RESEARCH ARTICLE

Cardiomyopathy-associated mutations in the RS domain affect nuclear localization of RBM20

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Abstract
Mutations in RBM20 encoding the RNA-binding motif protein 20 (RBM20) are associated with an early onset and clinically severe forms of cardiomyopathies. Transcriptome analyses revealed RBM20 as an important regulator of cardiac alternative splicing. RBM20 mutations are especially localized in exons 9 and 11 including the highly conserved arginine and serine-rich domain (RS domain). Here, we investigated in several cardiomyopathy patients, the previously described RBM20 mutation p.Pro638Leu localized within the RS domain. In addition, we identified in a patient the novel mutation p.Val914Ala localized in the (glutamate-rich) Glu-rich domain of RBM20 encoded by exon 11. Its impact on the disease was investigated with a novel TTN- and RYR2-splicing assay based on the patients' cardiac messenger RNA. Furthermore, we showed in cell culture and in human cardiac tissue that mutant RBM20-p.Pro638Leu is not localized in the nuclei but causes an abnormal cytoplasmic localization of the protein. In contrast the splicing deficient RBM20-p.Val914Ala has no influence on the intracellular localization. These results indicate that disease-associated variants in RBM20 lead to aberrant splicing through different pathomechanisms dependent on the localization of the mutation. This might have an impact on the future development of therapeutic strategies for the treatment of RBM20-induced cardiomyopathies.

KEYWORDS
cardiomyopathy, mutation, pathomechanisms, RBM20, splicing

1 | INTRODUCTION

Dilated cardiomyopathy (DCM) is a heterogeneous disease and the third frequent cause of heart failure (HF) with an estimated prevalence of 1 in 200-500 people (Hershberger, Hedges, & Morales, 2013). DCM is the most common indication for heart transplantation (HTx), especially in pediatric patients (Khush et al., 2018; Rossano et al., 2018). Regardless of its etiology, DCM is best described as a progressive ventricular wall thinning and dilatation combined with systolic dysfunction (McMurray et al., 2012).

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Recently, we found that in patients with severe forms of DCM, more than 50% of the HTx candidates might be affected by a genetic etiology (Klauke et al., 2017). Until now, rare variants in at least 40 different genes have been shown to be associated with DCM (Brodehl et al., 2019; Horvat et al., 2018).

Time- and cost-effective next-generation sequencing (NGS) techniques have transformed clinical genetic testing as several dozens of genes can now be sequenced in parallel (Lakdawala et al., 2012; Teekakirikul, Kelly, Rehm, Lakdawala, & Funke, 2013). However, due to often missing functional tests and rare cosegregation data, clinical interpretation of genetic sequence variants and the elucidation of the corresponding pathomechanisms still remains challenging (Hershberger et al., 2018; Richards et al., 2015).

In 2009, RBM20 was established as a novel DCM-associated gene (Brach et al., 2009; MIM®#613172). RBM20 is predominantly expressed in striated muscle with the highest expression in the heart (Li, Guo, Dewey, & Greaser, 2013; Maatz et al., 2014). RBM20 belongs to the category of serine- and arginine-rich (SR) like proteins and contains domains typically also found in other splicing factors: a proline-rich region, a ribonucleic acid recognition motif (RRM), an RS domain, a Glu-rich region, and a U1 zinc finger domain (Guo et al., 2012). The RRM and RS domains of RBM20 are highly conserved between orthologues and are essential for nuclear retention of the protein (Filippello, Lorenzi, Bergamo, & Romanelli, 2013; Weeland, van den Hoogenhof, Beqqali, & Cremers, 2015). Mutations in RBM20 are associated with an early onset, highly penetrant, and clinically aggressive form of DCM, with an increased risk of malignant ventricular arrhythmias (Parikh et al., 2019; van den Hoogenhof et al., 2018). Transcriptome analyses revealed RBM20 as a prominent splicing regulator of the TTN-transcripts influencing the myocardial isoform composition of the giant sarcomeric protein titin (Guo et al., 2012; Maatz et al., 2014). Aberrant splicing of TTN is believed to contribute to the DCM phenotype in RBM20-mutation carriers. However, aberrant TTN splicing is not likely to explain the increased risk of arrhythmias (van den Hoogenhof et al., 2018). RBM20 also controls tissue-specific isoform expression of many other genes, including sarcomeric genes like MYOM1, but also genes encoding Ca²⁺- and ion-handling proteins such as CAMK2D and RYR2 (Guo et al., 2012; Maatz et al., 2014). Studies in knockout mice revealed a disturbed Ca²⁺-handling induced by dysfunctional Rbm20, which potentially underlies the increased risk of arrhythmias in patients with RBM20 mutations (van den Hoogenhof et al., 2018). Parikh et al. (2019) found two regions in RBM20 (in exons 9 and 11), which are significantly enriched for cardiomyopathy-associated mutations. Arrhythmias and risk for sudden cardiac death (SCD) are frequently found in patients harboring RBM20 mutations within these regions (Parikh et al., 2019). Disease expression associated with pathogenic RBM20 mutations is especially severe in males (Hey et al., 2019). Until now, most of the disease-associated RBM20 mutations have been identified in the highly conserved RS domain of the protein (Beqqali et al., 2016).

We identified in HTx candidates the previously described RBM20-mutation p.Pro638Leu (Brach et al., 2009) localized within the conserved RS domain and the novel mutation p.Val914Ala localized in the Glu-rich domain in two different German families. Functional consequences were analyzed by investigating the myocardial splicing of TTN and RYR2 in the mutation carriers in comparison to non-failing donor and DCM hearts without RBM20 mutations. In addition, we analyzed the myocardial splicing of these genes in patients with uncharacterized RBM20 variants. Furthermore, we show that mutations affecting the conserved Pro638 within the RS domain lead to mislocalization of RBM20 in human myocardial tissue and cell culture. In contrast, the disease-associated mutation p.Val914Ala has no influence on the intracellular localization of RBM20 in human myocardium.

In summary, our study reveals the clinical impact of splice defect analysis of RYR2 and TTN in human myocardium to prove the pathogenicity of novel RBM20 variants.

2 | MATERIAL AND METHODS

2.1 | Patient cohort

All individuals underwent comprehensive clinical examinations at the Heart and Diabetes Centre NRW (Bad Oeynhausen, Germany) including 12-lead electrocardiogram, echocardiography, and a coronary angiogram if indicated. The diagnosis of familial DCM was based on previously described diagnostic criteria (Mestroni et al., 1999). All patients with manifest DCM received guideline-based drug treatment for HF in accordance with their New York Heart Association (NYHA) class and their left ventricular ejection fraction (LVEF). Furthermore, implantable cardioverter defibrillators (ICDs) were implanted for primary or secondary prophylaxis when necessary (Ponikowski et al., 2016; Priori et al., 2015). As controls, we included HTx patients with no variants or with RBM20 variants of unknown significance and rejected donor’s hearts. All but one sample included in this study were genotyped by an NGS gene panel covering 174 HF-associated genes. For an overview of the clinical baseline characteristics see Table S1.

2.2 | Clinical description of the patients

Family 1 (Figure 1a) is a large German family with 14 affected members. An early onset DCM and/or arrhythmias and a transmission in an autosomal dominant trait were found. SCD was reported for three family members (III.2, III.3, III.4), which were, therefore, not available for genotyping.

Index patient IV.2 had at the age of 11 years, a dilated left ventricle, a latent contraction disorder, and pronounced sinus arrhythmias. He received beta-blockers. Additionally, the patient has familial homocysteinemia. At the age of 20 years, the patient received a left ventricular assisted device (LVAD) and HTx was performed at the age of 24 years. The patient’s elder brother (IV.3) was also diagnosed with DCM and reduced systolic function in childhood. At the age of 26 years, he received a single-chamber ICD. He
received HTx at the age of 28 years. The patient’s brother (IV.1) showed no cardiac abnormalities and was not genotyped for personal reasons. The patients’ father (III.2) was listed for HTx and died aged 28 years. The children of patient IV.3 were diagnosed with DCM at the age of 2 years (V.1) or 9 months (V.2) with mild left ventricular dilatation but without further clinical symptoms. Index patient IV.12 was diagnosed at the age of 24 years with left ventricular dilatation in combination with systolic dysfunction. Additionally, the patient has hypothyreosis. Aged 28 years, the patient received a single-chamber ICD, and at 32 years a device for cardiac contractility modulation (CCM). HTx was performed at the age of 32 years due to severe DCM. The patient’s father (III.8) had also DCM and was transplanted aged 39 years. The patient’s cousin (IV.7, 23 years) received a single-chamber ICD at the age of 20 years and mechanical unloading by LVAD support at the age of 21 years. Patient III.7 showed a slightly impaired left ventricular function at the age of 36 years. At the age of 47 years, she showed episodes of tachycardia, palpitations, and dizziness with preserved ejection fraction as diagnosed by magnetic resonance imaging. Within a further 2 years, left ventricular function declined with mid-range 

FIGURE 1  Pedigrees of cardiomyopathy families with (likely) pathogenic RBM20 mutations. Circles represent females, squares males, and slash denotes deceased. Index patients are marked with arrows. Available myocardial tissue of patients with end-stage heart failure, who were transplanted (HTx) or received mechanical circulatory support (VAD) is indicated by heart symbols. Affected members (cardiomyopathy, arrhythmias, or SCD) are given as black filled symbols and obligate mutation carriers are shown in brackets. The current age (year [y]) of the patients or their age at SCD, HTx, VAD, or implantable cardioverter defibrillator (ICD) implantation is specified. Patients with suspected disease phenotype are presented with hatched symbols. (a) The heterozygous (+/−) pathogenic mutation RBM20 p.Pro638Leu reveals an aggressive and early-onset dilated cardiomyopathy. (b) Pedigree of the RBM20 p.Val914Ala carriers. HTx, heart transplantation; SCD, sudden cardiac death; VAD, ventricular assist device
ejection fraction most recently diagnosed. In consideration of repeatedly observed nonsustained ventricular tachycardia, the deterioration of the left ventricular function in a relatively short period of time and knowledge of the high risk for sustained ventricular arrhythmias or SCD, this patient received an ICD at the age of 49 years, irrespective of the required criteria according to the actual ESC guidelines for ICD implantation.

In the second family (Figure 1b) index patient III.6 received a single-chamber ICD at the age of 35 years due to ventricular arrhythmias and HTx was performed at the age of 44 years. The patient died at 45 years. His father (II.3) was diagnosed with DCM and died aged 35 years. Two siblings (III.5 and III.7) were not available for clinical examination or genotyping because of personal reasons.

The cousin (III.2) of the index patient showed the first cardiac symptoms at the age of 39 years. An ICD was implanted at the age of 50 years and he received an upgrade to a cardiac resynchronization therapy device at 56 years. The patient had several appropriate shocks (syncopes and ventricular tachycardia; ventricular fibrillation). Therefore, he was listed for HTx but died at the age of 56 years due to multiple organ failure. His father (II.2) died from HF at the age of 56 years due to irreversible atriation of the left ventricular function in a relatively short period of time and knowledge of the high risk for sustained ventricular arrhythmias or SCD, this patient received an ICD at the age of 49 years, irrespective of the required criteria according to the actual ESC guidelines for ICD implantation.

2.3 | Genetic analyses

Molecular genetics was performed after oral and written informed consent. The local ethics committee approved the study protocol (Reg. No. 2018-330). DNA was isolated from blood using standard techniques (High Pure PCR Template Preparation Kit®, Roche Diagnostics GmbH, Mannheim, Germany) and prepared for cardiac gene enrichment re-sequencing on a MiSeq® next-generation sequencing system according to manufacturer’s instructions (TruSight™ Rapid Capture Sample Preparation Kit; Illumina, San Diego). Index patients and controls were screened for variants in 174 genes associated with inherited cardiac conditions using the TruSight™ Cardio gene panel (Illumina). Evidence criteria for mutation classification were 95°C, 10 min for initial denaturation, 40 cycles 60°C, 1 min/95°C, 15 s using Maxima Probe/ROX qPCR MasterMix (Thermo Fisher Scientific, Waltham, MA) after random priming with hexamers. Reverse transcription of myocardial RNA was performed using 250 ng of total RNA and 50 units of the enzyme Superscript II (Thermo Fisher Scientific, Waltham, MA) after random priming with hexamers. Relative quantification of RBM20, TTN, and RYR2 messenger RNA (mRNA) was done with 2 µl of the reverse transcription reaction. Hprt1 was used as a housekeeping gene as previously described (Thelin et al., 1999). The StepOnePlus™ real-time PCR system (Thermo Fisher Scientific) was used. All measurements were performed at least in duplicates. Relative quantification was evaluated using the comparative Ct-method (ΔΔCt; Livak & Schmittgen, 2001) of the StepOne™ software (v2.0; Thermo Fisher Scientific). Primer sequences are listed in Table S2. The conditions for the PCR-reaction were 95°C, 10 min for initial denaturation, 40 cycles 60°C, 1 min/95°C, 15 s using Maxima Probe/ROX qPCR MasterMix (Thermo Fisher Scientific). Relative quantity (RQ) of RBM20-mRNA was normalized to Hprt1 mRNA expression.

The RYR2-splice variant ratio was calculated from the RQ value of the RYR2-splice variant with an additional 24 bp exon against the regular RYR2-splice variant. The TTN-splice variant ratio was calculated from the RQ values of the TTN-N2B-splice variant to total TTN. Primers for the qRT-PCR assay were designed according to the RBM20-specific RYR2 and TTN-splicing targets suggested by Guo et al. (2012) and Maatz et al. (2014). For primer sequences and amplified transcripts see Table S2. The amplicon identity was proven by Sanger Sequencing (Macrogen, Amsterdam, Netherlands).

2.5 | Isolation of total RNA

Total RNA was isolated from 30 mg of left ventricular myocardium using a commercial kit (RNeasy, Qiagen, Hilden, Germany) as previously reported (Gaertner-Rommel et al., 2019). Purity and integrity were assessed by agarose gel electrophoresis.

2.6 | Quantitative real-time polymerase chain reaction (qRT-PCR)

Reverse transcription of myocardial RNA was performed using 250 ng of total RNA and 50 units of the enzyme Superscript II (Thermo Fisher Scientific, Waltham, MA) after random priming with hexamers. Relative quantification of RBM20, TTN, and RYR2 messenger RNA (mRNA) was done with 2 µl of the reverse transcription reaction. Hprt1 was used as a housekeeping gene as previously described (Thelin et al., 1999). The StepOnePlus™ real-time PCR system (Thermo Fisher Scientific) was used. All measurements were performed at least in duplicates. Relative quantification was evaluated using the comparative Ct-method (ΔΔCt; Livak & Schmittgen, 2001) of the StepOne™ software (v2.0; Thermo Fisher Scientific). Primer sequences are listed in Table S2. The conditions for the PCR-reaction were 95°C, 10 min for initial denaturation, 40 cycles 60°C, 1 min/95°C, 15 s using Maxima Probe/ROX qPCR MasterMix (Thermo Fisher Scientific). Relative quantity (RQ) of RBM20-mRNA was normalized to Hprt1 mRNA expression.

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2.7 | Plasmid construction

Human RBM20 complementary DNA (cDNA) was obtained by reverse transcription of total mRNA isolated from explanted cardiac
tissue using SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific). To generate plasmid coding for RBM20, cDNA (c.385-3684) was amplified using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and was cloned into the plasmids pEYFP-N1 or pECFP-N1 (Takara Bio, Mountain View, CA) via EcoR1 and BamHI restriction sites. RBM20 mutations and variants were introduced by the QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. The RBM20 coding parts of all generated plasmids were verified by Sanger sequencing (Macrogen).

### 2.8 Cell culture and transient transfection

C2C12 (ATCC, Manassas, VA) cells were cultivated in Dulbecco’s Modified Eagle Medium (High Glucose, l-Glutamin, HEPES, Phenol Red; Thermo Fisher Scientific) supplemented with 10% fetal calf serum (PAA; GE Healthcare, Chalfont Saint Giles, Great Britain), 100 U/ml Penicillin (Merck) and 0.1% (w/v) Streptomycin (Merck). Lipofectamine 2000 (Thermo Fisher Scientific) was used for cell transfections according to the manufacturer’s instructions. Cells were plated 24 hr before transfection. At transfection time, cells were 70–90% confluent. Transient transfections were performed with 750 ng RBM20-pEYFP-N1 or RBM20-pECFP-N1 plasmid DNA in a 24-well plate.

### 2.9 Immunohistochemistry

Frozen cardiac tissue was sliced using a cryomicrotome (Leica, Wetzlar, Germany) into 5 µm sections. After thawing, slides were blocked with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hr at room temperature (RT). Blocked sections were incubated with anti-RBM20 antibody (NB51-91002; Novus Biologicals, Littleton, CO) at a 1:100 dilution in BSA/PBS overnight at 4°C. After washing in BSA/PBS, slices were incubated with a Cy3-conjugated anti-rabbit IgG antibody (C2306; Sigma-Aldrich, St. Louis, MO) at a concentration of 1:100 in BSA/PBS for 1 hr at RT. Afterwards, slices were washed with PBS and incubated with 1 x 10^-7% (w/v) 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) solution (Carl Roth, Karlsruhe, Germany) in BSA/PBS for 5 min at RT. Sections were washed with distilled water and embedded with Mowiol 4-88 (Carl Roth).

### 2.10 Confocal microscopy

Cells were grown on coverglasses coated with 0.02% collagen R-solution (SERVA Electrophoresis, Heidelberg, Germany). Forty-eight hours after transfection cells were fixed with Roti®-Histofix 4% (Carl Roth) for 15 min at RT, washed with PBS, and incubated with DAPI (1 µg/ml) in PBS for 10 min at RT in the dark. Afterward, cells were washed with distilled water and embedded with Mowiol 4-88. Image acquisition was performed with a TCS SP8 confocal microscope (Leica). Image deconvolution was performed with Huygens Essential software (Scientific Volume Imaging B.V., Hilversum, Netherlands). For localization analysis, the subcellular distribution of each RBM20 variant was determined in approximately 300 cells from three independent transfection experiments in total.

### 2.11 Statistical analyses

Statistical analyses were performed using Prism v8.4.3 for Windows (GraphPad Software, San Diego, CA).

For the determination of the reference range, first tests for normal distribution (Anderson Darling Test, D’Agostino & Pearson Test, Shapiro–Wilk test, or Kolmogorov–Smirnov test) were performed for the reference groups (NF, DCM, and p.Pro638Leu). As the reference groups passed normality tests, a parametric approach for the definition of the reference range was used. For this, the 95% confidence interval (CI) of each reference group was calculated. The boxes for NF, DCM, and RBM20-p.Pro638Leu samples reflect the 25–75% percentiles, the whiskers the min and max percentiles. Medians are indicated by a line and means by crosses. RQ values for the technical replicates of single tested patients are shown as means ± standard error. One-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test was applied for the comparison of the NF, DCM, and RBM20-p.Pro638Leu groups.

For statistical analyses of the localization experiments, a one-way ANOVA with Tukey’s multiple comparisons test was performed.

### 3 RESULTS

We have identified (Klaue et al., 2017) the previously published RBM20 (NM_001134363.3) mutation c.1913C>T p.Pro638Leu (Brauch et al., 2009) in a large German family with 14 affected members (Figure 1a). Genetic analyses of the pedigree revealed a cosegregation of the variant with an early onset DCM and/or arrhythmias (see Section 2) and a transmission in an autosomal dominant trait with complete penetrance. SCD was reported in three family members who were therefore not available for genotyping (III.2, III.3, III.4). Patient IV.3 is additionally a carrier of the mutation MYBPC3 (NM_000256.3; MIM#600958) c.2373dupG, p.W792VfsX41, which was previously described as a pathogenic mutation associated with hypertrophic cardiomyopathy (HCM; Alders et al., 2003; Moolman et al., 2000; van Dijk et al., 2009). Explanted cardiac tissue was available from four mutation carriers.

In the second family (Figure 1b), we identified the novel mutation RBM20 c.2741T>C p.Val914Ala in patients III.6 and III.2 who both had DCM. Obligatory mutation carriers II.3 and II.2 died from HF according to the report of the family. Explanted cardiac tissue was available from patient III.6.

The genotypes of all patients with RBM20 variants are shown in Table S3. The RBM20 genotype of the controls is given in Table S1.
From the limited available data, we estimated an average clinical event-free (HTx, VAD, or death) survival of 28 years for family members carrying RBM20-p.Pro638Leu and an average event-free survival of 42 years for RBM20-p.Val914Ala carriers. These data show that the RBM20-p.Pro638Leu is associated with a poor prognosis.

3.1 | The ratio of RYR2- and TTN-splice variants can be used as a tool for the disease classification of uncharacterized RBM20 variants

As previously shown, cardiomyopathy-associated RBM20 mutations lead to an overexpression of an RYR2-splice variant containing an additional small exon (24 bp; Maatz et al., 2014). We used qRT-PCR to determine the ratio of this splice form and the regular RYR2-splice form (RYR2-ratio). This ratio did not differ significantly between NF (1.42 ± 0.41) and DCM (1.87 ± 0.57) control samples but was significantly increased in the RBM20-p.Pro638Leu group (5.61 ± 1.92; Figure 2a). Interestingly, analysis of the RBM20 p.Val914Ala sample also revealed an aberrant RYR2-ratio: the mean of the p.Val914Ala sample (5.89 ± 1.48) was out of the reference range (95% CI of each reference group) for the NF (1.13–1.71) and DCM (1.47–2.28) groups but within the reference range of the p.Pro638Leu pathogenic group (2.55–8.67), respectively. In contrast, the RQ means ± SEM of RBM20 variants of unknown significance (p.Val545Ile, p.Asp888Asn, and p.Pro1039Ser) showed an RYR2-ratio out of the p.Pro638Leu reference range and within the control reference range (Figure 2a, Tables S4 and S5). Interestingly, the RYR2-ratio of a patient carrying RBM20-p.Val914Ile was also within the control reference range and out of the aberrant splicing (p.Pro638Leu) reference range.

Splicing analysis revealed that the ratio of TTN-N2B to total TTN (TTN-ratio) is not significantly different between NF (0.77 ± 0.36) and DCM (0.76 ± 0.21) samples (Figure 2b). As expected from previous investigations using Rbm20-deficient rodents (Guo, Pleitner, Saupe, & Greaser, 2013; Murayama et al., 2018), human samples from RBM20-p.Pro638Leu carriers showed a TTN-ratio (0.07 ± 0.03), which significantly differs from the NF and DCM control groups. Interestingly, the TTN-splicing pattern in the samples of the patient with the novel RBM20-variant p.Val914Ala (0.07 ± 0.01) localized outside the conserved RS domain was within the reference range for the pathogenic mutation p.Pro638Leu (0.03–0.11) and outside the control groups reference range (0.51–1.03 for NF and 0.61–0.92 for DCM). Comparable to the RYR2-ratio, the TTN-ratio of patients carrying the RBM20 variants of unknown significance p.Val545Ile, p.Asp888Asn, and p.Pro1039Ser was out of the reference range for the pathogenic p.Pro638Leu mutation (Figure 2b). The TTN-ratio for the RBM20-p.Val914Ile was also out of the pathogenic reference range.

Our functional data obtained in the splicing assay support the pathogenicity of several RBM20 mutations and are therefore relevant for the diagnostic classification according to the guidelines of the American College of Medical Genetics and Genomics (ACMG; Richards et al., 2015). We, therefore, suggest that our qRT-PCR assay can be used as a tool to prove the pathogenic impact of unclassified RBM20 variants if myocardial biopsies are available.

For samples with a RYR2- and TTN-splicing pattern out of the control and within the p.Pro638Leu reference range, PS3 (evidence of pathogenicity: strong) according to the ACMG criteria was used. However for samples with an RYR2- and TTN-splicing pattern out of

FIGURE 2 qRT-PCR analysis of (a) RYR2- (b) TTN-splice variant ratios, and (c) relative quantification of RBM20 mRNA. Data from failing (DCM) and non-failing (NF) hearts (N = 10 in technical duplicates for each group) without RBM20 mutations and for patients with the pathogenic p.Pro638Leu mutation (N = 4 in technical quintuplicates (a,b) or duplicates (c)) are given as box and whiskers plots. Within the plots, means for each sample are shown. Whiskers include the min and max values. The TTN- and RYR2-splicing in p.Pro638Leu patients is significantly different from the NF and DCM control groups. Sample p.Val914Ala (blue) also shows aberrant splicing. Green dots represent samples with ratios of the splice products out of the p.Pro638Leu reference range. Dots show the means of measurements in quintuplicates ± SEM. (c) qRT-PCR of RBM20-mRNA indicates that the RBM20 mRNA of the failing myocardium is significantly upregulated compared to NF controls. There is no significant difference in RBM20-mRNA expression in the patients with the pathogenic mutation RBM20-p.Pro638Leu compared to the DCM or NF group. Dots show the means of measurements in duplicates ± SEM. For values see Tables S4 and S5. DCM, dilated cardiomyopathy; qRT-PCR, quantitative real-time polymerase chain reaction; mRNA, messenger RNA; SEM, standard error of the mean.
the p.Pro638Leu reference range and within the control reference range, BS3 (evidence of benign effect: strong) was used (Richards et al., 2015). For samples out of both reference ranges, no ACMG criterion was applied. For RBM20-p.Pro1039Ser, BS3 was fulfilled although the sample shows a TTN-splicing pattern out of control and p.Pro638Leu reference ranges. Nonetheless, the TTN splicing in this sample is shifted toward increased TTN-N2B levels which are not expected to be caused by RBM20 mutations.

The novel RBM20-mutation p.Val914Ala was classified according to the ACMG guidelines as a probably pathogenic mutation (class 4; Richards et al., 2015). In contrast, we reclassified the variant p.Asp888Asn as benign and the variant p.Pro1039Ser as likely benign according to the ACMG guidelines. These variants were classified as disease-causing mutation? or disease-causing mutation? in the Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/; Stenson et al., 2014). The RBM20 variants p.Val914Ile and p.Val545Ile are not listed in HGMD (Stenson et al., 2014) and were classified here as variants of unknown significance according to the ACMG guidelines (for details of the ACMG criteria see Table S6).

3.2 RBM20-mRNA expression level is not affected by DCM-associated RBM20 mutations

We analyzed the human myocardial samples on expression changes of the RBM20-mRNA, as the expression level might have an influence on the cardiac splicing. RBM20-mRNA expression in DCM patients was significantly increased in comparison to NF samples (RQ mean ± SED DCM 0.67 ± 0.16 vs. NF 0.45 ± 0.18; p < .01). However, there was no significant difference in RBM20-mRNA expression in the patients with the pathogenic RBM20-mutation p.Pro638Leu (0.61 ± 0.14) compared to the DCM or NF group, respectively. Interestingly, the expression level of all RBM20 mutants is within the reference range of NF (0.31–0.58) or DCM samples (0.56–0.78) (Figure 2c). Thus, differential RYR2 and TTN splicing is not due to aberrant RBM20-mRNA expression levels.

3.3 RBM20-p.Pro638Leu leads to an abnormal mislocalization in the cytoplasm

We analyzed the localization of wild-type and mutant RBM20-EYFP fusion proteins in C2C12 cells. As expected, wild-type RBM20 protein localized predominantly in the nuclei, whereas RBM20-p.Pro638Leu was found predominantly in the cytoplasm as aggregate-like structures (Figure 3). Although we identified an aberrant splicing effect for TTN- and RYR2-mRNA, which was comparable to the RBM20-p.Pro638Leu carriers, the mutant RBM20-p.Val914Ala showed regular nuclear localization comparable to the wild-type form indicating a different loss-of-function mechanism. The RBM20 variants with a regular splicing pattern p.Val545Ile, p.Asp888Asn, and p.Pro1039Ser were also found predominantly within the nucleus (Figure 3).

Recently, evidence was found that the murine mutations in Rbm20 corresponding to the human p.Arg634Trp, p.Ser635Ala and p.Ser637Ala within the RS domain of RBM20 might lead to a mislocalization of the protein and are not involved in the splicing process (Murayama et al., 2018). As it is assumed that mislocalization of RBM20 mutants within the RS domain might be caused by the lack of phospho-serines, we hypothesized that the mislocalization of RBM20-p.Pro638Leu is caused by an aberrant phosphorylation. We supposed that Pro638 is part of a kinase recognition motif necessary for

**FIGURE 3** Localization analyses of RBM20-EYFP wild type and variants in transfected C2C12 cells. (a) Quantitative analysis of the localization. Shown are the means from three independent transfection experiments ± standard deviation. (b) Representative images for each of the observed localization types (only nucleus/only cytoplasm/nucleus + cytoplasm) of transiently transfected cells are shown. In the overlay image, EYFP-labeled RBM20 is shown in yellow, DAPI stained nuclei in blue. Wild-type RBM20 and variants not localized in the RS domain are mainly found in the nuclei. Mutants concerning the RS domain of RBM20 are mainly localized in the cytoplasm. Scale bars = 10 µm. RBM20-EYFP-staining is only detectable in transfected cells. DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride
phosphorylation of Ser637. To test this hypothesis, we compared the localization of RBM20-p.Ser637Gly, p.Ser637Gly, and the phospho-
mimetic mutants p.Ser637Asp and p.Ser637Glu in C2C12 cells (Figure 3). We observed that the mutants p.Ser637Gly and p.Ser637Ala are localized predominantly outside the nuclei. Sub-
stitution against the negatively charged aspartate residue causes a shift in the localization of RBM20 (Figure 3). Nevertheless, the sub-
stitution of Ser637 by a phosphomimetic amino acid does not lead to a statistically significant localization difference compared to p.Ser637Gly and p.Ser637Ala or other non-phosphomimetic variants. From these experiments, we conclude that although a serine at po-
sition 637 is critical for nuclear localization of RBM20, the sub-
stitution of this serine by a phosphomimetic group is not sufficient for re-localization of the protein from the cytoplasm to the nucleus.

To analyze the hypothesis that the putative destruction of a kinase recognition motif might lead to a mislocalization of the p.Pro638Leu mutant, we generated double mutants in which Ser637 was exchanged in addition to the mutation p.Pro638Leu (Figure 3). We observed that the substitution of Ser637 by phosphomimetic amino acids did not lead to nuclear localization of RBM20 when additionally the mutation p.Pro638Leu was present.

### 3.4 RBM20 mutants leading to nuclear mislocalization have no dominant impact on the localization of the wild-type protein

We analyzed if the mislocalized mutant proteins might have an impact on the nuclear localization of the wild-type protein. The subcellular localization of the wild-type RBM20 was checked by cotransfection experiments with the mutant protein (Figure 4). We observed that cotransfection of wild-type RBM20 conjugated with two different

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**FIGURE 4** Localization analyses of RBM20 wild type and mutants in C2C12 cells. Shown are representative images of cells, which were cotransfected with RBM20-wt-ECFP and RBM20-wt-EYFP, p.Pro638Leu or p.Val914Ala-EYFP. Although localized outside the nucleus the RBM20-p.Pro638Leu-mutant has no influence on the localization of RBM20-wt-ECFP. In the overlay images, ECFP is shown in cyan and EYFP in yellow. Scale bars = 10 µm. DAPI staining was not performed due to the overlapping emission spectra of DAPI and ECFP. DAPI, 4',6-diamidine-2'-phenylindole dihydrochloride
fluorescent proteins led to nuclear localization of both forms. In contrast, cotransfection of both wild-type and RBM20-p.Pro638Leu conjugated with different fluorescence tags led to predominantly nuclear localization of the wild-type form whereas the mutant RBM20 was found in the cytoplasm. Thus, RBM20-p.Pro638Leu does not dominantly influence the localization of wild-type protein.

3.5 | RBM20 shows a mislocalization in explanted cardiac tissue of RBM20-p.Pro638Leu carriers

We also analyzed the localization of RBM20 in the cardiac tissue of patients with pathogenic RBM20 mutations (Figure 5). As controls samples from an NF sample, a DCM patient without RBM20 mutations and cardiomyopathy patients with nonpathogenic RBM20 variants were used. Comparable to the cell culture experiments, the controls and the pathogenic mutation p.Val914Ala showed localization in nuclei. Immunohistochemistry of myocardial samples from four patients with RBM20-p.Pro638Leu revealed nuclear staining, presumably from the wild-type allele, and cytoplasmic aggregate-like structures presumably caused by the mutant form.

4 | DISCUSSION

RBM20 regulates muscle-specific splicing of genes like TTN and RYR2 (Guo et al., 2012; Li et al., 2013; Maatz et al., 2014). Rbm20-deficient rats show ventricular dilatation and sudden death (Guo et al., 2012) mirroring the pathological features of RBM20-deficient humans (Brauch et al., 2009). Several mutations in RBM20 have been identified in humans associated with DCM (Beqqali et al., 2016; Brauch et al., 2009; Guo et al., 2012) or left ventricular noncompaction cardiomyopathy (Sedaghat-Hamedani et al., 2017). However, until now the splicing defect of RBM20 mutations was particularly analyzed only in rodent models (Guo et al., 2013; Murayama et al., 2018) and in human-induced pluripotent stem cell-derived cardiomyocytes (Streckfuss-Bomeke et al., 2017). Furthermore, there is only limited evidence on the pathomechanisms leading to RBM20-dependent missplicing (Dauksaite & Gotthardt, 2018; Murayama et al., 2018).

Here, we present first results on the splicing of TTN and RYR2 in the myocardium of human RBM20-mutation carriers compared to control samples of DCM patients and rejected donor hearts without pathogenic RBM20 mutations. Comparable to rodent models (Guo et al., 2013; Murayama et al., 2018), we confirmed that patients carrying RBM20-p.Pro638Leu showed aberrant splicing of TTN and RYR2. Furthermore, our splicing assay revealed that the novel mutation RBM20-p.Val914Ala in the Glu-rich domain can be classified as likely pathogenic due to its aberrant splicing. Interestingly, a different pathogenic RBM20 variant (p.Glu913Lys) localized in the Glu-rich domain causes DCM through missplicing of TTN-mRNA and impairment of the Frank–Starling mechanism (Beqqali et al., 2016). Furthermore, Parikh et al. (2019) identified the Glu-rich region encoded by exon 11 as a high confidence region of pathogenicity.

FIGURE 5  Localization analyses of RBM20 in cardiac tissue. Images of explanted myocardial tissue sections are shown, which were labeled with primary anti-RBM20 and Cy3-conjugated secondary antibodies. RBM20 is shown in yellow and the nuclei were labeled with DAPI (blue). Sections from the NF-patient, the DCM patient with no RBM20 mutation, and the patients with nonpathogenic variants p.Val545Ile, p.Pro1039Ser, and p.Asp888Asn show an exclusively nuclear localization of RBM20. Sections from four DCM patients carrying the pathogenic RBM20-p.Pro638Leu mutation show a localization of RBM20 in the nuclei and in cytoplasmic aggregate-like structures. Scale bars = 20 µm. Of note, RBM20 is specifically expressed in cardiomyocytes and is therefore not detectable in other cardiac cell types.

DAPI, 4',6-diamidine-2'-phenylindole dihydrochloride; DCM, dilated cardiomyopathy; NF, non-failing
Comparably, our results support the pathogenic significance not only of the RS domain (exon 9) but also of the Glu-rich domain (exon 11). Surprisingly, RBM20-p.Val914Le in also changing Val914 does not lead to an aberrant splicing pattern in our splicing assay. Valine, alanine, and isoleucine are hydrophobic amino acids. As the exchange of the valine through an isoleucine does not lead to a misfunction of RBM20 but the valine/alanine exchange does, it can be assumed that for appropriate splicing a branched-chain amino acid might be necessary at position 914 of the protein. It can be suggested that although the Glu-rich region in exon 11 is a region of pathogenicity, not every amino acid exchange leads to a pathogenic effect. Two other RBM20 variants were categorized as (likely) benign considering data of our splicing assay. As the clinical interpretation of novel RBM20 sequence variants, especially missense variants, might be challenging, we used a splicing assay to support the classification of novel RBM20 variants. The assay might have relevance for clinical interpretation of RBM20 mutations and genetic counseling of patients with RBM20 variants in the future. However, this assay can only be used when explanted myocardial tissue is available.

Patient IV.3 in family 1 carries two pathogenic mutations: RBM20-p.Pro638Leu and MYBPC3-p.Trp792ValfsTer41. However, the MYBPC3 mutation is associated with HCM (Alders et al., 2003; Moolman et al., 2000; van Dijk et al., 2009). Therefore, the contribution of this MYBPC3 mutation to disease development in patient IV.3 is currently unclear as he developed DCM instead of HCM, as previously described (Alders et al., 2003). In addition, the MYBPC3 mutation has an incomplete penetrance and might develop its clinical impact later in the life of the patient.

Maatz et al. (2014) showed a strong variation of RBM20 expression in HF samples, which was associated with effects on alternative splicing of several RBM20 target genes. They suggest that differences in RBM20 expression levels contribute to modulation of cardiac function under pathophysiological conditions. We found increased RBM20-mRNA expression in the myocardium of RBM20 wild type and mutation carriers with end-stage DCM compared to NF controls. However, it remains unclear whether the elevated RBM20-mRNA levels in DCM patients represent a genotype independent compensatory effect or contribute to the pathomechanism.

RBM20 contains like other SR-like RNA-binding proteins prototypical RRM and RS domains (Brauch et al., 2009). It was previously found that the amino acid stretch 491–658 in RBM20 including RRM and RS domains contributes to the full nuclear localization of the protein (Filippello et al., 2013). Of note, Filippello et al. (2013) found that even an N-terminal RBM20 protein fragment up to the amino acid 614 is completely localized in the nuclei. Recently published results furthermore reveal that mutations affecting arginines and serines within the RS domain lead to mislocalization of RBM20 (Murayama et al., 2018). Here, we discovered that also the substitution of a proline residue (p.Pro638Leu) in the RS domain leads to a nuclear mislocalization of RBM20, confirming the negative effect of missense mutations on the nuclear localization of mutant RBM20.

As most patients are heterozygous carriers, the nuclear mislocalization might lead to reduced nuclear RBM20 protein levels and consequently to a functional deficiency. Maatz et al. (2014) detected aberrant interaction with alternative spliceosomal proteins for the RBM20-p.Ser635Ala mutation which is also part of the RSRSP stretch. As shown by Murayama et al. (2018), the murine mutation p.Ser637Ala corresponding to human p.Ser635Ala leads to abnormal cytoplasmic localization of mutant RBM20. Thus, it remains unclear if mutations in the RSRSP stretch cause aberrant interactions in the splicing process or are not able to interact with the appropriate interaction partners due to their mislocalization.

In cell culture, we found that RBM20-p.Pro638Leu was localized in cytoplasmic aggregate-like structures, whereas wild-type RBM20 was detectable almost exclusively in the nucleus. It can be suggested that the assembly of the mutant protein in cytoplasmic aggregates might contribute to impaired transport of the wild-type protein. For amyloid-like cytoplasmic protein aggregates, it has been previously shown that they lead to mislocalization and sequestration of multiple factors of the import and export machinery (Woerner et al., 2016). Consequently, aggregate formation might lead to dysfunctional nuclear-cytoplasmic transport processes (Woerner et al., 2016). In our study, it was not tested whether the cytoplasmic RBM20-protein aggregates contain additional proteins or mRNA molecules. Nevertheless, cotransfection of wild-type and mutant RBM20 reveals that mutant RBM20 does not lead to wild-type mislocalization. The presence of cytoplasmic aggregates was also found in explanted cardiac tissue of RBM20-p.Pro638Leu carriers suggesting that the cell culture data are comparable to in vivo findings. These patients also showed nuclear RBM20 localization indicating that wild-type RBM20 is localized properly. This is again in accordance with the cotransfection experiments which also show no dominant negative effect of the mutant on the localization of the wild-type protein.

We used C2C12 cells in our RBM20 overexpression studies as these cells were shown to express RBM20 in the nucleus (Guo et al., 2012). Gene expression from strong constitutive promoters like the cytomegalovirus (CMV)-promotor, which we have used in our study can violate balanced gene dosage affecting protein folding, complex assembly, and downstream regulation (reviewed in Gibson, Seiler, & Veitia, 2013). These overexpression effects might also lead to localization artifacts. Therefore, we used for wild type and variants of RBM20, the same promoter suggesting that the cytoplasmic mislocalization of the respective variants is due to the amino acid exchange and is not caused by the overexpression. These findings are proven by the immunocytochemical analyses of explanted myocardial tissue of mutation carriers, which also show aggregate-like structures in the cytoplasm.

We and others (Murayama et al., 2018) found that a serine at position 637 is critical for the nuclear localization of RBM20. Murayama et al. showed that RBM20 is constitutively phosphorylated on multiple residues including Ser637, which is part of the RSRSP stretch. In this study, we inserted phosphomimetic amino acids at this position to analyze the subcellular distribution of RBM20. The substitution of Ser637 by an aspartate or glutamate does not lead to a significant nuclear relocalization of the protein, which presumably means that a single phosphomimetic group is not enough to mimic a phosphorylated serine in our experiment. A number of
proline-directed protein kinases (PDK) phosphorylate serine or threonine residues preceding a proline residue (Nigg, 1991; Pelech & Sanghera, 1992; Vulliet, Hall, Mitchell, & Hardie, 1989). Our phosphomimetic double mutants show no nuclear localization, which might be explained either by the fact that the proline-directed phosphorylation of Ser637 of RBM20 is alone not sufficient for nuclear localization or by the fact that a phosphomimetic amino acid does not perfectly mimic a phospho-serine at position 637 of RBM20. Interestingly, proline residues might introduce a backbone switch into a polypeptide chain by cis-trans isomerization of the prolyl bonds (Johnson, Noble, & Owen, 1996). Phosphorylation of proteins at Ser/Thr-Pro motifs can generate highly specific rearrangements of the conformation (Schutkowski et al., 1998). This process is catalyzed by peptidyl-prolyl cis/trans isomerases specific for phosphorylated Ser/Thr-Pro motifs (Lu, Hanes, & Hunter, 1996; Schutkowski et al., 1998). For this reason, it might be suggested that both the phosphorylation and isomerization at the motif Ser637/Pro638 is essential for the nuclear transport mechanisms of RBM20.

We found that RBM20-p.Val914Ala, which is localized outside the conserved RRM or RS domain has no effect on the nuclear localization in human myocardium. Nevertheless, aberrant splicing was observed for this mutant indicating that the nuclear mislocalization is not the only pathomechanism leading to myocardial missplicing caused by RBM20 mutations. A wild-type-like nuclear localization was also observed for the (likely) benign RBM20 variants (p.Val545Ile, p.Pro1039Ser) analyzed in this study. We found that RBM20-p.Pro638Leu is associated with an aggressive and early-onset cardiomyopathy, which appears to be different from the disease course of the mutant p.Val914Ala.

Due to a limited number of RBM20 cases available for a Kaplan–Meyer analysis, a valid calculation of the event-free survival was not possible. However, we estimated for the p.Pro638Leu mutation carriers that the young age of the event-free survival is comparable with the two p.Pro638Leu families described previously (Brauch et al., 2009). The diverging age for event-free survival for the two different pathogenic mutations presented in our study might be related to different pathomechanisms of mutant RBM20.

4.1 | Limitations of the study

The influence of further environmental, epigenetic, or other genetic factors might significantly modulate the time course and penetrance of the investigated cases of RBM20-related cardiomyopathies.

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CONFLICT OF INTEREST

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Alders, M., Jongbloed, R., Deelen, W., van den Wijngaard, A., Doevedrans, P., Ten Cate, F., ... Mannens, M. (2003). The 2373insG mutation in the MYBPC3 gene is a founder mutation, which accounts for nearly one-fourth of the HCM cases in the Netherlands. European Heart Journal, 24(20), 1848–1853. https://doi.org/10.1016/s0195-668x(03)00466-4
Beqgali, A., Bollen, I. A., Rasmussen, T. B., van den Hoogenhof, M. M., van Deutkom, H. W., Schafer, S., ... Pinto, Y. M. (2016). A mutation in the glutamate-rich region of RNA-binding motif protein 20 causes dilated cardiomyopathy through missplicing of titin and impaired Frank–Starling mechanism. Cardiovascular Research, 112(1), 452–463. https://doi.org/10.1093/cvr/cvw192
Brauch, K. M., Karst, M. L., Herron, K. J., de Andrade, M., Pellikka, P. A., Rodeheffer, R. J., ... Olson, T. M. (2009). Mutations in ribonucleic acid binding protein gene cause familial dilated cardiomyopathy. Journal of the American College of Cardiology, 54(10), 930–941. https://doi.org/10.1016/j.jacc.2009.05.038
Brodehl, A., Ebbinghaus, H., Deutsch, M. A., Gummett, J., Gartner, A., Ratnavadivel, S., & Milting, H. (2019). Human induced pluripotent stem-cell-derived cardiomyocytes as models for genetic cardiomyopathies. International Journal of Molecular Sciences, 20(18), 4381. https://doi.org/10.3390/ijms20184381
Dauksaite, V., & Gotthardt, M. (2018). Molecular basis of titin exon exclusion by RBM20 and the novel titin splice regulator PTB4. Nucleic Acids Research, 46(10), 5227–5238. https://doi.org/10.1093/nar/gky165
Filippello, A., Lorenzi, P., Bergamo, E., & Romanelli, M. G. (2013). Identification of nuclear retention domains in the RBM20 protein. FEBs Letters, 587(18), 2989–2995. https://doi.org/10.1016/j.febslet.2013.07.018
Gaertner-Rommel, A., Tiesmeier, J., Jakob, T., Strickmann, B., Veit, G., Bachmann-Mennenga, B., ... Milting, H. (2019). Molecular autopsy and family screening in a young case of sudden cardiac death reveals an unusually severe case of FHL1 related hypertrophic cardiomyopathy. Molecular Genetics & Genomic Medicine, 7(8), e841. https://doi.org/10.1002/mgg3.841
Gibson, T. J., Seiler, M., & Veitia, R. A. (2013). The transience of transient overexpression. Nature Methods, 10(8), 715–721. https://doi.org/10.1038/nmeth.2534
Guo, W., Pleitner, J. M., Saupe, K. W., & Greaser, M. L. (2013). Pathophysiological defects and transcriptional profiling in the RBM20−/− rat model. PLOS One, 8(12), e84281. https://doi.org/10.1371/journal.pone.0084281
Guo, W., Schafer, S., Greaser, M. L., Radke, M. H., Liss, M., Govindarajan, T., ... Gotthardt, M. (2012). RBM20, a gene for hereditary cardiomyopathy, regulates titin splicing. Nature Medicine (New York, NY, United States), 18(5), 766–773. https://doi.org/10.1038/nm.2693
Hersberger, R. E., Givertz, M. M., Ho, C. Y., Judge, D. P., Kantor, P. F., McBride, K. L., ... Ware, S. M. (2018). Genetic evaluation of cardiomyopathy—A Heart Failure Society of America practice guideline. Journal of Cardiac Failure, 24(5), 281–302. https://doi.org/10.1016/j.cardfail.2018.03.004
