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Structure and Function of Cardiac Troponin C (TNNC1): Implications for Heart Failure, Cardiomyopathies, and Troponin Modulating Drugs

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Abstract

In striated muscle, the protein troponin complex turns contraction on and off in a calcium-dependent manner. The calcium-sensing component of the complex is troponin C, which is expressed from the TNNC1 gene in both cardiac muscle and slow-twitch skeletal muscle (identical transcript in both tissues) and the TNNC2 gene in fast-twitch skeletal muscle. Cardiac troponin C (cTnC) is made up of two globular EF-hand domains connected by a flexible linker. The structural C-domain (cCTnC) contains two high affinity calcium-binding sites that are always occupied by Ca^{2+} or Mg^{2+} under physiologic conditions, stabilizing an open conformation that remains anchored to the rest of the troponin complex. In contrast, the regulatory N-domain (cNTnC) contains a single low affinity site that is largely unoccupied at resting calcium concentrations. During muscle activation, calcium binding to cNTnC favors an open conformation that binds to the switch region of troponin I, removing adjacent inhibitory regions of troponin I from actin and allowing muscle contraction to proceed. Regulation of the calcium binding affinity of cNTnC is physiologically important, because it directly impacts the calcium sensitivity of muscle contraction. Calcium sensitivity can be modified by drugs that stabilize the open form of cNTnC, post-translational modifications like phosphorylation of troponin I, or downstream thin filament protein interactions that impact the availability of the troponin I switch region. Recently, mutations in cTnC have been associated with hypertrophic or dilated cardiomyopathy. A detailed understanding of how calcium sensitivity is regulated through the troponin complex is necessary for explaining how mutations perturb its function to promote cardiomyopathy and how post-translational modifications in the thin filament affect heart function and heart failure. Troponin modulating drugs are being developed for the treatment of cardiomyopathies and heart failure.

Keywords

Hypertrophic cardiomyopathy; dilated cardiomyopathy; heart failure; calcium sensitizer; levosimendan; sarcomere modulators

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Biological context of troponin C

Muscle contraction is produced by the sliding of actin thin filaments against myosin thick filaments. In cardiac and skeletal muscle, thin and thick filaments are organized into highly polarized contractile units known as sarcomeres, which are linked in tandem and bundled together to form myofilaments. This arrangement gives these tissues their characteristic “striated” appearance.

The calcium-dependent contraction of striated muscle is controlled by the thin filament through the action of tropomyosin and the troponin complex, first discovered in 1964^{2,3}. The thin filament has a periodicity of about 38.5 nm¹, comprising fourteen actin monomers arranged like a twisted “double string of beads”. Tropomyosin is a homodimeric parallel coiled-coil that polymerizes in a head-to-tail manner along the entire length of the thin filament (except at the Z-disc)⁴, with each tropomyosin homodimer associated with one troponin complex and seven actin monomers. In the absence of troponin, tropomyosin lies along a positively charged groove^{5,6}, though it is able to shift or rotate considerably along the filament axis. At resting Ca²⁺ concentrations, the troponin complex anchors tropomyosin into a “blocked” position that sterically hinders the approach of myosin towards actin. An increase in free calcium concentration results in a conformational change within the troponin complex that releases tropomyosin into a “closed” position that allows weak actin-myosin interaction⁷. Strong binding of myosin to actin further shifts tropomyosin to the “open” position.

The troponin complex is made up of three components^{8–10}: the calcium binding subunit, troponin C (TnC); the inhibitory subunit, troponin I (TnI); and an elongated protein, troponin T (TnT), that binds both TnC and TnI and anchors the entire complex to tropomyosin. For each of the subunits, homologous genes encode isoforms specific to the different types of striated muscle: cardiac, slow-twitch (type I) skeletal, and fast-twitch (type II) skeletal. The TNNC1 gene (3p21.1) is expressed in both slow skeletal and cardiac tissue (we will refer to it as cardiac troponin C, cTnC), whereas the TNNC2 gene (20q12-q13.11) encodes fast skeletal troponin C (hereafter referred to as sTnC)^{11–15}. During development, both cTnC and sTnC are expressed in embryonic skeletal muscle, but expression of the cTnC gene is subsequently turned off during the transition to fast-twitch muscle^{16,17,18}.

The Ca²⁺-dependent regulation of striated muscle contraction by troponin has been a fascinating area for biochemical and biophysical study since its discovery more than 50 years ago. TnC expresses well in *E. coli* and is the only soluble globular protein of the sarcomeric thin filament. Moreover, its role within the thin filament has been extensively studied, using well-established methods to reconstitute actin, tropomyosin, and troponin into functional filaments^{10,19}. This review will focus on cardiac troponin C, highlighting its structure and function within the troponin complex. In recent times, the cardiac troponin complex has become important for understanding the pathogenesis, diagnosis, and treatment of cardiac diseases, especially as a target for the design of cardiac drugs. Underscoring its critical function, cTnC is highly conserved (96.8%–99.4% = 1–6 sequence differences) across 61 known TnC sequences that have been cloned from 41 vertebrate and invertebrate species to date²⁰.

Structure of cardiac troponin C

Troponin C is an 18-kDa member of the EF-hand Ca^{2+} -binding protein family, first described in the X-ray crystal structure of parvalbumin in 1973²¹. The family derives its name from a characteristic helix-loop-helix motif, in which six residues contribute oxygen ligands to define an octahedral Ca^{2+} -binding site: 1(X), 3(Y), 5(Z), 7(-Y), 9(-X), and 12(-Z). Most of the ligands are polar amino acid sidechains, whereas the residue in the -Y position contributes a backbone carbonyl oxygen, and the sidechain in the -X position often indirectly coordinates Ca^{2+} via a bridging water molecule. The -Z position is almost always glutamate, which provides the only bidentate ligand, a carboxyl group, which changes the coordination geometry from octahedral to pentagonal bipyramidal. Both sTnC and cTnC comprise four EF-hand helix-loop-helix motifs as potential Ca^{2+} -binding sites (I-IV), except that site I in cTnC is inactive due to an insertion (V28) and two key Ca^{2+} -binding amino acid substitutions (D29L and D31A) (see Figure 1).

The first three-dimensional structures of fast skeletal TnC were solved by X-ray crystallography in 1985, full-length turkey sTnC²² and full-length chicken sTnC²³. sTnC is organized into two domains, each containing two Ca^{2+} -binding EF-hands. In both structures, the two Ca^{2+} -binding sites of the N-terminal domain (sNTnC) were unoccupied, while two Ca^{2+} ions were bound to the C-terminal domain (sCTnC) (Figure 2A). Comparison of the two homologous N- and C-domains showed that sNTnC was in a closed state, while sCTnC was in an open state, leading to the suggestion that Ca^{2+} binding to sNTnC would lead to a structural transition²⁴, causing helices B+C to rotate away from helices N+A+D and exposing a large hydrophobic patch. A short anti-parallel β -sheet formed between EF-hands I and II (centered at position 8, see Figure 1) acts as a hinge for these sub-domain movements. This closed-to-open transition was confirmed in 1995 with the NMR solution structure of sTnC in the fully Ca^{2+} -saturated state^{25,26}.

Subsequent X-ray structures of sNTnC•2 Ca^{2+} and sTnC•4 Ca^{2+} have been reported^{27,28}. The X-ray structures provide precise details of the metal coordination sites not obtainable from solution NMR methods. The main difference between the X-ray and solution structures of sTnC is found in the central linker connecting the N- and C-domains. The linker forms a rigid α -helix in the crystal forms, but it is unstructured and flexible in solution, highlighting a key caveat in the interpretation of X-ray crystal structures, that crystal packing contacts can induce structure in otherwise flexible regions. There is extensive solution NMR data on isolated TnC to suggest that the two domains can adopt a wide range of orientations: ¹⁵N relaxation measurements showing that the linker region is very dynamic and that the two domains tumble independently²⁹, and paramagnetic relaxation enhancement (PRE) studies measuring the range of distances between domains³⁰.

The NMR solution structure of cTnC•3 Ca^{2+} was determined in 1997³¹. Like sTnC, cTnC also adopts a dumbbell shape with a flexible linker (Figure 2B). As expected the structural C-domain of cTnC binds two Ca^{2+} ions and adopts an open conformation. A comparison of the regulatory domain of cTnC (cNTnC) in both the apo and Ca^{2+} -bound states^{31,32} revealed that cNTnC remains strikingly closed in both the absence and presence of calcium, unlike sNTnC. This is likely a consequence of a defunct site I. In sNTnC, a site I-disrupting

mutation E41A similarly abolished the link between Ca^{2+} -binding and transition to the open state³³.

Structure and function of cardiac troponin C within the troponin complex

Binding of Ca^{2+} to the regulatory NTnC domain is the key event that links cytoplasmic calcium influx to muscle contraction. Ca^{2+} binding allows NTnC to interact with the switch region of troponin I, removing the adjacent inhibitory region of TnI from its binding site on actin. It is primarily the inhibitory region of TnI³⁴, and to a lesser degree, its C-terminal tail^{35,36}, that anchors the troponin-tropomyosin complex to the blocked position that prevents actin-myosin interaction and shuts off muscle contraction in the absence of Ca^{2+} .

The closed-to-open transition observed in sNTnC suggests a mechanism for Ca^{2+} -mediated regulation, whereby Ca^{2+} binding is coupled to the opening of a “sticky” hydrophobic surface. This is the central paradigm for calmodulin, the ubiquitous Ca^{2+} -sensing protein that binds and regulates a plethora of targets via its versatile hydrophobic surfaces³⁷. Indeed, sNTnC binds to the switch region of fast skeletal TnI via the corresponding hydrophobic surface exposed by Ca^{2+} binding³⁸. Despite its significant homology to sNTnC, it was initially uncertain whether cNTnC would bind its target in a similar mode, since its Ca^{2+} -bound form is predominantly closed. Nevertheless, the solution NMR structure of cNTnC• Ca^{2+} •cTnI₁₄₇₋₁₆₃ revealed an open state for cNTnC³⁹. The bound cTnI₁₄₇₋₁₆₃ switch peptide adopts an α -helical conformation spanning residues 149–159, which wedges into the AB helical interface and inserts Ile148 and Met153 into the hydrophobic pocket to stabilize the open conformation of cNTnC (Figure 3).

Most of the structural work on cTnC has focused on its place in the cardiac troponin complex. However, cTnC is also the isoform for troponin C in slow-twitch skeletal muscle, forming a complex with slow skeletal TnI (ssTnI) and slow skeletal TnT (ssTnT). In fact, ssTnI is also present in the fetal heart, where it is postulated to allow the troponin complex to function in a more acidic environment⁴⁰. The Ca^{2+} affinity of cTnC is reduced under acidic conditions through direct competition between H^+ and Ca^{2+} ions. However, replacement of cTnI in cardiac muscle with ssTnI restores calcium sensitivity at low pH⁴¹. This acid-resistance was found to depend on a single amino acid substitution: the replacement of Ala162 in cTnI with the corresponding His130 in ssTnI⁴². The positively charged imidazole ring of His130 forms a salt bridge with Glu19 on the surface of cNTnC, stabilizing the cNTnC-ssTnI complex and restoring Ca^{2+} -binding affinity under acidic conditions⁴³. In the complex, His130 has a pKa of ~ 6.7 ⁴⁴, becoming more positively charged as the intracellular pH drops below 7. Recent NMR studies of cNTnC• Ca^{2+} bound to fsTnI₁₁₅₋₁₃₁, as well as cNTnC• Ca^{2+} bound to A162H-cTnI₁₄₇₋₁₆₃, have confirmed the importance of this electrostatic interaction⁴⁵. The interaction is also present in the X-ray structure of the fast skeletal core troponin complex⁴⁶, suggesting that it contributes to pH insensitivity in fast skeletal muscle as well. Some have advocated cardiac gene therapy to introduce the TnI A162H mutation in patients with severe cardiac ischemia⁴⁷, since the Ca^{2+} sensitivity of the ischemic heart is reduced by cytosolic acidosis⁴⁰.

While the regulatory NTnC domain is generally accepted to play the crucial role of turning muscle contraction on and off in response to calcium, the structural CTnC domain keeps troponin C firmly anchored to the rest of the troponin complex throughout the cardiac cycle. The X-ray crystal structure of the cardiac troponin complex (Figure 4), $cTnC \cdot 3Ca^{2+} \cdot cTnI_{33-209} \cdot cTnT_{183-288}$ ⁴⁸, is indispensable for understanding the spatial relationships between troponin C, I, and T. (Note that the numbering of cTnI and cTnT is inconsistent throughout the literature. For cTnI, discrepancies arise because the N-terminal methionine residue is removed and replaced by an acetyl group, and it is variably included in the numbering. In this review, we will not include the initiator methionine so the first residue is Ala1. For cTnT, alternatively spliced forms result in varying lengths. We will use the numbering for the 288-residue version of human cTnT used in the crystal structure⁴⁸.) The overall core domain structure is dominated by α -helical elements, most notably the IT arm (consisting of cCTnC, cTnI₄₁₋₁₃₅ and cTnT₂₂₅₋₂₇₆). cTnI₄₁₋₁₃₅ forms two long α helices, cTnI₄₁₋₇₉ and cTnI₈₈₋₁₃₅. cTnI₄₁₋₆₀ binds to the hydrophobic cleft of cCTnC, and then residues 62–80 continue the long helix well beyond cCTnC. Residues 80–87 form a loop that interacts with cTnT, and then cTnI₈₈₋₁₃₅ proceeds back towards the opposite face of cCTnC while forming a coiled coil with cTnT₂₂₅₋₂₇₆. cTnT₂₂₅₋₂₇₆ is positioned between cCTnC and cTnI₈₈₋₁₃₅, with cTnT₂₅₉₋₂₇₀ binding extensively to the Ca²⁺-bound EF-hands III and IV of cTnC.

Limited proteolysis of troponin T (rabbit fast skeletal) yields two fragments, TnT1 and TnT2^{49,50}, with TnT2 featured in the troponin X-ray crystal structure. Binding studies of TnT2 have shown that both^{51,52} the region N-terminal⁵³ and C-terminal⁵⁴ to the IT arm (cTnT₂₂₅₋₂₇₆) contribute to the interaction with tropomyosin, suggesting that the IT arm is rigidly fixed to tropomyosin. The C-terminus of cTnT, cTnT₂₇₅₋₂₈₈, is also immediately adjacent to the cTnI₂₈₋₁₄₇ inhibitory region, and both are essential for maintaining the blocked state of actin-tropomyosin⁵⁵. On the other hand, one group has reported that the region corresponding to cTnT₁₈₂₋₂₁₅ interacts with actin and contributes to the activation of actomyosin ATPase⁵⁶. Thus, the IT arm and regions immediately adjacent to it are key for binding the troponin complex to tropomyosin as well as for stabilizing the inactive blocked state or the active open state of troponin-tropomyosin on actin.

Work performed prior to the publication of the X-ray structure of the cardiac troponin complex in 2003 suggested a possible interaction between the cTnI₂₈₋₁₄₇ inhibitory region and the structural cCTnC domain. An NMR structure of $cCTnC \cdot 2Ca^{2+} \cdot cTnI_{28-147}$ ⁵⁷ shows residues L134-K139 of cTnI forming a helix upon interaction with the E and H helices of $cCTnC \cdot 2Ca^{2+}$. However, since this arrangement of cTnI₂₈₋₁₄₇ on cCTnC would be displaced by cTnI₄₁₋₆₀, and cTnI₈₈₋₁₃₅ is positioned on the opposite side of cCTnC as part of the IT arm (Figure 4), the observed cCTnC-cTnI₂₈₋₁₄₇ interaction is unlikely to occur within the context of intact troponin complex. This example illustrates how binding studies on simplified systems need to be interpreted with caution.

Although N- and C-domains of troponin C are connected by a flexible linker, there are additional intermolecular interactions within the whole troponin complex that can fix the relative domain orientations of cTnC. The domain positions have recently been described

using Förster resonance energy transfer (FRET) in reconstituted cardiac troponin complex⁵⁸ as well as with fluorescence polarization in intact thin filament⁵⁹.

In cardiac TnC, the orientation of cNTnC relative to cCTnC is fixed by the direct interaction of the N-terminal 31-amino acid cardiac-specific region of cTnI with cNTnC^{60–62}. This region is critical to the regulation of calcium sensitivity in the cardiac sarcomere. Phosphorylation of cTnI in response to β -adrenergic sympathetic stimulation was first identified in 1976⁶³. β -adrenergic stimulation leads to production of cyclic AMP and activation of protein kinase A, which phosphorylates cTnI Ser22 and Ser23 (in addition to many other targets)^{65,66}. Other kinases have been found to phosphorylate this site as well including PKC, PKD1, and PKG⁶⁷, showing Ser22/Ser23 to be an important regulatory node for integrating multiple signaling pathways. Phosphorylation at this site has been consistently shown to decrease the calcium sensitivity of the cardiac sarcomere⁶⁴.

Recently, we have used solution NMR spectroscopy to study cTnI₁₋₇₃ in complex with cTnC • 3Ca²⁺⁶⁸. In this complex, cTnI₃₉₋₆₀ binds to cCTnC, stabilizing an α helix in cTnI₄₁₋₆₇ and a type VIII turn in cTnI₃₈₋₄₁ that brings cTnI₉₋₃₇ into close proximity with the negatively charged surface of cNTnC (opposite the hydrophobic surface that binds the switch peptide, cTnI₁₄₈₋₁₅₈). The interaction with cTnI₉₋₃₇ is electrostatic in nature, which allows cTnI₁₋₃₇ to maintain a disordered state even while in complex with cNTnC. In contrast to a previous model⁶⁹ for the cNTnC-cTnI₁₋₃₇ complex, the interaction does not induce secondary structure in cTnI₁₋₃₇, it does not favor the open state of cNTnC, and it does not directly affect the Ca²⁺-binding affinity of cNTnC. However, it does fix the positioning of cNTnC relative to the rest of the troponin complex. We propose that this domain orientation is optimal for cNTnC binding to the cTnI₁₄₈₋₁₅₈ switch region, and this is how cTnI₉₋₃₇ indirectly increases the Ca²⁺ affinity of cNTnC within the context of the cardiac thin filament. Binding of cTnI₁₄₈₋₁₅₈ stabilizes the Ca²⁺-bound open conformation of cNTnC, thus increasing its Ca²⁺ binding affinity. The cNTnC domain is released by protein kinase A (PKA) phosphorylation of cTnI Ser22 and Ser23, which disrupts the electrostatic interactions between cTnI₉₋₃₇ and cNTnC.

The domain positioning of cNTnC relative to cCTnC is stabilized by the electrostatic interaction between cNTnC and cTnI₉₋₃₇, but it is fully defined by additional weak interactions: a contact between Ala7 in the N helix of cNTnC and Ala42/Ser43 of the cTnI₄₁₋₈₀ helix bound to cCTnC, as well as an interaction between the negatively charged C-terminal residue Glu161 of cCTnC and positively charged residues in the cTnC interdomain linker. These interactions are present in the X-ray crystal structure of the cardiac troponin complex⁴⁸, and they bring together the N helix of cNTnC, the interdomain linker, and the C-terminus of cCTnC in the presence of unphosphorylated cTnI₉₋₄₃ to define the interdomain orientation of cTnC.

Energetics of binding and conformational transitions

The calcium binding affinity of cNTnC must be exquisitely tuned to the cytosolic Ca²⁺ fluctuations within the cardiomyocyte. The cardiomyocyte free cytosolic Ca²⁺ concentration

during diastole is about 100 nM, reaching a peak of about 1 μM during systole⁷⁰. The half-activating concentration of free Ca^{2+} in cardiac myofilaments is typically 1–4 μM ^{71,72}.

The calcium binding affinity of the regulatory cNTnC domain is determined by the relative stability of the Ca^{2+} -bound state versus the apo state. In Ca^{2+} -binding EF-hand proteins that undergo a closed-to-open conformational transition, Ca^{2+} binding is driven by electrostatic interactions as well as entropically favorable release of water molecules⁷³. This is offset by the exposure of a large hydrophobic patch upon transition to the open state. In cNTnC, the fact that EF-hand I is defunct means that there is no positive cooperativity of Ca^{2+} binding (at least for the isolated cNTnC domain), and the transition to the open state is less favorable, so that the closed state predominates in the absence of cTnI switch peptide. The structure of cNTnC in the closed conformation was recently highlighted by an X-ray structure of cNTnC in complex with Cd^{2+} , providing for the first time a high resolution model of EF-hand II metal ion coordination in the closed state. Most of the Ca^{2+} -coordinating interactions observed in the open state⁴⁸ (see Figure 1) were intact in the closed state, except that D67 (the 3Y position of EF-hand II) and S69 (position 5Z) do not participate in the coordination of Cd^{2+} ⁷⁴. This partial coordination may account for the increased rate of Ca^{2+} release in the closed state when compared to the cTnI switch peptide-stabilized open state (see below).

cNTnC is predominantly in the closed state when it is bound to Ca^{2+} . However, it is now apparent that Ca^{2+} -bound cNTnC exists in a dynamic equilibrium between closed and open forms (Figure 3). The opening involves movement of the BC helices as a unit away from the NAD helices unit. MD simulations have detected a concerted tilting motion of helices B and C away from helix A, corresponding to the opening motion of cNTnC• Ca^{2+} ⁷⁵. NMR amide proton relaxation dispersion experiments reveal that many residues in the hinge regions of cNTnC experience a conformational exchange process with a time scale of $\sim 30 \mu\text{s}$ ^{76,77}. It was estimated that the open state is populated at about 20%⁷⁸. This value is consistent with recent solution NMR paramagnetic relaxation enhancement (PRE) data from four spin-labeled monocysteine constructs of isolated cTnC, suggesting that the open state is populated $\sim 27\%$ in the calcium-saturated cTnC^{79,79}, compared with $\sim 0\%$ in the apo state. Together these data support a model in which Ca^{2+} binding creates a dynamic equilibrium between the ‘closed’ and ‘open’ structural states to prime cNTnC for subsequent binding of the cTnI₁₄₈₋₁₅₈ switch region.

Hydrophobic binding of cTnI₁₄₈₋₁₅₈ to the large hydrophobic pocket of cNTnC stabilizes its open state, increasing its Ca^{2+} binding affinity. Using fluorescent IAANS labeling at Cys35 and Cys84, a Ca^{2+} binding K_D of about 2 μM was determined for cNTnC in the context of isolated cTnC⁸⁰. When cTnC was combined with cTnI and cTnT to make full troponin complex, K_D of cNTnC became 0.3 μM , indicating about one order of magnitude tighter Ca^{2+} binding. This is due to a slowing of Ca^{2+} release, from $>1000 \text{ s}^{-1}$ from isolated cTnC at 15°C ⁸¹ to 122 s^{-1} in the presence of cTnI and $33\text{--}42 \text{ s}^{-1}$ in the presence of cTnI and cTnT⁸².

Solution NMR spectroscopy was also used to assess the binding of Ca^{2+} and cTnI₁₄₇₋₁₆₃ to cNTnC at 30°C ⁸³. K_D of $\sim 20 \mu\text{M}$ and $\sim 150 \mu\text{M}$ were determined for Ca^{2+} and cTnI₁₄₇₋

163 peptide binding, respectively. The Ca^{2+} release rate was estimated to be 5000 s^{-1} in the absence of cTnI. The off rate of cTnI₁₄₇₋₁₆₃ was about 5000 s^{-1} , suggesting a rapid equilibrium between bound and free states.

A subsequent fluorescence study suggested that IAANS labeling at Cys35 and Cys84 altered the Ca^{2+} -binding properties of cTnC, so a monocysteine C35S/T53C/C84S mutant was put forward⁸⁴. This mutant was used to study the Ca^{2+} binding characteristics of cTnC in the context of cardiac troponin complex (cTn) versus slow skeletal troponin complex (ssTn), as well as in the presence of actin, tropomyosin, and myosin S1 subfragment. In the isolated cardiac complex, a Ca^{2+} K_D of $0.65 \mu\text{M}$ was measured, compared with $0.25 \mu\text{M}$ in the slow skeletal complex. The higher Ca^{2+} affinity of the ssTn complex can be expected from the enhanced affinity of the ssTnI switch region for cTnC. Upon addition of thin filament (actin and tropomyosin), the Ca^{2+} K_D of cTn increased to $5 \mu\text{M}$, explainable by the binding of cTnI₁₂₈₋₁₄₇ to actin, making the cTnI₁₄₈₋₁₅₈ switch region less available for binding to cTnC. Addition of myosin S1 fragment (to troponin complex + actin + tropomyosin) changed the Ca^{2+} K_D of cTn to $0.78 \mu\text{M}$, likely because the formation of actomyosin rigor complexes shifts the thin filament to an open state that releases cTnI₁₂₈₋₁₄₇ from actin. These studies demonstrate how the Ca^{2+} binding affinity of cTnC is exquisitely dependent on the availability of cTnI₁₄₈₋₁₅₈ switch region for binding. cTnI₁₄₈₋₁₅₈ is tethered to actin via adjacent segments³⁴ when the thin filament is in the blocked state, but released and available for binding cTnC in the thin filament closed or open states. (Note that cTnC itself has closed and open states that are distinct from the thin filament states, for which “closed” refers to the positioning of tropomyosin when cTnC is bound to calcium, and the “open state” refers to a further shift of tropomyosin when myosin forms strong cross-bridges to actin.)

There are no known post-translational modifications of cTnC that modulate its calcium affinity. However, there are numerous phosphorylation sites on cTnI⁸⁵ that impact the calcium affinity of cTnC, the most important of which is Ser22/23 in humans. We propose that many of these phosphorylation sites, including Ser22/23, impact cTnC calcium binding by modulating the availability of cTnI₁₄₈₋₁₅₈ switch peptide for binding (the interaction between the unphosphorylated cardiac-specific N-terminal extension and cTnC orients it optimally for binding cTnI₁₄₈₋₁₅₈, thus increasing the effective concentration of cTnI₁₄₈₋₁₅₈). Moreover, modifications to TnT, tropomyosin, actin, or myosin can also impact the blocked-closed-open equilibrium of the thin filament to impact cTnI₁₄₈₋₁₅₈ availability and calcium sensitivity.

In contrast to cTnC, cCTnC binds Ca^{2+} with very high affinity. In isolated cTnC, the Ca^{2+} binding K_D is about 40 nM ⁸⁶, decreasing to about 3 nM in intact troponin complex⁸⁰. The very high affinity of cCTnC is a consequence of the instability of its apo state. A closed-to-open transition does not occur in skeletal or cardiac cCTnC, which appears to form a molten globule in the absence of Ca^{2+} ^{87,88}. Ca^{2+} binding induces the formation of well defined tertiary structure, demonstrating that the sequence of cCTnC is optimized for the open conformation, but not the closed. cTnI₃₉₋₆₀ binds to Ca^{2+} -saturated cCTnC with very high affinity^{83,89}. An affinity of 3 nM was measured for cTnI₃₈₋₅₇ via surface plasmon resonance

and ELISA⁹⁰. The affinity for intact cTnI in the presence of cTnT is likely even stronger, given the additional contacts formed within the IT arm.

Mg²⁺ is chemically similar to Ca²⁺ and able to compete for the same binding sites in many EF-hand-containing proteins⁹¹. A classic equilibrium dialysis study of Ca²⁺- and Mg²⁺-binding in fast skeletal TnC showed that Mg²⁺ competes for the high affinity Ca²⁺ binding sites in the structural CTnC domain, whereas the lower affinity sites in the regulatory NTnC are Ca²⁺-specific⁹². (The same study also identified two non-competitive Mg²⁺ sites.) More recent fluorescence studies, however, have demonstrated that Mg²⁺ is also able to compete with Ca²⁺ in the NTnC domain, reducing the apparent calcium affinity by about a factor of 2 at a Mg²⁺ concentration of 1–3 mM⁹³. Thus, the presence of Mg²⁺ in the muscle cytoplasm would have a calcium desensitizing effect, since Mg²⁺ itself does not trigger muscle contraction. Compared to Ca²⁺, Mg²⁺ binding tends to favor the closed conformation^{91,93}, so cNTnC cannot bind cTnI switch peptide (in the open conformation) in the absence of Ca²⁺. In contrast, cCTnC is able to adopt an open conformation and bind cTnI₃₃₋₈₀ when loaded with Mg²⁺ instead of Ca²⁺, as demonstrated by an NMR structure^{94,95}.

Hypertrophic cardiomyopathy-associated mutations

Hypertrophic cardiomyopathy (HCM) is a common genetic disease, having an estimated prevalence of ~1:500^{96,97}. Although there are some rare infiltrative causes, HCM is generally a disease of the sarcomere, with a sarcomeric mutation identifiable in about half of affected patients. Mutations in the myosin heavy chain β and cardiac myosin binding protein-C are most common, followed by mutations in cardiac troponin I and troponin T^{98,99}. One challenge in identifying definitive mutations is that many mutations have incomplete penetrance. As the name suggests, the most striking feature of HCM is abnormal hypertrophy of the ventricles, most classically prominent in the interventricular septum. The hypertrophy can be so severe that it causes a left ventricular outflow tract obstruction. The hypertrophied ventricles are also prone to ventricular arrhythmias that can cause sudden death. One challenge in the treatment of HCM is identifying patients at greatest risk of sudden death who would benefit from placement of an implantable cardioverter defibrillator. Other common complications include atrial fibrillation, diastolic dysfunction, myocardial ischemia, and mitral regurgitation¹⁰⁰. To date, 7 mutations associated with HCM (A8V, L29Q, A31S, C84Y, Q122AfsX30, E134D, D145E) and 7 mutations associated with dilated cardiomyopathy (DCM) (Y5H, Q50R, E59D/D75Y, M103I, D145E, I148V, G159D) in cTnC have been reported. A recent review¹⁰¹ has summarized the biochemical characterization of these mutations, but we will briefly survey them here.

The first publication of a TNNC1 mutation associated with cardiomyopathy was in 2001¹⁰². An L29Q mutation was identified in a 60-year-old male HCM patient with 15 mm thickness of the septal and posterior left ventricular wall (normal is <11 mm and 13 mm is the minimum in adults required for HCM diagnosis). Ca²⁺ sensitivity of the L29Q–cTnC mutant has been reported to be decreased¹⁰³, increased^{104,105}, or unchanged¹⁰⁶. These discrepancies have been suggested to be at least partly due to the different model systems used in these studies, although the same range of divergent results have also been reported for isolated

protein components. Given the wide range of experimental findings in the absence of further supportive genetic evidence, it is possible that L29Q represents an incidental polymorphism.

An important study describing HCM mutations in troponin C was published in 2008, in which 1025 HCM patients were screened for mutations in TNNC1, and all positive hits were further screened at fifteen other known HCM susceptibility loci¹⁰⁷. Four patients were identified with missense mutations in TNNC1 and no other locus, and these had left ventricular maximum wall widths ranging from 19 to 26 mm. The same authors later identified another mutation, A31S, in a five-year-old boy with a history of ventricular fibrillation and a mean ventricular wall thickness of 20 mm¹⁰⁸. None of these patients had affected family members with the same mutation. The five mutations, A8V, A31S, C84Y, E134D, and D145E were expressed recombinantly in troponin C and exchanged into porcine muscle fibers. All of the mutants except E134D were associated with increased calcium sensitivity^{109–112}, so E134D may be a clinically insignificant variant. The observed increases in calcium sensitivity are consistent with what is observed in HCM-associated mutations involving other thin filament proteins⁹⁹. It seems plausible that increased calcium sensitivity of the thin filament leads to increased muscle activation and exuberant muscle hypertrophy.

HCM-associated mutations in cNTnC highlight the potential mechanisms by which the calcium affinity of cNTnC can be increased. The A8V mutation was found to increase calcium sensitivity of reconstituted thin filaments, particularly when cTnI was unphosphorylated¹¹³. Ala8 is part of the N helix, which plays the key role in defining the domain orientation of cNTnC relative to the rest of the troponin complex, so it is possible that the A8V mutation stabilizes the positioning of the cNTnC domain. Ala31 is located in the loop region of EF hand I, so the A31S mutation may favor the conformation of this loop when cNTnC is in the open form. Cys84 contacts the switch region of cTnI in the open conformation and the BC loop in the closed conformation, so the C84Y mutation may either stabilize the open complex bound to cTnI or destabilize the closed conformation of cNTnC. In summary, we propose that the A8V, A31S, and C84Y increase the Ca²⁺-binding affinity of cNTnC by favoring the active form of cNTnC, either by stabilizing the activating orientation of the cNTnC domain, shifting the conformational equilibrium from the closed to open state, or directly enhancing binding to the cTnI switch peptide.

HCM-associated mutations identified in the cCTnC domain have far more destabilizing effects than those identified in the cNTnC domain. Asp145 is Ca²⁺-coordinating residue 5(Z) of EF-hand IV, and the D145E mutation was found to drastically reduce the Ca²⁺ binding affinity of cCTnC^{112,114}. Another HCM-associated mutation with a profound effect on the C-domain is Q122AfsX30, in which a frameshift mutation obliterates the last half of cCTnC and replaces it with 30 mistranslated residues¹¹⁵. This mutation was identified in a patient with histologically confirmed HCM after sudden death at the age of 19. The mutation was also identified in other family members with HCM. How these drastic mutations in the structural cCTnC domain contribute to HCM is currently unknown.

Dilated cardiomyopathy-associated mutations

Familial dilated cardiomyopathy (DCM) causes thin dilated ventricles associated with a decreased left ventricular ejection fraction. Compared with HCM, there is a wider variety of mutations, both sarcomeric and non-sarcomeric, that give rise to familial DCM, though DCM is more rare, with an estimated prevalence of ~1:5000^{99,117}.

G159D was the first DCM mutation reported¹¹⁸. It co-segregated with five affected family members, including the proband, who developed heart failure at the age of 21 and received a heart transplant two years later. A subsequent study identified a sixth family member who developed severe systolic heart failure requiring a heart transplant at the age of 3¹¹⁹. Thus, the genetic evidence supporting G159D as a causative mutation in DCM is the most extensive of any TNNC1 mutation. G159D-cTnC was shown to have similar Ca²⁺-binding affinity to wildtype-cTnC in isolation, but a reduced Ca²⁺ affinity was observed in intact muscle fiber experiments^{120–123}. Further studies demonstrated that G159D blunted the effect of cTnI phosphorylation at Ser22 and Ser23^{124–126}. Gly159 is located near the C-terminus of cTnC, where we postulate that the G159D mutation disrupts the delicate positioning of cNTnC relative to cCTnC.

The double mutant E59D/D75Y was identified in one patient¹²⁷. The same study also found that rat cardiomyocytes transfected with D75Y mutation, but not E59D, had greatly reduced contractility. Mutant D75Y-cTnC appears to have reduced Ca²⁺-binding affinity when tested in isolation, but the reduction in affinity appears more pronounced when it is incorporated into intact troponin complex or reconstituted thin filaments¹²³. Asp75 is located position 11 of EF-hand II, though it is not directly involved in coordinating Ca²⁺. It is possible that the D75Y mutation favors the closed conformation of EF-hand II.

The Q50R mutation was identified in a 16-month-old with dilated cardiomyopathy, whose mother also had peripartum cardiomyopathy. The maternal grandmother and two of her sisters had a history of dilated cardiomyopathy as well, and the mutation co-segregated with disease in at least four family members across three generations¹²⁸. Thus, the genetic evidence supporting Q50R as a DCM-causative mutation is strong, though no biochemical studies have been published to date. Q50 is located in the BC loop between EF-hands I and II, in a position where the Q50R mutation may produce an electrostatic repulsion with R147 of cTnI, disrupting binding of the switch region, cTnI_{148–158}.

The remaining DCM-associated mutations in TNNC1 were identified in a study¹²⁹ that examined a cohort of 312 patients with DCM. A total of 14 genes were sequenced, identifying mutations in up to 27% of the patients. There were four DCM-associated TNNC1 mutations identified: Y5H, M103I, D145E, and I148V. Y5H was detected in one patient, who developed heart failure at two weeks of age and later received a heart transplant at age of 15. Neither parent had DCM or the Y5H mutation. The father and the proband both had a mutation identified in MYH7, the beta myosin heavy chain, so it is possible that Y5H was only an incidentally observed mutation. All of the TNNC1 mutations identified in the study yielded small changes in calcium sensitivity, but Y5H had the largest Ca²⁺ desensitizing effect and also reduced the effect of PKA phosphorylation¹⁰⁹. Tyr5 is located

in helix N of cNTnC, where it packs against Arg83 of helix D. It is possible that the Y5H mutation creates an electrostatic repulsion that destabilizes helix N and disrupts positioning of the cNTnC domain. The M103I mutation was detected in a patient and a sister both with DCM¹²⁹. However, this family had two additional sisters without this mutation, but who had cardiac conduction abnormalities and/or syncope. Met103 is located in helix E of EF-hand III and binds to cTnI₅₇₋₆₄. It is interesting to note that there is an isoleucine at the homologous position in skeletal TnC, so the M103I mutation would not be expected to cause a major perturbation. I148V was identified in a single patient with DCM¹²⁹. Ile148 is located in the EF-hand IV loop region and is part of the small β -sheet at the base of the hydrophobic pocket. Other EF-hand proteins contain a valine at this site, so I148V is a relatively conservative substitution. Thus, M103I and I148V mutations would be expected to have only a minor impact on cCTnC structure and function, so it is uncertain whether these are truly clinically significant. In contrast, the D145E mutation, also independently identified in an unrelated HCM study (see section on HCM above)¹⁰⁷, drastically reduces the calcium affinity of cCTnC. It is possible that this mutation primarily causes HCM, but HCM can progress towards a DCM phenotype in later stages.

The genetic evidence behind some DCM-associated TNNC1 mutations is excellent. However, unlike thin filament HCM mutations, for which there is a consensus about increased myofilament calcium sensitivity, there is less agreement regarding the functional impact of DCM mutations. Although some studies document a decrease in calcium sensitivity, this has not been consistently demonstrated. One interesting observation in a number of DCM-associated mutations in the thin filament is an insensitivity to the effect of PKA-mediated phosphorylation of Ser22/Ser23 in cTnI¹³⁰. Phosphorylation of cTnI is an important mechanism by which the calcium sensitivity of the cardiac sarcomere is regulated. Dephosphorylation of cTnI has been observed in heart failure of various etiologies, including DCM and ischemic cardiomyopathy¹³¹. A non-phosphorylatable R21C mutation in cTnI (it abolishes the PKA RRXS phosphorylation motif) was associated with hypertrophic cardiomyopathy and diastolic dysfunction^{132,133}. It is possible that the insensitivity to cTnI phosphorylation seen in some DCM mutants is indicative of a structural derangement whereby positioning of the regulatory cNTnC domain by unphosphorylated cTnI is either unachievable or ineffective. In this case, the predominant defect would be a decrease in calcium sensitivity for which dephosphorylation of cTnI would be unable to compensate.

Drugs that bind the regulatory domain, cNTnC

A number of small molecules have been found to bind to cardiac troponin C (see Figure 5). Of these, levosimendan is the most widely studied to date, licensed in some South American and European countries for use as a positive inotropic agent in acute decompensated systolic heart failure. Levosimendan has been shown to improve cardiac output and decrease symptoms of heart failure¹³⁴. However, its use has been limited by associated cardiac arrhythmias and hypotension, and recent Phase III trials demonstrated no improvement in overall mortality^{135,136}, though some meta-analyses suggest some mortality benefit¹³⁷. Levosimendan has been suggested to bind to the regulatory domain of troponin C, cNTnC, but its exact mechanism of action has remained controversial^{138,139}. One study failed to

detect evidence of levosimendan binding to cTnC¹⁴⁰, though small amounts of covalent adduct were detected after prolonged incubation, likely formed through levosimendan's reactive malonitrile moiety (see Figure 5). It was later suggested that Cys84 is necessary for the covalent binding of levosimendan with cTnC^{139,141}, and this residue was changed to serine to produce a cysteineless mutant in some studies. Nevertheless, even with intact Cys84, no group has yet produced uniform stable cTnC•levosimendan complex for structural analysis. Moreover, the active metabolite of levosimendan, OR-1896¹⁴², does not possess the reactive malonitrile group and is unlikely to form a covalent conjugate. On the other hand, levosimendan does inhibit type 3 phosphodiesterase (PDE3) with nanomolar affinity¹⁴², and there are many other PDE3 inhibitors that act as positive inotropes and calcium sensitizers¹⁴³, all of which also cause hypotension and arrhythmias as undesirable side effects¹⁴⁴.

A levosimendan analogue (DFBP-O) was shown to bind to cNTnC-cTnI₁₄₇₋₁₆₃ and increase the Ca²⁺ sensitivity of skinned cardiac trabeculae¹⁴⁵. The structure of cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•DFBP-O shows that DFBP-O binds to the interface between cNTnC and cTnI₁₄₇₋₁₆₃ to enhance the binding of cTnI₁₄₇₋₁₆₃ to cNTnC (see Figure 6). Using the structure of cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•DFBP-O, a novel Ca²⁺ sensitizer 4-(4-(2,5-dimethylphenyl)-1-piperazinyl)-3-pyridinamine (NCI147866) was discovered using computational drug screening and verified by solution NMR¹⁴⁶. NCI147866 binds to both cNTnC•Ca²⁺ and the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃ complex. Its presence increases the affinity of switch peptide to cNTnC by approximately a factor of two. Its affinity for cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃ is comparable to that of DFBP-O (K_D 380 μM for both).

A CaM antagonist, W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide) was found to inhibit the maximum ATPase activity and tension development in both skeletal and cardiac muscle fibers^{147,148}. NMR titration studies have shown that W7 reduced the affinity of cTnI switch peptide for cNTnC from 154 μM to over 2000 μM¹⁴⁹. An NMR structure of the ternary complex cNTnC•Ca²⁺•cTnI₁₄₄₋₁₆₃•W7 (see Figure 6) revealed that W7 binding causes a considerable displacement of the cTnI switch peptide from its preferred binding site¹⁴⁹. Thus, W7 acts as a troponin inhibitor and Ca²⁺ desensitizer by increasing the off rate of the cTnI switch peptide. Its mechanism of action stands in contrast to DFBP-O and NCI147866, which both act as troponin activators by enhancing the binding of cTnI switch peptide to cNTnC.

Bepridil is a Ca²⁺ channel blocker and a calmodulin (CaM) antagonist previously marketed as an anti-anginal agent. Bepridil increases the Ca²⁺ sensitivity of myofilaments, increasing actomyosin ATPase activity and force generation¹⁵⁰. The X-ray structure of cTnC•3Ca²⁺•3bepridil provides detailed structural data on bepridil binding¹⁵¹. In this structure, three bepridil molecules stack together, but one molecule is bound to the hydrophobic pocket of cNTnC and another to the hydrophobic pocket of cCTnC, bringing the two domains together. The NMR structure of the cNTnC•Ca²⁺•cTnI₁₄₇₋₁₆₃•bepridil ternary complex reveals a binding site for cTnI₁₄₇₋₁₆₃ primarily located on the A/B interhelical interface and a binding site for bepridil in the hydrophobic core of cNTnC•Ca²⁺¹⁵². Bepridil seems to s

In summary, cNTnC is an eminently “druggable” target with a large hydrophobic cavity already known to bind a number of compounds, albeit at low affinity. Moreover, cNTnC-binding compounds are able to act as troponin activators or inhibitors, depending on their effect on cTnI switch peptide binding. (It is preferable to refer to these compounds as troponin modulators rather than calcium sensitizers/desensitizers to distinguish their mechanism of action from other compounds that act through signaling pathways, like levosimendan.) Cardiomyopathy-associated mutations in troponin demonstrate that minute changes in troponin can have a dramatic effect on cardiac function and phenotype, so it is very possible that drugs targeting troponin will play a role in treating cardiomyopathies as well as heart failure in general. For fast skeletal troponin, a number of troponin activators developed by Cytokinetics are already at clinical and pre-clinical stages. We are currently working to develop the first high affinity cardiac troponin modulators.

Compounds that bind to the structural domain, cCTnC

As noted above, bepridil also binds to the structural domain of cardiac troponin C, cCTnC, being a promiscuous molecule that also interacts with calmodulin and calcium channels. However, bepridil does not bind to cCTnC in the presence of cTnI. The hydrophobic patch of cCTnC is capable of binding a number of other compounds well known for their promiscuity: EMD 57033, resveratrol, and green tea catechin EGCg (epigallocatechin gallate) (see Figure 5). Of these, resveratrol and EGCg have been highlighted recently as an example of “PAINS” (pan-assay interference compounds)¹⁵³. Thus, although these compounds have demonstrated activity as calcium sensitizers or desensitizers, it is uncertain whether binding to cCTnC or some other target accounts for their observed activity.

EMD 57033 is the (+)-enantiomer of EMD 53998. While the (–)-enantiomer, EMD 57439, is a PDE3 inhibitor, EMD 57033, enhances the Ca²⁺-sensitivity of both myofibrillar ATPase and force development in skinned muscle fibers^{154–156}. EMD 57033 exerts a direct effect on actomyosin, increasing its ATPase activity. A recent study of EMD 57033 binding to myosin suggested that its binding site involved the SH3-like subdomain of the N-terminal domain¹⁵⁷. Direct binding of EMD 57033 to the C-domain of cTnC has been detected through tyrosine fluorescence¹⁵⁸ and solution NMR spectroscopy^{140,159}. In the high resolution structure of cCTnC•2Ca²⁺•EMD 57033⁸⁹, the drug molecule is oriented such that the chiral group of EMD 57033 fits deep in the hydrophobic pocket and makes several key contacts with the protein (see Figure 7). EMD 57033 is completely displaced by the binding of cTnI_{34–71}, but it is compatible with cTnI_{128–147} binding¹⁵⁹. This suggests that EMD 57033 does not bind to cTnC in the context of intact troponin complex, but its interaction may become a factor if cCTnC becomes dissociated from the rest of the IT arm. In such a circumstance, EMD 57033 may even act as a calcium sensitizer by stabilizing the open conformation of cCTnC and priming it to bind cTnI_{128–147}. However, there is currently no evidence that this ever occurs *in vivo*.

The polyphenolic compounds EGCg¹⁶⁰ and resveratrol¹⁶¹ were also found to bind to cCTnC, but not cNTnC. While EGCg is found in green tea, resveratrol is found in red wine. The NMR solution structures of cCTnC•2Ca²⁺•EGCg¹⁶⁰ and cCTnC•2Ca²⁺•resveratrol¹⁶¹ show that these compounds bind to a similar pocket as EMD 57033 (see Figure 7). EGCg

binding is more shallow than EMD 57033 binding, and unlike EMD 57033, EGCg can still bind cTnC in the presence cTnI₃₄₋₇₁, although the affinity is very weak, with a K_D of about 1.8 mM. An examination of the cTnC complexes presented in Figure 7 suggests that EGCg and cTnI₁₂₈₋₁₄₇ bind more superficially to cTnC than cTnI₃₄₋₇₁ and the other drugs.

Summary

Over the past 40 years, significant progress has been made toward understanding the structure and function of cardiac troponin C. cTnC plays a critical role in the heart, coupling its electrical pacing system to its mechanical apparatus through the binding of Ca^{2+} ions. Calcium plays a dual role in cTnC, stabilizing the structural C-domain (via two high affinity binding sites), as well as activating the regulatory N-domain (via a single low affinity binding site), the key calcium sensor of cardiac contraction. cTnC is a dynamic molecule, allowing for effective regulation of cardiac calcium sensitivity. First of all, cTnC undergoes a rapid closed-to-open equilibrium, with the open state being the biologically active form that binds the troponin I switch peptide. Troponin modulating drugs can act by binding the open state, with troponin activators enhancing the affinity for the switch peptide and troponin inhibitors blocking this interaction. The cTnC domain as a whole is dynamic, being connected to cTnI and the rest of the troponin complex by a flexible linker. Stabilizing or disrupting the orientation of cTnC via weak electrostatic interactions is an important regulatory mechanism readily impacted by phosphorylation of troponin I. Finally, the availability of the troponin I switch peptide as the thin filament alternates between blocked, closed, and open states, is an important downstream modulator of cTnC function that can be impacted by modifications in myosin, actin, tropomyosin, and the rest of the troponin complex. All of these dynamic processes (the closed-to-open equilibrium, domain positioning, and switch peptide availability) are readily impacted by cardiomyopathy-causing mutations. Understanding the dynamic structural biology of cardiac troponin C within the thin filament will impact not only the treatment of the heritable cardiomyopathies, but also the treatment of heart failure of any etiology.

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Highlights

- Cardiac troponin C (TNNC1) is the sarcomeric calcium sensor in cardiac and slow skeletal muscle
- The C-domain has two high affinity Ca^{2+} binding sites and is anchored to troponin I and T
- Ca^{2+} binding to the N-domain shifts muscle from relaxed to contracted states
- Mutations in TNNC1 are associated with hypertrophic or dilated cardiomyopathy
- Troponin modulators bind the open conformation of the N-domain and have therapeutic potential

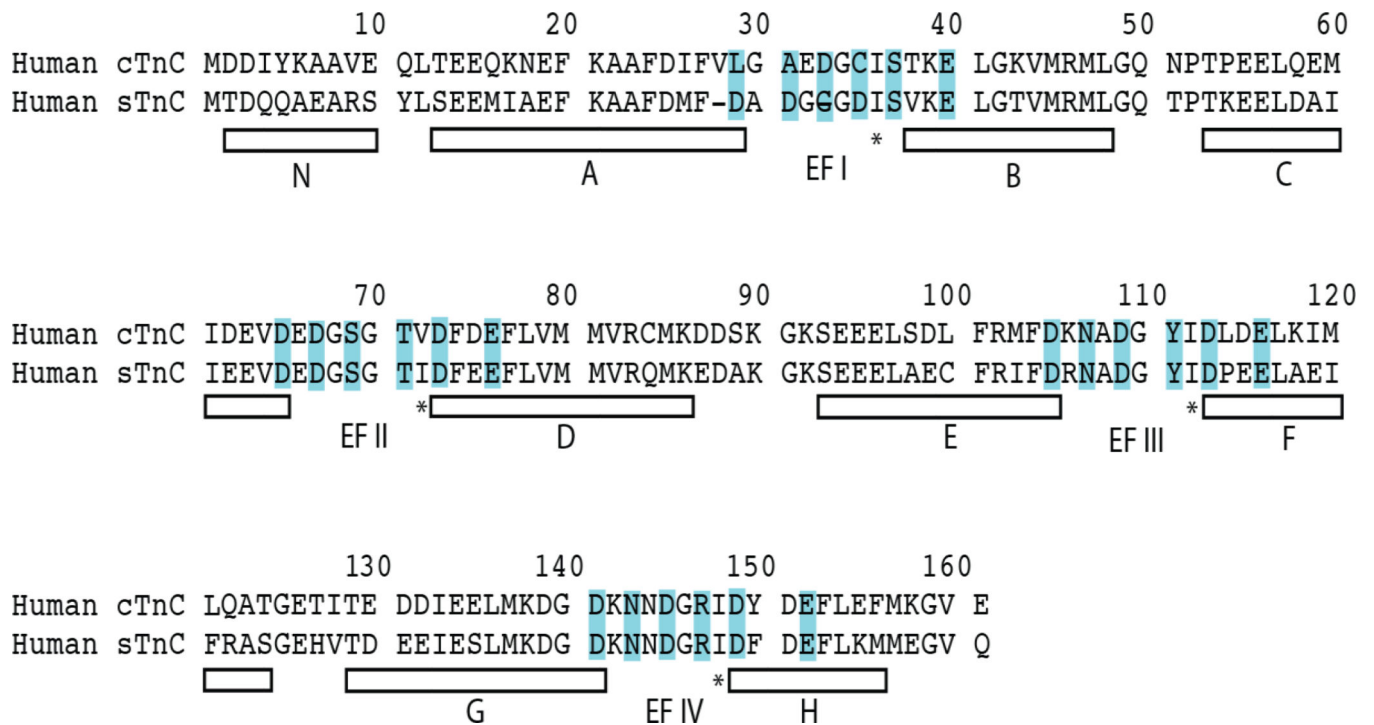


Figure 1.

Amino acid sequence comparison of human cTnC and sTnC. Helices are denoted by white bars below the sequences. In cTnC, residues 87–92 comprise the inter-domain helix. Calcium-coordinating EF-hand positions 1, 3, 5, 7, 9, and 12 are highlighted in cyan. Note, however, that EF-hand I in cTnC is defunct. Position 8 of each EF-hand is marked with an asterisk, denoting the hydrogen bonding central β -sheet-forming residue in each EF-hand.

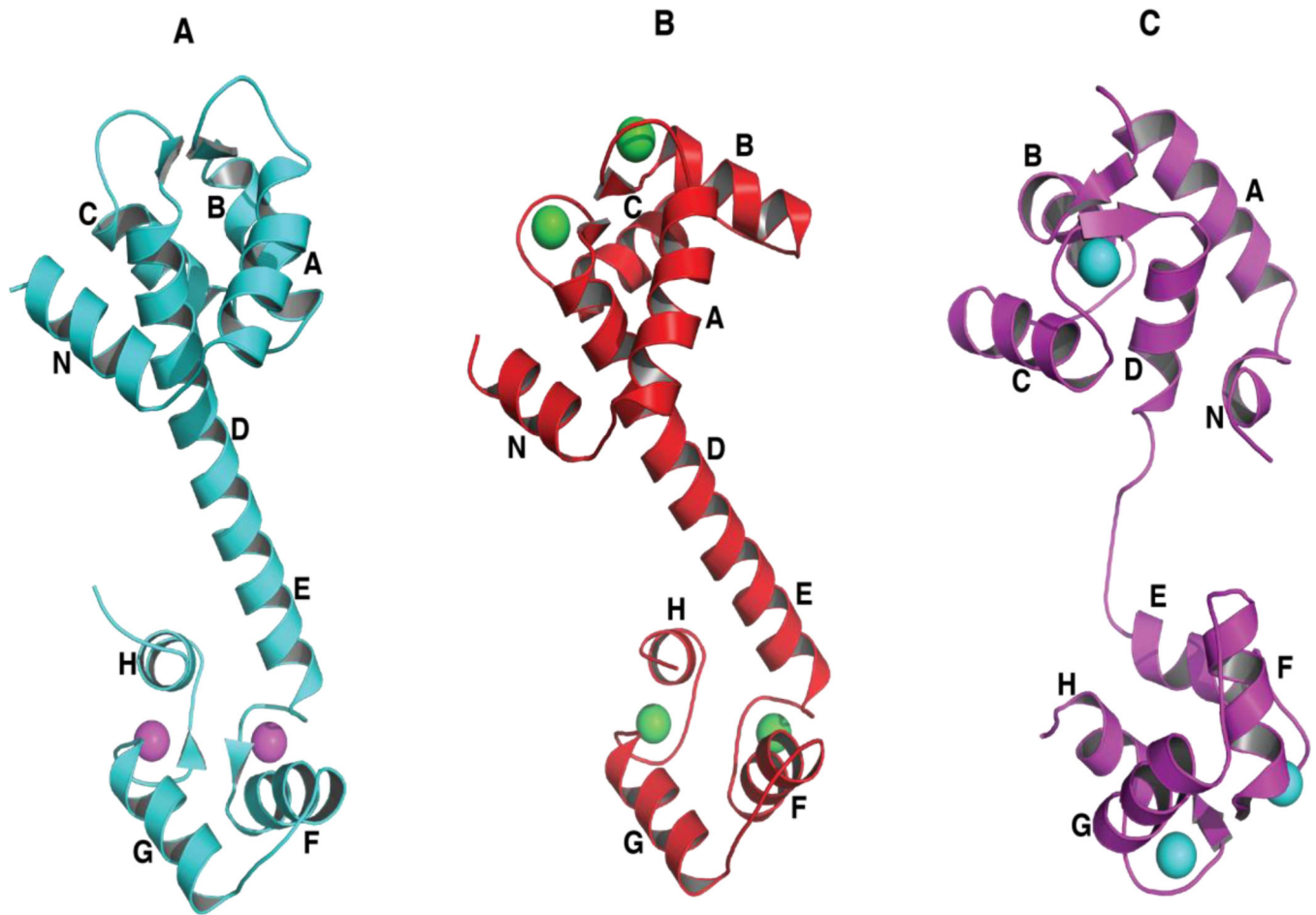
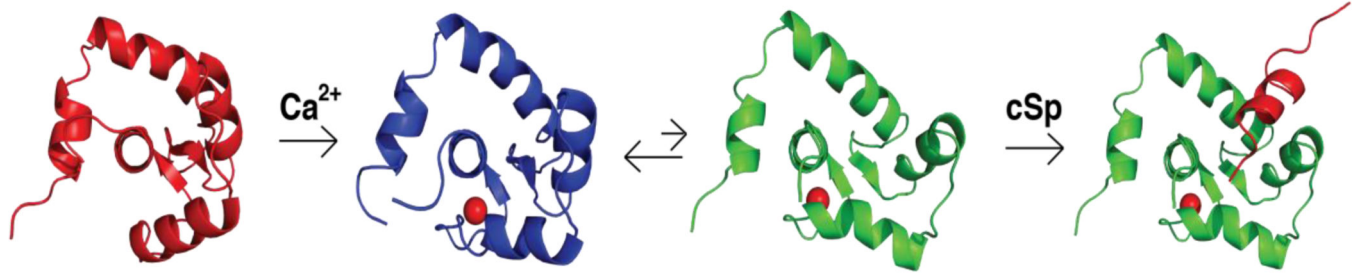
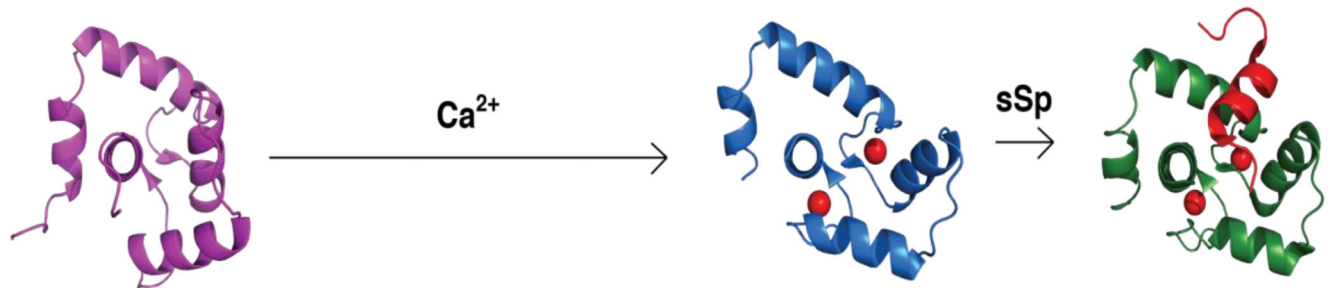


Figure 2. Structures of sTnC and cTnC: A. sTnC•2Ca²⁺ (X-ray, pdb 5TNC); B. sTnC•4Ca²⁺ (X-ray, pdb 2TN4); C. cTnC•3Ca²⁺ (NMR, pdb 1AJ4). Ca²⁺ ions are shown as spheres.

cNTnC



sNTnC

**Figure 3.**

Comparison of the Ca^{2+} - and cTnI switch peptide-induced structural changes in cNTnC and sNTnC. The pdb code for the structures are cNTnC•apo (1SPY), cNTnC• Ca^{2+} (1AP4), cNTnC• Ca^{2+} •cSp (1MXL), sNTnC•apo (1TNP), sNTnC• 2Ca^{2+} (1AVS), sNTnC• 2Ca^{2+} •sSp (1YTZ). Ca^{2+} ions are shown as spheres. Cardiac switch peptide (cSp) and fast skeletal switch peptide (sSp) are shown in red in the far right complexes.

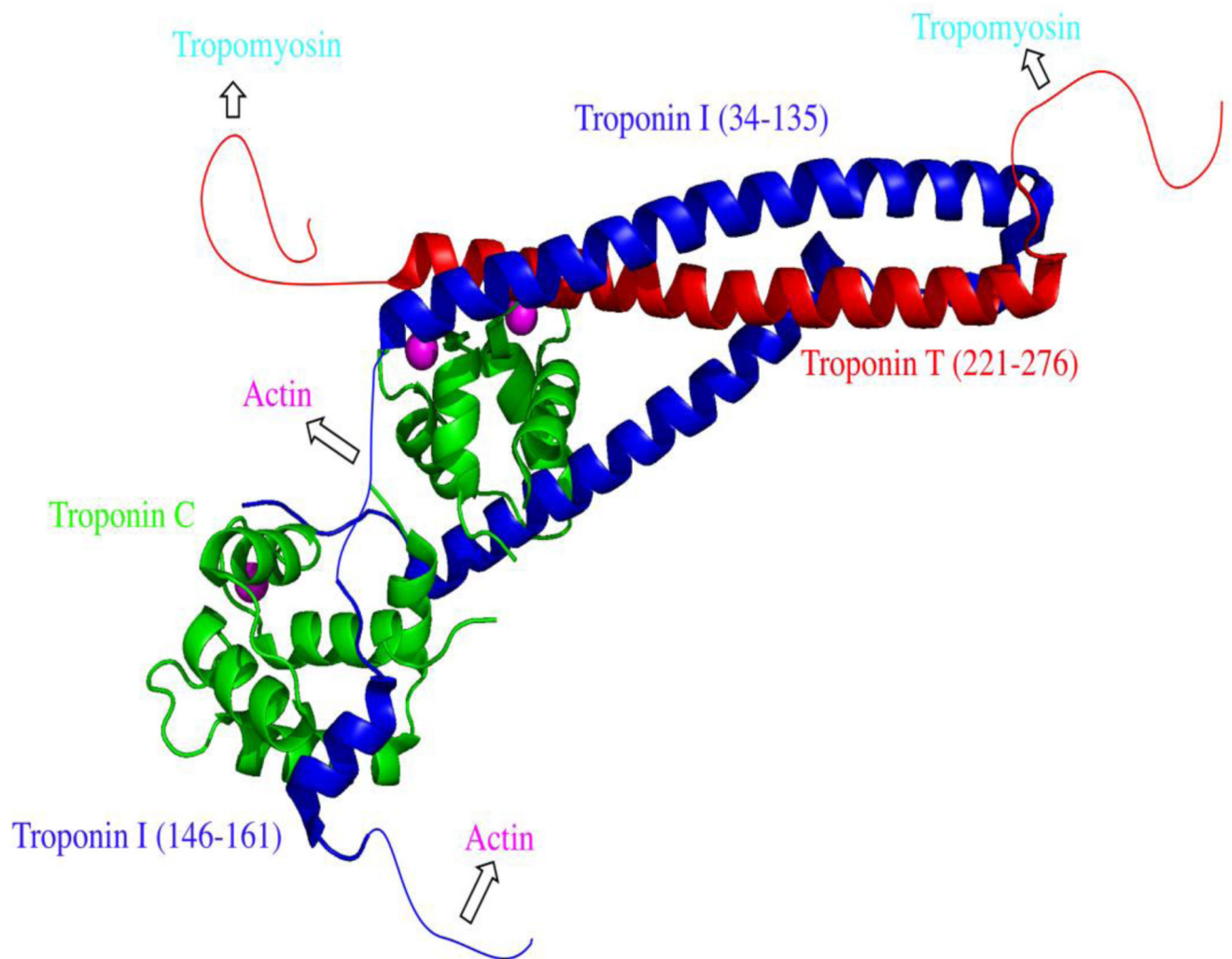


Figure 4.

X-ray structure of cardiac troponin core complex, $cTnC \cdot 3Ca^{2+} \cdot cTnI_{34-161} \cdot cTnT_{221-276}$ (pdb 1J1E). $cTnC$ is colored in green and the 3 bound Ca^{2+} are represented by magenta spheres, $cTnI$ is colored in blue, and $cTnT$ is colored in red. Regions N-terminal and C-terminal to $cTnT_{221-276}$ are known to anchor the entire complex to tropomyosin and have been drawn in as squiggles to show that their structures are unknown. $cTnI_{136-145}$ and $cTnI_{162-209}$ are known to interact with actin to maintain the blocked state of the thin filament. These regions are shown as blue squiggles to signify that they are unstructured in the calcium-activated state, but acquire an as-of-yet undetermined structure bound to actin in the blocked state.

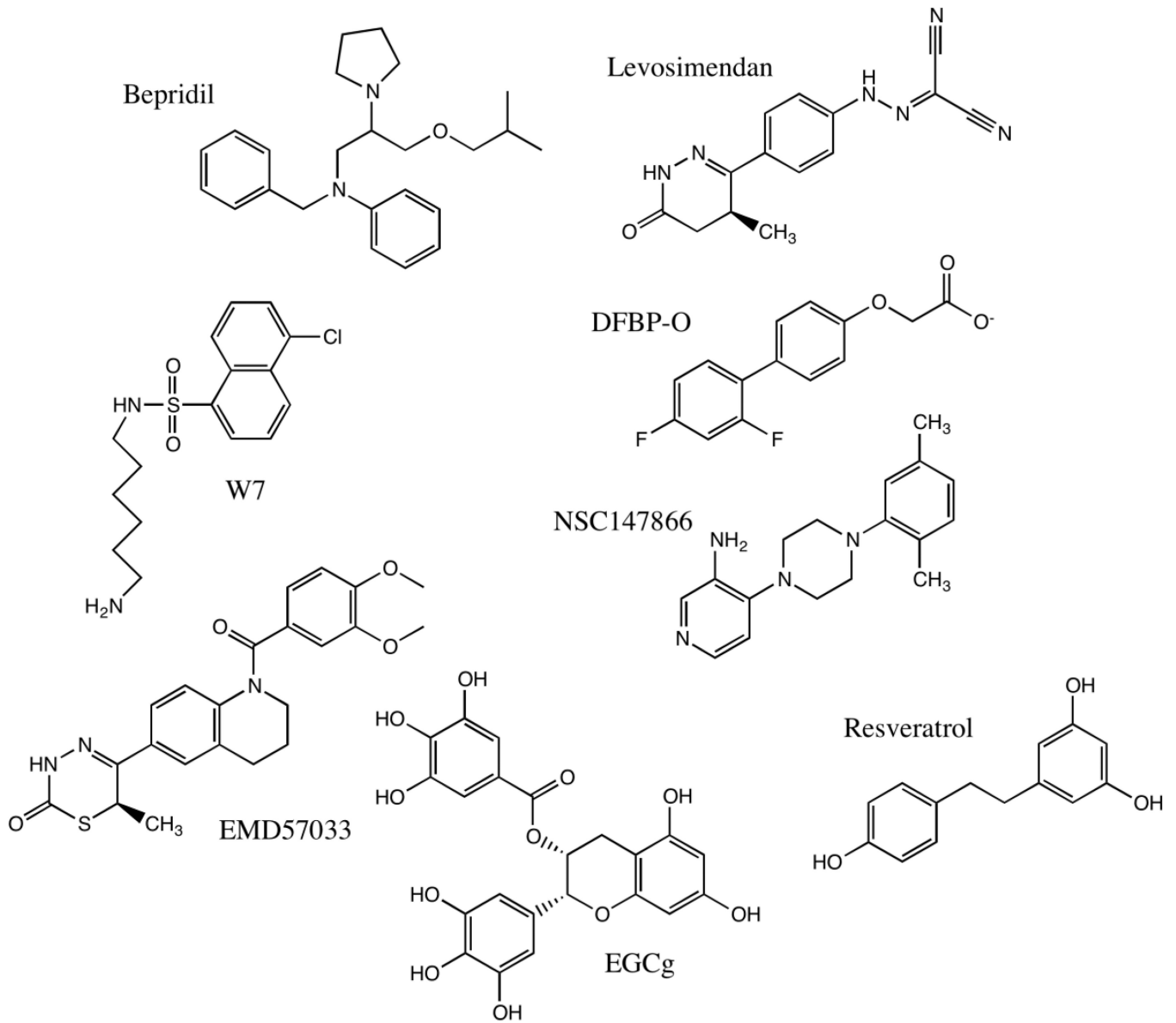


Figure 5.
Chemical structures of compounds known to bind to cardiac troponin C.

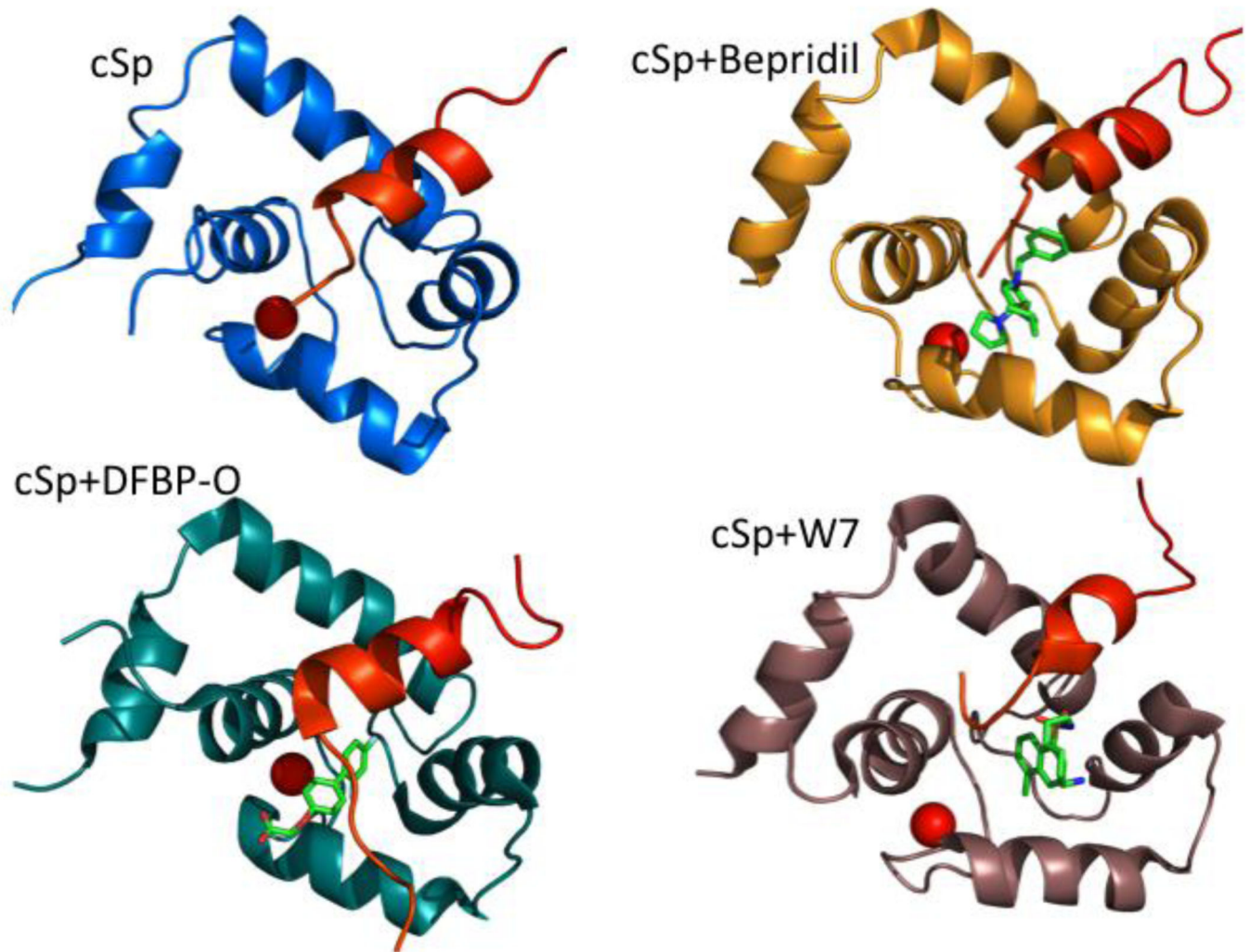


Figure 6.

Structures of cTnC in complex with drugs and/or cTnI peptides: cTnC•Ca²⁺•cSp (pdb 1MXL) is colored blue, cTnC•Ca²⁺•cSp•bepridil (pdb 1LXF) is colored gold, cTnC•Ca²⁺•cSp•DFBP-O (pdb 2L1R), cTnC is colored teal, and cTnC•Ca²⁺•cSp•W7 (pdb 2KRD) is colored brown. The cardiac switch peptide (cSp) is colored red, Ca²⁺ ions are shown as red spheres, and drugs are shown as sticks.

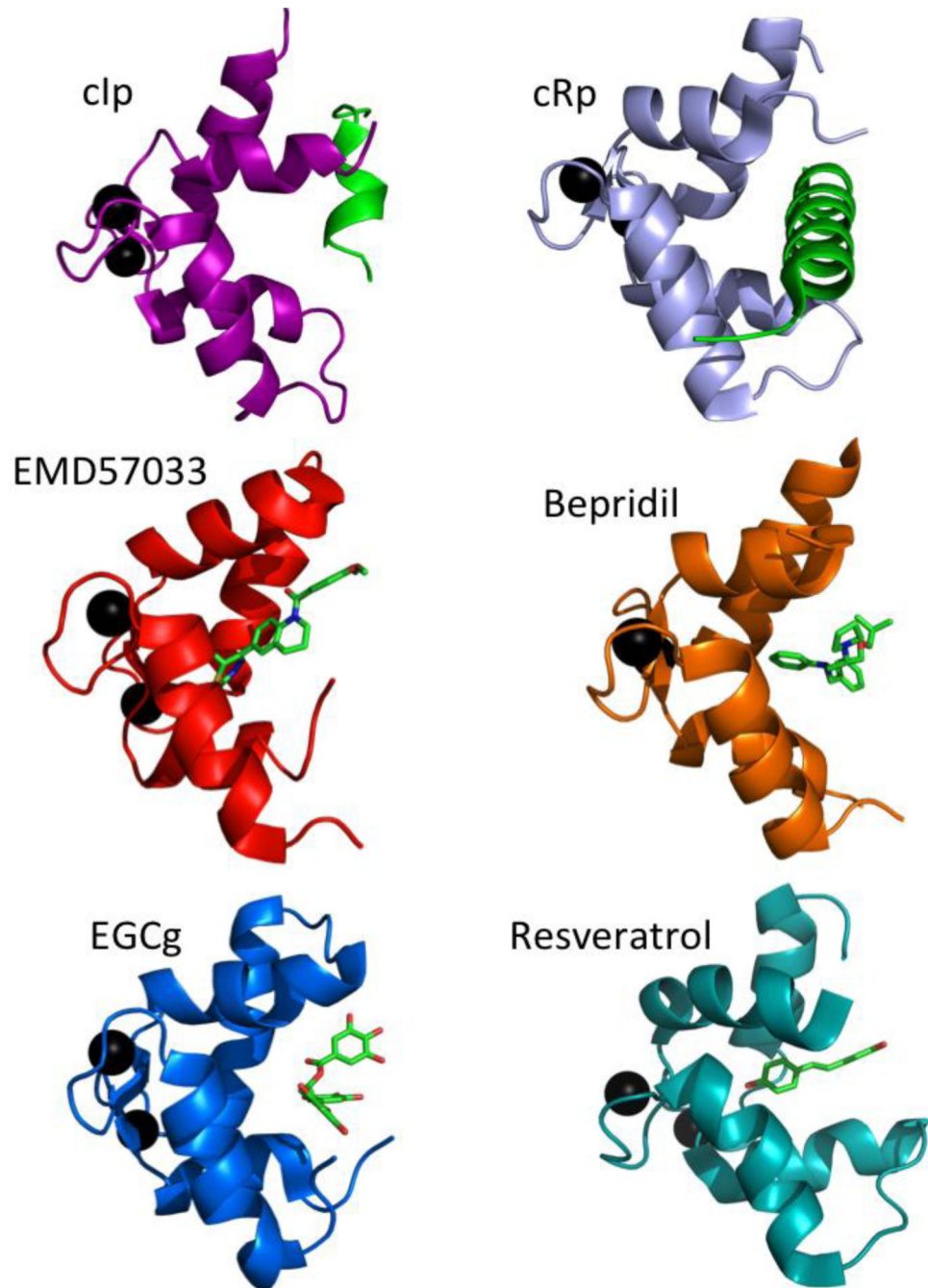


Figure 7. Structures of cTnC in complexes with drugs or cTnI peptides: cTnC•2Ca²⁺•cIp (pdb 1OZS) is colored magenta, cTnC•2Ca²⁺•cRp (pdb 1J1E) is colored slate, cTnC•2Ca²⁺•EMD57033 (pdb 1IH0) is colored red, cTnC•2Ca²⁺•bepridil (pdb 1DTL) is colored orange, cTnC•2Ca²⁺•EGCg (pdb 2KDH) is colored blue, and cTnC•2Ca²⁺•resveratrol (pdb 2L98) is colored cyan. Ca²⁺ ions are shown as black spheres,

inhibitory peptide (cIp, cTnI₁₂₈₋₁₄₇) and regulatory peptide(cRp, cTnI₃₄₋₇₁) are shown in green, and drugs are shown as sticks.

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