In Vivo Analysis of Troponin C Knock-In (A8V) Mice
Evidence that TNNC1 Is a Hypertrophic Cardiomyopathy Susceptibility Gene

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Background—Mutations in thin-filament proteins have been linked to hypertrophic cardiomyopathy, but it has never been demonstrated that variants identified in the TNNC1 (gene encoding troponin C) can evoke cardiac remodeling in vivo. The goal of this study was to determine whether TNNC1 can be categorized as an hypertrophic cardiomyopathy susceptibility gene, such that a mouse model can recapitulate the clinical presentation of the proband.

Methods and Results—The TNNC1-A8V proband diagnosed with severe obstructive hypertrophic cardiomyopathy at 34 years of age exhibited mild-to-moderate thickening in left and right ventricular walls, decreased left ventricular dimensions, left atrial enlargement, and hyperdynamic left ventricular systolic function. Genetically engineered knock-in (KI) mice containing the A8V mutation (heterozygote=KI-TnC-A8V+/−; homozygote=KI-TnC-A8V+/+) were characterized by echocardiography and pressure–volume studies. Three-month-old KI-TnC-A8V+/+ mice displayed decreased ventricular dimensions, mild diastolic dysfunction, and enhanced systolic function, whereas KI-TnC-A8V−/− mice displayed cardiac restriction at 14 months of age. KI hearts exhibited atrial enlargement, papillary muscle hypertrophy, and fibrosis. Liquid chromatography–mass spectroscopy was used to determine incorporation of mutant cardiac troponin C (∼21%) into the KI-TnC-A8V−/− cardiac myofilament. Reduced diastolic sarcomeric length, increased shortening, and prolonged Ca2+ and contractile transients were recorded in intact KI-TnC-A8V−/− and KI-TnC-A8V+/+ cardiomyocytes. Ca2+ sensitivity of contraction in skinned fibers increased with mutant gene dose: KI-TnC-A8V+/+>KI-TnC-A8V−/−>wild-type, whereas KI-TnC-A8V−/− relaxed more slowly on flash photolysis of diazo-2.

Conclusions—The TNNC1-A8V mutant increases the Ca2+ binding affinity of the thin filament and elicits changes in Ca2+ homeostasis and cellular remodeling, which leads to diastolic dysfunction. These in vivo alterations further implicate the role of TNNC1 mutations in the development of cardiomyopathy. (Circ Cardiovasc Genet. 2015;8:653-664. DOI: 10.1161/CIRCGENETICS.114.000957.)

Key Words: Ca handling ■ calcium sensitization ■ cardiomyopathy ■ hypertrophic cardiomyopathy ■ knock-in mouse model ■ mouse ■ myofilament protein ■ TNNC1 ■ troponin ■ troponin C

When intracellular Ca2+ levels saturate cardiac troponin C’s (cTnC) single low-affinity regulatory Ca2+-binding site II, a series of conformational changes occur among the proteins that make up the thin filament, allowing myosin to cycle faster with actin, thus leading to force generation.1 Troponin C is part of a regulatory trimeric protein troponin complex (Tn) situated at regular intervals along the thin filament. Tn also comprises troponin I (TnI), which regulates Ca2+ sensitivity of contraction and negatively regulates myofilament responsiveness to Ca2+.2,3

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Inhibits cross bridge formation between myosin and actin and troponin T, which is important for myofilament activation and anchoring of the Tn complex to the thin filament.2,3 Fluctuations in intracellular Ca2+ during excitation–contraction coupling control the contractile and relaxation phases of cardiac contraction.1 Hence, perturbations in the Ca2+ affinity for tropomyosin inhibits cross bridge formation between myosin and actin and troponin T, which is important for myofilament activation and anchoring of the Tn complex to the thin filament.2,3

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Circ Cardiovasc Genet is available at http://circgenetics.ahajournals.org DOI: 10.1161/CIRCGENETICS.114.000957

Received November 10, 2014; accepted August 4, 2015.
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of cTnC can modify myofilament function, as demonstrated by in vitro studies.4,5

In an effort to determine whether the TNNC1 (cTnC gene) might be associated with hypertrophic cardiomyopathy (HCM), a large cohort of unrelated HCM patients was screened for mutations, which revealed the existence of 4 rare cTnC variants.6,7 Recently, 2 other TNNC1 mutations have been identified in HCM patients.7,8 Direct evidence that points toward causality for these mutations is lacking. The absence of positive family history of HCM disease or comprehensive linkage analysis has hindered the designation of TNNC1 as an established HCM susceptibility gene.9 Therefore, what has remained elusive is an established link between the variants found in TNNC1 and the disease processes that lead to the development of HCM.

Our laboratory has used in vitro tools to define cTnC variants as disease causing by assaying for functional changes and determining whether they fit within parameters of known, established pathogenic thin filament HCM mutations.6,9,10 In this regard, a rare heterozygote variant A8V in TNNC1 was identified in an HCM patient who was genotype-negative for mutations in 8 other sarcomeric protein genes.6 The substitution of alanine for valine at position 8 in cTnC is located in the N-terminal region, which is known to modulate Ca2+-binding affinity of both the C- and N-terminal domains.11,12 Assessments were made as to whether cTnC-A8V led to disease-like properties in functional tests that included porcine-skinned fibers and reconstituted actomyosin ATPase assays.6,9 In reconstituted functional assays, the cTnC-A8V mutant demonstrated an ability to sensitize the myofilament to Ca2+.3,5 Although this compilation of findings strongly suggested that the cTnC-A8V mutant was the cause of HCM in the patient, additional proof was still necessary. The proband had a negative family history of HCM, and therefore, the rare variant could not be shown to segregate with the disease.

Here, we describe the functional consequences of the first cTnC animal model generated to date, a mouse bearing the mutation A8V inserted via a knock-in strategy. As in the TNNC1-A8V proband, the knock-in (KI) mouse containing the cTnC-A8V (KI-TnC-A8V) also developed mild to moderate left- and right ventricular hypertrophy, hyperdynamic systolic function, and atrial enlargement. Furthermore, we explored the underlying cellular and molecular mechanisms governing the disease process in the KI-TnC-A8V mice. Altered mechanical function and Ca2+ handling in intact cardiomyocytes were accompanied by increased Ca2+ sensitivity of contraction, likely contributing to cardiac dysfunction. Profound clinical similarities regarding the manifestation of disease in the patient and animal model provide evidence that increased cTnC N-domain Ca2+ affinity is an underlying mechanism of HCM and that TNNC1-A8V is a definite HCM-causative mutation. Altogether, these findings further establish that TNNC1 is a HCM susceptibility gene.

Methods

For further details, see Methods in Data Supplement.

Clinical Data

The proband was presented to Mayo Clinic for clinical evaluation and management of HCM and consented to participate in an IRB-approved protocol.6

Mice

All protocols and experimental procedures followed National Institutes of Health guidelines and were approved by University of Miami, Florida State University, and Wayne State University Animal Care and Use Committee.

Statistical Analysis

All values are presented as mean±SE. Significance for echocardiography (ECHO), gene expression levels, Ca2+ and contractile transients in intact cardiomyocytes, Ca2+ sensitivity of contraction and flash photolysis in skinned fibers, and immunoblot data were determined by 1-way ANOVA using Fisher least significant difference or Bonferroni post hoc analysis. Significance for body weight (BW), heart weight (HW)/BW, atrial weight, and atrial/HW were determined by 1-way ANOVA with Tukey post hoc analysis, whereas 2-way or 1-way ANOVA with Tukey post hoc analysis was used for pressure-volume (P-V) studies depending on the comparison indicated in the legend of Figure 2. A priori alpha level of P<0.05 was considered to be significant.

Results

Clinical Presentation of the Patient With the TNNC1-A8V Mutation

The proband was first examined at the Mayo Clinic in 2003 and, after consenting to a study, was found to be genotype-positive for the rare missense A8V variant in TNNC1. Clinically, he was diagnosed with severe obstructive HCM at age 34, exhibiting increased left and right ventricular wall thickness and a left ventricular outflow tract gradient of 117 mm Hg at rest with systolic anterior motion of the mitral valve. An ECHO (Figure 1A and 1B) was performed indicating (1) increased ventricular septal thickness of 28 mm (normal value: 9–12 mm) and posterior wall thickness of 15 mm (normal value: 8–12 mm); (2) decreased left ventricular dimensions 38 mm (normal value: 43–57 mm) during the diastolic interval and 21 mm (normal value: 26–37 mm) during the systolic interval; (3) mild–moderate left atrial (LA) enlargement (end-diastolic dimension 49 mm [normal value: 31–45 mm], LA volume 40 cc/mm2 [normal value: 17–27 cc/mm2]); and (4) hyperdynamic left ventricular systolic function (calculated ejection fraction [EF] 73%). ECG tracings obtained during the following years of evaluation (2003–2005; Figure 1C–1G) showed evidence of myocardial ischemia, left ventricular hypertrophy, and LA enlargement. The patient had a negative family history for HCM or sudden cardiac death after genetic testing of his parents, grandparents, and members of his extended family. At the time of his diagnosis (2003), the proband had 2 sons, aged 12 and 14 years, who were asymptomatic and clinically negative for HCM by screening ECHO. Over time, the proband’s symptoms progressed to New York Heart Association Class III with chest pain, dyspnea on exertion, and light headedness, and in May of 2004, he underwent surgical septal myectomy. Based on the severity of disease, an implantable cardioverter defibrillator was implanted in October of 2004. A 24-h Holter recording during this time showed a sinus rhythm with a wandering atrial pacemaker, with the rate varying from 61 to 109 bpm. The patient and his sons have not been seen at the Mayo Clinic since 2005.

Echocardiography of KI-TnC-A8V Mice

The KI mice were analyzed by ECHO to determine how the cTnC-A8V variant affected cardiac function in mice at 3, 9, and
14 months of age. A representative M-mode image of the left ventricle from a 14-month-old KI-TnC-A8V+/+ mouse heart is shown in Figure 2B, in which a narrowing of the cardiac chamber lumen can be observed compared with age-matched wild-type (WT; Figure 2A). The end-diastolic volume (μL), end-systolic volume, and left ventricular dimensions were reduced significantly in KI-TnC-A8V+/+ at all ages tested. Left ventricular dimensions were significantly reduced only at 14 month for KI-TnC-A8V+/− compared with WT mice (see Table). Because KI mouse hearts may undergo changes in size, we attempted to calculate relative wall thickness. As shown in Table, significant changes in relative wall thickness can be observed in KI-TnC-A8V+/− and KI-TnC-A8V+/+ hearts as early as 3 months compared with WT hearts. The EF was increased in KI-TnC-A8V+/+ mice at 3 and 9 months of age, suggesting hyperdynamic left ventricular systolic function that was also seen in the patient (Table). The isovolumic contraction time was found decreased for the KI-TnC-A8V+/+ mice at 9 and 14 months of age. Additional changes occurred in parameters related to diastolic function (increased isovolumic relaxation time (RT) and decreased mitral valve early peak flow velocity/atrial peak flow velocity) for KI-TnC-A8V+/+ at different ages, consistent with Stage I diastolic dysfunction. Clinical findings from the patient indicated a mitral valve early peak flow velocity/atrial peak flow ratio of 1.13 (normal values 0.9–2.5) in an ECHO performed in 2003.

**Pressure–Volume (P–V) Studies of KI-TnC-A8V Mice**

Cardiac function was examined in ex vivo working heart preparations at baseline and 3, 10, or 30 nmol/L isoproterenol in 12- to 13- month-old mice. Higher stroke volumes were seen when normalized to HW in KI-TnC-A8V+/− and KI-TnC-A8V+/+ compared with WT hearts (Figure 2C), indicating augmented systolic function. KI-TnC-A8V+/− hearts had higher LVPmax values compared with WT and KI-TnC-A8V+/+ hearts (Figure 2D), whereas the KI-TnC-A8V+/+ hearts displayed higher LVPmin values compared with KI-TnC-A8V+/− hearts (Figure 2E). The diastolic velocity (−dP/dt) of KI-TnC-A8V+/− hearts was decreased compared with WT and KI-TnC-A8V+/+ hearts (Figure 2G). Examination of baseline cardiac function revealed higher LVPmax values in KI-TnC-A8V+/− compared with KI-TnC-A8V+/+ and lower −dP/dt in KI-TnC-A8V+/− compared with KI-TnC-A8V+/+ and WT hearts, indicating impaired diastolic function (Figure 2E and 2G). The systolic velocity (+dP/dt) of KI-TnC-A8V+/− and KI-TnC-A8V+/+ hearts were similar to that of WT hearts (Figure 2F).

**Heart Weight of KI-TnC-A8V Mice**

In 12- to 13- month-old mice, no significant difference was found in the BW of KI-TnC-A8V+/− and KI-TnC-A8V+/+ compared with WT mice (Figure 3A). However, the HW to BW ratio (HW/BW) was significantly reduced in both KI-TnC-A8V+/− and KI-TnC-A8V+/+ mice (Figure 3B). LA mass in KI-TnC-A8V+/+ mice was dramatically increased as shown by the increased weight of the LA alone and when compared with the whole heart weight LA/HW ratio compared with that of WT hearts (Figure 3C and 3D), suggesting that adaptive remodeling occurred in response to impaired left ventricular diastolic function. No significant changes were observed in the right atria of these mice (data not shown).

**Cardiac Fetal Gene Expression in Mutant KI-TnC-A8V Mice**

Consistent with the previous findings indicating occurrence of a cardiomyopathic process, mRNA expression levels of brain natriuretic peptide and atrial natriuretic peptide by quantitative reverse transcriptase polymerase chain reaction were significantly increased in the left ventricle of 16- to 18-month-old KI-TnC-A8V+/− and KI-TnC-A8V+/+ mice compared with age-matched controls, whereas mRNA expression levels of myosin heavy chain β increased only in the KI-TnC-A8V+/− left ventricle (Figure 3E). In addition, we assessed the expression levels of fetal genes in the right ventricle. Only KI-TnC-A8V+/−
mice showed a significant increase in brain natriuretic peptide and atrial natriuretic peptide gene expression compared with WT mice (Figure 3F).

Assessment of Histopathology in KI-TnC-A8V Hearts

The hearts of 16- to 18-month-old mice were stained using Masson trichome and evaluated for changes in tissue...
architecture and fibrosis. Representative images are shown in Figure 4, and the black arrows indicate the location of the papillary muscle. Compared with WT hearts (Figure 4A and 4D), the hearts of KI-TnC-A8V+/− (Figure 4B and 4E) and KI-TnC-A8V+/+ (Figure 4C and 4F) exhibited papillary muscle hypertrophy, as well as interstitial fibrosis, consistent with findings of HCM. In addition, myofibrillar disarray, atrial enlargement, right ventricular wall, and apical hypertrophy were observed in both KI-TnC-A8V+/− and KI-TnC-A8V+/+ hearts as shown in Figure II A–III in the Data Supplement.

Determination of Mutant to WT Protein Ratio in the KI-TnC-A8V+/− Hearts

Because we are studying KI mice with either 1 or 2 alleles mutated, we attempted to determine the ratio of mutant to WT cTnC protein incorporated into the KI-TnC-A8V+/− hearts. Heart extract samples of 4-month-old mice were resolved by 15% SDS-PAGE, and the cTnC band was excised and extracted from the SDS-PAGE gel, followed by trypsinization. The samples were then analyzed by liquid chromatography mass spectrometry quantification (see Methods in the Data Supplement for further details). Cardiac TnC was digested at the residue number 8 of cTnC. Figure 4G shows the retention ratio of mutant cTnC to WT cTnC-A8V+/− and KI-TnC-A8V+/+ cardiac myocytes contributed to an increased contractile percentage at all frequencies of stimulation. Cardiac myocytes compared with those of WT at all stimulation frequencies. Next, we evaluated the contractility percentage and found that the cTnC-A8V mutation present in both KI-TnC-A8V+/− and KI-TnC-A8V+/+ hearts, and the intracellular Ca2+ and sarcomere length (SL) parameters were recorded at 4 different stimulation frequencies (1, 2, 4, and 6 Hz). From these experiments, we sought to understand how hemodynamics was changed and whether function was impaired at the cellular level because of the incorporation of mutant cTnC into the myofilament. Figure 5A shows that diastolic SL was significantly shorter in KI-TnC-A8V+/− and KI-TnC-A8V+/+ mouse hearts, and the diastolic Ca2+ levels only at 6 Hz (Figure 5C). Furthermore, the peak Ca2+ to baseline measured as a percentage was monitored by Fura-2 pentakis (acetoxymethyl) ester. It was found that KI-TnC-A8V+/− cardiomyocytes possess higher diastolic Ca2+ levels at all pacing frequencies compared with those of WT at all stimulation frequencies. Next, we evaluated the contractility percentage and found that the cTnC-A8V mutation present in both KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes contributed to an increased contractile percentage at all frequencies of stimulation (Figure 5B). This finding is consistent with the hypercontractile phenotype as evidenced by the increased stroke volumes for KI-TnC-A8V+/− and KI-TnC-A8V+/+ and EF for KI-TnC-A8V+/+. Table further measurements were conducted to determine resting intracellular Ca2+ levels, as monitored by Fura-2 pentakis (acetoxymethyl) ester. It was found that KI-TnC-A8V+/− cardiomyocytes possess lower diastolic Ca2+ levels at all pacing frequencies compared with age-matched WT cardiomyocytes, whereas KI-TnC-A8V+/+ showed lower diastolic Ca2+ levels only at 6 Hz (Figure 5C). Furthermore, the peak Ca2+ to baseline measured as a percentage was significantly diminished in both KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes compared with WT cardiomyocytes, as shown in Figure 5D. The gene-dosage effect, however, is evident in the diastolic SL and peak Ca2+ measurements.

Table. Summary of Echocardiographic Data From Wild-Type (WT) and Both Heterozygous (HT) and Homozygous (HM) KI-TnC-A8V Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3 mo</th>
<th>9 mo</th>
<th>14 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n=10)</td>
<td>HT (n=12)</td>
<td>HM (n=14)</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>458±6</td>
<td>448±12</td>
<td>447±8</td>
</tr>
<tr>
<td>EDV, µL</td>
<td>55±4</td>
<td>51±6</td>
<td>37±2†</td>
</tr>
<tr>
<td>ESV, µL</td>
<td>17±3</td>
<td>14±2</td>
<td>8±1‡</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.6±0.1</td>
<td>3.4±0.1</td>
<td>3.1±0.1*</td>
</tr>
<tr>
<td>LVEESD, mm</td>
<td>2.1±0.1</td>
<td>1.9±0.1</td>
<td>1.6±0.1*</td>
</tr>
<tr>
<td>EF, %</td>
<td>71±3</td>
<td>73±3</td>
<td>78±3‡</td>
</tr>
<tr>
<td>AET, ms</td>
<td>50±3</td>
<td>54±2</td>
<td>52±2</td>
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<tr>
<td>DecelTime, ms</td>
<td>15±2</td>
<td>14±1</td>
<td>14±1</td>
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<tr>
<td>IVRT, ms</td>
<td>20±1</td>
<td>20±1</td>
<td>27±2§</td>
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<tr>
<td>IVCT, ms</td>
<td>17±2</td>
<td>18±2</td>
<td>14±1</td>
</tr>
<tr>
<td>MV E/A</td>
<td>1.6±0.2</td>
<td>1.6±0.1</td>
<td>1.1±0.5‡</td>
</tr>
<tr>
<td>RWT, %</td>
<td>51±5</td>
<td>61±3‡</td>
<td>71±1*§</td>
</tr>
<tr>
<td>LV mass, mg</td>
<td>145±14</td>
<td>149±10</td>
<td>103±6†</td>
</tr>
</tbody>
</table>

Data are mean±SEM. A indicates atrial peak flow velocity; AET, aortic ejection time; CO, cardiac output; DecelTime, deceleration time; E, early peak flow velocity; EDD, end diastolic diameter; EDV, end-diastolic volume; EF, ejection fraction; ESD, end systolic diameter; ESV, end-systolic volume; FS, fractional shortening; HR, heart rate; IVCT, isovolumetric contraction time; IVRT, isovolumetric relaxation time; LV, left ventricular; MV, mitral valve; PWTd, posterior wall thickness during diastole; RWT, relative wall thickness; and SV, stroke volume. RWT was calculated as follows: ((2×PWTd)/LVEDD)×100) ANOVA with least significant difference post hoc test.

*p<0.01 HT or HM vs WT within age timepoint. †p<0.05 HM vs HT within age timepoint. ‡p<0.05 HT or HM vs WT within age timepoint. §p<0.01 HM vs WT within age timepoint.
Kinetics of SL Shortening and Ca²⁺ Transient in Intact Cardiomyocytes From KI-TnC-A8V

To further understand the cellular mechanisms underlying development of HCM elicited by introduction of the cTnC-A8V mutant in mice, we evaluated the kinetics of SL shortening and intracellular Ca²⁺ transients in isolated cardiomyocytes (the representative raw and normalized data traces are shown in Figure IIIA in the Data Supplement). This experiment allowed us to investigate the effects of the mutant in an intact cellular system, without the complications of autonomic modulation. The experiment was conducted and recorded using a range of frequencies, from 1 to 6 Hz. In Figure 6A and 6B, the SL shortening and intracellular Ca²⁺ kinetics parameters were recorded using 2 higher pacing frequencies (4 and 6 Hz) to evoke outcomes more consistent with cardiac mouse physiology. As shown in Figure 6A (top panels), the $T_{50}$ RT is determined to be 50% of the time it takes for the SL to return from peak to baseline, and was prolonged in KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes at 4 and 6 Hz stimulation frequencies compared with WT cardiomyocytes. Note that at 4 Hz, the $T_{50}$ RT for SL in KI-TnC-A8V+/+ cardiomyocytes was also significantly prolonged compared with that in KI-TnC-A8V−/− cardiomyocytes. These results suggest that the kinetics of cardiomyocyte relaxation was affected in a gene dose–dependent fashion. The rates of cell relengthening at 4 and 6 Hz were consistent with results obtained for $T_{50}$ RT, excluding the possibility that a delay in SL relaxation could be caused by excessive cell shortening (Figure 6A, middle panels). The SL shortening...
time from baseline to peak was found to be significantly prolonged in KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes at 4 and 6 Hz compared with WT cardiomyocyte. Note that KI-TnC-A8V+/+ cardiomyocytes was also significantly prolonged compared with KI-TnC-A8V+/− at both frequencies (Figure 6A, bottom panels). Next, we investigated whether the slower SL shortening and relengthening kinetics found in KI-TnC-A8V cardiomyocytes correlated with changes in the Ca²⁺ transient. The \( T_{50} \) for Ca²⁺ decay (50% of the time for the intracellular Ca²⁺ to return from peak to baseline) was found to be significantly delayed in KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes compared with WT cardiomyocyte (Figure 6B, top panels). The time from baseline to peak for intracellular Ca²⁺ was also investigated and found to be delayed in KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes when paced at 4 and 6 Hz (Figure 6B, bottom panels). The SL \( T_{50} \) RT was found to be prolonged similarly in both KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes when stimulated at lower frequencies compared with WT cardiomyocyte, whereas the effect of gene dosage on the delay in \( T_{50} \) of Ca²⁺ decay was also seen at 1 Hz (Figure IIIB in the Data Supplement).

**Ca²⁺ Sensitivity of Contraction and Rate of Relaxation in Skinned Fibers From KI-TnC-A8V**

To evaluate whether the TnC-A8V mutant elicited cellular, morphological, and hemodynamic changes in the heart, time from baseline to peak was found to be significantly prolonged in KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes at 4 and 6 Hz compared with WT cardiomyocyte. Note that KI-TnC-A8V+/− cardiomyocytes was also significantly prolonged compared with KI-TnC-A8V+/− at both frequencies (Figure 6A, bottom panels). Next, we investigated whether the slower SL shortening and relengthening kinetics found in KI-TnC-A8V cardiomyocytes correlated with changes in the Ca²⁺ transient. The \( T_{50} \) for Ca²⁺ decay (50% of the time for the intracellular Ca²⁺ to return from peak to baseline) was found to be significantly delayed in KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes compared with WT cardiomyocyte (Figure 6B, top panels). The time from baseline to peak for intracellular Ca²⁺ was also investigated and found to be delayed in KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes when paced at 4 and 6 Hz (Figure 6B, bottom panels). The SL \( T_{50} \) RT was found to be prolonged similarly in both KI-TnC-A8V+/− and KI-TnC-A8V+/+ cardiomyocytes when stimulated at lower frequencies compared with WT cardiomyocyte, whereas the effect of gene dosage on the delay in \( T_{50} \) of Ca²⁺ decay was also seen at 1 Hz (Figure IIIB in the Data Supplement).

**Figure 4.** Histopathologic analysis of KI-TnC-A8V mouse hearts at 16 to 18 months of age. Representative Masson trichrome stained sections were imaged at 10× magnification. A–C, The black arrows indicate the location of papillary muscles. Papillary muscle hypertrophy can be seen in KI-TnC-A8V+/− and KI-TnC-A8V+/+ mouse hearts. Evidence of papillary muscle hypertrophy can also be seen at lower magnification in Figure IIA–IIC in the Data Supplement. E–F, Interstitial fibrosis can be seen in heart sections obtained from the same group of mice. G, The retention time of the cTnC peptides separated by liquid chromatography mass spectrometry. The graphs show the ratio cTnC A8V:WT in the KI-TnC-A8V+/− hearts quantified by the abundance of 15 and 11 amino acid peptides. n=3.
because of the fundamentally increased Ca\textsuperscript{2+} affinity of the myofilament, the Ca\textsuperscript{2+} sensitivity of force development in papillary skinned fibers obtained from KI-TnC-A8V +/− and KI-TnC-A8V+/+ mice was tested at 2 different ages (4 months and 14–16 months). The 4-month-old KI-TnC-A8V +/− (pCa\textsubscript{50}=6.01±0.02) and KI-TnC-A8V+/+ (pCa\textsubscript{50}=6.28±0.02) skinned fibers demonstrated significantly increased Ca\textsuperscript{2+} sensitivity of contraction compared with age-matched WT (pCa\textsubscript{50}=5.88±0.03) as shown in Figure 7A. The cooperativity of thin filament activation (n\textsubscript{Hill}) was significantly decreased in the KI-TnC-A8V+/+ (n\textsubscript{Hill}=1.79±0.06) skinned fibers compared with that from WT (n\textsubscript{Hill}=2.59±0.14) and KI-TnC-A8V +/− (n\textsubscript{Hill}=2.26±0.14), whereas the n\textsubscript{Hill} of the KI-TnC-A8V +/− skinned fibers remained unchanged compared with that of WT (Figure 7A). The same trend of increased Ca\textsuperscript{2+} sensitivity appeared to occur in a gene-dose–dependent fashion when the experiments were conducted with 14- to 16-month-old mice. The n\textsubscript{Hill} from the 14- to 16-month-old mice also followed the same trend as that of the 4-month-old group (Figure IV in the Data Supplement). Flash photolysis of the diazo-2–loaded skinned fibers indicated that the rate of relaxation was significantly slower for KI-TnC-A8V+/+ compared with WT (Figure 7B). The rate constants and amplitudes are included in the figure legend.

Immunoblotting for Detection of Key Ca\textsuperscript{2+}-Handling Proteins in KI-TnC-A8V Hearts

The proteins responsible for loading the sarcoplasmic reticulum (SR) were assessed to determine whether they underwent adjustments to maintain cellular Ca\textsuperscript{2+} homeostasis. Figure 7C shows the representative immunoblots for 9-month-old hearts. The rationale for conducting these experiments at this age is to identify early molecular markers for the disease in KI-TnC-A8V/+ mice because morphological changes are still not evident. Sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2) protein levels were reduced in heart extracts obtained from KI-TnC-A8V+/+ and KI-TnC-A8V+/+ mice (Figure 7D). The phosphorylation levels of serines 23,24 in cTnI (TnI-P) were also reduced in both KI-TnC-A8V compared with WT hearts, whereas the phosphorylation levels of serine 16 in PLN (PLN-P\textsuperscript{Ser16}) were significantly decreased only in KI-TnC-A8V+/+ hearts (Figure 7D). The protein levels of phospholamban (PLN-T), calsequestrin (CASQ2), and Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchanger (NCX1) were found unaltered in KI-TnC-A8V+/+ and KI-TnC-A8V+/+ heart extracts (Figure 7D). We also looked for changes that occurred in protein expression and phosphorylation of SERCA2 and PLN, respectively, in younger (4-month-old) and older (16- to 18-month-old) groups of mice (Figure V in the Data Supplement). Interestingly, the phosphorylation levels of threonine 17 in PLN (PLN-P\textsuperscript{Thr17}) were statistically lower in both KI-TnC-A8V/+ and KI-TnC-A8V+/+ hearts throughout the 3 ages tested. The levels of PLN-P\textsuperscript{Ser16} in KI-TnC-A8V/+ hearts were reduced at 4 months, unchanged at 9 months, and again reduced at 16 to 18 months compared with WT hearts, suggesting that some adaptation process to disease has occurred by midage. In KI-TnC-A8V+/+ hearts, the levels of PLN-P\textsuperscript{Ser16} were lower at all the different ages tested. The levels of PLN-T were lower in both KI-TnC-A8V genotypes.
only at 16 to 18 months, whereas SERCA2 was lower at 9 months and 16 to 18 months (Figure V in the Data Supplement). These data suggest that decreased levels of SERCA2 and PLN-PThr17 could provide early molecular indicators of disease in KI-TnC-A8V+/− hearts.

**Discussion**

The objectives of this work were (1) to provide conclusive evidence that TNNC1 is an uncommon but definitive HCM-susceptibility gene; (2) to determine whether changes in the N-terminal domain Ca²⁺-binding affinity of cTnC in a KI mouse containing...
the human HCM-associated A8V mutation are sufficient to induce cardiac remodeling that resembles cardiomyopathic human diseases; and (3) to investigate the cellular and molecular mechanism underlying the morphological changes in the heart and diastolic dysfunction caused by the aforementioned mutation, while correlating all these results with the clinical phenotype of the proband.

On initial evaluation, the proband reported a negative family history of HCM because his children had not developed disease at this point. Therefore, the lack of unambiguous linkage studies for the cTnC variants discovered has thus far prevented the firm establishment of TNNC1 as an HCM-susceptibility gene. To date, there are 7 cTnC mutations described that are associated with HCM, and only 2 have demonstrated inheritance among family members.6–8,13 Because of the lack of co-segregation studies, in vivo studies, such as the one presented here, are therefore of utmost importance. Here we report the cardiac phenotype obtained in the first mouse model specifically designed to test the role of TNNC1 in HCM-associated disease processes.

Similar to the KI-TnC-A8V mouse model presented here, animal models bearing troponin T or TnI mutations associated with HCM either had unchanged or decreased HW/BW ratio.14–17 Although mouse models do not entirely recapitulate human disease processes, some disease traits are still manifested, for example, diastolic dysfunction and predisposition to arrhythmias.14,18,19 In this study, we attempted to correlate morphological changes in the heart of the human patient with those of the mouse model containing the TNNC1-A8V mutation. The KI-TnC-A8V mouse mirrored the cardiac remodeling seen in the patient, who displayed mild to moderate right and left ventricular hypertrophy and LA enlargement as shown by relative wall thickness and histopathologic studies.20 The LA enlargement seen in the TNNC1-A8V patient and in the KI mouse suggest that this mutation may also be implicated in the development of a restrictive cardiomyopathy (RCM)-like phenotype. This mixed phenotype has become acknowledged in the contemporary classification of inherited cardiomyopathy, which highlights the clinical overlap in the presentation of cardiomyopathies, such as HCM and RCM, defined previously as distinct clinical entities.21

Diastolic dysfunction is a clinical hallmark of HCM. Therefore, an important question to address is the source of impaired left ventricular relaxation. On studying the KI-TnC-A8V mouse, we identified interstitial fibrosis and myofibrillar disarray. These changes in tissue organization may contribute to increased stiffness and impaired muscle relaxation in HCM patients.20 The LA enlargement seen in the TNNC1-A8V patient and in the KI mouse suggest that this mutation may also be implicated in the development of an RCM-like phenotype. This mixed phenotype has become acknowledged in the contemporary classification of inherited cardiomyopathy, which highlights the clinical overlap in the presentation of cardiomyopathies, such as HCM and RCM, defined previously as distinct clinical entities.21

Experiments performed at the cellular level with KI-TnC-A8V cardiomyocytes indicated a delay in mechanical...
relaxation and intracellular Ca\textsuperscript{2+} decay times. The molecular explanation for this phenomenon may be that the A8V substitution increases the Ca\textsuperscript{2+}-binding affinity of cTnC and consequently delays the Ca\textsuperscript{2+} dissociation rate from the thin filament, which we have previously shown in reconstituted systems.\textsuperscript{23,24} The drastic Ca\textsuperscript{2+} sensitization observed in reconstituted porcine cardiac fibers (+0.36 pCa units) supported our rationale to subsequently develop the KI-TnC-A8V mouse because this cTnC mutant elicited the largest shift in this parameter among the other cTnC HCM mutants tested.\textsuperscript{8} Measurements of the Ca\textsuperscript{2+} sensitivity of contraction in the KI-TnC-A8V skinfied fibers recapitulated our previous findings in reconstituted fibers. These data suggest that increased myofilament Ca\textsuperscript{2+} sensitization causes the impaired mechanical relaxation and delayed Ca\textsuperscript{2+} reuptake times in the intact KI-TnC-A8V cardiac myocytes, thus fitting a common pathogenetic process seen in TnI and troponin T models of HCM.\textsuperscript{14,16,26} More recently, we and others have shown that genetically engineering the myofilament to desensitize it to Ca\textsuperscript{2+} can potentially reverse the aberrant phenotypes associated with HCM and RCM caused by troponin and tropomyosin mutations.\textsuperscript{25,26}

We found that KI-TnC-A8V cardiomyocytes have shortened SLs during diastole and reduced intracellular Ca\textsuperscript{2+} oscillation between diastole and systole. These findings led us to hypothesize that under resting conditions, the cardiac myofilaments containing cTnC-A8V are preactivated.\textsuperscript{32,37} Thus, only a small increase in intracellular Ca\textsuperscript{2+} levels (lower than normal physiological requirements) is sufficient to trigger muscle activation equivalent to or greater than that occurring during systole because EF and SL percentage of contraction in cardiomyocytes was increased. We and others have reported that HCM and RCM troponin mouse models may have a basal activation of contraction under resting conditions in skinfied fibers and intact cells. This, in turn, impairs the relaxation phase in diastole.\textsuperscript{14,16,28}

Adjustments to the levels of Ca\textsuperscript{2+}-handling proteins at the plasma membrane and the SR are known to occur during heart disease. Consistent with other mouse models of HCM, levels of SERCA2 were found to be decreased in the KI-TnC-A8V hearts, suggesting that the SR is unable to properly buffer intracellular Ca\textsuperscript{2+} levels. Therapeutics that increase Ca\textsuperscript{2+} buffering in cardiomyocytes during active disease states, such as HCM and RCM, may have a basal activation of contraction under resting conditions in skinfied fibers and intact cells. This, in turn, impairs the relaxation phase in diastole.\textsuperscript{14,16,28}

The decreased levels of PLN-T seen only at 16 to 18 months may indicate that the disease may have progressed to a more advanced stage and a combination of disease and effects of aging. The decreased levels of cTnI-P observed in KI-TnC-A8V mouse hearts is suggested to be a hallmark of the disease process because it was previously found that cardiac samples obtained from HCM patients who harbor mutations in sarcomeric proteins have decreased levels of cTnI-P.\textsuperscript{35} The decreased phosphorylation levels of cTnI may be explained by the increased activity of phosphatases seen in patients transitioning into different stages of disease, such as heart failure.\textsuperscript{32}

Herein, we describe the functional consequences of the first cTnC animal model generated to date, a mouse that bears the human heterozygous mutation A8V inserted via a knock-in strategy. The heterozygote and homozygote mice displayed functional and morphological changes that were consistent overall with the HCM phenotype, presenting a striking correlation to the phenotype of the human proband. In conclusion, this study provides compelling evidence that mutation in TNNCI is a pathogenic mechanism that underlies the development of HCM.

Acknowledgments
We thank Ana Rojas, Edda Ruiz, Jingsheng Liang, and Brittany Griffin for the technical and animal husbandry assistance. The liquid chromatography mass spectrometry experiment was performed in the Translational Science Laboratory, Florida State University (FSU) College of Medicine. We thank Dr Roger Mercer, Director of Translational Science Laboratory, for providing rapid and efficient service.

Sources of Funding
This research was supported by National Heart, Lung, and Blood Institute of the National Institutes of Health Grant HL103840 to Dr Pinto, HL098945 to Dr Jin, American Heart Association Postdoctoral Fellowship 09POST230030 to Dr Parvatiyar, Florida Heart Research Institute to Dr Pinto, and the Mayo Clinic Windland Smith Rice Comprehensive Sudden Cardiac Death Program to Dr Ackerman. Dr Pinto acknowledges generous start-up support from the FSU College of Medicine.

Disclosures
Dr Ackerman is a consultant for Boston Scientific, Gilead Sciences, Medtronic, and St Jude Medical and also receives royalties from Transgenomic for FAMILION-Long QT Syndrome and FAMILION-Catecholaminergic Polymorphic Ventricular Tachycardia testing. However, none of these companies had anything to do with this study. The other authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

This study is the first to directly validate the pathogenicity of a *TNNC1* variant A8V found in a 34-year-old patient diagnosed with severe obstructive hypertrophic cardiomyopathy (HCM). The patient displayed mild-to-moderate thickening of both ventricular walls, decreased ventricular dimensions, left atrial enlargement, and hyperdynamic left ventricular systolic function. Since the discovery of *TNNC1* mutations in HCM patients, in vitro methods suggested that mutant cardiac troponin C (*cTnC*) protein incorporated into myofilaments could cause disease. In response, *TNNC1* has been included in commercially available HCM gene panels for patient screening. The absence of linkage and co-segregation studies in patients carrying mutations in *TNNC1*, however, has complicated efforts to establish pathogenicity. To address this disconnect between diagnosis and causation, we developed a knock-in mouse model containing the *TNNC1-A8V* mutation and here report the physiological consequences. *TNNC1-A8V* mice recapitulated significant aspects of human HCM disease. Pathogenesis was elicited by alterations in calcium-binding affinity of the thin filament that affected overall calcium homeostasis and led to cardiac remodeling and development of cardiomyopathy. The knowledge obtained from in vivo studies, such as this, may help identify targetable regions in *cTnC* that could be used to normalize thin filament function and thereby alleviate symptoms of HCM disease.
In Vivo Analysis of Troponin C Knock-In (A8V) Mice: Evidence that TNNC1 Is a Hypertrophic Cardiomyopathy Susceptibility Gene


Circ Cardiovasc Genet. 2015;8:653-664; originally published online August 24, 2015; doi: 10.1161/CIRCGENETICS.114.000957

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circgenetics.ahajournals.org/content/8/5/653

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SUPPLEMENTAL MATERIAL

Expanded Methods and Results

Clinical data - Following written informed consent, DNA was obtained upon venipuncture and subjected to genetic testing as previously described. Clinical data was obtained after reviewing the electronic medical record.

Construction of the KI-TnC vector - A genomic clone encompassing the entire murine cTnC gene was isolated from a mouse 129/SV fix DNA library (Stratagene). The cTnC mutant A8V was generated using sequential overlapping PCR-based methods. The “Targeting Vector” construct was generated in the vector Osdupdel (a gift from O. Smithies, Univ. of North Carolina), which contains MCI neo flanked by loxP and thymidine kinase under the control of 3’ phosphoglycerate kinase (PGK). The strategy for mutating murine cTnC was the following; a 3.2 kb fragment with Kpn1 /Xho1 sites from the 5’ of the cTnC gene was ligated into the vector downstream of the neo gene that was homologous to the 5’ region. A 5.4 kb fragment of the targeting gene with an Xho1 site containing the mutation was ligated into the Nhe1 site between the neo and thymidine kinase gene (tk) to form the 3’ homology region. Ligation orientation was confirmed by restriction enzyme digestion and sequencing. Supplemental Figure 1A presents a schematic outlining the strategy used to generate the A8V mice. This figure depicts “The TnC Locus” the restriction enzyme map of the wild-type cTnC (WT) gene and the organization of exon-introns 1-6. In addition, the targeting construct is shown which was used to introduce the loxP flanked neo cassette into intron 1 and the A8V mutation into exon 1. The “Targeted TnC” portion of Figure 1A shows the mutated cTnC allele after homologous recombination in ES cells. The probe and digested DNA fragment used for Southern blot analysis to screen the targeted ES cells are shown in Figure 1B. The “Neo Deletion” section illustrates the single loxP site that remains in intron 1, of cTnC, after deletion of the neo gene.
Selection of the targeted ES clones and generation of KI mice - Culturing and selection of the embryonic stem (ES) cells and the screening of the targeted clones was performed as described by using negative (gancyclovir) and positive (G-418) selection. Briefly, Tc1 ES cells were grown in ES cell medium on a layer of irradiated embryonic fibroblasts. The construct DNA was linearized by Not 1 digestion and purified on a Qiagen column, ethanol precipitated and dissolved in TE to a final [1 µg/µl]. ES cells were resuspended in 0.8 µl PBS and electroporated with 25 µg of linearized construct DNA at 250V, 500 µF using a Bio-Rad Gene Pulser. After electroporation, the cells were incubated on ice for 20m. Each set of electroporated cells was plated on 4 plates (100 mm). At 48h post electroporation, 350 µg/ml G418 and 2 µm GANC were added to the ES medium for selection of recombinant ES cells. The drug resistant clones were selected after 7-12 days of selection. Once the drug resistant clones became nearly confluent in the 48 well plates, the DNA was prepared for digestion using the appropriate restriction enzymes. These samples were then analyzed by Southern blot analysis for identification of the gene targeted clones in Supplemental Figure 1B.

Two independent ES clones that underwent successful homologous recombinant of the A8V mutation were injected into C57/BL/6 blastocysts. Male progeny with a high percentage of coat color chimerism were bred to C57BL/6 females to establish germ line transmission. The mouse genotypes were identified by Southern blot of tail DNA digested with SalI and probed with the 5’ probe. Heterozygote mice (KI-TnC-A8V+/−) were mated with MHC-Cre transgenic mice to delete the neo marker and the resulting heterozygote mice were shown to contain the mutation. The homozygote mice KI-TnC-A8V+/+ were obtained by intercrossing the A8V heterozygote mice. The mouse genotypes were identified by PCR analysis of tail DNA using the primer pair P1 and P2.

Echocardiography (ECHO) measurements - ECHO was performed using a Vevo 2100 high-resolution in vivo imaging system as previously described. The WT, KI-TnC-A8V+/− and KI-TnC-A8V+/+ mice were lightly anesthetized with isoflurane (2%). M-mode imaging of the parasternal short axis view allowed evaluation of systolic and diastolic dimensions as well as measurement of posterior wall thickness. M-
mode imaging was also utilized to calculate fractional shortening (FS) and ejection fraction (EF). The mitral flow parameters were acquired while imaging the four-chamber view. Pulsed-wave spectral doppler was used to measure mitral valve inflow including its two main waves, early (E wave) and late atrial (A wave), from which deceleration time (DT) and isovolumetric relaxation time (IVRT) were derived. Mitral valve inflow parameters were used as surrogates of diastolic function.

_Ex-vivo working heart studies_ - Isolated ex-vivo working heart function was measured in 12-13m KI mouse hearts as previously described. Briefly, after the mice were heparinized for 30 min (100 Unit heparin i.p.), they were anesthetized by injection of pentobarbital (100 mg/kg body weight, i.p.). After mice were fully anesthetized, the hearts were isolated rapidly (within 3 min) after opening the chest cavity. The aortas were cannulated with a modified 18-gauge needle and Langendorff retrograde perfusion was immediately begun. Aortic pressure was monitored by a pressure sensor (MLT844 pressure transducer, Capto, Horten, Norway) at the equivalent level found in the heart. A 0.5 mL air bubble was placed in the aortic trap to mimic _in vivo_ arterial compliance. A reservoir was connected to the pulmonary vein with a 16-gauge needle to perfuse the heart via the left atrium in the working mode. The coronary effluent from the right ventricle was monitored through a cannula via the pulmonary artery. A 1.2-Fr pressure-volume (P-V) catheter (Scisense, London, ON, Canada) was inserted into the left ventricle through a path made by puncturing the apex with a 30-gauge needle. The temperature of the heart and the perfusate was maintained at 37°C. After cannulation was established, a pair of custom-modified platinum wires was attached to the surface of the right atrium to pace the heart rate via an isolated stimulator (A365, World Precision Instruments). The working heart mode was started by instigating left atrial preload perfusion. The perfusion medium was a modified Krebs-Henseleit bicarbonate buffer equilibrated with 95% O₂-5% CO₂, containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 2.25 mM MgSO₄, 2.25 mM CaCl₂, 0.32 mM EGTA, 2 mM pyruvate, and 15 mM D-glucose. NaHCO₃ was added to adjust the pH to 7.4 at 37°C. The perfusion buffer was filtered through a 0.45-μm membrane and used only once. Aortic and coronary effluent volumes were recorded in real time using a calibrated counting drop method.
to quantify the outflow. Pressure and volume development data were collected at a sampling rate of 1 kHz with a 100-Hz filter using a Powerlab 16-channel analog-to-digital interface and Chart 5.0 software (AD Instruments). Preload was set at 10 mmHg and afterload was set at 55 mmHg. After equilibration at 480 bpm for 30 min, isoproterenol was administrated from the preload reservoir at 3, 10 and 30 nM to examine the \( \beta \)-adrenergic response. After the pressure-volume studies, the hearts were excised and their total and atrium weights were recorded.

**RNA Extraction and cDNA preparation** - WT and KI-TnC-A8V hearts were excised and the left and right ventricles were separated using sterile aseptic technique. Each set of WT, KI-TnC-A8V\(^{+/+}\) and KI-TnC-A8V\(^{+/-}\) left and right ventricles was immersed in 800 \( \mu \)l of RNA-Bee (Tel-Test, Inc) and immediately stored at \(-80^\circ\)C until homogenization. Samples were homogenized using the Power Gen 500 homogenizer (Fisher Scientific\(^\circ\)). RNA was extracted by addition of chloroform to the homogenized samples at a 1:5 ratio, mixed, incubated on ice for 5 min, and centrifuged for 15 min at 12000 rpm at \(4^\circ\)C. The aqueous solutions were further processed using the RNeasy kit (Qiagen, Inc.). The obtained RNA was analyzed using the 2100 Bioanalyzer (Agilent\(^\circ\)). The Biorad iScript cDNA synthesis kit (BioRad\(^\circ\)) was used to synthesize the cDNA using iScript reverse transcriptase from 1 \( \mu \)g RNA obtained from each ventricle. The cDNA concentrations were obtained and diluted to 100 ng/\( \mu \)l.

**Quantitative RT-PCR analysis of cardiac fetal gene expression** – Real Time PCR was performed using the C1000 Touch Thermal Cycler (Biorad CFX96 Real-Time System). Primers from IDT (Integrated DNA Technologies) Cyclophilin Primers (Fw-5’-TTGTGACTTTACACGCCATAA-3’; Rv – 5’-CCATCCAGCCATTCAGTCTT-3’) were used as an endogenous control. We examined changes in expression of the hypertrophic markers ANP; Primers (Fw 5’-TTTGGCTTCCAGGCCATATT-3’; Rv 5’-CATCTTCTACCGGCATCTTTC-3’), BNP; Primers (Fw- 5’-ACTCCTATCCTCTGGAAGTC-3’; Rv- 5’GCTGTCTCTGGGGGCAATT-3’), MHC \( \beta \)- Myosin Heavy Chain \( \beta \) Primers (Fw– 5’-
CCACCCAAGTTGCAAGAT-3'; Rv–5’-AAGAGGCCCGAGTAGGTATAG-3’). For each RT-PCR reaction PerfeCTa® SYBR® Green SuperMix, Low ROX™ was used. The PCR run conditions included a denaturation temperature of 95 °C and annealing temp of 54.3 °C, with each step lasting 30s. The results were reported as fold change relative to WT group. PCR products were checked for specificity by measuring their melting temperature.

**Histopathological studies** – Sixteen-month old male WT, KI-TnC-A8V+/- and KI- TnC-A8V+/+ mouse hearts were excised and immersed in a 10% buffered formaldehyde solution. The hearts were sliced and stained by IDEXX Inc. (Boston MA, USA) and examined using a Zeiss microscope with the 40x/0.65 Plan Apo S objective.

**Liquid chromatography mass spectrometry (LCMS) for determination of the ratio of mutant to WT in the KI-TnC-A8V+/- hearts** - In-gel digest was performed of cardiac tissue obtained from 4m old mice using the ProteoExtract All-in-One Trypsin Digestion Kit (Cat. No. 650212 Calbiochem) according to manufacturer's instructions. Briefly, carefully excised gel pieces were destained with wash buffer and dried at 90°C for 15 min. Gels were rehydrated with digest buffer, reduced with reducing agent for 10 min at 37°C. Samples were cooled to room temperature and then blocked using the blocking reagent for 10 min at room temperature. Trypsin at a final concentration of 8 ng/µl was added and incubated for 2 hrs at 37°C while shaking. Peptides were eluted in 50µL 0.1% formic acid and run on LCMS as follows: An externally calibrated Thermo LTQ Orbitrap Velos (high-resolution electrospray tandem mass spectrometer) was used with the following parameters: nLC-MS/MS was run in technical triplicate to enable normalization and analysis. A 2cm, 100 µm i.d. trap column (SC001 Easy Column from Thermo-scientific) was followed by a 10cm analytical column of 75 µm i.d. (SC200 Easy Column from Thermo-scientific). Both the trap column and analytical column had C18-AQ packaging. Separation was carried out using Easy nanoLC II (Thermo-Scientific) with a continuous, vented column configuration. A 5 µL sample was aspirated into a 20 µL loop and loaded onto the trap. The flow rate was set to 300 nL/min for
separation on the analytical column. Mobile phase A was composed of 99.9% water (EMD Omni Solvent), and 0.1% formic acid and mobile phase B was composed of 99.9% acetonitrile, and 0.1% formic acid. A 60 min linear gradient from 5% to 35% B was performed. The liquid chromatography eluent was directly nanosprayed into an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). During chromatographic separation, the LTQ Orbitrap Velos was operated in a data-dependent mode and under direct control of the Xcalibur 2.2 Sp1 software (Thermo Scientific). The mass spectrometry data were acquired using the following parameters: 10 data-dependent collisional-induced-dissociation (CID) MS/MS scans per full scan (400 to 2000 m/z). All measurements were performed at room temperature. Resultant Raw files were searched with Proteome Discoverer 1.4 using SequestHT, while the search engine used a modified mouse FASTA database that included the mutant troponin C protein sequence. Resultant msf files were quantitatively analyzed by Skyline 3.1 using MS1 filtering method.

*Cardiomyocyte isolation and kinetics experiments* - Cardiomyocytes were isolated from 3m old mice using the Langendorff method and experiments performed as previously described.² WT, KI-TnC-A8V+/− and KI-TnC-A8V+/+ mice were injected intraperitoneally with 0.5 ml heparin diluted to 100 IU/ml in phosphate-buffered saline (PBS). At 15 min post-injection the mice were euthanized by cervical dislocation. The hearts were excised rapidly and placed into a chamber containing 120 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 1.2 mM Na₂HPO₄, 5.6 mM glucose, 20 mM NaHCO₃, 20 mM 2, 3 butanedione monoxime (Alfa Aesar), and 5 mM taurine (Sigma), at 95% O₂ / 5% CO₂, and the aortic arch was exposed by rapid excision of fat deposits from the hearts. The hearts were connected to a 22G cannula which was inserted into the descending aortic arch and perfused with a Ca²⁺ free buffer, to remove remaining blood in the coronary arteries and veins. Next, the hearts were perfused with an enzymatic digestion solution composed of 1 mg/mL collagenase type II (Worthington) and 0.1 mg/mL protease type XIV (Sigma) for a total of 7-10m. Cardiomyocytes were obtained from the digested cardiac tissue by trituration gently with a plastic pipette. Subsequently, the cardiomyocytes were transferred to a conical tube and filtered through a cell strainer. To obtain an ideal Ca²⁺ concentration for culture media of 1.8 mM, Ca²⁺ was incrementally
added to achieve the following reconstitution steps: 0.25 mM, 0.50 mM, 0.75 mM and 1 mM. Each reconstitution step lasted for 15 min and after the final Ca\(^{2+}\) addition, the cardiomyocytes were incubated in a 37°C incubator, under 95% O\(_2\)/5% CO\(_2\) during the remainder of the experimental procedures.

For the Ca\(^{2+}\) measurements, cells were incubated with 1.5 µM Fura-2 acetoxyethyl ester (Molecular Probes) for 25 min and protected from light. Next, the cells were transferred to laminin coated slides and attached to the stage of an inverted Motic AE31 microscope, with the chamber temperature was controlled at 37°C. Sarcomere and Ca\(^{2+}\) transients were recorded at 1, 2, 4 and 6 Hz pacing stimulation frequencies, with MyoPacer Field Stimulator (IonOptix MA, USA). During the intracellular Ca\(^{2+}\) measurements, cardiomyocytes were incubated with the dye Fura2 AM and subjected to dual excitation (340/380 nm) using a fluorescence photomultiplier system (IonOptix MA, USA) equipped with a xenon lamp. The emission fluorescence was collected at 510 nm.

**Skinned Fiber Experiments** - Skinned cardiac fibers were obtained from 4 and 14-16m-old WT, KI-TnC-A8V\(^{+/+}\) and KI-TnC-A8V\(^{+/-}\) mice and prepared according to established protocols.\(^4\) Papillary muscle fibers were isolated from the left ventricles, skinned using 1% Triton X-100, rinsed and then stored in 50% glycerol (v/v) at −20°C. Fibers were mounted onto a force transducer and immersed in a relaxing solution (10\(^{-8}\) M free Ca\(^{2+}\), 150 mM ionic strength, 2.5 mM MgATP\(^2-\)). The Ca\(^{2+}\) dependence of tension was measured in a series of solutions with increasing Ca\(^{2+}\) concentrations at room temperature (~20°C). Methods for solving the free and bound metal ion equilibria in our pCa solutions were provided by the computer program pCa Calculator.\(^5\)

**Flash Photolysis of Skinned Fibers** - To monitor the rate of relaxation, diazo-2 was applied to cardiac skinned fibers obtained from 4m-old WT and KI-A8V\(^{+/+}\) mice as previously described.\(^2\) Upon photolysis, Diazo-2 rapidly chelates Ca\(^{2+}\) since it converts from a low Ca\(^{2+}\) affinity (K\(_d\)=2.2 µmol/l) to a high Ca\(^{2+}\) affinity (K\(_d\)=0.073 µmol/l) state. After a test of the steady-state force, the fibers were immersed in a solution containing 2 mM diazo-2, 0.5 mM CaCl\(_2\), 60 mM TES, pH 7.0, 5 mM MgATP, 1 mM [Mg\(^{2+}\)]
and 10 mM creatine phosphate along with 15U/ml creatine phosphokinase, ionic strength = 200 mM. Initially, this solution supplies an activating concentration of Ca\(^{2+}\); therefore, fibers produce an initial force (~80% of the maximal force). When the steady-state force reaches equilibrium, the fibers are then exposed to a laser beam. The photolysis of diazo-2 increases its affinity for Ca\(^{2+}\), thereby reducing the free [Ca\(^{2+}\)] and promoting relaxation of the fiber. The rate constants of relaxation were calculated according to the equation:

\[ Y = A_1 e^{B_1X_i} + A_2 e^{B_2X_i}; \]

where \( Y \) is the quantity (i.e. relative force) at time \( X_i \), \( A_1 \) and \( A_2 \) are the % amplitudes for both phases of the curve; \( B_1 \) and \( B_2 \) are the corresponding rate constants.

**Immunoblot Analysis** - The Western blots were performed as previously described.\(^2\) Left ventricles were obtained from 4, 9 and 16-18m WT, KI-A8V\(^{+/+}\) and KI-A8V\(^{+-}\) mice, the tissue was ground in liquid nitrogen, and lysed in RIPA buffer containing protease inhibitors. RIPA lysates were quantified using the Nanodrop 2000c system. The samples (15-20µg) were loaded onto 10% or 15% SDS polyacrylamide gels (SDS-PAGE). Proteins were transferred onto nitrocellulose membranes (Amersham Hybond ECL GE 0.45 µm) in the cold room for 2 hr at 100V. Membranes were blocked using 5% non-fat milk in PBS for an hour. Primary antibodies were diluted in PBS containing 5% non-fat milk. Immunoblot antibody detection was performed using primary antibodies against: sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) (Santa Cruz Biotechnology, INC; 1:500), calsequestrin (CASQ2) (Upstate Biotechnology; 1:500), total phospholamban (PLN-T) (Millipore;1:1000), PLN phosphorylated at Serine16 (PLN-P\(^{\text{Ser16}}\)) (Millipore; 1:1000); PLN phosphorylated at Threonine 17 (PLN-P\(^{\text{Thr17}}\)) (Badrilla Ltd.; 1:1600); cardiac troponin I phosphorylated at Serines 22 and 23 (TnI-P) (Cell Signaling Technology; 1:1000), Na\(^{+}\)Ca\(^{2+}\)-exchanger (NCX1) (Swant; 1:200) and α/βTubulin (Cell Signaling Technology; 1:1000) as a loading control. Primary antibodies were incubated overnight at 4°C. The secondary antibodies utilized were anti-mouse, anti-goat, anti-sheep and anti-rabbit immunoglobulins (IRDye® LI-COR, USA), at a dilution of
1:10,000-20,000. Odyssey Blocking Buffer with the addition of 0.1% sodium azide was used to dilute the secondary antibodies. The proteins were incubated with the secondary antibodies for 1 hr at RT. Antigen detection was performed using the infrared Odyssey system (Odyssey LI-COR, USA). Protein signal intensities were quantified using the Odyssey system (Odyssey LI-COR, USA/Image Studio 2.1 software, and first normalized to tubulin signal intensity. Protein signals corresponding to KI-A8V+/− and KI-A8V+/+ mice were further normalized to WT signal intensities.

Statistical Analysis - The assumptions for ANOVA normality and homoscedasticity were tested by means of the Shapiro-Wilk and Levene’s tests, respectively, except for the experiments that the data were normalized to fold change relative to WT (gene expression levels and immunoblot) that the normality test was not considered. In the flash photolysis experiments the following parameters $A_1$, $A_2$ and $k_2$, either the WT or KI-TnC-A8V+/+ group failed the normality test ($p < 0.05$), thus the significance was determined by the Mann-Whitney Rank Sum Test.

References

5. Dweck D, Reyes-Alfonso A, Jr., Potter JD. Expanding the range of free calcium regulation in 
Supplemental Figure 1. Construction of the gene targeting vector for introduction of the A8V mutation into the KI-A8V mice. A) A phage clone containing the entire cTnC sequence has been isolated from a 129/Sv fix library. TnC locus - shows the TnC exon and intron organization and a simplified restrictive map used for gene targeting. Targeting vector – depicts the construct used for targeting the allele of cTnC in which the neo gene, tk gene and loxP was inserted into the gene. A 3.2 kb Kpn1/Xho1 fragment containing Exon 1 was cloned to form the 5’ homologous region. A 5.4 kkb Xho1 fragment containing intron 1 through intron 4 was cloned to form the 3’ homologous region. The Neo gene is flanked by two loxP sites and inserted into intron 2. Targeted TnC - describes the homologous recombination in ES cells. Neo deletion - indicates the targeting allele of TnC where the gene is targeted by expression of the Cre DNA recombinase in heterozygote mice. B) Southern blot analysis of
ES cell DNA digested with Sal 1 and probed with the 5’ probe to screen recombinant ES cells DNA with Sal 1 and probed with the 5’ probe to screen recombinant ES cell clones to detect the TnC mutation. Lanes 1-2 WT ES cell clones; Lane 3 is the recombinant ES A8V clone. E=Exon, H=Hind III, K=Kpn 1, Nh=Nhe 1, Sa=Sal 1, Xh=Xho 1.
Supplemental Figure 2: Pathophysiology of the progression of cardiomyopathy. Representative masson trichrome stained sections were imaged at 4.03X (A-F) and 40X (G-I) magnification. Panels A-C) representative figures of hearts showing atrial enlargement and apical hypertrophy obtained from 16-18m-old mice; horizontal black arrows point toward the apical hypertrophic region (B and C). In addition, papillary muscle hypertrophy is clearly observed in both panels B and C. In D-F) representative figures of right ventricular wall hypertrophy (indicated by
black arrows and circles in E and F) in hearts at 16-18m of age. In panels G-I) representative figures displaying cardiac myofibrillar disarray (black arrows) from the same group of mice.
Supplemental Figure 3. Kinetics of contractility and Ca\(^{2+}\) transients in intact cardiomyocytes electrically stimulated at 1 and 2Hz. A) The left panels show representative raw data of SL shortening and intracellular Ca\(^{2+}\) transients at 1Hz. The right panels show a single normalized SL shortening and intracellular Ca\(^{2+}\) transient. B) SL T\(_{50}\) RT, the time for the SL to return to 50% of baseline; Ca\(^{2+}\) T\(_{50}\) Decay, the time for intracellular Ca\(^{2+}\) to return to 50% of baseline. Data are shown as mean ± S.E., n = 4-6 mice. *p<0.05 KI vs WT; #p<0.05, KI-TnC-A8V\(^{+/+}\) vs KI-TnC-A8V\(^{+/+}\).
### 4 Months Old

**ΔpCa_{50} (KI-TnC-A8V pCa_{50} - WT pCa_{50})**

- WT
- +/-
- +/-

**n_{Hill}**

- WT
- +/-
- +/-

### 14-16 Months Old

**ΔpCa_{50} (KI-TnC-A8V pCa_{50} - WT pCa_{50})**

- WT
- +/-
- +/-

**n_{Hill}**

- WT
- +/-
- +/-

# Significant Differences

- WT vs. +/-: 
- WT vs. +/-: 
- +/- vs. +/-: 

*Significance codes:* 
- # indicates significant difference from WT
- * indicates significant difference from WT
Supplemental Figure 4. The ΔpCa50 and cooperativity of thin-filament activation ($n_{Hill}$) calculated from the Ca$^{2+}$ sensitivity measurements in cardiac skinned fibers at two different ages. Data for ΔpCa50 are shown as the difference between pCa50 averages. At 14-16m of age the pCa50 values were 5.48 ± 0.02, 5.57 ± 0.02 and 6.07 ± 0.03 for WT, KI-TnC-A8V+/- and KI-TnC-A8V+/+, respectively (n = 8). At 14-16m of age the pCa50 of KI-TnC-A8V+/+ was statistically significant different from WT and KI-TnC-A8V+/-; and the pCa50 of KI-TnC-A8V+/- was statistically significant different from WT. Data for $n_{Hill}$ are shown as mean ± S.E., n = 9-11 (4m) and n = 8 (14-16m). *p<0.05 KI vs WT; #p<0.05, KI-TnC-A8V+/- vs KI-TnC-A8V+/+. WT, wild-type; +/-, KI-TnC-A8V+/-; and +/+, KI-TnC-A8V++.
Supplemental Figure 5. Immunoblot of SERCA2, PLN-T and PLN-P in KI-TnC-A8V hearts at different ages. Representative immunoblots for the detection of SERCA2, PLN-T, PLN-P at serine 16 (PLN-P^{Ser16}) and PLN-P at threonine 17 (PLN-P^{Thr17}) in the left ventricle of WT, KI-TnC-A8V^{+/-} (+/-) and KI-TnC-A8V^{+/+} (+/) mice. For all experiments, tubulin was used as an internal control. The graphs show the quantification of the protein expression and phosphorylation levels. The same immunoblot membrane was incubated with primary antibodies against PLN-P^{Thr17} and TnI-P at 9M (Figure 7C),
therefore the loading control bands (tubulin) for these two proteins are shown the same. Data are shown as mean ± S.E., n = 6 mice. *p<0.05 KI vs WT; 
#p<0.05, KI-TnC-A8V+/− vs KI-TnC-A8V+/+. 

Supplemental Table 1: Summary of sarcomere length parameters and intracellular Ca²⁺ levels in 3m KI-TnC-A8V intact cardiomyocytes at different frequencies of stimulation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1 Hz</th>
<th>2 Hz</th>
<th>4 Hz</th>
<th>6 Hz</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>HT</td>
<td>HM</td>
<td>WT</td>
</tr>
<tr>
<td>Sarcomere Length (µm)</td>
<td>1.801±</td>
<td>1.773±</td>
<td>1.722±</td>
<td>1.795±</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>0.002*</td>
<td>0.006*</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>0.160</td>
<td>0.231*</td>
<td>0.243*</td>
<td>0.183</td>
</tr>
<tr>
<td></td>
<td>0.012</td>
<td>0.007*</td>
<td>0.009†</td>
<td>0.011</td>
</tr>
<tr>
<td>Peak Ca²⁺ % Baseline</td>
<td>22.31±</td>
<td>18.30±</td>
<td>17.47±</td>
<td>22.93±</td>
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<tr>
<td></td>
<td>0.63</td>
<td>0.39*</td>
<td>0.32*</td>
<td>0.59</td>
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</table>

Values are given as mean ± S.E.M. (measurements were taken from 3-4 mice per group; 10-32 cells per group were studied (except HT and HM at 6Hz where only 5-10 cells could be analyzed). 

WT = wild-type; HT = heterozygous (KI-TnC-A8V+/−); HM = homozygous (KI-TnC-A8V+/+)

*Sarcomere Shortening (%) and Peak Ca²⁺ % Baseline represents the amplitude of increase of Sarcomere Length and [Ca²⁺] shortening that occurs during systole and is calculated using the following equation, (|x − y| ÷ y) • 100; where x is the peak systolic Sarcomere Length (or Fura-2 ratio), and y is the diastolic Sarcomere Length (or Fura-2 ratio).

#Fura-2 was excited at 340 and 380 nm and the ratio of their emissions at 510 nm; i.e., Fura-2 ratio is a representative of the intracellular Ca²⁺ levels.

*p<0.05 HT or HM vs WT at the same frequency of stimulation.

†p<0.05 HM vs HT at the same frequency of stimulation.
Supplemental Table 2: Summary of Ca\(^{2+}\) and contractile transient parameters measured from cardiomyocytes isolated from 3m old WT and KI-TnC-A8V mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>4 Hz</th>
<th>6 Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL T(_{50}) RT (Sec.)(^a)</td>
<td>WT</td>
<td>HT</td>
</tr>
<tr>
<td></td>
<td>0.0592 ± 0.0005</td>
<td>0.0700 ± 0.0008*</td>
</tr>
<tr>
<td></td>
<td>0.0572 ± 0.0004</td>
<td>0.0639 ± 0.0005*</td>
</tr>
<tr>
<td>-dL/dt (µm/sec)</td>
<td>251.38 ± 10.39</td>
<td>176.66 ± 5.30*</td>
</tr>
<tr>
<td></td>
<td>273.56 ± 6.36</td>
<td>198.22 ± 4.60*</td>
</tr>
<tr>
<td>SL Baseline to Peak Time (Sec.)</td>
<td>0.0318 ± 0.0003</td>
<td>0.0413 ± 0.0004*</td>
</tr>
<tr>
<td></td>
<td>0.0326 ± 0.0002</td>
<td>0.0385 ± 0.0002*</td>
</tr>
<tr>
<td>Ca(^{2+}) T(_{50}) Decay (Sec.)(^a)</td>
<td>0.0662 ± 0.0005</td>
<td>0.0778 ± 0.0004*</td>
</tr>
<tr>
<td></td>
<td>0.0586 ± 0.0005</td>
<td>0.0680 ± 0.0005*</td>
</tr>
<tr>
<td>Ca(^{2+}) Baseline to Peak Time (Sec.)</td>
<td>0.0162 ± 0.0002</td>
<td>0.0160 ± 0.0002</td>
</tr>
<tr>
<td></td>
<td>0.0157 ± 0.0003</td>
<td>0.0156 ± 0.0002</td>
</tr>
</tbody>
</table>

Values are given as mean ± S.E.M. (measurements were taken from 3-4 mice per group; 10-32 cells per group were studied (except HT and HM at 6Hz where only 5-10 cells could be analyzed)).

WT = wild-type; HT = heterozygous (KI-TnC-A8V+/-); HM = homozygous (KI-TnC-A8V+/+)

\(^a\)SL T\(_{50}\) RT and Ca\(^{2+}\) T\(_{50}\) Decay, denotes the time it takes for the Sarcomere Length and Ca\(^{2+}\) amplitudes to decrease by 50%.

\(^*\)p<0.05 HT or HM vs WT at the same frequency of stimulation.

\(^†\)p<0.05 HM vs HT at the same frequency of stimulation.