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The Effect of Cardiomyopathy Mutation (R97L) in Mouse Cardiac Troponin T on the Muscle Length-mediated Recruitment of Crossbridges is Modified Divergently by α - and β -myosin Heavy Chain

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Abstract

Hypertrophic cardiomyopathy mutations in cardiac troponin T (TnT) lead to sudden cardiac death. Augmented myofilament Ca^{2+} sensitivity is a common feature in TnT mutants, but such observations fail to provide a rational explanation for severe cardiac phenotypes. To better understand the mutation-induced effect on the cardiac phenotype, it is imperative to determine the effects on dynamic contractile features such as the muscle length (ML)-mediated activation against α - and β -myosin heavy chain (MHC) isoforms. α - and β -MHC are not only differentially expressed in rodent and human hearts, but they also modify ML-mediated activation differently. Mouse analog of human TnT_{R94L} (TnT_{R97L}) or wild-type TnT was reconstituted into demembranated muscle fibers from normal (α -MHC) and transgenic (β -MHC) mouse hearts. TnT_{R97L} augmented myofilament Ca^{2+} sensitivity by a similar amount in α - and β -MHC fibers. However, TnT_{R97L} augmented the negative impact of strained crossbridges on other crossbridges (γ) by 22% in α -MHC fibers, but attenuated γ by 21% in β -MHC fibers. TnT_{R97L} decreased the magnitude of ML-mediated recruitment of crossbridges (E_R) by 37% in α -MHC fibers, but increased E_R by 35% in β -MHC fibers. We provide a mechanistic basis for the TnT_{R97L}-induced effects in α - and β -MHC fibers and discuss the relevance to human hearts.

Keywords

cardiac troponin T; hypertrophic cardiomyopathy mutation; myosin heavy chain; thin filament cooperativity; length-mediated activation; sudden cardiac death

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INTRODUCTION

Although increased myofilament Ca^{2+} sensitivity has been identified as the hallmark of many hypertrophic cardiomyopathy (HCM)-related mutations in contractile regulatory proteins [1–3], molecular mechanisms underpinning the associated cardiac contractile dysfunctions remain unresolved. The lack of understanding of how mutations lead to severe cardiac phenotypes is a major problem in the case of cardiac troponin T (TnT) because many mutations in TnT have been linked to sudden cardiac death, with little or no evidence of ventricular hypertrophy [4, 5]. This leads to a conclusion that underpins the fundamental role of TnT in cardiac muscle function. Because various mutations are distributed in different structural/functional regions of TnT, it raises important questions such as: 1) if some mutations affect more than one contractile parameter; and 2) if such effects enable us to make a strong correlation between the mutant effect and severe cardiac phenotype.

The focus of this study is the HCM-related mutation, R94L [6], which lies within the central region (CR; 80–180 residues) of human TnT. Previous studies have demonstrated that the CR of TnT tunes both the magnitude and dynamics of myofilament activation by modulating the T_m -dependent functions, as well as the crossbridge (XB)-mediated effects on the thin filament [7–9]. Because there is significant interplay between the TnT- and XB-mediated effects on the thin filament [10–13], the expression of different types of cardiac MHC isoforms in different animal models adds another level of complexity to our understanding of HCM in humans. Different forms of XBs — as in α - and β -MHC — are likely to have different effects on contractile dynamics because α - and β -MHC have been shown to divergently modify the ML-mediated activation of cardiac myofilaments [14–16]. Thus, the effects of TnT mutations on the thin filament is likely to be modified differentially by α - and β -MHC isoforms. Whether the mutation-induced effects go beyond changes in Ca^{2+} sensitivity to alter the dynamics of ML-mediated activation will help us better understand the severity of cardiac phenotypes in humans. This is an important issue to address because an increase in myofilament Ca^{2+} sensitivity, which is a universal finding of most HCM mutations in TnT [1–3], including R94L mutation [17, 18], does not explain the malignant functional phenotype and high mortality rate in humans harboring R94L mutation.

To test our hypothesis that the R94L-mediated effects are differentially modulated by the varied length-sensing mechanisms of α - and β -MHC isoforms, we generated a recombinant mouse TnT analog of the human R94L mutation (TnT_{R97L}) [6] and tested its effects in muscle fibers extracted from normal (α -MHC) and transgenic (TG; β -MHC) mouse hearts. The effects of TnT_{R97L} on the negative impact of strained XBs on other XBs (γ) and the magnitude of ML-mediated recruitment of new force-bearing XBs (E_R) were divergently altered by α - and β -MHC. Our discussion will focus on the mechanistic basis for the TnT_{R97L}-induced effects in α - and β -MHC fibers and the relevance of our findings to human hearts.

MATERIALS AND METHODS

Animal Treatment Protocols

3–4 month old male WT C57BL/6N (non-TG, NTG) expressing α -MHC and TG mice overexpressing β -MHC were used in this study. NTG mice were acquired from Simonsen's laboratories (Gilroy, CA), while β -MHC TG mice were a gift from Dr. Jil Tardiff, University of Arizona, Tucson, AZ. The generation and characterization of β -MHC TG mice was as described previously [19–21]. The line was maintained by backcrossing β -MHC TG mice to NTG mice. PCR was used to genotype offsprings [21]. The treatment of animals followed the established guidelines of the Washington State University Institutional Animal Care and Use committee.

Purification of Recombinant Cardiac Tn subunits

The generation and purification of recombinant *c-myc* tagged mouse TnT (TnT_{WT} and TnT_{R97L}), mouse TnI, and rat TnC (100% identical to mouse TnC sequence), were as described previously [22–26]. All recombinant proteins were cloned into the T7 promoter-based pSBETa vector and expressed in BL21*DE3 cells (Novagen, Madison, WI) for protein synthesis. TnT_{WT} and TnT_{R97L} were purified by an ion-exchange chromatography on a DEAE-fast sepharose column [7, 22], TnI by an ion exchange chromatography on a CM sepharose column [23–25], and TnC by an ion exchange chromatography on a DE-52 anion-exchange column [23, 25, 26]. Samples from eluted fractions were run on a 12.5% SDS gel to assess their purity. Pure fractions were pooled and dialyzed extensively against deionized water containing 15 mM β -mercaptoethanol, lyophilized, and stored at -80°C .

Preparation of Detergent-skinned Cardiac Muscle Fibers and Tn Reconstitution

Mice were deeply anesthetized using isofluorane and their hearts were quickly isolated and placed in an ice-cold high relaxing (HR) solution. The composition of HR solution is listed in our previous works [11, 13]. Left ventricular (LV) papillary muscle bundles were extracted and further dissected into smaller muscle bundles (~0.15 mm in cross-section and 2.0 mm in length) and chemically-skinned overnight at 4°C in HR solution containing 1% Triton X-100.

Whole Tn was reconstituted into detergent-skinned muscle fibers, as described elsewhere [11, 27]. Briefly, TnT_{WT} or TnT_{R97L} (0.9 mg/ml, W/V) and TnI (1.0 mg/ml, W/V) were dissolved in an extraction buffer (*buffer 1*: 50 mM Tris-HCl (pH 8.0), 6 M urea, 1.0 M KCl, and a cocktail of protease inhibitors). High salt and urea in the extraction buffer were removed by successive dialysis against various buffers in the following order: *buffer 2* (50 mM Tris-HCl (pH 8.0 at 4°C), 4 M urea, 0.7 M KCl), *buffer 3* (50 mM Tris-HCl (pH 8.0 at 4°C), 2 M urea, 0.5 M KCl), and *buffer 4* (50 mM BES (pH 7.0 at 20°C), 200 mM KCl, 10 mM BDM, 6.27 mM MgCl_2 , 5 mM EGTA). All *buffers 2–4* included a fresh cocktail of protease inhibitors (0.2 mM PMSF, 2 mM benzamidine-HCl, 1 mM DTT, and 0.01% NaN_3). Any undissolved protein was removed by spinning the protein solution at 3000 rpm for 15 minutes. Detergent-skinned fibers were treated with the extraction buffer containing TnT_{WT}+TnI or TnT_{R97L}+TnI for ~3 hours at 20°C with gentle stirring. Muscle fibers were

then washed twice (10 min each) using *buffer 4* at 20°C and were then incubated in a solution containing TnC (3.0 mg/ml, W/V) overnight at 4°C.

Western blot analysis

Reconstituted muscle fibers were solubilized in a 2.5% SDS solution and their final concentrations were adjusted to 2 mg/ml using protein loading dye (125 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 0.01% bromophenol blue, and 50 mM β -mercaptoethanol). Equal amounts (2 μ g) of protein samples were loaded and run on an 8% SDS-gel for optimal separation of TnT [8, 25, 28]. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane and TnT was probed using a monoclonal anti-TnT primary antibody (M401134, Fitzgerald Industries Int, Concord, MA), followed by HRP-labeled anti-mouse secondary antibody (RPN 2132, Amersham Biosciences, Piscataway, NJ). Densitometric analysis of the Western blot was performed as described previously [8, 28].

Measurements of Steady-state Isometric Tension and ATPase Activity

Steady-state isometric tension and ATPase activity were measured, as described previously [27, 29, 30]. T-shaped aluminum clips were used to attach muscle fibers between a motor arm (322C, Aurora Scientific Inc., Ontario, Canada) and a force transducer (AE 801, Sensor One Technologies Corp., Sausalito, CA). The sarcomere length of the muscle fibers was set to 2.3 μ m under relaxing conditions using He-Ne laser diffraction technique. After two cycles of maximal activation (pCa 4.3; pCa = $-\log$ of $[\text{Ca}^{2+}]_{\text{free}}$) and relaxation (pCa 9.0), sarcomere length was readjusted to 2.3 μ m if necessary. The muscle fibers were then exposed to various Ca^{2+} solutions with ranging from 9.0 to 4.3 in a constantly-stirred chamber and the corresponding steady-state force values were measured. The compositions of various pCa solutions were based on the program by Fabiato and Fabiato [31], and these were listed in our earlier works [11, 13]. The pH and ionic strength of pCa solutions were adjusted to 7.0 and 180 mM, respectively. Measurements of steady-state ATPase activity under isometric conditions was based on an enzymatically coupled assay [27, 29, 30]. All measurements were made at 20°C.

Mechano-dynamic studies

A family of various amplitude step-like length perturbations (± 0.5 , ± 1.0 , ± 1.5 , and $\pm 2.0\%$ of the initial muscle length, ML) were imposed on the muscle fiber in the steady-state (pCa 4.3) and force responses were measured [32]. A nonlinear recruitment-distortion (NRD) model was fitted to family of force responses to estimate five NRD model parameters: the magnitude of instantaneous change in stiffness caused by a sudden ML change (E_D); the rate by which the sudden ML-induced strain within force-bearing XBs dissipates to a steady-state level (c); the parameter that characterizes the XB strain-mediated negative impact on other force-bearing XBs (γ); the rate by which new XBs are recruited into the force-bearing state due to a change in ML (b); and the magnitude of change in the steady-state stiffness caused by the ML-mediated change in the number of newly-recruited force-bearing XBs (E_R). More details on step-like ML perturbation protocol and the significance of NRD model parameters are in our previously published articles [13, 28, 32].

Data analysis

Normalized pCa-tension data were fitted to the Hill equation to derive pCa_{50} (a measure of myofilament Ca^{2+} sensitivity) and n_H (a measure of myofilament cooperativity). Because our experimental model involved two factors, TnT (TnT_{WT} and TnT_{R97L}) and MHC (α - and β -MHC), we used a two-way ANOVA to analyze our data. A significant MHC-TnT interaction effect suggested that the effects of TnT_{R97L} were dissimilar in α - and β -MHC fibers. When MHC-TnT interaction effect was not significant, we interpreted the main effects due to MHC or TnT. To probe the cause for a significant MHC-TnT interaction effect or main effect (MHC or TnT), we conducted multiple post-hoc tests using uncorrected Fisher's Least Significant Difference (LSD) method. The criterion for statistical significance was $P < 0.05$. Data are expressed as mean $\pm$ standard error (SE).

RESULTS

Western Blot Analysis of Recombinant TnT Incorporation in α - and β -MHC Fibers

Previously, we demonstrated that the level of β -MHC expression is $\sim 70\%$ of the total MHC in TG mouse hearts [11]. Moreover, β -MHC expression did not alter the stoichiometry or the phosphorylation status of other sarcomeric proteins [11]. *c-myc* tagged TnT and the endogenous TnT migrated differently on the SDS gel. Differential migration of *c-myc* tagged TnT enabled us to quantify the incorporation levels in reconstituted muscle fibers. The *c-myc* tag in TnT does not alter cardiac function [33, 34]. Densitometric analysis of the Western blot (Fig. 1) confirmed that the levels of TnT_{WT} and TnT_{R97L} in α -MHC fibers were 79% and 71%, while those in β -MHC fibers were 78% and 70%, respectively. These results confirmed that the level of TnT_{R97L} was similar to that of TnT_{WT}.

Effect of TnT_{R97L} on Steady-state Maximal Tension and ATPase activity in α - and β -MHC Fibers

We compared the steady-state tension and ATPase data among various groups at maximal Ca^{2+} activation (pCa 4.3). Two-way ANOVA did not reveal a significant TnT_{R97L}-MHC interaction effect on both maximal tension ($P=0.59$) and maximal ATPase activity ($P=0.42$). Likewise, our analysis did not show a significant main effect of TnT_{R97L} on either maximal tension ($P=0.22$) or ATPase activity ($P=0.69$). Post-hoc *t*-tests confirmed that TnT_{R97L} had no impact on both maximal tension and ATPase activity, whether its effect was tested in α - or β -MHC fibers (Table 1). Our observation that TnT_{R97L} does not affect maximal activation is consistent with another study of human TnT_{R94L} [17].

Effect of TnT_{R97L} on pCa-tension Relationship in α - and β -MHC Fibers

A comparison of pCa-tension relationships shows that TnT_{R97L} not only induces a leftward shift in the pCa-tension relationship, but also decreases the steepness of this relationship in both α -MHC (Fig. 2A) and β -MHC (Fig. 2B) fibers. These observations suggest that TnT_{R97L} sensitizes thin filaments to Ca^{2+} , but attenuates thin filament cooperativity, regardless of the MHC isoform. The magnitude of such TnT_{R97L}-induced effects on pCa-tension relationship were assessed using the Hill model-derived parameters, pCa_{50} and n_H . Two-way ANOVA did not reveal a significant TnT_{R97L}-MHC interaction effect on either

pCa₅₀ ($P=0.54$) or n_H ($P=0.84$). However, our analysis showed a significant main effect of TnT_{R97L} on both pCa₅₀ ($P<0.001$) and n_H ($P<0.001$). Post-hoc t -tests revealed that TnT_{R97L} significantly increased pCa₅₀ by 0.12 pCa units in α -MHC fibers ($P<0.001$; Fig. 2C) and by 0.14 pCa units in β -MHC fibers ($P<0.001$; Fig. 2D). In contrast, TnT_{R97L} significantly decreased n_H by 24% in α -MHC fibers ($P<0.001$; Fig. 2E) and by 25% in β -MHC fibers ($P<0.001$; Fig. 2F).

Effect of TnT_{R97L} on XB Detachment Kinetics in α - and β -MHC Fibers

To determine if TnT_{R97L} altered the rate of XB detachment, g , in a MHC-specific manner, we measured tension cost and the rate constant of XB distortion dynamics (c) among groups. Previous studies have correlated tension cost to c and demonstrated that both are useful measures of g [11, 12, 35, 36]. Thus similar effects in both these parameters would validate if TnT_{R97L} altered g . Two-way ANOVA did not show a significant TnT_{R97L}-MHC interaction effect on either tension cost ($P=0.67$) or c ($P=0.52$). Likewise, our analysis did not show a significant main effect of TnT_{R97L} on either tension cost ($P=0.17$) or c ($P=0.31$). Thus, TnT_{R97L} does not affect XB detachment kinetics in α - or β -MHC fibers (Table 1).

Effect of TnT_{R97L} on the rate of Recruitment of Strong XBs in α - and β -MHC Fibers

To determine if TnT_{R97L} modulated the rate of recruitment of strong XBs, we analyzed differences in the rate constant of ML-mediated recruitment of strong XBs (b) among different groups. Figures 3A and 3B show the effect of TnT_{R97L} on force responses to a sudden 2% stretch in α - and β -MHC fibers, respectively. It is evident that TnT_{R97L} attenuates the rate of delayed force rise in α -MHC fibers (Fig. 3A), whereas it shows no effect in β -MHC fibers (Fig. 3B). This suggests that TnT_{R97L} slows b in α -MHC fibers but not in β -MHC fibers. Two-way ANOVA revealed a significant TnT_{R97L}-MHC interaction effect on b ($P<0.05$), suggesting that the effect of TnT_{R97L} on b is differently altered by α - and β -MHC. Post-hoc t -tests confirmed that TnT_{R97L} significantly decreased b by 11% in α -MHC fibers ($P<0.01$; Fig. 3C), but showed no effect in β -MHC fibers ($P=0.89$; Fig. 3D). These observations suggest that TnT_{R97L}-induced changes in thin filaments interact differently with those induced by α - and β -MHC to result in differential effects on the rate of strong XB recruitment.

Effect of TnT_{R97L} on γ in α - and β -MHC Fibers

γ represents allosteric/cooperative (nonlinear) mechanisms in the thin filament by which strained XBs negatively impact other force-bearing XBs [32]. Thus, a change in γ suggests an alteration in the thin filament-based regulatory mechanisms that mediate XB-RU and/or XB-XB cooperative mechanisms. A cursory look at the comparisons of force responses to sudden 2% stretch suggest that TnT_{R97L} augments γ in α -MHC fibers (Fig. 3A), but attenuates γ in β -MHC fibers (Fig. 3B). Two-way ANOVA revealed a significant TnT_{R97L}-MHC effect on γ ($P<0.01$), suggesting that the effects of TnT_{R97L} on γ were differentially altered by α - and β -MHC isoforms. Post-hoc t -tests revealed that TnT_{R97L} significantly increased γ by 22% in α -MHC fibers ($P<0.01$; Fig. 4A), but it decreased γ by 21% in β -MHC fibers ($P<0.05$; Fig. 4B). These observations demonstrate the TnT_{R97L}-induced effects on thin filament-based regulatory mechanisms — which mediate XB-RU/XB-XB cooperativity — are differently modified by α - and β -MHC isoforms.

Effect of TnT_{R97L} on E_D and E_R , in α - and β -MHC Fibers

To determine whether TnT_{R97L} differentially altered the magnitude of strong XB recruitment in α - and β -MHC fibers, we assessed the differences in the stiffness parameters, E_D and E_R , among groups. E_D is an approximate measure of the number of strong XBs in the isometric steady-state prior to a change in the ML. E_D is derived from the slope of the linear relationship between $(F_1 - F_{ss})$ and L (see Figs. 3A–B).

Instantaneous force, F_1 , results from the stretch of elastic elements within bound XBs. Thus, F_1 increases when the number of strong XBs is higher prior to ML change. TnT_{R97L} has no effect on F_1 in either α -MHC (Fig. 3A) or β -MHC fibers (Fig. 3B), suggesting no impact on E_D . Two-way ANOVA did not show a significant TnT_{R97L}-MHC interaction effect ($P=0.33$) or a main effect of TnT_{R97L} ($P=0.87$) on E_D . Post-hoc t -tests confirmed that TnT_{R97L} had no effect on E_D in either α -MHC ($P=0.44$; Table 1) or β -MHC fibers ($P=0.54$; Table 1). We have previously demonstrated that E_D is strongly correlated to maximal tension [¹³, 25, 32, 36]. Similar observations in E_D and maximal tension (Table 1) substantiate our conclusion that TnT_{R97L} does not affect maximal activation regardless of the MHC isoform.

E_R is an approximate measure of the ML-mediated change in the number of newly-recruited strong XBs, following a change in the ML [³², 35]. E_R is derived from the slope of the linear relationship between $(F_{nss} - F_{ss})$ and L (see Figs. 3A–B). The level of force is higher in F_{nss} (new steady state; Figs 3A–B) because additional XBs are recruited into the force-bearing state. TnT_{R97L} attenuates F_{nss} in α -MHC fibers (Fig. 3A), but it augments F_{nss} in β -MHC fibers (Fig. 3B). A comparison of $(F_{nss} - F_{ss})$ - L plots also confirm that TnT_{R97L} decreases the slope of $(F_{nss} - F_{ss})$ - L relationship in α -MHC fibers (Fig. 5A), while it increases the slope of this relationship in β -MHC fibers (Fig. 5B). These observations suggest that TnT_{R97L} attenuates E_R in α -MHC fibers, whereas it augments E_R in β -MHC fibers. Two-way ANOVA revealed a significant TnT_{R97L}-MHC interaction effect ($P<0.001$) on E_R , indicating that the effect of TnT_{R97L} on E_R significantly varied with the MHC isoform. Post-hoc t -tests showed that TnT_{R97L} significantly decreased E_R by 37% in α -MHC fibers ($P<0.01$; Fig. 5C), while it increased E_R by 35% in β -MHC fibers ($P<0.05$; Fig. 5D). Thus, the TnT_{R97L}-induced changes in the thin filament interact differently with those induced by α - and β -MHC to contrastingly modify the magnitude of ML-mediated recruitment of strong XBs.

DISCUSSION

One striking observation in our study is that TnT_{R97L} negatively affects the ML-mediated activation in α -MHC fibers, while such an effect is counteracted in β -MHC fibers. Supporting evidence is provided by three different contractile parameters — γ (Fig. 4), b (Fig. 3), and E_R (Fig. 5). These observations demonstrate that TnT_{R97L}- and α -MHC-induced changes in the thin filament interact to attenuate the ML-mediated activation of thin filaments, while those induced by TnT_{R97L} and β -MHC interact to augment the ML-mediated activation of thin filaments. Interestingly, TnT_{R97L} induced a similar increase in myofilament Ca^{2+} sensitivity (pCa_{50} ; Fig. 2C–D) and a similar decrease in myofilament cooperativity (η_H ; Fig. 2E–F) in both α - and β -MHC fibers; this suggests that at submaximal

Ca^{2+} activations, differential effects of α - and β -MHC were minimized by the dominating effect of TnT_{R97L} on thin filaments.

The Ca^{2+} -sensitizing effect of TnT_{R97L} on myofilaments may be attributed to its effect on: (1) Ca^{2+} -binding to Tn that influences the regulatory unit (RU; Tn-Tm complex) *off/on* kinetics; and/or (2) XB cycling kinetics. Our measurements at maximal Ca^{2+} activation (pCa 4.3), when interpreted in the context of the two-state XB model [37], suggest that TnT_{R97L} does not affect XB cycling kinetics. Two independent measures of g in our study, tension cost and c (Table 1), confirmed that TnT_{R97L} had no impact on g in either α - or β -MHC fibers. Our observation that g is unaffected by TnT_{R97L} is consistent with another finding [18]. Because g is independent of Ca^{2+} concentration [38, 39], our observations at maximal Ca^{2+} activation are also valid at submaximal Ca^{2+} activations. Similarly, unaltered maximal tension (Table 1) and E_{D} (Table 1) suggest that TnT_{R97L} had no impact on the fraction of attached XBs, $f/(f+g)$, in either α - or β -MHC fibers. Therefore, we believe that both f and g are unaffected by TnT_{R97L} in α - and β -MHC fibers. Collectively, these observations suggest that TnT_{R97L} sensitizes thin filaments to Ca^{2+} by modifying the RU *off/on* kinetics, with no effect on XB cycling kinetics.

The notion that TnT_{R97L} may sensitize thin filaments to Ca^{2+} by decreasing the affinity of TnT to Tm comes from a previous study on TnT_{R94L} [40]. Weaker TnT-Tm interactions may push the RU *off/on* equilibrium more towards the *on* state [41]. A consequence of such action by TnT_{R97L} is that pCa_{50} is augmented in both α - and β -MHC fibers. Because this leaves fewer RUs in the *off* state and fewer XBs in the non-cycling pool, we expect that strong XB-based cooperative mechanisms are less operative in thin filaments containing TnT_{R97L} . Such notion is supported by our observations that TnT_{R97L} induces a similar increase in pCa_{50} and a similar decrease in η_{H} in both α - and β -MHC fibers (Fig. 2A–F).

The ML-mediated activation of thin filaments at maximal Ca^{2+} activation is contrastingly affected by α - and β -MHC. The following two sources of cooperativity may contribute to differential effects of α - and β -MHC on the ML-mediated activation of thin filaments: (1) cooperative effects that exist within the thin filament at the onset of ML change; and (2) additional ML-sensing effects induced by α - and β -MHC in the thin filament. Valuable insights into how such TnT_{R97L} -induced cooperative effects are differentially modified by α - and β -MHC may be gleaned from comparisons of γ because it represents nonlinear thin filament-based mechanisms by which strained XBs (mediated by ML change) negatively impact other force-bearing XBs [11, 15, 32]. Augmentation of γ in $\text{TnT}_{\text{R97L}}+\alpha$ -MHC fibers (Figs. 3A and 4A) suggests that TnT_{R97L} enhances XB-XB cooperativity at the onset of ML change; this enhanced XB-XB cooperativity causes strained XBs to cooperatively deactivate other force-bearing XBs in α -MHC fibers, leading to a larger force decline (greater γ). This observation is validated by our observation that TnT_{R97L} significantly attenuates b in α -MHC fibers (Fig. 3C). Previous studies have shown that enhanced XB-XB cooperativity attenuates the speed of strong XB recruitment [41, 42]. Enhanced XB-XB cooperativity is also expected to increase E_{R} — an index of LDA — because XB-based (XB-RU/XB-XB) cooperativity dominates LDA in cardiac muscle [43–46]. However, our observation that E_{R} significantly decreases in $\text{TnT}_{\text{R97L}}+\alpha$ -MHC fibers suggests that attenuation of XB-RU cooperativity may be a source of this effect. Our data suggests that the effect of attenuated

XB-RU cooperativity on the thin filament activation is strong enough to negate the positive effect of increased XB-XB cooperativity, leading to an attenuation of E_R in TnT_{R97L}+ α -MHC fibers. γ decreases in TnT_{R97L}+ β -MHC fibers because XB-RU cooperativity is increased, without affecting XB-XB cooperativity (unaltered b ; Fig. 3D). Evidence for an increase in XB-RU cooperativity is provided by an increase in E_R in TnT_{R97L}+ β -MHC fibers (Fig. 5D).

While different initial conditions partly underlie differential ML-mediated responses, there is sufficient evidence to suggest that specialized features related to ML-sensing mechanisms may also enable α - and β -MHC to differently modify allosteric mechanisms within the thin filament. Two independent studies [15, 16] demonstrated that an increase in ML had no impact on the rate constant of tension redevelopment (k_{tr}) in α -MHC fibers, but decreased k_{tr} in β -MHC fibers. Interestingly, a decrease in k_{tr} observed at long ML in β -MHC fibers was also accompanied by a decrease in XB detachment rate (g) [15], suggesting that the observed decrease in k_{tr} was reflective of a decrease in g . Thus, the longer dwell time of β -MHC XBs — ascribed to a decrease in g at long ML — amplifies thin filament-based cooperative mechanisms by prolonging the XB-based feedback effect on thin filaments. In the osmotic compression (2% dextran) study, β -MHC fibers responded with steeper changes in XB cycling kinetics [16]. Electron microscopy and optical diffraction studies documented differences in the azimuthal flexibility/radial extension between α - and β -MHC XBs in rat cardiac myofilaments [47]. Unperturbed radial/azimuthal flexibility of XBs, in response to an increase in ML, may allow β -MHC to exhibit steeper ML-dependent changes in XB cycling kinetics [16]. These diverse set of observations from previous studies — along with our own findings on γ , b , and E_R — strongly suggest that ML-related features of α - and β -MHC enable them to divergently modify TnT_{R97L}-mediated effects on thin filaments.

Relevance of our Findings to Human Hearts Harboring R94L

Myofilament Ca^{2+} sensitivity and E_R are augmented in TnT_{R97L}+ β -MHC fibers. Enhanced myofilament Ca^{2+} sensitivity by TnT_{R97L} may attenuate the rate of dissociation of Ca^{2+} from thin filaments; thereby impairing LV relaxation by prolonging cytosolic Ca^{2+} transients during early diastole. However, a normal echo in two young patients (6- and 7-year old) harboring TnT_{R94L} presented no diastolic abnormalities [6], suggesting that compensations may have occurred to restore the LV diastolic function to a normal level. Interestingly, the only surviving adult displayed an exercise-induced syncope [6], suggesting an impaired cardiovascular response to exercise.

Our finding that E_R is augmented in TnT_{R97L}+ β -MHC fibers suggests that during exertion β -MHC may impose additional length-dependent effects on top of the TnT_{R97L}-mediated increase in myofilament Ca^{2+} sensitivity. Such synergistic actions of TnT_{R97L} and β -MHC may exacerbate the slowing effect on relaxation during exertion. Supporting evidence is provided by another study which showed greater elevation of diastolic Ca^{2+} in TG mouse hearts expressing HCM-linked TnT_{I79N} both at higher pacing rates, as well as to isoproterenol-stress tests [48]. Such elevation of diastolic Ca^{2+} may dramatically increase the time required for the LV pressure to fall below that of the left atrium; thereby reducing

the time for subsequent filling. This may be one of the reasons for the sudden cardiac failure in some of the HCM patients harboring TnT_{R94L} [6].

Abbreviations used

ANOVA	analysis of variance
CR	central region
HCM	hypertrophic cardiomyopathy
HR	high relaxing
LSD	least significant differences
MHC	myosin heavy chain
ML	muscle length
NRD	nonlinear recruitment distortion
RU	regulatory unit
SE	standard error
TG	transgenic
NTG	non-transgenic
Tm	tropomyosin
Tn	troponin
TnC	troponin C
TnI	troponin I
TnT	troponin T
WT	wild-type
XB	crossbridge

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- R97L mutation in mouse cardiac troponin T is linked to sudden cardiac death
- Different effects of α - β -myosin heavy chain (MHC) on R97L shed light on phenotype
- R97L reduces crossbridge (XB) recruitment in α -MHC but augments in β -MHC fiber
- R97L reduces XB-regulatory unit cooperativity in α -MHC but augments in β -MHC fiber

Figure 1. Western Blot Analysis of Reconstituted Muscle Fibers

α - and β -MHC fibers reconstituted with TnT_{WT} or TnT_{R97L} were solubilized using 2.5% SDS solution. 2 μ g of each standardized (2 mg/ml) protein sample were loaded and run on a small 8% SDS-gel for optimal separation of TnT. Proteins were then transferred onto a PVDF membrane and TnT was probed using anti-TnT primary and secondary antibodies (see Methods).

Figure 2. Impact of TnTr97L on pCa-tension Relationships in α- and β-MHC Fibers

Steady-state tensions at various pCa, normalized by the corresponding value at pCa 4.3, were plotted against pCa to construct pCa-tension relationships. The Hill model was fitted to the pCa-tension relationships to derive pCa₅₀ and n_H. Panels **A** and **B** show the effect of TnTr97L on the averaged pCa-tension relationship in α- and β-MHC fibers, respectively. Panels **C** and **D** show the effect of TnTr97L on pCa₅₀ in α- and β-MHC fibers, respectively. Panels **E** and **F** show the effect of TnTr97L on n_H in α- and β-MHC fibers, respectively. Statistical differences were analyzed by two-way ANOVA and post-hoc multiple pairwise

comparisons using Fisher's LSD method. Asterisks indicate significant differences relative to respective TnT_{WT} (***) ($P < 0.001$). The number of fibers measured in each group is: 13 for α -MHC+TnT_{WT} (3 hearts), 16 for α -MHC+TnT_{R97L} (3 hearts), 14 for β -MHC+TnT_{WT} (3 hearts), 17 for β -MHC+TnT_{R97L} (3 hearts). Data are presented as mean $\pm$ SE.

Figure 3. Impact of TnTR97L on b in α - and β -MHC Fibers

Panels A and B show the effect of TnTR97L on force responses to sudden 2% stretch in α - and β -MHC fibers, respectively. Forces are normalized by the isometric steady-state force, F_{ss} , prior to the muscle length (ML) change. F_1 represents the instantaneous increase in force due to sudden increase in ML, c describes the rate by which the sudden ML-induced strain within force-bearing XBs dissipates to a steady-state level, γ characterizes the XB strain-mediated negative impact on other XBs, b governs the rate of delayed force rise following an increase in ML, and F_{nss} represents the new steady-state force corresponding to an increase in ML. Panels C and D show the effect of TnTR97L on b in α - and β -MHC fibers, respectively. Statistical differences were analyzed by two-way ANOVA and post-hoc multiple pairwise comparisons using Fisher's LSD method. Asterisks indicate significant differences relative to respective TnT_{WT} (** $P < 0.01$; NS, not significant). The number of fibers measured in each group is: 13 for α -MHC+TnT_{WT} (3 hearts), 16 for α -MHC+TnTR97L (3 hearts), 14 for β -MHC+TnT_{WT} (3 hearts), 17 for β -MHC+TnTR97L (3 hearts). Data are presented as mean $\pm$ SE.

Figure 4. Impact of TnTR97L on γ in α - and β -MHC fibers

γ represents allosteric/cooperative mechanisms in the thin filament that mediate the effect of strained XBs on other force-bearing XBs [32]. γ was determined as described in Materials and Methods. A greater γ suggests larger magnitude of force decline to the minimum point (nadir; see dashed trace in Fig. 3A), while a lower γ reflects a smaller magnitude of force decline (see dashed trace in Fig. 3B). Panels **A** and **B** show the effect of TnTR97L on γ in α - and β -MHC fibers, respectively. Statistical differences were analyzed by two-way ANOVA and post-hoc multiple pairwise comparisons using Fisher's LSD method. Asterisks indicate significant differences relative to respective TnT_{WT} (* P <0.05; ** P <0.01; NS, not significant). The number of fibers measured in each group is: 13 for α -MHC+TnT_{WT} (3 hearts), 16 for α -MHC+TnTR97L (3 hearts), 14 for β -MHC+TnT_{WT} (3 hearts), 17 for β -MHC+TnTR97L (3 hearts). Data are presented as mean $\pm$ SE.

Figure 5. Impact of TnTr97L on E_R in α - and β -MHC Fibers

E_R was estimated as the slope of a linear relationship between ($F_{nss}F_{ss}$) elicited by muscle fibers to imposed ML changes, ΔL (also see Figs. 3A–B). Panels **A** and **B** show the effect of TnTr97L on ($F_{nss}F_{ss}$)- ΔL relation in α - and β -MHC fibers, respectively. Panels **C** and **D** show the effect of TnTr97L on E_R in α - and β -MHC fibers, respectively. Statistical differences were analyzed by two-way ANOVA and post-hoc multiple pairwise comparisons using Fisher's LSD method. Asterisks indicate significant differences relative to respective TnT_{WT} (* P <0.05; ** P <0.01; NS, not significant). The number of fibers measured in each group is: 13 for α -MHC+TnT_{WT} (3 hearts), 16 for α -MHC+TnTr97L (3 hearts), 14 for β -MHC+TnT_{WT} (3 hearts), 17 for β -MHC+TnTr97L (3 hearts). Data are presented as mean $\pm$ SE.

Table 1Effects of TnT_{R97L} on various contractile parameters in α - and β -MHC fibers.

	α -MHC		β -MHC	
	TnT _{WT}	TnT _{R97L}	TnT _{WT}	TnT _{R97L}
Maximal Tension (mN·mm ⁻²)	46.23±0.91	49.21±0.91	48.98±1.92	50.76±1.65
Maximal ATPase (pmol·mm ⁻³ ·s ⁻¹)	283±10	271±11	137±7	142±9
E_D (mN·mm ⁻³)	1045±71	969±33	1022±53	1071±36
Tension cost (pmol·mN ⁻¹ ·mm ⁻¹ ·s ⁻¹)	5.94±0.25	5.59±0.22	2.86±0.11	2.65±0.14
c (s ⁻¹)	43.46±1.86	47.01±2.02	18.82±0.95	19.20±0.98

Steady-state isometric tension and ATPase activity were measured at pCa 4.3, as described previously [29, 30]. E_D was estimated as the slope of the linear relationship between (F_l - F_{ss}) values and the corresponding ML changes (ΔL) imposed on muscle fibers (see Figs. 3A–B). Tension cost was estimated as the slope of the linear relationship between steady-state tension and ATPase data at various pCa. c represents the rate of force decay to a steady-state level, following a sudden ML change (see Figs. 3A–B), and was estimated as described in Materials and Methods. Statistical differences were analyzed by two-way ANOVA and post-hoc multiple pairwise comparisons using Fisher's LSD method. When compared to respective TnT_{WT}, TnT_{R97L} showed no effect on all of the above listed parameters in either α -MHC or β -MHC fibers. The number of fibers measured in each group is: 13 for α -MHC+TnT_{WT} (3 hearts), 16 for α -MHC+TnT_{R97L} (3 hearts), 14 for β -MHC+TnT_{WT} (3 hearts), 17 for β -MHC+TnT_{R97L} (3 hearts). Data are presented as mean±SE.