Biochemical and Biophysical Research Communications 444 (2014) 445–450

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The RNA recognition motif domains of RBM5 are required for RNA binding and cancer cell proliferation inhibition

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ARTICLE INFO

Article history:
Received 15 January 2014
Available online 29 January 2014

Keywords:
RBM5
RNA recognition motif
Cell proliferation
Apoptosis
RNA binding
Alternative splicing

ABSTRACT

RBM5 is a known putative tumor suppressor gene that has been shown to function in cell growth inhibition by modulating apoptosis. RBM5 also plays a critical role in alternative splicing as an RNA binding protein. However, it is still unclear which domains of RBM5 are required for RNA binding and related functional activities. We hypothesized the two putative RNA recognition motif (RRM) domains of RBM5 spanning from amino acids 98–178 and 231–315 are essential for RBM5-mediated cell growth inhibition, apoptosis regulation, and RNA binding. To investigate this hypothesis, we evaluated the activities of the wide-type and mutant RBM5 gene transfer in low-RBM5 expressing A549 cells. We found that, unlike wild-type RBM5 (RBM5-wt), a RBM5 mutant lacking the two RRM domains (RBM5-ΔRRM), is unable to bind RNA, has compromised caspase-2 alternative splicing activity, lacks cell proliferation inhibition and apoptosis induction function in A549 cells. These data provide direct evidence that the two RRM domains of RBM5 are required for RNA binding and the RNA binding activity of RBM5 contributes to its function on apoptosis induction and cell growth inhibition.

1. Introduction

RNA-binding motif protein 5 (RBM5) is a putative tumor suppressor gene, which maps to the human lung cancer tumor suppressor locus 3p21.3 [1,2]. Previous studies have shown that RBM5 was down-regulated in ras-transformed Rat-1 embryonic fibroblastic cells [3], human vestibular schwannoma cells [4], clinical breast cancer samples [5] and non-small cell lung cancer tissues [6]. Overexpression of RBM5 inhibits cell proliferation by promoting apoptosis in human lung cancer cells [7,8], human prostate cancer cells [9], Jurkat T cells [10] and breast carcinoma cells [11]. Together, these observations suggest that RBM5 inhibits cancer cell growth by promoting apoptosis.

RBM5 also plays a role in alternative splicing as an RNA binding protein. Structural analysis suggests that RBM5 contains two RNA recognition motif (RRM) domains (aa98–178 and aa231–315)[12]. Functional studies have demonstrated that RBM5 protein preferentially bound to poly(G) RNA homopolymer in vitro [13]. Moreover, RBM5 was reported to regulate the ratio of proapoptotic to antiapoptotic casp-2 splicing isoforms by directly binding to casp-2 pre-mRNA [14].

So far, it is not clear which domains of RBM5 are required for its RNA binding and apoptosis regulation activities. In this study, we hypothesized that the two putative RRM domains of RBM5 are required for RBM5-mediated cell growth inhibition, apoptosis regulation, and RNA binding. To investigate this hypothesis, we biochemically determined the role of the RRM domains in these RBM5-mediated functions by analyzing the effects of distinct deletion. Our data showed that the two RRM domains are required for RNA binding and the regulation of caspase-2 alternative splicing, which leads to the induction of apoptosis and cancer cell growth inhibition.

2. Materials and methods

2.1. Materials and plasmid constructions

The human RBM5 low expressing lung cancer cell lines, A549, was obtained from ATCC and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS. The DNA fragment encoding wild-type RBM5 (RBM5-wt, 815 aa) was cloned from MCF7 cDNA. The two RRM domains-deleted RBM5 (RBM5-ΔRRM, 650 aa), which lacked aa98–178 and aa231–315, was

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http://dx.doi.org/10.1016/j.bbrc.2014.01.102
0006-291X/

generated by overlap extension PCR. For mammalian expression, RBM5-wt and RBM5-ΔRRM were cloned into the Lenti-X pLVX-Puro vector (BD Biosciences Clontech). To generate the bacterial GST tagged fusion protein, RBM5-wt and RBM5-ΔRRM were cloned into pGEX-2T (Amersham Biosciences). HA-tagged expression vectors for RBM5-wt and RBM5-ΔRRM were generated by inserting the corresponding cDNA fragments into the correct reading frame of pCMV-HA (BD Biosciences Clontech).

2.2. Lentivirus production

For production of the lentivirus particles, 293T cells were transfected with the viral vector expressing RBM5 or RBM5-ΔRRM following the Lenti-X™ Lentiviral Expression Systems User Manual. At 5 h post-transfection, the medium was replaced with fresh DMEM containing 10% FBS, and cells were grown for an additional 48 h. Then, the culture suspension was harvested and filtered through a 0.45 μm filter before storage at −80 °C.

2.3. Establishment of stable transgenic cell lines

A549 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). At ~60% cell confluence, the medium was replaced with 10% FBS-DMEM containing the lentivirus and polybrene at 6.0 μg/ml for overnight transduction. At 12 h post-infection, the media was changed to fresh DMEM containing 10% FBS. After 24 h of incubation, cells were passaged three times with 10% FBS-DMEM containing 2 μg/ml puromycin. The positively screened cell lines were then expanded and RBM5 expression was detected via Western blot.

2.4. Western blot analysis

Western blot analysis was done according to the standard protocol [15]. The anti-RBM5 mouse pAb (Cat. No. SAB1406941) was purchased from Sigma–Aldrich. The anti-Bax RabMAb (Cat. No. 1063-1) and anti-Bcl-2 RabMab (Cat. No.1017-1) for apoptotic pathways were purchased from Epitomics.

2.5. Cell counts and soft agar assay

Cells were plated in triplicate in six-well plates (5000/well). After plating (day 0), and cells were counted at days 2, 4, 6 and 8 by TC10™ Automated Cell Counter. For soft agar assay, the cells were suspended in 1 ml of 0.3% melted agar in DMEM containing 10% FBS and plated in six-well plates (5000/well) overlayed with 0.5% agar in the same medium and were incubated for up to 3 weeks before staining with nitroblue tetrazolium and photography.

2.6. Immunofluorescence staining

For Edu labeling, Cells were seeded at 1000 cells/cm^2 on collagen-coated coverslips in 24-well plates and grew overnight. Cells were then incubated with BrdU (50 μM) for 2 h and fixed in 4% paraformaldehyde for 15 min at room temperature. After that, EdU and DAPI was stained using the Click-iT™ EdU imaging kit (Invitrogen, Carlsbad, CA) with Alex Fluor 555 according to the manufacturer's protocol.

For Actived caspased-3 labeling and Hoechst staining, cells were seeded at 1000 cells/cm^2 on collagen-coated coverslips in 24-well plates. After 24 h incubation, the cells were fixed for 10 min with formaldehyde (4%, v/v) and permeabilized with 0.05% (w/v) Triton X-100 in PBS for 10 min at room temperature. After that, cells was stained using the Anti-ACTIVE® caspase-3 pAb (Promega) with Alex Fluor 488 according to the manufacturer's protocol. To visualize the nucleus and cell morphology, the cells were stained with 5 μg/ml of Hoechst 33342 and 0.2 μM TRITC-phalloidin for 20 min at room temperature in PBS–BSA.

Images were taken using a 20× objective by the Leica DM2500 microscope. At least ten randomly chosen areas in every slide were taken. The numbers of stained cells (blue or green) that exhibited apoptotic features were determined by an observer blinded to experimental conditions for 300 cells for each cell pool.

2.7. Annexin V assay

Cells were plated at 2×10^6 cells/100-mm-diameter tissue culture dish, and grown overnight. Cell apoptosis was assessed by Annexin V-FITC staining using Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Cat. No. V13241, Invitrogen, Carlsbad, CA). The stained cells with Annexin V-FITC/propidium iodide were analyzed by BD Accuri C6 Flow cytometer (BD Biosciences) and FlowJo analysis software (Tree Star, Ashland, OR, USA).

2.8. Expression and purification of recombinant proteins

The recombinant plasmid pGEX-2T-RBM5 or pGEX-2T-RBM5ΔRRM was transformed into *Escherichia coli* BL21(DE3) competent cells (Novagen). Protein expression was induced with IPTG at a final concentration of 0.8 mM at 18 °C for overnight. The GST-tagged recombinant proteins were purified by 0.5 ml GST Trap HP column (GE Healthcare) according to the manufacturer's instructions. The purified fusion proteins were verified by Western blotting with the anti-GST mAb (Epitomics, China).

2.9. RNA binding assay

GST pull down and RT-PCR were combined to assess the RNA-binding potential of RBM5-wt and RMB5-ΔRRM in vitro according to a previously described protocol [16]. The resulting reverse transcriptase PCR products were subjected to electrophoresis through agarose gel and stained with Gelred (Biotium, Hayward, CA, USA).

2.10. RNA immunoprecipitation

For each RNA-IP experiment, 2×10^7 A549 cells were transiently transfected with plasmid expressing HA-RBM5 or HA-RBM5-ΔRRM. At 36 h post-transfection, Cells were then washed three times in ice cold PBS and pelleted. The RNA immunoprecipitation was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Proteins isolated from the beads were detected by Western blot analysis with the anti-HA mAb (Sigma–Aldrich). The binding RNA was extracted from beads using TRIzol (invitrogen), and detected by qRT-PCR. Primers for qPCR are as follows: Forward caspase-2 In100 (5′-CTT CCT TTC TGA GAA CTC T-3′), Reverse caspase-2 In100 (5′-AAA AAG ACA GAT CAA AAA TCC G-3′), Forward β-actin (5′-CTC CTC CCT GGA GAA GAG CTA-3′), Reverse β-actin (5′-CCT TCT GCA TCC TGT CGG CAA-3′). The data of retrieved RNAs is calculated as the ratio of IP/input using BioRad iQ5 software (for each experiment, $n > 3$).

2.11. Caspase-2 splicing assays

Cells were plated at 2×10^6 cells/100-mm-diameter tissue culture dishes and grown overnight. Total RNA were extracted using TRIzol (invitrogen). 2 μg total RNA was reverse-transcribed into cDNA. The PCR assay was performed using 1 μl of the cDNA with Forward caspase-2 (5′-TTG CAC AGT TAC CTG CAC ACC-3′) and Reverse caspase-2 (5′-GGTTCTTTCCATCTTGTTGGTC-3′). Amplified PCR

products were analyzed on 3% agarose gel. The images were captured and analyzed on Alpha-Imager system (Alpha-Innotech, USA).

3. Result

3.1. The RRM domains of RBM5 are essential for cell proliferation inhibition

The diagrams of RBM5-wt and RBM5-ΔRRM were shown in Fig. 1A. Three stable A549 cell lines expressing RBM5-wt (A549/RBM5-wt), RBM5-ΔRRM (A549/RBM5-ΔRRM) and vector control (A549/control) were established by lentivirus infection and puromycin selection. A549 cells have been shown to have very low RBM5 expression [7]. RBM5 expression levels in each stable cell line were analyzed by Western blot and shown in Fig. 1B.

To measure proliferation rates, proliferation curves were generated for the three stable cell lines described above over an 8-day period. By day 8, proliferation of A549/RBM5-wt cells was reduced by 76%, compared with A549/control cells. However, there was no significant difference in cell proliferation between A549/RBM5-ΔRRMs cells and A549/control cells at any of the analyzed time points (Fig. 1C).

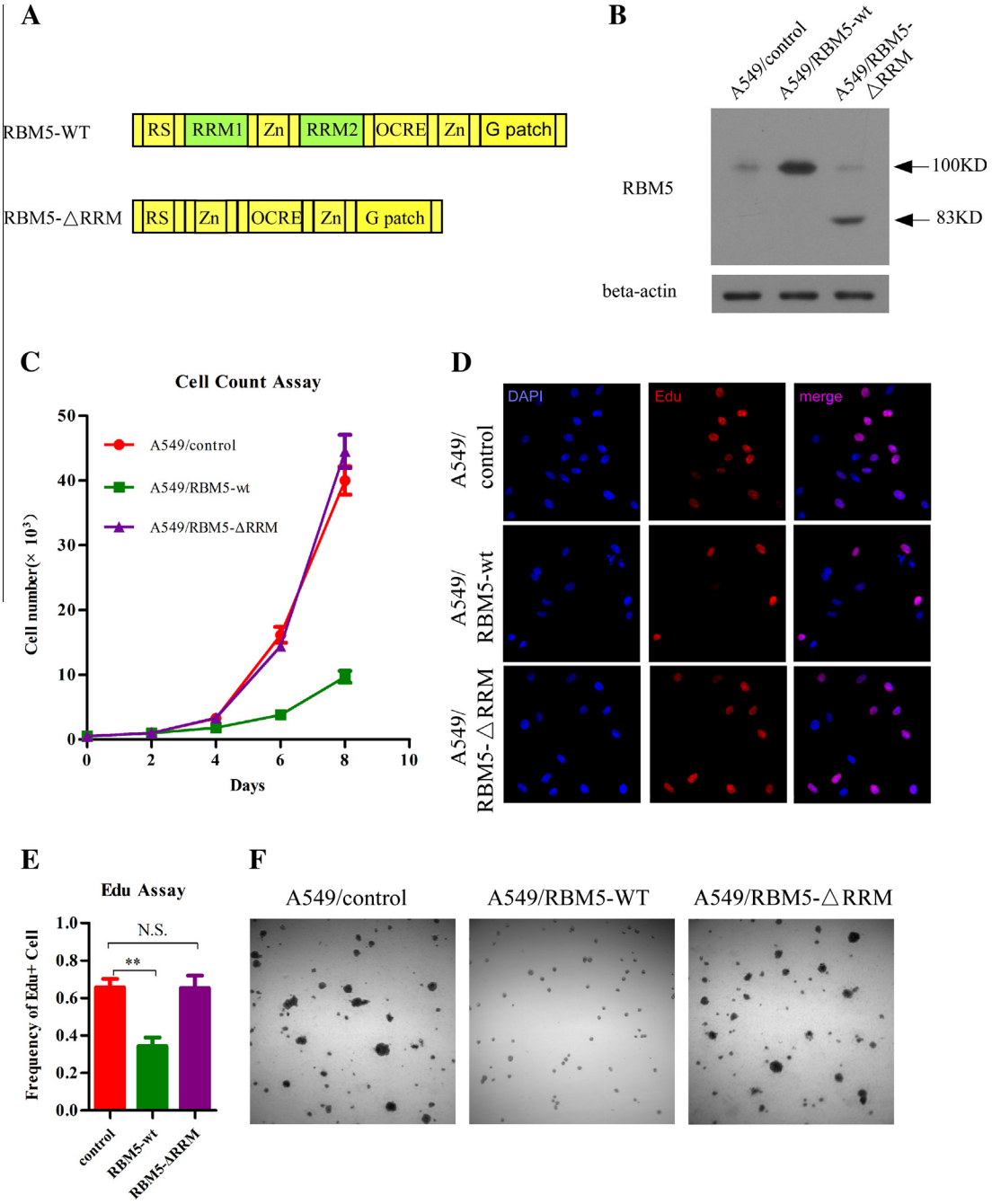

Fig. 1. The RRM domains of RBM5 are essential for cell proliferation inhibition. (A) Schematic diagram of RBM5 and the deletion mutant (RBM5-ΔRRM). (B) Western blot analysis of A549 cells stably transduced with empty vector (A549/control), RBM5-wt (A549/RBM5-wt) or RBM5-ΔRRM (A549/RBM5-ΔRRM) using anti-RBM5(upper panel) or anti-actin(lower panel) antibody. (C) Cell count assay was performed using A549 stable cell lines. (D) EdU incorporation assay was performed to identify proliferating cells in A549 stable cell lines. (E) Histogram shows percentages of Edu positive cells. Mean ± SE; $n > 3$; $^{**}p < 0.01$; N.S., no significance. (F) Colony formation of A549 stable cell lines in agar. The agar was seeded with 5×10^3 cells and solidified in six-well plates, and the colonies were stained and photographed after 3 weeks.

To further confirm the requirement for the RRM domains of RBM5 in cell poliferation, EdU incorporation assay was employed to identify proliferating cells by labeling newly synthesized DNA. The EdU-positive cells were defined as proliferating cells. As shown in Fig. 1D, the staining results showed that the lowest numbers of EdU positive cells were found in A549/RBM5-wt group. Quantitative analysis result (Fig. 1E) showed that the percentage of EdU-positive cells in A549/RBM5-ΔRRM group (56.74%) was not statistically significantly different from that in A549/control group (52.62%).

To evaluate whether the RRM domains of RBM5 are essential for its suppression of clonogenicity, we compared the ability of the three stable cell lines to form colonies in soft agar. This assay has been previously used to demonstrate tumor suppressor function of wild-type RBM5 [17]. After a 4-week observation period, the colonies formed by A549/RBM5-wt cells were less numerous and much smaller than those formed by A549/control cells and A549/RBM5-ΔRRM cells (Fig. 1F). These data demonstrated that RBM5-ΔRRM, unlike RBM5-wt, lacks cell proliferation inhibition function in human lung cancer line A549 cells. Together, these data indicated that the two RRM domains of RBM5 are required for its cell proliferation inhibition function.

3.2. The RRM domains of RBM5 are essential for apoptosis induction

Previous reports showed that RBM5 inhibited cell proliferation by promoting apoptosis in human lung cancer cells [7,8]. To evaluate whether the RRM domains of RBM5 are indispensible for cell apoptosis, Hoechst 33342 staining and active caspase-3 labeling were employed to detect apoptotic cells. Fig. 2A shows fluorescence images of cells stained with Hoechst 33342, Anti-ACTIVE® caspase-3 pAb and TRITC-phalloidin. As shown in the left panel of Fig. 2A, condensed and fragmented nuclei were observed in A549/RBM5-wt cells, which is the hallmark of the cells undergoing apoptosis. In contrast, both A549/control cells and A549/RBM5-ΔRRM cells remained healthy with normal nuclear structure with only rare chromatin condensation. We further confirmed this phenomenon using a more specific activated caspase-3 assay. In this assay, Anti-ACTIVE® caspase-3 pAb was used to detect activated caspase-3 in apoptotic cells. The result showed that apoptotic signals were found in cells of A549/RBM5-wt group, but not A549/RBM5-ΔRRM and A549/control groups. Furthermore, the apoptosis signals of the two assays were found in the same cells (Fig. 2A, middle panel). The detection results of apoptotic cells with Hoechst 33342 (Fig. 2B) and activated caspase-3 (Fig. 2C) staining analysis were quantified, and showed that RBM5-wt significantly induced apoptosis as compared with RBM5-ΔRRM and control group.

To confirm the microscopic observations, we analyzed the cells by flow cytometry after Annexin V and PI staining (Fig. 2D). The results showed that the number of Annexin V positive but PI-negative cells increased in A549/RBM5-wt group (15.2%) but not in A549/RBM5-ΔRRM group (3.77%) as compared with in A549/control group (3.66%). These data demonstrated that RBM5-ΔRRM, unlike wild-type RBM5, lacks cell apoptotic induction function in human lung cancer line A549 cells.

The ratio of pro-apoptotic (BAX) to anti-apoptotic (BCL-2) is a major checkpoint in the mammalian cell death pathway. Overexpression of wild-type RBM5 has been previously reported to up-regulate Bax and down-regulate Bcl-2 in A549 cells [7,8]. To further explore the effect of the RRM domains of RBM5 in inducing cell apoptosis, we next tested the expression levels of Bax and Bcl2 in cells overexpressing RBM5-wt, RBM5-ΔRRM and vector control. Similar to previous report, the overexpresion of wild-type RBM5 significantly up-regulated Bax and down-regulated Bcl2 as compared with their levels in A549/control cells. However, in contrast, the expression of Bax and Bcl2 had no significant difference between A549/RBM5-ΔRRM cells and A549/control cells (Fig. 2E). The unchanged ratio of BAX to BCL-2 in A549/RBM5-ΔRRM cells is consistent with its unnoticeable apoptosis induction. Together, these data demonstrated that the two RRM domains of RBM5 are required for its apoptosis induction function.

3.3. The RRM domains of RBM5 are essential for RNA binding

As noted in the introduction, RBM5 was believed to regulate caspase-2 alternative splicing by binding directly to casp-2 pre-mRNA, which regulated the apoptosis induction function of RBM5 [14]. Given the lack of apoptosis inducing and cell growth inhibition function of RBM5-ΔRRM, we hypothesized that RBM5-ΔRRM is defective in RNA binding, and RBM5-associated caspase-2 alternative splicing.

Thus, we employed GST pull down assay to isolate the RNA pool that binds to wild-type RBM5 or RBM5-△RRM in vitro. Reconstructed GST (negative control), GST-RBM5 and GST-RBM5-ΔRRM fusion proteins were expressed and purified from bacteria (Fig. 3A), bound to GST-agarose beads, and combined with total RNA isolated from A549 cells. The beads were extensively washed, and the RNA was extracted to make cDNA, which was then amplified by PCR and separated on agarose gels. The results showed that PCR products were presented in GST-RBM5 lane but not in GST-RBM5-ΔRRM lane (Fig. 3B), indicating that the two RRM domains of RBM5 are essential for RNA binding in vitro.

3.4. The RRM domains of RBM5 are essential for caspase-2 alternative splicing

For further verification, we evaluated the ability of wild-type RBM5 and RBM5-ΔRRM to coimmunoprecipitate casp-2 pre-mRNA in A549 cells. Casp-2 premRNA is the only known target RNA that directly interacts with RBM5. Consistant with previous data in vitro [14], immunoprecipitation (IP) of HA-tagged RBM5 from A549 cells specifically retrieved endogenous casp-2 pre-RNA with almost 10-fold enrichment to IgG IP, the negative control. However, in contrast, casp-2 pre-RNA failed to retrieve with HA-tagged RBM5-ΔRRM in A549 cells. IP of neither HA-tagged RBM5 nor HA-tagged RBM5-ΔRRM specifically retrieved β-actin RNA, a cytosolic mRNA that served as a negative control (Fig. 4A).

Previous study indicated that wild-type RBM5 binds to casp-2 pre-mRNA and regulates the balance of casp-2L versus casp-2S splicing isoform [14]. To investigate the function of the RRM domains of RBM5 in casp-2 alternative splicing, we employed RT-PCR to measure Casp-2 alternative splicing in A549/control cells, A549/RBM5-wt cells, and A549/RBM5-ΔRRM cells (Fig. 4B–D). The Casp-2L/Casp-2S ratio in A549/RBM5-ΔRRM cells was comparable to the level observed in A549/control cells, which indicates that there was no Casp-2 alternative splicing activity specifically associated with RBM5-ΔRRM. In contrast, there is a remarkable increase in A549/RBM5-wt cells as compared with in A549/RBM5-ΔRRM cells or in A549/control cells. These finding demonstrate that the two RRM domains of RBM5 are essential for caspase-2 alternative splicing.

4. Discussion

RBM5 is a putative tumor suppressor, which plays a critical role in the regulation of cell proliferation, apoptosis and cancer. In the past several years, extensive work has been done to elucidate the molecular mechanisms of RBM5 function. In 2006, Oh et al. proposed a model for the role of RBM5 in tumor suppression, suggesting that RBM5 may function as a post-transcriptional regulator in

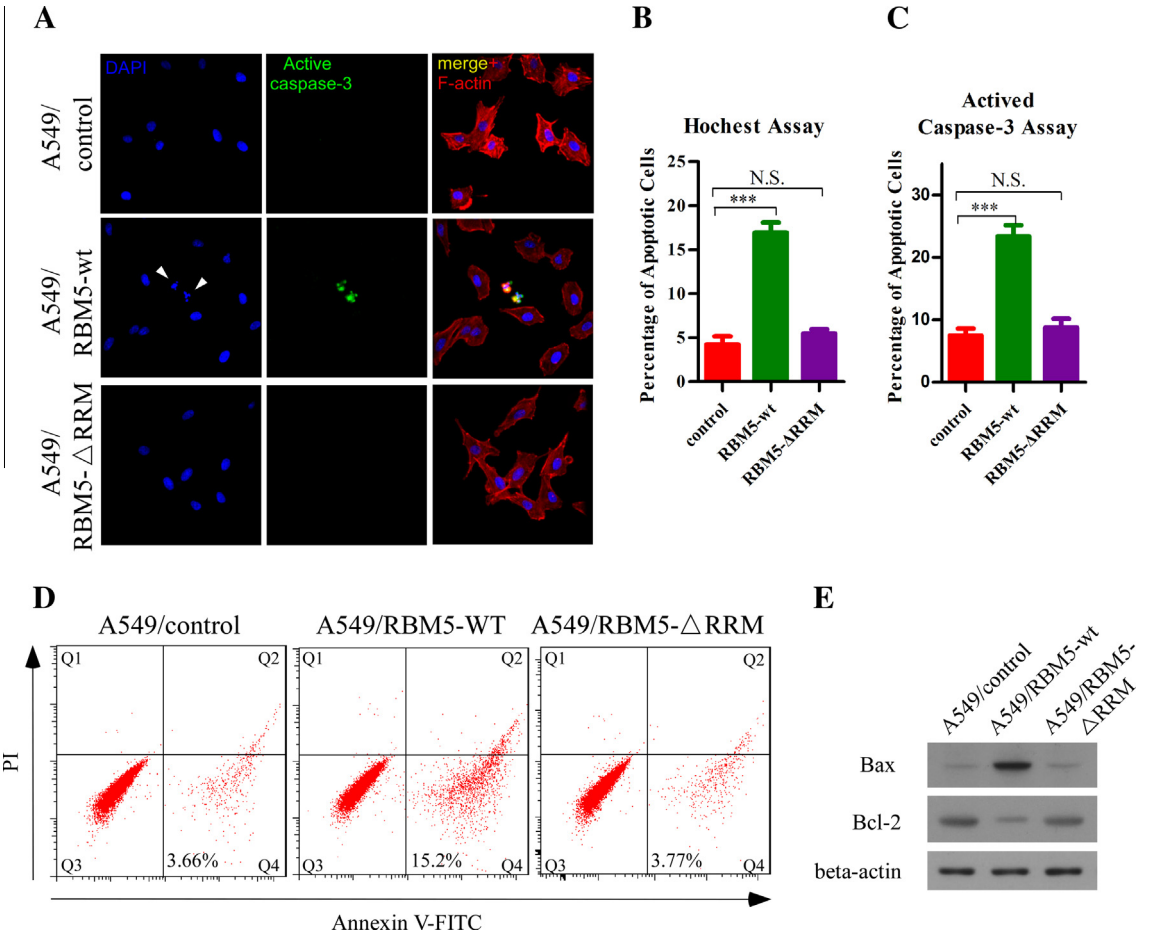

Fig. 2. The RRM domains of RBM5 are essential for apoptosis induction. (A) Analysis of apoptosis by Actived caspase-3 labeling and Hoechst staining. A549/control cells, A549/RBM5-wt cells and A549/RBM5-ΔRRM cells were labeled with Hoechst 33258 and anti-Actived caspase-3 antibody, then visualized with fluorescence microscopy. Both DNA fragment (arrows) and Actived caspase-3 positive (green) are illustrated as signs of apoptosis. (B) Histogram shows percentages of DNA fragment positive cells. (C) Histogram shows percentages of Actived caspase-3 positive cells. (D) Flow cytometric analysis of apoptosis after Annexin V and PI staining. A549/control cells, A549/RBM5-wt cells and A549/RBM5-ΔRRM cells were subjected to Annexin V and PI staining, followed by flow cytometry. Q1, normal cells. Q2, early apoptotic cells. Q3, late apoptotic cells. Q4, necrotic cells. (E) Western blot analysis was done to compare expression levels of Bax (top panel) and Bcl-2 (middle panel) protein in A549 stable cell lines. β-Actin was used as a protein loading control. (B and C) Mean ± SE; $n > 3$; $^{***}p < 0.001$; N.S., no significance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

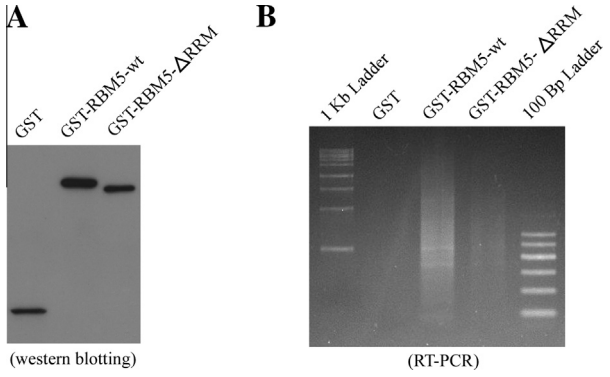

Fig. 3. The RRM domains of RBM5 are essential for RNA binding. (A) Western blot analysis of GST, GST-RBM5 and GST-RBM5-ΔRRM fusion proteins purified from *E. coli*. (B) RNA binding analysis. Reverse transcriptase PCR products of RNA pools bound by RBM5 GST fusion proteins encompassing the RBM5-wt and RBM5-ΔRRM were analyzed with an 1.5% agrose gel. The negative control is GST alone.

pre-mRNA splicing [7]. In support of this concept, Bonnal et al. demonstrated that RBM5 regulated the ratio of proapoptotic/anti-apoptotic Fas splicing isoforms by binding to the splicing factor U2AF [18]. Moreover, Fushimi et al. reported RBM5 binds to casp-2 pre-mRNA and regulates the balance of casp-2L versus casp-2S splicing isoforms [14]. However, it is still unclear about which domains of RBM5 are responsible for RNA binding and related functional activities. In the work described here, we hypothesized the two putative RNA recognition motif domains of RBM5 play important roles in RBM5-mediated cell growth inhibition, apoptosis regulation, and RNA binding.

To investigate this hypothesis, we constructed two recombinant proteins, wild-type RBM5 (RBM5-wt) and RBM5 with two RRM domains deleted (RBM5-ΔRRM), and elucidated the roles of the two RRM domains in RNA binding, apoptosis regulation, and cell growth inhibition. Our data indicated that the two RRM domains (aa98–178 and aa231–315) of RBM5 are essential for its ability to mediate cell proliferation inhibition (Fig. 1), induction apoptosis (Fig. 2), RNA binding (Fig. 3), and casp-2 alternative splicing activity (Fig. 4).

Our observation is consistent with the model that casp-2 pre-mRNA binding by RBM5 may contribute to its caspase-2 alternative splicing activity [14]. This model has important implications for understanding the molecular mechanism by which the RRM domains of RBM5 functions in apoptosis regulation. Since the impaired apoptosis regulation of RRM domains deleted RBM5

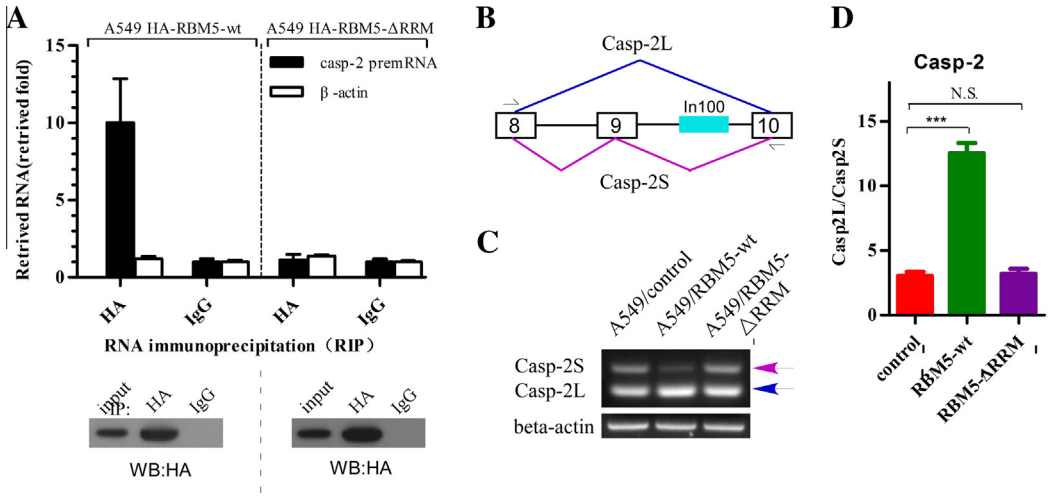

Fig. 4. The RRM domains of RBM5 are essential for caspase-2 alternative splicing. (A) qPCR analysis of caspase-2 premRNA retrieved by immunoprecipitation with HA-RBM5 or HA-RBM5-ΔRRM. β-Actin RNA was used as non-specific RNA control. Data (mean ± SE, $n = 3$) was relative to mock-IP (IgG). Bottom panel shows Western blot analysis of the immunoprecipitation of HA-RBM5 and HA-RBM5-ΔRRM. (B) Schematic diagram of the caspase-2 illustrating the formation of Casp-2L and Casp-2S splicing isoforms (exon, black box; intron, black line; blur box, RBM5-binding clusters). (C) In A549 stable cell lines, endogenous Casp-2 splicing isoforms and expression of β-actin (as a RNA loading control) were detected by RT-PCR with the Caspase-2 specific primers or β-actin primers. (D) Histogram represents quantification of casp-2 isoform ratio in A549 stable cell lines. Mean ± SE; $n > 3$; $^{***}p < 0.001$; N.S., no significance.

correlates with its failure to bind to RNA, and its inability to regulate Casp-2 alternative splicing, the up-regulation of A549 apoptosis activities by enforced RBM5 expression is likely associated with its RNA binding and alternative splicing modulation function. Similar to our result, Bonnal et al. reported that deletion of the carboxy-terminal region of RBM5 compromised both the association of RBM5 to U2AF complexes and the ability to induce Fas alternative splicing [18]. Based on these two observations, we speculate that RBM5 may link U2AF complexes and the target pre-mRNA by acting as a modular scaffold, assembling the prespliceosome A complex to the specific cis-acting element in pre-mRNA. Further studies are required to test this possibility.

In conclusion, we dissected the roles of the two RRM domains of RBM5 in its cell growth inhibition, apoptosis induction and RNA binding. The results demonstrated that the two RRM domains of RBM5 are responsible for RNA binding, and are indispensible for RBM5-mediated cancer cell proliferation inhibition. These findings provided more detailed molecular mechanism for the functions of RBM5.

Acknowledgments

This work was supported by National Natural Science Foundation of China Grants 31371325 and 81301946, Chinese 54th postdoctoral fund 2013M540467.

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