RBM20 Mutations Induce an Arrhythmogenic Dilated Cardiomyopathy Related to Disturbed Calcium Handling

RBM20 mutations induce an arrhythmogenic dilated cardiomyopathy related to disturbed calcium handling

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Running title: Rbm20 impacts cardiac Ca^{2+} handling

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ABSTRACT:

BACKGROUND: Mutations in RBM20 cause a clinically aggressive form of dilated cardiomyopathy (DCM), with an increased risk of malignant ventricular arrhythmias. RBM20 is a splicing factor that targets multiple pivotal cardiac genes, such as Titin (TTN) and Calcium/calmodulin-dependent kinase II delta (CAMK2D). Aberrant TTN splicing is thought to be the main determinant of RBM20-induced DCM, but is not likely to explain the increased risk of arrhythmias. Here, we investigated the extent at which RBM20 mutation carriers have an increased risk of arrhythmias and explore the underlying molecular mechanism.

METHODS: We compared clinical characteristics of RBM20 and TTN mutation carriers and used our previously generated Rbm20 knockout (KO) mice to investigate downstream effects of Rbm20-dependent splicing. Cellular electrophysiology and Ca^{2+} measurements were performed on isolated cardiomyocytes from Rbm20 KO mice to determine the intracellular consequences of reduced Rbm20 levels.

RESULTS: Sustained ventricular arrhythmias were more frequent in human RBM20 mutation carriers than in TTN mutation carriers (44% vs 5%, respectively, p=0.006). Splicing events that affected Ca^{2+} and ion handling genes were enriched in Rbm20 KO mice, most notably in the genes CamkIIδ and RyR2. Aberrant splicing of CamkIIδ in Rbm20 KO mice resulted in a remarkable shift of CamkIIδ towards the δ-A isoform which is known to activate the L-type Ca^{2+} current ($I_{Ca,L}$). In line with this, we found an increased $I_{Ca,L}$, intracellular Ca^{2+} overload and increased sarcoplasmic reticulum (SR) Ca^{2+} content in Rbm20 KO myocytes. Additionally, not only complete loss of Rbm20, but also heterozygous loss of Rbm20 increased spontaneous SR Ca^{2+} releases, which could be attenuated by treatment with the $I_{Ca,L}$ antagonist verapamil.

CONCLUSIONS: We show that loss of Rbm20 disturbs Ca^{2+} handling and leads to more pro-arrhythmic Ca^{2+} releases from the SR. Patients that carry a pathogenic RBM20 mutation have more ventricular

arrhythmias despite a similar LV function, compared to patients with a TTN mutation. Our experimental data suggests that RBM20 mutation carriers may benefit from treatment with an $I_{Ca,L}$ blocker to reduce their arrhythmia burden.

Keywords: RBM20, alternative splicing, dilated cardiomyopathy, calcium handling, arrhythmia, L-type calcium channel

Clinical perspective:

What is new?

- Patients that carry a pathogenic RBM20 mutation have more ventricular arrhythmias than patients with a TTN mutation, despite similarly depressed LV function.
- Targets of RBM20 splicing are enriched for Ca^{2+} and ion handling genes, most notably CamkIIδ and RyR2.
- Loss of Rbm20 induces an increased L-type Ca^{2+} current density, intracellular Ca^{2+} overload, increased SR Ca^{2+} content, and increased spontaneous Ca^{2+} releases, which can be attenuated by treatment with an L-type Ca^{2+} channel blocker.

What are the clinical implications?

- RBM20 mutation carriers may benefit from treatment with an $I_{Ca,L}$ blocker to reduce their arrhythmia burden.
- RBM20 mutation carriers should be closely monitored for potential electrical disturbances and cardiac arrhythmias, even in the early stages of the disease.

INTRODUCTION

Mutations in the gene encoding RNA-binding motif protein 20 (RBM20) are known to cause dilated cardiomyopathy (DCM)[1-3]. In fact, disease-causing mutations in RBM20 are relatively often found in familial DCM, as it accounts for about 3% of the familial DCM cases[4]. Recent studies suggest that loss of RBM20 causes DCM by missplicing of the gene Titin (TTN)[5, 6]. TTN is a giant sarcomeric protein which acts as a molecular spring in the sarcomere, and as such, defines the passive stiffness of the cardiomyocyte. Loss of Rbm20 leads to aberrant splicing of TTN, reflected by increased expression of very large and compliant TTN isoforms in the heart, which is believed to underlie the DCM phenotype in RBM20 mutation carriers. However, clinical observations challenge the idea that mutations in RBM20 cause DCM only via dysfunctional TTN. RBM20 mutation carriers are known to present with a clinically aggressive form of DCM, associated with young age at diagnosis, fast progression of heart failure, increased risk of arrhythmias, and high mortality[1, 2, 4], while TTN mutations are not associated with such an aggressive clinical course[7, 8].

RBM20 is a heart- and skeletal muscle-enriched splicing factor which controls tissue-specific isoform expression of many other genes besides TTN, including sarcomeric genes such myomesin 1, but also Ca^{2+} and ion handling genes such as calcium/calmodulin-dependent kinase II-delta (CAMK2D) and Ryanodine Receptor 2 (RYR2)[5, 6]. In that regard, it is of interest that human RBM20 mutation carriers present with an increased risk of arrhythmias, which is also observed in Rbm20 deficient rats[4, 5]. Taken together, this indicates that the missplicing of TTN alone is unlikely to fully explain the severe heart failure phenotype found in RBM20 mutations carriers, and that additional Rbm20 targets may be of clinical importance. Indeed, *in vitro* analysis of cardiomyocytes derived from induced pluripotent stem cells of an RBM20 mutation carrier recently provided the first evidence that Rbm20 regulates Ca^{2+} handling[9].

Here we investigated the underlying mechanism of arrhythmias in RBM20 mutation carriers, by using Rbm20 knockout (KO) mice and performing electrophysiological studies and Ca^{2+} measurements. We recorded L-type Ca^{2+} current ($I_{Ca,L}$) in wildtype and Rbm20 KO cardiomyocytes, and found an almost 2-fold increase in current density. Ca^{2+} measurements revealed a severe intracellular Ca^{2+} overload, and a propensity to spontaneous Ca^{2+} releases from the sarcoplasmic reticulum (SR) in both heterozygous and homozygous Rbm20 KO cardiomyocytes. Blockade of $I_{Ca,L}$ with verapamil rescued the Ca^{2+} releases from the SR, indicating that increased $I_{Ca,L}$ density is, at least in part, responsible for Ca^{2+} overload in Rbm20 KO cells. Our data indicates that this disturbed Ca^{2+} handling induced by dysfunctional Rbm20 underlies the increased risk of arrhythmias in RBM20 mutation carriers, and therefore this study provides new avenues to treat arrhythmias in RBM20 mutation carriers.

METHODS

The data, analytic methods, and study materials will be made available upon reasonable request to other researchers for purposes of reproducing the results or replicating the procedure. Extended methods are given in Supplemental File 1.

Patient inclusion

The genetic test results of all patients with (suspected) DCM who were referred to the Department of Clinical Genetics of the Academic Medical Center (Amsterdam, Netherlands) between 2005 and 2016 were retrospectively reviewed. All patients with a (likely) pathogenic variant in the RBM20 gene (n=18) or the TTN gene (n=22) and no other variants in genes associated with DCM were included. Additional information on patient inclusion can be found in Supplemental File 1, and the mutations included in this study can be found in Supplemental Table 1. Informed consent was obtained from all individuals and the study confirmed to the ethical guidelines of the 1975 Declaration of Helsinki.

Rbm20 KO mice

Rbm20 KO mice were previously generated[10]. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam, and in accordance with the guidelines of this institution and the Directive 2010/63/EU of the European Parliament.

Ca^{2+} measurements

Left ventricular cardiomyocytes from 8 to 12-week old wildtype (WT), heterozygous (+/-), and homozygous (-/-) Rbm20 KO mice were isolated by enzymatic dissociation on a Langendorff set-up, loaded with the Ca^{2+} sensitive dye Indo-1-AM (Molecular Probes, Eugene, OR, USA), and Ca^{2+} transients were measured as previously described[11].

Cellular electrophysiology

Action potentials (APs) and $I_{Ca,L}$ were recorded in isolated left ventricular (LV) cardiomyocytes from 8 to 12-week old WT and Rbm20 KO mice at 36±0.2°C using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Voltage control, data acquisition, and analysis were realized with custom software, and potentials were corrected for the calculated liquid junction potentials[12]. Cell membrane capacitance (C_m) was calculated by dividing the time constant of the decay of the capacitive transient after a -5 mV voltage step from –40 mV by the series resistance. Signals were low-pass-filtered with a cutoff of 5 kHz and digitized at 40 and 10 kHz for APs and $I_{Ca,L}$, respectively. Series resistance was compensated by ≥80%.

Statistics

Data are presented as mean ± sem, and were analyzed with appropriate statistical tests, as indicated in the respective figure legends. A value of $p < 0.05$ was considered statistically significant.

RESULTS:

Increased prevalence of malignant ventricular arrhythmias in RBM20 mutation carriers

Patients with heterozygous RBM20 mutations present with DCM, but often also experience cardiac arrhythymias. We compared arrhythmia burden of DCM patients with RBM20 mutations (n=18) to DCM patients with TTN mutations (n=22) (Table 1). Since TTN is the most prominently studied target of RBM20, and is considered the main determinant of the DCM phenotype of RBM20 mutation carriers, we used DCM patients with TTN mutations as a reference group. Both groups had similarly decreased LV chamber function ($LVEF_{RBM20vsTTN}$; 37±17 vs 37±13), dilation of the LV ($LVEDD_{RBM20vsTTN}$; 60±11 vs 59±9 and $LVEDV_{RBM20vsTTN}$; 269±92 vs 246±73), and similar ECG timing intervals (Table 1). Despite comparable cardiac function and remodeling in both patient groups, RBM20 mutation carriers experienced more malignant ventricular arrhythmias, as 44% of the RBM20 mutation carriers had sustained ventricular arrhythmias (i.e. sustained/symptomatic ventricular tachycardia or ventricular fibrillation), compared to only 5% in the TTN group (p=0.006). The increased risk of arrhythmias was also reflected by the increased number of ICD implantations in the RBM20 group ($ICD_{RBM20vsTTN}$; 61% vs 9%, p=0.002). There were no differences in the pharmacological treatment of patients with RBM20 mutations and patients with TTN mutations. Finally, RBM20 mutation carriers tended to have more often a familial history of sudden cardiac death (SCD) ($SCD_{RBM20vsTTN}$; 72% vs 36%, p=0.052). In conclusion, these data indicate that patients with RBM20 mutations have an increased risk of malignant ventricular arrhythmias as compared to TTN mutation carriers, even though cardiac dilation and function is comparable in both patients groups. This also suggests that the cardiac pathology of RBM20 mutation carriers is only partly explained by altered TTN function, and that other factors contribute to the arrhythmia susceptibility.

Characterization of Rbm20 KO mice

In order to investigate the molecular mechanisms underlying the increased risk of arrhythmias in RBM20 mutation carriers, we used Rbm20 KO mice that were previously generated by targeted disruption of exon 4 and 5 of the Rbm20 gene (Figure 1A)[10]. Rbm20 KO mice were born in normal Mendelian ratios, were viable, and did not exhibit obvious abnormalities. We validated the loss of the targeted Rbm20 allele by qPCR (all primer sequences can be found in Supplemental Table 2), and observed an approximate 50% reduction of Rbm20 mRNA in Rbm20+/- hearts and a complete loss of Rbm20 mRNA in Rbm20-/- hearts (Figure 1B). qPCR analysis of the stress markers ANF and BNP revealed an upregulation of both markers in the hearts of Rbm20-/- mice, but not in Rbm20+/- mice (Figure 1C-D). Loss of Rbm20 resulted in disrupted splicing of Ttn and LIM-domain Binding 3 (Ldb3) in the hearts Rbm20+/- and Rbm20-/- mice (Figure 1E). This recapitulates previously described splicing effects of Rbm20-deficiency in mice and rats[5, 13]. Next, we investigated cardiac function and morphology of these mice. Heart weight/body weight ratios were not different between wildtype, Rbm20+/-, and Rbm20-/- mice (Figure 1F), but M-mode echocardiography revealed decreased fractional shortening and increased systolic diameters in both Rbm20+/- and Rbm20-/- mice, indicative of a cardiomyopathic phenotype (Figure 1G-I). Surface electrocardiograms (ECG) showed a prolonged PR and QTc interval in Rbm20-/- mice, starting at 6 weeks of age, which remained until at least 25 weeks of age (Figure 1J-K). No difference in QRS duration and RR interval were observed (Supplemental Figure 1). The differences between 6 and 8 weeks can be explained by growth and maturation of the mice. Rbm20-/- hearts showed moderate fibrosis, as was evidenced by Sirius Red stained sections of the hearts of 25 weeks old mice (Figure 1L-M). Consistently, the expression of collagen 1a1 and collagen 3a1 mRNA was increased in Rbm20-/- hearts (Supplemental Figure 2). In summary, Rbm20 KO mice develop cardiomyopathy, have ECG abnormalities, increased cardiac fibrosis, and disturbed splicing of previously described Rbm20-targets.

Rbm20 KO mice display splicing abnormalities in Ca^{2+} and ion handling genes

To uncover splicing targets of Rbm20 that could explain the increased risk of arrhythmias in RBM20 mutation carriers, we used next-generation RNA-sequencing on wildtype and Rbm20-/- mice, 25 weeks of age, and analyzed their transcriptomes on differential splicing events using DEXseq[14]. Gene ontology enrichment analysis on the differentially spliced genes revealed that genes involved in '*cardiac muscle contraction*' were enriched in the hearts of Rbm20-/- mice (Supplemental File 2). Interestingly, genes in the biological process '*cardiac muscle contraction*' encompass two types of genes; sarcomeric genes, such as Ttn, Tnnt2, and Myh6/7, and Ca^{2+} and ion handling genes, such as Slc8a1, RyR2, and CamkIIδ (Figure 2A). We validated the differential splicing events using endpoint RT-PCR in wildtype, Rbm20+/-, and Rbm20-/- mice, and were able to verify the splicing changes in CamkIIδ, RyR2, Scn5a (which encodes the cardiac Na+ channel NaV1.5), and Cacna1c (which encodes the L-type calcium channel subunit CaV1.2) (Figure 2B, Supplemental Figure 3A-C).

The atypical splicing of CamkIIδ in the Rbm20 KO hearts is particularly relevant for cardiomyocyte biology. CamkIIδ is a multifunctional Ser/Thr protein kinase and is an important regulator of excitation-contraction coupling by phophorylation of its substrates phopholamban (PLB), the ryanodine receptor (RyR), and the L-type Ca^{2+} channel (LTCC)[15-17]. CamkIIδ exists in multiple isoforms in the adult heart, most notably δ-A, δ-B, δ-C, and δ-9[18]. In the healthy adult heart, the highest expressed isoforms are δ-B and δ-C, but loss of Rbm20 induced a switch towards the bigger δ-A and δ-9 mRNA isoforms (Figure 2B). The smallest isoform, δ-C lacks all alternative exons, and is located in the cytoplasm where it has been shown to phosphorylate RyR2 and PLB[15]. In the δ-B isoform, exon 14 with a functional nuclear localization signal is spliced into the transcript, thereby targeting CamkIIδ to the nucleus, where it specifically associates with histone deacetylases to regulate transcription[19, 20]. The δ-A isoform results from inclusion of exon 15 and 16, and is associated with the intercalated disc and T-tubules, where Ca^{2+} channels and activated

CamKIIδ are concentrated[16, 21]. The exact function of δ-9 is currently unknown, but as it is the isoform that resembles δ-A the most (it only lacks exon 15), it likely fullfils a similar function as δ-A.

The nearly complete switch of CamKIIδ to the δ-A isoform led us to investigate the consequences of aberrant splicing on CamKIIδ expression and localization in the Rbm20 KO hearts. Western blotting further provided evidence of the isoform switch, as we detected a larger CamKIIδ protein in Rbm20+/- and Rbm20-/- hearts (Figure 2C, Supplemental Figure 3E). We did not find a difference in overall mRNA expression of CamkIIδ in wildtype, Rbm20+/-, and Rbm20-/- mice (Supplemental Figure 3D). We performed immunohistochemistry on sections of wildtype and Rbm20-/- hearts and found that CamkIIδ colocalizes with the LTCC in the t-tubules, as indicated by costaining with Cacnb1 (Figure 2D, and supplemental Figure 4). We did not find increased CamkIIδ localization in the t-tubules in Rbm20-/- hearts as compared to wildtype hearts, but this difference might be subtle and not readily identifiable with immunostainings. Interestingly, we detected increased expression of CamKIIδ in the intercalated discs of Rbm20-/- cardiomyocytes, again indicating a switch to the δ-A isoform[21]. Since the switch in CamkIIδ isoform could potentially impact phosphorylation targets of CamkIIδ, we performed western blotting for phosphorylated RyR2 (at Serine 2815) and phosphorylated Cacnb2 (at Threonine 498) in Rbm20-/- hearts. In both cases, we did not observe a difference in phosphorylation status at these specific amino acids (Supplemental Figure 5).

The differential splicing event in RyR2 involves inclusion of a 24-bp exon, which targets RyR2 to the nucleus instead of the SR, which may interfere with proper Ca^{2+} handling[22]. The splicing change that we observed in Scn5a represents alternative first exon usage with yet unknown significance[23]. The splicing change in Cacna1c, which encodes a subunit of the LTCC, in Rbm20 -/- hearts represents the inclusion of exon 9*, and this splicing event has been associated with hyperpolarization of the LTCC[24]. With respect to the differential splicing events in Slc8a1 (Ncx1), we found increased inclusion of exon 2A, indicative of

increased expression of the NCX1.1 splice variant[25]. The increased expression of the NCX1.1 splice variant could indicate that Na^+-dependent inactivation of NCX is reduced[25], but the functional consequences in Rbm20 KO hearts are unclear.

To rule out that the differential splicing events are not *secondary* to the DCM, but due to loss of Rbm20, we used GapmeRs to knockdown Rbm20 in neonatal rat cardiomyocytes (NRCM), which resulted in a ~80% decrease in Rbm20 mRNA expression (Figure 2E). Using endpoint RT-PCR we observed similar splicing changes in Ttn, CamkIIδ, and RyR2 as in the Rbm20 KO hearts (Figure 2F). These in vitro data indicate that Rbm20 directly regulates alternative splicing of TTN, CamKIIδ and RyR2 and that these isoform switches are not secondary to the DCM.

To investigate whether these Rbm20-dependent splicing changes also occur in human RBM20 mutation carriers we made use of an RNAseq dataset of a previously described DCM patient with a heterozygous RBM20 (p.E913K) mutation that we compared to an idiopathic DCM patient and 10 healthy individuals[26, 27]. We calculated the percentage-spliced-in (PSI) of the alternative exons and found that also in this RBM20 mutation carrier, but not in the DCM patient, alternative splicing of CAMK2D and RYR2 was similarly affected (Figure 2G, Supplemental Figure 6). We did not observe splicing differences in SCN5A and CACNA1C in the RBM20 patient compared to the DCM patient (data not shown).

With respect to the CAMK2D splicing in the human RBM20 mutation carrier, it is remarkable that δ-9 was greatly induced, and not δ-A, as we found in the Rbm20 KO mice and in NRCM with reduced Rbm20 levels. Indeed, it was shown earlier that exon 15 (included in δ-A, but excluded in δ-9) is hardly expressed in human heart[5, 6]. In conclusion, these data reveal splicing abnormalities in Ca^{2+} and ion handling genes such as CamkIIδ and RyR2 after loss of Rbm20 in human, rat, and mouse.

The L-type Ca^{2+} current ($I_{Ca,L}$) is increased in Rbm20 KO myocytes

To investigate whether the splicing abnormalities in CamKIIδ and Cacna1c in Rbm20-/- hearts would affect $I_{Ca,L}$ and APs, we performed patch-clamp electrophysiological studies in isolated cardiomyocytes of 8 to 12-week old mice, representing the early phase in the development of the cardiomyopathy. We found that the action potential duration at 90% of repolarization (APD_{90}) was prolonged in Rbm20-/- cardiomyocytes by approximately 50% (Figure 3A-B). The upstroke of the AP, as indicated by the V_{max} and AP amplitude (APA), and the resting membrane potential (RMP) were not signficantly different (Figure 3C-D). Since the AP duration is importantly regulated by $I_{Ca,L}$ and since the isoform switch of CamkIIδ is expected to increase the activity of the LTCC, we recorded $I_{Ca,L}$. Figure 3E shows representative $I_{Ca,L}$ recordings upon a depolarizing step from -60 to 0 mV, and the right panel shows the same recordings but scaled to the $I_{Ca,L}$ of wildtype cardiomyocytes. $I_{Ca,L}$ was significantly larger in Rbm20 -/- cardiomyocytes, and inactivation was faster, as analyzed with biexponential fits. Time constants of both fast and slow inactivation were significantly lower in Rbm20-/- cardiomyocytes (τ_s: 31.7 ± 3.4 vs 22.2 ± 1.4 ms; τ_f : 5.6 ± 0.5 vs 3.2 ± 0.2 ms). Most strikingly, the average current-voltage (I-V) relationships were significantly larger (~82% at 0 mV) in Rbm20-/- cardiomyocytes (Figure 3F). Neither voltage-dependency of activation nor voltage-dependency of inactivation were different between wildtype and Rbm20-/- cardiomyocytes (Figure 3G). The increase in $I_{Ca,L}$ density and action potential duration could not be explained by altered expression of Ncx or Cacna1c (Supplemental Figure 5). In conclusion, Rbm20-/- cardiomyocytes display increased $I_{Ca,L}$ density, resulting in AP prolongation.

Rbm20 KO cardiomyocytes exhibit intracellular Ca^{2+} overload and are prone to spontaneous SR Ca^{2+} releases

To investigate whether the increased $I_{Ca,L}$ in Rbm20 KO cardiomyocytes further leads to Ca^{2+} handling abnormalities, we analyzed Ca^{2+} transients in isolated 8 to 12-week old wildtype, Rbm20+/-, and Rbm20-

/- cardiomyocytes at 2, 4, and 6 Hz of field stimulation. We found that both Rbm20+/- and Rbm20-/- cardiomyocytes exhibit remarkably large Ca^{2+} transients, and this was most pronounced at 6 Hz (Supplemental Figure 7). Figure 4A shows representative Ca^{2+} transients of a wildtype, Rbm20+/-, and Rbm20-/- cardiomyocyte at 6 Hz of stimulation. Diastolic Ca^{2+} was increased ~2-fold (Diastolic $Ca^{2+}_{WTvsHETvsKO}$: 79.9 ± 7.3 vs 184.4 ± 22.1 vs 163 ± 11.9 nM) and the peak amplitude was increased ~3-fold (Peak $amplitude_{WTvsHETvsKO}$: 101 ± 10.5 vs 333.9 ± 60.8 vs 308.9 ± 58.8 nM) in Rbm20+/- and Rbm20-/- cardiomyocytes (Figure 4A-C). Next, we examined SR Ca^{2+} content by rapid cooling and found that it was increased ~3.5-fold in Rbm20+/- and Rbm20-/- cardiomyocytes (SR Ca^{2+} $_{WTvsHETvsKO}$: 214.4 ± 21.3 vs 791.8 ± 170.7 vs 690.8 ± 79.7 nM) (Figure 4D, representative traces in Supplemental Figure 8). Both increased SR Ca^{2+} content and elevated diastolic Ca^{2+} increase the open probability of the RyR-channels, resulting in an increase of spontaneous SR Ca^{2+} releases, which in turn can trigger delayed after depolarizations (DADs) that are pro-arrhythmic[28]. To examine spontaneous SR Ca^{2+} releases, we stopped stimulation (6 Hz) of cardiomyocytes, after which spontaneous activity was recorded for 10 seconds. At baseline, both Rbm20+/- and Rbm20-/- cardiomyocytes showed more spontaneous Ca^{2+} releases than wildtype cardiomyocytes, and this effect was exacerbated after application of 50 nM noradrenaline (Figure 4E, representative traces in Supplemental Figure 9). In conclusion, out data shows that Rbm20 KO cardiomyocytes display Ca^{2+} overload, increased SR Ca^{2+} content, and more spontaneous Ca^{2+} releases, especially after β-adrenergic stimulation. Interestingly, Ca^{2+} handling was as severely affected in Rbm20+/- as in the Rbm20-/- cardiomyocytes.

Inhibition of L-type Ca^{2+} current with verapamil decreases pro-arrhythmic spontaneous Ca^{2+} releases

Since we suspect that the increased $I_{Ca,L}$ underlies the increased intracellular Ca^{2+} levels, the SR Ca^{2+} overload, and the spontaneous Ca^{2+} releases in the Rbm20 KO cells, we hypothesized that inhibiting $I_{Ca,L}$ with verapamil, a well-known LTCC-antagonist, would decrease the number of spontaneous Ca^{2+}

releases. We incubated Rbm20-/- cardiomyocytes with 1 µM verapamil, a concentration that has been shown to decrease $I_{Ca,L}$ by approximately 30%[29], and again recorded spontaneous Ca^{2+} releases. Strikingly, incubation of Rbm20-/- cardiomyocytes with verapamil decreased the number of spontaneous Ca^{2+} releases after the addition of noradrenaline to wildtype levels (Figure 4E, and Supplemental Figure 9). These data indicate that the increased $I_{Ca,L}$, at least in part, contributes to pro-arrhythmic SR Ca^{2+} releases in Rbm20-/- cardiomyocytes. Moreover, our results indicate that treatment of RBM20 mutation carriers with the LTCC-antagonist verapamil might prove beneficial to decrease the number of malignant ventricular arrhythmias that RBM20 mutation carriers experience. Figure 5 depicts the proposed model of how Rbm20 deficiency leads to pro-arrhythmic spontaneous Ca^{2+} releases.

DISCUSSION

This study shows that RBM20 mutation carriers have more severe ventricular arrhythmias than TTN mutation carriers, despite equally severe DCM. This suggests that other mechanisms besides missplicing of TTN may contribute to the aggressive clinical course of RBM20-induced DCM. We addressed whether this relates to missplicing of Ca^{2+} handling genes. We investigated this in Rbm20 KO mice, and revealed that Rbm20 KO cardiomyocytes have severely disturbed Ca^{2+} handling, with increased Ca^{2+} transients, increased diastolic Ca^{2+}, increased SR Ca^{2+} and more spontaneous Ca^{2+} releases from the SR. Furthermore, electrophysiological studies revealed that the AP is prolonged in Rbm20 KO cardiomyocytes. Alternative splicing changes in two Rbm20 target genes, i.e. CamkIIδ and Cacna1c, pointed us to the LTCC as the potential culprit for the observed Ca^{2+} overload in Rbm20 KO cardiomyocytes. We recorded $I_{Ca,L}$ and show that LTCC activity is substantially increased in Rbm20 KO cardiomyocytes. Moreover, blockade of $I_{Ca,L}$ with the LTCC-antagonist verapamil completely rescued the noradrenaline-induced spontaneous Ca^{2+} releases from the SR, indicating that increased $I_{Ca,L}$ density enhances Ca^{2+} influx and, in turn, causes intracellular and SR Ca^{2+} overload in Rbm20 KO cells (Figure 5).

Our data indicates that this function of Rbm20 likely underlies the increased risk of arrhythmias in RBM20 mutation carriers, and therefore this study provides new avenues to investigate the treatability of arrhythmias in RBM20 mutation carriers. Furthermore, our findings underscore the necessity for careful clinical monitoring of potential electrical disturbances and cardiac arrhythmias in RBM20 mutation carriers, even during the early stages of the disease.

It has been proposed that regulating TTN splicing, by means of modulating RBM20 levels, could be beneficial for the heart, especially in the setting of heart failure with preserved ejection fraction (HFpEF)[13, 30, 31]. Methawasin et al. have shown that in heterozygous Rbm20 KO mice beneficial effects of more compliant Ttn (i.e. reduction in diastolic chamber stiffness) dominate over disadvantageous effects (i.e. depressed end-systolic elastance)[13]. In a mouse model of HFpEF, these beneficial effects were even more pronounced[30]. However, our current study reveals that although it may be advantageous to modulate Ttn splicing to decrease passive stiffness in certain types of heart disease where passive stiffness is increased, the effect on other Rbm20 targets must be carefully evaluated. The fact that a 50% reduction in Rbm20 levels (i.e. in Rbm20+/- mice) already induces a clear shift in CamkIIδ towards the δ-A and δ-9 isoform, with concomitant Ca^{2+} handling abnormalities, argues for careful examination of using RBM20 modulation as a therapeutic option. The exquisite sensitivity of isoform expression by levels of RBM20 was also observed in the study of Maatz et al. who showed that RBM20 levels closely correlate with the extent of splicing in RBM20 targets such as TTN, CAMK2D, RYR2 and LDB3.[6]

The observed increase in LTCC density and intracellular Ca^{2+} overload in Rbm20 KO hearts can not be explained by an underlying cardiomyopathy since it sharply contrasts to what is generally observed in DCM or heart failure, where LTCC density is either unaltered or reduced, and intracellular Ca^{2+} and SR Ca^{2+} content are decreased[32, 33]. Therefore, it is very unlikely that the Ca^{2+} handling abnormalities in the

Rbm20 KO hearts are secondary to the DCM, but rather directly caused by the loss of Rbm20 and its effects on splicing of Ca^{2+} handling genes. We show that $I_{Ca,L}$ inhibition with verapamil in Rbm20-/- cardiomyocytes decreases the number of pro-arrhythmic Ca^{2+} releases, and this suggests that verapamil may be used as therapy for RBM20 mutation carriers to reduce their arrhythmia burden. Verapamil is a class IV anti-arrhythmic agent, and acts by blocking voltage-dependent Ca^{2+} channels. It is currently used to treat hypertension and migraines, but is contra-indicated in the treatment of general heart failure. This is due to the fact that verapamil has a negative inotropic effect since it lowers intracellular Ca^{2+}, which in most forms of heart failure is already decreased[32, 33]. In addition, the few small clinical trials in heart failure patients that have been carried out, have not shown a beneficial effect of this drug[34]. However, since in heart failure cardiac Ca^{2+} cycling is generally diminished, it is not surprising that verapamil was not found to have a beneficial effect (it could even worsen cardiac function by further decreasing intracellular Ca^{2+} and thereby contractility). Since cardiac Ca^{2+} transients are increased in RBM20 mutation carriers, treatment with verapamil could be beneficial for this subgroup of heart failure patients. Besides verapamil, other ways to therapeutically decrease intracellular Ca^{2+} and SR Ca^{2+} content, might be worthwile to explore in RBM20 mutation carriers as well.

In recent years, the importance of alternative splicing, and how abnormal splicing contributes to heart disease, has become increasingly clear[35, 36]. Several splicing factors, including Rbm20, Rbm24, Rbfox, and SF3B1, were shown to have critical roles in the developing or adult heart[5, 37-39]. Interestingly, the isoform switch in CamkIIδ to the δ-A and δ-9 isoform that we observed in the Rbm20 KO mice is also seen after cardiac-specific deletion of yet another splicing factor, ASF/SF2, leading to similar changes in cardiac Ca^{2+} handling[21]. Furthermore, the same study also provided evidence that transgenic overexpression of CamkIIδ-A alone phenocopies the Ca^{2+} handling defects observed in the ASF/SF2 KO mice, indicating that the shift in CamkIIδ isoforms largely underlies the Ca^{2+} handling defects in the ASF/SF2 KO[21]. Surprisingly,

a complete loss of CamkIIδ in mycocytes also results in an increase in $I_{Ca,L}$, albeit to a lesser extent (15-18% in CamkIIδ KO vs 82% in Rbm20 KO)[40]. However, opposite to what is seen in ASF/SF2 KO (and in Rbm20 KO), CamkIIδ KO cardiomyocytes have decreased intracellular Ca^{2+} and SR Ca^{2+} content. Overall, these studies show that changes in CamkIIδ isoforms are more detrimental than complete loss of CamkIIδ[15, 18, 21, 41, 42]. Particularly the observation that transgenic overexpression of the δ-A isoform of CamkIIδ phenocopies the Ca^{2+} handling defects that we observed in the Rbm20 KO hearts, suggests that the isoform switch in CamKIIδ in Rbm20 KO hearts is largely responsible for the Ca^{2+} defects. We did not observe increased phosphorylation of Thr498 in Cacnb2 (a reported CamKIIδ target[16]) in Rbm20 KO hearts, indicating that phosphorylation of this specific residue is not the reason why the LTCC is more active. Therefore, how increased expression of CamkIIδ-A increases L-type calcium current, be it through increased LTCC phosphorylation[16, 17] or a different mechanism, remains to be investigated.

Naturally, we can not exclude the contribution of Rbm20-dependent splicing events in RyR2, Cacna1c, Ncx1, and Scn5a to the Ca^{2+} phenotype, but the function of these splice isoforms are less well-studied. It is for example known that inclusion of the Rbm20-dependent 24-bp exon in RyR2 targets RyR2 to an intranuclear Golgi-apparatus, instead of the SR[22]. Reduced RyR2 in the SR could also impact cardiac Ca^{2+} handling, but the contribution of this differential splice event in Rbm20 KO cardiomyocytes remains to be determined. The same holds true for the splicing changes in Cacna1c, Ncx1, and Scn5a, as these could also impact Ca^{2+} handling, either directly or indirectly. However, we did not observe hyperpolarization of the LTCC due to increased inclusion of exon 9* in Cacna1c, as was previously reported[24], indicating that this specific splicing event does not affect the LTCC in mouse cardiomyocytes, or that the increase was not sufficient to induce the hyperpolarization (Fig 3G).

Familial DCM is often caused by mutations in sarcomeric genes[4], but in RBM20 mutation carriers the DCM is caused by a dysfunctional splicing factor, which makes the disease phenotype likely much more complex. Dysfunctional RBM20 affects a multitude of genes, such as sarcomeric and Ca^{2+} handling genes, which together are responsible for the disease phenotype in RBM20 mutation carriers. In that sense, it is likely that the disease (and arrhythmogenic) phenotype is only partly explained by the CamkIIδ-LTCC pathway, and that other factors also contribute. The splicing changes in CamkIIδ likely also affect other target proteins, such as PLB or RyR2, and although we did not observe a difference in CamkII-dependent phosphorylation of RyR2 on S2815, we cannot rule out that altered phosphorylation status of these targets might have contributed to the calcium disturbances. Additionally, loss of the nuclear CamKIIδ-B isoform in the Rbm20 KO hearts may lead to hampered nuclear export of histone deacetylases, thereby potentially repressing MEF2-dependent gene expression[20].

Limitations

There are some limitations to this study. First, with regard to the clinical data, it must be noted that we had access to only a limited number of RBM20 and TTN mutation carriers, and therefore these results can merely serve as an indication of the arrhythmia burden of these two groups of DCM patients. It will be important to validate these findings in larger cohorts. In addition, although it is likely that TTN is misspliced in the RBM20 mutation carriers, we do not have acces to their cardiac tissue, and could not validate that TTN is similarly affected in all patients. Therefore, we need to be cautious about the conclusions drawn from this table. Nevertheless, the table shows that dysfunctional TTN does not lead to an arrhythmogenic phenotype, while dysfunctional RBM20 does. In addition, the occurrence of sustained VT/VF in 50% in RBM20 mutation carriers indicates that RBM20 mutations carriers have an arrhythmia burden comparable to LMNA mutation carriers, which are known to have an arrhythmogenic phenotype[4].

Second, there are differences between the human RBM20 mutation carriers and our Rbm20 KO mouse model. The mouse model represents a true loss-of-function (and 50% Rbm20 protein levels in the heterozygotes), while most human RBM20 mutation carriers have a missense mutation in the RNA binding region in only one allele. It is conceivable that these missense mutations do not lead to protein instability or nonsense mediated decay, but rather to disruption of RBM20 binding to RNA through potentially dominant-negative mechanisms. Mouse models in which specific human RBM20 mutations are introduced are awaited with great interest to further study the underlying pathophysiological mechanism. Third, the electrophysiological properties of human and mouse cardiomyocytes are not entirely similar. For example, the differences in potassium currents between human and mouse may explain why we did not observe QTc prolongation in RBM20 mutation carriers, while we did see QTc prolongation in Rbm20-/- mice[43]. However, the basic calcium handling machinery is comparable, and human iPSC-CM derived from an RBM20 mutation carrier show similar calcium handling abnormalities[9].

In conclusion, much of the research regarding RBM20-induced cardiomyopathy has focussed on TTN, but aberrant TTN splicing only partly explains the clinical characteristics of RBM20 mutation carriers. Here, we provide evidence that Rbm20 controls Ca^{2+} handling by regulating the activity of $I_{Ca,L}$. This function of Rbm20 likely underlies the increased risk of arrhythmias in RBM20 mutation carriers, and therefore we provide proof-of-concept of a potential therapy to relieve RBM20 mutation carriers from their arrhythmia burden by treatment with LTCC antagonists.

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Disclosures

None.

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Table 1. Comparison of patients with *RBM20* mutations to patients with *TTN* mutations.

Parameter	RBM20 (n=18)	TTN (n=22)	*P* value
Patient characteristics			
Age (yrs)	42 ± 14	45 ± 16	0.486
Male sex, n (%)	8 (44)	9 (41)	0.923
Proband, n (%)	11 (61)	14 (64)	0.870
FH SCD, n (%)	13 (72)	8 (36)	0.052
Follow-up (months)	71±65	62±34	0.098
Cardiac function			
LVEF (%)	37 ± 17	37 ± 13	0.933
LVEDD (mm)	60 ± 11	59 ± 9	0.828
LVEDV (ml)	269 ± 92	246 ± 73	0.447
LVEDV index (ml/BSA)	132 ± 32	120 ± 7	0.201
ECG parameters			
Heart rate (bpm)	72 ± 20	71 ± 13	0.884
PR (ms)	149 ± 14	165 ± 29	0.056
QRS (ms)	101 ± 20	98 ± 19	0.570
QTc (ms)	414 ± 34	411 ± 40	0.828
Arrhythmias			
Atrial fibrillation, n (%)	6 (33)	8 (36)	0.870
Non-sustained VT, n (%)	5 (28)	7 (32)	0.945
Sustained VT or VF, n (%)	8 (44)	1 (5)	0.006
ICD treatment, n (%)	11 (61)	2 (9)	0.002
Pharmacological therapy			
Beta-blocker, n (%)	14 (78)	14 (64)	0.533
ACE inhibitor or ATII antagonist, n (%)	12 (67)	15 (68)	0.812
MRA, n (%)	8 (44)	8 (36)	0.846
Diuretics, n (%)	7 (39)	6 (27)	0.659
Digoxin, n (%)	1 (6)	3 (14)	0.613
Class III antiarrhythmic drug, n (%)	2 (11)	4 (18)	0.673

ACE, angiotensin converting enzyme; ATII, angiotensin receptor II; FH SCD, family history of sudden cardiac death <45 years; ICD, internal cardioverter defibrillator; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volume; LVEDV index, left ventricular end-diastolic diameter indexed to body surface area (BSA); MRA, mineralocorticoid receptor antagonist; QTc, heart-rate corrected QT interval; VT, ventricular tachycardia; VF, ventricular fibrillation. N indicates number of patients. Data are presented as mean ± standard deviation (SD). To test for statistically significant differences between the groups, two-tailed Students t-test was used for continuous variables and Fisher exact test or χ^2 test for categorical variables.

Figure 1: Characterization of Rbm20 KO mice. A. Gene targeting strategy of Rbm20 KO mice. Mice carrying the targeted allele were crossed with Flp-mice to remove the LacZ and neomycin cassette and subsequently with CMV-Cre mice to genetically delete exon 4 and 5 of Rbm20. B-D. qPCR in hearts of wildtype (n=6), Rbm20 +/- (n=8), and Rbm20 -/- (n=8) mice of respectively Rbm20, ANF, and BNP. E. RT-PCR analysis of Ttn and Ldb3 splicing in the hearts of 3 wildtype, 3 Rbm20 +/-, and 3 Rbm20 -/- mice. Largest PCR product of TTN represents the inclusion of additional PEVK exons, indicative of the N2BA-G isoform. F. Heart weight/body weight ratios of wildtype (n=6), Rbm20 +/- (n=8), and Rbm20 -/- (n=8) mice. G-I. Echocardiographic analysis of wildtype (n=7), Rbm20 +/- (n=9), and Rbm20 -/- (n=8). LVESD, Left Ventricular End-Systolic Diameter. J-K. Surface electrocardiograms of wildtype (n=7), Rbm20 +/- (n=9), and Rbm20 -/- (n=8) mice. QTc, heart-rate corrected QT interval. L. H&E and Picrosirius Red staining of wildtype, Rbm20 +/- , and Rbm20 -/- heart sections. M. Quantification of fibrosis in wildtype (n=6), Rbm20 +/- (n=6), and Rbm20 -/- (n=8) hearts. All analyses were done in 25 weeks old mice. One-way ANOVA with Least Significance Difference (LSD) post-hoc correction was used to test for statistical significance. * means $p < 0.05$ vs wildtype, # means $p < 0.05$ vs Rbm20 +/-.

Figure 2. Rbm20 regulates splicing of Ca^{2+} and ion handling genes. A. Two biological categories were identified in the most enriched process (cardiac muscle contraction) after gene ontology enrichment analysis (DAVID) of differentially spliced genes in Rbm20 KO hearts: sarcomeric genes and ion handling genes. *Cacna1c splicing changes did not pass the adjusted p-value in this n=3 experiment, but has previously been shown to be an Rbm20-target[5]. B. RT-PCR validation of differential splicing events in CamKIIδ, RyR2, Scn5a, Cacna1c in hearts of wildtype, Rbm20 +/-, and Rbm20 -/- hearts. C. Western blot of CamkIIδ in hearts of wildtype, Rbm20 +/-, and Rbm20 -/- mice. D. Immunohistochemistry of CamkIIδ (red) in hearts of wildtype and Rbm20 -/- mice. Cacnb2 (green) was used to stain LTCCs, DAPI (blue) was used to stain nuclei. Scale bar = 20 µm. The analyses were done on hearts of 25 weeks old mice. E. qPCR of Rbm20 mRNA levels in NRCM 48h after GapmeR-mediated knockdown of Rbm20. SCR = Scrambled, KD = Knockdown F. RT-PCR of Ttn, CamkIIδ, and RyR2 splicing after Rbm20 knockdown in NRCM. G. DeltaPSI-plots of CamkIIδ and RyR2 of a DCM-patient (black line) and an RBM20 mutation carrier (red line) as

compared to the mean of 10 healthy control hearts. PSI = Percentage Spliced In. Two-tailed Students t-test was used to test for statistical significance.

Figure 3. Rbm20 KO cardiomyocytes display action potential prolongation and increased L-type calcium current. A. Typical action potentials of wildtype and Rbm20 -/- cardiomyocytes. B. Action potential duration at 20%, 50%, and 90% of repolarization of wildtype and Rbm20 -/- cardiomyocytes. C. Action potential amplitude (APA) and resting membrane potential (RMP) of wildtype and Rbm20 -/- cardiomyocytes. D. Average Vmax of wildtype and Rbm20 -/- cardiomyocytes. E. Left panel, representative recordings of current traces following depolarizing pulses from -60 to 0 mV. Right panel, normalized currents. F. Average current-voltage relationships of $I_{Ca,L}$. G. Activation and inactivation relationships. Solid lines are Boltzmann fits. Inset, Voltage clamp protocol. Cells were isolated from 8-12 weeks old mice. Unpaired t-test or Two-Way Repeated Measures ANOVA followed by pairwise comparison using the Student-Newman-Keuls test were used to test for statistical significance.

Figure 4. Rbm20 KO cardiomyocytes display intracellular Ca^{2+} overload and are prone to spontaneous Ca^{2+} releases. A. Representative calcium transients of wildtype, Rbm20 +/-, and Rbm20 -/- cardiomyocytes. B. Average of diastolic calcium in wildtype (n=23), Rbm20 +/- (n=21), and Rbm20 -/- (n=18) cardiomyocytes at 6 Hz. C. Average peak transient in wildtype (n=23), Rbm20 +/- (n=21), and Rbm20 -/- (n=18) cardiomyocytes at 6 Hz. D. Average SR calcium load in wildtype (n=20), Rbm20 +/- (n=16), and Rbm20 -/- (n=18) cardiomyocytes. E. Average number of spontaneous Ca^{2+} releases during 10 seconds after stimulation at 6 Hz was stopped, with and without 50 nM Noradrenaline in wildtype, Rbm20 +/-, and Rbm20 -/- cardiomyocytes, and in Rbm20 -/- cardiomyocytes treated with 1 µM verapamil. Cells were isolated from 4-5 different wildtype, Rbm20 +/-, and Rbm20 -/- mice of 8-12 weeks of age. One-way ANOVA with LSD posthoc correction (B-D) and Mann-Whitney U test (unpaired samples) or Wilcoxon rank test (paired samples) (E) were used to test for statistical significance. * means $p < 0.05$ vs wildtype or vs Rbm20 -/- (in case of verapamil treatment).

Figure 5. Proposed model of how Rbm20 deficiency leads to pro-arrhythmic spontaneous Ca^{2+} releases.

Rbm20 deficiency increases inclusion of exon 15 and 16 in CamkIIδ, resulting in the expression of the CamkIIδ-A isoform. CamkIIδ-A is preferentially localized at the t-tubules[21], where the LTCC is also localized. An increased $I_{Ca,L}$ subsequently enhances intracellular [Ca^{2+}] and SR Ca^{2+} content. The increase in SR Ca^{2+} content drives spontaneous Ca^{2+} releases from the SR. Inhibition of $I_{Ca,L}$ with the LTCC-antagonist verapamil decreases the number of spontaneous Ca^{2+} releases. LTCC, L-type calcium channel. p-LTCC, phosphorylated L-type calcium channel. RyR2, Ryanadine Receptor 2. SR, sarcoplasmic reticulum. N, nucleus. $I_{Ca,L}$, L-type calcium current.