













Taken together, the activity of RBM20 depends on the RRM and RS domains, necessary but not sufficient for exon exclusion. The addition of the C-terminus including the ZnF2 is sufficient for the repressor activity of RBM20.

### RBM20-RRM binds to sequences within the minimal repression segment

The RNA binding motif of RBM20 contains a UCUU core (15). In a systematic approach we used gel-shift assays on serial truncations of the reporter construct to identify the minimal repression segment. The *in vitro* transcribed and uniformly radiolabeled RNA was exposed to increasing amounts of purified recombinant protein containing the RBM20-RRM domain (RBM20 $\Delta$ 2, residues 511–601; Supplementary Figure S3A) followed by separation on non-denaturing acrylamide gels. The 994 nt RBM20-responsive RNA segment was divided into the upstream intron transcript (A), the exon (B) and serial downstream intron transcripts (Figure 4A). RBM20 $\Delta$ 2 bound with highest affinity to transcripts C and D. RBM20 $\Delta$ 2 did not bind any other segments— independent of the number of consensus sites. A minor shift of transcript B when the highest RBM20 $\Delta$ 2 concentration was applied (Figure 4B) was reevaluated using shorter B-derived transcripts, but none of them bound RBM20 $\Delta$ 2 (Supplementary Figure S4B). C and D transcripts are situated immediately downstream the regulated exon and contain the most of the predicted RBM20 binding motifs (Figure 4A). Thus, RBM20 binds predominantly to an ~300 nt RNA segment downstream of the regulated exon 242, which contains eight potential RBM20 binding motifs. The UCUU motif is sufficient for RBM20 $\Delta$ 2 binding specificity (Supplementary Figure S4C). To support our identification of the relevant motives, we introduced point mutations in TTN intron 242–243 eliminating the four UCUU motifs proximal to the 5'SS as potential RBM20 binding sites (Supplementary Figure S4D). Elimination of the first UCUU motif (mut1) had the strongest effect on RBM20-dependent alternative exon exclusion (Supplementary Figure S4E). However, this mutation was not sufficient to eliminate RBM20-mediated exon exclusion (Supplementary Figure S4E). The remaining mutations (mut2, mut3

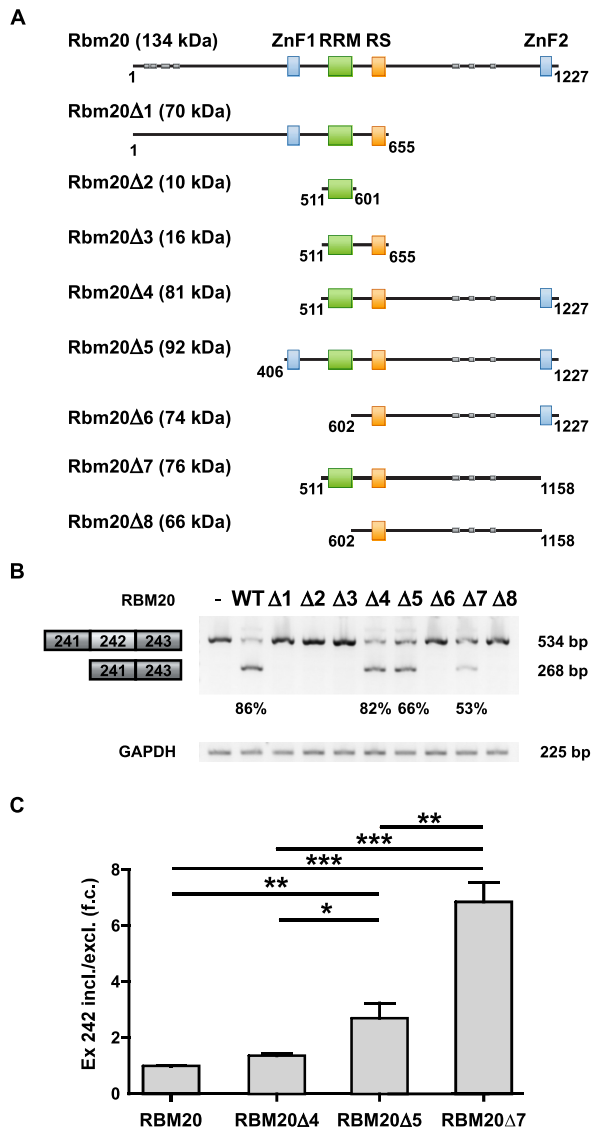
and mut4) had minor or negligible effects. RT-PCR results were confirmed by qRT-PCR (Supplementary Figure S4F).

### RBM20 prevents exon 242 inclusion by interfering with the removal of the upstream and downstream introns

So far, we demonstrated that exonic and flanking intronic sequences are necessary and addition of RBM20 is sufficient to cause exon 242 skipping. Based on thesis findings, we hypothesized that RBM20 simultaneously represses splicing of both individual flanking introns to exclude the alternative exon.

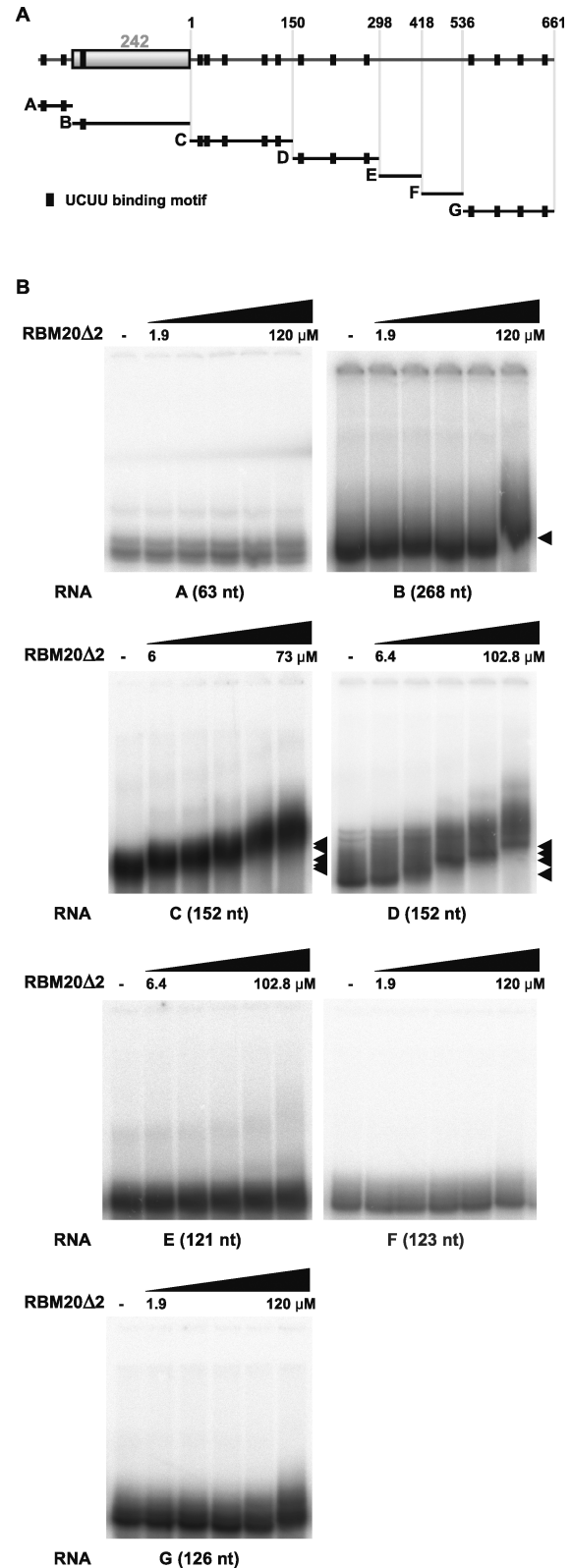
Building on established protocols (20), we used transient transfection of the TTN<sup>241-3</sup> splicing reporter into the HEK293 cells followed by qRT-PCR to quantify splicing intermediates, where only one of two exon–exon junctions are generated (Figure 5A). Addition of RBM20 prevented splicing of both introns (Figure 5B and C). After addition of RBM20 the splicing intermediate lacking the down- or upstream intron were decreased 2- or 8-fold, respectively (Figure 5B and C). In contrast, addition of PTB4, which binds the same consensus motif as RBM20, differentially affected the splicing of the introns flanking titin exon 242: PTB4 increased the splicing intermediate lacking the downstream intron ~6-fold (Figure 5D) with a minor decrease of the intermediate lacking the upstream intron that did not reach significance (Figure 5E). As the size of RBM20 and the minimal splicing reporter precluded their use in an *in vitro* splicing reaction (compare supplementary methods and Supplementary Figure S5A), we analyzed the accumulation of mRNA products, resulting from the cotransfection of TTN<sup>241-3</sup> splicing reporter with RBM20, PTB4 or control into the HEK293 cells (Figure 5F and G). The mRNA was enriched, reverse transcribed and used for primer extension analysis (Figure 5F). The size of the resulting product was consistent with exclusion of the alternative exon 242 after addition of RBM20, but not after addition of PTB4 (Figure 5G). RBM20 dramatically reduced accumulation of the primer extension product over the downstream intron and slightly reduced upstream intron primer extension product accumulation, while PTB4 had no or the opposite effect. The accumulation of splicing intermediates and products, resulting from the cotransfection of TTN<sup>241-3</sup> splicing re-

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 exon was determined for each mutant. Inclusion of intron segment 118–598 restored >20% of RBM20 response (X4). The additional inclusion of 598 to 663 restored >50% (X5) (quantification of three independent transfections). (C) Human titin/ $\beta$ -globin hybrid splicing reporters with TTN (gray) and  $\beta$ -globin sequences (blue) to evaluate the contribution of exon 242 and the upstream intron sequence to RBM20-mediated splicing repression. The black bar indicates the titin region not required for RBM20 responsiveness. (D) RT-PCR validation of RBM20 effects on alternative splicing of the human BG-TTN hybrid splicing reporters X7–X8. Constructs that contained the titin downstream intron or only the alternative exon with flanking exon definition sites did respond to RBM20 (quantification of three independent transfections). (E) Human titin/ $\beta$ -globin hybrid splicing reporters generated to evaluate the intron sequences proximal to the 5' splice site for splice repression activity. The 153 nt mid intron segment (illustrated in thin black line) was positioned at indicated distances from the 5' ss. (F) RT-PCR validation of RBM20-mediated changes in alternative splicing of the human titin/ $\beta$ -globin hybrid splicing reporters X9–X11. Adding the majority of the 3' intron to the alternative exon with immediate flanking exon definition sites transfers RBM20 responsiveness to the  $\beta$ -globin splice reporter (quantification of three independent transfections). (G) Human titin/ $\beta$ -globin hybrid splicing reporters generated to evaluate the contribution of exon 242 and the upstream intron sequence to RBM20-mediated splicing repression. (H) RT-PCR validation of RBM20 effects on splicing of the human titin/ $\beta$ -globin hybrid splicing reporters X12–X15. Only the combination of complete exonic sequence, flanking region and 750 bp in the downstream intron restore RBM20 responsiveness (included/excluded >84%, quantification of three independent transfections). (I) Human titin/ $\beta$ -globin hybrid splicing reporters to evaluate the length of exon 242 required for RBM20-dependent splicing repression. The residual length of original exon sequence is indicated above the exon box. (J) RT-PCR validation of RBM20-mediated changes in alternative splicing of the human titin/ $\beta$ -globin hybrid splicing reporters X16–X19. None of the shortened constructs responded efficiently to RBM20 (quantification of three independent transfections). (K) Human titin/ $\beta$ -globin/*lacZ* hybrid splicing reporters generated to evaluate the sequence of exon 242 required in RBM20 mediated splicing repression. (L) RT-PCR validation of RBM20-mediated changes in alternative splicing of the human titin/ $\beta$ -globin/*lacZ* hybrid splicing reporters indicate that exonic sequence and size contribute to RBM20 responsiveness (quantification of three independent transfections).

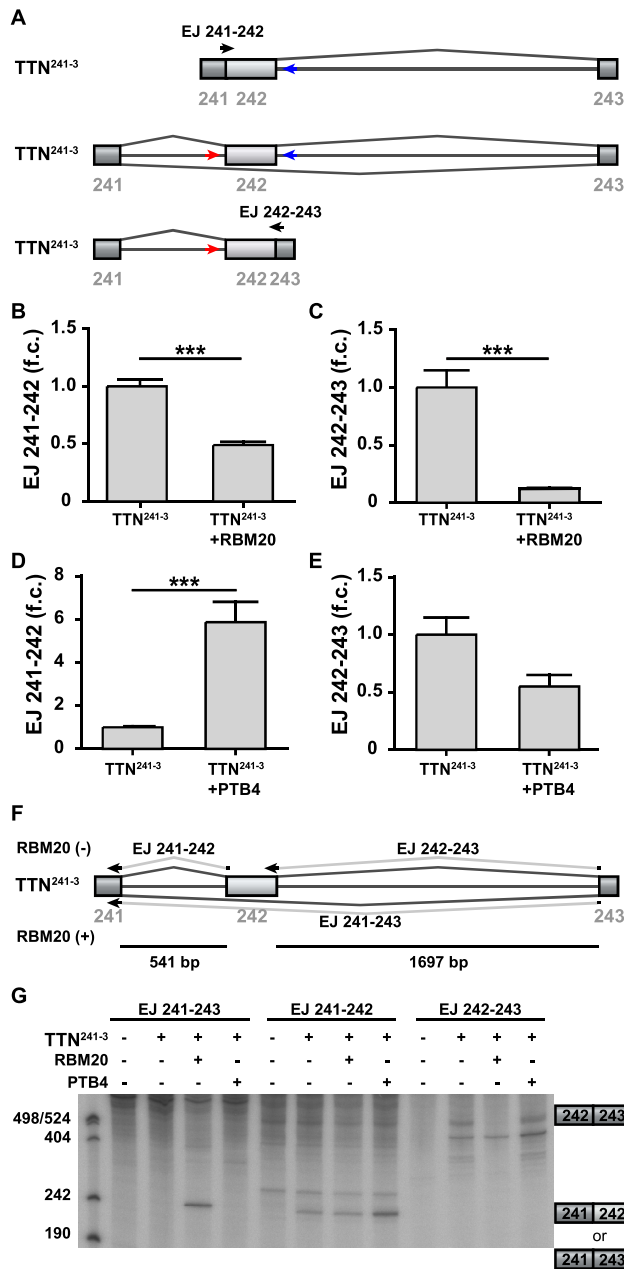


**Figure 3.** The RBM20 RRM and C-terminus are sufficient for titin splicing repression. (A) Schematic representation of RBM20 and the deletion mutants. The two zinc finger (ZnF) domains are depicted in blue, the RRM green, the RS-like domain in orange. Unstructured regions are indicated as small gray boxes. The WT protein and all deletion mutants carry C-terminal myc- and his-epitope tags. RBM20 amino acid residues are indicated underneath the schemes and the corresponding molecular weight of proteins is depicted in brackets. (B) RT-PCR validation of RBM20-mediated changes in processing the human titin  $TTN^{241-3}$  splicing reporter in HEK293 cells. Gray boxes represent mRNA species with and without the alternative exon 242 (top and bottom band, respectively). Quantification of three independent transfections are shown as percentages of mRNA excluding the alternative exon. (C) qRT-PCR (SYBR Green) analysis of HEK293 cells transfected with the  $TTN^{241-3}$  splicing reporter and the RBM20 expression constructs with activity in panel B:  $n = 3$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

porter with RBM20 or PTB4 into the HEK293 cells was examined using the S1 nuclease assay. This analysis revealed that the exon 242–243 product is increased upon addition of PTB4 (Supplementary Figure S5C), while the exon 241–243 product is increased upon addition of RBM20 (Supplementary Figure S5D). These findings are consistent with a role



**Figure 4.** RBM20-RRM binds to the intron regions downstream of the alternative exon. (A) Schematic representation of the RNA transcripts used to study RBM20Δ2 binding. Titin transcripts were derived from the minimal region necessary for RBM20 function (labeled A–G). Black boxes indicate UCUU binding motifs—consensus sites for RBM20 binding. (B) RN-protein complex formation as determined by gel-shift assays. The same batch of recombinant RBM20Δ2 was used to allow direct comparisons of affinities. Black arrows indicate the RNA-protein complexes.



**Figure 5.** Analysis of splicing intermediates of the RBM20-regulated TTN<sup>241-3</sup> splicing reporter. (A) Schematic representation of intermediate splicing products and arrangement of primer pairs used to quantify splicing intermediates upon RBM20 addition on TTN<sup>241-3</sup> splicing reporter. The pre-mRNA (depicted in the middle) was used for normalization (primers in red and blue). Primers specific for the intermediate RNAs were arranged at indicated exon–exon junctions (depicted in black). (B–E) RT-PCR (SYBR Green) validation of splicing intermediates upon transient transfection of TTN<sup>241-3</sup> splicing reporter with/without RBM20/PTB4-expression plasmid into HEK293 cells. Splicing intermediates were normalized to the pre-mRNA.  $n = 3$ ; \*\*\* $P < 0.001$ . (F) Splice junction oligonucleotides to detect the alternative splicing products. (G) Alternative splicing with or without RBM20 and PTB was analyzed by primer extension. The expected products of primer extension are indicated. RBM20 suppresses and PTB4 enhances inclusion of titin exon 242.

of RBM20 in repressing the splicing of both upstream and downstream introns.

### Repressor activity of RBM20 is counteracted by PTB4

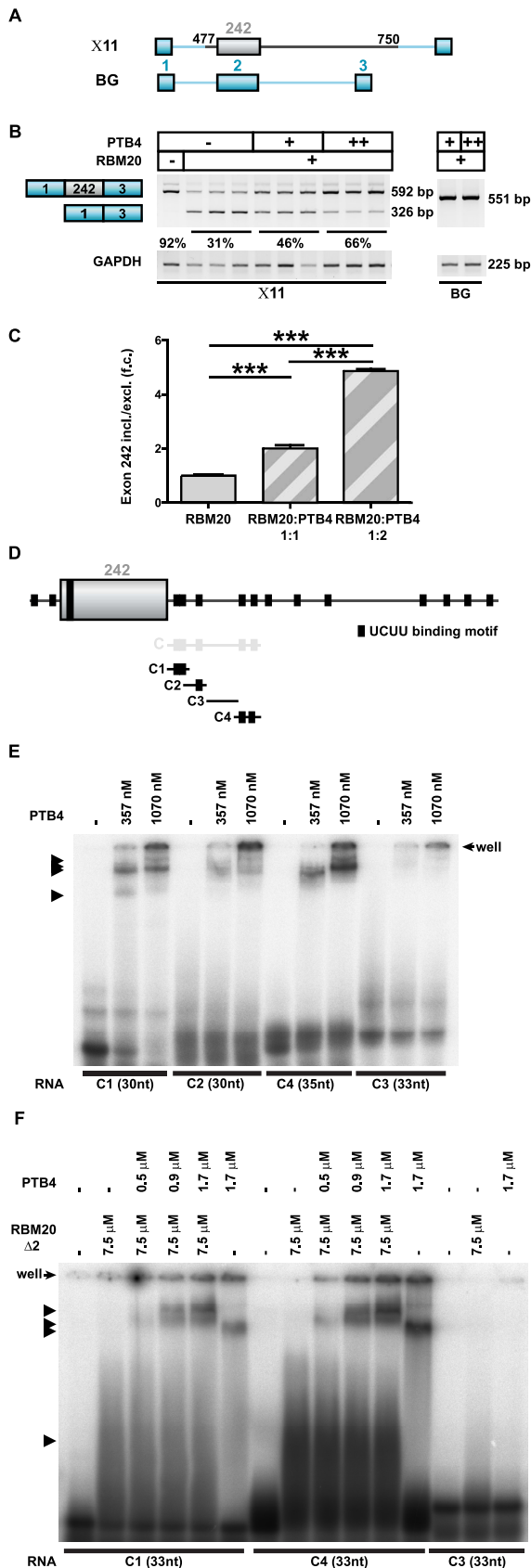
To investigate whether Polypyrimidine tract-binding protein (PTB) could regulate alternative splicing of titin-derived substrates, we first performed transient co-transfections of PTB and RBM20 together with a titin/ $\beta$ -globin mutant splicing reporter X11 (Figure 6A) in HEK293 cells and evaluated results by the RT-PCR (Figure 6B). We included RBM20-expressing plasmids in our transient transfection at 1:1 ratio with the reporter, to facilitate detection of the potential enhancing activity of the test protein. PTB exists in three alternatively spliced isoforms, PTB1, PTB2 and PTB4, which have distinct effects on alternative splicing of  $\alpha$ -tropomyosin (TM) (21). As PTB2 had an intermediate effect in the repressive hierarchy (in the transfected smooth muscle cells), we restricted ourselves in cloning and testing only PTB1 and PTB4 isoforms. PTBP1 is expressed in HEK293 cells and in developing rat cardiomyocytes (Supplementary Figure S6I and J).

Increasing concentrations of transfected PTB4 significantly enhanced inclusion of the regulated middle exon in the X11 splicing reporter (Figure 6B and C; quantification in Supplementary Figure S6A and B). Similarly, co-transfection of equivalent or higher molar ratios of PTB1 did increase inclusion of the regulated exon 242 into the X11 splicing reporter (Supplementary Figure S6C–G). Toward understanding the molecular basis of PTB4 dependent titin splicing, we performed *in vitro* binding assays. Building on our RBM20 $\Delta$ 2 interaction data, we divided TTN 242–243 intron transcript C sequence into  $\sim 30$  nt RNAs with single, double or no binding motif UCUU (Figure 6D). Increasing amounts of recombinant PTB4 protein were incubated with those RNAs and formed mixtures were separated on a native gel (Figure 6E). PTB4/RNA complexes were formed only with RNAs containing UCUU motifs (C1, C2 and C4 versus C3). Adding increasing amounts of PTB4 to complexes of RBM20 $\Delta$ 2 and RNA containing two UCUU motifs or only a single UCUU, resulted in complexes of higher molecular weight than complexes with RBM20 or PTB4 alone. This suggests simultaneous binding of PTB4 and RBM20 to transcripts (Figure 6F).

We conclude that both PTB4 and RBM20 bind the intron downstream of the alternative TTN exon 242 where PTB4 overcomes the repression by RBM20 as summarized in Figure 7.

### DISCUSSION

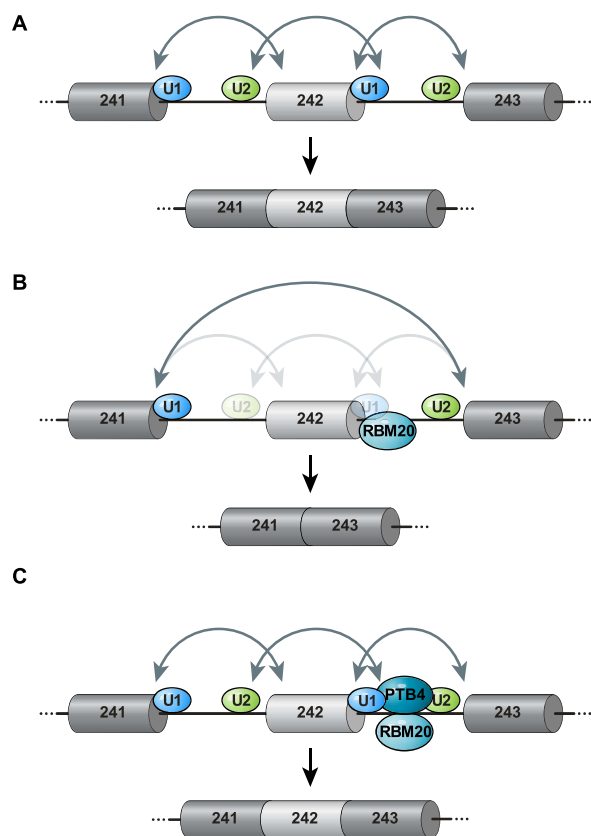
In this study, we investigated several aspects of alternative splicing regulation by the RBM20 protein and identified a 994-nt segment in the TTN pre-mRNA, which is necessary and sufficient to regulate exon 242 splicing by RBM20. The segment contains 64 nt from the upstream intron, the regulated exon 242 (267 nt) and 663 nt from the downstream intron. Additional truncation of this segment reduced or abolished the RBM20 repressor activity (Figures 1 and 2), indicating that the whole genomic segment



**Figure 6.** PTB4 enhances TTN exon 242 inclusion dependent on the downstream intron. (A) Schematic representation of the human TTN

contributes to splicing regulation. Interaction of the 994 nt segment with the RBM20-RRM domain involves a region immediately downstream of exon 242 which contains multiple UCUU binding motifs. A stretch of these motifs is required to mediate RBM20-mediated exon repression as demonstrated with the deletion analysis of the titin genomic segment (Figure 2A and B) and confirmed by mutagenesis gradually eliminating UCUU binding motifs in TTN<sup>241-3</sup> downstream intron (Supplementary Figure S4E). Remarkably, the RBM20-RRM concentration required to achieve a gel-shift is lower for region C (immediately downstream of the alternative exon) versus region D (3' of region C), which might indicate that sequences in the proximity to the 5'ss are bound with the higher affinity than more distal intronic sequences. This is consistent with the increased presence of UCUU motives and a cluster toward the 5'ss. Among the transcriptome-wide RBM20 binding sites in heart-specific transcripts identified using HITS-CLIP, intronic RBM20-binding positions flanking alternative exons correlate with splicing repression (15). In particular, RBM20 binding sites were significantly over-represented in a window spanning 400 bp upstream/downstream from the 3'/5' splice sites of differentially spliced exons and RBM20 binding was found to peak 50 nt upstream and 100 nt downstream of repressed exons. Using RBM20-RRM in our binding assays we identified the region downstream of the repressed exon to be most strongly bound by RBM20 and no significant binding to the upstream intron of the repressed exon (Figure 4). This might relate to the averaging of consensus sites over the transcriptome versus the analysis of an individual exon: RBM20 might employ slightly different mechanisms for alternative exon repression versus mutually exclusive exons selection. Indeed, the presence of additional upstream RBM20 binding sites could thus relate to alternative splicing of mutually exclusive exons. An alternative explanation might be the differential binding of full length RBM20 versus the truncated isoform. Nevertheless, RNA-binding and repression are not directly connected, as exon 242 contains binding sites for RBM20 interacting proteins that can support the repressor activity of RBM20. An example is the

(gray)/β-globin (blue) hybrid splicing reporters used to evaluate PTB4 effect on titin splicing regulation. (B) PTB4-mediated changes in the alternative splicing of the human titin/β-globin hybrid splicing reporter X11. Changes in X11 alternative splicing were assayed in the background of RBM20. Increasing concentrations of PTB4 reverted the repressor activity of RBM20 on X11 splicing reporter. Titin-derived sequences missing splicing reporter (BG) was not responding to RBM20 or PTB4 (indicated on the right). (C) qRT-PCR (SYBR Green) quantification of PTB4-regulated alternative splicing events in the titin/β-globin mutant splicing reporter:  $n = 3$ ;  $*** P < 0.001$ . (D) Schematic representation of the RNA transcripts used to study PTB4 binding. Black boxes indicate UCUU binding motifs. C1 transcript contains two UCUU binding motifs, organized in tandem, C2 contains one UCUU binding motif, C3 contains no UCUU binding motif and the C4 transcript contains two separated UCUU binding motifs. (E) RNA-protein complex formation as analyzed in a gel-shift assay. Black arrows indicate the observed formation of RNA-protein complexes. PTB4 binds all transcripts except for C3 that does not contain the consensus site. (F) RNA-protein complex formation analyzed by gel-shift assay using RBM20Δ2 and PTB4 protein. Black arrows indicate the formation of RNA-protein complexes. Increasing the concentration of PTB4 promotes the generation of additional RNA/protein complexes and leads to a reduction of RBM20Δ2/RNA complexes.



**Figure 7.** Model for the regulation of  $TTN^{241-3}$  splicing by RBM20 and PTB4. (A) In the absence of RBM20, spliceosomal complexes form over the exon and over the both introns (solid arrows) to include titin exon 242. Constitutive exons in dark gray, alternative exon 242 light gray and introns are indicated as thin lines. (B) Model of RBM20 mediated exclusion of exon 242: RBM20 binds downstream intron sequences and interferes with exon 242 5'ss recognition by the U1 snRNP resulting in reduced recognition of the downstream intron. This interaction destabilizes the upstream 3'ss recognition by the U2 snRNP and interferes with the exon definition complex and upstream intron recognition. As a result, the spliceosome recognizes only the external 5' and 3' splice sites and exon 242 is excluded. Transparent arrows indicate absent or incomplete communication between splice sites. (C) Addition of PTB4 overcomes the negative effect of RBM20 on exon retention as both factors bind the downstream intron, where they differentially interfere with U1 snRNP and 5'ss interactions, formation of the exon definition complex and subsequent inclusion of the alternative exon.

RBM20-interacting protein FUS (15), which is predicted to bind titin exon 242 (RBPDB search (22)). A similar arrangement was recently described for splicing regulator RBfox that acts as part of a large complex of RNA-binding proteins (LASR) to stimulate splicing repression (23).

We found that the 5' and 3' splice sites of the flanking exons 241 and 243 did not contribute to the alternative splicing of exon 242, as their exchange for  $\beta$ -globin exons did not affect RBM20-dependent isoform expression (Figure 2). The minimal genomic segment required for RBM20-mediated exon repression includes the alternative exon and flanking intronic region. Together with the information on RBM20 binding, this data would be consistent with the molecular mechanism summarized in Figure 7. Binding of RBM20 to the downstream intron suggests that repression is achieved by interfering with intron definition. We find the

3'ss of exon 242 and the upstream intron sequences necessary for the repression, implying an even earlier role of RBM20—already at the level of spliceosome assembly during exon definition. This model suggests that inefficient recruiting of the spliceosome to the 5'ss of exon 242 would affect recognition of the upstream 3'ss needed to define the exon. Here, splicing repression would result from the poorly defined exon 242 3'ss and involve the inhibition of spliceosome assembly over the upstream intron (Figure 7B).

Diverse mechanisms of splicing repression have been described. Among them is binding of PTB at sites overlapping with splicing signals (polypyrimidine tract or branch point) and steric blockage of splicing factor access (24,25). Another scenario of PTB splicing repression requires binding to sequences located in both flanking introns and cooperative interaction between PTB molecules, with subsequent looping out of the intervening region or to its silencing by coating it with PTB molecules (19,26). Binding to an exonic splicing silencer and targeting molecular events that lead to exon definition (inhibiting the association of U2AF and U2 snRNP with the upstream 3'ss, without affecting recognition of the downstream 5'ss by the U1 snRNP) is used by PTB to exclude Fas exon 6 and generate mRNA encoding a soluble isoform of a receptor (18). Neuron-specific alternative splicing factors (Nova proteins) block U1 snRNP binding and inhibit exon inclusion by binding to an exonic YCAY cluster (20). Muscleblind-like 1 (MBNL1) protein binds to a structured intronic silencer to compete with U2AF65 binding and to repress cardiac troponin T exon 5 (22). hnRNP L together with hnRNP A1 repress spliceosome assembly and subsequently splicing of CD45 exon 4 by promoting aberrant U1 snRNA binding with exonic sequences upstream of the 5'ss (24). For RBM20 the details of the exon repression mechanism were unknown.

Here, we uncovered that RBM20 upon binding its functional response element in the downstream intron prevents removal of upstream and downstream introns and thus inhibits alternative exon inclusion. A similar mechanism has been described for activation of alternative exon inclusion by MBNL1 as binding of MBNL1 to its functional response element in the downstream intron and enhancing U2AF65 binding and splicing of the upstream intron leads to the inclusion of insulin receptor exon 11 (27). This mechanism relates to activation of exon inclusion, but has—to our knowledge—so far not been described for inhibition as described here for RBM20.

Toward understanding how splice factors concertedly shape cardiac isoform expression, we identified PTB4 as a novel titin splicing regulator that counteracts the splice repressor activity of RBM20. Interestingly, PTBP1 and RBM20 are both expressed from the late embryonic stages to the adulthood (Supplementary Figure S6J and (10)). In addition, we find that PTB4 and RBM20 bind the same motive on the 5'SS downstream of the alternative exon. Thus, we do not only show for the first time that PTB isoforms bind to titin-derived mRNA, but provide a possible mechanism of regulating titin isoform expression through additive binding of PTB and RBM20 to the downstream intron. Building on these findings, our future work will compare the titin splice regulators RBM20 and PTB4 as therapeutic

tic targets to adapt cardiac isoform expression and improve diastolic function in patients with heart disease.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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*Author Contribution:* V.D. designed and conducted experiments and analyzed the data. M.G. designed the project and experiments. V.D. and M.G. wrote the manuscript. We thank Beate Goldbrich-Hannig for excellent technical assistance. Michael Radke supported the initial cloning of the TTN<sup>241-3</sup> splicing reporter.

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