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Structural Basis for the Activation of Muscle Contraction by Troponin and Tropomyosin

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Abstract

The molecular regulation of striated muscle contraction couples the binding and dissociation of Ca^{2+} on troponin to the movement of tropomyosin on actin filaments. In turn, this process exposes or blocks myosin binding sites on actin, thereby controlling myosin crossbridge dynamics and consequently muscle contraction. Using 3D-EM, we recently provided structural evidence that a C-terminal extension of TnI is anchored on actin at low Ca^{2+} and competes with tropomyosin for a common site to drive tropomyosin to the B-state location, a constrained, relaxing position on actin that inhibits myosin-crossbridge association. Here, we show that release of this constraint at high Ca^{2+} allows a second segment of troponin, probably representing parts of TnT or the troponin core domain, to promote tropomyosin movement on actin to the Ca^{2+}-induced C-state location. With tropomyosin stabilized in this position, myosin binding interactions can then begin. Tropomyosin appears to oscillate to a higher degree between respective B- and C-state positions on troponin-free filaments than on fully regulated filaments, suggesting that tropomyosin positioning in both states is troponin dependent. By biasing tropomyosin to either of these two positions, troponin appears to have two distinct structural functions; in relaxed muscles at low Ca^{2+}, troponin operates as an inhibitor, while in activated muscles at high Ca^{2+}, it acts as a promoter to initiate contraction.

Keywords

actin; troponin; tropomyosin; calcium; electron microscopy

Contraction in all muscles results from the relative sliding of thick and thin filaments. The process is driven by the myosin-crossbridge motors projecting from thick filaments and interacting cyclically with actin subunits on the thin filament molecular track. In skeletal and cardiac muscles, contraction is switched on and off by changes in sarcoplasmic free Ca^{2+}

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concentration and by the corresponding binding and dissociation of Ca^{2+} from the troponin complex, located on thin filaments. Crossbridge dynamics is a function of the myosin ATPase, which itself is activated by myosin-crossbridge binding to actin. Thus, any modulation of actin-activation would, in turn, serve to regulate contraction. Indeed, at low Ca^{2+}, actin-myosin interaction is inhibited because myosin binding sites on actin become inaccessible. It is generally recognized that this inhibition occurs when Ca^{2+}-free troponin impinges on elongated tropomyosin to then block myosin binding (reviewed in[1,2]). In contrast, the involvement of troponin in muscle activation is less well characterized. The crossbridge cycle may turn on simply because the troponin constraint is released at high Ca^{2+} and tropomyosin defaults to an unstrained, non-blocking position on actin. Alternatively, Ca^{2+}-saturated troponin may play a more dynamic role and facilitate regulatory switching by actively promoting tropomyosin movement away from the blocking position. We have investigated these possibilities using electron microscopy and 3D reconstruction of thin filaments, and report here on troponin's structural influence over tropomyosin at high Ca^{2+}.

Tropomyosin is a ~40 nm long "coiled-coil" α-helical protein, which lies along the long-pitch double helical array of actin monomers on thin filaments[3–5]. Tropomyosin molecules associate together in an end-to-end fashion[6] to form a continuous strand, with each tropomyosin spanning 7 successive actin molecules. This arrangement is possible because tropomyosin possesses a series of 7 quasi-repeating motifs designed to bind to neighboring actin monomers along filaments[7–9]. In turn, troponin, consisting of 3 subunits (TnT, TnI, and TnC), binds to tropomyosin at specific points along the tropomyosin molecule[10–13]; troponin complexes therefore assume the 40 nm periodicity of tropomyosin on thin filaments. Troponin is thought to have multiple, compartmentalized functions, with each subunit having a particular role in binding tropomyosin or Ca^{2+} or in inhibiting actomyosin ATPase[14]. TnC is well characterized and functions as the Ca^{2+} receptor. After binding Ca^{2+}, it neutralizes the inhibition of actomyosin ATPase imposed by TnI (the inhibitory subunit). TnT, a fairly long asymmetric molecule[15] (~19 nm), links the entire troponin complex to tropomyosin[14,16,17]. The N-terminal "tail" of TnT binds alongside tropomyosin on thin filaments, bridging the head-to-tail joint between adjacent tropomyosin molecules. However, the C-terminal part of TnT converges on TnC and TnI and interacts with them to form the core domain of troponin[18,19]. Other than providing a scaffold for TnI and TnC, its actions near or within the core domain are obscure. The view of TnT solely as a structural intermediary between troponin subunits and tropomyosin may be an oversimplification. In this regard, biochemical approaches have shown that in concert with tropomyosin and the rest of the troponin complex, TnT enhances actomyosin ATPase at high Ca^{2+}(2,[20,21]), suggesting that it and the troponin core domain play more than just a permissive role during activation. Thus, troponin subunits may have a dual role in thin filament regulation, with TnI being linked to inhibition at low Ca^{2+} and TnT and the rest of the complex linked to activation at high Ca^{2+}(22).

In the complete absence of other factors, tropomyosin is thought to be able to oscillate laterally over a narrow region of the flat surface of actin[23–28]. It is generally assumed, although not explicitly established, that this intrinsic ability to shift azimuthally around actin filaments at low energy cost is inherent to and necessary for the thin filament regulatory mechanism[23–25]. Presumably, tropomyosin location becomes biased towards specific regulatory positions on actin[25,29] in the presence of troponin and/or myosin, and depending on levels of Ca^{2+} binding to troponin and myosin binding to actin. At low Ca^{2+}, tropomyosin localizes over the outer domain of actin (on actin subdomains 1 and 2, covering myosin binding sites; the B-state position). At high Ca^{2+}, tropomyosin moves to actin's inner domain (to the edge of subdomains 3 and 4, exposing most but not all of the myosin binding site; the C-state position), and after myosin binding it moves further onto the inner domain (exposing the entire myosin binding site; the M-state position)[29]. Thus the Ca^{2+}-induced movement of tropomyosin is thought to

increase the probability of myosin binding, and the resulting myosin interaction leads to a further tropomyosin shift and full activation of the thin filament[29].

We recently showed that the C-terminal end of TnI (cTerm-TnI) drives tropomyosin to the B-state blocking position on the actin outer domain at low Ca^{2+}([30,31]). Here we have extended these studies to characterize how troponin influences tropomyosin in the presence of Ca^{2+}. Our results demonstrate that a troponin extension, likely to involve TnT and/or parts of the troponin core domain complex, promotes tropomyosin movement away from the blocking position and stabilizes it in the C-state position. Our work therefore supports the view that troponin exhibits dual function, inhibiting actin-myosin interaction at low Ca^{2+} and facilitating interaction at high Ca^{2+}. Our results suggest further that troponin transforms tropomyosin's fundamentally ambiguous position on striated muscle actin by dampening tropomyosin's oscillatory behavior, while actively promoting its movement to B- or C-state configurations.

3D reconstruction of Ca^{2+}-treated thin filaments

Thin filaments were reconstituted from F-actin, cardiac troponin and tropomyosin under conditions known to saturate the filaments with the regulatory proteins[25,26]. Filaments were then negatively stained[25,26]. EM images of the reconstituted thin filaments showed characteristic double-helical arrays of actin monomers, tropomyosin strands, and troponin densities repeating with a 40 nm periodicity (Fig. 1). The visually apparent, in-register binding of troponin on each helical strand of F-actin at 40 nm intervals has been directly quantified by analysis of class averaged 2D projections of these same filaments[32], corroborating that troponin-tropomyosin binds with the same structural arrangement and molar stoichiometry displayed by native thin filaments isolated directly from muscle. Troponin densities were absent in actin-tropomyosin controls (Fig. 1).

Helical reconstructions of Ca^{2+}-treated filaments showed actin subunits and densities that were attributable to tropomyosin, as previously observed (Fig. 2c, d). As expected, the longitudinally continuous tropomyosin strands were well defined and localized over the outer part of the inner domain of actin. This position is distinctly different from that assumed by tropomyosin on the actin outer domain in reconstructions of EGTA-treated filaments (Fig. 2e, f, *cf.*[25,26]). In an effort to identify material derived from troponin, the threshold density cutoff was reduced to 1.5 to 3.0 sigma standard deviation units above the mean density (normally >5.0 σ is used). Additional densities, not previously detected, were then evident in the maps of high Ca^{2+} filaments, but were absent at low Ca^{2+} at the same or considerably lower threshold density levels. The densities emerged from the center of actin subdomain 1 and traversed the face of the domain in a path that approached and then abutted the C-state tropomyosin (Fig. 2d); i.e. they bridged sites on actin normally occupied by B-state tropomyosin at low Ca^{2+}. As these densities were not seen in actin-tropomyosin controls (Fig. 2b), we attribute them to troponin. As they were also not seen in reconstructions of low Ca^{2+}-treated troponin-tropomyosin filaments (Fig. 2e), we ascribe them to a part of troponin that is visualized only in the Ca^{2+}-saturated conformation.

Densities specific to the high Ca^{2+} filaments are statistically significant

Helical reconstruction is an averaging technique that treats each successive actin along thin filaments with its associated proteins as equivalent units. Thus well-ordered troponin domains present only on every seventh actin along thin filaments will distribute as if they derived from every single actin and would be expected to appear so in reconstructions. The distribution process dilutes the troponin signal which becomes comparatively low in amplitude relative to actin and tropomyosin. In this case, the amplitude of each extra density observed on every actin in the reconstructions of Ca^{2+}-treated filaments at best may represent one seventh that of the original troponin mass. Nonetheless, the troponin densities observed in the reconstructions

were quite robust and displayed high statistical significance at greater than the 99% confidence level (Fig. 3).

The position of striated muscle tropomyosin is indeterminate in the absence of troponin

In order to assess the impact of troponin on the structure of thin filaments further, we examined the structural interactions of tropomyosin on troponin-free actin, particularly since biochemical studies suggest that cardiac tropomyosin binding is weakened when troponin is absent[20]. Helical reconstruction of 500 to 1000 nm stretches of actin-tropomyosin (free of troponin) revealed that the mean location of tropomyosin was midway between B- and C-state configurations, possibly reflecting an assemblage of filaments with variable tropomyosin position (Fig. 4a, b). Inspection of reconstructions of the individual filaments contributing to the average map confirmed this possibility. About half the filament reconstructions analyzed could be clearly identified by eye (as well as by fitting protocols) as either being distinctly B- or C-state examples of tropomyosin association. Dividing these two filament classes into separate data sets and averaging each set independently yielded two distinctly different reconstructions where C- and B-state modes were each indistinguishable from those displayed by troponin-tropomyosin filaments at corresponding high or low Ca^{2+} (Fig. 4c, d; *cf.*[25–27]). This demonstrates that approximately half the filaments possessed long stretches that on average were in one or another well-defined regulatory state. Hence, a mixed population of B- and C-state conformations does coexist in these cardiac filament preparations, in part explaining the diffuse localization of cardiac muscle tropomyosin in averaged data[26]. The more ambiguous positioning of tropomyosin, seemingly in between B- and C-state positions on remainder of the filaments, may reflect a disconnect between the tropomyosin position on the two opposing actin helical strands and/or a disordering of tropomyosin position resulting from a dynamic fluctuation between states.

Previous single particle reconstructions of 40 nm long segments of actin – cardiac tropomyosin filaments suggested a similar positional ambiguity for tropomyosin[26]. Attempts were made here to classify segments from the above set of filaments showing poorly localized tropomyosin. Cross-correlation against B-, C- or intermediate state models did not succeed in sorting the data into demonstrably separate categories and reconstructions generated showed no uniquely distinct tropomyosin positional states. Thus, single particle methods and helical analysis both identified a set of filaments characterized by an apparent local disordering of cardiac tropomyosin on actin in the absence of troponin.

By contrast, marked variability in tropomyosin position has not been observed for preparations of filaments composed of cardiac troponin-tropomyosin; without exception, helical reconstruction showed that on average tropomyosin along all single filaments examined at low Ca^{2+} could be readily classified as belonging to the B-state mode and analogously tropomyosin along all filaments in high Ca^{2+} to the C-state configuration. Similarly, single particle analysis of short segments of these filaments indicated that while localized oscillation between positional modes appears to occur, tropomyosin occupied characteristic regulatory state positions along roughly 80 percent of filament lengths[26].

Different tropomyosin isoforms interact differently on F-actin

The precision with which tropomyosin is positioned is not simply a matter of troponin being present or absent, but rather also depends on the isoform of tropomyosin examined. For example, polymorphic positioning of tropomyosin was not observed here or previously[25,33] on thin filaments containing smooth muscle tropomyosin (without troponin), where a single mode of binding interaction was detected. In fact, the variance associated with densities

connecting cardiac tropomyosin to actin is higher than that for the smooth muscle tropomyosin connection, particularly near subdomain 1 (Fig. 5).

The location of tropomyosin on actin is defined by weak electrostatic contacts[8]. In turn, both the collective strength of these weak interactions and the stiffness of the tropomyosin strand on actin will influence tropomyosin's responsiveness to mechanical or chemical perturbations. Maytum *et al*.[28] (2008) likened the behavior of different tropomyosin isoforms to variably vibrating guitar strings being held taut or not, where the additional displacement of the guitar strings by a pick, would be the counterpart of troponin or other proteins disturbing preset average equilibrium positions. Thus in a case in which tropomyosin is well localized on actin, for example in smooth muscle filaments[25], the tropomyosin in effect is taut and well positioned by electrostatics. Non-muscle tropomyosins, also well localized on actin, could be similarly described[25,28,34]. A high degree of chemomechanical specificity may be required in these cases of troponin-free filaments, in order to maximally stabilize tropomyosin on actin. In contrast, we have shown here that the position of cardiac striated muscle tropomyosin on actin is less well defined, as if the isoform were held more loosely on actin, at least in the absence of troponin (*cf*.21). Given this extra degree of plasticity, limited as it might be to yielding azimuthal oscillations amounting to between 15 Å and 25 Å(27), the behavior of tropomyosin on actin filaments may be more easily fine-tuned by troponin.

We have previously demonstrated that the mechanochemical equilibrium balance of tropomyosin that determines its positional state is easily perturbed by small changes in electrostatic interactions between actin and tropomyosin[25]. We also showed that the content of αα-, ββ-, or αβ-tropomyosin isoforms may strongly influence the equilibrium balance of tropomyosin on actin[25]. Less well characterized variation in the end-to-end contacts between successive tropomyosins along tropomyosin and their degree of phosphorylation may also influence tropomyosin interactions. Whether such subtleties affect tropomyosin positioning *in vivo* and are physiologically meaningful is not known.

Troponin promotes tropomyosin movement on actin

We have recently indicated that at low Ca^{2+}, a C-terminal domain of TnI (cTerm-TnI) binds to actin, displaces tropomyosin from the C-state and stabilizes it in the B-state position[31]. This would account for the unambiguous position of tropomyosin in low Ca^{2+}-treated thin filaments. We suggest here that at high Ca^{2+}, C-terminal domains of TnT or parts of the troponin core domain complex move over the B-state binding sites, promote tropomyosin movement and then stabilize tropomyosin in the C-state, thus accounting for tropomyosin's positional fidelity in Ca^{2+}-treated filaments. Hence the presence of troponin appears to diminish the positional promiscuity of cardiac tropomyosin at both low and high Ca^{2+}. We also previously indicated that the N-terminal TnT tail domain stabilizes tropomyosin in a B-state position[35]. Thus the TnT tail alone cannot promote the transition of tropomyosin to the C-state at high Ca^{2+}. Clearly additional parts of troponin, whose domain structures are yet to be identified, are required for inducing tropomyosin movement to the C-state.

We have suggested that the blocked, B-state is brought about and muscle relaxation ensues at low Ca^{2+} because tropomyosin is wedged in an inhibitory position between the cTerm-TnI regulatory domain on one side and TnT and the troponin core domain on the other[31]. This configuration may compress the C-terminal end of TnT against the troponin core domain complex. At low Ca^{2+}, the core domain may also become constricted and thus more limited in its binding to targets on actin. It follows that once cTerm-TnI dissociates from actin at high Ca^{2+}, such strain will be released, thrusting tropomyosin toward the C-state position (Fig. 6). The extra density seen in high Ca^{2+} reconstructions is likely to represent an obliquely oriented segment of troponin that associates closely with actin and drives this process. Comparable

densities are not resolved from tropomyosin in low Ca^{2+}-reconstructions, possibly because they may be compressed linearly against the elongated protein or become disordered by a radial displacement from actin.

Our results support the hypothesis that troponin is intimately involved in both the inhibition and the activation of muscle contraction. We propose that the counterpunching action of the mobile parts of troponin determines the positions of tropomyosin at low and high Ca^{2+}, thereby regulating the thin filament. We argue further that the inherent positional ambiguity of striated muscle tropomyosin on actin is an adaptation attuned to troponin function. While we have not defined how the entire troponin complex reconfigures during muscle activation and inhibition, our results indicate that key structural domains of troponin bring about striking steric effects that control actin-activation of myosin ATPase and consequently contraction.

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Abbreviations

TnI
: the inhibitory subunit of troponin

cTerm-TnI
: the C-terminal 80 amino acid domain of TnI that links to actin at low Ca^{2+}

TnC
: the Ca^{2+}-sensor of troponin that releases inhibition

TnT
: the element linking troponin to tropomyosin

B-state
: the blocked state

C-state
: the closed state

M-state
: the open state

EM
: electron microscopy

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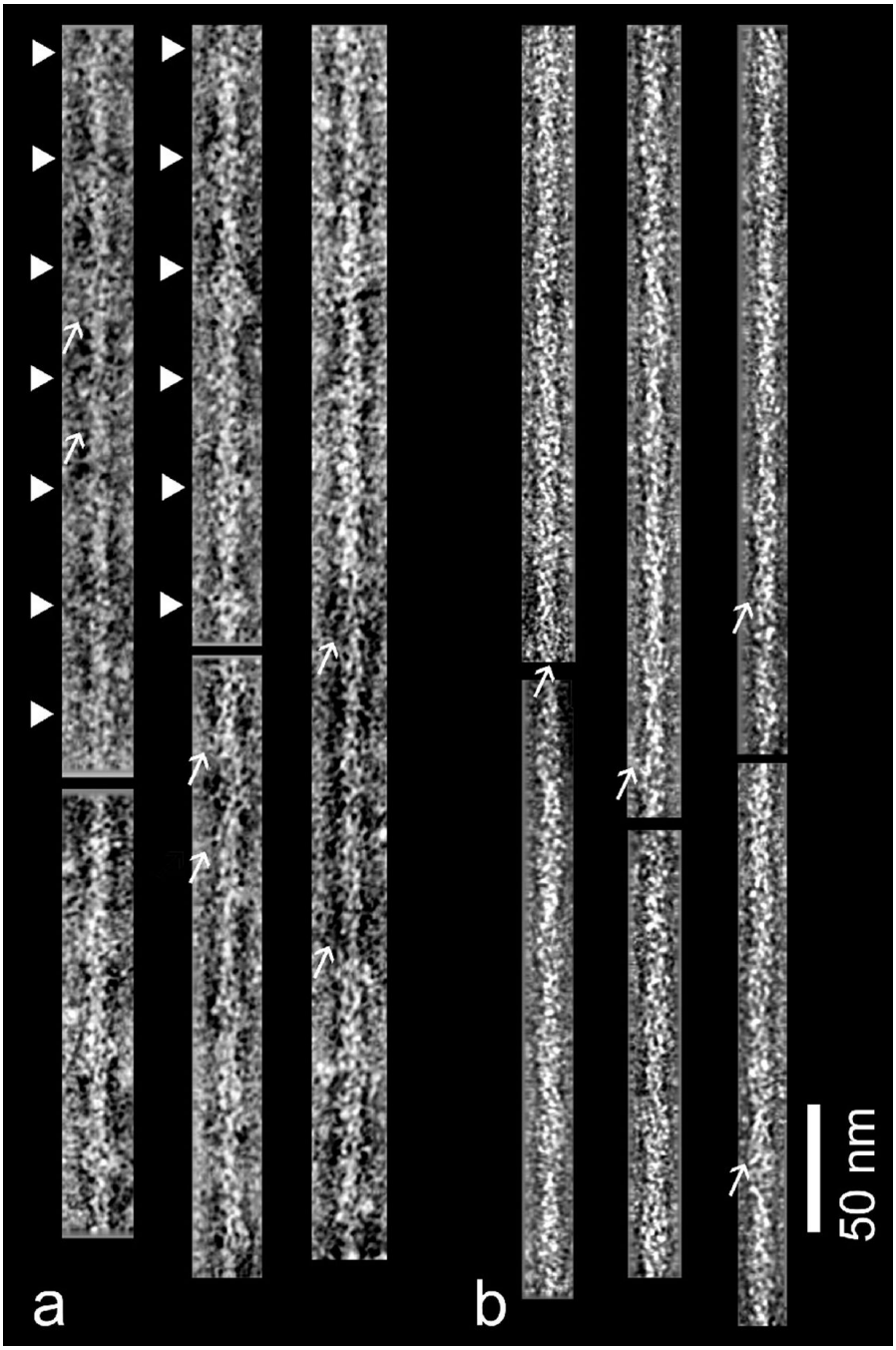

Fig. 1.

Electron micrographs of negatively stained filaments. (a) F-actin-troponin-tropomyosin, (b) F-actin-tropomyosin (b). Note that the presence of troponin (arrowheads), distributed with a 40 nm periodicity in (a) increases the maximum width of thin filaments; also note obliquely oriented tropomyosin strands in (a) and (b) (arrows). Filaments are shown with their pointed ends facing up; polarity was determined by alignment tools in reference[36]. Scale bar = 50 nm. *Preparation of proteins*: F-actin, bovine cardiac troponin and tropomyosin were purified as previously[37]. SDS-PAGE indicated that the cardiac tropomyosin consisted of greater than 90% aa-isoform and the remainder αβ-isoform; it was presumed to be 20 to 30 percent phosphorylated, as is the tropomyosin in adult cardiac tissue of other mammals[38,39]. The

cardiac troponin as isolated was monophosphorylated and not biphosphorylated as occurs after adrenergic stimulation.

EM and image processing: We have previously shown that a moderate excess of troponin-tropomyosin relative to actin is needed to saturate filaments at the low protein actin concentrations required for successful EM work[25,26]. Filaments were thus prepared by mixing a two-fold molar excess of tropomyosin or troponin–tropomyosin (40 μM) with F-actin (20 μM) to optimize binding, in 100mM NaCl, 3mM $MgCl_2$, 1mM NaN_3, 0.2mM EGTA, 1mM dithiothreitol, 5mM sodium phosphate/5mM Pipes buffer (pH 7.0) at 25°C[25,26]. The mixture was diluted 20-fold, applied to carbon-coated grids and negatively stained with 1% uranyl acetate[25,26]. Filaments from two preparations of F-actin-troponin-tropomyosin and three preparations of F-actin-tropomyosin were recorded and processed in these studies. EM was done on a Philips CM120 EM at a magnification of X60,000 under low dose conditions (~12 e^-/Å). Helical reconstruction[36] was performed by standard methods as previously[25,26]. Helical reconstruction was particularly well suited to identify the relatively low density troponin signal observed, as the method takes advantage of layer line indexing information to filter out noise, which otherwise would have obscured the troponin density observed. In addition, robust statistical programs accompany the helical reconstruction package, allowing the significance of weak densities to be assessed by Student's t-test methodology[40,41]. Real space reconstruction methods[42], treating filament segments as single particles, were also used to confirm results obtained by helical reconstruction and to attempt to sort filament segments according to their tropomyosin positional modes, as previously[26].

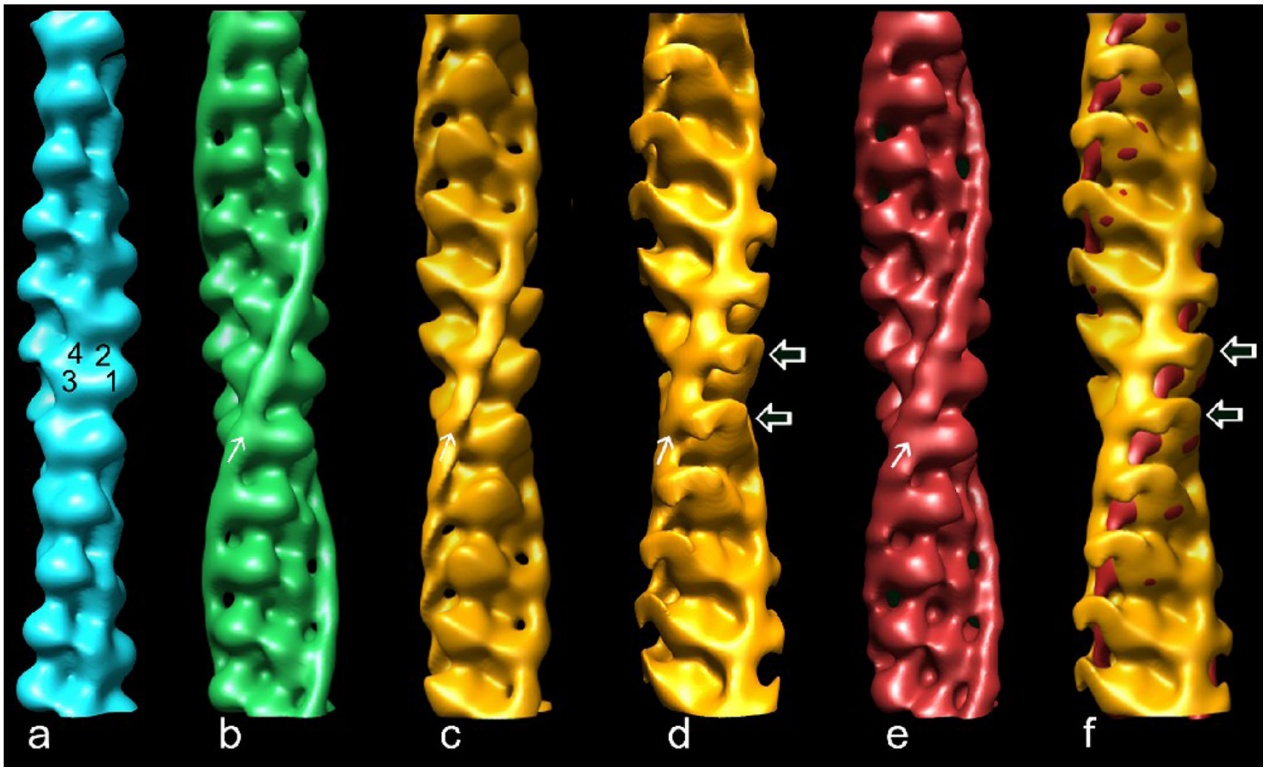

Fig. 2.

Surface views of thin filament reconstructions showing the position of the high Ca^{2+} troponin extension. Reconstructions of: (a) F-actin (cyan, subdomains numbered on one actin subunit) and (b) F-actin-tropomyosin (green) controls, (c, d, e) F-actin-troponin-tropomyosin: (c, d) high Ca^{2+} (gold), (e) low Ca^{2+} (maroon). Note tropomyosin positions indicated by arrows. High Ca^{2+} reconstructions displayed at two density cutoff thresholds ((c) 5 σ, (d) 2.5 σ greater than the mean density). Note the presence of extra densities in (d) emerging from the centers of subdomain 1, which traverse the face of the domain to abut C-state tropomyosin (open arrowheads). Note also that these densities are absent from F-actin-tropomyosin controls (b) and low Ca^{2+} F-actin-troponin-tropomyosin (e) (each displayed at 2.5 σ). (f) Reconstructions in (d) and (e) superimposed for comparison. Note that the respective tropomyosin strands occupy different positions, with tropomyosin from the high and low Ca^{2+} maps in C- and B-states. Also note that Ca^{2+} specific extra density attributed to troponin (open arrows) crosses the B-state tropomyosin position. All of the reconstructions of thin filaments obtained were aligned to each other and are shown with filament pointed ends facing up. Each map shows densities with amplitudes that are at a threshold of 2.5 sigma over the mean density (with the exception of the map in (c)).

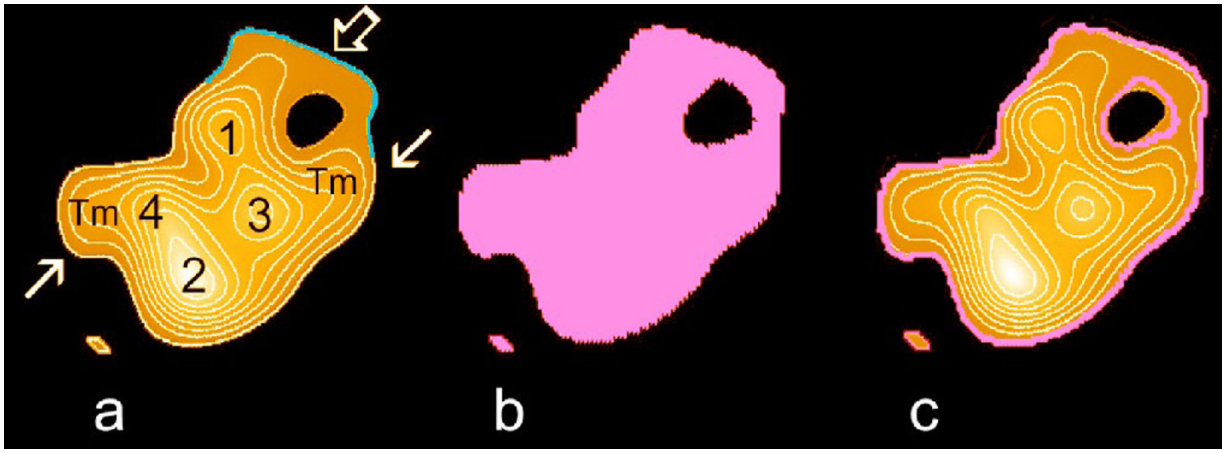

Fig. 3.

Statistical significance of densities contributing to reconstructions of Ca^{2+}-treated F-actin-troponin-tropomyosin. (a) z-section of reconstruction shown in figure 2d (gold). Note actin subdomains (numbered) and tropomyosin positions (indicated by arrows and labeled Tm) on the inner domain of actin over subdomains 3 and 4. Note also the extra density emerging from subdomain 1 and connecting to tropomyosin (indicated by open arrowhead; the outer edge of the extra density is highlighted in cyan). (b) Densities associated with the z-section in (a) that have a confidence level greater than 99 % (pink). (c) The z-section in (a) superimposed on the statistical map in (b), showing that all densities in (a), including the extra density attributable to troponin, have a high level of statistical significance.

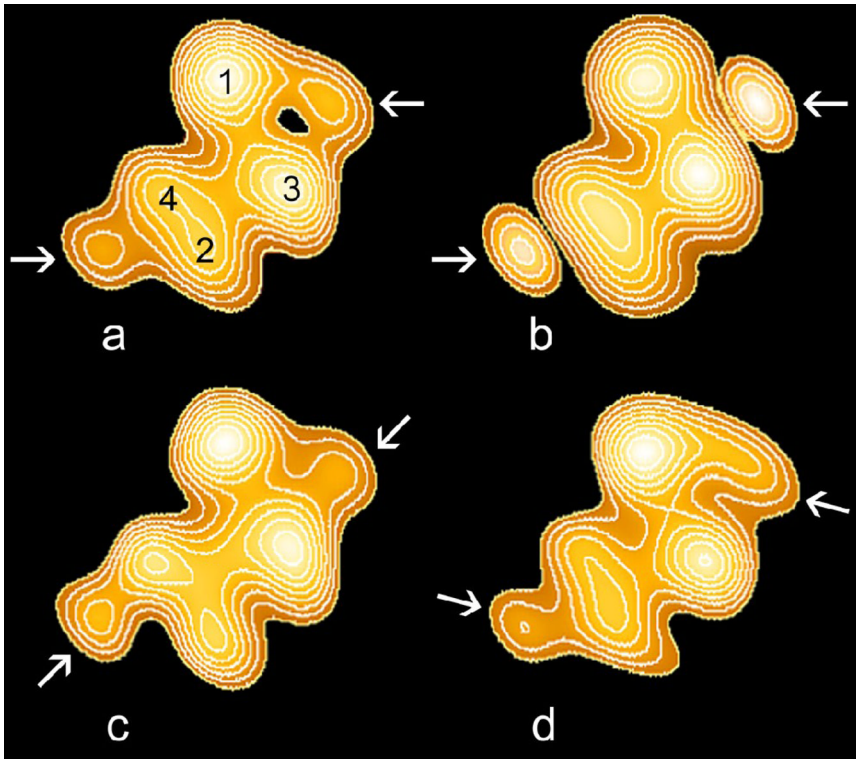

Fig. 4.

Reconstructions of troponin-free actin – tropomyosin. (a) z-section of reconstruction shown in figure 2b of cardiac tropomyosin bound to F-actin (no troponin). Note that tropomyosin (arrows) occupies a position mid-way between the inner and outer domains of actin, i.e. between actin subdomains 1 and 3 on one side of the filament and between subdomains 2 and 4 on the other side. (b) Tropomyosin densities determined by subtracting densities in the F-actin reconstruction from those of the map in (a) and then superimposing them on the corresponding z-section of F-actin in order to highlight the position of tropomyosin. (c, d) the filaments contributing to the average density map in (a) were sorted according to their best alignment to B- or C-state models. (c) Reconstruction of the sorted filaments that were in the C-state mode; note tropomyosin densities on the actin inner domain (i.e. associated with actin subdomains 3 and 4). (d) Reconstruction of the sorted filaments that were in the B-state mode, with tropomyosin densities on the outer domain (i.e. associated with subdomains 1 and 2). Comparable results were obtained when the skeletal muscle isoform of tropomyosin was examined on troponin-free F-actin, *viz.* an average position midway between B- and C-state, but here individual filament reconstructions showed a slightly greater bias for the C-state mode than with a found will cardiac tropomyosin.

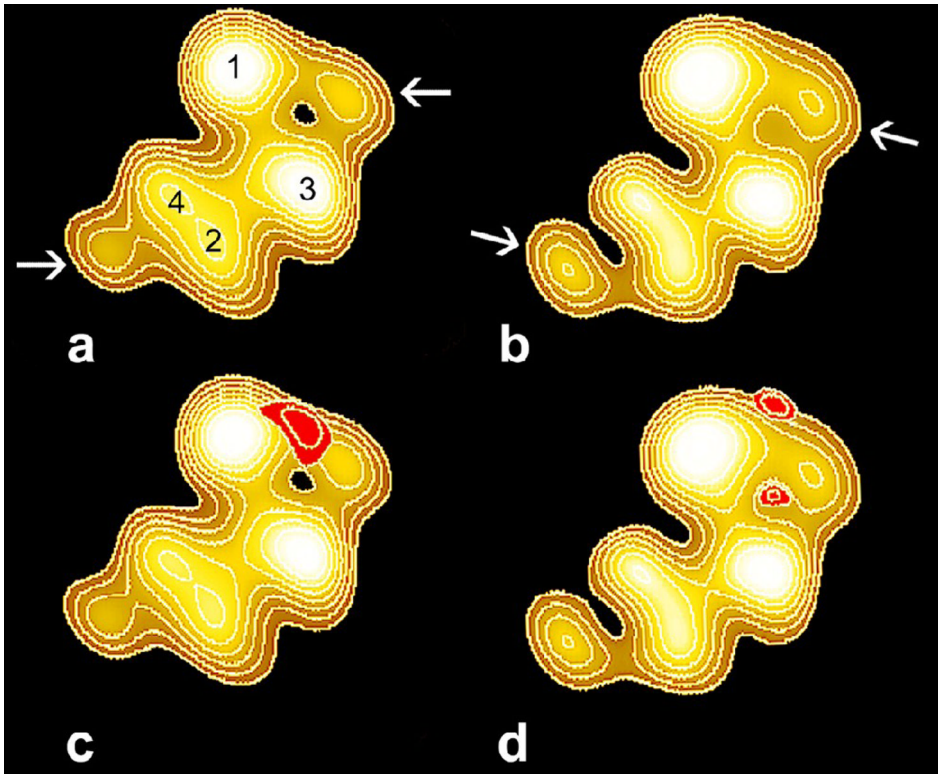

Fig. 5.

Comparison of cardiac and smooth muscle tropomyosin positions on actin. z-sections of reconstructions of F-actin combined with cardiac muscle tropomyosin (a) and aortic smooth muscle tropomyosin (b). Note the closer association of the smooth muscle tropomyosin density with the actin outer domain (compare white arrows in (a) and (b)). (c) Maps plotting the variance associated with the contributing density points[40,41] in the reconstruction in (a) show that a high variance (red) occurs over the site where tropomyosin normally makes contact with actin in the B-state. (d) In contrast, corresponding variance maps of actin – smooth muscle tropomyosin reconstructions show low variance at this site and only comparably high variance (red) at the very edges of the tropomyosin density.

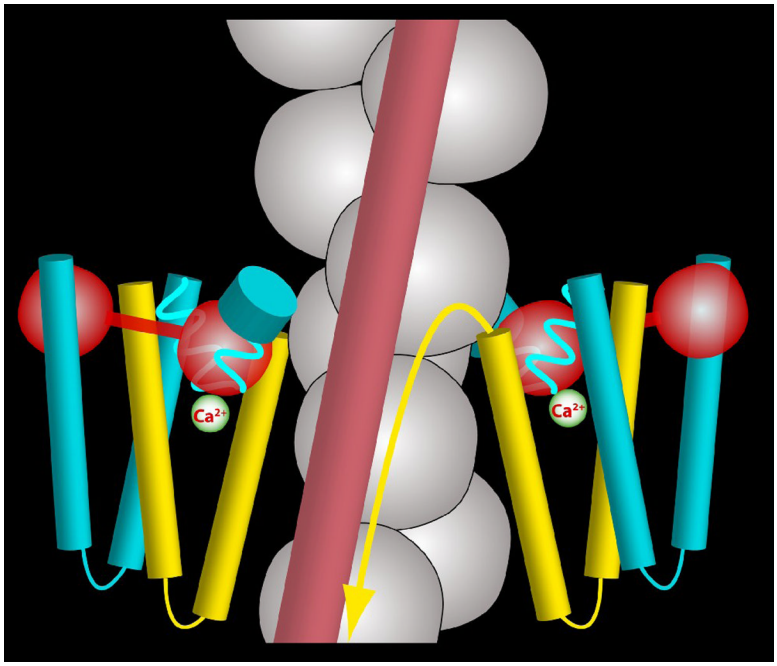

Fig. 6.

Cartoon representation of the organization of the thin filament at high Ca^{2+}. As previously[31], the troponin core domain complexes on either side of F-actin are depicted as W-shaped TnIT structures supporting dumbbell-shaped TnC[18,19]; actin, grey; tropomyosin, salmon; TnI, cyan; TnC, red; TnT, yellow. At high Ca^{2+}, cTerm-TnI dissociates from actin and binds to the N-terminal lobe of TnC. We argue that this releases chemomechanical constraints on troponin and tropomyosin. Troponin then unfurls, here depicted for simplicity as TnT propelling tropomyosin to the C-state position. Other related depictions of troponin on thin filaments can be found in references[18,19,43].