BMB Reports - Manuscript Submission

Manuscript Draft

Manuscript Number: BMB-16-118

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Article Type: Article

Keywords: SR protein; pre-mRNA splicing; CD44; V6 exon

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Title:

SR proteins regulate V_6 exon splicing of CD44 pre-mRNA

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Running title:

SR proteins regulate V_6 exon splicing of CD44 pre-mRNA

Keywords:

SR protein, pre-mRNA splicing, CD44, V_6 exon

Abstract

CD44 pre-mRNA includes 20 exons, among which exons1-5 (C_1-C_5) and exons16-20 (C_6-C_{10}) are constant exons, whereas exons 6-15 (V_1-V_{10}) are variant exons. V_6 exon containing isoforms has been known to be implicated in tumor cell invasion and metastasis. In the present study, we performed SR protein screen for CD44 V_6 splicing using overexpression and lentivirus-mediated shRNA treatment. Using CD44 V_6 minigene, we demonstrate that increased SRSF3 and SRSF4 expression do not affect V_6 splicing, but increased expression of SRSF1, SRSF6 and SRSF9 inhibit V_6 splicing significantly. In addition, using constitutive exon specific primer set, we could not detect alteration of CD44 splicing after SR protein-targeting shRNA treatment. However, using V_6 specific primer, we identified that reduced SRSF2 expression significantly reduced V_6 isoform, but increased $V_{6\text{-}10}$ and $V_{6,7\text{-}10}$ isoforms. Our results indicate that SR proteins are important regulatory proteins for CD44 V_6 splicing.

Introduction

Pre-mRNA splicing is an important gene regulatory process in which introns are removed and exons are ligated together to produce mRNA (1). Importantly, alternative splicing produces multiple proteins from a single gene. At least 95% of human genes are alternatively spliced, thus regulation of alternative splicing plays key roles in signal transduction and development. Aberrant splicing causes various genetic diseases and cancer (2-4). Alternative splicing is regulated by cis- and trans-acting elements. Cis-acting elements are splicing enhancers or inhibitors that are located at exons or introns. Trans-acting elements are proteins that regulate alternative splicing. The best known trans-acting elements are Serine-Arginine rich (SR) proteins and heterogeneous nuclear ribonucleoprotein (hnRNP) (5, 6). Pre-mRNA splicing is processed by a ribonucleoprotein (RNP) complex called spliceosome that is composed of U small nuclear RNP particles (snRNPs) and many proteins.

CD44 receptor is a cell adhesion membrane glycoprotein, which mediates communication and adhesion between adjacent cells as well as between cells and the extracellular matrix, and directs intracellular signaling for growth and motility. The function of CD44 depends on its ligands, whereas Hyaluronic acid mediates the tumor suppressor function of CD44, growth

factors regulate growth promotion function of CD44 (7). CD44 pre-mRNA includes 20 exons, among which exons1-5 (C_1-C_5) and exons16-20 (C_6-C_{10}) are constant exons, whereas exons6-15 (V_1-V_{10}) are variant exons (8). Alternative splicing of CD44 variant exons produces a large number of mRNA isoforms encoding for proteins with different post-transcriptional modifications and diverse ligand-binding properties (9-11). Variant exons are differently included or skipped to generate a large variety of splicing variants. CD44 proteins sizes are ranged from 85 kDa (CD44s) to 250 kDa (CD44 V_3-V_{10}). V_6 exon containing isoforms play important roles in tumor cell invasion and metastasis. V_6 exon has been shown to be highly expressed in tumors compared with normal tissues. V_6 containing isoform forms a complex with hepatocyte growth factor (HGF) and tyrosine kinase receptor Met to activate met-dependent Ras signaling through association of ezrinradixin-moesin (ERM) to the cytoplasmic tail of CD44 (12, 13).

SR proteins are a protein family that includes 13 members - SRSF1~12 and tra2β. SR proteins are composed of RNA recognition motif (RRM) domain and RS domain (5). SR proteins have important roles both in constitutive and alternative splicing. In the constitutive splicing, SR proteins are known to promote spliceosome assembly including U1 snRNP binding to 5′ splice-site and U2 snRNP binding to branch-point (14, 15). In alternative splicing, SR proteins are shown to antagonize

hnRNP functions (16). SR proteins could promote exon inclusion or skipping through interacting with exons or introns. In addition to the roles in RNA splicing, SR proteins also functions in transcription elongation, RNA stability, mRNA transport and mRNA translation (17).

In the present study, we performed SR protein screen for CD44 V_6 splicing using overexpression and lentivirus-mediated shRNA treatment. Using CD44 V_6 minigene, we demonstrate that SRSF3 and SRSF4 do not affect V_6 splicing, SRSF1, SRSF6 and SRSF9 inhibit V_6 splicing significantly. In addition, using constitutive exon specific primer set, we could not detect alteration of CD44 splicing after SR protein-targeting shRNA treatment. Using V_6 specific primer, we identified that reduced SRSF2 expression significantly reduced V_6 isoform, but increased $V_{6\text{-}10}$ and $V_{6,7\text{-}10}$ isoforms. Our results indicate that SR proteins are important regulatory proteins for CD44 V_6 splicing

Results

SRSF3 and SRSF4 did not affect V_6 exon splicing of CD44 pre-mRNA

In order to identify the SR proteins that affect V_6 exon splicing of CD44 pre-mRNA, we applied the MCF7 stable cell line that expresses pFlare-V_6 plasmid (18). As previously

described, in the pFlare-V_6 plasmid, V_6 exon and its flanking introns are inserted between β-globin exon 1 and GFP exon (figure 1A). GFP is expressed when V_6 is skipped, and then RFP is expressed when V_6 is included. To detect V_6 exon splicing, we used a primer set that basepair with β-globin and GFP exon (figure 1A). Consistent with the previous results (18), V_6 included isoform was dominantly expressed, whereas V_6 excluded isoform was expressed in a much less significant level (lane 1, figure 1B). It was also consistent with previously published conclusion (18), that SRSF2 significantly promotes V_6 skipped isoform and inhibits V_6 inclusion. Next we tested the function of SRSF3 and SRSF4 on V_6 splicing. Although V_6 exon and flanking introns include a number of potential binding sites for SRSF3 and SRSF4, figure 1B shows that neither SRSF3 nor SRSF4 affected V_6 splicing. Thus we conclude that SRSF3 and SRSF4 are not regulatory factors for V_6 exon splicing of CD44 pre-mRNA.

SRSF1, SRSF6 and SRSF9 inhibit V_6 exon splicing

We further asked whether other SR proteins regulate V_6 exon splicing. We also noticed that V_6 exon and flanking introns contains a significant numbers of potential binding sequences for SRSF1, SRSF6 and SRSF9. These sequences provide potentials that these proteins regulate V_6 exon splicing. To this aim, we expressed SRSF1 or SRSF6 or SRSF9 in the pFlare-V_6 cell line.

Figure 2 shows that, by contrast to SRSF3 and SRSF4, treatment of these proteins induced the V_6 skipped isoform significanty (~44%, ~36% and ~46% independently). Therefore we conclude that SRSF1, SRSF6 and SRSF9 inhibit V_6 exon splicing.

Using primer set that basepair with constitutive exons could not detect the induction of any various exon-included isoforms by reduced SR protein expression

We next wondered whether reduced expression of SR proteins could induce alteration of endogenous CD44 splicing. To address this question, we treated MCF7 cells using lentivirus-mediated shRNA and then extracted RNA from cells. The standard primers that basepair with constitutive C5 and C6 exons were used to detect both the isoform that includes only constitutive exons (C) and the isoforms that includes any variant isoform (V) (lower panel, figure 3). Consistent with the previously reported results (19), RT-PCR results for CD44 splicing using these primers show that C isoform was predominantly detected, whereas V isoforms were not detected (lane 1, figure 3). Moreover, non-silencing (NS) shRNA treatment did not induce any alteration of CD44 splicing (lane 2), suggesting that the NS shRNA can be used as a negative control. The results using shRNAs that target different SR proteins suggest that reduced expression of SRSF1, SRSF2, SRSF3, SRSF4 and SRSF9 did not induce production of any V

isoform (lanes 3-7). The results is consistent with the conclusion that SRSF3 and SRSF4 did not affect CD44 splicing, but not with the conclusion that SRSF1, SRSF2 and SRSF9 regulate V_6 exon splicing.

Using V_6 exon specific primer could detect the induction of various exon-included isoforms by reduced SRSF2

As the primers could not detect various isoform containing isoforms, we determined to use one primer that basepair with V_6 exon and the other that basepair with C6 exon (lower panel, figure 4). The primers could detect $V_{6\text{-}10}$ exon combinations, but not V_2-V_5. The figure 4 results show that the isoform that includes only V_6 isoform among $V_{6\text{-}10}$ was dominantly detected (V_6, lane 1). In addition, an isoform that include V_6, V_7, V_8, V_9 and V_{10} ($V_{6\text{-}10}$) and an isoform that includes V_6, V_8, V_9 and V_{10} ($V_{6,7\text{-}10}$) were produced in less significant levels. We next asked whether reduced expression of SR proteins affects expression of these CD44 isoforms. Figure 4 results demonstrate that reduced expression of SRSF3 SRSF1 caused a decrease of $V_{6\text{-}10}$ and $V_{6,7\text{-}10}$ isoforms (lanes 1 and 6). Moreover, reduced SRSF9 and SRSF4 expression did not induce significant change of CD44 isoforms. Most significantly, reduced expression of SRSF2 induced decreased expression of V_6, but increased expression of both $V_{6\text{-}10}$ and $V_{6,7\text{-}10}$ expression. Our results suggest that SRSF2 is a key player in CD44 V_6 splicing.

Discussion

CD44 pre-mRNA splicing is one of most complicated splicing events in human genes. CD44 pre-mRNA includes 10 constant exons, exons 1-5 (C_1-C_5) and 16-20 (C_6-C_{10}), and 10 various exons, exons 6-15 (V_1-V_{10}) (18, 19). In this manuscript, we studied the function of SR proteins on V_6 exon splicing of CD44 pre-mRNA. First, in the overexpression of SR proteins into the pFlare-V_6 minigene harboring MCF7 stable cell line, we demonstrated that SRSF1, SRSF6 and SRSF9 but not SRSF3 and SRSF4 inhibit V_6 exon splicing. Next, we analyzed the SR proteins function by reducing their expression through shRNA treatment. We found that using the primer set that basepair with the constitutive exons of CD44 pre-mRNA, the changes of alternative splicing by SR proteins were not detectable. However, Using the primer that basepair with V_6 exon, we show that SRSF2-targeting shRNA decreased V_6 isoform significantly, but increased $V_{6\text{-}10}$ and $V_{6,7\text{-}10}$ isoforms. Our results indicate that CD44 V_6 splicing is regulated by SR proteins.

SR proteins have been known to function through binding to the enhancer to promote spliceosome assembly (20-22). Recently it was also reported that SR proteins can either promote or inhibit exon inclusion (23-25). In addition, using tethered SR proteins it was demonstrated that splicing activation and repression by SR proteins depends on the location of their

binding (26). Our results demonstrate that although all of the SR proteins we analyzed could potentially interact with V_6 exon and flanking introns, only some of them could inhibit V_6 splicing. Furthermore, the locations of SR protein binding did not affect their functions. The results can be explained that various potential binding locations of SR proteins on V_6 exon and flanking introns could possibly function through combinatory or synergistically. How these combination or synergistic effects regulate alternative splicing has not been well understood. One of our most striking results is that reduced SRSF2 expression could induce various V_6 exon containing isoforms. How the proteins encoded by these mRNA isoforms function need to be determined.

Our results indicate that the the SR proteins, whose overexpression showed inhibitory effects on V_6 splicing, did not demonstrate significant effects as their expressions were reduced. This kind of quantitative differences was also shown before (19, 27). Another possibility is the difference of the assay systems in two experiments: whereas overexpression experiments were performed using minigene-harboring stable cell line, shRNA treatments were performed by analyzing endogenous CD44 splicing. Another different results caused by assay systems are the differences in primer for analyzing V_6 splicing of CD44 pre-mRNA: only V_6 specific primers but not constitutive exon specific primers could detect the alteration

of V_6 splicing. The results indicate that various exons in CD44 pre-mRNA should be detected using the primer that basepair with itself.

Materials and methods

Cell culture

MCF7 cells was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone) supplemented with antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) and 10% of Fetal Bovine Serum (FBS; Hyclone) in a humidified 5% CO2 condition at 37°C. The stable pFlare-V_6 stable cell was obtained as previous described (18). Different SR proteins plasmids were transfected into the stable cells using polyethyleneimide (PEI) Reagent according to the manufacturer's protocol.

RT-PCR

Total RNAs were extracted using RiboEx reagent (GeneAll) following manufacturer's protocol. RT-PCR was conducted as previously described (19). For the endogenous CD44 pre-mRNA splicing, RT-PCR was conducted as described previously (28). A specific primer, CD44RT (5'-ATG CAA ACT GCA AGA ATC-3') was used for reverse transcription. Following primers were used to detect CD44 splicing: primers for detection of pFlare-V_6 stable cells [pFlarev6 Fwd (5'- GGA AGA GTT GGT GGT GAG G-3'), pFlarev6 Rev (5'-GGT GCA GAT GAA CTT CAG G-3')], endogenous CD44 splicing [For (5'- AAG ACA TCT ACC CCA GCA AC-3'), Exon C_7 Rev (5'- TTT GCT CCA CCT TCT TGA CTC C -3')], V_6 splicing [Fwd

(5′-TCC AGG CAA CTC CTA GTA GT-3′), Exon C_7 Rev (5′-TTT GCT CCA CCT TCT TGA CTC C-3′)].The endogenous RT-PCR products were confirmed by sequencing.

shRNA treatment

shRNA lenti-virus was prepared using different SR protein shRNA plasmi as previous described (19). Knockdown of SR proteins were performed by treating cells with the virus for 72 h.

Acknowledgements

This work was supported by the NRF-2015R1A2A1A15054247 grant to Haihong Shen, the NRF-2016R1A2B1007135 grant to Xuexiu Zheng and Cell Logistics Research center (2016R1A5A1007318) funded by the National Research Foundation (NRF) of Korea, and an integrative aging research grant at the Gwangju Institute of Science and Technology (GIST).

Figure legends

Figure 1. SR proteins regulate V_6 exon splicing of CD44 pre-mRNA. (A) pFlare-V_6 minigene is shown. V_6 exon is shown with black box, β-globin and GFP/RFP exons are shown with white/gray boxes. Introns that flanks V_6 are shown with thicker lines, introns of β-globin and GFP are shown with thinner

lines. (B) RT-PCR analysis using RNAs from pFlare-V_6 MCF7 cells treated with pcDNA3.1+ or SRSF2 or SRSF3 or SRSF4. Quantitation results by Image J from three independent experiments are shown at bottom. The significant change was evaluated by Student's *t-test*. The error bars represent standard deviation of the repeats.

Figure 2. SRSF1, SRSF6 and SRSF9 inhibit V_6 exon splicing. RT-PCR analysis using RNAs from pFlare-V_6 MCF7 cells treated with pcDNA3.1+ or SRSF1 or SRSF6 or SRSF9. Quantitation results by Image J from three or more independent experiments are shown at bottom. The significant change was evaluated by Student's *t-test*. Results are expressed as percentages of ratio skipping V_6 /total. The error bars represent standard deviation of the repeats.

Figure 3. Using primer set that basepair with constitutive exons could not detect the induction of any various exon-included isoforms by reduced SR protein expression. (Upper panel) RT-PCR analysis using RNAs from cells treated with shRNA viruses that target SRSF3, SRSF9, SRSF2, SRSF4 or SRSF1. Non-silencing shRNA was used as a control. The identities of spliced products are shown at right. (Lower panel) primers used in RT-PCR analysis are shown with arrows.

Figure 4. Using V_6 exon specific primer could detect the induction of various exon-included isoforms by reduced SRSF2. (Upper panel) RT-PCR analysis using RNAs from SR protein-

targeting shRNA viruses treated cells. The identities of the spliced products are shown at right. (Lower panel) primers used in the RT-PCR analysis are shown with arrows.

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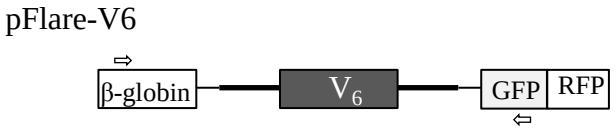
Figure 1.

A.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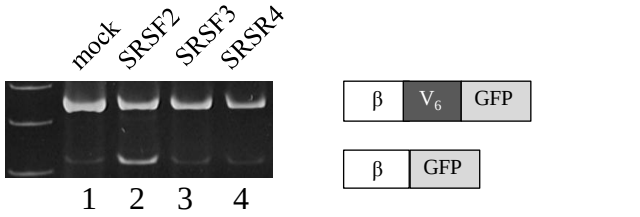
β-globin
V6
GFP
RFP

B.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SRSF3
SRSR4
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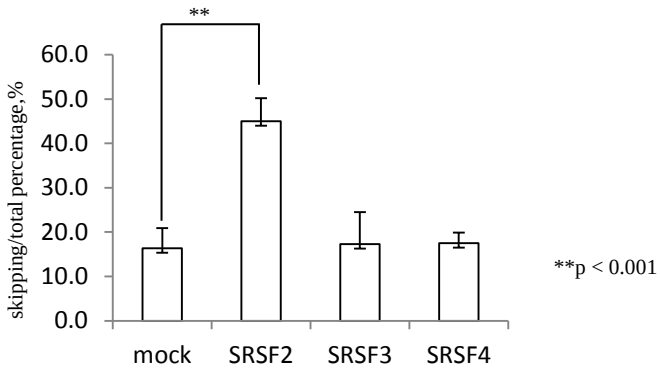
β
V6
GFP
β
GFP


**
60.0
50.0
40.0
30.0
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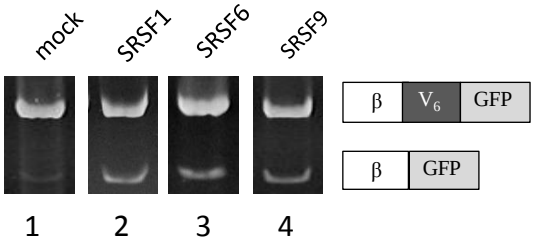
0.0
skipping/total percentage,%
mock
SRSF2
SRSF3
SRSF4
**p < 0.001

Figure 2.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GFP
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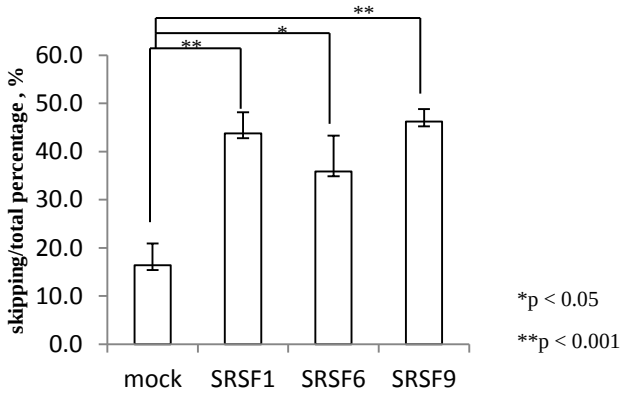
2
3
4


skipping/total percentage , %
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50.0
40.0
30.0
20.0
10.0
0.0
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*
**
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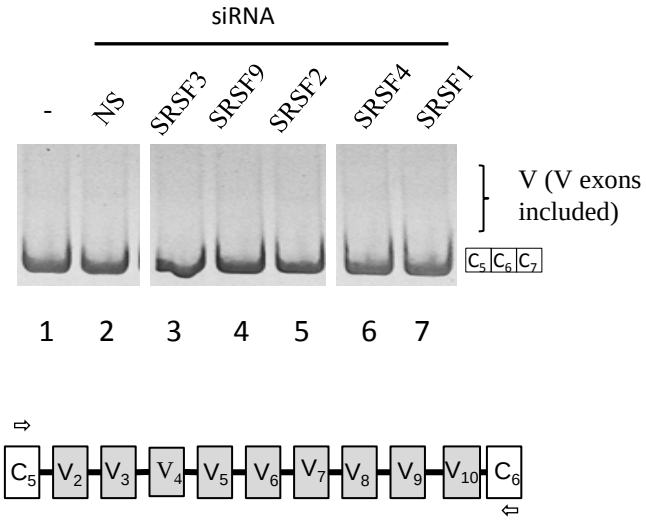
SRSF1
SRSF6
SRSF9
*p < 0.05
**p < 0.001

Figure 3.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SRSF9
SRSF2
SRSF4
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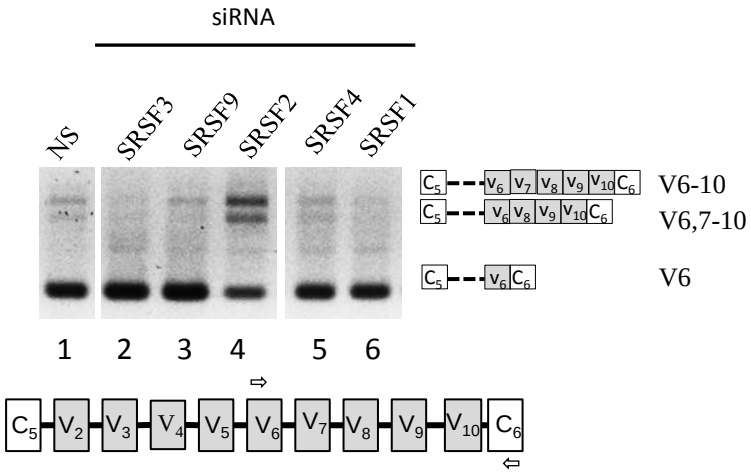
C5 V2 V3 V4 V5 V6 V7 V8 V9 V10 C6

Figure 4.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SRSF2
SRSF4
SRSF1
V6-10
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V6
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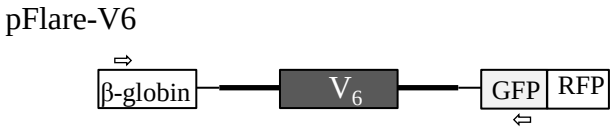
C5 V2 V3 V4 V5 V6 V7 V8 V9 V10 C6

Figure 1.

A.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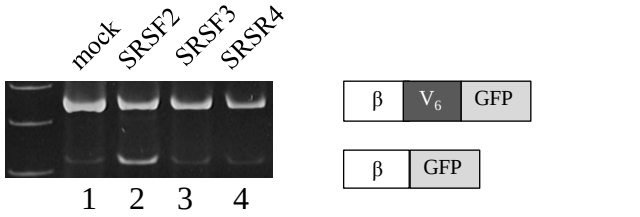
β-globin
V6
GFP
RFP

B.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SRSF3
SRSR4
1 2 3 4
β
V6
GFP
β
GFP

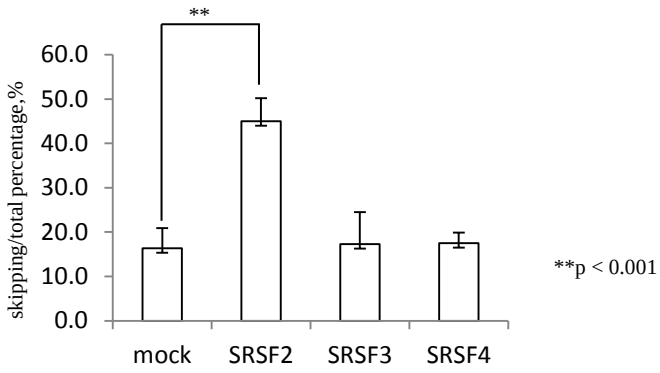
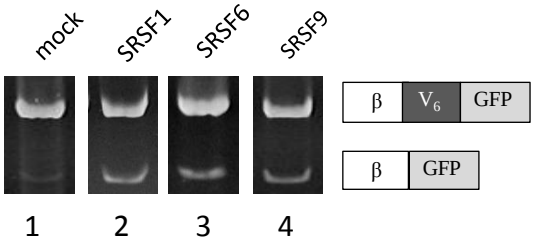

**
60.0
50.0
40.0
30.0
20.0
10.0
0.0
skipping/total percentage,%
mock
SRSF2
SRSF3
SRSF4
**p < 0.001

Figure 2.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SRSF1
SRSF6
SRSF9
β
V6
GFP
β
GFP
1
2
3
4

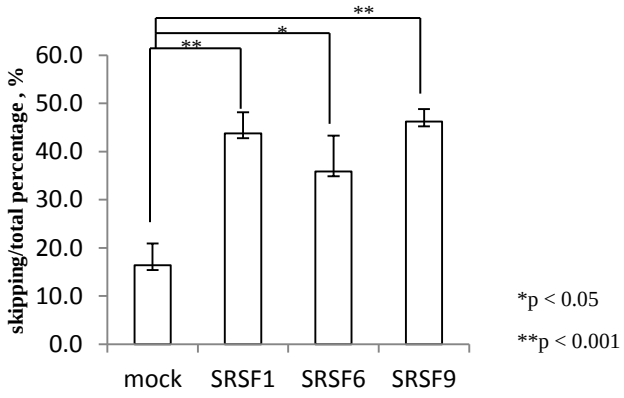

skipping/total percentage , %
60.0
50.0
40.0
30.0
20.0
10.0
0.0
**
*
**
mock
SRSF1
SRSF6
SRSF9
*p < 0.05
**p < 0.001

Figure 3.

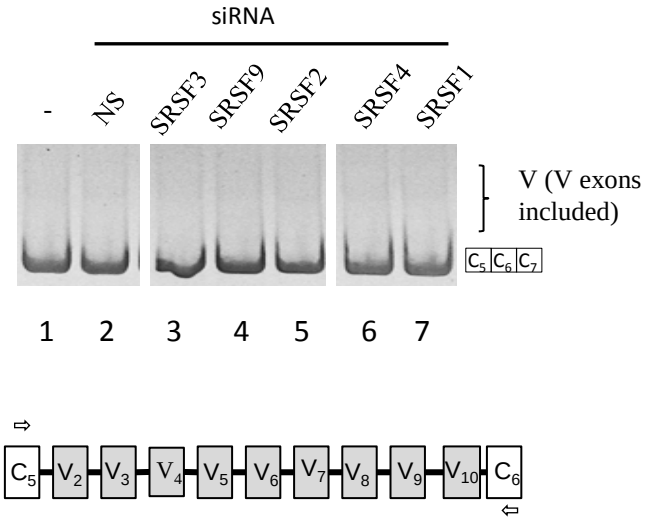
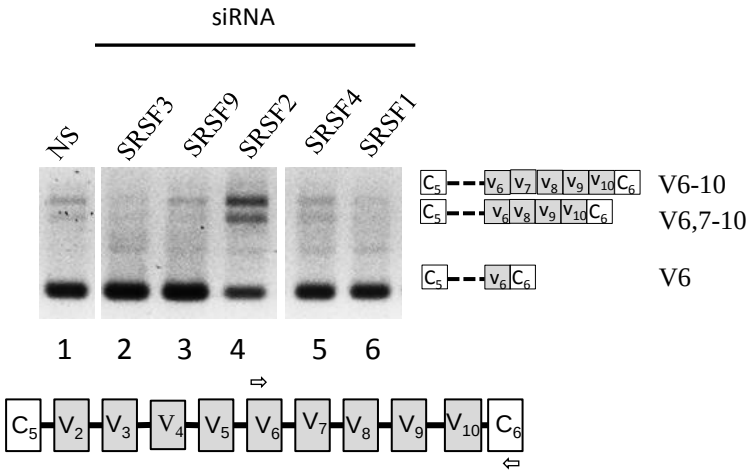

siRNA
-
NS
SRSF3
SRSF9
SRSF2
SRSF4
SRSF1
V (V exons included)
C5 C6 C7
1 2 3 4 5 6 7
C5 V2 V3 V4 V5 V6 V7 V8 V9 V10 C6

Figure 4.


siRNA
NS
SRSF3
SRSF9
SRSF2
SRSF4
SRSF1
V6-10
V6,7-10
V6
1 2 3 4 5 6
C5 V2 V3 V4 V5 V6 V7 V8 V9 V10 C6