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Cardiac circRNAs arise mainly from constitutive exons rather than alternatively spliced exons

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Abstract

Circular RNAs (circRNAs) are a relatively new class of RNA molecules, and knowledge about their biogenesis and function is still in its infancy. It was recently shown that alternative splicing underlies the formation of circular RNAs (circRNA) arising from the Titin (*TTN*) gene. Since the main mechanism by which circRNAs are formed is still unclear, we hypothesized that alternative splicing, and in particular exon skipping, is a major driver of circRNA production. We performed RNA sequencing on human and mouse hearts, mapped alternative splicing events and overlaid these with expressed circRNAs at exon-level resolution. In addition, we performed RNA sequencing on hearts of Rbm20 KO mice to address how important Rbm20-mediated alternative splicing is in the production of cardiac circRNAs. In human and mouse hearts, we show that cardiac circRNAs are mostly (~90%) produced from constitutive exons and less (~10%) from alternatively spliced exons. In Rbm20 KO hearts, we identified 38 differentially expressed circRNAs of which 12 were produced from the *Ttn* gene. Even though *Ttn* appeared the most prominent target of Rbm20 for circularization, we also detected Rbm20-dependent circRNAs arising from other genes including *Fan1*, *Stk39*, *Xdh*, *Bcl2l13* and *Sorbs1*. Interestingly, only *Ttn* circRNAs seemed to arise from Rbm20-mediated skipped exons. In conclusion, cardiac circRNAs are mostly derived from constitutive exons, suggesting that these circRNAs are generated at the expense of their linear counterpart and that circRNA production impacts the accumulation of the linear mRNA.

Introduction

Circular RNAs (circRNAs) have only recently been added to the family of regulatory RNAs, despite their discovery over 20 years ago (Nigro et al. 1991). This species of RNA molecules was largely ignored due to their unusual splicing behaviour in which exons are joined at consensus splice sites, but in a scrambled order relative to the primary transcript, and due to their low expression levels compared to mRNAs. Decades later, with the advent of next generation sequencing, thousands of endogenous circRNAs were found to be expressed in various tissues and cells types (Salzman et al. 2012; Memczak et al. 2013; Jeck and Sharpless 2014), including the heart (Khan et al. 2016; Tan et al. 2017; Jakobi et al. 2016; Werfel et al. 2016). Recent studies have revealed that circRNAs can regulate gene expression by different mechanisms; specifically, by serving as miRNA sponges (Memczak et al. 2013; Zheng et al. 2016; Hansen et al. 2013), facilitating transcription of their host gene by directly associating with RNA polymerase II (Zhang et al. 2013) or forming platforms for protein interactions (Du et al. 2016). Emerging evidence also suggests that some circRNAs can act as templates for protein synthesis (Pamudurti et al. 2017; Legnini et al. 2017; Yang et al. 2017).

The biogenesis of circRNAs is linked to pre-mRNA splicing, a process carried out by the spliceosome (Starke et al. 2015; Ashwal-Fluss et al. 2014). Pre-mRNA splicing represents the process of removal of introns and joining of exons in a linear fashion to produce a mature mRNA. CircRNAs, however, are formed by a back-splicing event, in which a donor site of an exon is not connected with an acceptor site of a downstream

exon as observed in linear splicing, but rather with an acceptor site of an upstream exon (or with the acceptor site of the same exon in case of a single-exon circRNA). This gives rise to a single-stranded RNA loop with a unique exon-exon junction not present in the linear transcript. Mechanistically, back-splicing requires that the donor and acceptor site of the back-spliced exons are brought in close proximity to each other, which can be accomplished by direct RNA base-pairing of reverse complementary sequences in the introns flanking the back-spliced exons, or by the interaction of RNA binding proteins that dock on these flanking introns (Ashwal-Fluss et al. 2014; Conn et al. 2015; Zhang et al. 2014).

Ashwal-Fluss et al. (2014) demonstrated that there is strong competition between linear splicing and circRNA biogenesis. By introducing strong 5' and 3' splice sites in the exons that flank the circRNAs they observed a dramatic reduction in circularization efficiency in minigene experiments in HEK293 cells. Moreover, flies carrying a mutation in RNA polymerase II, which has been shown to increase co-transcriptional splicing efficiency, produced significantly lower numbers of circRNAs. Both interventions decreased circRNA abundance in a context where linear splicing was more efficient, indicating that inclusion of exons in the linear transcript competes with back-splicing events of the same exons, thereby inhibiting circRNAs production. Thus circRNA production seems to have a negative effect on linear splicing and therefore on gene expression (Ashwal-Fluss et al. 2014). Furthermore, Liang et al. (2017) recently showed that the steady-state output of protein-coding genes shifts to circular RNAs when canonical pre-mRNA splicing events are inhibited.

How RNA circularization is mechanistically connected to *alternative* splicing remains largely unknown, but splicing factors and RNA binding proteins (RBPs), such as Muscleblind, Quaking (Ashwal-Fluss et al. 2014; Conn et al. 2015), RNA-binding domain containing immune factors NF90/NF110, hnRNPs and SR proteins (Kramer et al. 2015; Li et al. 2017) have been shown to regulate back-splicing and circRNA biogenesis. RNA Binding Motif protein 20 (RBM20) is yet another splicing factor that has recently been implicated in circRNA production, specifically from the *TTN* gene (Khan et al. 2016). RBM20 is highly expressed in the heart and regulates alternative splicing of a large number of genes, including titin (*TTN*); a structural component of sarcomeres (Guo et al. 2012; Labeit and Kolmerer 1995). Mutations in the gene encoding RBM20 have been implicated in a clinically aggressive form of dilated cardiomyopathy (DCM), characterised by progressive dilation and dysfunction of the left ventricle (Brauch et al. 2009; Beqqali et al. 2016). The missplicing of *TTN* is considered an important reason why RBM20 mutation carriers develop DCM. We recently discovered that a remarkably large number of circRNAs are produced from the *TTN* gene and that RBM20 is required for the production of a subset of these *TTN* circRNAs (Khan et al. 2016).

In the current study, we hypothesised that alternative splicing, and in particular exon skipping, drives the formation of cardiac circRNAs, and that Rbm20-mediated alternative splicing drives the formation of circRNAs that arise from Rbm20 target genes. To investigate to what extent alternative splicing underlies circRNA formation in the heart, we performed RNA sequencing on human and mouse hearts, and on the hearts of Rbm20 knockout (KO) mice. We integrated *in silico* analyses of alternative

splicing and circRNA production in human heart and revealed that ~90% of cardiac circRNAs are produced from constitutive exons and ~10% from alternatively spliced exons. Strikingly, of the circRNAs that were formed from alternatively spliced exons, a large fraction (~26%) stemmed from the *TTN* gene. In addition, we show that the strong correlation observed between exon skipping and circular RNA formation in the *Ttn* gene does not appear as a common mechanism for other Rbm20 target genes, suggesting that circRNA formation is not a general function of Rbm20. In conclusion, we show that alternative splicing drives the formation of only a small subset of cardiac circRNAs, as most cardiac circRNAs arise from exons that are constitutively spliced.

Results

Identification of circRNAs in mouse hearts

To detect circRNAs in the mouse heart, ribosomal-depleted RNA obtained from left ventricles of three wild-type and three Rbm20 KO mice was used for whole transcriptome sequencing (Fig. 1A). The MapSplice tool was used to identify back-splice junctions in the RNA-seq data (Wang et al. 2010; Khan et al. 2016). We detected a total of 1283 unique circRNAs in these 6 mouse samples (Table 1; Supplemental Table S1). However, the expression of the circRNAs was rather variable between hearts, as there were only 156 circRNAs commonly detected in all three wild-type hearts (Table 1). Most circRNAs were predicted to comprise between 2 and 6 exons within the back-spliced exons, but we also detected circRNAs potentially harbouring as many as 30 to 100 exons within the back-spliced exons, albeit less frequently (Supplemental Fig. S1A). We confirmed the previous observation (Salzman et al. 2012)

that particularly exon 2 of the host gene is overrepresented in circRNAs (Supplemental Fig. S1B). Furthermore, half of the host genes were predicted to produce a single circRNA, while the other half generated between 2 to 9 different circRNAs, with the exception of Ttn, which generated a total of 38 putative circRNAs (Supplemental Fig. S1C). These Ttn specific circRNAs were mostly formed from the I-band region of the gene; a region known to undergo complex alternative splicing. This is in line with our previous report showing robust circRNA production from titin's I-band region in human hearts (Khan et al. 2016) (Fig. 1B).

Only a small subset of circRNAs expressed in the heart are evolutionarily conserved

We have previously identified a total of 7130 unique circRNAs in human hearts, of which 3478 were found in hearts of healthy individuals. In the current study, using the exact same methodology, we detected a total of 1283 circRNAs in the mouse hearts. Overall, we found that ~9% and ~3% of the genes expressed in the human and mouse hearts produced circRNAs, respectively. To investigate to what extent circRNAs in human and mouse hearts are evolutionarily conserved, we converted mouse circRNA back-splice genomic coordinates to human coordinates and compared the genomic positions of circRNAs expressed in human and mouse hearts. We found that only 5% of human circRNAs were conserved in the mouse (Fig. 1C; Supplemental Table S2). Interestingly, when filtering human circRNAs for high expression levels (≥ 70 reads), the overlap with mouse circRNAs increases from 5% to 19% (Fig. 1C). This indicates that the highly expressed human circRNAs show a higher degree of conservation. Vice

versa, up to 50% of the mouse circRNAs were also identified in human hearts (Supplemental Table S3).

Mouse cardiac circRNAs are flanked by Reverse Complementary Sequences (RCS)

There is a consensus that the presence of RCS in introns flanking back-spliced exons is associated with the formation of circRNAs (Dubin et al. 1995; Zhang et al. 2014; Ivanov et al. 2015). To investigate the presence of these RCS, we retrieved 1000 bp of intronic sequence flanking each of the 1283 mouse circRNAs and investigated complementarity (Supplemental Fig. S2A). We selected the best local alignment found between each circRNA-flanking intron pair and compared it with randomly selected introns to assess the statistical significance (see methods). All the introns flanking the detected circRNAs had RCS ranging from 9 to 332 bp, but only 802 were statistically significant ($FDR \leq 0.05$, at least 67% identity) (Supplemental Table S4). Analysis of the identified RCS showed that ~13% of them share sequence similarity with inverted B1 elements (mouse equivalent of Alu-repeats in human) (Fig. 1D), ~10% with other elements like short interspersed sequence elements (SINEs) B2-B4, long interspersed sequence elements (LINEs) and simple repeats, and ~77% with none of the known elements (Supplemental Fig. S2B). The identification of the above repeats is in line with other groups who revealed that intronic repeats are necessary and sufficient for circularisation (Starke et al. 2015; Liang and Wilusz 2014; Zhang et al. 2014; Ivanov et al. 2015). The sequences of our identified RCS are shown in Supplemental Table S4. Experimental studies are

needed to provide evidence whether base-pairing events of the identified sequences are necessary and/or sufficient for circularization of these mouse circRNAs.

38 circRNAs are differentially expressed in hearts of RBM20 knockout mice

To identify Rbm20-dependent circRNAs expressed in the heart, we performed differential expression analysis by comparing circRNAs in Rbm20 KO with wild-type mice. We identified 38 out of 1283 circRNAs to be differentially expressed, of which 26 were down-regulated and 12 were up-regulated in Rbm20 KO mice compared to wild-type mice (Fig. 2A; Supplemental Table S1). Notably, our RNA-seq data showed that a subset of 19 circRNAs was completely absent in Rbm20 KO mouse hearts, while they were readily expressed in wild-type hearts. Eleven of those circRNAs were generated from the *Ttn* gene and the remaining from the host genes *Unc13b*, *Stk39*, *Arhgap10*, *Tfdp2*, *Sorbs1*, *Fan1*, *Insr* and *Xdh* (Table 2). A subset of 7 up-regulated circRNAs was uniquely expressed in the hearts of Rbm20 KO mice, and they were generated from the host genes *Sorbs1*, *D19Ert386e*, *Ehmt1*, *Gcom1*, *Bcl2l13*, *Smad1* and *Ttn* (Table 2).

To validate these differentially expressed circRNAs experimentally, we selected 6 circRNA candidates, based on p-value and expression level, that were down-regulated (circTtn_2, circTtn_3, circTtn_4, circFan1, circStk39 and circXdh) and 4 circRNA candidates that were up-regulated (circTtn_7, circSorbs1, circBcl2l13, circEhmt1) in Rbm20 KO hearts (Fig. 2B; Table 2). We designed divergent primers flanking the circRNA-specific back-spliced junctions and performed RT-PCR on RNA isolated from hearts of 6 wild-type, 6 heterozygous and 6 Rbm20 KO mice. Sanger sequencing confirmed the presence of back-splice junctions in the amplicons, and RT-PCR on

poly(A)-negative and -positive RNA, and on RNase R-treated RNA and mock samples revealed that we detected bona fide circRNAs (Supplemental Fig. S3A; Supplemental Table S5). In line with our previous report (Khan et al. 2016), we confirmed the loss of the three Ttn circRNAs in the Rbm20 KO hearts, but also the downregulation of circRNAs produced from *Fan1* and *Stk39*. Interestingly, the circRNA derived from the *Fan1* gene seemed to depend critically on Rbm20 expression, as we found that circFan1 was substantially reduced in the heterozygotes and completely absent in the Rbm20 KO hearts (Fig. 2B). Of the circRNAs that were up-regulated in the Rbm20 KO hearts, we could experimentally validate circTtn_7, circSorbs1 and circBcl2l13. Based on RT-PCR, expression of circXdh and circEhmt1 did not seem to be affected in the absence of Rbm20 in this set of 18 mouse hearts. Finally, we performed quantitative RT-PCR for circFan1, circStk39, circXdh, circEhmt1, circTtn3 and circTtn7 and further confirmed the differential expression of these circRNAs, except for circEhmt1 (Supplemental Fig. S3B; Supplemental Table S5). Overall, 9 out of 10 differentially expressed circRNAs could be experimentally validated.

Furthermore, expression analysis of the host genes of the 38 differentially expressed circRNAs revealed that there were only 3 genes differently expressed (when considering an absolute fold-change ≥ 1.5), indicating that the differential expression of the circRNAs was not due to dysregulation of the corresponding host genes (Supplemental Table S6).

Rbm20 binding site enrichment analysis showed that introns flanking the 38 differentially expressed circRNAs are 2-fold enriched for Rbm20 binding sites compared

to a random set of introns; with the most significant enrichment occurring within a flanking distance of 100bp ($p \leq 0.001$) (Supplemental Fig. S4). Interestingly, even after removing *Ttn* circRNAs from the analysis, a significant enrichment remained. For instance, we identified a total of 4 potential Rbm20 binding sites in the introns flanking circFan1 (Supplemental Fig. S5A). In conclusion, the RNA-seq in wild-type and Rbm20 KO hearts in combination with the Rbm20 binding site prediction revealed that the *Ttn* gene seems to be the most prominent target of Rbm20-regulated circRNA production. Nevertheless, of the 38 differentially expressed circRNAs, 26 originated from other genes, suggesting that circRNAs arising from *Ttn* gene are not the only ones that are regulated by Rbm20.

Rbm20-dependent alternative splicing and circRNA production

We have previously shown that Rbm20-dependent exon skipping within *Ttn*'s I-band region is associated with circRNA formation (Khan et al. 2016). To assess whether Rbm20-controlled alternative splicing and circRNA formation are connected beyond the *Ttn* gene, we calculated the Percentage Spliced In (PSI) (Schafer et al. 2015; Kakaradov et al. 2012) of the back-spliced exons of all 38 differentially expressed circRNAs. The PSI indicates the efficiency of splicing of a specific exon into the transcript population of a gene. It ranges from 0 to 1, indicating an exon is never (0) to always (1) spliced into the expressed transcripts of a gene. In Figure 3A and B, we plotted the PSI of back-spliced exons of the 38 differentially expressed circRNAs against the expression of the corresponding circRNAs. Figure 3A shows that particularly the circRNAs from the *Ttn* gene arise from exons that are spliced out of the linear

transcripts. The back-spliced exons of almost all other differentially expressed circRNAs, except those arising from *Arhgap10* and *2310046A06Rik* host genes, have a PSI close to 1, indicating that these exons are always included in the linear transcripts. In Figure 3B, where the PSI is plotted against the expression of the circRNAs in the *Rbm20* KO hearts, it can be appreciated that PSI levels of the *Ttn* back-spliced exons increase to 1, indicating that these exons are now included in the linear *Ttn* transcripts. Alternative splicing changes for *Ttn* and *Arhgap10* in wild-type, heterozygous and *Rbm20* KO hearts were confirmed by semi-quantitative RT-PCR (Supplemental Figure S6; Supplemental Table S5). To further quantify the difference in exon skipping between *Rbm20* KO and wild-type mice, we calculated the delta-PSI of the back-spliced exons and plotted these against the expression changes of the circRNAs (Fig. 3C, Supplemental Table S7). Again, this shows that the exons used for circularization are mostly constitutive exons since the exons that are differentially back-spliced upon *Rbm20* loss are not differentially spliced in the linear transcript. Also, in this approach, the most notable exceptions are *Ttn* circRNAs.

In addition to these PSI calculations, we used an alternative strategy to assess whether circRNA formation and alternative splicing are concurrently regulated. We used MapSplice to retrieve the linear junction reads (lj reads) stemming from the back-spliced exons in the *Rbm20* KO and wild-type mice. We then plotted the fold changes of the linear junction reads against the fold changes of back-spliced junction reads (bsj reads) in *Rbm20* KO compared to wild-type mice (Supplemental Fig. S7). This also shows that reduced numbers of *Ttn* circRNAs in *Rbm20* KO hearts associates with increased linear

junction read counts, indicative of a negative correlation between circRNA production and linear exon usage.

Besides regulating *Ttn* splicing, Rbm20 is known to regulate linear splicing of many other cardiac genes, including *Camk2d*, *Ldb3* and *Myh7* (Maatz et al. 2014). We next performed a dedicated search for circRNAs that may have arisen by exon skipping events in these other Rbm20 target genes. Genome-wide differential exon usage analysis (using the R Bioconductor package DEXSeq) in our Rbm20 mouse model confirmed that loss of Rbm20 leads to missplicing of several genes, including *Ttn*, *Myh7*, *Obscn*, *Slc8a1*, *Sorbs2*, *Camk2d*, *Ldb3* and *Rtn4* (Supplemental Table S8). We retrieved the exons that are alternatively spliced by Rbm20 and overlapped these with the back-spliced exons to assess whether these exons generated circRNAs. Interestingly, the exons that were found to be differentially spliced in the Rbm20 KO mice do not produce detectable circRNAs, except the ones arising from the *Ttn* gene (Supplemental Table S9). As described in the previous section we identified circFan1 as a circRNA that also critically depends on Rbm20 expression. To investigate whether Rbm20-dependent alternative splicing may underlie circFan1 production we generated a splicing plot for the *Fan1* gene showing exon usage in Rbm20 KO and wild-type mice. As shown in Supplemental Figure S5B, several exons in the *Fan1* mRNA are alternatively spliced but not the two exons that circularise (Supplemental Table S10). Therefore, it remains unclear at this point how Rbm20 regulates the production of circFan1. In conclusion, Rbm20-mediated exon skipping results in circRNA production from the *Ttn* gene, but not from other bona fide splicing targets of Rbm20.

Genome-wide analysis of alternative splicing and circRNA production in the human heart

With the availability of RNA datasets of the mouse and human heart, we have the opportunity to investigate the connection between alternative splicing and circRNAs formation, in an unbiased and genome-wide manner. Therefore, we calculated the PSI of the back-spliced exons of all circRNAs passing the filtering step (read count ≥ 2 in both samples) in 2 control human hearts from our previous study (Khan et al. 2016). Figure 4A shows the PSI of back-spliced exons of the 1211 circRNAs detected in human hearts, plotted against the expression level of the corresponding circRNAs. We considered exons to be constitutive if they were included in the linear mRNA in at least 90% of the transcripts (PSI >0.90) (Schafer et al. 2017). The results show that only ~10% of the circRNAs had back-spliced exons with a PSI ≤ 0.90 and thus may have been derived from exon skipping events (Supplemental Table S11). Of note, when we use a more stringent filtering step (read count ≥ 8 in both samples) for circRNA expression, the percentage of alternative back-spliced exons and constitutive back-spliced exons does not change, suggesting that our PSI-findings are not biased by lowly expressed circRNAs or false-positive circRNA predictions (Supplemental Table S12).

We also calculated the ratio of constitutive vs. alternative exons in the complete human and mouse transcriptome, and found that, after correcting for the ratio of constitutive vs alternative exons in the transcriptome, a 3.3-2.1-fold enrichment of constitutive exons in the group of back-spliced exons was still present (Supplemental Table S13). This

confirms our observation that alternative splicing is not a major driver of circRNA production.

Thus, these findings indicate that most circRNAs are not derived from alternatively spliced exons but from constitutive exons. Of the 10% of the circRNAs that we found to associate with alternative splicing, a large proportion (26%) were derived from the *TTN* gene (Fig. 4A, blue dots). Interestingly, we also found several other genes where exon skipping was associated with circRNA production. To visualise the relation between exon skipping and circRNA production in greater detail, we plotted the PSI of four of these genes, *PALM2*, *YAF2*, *CUX1*, and *ZEB1* and marked the exons used for circularization in orange (Fig. 4B-E). In the mouse hearts around 13% of the circRNAs were associated with exon skipping events, when considering a PSI of 0.90 as a cutoff (Supplemental Fig. S8; Supplemental Table S14). In conclusion, many of the cardiac circRNAs that coincide with alternative splicing are derived from the *Ttn* gene. However, particularly in the human heart, we found several examples of non-*TTN* circRNAs that were produced from regions that undergo extensive alternative splicing.

Based on these results we propose a model in which there are two mechanisms for circRNA production: competition-based and exon skipping-based circRNA production (Fig. 4F). In the competition-based mechanism, which is the most frequently observed mechanism (~90% of the circRNAs), a pre-mRNA is used to produce a circRNA, but not a mature linear mRNA. In this case, circRNA biogenesis may be an important regulator in the control of gene expression and depending on the gene in question; there might be a favoured production of linear mRNAs or circular RNAs. In the exon skipping-based

mechanism, which is less frequently observed (~10%), pre-mRNA undergoes alternative splicing and results in the formation of both a linear exon-skipped mRNA and a circular RNA.

Discussion

It is known that the splicing machinery can circularise exons and that linear splicing is directly involved in circRNA production (Zaphiropoulos 1996). However, to what extent alternative splicing, and in particular exon skipping, drives circRNA formation is currently unknown. Here, we analysed RNA-seq data from mouse and human hearts on alternative splicing and circRNA production and show in humans that ~90% of the circRNAs are derived from exons that are typically not alternatively spliced (i.e. constitutive exons). This suggests that these circRNAs are mostly generated at the expense of linear mRNA isoforms, and that circRNA production competes with the accumulation of linear mRNA. Conversely, ~10% of the human circRNAs may have been produced from exon skipping events, as these circRNAs are derived from exons that are subjected to alternative splicing (Figure 4F). Interestingly, a large proportion (~26%) of the circRNAs that are associated with exon skipping events in the human heart is derived from the TTN gene.

Our finding that circRNAs can be derived from exon-skipping events is in line with other studies (Salzman et al. 2012; Jeck et al. 2013; Barrett et al. 2015; Kelly et al. 2015), although the described frequency of these events differs. Jeck et al. (2013) determined exon-skipped linear transcripts from human fibroblasts and found that 45% of the expressed circRNAs are derived from linear splicing reads with characteristics of an

exon-skipping event. Salzman et al. (2012) provided circumstantial evidence that in a variety of normal and malignant human cells, 2% of the circRNAs are derived from exons predicted to undergo alternative splicing. In the current study, we used an integrated approach to evaluate alternative splicing and circRNA production by calculating PSI, back-spliced junction reads, and linear junction reads and provide evidence that at most 10% of the circRNAs are derived from exon skipping events. This difference in frequencies between the studies may be explained by differences in examined tissue, or the difference in methods / cut-offs to detect exon skipping. Unfortunately, we were unable to examine the underlying differences since it is unclear what methods and cut-offs were exactly used for the detection of alternative splicing in the other two studies. We were rather strict in setting the cut-off for the detection of alternative splicing ($PSI \leq 0.90$); with a more lenient cut-off for exon skipping ($PSI \leq 0.98$), we find that up to 27% of the circRNAs can be derived from exon skipping. In contrast to the studies mentioned above and our current study, Kelly et al. (2015) presented an extraordinarily high correlation between exon skipping and circRNA production. However, two fundamental problems seem to underlie their analyses. Firstly, they express the abundance of back-spliced exons within circRNAs as expression ratios (back-spliced junction reads in circRNAs / back-spliced exon aligning reads in linear mRNA). The expression of the back-spliced exons in the linear mRNA were subsequently plotted against these ratios and used to calculate correlations. However, since a common factor (i.e reads aligning to the back-spliced exons in linear mRNA) is present in both correlation variables, this will automatically, but falsely result in a high correlation. Secondly, to assess exon skipping, Kelly et al. only measured the

abundance of exonic reads in linear RNA. However, simply measuring reduced exonic reads does not provide evidence for exon skipping. To be included as a possible product of exon skipping, they should have selected only those linear reads that completely traverse the back-spliced exons. In our study, we calculated the PSI, in which the efficiency of splicing of a specific exon within a transcript population of a gene is calculated based on exon-skipped junctional reads, which to our opinion is a more appropriate approach for this purpose.

A limitation of the current study is that we can not rule out that post-transcriptional degradation processes, such as nonsense-mediated decay (NMD), clear away mRNAs that have skipped exons that yield circRNAs (and hence the alternatively spliced mRNAs would not be detected in RNA-seq). Furthermore, Conn et al. (2017) showed that low-abundance splice variants may be missed in RNA-seq experiments, at least in plants. Both events may have led to an underestimation of the proportion of exon skipping-based circRNAs. In regard to the first point, it would be interesting to block NMD, for example pharmacologically with drugs like emetine, and then examine the expression of linear mRNA transcripts arising from the circRNA host genes to ascertain the proportion of exon skipping-based circRNAs that lead to the degradation of their linear counterpart. A related point is that current circRNA profiling studies typically examine steady-state levels of circRNAs and mRNAs and do not provide information on nascent RNA. It has recently been shown that back-splicing is less favorable than canonical splicing, and that the expression of circRNAs for a large part depends on accumulation of synthesized circRNAs rather than increased synthesis (Zhang et al. 2016), and in that sense, the effect of circRNA biogenesis on linear mRNA expression

can be only minor. Therefore, experiments in which newly transcribed RNAs are labeled metabolically (for instance using 4-thiouridine (Fuchs et al. 2015)) to study the kinetics of circRNA biogenesis and nascent RNA splicing in cardiac cells are awaited with great interest.

We recently discovered a causal link between exon skipping regulated by the splice factor RBM20 and circular RNA formation in the human *TTN* gene (Khan et al. 2016). These results led us to hypothesise that this phenomenon may not be restricted to a single gene but that RBM20 may also regulate the formation of circRNAs arising from other RBM20 target genes. Therefore, we designed this study to investigate whether Rbm20 is required for the production of circRNAs beyond those of the *Ttn* gene. We identified 38 differentially expressed circRNAs in Rbm20 KO hearts, and the *Ttn* gene appeared as the most prominent target of Rbm20 for circRNA production, as we found that 11 *Ttn* circRNAs were completely absent in Rbm20 KO hearts. We next investigated whether the set of 38 circRNAs that were regulated in the Rbm20 KO hearts could have been formed by exon skipping events. We used two different strategies (PSI and linear junction read count of back-spliced exons) to interrogate alternative splicing of linear transcripts, and both methods similarly showed that predominantly the circRNAs from the *Ttn* gene are associated with exon skipping events. This is a remarkable finding since Rbm20 is known to act as a splicing repressor of many cardiac genes, including *Camk2d*, *Ldb3*, *Sorbs1* and *Myh7* (Maatz et al. 2014). Dedicated searches for circRNAs in the mapped reads of these well-described Rbm20 target genes did not yield any evidence of back-spliced junctions in the exons that are targeted by Rbm20 for splicing. Also, the *Sorbs1* and *Ryr2* circRNAs

that we found to be differentially expressed in the Rbm20 KO hearts were derived from different exons than the ones that are normally repressed for splicing by Rbm20. A differential exon usage plot of the *Fan1* gene in wild-type and Rbm20 KO hearts revealed that Rbm20 regulates alternative splicing (i.e. exon inclusion) in the region of the back-spliced exons, but not in the back-spliced exons specifically. The presence of 4 Rbm20 binding sites in the introns flanking the back-spliced exons of circFan1 further suggest that Rbm20 underlies circRNA formation from this gene directly, however, the precise mechanism remains elusive. Overall, the tight correlation between exon skipping and circular RNA formation observed in *Ttn* gene does not appear a common mechanism for Rbm20 target genes indicating that circRNA formation is not a general function of Rbm20.

We performed a comparative analysis and showed that the normal human heart expresses 4 times more circRNAs than the mouse heart (3478 circRNAs in humans and 848 in mice). This is in line with a study by Tan et al. (2017), where 5 times more circRNAs were detected in the human heart compared to the mouse heart. Werfel et al. (2016) only found 1.6 times more circRNAs in human hearts compared to the mouse hearts; which is probably caused by the relatively high number of circRNAs they detected in the mouse hearts. We further interrogated evolutionary conservation of the circRNAs by converting the mouse genomic coordinates of the back-spliced junctions to the human genome. We show that only 184 (i.e. 5%) of the human circRNAs are also expressed in mouse hearts. When discarding the lowly expressed human circRNAs, the overlap with mouse circRNAs increased from 5% to 19%. This level of evolutionary conservation is in agreement with the study of Werfel et al. (2016), who showed that

approximately 13% of the human cardiac circRNAs are conserved to mouse and rats. Vice versa, due to the lower numbers of circRNAs expressed in the mouse heart, a much higher percentage (between 20-50%) of the mouse circRNAs are conserved to human. This suggests that during evolution, an elusive mechanism has led to an astounding increase in the number of circRNAs in the human transcriptome. The observation that the expression of circRNAs is highly variable between hearts from the same species suggests that occasional circularization of exons is easy to evolve and may provide a mechanism for rapid evolution of stably expressed circRNAs. It has been shown that inverted *Alu* repeats in the introns flanking back-spliced exons are associated with the formation of circRNAs (Jeck et al. 2013). *Alu* repeats are primate-specific SINE retrotransposons and thus not present in the mouse genome (Kriegs et al. 2007). In the mouse genome, the B1 element is the most abundant class of repetitive elements. They belong to the SINE family and, like *Alu* repeats in human, originated from an initial duplication of the 7SL RNA and amplified and duplicated independently in the two genomes while accumulating specific mutations (Quentin 1994). The discrepancy in the numbers of identified circRNAs in human and mouse hearts may relate to the fact that regulatory sequences for circularization (for instance *Alu* and B1 elements) are not well conserved (Tsirigos and Rigoutsos 2009). Analysis of the introns flanking the back-spliced exons of the mouse circRNAs revealed besides the B1 elements, also reverse complementary sequences that do not share any sequence similarity with transposable elements. Further investigation is required to evaluate whether the identified sequences are necessary for circularization.

In conclusion, this study revealed that the splice factor Rbm20 is not a global regulator of circRNA formation in the heart. The tight correlation between exon skipping and circular RNA formation that we observed for the *Ttn* gene does not appear a common mechanism for other Rbm20 target genes. In addition, we have shown that cardiac circRNAs are mostly (~90%) produced from constitutive exons, indicating that these circRNAs are generated at the expense of their linear counterpart. Future studies investigating how circRNA production from these constitutive exons affect gene expression and protein production of the host gene will be awaited with great interest.

Materials and Methods

Rbm20 knockout mice

Generation of the Rbm20 KO mice has been described previously (Khan et al. 2016). In line with other Rbm20 KO models (Guo et al. 2012; Methawasin et al. 2014), homozygous Rbm20 knockout mice revealed abnormal *Ttn* splicing and a DCM phenotype, which is manifested by LV dilatation and impaired cardiac function. Total RNA was extracted from LV tissue samples of 6 months old mice (3 wild-type and 3 Rbm20-KO) with TRI Reagent (Sigma-Aldrich) according to manufacturer's protocol.

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.

Library preparation and whole transcriptome RNA sequencing

RNA quality was assessed with the Agilent 2100 Bioanalyzer. All samples had a RIN score of ~8. Total RNA samples (500 ng) were treated with biotin-streptavidin-based bead systems (Exiqon) to minimise ribosomal contamination. Ribosomal-depleted RNA libraries were sequenced on an Illumina NextSeq 500 platform in paired-end mode and with a read length of 101bp at Exiqon A/S (Vedbaek, Denmark). Sequencing depth was approximate 70 million raw reads per sample (Supplemental Table S15). Base-calling was performed using the bcl2fastq 2.0 Conversion Software from Illumina.

Quality Control, processing and mapping of RNA-Seq data

Quality control of fastq files was performed using FASTQC (<http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/>). Trimmomatic version 0.351 (Bolger et al. 2014) was used to remove Illumina adapters and low quality bases, using a Phred score cut-off of 30 whilst discarding reads with a length below 25 bases. The paired-end RNA-seq reads passing the quality controls from the 3 Rbm20 KO mice and wild type mice were then aligned against the mouse genome, Gencode annotation release M1 (NCBI37), using TopHat2 (Kim et al. 2013) version 2.0.14 with default values.

Conservation analysis between human and mouse circRNAs

To determine homologous exons groups, we used UCSC liftOver (Karolchik et al. 2004) to convert mouse splice coordinates (mm9) to human coordinates (hg19). For the analysis, we used the 848 unique circRNAs detected in wild-type mice (1 read counts in at least 1 mouse) and the 3487 unique circRNAs detected in human controls (1 read counts in at least 1 control) from our previous work (Khan et al. 2016). For a more

stringent analysis, we filtered the human and mouse circRNAs for the expression level using the cut-off of at least 2, 10, 40 and 70 normalised read counts in at least 2 samples.

Reverse complementary sequences (RCS) analysis in the flanking introns of the predicted circRNAs

For each circRNA we aligned the downstream and reversed complement of the upstream intron, using the `pairwiseAlignment` function in the R package `Biostrings` and retrieved the score of the best local alignment. To calculate a p-value associated with each match, we compared the actual score to the distribution of scores obtained by aligning the sequence of the best local alignment (without gap) with 1000 random introns. The p-value was then adjusted for multiple testing using the FDR methods. The following alignment parameters were used: match = 2; mismatch = -3, gap opening = -5, gap extension = -2. To calculate the percent sequence identity for a pairwise sequence alignment we used the following formula: $100 * (\text{identical positions}) / (\text{aligned positions} + \text{internal gap positions})$. We repeated the procedure for each pair intron flanking circRNA. As the last step, we blasted the identified RCS with Repeat Masker Database using *ABblast* algorithm (Smit, AFA, Hubley, R & Green, P.).

Identification of back-spliced and linear junction reads – MapSplice

Back-spliced and linear junction reads were identified using MapSplice version 2.2.0 (Wang et al. 2010). For circRNAs detection we set the following options `--min-fusion-distance 200` (as suggested by the authors), `--filtering 1` and `--min-map-len 25`. The

reported linear and back-spliced junction coverage for each sample were then formatted into matrices where the value in the i -th row and the j -th column of each matrix represented the total number of reads successfully mapped to the back-splice or linear junctions of the host gene i in sample j . The raw counts were then normalised using the R Bioconductor package DESeq2 (Love et al. 2014). GenomicRanges function and custom R scripts were used to overlay linear splicing events with back-splicing events.

Differential circular RNA expression analysis – DESeq2

Differential expression analysis of circRNAs identified by MapSplice in the previous step was performed using the R Bioconductor package, DESeq2 (Love et al. 2014). To investigate circRNAs expression in Rbm20 KO and wild-type mice, 2 sets of differential expression analyses were conducted. The first analysis was performed using the total number of circRNAs detected in the hearts of wild-type mice and Rbm20 KO mice. To increase sensitivity, the second analysis was performed considering only circRNAs detected in at least two wild-type or two Rbm20 KO mice with normalised reads counts ≥ 2 . In the end, only those circRNAs with an absolute \log_2 fold change cut-off ≥ 1 together with a p-value cut-off ≤ 0.05 were deemed significantly differentially expressed. Owing to the limited number of samples ($n=3$ per group), the adjusted p-value was not used as a criterion to select candidates and the final set comprised of 38 circRNAs, which were selected for downstream analyses.

Differential gene expression analysis – DESeq2

Differential gene expression analysis was performed using the R Bioconductor package, DESeq2 (Love et al. 2014). GenomicRanges infrastructure was used to count the number of aligned reads overlapping with each gene. The expression of the host genes of the 38 differentially expressed circRNAs was retrieved. Only those circRNA host genes with an absolute log₂ fold change cut-off ≥ 1 together with a adjusted p-value cut-off ≤ 0.05 were deemed significantly differentially expressed.

Rbm20 binding site enrichment analysis

Genomic sequences of introns flanking the differentially expressed circRNAs were obtained from the UCSC genome table browser. Gregexpr2 function in the R package Biostrings and custom R scripts were then used to determine for each candidate circRNA, the genomic positions of the Rbm20 motif (UCUU) occurring within introns flanking 100bp upstream and downstream of the back-splicing acceptor and donor sites respectively. Rbm20 motifs occurring in flanking introns of the circRNAs were then tallied. An equal number of randomly selected introns was utilised as a control set. The number of Rbm20 motifs occurring within introns flanking 100bp upstream and downstream of these 'control' introns pairs were tallied.

Differential exon usage analysis- DEXSeq

To test for exon usage differences between Rbm20 KO and wild-type mice we used the R Bioconductor package DEXSeq version 1.20.0 (Anders et al. 2012), using GenomicRanges infrastructure to count the number of aligned reads overlapping with each exonic region. Only those exons with an absolute log₂ fold change cut-off ≥ 1

together with a adjusted p-value cut-off ≤ 0.05 were deemed significantly differentially expressed.

Percentage Spliced In of the back-spliced exons – PSI

The ‘percentage spliced in’ index (PSI) was computed by executing the script PSI.sh of Schafer et al. (Schafer et al. 2015) on exonic parts derived from the Gencode annotation release M1 (NCBIM37) for mouse and Gencode annotation release 19 (GRCh37.p13) for human. GenomicRanges function and custom R scripts were used to overlay the coordinates of the back-spliced exons with the coordinates of the exon bins identified during the PSI calculation. In mice we found the corresponding PSI of back-spliced exons for 1148 on a total of 1283 detected circRNAs. In human of the 3487 detected circRNAs we found the the corresponding PSI of back-spliced exons for 3401 of them. Back-spliced exons of circRNAs detected in at least two samples, wild-type or Rbm20 KO mice, and two human controls with normalised reads counts ≥ 2 or ≥ 8 (high-confidence expressed circRNAs (Zhang et al., Cell, 2014) with RPM ≥ 0.1 correspond to 8 reads in our sample with the lowest number of mapped reads) were used in the analysis. If an exon’s boundary is not the same in all transcripts, the exon is cut into two or more parts, referred to as exon bins. We considered exons to be constitutive if they were included in the linear mRNA in at least 90% of the transcripts (PSI 0.9) (Schafer et al. 2017).

Data visualisation and statistical analysis

All results generated from differential circRNA analysis were visualised using the R Bioconductor package, ggplot2. The graphical representation of the *TTN* gene and circRNAs were constructed using the R package, GenomicRanges and custom R scripts, respectively. Differential exon usage and differential expression analyses of circRNAs and circRNA host genes across conditions, were statistically analysed using the negative binomial test in the R Bioconductor package, DESeq2. All correlation tests were performed in R, using the Spearman's correlation as the method of choice. Differences in the enrichment of Rbm20 sites between circRNAs and control sequences were compared using the Fisher-exact test. To be able to analyse circRNAs that were not expressed in the RBM20 KO mice or wild-type mice, a count of 1 was added to avoid problems with infinite values when log transforming. The p-value for delta PSI difference was calculated using the Student's t-test. Scatter plots were used to plot the mean values of the samples. qRT-PCR data were analyzed using LinRegPCR quantitative PCR data analysis software (Ruijter et al. 2009) and normalized with Hprt. Values are expressed as mean \pm standard error. Differences between groups were compared using the Student's t-test.

Preparation of poly(A)-positive and poly(A)-negative RNA fractions

Poly(A)-positive and poly(A)-negative RNA fractions were generated as described previously (Khan et al. 2016). Briefly, 6 μ g total RNA from three wild-type mouse hearts and 200 μ l oligo d(T)25 Magnetic Beads (NEB) were used to generate poly(A) enriched and depleted fractions. The beads were washed first and subsequently incubated with the RNA in a 100 μ l reaction for 10 minutes with gentle agitation. Afterwards, the poly(A)

negative fraction was collected as supernatant by pulling the beads to the side of the tube. After three times washing, beads were incubated in 100 μ l elution buffer at 50°C for 2 minutes to recover the poly(A) positive RNA fraction. The poly(A) negative RNA fraction was also treated at 50°C for 2 minutes.

RNase R treatment

RNase R treatment was performed as described previously (Khan et al. 2016). Briefly, 1 μ g total RNA from three wild-type mouse hearts was treated with or without 5 units of RNase R (Epicentre) at 37°C for 10 minutes in a 20 μ l reaction followed by heat inactivation at 95°C for 3 minutes.

RT-PCR and qRT-PCR

RT-PCR and qRT-PCR were performed as described previously (Khan et al. 2016). Briefly, 1 μ g of poly(A) enriched/depleted or RNase R treated/non-treated RNA was DNA depleted with DNase I (Invitrogen) and subsequently used to generate cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamers (Invitrogen). 2 μ l of 5-times diluted cDNA was amplified for 30-35 cycles with HOT FIREpol DNA polymerase together with divergent primers for circRNA detection or normal convergent primers for mRNA detection. Primers are designed to amplify the smallest band depicted on the gels. Sanger sequencing confirmed the presence of the expected back-splice junctions in these bands. For qRT-PCR, 2 μ l of 5-times diluted cDNA was amplified for 45 cycles with SYBR Green I Master (Roche) on a LightCycler480 system II (Roche). Primer sequences are included in Supplemental Table S5.

Data deposition

Raw RNA sequencing data are available at NCBI BioProject accession number PRJNA417769.

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Tables

Table 1. Summary of the circRNAs detected in mouse hearts.

	wt1	wt2	wt3	ko1	ko2	ko3
Unique circRNAs identified in each sample	451	461	421	550	180	525
Commonly identified circRNAs per genotype	156			105		
Unique circRNAs identified in all samples	1283					

Number of circRNAs identified in each sample, per genotype (at least 1 read in all 3 samples) and in all samples (at least 1 read in at least 1 sample).

Table 2. Normalized read counts of differentially expressed circRNAs in hearts of Rbm20 knock-out mice.

circRNA	gene	Downregulated						Log2 FC *	p-value	circRNA id (mm9)
		wt1	wt2	wt3	ko1	ko2	ko3			
circTtn_4	Ttn	906	1379	1649	0	0	0	-11,3	1,3E-27	chr2:76695961-76719896
circTtn_3	Ttn	137	122	105	0	0	0	-8,3	1,1E-14	chr2:76695961-76722254
	Ttn	25	38	15	0	0	0	-6,1	4,0E-07	chr2:76685445-76719896
	Ttn	18	15	28	0	0	0	-5,7	4,8E-06	chr2:76688627-76719896
circTtn_2	Ttn	15	23	11	0	0	0	-5,3	5,6E-05	chr2:76640288-76656873
	Ttn	11	13	18	0	0	0	-5,0	2,6E-04	chr2:76700407-76719896
	Ttn	16	15	9	0	0	0	-4,9	3,4E-04	chr2:76688627-76722254
	Tfdp2	15	12	10	0	0	0	-4,8	7,6E-04	chr9:96147507-96102550
circStk39	Stk39	5	19	8	0	0	0	-4,5	2,5E-03	chr2:68196871-68248198
	Ttn	9	9	13	0	0	0	-4,5	2,4E-03	chr2:76695961-76731234
circFan1	Fan1	10	11	2	0	0	0	-3,8	1,6E-02	chr7:71506826-71516545
circXdh	Xdh	6	7	8	0	0	0	-3,8	1,8E-02	chr17:74255350-74257085
	Ttn	0	7	17	0	0	0	-3,7	2,7E-02	chr2:76700857-76719896
	Ttn	0	14	9	0	0	0	-3,6	2,8E-02	chr2:76699154-76719896
	Unc13b	0	8	15	0	0	0	-3,6	2,9E-02	chr4:43128032-43100775
	Arhgap10	10	3	8	0	0	0	-3,6	2,7E-02	chr8:79868539-79875571
	Ttn	0	18	4	0	0	0	-3,4	4,1E-02	chr2:76640288-76655920
	Insr	16	5	0	0	0	0	-3,4	4,7E-02	chr8:3158696-3174888
	Sorbs1	5	6	6	0	0	0	-3,3	4,3E-02	chr19:40411476-40439656

circRNA	gene	Upregulated						Log2 FC	pvalue	circRNA id
		wt1	wt2	wt3	ko1	ko2	ko3			
circEhmt1	Ehmt1	0	0	0	10	11	3	4,2	1,00E-02	chr2:24669047-24680587
	Smad1	0	0	0	9	13	0	3,8	2,00E-02	chr8:81878732-81879240
circSorbs1	Sorbs1	0	0	0	14	9	0	3,8	2,00E-02	chr19:40437692-40439656
circTtn_7	Ttn	0	0	0	9	7	3	3,7	2,00E-02	chr2:76700169-76700484
	D19Ert386e	0	0	0	10	0	11	3,8	2,00E-02	chr19:42658033-42637042
	Gcom1	0	0	0	14	0	4	3,4	5,00E-02	chr9:71362876-71403544
circBcl2l13	Bcl2l13	0	0	0	6	7	3	3,4	5,00E-02	chr6:120826364-120820784

*A finite estimate was added to the zero count to allow fold change calculation.

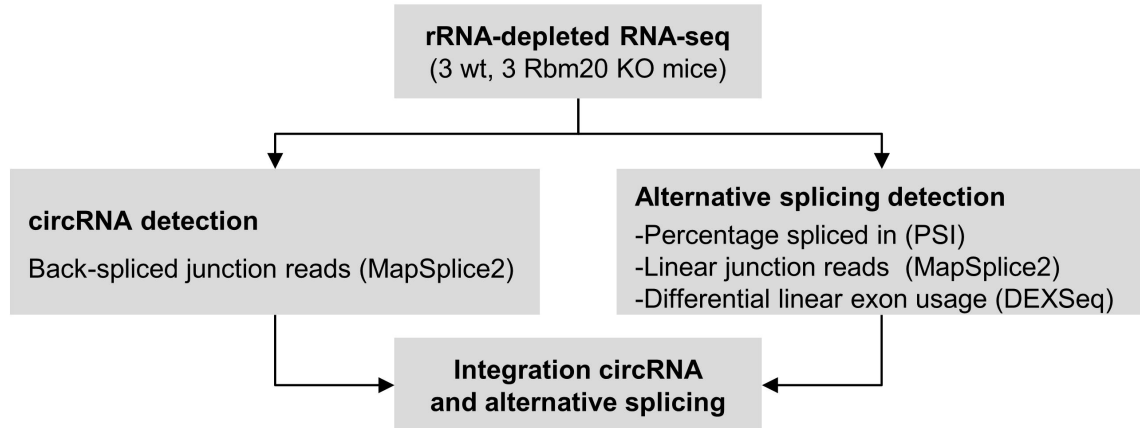
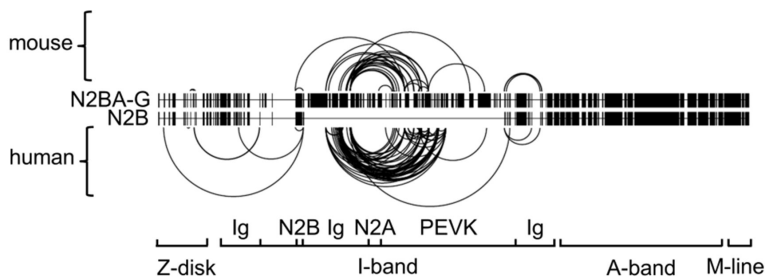
Figure legends

Figure 1: Characterization of circRNAs in mouse hearts. (A) Strategy used to elucidate the relation between the circRNAs formation and alternative splicing. (B) Schematic representation of two splice isoforms of the *Ttn* gene (N2BA-G and N2B) and the location of the 38 identified circRNAs in the mouse hearts and the previously identified TTN circRNAs in human hearts (Khan et al. 2016) (C) Table showing conservation of cardiac circRNAs derived from mouse and human after filtering the human circRNAs for the expression level. Between brackets the number of circRNAs passing the filter are indicated. (D) Scatter plot showing the relation between the percentage of complementarity and the length of the identified reverse complementary sequences (RCS) passing the p-value cut-off ($FDR \leq 0.05$). RCS sharing high sequence similarity with the mouse B1 elements are depicted in black.

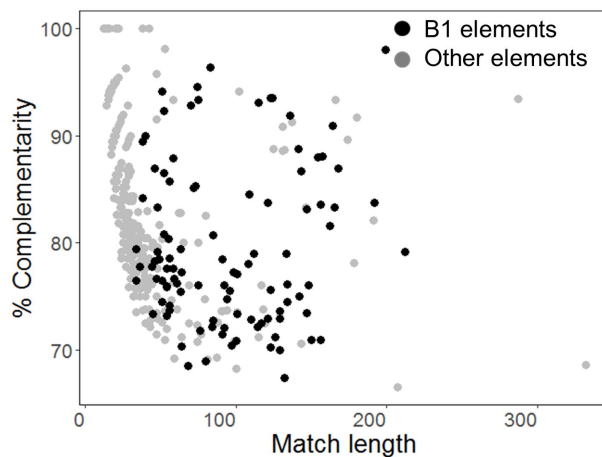
Figure 2: A subset of circRNAs are differentially expressed in hearts of Rbm20 knock-out mice. (A) Volcano plot showing differentially expressed circRNAs in Rbm20 KO hearts compared to wild-type hearts. Differentially expressed circRNAs ($1 \geq \text{Log}_2$ fold change ≥ 1 and p-value ≤ 0.05) are marked in grey. (B) RT-PCR for circRNAs on wild-type (WT), heterozygous (HET) and Rbm20 knockout (KO) mouse hearts. GAPDH was used as input control.

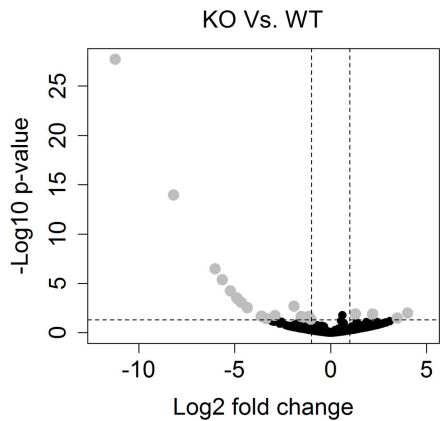
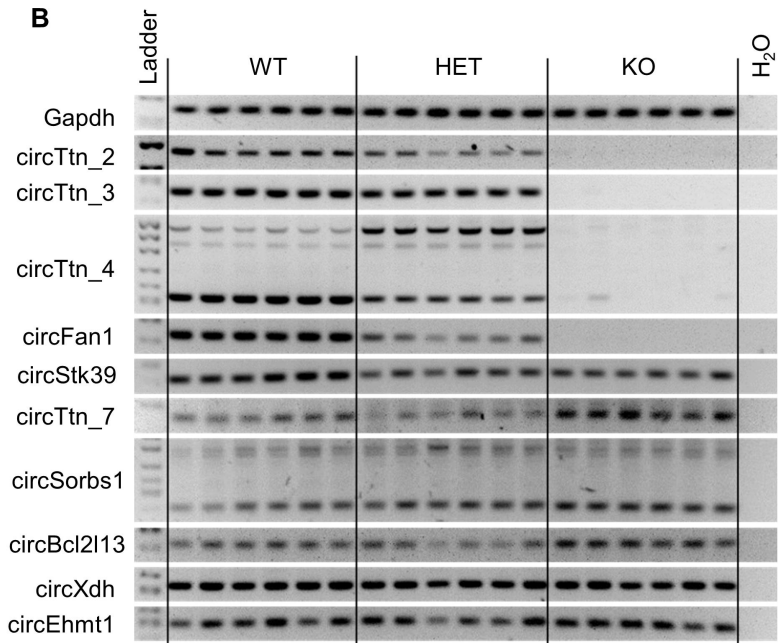
Figure 3: Rbm20-dependent alternative splicing and circRNA production. Scatterplots showing the relation between the PSI of the back-spliced exons and the expression of the circRNAs (Log_2 bsj reads) in (A) wild-type mice and (B) Rbm20 KO mice. Only the 38 differentially expressed circRNAs are depicted. The names of the circRNA host genes are plotted. (C) Scatterplot showing the relation between the dPSI of the back-spliced exons of the 38 differentially expressed circRNAs (dPSI back-spliced exons) and the expression fold changes (Log_2 bsj reads FC) of the corresponding circRNAs between Rbm20 KO hearts and WT hearts.

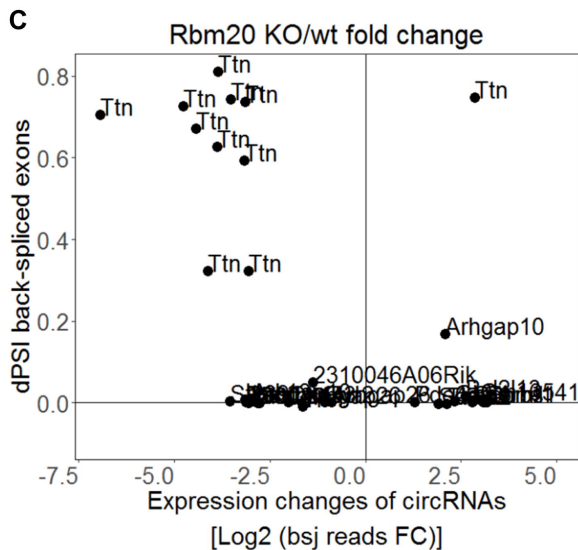
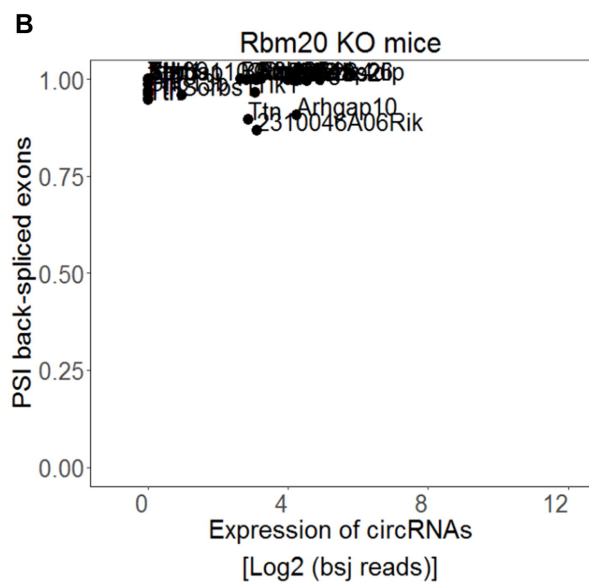
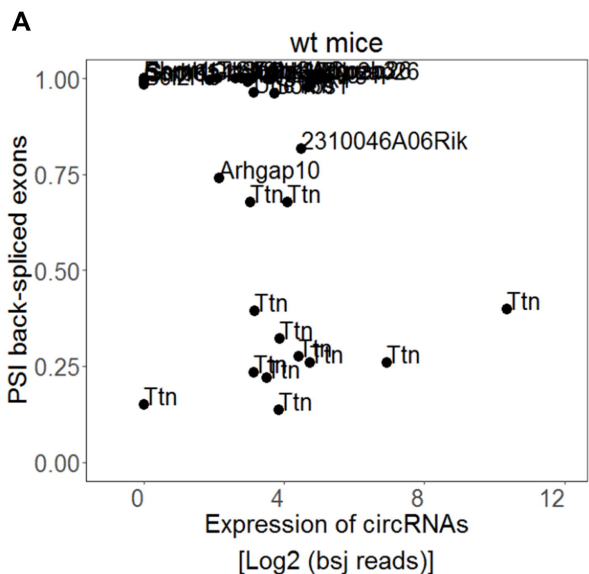
Figure 4: CircRNAs in the human heart are derived from exons that are typically not alternatively spliced. (A) Scatterplot showing the relation between the PSI of the back-spliced exons with the expression of the corresponding circRNAs (Log_2 bsj reads) in the human hearts. Grey dots represent back-spliced exons with $PSI > 0.90$. Blue dots represent back-spliced exons from TTN gene. Black dots represent back-spliced exons with $PSI \leq 0.90$. (B-E) The PSI of the 4 host genes (PALM2, YAF2, CUX1 and ZEB1, indicated in orange in Figure A) are plotted, in which exon skipping is connected with circRNA production. Black dots represent all exon bins of the corresponding genes and orange dots represent the exon bins that give rise to the circRNAs (e.g. back-spliced exons). (F) Proposed model showing two mechanisms for circRNA production: competition-based and exon skipping-based circRNA production.

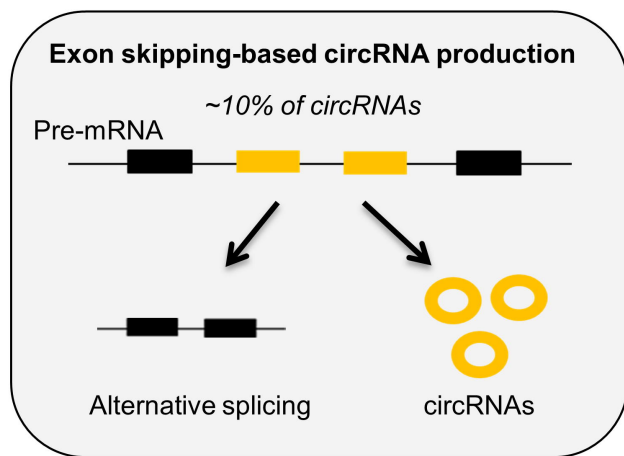
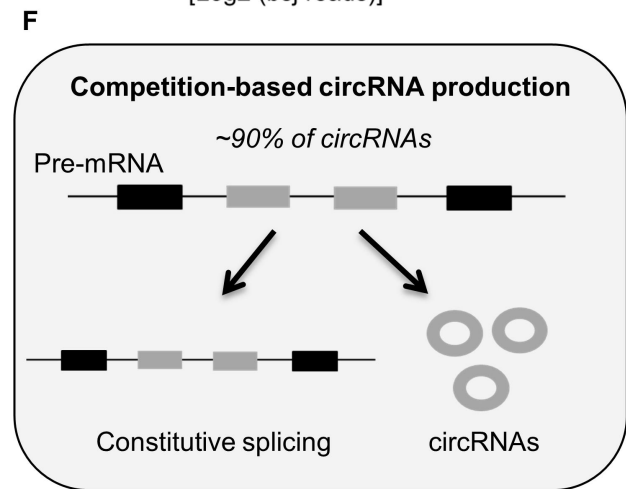
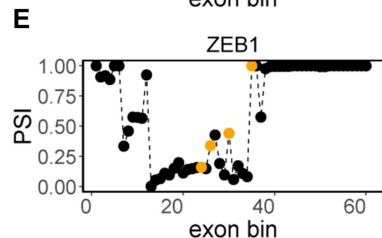
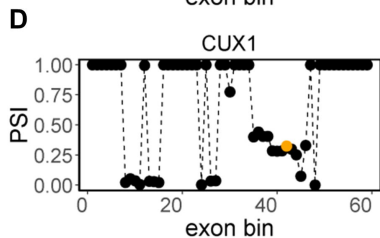
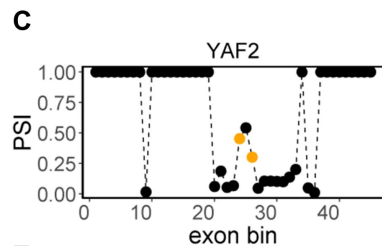
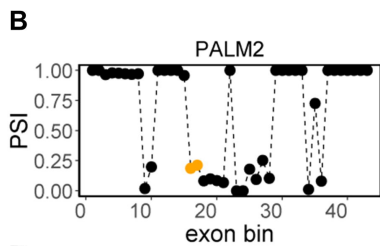
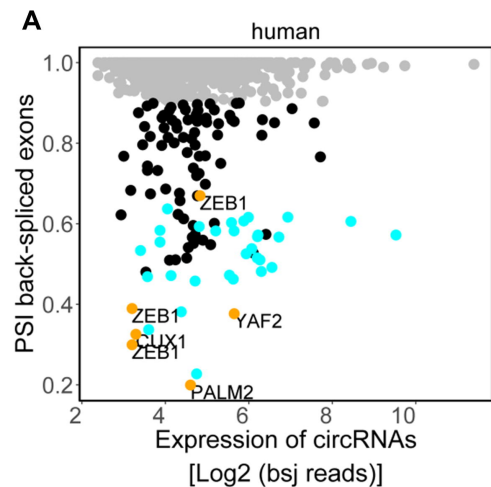
A**B****C**

Selection of human circRNAs (# read counts)	% Conservation (human Vs. mouse)
All (3487)	5%
≥2 (1289)	8%
≥10 (919)	10%
≥40 (203)	17%
≥70 (79)	19%

D

A**B**







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Simona Auffero, Maarten M. G. van den Hoogenhof, Yolan J. Reckman, et al.

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