Functional Analysis of a Unique Troponin C Mutation, GLY159ASP, that Causes Familial Dilated Cardiomyopathy, Studied in Explanted Heart Muscle

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Background—Familial dilated cardiomyopathy can be caused by mutations in the proteins of the muscle thin filament. In vitro, these mutations decrease Ca2+ sensitivity and cross-bridge turnover rate, but the mutations have not been investigated in human tissue. We studied the Ca2+-regulatory properties of myocytes and troponin extracted from the explanted heart of a patient with inherited dilated cardiomyopathy due to the cTnC G159D mutation.

Methods and Results—Mass spectroscopy showed that the mutant cTnC was expressed approximately equimolar with wild-type cTnC. Contraction was compared in skinned ventricular myocytes from the cTnC G159D patient and nonfailing donor heart. Maximal Ca2+-activated force was similar in cTnC G159D and donor myocytes, but the Ca2+ sensitivity of cTnC G159D myocytes was higher (EC50 G159D/donor=0.60). Thin filaments reconstituted with skeletal muscle actin and human cardiac troponymosin and troponin were studied by in vitro motility assay. Thin filaments containing the mutation had a higher Ca2+ sensitivity (EC50 G159D/donor=0.55±0.13), whereas the maximally activated sliding speed was unaltered. In addition, the cTnC G159D mutation blunted the change in Ca2+ sensitivity when TnI was dephosphorylated. With wild-type troponin, Ca2+ sensitivity was increased (EC50 P/unP=4.7±1.9) but not with cTnC G159D troponin (EC50 P/unP=1.2±0.1).

Conclusions—We propose that uncoupling of the relationship between phosphorylation and Ca2+ sensitivity could be the cause of the dilated cardiomyopathy phenotype. The differences between these data and previous in vitro results show that native phosphorylation of troponin I and troponin T and other posttranslational modifications of sarcomeric proteins strongly influence the functional effects of a mutation. (Circ Heart Fail. 2009;2:456-464.)

Key Words: cardiomyopathy ■ contractility ■ heart failure

Dilated cardiomyopathy (DCM) is a common cause of sudden death and heart failure, and it is estimated that 20% to 30% of cases of DCM are caused by mutations in specific proteins.1 Many cases of inherited “pure” DCM that are not associated with other symptoms, such as conduction disease, are caused by mutations in contractile proteins, including actin, myosin, tropomyosin, and all 3 subunits of troponin.1,2 These cardiomyopathy-causing mutations present a unique opportunity to link genotype with phenotype. Because mutations at different sites in several different contractile proteins can produce a single phenotype, it has been proposed that mutations causing the same phenotype alter the contractile mechanism in the same way. In support of this hypothesis, a number of DCM mutations have been shown to cause decreased Ca2+ sensitivity linked to reduced troponin C Ca2+-binding affinity and decreased cross-bridge turnover rate in vitro.3-9 However, recent work, studying both recombinant proteins and intact myofibrils, has provided results that contradict the simple hypothesis and this raises doubts about the physiological relevance of the early studies.10-13

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It has been noted previously that the functional effect of cardiomyopathic mutations in sarcomeric proteins depends on a number of extrinsic factors. Predominant among these are the level of expression of the mutant protein, the species and isoform of the partner proteins used in reconstituted systems,4,14 and the posttranslational modifications, particularly phosphorylation, of both the mutated and the partner proteins.11,15-19 These factors are often unknown and all of them are difficult to reproduce in the synthetic systems.
Currently studied. Consequently, unraveling the links between genotype and phenotype in familial cardiomyopathies demands the study of myopathic human cardiac tissue. The availability of such material from septal myectomy operations on hypertrophic cardiomyopathy patients has yielded some information about phenotype-genotype relationships in MYH7 and MYBPC3 mutations, but no studies on familial DCM mutations have been reported.

In this article, we report the first functional investigation of cardiac myocytes and isolated troponin from the explanted heart of a patient with familial DCM caused by the mutation of glycine 159 to aspartic acid in troponin C (TNNC1 gene). This is the only DCM-causing mutation that has been found in TNNC1, and as a result, it has been extensively investigated in vitro. In experiments using filaments reconstituted from recombinant proteins, the mutation has been shown to cause the typical DCM molecular phenotype of reduced Ca$^{2+}$ sensitivity and slower cross-bridge turnover, but interestingly, there is already evidence from studies using recombinant troponin C exchanged into more organized systems, such as skinned skeletal or cardiac muscle fibers, that the effects of this mutation on muscle function depend on partner proteins and on their phosphorylation levels. We have obtained a tissue sample from a patient with this mutation and have therefore been able to investigate this disease-causing mutation in a completely native context.

This has enabled us to clarify the basic mechanism by which this mutation, and by analogy other DCM-causing mutations, may cause DCM in man.

**Methods**

**Human Heart Muscle Samples**

The TNNCI G159D mutation associated with DCM was identified in a single family by Mogensen et al. A 3-year-old child from this family presented with DCM (left ventricular end diastolic diameter 54 mm, left ventricular end-systolic diameter 50 mm, ejection fraction 20%) and was given a heart transplant. This case has been described in detail by Kaski et al. The explanted heart muscle was cut into 0.5-g pieces, rapidly frozen in liquid nitrogen, and stored for further analysis. Local ethical approval was obtained from University College London Hospitals and the Brompton, Harefield, and National Heart and Lung Institute ethics committees for collection and use of tissue samples. Control heart muscle from 4 nonfailing donor hearts was supplied by C. Dos Remedios, University of Sydney (Sydney, Australia). Ethical approval was obtained from the Brompton, Harefield, and National Heart and Lung Institute Research Ethics Committee (London, United Kingdom) and St Vincent’s Hospital (Sydney, Australia). The donor heart tissue was obtained from hearts where no suitable transplant recipient was found. The donor patients had no history of cardiac disease, a normal cardiac examination, normal ECG, and normal ventricular function on echocardiography within 24 hours of heart explantation. Clinical and functional characteristics of the donor heart samples have been reported previously.
Skinned Myocytes

Skinned myocyte experiments were performed as described previously, with minor modifications (see online-only Data Supplement for details).

Human Cardiac Troponin

Human cardiac troponin was prepared from heart myofibrils, using an anti-TnI antibody affinity column, as described by Messer et al, and analyzed by mass spectroscopy and SDS-PAGE (see Data Supplement for details).

In Vitro Motility Assay

In vitro motility measurements were made in paired cells within 2 days of preparation of troponin, as previously described (see Data Supplement for details). For measurements of Ca\(^{2+}\)-dependence of motility, we found variability between the absolute values of EC\(_{50}\) obtained with different troponin and heavy meromyosin (HMM) preparations; however, the ratio of the EC\(_{50}\) values in donor and G159D cTnC samples measured in paired experiments is consistent. We used the single group t test to determine whether the ratio was significantly different from 1. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Identification and Characterization of Troponin With TnC G159D Mutation

The presence of the G159D cardiac troponin C (cTnC) mutation in troponin isolated from the patient sample was confirmed by matrix-assisted laser deorption/ionization mass spectrometry (MALDI-MS) spectroscopy (Figure 1). MALDI-MS analysis of pure recombinant wild-type cTnC generated a peak at m/z 18410, whereas the G159D cTnC generated a peak at m/z 18462 (Figure 1A). These peaks represent singly charged cTnC ions (calculated molecular weights for wt and G159D cTnC being 18402 and 18460, respectively). Spectra of the affinity-purified heart troponin showed major peaks at m/z = 18495, 23956, and 34566, consistent with singly charged ions of cTnC, TnI, and TnT, respectively (Figure 1B). Comparison of the cTnC peaks of the troponin isolated from donor heart and from the patient's heart muscle clearly showed the presence of additional peaks in the patient sample. The 2 peaks were separated by approximately m/z 58, consistent with the 58 Da increase in mass introduced by the glycine to aspartic acid mutation (Figure 1C).

The peak intensity of the mutant component of cTnC in the patient sample was 86% of the wild-type component (46% of total). Spectra of mixtures of recombinant cTnC and G159D cTnC were observed to be additive (supplemental Figure I); therefore, we can obtain a more accurate estimate of the fraction of mutant cTnC in the tissue sample troponin by comparison with synthetic spectra of mixtures at varying ratios. The results of this calculation (Figure 1D) show that the best fit was obtained with 40% mutant protein.

The spectrum of troponin C was also measured in donor and mutant heart troponin by Fourier transform ion cyclotron resonance mass spectroscopy. The mutant troponin spectrum showed the presence of 2 families of peaks differing in mass by 58 Da, with an apparent proportion of 60% wild type: 40% G159D cTnC (see Data Supplement).
The contractile protein composition of myofibrils and isolated troponin from donor and G159D cTnC hearts were indistinguishable, and in particular, the troponin C content of myofibrils was the same in all the samples we examined (Figure 2A). Comparison of phosphorylation of MyBP-C, troponin T, troponin I, and MLC-2 in myofibrils showed no significant difference between donor and patient troponin (Figure 2A). Comparison of phosphorylation of MyBP-C, myofibrils was the same in all the samples we examined indistinguishable, and in particular, the troponin C content of isolated troponin from donor and G159D cTnC hearts were visible difference in the sarcomeric structure between skinned donor hearts and from the G159D cTnC patient. We found no visible difference in the sarcomeric structure between skinned donor and G159D cTnC myocytes. A range of submaximal Ca$^{2+}$-activating solutions was used to determine myofibrillar Ca$^{2+}$ sensitivity. There was consistency between the force-pCa curves in several myocytes from each patient, and there was no difference in mean $EC_{50}$ values between the 4 donor patients ($P=0.11$; 1-way ANOVA). The data from all donor patients were therefore combined to give the average force-pCa curve for skinned human donor myocytes (Figure 3). We found that the Ca$^{2+}$ sensitivity of G159D cTnC myocytes was significantly greater than for donor myocytes (Figure 3; Table ), with mean tension $EC_{50}$ values of 0.49±0.04 mmol/L (n=9 myocytes) and 0.80±0.04 mmol/L (n=22), respectively ($P<0.001$, unpaired $t$ test), giving a mean ratio for $EC_{50}$ G159D/EC$_{50}$ donor of 0.60. The steepness of the force-Ca$^{2+}$ relationship was also lower in human G159D cTnC than in donor myocytes, with Hill n values of 1.76±0.12 and 2.99±0.18, respectively ($P<0.001$, unpaired $t$ test). As shown in the inset to Figure 3, maximum force was found to be similar in G159D cTnC and donor myocytes ($P=0.66$, unpaired $t$ test).

Thin Filament Regulation Measured by In Vitro Motility Assay

Thin filaments containing human cardiac troponin and tropomyosin were studied. G159D cTnC and donor troponin were equally effective at regulating actin-tropomyosin filaments in the in vitro motility assay. At low [Ca$^{2+}$] (1 nmol/L), the thin filament-based contractile function in human skinned ventricular myocytes prepared from 4 donor hearts and from the G159D cTnC patient. We investigated the myofilament-based contractile function in human heart tropomyosin, recombinant troponin and tropomyosin, and Dyer et al Gly159Asp Mutant Troponin C From Explanted Heart 459

**Contractility of Skinned Myocytes**

**Table. Effect of the G159D cTnC Mutation on Ca$^{2+}$ Sensitivity of Thin Filaments From Patient Muscle and in Reconstituted Systems**

<table>
<thead>
<tr>
<th>System</th>
<th>Method</th>
<th>Donor</th>
<th>G159D</th>
<th>n (Paired Experiments)</th>
<th>Ratio $EC_{50}$ G159D/Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human heart muscle</td>
<td>Skinned myocytes</td>
<td>0.80±0.01</td>
<td>0.48±0.01</td>
<td>(n=22)</td>
<td>0.60*</td>
</tr>
<tr>
<td>Human heart troponin and tropomyosin</td>
<td>Motility of thin filaments</td>
<td>0.13±0.11</td>
<td>0.04±0.04</td>
<td>4</td>
<td>0.27±0.11†</td>
</tr>
<tr>
<td>Deyphosphorylated human heart troponin and tropomyosin</td>
<td>Motility of thin filaments</td>
<td>0.14±0.06</td>
<td>0.08±0.01</td>
<td>6</td>
<td>0.55±0.13‡</td>
</tr>
<tr>
<td>Human heart troponin, recombinant tropomyosin</td>
<td>Motility of thin filaments</td>
<td>0.13±0.01</td>
<td>0.08±0.02</td>
<td>3</td>
<td>0.61±0.09‡</td>
</tr>
<tr>
<td>Recombinant Troponin (50%), human heart troponin</td>
<td>Motility of thin filaments</td>
<td>0.19±0.06</td>
<td>0.38±0.10</td>
<td>4</td>
<td>2.13±0.68†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15±0.04</td>
<td>0.29±0.14</td>
<td>4</td>
<td>1.89±0.70‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.32±0.19</td>
<td>0.50±0.1</td>
<td>3</td>
<td>1.5±0.2‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25±0.03</td>
<td>0.40±0.03</td>
<td>3</td>
<td>1.5±0.1‡</td>
</tr>
</tbody>
</table>

ND indicates not determined.

*P<0.001.
†P<0.01.
‡P<0.05.
filament motility for both G159D cTnC and donor troponin was switched off by 40 nmol/L troponin, with the fraction motile falling to ~0.05. The sliding speed was reduced by 35±5% for G159D cTnC troponin and 38±7% for donor troponin (means of 7 preparations, P=0.75 in paired t test). At high [Ca\textsuperscript{2+}] (3.9 μmol/L), 40 nmol/L human cardiac troponin increased sliding speeds by 7% over actin-tropomyosin as has been previously observed.\textsuperscript{31} In these experiments, the increase was not statistically significant. The mean fraction motile at high Ca\textsuperscript{2+} was not significantly different for the 2 troponin species (G159D cTnC and donor troponin) (mean n=7, P=0.61). Sliding speed was similarly unaffected by the presence of the mutant cTnC (P=0.92).

When we measured Ca\textsuperscript{2+} sensitivity of thin filaments reconstituted with G159D cTnC and donor troponin, we found that the Ca\textsuperscript{2+} sensitivity of thin filaments containing G159D cTnC troponin was higher than donor, for both the fraction motile and sliding speed parameters when they were compared in the same dual chamber motility cell. As previously found with this assay method, there was considerable variation in the absolute value of EC\textsubscript{50} between assays with different HMM and actin preparations, but paired measurements in the same motility cell always produced a consistent difference between G159D cTnC and donor troponin. In the example in Figure 4, showing the results from 1 troponin preparation, the G159D cTnC curve was shifted to the left and the calculated Ca\textsuperscript{2+} sensitivity was 1.5-fold higher than donor troponin. The results from 6 pairs of troponin preparations, shown in detail in supplemental Table I and summarized in the Table, confirm the higher Ca\textsuperscript{2+} sensitivity with the mutant troponin, with the mean ratio EC\textsubscript{50} G159D/donor for fraction motile=0.55±0.13 and sliding speed=0.20±0.04. The calculated Hill coefficient was very variable and was not significantly different between G159D cTnC and donor troponin (mean n=14±0.2, n=12, all assays pooled).

**The Effect of Changing Troponin Phosphorylation Levels**

Troponin I and troponin T were natively phosphorylated in G159D cTnC and donor troponin (Figure 2), but our previous studies with this mutation used unphosphorylated recombinant troponin.\textsuperscript{8} Therefore, we determined the effect of complete dephosphorylation of troponin, using acid phosphatase. Figure 5 shows 2 typical measurements. With donor troponin, we observed higher Ca\textsuperscript{2+} sensitivity and decreased sliding speed with dephosphorylated troponin, as previously reported.\textsuperscript{27} In contrast, native and dephosphorylated G159D cTnC troponins exhibited similar Ca\textsuperscript{2+} sensitivities, and there was no significant effect of dephosphorylation on the maximum sliding speed. Thus, the dephosphorylated G159D cTnC containing thin filaments were not responsive to the phosphorylation change shown by the donor troponin. In pooled data from paired measurements with 5 troponin preparations, the EC\textsubscript{50} G159D cTnC/dephosphorylated (dP) G159D cTnC was 1.18±0.09 (supplemental Table II). When dephosphorylated G159D cTnC and donor troponins were compared directly in paired experiments, the Ca\textsuperscript{2+} sensitivity of G159D cTnC remained greater than donor (EC\textsubscript{50} G159 D cTnC dP/donor dP=0.61±0.09, P=0.024; Table and supplemental Table III). Unfortunately, attempts to produce similar dephosphorylation of native troponin in skinned myocytes proved unsuccessful, either due to degradation of cell structure (after incubation in acid or alkaline phosphatases) or due to lack of substantial dephosphorylation of TnI (after incubation in PP2A catalytic subunit).

**The Effect of Changing Tropomyosin**

Previous studies using unphosphorylated recombinant proteins showed a lower Ca\textsuperscript{2+} sensitivity with G159D cTnC as well as with other DCM mutations in troponin T and tropomyosin.\textsuperscript{8} In contrast, the patient sample of G159D cTnC showed a higher Ca\textsuperscript{2+} sensitivity than donor cTnC, when studied using native human troponin and tropomyosin, and its Ca\textsuperscript{2+} sensitivity was independent of phosphorylation (Table, Figures 3 through 5). To test whether this effect is related to partner proteins, we substituted the native human cardiac tropomyosin used in these studies with a recombinant α-tropomyosin expressed in *Escherichia coli* with an N-terminal Ala-Ser extension to compensate for the absence of the native N-terminal acetylation (AS-α-tropomyosin).\textsuperscript{32}
Recent studies show that this tropomyosin species is functionally different from native cardiac tropomyosin. In the presence of AS\textsuperscript{-}/H\textsubscript{9251}-tropomyosin, we observed a 1.9-fold lower Ca\textsuperscript{2+}/H\textsubscript{11001} sensitivity with G159D cTnC troponin compared with donor troponin, similar to previous experiments using recombinant troponin subunits (Figure 6, Table, and supplemental Table IV).

Discussion

Mutations that cause familial DCM without complications, such as conduction disease, have been found in most of the proteins of the cardiac muscle sarcomere; however, only 1 DCM-associated mutation has so far been identified in TNNC1. The cTnC G159D mutation was found in a single family, and genotypes were obtained over 5 generations. This seems to be a particularly malignant mutation, because every individual with the mutation has been affected, and in particular, the individual studied here required a heart transplant at age 3. Despite the young age of the patient, the tissue sample contained adult isoforms of contractile proteins and high levels of contractile protein phosphorylation similar to adult donor heart (Figure 2). The mechanism by which mutations in thin filament proteins cause DCM has been extensively studied using recombinant proteins, and a consistent pattern of decreased Ca\textsuperscript{2+} sensitivity and slower cross-bridge turnover has emerged. This article reports the first study of mutated troponin from human heart, and it is evident that the functional effects of the mutation are quite strikingly different from the same mutation studied in synthetic systems.

Mass spectroscopy of the troponin from the heart muscle sample shows clearly that the cTnC G159D mutation predicted from genetic studies is expressed in heart as \~40% of total cTnC (Figure 1), and SDS-PAGE indicates normal levels of all 3 troponin subunits in the isolated troponin we have studied (Figure 2), therefore, it is likely that the G159D cTnC mutation acts as a poison peptide by changing thin filament regulation. In functional measurements at both the myocyte and myofilament level, the mutation causes an increase in Ca\textsuperscript{2+} sensitivity with no change in cross-bridge turnover rate, as assessed by the maximum sliding speed (Figures 3 and 4; Table). These findings are in contrast to that previously found with recombinant DCM mutations, where Ca\textsuperscript{2+} sensitivity and cross-bridge turnover rate were found to be decreased. On the other hand, as previously reported for motility and ATPase measurements with thin filaments containing recombinant DCM mutations, cooperativity of activation was reduced in skinned myocytes, although this was not consistently observed in isolated thin filaments. Increased Ca\textsuperscript{2+} sensitivity is a characteristic of mutations associated with hypertrophic cardiomyopathy, but the patient showed no symptoms of hypertrophy preceding heart failure, and this mutation has been consistently linked to the DCM pheno-
relationship between phosphorylation and Ca\(^{2+}\) sensitivity is the primary cause of this DCM phenotype, at least for this mutation. This provides a novel mechanism for initiation of DCM; if thin filaments do not respond to protein kinase A phosphorylation, the contractile response to β-adrenergic stimulation (increased relaxation rate and power output) will be blunted. This would cause cardiac relaxation rate and contractile reserve to be reduced in the same way as they are in acquired heart failure, where troponin I becomes dephosphorylated, resulting in a higher than normal Ca\(^{2+}\) sensitivity. The combination of high Ca\(^ {2+}\) sensitivity, which has not been seen with other DCM-causing mutations,10,13,16 and blunted response to adrenergic stimulation may account for the severity and early onset of DCM with the G159D cTnC mutation.1,25

The findings from this human biopsy study of G159D cTnC are compatible with recently published animal studies that used recombinant G159D cTnC in skinned muscle.11,19 Biesiadecki et al11 reported that G159D cTnC specifically blunted the phosphorylation-induced decrease in Ca\(^{2+}\)-sensitive tension development, without altering the increase in cross-bridge cycling, when the mutant troponin was exchanged into rat heart trabeculae. Moreover, measurements of Ca\(^ {2+}\) binding by TnI-TnC complexes in vitro have shown that the dependence of Ca\(^ {2+}\)-binding affinity on troponin I phosphorylation was lost in the presence of the G159D cTnC mutation.11,36

In contrast to the results from G159D cTnC using native troponin, reported here, this mutation previously produced a decrease in Ca\(^ {2+}\) sensitivity or Ca\(^ {2+}\) affinity when tested with recombinant troponin subunits.8,9 We also found a lower Ca\(^ {2+}\) sensitivity with native troponin containing G159D cTnC when we used recombinant AS-α-tropomyosin in place of native human tropomyosin (Figure 6; Table). These findings emphasize the importance of studying the subtle functional consequences of cardiomyopathy-causing mutations in a native context. We have already shown that the effect of a mutation depends on the fraction of mutant protein present and also on the species origin of partner proteins.14 The current work emphasizes that phosphorylation of troponin I and troponin T and posttranslational modifications of tropomyosin may strongly influence the functional effect of a mutation.37 It is, therefore, vitally important to conduct experiments in as near-native conditions as possible and also, when possible, to check in vitro experiments against the results obtained using native tissue from patients with the corresponding cardiomyopathy.

Could uncoupling of the TnI phosphorylation effect on EC\(_{50}\) be a common mechanism of familial DCM? We have shown that this mechanism explains the G159D data, and it is also a likely explanation for the results from the ACTC E361G transgenic mouse.8 In addition, the hypothesis is not incompatible with published work, because the relationship between the effect of a mutation and troponin phosphorylation has not yet been tested with human sequence actin, tropomyosin, and troponin for any other DCM mutations.8–10,12,13

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**Figure 6.** Ca\(^ {2+}\) sensitivity of thin filaments reconstituted with AS-α-tropomyosin and donor or G159D cTnC troponin. The figure shows the sliding speed (top) and fraction of filaments motile (bottom) derived from the same motility experiment. Points are mean and standard error of 4 measurements made in a single motility cell. ○ and dotted line indicate thin filaments containing AS-α-tropomyosin and donor troponin. ● and solid line indicates thin filaments containing AS-α-tropomyosin and G159D cTnC troponin. Hill equation fits to fraction motile, and sliding speed data are shown in Data Supplement, Table 4, experiment 4.
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Disclosures
None.

References

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**CLINICAL PERSPECTIVE**

Familial cardiomyopathies are the commonest cause of sudden cardiac death in the young. In 2004, a novel mutation in the gene encoding troponin C was identified in a family with dilated cardiomyopathy (DCM). In this manuscript, we report the first functional investigation of cardiac muscle and isolated troponin from the explanted heart of a patient with familial DCM due to the mutation of glycine 159 to aspartic acid in troponin C. We believe that this is one of the first longitudinal studies of its kind, which encompasses both clinical characterization of an affected individual with familial DCM and the basic science of how the mutated protein functions and ultimately contributes to the resultant clinical phenotype. Mass spectroscopy of the heart muscle shows that the mutation is expressed in the heart and therefore acts as a poison peptide by changing thin filament regulation, highlighting the clinical importance of early detection of patients with DCM, and routine screening of patients for sarcomeric protein mutations since the mutation is expressed throughout life and early detection and medical treatment of asymptomatic patients may improve prognosis. The uncoupling of the relationship between phosphorylation and Ca^{2+} sensitivity provides a novel mechanism for initiation of DCM. Current therapies for DCM are not always applicable to every patient, but by knowing the effect of a genetic mutation, we hope this will ultimately help future prognosis and treatment of specific patients. Novel approaches are required to ultimately treat the specific underlying molecular mechanisms involved in heart failure and its progression in a given patient.
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SUPPLEMENTAL MATERIAL:

FUNCTIONAL ANALYSIS OF A UNIQUE TROPONIN C MUTATION, GLY159ASP, THAT CAUSES FAMILIAL DILATED CARDIOMYOPATHY, STUDIED IN EXPLANTED HEART MUSCLE.

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SUPPLEMENTAL METHODS SECTION

Skinned myocytes

Skinned myocyte experiments were carried out as described previously \(^1\), with minor modifications noted below. In brief, on the day of use, a section of the human tissue sample was removed from storage in liquid nitrogen and was thawed at 4°C in Ca\(^{2+}\)-free relaxing solution containing protease inhibitors (Roche Complete Mini protease inhibitor cocktail tablets, 100\(\mu\)g/L). The tissue was homogenised in a mechanical blender, resulting in a suspension of single myocytes, cell fragments and small groups of cells. Myocytes were permeabilized by treatment with relaxing solution containing 1% Triton X-100 for 15 min. The myocyte pellet was washed three times in relaxing solution and stored on ice for up to 10 hours.

A single myocyte was attached between a force transducer and a high-speed servomotor with silicone glue \(^2\) and was stretched to a resting sarcomere length of 2.0 mm. Myocytes were gravity-superfused using a 3-barrel pipette attached to a stepper motor \(^3\). Isometric force was measured as the difference between the steady force during Ca\(^{2+}\) activation and the zero force
reached during the period when the myocyte was made slack during activation by rapid shortening the myocyte by 20% for 40 ms. All measurements were made at 15°C. To determine the Ca\(^{2+}\)-sensitivity of force, isometric force measured in each submaximal Ca\(^{2+}\)-activation solution was expressed as a fraction of maximal Ca\(^{2+}\)-activated force (determined at 30 µmol/L Ca\(^{2+}\)). The force – [Ca\(^{2+}\)] relationship data for each myocyte was fitted by a modified Hill equation. Maximal force was normalised to cross-sectional area. Cross-sectional was calculated using the width and depth of the myocyte assuming that the cell had an oval cross-section. We attempted to dephosphorylate troponin I in the skinned myocytes using potato acid phosphatase (Sigma P0157, dialysed with relaxing solution), 1U/µL alkaline phosphatase (calf intestinal, New England Biolabs #M0290S), or 0.75U/µL or 1.5U/µL PP2A (Upstate #14-111 or bovine kidney, Calbiochem) for 60 min in relaxing solution at 30°C, as described by Zaremba et al\(^4\).

**Human cardiac troponin**

Human cardiac troponin was prepared from heart myofibrils using an anti-TnI antibody affinity column as described by Messer et al\(^5\). Phosphorylation of troponin I and troponin T was measured in SDS-PAGE using Pro-Q Diamond phosphoprotein specific stain as described previously\(^5\). Troponin was dephosphorylated by treatment with potato acid phosphatase as previously described\(^5\). Ca\(^{2+}\)-regulation of thin filaments containing human cardiac troponin was studied using the quantitative *in vitro* motility assay\(^5-7\). Since human cardiac troponin is unstable donor and G159D troponin were isolated in parallel. In vitro motility measurements were made in paired cells as previously described within two days of preparation of troponin. For measurements of Ca\(^{2+}\)-concentration dependence of motility we found variability between the absolute values of EC\(_{50}\) obtained with different troponin and
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Comparison of Ca²⁺ sensitivity of thin filaments containing donor heart or G159D heart troponin

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<th>Expt. No.</th>
<th>EC₅₀ Sliding Speed, µmol/L</th>
<th>EC₅₀ G159D EC₅₀ donor</th>
<th>EC₅₀ Fraction motile, µmol/L</th>
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<tr>
<td></td>
<td>Non-Failing</td>
<td>G159D</td>
<td>Non-Failing</td>
<td>G159D</td>
</tr>
<tr>
<td>1</td>
<td>0.025 ± 0.018</td>
<td>0.005 ± 0.005</td>
<td>0.2</td>
<td>0.044 ± 0.002</td>
</tr>
<tr>
<td>2</td>
<td>0.066 ± 0.018</td>
<td>0.013 ± 0.013</td>
<td>0.19</td>
<td>0.201 ± 0.080</td>
</tr>
<tr>
<td>3</td>
<td>0.264 ± 0.155</td>
<td>0.024 ± 0.065</td>
<td>0.09</td>
<td>0.177 ± 0.029</td>
</tr>
<tr>
<td>4</td>
<td>0.159 ± 0.026</td>
<td>0.097 ± 0.016</td>
<td>0.61</td>
<td>0.101 ± 0.022</td>
</tr>
<tr>
<td>5</td>
<td>nd</td>
<td>nd</td>
<td>0.120 ± 0.02</td>
<td>0.110 ± 0.008</td>
</tr>
<tr>
<td>6</td>
<td>nd</td>
<td>nd</td>
<td>0.18 ± 0.10</td>
<td>0.140 ± 0.11</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.128 ± 0.106</td>
<td>0.035 ± 0.042</td>
<td><strong>0.27</strong> ± <strong>0.11</strong> p=0.008</td>
<td>0.137 ± 0.060</td>
</tr>
</tbody>
</table>
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Comparison of Ca$^{2+}$ sensitivity of thin filaments containing native or dephosphorylated G159D heart troponin

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<thead>
<tr>
<th>Expt. No.</th>
<th>EC$_{50}$ Fraction motile, µmol/L</th>
<th>EC$_{50}$ native G159D</th>
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<td>[Native]</td>
<td>[Dephosphorylated]</td>
<td></td>
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<tr>
<td>1</td>
<td>0.036±0.014</td>
<td>0.033±0.009</td>
<td>1.09</td>
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<td>2</td>
<td>0.019±0.005</td>
<td>0.018±0.003</td>
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</tr>
<tr>
<td>3</td>
<td>0.033±0.018</td>
<td>0.021±0.010</td>
<td>1.57</td>
</tr>
<tr>
<td>4</td>
<td>0.065±0.021</td>
<td>0.063±0.007</td>
<td>1.03</td>
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<td>5</td>
<td>0.109±0.030</td>
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<td>1.18</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.052±0.036</td>
<td>0.045±0.032</td>
<td>1.18±0.09 p=0.0003</td>
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Comparison of Ca$^{2+}$ sensitivity of thin filaments containing dephosphorylated donor heart or dephosphorylated G159D heart troponin

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<tr>
<th>Expt. No.</th>
<th>EC$_{50}$ Fraction motile, µmol/L</th>
<th>EC$_{50}$ Non-Failing G159D</th>
<th>EC$_{50}$ G159D</th>
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<tbody>
<tr>
<td>1</td>
<td>0.132±0.041</td>
<td>0.064±0.031</td>
<td>0.48</td>
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<td>2</td>
<td>0.137±0.021</td>
<td>0.077±0.006</td>
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<td>3</td>
<td>0.124±0.018</td>
<td>0.100±0.009</td>
<td>0.80</td>
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<tr>
<td>Mean ± SD</td>
<td>0.131±0.009</td>
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<tr>
<th>Expt. No.</th>
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<tr>
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<td>Non-Failing</td>
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<tr>
<td>1</td>
<td>0.165±0.039</td>
<td>0.241±0.121</td>
<td>1.46</td>
<td>0.126±0.006</td>
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<tr>
<td>2</td>
<td>0.257±0.06</td>
<td>0.443±0.18</td>
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<td>0.199±0.055</td>
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<td>3</td>
<td>0.201±0.068</td>
<td>0.473±0.31</td>
<td>2.35</td>
<td>0.140±0.025</td>
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MALDI-MS spectra of mixtures of recombinant wild-type (black lines) and G159D mutant cardiac troponin C (red lines).

cTroponin C shows a major and a minor peak. Both peaks are displaced by 55-60 Da in the G159D mutant troponin C.
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6. Fraser ID, Marston SB. In vitro motility analysis of actin-tropomyosin regulation by troponin and calcium. The thin filament is switched as a single cooperative unit. 1995;270:7836-7841.

SUPPLEMENTAL MATERIAL:

FUNCTIONAL ANALYSIS OF A UNIQUE TROPONIN C MUTATION, GLY159ASP, THAT CAUSES FAMILIAL DILATED CARDIOMYOPATHY, STUDIED IN EXPLANTED HEART MUSCLE.

Emma C Dyer, Adam M Jacques, Anita C Hoskins, Douglas G Ward, Clare E Gallon, Andrew E Messer, Juan Pablo Kaski, Michael Burch, Jonathan C Kentish, Steven B Marston

SUPPLEMENTAL METHODS SECTION

Skinned myocytes

Skinned myocyte experiments were carried out as described previously, with minor modifications noted below. In brief, on the day of use, a section of the human tissue sample was removed from storage in liquid nitrogen and was thawed at 4°C in Ca^{2+}-free relaxing solution containing protease inhibitors (Roche Complete Mini protease inhibitor cocktail tablets, 100µg/L). The tissue was homogenised in a mechanical blender, resulting in a suspension of single myocytes, cell fragments and small groups of cells. Myocytes were permeabilized by treatment with relaxing solution containing 1% Triton X-100 for 15 min. The myocyte pellet was washed three times in relaxing solution and stored on ice for up to 10 hours.

A single myocyte was attached between a force transducer and a high-speed servomotor with silicone glue and was stretched to a resting sarcomere length of 2.0 mm. Myocytes were gravity-superfused using a 3-barrel pipette attached to a stepper motor. Isometric force was measured as the difference between the steady force during Ca^{2+} activation and the zero force
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p=0.008, p=0.008, p=0.017
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<td>0.077±0.006</td>
<td>0.56</td>
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<td>3</td>
<td>0.124±0.018</td>
<td>0.100±0.009</td>
<td>0.80</td>
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<tr>
<td>Mean ± SD</td>
<td>0.131±0.009</td>
<td>0.080±0.018</td>
<td>0.61±0.09 p=0.024</td>
</tr>
</tbody>
</table>
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Comparison of Ca2+ sensitivity of thin filaments containing donor heart or G159D heart troponin and AS-α tropomyosin

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>EC$_{50}$ Sliding Speed, µmol/L</th>
<th>EC$_{50}$ G159D</th>
<th>EC$_{50}$ Fraction motile, µmol/L</th>
<th>EC$_{50}$ G159D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Failing G159D</td>
<td>non-Failing</td>
<td>G159D donor</td>
<td>G159D donor</td>
</tr>
<tr>
<td>1</td>
<td>0.165±0.039 0.241±0.121</td>
<td>1.46</td>
<td>0.126±0.006 0.180±0.018</td>
<td>1.43</td>
</tr>
<tr>
<td>2</td>
<td>0.257±0.06 0.443±0.18</td>
<td>1.72</td>
<td>0.199±0.055 0.450±0.18</td>
<td>2.26</td>
</tr>
<tr>
<td>3</td>
<td>0.201±0.068 0.473±0.31</td>
<td>2.35</td>
<td>0.140±0.025 0.166±0.020</td>
<td>1.18</td>
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<tr>
<td>4</td>
<td>0.120±0.081 0.360±0.15</td>
<td>3.00</td>
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<td>Mean ± SD</td>
<td>0.186±0.058 0.379±0.103</td>
<td><strong>2.13±0.68</strong></td>
<td>0.146±0.036 0.289±0.139</td>
<td><strong>1.89±0.70</strong></td>
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</table>

p=0.008
p=0.01276
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