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HCM-Linked Δ 160E Cardiac Troponin T Mutation Causes Unique Progressive Structural and Molecular Ventricular Remodeling in Transgenic Mice

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Abstract

Hypertrophic cardiomyopathy (HCM) is a primary disease of cardiac muscle, and one of the most common causes of sudden cardiac death (SCD) in young people. Many mutations in cardiac troponin T (cTnT) lead to a complex form of HCM with varying degrees of ventricular hypertrophy and ~65% of all cTnT mutations occur within or flanking the elongated N-terminal TNT1 domain. Biophysical studies have predicted that distal TNT1 mutations, including Δ 160E, cause disease by a novel, yet unknown mechanism as compared to N-terminal mutations. To begin to address the specific effects of this commonly observed cTnT mutation we generated two independent transgenic mouse lines carrying variant doses of the mutant transgene. Hearts from the 30% and 70% cTnT Δ 160E lines demonstrated a highly unique, dose-dependent disruption in cellular and sarcomeric architecture and a highly progressive pattern of ventricular remodeling. While adult ventricular myocytes isolated from Δ 160E transgenic mice exhibited dosage-independent mechanical impairments, decreased sarcoplasmic reticulum calcium load and SERCA2a calcium uptake activity, the observed decreases in calcium transients were dosage-dependent. The latter findings were concordant with measures of calcium regulatory proteins abundance and phosphorylation state. Finally, studies of whole heart physiology in the isovolumic mode demonstrated dose-dependent differences in the degree of cardiac dysfunction. We conclude that the observed clinical severity of the cTnT Δ 160E mutation is caused by a combination of direct sarcomeric disruption coupled to a profound dysregulation of Ca^{2+} homeostasis at the cellular level that results in a unique and highly progressive pattern of ventricular remodeling.

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Disclosures:

None

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Keywords

hypertrophic cardiomyopathy; cardiac troponin T; calcium regulation; impaired relaxation; contractile performance; transgenic mouse models

1. Introduction

Hypertrophic cardiomyopathy (HCM) is a primary disease of cardiac muscle, and is one of the most common cardiac genetic disorders [1]. Hundreds of known mutations in at least eight genes of the cardiac sarcomere have been implicated in this autosomal-dominant disorder [1–9]. Of note, the recent advent of expanded genotype screening in HCM has greatly expanded the definition of the disease, especially with respect to the degree and nature of the observed ventricular remodeling and the complex, progressive changes in cardiac function that follow [10]. It is thus important to view HCM not merely as a “hypertrophic” form of cardiac disease, but one that results in a progressive spectrum of changes in ventricular geometry and function over time. Regarding the complexity of linking genotype to phenotype the clinical phenotypes of many of the known mutations, are highly variable with respect to the degree of hypertrophy, fibrosis, and incidence of sudden cardiac death, though insufficient clinical data exists to directly determine the outcome for specific mutations [11–13]. The observed phenotypic heterogeneity suggests that individual mutations give rise to specific changes at the molecular level, triggering alternative downstream effects that lead to complex ventricular remodeling. The molecular mechanisms that give rise to these phenotypic variations remain unclear, which adds to the complexity in designing novel treatment approaches. Determination of more robust genotype-to-phenotype links will reveal the direct effect of the mutation at the molecular level, and identify its downstream effectors as potential therapeutic targets.

Cardiac TnT is a part of the trimeric troponin complex that binds to tropomyosin and together these thin filament proteins regulate calcium-dependent myofilament activation [14, 15]. Many mutations in cTnT cause a malignant form of HCM associated with variable ventricular hypertrophy and often a poor prognosis [3, 16–18]. Sixty-five percent of cTnT mutations fall within or flanking TNT1, the extended N-terminal tropomyosin-binding domain of cTnT. TNT1 mutations tend to cluster at the N- and C-terminal ends of the domain, with mutational hotspots occurring at residues 92–96 and 160–163. Despite their location in the same domain, these mutational hotspots exhibit significant differences in their molecular and clinical phenotypes [13, 19–22]. It has been previously shown that N-terminal and C-terminal TNT1 mutations have distinctly different biophysical effects on TNT1-tropomyosin interactions [21, 23]. While the structure of the N-terminal domain of cTnT was not included in the extant high-resolution crystal structure published by Takeda, et al, subsequent biophysical studies in several labs have established the extended alpha-helical nature of the region from ~90 – 150 and its role in stabilizing the crucial interaction with tropomyosin [19, 24, 25]. The C-terminal region of TNT1 (distal to residue 150 and extending to approximately residue 200), however, has not been well-defined at the structural level. While residues 180–200 have been posited to form a “flexible loop” structure immediately proximal to the H1 helix of the TNT2 domain, no structural information is available for residues 150–180 [25]. This region is highly conserved across species at the amino acid level and identical between humans and mice. It contains a highly charged, likely solvent-accessible region from ~160 – 172 that includes the mutational hotspot. Both the high conservation and severity of the human mutations argue for a central functional role for this extended linker region.

The 160E mutation in cTnT is caused by the in-frame deletion of three nucleotides encoding a glutamic acid at residue 160 (also reported as residue 163, as residues 160–163 are identical) and results in a severe form of HCM with several families exhibiting a high frequency of early sudden cardiac death [3, 13, 26]. It represents one of the most common disease-causing mutations in cTnT. While the 160E mutation has been studied *in vitro* in a variety of experimental systems, no consensus has been reached regarding disease mechanism. Expression of 160E hcTnT in quail myotubes demonstrated regional disruptions in structure as well as a reduction in maximal force production and decreased calcium sensitivity [27]. Reconstitution studies performed by several groups revealed that 160E cTn increased calcium sensitivity (to varying degrees) and affected TM binding [19, 23]. However, a study of hcTnT fragments including residues 70–170 demonstrated that 160E did not affect any of the tropomyosin-dependent functions of TnT including TM binding, stabilizing the TM head-to-tail overlap, or promoting actin-TM binding [21].

To begin to surmount the lack of mechanistic consensus among the prior studies, a transgenic murine model was created to provide a direct, *in vivo* system to determine the pathogenic mechanism of the cTnT 160E mutation. Transgenic mice expressing 160E cTnT at 35% of total demonstrated an energy-tension mismatch and increased calcium sensitivity in isolated fibers compared to Non-Transgenic siblings [22]. A study of the downstream effects of this mouse line demonstrated decreases in SR calcium load and uptake that correspond to calcium and mechanical impairments in isolated myocytes, as well as alterations in expression of calcium handling proteins [20]. Additionally, these mice demonstrated conduction abnormalities and ventricular ectopy [28]. While these *in vivo* studies of the 35% 160E cTnT mice suggested that multiple pathways of myocellular function were disrupted, the origin of the unique ventricular remodeling pattern remained unclear. Given the observed severity and relatively high penetrance of the human phenotype in patients with the cTnT 160E mutation, our hypothesis is that this complex, progressive myocellular response in the animal models is directly related to the extent and degree of overall ventricular remodeling, an important determinant of disease outcome.

To directly address this hypothesis, we now examine the effect of varying 160E cTnT protein levels on the cellular and whole heart phenotypes to further understand the link between the primary sarcomeric effect and myocellular pathophysiology especially with respect to the longitudinal, dynamic changes in Ca^{2+} handling and ventricular remodeling. We demonstrate the highly progressive nature of 160E cTnT – linked HCM is caused by a unique and likely additive effect of primary, dosage-dependent sarcomeric disruption and severe alterations in downstream Ca^{2+} handling that cause a uniquely aggressive pattern of ventricular remodeling.

2. Material and Methods

2.1 Transgenic mouse models

Sixteen- to 24-week-old C57Bl/6 mice bearing a *c-myc*-tagged murine cTnT with deletion of Glu160 (160E) were generated as previously described [20, 22]. Two independent lines were created that each expressed the transgene at a different percentage of total cTnT, 160E-35% and 160E-70%, yielding a low and high expressing line respectively. Each line was backcrossed to C57Bl/6 wildtype mice for 8–10 generations and protein expression verified at each generation. Each animal was genotyped via PCR-amplified tail DNA. Sibling mice were used to provide Non-Transgenic controls (paired for each set of experiments). Experimental protocols were approved by the Institute of Animal Studies at the Albert Einstein College of Medicine, and animal maintenance followed current NIH guidelines.

2.2 Cardiac protein isolation and Western Analysis

Myofibril isolation and subsequent SDS-PAGE/Western/Densitometry analysis to determine transgene expression levels were performed as described previously [29]. Semi-quantitative immunoblotting of whole cardiac homogenates was carried out as described previously [30].

2.3 Light and Ultrastructural Tissue Analysis

Tissue sections for sarcomeric ultrastructural analysis were isolated, fixed and stained with Uranyl-acetate and Lead-citrate and examined with a JEOL-100CX electron microscope as described in [31]. Histological analysis was performed on tissue sections from 6-month-old male mice. Both ultrastructural and light microscopic evaluation were performed in a blinded fashion and repeated 3 independent times.

2.4 Cardiac Myocyte Isolation

Ventricular myocytes were dissociated from 4–6-month old mice using a modified protocol as described previously [20]. In brief, heparinized mice were cervically dislocated and hearts were rapidly excised. The heart was cannulated via the aorta and perfused with Ca^{2+} -free Tyrode solution at 3 ml/min for 4 minutes. The heart was then perfused with a digestion solution (Ca^{2+} -free Tyrode) containing Liberase Blendzyme 1 (Roche), trypsin, and 12.5 μM CaCl_2 for 8–10 minutes. The left ventricle was cut into small pieces and gently triturated, allowing the myocytes to be dispersed. Cell suspension was filtered through a 250 μm mesh collector into a Myocyte Stopping Buffer of Ca^{2+} -free Tyrode with Bovine Calf Serum and 12.5 μM CaCl_2 . Cells were allowed to settle for 3–7 minutes, supernatant removed, and the cells were resuspended again in Myocyte Stopping Buffer with 200 μM CaCl_2 . After 6 minutes, supernatant was removed and the cells were resuspended in Ca^{2+} containing, BDM wash buffers, with Ca^{2+} concentrations gradually increasing from 0.2 to 1.0 mM Ca^{2+} . Cells were kept in the 1.0 mM Ca^{2+} solution until incubation on a flow chamber for contractility measurements.

Samples from each myocyte isolation were fixed and examined for transgene protein localization as described in [32]

2.5 Measurement of Myocellular Mechanics, intracellular Ca^{2+} transients, and SR Ca^{2+} load

Sarcomere length, contraction/relaxation measurements, intracellular Ca^{2+} and SR Ca^{2+} load were measured as previously described with the following modifications [20, 30]. For the measurement of SR Ca^{2+} load, Fura-2 loaded myocytes were stimulated at least ten times at 1 Hz (at 25°C) in 1.2 mM Ca^{2+} Tyrode solution containing probenecid in order to bring the cellular Ca^{2+} content to a steady state. Stimulation was paused for 1 second and the perfusate was rapidly switched to 0Na0Ca Tyrode for 30 seconds and subsequently to 0Na0Ca Tyrode containing 20 mM caffeine for 30 seconds. The caffeine was administered without field stimulation and resulted in a Ca^{2+} transient that corresponds to the total Ca^{2+} release from the sarcoplasmic reticulum (SR). The difference between the basal and peak total intracellular Ca^{2+} in the presence of caffeine corresponds to all the available SR Ca^{2+} .

2.6 Measurement of SR Ca^{2+} Uptake

SR Ca^{2+} uptake was determined as previously described [30].

2.7 Isovolumic Studies

Hearts from 4–6 month old mice were cannulated as described in the protocol above for isolating myocytes. The heart was perfused at 3 ml/min with a 37 °C, pH 7.4 modified Krebs-Henseliet solution (in mmol/L: NaCl 137, KCl 5.4, NaHCO_3 10, MgSO_4 2, glucose 10, CaCl_2 1.8) that was aerated with 95% O_2 / 5% CO_2 . An incision was made on the left

atria and to drain thebesian flow in the left ventricular cavity, a one-inch piece of PE-50 tubing with one end partially melted was passed through the mitral valve to pierce the apex of the heart. A custom-made balloon made from polyvinyl chloride film was passed through the mitral valve and inflated in the left ventricle to set an end-diastolic pressure of ~10 mmHg and was held constant for the duration of the recording. The balloon was tied to one end of a 12-inch piece of PE-50 tubing which has 23-gauge cannula attached to the other end. The balloon is filled with degassed sterile water and connected to a clip-on dome with a silicone base that was directly connected to pressure transducer (ADInstruments). A 100 μ l gas tight Hamilton syringe was also attached to the balloon-dome setup and was used to inflate and deflate the balloon. The balloon-dome setup was calibrated at the beginning of each experiment. Platinum wires were placed on the right atrium to electrically pace the heart at 400 bpm using 2 mA of current. The heart was then submerged in a bath containing perfusion solution to maintain the temperature at 37 °C for the duration of the experiment. After 20 minutes of perfusion to allow the heart to equilibrate to its environment, increasing bolus doses (0.01, 0.1, 1.0, 10, 100 μ mol/L) of dobutamine were administered every 5 min to the heart via an inline injection port. Pressure development over time was acquired via the the Powerlab ChartPro version 7.0 software system (ADInstruments, Colorado Springs) at a sampling rate of 200 Hz. The Blood Pressure module version 2.0 was used to compute the derivative of the pressure signal from the average of 10 consecutive tracings to compute peak positive change in pressure over time (+dP/dt) and peak negative change in pressure over time (−dP/dt).

2.8 Statistical Analysis

Values are presented as means $\pm$ SEM. Statistical analyses were performed using One-way ANOVA followed by Newman-Keuls post hoc analysis. A level of $p < 0.05$ was considered significant throughout.

3. Results

3.1. Characterization of cTnT-cMyc- Δ 160E transgenic mice with high and low levels of transgene expression

In the current study, we examined the Δ 160E mutation in mouse lines that express the mutant protein in the heart as 35% or 70% of total cTnT (Figure 1A). Δ 160E-cTnT was myc-tagged at its amino-terminus and expressed under the cardiac myocyte specific promoter of the rat β -myosin heavy chain gene as previously described [22, 29]. As noted in earlier models, transgene expression did not alter the overall expression of cTnT or its incorporation into the cardiac sarcomere (Figure 1B). The 70% line expressing twice the amount of mutant protein as the 35% line was developed to determine the direct effect of an increase in protein load as a determinant of the progressive remodeling caused by this mutation.

3.2. Δ 160E causes dose-dependent disruption in cellular architecture and sarcomere structure

Hematoxylin and eosin staining of sections taken from the left ventricle of both Δ 160E transgenic mouse lines revealed cellular hypertrophy and disarray in the left ventricle as well as myocyte degeneration and a general increase in disorganization at 70% transgene dosage (Figure 1C). Extensive fibrosis was not observed in either of the 6-month samples from the transgenic lines, though LV sections from the 70% Δ 160E hearts at 12 months showed more prominent (largely perivascular) fibrosis as compared to the 30% line (data not shown). These findings were consistent with the myocellular disarray observed in HCM patients. Ultrastructural analysis of left ventricular samples taken from transgenic and Non-Transgenic mice demonstrated that incorporation of Δ 160E cTnT caused myofibrillar

disarray, and Z-band misregistration in transgenic mouse hearts. Again, the structural disruption was qualitatively greater in the 70% line where sarcomeric hypercontraction and collapse was commonly observed (Figure 1D).

3.3. Progressive remodeling in $\Delta 160E$ transgenic hearts

In addition to dose-related changes in sarcomeric structure, $\Delta 160E$ mouse hearts exhibited extensive remodeling over time when compared to Non-Transgenic siblings. As noted in prior studies, both the 30 and 70% lines exhibited a decrease in overall size (Figure 2A) and cardiac mass, quantified as heart weight/body weight ratios (Figure 2B) [33]. Of note, no significant differences in body weight were observed among the different groups (data not shown). At 6 months, the two transgenic lines exhibited a similarly significant decrease in cardiac mass in comparison to Non-Transgenic. However, at 12 months, the 35% line had an even greater decrease in cardiac mass indicating progressive remodeling in the heart over time. The cardiac mass of the higher expressing line was decreased compared to Non-Transgenic, but was significantly higher than the 35% expressing line at the same age. On gross examination, while the 70% expressing hearts appeared larger at 12 months than those at 6 months of age, the overall mass remained the same due to the onset of early ventricular dilatation in the 70% line alone. These results suggest that this mutation causes a progressive ventricular remodeling in mice that increases in severity with transgene dose. The 70% hearts at 6 months resemble the 35% hearts at 12 months, consistent with an acceleration in ventricular remodeling caused by an increase in mutant cTnT protein load, an observation that could account for the oft-noted variation in clinical phenotype in HCM.

3.4. Cardiac myocyte mechanics are impaired in $\Delta 160E$ transgenic mice

In order to determine the effect of $\Delta 160E$ dosage on myocellular contractility, adult ventricular myocytes were isolated from hearts of both of the $\Delta 160E$ transgenic mouse lines and age matched, Non-Transgenic siblings and were field stimulated at 1 Hz. Both transgenic lines demonstrated impairments in both contraction and relaxation to a similar extent for most of the measured parameters. Both $\Delta 160E$ -35% and $\Delta 160E$ -70% exhibited shortened baseline sarcomere lengths (μm) compared to Non-Transgenic (1.67 ± 0.009 vs. 1.67 ± 0.008 vs. 1.78 ± 0.008), respectively (Figures 3A and B). This is consistent with previous measurements as well as findings in BDM-treated $\Delta 160E$ myocytes where mutants had a higher baseline level of sarcomeric activation [20]. Similar decreases occur in percent shortening (4.04 ± 0.21 vs 4.37 ± 0.23 vs 6.17 ± 0.37) (Figure 3C) and peak rate of contraction (1.03 ± 0.06 vs 1.29 ± 0.07 vs 1.72 ± 0.11) (Figure 3D), indicative of impaired contractility. Additionally, decreases in peak rate of relaxation (0.67 ± 0.06 vs 0.87 ± 0.07 vs 1.36 ± 0.14) and time to 90% relaxation (0.39 ± 0.02 vs 0.34 ± 0.01 vs 0.27 ± 0.01) were observed in both $\Delta 160E$ lines compared to Non-Transgenic, indicating impaired relaxation. Thus, the presence of the $\Delta 160E$ mutation in cTnT leads to significant alterations in both contraction and relaxation and the magnitude is independent of transgene dose.

3.5. $\Delta 160E$ ventricular myocytes exhibit dose-dependent impairments in calcium handling

Fura-2 was loaded into isolated myocytes and Fura-2 ratios were used as an index of intracellular calcium and transients in order to determine the effect of transgene dosage on myocellular calcium kinetics. The $\Delta 160E$ cells showed impairments in both systolic and diastolic calcium handling, concordant with the impaired contractility and relaxation seen in the mechanical measurements. Both 35%- and 70%-expressing mutants show significant decreases in resting intracellular calcium level versus Non-Transgenic (1.87 ± 0.083 vs 1.95 ± 0.135 vs 2.48 ± 0.085) (Figures 4A and B). The peak amplitude of the calcium transient decreased with transgene dosage compared to Non-Transgenic (35.26 ± 2.35 vs 17.37 ± 1.16 vs 50.22 ± 2.609) (Figure 4C), as did the peak rate of calcium rise (16.75 ± 1.32 vs $9.10 \pm$

0.925 vs 30.58 ± 1.72), consistent with the mechanical impairments in contraction (Figure 4D).

Diastolic impairments in calcium handling, e.g. the decreased ability to remove calcium from the cytoplasm, were also dose-dependent. The rates of calcium decline were decreased in 160E-35% and 70% compared to Non-Transgenic (3.63 ± 0.36 vs 1.80 ± 0.24 vs 8.32 ± 0.62) (Figure 4E). Complimentary to this measurement, the times to 90% calcium decline increased similarly (0.61 ± 0.014 vs 0.68 ± 0.018 vs 0.47 ± 0.012) (Figure 4F). Tau, a measurement of the exponential decay of transient Ca^{2+} decline from contraction to contraction, also indicated impaired relaxation with an increase in 160E-35% and 160E-70% compared to Non-Transgenic (0.26 ± 0.014 , 0.40 ± 0.026 vs 0.16 ± 0.005) (Figure 4G). This is consistent with an increase in residual cytoplasmic Ca^{2+} from beat to beat, and the increased cytoplasmic Ca^{2+} likely contributes to the observed impairment in relaxation. Interestingly, unlike most of the mechanical parameters, the affect on 160 E myocyte calcium transients were dose-dependent.

3.6. Transgenic myocytes exhibit a decrease in calcium load

To determine the contribution of SR calcium load to calcium handling defects, caffeine was rapidly administered to isolated myocytes to stimulate the total release of calcium from the sarcoplasmic reticulum. The amplitude of the peak Fura-2 ratio is a quantitative measurement of the total calcium load in the SR. However, while the SR calcium load in the transgenic myocytes was decreased compared to Non-Transgenic, there was no significant dosage effect between the two 160E lines (0.91 ± 0.17 vs 0.99 ± 0.14 vs 1.70 ± 0.15) (Figure 5). Therefore, primary changes in SR calcium load do not account for the observed dosage dependent differences in myocyte calcium handling.

3.7. SERCA2a activity is impaired in $\Delta 160\text{E}$ transgenic mice

We next measured SR calcium uptake, (mediated by SERCA2a and its regulatory protein phospholamban), and in both 160E lines, observed a marked decrease in SERCA2a function (Figures 6A and B). Maximum velocities in nmol/mg/min were significantly decreased for 160E-35% (476 ± 26) and 160E-70% (451 ± 34) compared to Non-Transgenic (682 ± 16). There were no changes in the calcium sensitivity or EC_{50} of uptake in 160E-35% (6.68 ± 0.08) and 160E-70% (6.70 ± 0.12) compared to Non-Transgenic (6.67 ± 0.03), indicating that the decrease in uptake was a result of reduced SERCA activity specifically, and not due to a change in the phosphorylation status of PLB (Figure 6C).

3.8. Expression and phosphorylation potential of $\Delta 160\text{E}$ hearts are altered in a dose-dependent manner

There was a trend toward a decrease in SERCA/PLB levels with increasing transgene expression, and the 160E-70% ratio was significantly decreased compared to Non-Transgenic (Figures 7A – D). PLB phosphorylation followed a similar expression pattern for both the PKA and CaMKII substrate sites, Ser16 and Thr17 respectively. Transgenic mice exhibited a trend toward decreased phosphorylation in 160E-35% cells and toward levels similar to or higher than Non-Transgenic in the 160E-70% myocytes (Figures 7E – G). Of note, there was a significant difference between the 30% and 70% lines when compared to each other. Finally, expression levels of the $\text{Na}^{+}/\text{Ca}^{2+}$ -Exchanger Channel (NCX) were significantly elevated only in the 160E-70% mice as compared to both Non-Transgenic and 160E-35% (Figures 7H and I). These cumulative findings are consistent with an accelerated development of a “molecular failure” pattern of intracellular Ca^{2+} kinetics in 160E-70% myocytes that closely correlates with the accelerated progression of ventricular remodeling in the higher expressing line.

3.9. Cardiac Performance in the Isovolumic Mode

Figure 8 illustrates the effects of the 160E mutation on whole heart physiology at baseline and in response to increased cardiac demand using bolus dobutamine injections (values and statistics are summarized in Table 1). In these studies we found that the major functional impairment in the 160E mice was diastolic. While there was no significant difference in the three lines at baseline, the 160E-70% hearts were significantly impaired compared to Non-Transgenic siblings in response to an inotropic challenge. Although the 160E-35% hearts were not significantly different than either the Non-Transgenic or the 70% expressing hearts, the values of +dP/dt and -dP/dt lie almost midway between the two, supporting the dose-dependent trend of impaired relaxation.

4. Discussion

In the present study, we have examined the cTnT- 160E mutation that is unique among the other TNT1 domain-HCM mutations due to its extensive structural, biophysical and physiologic effects. We have shown that the 160E mutation causes dramatic and progressive geometric, ultrastructural, and molecular ventricular remodeling. These differences, particularly regarding sarcomeric structure are likely due to the position of this mutation in a flexible loop at the C-terminus of the TNT1 domain. In the 160E mice, the transgene dose has a significant 2-tiered impact on the severity of disease. The primary effect is the impact of the mutation on the structure and orientation of cTnT within the troponin complex that appears to disrupt the integrity of the thin filament. The presence of increasing levels of mutant cTnT further disrupts sarcomeric structure and leads to the eventual collapse of sarcomeres as observed in the 160E-70% line. The secondary effect is the impact of the mutation on myocellular Ca^{2+} kinetics. While both of the 160E transgenic lines exhibit a decreased sarcoplasmic reticular Ca^{2+} load compared to Non-Transgenic, there is no significant difference in load between the two transgenic lines. There are, however, dose-dependent changes in the intracellular calcium release and uptake velocities and amplitudes. These additive effects on Ca^{2+} dynamics, coupled to the greater degree of structural disruption in the 160E-70% line are likely directly responsible for the observed accelerated ventricular remodeling and eventual dilatation.

Along similar lines, it is important to note that the murine models presented here require a significantly lower amount of transgene expression to elicit a pathologic response as compared to our previously published R92 mice (35 vs 50%) [32]. A much lower maximal transgene expression level is tolerated in the 160E mice (70%) as compared to another model based on a severe human mutation (R92Q-92%)[3, 31]. At low transgene doses the 160E mutation has a profound effect on sarcomeric structure not observed in R92 models even at very high transgene doses, suggesting that this mutation has a more severe, direct effect on myofilament structure, consistent with a unique primary mechanism as originally postulated by Palm, et al [21]. Taken together, mutations causing a primary structural disruption are tolerated at a much lower dose. This includes the most extreme case, the cTnT exon 15/16 truncation where murine models did not tolerate greater than 5% mutant protein [29].

As shown in Figure 2A, the observed end effect of increases in mutant protein levels in these models is to alter both the degree of remodeling and the speed with which it occurs. Thus, mice from either line that are studied at a given time point would be expected to exhibit clear phenotypic variability based on dose. This raises the question of whether similar mechanisms occur in human genetic HCM cohorts where variations in clinical phenotype are the rule rather than the exception. It has long been assumed that both mutant and normal alleles would be co-dominantly expressed in patients with HCM and thus the “effective” mutant protein dose would be expected to be 50%. This is consistent with the observed

autosomal dominant mode of inheritance. Exceptions have been previously noted, for example, mutations in Myosin Binding Protein-C (*MYBPC3*) exhibit variable expressivity at the protein level [33, 34]. More recently, Tripathi et al., demonstrated a striking degree of deviation from the expected 50:50 wild-type to mutated protein levels in cardiac and soleus muscle biopsies from patients with 5 independent HCM-linked myosin heavy chain mutations (MYH7) [35]. Protein expression levels ranged from 12 to 70% of total MyHC and were largely consistent for each mutation. While the patient sample sizes were not large, for several of the mutations a clear correlation between the amount of mutant MyHC at the protein level and the time course and severity of the clinical phenotype were observed. This “allelic imbalance” represents an added component to the clinical variability seen in patients with HCM and our cTnT-160E mouse models mirror the human results, thus providing a unique opportunity to longitudinally study the potential effects of varying mutant protein dosage on HCM pathogenesis.

It has long been noted that patients with HCM often exhibit impaired relaxation that is “out of proportion” to the degree of left ventricular hypertrophy [36, 37]. One potential intracellular mechanism for the observed decrease in compliance is an alteration in myocellular Ca^{2+} homeostasis. Multiple studies have documented significant dynamic changes in Ca^{2+} kinetics in animal models of HCM and recently, Schober, et al were able to link the oft-noted increase in the Ca^{2+} sensitivity of myofilament activation to an increase in cytosolic Ca^{2+} binding that they suggest may contribute to a decrease in the arrhythmia threshold [38]. The disruption in Ca^{2+} handling in the 160E mouse lines is complex and, unlike the R92W hearts, there is little evidence for an early temporal compensatory response, consistent with a mutation-specific mechanism. Given the profound effects on diastolic function at the whole heart level, we focused on the calcium handling proteins that are responsible for Ca^{2+} sequestration and extrusion from the cytosol. Note that while the current studies were performed at the 6 month time point, initial measurements at 2 months were identical (data not shown). While this early onset of Ca^{2+} dysregulation was also observed in the cTnT R92W line, both the magnitude of the changes and the lack of any significant early compensatory myocellular response were unique to the 160E lines [30]. The most consistent finding was a nearly 50% decrease in SERCA2a activity for both lines, with a concomitant decrease in SR Ca^{2+} load. While the mechanism of this shared decrease in the ability of the 160E myocytes to re-sequester Ca^{2+} in the SR was due, in part to a decrease in the SERCA2a/PLB ratio, there was clear evidence of a more accelerated molecular “failure” response in the 160E-70% line alone including a 2-fold increase in NCX expression and an increase in PLB Ser-16 phosphorylation. These observations for the 160E – 70% are consistent with a compensatory molecular response triggered by the accelerated remodeling. These alterations are not sufficient to be able to restore normal function at either the cellular or whole heart levels. Stepwise administration of dobutamine in isovolumic heart studies revealed significant and dosage-dependent impairments in relaxation and a blunting of the inotropic response for the 160E-70% line, again consistent with a more severe degree of molecular remodeling. We have previously shown that there is an energy-tension mismatch in 160E skinned fibers, and the present data similarly suggest that under stress the heart cannot accommodate the demand because of a lack of contractile reserve [22].

While direct comparisons to the human disorder should be made with caution, all of the available clinical data indicate that the cTnT-160E mutation causes a severe form of HCM in patients [3, 13, 26]. The results from the two independent 160E mouse models presented here suggest a complex multi-tiered disease mechanism encompassing both primary and secondary pathways. The loss of a single glutamic acid at residue 160 of cTnT within the extended linker at the C-terminus of the domain may cause an alteration in the orientation of the protein with respect to other components of the thin filament. Troponin T has been

shown to be essential in sarcomere assembly and thus this mutation may alter the dynamics of sarcomere turnover and act as a constant structural “stress” on the sarcomere that promotes the progressivity of the remodeling [39, 40]. Increasing the proportion of abnormal cTnT would be expected to cause a proportionally greater effect as we observed. Interestingly the impact of the higher mutant protein dose accelerated both the timing and the magnitude of the remodeling, potentially a significant factor in the human disorder. Of note, a recent report by Sun et al utilized induced pluripotent stem cells (iPSCs) isolated from patients carrying a cTnT mutation (R173W) in the same region of TNT1 that was previously linked to dilated cardiomyopathy [41]. Their findings were highly concordant with our murine models as the iPSCs exhibited not only a striking degree of sarcomeric disruption via EM but also evidence for altered Ca²⁺ handling. These results support the hypothesis that the C-terminal domain of TNT1 play an important structural role within the cardiac thin filament.

We and others have previously shown that the 160E mutation increases the Ca²⁺ sensitivity of myofilament activation in skinned fibers [22, 42]. Of note the magnitude of the shift was not significantly different between the two transgenic lines studied here (data not shown). This increase in Ca²⁺ sensitivity would both impair relaxation and increase the Ca²⁺ buffering potential of the sarcomeres, in this case, however, while the dose plays less of a role in the timing of the onset of the observed changes in Ca²⁺ dysregulation, it is likely that the additive effects of the more severe structural disruption play an significant role in the more rapid progression of the remodeling at all levels.

In the main, the work presented here clearly establishes the complex cellular mechanisms that drive the severe remodeling observed in patients with the cTnT 160E mutation. While the two-tiered effects of disruptions in both structure and Ca²⁺ homeostasis are unique to this mutation, the importance of understanding all of the potential myocellular “contributors” to the end phenotype cannot be underemphasized and has clear clinical utility. Not only with respect to the potential effects of allelic imbalance and the potential for clinical variability, but also for the eventual design of therapeutic interventions. For example, in the case of the 160E mutation, drugs designed to alter Ca²⁺ handling would be predicted to only partially arrest the pathogenic remodeling and would have to be instituted early in the course of the disorder. Finally, this work strongly supports the clinical findings by Tripathi, et al, suggesting that the level of mutant protein in HCM may play a significant role in disease severity and may represent a novel pathogenic factor [35].

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Highlights

- A novel cTnT-linked hypertrophic cardiomyopathy mouse model was generated
- Mutations in the unstructured linker domain of TNT1 cause sarcomeric disruption
- Severe alterations in myocellular Ca²⁺ handling contribute to pathogenesis
- Left ventricular remodeling was severe and dosage-dependent
- These findings are consistent with clinical and iPSC-based results and provide a unique model system

Figure 1. Characterization of cmyc-cTnT- $\Delta 160E$ transgenic mice

(A) Western blot analysis of heart homogenates from $\Delta 160E$ -35%, -70%, and Non-Tg mice probed with antibodies, as indicated. TnT upper band represents c-myc tagged transgenic protein and lower band represents endogenous cTnT. (B) Immunofluorescence staining of isolated myocytes against TnT (Non-Tg) and c-myc ($\Delta 160E$ lines) (C) Representative Hematoxylin and eosin staining of LV tissue non-transgenic (left), $\Delta 160E$ -35% (center), and $\Delta 160E$ -70% (right) mice at 6 months of age (D) Representative electron micrographs of left ventricular tissue sections from 6-month old mice are shown at 10,000 $\times$ magnification.

Figure 2. Gross morphology and size of transgenic hearts at 6 and 12 months

(B) Representative whole hearts (left panel) and ventricular cross-sections (right panel) from each transgenic line at two time-points. Non-Tg control was sibling-matched. Bar represents 5 mm. (C) Cardiac mass is represented as heart weight/body weight (HW/BW) ratios. Values are represented as mean $\pm$ SEM. ANOVA and Newman-Keuls post hoc analysis were used for statistical comparison to age-matched Non-Tg * $p < 0.05$, ** $p < 0.005$, NS not significant

Figure 3. Contraction and relaxation measurements of field-stimulated ventricular myocytes (A) Representative contraction/relaxation recordings from Non-Tg, $\Delta 160E-35\%$, and $\Delta 160E-70\%$ myocytes. (B–F) Cardiac myocyte mechanical measurements. An n of 4–6 animals were used for each group with at least 40 cells analyzed in total. Values are expressed as mean $\pm$ S.E.M. ANOVA and Newman-Keuls post hoc analysis were used for statistical comparison to Non-Tg. * $p < 0.05$ ** $p < 0.01$

Figure 4. Intracellular Ca^{2+} transient measurements of field-stimulated ventricular myocytes (A) Representative tracings of calcium transients from Non-Tg, 160E-35%, and 160E-70% myocytes. (B–G) Measurements of intracellular calcium transients represented as Fura-2 ratios. An n of 4–6 animals were used for each group with at least 40 cells analyzed. Values are expressed as mean $\pm$ S.E.M. ANOVA and Newman-Keuls post hoc analysis were used for statistical comparison to non-transgenic. ** $p < 0.01$

Figure 5. Caffeine-induced measurement of SR calcium load

(A) Representative tracings of myocytes from each group of mice include transients followed by a period of rest for 30 seconds then caffeine-stimulated total release of calcium from the SR. (B) Averages of peak amplitude of caffeine-induced SR calcium release. Data expressed as mean $\pm$ S.E.M. ANOVA and Newman-Keuls post hoc analysis were used for statistical comparison to Non-Tg ** $p < 0.01$

Figure 6. Sarcoplasmic reticulum calcium uptake

(A) Curves represent the best non-linear fits through the average calcium oxalate uptake velocities of 5 mice per group, each performed in triplicate. (B) The average values of calculated V_{max} for each group. (C) The average values of EC_{50} for each group. Data expressed as mean $\pm$ S.E.M. ANOVA and Newman-Keuls post hoc analysis were used for statistical comparison to Non-Tg *** $p < 0.0001$

Figure 7. Expression and phosphorylation levels of calcium handling proteins in 160E transgenic lines

(A) Representative blots probed with SERCA and PLB (B-D) Graphs summarizing SERCA and PLB expression levels (E) Representative P-PLB and PLB blots (F-G) Graphs summarizing PLB phosphorylation levels (H) Representative blots probed for NCX (I) Graphs summarizing NCX expression levels. $n = 5-7$ mice. Data expressed as mean $\pm$ S.E.M. ANOVA and Newman-Keuls post hoc analysis were used for statistical comparison to Non-Tg.

Figure 8. Contractile performance of 160E-35% and -70% hearts at baseline and in response to dobutamine

N= 7-9 mice for each group. Dobutamine given in bolus injections increasing as indicated.

Table 1

Values and Statistical Significance of Isovolumic Performance

	Non-Tg	160E-35%	160E-70%
<i>+dp/dt</i>			
Dobutamine (μM)			
0	2700 ± 94	3059 ± 136	3011 ± 163
0.01	4027 ± 229	3851 ± 219	3997 ± 261
0.1	3847 ± 210	4007 ± 157	3834 ± 259
1.0	3997 ± 241	3971 ± 253	3942 ± 333
10	5647 ± 460	5810 ± 237	4908 ± 339
100	6736 ± 617	6548 ± 487	5391 ± 329 *
<i>-dp/dt</i>			
Dobutamine (μM)			
0	-2173 ± 78	-2088 ± 132	-1793 ± 43
0.01	-2910 ± 116	-2390 ± 152*	-1970 ± 73***
0.1	-2770 ± 108	-2409 ± 171	-1909 ± 71***
1.0	-3000 ± 165	-2527 ± 126	-2219 ± 130***
10	-4379 ± 143	-4224 ± 149	-3447 ± 123 ***, ^{†††}
100	-4732 ± 164	-4666 ± 273	-3724 ± 123 ***, ^{†††}