HHS Public Access

Author manuscript

J Cell Signal. Author manuscript; available in PMC 2021 September 16.

Published in final edited form as:

J Cell Signal. 2021 ; 2(3): 162–171.

NH_2-Terminal Cleavage of Cardiac Troponin I Signals Adaptive Response to Cardiac Stressors

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Abstract

Cardiac sarcomeres express a variant of troponin I (cTnI) that contains a unique N-terminal extension of ~30 amino acids with regulatory phosphorylation sites. The extension is important in the control of myofilament response to Ca^{2+}, which contributes to the neuro-humoral regulation of the dynamics of cardiac contraction and relaxation. Hearts of various species including humans express a stress-induced truncated variant of cardiac troponin I (cTnI-ND) missing the first ~30 amino acids and functionally mimicking the phosphorylated state of cTnI. Studies have demonstrated that upregulation of cTnI-ND potentially represents a homeostatic mechanism as well as an adaptive response in pathophysiology including ischemia/reperfusion injury, beta adrenergic maladaptive activation, and aging. We present evidence showing that cTnI-ND can modify the trigger for hypertrophic cardiomyopathy (HCM) by reducing the Ca^{2+} sensitivity of myofilaments from hearts with an E180G mutation in α-tropomyosin. Induction of this truncation may represent a therapeutic approach to modifying Ca^{2+}-responses in hearts with hypercontractility or heat failure with preserved ejection fraction.

Keywords

Sarcomeres; Ca^{2+}-sensitivity; Hypertrophic cardiomyopathy; Heart failure; Tropomyosin

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#These authors made equal contributions to this manuscript

Author Contributions

CMW: Writing-Original Draft, Writing- Review & Editing, Methodology, Investigation; MH: Data Collection and Analysis, Writing- Review & Editing; HZF: Resources, Writing- Review & Editing; BMW: Writing- Review & Editing Funding acquisition; JPJ: Conceptualized, Funding acquisition, Writing- Review & Editing; RJS: Writing-Original Draft, Writing- Review & Editing, Conceptualized, Funding acquisition.

Declaration of Interests

R. John Solaro is a member of the Scientific Advisory Board of Cytokinetics, Inc., a consultant to Pfizer, Inc. and Myokardia/Bristol Myers Squibb. The authors declare no additional competing financial interests.

Introduction

In evidence presented here, we discuss a novel adaptive role for physiological and pathological restricted proteolysis of cardiac troponin I (cTnI), which is uniquely expressed in cardiac sarcomeres. cTnI is the inhibitory unit of the heterotrimeric troponin complex (cTn) that controls Ca^{2+}-dependent activation of sarcomeres. Early evidence demonstrated a release of cardiac troponin I (cTnI) into serum in myocardial infarction (MI). This finding led to the development of specific high affinity antibodies for detection of serum cTnI, and led to a vast literature on their use as the preferred marker for MI as well as other cardiac related pathologies (see Solaro and Solaro [1]; Katrukha and Katrukha [2] for recent reviews). Adding to the complexity of its use as a biomarker is evidence that there is a proteolysis of cTnI occurring in the necrosis and necroptosis in these pathologies. Understanding the nature of these products of proteolysis in serum and correlating these data with clinical profiles has been proposed to be important in diagnosis, prognosis, and stratification of patients [1,2].

In contrast to the pathological proteolysis of cTnI, evidence reviewed here has demonstrated an adaptive role for stress-induced, restricted proteolysis of cTnI generating an NH_2-deletion (cTnI-ND) of 27–30 amino acids without affecting the core structure in the rest of the molecule [3–5]. As illustrated in Figure 1, cTnI was named for its action as the inhibitory unit of the heterotrimeric Tn complex, which together with cardiac troponin T (cTnT), the tropomyosin (Tm) binding unit, and cardiac troponin C (cTnC), the Ca^{2+}-binding unit, act to modify the of Tm-actin interaction in regulating the on-off state of thin filaments in sarcomere activation and relaxation [6,7]. In diastole, cTnI structures located in an inhibitory peptide (Ip) and at the mobile COOH-domain interact strongly with actin and Tm and promote protein-protein interactions with cTnT-Tm to establish an inhibited state with Tm blocking the reaction of cross-bridges with actins (Figure 1). With the release of Ca^{2+} into the sarcomere space, Ca^{2+} binds to the NH_2 lobe of cTnC exposing a hydrophobic patch of amino acids that react with a cTnI switch peptide (Sw) thereby releasing the thin filament from its inhibited state. These actions of cTnI are modulated by phosphorylation of cTnI contained in its unique NH_2 extension (Figure 1) [8]. The major regulatory phosphorylation sites at S23 and S24 are substrates for protein kinase A [8,9]; a tyrosine kinase phosphorylates cTnI at Tyr 26 [10]. These phosphorylations depress myofilament Ca^{2+} sensitivity of tension development in detergent extracted (skinned) fiber preparations. Compared to the phosphorylated state, unphosphorylated cTnI NH_2 stabilizes the cTnI-Sw-cTnC activated state by binding relatively tightly to cTnC regulatory site II and the region of Sw binding (Figure 1). When cTnI is phosphorylated, binding of its NH_2-terminus with cTnC is modified inducing a weakening of the cTnI-Sw-cTnC interaction [11]. Structure-function and cross-linking studies demonstrated that when cTnI is phosphorylated, the acidic domain in the highly mobile cTnI-NH_2-terminus is free to interact in an intra-molecular interaction with the basic Ip [12,13]. Moreover, epitope mapping studies have reported that these NH_2-terminal interactions induce long range protein-protein interactions modifying the thin filament including regions that interact with cTnT and the Ip [12,14]. The result of this phosphorylation induced weakening of cTnI binding to cTnC is a desensitization of the sarcomeres to the rising Ca^{2+} in systole and faster relaxation as Ca^{2+} falls towards

the diastolic state. These effects of cTnI phosphorylation during adrenergic stimulation and activation of PKA signaling induce a relaxant effect and abbreviate the cycle time of the heartbeat to tune cardiac function to the prevailing heartbeat [8,15].

Adaptive Restrictive Proteolysis of Cardiac Troponin I

As expected, truncation of cTnI by restrictive proteolysis removes these effects of interactions at its NH_2-terminus with the sarcomere creating a sustained state of sarcomere inhibition with increased cardiac dynamics that mimics the effects of phosphorylation [8,16,17]. Yu et al. [3,4] first reported that a restrictive proteolysis of cTnI removing the NH_2-terminal extension occurred in rats subjected to hind limb suspension, simulating micro-gravity. These studies demonstrated that the generation of cTnI-ND did not affect the stoichiometry of the sarcomere proteins, maintained sarcomere Ca^{2+}-response, reduced sarcomere tension generation, and appeared to protect the heart from the elevated neurohumoral signaling and hemodynamic stress in micro-gravity.

Importantly, cTnI-ND was present in the normal sarcomeres in various species including human hearts suggesting a physiological role in modulating contraction and relaxation reserve. Consideration of cTnI-ND as a novel adaptive response was a major conclusion from this early study. There is also evidence that compared to controls myofibrils partially regulated by cTnI-ND show a potential modification of Ca^{2+}-response of the actomyosin ATPase cycle at the level of the rate limiting phosphate release step controlling relaxation [23]. In comparison to control unloaded isolated ventricular cardiac myocytes, cTnI-ND expressing myocytes had shorter resting lengths whereas the diameter and resting sarcomere length were unchanged. With activation there was an increase in the amplitude of contraction and faster shortening and re-lengthening, which are non-additive with the effect of isoproterenol treatment [26]. Moreover, there was an increase in early rates of fall of the Ca^{2+} transient with no change in peak amplitude or basal levels suggesting an enhanced release of Ca^{2+} from cTnC during relaxation [18,26]. Transgenic mice expressing cTnI-ND in the cardiac compartment confirmed its role in modulating diastolic function in the intact heart [17].

The discovery that cTnI-ND is normally present and functional in myofibrils indicates a potential to modulate response to β-adrenergic stimulation. Interestingly the relative amounts of cTnI-ND expressed are low in cardiac myocytes with relatively high prevailing β-adrenergic stimulation and PKA dependent phosphorylation and higher in myocytes with reduced PKA dependent phosphorylation [18]. An important question is the proteolytic process generating cTnI-ND. Likely candidates are mu-calpain and matrix metalloproteinases (MMPs) both of which have been identified to be present in association with cardiac myofibrils. Mu-calpains have been reported to be important in the proteolytic NH_2 degradation of cTnT [19] and MyBP-C [20]. Mu-calpains are also responsible for degradation of cTnI, but have not been specifically identified in the adaptive generation of cTnI-ND. There is evidence that treatment of cTn with MMP cleaves at multiple locations including those in the generation of cTnI-ND in animal models (Figure 1) [21]. MMPs have been shown to be upregulated in HCM [22], but there have been no studies of whether early increases in MMP activity represent an adaptive process. Our hypothesis is that as with

many control mechanisms there is a homeostatic range of proteolytic activity that is adaptive and elevated activities outside this range are maladaptive.

Restricted Proteolysis of cTnI and Cardiac Remodeling

Evidence has been developed indicating the potential significance of the generation of cTnI-ND in chronic physiological and pathological remodeling of the myocardium. Results of our studies comparing aging related modifications in cardiac and sarcomere function in controls and Tg-cTnI-ND mice emphasize a role of restricted proteolysis of cTnI in physiological adaptation [23]. Intra-ventricular pressure measurements in hearts at 4 months of age showed control mice already had depressed diastolic function compared to Tg-cTnI-ND hearts. Measurement of LV end-diastolic pressure that was relatively high and −dp/dt that were relatively low in the 4-month controls were significantly improved in the 4-month old Tg-cTnI-ND hearts. Working heart preparations showed a prolonged diastolic duration and abbreviated systolic duration in Tg-cTnI-ND compared to controls. Investigations of the associated changes in sarcomere Ca^{2+}-response together with measurements of ATP hydrolysis showed that compared to controls skinned fibers from hearts of cTnI-ND hearts were less sensitive to Ca^{2+}, with a steeper response to Ca^{2+}, and an increase in cross-bridge kinetics as determined from the tension cost (ratio of unit ATP hydrolyzed/unit of tension) [24]. Importantly skinned fibers from controls and Tg-cTnI-ND mice had no differences in the population of the alpha-myosin heavy chain (α-MHC) with fast kinetics and the slow β-MHC with slow kinetics. Echocardiography studies comparing hearts of these mice beating *in situ* at 16 months of age reported significant diastolic abnormalities in ejection fraction, cardiac output, and intra-ventricular relaxation times in the controls which were prevented in the Tg-cTnI-ND mice [24].

There are also studies indicating a role for the generation of cTnI-ND in chronic remodeling in cardiac disorders. Compared to controls, myocytes from human hearts in failure commonly demonstrate diastolic abnormalities associated with low levels of cTnI phosphorylation at the PKA sites and increased myofilament response to Ca^{2+} [25]. Thus, it seemed reasonable to speculate that under these conditions the presence of cTnI-ND would be beneficial in restoring relaxation kinetics. In a study interrogating the possible up regulation of cTnI-ND in a heart failure model induced by β-adrenergic signaling deficiency (Gsα-DF) developed by a conditional cardiac specific deficiency of Gsα, Feng et al. [5] demonstrated an increase in expression of cTnI-ND from 10% of total cTnI in controls to 30% in the Gsα-DF mouse hearts. To show that this restricted proteolysis was adaptive, Feng et al. [5] crossed the Gsα-DF mice with mice expressing cardiac cTnI-ND. Double transgenic mice were protected from the progression of heart failure occurring in the single transgenic Gsα-DF mice. An application of sarcomere inhibition by cTnI-ND is in familial hypertrophic cardiomyopathy (HCM) in which there is hyper-contractility mainly associated with mutations of sarcomere proteins inducing an increase in myofilament Ca^{2+}-sensitivity thought to be the biophysical trigger for the clinical course of the disorder. In the case of the diastolic abnormality associated with expression of a mutant cTnI (cTnI-R193H) linked to restrictive cardiomyopathy (RCM), Li et al. [26] reported that cross-breeding Tg-cTnI-ND mice with the Tg-cTnI-R193H mice was able to attenuate the effect of cTnI-R193H, reduce the increased Ca^{2+}-response, and partially restore diastolic function close to

control levels. Although these data demonstrate a minimization of diastolic abnormalities in hearts of TG-cTnI-R193H when crossed with cTnI-ND [26], the mutant cTnI-R193H was partially replaced by cTnI-ND. Thus, it was difficult to know whether the improvement in function was due directly to a general desensitization of the myofilaments. Along these lines, we tested whether sustained increased sarcomere inhibition and relaxation associated with transgenic expression of a phospho-mimetic cTnI-S23D, S24D (cTnI-DD) was able to modify disease progression in an HCM mouse model (Tg-αTmE180G) expressing a mutant of another sarcomere protein other than a cTnI mutant, which increased Ca^{2+}-response [27]. We crossed Tg-cTnI-DD mice with Tg-αTmE180G mice and demonstrated a significant improvement in diastolic dysfunction as well as significantly reduced fibrosis [28]. This has led us to test whether cTn-ND can modify the pathologic trigger associated with a maladaptive response in the myofilaments regulated by αTm-E180G (Tm-180).

cTnI-ND Reduces the Biophysical Trigger in Sarcomeres Expressing a Mutant Tm Linked to HCM

To test the ability of cTnI-ND to reduce the increased response to Ca^{2+} in myofilaments controlled by mutant sarcomere proteins other than mutant cTnI, we employed detergent-extracted (skinned) fiber bundles dissected from papillary muscles of WT mice and transgenic mice expressing the HCM linked mutation α-Tm-E180G (Tm-180) [27]. Materials and methods used in these experiments are detailed in the supplement. Figure 2 shows data confirming the increased response to Ca^{2+} in skinned fibers isolated from control hearts and hearts expressing Tm-180. Figure 2A illustrates the relation between free Ca^{2+} expressed as pCa (−log [Ca]) and steady-state tension developed by WT fibers and Tm-180 fibers. The left shift of the pCa-tension relation of the Tm-180 fibers compared to WT indicates an increase in Ca^{2+}-response considered the trigger for HCM. Figure 2B demonstrates this significant increase in Ca^{2+}-response of the Tm-180 fibers showing that half-maximal tension occurred at a higher pCa_{50} (pCa at half-maximum tension) i.e. lower free Ca^{2+} concentration compared to the WT fibers. Data in Figure 2C shows that Tm-180 fibers developed a lower maximum tension than the the WT fibers. To illustrate the relative steepness of the pCa-tension relations, Figure 2D shows the value of Hill coefficients (Hill Slope), a measure of cooperative activation of the myofilaments. Tm-180 fibers show less cooperative activation than the WT fibers (Figure 2D).

For incorporating WT cTnI and cTnI-ND into the Tm-180 myofilaments, we generated WT cTnI and cTnI-ND in a bacterial expression system and reconstituted troponin complexes containing either wild-type cTnI-WT or cTnI-ND, expressed Myc-labeled cardiac troponin T (cTnT) as a marker, and expressed TnC-WT. As previously demonstrated [28], addition of an excess of these reconstituted complexes to skinned fibers under optimal conditions induces an exchange of the exogenous complex with native cTn. We determined the exchange efficiency by expression of the myc-tagged cTnT during the production of the exogenous cTn complex, which allowed us to separate the exogenous *vs.* endogenous troponin based on molecular weight. Importantly there was no significant difference in exchange efficiency between the Tm180 fibers exchanged with cTnI-ND *vs.* Tm180 fiber exchanged with cTnI-WT containing Tn complex (Figure 3A, 3B).

The exchange efficiency for the Tm-WT fiber exchanged with cTnI-ND was about 78% whereas the Tm-WT fiber exchanged with cTnI-WT was significantly higher at 85% (Figure 3A, 3B). Tm-WT vs Tm180 exchanged with cTnI-WT was significantly increased by 8% (Figure 3A, 3B). Furthermore, Tm-WT vs Tm180 exchanged with cTnI-ND was also significantly increased by 17% (Figure 3A, 3B). However, there were no significant differences between Tm180 fibers exchanged with either cTnI-WT or cTnI-ND, which was important to analyze the effects of Ca^{2+} sensitivity on the background of this mutation (Figure 3A, 3B). In the data reported here, Tm-WT skinned fibers controlled by either cTnI-WT or cTnI-ND showed no differences in the pCa-tension relation (Figure 3C, 3D, Table 1). In contrast to this finding with an exchange of cTnI-WT with cTnI-ND, our previous studies in skinned fibers from hearts of TG-cTnI-ND mice [24] demonstrated a desensitization compared to controls. This difference suggests that other modifications may occur *in situ*. One possibility is the limited expression of α-smooth muscle actin (α-SMA) in myofibrils, which has been reported to occur in young TG-cTnI-ND mouse hearts [29], which was not present in the current study. There are also possible post-translational modifications occurring *in vivo* in the transgenic hearts. Nevertheless our objective was to show that the presence of cTnI-ND can alter the biophysical triggering of HCM signaled by sarcomere protein mutations.

As expected, the Ca^{2+} sensitivity was significantly increased in the Tm180 fiber exchanged with cTnI-WT vs Tm-WT fibers exchanged with cTnI-WT (Figure 3C, 3D, Table 1), recapitulating the results found with non-exchanged fibers (Figure 2) and further validating the exchange fiber approach [30–32]. The Tm180 fibers exchanged with cTnI-ND *vs.* cTnI-WT showed a significant decrease in the myofilament's response to Ca^{2+} (Figure 3C, 3D, Table 1), indicating the presence of cTnI-ND in the Tn complex is sufficient to partially restore myofilament Ca^{2+} sensitivity in the etiology of a myofilament mutation disease. The effects of cTnI-ND and cTnI-S23D, S24D to correcte myofilament response to Ca^{2+} inform the identifications of targets for small molecules for use in HCM. Targeting the cTnI-cTnC interface with such compounds is also aided by earlier work [12,13,33] and recent findings [11] identifying the regions of interaction of the unphosphorylated NH_2 region of cTnI with the cTnI switch-peptide binding region as well as the Ca^{2+} binding site II of cTnC.

Conclusions and Future Directions

Ample evidence indicates a novel mechanism of control of cardiac contractility by a restrictive proteolysis of cTnI removing its unique regulatory NH_2 terminal extension. These findings also support the idea that a general mechanism of control of cardiac sarcomeres is restrictive proteolysis, which has been reported in proteolytic degradation of cTnT [20]. Studies in aging-induced and cardiac disorder-induced diastolic abnormalities indicate the potential for therapeutic applications of our findings. The effects of cTnI-ND provide information for targeting the sarcomere with small molecules or peptides such as that derived the COOH-terminus of cTnI [34]. The success in the use of small molecules for inhibition of the hypercontractility in the clinical course of advanced HCM [35] indicates the importance of detection of new targets to address the diverse responses to triggering mutations in HCM [36]. Approaches that identify mechanisms for specific activation of

proteolytic cascades to generate cTnI-ND also provide an intriguing challenge to future investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants RO1 HL128468 (to BMW and RJS), PO1 HL062426 Project 1 and Core C (to RJS, BMW, and CMW), RO1 HL127691 (to JPJ and RJS).

Abbreviations:

cTnI-ND	N-terminal truncated cardiac troponin I
Tm180	α-tropomyosin mutation at E180G
Tm-WT	α-Tropomyosin Wild Type
cTnI-WT	Cardiac Troponin I Wild-Type
cTnI	Cardiac Troponin I
cTn	Cardiac Troponin complex
cTnT	Cardiac Troponin T
cTnC	Cardiac Troponin C
HCM	Hypertrophic Cardiomyopathy

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Highlights

- Evidence shows there is a stress induced proteolysis truncating the N-terminus of cTnI
- N-terminal deleted cardiac TnI promotes adaptive responses to stresses
- The truncated cardiac TnI decreased elevated Ca^{2+}-sensitivity in an HCM model

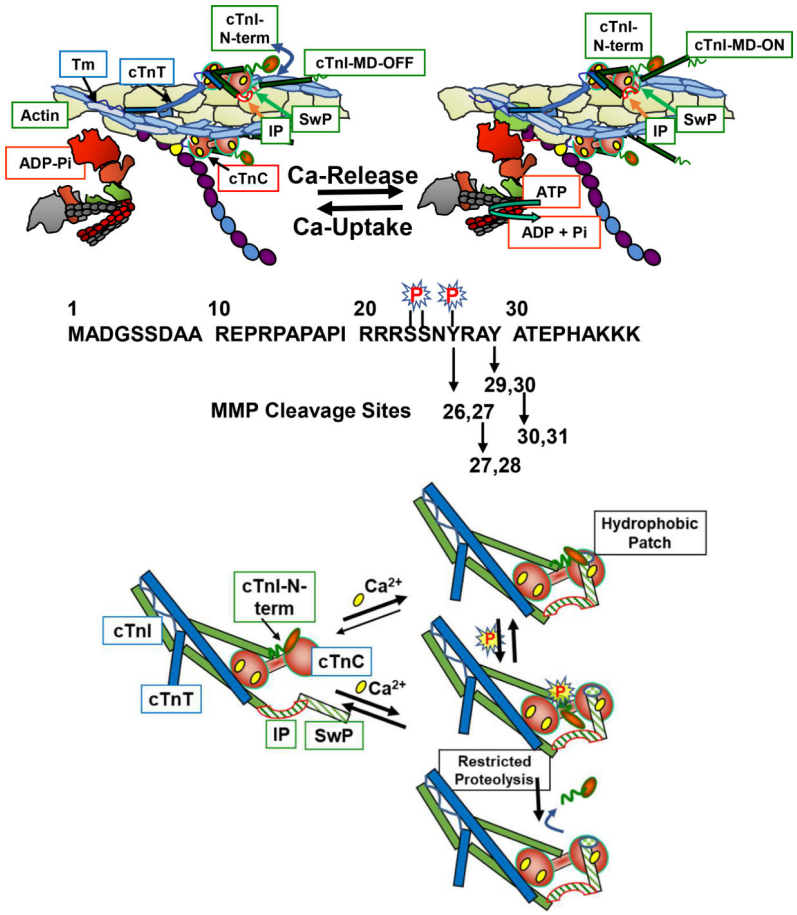

Figure 1: The Unique Cardiac Troponin I NH_2 Extension and the Structure Function Relations of a Regulatory Unit of Cardiac Sarcomeres.

The upper panels show a sarcomere with a thin filament regulatory unit consisting of actin, Tm, and the cTn complex in a 7:1:1 ratio. The upper left panel shows the diastolic state with a relaxed cross-bridge with light chains. Also shown is myosin binding protein C (MyBP-C), which reacts with both the thin filament and the cross-bridge depending on its state of phosphorylation. In diastole the thin filament is in an inhibited state with immobilization of Tm in a position blocking the actin-cross-bridge reaction. The inhibited state is induced by the interaction of a cTnI inhibitory peptide (Ip) and mobile domain (MD) with actin-Tm and cTnT with Tm-actin. The upper right panel shows the systolic state in which Ca^{2+}release and binding to cTnC induces a hydrophobic patch on the NH_2-lobe of cTnC that interacts with the cTnI switch peptide (Sw) thereby releasing the thin filament from inhibition and promoting the force generating reaction of myosin cross-bridges with actin. As more fully discussed in the text, these control mechanisms are modulated by the unique cTnI-NH_2 extension. The middle panels show the amino acid sequence of the cTnI NH_2-terminus identifying major phosphorylation sites at S23, S24 and Y26 and indicating matrix metalloproteinase (MMP) cleavage sites. The lower left panel shows diastolic cTn protein-protein interactions with the cTnI-NH2 terminal peptide bound to cTnC. Panels to the lower right show Ca^{2+}-activation of the cTn complex in various states of the cTnI-NH2 terminus. In the dephosphosphorylated state binding of the

NH2 terminus to cTnC stabilizes the activated state. Stabilization of the activated state is lost with phosphorylation of the NH_2-terminus weakening the interactions of the Sw with cTnC, an intra-molecular interaction with the Ip, and long range allosteric effects modifying the cTnT-cTnI interaction. The result is an enhanced release of Ca^{2+} from cTnC during relaxation, and a desensitization to Ca^{2+}, which promotes relaxation and abbreviates the cycle time of contraction/relaxation. These effects of phosphorylation are mimicked by restrictive proteolysis removing the NH_2-terminus generating the truncated cTnI-ND. See text for further discussion.

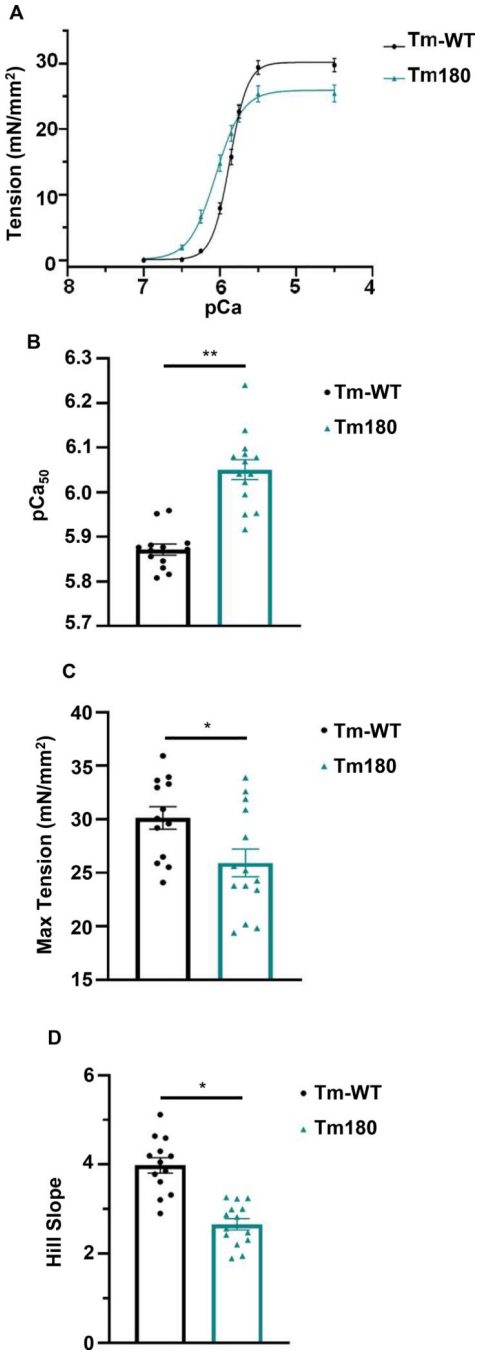

Figure 2: Dysfunction of Ca^{2+} -activated tension in Tm180 transgenic skinned fibers.

Data represented as mean ± SEM from (n= 13–14). Tm-WT, non-transgenic littermates expressing wildtype tropomyosin; Tm180, transgenic littermates expressing point mutant E180G of tropomyosin. pCa_{50}, $-\log_{10}$ of the $[Ca^{2+}]$ required to develop 50% of the maximal tension. **(A)** pCa ($-\log_{10}[Ca^{2+}]$) -force relations in Tm-WT and Tm180 mouse skinned papillary muscles. **(B)** Scatter plot of pCa_{50}. **, P<0.0001. **(C)** Scatter plot of max tension (mN/mm^2). *, P<0.05. **(D)** Scatter plot of Hill slope, **, P<0.0001.

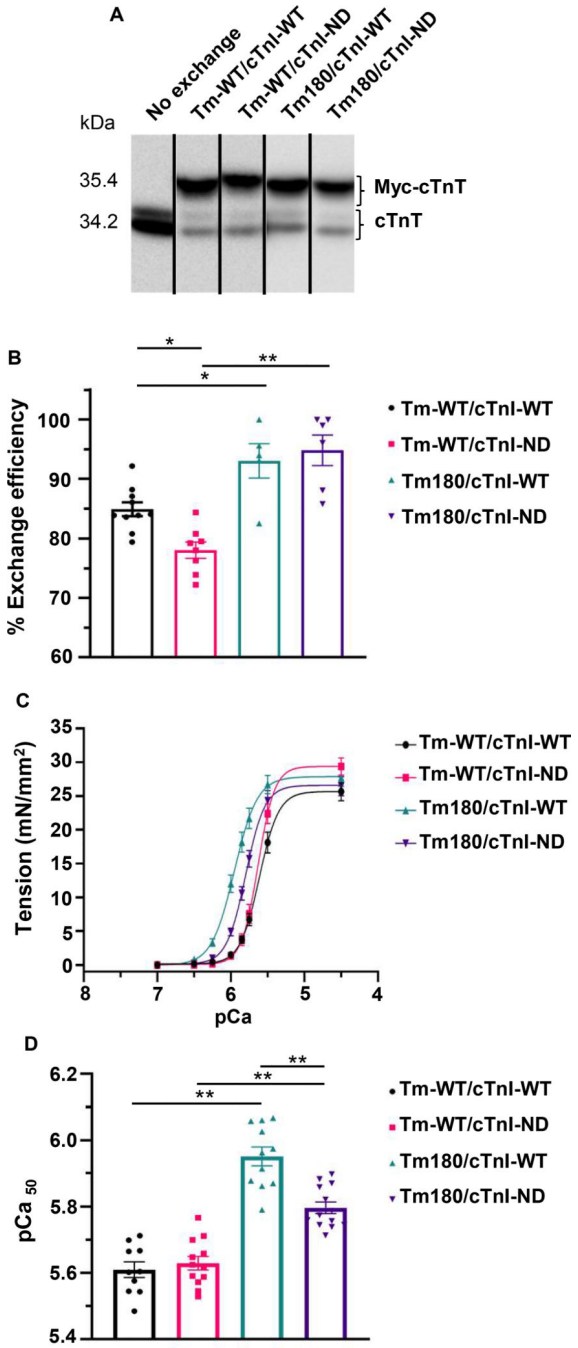

Figure 3: Significant restoration of Ca^{2+}-activated tension in Tm180 transgenic skinned fibers with cTnI-ND.

The groups compared were: Tm-WT/cTnI-WT: Tropomyosin Wildtype exchanged with Wildtype cTnI containing Troponin; Tm-WT/cTnI-ND: Tropomyosin Wildtype exchanged with N-terminal truncated cTnI containing Troponin; Tm180/cTnI-WT: Tropomyosin with E180G mutation exchanged with Wildtype cTnI containing Troponin; Tm180/cTnI-ND: Tropomyosin with E180G mutation exchanged with N-terminal truncated cTnI containing Troponin. Only biologically relevant significance is shown. **(A)** Representative Western blot of cTnT indicating relative amounts of endogenous cTnT vs exogenous myc-tagged cTnT.

No exchange, control fiber with no troponin exchanged. **(B)** Scatter plot of quantitative data from panel A indicating percent exchange efficiency. Data represented as mean ± SEM, n=5–10 exchanged fibers, *, P<0.05, **, P<0.0001. **(C)** pCa ($-\log_{10}[Ca^{2+}]$) -force relations in skinned papillary muscles. **(D)** Scatter plot of pCa_{50}, $-\log_{10}$ of the $[Ca^{2+}]$ required to develop 50% of the maximal tension. Data represented as mean ± SEM, n=10–13 skinned fibers **, P<0.0001.

Table 1:

Ca^{2+} -activated tensión parameters for exchanged fibers.

Groups	Max Tension (mN/mm^2)	Hill Slope (nH)	pCa_{50}	n = skinned fibers
Tm-WT/cTnI-WT	25.68 ± 1.36	3.50 ± 0.15	5.61 ± 0.02^	10
Tm-WT/cTnI-ND	29.35 ± 1.26	4.30 ± 0.22 *,#	5.63 ± 0.02$	12
Tm180/cTnI-WT	27.88 ± 1.33	2.99 ± 0.10	5.95 ± 0.03†	11
Tm180/cTnI-ND	26.55 ± 1.41	3.38 ± 0.07	5.80 ± 0.02	13