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Distinct conformational and functional effects of two adjacent pathogenic mutations in cardiac troponin I at the interface with troponin T

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A B S T R A C T

The α-helix in troponin I (TnI) at the interface with troponin T (TnT) is a highly conserved structure. A point mutation in this region, A116G, was found in human cardiac TnI in a case of cardiomyopathy. An adjacent dominantly negative mutation found in turkey cardiac TnI (R111C, equivalent to K117C in human and K118C in mouse) decreased diastolic function and blunted beta-adrenergic response in transgenic mice. To investigate the functional importance of the TnI–TnT interface and pathological impact of the cardiac TnI mutations, we engineered K118C and A117G mutations in mouse cardiac TnI for functional studies. Despite their adjacent locations, A117G substitution results in faster mobility of cardiac TnI in SDS–PAGE whereas K118C decreases gel mobility, indicating significant and distinct changes in overall protein conformation. Consistently, monoclonal antibody epitope analysis demonstrated distinct local and remote conformational alterations in the two mutant proteins. Protein binding assays showed that K118C, but not A117G, decreased the relative binding affinity of cardiac TnI for TnT. K118C mutation decreased binding affinity for troponin C in a Ca^{2+}-dependent manner, whereas A117G had a similar but less profound effect. Protein kinase A phosphorylation or truncation to remove the cardiac specific N-terminal extension of cardiac TnI resulted in similar conformational changes in the region interfacing with TnT and minimized the functional impacts of the mutations. The data demonstrate potent conformational and functional impacts of the TnT-interfacing helix in TnI and suggest a role of the N-terminal extension of cardiac TnI in modulating TnI–TnT interface functions.

1. Introduction

The troponin complex plays a central role in the Ca^{2+} regulation of cardiac and skeletal muscle contraction. The binding of Ca^{2+} to troponin subunit C (TnC) induces a series of conformational changes in troponin complex and sarcomeric actin thin filament to activate cross bridge cycling between myosin and actin and muscle contraction [1]. Troponin I (TnI) is the inhibitory subunit of troponin [2]. An α-helix in TnI (amino acids 90–135 in human cardiac TnI) [3] forms a coiled coil interface with troponin T (TnT) (Fig. 1A), the tropomyosin anchoring subunit of troponin

[4]. This segment of TnI is a relatively rigid structure [5] with amino acid sequence highly conserved in muscle type-specific TnI isoforms and across vertebrate species [6–8].

Very few pathogenic mutations have been found in this highly conserved region of cardiac TnI. A single amino acid substitution, A116G, was reported in association with cardiomyopathy [9]. An adjacent single amino acid substitution was found in the TnT-interfacing helix of wild turkey cardiac TnI (R111C, as the position equivalent to K117 in human cardiac TnI) in concurrence with an abnormally spliced myopathic cardiac TnT [6]. The substitution of Arg_{111} with Cys in turkey cardiac TnI reduced binding affinity for TnT, compensating for the effect of the abnormally spliced cardiac TnT. Overexpression of the corresponding mutation (K118C) in mouse cardiac TnI (McTnI$_{K118C}$) reduced diastolic function and blunted β-adrenergic response in transgenic mouse hearts [10]. Isolated adult cardiomyocytes from McTnI$_{K118C}$ transgenic mice exhibited lower contractile amplitude and slower velocities of shortening and re-lengthening than wild type controls with lower responses to β-adrenergic stimulation [11]. In the mean time,

ventricular cardiomyocytes of young $McTnI_{K118C}$ mice were significantly longer than cells isolated from age- and sex-matched wild type mice [11]. Such early remodeling of $McTnI_{K118C}$ cardiomyocytes prior to the development of clinical heart failure indicates potent effects of K118C mutation on myocardial remodeling. These two mutations with dominantly negative impacts on cardiac muscle function indicate the functional importance of the TnI–TnT interface in the troponin complex.

Different from slow and fast skeletal muscle TnI, cardiac TnI has an ∼30 amino acids N-terminal extension [2,12,13]. An adult heart-specific regulatory structure, the N-terminal extension contains β-adrenergic activated protein kinase A (PKA) phosphorylation sites $Ser_{23/S24}$ [14]. Phosphorylation of $Ser_{23/S24}$ modulates the function of cardiac TnI [12,15–17], involving long-range conformational modulations [18]. The N-terminal extension of cardiac TnI is also regulated by a selective deletion via limited, proteolysis, occurring in cardiac adaptation to hemodynamic stress and β-adrenergic deficiency [19–21]. PKA phosphorylation at $Ser_{23/S24}$ in the N-terminal extension or truncation of the entire N-terminal extension lowers myofilament Ca^{2+} sensitivity and facilitates cardiac muscle relaxation. Their similar and non-additive regulatory effects [22] implicate a common down-stream mechanism for the N-terminal extension to regulate the function of cTnI [22,23].

Consistent with this hypothesis, PKA phosphorylation and N-terminal truncation result in similar conformational modulations in cardiac TnI in the region interfacing with TnT and produce similar effects on the binding affinity of cardiac TnI for TnT and TnC [18]. Supporting the structural importance and sensitivity of the TnT interface of TnI, mutations in the C-terminal end segment of cardiac TnI (R193H and R205H), which cause restrictive cardiomyopathy, also result in conformational changes in this region [24].

To further understand the function and regulation of the TnT interface structure of TnI, here we characterized the conformational and functional effects of A117G and K118C mutations in intact as well as in N-terminal truncated mouse cardiac TnI. The results showed that the two adjacent TnI–TnT interface mutations produced distinct conformational and functional changes in cardiac TnI. The effects of N-terminal phosphorylation and truncation on the function of the mutant cardiac TnI were investigated for regulatory and compensatory impacts. The data demonstrate the critical role of the TnI–TnT interface in the regulation of muscle contraction and cardiac function.

2. Results

2.1. A117G and K118C mutations produced opposite effects on the mobility of cardiac TnI in SDS–PAGE

Wild type and mutant mouse cardiac TnI protein, intact or with the N-terminal extension truncated (Fig. 1B), were successfully expressed in *Escherichia coli* and purified (Fig. 2A and B). In addition to sequencing verification of the cDNA constructs, Western blots using mAb TnI-1 against a conserved C-terminal epitope of TnI confirmed the authenticity of recombinant proteins (Fig. 2A and B).

A striking finding is that $McTnI_{A117G}$ exhibits faster gel mobility than that of wild type mouse cardiac TnI in SDS–PAGE, whereas $McTnI_{K118C}$ exhibits slower SDS–gel mobility than that of wild type mouse cardiac TnI (Fig. 2A). Implication of the results is twofold: First, these single amino acid substitutions in the TnT interface helix of cardiac TnI corresponding to minimum changes in molecular mass (M_r for McTnI = 24,258, $McTnI_{A117G}$ = 24,245 and
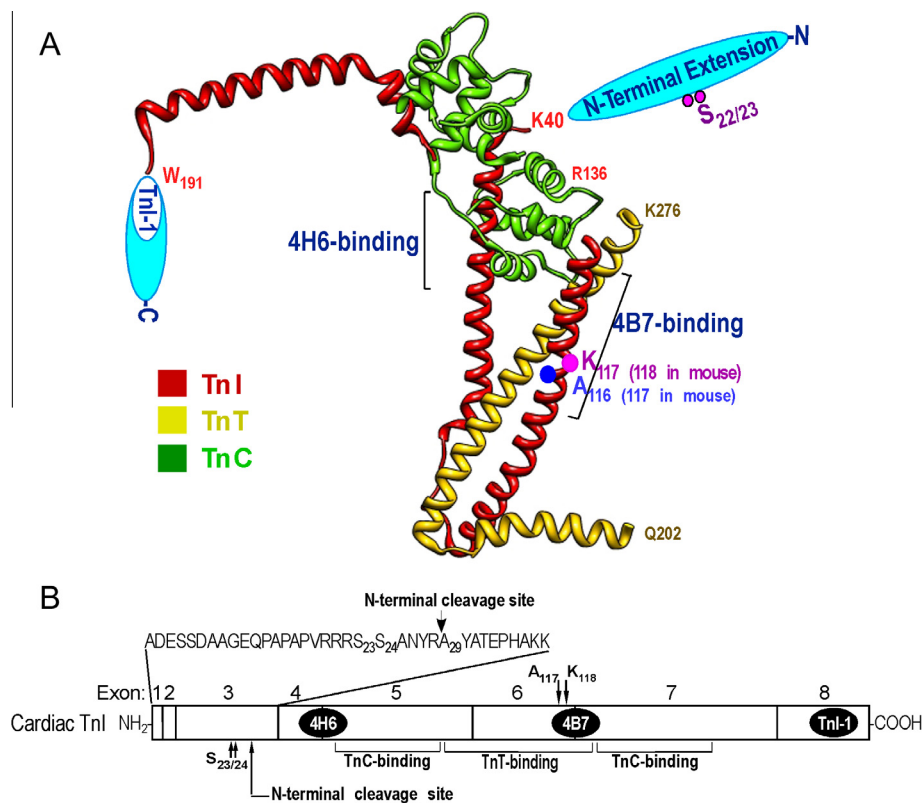
Fig. 1. Cardiac TnI mutations in the helix interfacing with TnT. (A) The residues A116 (A117 in mouse) and K117 (K118 in mouse) in human cardiac TnI at the TnT interface, the PKA phosphorylation sites $Ser_{23/24}$ in the N-terminal extension, and the epitopes for mAbs 4H6 and 4B7 are highlighted in the partial crystal structure of human cardiac troponin complex (redrawn from published data) [3]. (B) The linear map of mouse cardiac TnI illustrates the locations of PKA phosphorylation sites $Ser_{23/24}$, a naturally occurring cleavage site that selectively removes the cardiac-specific N-terminal extension to modify contractility, the TnT and TnC binding regions, the epitopes recognized by mAbs 4H6 and 4B7, and the A117G and K118C mutations.

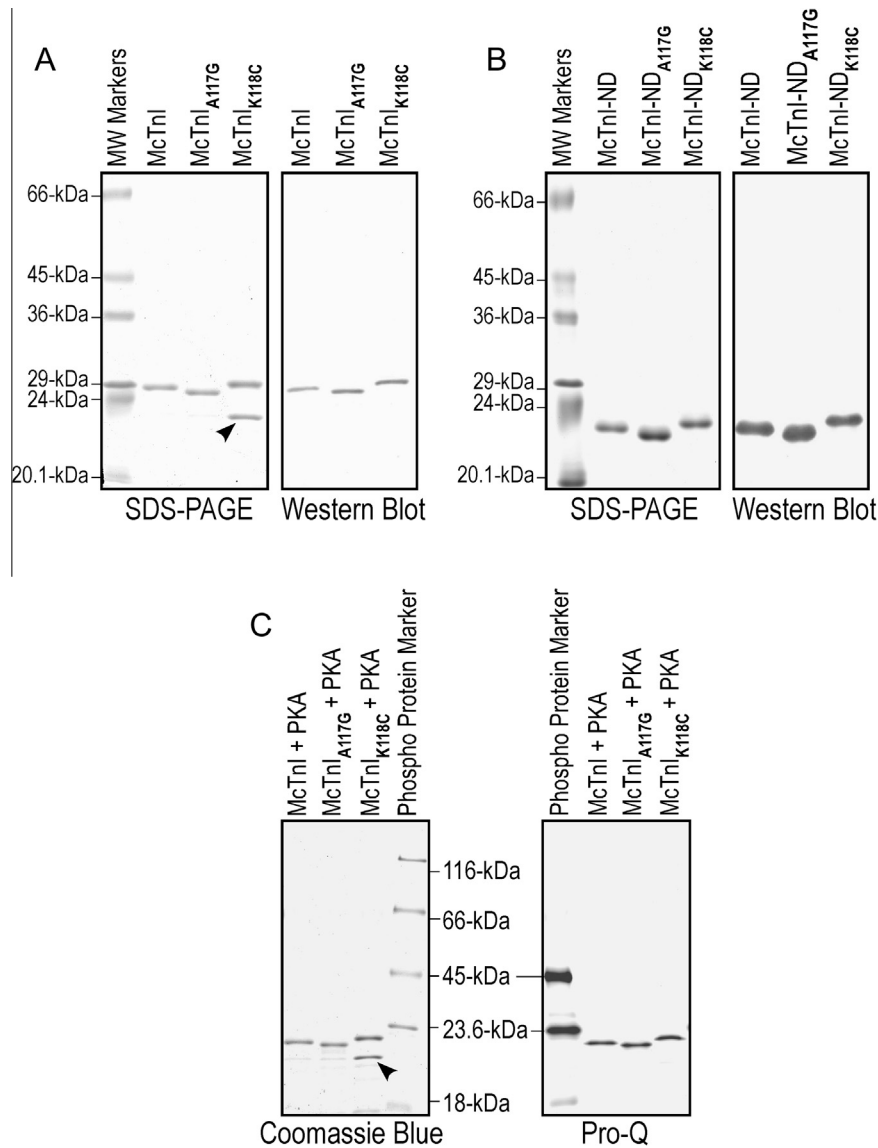**Fig. 2.** A117G and K118C mutations have opposite effects on the mobility of cardiac TnI in SDS–PAGE. (A) The SDS–PAGE and mAb TnI-1 Western blots showed that A117G mutation increased and K118C mutation decreased the gel mobility of intact mouse cardiac TnI in SDS–PAGE as compared with wild type control. There was a contaminant protein in the McTnI$_{K118C}$ preparation (pointed by the arrowhead), which however, did not affect the coating of McTnI$_{K118C}$ to ELISA plate for conformational and protein binding studies. (B) SDS–PAGE and mAb TnI-1 Western blots showed that removal of the N-terminal extension did not have qualitative effect on this feature of the mutations. (C) Pro-Q Diamond and Coomassie Blue staining of McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ showed effective phosphorylation after PKA treatment. The gel mobility of McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ decreased slightly upon phosphorylation, implicating an effect on the overall molecular conformation and the binding of SDS [29]. Different from the molecular weight makers used in (A) and (B), PeppermintStick phosphoprotein molecular weight marker was used as a control in Panel C.

McTnI$_{K118C}$ = 24,234, respectively) both result in profound structural changes readily detectable as mobility changes in SDS–gel; and second, A117G and K118C mutations produce distinct structural changes leading to the opposite effects on SDS–gel mobility independent of the trivial changes in molecular mass (Fig. 2A). In comparison with wild type cardiac TnI, the faster gel mobility of McTnI$_{A117G}$ may indicate a more compact or more compliant overall molecular conformation whereas McTnI$_{K118C}$ may have produced a more open or more rigid conformation.

N-terminal truncation that is known to result in long-range conformational modulations in cardiac TnI [18] did not change the gel mobility features of McTnI$_{A117G}$ and McTnI$_{K118C}$ (Fig. 2B). Therefore, the effects of the two point mutations at the TnT-binding interface on the molecular conformation of cardiac TnI appear to be dominant traits.

PKA catalyzed phosphorylation at Ser$_{23/24}$ in the N-terminal extension was at similar extents in McTnI$_{A117G}$, McTnI$_{K118C}$, and wild type McTnI as visualized by Pro-Q Diamond staining (Fig. 2C). The results also showed that PKA phosphorylation slightly decreased the mobility of McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$, consistent with the conformational modulation role of the N-terminal extension [18].

2.2. Conformational effects of A117G and K118C mutations on functional sites of cardiac TnI

ELISA epitope analysis using two anti-cardiac TnI mAb probes, 4B7 that recognizes an epitope in the helix interfacing with TnT and 4H6 that recognizes an epitope in the TnC-binding helix 1 (Fig. 1A), identified local and remote conformational changes resulting from A117G and K118C mutations of cardiac TnI.

The results in Fig. 3 showed that mAb 4B7 detected a statistically significant change in the local conformation of the TnT-interfacing helix in McTnI$_{A117G}$ but not McTnI$_{K118C}$. The results

demonstrate that although the two mutations are located next to each other in an α-helix (Fig. 1A), they had rather distinct effects on the local conformation.

Monoclonal antibody 4H6 detected a remote conformational change in the TnC-binding helix 1 of cardiac TnI resulting from the A117G mutation (Fig. 4). In contrast, the adjacent K118C mutation did not convey such remote conformational change (Fig. 4). The results further demonstrate that the two mutations also had distinct remote conformational effects.

2.3. Mutation K118C, but not A117G, lowers the relative binding affinity of cardiac TnI for TnT

Solid phase protein binding assay [25] revealed that the K118C mutation in the TnT interface helix resulted in a statistically significant decrease in the relative binding affinity of cardiac TnI for TnT (Fig. 5). On the other hand, the adjacent mutation A117G did not significantly alter the binding affinity for TnT (Fig. 5).

2.4. A117G and K118C mutations both decrease the relative binding affinity of cardiac TnI for TnC at pCa 4

ELISA solid phase protein binding assay demonstrated that at pCa 9, TnC binds TnI with low affinity (Fig. 6A), consistent with that shown in previous studies in solution or using affinity chromatography [26,27]. Neither A117G nor K118C mutation had detectable effect on the binding affinity of cardiac TnI for TnC at pCa 9 (Fig. 6A).

At pCa 4, the binding affinity of TnC for TnI was significantly higher than that at pCa 9 (Fig. 6B), also consistent with previous studies using traditional protein binding assays [27]. The K118C mutation significantly decreased the relative binding affinity of cardiac TnI for TnC at pCa 4 (Fig. 6B). The adjacent mutation A117G also produced a detectable decrease in the relative binding affinity for TnC at pCa 4, although much less than that caused by K118C (Fig. 6B).

2.5. Effects of N-terminal modifications on relative binding affinity of the mutant cardiac TnI for TnT

The N-terminal extension of cardiac TnI is an adult heart-specific regulatory structure [28] that is known to fine-tune the func-
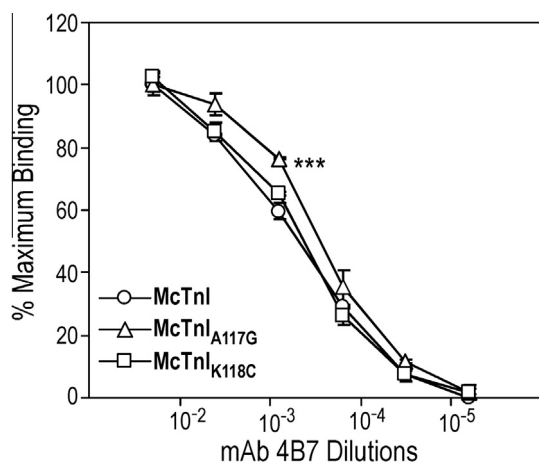

Fig. 4. Mutation A117G, but not K118C, of cardiac TnI in the TnT interface alters the conformation of the TnC-binding helix 1. ELISA epitope analysis using mAb 4H6 recognizing an epitope at the TnC binding helix 1 in cardiac TnI detected a significant conformational change caused by the A117G mutation (***$P < 0.00001$), but the adjacent K118C mutation did not have significant effect. Statistical analyses were done using Fisher test in Two Way ANOVA with Origin Pro 8 software.
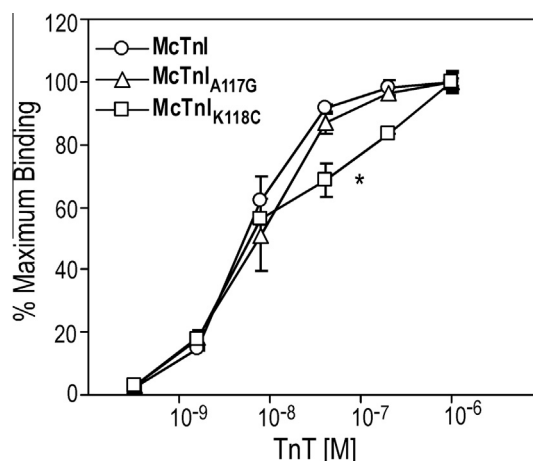
Fig. 5. Mutation K118C, but not A117G, in the TnT interface of cardiac TnI decreases the binding affinity for TnT. Protein binding assay showed that mutation K118C, but not A117G, caused a decrease in the binding affinity of cardiac TnI for TnT (*$P < 0.01$ as comparing data points in the binding curves corresponding to TnT concentrations from 8×10^{-9} M to 1×10^{-6} M). Statistical analyses were done using Fisher test in Two Way ANOVA with Origin Pro 8 software.

tion of troponin via phosphorylation of $Ser_{23/S24}$ by PKA [12,15–17] and truncation by limited proteolysis [19,21,22]. ELISA solid phase protein binding assay showed that PKA phosphorylation of $Ser_{23/24}$ in the N-terminal segment and deletion of the entire N-terminal segment of wild type cardiac TnI resulted in similarly small but statistically significant decreases in the relative binding affinity for TnT (Fig. 7A).

Neither PKA phosphorylation nor N-terminal truncation altered the TnT binding affinity of $McTnI_{A117G}$ (Fig 7B). In contrast, N-terminal truncation, but not PKA phosphorylation, resulted in a small but statistically significant decrease in the TnT binding affinity of $McTnI_{K118C}$ (Fig. 7C).

To directly compare the effects of N-terminal truncation on the function of cardiac TnI A117G and K118C mutations, a separate set of binding studies showed that $McTnI-ND_{A117G}$ had higher affinity for TnT as compared with that of McTnI-ND and $McTnI-ND_{K118C}$ (Fig. 7D). A117G mutation in intact cardiac TnI had minimum effect on TnT binding affinity (Fig. 5). Therefore, the result in

Fig. 3. A117G and K118C mutations result in local conformational changes. ELISA epitope analysis using mAb 4B7 recognizing an epitope at the TnT interfacing helix of cardiac TnI detected a significant conformational change produced by the A117G mutation (***$P < 0.00001$), where as the adjacent K118C mutation had no significant effect. Statistical analyses were done using Fisher test in Two Way ANOVA with Origin Pro 8 software.
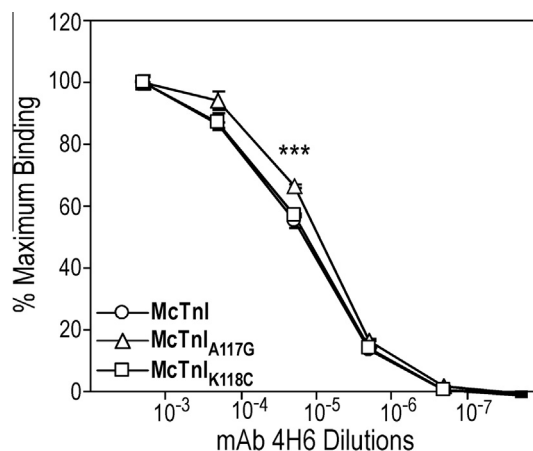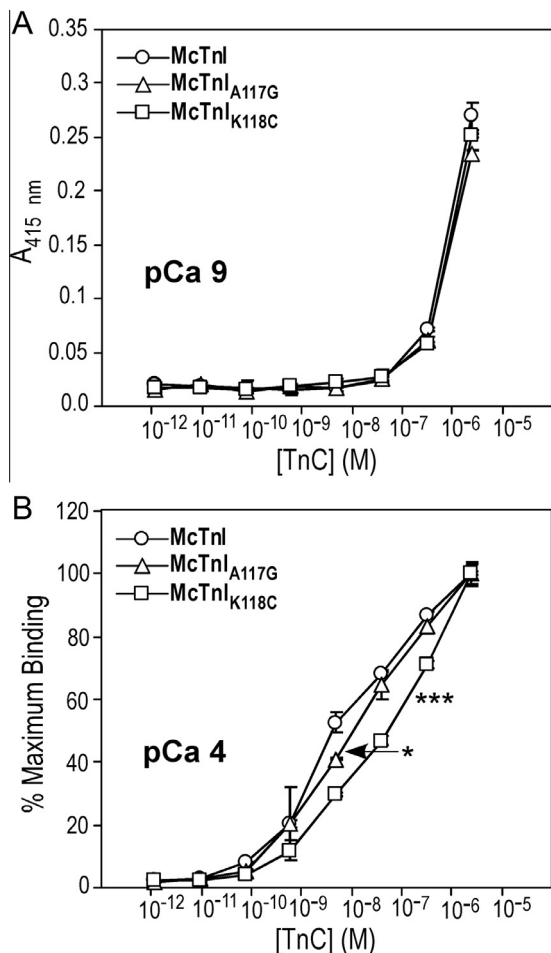
Fig. 6. Mutations in the TnT interface helix decreased the binding affinity of cardiac TnI for TnC at pCa 4. Protein binding experiments showed that (A) A117G and K118C mutations did not cause any detectable change in the binding affinity of cardiac TnI for TnC at pCa 9. (B) At pCa 4, K118C mutation caused a significant decrease in the binding affinity of cardiac TnI for TnC while the adjacent mutation A117G also had a much less but still statistically significant effect (*$P < 0.001$, ***$P < 0.000001$). Statistical analyses were done using Fisher test in Two Way ANOVA with Origin Pro 8 software.

Fig. 7D indicates that the conformational modulation by N-terminal truncation [18] augmented the functional effect of A117G mutation. In contrast, the significant effect of K118C mutation in intact cardiac TnI on the binding affinity for TnT (Fig. 5) was minimized by N-terminal truncation (Fig. 7D). Altogether, the data demonstrate that the two adjacent single amino acid substitutions in the TnT interface of cardiac TnI had differential responses to the modulatory effect of N-terminal modifications.

2.6. Effects of N-terminal modifications on the relative binding affinity of mutant cardiac TnI for TnC in the Ca²⁺-depleted state

ELISA protein binding assay showed that at pCa 9, PKA phosphorylation and N-terminal truncation did not produce any detectable change in the binding affinities of wild type cardiac TnI for TnC at Ca²⁺-depleted state (Fig. 8A). In contrast, N-terminal truncation, but not PKA phosphorylation, produced an increase in the relative binding affinity of McTnI$_{A117G}$ for TnC at pCa 9 (Fig. 8B). N-terminal truncation of McTnI$_{K118C}$ produced a similar increase in the binding affinity of McTnI$_{A117G}$ for TnC at pCa 9 while no change in affinity was detectable upon PKA phosphorylation (Fig. 8C).

To directly compare the effect of N-terminal truncation on the binding affinities of A117G and K118C mutants of cardiac TnI for

TnC in Ca²⁺-depleted state, a separate set of protein binding assay showed that McTnI-ND$_{A117G}$ and McTnI-ND$_{K118C}$ both had higher binding affinity for TnC than that of McTnI-ND at pCa 9 (Fig. 8D). Since intact wild type and mutant cardiac TnI had similar binding affinities for TnC at pCa9 (Fig. 6A), the results in Fig. 8D indicate that N-terminal truncation, but not PKA phosphorylation, augmented the effects of the TnT interface mutations on the relative binding affinity for TnC in the Ca²⁺-depleted state.

2.7. Effects of N-terminal modifications on the relative binding affinity of mutant cardiac TnI for TnC in the Ca²⁺-saturated state

ELISA solid phase protein binding assay showed that PKA phosphorylation of Ser$_{23/24}$ in the N-terminal segment or truncation of the N-terminal extension of cardiac TnI increased the binding affinity of wild type cardiac TnI for TnC at pCa 4 (Fig. 9A). Similar effects were seen for McTnI$_{A117G}$ at pCa 4, whereas the effect of N-terminal truncation was more profound than that of PKA phosphorylation (Fig. 9B). In contrast, N-terminal truncation, but not PKA phosphorylation, produced a significant increase in the relative binding affinity of McTnI$_{K118C}$ for TnC at pCa 4 (Fig. 9C).

To directly compare the effects of N-terminal truncation on the binding affinities of the A117G and K118C mutants of cardiac TnI for TnC in Ca²⁺-saturated state, a separate set of binding assays showed that in comparison with wild type control, N-terminal truncation partially reversed the decreased binding affinity of McTnI$_{K118C}$ for TnC at pCa 4 (Fig. 6B) with no effect (Fig. 9D) on the less profoundly decreased binding affinity of McTnI$_{A117G}$ for TnC at pCa 4 (Fig. 6B). The reduced decrease in the affinity of McTnI$_{K118C}$ for TnC at pCa 4 upon N-terminal truncation indicates a rescuing effect to minimize the impact of this dominantly negative mutation [10].

3. Discussion

TnI–TnT interface is a pivotal structure in the troponin complex, formed by coiled coils of α-helices. To investigate the function of the TnI–TnT interface, the present study characterized two pathogenic mutations of single amino acid substitution, A117G and K118C, in the TnT-interfacing helix of cardiac TnI for their structural and functional impacts. Summarized in Table 1, the results contribute to the following insights into the structure–function relationship of cardiac TnI.

3.1. K118C and A117G single amino acid substitutions produce distinct conformational alterations

Molecular modeling showed that substitution of Cys for Lys$_{117}$ in human troponin complex (K118 in mouse) would not disrupt the overall α-helical structure of TnI that interacts with TnT but leads to a new hydrogen bond with Ile$_{114}$ to generate a structural alteration in the TnI–TnT interface [6]. In our present study, A117G and K118C mutations both altered the mobility of cardiac TnI in SDS–PAGE, in which proteins are largely unfolded. A possible explanation is that altered tertiary structure of protein may affect the binding of SDS [29]. Although both mutations are single amino acid substitutions with smaller residues, McTnI$_{K118C}$ exhibits slower gel mobility whereas McTnI$_{A117G}$ has faster mobility than wild type control (Fig. 2). The mobility changes may indicate changes molecular conformation, which affected the binding of SDS to cardiac TnI.

We previously demonstrated that N-terminal modification by PKA phosphorylation of Ser$_{23/24}$ or truncation of the N-terminal extension results in long-range conformational modulations in cardiac TnI [18]. The effects of McTnI$_{A117G}$ and McTnI$_{K118C}$ mutations on SDS–gel mobility remained after truncation of the N-terminal
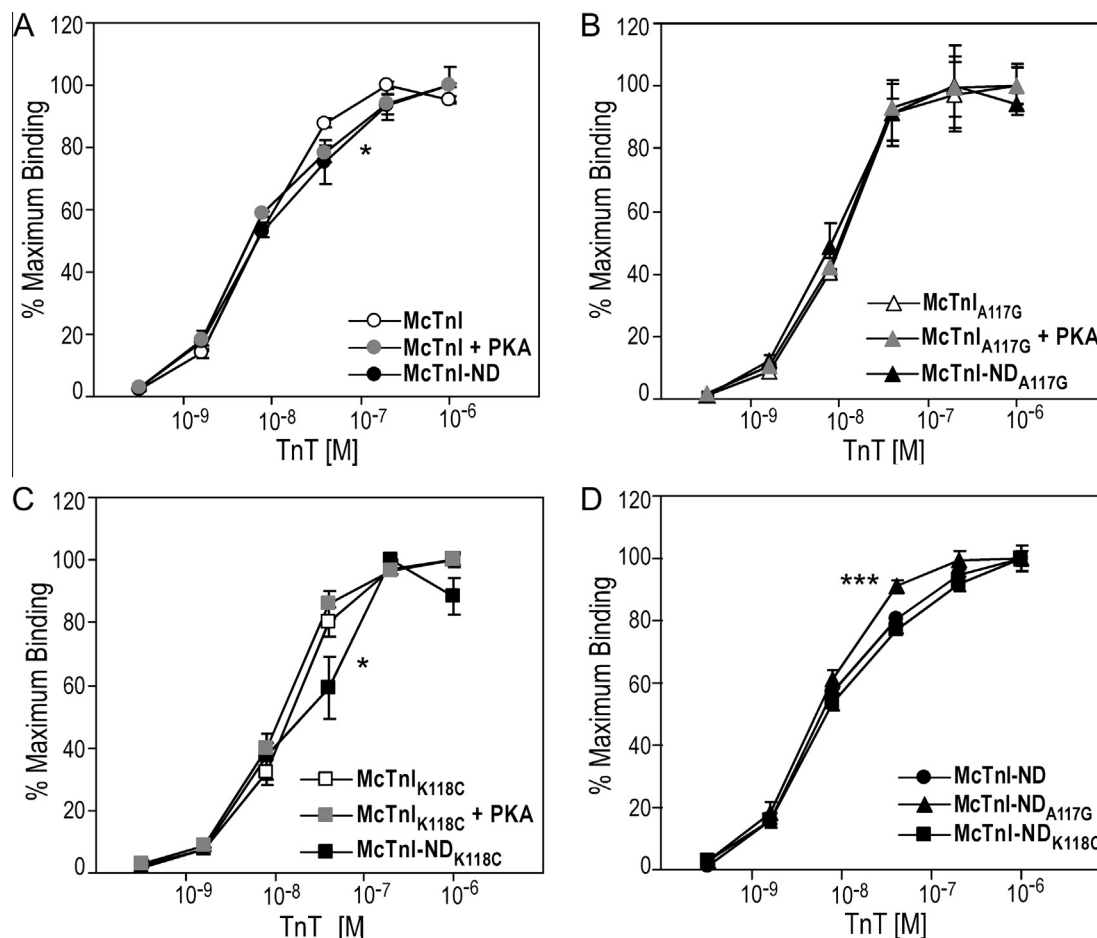
Fig. 7. Effects of N-terminal modification on TnT binding affinity of the mutant cardiac TnI. Protein binding assay showed that (A) both PKA phosphorylation and N-terminal truncation caused small but statistically significant decreases in the binding affinity of wild type mouse cardiac TnI for TnT (*$P < 0.05$ as comparing data points in the binding curves corresponding to TnT concentrations from 1.6×10^{-9} M to 2×10^{-7} M). (B) PKA phosphorylation or N-terminal truncation did not affect the binding affinity of McTnI$_{A117G}$ for TnT. (C) N-terminal truncation, but not PKA phosphorylation, caused a small decrease in the binding affinity of McTnI$_{K118C}$ for TnT (*$P < 0.02$). (D) N-terminal truncation minimized the effect of K118C mutation on TnT binding and increased the binding affinity of A117G mutation for TnT (***$P < 0.000001$). Statistical analyses were done using Fisher test in Two Way ANOVA with Origin Pro 8 software.

segment (Fig. 2). This observation indicates that the effects of A117G and K118C mutations on molecular conformation are dominant traits over the N-terminal modification-mediated regulatory effect.

While the K118C mutation caused clear gel mobility change that may reflect tertiary structure changes affecting the binding of SDS [29], we detect only a small trend of local conformational change in cTnI$_{K118C}$ mutation using mAb 4B7 and no remote change was detected with mAb 4H6. Therefore, the gel mobility change may indicate conformational changes in regions not probed by the two mAbs. The opposite effects of the two adjacent mutations in the TnT interface of cardiac TnI implicate their distinct structural impacts, which is worth further investigation in order to understand the structural and functional role of the TnI–TnT interface in the troponin complex.

3.2. Monoclonal antibody epitope mapping of conformational effects of cardiac TnI mutations

Monoclonal antibodies provide conformational probes to detect alterations in three-dimensional epitope structure and molecular conformation [30]. Changes in mAb affinity can be quantified to detect local and long-range conformational changes in TnI and TnT as shown in our previous studies [18,24,28,31]. In the present

study, ELISA epitope analysis using mAb probe 4B7 recognizing an epitope in the segment of cardiac TnI interfacing with TnT [18] detected changes in the local conformation resulting from McTnI$_{A117G}$ and McTnI$_{K118C}$ mutations (Fig. 3). Consistent with their distinct effects on SDS–gel mobility, the local conformational change resulting from McTnI$_{A117G}$ was different from that from McTnI$_{K118C}$, evident by the higher affinity of mAb 4B7 to McTnI$_{A117G}$ than that to McTnI$_{K118C}$.

Another mAb probe 4H6 recognizing an epitope in the TnC binding helix 1 of cardiac TnI [18] was employed to examine remote conformational changes produced by the TnT interface mutations. 4H6 detected conformational alteration in McTnI$_{A117G}$ but not in McTnI$_{K118C}$ (Fig. 4). The distinct remote conformational effects of McTnI$_{A117G}$ and McTnI$_{K118C}$ further support the observation that the two adjacent mutations in the TnT interface of cardiac TnI have distinct effects on overall molecular conformation.

It is worth noting that more quantitative measurements, such as circular dichroism studies of α-helical contents and fluorescence reporter-based protein binding assays in solution, may provide more insights into the structure–function relationship of the cardiac TnI mutations. The results from our ELISA epitope analysis and solid phase protein binding studies have laid groundwork for the future investigations.
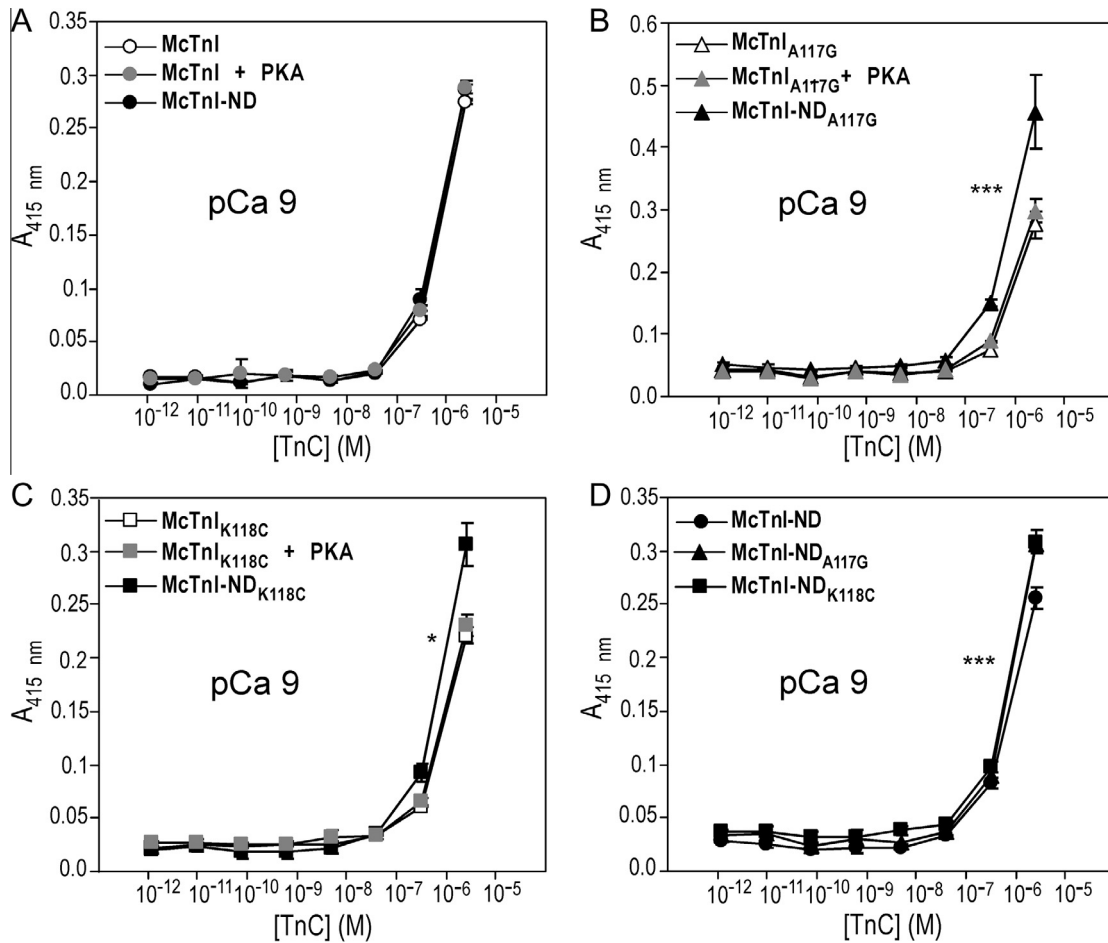
Fig. 8. Effect of N-terminal modifications on the binding affinities of cardiac TnI and mutants for TnC in the Ca^{2+} depleted state. Protein binding assay showed that (A) PKA phosphorylation or N-terminal truncation did not result in detectable change in the affinity of wild type mouse cardiac TnI for TnC at pCa 9. (B) N-terminal truncation, but not PKA phosphorylation, increased the binding affinity of $McTnI_{A117G}$ for TnC at pCa 9 (***$P < 0.00001$). (C) N-terminal truncation caused a similar increase in the binding affinity of $McTnI_{K118C}$ for TnC at pCa 9 (*$P < 0.05$) whereas PKA phosphorylation had no detectable effect. (D) N-terminal truncation increased the binding affinity of both McTnI-ND_{A117G} and McTnI-ND_{K118C} for TnC at pCa 9 as compared with wild type control (***$P < 0.000001$). Statistical analyses were performed using Fisher test in Two Way ANOVA with Origin Pro 8 software.

3.3. Effects of the myopathic mutations of cardiac TnI on interactions with TnT and TnC

In agreement with previously reported lower binding affinity of the R111C mutation of turkey cardiac TnI for TnT [6], ELISA protein binding assay showed that K118C mutation decreased the relative binding affinity of mouse cardiac TnI for TnT, whereas mutation A117G did not produce a detectable change in TnT binding affinity (Fig. 5). The distinct effects of K118C and A117G mutations on TnT binding may be based on their distinct conformational effects and suggest that the TnI–TnT interactions are not only determined by the structure at the binding site but also dependent on the overall molecular conformation.

Our results further showed that $McTnI_{K118C}$ exhibited lower binding affinity for TnC at pCa 4 as compared to that of wild type cardiac TnI, whereas $McTnI_{A117G}$ showed less effect (Fig. 6). The altered binding affinity for TnC due to mutations in TnT interface structure demonstrates remote functional effects, supporting a role of the TnI–TnT interface in the overall conformation and allosteric function of troponin in the regulation of muscle contraction. The different effects of A117G and K118C on the binding affinity of cardiac TnI for TnC also support the distinct remote conformational effects of the two adjacent TnT interface mutations.

Although the two dominantly negative mutations that we have studied caused relatively mild changes in the binding affinity for the other subunits of troponin, the TnI–TnT interface structure plays a pivotal role in transmitting the conformational signals in the troponin complex initiated from Ca^{2+}-binding to TnC and weakened binding between TnI and TnT may have an impact on this signal transduction. Weaker or stronger binding of TnI to TnC may also alter myofilament Ca^{2+} sensitivity. The conformational change detected in $McTnI_{A117G}$ using mAb 4H6 probe specific to the TnC binding helix 1 that interacts with the C domain of TnC could be responsible for the altered affinity of TnI for TnC since this region is in close vicinity to the TnI–TnT interface where the A117G mutation is located.

3.4. Effects of the dominantly negative mutations of cardiac TnI are modulated by N-terminal modifications

The N-terminal extension of cardiac TnI is an adult heart-specific regulatory structure not present in the two skeletal muscle isoforms of TnI [2,28]. The regulation of the N-terminal extension can be mediated through two posttranslational modifications: Phosphorylation of $Ser_{23/24}$ by PKA and limited proteolysis. Proteolytic truncation to selectively remove the N-terminal extension of cardiac TnI in adaption to hemodynamic stress or β-adrenergic deficiency [19–21] and PKA phosphorylation produce similar conformational modulations in the region interfacing with TnT and similar effects on the binding affinities for TnT and TnC [18].
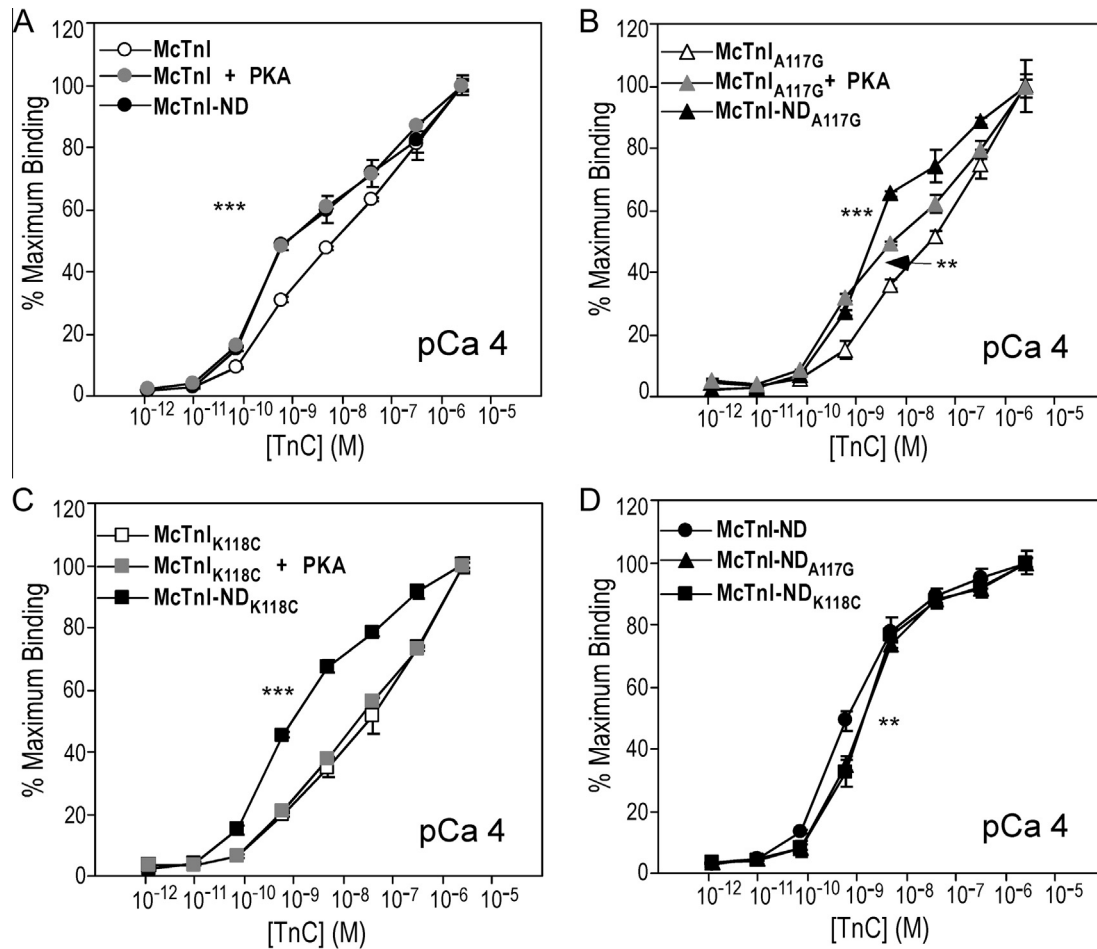
Fig. 9. Effect of N-terminal modifications on the binding affinity of cardiac TnI and mutants for TnC in the Ca^{2+}-saturated state. Protein binding assay showed that (A) PKA phosphorylation or N-terminal truncation increased the binding affinity of wild type mouse cardiac TnI for TnC at pCa 4 ($^{***}P < 0.00001$). (B) PKA phosphorylation increased the binding affinity of McTnI$_{A117G}$ for TnC at pCa 4 and N-terminal truncation had a more profound effect ($^{**}P < 0.002$, $^{**}P < 0.00001$). (C) N-terminal truncation, but not PKA phosphorylation, resulted in significantly higher binding affinity of McTnI$_{K118C}$ for TnC at pCa 4 ($^{***}P < 0.000001$). (D) N-terminal truncation decreased the affinity of McTnI$_{A117G}$ and McTnI$_{K118C}$ for TnC at pCa 4 as compared to the wild type control ($^{**}P < 0.001$). Statistical analyses were done using Fisher test in Two Way ANOVA with Origin Pro 8 software.

Table 1
Comparison of the effects of A117G and K118C mutations of cardiac TnI on the interactions with TnT and TnC and the modulation by N-terminal modifications.

(A)	McTnI	McTnI$_{A117G}$ (comparing with McTnI)	McTnI$_{K118C}$ (comparing with McTnI)
TnT	Reference point	=	↓
TnC (pCa 4)	Reference point	↓	↓
TnC (pCa9)	Reference point	=	=
(B)	McTnI$_{PKA}$ (comparing with McTnI)	McTnI$_{A117G+PKA}$ (comparing with McTnI$_{A117G}$)	McTnI$_{K118C+PKA}$ (comparing with McTnI$_{K118C}$)
TnT	↓	=	=
TnC (pCa 4)	↑	↑	=
TnC (pCa 9)	=	=	=
(C)	McTnI-ND (comparing with McTnI)	McTnI-ND$_{A117G}$ (comparing with McTnI$_{A117G}$)	McTnI-ND$_{K118C}$ (comparing with McTnI$_{K118C}$)
TnT	↓	=	↓
TnC (pCa 4)	↑	↑	↑
TnC (pCa 9)	=	↑	↑
(D)	McTnI (ND/PKA)	McTnI$_{A117G}$ (ND/PKA)	McTnI$_{K118C}$ (ND/PKA)
TnT	=	=	<1
TnC (pCa 4)	=	>1	>1
TnC (pCa 9)	=	>1	>1

(A) McTnI$_{A117G}$ and McTnI$_{K118C}$ are compared for TnT and TnC binding affinities with wild type McTnI control as the reference point. (B) TnT and TnC binding affinities of PKA phosphorylated wild type McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ are compared with non-phosphorylated control as the reference point. (C) TnT and TnC binding affinities of N-terminal truncated wild type McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ are compared with their intact protein control as the reference point. ↑, increased and ↓, decreased affinity in the comparisons; =no difference. (D) TnT and TnC binding affinities of N-terminal truncated McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ are compared with that of PKA phosphorylation (ND/PKA ratio >1 or <1 indicates that N-terminal truncation had larger or smaller effect than that of PKA phosphorylation).

Our present study showed that N-terminal phosphorylation and truncation resulted in decreases in the binding affinity of wild type cardiac TnI for TnT (Fig. 7A). N-terminal truncation, but not PKA phosphorylation, decreased the binding affinity of McTnI$_{K118C}$ for TnT (Fig. 7C), whereas neither had detectable effect on the binding affinity of McTnI$_{A117G}$ for TnT (Fig. 7B). N-terminal truncation rescued the decreased affinity of McTnI$_{K118C}$ for TnT (Fig. 7D). While McTnI$_{A117G}$ had only minimal effect on the affinity for TnT (Fig. 5), N-terminal truncation increased its TnT binding affinity as compared to the effect on wild type McTnI (Fig. 7D). Altogether, the results indicate that the conformational effect of N-terminal truncation [18] is able to modulate the function of TnI–TnT interface.

In the calcium depleted state at pCa 9, the binding affinity of cardiac TnI and the mutants for TnC was low in our ELISA solid phase protein binding experiments, consistent with that established in previous studies [32]. N-terminal truncation, but not PKA phosphorylation, increased the binding affinity of McTnI$_{A117G}$ for TnC at pCa 9 (Fig. 8B). N-terminal truncation similarly increased the binding affinity of McTnI$_{K118C}$ for TnC at pCa 9 while phosphorylation had no detectable effect (Fig. 8C). Although the TnC binding affinities of intact cardiac TnI and the mutants were similar at pCa 9 (Fig. 6), N-terminal truncation produced detectably higher TnC binding affinities of McTnI-ND$_{A117G}$ and McTnI-ND$_{K118C}$ at pCa 9 than that produced in wild type cardiac TnI (Fig. 8D). The results indicate that N-terminal truncation, but not PKA phosphorylation, selectively affects the function of the mutant, but not wild type cardiac TnI.

High affinity binding of cardiac TnI and TnC was demonstrated at pCa 4 in our ELISA solid phase binding assays, also consistent with that established in previous studies [32]. N-terminal truncation or PKA phosphorylation increased the binding affinity of wild type cardiac TnI for TnC at pCa 4 (Fig. 9A). The N-terminal modifications produced similar responses in McTnI$_{A117G}$ at pCa 4, in which N-terminal truncation had a more potent effect (Fig. 9B). In contrast, only N-terminal truncation but not PKA phosphorylation, increased the affinity of McTnI$_{K118C}$ for TnC at pCa 4 (Fig. 9C). At pCa 4, N-terminal truncation partially corrected the decreased TnC-binding affinity of McTnI$_{K118C}$ but did not change the weaker effect of McTnI$_{A117G}$ (Fig. 6B versus Fig. 9D).

Together with the TnT binding studies, the similar but yet distinct modulatory effects of PKA phosphorylation and N-terminal truncation on the functional impact of the A117G and K118C mutations indicate that these two N-terminal regulatory modifications of cardiac TnI have distinct effects on the function of the TnI–TnT interface. It is worth further investigating the use of N-terminal modifications, especially N-terminal truncation, to reduce myopathic impact of TnI–TnT interface mutations.

3.5. Proposed mechanistic insights

In McTnI$_{A117G}$ (A116G in human) a hydrogen in Gly replaced the methyl group in Ala side chain in wild type McTnI to slightly decrease the molecular mass without changing charge (10.39 at pH 7) or isoelectric point (9.57). The conservative minimal change in physical property is in agreement with the data that although A117G mutation altered the local conformation in the TnT interfacing helix of cardiac TnI and caused a remote conformational modulation in the TnC binding helix 1, it did not alter the binding affinity for TnT and only slightly reduced the binding affinity for TnC at pCa 4. Nonetheless, patients with A116G mutation in cardiac TnI exhibited dilated cardiomyopathy, indicating the functional importance of the TnI–TnT interface that is highly sensitive to structural alterations.

On the other hand, in McTnI$_{K118C}$ neutral side chain of Cys replaces positively charged side chain of Lys to cause an acidic shift of charge of McTnI$_{K118C}$ (9.36 vs 10.39 at pH 7) along with a change

in isoelectric point (from 9.57 to 9.42), which is a larger change than that in McTnI$_{A117G}$. Molecular modeling previously showed that substitution of Cys for Lys at this position does not result in significant change in the local structure [6], which supports our observation of the small conformational change detected using mAb 4B7 probe. The modeling of the K117C mutation further predicted an additional hydrogen bond between Cys^{117} and Ile^{114}. Therefore, alteration in side chain interactions in the TnT interface helix may alter the overall conformation of McTnI$_{K118C}$ to cause functional changes.

In summary, the present study of two adjacent pathogenic mutations of cardiac TnI provides evidence for a critical role of the TnI–TnT interface in the function of troponin. While more investigation is needed to relate these findings to the development of cardiomyopathy, alterations in calcium-sensitivity or the dynamics of calcium activation within the troponin complex might underlie the mechanisms for the TnI–TnT interface mutations to cause contractile dysfunction. These protein level phenotypes laid groundwork for further studies of the pathogenesis and differences of these mutations in higher order experimental systems, such as muscle strips and transgenic mouse hearts.

4. Materials and methods

4.1. Construction of expression vectors encoding A117G and K118C mutants of mouse cardiac TnI

A cDNA encoding the K118C mutant of mouse cardiac TnI (McTnI$_{K118C}$) was amplified using polymerase chain reaction (PCR) from a transgene construct described previously [22]. The amplified cDNA was cut with restriction enzymes NdeI and XhoI and ligated to compatibly cut pAED4 plasmid vector [33].

A cDNA encoding the A117G mutant of mouse cardiac TnI (McTnI$_{A117G}$, equivalent to the A116G mutation in human cardiac TnI) was de novo constructed with synthetic PCR primers and GENEART site-directed mutagenesis kit (Invitrogen) using the pAED4 McTnI$_{K118C}$ expression plasmid as template.

The recombinant plasmid DNA was purified and sequenced to verify the constructions.

4.2. Construction of expression vectors encoding mutants of N-terminal truncated mouse cardiac TnI

As described previously for the construction of N-terminal truncated mouse cardiac TnI with the deletion of amino acids 1–28 [22], recombinant PCR was applied to generate cDNAs encoding N-terminal truncated McTnI$_{A117G}$ and McTnI$_{K118C}$ (Fig. 1B). Using McTnI$_{A117G}$ and McTnI$_{K118C}$ recombinant pAED4 plasmids as templates, a forward primer (5′-CAACCATATGGCCTATGCCACCGA-3′) containing an NdeI restriction enzyme site (underlined) and an added Met initiation codon was used together with the 3′ flanking T7 primer in PCR to produce N-terminal truncated cDNA templates. The amplified cDNA was digested with NdeI and XhoI and ligated to compatibly cut pAED4 vector. Recombinant plasmid DNAs encoding N-terminal truncated McTnI$_{A117G}$ (McTnI-ND$_{A117G}$) and McTnI$_{K118C}$ (McTnI-ND$_{K118C}$) were sequenced to verify the constructions.

4.3. SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting

Protein samples of wild type, A117G, and K118C mouse cardiac TnI, intact and N-terminal truncated, were dissolved in SDS–gel sample buffer containing 2% SDS and 150 mM DTT for electrophoresis on SDS–gel (14% gel with acrylamide/bisacrylamide ratio of

180:1) as described previously [11]. The resulting gels were stained with Coomassie Blue R250 to visualize the resolved protein bands.

Duplicate gels were electrophoretically transferred to nitrocellulose membranes for Western blot analysis. After blocking in 1% bovine serum albumin (BSA), the membranes were incubated with an anti-TnI monoclonal antibody (mAb) TnI-1 [34] at 4 °C overnight. The blots were washed with Tris-buffered saline containing 0.5% triton X-100 and 0.05% SDS, further incubated with alkaline phosphatase-labeled second antibody (Santa Cruz), followed by final washes and 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium substrate reaction.

4.4. Expression and purification of intact cardiac TnI and mutants

Competent BL21(DE3)pLysS *E. coli* cells were transformed with recombinant pAED4 plasmids encoding wild type mouse cardiac TnI (McTnI), $McTnI_{A117G}$ and $McTnI_{K118C}$ and cultured in 2X TY media containing carbenicillin (50 µg/mL) and chloramphenicol (12.5 µg/mL) at 37 °C with vigorous shaking. Isopropyl-1-thiol-β-galactoside (IPTG) was added when $O.D._{600}$ of the culture reached ∼0.3 to induce the expression of recombinant protein. Three hours after induction, the bacterial cells were harvested by centrifugation and re-suspended in 50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 15 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by passing through a French press for three or more times.

After centrifugation to clarify the cell lysate, the supernatant was fractionated with ammonium sulfate precipitation at 0 °C. The fractions were dialyzed at 4 °C against 10 mM citrate buffer, pH 6.0, containing 0.1 mM EDTA and examined together with the total lysate using SDS–PAGE and Western blot to determine the fraction enriched with the recombinant mouse cardiac TnI. The fraction between 30% and 50% saturation containing the highest amount of mouse cardiac TnI was added with urea to a final concentration of 6 M, β-mercaptoethanol to 15 mM and PMSF to 0.5 mM. Centrifuged at high speed to remove any insoluble materials, the supernatant was loaded to a CM52 cation exchange column equilibrated at 4 °C in the same buffer. Following washing with the equilibration buffer, the bound protein was eluted with 0–500 mM linear gradient of KCl.

Protein peaks of the CM52 column fractions with A280 absorbance were analyzed by SDS–PAGE and Western blot to identify fractions that contained cardiac TnI or mutants. After dialysis at 4 °C against 0.1 mM EDTA and concentrated by lyophilization, the positive fraction was further fractionated at 4 °C on a Sephadex G75 gel filtration column in 6 M urea, 0.5 M KCl, 0.1 mM EDTA, 6 mM β-mercaptoethanol, and 10 mM imidazole, pH 7.0, as described previously [6]. The G75 fractions containing purified McTnI, $McTnI_{A117G}$ and $McTnI_{K118C}$ were identified using SDS–PAGE and verified with Western blot, dialyzed against 2% formic acid, and lyophilized.

4.5. Expression and purification of N-terminal truncated cardiac TnI and mutants

The transformation and culture of bacteria for the expression of N-terminal truncated wild type mouse cardiac TnI (McTnI-ND), $McTnI-ND_{A117G}$ and $McTnI-ND_{K118C}$ were same as that for the intact cardiac TnI. McTnI-ND, $McTnI-ND_{A117G}$ and $McTnI-ND_{K118C}$ expressed in BL21(DE3)pLysS cells formed inclusion bodies that were collected from cell lysate with centrifugation. Washed with 50 mM Tris–HCl, 5 mM EDTA, pH 8.0, and again with 10 mM citrate, 0.1 mM EDTA, the inclusion bodies were dissolved in ice-cold 6 M urea in 10 mM citrate, 0.1 mM EDTA, 15 mM β-mercaptoethanol and 0.5 mM PMSF, pH 6.0. The protein extract was

centrifuged at high speed and the supernatant was loaded on a CM52 column equilibrated at 4 °C in the same buffer. The rest of the purification procedure was the same as described for the intact mouse cardiac TnI and mutants.

4.6. Other myofilament proteins

Bovine cardiac TnT was purified from adult left ventricular muscle as described previously [35]. Bovine and mouse cardiac TnTs have very high degree of similarity in amino acid sequence, especially in the TnI interface helix. Therefore, we used bovine instead of mouse cardiac TnT in binding studies with mouse cardiac TnI and mutants.

Chicken fast TnC was purified from *E. coli* expression as described previously [36]. The main difference between cardiac/slow TnC and fast TnC is in the N domain, in which the metal binding site I is inactive in cardiac/slow TnC. The two TnC isoforms have very similar structures in the C domain that binds TnI near the TnI–TnT interface. For our study of the effect of the two cardiac TnI mutations on the binding affinity for TnC, the use of fast TnC instead of cardiac/slow TnC should not cause qualitative discrepancies.

4.7. Epitope analysis of conformational changes in mutant cardiac TnI

The binding of a protein antigen and a specific antibody is a protein–protein interaction based on conformational fit between three-dimensional structures at the antigenic epitope and the antibody paratope. Therefore, mAbs that recognize specific epitopes can be utilized as site-specific structural probes to detect and monitor changes in local structure as well as in overall molecular conformation of a protein [28,31]. As described previously, we applied mAb probes recognizing specific regions of cardiac TnI in microtiter plate enzyme linked immunosorbent assay (ELISA) [18,31,36,37] to investigate the conformational effects of the TnT interface mutations in cardiac TnI under native conditions [28,31].

McTnI, $McTnI_{A117G}$ or $McTnI_{K118C}$ proteins was dissolved in 100 mM KCl, 3 mM $MgCl_2$, 20 mM PIPES, pH 7.0 (Buffer A) as 1 µg/mL for coating on 96-well microtiter plates (Immulon 2HB, Thermo Scientific) at 100 µl/well at 4 °C overnight. The similar coating of McTnI, $McTnI_{A117G}$ and $McTnI_{K118C}$ on ELISA plate was verified by direct titration using TnI-1 mAb that has same affinity for the three protein variants (data not shown).

Following washes with Buffer A containing 0.05% Tween-20 (Buffer T) to remove unbound protein and block the remaining plastic surface, the wells were incubated with serial dilutions of anti-TnI mAbs TnI-1, 4H6 [31], or 4B7 [31] in Buffer A containing 0.1% BSA (Buffer B) at room temperature for 2 h. Washed with Buffer T to remove unbound antibody, the plates were further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG second antibody (Santa Cruz) in Buffer B. After washes to remove the unbound second antibody, the plates were developed with H_2O_2-ABTS (2-2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) substrate in 0.1 M citrate buffer, pH 4.0. A_{415nm} of each assay well was monitored with an automated microplate reader (BioRad Benchmark). Data within the linear course of the color development were used to plot the titration curves. All data points were obtained as the average of triplicate wells and each assay was repeated three times.

4.8. Phosphorylation of recombinant mouse cardiac TnI and mutants

Purified recombinant McTnI, $McTnI_{A117G}$ and $McTnI_{K118C}$ (160 µg/mL) were incubated with 25 µg/mL rabbit skeletal muscle cAMP-dependent PKA (Sigma Chemical Co.) in a buffer containing 0.1 M NaCl, 10 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.1 mM cAMP,

50 mM Tris–HCl, pH 7.5, at 30 °C for 135 min. The reaction mixture was frozen at −80 °C to terminate the reaction. PKA-catalyzed phosphorylation of McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ was visualized with Pro-Q Diamond (Invitrogen) staining of SDS–PAGE gel and quantified using ImageJ software. The same gel or a parallel gel was stained with Coomassie Brilliant Blue R250 to determine the amount of total cardiac TnI in the samples. PeppermintStick phosphoprotein markers (Invitrogen) were run in the SDS–PAGE along with the PKA-treated McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ samples as control.

4.9. Solid phase protein binding assay

ELISA solid phase protein binding assay was carried out as described for binary protein binding studies [6,18,36]. Wild type or mutant cardiac TnI, intact, N-terminal truncated or PKA phosphorylated, was dissolved in Buffer A containing 1 mM DTT at 2.5 μg/mL for coating on microtiter plate at 100 μl/well. After removal of excess protein by washing with Buffer T containing 1 mM DTT, the wells were blocked with Buffer T containing 1% BSA and 1 mM DTT at room temperature for 1 h. Following washes with Buffer T containing 1 mM DTT, the wells were incubated with serial dilutions of bovine cardiac TnT in Buffer B containing 1 mM DTT at room temperature for 2 h. The plates were then washed with Buffer T to remove unbound TnT and DTT, incubated with an anti-TnT mAb CT3 [38] at room temperature for 1 h. The plates were washed again and further incubated with HRP-conjugated anti-mouse immunoglobulin secondary antibody at room temperature for 45 min. After washes to remove excess secondary antibody, substrate reaction and A_{415nm} recording were carried out as described above in the ELISA epitope studies.

In TnC binding experiments, microtiter plates coated with various cardiac TnI proteins were incubated with chicken fast TnC in Buffer B containing 1 mM DTT, 5 mM EGTA, 5 mM CaCl$_2$ (pCa 4) or 11 μM CaCl$_2$ (pCa 9). The binding of TnC to immobilized TnI was detected using an anti-TnC mAb 2C3 [36]. The blocking, washing and antibody dilution buffers were maintained at either pCa 4 or pCa 9.

Data within the linear course of the color development were used to plot the binding curves. All data points were obtained as the average of triplicate wells and each assay was repeated three times.

The solid phase protein binding assay involves two phases: The initial binding step reaches equilibrium binding of the protein in solution to the immobilized protein; and the subsequent washing separation depends on the coupling strength of the binding in a non-equilibrium manner [25]. Therefore, we used the concentration of the protein in solution, which reached 50% maximum binding together with curve shape to evaluate relative binding affinities to compare the binding between TnI and other troponin subunits.

4.10. Data analysis

Protein concentrations were determined using Bradford assay and verified by quantification of TnI protein bands in Coomassie Blue-stained SDS-gels using ImageJ software. Similar coating of McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ on ELISA plate for binding assay was verified by titration with TnI-1 mAb that has identical affinity for McTnI, McTnI$_{A117G}$ and McTnI$_{K118C}$ (data not shown). Absorbance of triplicate ELISA wells was used for analysis. All quantitative data are presented as mean ± SD. Statistical analyses were performed using Fisher test in Two Way ANOVA with Origin Pro 8 software.

Author contribution statement

S.A. designed and carried out the experiments, drafted the paper and figures, edited the text and figures, and approved the submission. J.P.J. conceived the research, designed the experiments, edited the paper and figures, and approved the submission.

Acknowledgments

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