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The Missing Links within Troponin

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Abstract

The cardiac contraction-relaxation cycle is controlled by a sophisticated set of machinery. Of particular interest, is the revelation that allosteric networks transmit effects of binding at one site to influence troponin complex dynamics and structural-mediated signaling in often distal, functional sites in the myofilament. Our recent observations provide compelling evidence that allostery can explain the function of large-scale macromolecular events. Here we elaborate on our recent findings of interdomain communication within troponin C, using cutting-edge structural biology approaches, and highlight the importance of unveiling the unknown, distant communication networks within this system to obtain more comprehensive knowledge of how allostery impacts cardiac physiology and disease.

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Written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Keywords

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Current understanding of troponin regulation of thin filament function

Regulation of the thin filament depends on the well-coordinated function of the troponin (Tn) protein complex. Striated muscle contraction relies on the interaction between the two filamentous systems - the thick filament is comprised of myosin and its auxiliary proteins, and the thin filament (TF) composed of filamentous actin (F-actin), tropomyosin (Tm) along with the Tn complex. In the heart, the Tn complex, a trimer comprised of troponin C (cTnC), troponin T (cTnT), and troponin I (cTnI), is situated at regular intervals along the TF and is responsible for Ca^{2+} -dependent positioning of cable-like Tm upon the surface of F-actin (Fig. 1). TF activation requires coordinated movement of Tm, which normally disallows formation of cross-bridges between thick and TFs. When intracellular Ca^{2+} levels saturate the low-affinity regulatory cTnC Ca^{2+} binding Site II (Fig. 1), its interactions with cTnI are reinforced. This induces changes in cTn that translocate Tm away from the myosin-binding sites on F-actin [1, 2], allowing the formation of force-generating cross-bridges between actin and myosin [3]. Cycling of intracellular free Ca^{2+} levels between 10^{-7}M and 10^{-5}M in cardiac muscle control the contractile and relaxation phases [4, 5]. Therefore, perturbations in cTnC Ca^{2+} binding affinity can modify TF function [6, 7]. Furthermore, one remarkable feature of the cTn is its dynamic flexibility [8-11], which in part has thwarted attempts to obtain an accurate and complete view of the entire complex structure.

Our current understanding of Tn regulation has been derived from studies conducted since the pioneering discoveries of Ebashi [12]. In 1995 Farah and Reinach outlined compelling new technologies destined to yield insights on Tn subunit interactions including: bacterial expression of Tn mutants, cross-linking and fluorescence techniques, use of synthetic, recombinant, and Tn fragments in nuclear magnetic resonance (NMR) and X-ray crystallographic studies [1]. This review expands on these advances in both technology and the understanding of factors regulating TF and muscle function. Since 1995, numerous studies have uncovered major TF segments involved in its regulatory activity. While these contributions have yielded an abundance of knowledge of functional regions within the TF, our current knowledge of protein structure, which ultimately dictates its function, is still lacking. To date, a crystal structure is available of the cTn core domain [10] and another of skeletal troponin (sTn) [11], however still little is known about the actionable portions of the complex within the context of the TF.

The current model of muscle regulation is that Tm limits myosin interactions with F-actin during relaxing conditions. Whereas at activating conditions (high- Ca^{2+}) Tm translocation only partially exposes F-actin myosin binding sites. Therefore, binding of rigor-state myosin is required to fully activate the TF. Our most comprehensive knowledge of Tn-Tm structure has been derived from cryoelectron microscopy (cryo-EM) reconstruction [13]. More mechanistic insights regarding the interactions of F-actin, myosin, and TF components are necessary at near-atomic resolution. The first subnanometer-resolution structure of the actin-

Tm-myosin complex in the rigor (nucleotide-free) state, provides structural evidence for direct myosin and Tm interactions [14]. Revelations such as this redefine our view of the interactions between thick and TF. The previously unknown tight interactions between the three proteins (F-actin, myosin, and Tm), may in part explain the severe effects of α -actin and β -myosin HCM-linked mutations.

While high resolution NMR and X-ray crystal structures exist for the Tn complex, none are of the complete cTn and to date no high resolution structures exist of the cTn within the TF context. Single particle reconstruction of negatively stained cardiac TF showed the densities of F-actin, Tm, and Tn at a resolution of 25 Å [15]. Overall, the density of cTn within the filament closely resembled the shape defined in Tn crystallographic structures, therefore it was possible to determine the polarity of Tn. Absent in the reconstruction, however, was sufficient resolution to distinguish changes in rotation of the TnC N-lobe within the core domain or pinpoint residue contacts existing between Tn and F-actin or Tm. Importantly, this study did confirm spectroscopic experiments assessing TF structure [16], indicating in the blocked state Tm is wedged between two Tn complexes, on one side by the TnI C-terminal domain (and TnT) and on the other side by the Tn core domain, suggesting functional interaction between two sides of the TF. During muscle activation, structural inhibition by Tm is reversed upon Ca^{2+} binding to the TnC N-lobe. The TnI mobile domain binds the TnC N-lobe with less affinity for F-actin, favoring Tm release from the blocked position, subsequently allowing cross-bridge cycling through interactions between myosin and F-actin [17, 18]. A single particle cryo-EM study by Risi et al., applied helical reconstruction of frozen hydrated native cardiac TF in both relaxing and activating conditions [19]. The study revealed that upon Ca^{2+} activation, Tm exhibits azimuthal movement on the surface of native cardiac TF. Therefore, either under relaxing or activating conditions, Tm exists between three different structural positions on the TF. These findings allow more precise structural interpretation of the three state model and understanding of the basis of enhanced activation of cardiac compared to skeletal muscle [19].

Revelations from current troponin structures

The lack of information underlying the plethora of dynamic events within macromolecular complexes, represents one of the complications in developing a clear understanding of systems behavior. Thus, efforts from different structural techniques aim to surmount the current pitfalls while we attempt to explain mechanistic insights. Concerning the structural changes triggered by the binding of Ca^{2+} to the Tn complex within the TF, much contribution has been made by NMR spectroscopy, X-ray crystallography, cryo-EM, and molecular dynamics simulations. This section summarizes some achievements and limitations of these methodologies to understand cTn as a dynamic structure with an essential role in muscle physiology and cardiac disease.

NMR spectroscopy is a powerful tool to determine the three-dimensional (3D) structure of macromolecules in solution and for dynamic measurements at different timescales. Sykes and coauthors provided a valuable contribution by using solution NMR to solve the 3D structure of the cTnC regulatory domain [20]. Using this approach, the authors identified a distinct Ca^{2+} -induced conformation within cTnC to that observed in the skeletal isoform

[21], thereby providing the mechanistic basis to explain the dissimilarities between cardiac and skeletal contraction. Solid state NMR offers advantages in resolving structures of filamentous proteins such as the cTF. However, it requires a prohibitive amount of sample and poses challenges in determining helical parameters. This makes this structural method not as straightforward as the cryo-EM iterative helical real space reconstruction method [22].

It is worth mentioning the unique advantage that solution NMR possesses to assess the dynamic status of cTnC and its binding partners. Our group has been studying cardiomyopathy-related mutants using dynamic NMR measurements to understand how both cTnC domains communicate with each other and transmit the contraction signal to other partners within the Tn complex and the TF [23, 24]. NMR demands laborious isotope labeling and is limited by molecular size, a constraint that has hampered the generation of 3D structures of the whole Tn complex. Information about interaction sites within this complex were only feasible by NMR when cTnI was cut into small peptides and systematically titrated against TnC domains [25-27]. Limitations of working with the aforementioned approach involves the sensitivity of NMR chemical shifts to subtle changes in solution and the impossibility of revealing the hierarchy of interactions within the Tn multi-binding complex.

The problem of capturing multiple-binding sites within the cTn complex was partially solved using X-ray crystallography [10]. Although incredibly helpful in the last 60 years, crystallography requires an ordered crystallized macromolecule, which is sometimes difficult to obtain. Flexible and disordered segments plague crystallization trials and are not observable in electron density maps. Rational crystallography structures very often exclude flexible segments as the case for the N- and C-terminal regions of cTnI and cTnT of Takeda's troponin model [10]. To overcome the lack of information regarding flexible segments and linkers, the use of low resolution approaches such as the ensemble optimization method from small-angle X-ray scattering may provide some sources of information, but naturally imposes limitations on interpreted results [28].

The seminal discoveries that an amorphous solid glass state is formed when water is frozen extremely quickly, together with the development of semiconductor chips to directly detect electrons has poised cryo-EM as the current state-of-the-art technique for solving near-atomic density maps of large macromolecular assemblies. Negative staining and cryo-EM reconstructions assisted in defining Tn organization within cardiac TFs [15] and expanded our understanding of allosteric propagation within the TF [19]. The positions of the Tn complex along TFs naturally limits the reconstruction of near-atomic density maps of the Tn-Tm-actin complex as many different views of Tn along F-actin ultimately blurs Tn resolution by helical reconstruction methods.

Molecular dynamic (MD) simulation is another useful tool for elucidating the dynamics of Ca^{2+} -induced conformation changes and the allosteric propagation within cardiac TFs. Although the trajectories of biologically relevant events may be computationally consuming, MD simulation has been successfully used to capture the details of the cTnI switch peptide binding to the regulatory domain of cTnC [29, 30]. By using a fully atomistic cardiac TF model and computational methods, the allosteric transmission within the cTn complex has

been observed as changes induced by a cTnT mutant propagated via cTnI to cause functional changes to cTnC [31].

Interdomain communication within troponin C

Macromolecules require a delicate tuning between active and inactive conformations and the equilibrium shift toward either state depends on overcoming energy barriers and formation of transient switchable forms [32]. In the Tn complex, theoretical underpinnings have correlated TnC Ca^{2+} -binding as the trigger instigating muscle contraction. In fact, a sweeping view can be derived through the understanding that the Tn complex is an extensive allosteric network [33]. The classical view of allostery presents a conformational switch between two states, for example, active or inactive, and is regulated at a distance by small effectors or protein binding. Since the 1960's, understanding allostery from the viewpoint of its influence on protein dynamics has attracted tremendous research efforts [34, 35]. Up to now, there are three models that have been proposed to explain such long-range cooperativity that commonly occurs in essential biomolecular machines; including the “induced-fit” model, the “population-shift” (conformation selection) model, and the recent “dynamic allostery” model [36]. Among these proposals, the “induced-fit” model and the “population-shift” model indeed respectively correspond to the “KNF” [37] and the “MWC” [38] kinetics models that were originally proposed to assist understanding of the first observation of cooperativity in hemoglobin absorption of oxygen molecules. In fact, biomolecules exist in a continuum of closely related conformational states called ensemble. Therefore, allostery is the response of any perturbation that changes the energy landscape of an ensemble and leads to different population distribution. Moreover, allostery is a universal phenomenon that can be related to both structural and dynamic events [36]. A more enlightened view relies on the understanding of the thermodynamic, structural and dynamic elements required to reach different conformational ensembles and functional states. One example of a structural element is the presence of conserved motifs existing in allosteric networks of macromolecules [33, 39].

The evolution of TnC sequencing has revealed conserved motifs (Fig. 2a and b) [40]. The atomic model of cTnC reveals a dumbbell-shaped structure comprising two Ca^{2+} binding domains connected by a flexible linker. It implies the predominance of low transition barriers to populate the active conformational state and allosteric events between both domains [41]. For instance, ^{15}N relaxation measurements revealed nanosecond timescale motions in the linker region, allowing a wide range of conformational heterogeneity [42]. Paramagnetic relaxation enhancement (PRE) experiments demonstrated approximation between the two domains, suggesting a pathway for signal transmission [43]. Besides, our group revealed new allosteric features in cTnC upon insertion of an extra methylene group in position D145 (D145E) of the conserved Ca^{2+} -binding site IV.

Through extensive structural analysis, the cTnC D145E mutant increases its linker flexibility, alters the αH helix dynamics and promotes different hydrogen bond interactions with the solvent at physiological temperatures (Fig. 2c), supporting new elements for allosteric communication within cTnC [23, 24]. The flexibility implies that the conformations that will be sampled and the dynamic allostery model primarily accounts for

the normalization contribution of disordered populations of a protein to the relative equilibrium of functional conformers. To some extent, dynamics can be considered as an additional effect within a “population-shift” process and such effect can plausibly play a role in a “conformation selection” process. Our data obtained with D145E in cTnC can further exemplify this effect. This mutant affected its surrounding environment as well as distant sites in the N-domain. Moreover, the hydrophobic exposure of cTnC is altered, as well as N-domain Ca^{2+} affinity in the isolated state and within reconstituted fibers [23, 24]. The structural shuffling in the EF-hand motif disrupts its Ca^{2+} chelating function and completely shifts the fast-exchange motion to the intermediate range within the C-domain. ^{15}N relaxation experiments revealed the existence of disturbed residues outside the nanosecond timescale within the C-domain. To capture quantitative information about excited states, ^{15}N CPMG relaxation dispersion measurements reported that affected residues were clustered close to the Ca^{2+} -binding site III loop, along with the αH helix, and the extreme C-terminus. Our relaxation dispersion measurements have captured in the cTnC D145E mutant protein a conformational exchange state that exists between the native state (N) and a high energy state that occurs with exchange rates of $K_{\text{ex}} = 1276.7 \pm 173 \text{ s}^{-1}$ and a population frequency of $1.89 \pm 0.16\%$ for the high energy conformational state [23]. Moreover, it is very likely that D145E mutant shifts the equilibrium of a hidden conformation, which only occupies a minor population of the entire conformational space, to be the dominant state; thereby it leads to a significant allosteric effect. Combining NMR measurements and powerful molecular dynamics simulation techniques to characterize such “hidden” conformations hold great promise, providing the ability to elucidate the secrets underlying such allostery.

Surprisingly, the ability of cTnC to anchor to cTnI persisted and even increased the affinity of the cTnC D145E mutant protein to the TF, even in the absence of Ca^{2+} binding to sites III and IV. Traditionally, a functional C-terminal domain was believed essential for structurally anchoring the Tn complex to the TF [44]. These observations suggest an allosteric compensatory mechanism within cTnC. In contrast, the G159D mutant located in the αH helix does change cTnC dynamics but weakens the anchoring interaction between cTnC and cTnI [45], reinforcing the concept that evolutionarily conserved networks are responsible for mediating allosteric communication.

Likewise, site I of cTnC is quite conserved throughout evolution, its overall fold maintained, and it has been found to be a structural element within the cTnC allosteric network. A substitution L29Q within this site modifies Ca^{2+} sensitivity at site II. Moreover, L29Q increases backbone flexibility and the solvent accessible area in the αA helix and decreases the stability of the αN helix. Because the αA helix plays a role in opening of the N-domain, L29Q may also promote alterations in the cTnC allosteric regulation within this domain [46, 47].

As conserved residues are crucial for allosteric transmission in the cTnC, singularities between different isoforms may also affect their allosteric profile. sTnC displays two main local differences: two active Ca^{2+} binding sites in the N-domain and a rigid linker. Unlike cTnC, Ca^{2+} binding to sTnC N-domain promotes structural rearrangements required for binding of the sTnI switch peptide and the release of sTnI actin binding sites on actin triggering contraction [1]. The intramolecular coupling between the N- and C-domains was

reported for isolated sTnC upon Ca^{2+} binding and was shown to increase the overall stability of the isolated Tn complex [48]. Moreover, the presence of a less dynamic helical linker in sTnC, may represent a relevant constraint for stability and maintenance of the allosteric control between both isoforms during contraction. In the absence of Ca^{2+} , the C-domain has only a small effect on the N-domain structure. However, in the Ca^{2+} -bound form, folding free energy barriers revealed that stability of the N-domain is affected by the presence of the C-domain confirming that both domains communicate with each other [48].

A cell-wide view may drive the questions, for instance, of how allosteric changes in a single protein can affect other partners within macromolecular complexes that ultimately alter distinct physiological roles such as muscle contraction? What would be the influence of a mutant that disrupts an essential allosteric effect? Proteins mediate transmission of signals along a vast majority of intercellular and intracellular pathways and fluctuate among many states. To accomplish their evolutionary role, a hierarchical and cadenced symphony of dynamic tones oscillate at frequencies ranging from picoseconds to milliseconds or even seconds [36, 49]. Although quantitative descriptions of allosteric interconversion states are still challenging, NMR relaxation dispersion methods are useful in the case of flexible segments within the Tn complex. For instance, cTnI has intrinsically disordered regions (IDRs) that are essential sites for signaling transmission and allosteric regulation [50]. IDRs are typically associated with the structural plasticity, high specificity for multiple targets and fast association and dissociation turnover rates [41]. Moreover, cTnT presents two well-defined domains with highly conserved residues and a hypervariable region that plays a critical role in defining T_m affinity. In this sense, mutants may redistribute protein ensembles by populating on- or off-states and changing the dynamic landscape, a condition that might redefine protein-protein interaction patterns. Due to their important regulatory roles, cTn mutants can affect the proper regulation of the Tn complex and downstream partners, leading to the development of diseases.

Upon cracking the genetic code, Crick and colleagues proposed that the secret of life, the central dogma of molecular biology is that information contained in genes can flow into proteins. Likewise, allostery alludes to "the second secret of life" [51] and is a critical biological phenomena for the understanding of biological systems, disease, and has established a new paradigm for drug discovery. The cTnC allosteric events captured in our studies bring new insights into the understanding of muscle contraction and communication within this intricate machinery. The knowledge of the short- and long-term allosteric maps will certainly help to unveil essential processes in cardiac contraction and disease.

Novel insights to integrate Tn structure with its function

We provide a fundamental basis to launch investigations into the role of undefined or novel regions of the cTn complex and insights into how the cTn communicates through long-range interactions. Evidence of communication through far reaching networks has been previously shown between closely interacting proteins. For example intra-molecular coupling has been reported in skeletal and cTnC [48, 52] and the bending of the D/E linker is anticipated to be a mechanism augmenting interdomain cross-talk [53] which is pronounced in the cardiac isoform [54]. Some is known about the "semi-fixed" structural subdomains within cTn, but

much less information exists about their relationship to each other since they are connected by “flexible” linkers [10]. We need to fully understand how this built-in flexibility instills a means to fine-tune muscle function, regulate allostery and provides a novel basis for developing therapeutic interventions.

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Figure 1. Schematics showing structural organization of TFs.

The density map of F-actin-Tm (EMDB code 8162), atomic models of F-actin-Tm (PDB code 5j1f), and cTn (PDB code 1j1e) were fit as rigid bodies and may not represent real positioning of cTn within TFs. The residues participating on Ca²⁺-binding at sites II and IV are shown as sticks and Ca²⁺ ions are indicated as green spheres. cTnC helices (αN-αH) are labeled within rectangles.

Figure 2. Conserved motifs and allosteric elements within TnC.

(a) Conserved motif distribution within human cTnC (PDB code 1J1E). The percentage of sequence conservation are colored from red to blue. (b) Alignment of 38 skeletal and cTnC sequences from different organisms used to generate the conservation map in (a). Non-similar residues are colored in white/black; Conserved residues are cyan/blue; Strongly similar are green/black; Identical residues are indicated as yellow/red; Weakly similar residues are shown as white/green. Similarity is based upon the residues physicochemical properties. (c) Schematics showing new allosteric elements captured in our studies using the D145E mutant. Hydrogen bonds are shown as white dotted lines and residues presenting distinct H-bond patterns are shown in magenta. Water molecules are labeled as red/white spheres and Ca^{2+} ions as green spheres.