Myofibrillar Ca\(^{2+}\) sensitivity is uncoupled from troponin I phosphorylation in hypertrophic obstructive cardiomyopathy due to abnormal troponin T

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Aims
We studied the relationship between myofilament Ca\(^{2+}\) sensitivity and troponin I (TnI) phosphorylation by protein kinase A at serines 22/23 in human heart troponin isolated from donor hearts and from myectomy samples from patients with hypertrophic obstructive cardiomyopathy (HOCM).

Methods and results
We used a quantitative in vitro motility assay. With donor heart troponin, Ca\(^{2+}\) sensitivity is two- to three-fold higher when TnI is unphosphorylated. In the myectomy samples from patients with HOCM, the mean level of TnI phosphorylation was low: 0.38 ± 0.19 mol Pi/mol TnI compared with 1.60 ± 0.19 mol Pi/mol TnI in donor hearts, but no difference in myofilament Ca\(^{2+}\) sensitivity was observed. Thus, troponin regulation of thin filament Ca\(^{2+}\) sensitivity is abnormal in HOCM hearts. HOCM troponin (0.29 mol Pi/mol TnI) was treated with protein kinase A to increase the level of phosphorylation to 1.56 mol Pi/mol TnI. No difference in EC\(_{50}\) was found in thin filaments containing high and low TnI phosphorylation levels. This indicates that Ca\(^{2+}\) sensitivity is uncoupled from TnI phosphorylation in HOCM heart troponin. Coupling could be restored by replacing endogenous troponin T (TnT) with the recombinant TnT T3 isoform. No difference in Ca\(^{2+}\) sensitivity was observed if TnI was exchanged into HOCM heart troponin or if TnT was exchanged into the highly phosphorylated donor heart troponin. Comparison of donor and HOCM heart troponin by mass spectrometry and with adduct-specific antibodies did not show any differences in TnT isoform expression, phosphorylation or any post-translational modifications.

Conclusion
An abnormality in TnT is responsible for uncoupling myofibrillar Ca\(^{2+}\) sensitivity from TnI phosphorylation in the septum of HOCM patients.

Keywords
Troponin I • Troponin T • Phosphorylation • Hypertrophic cardiomyopathy • Cardiac muscle contractility

1. Introduction

Hypertrophic cardiomyopathy (HCM) is the most prevalent cardiovascular single-gene disorder, affecting up to 0.2% of the population. The disease is characterized morphologically by left ventricular wall and septal hypertrophy (>15 mm septal thickness is a common diagnostic criterion) leading to decreased ventricular volume and diastolic dysfunction.\(^1\) Obstruction of the left ventricular outflow tract is present at rest in ~25% of patients and in more during exercise. In histology sections, myocyte disarray and interstitial fibrosis are commonly observed. At the myofilament level, HCM heart muscle is initially hypercontractile but pressure overload, due to the...
outflow tract obstruction, and impaired relaxation cause a secondary progression towards heart failure and an increased probability of ventricular arrhythmia that can lead to sudden cardiac death.

The vast majority of single mutations that have been demonstrated as causes of HCM are in the contractile proteins of the sarcomere: mutations in the myosin heavy chain (MYH7) and myosin-binding protein C (MYBPC3) genes make up 80% of known mutations, but HCM is also caused by mutations in cardiac troponin T (TnT) (TNNT2), TnI (TNNI3), troponin C (TNNC1), α-tropomysin (TPM1), and actin (ACTC) genes.1–6

The molecular events that link the mutation in a sarcomeric protein to HCM have mainly been studied using recombinant pure contractile proteins or in transgenic mouse models, studies in human heart tissue being relatively few.7–12 The consensus from such studies is that the primary effect of HCM-causing mutations is a two- to three-fold increase in myofibrillar Ca\(^{2+}\) sensitivity, which produces a hypercontractile phenotype with impaired relaxation that corresponds to the diastolic dysfunction characteristic of HCM.6,13–15

It is not clear whether the same molecular abnormalities are manifested in the human heart, nor is it certain from animal experimentation how higher Ca\(^{2+}\) sensitivity leads to the longer term consequences of hypertrophy; myocyte disarray, fibrosis, and an increased risk of arrhythmias, which could lead to sudden cardiac death. The physiology of mice and that of humans are very different; however, HCM is a chronic disease that is often not symptomatic until the second or the third decade of life. In order to investigate the relationship between the primary molecular abnormality and the development of the disease, it is necessary to study hypertrophied human heart tissue directly.

In a number of studies this has been approached using muscle samples from patients with hypertrophic obstructive cardiomyopathy (HOCM) who undergo septal myectomy operations to relieve left ventricular outflow tract obstruction (LVOTO).16–18 Up to 5 g of the hypertrophied septum is removed in this operation and it provides a valuable source of fresh HCM heart muscle, although myectomy samples can only give a snapshot of the muscle in an advanced stage of disease.6,9,19,20

Investigation of myectomy samples has shown that contractility is abnormal and in many respects resembles end-stage failing heart muscle. Isometric force is reduced2 and in myocytes, amplitude and rates of contraction and relaxation are reduced8; there is increased expression of the skeletal isoform of actin21 and low levels of MyBP-C and TnI phosphorylation.22,23

For functional studies of contractile proteins in human heart samples, we have developed techniques to isolate individual contractile proteins from small muscle samples and have studied Ca\(^{2+}\) regulation in individual reconstituted thick and thin filaments, using a quantitative in vitro motility assay (IVMA).8,12,24,25 Applying these techniques to contractile proteins from HOCM heart muscle, we found evidence that some of the contractile proteins themselves are abnormal independently of the disease-causing mutation; for instance, there was a 10% lower fraction of thin filaments moving in the motility assay with myosin from HOCM tissue.8 The most prominent contractile protein abnormality in the septum of HOCM hearts is in the Ca\(^{2+}\)-regulated protein complex, tropoinin.26

In this study, we have examined troponin from HOCM heart muscle in comparison with proteins from donor hearts. We found that the modulation of Ca\(^{2+}\) sensitivity by the level of TnI phosphorylation by protein kinase A, which is an essential component of the lusitropic response to β-adrenergic stimulation, is absent in HOCM heart muscle. Further analysis shows that the uncoupling of the relationship between Ca\(^{2+}\) sensitivity and TnI phosphorylation in HOCM hearts is due to a mutation-independent abnormality in TnT.

2. Methods

2.1 Collection and storage of human myocardium

Human myocardial samples were obtained from patients with HOCM undergoing surgical septal myectomy for relief of LVOTO. The samples were snap-frozen in liquid nitrogen within 5 min of excision and stored for later use. Local ethical approval was obtained from University College London Hospitals and the Brompton, Harefield, and NHLI ethics committees for collection and use of tissue samples. All the patients had cardiac investigations, including 12-lead ECG, chest X-ray, Holter monitor, cardio-pulmonary exercise test, two-dimensional transthoracic echocardiography, transoesophageal echocardiography, cardiac catheterization, and coronary angiography (for clinical details, see Supplementary material online, Table SIA). In addition, we studied troponin from the explanted heart of an HCM patient with the homozygous TNNT2 K280N mutation who had previously had a myectomy operation.

Donor and failing heart muscle tissue samples were collected by Prof. C. dos Remedios, University of Sydney, Australia. Ethical approval was obtained from the Brompton, Harefield, and NHLI Research Ethics Committee, London, and the Australian Red Cross Blood Service and St Vincent’s Hospital, Sydney. The investigation conformed to the principles outlined in the Declaration of Helsinki. The donor heart tissue was obtained from hearts where no suitable transplant recipient was found. The patients had no history of cardiac disease, a normal cardiac examination, normal ECG, and normal ventricular function on echocardiography within 24 h of heart explantation. The clinical details are shown in Supplementary material online, Table SIB: the functional characteristics of some of the donor heart samples have been previously reported.25

2.2 Human heart troponin

Troponin was isolated from human heart muscle, using an anti-cTnI monoclonal antibody affinity column as described by Messer et al.;25 this yields pure and active troponin, which is usable for 3 days in an in vitro motility assay. TnI phosphorylation was measured in purified troponin by phosphate affinity SDS–PAGE as described by Messer et al.23 Where appropriate, troponin was dephosphorylated by treatment with shrimp alkaline phosphatase and phosphorylation levels were increased by treatment with protein kinase A catalytic subunit or recombinant troponin subunits were exchanged into human troponin as previously described25 (for detailed protocols, see Supplementary material online, Methods).

2.3 Quantitative in vitro motility assay

Thin filaments were reconstituted with rabbit skeletal muscle α-actin (labelled with TRITC phalloidin)27 and human heart muscle tropomyosin and troponin, and Ca\(^{2+}\) regulation of filaments was studied by the quantitative in vitro motility assay.25,28 Thin filament movement over a bed of immobilized rabbit fast skeletal muscle heavy meromyosin (100 μg/mL) was compared in paired motility cells differing in a single variable (tissue source, phosphorylation state, or troponin subunit exchange). Filament movement was recorded and analysed as previously described,29 yielding two parameters, the fraction of filaments moving and the speed of the moving filaments. In our motility system, both the speed of sliding and the fraction of thin filaments moving are regulated by Ca\(^{2+}\). The Ca\(^{2+}\)-dependent change in sliding speed was more variable, as previously noted,30 and therefore, we were not always able to
obtain a satisfactory fit of the speed data to the Hill equation. Nevertheless, when the EC50 for both speed and fraction motile parameters was obtained, they always gave the same result (see Supplementary material online, Part II).

Fraction motile and sliding speed was measured over a range of Ca2+ concentrations to generate Ca2+-activation curves. Each experiment involved the preparation of test and control troponins at the same time. In the experiment, thin filaments were reconstituted by mixing with the same tropomyosin and actin. Test and control mixes were made together and for each Ca2+-concentration point, they were pipetted into two channels of the dual motility cell. Motility was then measured within a couple of minutes of each other. Each pair of Ca2+ curves (yielding the data in one row in the tables of the supplement or one of the graphs in Figure 2) took ~2 h to complete. Intra-experiment variability is kept as low as possible by this methodology. The data were fitted to the four-variable Hill equation to yield a value for EC50 (for detailed protocols, see Supplementary material online, Methods). EC50 values from replicate experiments were analysed by paired and unpaired t-test, since the distribution of EC50 has been shown to be normal.25,28

2.4 Antibodies

Antibodies were used to investigate post-translational modifications in troponin. Several anti-nitrosylotroponin antibodies were used: Santa Cruz SC-32731, 1:200 dilution; Sigma N4009, 1:1000 dilution; and Upstate 05-233, 1:1000 dilution. All used nitrated albumin as a positive control. To test for S-nitrosylated cysteines, an antibody for S-Nitroso-Cysteine (SNO-Cys) was used: Sigma NS411, 1:1000 dilution and used nitrated albumin as a positive control. For O-GlcNac modification, we used the anti-O-linked N-acetylgalactosamine monoclonal antibody (Thermo Scientific RL-2) at a 1:1000 dilution and used CandyCane Glycoprotein Molecular Weight standards (Molecular Probes C21852) as a positive control normal.31 For lysine acetylation, anti-acetyl lysine (Upstate 06-933) at a 1:1000 dilution was used with acetylated bovine serum albumin (AcBSA) used as a positive control and AcBSA pre-treated with antibody as a negative control. For arginine methylation, we used an antibody against asymmetric dimethyl-Arginine, ASYM24 (Millipore 07-414) at a 1:500 dilution, with the positive control being a Jurkat cell lysate. To investigate protein oxidation, the OxyBlot Protein Oxidation Detection Kit from Millipore was used (Millipore S7150) with the positive control being a Jurkat cell lysate. Weight standards (Molecular Probes C21852) as a positive control.

In the IVMA, test and control thin filaments are made together, and for each Ca2+-concentration point, they are pipetted into the two channels of the dual motility cell. Motility is then measured within minutes of each other and in this way intra-experiment variability is kept as low as possible. However, variability between different motility cells day to day and on the same day is greater, and therefore, the absolute Ca2+-sensitivity is variable in these quantitative assays. Thus, need to calculate the ratio of EC50 values to increase accuracy.

In a preliminary series of experiments, we measured the Ca2+-sensitivity of thin filaments containing troponin from donor and explanted failing hearts compared with troponin from HOCM heart muscle. We found that, for donor and failing heart troponin, Ca2+-sensitivity and sliding speed at 3.9 μM Ca2+ are related to the TnI phosphorylation level and a low level of phosphorylation is associated with a two- to three-fold higher Ca2+-sensitivity than donor heart troponin, as previously documented (Figure 1, Supplementary material online, Figure S1IA); however, troponin from HOCM heart did not conform to this relationship. We therefore investigated the basis of this HOCM-specific abnormality.

When we measured Ca2+-activation curves for thin filaments containing human heart troponin, using the quantitative IVMA, we always found that donor heart and HOCM heart troponin had almost the same sensitivity to Ca2+. The absolute Ca2+ sensitivity was almost the same for both donor and failing heart troponin.

### Table 1 Mean values of EC50 for replicates of the experiments illustrated in Figure 2

<table>
<thead>
<tr>
<th>Experiment (test vs. control)</th>
<th>EC50 μM (test vs. control)</th>
<th>Ratio (EC50 test/EC50 control) ± SEM</th>
<th>n</th>
<th>P-value</th>
<th>TnI phosphorylation level, molsPi/mol (test vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>HOCM vs. Donor</td>
<td>0.13 ± 0.02 0.15 ± 0.02</td>
<td>0.90 ± 0.05</td>
<td>14</td>
<td>0.05</td>
</tr>
<tr>
<td>B</td>
<td>uP HOCM vs. uP Donor</td>
<td>0.16 ± 0.02 0.09 ± 0.02</td>
<td>1.86 ± 0.50</td>
<td>4</td>
<td>0.02</td>
</tr>
<tr>
<td>C</td>
<td>P HOCM vs. uP HOCM</td>
<td>0.15 ± 0.02 0.16 ± 0.02</td>
<td>0.99 ± 0.08</td>
<td>8</td>
<td>0.9</td>
</tr>
<tr>
<td>D</td>
<td>P Donor vs. uP Donor</td>
<td>0.14 ± 0.03 0.05 ± 0.01</td>
<td>3.09 ± 0.55</td>
<td>10</td>
<td>0.004</td>
</tr>
<tr>
<td>E</td>
<td>HOCM vs. HOCM XT</td>
<td>0.13 ± 0.02 0.08 ± 0.01</td>
<td>1.72 ± 0.12</td>
<td>5</td>
<td>0.004</td>
</tr>
<tr>
<td>F</td>
<td>P HOCM XT vs. uP HOCM XT</td>
<td>0.14 ± 0.01 0.08 ± 0.007</td>
<td>1.82 ± 0.13</td>
<td>5</td>
<td>0.003</td>
</tr>
<tr>
<td>G</td>
<td>HOCM vs. HOCM XI</td>
<td>0.15 ± 0.03 0.13 ± 0.03</td>
<td>0.99 ± 0.04</td>
<td>4</td>
<td>0.7</td>
</tr>
<tr>
<td>H</td>
<td>Donor vs. Donor XT</td>
<td>0.12 ± 0.01 0.12 ± 0.02</td>
<td>1.00 ± 0.02</td>
<td>5</td>
<td>0.8</td>
</tr>
</tbody>
</table>

For each type of experiment, mean EC50 ± SEM for test and control, measured by IVMA, is shown, together with the measured level of TnI phosphorylation in test and control thin filaments. The mean ratio of EC50 test/EC50 control together with SEM, n, and P-value determined by Student’s t-test (single value compared with 1) is also shown. Full details of individual experiments and statistics are shown in Supplementary material online, Part II.
same Ca$^{2+}$ sensitivity and sliding speed at 3.9 μM Ca$^{2+}$ despite very different phosphorylation levels; on average, the HOCM troponin was 10% more Ca$^{2+}$ sensitive than donor and this difference was statistically significant (Figure 2A, Table 1). Thus, troponin from HOCM heart is abnormal, since it confers a low Ca$^{2+}$ sensitivity (EC$_{50}$ 0.1–0.15 μM) on thin filaments with a low level of phosphorylation. In contrast native troponin from donor heart in which TnI is phosphorylated (open square), failing heart troponin (open square), unphosphorylated (phosphatase treated) donor heart troponin (open triangle), unphosphorylated recombinant Tnl exchanged into donor heart troponin (XII) (open diamond), protein kinase A-treated recombinant Tnl exchanged into donor heart troponin (XII) (filled diamond). Troponin from myectomy samples from HOCM patients (open circle).

Figure 1 The relationship between Tnl phosphorylation and Ca$^{2+}$ sensitivity of thin filaments containing human cardiac troponin. Troponin phosphorylation was measured by phosphate affinity SDS–PAGE (data from Dyer et al.$^{24}$). EC$_{50}$ for Ca$^{2+}$ activation of thin filament motility was measured using a quantitative in vitro motility assay. Natively phosphorylated donor heart troponin (filled square), failing heart troponin (open square), unphosphorylated (phosphatase treated) donor heart troponin (open triangle), unphosphorylated recombinant Tnl exchanged into donor heart troponin (XII) (open diamond), protein kinase A-treated recombinant Tnl exchanged into donor heart troponin (XIP) (data from Jacques et al.$^{25}$) (filled diamond). Troponin from myectomy samples from HOCM patients (open circle).

(figure 2D and Table 1), while for HOCM troponin, phosphorylation levels were raised, from 0.1 mol P/mol Tnl to 1.6 mol P/mol Tnl, by treatment with protein kinase A catalytic subunit. We found that the Ca$^{2+}$ sensitivity of HOCM troponin was insensitive to the Tnl phosphorylation level (Figure 2G) in contrast to the 3.1-fold difference in EC$_{50}$ for donor heart troponin (Figure 2D, Table 1). This pattern of results was observed in all the myectomy samples and includes disease-causing mutations in β-myosin heavy chain and MyBP-C and samples with no detectable sarcomeric mutation (Supplementary material online, Part II, Table D, summarized in Table 1). We concluded that in HOCM heart thin filaments, Ca$^{2+}$ sensitivity is uncoupled from changes in the level of Tnl phosphorylation.

To determine which subunit or subunits of troponin were responsible for the abnormal behaviour of HOCM troponin, we replaced Tnl (XI) or TnT (XT) in human cardiac troponin with recombinant troponin subunits by an exchange process (see Supplementary material online, Methods). The extent of exchange was determined from the reduction in Tnl or TnT phosphorylation measured in SDS–PAGE gels stained with Pro-Q Diamond phosphoprotein stain, since the recombinant protein was not phosphorylated (insets to Figure 2E and G). The efficiency of exchange was 89% for TnT and 91% for Tnl replacement. Exchange of recombinant (unphosphorylated) Tnl into HOCM troponin (HOCM XI) had no effect on Ca$^{2+}$ sensitivity (Figure 2G, Table 1), but exchange of recombinant TnT (T3 isoform, HOCM XT) resulted in a 1.7-fold higher Ca$^{2+}$ sensitivity as would be expected for troponin with a low level of Tnl phosphorylation (Figures 1 and 2E, Table 1; the full data set is shown in Supplementary material online, Part II, Tables E and G). These results indicate that the abnormal function of HOCM troponin is confined to the TnT subunit. We confirmed this conclusion by an experiment where TnT was exchanged into HOCM troponin (uP HOCM XT) and subsequently the native Tnl was phosphorylated with protein kinase A (P HOCM XT). We observed a restoration of the relationship between Ca$^{2+}$ sensitivity and Tnl phosphorylation following replacement of TnT, although the change in sliding speed at 3.9 μM Ca$^{2+}$ was not restored (Figure 2F, Table 1, Supplementary material online, Part II, Tables D and F and Figure SIIA). The Ca$^{2+}$-dependent increase in cross-bridge turnover rate has been shown to be a property of TnT in skeletal muscle,$^{32,33}$ however, in human cardiac muscle this appears to be a rather labile phenomenon. In a final control, we exchanged recombinant TnT into donor heart troponin (Donor XT) and observed that there was no change in Ca$^{2+}$ sensitivity, thus confirming that recombinant TnT behaves like native TnT in the normal donor heart context, even though it is not phosphorylated (Figures 2H and 3, Table 1).

Using MALDI mass spectrometry, we found that the functional abnormality of TnT in HOCM muscle was not related to an isoform change. Comparison of dephosphorylated human cardiac TnT with recombinant TnT isoforms by MALDI-TOF-MS showed that it was exclusively TNN2 isoform 6 (P45379-6), commonly referred to as the T3 isoform according to the notation introduced by Anderson et al.$^{34,35}$ (Figure 3A). Native TnT, prior to treatment with alkaline phosphatase, was 80 Da heavier indicating that the majority of TnT is monophosphorylated (Supplementary material online, Figure SIIA). MALDI spectra obtained from four separate troponin purifications each from several donor and HOCM hearts showed the same mass for the major TnT peak and additional minor peaks at 80 and −128 Da comprising ~22 and 11% of the total TnT signal intensity, respectively. The −80 Da peak corresponds to unphosphorylated T3 and the −128 Da peak was predicted to correspond to the monophosphorylated T3 splice-
Figure 2 The effects of TnI phosphorylation and exchange of troponin subunits on the $\text{Ca}^{2+}$ sensitivity of thin filaments containing donor and HOCM troponin. $\text{Ca}^{2+}$ activation of the fraction of thin filament motility was measured using the quantitative in vitro motility assay. $\text{Ca}^{2+}$ regulates the fraction of filaments motile and their sliding speed in a similar manner; the fraction motile parameter is plotted here; the complete analysis of individual experiments is shown in the Supplementary material online, part II. Typical comparison experiments done in paired motility cells; The points are the mean and SEM of four measurements made in a single motility cell and the lines are 4-parameter fits to the Hill equation. Mean results from several replicates of each experiment and the measured TnI phosphorylation levels are shown in Table 1. Solid lines and points, high TnI phosphorylation level; dotted lines and open points, low TnI phosphorylation level. (A) Thin filaments containing troponin from donor heart (1.6 mol Pi/mol TnI) and HOCM heart (0.3 mol Pi/mol TnI). Inset: phosphate affinity SDS–PAGE showing TnI phosphorylation levels. (B) Thin filaments containing unphosphorylated troponin from phosphatase-treated donor (0.2 mol Pi/mol TnI) or HOCM heart (0.3 mol Pi/mol TnI). Inset: phosphate affinity SDS–PAGE showing TnI phosphorylation level. (C) Thin filaments containing troponin from a HOCM heart (0.1 mol Pi/mol TnI) or protein kinase A-treated HOCM heart troponin (1.6 mol Pi/mol TnI), Inset: phosphate affinity SDS–PAGE showing TnI phosphorylation levels. (D) Thin filaments containing natively phosphorylated donor heart troponin (1.6 mol Pi/mol TnI) or phosphatase-treated donor troponin (0.2 mol Pi/mol TnI). Inset: phosphate affinity SDS–PAGE showing TnI phosphorylation levels. (E) Thin filaments containing HOCM troponin compared with HOCM troponin with TnT replaced by exchange (HOCM XT; both 0.3 mol Pi/mol TnI). Inset: Pro-Q Diamond-stained gels demonstrating the replacement of TnT. (F) Thin filaments containing unphosphorylated (uP HOCM XT; 0.2 mol Pi/mol TnI) or protein kinase A-phosphorylated HOCM troponin (P HOCM XT; 1.6 mol Pi/mol TnI) with TnT replaced by exchange in both samples. Inset: phosphate affinity SDS–PAGE showing TnI phosphorylation levels. (G) Thin filaments containing HOCM troponin (0.3 mol Pi/mol TnI) compared with HOCM troponin with TnI replaced by exchange (HOCM XI; 0.2 mol Pi/mol TnI). Inset: shows Pro-Q Diamond-stained gels demonstrating the replacement of TnI. (H) Thin filaments containing donor heart troponin compared with donor heart troponin with TnT replaced by exchange (both 1.7 mol Pi/mol TnI).
variant lacking glutamine 191 (34 431.4 Da) previously described in rat heart. The presence of this variant was confirmed by MS and MS/MS of glu-C digests of TnT (Supplementary material online, Figure SIIIC). Levels of the splice variant and phosphorylation in donor and HOCM troponin were not significantly different when analysed by MALDI-TOF-MS (Supplementary material online, Table SIIIA).

In addition to the MALDI spectra, high-resolution spectra were obtained by electrospray-Fourier transform mass spectrometry (ESI-FT-MS) of TnC, TnI, and TnT from one donor and one HOCM troponin sample (Figure 3B, Supplementary material online, Figure SIIIB). In agreement with the MALDI spectra, the major species of human cardiac TnT is monophosphorylated T3 isoform (hcTnT3) with an experimentally determined monoisotopic mass of 34 559.5 Da (calculated mass for monophosphorylated hcTnT3 lacking the N-terminal methionine and with N-terminal acetylation = 34 560.4). We also observed peaks corresponding to unphosphorylated hcTnT3 and monophosphorylated hcTnT3ΔQ in both donor and HOCM heart troponin (measured masses 34 431.5 and 34 479.5, respectively), with no detectable differences between donor and HOCM TnT. TnC had an identical mass in donor and HOCM troponins (18 432.7 Da) and Tnl (23 903.8 Da) differed only in the extent of phosphorylation (see Figure 2A).

Comparative measurements using the Pro-Q Diamond phosphoprotein stain in SDS–PAGE confirmed no consistent difference in the level of TnT phosphorylation over the full set of myectomy samples (phosphorylation in HOCM TnT was 99 ± 1% of donor heart level, n = 28, P = 0.35). Using antibodies specific to putative covalent post-translational modifications, we were unable to find any evidence for any other altered post-translational modifications that may not be detectable in mass spectrometry because of low abundance or instability, including sulphhydryl oxidation (TnT T3 has no cysteines), nitrotyrosine, O-GlcNAc modification, and lysine acetylation.

Figure 2 (Continued).
4. Discussion

Investigation of hypertrophic human heart tissue obtained from the interventricular septum in myectomy operations has shown that contractility is abnormal in all samples and in many aspects resembles end-stage failing heart muscle. Isometric force is reduced and in myocytes, amplitude and rates of contraction and relaxation are reduced; there is increased expression of the skeletal isoform of actin and low levels of MyBP-C and TnI phosphorylation. Functional investigation of the individual contractile proteins showed that cardiac myosin was partially inactivated in HOCM tissue but actin and tropomyosin function was the same as in donor tissue.

The most prominent contractile protein abnormality in the septum of HOCM hearts is in troponin. By using fully reconstituted thin filaments and the quantitative in vitro motility assay, we have shown that the myofilament Ca\textsuperscript{2+} sensitivity is independent of the level of TnI phosphorylation and is generally close to that of donor heart thin filaments, despite the very low level of TnI phosphorylation. This 'uncoupling' phenomenon was consistently observed and was not related to the disease-causing mutations, since it was observed with HCM-causing mutations in MYBPC3, MYH7, and TNNT2 and even when no mutation could be identified. The origin of the mutation-independent abnormality in HOCM heart troponin is therefore quite different from that found in previous reports on uncoupling that was a direct consequence of a mutation in one of the thin filament proteins; this has been observed for familial dilated cardiomyopathy (DCM) mutant proteins and also in several cases of HCM mutations.

Tnl uncoupling in HOCM is clearly a secondary effect but it is quite different from our observations of end-stage heart failure, where troponin function is normal, but the level of protein, kinase A-dependent phosphorylation is very low (Figure 1). At present, secondary uncoupling has been observed only in heart muscle troponin from cases of HCM that also suffer from severe pressure overload due to LVOTO (see Supplementary material online, Table S1A), and it is possible that uncoupling is triggered by the pressure overload itself. It would be very interesting to investigate whether troponin from the hearts of the 75% of HCM patients without LVOTO or from patients with LVOTO and pressure overload not caused by HCM, for instance aortic stenosis, shows uncoupling. However, it is unlikely that the relevant human heart muscle samples could be obtained.

By exchanging the subunits of troponin, we demonstrated that the altered functional properties of HOCM troponin are due to a functional abnormality in TnT; however, we were unable to find any modification of cTnT isoform expression or post-translational modifications in HOCM heart troponin that could account for the abnormality. Mass spectrometry clearly shows that cTnT is exclusively the T3 isoform (contains exon 4 but not exon 5) and that the DQ191 isoform (splice variant of exon 13) is present at a low level in both donor and HOCM TnT. The peak of HOCM and donor TnT corresponds to the monophosphorylated T3 isoform. A small amount of the DQ191 splice variant was observed in both donor and HOCM TnT. In addition to the major protein species indicated, adducts were seen at +17 Da in the TnC spectra and +98 and +113 in both the Tnl and the TnT spectra.

Figure 3 Mass spectrometry of intact troponin subunits. (A) Identification of TnT isoform. The MALDI-TOF spectra show the mass of the TnT peak obtained from troponin purified from donor and HOCM heart and dephosphorylated with alkaline phosphatase. For reference, we also show spectra of recombinant T1, T3, and T4 isoforms of hcTnT. The peak of HOCM and donor TnT corresponds to the T3 isoform with no evidence for any T1 or T4 isoforms. A small amount of the DQ191 splice variant was observed in both donor and HOCM TnT. (B) FTICR mass spectrometry of native HOCM and donor TnT. High-resolution electrospray mass spectra of TnC (16+ ion), Tnl (33+ ion), and TnT (48+ ion) isolated from donor and HOCM heart. The peak of HOCM and donor TnT corresponds to the monophosphorylated T3 isoform. A small amount of the DQ191 splice variant was observed in both donor and HOCM TnT. In addition to the major protein species indicated, adducts were seen at +17 Da in the TnC spectra and +98 and +113 in both the Tnl and the TnT spectra.
Abnormal troponin in heart muscle from HOCM patients

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Figure 1. Positions of the amino acid substitutions in actin and troponin T.

Figure 2. Distribution of the amino acid substitutions among patients and the number of actin and troponin T mutations in each group.

Figure 3. Distribution of the troponin T mutations among patients with HOCM and DCM.

Figure 4. Distribution of the troponin T mutations among patients with HOCM and DCM.

Figure 5. Distribution of the troponin T mutations among patients with HOCM and DCM.

Figure 6. Distribution of the troponin T mutations among patients with HOCM and DCM.

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Figure 111. Distribution of the troponin T mutations among patients with HOCM and DCM.

Figure 112. Distribution of the troponin T mutations among patients with HOCM and DCM.


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