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The splicing factor RBM4 controls apoptosis, proliferation, and migration to suppress tumor progression

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Summary

Splicing dysregulation is one of the molecular hallmarks of cancer. However, the underlying molecular mechanisms remain poorly defined. Here we report the splicing factor RBM4 suppresses proliferation and migration of various cancer cells by specifically controlling cancer-related splicing. Particularly, RBM4 regulates Bcl-x splicing to induce apoptosis, and co-expression of Bcl-xL partially reverses the RBM4-mediated tumor suppression. Moreover, RBM4 antagonizes an oncogenic splicing factor, SRSF1, to inhibit mTOR activation. Strikingly, RBM4 expression is dramatically decreased in cancer patients, and RBM4 level is positively correlated with improved survival. In addition to providing mechanistic insights of cancer-related splicing dysregulation, this study establishes RBM4 as a tumor suppressor with therapeutic potentials and clinical values as a prognostic factor.

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Author Contributions

Y.W. and Z. W. designed experiments, interpreted the results and wrote the manuscript. Y.W., D. C., and H. Q. performed experiments. S. S. and Q. L. help to interpreted the data. Y. T., D. D., and Y.W. analyzed the RNA-seq data.

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Introduction

As one of the most prevalent mechanisms of gene regulation, alternative splicing (AS) plays a vital role in intricate regulation of protein function, and splicing dysregulation is closely associated with human cancers (David and Manley, 2010; Oltean and Bates, 2013; Venables, 2006). Accumulating evidence suggests that aberrant AS elicits control over major hallmarks of cancer, including apoptosis (Schwerk and Schulze-Osthoff, 2005), epithelial-mesenchymal transition (Warzecha et al., 2010), and tumor invasion and metastasis (Ghigna et al., 2008). The “cancerous” splicing variants of specific genes can serve as molecular markers of cancer (e.g. CD44, WT1) (Venables et al., 2008) or directly mediate cancer pathogenesis (e.g. BRCA1, p53) (Venables, 2006). However, mechanistic details underlying deregulated splicing in cancer are still limited.

AS is generally regulated by multiple *cis*-elements that recruit splicing factors to affect adjacent splice sites (ss) via various mechanisms (Matera and Wang, 2014; Matlin et al., 2005; Wang and Burge, 2008). Common splicing factors include serine/arginine-rich (SR) proteins that promote splicing by binding to exons but inhibit splicing by binding to introns (Erkelenz et al., 2013; Wang et al., 2013), and hnRNPs that positively or negatively control splicing in different pre-mRNA regions (Wang et al., 2012). The expression level, localization, and activity of splicing factors generally determine splicing outcomes in different tissues and cellular conditions. Therefore altered splicing factor activity is believed to be a main cause of splicing dysregulation in cancer (Bechara et al., 2013; Shkreta et al., 2013). For example, SRSF1 is a proto-oncogene that controls splicing of several cancer related genes including those in mTOR pathway (Blaustein et al., 2005; Karni et al., 2007). Since splicing dysregulation is one of molecular hallmarks of cancer (Oltean and Bates, 2013), specifically targeting splicing factors opens potential new avenues for cancer therapy (Dehm, 2013).

We have previously identified RNA-binding motif 4 (RBM4) as a binding factor for a group of intronic splicing regulatory elements that control AS of human genes (Wang et al., 2012). Initially identified by sharing nuclear import pathway with SR proteins, RBM4 shuttles between cytoplasm and nucleus but is mostly found in nuclear speckles (Lai et al., 2003) where most splicing events occur. Consistently RBM4 was shown to control AS of Tau and α-tropomyosin (Kar et al., 2006; Lin and Tarn, 2005). In addition, RBM4 was found to affect translation (Lin and Tarn, 2009; Uniacke et al., 2012). Multiple physiological functions were reported for RBM4, including mediating differentiation of muscle and pancreas cells (Lin et al., 2007; Lin et al., 2013). However, the involvement of RBM4 in tumorigenesis has not been reported. Here we systematically analyzed RBM4-mediated changes of transcriptome and assessed the role of RBM4 in cancer progression.

Results

RBM4 is a sequence-specific splicing inhibitor that regulates various AS events

Previously we identified several groups of intronic splicing regulatory elements and their cognate splicing factors (Wang et al., 2012; Wang et al., 2013). Among these factors, we demonstrated that RBM4 specifically binds to the GTAACG motif to inhibit splicing from

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introns (Wang et al., 2012). In addition, another RBM4 binding motif (CGG repeats) was also identified with CLIP-seq (Uniacke et al., 2012). Since AS is usually regulated in a context dependent manner, we sought to examine how RBM4 controls splicing when bound to distinct RNA motifs in different pre-mRNA contexts.

We generated four splicing reporters with candidate RBM4-binding motifs (GTAACG or CGGCGG) inserted in different regions to examine if RBM4 can specifically alter their splicing (Figure 1). First, we found that RBM4 specifically inhibited the inclusion of a cassette exon containing its cognate binding sites, whereas the control reporter was not affected (Figure 1A). Furthermore, RBM4 specifically suppressed inclusion of reporter exon with a downstream RBM4 binding site (Figure 1B). These results suggest that RBM4 functions as a general splicing inhibitor to specifically suppress splicing from both exonic and intronic contexts. Such activities are in contrast to DAZAP1, a splicing factor that recognizes the same GTAACG site but functions as a splicing activator (Choudhury et al., 2014). Interestingly, DAZAP1 does not affect splicing of exons containing a nearby CGGCGG site (Figure S1A and S1B), suggesting a partial overlap of binding specificity and an incomplete functional competition between RBM4 and DAZAP1.

Using splicing reporters containing RBM4-binding motifs between alternative 5′ ss or 3′ ss, we found that RBM4 reduced the use of the downstream 5′ ss (Figure 1C) or upstream 3′ ss (Figure 1D). The inhibition of distal alternative ss is again sequence specific, as RBM4 showed no effect on the control reporters (Figure 1C and 1D). Consistently, knockdown of RBM4 with shRNA had opposite effects by increasing exon inclusion of the same splicing reporters that contain RBM4-binding sites in various locations (Figure S1C–F). In addition, similar results were obtained in a different cell type (e.g. HeLa cells), indicating that the splicing regulation activity of RBM4 is not limited to a specific cell line (Figure S1G–J). Together these data demonstrated that RBM4 is a general splicing inhibitor that controls different types of AS when specifically binding to pre-mRNA.

Like many canonical splicing factors, RBM4 has a modular domain configuration. The N-terminus contains two RNA recognition motifs (RRMs) and a CCHC-type zinc finger that can specifically bind RNAs, while the C-terminus contains a low-complexity region (i.e. Ala-rich stretches) that can interact with other proteins (Lin and Tarn, 2009) (Figure 1E). To examine if RBM4 has a modular activity in splicing regulation, we fused the full-length, N- or C-terminal fragments of RBM4 to another RNA binding domain PUF (Wang et al., 2009). We co-expressed the fusion proteins with splicing reporters containing cognate PUF targets inside an alternative exon (Figure 1F) or at downstream intron (Figure 1G) and measured how splicing is affected. As expected, tethering the full length RBM4 to a target site inside an alternative exon suppressed exon inclusion. Surprisingly, tethering either the N- or C-terminal domain of RBM4 partially inhibited exon inclusion (Figure 1F), suggesting that the RNA binding fragment and the low-complexity domain both serve as functional modules. Such effect is sequence-specific, as these fusion proteins had no effect on control reporters with a non-cognate target. Consistently, the full length RBM4 inhibited exon inclusion when tethered to downstream of a cassette exon (Figure 1G). Interestingly, the N-terminal fragment partially inhibited splicing from an intron, whereas the C-terminus showed a slight splicing inhibitory activity (Figure 1G). Altogether, the N-terminal RNA-

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binding fragment and C-terminal low-complexity domain of RBM4 function cooperatively to control different types of AS events in a sequence specific manner.

Global regulation of transcriptome by RBM4 in cancer-related genes

To gain further insight into RBM4-regulated AS events and thereby its physiological functions, we conducted mRNA-seq with H157 cells expressing RBM4. With ~80 million 100-nt paired-end reads, we identified 473 RBM4-regulated AS events with an obvious change of percent-spliced-in (PSI) values (PSI ≥ 0.15). Figure 2A shows the read tracks of two examples. We found that various types of AS can be regulated by RBM4, including skipped exon (SE), alternative 5′ ss exon (A5E), alternative 3′ ss exon (A3E), retained intron (RI), mutually exclusive exons (MXE), and tandem UTR (TUTR) (Figure 2B, Table S1). Subsequent analysis indicated that most of the AS events were negatively regulated by RBM4 (decreased PSI value by RBM4 expression) (Figure 2C), consistent with our finding that RBM4 suppressed splicing when directly binding to its pre-mRNA targets (Figure 1A to 1D).

We further analyzed RNA motifs in RBM4-regualted pre-mRNAs by extracting the sequences near the RBM4-regulated SEs or between alternative 5′ ss of A5E. The relative abundance of RBM4 binding motifs (GTAACG and CGGCGG) in these regions was compared to control exons unaffected by RBM4 (Fairbrother et al., 2002). We found that RBM4-binding motifs are enriched near the SEs or A5Es negatively regulated by RBM4 (Figure 2D), consistent with the model that RBM4 directly recognizes these pre-mRNAs to control splicing. The AS events apparently promoted by RBM4 are likely due to indirect effects, as these exons lack known RBM4 binding motifs (Figure 2D).

When analyzing cellular functions of RBM4-regulated AS events using gene ontology, we found that RBM4 affects genes in RNA processing pathway including translation control, RNA processing, and mRNA metabolic process (Figure 2E). Such functional enrichment is not surprising since RBM4 is an RNA binding factor known to regulate splicing and translation. Intriguingly, RBM4 targets are also enriched with cancer-related functions such as regulation of NF-κB cascade and cell cycle. In addition, several RBM4-regulated AS events were found to regulate the apoptotic pathway. Although this enrichment of apoptosis is slightly below our significance cutoff, the changes of PSI value are fairly large and thus may have significant functional consequences. Many of the RBM4-regulated splicing targets were functionally connected into well-linked interaction networks as judged by STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) (Figure 2F). As expected, two large subgroups of RBM4 targets contain genes in translation control and RNA processing. Surprisingly, the other subgroup includes many genes in cell migration and adhesion (Figure 2F). Taken together, these results suggest that the biological processes affected by RBM4 are related to apoptosis, proliferation, migration and tumorigenesis.

We subsequently validated mRNA-seq results by measuring splicing change of 10 newly identified targets, which were arbitrarily selected to include genes with cancer related function. We confirmed that RBM4 either positively or negatively controls all endogenous AS events tested (Figure 2G), and that the relative changes of PSIs obtained from RT-PCR are highly correlated to those observed by mRNA-seq (Figure S2A, $R^2 = 0.6$). These events

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were also validated in another cell line (HeLa) (Figure S2B), suggesting that RBM4 can regulate AS with consistent activity across different cell types. In addition, we found that knockdown of RBM4 caused opposite changes of splicing in endogenous RBM4 targets, further confirming the reliability of our analyses (Figures S2C and S2D).

We also analyzed how RBM4 affects global gene expression. 185 genes were identified with significant expression change (>2-fold with adjusted $p < 0.05$) (Table S2). These genes are significantly associated with cancer related functions as judged by gene ontology (including DNA replication, chemotaxis, cell proliferation, response to wounding, cell cycle, and cell migration, Figure 2H), again suggesting that RBM4 is involved in cancer cell proliferation and migration. Many RBM4-regulated genes were also functionally connected into a densely-linked network that contains genes in regulating cell proliferation, wound healing, cell cycle and DNA damage (Figure 2I). The selected RBM4 targets were further validated with real time RT-PCRs in all events tested (Figure 2J). Taken together, all these data imply that RBM4 may be a key regulator of cell proliferation and migration, thus to control cancer progression.

RBM4 inhibits cancer cell proliferation and migration

To examine this possible role of RBM4 in cancer progression, we stably expressed RBM4 in a panel of human cancer cells, including H157 (lung cancer), MDA-MB-231 (breast cancer), SKOV3 (ovarian cancer), Panc-1 (pancreatic cancer), HepG2 (liver cancer), and PC-3 (prostate cancer) (Figure S3A). Strikingly, in all cancer cells tested, RBM4 inhibited anchorage dependent or independent growth as judged by colony formation or soft agar assay (Figure 3A). In addition, RBM4 inhibited cell migration of these cells in a would-healing assay (Figure 3B). Together the inhibition of cancer cell proliferation and migration by RBM4 suggests that it may function as a tumor suppressor.

We further analyzed how RBM4 affects cancer progression using NSCLC cells, which represents one of the most prevalent human cancers. The RBM4 levels are markedly decreased in a panel of NSCLC cells compared to normal bronchial cells (Figure 3C). Consistently, when re-expressed in a NSCLC cell line H157, RBM4 significantly inhibited cell growth (Figure 3D, $p=0.02$ by t-test). Similar growth inhibition by RBM4 was observed in 293T cells (Figure S3B and S3C). Interestingly, although both N- and C-terminus of RBM4 partially regulate splicing, lung cancer cell expressing either domain (1–177aa or 178–364aa of RBM4) displayed normal growth rates (Figure 3E), suggesting that both domains are required to suppress tumorigenesis.

To further assess whether RBM4 affects cancer growth *in vivo*, we determined if RBM4 re-expression can suppress tumor growth in a xenograft mouse model. We generated H157-luc-RBM4 cells and control cells with lentiviral vectors, and subcutaneously injected them into flanks of nude mice (left flank: RBM4; right flank: control). The growth of tumors was measured every three days for five weeks, and xenograft tumors were removed for final analysis. Consistent with the *in vitro* results, cells expressing RBM4 developed smaller tumors as compared to control cells (Figure 3F and 3G). In addition, the xenograft tumors with RBM4 re-expression grew much slower than controls (Figure 3H), suggesting that RBM4 substantially inhibits cancer progression *in vivo*. Altogether these findings indicate

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that RBM4 is a potent tumor suppressor that inhibits lung cancer progression both in cultured cells and in a tumor xenograft model.

RBM4 induces cancer cell apoptosis via regulating AS of Bcl-x

To determine the mechanisms of how RBM4 affects cancer progression, we focused on an RBM4 target gene, Bcl-x, an apoptosis regulator that produces two splicing isoforms with opposite functions. By alternative use of 5′ ss, Bcl-x is spliced as an anti-apoptotic isoform (Bcl-xL) or a pro-apoptotic isoform (Bcl-xS) (Adams and Cory, 2007). RBM4 expression appeared to shift the Bcl-xL into the Bcl-xS (Figure 2G). Such shift requires an entire RBM4, as neither the N- nor C-terminus can affect Bcl-x splicing by itself (Figure S4A). We identified a potential RBM4 binding site (CGGCGG) between the two alternative 5′ ss (Figure 4A), implying that RBM4 may control splicing through directly binding to Bcl-x pre-mRNA. Consistently, with an RNA-immunoprecipitation assay, we found that RBM4 indeed directly binds to the endogenous Bcl-x pre-mRNA but not the control pre-mRNA of another alternatively spliced apoptotic gene (Mcl1) (Figure 4B). Using a splicing reporter containing Bcl-x pre-mRNA, we found that the RBM4 binding is indeed dependent on the CGGCGG site, as mutation of this site abolished RNA-protein interaction (Figure 4C). Replacing the mutated sequence with the other RBM4-binding site (GTAACG) restored the interaction, confirming that RBM4 directly recognizes the exon extension region of Bcl-x.

In addition to H157 cells, an inducible expression of RBM4 also shifted splicing of Bcl-x in 293 cells (Figure 4D). This shift caused a rapid and robust decrease of Bcl-xL protein as judged by western blot (Figure S4B). To determine if the binding by RBM4 is responsible for observed splicing shift, we co-transfected RBM4 with a series of Bcl-x reporters containing various mutations near the alternative 5′ ss (Figure 4A). We found that RBM4 shifted the splicing of wild-type reporter by reducing Bcl-xL, and such regulation was not affected by three exonic mutations (mutations 1 to 3) (Figure 4E). However, the mutation of RBM4 binding site (mut 4) completely abolished the splicing regulation through RBM4, indicating that the RBM4 binding motif (CGGCGG) is indeed responsible for Bcl-x splicing switch. Importantly, replacing CGGCGG with another RBM4 binding site (mut 5) restored the regulation by RBM4 (Figure 4E), suggesting that binding of RBM4 to Bcl-x pre-mRNA is sufficient to shift splicing.

The two splicing isoforms of Bcl-x have opposite functions in controlling apoptosis (Adams and Cory, 2007). The Bcl-xL is the predominant isoform in cancer, and RNAi of Bcl-xL was shown to induce apoptosis in several cancer cell lines (Mercatante et al., 2001; Zhu et al., 2005). We found that expression of RBM4 in H157 cells substantially reduced the level of Bcl-xL protein, resulting in the cleavage of caspase 3 and PARP, two molecular markers of apoptosis (Figure 4F). Consistently, RBM4 dramatically increased spontaneous apoptosis as judged by flow cytometry (Figure 4G and Figure S4C). These results support the model that sequence-specific binding of RBM4 to Bcl-x pre-mRNA shifts its splicing from anti-apoptotic Bcl-xL to pro-apoptotic Bcl-xS, thereby promoting cancer cell death.

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RBM4 suppresses tumor progression in part through Bcl-x

Since RBM4 may inhibit cancer proliferation through modulating Bcl-x splicing, we next examined if co-expression of Bcl-xL, but not other similar apoptotic regulators, can overturn the tumor suppressor activity of RBM4. We stably transfected the parental H157 line containing re-expressed RBM4 with Bcl-xL or another apoptotic inhibitor Mcl-1 (Figure 5A), generating a cell line with partially restored Bcl-xL/Bcl-xS ratio and reduced PARP cleavage (Figure 5B). We found that cells expressing RBM4/Bcl-xL grew much faster than those expressing RBM4 alone, although the growth rate was not fully restored compared to control (Figure 5C). However cells expressing RBM4/Mcl-1 showed a similar growth rate as compared to cells expressing RBM4 alone (Figure 5C), indicating such phenotypical rescue is specific for Bcl-xL. In addition, cancer cells expressing RBM4/Bcl-xL migrated significantly faster than cells expressing RBM4 alone or RBM4/Mcl-1 (Figure 5D), again suggesting that restoring Bcl-xL level partially reversed the RBM4 phenotype. Consistently, the xenograft tumors generated from RBM4/Bcl-xL cells are significantly larger than those from RBM4/vector cells, indicating that reducing Bcl-xL level is partially responsible for RBM4-mediated tumor suppression *in vivo* (Figure 5E). This phenotypic rescue is robust and statistically significant, although it could not fully restore tumor progression probably due to partial reverse of Bcl-xL/Bcl-xS ratio (Figure 5B).

We further applied a specific Bcl-xL inhibitor (WEHI-539) in cells expressing RBM4 and examined its effect on cell growth. Consistent with previous reports (Lessene et al., 2013), WEHI-539 did not significantly affect the viability of control cells. However, WEHI-539 treatment inhibited the proliferation of RBM4-expressing cancer cells as compared to untreated cells (Figure 5F and 5G). Such apparent synergistic effect may reflect two mechanisms that are not mutually exclusive: (1) Through splicing regulation, RBM4 reduces the level of Bcl-xL to the extent where the WEHI-539 can have a detectable effect; (2) RBM4 inhibits cell proliferation through other mechanisms in addition to reducing anti-apoptotic Bcl-xL, whereas WEHI-539 specifically inhibits Bcl-xL. By targeting parallel pro-survival pathways, the combination of RBM4 and WEHI-539 synergistically suppressed cancer cell proliferation.

Consistently, we found an increased expression of Bcl-xL in lung cancers, breast cancers and pancreatic cancers, which is inversely correlated to RBM4 level (Figure 5H, S5A and S5B). This finding further supported that RBM4 inhibits tumor progression (at least partially) via controlling Bcl-x splicing.

RBM4 antagonizes oncogenic SRSF1 to inhibit mTOR activation

Although our data clearly demonstrate that RBM4 suppresses cancer progression by modulating Bcl-x splicing, this may not be the only mechanism as co-expression of Bcl-xL partially reversed the phenotype of RBM4. To eliminate the apoptosis effect, we treated cells with a pan-caspase inhibitor, Z-VAD. We found that, even when the apoptosis was strongly inhibited (Figure 6A), proliferation and migration of cancer cells were still significantly suppressed by RBM4 (Figure 6B). This observation suggests that RBM4 might also inhibit cancer progression through other mechanisms besides regulating apoptosis.

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It was previously reported that the general splicing factor SRSF1 functions as proto-oncogene to transform rodent fibroblasts (Karni et al., 2007). We found that RBM4 interacted with SRSF1 in a co-IP assay (Figure S6A). Remarkably, RBM4 can reduce the protein level of SRSF1 in a dose dependent manner (Figure 6C). Such inhibition is specific to SRSF1, as two other splicing factors, DAZAP1 and hnRNPA1, were not affected (Figure 6C). Similar results were also obtained in a cell line with inducible expression of RBM4 (Figure S6B). Since SRSF1 is a well-characterized oncogenic factor to promote tumorigenesis through multiple pathways (Anczukow et al., 2012; Karni et al., 2007), our observation suggests that RBM4 may also inhibit cancer progression via antagonizing SRSF1.

SRSF1 was known to control multiple AS events that promote tumorigenesis (Anczukow et al., 2012; Karni et al., 2007). For example, BIN1 is a tumor suppressor that binds to MYC (Sakamuro et al., 1996), and SRSF1 promotes inclusion of BIN1 exon 12a to generate a BIN1+12 isoform that lacks tumor suppressor activity (Karni et al., 2007); SRSF1 also inhibits the exclusion of exon 11 in RON, generating RONΔ11 that promotes cell migration and invasion (Anczukow et al., 2012). We examined whether RBM4 could affect the splicing of cancer-related SRSF1 targets using cells stably expressing SRSF1, RBM4, or SRSF1/RBM4. As expected, RBM4 regulated splicing of both BIN1 and RON in an opposite fashion to SRSF1, shifting their splicing towards anti-oncogenic isoforms (Figure 6D and S6C).

SRSF1 was also reported to activate mTOR pathway by increasing phosphorylation of S6K1 and 4E-BP1, as well as promoting oncogenic S6K1 splicing isoform 2 (Karni et al., 2007; Karni et al., 2008). Co-expression of RBM4 with SRSF1 substantially inhibited SRSF1-induced mTOR activation, as judged by the dramatic reduction in the phosphorylation of S6K1 and 4E-BP1 (Figure 6E). However, phosphorylation of two upstream components of mTOR signaling pathway, Akt and Erk, are not affected by RBM4. Interestingly, co-expression of RBM4 also reduced the S6K1 isoform 2 (Figure 6E and S6D), an SRSF1-induced oncogenic variant that induces cell transformation through activating mTOR pathway (Ben-Hur et al., 2013).

In addition, expression of RBM4 alone also inhibits the insulin-induced mTOR activation as judged by reduced phosphorylation of S6K1, 4E-BP1, and mTOR itself (Figure 6E). The phosphorylation of Erk and Akt was not affected, suggesting that RBM4 controls downstream stages of mTOR activation. This observation indicates that RBM4 can also directly inhibit mTOR activation. Since mTOR pathway plays key roles in promoting cell proliferation, inhibition of mTOR by RBM4 probably contribute to its tumor suppressor activity.

SRSF1 is also predicted to bind the same CGGCGG site in Bcl-x pre-mRNAs (Figure S6E and 4A), indicating that RBM4 and SRSF1 may counteract with each other in tuning the Bcl-x splicing switch. We co-expressed the Bcl-x splicing reporters (wild-type and CGGCGG mutation) with RBM4, SRSF1, or RBM4/SRSF1. As expected, SRSF1 promoted the anti-apoptotic Bcl-xL isoforms, whereas RBM4 reduced Bcl-xL. In addition, co-expression of RBM4 can overturn the activity of SRSF1 to increase the pro-apoptotic Bcl-xS

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isoform (Figure 6F). Consistently, when the binding site (CGGCGG) was mutated, the splicing regulation of Bcl-x by both RBM4 and SRSF1 were abolished, confirming that they control Bcl-x splicing through competing the same regulatory element.

We further applied colony formation and soft agar assays with cancer cells expressing SRSF1 and SRSF1/RBM4. Compared to controls, H157 cells expressing SRSF1 formed more and larger colonies in both assays, whereas co-expression of RBM4 with SRSF1 significantly reduced colony number and size (Figure 6G), indicating that RBM4 could inhibit cancer cell proliferation through antagonizing SRSF1.

RBM4 is reduced in NSCLC patients and positively correlated with survival

To further study the role of RBM4 as a tumor suppressor, we examined its clinical relevance in cancer patients. We first analyzed the microarray dataset from various large-scale studies (Buchholz et al., 2005; Finak et al., 2008; Selamat et al., 2012), and found that tumors collected from NSCLC patients have significantly decreased RBM4 level compared to normal controls (Figure 7A). Similar reduction of RBM4 was also observed in other cancers, including breast and pancreatic cancer (Figures S7A and S7B), consistent with the notion that RBM4 is a tumor suppressor in various human cancers.

Next we surgically collected paired NSCLC samples and adjacent normal tissues from seven patients to measure RBM4 levels. We found that, compared to the paired normal tissues, all 7 primary NSCLC specimens have substantially reduced RBM4 expression in the level of mRNA and protein (Figure 7B and 7C). Such reduction was independently validated by immunohistochemistry assay of 110 clinical samples (Figure 7D and 7E). We found that, compared to non-cancerous tissues (n=40), RBM4 was noticeably reduced in both lung squamous cell carcinoma (n=30) and adenocarcinoma (n=40): The RBM4 staining was undetectable in 57 of 70 (81%) NSCLC samples, and weak staining was detected in 13 tumors (19%). In contrast, most normal lung samples (39 of 40) exhibited strong or weak staining for RBM4.

Compared to paired normal tissues, the splicing of Bcl-x is shifted in tumor samples with Bcl-xL being the predominant isoform (Figure 7F). In addition, the protein levels of Bcl-xL and SRSF1 are substantially increased in NSCLC samples as compared to paired normal tissues (Figure 7G), further supporting our findings using cultured cancer cells. Taken together, these clinical observations strongly support the RBM4-mediated tumor suppression model derived from cell culture and animal studies.

To further investigate the clinical significance of RBM4 in lung cancers, we used a survival analysis tool, Kaplan-Meier Plotter, to analyze the overall survival of cancer patients with different RBM4 levels using datasets from GEO, EGA and TCGA consortium (Gyorffy et al., 2012). Strikingly, higher expression of RBM4 was closely associated with improved overall survival in patients with lung cancers (Figure 7H), breast cancers (Figure S7C), and ovarian cancers (Figure S7D), indicating that RBM4 might be recognized as an independent prognostic factor for cancer survival. This result validated the mechanistic link between reduced expression of RBM4 and cancer progression, supporting the conclusion that reduced RBM4 expression affects human cancer progression and patient survival.

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Discussion

Previously RBM4 was reported to promote inclusion of alternative exons in tau and α-tropomyosin (Kar et al., 2006; Lin and Tarn, 2005). Using various reporters we demonstrate that RBM4 predominantly functions as a splicing suppressor when directly bound to pre-mRNA. Over-expression of RBM4 in H157 cells inhibited exon inclusion in ~60% of cassette exons detected, further supporting RBM4's role as a splicing suppressor. However, a smaller fraction of AS events are positively regulated by RBM4, and we speculate that the splicing enhancement by RBM4 is likely a result of more complicated mechanisms other than direct binding between pre-mRNA and RBM4 (such as indirect effects through other genes, RNA structures, or by functioning together with other splicing factors). For example, RBM4 promoted exon inclusion of RON (Figure 6D and S6C), which probably is due to an indirect effect resulted from reduction of SRSF1 by RBM4.

Both N-terminal RNA-binding fragment or C-terminal low-complexity domain of RBM4 were sufficient to inhibit splicing from exons, while the N-terminal fragment had partial splicing inhibitory activity from introns. These results present an unusual regulatory mode compared to typical splicing factors. Many splicing factors, like SR proteins, hnRNP A1, and DAZAP1, contain a separate RNA binding module to recognize targets and an independent functional module to control splicing (Choudhury et al., 2014; Del Gatto-Konczak et al., 1999; Graveley and Maniatis, 1998). RBM4 may represent another class in which both fragments can inhibit splicing but the entire protein harbors a stronger activity, indicating that the two fragments cooperatively control splicing rather than function in a modular fashion.

Besides splicing regulation, RBM4 was also shown to regulate translation by suppressing cap-dependent translation (Lin and Tarn, 2009), activating internal ribosomal entry site (IRES)-mediated translation under cell stress (Lin et al., 2007), or mediating an oxygen-regulated translation switch (Uniacke et al., 2012). As an RNA binding protein shuttling between the nucleus and cytoplasm, it is possible that RBM4 may affect cell growth through controlling both translation and splicing. Future investigations should be conducted to determine whether the translation regulation by RBM4 contributes to its tumor suppression and how such process interacts with splicing regulation.

Our results support a model that RBM4 shifts splicing of Bcl-x to control the balance between pro- and anti- apoptotic pathways, and thus suppresses cancer progression. Consistently, the shift of Bcl-x splicing is correlated with RBM4 reduction in cancer patients, and co-expressing the anti-apoptotic variant Bcl-xL partially reverses the tumor suppression by RBM4 *in vitro* and in mouse models. Since the splicing switch of Bcl-x is associated with many cancers to protect cancers against apoptotic signals (Danial, 2007), modulating Bcl-x splicing has been shown to be a potential therapeutic intervention for cancer (Bauman et al., 2010; Shkreta et al., 2008; Villemaire et al., 2003).

Other RBM4-regulated events may also contribute to tumor pathogenesis and progression. With genomic analyses we found RBM4 affected many other AS events in cell metabolism and cell cycle regulation. For example, RBM4 affects splicing of CD44, which is known to

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mediate cell migration, tumor progression and metastasis (Williams et al., 2013). The choice of CD44 splicing variants has long been found with oncogenic roles in different cancers (Brown et al., 2011; Yae et al., 2012). These results suggest that RBM4 mediates multiple AS events critical to cancer progression. As a master regulator of cancer-related AS, RBM4 potentially inhibits tumorigenesis through multiple oncogenic pathways, which helps to explain why co-expression of Bcl-xL partially reversed the phenotype of restoring RBM4 level in cancer.

In addition to regulating Bcl-x splicing, RBM4 probably also inhibits tumor progression by antagonizing SRSF1 that is known to promote cell proliferation and delay apoptosis. The reduction of SRSF1 by RBM4 inhibits the oncogenic activity of several SRSF1 targets, including the proto-oncogene RON and tumor suppressor BIN1. More importantly, RBM4 counteracts with SRSF1 to mediate the activation of mTORC1 pathway. Taken together, the antagonism between two splicing factors, tumor-suppressor RBM4 and proto-oncogene SRSF1, presents a delicate functional balance that controls multiple splicing targets and signaling pathways critical to cancer proliferation.

Splicing dysregulation was recently been recognized as a major molecular hallmark of human cancer (David and Manley, 2010; Oltean and Bates, 2013). Some dysregulated AS events can serve as molecular markers of cancer, while others may be directly responsible for tumorigenesis. Therefore a mechanistic study of splicing mis-regulation will provide new insights in cancer. This study represents an important example of how a splicing factor can control critical AS events in cancer progression. In addition, re-expression of RBM4 can inhibit tumor growth both in cell culture system and in mouse models, suggesting that restoration of RBM4 activity may provide an attractive route for future therapy.

Experimental Procedures

Cell culture and splicing assay

HEK 293T, HeLa, and H157 cells were maintained at standard culture conditions (37°C, 5% CO_2) in culture medium recommended by ATCC. To generate stable cells that express RBM4, the ORF was cloned into pcDNA5/FRT/TO and resulting vector was co-transfected with pOG44 plasmid into the Flp-In T-REx 293 cells (Life Technologies). Similarly stable H157 cells were created using lentiviral vector (pCDH cDNA cloning and expression lentivectors, System Biosciences, Inc.). Semi-quantitative RT-PCR was performed as described previously (Wang et al., 2013), and were quantified using Image Quant Software™ (GE Health Care). See supplementary methods for more details.

Clinical tissues samples collection

Fresh tumor tissues and normal adjacent tissues were collected from patients with pathologically and clinically confirmed carcinomas. All human tumor tissues were obtained with written informed consent from patients or their guardians prior to participation in the study. The Institutional Review Board of the Dalian Medical University approved use of the tumor specimens in this study. Most of tissue samples were fixed in formalin, embedded in paraffin, and sectioned at 5 μm. One section was stained with H&E for histological

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examination, and the others were used for immunohistochemistry staining. A portion of tissue specimens were kept in liquid nitrogen and sectioned for protein or mRNA extraction.

Xenograft Assays

The Institutional Animal Care and Use Committee of the Dalian Medical University approved the experimental protocols performed on the animals. Female nude mice were purchased from Vital River Laboratories (VRL) for subcutaneous xenograft experiments. H157-luc-RBM4 and control cells were injected subcutaneously (1×10^6 cells). Tumor size was measured by caliper every three days.

RNA seq analysis

Cell lines stably expressing RBM4 or control vector were created, and the total RNA was purified from the cells using TRIzol reagents. The polyadenylated RNAs were purified from the cell lines for construction of sequencing library using Illumina TruSeq Total RNA Sample Prep kits (UNC High Throughput Sequencing Facility). The paired-end reads were generated by the Illumina Hi-Seq 2000 platform and mapped to human genome. Changes of splicing isoforms were analyzed by MISO pipeline. The sequencing data were deposited in GEO repository with accession number GSE58594.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- RBM4 controls various types of splicing in a sequence-specific manner
- RBM4 regulates various cancer-related genes and suppresses tumorigenesis
- RBM4 inhibits cancer by antagonizing oncogenic SRSF1 and regulating Bcl-x splicing
- RBM4 is reduced in cancer patients and positively correlated with improved survival

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Significance

Aberrant splicing is closely associated with human cancers, however the mechanistic details underlying these connections are largely unknown. Investigating splicing factors that play vital roles in cancer progression would enable therapeutic targeting of deregulated splicing and open new avenues for cancer therapy. Here we systematically dissect an RBM4-mediated splicing regulation pathway that is closely related to cancer progression. We uncover that the splicing factor RBM4 suppresses tumor progression by balancing the pro- and anti-apoptotic signals through splicing regulation and by antagonizing the oncogenic splicing factor SRSF1. The clinical relevance of such regulation is further revealed. This study represents a detailed mechanism of cancer-related splicing dysregulation, and establishes RBM4 as a tumor suppressor with therapeutic potentials.

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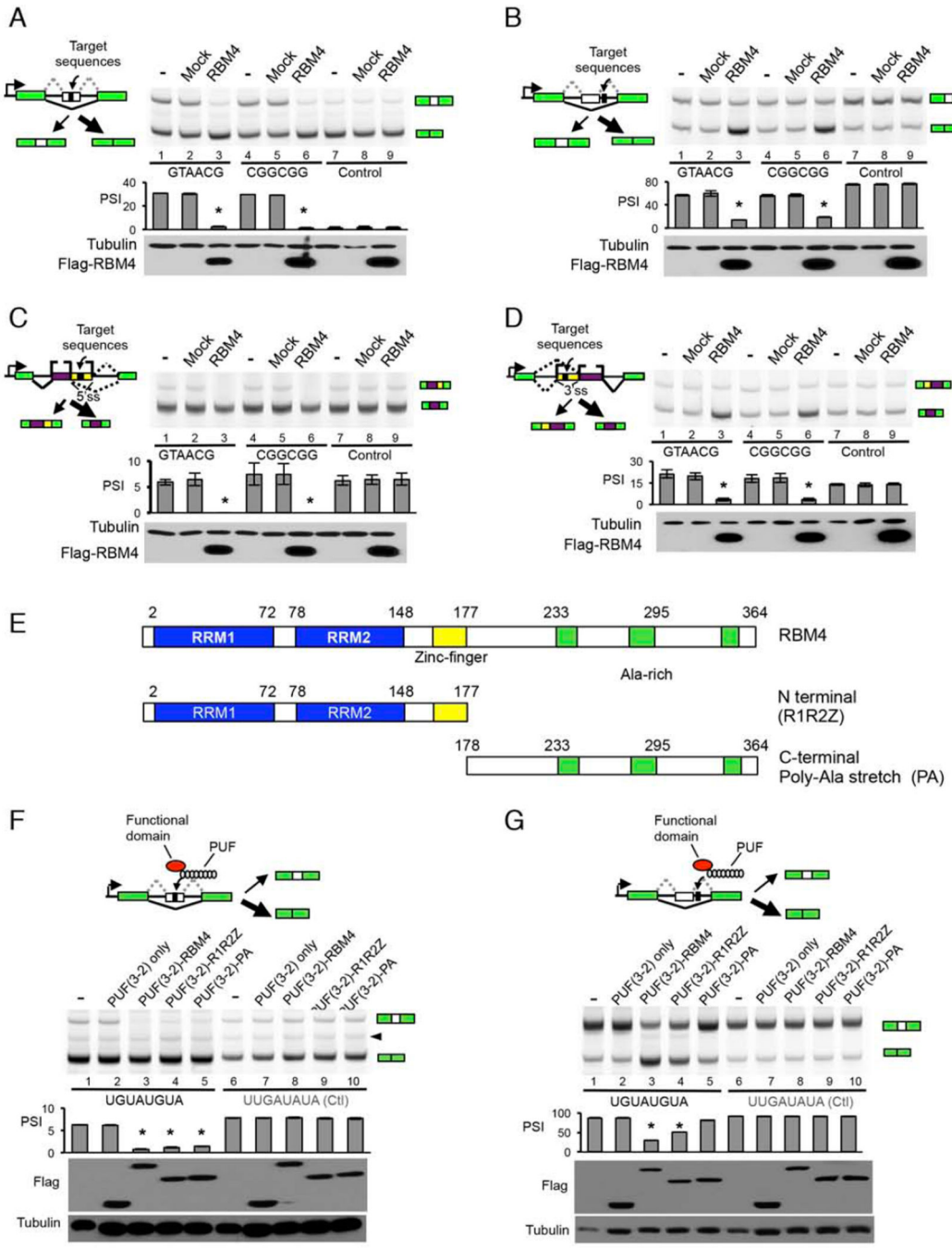

Figure 1. Splicing regulation by RBM4

(A) The RBM4 binding site and a control (GAATTG) were inserted into splicing reporter pGZ3 that were co-transfected with RBM4 expression vector or empty vector (mock) into 293T cells. Splicing changes were examined by electrophoresis of RT-PCR products. **(B)** The same set of sequences analyzed in A were inserted downstream of a cassette exon in the pZW2C reporter to measure splicing changes as in A. **(C–D)** The same set of RBM4-binding sequences analyzed in A were inserted into the splicing reporters between two tandem sites with competing 5′ (C) or 3′ ss (D) and splicing changes were measured as in A.

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(E) Schematics of RBM4 domains. R1R2Z fragment contains two RRM domains and a Zinc-finger domain. The PA fragment contains poly-alanine stretch. **(F–G)** Different RBM4 fragments were fused to a PUF domain, PUF(3–2), which specifically binds to its target RNA. The fusion proteins were co-transfected with a splicing reporter containing PUF binding site or a control (Ctl) site in a cassette exon **(F)** or at downstream intron **(G)** and splicing changes were measured as in A. In all panels measuring changes in splicing, expression of exogenous protein was confirmed by western blots. Tubulin served as a protein loading control. Three independent experiments were conducted, with the mean +/− SD of PSIs plotted below representative gels. The arrowhead indicates a non-specific product. Asterisks indicate $p<0.05$ by t-test. See also Figure S1.

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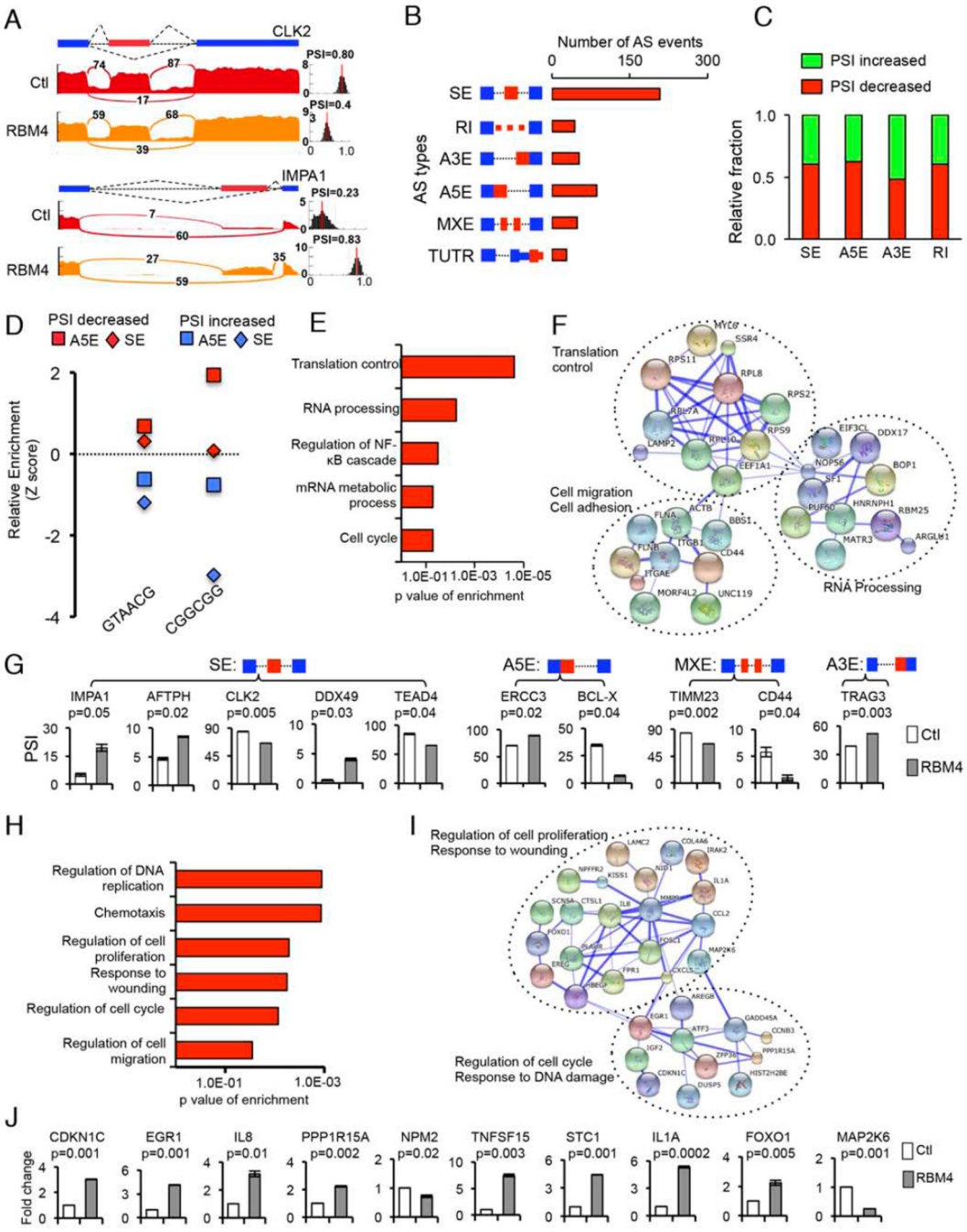

Figure 2. Global splicing and transcriptional regulation by RBM4

(A) Examples of alternative exons affected by RBM4. Genes were chosen to represent both an increase and a decrease of PSI, and numbers of exon junction reads were indicated. **(B)** Quantification of the different AS events affected by RBM4. **(C)** The relative fraction of each AS event positively or negatively affected by RBM4. **(D)** Relative enrichment of the indicated RNA motifs bound by RBM4. Enrichment scores were computed by comparing RBM4-regulated SEs or A5Es with control AS events unaffected by RBM4. The AS events with increased or decreased PSI values upon RBM4 expression were analyzed separately.

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(E) Gene ontology of RBM4-regulated AS targets. Fisher exact p-values were plotted for each enriched functional category. **(F)** Functional association network of RBM4-regulated AS targets. Genes in panel E were analyzed using the STRING database, and subgroups are marked according to their functions. **(G)** Validation of different types of RBM4-regulated AS events by semi-quantitative RT-PCR using H157 cells transfected with RBM4 or control vectors. The mean +/− SD of PSIs from three experiments were plotted (p values from paired t-test). **(H)** Gene ontology analyses of RBM4-regulated gene expression events. Fisher exact p values were plotted for each category. **(I)** The functional association networks of RBM4-regulated genes were analyzed using the STRING database, with subgroups marked by their functions. **(J)** Validation of gene expression changes by real-time RT-PCR. The mean +/− SD of relative fold changes from triplicate experiments were plotted with p values calculated by paired t-test. See also Figure S2 and Tables S1 and S2.

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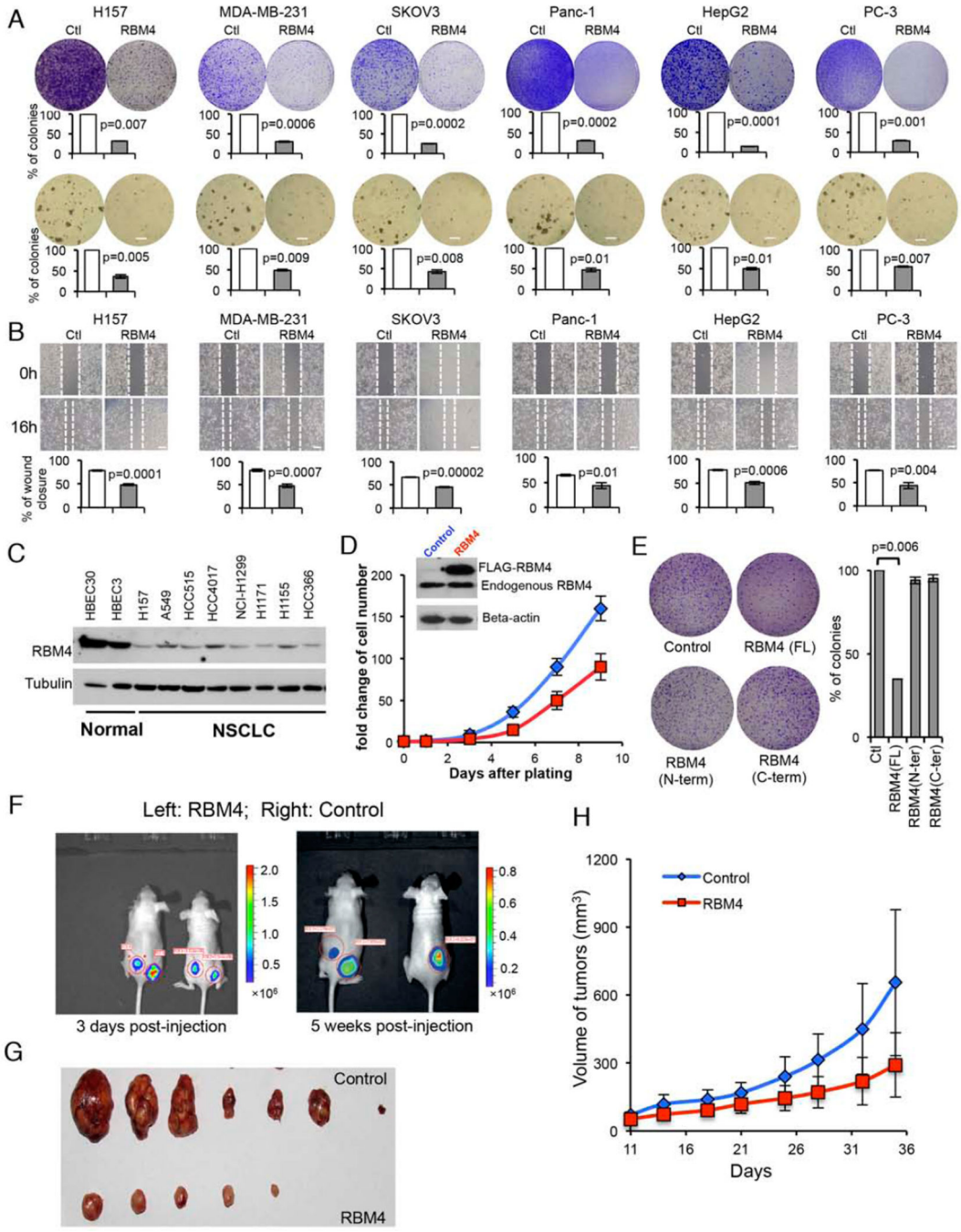

Figure 3. RBM4 inhibits cancer progression

(A) RBM4 effects on the proliferation of various cancer cells, including H157, MDA-MB-231, SKOV3, Panc-1, HepG2, and PC-3. The cells were stably transfected with RBM4 or vector control and analyzed by colony formation (upper panel) or soft agar (lower panel) assays. All experiments were performed in triplicates, with mean +/− SD of relative colony numbers plotted (p values from t-test). Scale bar = 100 μm in lower panels. Images of the whole plate are shown in upper panel. **(B)** Different cancer cell lines expressing RBM4 or vector control were analyzed by wound healing assay. Percent of wound closure was

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measured from triplicate experiments, with mean +/− SD plotted (p values from t-test). Scale bar = 200 μm. **(C)** Levels of RBM4 in the indicated NSCLC cell lines and normal bronchial cells were measured by western blot. **(D)** H157 cells stably expressing RBM4 or vector control were grown for 9 days, with cell numbers counted every two days. The changes of cell numbers were compared to day 0. The mean +/− SD from three experiments was plotted. **(E)** H157 cells expressing full length (FL), or the N- or C-terminal fragments of RBM4 were analyzed by colony formation assay. Representative pictures of the whole plates from triplicate experiments are shown. The mean +/− SD of relative colony numbers were plotted, with p values calculated by t-test. **(F)** H157-luc-RBM4 and control cells were subcutaneously injected into left and right flank of seven nude mice. The growth of xenograft tumors was monitored by bioluminescence imaging on day 3 and 35, and pictures of two representative mice were shown. **(G)** Pictures of the tumors removed after 35 days. **(H)** The average sizes of xenograft tumors measured every three days (n=7, error bars indicate +/− SD, $p<0.05$ by t-test). See also Figure S3.

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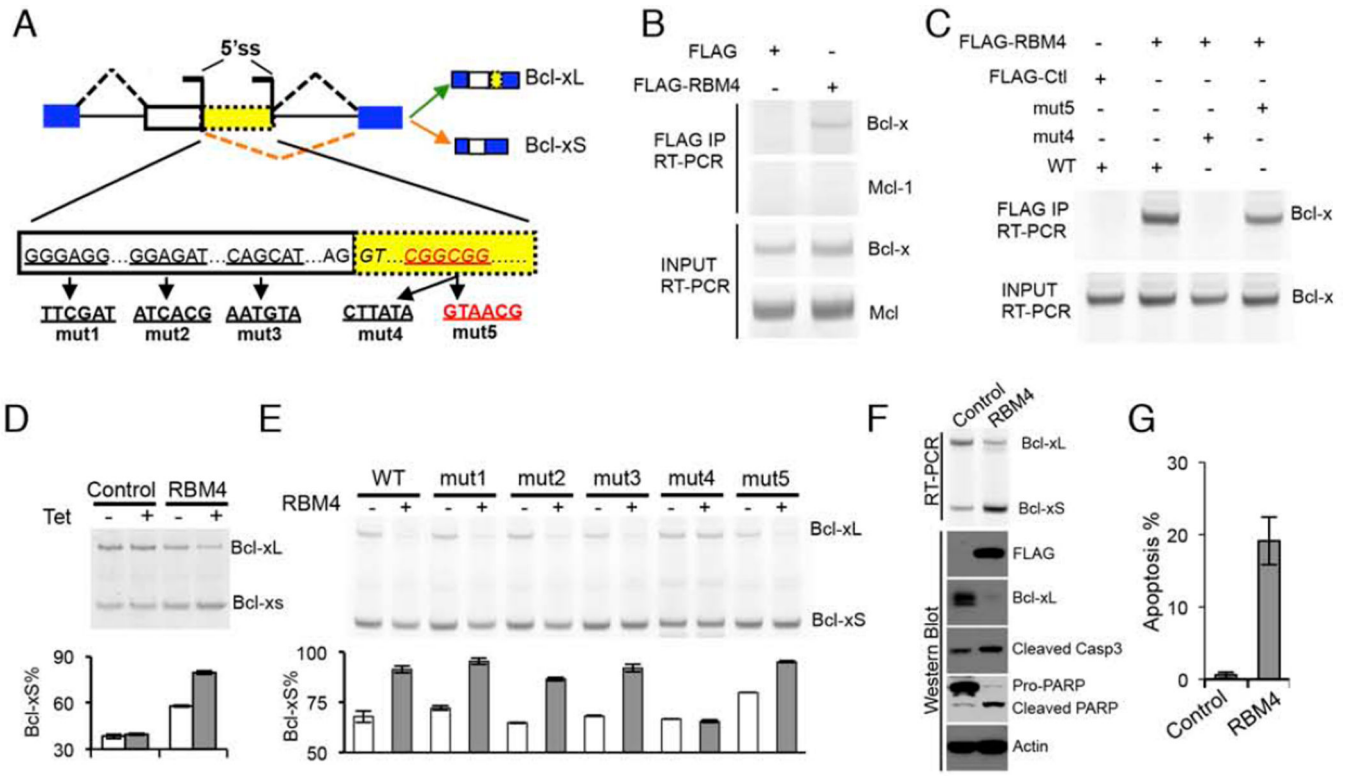

Figure 4. RBM4 regulates Bcl-x splicing to induce apoptosis

(A) The schematic of Bcl-x pre-mRNA where the potential RBM4-binding site in red. Bcl-x splicing reporters with the indicated mutations (mut1 to 5) were generated. **(B)** Binding of Bcl-x pre-mRNAs with RBM4 is detected by RNA-immunoprecipitation assay in cells exogenously expression FLAG-RBM4 or vector control. The binding of Mcl-1 mRNA was used as specificity control. (**C**) 293 cells were co-transfected with Flag-RBM4 or vector control and the indicated mutant or wild-type (WT) Bcl-x reporters, and then immunoprecipitated with anti-Flag antibody. The co-precipitated RNAs were detected by RT-PCR. **(D)** 293 cells containing tetracycline-inducible RBM4 or vector control were used to measure Bcl-x splicing. Increased levels of Bcl-xS in uninduced cells are likely due to expression leakage. The mean +/− SD of PSI from triplicate experiments were plotted. **(E)** Bcl-x splicing reporters containing various mutations were co-expressed with RBM4 or vector control in 293T cells to assay for the splicing change of Bcl-x. The mean +/− SD of Bcl-xS% was plotted. A representative gel from triplicate experiments was shown. **(F)** H157 cells expressing RBM4 or vector control were used to examine apoptotic markers including Bcl-xL, cleaved caspase 3 and PARP. (**G**) Expression of RBM4 promotes apoptosis. H157 cells expressing RBM4 or control were stained with propidium iodide and the apoptotic cells were detected by flow cytometry. The mean +/− SD of percentage of apoptotic cells from triplicate experiments were plotted. See also Figure S4.

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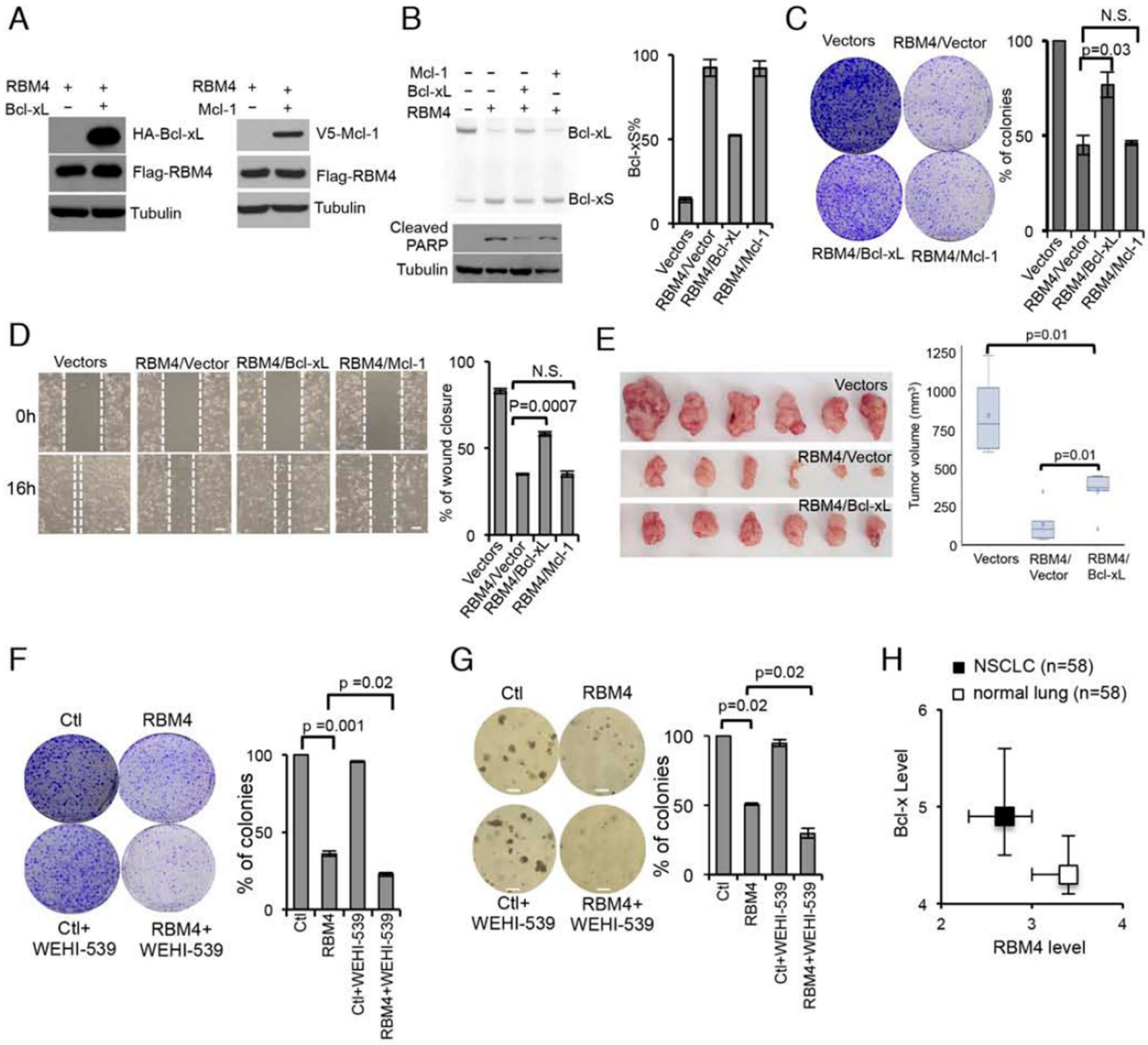

Figure 5. RBM4 regulates Bcl-x splicing to inhibit cancer progression

(A) H157 cells with stable co-expression of RBM4/Bcl-xL, RBM4/Mcl-1 or RBM4/vector control were generated. The protein expression was confirmed by western blot. **(B)** Splicing of Bcl-x in H157 cells expressing RBM4/Bcl-xL, RBM4, RBM4/Mcl-1 or vector control was measured by semi-quantitative RT-PCR. The mean +/− SD of Bcl-xS% from three experiments was plotted. PARP cleavage was examined by western blot. **(C)** Colony formation assays using H157 cells expressing RBM4, RBM4/Bcl-xL, RBM4/Mcl-1 or vector control. Images of the whole plate are shown. Three experiments were carried out with mean +/− SD of relative colony numbers plotted (p values were determined by t-test). N.S. = not significant. **(D)** Wound healing assay of H157 cells expressing RBM4, RBM4/ Bcl-xL, RBM4/Mcl-1 or vecor control. The means +/− SD of wound closure were plotted (p

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values were calculated by t-test). Scale bar = 200 μm. **(E)** Xenograft tumors were generated using nude mice injected with H157-luc-control, H157-luc-RBM4/Bcl-xL, or H157-luc-RBM4 cells. Tumors were removed after five weeks, and tumor volume was quantified. The median, upper and lower quartiles of tumor volume were plotted as box plot, with whiskers indicating data range. The points that are >1.5×interquartile range are marked outliers. **(F–G)** H157 cells expressing RBM4 or vector control were treated with or without the Bcl-xL inhibitor WEHI-539. The resulting cells were analyzed by colony formation **(F)** and anchorage-independent growth **(G)** assays. Three experiments were carried out with the mean +/− SD of relative colony numbers plotted (p values from t-test). Images of the whole plate are shown in panel F. Scale bar = 100 μm in panel G. **(H)** Inverse correlation of Bcl-x and RBM4 in lung cancer patients. Oncomine was used to analyze expression data. The mean levels of Bcl-xL and RBM4 were plotted, error bars indicate upper and lower quartile. See also Figure S5.

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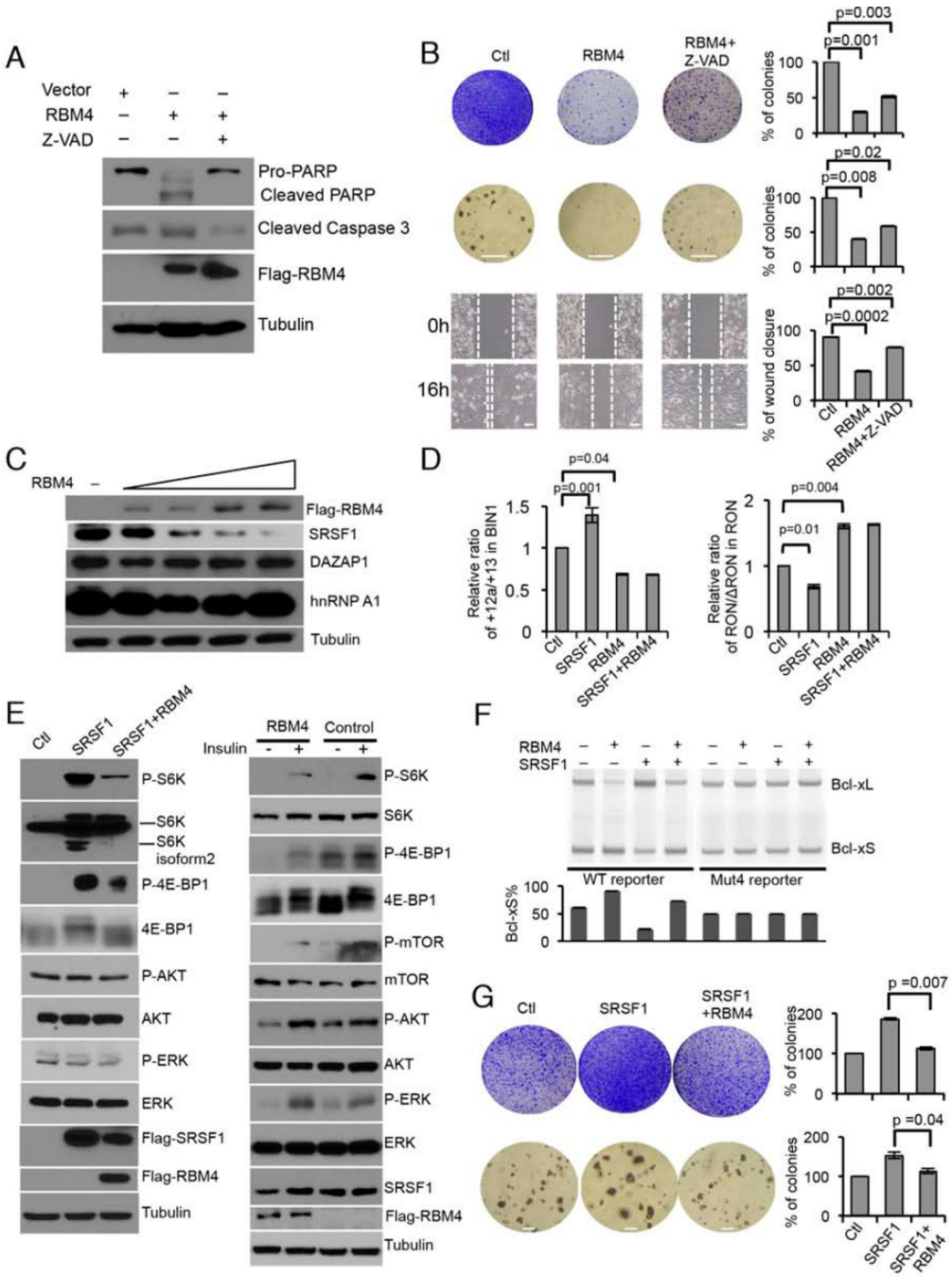

Figure 6. RBM4 antagonizes SRSF1 to inhibit cancer cell growth

(A) H157 cells expressing RBM4 were treated with Z-VAD or DMSO control. The apoptotic markers, cleaved PARP and caspase 3, were examined by western blot. **(B)** H157 cells expressing RBM4 with or without Z-VAD treatment were analyzed by colony formation, soft agar, and wound healing assays. The experiments were performed in triplicate, and representative pictures are shown. The p values calculated by t-test. Images of the whole plate are shown in upper panel. Scale bar = 200 μm. **(C)** H157 cells were transiently transfected with increasing amounts of RBM4 and examined by western blot for

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protein levels of SRSF1, DAZAP1, hnRNP A1, and RBM4. **(D)** H157 cells expressing SRSF1, SRSF1/RBM4, or control were collected to examine splicing of SRSF1 targets, BIN1 (left) and RON (right), by semi-quantitative RT-PCR. **(E)** The levels of mTOR and various mTOR targets were examined in H157 cells expressing SRSF1, SRSF1/RBM4, or control vector using western blots. Cells expressing RBM4 or control were also treated with insulin for 30 min to measure activation of mTOR pathway. **(F)** Bcl-x splicing reporters (WT and mut4 in figure 4A) were co-expressed with RBM4, SRSF1, or both to examine its splicing by RT-PCR. **(G)** The colony formation and soft agar assays using H157 cells expressing SRSF1, SRSF1/RBM4, or control. Three experiments were carried out. The p values were calculated by t-test. Images of the whole plate are shown in upper panel. Scale bar = 100 μm. In panels B, D, F, and G, the means +/− S.D. are plotted. See also Figure S6.

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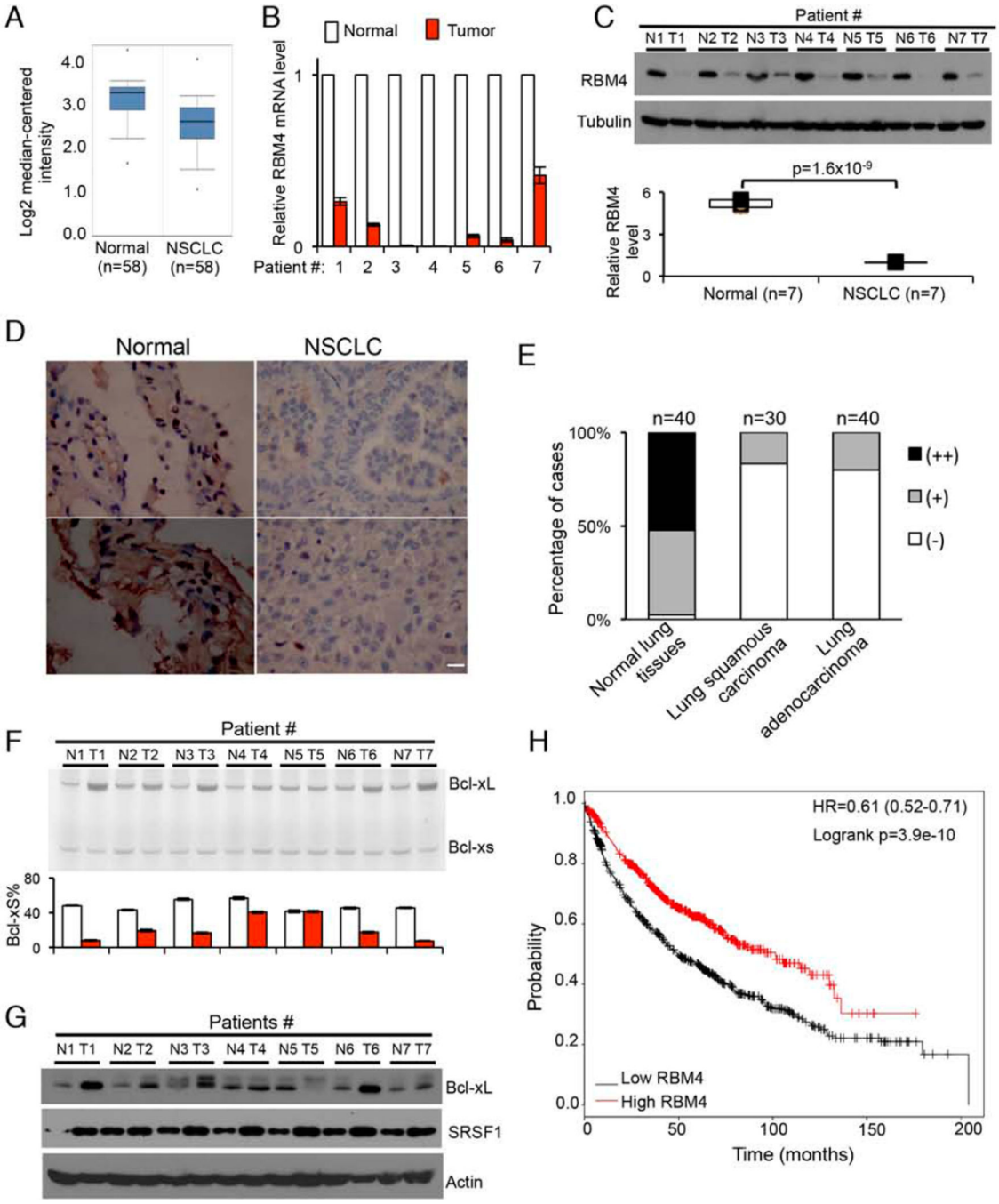

Figure 7. RBM4 in NSCLC patients

(A) RBM4 mRNA level in lung cancer patients as reported from Oncomine. The median, upper and lower quartiles were plotted, and the whiskers indicate the data range. The points that are >1.5×interquartile range are marked outliers. **(B)** Total RNA isolated from paired NSCLC tumors and adjacent normal tissues were assayed by real-time RT-PCR. **(C)** RBM4 levels from seven paired NSCLC tumors (T) and normal (N) tissues were analyzed by western blot. The p value was calculated by t-test. **(D)** Normal lung tissues and NSCLC samples were collected and subjected to immunohistochemical staining with an RBM4

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antibody. Scale bar = 40 μm. **(E)** The quantification of RBM4 protein levels in normal lung, lung squamous carcinoma and lung adenocarcinoma. The RBM4 levels were classified into three grades (negative, weak positive, strong positive) by results from immunohistochemical staining and plotted. **(F)** Splicing of Bcl-x in paired NSCLC tumor and adjacent normal tissue. (**G**) Protein levels of Bcl-xL and SRSF1 in the paired tumor and adjacent normal tissues. **(H)** Kaplan-Meier curve showing overall survival of lung cancers with high or low RBM4 expression (p=3.9e-10 by log-rank test). Error bars indicate +/− S.D. in panels C and F. See also Figure S7.