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## Cardiac Myosin-Binding Protein C Mutations and Hypertrophic Cardiomyopathy Haploinsufficiency, Deranged Phosphorylation, and Cardiomyocyte Dysfunction

Sabine J. van Dijk, MSc; Dennis Dooijes, PhD; Cris dos Remedios, PhD; Michelle Michels, MD, PhD; Jos M.J. Lamers, PhD; Saul Winegrad, PhD; Saskia Schlossarek, PhD; Lucie Carrier, PhD; Folkert J. ten Cate, MD, PhD; Ger J.M. Stienen, PhD; Jolanda van der Velden, PhD

**Background**— Mutations in the *MYBPC3* gene, encoding cardiac myosin-binding protein C (cMyBP-C), are a frequent cause of familial hypertrophic cardiomyopathy. In the present study, we investigated whether protein composition and function of the sarcomere are altered in a homogeneous familial hypertrophic cardiomyopathy patient group with frameshift mutations in *MYBPC3* (*MYBPC3<sub>mut</sub>*).

**Methods and Results**— Comparisons were made between cardiac samples from *MYBPC3* mutant carriers (c.2373dupG, n=7; c.2864\_2865delCT, n=4) and nonfailing donors (n=13). Western blots with the use of antibodies directed against cMyBP-C did not reveal truncated cMyBP-C in *MYBPC3<sub>mut</sub>*. Protein expression of cMyBP-C was significantly reduced in *MYBPC3<sub>mut</sub>* by  $33 \pm 5\%$ . Cardiac MyBP-C phosphorylation in *MYBPC3<sub>mut</sub>* samples was similar to the values in donor samples, whereas the phosphorylation status of cardiac troponin I was reduced by  $84 \pm 5\%$ , indicating divergent phosphorylation of the 2 main contractile target proteins of the  $\beta$ -adrenergic pathway. Force measurements in mechanically isolated Triton-permeabilized cardiomyocytes demonstrated a decrease in maximal force per cross-sectional area of the myocytes in *MYBPC3<sub>mut</sub>* ( $20.2 \pm 2.7$  kN/m<sup>2</sup>) compared with donor ( $34.5 \pm 1.1$  kN/m<sup>2</sup>). Moreover, Ca<sup>2+</sup> sensitivity was higher in *MYBPC3<sub>mut</sub>* ( $pCa_{50} = 5.62 \pm 0.04$ ) than in donor ( $pCa_{50} = 5.54 \pm 0.02$ ), consistent with reduced cardiac troponin I phosphorylation. Treatment with exogenous protein kinase A, to mimic  $\beta$ -adrenergic stimulation, did not correct reduced maximal force but abolished the initial difference in Ca<sup>2+</sup> sensitivity between *MYBPC3<sub>mut</sub>* ( $pCa_{50} = 5.46 \pm 0.03$ ) and donor ( $pCa_{50} = 5.48 \pm 0.02$ ).

**Conclusions**— Frameshift *MYBPC3* mutations cause haploinsufficiency, deranged phosphorylation of contractile proteins, and reduced maximal force-generating capacity of cardiomyocytes. The enhanced Ca<sup>2+</sup> sensitivity in *MYBPC3<sub>mut</sub>* is due to hypophosphorylation of troponin I secondary to mutation-induced dysfunction. (*Circulation*. 2009;119:1473-1483.)

**Key Words:** cardiomyopathy ■ myocardial contraction ■ myocytes ■ mutation ■ proteins

Familial hypertrophic cardiomyopathy (FHCM) is the most frequent inheritable cardiac disease with a prevalence of 0.2%.<sup>1,2</sup> FHCM-causing mutations are identified in 13 genes encoding sarcomeric proteins.<sup>3</sup> Mutations in the *MYBPC3* gene encoding cardiac myosin-binding protein C (cMyBP-C) represent >40% of all FHCM cases.<sup>4</sup> Most *MYBPC3* mutations are predicted to produce C-terminally truncated proteins, lacking titin and/or major myosin-binding sites.<sup>4-6</sup> Studies in FHCM patients carrying a *MYBPC3* mutation failed to reveal truncated cMyBP-C protein,<sup>7-9</sup> suggesting that *MYBPC3* mutations may lead to haploinsufficiency.

### Clinical Perspective p 1483

Evidence suggests that the first step in the pathogenesis of FHCM involves mutation-induced sarcomeric dysfunction.<sup>2,3</sup> Myocardial dysfunction in this group of patients has been attributed at least partly to myocyte hypertrophy, disarray, and interstitial fibrosis.<sup>10</sup> However, direct evidence for both reduced cMyBP-C expression and sarcomeric dysfunction in *MYBPC3* mutant carriers is missing.

Approximately 35% of the FHCM patients in the Netherlands have founder mutations in the *MYBPC3* gene

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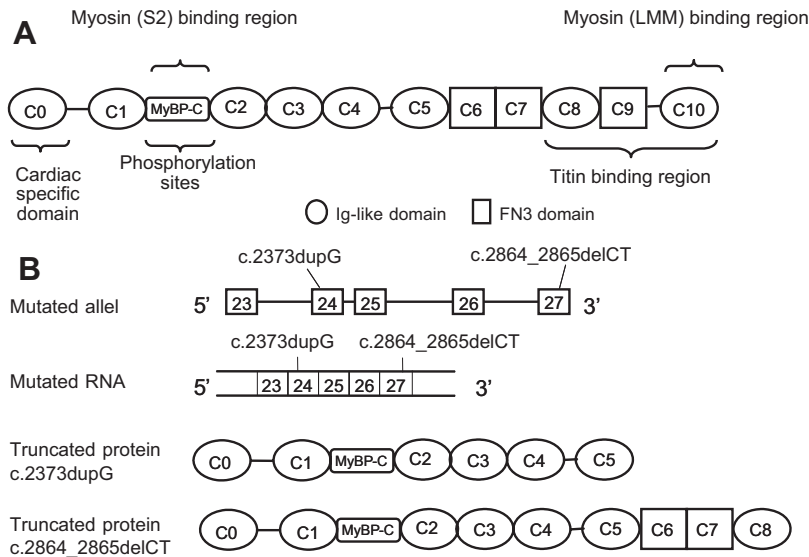
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**Figure 1.** A, Schematic representation of cMyBP-C structure and MYBPC3 mutations. cMyBP-C consists of 8 immunoglobulin (Ig)-like and 3 fibronectin (FN3) domains,<sup>15,16</sup> with binding sites for myosin and titin. The N-terminal C0 is specific for cMyBP-C, as is the MyBP-C-like motif between C1 and C2, which contains 3 phosphorylation sites. B, Localization of the mutations in cMyBPC. The c.2373dupG mutation creates a splice donor site, resulting in reading frame shift.<sup>8</sup> Consequently, the C-terminus of the protein is altered and terminated after the C5 region, leading to a protein with a predicted weight of ≈93 kDa. The CT deletion in exon 27 (c.2864\_2865delCT)<sup>9</sup> creates a premature termination codon in exon 29, which is located 42 bp upstream of the 3' end. The expected truncated protein of 116 kDa contains 1049 amino acids including 89 new ones and a termination of the translation at the end of the C8 domain.

(c.2373dupG and c.2864\_2865delCT)<sup>11</sup> that both are predicted to encode C-terminally truncated proteins (Figure 1).<sup>8,9</sup> This allowed us to investigate whether truncating mutations in the MYBPC3 gene alter sarcomeric protein composition and function in a rather homogeneous patient group. Cardiac MyBP-C mRNA and protein expression and phosphorylation status of sarcomeric proteins were analyzed in concert with cardiomyocyte function in MYBPC3 mutation carriers and compared with nonfailing donor samples.

**Methods**

**Cardiac Biopsies**

Cardiac tissue was obtained from the left ventricular (LV) septum of 11 patients with a founder mutation<sup>11,12</sup> in the MYBPC3 gene encoding cMyBP-C (MYBPC3<sub>mut</sub>: c.2373dupG, n=7; 32 to 69 years of age, mean 50±5 years; 2/5 male/female; c.2864\_2865delCT, n=4; 32 to 62 years of age, mean 44±6 years; 2/2 male/female), who underwent alcohol ablation or myectomy to relieve LV outflow obstruction. Echocardiographic and clinical data of the patients are given in the Table. Hypertrophic obstructive cardiomyopathy was

evident from increased septal thickness (21±1 mm; normal value <13 mm)<sup>13</sup> and high LV transaortic pressure gradient (78±6 mm Hg; normal value <30 mm Hg).<sup>14</sup> LV ejection fraction was moderately depressed (44±2%). Both mutations encode for slightly different C-terminally truncated proteins with a theoretical mass of 93 and 116 kDa for c.2373dupG<sup>8</sup> and c.2864\_2865delCT,<sup>9</sup> respectively (Figure 1).<sup>15,16</sup>

Nonfailing cardiac tissue from the free LV wall was obtained from donor hearts (n=13; 13 to 65 years of age, mean 34±5 years; 10/3 male/female) when no suitable transplant recipient was found. The donors had no history of cardiac disease, a normal cardiac examination, normal ECG, and normal ventricular function on echocardiography within 24 hours of heart explantation. It should be noted that the donor group was slightly younger and included relatively more males than the FHCM group.

All samples were immediately frozen and stored in liquid nitrogen. The study protocol was approved by the local ethics committees, and written informed consent was obtained.

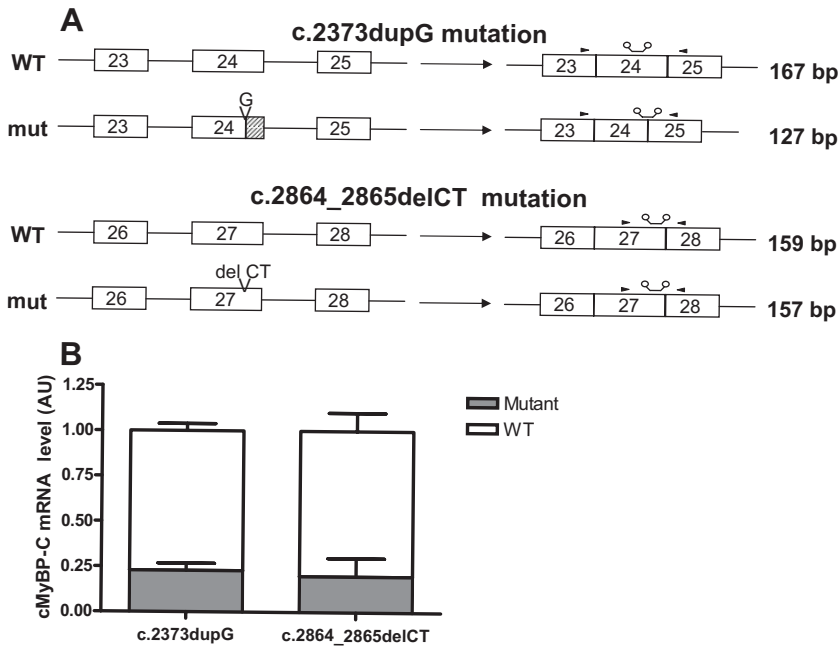
**Quantitative mRNA Analyses**

Total RNA was extracted from 5 to 40 mg of 4 nonfailing and 4 FHCM frozen cardiac tissues with the use of the SV Total RNA

**Table. Patient Characteristics**

Mutation	Age, y	Sex, M/F	LV Transaortic Pressure				LVEF, %	Medication
			Gradient, mm Hg	ST, mm	LVEDD, mm	LVESD, mm		
c.2373dupG	32	F	100	30	40	20	50	β-Blocker
c.2373dupG	39	F	60	20	38	21	45	CCB, diuretics
c.2373dupG	45	F	94	20	40	20	50	CCB
c.2373dupG	62	M	64	23	39	22	44	ATII, β-blocker, statins
c.2373dupG	44	F	60	17	42	24	43	CCB
c.2373dupG	69	M	74	19	44	30	32	β-Blocker, diuretics, statins
c.2373dupG	57	F	74	24	41	20	51	β-blocker
c.2864_2865delCT	39	M	116	23	48	26	46	CCB, vitamin D, calcium
c.2864_2865delCT	44	M	70	20	40	25	38	CCB, β-blocker
c.2864_2865delCT	62	F	67	15	39	24	38	β-Blocker, diuretics, statins, antiarrhythmic agent
c.2864_2865delCT	32	F	121	23	44	21	52	β-Blocker, CCB, diuretics

M/F indicates male/female; ST, septal thickness; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVEF, LV ejection fraction, calculated as (LVEDD—LVESD)/LVEDD×100%; CCB, calcium channel blocker; and ATII, angiotensin II receptor antagonist.



**Figure 2.** Detection and quantification of mRNA by real-time polymerase chain reaction. A, Scheme illustrating primers and probes used in real-time polymerase chain reaction with the Taqman system. mut indicates mutant. B, The amount of mutant mRNA was  $\approx 20\%$  of total cMyBP-C mRNAs in both patient groups.

Isolation kit (Promega, Madison Wis) according to the manufacturer's instructions. RNA concentration, purity, and quality were determined with the use of the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, Mass). Reverse transcription was performed with the use of oligo-dT primers with the Superscript III (Invitrogen, Carlsbad, Calif) from 50 to 100 ng RNA. Quantitative determination of wild-type (WT) and mutant cMyBP-C mRNAs was performed by real-time polymerase chain reaction with the TaqMan ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, Calif) and TaqMan probes and primers specified as follows (Figure 2A). For the c.2373dupG mutation, primers were designed in exons 23 (F5'-CCT CAC AGT CAA GGT CAT CG-3') and 25 (R5'-TCC ACC GGT AGC TCT TCT TC-3'). Specific TaqMan probes were designed to recognize either the WT mRNA in exon 24 (F5'-GAG CCG CCT GCC TAC GAT-3') or the mutant mRNA at the junction between the smaller exon 24 (-40 bp due to the new cryptic donor splice site) and exon 25 (F5'-GCA CAG TAC AGG CTA CAT CCT G-3'). For the c.2864\_2865delCT mutation, primers were designed in exons 27 (F5'-AGT GCG GGC ACA CAA TAT G-3') and 28 (R5'-GGG ATG AGA AGG TTC ACA GG-3'). The WT probe recognized a WT sequence in exon 27 (F5'-TGG AGC CCC TGT TAC CAC C-3'), and the mutant probe recognized a mutant sequence (deleted of CT) in exon 27 (F5'-CTG GAG CCC GTT ACC ACC A-3'). GAPDH was used as endogenous control to normalize the quantification of the target mRNAs for difference in the amount of cDNA added to each reaction. All analyses were performed in triplicate with the software ABI 7900HT SDS 2.2. The mRNA amount was estimated according to the comparative Ct method with the  $2^{-\Delta\Delta Ct}$  formula. The amount of both WT and mutant mRNA was reported as the mean of the WT obtained from the 4 nonfailing samples for each exon amplification.

### Protein Analysis

Cardiac samples (11 MYBPC3<sub>mut</sub>, 8 donor) were treated with trichloroacetic acid before protein analysis to preserve the endogenous phosphorylation status of the sarcomeric proteins.<sup>17</sup>

### Western Immunoblotting

Proteins were separated by 1-dimensional gel electrophoresis on a 15% polyacrylamide SDS gel and subsequently transferred to nitrocellulose paper by wet blotting. Polyclonal antibodies (diluted 1:1000) raised against recombinant C0C2, C5, and C8C9 produced

from human cDNA encoding cMyBP-C<sup>15</sup> (Figure 1) were used for detection of cMyBP-C (Dr S. Winegrad, University of Pennsylvania, Philadelphia). Primary antibody binding was visualized with a secondary goat anti-rabbit antibody (diluted 1:2000) and enhanced chemiluminescence (Amersham, GE Healthcare, Chalfont St. Giles, UK).

To detect truncated cMyBP-C, 2 antibodies were used, which are directed to the N-terminal part of cMyBP-C (C0C2) and the middle region of cMyBP-C (C5) (Figure 1). The sensitivity of the 2 antibodies to detect low amounts of truncated cMyBP-C under the experimental conditions used was assessed with a dilution series of a nonfailing donor sample (0.04 to 5  $\mu\text{g}$ ). The dilution at which the cMyBP-C band was still discernible was defined as the lower detection limit and amounted to 1.6% (0.08  $\mu\text{g}$ ) for the C0C2 antibody and 3.2% (0.16  $\mu\text{g}$ ) for the C5 antibody (data not shown).

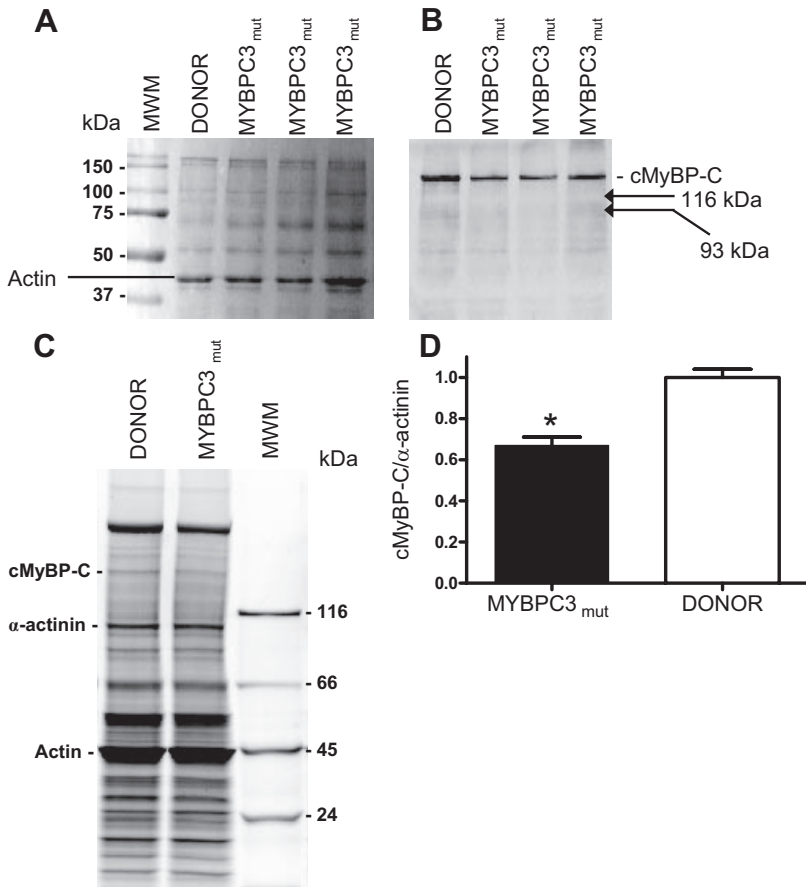
In addition, phosphorylation of cMyBP-C at Ser282 (P-cMyBP-C; dilution 1:1000) and bisphosphorylation of cardiac troponin I (cTnI) at Ser23/24 (ie, protein kinase A [PKA] sites, rabbit polyclonal antibody; dilution 1:500; Cell Signaling, Danvers, Mass) were analyzed and normalized to cMyBP-C (C0C2 antibody) and cTnI (8I-7, mouse monoclonal antibody; dilution 1:6000, Spectral Diagnostics), respectively, to correct for differences in protein loading.

### SYPRO Ruby and ProQ Diamond Staining of Gradient Gels

Proteins were separated on 4% to 15% precast Tris-HCl gels (Bio-Rad Laboratories, Hercules, Calif) and stained with SYPRO Ruby and ProQ Diamond to determine sarcomeric protein levels and phosphorylation, respectively, as described previously.<sup>17</sup> The phosphorylation status of sarcomeric proteins was expressed relative to  $\alpha$ -actinin, except when noted otherwise. Protein values of MYBPC3<sub>mut</sub> are given as fraction (or percentage) of the value found for donor samples, which was set to 1 (or 100%).

### Isometric Force Measurements

Cardiomyocytes were mechanically isolated from small tissue samples as described previously.<sup>18</sup> Triton-permeabilized cardiomyocytes were glued between a force transducer and a piezoelectric motor and stretched to a sarcomere length of  $\approx 2.2 \mu\text{m}$ . Force measurements were performed at various calcium concentrations (pCa,  $-\log_{10}[\text{Ca}^{2+}]$ , values ranging from 4.5 to 6.0) as described previously.<sup>18,19</sup> Force measurements were performed in single cardiomyo-



**Figure 3.** cMyBP-C protein levels. A, Western immunoblots were stained with Ponceau to visualize proteins. MWM indicates molecular weight marker. B, Western immunoblot using a C0C2 antibody illustrates reduced expression of full-length cMyBP-C ( $\approx 140$  kDa) in cardiac biopsies from MYBPC3<sub>mut</sub> patients compared with donor. No traces were found of truncated cMyBP-C (theoretical mass: 93 kDa [c.2373dupG]; 116 kDa [c.2864\_2865delCT]). C, Representative cardiac samples on a gradient gel stained with SYPRO Ruby (donor vs MYBPC3<sub>mut</sub>). D, Relative expression of cMyBP-C is significantly lower in MYBPC3<sub>mut</sub> ( $n=11$ ) than in donor ( $n=8$ ).  $*P<0.05$ .

cytes isolated from 10 MYBPC3<sub>mut</sub> (34 cardiomyocytes) and 13 donor samples (47 cardiomyocytes). On average, 2 to 5 cardiomyocytes were studied for each patient/donor. One MYBPC3<sub>mut</sub> biopsy was too small to isolate cardiomyocytes and was used for protein analysis only. Cross-sectional area ( $\text{width} \times \text{depth} \times \pi/4$ ) of the cardiomyocytes determined at a sarcomere length of  $2.2 \mu\text{m}$  was significantly higher in MYBPC3<sub>mut</sub> ( $508 \pm 67 \mu\text{m}^2$ ) than in donor ( $374 \pm 26 \mu\text{m}^2$ ). Length between the attachments did not significantly differ and amounted to  $62 \pm 5 \mu\text{m}$  in MYBPC3<sub>mut</sub> and  $72 \pm 4 \mu\text{m}$  in donor. Passive tension ( $F_{\text{passive}}$ ) was determined by shortening the cell in relaxation solution (pCa 9.0) by 30% and immediately restretching it to its original length. Maximal calcium activated tension ( $F_{\text{active}}$  [ie, maximal force/cross-sectional area]) was calculated by subtracting  $F_{\text{passive}}$  from the total force ( $F_{\text{total}}$ ) at saturating  $[\text{Ca}^{2+}]$  (pCa 4.5).  $\text{Ca}^{2+}$  sensitivity is denoted as pCa<sub>50</sub> (ie, pCa value at which 50% of  $F_{\text{active}}$  is reached). Force measurements were repeated after incubation of cells for 40 minutes at  $20^\circ\text{C}$  in relaxing solution containing the catalytic subunit of PKA (100 U/mL, Sigma) or with the catalytic domain of protein kinase C (PKC) (0.25 U/mL, Sigma).

### Data Analysis

Data are presented as mean  $\pm$  SEM. Cardiomyocyte force values were averaged per sample, and mean values for MYBPC3<sub>mut</sub> and donor samples were compared with unpaired Student *t* tests. Effects of PKA/PKC were tested with 2-way ANOVA.  $P<0.05$  was considered significant. Asterisks denote significant difference between MYBPC3<sub>mut</sub> and donor, and daggers denote significant difference before versus after PKA/PKC treatment.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

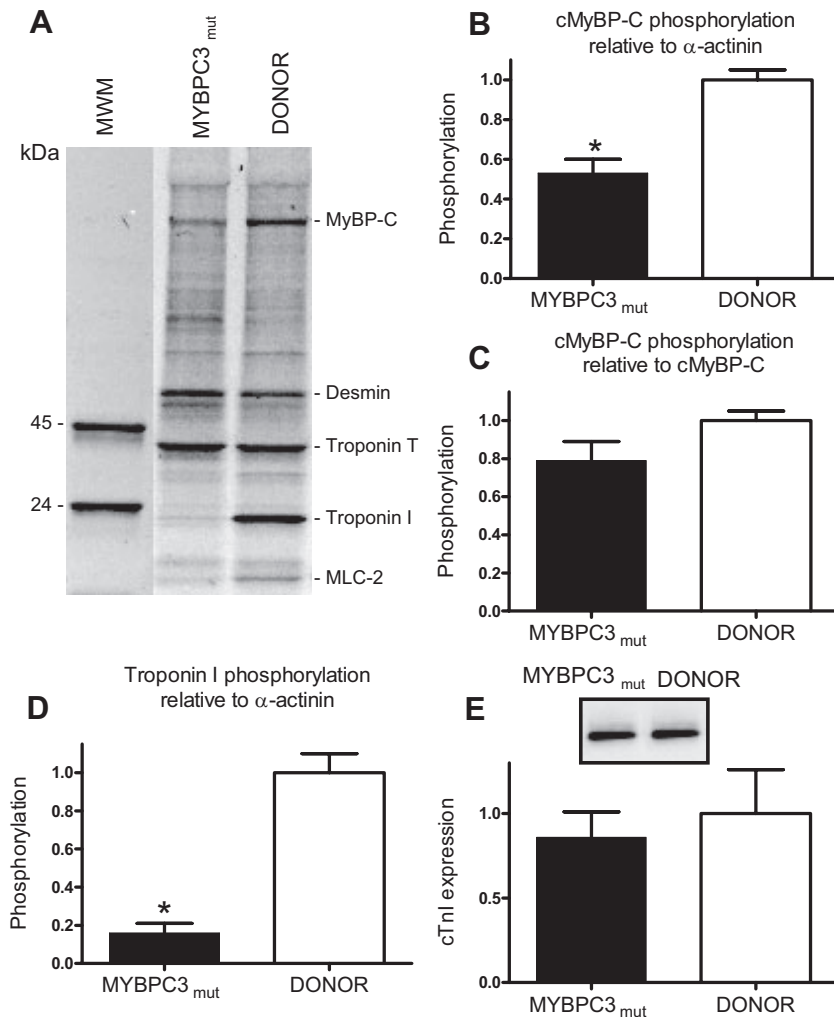
## Results

### Reduced Mutant cMyBP-C mRNA Level in FHCM Ventricular Tissue

To investigate whether both WT and mutant cMyBP-C mRNAs were transcribed in FHCM ventricular tissues, real-time reverse transcription polymerase chain reaction was performed with the use of specific Taqman probes and primers for each mutation (Figure 2A). The c.2373dupG mutation was expected to induce a cryptic donor splice site in exon 24 and the skipping of 40 nucleotides (Figure 1B). We therefore designed WT and mutant probes accordingly. Similarly, a specific mutant probe was designed for the 2864 to 2865 CT deletion in exon 27 and the corresponding WT probe (Figure 2A). Mutant mRNA represented 23% and 20% of the total cMyBP-C mRNA in c.2373dupG and c.2864\_2865delCT groups, respectively (Figure 2B). Because both mutations result in a frameshift and a premature termination codon (Figure 1B), the data suggest that both nonsense mutant mRNAs are partially subjected to degradation by the nonsense-mediated mRNA decay.<sup>20</sup>

### Reduced cMyBP-C Protein Level in FHCM Ventricular Tissue

The presence of truncated cMyBP-C in MYBPC3<sub>mut</sub> patients was examined by Western immunoblotting. After separation by gel electrophoresis, proteins were transferred to nitrocellulose and visualized with Ponceau (Figure 3A). Figure 3B



**Figure 4.** Phosphorylation status of cMyBP-C and cTnI. A, ProQ Diamond-stained gradient gel. MWM indicates molecular weight marker. B, The phosphorylation status of cMyBP-C (relative to SYPRO Ruby-stained  $\alpha$ -actinin) is lower in MYBPC3<sub>mut</sub> (n=11) than in donors (n=8), whereas phosphorylation of cMyBP-C normalized to its own expression level is comparable between MYBPC3<sub>mut</sub> and donors (C). D, The phosphorylation status of cTnI (relative to SYPRO Ruby-stained  $\alpha$ -actinin) is lower in MYBPC3<sub>mut</sub> than in donors. E, cTnI relative to Ponceau-stained actin did not differ between MYBPC3<sub>mut</sub> and donor myocardium. \* $P < 0.05$ .

shows results with the use of an antibody directed against the C0C2 region of cMyBP-C. None of the antibodies used (against C0C2, C5, or C8C9) revealed truncated cMyBP-C (predicted mass at 93 or 116 kDa) in any of the samples. On the basis of the sensitivity of our Western immunoblot analysis, levels of truncated cMyBP-C  $< 1.6\%$  could not be detected. Hence, we cannot completely exclude the presence of trace amounts of truncated cMyBP-C in MYBPC3<sub>mut</sub>. However, overloading of MYBPC3<sub>mut</sub> samples (40  $\mu$ g; 8 $\times$  higher concentration) did not reveal protein bands at the predicted mass (not shown), indicating the trace amounts of truncated protein, if present, would be even  $< 0.2\%$ .

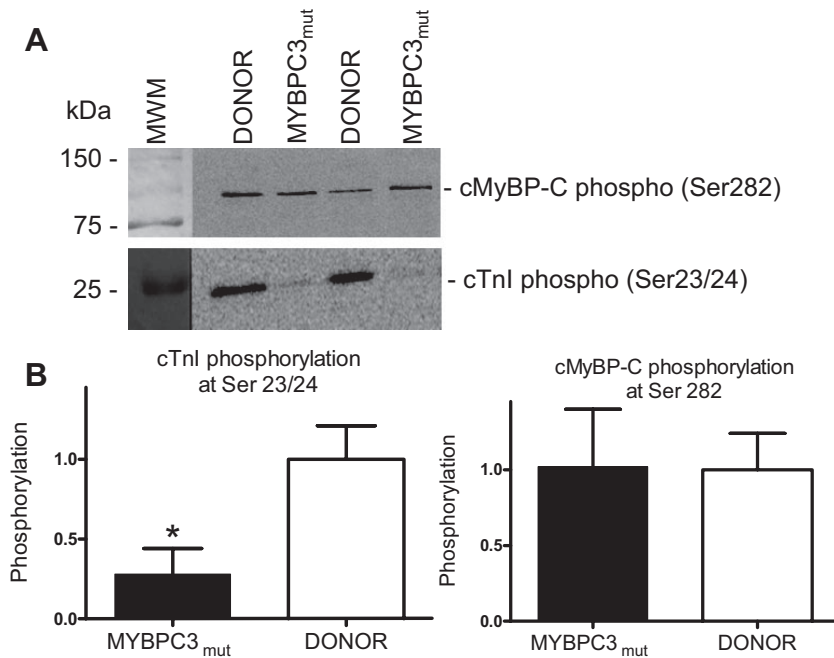
To determine the levels of full-length cMyBP-C, proteins were separated by 1-dimensional gel electrophoresis and stained with SYPRO Ruby (Figure 3C). The  $\alpha$ -actinin intensity was used as loading control. The cMyBP-C/ $\alpha$ -actinin protein ratio on the SYPRO Ruby-stained gels was 33% lower in MYBPC3<sub>mut</sub> (n=11) than in donor (n=8) (Figure 3D). Western immunoblot analysis confirmed a reduced level of full-length cMyBP-C (Figure 3B) in MYBPC3<sub>mut</sub> compared with donor myocardium. On average, Western blot data with the use of the C0C2 antibody showed a 23% lower amount of cMyBP-C (normalized to Ponceau-stained actin on the same blot) in MYBPC3<sub>mut</sub> compared with donor. How-

ever, the coefficient of variation of the Western immunoblot analysis was much higher than that of SYPRO Ruby staining (56.3% and 25.8%, respectively). Therefore, the SYPRO values are considered to represent the protein levels more accurately.

### Deranged Phosphorylation

The SYPRO Ruby-stained gels were also stained with ProQ Diamond, which selectively stains phosphorylated serine, threonine, and tyrosine residues (Figure 4A). Phosphorylation of cMyBP-C normalized to  $\alpha$ -actinin was reduced by  $47 \pm 7\%$  in the MYBPC3<sub>mut</sub> compared with donor myocardium ( $P < 0.0001$ ; Figure 4B). Interestingly, phosphorylation of cMyBP-C normalized to its own protein level (determined with SYPRO) was similar between MYBPC3<sub>mut</sub> and donor myocardium ( $79 \pm 11\%$  versus  $100 \pm 5\%$ , respectively;  $P = 0.14$ ; Figure 4C).

In addition, massive dephosphorylation of cTnI was observed in MYBPC3<sub>mut</sub> relative to donor by  $84 \pm 5\%$  ( $P < 0.0001$ ; Figure 4D). Because the reduced ProQ Diamond signals for cTnI in MYBPC3<sub>mut</sub> may be due to reduced cTnI expression, we analyzed the steady state level of cTnI by Western immunoblot. The amount of cTnI relative to Ponceau-stained actin did not differ between MYBPC3<sub>mut</sub> and



**Figure 5.** A, Western immunoblot analysis with the use of a specific antibody against cTnI phosphorylated at PKA sites (Ser23/24) revealed reduced cTnI phosphorylation in MYBPC3<sub>mut</sub> compared with donor myocardium, whereas phosphorylation of cMyBP-C at Ser282 did not differ between groups. MWM indicates molecular weight marker. B, Phosphorylation levels of cTnI and cMyBP-C were normalized to total cTnI and cMyBP-C protein expression, respectively. \* $P < 0.05$  MYBPC3<sub>mut</sub> vs donor.

donor myocardium (Figure 4E). Hence, the reduced ProQ Diamond signals represent reduced phosphorylation of cTnI in MYBPC3<sub>mut</sub> compared with donor. Phosphorylation of other sarcomeric proteins desmin, cardiac troponin T, and myosin light chain 2 was also significantly reduced in MYBPC3<sub>mut</sub> by  $24 \pm 8\%$ ,  $41 \pm 7\%$ , and  $61 \pm 4\%$ , respectively, relative to donor samples.

Western immunoblot analysis (Figure 5) revealed significantly lower bisphosphorylation of PKA sites (Ser23/24) in cTnI in MYBPC3<sub>mut</sub> compared with donors, whereas phosphorylation of cMyBP-C at Ser282 was similar in MYBPC3<sub>mut</sub> and donor myocardium. This confirms the data obtained with ProQ Diamond stain (Figure 4).

### Depressed Force Development

Functional implications of the MYBPC3 mutations were investigated by cardiomyocyte force measurements in c.2373dupG ( $n=7$ ; 25 cells) and c.2864\_2865delCT ( $n=3$ ; 9 cells). A representative cardiomyocyte and a recording at saturating  $\text{Ca}^{2+}$  concentration (pCa 4.5) from a MYBPC3<sub>mut</sub> patient are shown in Figure 6A and 6B. Maximal force development ( $F_{\text{active}}$ ) was significantly depressed in MYBPC3<sub>mut</sub> ( $20.2 \pm 2.7$  kN/m<sup>2</sup>) compared with donor ( $34.5 \pm 1.1$  kN/m<sup>2</sup>;  $n=13$ ; 47 cells) (Figure 6C). Passive force ( $F_{\text{passive}}$ ) in MYBPC3<sub>mut</sub> ( $3.8 \pm 0.7$  kN/m<sup>2</sup>) was somewhat elevated compared with donor ( $3.4 \pm 0.4$  kN/m<sup>2</sup>), but the difference was not significant (Figure 6D). Interpatient variation in  $F_{\text{active}}$  and  $F_{\text{passive}}$  in the MYBPC3<sub>mut</sub> group was larger than in the donor group (Figure 6E, 6F).  $F_{\text{active}}$  and  $F_{\text{passive}}$  were lower in c.2864\_2865delCT than in c.2373dupG ( $P=NS$ ).

Cells from MYBPC3<sub>mut</sub> (c.2373dupG,  $n=4$ , 9 cells; c.2864\_2865delCT;  $n=2$ ; 6 cells) and donors ( $n=6$ ; 18 cells) were incubated with PKA,<sup>21</sup> after which  $F_{\text{active}}$  and  $F_{\text{passive}}$  measurements were repeated to determine whether  $\beta$ -adrenergic stimulation could correct depressed force in

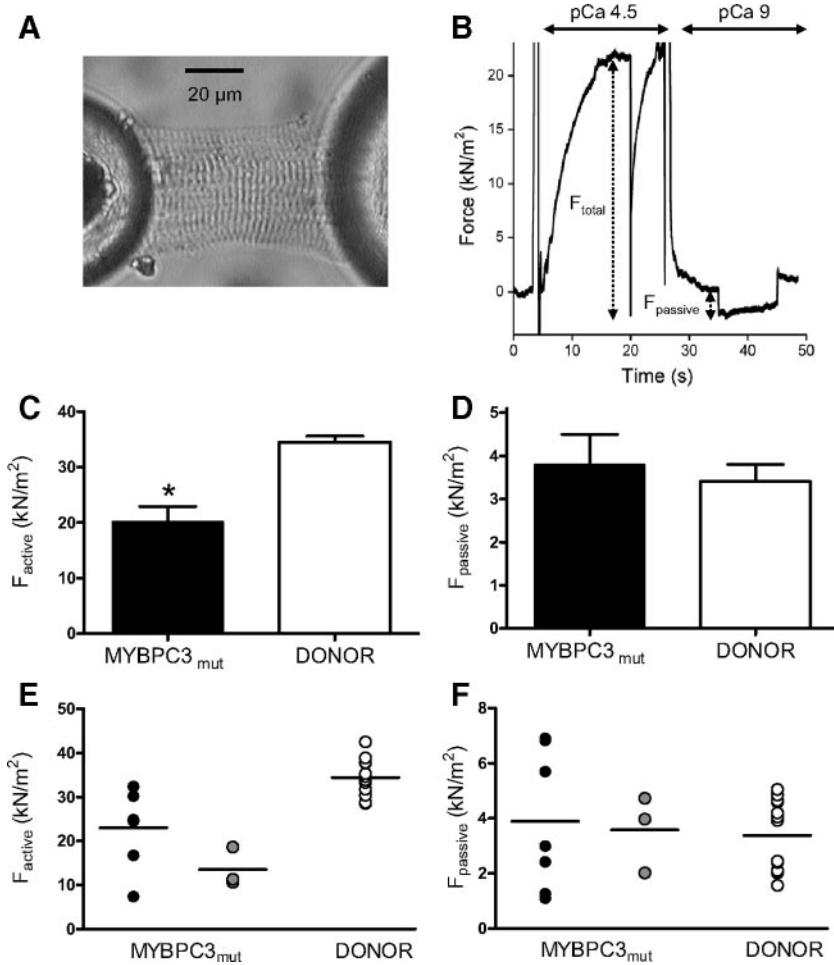
MYBPC3<sub>mut</sub>. PKA treatment resulted in a minor decrease in  $F_{\text{active}}$  in MYBPC3<sub>mut</sub> (before versus after:  $14.2 \pm 2.9$  and  $13.5 \pm 2.5$  kN/m<sup>2</sup>), which was similar to the PKA effect in donor (before versus after:  $27.9 \pm 3.5$  and  $25.8 \pm 3.2$  kN/m<sup>2</sup>) ( $P=0.03$ , 2-way ANOVA). Moreover, PKA significantly decreased  $F_{\text{passive}}$  in both groups (MYBPC3<sub>mut</sub> before versus after:  $2.3 \pm 0.3$  and  $2.1 \pm 0.2$  kN/m<sup>2</sup>; donor before versus after:  $3.3 \pm 0.5$  and  $2.7 \pm 0.4$  kN/m<sup>2</sup>) ( $P=0.001$ , 2-way ANOVA).

Because increased PKC activity/expression has been associated with depressed maximal force generating capacity of myofilaments,<sup>22</sup> force measurements were repeated after incubation with the catalytic domain of PKC.  $F_{\text{active}}$  in MYBPC3<sub>mut</sub> (c.2373dupG;  $n=3$ ; 5 cells) was significantly reduced after PKC by  $2.6 \pm 0.6$  kN/m<sup>2</sup>. A similar decrease ( $3.3 \pm 0.7$  kN/m<sup>2</sup>) was found in cardiomyocytes from donor hearts ( $n=5$ ; 9 cells). PKC slightly decreased  $F_{\text{passive}}$  in both groups, although the effect was not significant (data not shown).

### Enhanced $\text{Ca}^{2+}$ Sensitivity

$\text{Ca}^{2+}$  sensitivity of the sarcomeres was significantly higher in MYBPC3<sub>mut</sub> ( $\text{pCa}_{50}=5.62 \pm 0.04$ ) than in donor cells ( $\text{pCa}_{50}=5.54 \pm 0.02$ ). The average force-pCa relationships obtained in MYBPC3<sub>mut</sub> and donor cardiomyocytes are shown in Figure 7A.  $\text{Ca}^{2+}$  sensitivity was similar in c.2864\_2865delCT ( $\text{pCa}_{50}=5.63 \pm 0.05$ ) and c.2373dupG ( $\text{pCa}_{50}=5.60 \pm 0.09$ ). Figure 7B illustrates that interpatient variation in  $\text{pCa}_{50}$  was larger in the MYBPC3<sub>mut</sub> than in the donor group.

Treatment with exogenous PKA significantly reduced  $\text{Ca}^{2+}$  sensitivity in both groups. The reduction in  $\text{pCa}_{50}$  was significantly larger in MYBPC3<sub>mut</sub> ( $\Delta\text{pCa}_{50}=0.18 \pm 0.03$ ) than in donor ( $\Delta\text{pCa}_{50}=0.06 \pm 0.01$ ) cells (Figure 7C). PKA treatment abolished the initial difference in  $\text{Ca}^{2+}$  sensitivity between MYBPC3<sub>mut</sub> and donor (Figure 7D). ProQ Diamond staining of a MYBPC3<sub>mut</sub> sample that was incubated without



**Figure 6.** Force measurements. Permeabilized cardiomyocyte (A) and force recording (B) of a MYBPC3<sub>mut</sub> sample. When a maximal steady force level was reached at saturating [Ca<sup>2+</sup>] (pCa4.5), the cell was quickly shortened by 30% and immediately restretched to its original length to determine the baseline of the force recording and total force ( $F_{total}$ ). Subsequently, the cell was transferred to relaxing solution (pCa 9.0) and shortened for a period of 10 seconds to determine passive force ( $F_{passive}$ ). C, Active force ( $F_{active} = F_{total} - F_{passive}$ ) in MYBPC3<sub>mut</sub> was significantly depressed, and  $F_{passive}$  (D) was slightly but not significantly elevated compared with donor. E and F, Variation among mean force parameters of individuals among the MYBPC3<sub>mut</sub> group (c.2373dupG, black symbols; c.2864\_2865delCT, gray symbols) compared with the donor group (open symbols). \* $P < 0.05$ .

(control incubation) and with PKA showed a 4-fold increase in cTnI phosphorylation, whereas the increase in cMyBP-C phosphorylation was small (Figure 7E).

Similar to PKA, PKC significantly reduced pCa<sub>50</sub> in both groups. However, in contrast to PKA, the PKC-induced shift in pCa<sub>50</sub> did not significantly differ between MYBPC3<sub>mut</sub> ( $\Delta pCa_{50} = 0.11 \pm 0.04$ ) and donor cells ( $\Delta pCa_{50} = 0.08 \pm 0.01$ ) and thus does not explain the baseline difference in Ca<sup>2+</sup> sensitivity between MYBPC3<sub>mut</sub> and donor myocardium.

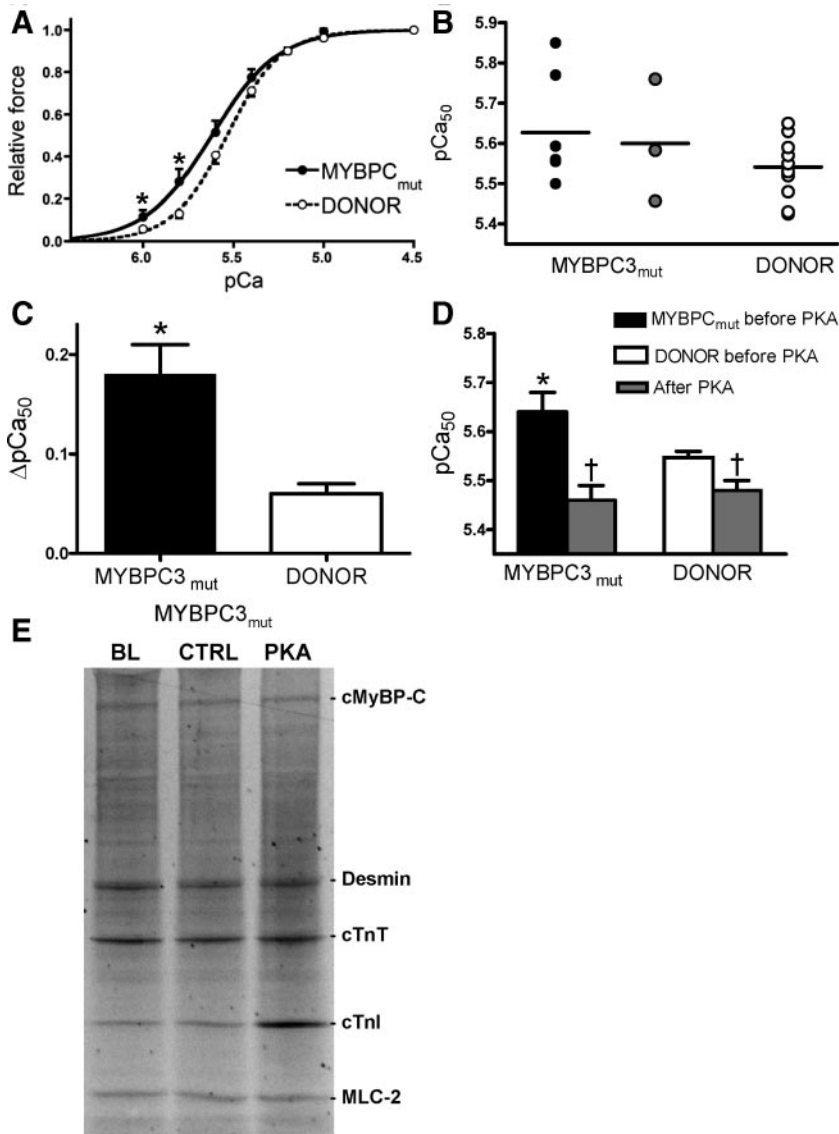
### Discussion

Our study provides direct evidence for reduced cMyBP-C protein level and contractile dysfunction in a group of FHCM patients with MYBPC3 frameshift mutations. Consistent with previous studies,<sup>7-9</sup> no truncated cMyBP-C protein was detected, and the amount of full-length cMyBP-C was 33% lower in FHCM than in donor myocardium. Our data therefore indicate that the pathomechanism involves haploinsufficiency rather than a poison polypeptide. Using adenoviral gene transfer of cardiomyocytes, Sarikas et al<sup>23</sup> showed rapid and quantitative degradation of truncated forms of cMyBP-C by the ubiquitin-proteasome system, which could in turn inhibit ubiquitin-proteasome system-mediated degradation of other cellular proteins.<sup>23</sup> Thus, the absence of truncated cMyBP-C in FHCM patients in the present study suggests

that the ubiquitin-proteasome system may degrade truncated cMyBP-C. The full-length C-protein, in contrast, compensates for the absence of truncated protein. The fact that mutant mRNAs were detected in both FHCM groups supports the involvement of the ubiquitin-proteasome system in the degradation of truncated protein. On the other hand, the lower level of mutant versus WT cMyBP-C mRNA in both FHCM groups suggests partial instability of nonsense mutant mRNA, which could be degraded by the nonsense-mediated mRNA decay.<sup>20</sup>

The maximum force-generating capacity (ie,  $F_{active}$ ) of cardiomyocytes from MYBPC3<sub>mut</sub> carriers was significantly reduced by 42% compared with nonfailing myocardium. Recent studies<sup>24-28</sup> revealed an important role for cMyBP-C in cross-bridge kinetics. Loss of cMyBP-C accelerates cross-bridge cycling and impairs kinetics of contraction and relaxation.<sup>24,25,27</sup> Complete knockout of cMyBP-C resulted in profound cardiac hypertrophy and impaired contractile function in mice.<sup>29,30</sup> Surprisingly, transgenic mice harboring only 40% of the normally expressed full-length cMyBP-C did not have LV hypertrophy and showed preserved cardiac function.<sup>26</sup> In contrast, our study shows that an  $\approx 33\%$  reduction of full-length cMyBP-C level is sufficient to trigger LV hypertrophy and contractile dysfunction in human. Intriguingly, reduced cMyBP-C levels per se do not seem to explain the decline in maximum force in MYBPC3<sub>mut</sub> because  $F_{active}$





**Figure 7.** A, Force measurements were performed at maximal and submaximal  $[Ca^{2+}]$  (pCa range 4.5 to 6). Force values obtained in solutions with submaximal  $[Ca^{2+}]$  were normalized to the maximal force level at pCa 4.5. Force-pCa relations were fit to a modified Hill equation.  $Ca^{2+}$  sensitivity of the sarcomeres (pCa<sub>50</sub>) was significantly higher in MYBPC<sub>3mut</sub> than in donor. B, Variation among mean pCa<sub>50</sub> parameters of individuals among the MYBPC<sub>3mut</sub> group (c.2373dupG, black symbols; c.2864\_2865delCT, gray symbols) compared with the donor group (open symbols). C, Treatment of cardiomyocytes with exogenous PKA induced a larger reduction in pCa<sub>50</sub> in MYBPC<sub>3mut</sub> than in donor. D, PKA treatment abolished the initial difference in  $Ca^{2+}$  sensitivity between MYBPC<sub>3mut</sub> and donor. \* $P < 0.05$  MYBPC<sub>3mut</sub> vs donor; † $P < 0.05$  before vs after PKA treatment. E, ProQ Diamond staining of MYBPC<sub>3mut</sub> myocardium, which was directly frozen (BL indicates baseline) or incubated without PKA (CTRL indicates control) or with PKA (100 U/mL). Phosphorylation of cTnI increased on incubation with PKA. cTnT indicates cardiac troponin T; MLC-2, myosin light chain 2.

did not correlate with the level of full-length cMyBP-C (not shown) but may rather involve reduced expression and altered phosphorylation of cMyBP-C.

Cardiac MyBP-C is phosphorylated by PKA on adrenergic stimulation.<sup>31</sup> Apart from PKA, cMyBP-C can be phosphorylated by  $Ca^{2+}$ -calmodulin-dependent kinase (CaMK)<sup>32,33</sup> and PKC.<sup>34,35</sup> Transgenic mice hearts in which the phosphorylation sites of cMyBP-C were changed to nonphosphorylatable alanines displayed reduced contractility and altered sarcomeric structure, indicating that phosphorylation of cMyBP-C is essential for normal cardiac function.<sup>36</sup> Reduced cMyBP-C phosphorylation has been observed in animal models of cardiac hypertrophy and failure<sup>36,37</sup> and in humans with end-stage idiopathic and ischemic cardiomyopathy.<sup>21,37</sup> The discrepant phosphorylation levels of cMyBP-C and cTnI in MYBPC<sub>3mut</sub> are in contrast to previous observations in non-FHCM (idiopathic and ischemic cardiomyopathy) and donor myocardium, which revealed parallel changes in the main target proteins of the  $\beta$ -adrenergic pathway.<sup>21,37</sup> Hence, it is possible that this discrepancy causes contractile dysfunction.

Because PKA did not correct the reduction in  $F_{active}$ , other (mal)adaptive signaling routes are responsible for divergent phosphorylation of cMyBP-C and cTnI and sarcomeric dysfunction. In a recent study, increased PKC expression level in 2 models of heart failure (pressure overload and ischemic) in rat was associated with reduced maximal force-generating capacity of myofilaments.<sup>22</sup> To test whether this applies to human tissue, force measurements were performed in single human cardiomyocytes from MYBPC<sub>3mut</sub> and donor hearts before and after incubation with PKC. The effects of PKC on cardiomyocyte force parameters ( $F_{active}$ ,  $F_{passive}$ , and pCa<sub>50</sub>) were similar in MYBPC<sub>3mut</sub> and donor cardiomyocytes, indicating that impaired myofilament function in MYBPC<sub>3mut</sub> does not seem to be related to a difference in PKC-mediated phosphorylation of myofilament proteins. Interestingly, Yuan et al<sup>38</sup> revealed differential phosphorylation of cMyBP-C on myocardial stunning and suggested a role for altered calcium handling and activation of CaMK. Thus, in combination with the evidence presented in the literature, our experiments suggest that the reduced

maximal force-generating capacity of cardiomyocytes is not a direct consequence of haploinsufficiency but rather might be caused by differential phosphorylation of cMyBP-C resulting from (mal)adaptive neurohumoral signaling in hearts of MYBPC3<sub>mut</sub> carriers.

Similarly, the higher Ca<sup>2+</sup> sensitivity of force development in MYBPC3<sub>mut</sub> patients may be either a direct or an indirect consequence of the cMyBP-C haploinsufficiency. Previous studies of FHCM mutations in the thin-filament proteins troponin and tropomyosin reported enhanced myofilament Ca<sup>2+</sup> sensitivity in contrast to a reduction in Ca<sup>2+</sup> sensitivity, which is considered characteristic for mutations found in familial dilated cardiomyopathy.<sup>39–41</sup> Robinson et al<sup>41</sup> proposed that the mutant-induced enhanced Ca<sup>2+</sup> sensitivity reflects changes in Ca<sup>2+</sup> binding affinity, which may directly alter Ca<sup>2+</sup> transient and trigger hypertrophic signaling routes.<sup>42</sup> Extraction of cMyBP-C by ≈30% to 70% from rat cardiomyocytes resulted in an increase in Ca<sup>2+</sup> sensitivity.<sup>43,44</sup> Similarly, a greater myofilament Ca<sup>2+</sup> sensitivity was found in skinned myocytes at short sarcomere length from cMyBP-C knockout mice,<sup>45</sup> and a greater sensitivity to external Ca<sup>2+</sup> was found in cMyBP-C knockout intact atrial tissue.<sup>25</sup> Hence, the frameshift MYBPC3 mutations inducing cMyBP-C haploinsufficiency may directly increase Ca<sup>2+</sup> sensitivity. On the other hand, increased myofilament Ca<sup>2+</sup> sensitivity has also been found in end-stage failing human myocardium (idiopathic dilated cardiomyopathy) without known mutations in sarcomeric proteins.<sup>19,46,47</sup> This enhanced Ca<sup>2+</sup> sensitivity has been ascribed to hyperactivation of the β-adrenergic signaling pathway in response to reduced cardiac pump function. Chronic activation of the β-adrenergic receptor pathway results in downregulation and desensitization of the receptors in failing myocardium and a subsequent parallel reduction in phosphorylation of the PKA target proteins cMyBP-C and cTnI.<sup>17,21,37</sup> In healthy myocardium, the main effect of PKA-mediated phosphorylation of cTnI is reduced Ca<sup>2+</sup> sensitivity, which contributes to appropriate myocardial relaxation.<sup>27,48,49</sup> Reduced phosphorylation of cTnI and PKA-mediated increase in cTnI phosphorylation and correction of Ca<sup>2+</sup> sensitivity to donor values in MYBPC3<sub>mut</sub> suggest that heart failure-induced β-adrenergic desensitization underlies the increase in Ca<sup>2+</sup> sensitivity. Hence, on the basis of our data, we postulate that the enhanced Ca<sup>2+</sup> sensitivity of sarcomeres in MYBPC3<sub>mut</sub> is a secondary consequence of the frameshift mutation-induced cardiac dysfunction, which triggers adrenergic hyperactivation. The ensuing defects in β-adrenergic signaling may impair phosphorylation of Ca<sup>2+</sup> handling proteins, and subsequent alterations in cellular Ca<sup>2+</sup> transient may activate kinases, such as CaMK, involved in differential phosphorylation of cMyBP-C and cTnI.

Our data may be confounded by differences in medication and in the origin of LV tissue (septum versus free wall). Moreover, we cannot exclude that age and sex differences affected our analysis. However, the unique MYBPC3 founder mutations allowed us to characterize contractile properties in a relatively homogeneous group of FHCM patients. The combined analysis of sarcomere protein composition and function revealed haploinsufficiency and reduced contractil-

ity in patients carrying a frameshift MYBPC3 mutation. The sarcomeric phenotype in MYBPC3<sub>mut</sub> is the complex resultant of the mutation and secondary alterations in the sarcomeric phosphoproteome due to maladaptive alterations in neurohumoral signaling and/or Ca<sup>2+</sup> homeostasis. Therefore, our data support the concept that contractile dysfunction is a pivotal link between the mutant sarcomeric protein and pathological hypertrophic cardiomyopathy.

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## Disclosures

None.

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### CLINICAL PERSPECTIVE

Familial hypertrophic cardiomyopathy is the most frequent inheritable cardiac disease, with a prevalence of 1/500. Mutations in the *MYBPC3* gene encoding cardiac myosin-binding protein C (cMyBP-C) represent >40% of all familial hypertrophic cardiomyopathy cases. Cardiac MyBP-C is bound to the thick myosin filament and may influence binding of the myosin head to actin and thereby force generation and pressure development of the heart. cMyBP-C function is influenced by  $\beta$ -adrenergic stimulation through protein kinase A-mediated phosphorylation. Most *MYBPC3* mutations are predicted to produce C-terminally truncated proteins, lacking titin and/or major myosin binding sites. Approximately 35% of the familial hypertrophic cardiomyopathy patients in the Netherlands have founder mutations in the *MYBPC3* gene that encode C-terminally truncated proteins. This allowed us to investigate whether truncating mutations in *MYBPC3* alter sarcomeric protein composition and function in a rather homogeneous patient group. No truncated cMyBP-C was detected, full-length cMyBP-C was reduced by 33% compared with nonfailing myocardium, and cMyBP-C phosphorylation was preserved. Our data therefore indicate that the pathomechanism involves haploinsufficiency rather than a poison polypeptide. Force measurements in single cardiomyocytes revealed reduced maximal force-generating capacity compared with healthy cells. In addition,  $\text{Ca}^{2+}$  sensitivity of the contractile apparatus was increased because of hypophosphorylation of troponin I, another target of protein kinase A. We conclude that the sarcomeric phenotype in familial hypertrophic cardiomyopathy with *MYBPC3* mutations includes a primary contractile sarcomeric defect causing deranged secondary alterations in protein phosphorylation. Our data therefore suggest that contractile dysfunction is a pivotal link between the mutant sarcomeric protein and pathological hypertrophic cardiomyopathy.