The functional effect of dilated cardiomyopathy mutation (R144W) in mouse cardiac troponin T is differently affected by α- and β-myosin heavy chain isoforms

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Gollapudi SK, Tardiff JC, Chandra M. The functional effect of dilated cardiomyopathy mutation (R144W) in mouse cardiac troponin T is differently affected by α- and β-myosin heavy chain isoforms. Am J Physiol Heart Circ Physiol 308: H884–H893, 2015. First published February 13, 2015; doi:10.1152/ajpheart.00528.2014.—Given the differential impact of α- and β-myosin heavy chain (MHC) isoforms on troponin T (TnT) modulates contractile dynamics, we hypothesized that the effects of dilated cardiomyopathy (DCM) mutations in TnT would be altered differently by α- and β-MHC. We characterized dynamic contractile features of normal (α-MHC) and transgenic (β-MHC) mouse cardiac muscle fibers reconstituted with a mouse TnT analog (TnTR144W) of the human DCM R141W mutation. TnTR144W did not alter maximal tension but attenuated myofilament Ca2+ sensitivity (pCa50) to a similar extent in α- and β-MHC fibers. TnTR144W attenuated the speed of cross-bridge (XB) distortion dynamics (c) by 24% and the speed of XB recruitment dynamics (b) by 17% in α-MHC fibers; however, both b and c remained unaltered in β-MHC fibers. Likewise, TnTR144W attenuated the rates of XB detachment (g) and tension redevelopment (k0) only in α-MHC fibers. TnTR144W also decreased the impact of strained XBs on the recruitment of new XBs (γ) by 30% only in α-MHC fibers. Because c, b, g, k0, and γ are strongly influenced by thin filament-based cooperative mechanisms, we conclude that the TnTR144W- and β-MHC-mediated changes in the thin filament interact to produce a less severe functional phenotype, compared with that brought about by TnTR144W and α-MHC. These observations provide a basis for lower mortality rates of humans (β-MHC) harboring the TnTR141W mutant compared with transgenic mouse studies. Our findings strongly suggest that some caution is necessary when extrapolating data from transgenic mouse studies to human hearts.

DILATED CARDIOMYOPATHY (DCM), a disease caused by mutations in many sarcomeric proteins, including cardiac troponin T (TnT), is characterized by systolic dysfunction and dilated ventricles. Our understanding of the molecular mechanisms by which DCM mutations lead to cardiac pathogenesis remains poorly understood (45). This is complicated by the following two reasons: 1) previous studies are done under steady-state conditions, which do not address the effects of the mutation on the dynamic aspects of heart muscle contraction; and 2) the functional effects of DCM mutations in transgenic mouse models are primarily investigated against the α-myosin heavy chain (MHC) isoform, as opposed to the β-MHC isoform in the human heart (33, 36).

The divergent effects of α- and β-MHC isoforms on thin filament cooperativity and contractile dynamics suggest that the TnT mutation-mediated effect on contractile function may be differentially modulated by α- and β-MHC isoforms. For a meaningful assessment of a given TnT mutant effects in humans, it is imperative that the study is carried out against a β-MHC background.

Our study focuses on the DCM mutation R141W in human TnT (26). The R141W mutation lies within the central region (CR; 80–180 residues) of human TnT, which interacts strongly with tropomyosin (Tm). Recently, we demonstrated that the CR-Tm interactions tune cross-bridge (XB) recruitment dynamics by modulating strong XB-mediated cooperative feedback effect on the thin filament (14). Therefore, point mutations in the CR of TnT have the potential to alter cardiac contractile dynamics by modifying the interplay between the TnT- and MHC-mediated effects on thin filament cooperativity (12, 49). Therefore, an important question that follows is: how is the interplay between the mutant TnT- and MHC-mediated impact on thin filaments altered by different MHC isoforms? The ability of a given MHC isoform to alter the TnT-mediated function is related to its innate kinetic properties. Due to the longer XB dwell time, a slower cycling β-MHC isoform (10, 41, 43) may modify thin filament-based cooperative mechanisms and dynamic contractile behavior (4, 10, 29, 30, 43).

Because the heart muscle comprises dynamically interacting components, it is expected that the mutant TnT- and the MHC-induced changes in the thin filament will interact to yield properties that are abnormal.

It is now widely appreciated that dynamic aspects, rather than steady-state aspects of the force-pCa relationship, dominate in physiological conditions under which cardiac muscle functions. Therefore, we hypothesized that α- and β-MHC have a different impact on contractile dynamics mediated by the DCM-causing mutation in TnT. To test our hypothesis, we generated a recombinant mouse TnT analog (TnTR144W) of the human DCM mutation, R141W. TnTR144W and wild-type mouse TnT (TnTW1) were reconstituted into detergent-skinned cardiac papillary muscle fibers extracted from normal mouse expressing α-MHC and transgenic (TG) mouse expressing β-MHC. Steady-state and dynamic contractile parameters were measured in TnTW1- and TnTR144W-reconstituted fibers expressing either the α- or β-MHC isoform. We will discuss the molecular mechanisms by which TnTR144W and β-MHC-mediated changes in the thin filament interact to produce a differential effect on contractile dynamics, compared with those brought about by TnTR144W and α-MHC.
METHODS

Animal protocols and generation of β-MHC TG mice. Wild-type (WT) C57BL/6N (non-TG, NTG) mice expressing α-MHC were obtained from Simonsen’s laboratories (Gilroy, CA). The generation and characterization of β-MHC TG mice were as described previously (23–25, 40). The line was maintained by backcrossing to C57Bl/6N mice. PCR was used to genotype offspring (25). All animals (4- to 5-mo-old male mice) used in this study received proper care and treatment, and our experimental protocols were approved by the Washington State University Institutional Animal Care and Use Committee. The procedures for euthanizing mice conform to the recommendations of Euthanasia of the American Veterinary Medical Association.

Recombinant mouse cardiac Tn subunits. A recombinant c-myc-tagged mouse TnT mutant (R144W mutation; TnTR144W) was generated; this mutation corresponds to the R141W mutation in human TnT (26). c-myc-Tagged WT mouse TnT (TnTRWT) served as the control. Recombinant TnT (TnTR144W or TnTRWT), TnI, and TnC were all cloned into the T7 promoter-based pSBETa vector, expressed in BL21*DE3 cells (Novagen, Madison, WI) for protein synthesis and were purified using ion-exchange chromatography techniques (3, 17, 38). TnTWT and TnTR144W were purified as follows: a culture preparation using pellets obtained from 60% ammonium sulfate cut was loaded onto a DEAE fast Sepharose column, and TnT was eluted using a linear NaCl gradient (3). TnI was purified on a CM fast Sepharose cation exchange column and eluted using a linear NaCl gradient, as described before (17). TnC was partially purified using a DE-52 anion-exchange column and then further purified on a phenyl Sepharose column (38). All fractions containing pure proteins were pooled and dialyzed thoroughly against deionized water containing 15 mM β-mercaptoethanol, lyophilized, and stored at −80°C.

Detergent-skinned mouse cardiac muscle fibers. Left ventricular papillary muscle fibers from mouse hearts were isolated and detergent skinned, as described previously (4, 5). Briefly, mice were deeply anesthetized using isoflurane, and their hearts were quickly excised and placed into an ice-cold high-relaxing (HR) solution of pCa 9.0 (pH of 7.0). The HR solution contained the following (in mM concentrations): 20 2,3-butanedione monoxime (BDM), 50 TnC, and 50 Na2ATP. This solution was then extensively dialyzed against various solutions with pCa ranging from 4.3 to 9.0, the compositions of which were based on the program (43). In brief, the two ends of the muscle fiber were first clipped using T-shaped aluminum foil clips and the fiber was mounted between the force transducer (model AE-801; Sensor One Technologies, Sausalito, NJ) to estimate the levels of mutant TnT incorporation in reconstituted muscle fibers. The level of mutant TnT incorporation in reconstituted muscle fibers was quantified using the Western blot. We added an 11-amino acid c-myc tag at the NH2 terminus of recombinant TnT proteins so that they could be distinguished from the native TnT on SDS gels. We have previously shown that the insertion of this c-myc tag has no effect on TnT-mediated function in cardiac muscles (6, 34, 46). For the Western blot, 2 μg of each standardized sample were loaded and ran on a 8% SDS gel. Proteins were then transferred to a polyvinylidene difluoride membrane and TnT was probed using an anti-TnT primary antibody (M401134; Fitzgerald Industries, Concord, MA) and an anti-mouse secondary antibody (RPN2132; Amersham Pharmaccia, Piscataway, NJ) to estimate the levels of mutant TnT incorporation in reconstituted muscle fibers (14, 15).

Reconstitution of recombinant proteins into detergent-skinned muscle fibers. Recombinant Tn subunits were reconstituted into skinned fibers, as described previously (4, 5, 13). Briefly, excess amounts of TnT (1.5 mg/ml, wt/vol) and TnI (1.0 mg/ml) were dissolved in a buffer containing 50 mM Tris-HCl (pH 8.0), 6 M urea, 1.0 M KCl, and 1 mM DTT. High salt and urea in the extraction solution were gradually removed by successive dialysis at 4°C against two buffers that contained decreasing amounts of salt and urea (4 M urea, 0.7 M KCl; 2 M urea, 0.5 M KCl). The solution was then extensively dialyzed against a buffer containing 50 mM BES (pH 7.0 at 20°C), 200 mM KCl, 10 mM BDM, 5 mM EGTA, and 2.77 mM MgCl2. All buffers used for protein dialysis contained a cocktail of protease inhibitors. Skinned fibers were first treated with this extraction solution containing TnT and TnI for 3 h at room temperature, with gentle shaking. Fibers were then incubated with mouse Trc3 (3 mg/ml in HR) overnight at 4°C. For simplicity, we will refer to NTG mouse cardiac muscle fibers expressing α-MHC as “α-MHC fibers,” and the TG mouse cardiac fibers expressing β-MHC as “β-MHC fibers.” α-MHC fibers reconstituted with TnTWT + TnI + TnC and TnTR144W + TnI + TnC will be referred to as “α-MHC + TnTWT” and “α-MHC + TnTR144W” fibers, while those reconstituted in β-MHC fibers will be referred to as “β-MHC + TnTWT” and “β-MHC + TnTR144W” fibers, respectively.

Determination of MHC composition and phosphorylation status of sarcomeric proteins in NTG and TG mouse hearts. Ventricular tissues from NTG and TG mouse hearts were finely pulverized in liquid nitrogen and solubilized using a muscle protein extraction buffer: 2.5% SDS, 10% glycerol, 50 mM Tris base (pH 6.8 at 4°C), 1 mM DTT, 4 mM benzamidine HCl, and a cocktail of phosphatase and protease inhibitors. For analyzing the MHC isoform composition, 20 μg of each standardized sample were loaded and ran on a 5% large SDS gel (12, 15). Proteins were visualized by staining the gel with Coomassie brilliant blue (R-250; Bio-Rad Laboratories, Hercules, CA). Densitometric analysis of the MHC protein profiles was performed using the ImageJ software (available on the National Institutes of Health web site), as described previously (14, 15, 30). For assessing the phosphorylation status of sarcomeric proteins, 10 μg of each standardized sample were loaded and ran on a 12.5% small SDS gel. The gel was then treated with Pro-Q diamond stain and destain (P33300 and P33310; Life Technologies, Grand Island, NY) as described in the Life Technologies manual to analyze the levels of phosphorylated proteins in NTG and TG mouse hearts (12, 15).

Western blot analysis of reconstituted muscle fibers. The level of mutant TnT incorporation in reconstituted muscle fibers was quantified using the Western blot. We added an 11-amino acid c-myc tag at the NH2 terminus of recombinant TnT proteins so that they could be distinguished from the native TnT on SDS gels. We have previously shown that the insertion of this c-myc tag has no effect on TnT-mediated function in cardiac muscles (6, 34, 46). For the Western blot, 2 μg of each standardized sample were loaded and ran on a 8% SDS gel. Proteins were then transferred to a polyvinylidene difluoride membrane and TnT was probed using an anti-TnT primary antibody (M401134; Fitzgerald Industries, Concord, MA) and an anti-mouse secondary antibody (RPN2132; Amersham Pharmaccia, Piscataway, NJ) to estimate the levels of mutant TnT incorporation in reconstituted muscle fibers (14, 15).

Simultaneous measurement of tension and ATPase activity. Force and ATPase activity were measured as described previously (8, 44). In brief, the two ends of the muscle fiber were first clipped using T-shaped aluminum foil clips and the fiber was mounted between the force transducer (model AE-801; Sensor One Technologies, Sausalito, CA) and the servo motor (model 322C; Aurora Scientific, Aurora, ON, Canada). The sarcomere length (SL) of the muscle fiber was adjusted to 2.3 μm in pCa 9.0 solution using the He-Ne laser diffraction technique. pH-ATPase relationships were measured by sequentially bathing the fiber in various solutions with pCa ranging from 4.3 to 9.0, the compositions of which were based on the program developed by Fabiato and Fabiato (9). The relaxation (pCa 9.0) solution contained the following (in mM concentrations): 50 BES, 5 NaN3, 10 phosphoenol pyruvate (PEP), 10 EGTA, 0.024 CaCl2, 6.87 MgCl2, 5.83 Na2ATP, and 51.14 K-propionate, while the maximal Ca2+ (pCa 4.3) solution contained the following (in mM concentrations): 50 BES, 5 NaN3, 10 PEP, 10 EGTA, 0.111 CaCl2, 6.61 MgCl2, 5.95 Na2ATP, and 31 K-propionate. In addition, 2 mM pyruvate kinase, 0.2 mM lactate dehydrogenase, and a fresh cocktail of protease inhibitors were also added to pCa 9.0 and pCa 4.3 solutions. The pH and the ionic strength of each pCa solution were adjusted to 7.0 and 180 mM, respectively. All experiments were performed at 2°C.

The measurement of ATPase activity was based on a coupled enzymatic assay (8, 44). In brief, a near ultraviolet (UV) light was projected through the muscle chamber and was split 50:50 by a beam splitter for intensity detection at 340- and 400-nm wavelengths. The UV absorbance signal at 340 nm was sensitive to the amount of NADH in the pCa solution, while that at 400 nm was insensitive and served as the reference signal. Because the oxidation of NADH is linked to the ATP consumption in this assay, changes in ATPase

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activity resulted in changes in the amount of NADH consumed in the bathing pCa solution. An analog divider and log amplifier produced a UV absorbance signal proportional to changes in the ATP consumption. The resulting changes in the signal were transferred to a molar ATP consumption by multiple rapid injections of 250 pmol of ADP into the bathing solution using a motor-controlled micro syringe. Tension cost was determined as the slope of the relationship between tension and ATPase (8, 44).

Rate of tension redevelopment. The rate of tension redevelopment ($k_{tr}$) was estimated using a modification to the large slack/restretch maneuver formerly described by Brenner and Eisenberg (1). The modified procedure has been described before (10, 14, 15). In brief, the maximally activated muscle fiber was rapidly (0.5 ms) slackened by 10% of its initial muscle length (ML) via a servo motor and was allowed to briefly shorten at the increased length for 25 ms. The fiber was then quickly stretched past its ML by 10%, following which it was rapidly brought back to its initial ML and allowed to redevelop force. $k_{tr}$ was estimated by fitting the following monoequational function to the force recovery phase:

$$F(t) = (F_{s0} - F_{res})(1 - e^{-kt}) + F_{res}$$

where $F_{s0}$ is the steady-state isometric force and $F_{res}$ is the residual force from which the fiber starts to redevelop force.

Mechanodynamic studies. Force responses to various amplitude quick stretches and releases ($\pm 0.5, \pm 1.0, \pm 1.5, \pm 2.0$% of the initial ML) were measured as described previously (11). In brief, the maximally activated fiber was first subjected to a quick (0.5 ms) stretch by 0.5% of ML, was held at this increased length for 5 s, and was then brought back to its initial ML by applying a quick release by 0.5%. After 5 s, this procedure was repeated with 1.0, 1.5, and 2.0% ML quick stretches/releases and the corresponding force responses were recorded. A nonlinear recruitment-distortion (NRD) model was fitted to the family of force responses to estimate five model parameters: the magnitude of instantaneous stiffness increase caused by a sudden stretch-mediated increase in force ($E_a$); the rate by which the sudden stretch-mediated strain in strongly bound Xbs dissipates to the steady-state level ($c$); the rate by which new Xbs are recruited into the force-bearing state due to an increase in ML ($b$); the magnitude of the stiffness increase caused by the ML-mediated increase in the number of newly recruited force-bearing Xbs ($E_b$); and the nonlinear interaction parameter that describes how strain within strongly bound Xbs influences the recruitment of new Xbs ($\gamma$). More details on the characteristic features of force responses to step-like ML changes and the significance of the NRD model parameters can be found in our previously published articles (11, 15).

Data analysis. Normalized pCa-tension data were fitted to the Hill equation to derive $pC_{50}$ (a measure of myofilament Ca$^{2+}$ sensitivity) and $n_H$ (a measure of myofilament cooperativity). Contractile parameters were analyzed using a two-way ANOVA, with TnT (TnTWT and TnTR144W) and MHC (a-MHC and b-MHC) as the two factors. A significant TnT-MHC interaction effect suggested that the effects of TnT were dissimilar in a-MHC and b-MHC fibers. When the TnT-MHC interaction effect was not significant, we interpreted the main effects due to either TnT or MHC. To probe the cause for either a significant interaction effect or a main effect, we conducted post hoc multiple comparisons using uncorrected Fisher’s least significant difference method. The criterion for statistical significance was $P < 0.05$. Data are expressed as means $\pm$ SE.

RESULTS

Analysis of MHC composition in NTG and TG mouse hearts. To determine the relative composition of MHC isoforms, SDS-solubilized muscle protein samples from NTG (a-MHC) and TG (b-MHC) mouse hearts were analyzed on a large 5% SDS-gel (4, 10). Figure 1A shows a representative gel demonstrating the MHC composition in one NTG and one TG mouse heart. Values from several preparations showed that the NTG mouse hearts expressed 100% a-MHC ($n = 4$; Fig. 1A), while the TG mouse hearts expressed 69.4 $\pm$ 0.8% b-MHC ($n = 4$; Fig. 1A), thus providing a good model to understand the interplay between the MHC- and TnT-mediated effects on myofilament function.

Phosphorylation status of sarcomeric proteins in NTG and TG mouse hearts. The representative gel shown in Fig. 1B demonstrates that the phosphorylation levels of proteins, myosin-binding protein C, desmin, TnT, Tm, and Tni, are similar in NTG and TG mouse hearts, with one minor exception; TG mouse hearts (expressing b-MHC) showed slightly lower phosphorylation levels of myosin light chain (MLC)-1 and MLC-2. Small differences in phosphorylation levels of MLC-1 and MLC-2 are not expected to affect our findings because 1)
observed differences in contractile parameters (maximal tension, pCa50, tension cost, c, b, kHc, etc.) between α- and β-MHC fibers are similar to those reported by previous studies (10, 12, 29, 30, 49); and 2) the effect of TnTR144W is assessed relative to TnTWT in each group, i.e., α-MHC + TnTR144W vs. α-MHC + TnTWT or β-MHC + TnTR144W vs. β-MHC + TnTWT.

Western blot analysis of reconstituted muscle fibers. Western blot analysis was performed to determine the efficacy of Tn reconstitution. As we have demonstrated previously, the endogenous Tn complex is replaced as when a vast excess of exogenously added TnT competes with the endogenous Tn (3, 5, 14). Therefore, the extent of TnTWT or TnTR144W incorporation could be used as an index to determine the extent of Tn replacement in muscle fibers. The differential migration pattern of the c-myc-tagged mutant TnT, compared with the endogenous TnT, enabled us to assess the level of its incorporation. A representative Western blot showing the level of mutant TnT incorporation in α- and β-MHC fibers is shown in Fig. 1C. Densitometric analysis of TnT band profiles demonstrated that the extent of TnTWT incorporation in α-MHC fibers was 77 ± 3% (n = 3; lane 1 of Fig. 1C), while that of TnTR144W was 75 ± 1% (n = 3; lane 2 of Fig. 1C). The extent of TnTWT incorporation in β-MHC fibers was 75 ± 3% (n = 3; lane 3 of Fig. 1C), while that of TnTR144W was 73 ± 3% (n = 3; lane 4 of Fig. 1C).

Effects of α- and β-MHC isoforms on the TnTR144W-mediated impact on Ca2+-activated tension and ATPase activity. The effects of TnTR144W on Ca2+-activated maximal tension and ATPase activity (pCa 4.3) were measured in α- and β-MHC fibers. Two-way ANOVA did not reveal a significant MHC-TnT interaction effect on the steady-state maximal tension. This is because α- and β-MHC fibers reconstituted with either TnTWT or TnTR144W did not show any differences in the maximal tension (Table 1). In contrast, our analysis revealed a significant MHC-TnT interaction effect (P < 0.01) on maximal ATPase activity, indicating that the effects of TnTR144W on ATPase activity were dissimilar in the presence α- and β-MHC isoform. Post hoc multiple comparisons demonstrated that TnTR144W decreased ATPase activity by 13% (P < 0.01; Table 1) in α-MHC fibers, while it increased ATPase activity by 18% (P < 0.05; Table 1) in β-MHC fibers. Thus α- and β-MHC isoforms had contrasting effects on how TnTR144W modulated ATPase activity.

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<td>Steady-state contractile parameters</td>
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<td>1.036 ± 77</td>
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Data are presented as means ± SE. The number of fibers is as follows: 14 (from 3 hearts) for α-myosin heavy chain (MHC) + troponin T (TnTWT), 14 (from 3 hearts) for α-MHC + TnTR144W, 18 (from 3 hearts) for β-MHC + TnTWT, and 16 (from 3 hearts) for β-MHC + TnTR144W. Steady-state tension and ATPase activity were measured simultaneously (pCa 4.3), as described previously (8, 44). The magnitude of instantaneous stiffness increase caused by a sudden stretch-mediated increase in force (E₁ₑ) and the magnitude of the stiffness increase caused by the muscle length-mediated increase in the number of newly recruited force-bearing cross-bridges (E₀) were estimated using the nonlinear recruitment-distortion model (11). Statistical differences were analyzed by two-way ANOVA and the subsequent post hoc multiple comparisons (Fishers least significant difference test). *P < 0.05, †P < 0.01, statistically significant result compared with TnTWT fibers.

Table 1. Effect of TnTR144W on various contractile parameters

Effects of α- and β-MHC isoforms on the TnTR144W-mediated impact on myofilament Ca2⁺ sensitivity and cooperativity. Figure 2, A and B, shows the effect of TnTR144W on the pCa-tension relationships in α- and β-MHC fibers, respectively. It is evident from these comparisons that TnTR144W induces a rightward shift in pCa-tension relationships of both α- and β-MHC fibers, suggesting a decrease in myofilament Ca2⁺ sensitivity (pCa50). Two-way ANOVA showed no significant MHC-TnT interaction effect on pCa50, but the main effect of TnT was significant (P < 0.001). Post hoc multiple comparisons showed that TnTR144W decreased pCa50 by 0.12 pCa units (P < 0.001; Fig. 2C) in α-MHC fibers and by 0.11 pCa units (P < 0.001; Fig. 2C) in β-MHC fibers. These observations confirm that myofilament Ca2⁺ sensitivity is attenuated by TnTR144W, regardless of the MHC isoform. A cursory look at pCa-tension relations in Fig. 2, A and B, suggests that TnTR144W does not affect the steepness (nH) of pCa-tension relationships in α-MHC fibers (Fig. 2A) but decreases nH in β-MHC fibers (Fig. 2B). Two-way ANOVA showed a significant MHC-TnT interaction effect on nH. Post hoc multiple comparisons demonstrated that TnTR144W had no effect (P = 0.38; Fig. 2D) on nH in α-MHC fibers but significantly decreased nH by 17% (P < 0.05; Fig. 2D) in β-MHC fibers. These observations demonstrate that α- and β-MHC isoforms have a diverse impact on how TnTR144W modulated myofilament cooperativity.

Effects of α- and β-MHC isoforms on the TnTR144W-mediated impact on XB detachment rate. Previous studies have shown that TnTR144W slows XB detachment rate (g) in α-MHC containing muscle fibers (32, 50, 51) by affecting the structure of the thin filament. Because α- and β-MHC isoforms also have a different impact on the structure of thin filaments (10, 12, 29, 30, 49), we predicted that the TnTR144W-mediated impact on g would be differently modulated by α- and β-MHC isoforms. To assess the effects of TnTR144W on XB detachment kinetics, we used estimates of tension cost and c. Previous studies have shown that tension cost is correlated to c and that both parameters are measures of XB detachment rate, g (2). A comparison of tension-ATPase plots suggests that TnTR144W decreases tension cost in α-MHC fibers (Fig. 3A) but shows no effect in β-MHC fibers (Fig. 3B). Two-way ANOVA revealed a significant MHC-TnT interaction effect (P < 0.01), suggesting a differential impact of α- and β-MHC on how TnTR144W
altered tension cost. Post hoc analysis showed that TnTR144W significantly decreased tension cost by 16% ($P < 0.01$; Fig. 3C) in α-MHC fibers but had no effect ($P = 0.46$; Fig. 3D) in β-MHC fibers. A small but significant increase in maximal ATPase activity of TnTR144W-α-MHC fibers coincided with a slight increase in maximal tension (see Table 1), resulting in an unaltered tension cost compared with TnTWT-α-MHC fibers. The effects of TnTR144W on tension cost were also corroborated by our estimates of $c$. For example, representative force traces shown in Fig. 4, A and B, suggest that TnTR144W attenuates the rate of force decay in α-MHC fibers (notice a rightward shift) but shows no affect in β-MHC fibers. A slower rate of force decay reflects a decrease in the rate of XB detachment by TnTR144W in α-MHC fibers. Two-way ANOVA showed a significant MHC-TnT interaction effect ($P < 0.001$) on $c$. Post hoc analysis demonstrated that TnTR144W signifi-
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...cantly decreased $c$ by 24% ($P < 0.001$; Fig. 4C) in α-MHC fibers but had no effect ($P = 0.20$; Fig. 4D) in β-MHC fibers. Our observations demonstrate that the TnTR144W-mediated impact on $g$ is modulated differently by α- and β-MHC isoforms. Therefore, the interplay between the MHC- and TnTR144W-mediated effects on the thin filament alters XB detachment rate, $g$.

Effects of α- and β-MHC isoforms on the TnTR144W-mediated impact on the rate of XB recruitment. The R144W mutation lies within the strong Tm-binding region of TnT, which plays an important role in tuning XB recruitment dynamics (14). To determine whether the structural change induced by R144W affects XB recruitment dynamics, and whether such an effect is MHC-isoform dependent, we assessed two independent contractile rate parameters, $b$ and $k_{tr}$. $b$ Governs the rate of delayed force rise, following a sudden increase in ML (11), whereas $k_{tr}$ governs the rate of force rise, following a large slack-restretch maneuver (1). A comparison of force responses to a sudden 2% stretch suggests that TnTR144W attenuates $b$ in α-MHC fibers (Fig. 4A) but shows no effect in β-MHC fibers (Fig. 4B). Two-way ANOVA revealed a significant MHC-TnT interaction effect on $b$ ($P < 0.01$), suggesting a differential impact of α- and β-MHC isoforms on how TnTR144W altered $b$. Post hoc multiple comparisons confirmed that TnTR144W significantly decreased $b$ by 17% ($P < 0.001$; Fig. 5A) in α-MHC fibers but had no effect ($P = 0.70$; Fig. 5B) in β-MHC fibers. In agreement with the data on $b$, TnTR144W attenuated the rate of force redevelopment in α-MHC fibers (notice a rightward shift; Fig. 5C) but showed no effect in β-MHC fibers (Fig. 5D). Two-way ANOVA revealed a significant MHC-TnT interaction effect on $k_{tr}$ ($P < 0.001$). Post hoc multiple comparisons confirmed that TnTR144W significantly decreased $k_{tr}$ by 15% ($P < 0.001$; Fig. 5E) in α-MHC fibers but showed no effect ($P = 0.39$; Fig. 5F) in β-MHC fibers. Although $b$ and $k_{tr}$ were estimated using force responses of muscle fibers under different mechanical conditions, both represent similar underlying molecular processes that govern XB turnover rates (2). Therefore, similar observations in $b$ and $k_{tr}$ reinforce our conclusion that TnTR144W attenuates XB turnover rate in α- but not in β-MHC fibers. Here again, our data demonstrate that the TnTR144W-mediated impact on XB recruitment rate is modulated differently by α- and β-MHC isoforms.

Effects of α- and β-MHC isoforms on the TnTR144W-mediated impact on $c$. The mechanism by which strained XBs modulate recruitment of other XBs involves an effect that is transduced via the thin filament regulatory systems and is described by the NRD model parameter, $γ$ (11). Thus changes in $γ$ reflect changes in thin filament cooperativity. A larger decline in force suggests that strained XBs (sudden stretch-mediated) have a greater negative impact on thin filament cooperativity and XB recruitment that leads to a larger decline in force (more prominent nadir; see WT in Fig. 4A). Thus novel insight into how TnTR144W may alter thin filament cooperativity and how such effects are modulated differently by α- and β-MHC isoforms may be gleaned from the magnitude of $γ$. Representative force traces reveal that TnTR144W elicits a lower $γ$ in α-MHC fibers (less prominent nadir; see R144W in Fig. 4A) but shows no effect in β-MHC fibers (Fig. 4B). Two-way ANOVA revealed a significant MHC-TnT interaction effect on $γ$. Post hoc multiple comparisons confirmed that TnTR144W decreased $γ$ by 30% ($P < 0.01$; Fig. 6) in α-MHC fibers but had no effect ($P = 0.61$; Fig. 6) in β-MHC fibers. These observations demonstrate that α- and β-MHC isoforms have a different impact on how TnTR144W alters thin filament-based cooperative mechanisms.

Fig. 4. Effect of TnTR144W on $c$ in α- and β-MHC fibers. A family of step-like length perturbations was used to elicit force responses from muscle fibers, and the nonlinear recruitment-distortion (NRD) model was fitted to these force responses to estimate $c$. A: effect of TnTR144W on the force response to 2% stretch in muscle length (ML) in α-MHC fibers. B: effect of TnTR144W on the force response to 2% stretch in ML in β-MHC fibers. C: effect of TnTR144W on $c$ in α-MHC fibers. D: effect of TnTR144W on $c$ in β-MHC fibers. Statistical differences were analyzed by two-way ANOVA and subsequent post hoc multiple pairwise comparisons (Fisher’s LSD method). ***(P < 0.001, statistically significant result compared with TnTWT fibers. The number of fibers is as follows: 14 (from 3 hearts) for α-MHC + TnTWT, 14 (from 3 hearts) for α-MHC + TnTR144W, 18 (from 3 hearts) for β-MHC + TnTWT, and 16 (from 3 hearts) for β-MHC + TnTR144W. Data are presented as means ± SE. [10.1152/ajpheart.00528.2014 • www.ajpheart.org]
Effects of α- and β-MHC isoforms on the TnTR144W-mediated impact on $E_D$ and $E_R$. To investigate whether the TnTR144W-mediated effects on the magnitude of XB recruitment were differently modified by α- and β-MHC, we assessed the stiffness parameters, $E_D$ and $E_R$. We have previously demonstrated that $E_D$ is an approximate measure of the number of strongly bound XBs, while $E_R$ is an approximate measure of the number of newly recruited force-bearing XBs, following a change in ML (2, 11). Two-way ANOVA did not reveal a significant MHC-TnT interaction effect on $E_D$. This is because α- and β-MHC fibers reconstituted with either TnTWT or TnTR144W did not show any significant differences in $E_D$ (Table 1). Similar effects were also observed in $E_R$ (Table 1). Thus the TnTR144W-mediated effects on the number of force-bearing XBs were not altered differently by α- and β-MHC.

**DISCUSSION**

Because of the divergent impact of α- and β-MHC isoforms on the NH2 terminus of TnT (12, 29, 49), we predicted that the effects of the DCM mutation in TnT would be altered by the type of MHC isoform present. To our knowledge, this is the first study that addresses how the effects of the mouse TnT analog (TnTR144W) of the human DCM mutation (R141W) on cardiac contractile dynamics are differently altered by α- and β-MHC isoforms. Novel findings from our study will be discussed in terms of the underlying molecular mechanisms by which the interplay between the TnTR144W- and MHC-mediated effects on the thin filament brings about unique function.

A shift from α- to β-MHC isoform neutralizes the attenuating effect of TnTR144W on the XB turnover rate. A major finding in our study is that TnTR144W attenuates the XB turnover rate in the presence of α-MHC, but not β-MHC, as demonstrated by effects observed in two independent contractile rate parameters, $b$ (Fig. 5, A and B) and $k_{tr}$ (Fig. 5, C–F). This observation raises the question: “how does TnTR144W attenuate XB turnover rate only in the presence of α-MHC?” Such an effect of TnTR144W on XB turnover rate may be ascribed to the attenuation of 1) myofilament Ca2+ affinity, which influences the off/on kinetics of the regulatory unit (RU; Tn-Tm complex); 2) XB cycling kinetics; and 3) a combinatorial effect of 1 and 2.

Ca2+ activation of RUs and the strength of RU-RU interactions depend on the initial conditions (cooperative effects) of the thin filament from which force development occurs (39). Such initial conditions, in turn, depend on the strength of TnT-Tm interaction at the head-to-tail overlap region of contiguous Tm dimers (15, 18, 37). Two previous studies have shown that the R141W mutation enhances the binding of human TnT to α-Tm (27, 28). Thus one expectation is that a
stronger TnT-Tm interaction may hinder the TnTR144W-induced azimuthal shift of Tm on actin, leading to a decrease in the number of RUs turned on at submaximal activations. Inactive RUs, which result from the actions of TnTR141W, may make it difficult for the near-neighbor RUs to bind Ca\(^{2+}\) because of strong RU-RU interactions. However, such effects are expected to be more prominent at submaximal Ca\(^{2+}\) activation, conditions under which cooperativity is more optimally expressed (39). Just as expected, TnTR144W caused a similar decrease in \(pC_{50}\) in \(\alpha\)-MHC (Fig. 2A) and \(\beta\)-MHC (Fig. 2B) fibers, suggesting a dominant effect of TnTR144W on the off/on kinetics of RUs at submaximal activations, regardless of the MHC isoform. Because the off/on equilibrium of RUs is shifted more towards the on state at maximal activation, we expect that the negative effect of TnTR144W is minimized at pCa 4.3. This was substantiated by our observations that the Ca\(^{2+}\)-activated maximal tension and \(E_{0}\) remained unaltered whether the TnTR144W effect was tested in \(\alpha\)- or \(\beta\)-MHC fibers (Table 1).

It is well documented that full activation requires the XB feedback effect on thin filaments (16, 35). Several studies show that this effect of MHC on the thin filament is sensitive to structural changes within RUs. Coffee Castro-Zena and Root (7) reported that the myosin lever arm prefers the prepower stroke orientation when located closer to the Tn complex; however, the lever arm prefers the postpower stroke orientation when constrained distal to the Tn complex. Their data (7) are strongly indicative of the structural effect of Tn on the dynamics of XB interactions with actin. The notion that the mutant Tn may exert a modulatory role on MHC-dependent function also comes from the study of Midde et al. (31), which shows that cardiomyopathy-related mutations in Tn alter the disposition of XBs. Other studies also suggest that the DCM-related mutations in human TnT (R141W, R131W, and ΔK210) alter XB cycling kinetics, without affecting the maximal tension generating ability of the cardiac muscle (21, 32, 50). These observations, including a diverse set of data from our current study, strongly support the notion that TnTR144W alters thin filament-based allosteric mechanisms. Because the XB turnover rate is attenuated by TnTR144W only in \(\alpha\)-MHC fibers, we conclude that the TnTR144W-mediated changes in the thin filament are affected differently by \(\alpha\)- and \(\beta\)-MHC.

**Fig. 6.** Effect of TnTR144W on \(\gamma\) in \(\alpha\)- and \(\beta\)-MHC fibers. \(\gamma\) was estimated by fitting the NRD model to the family of forces responses to various amplitude step-like length perturbations (11). Statistical differences were analyzed by two-way ANOVA and subsequent post hoc multiple pairwise comparisons (Fisher’s LSD method). Also, see experimental force traces showing the effect on \(\gamma\) in Figs. 3, A and B. * * * * \(P < 0.01\), statistically significant result compared with TnTTWT fibers. The number of fibers is as follows: 14 (from 3 hearts) for \(\alpha\)-MHC + TnTTWT, 14 (from 3 hearts) for \(\alpha\)-MHC + TnTR144W, 18 (from 3 hearts) for \(\beta\)-MHC + TnTTWT, and 16 (from 3 hearts) for \(\beta\)-MHC + TnTR144W. Data are presented as means \(\pm\) SE.

A shift from \(\alpha\)- to \(\beta\)-MHC isoform neutralizes the attenuating effect of TnTR144W on the XB detachment rate. Another major finding from our study is that TnTR144W decreases the rate of XB detachment in the presence of \(\alpha\)-MHC, but not \(\beta\)-MHC, as indicated by changes in tension cost (Fig. 3, A–D) and c (Fig. 4, A–D). This evidence confirms that the interplay between the effects mediated by TnTR144W and \(\beta\)-MHC modulates the activation of thin filament such that the rate of transition of XBs from the strongly bound to the detached state is attenuated. The mechanism by which a structural modification in the CR of TnT attenuates g is at present unclear but may potentially involve a slowing effect of TnTR144W on the transition of XBs from the weakly to strongly bound states. This claim is based on our argument that a TnTR144W-induced decrease in g alone should increase force produced in \(\alpha\)-MHC fibers because, under isometric conditions, the fraction of attached XBs in a simple two-state XB model is proportional to \(f(f + g)\) (20). However, TnTR144W showed no effect on maximal tension and \(E_{D}\) of \(\alpha\)-MHC fibers. Therefore, at maximal activation, f may decrease in proportion to g, such that \(f(f + g)\) and, therefore, maximal tension and \(E_{D}\), are unaltered by TnTR144W in \(\alpha\)-MHC fibers. Such attenuation of both f and g may be brought about by a direct effect of TnT on Tm (27, 28) or an indirect effect on Tnl, Tnc, and actin (27, 28, 32).

Regardless of the mechanism, the consequence is an increase in the time XBs spend in the strongly bound state, which provides a reasonable explanation for a decrease in ATPase activity (Table 1), tension cost (Fig. 3), and c (Fig. 4) in TnTR144W + \(\alpha\)-MHC fibers. Because the TnTR144W-mediated changes in the thin filament interact differently with those induced by \(\alpha\)-MHC and \(\beta\)-MHC, we predict that XB-mediated recruitment of other XBs (XB-XB-based cooperativity) is enhanced in TnTR144W + \(\alpha\)-MHC fibers. TnTR144W-induced augmentation of November 10, 2017
β-MHC fibers (Fig. 6). This observation suggests that the rate-limiting step in the closed- to open-state transition, when XB-XB-based cooperativity is more prominent, of the thin filament in TnTR144W + β-MHC fibers is dominated by the slower kinetics of β-MHC. Thus the state of the equilibrium between nonforce- and force-bearing XBs is determined solely by the effect of β-MHC. This conjecture is substantiated by our observations that the TnTR144W-mediated effects on the thin filament attenuate XB kinetics (tension cost, $c$, $k_t$, and $b$) only in α-MHC (Figs. 3–5). Collectively, a logical conclusion that follows is that α- and β-MHC isoforms have different impacts on how TnTR144W modulates cardiac contractile function.

Relevance to pathological cardiac function and phenotype in human patients harboring R141W mutation. We demonstrate that all dynamic contractile parameters are attenuated by TnTR144W in the presence of α-MHC but not β-MHC; this demonstrates that the mutant exhibits a more severe functional phenotype when α-MHC is present. Thus a logical expectation is that the impact of its human analog, R141W mutation, would be less severe in human hearts (β-MHC) but more severe in transgenic mouse hearts (α-MHC). This assertion is supported by what is known in the current literature. For example, transgenic mouse studies of human TnT R141W mutation demonstrated increased mortality (22, 27, 52), compared with humans containing this mutation (26). One study reported that 29% of transgenic mice containing the human TnT R141W mutation died within 1 yr of age, while 100% died within 2 yr (27). Another transgenic mouse study also confirmed a 30% increase in the mortality of transgenic mice expressing the human TnT R141W mutation by the age of 7 mo (52). Collectively, these observations, including our own findings, suggest that some caution is necessary when extrapolating data from transgenic mouse studies to human hearts because the resultant phenotype depends on the type of MHC isoform present.

Our data from β-MHC fibers also suggest that the major effect of human R141W mutation would be to desensitize thin filaments to Ca$^{2+}$ in the human heart. Because cardiac muscle cells operate under a very narrow range of submaximal Ca$^{2+}$ concentrations, decreased thin filament responsiveness to Ca$^{2+}$ would attenuate tension at a given Ca$^{2+}$ level. This effect translates to an attenuation of the pressure generated by the myocardium, leading to a diminished ejection. Consequently, the stroke volume and the ejection fraction decrease significantly, leaving behind a larger than normal amount of blood in the ventricles at the end of systole. As a result, assuming no changes in the venous return, the preload increases in the next beat, which stretches the myocardium. The Frank-Starling mechanism may partly compensate for the decreased stroke volume, but it comes at a heavy cost. Under basal conditions, the heart of a DCM patient may attempt to maintain a normal stroke volume, but it does so at an elevated end-diastolic volume and an elevated end-diastolic pressure (42). Chronic mechanical stress on the myocardium, caused by a constant volume overload, may trigger cellular responses that lead to ventricular dilatation in severely affected individuals.

Limitations. A limitation of our study is that the differential impact of α- and β-MHC on the R141W mutation-mediated effect on contractile function was studied in reconstituted muscle fibers. To our knowledge, there is no study that investigated a mouse model expressing both the TnT R141W mutation and β-MHC in vivo. Despite this limitation, it is worth noting that our findings demonstrate how a TnT mutation-mediated contractile function is altered differently by disparate MHC isoforms. Interestingly, our findings also provide a basis for why humans (β-MHC) containing the TnT R141W mutation show lower mortality rates when compared with transgenic mice (α-MHC) that express TnT R141W. The assessment of cardiac function in a transgenic mouse model coexpressing both the TnT R141W mutant and β-MHC will not only complement our findings but will also provide other important details pertaining to cardiac phenotypes and whole heart mechanics.

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AUTHOR CONTRIBUTIONS
Author contributions: S.K.G. and M.C. conception and design of research; S.K.G. performed experiments; S.K.G. and M.C. analyzed data; S.K.G., J.C.T., and M.C. interpreted results of experiments; S.K.G. prepared figures; S.K.G. and M.C. drafted manuscript; S.K.G., J.C.T., and M.C. edited and revised manuscript; S.K.G., J.C.T., and M.C. approved final version of manuscript.

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