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Troponin I modulation of cardiac performance: Plasticity in the survival switch

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Abstract

Signaling complexes targeting the myofilament are essential in modulating cardiac performance. A central target of this signaling is cardiac troponin I (cTnI) phosphorylation. This review focuses on cTnI phosphorylation as a model for myofilament signaling, discussing key gaps and future directions towards understanding complex myofilament modulation of cardiac performance. Human heart cTnI is phosphorylated at 14 sites, giving rise to a complex modulatory network of varied functional responses. For example, while classical Ser23/24 phosphorylation mediates accelerated relaxation, protein kinase C phosphorylation of cTnI serves as a brake on contractile function. Additionally, the functional response of cTnI multi-site phosphorylation cannot necessarily be predicted from the response of individual sites alone. These complexities underscore the need for systematically evaluating single and multi-site phosphorylation on myofilament, cellular and *in vivo* contractile function. Ultimately, a complete understanding of these multi-site responses requires work to establish site occupancy and dominance, kinase / phosphatase signaling balance, and the function of adaptive secondary phosphorylation. As cTnI phosphorylation is essential for modulating cardiac performance, future insight into the complex role of cTnI phosphorylation is important to establish sarcomere signaling in the healthy heart as well as identification of novel myofilament targets in the treatment of disease.

Keywords

troponin I; phosphorylation; cardiac; contraction; relaxation; signaling

Cardiac performance is determined by rhythmic cycles of systolic contraction and diastolic relaxation, to pump blood to meet the energy demands of peripheral tissues[1]. Nearly 80% of protein within the contractile cardiac myocyte is organized into sarcomeres, assembled in

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parallel arrays of myofilaments. Each sarcomere is composed of myosin-containing thick filaments and regulatory thin filaments (TFs) ultimately responsible for contraction and relaxation in response to intracellular Ca^{2+} transients. The cardiac TF is composed of actin, tropomyosin and a heterotrimeric troponin (cTn), consisting of the calcium-binding subunit, troponin C (cTnC); the tropomyosin-binding subunit, troponin T; and the inhibitory subunit troponin I (cTnI). When intracellular Ca^{2+} levels are low, cTn and tropomyosin sterically inhibits actin interactions with myosin. With each heartbeat, action potential-induced increases in intracellular Ca^{2+} bind to cTn inducing TF conformational changes, allowing myosin to strongly bind actin and generate force. During relaxation, Ca^{2+} dissociates from cTn as intracellular Ca^{2+} levels decrease, and the interaction between actin and myosin is once again blocked by cTn[2].

Signaling mechanisms are critical to modulate each component of enhanced cardiac pump performance in response to increased peripheral energy demand. Several earlier reviews have summarized modulatory mechanisms of myosin[3] and Ca^{2+} [4]. This review focuses on phosphorylation of the TF molecular switch protein cTnI, which was the first reported sarcomere-based cardiac post-translational modification (PTM)[5, 6]. As an increasing number of signaling- and stress- pathways targeting cTnI for phosphorylation at multiple residue sites are identified[7], it has become appreciated that the phosphorylation of cTnI modulates contractile function to meet bodily demand much like a rheostat. Understanding cTnI-mediated modulation of contraction and relaxation has reached a crossroads that requires integrated insight into the functional role played by these multiple phosphorylation sites. The functional response of cTnI phosphorylation is accordingly expected to serve as a template for gaining insight into PTM of other TF proteins. We therefore review the established mechanisms and new directions of cTnI-mediated cardiac modulation mediated via phosphorylation. The influences of other TF PTMs are also important, and are covered in more detail in earlier reviews.[7]

Cardiac troponin I Ser23/24 phosphorylation: Historical perspective

Phosphorylation of cTnI was first described in the early 1970's[5, 6], and its physiological significance was established in studies showing cTnI phosphorylation closely correlated in time with the *in vivo* response to β-adrenergic receptor (β-AR) activation of protein kinase A (PKA) and the increase in cardiac pump performance[8, 9]. Later biochemical and biophysical work demonstrated PKA phosphorylates Ser23 and Ser24 residues in the cardiac-specific, N-terminal extension of human cTnI (Fig. 1A, B; all residues discussed are numbered according to the human cTnI sequence containing the initial methionine; Uniprot P19429), to increase Ca^{2+} dissociation from cTnC and reduce the Ca^{2+} sensitivity of myofilament force[10–12]. These changes in myofilament function translated into the important accelerated relaxation, or positive lusitropic response observed in response to PKA activation[13]. Extensive studies subsequently demonstrated cTnI Ser23/24 phosphorylation accelerates *in vivo* relaxation[14–17]. The replacement of endogenous cTnI with phospho-mimetic cTnI Ser23/24Asp in transgenic animal models provided direct evidence showing cTnI mediated Ser23/24 phosphorylation and the shift in myofilament Ca^{2+} sensitivity significantly contributes to the accelerated relaxation in response to β_1-AR-mediated activation of cardiac PKA[14, 15]. Further support for this idea was demonstrated by

the absence of a PKA-induced change in myofilament Ca^{2+} sensitivity of knock-in cTnISer23/24Ala mice[18, 19]. The transduction mechanism for cTnI Ser23/24 phosphorylation mediated accelerated relaxation resulted from the reduced ability of the cTnI H3 helix "switch peptide" to insert into the hydrophobic patch of cTnC in response to Ca^{2+} [16] which accelerates the release of cTnI from cTnC[20] (Figure 1B)

Under physiological conditions, cTnI Ser24 is more likely to be constitutively phosphorylated in the myocardium[21,22] and is phosphorylated more quickly than Ser23[12, 21]. Until recently, accelerated relaxation was believed to be dependent on both Ser23 and Ser24 phosphorylation [12, 22]. More recent work demonstrated this accelerated relaxation is possible when only one of the Ser sites is phosphorylated, however a complete understanding of the *in vivo* role of this single phosphorylation has yet to be determined[23]. In addition to physiological regulation, cTnI Ser23/24 phosphorylation is also important in pathophysiological conditions with Ser23/24 phosphorylation increasing during ischemia[24, 25] but decreasing in human and animal models of decompensated heart failure[26–28]. In heart failure the down regulation of β-AR signaling reduces cTnISer23/24 phosphorylation which in turn slows myocardial relaxation to further deteriorate performance[29, 30].

Dynamic modulation of the sarcomere: Multiple kinases and cTnI target residues

In addition to PKA, the cTnI Ser23/24 phosphorylation cluster also is a target for other AGC kinases PKG and PKC[31, 32], as well as kinases from other kinome families, including PKD[33], ROCKII[34], RSK[35, 36], and PAK1[37]. There is also evidence AMPK and phosphorylase kinase phosphorylate the Ser23 residue[38–40]. Moreover, human cTnI residues are phosphorylated at 12 additional sites including Ser5, Ser6, Tyr26, Ser42, Ser44, Thr51, Ser77, Thr78, Thr143, Ser166, Thr181, Ser199, and residues equivalent to Tyr31 and Ser150 are phosphorylated in mammalian hearts[39, 41, 42] (Figure 1A). Unlike Ser-23/24, the kinases responsible for phosphorylation at these sites are often not known or incomplete.

The functional output for most of these other cTnI phosphorylation sites remains to be thoroughly investigated, especially at the cellular and *in vivo* level. Classically, there is a dogma that cTnI phosphorylation functions as an "*accelerator*" to enhance relaxation during periods of enhanced cardiac performance, which is largely based upon extensive work showing Ser23/24 phosphorylation accelerates diastolic relaxation[13–15, 17]. However, it is becoming increasingly clear that phosphorylation at other cTnI sites can act as a "brake" to ultimately slow and/or reduce pump function[43–45]. Thus, multiple kinases can target a single cTnI site, such as Ser23/24, and sites other than Ser23/24 within cTnI to produce different functional responses. As a result, there is growing recognition that cTnI modulation of the sarcomere is dynamic and requires an integrated understanding of the role that multiple phosphorylated sites play within cTnI as well as coordination at a single site by multiple kinases and phosphatases.

PKC phosphorylation of cTnI: An example of cTnI phosphorylation acting as a “brake”

In contrast to the accelerated relaxation produced by cTnI Ser23/24 phosphorylation, Ser42/44 or Thr143 phosphorylation responses are better defined as “brakes”. Protein kinase C (PKC) targets these 2 clusters in addition to Ser23/24 for phosphorylation[32, 46](Figure 1B). Increased phosphorylation of these sites is reported under physiological and pathophysiological conditions[41, 47–51], although technical issues have made it difficult to precisely establish Ser42 versus Ser44 phosphorylation levels. Experimental phosphorylation of cTnI Ser42/44 and phospho-mimetic substitutions each cause reduced myofilament Ca^{2+} sensitivity of tension, which is likely explained by conformational interactions between cTnI and cTnC that produce a higher dissociation constant (K_d) and faster Ca^{2+} off rate from cTn[45, 52–54]. In addition, phospho-mimetic cTnI Ser42/44 reduces peak tension and slows sliding speed in motility assays[45]. Studies on intact myocytes serve as an important bridge for integrating *in vitro* and *in vivo* studies, by showing that cTnI-Ser42Asp or –Ser44Asp each act as a brake to reduce the amplitude and rate of myocyte shortening and re-lengthening, although their influence is not additive[43]. Both cTnI-Ser42Asp and -Ser44Asp appear to dominantly reduce contractile function, even in the presence of equal amounts of phospho-mimetic Ser23/24Asp[44].

Similar to Ser-42/44, biochemical studies demonstrate Thr143 phosphorylation decreases the Ca^{2+} sensitivity of *in vitro* sliding speed, but not isometric tension[45, 55]. In addition, phospho-mimetic Thr143 has little influence or decreases the thin filament Ca^{2+} off rate[53, 55], indicating Thr143 works via a different mechanism than Ser42/44. Unlike Ser23/24, the conformational changes responsible for the responses to Ser42/44 or Thr143 are not well defined[45, 55]. Mechanistic insight often depends on the use of phospho-mimetic as well as phospho-null substitutions, but these approaches have proven challenging in the study of these sites as the typical use of phospho-null Ala substitutions at cTnI Ser42/44 is not functionally conservative and instead reduces myofilament Ca^{2+} sensitivity[19, 54, 56]. However, intact myocyte work shows that myofilament function is conserved when using a polar Asn substitution as a phospho-null substitution at Ser42/44[56], which will be helpful for gaining mechanistic insight in the future. In addition, these results highlight the need to validate phospho-mimetic and phospho-null substitutions at each phosphorylated residue.

The myofilament responses to Ser42/44 and Thr143 phosphorylation predict that *in vivo* models with phospho-mimetic substitutions should develop a reduction in pump function. Instead, complex and divergent phenotypes develop in mice expressing combinations of phospho-mimetic or phospho-null substitutions at the PKC-targeted cTnI Ser23/24, Ser42/44, and Thr143[57–59]. Moreover, there is a wide range of exogenous cTnI replacement levels observed in these mice. For example, complete replacement with phospho-mimetic cTnISer23/24/42/44/Thr143 produces only minimal reductions in diastolic function while <10% replacement with the cTnI Ser42/44/Thr143 phospho-mimetic results in severe systolic and diastolic dysfunction[58, 59]. To date, there are no published models with only Ser42/44 or Thr143 phospho-mimetics, or functionally conservative phospho-null

substitutions to resolve the ongoing debate about the roles these residues play in modulating cardiac performance.

Dynamic sarcomere modulation by cTnI phoshorylation: Current insights and gaps

To date, the majority of cTnI phosphorylation-mediated functional effects have been determined based on phosphorylation changes in isolation at an individual site, yet cTnI phosphorylation in the human heart does not solely occur at an individual site but rather occurs at multiple sites[41]. As the signaling cascades causing cTnI phosphorylation become better understood, we have begun to appreciate that kinase mediated signaling results in "phosphorylation clusters", or phosphorylation of multiple sites by a given kinase. For example, PKC-associated cellular signaling can lead to the phosphorylation of cTnI Ser23/24, Ser42, Ser44 and Thr143[32, 46]. Understanding the effect of multiple cTnI site phosphorylation is further complicated by the presence of multiple signaling pathways that can become simultaneously activated and also result in phosphorylation at multiple clusters[60, 61]. For example, PKA mediated phosphorylation of cTnI Ser23/24 and AMPK-induced phosphorylation at Ser150[39]. Finally, each of these additional phosphorylation events occur on top of the basal cTnI phosphorylation. A significant portion of cTnI in the heart is basally phosphorylated and cTnI Ser23/24 comprises ~40% of this basal phosphorylation[62, 63], any additional phosphorylation will occur at Ser23/24 as well as other sites. This integrated impact of basal TnI phosphorylation, together with transient signal- and stress-activated kinase activation and downstream targeting of cTnI phosphorylation clusters is important as it allows for optimal fine-tuning of cardiac performance. However, this flexibility also raises the need for studies investigating the complex myofilament, cellular, and *in vivo* responses and/or phenotypes produced by a phosphorylated cluster.

A difficulty towards understanding the integrated effects of multiple cTnI phosphorylation is that the functional response for a specific phosphorylation or cluster cannot necessarily be predicted based on the impact of each individual cTnI phosphorylation site. For example, phospho-mimetic cTnI Ser150Asp alone increases myofilament Ca^{2+} sensitivity, cooperativity and slows Ca^{2+} dissociation[39, 40]. Based on the similarly opposing impact of cTnI Ser23/24 and Ser150 phosphorylation on Ca^{2+} sensitivity and TF Ca^{2+} dissociation, cTnISer23/24/150Asp is not predicted to change Ca^{2+} sensitivity or Ca^{2+} dissociation from basal control values. However, while Ser150Asp attenuated the ability of cTnI Ser23/24Asp to decrease myofilament Ca^{2+} sensitivity, the Ca^{2+} dissociation remained accelerated similar to that of Ser23/24Asp alone [20, 39]. A mechanism for Ser150 phosphorylation is not definitively established but likely involves a decrease of cTnI C-terminal binding to actin and increased binding to cTnC to slow Ca^{2+} dissociation that differs from the Ser23/24 mechanism and includes modified cooperativity[20, 64] (Figure 1B). While the *in vivo* effect has yet to be determined, these results suggest that combination of cTnI Ser150 on top of basal Ser23/24 phosphorylation can enhance myocardial force production without altering Ca^{2+} transients and/or causing diastolic dysfunction in intact hearts, which could not be anticipated based on the *individual* effects of each phosphorylation site. Integrated cTnI Ser150 and Ser23/24 phosphorylation may therefore be significant during myocardial

ischemia when phosphorylation at both sites is elevated[25] and to improve cardiac performance during pathological systolic dysfunction.

Another example of integrated cTnI phosphorylation functional effects that would not be anticipated from the effects of the individual sites alone is observed from the function of combined cTnI Tyr26 and Ser23/24 phosphorylation. Isolated Tyr26 phosphorylation (Figure 1B), Tyr26Glu and Tyr26Asp each decreased myofilament Ca^{2+} sensitivity to a similar extent as Ser23/24Asp and Tyr26Glu accelerated TF Ca^{2+} dissociation similarly to Ser23/24Asp[65]. In contrast, the combination of the Ser23/24Asp/Tyr26Glu phospho-mimetic resulted in the expected non-additive reduction in Ca^{2+} sensitivity, but there was also a further doubling in the acceleration of TF Ca^{2+} dissociation. These findings suggest that the combination of cTnI Tyr26 on top of basal Ser23/24 phosphorylation further enhances diastolic relaxation beyond that of either phosphorylation alone, without further depressing systolic function. Increasing both cTnI Ser23/24 and Tyr26 phosphorylation therefore appears to be more effective at improving diastolic dysfunction in cardiac disease, such as heart failure with preserved ejection fraction, compared to either individual phosphorylation alone.

This integration of phosphorylation clusters is of further significance as it also extends beyond intra-cTnI interactions and includes evidence for communication between myofilament proteins[44, 66]. Together with the functional response to PKC –targeted cTnI presented above, these examples illustrate the important point that the independent functional effects of each phosphorylation site alone may not predict the response resulting from the integrated effects of multiple cTnI phosphorylations. The importance of this concept is further highlighted as the transduction mechanisms of phosphorylation integration within cTn is largely unknown for phosphorylation sites other than Ser23/24.

Our knowledge of integrated cTnI phosphorylation is further limited by our lack of understanding as to how phosphorylation occupancy level and functional dominance among phosphorylation sites modulate contractile function. While there is limited information about the functional dominance of one cTnI phosphorylation site compared to another, emerging evidence is describing the percent occupancy of individual cTnI phosphorylation sites under specific conditions[41]. An accurate quantification of the occupancy at any given site and the relative functional dominance of a site at a given occupancy are however challenging to determine and have yet to be systematically investigated. Moreover, it is unclear whether the same residues are phosphorylated on each cTnI or instead may be more heterogeneously dispersed either spatially or temporally across the population of cTnI within a sarcomere. Even though these important issues have yet to be resolved, it is clear that there is constitutive phosphorylation at multiple sites within cTnI that contributes to a basal state of contractile function[41]. Both kinase and phosphatase activity modulate contractile function by changing cTnI phosphorylation[7], and the further participation of both kinases and phosphatases are essential for oscillatory or dynamic signaling[67, 68]. As an end target for multiple kinases and phosphatases, cTnI phosphorylation is therefore a central target for fine-tuning cardiac performance in response to physiological and environmental factors as well as during stress and pathophysiological conditions. Dynamic modulation via cTnI is essential for compensation during chronic dysfunction and a reduction in the ability of

different cTnI phosphorylation sites to respond could accelerate or enhance contractile dysfunction. Thus, efforts to systemically address concepts such occupancy and dominance among the multiple cTnI phosphorylation sites is needed in the future.

A final issue when considering the integration of multiple phosphorylation sites is the growing evidence that sustained phosphorylation at one cTnI site triggers secondary signaling at other sites. This idea of a "secondary phosphorylation" response was first reported in mice expressing cTnISer42/44Ala[69]. Subsequently, the potential for secondary phosphorylation to play an adaptive functional role was demonstrated in isolated myocytes expressing cTnI Ser42Asp or Ser44Asp phospho-mimetics[43, 44] In these studies, the initial reduction in shortening rate resulting from expression of these phospho-mimetics partially returned to baseline over time even though Ser42Asp or Ser44Asp replacement continued to increase. This partial return of contractile function concurrent with secondary phosphorylation at other cTnI sites and myofilament proteins[43, 44] led to the conclusion that the onset of secondary phosphorylation serves an adaptive role to return function back toward a basal steady state or "set-point", the absence of which produces a stress that results in disease[70]. This conclusion is consistent with the variable systolic and diastolic phenotypes reported in mice expressing cTnISer42/44 combined with Ser23/24 and/or Tl43 phosphomimetics[57–59, 69]. Adaptation of function in response to secondary cTnI phosphorylation adds another level of complexity, but also establishes cTnI as an important contributor to fine-tune sarcomere function. The consistent association between heart disease and elevated PKC activity, which targets Ser42/44 and Thr143[32, 49, 54, 71] also suggests that this secondary phosphorylation may be particularly important during cycles of stress or under chronic pathological conditions. As a result, the absence or loss of the secondary phosphorylation response over time may push the sarcomere further from its set-point causing a faster and/or more severe deterioration in cardiac function. Alternately, maintaining or initiating additional secondary phosphorylation may restore the sarcomeric set-point and delay disease progression as demonstrated by expression of the desensitizing TnI Ser23/24Asp phospho-mimetic in a sensitized, genetic hypertrophic model[70]. Thus, future systematic studies are critical to understand spatial and temporal secondary phosphorylation and their impact on myocyte and *in vivo* adaptive function after primary cTnI phosphorylation events or pathological stress.

Future directions

The dynamic nature of cTnI phosphorylation discussed above shows that this molecular switch protein plays an essential role in modulating sarcomere function in both health and disease. Specifically, modulation via cTnI helps maintain basal contractile function under physiological conditions, and could also play a central role in adaptive phosphorylation during the compensatory phase of heart disease. Thus, it is critical to fully understand the impact of multiple integrated cTnI phosphorylation sites and clusters on cardiac performance. To achieve this goal requires future research utilizing multidisciplinary approaches to define the signaling cascades that target cTnI, their sites of phosphorylation and their temporal activation in response to environmental and/or pathophysiological conditions. The transduction mechanism(s) resulting from phosphorylation events and their functional affects also need to be defined at both the myofilament and cellular level. Insight

into the integrated effects of multiple phosphorylations and the dynamic component of a cTnI signal is equally important and requires both cellular and *in vivo* approaches to evaluate the response to primary phosphorylation alone, the resulting changes in signaling and/or secondary phosphorylation events and pathological induced stress. These responses should also address whether a phosphorylation cluster observed under physiological conditions is changed under pathophysiological conditions or in response to therapeutic drugs and devices. The resulting work will fill significant gaps in our understanding of cTn and TF function, provide significant insight into the dynamic nature of the sarcomere and its regulation as well as ultimately make available a more complete understanding of the disease process and novel areas to intervene.

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Abbreviations

TFs	thin filaments
cTn	cardiac troponin
cTnC	cardiac troponin C
cTnI	cardiac troponin I
PTM	post-translational modification
β-AR	β-adrenergic receptor
PKA	protein kinase A
PKC	protein kinase C

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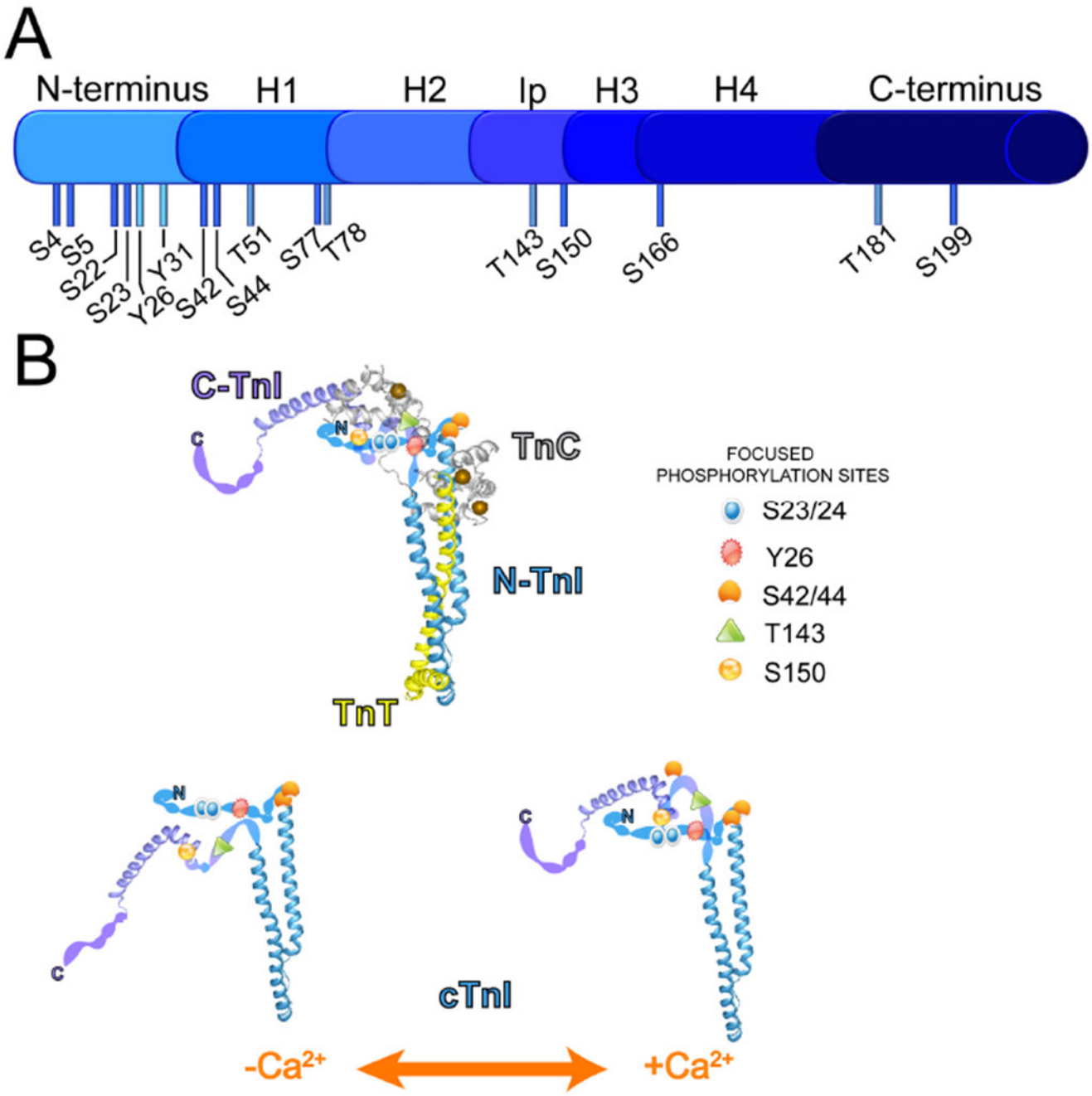

FIGURE 1.

A. Location of known Ser (S), Thr (T), and Tyr (Y) phosphorylated residues on cardiac troponin I (cTnI). **B.** Location of phosphorylation sites within the troponin complex composed of cTnI, troponin T (TnT) and troponin C (TnC) and in the proposed TnI protein structure in the absence ($-Ca^{2+}$) and presence ($+Ca^{2+}$) bound to TnC. N-TnI refers to the amino-terminus and C-TnI to the carboxy-terminus of TnI. Structures in B are adapted from Takeda et al., 2003 (ref 72).