






**RESEARCH ARTICLE**

# The homozygous K280N troponin T mutation alters cross-bridge kinetics and energetics in human HCM

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**Hypertrophic cardiomyopathy (HCM) is a genetic form of left ventricular hypertrophy, primarily caused by mutations in sarcomere proteins. The cardiac remodeling that occurs as the disease develops can mask the pathogenic impact of the mutation. Here, to discriminate between mutation-induced and disease-related changes in myofibril function, we investigate the pathogenic mechanisms underlying HCM in a patient carrying a homozygous mutation (K280N) in the cardiac troponin T gene (*TNNT2*), which results in 100% mutant cardiac troponin T. We examine sarcomere mechanics and energetics in K280N-isolated myofibrils and demembrated muscle strips, before and after replacement of the endogenous troponin. We also compare these data to those of control preparations from donor hearts, aortic stenosis patients (LVH<sub>ao</sub>), and HCM patients negative for sarcomeric protein mutations (HCM<sub>smn</sub>). The rate constant of tension generation following maximal Ca<sup>2+</sup> activation ( $k_{ACT}$ ) and the rate constant of isometric relaxation (slow  $k_{REL}$ ) are markedly faster in K280N myofibrils than in all control groups. Simultaneous measurements of maximal isometric ATPase activity and Ca<sup>2+</sup>-activated tension in demembrated muscle strips also demonstrate that the energy cost of tension generation is higher in the K280N than in all controls. Replacement of mutant protein by exchange with wild-type troponin in the K280N preparations reduces  $k_{ACT}$ , slow  $k_{REL}$ , and tension cost close to control values. In donor myofibrils and HCM<sub>smn</sub> demembrated strips, replacement of endogenous troponin with troponin containing the K280N mutant increases  $k_{ACT}$ , slow  $k_{REL}$ , and tension cost. The K280N *TNNT2* mutation directly alters the apparent cross-bridge kinetics and impairs sarcomere energetics. This result supports the hypothesis that inefficient ATP utilization by myofibrils plays a central role in the pathogenesis of the disease.**

## Introduction

Hypertrophic cardiomyopathy (HCM) is a primary disorder of cardiac muscle. Traditionally, it has been characterized by the presence of unexplained left ventricular (LV) hypertrophy, which occurs in the absence of any other known etiology. HCM is often familial with autosomal dominant inheritance. Linkage studies in the 1980s led to the discovery of pathogenic mutations in genes that encode different components of the contractile apparatus, which has been confirmed by recent large-scale whole-exome sequencing analysis (Alfares et al., 2015).

This discovery established the paradigm that HCM is a disease of the sarcomere.

With the notable exception of the numerous truncation mutations in the gene encoding the thick filament protein cardiac myosin binding protein C, for which evidence of haploinsufficiency has been generated (Marston et al., 2009; van Dijk et al., 2009), most HCM mutations result in stable mutant proteins that are incorporated into the sarcomere and may act in a dominant fashion on the contractile performance (Cuda et al., 1993; Bottinelli

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This work is part of a special collection on myofibril function.

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et al., 1998b). The pathogenic impact of sarcomeric HCM mutations has been varying attributed to aberrant cross-bridge dynamics, leading to decreased/increased contractility (Cuda et al., 1993; Palmiter et al., 2000; Belus et al., 2008; Palmer et al., 2008), increased intrinsic force of the myosin motor (Seebohm et al., 2009; Sommese et al., 2013), and increased sarcomeric  $\text{Ca}^{2+}$  sensitivity (Bottinelli et al., 1998a; for a review, see Marston et al., 2012). To reconcile the lack of consistent contractility changes in HCM, it has been proposed that HCM sarcomere mutations may lead to increased energy cost of force production through inefficient or excessive ATPase activity and that this ultimately results in an energy deficiency that contributes to the pathogenesis of the disease (Ashrafian et al., 2003). Several studies on HCM disease mechanisms in patient hearts and animal models support this hypothesis (Jung et al., 1998; Crilley et al., 2003; Javadpour et al., 2003; Chandra et al., 2005; He et al., 2007; Belus et al., 2008; Luedde et al., 2009; Witjas-Paalberends et al., 2014a,b; Ferrantini et al., 2017). Though collectively these studies suggest that energy deficiency is a primary consequence of the underlying mutation, perturbations occurring in cardiomyocyte signaling pathways during disease development can lead to cardiac remodeling that may exacerbate or counteract mutation-induced alterations.

Homozygous HCM-causing mutations are rare and usually have more severe symptoms than heterozygous mutations (Ho et al., 2000; Keller et al., 2004). In this study, we investigated sarcomere mechanics and energetics in isolated myofibrils and demembranated multicellular cardiac muscle strips from a unique HCM patient with a homozygous charge mutation (K280N) in the *TNNT2* gene (Sequeira et al., 2013; Messer et al., 2016). As shown here, this homozygous mutation results in 100% mutant cardiac troponin T (cTnT), and therefore, it represents a unique means to avoid the problems of estimating the extent and the variability of the expression of the mutant versus WT alleles between and within cardiomyocytes (Montag et al., 2018). In addition, with 100% mutant cTnT, it becomes easier to assess, by the use of troponin exchange experiments, which changes in contractile function are directly due to the presence of the mutant protein, rather than secondary to cardiac myofilament remodeling. This also has the potential to assess how much mutant protein is required to significantly perturb sarcomere function.

In this study, experimental data from one single patient have been compared with those from different control groups made of several subjects. The challenge of performing meaningful statistics has been faced using a multistep approach in which the descriptive statistics and the comparison among groups were followed by a sensitivity analysis, where we analyzed subject-specific random effects, and we individually compared the K280N data with those from each control patient.

## Materials and methods

Genotyping of a 26-yr-old male HCM patient who underwent cardiac transplantation surgery identified a homozygous mutation in *TNNT2*, the gene encoding cTnT that translates into the missense mutation K280N in the predominant cardiac muscle isoform (T3; Uniprot ID P45379-6). The heart muscle sample was provided by the Sydney Heart Bank (ID 3.166; Li et al., 2013). Tis-

sue from the free LV wall and interventricular septum (IVS) of the explanted heart was collected in cardioplegic solution and immediately frozen in liquid nitrogen. Samples of nonfailing LV or IVS tissue were obtained from five healthy donor hearts, nine patients who underwent aortic valve replacement due to aortic stenosis (LVH<sub>ao</sub> group), and nine HCM patients without an identified sarcomeric gene mutation after screening of nine sarcomere genes who underwent myectomy to relieve LV outflow tract obstruction (sarcomere mutation-negative; HCM<sub>smn</sub> group). Written informed consent from each patient was obtained before surgery, and the study was approved by the local Ethics Committees. Clinical details of all patients together with an extended version of Materials and methods are given in the Supplemental material (Table S1).

### Mutant expression

Total RNA was extracted from the human heart biopsies, and cTnT mRNA was determined by real-time PCR.

TnT was digested with glu-C, and the peptides were analyzed by MALDI-TOF/TOF mass spectrometry. Data were searched against a human sequence database containing WT and K280N TnT sequences using MASCOT.

SDS PAGE and Western blot analysis were used to estimate cTnT protein content relative to actin.

### Myofibril experiments

To isolate myofibrils from frozen human cardiac samples and perform mechanical experiments, we used previously published techniques (Piroddi et al., 2007; Belus et al., 2008). Bundles of few myofibrils (25–80- $\mu\text{m}$  long and 1–4- $\mu\text{m}$  wide), mounted in a force recording apparatus (15°C; initial sarcomere length,  $\sim 2.2 \mu\text{m}$ ), were activated and relaxed by rapidly (<5 ms) translating the interface between two flowing streams of activating (pCa 4.5) and relaxing (pCa 8.0) solutions across the preparation (Colomo et al., 1998). Maximal isometric tension ( $P_0$ ) and kinetic parameters of maximal tension generation ( $k_{\text{ACT}}$ , rate constant of tension generation following  $\text{Ca}^{2+}$  activation;  $k_{\text{TR}}$ , rate constant of tension redevelopment following release–stretch applied to the myofibril under steady-state conditions of force generation) were estimated. Specifically, to estimate  $k_{\text{TR}}$ , a sudden (1–2 ms) decrease in length (20%  $l_0$ ) was imposed on myofibrils during the steady contraction, bringing the initial length of the preparation below the slack value. After a period of unloaded shortening, which reduced the number of cross-bridges and before unloaded ventricular myofibrils could take up the slack ( $\sim 80$  ms), myofibrils were rapidly (1–2 ms) stretched back to their original length to mechanically dissociate the residual acto-myosin bonds. Relaxation parameters were measured as follows:  $D_{\text{slow}}$ , duration of the slow isometric phase of relaxation following sudden  $\text{Ca}^{2+}$  removal, measured from the start of the solution switch to the tension “give”; slow  $k_{\text{REL}}$ , rate of the slow isometric phase of relaxation estimated from the normalized slope of the linear fit to the force trace; fast  $k_{\text{REL}}$ , rate of the fast phase of relaxation estimated from the time constant of the exponential fit to the force trace. IVS samples were used to prepare myofibrils from the LVH<sub>ao</sub> and HCM<sub>smn</sub> patients. Both IVS and LV free wall samples were used to isolate

myofibrils from the K280N patient and donor hearts. Since no differences were found between the parameters measured from IVS and LV myofibrils, data from IVS and LV myofibrils of each group were pooled.

### Tension cost measurement in cardiac strips

The experimental procedures, solutions, and equipment used to simultaneously measure maximal tension and ATPase in human permeabilized strips from IVS samples of the K280N, HCM<sub>smn</sub>, and LVH<sub>ao</sub> patients were as described previously (Narolska et al., 2005; He et al., 2007; Wijnker et al., 2014b; Witjas-Paalberends et al., 2014b). Maximal isometric tension and ATP consumption were measured at saturating [Ca<sup>2+</sup>] and 20°C. The length of the preparations was adjusted to a sarcomere length of ~2.2 μm (Narolska et al., 2005). The maximal Ca<sup>2+</sup>-activated ATPase activity was normalized to the volume of the muscle strip and subtracted of the basal ATPase activity in relaxing solution.

### Recombinant cTn preparation and exchange in myofibrils and muscle strips

Recombinant WT human cTnT containing an N-terminal myc-tag, human K280N mutant cTnT and human WT cTnI and cTnT complexes were reconstituted according to previously described techniques (Robinson et al., 2002; Wijnker et al., 2014a,b). Whole troponin complex exchange was achieved in human cardiac myofibrils and multicellular muscle strips by mass displacement following protocols adapted from previously described techniques (Piroddi et al., 2003; Narolska et al., 2006). Myofibrils were always exchanged overnight (o/n) while cardiac strips were exchanged for 4 h or o/n. Preparations incubated for the same time in the exchange solution without the exogenous cTn complex were used as sham-treated controls.

### Determination of degree of troponin exchange

The degree of Tn exchange in K280N and donor myofibrils exchanged with WT Tn containing recombinant cTnT labeled with a Myc-tag was estimated using one-dimensional SDS-PAGE electrophoresis with 15% acrylamide. Densitometric measurements of the ratio between the intensities of native cTnT, and α-actinin bands were compared in sham-treated and exchanged myofibrils.

In the muscle strips, the amount of endogenous K280N cTn complex exchanged with the exogenous WT cTn complex was assessed using 13% SDS-polyacrylamide gel and immunoblotting.

The recombinant K280N mutated cTnT did not include a Myc-tag. However, the phosphorylated endogenous cTn complex in the HCM<sub>smn</sub> was exchanged for the nonphosphorylated exogenous recombinant K280N. The amount of exchange was determined using ProQ-Diamond phospho-stained gels as described previously (Zaremba et al., 2007). The phosphorylation signals of the ProQ-Diamond-stained cTnT were normalized to the intensities of the SYPRO ruby-stained cTnT to correct for protein loading.

### Statistics

Data from myofibrils and muscle strips are expressed in Table 1, Table 2, and Table 3 as means ± SEMs. Statistical analysis, taking

into account non-Gaussian distribution, inequality of variances, and within-subject correlation, was performed as detailed in the Supplemental material. In brief, data from one single HCM patient, i.e., the K280N cTnT homozygous patient (Group 1), were compared with those from different groups of control patients (HCM<sub>smn</sub>, Group 2; donors, Group 3; and LVH<sub>ao</sub>, Group 4). For each measured parameter, statistical analysis was initially performed by comparison among groups. Repeated measurement ANOVA and Kruskal-Wallis rank-sum test were used to evaluate differences among the four groups. Post-hoc multiple comparisons were performed when ANOVA indicated the presence of significant differences among groups (Graph S1[1]). We used the Benjamini-Hochberg approach (Benjamini and Cohen, 2017) and calculated the corresponding adjusted P values. A false discovery rate (FDR) of 0.05 was used as a cut off for rejection. When comparing K280N data with the control groups in the repeated measurement ANOVA, we assumed homogeneous within-subject correlation because the K280N group was constituted by a single subject. Thus, for sensitivity analysis, we fitted a linear mixed model with patient-specific random effect and omitted the group membership indicator. We analyzed subject-specific random effect residuals from the fitted linear mixed model data to identify differences between subjects that were not related to group belonging (Graph S2[1]). Finally, to better investigate the differences between the K280N patient and the other subjects that were not group related, we treated patient indicator as a fixed effect and fitted a one-way ANOVA with post-hoc comparison. As above, to correct for multiple testing, we used the Benjamini-Hochberg approach (Benjamini and Cohen, 2017) and calculated the corresponding adjusted P values. An FDR of 0.05 was used as a cut off.

In the cTn exchange experiments, differences between groups of myofibrils or muscle strips (exchanged vs. sham-treated unexchanged controls) were analyzed using Student's *t* test for unpaired observations.

### Online supplemental material

Supplemental text, graphs, tables, and figures are included in a separate Microsoft Word file. In the Online supplemental material, clinical details of all patients used in this study are given together with an extended version of Materials and methods and a detailed description of the statistical analysis.

## Results

### Clinical characteristics of patient and identification of homozygous cTnT K280N mutation

A 23-yr-old man with progressive worsening of dyspnea on effort was diagnosed with HCM. Because of severe resting outflow tract obstruction, he underwent surgical septal myectomy. In the next few years, he became severely symptomatic, and at 26-yr of age, he was transplanted. Pathological examination of the explanted heart confirmed the diagnosis of severely obstructive HCM. The heart displayed marked asymmetric hypertrophy of the IVS (maximum thickness, 37 mm) and posterior LV wall (maximum thickness, 27 mm). Right ventricular wall was up to 10-mm thick. An adverse-restrictive evolution was observed, characterized

**Table 1. Mechanical and kinetic parameters of myofibrils from the homozygous K280N cTnT HCM patient and different groups of control patients (top four rows of data) and impact of cTn exchange in K280N and donor myofibrils (bottom six rows of data)**

Myofibril type	RT	P <sub>0</sub>	k <sub>ACT</sub>	D <sub>slow</sub>	slow k <sub>REL</sub>	fast k <sub>REL</sub>
	kN m <sup>-2</sup>	kN m <sup>-2</sup>	s <sup>-1</sup>	ms	s <sup>-1</sup>	s <sup>-1</sup>
Donors (N = 5)	10.4 ± 0.6 (n = 98)	111 ± 5 (n = 96)	0.85 ± 0.02 (n = 118)	186 ± 5 (n = 100)	0.29 ± 0.02 (n = 95)	4.18 ± 0.14 (n = 101)
LVH <sub>ao</sub> (N = 7)	9.8 ± 1.1 (n = 48)	96 ± 6 (n = 58)	0.71 ± 0.04 (n = 50)	248 ± 13 (n = 54)	0.20 ± 0.02 (n = 54)	3.15 ± 0.20 (n = 54)
HCM <sub>smn</sub> (N = 3)	8.2 ± 0.9 (n = 44)	87 ± 7 (n = 43)	0.74 ± 0.03 (n = 45)	206 ± 7 (n = 44)	0.30 ± 0.02 (n = 44)	4.62 ± 0.20 (n = 44)
K280N (N = 1)	14.9 ± 1.4* (n = 43)	87 ± 5 (n = 43)	1.73 ± 0.07* (n = 39)	226 ± 11 (n = 43)	0.63 ± 0.05* (n = 42)	3.76 ± 0.16 (n = 42)
<b>Tn-exchanged batches</b>						
K280N sham treated (N = 1)	15.9 ± 2.9 (n = 12)	89 ± 9 (n = 12)	1.69 ± 0.08 (n = 17)	240 ± 16 (n = 16)	0.69 ± 0.08 (n = 16)	3.90 ± 0.27 (n = 16)
K280N WT Tn exch (N = 1)	15.7 ± 2.0 (n = 13)	86 ± 7 (n = 14)	1.00 ± 0.09** (n = 16)	259 ± 27 (n = 15)	0.31 ± 0.05** (n = 16)	3.13 ± 0.19* (n = 16)
Donors sham treated (N = 2)	10.5 ± 0.83 (n = 64)	114 ± 7 (n = 62)	0.86 ± 0.03 (n = 70)	173 ± 6 (n = 62)	0.28 ± 0.02 (n = 58)	4.26 ± 0.18 (n = 63)
Donors WT Tn exch (N = 2)	10.1 ± 0.7 (n = 72)	91 ± 6* (n = 63)	0.84 ± 0.03 (n = 70)	179 ± 8 (n = 59)	0.30 ± 0.02 (n = 66)	4.65 ± 0.19 (n = 65)
Donor sham treated (N = 1)	10.8 ± 1.1 (n = 14)	94 ± 10 (n = 14)	0.81 ± 0.04 (n = 15)	214 ± 7 (n = 15)	0.33 ± 0.04 (n = 15)	3.78 ± 0.32 (n = 15)
Donor K280N Tn exch (N = 1)	15.9 ± 1.9* (n = 12)	77 ± 9 (n = 10)	1.04 ± 0.08** (n = 12)	186 ± 9* (n = 12)	0.60 ± 0.07** (n = 13)	4.18 ± 0.62 (n = 13)

Means ± SE; N, number of patients in the group; n, number of myofibrils; P<sub>0</sub>, maximal Ca<sup>2+</sup>-activated tension. For the statistics of the comparison between groups in the first four rows, see Supplemental materials; \*, K280N parameters for which the statistical analysis shows a significant difference versus all control groups. For the statistics of the cTn exchange experiments (bottom six rows): \*, P < 0.05; \*\*, P < 0.01 Student's *t* test for unpaired observations (exchanged myofibrils vs. sham-treated unexchanged myofibrils).

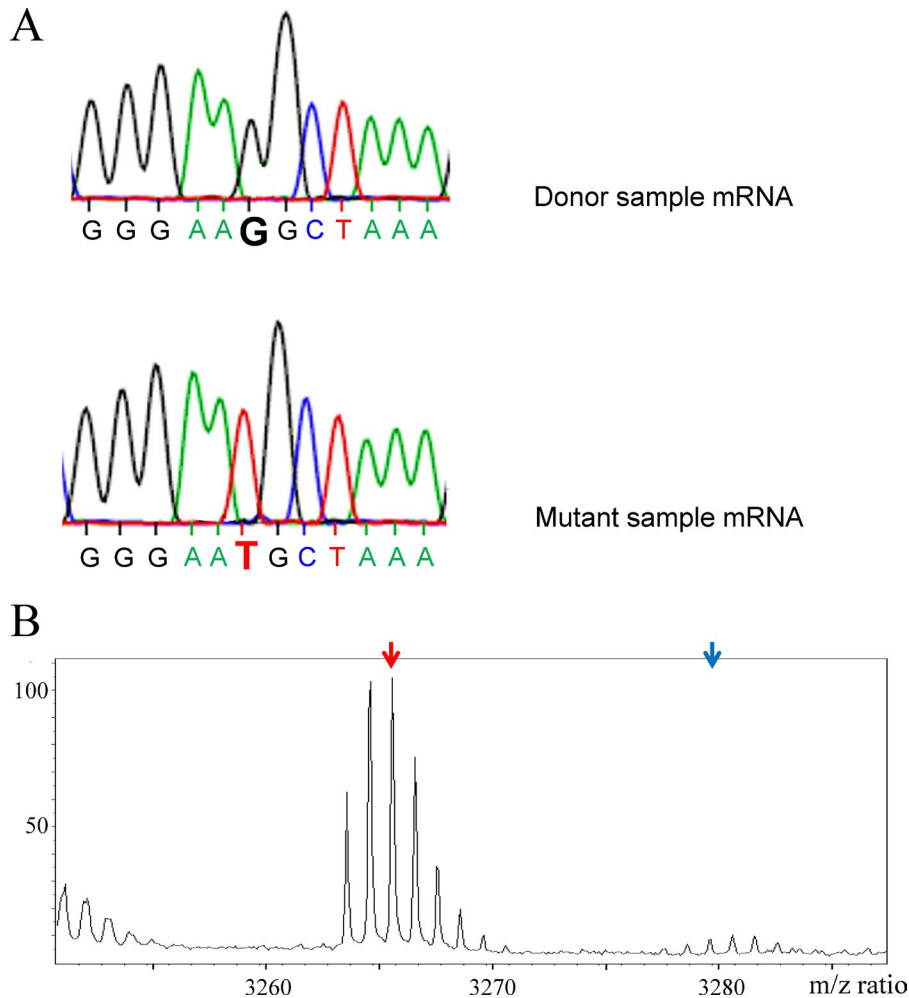
by small-sized LV cavity (i.e., LV end diastolic diameter was 46 mm; LV end systolic diameter was 23 mm). Despite resting LV ejection fraction measurements showing supernormal values (>70%), cardiac stroke volume was extremely reduced (<50 ml/beat), leading to severe heart failure symptoms (New York Heart Association Heart Failure Classification IV). Histological sections showed diffuse cardiomyopathic features: moderate fiber disarray in the septum, a moderate degree of anisocytosis (2+), mild myocytolysis (1+), and a moderate diffuse degree of interstitial fibrosis (2+). There was no indication of inflammation, and stains for iron and amyloid were negative.

Genotyping indicated a novel homozygous missense mutation c.804G>T in the *TNNT2* gene predicted to produce a K280N mutant in cTnT. The homozygous mutation was confirmed at the mRNA level (Fig. 1 A) and at the protein level by mass spectrometry, where the K280N variant was detected, and the WT protein was undetectable (Fig. 1 B). Western blot analysis showed that the protein content of cTnT relative to actin was indistinguishable in K280N and donor samples indicating that there was no haploinsufficiency and that the mutant cTnT could act as a poison peptide (Fig. 2).

### Impact of K280N cTnT mutation on myofibril mechanics and kinetics

Fig. 3 shows representative traces of tension responses to maximal Ca<sup>2+</sup> activation by fast solution, switching for K280N and aortic stenosis (LVH<sub>ao</sub>) myofibrils. Average data for both myofibril groups are shown in Table 1, together with data of myofibrils

taken from donors and HCM<sub>smn</sub> patients (for statistics, see tables and graphs in Supplemental statistics). In the K280N myofibrils resting tension (RT) was higher, compared with all control myofibril groups, although the difference between K280N and LVH<sub>ao</sub> myofibrils was uncertain because of the rather large variability among patients of the LVH<sub>ao</sub> group (Graph S2[1] and Table S2[1]). Maximal active tension (P<sub>0</sub>) of K280N myofibrils was not significantly different from that of the three control groups. The kinetics of tension generation, k<sub>ACT</sub> (as well as k<sub>TR</sub>; see Tables S1[4] and S2[4] and Graphs S1[4] and S2[4]), of K280N myofibrils were markedly faster compared with those of the three control myofibril groups (Table 1 and Fig. 2 C), indicative of faster cross-bridge turnover rate. The kinetics of full tension relaxation with step reduction of [Ca<sup>2+</sup>] are shown on a faster time scale in Fig. 2 D for LVH<sub>ao</sub> and K280N myofibrils. As previously described (Piroddi et al., 2007; Belus et al., 2008), the time course of force relaxation in human cardiac myofibrils was biphasic. It has been shown (Poggesi et al., 2005; Stehle et al., 2009) that the early slow relaxation phase occurs while sarcomeres are isometric, and its rate constant (slow k<sub>REL</sub>) is predominantly the apparent rate with which attached cross-bridges leave force-generating states. The fast exponential phase follows the “give” of a few sarcomeres and is dominated by intersarcomere dynamics. Slow k<sub>REL</sub> was markedly faster in the K280N myofibrils than in all three control groups (Table 1), indicative of faster cross-bridge detachment under isometric conditions. The duration of the slow relaxation phase and the rate constant of the fast relaxation phase (fast k<sub>REL</sub>), instead, were not significantly different in the K280N



**Figure 1. Identification of homozygous cTnT K280N mutation. (A)** Sequence of the *TNNT2* mutation at the mRNA level. The complete substitution of guanine residue at position 840 predicts a homozygous change of amino acid from a lysine (K) to an asparagine (N) at position 280. **(B)** MALDI-MS mass spectrometry of purified troponin from the HCM sample showing TnT part of the spectrum. The predicted WT human TnT peptide corresponds to an  $m/z$  ratio of 3,279.8984; the predicted mutated peptide sequence corresponds to a peptide ion with  $m/z$  ratio of 3,265.8464. The spectrum shows peaks for the reporter mutated peptide at 3,265 (red arrow) and the virtual absence of signal at 3,279 (blue arrow) for WT peptide. Tandem mass spectrometry analysis confirmed the mutated peptide sequence. The absence of the predicted WT peptide indicates that the sample has a homozygous mutation. Modified from Messer et al. (2016) supplementary data.

myofibrils compared with the three control groups (Table 1 and Supplemental statistics).

Replacement of most mutant cTnT (~70%; Fig. 4 A) by exchange of the endogenous Tn complex for a recombinant human WT cTn complex into the homozygous K280N myofibrils reduced  $k_{ACT}$  and slow  $k_{REL}$  close to control values without affecting myofibril active and resting tension ( $P_0$  and RT; Figs. 4, B and C; and Table 1). As previously reported, replacement of the endogenous cTn with the recombinant human WT complex into donor myofibrils had no effects on force kinetics (Figs. 4, B and C), though it could slightly reduce maximal tension (Table 1).

Replacement by exchange of the endogenous cTn complex for a recombinant human cTn complex containing the K280N mutant cTnT into donor myofibrils significantly accelerated  $k_{ACT}$  and increased slow  $k_{REL}$  and RT close to the values found in the myofibrils from the K280N patient (Figs. 4, B and C; and Table 1).

This result obtained from myofibrils demonstrates that the kinetic changes observed in the homozygous K280N human sample are directly related to the presence of the mutant protein. Together with  $k_{ACT}$  (as well as  $k_{TR}$ ), slow  $k_{REL}$  is especially affected by the K280N cTnT form suggesting that faster cross-bridge detachment rate, and therefore, increased energy cost of tension generation are primary effects of the mutation.

#### Impact of the K280N TnT mutation on sarcomeric tension cost

Direct demonstration of the impact of the K280N mutation on the energy cost of tension generation was given with a series of experiments in which tension and ATPase activity were simultaneously measured in demembrated muscle strips from the K280N homozygous HCM patient, from two donors, from three LVH<sub>ao</sub> patients, and from six HCM<sub>smn</sub> patients. The ratio between maximal ATPase activity and active tension generation, representing the energetic cost of tension generation, was significantly higher in the K280N compared with control muscle strips (Table 2 and Table S2[8]). While the sensitivity analysis for the parameter tension cost confirmed the presence of a systematic increase in K280N compared with both donors, four over six HCM<sub>smn</sub> and two over three LVH<sub>ao</sub>, statistical differences in maximal tension and maximal ATPase activity were uncertain because of variability issue (see Supplemental statistics). No systematic differences were found in the resting ATPase activity between the K280N and the control groups (Table 2 and Supplemental statistics).

Replacement of the mutant cTnT for the WT cTnT by cTn exchange in the K280N muscle strips lowered the maximal ATPase activity without affecting maximal tension (Figs. 5, A and B). As a result, the high tension cost of the K280N cardiac muscle was rescued to values closer to those measured in LVH<sub>ao</sub>

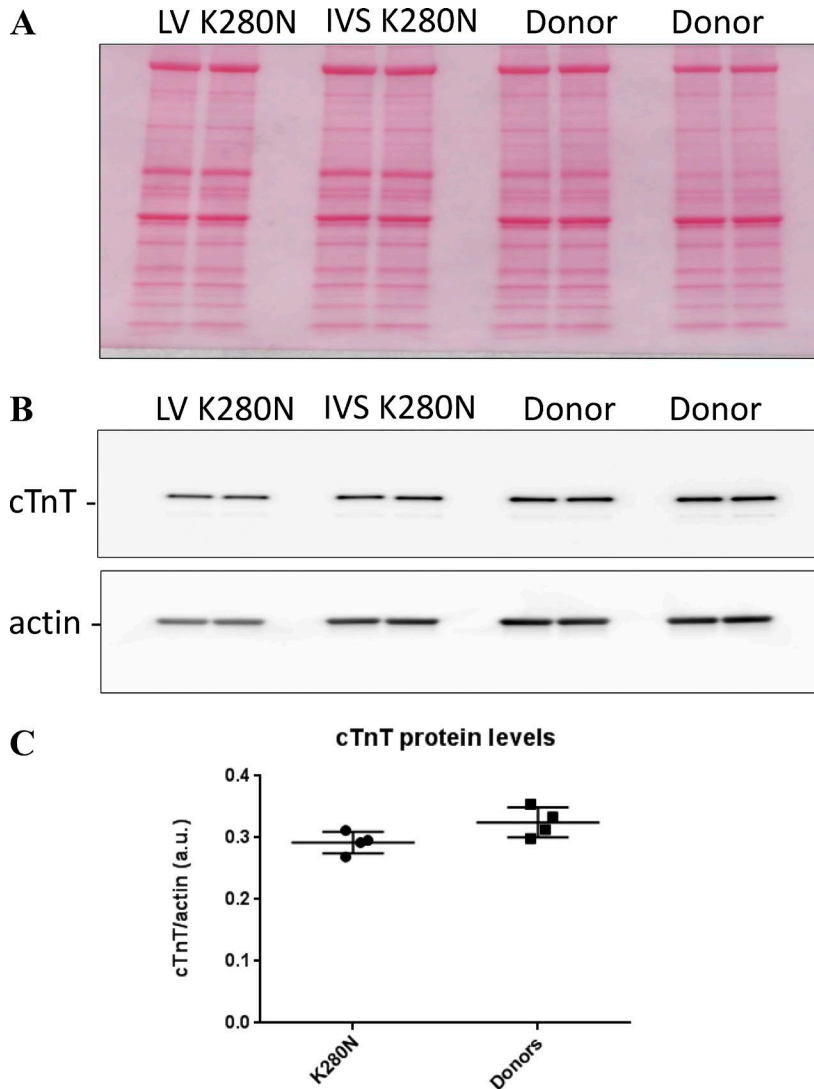


Figure 2. Western blot analysis to determine the level of cTnT in the K280N sample (LV and IVS; both in duplicate) compared with donor samples ( $n = 2$  in duplicate). (A) The Ponceau-stained blot shows equal loading of samples. (B and C) The blot was stained with anti-actin and anti-troponin T antibody to calculate the ratio between cTnT and actin, which did not differ between K280N and donor samples (C). a.u., arbitrary units.

and HCM<sub>smn</sub> preparations (Fig. 5 C and see Table 2). The effect reached significance in case of the o/n exchange procedure. The amount of endogenous K280N cTnT replaced by exogenous WT cTnT was ~50% after 4 h exchange and >80% after o/n exchange (Fig. 5 D).

Replacement of the endogenous cTnT by the exogenous recombinant K280N cTnT by cTn exchange in HCM<sub>smn</sub> preparations did not significantly modify maximal tension and maximal ATPase activity (Figs. 5, E and F), but tended to increase tension cost in the 4 h exchanged HCM<sub>smn</sub> muscle strips (Fig. 5 G). The amount of endogenous cTnT in the HCM<sub>smn</sub> muscle strips exchanged after 4 h by the recombinant exogenous K280N cTnT yielded ~50% based on cTnT phosphorylation differences (Fig. 5 H).

All together Tn replacement experiments in the strips indicate that >50% of the mutant protein is needed to significantly increase tension cost.

## Discussion

Previously reported data from the same homozygous patient (Sequeira et al., 2013; Messer et al., 2016) had a different focus,

i.e., the impact of HCM mutations on length dependent activation and post-translational changes of regulatory proteins. Here, we focus on kinetic and energetic parameters measured at maximal Ca<sup>2+</sup> activation and optimum myofilament overlap that are either unaffected or are scarcely affected by post-translational changes of regulatory proteins. We compared human cardiac sarcomeres expressing 100% of the K280N cTnT mutation with those from several reference groups of patients and found that the mutation is specifically associated with large changes in cross-bridge kinetics. These changes do not affect maximal tension, but lead to increased energy cost of tension generation. Tn replacement experiments show that the sarcomere kinetic and energetic changes are primary effects of the HCM-associated K280N cTnT mutation rather than secondary effects of disease-related remodeling. Faster cross-bridge detachment under isometric conditions and higher energy cost of tension generation may be a common feature to several HCM-associated mutations (Belus et al., 2008; Ferrantini et al., 2009; Luedde et al., 2009; Witjas-Paalberends et al., 2013, 2014b).

The K280N is a novel mutation in cTnT. The clinical features of the patient and the biophysical phenotype of his cardiac tissue

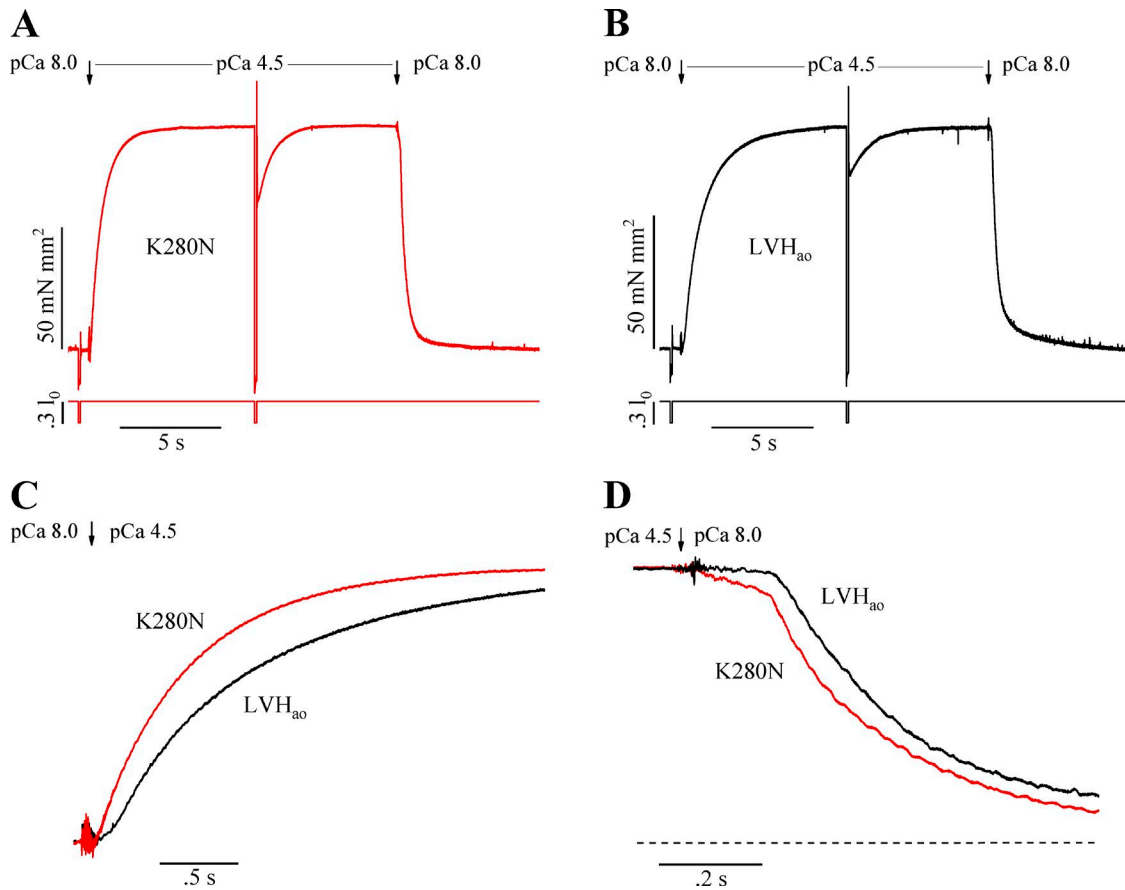


Figure 3. **Impact of K280N cTnT mutation on myofibril mechanics and kinetics.** (A and B) Representative records of maximum tension activation and full relaxation in response to sudden pCa changes by fast solution switching in K280N (A) and LVH<sub>ao</sub> (B) myofibril preparations. pCa changes at arrows as indicated. Lower traces show fast release–restretch protocol for  $k_{TR}$  registration at steady Ca<sup>2+</sup>-activation. (C and D) Time courses of tension activation (C) and relaxation (D) of K280N and LVH<sub>ao</sub> myofibrils superimposed after normalization to maximal tension (same traces as in A and B on faster time base).

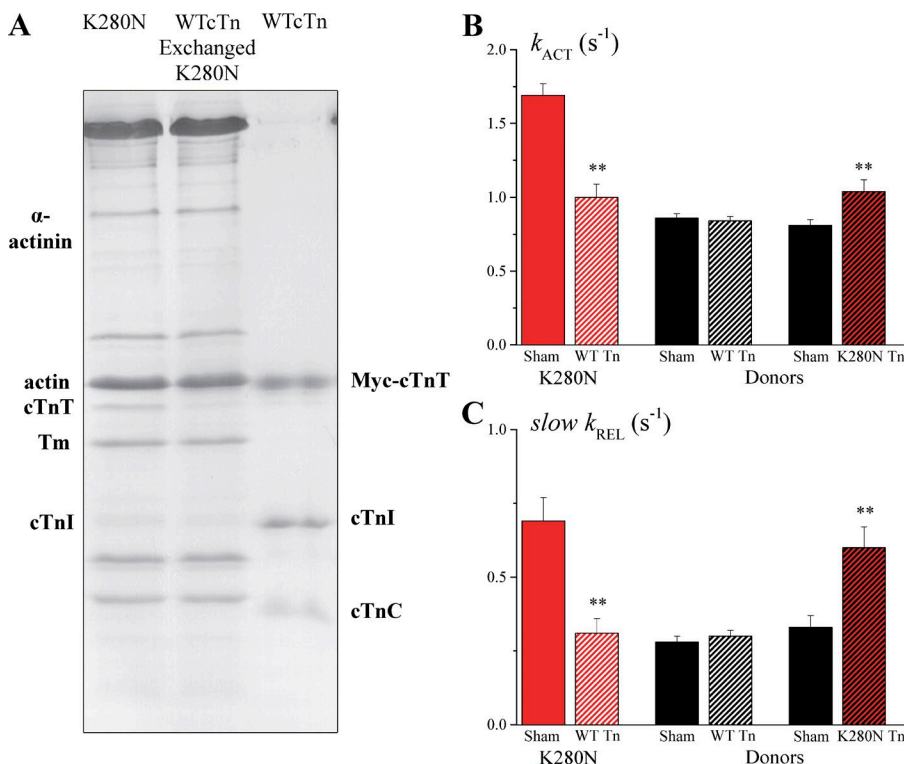


Figure 4. **cTn replacement in K280N myofibrils.** (A) 15% SDS-PAGE gel of unexchanged K280N myofibrils (first lane), WTcTn exchanged K280N myofibrils (second lane), and WT recombinant cTn with Myc-tag cTnT (third lane). The extent of the exchange estimated from the intensity ratio of the endogenous cTnT band to the α-actinin band in the first two lanes was around 70%. (B and C) Effects of Tn replacement by exchange on  $k_{ACT}$  (B) and slow  $k_{REL}$  (C) of K280N (first two columns in each panel) and donor myofibrils (last four columns in each panel). Donor myofibrils were exchanged either with WT Tn (third and fourth column) or K280N Tn (last two columns). Bars are SEM. \*\*,  $P < 0.01$ . Student's  $t$  test for unpaired observations (exchanged myofibrils vs. sham-treated unexchanged myofibrils).

Table 2. Mechanical and energetic parameters of permeabilized ventricular strips from the homozygous K280N cTnT HCM patient and three groups of control patients

	K280N (N = 1, n = 16)	Donors (N = 2, n = 12)	LVH <sub>ao</sub> (N = 3, n = 13)	HCM <sub>smin</sub> (N = 6, n = 29)
Maximal tension (kN m <sup>-2</sup> )	13.5 ± 1.8*	38.7 ± 1.2	21.3 ± 1.5	25.6 ± 1.6
Maximal ATPase activity (μmol L <sup>-1</sup> s <sup>-1</sup> )	38.4 ± 4.8	91.0 ± 4.7	52.2 ± 6.3	45.2 ± 2.7
Tension cost (μmol L <sup>-1</sup> s <sup>-1</sup> / kN m <sup>-2</sup> )	3.1 ± 0.3*	2.3 ± 0.1	2.5 ± 0.3	1.8 ± 0.1
Basal ATPase activity (μmol L <sup>-1</sup> s <sup>-1</sup> )	4.6 ± 0.8	10.9 ± 2.1	10.3 ± 2.2	7.2 ± 1.0

Means ± SE; N, number of patients in the group; n, number of muscle strips. For the statistics of the comparison between groups, see Supplemental materials; \*, K280N parameters for which the statistical analysis shows a significant difference versus control groups.

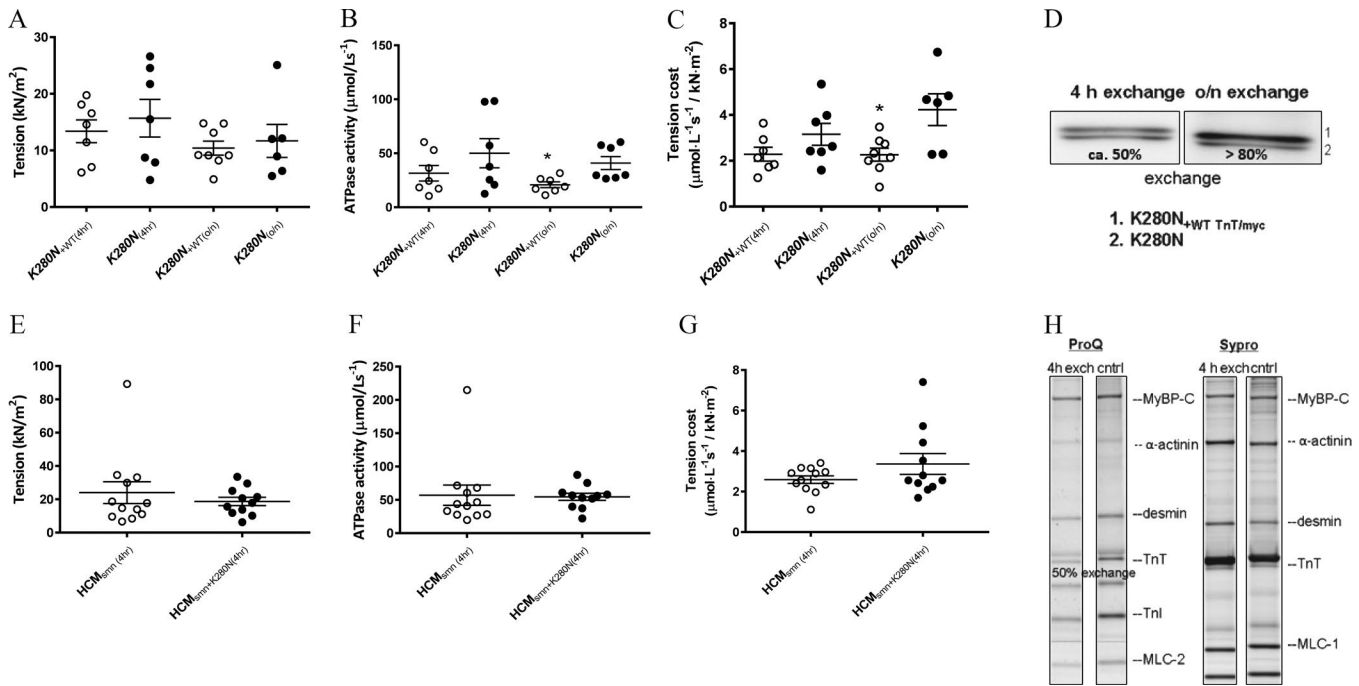


Figure 5. Impact of K280N cTnT mutation on skinned myocardium mechanics and energetics. (A–C) Tn replacement by exchange with WT Tn in K280N demembrated muscle strips. Impact of 4-h and o/n exchange protocols on maximal tension (A), maximal ATPase (B), and tension cost (C). Tn-exchanged preparations (K280N<sub>WT Tn</sub>) are compared with the corresponding sham-treated unexchanged preparations (K280N). \*, P < 0.05. Student's *t* test for unpaired observations. (D) Replacement of the endogenous mutant Tn with WT Tn containing cTnT labeled with a Myc-tag was assessed in K280N muscle strips after 4 h and o/n exchange using 13% SDS-polyacrylamide gel and immunoblotting. (E–G) Tn replacement by exchange with K280N Tn in HCM<sub>smin</sub> demembrated muscle strips. Impact of 4-h exchange protocol on maximal tension (E), maximal ATPase (F), and tension cost (G). K280N Tn-exchanged preparations (HCM<sub>smin</sub>+K280N Tn) are compared with sham-treated unexchanged HCM<sub>smin</sub> preparations (HCM<sub>smin</sub>). (H) The amount of exchange was determined using ProQ-Diamond phospho-stained one-dimensional gels (left). The phosphorylation signals of the ProQ-Diamond-stained cTnT were normalized to the intensities of the SYPRO Ruby stained cTnT (right) to correct for protein loading.

that can be rescued by Tn replacement strongly suggest that the novel mutation is pathogenic for HCM. HCM is generally caused by the autosomal dominant action of heterozygous mutations and the finding of homozygous mutations is very rare. Homozygous carriers of HCM sarcomeric mutations have been previously reported to develop more severe phenotypes compared with heterozygous carriers (Ho et al., 2000; Lin et al., 2000; Richard et al., 2000; Knöll et al., 2010). In particular, there have been two previous reports of homozygous mutations in *TNNT2* (Ho et al., 2000; Lin et al., 2000). In the case of the S179F cTnT mutation (Ho et al., 2000), the single affected individual had severe HCM with sudden death at age 17, and three heterozygous siblings and

one parent were asymptomatic (the other parent had exertional chest pain). In the second case, 11 members of a Japanese family carried the F110I cTnT mutation (Lin et al., 2000). Cardiac hypertrophy and other clinical features were severe in the two subjects homozygous for the mutation, while the nine heterozygous subjects either suffered from less severe disease or showed no clear evidence of hypertrophy. Unfortunately, family information is not available for the K280N proband in this study. However, the early and severe clinical phenotype of the K280N patient seems to confirm previous observations of the severity of homozygous mutations. The demonstration that the homozygous K280N mutation resulted in 100% mutant cTnT with no evidence of haplo-



Table 3. Estimate of the apparent rate of cross bridge attachment ( $f_{app}$ ) and duty ratio (proportional to maximal tension) from myofibril kinetic measurements

Myofibril type	$g_{app}$ ( $\alpha Tc$ )	$f_{app}$	Estimated duty ratio ( $\alpha P_0$ )
	slow $k_{REL}$	( $k_{ACT}$ -slow $k_{REL}$ )	( $f_{app}/f_{app}+g_{app}$ )
Donors (N = 5)	0.29 ± 0.02 (95)	0.55 ± 0.03 (93)	0.63 ± 0.02 (93)
L <sub>VH</sub> <sub>ao</sub> (N = 7)	0.20 ± 0.02 (54)	0.39 ± 0.04 (34)	0.675 ± 0.05 (34)
HCM <sub>snn</sub> (N = 3)	0.30 ± 0.02 (44)	0.43 ± 0.04 (42)	0.56 ± 0.04 (42)
K280N (N = 1)	0.63 ± 0.05* (42)	1.14 ± 0.08 (38)*	0.635 ± 0.03 (38)

Means ± SE; N, number of patients in the group; numbers in parenthesis are the numbers of myofibrils in each group. \*,  $f_{app}$  is significantly higher in K280N myofibrils versus all control groups.

insufficiency further supports the idea of a gene dose-dependent effect of HCM mutations on the severity of the phenotype.

For comparison with the data from the K280N myofibrils here we used tissue from different “control” groups. Samples from donor hearts could be taken, in principle, as the example of healthy nonhypertrophic nonfailing cardiac muscle. However, potential problems related to the use of cardiac samples from explanted hearts that are not used for transplantation for technical reasons have raised some concerns (Marston and de Tombe, 2008). The variability among different donors in the parameters describing overall myofibril relaxation ( $D_{slow}$  and fast  $k_{REL}$ ) may be related to some of those concerns (see Supplemental statistics). L<sub>VH</sub><sub>ao</sub> patients present with hypertrophic phenotype, and their IVS tissue may share some features of the cardiac muscle remodeling observed in HCM patients. L<sub>VH</sub><sub>ao</sub> seems to be the most heterogeneous of the control groups; several parameters (e.g., myofibril RT and kinetic parameters) show a variability among patients that is larger than that observed in the other control groups (see Supplemental statistics). The HCM<sub>snn</sub> patients do not harbor a sarcomeric gene mutation after thoroughly screening nine genes, but present with a similar clinical phenotype as the HCM patients carrying myofilament protein mutations. This suggests they could be the best reference group when seeking for the direct impact of a mutant protein associated with HCM. Independent of the above considerations, the kinetics of force generation ( $k_{ACT}$ ) and isometric relaxation (slow  $k_{REL}$ ) of the K280N myofibrils significantly differed from those of each patient of all control groups (see Supplemental statistics).

The markedly enhanced slow  $k_{REL}$  found in the K280N myofibrils indicates that the rate of cross-bridge detachment under isometric conditions is accelerated by the mutation (Poggesi et al., 2005; Stehle et al., 2009) though other relaxation parameters ( $D_{slow}$  and fast  $k_{REL}$ ) are unaffected (Fig. 3 and Table 1). Cross-bridge detachment rate can in principle affect all relaxation parameters, but a strict correlation between slow  $k_{REL}$ ,  $D_{slow}$ , and fast  $k_{REL}$  is not necessarily expected. The duration of the slow initial phase of relaxation is dependent on the ability of the sarcomeres to remain isometric, and changes in sarcomere homogeneity can affect both the time of appearance of the tension shoulder ( $D_{slow}$ ) and the intersarcomere dynamics that dominate fast  $k_{REL}$ . In addition, both  $D_{slow}$  and fast  $k_{REL}$ , at variance with slow  $k_{REL}$ , are sensitive to the possibility that a few force generating cross-bridges can still be formed after sudden  $Ca^{2+}$  removal because of im-

paired thin filament switch off mechanisms (Poggesi et al., 2005; Stehle et al., 2009). The K280N cTnT mutation appears to impact myofibril RT that is consistently higher in the mutant myofibrils compared with controls (Table 1). The mechanism underlying the increase in RT associated with the K280N mutation has not been investigated in the present study, but residual cross-bridge attachment may contribute to increase myofibril tension in the virtual absence of  $Ca^{2+}$ . Replacement of most mutant protein for the WT form in the K280N myofibrils was unable to reduce RT (Table 1). However, replacement of the endogenous WT Tn with the K280N mutant complex into donor myofibrils did significantly increase RT (Table 1). These apparently contrasting results suggest that a modest amount of the mutant protein is enough to impair contraction switch off and increase myofibril tension in the virtual absence of  $Ca^{2+}$ . The amount of Tn replacement was never 100% and varied with experimental conditions (e.g., Figs. 4, D and H; and Fig. S1). Specific changes due to the mutation may be differently related to the amount of mutant protein present in the sarcomere. Further work, however, is required to better investigate this issue.

The K280N mutation in cTnT accelerates cross-bridge kinetics, which is supported by the markedly enhanced  $k_{ACT}$  and slow  $k_{REL}$  reported here, but does not change myofibril maximal force (Fig. 3 and Table 1). According to a simple two-state cross-bridge model (Brenner, 1988), force-activation kinetics reflect cross-bridge turnover rate, and under the present experimental conditions (nominally zero  $[P_i]$ ),  $k_{ACT}$  reports the sum of the apparent rate constants  $f_{app}$  and  $g_{app}$  that rate limit the transition of cross-bridges from nonforce-generating to force-generating states and from force-generating to nonforce-generating states, respectively. In terms of cross-bridge turnover kinetics, slow  $k_{REL}$  is a measure of  $g_{app}$  that in the model is also equal to the tension cost (isometric ATPase per unit force). Force is proportional to the apparent duty ratio [ $f_{app} / (f_{app} + g_{app})$ ], and an increase in  $g_{app}$  should lead to a decline in the force generating capacity of the sarcomere, unless the increase in  $g_{app}$  is compensated for by an increase in  $f_{app}$ . Estimating  $f_{app}$  from the difference between  $k_{ACT}$  and slow  $k_{REL}$  and  $g_{app}$  from slow  $k_{REL}$  yields duty ratios that are not significantly different in K280N versus all control groups of myofibrils (Table 3). Our analysis reveals that the estimated  $f_{app}$  is significantly higher in the K280N myofibrils than in the myofibrils of all control groups. Myofibril kinetic data support the idea that the K280N mutation does increase  $f_{app}$  (two- to threefold)

besides increasing  $g_{app}$  (two- to threefold) with no impact on sarcomere force generation.

Following  $Ca^{2+}$ -activation,  $f_{app}$  (the probability of the cross-bridge transition to force-producing states) behaves as a second order rate constant that can be regarded as the product of the fraction of acto-myosin complexes in a preforce-generating state available for force generation multiplied by the rate constant of the forward transition from this preforce state to force states. One possibility is that the K280N cTnT mutation increases the fraction of the acto-myosin complexes available for force generation at maximal  $Ca^{2+}$  activation rather than their forward transition rate constant. As to the impact of the mutation on  $g_{app}$  shown by present results, pioneer studies (Potter et al., 1995; Bing et al., 1997; Sweeney et al., 1998) reviewed by Gordon et al. (2000) reported that HCM-associated cTnT mutations may cause changes in regulated acto-S1 ATPase and unloaded thin filament sliding speed. This implies that cTnT can modulate the cross-bridge kinetics of the strongly bound state in addition to its ability to control the attachment of cross-bridges to the thin filament. Potential molecular mechanisms of the effects of the K280N mutation remain, however, unclear also because the extreme C-terminal domain of cTnT could not be resolved in the extant structures (Takeda et al., 2003), because of the high degree of inherent protein flexibility required to modulate the physical position of Tm in response to  $Ca^{2+}$  binding.

In K280N multicellular strips, at variance with myofibrils, maximal force was lower than in HCM<sub>smn</sub> and LVH<sub>ao</sub> (though sensitivity analysis makes the difference vs. the LVH<sub>ao</sub> group uncertain). Myocardial remodeling such as cellular hypertrophy, lower myofibrillar density, and fibrosis may contribute to lower maximal force in the K280N multicellular preparations. Indeed, a decrease in myofibrillar density has previously been correlated with the decrease in the maximal force generating capacity of skinned cardiomyocytes from HCM patients carrying different mutations (Witjas-Paalberends et al., 2013). However, tension cost measurements and the results of Tn replacement experiments in skinned strips of cardiac muscle substantiate the idea that the K280N mutation primarily increases the energetic cost of tension generation by increasing the rate of force generating cross-bridge detachment.

### Study limitations

One limitation of the present study is the fact that a single HCM patient carrying a novel pathogenic cTnT mutation has been investigated. At present, no possibility to recruit additional homozygous patients with this K280N mutation exists. Performing a meaningful statistics to compare the study group (K280N) constituted by a single patient with control groups (donors, HCM<sub>smn</sub>, and LVH<sub>ao</sub>) constituted by a variable number of subjects is challenging and we had to use a multistep procedure, illustrated in details in the Supplemental material and summarized in the Materials and methods. As a final step of this statistic procedure, we performed a sensitivity analysis, and we looked for the presence of individual differences between subject 1 (K280N) and the several control subjects, not related to group identity. This sensitivity analysis allowed us to conclude for a systematic difference between K280N and controls for three parameters:  $k_{ACT}$ , slow  $k_{REL}$ , and tension cost.

Sarcomere post-translational changes were not investigated in this study. In particular, differences in the phosphorylation status of the sarcomeric proteins may alter the kinetics of activation and relaxation (Rao et al., 2014), though protein kinase A treatment seems unable to affect the kinetics of force generation and relaxation of human cardiac myofibrils (Walker et al., 2011). Similar cTnI phosphorylation levels were observed in the K280N and donor samples (unpublished data), suggesting that the large kinetic differences found in the K280N myofibrils compared with donors cannot be explained by differences in the sarcomere phosphorylation status. Importantly, the lack of impact of recombinant unphosphorylated WT Tn exchanged into donor myofibrils on force kinetics argues against a significant effect of cTnI phosphorylation on human cardiac myofibril kinetics. Among control groups used in the present study, we had previously reported differences in the phosphorylation status (van Dijk et al., 2012) that do not result here into force kinetic changes of maximally activated myofibrils. For instance, protein kinase A phosphorylation of cTnI in HCM<sub>smn</sub> samples was significantly lower compared with donors (van Dijk et al., 2012), while  $k_{ACT}$  and slow  $k_{REL}$  were the same (Table 1). Our results indicate that cTnI phosphorylation status does not alter kinetics and tension cost of maximally activated cardiac preparations, which is in line with results of Walker et al., 2011. In human septal HCM samples from patients with thick-filament mutations, we previously identified significant post-translational modifications in Excitation Contraction Coupling and sarcolemmal proteins caused by a maladaptive change in calcium-/calmodulin-dependent protein kinase II (CaMKII) signaling (Coppini et al., 2013). Similar variations of CaMKII activity were observed in two HCM mouse models carrying different *TNNT2* mutations (R92Q and E163R; Coppini et al., 2017; Ferrantini et al., 2017). We cannot exclude CaMKII-related changes in the K280N myocardium, but it is unlikely they are responsible for the sarcomere kinetic and energetic alterations reported in the present work.

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