A Mutation in the Glutamate-rich Region of RBM20 Causes Dilated Cardiomyopathy through Missplicing of Titin and Impaired Frank-Starling Mechanism

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Abstract

Aim
Mutations in the RS-domain of RNA-binding motif protein 20 (RBM20) have recently been identified to segregate with aggressive forms of familial dilated cardiomyopathy (DCM). Loss of RBM20 in rats results in missplicing of the sarcomeric gene titin (TTN). The functional and physiological consequences of RBM20 mutations outside the mutational hotspot of RBM20 have not been explored to date. In this study we investigated the pathomechanism of DCM caused by a novel RBM20 mutation in human cardiomyocytes.

Methods and Results
We identified a family with DCM carrying a mutation (RBM20E913K/) in a glutamate-rich region of RBM20. Western blot analysis of endogenous RBM20 protein revealed strongly reduced protein levels in the heart of a RBM20E913K/ carrier. RNA deep-sequencing demonstrated massive inclusion of exons coding for the spring region of titin in the RBM20E913K/ carrier. Titin isoform analysis revealed a dramatic shift from the less compliant N2B towards the highly compliant N2BA isoforms in RBM20E913K/ heart. Moreover, an increased sarcomere resting-length was observed in single cardiomyocytes and isometric force measurements revealed an attenuated Frank-Starling mechanism (FSM), which was rescued by protein kinase A treatment.

Conclusion
A mutation outside the mutational hotspot of RBM20 results in haploinsufficiency of RBM20. This leads to disturbed alternative splicing of TTN, resulting in a dramatic shift to highly compliant titin isoforms and an impaired FSM. These effects may contribute to the early onset, and malignant course of DCM caused by RBM20 mutations. Altogether, our results demonstrate that heterozygous loss of RBM20 suffices to profoundly impair myocyte biomechanics by its disturbance of TTN splicing.
**Introduction**

Dilated cardiomyopathy (DCM) is characterized by cardiac dilatation and systolic dysfunction, which is the leading cause of heart transplantation. 25-50% of DCM cases are familial and causative mutations have been described in >50 genes encoding mostly structural components of cardiomyocytes.\(^1\)

Recently, mutations in the RNA-binding protein RBM20 were shown to cause an early onset and clinically aggressive form of DCM.\(^2, 3\) Subsequently, next generation sequencing (NGS) in a large cohort of idiopathic DCM (iDCM) patients revealed that RBM20 belongs to the most frequently affected genes in DCM.\(^4\)

Studies in rodents demonstrated that RBM20 expression is highly enriched in the heart and regulates the alternative splicing of a set of genes of which titin (TTN) is its most prominent target.\(^5\)-\(^9\)

Altered splicing of TTN occurs in a number of cardiac diseases such as heart failure, ischemic heart disease and hypertrophic cardiomyopathy (HCM).\(^10\) Studies have shown a shift in expression from the stiff N2B isoform of titin towards the compliant N2BA isoform in human cardiomyopathies. This shift has been associated with reduced myofibrillar stiffness in DCM patients,\(^11, 12\) which has been proposed to improve diastolic filling.\(^7, 13\) In addition, an increase in compliant titin has also been suggested to impair systolic performance by affecting the Frank-Starling mechanism (FSM), i.e. the ability of the sarcomere to increase contractile force in response to stretch.\(^13\)

RBM20 knock out rats showed altered splicing of TTN and developed cardiomyopathy, which provides additional evidence for its causal role in disease development. The functional and physiological consequences of human RBM20 mutations at the cardiomyocyte and sarcomere level are unknown. Here, we show that a novel mutation outside the proposed mutational hotspot,\(^2, 3\) leads to missplicing of TTN resulting in increased compliance of cardiomyocytes with subsequent Ca\(^{2+}\)-sensitized sarcomeres, and an impaired FSM in human cardiomyocytes. Treatment of RBM20 mutant cardiomyocytes with the downstream kinase of the β-adrenergic receptor restored the FSM, while active and passive force at submaximal, physiologic calcium concentrations were lower compared to controls. Our data indicate that heterozygous loss of RBM20 decreases its levels sufficiently to shift TTN splicing towards highly compliant isoforms and thereby lower force generated by sarcomeres.
Methods

Clinical and genetic investigations
All individuals underwent clinical examinations in a dedicated cardiomyopathy clinic at Aarhus University Hospital including 12-lead ECG, two-dimensional echocardiography, and a coronary angiogram if indicated. Diagnosis of familial DCM was based on diagnostic criteria. The proband was included in the INHERITANCE (Integrated Heart Research In Translational Genetics of Cardiomyopathies in Europe) project after oral and written informed consent. The local ethics committee approved the study protocol (M-20110014).

Myocardial tissue
Myocardial biopsies of the proband’s explanted heart were obtained immediately after removal from the patient and were snap-frozen in liquid nitrogen and stored at -80°C or fixated in 4% paraformaldehyde until further processing. Left ventricular samples from end-stage heart failure patients with idiopathic dilated cardiomyopathy were obtained during heart transplant surgery. Tissue from donor hearts served as reference for non-failing myocardium (controls). Samples were obtained after informed consent and with approval of the local Ethical Committee (St Vincent’s Hospital Human Research Ethics committee, Sydney, Australia: File number: H03/118; Title: Molecular analyses of human Heart Failure) and by The University of Sydney HREC number 12146. The investigation conforms with the principles outlined in the Declaration of Helsinki (1997).

Plasmid constructs
Full-length Rbm20 cDNA with an N-terminal FLAG-tag was amplified by PCR from mouse heart cDNA and cloned into pCRII-TOPO (Invitrogen). Next, cDNA encoding Rbm20 with a N-terminal FLAG epitope was subcloned into the pcDNA3.1 (Invitrogen) expression vector.

Mutagenesis was performed using the QuickChange II Site-directed mutagenesis kit (Agilent technologies) according to the manufacturer’s instructions. All cloning products were confirmed by sequencing. PCR primers for cloning were as follows:

FLAG-Rbm20_Fwd: 5’-CACCATGGACTACAAGGATGACGACGACAAGGTGCTGGCAGTAGCCATGAGC-3’

Rbm20_rev: 5’-TCATAGCTTCTTCTTCTTCCAAG-3’

Primers for mutagenesis were as follows:
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Rbm20E913K_fwd: 5’-TTGGTCACAGTGCCAGGACAAAGTAGGCCCGAGGAAG-3’
Rbm20E913K_rev: 5’-CTTCCCTCGCCTACTTTGTCCACTGTAACCA-3’

Cell culture and transient transfection

U2-OS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Life technologies) containing 10% fetal calf serum (FCS) (Life technologies). For Western blot analysis cells were plated in 6-well plates one day before transfection. For immunocytochemistry cells were plated on glass coverslips in a 12-well plate one day before transfection. U2-OS cells were transiently transfected using Lipofectamin 2000 (Invitrogen) according to the manufacturers protocol and analyzed 48 hours after transfection.

Myocyte isolation and GapmeR transfection

Neonatal rat cardiomyocytes (NRCMs) were isolated as previously described.15 NRCMs were plated on fibronectin-coated (Corning, 356008) 6-well plates (750,000 cells/well). Cells were serum-starved for 24 hours, after which they were transfected with GapmeRs (Exiqon, final concentration 25 nM) against Rbm20 (Rbm20-1: 5’-GTAATGATTGACGAG-3’ Rbm20-2: 5’-ACTTAGCTTTAGTCT-3’) or a negative control (300610) using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer’s protocol. Cells were harvested 48 hours after transfection and RNA was processed for RT-PCR and qRT-PCR as described below.

Western blot

Cells were lysed in ice-cold RIPA-buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 0.1%SDS) supplemented with DTT, PMSF and protease inhibitor cocktail (Roche). Cell lysates were cleared by centrifuging 10,000xg for 15 min at 4 ºC. Left ventricular tissue was lysed in Urea/thiourea buffer (8M Urea, 2M thiourea, 0.05M Tris-HCl pH6.8, 0.075M Dithiothreitol, 3% SDS, 0.03% bromophenol blue, 50% glycerol) supplemented with protease inhibitor cocktail (Roche). Western blotting was performed according to standard protocols. Briefly, protein concentrations were determined using the BCA protein assay (Pierce) and proteins were resolved by SDS-PAGE and transferred to Polyvindyline difluoride (PVDF) membrane (Bio-Rad). PVDF membranes were incubated overnight at 4ºC with the following primary antibodies: rabbit anti-FLAG (1:1000, Thermo scientific), mouse anti-GAPDH (1:5000, Bioconnect). Horseradish peroxidase-conjugated secondary antibodies (Amersham) were used for detection and were incubated for 1 hour at room temperature. Western blots were developed with ECL prime western blotting detection reagent (Amersham) and images were acquired using
the ImageQuant LAS4000 (GE Healthcare). Densitometric analysis of Western blots was performed using Image J software.

**Quantitative Real Time PCR (qPCR)**

RNA was isolated from tissue or cells using TRIzol (Invitrogen) according to the manufacturers protocol. Subsequently, 1 μg RNA was treated with DNase I (Invitrogen) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). qPCR was performed on a Lightcycler 480 (Roche) using SYBR green (Roche). Gene expression was normalized to *Gapdh* expression. Analysis of qPCR data was performed using LinRegPCR analysis software. Primers (annealing temperature of 60ºC) used for qPCR were as follows:

- Rbm20 fwd: 5’-ACCTACCACATATCTGCAGC-3’
- Rbm20 rev: 5’-TCCTGAAGCAGTATACTCC-3’
- HsTTN exon 59Fw: 5’-GCCACAGTCACAAGATGTCA-3’
- HsTTN exon 59Rv: 5’-TGCTGTGCAACACATCTGC-3’
- HsTTN exon311Fw: 5’-ACCCAGCAAACCCACAGAAT-3’
- HsTTN exon312Rv: 5’-CTTTGGAGCCGAGCCAGATCA-3’
- HsTTN exon360Fw: 5’-AAATCAGTGCGAGCCAGATCC-3’
- HsTTN exon361Rv: 5’-CGCTGACTGATGCATTTCGG-3’

**GAPDH**

- GAPDH fwd: 5’-ACCCACTCCTCCACCTTTGAC-3’
- GAPDH rev: 5’-ACCCTGTTGCTGTAGCCAAATT-3’

To confirm TTN splicing by qRT-PCR we normalized to exons within the same gene that were not differentially spliced (exon 360-361)

**Immunocytochemistry**

Cells were fixed in 2% paraformaldehyde for 30 minutes, washed 3 times in PBS and permeabilized in 0.1% Triton-X/PBS for 8 minutes. Cells were blocked in 4% normal goat serum (NGS) for 1 hour at room temperature (RT) and then incubated with first antibody in 4% NGS overnight at 4ºC. Secondary antibody incubation occurred in 4% NGS for 1 hour at RT. Nuclear staining was performed as a last step using DAPI (Molecular Probes). Coverslips were then mounted on glass slides with Mowiol (Calbiochem) and images were captured using confocal microscopy (Leica SP8). Primary antibodies were as follows: rabbit anti-FLAG (1:200, Thermo Scientific). Alexa Fluor® 488 conjugated secondary antibody was used at (1:250, Invitrogen).
Immunohistochemistry
Human left ventricular biopsies were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Antigen retrieval was performed by microwave heating of tissue sections (7µm) in citrate buffer (pH 6) after which primary antibody incubation occurred overnight at 4 °C. The following primary antibodies were used: custom made Rabbit anti-RBM20 (1:100, Eurogentec) and mouse anti-alpha actinin (1:800, Sigma-Aldrich). Alexa Fluor® 488 and Alexa Fluor® 568 conjugated secondary antibodies were used at 1:250 (Invitrogen). Nuclear staining was performed as a last step using DAPI (Molecular Probes) and images were captured using confocal microscopy (Leica SP8).

RNA sequencing and splicing analyses
Total RNA was isolated with TRIzol (Life Technologies) according to manufacturers instructions. RNA integrity was verified with the Agilent Bioanalyzer (Agilent Biotechnologies) before RNA-seq libraries were prepared with the TruSeq RNA sample Preparation Kit (Illumina). RNA sequencing on a HiSeq 2000 instrument (Illumina) and analysis were performed as described before. 5,6

RT-PCR
Total RNA was isolated from cells using Trizol reagent (Invitrogen) according to standard protocol. DNaseI digestion was performed to ensure elimination of genomic DNA. Total RNA (1 µg) was transcribed by reverse transcriptase (SuperScript™ II RT, Invitrogen) and used for PCR using HOT FIREPol DNA polymerase (Solis Biodyne). For each PCR reaction 1 µl cDNA was used in a reaction volume of 50 µl. The cycling parameters were 95°C for 15 s; 58°C, 30 s; and 72°C, 45 s, for 30 cycles. The PCR cycles were preceded by an initial denaturation of 15 minutes at 95°C and followed by a final extension of 7 minutes at 72°C. Glyceraldehyde phosphate dehydrogenase (Gapdh) was used as RNA input control. As a negative control total RNA (-RT control), and a water sample were used directly for PCR. Primers used for RT-PCR of the RBM20 dependent splicing of the PEVK region in human and rat were as follows:

Hs TTN exon 114 Fwd: 5’-AAGCAAGCAGTCCACAAGGA-3’
Hs TTN exon 122 Rev: 5’-TCTTTGCAACAGGAACGGGA-3’
Rno Ttn PEVK fwd: 5’-CAGGAGCAGGTTTCTTTTGG-3’
Rno Ttn PEVK rev: 5’-GAGCCGTATGAGGAAACCGTA-3’

The PEVK splice RT-PCR primers for rat have been described before by Guo et al.5.
Protein isoform analysis

Titin isoforms were separated on 1% agarose gel and stained with SYPRO Ruby protein stain as described previously\textsuperscript{17} and measured in triplo. Titin size was estimated as previously described\textsuperscript{17,18} by comparison of the relative mobility to myosin heavy chain (MHC) of adult mouse soleus, human control left ventricular (LV) tissue and DCM RBM20 mutant and was measured in triplo. Titin isoform ratio of RBM20 E913K was compared to idiopathic dilated cardiomyopathy samples (iDCM; N=4) and control hearts (N=3). All values indicated for titin isoform ratio and titin N2BA size are mean of triplo measurements. Raw data of titin isoform measurements is available as Supplementary data file S1 online on the website of the journal Cardiovascular Research, publisher of this article.

Membrane-permeabilized cardiomyocyte measurements

Maximal and passive force of sarcomeres was measured in single membrane-permeabilized cardiomyocytes mechanically isolated from heart tissue as previously described.\textsuperscript{19} Length-dependent activation and protein kinase A (PKA) incubations were performed as previously described\textsuperscript{20}. Data is shown as mean ± SEM. N= number of patients, n= number of cardiomyocytes. Raw data of cardiomyocyte measurements is available as Supplementary data file S1 online on the website of the journal Cardiovascular Research, publisher of this article.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism (GraphPad Software), SPSS (IBM Statistics) and Excel (Microsoft). Data sets that follow a normal distribution were statistically compared with unpaired t-test e.g. Western blot quantifications (Figure 2A, B) and nuclear/cytoplasmic ratio quantifications in online Figure S2. When the E913K patient N=1 was compared to control group, Z-score was inferred and a subsequent probability was calculated of which a p-value of <0.05 was considered significant. In case of non-parametric data a Wilcoxon one-sample test was used. A one-way ANOVA was used to compare resting sarcomere lengths of cardiomyocytes of control (N=3, n=32), iDCM (N=3, n=49) and E913K RBM20 (N=1, n=45) cardiomyocytes after normal distribution was confirmed by Kolmogorov-Smirnov test. For passive force measurements normal distribution was confirmed by Kolmogorov-Smirnov test after which a one-way ANOVA with Holm-Sidak posthoc test was used to compare means between groups. The means of calcium sensitivity were compared with the non-parametric Kruskal-Wallis with Dunn's post-hoc test. For statistically significant differences the p-value is noted, for differences that were not statistically significant
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(N.S.) is noted in figure legends. Raw data are available as Supplementary data file S1 online on the website of the journal Cardiovascular Research, publisher of this article. A P-value of <0.05 was considered significant. All data are expressed as mean ± SEM. The significance level was indicated as follows: P<0.05=*; P0.01=**; P<0.001=***

Results

Identification of a novel RBM20 variant in a family with DCM

From a recently published screen in a European cohort of 639 idiopathic DCM (iDCM) patients we identified a novel heterozygous variant in RBM20 (NM_001134363.1 c.2737G>A). Genetic analyses of the pedigree showed that the variant co-segregated with DCM and transmitted as an autosomal dominant trait with complete penetrance (Figure 1A). The NGS results were confirmed with Sanger sequencing (Figure 1B). No other mutations were identified in 83 DCM genes, including TTN. The variant was absent in more than 60,000 exomes (Exome Aggregation Consortium (ExAC), Cambridge, MA, URL: http://exac.broadinstitute.org January, 2015), however it is annotated in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html) as a variant of uncertain significance (rs397516607) with no frequency data available.

The index patient (III-3) presented with decompensated heart failure at the age of 35. An echocardiogram showed a LVEF of 10% and a restrictive LV filling pattern in absence of coronary atherosclerosis. Endomyocardial biopsies were consistent with DCM and demonstrated hypertrophy and fibrosis without signs of myocarditis. Medical heart failure therapy was initiated following a recovery of systolic function to a stable LVEF of 45% eleven years after initial diagnosis. The brother (III-1) and the mother (II-2) both died of heart failure at the ages of 29 and 72, respectively. Evaluation of remaining family members identified six individuals with clinical signs of DCM, including the proband (IV-5) who despite medical therapy developed terminal heart failure and required heart transplantation (Figure 1A). Other family members were less affected and had only mild impairment of LVEF. Detailed clinical characteristics of the proband and relatives are shown in Supplementary material online, Table S1.

The identified variant is located in exon 11, and leads to an amino acid change at position 913 from a negatively charged and acidic glutamate to a positively charged and basic lysine residue (Fig. 1C). We examined the putative functional effect of the p.E913K mutation by using the prediction algorithms Polyphen-2 and SIFT which predicted this amino acid change to be “probably damaging” (HVAR score 0.994) and “deleterious” (SIFT score: 0) respectively.
Figure 1. Identification of a novel RBM20 mutation in a family with DCM

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Figure 2. E913K mutation leads to lower RBM20 protein levels

**A:** Immunoblot for endogenous RBM20 in control and RBM20<sup>E913K/+</sup> heart with quantification of Western blot (right panel, control N=8, RBM20<sup>E913K/+</sup> N=1 in triplicate). **B:** qPCR analysis of RBM20 mRNA in control (N=5), iDCM (N=3) and RBM20<sup>E913K/+</sup> (N=1) left ventricular tissue. **C:** Immunoblot of U2-OS cells transfected with FLAG-RBM20 and FLAG-RBM20<sup>E913K</sup>. GAPDH was used as loading control. Quantification of immunoblot for FLAG-RBM20 and mutant as determined by densitometry and normalized for RBM20 mRNA (values represent the averages from three independent experiments) T-test ***P<0.001 **D:** qPCR analysis of RBM20 mRNA in transfected U2-OS cells (N=3 independent experiments) **E** Subcellular localization of endogenous RBM20 in control and RBM20<sup>E913K/+</sup> heart (green). White arrows indicate the nuclear localization of RBM20 aggregates. Cardiomyocytes were stained for alpha-actinin (red) and nuclei were stained with DAPI (blue), scalebar: 10 μm.
When aligning the amino acid sequence of RBM20 in different species, we observed that the affected glutamate residue lies within an evolutionary highly conserved glutamate-rich (34%) region, which likely represents a domain of functional importance (Figure 1D).

**E913K mutation decreases stability of the RBM20 protein**

To assess whether the RBM20 mutation acts as dominant-negative or leads to haploinsufficiency, we investigated endogenous RBM20 protein levels in cardiac tissue of subject IV-5 who underwent heart transplantation at 19 years of age. Western blot analysis showed a strong downregulation of RBM20 protein levels in the patient as compared to the control hearts (Figure 2A). Quantitative PCR analysis demonstrated that RBM20 mRNA levels were not downregulated in the RBM20 patient as compared to healthy controls and idiopathic DCM samples suggesting that the mRNA stability is not affected by the c.2737G>A variant (Figure 2B).

To further investigate whether the E913K mutation has an effect on RBM20 protein stability we generated a FLAG-tagged construct of the mutant RBM20 and transfected it in U-2 OS cells. Western blot analysis showed a lower RBM20 protein level for the E913K mutant compared to wildtype RBM20 (Figure 2C). Quantitative PCR (qPCR) on the transfected cells revealed no sign of RBM20 mRNA degradation, which rules out that the observed decrease in RBM20 protein is due to differences in transfection efficiency (Figure 2D). Densitometric analysis revealed an 80% lower RBM20 protein to RBM20 mRNA ratio in the E913K mutant-transfected cells (Figure 2C).

We did not observe an effect of the E913K mutation on the subcellular localization of RBM20 in histological sections of the RBM20E913K/+ heart (Figure 2E, mutant RBM20 fluorescent signal was digitally enhanced for better visualization). RBM20 was located predominantly in sub-nuclear foci of cardiomyocytes, as evidenced by co-staining with the cardiomyocyte marker alpha-actinin. The weak fluorescent signal for RBM20 in the RBM20E913K/+ hearts supported our findings of RBM20 haploinsufficiency.

Furthermore, we investigated subcellular distribution of wildtype and mutant RBM20 in the easy to transfect cell line U-2 OS. Although RBM20 has been described as exclusively nuclear, we also observed cytoplasmic localization in these cells (Supplementary Figure S1A, fluorescent signal of RBM20-E913K was digitally enhanced for better visualisation). To assess whether the mutant RBM20 has influence on the nucleus to cytoplasm ratio of RBM20, we quantified subcellular immunofluorescence and found no significant changes between the wildtype and RBM20E913K transfected cells (Supplementary Figure S1B). Together, these findings suggest that RBM20E913K/+ drives pathogenesis through a mechanism mediated by haploinsufficiency.
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RBM20\textsuperscript{E913K/+} mutation affects TTN splicing

To determine whether RBM20\textsuperscript{E913K/+} affects TTN splicing we performed RNA sequencing (RNA-seq) on LV tissue of subject IV-5. Splicing analysis revealed that long stretches of exons in TTN were misspliced in RBM20\textsuperscript{E913K/+} LV compared to a control subject and a non-RBM20 DCM patient. In particular, a higher percentage (expressed as ‘Percentage Spliced In’) of the exons encoding the elastic PEVK (PSI 85-95% in RBM20\textsuperscript{E913K/+} vs 70-80% in control) and immunoglobulin-rich region (PSI 60-90% in RBM20\textsuperscript{E913K/+} vs 5-80% in control) was included in the titin transcripts of the RBM20\textsuperscript{E913K/+} patient (Figure 3A).

We confirmed the inclusion of exons in TTN by RT-PCR in a region that encodes part of the PEVK region that was shown to be subject to RBM20-dependent splicing.\textsuperscript{5} In myocardial samples of iDCM hearts, splicing of TTN in this region appeared similar to control hearts (Figure 3B). In addition, we confirmed the increase in exon inclusion in the middle Ig region of titin by qPCR (Supplementary Figure S2A). Interestingly, the RNA-seq results showed regions of exon inclusion in the RBM20\textsuperscript{E913K/+} heart, which we confirmed with qPCR (Supplementary Figure S2B).

To assess whether the differential splicing pattern of TTN is indeed a result of reduced RBM20 levels, we performed Rbm20 knockdown experiments in neonatal rat cardiomyocytes. Splicing of the PEVK region of rat titin was similar to that of the RBM20\textsuperscript{E913K/+} patient, as shown by splice RT-PCR (Figure 3C).

To determine whether these splicing changes are also reflected in the titin protein, we investigated titin isoforms by SDS-agarose gel electrophoresis. Titin isoforms in the RBM20\textsuperscript{E913K/+} patient showed a dramatic shift towards more compliant titin isoforms evident from a higher N2BA/N2B ratio (7.45) compared to 0.54 ± 0.04 and 0.96 ± 0.27 in control and iDCM hearts respectively (Figure 4A).

Also, a wider N2BA band was visible (Figure 4A) compared both to donor and iDCM samples, indicating alternative N2BA products at protein level. Therefore, the mobility of the compliant titin of the RBM20\textsuperscript{E913K/+} sample was compared to that of samples with various proteins of known size (nebulin and N2A of an adult mouse soleus muscle and N2B of a human non-failing control left ventricle) during simultaneous gel electrophoresis. We constructed a reference line of molecular weight in relation to the mobility relative to myosin heavy chain (MHC). Titin size estimation showed titin products in the RBM20\textsuperscript{E913K/+} LV of 2849 kDa (N2B) and a predominant 3390–3921 kDa (N2BA) isoform (Figure 4B). This is a larger so-called giant N2BA (N2BA-G) compared to the conventional N2BA isoform of ~3300 kDa.\textsuperscript{23} These findings suggest that the RBM20\textsuperscript{E913K/+} mutation strongly alters TTN splicing and protein isoform composition leading to an increase in larger and more compliant titin isoforms.
Figure 3. RBM20<sup>E913K/+</sup> affects titin mRNA splicing

A: Percentage Spliced In (PSI) plot of titin mRNA as determined by RNA-seq on control (N=1), iDCM (N=1) and RBM20<sup>E913K/+</sup> (N=1) heart. Titin isoforms are indicated in different colors. Exons that are validated by PCR are indicated with red dashed lines. B: RT-PCR of titin PEVK region spanning exons 114 to 122 on control (N=3), iDCM (N=3) and RBM20<sup>E913K/+</sup> heart (N=1). C: Upper panel: qPCR for Rbm20 in neonatal rat cardiomyocytes transfected with gapmers to induce Rbm20 knockdown. Lower panel: splice-RT PCR of titin PEVK region in Rbm20 knockdown cardiomyocytes versus control gapmers and untransfected cardiomyocytes.
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Figure 4. RBM20^{E913K/+} results in larger titin protein isoforms production

A: Representative SDS-agarose gel of titin isoforms in controls, iDCM and RBM20^{E913K/+} patient. Right panel: Quantification of N2BA/N2B ratio as determined by densitometry analysis in controls (N=3), iDCM (N=4) and RBM20^{E913K/+} patient (N=1). Values shown are mean of triplo measurements. B: Molecular weight estimation of titin isoforms in RBM20^{E913K/+} patient as determined by relative mobility assay.

The Frank-Starling mechanism is impaired in RBM20^{E913K/+} cardiomyocytes

We hypothesized that the RBM20^{E913K/+}-induced shift towards more compliant titin affects cardiomyocyte stiffness. Correspondingly, a significantly higher resting sarcomere length (P<0.0001) of the RBM20^{E913K/+} cardiomyocytes was observed after cell isolation compared to both control and iDCM (Figure 5A). In addition, due to the highly compliant N2BA-G cardiomyocytes could easily be stretched to a sarcomere length of 2.4µm and measure isometric force in the RBM20^{E913K/+} cardiomyocytes (Figure 5B). Functional measurements of maximal force at saturating calcium concentrations showed no difference between RBM20^{E913K/+} compared to control hearts (data not
shown). Passive stiffness measured over a range of sarcomere lengths, revealed a similar passive force development in RBM20\(^{6913K/+}\) cardiomyocytes at shorter sarcomere lengths while a trend towards a decreased passive tension in RBM20\(^{6913K/+}\) cardiomyocytes at the higher sarcomere lengths 2.3 and 2.4 \(\mu m\) was observed (N.S.) compared to both control and iDCM hearts (Figure 5C).

![Figure 5. Sarcomere length is increased in RBM20\(^{6913K/+}\) cardiomyocytes](image)

**Figure 5.** Sarcomere length is increased in RBM20\(^{6913K/+}\) cardiomyocytes  

**A:** Slack sarcomere length of RBM20\(^{6913K/+}\) (N=1, n=45) cardiomyocytes was significantly higher compared with control (N=3, n=32) and iDCM (N=3, n=49) cardiomyocytes (**,** P<0.0001) **B:** RBM20\(^{6913K/+}\) cardiomyocyte at a sarcomere length of 2.4\(\mu m\). **C:** Passive force in cardiomyocytes of control (N=3, n=16), iDCM (N=3, n=19) and RBM20\(^{6913K/+}\) (N=1, n=6) at different sarcomere lengths as determined by isometric force measurements were not different (N.S.) between groups. **D:** Length-dependent activation in iDCM (N=3, n=10) and RBM20\(^{6913K/+}\) (N=1, n=9) compared with control (N=2, n=4) cardiomyocytes.

As changes in titin isoform composition may alter the FSM,\(^{13}\) we measured force development at various \([Ca^{2+}]\) and increasing sarcomere lengths. RBM20\(^{6913K/+}\) cardiomyocytes showed increased myofilament \(Ca^{2+}\)-sensitivity and decreased slope compared to control cardiomyocytes at a sarcomere length of 2.2\(\mu m\) (Supplementary Figure S3). The \(Ca^{2+}\)-sensitivity is expressed as \(EC_{50} \) defined as the \([Ca^{2+}]\) needed to achieve 50% of maximal force development. The RBM20\(^{6913K/+}\) cardiomyocytes showed
higher Ca\(^{2+}\)-sensitivity at all sarcomere lengths compared to control hearts and a blunted FSM (Figure 5D), evident from a smaller length-dependent increase in Ca\(^{2+}\)-sensitivity ($\Delta EC_{50} = 0.83\pm0.27$ and $0.30\pm0.04$ in control and RBM20\(^{E913K/+}\) respectively, Figure 5D). Myofilament Ca\(^{2+}\)-sensitivity was also higher (Figure 5D) and FSM smaller ($\Delta EC_{50} = 0.49\pm0.14$) in iDCM compared to control cells. This indicates that a common protein modification, such as lower protein phosphorylation, underlies the differences in myofilament functional properties between DCM and control samples. PKA-mediated sarcomere protein phosphorylation in length-dependent sarcomere activation enhances the length-dependent shift in the force-Ca\(^{2+}\) relation.\(^{24}\)

To test whether high Ca\(^{2+}\)-sensitivity and the blunted length-dependent myofilament activation could be corrected by PKA-mediated phosphorylation, force measurements were performed after treatment with exogenous PKA. PKA treatment unmasked lower passive force development in RBM20\(^{E913K/+}\) compared to both controls and iDCMs (Figure 6A). $EC_{50}$ increased over the entire range of sarcomere lengths in RBM20\(^{E913K/+}\) to levels above control and iDCM hearts (Figure 6B,C), indicating lower myofilament Ca\(^{2+}\)-sensitivity in RBM20\(^{E913K/+}\) after PKA treatment. Higher $EC_{50}$ indicates a lower Ca\(^{2+}\)-sensitivity of myofilaments in RBM20\(^{E913K/+}\) compared to controls and iDCMs after PKA treatment (Figure 6D). Moreover, after PKA the slope of Ca\(^{2+}\)-sensitivity values at various sarcomere lengths of RBM20\(^{E913K/+}\) increased in a manner similar to that observed in controls indicating restoration of the FSM (Figure 6B).
Figure 6. The Frank-Starling mechanism is impaired in RBM20E913K/+ cardiomyocytes

A: PKA treatment unmasked decreased passive force (statistically significant at sarcomere length 2.4 μm in RBM20 E913K compared with iDCM, *P=0.0373) in cardiomyocytes of RBM20E913K/+ (N=1, n=6) compared with control (N=3, n=14) and iDCM (N=3, n=16) cardiomyocytes at different sarcomere lengths B; Length-dependent activation in iDCM (N=2, n=8) and RBM20E913K/+ (N=1, n=5) cardiomyocytes was rescued after PKA treatment to control (N=1, n=3) levels. C: Calcium sensitivity at baseline was significantly increased in RBM20E913K/+ (*P=0.0324) and iDCM (*P=0.0201) compared with control at a sarcomere length of 2.2 μm. Calcium sensitivity was decreased (ns) in RBM20E913K/+ compared with iDCM and control after PKA treatment at a sarcomere length of 2.2 μm. D: Length dependent activation before and after PKA treatment.
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Discussion

Mutations in RBM20 have recently been identified to segregate with DCM in humans and loss of RBM20 in rats induces altered splicing of titin. Here, we demonstrated that a novel mutation in RBM20 in a yet uncharacterized glutamate-rich domain decreased the stability of RBM20 protein and resulted in an abnormal inclusion of exons coding for the extensible I-band region of titin. This led to a massive shift from the N2B to highly compliant N2BA-titin isoforms. RBM20E913K/+ cardiomyocytes had decreased length-dependent activation, which was rescued by PKA treatment. Moreover, PKA-mediated protein phosphorylation revealed lower passive force development and decreased calcium sensitivity at physiological [Ca^{2+}] in RBM20E913K/+ cardiomyocytes compared to control and iDCM cardiomyocytes over a range of sarcomere lengths. A limitation in our study was the availability of human cardiac tissue of only one patient. Although this was the case, we believe our data sheds new light on the molecular mechanism involved in RBM20-related DCM in human cardiomyocytes.

The c.2737G>A mutation is located in exon 11 of the RBM20 gene, outside the previously described hotspot in exon 9, and alters an amino acid in an evolutionary conserved glutamate-rich region. Recent next generation sequencing studies described probands harbouring potential causal missense variants outside the RS domain of RBM20. Although mutations in RBM20 are known to segregate with DCM, their effect on protein stability and function remains elusive. It is unknown whether these mutations act via dominant negative or rather via haploinsufficiency mechanisms. We showed that the E913K mutation strongly decreases endogenous and exogenous RBM20 protein levels without affecting its subcellular localization. This mutation could possibly lead to protein misfolding followed by proteasomal degradation. Interestingly, we observed a reduction of more than 50% of RBM20 protein levels in the RBM20E913K/+ patient. This indicates that there might be a secondary mechanism that also influences the levels of the wildtype RBM20 protein. Although our findings strongly indicate a haploinsufficiency-mediated mechanism of disease, a dominant negative scenario cannot be excluded and would require mutation specific antibodies or mass spectrometry approaches to discriminate between the wildtype and RBM20 mutant protein.

In rats, RBM20 regulates titin splicing by acting as a splicing repressor on exons coding for the middle Ig region and PEVK domain in the extensible I-band region of titin. Here, we demonstrated that repression of these exons is greatly reduced in the LV of a RBM20E913K/+ patient, leading to erroneous inclusion of many exons in the mRNA of titin. Interestingly, titin splicing observed in the E913K patient is different from a previously reported mutation (S635A) in the RS-domain of RBM20, which results in titin exon inclusion only. Since the RS-domain is predicted to be involved in protein–
protein interaction, this mutation may affect the ability of RBM20 to interact with other spliceosome proteins, thus disrupting the normal RNA splicing process. However, RBM20\(^{E913K/+}\) induces haploinsufficiency, resulting in both titin exon inclusion and exclusion, suggesting an additional role for RBM20 as a splice enhancer. Importantly, the RBM20 DCM patient did not carry a rare variant in \(TTN\) and 83 other DCM related genes that could explain the aberrant alternative splicing in \(TTN\). Overall, our results indicate that the amount of available wildtype RBM20 protein is critical to ensure proper splicing of titin.

The disturbed alternative splicing of titin in the RBM20\(^{E913K/+}\) patient was reflected by the dramatic increase in titin N2BA/N2B isoform ratio. Previous studies showed that this ratio in healthy controls ranged from 0.4-0.8 while in iDCM and HF it ranged between 0.5-1.7. \(^{11,12,28}\) Here, we confirmed these ratios in the healthy donor and iDCM group. Strikingly, the RBM20\(^{E913K/+}\) patient had a N2BA/N2B ratio of 7.45. The much higher level of the N2BA giant isoforms of titins could explain the observed increase in slack sarcomere length in the RBM20\(^{E913K/+}\) cardiomyocytes. An increase in slack sarcomere length was also found in a very recent study by Wyles et al. using human induced puripotent stem cell-derived cardiomyocytes (hIPSC) from a RBM20\(^{R636S/+}\) DCM patient.\(^{29,30}\) This highlights the importance of RBM20 in determining the N2BA/N2B ratio in the heart.

An increased N2BA/N2B ratio in disease was shown to correlate with a decreased passive stiffness of cardiomyocytes.\(^{11,12}\) Although the N2BA/N2B ratio was much higher than previously reported for other types of heart disease, this did not translate to a proportional decrease of passive stiffness at short sarcomere lengths. Only at sarcomere lengths 2.3 and 2.4 \(\mu\)m a trend towards decreased passive force was observed. A possible explanation for this discrepancy could be that passive stiffness is not only determined by the ratio of compliant versus stiff isoforms, but also by posttranslational mechanisms such as protein phosphorylation. Indeed, after PKA treatment, which phosphorylates sarcomere target proteins such as titin and troponin I, passive forces were lower in the RBM20\(^{E913K/+}\) patient at all measured sarcomere lengths. While exogenous PKA decreased passive force in the RBM20\(^{E913K/+}\) patient, this was not the case in the iDCM patients. Reduced phosphorylation of myofilament proteins in heart failure has been observed due the impairment of the beta-adrenergic receptor signaling and subsequent decreased PKA activity.\(^{31}\) We show that the RBM20\(^{E913K/+}\) patient reacts differently to exogenous PKA than iDCM patients with respect to passive force development.
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PKA-treated cells showed a clear trend towards decreased Ca\(^{2+}\)-sensitivity in RBM20\(^{E913K/+}\) while it had no effect on control cardiomyocytes. Remarkably, Ca\(^{2+}\)-sensitivity in E913K cardiomyocytes was even lower after PKA than in control and iDCM cardiomyocytes indicating that the mutation has profound effects on both phosphorylation status and sarcomere function. Thus, apart from differences in passive tension, the E913K mutation showed a larger response to PKA with respect to calcium sensitivity compared with iDCM patients. The introduction of a negatively charged phosphate group on a protein can induce a conformational change leading to altered protein function. A possible explanation for the altered response of the RBM20\(^{E913K/+}\) patient compared to IDCM might be that the additional length in the protein interferes with the induced conformational changes upon phosphorylation. If and how these additional domains interfere with conformational changes cannot be concluded from this study and additional research into this would be valuable.

The blunted FSM in E913K cardiomyocytes at baseline is in accordance with previously reported data in RBM20\(^{ΔRRM}\) mice,\(^7\) although the effect of PKA was not investigated in these mice. The high myofilament Ca\(^{2+}\)-sensitivity and blunted FSM may contribute to disease progression in DCM. Phosphorylation of myofilament proteins can also play an important role in length-dependent activation.\(^{32}\) In this study we show that the length-dependent activation was indeed impaired in the RBM20\(^{E913K/+}\) patient, but was restored after exogenous PKA treatment. This indicates that in this patient, phosphorylation deficits underlie the reduced length-dependent activation and may be a secondary mutation-induced effect in disease development. Furthermore, since PKA is a downstream effector of beta-adrenergic receptor signaling, beta-blocker treatment (and thus lowering PKA activity) could be detrimental to patients with an RBM20 mutation and its effectiveness should therefore be carefully investigated. Although missplicing of titin alone may explain the early onset and clinically aggressive RBM20-related DCM, we cannot exclude a contribution of missplicing of other direct RBM20 target genes that have previously been identified.\(^6\) However, RNA binding studies revealed that titin mRNA was the most prominent RBM20-bound target in cardiomyocytes.\(^5,6\) In addition, the majority of RBM20 protein was localized in nuclear aggregates that associated with titin mRNA.\(^{26}\) Altogether, this suggests that titin missplicing caused by a defect in RBM20 has a major contribution in disease onset and progression.

In conclusion, our data supports a model in which haploinsufficiency of RBM20 results in more compliant titin isoforms and a disturbed FSM. In addition, we show the importance of phosphorylation on myofilament function. While passive tension was normal and calcium sensitivity was increased at baseline, exogenous PKA could rescue the impaired FSM and unmasked decreased passive tension and decreased calcium sensitivity in the RBM20\(^{E913K/+}\) patient compared to control and iDCM patients. These
findings, may at least in part explain the depressed contractile function in the failing heart of RBM20<sup>E5913K/+</sup> patients. The aggressive and often malignant course of DCM caused by RBM20 mutations underscore the importance of clinical screening for this gene and identifying the underlying pathophysiology to design possible ways of treatment.

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References


Chapter 5


Supplemental Figures

Supplementary Figure S1. Subcellular distribution of RBM20<sup>E913K</sup>

**A**: Immunofluorescent staining of FLAG-RBM20 and FLAG-RBM20<sup>E913K</sup> transfected U-2 OS cells. Nuclear foci of RBM20 are indicated with white arrowheads. Nuclei were stained with DAPI (blue). Scale bar 25 μm. **B**: Quantification of the nucleo/cytoplasmic ratio of FLAG-RBM20 fluorescence. FLAG-RBM20 transfected cells (N=138) FLAG-RBM20<sup>E913K</sup> (N=135).
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Supplementary Figure S2. Validation of TTN splicing in RBM20 E913K/+ heart.

A: qPCR of TTN middle Ig exon 59 confirming increased exon inclusion in the RBM20 E913K/+ heart. B: qPCR of TTN A-band exon 311-312 indicating exon exclusion in RBM20 E913K/+ heart. P > 0.05 N.S.; mRNA levels were normalized to GAPDH.

Supplementary Figure S3. Calcium-force relation at a sarcomere length of 2.2 μm