Genotype-specific pathogenic effects in human dilated cardiomyopathy

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Key points

- Mutations in genes encoding cardiac troponin I (TNNI3) and cardiac troponin T (TNNT2) caused altered troponin protein stoichiometry in patients with dilated cardiomyopathy.
- TNNI3p.98trunc resulted in haploinsufficiency, increased Ca²⁺-sensitivity and reduced length-dependent activation.
- TNNT2p.K217del caused increased passive tension.
- A mutation in the gene encoding lamin A/C (LMNAp.R331Q) led to reduced maximal force development through secondary disease remodelling in patients suffering from dilated cardiomyopathy.
- Our study shows that different gene mutations induce dilated cardiomyopathy via diverse cellular pathways.

Abstract Dilated cardiomyopathy (DCM) can be caused by mutations in sarcomeric and non-sarcomeric genes. In this study we defined the pathogenic effects of three DCM-causing mutations: the sarcomeric mutations in genes encoding cardiac troponin I (TNNI3p.98truncation) and cardiac troponin T (TNNT2p.K217deletion; also known as the p.K210del) and the non-sarcomeric gene mutation encoding lamin A/C (LMNAp.R331Q). We assessed sarcomeric protein expression and phosphorylation and contractile behaviour in single membrane-permeabilized cardiomyocytes in human left ventricular heart tissue. Exchange with recombinant troponin complex was used to establish the direct pathogenic effects of the mutations in TNNI3 and TNNT2. The TNNI3p.98trunc and TNNT2p.K217del mutation showed reduced expression of troponin I to 39% and 51%, troponin T to 64% and 53%, and troponin C to 73% and 97% of controls, respectively, and altered stoichiometry between the three cardiac troponin subunits. The TNNI3p.98trunc showed pure haploinsufficiency, increased Ca²⁺-sensitivity and impaired length-dependent activation. The TNNT2p.K217del mutation showed a significant increase in passive tension that was not due to changes in titin isoform composition or phosphorylation. Exchange with wild-type troponin complex corrected troponin protein levels to 83% of controls in the TNNI3p.98trunc sample. Moreover, upon exchange all functional deficits in the TNNI3p.98trunc and TNNT2p.K217del samples were normalized to control values confirming the pathogenic effects of the troponin mutations. The LMNAp.R331Q mutation resulted in reduced maximal force development due to disease remodelling. Our study shows that different gene mutations induce DCM via diverse cellular pathways.
The troponin complex consists of three different troponin proteins; cardiac troponin T (cTnT), cardiac troponin I (cTnI) and cardiac troponin C (cTnC). The involvement of troponin and tropomyosin in force generation has been described in the three state model of the thin filaments (McKillop & Geeves, 1993). The role of cTnI is to inhibit actin–myosin interaction and, through its interaction with cTnC, plays an important role in the Ca\(^{2+}\)-sensitivity of sarcomere activation (Westfall et al. 1999). The troponin complex can lock tropomyosin in the blocked state (so called B-state) at low Ca\(^{2+}\) concentrations during which contraction does not occur since tropomyosin sterically hinders the interaction between myosin and actin. During contraction calcium binds to cTnC, which leads to a conformational change that enhances binding of cTnC to cTnI. This results in a large conformational change in cTnI, which leads to displacement of its inhibitory domains away from actin and thereby releasing its inhibitory effect on actin–myosin interaction (Spyracopoulos et al. 1997; Stone et al. 1998). Tropomyosin moves and transits into the closed state (C-state), which enables myosin to bind to actin and subsequently cause force generation. The open state (M-state) is the final shift of tropomyosin after myosin has bound and facilitates the formation of a strong cross-bridge. By binding to both cTnC and tropomyosin (Li et al. 2002), cTnT regulates ATPase activity during contraction, but also serves as an anchor on the thin filaments for the tropinin complex. The different troponin proteins as a complex and the location of the mutations studied are shown in a schematic representation in Fig. 1. The 292C→T transition in the TNNI3 gene encoding cTnI is predicted to result in a premature stop codon at amino acid 98. Truncation in this part of the protein would cause loss of the cTnC and two actin-binding domains of cTnI (Mogensen et al. 2015). The p.K217del (also known as p.K210del; Otten et al. 2010) mutation in the TNNT2 gene has been reported across the world in unrelated families and is associated with high mortality and disease onset at a young age (~33 years) (Kamisago et al. 2000; Mogensen et al. 2004; Hershberger et al. 2009; Otten et al. 2010). Mutations in the non-sarcomeric gene LMNA, encoding the inner nuclear protein lamin A/C, have been found in 6% of DCM patients (Parks et al. 2008). Many patients carrying a LMNA mutation show

### Introduction

Dilated cardiomyopathy (DCM) is a cardiac disease characterized by dilatation of the left ventricle (LV) and a reduced systolic function. Initially, the prevalence of DCM was determined to be 1:2500 based on phenotypic screening, but recent studies suggested that it could be as high as 1:250 (Hershberger et al. 2013). DCM can be caused by environmental factors (viral infection, alcohol abuse, drug toxicity) or have a genetic basis. With current genetic screening, a genetic cause is found in 20–50% of DCM patients (Hershberger et al. 2010; Herman et al. 2012; van Spaendonck-Zwarts et al. 2013). Over 30 genes have been found to harbour mutations that are likely to cause DCM (Hershberger et al. 2013). The Exome Aggregation Consortium (ExAC) recently reported that many rare variants in various sarcomeric and non-sarcomeric genes, which were assumed to be disease-causing, only have limited pathogenic burden as no or limited excess variation was found in a DCM population compared with ~60,000 reference samples (Walsh et al. 2017). On the other hand, the presence of rare variants of uncertain significance was reported to be significantly higher in the DCM population compared with the ExAC reference samples indicating an overly conservative estimation of pathogenicity of these variants (Walsh et al. 2017). Among the various genes implicated in DCM are genes encoding sarcomeric proteins such as cardiac troponin I (encoded by TNNI3) (Carballo et al. 2009; van Spaendonck-Zwarts et al. 2013), cardiac troponin T (encoded by TNNT2) (Hershberger et al. 2009; van Spaendonck-Zwarts et al. 2013; Walsh et al. 2017) and titin (encoded by TTN) (Herman et al. 2012; Walsh et al. 2017), and genes encoding for non-sarcomeric proteins such as lamin A/C (encoded by LMNA), a protein involved in nuclear stability (Parks et al. 2008; van Spaendonck-Zwarts et al. 2013; Walsh et al. 2017). The fact that mutations in proteins of such diverse function can cause DCM implies that multiple pathomechanisms can lead to cardiac dilatation and associated cardiac dysfunction. In this study we defined the pathological effects on cardiomyocyte function of three different DCM-causing mutations in genes encoding sarcomeric (TNNI3, TNNT2) and non-sarcomeric (LMNA) proteins.
conduction abnormalities and arrhythmias (Parks et al. 2008; Perrot et al. 2009). The LMNA\textsubscript{p,R331Q} mutation is located in the coil 2B domain, which is important for homodimerization. The LMNA\textsubscript{p,R331Q} mutation is predicted to cause loss of salt-bridge interaction and thereby affect lamina stability (Gangemi & Degano, 2013).

Apart from the direct mutation-mediated changes in cardiac function, secondary disease remodelling plays an important role in DCM pathogenesis (Kötter et al. 2013; Beqqali et al. 2016). Phosphorylation by protein kinase A (PKA) of cTnI can fine-tune Ca\textsuperscript{2+}-sensitivity and length-dependent activation (LDA) of sarcomeres (Konhilas et al. 2003; Sequeira et al. 2013). PKA-mediated phosphorylation of cTnI upon activation of the β-adrenergic receptors by adrenaline reduces myofilament Ca\textsuperscript{2+}-sensitivity and enhances LDA (Konhilas et al. 2003). In heart failure, the β-adrenergic receptor system is chronically stimulated leading to down-regulation and desensitization of the β-adrenergic receptors and subsequently decreased PKA-mediated phosphorylation (Harding et al. 1994). Therefore, it is important to distinguish between the direct effects of mutations on sarcomere function and the indirect effects through changes in the β-adrenergic system.

Even though various genes are implicated in DCM, these mutations ultimately result in a dilated heart and cardiac dysfunction. Mutations can induce different cellular changes depending on their effect on protein function. Therefore, patients could have different disease mechanisms leading to DCM. Our studies in human cardiac tissue of DCM patients showed reduced expression of the troponin complex in samples harbouring sarcomeric mutations in TNNI3\textsubscript{p,98trunc} and TNNT2\textsubscript{p,K217del}. In the TNNI3\textsubscript{p,98trunc} sample we did not

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**Figure 1. Schematic representation of the troponin complex**

cTnT is shown in yellow, cTnI in blue and cTnC in red. The letters N and C indicate the N- and C-terminus, respectively. The upper diagram shows the troponin complex in the presence of Ca\textsuperscript{2+} while the lower diagram shows the troponin complex without Ca\textsuperscript{2+}. Location of the studied mutations are indicated with stars and an arrow in the upper panel. The letter H indicates a helix structure. IR, inhibitory region.
find a truncated protein, and the haploinsufficiency led to increased Ca\(^{2+}\)-sensitivity and reduced LDA. The TNN\(_{2p,K217del}\) mutation caused increased passive tension (\(F_{\text{pass}}\)) and a non-significant mild reduction in Ca\(^{2+}\)-sensitivity. Upon exchange with wild-type (WT) troponin complex, all parameters normalized to control confirming the pathogenicity of these sarcomeric mutations. The non-sarcomeric mutation LMNA\(_{p,R331Q}\) showed decreased maximal force (\(F_{\text{max}}\)) development and increased Ca\(^{2+}\)-sensitivity of sarcomeres, which were both attributed to secondary disease remodelling. Also idiopathic DCM (IDCM) samples showed increased myofilament Ca\(^{2+}\)-sensitivity and reduced LDA, which could be attributed to secondary disease remodelling. Therefore, mutations in genes encoding proteins of diverse functions can cause DCM, which implies that changes in different cellular pathways can lead to cardiac dilatation and dysfunction.

**Methods**

**Ethical approval**

Left ventricular (LV) tissue was obtained from DCM patients who underwent cardiac transplantation, two samples of patients who carried the LMNA\(_{p,R331Q}\) mutation were derived from a biopsy taken prior to LV assist device (IVAD) implantation. The other LMNA\(_{p,R331Q}\) sample was derived from a cardiac transplantation of a heart that had been supported by a IVAD prior to transplantation. Most DCM patient samples used in this study were acquired from the Biobank of the University Medical Centre Utrecht, the Netherlands. This study was approved by the Biobank Research Ethics Committee, University Medical Centre Utrecht, Utrecht, the Netherlands (protocol number WARB 12/387). Written informed consent was obtained. Samples were obtained from regions halfway between the atrioventricular valves and the apex. As control samples we used explanted LV heart tissue of healthy donors – people who had died from a non-cardiac cause, typically motor vehicle accidents. These healthy donor samples and three DCM were acquired from the University of Sydney, with the ethical approval of the Human Research Ethics Committee no. 2012/2814. The control samples used were 3.160, 4.049, 6.042, 3.162, 5.128, 6.020, 7.044, 3.164, 3.141, 6.008, 5.086, 8.004, 7.054, and the DCM samples used were 4.036, 3.107 and 2.082. All samples were stored in liquid nitrogen or at −80°C until use.

**Cardiomyocyte force measurements**

\(F_{\text{max}}\) and \(F_{\text{pass}}\) of sarcomeres were measured at pCa 4.5 and pCa 9.0, respectively, in single membrane-permeabilized cardiomyocytes mechanically isolated from heart tissue as previously described (van Dijk et al. 2012). LDA experiments and PKA incubations were performed as previously described (van der Velden et al. 2000). Ca\(^{2+}\)-sensitivity was measured as the [Ca\(^{2+}\)] needed to achieve 50% of \(F_{\text{max}}\) (EC\(_{50}\)) and LDA was measured as the shift in EC\(_{50}\) (ΔEC\(_{50}\)) at a sarcomere length of 1.8 μm and 2.2 μm.

**Protein expression and phosphorylation**

**Titin.** Titin isoforms were separated on a 1% (w/v) agarose gel and stained with SYPRO Ruby protein stain (Invitrogen, Carlsbad, CA, USA) as described previously (Warren et al. 2003) and samples were measured in triplicate. Phosphorylation of titin was assessed as previously described (Kötter et al. 2016). For titin phosphorylation, site-specific antibodies directed to Ser4010 (N2Buniqule sequence (N2Bus) domain; PKA and extracellular signal-regulated kinase 2 (ERK2) target), and Ser12022 and Ser11878 (PEVK domain; protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKIIδ) target) were used.

**Troponin.** The troponin proteins were separated by 12% polyacrylamide and 4–15% precast gradient gels (BioRAD, Hercules, CA, USA) gel electrophoresis and Western blots were stained with specific antibodies (cTnI: Abcam, Cambridge, UK, ab10231; cTnT: Sigma, St. Louis, MO, USA, T6277; cTnC: Santa Cruz, Dallas, TX, USA, sc48347) to determine their expression, which was corrected by expression of other cellular proteins (glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Cell signaling, 2118S, Cell signaling, Danvers, MA, USA; α-actinin: Sigma, A7811). The TNN\(_{3p,K217del}\) and TNN\(_{2p,K217del}\) samples were measured in duplicate of which the average is shown. Phosphorylation of cTnI was assessed as previously described (Zaremba et al. 2007).

**Troponin exchange**

The troponin complex was exchanged in membrane-permeabilized cardiomyocytes as previously described (Wijnker et al. 2013). The recombinant WT or TNN\(_{2p,K217del}\) troponin complex was added to the cells in a concentration of 1 mg ml\(^{-1}\). The recombinant troponin complexes could be distinguished from highly phosphorylated endogenous troponin since the recombinant troponins were not phosphorylated. Quantification of the exchange rate was performed by phos-tag analysis in which non-, mono- and bis-phosphorylated cTnI (Pierce, Rockford IL, USA, MA1-22700) were separated by polyacrylamide-bound Mn\(^{2+}\)-phos-tag gel electrophoresis Western blotting as previously described (Najafi et al. 2015). The percentage of recombinant troponin complex present after exchange was quantified as the percentage of non-phosphorylated cTnI.
to the total of non-, mono- and bis-phosphorylated cTnI levels. Total cTnI levels after exchange were quantified by cTnI (Abcam, ab10231) and corrected for myosin light chain-2 (MLC2) (Enzo, Farmingdale, NY, USA, ALX-BC-1150).

Statistics

Graphpad Prism software was used for statistical analysis. $F_{\text{max}}$ of DCM cardiomyocytes compared to control cardiomyocytes was compared with one-way ANOVA and Tukey's post hoc test. LDA was calculated as $\Delta E_{\text{Ca}}$. Ca$^{2+}$-sensitivity and passive tension in DCM cardiomyocytes were compared to control cardiomyocytes by two-way ANOVA. All values are shown as mean ± standard error of the mean. A $P$ value <0.05 was considered to represent a significant difference and is indicated with an asterisk in figures. The 95% confidence intervals (CI) of the control group are indicated with a dotted line in the graphs and was used to assess the difference of the sample of interest compared to controls in situations where only a single data point of the sample of interest could be used.

Results

Diverse functional myofilament changes in DCM with sarcomeric and non-sarcomeric mutations

The 12 control samples used for experiments consisted of four females and eight males with a mean age of 44.5 ± 3.4 years. The patient with $TNNI3_{p.98\text{trunc}}$ mutation was a 46-year-old male and the patient with $TNNT2_{p.K217\text{del}}$ mutation was a 19-year-old male. Three patients with $LMNA_{p.R331Q}$ were studied of which two were male and one female with a mean age of 45.3 ± 3.4 years. The IDC samples consisted of four males and one female with a mean age of 54.6 ± 3.2 years. To determine the functional properties of human DCM samples with sarcomeric and non-sarcomeric protein mutations, sarcomere function was measured in single isolated membrane-permeabilized cardiomyocytes at various [Ca$^{2+}$] to assess passive and active properties of the sarcomeres. No difference in $F_{\text{max}}$ (Fig. 2A) was observed in IDC, $TNNI3_{p.98\text{trunc}}$ and $TNNT2_{p.K217\text{del}}$ cardiomyocytes, while $F_{\text{max}}$ was significantly lower in cardiomyocytes with the $LMNA_{p.R331Q}$ mutation (17.9 kN m$^{-2}$, data from Hoorntje et al. 2016) compared to controls (28.2 kN m$^{-2}$). A previous study showed that this decreased $F_{\text{max}}$ was due to decreased myofilibril density (Hoorntje et al. 2016). $F_{\text{pass}}$ is an important determinant of diastolic function which was measured at low [Ca$^{2+}$] (pCa 9.0) over a range of sarcomere lengths (Granzier & Irving, 1995). IDC samples (Fig. 2B), $TNNI3_{p.98\text{trunc}}$ (Fig. 2C) and $LMNA_{p.R331Q}$ (Fig. 2E) showed a comparable $F_{\text{pass}}$ development over the range of sarcomere lengths compared to controls. The $TNNT2_{p.K217\text{del}}$ cardiomyocytes showed a significant increase in $F_{\text{pass}}$ compared to controls, which was most pronounced at longer sarcomere lengths (Fig. 2D). In addition to changes in $F_{\text{pass}}$, stretching of cardiomyocytes during filling of the heart increases active force development. This LDA of myofilaments is the cellular basis of the Frank–Starling mechanism (Sequeira et al. 2013; Beqqali et al. 2016). We measured active force development over a range of [Ca$^{2+}$] at sarcomere lengths 1.8 μm and 2.2 μm to study LDA. IDC samples showed an increased Ca$^{2+}$-sensitivity and reduced LDA compared to controls (Fig. 2F). The $TNNI3_{p.98\text{trunc}}$ cardiomyocytes showed Ca$^{2+}$-sensitivity was increased and LDA was blunted (Fig. 2G) compared to controls. $TNNT2_{p.K217\text{del}}$ cardiomyocytes showed only a minor and non-significant decrease in Ca$^{2+}$-sensitivity and LDA was preserved (Fig. 2H). The Ca$^{2+}$-sensitivity of $LMNA_{p.R331Q}$ cardiomyocytes was significantly increased compared to controls, while LDA was preserved (Fig. 2J). Overall these data illustrate that changes in passive and active myofilament properties differ between different sarcomeric gene mutations and between sarcomeric and non-sarcomeric mutations.

Haploinsufficiency and altered stoichiometry of troponin proteins in DCM with $TNNI3_{p.98\text{trunc}}$ and $TNNT2_{p.K217\text{del}}$ mutations

Since the troponin complex and the interactions between the various troponin proteins is important for adequate contractile behaviour of the myofilaments, we studied the composition of the troponin complex in the $TNNI3_{p.98\text{trunc}}$ and $TNNT2_{p.K217\text{del}}$ samples. We observed that cTnI protein level was decreased in both the $TNNI3_{p.98\text{trunc}}$ and $TNNT2_{p.K217\text{del}}$ samples compared to controls when normalized to the cytoplasmic housekeeping protein GAPDH (Fig. 3A and B). If the truncated cTnI protein is present in the $TNNI3_{p.98\text{trunc}}$ sample an antibody raised against the N-terminus of cTnI is expected to show two bands: the native protein and the truncated protein. However, only one band was visible at the height of the native cTnI protein (Fig. 3A). This indicates that the mutant protein is either not expressed or efficiently degraded resulting in cTnI haploinsufficiency. The level of cTnI relative to the sarcomeric housekeeping gene α-actinin was also decreased in both $TNNI3_{p.98\text{trunc}}$ and $TNNT2_{p.K217\text{del}}$ samples (Fig. 3C and D), which indicates that less cTnI is present within the sarcomeres itself. The reduction in cTnI levels was accompanied by a less pronounced decrease in cTnT (Fig. 3E and F) and near normal cTnC levels (Fig. 3G and H). This indicates that the $TNNI3_{p.98\text{trunc}}$ and $TNNT2_{p.K217\text{del}}$ Samples have altered stoichiometry of the three troponin proteins since the decrease in cTnI, cTnT and cTnC is not to the same extent.

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Hypophosphorylation of cTnI underlies increased Ca\(^{2+}\)-sensitivity in DCM with the LMNA\(_{p.R331Q}\) mutation, but does not underlie myofilament defects in DCM with TNNI3\(_{p.98trunc}\) and TNNT2\(_{p.K217del}\) mutations

The troponin complex was reduced in both the TNNI3\(_{p.98trunc}\) and TNNT2\(_{p.K217del}\) samples, while cardiomyocytes from these samples showed different myofilament properties. We therefore studied phosphorylation of cTnI using phos-tag analysis. Control samples showed prominent bis- and mono-phosphorylated cTnI and low non-phosphorylated cTnI (Fig. 4A and B). IDCM samples showed reduced cTnI phosphorylation as is evident by prominent non- and mono-phosphorylated cTnI bands and a weak bis-phosphorylated cTnI band (Fig. 4A and B). In the TNNI3\(_{p.98trunc}\) and TNNT2\(_{p.K217del}\) samples cTnI was highly phosphorylated indicated by an intense bis-phosphorylated cTnI band and very weak non- and mono-phosphorylated cTnI bands (Fig. 4A and B). On the contrary, the LMNA\(_{p.R331Q}\) samples showed decreased cTnI phosphorylation evident from prominent non- and mono-phosphorylated cTnI bands (Fig. 4A and B). Low phosphorylation of cTnI has been reported previously in DCM (Wijnker et al. 2014) and may underlie increased Ca\(^{2+}\)-sensitivity (Konhilas et al. 2003; Wijnker et al. 2014) and a blunted LDA (Konhilas et al. 2003; Wijnker et al. 2014). Indeed, after incubation with exogenous...
Figure 3. Expression of troponin in troponin mutants

A and B, cTnI levels measured with an antibody directed to the N-terminal of cTnI and normalized to GAPDH were decreased to 46% in the TNNI3_p.98trunc (0.28) and to 40% in TNNT2_p.K217del (0.24) samples compared to controls (N = 8, mean = 0.60, CI = 0.43–0.77). A, corresponding gel image showed no additional bands indicative of a truncated cTnI protein. C and D, cTnI levels were decreased to 39% in TNNI3_p.98trunc (0.34) and to 51% in TNNT2_p.K217del (0.44) samples compared to controls (N = 8, mean = 0.87, CI = 0.59–1.15) when normalized for α-actinin. E and F, cTnT levels normalized to α-actinin were also decreased to 64% in TNNI3_p.98trunc (0.70) and to 51% in TNNT2_p.K217del (0.59) samples compared to controls (N = 8, mean = 1.11, CI = 0.83–1.38). G and H, cTnC levels normalized to α-actinin were slightly decreased to 73% in TNNI3_p.98trunc sample (0.66) but still within the 95% CI of controls (N = 8, mean = 0.91, CI = 0.67–1.15). TNNT2_p.K217del showed normal (0.88, 97% of controls) cTnC levels. [Colour figure can be viewed at wileyonlinelibrary.com]
PKA we observed normalization of Ca\(^{2+}\)-sensitivity to controls in IDCM (Fig. 4C) and in the LMAN_{p.R331Q} cardiomyocytes (Fig. 4D). Incubation with exogenous PKA did not change Ca\(^{2+}\)-sensitivity in controls. Also LDA was restored in IDCM samples compared to controls after incubation with exogenous PKA (Fig. 4C).

However, Ca\(^{2+}\)-sensitivity was still significantly increased compared to controls after incubation with exogenous PKA in the TNNT2_{p.R98trunc} cardiomyocytes (Fig. 4E). These experiments confirm that impaired \(\beta\)-adrenergic receptor signalling, and subsequent hypophosphorylation of cTnI, is the cause of the increased Ca\(^{2+}\)-sensitivity in IDCM.

**Figure 4. Secondary disease remodelling and direct mutation effects**

A, phos-tag analysis showed separation of non- (0P), mono- (1P) and bis- (2P) phosphorylated cTnI. B, phosphorylation of cTnI was increased in TNNT2_{p.R98trunc} and TNNT2_{p.K217del} samples compared to controls (\(N = 7\)) while cTnI phosphorylation in LMAN_{p.R331Q} (\(N = 3\)) and IDCM (\(N = 3\)) was decreased compared to controls. C, Ca\(^{2+}\)-sensitivity was normalized in IDCM cardiomyocytes (\(N = 5, n = 12\)) compared to control cardiomyocytes (\(N = 6, n = 14\)) after incubation with exogenous PKA. D, Ca\(^{2+}\)-sensitivity was normalized in LMAN_{p.R331Q} cardiomyocytes (\(N = 3, n = 7\)) compared to control cardiomyocytes (\(N = 6, n = 14\)) after incubation with exogenous PKA. E, after incubation with exogenous PKA, Ca\(^{2+}\)-sensitivity in TNNT2_{p.R98trunc} cardiomyocytes (\(N = 1, n = 7\)) remained significantly increased (\(P < 0.01\)) compared to control cardiomyocytes (\(N = 6, n = 14\)). F, exchange with WT troponin complex restored cTnI levels in the TNNT2_{p.R98trunc} sample to 83\% of that of controls exchanged with WT troponin complex (H). G, phos-tag gel analysis showed high phosphorylation of native troponin complex prior to exchange (NE) and incorporation of unphosphorylated recombinant protein after exchange. H, the 83\% was composed of 46\% recombinant troponin and 37\% native troponin in the TNNT2_{p.R98trunc} sample compared with 43\% recombinant troponin in the control exchanged with WT troponin complex. I, Ca\(^{2+}\)-sensitivity and LDA were restored in TNNT2_{p.R98trunc} cardiomyocytes (\(N = 1, n = 9\)) compared to control (\(N = 2, n = 11\)) after exchange with WT troponin complex and incubation with exogenous PKA. N, number of samples; n, number of total cardiomyocytes measured.
and the \(LMNA_{p.R331Q}\) cardiomyocytes, while the observed increased \(Ca^{2+}\)-sensitivity and impaired LDA is a direct mutation effect in the \(TNNI3_{p.98trunc}\) cardiomyocytes.

**Correction of high \(Ca^{2+}\)-sensitivity and blunted LDA in DCM with \(TNNI3_{p.98trunc}\) by human recombinant WT troponin**

Next we aimed to assess whether the observed haploinsufficiency of cTnI, and reduced cTnT and cTnC, caused increased \(Ca^{2+}\)-sensitivity and reduced LDA in the \(TNNI3_{p.98trunc}\) cardiomyocytes. We exchanged the endogenous troponin complex with the recombinant WT troponin complex in order to restore total troponin levels. The level of cTnI increased to 83% after exchange (Fig. 4F and H), relative to the cTnI level in control cells exchanged with exogenous recombinant WT troponin complex. The 83% cTnI in the \(TNNI3_{p.98trunc}\) sample after exchange consisted of 46% recombinant troponin complex and 37% native troponin complex, as determined by phos-tag gel analysis (Fig. 4G and H). In the control sample 43% of total cTnI levels was derived from the recombinant troponin complex (Fig. 4G and H). This indicates that we exchanged endogenous troponin complex with recombinant WT complex, but also added additional recombinant WT troponin complex in the exchange process thereby largely overcoming the haploinsufficiency in the \(TNNI3_{p.98trunc}\) cardiomyocytes. Since the recombinant troponin complex is unphosphorylated we incubated the exchanged cells with exogenous PKA prior to functional cell measurements. Upon exchange with the WT troponin complex both \(Ca^{2+}\)-sensitivity as well as LDA were normalized to control values in the \(TNNI3_{p.98trunc}\) cardiomyocytes (Fig. 4I).

**High passive force in DCM with \(TNNT2_{p.K217del}\) is caused by the mutation and not by changes in isoform composition or phosphorylation of titin**

We next set out to determine the cause of the increased \(F_{pass}\) in the \(TNNT2_{p.K217del}\) sample. An important determinant of \(F_{pass}\) is titin isoform composition (Makarenko et al. 2004; Nagueh et al. 2004). Titin can exist as a stiff isoform (N2B) or a larger, more compliant isoform (N2BA). All DCM groups showed an increase in compliant titin compared to controls independent of the type of mutation (Fig. 5A and B). The observed increase in N2BA/N2B ratio cannot explain the high \(F_{pass}\) in \(TNNT2_{p.K217del}\). Therefore, we examined phosphorylation of titin at three well-established phosphorylation sites in the elastic I-band region. Phosphorylation of Ser4010 on titin, a target of PKA, is known to decrease \(F_{pass}\) (Kötter et al. 2013), while PKC-mediated phosphorylation of Ser12022 and Ser11878 results in increased \(F_{pass}\) (Hidalgo et al. 2009). While phosphorylation at Ser4010 was lower in the IDCm, \(TNNI3_{p.98trunc}\) and \(LMNA_{p.R331Q}\) samples compared to controls, a preserved or even slightly increased Ser4010 phosphorylation was observed in the \(TNNT2_{p.K217del}\) sample (Fig. 5C and D). Ser12022 (Fig. 5E and F) and Ser11878 (Fig. 5G and H) phosphorylation, which would increase \(F_{pass}\), was within the 95% CI of controls in IDCm but lower in samples carrying mutations compared to controls. Therefore, the increase in \(F_{pass}\) in the \(TNNT2_{p.K217del}\) cardiomyocytes is not caused by alterations in titin phosphorylation at the investigated sites. The increase in \(F_{pass}\) was not due to impaired PKA-mediated phosphorylation of titin since \(F_{pass}\) remained significantly higher in \(TNNT2_{p.K217del}\) cardiomyocytes compared to controls after incubation with exogenous PKA (Fig. 6A). Exchange with the WT troponin complex led to a 59% incorporation of recombinant troponin complex in the \(TNNT2_{p.K217del}\) sample (Fig. 6B and C). The exchange normalized \(F_{pass}\) in the \(TNNT2_{p.K217del}\) cardiomyocytes to control level (Fig. 6D). In addition, after exchange of the mutant \(TNNT2_{p.K217del}\) troponin complex into a healthy control sample we observed that only 34% of total troponin present after exchange was recombinant (Fig. 6B and C). However, this was sufficient to cause a significant increase in \(F_{pass}\) (Fig. 6D). The increase in \(F_{pass}\) upon exchange with \(TNNT2_{p.K217del}\) in a control sample was not due to impaired PKA-mediated phosphorylation since after incubation with exogenous PKA \(F_{pass}\) remained significantly increased (Fig. 6E). These results indicate that the \(TNNT2_{p.K217del}\) mutant protein itself increases \(F_{pass}\).

**Discussion**

Mutations in various sarcomeric and non-sarcomeric genes can induce DCM. In this study we aimed to define the pathogenic effects of the sarcomeric \(TNNI3_{p.98trunc}\) and \(TNNT2_{p.K217del}\) mutations, and the non-sarcomeric \(LMNA_{p.R331Q}\) mutation. Our study provides proof that the two sarcomere mutations cause myofilament dysfunction, while changes in myofilament properties in IDCm and the non-sarcomeric mutation samples are the result of secondary disease remodelling. One of the \(LMNA_{p.R331Q}\) samples showed a smaller decrease in cTnI phosphorylation compared to the other two samples. This was the sample obtained from a patient who used a LVAD prior to transplantation. It is therefore possible that the LVAD has partly reversed the secondary remodelling (Sakamuri et al. 2016). However, this patient did not show deviations from the other two \(LMNA_{p.R331Q}\) patients in other protein analyses.

**Haploinsufficiency and altered stoichiometry of troponin proteins in human DCM**

We show that the \(TNNI3_{p.98trunc}\) sample does not lead to a truncated protein but causes haploinsufficiency.
Figure 5. Alterations in titin isoform composition and phosphorylation in DCM mutants

A, titin isoforms, N2BA and N2B, separated by agarose gel electrophoresis. B, titin N2BA/N2B ratios were increased in IDCM (0.99 ± 0.20, N = 5), TNNI3p.98trunc (0.78, N = 1), TNNT2p.K217del (0.82, N = 1) and LMNAp.R331Q (0.99 ± 0.38, N = 3) samples compared to controls (0.50 ± 0.02, N = 12, CI = 0.49–0.55).

C, phosphorylated Ser4010 compared to total titin levels. D, titin phosphorylation at Ser4010 was decreased in IDCM (N = 5), TNNI3p.98trunc (N = 1) and LMNAp.R331Q (N = 3) compared to controls (N = 10, CI = 0.93–1.17), while TNNT2p.K217del (N = 1) showed slight increased phosphorylation of Ser4010 compared to control.

E, phosphorylated Ser12022 compared to total titin levels. F, titin phosphorylation at Ser12022 was decreased in TNNI3p.98trunc (N = 1), TNNT2p.K217del (N = 1) and LMNAp.R331Q (N = 3), compared to control (N = 9, CI = 0.58–1.66), while phosphorylation at Ser12022 was within the 95% CI of controls in IDCM (N = 5).

G, phosphorylated Ser11878 compared to total titin levels. H, titin phosphorylation at Ser11878 was decreased in TNNI3p.98trunc (N = 1), TNNT2p.K217del (N = 1) and LMNAp.R331Q (N = 3), compared to control (N = 11, CI = 0.62–1.49) while phosphorylation at Ser11878 was within the 95% CI of controls in IDCM (N = 5).
Genotype-specific pathogenic effects in dilated cardiomyopathy

Figure 6. *TNNT2*<sub>p.K217del</sub> increases passive tension

A, $F_{\text{pass}}$ remained significantly increased ($P < 0.0001$) in *TNNT2*<sub>p.K217del</sub> cardiomyocytes ($N = 1, n = 9$) compared to controls ($N = 4, n = 13$) after incubation with exogenous PKA. B, phos-tag gel analysis showed high phosphorylation of native troponin complex prior to exchange (NE) and incorporation of unphosphorylated recombinant protein after exchange. C, after exchange 43% of present troponin complex in controls was recombinant WT cTnI while in the *TNNT2*<sub>p.K217del</sub> sample this was 59% and in control exchanged with *TNNT2*<sub>p.K217del</sub> mutant troponin complex this was 34%. D, upon exchange with WT troponin complex, cardiomyocytes of *TNNT2*<sub>p.K217del</sub> ($N = 1, n = 11$) showed restoration of $F_{\text{pass}}$ compared to controls exchanged with WT troponin complex ($N = 2, n = 8$) while $F_{\text{pass}}$ was significantly increased ($P = 0.001$) in control cardiomyocytes exchanged with mutant *TNNT2*<sub>p.K217del</sub> troponin complex ($N = 2, n = 7$). E, after incubation with exogenous PKA, cardiomyocytes of *TNNT2*<sub>p.K217del</sub> exchanged with recombinant WT troponin complex ($N = 1, n = 13$) showed normalization of $F_{\text{pass}}$ compared to control cardiomyocytes exchanged with WT troponin complex ($N = 2, n = 9$) while $F_{\text{pass}}$ was significantly increased ($P < 0.0001$) in control cardiomyocytes exchanged with mutant *TNNT2*<sub>p.K217del</sub> troponin complex ($N = 2, n = 7$). N, number of samples; n, number of total cardiomyocytes measured.
This is in line with Kostareva et al. who showed that a truncation in the TNNI3 gene at the 176th amino acid in a patient with restrictive cardiomyopathy did not lead to a truncated protein, but instead to a 50% reduction of cTnI (Kostareva et al. 2009). The decrease in cTnI in our patient was associated with decreased levels of cTnT, while cTnC levels remained near normal. Also in the TNNT2p.K217del sample reduced levels of cTnI and cTnT were observed albeit to a smaller extent than observed in the TNNI3p.98trunc sample. While cTnT was most reduced in both samples, this was accompanied by a less pronounced decrease in cTnT, while cTnC levels remained near normal leading to altered stoichiometry. Since cTnT itself can bind to the thin filaments through tropomyosin and serves as an anchor for the whole troponin complex, this might explain why we observed a smaller decrease in cTnT protein levels compared to cTnI, which is more dependent on the formation of the whole troponin complex to attach to the thin filaments. This is in line with a study by Feng et al. in which they showed that an expression level of 25% of cTnI was accompanied by a 53% decrease in cTnT (Feng et al. 2009).

**A TNNI3 truncation mutation in human DCM causes haploinsufficiency, high Ca\(^{2+}\)-sensitivity and impaired LDA of myofilaments**

Various mutations in TNNI3 have been shown to increase Ca\(^{2+}\)-sensitivity and subsequently impair cardiac relaxation leading to hypertrophic cardiomyopathy (HCM) (Takahashi-Yanaga et al. 2001). These observations have been attributed to the possibility that the mutations act as a poison peptide and ‘lock’ tropomyosin in the C- or M-state. They are suggested to increase the stability of the Ca\(^{2+}\)-bound form of the thin filaments or destabilize the Ca\(^{2+}\)-free form of the thin filaments (Kobayashi & Solaro, 2006). In this study, we show that the TNNI3p.98trunc mutation in DCM patient cardiomyocytes also increased Ca\(^{2+}\)-sensitivity and in addition impaired LDA, which could not be corrected with exogenous PKA (Figs 2G and 4E) while the increased Ca\(^{2+}\)-sensitivity and impaired LDA in IDC could be corrected by exogenous PKA (Figs 2F and 4C). This is in line with Sequeira et al. who reported that the HCM-causing TNNI3p.R145W mutation impaired LDA, which could not be rescued with exogenous PKA (Sequeira et al. 2013). However, it has been heavily debated if mutations in TNNI3 and TNNT2 are able to cause DCM or HCM through haploinsufficiency. Homozygous TNNT2 knock out (KO) mice are embryonically lethal, while heterozygous TNNT2 KO mice have reduced cTnT mRNA levels, but normal cTnT protein levels, and show no cardiac phenotype (Ahmad et al. 2008). The TNNT2 gene apparently has robust compensatory mechanisms in order to maintain protein levels. In addition, full TNNI3 KO in mice is lethal around 18 days of age (Liu et al. 2007; Feng et al. 2009), while heterozygous TNNI3 KO mice survive without detectable phenotype (Feng et al. 2009). Feng et al. suggested that a cTnI threshold of 25% WT protein exists for the mice to survive. They also showed that cTnI is likely to be produced in excess amounts under healthy conditions (Feng et al. 2009). TNNI3 KO mice were characterized by impaired diastolic function as an early cardiac phenotype, followed by enlarged cardiac dimensions and overt heart failure (Liu et al. 2007; Feng et al. 2009). In support of impaired diastolic dysfunction, an increased resting tension in isolated ventricular myocytes of TNNI3 KO mice has been found (Huang et al. 1999). However, we did not find any alterations in F\(_{pass}\) in the TNNI3p.98trunc cardiomyocytes. In the early postnatal life of TNNI3 KO mice, slow skeletal TnI (ssTnI) production was maintained in order to compensate for the absence of cTnI (Huang et al. 1999; Liu et al. 2007; Feng et al. 2009). Although ssTnI was elevated in KO mice for a longer period than in WT littermates, it also decreased over time and the compensatory effect was gradually lost. Ca\(^{2+}\)-sensitivity decreased in TNNI3 KO mice along with the decrease of ssTnI. However, compared to WT littermates of the same age, the TNNI3 KO mice showed an increased Ca\(^{2+}\)-sensitivity (Huang et al. 1999). This is in line with the increased Ca\(^{2+}\)-sensitivity we observed in the TNNI3p.98trunc cardiomyocytes, and with the increased Ca\(^{2+}\)-sensitivity of ATPase activity in rabbit skeletal muscle upon extraction of TnI that has been reported previously (Shiraishi & Yamamoto, 1994). Using troponin exchange experiments in single human cardiomyocytes, we were able to increase cTnT levels close to control levels and normalize Ca\(^{2+}\)-sensitivity and LDA in the TNNI3p.98trunc cardiomyocytes. Our data prove that the increased Ca\(^{2+}\)-sensitivity and impaired LDA were directly caused by the mutation-induced haploinsufficiency.

**Secondary disease-related changes in DCM with sarcomeric and non-sarcomeric mutations**

In line with previous reports in human DCM (Makarenko et al. 2004; Nagueh et al. 2004; Beqqali et al. 2016), all DCM patients showed an increase in compliant titin, indicated by a higher N2BA/N2B ratio compared to controls (Fig. 5A and B). The increase in compliant titin therefore seems to be a general hallmark of DCM and not a specific effect of the mutations studied. Despite the increase in compliant titin, F\(_{pass}\) was similar to controls in IDC, the LMA\(_{p.R331Q}\) and TNNI3p.98trunc cardiomyocytes. Interestingly, PKA-mediated phosphorylation titin was unaltered in the TNNT2p.K217del sample. In addition, cTnI phosphorylation was also not affected in the TNNT2p.K217del and TNNI3p.98trunc samples suggesting that these specific mutations do not lead to defects in β-adrenergic receptor signalling. This is contrary to what...
we observed in the IDC samples and to what has been reported in other DCM samples (Wijnker et al. 2014) and might indicate that mutations in troponin can impair phosphorylation through local signalling.

The \textit{TNNT2\textsubscript{p.K217del}} mutation causes high passive stiffness in human cardiomyocytes

The \textit{TNNT2\textsubscript{p.K217del}} cardiomyocytes showed increased $F_{\text{pass}}$ (Fig. 2D). Since the troponin levels in the \textit{TNNI3\textsubscript{p.98trunc}} and \textit{TNNT2\textsubscript{p.K217del}} sample were reduced in a similar fashion we expect that the poison peptide of \textit{TNNT2\textsubscript{p.K217del}} and not reduced troponin complex was the cause of the high $F_{\text{pass}}$. We hypothesize that the mutant \textit{TNNT2\textsubscript{p.K217del}} troponin complex is less likely to incorporate in the sarcomeres than the WT troponin complex. Also the observed decreased cTnT levels in \textit{TNNT2\textsubscript{p.K217del}} might indicate the mutant protein is not as stable as healthy cTnT. We observed a low exchange rate of the mutant \textit{TNNT2\textsubscript{p.K217del}} protein complex in a control sample (34%) and a high exchange rate of the WT troponin complex in the \textit{TNNT2\textsubscript{p.K217del}} sample (59%) compared to the exchange rate of WT in a control sample (43%) (Fig. 6C). Most models have high incorporation of the mutant with values reported to be 79% (Michael et al. 2016) and an estimated incorporation of ~55% (Morimoto et al. 2002). The limited incorporation of the mutant cTnT in our study in combination with the decrease in total troponin levels we observed have important implications. The mutant protein levels are probably higher in exchange experiments in healthy tissue, knock in (KI) or transgenic mouse models than in human patients. In addition, total troponin levels might not be affected in these models while we show they can be decreased in human patient tissue. Therefore, cardiomyocytes of DCM patients with the \textit{TNNT2\textsubscript{p.K217del}} mutation might have different contractile performance than reported in previously published animal models. In support of this, a transgenic mouse model of the \textit{TNNT2\textsubscript{p.K217del}} mutation showed that the severity of DCM is related to the ratio of mutant vs WT transcript (Ahmad et al. 2008). Inoue et al. also showed an increase in $F_{\text{pass}}$ in a mouse KI model of this mutation (Inoue et al. 2013). They indicated that part of the $F_{\text{pass}}$ increase was titin based, but they did not find an increase in N2B titin.

Inoue et al. proposed that the increase in $F_{\text{pass}}$ might
be due to increased PKC-mediated phosphorylation of titin although they did not assess titin phosphorylation. We observed an increase in compliant titin and lower PKC-mediated phosphorylation in the TNNT2<sub>p,K217del</sub> sample (Fig. 5), neither of which could explain the high <i>F<sub>pass</sub></i>. Our troponin exchange experiments provided proof that the TNNT2<sub>p,K217del</sub> mutation itself causes a significant increase in <i>F<sub>pass</sub></i>, irrespective of PKA-mediated phosphorylation. To our knowledge we are the first to show that the TNNT2<sub>p,K217del</sub> mutation causes a profound increase in <i>F<sub>pass</sub></i> in human heart tissue. The lysine at 217 is part of the H1 helix of cTnT which directly interacts with tropomyosin. The interaction between cTnT and tropomyosin might therefore be affected by the TNNT2<sub>p,K217del</sub> mutation. The TNNT2<sub>p,K217del</sub> might cause tropomyosin to be available for residual cross-bridge interaction even at low calcium concentrations resulting in high <i>F<sub>pass</sub></i>. We observed a mild, though non-significant decrease in Ca<sup>2+</sup>-sensitivity in TNNT2<sub>p,K217del</sub> cardiomyocytes compared to controls. The lysine at 217 in cTnI is believed to be involved in calcium-sensitive cTnC binding (Tanokura et al. 1983). Decreased Ca<sup>2+</sup>-sensitivity has been reported in various studies that exchanged human WT or TNNT2<sub>p,K217del</sub> in various animal models (Morimoto et al. 2002; Venkatraman et al. 2003; Michael et al. 2016), while another study showed no effect on Ca<sup>2+</sup>-sensitivity (Bai et al. 2013). In addition, a KI mouse model (Du et al. 2007; Inoue et al. 2013; Memo et al. 2013) and a heterozygous KO mouse model with transgenic expression of TNNT2<sub>p,K217del</sub> also showed decreased Ca<sup>2+</sup>-sensitivity (Ahmad et al. 2008). An impaired interaction of cTnT with cTnI and cTnC due to the TNNT2<sub>p,K217del</sub> mutation has been reported (Mogensen et al. 2004), while another study showed no significant difference in the secondary structure of TNNT2<sub>p,K217del</sub> measured as α-helical content (Venkatraman et al. 2003). We hypothesize that we only observed a minor decrease in Ca<sup>2+</sup>-sensitivity in the TNNT2<sub>p,K217del</sub> cardiomyocytes due to the reduced level of total troponin complex. In the TNNI3<sub>p,98trunc</sub> sample we observed an increased Ca<sup>2+</sup>-sensitivity, which was corrected upon troponin exchange which increased troponin complex levels. Decreased troponin complex levels combined with the Ca<sup>2+</sup>-desensitizing effect of the TNNT2<sub>p,K217del</sub> mutation might have counteracted each other leading to a negligible effect on myofilament Ca<sup>2+</sup>-sensitivity. Reports about <i>F<sub>max</sub></i> in TNNT2<sub>p,K217del</sub> mutants range from no effect (Morimoto et al. 2002; Du et al. 2007; Ahmad et al. 2008; Inoue et al. 2013; Michael et al. 2016) to a decrease (Venkatraman et al. 2003; Bai et al. 2013). In our study we did not find a decrease in <i>F<sub>max</sub></i> in the TNNT2<sub>p,K217del</sub> sample (Fig. 2A). Inoue et al. also showed a depressed Frank–Starling mechanism (Inoue et al. 2013) which we could not confirm in the human patient tissue and was also not observed in a transgenic mouse model of TNNT2<sub>p,K217del</sub> (Ahmad et al. 2008). A KI mouse model, transgenic mouse model, or exchange experiments might give rise to different levels of mutant protein in the sarcomeres and explain the different results on force-generating capacity. Differences in the ability of the body to degrade the mutant protein and to compensate with the healthy allele might cause variable penetrance, age of onset and severity in human patients.

Conclusion

Mutations in different sarcomeric and non-sarcomeric genes lead to DCM and we have shown that these mutations trigger different pathological routes leading to end-stage dilated hearts (Fig. 7). In this study we show that the sarcomeric mutations TNNI3<sub>p,98trunc</sub> and TNNT2<sub>p,K217del</sub> cause reduced expression of the troponin complex and altered stoichiometry between the troponin subunits. In the TNNI3<sub>p,98trunc</sub> cardiomyocytes this led to increased Ca<sup>2+</sup>-sensitivity, which could not be corrected with exogenous PKA but was normalized to control levels upon exchange with WT troponin complex. The TNNT2<sub>p,K217del</sub> mutation caused a mild, non-significant, reduction in Ca<sup>2+</sup>-sensitivity and significantly increased <i>F<sub>pass</sub></i>, which could not be corrected by PKA but was normalized to control levels upon exchange with WT troponin complex. In addition, incorporation of the TNNT2<sub>p,K217del</sub> mutant troponin complex in a control sample confirmed the mutant protein itself causes increased <i>F<sub>pass</sub></i>. This implies that even mutations in the genes encoding for the troponin proteins have different effects on myofilament function. In contrast, the LMNA<sub>p,331Q</sub> mutation caused reduced maximal force development and increased Ca<sup>2+</sup>-sensitivity due to secondary disease remodelling. Also IDCM samples showed an increased Ca<sup>2+</sup>-sensitivity due to secondary disease remodelling. We show that although DCM patients present general hallmarks, the causative mutations underlie different cellular changes. Based on our studies, we propose that different mutations cause DCM via diverse pathways.

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**Additional information**

**Competing interests**

None declared.

**Author contributions**

I.A.E.B., D.W.D.K. and J.V.D.V. conceived, designed and coordinated the study and wrote the paper. I.A.E.B. created Figs 1 and 7 and performed and analysed the experiments shown in Figs 2, 4, 5 and 6. M.S. performed and analysed the experiments shown in Figs 4 and 6. M.H., A.V. and F.W.A. were involved in patient data and material acquisition. J.R.P. created recombinant protein complexes used in Figs 4 and 6. M.K. provided antibodies and supervision for experiments in Fig. 5. Experiments shown in Figs 2, 3, 4, 5A,B and 6 were performed at the Department of Physiology at the VU University Medical Center in Amsterdam, the Netherlands. Experiments shown in Fig 5 C-H were performed at the Institute for Cardiovascular Physiology at the Heinrich-Heine University in Düsseldorf, Germany. All authors critically revised the manuscript, reviewed the results and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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