Recent clinical studies have revealed a new hypertrophic cardiomyopathy–associated mutation (F87L) in the central region of human cardiac troponin T (TnT). However, despite its implication in several incidences of sudden cardiac death in young and old adults, whether F87L is associated with cardiac contractile dysfunction is unknown. Because the central region of TnT is important for modulating the muscle length–mediated recruitment of new force-bearing cross-bridges (XBs), we hypothesize that the F87L mutation causes molecular changes that are linked to the length-dependent activation of cardiac myofilaments. Length-dependent activation is important because it contributes significantly to the Frank–Starling mechanism, which enables the heart to vary stroke volume as a function of changes in venous return. We measured steady-state and dynamic contractile parameters in detergent-skinned guinea pig cardiac muscle fibers reconstituted with recombinant guinea pig wild-type TnT (TnTWT) or the guinea pig analogue (TnTF88L) of the human mutation at two different sarcomere lengths (SLs): short (1.9 µm) and long (2.3 µm). TnTF88L increases pCa50 (-log [Ca2+]free required for half-maximal activation) to a greater extent at short SL than at long SL; for example, pCa50 increases by 0.25 pCa units at short SL and 0.17 pCa units at long SL. The greater increase in pCa50 at short SL leads to the abolishment of the SL-dependent increase in myofilament Ca2+ sensitivity (ΔpCa50) in TnTF88L fibers, ΔpCa50 being 0.10 units in TnTWT fibers but only 0.02 units in TnTF88L fibers. Furthermore, at short SL, TnTF88L attenuates the negative impact of strained XBs on force-bearing XBs and augments the magnitude of muscle length–mediated recruitment of new force-bearing XBs. Our findings suggest that the TnTF88L–mediated effects on cardiac thin filaments may lead to a negative impact on the Frank–Starling mechanism.

Cardiomyopathy mutation (F88L) in troponin T abolishes length dependency of myofilament Ca2+ sensitivity

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Introduction

Hypertrophic cardiomyopathy (HCM) mutations in the central region (CR; residues 80–180) of cardiac troponin T (TnT) are not well tolerated, perhaps because of their negative impact on thin filament cooperativity and length-dependent activation of cardiac myofilaments. Previous work from our laboratory has demonstrated that the structural integrity of CR is important for thin filament cooperativity and length-dependent activation of cardiac myofilaments (Ford et al., 2012; Gollapudi and Chandra, 2016a,b). Recent studies have suggested that impairment of length-dependent activation may be one of the primary mechanisms by which HCM mutations lead to altered cardiac phenotype and deleterious complications associated with HCM (Sequeira et al., 2013). Clinical data have recently discovered a mutation in the CR of human TnT (F87L) that is associated with HCM and high incidences of sudden cardiac death in young and old adults (Gimeno et al., 2009). However, cardiac contractile dysfunction associated with the F87L mutation in human TnT is unknown.

Mutation-mediated impairment of length-dependent activation may have significant implications for intact heart function because length-dependent activation underlies the molecular basis for the Frank–Starling mechanism, which describes the ability of the heart to regulate cardiac output in response to beat-to-beat variations in end-diastolic volume (Allen and Kentish, 1985; Plotnick et al., 1986; Holubarsch et al., 1996; Konhilas et al., 2002a; Nowak et al., 2007; Abraham et al., 2016). Indeed, impairment of length-dependent activation has been observed in several cases of cardiomyopathies (van Dijk et al., 2012) and failing human hearts (Schwinger et al., 1994; Sequeira et al., 2013). At the myofilament level, cardiac muscle exhibits pronounced length-dependent activation, whereby an increase in sarcomere length (SL) augments myofilament Ca2+ sensitivity (Allen and
Korte and McDonald, 2007; Ford et al., 2012). Steady-state and SL-dependent changes in dynamic contractile parameters cle fibers expressing β-MHC have been shown to exhibit steeper on the type of MHC isoform (Ford et al., 2012). In addition, mus-
TnT-mediated changes on length-dependent activation depends not in α-MHC–expressing fibers, suggesting that the effect of mutations in the CR because the effects of CR on con-
tractile function are modulated differently by α- and β-MHC isoforms (Ford et al., 2012; Gollapudi et al., 2016a,b). For example, a previous study has demonstrated that the R92L mutation in the CR of mouse TnT enhances myofilament Ca2+ sensitivity but abolishes the SL-mediated increase in myofilament Ca2+ sens-
sitivity (Ford et al., 2012). Because thin filament cooperativity and muscle length (ML)-mediated XB recruitment dynamics are significantly modulated by the CR of TnT (Gollapudi et al., 2013), HCM-associated mutations in the CR are expected to have a neg-
ative impact on length-dependent activation.

To investigate the effect of F87L on contractile function and whether such effects varied in an SL-dependent manner, recombinant guinea pig wild-type TnT (TnTWT) and mutant TnT (TnTF88L) were generated and reconstituted into detergent-skinned papillary muscle fibers isolated from guinea pig left ventricles. Guinea pig cardiac tissue was preferred because guinea pigs, like humans (Narolska et al., 2005a,b), predomi-
nantly express the β-myosin heavy chain (β-MHC) isoform in the myocardium (van der Velden et al., 1998). MHC isoform becomes an important aspect to consider when assessing the effect of mutations in the CR because the effects of CR on con-
tactile function are modulated differently by α- and β-MHC isoforms (Ford et al., 2012; Gollapudi and Chandra, 2016a,b). For example, R92L in TnT abolishes the length-dependent increase in myofilament Ca2+ sensitivity in β-MHC-expressing fibers, but not in α-MHC-expressing fibers, suggesting that the effect of TnT-mediated changes on length-dependent activation depends on the type of MHC isoform (Ford et al., 2012). In addition, muscle fibers expressing β-MHC have been shown to exhibit steeper SL-dependent changes in dynamic contractile parameters (Korte and McDonald, 2007; Ford et al., 2012). Steady-state and dynamic contractile parameters were measured in TnTWT and TnTF88L muscle fibers at two different SLs (1.9 μm and 2.3 μm). Our results demonstrated that TnTF88L abolished the SL-medi-
ated increase in cardiac myofilament Ca2+ sensitivity. Moreover, the magnitude of ML-mediated recruitment of XBs (ϕ) and the negative impact of strained XBs on recruitment of force-bearing XBs (γ) were altered in an SL-dependent manner.

Materials and methods
Animal protocols
8–10-mo-old male Dunkin-Hartley guinea pigs (Cavia porcellus) acquired from Charles River were used in this study. All animals were housed in environmentally controlled rooms of an Asso-
ciation for Assessment and Accreditation of Laboratory Animal Care–accredited facility under 12-h light and dark cycles. All animals received proper care and treatment in accordance with the preapproved protocols by Washington State University Insti-
tutional Animal Care and Use Committee. The procedures for euthanizing guinea pigs conformed to the recommendations of the American Veterinary Medical Association, as outlined in the Guidelines for the Euthanasia of Animals.

Expression and purification of recombinant guinea pig cardiac troponin subunits
Recombinant c-myc-tagged guinea pig TnT (TnTWT and TnTF88L), guinea pig WT troponin I (TnI), and guinea pig WT troponin C (TnC) were generated and cloned into a pSBETa vector (GenScript USA). The inclusion of the c-myc tag has been previously shown to have no effect on contractile function (Tardiff et al., 1998; Montgomery et al., 2001; Chandra et al., 2005). Recombinant DNA was transformed and expressed in BL21®DE3 cells (Invitro-
gen). In brief, TnTWT and TnTF88L were purified by ion-exchange chromatography on a DEAE-Fast Sepharose column (GE Health-
care Biosciences). TnI was purified using a CM Sepharose ion-ex-
change column, and TnC was purified using a DE-52 column and phenyl Sepharose column. Details on protein purification can be found in the Supplemental materials and methods.

Reconstitution of recombinant troponin complexes in detergent-skinned guinea pig cardiac muscle fibers
Left ventricular guinea pig papillary muscle fibers were pre-
pared and detergent skinned, as described in the Supplemental materials and methods. Reconstitution of recombinant troponin into cardiac muscle fibers was done as previously described (Chandra et al., 1999; Gollapudi et al., 2012; Mamidi et al., 2013). Recombinant TnTWT or TnTF88L (0.9 mg/ml, wt/vol) and TnI (1.0 mg/ml, wt/vol) were dissolved in an exchange buffer containing the following: 50 mM Tris-HCl, 6 M urea, and 1.0 mM KCl, pH 8.0, at 4°C (buffer 1). To remove high salt and urea, proteins were successively dia lyzed against 50 mM Tris-HCl, 4 M urea, and 0.7 M KCl, pH 8.0, at 4°C (buffer 2); 50 mM Tris-HCl, 2 M urea, and 0.5 M KCl, pH 8.0, at 4°C (buffer 3); and 50 mM N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 200 mM KCl, 10 mM 2,3-butanedione monoxime (BDM), 6.27 mM MgCl2, and 5 mM EGTA, pH 7.0, at 22°C (buffer 4). All buffers included a cocktail of protease inhibitors (0.2 mM PMSF, 2 mM benzami-
dine-HCl, 1 mM dithiothreitol (DTT), and 0.01% sodium azide). Any undissolved protein in the exchange buffer was removed by centrifugation at 3,000 rpm for 15 min. Detergent-skinned fiber bundles were treated with the exchange buffer containing TnTWT + TnI or TnTF88L + TnI for ~3 h at room temperature (22°C) with gentle stirring. Muscle fiber bundles were then washed twice (10 min each) with buffer 4 to remove remnants of exchange buffer, followed by overnight incubation at 4°C in high-relaxing solution containing TnC (3.0 mg/ml, wt/vol).

Measurement of isometric steady-state tension and ATPase activity
Steady-state isometric tension was measured in muscle fibers at various pCa values (∼log10 of [Ca2+]free), as previously described
The force-bearing state at the new ML (b); and the magnitude force-bearing XBs (γ); the rate by which XBs are recruited into term representing the negative effect of strained XBs on other force-bearing XBs; b, the rate of delayed force rise after (phase 2); γ, a nonlinear interaction term representing the negative impact of strained XBs on other force-bearing XBs; b, the rate by which force drops because of dissipation of strain within bound XBs (c); a nonlinear interaction term representing the negative effect of strained XBs on other force-bearing XBs; b, the rate of delayed force rise after (phase 2). Figure 1. Representative force response to a sudden 2% stretch. (A) A representative 2% sudden stretch in ML imposed on a TnTWT muscle fiber at maximal Ca$^{2+}$ activation (pCa 4.3). (B) The corresponding force response normalized to the isometric steady-state force (Fss) before ML change. The NLRD model was fitted to the family of force responses to steplike changes in ML (±0.5, ±1.0, ±1.5, and ±2.0% of initial ML) to estimate various model parameters (Ford et al., 2010). The different phases (dashed lines) from which the respective parameters were estimated are highlighted. F0, the instantaneous increase in force caused by sudden increase in ML (phase 1); c, the rate by which force decays to a minimum point after a sudden stretch in ML (phase 2); γ, a nonlinear interaction term representing the negative impact of strained XBs on other force-bearing XBs; b, the rate of delayed force rise after an increase in ML (phase 3); Fnew, the new steady-state force corresponding to an increase in ML.

Measurement of muscle fiber mechanodynamics
Upon attainment of maximal steady-state activation, the motor arm was commanded to elicit various amplitude stretch/release perturbations (±0.5, ±1.0, ±1.5, and ±2.0% of the initial ML) to the attached muscle fibers (Ford et al., 2010). Each length perturbation was maintained for 5 s, and the corresponding force responses were recorded to highlight three different phases. As described previously (Ford et al., 2010), a nonlinear recruitment-distortion (NLRD) model was fitted to the force response phases to estimate five model parameters: the magnitude of the instantaneous increase in force caused by a sudden increase in ML (E0); the rate by which force drops because of dissipation of strain within bound XBs (c); a nonlinear interaction term representing the negative effect of strained XBs on other force-bearing XBs (γ); the rate by which XBs are recruited into the force-bearing state at the new ML (b); and the magnitude of increase in steady-state force caused by recruitment of additional force-bearing XBs at the increased ML (E0). Fig. 1 depicts the length protocol of 2% sudden stretch (Fig. 1 A) and the corresponding force response (Fig. 1 B) from a muscle fiber. Details on the physiological significance of each model parameter can be found in the Supplemental materials and methods.

Rate constant of tension redevelopment
The rate constant of tension redevelopment ($k_{tr}$) was estimated using a modified version of the large slack/restretch maneuver originally designed by Brenner and Eisenberg (1986) and is described in the Supplemental materials and methods.

Statistical analysis
Our experimental model investigated the effects of two factors, TnT (TnTWT and TnTF88L) and SL (1.9 and 2.3 µm). Thus, two-way ANOVA was constructed to analyze the effect of TnT on each contractile parameter at a given SL. A significant TnT–SL interaction effect suggested that the effect of TnTF88L on a given parameter...
was dissimilar at different SLs. When the interaction was not significant, we assessed the main effect of TnT at different SLs. To determine the source of significant interaction or main effect, post hoc t tests using Fisher’s least significant difference (LSD) method were analyzed. The criterion for statistical significance was set to $P < 0.05$. Data are presented as mean ± SEM.

Online supplemental material
Details regarding expression and purification of recombinant guinea pig cardiac troponin subunits, preparation of guinea pig cardiac muscle fibers, Western blot analysis of reconstituted muscle fiber samples, composition of pCa solutions, NLRD model parameters, and measurement of $k_v$ can be found in the supplemental materials and methods. Western blot analysis of reconstituted muscle fibers (Fig. S1) and contractile dynamic data regarding the effect of TnT$_{F88L}$ at submaximal activation (Table S1) are also included.

Results
Western blot analysis of recombinant TnT incorporation in guinea pig cardiac muscle fibers
To assess the level of recombinant TnT incorporation, reconstituted TnT$_{WT}$ or TnT$_{F88L}$ muscle fibers were solubilized in 2.5% SDS solution and run on 8% SDS gel to separate endogenous and recombinant (c-myc–tagged) TnT, as described in Supplemental materials and methods. The inclusion of the c-myc tag at the N terminus allowed us to separate recombinant TnT (TnT$_{WT}$ or TnT$_{F88L}$) from the endogenous TnT via different migration on SDS gel. We and others have previously shown that the c-myc epitope at the N terminus of TnT does not affect cardiac function (Tardiff et al., 1998; Montgomery et al., 2001; Chandra et al., 2005). To quantify the level of incorporation of recombinant TnT (i.e., the amount of total endogenous TnT that was replaced by recombinant TnT) in reconstituted muscle fibers, we used Western blot analysis. Densitometric analysis of the band profiles from the Western blot revealed that the incorporation level of c-myc–tagged TnT$_{WT}$ was 77 ± 6%, and that of c-myc–tagged TnT$_{F88L}$ was 72 ± 7% (Fig. S1). Values reported as mean ± SD; $n = 3$.

Table 1. Effect of TnT$_{F88L}$ on various contractile parameters at short and long SLs

| Parameter | 1.9 µm | 2.3 µm |
|-----------|--------|--------|
| Maximal tension (mN · mm$^{-2}$) | TnT$_{WT}$ | TnT$_{F88L}$ | TnT$_{WT}$ | TnT$_{F88L}$ |
| 29.72 ± 1.25 | 31.63 ± 1.52 | 49.07 ± 1.24 | 48.25 ± 0.93 |
| Tension cost (pmol · mN$^{-1}$ · mm$^{-1}$ · s$^{-1}$) | 2.56 ± 0.17 | 2.34 ± 0.11 | 1.26 ± 0.06 | 1.32 ± 0.07 |
| c (s$^{-1}$) | 12.17 ± 0.42 | 10.98 ± 0.89 | 7.95 ± 0.13 | 8.10 ± 0.38 |
| b (s$^{-1}$) | 4.32 ± 0.09 | 4.19 ± 0.09 | 4.33 ± 0.11 | 4.37 ± 0.09 |
| $k_v$ (s$^{-1}$) | 1.99 ± 0.05 | 1.83 ± 0.11 | 1.61 ± 0.10 | 1.57 ± 0.05 |

Maximal tension was measured by exposing muscle fibers to saturating Ca$^{2+}$ concentration (pCa 4.3) in a constantly stirred chamber. Tension cost was derived from the ATPase/tension relationship, as previously described (de Tombe and Stienen, 1995; Chandra et al., 2015). Parameters c and b were estimated by fitting the NLRD model to the force response phases to various amplitude stretch/release perturbations (Fig. 1; Ford et al., 2010). $c$ represents the rate of force decay to a minimum force point following a sudden stretch in ML, and b represents the rate at which force rises at the new ML. $k_v$ was estimated by fitting a monoexponential function to the rising phase of the force response to a large slack-restretch length maneuver and represents the rate of tension redevelopment (Brenner and Eisenberg, 1986). Estimates from several muscle fibers per group were averaged and presented as mean ± SEM. Statistical differences were analyzed using two-way ANOVA and subsequent post hoc multiple pairwise comparisons (Fisher’s LSD method). The number of fibers measured (from three hearts) for all groups was 10.

Table 1. Effect of TnT$_{F88L}$ on various contractile parameters at short and long SLs

Effect of TnT$_{F88L}$ on Ca$^{2+}$-activated tension and $E_D$ at short and long SLs
To determine whether TnT$_{F88L}$ altered maximal tension and whether this effect varied in an SL-dependent manner, we assessed steady-state tension at maximal Ca$^{2+}$ activation at both short and long SLs. Two-way ANOVA did not show a significant TnT–SL interaction effect ($P = 0.28$) or a significant TnT main effect ($P = 0.66$) on maximal tension (Table 1). To corroborate our findings on maximal tension, we assessed $E_D$. Previous studies have shown that $E_D$ is strongly correlated to maximal tension and is therefore an index of the number of strongly bound XBs (Campbell et al., 2004; Ford et al., 2010). Similar to our findings on maximal tension, two-way ANOVA did not show a significant TnT–SL interaction effect ($P = 0.22$) or a significant TnT main effect ($P = 0.74$) on $E_D$. At short SL, $E_D$ was 693 mN/mm$^2$ for TnT$_{WT}$ fibers and 690 mN/mm$^2$ for TnT$_{F88L}$ fibers. At long SL, $E_D$ was 897 mN/mm$^2$ for TnT$_{WT}$ fibers and 867 mN/mm$^2$ for TnT$_{F88L}$ fibers. Observed changes in maximal tension and $E_D$ suggest that TnT$_{F88L}$ does not alter maximal activation.

Although TnT$_{F88L}$ did not alter maximal activation, we observed a substantial effect on tension at submaximal activations (pCa 5.6). At short SL, tension at pCa 5.6 was 4.59 mN/mm$^2$ for TnT$_{WT}$ fibers and 47.33 mN/mm$^2$ for TnT$_{F88L}$ fibers. At long SL, tension at pCa 5.6 was 14.02 mN/mm$^2$ for TnT$_{WT}$ fibers and 27.16 mN/mm$^2$ for TnT$_{F88L}$ fibers. Two-way ANOVA of tension at pCa 5.6 did not show a significant interaction effect ($P = 0.85$), but the main effect of TnT was significant ($P < 0.001$). TnT$_{F88L}$ increased tension at pCa 5.6 to a greater extent at short SL (278%, $P < 0.001$) than at long SL (93%; $P < 0.001$). Because of this greater increase in tension at short SL, the SL-dependent increase in tension at pCa 5.6 was ∼3.6-fold less in TnT$_{F88L}$ fibers compared with TnT$_{WT}$ fibers. The SL-mediated increase in tension at pCa 5.6 was 207%
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F88L in TnT attenuates length-dependent activation

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in TnTWT fibers (P < 0.001), whereas it was only 57% in TnT_F88L fibers (P < 0.001). Our data on submaximal tension demonstrates that the SL-mediated increase in force is significantly attenuated by TnT_F88L, suggesting that TnT_F88L may blunt ML-mediated increase in force production in heart muscle, which normally operates under submaximal activating conditions.

Effect of TnT_F88L on pCa50 and nH at short and long SLS

A comparison of pCa–tension relationships shows a leftward shift in the pCa–tension relationship of TnT_F88L muscle fibers at both short SL (Fig. 2 A) and long SL (Fig. 2 B). A cursory look indicates that the magnitude of leftward shift in pCa–tension relationship is greater at short than at long SL, suggesting that TnT_F88L increases myofilament Ca2+ sensitivity to a greater extent at short SL than at long SL. To quantify the magnitude of TnT_F88L-mediated effect on pCa–tension relationship, we used Hill model–derived parameters, pCa50 and nH. Two-way ANOVA revealed a significant TnT–SL interaction effect (P = 0.003) on pCa50, suggesting that the effect of TnT_F88L on pCa50 is dissimilar at different SLS. Post hoc t tests indicated that TnT_F88L significantly increased pCa50 to a greater extent at short SL (0.25 units; P < 0.001; Fig. 2 C) than at long SL (0.17 units; P < 0.001; Fig. 2 C). This differential effect on pCa50 impacted the SL dependence of pCa50 in TnT_F88L muscle fibers, such that pCa50 was not different between short and long SL in TnT_F88L muscle fibers. Although increasing SL from 1.9 to 2.3 µm significantly increased pCa50 by 0.10 units in TnTWT fibers (P < 0.001; Fig. 2 C), it did not significantly increase pCa50 in TnT_F88L fibers (P = 0.22; Fig. 2 C), indicating that TnT_F88L abolished the length-mediated increase in Ca2+ sensitivity, a major mechanism underlying cardiac length-dependent activation. With regard to nH, two-way ANOVA did not reveal a significant TnT–SL interaction effect on nH (P = 0.84); however, the main effect of TnT was significant (P < 0.001). Post hoc multiple pairwise comparisons (Fisher’s LSD) were used to determine significant differences between groups. Asterisks indicate significant difference from TnTWT at a given SL (*, P < 0.05; **, P < 0.01; ***, P < 0.001). The numbers of fibers measured (from three hearts) for TnTWT and TnT_F88L at short SL were 10 and 10, and those at long SL were 9 and 10, respectively.

Effect of TnT_F88L on the rate of XB detachment at short and long SLS

Previous studies have shown that mutations in TnT may alter the rate of XB detachment, g (Chandra et al., 2015; Gollapudi and

Figure 2. Effect of TnT_F88L on pCa–tension relationship at short and long SLS. (A and B) A comparison of pCa–tension relationship between TnTWT and TnT_F88L muscle fibers at short SL (A) and long SL (B). Steady-state tensions at various pCa values were normalized to the corresponding value at pCa 4.3 and plotted against pCa to derive pCa–tension data. Normalized pCa–tension data measured from several muscle fibers per group were averaged and presented as mean ± SEM. Bar graphs showing the effect of TnT_F88L on myofilament Ca2+ sensitivity (C; pCa50) and cooperativity (D; nH) at short and long SLS. Normalized pCa–tension data from each muscle fiber was individually fitted to Hill’s model to derive pCa50 and nH. Estimates from several fibers per group were averaged and presented as mean ± SEM. Two-way ANOVA revealed a significant TnT–SL interaction effect (P = 0.003) on pCa50. Two-way ANOVA did not show a significant TnT–SL interaction effect on nH (P = 0.84); however, the main effect of TnT was significant (P < 0.001). Post hoc multiple pairwise comparisons (Fisher’s LSD) were used to determine significant differences between groups. Asterisks indicate significant difference from TnTWT at a given SL (*, P < 0.05; **, P < 0.01; ***, P < 0.001). The numbers of fibers measured (from three hearts) for TnTWT and TnT_F88L at short SL were 10 and 10, and those at long SL were 9 and 10, respectively.
findings on c and tension cost strongly suggest that TnT F88L attenuates length-dependent activation of TnT (P = 0.65; Table 1). Two-way ANOVA of \( k_{tr} \) did not show a significant TnT–SL interaction effect (P = 0.49) or a significant main effect of TnT (P = 0.24; Table 1). Collectively, our observations suggest that TnT F88L does not alter XB turnover rate.

Effect of TnTF88L on \( \gamma \) at short and long SLs

In our NLRD model, parameter \( \gamma \) represents the nonlinear interaction term, which is formulated as an effect by which the distortion of bound XBs influences the recruitment of other XBs. \( \gamma \) is estimated by fitting the NLRD model to a family of force responses to various amplitude length perturbations (Ford et al., 2010). From a physiological standpoint, \( \gamma \) is an indicator of the negative effect that strained XBs have on other force-bearing XBs. For example, when the negative impact of strained XBs on the state of other force-bearing XBs is less pronounced, the magnitude of force decline brought about by detachment of strained XBs is lesser (less prominent nadir), and thus \( \gamma \) is lower. Because such XB-based interactions are mediated by cooperative interactions within the thick and thin filaments, \( \gamma \) is thought to be influenced by allosteric/cooperative mechanisms in the myofilaments. A comparison of force responses to 2% sudden stretch shows that TnTF88L induces a less pronounced force decline at short SL but has no effect at long SL (Fig. 4, A and B). Two-way ANOVA of \( \gamma \) showed a marginally significant TnT–SL interaction effect (P = 0.051), suggesting that TnTF88L altered \( \gamma \) differently at short and long SLs. Post hoc analysis showed that TnTF88L significantly decreased \( \gamma \) at short SL (16%; P = 0.015; Fig. 4 C); however, TnTF88L had no effect at long SL (P = 0.77; Fig. 4 C) when compared with TnT WT. Differential effects on \( \gamma \) suggest that the TnTF88L-mediated impact on thin filament–based allosteric/cooperative processes—which mediate XB-based cooperativity—is different at short and long SLs. Such differential effects attenuate the SL dependence of \( \gamma \) in TnTF88L fibers; this is demonstrated by the observation that when SL was increased from 1.9 to 2.3 \( \mu \)m, \( \gamma \) decreased by 36% (P < 0.001; Fig. 4 C) in TnT WT fibers but decreased by only 21% (P = 0.006; Fig. 4 C) in TnTF88L fibers, suggesting that SL-dependent effects on cooperative mechanisms that govern \( \gamma \) are attenuated by TnTF88L. We also assessed \( \gamma \) at comparable levels of force generation in TnT WT and TnTF88L fibers during submaximal activations. Our data at submaximal activations corroborate our findings at maximal activation; TnTF88L significantly decreased \( \gamma \) only at short SL (Table S1).

Effect of TnTF88L on the magnitude of ML-mediated XB recruitment at short and long SLs

To determine the TnTF88L-mediated effect on the magnitude of ML-mediated XB recruitment, we assessed estimates of \( E_R \) at maximal Ca2+ activation (pCa 4.3). \( E_R \) represents the magnitude of delayed force rise in response to an increase in ML, an effect that is mediated by XB-based cooperative mechanisms (Campbell et al., 2004; Campbell and Chandra, 2006; Stelzer et al., 2006). \( E_R \) is estimated by fitting the NLRD model to a family of force responses to various amplitude length perturbations (Ford et al., 2010). Two-way ANOVA revealed a significant TnT–SL interaction effect on \( E_R \) (P = 0.031), suggesting that the effect of TnTF88L on \( E_R \) was different at short and long SLs. Post hoc analysis confirmed that TnTF88L increased \( E_R \) by 39% at short SL (P = 0.043;
Discussion

Data presented in this study provide novel insight regarding the effect of HCM-linked TnTF88L on contractile function and myofilament length-dependent activation. The major finding in our study is that the effect of TnTF88L mutation varies in an SL-dependent manner. Notably, TnTF88L increases pCa50 to a greater extent at short SL than at long SLs (Fig. 3), indicating an impact on mechanisms governing cardiac length-dependent activation. Given that Figs. 4 and 5 but had no effect on E_R at long SL (P = 0.25; Fig. 5), indicating that cooperative mechanisms governing E_R were augmented at short SL. This dissimilar effect on E_R at short and long SLs impacted the SL-mediated increase in E_R; for example, post hoc analysis showed that increasing SL increased E_R by 207% (P < 0.001; Fig. 5) in TnTWT fibers, but by only 105% (P < 0.001; Fig. 5) in TnTF88L fibers. Such an observation suggests that TnTF88L attenuates SL-dependent effects on cooperative mechanisms underlying E_R. Here again, our data at submaximal activations corroborate our findings at maximal activation; TnTF88L significantly increased E_R only at short SL (Table S1).

Fig. 5. Effect of TnTF88L on E_R at short and long SLs. E_R corresponds to the ML-mediated increase in steady-state force and represents the magnitude of ML-mediated XB recruitment. E_R is estimated as the slope of the linear relationship between (F_{nss}−F_{ss}) and ML changes, ΔL (see Figs. 1 and 4, A and B). Estimates from several muscle fibers per group were averaged and presented as mean ± SEM. Two-way ANOVA revealed a marginally significant TnT–SL interaction effect (P = 0.051) on E_R. Post hoc multiple pairwise comparisons (Fisher’s LSD) were used to determine significant differences between groups. Asterisks indicate significant difference from TnTWT at a given SL (*, P < 0.05). The numbers of fibers measured (from three hearts) for TnTWT and TnTF88L at short SL were 9 and 10, and those at long SL were 10 and 10, respectively.
length-dependent activation is a significant contributor to the Frank–Starling mechanism and that attenuated Frank–Starling mechanism is a hallmark of failing human hearts (Schwinger et al., 1994; van Dijk et al., 2012; Sequeira et al., 2013), our findings have important implications regarding cardiac dysfunction in patients harboring F87L.

Our observation that TnTF88L increases myofilament Ca\(^{2+}\) sensitivity to a greater extent at short SL than at long SL raises two important questions: (1) how does TnTF88L augment thin filament Ca\(^{2+}\) sensitivity, and (2) why is such an effect greater at short SL than at long SL? To address these questions, we must first consider mechanisms by which mutations in the CR of TnT may augment intrinsic thin filament responsiveness to Ca\(^{2+}\). TnTF88L is located in the CR, a region of TnT involved in allosteric/cooperative interactions that regulate Ca\(^{2+}\)-mediated activation of thin filaments. Therefore, structural perturbations caused by mutations in the CR may affect processes regulating thin filament activation. In this regard, several lines of evidence show that mutations in the CR alter Ca\(^{2+}\) sensitivity of Tn by modifying allosteric interactions within the thin filament (Liu et al., 2012; Sommese et al., 2013; Williams et al., 2016); for example, I79N and E163K mutations in TnT increase Ca\(^{2+}\) affinity for Tn in reconstituted thin filament preparations containing actin:Tm:Tn (Sommese et al., 2013). However, I79N and E163K do not alter Ca\(^{2+}\) affinity for Tn in vitro preparations containing TnT + TnI + TnC, suggesting two important points: (1) mutation-mediated effect on thin filament Ca\(^{2+}\) sensitivity is mediated by cooperative interactions within the thin filament, and (2) mechanisms other than a direct effect of the mutant on Tn Ca\(^{2+}\) sensitivity may be involved in altering thin filament Ca\(^{2+}\) sensitivity. Further support for this notion is provided by the observation that, although both R92L and R92W mutations in CR increase myofilament Ca\(^{2+}\) sensitivity in muscle fibers (Ford et al., 2012), the rate of Ca\(^{2+}\) dissociation from the isolated TnT + TnI + TnC complex is decreased by R92L but increased by R92W (Williams et al., 2016), suggesting that proper manifestation of the effect of CR mutations on myofilament Ca\(^{2+}\) sensitivity requires participation of allosteric/cooperative processes within the thin filament.

Structural changes in the CR of TnT may alter thin filament Ca\(^{2+}\) sensitivity by altering cooperative processes that affect the equilibrium between off/on states of regulatory unit (RU; Tn-Tm) located on actin filaments. CR not only promotes the binding of Tm to actin, but also aids in the assembly of Tm on the actin filament by promoting head-to-tail interactions between two contiguous Tms (Jackson et al., 1975; Heeley et al., 1987; Lehrer and Geeves, 1998; Palm et al., 2001). Therefore, CR plays a key role in mediating cooperative interactions between two neighboring RUs, which defines RU–RU cooperativity (Razumova et al., 2000). Thus, it is not surprising that mutations in CR modify RU–RU cooperativity by altering the coupling between CR and Tm (Palm et al., 2001; Hinkle and Tobacman, 2003). Because RU–RU cooperativity has the greatest effect on nRU (Razumova et al., 2000), significant attenuation of nRU is suggestive of a decrease in RU–RU cooperativity, which is expected to increase myofilament Ca\(^{2+}\) sensitivity (Razumova et al., 2000). To elaborate, at lower levels of Ca\(^{2+}\), when the majority of RUs are in the off state, cooperativity between two neighboring RUs tends to stabilize RUs in the off state. A consequence of this effect is that it requires more Ca\(^{2+}\) to activate RUs, which results in decreased Ca\(^{2+}\) sensitivity. When cooperative stabilization between neighboring RUs is decreased by TnTF88L, it decreases the threshold for Ca\(^{2+}\) to activate RUs, leading to increased Ca\(^{2+}\) sensitivity of thin filaments. Such attenuation of RU–RU cooperativity may result from the weakening of TnT–Tm interactions (Gangadharan et al., 2017) and/or an effect on Tm–Tm overlap junction; in theory, this would lower the effective stiffness of the Tm chain and decrease cooperative communication between RUs. This line of reasoning is consistent with previous findings that have suggested that one primary mechanism by which HCM-linked thin filament mutations enhance myofilament Ca\(^{2+}\) sensitivity is through attenuation of RU–RU cooperativity (Palm et al., 2001; Hinkle and Tobacman, 2003). Thus, a substantial increase in Ca\(^{2+}\) sensitivity in TnTF88L fibers may be attributed to alterations in thin filament allosteric/cooperative mechanisms brought about by structural changes in RU.

Although the above explanation provides a molecular basis for the TnTF88L-mediated effect on thin filament Ca\(^{2+}\) sensitivity, it does not explain the differential effects observed at short and long SLs. Differential effects on tension at short and long SLs during submaximal activation may help explain the lack of SL-dependent effect on myofilament Ca\(^{2+}\) sensitivity in TnTF88L fibers. Notably, at pCa 5.6, TnTF88L augments tension by 278% at short SL and by 93% at long SL, demonstrating that the ability of TnTF88L to increase the number of force-bearing XBs at submaximal activations is substantially greater at short SL. A consequence of such an increase in the number of strongly bound XBs at submaximal activation is that TnTF88L enhances XB-based cooperative mechanisms (XB–XB and XB–RU) more at short SL. Therefore, we posit that TnTF88L enhances XB-based cooperative feedback effect on thin filament to a greater degree at short SL. Because an increase in XB-based cooperativity is important for the SL-dependent increase in myofilament Ca\(^{2+}\) sensitivity (Allen and Kentish, 1985; Wang and Fuchs, 1994; Fitzsimons and Moss, 1998; Konhilas et al., 2002b; Smith et al., 2009), the lack of an increase in Ca\(^{2+}\) sensitivity between short and long SL suggests that XB-based cooperative mechanisms are saturated at short SL in TnTF88L fibers. A consequence of proper manifestation of XB-based cooperativity is that the SL-mediated increase in tension (that is, an increase in tension associated with an increase in SL from 1.9 to 2.3 µm) is more pronounced at lower Ca\(^{2+}\) concentrations (Campbell, 1997; Razumova et al., 2000). Indeed, the SL-dependent increase in tension in TnTF88L fibers is more pronounced at lower Ca\(^{2+}\) activations because the SL-dependent increase in XB-based cooperativity is normal in TnTF88L fibers (Fig. 3). However, this SL-mediated increase in tension is absent in TnTF88L fibers at all Ca\(^{2+}\)-concentrations tested, suggesting that XB-based cooperative mechanisms are saturated at short SL in TnTF88L fibers. To clarify, the TnTF88L-mediated increase in XB-based cooperativity, combined with an increase in RU activation, causes a depletion in the number of RUs and XBs available for cooperative recruitment. Consequently, it limits the number of RUs and XBs that can be effectively engaged to enhance XB-based cooperative mechanisms as SL increases.

Additional evidence for increased XB-based cooperativity at short SL and disruption of SL-mediated effects on XB-based
cooperativity may be gleaned from our observations on two other contractile dynamic parameters, \( \gamma \) and \( E_g \). Parameter \( \gamma \) represents thin filament–based regulatory mechanisms by which strained XBs negatively impact other force-bearing XBs (Ford et al., 2010). A significant decrease in \( \gamma \) at short SL in TnT\textsubscript{F87L} fibers suggests that the negative effect of strained XBs on force-bearing XBs is attenuated, a likely consequence of greater expression of XB-based cooperativity. We posit that increased XB-based cooperativity counters the negative impact of strained XBs on force-bearing XBs, leading to a decrease in \( \gamma \) at short SL. The augmenting effect of TnT\textsubscript{F87L} on XB-based cooperativity at short SL is also supported by the magnitude of ML-mediated XB recruitment, \( E_g \), which is sensitive to changes in XB-based cooperativity (Campbell et al., 2004; Campbell and Chandra, 2006; Stelzer et al., 2006; Gollapudi et al., 2017). TnT\textsubscript{F87L} significantly increases \( E_g \) at short SL (59%) but not at long SL, suggesting that TnT\textsubscript{F87L} imparts a greater effect on XB-based cooperativity at short SL. Just as it did in tension experiments, this saturation of XB-based cooperativity at short SL blunted the SL-mediated effects on \( \gamma \) (Fig. 4) and \( E_g \) (Fig. 5) in TnT\textsubscript{F87L} fibers.

With respect to XB-based feedback effects (XB–XB and XB–RU cooperativity) and changes in Ca\textsuperscript{2+} sensitivity, the consequence of augmented XB–RU cooperativity is of importance, because changes in XB–RU cooperativity are most prominently reflected by changes in Ca\textsuperscript{2+} sensitivity (Razumova et al., 2000). In this context, a consequence of increased XB–RU cooperativity at short SL is that it engages more RUs in XB–RU population, causing the depletion of RU pool and limiting the scope of XB–RU population to increase as SL increases. Therefore, lack of SL-mediated increase in XB–RU cooperativity in TnT\textsubscript{F87L} fibers would explain why an increase in SL results in no further increase in myofilament Ca\textsuperscript{2+} sensitivity (Fig. 2 C). Although we cannot definitively distinguish between the two types of XB-based cooperativity, findings from previous modeling simulations (Razumova et al., 2000) — in conjunction with data from our study — provide clues about the effect of TnT\textsubscript{F87L} on XB-based cooperativity. Previous modeling studies have suggested that changes in XB–XB cooperativity have an impact on maximal tension, \( k_m \), and \( pC_{a,0} \) such that an increase in XB–XB cooperativity augments maximal tension, slows \( k_m \), and decreases \( pC_{a,0} \) (and vice versa; Razumova et al., 2000). In this context, our observation that TnT\textsubscript{F87L} does not alter maximal tension and \( k_m \), and significantly increases \( pC_{a,0} \), allows us to speculate that TnT\textsubscript{F87L} may have little to no impact on XB–XB cooperativity.

Conclusion

TnT\textsubscript{F87L} increases myofilament Ca\textsuperscript{2+} sensitivity at both short and long SLs; however, the effect is disproportionately greater at short SL, which results in abolishment of length–dependent activation. Under submaximal activating conditions, increased myofilament Ca\textsuperscript{2+} sensitivity, combined with an increase in \( E_g \), may increase ventricular force output during systole and prolong systolic ejection time (Davis et al., 2001; Stelzer et al., 2006; Stelzer and Moss, 2006), leading to a delay in ventricular relaxation. Slowed ventricular relaxation would offer greater resistance to ventricular filling in late diastole such that ventricular diastolic pressure is elevated; this may lead to dyspnea, as observed in patients harboring F87L (Gimeno et al., 2009). Impairment of length–dependent activation suggests that the Frank–Starling mechanism may be significantly impaired in intact hearts containing F87L. Under increased hemodynamic demands, attenuation of the Frank–Starling mechanism may have severe consequences on heart function because it limits the ability of the heart to increase its stroke volume in response to an increase in venous return. In some cases, however, a substantial increase in myofilament Ca\textsuperscript{2+} sensitivity and subsequent increase in myocardial force development may offer a benefit to F87L patients who are at advanced stages of heart failure. Patients at advanced stages of heart failure are likely to minimize high stress workloads and thus operate at lower end-diastolic volumes; under such conditions, increased force at short SL may improve contraction.

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