Familial dilated cardiomyopathy mutations uncouple troponin I phosphorylation from changes in myofibrillar Ca\textsuperscript{2+} sensitivity

Massimiliano Memo\textsuperscript{1}, Man-Ching Leung\textsuperscript{1}, Douglas G. Ward\textsuperscript{2}, Cristobal dos Remedios\textsuperscript{3}, Sachio Morimoto\textsuperscript{4}, Lianfeng Zhang\textsuperscript{5}, Gianina Ravenscroft\textsuperscript{6}, Elyshia McNamara\textsuperscript{6}, Kristen J. Nowak\textsuperscript{6}, Steven B. Marston\textsuperscript{1}, and Andrew E. Messer\textsuperscript{1*}

\textsuperscript{1}Myocardial Function, NHLI, Imperial College London, London, W12 0NN, UK; \textsuperscript{2}School of Cancer Sciences, University of Birmingham, Birmingham, B15 2TT, UK; \textsuperscript{3}Bosch Institute, University of Sydney, Sydney, Australia; \textsuperscript{4}Kyushu University Graduate School of Medicine, Fukuoka, Japan; \textsuperscript{5}Institute of Laboratory Animal Science, Beijing, China; and \textsuperscript{6}Centre for Medical Research, University of Western Australia, WA Institute for Medical Research, Nedlands, Australia

Received 7 December 2012; revised 13 March 2013; accepted 18 March 2013; online publish-ahead-of-print 27 March 2013

Time for primary review: 34 days

Aims
The pure form of familial dilated cardiomyopathy (DCM) is mainly caused by mutations in genes encoding sarcomeric proteins. Previous measurements using recombinant proteins suggested that DCM mutations in thin filament proteins decreased myofibrillar Ca\textsuperscript{2+} sensitivity, but exceptions were reported. We re-investigated the molecular mechanism of familial DCM using native proteins.

Methods and results
We used the quantitative in vitro motility assay and native troponin and tropomyosin to study DCM mutations in troponin I, troponin T, and \(\alpha\)-tropomyosin. Four mutations reduced myofilament Ca\textsuperscript{2+} sensitivity, but one mutation (TPM1 E54K) did not alter Ca\textsuperscript{2+} sensitivity and another (TPM1 D230N) increased Ca\textsuperscript{2+} sensitivity. In thin filaments from normal human and mouse heart, protein kinase A (PKA) phosphorylation of troponin I caused a two- to three-fold decrease in myofibrillar Ca\textsuperscript{2+} sensitivity. However, Ca\textsuperscript{2+} sensitivity did not change with the level of troponin I phosphorylation in any of the DCM-mutant containing thin filaments (E40K, E54K, and D230N in \(\alpha\)-tropomyosin; R141W and DK210 in cardiac troponin T; K36Q in cardiac troponin I; G159D in cardiac troponin C, and E361G in cardiac \(\alpha\)-actin). This ‘uncoupling’ was observed with native mutant protein from human and mouse heart and with recombinant mutant protein expressed in baculovirus/Sf9 systems. Uncoupling was independent of the fraction of mutated protein present above 0.55.

Conclusion
We conclude that DCM-causing mutations in thin filament proteins abolish the relationship between myofilament Ca\textsuperscript{2+} sensitivity and troponin I phosphorylation by PKA. We propose that this blunts the response to \(\beta\)-adrenergic stimulation and could be the cause of DCM in the long term.

Keywords
Familial dilated cardiomyopathy • Troponin phosphorylation • Ca\textsuperscript{2+} regulation of contractility • Mutations • In vitro motility assay

1. Introduction
Idiopathic dilated cardiomyopathy (DCM) has a prevalence of 1 in 2500 and is a common cause of heart failure, especially in the young. The disease is characterized by an unexplained left ventricular dilatation and impaired systolic function and is believed to be caused by genetic mutations in up to 50% of cases. Typical echocardiographic criteria are left ventricular end-diastolic diameter >117% of expected for age and body weight and fractional shortening <25%.

Mutations associated with the familial DCM phenotype have been found in genes encoding proteins located in a wide range of organelles of the cardiomyocyte that include the sarcomeres, Z-discs, costameres, and cytoskeleton. The molecular mechanisms proposed for familial DCM include defects in force production, force...
transmission, and stress-dependent cell signalling. This diversity contrasts with hypertrophic cardiomyopathy, where there is a strong association between mutations in sarcomeric proteins and higher myofibrillar Ca$^{2+}$ sensitivity.\textsuperscript{4,5} 

Mutations in the genes for the force generating proteins of the cardiac muscle sarcomere, actin (ACTC), myosin (MYH7), tropomyosin (TPM1), troponin I (Tni) (TNNT3), troponin T (TNNT2), troponin C (TNNC1), and titin (TTN) were observed to be associated with a specific ‘pure’ cardiac phenotype without any additional syndromes, such as hypertrophy, conduction disease, or dystrophy and with a high prevalence in young people.\textsuperscript{1,6–8} The common phenotype of mutations in sarcomeric proteins with related functions suggests that for this subset of DCM-causing mutations there could be a common causative molecular mechanism.

Initial studies of DCM-causing mutations in sarcomeric proteins at the myofilament and myofibrillar levels suggested that there could be a common defect in Ca$^{2+}$ regulation, with a decrease in myofibrillar Ca$^{2+}$ sensitivity, Ca$^{2+}$-binding affinity for troponin C, and a slower cross-bridge cycling rate at maximally activating [Ca$^{2+}$], and this was proposed to be sufficient to induce the DCM phenotype.\textsuperscript{2,9,10} However, exceptions to this pattern of results have been reported: for example, α-tropomyosin E54K did not change Ca$^{2+}$ sensitivity and increased Ca$^{2+}$-binding affinity, troponin T (cTnT) ΔK210 increased Ca$^{2+}$ sensitivity at 100% but decreased Ca$^{2+}$ sensitivity when present at 50% of cTnT\textsuperscript{9,10} and the cardiac α-actin E361G mutation did not alter Ca$^{2+}$ sensitivity.\textsuperscript{11} Further exceptions have been recently reported.\textsuperscript{12,13} The original studies mostly used recombinant, unphosphorylated troponin subunits and did not take into account the effect of troponin I (cTnI) phosphorylation in modulating Ca$^{2+}$ sensitivity. In normal heart muscle, protein kinase A (PKA) phosphorylates cTnI which leads to a two- to three-fold reduction in Ca$^{2+}$ sensitivity and an increased rate of Ca$^{2+}$ dissociation from troponin C (cTnC); this process plays a key role in the lusitropy induced by β-adrenergic stimulation in the heart.\textsuperscript{14–16}

We have developed methodologies for measuring both myofilament Ca$^{2+}$ sensitivity (by quantitative in vitro motility assay) and cTnI phosphorylation (by phosphate affinity SDS–PAGE) in troponin from human and mouse hearts.\textsuperscript{17,18} Studies on two DCM-causing mutations, cTnC G159D (TNNT3) and cardiac α-actin E361G (ACTC) found that isolated mutant thin filament Ca$^{2+}$ sensitivity was not related to the level of phosphorylation of cTnI and a similar phenomenon was noted in other experimental systems.\textsuperscript{11,19–21} On the basis of these results, we proposed an alternative mechanism for the familial DCM phenotype: DCM mutations uncouple cTnI phosphorylation from the change in myofilament Ca$^{2+}$ sensitivity and blunt the heart’s response to β-adrenergic stimulation, leading to a reduced cardiac reserve with consequent contractile dysfunction under stress, leading to DCM.\textsuperscript{4}

This hypothesis is currently based on studies of only two mutations which may be special cases. In this manuscript, we have investigated Ca$^{2+}$ sensitivity and its modulation by cTnI phosphorylation in a further six DCM-causing mutations, including a unique human heart sample with a previously characterized DCM mutation in the TNNI3 gene, cTnI K36Q;\textsuperscript{22} two mutations in cTnT (TNNT2 gene) extracted from transgenic mouse hearts (R141W and ΔK210);\textsuperscript{23,24} Three mutations in the TPM1 gene coding for α-tropomyosin E40K, E54K, and the recently reported D230N\textsuperscript{25,26} were expressed in a baculovirus/Sf9 system so as to include native post-translational modifications that can affect function.\textsuperscript{9,10,27} Taken together with previous measurements, we show that myofibrillar Ca$^{2+}$ sensitivity is not correlated with the DCM phenotype, while Ca$^{2+}$ sensitivity is uncoupled from TnI phosphorylation in all cases of DCM due to mutations in thin filament proteins.

2. Methods

2.1 Sources of contractile proteins

Troponin was isolated from 100 mg of heart muscle using an anti-cTnI monoclonal antibody affinity column as described by Messer et al.\textsuperscript{18} Control troponin was obtained from eight human donor heart samples. The heart donors had no history of cardiac disease, and normal ECG and ventricular function were obtained when no suitable transplant recipient was found. The heart sample with the cTnI (TNNT3) K36Q mutation, described by Carballo et al.\textsuperscript{22} (patient III:2), was obtained from an explanted heart. Ethical approval was obtained from The Royal Brompton and Harefield Hospital, London and St Vincent’s Hospital, Sydney, Australia. The investigation conforms to the principles of the Declaration of Helsinki. The cTnT R141W and ΔK210 mutant troponins were obtained from frozen hearts of transgenic and knock-in (KI) mouse models.\textsuperscript{22,23} Native α-tropomyosin (TPM1) was isolated from human heart muscle\textsuperscript{29} and wild-type α-tropomyosin, and the mutants E40K, E54K, and D230N were expressed in a baculovirus/Sf9 system with a protocol based on that of Akkari et al.\textsuperscript{29} (see Supplementary material online, Methods). The quantity of mutant relative to wild-type protein present in native troponin preparations was determined by mass spectrometry (see Supplementary material online, Part SC). Phosphorylation of TnI with PKA and dephosphorylation of troponin with shrimp alkaline phosphatase are described in the Supplementary material online, Methods.

2.2 Measurement of phosphorylation in situ by phosphate affinity SDS–PAGE

Cardiac myofilament protein extract from human heart tissue and pure troponin gel samples were prepared in sample buffer containing 8 M urea, 2 M thiourea, 0.05 M Tris–HCl, pH 6.8, 75 mM dithiothreitol, 3% SDS, and 0.05% bromophenol blue using the methods of Layland et al.\textsuperscript{21} TnI phospho species were separated by phosphate affinity SDS–PAGE.\textsuperscript{17}

Discontinuous SDS–PAGE gels were hand-cast and run using the MiniPROTEAN® 3 (Bio-Rad Laboratories) system. Gel formulations were as follows: stacking gel: 4% acrylamide (29:1 acrylamide: bis-acrylamide), Phos-tag\textsuperscript{TM} resolving gel: 10% acrylamide (29:1 acrylamide: bis-acrylamide), 50 μM Mn-Phos-tag\textsuperscript{TM}-Acrylamide (Phos-tag Consortium).\textsuperscript{31} Western blots of gels were probed with anti-TnI 14G5 (Abcam, 1/2000 dilution).

2.3 Quantitative in vitro motility assay

Thin filaments were reconstituted with 10 nM rabbit skeletal muscle α-actin (labelled with TRITC phalloidin),\textsuperscript{32} human heart muscle tropomyosin (40–60 nM), and troponin (20–60 nM) to study Ca$^{2+}$ regulation of filament motility by the quantitative in vitro motility assay.\textsuperscript{18,33} Thin filament movement over a bed of immobilized rabbit fast skeletal muscle heavy meromyosin (100 μg/mL) was compared in paired motility cells in which troponin varied by a single factor (mutation or phosphorylation state). Filament movement was recorded and analysed as previously described,\textsuperscript{34} yielding two parameters, the fraction of filaments moving and the speed of moving filaments. In our motility system, both these parameters are regulated by Ca$^{2+}$. The fraction motile changes from <0.1 to >0.8 in the range 1 nmol/L to 3.7 μmol/L free Ca$^{2+}$.\textsuperscript{9,10,27}
The Ca\textsuperscript{2+}-dependent change of sliding speed was less and more variable (0–50% change), as previously noted.\textsuperscript{21}

Fraction motile and sliding speed was measured over a range of Ca\textsuperscript{2+} concentrations to generate Ca\textsuperscript{2+} activation curves. Each experiment involved the preparation of test and control troponins at the same time. To keep intra-experiment variability as low as possible, test and control thin filament were reconstituted together and for each Ca\textsuperscript{2+}-concentration point they were pipetted into two channels of the dual chamber motility cell. Motility was then measured in each chamber within a couple of minutes of each other. Each pair of Ca\textsuperscript{2+} curves (as shown in Figures 1 and 4) took \~2 h to complete; troponin had a lifetime of 3 days and each preparation yielded up to 4 Ca\textsuperscript{2+}-activation curves. The data were fitted to the four-variable Hill equation to yield a value for EC\textsubscript{50}. EC\textsubscript{50} values from replicate experiments were analysed by the paired t-test since the distribution of EC\textsubscript{50} has been shown to be normal.\textsuperscript{18,33} The absolute value of EC\textsubscript{50} was variable between different troponin and myosin preparations, but the ratio of control to test was very consistent.

### 3. Results

#### 3.1 Effect of DCM-causing mutations on myofilament Ca\textsuperscript{2+}-sensitivity

The quantity of mutant protein expressed in mouse or human hearts was determined by mass spectrometry (see Supplementary material online, Part SC). Troponin from cTnT R141W mice contained 60% mutant troponin, troponin from heterozygous cTnT ΔK210 KI mice contained 40% mutant troponin, while troponin from homozygous cTnT ΔK210 KI mice contained 100% mutant troponin. In the human sample with the cTnI K36Q mutation, both wild-type and mutant sequences were detected and we estimated that >86% of the cTnI was present as the mutant form (see Supplementary material online, Table SA). The native cTnI phosphorylation levels of the mutant mouse troponins were the same as wild-type littermates (range 1.1–1.3 molsPi/mol cTnI) but the TNNI2 K36Q troponin, obtained from a

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/99/1/65/323722)

**Figure 1** Comparison of Ca\textsuperscript{2+} regulation of thin filaments containing wild-type and DCM-mutant troponin or tropomyosin. Thin filament motility was measured by the motility assay over a range of [Ca\textsuperscript{2+}] in paired cells. The fraction of filaments motile is plotted as a function of [Ca\textsuperscript{2+}] for a typical experiment. The points are the mean of four determinations of fraction motile measured in one motility cell ± SEM. The curves are fits of the data to the Hill equation. Dashed lines and solid points, wild-type thin filaments, solid lines and open points, DCM-mutant thin filaments. The mean values of EC\textsubscript{50} and the ratio of EC\textsubscript{50} mutant:wild-type from replicate experiments is plotted in Figure 2A and shown in Supplementary material online, Table SA. (A) Thin filaments containing human heart troponin with TnI K36Q mutation (PKA treated) compared with donor heart troponin; human heart tropomyosin; and rabbit skeletal muscle α-actin. (B) Thin filaments containing transgenic TnT R141W (40%) mouse troponin compared with non-transgenic mouse troponin; human heart tropomyosin; and rabbit skeletal muscle α-actin. (C) Thin filaments containing heterozygous TnT ΔK210 (60%) KI mouse troponin compared with non-transgenic mouse troponin; human heart tropomyosin; and rabbit skeletal muscle α-actin. (D) Thin filaments containing α-tropomyosin E40K (100%) compared with wild-type tropomyosin (both expressed in the baculovirus/SF9 system); human donor heart troponin and rabbit skeletal muscle α-actin. (E) Thin filaments containing α-tropomyosin E54K (100%) compared with wild-type tropomyosin (both expressed in the baculovirus/SF9 system); human donor heart troponin; and rabbit skeletal muscle α-actin. (F) Thin filaments containing α-tropomyosin D230N (100%) compared with wild-type tropomyosin (both expressed in the baculovirus/SF9 system); human donor heart troponin; and rabbit skeletal muscle α-actin.
Interestingly, when tested using skeletal muscle troponin, α-tropomyosin D230N decreased Ca\(^{2+}\) sensitivity (Supplementary material online, Table SB2). Unchanged Ca\(^{2+}\) sensitivity was previously observed with the α-actin E361G mutation and increased Ca\(^{2+}\) sensitivity was reported for the cTnC G159D mutation. These findings do not contradict previous studies, since when we compared fully dephosphorylated thin filament proteins we found that all the DCM mutations decreased Ca\(^{2+}\) sensitivity (Supplementary material online, Table SB; cTnC G159 appears to be an exception). These inconsistent results suggested that the effect of DCM-causing mutations may be more closely related to TnI phosphorylation than absolute Ca\(^{2+}\) sensitivity.

### 3.2 DCM-causing mutations in thin filament proteins uncouple Ca\(^{2+}\)-sensitivity changes from TnI phosphorylation

In normal cardiac muscle, phosphorylation of cTnI by PKA modulates myofibrillar Ca\(^{2+}\) sensitivity.\(^{15,16,20}\) When we compared Ca\(^{2+}\) regulation in thin filaments containing natively phosphorylated or unphosphorylated troponin, using the quantitative in vitro motility assay, the Ca\(^{2+}\) sensitivity of phosphorylated cTnI was lower than with unphosphorylated cTnI (3.0 ± 0.03-fold change in EC\(_{50}\) for human and 2.0 ± 0.2-fold change in EC\(_{50}\) for mouse heart, see Figure 2 and Table 1).\(^{18,21}\)

Figure 3 shows a titration of the EC\(_{50}\), measured by in vitro motility assay, against the level of TnI phosphorylation in thin filaments containing human cardiac troponin. To reduce cTnI phosphorylation, while leaving all the other troponin subunits unaltered, we exchanged recombinant, unphosphorylated cTnI into donor heart troponin, and to increase the level of phosphorylation, we treated troponin with PKA. EC\(_{50}\) increased (Ca\(^{2+}\) sensitivity decreased) with increasing bis-phosphorylation of cTnI up to ~1.5 molsPi/mol cTnI, but was unchanged at higher levels of phosphorylation.

It was previously noted that the Ca\(^{2+}\) sensitivity of thin filaments containing the DCM-causing mutants cardiac α-actin E361G or cTnC G159D was independent of the level of cTnI phosphorylation.\(^{15,19,21}\) We, therefore, tested whether this ‘uncoupling’ of the Ca\(^{2+}\) sensitivity–TnI phosphorylation relationship was also a property of DCM-causing mutations in cTnl, cTnT, and α-tropomyosin. The Ca\(^{2+}\) sensitivity of thin filaments containing phosphorylated cTnI (native phosphorylation levels were ~1.5 molsPi/mol cTnI) and unphosphorylated cTnI (<0.3 molsPi/mol cTnI) was compared. Unphosphorylated cTnI was obtained by treating wild-type and mutant mouse or human heart troponin with a phosphatase. The three tropomyosin mutants were tested with natively phosphorylated and unphosphorylated human donor heart troponin. The native phosphorylation level of cTnI K36Q troponin was lower than donor heart troponin; accordingly this was treated with either phosphatase to reduce phosphorylation or PKA to increase phosphorylation levels. Table 1 lists the measured phosphorylation levels and EC\(_{50}\) of the phosphorylated/unphosphorylated thin filament pairs we studied. Representative experiments are shown in Figure 4 and results from several replicates are summarized in Figure 2B.

In contrast to native thin filaments, when we compared the Ca\(^{2+}\) sensitivity of thin filaments containing DCM-causing mutations in the phosphorylated and unphosphorylated states, there was no difference in Ca\(^{2+}\) sensitivity for any of them (Figures 2B and 4 and Table 1). The uncoupling phenomenon is expected to require

---

**Figure 2** The effect of DCM-causing mutations on thin filament Ca\(^{2+}\) sensitivity and on the relationship between TnI phosphorylation and EC\(_{50}\) (A) EC\(_{50}\) of mutant thin filaments relative to EC\(_{50}\) of wild-type troponin-tropomyosin is plotted for eight DCM-causing mutations. Data from Supplementary material online, Table SA. (B) EC\(_{50}\) of phosphorylated troponin relative to EC\(_{50}\) of unphosphorylated troponin is plotted for wild-type thin filaments and thin filaments containing DCM-causing mutations. Data from Table 1. Error bars show SEM of up to seven replicate comparative measurements. *P < 0.05; **P < 0.01; ***P < 0.001
The results obtained with eight DCM-causing mutations and wild-type human and mouse troponin controls are shown. EC50 for Ca2+ regulation of the fraction motile parameter of phosphorylated and unphosphorylated thin filaments moving in the in vitro motility assay and the ratios of EC50P/unP were compared. Representative individual experiments are shown in Figure 4 and the mean results from several replicate experiments are shown in Figure 2. Het, heterozygous; homo, homozygous. Mean and standard error for independent measurements are shown with \( P \)-value (student t test). Troponin I phosphorylation levels were measured by phosphate affinity SDS–PAGE. **, \( P < 0.01 \).

### Table 1

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Source of mutant protein</th>
<th>Source of troponin</th>
<th>EC50 of thin filaments containing phosphorylated troponin, ( \mu \text{M} \pm \text{SEM} )</th>
<th>EC50 of thin filaments containing un-phosphorylated troponin, ( \mu \text{M} \pm \text{SEM} )</th>
<th>( P ), paired t-test</th>
<th>Ratio of ( \text{EC50} ) of phosphorylated/unphosphorylated troponin, ( \mu \text{M} \pm \text{SEM} )</th>
<th>n and ( P ), paired t-test</th>
<th>Phosphorylation level of native phosphorylated troponin, mols Pi/mol Tn I, ( \mu \text{M} \pm \text{SEM} )</th>
<th>Phosphorylation level of the un-phosphorylated troponin, mols Pi/mol Tn I, ( \mu \text{M} \pm \text{SEM} )</th>
<th>Ratio of sliding speed at 3.9 ( \mu \text{M} ) Ca2+ level of the phosphorylated/unphosphorylated troponin, mols Pi/mol Tn I, ( \mu \text{M} \pm \text{SEM} )</th>
<th>n and ( P ), paired t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNNI3 K36Q</td>
<td>Human transplant</td>
<td>Human transplant</td>
<td>0.18 ± 0.02</td>
<td>0.24 ± 0.05</td>
<td>( P = 0.48 )</td>
<td>0.98 ± 0.05</td>
<td>( n = 3, P = 0.69 )</td>
<td>0.33 ± 0.10</td>
<td>0.09 ± 0.004</td>
<td>1.00 ± 0.03</td>
<td>( n = 3, P = 0.92 )</td>
</tr>
<tr>
<td>TNNI3 K36Q</td>
<td>PKA-treated</td>
<td>TG mouse</td>
<td>0.22 ± 0.03</td>
<td>0.23 ± 0.04</td>
<td>( P = 0.41 )</td>
<td>1.02 ± 0.04</td>
<td>( n = 7, P = 0.41 )</td>
<td>1.31 ± 0.01</td>
<td>0.09 ± 0.004</td>
<td>1.02 ± 0.02</td>
<td>( n = 7, P = 0.30 )</td>
</tr>
<tr>
<td>TNNI3 K36Q</td>
<td>Human transplant</td>
<td>Human transplant</td>
<td>0.17 ± 0.10</td>
<td>0.18 ± 0.01</td>
<td>( P = 0.48 )</td>
<td>0.95 ± 0.06</td>
<td>( n = 3, P = 0.46 )</td>
<td>1.15 ± 0.04</td>
<td>0.19 ± 0.07</td>
<td>0.99 ± 0.01</td>
<td>( n = 3, P = 0.42 )</td>
</tr>
<tr>
<td>TNNI3 K36Q</td>
<td>Human transplant</td>
<td>Human transplant</td>
<td>0.17 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>( P = 0.30 )</td>
<td>0.98 ± 0.02</td>
<td>( n = 6, P = 0.51 )</td>
<td>1.15 ± 0.02</td>
<td>0.23 ± 0.04</td>
<td>1.01 ± 0.01</td>
<td>( n = 6, P = 0.17 )</td>
</tr>
<tr>
<td>TNNI3 K36Q</td>
<td>Human transplant</td>
<td>Human transplant</td>
<td>0.15 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>( P = 0.87 )</td>
<td>1.00 ± 0.06</td>
<td>( n = 6, P = 0.97 )</td>
<td>1.06 ± 0.02</td>
<td>0.21 ± 0.04</td>
<td>1.00 ± 0.03</td>
<td>( n = 6, P = 0.65 )</td>
</tr>
<tr>
<td>TPM1 E40K</td>
<td>Baculovirus/Sf9</td>
<td>Human donor</td>
<td>0.18 ± 0.04</td>
<td>0.18 ± 0.04</td>
<td>( P = 1 )</td>
<td>1.00 ± 0.001</td>
<td>( n = 5, P = 1 )</td>
<td>1.60 ± 0.04</td>
<td>0.09 ± 0.02</td>
<td>0.96 ± 0.04</td>
<td>( n = 5, P = 0.38 )</td>
</tr>
<tr>
<td>TPM1 E54K</td>
<td>Baculovirus/Sf9</td>
<td>Human donor</td>
<td>0.14 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>( P = 0.36 )</td>
<td>1.05 ± 0.04</td>
<td>( n = 6, P = 0.36 )</td>
<td>1.79 ± 0.04</td>
<td>0.08 ± 0.03</td>
<td>0.99 ± 0.02</td>
<td>( n = 6, P = 0.78 )</td>
</tr>
<tr>
<td>TPM1 D230N</td>
<td>Baculovirus/Sf9</td>
<td>Human donor</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>( P = 0.21 )</td>
<td>0.97 ± 0.02</td>
<td>( n = 5, P = 0.24 )</td>
<td>1.60 ± 0.04</td>
<td>0.24 ± 0.02</td>
<td>0.99 ± 0.004</td>
<td>( n = 5, P = 0.19 )</td>
</tr>
<tr>
<td>TNNC1 G159D</td>
<td>Human transplant</td>
<td>Human transplant</td>
<td>0.52 ± 0.02</td>
<td>0.45 ± 0.01</td>
<td>( P = 0.092 )</td>
<td>1.18 ± 0.09</td>
<td>( n = 5, P = 0.54 )</td>
<td>1.53 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>1.01 ± 0.02</td>
<td>( n = 5, P = 0.64 )</td>
</tr>
<tr>
<td>ACTC E361G</td>
<td>Human transplant</td>
<td>Human transplant</td>
<td>0.31 ± 0.08</td>
<td>0.31 ± 0.09</td>
<td>( P = 0.75 )</td>
<td>1.04 ± 0.07</td>
<td>( n = 5, P = 0.58 )</td>
<td>1.33 ± 0.09</td>
<td>0.07 ± 0.02</td>
<td>0.99 ± 0.01</td>
<td>( n = 5, P = 0.34 )</td>
</tr>
<tr>
<td>Human donor</td>
<td>Human transplant</td>
<td>Human donor</td>
<td>0.24 ± 0.07</td>
<td>0.08 ± 0.02</td>
<td>( P = 0.004 )</td>
<td>3.0 ± 0.03**</td>
<td>( n = 7, P = 0.003 )</td>
<td>1.60 ± 0.04</td>
<td>0.12 ± 0.01</td>
<td>0.97 ± 0.04</td>
<td>( n = 3, P = 0.55 )</td>
</tr>
</tbody>
</table>
| Mouse WT       | Human transplant         | Human donor        | 0.28 ± 0.04                         | 0.15 ± 0.03                         | \( P = 0.005 \) | 2.0 ± 0.2**                         | \( n = 5, P = 0.005 \) | 1.30 ± 0.09                                 | 0.05 ± 0.02                                | **Dyer et al.11** \n**Song et al.21**
The Ca\textsuperscript{2+} and 40% of mutant protein in the thin filaments, respectively. We activity when studied with native proteins (2,9,10,35). However, when we tested eight DCM-causing mutations in thin filament proteins was a lower Ca\textsuperscript{2+} sensitivity and slower maximally activated unloaded cross-bridge turnover. Every DCM mutation we tested, independent of the gene mutated or the origin of the mutated protein (recombinant or native, from mouse or human heart, see Figure 2 and Table 1). Moreover, uncoupling needed only 50% mutant protein, a ratio that corresponds to the amount of mutant protein expressed in heart tissue of DCM patients (Figure 5A).

For the ACTC and the three TPM1 mutations, we can confidently conclude that the uncoupling is a direct effect of the mutation, since the troponin used in the tests is from human donor hearts that showed a phosphorylation-dependent Ca\textsuperscript{2+}-sensitivity change with wild-type \(\alpha\)-actin and \(\alpha\)-tropomyosin. Uncoupling has also been reported for a DCM-causing mutation in cTnI and several mutations in cTnC. (19,20,36)

### 4.1 DCM-causing mutations in thin filament proteins uncouple Ca\textsuperscript{2+} sensitivity changes from TnI phosphorylation

Phosphorylation of TnI by PKA is well known to modulate myofibrillar Ca\textsuperscript{2+} sensitivity, as demonstrated for our assay system by the titration of EC\textsubscript{50} against TnI bis-phosphorylation (Figure 3), but we observed that there was no modulation of myofibrillar Ca\textsuperscript{2+} sensitivity by PKA-dependent phosphorylation of cTnI in thin filaments containing DCM-causing mutations. This ‘uncoupling’ was observed in every DCM mutation we tested, independent of the gene mutated or the origin of the mutated protein (recombinant or native, from mouse or human heart, see Figure 2 and Table 1). Moreover, uncoupling needed only 50% mutant protein, a ratio that corresponds to the amount of mutant protein expressed in heart tissue of DCM patients (Figure 5A).

For the ACTC and the three TPM1 mutations, we can confidently conclude that the uncoupling is a direct effect of the mutation, since the troponin used in the tests is from human donor hearts that showed a phosphorylation-dependent Ca\textsuperscript{2+}-sensitivity change with wild-type \(\alpha\)-actin and \(\alpha\)-tropomyosin. Uncoupling has also been reported for a DCM-causing mutation in cTnI and several mutations in cTnC. (19,20,36)

### 4.2 Uncoupling of Ca\textsuperscript{2+} sensitivity from TnI phosphorylation as a default state in cardiomyopathy

A structural model of the cardiac-specific N-terminal extension of TnI complexed with cTnC in the unphosphorylated state (high Ca\textsuperscript{2+} sensitivity) shows an ordered interaction between TnI amino acids 30–35 and the Ca\textsuperscript{2+} binding loop of TnC that is allosterically destabilized by phosphorylation of cTnI at Serines 22 and 23, thus accounting for the lower Ca\textsuperscript{2+} sensitivity of phosphorylated troponin. (14,37) On average we found that the Ca\textsuperscript{2+} sensitivity of thin filaments containing DCM-causing mutations was the same as, or lower than, the Ca\textsuperscript{2+} sensitivity of natively phosphorylated wild-type thin filaments, suggesting they are representative of the destabilized state. Thus, it seems probable that DCM-causing mutations in any component of the thin filament act by allosterically destabilizing the interaction between the TnI N-terminus and the TnC Ca\textsuperscript{2+}-binding EF hand, resulting in a default state having Ca\textsuperscript{2+} regulatory properties similar to phosphorylated wild-type troponin, irrespective of the cTnI phosphorylation level (illustrated in Figure 5B).

It is interesting to note that, in addition to DCM, uncoupling has also been reported for mutations in cardiac \(\alpha\)-actin and cTnI that cause hypertrophic cardiomyopathy, as well as secondary abnormality in troponin isolated from an interventricular septum of patients with hypertrophic obstructive cardiomyopathy, where it is not
4.3 The physiological significance of uncoupling

In the normal heart, β-adrenergic stimulation leads to activation of PKA and increased phosphorylation of cTnI. The resulting decrease in myofilament Ca\(^{2+}\) sensitivity and the associated increase in the rate of Ca\(^{2+}\) dissociation from cTnC is a key component of the lusitropic response, since Ca\(^{2+}\) release is likely to be a rate limiting step for relaxation in vivo.\(^{16}\) We can measure the modulation of Ca\(^{2+}\) sensitivity by TnI phosphorylation in our in vitro motility assay system (Figure 3) and demonstrate that the modulation is uncoupled by DCM-causing mutations (Figure 2). We have also demonstrated uncoupling of the Ca\(^{2+}\) sensitivity of isometric force production in phosphorylated and unphosphorylated single myofibrils with the α-actin E361G DCM-causing mutation.\(^{42}\)

Faster relaxation (lusitropy) is a pre-requisite for the faster heart rate and increased contractile force (inotropy) induced by β-adrenergic stimulation. Therefore, it is likely that uncoupling due to DCM-causing mutation blunts the response to β-adrenergic stimulation, leading to a reduced cardiac reserve.\(^{43}\) A significantly reduced response to acute β-adrenergic agonists has been demonstrated in several DCM-mutant mice (ACTC E361G, TNNT2 ΔK210, and TPM1 E54K).\(^{21,24,44}\) The resulting contractile dysfunction under stress would predispose the heart to DCM in the long term as has been found with DCM-mutant transgenic mice.\(^{45}\) Mutation-dependent decreases in absolute myofibrilar Ca\(^{2+}\) sensitivity may also contribute, but the lack of a correlation (Figures 1 and 2) indicates that they are not necessary for the DCM phenotype.
Figure 5  (A) The effect of changing the ratio of wild-type: E40K mutant α-tropomyosin on the relationship between Ca\(^{2+}\)-sensitivity and cTnI phosphorylation. EC\(_{50}\) was compared, as in Figure 4E, with natively phosphorylated and unphosphorylated human donor heart troponin, rabbit skeletal muscle α-actin and α-tropomyosin consisting of varying ratios of wild-type and E40K α-tropomyosin. The ratio of EC\(_{50}\) for natively phosphorylated and unphosphorylated thin filaments is plotted. The original data are shown in Supplementary material online, Part S8.1. The ratio was 2.9 for wild-type α-tropomyosin; a ratio of one indicates complete uncoupling (dotted line). The arrows indicate the measured fraction of mutant protein in heterozygous cTnT\(\Delta K210\) (40%), cTnC G159D (45%), α-actin E361G (50%), and cTnT R141W (60%) hearts, respectively, (see Supplementary material online, SC). (B) A molecular model of the complex between troponin I N-terminus and the Ca\(^{2+}\)-binding loop of troponin C in the unphosphorylated and phosphorylated states. Structures drawn with PyMol using the pdb files calculated by Howarth et al., 2007.22,37 The model shows the N-terminal lobe of cTnC (1–95) and the N-terminal 50 amino acids of cTnI, including the cardiac-specific extension. The structure is orientated so that the Ca\(^{2+}\)-binding EF hand II of cTnC and the interacting cTnI sequence (31–35) are in the plane of the paper, thus showing the true separation between the peptides. Phosphorylation of Ser 22 and 23 causes the extension of an alpha helix (cTnI 21–30) that leads to the bending of the chain through \(\sim 90^\circ\) away from its interaction site with cTnC. We propose that DCM-causing mutations also interfere with the specific interaction of cTnI with cTnC due to allosteric conformational changes.

Table 2  Variability of the effect of DCM-causing mutations on Ca\(^{2+}\) sensitivity in native and synthetic thin filaments

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Ratio EC(_{50}) mut/wt for native thin filaments</th>
<th>References</th>
<th>Ratio EC(_{50}) mut/wt for synthetic thin filaments</th>
<th>Synthetic system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNNT2 (\Delta K210) (homozygous)</td>
<td>1.6</td>
<td>Du et al(^{24})</td>
<td>0.65</td>
<td>Recombinant troponin, AS(\alpha)-tropomyosin</td>
<td>Mirza et al(^9)</td>
</tr>
<tr>
<td>TNNC1 G159D</td>
<td>2.2</td>
<td>This work</td>
<td>0.99</td>
<td>Recombinant troponin, AS(\alpha)-tropomyosin</td>
<td>Mirza et al(^9)</td>
</tr>
<tr>
<td></td>
<td>0.55</td>
<td>Dyer et al(^{11})</td>
<td>1.8</td>
<td>Mouse heart fibres exchanged with recombinant TnC</td>
<td>Besiadeccki et al(^{19})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Human heart troponin, AS(\alpha)-tropomyosin</td>
<td>Dyer et al(^{11})</td>
</tr>
<tr>
<td>TPM1 E54K</td>
<td>1.0</td>
<td>This work</td>
<td>1.7</td>
<td>Recombinant troponin, AS(\alpha)-tropomyosin</td>
<td>Mirza et al(^9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Recombinant troponin, AS(\alpha)-tropomyosin</td>
<td>Robinson et al(^{10})</td>
</tr>
<tr>
<td>TPM1 D5230N</td>
<td>0.43</td>
<td>This work</td>
<td>1.7</td>
<td>Recombinant troponin, AS(\alpha)-tropomyosin</td>
<td>Lakdawala et al(^{26})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Skeletal muscle troponin, native (\alpha)-tropomyosin</td>
<td>This work</td>
</tr>
<tr>
<td>ACTC E361G</td>
<td>0.95</td>
<td>Song et al(^{21})</td>
<td>3.3</td>
<td>Skeletal muscle troponin, native (\alpha)-tropomyosin</td>
<td>Song et al(^{21})</td>
</tr>
</tbody>
</table>

Ratio EC\(_{50}\) mut/wt >1 means reduced Ca\(^{2+}\) sensitivity, ratio EC\(_{50}\) mut/wt <1 means increased Ca\(^{2+}\) sensitivity. AS\(\alpha\)-tropomyosin is recombinant tropomyosin with Ala-Ser in place of native N-terminal acetylation.
DCM mutations abolish effects of troponin I phosphorylation

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

Funding

S.B.M., A.E., M.-C.L., and M.M. acknowledge funding from The British Heart Foundation (PG/08/077/25587, RG/08/10/25918, FS/07/057/23834). K.J.N. is supported by an Australian Research Council Future Fellowship (FT100100734). D.G.W. and the mass spectrometry equipment was funded by Birmingham Science City.

References


