Chapter 6

A novel HCM mutation in cardiac troponin T primarily alters cross-bridge kinetics and increases the energetic cost of tension generation in human cardiac tissue

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Abstract

A novel homozygous mutation in the *TNNT2* gene encoding cardiac troponin T (cTnT K280N) was identified in one hypertrophic cardiomyopathy (HCM) patient undergoing cardiac transplantation. Mass spectrometry, protein and mRNA analyses revealed expression of the mutant alleles without evidence of haploinsufficiency. Kinetics of contraction and relaxation of myofibrils from a frozen left ventricular sample of the K280N HCM patient were compared with those of control myofibrils from donor hearts, from aortic stenosis patients (LVH_{ao}), and from HCM patients negative for sarcomeric protein mutations (HCM_{smn}). Preparations, mounted in a force recording apparatus (15 °C), were maximally Ca^{2+}-activated (pCa 4.5) and fully relaxed (pCa 8) by rapid (<10 ms) solution switching. The rate constant of active tension generation following maximal Ca^{2+} activation (k_{act}) was markedly faster in K280N myofibrils compared with all control groups. The rate constant of isometric relaxation (slow k_{rel}) was 2-3-fold faster in K280N myofibrils than in all controls. This indicates that the apparent rate with which cross-bridges leave the force generating states is accelerated in the mutant preparations. The results suggest that the energetic cost of tension generation is increased in the K280N sarcomeres. Simultaneous measurements of maximal isometric ATPase activity and Ca^{2+}-activated force in Triton-permeabilized left ventricular muscle strips from the K280N sample demonstrated that tension cost (TC) was markedly higher in the K280N than in LVH_{ao} and HCM_{smn} myocardium. Replacement of the mutant protein by exchange with wild-type recombinant human cTn in the K280N preparations reduced both slow k_{rel} and TC close to control values. In donor myofibrils and in HCM_{smn} demembranated strips, replacement of endogenous cTn by exchange with cTn containing the K280N mutant cTnT increased or tended to increase both slow k_{rel} and TC. This demonstrates that the HCM-associated *TNNT2* mutation K280N primarily alters cross-bridge kinetics and impairs sarcomere energetics.

Introduction

Hypertrophic cardiomyopathy (HCM) is a primary disorder of cardiac muscle. Traditionally, it has been characterized by the presence of unexplained left ventricular hypertrophy which occurs in the absence of any other known etiology. HCM is often familial with autosomal dominant inheritance. Genome-wide linkage studies in the 1980s led to the discovery of pathogenic mutations in genes that encode different components of the contractile apparatus. 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 myosin binding protein C (*MYBPC3*) for which evidence of haploinsufficiency has been generated[1,2], most HCM mutations appear to act as dominant negatives and the mutant proteins are incorporated into the sarcomere where they may directly alter contractile performance.[3,4] The pathogenic impact of sarcomeric HCM mutations has been varyingly attributed to aberrant cross-bridge dynamics leading to decreased or increased contractility[3,5–7], increased intrinsic force of the myosin motor [8,9], increased sarcomeric Ca^{2+}-sensitivity.[10] To reconcile the lack of consistent contractility changes in HCM, it has been proposed that HCM sarcomere mutations may lead to increased energetic 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.[11] Several studies on HCM disease mechanisms in patient hearts and animal models support this hypothesis.[5,12–18] Though collectively these studies suggest that energy deficiency is a primary event and 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.

To discriminate between mutation-induced and disease-related changes in myofilament function, sarcomere mechanics and energetics were analyzed here in isolated myofibrils and demembranated multicellular cardiac muscle strips from an HCM patient carrying a homozygous charge mutation (K280N) in the *TNNT2* gene and compared with preparations of non-failing donors, sarcomere mutation-negative HCM patients (HCM_{smn}) and patients with left ventricular (LV) hypertrophy due to aortic stenosis (LVH_{ao}).

Methods

Cardiac tissue

Genotyping of a 26 year old male HCM patient who underwent cardiac transplantation surgery identified a homozygous missense mutation (K280N) in the gene encoding cTnT. Tissue from the free left ventricular wall and interventricular septum (IVS) of the explanted heart was collected in cardioplegic solution and immediately frozen in liquid nitrogen. Samples of non-failing LV or IVS tissue were obtained from 5 donor hearts, 9 patients who underwent aortic valve replacement due to aortic stenosis (LVH_{ao} group), 9 HCM patients without an identified sarcomeric gene mutation after screening of 8 sarcomere genes who underwent myectomy to relieve LV outflow tract obstruction (sarcomere mutation-negative; HCM_{smn} group). Clinical details of the homozygous cTnT mutant patient and all other

patients are given in Table 1. Written informed consent from each patient was obtained before surgery and the study was approved by the local Ethics Committees.

Table 1: Patient characteristics.

Patient	Age	Sex	Surgery	LVOT	ST
Donor					
1	24	F	Explantation		
2	39	M	Explantation		
3	65	M	Explantation		
4	40	M	Explantation		
5	19	M	Explantation		
LVH_{ao}					
1	65	F	AoV repl.		
2	75	M	AoV repl.		
3		F	AoV repl.		
4	76	F	AoV repl.		
5	66	F	AoV repl.		
6	40	M	AoV repl.	115	15
7	76	F	AoV repl.	56	14
8	74	F	AoV repl.	61	14
9	75	F	AoV repl.		
HCM_{smn}					
1	35	M	Myectomy	76	30
2	54	M	Myectomy	100	33
3	42	M	Myectomy	75	26
4	52	M	Myectomy	169	22
5	65	F	Myectomy	85	19
6	72	F	Myectomy	88	24
7	49	M	Myectomy	61	20
8	46	M	Myectomy	81	19
9	59	M	Myectomy	85	18
K280N					
1	26	M	Transplantation		

M, male; F, female; LVOT, left ventricular outflow tract pressure gradient in mmHg; ST, septal thickness in mm; AoV repl., Aortic Valve replacement surgery.

Identification of mutation

Total RNA was extracted from the human heart biopsies using the SV Total RNA Isolation kit (Promega) according to the manufacturer's instructions. RNA concentration, purity and quality were determined using the NanoDrop® ND-1000 spectrophotometer. Reverse transcriptase was performed using oligo-dT primers with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) from 100 ng RNA. The quantitative determination of cTnT mRNA was performed by real-time PCR using the Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) and the primers F: 5'-TCGACCTGCAGGAGAAGTT-'3 and R: 5'-GAGCGAGGAGCAGATCTTTG-'3. GAPDH (F: 5'-ATGTTCGTCATGGGTGTGAA-'3 and R: 5'-TGAGTCCTTCCACGATACCA-'3) was used as endogenous referent gene. Experiments were performed on the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). The mRNA amount was estimated according to the comparative Ct method with the 2-ΔΔCt formula.

Myofibril experiments

To isolate myofibrils from frozen human cardiac samples and perform mechanical experiments we used previously published techniques.[5,19] Thin strips dissected from the ventricular samples were permeabilized overnight in ice cold rigor solution (pH 7.1; 132 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris, 5 mM EGTA) added with 1% Triton-X100. Demembranated strips were then homogenized in rigor solution to produce myofibril suspensions.

Single myofibrils or bundles of few myofibrils (25–80 µm long, 1–4 µm wide) were mounted horizontally between two glass microtools in a temperature-controlled chamber (15°C) filled with relaxing solution (pCa 8.0). One tool was connected to a length-control motor that could produce rapid (<1ms) length changes. The second tool was a calibrated cantilevered force probe (2–6 nm/nN; frequency response 2–5 kHz). Force was measured from the deflection of the image of the force probe projected on a split photodiode. Average sarcomere length and myofibril diameter were measured from video images (ca 1800x). The initial sarcomere length of the preparations was set around 2.2 µm. Myofibrils were activated and relaxed by rapidly translating the interface between two flowing streams of activating (pCa 4.5) and relaxing (pCa 8.0) solutions across the length of the preparation. The solution change was complete in less than 5 ms.

Activating and relaxing solutions were at pH 7.00 and contained 10mM total EGTA (CaEGTA/EGTA ratio set to obtain pCa 8.0 and 4.5), 5 mM MgATP, 1 mM free Mg^{2+}, 10 mM Mops, propionate and sulphate to adjust the final solution to an ionic strength of 200 mM and monovalent cation concentration of 155 mM. Creatine phosphate (CP; 10mM) and creatine kinase (200 unit/ml) were added to all solutions. Contaminant inorganic phosphate (P_i) from spontaneous breakdown of MgATP and CP was reduced to less than 5 µM by a P_i scavenging system (purine-nucleoside-phosphorylase with substrate 7-methyl-guanosine). All solutions to which the samples and myofibrils were exposed contained a cocktail of protease inhibitors including leupeptin (10 µM), pepstatin (5 µM), PMSF (200 µM) and E64 (10 µM), as well as NaN_3 (500 µM) and DTE (2 mM).

Measurements in multicellular cardiac muscle strips

Multicellular cardiac muscle strips were cut parallel to the long axis of the cardiomyocytes to minimize damage in cold relaxing solution (pH 7.0; 1 mM free Mg^{2+}, 145 mM KCl, 2 mM EGTA, 4 mM ATP, 10 mM imidazole). Dissected muscle strips were permeabilized overnight in relaxing solution with 1% Triton at 4°C. The experimental procedures, solutions and equipment used to measure maximal tension and ATPase in human cardiac strips were as described previously.[18,20,21] The muscle strips were stretched to a sarcomere length of 2.2 µm. Maximal isometric force and ATPase activity were measured at saturating [Ca^{2+}] at 20°C. Maximal force was determined after the force signal reached a plateau and normalized to the cross-sectional area (CSA) of the muscle strip to calculate tension. CSA of the preparation was estimated assuming an elliptical shape, CSA = (width x depth x π)/4. ATPase activity was measured using an enzyme coupled assay in which ATP regeneration from ADP and phosphoenol-pyruvate by the enzyme pyruvate kinase is coupled to the oxidation of NADH to NAD^+ and the reduction of pyruvate to lactate by L-lactic dehydrogenase. NADH oxidation was photometrically measured from the absorbance at 340 nm of near-UV light. The maximal Ca^{2+}-activated ATPase activity was calculated by normalizing the maximal NADH oxidation to the volume of the muscle strip and subtraction of the resting NADH oxidation (resting ATPase activity) normalized to muscle strip volume. Resting NADH oxidation was measured in relaxing solution.

Recombinant cTn preparation and exchange in myofibrils and muscle strips

We developed recombinant wildtype (WT) and mutated K280N cTnT which was build into a cTn construct with troponin C (cTnC) and troponin I (cTnI) as described previously.[22] The whole troponin complex exchange was achieved in human cardiac myofibrils and multicellular muscle strips by mass displacement following protocols adapted from previously described techniques.[23–25] K280N and donor myofibrils were pelleted by centrifugation and re-suspended in a high Ca^{2+} rigor buffer (10 mM imidazole, 170 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EGTA and 3 mM $CaCl_2$) containing 0.5 mg/ml of the exogenous Tn complex (containing either WT cTnT or K280N cTnT). The exchange was allowed overnight at +4°C. Before being used for mechanical investigation and exchange quantification the myofibrils were washed 2 times and re-suspended in usual rigor solution.

The K280N, donor and HCM_{smn} demembranated muscle strips were washed twice with rigor solution and once with exchange buffer (10 mM imidazole, 200 mM KCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mM DTT). Subsequently, the muscle strips were exchanged for either 4 hours or overnight (o/n) (to check differences in amount of exchange over time) in the same exchange buffer containing the exogenous cTn (1 mg/mL in the case of WT cTn exchanged into the K280N preparations, 0.250 mg/mL in the case of cTn containing the K280N mutant TnT exchanged into the HCM_{smn} preparations) with the addition of (in mM) $CaCl_2$ 4, KOH 4.8, DTT 4 and 5 µl/mL protease inhibitor cocktail (PIC, Sigma, P8340). After the exchange the muscle strips were washed twice with rigor solution and once with relaxing solution and used to investigate sarcomere energetics. K280N and HCM_{smn} muscle strips incubated for either 4 hours or o/n in the exchange solution without the exogenous cTn complex were used as controls.[22]

Determination of degree of troponin exchange

Myofibrils

The degree of Tn exchange in K280N and donor myofibrils exchanged with WT Tn containing recombinant cTnT labelled with a Myc-tag was estimated using one-dimensional SDS-PAGE electrophoresis with 15 % acrylamide. The recombinant K280N mutant cTnT exchanged into donor myofibrils did not carry a Myc-tag and we were unable to estimate the extent of the exchange. The composition of the separating gel, staking gel, running and sample buffers were as follows:

Separating gel: acrylamide/bis-acrylamide (200:1) 7.5 ml, 3 M Tris (pH 9.3) 3.75 ml, H_2O 2.05 ml, glycerol 1.55 ml, 10 % SDS 0.15 ml, 10 % APS 0.045 ml and Temed 0.018 ml. *Stacking gel:* acrylamide/bis-acrylamide (20:1) 0.63 ml, 0.5 M Tris (pH 6.8) 1.5 ml, H_2O 3.17 ml, glycerol 0.6 ml, 10 % SDS 0.06 ml, 10 % APS 0.024 ml and Temed 0.0204 ml. *Running buffer:* Tris 18 g, glycine 86.4 g, SDS 4g, H2O 6000 ml. *Sample buffer (Laemli)*: 62.5 mM Tris (pH 8.8), 10 % glycerol, 2.3 % SDS, 5% bromphenolblue, 5 % β-mercaptoethanol.

Before loading, samples were boiled for 90 sec and centrifuged at 4000g for 10 min at room temperature to remove undissolved particles. Gels were run for 4.5 hours at constant current 16 mA (air conditioning on, stirring of the lower buffer). Subsequently, gels were fixated for 30 min in fixating solution (50 % methanol, 12 % acetic acid) and stained with 0.1 % Coomassie solution (40 % methanol, 10 % acetic acid) for one hour. Gels were destained by rapid destaining solution (40% methanol, 7% acetic acid) for 30 min and slow destaining solution (5% methanol, 7% acetic acid) for 5-6 hours.

To evaluate the extent of the exchange, densitometric measurements of the ratio between the intensities of native cTnT and α-actinin were compared in sham treated and exchanged myofibrils. In exchanged myofibrils this ratio decreased since endogenous cTnT was replaced by the Myc-tagged WT cTnT that migrated overlapped to the actin band. The α-actinin band was chosen as a reference to avoid problems related to signal saturation as its intensity was close to that of TnT. This approach, based on estimate of residual native proteins, also avoids possible artifacts from incomplete wash out of the exogenous proteins from myofibril suspensions after the exchange and/or a specific binding to the myofibrils.

Multicellular muscle strips

After the functional experiments the muscle strips were treated with the 2D-clean-up kit (GE Healthcare) according to the manufacturer protocol. After this treatment the muscle strips were homogenized in sample buffer containing 15% glycerol, 62.5 mM Tris (pH 6.8), 1% (w/v) SDS and 2% (w/v) DTT. The protein concentration was measured with the RCDC Protein Assay Kit II (Biorad). The amount of endogenous cTn complex with the K280 mutation exchanged with the exogenous WT cTn complex was assessed by immunoblotting. The recombinant WT cTnT was labelled with a Myc-tag to discriminate between the endogenous and exogenous recombinant cTnT. The proteins were separated on a one-dimensional 13% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond) in 1 hour at 75V. The endogenous and exogenous recombinant cTnT was detected,

using the specific monoclonal antibody against cTnT, Clone JLT-12, in a 1:1250 dilution (Sigma), by chemiluminescence (ECL, Amersham Biosciences).[22] The recombinant K280N mutated cTnT did not carry a Myc-tag. However, the phoshorylated endogenous cTn complex in the HCM_{smn} is exchanged by the non-phosphorylated exogenous recombinant K280N. Therefore, the amount of endogenous cTn complex exchanged with the exogenous recombinant K280N was analyzed, using ProQ-Diamond phospho-stained 1D-gels as described previously.[26] 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 analysis and statistics were performed using Prism version 5.0 (Graphpad Software, Inc., La Jolla, Ca) and SPSS version 20.0 (IMB, Armonk, NY, USA). Statistical analysis of the mRNA data comprised a Student's t-test with $P<0.05$ considered significant.

Data from myofibrils and muscle strips are expressed as mean ± SEM. Statistical analysis, taking into account non-Gaussian distribution, inequality of variances and within-subject correlation was performed. In brief, myofibril data from a single case HCM patient carrying cTnT K280N mutation (K280N, group 1) were compared with those from several control patients belonging to 3 different groups (2 in the case of muscle strips):

- Sarcomere mutation negative HCM patients (HCM_{smn}, group 2),
- Healthy donors (Donors, group 3)
- Patients with aortic stenosis (LVH_{ao}, group 4).

When comparing K280N with the three control groups (comparison of groups 1-2, 1-3, 1-4), within-subject correlation could not be estimated because the K280N group is represented by a single subject. To exclude random-effects due to subject-related variability, K280N data were first individually compared with those of each control patient (belonging to group 2, 3 or 4), performing residual analysis with linear mixed models (by subject) and calculating corresponding P-values with one-way ANOVA. If data from the single K280N patient were statistically significant versus each individual patient of a control group no further analysis was performed and K280N was considered significantly different from that control group. Whenever data from the K280N patient did not reach significance versus all subjects of a control group but the differences estimated by the residual analysis were all in the same direction, data were compared by ANOVA with correction for nested variances, accounting for within-subject correlation in the control group.

When comparing the control groups one another (2-3, 2-4, 3-4), multilevel analysis was performed with linear mixed models as previously described,[18,27–29] since each group was constituted by more than one subject and allowed estimation of within-subject correlation. Differences were considered statistically significant for $P<0.05$.

In the cTn exchange experiments, differences between groups of myofibrils or muscle strips (exchanged vs unexchanged sham treated) were analyzed using Student's *t*-test for unpaired observations. $P<0.05$ was considered significant.

Results

Identification of the homozygous cTnT K280N mutation in human heart tissue

Genotyping indicated a novel potentially disease-causing homozygous missense mutation c804G>T in the *TNNT2* gene predicted to produce a K280N mutation in cardiac troponin T (Figure 1). The homozygous mutation was confirmed at the cDNA level and at the protein level by mass spectrometry, where the K280N variant was detected and the wild-type protein was absent (not shown). In 1D gels, the protein content of cTnT relative to actin was indistinguishable from wild-type (Figure 2) indicating that there was no haploinsufficiency and the mutation could act as a poison peptide.

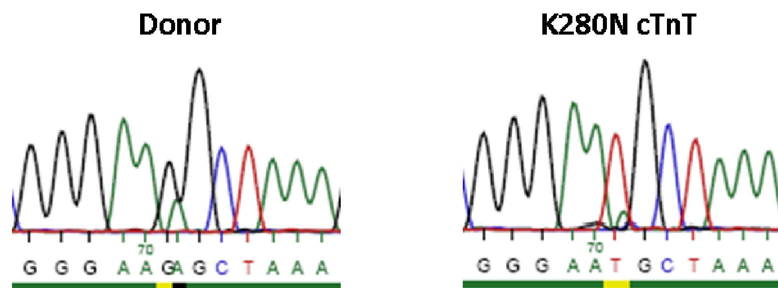

Figure 1. Genotyping. The G>T transition at position 804 in K280N cTnT

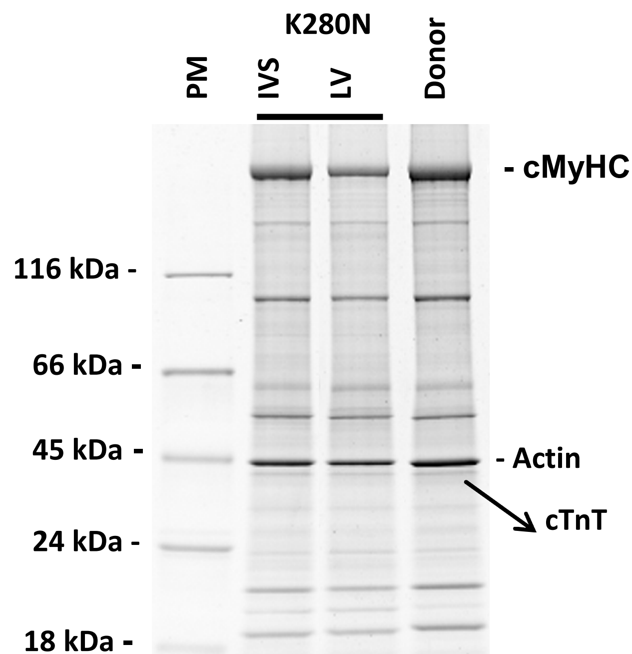

Figure 2. Protein analysis. PM, protein marker.

Impact of K280N cTnT mutation on myofibril mechanics and kinetics

Figure 3 shows, for both K280N and HCM_{smn} myofibrils, representative traces of tension responses to maximal Ca^{2+} activation by fast solution switching. Average data for both myofibril groups are shown in Table 2 together with data of myofibrils taken from donors and HCM_{smn} patients. In the K280N myofibrils, resting tension (RT) was higher or tended to be higher compared to all control groups whereas maximal active tension was lower versus donors but not significantly different from those of LVH_{ao} and HCM_{smn} myofibrils. The kinetics of tension generation, k_{act} (as well as k_{tr}, data not shown), of K280N myofibrils were markedly faster compared to all control groups (Table 1 and Figure 3C), indicative of faster cross-bridge turnover rate. The kinetics of full tension relaxation with step reduction of $[Ca^{2+}]$ are shown on a faster time scale in Figure 3D for LVH_{ao} and K280N myofibrils. As previously described,[19] the time course of force relaxation in human cardiac myofibrils started with a slow, seemingly linear, force decay followed by a fast, exponential, relaxation phase. Slow k_{rel} was markedly faster in the K280N myofibrils than in all control groups (Table 2), indicative of faster cross-bridge detachment under isometric conditions. The rate constant of the fast relaxation phase (fast k_{rel}), instead, was not different in the K280N myofibrils compared to donors and HCM_{smn} whereas it tended to be slower in the LVH_{ao} group than in the other groups (Table 2).

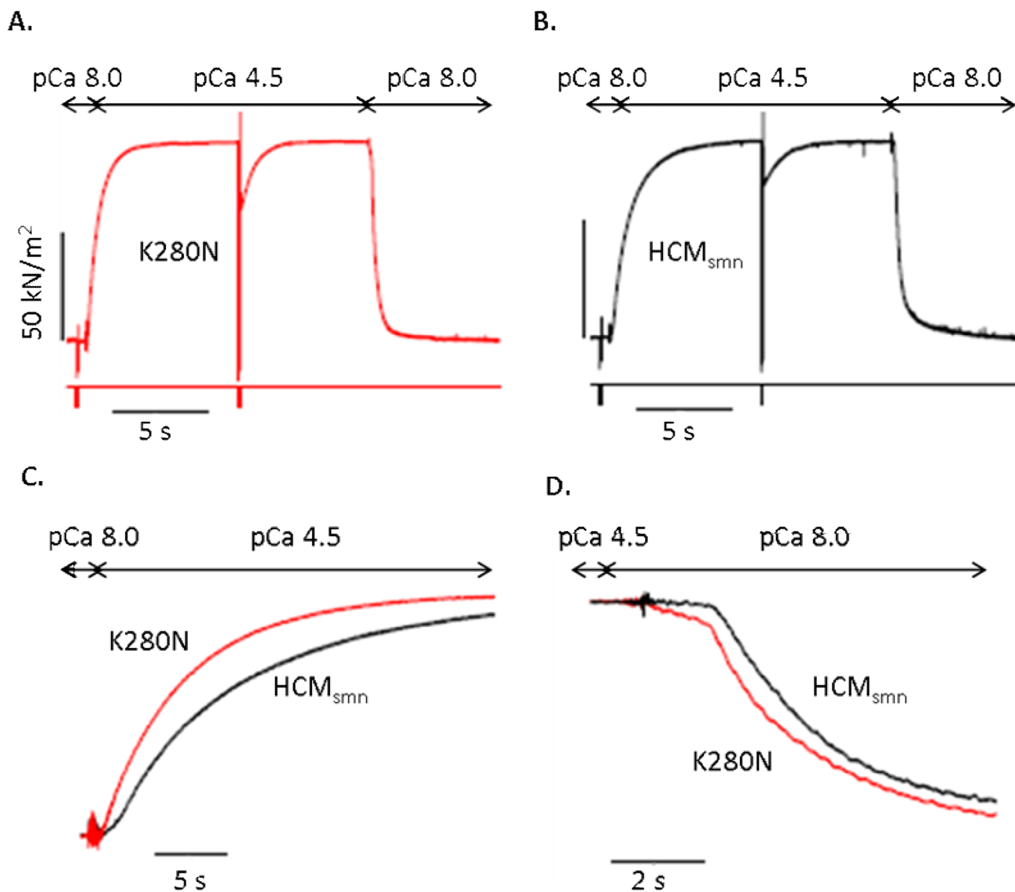

Figure 3. Original registrations of a myofibril preparation. A, B. Contraction-relaxation cycles of HCM_{smn} and K280N myofibril preparations. **C.** Myofibril tension activation *(k_{act})*. **D.** Myofibril tension relaxation (k_{rel}).

Table 2: Mechanical and kinetic parameters of myofibrils from different groups of control patients and from the homozygous K280N cTnT HCM patient.

Myofibril type	RT (kN/m^2)	Tension (kN/m^2)	k_{act} (s^{-1})	Slow k_{rel} (s^{-1})	Fast k_{rel} (s^{-1})
Donors (N=5)	10.4±0.6 (n= 98)	111±5 (96)	0.85±0.02 (118)	0.29±0.02 (95)	4.18±0.14 (101)
LVH_{ao} (N=7)	9.8±1.1 (48)	96±6 (58)	0.71±0.04 (50)	0.20±0.02 (54)	3.15±0.20 (54)
HCM_{smn} (N=3)	8.2±0.9 (44)	87±7 (43)	0.74±0.03 (45)	0.30±0.02 (44)	4.62±0.20 (44)
K280N (N=1)	14.9±1.4 (43)*	87±5 (43)*(vs donors only)	1.73±0.07 (39)*	0.63±0.05 (42)*	3.76±0.16 (42)
Tn exchanged batches					
K280N sham treated (N=1)	15.9±2.9 (12)	89±9 (12)	1.69±0.08 (17)	0.69±0.08 (16)	3.90±0.27 (16)
K280N WT Tn exch (N=1)	15.7±2.0 (13)	86±7 (14)	1.00±0.09 (16)*	0.31±0.05 (16)*	3.13±0.19 (16)*
Donors sham treated (N=2)	10.5±0.83 (64)	114±7 (62)	0.86±0.03 (70)	0.28±0.02 (58)	4.26±0.18 (63)
Donors WT Tn exch (N=2)	10.1±0.7 (72)	91±6 (63)*	0.84±0.03 (70)	0.30±0.02 (66)	4.65±0.19 (65)
Donor sham treated (N=1)	10.8±1.5 (10)	99±10 (10)	0.81±0.04 (15)	0.33±0.04 (15)	3.78±0.32 (15)
Donor K280N Tn exch (N=1)	15.9±3.6 (6)	74±14 (6)	1.04±0.08 (12)*	0.60±0.07 (13)*	4.18±0.62 (13)

N= number of patients in the group; n= number of myofibrils; RT, resting tension; k_{act} rate of force generation following maximal Ca^{2+}-activation; slow k_{rel} rate of the slow isometric phase of relaxation estimated from the normalized slope of the linear fit to the force trace; fast k_{rel}, rate of the fast phase of relaxation estimated from the time constant of the exponential fit to the force trace. $^*P<0.05$ vs the corresponding control group(s).

Replacement of most mutant cTnT (ca. 70%, Figure 4A) by exchange of the endogenous Tn complex for a recombinant human WT cTn complex containing the WT cTnT into the homozygous K280N myofibrils reduced k_{act} and slow k_{rel} close to donor values without affecting myofibril resting and active tension (Figure 4B&C and Table 2). The replacement also decreased fast k_{rel} to values closer to those of LVH_{ao} myofibrils and tended to slightly prolong the duration of the isometric relaxation phase. As previously reported[25] replacement of the endogenous cTn with the recombinant human WT complex into donor myofibrils had no effects on force kinetics (Figure 4B&C) while the exchange protocol tended to depress maximal tension (Table 2). 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} (Figure 4B) and increased slow k_{rel} close to the values found in the myofibrils from the K280N patient (Figure 4C). The replacement also reduced the duration of the isometric phase of relaxation and tended to accelerate fast k_{rel} (Table 2). Resting tension tended to increase whereas maximal tension tended to decrease following cTn replacement with the mutant cTnT (Table 1). Results in myofibrils demonstrate

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}, 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.

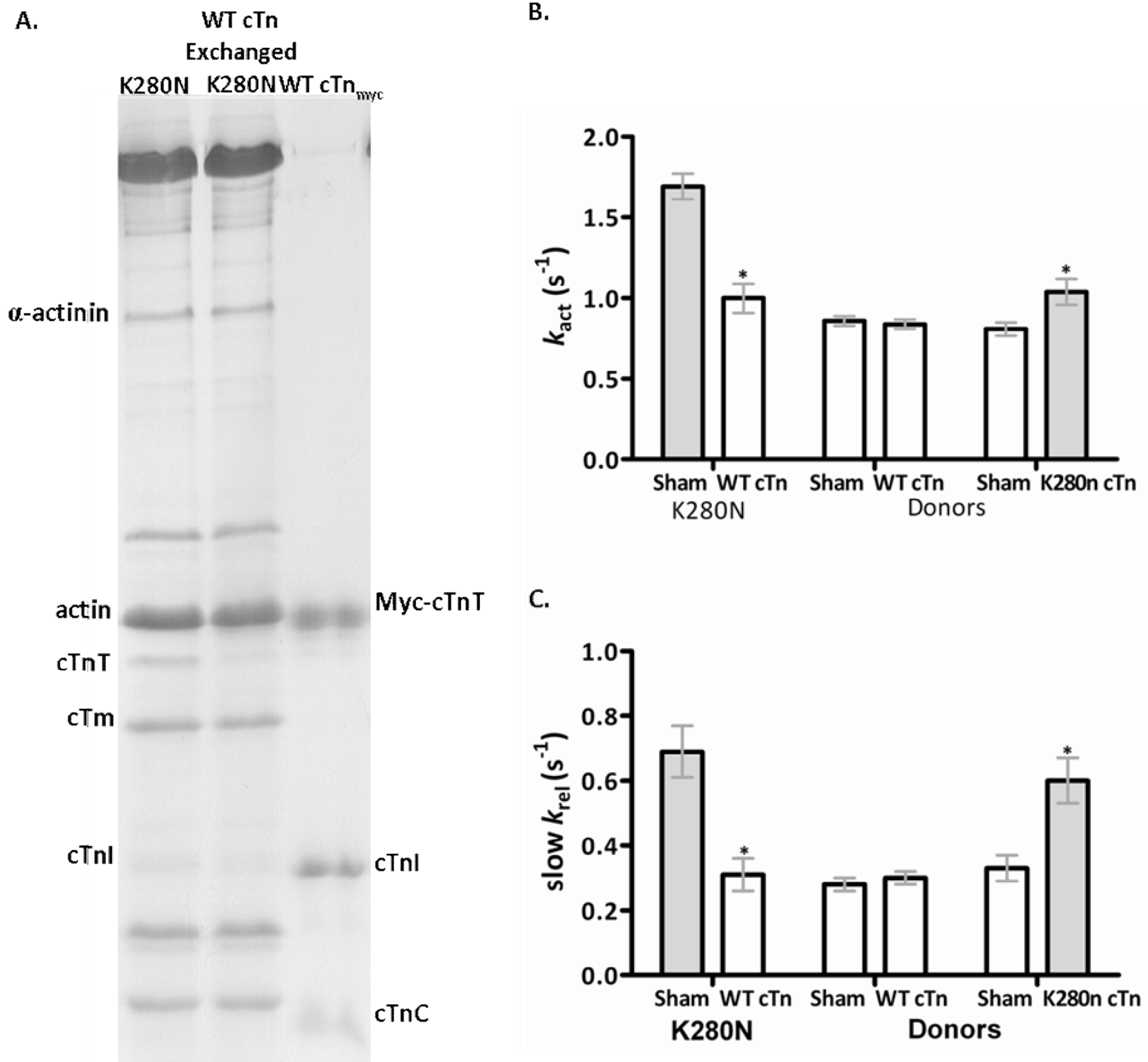

Figure 4. cTn replacement experiments in K280N and control myofibrils. A. Exchange determination in K280N myofibrils. 15% SDS-PAGE gel of unexchanged (1st lane), WTcTn exchanged (2nd lane) K280N myofibrils and WT recombinant cTn with Myc-tag cTnT (3rd lane). The extent of the exchange estimated from the intensity ratio of the endogenous cTnT band to the α-actinin band in the first two lanes was around 70%. **B**. K_{act} of sham & exchanged K280N myofibrils, sham & exchanged WT cTn donor myofibrils and sham & exchanged K280N cTn donor myofibrils. **C.** Slow k_{rel} of sham vs. exchanged K280N myofibrils, sham vs. exchanged WT cTn donor myofibrils and sham vs. exchanged K280N cTn donor myofibrils. $^{*}P<0.05$.

Impact of the K280N TnT mutation on sarcomeric TC

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 demembranated muscle strips from the K280N homozygous HCM patient, from 3 LVH_{ao} patients and from 6 HCM_{smn} patients. Maximal Ca^{2+}-activated tension was significantly lower in K280N muscle strips compared to LVH_{ao} and HCM_{smn} preparations (Table 3). Maximal ATPase activity was significantly lower in the K280N compared to LVH_{ao} muscle strips (Table 3), but did not differ with HCM_{smn}. The ratio between maximal ATPase activity and tension generation, representing TC, was significantly higher in the K280N and LVH_{ao} muscle strips compared to HCM_{smn} muscle strips (Table 3) and tended to be higher in the K280N compared to the LVH_{ao} muscle strips. Resting ATPase activity was significantly lower in the K280N compared with the LVH_{ao} muscle strips (Table 3).

Table 3. Functional data of the multicellular muscle strips.

	Maximal tension (kN/m^2)	**Maximal ATPase activity ($\mu mol/L \cdot s^{-1}$)**	**Maximal TC ($\mu mol \cdot L \cdot s^{-1}/kN/m^2$)**	**Resting ATPase activity ($\mu mol/L \cdot s^{-1}$)**
LVH_{ao} (N=3, n=16)	21.3±1.5	52.2±6.3	2.5±0.3*	10.3±2.2
HCM_{smn} (N=6, n=29)	25.6±1.6	45.2±2.7	1.8±0.1	7.2±1.0
K280N (N=1, n=16)	13.5±1.8*#	38.4±4.8#	3.1±0.3*	4.6±0.8#

*$P<0.05$ vs. HCM_{smn} #$P<0.05$ vs. LVH_{ao} N= number of patients, n= number of muscle strips

Replacement of the mutant cTnT for the WT cTnT by cTn exchange in the K280N muscle strips lowered the maximal ATPase activity ($P=0.0097$ in o/n exchange) without affecting maximal tension (Figure 5A&B). As a result the high TC of the K280N cardiac muscle was at least partially rescued to values closer to those measured in LVH_{ao} and HCM_{smn} preparations (Table 3 and Figure 4C) and reached significance in case of the o/n exchange procedure ($P=0.0135$, Figure 4C). The amount of endogenous K280N cTnT replaced by exogenous WT cTnT was around 50% after 4 hrs exchange and approximately 95% after o/n exchange showing a clear time effect on amount of exchange (Figure 4G). Replacement of the endogenous cTnT by the exogenous recombinant K280N cTnT by cTn exchange in HCM_{smn} preparations did not significantly modify maximal tension and maximal ATPase activity (Figure 4E&F), but tended to increase TC in the exchanged HCM_{smn} muscle strips (Figure 4G). The amount of endogenous cTnT in the HCM_{smn} muscle strips exchanged by the recombinant exogenous K280N cTnT yielded approximately 50% based on cTnT phosphorylation differences after 4 hrs exchange (Figure 4H). Amount of exchange in the o/n exchange of recombinant K280N cTnT in HCM_{smn} was not possible as the obtained amount of homogenate after the clean-up procedure (see methods) was not sufficient.

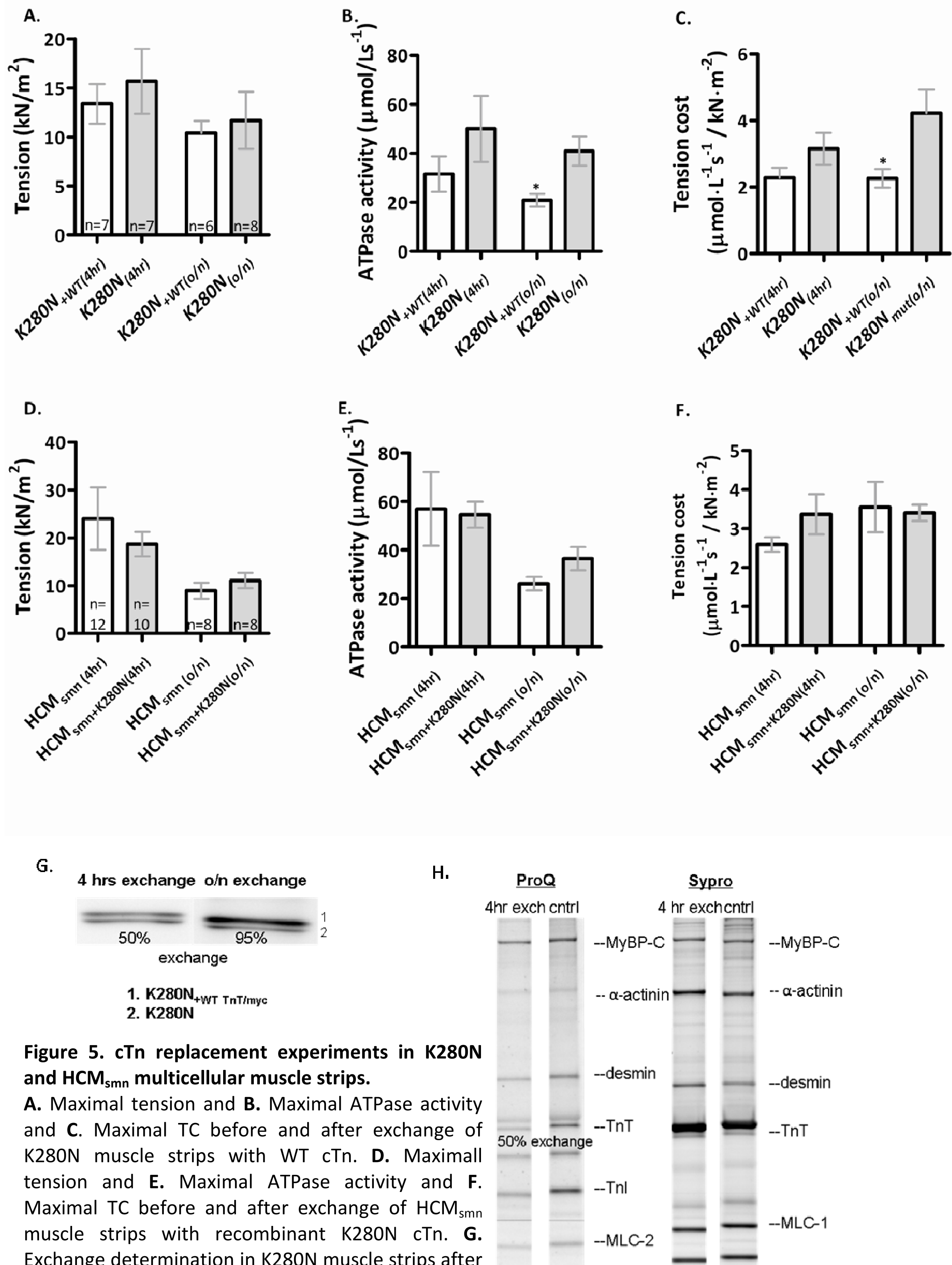

Figure 5. cTn replacement experiments in K280N and HCM_{smn} multicellular muscle strips.
A. Maximal tension and **B.** Maximal ATPase activity and **C**. Maximal TC before and after exchange of K280N muscle strips with WT cTn. **D.** Maximall tension and **E.** Maximal ATPase activity and **F.** Maximal TC before and after exchange of HCM_{smn} muscle strips with recombinant K280N cTn. **G.** Exchange determination in K280N muscle strips after both 4 hrs and o/n and **H.** HCM_{smn} muscle strips after 4 hrs exchange. $^{*}P<0.05$

Discussion

We showed that human cardiac sarcomeres expressing 100% of the K280N cTnT mutation exhibit faster cross-bridge kinetics leading to increased energetic cost of tension generation. Tn replacement experiments with WT cTn complex in the K280N tissue rescued this kinetic and energetic phenotype, while replacement experiments of recombinant K280N in control tissue directly showed the negative impact of K280N mutated cTnT. Therefore, the effects of cross-bridge kinetics and energetic cost of tension generation are primary effects of the HCM-associated K280N cTnT mutation, rather than secondary effects of disease related remodeling.

K280N

Our data extend previous observations in transgenic animal models harboring *TNNT2* mutations[14–16], in which an increase in energetic cost has been associated with the mutation as well. Although rodent models may resemble the cardiac phenotype observed in human, studies in human cardiac tissue are crucial to extrapolate and validate observations from *in vitro* studies and transgenic animal models to human. We had the unique opportunity to investigate functional sarcomeric properties in cardiac tissue from a HCM patient with the homozygous K280N cTnT mutation. K280N is a mutation by which a lysine is replaced by asparagine, and thereby alters electrical charge of the amino acid number 280. Therefore, it is likely that this charge mutation changes protein conformation. Moreover, as the C-terminal part of cTnT is known to interact with cTm[30–33], cTnC[34] and TnI[34], K280N may alter regulatory properties of the cardiac cTn-cTm complex (Figure 6).

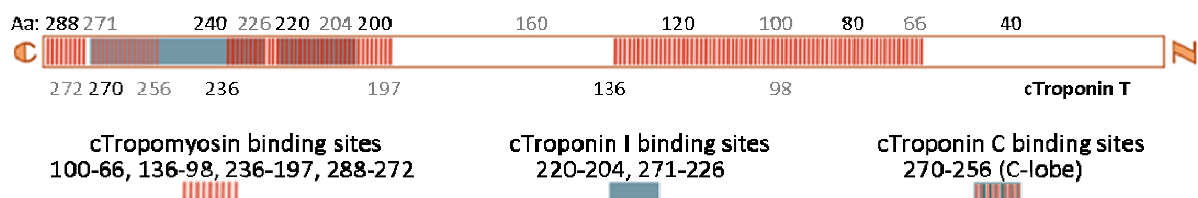

Figure 6. Aminoacid structure of cTroponin T. Aa., amino acid.

K280N and cross-bridge function

The incorporation of K280N in the sarcomere resulted in an increased cross-bridge activation k_{act} and slow relaxation k_{rel} in single myofibrils compared with control groups (Table 2). The interaction of myosin with actin can be described by a 2-stage model in which the transition of cross-bridges into the force-generating states is represented by the apparent rate constant, f_{app}, and the transition back to non-force generating states by the apparent rate constant g_{app}.[35] F_{app} is related to k_{act} via the k_{tr}. G_{app} is related to slow k_{rel} as it has been shown[36,37] that the slow linear force decay occurs while sarcomeres are isometric and slow k_{rel} 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. Therefore, the incorporation of K280N seems to

accelerate cross-bridge cycling kinetics trough fast attachment and fast detachment of cross-bridges.

Moreover, the detachment rate constant of cross-bridges, g_{app}, is proportional to the energetic cost of tension generation[19,35], suggesting an increased TC in K280N tissue through the increase in slow k_{rel}. Indeed, simultaneous measurements of tension generation and ATPase activity in multicellular muscle strips revealed an increase in TC for the K280N muscle trips compared with HCM_{smn}. This is in line with our previous findings regarding the direct positive correlation between cross-bridge kinetics and energetic cost of contractions in tissue with the heterozygous R403Q mutation in the gene encoding myosin heavy chain (*MYH7*)[38] and increased TC in HCM tissue with thick filament mutations compared with HCM_{smn}.[18] Therefore, faster cross bridge detachment under isometric conditions and higher TC may be a common feature to several HCM-associated mutations.

K280N results in an intrinsic sarcomere defect

Interestingly, this homozygous mutation was expressed 100% in the sarcomere, which is in contrast to heterozygous missense mutations in for example *MYH7* (myosin heavy chain) resulting in allelic imbalance[39] or truncation mutations in *MYBPC3* leading to haploinsufficiency[1,2] of an entire protein (myosin-binding protein C). Therefore, tissue with the K280N mutation represents a unique tool to assess by the troponin exchange experiments which sarcomere changes are primarily due to the mutant protein rather than being induced by disease-related cardiac myofilament remodeling. In both the single myofibrils and the multicellular muscle strips the defect in kinetics and energetics could be rescued by exchange of WT cTn complex in the mutated tissue (Table 2, Figures 4&5C). Exchange of the endogenous cTn complex in control tissue by recombinant K280N cTn complex showed that the defect could be induced as well (Table 2, Figures 4&5F). This clearly shows that the K280N mutation interferes directly with sarcomere function. The amount of neither exchange of the WT exogenous cTn in the K280N mutant, nor the exchange of recombinant K280N cTn complex in control tissue was 100% (Figures 4A, 5G&H). However, the effect on sarcomere function was clear. Therefore, further research is necessary to assess how much mutant K280N is required to significantly perturb sarcomere function.

In conclusion, the homozygous K280N *TNNT2* mutation results in a 100% expression of mutated cTnT in the cardiac sarcomere. It induces increased cross-bridge cycling kinetics and increased energetic cost of contraction. As these changes can either be rescued or induced, the K280N mutation directly affects sarcomere function.

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