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Allosteric effects of cardiac troponin TNT1 mutations on actomyosin binding: A novel pathogenic mechanism for hypertrophic cardiomyopathy

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Abstract

The majority of hypertrophic cardiomyopathy mutations in (cTnT) occur within the alpha-helical tropomyosin binding TNT1 domain. A highly charged region at the C-terminal end of TNT1 unwinds to create a flexible “hinge”. While this region has not been structurally resolved, it likely acts as an extended linker between the two cTnT functional domains. Mutations in this region cause phenotypically diverse and often severe forms of HCM. Mechanistic insight, however, has been limited by the lack of structural information. To overcome this limitation, we evaluated the effects of cTnT 160–163 mutations using regulated *in vitro* motility (R-IVM) assays and transgenic mouse models. R-IVM revealed that cTnT mutations 160E, E163R and E163K disrupted weak electrostatic actomyosin binding. Reducing the ionic strength or decreasing Brownian motion rescued function. This is the first observation of HCM-linked mutations in cTnT disrupting weak interactions between the thin filament and myosin. To evaluate the *in vivo* effects of altering weak actomyosin binding we generated transgenic mice expressing 160E and E163R mutant cTnT and observed severe cardiac remodeling and profound myofilament disarray. The functional changes observed *in vitro* may contribute to the structural impairment seen *in vivo* by destabilizing myofilament structure and acting as a constant pathophysiologic stress.

Keywords

Troponin; Thin filaments; Hypertrophic cardiomyopathy; Weak binding

Introduction

Cardiac muscle contraction is dependent on the interaction of myosin heads with actin filaments to form force-generating crossbridges. This interaction is regulated by the thin filament proteins tropomyosin (Tm) and troponin (cTn), a trimeric complex consisting of cTnC, the Ca²⁺ binding subunit, cTnI, the inhibitory subunit, and cTnT, the tropomyosin

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binding subunit. Regulation of the actomyosin crossbridge cycle by these proteins is described by the three-state model of myofilament activation, whereby the position of the Tm coiled-coil dimer is in dynamic equilibrium between three positions on actin [1–3]. Despite our understanding of the basic biophysics of thin filament function, the precise mechanisms that underlie the development of often severe human cardiomyopathies caused by mutations in independent protein components of the complex have proven elusive [reviewed in [4]]. A primary limitation to developing mechanistic insight into the pathogenesis of these not uncommon disorders resides in the multiple levels of experimental resolution required for rigorously coupling changes in the efficiency of myofilament activation to whole heart remodeling, a problem that is further compounded by the difficulty in linking structure and function within the highly allosteric actomyosin complex. To directly address these limitations, in the current study we have utilized a coupled *in vitro*–*in vivo* system to examine the effects of these clinically severe mutations, with a long-term goal of applying this integrated approach to develop a basic paradigm for studying the mechanism(s) of sarcomeric protein function in health and disease.

Hypertrophic cardiomyopathy (HCM)² is a primary genetic disease of the cardiac sarcomere [5]. Approximately 15% of HCM mutations are found in the thin filament protein cTnT [6]. Approximately 65% of cTnT mutations are found within or flanking TNT1, the N-terminal tropomyosin-binding domain of cTnT. Far UV circular dichroism studies showed that TNT1 is highly alpha-helical however the structural details of this functionally important domain are unknown, as it was excluded from the crystal structure of the Ca²⁺-activated human cTn complex and subsequent cryo-EM reconstructions [7–10]. This lack of structural data limits our ability to understand the molecular pathogenesis of this complex disorder.

TNT1 mutations tend to cluster at the N- and C-terminal ends of the domain, with 2 of several mutational hotspots occurring at residues 92 (R92Q, R92L, R92W) and 160–163 (160E, E163R, E163K). Two of these mutations, R92Q and 160E, are consistently associated with a particularly poor clinical prognosis [11–13]. Interestingly, these mutations show significant differences in their molecular phenotypes despite being located within the same functional domain [7,14,15]. Mutation-specific alterations in myofilament activation and myocellular Ca²⁺ homeostasis *in vivo* differ between 160E and R92 mutations [14,16]. Moreover, it has been shown that N-terminal and C-terminal TNT1 mutations have distinct biophysical effects on TNT1–Tm interactions. R92 mutations reduce the affinity of cTnT for Tm and impair the ability of TNT1 to promote Tm binding to actin and stabilize the head-to-tail Tm overlap complex [15,17]. In contrast, the C-terminal mutation 160E (an in-frame single amino acid deletion) has no effect on the Tm dependent functions of TNT1, leading to the hypothesis that the C-terminal mutations must cause disease by another, unknown mechanism [15].

The mutational hotspot at residues 160–163 is located within a conserved, highly charged region (158-RREEENRR-166) (Fig. 1A and B). We hypothesize that this putative solvent accessible region unwinds to create a flexible hinge that is necessary for normal thin filament regulation, and mutations in this region alter its flexibility and lead to HCM. The

²Abbreviations used: HCM, hypertrophic cardiomyopathy; HMM, heavy meromyosin; R-IVM, regulated *in vitro* motility.

flexibility of this region may be important for the transduction of a Ca^{2+} activated signal from the core domain of cTnT to the Tm-binding TNT1 domain, fully required for activation of the thin filament.

The present work investigates the effects of known HCM mutations 160E, E163R, and E163K (mutations 160–163) using an integrative approach that utilizes both *in vitro* and *in vivo* techniques. The 160E is a single amino acid in-frame deletion of a glycine residue at residue 160 while E163R/K represents either an arginine or lysine substitution for the native glycine at residue 163. We examine the functional effects of these mutations on Ca^{2+} sensitivity and ATPase activity via regulated *in vitro* motility assays, and the *in vivo* structural consequences of 160E and E163R in transgenic mouse models. We present evidence of a deficiency in the weak binding interactions of the partially activated “closed” state of the 3-state model of myofilament activation as a novel molecular disease mechanism associated with C-terminal TNT1 HCM mutations.

Methods

Human cardiac TnT sequence alignment

Sequence alignment of the TNT1 domain of several species was performed using ClustalW. Residues 40–200 of the hcTnT cDNA sequence were aligned with homologous mRNA sequences from human (NM_000364), mouse (NM_001130174), feline (NM_001009347), xenopus (NM_001089041), and zebrafish (AF282384).

Site-directed mutagenesis

160E, E163R, and E163K mutations were introduced into human cTnT (hcTnT) cDNA in a pET3D expression vector (provided by Dr. J.D. Potter) using the Quick-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Primers used for mutagenesis are listed in Supplemental Table 1. The identity of each mutant construct was verified by direct DNA sequencing.

Protein expression and purification

cDNA for human cardiac TnI (hcTnI) in a pET11d expression vector (Novagen) and human cardiac TnC (hcTnC) in a pET3d vector (provided by Dr. J.D. Potter). For protein expression, constructs were transformed into BL21 competent cells (Stratagene). hcTnT constructs were transformed into Rosetta II (DE3) competent cells (Novagen). Recombinant cTn proteins were purified by ion exchange and, in the case of cTnI, TnC affinity chromatography as previously described [18,19]. Purified cTn subunits were flash frozen, lyophilized, and stored at -80°C .

Recombinant cTn complex reconstitution

The recombinant cTn complex was reconstituted as previously described [18]. Equimolar amounts of hcTnT, hcTnI and hcTnC were mixed in solubilization buffer containing 6 M urea, 50 mM Tris, pH 8.0, 1 M NaCl, 5 mM MgCl_2 , 1 mM CaCl_2 , 1 mM DTT. The solution was dialyzed against 0.7 M NaCl, 0.4 M NaCl, and finally 0.2 M NaCl in the above buffer minus urea. The cTn complex was separated from the monomeric troponins by anion

exchange on a RESOURCE-Q column (Amersham Biosciences) with an AKTA FPLC system (GE Biosciences). cTn complex purity was verified by 12% SDS-PAGE.

Myosin preparation

All experimental protocols followed current NIH guidelines and were approved by The Institute of Animal Studies at the University of Arizona. Myosin isolation and purification from rabbit fast skeletal muscle was performed as previously described [20]. Myosin was stored in a 50% glycerol solution at -20°C . Heavy meromyosin (HMM) was prepared from glycerinated myosin by chymotryptic digestion as previously described [21]. HMM is stored at 4°C on ice for use in IVM assays for up to 1 week.

Tropomyosin (Tm) preparation

Tm was prepared from rabbit fast skeletal muscle acetone powder as previously described [22]. For use in *in vitro* motility assays, purified Tm was dissolved in AB solution (20 mM KCl, 10 mM MOPS, 2 mM MgCl_2 , 1 mM DTT) to $1\text{ }\mu\text{M}$.

Actin preparation and labeling

20 M F-actin was prepared from rabbit skeletal g-actin (Cytoskeleton Inc., Denver CO) in Buffer A (5 mM Tris, 0.2 mM ATP, 0.2 mM CaCl_2 , 1 mM DTT) with 2 mM MgATP, 1 mM DTT and KMEI (500 mM KCl, 25 mM MgCl_2 , 250 mM EGTA, 5 mM NaN_3 , 100 mM DTT). G-actin was allowed to polymerize at 4°C for at least 30 min. F-actin was diluted to 2 M and labeled with 2 M Rhodamine-Phalloidin (RPh, Sigma) in 25 mM KCl, 2 mM MgCl_2 , 2 mM EGTA, 25 mM MOPS, 5 mM DTT.

Regulated *in vitro* motility (R-IVM) assays

In vitro motility assays were performed as previously described [23,24]. 54 mM ionic strength motility solution was used as a standard condition (40 mM MOPS, 1 mM free Mg, 3 mM MgATP, 2 mM EGTA, 50 mM DTT, 100 nM hcTn, 100 nM rsTM, 20 nM rhodamine-phalloidin). The Ca^{2+} concentration of the solutions were varied from pCa9 to pCa5 by varying ratios of K_2EGTA - and Ca_2EGTA -containing motility solutions. Ratios were calculated using a QuickBasic based program provided by E. Homsher [25].

Motility analysis

Motility was viewed using an Olympus IX-71 inverted microscope under fluorescence illumination and a $100\times$ objective. Thin filament movement was recorded using a Hamamatsu ORCA-ER cooled CCD. 8–12 sites were recorded per slide for 25 s each. Using MetaMorph 7 (Molecular Devices), the movement of each filament in the field of view was recorded using frame grabbing rates of 10 frames/s. The Track Points program within the MetaMorph system was used to manually track the centroid of each filament from frame to frame to measure sliding filament speed. The lengths of moving filaments varied from 0.4 to 20 μm and each filament was analyzed for at least 10–20 frames. Minimums of 100 filaments per condition were analyzed. The quality of the filament movement was determined as previously described [23]: filaments not moving had an average speed of $<0.8\text{ m/s}$; filaments moving at uniform speed had a frame-to-frame standard deviation (SD) of <0.5 of the

filament's mean speed; erratically moving filament's frame-to-frame SD was >0.5 of its mean speed.

ATP-free in vitro motility assays

By excluding Mg-ATP from the motility solutions, we observed strong actomyosin binding. 54 mM motility solutions with and without Mg-ATP were prepared at pCa9 and pCa5. Motility assays were performed with the following modifications: ATP-free motility solution was injected into motility chamber first and movies recorded. Immediately following, a motility solution of the same pCa containing ATP was injected into the same chamber and recorded.

Low ionic strength, methylcellulose and high [HMM] in vitro motility assays

Conditions of the *in vitro* motility assays were altered to attempt to facilitate weak binding interactions and increase the number of thin filaments bound to the surface. Low ionic strength assays were performed as described with the ionic strength of the motility solution lowered to 18.8 mM by reducing MOPS concentration to 10 mM and MgATP to 1 mM. Ionic strengths were calculated with a QuickBasic based program [25]. Motility assays were also performed under standard 54 mM conditions with 0.7% methylcellulose (w/v) added to the motility solutions [26]. Finally, HMM concentrations of 0.3, 0.6, 0.8, and 1 mg/ml were applied to the motility surface, and regulated motility assays were performed as described at 54 mM ionic strength.

Transgenic mice-clone construction and screening

160E and E163R transgenic constructs were generated via pronuclear injection as previously described [27]. A c-myc tag engineered onto the N-terminal end of the construct is used to identify the transgene via PCR (forward primer:

TCCTCTTCAGAGATGAGCTTT; reverse primer: ACCTAGAGGGAAAGTGTCTT).

160E and E163R mutations were introduced via the QuickChange Site Directed Mutagenesis kit (Stratagene). Transgenic lines expressing a mid level of transgenic protein (35% 160E and 50% E163R) were backcrossed into C57Bl/6 background for at least 7 generations.

Cardiac protein isolation and Western analysis

Crude cardiac protein isolation, SDS-PAGE, and Western analysis were performed as previously described [28].

Ultrastructural analysis

Tissue preparation—20-week-old female mice (Non-Tg, 160E-35% and E163R-50%) were anesthetized with isofluorane and the hearts were perfused in situ with 3 ml Ringer's solution + 30 mM KCl and 3 mM BDM to induce cardiac arrest. The arrested hearts were immediately perfused with 10 ml fixative (3% glutaraldehyde in Ringer's solution), isolated and fixed overnight at 4 °C with 3% glutaraldehyde in Ringer's solution. After three quick rinses with cold Ringer's solution, the papillary muscles and the fibers of the mid myocardial wall were collected from the left ventricle. The tissue was immersed in PIPES

buffer (0.1 M, pH 7.4) for 24 h at 4 °C then postfixed for 1 h with 2% osmium tetroxide in PIPES at room temperature. After three five-minute washes with deionized water, the tissue was stained with 2% uranyl acetate for 20 min at room temperature and dehydrated in ascending ethanol solutions (50%, 70% and 90%; 10 min each and 100% ethanol; three ten-minute washes). The samples were infiltrated with a 50:50 mixture of ethanol/Spurr's resin for 2 h then with 100% Spurr's resin (3× 1 h) at room temperature prior to embedding in a fresh 100% Spurr's resin. Tissue blocks were polymerized overnight at 60 °C, cut into 70 nm-thick sections and mounted on 150 mesh copper grids. Finally, the grids were stained with 2% lead citrate for 2 min followed by a brief wash with deionized water and examined with a CM12 TEM transmission electron microscope (Philips, Eindhoven, Netherlands). A total of 3 mice per line were prepared.

Z disk alignment measurements—Electron micrographs of the longitudinal mid myocardial fibers were analyzed for Z disk misalignment (3–4 images per mouse strain; 8200× magnification). Z disks were counted and classified as “normally aligned”, if they had perfect alignment with the Z disks of adjacent sarcomeres, “moderately misaligned”, if they overlap with the I band of at least one adjacent sarcomere or “severely misaligned” if they overlap with the A band of at least one adjacent sarcomere. Statistical analysis was performed using the Chi-Square test (Prism 6; GraphPad Software, Inc.). A level of $p < 0.05$ was considered as statistically significant.

Statistical analysis

All values are reported as mean + S.E.M. Statistical analysis was performed in GraphPad Prism 6. Student's t-test was used to make comparisons between wild type (WT) and mutant measurements. A p-value of less than 0.05 was accepted as statistically significant.

Results and discussion

The C-terminal TNT1 domain: a highly conserved, extended linker

HCM-linked mutations in the C-terminal TNT1 domain, particularly 160E, confer a poor prognosis and high incidence of sudden cardiac death [12,13]. While it has been previously shown that mutations at the N-terminal end of TNT1 disrupt Tm interactions, the molecular pathogenesis for the C-terminal hotspot mutations remains unclear [15]. Alignment of human cTnT residues 140–200 reveals the C-terminal end of TNT1 to be highly conserved across vertebrate species (Fig. 1C). At residues where the amino acid sequence was not 100% identical the charge was frequently conserved. The proposed hinge region (residues 158–166) was completely conserved across mammalian species, and mutational hotspot residues 160–163 were 100% identical across all species analyzed. The “flexible loop” region spanning hcTnT residues 183–202 was similarly conserved, interestingly, this region contains no known disease causing mutations or polymorphisms [9]. The high degree of sequence conservation of this region across vertebrate species and the complexity of the disease-causing mutations within the putative hinge support the structural and functional importance of this region. Flexible linker regions connecting two α -helical domains are a common motif in thin filament proteins. The putative hinge and flexible loop regions in TNT1 differ from these common linkers in a few key areas. In particular, common linkers

are relatively short (5–10 amino acids) compared to the roughly 50 amino acids that make up the putative hinge and flexible loop regions, and are generally not highly conserved across species. Based in part on the highly charged nature of the region, this portion of the domain is likely a solvent accessible flexible “hinge” and plays an important role in thin filament regulation of the crossbridge cycle.

Human cTnT 160–163 mutations disrupt HMM binding

Under standard assay conditions (54 mM ionic strength) native (WT) thin filaments were fully regulated and exhibited the expected cooperative Ca^{2+} sensitivity of myofilament interaction. In contrast, substituting hcTnT carrying any of the 160, E163R and E163K mutations did not bind normally to HMM on the motility surface at any of the measured Ca^{2+} concentrations (from pCa9 to pCa5). This result was unexpected and reproducible across a range of HMM concentrations from standard (0.3 mg/ml) to high (0.6–1.0 mg/ml). The disruption in actomyosin interaction could occur at two points in the actomyosin crossbridge cycle: the weak binding of the thin filament to myosin (corresponding to the “closed state” of the 3-state model), or the formation of strong actomyosin crossbridges (the “open state”). Our initial observations were similar to those seen with high ionic strength motility solutions (>60 mM) and low HMM concentrations (>6.5 lg/ml), conditions where weak binding interactions are insufficient to hold WT filaments to the motility surface [25,26,29]. Simply increasing the density of HMM available to bind with actin was not sufficient to restore normal activity, consistent with a direct impairment in weak binding as a primary biophysical disease mechanism.

Human cTnT 160–163 mutations do not alter strong binding to HMM

In the absence of ATP, actin is strongly bound to myosin and unable to dissociate, halting the actomyosin crossbridge cycle in a rigor state. To test whether the hcTnT 160–163 mutations altered strong binding, motility assays were performed in the absence of Mg-ATP. This mimics a rigor state in which the actin filament remains strongly bound to HMM. With WT hcTn, filaments bound tightly to the HMM coated surface and did not slide at any Ca^{2+} concentration. As expected, normal sliding behavior was restored to the WT filaments by the addition of Mg-ATP. In the case of the 160E, E163K and E163R cTn, strong binding similar to WT was restored in the absence of Mg-ATP and lost with the subsequent addition of Mg-ATP, confirming that strong actomyosin binding can be achieved in the presence of hcTnT 160–163 mutants and suggesting that subsequent conformational changes induced by the binding of Mg-ATP to the mutant filaments were sufficient to detach them from the HMM surface. Thus, strong, rigor state binding is intact in the 160–163 mutants. In the absence of ATP, however, we cannot definitively conclude that strong binding interactions are completely unaffected, since several nucleotide-bound and unbound strong binding states exist [30].

Low ionic strength conditions rescue cTn 160–163 filament sliding

It has been previously shown that lowering the ionic strength of R-IVM motility conditions results in decreased overall sliding filament velocities [25,31]. Lowering the ionic strength reduces the ionic screening effect, thereby increasing the strength of the native weak electrostatic interactions between HMM and actin. As expected, the maximal sliding

filament speed of WT thin filaments is decreased under these conditions (Fig. 2A). In comparison to the standard ionic strength, WT thin filaments at 18.8 mM exhibit a similar sensitivity to Ca^{2+} , but a maximum sliding filament velocity (V_f) that is 2.8 times lower. Under reduced ionic strength conditions, normal filament attachment and the ability to slide were fully rescued in all cTn 160–163 mutant proteins. Facilitating thin filament sliding allowed for the measurement of Ca^{2+} sensitivity, shown in Fig. 2 and Table 1. E163R and E163K exhibit a slightly decreased Ca^{2+} sensitivity of filament activation when compared to WT, while the Ca^{2+} sensitivity of 160E is increased. These results are consistent with previous reports where 160E and E163K reconstituted into skinned fibers showed similar changes in the force- Ca^{2+} sensitivity relationship [8,32]. In comparison to WT, for thin filaments at low ionic strength, the maximum V_f of all 160–163 mutants was significantly increased. E163R and E163K V_f were also significantly increased over 160E (Fig. 2B).

160E has a maximum V_f 1.5 times greater than WT, while E163R and E163K exhibit maximum V_f of approximately 2.5 times greater than WT. Thus, while low ionic strength conditions were able to qualitatively rescue thin filament binding and sliding in the presence of the hcTnT 160–163 cTn, a full WT-like response was not observed (Table 1). V_f is analogous to unloaded filament shortening velocity, and is a correlate of ATPase activity [33]. Of note, the decrease in V_f typically observed with WT thin filaments at 18 mM could partially be explained by an enhancement of weak binding producing a retarding force against which force-generating crossbridges must work. Previous studies by Homsher et al. suggested that a reduction in ionic strength could alter actomyosin–ATP kinetics, causing either a reduction in the rate of ATP binding to actomyosin rigor heads, or a decrease in the rate of release of ADP from actomyosin crossbridges [25]. Both would be predicted to slow the crossbridge cycle [34,35]. In the case of the cTnT 160–163 mutations, the enhanced motility seen under the low ionic strength conditions may be indicative of a change in crossbridge-ATP kinetics. This is consistent with increased tension-dependent ATP consumption observed in cardiac muscle fiber studies of transgenic 160E mice, as well as increased ATPase activity in fibers reconstituted with E163K [32,36].

Abrogating the requirement for weak binding rescues hcTnT 160–163 mutants

To further assess whether facilitating the weak actomyosin interaction could restore normal filament behavior in hcTn 160–163 mutants, R-IVM assays were performed under 54 mM ionic strength (normal) conditions in 0.7% methylcellulose. Methylcellulose increases solution viscosity, reducing Brownian motion and lateral diffusion of filaments in R-IVM assays, and has been used in studies requiring a low density of myosin or ionic strengths above 60 mM [25,26]. *In vivo*, thick and thin filaments are held in close proximity to one another by the highly ordered structure of the sarcomere. In the absence of the normal scaffold, as is the case with R-IVM, intact weak interactions are required to hold the actin and myosin together on the motility surface. At ionic strengths higher than 60 mM (closer to physiologic values), these interactions become too weak to hold actin filaments to myosin, thus, assays are conducted at sub-physiologic ionic strength to facilitate weak electrostatic binding. Increasing solution viscosity essentially eliminates this dependence of the assay on weak binding. Under these conditions, filament binding and sliding were again restored and, in addition, a near-WT V_f -pCa relationship was obtained for all mutants (Fig. 3 and Table 2). Of note, the maximal V_f of E163R is significantly increased compared to WT, while the

160E and E163K mutants show slight, albeit significant, decreases in maximal V_f (Fig. 3B). Thus, R-IVM assays at 54 mM ionic strength in the presence of methylcellulose fully rescued *in vitro* function of the cTn 160–163 mutant proteins, consistent with the hypothesis that an impairment in weak actomyosin binding is the primary molecular disease mechanism.

Cardiac cTnT 160E and E163K disrupt sarcomeric structure in vivo

In order to correlate our *in vitro* findings to *in vivo* phenotype, transgenic mouse lines expressing 160E and E163R were generated by pronuclear injection of a transgene construct as depicted in Fig. 4A. Transgenic protein expression levels of the 160E and E163R lines were determined to be ~35% and 50% mutant forms, respectively (Fig. 4B). The stoichiometry of the cardiac sarcomere is tightly regulated, and it has been previously shown that the expression levels seen in transgenic animals generated with this construct result in a replacement of WT protein and not simply an overexpression [27,37]. Of note, while several transgenic founder animals of cTnT E165R mice expressing greater than 90% transgene were obtained, 160E founders expressing greater than 70% transgene could not be stably established, consistent with the more severe effects on myofilament activation observed in the 160E R-IVM studies. Both transgenic lines showed structural changes at the whole heart and sarcomeric levels. At 5 months of age, observation at the whole heart level revealed a slight decrease in overall heart mass and extensive cardiac remodeling in both E163R and 160E compared to sibling matched Non-Transgenic (Non-Tg) controls (Fig. 4C). While both lines exhibited ventricular remodeling, 160E hearts were consistently more severely affected despite expressing a lower transgene protein level. In addition, E163R hearts alone exhibited pronounced atrial enlargement. Ultrastructural analysis of left ventricular tissue after KCl-arrest from 5-month old 160E and E163R animals showed significant abnormalities in sarcomeric structure in comparison to Non-Tg (Fig. 5A). Extensive misregistration of thin filament orientation was seen at the Z-disk with myofibrillar lysis and disarray. M-lines were poorly visualized and the I-A borders are irregular as compared to Non-Tg controls. As shown in Fig. 5B, measurement of Z-disk alignment revealed that sarcomeric structural disarray was more prominent in 160E sections despite the higher level of transgene expression in E163R, further evidence for the highly mutation-specific effects of these closely related mutations.

The extensive sarcomere disruption and myofilament disarray observed in transgenic sarcomeres with cTnT 160E and E163R strongly suggest that there is a structural impairment in these transgenic hearts, supporting our hypothesis of a structural change in the mutant proteins. This degree of sarcomeric disruption is unique to C-terminal TNT1 HCM mutations, as it is not observed in our N-terminal HCM mutant models [28,37]. The observed structural changes may be the result of destabilization of the thin filament due to an alteration in the structure of the mutant cTnT. It has been previously shown that cTnT is essential for sarcomere assembly. Cardiac TnT knockout models in mouse and zebrafish exhibit a sarcomeric phenotype similar to that seen in 160E and E163R transgenic mice [38–40]. Cardiac TnT also plays important roles in anchoring cTn to the thin filament and promoting the binding of Tm to actin [15,17,41]. A change in the flexibility and/or structure of the putative hinge region in TNT1 by cTnT 160–163 mutations could result in modulation

of these dynamic interactions. This may lead to displacement of Tm during assembly or crossbridge cycling, destabilizing the sarcomere and leading to disruption. Another sarcomeric structural protein, Nexilin, has been shown to cause changes in sarcomere ultrastructure. Mutations in Nexilin, an actin-binding protein that plays a role in Z-disk stabilization, are a known cause of dilated cardiomyopathy [42]. Nexilin mutations in humans and zebrafish knockouts cause destabilization of sarcomeric units. Initial assembly of sarcomeres in the knockouts was not affected, but ultrastructural damage increased over time, at least in part due to mechanical stress imposed by the workload of the heart. C-terminal TNT1 mutations may have similar destabilizing effects on the myofilament, resulting in local areas along the sarcomere that exhibit dysregulation of the crossbridge interaction that contributes to myofibrillar instability. This, along with the increased energetic requirement, may trigger a pathologic signaling response that leads to the whole-heart remodeling observed in the E163R and 160E mice.

Conclusion

Our coupled *in vitro*–*in vivo* studies have revealed a unique potential primary mechanism for C-terminal TNT1 mutations that cause a severe form of HCM. Utilizing the regulated *in vitro* motility assay under varying experimental conditions showed that mutations within the highly charged, likely solvent accessible region spanning cTnT residues 158–166 altered the weak electrostatic binding conditions that comprise the transition from the blocked to closed state. Moreover, clear mutation-specific differences were observed, especially with respect to V_f under low ionic strength conditions. These findings are consistent with a mutation-specific primary change in the local structure and/or geometry in the hinge-flexible loop domain that alters the position of this highly conserved region with respect to actin and directly blocks the formation of weak binding crossbridges. It is important to note that it is now well-established that the functional and structural effects of mutations within thin filament proteins can be propagated to distant portions of the molecule and thus have complex and unexpected impacts on myofilament function [43–45]. Further molecular level understanding awaits additional high resolution structural details. Extension of our studies to the *in vivo* level via the generation of cardiac-specific transgenic mouse models revealed a unique, highly progressive pattern of cardiac remodeling and sarcomeric disruption at the ultrastructural level. The apparent myofibrillar disarray and lysis are consistent with abnormalities in either the ability of the mutant thin filaments to properly assemble or a subsequent structural instability that leads to dysfunction and lysis. While similar findings have been demonstrated in troponin knock-out models of both mice and zebrafish, where it has been proposed that normal actomyosin binding is required for sarcomeric integrity, our work is the first to both recapitulate these results with a point mutation and localize the effects to the weak crossbridge formation step [40,46]. Finally, our results at both the biophysical and whole-heart levels demonstrate clear mutation-specific phenotypes. In particular, the 160E cTnT mutation exhibited a more severe phenotype, both with respect to the effects on V_f in the R-IVM and the severity of the remodeling and ultrastructural disarray. The later was particularly striking given that the mutant protein expression level was lower in the 160E hearts as compared to the E163R. This is likely explained by the predicted greater structural effect of a single amino acid deletion as compared to a missense

substitution. Taken together, our results have identified a novel primary disease mechanism at two levels of investigative resolution, a validation of an integrative approach to this complex genetic cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.abb.2014.01.016>.

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Fig. 1.

Human cTnT based on the crystal structure of the calcium-activated core domain. (A) Full structure, inset represents the core domain. (B) Cardiac TnT. Residues 160 and 163 (starred) are a mutational hotspot at the C-terminal end of TNT1. Residues 158–166 constitute a highly charged putative hinge region. (C) The C-terminal TNT1 domain is highly conserved across vertebrate species. The black bar indicates the highly charged putative hinge region. [*] = 100% conservation; [:] = Charge conservation; [.] = Mammalian conservation.

Fig. 2. Calcium sensitivity of thin filament activation measured by regulated *in vitro* motility assays. Ionic strength was reduced from the standard 54 mM to 18.8 mM. (A) Calcium sensitivity of filament sliding speed. (B) Maximum filament sliding speeds. (C) Normalization of the percent of filaments moving uniformly at a given pCa. Values are represented as mean + SEM. *** $p < 0.001$, # $p < 0.001$ compared to E163R.

Fig. 3.

Calcium sensitivity of thin filament activation measured by regulated *in vitro* motility assays. Experiments were performed at the standard 54 mM ionic strength supplemented with 0.7% methylcellulose. (A) Calcium sensitivity of filament sliding speed. (B) Maximum filament sliding speeds. (C) Normalization of the percent of filaments moving uniformly at a given pCa, representative of the calcium sensitivity of thin filament activation. Values are represented as mean + SEM. *** $p < 0.001$ compared to WT; # $p < 0.0001$ compared to Delta160E.

Fig. 4. E163R and Delta160E transgenic mouse models (A) Cardiac-specific transgenic construct. (B) Western blot analysis of transgene expression. cMyc-TnT (upper band) is transgenic protein, TnT (lower band) is exogenous protein. (C) Whole heart remodeling in 5-month mice. Bar-5 mm Scale is equivalent for all boxes.

Fig. 5. EM Ultrastructural Analysis. (A) Representative EM images of LV tissue from 5-month old mice. Magnification is listed to the right of the images. (B) Measurement of Z-disk alignment as a marker of structural disruption.

Table 1

Velocity–pCa relationship at low ionic strength.

	WT	160E	E163R	E163K
Max V_f ($\mu\text{m/s}$)	1.67 ± 0.22	2.77 ± 0.15	4.15 ± 0.44	4.32 ± 0.58
EC_{50}	7.58 ± 0.01	7.99 ± 0.02	6.93 ± 0.01	7.28 ± 0.01
n_h (Hill Coeff)	2.6 ± 0.04	2.4 ± 0.01	1.60 ± 0.04	1.60 ± 0.01

Table 2

Velocity–pCa relationship with 0.7% methylcellulose.

	WT	160E	E163R	E163K
Max V_f ($\mu\text{m/s}$)	5.14 ± 0.47	4.59 ± 0.67	4.61 ± 0.48	4.58 ± 0.64
EC_{50}	7.25 ± 0.02	7.15 ± 0.01	6.87 ± 0.01	7.16 ± 0.01
n_h (Hill Coeff)	1.7 ± 0.09	1.5 ± 0.06	1.8 ± 0.04	1.9 ± 0.07