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Variant R94C in TNNT2-Encoded Troponin T Predisposes to Pediatric Restrictive Cardiomyopathy and Sudden Death Through Impaired Thin Filament Relaxation Resulting in Myocardial Diastolic Dysfunction

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Background—Pediatric-onset restrictive cardiomyopathy (RCM) is associated with high mortality, but underlying mechanisms of disease are under investigated. RCM-associated diastolic dysfunction secondary to variants in TNNT2-encoded cardiac troponin T (TNNT2) is poorly described.

Methods and Results—Genetic analysis of a proband and kindred with RCM identified TNNT2-R94C, which cosegregated in a family with 2 generations of RCM, ventricular arrhythmias, and sudden death. TNNT2-R94C was absent among large, population-based cohorts Genome Aggregation Database (gnomAD) and predicted to be pathologic by in silico modeling. Biophysical experiments using recombinant human TNNT2-R94C demonstrated impaired cardiac regulation at the molecular level attributed to reduced calcium-dependent blocking of myosin’s interaction with the thin filament. Computational modeling predicted a shift in the force-calcium curve for the R94C mutant toward submaximal calcium activation compared within the wild type, suggesting low levels of muscle activation even at resting calcium concentrations and hypercontractility following activation by calcium.

Conclusions—The pathogenic TNNT2-R94C variant activates thin-filament–mediated sarcomeric contraction at submaximal calcium concentrations, likely resulting in increased muscle tension during diastole and hypercontractility during systole. This describes the proximal biophysical mechanism for development of RCM in this family. (J Am Heart Assoc. 2020;9:e015111. DOI: 10.1161/JAHA.119.015111.)

Key Words: heart failure • myocardial biology • pediatrics • restrictive cardiomyopathy • sudden cardiac death

Restrictive cardiomyopathy (RCM) is a primary disorder of increased myocardial stiffness and diastolic dysfunction with often normal to slightly hypertrophied ventricular wall thickness.1 This disease typically manifests with marked atrial dilation, attributable to transmission of elevated pressures during ventricular relaxation, with preserved ventricular systolic function. The etiologies of RCM are broad, including inherited and acquired causes, and the prognosis is poor.2,3 RCM comprises one of a group of primary muscle diseases of the heart collectively called cardiomyopathies. Once thought to be separate entities, cardiomyopathies are now thought to be a spectrum of diseases with the presence of restrictive and hypertrophic features varying across the phenotypes. Hypertrophic cardiomyopathy (HCM) is common and is associated with mutations in sarcomere protein-encoding genes.4–7 RCM and HCM patients often share the feature of diastolic...
Clinical Perspective

What Is New?

- TNNT2-encoded cardiac troponin T TNNT2-R94C is a heritable cause of restrictive cardiomyopathy and sudden death.
- This pathologic variant leads to initiation of cardiac muscle contraction at resting calcium levels.
- Computational modeling predicts that this causes increased basal muscle tension and likely leads to diastolic cardiac dysfunction.

What Are the Clinical Implications?

- Pediatric restrictive cardiomyopathy is a rare cause of sudden cardiac death in children.
- This study demonstrates the pathological mechanism of the TNNT2-R94C variant.
- Patients with this variant should be followed closely for development of cardiomyopathy and/or arrhythmias, and familial screening should be performed when the variant is discovered in an individual.

dysfunction, and a subset of patients with RCM demonstrate mild ventricular hypertrophy. The genetic mechanisms underlying RCM, corresponding clinical phenotypes, and subclass of RCM with hypertrophic features have not been well described.

To date, >1000 variants associated with cardiomyopathy have been identified in sarcomeric genes, including TNNT2-encoded cardiac troponin T (TNNT2).7,8 TNNT2 combines with the calcium-binding proteins troponin C and troponin I to form the troponin complex. This troponin complex is integrated into the thin filament of the sarcomere and, in combination with tropomyosin, coordinates contraction of the cardiac muscle by regulating the calcium-dependent interaction between myosin and the thin filament. TNNT2-specific gene variants are a known, rare cause of HCM, found in 3% to 5% of patients with HCM.9 They have also been associated with the development of ventricular arrhythmias.10–13 Recent studies have suggested that variants in TNNT2 may also be associated with development of RCM.14–16 For example, the TNNT2 variant, I79N, has been shown to cause RCM and HCM within the same family.17 Previous biochemical studies have suggested that TNNT2 variants identified in patients with HCM and dilated cardiomyopathy result in alterations in calcium sensitivity, leading to systolic dysfunction.18,19 Based on these findings, this molecular mechanism has been extrapolated to disorders of diastolic dysfunction. To confirm this hypothesis, variants known to produce a clinical phenotype of primary diastolic dysfunction and RCM should be studied to confirm the underlying molecular mechanisms.

Herein, we identify a TNNT2 variant, TNNT2-R94C, in a family with multiple members affected with a range of clinical presentations, including arrhythmias, cardiomyopathy with predominantly restrictive physiology, and sudden death. We used biochemical techniques and computational modeling to demonstrate that this variant is likely pathogenic, and we determined its molecular mechanism. We show that this variant causes activation of the thin filament at submaximal calcium concentrations, contributing to diastolic dysfunction as observed in this family.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Clinical Evaluation

This study received approval from the institutional review boards at Baylor College of Medicine and Duke University Health System. Available clinical data from the identified proband and relatives were collected, which included pertinent personal and family history, physical examination, standard 12-lead ECG analysis, echocardiographic testing, cardiac catheterization data, and genetic testing. An autopsy was performed on the brother of the proband by the Medical Examiner Service of the Harris County, Texas, Institute of Forensic Sciences (Houston, TX) following his death. The family underwent a full evaluation by pediatric cardiomyopathy and heart failure specialists.

Genetic Analysis

The proband underwent clinical genetic testing using the Famillion (New Haven, CT) HCM panel test, which involves sequencing 9 sarcomeric genes (ACTC, MYBPC3, MYH7, MYL2, MYL3, TNNT2, TNNI3, TNNC1, and TPM1) and 3 metabolic genes (GLA, LAMP2, and PKRG2). Clinical genetic testing was performed on family members using the same HCM panel test. Subsequently, Sanger sequencing was utilized for confirmatory testing. For this, genomic DNA was isolated using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA) from peripheral whole blood as well as postmortem blood spot. To confirm the absence of the identified presumed pathogenic variant in ostensibly healthy individuals, the publicly available Genome Aggregation Database (gnomAD) was used as a control cohort, which is comprised of a total of 15 708 genomes and 125 748 exomes from 141 456 individuals.20 Though the gnomAD database is comprised partly of various disease-specific cohorts in addition to population genetics studies, it excludes individuals known to have severe pediatric disease or severe disease in their first-degree relatives. These individuals were therefore utilized as “control” or “reference” alleles. Variant
pathogenicity classification was also assigned based on 2015 American College of Medical Genetics criteria\textsuperscript{21} and ClinVar (National Center for Biotechnology Information).\textsuperscript{22}

**Sequencing Conservation**

Sequence conservation analysis was performed using the primary TNNT2 sequence obtained from the National Center for Biotechnology Information (NP_001001430.1).\textsuperscript{23} Multialign alignment algorithms were utilized to determine sequence conservation across species.\textsuperscript{24,25}

**In Silico Variant Pathogenicity Modeling**

In silico variant pathogenicity prediction was performed using GenMAPP,\textsuperscript{26} PolyPhen-2,\textsuperscript{27} PredictSNP,\textsuperscript{28} SIFT,\textsuperscript{29} SNAP,\textsuperscript{30} and PANTHER\textsuperscript{31} prediction tools. Variants were considered pathogenic if they carried the designation of "damaging or probably damaging," "deleterious," "disease-related," and/or "disease-associated." Variants were considered benign if they carried designation of "tolerated," "benign," or "neutral."

**Purification of Cardiac Myosin and Actin**

Porcine cardiac ventricular myosin and actin were purified from cryoground tissue as previously described.\textsuperscript{32–34} Myosin subfragment 1 was prepared by chymotryptic digestion as previously described,\textsuperscript{32} using standard techniques,\textsuperscript{35,36} and the purity of the protein was assessed by SDS-PAGE. The concentration of myosin subfragment 1 was determined by absorbance at 280 and 320 nm. Pyrene-labeled actin was prepared from acetone powder\textsuperscript{37} and labeled with the dye, N-(1-pyrenyl)iodoacetamide (pyrene), as described previously.\textsuperscript{38,39} Concentration of pyrene actin was determined by absorbance at 290 and 344 nm. Before use, all actin was stabilized by incubating with equimolar concentrations of phalloidin.

**Preparation of Recombinant Human Troponin and Tropomyosin**

Human cardiac tropomyosin was expressed in BL21-CodonPlus cells (Agilent Technologies), purified, and complexed using established protocols.\textsuperscript{43}

**Determination of $K_B$ Using Stopped Flow Transient Kinetics**

To determine the equilibrium constant between the blocked and closed states, $K_B$, a stopped flow approach developed by McKillop and Geeves\textsuperscript{32,44} was utilized. This method analyzes the rate of myosin binding to reconstituted thin filaments in the presence and absence of calcium. Pyrene-actin was excited at 365 nm, and fluorescence emission was detected using a 390 nm long-pass filter. Reconstituted thin filaments (5 μmol/L of phalloidin-stabilized pyrene actin, 2 μmol/L of tropomyosin, and 2 μmol/L of troponin) and 0.04 U/mL of apyrase were rapidly mixed with 0.5 μmol/L of subfragment 1 myosin and 0.04 U/mL of apyrase at 20°C in an SX-20 stopped flow apparatus (Applied Photophysics, Leatherhead, UK). The high calcium (pCa 4) buffer contained 200 mmol/L of KCl, 5 mmol/L of MgCl\textsubscript{2}, 60 mmol/L of MOPS, 2 mmol/L of EGTA, 1 mmol/L of DTT, and 2.15 mmol/L of CaCl\textsubscript{2}. The low calcium (pCa 9) buffer contained 200 mmol/L of KCl, 5 mmol/L of MgCl\textsubscript{2}, 60 mmol/L of MOPS, 2 mmol/L of EGTA, 1 mmol/L of DTT, and 5.2 μmol/L of CaCl\textsubscript{2}. Myosin strong binding to pyrene-labeled actin in reconstituted thin filaments quenches pyrene fluorescence. Fluorescence transients were collected for at least 3 separate mixes, and a single exponential function was fit to the transient. $K_B$ is calculated from the ratio of the rates of myosin binding to the reconstituted thin filaments at high and low calcium:\textsuperscript{45}

$$\frac{k_{\text{obs}}(-\text{Ca}^{2+})}{k_{\text{obs}}(+\text{Ca}^{2+})} \approx \frac{K_B}{1 + K_B}$$

The average $K_B$ was calculated from 3 different experiments, and the $P$ value was calculated from a 2-tailed Student $t$ test.

**Determination of $K_W$, $K_T$, and $n$ From Fluorescence Titrations**

Values of the equilibrium constant between the open and weakly bound myosin states, $K_W$, the equilibrium constant between the closed and open states $K_T$, and the size of the cooperative unit (ie, the number of binding sites on the thin filament opened by myosin binding), $n$, were determined by measuring the steady-state binding of myosin to pyrene-labeled regulated thin filaments.\textsuperscript{44} Fluorescence titrations were carried out at 20°C in an Applied Photophysics SX-20. Myosin S1 was added at 1-minute intervals to a stirred cuvette containing 0.5 μmol/L of pyrene-actin, 0.27 μmol/L of troponin, and tropomyosin up to a final concentration of 10 μmol/L of
subfragment 1. Buffers contained 200 mmol/L of KCl, 5 mmol/L of free MgCl₂, 60 mmol/L of MOPS, 2 mmol/L of EGTA, 1 mmol/L of DTT, and 2 mmol/L of ADP and the desired free concentration of free calcium. Any contaminating ATP was eliminated by adding 50 μmol/L of P1,P5-di(adenosine-5’) pentaphosphate, 2 mmol/L of glucose, and 1 μmol/L of hexokinase. Titration were performed at 3 calcium concentrations: low (2 mmol/L of EGTA), intermediate (pCa 6.25), and high (pCa 3). Five technical replicates were performed. Data was analyzed as previously described.32

To quantify differences in myosin binding of the wild-type (WT) and mutant proteins, titration were fit to the fractional change in the fluorescence, \(a\), as a function of myosin, \([M]\), given by:

\[
a = \frac{F_0 - F}{F_0 - F_\infty} = \frac{K_w[M]P^n-1(K_1 + K_S)^n + 1}{(K_1)^n + Q^1 + \frac{1}{K_S}}(1 + K_S)^n-1
\]

where \(F\) is the measured fluorescence, \(F_0\) is the fluorescence of the pyrene in the absence of myosin binding, \(F_\infty\) is the fluorescence at saturating myosin concentrations, \(n\) is the size of the cooperative unit, \(P = 1 + K_w[M](1 + K_S)\), and \(Q = 1 + K_w[M]\). For the fitting, the equilibrium constant between the closed and open states (\(K_1\)), the equilibrium constant between the open and myosin weakly bound state (\(K_w\)), and \(n\) were fitted parameters. Titration curves were globally fit to extract parameter values, 95% CIs by bootstrapping simulations, and \(P\) values were determined as described.32 \(K_S\) was fixed based on stopped flow experiments. The equilibrium constant between the states in which myosin is weakly and strongly bound to the thin filament (\(K_S\)) was fixed based on previous studies.45

Computational Modeling of the Variants on Thin Filament Regulation

To quantitatively model the impacts of the variant on overall calcium-force production relationship in the heart, we utilized a computational model developed by the McCulloch laboratory, which uses the measured equilibrium constants to calculate the expected force per sarcomere as a function of calcium.46 Model parameters for the mutant protein were adjusted to be proportional to our measured parameters, and we used these parameters to simulate a force-calcium curve. For the WT protein, we used the default model parameters.

Statistical Analysis

A Student \(t\) test was performed to determine statistical significance between 2 groups. \(P<0.05\) was considered significant, unless otherwise noted.
total of 8 kindred were consented and subjected for confirmatory genetic testing. In addition to the proband (IV.3), postmortem genetic testing of the deceased brother (IV.2) also demonstrated presence of the heterozygous TNNT2-R94C variant. While a first trimester fetal death (IV.4) was also identified from the mother (III.4), no fetal tissue was available for genetic analysis. The father (III.3) was also found to carry the TNNT2-R94C, which was absent in both paternal grandparents (II.1 and II.2) and paternal sibling (III.2), suggesting a de novo variant that was subsequently passed in an autosomal-dominant fashion. This is summarized in Figure 3. In silico prediction modeling was performed and demonstrated a prediction of deleterious impact from 5 of 6 models and neutral from 1 model, with an average confidence of 74.6% (Table). This variant was designated as pathologic in ClinVar, and there is strong evidence of pathogenicity based on 2015 American College of Medical Genetics criteria21 for classifying pathogenic variants.

**R94C Destabilizes the Blocked State of Tropomyosin and Increases the Equilibrium Constant for Myosin Weak Binding to the Thin Filament**

Cardiac troponin T is part of the machinery that regulates the calcium-dependent interactions between myosin and the thin filament (Figure 4A). To determine the molecular consequences of the TNNT2-R94C variant on cardiac contractile regulation, we expressed human WT (TNNT2WT) and mutant troponin (TNNT2R94C) and determined the biochemical impacts on thin filament regulation.

The first step in muscle activation is the calcium-induced change in positioning of tropomyosin along the thin filament, moving it from the blocked to the closed state. The equilibrium constant that defines this transition, $K_B$, was calculated by measuring the rate of myosin binding to reconstituted thin filaments containing pyrene-labeled actin at high- and low-calcium concentrations.32,44 The pyrene fluorescence is quenched upon myosin binding, and the rate of myosin binding to the thin filament can be calculated by fitting an exponential function to the fluorescence transient (Figure 4B). In TNNT2WT, the rate of binding at high calcium (pCa 4) is faster than at low calcium (pCa 9) given that the blocked state is scarcely populated at high calcium. Whereas the fluorescence transients collected at high calcium are similar for the TNNT2WT and TNNT2R94C at low calcium, TNNT2R94C binds faster than the TNNT2WT, consistent with less inhibition at low calcium in TNNT2R94C. Consistent with this notion, we found that $K_B$ for TNNT2R94C (0.67±0.17) is significantly larger than $K_B$ for the WT (0.40±0.15; $P<0.04$). This result demonstrates that the inhibitory blocked state is less populated in TNNT2R94C at low calcium compared with
the TNNT2WT. This loss of inhibition at low calcium would lead to hypercontractility (ie, increased population of force-generating states) and create a predisposition toward myocardial stiffness and ultimately diastolic dysfunction.

Next, we examined the effects of TNNT2R94C on the closed, open, and myosin-bound states of the thin filament (Figure 4A) by performing steady-state titrations of myosin binding to reconstituted pyrene-labeled thin filaments at high (pCa 3), low (2 mmol/L of EGTA), and intermediate calcium concentrations (pCa 6.25)32,44 (Figure 4C and 4D). Qualitatively, the myosin binding isotherms for the TNNT2WT and TNNT2R94C thin filaments are similar at high and intermediate calcium concentrations; however, at low calcium, myosin binding to TNNT2R94C thin filaments is increased at low myosin concentrations compared with TNNT2WT. These findings are consistent with the stopped flow data showing less inhibition to myosin binding at low calcium. Fitting of the data (see Table S1 for details) demonstrates that TNNT2R94C has an increased equilibrium constant for myosin weak binding, $K_W$ (0.20 $-0.03/0.16$ for TNNT2R94C versus 0.13 $-0.01/0.01$ for the WT; $P=0.002$). The variant does not significantly change the equilibrium constant between the closed and open states. This includes $K_T$, (0.11 $-0.08, 0.12$ for TNNT2R94C versus 0.06 $-0.03, 0.06$ for the WT at low calcium, $P=0.43$; 0.07 $-0.04, 0.05$ for TNNT2R94C versus 0.08 $-0.04, 0.04$ for the WT at intermediate calcium, $P=0.45$; and 0.14 $-0.08, 0.09$ for TNNT2R94C versus 0.18 $-0.07, 0.08$ for the WT at high calcium, $P=0.37$). Furthermore, cooperativity of activation was unchanged between TNNT2R94C versus TNNT2WT, n (4.47 $-1.66, 2.18$) for TNNT2R94C versus TNNT2WT; $P=0.18$). These results are summarized in Figure 5A and 5B.

**Figure 2.** R94C localizes to a highly conserved region of TNNT2. (A) Sanger sequencing chromatograms of heterozygous mutant TNNT2-R94C (obtained from subject III-3) and wild-type genotypes. (B) The topological map of TNNT2 with primary sequence alignment from multiple divergent species is shown. The variant localizes to the N-terminal segment of TNNT2 in the tropomyosin binding domain I. This region is highly conserved across species. AH indicates actin helix; BD, binding domain; TnC, troponin C; Tnl, troponin I; TNNT2, TNNT2-encoded cardiac troponin T; WT, wild type.
Computational Modeling Reveals Hypercontractility in TNNT2<sup>R94C</sup>

These biochemical experiments identified the primary effects of TNNT2<sup>R94C</sup> on thin filament regulation as 2-fold increases in the values for both $K_B$ and $K_W$. Computational modeling was used to predict how these changes in equilibrium constants would affect the force-calcium curve in cardiac muscle. Modeling demonstrates a shift in the force-pCa curve for TNNT2<sup>R94C</sup> toward submaximal calcium activation compared with TNNT2<sup>WT</sup>, consistent with molecular hypercontractility. The model also predicts that even at resting calcium concentrations (100–150 nmol/L), there could be some basal level of activation with TNNT2<sup>R94C</sup> (Figure 5C). Taken together, these data demonstrate that TNNT2<sup>R94C</sup> would yield less calcium-induced inhibition to contraction, leading to hypercontractility and potentially an increase in basal muscle tension. These contractile defects could lead to diastolic dysfunction, culminating in restrictive cardiac physiology.

**Discussion**

**Spectrum of Cardiomyopathy Phenotypic Presentation**

Recent advances in high-throughput sequencing have facilitated the identification of a number of novel cardiomyopathy-
Figure 4. Measurement of the biochemical steps involved in thin filament activation. (A) Kinetic scheme for thin filament activation. Tropomyosin lies along the thin filament in 3 positions: blocked (red), closed (yellow), and open (green). Positioning of tropomyosin depends both on calcium and myosin binding. Myosin binds weakly to the thin filament before undergoing an isomerization to the strong-binding state, where force is generated. (B) Measurement of $K_B$, the equilibrium constant between the blocked and closed states, was measured by rapidly mixing myosin with pyrene-labeled thin filaments at low (pCa 9) and high calcium (pCa 4). Myosin binding quenches fluorescence. The ratio of the rates of binding at high and low calcium can be used to calculate $K_B$ (see Methods). There is significantly less blocking in the R94C mutant than the WT at low calcium, as evidenced by the faster binding of the mutant at low calcium. (C and D) Equilibrium titrations of myosin with pyrene-labeled regulated thin filaments enable the calculation of several equilibrium constants (see Methods). Experiments were conducted with thin filaments containing either (C) TNNT2WT or (D) TNNT2R94C. TNNT2 indicates TNNT2-encoded cardiac troponin T.

associated genes, with the number identified as pathogenic increasing significantly over the last few years.5 Because of the increased utilization of this technology, it is now possible to identify shared genetic variants among phenotypically dissimilar individuals, whose clinical presentations have not previously been associated with specific genes.5–1 This has increased understanding of the role of genetic variants in the predisposing pathophysiological mechanisms of atypical forms of cardiomyopathy.52 Here, we describe a variant associated with relatively mild hypertrophy and clear evidence of restrictive physiology consistent with RCM. It is well established that cardiomyopathic diseases, including RCM, have variable expressivity of disease frequently yielding a wide spectrum of phenotypes.53,54 This study is supportive of these previous findings.

We report on a family in which a single variant in TNNT2 is associated with restrictive cardiomyopathy and sudden cardiac death. The TNNT2-R94C variant was not observed in control populations and was predicted to be deleterious by in silico modeling, ClinVar, and current American College of Medical Genetics guidelines. Our biochemical data show a clear molecular phenotype that would be consistent with hypercontractility and diastolic dysfunction, supporting the concept that this variant is causative for the disease. There are a total of 5 previous reports of the TNNT2-R94C variant (National Center for Biotechnology Information ClinVar database), all with the designation of “pathogenic” or “likely pathogenic” and associated with familial HCM.13,55–58 There has only been 1 previous report of a TNNT2 variant causing restrictive physiology by autosomal-dominant inheritance.13 Although the TNNT2-R94C variant has been previously associated with HCM, the spectrum of phenotypic features associated with TNNT2-R94C, including RCM, has not been well described.

Contractile Defects Caused by the R94C Variant

Overall, the clinical phenotype of RCM consists of severe diastolic dysfunction leading to atrial enlargement and, at most, mild ventricular hypertrophy. The molecular mechanisms of contractile defects causing diastolic disease remain largely unknown. Reflecting this, genetic causes of pediatric RCM cases remain unexplained, with only rare patients hosting variants within the thin filament. Here, we put forward TNNT2-R94C as a likely causative of disease. TNNT2 is part of the troponin complex that regulates calcium-dependent interactions between the molecular motor myosin and thin
filament. R94 is located in the highly conserved region of the TNNT2 N-terminal segment, and it is located near other residues that have been associated with other forms of cardiomyopathy, such as R92.65–66 Currently, there is no high-resolution molecular structure of the thin filament or this region of TNNT2, so it is not possible to determine the exact structural basis of the biophysical changes that we observe; however, it has been proposed that this region plays an important role in mediating interaction between tropomyosin and the troponin complex. HCM variants in this region have been associated with reduced affinity of troponin for tropomyosin,65,66 leading to the proposal that these variants cause net hypercontractility and diastolic dysfunction. The phenotype observed in R94C patients, consisting of a primarily restrictive phenotype with mild hypertrophy, would be consistent with this hypothesis.

Limitations

Although we illustrate a novel genetic cause of RCM, our findings are based around a single family and may limit study generalizability. Our classification of this variant as causative for the disease is supported by our biochemical studies demonstrating that the introduction of this single point variant alone causes hypercontractility and features of diastolic dysfunction at the molecular scale. Further studies are warranted to determine how the findings presented here translate to cellular and organ levels; however, our study clearly identifies the initial lesion that leads to downstream changes with disease progression. Clinical genetic testing for patients with cardiomyopathic disease is routinely limited to genes of the sarcomere and metabolism, which are known causes of cardiomyopathy. It is possible that another variant or variants may be contributing to the severe phenotype observed in the family. This is unlikely, considering that the results of our biochemical experiments clearly demonstrate a pathological mechanism of disease.

Moreover, caution should be used when extrapolating the results here to the disease progression observed in human patients. Our biochemical experiments and modeling were
performed using all mutant troponin; however, most patients with the disease are heterozygous for the variant, and the exact proportion of protein that is expressed and incorporated into the sarcomere can vary with the variant. Moreover, as the disease progresses, there are changes in gene expression, fibrosis, and cellular organization that occur over the course of years. Although our modeling cannot capture the full complexity of the disease progression, it clearly demonstrates the initial contractile defects that lead to the disease phenotype. Future studies will be needed to identify the connection between the initial defect and disease phenotype observed in patients in the later stages of the disease.

Conclusions
The R94C variant in TNNT2 is a novel genetic cause of RCM occurring in a conserved domain which causes dysfunction of calcium-based regulation of cardiac contraction and likely yields significant myocardial diastolic dysfunction. This variant, when compared with control cohorts, has a high likelihood of producing a malignant phenotype. Furthermore, this work demonstrates the power of using in vitro biochemical studies as well as computational modeling to support disease causality.

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Disclosures
None.

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SUPPLEMENTAL MATERIAL
| Individual | Age (years) | Sex | Status | TNNT2-R94C Status | Cardiovascular Phenotype |
|------------|-------------|-----|--------|-------------------|--------------------------|
| I.1        | 78          | M   | Deceased | Unknown           |                          |
| I.2        | 83          | F   | Deceased | Unknown           |                          |
| I.3        | 81          | M   | Deceased | Unknown           |                          |
| I.4        | 83          | F   | Alive    | Unknown           |                          |
| II.1       | 59          | M   | Alive    | +/-               | Atrial fibrillation      |
| II.2       | 60          | F   | Alive    | +/-               |                          |
| II.3       | 57          | M   | Deceased | Unknown           |                          |
| II.4       | Unknown     | F   | Alive    | Unknown           |                          |
| III.1      | 33          | M   | Alive    | Unknown           |                          |
| III.2      | 31          | F   | Alive    | +/-               | Supraventricular tachycardia |
| III.3      | 35          | M   | Alive    | +/-               | Ventricular tachycardia  |
| III.4      | 36          | F   | Alive    | +/-               |                          |
| IV.1       | 4           | M   | Alive    | +/-               |                          |
| IV.2       | 4           | M   | Deceased | +/-               | Hypertrophic cardiomyopathy with restrictive features, sudden cardiac arrest |
| IV.3       | 2           | F   | Deceased | +/-               | Restrictive cardiomyopathy with mild hypertrophy, sudden cardiac arrest |
| IV.4       | Fetus       | Unknown | Deceased | Unknown           | First trimester fetal death |