

# RBM4 down-regulates PTB and antagonizes its activity in muscle cell–specific alternative splicing

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**A**lternative splicing contributes largely to cell differentiation and functional specification. We previously reported that the RNA-binding protein RBM4 antagonizes the activity of splicing factor PTB to modulate muscle cell–specific exon selection of  $\alpha$ -tropomyosin. Here we show that down-regulation of PTB and its neuronal analogue nPTB during muscle cell differentiation may involve alternative splicing-coupled nonsense-mediated mRNA decay. RBM4 regulates PTB/nPTB expression by activating exon skipping of their transcripts during myogenesis. Moreover, RBM4 and PTB target a common set of transcripts that undergo muscle

cell–specific alternative splicing. Overexpression of RBM4 invariably promoted expression of muscle cell–specific isoforms, which recapitulated in vivo alternative splicing changes during muscle differentiation, whereas PTB acted oppositely to RBM4 in expression of mRNA isoforms specific for late-stage differentiation. Therefore, RBM4 may synergize its effect on muscle cell–specific alternative splicing by down-regulating PTB expression and antagonizing the activity of PTB in exon selection, which highlights a hierarchical role for RBM4 in a splicing cascade that regulates myogenesis.

## Introduction

Gene expression can be greatly diversified by alternative splicing of precursor mRNAs (pre-mRNAs) in higher eukaryotic cells. Cell type–specific and developmental regulation of alternative splicing plays an important role in cell differentiation and functional specification. Although the vast majority of mammalian genes undergo alternative splicing, not every resultant transcript functionally encodes protein. Genomics studies have indicated that  $\sim 35\%$  of alternatively spliced transcripts acquire premature termination codons due to reading frame shift and are thereby eliminated by the nonsense-mediated decay (NMD) pathway (Lejeune and Maquat, 2005; McGlincy and Smith, 2008). This alternative splicing–coupled NMD (AS-NMD) regulation not only allows manipulating mRNA levels at the post-transcriptional level, but also provides a means of gene expression control in a regulatory loop.

Alternative splicing is regulated by a variety of RNA-binding proteins. Interplay between these splicing regulatory factors and corresponding cis-elements determines splice site utilization (Wang and Burge, 2008; Chen and Manley, 2009;

Tejedor and Valcárcel, 2010). In addition, cooperative or antagonistic action between the regulatory factors modulates alternative splicing. Because most trans-acting factors are ubiquitously expressed, their relative abundance or activity in a cell is thus important for alternative splicing regulation (Long and Cáceres, 2009; Licatalosi and Darnell, 2010). In principal, the activity, stability, or subcellular localization of splicing factors may be modulated by their post-translational modification upon induction of cellular signaling pathways (Blaustein et al., 2007; Lynch, 2007; Stamm, 2008). Besides, expression of a substantial pool of splicing factors is post-transcriptionally regulated via AS-NMD (Wollerton et al., 2004; Boutz et al., 2007b), and therefore, their abundance may be controlled by an autoregulatory loop or by other splicing regulators (Stamm 2008; Sun et al., 2010).

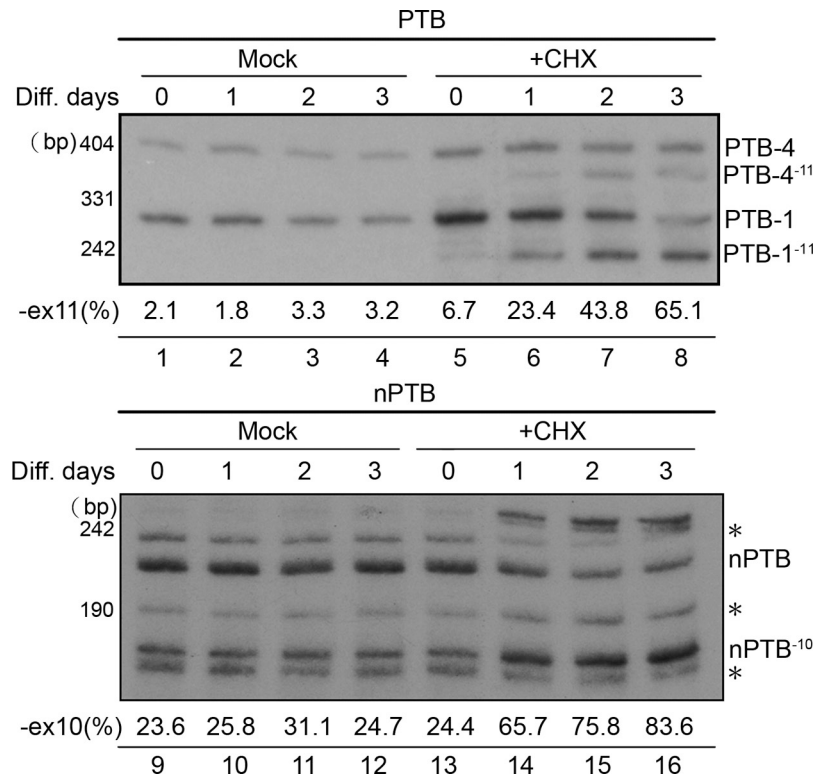
The polypyrimidine tract-binding (PTB) protein is a master regulator of alternative splicing in mammalian cells and also functions at several other steps of mRNA metabolism (Sawicka et al., 2008). PTB contains four RNA recognition motifs

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Abbreviations used in this paper:  $\alpha$ -TM,  $\alpha$ -tropomyosin; cTnT, cardiac troponin T; fTnT, fat skeletal TnT; NMD, nonsense-mediated decay; premRNA, precursor mRNA; PTB, polypyrimidine tract-binding; RBM, RNA-binding motif; UCE, ultra-conserved element.

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**Figure 1. AS-NMD of PTB/nPTB transcripts occurs during myogenesis.** C2C12 cells were cultured under differentiation conditions for 0–3 d. Before harvest, cells were mock treated or treated with cycloheximide (CHX). Total RNA was isolated for RT-PCR analysis followed by probing with <sup>32</sup>P-labeled primers specific to PTB exon 10 (lanes 1–8) or nPTB exon 9 (lanes 9–16). The identity of the transcripts is indicated. Asterisks represent nonspecific bands. Exon exclusion efficiency (percentage) is represented by the ratio of exon-skipped transcripts (PTB-1<sup>-11</sup> and PTB-4<sup>-11</sup> for PTB; nPTB<sup>-10</sup> for nPTB) to total RNA species; the averaged values shown below the lanes were obtained from three independent experiments.



(Oberstrass et al., 2005) that confer RNA binding activity and mediate PTB homodimer formation (Oberstrass et al., 2005; Lamichhane et al., 2010). PTB favors binding to UUCU elements that often exist within the polypyrimidine tract of an intron; therefore, PTB usually functions as a splicing suppressor by competing off the splicing factor U2AF and the U2 small nuclear ribonucleoprotein complex from the 3' end of the intron (Sharma et al., 2005). Moreover, PTB modulates alternative splicing of a large number of transcripts (Amir-Ahmady et al., 2005; Gama-Carvalho et al., 2006). Thus, control of PTB expression level is important for splicing regulation in cells (Boutz et al., 2007b; Makeyev et al., 2007; Spellman et al., 2007). Indeed, PTB is particularly down-regulated via various mechanisms during neuron and muscle development (Boutz et al., 2007a; Makeyev et al., 2007). Notably, PTB has an autoregulatory activity; overexpression of PTB activates the skipping of exon 11 of its own premRNA, yielding premature termination codon-containing isoforms that are subsequently targeted to NMD (Wollerton et al., 2004). Analogously, PTB cross-regulates the expression of its neuronal analogue nPTB by promoting exon 10 exclusion in nPTB transcripts (Makeyev et al., 2007; Spellman et al., 2007). However, whether PTB can be regulated by any other splicing regulatory factors via AS-NMD is not yet known.

The RNA-binding motif 4 (RBM4) protein is a multifunctional protein that has been shown to act at least in premRNA splicing regulation and translation control (Lin and Tarn, 2005, 2009; Lin et al., 2007). We have previously screened for target mRNAs of human RBM4 and verified that RBM4 could specifically promote the selection of skeletal muscle-specific exons in  $\alpha$ -tropomyosin ( $\alpha$ -TM; Lin and Tarn, 2005). In this regard, RBM4 competes with PTB binding to intronic CU-rich

elements adjacent to the regulated exons and thereby antagonizes the suppressive effect of PTB in  $\alpha$ -TM exon selection (Lin and Tarn, 2005). Although RBM4 is ubiquitously expressed, its abundance is particularly high in muscle and heart (Lin and Tarn 2005). Upon induction of differentiation in mouse C2C12 myoblasts, RBM4 is phosphorylated and a proportion of total RBM4 transiently translocates to the cytoplasm, where it may function in translation control (Lin et al., 2007). Nevertheless, RBM4 may have nuclear functions during muscle cell differentiation. The observation that RBM4 regulates exon selection of  $\alpha$ -TM provides a hint that it has potential in modulating muscle cell type-specific splicing of other transcripts during myogenesis. Moreover, the expression of RBM4 in muscle cells prompted us to investigate whether it participates in regulation of PTB expression.

## Results

### AS-NMD-mediated down-regulation of PTB/nPTB during myogenesis

In neurons, expression of PTB and nPTB is mutually exclusive, with PTB primarily expressed in precursor cells and glia, and nPTB in post-mitotic neurons (Boutz et al., 2007b). Suppression of nPTB expression in nonneuronal cells primarily results from PTB-mediated AS-NMD of the nPTB transcript (Spellman et al., 2007). In differentiated muscle cells, however, expression of both PTB and nPTB is suppressed (Boutz et al., 2007b). A recent report indicated that nPTB down-regulation in differentiated muscle cells is in part controlled by microRNAs (Boutz et al., 2007b). However, whether the AS-NMD pathway is also responsible for down-regulation of nPTB and

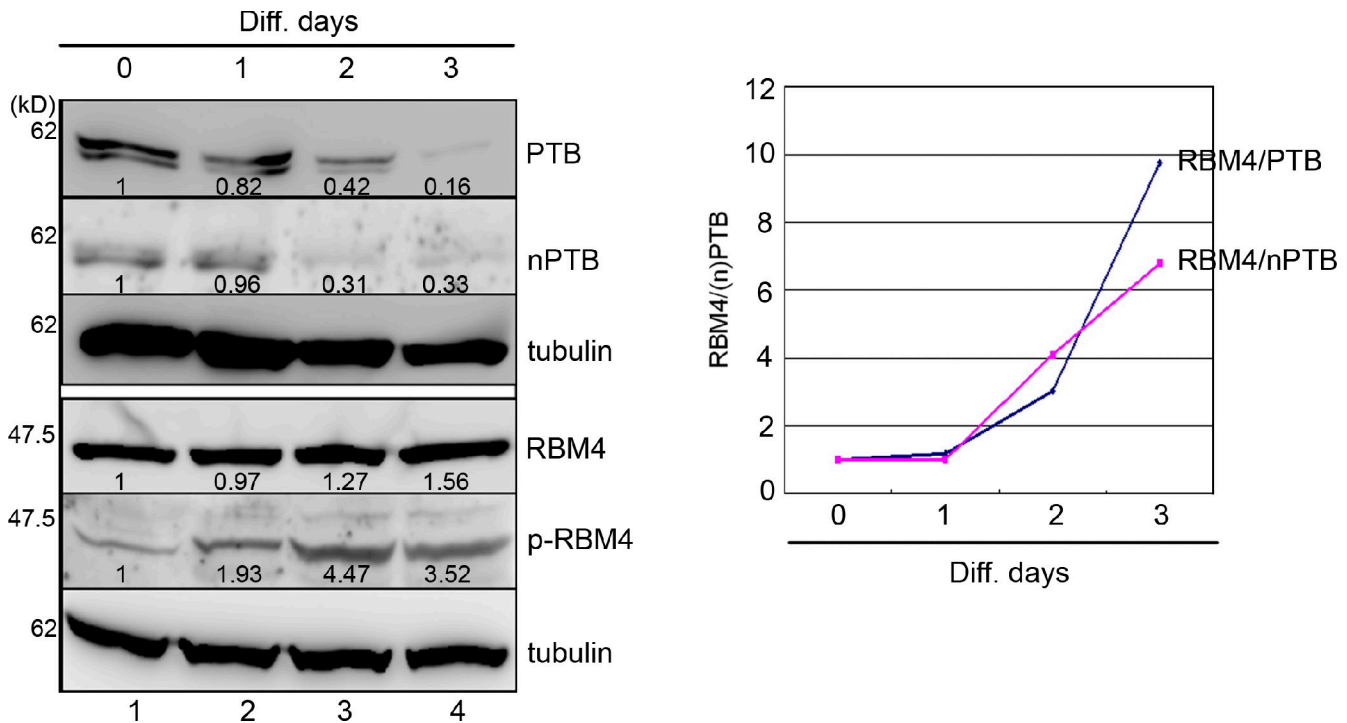


Figure 2. **Differential expression of RBM4 and PTB/nPTB proteins during muscle cell differentiation.** C2C12 cells were cultured under differentiation conditions for 0–3 d. Protein samples were fractionated on separate gels followed by immunoblotting sequentially with antibodies specific for PTB, nPTB, and tubulin (top) or with RBM4, phosphorylated RBM4, and tubulin (bottom). Values given below the bands represent relative protein expression levels of each protein throughout differentiation. The graph shows the change in the ratio of total RBM4 to (n)PTB during cell differentiation.

even PTB in muscle cells has not been excluded. To assess this possibility, we performed RT-PCR to detect PTB and nPTB transcripts in C2C12 cells. Two major PTB transcripts, i.e., full-length PTB-4 and exon 9-skipped PTB-1, were expressed in non-differentiated C2C12 myoblasts (Fig. 1, lane 1). The expression level of these two transcripts did not change significantly or was minimally reduced as cells differentiated (lanes 2–4). Although NMD was blocked by translation inhibitor cycloheximide or overexpression of the dominant-negative mutant of the NMD factor Upf1 (Upf1-DN) in cells (Banihashemi et al., 2006), the exon 11-skipped products, PTB-1<sup>-11</sup> and PTB-4<sup>-11</sup>, were detectable, and their expression level was increased throughout differentiation (Fig. 1, lanes 5–8; and Fig. S1 A). Although the exon 10-skipped mRNA isoform of nPTB could be detected in nondifferentiated cells, its abundance also gradually increased throughout differentiation in the presence of cycloheximide (Fig. 1, lane 9–16). This result suggested that AS-NMD contributes at least in part to controlling PTB and nPTB expression during muscle cell differentiation.

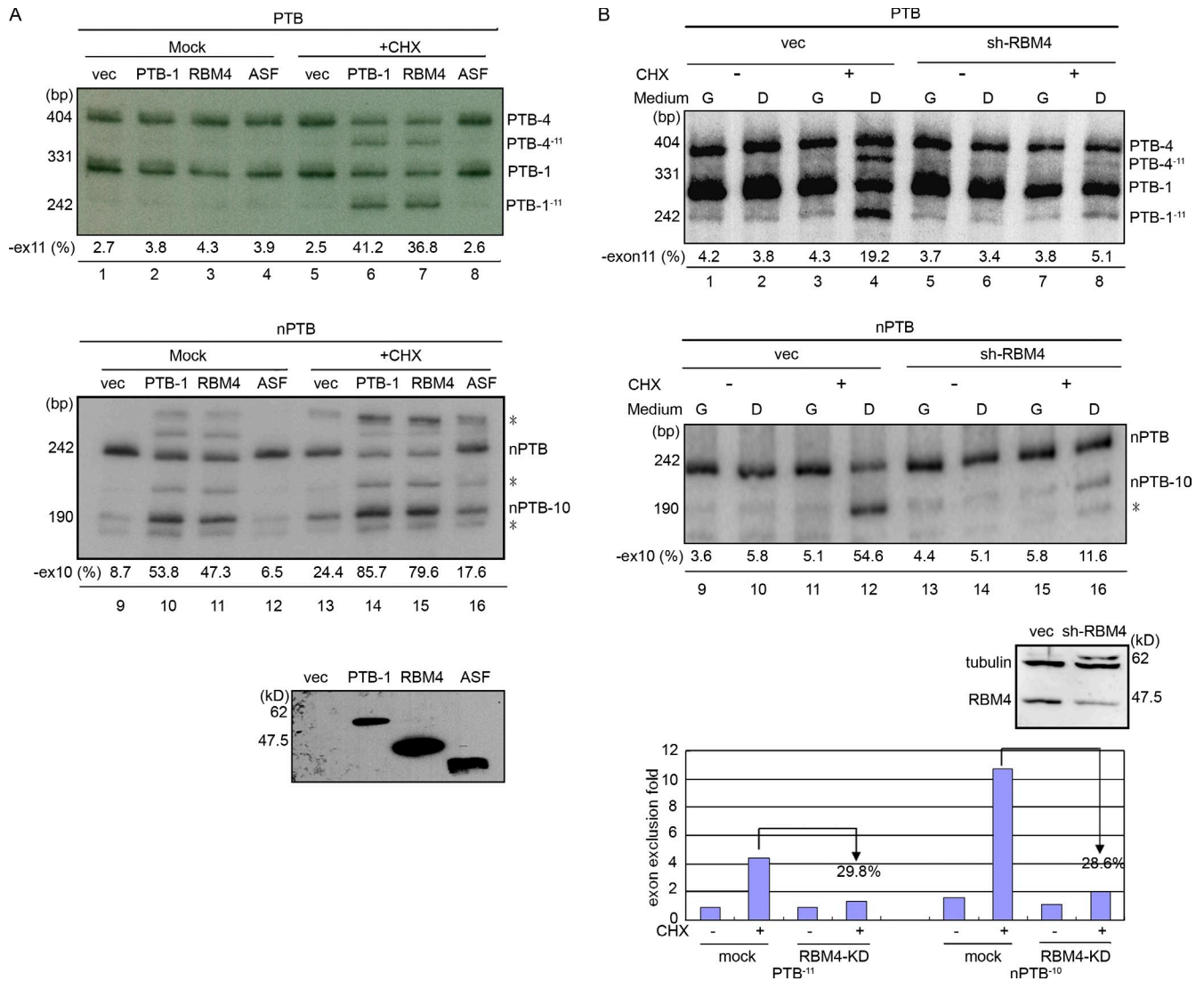
#### Expression of RBM4 and PTB/nPTB proteins in differentiating myoblasts

Using immunoblotting, we observed that the protein level of both PTB and nPTB gradually diminished throughout C2C12 cell differentiation (Fig. 2), which was consistent with previous reports (Boutz et al., 2007a). We thus assumed that AS-NMD of PTB and nPTB transcripts is activated or maintained by a splicing factor(s) expressed in differentiated muscle. Because RBM4 is expressed in differentiated C2C12 cells and binds

preferentially to CU-rich sequences (Lin et al., 2007; Lin and Tarn, 2009), it might serve such a role. Indeed, RBM4 was detectable, and its expression level even increased slightly throughout differentiation in concert with diminished expression of PTB/nPTB (Fig. 2; total RBM4). Moreover, phosphorylation of RBM4 was significantly enhanced during C2C12 differentiation (Fig. 2; phos-RBM4), as reported (Lin et al., 2007). Therefore, unlike PTB/nPTB, RBM4 may act as a functional splicing regulatory factor in muscle cells. If so, any substantive increase in the molar ratio or the relative activity of RBM4 to PTB/nPTB might profoundly change alternative splicing patterns in differentiating muscle cells.

#### RBM4 modulates alternative splicing of PTB and nPTB

In nonneuronal cells, PTB suppresses nPTB expression by inducing exon 10 skipping in nPTB premRNA (Spellman et al., 2007). Indeed, excess PTB can also modulate its own premRNA splicing (Wollerton et al., 2004). Our notions that RBM4 shares similar binding elements with PTB (Lin and Tarn, 2005) and that their expression and/or activity might be reciprocally regulated during C2C12 cell differentiation (Fig. 2) prompted us to examine whether RBM4 may regulate alternative splicing of PTB/nPTB. We overexpressed FLAG-tagged RBM4 in C2C12 cells and treated cells with cycloheximide. RT-PCR analysis showed that RBM4, like PTB, increased the abundance of two exon 11-skipped transcripts of endogenous PTB (Fig. 3 A, lanes 6 and 7), and promoted PTB exon 11 skipping in a dose-dependent manner (Fig. S2 A). Analogously, both RBM4 and



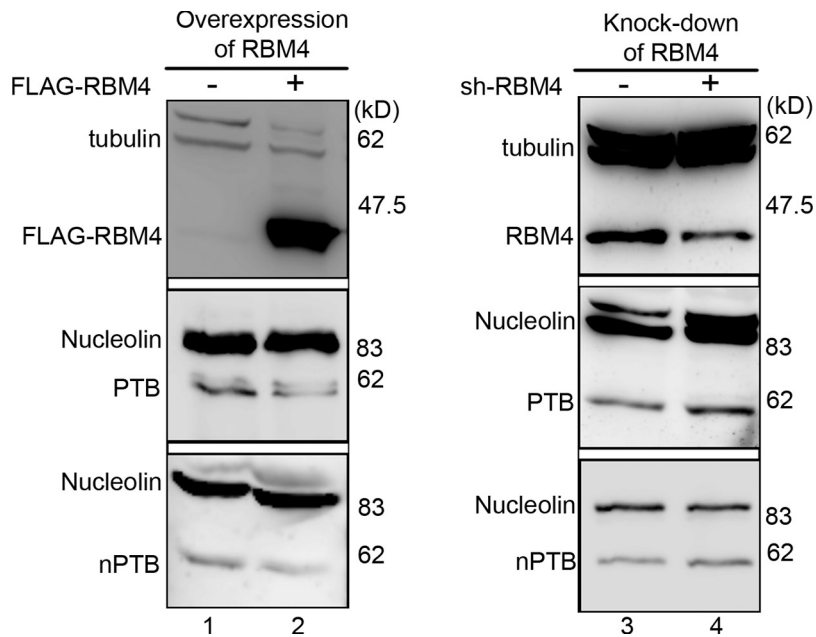
**Figure 3. RBM4 activates exon skipping of PTB/nPTB premRNAs and is involved in differentiation-induced alternative splicing of PTB/nPTB.** (A) C2C12 cells were transiently transfected with the mock vector or vector expressing FLAG-tagged PTB, RBM4, or ASF. (B) C2C12 cells were transfected with the mock vector (lanes 1–4) or RBM4-targeting shRNA expression vector (lanes 5–8), and then cultured in growth (G) or differentiation (D) medium. Before harvest, cells were mock treated or treated with cycloheximide (CHX). RT-PCR analysis and calculation of exon exclusion efficiency were performed in Fig. 1. Asterisks represent nonspecific bands. Immunoblotting was performed with anti-FLAG (A) and with anti-RBM4 and anti-tubulin in parallel on the same membrane (B).

PTB could activate exon 10 skipping of endogenous nPTB (Fig. 3 A, lanes 14 and 15). Nevertheless, ASF had no effect on PTB or nPTB alternative exon selection (lanes 8 and 16). This result provided a hint that the presence of RBM4 during muscle cell differentiation could potentially activate PTB/nPTB exon skipping, which may result in down-regulation of their protein expression. Because RBM4 is significantly phosphorylated upon differentiation induction (Fig. 2; Lin et al., 2007), we asked whether phosphorylation of RBM4 has any effect on its splicing activity. As shown in Fig. S2, both nonphosphorylatable (SA) and phosphomimetic (SD) RBM4 proteins could activate exon skipping of PTB/nPTB (Fig. S2 B). However, the slightly lower activity of RBM4-SD was perhaps due to its relative high abundance in the cytoplasm as compared with the wild-type RBM4 (Fig. S2 C). Nevertheless, we apparently concluded that phosphorylation of RBM4, which occurs during

muscle cell differentiation, may not be a critical factor for inducing PTB/nPTB exon skipping.

Next, we examined whether RBM4 is essential for PTB/nPTB splicing regulation during muscle cell differentiation. Transient expression of a short hairpin RNA targeting mouse RBM4 mRNA depleted ~60% of endogenous RBM4 protein in C2C12 cells (Fig. 3 B, immunoblot). Such partial depletion of RBM4 reduced differentiation-induced exon skipping of PTB and nPTB by ~35 and ~26%, respectively (Fig. 3 B, lanes 8, 16, and graph). A similar result was obtained by using another RBM4 shRNA (shRNA2) (Fig. S3 A). This result indicated that RBM4 participates in alternative splicing regulation of PTB/nPTB during C2C12 differentiation.

Moreover, using immunoblotting, we observed that the levels of PTB and nPTB proteins decreased upon overexpression of FLAG-tagged RBM4 (Fig. 4, lane 2), but they increased



**Figure 4. RBM4 modulates PTB/nPTB protein expression levels in C2C12 cells.** C2C12 cells were mock transfected (lanes 1 and 3) or transfected with vector expressing FLAG-RBM4 (lane 2) or RBM4 shRNA (lane 4). Immunoblotting was performed on independent membranes (separated by blank space) with pairs of antibodies in parallel that were specific for the FLAG epitope (FLAG-RBM4) or endogenous proteins as indicated.

when RBM4 was down-regulated by either one of the tested shRNAs (Fig. 4, lane 4; Fig. S3 B). This result coincided with the effect of RBM4 in promoting alternative splicing of PTB/nPTB to yield potential NMD substrates (Fig. 3). Overall, our results suggested a role for RBM4 in controlling PTB/nPTB expression by modulating the alternative splicing of PTB/nPTB transcripts during muscle cell differentiation.

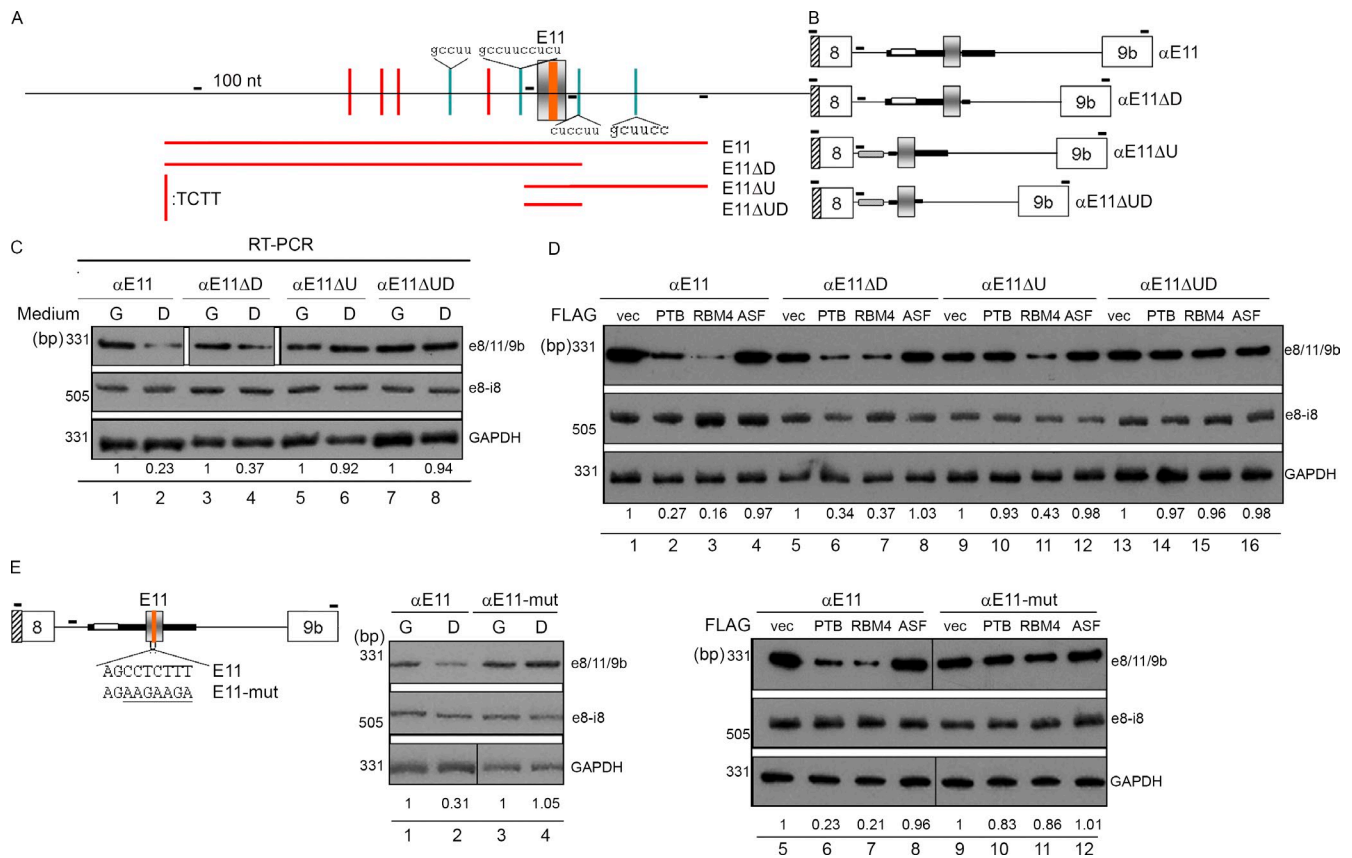
#### **RBM4 regulates PTB exon selection by binding to CU-rich elements**

Next, we attempted to explore the mechanism of RBM4-mediated PTB splicing regulation by using minigene assay. We observed that overexpression of RBM4 could result in exon 11 skipping of a PTB minigene transcript that encompassed exon 10–12 of the mouse PTB (Fig. S4). Because RBM4 favors to bind CU-rich sequences, we suspected that RBM4 regulates PTB exon 11 selection via its surrounding CU-rich ultraconserved elements (UCEs; Wollerton et al., 2004; Ni et al., 2007). Indeed, an intronic CU-rich element immediately upstream of PTB exon 11 most resembles the consensus binding site for RBM4 (Fig. 5 A; Lin and Tarn, 2005). Therefore, to evaluate whether the UCEs surrounding PTB exon 11 are necessary and sufficient for RBM4-mediated splicing regulation in a heterologous sequence context, we inserted PTB exon 11 with or without flanking intronic sequences into the  $\alpha$ -TM reporter to replace its RBM4-responsive exon 9a (Fig. 5 B; Lin and Tarn, 2005). Because  $\alpha$ -TM exons 9a and 9b are concomitantly included or excluded (Lin and Tarn, 2005), we performed RT-PCR using two sets of primers (Fig. 5 B) to assess the expression of the resulting  $\alpha$ -TM–PTB chimeric reporters in C2C12 cells. One RT-PCR product spanning the 5' SV40 sequence and  $\alpha$ -TM intron 8 (e8/i8) represented the reporter premRNAs, whereas another product amplified by using SV40 and  $\alpha$ -TM exon 9b primers corresponded to the spliced products (e8/i1/9b). The result showed that all four tested reporter transcripts included PTB exon 11 and following  $\alpha$ -TM exon 9b with similar

efficiency in growing C2C12 cells (Fig. 5 C, lanes 1, 3, 5, and 7). As anticipated, these two exons were excluded in differentiated C2C12 cells (lane 2, reporter  $\alpha$ E11), suggesting that PTB exon 11 with its upstream and downstream intronic UCEs could properly respond to differentiation signals in a heterologous reporter. Further, such an exon exclusion was only minimally impaired in the reporter  $\alpha$ E11 $\Delta$ D (lane 4) but was completely abolished in reporters  $\alpha$ E11 $\Delta$ U and  $\Delta$ DU (lanes 6 and 8). This result suggested that the upstream intronic sequence was most important for differentiation-induced exon skipping, whereas the sequence downstream of exon 11 contributed less to such splicing regulation activity.

As anticipated and observed under cell differentiation conditions, overexpression of either RBM4 or PTB could result in exclusion of PTB exon 11 and  $\alpha$ -TM exon 9b in the  $\alpha$ E11 reporter mRNA (Fig. 5 D, lanes 2 and 3). RBM4 overexpression could suppress such exon inclusion of both  $\alpha$ E11 $\Delta$ D and  $\alpha$ E11 $\Delta$ U, albeit with reduced efficiency (lanes 7 and 11), whereas only  $\alpha$ E11 $\Delta$ D but not  $\alpha$ E11 $\Delta$ U was still responsive to PTB-mediated exon 11 exclusion (lanes 6 and 10). Therefore, both upstream and downstream introns of exon 11 might contain the elements responsive to RBM4-mediated regulation, whereas the activity of PTB largely depended on the upstream elements. Nevertheless, ASF had no effect on either reporter examined (lanes 4, 8, 12, and 16). The notion that splicing of  $\alpha$ E11 $\Delta$ U could be modulated by RBM4 but not by differentiation (Fig. 5 C, lane 6; and Fig. 5 D, lane 11) was somewhat intriguing. We apparently reasoned that overexpressed RBM4 might force exon 11 skipping via the elements in the downstream intron or even in exon 11 (see following paragraph).

It has been predicted that the CU-rich exon 11 of PTB harbors a PTB recognition motif (Wollerton et al., 2004), which appeared to coincidentally overlap with a putative RBM4 binding site (Fig. 5 A). Therefore, to test whether RBM4 and PTB act on such a composite element, we mutated its sequence in the  $\alpha$ E11 reporter (Fig. 5 E, diagram). RT-PCR analysis showed



**Figure 5. Ultraconserved elements of PTB are responsible for RBM4-mediated regulation of PTB AS-NMD.** (A) Diagram showing the mouse PTB genomic fragment encompassing exon 11 and a part of its adjacent introns. Putative intronic RBM4 and PTB binding sites are indicated by red and blue vertical lines, respectively. The CU-rich element in exon 11 is indicated by a thick orange line. The PTB genomic fragments of the  $\alpha$ E11 and its derived reporters (see panel B) are depicted. (B) Diagrams showing the chimeric reporters in which exon 9a of human  $\alpha$ -TM was replaced by mouse PTB exon 11 with different lengths of the flanking intron sequences. (C) Each reporter, as indicated, was transiently transfected into C2C12 cells. Cells were cultured under growth (G) or differentiation (D) conditions for 1 d. Cells were treated with cycloheximide before harvest. For C–E, total RNA was isolated from the transfectants and subjected to RT-PCR using a forward primer specific to SV40 and reverse primers specific to  $\alpha$ -TM intron 8 (i8) and exon 9b (9b) to yield products e8/11/9b and e8-i8, respectively. The PCR products were probed with  $^{32}$ P-labeled SV40 primer. Exon 11 exclusion of the reporter transcripts is represented by the ratio of e8/11/9b to e8-i8; the averaged values shown below the lanes were obtained from three independent experiments, and then normalized to that of mock transfectants. GAPDH was used as the control. (D) Each indicated reporter was cotransfected with an expression vector encoding FLAG-tagged PTB, RBM4, or ASF. (E) Diagram showing the mutant  $\alpha$ E11 reporter in which the CU-rich sequence of exon 11 was mutated. The reporter assay for lanes 1–4 and 5–12 was performed as in C and D, respectively.

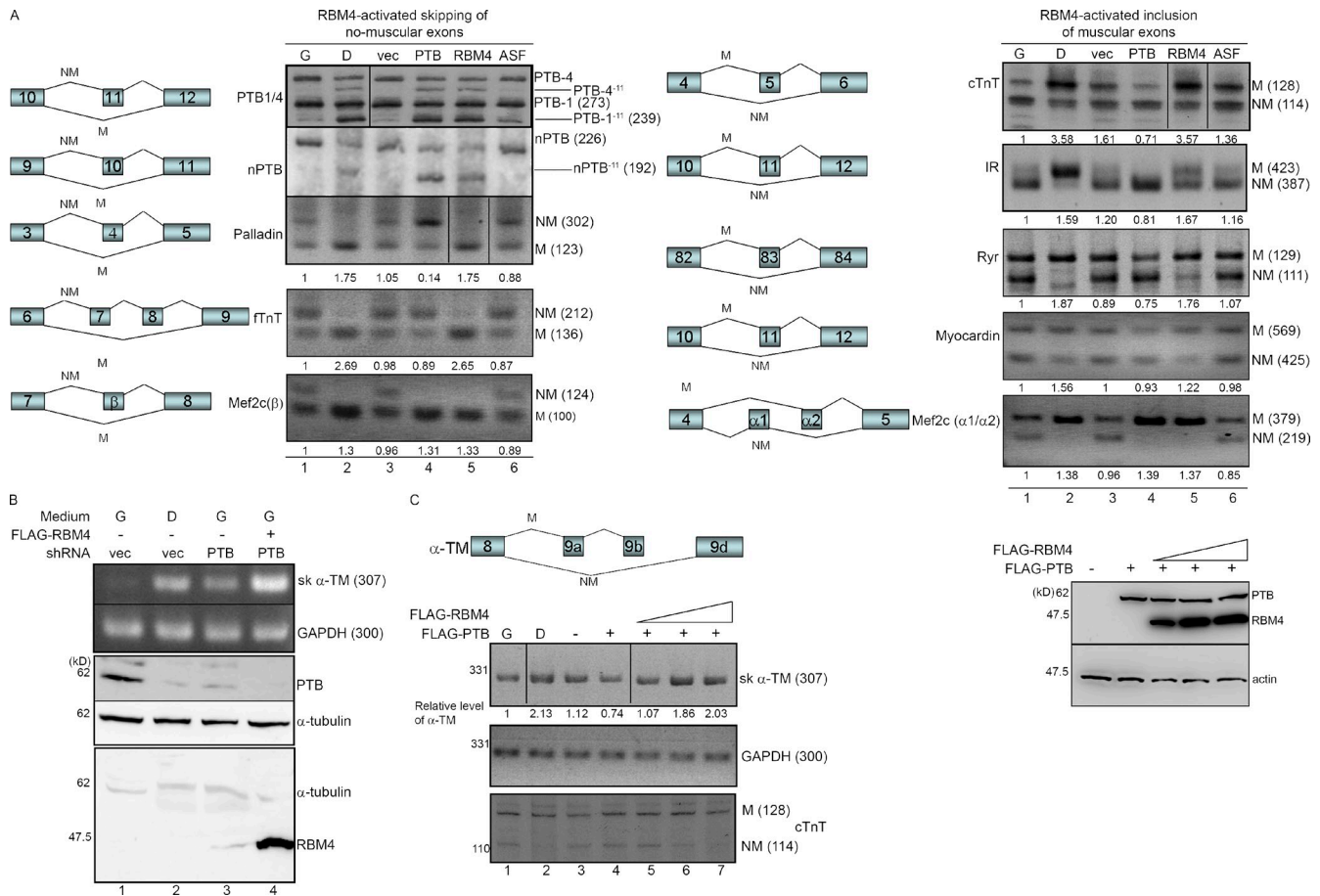
that the mutant exon 11 was no longer skipped in differentiated C2C12 cells (not depicted) and, accordingly, was not responsive to RBM4 or PTB-mediated control (Fig. 5 E, RT-PCR).

An immunoprecipitation RT-PCR analysis revealed that RBM4 associated with the  $\alpha$ -TM transcripts in cells (unpublished data). Moreover, recombinant RBM4 might directly bind PTB exon 11 and its upstream and downstream CU-rich sequences (Fig. S5). Therefore, our results together indicated that RBM4 and PTB may regulate alternative exon selection in PTB mRNA via binding to respective recognition sites throughout the entire UCs of PTB, including exon 11 and its flanking introns.

#### RBM4 and PTB differentially regulate muscle-specific alternative splicing

To this point our data indicated that RBM4 down-regulates PTB/mPTB expression in differentiated C2C12 cells. Indeed, RBM4 and PTB oppositely regulate skeletal muscle-specific  $\alpha$ -TM splicing via competing for binding to common intronic

CU-rich elements (Lin and Tarn, 2005). We assumed that RBM4 and PTB may antagonistically regulate some other muscle-specific splicing events and that an increase in the RBM4/PTB ratio throughout myoblast differentiation may have functional and physiological significance. To test this possibility, we examined the effect of RBM4 on several premRNAs regulated by PTB (Amir-Ahmady et al., 2005; Gama-Carvalho et al., 2006). FLAG-tagged RBM4, PTB, or ASF was overexpressed in C2C12 cells followed by RT-PCR analysis using primers specific for validated PTB targets, including palladin, cardiac troponin T (cTnT), insulin receptor (IR), and ryanodine receptor (RyR; Fig. 6 A, diagram). Indeed, all these mRNAs underwent alternative exon utilization upon myoblast differentiation to generate muscle cell-specific isoforms (Fig. 6 A, lane 2). Interestingly, overexpression of RBM4 promoted splicing toward differentiation-induced isoforms of all tested transcripts (lane 5). However, PTB acted oppositely to RBM4 in alternative exon selection of these myocyte-expressed transcripts (lane 4). ASF had no significant effect on the examined reporters except for



**Figure 6. RBM4 and PTB differentially modulate alternative splicing of muscle cell-specific exon selection.** (A) Diagrams showing the alternatively spliced regions of premRNAs encoding mouse PTB, nPTB, palladin, fTnT, MEF2c (exons  $\alpha 1/2$  and  $\beta$ ), cTnT, IR, Ryr, and myocardin. M and NM represent muscle-enriched and nonmuscle-enriched mRNA isoforms, respectively. C2C12 cells were cultured in growth (lane 1) or differentiation (lane 2) medium for 3 d or transfected with the mock vector (lane 3) or expression vector of FLAG-tagged PTB, RBM4, or ASF (lanes 4–6). Total RNA was subjected to RT-PCR using gene-specific primers (Table II), and the PCR products were blotted for probing with a  $^{32}\text{P}$ -labeled forward primer. Efficiency of muscle-enriched isoform production is reflected by the ratio of M/NM; the values shown below the lanes are averaged from three independent experiments. (B) C2C12 cells were transfected with the empty vector (lanes 1 and 2) or the PTB shRNA expression vector (lanes 3 and 4) and cultured under growth (lanes 1, 3, and 4) or differentiation (lane 2) conditions for 2 d. RT-PCR was performed to examine terminal exon inclusion of  $\alpha$ -TM as described in Lin and Tarr (2005). GAPDH was used as the control. Protein samples were fractionated on separate gels followed by immunoblotting sequentially with anti-PTB and anti-tubulin (middle) or with anti-RBM4 and anti-tubulin in parallel (bottom). (C) C2C12 cells were transfected with the  $\alpha$ -TM reporter and then cultured under growth (lane 1) or differentiation (lane 2) conditions for 3 d. In addition to the  $\alpha$ -TM reporter, C2C12 cells were also transfected with an empty vector (lane 3) or the FLAG-PTB expression vector alone (lane 4) or together with increasing amounts of the FLAG-RBM4 expression vector (lanes 5–7).  $\alpha$ -TM and cTnT transcripts were detected as in B and A, respectively. GAPDH was used as the control. Values represent the relative level of  $\alpha$ -TM normalized to GAPDH in for each sample. Immunoblotting was performed using anti-FLAG. In all panels, band sizes for RT-PCR products are shown in parentheses.

cTnT (lane 6). Next, to examine whether RBM4 may directly regulate the above tested PTB substrates, we knocked down PTB by using the shRNA strategy (Boutz et al., 2007b). We first evaluated alternative splicing of  $\alpha$ -TM in C2C12 cells under growth and differentiation conditions. The result showed that inclusion of the terminal exons 9a and 9b of  $\alpha$ -TM was significantly induced upon differentiation (Fig. 6 B, lane 2), which allowed expression of the skeletal muscle-specific isoform. Knockdown of PTB could somewhat relieve the suppression of exon 9a/9b inclusion in proliferating C2C12 cells (lane 3). Nevertheless, overexpression of RBM4 in the absence of PTB greatly promoted exon 9a/9b inclusion (lane 4), suggesting a likely direct role RBM4 in  $\alpha$ -TM exon selection. Furthermore, we demonstrated that overexpression of RBM4 could reverse the effect of PTB-induced exclusion of  $\alpha$ -TM exon 9a/9b and cTnT exon 5 in C2C12 cells, supporting a role for RBM4 in

antagonizing the suppressive effect of PTB, irrespective of exon exclusion during muscle cell differentiation (Fig. 6 C).

Finally, to understand more about how RBM4 and PTB modulate muscle cell-specific alternative splicing, we searched for candidate genes that encode CU-rich elements near the muscle cell-specific exons. We chose the genes encoding fast skeletal TnT (fTnT), myocardin, and MEF2c for further analysis. RBM4 promoted skipping of fTnT exons 7 and 8 but included exon 11 of myocardin (Fig. 6 A, lane 5), confirming that RBM4 promoted muscle-specific isoform expression. MEF2c undergoes alternative splicing in differentiated muscle cells to select exon  $\alpha 2$  but exclude exon  $\beta$  (Hakim et al., 2010). We observed the same results in C2C12 cells after induction of differentiation (lane 2), as reported (Hakim et al., 2010), and also in cells that overexpressed RBM4 or PTB (lanes 4 and 5). Therefore, in the case of MEF2c, PTB functioned similarly to RBM4; the

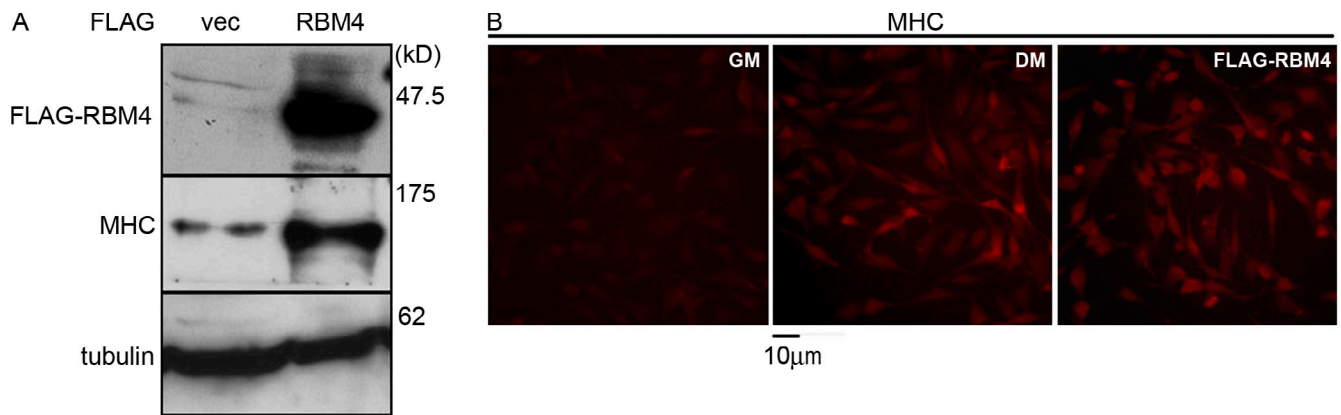


Figure 7. **Overexpression of RBM4 promotes muscle cell differentiation.** (A) C2C12 cells were mock transfected or transiently transfected with the expression vector encoding FLAG-tagged RBM4. Immunoblotting was performed with antibodies against myosin heavy chain (MHC), FLAG-epitope, and  $\alpha$ -tubulin. (B) C2C12 cells were cultured under growth (G) or differentiation (D) conditions or transiently overexpressed FLAG-tagged RBM4. Indirect immunofluorescence was performed with anti-MHC. Bar, 10  $\mu$ m.

implication of differential regulation of exon selection by PTB is discussed below. In total, our results suggest that RBM4 and PTB regulate the same set of transcripts but generally act oppositely to control muscle cell-specific exon selection.

#### Overexpression of RBM4 promotes muscle cell differentiation

The above data indicate that RBM4 modulates alternative splicing of muscle cell transcripts to produce skeletal muscle-specific isoforms. We therefore examined whether RBM4 acts as a muscle cell differentiation-promoting factor. Immunoblotting analysis showed that transient overexpression of FLAG-tagged RBM4 in C2C12 myoblasts increased the level of myosin heavy chain (MHC), a differentiation marker of muscles (Fig. 7 A). Immunofluorescence coincidentally showed significant high abundance of MHC in RBM4-overexpressed cells (Fig. 7 B). Moreover, we observed that cells underwent morphological change toward myocyte phenotype upon RBM4 overexpression (unpublished data). However, the possibility that RBM4 promotes muscle cell differentiation not only via alternative splicing but also via other steps of mRNA metabolism cannot be completely excluded.

## Discussion

Here we report that down-regulation of PTB/nPTB in differentiated muscle cells may, at least in part, involve the AS-NMD pathway. RBM4 activated alternative exon skipping of PTB/nPTB transcripts and was responsible for differentiation-induced alternative splicing of PTB/nPTB in myoblasts. Moreover, RBM4 and PTB shared a common set of targets but differentially modulate their exon selection. Therefore, the observed increase in the RBM4/(n)PTB ratio during muscle cell differentiation might result in a change in global alternative splicing patterns.

Cell differentiation involves not only transcriptional reprogramming but also regulated alternative splicing (Kuyumcu-Martinez and Cooper, 2006; Fu et al., 2009; Loya et al., 2010). The latter may require cell type-specific splicing regulators;

expression of some of these factors may be regulated at the post-transcriptional level. Recent findings that the expression of a set of UCE-containing mammalian splicing regulatory factors is regulated by AS-NMD suggest the existence of splicing regulation cascades (Ni et al., 2007). Therefore, the levels of these factors in cells can be controlled by regulated splicing.

PTB is ubiquitously expressed in mammalian tissues but is replaced by nPTB in neurons. PTB expression is down-regulated in neuronal cells by both transcriptional silencing and microRNA 124-mediated translation suppression (Makeyev et al., 2007). In nonneuronal cells, PTB suppresses nPTB expression via AS-NMD (Spellman et al., 2007). However, a reduction of PTB level in neurons allows reactivation of nPTB expression (Boutz et al., 2007b; Makeyev et al., 2007). Because PTB and nPTB regulate the expression of overlapping but distinct sets of genes, the switch from PTB to nPTB provides an important role in reprogramming alternative splicing during neuronal development. In differentiated muscle cells, both PTB and nPTB are down-regulated (Boutz et al., 2007b). Although it has been demonstrated that muscle-specific microRNAs are responsible for PTB/nPTB suppression in muscles (Boutz et al., 2007a), we detected exon 10/11-skipped PTB/nPTB transcripts in differentiated C2C12 cells upon NMD inactivation (Fig. 1). Therefore, differentiation-induced PTB/nPTB suppression might also involve AS-NMD. Note that expression of RBM4 continued during muscle cell differentiation (Fig. 2; Lin et al., 2007). Overexpression of RBM4 promoted PTB/nPTB exon skipping, similar to that observed with cell differentiation, whereas knock-down of RBM4 suppressed exon skipping and accordingly increased the levels of PTB and nPTB (Figs. 3 and 4). Our result indicates that RBM4 contributes at least in part to the reduction of PTB and nPTB levels during C2C12 cell differentiation. Therefore, analogous to the scenario that PTB suppresses nPTB expression in nonneuronal cells, RBM4 down-regulates its functional antagonists, namely PTB and nPTB, also by AS-NMD during muscle cell differentiation. Moreover, RBM4-mediated splicing control of PTB and nPTB reveals a novel splicing cascade in mammalian cells.

We have previously shown that overexpression of RBM4 promotes the distal 5' splice site utilization of the adenovirus



Table I. **RBM4 and PTB differentially modulate alternative splicing of a set of muscle-specific transcripts**

Transcript	AS exon <sup>1</sup>	Muscle isoform	CU-rich motifs	
			Exon	Intron
RBM4 and PTB show the same effect; both promote the expression of muscle-specific isoforms.				
PTB	e11	Skip	+	+
nPTB	e10	Skip	+	+
MEF2c	$\alpha 1/\alpha 2$	Include $\alpha 2$	+ ( $\alpha 2$ )	+ ( $\alpha 2$ )
MEF2c	$\beta$	Skip	-	+
RBM4 and PTB show the opposite effect; RBM4 promotes the expression of muscle-specific isoforms				
<i>RBM4 skips; PTB includes</i>				
Palladin	e4	Skip	-	+
fTnT	E6 and 7	Skip	+ <sup>2</sup>	+
$\alpha$ -Actinin <sup>3</sup>	e19	Skip	+	+
<i>RBM4 includes; PTB skips</i>				
$\alpha$ -Tropomyosin	e9a	Include (sk <sup>4</sup> )	-	+
cTnT	e5	Include	-	+
IR	e11	Include	-	+
Ryr	e83	Include	-	+
Myocardin	e11	Include	+	-
Vinculin <sup>3</sup>	e19	Include	-	+

<sup>1</sup>AS exon, alternatively spliced exon.

<sup>2</sup>The CU-rich sequence is located between exons 6 and 7, but is not present in the exons.

<sup>3</sup>Data for the RT-PCR analysis are not shown in Fig. 6.

<sup>4</sup>sk, skeletal muscle cell.

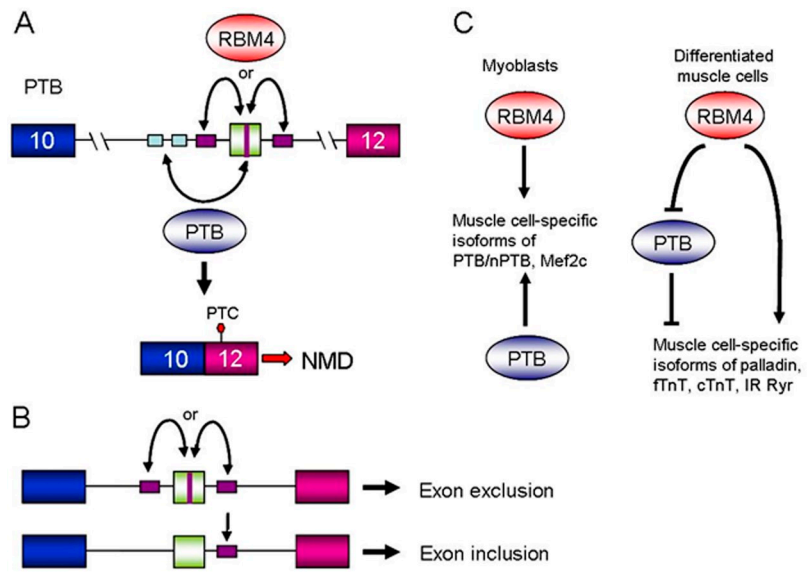
E1a transcript (Lai et al., 2003). In this regard, RBM4 and the SR protein ASF act oppositely on 5' splice site selection, but the underlying mechanism has not been investigated. A search for cellular targets of RBM4 has suggested that it has preference for CU-rich sequences (Lin and Tarn, 2005; Lin et al., 2007). Indeed, RBM4 activates skeletal muscle-specific exons of  $\alpha$ -TM by competing with PTB for binding to the CU-rich elements downstream of its regulated exons (Lin and Tarn 2005). Consistent with previous observations (Lin and Tarn, 2005; Lin et al., 2007), RBM4 may bind multiple CU-rich elements within and adjacent to exon 11 of PTB (Fig. 5 and Fig. S5). Using truncated and mutant PTB reporters, we observed that that RBM4 perhaps binds simultaneously to exon 11 and its upstream or downstream intron (Figs. 5 and 8 A). However, in contrast to its effect on  $\alpha$ -TM exon selection, RBM4 excluded exon 11 from PTB transcripts. As compared with PTB exon 11,  $\alpha$ -TM exon 9a appears to lack CU-rich motifs. Consistent with this observation, most of the alternative exons examined that are included upon overexpression of RBM4, such as in the premRNAs for cTnT, IR, Ryr, and vinculin, also lack potential RBM4 binding sites (Table I). The exception is myocardin, in which the alternative exon 11 harbors CU-rich motifs but flanking introns are deficient in C/U residues. We thus assumed that, in addition to out-competing negative regulators, when RBM4 binds to intronic elements nearby a regulated exon, it may promote exon inclusion by facilitating association of the spliceosome with the adjacent splice sites (Fig. 8 B). However, when RBM4 binds simultaneously to the alternative exon and either flanking intron, it may interfere with the nearby spliceosome association with the splice sites and thus excludes the exon (Fig. 8 B). By this

mechanism, RBM4 perhaps activates exon skipping of PTB/nPTB as well as fTnT and  $\alpha$ -actinin (Table I). These and other possibilities remain to be investigated.

Recent studies have globally mapped the binding sites of several splicing regulatory factors including PTB on mRNA targets, facilitating a clearer picture of splicing regulation (Sharma and Black, 2006; Ule et al., 2006). PTB in general functions as a splicing suppressor to activate exon skipping via binding to intronic CU-rich elements near regulated exons (Valcárcel and Gebauer, 1997; Wagner and Garcia-Blanco, 2001; Spellman et al., 2005). Because these elements are often located within or near the polypyrimidine tract, the binding of PTB likely perturbs spliceosome assembly at the 3' end of the intron. While binding to both upstream and downstream introns, multimerized PTB may loop out the exon and hence prevent exon recognition by the spliceosome (Lamichhane et al., 2010). Further, PTB can activate exon utilization when its binding sites are located distal to regulated exons (Ni et al., 2007; Castle et al., 2008; Sawicka et al., 2008). Our reporter assay indicated that PTB suppresses its own exon 11 inclusion likely via binding to both exon 11 and its upstream intron (Fig. 5), which conforms to the rule of PTB-mediated exon exclusion (Ni et al., 2007; Castle et al., 2008). However, PTB also promoted inclusion of the alternative exons of palladin, fTnT, and  $\alpha$ -actinin (Fig. 5 and unpublished data), but, intriguingly, these exons have similar features to those that are skipped by PTB. Therefore, how PTB distinguishes various exons and determines their fate remains an enigma and still requires future investigation.

This study shows that RBM4 regulates a number of muscle-specific exons, which are also the targets of PTB (Fig. 6).

**Figure 8. Model for RBM4-mediated control of muscle cell-specific alternative splicing.** (A) RBM4 activates exon 11 exclusion via binding to exon 11 together with its upstream or downstream intron. The resulting premature termination codon-containing PTB transcripts are targeted for degradation through NMD. PTB activates exon 11 skipping of its own transcripts via binding to exon 11 as well as upstream intronic elements. Purple and blue rectangles depict representative RBM4 and PTB binding sites, respectively. RBM4 and PTB may share a part of CU-rich binding sites. (B) RBM4 activates alternative exon skipping when it binds simultaneously to both exonic and intronic elements, whereas it may promote exon inclusion via binding only to (downstream) intronic elements. (C) In myoblasts, RBM4 and PTB may function similarly in splicing regulation of the transcripts (such as MEF2c) that are expressed at an early stage of differentiation to promote muscle cell-specific isoform expression. However, overexpression of PTB in general suppresses the production of mRNA isoforms that are specific to late stages of differentiation. RBM4 down-regulates PTB via AS-NMD and meanwhile acts oppositely to PTB or antagonizes its suppressive activity in alternative splicing control to achieve maximal yield of muscle cell-specific mRNA isoforms.



Overexpression of RBM4 induced either skipping or inclusion of the regulated exons, but invariably promoted expression of muscle-specific isoforms. Interestingly, except for PTB/nPTB and MEF2c transcripts, RBM4 and PTB functioned in opposition to modulate muscle-specific exon selection. The MEF2 family members are transcriptional enhancers and function upstream of myogenin and MyoD in a transcriptional cascade during myogenesis (Berkes and Tapscott, 2005, Keren et al., 2006). Therefore, it is reasonable that RBM4 and PTB, both of which are expressed in undifferentiated myoblasts, show the same effect to promote the initial steps of myogenesis (Fig. 8 C). However, a large fraction of the RBM4/PTB targets we examined function in fully differentiated myocytes, including those encoding cytoskeletal proteins of actin filaments and receptors that modulate muscle cell physiology (Table I). RBM4 and PTB exert opposite effects in modulating alternative splicing of these common targets, as we previously observed with  $\alpha$ -TM exon selection (Lin and Tarn, 2005). Therefore, in order to enhance its effect on promoting muscle cell-specific isoform expression, RBM4 down-regulates the expression of its functional antagonist, PTB, as myoblasts commit to terminal differentiation (Fig. 8 C).

In conclusion, our results indicated that although the expression level of RBM4 does not increase greatly during muscle differentiation, it might strategically down-regulate PTB and nPTB in order to raise its level relative to such antagonizing factors and thereby potentiate its effect toward expression of muscle cell-specific isoforms. Moreover, our data coincidentally suggested that RBM4 functions positively in muscle cell differentiation in part via its role in alternative splicing.

## Materials and methods

### Plasmid construction and primers

The mammalian expression vectors for FLAG-tagged RBM4 (wild-type and phosphorylation site mutants) and FLAG-ASF and the bacterial expression vector of His-tagged RBM4 were described previously (Lin and Tarn, 2005). The expression vectors for FLAG-tagged PTB, nPTB, and dominant-negative mutant Upf1 were kind gifts of D. Black (University of California, Los Angeles, Los Angeles, CA) and J. Lykke-Andersen (University of California,

San Diego, La Jolla, CA), respectively. The His-tagged PTB expression vector was constructed by cloning the human PTB-coding region into pET-32a (QIAGEN). The  $\alpha$ TM-8/9a/9b reporter (hereafter abbreviated as  $\alpha$ TM) containing a fragment of the human  $\alpha$ -tropomyosin gene was described previously (Lin and Tarn, 2005). The mouse PTB genomic fragments containing exon 11 flanked by different lengths of its adjacent intron sequences were used to replace exon 9a of  $\alpha$ TM. The resulting vectors were full-length  $\alpha$ E11, and  $\alpha$ E11 $\Delta$ D,  $\Delta$ U, and  $\Delta$ UD containing truncated introns. The  $\alpha$ E11-mut vector containing the mutagenized CU-rich element of PTB exon 11 was generated via a site-directed mutagenesis system (QuikChange; Agilent Technologies). To construct the PTB minigene vector, a mouse genomic region spanning from exon 10 to exon 12 of PTB was obtained by PCR amplification using genomic DNA from ES cells as template, and then the PCR product was subcloned into pCH110 (GE Healthcare) to replace the  $\beta$ -galactosidase gene. All primers used in this study are listed in Table II.

### Cell culture, differentiation of myoblasts, and cycloheximide treatment

Mouse C2C12 cells were maintained in DME (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). To induce C2C12 cell differentiation, the medium contained 2% horse serum and 20  $\mu$ g/ml insulin. To block the NMD pathway, the cells were treated with 100  $\mu$ M cycloheximide for 2 h before harvest.

### Transfection and in vivo splicing assay

C2C12 cells were grown to 50–60% confluency in 6-well plates and transfected using Lipofectamine 2000 (Invitrogen). In general, 0.5  $\mu$ g of each reporter alone or together with 2  $\mu$ g of expression vector encoding the FLAG-tagged splicing effector was transfected into C2C12 cells for 24 h. To examine the effect of cell differentiation or trans-acting factors on splicing of endogenous PTB and nPTB as well as other muscle-specific pre-mRNAs, differentiation was induced for 24 h or 4  $\mu$ g of effector expression vector was transiently transfected for 24 h before cell harvest. Total RNA was collected from C2C12 cells using Trizol (Invitrogen). For RT-PCR analysis, 2  $\mu$ g of RNA was reverse transcribed by SuperScriptase III (Invitrogen) in a 10- $\mu$ l reaction. RT-PCR analysis was performed using appropriate sets of primers, and the resulting products were blotted and probed with  $^{32}$ P-labeled specific primers (see figure legends). Knockdown of RBM4 was performed by transient transfection of the vector expressing RBM4-targeting shRNA1 (Lin and Tarn, 2009) or shRNA2 (complementary to nucleotides 150–172 of human RBM4a). Knockdown of PTB was performed using pBS-sh1-shPTB (Boutz et al., 2007b).

### Immunoblotting

Immunoblotting analysis was conducted as recommended using the enhanced chemiluminescence (ECL) system (Millipore), and the images were analyzed by the LAS-3000 imaging system (Fujifilm). Primary antibodies used in this study include polyclonal anti-RBM4 and anti-pS309, which recognize total and phosphorylated RBM4, respectively (Lin et al., 2007); polyclonal

Table II. List of PCR primer sets

Gene	Accession no.	Forward primer	Reverse primer
PTB	NM_001077363	5'-AAGAGCAGAGACTACACTCGA-3'	5'-CTGCCGTCTGCCATCTGCACAA-3'
nPTB	NM_019550	5'-GCATTTGCCAAGGAGACATCC-3'	5'-CGCTGCACATCTCCATAAACAC-3'
Mef2c ( $\alpha 1/\alpha 2$ )	NM_025282	5'-GTGCTGTGCGACTGTGAGAT-3'	5'-CCAGTGTGCTGACAGGATTG-3'
Mef2c ( $\beta$ )	–	5'-CTGGCAGCAAGAACACGAT-3'	5'-AGGAGTTGCTACGGAACCA-3'
Palladin	NM_001081390	5'-CATCCAGAACTGAGGAGCC-3'	5'-AGCTTTCGCTGCAGAGTCC-3'
fTnT	NM_011620	5'-ATGTCTGACGAGGAAAC-3'	5'-ATCAAAGTCTACTTTCTCC-3'
$\alpha$ -Tropomyosin	NM_000366	5'-CGGAGAGGTCAGTAAC-3'	5'-CACTGGGCGAATTGCTTC-3'
cTnT	NM_011619	5'-GCCGAGGAGTGGTGGTGGAGGAGTA-3'	5'-GTCTCAGCCTCACCTCAGGCTCA-3'
IR	NM_010568	5'-GAGGATTACTGCACAACG-3'	5'-TTCCTTTGGCTCTTGCCAC-3'
Ryr	NM_177652	5'-CGAGAGGCAGAACAAAGGCAG-3'	5'-GGTCTGTGTGAACTCGTCA-3'
Myocardin	NM_145136	5'-TCACTGTGTGGAGTCTCAGGTC-3'	5'-TGGCATCGGCTGGCAITTC-3'
p $\alpha$ Ex11	–	5'-TTTTGGAGGCCTAGGCTTTT-3'	5'-CTGCCGTCTGCCATCTGCACAA-3'

anti-nPTB (a kind gift from D. Black) and monoclonal anti-PTB (EMD); anti-FLAG (Sigma-Aldrich); and anti-tubulin (Thermo Fisher Scientific).

### Immunofluorescence

Indirect immunofluorescence was performed essentially according to the procedure described in Lai et al. (2003) with monoclonal anti-MHC (Abcam) as primary antibody. The specimens were observed using the Radiance 2100 confocal microscopy system (Bio-Rad Laboratories).

### Electrophoretic mobility shift assay

Recombinant His-tagged proteins were prepared as described previously (Lin et al., 2007). RNA probes used were PTB exon 11 (E11 $\Delta$ UD, 57 nucleotides) or exon 11 plus its upstream (E11 $\Delta$ D, 382 nucleotides) or downstream (E11 $\Delta$ U, 237 nucleotides) intron. Intronic sequences were in vitro transcribed and used as probes. For RNA-protein interaction, 1  $\mu$ g of each recombinant protein was incubated with  $2 \times 10^5$  cpm of  $^{32}$ P-labeled transcript ( $3 \times 10^5$  cpm/pmol) as described previously (Lin et al., 2007). The reactions were analyzed by electrophoresis on a 4 or 6% nondenaturing polyacrylamide gel in TBE buffer (45 mM Tris-HCl, 45 mM boric acid, and 1 mM EDTA, pH 8.0).

### Online supplemental material

Fig. S1 shows that blockage of NMD by overexpression of a dominant-negative mutant of Upf1 caused accumulation of exon 11-skipped PTB isoforms that were induced by either differentiation or RBM4 overexpression. Fig. S2 shows that RBM4 activated PTB exon 11 skipping in a dose-dependent manner, and that both nonphosphorylatable (SA) and phosphomimetic (SD) forms of RBM4 could induce PTB exon 11 skipping. However, the slightly lower activity of RBM4-SD was perhaps due to its higher tendency to cytoplasmic distribution. Fig. S3 shows that knock-down of RBM4 compromised differentiation-induced PTB exon 11 skipping. Fig. S4 shows that RBM4 could activate exon 11 skipping of a PTB minigene transcript. Fig. S5 shows that recombinant RBM4 could bind to different PTB mRNA fragments containing exon 11 and might even form multimers. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201007131/DC1>.

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