Review

Neuron-specific splicing

Nor Hakimah Ab Hakim[1,*], Burhanuddin Yeop Majlis[1], Hitoshi Suzuki[2], Toshifumi Tsukahara[2]

[1] *Institute of Microengineering and Nanoelectronics (IMEN), Universiti Kebangsaan Malaysia, Selangor Darul Ehsan, Malaysia;*
[2] *School of Materials Science, Japan Advanced Institute of Science and Technology, Nomi, Ishikawa, Japan.*

Summary	During pre-mRNA splicing events, introns are removed from the pre-mRNA, and the remaining exons are connected together to form a single continuous molecule. Alternative splicing is a common mechanism for the regulation of gene expression in eukaryotes. More than 90% of human genes are known to undergo alternative splicing. The most common type of alternative splicing is exon skipping, which is also known as cassette exon. Other known alternative splicing events include alternative 5′ splice sites, alternative 3′ splice sites, intron retention, and mutually exclusive exons. Alternative splicing events are controlled by regulatory proteins responsible for both positive and negative regulation. In this review, we focus on neuronal splicing regulators and discuss several notable regulators in depth. In addition, we have also included an example of splicing regulation mediated by the RBFox protein family. Lastly, as previous studies have shown that a number of splicing factors are associated with neuronal diseases such as Alzheime's disease (AD) and Autism spectrum disorder (ASD), here we consider their importance in neuronal diseases wherein the underlying mechanisms have yet to be elucidated.

Keywords: Alternative splicing, gene regulation, RNA splicing, splicing regulator

1. Introduction

Posttranscriptional regulation is vital for gene expression. Furthermore, posttranscriptional regulation provides a mechanism for rapid changes in the protein composition of cells. In eukaryotic cells, the conversion of the initial primary transcript synthesized by RNA polymerase II into a functional mRNA involves three major events: 5′-capping, 3′-cleavage/polyadenylation, and RNA splicing. The addition of these specific modifications to both the 5′ and 3′ ends of the pre-mRNA is crucial, as they protect mRNAs from enzymes that will quickly digest uncapped RNAs generated by RNA processing, such as RNA transcribed downstream from a polyadenylation site and spliced-out introns. Moreover, in the nucleus, the 5′-cap and 3′-poly(A) tail distinguish pre-mRNA molecules from various other kinds of RNA (*1,2*).

As the nascent mRNA precursor is being transcribed, the pre-mRNA processing events (*i.e.*, capping, polyadenylation, and splicing) take place in the nucleus. Therefore, pre-mRNA processing occurs co-transcriptionally. The 5′-end is instantly modified as the RNA emerges from the surface of RNA polymerase II by the addition of the 5′-cap structure found on all mRNA molecules, and is immediately bound by members of a complex group of RNA-binding proteins as the nascent pre-mRNA continues to develop. These RNA-binding proteins subsequently assist in both RNA splicing and the exportation of fully processed mRNA molecules into the cytoplasm through nuclear pore complexes. In the cytoplasm, some of these proteins remain associated with the mRNA. However, most of them either remain in the nucleus or shuttle back into the nucleus shortly after the mRNA is exported to the cytoplasm (*1,2*).

In addition, it is important to note that mRNAs never occur as free RNA molecules in the cell, as they are always associated with proteins as ribonucleoprotein complexes (RNP). First, they exist as nascent pre-mRNPs that are capped and spliced as they are transcribed, and

Address correspondence to:
Dr. Nor Hakimah Ab Hakim, Institute of Microengineering and Nanoelectronics (IMEN), Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan, Malaysia.
E-mail: hakimah_nor@ukm.edu.my

then become nuclear mRNPs following cleavage and polyadenylation. Finally, following the exchange of proteins that accompanies the export to the cytoplasm, they are referred to as cytoplasmic mRNPs (*1,2*).

2. Splicing

Pre-mRNA splicing is a process where introns are removed from the pre-mRNA and the remaining exons are connected to form a single continuous molecule. Introns are regions on RNA transcripts that do not code for protein. Transcripts with many exons can be spliced co-transcriptionally, although most pre-mRNA splicing events take place after the completion of mRNA synthesis. Other transcript modifications by RNA processing, such as end-capping of the pre-mRNA, assist pre-mRNA splicing. A large protein complex, known as the spliceosome, catalyses the splicing reaction. Proteins and small nuclear RNA molecules form the spliceosome that recognizes splice sites in pre-mRNA (*1,2*).

2.1. *Alternative splicing*

Alternative splicing is a common mechanism used to regulate gene expression in eukaryotes (*3-7*). It is recognized as a major contributor of proteomic diversity because it allows for the generation of multiple proteins from a single gene (*8,9*). It is known that 90% of human genes undergo alternative splicing (*10*). One notable example is the *Drosophila Dscam*, which can encode more than 38,000 proteins from a single gene (reviewed in *11*).

2.2. *Types of alternative splicing*

There are several types of alternative splicing, the most common type being "exon skipping" (also known as "cassette exon"). Other known alternative splicing events are: alternative 5′ splice sites, alternative 3′ splice sites, intron retention, and mutually exclusive exons (reviewed in *11,12*). Owing to alterations in exon combinations during alternative splicing events, the resulting protein isoforms may have different functions or activities.

3. Regulation of gene expression through alternative splicing

Regulation of splicing involves both *trans*- and *cis*-components, including sequences in pre-mRNA as well as cellular factors (RNA or protein, respectively) (*12*). In most cases, the *trans*-factors and cis-elements involved in a particular alternative splicing event are difficult to predict. Similarly, because many pseudo-exons that are flanked by predicted splice sites are not spliced, it is challenging to predict exons from the

genome sequence information alone in the absence of transcript sequence information. It has been previously demonstrated that the presence of a pair of strong splice sites was not sufficient to define an exon (*12*).

To gain a better understanding of the underlying mechanisms, global analyses have been performed and the results indicate that relative enrichment in exon splicing enhancers (ESEs) or exon splicing silencers (ESSs) can help distinguish authentic exons from pseudo-exons. Despite extensive variation in the sequence of auxiliary splicing elements, they are crucial to the determination of alternative exons (*12*). Many ESEs were reported to contain binding sites for members of the serine-arginine rich (SR) protein family. SR proteins play important roles in the assembly of the spliceosome and function as both essential splicing and regulatory factors. Members of this protein family have a modular structure with one or two N-terminal RNA recognition motif (RRM)-type domains that bind RNA, in addition to C-terminal domains that are enriched in serine and arginine residues (RS domains). Additionally, RS domains are found in other core splicing factors, such as U2AF65 and U2AF35. They function to facilitate both protein-protein and protein-RNA interactions (*12*).

4. Neuronal splicing regulators

Certain pre-mRNA-binding regulatory proteins direct changes in splicing patterns and consequently enhance or silence particular splicing options. Previous studies have shown that these factors can be classified as positive or negative factors. SR proteins, as well as their related proteins, are among the most well-known and best characterized positive factors. These proteins bind to elements on ESEs and promote exon inclusion. Negative factors include members of the heterogeneous nuclear ribonucleoprotein (hnRNP) group of RNA-binding proteins, such as hnRNPA1 and the polypyrimidine tract-binding protein (PTB/PTBP1) (reviewed in *5,13*).

In addition, several proteins have the ability to either enhance or repress exon inclusion, depending on the position of their binding sites relative to the target exon. These RNA-binding proteins maintain developmentally regulated splicing patterns, albeit partially; however, the proteins that mediate changes in splicing in response to neuronal activity are largely unknown. Certain splicing factors are exclusively expressed in neurons for instance PTBP1, RBFox proteins and Nova 1, while others are enriched in neurons in the brain such as PTBP2 (nPTB) and RBFox proteins. However, splicing factors that are enriched in neurons in the brain can also be found in other tissues. Additionally, other factors can determine neuronal splicing patterns by their absence in neurons. Several proteins have been shown to control various groups of target transcripts in a coordinated

Table 1. Proteins known to regulate splicing in the nervous system

Neuronal splicing regulators	Homologues	Binding motifs	Tissue specificity	Known targets	References
PTBP1	Not applicable	UCUCU UUCU	Widespread / ubiquitous	Many, including GABA, α-actinin, α-tropomyosin, FGFR1 and 2	*5,20*
PTBP2 (nPTB)	Not applicable	CUCUCU	Brain	Many, including Bin 1, PMCA, Mef2, Nasp, and SRC	*5,20*
Nova1	Nova2	UCAUY	Neurons	Many, including GABAAγ2 and GlyRα2	*5,22,23*
RBFox1	RBFox3	UGCAUG	Brain, heart, neurons and skeletal muscle	α-actinin, EWS, FGFR2, fibronectin, and SRC	*5,27,28,32*
RBFox2	RBFox3	UGCAUG	Brain, heart, neurons, skeletal muscle and embryo	FGFR2, fibronectin, non-muscle myosin LC, and SRC	*5,27,28,29, 32*
KHDRBS1	Not applicable	UAAA UUUA	Nucleus	SMN2	*31*

Y indicates C or U; R indicates A or G; V indicates A, C, or G; H indicates A, C, or U. This table has been modified from Li Q, *et al.* (*5*).

manner, despite exon regulation by multiple factors (*5*).

5. Splicing regulators

As mentioned above, changes in splicing pattern are directed by positive and negative regulatory factors that bind to pre-mRNA and enhance or silence particular splicing choices (reviewed in *5,12,14*). Notable regulatory proteins that participate in neuronal alternative splicing are reviewed below and summarized in Table 1.

5.1. *PTBP1 and PTBP2*

Polypyrimidine tract-binding proteins are RNA-binding proteins that contain four RNA-binding domains of the RRM type (*15*). They are abundantly and widely expressed nuclear RNA-binding proteins. In addition, PTBP1 (Figure 1) has been shown to be involved in various cellular processes that affect gene expression, specifically alternative splicing (*14-17*). Furthermore, PTBP1 was reported to bind short RNA elements containing mixtures of U and C, such as UCUCU and UUCU (*15,18-20*). PTBP1 and hnRNP A1 are classified as hnRNP proteins. They have been implicated in repressing certain splicing events, thereby creating a layer of negative regulation. Moreover, PTBP1 has been recognized as a key splicing repressor in mammalian cells (*13*).

In contrast, the paralogue protein PTBP2, or nPTB, is present in the nervous system and testis (*21*). The function of PTBP2 in the brain remains unclear, although PTBP1 has been suggested to act as a negative regulator of neuron-specific exons in non-neuronal tissues (*21*). PTBP2 has been shown to be expressed in neuronal progenitors and it was detected in differentiated neurons and some astrocytes. Moreover, PTBP2 has been shown to be critical for postnatal survival and proper splicing of hundreds of mRNAs in the brain (*21*).

5.2. *Nova1*

The neuro-oncological ventral antigen (Nova1) is a neuron-specific RNA-binding protein that binds to the RNA sequence UCAUY. Nova1 has been reported to regulate the alternative splicing of several genes, including the glycine receptor a2 (*22,23*). In humans, the *NOVA1* gene is highly expressed in motor neurons. In contrast, *NOVA2* is abundantly expressed in neurons of the cortex, dorsal spinal cord, and hippocampus. Both factors were first identified in patients with paraneoplastic opsoclonus-myoclonus-ataxia (POMA) as autoantigens (reviewed in *5*).

5.3. *RBFox proteins*

Originally identified in *Caenorhabditis elegans*, the feminizing locus on X (*RBFox1*) gene functions as the numerator element to determine the number of X chromosomes relative to the ploidy. Additionally, *RBFox1* plays a role in the determination of male or hermaphrodite development (*24-26*). RBFox1 is an RNA-binding protein that contains an RRM. RBFox1 was reported to be expressed in the brain, heart, and skeletal muscle in mouse (*27*). Furthermore, Jin Y., *et al.* (*28*) showed that the zebrafish RBFox1 protein binds specifically to the pentanucleotide sequence GCAUG in their SELEX experiments.

In mammals, three members of the Fox family

proteins – RBFox1, RBFox2, and RBFox3 – have been identified. RBFox1 and RBFox2 are closely related because they share an identical RRM domain (illustrated in Figure 2) (*29*). RBFox1 and RBFox2 are also known as the ataxin-2 binding protein 1 (A2BP1) and RNA-binding motif protein 9 (RBM9), respectively. The latest addition to the family, RBFox3, has not been extensively characterized and its role is still not very well understood.

5.4. *KHDRBS1*

KH domain-containing, RNA binding, signal transduction-associated 1 (KHDRBS1), previously known as Src-associated in mitosis 68 kDa protein (SAM68), is a nuclear RNA-binding protein. KHDRBS1 has been reported to be involved in numerous mRNA metabolism events, including nuclear export, translation, and somatodendritic transport, as well as splicing (*30,31*). KHDRBS1 belongs to the signal transduction and activation of RNA (STAR) domain protein family. KHDRBS1 possesses a GSG domain that binds to A- or U-rich RNA sequences,
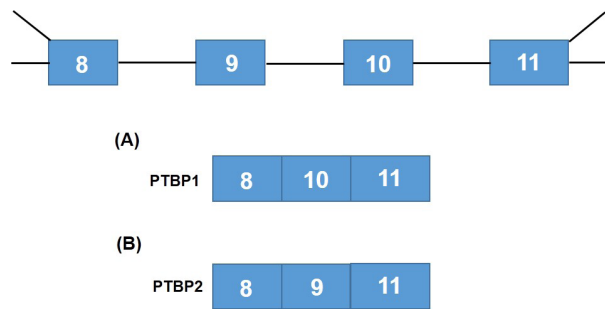
such as UAAA or UUUA, and forms homodimers. Consistent with its role in alternative splicing, KHDRBS1 is primarily localized in the nucleus (*30,31*).

6. An example of splicing regulation mediated by the RBFox protein family

In this section, we describe a mechanism for neuronal alternative splicing based on our previously published report (*32*), consistent with several others (summarized in Figure 3). The RBFox1 protein family functions as either a splicing enhancer or a repressor in the regulation of alternative splicing (*27,33*). The RBFox1 protein family specifically binds to the (U)GCAUG target sequence. It functions as an exon repressor when the target pentanucleotide is located in the upstream intronic flanking (UIF) region of the regulated exon. In contrast, the RBFox1 protein family promotes the inclusion of the skipping exon (cassette exon) if the (U)GCAUG element is located in the downstream intronic flanking (DIF) region.

As an example, in many heterologous cells, RBFox1 expression promotes skipping of exon 9 in the F1γ mini-gene by specifically binding to the GCAUG stretches located within the UIF region (*28,33*). Another example of RBFox-mediated regulation was demonstrated in the CaV1.2 L-type calcium channel gene, where the expression of RBFox1 and RBFox2 repressed exon 9* through (U)GCAUG motifs in the intronic and exonic regions (*34*).

As previously mentioned, the RBFox1 family can also mediate the inclusion of skipping exon (cassette exon) *via* (U)GCAUG sequences located within the DIF region. In the rat fibronectin gene, RBFox1 expression promoted the inclusion of an EIIIB exon *via* highly repeated and evolutionarily conserved (U)GCAUG elements in the DIF region. Additionally, several studies have shown that RBFox2 can positively regulate the

Figure 1. Schematic diagrams of *PTBP1* and *PTBP2*. Figure shows comparison between **(A)** *PTBP1* gene and **(B)** *PTBP2* gene structure.
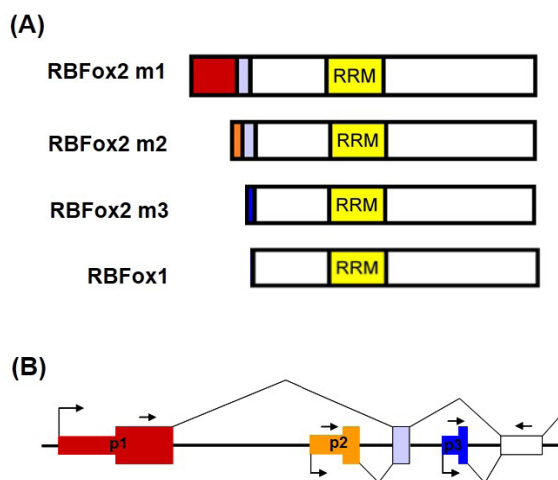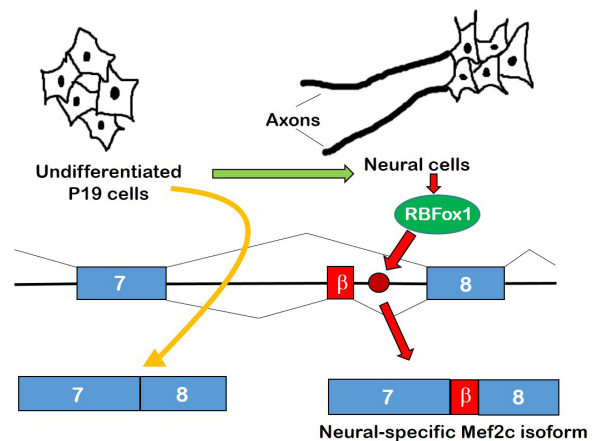

Figure 2. Alternative promoters in RB*Fox2*. Schematic diagrams of **(A)** RBFox2 isoforms and **(B)** *RBFox2* gene structure. The *RBFox2* gene has at least three alternative splicing promoters expressed in P19 cells. This figure has been modified from Hakim NHA, *et al*. (*29*).

Figure 3. A model for the regulation of *Mef2c* exon β alternative splicing by RBFox1. Findings from our previous study suggested that RBFox1, which is specifically expressed in the neuronal cell stage, promoted Mef2c exon β inclusion *via* the GCAUG motif (*32*).

inclusion of exon 16 in the protein 4.1 R pre-mRNA during late erythroleukemia cell differentiation and in heterologous HeLa cells *via* conserved triple (U)GCAUG repeats within the DIF region (*33,35,36*).

7. A comprehensive analysis of alternative splicing and functionality in the neuronal differentiation of P19 cells

In order to thoroughly investigate the neural splicing event in the P19 mouse embryonic carcinoma cell line, we previously performed a GeneChip exon array study using total RNA purified from undifferentiated P19 cells (Day 0), neuronal-differentiated cells (Day 7), and cells from the glial cell stage (Day 10) (*37*). We employed nine filtering conditions for probe sets based on annotations, estimated gene expression levels, splicing index (SI), and detection above background (DABG) values, as well as alternative splicing predictions. We extracted 262 differentially alternatively spliced (DAS) candidate exons for neural splicing. Among them, 30 exons were validated by RT-PCR. Results from RT-PCR suggested that 87% of these exons were altered between undifferentiated and neuronal-differentiated cells. Furthermore, bioinformatics approaches, such as gene ontology (GO) analysis, text-mining, and pathway analysis, also suggested that many candidate exons were associated with neural events. In addition to the gene-level informatics analyses, we also performed text-mining for the alternative isoforms. This type of analysis is a new approach to detecting exon-level annotations (*37*).

In order to test whether the 262 DAS exons of 236 DAS genes were involved in neural events, GO analysis was performed using the Biological Process category. These terms were categorized into 10 groups: neural-related process, differentiation and development, signalling, post-translational regulation, transcription, cytoskeleton and cell adhesion, cellular transport, cell cycle and proliferation, apoptosis, and others. The most enriched category was neural-related process (20.8%), followed by differentiation and development (12.5%), cytoskeleton and cell adhesion (12.5%), and signalling (9.7%) (*37*).

The functions (cell processes) of 151 of the 236 DAS genes have been previously described; however, the remaining 85 genes were classified as unknown genes. Because their functions in neural cells or organs have been previously reported, 66 of the 151 DAS genes with known functions were categorized as well-known genes in neural processes. The remaining 85 genes were categorized as functional DAS genes owing to limited evidence for their neural regulatory roles. Our results suggested that many DAS genes are associated with neural events, which was also demonstrated by the GO analyses. Because the 66 well-known genes have been studied in neural cells

or organs, further studies on functional differences between the alternative isoforms may reveal their functions in non-neural cells or organs (*37*).

We subsequently identified biological relationships between the 189 cell processes and 85 functional DAS genes. The analysis showed that 47 DAS genes were linked to these cell processes. These relationships are potentially important because isoforms created by neural splicing may have novel functions that have not been previously reported in neural cells. Furthermore, 11 of the 47 genes were involved in cell cycle-related events, as well as in G1 and/or S phase processes (*37*).

In addition, we also performed GO analysis on the 66 genes whose functions are known in neural cells and tissues. Surprisingly, RNA splicing was the most frequent GO term for these genes. Remarkably, the *RBFox1* homologue was among the 66 genes. The exon array analysis results also suggested that *RBFox2* has one alternative exon, which was altered in neuronal differentiation (*37*).

8. Alternative splicing and disease

In certain regulators, such as RBFox2, hnRNP, and SR proteins, changes in the nuclear level occur frequently in cancer (*39*). Furthermore, previous studies have shown that a number of splicing factors, including SR, RBFox, and hnRNPA1 proteins, are associated with Alzheimer's disease (AD) (*39*). Technically, the expression is not misregulated in the disease, but in the patient that has the disease. On the other hand, splicing components (*e.g.*, U1 snRNP) seem to be depleted from the nucleus, thus forming cytoplasmic aggregates (*39*).

RBFox proteins have been known to play an important role in brain development and function. Thus, the haploinsufficiency of this protein has been implicated in various neuropsychiatric disorders, including Autism spectrum disorder (ASD). Since RBFox proteins affect other pathways in transcription in addition to RNA processing, it is challenging to identify clinically relevant sets of splicing events. In addition, there are three highly related RBFox proteins expressed in the brain, with partially overlapping functions (*39*).

9. Conclusions

Alternative splicing is a major contributor to proteomic diversity. This mechanism allows multiple proteins to be generated from a single gene. In addition, the resulting isoforms may have different functions or activities, as exon combinations are altered in alternative splicing events. Changes in splicing patterns are influenced by regulatory proteins that bind to pre-mRNA and enhance or silence particular splicing choices. In addition, it has been noted that some splicing factors have been linked to neuronal diseases for instance AD and ASD as well as cancer (*39*).

Acknowledgements

References

1. Lodish H, Berk A, Kaiser CA, Krieger M, Scott, MP, Bretscher A, Ploegh H, Matsudaira P. Molecular cell biology. W. H. Freeman, New York, USA, 2008; pp. 325-335.
2. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular biology of the cells. Garland Science, New York, USA, 2007; pp. 479-480.
3. Suzuki H, Tsukahara T. A view of pre-mRNA splicing from RNase R resistant RNAs. Int J Mol Sci.. 2014; 15:9331-9342.
4. Sharma S, Kohlstaedt LA, Damianov A, Rio D, Black DL. Polypyrimidine tract binding protein controls the transition from exon definition to an intron defined spliceosome. Nat Struct Mol Biol. 2008; 15:183-191.
5. Li Q, Lee JA, Black DL. Neuronal regulation of alternative pre-mRNA splicing. Nat Rev Neurosci. 2007; 8:819-831.
6. Shin C, Manley JL. Cell signalling and the control of pre-mRNA splicing. Nat Rev Mol Cell Biol. 2004; 5:727-738.
7. Smith CW, Valcarcel J. Alternative pre-mRNA splicing: The logic of combinatorial control. Trends Biochem Sci. 2000; 25:381-388.
8. Black DL. Protein diversity from alternative splicing: A challenge for bioinformatics and post – genome biology. Cell. 2000; 103:367-370.
9. Graveley BR. Alternative splicing: Increasing diversity in the proteomic world. Trends Genet. 2001; 17:100-107.
10. Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science. 2003; 302:2141-2144.
11. Blencowe BJ. Alternative splicing: New insights from global analyses. Cell. 2006; 126:37-47.
12. Matlin AJ, Clark F, Smith CW. Understanding alternative splicing: Towards a cellular code. Nat Rev Mol Cell Biol. 2005; 6:386-398.
13. Wagner EJ, Garcia-Blanco MA. Polypyrimidine tract binding protein antagonizes exon definition. Mol Cell Biol. 2001; 21:3281-3288.
14. Black DL. Mechanisms of alternative pre-messenger RNA splicing. Annu Rev Biochem. 2003; 72:291-336.
15. Amir-Ahmady B, Boutz P, Markovtsov V, Phillips ML, Black DL. Exon repression by polypyrimidine tract binding protein. RNA. 2005; 11:699-716.
16. Valcárcel J, Gebauer F. Post-transcriptional regulation: The dawn of PTB. Curr Biol. 1997; 7:R705-708.
17. Smith CW, Valcárcel J. Alternative pre-mRNA splicing: The logic of combinatorial control. Trends Biochem Sci. 2000; 25:381-388.
18. Singh R, Valcárcel J, Green MR. Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract binding proteins. Science. 1995; 268:1173-1176.
19. Pérez I, McAfee JG, Patton JG. Multiple RRMs contribute to RNA binding specificity and affinity for polypyrimidine tract-binding proteins. Biochemistry. 1997; 36:11881-11890.
20. Simpson PJ, Monie TP, Szendroi A, Davydova N, Tyzack JK, Conte MR, Read CM, Cary PD, Svergun DI, Konarev PV, Curry S, Matthews S. Structure and RNA interactions of the N-terminal RRM domains of PTB. Structure. 2004; 12:1631-1643.
21. Licatalosi DD, Yano M, Fak JJ, Mele A, Grabinski SE, Zhang C, Darnell RB. Ptbp2 represses adult-specific splicing to regulate the generation of neuronal precursors in the embryonic brain. Genes Dev. 2012; 26:1626-1642.
22. Jensen KB, Musunuru K, Lewis HA, Burley SK, Darnell RB. The tetranucleotide UCAY directs the specific recognition of RNA by the Nova K-homology 3 domain. Proc Natl Acad Sci U S A. 2000; 97:5740-5745.
23. Dredge BK, Darnell RB. Nova regulates GABAA receptor $\gamma2$ alternative splicing *via* a distal downstream UCAU-rich intronic splicing enhancer. Mol Cell Biol. 2003; 23:4687-4700.
24. Auweter SD, Fasan R, Reymond L, Underwood JG, Black DL, Pitsch S, Allain H. Molecular basis of RNA recognition by the human alternative splicing factor Fox -1. The EMBO J. 2006; 25:163-173.
25. Hodgkin J, Zellan J, Albertson D. Identification of a candidate primary sex determination locus, fox-1, on the X -chromosome of Caenorhabditis elegans. Development. 1994; 120:3681-3689.
26. Skipper M, Milne CA, Hodgkin J. Genetic and molecular analysis of fox-1, a numerator element involved in *Caenorhabditis elegans* primary sex determination. Genetics. 1999; 151:617-631.
27. Fukumura K, Kato A, Jin Y, Ideue T, Hirose T, Kataoka N, Fujiwara T, Sakamoto H, Inoue K. Tissue-specific splicing regulator Fox-1 induces exon skipping by interfering E complex formation on the downstream intron of human *F1γ* gene. Nucleic Acids Res. 2007; 35:5303-5311.
28. Jin Y, Suzuki H, Maegawa S, Endo H, Sugano S, Hashimoto K, Yasuda K, Inoue K. A vertebrate RNA-binding protein Fox-1 regulates tissue-specific splicing *via* the pentanucleotide GCAUG. EMBO J. 2003; 22:905-912.
29. Hakim NHA, Majlis BY, Suzuki H, Tsukahara T. Expression Analysis of Fox-2 Alternative Isoforms during Neuronal Differentiation. Int'l Journal of Advances in Chemical Engineering & Biological Sciences (IJACEBS). 2016; 3:102104
30. Chawla G, Lin CH, Han A, Shiue L, Ares M, Black DL. Sam68 regulates a set of alternatively spliced exons during neurogenesis. Mol Cell Biol. 2009; 29:201-213.
31. Pedrotti S, Bielli P, Paronetto MP, Ciccosanti F, Fimia GM, Stamm S, Manley JL, Sette C. The splicing regulator Sam68 binds to a novel exonic splicing silencer and functions in SMN2 alternative splicing in spinal muscular atrophy. EMBO J. 2010; 29:1235-1247.
32. Hakim NH, Kounishi T, Alam AH, Tsukahara T, Suzuki H. Alternative splicing of Mef2c promoted by Fox-1 during neuronal differentiation in P19 cells. Genes Cells. 2010; 15:255-267.
33. Kuroyanagi H. Fox-1 family of RNA-binding proteins. Cell Mol Life Sci. 2009; 66:3895-3907.

34. Yang L, Embree LJ, Hickstein DD. TLS-ERG leukemia fusion protein inhibits RNA splicing mediated by serine-arginine proteins. Mol Cell Biol. 2000; 20:3345-3354.

35. Ponthier JL, Schluepen C, Chen W, Lersch RA, Gee SL, Hou VC, Lo AJ, Short SA, Chasis JA, Winkelmann JC, Conboy JG. Fox-2 splicing factor binds to a conserved intron motif to promote inclusion of protein 4.1 R alternative exon 16. J Biol Chem. 2006; 281:12468-12474.

36. Newman A. Molecular biology. RNA enzymes for RNA splicing. Nature. 2001; 413:695-696.

37. Suzuki H, Osaki K, Sano K, Alam AH, Nakamura Y, Ishigaki Y, Kawahara K, Tsukahara T. Comprehensive analysis of alternative splicing and functionality in neuronal differentiation of P19 cells. PLoS One. 2011; 6:e16880.

38. Yang G, Huang SC, Wu JY, Benz EJ Jr. Regulated Fox-2 isoform expression mediates protein 4.1 R splicing during erythroid differentiation. Blood. 2008; 111:392-401.

39. Chabot B, Shkreta L. Defective control of pre-messenger RNA splicing in human disease. J Cell Biol. 2016; 212:13-27.