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RBM5 promotes exon 4 skipping of *AID* pre-mRNA by competing with the binding of U2AF65 to the polypyrimidine tract

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ABSTRACT

Alternative splicing is involved in functional regulation of the mutagenic enzyme activation-induced cytidine deaminase (AID). However, the molecular basis for AID splicing regulation remains undefined. Using a mini-gene-based screen in HeLa cells, we found that overexpression of RNA binding motif protein 5 (RBM5, or LUCA-15/H37) significantly promoted *AID* exon 4 skipping by suppressing the splicing of intron 3. The inhibitive effect of RBM5 on intron 3 splicing required a weak 3′-splice site (ss). Indicative of the underlying mechanism, RBM5 interfered with the binding of U2AF65 to the polypyrimidine tract at the 3′-ss in vitro. Our findings thus not only shed lights on the regulatory mechanism of *AID* exon 4 skipping, but also provide new insights into how RBM5 functions in splicing regulation.

1. Introduction

Activation-induced cytidine deaminase (AID) is essential to the generation of human immunoglobulin (Ig) diversification in two different processes, somatic hypermutation (SHM) and class switch recombination (CSR) [1,2]. AID is believed to induce DNA double-strand breaks (DSBs) in both Ig and non-Ig genes [3,4]. Deregulated expression of AID has indeed been reported to account for the accumulation of oncogenic mutations and chromosome translocations in various B-cell lymphomas [5,6].

Alternative splicing is a process occurred in nearly 95% of human multi-exon genes, by which diverse mRNA variants are generated from a single gene [7]. Several recent studies have indicated that alternative splicing is involved in functional regulation of AID by generating mRNA variants that encode proteins containing different domains [8–10]. For example, exclusion of exon 4 results in expressing a carboxyl-terminal truncated isoform of AID with hyperactivity in somatic hypermutation [9–11]. Interestingly, one study shows that AID splice variants are singly expressed at individual cell level [10], indicating that splicing regulation plays critical roles in modulating the activity of AID. However, the molecular basis for AID alternative splicing regulation still remains unclear.

Pre-mRNA splicing is started from the recognition of the 5′-ss by U1 snRNP and the binding of U2AF65/35 heterodimer to the 3′-ss [12,13]. Association of protein factors to pre-mRNAs that facilitates or impairs the binding of U1 snRNP and/or U2AF to the splice sites is a major mechanism of alternative splicing regulation [12,14]. In addition, decision of splice sites selection can also be influenced by dynamic rearrangements involving both RNAs and proteins during spliceosome assembly [15], and even affected by elongation rates of transcription [16].

In this study, we found that overexpression of RNA binding motif protein 5 (RBM5) stimulated exon 4 skipping in a human *AID* mini-gene. We further showed that the effect of RBM5 most likely resulted from a direct competition with U2AF65 for binding to the polypyrimidine tract at the 3′-ss of *AID* intron 3. Our findings provide insights into the molecular basis of *AID* splicing regulation, and also extend our understanding on the regulatory roles of RBM5 in alternative splicing.

2. Materials and methods

2.1. Cell culture and transfection

HeLa and HEK293 cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in presence of 5% CO_2. Cell transfection was performed using Lipofectamine 2000 transfection reagents according to the manufacturer's instructions (Invitrogen, USA).

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2.2. Plasmid construction

For *AID* mini-gene construct, a 1.4 kb genomic DNA fragment, spanning from exon 3 to a portion of exon 5 of human *AID* gene, was amplified from HeLa cells and inserted into HindIII/BamHI sites of pcDNA3 vector (Invitrogen, USA). For point mutations, we followed a PCR-driven overlap extension (OE-PCR) strategy [17]. Full-length cDNAs of human *PTBP1*, *RBM5*, *RBM6*, *RBM10*, *ROD1*, *SPF45*, *SRP20*, *hnRNPA1*, and *RNPS1* genes were amplified from HeLa cDNA library using Pfu Turbo DNA Polymerase (Stratagene, USA), and inserted into EcoRI/XhoI sites of the pcDNA3-flag vector. RBM5 expression construct with Glutathione-*S*-transferase (GST) and histidine dual tags was obtained by subcloning of *RBM5* cDNA into the pGEX-6P-1 vector (GE Healthcare).

2.3. RNA purification and reverse transcription polymerase chain reaction (RT-PCR)

Cells were harvested at 24 h post-transfection. Total RNAs were extracted using RNAiso plus (TaKaRa, Japan) according to the manufacturer's instructions. 2 μg of total RNAs were used for the first-strand cDNA synthesis by using reverse transcriptase M-MLV (Invitrogen, USA) and oligo$(dT)_{16}$. 1/10 of the synthesized cDNAs were used as templates in PCR reaction with following conditions: 94 °C, 3 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 30s at 72 °C. Amplified DNAs were analyzed on 2% agarose gel. The images were captured and analyzed on Alpha-Imager® system (Alpha-Innotech, USA).

2.4. Purification of recombinant protein from Escherichia coli

Expression constructs were transformed into *E. coli* BL21 (DE3) strain. Cells were grown in the presence of 0.05 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 h at 10 °C. Recombinant proteins were purified as described [18].

2.5. Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed essentially as described [19]. In brief, RNA probes were synthesized by in vitro transcription reactions using T7 RNA polymerase in the presence or absence of [γ-^{32}P]-UTP. The radiolabeled RNA probes ($\sim 1 \times 10^5$ cpm) were incubated with 0.2 pmol of recombinant RBM5 or GST control proteins in a 10 μl reaction. RNA–protein complexes were separated by 6% native PAGE followed by autoradiography.

3. Results

3.1. Overexpression of RBM5 promotes AID exon 4 skipping

To understand the regulatory mechanisms underlying alternative splicing event involving *AID* exon 4, we constructed a CMV promoter-driven *AID* mini-gene, spanning its genomic sequence between exon 3 and 5 (Fig. 1A, top panel). Twenty four hours after transient transfection, three alternatively spliced variants from the transcripts of mini-gene were detected in both HeLa and HEK293

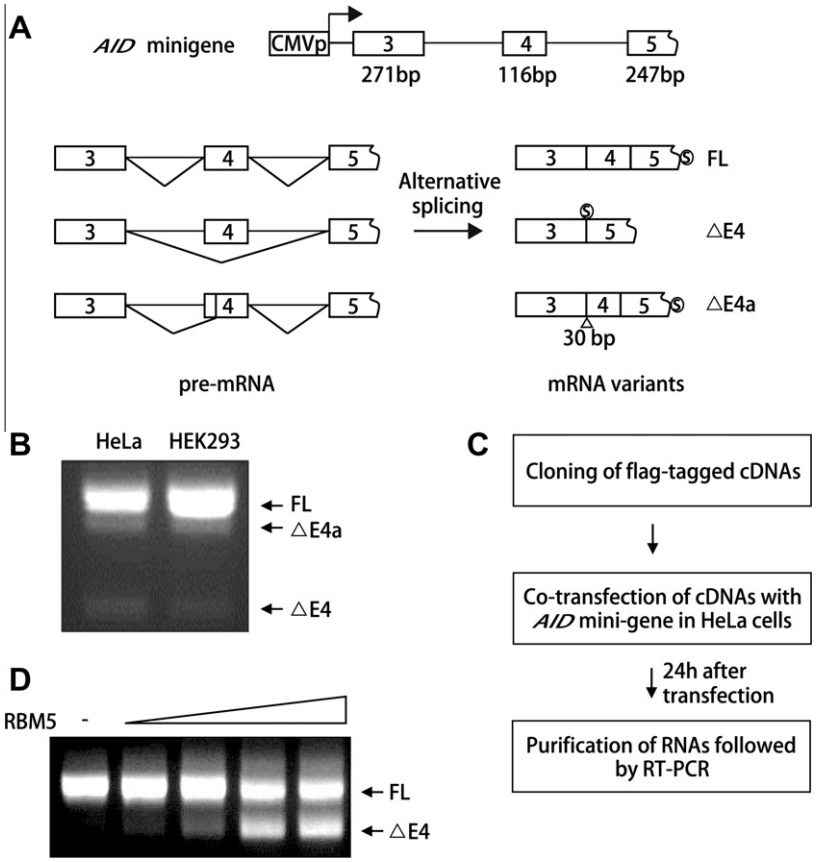

Fig. 1. Identification of splicing factor(s) promoting *AID* exon 4 skipping. (A) Alternative splicing pattern of transcripts from *AID* mini-gene. Upper panel: schematic diagram of *AID* mini-gene. Boxes represent exons, and lines represent introns. Lengths of exons are indicated. Lower panel: schematic diagram of alternative splicing patterns of *AID* mini-gene. FL: full-length spliced product; △E4: splice variant lacking exon 4; △E4a: splice variant lacking the first 30 bp of exon 4. Stop codon is indicated by a circled S. (B) Detection of splice variants from *AID* mini-gene by RT-PCR in HeLa and HEK293 cells. (C) Strategy of a candidate based screen for splicing regulator(s) promoting exon 4 skipping. (D) Overexpression of RBM5 induces exon 4 skipping in a dose-dependent manner. For 10^5 cells, 100 ng *AID* mini-gene was co-transfected with 0, 100, 300, 500 or 700 ng of RBM5 expression construct.

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cells by RT-PCR (Fig. 1A and B), analogous to what are generated from the endogenous *AID* pre-mRNA in human B cells [8–10].

We next set out to establish a candidate-based screen for potential splicing regulators that could promote exon 4 skipping in *AID* mini-gene (Fig. 1C). HeLa cells were co-transfected with *AID* mini-gene and individual splicing factor expression construct (PTBP1, RBM5, RBM6, RBM10, ROD1, SPF45, SRP20, hnRNPA1, and RNPS1). At 24 h after transfection, splice variants were monitored by RT-PCR. Among nine candidates, we found that overexpression of RBM5 markedly promoted exon 4 skipping of *AID* pre-mRNA in a dose-dependent manner (Fig. 1D).

3.2. RBM5 suppresses splicing of AID intron 3, but not intron 4

To gain more insight into the molecular basis for the effect of RBM5 on exon 4 skipping, we analyzed the splice variants of *AID* mini-gene by RT-PCR with primers specific for individual introns flanking exon 4 (Fig. 2A, upper panel). Consistent with its stimulative effect on exon 4 exclusion, overexpression of RBM5 resulted in a sharp increase on the retention of intron 3. However, no detectable effect on the splicing of intron 4 was observed upon RBM5 overexpression (Fig. 2A, lower panel). These data indicate that RBM5 promotes exon 4 skipping by suppressing the splicing of intron 3, but not intron 4.

3.3. A weak 3′-ss is required for RBM5-stimulated exon 4 skipping

We next analyzed the influence of splice site strength on RBM5-stimulated exon 4 skipping. Mutagenesis was first introduced at the 5′-ss to improve the base-pairing between 5′-ss and U1 snRNA (Fig. 2B, upper panel). Our data showed that strengthening the 5′-ss did not affect the activity of RBM5 (Fig. 2B, lower panel). In contrast, a point mutation from an adenine to a pyrimidine (U/C) at -3 position or a mutation from an adenine to a guanine at +1 position of the 3′-ss sharply compromised the stimulative effect of RBM5 on exon 4 skipping (Fig. 2C, lane 3–8). These effects were specific as a point mutation from adenine to guanine at -3 position had no effect on RBM5 function (Fig. 2C, lane 9 and10). Notably, all mutants except for the last one generate strong binding sites for U2AF35 at the 3′-ss of intron 3 [20], indicating that a weak 3′-ss in intron 3 is required for RBM5-stimulated exon 4 skipping.

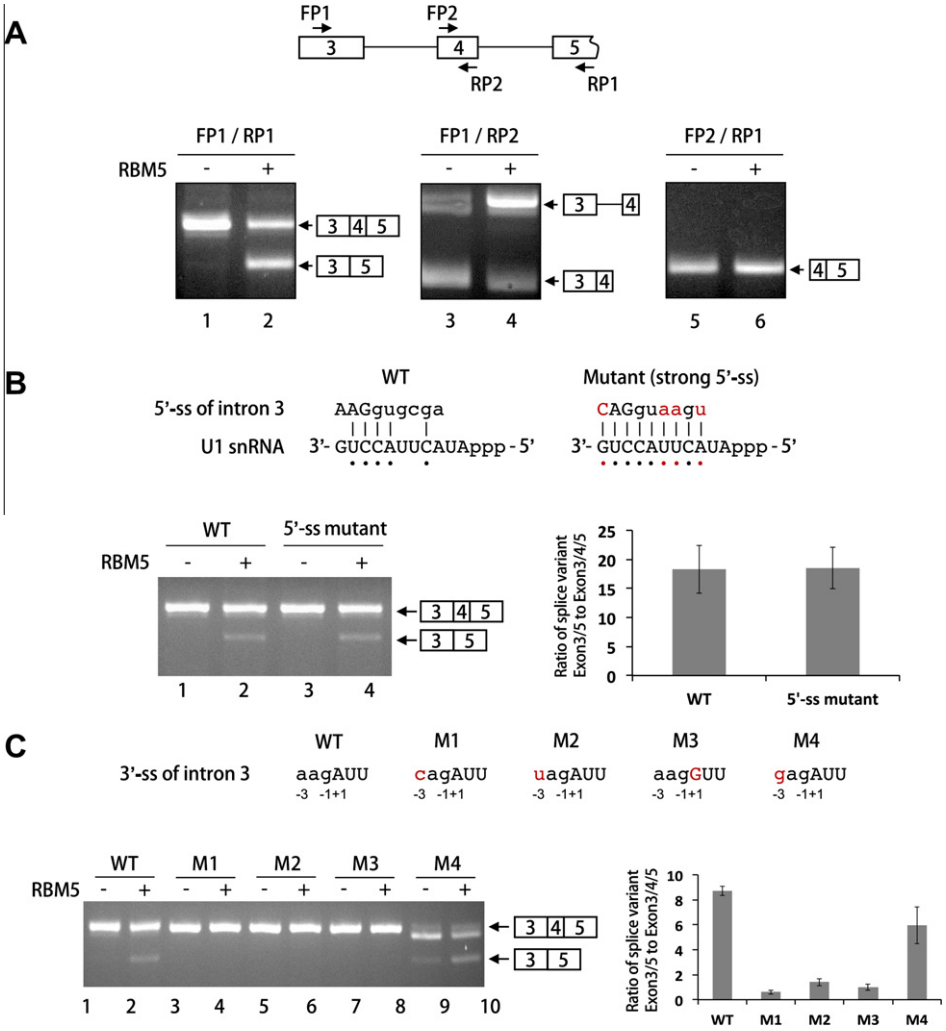

Fig. 2. A weak U2AF35 binding site is required for the suppressive effect of RBM5 on the splicing of *AID* intron 3. (A) RBM5 suppresses splicing of *AID* intron 3, but not intron 4. Locations of PCR primers are indicated in the upper panel. Alternative splicing events across the indicated introns were analyzed by RT-PCR. (B) Increase of the base pairing between U1 snRNA and the 5′-ss of intron 3 has no effect on RBM5-mediated exon 4 skipping. Sequences of 5′-ss in wild type (WT) and mutant mini-genes with their U1 snRNA base-pairing potential are shown in the upper panel. Exonic and intronic regions are indicated in uppercase and lowercase, respectively. Splice variants were analyzed by RT-PCR (lower panel). (C) A weak U2AF35 binding site at 3′-ss is required for suppressive effect of RBM5 on intron 3 splicing. Point mutations are introduced around the 3′-ss of intron 3 (upper panel). Alternative splicing events were monitored by RT-PCR (lower panel). Representative RT-PCR results are presented in lower left panel. Effect of RBM5 overexpression on AID exon 4 skipping is quantified by the amount of exon 4 skipped splice variant, which was normalized among samples by comparison with that of the RNA containing exon3/4/5 (lower right panel). Error bars represent standard deviations from at least three independent experiments.

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3.4. RBM5 directly interacts with AID pre-mRNA

RBM5 protein contains two putative RNA recognition motifs (RRMs) [21]. We next asked whether RBM5 regulates exon 4 exclusion by interaction with *AID* pre-mRNA. The RT-PCR results showed that mutagenesis at the conserved phenylalanine (F) residues to alanine (A) within the two RRMs significantly compromised the function of RBM5. Moreover, the N-terminal truncation mutant lacking of both RRMs entirely blocked the effect of RBM5 (Fig 3A and B).

To further confirm that RBM5 directly binds to *AID* pre-mRNA, we performed in vitro EMSA with recombinant RBM5 protein and individual radiolabeled RNA probe covering either entire mini-gene (p1), individual introns (p2 and p3) or exon 4 (p4) (Fig. 3C and D). Our data showed that RNA probes p1, p2 and p3 (at a less degree), but not p4, directly interacted with RBM5 (Fig. 3E). The interaction between RBM5 and *AID* pre-mRNA was further validated by EMSA in the presence of excessive amounts (20×) of unlabeled RNA probes as competitors (Fig. 3F). Notably, unlabeled p2 RNA probe competed with radiolabelled p1 probe in complex formation with RBM5 in the most efficient manner.

3.5. RBM5 competes with U2AF65 for binding to the polypyrimidine tract at the 3′-ss of AID intron 3 in vitro

Our data showed that overexpression of RBM5 suppressed splicing of AID intron 3, but not intron 4. We therefore decided to further characterize the role of the interaction between RBM5 and intron 3 in AID splicing. We found that shortening of intron 3 to an 80-nt region (p5) immediately upstream of exon 4 did not significantly affect the interaction between RBM5 and RNA (Fig. 4A and B). It has been demonstrated that RBM5 interacts with an intronic U/C-rich sequence in *caspase-2* pre-mRNA [19]. To

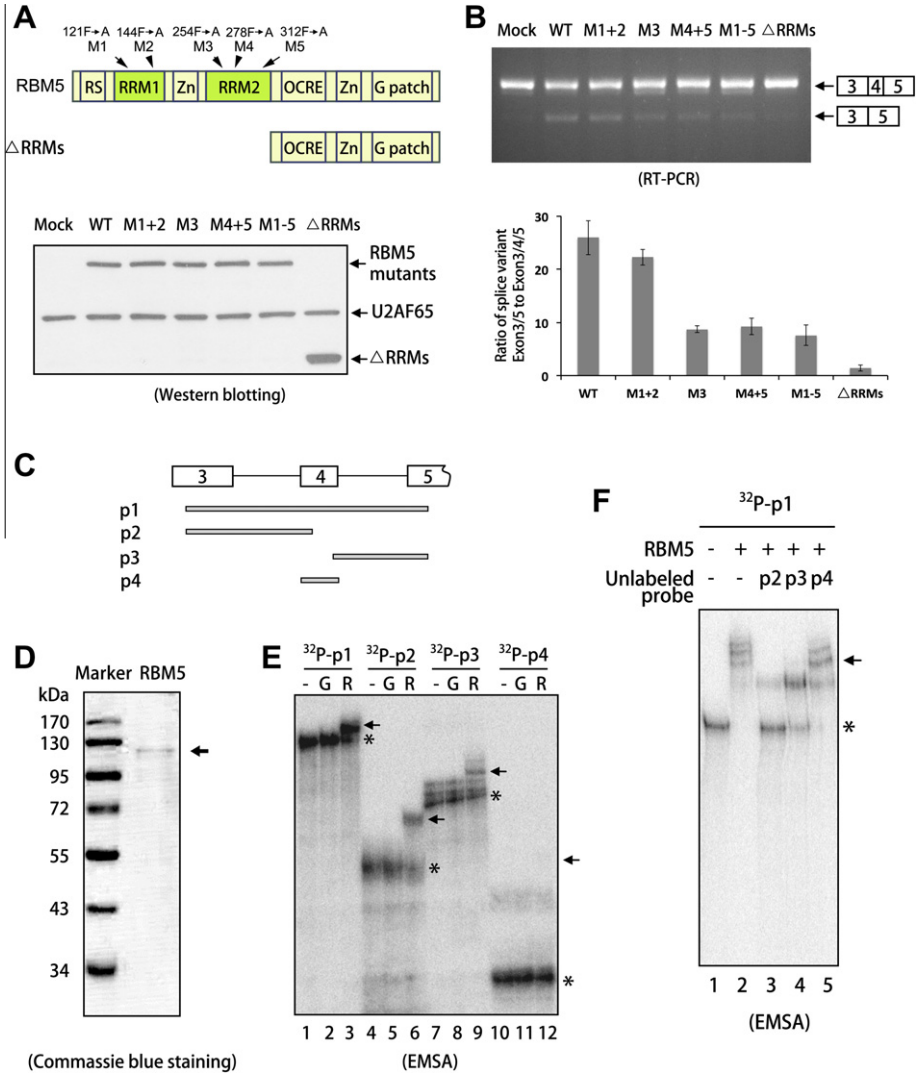

Fig. 3. RBM5 directly interacts with intron 3 of *AID* pre-mRNA in vitro. (A) Mutations of RBM5. Domain architecture of RBM5 and mutations (upper panel). Expression levels of the RBM5 mutants were detected by western blotting in transiently transfected HeLa cells (lower panel). (B) Mutations in RRMs compromised the effect of RBM5 on *AID* exon 4 skipping. Representative RT-PCR result is shown in the upper panel, and quantifications are shown in the lower panel. The effects of overexpression of RBM5 mutants on *AID* exon 4 skipping are quantified as described in Fig. 2. Error bars represent standard deviations from at least three independent experiments. (C) Schematic diagram of RNA probes used in EMSA. (D) Coomassie blue staining of recombinant RBM5 protein purified from *E. coli*. (E) Detection of RBM5-binding region in *AID* pre-mRNA by EMSA. The radiolabeled RNA transcripts were incubated with purified RBM5 protein (R), GST control protein (G) or buffer (−), respectively. The RNA–protein complexes were separated by native gel electrophoresis followed by autoradiography (lanes 1–12). (F) Competition EMSA. RBM5 protein was incubated with radiolabeled RNA probe p1 in the presence of excessive amounts (10X) of non-radiolabeled RNA transcripts as competitors. EMSA was performed as described in (E). RBM5–RNA complexes and free probes are indicated by arrows and asterisks, respectively.

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define RBM5 binding site further, we performed competition EMSA by using unlabeled RNA probes carrying either the wild type (p5) or U/C to G/A mutations at three different regions (m1–m3) within the 80-nt RBM5 binding sequence (Fig. 4A). Our data showed that, compared to m1 and m2 RNAs, U to G mutations in m3 markedly impaired its capacity in competing with wild type p5 RNA in RBM5 binding (Fig. 4B, lane 6). These data indicate that the U-rich element, mutated in m3, is critical for RBM5 binding although we would certainly not rule out the possibility that additional nucleotides might also be involved in RBM5 binding.

Notably, mutated U-rich element in m3 is overlapped with the polypyrimidine tract of intron 3. Binding of U2AF65 to the polypyrimidine tract is indispensible for 3′-ss definition [7]. By using EMSA, we showed that both RBM5 and GST-U2AF65 (Fig. 4C, lane 2 and 3), but not GST control protein (Fig. 4C, lane 7), formed RNP complexes with p5 RNA probe in vitro. Interestingly, existence of increasing amounts of RBM5 protein (lane 3–6), but not GST control protein (lane 8), led to a dramatic decrease of U2AF65–RNA complex formation (Fig. 4C), indicating that RBM5 directly competes with U2AF65 for binding to the polypyrimidine tract at the 3′-ss of intron 3 in vitro.

4. Discussion

By employing a mini-gene-based screen in HeLa cells, we demonstrated here that overexpression of RBM5 significantly promotes *AID* exon 4 skipping by suppressing the splicing of intron 3. The

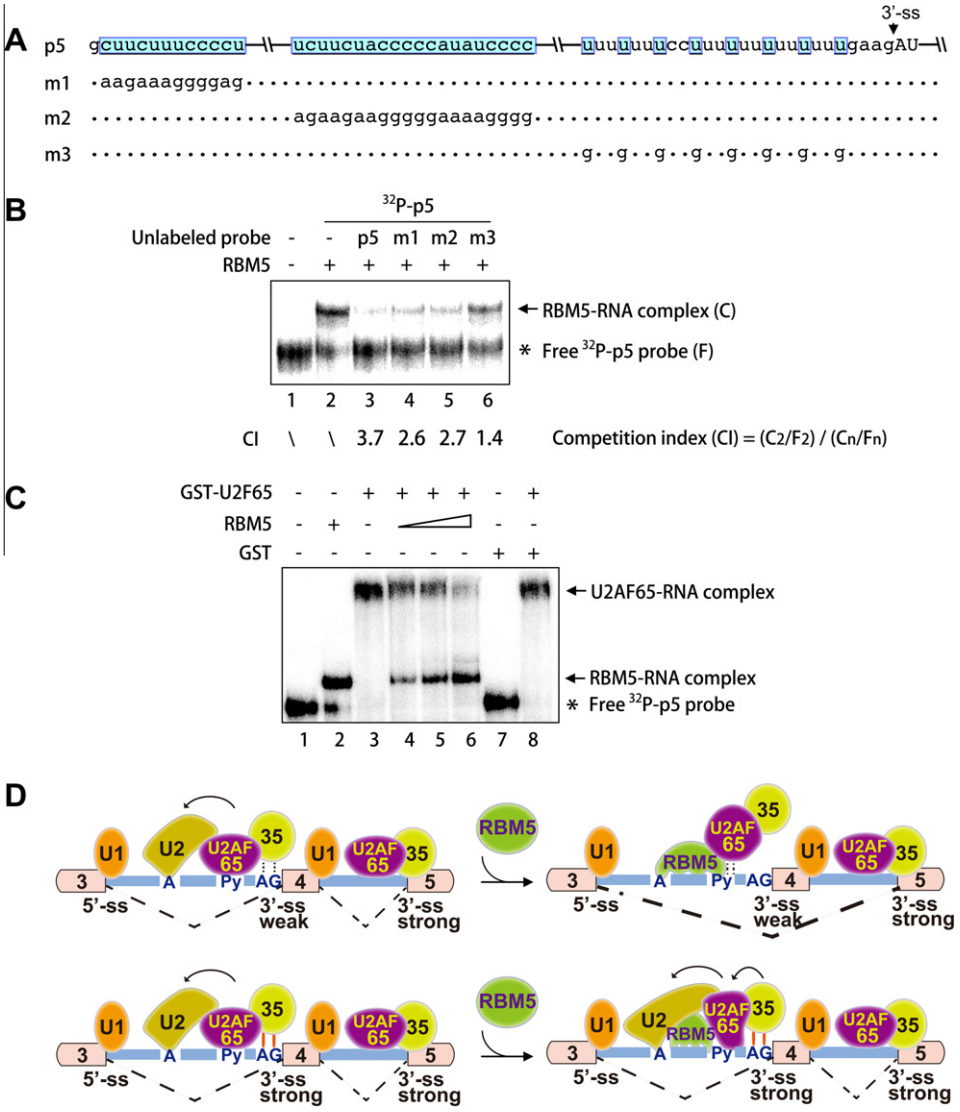

Fig. 4. RBM5 competes with U2AF65 for binding to the polypyrimidine tract. (A) Sequences of RNA probes containing the 3′-ss of *AID* intron 3. Mutated nucleotides and the position of the 3′-ss are indicated. (B) Competition EMSA was performed in the presence of radiolabeled probe p5, purified RBM5 protein and excessive amounts (20×) of unlabeled wild type (p5) or mutated (m1, m2 and m3) RNA probes as competitors. The representative photograph of the corresponding autoradiograph is shown, and the definition of competition index (CI) is depicted. (C) RBM5 prevents the interaction of U2AF65 with RNA probe p5. The radiolabeled p5 transcript was incubated with RBM5 (lane 2), GST (lane 7) alone or with 2 pmol of GST-U2AF65 in the presence of 0, 0.1, 0.2, or 0.4 pmol of recombinant RBM5 protein (lane 3–6) or 4 pmol of GST control protein (lane 8). Positions of RNA–protein complexes and free-labeled p5 RNA are indicated by arrows and asterisks, respectively. (D) A model for RBM5 function in promoting *AID* exon 4 skipping. Recruitment of U2AF65/35 heterodimer to the polypyrimidine tract and 3′ terminal AG dinucleotides of an intron is required for the recognition of 3′-ss and the binding of U2 snRNP at the branch point during prespliceosome assembly. RBM5 competes with U2AF65 for binding to the polypyrimidine tract of *AID* intron 3. This attenuates the association between U2AF35 and a weak binding site at the 3′-ss, affects prespliceosome formation across exon 4, impairs the recruitment of U2 snRNP to the branch point, and consequently promotes *AID* exon 4 skipping. Point mutations that generate strong binding sites for U2AF35 at the 3′-ss of *AID* intron 3 significantly antagonized the inhibitive effect of RBM5 on the splicing of intron 3 most likely due to stabilization of U2AF heterodimer at the 3′-ss.

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inhibitive effect of RBM5 on intron 3 splicing requires a weak U2AF35 binding site at the 3′-ss. Indicative of the underlying mechanism, RBM5 directly competes with U2AF65 for binding to the polypyrimidine tract in vitro. Recruitment of U2AF heterodimer to 3′-ss is a major determinant for 3′-ss selection [22]. Our data thus support a model that *AID* exon 4 exclusion can be modulated by RBM5 at early stage of spliceosome assembly by competing with U2AF65 binding at the polypyrimidine tract (Fig. 4D).

Two research groups have recently reported regulatory activities of RBM5 in alternative splicing of two proapoptotic genes, *Fas* and *Caspase-2* (*casp-2*) [19,23]. Both studies indicate that RBM5 regulates paring of splice sites after prespliceosome assembly via distinct mechanisms. In the case of *Fas* exon 6 splicing, RBM5 does not directly interact with pre-mRNA, but affects the U4/5/6 tri-snRNP recruitment most likely through protein–protein interactions [23]. In *Caps-2* exon 9 splicing, RBM5 directly binds to a U/C-rich RNA sequence in intron 9, similar to what was observed in *AID* splicing [19]. However, the binding site of RBM5 in C*asp-2* pre-mRNA is located immediately upstream of a well-defined intronic regulatory element In100, which contains a decoy 3′-ss [19]. It has been proposed that binding of RBM5 upstream to In100 might facilitate the formation of a pseudo-spliceosome complex between In100 and 5′-ss of intron 9. This could block spliceosome assembly across intron 9 and consequently promote the splice sites pairing between exon 8 and exon 10 [19]. Here we show that RBM5 is able to interfere with the binding of U2AF65 to the 3′-ss. Recruitment of U2AF complex at the 3′-ss is involved in a process termed exon definition, which is required for the initiation of prespliceosome complex formation across the spliced exon [12]. Our findings thus support a new model whereby RBM5 modulates splicing site selection by targeting molecular events that affect exon definition and prespliceosome assembly.

A similar mechanism for regulation of exon skipping has been reported in chicken *β-Tropomyosin* (*β-Tm*) pre-mRNA splicing [24]. In this case, the polypyrimidine tract binding protein (PTB), which also prefers binding to U-rich sequences within a pyrimidine-rich context, represses splicing of *β-Tm* exon 6b by directly interfering with the binding of U2AF65 to the polypyrimidine tract [24]. However, while both proteins have the capacities to compete U2AF65 binding, overexpression of PTB had only marginal effect on *AID* exon 4 skipping (data not shown). These results argue that RBM5 and PTB function through distinct molecular mechanisms in splicing regulation, consistent with our recent findings that RBM5, but not PTB, specifically interacts with and stimulates the helicase activity of DHX15, a component of U2 snRNP [18].

RBM5 has long been proposed as a candidate tumor suppressor located at cytoband 3p21.3, a chromosomal region frequently deleted in various cancer cells [25,26]. RBM5 can modulate expression of multiple genes controlling both apoptosis and cell proliferation [27,28]. However, up-regulation of RBM5 is also observed in some human neoplasms, suggestive of a potential promoting role of RBM5 in tumor progression [29,30]. Here we show that overexpression of RBM5 induces the expression of a putative oncogenic isoform of AID by promoting exon 4 skipping. These results provide insight for possible mechanism underlying a tumor-promoting function of RBM5. It will be of interest to further elucidate the potential pathological significance of RBM5-regulated alternative splicing events in future studies.

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