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The effect of tropomyosin variants on cardiomyocyte function and structure that underlie different clinical cardiomyopathy phenotypes

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ABSTRACT

Background – Variants within the alpha-tropomyosin gene (*TPM1*) cause dominantly inherited cardiomyopathies, including dilated (DCM), hypertrophic (HCM) and restrictive (RCM) cardiomyopathy. Here we investigated whether *TPM1* variants observed in DCM and HCM patients affect cardiomyocyte physiology differently.
Methods – We identified a large family with DCM carrying a recently identified *TPM1* gene variant (T201M) and a child with RCM with compound heterozygote *TPM1* variants (E62Q and M281T) whose family members carrying single variants show diastolic dysfunction and HCM. The effects of *TPM1* variants (T201M, E62Q or M281T) and of a plasmid containing both the E62Q and M281T variants on single-cell Ca^{2+} transients (CaT) in HL-1 cardiomyocytes were studied. To define toxic threshold levels, we performed dose-dependent transfection of *TPM1* variants. In addition, cardiomyocyte structure was studied in human cardiac biopsies with *TPM1* variants.
Results – Overexpression of *TPM1* variants led to time-dependent progressive deterioration of CaT, with the smallest effect seen for E62Q and larger and similar effects seen for the T201M and M281T variants. Overexpression of E62Q/M281T did not exacerbate the effects seen with overexpression of a single *TPM1* variant. T201M (DCM) replaced endogenous tropomyosin dose-dependently, while M281T (HCM) did not. Human cardiac biopsies with *TPM1* variants revealed loss of sarcomeric structures.
Conclusion – All *TPM1* variants result in reduced cardiomyocyte CaT amplitudes and loss of sarcomeric structures. These effects may underlie pathophysiology of different cardiomyopathy phenotypes.

1. Introduction

Cardiomyopathies are a heterogeneous group of diseases of the myocardium that are associated with mechanical and/or electrical dysfunction and frequently caused by a genetic defect [1]. The working group on Myocardial and Pericardial Diseases of the European Society of Cardiology proposed a clinically oriented classification system in which heart muscle disorders are grouped into the following subtypes according to ventricular morphology and function: hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), restrictive cardiomyopathy (RCM), arrhythmogenic right ventricular cardiomyopathy (ARVC) and unclassified cardiomyopathies [1,2].

Pathogenic variants in sarcomere genes are a frequent cause of cardiomyopathies and may lead to different cardiomyopathy subtypes [3]. One of these sarcomere genes is *TPM1*, which encodes alpha-tropomyosin and causes HCM (10 *TPM1* gene variants), DCM (11 *TPM1* variants), cardiomyopathy (2 *TPM1* variants) and left-ventricular non-compaction (3 *TPM1* variants) according to ClinVar database (January 2020) [4]. All reported *TPM1* variants are missense variants that result in a single amino acid substitution, and they account for 1.5% of all likely pathogenic and pathogenic gene variants in HCM and 1.9% in DCM [5].

Since distinct variants in the same sarcomere gene have been observed to cause either HCM or DCM, it has been proposed that these gene variants have to trigger two different series of events to culminate

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in the distinct cardiomyopathy subtypes [6]. Several studies have been published on the functional consequences of *TPM1* variants, adding to our understanding of the different pathophysiological processes that lead to distinct forms of cardiomyopathy [7–9].

Here we describe a large family with DCM carrying a *TPM1* variant that was previously reported in a large Dutch cohort study [10]. We also identified compound heterozygote *TPM1* variants in a child with RCM, with family members carrying one of the two variants and showing HCM(−like) phenotypes. As changes in calcium-handling have been suggested to be central in determining the type of remodeling that occurs in genetic cardiomyopathies [11], we expressed the three *TPM1* variants in HL-1 cardiomyocytes and measured the resulting Ca^{2+} transients (CaT).

2. Methods

The comprehensive method section is available in the Data Supplement.

3. Results

3.1. Clinical characteristics of DCM family members

Two probands (III:20 and IV:5) were investigated separately at the outpatient clinic and diagnosed with DCM. Clinical data of members of this DCM family (Fig. 1A) are listed in Table 1. Individual III:20 complained of progressive dyspnea, had a dilated LV and a LV ejection fraction (LVEF) of 15%, leading to a diagnosis of DCM at age 31. She received an implantable cardioverter defibrillator (ICD) at age 40. Ten years later, her LVEF was 18%. Genetic testing revealed that she carried the c.602C > T (p.(Thr201Met)) variant in exon 6 of *TPM1* (referred to as T201M), which had been previously identified in a large Dutch cohort study of DCM patients [10]. We classified T201M as likely pathogenic because it fulfills one strong and two moderate criteria (Table SI). This variant has not been further investigated so far.

This proband's son (IV:17) died at age 24 from heart failure that had worsened after a high energy trauma (car accident) two months before his death. Her sister (III:22) was diagnosed with DCM at age 38 following family screening, but no follow-up is available. Two other siblings tested negative for the familial *TPM1* variant. The father of the proband (II:11) was diagnosed with DCM at age 62. At age 66, he received an ICD, which was replaced at age 84. No ICD shocks were recorded.

The other proband in this family (IV:5) was diagnosed with DCM (Fig. 1B-C) and received an ICD at age 21. After 13 years of follow-up, he had not experienced any ICD shocks and his LVEF was 40%. His mother (III:5) died at age 44 in a traffic accident. His maternal aunt (III:7) was diagnosed with DCM at age 53 following family screening. His maternal grandfather (II:4), who had not undergone prior cardiac evaluations, was diagnosed with DCM and atrial fibrillation at age 84 following family screening.

Both probands were linked to the same ancestral couple (I:1 and I:2). After cascade family screening, 29 family members were identified as carriers of the familial *TPM1* variant. The phenotypic variability and age-related penetrance is further illustrated by III:24 and III:25, who were asymptomatic and without signs of DCM at ages 56 and 53, respectively. The penetrance, as indicated by age at DCM diagnosis, is shown in Fig. SI. The youngest age at diagnosis was 17 (V:1), though this individual was asymptomatic at that time. At age 60, a diagnosis of DCM was made in 55% of the family members who carried the T201M variant.

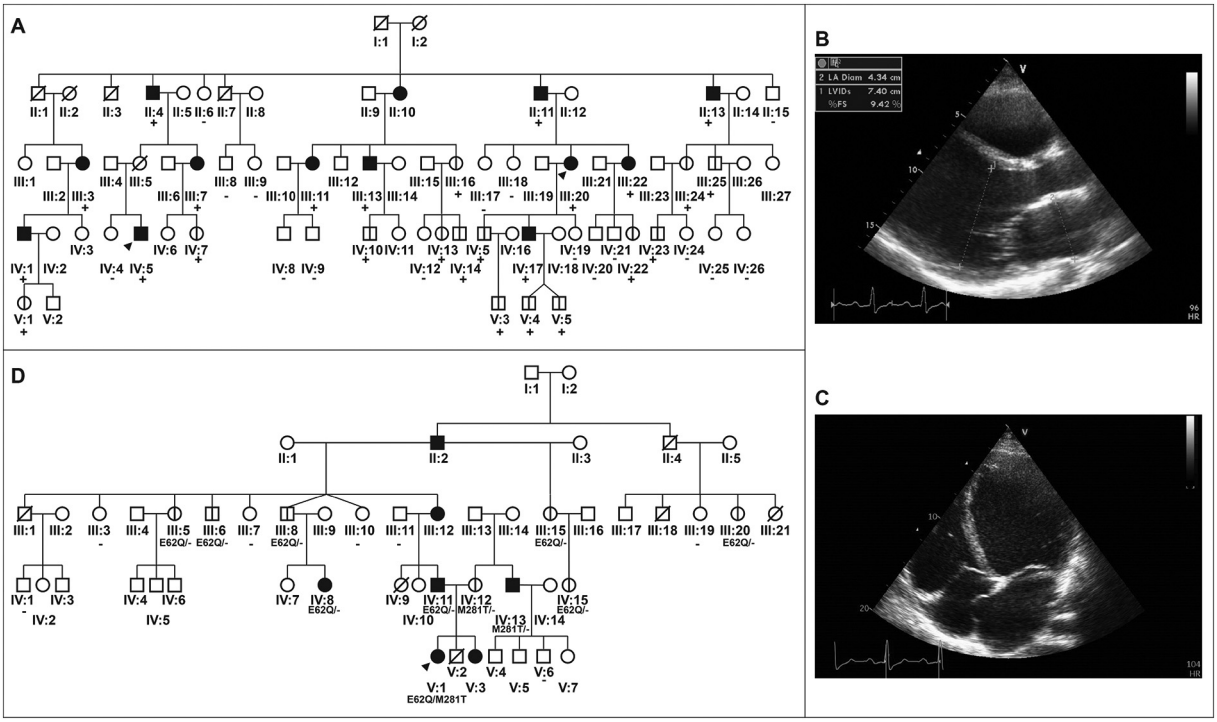

Fig. 1. Pedigree of *TPM1* variant carrier and echocardiographic images of a DCM patient. (**A**) Pedigree of the DCM family. Presence (+) or absence (−) of the *TPM1* variant c.602C > T p.(Thr201Met) is indicated for the analyzed subjects. (**B**) Parasternal long axis view and (**C**) apical 4-chamber view of patient IV:5 (DCM family) at presentation. The left ventricle is dilated (normal value <58 mm) and fractional shortening (FS) is reduced (normal value >25%). The left atrium is also enlarged (normal value <40 mm). (**D**) Pedigree of the family with the RCM patient. Presence (E62Q and/or M281T/−) or absence (−) of the *TPM1* variants c.184G > C p.(Glu62Gln) and c.842 T > C (p.Met281Thr) is indicated for the analyzed subjects. Squares represent males and circles represent females. Filled symbols indicate those with a diagnosis of (**A**) DCM and (**D**) RCM or HCM. Vertical lines indicate those who are asymptomatic or with mild signs or features of cardiomyopathies. Slashed symbols indicate deceased subjects. Arrowhead mark the index cases.

Table 1
Clinical data of members of DCM family.

Pedigree	*TPM1* genotype	1st presentation Age (years) and reason		Echocardiogram at presentation LVEDd (mm)	LVESd (mm)	FS (%)	LVEF (%)	Phenotype & follow-up
II:1	**p.(Thr201Met)***	NA						D/83 yr
II:4	**p.(Thr201Met)**	84	screening	62	44	29	NA	DCM, atrial fibrillation
II:10	**p.(Thr201Met)***	NA						D/63 yr, "enlarged heart"
II:11	**p.(Thr201Met)**	62	dyspnea	59	40	32	35	DCM, ICD at 66 yr, replaced at 84 yr, nsVTs
II:13	**p.(Thr201Met)**	70	screening	61	50	18	35	DCM, atrial fibrillation at 75 yr, no ICD
III:3	**p.(Thr201Met)**	53	CVA	60	45	25	NA	DCM, no follow-up available
III:5	**p.(Thr201Met)***	NA						D/44 yr, traffic accident
III:7	**p.(Thr201Met)**	53	screening	56	40	29	44	DCM, no follow-up available
III:11	**p.(Thr201Met)**	43	dyspnea	62	54	13	36	At 46 yr discharged from follow-up after normalization echo
III:13	**p.(Thr201Met)**	53	screening	52	37	29	50	No follow-up available
III:16	**p.(Thr201Met)**	48	screening	42	29	31	55	At 57 yr mild diastolic dysfunction, no DCM
III:20	**p.(Thr201Met)**	31	dyspnea	67	60	10	15	ICD at 40 yr, LVEF 18% at 50 yr
III:22	**p.(Thr201Met)**	38	screening	58	47	19	28	DCM, no follow-up available
III:24	**p.(Thr201Met)**	47	screening	48	30	38	60	Asymptomatic, no signs of DCM at 56 yr
III:25	**p.(Thr201Met)**	45	screening	51	34	33	55	Asymptomatic, no signs of DCM at 53 yr
IV:1	**p.(Thr201Met)**	33	screening	59	45	24	NA	No follow-up available
IV:5	**p.(Thr201Met)**	21	dyspnea	82	74	10	12	ICD at 21 yr, at 34 yr no ICD shocks, LVEF 40%
IV:7	**p.(Thr201Met)**	24	screening					No signs of DCM, no follow-up available
IV:10	**p.(Thr201Met)**	18	screening	52	37	29	60	Asymptomatic, LVEF 54% at 25 yr
IV:13	**p.(Thr201Met)**	14	screening	51	32	37	68	Asymptomatic, no signs of DCM at 22 yr
IV:14	**p.(Thr201Met)**	12	screening	51	33	35	62	Asymptomatic, no signs of DCM at 19 yr
IV:15	**p.(Thr201Met)**	21	screening				41	Mild DCM, no follow-up available
IV:17	**p.(Thr201Met)**	13	screening	48	32	33	62	Died of heart failure at 24 yr
IV:22	**p.(Thr201Met)**	7	screening	41	29	29	55	Asymptomatic, LVEF 50% at 19 yr
IV:23	**p.(Thr201Met)**	19	screening	58	37	36	60	Asymptomatic, no signs of DCM at 27 yr
V:1	**p.(Thr201Met)**	6	screening	41	28	32	62	Asymptomatic, LVEF 41% at 17 yr
V:3	**p.(Thr201Met)**	6	screening	39	26	33	NA	No signs of DCM at 6 yr
V:4	**p.(Thr201Met)**	3	screening	36	23	36	NA	No signs of DCM at 3 yr
V:5	**p.(Thr201Met)**	3	screening	37	22	41	NA	No signs of DCM at 3 yr

CVA, cerebrovascular accident; D/, death; DCM, dilated cardiomyopathy; echo, echocardiogram; FS, fractional shortening; ICD, implantable cardioverter-defibrillator; LVEDd, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVESd, left ventricular end-systolic diameter; NA, not available; nsVT, non-sustained ventricular tachycardia; yr, years.
* obligate carrier.

3.2. Clinical characteristics of RCM family members

We describe, for the first time, compound heterozygote *TPM1* variants in a child with RCM, with family members carrying one of the two variants and showing HCM(−like) phenotypes (Fig. 1D).

An asymptomatic 6-year-old girl (V:1) was referred to the pediatrician after the sudden death of her brother (V:2) at 22 months of age. No autopsy was performed, and no DNA was available for genetic testing. At age six, the proband's cardiac evaluation was normal. At age nine, she was referred to a pediatric cardiologist because she reported chest pain during exercise. Cardiac evaluation showed bi-atrial dilatation with near-normal LV volume and raised ventricular end-diastolic pressures at cardiac catheterization. There was no evidence of LV hypertrophy or constrictive pericarditis. Based on these findings, a diagnosis of RCM was made in this patient. At age 12, she received an orthotopic cardiac transplantation, and she reached adult age in good clinical condition. Genetic screening identified two missense variants in *TPM1*: c.184G > C (p.(Glu62Gln)) (referred to as E62Q) and c.842 T > C (p.(Met281Thr)) (referred to as M281T). Both variants had previously been identified in HCM patients [12,13]. We classified both variants as likely pathogenic, with E62Q fulfilling one strong and two moderate criteria and M281T fulfilling two moderate and ≥ 2 supporting criteria (Table SI).

The proband's sister (V:3) had a normal cardiac evaluation at age seven and has so far not undergone genetic testing. The proband's father (IV:11), a carrier of the E62Q variant, had minor abnormalities suggestive for RCM, with mild LV diastolic dysfunction on echo. Her mother (IV:12), a carrier of the M281T variant, was asymptomatic but had a mildly enlarged left atrium at age 40. The maternal uncle (IV:13), also a carrier of the M281T variant, was asymptomatic, but echocardiography at age 40 showed mild LV hypertrophy (13 mm). The paternal grandmother (III:12) and other carriers in her generation (III) who were positive for the E62Q variant showed stable diastolic dysfunction and atrial dilatation in an age range of 51–69 years. Notably, a cousin of the father (IV:8), a carrier of the E62Q variant, was diagnosed with HCM at age 17 with an LV wall thickness of 18 mm.

3.3. Time-dependent reduction of CaT amplitude by TPM1 variants

Variants in *TPM1* may lead to disturbed intracellular calcium handling that contributes to contractile dysfunction [14], and different changes in calcium handling have been linked to the development of either DCM or HCM [11]. We therefore overexpressed the three identified *TPM1* variants (untagged, 0.75 μg/μL) in HL-1 cardiomyocytes and determined the effect on CaT. All three *TPM1* variants significantly reduced CaT amplitude 48 h post-transfection when compared to HL-1 cardiomyocytes overexpressing untagged wild-type *TPM1* (Fig. 2A, G and SII). The reduction in CaT amplitude was significantly larger for $TPM1_{T201M}$ and $TPM1_{M281T}$ compared to $TPM1_{WT}$, with means ± SEM of 34.90% ± 0.05 and 35.78% ± 0.04, respectively, while the reduction for the $TPM1_{E62Q}$ variant was smaller (19.33% ± 0.07). As the RCM patient carried both the E62Q and M281T variants ($TPM1_{E62Q/M281T}$), we studied if expression of both variants exacerbated the phenotype and found that $TPM1_{E62Q/M281T}$ expression did not significantly decrease CaT (24.64% ± 0.06) in comparison to the reductions we saw for the individual variants. Longer overexpression (72 h) of all *TPM1* variants further reduced the CaT amplitude, but did not change the variant-specific effects that we had observed 48 h post-transfection (Fig. 2B and E). With the exception of $TPM1_{E62Q/M281T}$ 48 h post-transfection, no significant differences in calcium re-uptake kinetics were observed between the wild-type and variant *TPM1* forms 48 h and 72 h post-transfection (Fig. 2C,D,F and G).

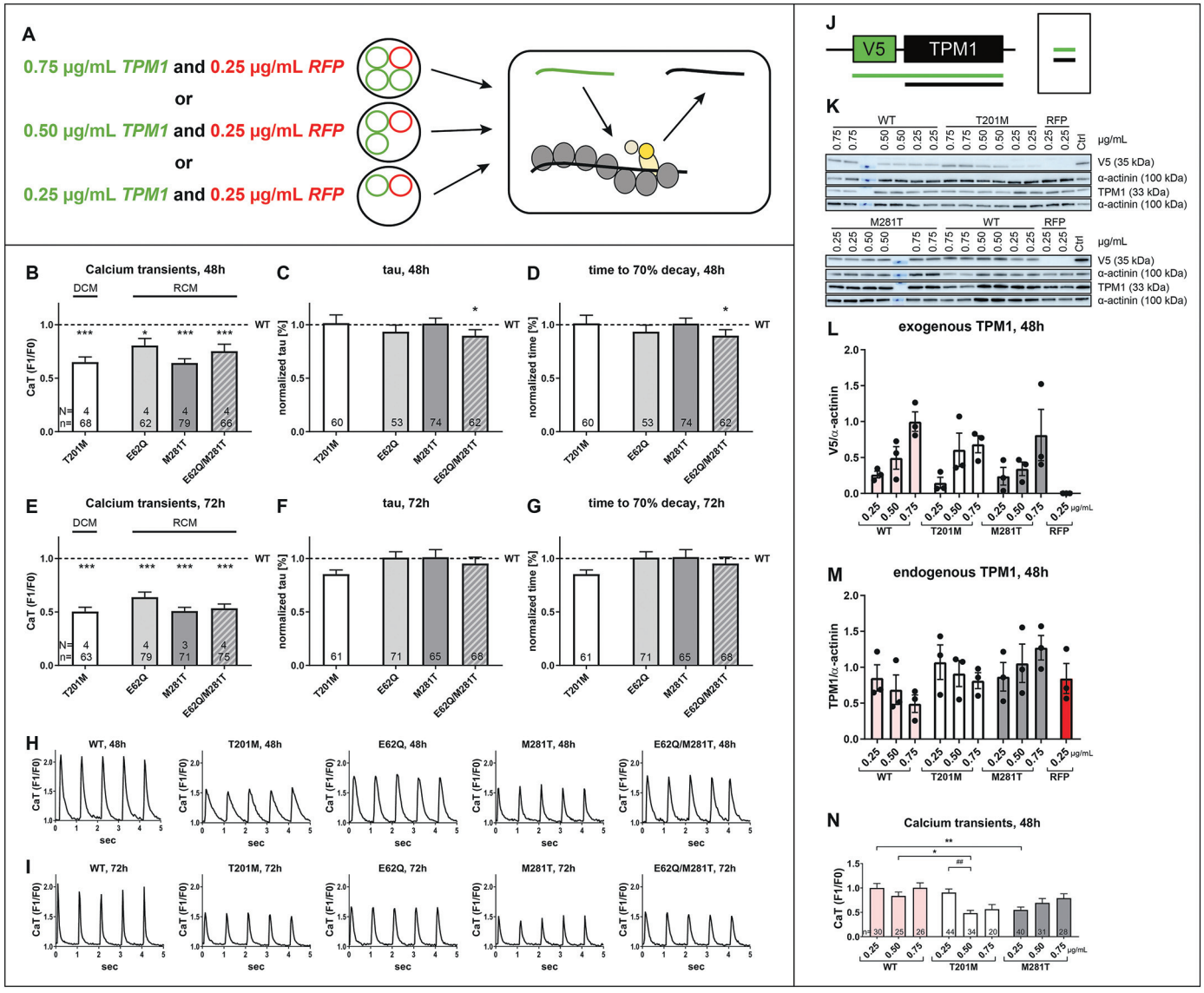

Fig. 2. Time- (B—I) and dose-dependent (J-N) transfection of *TPM1* variants. (A) Schematic representation of the ratio of double transfected DNA. 0.25 µg/mL DNA encoding RFP (red circle) was co-transfected with 0.75 (used in **B—I**), 0.50 or 0.25 µg/mL DNA encoding TPM1 (green circle). Green circles: *TPM1* plasmids. Red circle: *RFP* plasmid. Thick green line: exogenous TPM1 encoded by *TPM1* plasmid. Thick black line: endogenous TPM1. Grey circles: actin. Complex of yellow circles: troponin complex. (**B**) Quantified CaT amplitude data of single HL-1 cardiomyocytes 48 h post-transfection. N = number of transfections; n = number of measured HL-1 cardiomyocytes that were *RFP*-positive transfected. Exponential curve fitting was applied to obtain (**C**) the mean time constant of CaT decay (tau) and (**D**) the time to 70% decay. (**E**) Quantified CaT amplitude data of single HL-1 cardiomyocytes 72 h post-transfection, (**F**) tau and (**G**) time to 70% decay. Representative CaT traces detected (**H**) 48 h and (**I**) 72 h post-transfection. (**J**) Due to the amino-terminal V5-tag, exogenous TPM1 (green line) was larger and therefore displayed migration on western blot that was distinguishable from endogenous TPM1 (black line). (**K**) Representative blot images for endogenous (TPM1-antibody) and exogenous (V5-antibody) TPM1 expression level after dose-dependent double transfection of *TPM1* and *RFP*. Ctrl: Control cell lysate to compare different membranes. (**L**) Dose-dependent expression of exogenous V5-tagged $TPM1_{WT}$, $TPM1_{T201M}$ and $TPM1_{M281T}$. Each dot represents one transfection and each value was normalized to the mean of $TPM1_{WT\_0.75µg/mL}$. (**M**) Endogenous TPM1 levels after dose-dependent transfection. (**N**) Quantified CaT amplitude data of single HL-1 cardiomyocytes 48 h after dose-dependent double transfection ($N = 2$) of untagged wild-type/variant *TPM1* and *RFP*. Per transfection, each CaT value was normalized to the mean value of overexpressed (**B—I**) $TPM1_{WT}$ and (**N**) $TPM1_{WT\_0.25µg/mL}$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus $TPM1_{WT}$ and $^{\#\#}p < 0.01$ versus $TPM1_{T201M\_0.25µg/µL}$.

3.4. Dose-dependent effect of TPM1 variant expression

Since overexpression of $TPM1_{T201M}$ and $TPM1_{M281T}$ 48 h post-transfection resulted in a strong reduction in CaT amplitudes, we wanted to see if this effect is dose-dependent. Using different ratios of *RFP* (0.25 µg/mL) and V5-tagged *TPM1* (0.25, 0.50 and 0.75 µg/mL (used in previous experiment)), we were able to express TPM1 in a dose-dependent manner (Fig. 2J-L and SIII). Transfection of $TPM1_{WT}$ and $TPM1_{T201M}$ resulted in partial replacement of endogenous TPM1 (Fig. 2M). Notably, endogenous TPM1 levels increased with higher doses of transfected $TPM1_{M281T}$. Dose-dependent transfection of untagged *TPM1* variants and CaT assessment 48 h post-transfection showed that calcium handling dysfunction was influenced by the dose of $TPM1_{T201M}$ (Fig. 2N), showing significant effects at 0.5 µg/mL and a trend toward significance at 0.75 µg/mL $TPM1_{T201M}$ levels. Transfection of 0.25 µg/mL $TPM1_{T201M}$, however, had no detrimental effect on CaT amplitude, while transfection of 0.25 µg/mL $TPM1_{M281T}$ already significantly decreased CaT amplitude. A lower transfection dose of $TPM1_{M281T}$ reduced CaT amplitude more than a higher one.

3.5. TPM1 variants have no effect on autophagy and F-actin

Based on the increase in endogenous TPM1 after high-dose transfection of *TPM1*$_{M281T}$, we defined the effect of *TPM1* variants on components of cellular protein quality control. Immunofluorescence staining of V5-tagged TPM1 revealed no difference in localization of TPM1 variants 48 h post-transfection, nor the formation of aggregates (Fig. SIVA). We also observed unaltered levels of markers for cardiac-specific proteins (α-actinin and myosing heavy chain (MHC)), autophagy (LC3B and p62), endoplasmic reticulum stress (phosphorylation of translation-initiation factor eIF2α) and stress fibers (F-actin) in HL-1 cardiomyocytes transfected with TPM1 variants as compared to wild-type TPM1-transfected cardiomyocytes (Fig. SIV-V).

3.6. Derailed sarcomeric structures in human tissue biopsies with TPM1 variants

As HL-1 cardiomyocytes do not show the highly organized myofilament structure of adult cardiomyocytes [15,16], we determined the localization of TPM1 in the myocardium of patients carrying either the *TPM1*$_{T201M}$ variant or the *TPM1*$_{E62Q/M281T}$ variants. We stained small endomyocardial biopsies of patient samples that were embedded in paraffin and obtained for diagnostic purposes (Fig. SVIA) with antibodies against TPM1 and α-actinin. Both biopsies showed a loss of cardiomyocyte-typical architecture indicated by a loss of sarcomere striations and a loss of intact cardiac muscle fibers (Fig. 3). TPM1 epitopes appeared disrupted and were not organized in the characteristic contractile filaments, and this was accompanied by an almost complete loss of α-actinin (Fig. SVIB). Biopsies from end-stage ischemic heart failure and non-failing subjects were used as controls, and these show the expected sarcomere striation and distribution of TPM1 and α-actinin.

4. Discussion

Gene variants in thin filament components, including alpha-tropomyosin, are known to cause a complex heterogeneous pattern of progressive ventricular remodeling and clinical phenotypes [17]. Our study confirms this clinical heterogeneity in two families with *TPM1* variants and different cardiomyopathy subtypes, namely *TPM1*$_{T201M}$ (DCM) and *TPM1*$_{E62Q}$ and *TPM1*$_{M281T}$ (HCM; and in combination RCM). We also describe, for the first time, compound heterozygote *TPM1*$_{E62Q/M281T}$ variants in a child with RCM. Overall, the genotypes and diverse clinical characteristics of the family members of the DCM and RCM probands suggest that additional causal factors are necessary for the development of cardiac abnormalities in carriers of a heterozygous *TPM1* variant, whereas compound heterozygosity for *TPM1* variants causes severe cardiomyopathy in childhood.

4.1. Impaired cardiac performance due to reduced CaT amplitudes

In thin filament cardiomyopathies, altered myocellular Ca^{2+} homeostasis may trigger events that remodel the cardiomyocyte and ultimately perturb cardiac structure [17,18]. Data from experiments in which adenovirus was used to express human HCM-causing variants in isolated guinea pig left ventricular cardiomyocytes showed that altered CaT were one of the primary effects of HCM-causing variants expression [14]. We therefore measured CaT amplitudes in HL-1 cardiomyocytes. Cardiac muscle cell properties have been measured in this model system before [19,20]. In our experimental model system, production of all *TPM1* variants resulted in reduced CaT amplitudes that were independent of the clinical cardiomyopathy phenotype. Previous studies on HCM- and DCM-related thin filament gene variants have shown opposing changes in myofilament Ca^{2+}-sensitivity, specifically an increase for HCM variants and a decrease for DCM variants (reviewed in [21]). Tardiff and colleagues suggested that the observed primary alterations in Ca^{2+}-sensitivity at the myofilament level indicate a disruption of the Ca^{2+}-dependent sarcomeric activation by thin filament gene variants [17]. These Ca^{2+}-sensitivity changes were associated with similar opposite effects in the Ca^{2+} affinity of myofilaments, i.e. an increase for HCM variants and a decrease for DCM variants [14]. These opposing changes may alter cytosolic Ca^{2+} buffering in different ways, and thereby alter cellular CaT. Davis and colleagues provided evidence for diverse changes in developed tension and CaT when comparing HCM- and DCM-related cardiac troponin C variants, a finding they related to diverse activation of signaling pathways leading to either concentric or eccentric remodeling [11]. While previous studies on *TPM1* variants have also reported a similar opposite effect on the Ca^{2+}-sensitivity of myofilaments for HCM- and DCM-associated variants [8], two DCM-related *TPM1* variants have shown opposite effects: the Glu40Lys variant decreased Ca^{2+} affinity, while the Glu54Lys variant increased Ca^{2+} affinity (but at 100% variant level) [14]. Importantly, no effect on Ca^{2+} affinity was observed when a mixture of 50% variant:50% wild-type TPM1 was studied [14].

In contrast, we observed a decrease in CaT for both HCM- and DCM-related *TPM1* variants. The previous study and our current data indicate that *TPM1* variant-related changes in Ca^{2+} affinity and CaT are independent of the type of cardiac remodeling, which suggests that the diverse clinical cardiac phenotypes seen in affected subjects may rather involve modifier genes and/or environmental factors [17].

Reduced CaT amplitudes may affect tension development of cardiomyocytes. Comparing the CaT amplitudes at 48 h and 72 h post-transfection revealed that longer overexpression of all *TPM1* variants further reduced CaT amplitude, suggesting a progressive pathogenic process. This is in line with a series of longitudinal studies of patients with thin filament variants in whom the ventricle, in particular, was progressively remodeled [22,23]. Reduced CaT amplitudes have been associated with impaired cell-shortening of HL-1 myocytes, indicating contractile dysfunction [19]. Taken together, reduced CaT amplitudes may contribute to the poor cardiac contraction seen in affected individuals.

4.2. Dose-dependent transfection: Maintenance of TPM1 stoichiometry

Previous studies in several transgenic mouse models revealed that increasing the expression of *Tpm1* variants causes a concomitant decrease in wild-type TPM1 levels, histopathological changes and severe impairment of both contractility and relaxation [24,25]. Given the large decrease in CaT amplitude 48 h post-transfection induced by production of TPM1$_{T201M}$ (DCM) and TPM1$_{M281}$ (HCM) (Fig. 2B), and the previously reported dose-effects of *TPM1* variants, we studied the effect of dose-dependent transfection of these particular variants. A dose-dependent decrease in endogenous TPM1 was observed upon transfection with *TPM1*$_{T201M}$, and this coincided with a reduction in CaT. These data illustrate that a heterozygous gene variant, which results in a mixture of the variant and wild-type TPM1, is sufficient to suppress CaT. We therefore propose for *TPM1*$_{T201M}$ that total TPM1 levels seem to be kept constant to preserve stoichiometry of sarcomere proteins. Furthermore, "threshold" levels of TPM1$_{T201M}$ seem to have been reached when *TPM1*$_{T201M}$ was transfected between 0.25 and 0.50 μg/mL since functional compensation by wild-type TPM1 became insufficient. This is in line with *Tpm1* transgenic mouse experiments indicating that there may be "threshold" levels of TPM1 expression that lead to defined phenotypes rather than a gradient of physiological or pathological conditions [24,25]. While the total amount of TPM1 protein remained unchanged in these *Tpm1* transgenic mice, wild-type TPM1 levels were reduced by 35 to 65% [24,25].

In contrast to the *TPM1*$_{T201M}$-induced reduction of endogenous wild-type TPM1, higher transfection doses of the *TPM1*$_{M281T}$ variant did not lower endogenous TPM1, instead resulting in higher levels of wild-type TPM1. This indicates that synthesis of TPM1 is increased and/or its degradation decreased in the presence of an increasing dose of the *TPM1*$_{M281T}$ variant. The largest drop in CaT was observed at the lowest dose of *TPM1*$_{M281T}$ transfection, which coincided with the lowest level of wild-type TPM1.

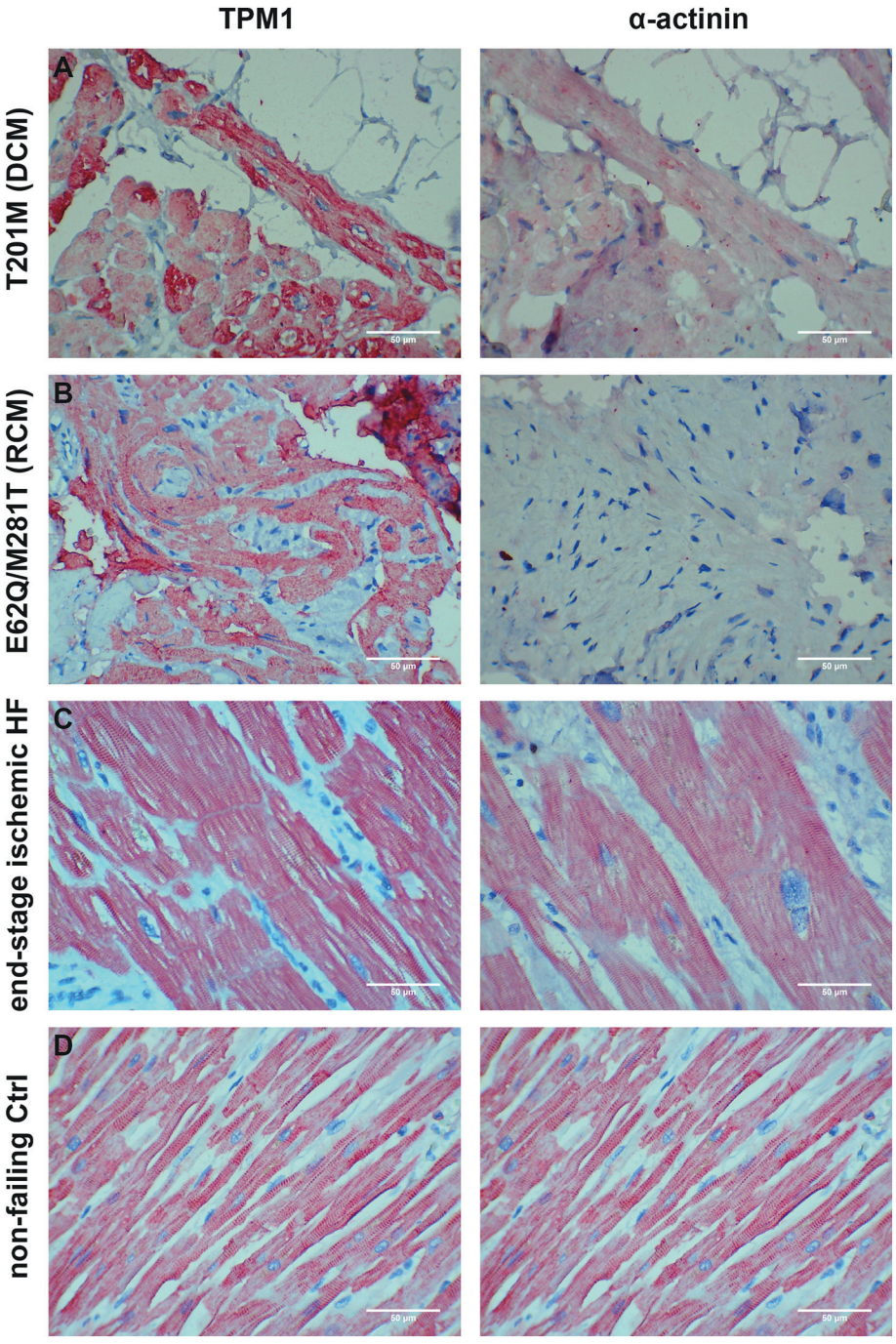

Fig. 3. Representative images of immunohistochemical staining for TPM1 (left column) and α-actinin (right column) of endomyocardial biopsies. (**A**) Subject (III:11) with *TPM1* T201M variant, (**B**) proband (V:1) with *TPM1* E62Q/M281T variants, (**C**) remote area biopsies from the left ventricle of one of three patients with end-stage ischemic heart failure (HF) and (**D**) septal tissue from one of four subjects that died due to a non-cardiac cause of death (non-failing Ctrl). Scale bar represents 50 µm.

Overall, we observed that the dose-dependent effect on CaT may be determined by the mixture of variant and wild-type TPM1. DCM- and HCM-associated *TPM1* variants led to opposite effects, and this difference may be related to the ability to replace/reduce endogenous sarcomeric tropomyosin.

4.3. Destruction of human cardiomyocytes in tissue biopsies with long-term expression of TPM1 variants

In contrast to our observations in short-term *TPM1* overexpression experiments and previous findings suggesting that *Tpm1* variants expressed in isolated rat cardiomyocytes or 12-month-old transgenic mice were normally incorporated into the sarcomeres without altering sarcomeric structures [26,27], cardiomyocytes in the respective human tissue biopsies displayed obvious structural changes. To our knowledge this is the first time that cardiac biopsies with these *TPM1* variants have been immunohistochemically analyzed for intracellular localization of TPM1 and α-actinin. We used α-actinin as a *Z*-disc marker to assess normal myofilament structure. The combination of chronic expression of *TPM1* variants and cardiomyopathy-associated pathological remodeling may have been responsible for the loss of cardiomyocyte-typical architecture. Cardiac histopathologic findings of myocyte disarray and

interstitial fibrosis have also been reported in an affected subject with the $TPM1_{D175N}$ variant [28], and myofibril loss has been observed in subjects with HCM, DCM and RCM [29–31]. Reduced myofibril density may subsequently impair contractility and enhance the respective cardiomyopathy-associated pathomechanisms.

Since the TPM1 epitopes appeared disrupted and not organized in the characteristic contractile filaments and α-actinin was almost completely lost, we hypothesize that these *TPM1* variants affect the sarcomeric organization, especially assembly of α-actinin. *TPM1*-deficient cardiomyocytes have been shown to assemble aberrant F-actin fibrils with α-actinin puncta dispersed irregularly along their lengths [32]. Moreover, disrupting the tropomodulin–TPM1 interaction in chicken cardiac myocytes led to thin filament depolymerization and disassembly that was accompanied by perturbation of *Z*-lines, thick filaments and titin filaments [33]. Likewise, the *TPM1* variants we studied may disrupt interactions of TPM1, leading to its depolymerization and, as a consequence, to disassembly of α-actinin.

4.4. Limitations of HL-1 cardiomyocytes and endomyocardial biopsies

As sarcomeric structures are immature [15,16], HL-1 cardiomyocytes present with limited contractility [16]. We therefore focused on the effect on Ca^{2+} transients since disturbed Ca^{2+} handling will impair contractility. CaT data in HL-1 cardiomyocytes has been shown to be similar to that of isolated rat and dog atrial cardiomyocytes, indicating that HL-1 cardiomyocytes represent a cardiac cell model to trace changes in Ca^{2+} transients in relation to cardiac diseases [19,34].

HL-1 cardiomyocytes are immortalized cells of mouse atrial origin and therefore do not precisely recapitulate a cellular milieu that reflects the ventricular cardiac myocytes in human or of acutely isolated ventricular cardiomyocytes [35]. In future, it is important to test these *TPM1* variants in a human cell-based model such as human iPSC-derived cardiomyocytes.

Paraffin-embedded endomyocardial biopsies were collected in the context of thorough diagnostic evaluation and all available endomyocardial samples were included in this study. The rareness and the sectional planes (Fig. SVIA) makes it difficult to make firm statements about the effects of these *TPM1* variants in other patients. For future studies, we suggest to use electron microscopy to clarify sarcomere ultrastructure.

5. Conclusion

In conclusion, various *TPM1* variants lead to heterogeneous clinical manifestations of different cardiomyopathy subtypes. Overexpression of *TPM1* variants led to time-dependent progressive deterioration of cardiomyocyte CaT amplitudes. The dose-dependent effect on CaT was opposite for DCM- and HCM-associated *TPM1* variants. Overall, our data indicate that reduced cardiomyocyte CaT amplitudes and loss of sarcomeric structures are independent of the *TPM1* variant and of the clinical cardiomyopathy phenotype.

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Author statement.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2020.08.101.

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