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Functional Characterization of *TNNC1* Rare Variants Identified in Dilated Cardiomyopathy*

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TNNC1*, which encodes cardiac troponin C (cTnC), remains elusive as a dilated cardiomyopathy (DCM) gene. Here, we report the clinical, genetic, and functional characterization of four *TNNC1* rare variants (Y5H, M103I, D145E, and I148V), all previously reported by us in association with DCM (Hershberger, R. E., Norton, N., Morales, A., Li, D., Siegfried, J. D., and Gonzalez-Quintana, J. (2010) *Circ. Cardiovasc. Genet.* 3, 155–161); in the previous study, two variants (Y5H and D145E) were identified in subjects who also carried *MYH7* and *MYBPC3* rare variants, respectively. Functional studies using the recombinant human mutant cTnC proteins reconstituted into porcine papillary skinned fibers showed decreased Ca^{2+} sensitivity of force development (Y5H and M103I). Furthermore, the cTnC mutants diminished (Y5H and I148V) or abolished (M103I) the effects of PKA phosphorylation on Ca^{2+} sensitivity. Only M103I decreased the troponin activation properties of the actomyosin ATPase when Ca^{2+} was present. CD spectroscopic studies of apo (absence of divalent cations)-, Mg^{2+}-, and Ca^{2+}/Mg^{2+}-bound states indicated that all of the cTnC mutants (except I148V in the Ca^{2+}/Mg^{2+} condition) decreased the α-helical content. These results suggest that each mutation alters the function/ability of the myofilament to bind Ca^{2+} as a result of modifications in cTnC structure. One variant (D145E) that was previously reported in association with hypertrophic cardiomyopathy and that produced results *in vivo* in this study consistent with prior hypertrophic cardiomyopathy functional studies was found associated with the *MYBPC3* P910T rare variant, likely contributing to the observed DCM phenotype. We conclude that these rare variants alter the regulation of contraction in some way, and the combined clinical, molecular, genetic, and functional data reinforce the importance of *TNNC1* rare variants in the pathogenesis of DCM.

The dilated cardiomyopathy (DCM)[3] phenotype is manifested by a decrease in left ventricular contractility, ventricular wall thinning, and dilation of the ventricular chamber, all of which lead to a reduction in the ejection fraction (1). Adverse consequences of DCM include heart failure, arrhythmia, and sudden cardiac death (2). Although DCM can be caused by a variety of factors, genetic predisposition remains an important cause (3). Continued efforts are necessary to discover the heritable causes of DCM in patients and, when identified, substantiate their significance with relevant functional studies (3).

DCM-causing mutations have been found in several sarcomeric proteins, but only a few have been identified in *TNNC1*, which encodes cardiac troponin C (cTnC), a Ca^{2+} sensor and key regulator of contraction (4). TnC plays an important role in excitation-contraction coupling and is the primary target of compounds such as levosimendan that augment Ca^{2+} responsiveness of the thin filament (5). Functionally, DCM troponin mutations manifest distinctively as decreased Ca^{2+} sensitivity, reduced thin filament Ca^{2+} affinity, and decreased *in vitro* cross-bridge cycling rate (4). The first DCM-causing mutation identified in *TNNC1*, G159D, was found in three families (6, 7). In functional studies, G159D showed little-to-no decrease in myofilament Ca^{2+} sensitivity (8–10) but an ablated response to PKA phosphorylation (9). It is known that cardiac β-adrenergic stimulation activates PKA in the myocardium, and phosphorylation of its targets in the myofilament leads to its decreased Ca^{2+} sensitivity (11–13). Structural studies have elucidated the molecular impact of the G159D mutation. By NMR spectroscopy, it was determined that the C-terminal domain of cTnC containing the G159D mutation has lower affinity for the cTnI-(37–71) peptide (14). In addition, the combinatory purported mutations E59D and D75Y were identified in a patient with idiopathic DCM, and functional characterization also suggested their potential relevance (15, 16).

Knock-out of cTnC in adult zebrafish using an inducible antisense strategy confirmed that the loss of TnC function leads to a phenotype consistent with DCM (17). Another recent human study identified a mutation in *TNNC1* that segregated with familial DCM (including a member with peripartum cardiomyopathy), adding to the genetic evidence of this disease (18). In this study, we present clinical, pedigree, and functional studies of the Y5H, M103I, D145E (this mutation was previ-

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[3] The abbreviations used are: DCM, dilated cardiomyopathy; cTn, cardiac troponin; HCM, hypertrophic cardiomyopathy; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; Tm, tropomyosin.

ously studied as a hypertrophic cardiomyopathy (HCM)-linked mutation), and cTnC I148V genetic variants in human DCM and show that two of these novel rare variants manifested functional properties typical of DCM (decreased Ca^{2+} sensitivity), accompanied by altered responsiveness to PKA phosphorylation in all the mutants.

EXPERIMENTAL PROCEDURES

Patient Population

Written informed consent was obtained from all subjects, and the Institutional Review Boards of the Oregon Health & Science University and the University of Miami Miller School of Medicine approved the project. The study was a subset of a previous publication (19) that used methods of clinical categorization of subjects with DCM as described previously (19–21).

Genetic Analysis

Genomic DNA was extracted from whole blood and sequenced in both directions to detect nucleotide variants in *TNNC1* (cTnC) as previously described (19, 21). All exons and intron/exon boundaries were PCR-amplified by standard methods at SeattleSNPs. Samples from probands identified by the resequencing service as carriers of protein-altering variants, as well as any available samples from their relatives, were resequenced in our laboratory for confirmation and segregation analysis. Nucleotide changes were evaluated only if they were absent in all 246 control samples analyzed at the resequencing center (186 white, 23 Yoruban, 19 Asian, and 18 Hispanic) as reported previously (19, 21).

Functional and Structural Studies

Recombinant Proteins—The recombinant TnC mutants Y5H, M103I, D145E, and I148V were cloned as previously described (22). Prior to expression and purification, all subcloned DNAs were sequenced to verify that the sequences were correct. *Escherichia coli*-derived BL21-CodonPlus(DE3) cells were transformed with pET-3d constructs containing human cTnC. The WT and mutant proteins were overexpressed and purified as described previously (22).

Fiber Preparation and Ca^{2+} Dependence of Force Development Measurements—Fresh cardiac tissue was obtained from slaughterhouse pigs. Strips of papillary were isolated from the left ventricle and skinned overnight (22). Briefly, a skinned fiber bundle ~75–100 μm in diameter was mounted using stainless steel clips to a force transducer and then immersed in a *p*Ca 8.0 relaxation solution as described (22). Native cTnC was depleted upon incubation of the fiber in 5 mM CDTA and 25 mM Tris (pH 8.4) for ~1.5 h. Fibers were then incubated with 55 μM mutant or WT cTnC diluted in *p*Ca 8.0 solution for 1 h (22). For the PKA experiments, after TnC reconstitution, the fibers were incubated with 500 units/ml PKA catalytic subunit (Sigma P2645) for 30 min under relaxing conditions. The Ca^{2+} dependence of force development was tested in the skinned fibers in various Ca^{2+} solutions. The following equation was used to analyze the data: % change in force $= 100 \times [Ca^{2+}]^n/([Ca^{2+}]^n + [Ca^{2+}{}_{50}]^n)$, where $[Ca^{2+}{}_{50}]$ is the free $[Ca^{2+}]$ that produces 50% force and n is the Hill coefficient.

Activation and Inhibition of the Actin-Tropomyosin-activated Myosin ATPase—Human cTnT and cTnI were overexpressed and purified as described (23). To form the troponin complexes, the individual troponin subunits 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 troponin subunits were mixed in a 1.3:1.3:1 TnT/TnI/TnC molar ratio. Then, the complexes were successively dialyzed against decreasing concentrations of KCl (0.7, 0.5, 0.3, 0.1, 0.05, and 0.025 M). The troponin complexes were centrifuged to remove any precipitated TnT and TnI. Prior to storage of the troponin complexes at −80 °C, SDS-PAGE was performed to determine the stoichiometry of the troponin subunits. Additional proteins utilized in this assay included porcine cardiac myosin, rabbit skeletal F-actin, and porcine cardiac tropomyosin (Tm), which were prepared as described previously (24). The protein concentrations utilized for actomyosin ATPase assays were as follows: 0.6 μM porcine cardiac myosin, 3.5 μM rabbit skeletal F-actin, 1 μM porcine cardiac Tm, and 0–2 μM preformed Tn complexes (described above). The ATPase inhibitory assay was performed using a 0.1-ml reaction mixture consisting of 3.4 mM $MgCl_2$, 0.13 μM $CaCl_2$, 1.5 mM EGTA, 3.5 mM ATP, 1 mM DTT, and 11.5 mM MOPS (pH 7.0) at 25 °C. The ATPase activation assay used the same 0.1-ml buffer mixture but with adjustments for 3.3 mM $MgCl_2$ and 1.7 mM $CaCl_2$. The reaction was initiated by the addition of ATP, and activity was quenched after 20 min with trichloroacetic acid to a final concentration of 35%. The precipitated assay proteins were removed by centrifugation, and the supernatant was analyzed according to the method of Fiske and SubbaRow (25) to determine the concentration of inorganic phosphate released by ATP hydrolysis.

Circular Dichroism Measurements—Far-UV CD spectra were collected using a 1-mm path length quartz cell in a Jasco J-720 spectropolarimeter with a bandwidth of 1 nm at a speed of 50 nm/min at room temperature (23). The mean residue ellipticity ($[\theta]_{MRE}$ in degrees·cm^2·dmol^{-1}) for the spectra was calculated with Jasco software using the following equation: $[\theta]_{MRE} = [\theta]/(10 \times C_r \times l)$, where $[\theta]$ is the measured ellipticity in millidegrees, C_r is the mean residue molar concentration, and l is the path length in centimeters. For all single experiments, 10 scans were collected and averaged without numerical smoothing, and the optical activity of the buffer was subtracted from relevant protein spectra. The experimental protein concentration for the WT and mutants was determined to be 0.2 mg/ml by the biuret reaction using bovine serum albumin as a standard. The CD experiments were performed under three different conditions: for apo, 1 mM EGTA, 20 mM MOPS, and 100 mM KCl (pH 7.0); for Mg^{2+}, 1 mM EGTA, 20 mM MOPS, 100 mM KCl, and 2.075 mM $MgCl_2$ (pH 7.0); and for Ca^{2+}/Mg^{2+}, 1 mM EGTA, 20 mM MOPS, 100 mM KCl, 2.075 mM $MgCl_2$, and 1.096 mM $CaCl_2$ (pH 7.0).

Statistical Analysis

The experimental results are reported as mean ± S.E. and were analyzed for significance using Student's t test at $p < 0.05$.

TABLE 1
Non-synonymous mutations in *TNNC1* (cTnC)

Pedigree	Molecular genetic data: UCSC hg18 coordinates	Molecular genetic data: Nucleotide change[a]	Molecular genetic data: Amino acid change	Clinical and pedigree data: Diagnosis, FDC[b] or IDC	Clinical and pedigree data: Race	Clinical and pedigree data: Disease-associated status in our prior report (19)	Clinical and pedigree data: Phenotype of variant reported previously (22)
A	chr3:52463059	2040T→C	Y5H	IDC	Caucasian	Possibly	
B	chr3:52460808	4291G→A	M103I	FDC	Caucasian	Likely	
C	chr3:52460466	4633C→A	D145E	IDC	Caucasian	Possibly	HCM
D	chr3:52460459	4640A→G	I148V	FDC	Caucasian	Possibly	

[a] Nucleotide numbering is per the SeattleSNPs resequencing service (gi:302313132).
[b] FDC, familial DMC; IDC, idiopathic DCM.

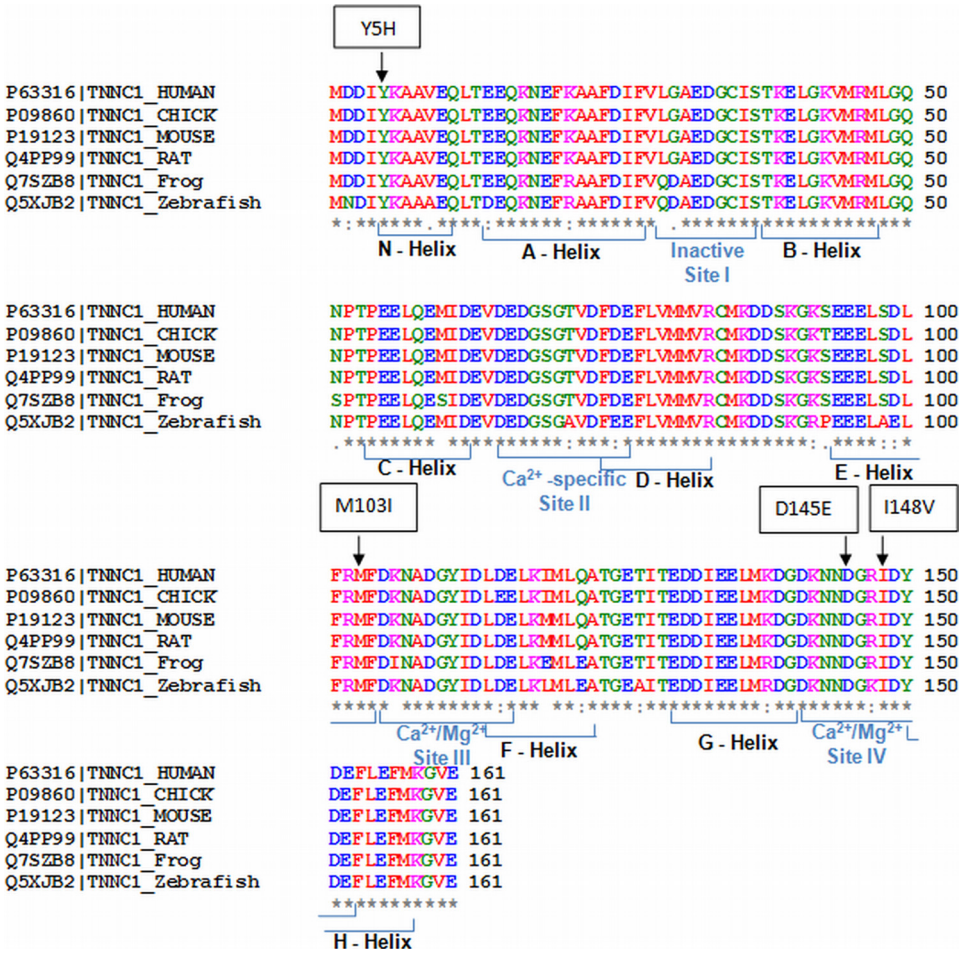

FIGURE 1. ***TNNC1* (cTnC) gene structure and amino acid conservation resulting from the identified rare variants.** The *TNNC1* gene shows a remarkable degree of conservation between mammalian, avian, amphibian, and fish species. Altered amino acids are indicated for each missense mutation in a *box* above the first line, and the human wild-type sequence is shown in the first line, followed by chicken, mouse, rat, frog, and zebrafish sequences. All variants were conserved throughout these various species. Information concerning the helices and sites can be found under UniProt accession number P63316.

RESULTS

Molecular Genetic Data—The *TNNC1* rare variants identified are shown in Table 1, and each was conserved among species (Fig. 1).

Clinical Data—Clinical assignments for each subject available for analysis and their family pedigrees are provided in Fig. 2 and Tables 1 and 2 according to the *TNNC1* rare variant identified. None of these variants were seen in control DNAs (246 DNAs, 492 chromosomes) in our initial report, providing support that these were rare variants (19). This conclusion is supported by a prior *TNNC1* study in HCM that sequenced 500 control DNAs (1000 control chromosomes) where only one synonymous variant was identified (22), suggesting that the cTnC protein is highly conserved.

Pedigree A—The proband in Pedigree A (A.3) was hospitalized with heart failure at 2 weeks of age and was diagnosed with congenital DCM. He received medical therapy but later developed heart failure at age 14 and received a heart transplant at age 15. Two missense variants (*TNNC1* Y5H and *MYH7* R1045C) not seen in 246 controls and 253 controls, respectively, were detected in the proband (19, 21). His mother, who was clinically unaffected (negative echocardiogram and electrocardiogram at age 47), was negative for both variants. His father was positive for the *MYH7* R1045C variant and negative

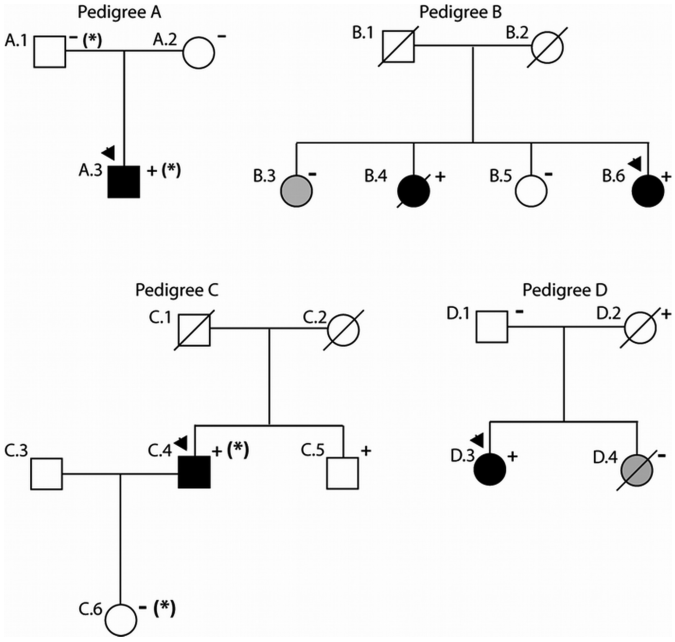

FIGURE 2. **Pedigrees of cTnC-associated DCM.** Pedigrees have been labeled by letters, which correspond to their respective mutation as shown in Fig. 1 and are given in Tables 1 and 2. *Squares* represent males, and *circles* represent females. *Arrowheads* denote the probands. *Diagonal line* mark deceased individuals. *Solid symbols* indicate DCM with or without heart failure; *shaded symbols* represent any cardiovascular abnormality; and *open symbols* represent unaffected individuals. The presence or absence of the family's *TNNC1* rare variant is indicated by + or −, respectively. Obligate carriers are noted in *parentheses* (+). The *asterisk* in Pedigree A denotes an *MYH7* R1045C rare variant and that in Pedigree C denotes an *MYBPC3* P910T rare variant thought to be possibly disease-causing as reported previously (19).

for the *TNNC1* Y5H variant. Thus, this *TNNC1* variant is likely a *de novo* mutation or results from germ line mosaicism in one of his parents. Clinical data are not available on this subject's father, although by patient report, he is asymptomatic with no history of heart disease, and he is currently 51 years old. No other clinical or genetic data are available for this family.

Pedigree B—The proband in Pedigree B (B.6) carried an M103I alteration in *TNNC1*, which segregated with disease in a sister (B.4) who was diagnosed with DCM at age 39 and died at age 48 of non-cardiac causes. The variant was absent in the two other sisters (B.3 and B.5). The proband (B.6) and her two unaffected sisters (B.3 and B.5) all have a history of conduction system disease and/or syncopal episodes, as well as a long QT interval.

Pedigree C—The proband in Pedigree C (C.4) (Fig. 2 and Table 2) was found to carry two missense variants (*TNNC1* D145E and *MYBPC3* P910T). The *TNNC1* variant was previously reported in a proband with familial HCM (22) who presented at age 57 with chest pain and dyspnea, with a maximal left ventricular wall thickness of 22 mm and a left ventricular outflow tract obstruction of 100 mm Hg; several other family members were affected with HCM. The proband in Pedigree C (C.4) had DCM without any evidence of an HCM phenotype (Table 2). A brother of the proband (Pedigree C; not shown) (Fig. 2) was reported by family history to have HCM; however, no medical records or genetic analysis is available for this family member to assess segregation of either variant with cardiomyopathy. Another brother (C.5) who had normal cardiac screening at age 63 without evidence of cardiomyopathy was found to carry the *TNNC1* variant only. The proband's daughter (C.6) (Fig. 2) carries only the *MYBPC3* variant seen in the proband and had normal cardiac screening at age 38.

Pedigree D—The *TNNC1* I148V nucleotide alteration in the proband of Pedigree D (D.3) (Fig. 2) is potentially disease-causing (Table 2), as it predicted the replacement of a conserved amino acid; however, no additional pedigree information was available to assess segregation with disease. A sister (D.4) died in her sleep at age 50 and was found to have left ventricular hypertrophy on autopsy; this individual did not carry the I148V *TNNC1* variant. The variant was inherited from the proband's mother, who died at age 74 with a history of a left bundle branch block as determined by an electrocardiogram at age 69 and coronary artery disease, but echocardiographic data and any further medical information are not available.

Skinned Cardiac Fibers—Skinned fibers reconstituted with cTnC mutants Y5H ($p\mathrm{Ca}_{50}$ = 5.532 ± 0.012) and M103I ($p\mathrm{Ca}_{50}$ = 5.548 ± 0.013) showed a statistically significant decrease in the Ca^{2+} sensitivity of force development compared with WT cTnC ($p\mathrm{Ca}_{50}$ = 5.613 ± 0.012) (Fig. 3*A* and Table 3). However, fibers reconstituted with I148V ($p\mathrm{Ca}_{50}$ = 5.573 ± 0.016) did not show a significant decrease in the Ca^{2+} sensitivity compared with WT cTnC (Fig. 3*A* and Table 3). We previously reported the Ca^{2+} sensitivity of force for skinned fibers reconstituted with D145E (22). In contrast to the other cTnC mutants, D145E ($p\mathrm{Ca}_{50}$ = 5.898 ± 0.008) displayed a leftward shift in the Ca^{2+} sensitivity compared with WT cTnC (Table 3). The Hill coefficient (n_H), which is an index of the cooperative activation of the myofilament, was not affected in any way by the incorporation of cTnC mutants (Table 3). It has been well established that PKA phosphorylation of cardiac myofilament proteins decreases their Ca^{2+} sensitivity of force and increases relaxation of cardiac muscle (13). Skinned fibers reconstituted with the cTnC mutants and incubated with PKA showed a reduced (Y5H and I148V) or abolished (M103I) effect of PKA phosphorylation on myofilament Ca^{2+} desensitization compared with WT cTnC (Fig. 3*B* and Table 3). The $\Delta p\mathrm{Ca}_{50}$ ($p\mathrm{Ca}_{50}$ PKA-treated − $p\mathrm{Ca}_{50}$ PKA-untreated) values for WT cTnC and mutants Y5H, M103I, and I148V were −0.117, −0.071, −0.018, and −0.054, respectively (Table 3). In contrast, D145E displayed an extensive Ca^{2+} desensitization upon PKA phosphorylation ($\Delta p\mathrm{Ca}_{50}$ = −0.343). The cTnC mutants did not affect the maximal force recovery in the presence or absence of PKA compared with WT cTnC (except for D145E in the absence of PKA (Fig. 3*B* and Table 3) as shown previously (22)).

Actomyosin-Tm-Tn ATPase Activity—We measured the ability of the mutant cTn complexes to activate (in the presence of Ca^{2+}) and inhibit (in the absence of Ca^{2+}) the actomyosin-Tm ATPase activity. cTn containing the M103I mutation was the only complex that was not able to fully activate the actomyosin-Tm ATPase activity compared with WT cTn (Fig. 4*A*). Inhibition of the actomyosin-Tm ATPase activity was not affected by any of the cTnC mutants (Fig. 4*B*). Activation and inhibition of actomyosin-Tm-Tn ATPase activity by D145E has been reported in our previous study (23). D145E increased activation of the actomyosin-Tm-Tn ATPase activity, although it

TABLE 2
Clinical characteristics

Subject	Age of diagnosis or screening	DCM	LVEDD (*Z*-score)[a]	LV septum, posterior wall thickness	Ejection fraction	ECG/arrhythmia	*TNNC1* mutation present?	Comment
	years		*mm*	*mm*	*%*			
Pedigree A: Y5H								
A.1	NA	Unk	NA	NA	NA	NA	No	Asymptomatic at 51 years, no clinical screening data
A.2	47	No	43 (−0.86)	7, 7	65	Unusual P axis, possible ectopic atrial rhythm	No	
A.3	2 weeks	Yes	72 (5.71)	5, 9	24	NSR, biatrial enlargement, possible RVH	Yes	Echo data at age 15 years, heart transplant at 15 years, also has *MYH7* R1045C rare variant
Pedigree B: M103I								
B.3	40	No	56 (3.11)	9, 9	60	1st degree AVB, SVT, ICD	No	LVE, syncope, palpitations, ICD age 41
B.4	39	Yes	59 (3.47)	11, 12	43	NSR, LAE, septal MI	Yes	
B.5	47	No	46 (−0.084)	7, 8	65	Sinus bradycardia, PVCs, QT prolongation	No	PM at age 47
B.6	47	Yes	60 (3.96)	10, 9	42	AF, RVR, LVH, PVCs, LAD, anterolateral infarction, long QT	Yes	ICD and PM after cardiac arrest at age 49
Pedigree C: D145E								
C.4	60	Yes	73	11, 12	10	LBBB, 1st degree AVB	Yes	Also has *MYBPC3* P910T rare variant
C.5	63	No	51 (−0.01)	11, 10	65	NSR, frequent PVCs, trigeminy	Yes	
C.6	38	No	40 (−2.44)	7, 8	65	NSR	No	Has *MYBPC3* P910T rare variant
Pedigree D: I148V								
D.1	NA	Unk	NA	NA	NA	NSR, RBBB, LVH, inferior MI pattern	No	No cardiovascular data except ECG available at age 70
D.2	NA	Unk	NA	NA	NA	NSR, LBBB, LVH	Yes	Coronary artery disease by angiogram at age 69, ECG at age 69
D.3	40	Yes	56 (3.40)	8, 10	30	NSR, LBBB	Yes	
D.4	40	Unk	49 (1.49)	8, 8	62	NA	No	Normal echo at age 40, died in sleep at age 50, autopsy (LVH)

[a] LVEDD, left ventricular end-diastolic dimension; LV, left ventricular; ECG, electrocardiogram; NA, data not available; Unk, unknown; NSR, normal sinus rhythm; RVH, right ventricular hypertrophy; echo, echocardiogram; 1st degree AVB, first degree atrioventricular block; SVT, supraventricular tachycardia; ICD, implantable cardiac defibrillator; LVE, left ventricular end; LAE, left atrial enlargement; MI, myocardial infarction; PVC, premature ventricular contraction; PM, pacemaker; AF, atrial fibrillation; RVR, rapid ventricular response; LVH, left ventricular hypertrophy; LAD, left axis deviation; LBBB, left bundle branch block; RBBB, right bundle branch block.

did not affect the inhibitory properties of the actomyosin ATPase (23).

Circular Dichroism—CD analyses were performed to address whether the mutations disrupt the normal secondary structure of the isolated protein. As shown in Fig. 5, the cTnC mutants decreased the amount of secondary structure under the apo (absence of cations) and Mg^{2+} conditions compared with WT cTnC. However, in the presence of Ca^{2+}/Mg^{2+}, I148V was the only mutant that did not show any significant rearrangements in secondary structure compared with WT cTnC (Fig. 5, *lower panel*). Table 4 lists the averages of the mean residue ellipticity in degrees·cm^2·$dmol^{-1}$ measured at 222 and 208 nm. The peaks at 222 and 208 nm are known to coincide with α-helix and β-sheets analyses, respectively. As reported previously, D145E showed a significant decrease in the amount of α-helix content in the apo- and Ca^{2+}/Mg^{2+}-bound states (23).

DISCUSSION

Three novel variants (Y5H, M103I, and I148V) showed functional properties typical of those reported previously in DCM associated with cTn mutations. These properties include decreased Ca^{2+} sensitivity of contraction, impaired response of the myofilament to undergo Ca^{2+} desensitization upon PKA phosphorylation, and often a diminished capacity to recover force in skinned fibers. Furthermore, reconstituted ATPase assays frequently show an impaired ability to activate the thin filament (8, 15, 26–29). With regard to PKA phosphorylation, these responses were altered in all of the novel DCM mutants tested here, consistent with what was shown previously for the DCM TnC mutation G159D (9). Further evidence of this phenomenon was provided when the cTnC mutations Y5H and I148V blunted PKA-mediated Ca^{2+} desensitization in skinned fibers, whereas M103I completely abolished the effects of PKA phosphorylation.

The fourth cTnC variant, D145E, previously reported in association with HCM (22), was observed in our study in an individual with a DCM phenotype who also harbored another sarcomeric rare variant in *MYBPC3*. With the D145E variant, we have previously observed enhanced myofilament Ca^{2+} sensitization, consistent with HCM (4, 22). We suggest that the sarcomeric mutation in *MYBPC3* may have modulated the otherwise likely HCM phenotype, based on prior reports, to the DCM phenotype observed in this patient. These results point to the complexity of mutation-based disease, where mutations in sarcomeric proteins such as cTnC may alter the proteins themselves, interactions with their associated proteins, or the ability to respond to higher order converging systems of regulation.

This study provides a valuable integration of clinical DCM data from different DCM-associated mutations to add to the

evidence that these rare variants are indeed disease-causing and to rigorously assess the fundamental basis of myofilament dysfunction. The precise molecular pharmacologic mechanisms of DCM are still unclear; however, most of the DCM mutations (including Y5H and M103I studied here) significantly decrease the Ca^{2+} sensitivity of skinned fibers (4). Another emerging concept is that the DCM mutations may affect the phosphorylation status/function of cTnI, and this may be an important parameter governing disease phenotypes (9, 30, 31). A number of thin filament mutations in *TNNI3*, *ACTC*, and *TNNC1* (also reported here) have been found to uncouple the effects of PKA phosphorylation on Ca^{2+} sensitivity, although these mutations do not always produce the same clinical phenotype (9, 30–32). This observation is based on a limited number of mutations that diminish lusitropy in the heart and impair heart rate acceleration and increased contractile force normally induced by β-adrenergic stimulation. Subsequently, this dysfunction causes the heart to lose its dynamic response to stress. In humans, it has been shown that DCM mutations involve primarily defective force transmission (33), which in functional studies is observed as decreased force recovery in skinned fibers with a corresponding reduction in ATPase activation (8, 15, 26, 27, 29). *In vitro* motility assays also indicated a decrease in Ca^{2+} sensitivity and a loss of thin filament sliding velocity (10). This dysfunction at the myofilament level may progress over time and lead to diastolic and systolic dysfunction, pathological remodeling, and ultimately heart failure.

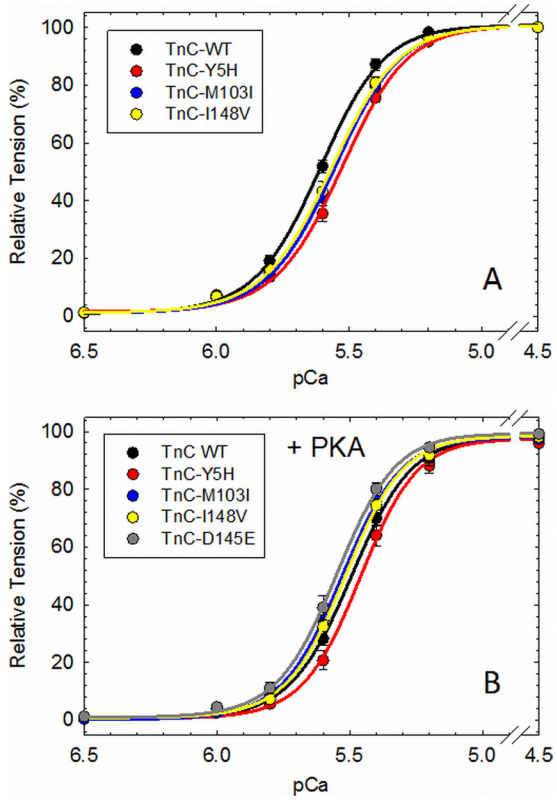

FIGURE 3. ***p*Ca-force relationship measured in porcine skinned papillary fiber containing DCM TnC mutants.** Native porcine TnC was extracted, and the skinned fibers were reconstituted with WT TnC or the DCM mutant. The Ca^{2+} sensitivity of force development was measured at pH 7.0 before (*A*) and after (*B*) PKA treatment. The fiber data are summarized in Table 3.

Mutations occurring in proteins such as phospholamban are normally accompanied by altered Ca^{2+} handling, a hallmark of DCM (34–36). We speculated that the decrease in myofilament Ca^{2+} affinity may affect cytosolic Ca^{2+} levels, interfering with the Ca^{2+} transient, thus leading to cardiac dysfunction and a predisposition to arrhythmia (37). We have previously shown in transgenic animal models bearing TnI Ca^{2+}-sensitizing mutations that they also display a simultaneous delay in Ca^{2+} transients in intact papillary muscle fibers (38, 39). This further highlights the importance of cTnC to the responsiveness of the thin filament to Ca^{2+}, the fundamental stimulus signaling contraction. Mutations in cTn may also diminish the contractile response, which could subsequently lead to a reduction in the ejection fraction seen in DCM patients.

The CD studies of isolated cTnC provide insight into whether the DCM mutations alter the global structure and capacity to transduce the Ca^{2+}-binding signal throughout the thin filament. Mutations Y5H, M103I, D145E, and I148V decrease the α-helix content of cTnC, which may be associated with a less coordinated response to Ca^{2+} binding and decreased Ca^{2+} sensitivity in skinned fibers (23, 28). Interestingly, Y5H is the second cTnC mutation found within the N-helix, known for its ability to modulate the Ca^{2+} affinity of Site II (40, 41). Conversely, the HCM cTnC mutation A8V, also located in the N-helix, increases the Ca^{2+} sensitivity and the amount of α-he-

TABLE 3

Summary of calcium-force relationship curves of TnC-extracted porcine papillary fibers reconstituted with WT TnC or DCM mutants

The pCa_{50}, n_H, percent maximal force, and percent Ca^{2+} unregulated force values are the average of a number of independent fiber experiments, and the errors are reported as S.E. values.

TnC	pCa_{50}	Hill coefficient (n_H)	ΔpCa_{50}[a]	ΔpCa_{50}[b]	Maximal force recovery	No. of experiments
					%	
WT	5.613 ± 0.012	3.678 ± 0.150			54.91 ± 2.34	12
Y5H	5.532 ± 0.012[c]	3.747 ± 0.231	−0.081		53.04 ± 1.94	15
M103I	5.548 ± 0.013[c]	3.705 ± 0.179	−0.065		49.28 ± 2.83	13
I148V	5.573 ± 0.016	3.728 ± 0.243	−0.040		54.97 ± 2.56	12
D145E[d]	5.898 ± 0.008[c]	-	+0.285		70.30 ± 1.40[c]	8
WT + PKA	5.496 ± 0.013[e]	3.988 ± 0.261		−0.117	54.39 ± 2.65	11
Y5H + PKA	5.461 ± 0.018[e]	4.269 ± 0.242	−0.035	−0.071	55.41 ± 1.64	12
M103I + PKA	5.530 ± 0.017	3.946 ± 0.220	+0.034	−0.018	53.74 ± 2.41	12
I148V + PKA	5.519 ± 0.012[e]	4.142 ± 0.269	+0.023	−0.054	55.62 ± 2.37	11
D145E + PKA	5.555 ± 0.017[c,e]	4.359 ± 0.249	+0.059	−0.343	47.36 ± 2.66	14

[a] ΔpCa_{50} = DCM TnC mutant pCa_{50} − WT TnC pCa_{50} (under the same conditions).
[b] ΔpCa_{50} = TnC pCa_{50} with PKA − TnC pCa_{50} without PKA.
[c] $p < 0.05$; the DCM TnC mutant is significantly different from WT TnC (under the same conditions).
[d] Values were obtained from a previous report (22).
[e] $p < 0.05$; WT TnC or DCM mutant in the presence of PKA was significantly different from the same TnC (WT or DCM mutant) without PKA.

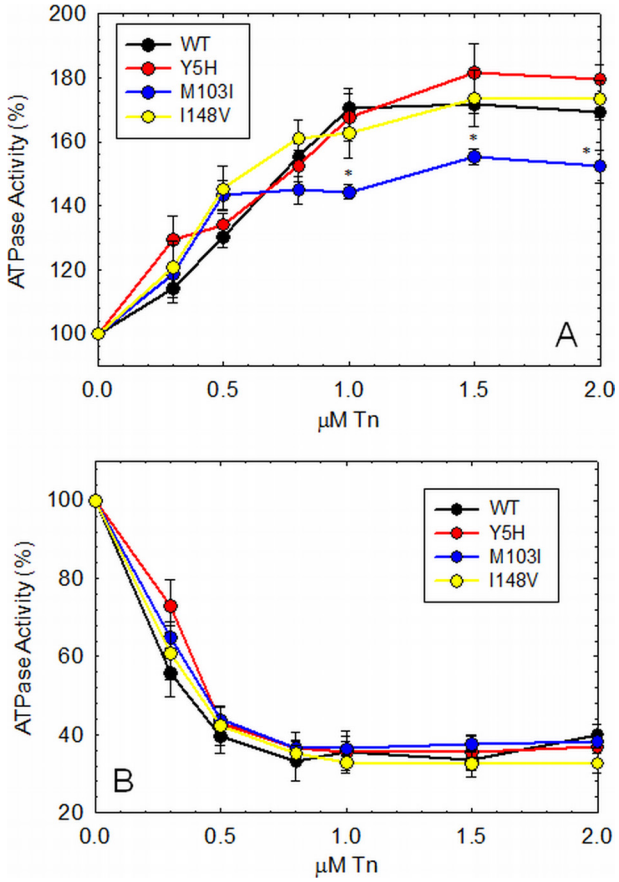

FIGURE 4. **Activation and inhibition of the actomyosin-Tm ATPase activity as a function of Tn.** *A*, activation of the ATPase activity is shown in the presence of Ca^{2+}. *B*, inhibition of the ATPase activity is shown in the absence of Ca^{2+}. Data are reported as means ± S.E. ($n = 6–8$).

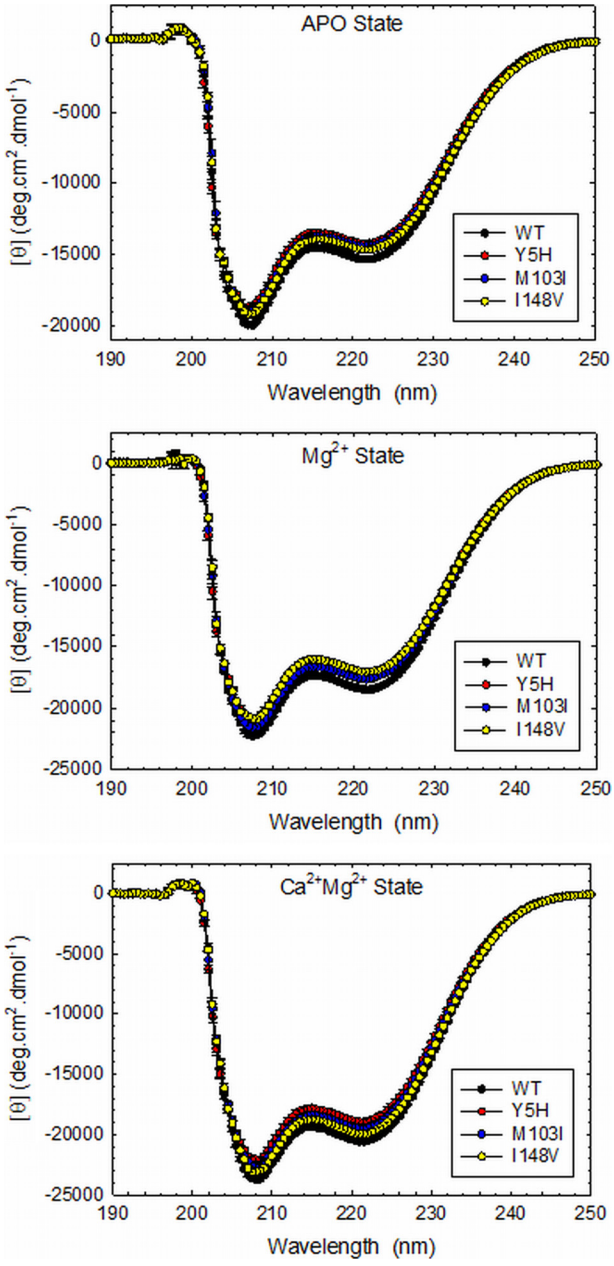

FIGURE 5. **Far-UV CD spectra.** Experiments were performed in apo-, Mg^{2+}-, and Ca^{2+}/Mg^{2+}-bound states. Spectra were recorded in a Jasco-720 spectropolarimeter at room temperature (21 °C). For each independent measurement, 10 scans were averaged. Data are summarized in Table 4. *deg*, degrees.

lix. These rare point mutations alter the structure of cTnC, which in turn affect the ability of the myofilament to bind Ca^{2+}.

A limitation of our study is that we were unable to simultaneously introduce the *MYBPC3* or *MYH7* mutations into our system(s) in which we evaluated the *TNNC1* mutations. Therefore, it is difficult to determine the additional functional consequences that these other mutations may confer. The cTnC D145E mutation had been previously described as an HCM mutation (22), and functional studies indicated that this replacement in cTnC corroborates its HCM phenotype (23). One possibility is that this TnC mutation in combination with *MYBPC3* P910T had an altered functional profile that led to the DCM phenotype instead of HCM as shown previously. Unfortunately, our physiological system did not permit us to address this question. Alternately, as described previously, some HCM patients develop DCM at later stages of disease (42–45). It is possible that an animal model for these mutations would better address these points. The cTnC Y5H mutation led to the development of a very severe clinical phenotype in the proband compared with the other cTnC mutations, with DCM onset at 2 weeks of age. The presence of the β-myosin heavy chain R1045C mutation accompanying cTnC Y5H may have potentiated the myofilament dysfunction and misregulation of the cardiac muscle. For example, the Y5H mutation did not show altered activation of the actomyosin ATPase, a trend found in most troponin mutations associated with DCM. However, we speculate that the absence of the additional mutation in our experimental system may have obscured a more complete functional profile of the double mutation. It has been shown that animal models bearing double mutations present a more severe phenotype (46). A murine model that contained the cTnI G203S and myosin R403Q mutations showed an increased mortality rate with survival of ~21 days (46). At 14 days of age, the double mutant mouse model developed the clinical and molecular features of cardiac hypertrophy such as increased heart/body weight ratio and interstitial myocardial fibrosis. Interestingly, by 16–18 days of age, the double mutant mice developed severe DCM associated with heart failure (46). It has been reported that the occurrence of double or triple sarcomeric

TABLE 4
Far-UV circular dichroism

TnC	$[\theta]_{222\ nm}$ Apo	$[\theta]_{222\ nm}$ Mg^{2+}	$[\theta]_{222\ nm}$ Ca^{2+}/Mg^{2+}	*n*
	degrees·cm²·dmol⁻¹			
WT	15,314.0 ± 29.4	18,496.6 ± 47.4	20,527.9 ± 114.3	4
Y5H	14,208.4 ± 208.5[a]	17,173.7 ± 196.7[a]	18,900.4 ± 261.1[a]	4
M103I	14,397.5 ± 212.2[a]	17,588.8 ± 106.8[a]	19,442.7 ± 343.3[a]	4
I148V	14,617.7 ± 173.5[a]	17,014.8 ± 46.1[a]	19,919.0 ± 225.7	4
	$[\theta]_{208\ nm}$			
WT	19,729.9 ± 74.6	22,208.2 ± 54.0	23,767.0 ± 121.6	4
Y5H	18,426.9 ± 134.5[a]	20,852.7 ± 230.9[a]	22,075.7 ± 299.2[a]	4
M103I	18,704.4 ± 295.8[a]	21,514.1 ± 123.8[a]	22,787.2 ± 354.0[a]	4
I148V	18,972.0 ± 298.2[a]	20,899.9 ± 5.0[a]	23,078.7 ± 299.1	4

Mean residue ellipticity ($[\theta]_{MRE}$ in degrees·cm²·dmol⁻¹) for the spectra was calculated utilizing the same Jasco software and the following equation: $[\theta]_{MRE} = [\theta]/(10 \times C_r \times l)$, where $[\theta]$ is the measured ellipticity in millidegrees, C_r is the mean residue molar concentration, and l is the path length in centimeters.
[a] $p < 0.05$ compared with WT TnC under the same conditions.

mutations in HCM patients might be associated with earlier onset of the disease and a more severe clinical phenotype (47).

Until recently, *TNNC1* was not considered a cardiomyopathy-associated gene (48). We recently demonstrated a set of mutations in *TNNC1* linked to HCM with the potential to disrupt myocardial contractility (23). Before this report, only two mutations in *TNNC1* had been linked to DCM (6, 7, 18). The G159D (6, 7) and Q50R (18) mutations have been shown to segregate among family members. These functional studies of G159D have strengthened our knowledge of its effects and potential to cause disease. Translational studies such as these provide a basis for comparison of the pathogenic phenotypes of DCM-associated mutations in the clinical setting with mechanistic analysis to further dissect the fundamental basis of myofilament dysfunction (49). In conclusion, these combined clinical, genetic, and functional data reinforce the importance of *TNNC1* rare variants in the pathogenesis of human DCM.

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