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Perturbed Length–Dependent Activation in Human Hypertrophic Cardiomyopathy With Missense Sarcomeric Gene Mutations

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Abstract

Rationale—High-myofilament Ca^{2+}-sensitivity has been proposed as trigger of disease pathogenesis in familial hypertrophic cardiomyopathy (HCM) based on in vitro and transgenic mice studies. However, myofilament Ca^{2+}-sensitivity depends on protein phosphorylation and muscle length, and at present, data in human are scarce.

Objective—To investigate whether high-myofilament Ca^{2+}-sensitivity and perturbed length-dependent activation are characteristics for human HCM with mutations in thick- and thin-filament proteins.

Methods and Results—Cardiac samples from patients with HCM harboring mutations in genes encoding thick (*MYH7, MYBPC3*) and thin (*TNNT2, TNNI3, TPM1*) filament proteins

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were compared with sarcomere mutation-negative HCM and nonfailing donors. Cardiomyocyte force measurements showed higher myofilament Ca^{2+}-sensitivity in all HCM samples and low phosphorylation of protein kinase A (PKA)-targets compared with donors. After exogenous PKA treatment, myofilament Ca^{2+}-sensitivity was either similar (*MYBPC3*$_{mut}$, *TPM1*$_{mut}$, sarcomere mutation-negative HCM), higher (*MYH7*$_{mut}$, *TNNT2*$_{mut}$), or even significantly lower (*TNNI3*$_{mut}$) compared with donors. Length-dependent activation was significantly smaller in all HCM than in donor samples. PKA treatment increased phosphorylation of PKA-targets in HCM myocardium and normalized length-dependent activation to donor values in sarcomere mutation-negative HCM and HCM with truncating *MYBPC3* mutations, but not in HCM with missense mutations. Replacement of mutant by wild-type troponin in *TNNT2*$_{mut}$ and *TNNI3*$_{mut}$ corrected length-dependent activation to donor values.

Conclusions—High-myofilament Ca^{2+}-sensitivity is a common characteristic of human HCM and partly reflects hypophosphorylation of PKA-targets compared with donors. Length-dependent sarcomere activation is perturbed by missense mutations, possibly via post-translational modifications other than PKA-hypophosphorylation or altered protein–protein interactions, and represents a common pathomechanism in HCM.

Keywords

calcium; cardiomyopathy; contractility; hypertrophy; myocardium

Myofilament contraction is initiated by interaction between the thin actin and thick myosin filaments. This actin–myosin interaction (ie, thin-filament-myosin cross-bridge binding) and the magnitude of myofilament force generation are tightly regulated by muscle length, Ca^{2+}-binding, and protein phosphorylation.[1] Defective proteins as a result of mutations in genes encoding sarcomeric proteins may directly impair regulation of muscle contraction and manifest themselves as phenotypic aberrations of the heart. Hypertrophic cardiomyopathy (HCM) reflects the pathological phenotype associated with sarcomeric gene mutations.[2,3] Affecting 1:500 individuals worldwide, HCM is the most common cause of sudden death in young people.[2] Genotyping studies have identified a disease-causing mutation in ≈70% of all patients with HCM.[4] Mutations in thick-filament–encoding genes, *MYH7* (β-myosin heavy chain [β-MyHC]) and *MYBPC3* (cardiac myosin–binding protein-C [cMyBP-C]), account for ≈80% of all identified sarcomere mutations, whereas ≈18% of the mutations are found in thin-filament–encoding genes, *TNNI3* (cardiac troponin I [cTnI]), *TNNT2* (cardiac troponin T [cTnT]), *TNNC1* (cardiac troponin C), *TPM1* (α–tropomyosin), and *ACTC1* (α-cardiac actin).[5] The remaining 2% is attributed to incidental mutations in the thick-filament genes, *MYL3* and *MYL2,* encoding the regulatory and essential myosin light chain, and the sarcomere-associated gene *TTN*, which encodes titin.[5] Based on the numerous sarcomeric gene mutations, HCM is referred to as disease of the sarcomere.

Both animal[6,7] and clinical[8–10] studies have shown that carriers of HCM-causing mutations demonstrate early signs of cardiac dysfunction, even before a hypertrophic phenotype is observed. Additionally, sudden cardiac death occurs in young individuals in the absence of clinically detectable cardiac hypertrophy.[11] This suggests that the initial defects in cardiac performance are triggered by mutant sarcomeric proteins rather than remodeling of the heart. Sarcomere mutations may directly impair myofilament function and contractile performance of the heart,[3] or indirectly via changes in intracellular Ca^{2+}-handling.[12] Because the myofilaments represent a major intracellular buffer of Ca^{2+}, any perturbation in myofilament Ca^{2+}-sensitivity may provide a substrate for cardiac arrhythmias. Only recently, the group of Knollmann[13] showed that *TNNT2* mutations alter intracellular Ca^{2+}-handling via myofilament Ca^{2+}-sensitization in transgenic mice models, which was

associated with altered action potential regulation and occurrence of arrhythmias. This implies that myofilament Ca^{2+}-sensitivity is central in HCM pathology.

Whether or not myofilament sensitization to Ca^{2+} is of relevance in human HCM is a matter of ongoing research.[14–16] Our recent studies in manifest HCM with *MYBPC3* mutations showed higher myofilament Ca^{2+}-sensitivity compared with nonfailing donor myocardium.[15,16] However, high Ca^{2+}-sensitivity coincided with low phosphorylation of target proteins of the β-adrenergic signaling pathway, cTnI, and cMyBP-C, and was normalized to donor values by exogenous protein kinase A (PKA) treatment.[15] This suggests that the high-myofilament Ca^{2+}-sensitivity observed in human HCM with *MYBPC3* mutations is attributable to secondary disease–related post-translational modifications rather than the mutation itself. In addition to post-translational protein modifications, muscle length represents an important determinant of myofilament Ca^{2+}-sensitivity. Recently, we observed impairment of length-dependent sarcomere activation evident from a blunted length–dependent increase in myofilament Ca^{2+}-sensitivity in HCM with *MYBPC3* mutations.[15] Under normal conditions, intracellular Ca^{2+} buffering will increase with increased myofilament Ca^{2+}-sensitivity on myofilament lengthening.[17,18] Perturbations in length-dependent myofilament activation will alter Ca^{2+} buffering by the sarcomeres and may provide a basis for altered Ca^{2+}-handling in HCM.

In the present study, we investigated whether perturbed length–dependent activation rather than high-myofilament Ca^{2+}-sensitivity is a common pathomechanism in human HCM with mutations in thick- and thin-filament genes. Measurements in sarcomere mutation-positive HCM were compared with sarcomere mutation-negative HCM (HCM_{smn}) and nonfailing donors. Our study shows that mutation-related changes in myofilament Ca^{2+}-sensitivity are diverse and depend on the affected gene. We observed impaired length–dependent sarcomere activation in all HCM samples. The blunted length–dependence of sarcomeres was attributable to low phosphorylation of PKA-targets compared with donor myocardium in patients with *MYBPC3* truncating mutations and HCM_{smn}, but a direct consequence of HCM missense mutations. Using troponin exchange in HCM cardiomyocytes with a homozygous *TNNT2* and a heterozygous *TNNI3* mutation, we provide evidence that <50% of poison peptide is sufficient to impair length-dependent activation of the sarcomeres. Because most patients carry a heterozygous sarcomere mutation that may result in relatively low levels of mutant (poison) peptide, impaired length–dependent sarcomere activation represents a pathomechanism in HCM.

Methods

An expanded version of the methods section can be found in the online-only Data Supplement.

Myocardial Samples

Left ventricular septum tissue was obtained from patients with HCM harboring thick- and thin-filament gene mutations during myectomy surgery to relieve left ventricular outflow obstruction. Hypertrophic obstructive cardiomyopathy was evident from the high-septal thickness (normal value <13 mm) and high-left ventricular outflow tract pressure gradient (normal value <30 mm Hg). Clinical characteristics and mutation information of patients with HCM are listed in Table 1. Our study included patients carrying heterozygous mutations in *MYBPC3* (n=21; $MYBPC3_{mut}$), *MYH7* (n=6; $MYH7_{mut}$), *TNNI3* (n=2; $TNNI3_{mut}$), and *TPM1* (n=1; $TPM1_{mut}$). The $MYBPC3_{mut}$ group consisted of patients with truncating (n=17) and missense (n=4) mutations. Data for these 2 $MYBPC3_{mut}$ groups are presented separately. Septum tissue was also obtained from 1 end-stage failing patient with HCM carrying a homozygous *TNNT2* mutation ($TNNT2_{mut}$). Septum myectomy tissue

from 7 patients with HCM in whom no mutation was found after screening of 8 genes (HCM_{smn}) and cardiac tissue from 12 nonfailing donors served as controls. Donors (age ranged 14–65 years; mean 39±5 years; 9/3 male/female, respectively) had no history of cardiac abnormalities, normal ECG, and normal ventricular function on echocardiography within 24 hours of heart transplantation. Samples were obtained after written informed consent, and the study protocol was approved by the local ethical committees.

Isometric Force Measurements and Protein Analysis

Analysis of single cardiomyocyte force measurements and sarcomeric protein phosphorylation was performed as described previously.[15,19,20]

Exchange of Recombinant Human Troponin Complex

Expression, purification, and reconstitution of recombinant human wild-type cardiac troponin in single cardiomyocytes from the $TNNT2_{mut}$ heart and 1 of the $TNNI3_{mut}$ hearts were performed as described previously.[21] Evaluation of the degree of exchange into cardiomyocytes was determined by labeling cTnT of wild-type recombinant cardiac troponin with a myc-tag to allow differentiation between endogenous and recombinant wild-type cTnT in Western blot analysis.

Data Analysis

Data are presented as mean±SEM of all single cardiomyocytes per patient/donor group. To take into account the repeated sample assessments within patient/donor groups, multilevel analysis was performed. Comparison between all groups was performed for Ca^{2+}-sensitivity at 2.2 μm sarcomere length and length-dependent activation of cardiomyocytes before and after PKA. Paired-group comparisons were performed for maximal developed force (F_{max}) at 1.8 and 2.2 μm sarcomere length before and after PKA.

Detailed explanation of the data analyses, significance levels (exact *P* values), and 95% confidence intervals for all comparisons are given in the online-only Data Supplement file.

Results

Myofilament Ca^{2+}-Sensitivity in HCM Compared With Nonfailing Donors

To assess myofilament Ca^{2+}-sensitivity, force-Ca^{2+} relations were constructed for all cardiac samples at a sarcomere length of 2.2 μm. Ca^{2+}-sensitivity for all HCM samples was significantly higher compared with donors, based on their lower EC_{50} values (Figure 1A). Because the high Ca^{2+}-sensitivity may be attributable to low-phosphorylation levels of PKA-target proteins, measurements were repeated after PKA treatment. The PKA-induced reduction in Ca^{2+}-sensitivity (ΔEC_{50}) was larger in all HCM groups compared with donors (with significant changes in $MYBPC3_{mut}$, $MYH7_{mut}$, and $TNNI3_{mut}$), except for the $TNNT2_{mut}$, which only showed a minor nonsignificant change (Figure 1B). After PKA treatment, myofilament Ca^{2+}-sensitivity was close to donor values in $MYBPC3_{mut}$, $TPM1_{mut}$, and HCM_{smn}, whereas Ca^{2+}-sensitivity remained significantly higher compared with donor values in $MYH7_{mut}$ and $TNNT2_{mut}$ (Figure 1C). Interestingly, after PKA treatment, Ca^{2+}-sensitivity was significantly lower in $TNNI3_{mut}$ compared with HCM_{smn} and donor values.

F_{max} at a sarcomere length of 2.2 μm was significantly lower in all HCM groups compared with donors and was not corrected by PKA treatment (Online Table I). In addition, the steepness of the force-Ca^{2+} relation was significantly lower and not corrected by PKA in all HCM groups compared with donors (data not shown).

Length-Dependent Myofilament Force Characteristics

Force development was measured at various [Ca^{2+}] and 2 sarcomere lengths to determine length-dependent activation of myofilaments (Figure 2A). After a sarcomere length increase from 1.8 to 2.2 μm at maximal Ca^{2+}-activation, donor samples showed a significant increase in F_{max} (Table 2), which is in accordance with the well-known effects of length on force development.[22] A significant length–dependent increase in F_{max} was observed in *MYBPC3*$_{mut}$, *MYH7*$_{mut}$, *TPM1*$_{mut}$, and HCM$_{smn}$ heart samples, whereas no significant increase in F_{max} was found for both *TNNT2*$_{mut}$ and *TNNI3*$_{mut}$. As illustrated in Table 2, the ΔF_{max} (difference in F_{max} between sarcomere lengths of 1.8 and 2.2 μm) was much lower in HCM with missense mutations compared with the other groups.

To investigate the length-dependent increase in myofilament Ca^{2+}-sensitivity, force at submaximal [Ca^{2+}] was normalized to the F_{max} to obtain normalized force-Ca^{2+} relations at both sarcomere lengths (Figure 2B). For all HCM and donor groups, the normalized force-Ca^{2+} relation shifted to the left as sarcomere length increased, indicative for increased Ca^{2+}-sensitivity. However, the increase in myofilament Ca^{2+}-sensitivity on an increase in sarcomere length (ΔEC_{50}) was significantly lower in all sarcomere mutation-positive HCM and HCM$_{smn}$ samples compared with donors (Figure 2C).

Stimulation of the β-adrenergic receptor pathway has been shown to enhance the length-dependent shift in the force-Ca^{2+} relation, suggesting a modulating role for PKA-mediated protein phosphorylation in length-dependent sarcomere activation.[23,24] As previous studies showed lower phosphorylation levels of myofilament PKA-target proteins in HCM compared with donors,[15,16] the blunted length–dependent activation in HCM compared with donors may be explained by a difference in protein phosphorylation level. Indeed, analysis of protein phosphorylation showed lower phosphorylation levels of both cTnI and cMyBP-C in all HCM groups compared with nonfailing donors, except in *TNNT2*$_{mut}$, in which phosphorylation of the PKA-target proteins did not differ from donor (Figure 3A).

To test whether the blunted length–dependent change in F_{max} and myofilament Ca^{2+}-sensitivity was corrected by PKA-mediated phosphorylation of cMyBP-C and cTnI, force measurements were performed after pretreatment with exogenous PKA in a subset of HCM and donor samples. PKA pretreated *MYBPC3*$_{mut}$, *MYH7*$_{mut}$, HCM$_{smn}$, and donor cells showed a significant increase in maximal force after a sarcomere length increase, similar to that observed in nontreated cardiomyocytes (Table 2). PKA did not restore the blunted length–dependent increase in F_{max} evident from the significantly lower ΔF_{max} in HCM with missense mutations compared with donor (Table 2). Pretreatment with exogenous PKA significantly enhanced the length-dependent shift in myofilament Ca^{2+}-sensitivity in HCM with truncating *MYBPC3*$_{mut}$ and HCM$_{smn}$ samples to values observed in donors, but did not correct the blunted length–dependent change in EC_{50} in all other HCM mutant groups harboring missense mutations (Figure 2D).

Because the *MYBPC3*$_{mut}$ and *MYH7*$_{mut}$ groups consisted of different mutations, we averaged functional data for each HCM mutation separately. Data are shown in the online-only Data Supplement material (Online Table II).

PKA-Mediated Protein Phosphorylation

To determine whether absence of an effect of PKA on sarcomere functional properties is attributable to a defect in PKA-mediated protein phosphorylation in HCM myocardium, phosphorylation status of PKA-target proteins was analyzed in HCM and donor samples incubated with exogenous PKA. Figure 3B shows HCM samples incubated without and with PKA separated by 1-dimensional gel electrophoresis and stained with ProQ-Diamond and SYPRO Ruby. PKA increased phosphorylation of both PKA-targets, cMyBP-C and cTnI, in

HCM samples. Phosphorylation levels (normalized to untreated donor samples, which were included on the gel and set to 1; dotted line) are depicted in Figure 3C and show that phosphorylation of cMyBP-C after PKA was close to values observed in donor samples in all HCM samples. Analysis of phosphorylation at specific PKA sites (Ser275 and Ser284) on cMyBP-C confirmed increased PKA–mediated phosphorylation in HCM samples (Figure 4). In addition to cMyBP-C, phosphorylation of cTnI increased by PKA in all HCM samples (Figure 3B), although values did not reach the level found in donor myocardium (Figure 3C). PKA treatment of donor samples increased cTnI phosphorylation by ≈25%. Similar data were obtained when cTnI phosphorylation was analyzed by Phos-Tag gel electrophoresis creating a pattern of un, mono-, and bisphosphorylated cTnI (Online Figure II). PKA-treated HCM samples showed increased bisphosphorylated cTnI levels, but some monophosphorylated cTnI remained. Previous Phos-Tag analysis of the donor samples (n=12) revealed a distribution pattern of 7% unphosphorylated, 27% monophosphorylated, and 66% bisphosphorylated cTnI.[15] On treatment of donor samples (n=2) with PKA, cTnI was mostly bisphosphorylated (Online Figure II).

Correction of Length-Dependent Activation in HCM With Mutant cTnT and Mutant cTnI by Human Recombinant Wild-Type Troponin

To determine whether mutant sarcomeric protein is the direct cause of the blunted length–dependent increase in myofilament Ca^{2+}-sensitivity, we performed troponin exchange experiments in cardiomyocytes from the *TNNT2*$_{mut}$ sample. The homozygous *TNNT2*$_{mut}$ necessarily results in 100% mutant cTnT and as such represents a unique tool to assess the level at which mutant protein perturbs sarcomere function. Exchange with increasing concentrations of wild-type human troponin complex (0.25, 0.5, and 1 mg/mL in the exchange solution) resulted in 62±2%, 78±1%, and 86±1% troponin exchange based on Western blot analyses of endogenous and myc-tag labeled wild-type cTnT (Figure 5A; left blot). In exchanged cells without PKA pretreatment, replacement of mutant troponin with unphosphorylated recombinant wild-type troponin did not restore the reduced length–dependent activation to donor values (Figure 5B). However, replacement of endogenous mutant troponin by unphosphorylated recombinant troponin reduces cTnI phosphorylation in the *TNNT2*$_{mut}$ cells. Therefore, measurements were also performed in the troponin-exchanged cells after PKA treatment to increase cTnI phosphorylation to donor levels. In cells in which 62% of mutant troponin was replaced by wild-type troponin, the length-dependent increase in myofilament Ca^{2+}-sensitivity was still significantly lower compared with donor values, indicating that 38% of mutant protein is sufficient to impair length-dependent activation. However, the blunted length–dependent activation was restored to donor values in *TNNT2*$_{mut}$-exchanged cells, harboring 22% and 14% endogenous mutant cTnT (Figure 5C). Similarly, exchange of mutant cTnI in 1 of the HCM samples harboring the R145W missense mutation in *TNNI3* by ≈90% unphosphorylated wild-type troponin complex (Figure 5A; right blot) corrected length-dependent activation only after PKA treatment (Figure 5D and 5E). Hence, normalization of length-dependent activation to donor values on exchange with wild-type cTn complex is only evident after PKA treatment of troponin-exchanged cells.

Discussion

Our study shows that high-myofilament Ca^{2+}-sensitivity in human HCM myocardium is independent of the presence of a sarcomere mutation and at least partly explained by protein hypophosphorylation. Sarcomere mutations may modify Ca^{2+}-sensitivity, but the direction and magnitude of the change depend on the affected gene. Impaired length–dependent activation of sarcomeres represents a common pathomechanism underlying HCM, and could not be corrected by PKA treatment in HCM with missense mutations in genes encoding

thick- and thin-filament proteins. Moreover, our troponin exchange experiments provide direct proof that mutant troponin impairs length-dependent activation. Our data indicate that mutant proteins resulting from missense mutations could perturb length-dependent sarcomere activation and underlie cardiac dysfunction observed at early stages of HCM disease development.

Myofilament Ca^{2+}-Sensitivity and Phosphorylation Background

Our studies in human HCM with mutations in both thick- and thin-filament proteins showed high-myofilament Ca^{2+}-sensitivity at a sarcomere length of 2.2 μm (Figure 1A). This is in agreement with previous studies in transgenic mouse models and in vitro studies with mutant proteins, which indicate that HCM sarcomere mutations sensitize myofilaments to calcium.[3,12,13,25] However, the higher Ca^{2+}-sensitivity in sarcomere mutation-positive HCM groups compared with nonfailing donors coincided with lower phosphorylation levels of cMyBP-C and cTnI (Figure 3A). The difference in myofilament Ca^{2+}-sensitivity between sarcomere mutation-positive HCM and nonfailing donor can thus be partly explained by hypophosphorylation of sarcomeric proteins rather than the sarcomere gene mutation itself.

A possible explanation for the low-phosphorylation levels may reside in a blunted β–adrenergic response in patients with HCM. A blunted response to isoproterenol, a β-adrenoreceptor agonist, has been reported in transgenic mice harboring *TPM1* and *MYH7* mutations.[26,27] Moreover, reduced β–adrenoreceptor density has been reported in patients with HCM.[28] Treatment of HCM samples with PKA increased phosphorylation of the PKA-target proteins (Figures 3 and 4 and Online Figure II) and normalized myofilament Ca^{2+}-sensitivity in $MYBPC3_{mut}$, $TPM1_{mut}$, and HCM_{smn} to values observed in nonfailing donors. In contrast, after PKA, higher myofilament Ca^{2+}-sensitivity was still present in $MYH7_{mut}$ and $TNNT2_{mut}$, suggesting a Ca^{2+}-sensitizing effect by these mutations. Interestingly, PKA treatment significantly lowered Ca^{2+}-sensitivity of the $TNNI3_{mut}$ R145W compared with donor. This observation contrasts with transgenic animal models and reconstituted thin-filaments using recombinant human mutant cTnI.[29–31] It has been suggested that the Ca^{2+}-sensitizing action of mutations in the inhibitory region of cTnI (residues 137–148) directly impairs the intrinsic inhibitory activity of cTnI.[29,30] A possible explanation for the contradicting results compared with our $TNNI3_{mut}$ samples may reside in the amount of endogenous mutant protein expression. Using adenovirus gene transfection to incorporate the cTnI R145W mutant into adult rat cardiomyocytes, Davis et al[32] observed no elevation of myofilament Ca^{2+}-sensitivity, which was attributed to poor incorporation (≈35%) of mutant protein into the sarcomeric structure compared with wild-type cTnI. In addition, the phosphorylation background of the sarcomeres may have been different among studies.

Overall, our data show that high Ca^{2+}-sensitivity is not a specific characteristic of human sarcomere mutation-positive HCM, as a similarly high-myofilament Ca^{2+}-sensitivity was found in HCM_{smn} compared with donors, which may be ascribed to reduced β–adrenergic signaling as part of cardiomyopathy development. Although PKA is the archetypical kinase involved in modulating Ca^{2+}-sensitivity through cTnI and cMyBP-C phosphorylation, it is by no means the only kinase that phosphorylates myofilament proteins. Both cTnI and cMyBP-C are targets for a whole range of kinases.[33–37] cMyBP-C phosphorylation is thought to mainly affect cross-bridge cycling kinetics,[38] although a role in mediating Ca^{2+}-sensitivity of force has been suggested.[39] Cardiac TnI is considered to be the key regulator of Ca^{2+}-sensitivity, and it is mainly through phosphorylation of Ser23 and Ser24 that PKA exerts its Ca^{2+}-desensitizing effect, although many other phosphorylation sites have been identified.[40] Phosphorylation is but one of many possible post-translational modifications. Recent reports have hinted at possible involvement of other post-translational modifications in the regulation of sarcomere function, such as oxidation and S-glutathionylation[41] or O-GlcNAcylation.[42] It would thus be an oversimplification to propose that a reduction in PKA-

phosphorylation of sarcomere proteins is solely responsible for the myofilament changes in HCM. However, the baseline Ca^{2+}-sensitivity seems to be dominated by the relatively low-phosphorylation levels of PKA myofilament target proteins compared with donors. Higher phosphorylation levels mimicked by exogenous PKA treatment, as would be induced during increased cardiac stress (eg, exercise), unveils a higher, similar, or even lower myofilament Ca^{2+}-sensitivity in HCM dependent on the affected gene. Our findings suggest diverse mutation–induced changes in Ca^{2+}-sensitivity, whereas high-myofilament Ca^{2+}-sensitivity is partly explained by secondary disease–related changes in protein phosphorylation.

Impairment of Length-Dependent Sarcomere Activation in HCM

Our data indicate that mutant sarcomeric proteins in HCM perturb length-dependent activation of myofilaments, which may contribute to early cardiac dysfunction observed in sarcomere mutation carriers. Studies in transgenic mouse models and troponin-exchange techniques with mouse tissue harboring HCM mutations were not conclusive as a reduced or normal length–dependent activation was found compared with controls.[27,43–46] The recent study of Ford et al[46] may shed some light on these previous observations as the authors studied length-dependent differences in mice expressing *TNNT2* mutations either in an α-MyHC (predominant in murines) or in a β-MyHC (predominant in healthy adult human hearts) background. It was observed that mice expressing the R92L mutation in the physiological α-MyHC background presented a normal length–dependent Ca^{2+}-activation, whereas in the presence of the slow cycling β-MyHC isoform, length dependence was lost.[46] Defects in length-dependent sarcomere properties were not similar in all HCM samples, because the blunted length–dependent increase in myofilament Ca^{2+}-sensitivity was corrected to donor values by exogenous PKA in truncating $MYBPC3_{mut}$ and HCM_{smn}, whereas it remained defective in HCM with missense mutations in *MYBPC3, MYH7*, *TNNT2*, *TNNI3,* and *TPM1.* Moreover, the increase in maximal force generating capacity on an increase in sarcomere length was almost entirely absent in HCM with troponin mutations (Table 2). Although PKA increased phosphorylation of cTnI and cMyBP-C and reduced myofilament Ca^{2+}-sensitivity at 2.2 μm in all HCM samples (except in the homozygous $TNNT2_{mut}$), it did not restore length-dependent activation in HCM samples with missense mutations. Intriguingly, a negative myofilament length–dependent activation was observed after PKA in the $TPM1_{mut}$ sample (Figure 2D), suggesting that length-dependent activation is even more impaired during increased cardiac stress.

The blunted length–dependent activation may be partly related to the relatively low phosphorylation of PKA-targets and high-baseline myofilament Ca^{2+}-sensitivity. The suggestion that PKA-mediated myofilament protein phosphorylation has a modulatory role in length-dependent activation comes from studies in ferret papillary muscles,[23] in which isoprenaline, a stimulator of the β-adrenergic receptor pathway, enhanced the length-dependent change in the force-Ca^{2+} relation. Studies in cardiac tissue in which cTnI was replaced by skeletal TnI, which lacks the PKA-target serines (Ser23/24), showed higher myofilament Ca^{2+}-sensitivity, but a significantly reduced length–dependent activation,[24,47] indicating a role for cTnI phosphorylation in length-dependent activation. A study by Cazorla et al[44] in transgenic mice lacking cMyBP-C demonstrated lower length–dependent activation than wild-type mice that could not be restored by exogenous PKA treatment, which suggests that cMyBP-C is needed for proper length–dependent sarcomere activation. In our human samples with truncating *MYBPC3* mutations, impaired length–dependent activation was corrected to donor values by PKA. Our previous study in HCM with truncating mutations in *MYBPC3* showed reduced expression of full-length cMyBP-C to ≈70% compared with donor values (ie, haploinsufficiency). Overall, our studies in human HCM with *MYBPC3* truncation mutations indicate that the presence of ≈70% of full-length

cMyBP-C protein in the sarcomere is sufficient to preserve the length-dependent properties of the sarcomeres.[15]

The perturbations in length-dependent activation in HCM with mutations in thin-filament genes may be explained by the 3-state model of filament transition, in which the troponin–tropomyosin complex has a central regulatory role. Myofilament contraction and force production are tightly regulated by the troponin–tropomyosin complex that regulates the interaction between the actin and myosin filaments. It is believed that the myofilaments exist in a dynamic equilibrium between 3 biochemical transitions (Figure 6) that reflect different interactions between actin and myosin, termed the blocked (B-state), closed (C-state), and open (M-state) states of thin-filament regulation.[48,49] In the B-state Ca^{2+} is not bound to cardiac troponin C, and tropomyosin sterically blocks myosin-binding sites on F-actin (Figure 6A). In the C-state, Ca^{2+} binds to cardiac troponin C, which changes conformation of the troponin–tropomyosin complex, resulting in nontension-generating cross-bridges, which bind weakly to F-actin (ie, weakly bound cross-bridges; Figure 6B).[49–51] The M-state involves strong binding of tension-generating cross-bridges, which results in myofilament contraction and force development (Figure 6C).[49–51] Because of the central roles of cTnT and cTnI in the transition from the B-state to the C-state,[52–54] it is likely that mutation-induced irregularities in protein interactions may translate into thin-filament abnormalities.

The C-terminal half of cTnI docks the troponin–tropomyosin complex onto the outer domain of F-actin at low-cytoplasmic [Ca^{2+}],[55] stabilizing the formation of the B-state. Interestingly, ≈86% of cTnI HCM–causing mutations (dashed red circle in Figure 6D), including the one present in our study, are found in the C-terminal half of cTnI (residues 137–210), a region responsible for actin binding.[25,56] Indeed, disruption of the B-state has been suggested in HCM-causing mutations not only affecting cTnI,[57] but also cTnT.[58,59] The relevance of the transition from the B- to the C-state for proper length–dependent activation has been shown by Smith and Fuchs[60] who were the first to provide evidence for a length-sensitive step in the transitions of thin-filament activation. A reduction in ionic strength (<0.05 mol/L), known to shift the B-state equilibrium toward a stable C-state,[61] coincided with impairment of length-dependent activation.[60] The blunted length–dependent increase in myofilament Ca^{2+}-sensitivity observed in the thin-filament mutation groups can thus be explained by disruption of the B-state and an increased number of weakly bound cross-bridges in the C-state.

Eleven HCM mutations have been identified in α–tropomyosin.[56] To the best of our knowledge, the present study is the first to analyze the effects of an HCM-causing mutation (M281T) in the overlap region of tropomyosin, which has a central role in cooperative activation of the thin filament.[62,63] The steepness of the force-Ca^{2+} relation, which is an indicator of the relative number of near-neighbor interacting sites, was significantly lower in $TPM1_{mut}$ compared with donors (1.98±0.37 and 3.33±0.11, respectively; Figure 2B), indicative for impairment of the cooperative response in activating the thin filament. Palm et al[64] demonstrated that tropomyosin overlap regions are required for proper formation of a ternary complex with the N-terminal tail of cTnT. Because the N-terminal region of cTnT is needed to maintain the thin filament in the B-state,[53,54] it is likely that mutations in the overlap region of α–tropomyosin structurally impair formation of the B-state and thereby impair length-dependent activation of myofilaments.

Previous studies indicated that myosin is not involved in the formation of the first 2 equilibrium states, that is, B- and C-states.[49–51] However, myosin is crucial for formation of the M-state (myosin-induced), because strong binding of tension-generating cross-bridges are required for thin-filament activation and force production.[49–51] Because 5 of the 6 samples used in our study have mutations in the myosin S1 domain, responsible for actin-

binding,[65] it is likely that the deleterious effects of *MYH7* mutations occur via perturbation of the M-state. A recent study by Farman et al[66] highlights the essential role of the orientation of the myosin heads that precede thin-filament activation for proper lattice spacing and length-dependent activation. Thus, altered myosin head orientation, as a result of mutations, may impair formation of the M-state and affect length-dependent activation.

The possible involvement of cMyBP-C in the modulation of thin-filament activity has been only recently addressed. Electron microscopy and 3-dimensional reconstruction of thin filaments with cMyBP-C suggest that the N-terminal extension of cMyBP-C could modulate the movement of tropomyosin on F-actin and interfere with actomyosin interactions, possibly involved in the regulation of thin-filament activation.[67] The *MYBPC3* missense mutations in our study are located along the N-terminal extension of cMyBP-C and may alter the tropomyosin–actin interaction and thereby impair length-dependent activation.

Study Limitations and Clinical Implications

Although we were able to study a large collection of human HCM samples with different mutations in thick- and thin-filament proteins, care must be taken to extrapolate our findings to all patients with HCM. Our HCM population consisted of patients with left ventricular outflow tract obstruction, and the patient with the homozygous *TNNT2* mutation had end-stage heart failure. Our data do highlight that mutation-induced changes in myofilament Ca^{2+}-sensitivity and length-dependent sarcomere activation are diverse and depend on the affected gene, and most likely location and type of the mutation in the affected protein. We provide evidence that mutant protein may impair sarcomere function at ≈38% expression (Figure 5C), which emphasizes the importance of studying the mutant protein level at which cardiac performance is impaired. Future studies in transgenic mice models and human myectomy samples are warranted to extend our findings to a broader set of sarcomere mutations and assess the toxic dose of mutant proteins.

Although sarcomere mutation-negative patients are commonly used as control group, we cannot completely rule out the presence of rare mutations. However, as our cardiomyocyte analyses revealed no functional impairments (PKA normalized length-dependent activation in HCM_{smn}), the likelihood of the presence of a rare mutation is low.

Our study revealed perturbed sarcomere length–dependent activation as a common mechanism underlying cardiac dysfunction in HCM. Pathological hypertrophy presumably reflects the compensatory response of the heart to counteract impaired sarcomere defects, such as the blunted length–dependent myofilament activation. The relatively low-force generating capacity of cardiomyocytes and the inability to increase force on an increase in sarcomere length may in part underlie cardiac dysfunction and initiate compensatory hypertrophy. Pak et al[68] showed a blunted end-systolic pressure volume relation in patients with HCM, suggesting that the hearts were unable to properly recruit preload to augment contractility. The latter observation may be explained by cardiac remodeling. However, likewise, the mutation-induced blunted length–dependent sarcomere activation may limit preload-mediated contractile reserve of the heart in patients with HCM. Our study in combination with others[6–10] supports the hypothesis that defective sarcomere function as a result of gene mutations is central to early stages of HCM disease and precedes development of hypertrophy. Moreover, the blunted increase in myofilament Ca^{2+}-sensitivity on an increase in length may alter Ca^{2+}-buffering in the cardiomyocytes and provide a substrate for arrhythmias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

B-state	blocked state
cMyBP-C	cardiac myosin–binding protein-C
C-state	Ca^{2+}-induced state
cTnI	cardiac troponin I
cTnT	cardiac troponin T
F_{max}	maximal developed force
HCM	hypertrophic cardiomyopathy
HCM_{smn}	sarcomere mutation-negative HCM
MyHC	myosin heavy chain
M-state	myosin-induced state
PKA	protein kinase A

Novelty and Significance

What Is Known?

- Hypertrophic cardiomyopathy (HCM) is commonly caused by mutations in genes encoding sarcomeric e proteins.
- Previous studies demonstrated early signs of myocardial dysfunction, in the absence of clinically detectable cardiac hypertrophy.
- In vitro and transgenic animal models expressing mutant thick- and thin-filament proteins in the heart implicate elevated myofilament Ca^{2+}-sensitivity as a major mechanism for the pathogenesis of HCM.

What New Information Does This Article Contribute?

- Cardiac myocytes isolated from the interventricular septal tissues of patients with hypertrophic obstructive cardiomyopathy show diverse and mutation-related changes in myofilament Ca2+-sensitivity, which are dependent on the affected gene.
- This increase in myofilament Ca^{2+}-sensitivity could be in part attributable to hypophosphorylation of protein kinase A-targets.
- Myofilament length–dependent activation is impaired in cardiac myocytes isolated from patients with HCM harboring missense mutations, and represents a mechanism underlying the development of HCM.

Data on myofilament length–dependent activation in transgenic animal models harboring HCM mutations are conflicting, and studies in human HCM are scarce. High-myofilament Ca^{2+}-sensitivity partly reflects hypophosphorylation of protein kinase A-targets, indicative for secondary disease–related alterations. Reduced length–dependent sarcomere activation is a common feature of human HCM caused by mutations in genes encoding thick- and thin-filament proteins. Pretreatment with protein kinase A did not totally correct impaired length–dependent sarcomere activation in HCM samples carrying missense mutations. Mutation-induced impaired length–dependent activation may limit the preload-mediated contractile reserve of the heart. Our findings, in combination with clinical studies, support the notion that sarcomere mechanical dysfunction is central to the pathogenesis of HCM and precedes the development of cardiac hypertrophy.

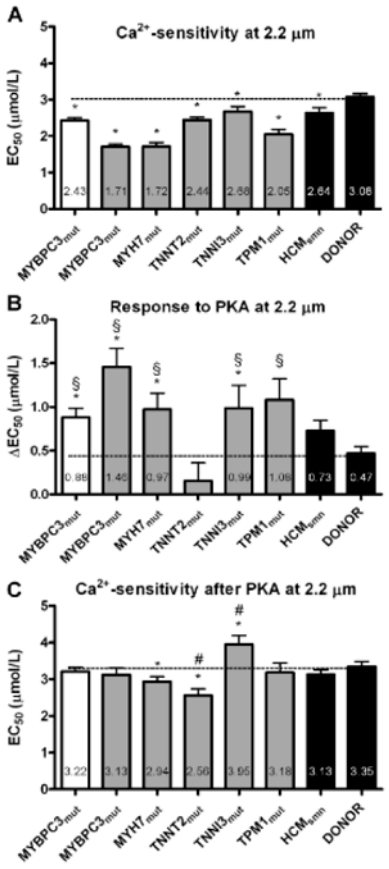

Figure 1. Myofilament Ca^{2+}-sensitivity at a sarcomere length of 2.2 μm

A, Myofilament Ca^{2+}-sensitivity (EC_{50}) was significantly higher in all hypertrophic cardiomyopathy (HCM) groups compared with donors. **B**, The protein kinase A (PKA)-induced reduction (ΔEC_{50}) in myofilament Ca^{2+}-sensitivity was larger in HCM groups compared with donors, except in the *TNNT2*$_{mut}$ sample, in which PKA had no significant effect. **C**, Myofilament Ca^{2+}-sensitivity was similar in *MYBPC3*$_{mut}$, *TPM1*$_{mut}$, sarcomere mutation-negative HCM (HCM_{smn}), and donor after treatment with PKA, whereas it was higher than donor in *MYH7*$_{mut}$ and *TNNT2*$_{mut}$. PKA-treated *TNNI3*$_{mut}$ cells showed a lower myofilament Ca^{2+}-sensitivity compared with HCM_{smn} and donor. Open bar graph represents *MYBPC3* truncating mutation; closed gray bar graphs represent missense mutations. *P<0.05 vs donor; §P<0.05 vs *TNNT2*$_{mut}$; #P<0.05 vs HCM_{smn}.

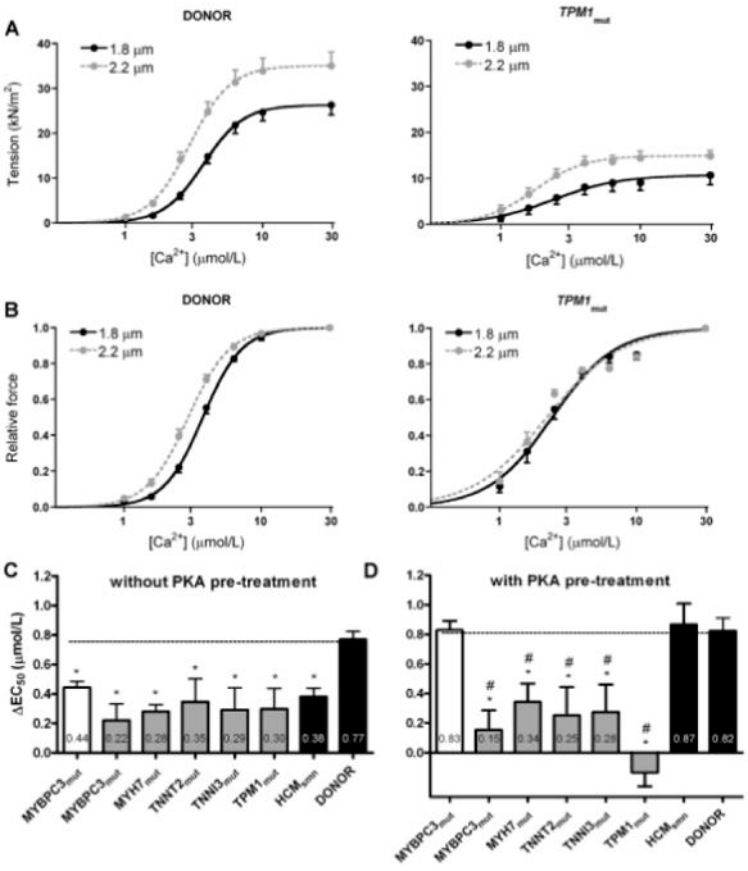

Figure 2. Myofilament length–dependent activation

A, Tension development as a function of [Ca^{2+}] at short (1.8 μm) and long (2.2 μm) sarcomere lengths for donor (**left**) and *TPM1*$_{mut}$(**right**) heart samples. **B**, Normalized force–Ca^{2+} relationships for donor (**left**) and *TPM1*$_{mut}$(**right**) heart samples. **C**, The length-dependent increase in myofilament Ca^{2+}-sensitivity was lower in all sarcomere mutation-positive hypertrophic cardiomyopathy (HCM$_{mut}$) compared with donors before protein kinase A (PKA) treatment. **D**, PKA pretreatment restored length-dependent activation to donor in HCM with truncating *MYBPC3*$_{mut}$ and sarcomere mutation-negative HCM (HCM$_{smn}$), but not in HCM$_{mut}$ with missense mutations. Open bar graph represents *MYBPC3* truncating mutations; closed gray bar graphs represent missense mutations.*P<0.05 vs donor; #P<0.05 vs HCM$_{smn}$.

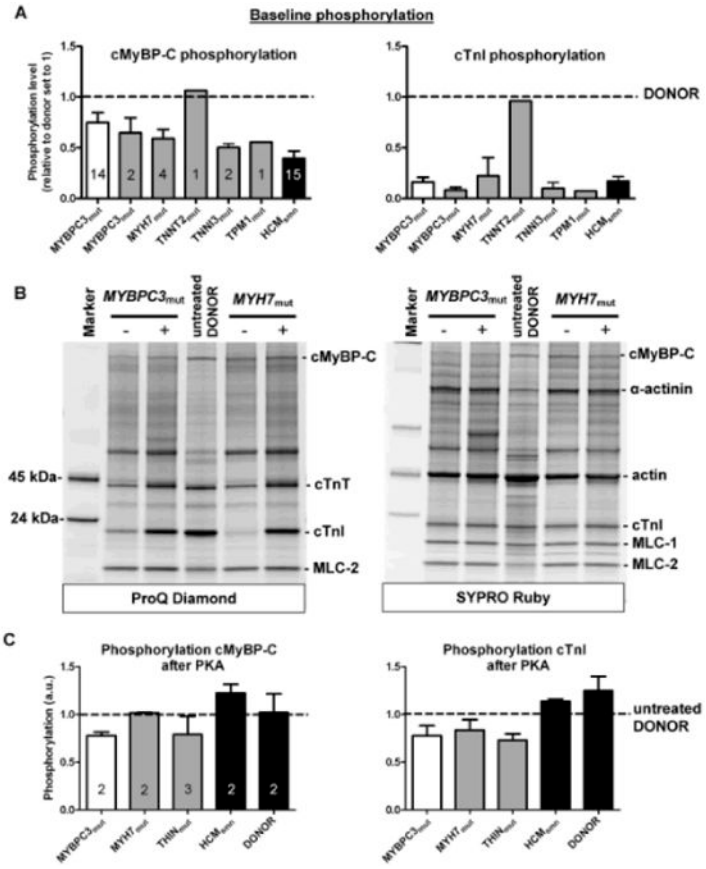

Figure 3. Phosphorylation of protein kinase A (PKA)-target proteins before and after PKA treatment

A, Protein phosphorylation values were corrected by the corresponding SYPRO-stained protein bands and normalized to the values found in donors, which were set to 1 (dotted line). Phosphorylation of cardiac myosin–binding protein-C (cMyBP-C; **left**) and cardiac troponin I (cTnI; **right**) was lower in all hypertrophic cardiomyopathy (HCM) samples compared with donors, except for the *TNNT2*$_{mut}$ sample, which showed relatively high phosphorylation. Open bar graph represents truncating *MYBPC3* mutations; closed gray bar graphs represent missense mutations. **B**, Cardiac samples before (−) and after (+) PKA treatment separated by 1-dimensional gel electrophoresis and stained with ProQ-Diamond (phosphorylation) and SYPRO Ruby (total protein stain). **C**, Thin-filament mutations were clustered in a single group (THIN$_{mut}$). ProQ-stained protein phosphorylation values of PKA-treated samples were corrected by the corresponding SYPRO-stained protein bands and normalized to values in nontreated donor samples, which were included on the gel and set to 1 (dotted line). PKA increased phosphorylation of both target proteins in all HCM groups. Numbers of samples included in the analyses are indicated in the bar graphs.

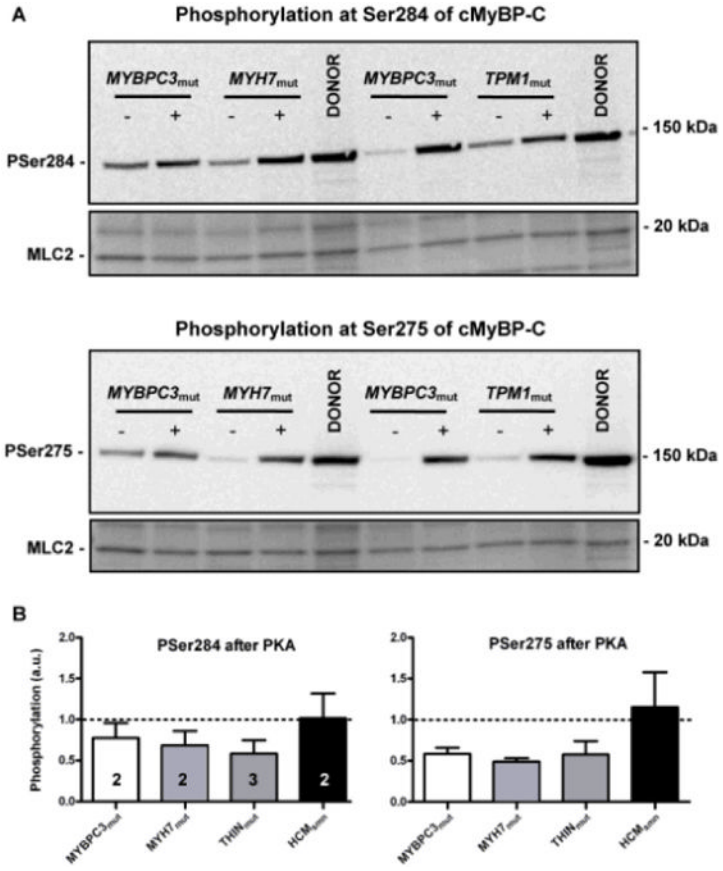

Figure 4. Site-specific phosphorylation of protein kinase A (PKA)-target sites of cardiac myosin–binding protein-C (cMyBP-C)

A, Western blot analysis of cMyBP-C phosphorylation with specific antibodies for PKA sites Ser284 (**top**) and Ser275 (**bottom**) before (−) and after (+) PKA treatment (phosphorylation values were corrected for minor differences in protein loading by Ponceau-stained MLC2, myosin light chain 2). **B**, Protein phosphorylation values were normalized to the values found in untreated donor samples, which were included on the blot and set to 1 (dotted line). Thin-filament mutations were clustered in a single group ($THIN_{mut}$). Numbers of samples included in the analyses are indicated in the bar graphs. All samples showed an increased phosphorylation at both sites on PKA treatment.

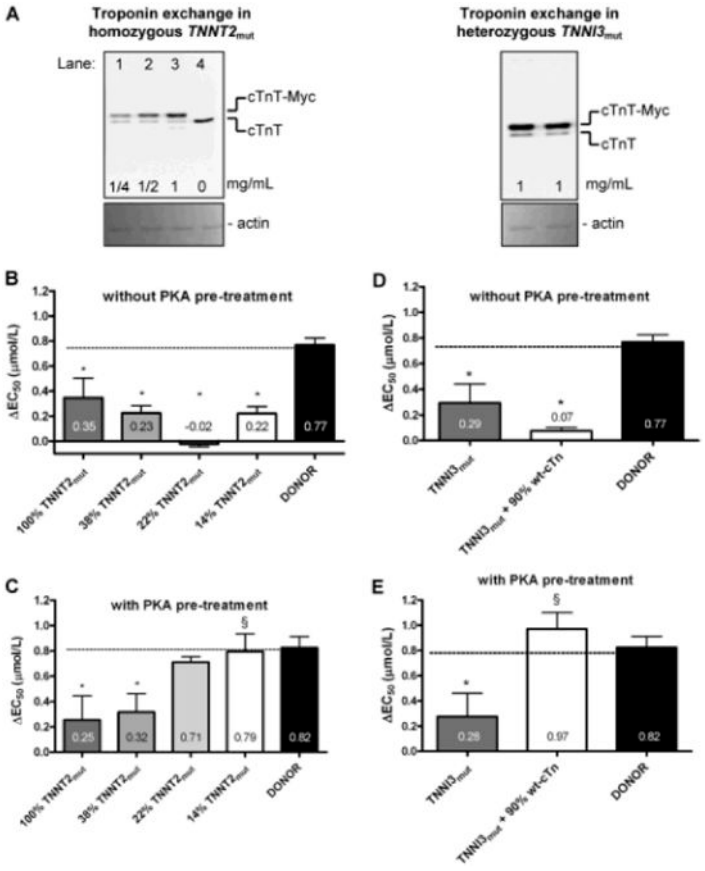

Figure 5. Replacement of endogenous mutant cardiac troponin by recombinant human wild-type troponin complex

A, Quantification of troponin exchange in cardiomyocytes from a *TNNT2*$_{mut}$ and a *TNNI3*$_{mut}$ heart. Immunoblots stained with an antibody against cardiac troponin T (cTnT) that recognizes both endogenous cTnT (lower band) and recombinant myc-tag labeled cTnT (cTnT-myc; upper band). **Left**, An example is shown of a suspension of cardiomyocytes from a *TNNT2*$_{mut}$ heart exchanged with increasing concentrations of wild-type human recombinant troponin complex. Exchange with 0.25 mg/mL (lane 1), 0.5 mg/mL (lane 2), and 1 mg/mL (lane 3) troponin complex. *TNNT2*$_{mut}$ heart without added recombinant troponin complex (lane 4). **Right,** A suspension of cardiomyocytes from a *TNNI3*$_{mut}$ heart exchanged with 1 mg/mL wild-type human recombinant troponin complex. Similar amounts were loaded on the blots (shown by Ponceau-stained actin) to allow cTnT analysis within the linear detection range. **B**, The length-dependent activation was significantly lower compared with donor in all *TNNT2*$_{mut}$ cells harboring varying amounts of mutant cTnT (100% without exchange and 38%, 22%, and 14% in cells exchanged with increasing concentrations of unphosphorylated recombinant wild-type troponin). **C**, Measurements were also performed in exchanged cardiomyocytes, which were subsequently treated with protein kinase A (PKA) to normalize cTnI phosphorylation. Pretreatment with exogenous PKA was not able to recover the blunted length–dependent activation in *TNNT2*$_{mut}$ cells with 38% of mutant cTnT, but did recover the reduced length–dependence of *TNNT2*$_{mut}$ exchanged with 78% and 86% wild-type troponin. **D**, The length-dependent activation was significantly lower in *TNNI3*$_{mut}$ cells exchanged with ≈90% unphosphorylated recombinant wild-type troponin (wt-cTn) compared with donor cells. **E**, Pretreatment with exogenous PKA restored the blunted length–dependent activation in *TNNI3*$_{mut}$ cells exchanged with wt-cTn complex to donor values. *P<0.05 vs donor; §P<0.05 vs *TNNT2*$_{mut}$ or *TNNI3*$_{mut}$.

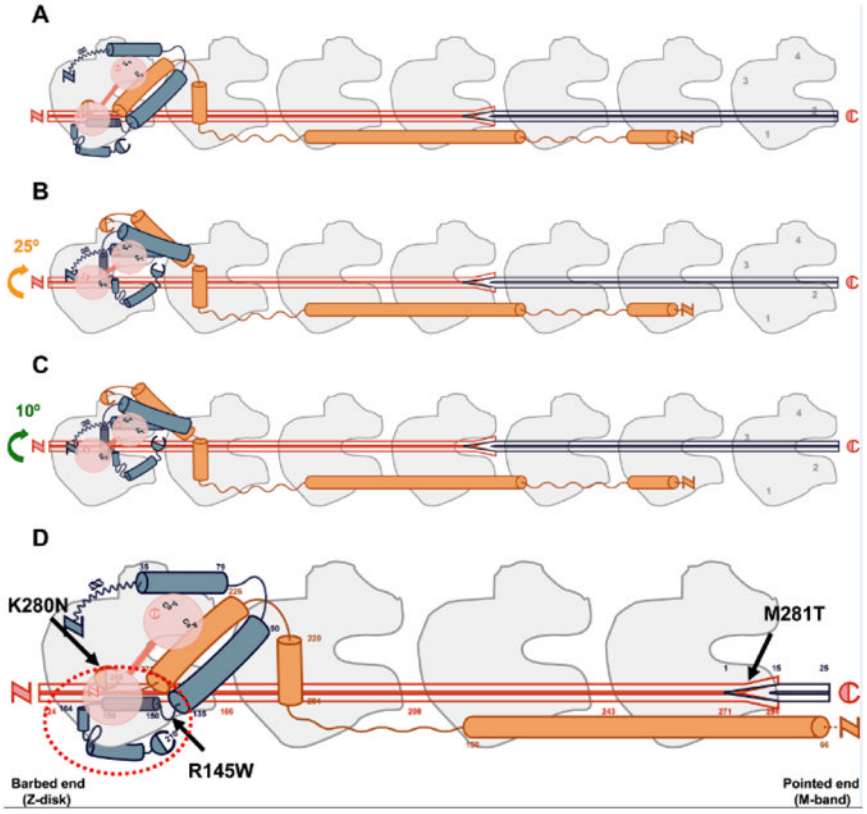

Figure 6. Schematic drawing of the thin-filament functional unit

Seven actin monomers (gray) spanned by 1 tropomyosin dimmer (red) and 1 troponin complex: cardiac troponin C (cTnC; pink), cardiac troponin I (cTnI; blue), and cardiac troponin T (cTnT; orange). Capitals N and C depict N- and C-terminal protein ends, respectively. Dark-blue tropomyosin depicts near-neighbor tropomyosin dimmer interaction.[69,70] The orientation of thin-filament proteins is as follows: the N-terminal region of cTnT points toward the pointed end (M-band), whereas the core domain of the troponin complex is oriented to the barbed end (Z-disk).[71] Interacting sites and structural location of actin–tropomyosin–troponin proteins are matched in accordance with available literature.[25,50,51,55,70,72] Cardiac TnI residues 1 to 34 are arbitrarily positioned. Our drawing follows the proposed mechanism for Ca^{2+}-regulation of contraction proposed by Murakami et al.[55] **A**, B-state (blocked); when ATP is present and cytoplasmic [Ca^{2+}] is low such that Ca^{2+} is not bound to cTnC, tropomyosin sterically blocks the myosin-binding sites on F-actin. **B**, C-state (Ca^{2+}-induced); cytoplasmic [Ca^{2+}] rises such that Ca^{2+} binds to cTnC inducing conformational changes of the troponin complex, resulting in an ≈25° movement of tropomyosin on the thin filament, thereby exposing most of the myosin-binding sites on F-actin. Note the movement of tropomyosin away from subdomains 1 and 2 of F-actin. In the C-state, the myofilament is not yet activated because nontension-generating cross-bridges bind weakly to F-actin. **C**, M-state (myosin-induced); involves the strong binding of tension-generating cross-bridges that induce an additional ≈10° movement of tropomyosin on F-actin, resulting in myofilament activation and contraction. Note the transition of tropomyosin into subdomains 3 and 4 of F-actin. **D**, Solid arrows depict the location of mutation sites on thin-filament proteins present in our human HCM samples. Cardiac TnT residues 1 to 65 are shortened to fit the enlarged scale.

Table 1

Mutations and Clinical Characteristics of Patients

	Mutation	Type	Age	Sex	LVOT	ST
***MYBPC3*$_{mut}$**		**Truncating mutations**				
1	c.927-2A>G	Splice site	37	M	61	19
2	c.927-2A>G	Splice site	48	M	82	18
3	c.927-2A>G	Splice site	22	M	71	30
4	c.1458-1G>C	Splice site	41	F	92	22
5	c.2373duplicationG	Insertion	32	F	100	30
6	c.2373duplicationG	Insertion	39	F	60	20
7	c.2373duplicationG	Insertion	45	F	94	20
8	c.2373duplicationG	Insertion	62	M	64	23
9	c.2373duplicationG	Insertion	44	F	100	17
10	c.2373duplicationG	Insertion	69	M	74	19
11	c.2373duplicationG	Insertion	57	F	74	24
12	c.2373duplicationG	Insertion	32	M	88	23
13	c.2373duplicationG	Insertion	60	M	77	23
14	c.2864.2865delCT	Deletion	42	M	116	23
15	c.2864.2865delCT	Deletion	45	M	100	20
16	c.2864.2865delCT	Deletion	62	F	67	15
17	c.3407.3409del	Deletion	55	M	UK	UK
MYBPC3$_{mut}$		Missense mutations				
18*	p.E258K	Missense	35	M	4	27
19	p.E258K	Missense	45	M	72	24
20	p.G531R	Missense	51	M	110	22
21	p.R597Q	Missense	47	F	85	20
MYH7$_{mut}$						
1	p.R403Q	Missense	25	M	85	34
2	p.V606M	Missense	46	F	79	17

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	Mutation	Type	Age	Sex	LVOT	ST
MYBPC3$_{mut}$		**Truncating mutations**				
3	p.S782R	Missense	30	F	128	29
4	p.R787H	Missense	61	M	UK	UK
5	p.T1377M	Missense	58	F	100	20
6	p.T1377M	Missense	43	M	UK	UK
TNNT2$_{mut}$						
1	p.K280N	Missense	26	M	UK	UK
TNNI3$_{mut}$						
1	p.R145W	Missense	46	M	100	23
2	p.R145W	Missense	66	M	100	16
TPM1$_{mut}$						
1	p.M281T	Missense	65	M	100	16
HCM$_{smn}$	Sarcomere mutation	Negative				
1			46	F	105	24
2			57	M	117	25
3			75	F	137	20
4			72	F	88	24
5			65	F	85	19
6			49	M	61	20
7			46	M	81	19

c indicates codon region; del, deletion; LVOT, left ventricular outflow tract pressure gradient in mm Hg; p, protein amino residue; ST, septal thickness in mm; and UK, unknown.

*Patient was operated because of extreme hypertrophy and small LV cavity.

Table 2

Effects of Sarcomere Length Increase on F_{max} Before and After PKA

	No PKA Pretreatment				PKA Pretreatment			
	F_{max}, kN/m^2				F_{max}, kN/m^2			
Sample	**1.8 μm**	**2.2 μm**	**ΔF_{max}**	**N/n**	**1.8 μm**	**2.2 μm**	**ΔF_{max}**	**N/n**
Truncating								
MYBPC3$_{mut}$	19.6±1.5	29.8±2.4 **	10.2±1.2	N=14/n=47	27.6±1.5	37.0±2.0 **	9.4±1.3	N=4/n=19
Missense								
MYBPC3$_{mut}$	13.0±1.3	15.5±1.7 **	2.5±0.8 *	N=4/n=12	11.4±1.0	13.9±1.5 **	2.5±1.0 *	N=4/n=12
MYH7$_{mut}$	15.1±1.6	19.1±1.8 **	3.9±1.0 *	N=6/n=32	18.8±2.6	22.6±3.1 **	3.8±1.1 *	N=4/n=15
TNNT2$_{mut}$	21.5±3.2	24.4±6.0	3.0±4.4	N=1/n=6	13.7±2.1	15.2±2.4	1.5±1.3 *	N=1/n=4
TNNI3$_{mut}$	9.8±2.5	10.1±2.8	0.3±1.1 *	N=2/n=8	8.4±1.4	9.5±1.9	0.83±0.8 *	N=2/n=10
TPMI$_{mut}$	10.7±2.1	17.0±3.0 **	6.3±1.5	N=1/n=6	9.1±2.3	9.6±1.7	0.58±0.7 *#	N=1/n=4
HCM$_{smn}$	18.2±1.4	28.3±2.6 **	10.1±1.7	N=7/n=31	26.8±2.3	38.7±2.7 **	12.0±1.4	N=3/n=12
DONOR	26.3±2.2	35.1±3.1 **	8.8±2.1	N=9/n=32	26.2±1.4	36.2±1.9 **	10.0±1.6	N=3/n=12

ΔF_{max} indicates difference in Fmax between sarcomere lengths of 1.8 and 2.2 μm; HCM, hypertrophic cardiomyopathy; N, no. of samples; n, no. of cardiomyocytes; and PKA, protein kinase A.

P<0.05 was considered significant;

* vs donor;

** 1.8 μm vs 2.2 μm;

ΔF_{max} (no PKA pretreatment) vs ΔF_{max} (PKA pretreatment).

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