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Identification of two new regions in the N-terminus of cardiac troponin T that have divergent effects on cardiac contractile function

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Key points

- To elucidate the cardiac-specific role of the highly acidic extended N-terminus of cardiac troponin T (cTnT), the following deletions were made in the N-terminus of mouse cTnT (McTnT): $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$.
- Thin-filament activation was assessed after reconstituting the deletion proteins into skinned non-transgenic mouse cardiac fibres expressing α-tropomyosin (Tm).
- Because the N-terminus of cTnT interacts with the overlapping ends of Tm, we also sought to understand how Tm isoforms modulate the functional effects of the N-terminus of cTnT. Thus, the deletion proteins were reconstituted into skinned transgenic mouse cardiac fibres expressing β-Tm.
- Maximal activation was decreased by $McTnT_{1-44\Delta}$ irrespective of the type of Tm background. Cooperativity was decreased by $McTnT_{45-74\Delta}$, an effect that was more pronounced under β-Tm background.
- We provide the first explicit evidence to show that the cardiac-specific extended N-terminus of cTnT contains two distinct regions that have divergent physiological roles in modulating cardiac thin-filament activation.

Abstract Cardiac troponin T (cTnT) has a highly acidic extended N-terminus, the physiological role of which remains poorly understood. To decipher the physiological role of this unique region, we deleted specific regions within the N-terminus of mouse cTnT (McTnT) to create $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ proteins. Contractile function and dynamic force–length measurements were made after reconstituting the McTnT deletion proteins into detergent-skinned cardiac papillary fibres harvested from non-transgenic mice that expressed α-tropomyosin (Tm). To further understand how the functional effects of the N-terminus of cTnT are modulated by Tm isoforms, McTnT deletion proteins were reconstituted into detergent-skinned cardiac papillary fibres harvested from transgenic mice that expressed both α- and β-Tm. $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, attenuated maximal activation of the thin filament. Myofilament Ca^{2+} sensitivity, as measured by pCa_{50} ($-$log of $[Ca^{2+}]_{free}$ required for half-maximal activation), decreased in $McTnT_{1-44\Delta}$ (α-Tm) fibres. The desensitizing effect of $McTnT_{1-44\Delta}$ on pCa_{50} was ablated in β-Tm fibres. $McTnT_{45-74\Delta}$ enhanced pCa_{50} in both α- and β-Tm fibres, with β-Tm having a bigger effect. The Hill coefficient of tension development was significantly attenuated by $McTnT_{45-74\Delta}$, suggesting an effect on thin-filament cooperativity. The rate of cross-bridge (XB) detachment and the strained XB-mediated impact on other XBs were augmented by $McTnT_{1-44\Delta}$ in β-Tm fibres. The magnitude of the length-mediated recruitment of XBs was attenuated by $McTnT_{1-44\Delta}$ in β-Tm

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fibres. Our data demonstrate that the 1−44 region of McTnT is essential for maximal activation, whereas the cardiac-specific 45−74 region of McTnT is essential for augmenting cooperativity. Moreover, our data show that α- and β-Tm isoforms have divergent effects on McTnT deletion mutant's ability to modulate cardiac thin-filament activation and Ca^{2+} sensitivity. Our results not only provide the first explicit evidence for the existence of two distinct functional regions within the N-terminus of cTnT, but also offer mechanistic insights into the divergent physiological roles of these regions in mediating cardiac contractile activation.

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Abbreviations cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; DTT, dithiothreitol; fsTnT, fast skeletal troponin T; HR, high-relaxing; McTnC, mouse cardiac troponin C; McTnI, mouse cardiac troponin I; McTnT, mouse cardiac troponin T; $McTnT_{NTE}$, mouse cardiac troponin T N-terminal extension; $McTnT_{WT}$, wild-type mouse cardiac troponin T; MfsTnT, mouse fast skeletal troponin T; ML, muscle length; NLRD, non-linear recruitment distortion; NTE, N-terminal extension; NTG, non-transgenic; PEP, phosphoenol pyruvate; PMSF, phenylmethylsulfonyl fluoride; RcTnT, rat cardiac troponin T; RU, regulatory unit; SL, sarcomere length; SU, structural unit; TG, transgenic; Tm, tropomyosin; Tn, troponin; XB, cross-bridge.

Introduction

In cardiac muscle contraction, the primary trigger for the movement of tropomyosin–troponin (Tm–Tn) complex on the actin filament comes from the binding of Ca^{2+} to cardiac troponin C (cTnC). In addition to cTnC, cardiac troponin T (cTnT) is also essential for the Ca^{2+}-regulated activation of acto-myosin interactions that generate force in striated muscle (Malnic & Reinach, 1994; Malnic *et al.* 1998; Gordon *et al.* 2000; Kobayashi & Solaro, 2005). cTnT not only promotes the binding of Tm to actin, but also aids in the assembly of Tm on the actin filament by promoting head-to-tail interaction between two contiguous Tms (Heeley *et al.* 1987; Lehrer & Geeves, 1998). This action of cTnT on Tm is considered to be important for full cooperative activation of myofilaments (Malnic & Reinach, 1994; Chandra *et al.* 1999).

Direct evidence for the effect of cTnT–Tm interactions on thin-filament activation comes from previous studies, which showed that deletion of the first 76 or 96 amino acids in cTnT resulted in a greater inhibition of force and ATPase activity by Tm (Chandra *et al.* 1999; Communal *et al.* 2002). Based on our study (Chandra *et al.* 1999), we concluded that cross-bridge (XB) recruitment was inhibited by the effect of mutant cTnT on the overlapping ends of contiguous Tms. Interestingly, the removal of the overlapping ends of contiguous Tms inhibited myosin S1-ADP binding to fully-regulated thin filaments (Walsh *et al.* 1985; Heeley *et al.* 1989), and modifications to the end-to-end interactions of Tm altered the contractile dynamics (Gaffin *et al.* 2006) and resulted in a 10-fold weakening of its interaction with actin (Coulton *et al.* 2008). Collectively, these observations suggest that the tail domain of cTnT (residues 1−191 in mouse sequence) has a significant effect on how Tm–actin interactions modulate strong XB recruitment.

There is experimental evidence (Brisson *et al.* 1986) to suggest that the N-terminal end of the tail domain of fast skeletal TnT (fsTnT) in one structural unit (SU; Tm_1–Tn_1–$actin_7$) may interact with the Tm from the neighbouring SU. Conclusions drawn from the study by Brisson *et al.* (1986) have major implications for cardiac muscle because the highly acidic tail domain of cTnT – in all species – has a unique cardiac-specific N-terminal extension (NTE) that extends deeper into the neighbouring SU (Gollapudi *et al.* 2012). For example, the NTE of cTnT extends by another 42 Å into the neighbouring SU, and is suggested to constrain the thin filaments more in the blocked state, thereby desensitizing the cardiac thin filaments to Ca^{2+} (Tobacman *et al.* 2002; Gollapudi *et al.* 2012). Our data also demonstrate that the NTE has an effect to modulate cooperative processes that govern non-linear effects during cardiac thin-filament activation (Gollapudi *et al.* 2012).

Because the extended N-terminus of cTnT is located around the head-to-tail overlapping region of two contiguous Tms, the presence of this highly acidic region in cTnT is strongly suggestive of a cardiac-specific functional role (Perry, 1998). The functional significance of the N-terminus of cTnT is further highlighted by the fact that numerous mutations, which are associated with familial hypertrophic cardiomyopathy, have been identified in the N-terminus of human cTnT (Willott *et al.* 2010). Moreover, some forms of human heart failure are associated with reexpression of embryonic isoforms that differ in the N-terminus of cTnT (Anderson *et al.* 1995). Furthermore, the existence of various Tm isoforms in different tissues suggests that the functional effect of the N-terminus of cTnT may be further modulated by the type of Tm isoform. For example, the α-Tm isoform predominates in the adult rat heart muscle, while the β-Tm isoform is expressed at low levels during cardiogenesis

and in the hearts of neonates (Muthuchamy *et al.* 1993). In contrast, fast twitch skeletal muscle of adult rat contains both α- and β-isoforms of Tm, but the γ-isoform of Tm is restricted to slow twitch skeletal muscle (Wolska & Wieczorek, 2003). Interestingly, these Tm isoforms differ significantly from each other in or near the Tm–Tm overlap regions, suggesting a causal link between the type of Tm isoform and variation in cooperative activation of the thin filaments (Wolska *et al.* 1999).

Exactly what specific region of the N-terminus is functionally important, and how its functions are further modulated by Tm isoforms has not been studied. Based on our recent mechano-dynamic studies on transgenic (TG) mouse expressing a cTnT variant in the heart (Gollapudi *et al.* 2012), we identified two specific regions in the N-terminus of mouse cTnT (McTnT) as the potential candidates for the functional effects of the N-terminus of cTnT on cardiac thin filaments (see Fig. S3 in the Supplemental Material). We deleted specific regions within the N-terminus of McTnT to create $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ proteins. The impact of deletions in McTnT on cardiac function was tested by reconstituting the proteins into detergent-skinned cardiac papillary fibres isolated from non-transgenic (NTG) mice expressing α-Tm. Because the N-terminus of cTnT interacts with the head-to-tail overlapping region of Tm, it is important to understand how Tm isoforms modulate the functional effects of the N-terminus of cTnT. Therefore, to test how the effects of the N-terminus of cTnT are modulated by Tm isoforms, McTnT deletion proteins were reconstituted into detergent-skinned cardiac papillary fibres isolated from TG mice that expressed ∼55% β-Tm and ∼45% α-Tm. The rationale for choosing β-Tm TG mice is that α- and β-Tm differ by 39 amino acids, 25 of which are confined to the carboxyl domain (Muthuchamy *et al.* 1997); this region of Tm interacts with the N-terminus of TnT (region of TnT that is being investigated in this study).

Contractile function and dynamic force–length measurements were made to delineate the effects of cTnT deletion mutants, and to test how their effects were further modulated by α- and β-Tm isoforms. Data presented here provide the first explicit evidence to demonstrate that the 1—44 region of cTnT is essential for maximal activation, while the 45—74 region of cTnT is essential for cooperative activation of cardiac myofibres. Moreover, our study reveals the divergent effects of α- and β-Tm isoforms on cTnT-mediated contractile activation of cardiac myofibres.

Methods

Ethical approval/animal protocols

TG mice (FVBN strain), expressing ∼55% β-Tm and ∼45% α-Tm in the myocardium, used in this study were previously generated and well characterized (Muthuchamy *et al.* 1995). NTG mice (FVBN strain), expressing α-Tm in the myocardium, were used as controls. All animals used in this study were properly handled to minimize the pain and suffering, according to the established guidelines of the Washington State University Institutional Animal Care and Use Committee.

Preparation of detergent-skinned cardiac muscle fibre bundles

Detergent-skinned cardiac papillary muscle fibres were prepared as previously described (Chandra *et al.* 2006, 2007). In brief, mice were deeply anaesthetized by inhalation of isoflurane. The depth of the anaesthesia was assessed by a lack of pedal withdrawal reflex. Hearts were quickly removed and placed into an ice-cold high-relaxing (HR) solution of pCa (pCa = −log of $[Ca^{2+}]_{free}$) 9.0 containing (in mM): 2,3-butanedione monoxime, 20; *N*,*N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES), 50; EGTA, 20; $MgCl_2$, 6.29; Na_2ATP, 6.09; potassium propionate, 30.83; sodium azide, 10; dithiothreitol (DTT), 1.0; benzamidine-HCl, 4. The pH of the solution was adjusted to 7.0 with KOH. A fresh cocktail of protease inhibitors (in μM: bestatin, 5; E-64, 2; leupeptin, 10; pepstatin, 1; phenylmethylsulfonyl fluoride (PMSF), 200) was added to the HR solution. Papillary muscle bundles collected from the left ventricles of the hearts were further dissected into smaller fibres of approximately 0.15 mm in width and 2 mm in length. Fibres were detergent-skinned overnight at 4°C in HR containing 1% Triton X-100.

Expression and purification of recombinant mouse cardiac Tn subunits

Recombinant *c-myc*-tagged McTnT (*c-myc* McTnT), mouse cardiac TnI (McTnI) and mouse cardiac TnC (McTnC), all cloned into pSBETa vector, were expressed in BL21*DE3 cells (Novagen, Madison, WI, USA) for protein synthesis (Chandra *et al.* 2006). McTnT 1—44 and McTnT 45—74 deletion ($McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$) genes were synthesized (GenScript USA Inc, Piscataway, NJ, USA) after codon optimization for enhanced protein expression, cloned into pSBETa vector and were expressed in BL21*DE3 cells. For all protein preparations, BL21*DE3 cells cultured in 6 litres of terrific broth were spun down and sonicated, to lyse the cells and release the expressed proteins, in a buffer containing 50 mM Tris base, 6 M urea, 5 mM EDTA (pH 8.0 at 4°C). The sonication buffer also contained a cocktail of protease inhibitors: 0.2 mM PMSF, 5 mM benzamidine-HCl, 10 μM leupeptin, 1 μM pepstatin, 5 μM bestatin, 2 μM E-64 and 1 mM

DTT. The sonicated preparation was then centrifuged to pellet out the insoluble cellular particulate material. The supernatant from the sonicated preparation was used for ammonium sulfate fractionation procedure. *c-myc* McTnT and $McTnT_{45-74\Delta}$ proteins were purified as follows: The pellet from 60% ammonium sulfate fractionation was dissolved in 50 mM Tris base (pH 8.0 at 4°C), 6 M urea, 1 mM EDTA, 0.2 mM PMSF, 5 mM benzamidine-HCl, 0.01% sodium azide and 1 mM DTT, and then purified by ion-exchange chromatography on a DEAE-fast Sepharose column (GE Healthcare Biosciences, Pittsburgh, PA, USA). *c-myc* McTnT and $McTnT_{45-74\Delta}$ proteins were eluted with a 0−0.4 M NaCl gradient. $McTnT_{1-44\Delta}$ protein was purified using a similar procedure used for *c-myc* McTnT, except that a CM Sepharose column (GE Healthcare Biosciences) was used for ion-exchange chromatography. McTnI protein was purified as follows: the supernatant from the sonicated preparation was loaded onto a CM Sepharose column and eluted with a 0−0.3 M NaCl gradient. The eluted fractions containing McTnI protein were pooled and dialysed against a buffer containing 50 mM Tris-HCl, 6 M urea, 0.2 M NaCl, 3 mM $CaCl_2$ and a cocktail of protease inhibitors (pH 8.0 at 4°C). The dialysed fractions were then loaded onto a cTnC affinity column. McTnI was eluted using a buffer containing 50 mM Tris-HCl, 6 M urea, 1 M NaCl, 2 mM EDTA and a cocktail of protease inhibitors (pH 8.0 at 4°C). McTnC protein was purified as follows: the supernatant from the sonicated preparation was loaded onto a DE-52 column (GE Healthcare Biosciences) and eluted with a 0–0.3 M NaCl gradient. The eluted fractions containing McTnC protein were pooled and dialysed against a buffer containing 50 mM Tris base, 6 M urea, 1 M NaCl, 5 mM $CaCl_2$ and a cocktail of protease inhibitors (pH 7.5 at 4°C). The dialysed fractions were loaded onto a Phenyl Sepharose column and eluted with a buffer containing 50 mM Tris base, 1 M NaCl, 10 mM EDTA and a cocktail of protease inhibitors. For all the preparations, the eluted protein fractions were run on a 12.5% SDS gel to assess their purity. Fractions containing the pure protein were pooled and dialysed thoroughly against deionized water containing 15 mM β-mercaptoethanol, lyophilized and stored at −80°C.

Reconstitution of recombinant mouse cardiac Tn subunits into detergent-skinned mouse cardiac muscle fibres

Reconstitution of recombinant Tn subunits into muscle fibres was performed as described previously (Chandra *et al.* 2007). *c-myc*-tagged wild-type McTnT ($McTnT_{WT}$) was used as the control. In brief, the extraction of native Tn subunits was carried out using an extraction solution containing a mixture of McTnT (*c-myc* McTnT or $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$) and McTnI proteins. *c-myc* $McTnT_{WT}$ was used in our procedure so that the incorporation of exogenously added Tn subunits in the control reconstitution could be assessed by observing a differential SDS gel migration pattern of *c-myc* McTnT when compared with the endogenous McTnT. It has been shown that the use of McTnT with an 11 amino acid *c-myc* epitope at the N-terminus does not affect the normal cardiac function (Tardiff *et al.* 1998; Montgomery *et al.* 2001). Replacement of endogenous Tn subunits was carried out using an extraction solution containing McTnT (*c-myc* McTnT or $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$; 1.5 mg ml^{-1}, W/V) and McTnI (1.0 mg ml^{-1}, W/V) in 50 mM Tris-HCl (pH 8.0), 6 M urea, 1.0 M KCl, 10 mM DTT and a cocktail of protease inhibitors. High salt and urea in the extraction solution were removed according to a protocol described previously (Chandra *et al.* 2006). Finally, the McTnT–McTnI-treated fibres were incubated with McTnC to complete the reconstitution procedure. Detergent-skinned fibres reconstituted with $McTnT_{1-44\Delta}$ + McTnI + McTnC are referred to as '$McTnT_{1-44\Delta}$', and those reconstituted with $McTnT_{45-74\Delta}$ + McTnI + McTnC are referred to as '$McTnT_{45-74\Delta}$'. Fibres reconstituted with *c-myc* McTnT + McTnI + McTnC are referred to as '$McTnT_{WT}$' and were used as controls.

The reconstituted fibres were resuspended in 2% SDS solution (10 μl $fibre^{-1}$) for SDS–PAGE, as described previously (Mamidi *et al.* 2012). SDS-digested fibres were mixed with an equal volume of protein loading dye that contained 125 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.01% bromophenol blue and 50 mM β-mercaptoethanol. Equal quantities of protein (15 μg of total protein for NTG and 10 μg of total protein for TG samples) from the digested fibres were loaded and separated on a SDS–PAGE (4% acrylamide stacking gel and 10% acrylamide separating gel) and stained with Bio-Safe Coomassie blue G-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to visualize the differential migration pattern of exogenously added McTnT proteins when compared with endogenous McTnT.

Proteins separated on the SDS gel were transferred onto a polyvinylidene fluoride membrane for Western blot analysis using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc). The incorporation of *c-myc* McTnT, $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$ was assessed using a monoclonal anti-TnT primary antibody (Sigma-Aldrich, ST Louis, MO, USA; clone JLT-12), followed by a HRP-labelled anti-mouse secondary antibody (Amersham Biosciences, Pittsburgh, PA, USA). Densitometric scanning of Western blots was performed using Image J software (acquired from NIH at: http://rsbweb.nih.gov/ij/).

Estimation of isoform levels and phosphorylation status of sarcomeric proteins in NTG and TG heart samples

Ventricles from NTG and TG mice were frozen in liquid nitrogen and thoroughly pulverized using a pestle and mortar as described previously (Ford *et al.* 2012). The pulverized tissue was resuspended in a protein extraction buffer (10 μl of buffer per 1 mg of tissue). The composition of the protein extraction buffers was: 2.5% SDS, 10% glycerol, 50 mM Tris base (pH 6.8 at 4°C), 1 mM DTT, 1 mM PMSF, 4 mM benzamidine-HCl, a cocktail of phosphatase inhibitors (PhosSTOP; Roche Applied Science, Indianapolis, IN, USA) and protease inhibitors (E-64, bestatin and leupeptin). The resuspended tissue was further homogenized using a tissue tearor and sonication in a water bath at 4°C. The sample was finally centrifuged at 10,000 rpm (7200g). Equal quantities (5 μg of total protein) of NTG and TG heart samples were loaded and separated on a 12.5% SDS gel. SDS gels were stained with either Coomassie blue or Pro-Q Diamond (Invitrogen, Carlsbad, CA, USA) to assess isoform expression levels or phosphorylation status of sarcomeric proteins.

Measurement of steady-state isometric tension and ATPase activity in reconstituted cardiac muscle fibres

Measurement of isometric steady-state tension and ATPase activity was according to a procedure described previously (Chandra *et al.* 2007, 2009). In brief, T-shaped aluminum clips were used to hold the muscle fibre between the motor arm (322C; Aurora Scientific Inc, Aurora, Ontario, Canada) and the force transducer (AE 801; Sensor One Technologies Corp, Sausalito, CA, USA). The resting sarcomere length (SL) was set to 2.3 μm using a laser diffraction pattern (Chandra *et al.* 2007, 2009). The muscle fibre was immersed in a constantly-stirred chamber containing Ca^{2+} solutions, the concentration of which ranged from pCa 4.3 to 9.0. The composition of various pCa solutions was calculated using methods described previously (Fabiato & Fabiato, 1979). The composition of the maximal Ca^{2+} activation solution (pCa 4.3) was (in mM): potassium propionate, 31; Na_2ATP, 5.95; $MgCl_2$, 6.61; EGTA, 10; $CaCl_2$, 10.11; BES, 50; sodium azide, 5; phosphoenol pyruvate (PEP), 10. The composition of the relaxing solution (pCa 9.0) was (in mM): potassium propionate, 51.14; Na_2ATP, 5.83; $MgCl_2$, 6.87; EGTA, 10; $CaCl_2$, 0.024; BES, 50; NaN_3, 5; PEP, 10. The activation and relaxing solutions also contained 0.5 mg ml^{-1} pyruvate kinase (500 U mg^{-1}), 0.05 mg ml^{-1} lactate dehydrogenase (870 U mg^{-1}), 20 μM diadenosine pentaphosphate, 10 μM oligomycin and a cocktail of protease inhibitors. The pH of the Ca^{2+} solutions was adjusted to 7.0 using KOH. The ionic strength was 180 mM. After two cycles of maximal activation and relaxation, the SL of the muscle fibre was again monitored and set to 2.3 μm if necessary. The muscle fibre was then immersed in a series of Ca^{2+} solutions (pCa 4.3−9.0), and the isometric steady-state tensions were digitally recorded on a computer.

Steady-state isometric ATPase activity was measured according to a protocol described previously (de Tombe & Stienen, 1995; Chandra *et al.* 2007). Briefly, near-UV light (340 nm) was projected through the muscle chamber, then split (50:50) via a beam splitter and detected at 340 nm (sensitive to changes in NADH) and 400 nm (insensitive to changes in NADH). ATPase activity was measured as follows: ATP regeneration from ADP was coupled to the breakdown of PEP to pyruvate and ATP catalysed by pyruvate kinase, which was linked to the synthesis of lactate catalysed by lactate dehydrogenase. The breakdown of NADH during the synthesis of lactate was proportional to the ATP consumption and was measured by changes in UV absorbance at 340 nm. The signal for NADH was calibrated by multiple injections of 0.25 nmol of ADP.

Measurement of muscle fibre mechano-dynamics in reconstituted cardiac muscle fibres

Muscle fibres were activated in pCa 4.3 solution and, once a steady-state isometric activation was attained, force responses to step-like length perturbations (±0.5%, ±1.0%, ±1.5% and ±2.0% of the muscle length (ML)) were recorded as described previously (Ford *et al.* 2010). A non-linear recruitment distortion (NLRD) model (Ford *et al.* 2010) was fit to the force responses to estimate the following model parameters: b, the rate by which new XBs are recruited due to a change in ML; c, the rate by which the strain of distorted XBs was dissipated following a change in ML; γ, a parameter that characterizes the XB strain-mediated effect on the recruitment of strong XBs; E_D, the instantaneous muscle fibre stiffness estimated from the immediate force response elicited due to a sudden change in ML; and E_R, the magnitude of increase, from an initial steady-state to a new steady-state, in muscle fibre stiffness due to length-mediated recruitment of additional XBs. For additional details on the NLRD model, please refer to the Supplemental Material.

Measurement of rate of tension redevelopment (k_{tr}) in reconstituted cardiac muscle fibres

Measurements of k_{tr} in maximally activated (pCa 4.3) muscle fibres was according to a protocol (Brenner & Eisenberg, 1986) described in the Supplemental Material.

Data analysis

Data were analysed using two-way ANOVA. One factor in this analysis was McTnT variant ($McTnT_{WT}$, $McTnT_{1-44\Delta}$

or $McTnT_{45-74\Delta}$), and the second was Tm isoform (α-Tm or β-Tm). Therefore, we used two-way ANOVA to test the hypothesis that the effect of the McTnT variant on a given contractile parameter depended on the type of Tm isoform (interaction effect). When the interaction effect was significant, it showed that the differences between effects of $McTnT_{WT}$, $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$ were dissimilar in the presence of different Tm isoforms. When the interaction effect was not significant, we interpreted the main effect due to McTnT variant or Tm isoform. Planned multiple pairwise comparisons were made using Fisher's LSD method to test the effects of McTnT variants or Tm isoforms on contractile parameters. Hill's equation was fitted to normalized pCa–tension data to estimate pCa_{50} ($-\log$ of $[Ca^{2+}]_{free}$ required for half-maximal activation) and Hill coefficient (n_H) values. Values are reported as mean ± SEM. The criterion for statistical significance was set at $P < 0.05$. Asterisks in the figures represent statistical significance using *post hoc* (Fisher's LSD) comparisons.

Results

Rationale for the generation of $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ deletion mutants

Based on our recent work (Gollapudi *et al.* 2012), we identified two specific regions in McTnT as the potential candidates for the functional effects of the N-terminal region on cardiac thin filaments: (1) the highly acidic region of McTnT (1−44 amino acids), which shares 50% sequence homology with its counterpart (1−41 amino acids) from the mouse fast skeletal TnT (MfsTnT); and (2) the peptide sequence corresponding to 45−74 amino acids of McTnT, which is only present in the cTnT (Fig. 1*A* and Fig. S3 in the Supplemental Material; Perry, 1998). These specific regions were deleted from McTnT to create $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ (Fig. 1*B*).

SDS–PAGE analysis of mouse cardiac muscle fibres to estimate the expression levels of β-Tm and the phosphorylation status of myofilament proteins

An equal quantity (5 μg of total protein) of samples from ventricular homogenates of NTG (α-Tm) and TG (β-Tm) mice was loaded and separated on a 12.5% SDS–PAGE, followed by staining with Coomassie blue. As shown in Fig. 2*A*, the amount of β-Tm was estimated to be ∼55% and the amount of α-Tm was ∼45% in the TG samples (Muthuchamy *et al.* 1995). No extra bands, apart from Tm, were found in TG samples, indicating that the isoform expression of other sarcomeric proteins such as myosin heavy chain, cardiac myosin binding protein-C, cTnT, cTnI and myosin light chain-1 were not different between NTG and TG samples. 12.5% SDS gels were stained with Pro-Q Diamond (Invitrogen) to estimate the phosphorylation status of sarcomeric proteins in NTG and TG samples (Ford *et al.* 2012). As shown in Fig. 2*B*, the phosphorylation status of sarcomeric proteins, apart from Tm, was not different between NTG and TG samples. TG samples had an extra phosphorylated band that corresponded to β-Tm.

SDS–PAGE and Western blot analysis of reconstituted fibres to estimate the extent of incorporation of exogenously added Tn subunits

$McTnT_{WT}$ was used as the control. In our reconstitution procedure, the exogenously added Tn complex consisted of McTnT (*c-myc* McTnT or $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$), McTnI and McTnC proteins. The

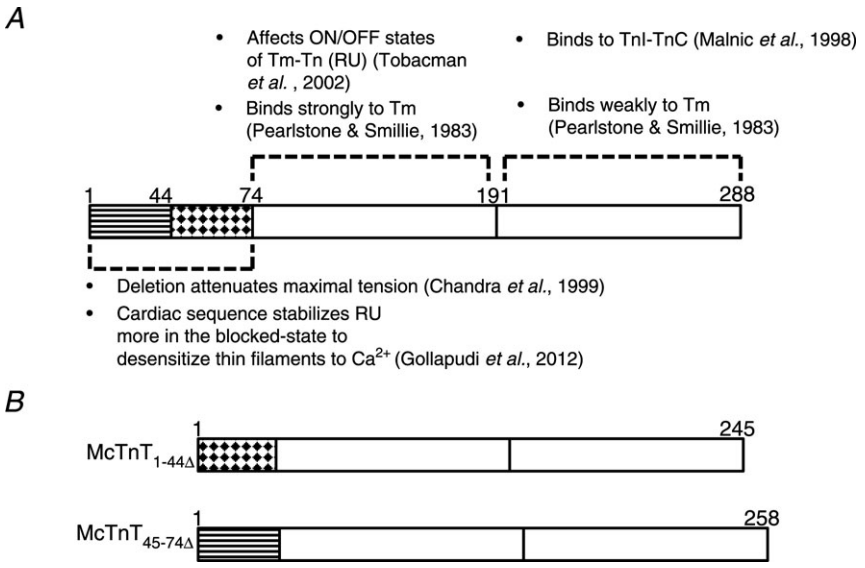

Figure 1. Rationale for the generation of mouse cardiac troponin T ($McTnT)_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ deletion mutants
Based on our recent results from TG experiments (Gollapudi *et al.* 2012), we have identified two important regions within the N-terminus of McTnT (see Fig. S3 in the Supplemental Material for details). *A*, schematic representation of McTnT sequence depicting the functional significance of its N-terminal end (residues 1–74), N-terminal region (residues 75–191) and C-terminal region (residues 192–288). Regions of McTnT that have been deleted in the present study are indicated by striped ($McTnT_{1-44\Delta}$) and dotted ($McTnT_{45-74\Delta}$) patterns, respectively. *B*, schematic representation of $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ sequences. Tm, tropomyosin; Tn, troponin; RU, regulatory unit.

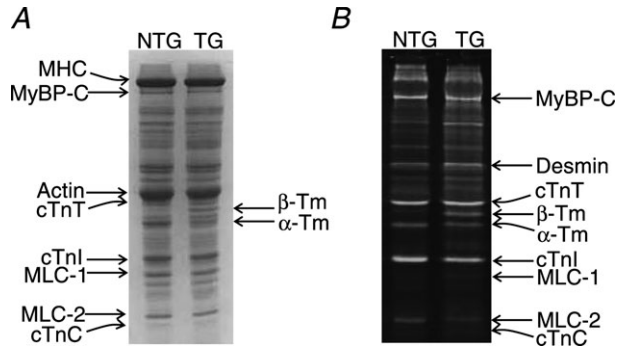

Figure 2. SDS–PAGE analysis of non-transgenic (NTG) and transgenic (TG) ventricular homogenates to estimate the expression level of β-tropomyosin (Tm) and phosphorylation status of sarcomeric proteins
Samples from NTG and TG ventricular homogenates were run on a 12.5% SDS gel. *A*, Coomassie blue-stained SDS gel showing an expression of ∼55% of β-Tm and ∼45% of α-Tm in TG hearts. There are no extra bands in the TG sample, apart from Tm, indicating that there are no changes in the isoform expression of other sarcomeric proteins between the NTG and TG samples. *B*, Pro-Q Diamond-stained SDS gel indicating that the phosphorylation status of sarcomeric proteins is not significantly different between NTG and TG samples. As expected, there is an additional band corresponding to β-Tm in the TG fibre lane. cTnC, cardiac troponin C; cTnI, cardiac troponin I; cTnT, cardiac troponin T; MHC, myosin heavy chain; MLC-1, myosin light chain-1; MyBP-C, myosin binding protein-C.

incorporation of $McTnT_{WT}$ was detected by the differential migration pattern of *c-myc*-tagged McTnT, when compared with the endogenous cTnT. The incorporation of $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$ was detected based on mobility differences when compared with the endogenous cTnT. As we have shown before (Chandra *et al.* 1999), the endogenous Tn complex is replaced as a whole when the molar excess of exogenously added cTnT competes with the endogenous cTnT. Therefore, the bands that correspond to McTnI and McTnC in the reconstituted fibres belong to the exogenously added components. First, the SDS-digested reconstituted fibre samples were run on an 8% SDS gel to visualize the mobility-based migration pattern of exogenously added McTnT proteins. Using SDS gels, we could not precisely quantify the extent of McTnT incorporation because the *c-myc*-tagged McTnT (WT) co-migrated with actin (lane *c* of Fig. 3*A*), while the presence of Tm bands hindered unambiguous quantification of $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$ (lanes *d* and *e* of Fig. 3*A*); hence, we used the Western blot analysis. Image J software was used to accurately quantify the optical intensities of the protein bands. The total optical band intensity (i.e. the total amount of cTnT in a single lane) was assumed to be the sum of the optical band intensities of endogenous McTnT and the incorporated McTnT (*c-myc*-tagged McTnT, $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$) bands. The extent of incorporation was then determined by dividing the optical band intensity of the exogenously added McTnT with the total band intensity. This approach allowed us to unambiguously estimate the extent of incorporation of different proteins: it was 90% for both *c-myc*-tagged McTnT (lane *c* of Fig. 3*B*) and $McTnT_{45-74\Delta}$ (lane *b* of Fig. 3*C*), but 97% for $McTnT_{1-44\Delta}$ (lane *c* of Fig. 3*C*). The extent of replacement of endogenous McTnT in the reconstituted TG fibre groups was similar to that in the reconstituted NTG fibre groups.

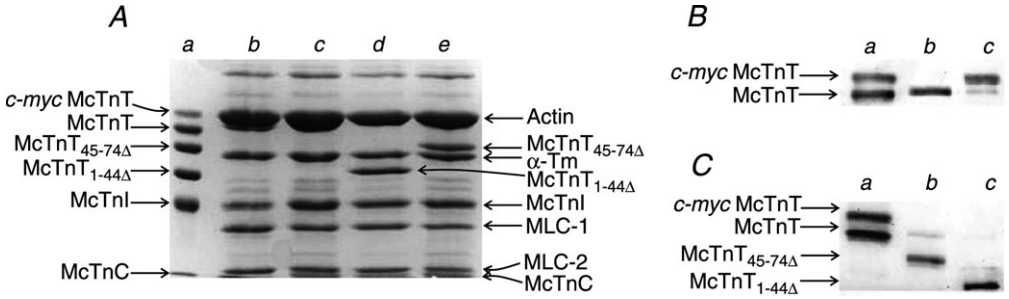

Figure 3. SDS–PAGE and Western blot analysis of reconstituted NTG (α-tropomyosin (Tm)) mouse fibres
Reconstituted NTG fibres were digested using 2% SDS (Mamidi *et al.* 2012). Digested samples were run on a 10% SDS gel to estimate the extent of replacement of endogenous Tn subunits. *c-myc*-tagged wild-type McTnT ($McTnT_{WT}$) was used as the control. Muscle fibre preparations from normal NTG hearts were used to show the extent of removal of endogenous cTnT in the reconstituted fibres. *A*, SDS gel showing the differential migration pattern of $McTnT_{WT}$, $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$ proteins in the reconstituted NTG fibre samples. Lane *a* represents purified recombinant proteins. Lanes *b*–*e* represent samples from normal NTG fibre, $McTnT_{WT}$, $McTnT_{1-44\Delta}$ or $McTnT_{45-74\Delta}$ reconstituted fibres. Lane *b* shows the presence of the endogenous McTnT, whereas lanes *c*–*e* show a near-complete replacement of endogenous McTnT in the reconstituted samples. *B*, Western blot analysis of the reconstituted fibre samples. Lane *a* represents purified recombinant proteins. Lanes *b* and *c* represent samples from normal NTG and $McTnT_{WT}$ reconstituted samples, respectively. *C*, Western blot analysis of $McTnT_{45-74\Delta}$ and $McTnT_{1-44\Delta}$ reconstituted samples. Lane *a* represents purified recombinant proteins. Lanes *b* and *c* represent samples from $McTnT_{45-74\Delta}$ and $McTnT_{1-44\Delta}$ reconstituted fibres, respectively. MLC-1, myosin light chain-1.

Effect of McTnT deletion mutants on the Ca^{2+}-activated maximal tension and ATPase activity in α- *vs.* β-Tm fibres

To assess the effect of McTnT deletion mutants on thin-filament activation, Ca^{2+}-activated maximal tension was measured at pCa 4.3 in reconstituted fibres. Two-way ANOVA revealed no significant interaction effect, but showed a significant main effect ($P < 0.0001$) of McTnT deletion mutants on the Ca^{2+}-activated maximal tension (Fig. 4*A* and *B*). To probe the determining factor for the significant main effect, subsequent *post hoc* tests were carried out. These *post hoc* tests using multiple planned pairwise comparisons revealed that $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, had a significant effect of decreasing maximal tension under both α- and β-Tm backgrounds. $McTnT_{1-44\Delta}$ decreased maximal tension by 36% in α-Tm fibres and by 30% in β-Tm fibres (Fig. 4*A* and *B*). Thus, whether it was under α- or β-Tm background, $McTnT_{1-44\Delta}$ attenuated the Ca^{2+}-activated maximal tension. In accord with our observations from Ca^{2+}-activated tension experiments, $McTnT_{1-44\Delta}$ attenuated the Ca^{2+}-activated maximal ATPase activity in α- and β-Tm fibres (Table 1). In agreement with our observations from Ca^{2+}-activated tension measurements, $McTnT_{45-74\Delta}$ had no effect on maximal ATPase activity in α- or β-Tm fibres (Table 1).

Effect of McTnT deletion mutants on the muscle fibre stiffness in α- *vs.* β-Tm fibres

To examine if the decrease in maximal tension by $McTnT_{1-44\Delta}$ was due to a decrease in the number of force-generating XBs, we measured the instantaneous muscle fibre stiffness (E_D) by imposing step-like length perturbations in constantly-activated muscle fibres (Ford *et al.* 2010). E_D is an approximate measure of stiffness of the population of strongly-bound XBs at the time of instantaneous ML change (refer to the Supplemental Material for details; and Ford *et al.* 2010). Two-way ANOVA showed no significant interaction effect, but

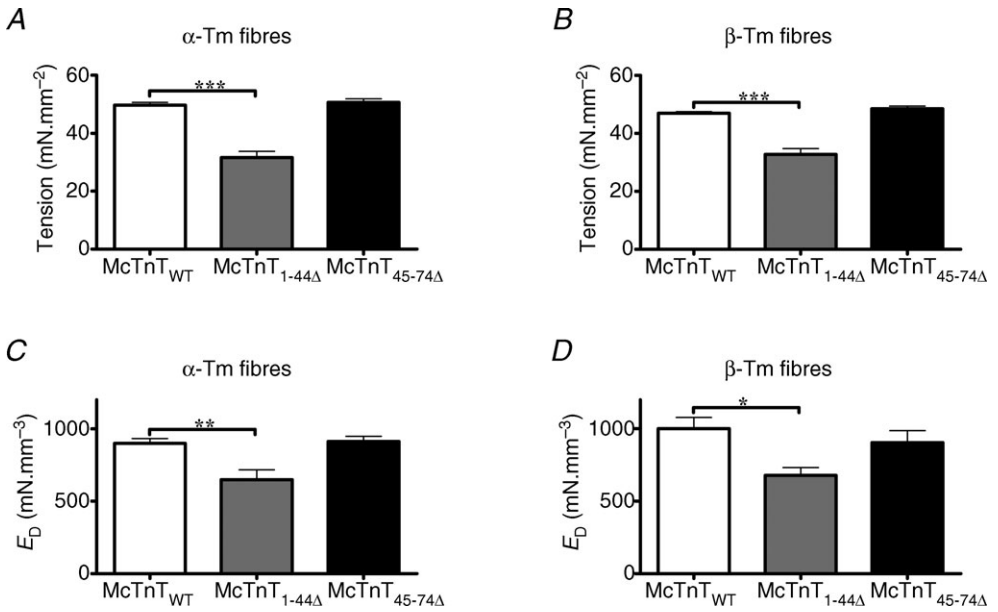

Figure 4. Effect of mouse cardiac troponin T (McTnT) deletion mutants on Ca^{2+}-activated maximal tension and muscle fibre stiffness (E_D) in α- *vs.* β-tropomyosin (Tm) fibres
Ca^{2+}-activated maximal tension was measured in reconstituted fibres at pCa 4.3 (de Tombe & Stienen, 1995). *A*, effect of McTnT deletions on Ca^{2+}-activated maximal tension in NTG fibres containing α-Tm. The number of determinations was 14, 13 and 14 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. *B*, effect of McTnT deletions on Ca^{2+}-activated maximal tension in TG fibres containing β-Tm. The number of determinations was 13, 14 and 14 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. Two-way ANOVA revealed no significant interaction effect, but revealed a significant main effect ($P < 0.0001$) of McTnT deletions on Ca^{2+}-activated maximal tension. *C*, effect of McTnT deletions on E_D in NTG fibres containing α-Tm. The number of determinations was 12, 7 and 13 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. *D*, effect of McTnT deletions on E_D in TG fibres containing β-Tm. The number of determinations was 14, 7 and 12 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. E_D represents muscle fibre stiffness and is an approximation of the number of strongly-bound XBs (Ford *et al.* 2010). E_D was estimated by subjecting maximally activated muscle fibres to step-like length changes (Ford *et al.* 2010). Two-way ANOVA revealed no significant interaction effect, but showed a significant main effect ($P < 0.001$) of McTnT deletions on E_D. Values are reported as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Table 1. k_{tr}, *b*, ATPase and pCa_{50} (ATPase) values in NTG (α-Tm) and TG (β-Tm) fibres

	$McTnT_{WT}$	$McTnT_{1-44\Delta}$	$McTnT_{45-74\Delta}$
NTG (α-Tm)			
k_{tr} (s^{-1})	12.87 ± 0.46 (14)	14.18 ± 0.47 (13)	14.05 ± 0.56 (14)
b (s^{-1})	31.81 ± 1.41 (12)	34.88 ± 3.20 (7)	33.83 ± 1.61 (13)
ATPase (pmol mm^{-3} s^{-1})	304.38 ± 9.36 (14)	225.33 ± 10.67 (13)***	303.79 ± 7.97 (14)
pCa_{50} (ATPase)	5.69 ± 0.02 (11)	5.58 ± 0.03 (12)**	5.99 ± 0.03 (7)***
TG (β-Tm)			
k_{tr} (s^{-1})	12.58 ± 0.50 (12)	13.84 ± 0.65 (14)	13.86 ± 0.52 (14)
b (s^{-1})	31.94 ± 1.33 (14)	35.42 ± 1.63 (7)	34.79 ± 1.13 (12)
ATPase (pmol mm^{-3} s^{-1})	258.01 ± 3.77 (10)	224.16 ± 8.31 (14)***	278.02 ± 8.44 (14)
pCa_{50} (ATPase)	5.74 ± 0.01 (8)	5.71 ± 0.01 (10)	6.18 ± 0.02 (7)***

The rate of tension redevelopment (k_{tr}) was determined using a large release and restretch protocol (Brenner & Eisenberg, 1986). The rate of XB recruitment (*b*) was determined by fitting the NLRD model to the force responses elicited by step-like changes in ML (Ford *et al.* 2010). The Hill equation was fitted to the normalized pCa–ATPase relationships to derive pCa_{50} (ATPase) values. The number of determinations for each group is presented in the brackets beside each estimate. Values are reported as mean ± SEM. $^{**}P < 0.005$; $^{***}P < 0.001$ when compared with $McTnT_{WT}$ within the respective groups. McTnT, mouse cardiac troponin T; $McTnT_{WT}$, wild-type mouse cardiac troponin T; NTG, non-transgenic; TG, transgenic; Tm, tropomyosin.

revealed a significant main effect ($P < 0.001$) of McTnT deletion mutants on E_D. Subsequent *post hoc* tests revealed that $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, had a significant effect of decreasing E_D in both α- and β-Tm fibres (Fig. 4*C* and *D*). For example, $McTnT_{1-44\Delta}$ decreased E_D by ∼28% in α-Tm fibres (Fig. 4*C*) and by ∼32% in β-Tm fibres (Fig. 4*D*). Thus, our data demonstrated that the decrease in Ca^{2+}-activated maximal tension in fibres reconstituted with $McTnT_{1-44\Delta}$ was due to reduced thin-filament activation caused by a decrease in the number of force-generating XBs. That the peptide segment 1−44 is essential for maximal activation is supported by our previous studies on the deletion of 1−76 amino acids in rat cTnT (RcTnT; Chandra *et al.* 1999) and the caspase-activated deletion of 1−96 amino acids in RcTnT (Communal *et al.* 2002). Although these two previous studies could not locate the specific segment responsible for the depression of thin-filament activation, our present study demonstrates that it is the first 44 amino acids of cTnT. This inference is supported by our observation that $McTnT_{45-74\Delta}$ mutant showed no effect on maximal activation because the peptide segment essential for maximal activation (1−44 amino acids) is still intact in the $McTnT_{45-74\Delta}$ mutant.

Effect of McTnT deletion mutants on myofilament Ca^{2+} sensitivity (pCa_{50}) in α- *vs.* β-Tm fibres

Normalized tension values were plotted against a range of pCa to estimate the pCa–tension relationships in the reconstituted fibres (Fig. 5*A* and *B*). The Hill equation was fitted to the pCa–tension relationships to estimate the pCa_{50} (Fig. 5*C*). Two-way ANOVA revealed a significant interaction effect ($P < 0.01$) on pCa_{50}, suggesting that the effect of McTnT deletions on pCa_{50} was influenced by the Tm isoform. In other words, these results demonstrate that the interplay between the effects of McTnT and Tm on thin filaments modulates Ca^{2+} sensitivity of tension development. In α-Tm fibres, $McTnT_{1-44\Delta}$ caused a significant decrease ($P < 0.001$) in pCa_{50}, as indicated by a rightward shift in the pCa–tension relationship (Fig. 5*A*). The $[Ca^{2+}]_{free}$ needed to produce half-maximal tension was ∼23% more in α-Tm fibres containing $McTnT_{1-44\Delta}$ when compared with the control $McTnT_{WT}$. Irrespective of the type of reconstitution, β-Tm fibres showed a higher pCa_{50} when compared with the corresponding group of α-Tm fibres (Fig. 5*C*). An interesting observation was that the decrease in pCa_{50} induced by $McTnT_{1-44\Delta}$ under an α-Tm background was ablated under a β-Tm background (indicated by arrows in Fig. 5*B*). This demonstrated that β-Tm maintained its ability to increase myofilament Ca^{2+} sensitivity even in the presence of a McTnT mutant protein that had the ability to decrease myofilament Ca^{2+} sensitivity – thus, resulting in attenuating the effect of $McTnT_{1-44\Delta}$ on myofilament Ca^{2+} sensitivity under β-Tm background.

In contrast, $McTnT_{45-74\Delta}$ had an opposite effect on myofilament Ca^{2+} sensitivity of tension (Fig. 5). For example, $McTnT_{45-74\Delta}$ induced a leftward shift in the pCa–tension relationship (Fig. 5*A* and *B*), causing a significant increase in pCa_{50} (Fig. 5*C*). The $[Ca^{2+}]_{free}$ needed to produce half-maximal tension was ∼55% less in α-Tm fibres containing $McTnT_{45-74\Delta}$ when compared with the control $McTnT_{WT}$. Another significant observation was that the increase in pCa_{50} induced by $McTnT_{45-74\Delta}$, under an α-Tm background, was further enhanced under a β-Tm background (Fig. 5*C*). Thus, the $[Ca^{2+}]_{free}$ needed to produce half-maximal tension was

~67% less in β-Tm fibres containing McTnT$_{45-74\Delta}$ when compared with the β-Tm fibres containing McTnT$_{WT}$. Results from pCa–ATPase relationships also showed similar effects (Table 1). These observations highlight the ability of β-Tm to increase myofilament Ca^{2+} sensitivity under very different conditions (Fig. 5*C*). Collectively, these results demonstrate that the Tm isoforms have divergent effects on McTnT deletion mutant's ability to modulate myofilament Ca^{2+} sensitivity.

Effect of McTnT deletion mutants on cooperativity of tension development (n_H) in α- *vs.* β-Tm fibres

The Hill equation was fitted to pCa–tension relationships to estimate n_H in the reconstituted fibres (Fig. 5*D*). Two-way ANOVA revealed no significant interaction effect, but showed a significant main effect ($P < 0.05$) of McTnT deletion mutants and Tm isoforms on n_H. *Post hoc* tests revealed that McTnT$_{45-74\Delta}$, but not McTnT$_{1-44\Delta}$, induced a significant decrease in n_H under both α- and β-Tm backgrounds (Fig. 5*D*). McTnT$_{45-74\Delta}$ decreased n_H by ~37% in α-Tm fibres, but the McTnT$_{45-74\Delta}$-induced decrease in n_H was greater under β-Tm background, as suggested by a ~46% decrease in n_H (Fig. 5*D*). It is interesting to note that while McTnT$_{1-44\Delta}$ showed no effect on n_H, it significantly attenuated maximal tension. On the other hand, McTnT$_{45-74\Delta}$ showed a significant attenuating effect on n_H, but it had no impact on maximal tension. This further highlights our observation that these two regions (1-44 and 45-74) of cTnT have discrete functional properties.

Effect of McTnT deletion mutants on XB detachment kinetics in α- *vs.* β-Tm fibres

XB detachment kinetics in the reconstituted fibres was assessed by estimating the tension cost and the rate of

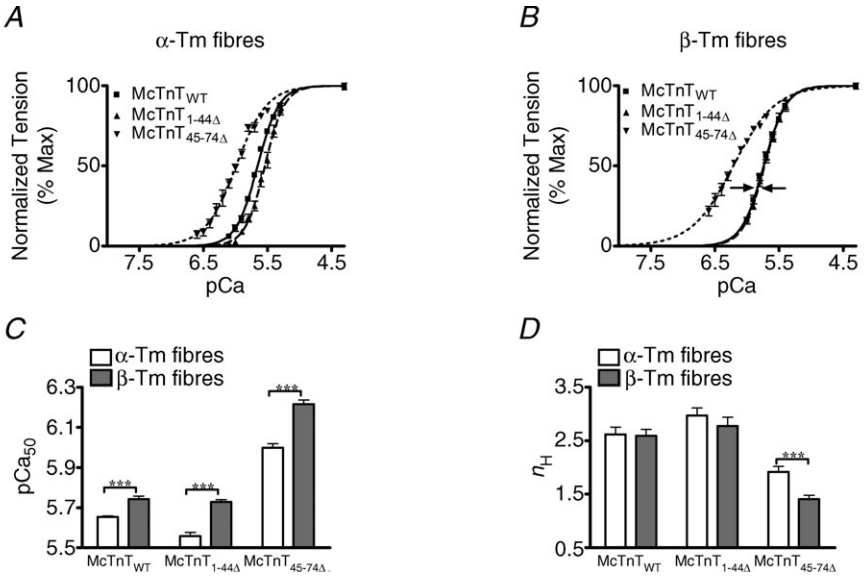

Figure 5. Effect of mouse cardiac troponin T (McTnT) deletion mutants on pCa–tension relationships, myofilament Ca^{2+}-sensitivity (pCa$_{50}$) and cooperativity of tension development (n_H) in α- *vs.* β-tropomyosin (Tm) fibres

Normalized tension values were plotted against pCa to derive the pCa–tension relationships. The Hill equation was then fitted to the pCa–tension relationships to estimate pCa$_{50}$ and n_H. *A*, effect of McTnT deletion mutants on pCa–tension relationships in NTG fibres containing α-Tm. *B*, effect of McTnT deletion mutants on pCa–tension relationships in TG fibres containing β-Tm. *C*, effect of McTnT deletion mutants on pCa$_{50}$ in α- and β-Tm fibres. *D*, effect of McTnT deletion mutants on n_H in α- and β-Tm fibres. Two-way ANOVA revealed a significant interaction ($P < 0.01$) on pCa$_{50}$, suggesting that the effect of McTnT deletion mutants on pCa$_{50}$ was influenced by the type of Tm isoform present. The main contributing factor for the significant interaction effect was the ablation of McTnT$_{1-44\Delta}$-induced reduction in pCa$_{50}$ under a β-Tm background (indicated by arrows in *B*). Irrespective of the type of reconstitution, TG (β-Tm) fibres exhibited a higher pCa$_{50}$ when compared with the corresponding group of NTG (α-Tm) fibres. Two-way ANOVA revealed no significant interaction effect, but revealed significant main effects due to McTnT deletion mutants ($P < 0.001$) and Tm isoforms ($P < 0.05$) on n_H. Subsequent *post hoc* tests revealed that McTnT$_{45-74\Delta}$ led to a decreased n_H under both α- and β-Tm backgrounds, with the decrease being more pronounced under a β-Tm background. The number of determinations for pCa–tension relationships, pCa$_{50}$ and n_H was 11, 12 and 14 for McTnT$_{WT}$, McTnT$_{1-44\Delta}$ and McTnT$_{45-74\Delta}$ groups of α-Tm fibres, respectively. The number of determinations for pCa–tension relationships, pCa$_{50}$ and n_H was 13, 10 and 14 for McTnT$_{WT}$, McTnT$_{1-44\Delta}$ and McTnT$_{45-74\Delta}$ groups of β-Tm fibres, respectively. Values are reported as mean ± SEM. ***$P < 0.001$.

XB distortion (c). To estimate tension cost values, the relationship between steady-state isometric tension and the corresponding ATPase activity was determined at various levels of Ca^{2+} activation (de Tombe & Stienen, 1995; Chandra *et al.* 2007). Two-way ANOVA revealed a significant interaction effect ($P < 0.05$), indicating the effect of McTnT deletion mutants on tension cost is influenced by the type of Tm isoform present. Subsequent *post hoc* tests revealed that $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, had a significant effect of increasing tension cost under β-Tm background (Fig. 6*A* and *B*). $McTnT_{1-44\Delta}$ increased tension cost by ∼29% in β-Tm fibres, when compared with the control β-Tm fibres reconstituted with $McTnT_{WT}$ (Fig. 6*B*). Because changes in tension cost reflect changes in the rate of XB detachment (Brenner, 1988), our data demonstrate that the rate of XB detachment is augmented by $McTnT_{1-44\Delta}$ in TG fibres containing β-Tm (Fig. 6*B*).

To substantiate our observations from tension cost experiments, we measured the rate of XB detachment kinetics, as estimated from XB distortion dynamics, c (Fig. 6*C* and *D*). Quick stretch-induced instantaneous increase in force (F_1 in Fig. S1 of Supplemental Material) decreases rapidly to a minimum with a characteristic dynamic rate. This rapid force decay in Phase 2 (Fig. S1) represents the dynamic detachment of strained XBs (Piazzesi *et al.* 1997; Campbell *et al.* 2004; Stelzer *et al.* 2006), and has been shown to be a measure of XB detachment rate (Campbell *et al.* 2004; Chandra *et al.* 2006). Two-way ANOVA showed that both the interaction and main effects were not significant. However, just as we observed in the tension cost experiments (Fig. 6*B*),

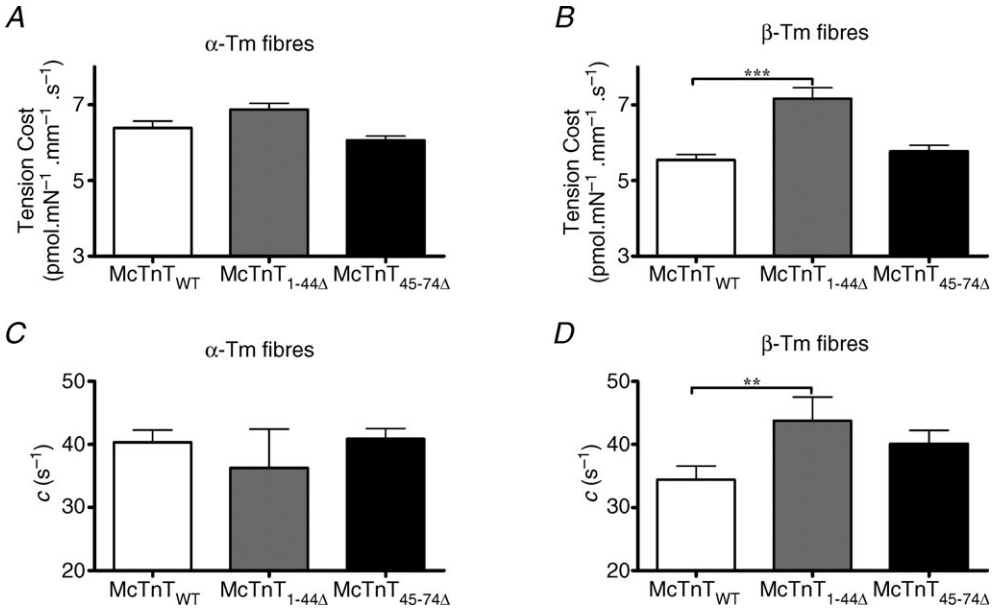

Figure 6. Effect of mouse cardiac troponin T (McTnT) deletion mutants on tension cost and rate of XB distortion dynamics (c) in α- *vs.* β-tropomyosin (Tm) fibres
Steady-state isometric tension and ATPase activity were measured simultaneously (de Tombe & Stienen, 1995; Chandra *et al.* 2007). Tension cost was derived from ATPase/tension relationship, as described previously (Chandra *et al.* 2006). c was estimated by fitting the NLRD model to force responses elicited by step-like changes in ML (Ford *et al.* 2010). Both tension cost and c represent the rate of XB detachment (Campbell *et al.* 2004). *A*, effect of McTnT deletion mutants on tension cost in NTG fibres containing α-Tm. The number of determinations was 12, 13 and 14 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. *B*, effect of McTnT deletion mutants on tension cost in TG fibres containing β-Tm. The number of determinations was 13, 14 and 14 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. Two-way ANOVA revealed a significant interaction effect ($P < 0.05$), indicating that the effect of McTnT deletion mutants on tension cost is influenced by the type of Tm isoform present. Subsequent *post hoc* tests revealed that $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, had a significant effect of increasing tension cost under a β-Tm background. *C*, effect of McTnT deletion mutants on c in NTG fibres containing α-Tm. The number of determinations was 12, 7 and 13 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. *D*, effect of McTnT deletion mutants on c in TG fibres containing β-Tm. The number of determinations was 14, 7 and 12 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups, respectively. Two-way ANOVA revealed no significant interaction effect and no significant main effects on c. However, similar to the effects observed for tension cost data, *post hoc* tests revealed that $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, had a significant effect of increasing c under a β-Tm background. Values are reported as mean ± SEM. $^{**}P < 0.05$; $^{***}P < 0.0001$.

subsequent *post hoc* tests showed that McTnT$_{1-44\Delta}$, but not McTnT$_{45-74\Delta}$, had a significant effect ($P < 0.001$) of increasing c under β-Tm background (Fig. 6*D*). For example, β-Tm fibres reconstituted with McTnT$_{1-44\Delta}$ showed a ∼27% increase in c, when compared with the control β-Tm fibres reconstituted with McTnT$_{WT}$ (Figs 6*D* and 8). Therefore, results from two independent and diverse experimental approaches demonstrate that the XB detachment kinetics is augmented by McTnT$_{1-44\Delta}$ in β-Tm fibres, but not in α-Tm fibres.

Effect of McTnT deletion mutants on XB strain-dependent impact on other XBs (γ) in α- *vs.* β-Tm fibres

Enhancement of XB detachment kinetics may affect the way strained XBs impact other XBs through an effect that is transduced via the thin-filament regulatory systems (Ford *et al.* 2010). As described in the Supplemental Material, the NLRD model parameter, γ, represents an effect by which strained XBs negatively impact other XBs via allosteric/cooperative mechanisms in the thin filament. Based on the augmenting effect of McTnT$_{1-44\Delta}$ on XB detachment kinetics (Fig. 6), we predicted that McTnT$_{1-44\Delta}$ would increase γ in the presence of β-Tm. Two-way ANOVA revealed no significant interaction effect, but showed a significant main effect ($P < 0.05$) of McTnT deletions on γ (Fig. 7*A* and *B*). The contributing factor for this significant main effect was determined by *post hoc* tests using multiple planned-pairwise comparisons. *Post hoc* analysis showed that McTnT$_{1-44\Delta}$, but not McTnT$_{45-74\Delta}$, induced a significant increase in γ under β-Tm background (Figs 7*B* and 8). For example, β-Tm fibres reconstituted with McTnT$_{1-44\Delta}$ showed a ∼58% increase in γ when compared with the control β-Tm fibres reconstituted with McTnT$_{WT}$ (Fig. 7*B*). These observations again highlight our findings that α- and β-Tm isoforms have divergent impact on myofilament function.

Effect of McTnT deletion mutants on the magnitude of the ML-mediated recruitment of new force-bearing XBs

McTnT$_{1-44\Delta}$-mediated effect on γ may decrease the magnitude of recruitment of new force-bearing XBs because its effect is to shift the equilibrium of XB recruitment away from the force-bearing XB population. Therefore, we measured the magnitude of increase in muscle fibre stiffness (E_R), resulting from the recruitment of additional XBs in response to an increase in ML (Ford *et al.* 2010). Thus, changes in the estimates of E_R can be correlated to changes in the SL-dependent activation of the muscle fibres. Stretch-activated force – resulting from a change in ML (Phase 3 of Fig. S1 in the Supplemental Material) – reaches a new steady-state level (F_{nss}). The difference between F_{nss} and the initial steady-state force (F_{ss}) represents the magnitude of newly-recruited force-bearing XBs. Two-way ANOVA revealed no significant interaction effect, but showed a main effect ($P < 0.01$) of McTnT deletion mutants on E_R. Subsequent *post hoc* tests revealed that E_R was unaltered by McTnT$_{1-44\Delta}$ under α-Tm background (Fig. 7*C*) and decreased significantly under β-Tm background (Fig. 7*D*). For example, McTnT$_{1-44\Delta}$ decreased E_R by ∼58% in β-Tm fibres (Fig. 7*D*). On the other hand, McTnT$_{45-74\Delta}$ had no effect on E_R in α- or β-Tm fibres (Fig. 7*C* and *D*).

Effect of McTnT deletion mutants on XB turnover rate in α- *vs.* β-Tm fibres

McTnT$_{1-44\Delta}$ augmented the negative effect of strained XBs on other XBs (γ), which suggested that the equilibrium of XB recruitment was shifted away from the force-bearing XB population. Therefore, we assessed the XB turnover rate by estimating the rate of tension redevelopment (k_{tr}) and the rate of XB recruitment (b) in maximally activated muscle fibres. As shown in Table 1, both McTnT$_{1-44\Delta}$ and McTnT$_{45-74\Delta}$ showed a general trend to increase k_{tr} and b in α- or β-Tm fibres. Two-way ANOVA revealed no significant interaction effect, but showed a significant main effect ($P < 0.05$) of McTnT deletions on k_{tr}. *Post hoc* tests using multiple planned-pairwise comparisons were performed to assess the contributing factors for the significant main effect of deletions in McTnT on k_{tr}. *Post hoc* tests showed that the McTnT$_{1-44\Delta}$-induced increase in k_{tr} under α-Tm background was only marginally not significant ($P = 0.057$), whereas McTnT$_{45-74\Delta}$ had no effect on k_{tr} in α- or β-Tm fibres. Two-way ANOVA revealed no significant interaction effect and no significant main effects on b. *Post hoc* tests showed that the McTnT deletion mutants had no effect on b in α- or β-Tm fibres. Because our *post hoc* tests showed marginal effects on k_{tr}, it precluded us from determining which of the McTnT deletion mutants was responsible for the main effect observed for k_{tr}.

Discussion

The dual role of cTnT in thin-filament activation by Ca^{2+} and XBs is most likely a function of its effects on Tm–actin interactions (Heeley *et al.* 1987; Malnic & Reinach, 1994; Gaffin *et al.* 2006; Coulton *et al.* 2008). However, very little is known as to which specific region of cTnT participates in modulating Tm–actin interactions within a SU (Tm_1–Tn_1–$actin_7$) or possibly between neighbouring SUs. In this study, we identify two key regions in the N-terminus of cTnT that have divergent effects on Ca^{2+}-activated maximal tension, myofilament

Ca^{2+} sensitivity and cooperativity of tension development. Furthermore, we provide new evidence to show that the interplay between cTnT and Tm also plays a key role in modulating the outcome of cardiac myofilament activation.

$McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ have divergent effects on Ca^{2+}-activated maximal tension

Within the 1-74 region of cTnT, we identify the specific region that is responsible for attenuating the Ca^{2+}-activated maximal activation in cardiac myofilaments (Fig. 4). For example, it was $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, that caused a significant decrease in Ca^{2+}-activated maximal tension and myofibre stiffness (E_D). Because both Ca^{2+}-activated maximal tension and E_D are a function of the number of strongly-bound XBs, a decrease in maximal activation can be attributed to a decrease in the number of strongly-bound XBs. Therefore, our data demonstrate that the amino acids 1-44 of McTnT are essential for maximal activation of cardiac myofilaments. Interestingly, changing the Tm background from α- to β-Tm did not alter the effects of $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ on Ca^{2+}-activated maximal tension (Fig. 4*B*) or myofibre stiffness (Fig. 4*D*). Thus, our data show that cTnT has a dominant effect on Ca^{2+}-activated maximal tension development in cardiac myofilaments.

Our data from tension measurements raise two important questions: (1) why is it that Ca^{2+}-activated maximal tension is attenuated by $McTnT_{1-44\Delta}$, but not by $McTnT_{45-74\Delta}$?; (2) why is the effect of $McTnT_{1-44\Delta}$ on maximal tension similar under both α- and β-Tm backgrounds? The attenuating effect on maximal tension by $McTnT_{1-44\Delta}$, but not by $McTnT_{45-74\Delta}$, can be understood in terms of the charge-related impact on cTnT–Tm interactions. For example, it is known that the region containing 258-284 residues of Tm – a key region involved in the formation of head-to-tail overlap of Tm – interacts with the N-terminus of TnT (Pearlstone & Smillie, 1983; Muthuchamy *et al.* 1997; Jagatheesan *et al.* 2010*b*) and carries a net charge of −3 (Jagatheesan *et al.* 2010*a*). In our study, the deletion of 1−44 residues amounted to a removal of 23 negative charges from the N-terminus of McTnT. Attenuation of electrostatic repulsion – caused by

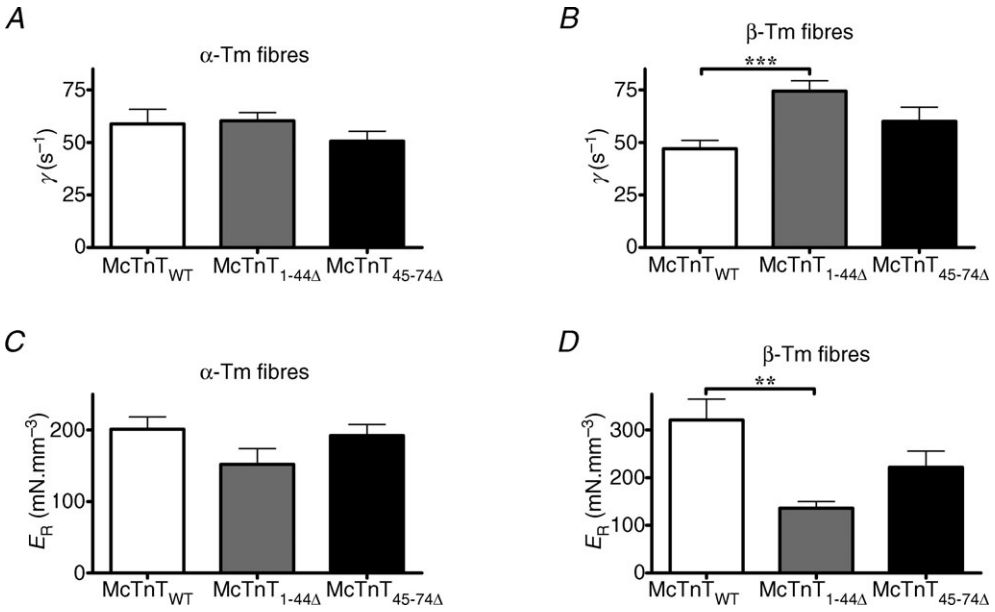

Figure 7. Effect of mouse cardiac troponin T (McTnT) deletion mutants on γ and E_R in α- *vs.* β-tropomyosin (Tm) fibres
γ and E_R were determined by fitting the NLRD model to the force responses elicited by the muscle fibres due to step-like length perturbations (Ford *et al.* 2010). *A*, effect of McTnT deletion mutants on γ in NTG fibres containing α-Tm. *B*, effect of McTnT deletion mutants on γ in TG fibres containing β-Tm. Two-way ANOVA revealed no significant interaction effect, and showed a main effect of McTnT deletions on γ. Subsequent *post hoc* tests showed that $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, induced a significant increase in γ under a β-Tm background. *C*, effect of McTnT deletion mutants on E_R in NTG fibres containing α-Tm. *D*, effect of McTnT deletion mutants on E_R in TG fibres containing β-Tm. Two-way ANOVA revealed no significant interaction effect, and showed a significant main effect of McTnT deletions on E_R. Subsequent *post hoc* tests revealed that $McTnT_{1-44\Delta}$ lead to a significant decrease in E_R under a β-Tm background. The number of determinations for γ and E_R was 12, 7 and 13 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups of α-Tm fibres, respectively. The number of determinations for γ and E_R was 14, 7 and 12 for $McTnT_{WT}$, $McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ groups of β-Tm fibres, respectively. Values are reported as mean ± SEM. $^{***}P < 0.001$; $^{**}P < 0.01$.

the removal of high density of negative charges – would be expected to enhance cTnT–Tm interactions (Chandra *et al.* 1999). Such enhanced cTnT–Tm interactions may act to stabilize the cardiac thin filament in a sub-maximally activated state, attenuating the Ca^{2+}-activated maximal tension. This cTnT effect on thin-filament activation may result from the effect of cTnT on the flexibility of Tm.

Recent studies (Li *et al.* 2012; Loong *et al.* 2012) suggested that an increase in the flexibility of Tm could lead to an increased activation of thin filaments. For example, the E180G Tm mutation-induced increase in myofilament Ca^{2+} sensitivity was shown to be associated with an increased Tm flexibility (Li *et al.* 2012; Loong *et al.* 2012). A flexible Tm would easily shift its azimuthal position on the actin filament to uncover the myosin binding sites: this would lead to a shift in the *on*/*off* equilibrium of the regulatory unit (RU; Tm–Tn) more toward the on-state, and thus cause an increase in Ca^{2+} sensitivity (Li *et al.* 2012). Therefore, a rigid Tm may shift the *on*/*off* equilibrium of the RU more toward the off-state, leading to a decreased activation of thin filaments. Because XB-mediated activation of thin filaments is more prominent in the cardiac muscle (Adhikari *et al.* 2004; Regnier *et al.* 2004), a rigid Tm-induced decrease in the number of strongly-bound XBs could further attenuate the activation of thin filaments. Thus, the net effect of a rigid Tm on the thin filament is that fewer RUs are turned on; leading to a decreased number of actin-bound XBs in $McTnT_{1-44\Delta}$ reconstituted fibres.

Unlike in the case of $McTnT_{1-44\Delta}$, the deletion of residues 45-74 amounted to a net removal of only six negative charges from the N-terminus of McTnT. Thus, $McTnT_{45-74\Delta}$ would be expected to have minor effects on the electrostatic interactions between McTnT and Tm. Both tension (Fig. 4*A* and *B*) and E_D (Fig. 4*C* and *D*) of $McTnT_{45-74\Delta}$ fibres remained unaltered, substantiating our observation that $McTnT_{1-44\Delta}$, but not $McTnT_{45-74\Delta}$, was responsible for attenuating the Ca^{2+}-activated maximal tension. The effect of $McTnT_{1-44\Delta}$ on maximal tension was similar under both α- and β-Tm backgrounds (Fig. 4*A* and *B*). Because both mouse α- and β-Tm isoforms possess a similar net charge of -3 in the region encompassing residues 258−284 (Jagatheesan *et al.* 2010*a*), $McTnT_{1-44\Delta}$ would be expected to have a similar effect on tension under either α- or β-Tm background. Different Tm isoforms had no effect on the $McTnT_{45-74\Delta}$ mutant's ability to generate normal tension, nor did they modulate the attenuating effect of $McTnT_{1-44\Delta}$, indicating that cTnT – but not Tm – had a dominant effect on the Ca^{2+}-activated maximal tension.

$McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ have divergent effects on myofilament Ca^{2+} sensitivity and cooperativity

$McTnT_{1-44\Delta}$ and $McTnT_{45-74\Delta}$ had contrasting effects on myofilament Ca^{2+} sensitivity under α- but not under β-Tm background. Under α-Tm background, $McTnT_{1-44\Delta}$ showed a significant decrease in pCa_{50}, while $McTnT_{45-74\Delta}$ showed a significant increase in pCa_{50} (Fig. 5*C*). Thus, it is likely that a synergistic effect of deleting the entire 1−74 region would be to result in unaltered Ca^{2+} sensitivity. Indeed, findings from our previous study (Chandra *et al.* 1999) showed that the deletion of 1−76 amino acids in rat cTnT resulted in an unaltered myofilament Ca^{2+} sensitivity.

One possible reason for the attenuating effect of $McTnT_{1-44\Delta}$ on pCa_{50} is that the thin filament harboring a rigid Tm would require more Ca^{2+} for full activation. On the other hand, $McTnT_{45-74\Delta}$ augmented pCa_{50}, with no effect on either the maximal tension or myofibre stiffness. The relationship of this work to our recent studies on a TG mouse – which expressed a McTnT N-terminal extension mutant ($McTnT_{NTE}$) in the heart – requires brief consideration (Gollapudi *et al.* 2012). The effect of $McTnT_{45-74\Delta}$ on myofilament Ca^{2+} sensitivity and tension has an uncanny similarity to the effect observed due to the deletion of $McTnT_{NTE}$ in which the first 1−73 amino acids of McTnT were replaced by 1−41 amino acids of MfsTnT (Gollapudi *et al.* 2012). In that study, we showed $McTnT_{NTE}$ increased myofilament Ca^{2+} sensitivity, without affecting the maximal tension or E_D. Based on our observations, we concluded that the cardiac-specific NTE of cTnT was important for stabilizing the thin filament more in the blocked-state (Gollapudi *et al.* 2012). Our results from the present study demonstrate that it is the 45−74 peptide segment that is responsible for the cardiac-specific effect on the thin filament. Interestingly, this extra peptide segment, albeit with minor amino acid variations, is present only in the cardiac variant of cTnT across all species (Perry, 1998).

Irrespective of the type of McTnT deletion mutant, fibres containing β-Tm exhibited a higher pCa_{50} than fibres containing α-Tm (Fig. 5*C*). Our present results, in conjunction with previous studies (Palmiter *et al.* 1996; Wolska *et al.* 1999), demonstrate that β-Tm sensitizes cardiac myofibres to Ca^{2+}. The Ca^{2+} sensitizing effect of β-Tm ablated the $McTnT_{1-44\Delta}$-induced decrease in pCa_{50}, which was observed under α-Tm background (indicated by arrows in Fig. 5*B*, also see Fig. 5*C*). This finding is in good agreement with a previous study which showed that the protein kinase A-mediated decrease in pCa_{50}, observed under α-Tm background, was abolished under β-Tm background (Palmiter *et al.* 1996). The Ca^{2+} sensitizing effect of β-Tm was also evident in fibres that contained $McTnT_{45-74\Delta}$. For example,

$McTnT_{45-74\Delta}$-mediated increase in pCa_{50} (α-Tm fibres) was further enhanced under β-Tm background (Fig. 5*C*).

Regardless of the type of Tm isoform, the Hill coefficient (n_H) was significantly attenuated by $McTnT_{45-74\Delta}$, suggesting an effect on thin-filament cooperativity (Fig. 5*D*). These results indicate that the region containing residues 45–74 in McTnT plays an important role in cooperative activation of thin filaments. Although the molecular basis of this altered cooperative mechanism is unclear, the source of this change is likely to be due to the deletion-induced effects on the Tm–Tn complex (RU), and possibly between two contiguous RUs. Because $McTnT_{45-74\Delta}$ did not affect maximal tension or E_D, it is likely that both Ca^{2+}- and XB-mediated activation of thin filaments were unaffected. Likewise, $McTnT_{45-74\Delta}$ did not affect the XB turnover rate as shown by an unaltered rate of tension redevelopment (k_{tr}) and the rate of XB recruitment (b; Table 1). Because k_{tr} is a measure of the rate of XB transition from the weak- to strong-binding states (Brenner & Eisenberg, 1986; Campbell, 1997), an unaltered k_{tr} indicates that there is no change in the equilibrium between the closed- and open-states of thin filaments. Because of its lack of effect on maximal activation and XB turnover rate, but an augmenting effect on Ca^{2+} sensitivity, we predict that the deletion of 45–74 amino acids in cTnT shifts the equilibrium of thin filaments from the blocked- to closed-state, thereby sensitizing the thin filament to Ca^{2+}. Supporting evidence for this conclusion comes from our recent data (Gollapudi *et al.* 2012), which demonstrated that $McTnT_{NTE}$ increased TG mouse cardiac fibre Ca^{2+} sensitivity, with no effect on maximal activation. Collectively, these observations demonstrate that the region 1–44 of McTnT is essential for maximal activation, whereas the region 45–74 of McTnT is essential for augmenting long-range cooperative/allosteric processes in the cardiac thin filament.

$McTnT_{1-44\Delta}$ shifts the equilibrium of XB recruitment away from the force-bearing population

The attenuating effect of $McTnT_{1-44\Delta}$ on maximal activation (Ca^{2+}-activated maximal tension and E_D) was similar under α- and β-Tm backgrounds (Fig. 4), suggesting that $McTnT_{1-44\Delta}$ shifted the equilibrium of XB recruitment away from the force-bearing population. But the mechanism by which $McTnT_{1-44\Delta}$ shifted the equilibrium differed depending on the type of Tm isoform present. For example, both tension cost (Fig. 6*B*) and c (Fig. 6*D*) were significantly greater in $McTnT_{1-44\Delta}$ reconstituted fibres under β-Tm background, but not under α-Tm background (Fig. 6*A* and *C*). These observations, in conjunction with experimental evidences presented above, are strongly suggestive of differential effects of α- and β-Tm on XB detachment dynamics and contractile function. Faster XB detachment kinetics (Figs 6*D* and 8) and a greater negative effect of distorted XBs (γ) on other bound XBs (Figs 7*B* and 8) suggest that $McTnT_{1-44\Delta}$ enhances the detachment of XBs from actin in β-Tm fibres. This effect of $McTnT_{1-44\Delta}$ on XBs would shift the equilibrium of XB recruitment away from the force-bearing population, leading to the attenuation of: (1) maximal tension by ∼30% (Fig. 4*B*); (2) E_D – which is an approximation of a number of strongly-bound XBs – by ∼32% (Fig. 4*D*); and (3) E_R – which reflects the ML-mediated effect on the recruitment of new XBs – by ∼58% (Figs 7*D* and 8). The decrease in E_R suggests that $McTnT_{1-44\Delta}$ blunts the mechanisms that underlie the SL-dependent recruitment of XBs under a β-Tm background. Regardless of the mode of action, $McTnT_{1-44\Delta}$ had an attenuating effect on thin-filament maximal activation. These data not only highlight the physiological significance of residues 1–44 in cTnT, but also demonstrate the divergent effects of Tm isoforms on cTnT-mediated cardiac function.

Conclusions

We provide the first explicit evidence to demonstrate the existence of two new functional regions within the N-terminus of cTnT and ascribe divergent physiological roles that were previously unknown: (1) amino acids 1-44 of McTnT modulate maximal activation of thin filaments, acting through a charge-dependent

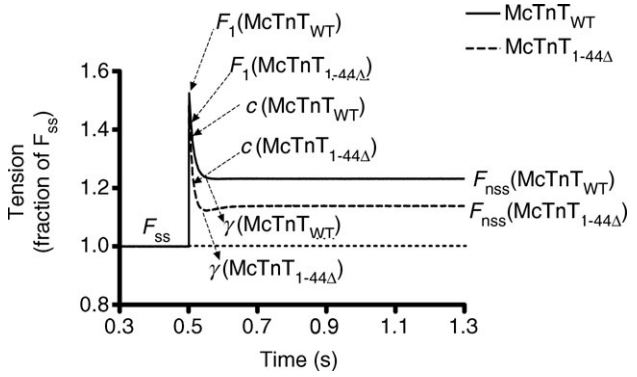

Figure 8. Effect of mouse cardiac troponin T ($McTnT)_{1-44\Delta}$ on c and γ in TG β-Tm fibres
Force responses normalized to the steady-state isometric force, F_{ss}, are shown for $McTnT_{WT}$ and $McTnT_{1-44\Delta}$ reconstituted β-Tm fibres. F_{nss} represents the new steady-state force attained by the muscle fibre due to a change in ML. Force responses to 2% stretch in ML show that the instantaneous force (F_1) is lower, while c is higher in β-Tm fibres reconstituted with $McTnT_{1-44\Delta}$. Force responses also show that γ is greater for $McTnT_{1-44\Delta}$ reconstituted fibres as indicated by a greater dip in the force response. The new steady-state force attained by the muscle fibre, F_{nss}, was lower in $McTnT_{1-44\Delta}$ reconstituted fibres, indicating that the ML-induced recruitment of additional XBs (E_R) is reduced.

mechanism on cTnT–Tm interactions; (2) amino acids 45-74 of McTnT desensitize cardiac thin filaments to Ca^{2+} through an effect on stabilizing the thin filament in the blocked-state; (3) cTnT, but not Tm, has a dominant effect on Ca^{2+}-activated maximal tension in cardiac myofilaments; and (4) interplay between the effects of cTnT and Tm on the thin filament modulates myofilament Ca^{2+} sensitivity of tension development. The physiological significance of amino acids 1-44 of cTnT stems from the observation that the N-terminus of cTnT undergoes changes – through alternative splicing – not only during development (Jin & Lin, 1988), but also in some forms of heart diseases (Akella *et al.* 1995; Saba *et al.* 1996). The functional relevance of our current study is further highlighted by the observation that the majority of the differences between the TnT isoforms — which are expressed in cardiac, slow and skeletal muscle fibre types — lie within the N-terminus of TnT (Wei & Jin, 2011). This suggests that structural differences in the N-terminus of TnT confer a regulatory mechanism to meet varied functional demands of different muscle fibre types. Our data also suggest that the presence of cardiac-specific residues 45-74 of cTnT – which is conserved across species – may be essential for augmenting long-range cooperative effects in cardiac thin filaments. Enhanced cooperative allosteric processes, combined with a desensitizing effect of cardiac-specific residues on Ca^{2+} sensitivity, may slow XB recruitment mechanisms such that it aids in tuning the dynamics of contraction to heart rate. Our study provides novel mechanistic insights that have significant implications for tissue- and disease-related changes in TnT isoform expression and muscle function.

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Author contributions

R.M. and S.L.M. were responsible for experimental design, data collection, analysing data, interpreting data, drafting and revising the manuscript. D.F.W. and M.C. were responsible for experimental design, analysing data, interpreting data, drafting and revising the manuscript. The experiments were carried out in the Department of VCAPP at Washington State University, Pullman, WA, USA. All authors approved the final version of the manuscript.

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