

A Mutation in *TNNC1*-encoded Cardiac Troponin C, *TNNC1*-A31S, Predisposes to Hypertrophic Cardiomyopathy and Ventricular Fibrillation*

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Background: Cardiac troponin C mutations are rare causes of HCM. A novel mutation in *TNNC1* gene was identified in a pediatric HCM patient.

Results: Functional characterization demonstrated increased myofilament Ca²⁺ affinity.

Conclusion: The proband presented with ventricular fibrillation, aborted sudden cardiac death associated with myofilament dysregulation.

Significance: The newly identified cardiac troponin C mutation predisposes to pathogenesis of a fatal arrhythmogenic subtype of HCM.

Defined as clinically unexplained hypertrophy of the left ventricle, hypertrophic cardiomyopathy (HCM) is traditionally understood as a disease of the cardiac sarcomere. Mutations in *TNNC1*-encoded cardiac troponin C (cTnC) are a relatively rare cause of HCM. Here, we report clinical and functional characterization of a novel *TNNC1* mutation, A31S, identified in a pediatric HCM proband with multiple episodes of ventricular fibrillation and aborted sudden cardiac death. Diagnosed at age 5, the proband is family history-negative for HCM or sudden cardiac death, suggesting a *de novo* mutation. TnC-extracted cardiac skinned fibers were reconstituted with the cTnC-A31S mutant, which increased Ca²⁺ sensitivity with no effect on the maximal contractile force generation. Reconstituted actomyosin ATPase assays with 50% cTnC-A31S:50% cTnC-WT demonstrated Ca²⁺ sensitivity that was intermediate between 100% cTnC-A31S and 100% cTnC-WT, whereas the mutant increased

the activation of the actomyosin ATPase without affecting the inhibitory qualities of the ATPase. The secondary structure of the cTnC mutant was evaluated by circular dichroism, which did not indicate global changes in structure. Fluorescence studies demonstrated increased Ca²⁺ affinity in isolated cTnC, the troponin complex, thin filament, and to a lesser degree, thin filament with myosin subfragment 1. These results suggest that this mutation has a direct effect on the Ca²⁺ sensitivity of the myofilament, which may alter Ca²⁺ handling and contribute to the arrhythmogenesis observed in the proband. In summary, we report a novel mutation in the *TNNC1* gene that is associated with HCM pathogenesis and may predispose to the pathogenesis of a fatal arrhythmogenic subtype of HCM.

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Hypertrophic cardiomyopathy (HCM),⁷ defined as left ventricular hypertrophy without a clinically identifiable origin, affects ~1 of 500 individuals and is the most common cause of sudden death in the young athlete (1–3). Thought to be of genetic origin, HCM is inherited typically in an autosomal dominant fashion. Investigations over the past 20 years have led to the identification of hundreds of mutations associated with dozens of genes that have been linked to the pathogenesis of HCM (4, 5). Principal among these are genes encoding components of the sarcomeric myofilament, which host the majority of HCM-associated mutations. Recently, mutations identified in *TNNC1*-encoded cardiac troponin C (cTnC), part of the sarcomeric thin filament, have been implicated as a rare cause of HCM (6–8). Despite this progress, little is known about the arrhythmogenic role of *TNNC1* mutations in the setting of car-

⁷ The abbreviations used are: HCM, hypertrophic cardiomyopathy; *TNNC1*, troponin C gene; TnC, troponin C; cTnC, cardiac TnC; QTc, QT interval corrected for heart rate; IAANS, 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid; S1, subfragment 1.

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diomyopathic disease. Furthermore, there is little mechanistic explanation for the increased risk of sudden cardiac death in some patients with this clinically heterogeneous disease (9).

Troponin C is part of the heterotrimeric regulatory troponin complex of the sarcomeric thin filament and serves as the Ca^{2+} sensor of muscle contraction. The Ca^{2+} -binding protein TnC works in concert with inhibitor troponin I (TnI) and troponin T (TnT), which provides a direct link to tropomyosin and assists in transducing the contractile signal to the rest of the thin filament. This process is initiated by the binding of cytosolic Ca^{2+} during Ca^{2+} -induced Ca^{2+} release, the beginning of the Ca^{2+} transient, which increases the binding affinity of TnC for TnI, thus pulling the TnI inhibitory domain away from its binding site on actin (10). The release of TnC allows the troponin-tropomyosin complex to move farther into the actin groove fully exposing the myosin binding sites on actin. The formation of active cross-bridges may then occur, allowing muscle tension to develop (10).

Cardiac TnC is a Ca^{2+} -binding protein that belongs to the EF-hand superfamily which consists of two globular functional domains attached by a flexible linker (11). The N-domain is considered the regulatory domain and has one active Ca^{2+} binding site (site II) that binds Ca^{2+} with low affinity ($\sim 10^5 \text{ M}^{-1}$). The C-domain is known as the structural domain and contains two $\text{Ca}^{2+}/\text{Mg}^{2+}$ binding sites (sites III and IV) that bind Ca^{2+} at low concentrations ($\sim 10^7 \text{ M}^{-1}$) while also competitively binding Mg^{2+} ($\sim 10^3 \text{ M}^{-1}$) (12). Therefore, only site II of the regulatory N-domain reversibly binds cytosolic Ca^{2+} during cardiac contraction, and mutations that affect the function of cTnC may alter its global structure thus modifying its Ca^{2+} affinity and/or interfering with protein-protein interactions necessary to appropriately transmit the Ca^{2+} binding signal. Recently, we identified a novel *TNNC1* mutation alanine substituted by serine at position 31 (A31S) in the non-functional Ca^{2+} binding site I. This mutation may alter Ca^{2+} binding to site II, which regulates the sensitivity of muscle contraction. Here, we investigated the source of the primary defect (increased Ca^{2+} sensitivity in skinned muscles and ventricular tachycardia in the patient) that occurs in the presence of this mutation.

In addition to initiating cardiac contraction, cTnC is an important Ca^{2+} buffer that assists in maintaining Ca^{2+} homeostasis in the myocyte, and increased Ca^{2+} binding affinity may result in arrhythmogenic Ca^{2+} mishandling (13). Traditionally associated with mutations in cardiac ion channels, ventricular tachycardia has been identified in mouse models hosting Ca^{2+} -sensitizing cTnT mutations (14). Furthermore, mouse models of a cTnI-R145G mutation have been shown to increase Ca^{2+} sensitivity and prolong Ca^{2+} transients (15). Despite these studies, the link between myofilament mutations and arrhythmogenesis, particularly in the context of HCM, remains relatively uncharacterized. To this end, we investigated the effects of a novel *TNNC1* mutation identified in a host demonstrating youthful HCM presentation and repeated episodes of medically refractory ventricular fibrillation.

EXPERIMENTAL PROCEDURES

Genetic Analysis

The proband underwent HCM genetic testing utilizing comprehensive direct sequencing analysis of the nine canonical

genes associated with sarcomeric HCM including *MYH7*-encoded β myosin heavy chain, *MYBPC3*-cardiac myosin binding protein C, *MYL2*-encoded regulatory myosin light chain, *MYL3*-encoded essential myosin light chain, *TNNT2*-, *TNNI3*-, *TNNC1*-, and *TPM1*-encoded α -tropomyosin, and *ACTC*-encoded α -cardiac actin (Transgenomic Inc, New Haven, CT). Genetic causes of cardiac disease, which can mimic HCM including *PRKAG2*-encoded γ -regulatory subunit of AMP-activated protein kinase, *GLA*-encoded α -galactosidase A, and *LAMP2*-encoded lysosome-associated membrane protein, were also comprehensively genotyped.

Study Control Cohorts

The absence of the mutation was confirmed in >26,600 reference alleles. Among these, were >800 unrelated, ethnically diverse, ostensibly healthy individuals recruited by Transgenomic Inc., 100 ostensibly health African American and 200 Caucasian American individuals from the Coriell Institute for Medical Research (Camden, NJ), and 200 additional Caucasian subjects with normal screening electro- and echocardiograms recruited from Olmsted County, Minnesota. Publicly available databases from the Exome Chip Project (12,031 exomes), including the 1000 Genome Project (1,128 exomes) and NHLBI, National Institutes of Health Exome Sequencing Project (4,260 exomes), were also searched for the presence of the identified mutation (16).

Clinical Evaluation of TNNC1-A31S Proband

Clinical data were collected including pertinent personal and family history, physical examination, 12-lead electrocardiogram analysis, QT interval corrected for heart rate (QTc) measurement, and echocardiographic testing to determine mean left ventricular wall thickness, maximum left ventricular outflow tract gradient, and other parameters.

Functional and Structural Studies

Cloning, Expression, and Purification of Human Cardiac Troponin T, Troponin I, and Troponin C Mutants—The cTnI and cTnT cDNAs were cloned as previously described (17). The cTnC cDNA was cloned previously from total RNA obtained from human heart tissue. The sequential overlapping PCR method was used to introduce the A31S mutation into the cDNA (18). Standard methods previously used in this laboratory were utilized for the expression and purification of wild-type and mutant cTnC (19).

Fiber Preparation and Ca^{2+} Dependence of Force Development Measurements—Fresh cardiac tissue was obtained from slaughterhouse pigs. Strips of papillary muscle 3–5 mm in diameter and ~ 5 mm in length were isolated from the left ventricle and skinned overnight in a 50% glycerol relaxing solution ($10^{-8} \text{ M} [\text{Ca}^{2+}]_{\text{free}}$) (6). Fibers were then transferred to a similar solution without Triton X-100 and stored at -20°C . Briefly, a skinned fiber bundle ~ 75 – $100 \mu\text{m}$ in diameter was mounted using stainless steel clips to a force transducer and then immersed in a pCa 8.0 relaxation solution (conditions described in Ref. 6). The Ca^{2+} dependence of force development was tested in skinned fibers at low, intermediate, and high concentration Ca^{2+} solutions (pCa 8.0–4.0) and calculated using

the pCa calculator program developed in our laboratory (20). The native cTnC was depleted upon incubation of the fiber in a 1,2-cyclohexylenenitilotetraacetic acid (CDTA) extracting solution (5 mM CDTA and 25 mM Tris (pH 8.4)) for ~ 1.5 h. Fibers were considered extracted of cTnC when residual tension remaining in the fiber in pCa 4.0 was 15% or below. Fibers were then incubated with 28 μ M concentrations of mutant or WT cTnC diluted in pCa 8.0 for 1 h. The following equation was used to analyze data: % change in force = $100 \times [\text{Ca}^{2+}]^n / ([\text{Ca}^{2+}]^n + [\text{Ca}^{2+}_{50}]^n)$, where $[\text{Ca}^{2+}_{50}]$ is the free $[\text{Ca}^{2+}]$ that produces 50% force, and n_{Hill} is the Hill coefficient. All fiber experiments were performed at room temperature.

Formation of Troponin Complexes—The purified individual troponin subunits including 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid (IAANS)-labeled cTnC were first dialyzed against 3 M urea, 1 M KCl, 10 mM MOPS, 1 mM DTT, and 0.1 mM phenylmethanesulfonyl fluoride and then twice against the same buffer excluding urea. The protein concentrations of the individual subunits were determined using the Coomassie Plus kit and then mixed in a 1.3:1.3:1 cTnT:cTnI:cTnC molar ratio. After 1 h, the complexes were successively dialyzed against solutions containing decreasing concentrations of KCl (0.7, 0.5, 0.3, 0.1, 0.05, 0.025 M). Precipitated excess proteins during complex formation were removed by centrifugation. Proper stoichiometry was verified by SDS-PAGE before storing the troponin complexes at -80°C . Ternary troponin complexes were utilized in the actin-tropomyosin-activated myosin-ATPase assays containing the cTnC mutant.

Actin-Tropomyosin-activated Myosin-ATPase Assays; Minimum and Maximum ATPase—Porcine cardiac myosin, rabbit skeletal F-actin, and porcine cardiac tropomyosin were prepared as previously described (18). The protein concentrations used for actomyosin ATPase assays were: 0.6 μ M porcine cardiac myosin, 3.5 μ M rabbit skeletal F-actin, 1 μ M porcine cardiac tropomyosin, and 0–2 μ M preformed troponin complexes as prepared above and were performed as single point assays that are linear over time (21). The proteins were in the following buffer conditions: myosin in 10 mM MOPS (pH 7.0), 400 mM KCl, 1 mM DTT; actin in 10 mM MOPS (pH 7.0), 40 mM KCl; tropomyosin in 10 mM MOPS (pH 7.0), 300 mM KCl, 1 mM DTT. The final ionic strength of the reactions was ~75 mM when considering the combined ionic contributions from all buffers. ATPase inhibition measurements were performed in a 0.1 ml reaction mixture: 3.4 mM MgCl_2 , 0.13 mM CaCl_2 , 1.5 mM EGTA, 3.5 mM ATP, 1 mM DTT, 11.5 mM MOPS (pH 7.0) at 25°C . The ATPase activation measurements were conducted using the same 0.1 ml buffer mixture with: 3.3 mM MgCl_2 and 1.7 mM CaCl_2 . ATP was added to initiate the reaction, which was quenched after 20 min using trichloroacetic acid at a final concentration of 35%. The precipitated assay proteins were removed by centrifugation. The amount of ATPase hydrolysis was determined by measuring the release of inorganic phosphate in the supernatant using methods established by Fiske and Subbarow (22).

Ca^{2+} Sensitivity of the ATPase—The pCa curves were performed using 1 μ M preformed troponin complex containing 100% WT or cTnC-A31S as well as 50:50 WT:cTnC-A31S. Also, the same concentrations of porcine β -myosin, rabbit skeletal

F-actin, and porcine cardiac tropomyosin were used as described above. The conditions of the assay varied slightly: 11.55 mM MOPS, 2 mM EGTA, 1 mM Nitrilotriacetic Acid, 3.7 mM MgCl_2 (pH 7.0 at 25°C) and the following pCa values: pCa 7.0 (0.448 mM CaCl_2), 6.5 (0.955 mM CaCl_2), 6.2 (1.293 mM CaCl_2), 6.0 (1.489 mM CaCl_2), 5.9 (1.574 mM CaCl_2), 5.7 (1.714 mM CaCl_2), 5.6 (1.770 mM CaCl_2), 5.4 (1.860 mM CaCl_2), 5.2 (1.929 mM CaCl_2), and 4.5 (2.140 mM CaCl_2).

Fluorescence Labeling of TNNC1—The TNNC1 was double-labeled with IAANS at Cys-35 and Cys-84 and mono-labeled at Cys-84. IAANS was obtained from Molecular Probes, Plano, TX. Fluorescent labeling and purification of IAANS-labeled cTnC was performed according to established methods (23).

Determination of Apparent Ca^{2+} Affinities by Fluorescence—IAANS-labeled cTnCs (WT and cTnC-A31S) were dialyzed into fluorescence buffer containing 2 mM EGTA, 5 mM Nitrilotriacetic Acid, 120 mM MOPS, 90 mM KCl. Before titration of isolated cTnC, 1.25 mM MgCl_2 and 1 mM freshly prepared DTT were added. For troponin complex formation and fluorescence experiments, fresh DTT was added before the titration because the dialysis buffer already contained 1.25 mM MgCl_2 that is needed for complex formation. Fluorescence measurements with isolated cTnC and the troponin complex were performed using double-labeled cTnC, with the IAANS label at Cys-35 and Cys-84. Steady state fluorescence measurements were performed using a Jasco 6500 spectrofluorimeter where IAANS fluorescence was excited at 330 nm, and emission was detected at 450 nm. The thin filaments were made according to Pinto *et al.* (24). The IAANS mono-labeling configuration was utilized for thin filament and thin filament + subfragment 1 (S1) measurements, where Cys-35 in cTnC was mutated to Ser; therefore, only Cys-84 would be labeled. Putkey *et al.* (23) first described this method for Ca^{2+} affinity measurements. The need for two labeling configurations, *i.e.* TnC labeled at both Cys-35 and -84 (double label) or with cTnC labeled only at Cys-84 (single label) is that in the presence of the other thin filament proteins, only one configuration responds to Ca^{2+} . Therefore, we used the labeling configuration that provides the largest change in fluorescent signal at each given level of thin filament complexity (*e.g.* cTnC combined with the other troponin subunits (ternary complex) in the presence of tropomyosin and actin). The concentration of proteins used for fluorescence measurements was 10 μ M for isolated cTnC, 0.5 μ M for troponin, 0.05 mg/ml for thin filament, and 0.02 mg/ml for S1. This gives an overall stoichiometry between the thin filament and S1 as 1:1.58, respectively. We have previously determined that this is the ideal concentration/ratio of S1 that caused the half-maximal change in fluorescence from thin filaments (24). The change in the fluorescence spectra was recorded during the titration of microliter amounts of CaCl_2 . The concentration of free Ca^{2+} and amounts of titrated Ca^{2+} were obtained using the pCa calculator program (20). The program made corrections for dilution effects that occur during titration of Ca^{2+} . The data were fit to a version of the Hill equation that accounted for the spectral changes that occur at a low Ca^{2+} concentration and plotted using SigmaPlot 11.0.

Circular Dichroism Measurements—Far UV circular dichroism spectra (CD) were collected using a 1-mm-path quartz cell

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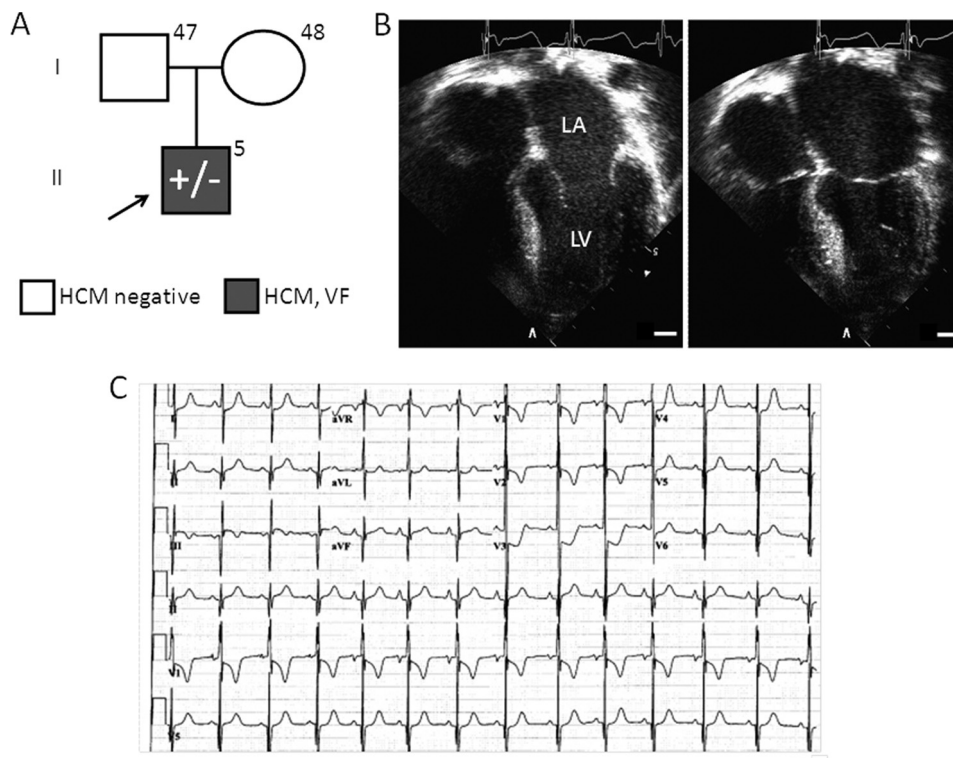


FIGURE 1. **TNNC1-A31S proband clinical characteristics.** A, shown is a pedigree depicting the family of the proband (arrow) who hosts the *TNNC1-A31S* heterozygous allele as well as his two parents, who are negative for a family history of HCM and sudden cardiac death and are unremarkable upon echocardiographic evaluation. LA, left atrium; LV, left ventricle; VF, ventricular fibrillation; bar, 10 mm. B, shown are representative echocardiographic images demonstrating asymmetric left ventricular hypertrophy and left atrial dilatation during end diastole (left image) and systole (right). C, shown is representative 12-lead electrocardiographic tracing demonstrating voltage criteria for HCM and QT prolongation. Bar, 0.4 s.

in a Jasco J-720 spectropolarimeter. Spectra were recorded at 195–250 nm with a bandwidth of 1 nm at a speed of 50 nm/min, whereas the resolution was 0.5 nm at room temperature. Ten scans were averaged, and numerical smoothing was not applied. The optical activity of the buffer was subtracted from relevant protein spectra. Mean residue ellipticity for the spectra was calculated utilizing the same Jasco system software using the following equation: $[\theta]_{\text{MRE}} = [\theta]/(10 \times Cr \times l)$, where $[\theta]$ is the measured ellipticity in millidegrees, Cr is the mean residue molar concentration, and l is the path length in cm (25). Protein concentrations were determined by the biuret reaction using bovine serum albumin as a standard. The CD experiments were performed using three different conditions: 1) apo state (divalent cation free) (1 mM EGTA, 20 mM MOPS, 100 mM KCl (pH 7.0)); 2) Mg^{2+} -bound state (1 mM EGTA, 20 mM MOPS, 100 mM KCl, 2.075 mM MgCl_2 (pH 7.0)); 3) Ca^{2+} -bound state (1 mM EGTA, 20 mM MOPS, 100 mM KCl, 2.075 mM MgCl_2 , 1.096 mM CaCl_2 (pH 7.0)). The experimental protein concentration for the WT and the A31S mutant cTnC was 0.2 mg/ml.

Three-dimensional Visualization—The cTnC-A31S mutation was visualized in the 1AJ4 Protein Data Bank file using PyMol software. PyMol is an open source molecular visualization program that allows manipulation of PDB files that contain molecular coordinates from x-ray crystallography- or nuclear magnetic resonance-based structures. The program allows mutagenesis of selected residues, portrays potential side chain interactions and potential for hydrogen bonding due to changes in the nature of and proximity of side chains.

Statistical Analysis—The experimental results were reported as $x \pm \text{S.E.}$ and analyzed for significance using Student's *t* test at $p < 0.05$.

RESULTS

Genetic Analysis—Comprehensive HCM genetic analysis identified a heterozygous *TNNC1* G → T mutation at nucleotide 91 resulting in a GCT → TCT alanine to serine alteration at residue 31 (*TNNC1-A31S*). This mutation was not identified in >26,600 reference alleles derived from ostensibly healthy individuals from a variety of racial and ethnic backgrounds. The proband did not host a compound mutation in eight other sarcomeric genes (*MYH7*, *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1*, and *ACTC*) as well as three HCM phenocopy-associated genes (*PRKAG2*, *GLA*, and *LAMP2*).

Clinical Evaluation—The *TNNC1-A31S* mutation was identified in a Caucasian male who was symptom-free until his sentinel event of ventricular fibrillation at 3.75 years of age while sleeping at night. He arose, expressed concern to his parents, became syncopal, and was defibrillated successfully by paramedics with an automatic external defibrillator from ventricular fibrillation. He underwent intracardioverter defibrillator implantation and was maintained on β blockade. Despite this therapy, he had five episodes of breakthrough ventricular fibrillation generally when emotionally excited and physically active, with single intracardioverter defibrillator shock restoring normal sinus rhythm in each case. He presented at age 5 to our institution for further evaluation.

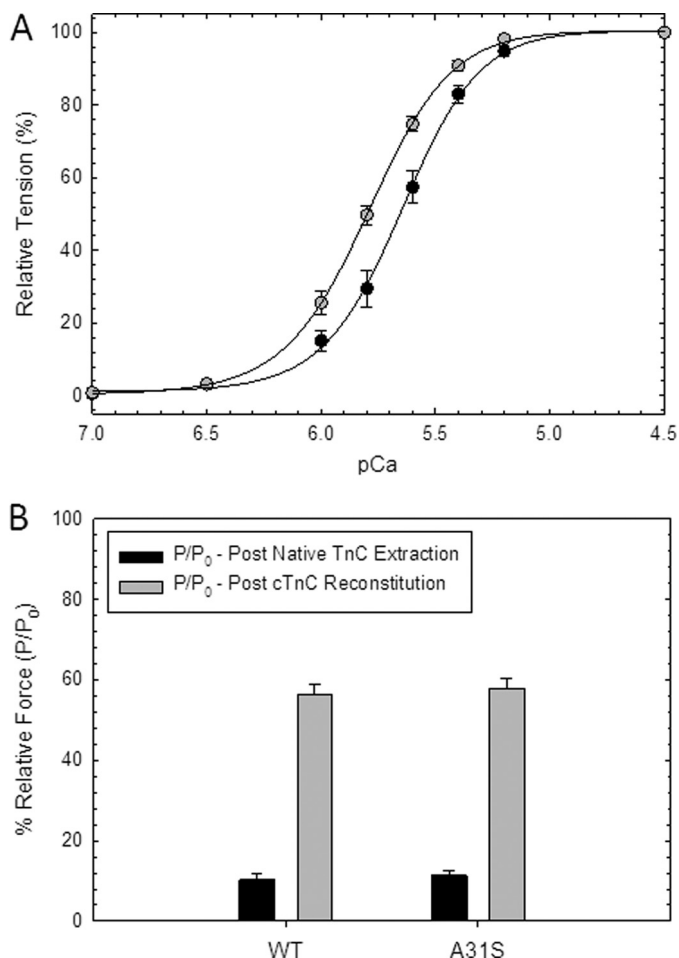


FIGURE 2. Skinned cardiac fiber experiments reconstituted with the HCM cTnC mutant. *A*, porcine cardiac fibers were reconstituted with mutant (gray circles) and WT cTnC (black circles), and Ca²⁺ sensitivity of force development was reported as pCa₅₀ values in Table 1; *B*, Force recovery values (*P*) obtained after reconstitution with mutant and WT cTnC were compared with the level of force present before extraction of native cTnC (*P*₀) and were reported as % (*P*/*P*₀). Data are reported as the mean ± S.E. (*n* = 8–10).

He had a negative family history for HCM and sudden cardiac death. Both parents, at 47 and 48 years of age, were negative for HCM by echocardiography (Fig. 1*A*). On echocardiographic examination, the proband demonstrated asymmetric septal wall hypertrophy with a mean left ventricular wall thickness of 20 mm (6–8 mm normal range) with reverse curve morphology, an ejection fraction of 65%, diastolic dysfunction, and no left ventricular outflow tract obstruction (Fig. 1*B*). He had moderate left atrial enlargement. Electrocardiographic analysis demonstrated significant voltage criteria for biventricular hypertrophy, ST segment depression in anterior leads, and borderline QT prolongation with a QTc of 460 ms (Fig. 1*C*).

Cardiac Skinned Fiber Experiments—To assess whether the A31S mutation perturbs myofilament function, the Ca²⁺ sensitivity and force recovery were evaluated using cTnC-depleted cardiac porcine fibers reconstituted with WT cTnC and cTnC-A31S. Incorporation of cTnC-A31S caused a leftward shift corresponding to an increase in Ca²⁺ sensitivity of 0.17 pCa units with a pCa₅₀ of 5.63 in the WT to 5.80 in the mutant (Fig. 2*A* and Table 1). The cooperativity of thin filament activation

TABLE 1
Summary of the Ca²⁺ sensitivity data obtained *in situ* and reconstituted assays

WT:A31S in ATPase measurements was 50% WT cTnC and 50% A31S cTnC mutant Tn complexes. ND, not determined.

TnC mutant	Skinned fiber				Reconstituted ATPase			
	pCa ₅₀		<i>n</i> _{Hill}		pCa ₅₀		<i>n</i> _{Hill}	
WT	5.63	0.01	2.71	0.14	5.79	0.02	2.35	0.16
A31S	5.80	0.02 ^a	2.45	0.11	6.17	0.02 ^a	1.95	0.18 ^a
WT:A31S	ND		ND		6.02	0.03 ^a	1.95	0.08 ^a

^a *p* < 0.05 compared with their respective WT.

(*n*_{Hill}) (Fig. 2*A* and Table 1) and force recovery % (*P*/*P*₀) (Fig. 2*B*) was unchanged.

Actomyosin ATPase Assays Using Reconstituted Troponin Complexes Containing cTnC-A31S—We next measured the ability of the troponin complex to activate or inhibit the actomyosin ATPase in the presence or absence of Ca²⁺, respectively. The activation of the ATPase (pCa 4) was measured with increasing amounts of preformed troponin complex (0–2.0 μM) (each point represents, *n* = 6, performed in triplicate). The troponin complexes containing cTnC-A31S demonstrated increased thin filament activation compared with WT upon increasing concentrations of troponin. Specifically, there was an increase in the level of activation in the mutant cTnC-A31S-reconstituted thin filament (~180%) compared with WT (~150%) at 1.0, 1.5, and 2.0 μM troponin (Fig. 3*A*). The inhibitory properties of the mutant cTnC-A31S complex was assessed at low Ca²⁺ concentrations (pCa 8) by monitoring the ability to inhibit ATPase activity *versus* WT. The A31S mutant inhibited the actomyosin ATPase in a manner similar to WT although at higher concentrations of troponin (1.0–2.0 μM) (each point represents *n* = 7, performed in triplicate). However, there was a statistically significant decrease in inhibition of the ATPase by the mutant troponin at the lower concentration range (~52% at 0.3–0.8 μM) compared with WT (~40%) as shown in Fig. 3*B*. The Ca²⁺ dependence of actomyosin ATPase activation was also evaluated, and the pCa₅₀ values were determined for the reconstituted thin filaments containing troponin complexes with either 100% WT, 100% cTnC-A31S, and 50:50 WT:cTnC-A31S (experiments performed *n* = 8). When 100% mutant A31S troponin was incorporated into the reconstituted thin filaments, and Ca²⁺ sensitivity was increased by +0.38 pCa units, whereas when 50% of the mutant A31S troponin complex was utilized, Ca²⁺ sensitivity increased by +0.23 pCa units (Fig. 3*C* and Table 1).

IAANS Fluorescence Measurements of cTnC Troponin, Thin Filament, and Thin Filament S1-containing cTnC-A31S—The Ca²⁺ affinity of isolated cTnC as measured by fluorescence obtained from IAANS, an extrinsic probe, indicated that the mutant cTnC-A31S (double label) had increased Ca²⁺ affinity of +0.17 pCa units compared with WT (Fig. 4*A* and Table 2). When additional constituents (tropomyosin and actin) of the thin filament were included, there was a substantial increase in thin filament Ca²⁺ affinity containing cTnC-A31S that increased +0.56 pCa units compared with WT as shown in Fig. 4*C* and Table 2. In addition, spectral fluorescence changes from isolated cTnC were detected from the low affinity C-terminal Ca²⁺ binding sites indicating that the probe picked up both sets

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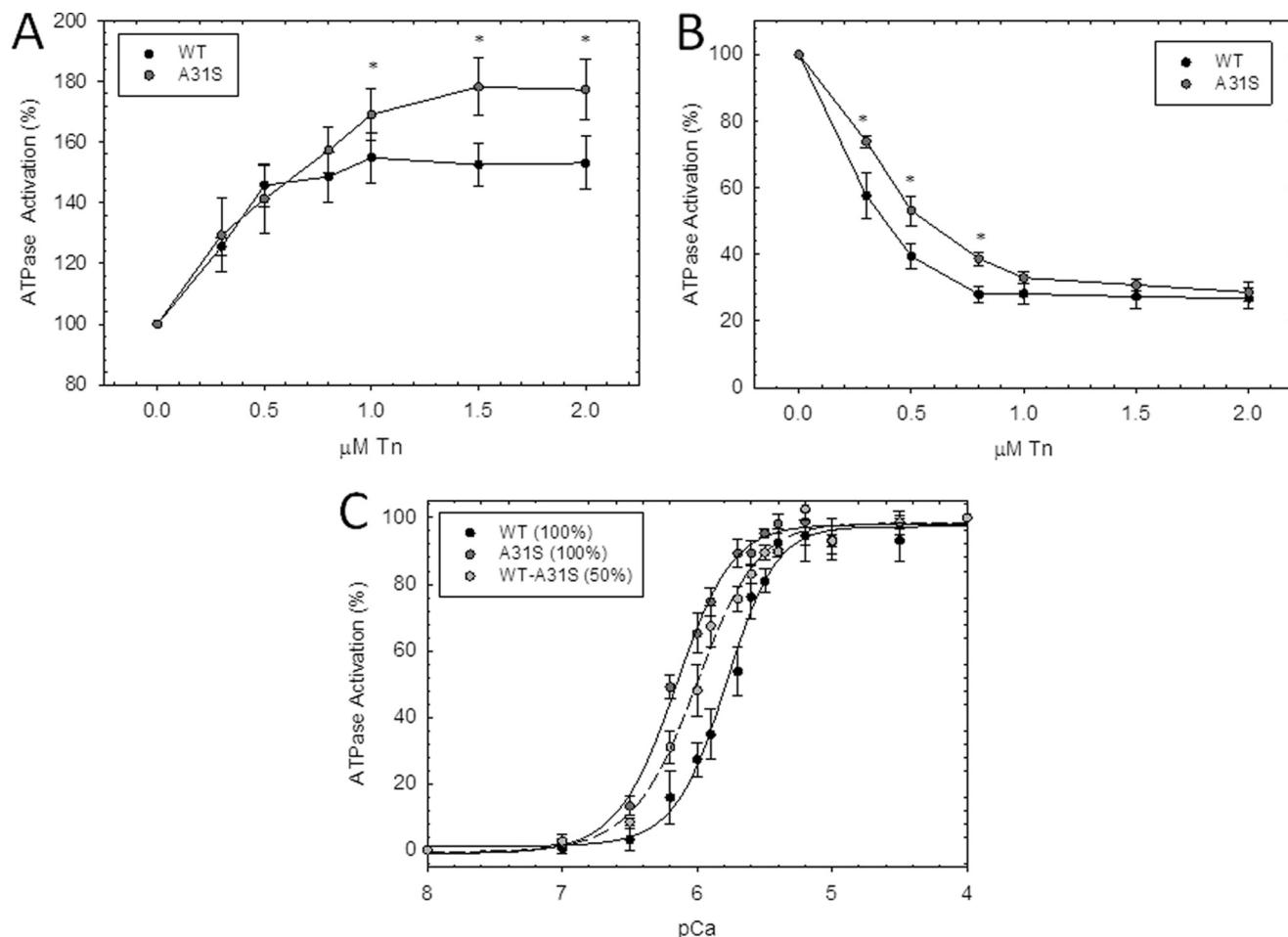


FIGURE 3. Effect of the HCM cTnC mutant on actin-tropomyosin-troponin-activated ATPase activity measurements. *A*, activation of actomyosin ATPase by preformed troponin WT and HCM-cTnC mutant complex at increasing ratios is indicated on the *abscissa* in the presence of Ca^{2+} . *B*, shown is inhibition of the actomyosin ATPase activity by increasing ratios of preformed troponin WT and HCM-cTnC complex in the absence of Ca^{2+} . For *A* and *B*, each point represents an average of six to seven experiments performed in triplicate and is expressed as the mean \pm S.E. *C*, shown is actin-tropomyosin-activated myosin ATPase activity of HCM-cTnC mutant as a function of pCa (each point represents an average of eight experiments, performed in triplicate and expressed as mean \pm S.E.). The *dark gray circles* indicate the presence of 100% cTnC mutant complexes, and *light gray circles with the dotted line* represent experiments performed with a 50:50 ratio of HCM-cTnC mutant to WT complexes. *Black circles* represent data obtained with WT alone. The myosin ATPase activity that occurs in the absence of troponin complex is considered 100% ATPase activity. The specific ATPase activity in the absence of troponin complexes was measured as $0.35 \text{ mol of P}_i \times \text{mol of myosin}^{-1} \times \text{s}^{-1}$.

of binding events (Fig. 4A). Next, the Ca^{2+} affinity of WT and cTnC-A31S was measured with the thin filament proteins (tropomyosin and actin) and myosin S1. Myosin S1 was added to each thin filament containing either WT or cTnC-A31S to determine whether the mutation further enhanced myofibril Ca^{2+} sensitivity when strong cross-bridges formed between S1 and actin. Subsequently, we found that the cTnC-A31S mutant, in the absence of MgATP, increased the Ca^{2+} affinity of the thin filament by +0.25 compared with WT (Fig. 4D and Table 2). In this way, the thin filament as well as cross-bridge binding may play an important role in modulating the Ca^{2+} sensitivity when the A31S mutation is present.

Circular Dichroism of the HCM cTnC Mutant—To determine whether cTnC-A31S had an effect on the structure of the protein in the isolated state, the amount of secondary structure was determined in three conditions: apo (unbound), Mg^{2+} -loaded, and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -loaded states (Fig. 5 and Table 3). Typically, the greatest change in α -helical content occurs when divalent cations, such as Mg^{2+} , bind to the C terminus. Thus,

the Mg^{2+} -bound state is expected to accurately indicate structural changes occurring within the C terminus. The cardiac TnC-A31S mutation did not significantly change the α -helical content of the states examined, although the IAANS probe present in the N terminus was able to detect Ca^{2+} binding events in the C terminus (Fig. 4A).

DISCUSSION

Mutations in Cardiomyopathic and Arrhythmic Disease—Although some HCM-associated mutations have been associated with sudden death susceptibility based on survival studies of the kindred hosting the mutations, there is a paucity of mechanistic insight into the arrhythmogenic state caused by these mutations (26). Early studies identifying “malignant mutations” in families, such as (β -myosin heavy chain) MYH7-R403Q and (cardiac troponin T) TNNT2-R92W, were associated with decreased Kaplan-Meier survival when compared with families hosting so-called “benign” HCM mutations; however, detailed

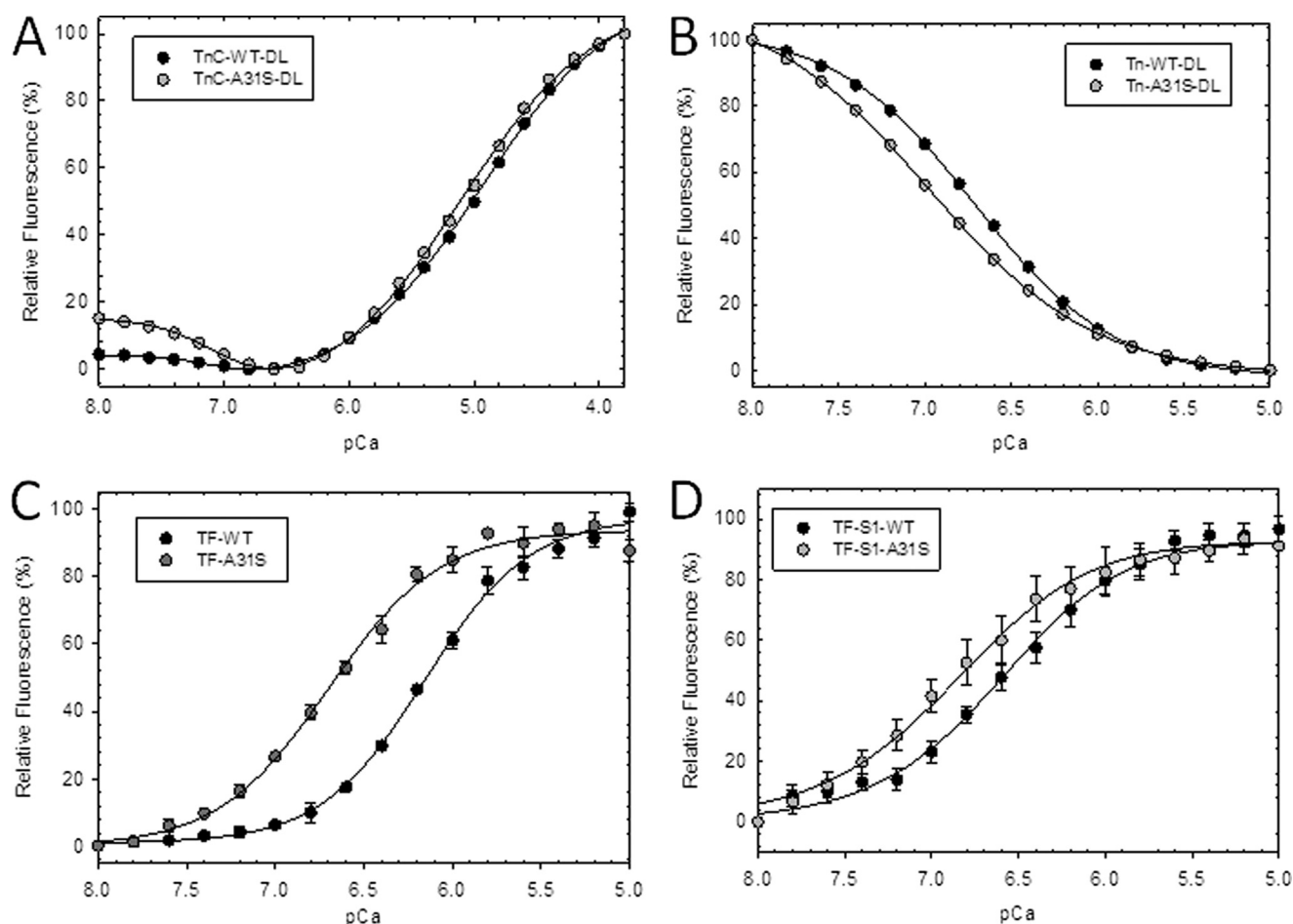


FIGURE 4. Steady state fluorescence to determine the apparent Ca^{2+} affinities of IAANS-labeled cTnC A31S mutant. A, isolated cTnC double-labeled with IAANS at Cys-35 and Cys-84 compare Ca^{2+} titration of A31S (dark gray circles) versus WT (black circles). B, troponin complex with cTnC-IAANS double-labeled at Cys-35 and Cys-84 compare A31S versus WT. C, thin filament with cTnC-IAANS single-labeled at Cys-84 of A31S versus WT is shown. D, thin filament with cTnC-IAANS single-labeled at Cys-84 in the presence of myosin S1 with conditions that favor strong cross-bridge formation ($-\text{ATP}$) is shown. Relative fluorescence values (%) are plotted as a function of Ca^{2+} concentrations in moles. Data are reported as mean \pm S.E. ($n = 4-7$).

TABLE 2

Summary of pCa_{50} of Ca^{2+} affinity measured by IAANS fluorescence at different levels of myofilament complexity

Mutant	cTnC		cTn		Thin filament		Thin filament + S1	
	pCa_{50}	n_{Hill}	pCa_{50}	n_{Hill}	pCa_{50}	n_{Hill}	pCa_{50}	n_{Hill}
WT	4.95 \pm 0.01	0.83 \pm 0.01	6.71 \pm 0.01	1.08 \pm 0.01	6.16 \pm 0.02	1.42 \pm 0.03	6.62 \pm 0.03	1.09 \pm 0.09
A31S	5.12 \pm 0.04 ^a	0.81 \pm 0.01	6.98 \pm 0.01 ^a	0.91 \pm 0.01 ^a	6.70 \pm 0.03 ^a	1.36 \pm 0.08	6.83 \pm 0.04 ^a	1.08 \pm 0.11

^a $p < 0.05$ compared with their respective WT.

mechanistic studies elucidating the sudden death susceptibility potentially caused by these mutations are needed (27, 28).

Significant progress has been made in elucidating the genetic underpinnings of HCM; however, only a handful of mutations in *TNNC1* have been identified. L29Q was one of the first mutations in *TNNC1* to be associated with HCM (7). Although the effects of this mutation remain controversial, functional analysis has suggested that it may be a non-pathogenic variant based on inconsistent findings of altered Ca^{2+} sensitivity (29–32). We previously characterized four additional missense mutations, *TNNC1*-A8V, C84Y, E134D, and D145E, identified in a large cohort of unrelated patients with HCM (6, 33). Cardiac fibers reconstituted with these HCM-associated cTnC mutants results in increased Ca^{2+} sensitivity through mutation-specific alterations of dynamic interactions between cTnC and other components of the cardiac myofilament (33).

None of the earlier reported HCM *TNNC1* mutations have been associated with arrhythmia, except that a recent study provides a link between the clinical outcome of sudden cardiac death and a *TNNC1* mutation (8). A mutation in *TNNC1*, c.363dupG or p.Gln122AlafsX30 (truncation at position 122; cTnC- Δ 122), has been identified in the proband age 19 in a small multi-generational family with HCM demonstrating reduced penetrance and variable expressivity (8). As with prior HCM-associated mutations, mechanistic studies identifying the pro-arrhythmic perturbation potentially imparted by this mutation have not been done to date (8). Therefore, we were unable to analyze shared functional properties between cTnC-A31S and the truncation mutant cTnC- Δ 122 that may induce arrhythmia. The functional properties of this cTnC- Δ 122 mutant are likely altered as it lacks Ca^{2+} binding site IV, eliminating the coupling that exists between sites of the same

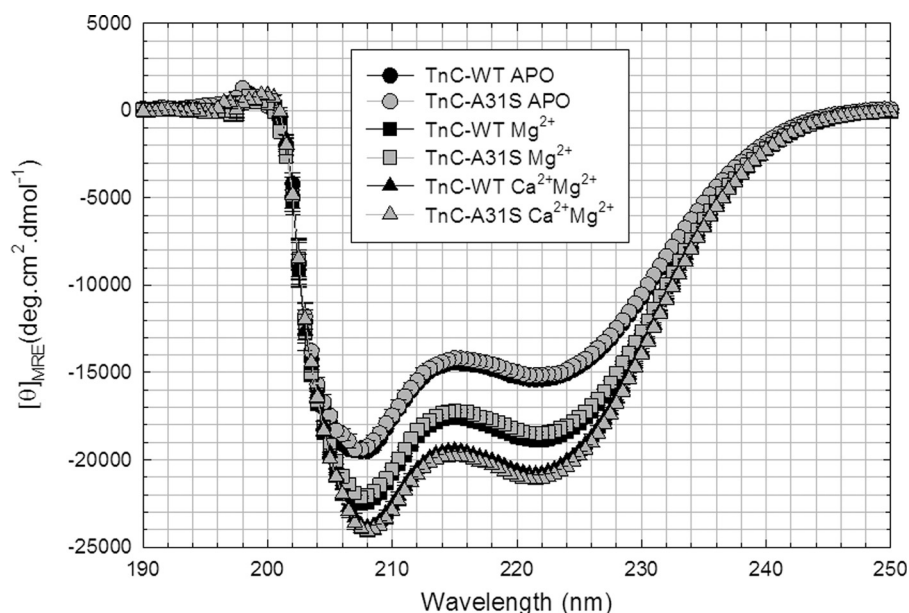


FIGURE 5. **Determination of secondary structural characteristics of HCM mutant and WT-cTnC by circular dichroism.** These spectra compare the changes in α -helical content of WT versus cTnC-A31S under different conditions. The graph compares cTnC in the apo, Mg^{2+} -bound, and Mg^{2+}/Ca^{2+} -bound states.

TABLE 3

Summary of circular dichroism results for HCM cTnC mutants

The CD spectrum is reported for apo (no divalent cations bound) and Mg^{2+} -bound and Ca^{2+}/Mg^{2+} -bound states, and error is reported as S.E. deg. degrees.

TnC	[θ] _{222 nm}			n
	Apo	Mg^{2+}	Ca^{2+}/Mg^{2+}	
		<i>deg.cm².dmol⁻¹</i>		
WT	-15,392.7 ± 106.8	-18,877.3 ± 86.5	-20,802.7 ± 167.0	4
A31S	-15,154.1 ± 290.0	-18,474.7 ± 101.7	-21,062.6 ± 274.9	4

TnC	[θ] _{208 nm}			n
	Apo	Mg^{2+}	Ca^{2+}/Mg^{2+}	
		<i>deg.cm².dmol⁻¹</i>		
WT	-19524.2 ± 194.8	-22420.2 ± 89.3	-23868.1 ± 193.9	4
A31S	-19237.7 ± 344.5	-22048.9 ± 96.1	-24082.8 ± 326.6	4

domain and affecting the Ca^{2+} buffering capacity of cTnC. Subsequently, the cTnC-A31S mutation located within the inactive Ca^{2+} binding site I is unlikely to have restored Ca^{2+} binding to any degree. However, in this case the A31S mutation may affect coupling between sites I and II of the same domain. Additional HCM-linked cTnC mutants may need to be identified and characterized to address how a cTnC mutant might cause arrhythmia.

In general, cardiomyopathic mutations that manifest significant physiological effects by an early age are considered most severe. In this study the patient presented significant cardiac symptoms involving ventricular fibrillation before the age of 4. In our previous study the *TNNC1* mutation C84Y caused mild symptoms (syncope on exertion) at 8.4 years and was successfully managed by β -blockade (6). Comparison of clinical presentation of disease in patients bearing *TNNC1* mutations with data measuring their functional consequences may provide additional insight.

Functional Properties of the HCM-associated cTnC-A31S Mutant—It has been hypothesized that a defining characteristic of *TNNC1* HCM-associated mutations is the increased, or possibly equivalent, Ca^{2+} sensitivity that may also alter the

maximal myocyte force generation (34). To determine whether the mutant increased Ca^{2+} sensitivity in a dominant-negative manner, we performed reconstituted actomyosin assays using 100% cTnC-A31S mutant and also in the “heterozygous” state using 50:50 cTnC-A31S:WT proteins to best represent the background of the patient. The intermediate pCa_{50} obtained with 50% mutant cTnC (compared with 100% mutant or 100% WT) better-approximated the pCa_{50} seen in the more intact skinned fiber system. This suggests that the mutation exerts its influence in a dominant negative manner, although the pCa_{50} value may be altered by different amounts of cTnC mutant incorporation.

The reconstituted thin filaments containing cTnC-A31S displayed a significant increase in activation levels compared with those containing WT (Fig. 3A). Enhanced thin filament activation coupled with increased Ca^{2+} sensitization of the myofilament may contribute toward the diastolic function seen in HCM patients. A likely scenario would be that the increase in Ca^{2+} sensitivity heightens the contractile response and subsequently impairs the degree of relaxation achieved during diastole. Despite the increase in the actomyosin ATPase activation, the maximal force recovery was unchanged in skinned fibers. However, actomyosin ATPase inhibition measurements performed under relaxing conditions (pCa 8.5) showed that cTnC-A31S inhibited ATPase activity to the same degree as WT when (1.0–2.0 μM cTn) was added to the thin filament. This is consistent with *bona fide* HCM cTn mutations, which usually do not alter ATPase inhibition (33). The basal force in skinned fibers was unaffected (data not shown) (35). Taken together, the functional data support the HCM phenotype found in the patient.

The *TNNC1-A31S* Proband—In addition to left ventricular hypertrophy, the proband QTc is at the far upper limits of normal at 460 ms for a prepubertal boy. Usually associated with long QT syndrome, it has been previously demonstrated that elevated QTc is directly, albeit weakly, associated with the

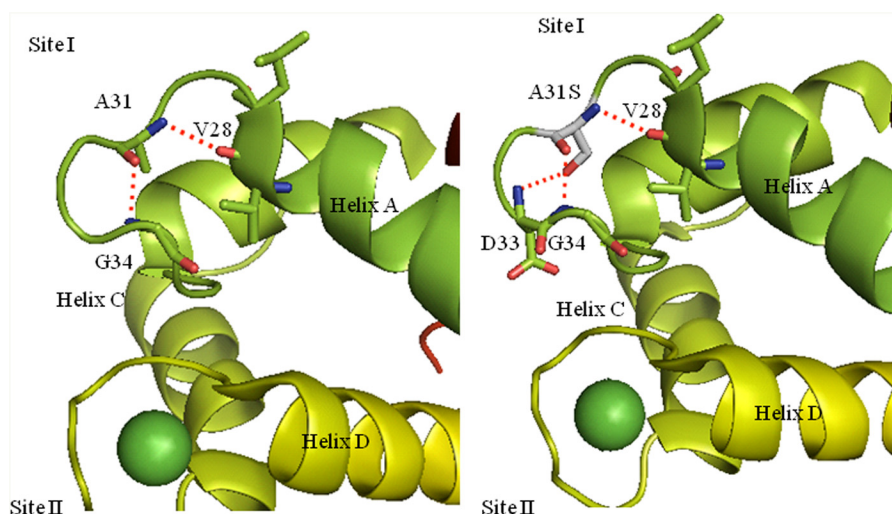


FIGURE 6. **Molecular visualization of the A31S mutation in the crystal structure 1AJ4.** *A*, the H-bonds made by Ala-31 to adjacent residues located in inactive Ca^{2+} binding site I are shown. *B*, shown are the putative H-bonds made when the A31S substitution is present.

degree of left ventricular wall thickness (36, 37). Although mutations in *KCNQ1*-encoded I_{Ks} potassium channel, *KCNH2*-encoded I_{Kr} potassium channel, and *SCN5A*-encoded I_{Na} sodium channel (NaV1.5, LQT3, gain-of-function), the three major genes associated with long QT syndrome, cannot be excluded from our genetic analysis, the elevated QTc observed in this proband is likely a reflection of profound hypertrophy rather than the presence of independent and concomitant long QT syndrome (38).

The etiologies of ventricular fibrillation are diverse. In a structurally normal heart, purely electrical diseases can serve as a pathogenic substrate for ventricular fibrillation and sudden cardiac death. This includes channelopathic diseases such as long QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, and short QT syndrome. The proband hosting the *TNNC1* A31S mutation demonstrated concurrent hypertrophic remodeling of the heart; thus, it is more likely that the ventricular fibrillation and QT prolongation is secondary to his significant underlying hypertrophic cardiomyopathy.

Thin Filament Destabilization and HCM-Associated Alterations in Calcium-Affinity—Our identification of a missense mutation associated with both HCM and sudden cardiac death may represent a novel mechanism of thin filament sensitization. To gain mechanistic insight into how the cTnC-A31S mutation induces the myofilament dysfunction that underlies the presentation of HCM in the patient, we examined the manner in which the mutation altered the Ca^{2+} affinity of the thin filament. These changes in Ca^{2+} affinity likely involve alterations in the Ca^{2+} binding capacity of cTnC that are translated into changes in Ca^{2+} sensitivity within the myofilament. Previously, we found that although mutations occur within cTnC, they can impact the Ca^{2+} affinity of the thin filament in a variety of ways; 1) directly (locally targeting the cTnC Ca^{2+} affinity at the level of cTnC or proteins in direct contact) or 2) indirectly but through local interactions (involving proteins that contact cTnC through its interacting partners *e.g.* cTnI or cTnT) or by occurrences that indirectly influence cTnC Ca^{2+} affinity such as strong cross-bridge formation (39, 40).

The cTnC-A31S mutant increased the Ca^{2+} affinity ($\Delta\text{pCa}_{50} = +0.17$) of the regulatory binding site II of isolated cTnC. In comparison, the previously characterized C84Y mutation also found in the N terminus did not measurably alter Ca^{2+} affinity ($\Delta\text{pCa}_{50} = -0.01$) of site II in isolated cTnC. The effects of the L29Q mutation on Ca^{2+} affinity of site II indicate that cTnC-L29Q was less sensitive to Ca^{2+} ($\Delta\text{pCa}_{50} = -0.12$) than the cTnC-A31S mutant reported here (29). In addition, the C-terminal mutant cTnC-D145E increased Ca^{2+} affinity of site II ($\Delta\text{pCa}_{50} = +0.11$) in a more complex manner, as it indirectly influences Ca^{2+} binding. The other HCM-linked cTnC mutants N-terminal A8V and C-terminal E134D did not statistically change Ca^{2+} affinity of site II in isolated cTnC (33).

Furthermore, we found that the cTnC-A31S mutant sensitized the thin filament to Ca^{2+} ($\Delta\text{pCa}_{50} = +0.54$) to a much larger degree than any cTnC HCM mutants tested to date. In comparison, the previously described HCM cTnC mutants A8V and D145E increased thin filament Ca^{2+} affinity with (ΔpCa_{50} s of +0.14 and +0.08), respectively (33). It is well known that the number of cross-bridges can modulate both Ca^{2+} sensitivity and TnC affinity for the thin filament (41–47). Therefore, the addition of myosin S1 was used to assess whether strong cross-bridge formation indirectly ($-$ ATP) modulated the Ca^{2+} affinity of the mutant containing thin and thick filament differently than the WT (41). S1 decreased the ΔpCa_{50} of the cTnC-A31S thin filament compared with when it was in a lower level of complexity (thin filament alone), which indicates that the thin filament may not always be the best predictive model. Subsequently, Ca^{2+} affinity of troponin with the addition of the thin filament and the catalytic myosin S1 more closely recapitulates what is seen in skinned fibers.

When combined, these data indicate that the A31S mutation increased Ca^{2+} affinity of cTnC of this increasingly complex system through a direct mechanism, such as increased affinity of the cTnC regulatory domain for Ca^{2+} or another known mechanism such as increased affinity of the mutant for cTnI. Potentially, a dramatically increased Ca^{2+} affinity of cTnC has a higher likelihood of perturbing its Ca^{2+} buffering role. The A31S mutant appears to substantially affect interactions

TNNC1-A31S in HCM and Ventricular Fibrillation

between thin filament proteins, and this level may serve as the primary source of increased Ca^{2+} sensitivity. In summary, the profoundly altered Ca^{2+} binding properties of the cTnC-A31S mutant in the isolated state, cTnC complex and thin filament coupled with early presentation of disease in the proband has never been shown before with a HCM-associated cTnC mutant.

Structural Characterization of the cTnC-A31S Mutant—Visualization of the troponin complex using the PyMol program suggested that A31S mutation may exert its Ca^{2+} -sensitizing effects by locally stabilizing the EF-hand structure of the inactive Ca^{2+} binding site I located in the N terminus (Fig. 6). The substitution of serine for alanine at position 31 introduces a polar amino acid into the Ca^{2+} binding loop. Using a mutagenic function of the program without energy minimalization, substitution of Ser-31 in the mutant cTnC introduces a reactive hydroxyl group in close proximity to the backbone NH_2 group of Asp-33 (see Fig. 6A). The program predicts formation of an additional H-bond that would provide three H-bonds to stabilize the structure of the inactive Ca^{2+} binding site (see Fig. 6B). It has been shown in skeletal troponin C that binding of Ca^{2+} to the EF hands is cooperative, where coordination exists between the Ca^{2+} binding sites of each domain. This same coordination has not been shown in the cTnC N terminus as Ca^{2+} cannot bind to Site I. Therefore, we speculate that this mutation helps to order the structure of site I in a manner similar to Ca^{2+} binding to site I in skeletal troponin C. This will need to be verified by established structural techniques that provide coordinates for the mutant protein compared with WT.

The CD data in the unbound (apo) state indicate that the mutation does not globally affect cTnC structure. No significant alterations were seen in the Mg^{2+} -bound cTnC structure, indicating that the N-terminal mutation does not perturb the structure of the C terminus, as Mg^{2+} binds primarily to the C terminus. The mutation also did not detectably alter the secondary structure of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -bound cTnC; this is not surprising as most of the spectral changes that occur when cTnC binds Ca^{2+} originate from the C terminus (48, 49). Therefore, we suggest that A31S in isolated cTnC sensitizes Ca^{2+} binding to site II by locally affecting coordination between the Ca^{2+} binding sites, which subtly affects overall structure, and results in a dramatic change in function.

In conclusion, the discovery of the TNNC1 mutation A31S represents one of the first cTnC mutants associated with verified episodes of ventricular fibrillation and aborted sudden cardiac death. This mutation may alter Ca^{2+} handling and result in both hypertrophic and electrophysiologic remodeling of the cardiomyocyte. From our characterization, it appears that the A31S mutation may increase the Ca^{2+} binding affinity of the regulatory site II of cTnC by stabilizing the N-domain structure. This in turn could bestow a tremendous capacity for Ca^{2+} sensitization of the mutant containing myofilament. This mechanistic analysis coupled with clinical data provides insight on the source of myofilament dysfunction and the pathogenic nature of the mutation. It is evident that additional TNNC1 mutations will be discovered in the future and that this study along with others is essential to establish defined functional

profiles characteristic of disease-causing mutations located within TNNC1.

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