Å resolution, has been validated extensively[7-13] Staining and subsequent microscopy was conducted on two independent protein preparations independently by the first and last authors.

Nomenclature

The construct $cTnI_{1-192}$ was named using a convention used by protein chemists that excludes the initiating methionine. Under this convention, human cTnI has 209 amino acids and our construct lacks the last 17 amino acids, specifically NIDALSGMEGRKKKFES. Using NCBI database numbering, the construct would encompass 1(Methionine) to 193 (Lysine). We have kept the $cTnI_{1-192}$ nomenclature to ensure consistency with prior work.

A Note on the Structures

Using the helical and iterative real-space reconstruction methods, the long-pitch image density observed spanning successive actin monomers is attributable to the tropomyosin molecule. Troponin, itself, is not visualized as its image density is averaged over 7 actin monomers. Differences between wild type and mutant data represent true conformational changes in tropomyosin, not changes in TnI density since the only difference between wt and mutant troponin experiments is 17 amino acids, whose small mass is distributed over 7 actin monomers (>2600 amino acids). Therefore, any difference in thin filament conformation caused by the loss of 17 amino acids, within the random coil domain at the C-terminus of cTnI, is due to its influence on tropomyosin, not the density difference due to the truncation itself (see references [9, 10] which address these points). That similar results were obtained across protein preparations by independent investigators, and across reconstruction algorithms, attests to the robustness of the final structures. In total, reconstructions were generated from 40 low-Ca control filaments, 37 low-Ca filaments containing TnI_{192}, 32 high-Ca control filaments and 36 high-Ca filaments containing TnI_{192}. Each reconstruction presented in the current paper, therefore, represents the respective average position of ~500 tropomyosin molecules lying over ~3500 actin monomers. Filament segments (40 nm) of any particular data set were sorted and classified into positional categories (Table 1) by cross-correlation to average 3D reconstructions representing B-state, C-state and "enhanced" C-state models[9].

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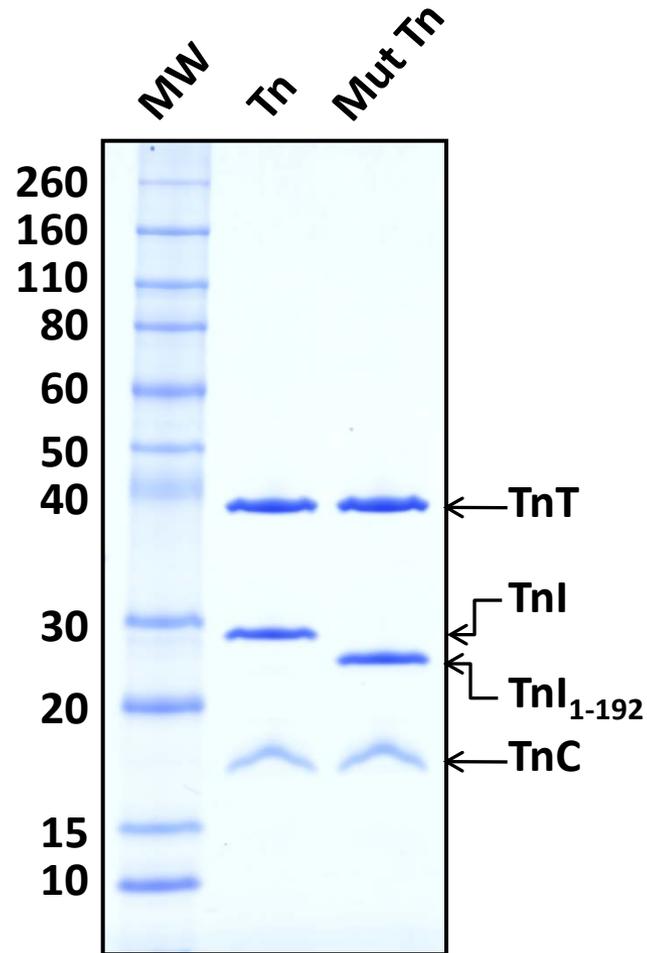
Online Figure I. **Purity and stoichiometry of the reconstituted troponin complexes.**
Following refolding of the wild-type and mutant complexes from their individual subunits, 3 µg of each complex was subjected to 12% Tris-glycine SDS-PAGE. Novex Sharp Prestained Markers were used as molecular weight standards. The gel was stained with Simply Blue (Invitrogen). Complexes were free of contaminants and proteolytic breakdown products. Comparable staining intensities of each of the troponin subunits between the complexes attests to proper stoichiometry of the subunits used for reconstitution.
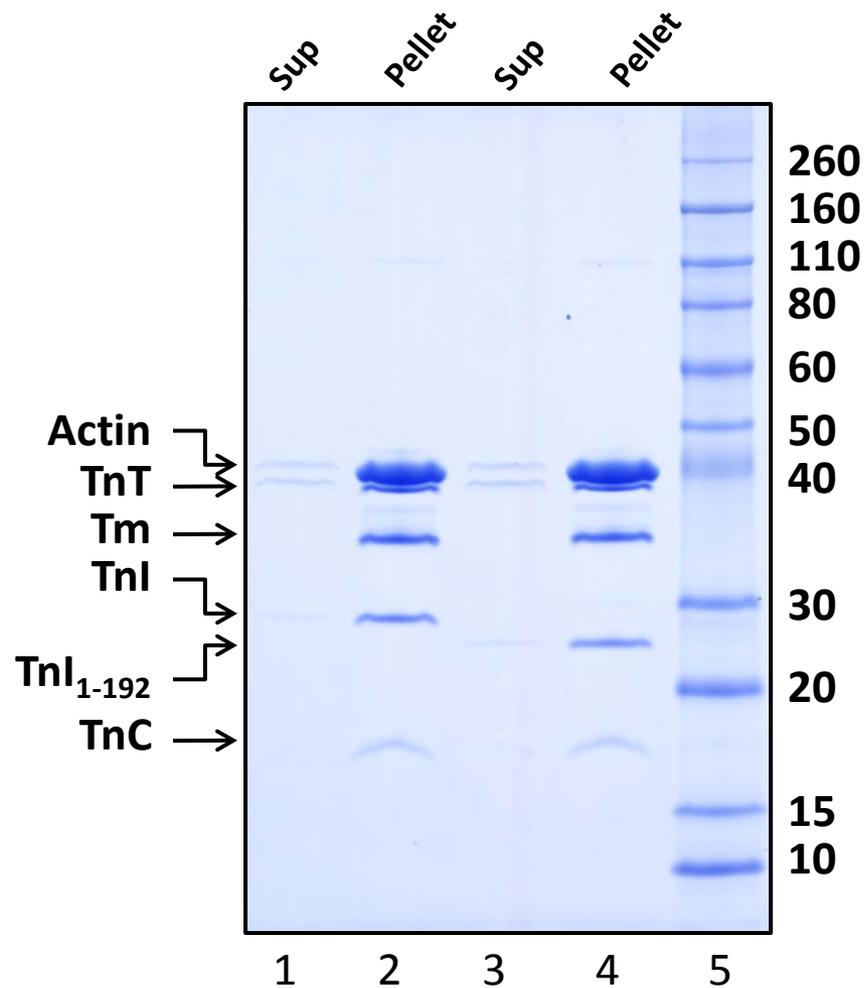
Online Figure II. Actin co-sedimentation control for thin filament reconstitution. F-actin (10 µM), tropomyosin (2.8µM) and troponin (2.8 µM) were incubated in EM buffer (see methods) for 20 min at room temperature prior to ultracentrifugation at 100 000 x g for 30 min. Supernatants were removed and pellets were redissolved in 1x SDS Laemmli sample buffer. Supernatants and pellets were subjected to 12% Tris-glycine SDS-PAGE. The gel was stained with Simply Blue (Invitrogen). Lanes 1 and 2 show wild-type troponin binding to actin-tropomyosin. Lanes 3 and 4 show binding of mutant troponin (containing TnI_{1-192}). Lane 5 contains Novex Sharp Pre-stained Protein Standards (Invitrogen). The degree of troponin binding under these conditions is similar in each case (lanes 2 and 4), indicative of proper thin filament assembly.
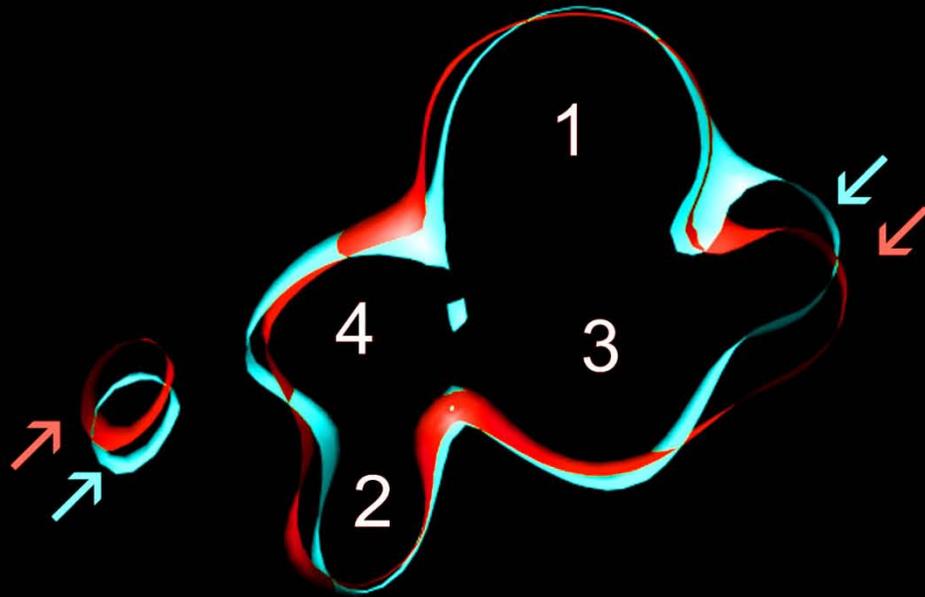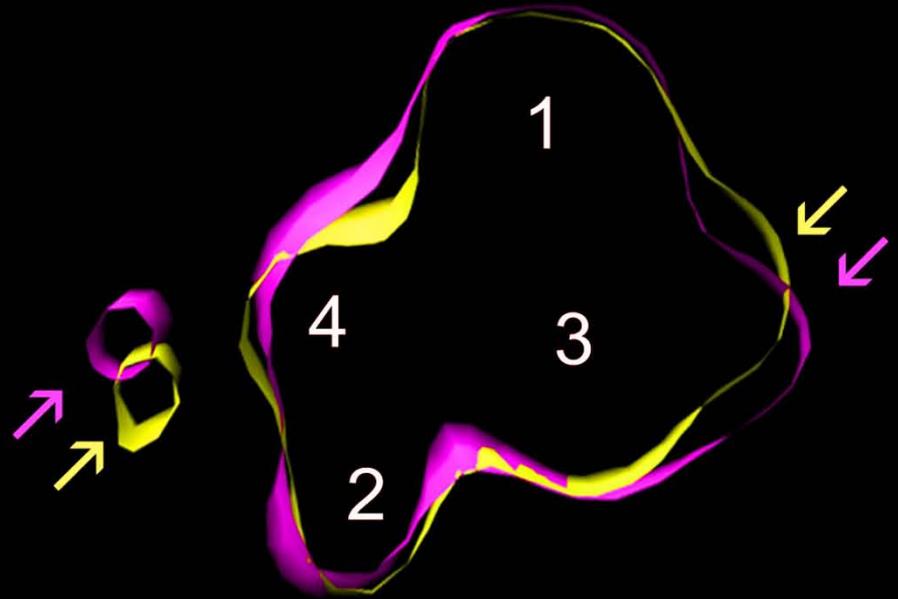
**Online Figure III. Altered thin filament structure is observed with two image reconstruction algorithms.** Panel A depicts superimposed cross-sections of actin-tropomyosin filaments, in Ca^{2+}, obtained by helical reconstruction using the Brandeis Helical Package. Tropomyosin regulated by mutant troponin (containing TnI_{1-192}) is shown in red. Tropomyosin regulated by wild-type troponin is shown in light blue. Panel B shows comparable superimposed cross-sections obtained by iterative helical real-space reconstruction (IHRSR) of filaments in the presence of Ca^{2+}. The position of mutant and wild-type regulated tropomyosin are shown in magenta and gold, respectively.
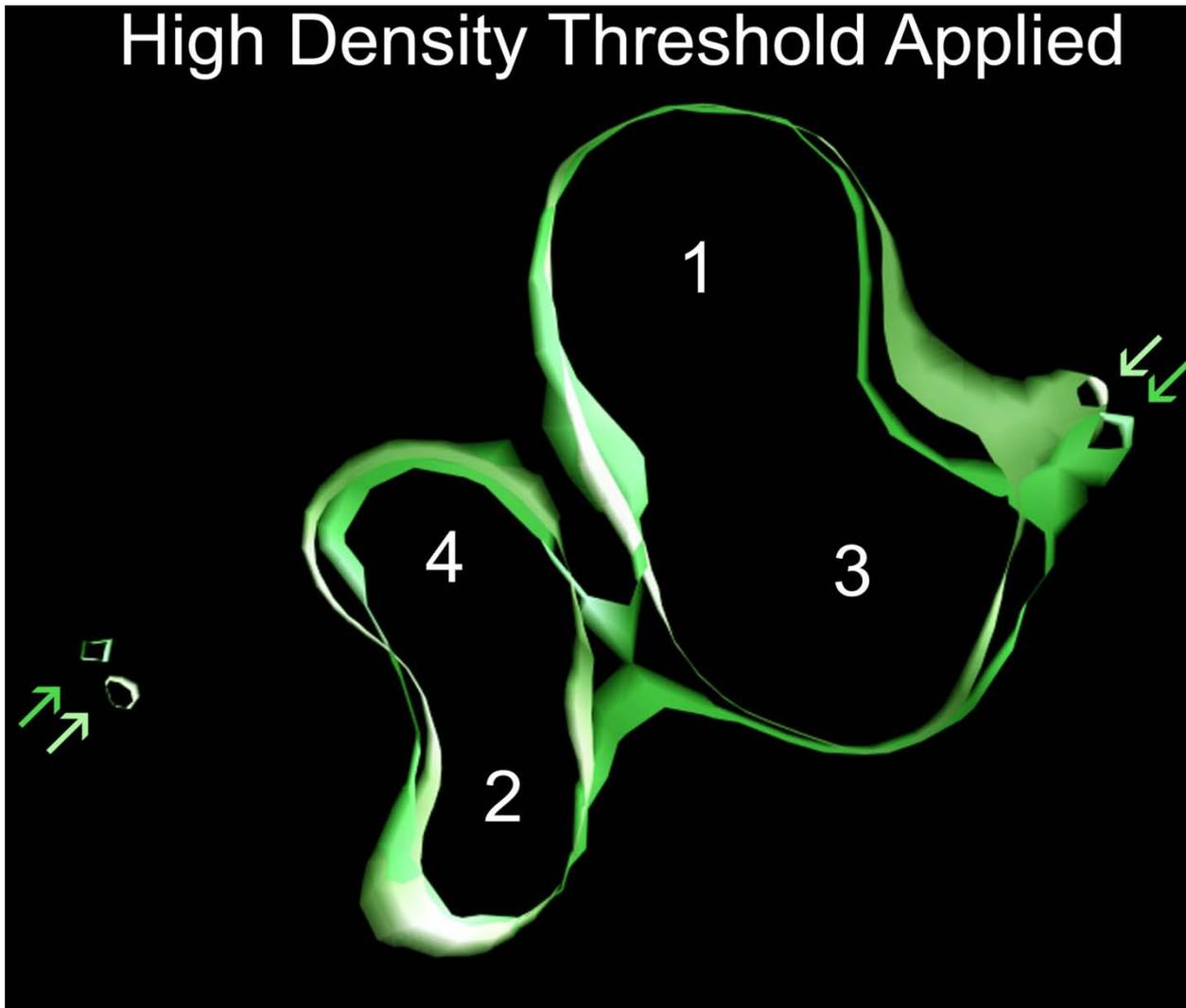
Online Figure IV. **High contour density cross-section of actin tropomyosin filaments regulated by wild-type and mutant Troponin (with TnI_{1-192}) in the presence of Ca^{2+}.** This view depicts the highest image densities (at 7 σ above the mean density) and therefore the most reliable data. Using the high threshold, the tropomyosin density reflects the average position of the central axis of the tropomyosin coiled-coil. The difference between the position of mutant Tn-controlled tropomyosin (dark green) and that of regular Tn-controlled tropomyosin (light green) is easily discernible.