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Recent Advances in Understanding of Alternative Splicing in Neuronal Pathogenesis

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1. Introduction

The nervous system is an intricate and highly specialized network of neurons. Neuronal differentiation involves complex reprogramming of gene expression. Alternative splicing of precursor mRNAs increases the complexity of transcriptomes and diversifies protein functions at the post-transcriptional level. Indeed, alternative splicing plays an important role in neuronal differentiation, axon guidance, synaptogenesis, synaptic transmission, and plasticity. Because the delicate structure and function of neurons make them particularly susceptible to dysregulation of splicing, aberrant expression or function of splicing factors may cause neuronal disorders. Therefore, it is important to improve our understanding of the mechanisms and physiological functions of alternative splicing regulation in neurons. Regulation of alternative splicing primarily involves the binding of regulatory factors to specific *cis*-elements of precursor mRNAs, and interplay between splicing factors may lead to fine tuning of splicing regulation, thereby diversifying the cadre of mature products. In addition, transcription rate and the availability of the basal splicing machinery may also influence alternative splicing. Recently, our understanding of the mechanisms underlying alternative splicing have been advanced from studies of several neuronal splicing factors; these studies have utilized genetic knockout or disease models as well as genome-wide analysis of mRNA isoforms. In this chapter, we review current understanding of alternative splicing in neurons.

2. Introduction to alternative splicing

Recent estimates have indicated that as many as 95% of human genes generate alternatively spliced mRNAs (Pan, et al., 2008; E. T. Wang, et al., 2008). Alternative splicing of precursor mRNAs (pre-mRNAs) may alter the coding sequence and hence change the function or stability of the encoded proteins. Moreover, alternative splicing may create pre-mature termination codons within the coding region due to frame shift, thereby inducing mRNA destruction via the nonsense-mediated decay pathway (Isken & Maquat, 2007; McGlincy & Smith, 2008; Moore & Proudfoot, 2009). Alterative splicing may also occur in the 3' untranslated region of a pre-mRNA and thus create or eliminate *cis*-regulatory elements that may change the kinetics of mRNA decay or translation (Khabar, 2010; Thiele, et al., 2006). Therefore, alternative splicing is a mechanism that not only increases protein diversity but

also may post-transcriptionally modulate the level of gene expression. Moreover, a growing body of evidence suggests that coordinated control of alternative splicing of functionally related transcripts allows for proper orchestration of cellular processes and thus the maintenance of homoeostasis (Allen, et al., 2010; Calarco, et al., 2011; Licatalosi & Darnell, 2010).

Alternative splicing plays a critical role in many fundamental biological processes, such as cell differentiation and specification during development and specificity of function in diverse cell types (Keren, et al., 2010; Nilsen & Graveley, 2010). The nervous system also adopts alternative splicing for cell differentiation, morphogenesis, and even for formation of complex neuronal networks and delicate synapse formation/plasticity (Calarco, et al., 2009; Grabowski, 2011; Li, et al., 2007). An extreme case of alternative splicing is the gene encoding *Drosophila* Down syndrome cell adhesion molecule (Dscam), which potentially could generate >38000 mRNA isoforms by mutually exclusive selection of cassette exons (Hattori, et al., 2008). Dscam encodes neuronal recognition proteins that act as axon guidance receptors. Homotypic interaction between identical Dscam isoforms on opposing membranes causes repulsion between sister neurites (J. W. Park & Graveley, 2007). Therefore, accurate alternative splicing control is critical for establishment of neural circuits in *Drosophila*. In the mammalian brain, alternative splicing is also an important regulatory mechanism for creating the remarkable capacity for plasticity and adaptation. For example, the splicing-mediated splicing of exon 21 in the ionotropic glutamate receptor *N*-methyl *D*-aspartate (NMDA) receptor subunit 1 mRNA affects the membrane trafficking of the NMDA receptor (Ares, 2007). Interestingly, inclusion of exon 21 can be suppressed, likely by the calmodulin-dependent protein kinase IV pathway, which is activated upon cell depolarization (Ares, 2007). Therefore, splicing control provides an intricate and rapid means for regulating mRNA isoform expression.

Alternative splicing is primarily controlled by splicing regulatory factors that bind to *cis*-elements within exons and/or introns of pre-mRNAs. Their binding may modulate the loading of the spliceosomal components to the splice sites and thereby influence alternative splice site utilization (Chen & Manley, 2009; Witten & Ule, 2011). Several neuron-specific splicing regulatory factors have been discovered; some of them, such as the neuro-oncological ventral antigen (Nova), have been studied intensively (Licatalosi & Darnell, 2010; C. Zhang, et al., 2010). In particular, identification of mRNA targets and potential binding sites of Nova have benefited greatly from recent genome-wide splicing arrays and sequencing technology. Therefore, the study of Nova has provided a detailed picture of a splicing regulatory network as well as the combinatorial action of multiple splicing factors (Nilsen & Graveley, 2010; Z. Wang & Burge, 2008).

The repertoire of splicing factors is adjusted during neuronal differentiation and perhaps for functional specification of different neuronal cell types. For example, the switch in the expression of the polypyrimidine tract-binding protein (PTB) to its neuronal homolog, nPTB, may tune neuronal transcriptomes during differentiation (Coutinho-Mansfield, et al., 2007; Tang, et al., 2011). In addition to RNA-binding factors, altered abundance of basic splicing machinery components may also modulate splice site selection (Calarco, et al., 2011; Saltzman, et al., 2011). The survival of motor neuron (SMN) protein is a key factor for the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). SMN deficiency may reduce snRNP abundance and thus influence splicing. Defective Nova and/or SMN proteins are associated with disease (Robert B. Darnell, 2011; Lorson, et al., 2010; Lukong, et al., 2008; G. H. Park, et al., 2010). Therefore, to understand how defects of Nova and SMN

induce pathological effects on neurons via misregulated splicing control is an interesting and important question. Therefore, a complete understanding of such diseases will require knowledge of how misregulated splicing control in Nova or SMN causes neuronal pathology.

We hereby discuss our current knowledge of Nova, PTB/nPTB, and SMN with respect to their splicing regulation mechanisms, physiological roles, and functions.

3. Nova

3.1 Nova proteins and expression

Nova was discovered as a target of the autoantibodies in cancer patients with paraneoplastic opsoclonus myoclonus ataxia, a type of rare paraneoplastic neurologic disease that causes motor disorders (Buckanovich, et al., 1993; R. B. Darnell & Posner, 2003; Yang, et al., 1998). The amino acid sequences of the two mammalian Nova proteins (1 and 2) are highly similar throughout their lengths, and both contain three heterogeneous nuclear RNP (hnRNP) K-homology (KH) RNA-binding domains (Buckanovich, et al., 1996; Yang, et al., 1998). In mice, Nova-1 expression is restricted in subcortical regions of developing and mature neurons, whereas Nova-2 is more broadly expressed in the central nervous system (Buckanovich, et al., 1993; Buckanovich, et al., 1996; Yang, et al., 1998). Moreover, Nova-1 and Nova-2 appear to be reciprocally expressed in the nervous system, i.e., the level of Nova-2 is generally low in regions where Nova-1 is abundant, which may reflect their distinct biological functions (Yang, et al., 1998). Nevertheless, Nova-1 and -2 primarily act as splicing regulatory factors and also play a role in alternative polyadenylation and mRNA trafficking in neuronal dendrites (Licatalosi, et al., 2008; Racca, et al., 2010).

3.2 RNA-binding specificity of Nova

The RNA-binding specificity of the Nova proteins has been examined by various methods. Initially, the results of *in vitro* systematic selection of ligands and RNP immunoprecipitation indicated that Nova-1 binds to long stem-loop RNAs containing UCAU repeats (Buckanovich & Darnell, 1997). Nova-2-selected RNA ligands also appear to form a stem-loop structure encompassing the UCAU motif (Yang, et al., 1998). Structural studies have shown that both Nova-1 and -2 bind to UCAU-containing sequences via their KH3 domains, of which the residues involved in UCAU interaction are conserved (Lewis, et al., 2000). Nevertheless, the KH1 and KH2 domains of both Nova proteins can also bind to the UCAU motif (Musunuru & Darnell, 2004). Perhaps cooperativity between several KH domains promotes protein binding to RNAs (Chmiel, et al., 2006; Valverde, et al., 2008). The similar RNA-binding properties of Nova-1 and Nova-2 suggest that they may regulate common RNA targets.

Next, ultraviolet cross-linking in conjunction with ribonucleoprotein immunoprecipitation (CLIP) was developed to identify *in vivo* targets of RNA-binding proteins. CLIP analysis has been applied to identify Nova-associated RNA fragments in mouse brain; approximately 340 Nova-1/-2 CLIP tags (~70 nucleotides each) contained, on average, four YCAY repeats (Y, pyrimidine) (Ule, et al., 2003). Detailed analysis of these tag sequences revealed an overrepresentation of YCAU tetramers flanked by pyrimidines. It is currently believed that YCAY repeats are the principal elements in the mRNAs to which Nova proteins bind. More recently, an unbiased method using CLIP combined with high-throughput sequencing (HITS) of RNA was used to identify genome-wide functional protein-RNA interactions (R.

B. Darnell, 2010; Ule, et al., 2005a). A HITS-CLIP analysis showed that Nova-2 tags in mouse cortex, as expected, also harbor ~3.6 YCAY repeats per tag (Ule, et al., 2005a). The results of all these CLIP analyses of Nova coincide with previous observations from RNA selection experiments (Licatalosi, et al., 2008).

Moreover, the aforementioned CLIP analyses also revealed that the largest set of Nova tags is located within introns and even flanking alternative exons (Licatalosi, et al., 2008), which is consistent with the primary role of Nova proteins in regulating pre-mRNA splicing (see below). In addition, Nova tags are also found in protein-coding regions and 3' untranslated region of mRNAs. The results of HITS-CLIP experiments have confirmed the high frequency of potential Nova-binding elements in 3' untranslated regions, thus disclosing a role for Nova in alternative polyadenylation and mRNA transport regulation in brain (Licatalosi, et al., 2008; Racca, et al., 2010).

3.3 Alternative splicing regulated by Nova

Earlier studies have shown that two neuronal transcripts, encoding the inhibitory glycine receptor α2 (GlyRα2) and Nova-1 itself, contain the Nova-1-binding elements adjacent to the alternatively spliced exons and indeed bound to the Nova-1 protein (Buckanovich & Darnell, 1997). The role of Nova-1 in splicing regulation was first analyzed by genetic knockout of Nova-1 in mice (Jensen, et al., 2000); in those mice, selection of GlyRα2 exon 3A is diminished. Further experiments demonstrated that Nova-1 binds to three consecutive YCAY repeats in the intron upstream of exon 3A and thereby increases exon 3A inclusion. Alternative exon selection in another neuronal ionotropic receptor, $GABA_A$ Rγ2, is also impaired in Nova-1 null mice (Dredge & Darnell, 2003; Jensen, et al., 2000). Nova-1 was subsequently shown to promote $GABA_A$ Rγ2 exon 9 inclusion via a distal downstream intronic YCAY-rich splicing enhancer. Notably, the Nova-1 gene itself harbors five YCAY repeats in its exon 4 that are indeed essential for Nova-1 autoregulation (Dredge, et al., 2005). Inclusion of exon 4 in Nova-1 transcripts was increased in haploinsufficient $Nova^{+/-}$ mice. Accordingly, exon 4 inclusion in a Nova-1 splicing reporter was suppressed upon Nova-1 overexpression. In this case, Nova-1 acts via binding to the YCAY repeats in the alternative exon. Therefore, Nova can function either as a positive or negative splicing regulator via binding to intronic or exonic YCAY elements. Further study by swapping of Nova-1-binding sites between various splicing substrates of Nova-1 indicated that the action of Nova-1 is determined by the position of the Nova-binding elements (Dredge, et al., 2005). This positional effect has also been found in other splicing regulators (see below).

Through CLIP and exon junction arrays, a large number of the potential targets of Nova, including some previously defined targets, have been identified (Ule, et al., 2003). Alternative splicing of several candidates was indeed altered in Nova knockout mice, confirming that those transcripts undergo Nova-mediated splicing control. This systematic and genome-wide identification also indicted that Nova may coregulate a set of synaptic and axonal transcripts by controlling alternative splicing. Such coordinated control of alternative exon usage may perhaps provide a powerful means to rapidly modulate synaptic function in response to stimuli.

More recently, HITS-CLIP analysis established functional RNA binding maps of Nova (Licatalosi, et al., 2008). That analysis predicted ~600 differentially spliced exons in brain that are potentially targeted by Nova-2. The transcripts harboring some of the identified exons showed a severe splicing defect in the neocortex of Nova-2 null mice, where Nova-2 is exclusively expressed. However, minor effects were seen in the spinal cord, cerebellum, and

midbrain perhaps owing to redundancy of Nova-1 and Nova-2 in these tissues (Licatalosi, et al., 2008). Other work has indicated that Nova proteins regulate alternative splicing across all members of the spectrin-ankyrin-protein 4.1-CASK scaffold complex and the Cav2.2 voltage-gated calcium channels and thereby may finely modulate synaptic function in a coordinated way (Licatalosi, et al., 2008; Ule, et al., 2005b). A genome-wide search in combination with gene ontology analysis revealed that Nova-2-regulated transcripts encode a group of synaptic proteins located at the cell membrane, most often at cell-cell junctions, and are implicated in synapse biogenesis and synaptic transmission. Therefore, Nova-1 and -2 may function analogously in regulating alternative splicing of pre-mRNAs encoded by functionally related genes in neurons.

Drosophila pasilla is the homolog of mammalian Nova proteins (Seshaiah, et al., 2001). Pasilla localizes primarily to nuclear puncta in *Drosophila* cells, indicating its role in splicing. Interestingly, identification of the mRNA targets of pasilla also revealed an enrichment of YCAY repeats near pasilla-regulated cassette exons (Brooks, et al., 2011). Like Nova, pasilla suppresses alternative exon inclusion when it binds predominantly upstream of the exon, whereas it activates splicing when it binds downstream of the exon. Therefore, the RNA-binding specificity and regulatory activity of Nova orthologs apparently have been preserved throughout evolution (Irimia, et al., 2011; Jelen, et al., 2007).

3.4 Mechanisms of Nova-mediated splicing regulation

The interplay between Nova proteins and *cis*-elements was revealed by CLIP-based analyses. CLIP followed by genome-wide detection has identified a considerable number of Nova-binding sites and Nova-regulated transcripts. Bioinformatics analysis revealed the mechanism of Nova-mediated alternative splicing regulation (Ule, et al., 2006). Consistent with previous reports using minigene splicing assays, the position of Nova-binding sites in pre-mRNAs determines the effect of Nova proteins on splicing (Dredge, et al., 2005). For a cassette exon, Nova binding to its downstream intronic YCAY clusters enhances exon inclusion, whereas exon skipping occurs when Nova binds either immediately upstream of or within the exon. Therefore, Nova binding to its target *cis*-elements may result in an asymmetric action on splicing regulation. Indeed, such a positional effect has also been observed for some other splicing regulatory factors (see below, (Konig, et al., 2010; Xue, et al., 2009; Yeo, et al., 2009)).

Bioinformatic analysis of the Nova HITS-CLIP data using Bayesian networks has further provided a comprehensive view of alternative splicing coordinately regulated by Nova and cofactors (C. Zhang, et al., 2010). This analysis initially predicted more than 600 Nova-regulated alternative splicing events. Gene ontology classification supported the hypothesis that Nova may regulate a subgroup of functionally coherent genes involved in synaptic plasticity. This analysis also revealed that the avidity with which Nova binds YCAY clusters may modulate how Nova affects splicing. Moreover, Nova binding to multiple regions may result in a different effect on alternative exon selection from binding to a single region (Figure 1). For example, Nova binding to both the regulated exon and its upstream intron increases the probability of exon exclusion. Moreover, it is estimated that ~15% of Nova targets harbor binding sites for the splicing factors Fox-1 and Fox-2, implying combinatory splicing regulation by Nova and Fox (C. Zhang, et al., 2010). Therefore, an intriguing issue is how the relative location of Nova and Fox binding sites dictates splicing regulation (Chen & Manley, 2009; Licatalosi & Darnell, 2010).

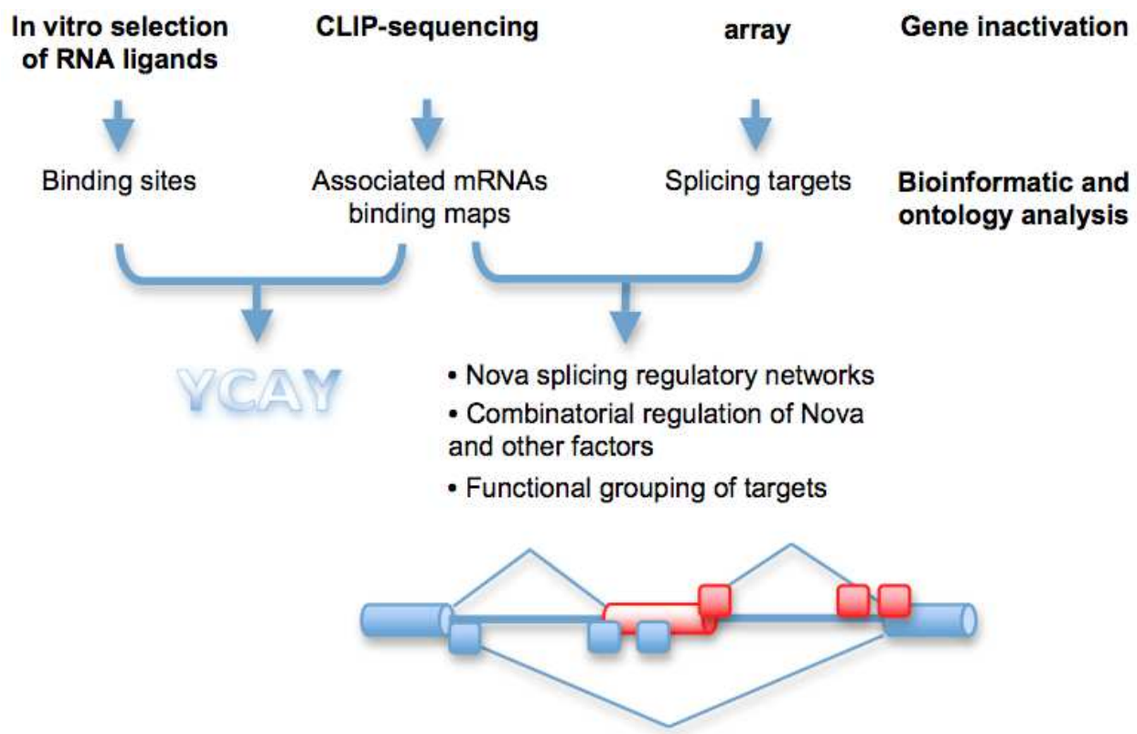

Fig. 1. **Establishment of a Nova regulatory network.** The binding sites and target mRNAs of Nova proteins are identified through *in vitro* RNA selection and *in vivo* crosslinking followed by sequencing or array analysis. In addition, comparative genome-wide transcriptome profiling of Nova knockout mutants also facilitates identification of the transcripts regulated by Nova. Bioinformatic and ontology analysis helps to establish binding and functional maps for Nova. Methods used are denoted by bold type. The bottom diagram shows a model of Nova-mediated splicing control. Positive and negative *cis*-elements that appear with high frequency in CLIP datasets are depicted by red and blue rectangles, respectively. Red and blue cylinders are alternative and constitutive exons, respectively.

Previous studies have also revealed how Nova proteins may modulate the activity of the spliceosome. For example, Nova binding to exons may interfere with U1 snRNP binding to the 5' splice site, thereby inhibiting splicing (Dredge, et al., 2005). When Nova binds to the downstream intron of a regulated exon, it may facilitate spliceosome assembly and promote exon inclusion (Dredge, et al., 2005). As described above, Nova and Fox proteins may coregulate alternative splicing of a considerable number of transcripts in a cooperative or antagonistic manner (C. Zhang, et al., 2010). Notably, neuronal depolarization can induce exon 19 exclusion of Fox-1, producing a Fox-1 isoform with higher splicing activity (Lee, et al., 2009). Therefore, under certain circumstances this Fox-1 isoform may function coordinately with Nova to modulate splicing of Fox-1/Nova-coregulated mRNAs. Moreover, the neuron-enriched nPTB can antagonize Nova action to increase inclusion of GlyRα2 exon 3A (Polydorides, et al., 2000). The physical and functional interactions between Nova and other splicing regulatory factors certainly complicate Nova splicing networks, but the detailed mechanisms remain to be investigated.

3.5 Cellular signaling pathways affect Nova expression level and activity

In contrast to the abundant information about Nova-target interactions, we have only rudimentary knowledge of whether Nova's function can be modulated by cellular signaling pathways. However, several reports have indicated that Nova expression may be regulated at different gene expression levels. It has been shown that the neuronal protein embryonic lethal abnormal visual (nELAV) can increase the stability of the Nova-1 mRNA via binding to its AU-rich elements in the highly conserved 3'-untranslated region (Ratti, et al., 2008; Rossi, et al., 2009). In addition, protein kinase C–induced phosphorylation of nELAV can promote Nova-1 translation (Ratti, et al., 2008). Therefore, nELAV can increase Nova-1 abundance. Moreover, nELAV can modulate the splicing activity of Nova-1 on its target pre-mRNAs (Ratti, et al., 2008). Glucocorticoids can also regulate Nova-1-mediated alternative splicing by downregulating Nova-1 (E. Park, et al., 2009). Moreover, cholinergic stimulation may decrease Nova-2 transcripts but increase Nova-1 transcripts in striatum (Jelen, et al., 2010). Therefore, the expression switch between these two Nova proteins may modify Nova activity in neurons. Finally, it is noteworthy that Nova-1 can autoregulate its exon 4 inclusion by acting as a splicing repressor (Dredge, et al., 2005). Because exon 4 contains multiple phosphorylation sites for serine/threonine kinases, it would be interesting to know whether Nova-1 may modulate its own activity via autoregulation of alternative splicing in response to activation of specific cellular signaling pathways.

3.6 Physiological function and pathological implications of Nova

Early studies of Nova-1 and -2 showed that these proteins have a reciprocal expression pattern in the neocortex and hippocampus in postnatal mouse brain and may have slightly different RNA-binding specificity and/or affinity (Buckanovich & Darnell, 1997; Yang, et al., 1998). Genetic knockout studies then provided further hints to their different physiological roles. Nova-1 knockout mice died 7-10 days after birth owing to a motor deficit caused by apoptotic death of spinal and brainstem neurons, indicating that Nova-1 is an essential gene in mice (Jensen, et al., 2000; Yang, et al., 1998). Nova-2 null mice died in the second postnatal week, whereas double Nova knockout caused perinatal death (Ruggiu, et al., 2009; Ule, et al., 2006). Microarray analysis revealed distinct splicing defects for Nova-1 vs. Nova-2 knockout mice (Ule, et al., 2005b; C. Zhang, et al., 2010). These results indicate that these two Nova genes have non-redundant physiological functions.

Identified targets of Nova have implicated a role for Nova in synaptic plasticity. As predicted, long-term potentiation induced by $GABA_B$ receptor-mediated slow inhibitory postsynaptic current in hippocampal neurons is abolished in Nova-2 knockout mice (Huang, et al., 2005). Moreover, a recent report showed a migration deficiency in cortical and Purkinje neurons in Nova-2 null mice (Yano, et al., 2010). These observations are consistent with the role of Nova-2 in alternative splicing regulation of GABA receptor subunits and of disabled-1, a regulatory factor of the reelin signaling pathway essential for cell positioning during neurogenesis. Therefore, Nova-2 can regulate neuronal migration and synaptic plasticity via its control of alternative splicing.

Certain Nova targets have been implicated in genetic disorders. For example, reelin-disabled-1 signaling may be associated with epilepsy, schizophrenia, and autism (Frotscher, 2010; Pardo & Eberhart, 2007). Interestingly, Fox-1, a functional partner of the Nova proteins, has been implicated in autism (Martin, et al., 2007; Smith & Sadee, 2011). Indeed, the genes coregulated by Nova and Fox appear to be more frequently associated with autism (C. Zhang, et al., 2010). Therefore, aberrant regulation of Nova proteins may contribute to autism.

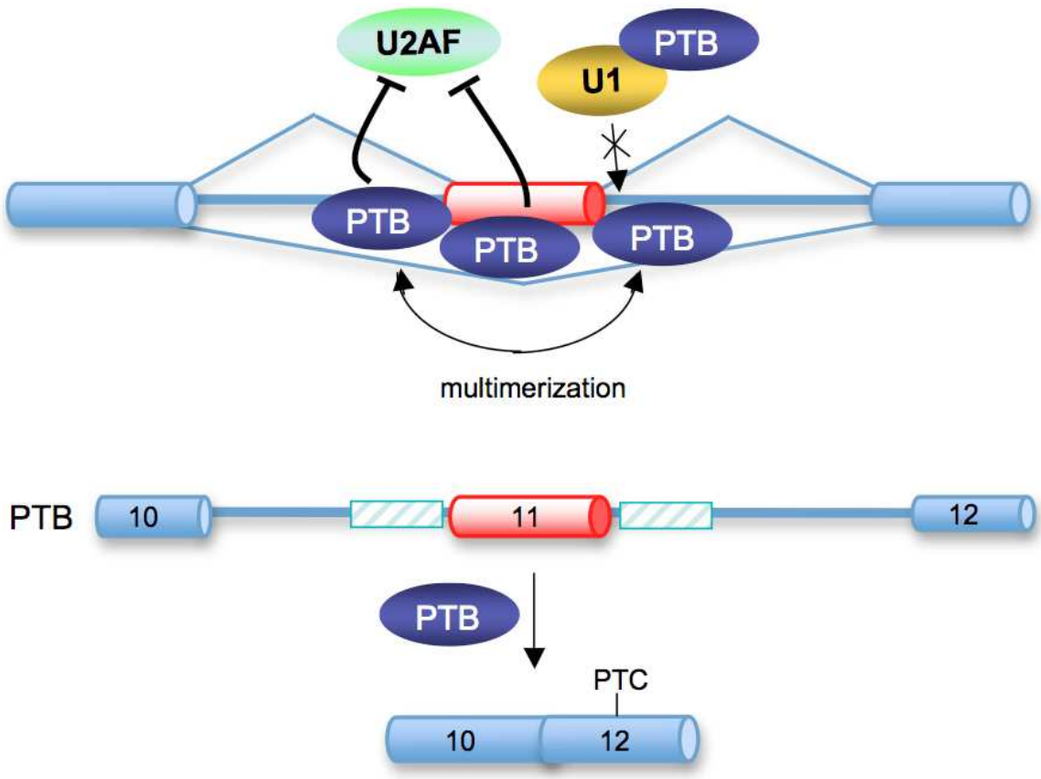

Fig. 2. **PTB-mediated splicing regulation and autoregulation.** Top diagram: PTB suppresses exon inclusion when bound to silencing elements located upstream or downstream of an alternative exon (red cylinder) or even within the exon. Blue cylinders represent constitutive exons. PTB may compete with U2AF for binding to the intron 3' end, prevent U1 snRNP recognition of the 5' splice site, or loop-out the regulated exon. However, PTB can promote exon inclusion when it binds downstream of an alternative exon or close to a strong constitutive splice site (not depicted). This model suggests a positional effect of PTB binding on splicing. (B) PTB activates exon 11 skipping in its own transcript via binding to phylogenetically conserved CU-rich sequences (green hatched boxes) surrounding exon 11, which fits well with the model shown in panel A. The resulting mRNA contains a premature termination codon (PTC) and is degraded by nonsense-mediated decay. Therefore, PTB downregulates the level of its own mRNA.

4. PTB and nPTB

4.1 PTB/nPTB proteins

PTB is a ubiquitously expressed RNA-binding protein containing four RNA recognition motifs with high affinity for CU-rich sequences (Xue, et al., 2009). nPTB is predominant in neurons although also present in other tissues and is remarkably similar to PTB in domain structure and RNA-binding specificity (Spellman, et al., 2007). Both PTB and nPTB primarily function as splicing regulators, and PTB can also regulate translation of specific mRNAs and internal ribosome entry site-mediated translation (Mitchell, et al., 2001; Sawicka, et al., 2008). PTB localizes primarily to the nucleus but can shuttle between the nucleus and cytoplasm

(Michael, et al., 1995). Protein kinase A-mediated phosphorylation of PTB can cause its accumulation in the cytoplasm, thereby promoting its cytoplasmic functions such as translation control and RNA transport (Ma, et al., 2007; Xie, et al., 2003).

4.2 Mechanisms of PTB/nPTB-controlled alternative spicing

Multiple mechanisms underlie PTB/nPTB-induced splicing regulation. In general, PTB and nPTB function as splicing inhibitors. Because CU-rich sequences frequently appear in the 3' end of most constitutive introns, binding of PTB to this region interferes with recognition of the 3' splice site by the essential splicing factor, U2AF, thus preventing spliceosome assembly (Sharma, et al., 2005). Moreover, CU-rich elements are also located in other discrete intronic regions surrounding alternative exons. PTB can loop out a regulated exon via binding to both its upstream and downstream intronic CU-rich sequences and forming homomultimers, which thus drives exon exclusion (Lamichhane, et al., 2010). Consistently, a recent genome-wide mapping of PTB-binding sites revealed a position effect for PTB-mediated splicing regulation (Sawicka, et al., 2008) (Figure 2). When PTB binds near an alternative exon, it generally induces exon skipping. However, PTB can also promote exon inclusion when it binds close to a strong constitutive splice site. Besides self-interaction, PTB can also interact with other splicing factors to form complexes that often compete with the splicing machinery, thereby interfering with splicing (Sharma, et al., 2011). A recent report showed that PTB can interact with a pyrimidine-rich loop of U1 snRNA and alter its recognition of the 5' splice site (Coutinho-Mansfield, et al., 2007). nPTB may use similar mechanisms as PTB to suppress exon inclusion. However, nPTB appears to be a weaker splicing suppressor compared with PTB, and nPTB may interact with additional transcripts that are implicated in neuronal activity (Spellman, et al., 2007). Thus, PTB and nPTB have distinct properties in regulating alternative splicing.

4.3 The PTB/nPTB switch during neuronal differentiation

Immunocytochemistry has shown that PTB is detected in neuronal precursor cells as well as non-neuronal lineages of the brain whereas nPTB is specifically expressed in post-mitotic neurons (Boutz, et al., 2007). Therefore, a switch in expression from PTB to nPTB likely occurs during neuronal differentiation. To date, two post-transcriptional mechanisms have been implicated in mutually exclusive expression of PTB and nPTB (Boutz, et al., 2007; Makeyev, et al., 2007). One mechanism involves alternative splicing-coupled nonsense-mediated decay, and the other involves microRNA-mediated translation control. Skipping of exon 11 of PTB and exon 10 of nPTB generates transcripts containing a premature termination codon that subsequently undergo nonsense-mediated decay. PTB is responsible for such exon suppression by binding to highly conserved CU-rich elements flanking the alternative PTB/nPTB exons (Figure 2). Through this activity, PTB may negatively autoregulate its own expression, perhaps to maintain appropriate levels of the protein and restrict the expression of nPTB in non-neuronal cells. In addition, the neuron-specific microRNA miR-124 can directly target to the PTB mRNA and suppresses its translation (Makeyev, et al., 2007). Therefore, PTB expression is down-regulated in neurons, which thus relieves nPTB suppression.

4.4 The PTB/nPTB switch reprograms specific splicing events

Although nPTB and PTB have overlapping function, their different spatial and timely expression patterns in neurons suggest that they have diverse physiological functions in

splicing regulation. Indeed, a recent report showed that ~25% of neuron-specific alternative splicing events may result from a decrease of PTB and increase of nPTB during neuronal differentiation (Boutz, et al., 2007). The possible distinct target specificity of PTB and nPTB may be important for establishing unique and neuron-specific splicing programs during neuronal differentiation. Therefore, the PTB/nPTB switch may have evolved as a post-transcriptional mechanism to fine tune the existing program and alter the transcriptome to promote cell differentiation.

As described above, Nova-1 and Nova-2, although having highly similar sequences, contribute to neuron-specific splicing in different types of neurons (C. Zhang, et al., 2010). Given that Nova-1 can drive its own exon 4 skipping, the Nova-1/Nova-2 reciprocal expression may in part proceed through a negative feedback control mechanism similar to that used by PTB/nPTB (Coutinho-Mansfield, et al., 2007; Dredge, et al., 2005). Therefore, the switch between two highly similar but still distinct splicing factors may provide a potent and rapid means to adjust cellular function in a specific environment.

5. SMN

5.1 SMN genes and expression

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by degeneration of lower motor neurons in the spinal cord with subsequent muscle atrophy (Pearn, 1978). SMA is caused by deletions or mutations of the survival of motor neuron 1 (SMN1) gene (Lefebvre, et al., 1995). In human, the SMN2 gene is almost identical to SMN1 but contains a C to T transition at position 6 in exon 7. This nucleotide change induces SMN2 exon 7 skipping during splicing and results in an unstable truncated SMN protein (Cho & Dreyfuss, 2010). Therefore, SMN2 fails to produce a sufficient amount of functional SMN protein to compensate for the loss of SMN1 (Lorson, et al., 1999; Monani, et al., 1999). Multiple factors have been proposed to regulate exon 7 inclusion/exclusion of the SMN transcripts. In principle, SMN1 exon 7 harbors a splicing enhancer for the splicing activator SF2/ASF, which promotes exon 7 inclusion in the SMN1 transcript, whereas the C to U change in SMN2 pre-mRNA disrupts the binding of SF2/ASF but creates a recognition site for the suppressor hnRNP A1 that excludes exon 7 (Cartegni, et al., 2006; Cartegni & Krainer, 2002; Kashima & Manley, 2003; Kashima, et al., 2007). Besides, other SMN regulators may function via direct binding to exon 7 or even to intronic elements or through a protein complex to modulate exon 7 selection (Doktor, et al., 2011; Nlend Nlend, et al., 2010; Pedrotti & Sette, 2010). SMN splicing regulation has been reviewed elsewhere; this section thus focuses on SMN function in pre-mRNA splicing and regulation.

5.2 SMN and its cellular localization

SMN expression is not restricted to neurons, and in fact it is expressed in all cell types Unlike Nova and PTB, SMN lacks a typical RNA-binding domain but contains a Tudor domain that mediates its interaction with the Sm proteins of spliceosomal snRNPs. Indeed, SMN participates in snRNP biogenesis, which is an important housekeeping function, and also in splicing, transcription, and neuronal mRNA trafficking (Burghes & Beattie, 2009; Coady & Lorson, 2011) (Figure 3). In the nucleus, SMN is particularly concentrated in discrete nuclear bodies that are very close to Cajal bodies (Carvalho, et al., 1999; Young, et al., 2000). This nuclear localization pattern suggests a role for SMN in nuclear snRNP maturation and regeneration. In neurons, SMN forms cytoplasmic granules in neurites and

growth cones and participates in active bidirectional transport of mRNAs (Fallini, et al., 2011; Todd, et al., 2010; H. Zhang, et al., 2006). SMN deficiency disrupts Cajal bodies and impairs mRNA trafficking in motor neuron axons (Girard, et al., 2006; Rossoll, et al., 2003; Shpargel & Matera, 2005), indicating that the diverse subcellular distribution of SMN is functionally important. Moreover, a recent observation that SMN associates and colocalizes with the α subunit of "coatomer", a protein coat for vesicles that mediate intracellular transport, indicates a role for Golgi-associated COPI vesicles in SMN transport (Peter, et al., 2011).

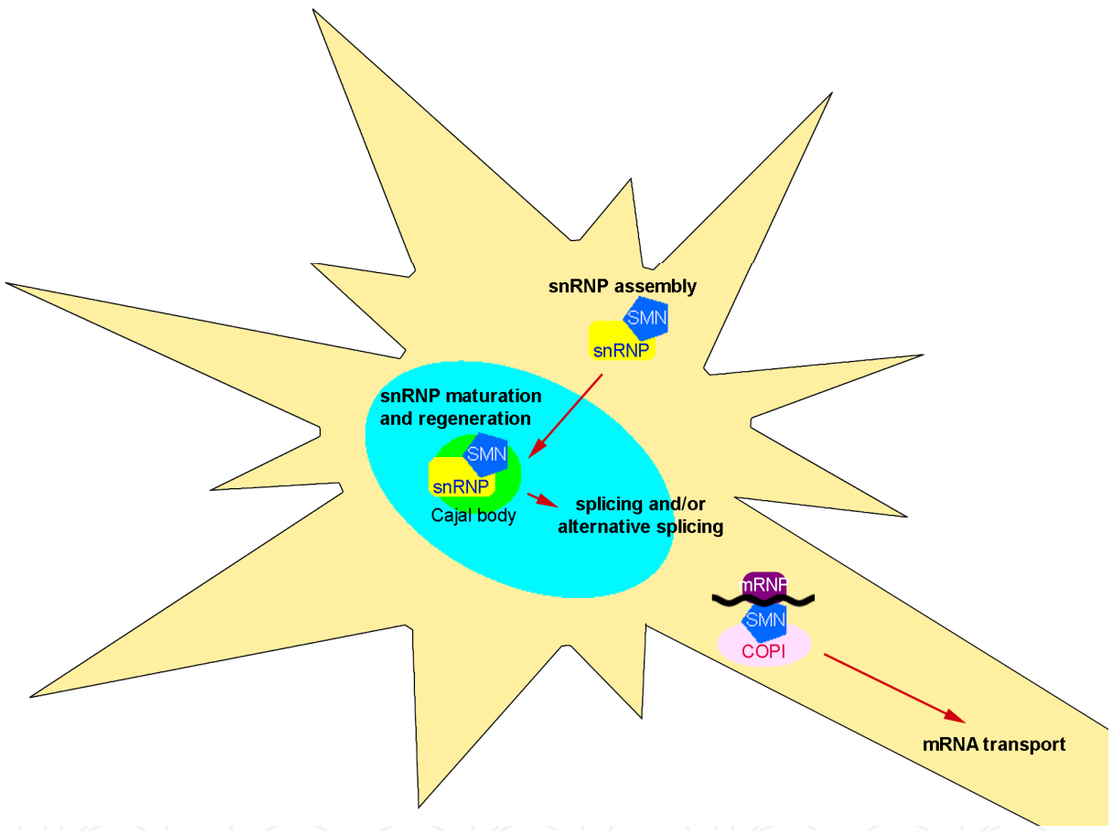

Fig. 3. **Functions of SMN in neurons.** In the cytoplasm, SMN forms a protein complex to facilitate snRNP biogenesis. Moreover, SMN along with other RNA-binding proteins participates in mRNA transport; the role of COPI-containing vesicles in SMN transport is unclear. In the nucleus, SMN is highly concentrated in Cajal bodies and possibly plays a role in snRNP maturation and multi-snRNP assembly. SMN deficiency may indirectly induce aberrant splicing.

5.3 SMN in snRNP biogenesis

The role of SMN in snRNP biogenesis has been well characterized. Assembly of the heptameric Sm cores with each spliceosomal snRNA occurs in a highly ordered manner (Burghes & Beattie, 2009; Cauchi, 2010; Coady & Lorson, 2011). Initially, the chaperon factor pICln brings methylated Sm protein subcomplexes to the SMN complex, which is composed of SMN, Gemin2-8, and unr-interacting proteins. The SMN complex facilitates the

arrangement of the Sm proteins on a single-stranded region of snRNA to form a ring-shaped structure of the snRNP. An initial report showed that ~80% depletion of SMN by RNA interference in cultured mammalian cells had no significant loss-of-function effect on snRNP assembly (Girard, et al., 2006). Perhaps excess SMN complex exists in cells to maintain normal levels of the basal splicing machinery. However, further reduction of SMN differentially impaired snRNP assembly (Gabanella, et al., 2007; Workman, et al., 2009; Z. Zhang, et al., 2008). In particular, the levels of several U12-type spliceosomal snRNAs decreased more significantly in SMA mice, and formation of the U12-type tri-snRNPs was also impeded in lymphoblasts derived from SMA patients (Gabanella, et al., 2007; Workman, et al., 2009; Z. Zhang, et al., 2008). The latter observation is also in accordance with the assumption that SMN is possibly involved in tri-snRNP assembly in Cajal bodies (Carvalho, et al., 1999; Novotny, et al., 2011; Young, et al., 2000). Although direct evidence for defective snRNP assembly or reduced snRNP levels in SMA pathogenesis is lacking, one hint has been provided by the observation that knockdown of SMN or other snRNP assembly factors in zebrafish causes motor axon defects (Winkler, et al., 2005).

5.4 SMN in pre-mRNA splicing

It is clear that SMN has an essential function for snRNP biogenesis, and possibly that insufficient SMN causes various degrees of snRNP assembly defects. However, whether loss of SMN affects splicing of a wide range or a specific set of transcripts and whether the effect of SMN in splicing, if any, indeed results from impaired snRNP assembly have just begun to be investigated.

Exon array analysis has shown that splicing of numerous transcripts is affected in various tissues of late-symptomatic SMA mice (Z. Zhang, et al., 2008). It is unclear whether such a widespread splicing defect is caused by reduced levels of SMN. It is suspected that the decrease in SMN level may affect splicing of specific transcripts in motor neurons that are most vulnerable to degeneration in SMA (Briese, et al., 2005; Liu, et al., 2010; Monani, 2005). A recent report showed that the tri-snRNP of the U12-type splicing machinery is most affected in SMA patients and, consistently, the splicing of a subgroup of U12-type introns is affected (Boulisfane, et al., 2011). Because U12-type introns are present in a number of genes involved in cytoskeletal organization, defects in their excision may impair motor neuron function. Finally, the error rate of exon inclusion/skipping is higher in fibroblasts of SMA patients, perhaps owing to poor recognition of the splice sites by a low abundance of functional snRNPs (Fox-Walsh & Hertel, 2009).

5.5 How does SMN deficiency cause SMA pathogenesis

To date, two plausible possibilities have been raised to explain how SMN deficiency causes specific neurological defects of motor neurons. First, as discussed above, inefficient snRNP assembly may affect the splicing of a specific set of transcripts that are critical to motor neuron functions. However, issues such as which transcripts are most sensitive to SMN deficiency and whether their splicing defects lead to SMA pathogenesis remain to be investigated. In addition, it has been shown that SMN forms RNA granules with other RNA-binding proteins such as hnRNP R to deliver β-actin mRNA in motor axons (Glinka, et al., 2010; Rossoll, et al., 2003). A recent report showed that clustering of Cav2.2 calcium channels is impaired in axonal growth cones of SMA animals, and such a defect can be restored by rescue of SMN expression (Jablonka, et al., 2007). It is possible that SMN plays a

role in actin filament formation via β-actin mRNA trafficking, and such an activity of SMN is critical for motor neuron functions.
SMA patients and animal models show a variable extent of defects in neuromuscular junction functions, axonal arborization, synaptic transport, and neurodevelopment. Such complexity may result from different residual levels of SMN in different systems as well as multiple cellular functions and interacting partners of SMN (Boyer, et al., 2010; Burghes & Beattie, 2009; Cauchi, 2010; Wu, et al., 2011). Nevertheless, restoration of SMN levels or function is certainly a primary therapeutic strategy for SMA treatment (Kolb & Kissel, 2011). For example, activation of SMN2 transcription and restoration of SMN2 splicing ameliorate symptoms of SMA mice and thus provide promise for future SMA treatment.

6. Conclusions

Our understanding of splicing regulation mechanisms and splicing regulatory networks has been advanced substantially by recent studies using gene inactivation techniques and genome-wide experimental and computational examination of alternative splicing events. In the past decade, CLIP in conjunction with various types of mRNA identification systems has been used extensively for *in vitro* study of splicing factors and their regulation mechanisms. Ablation of splicing factors in cultured cells by RNA interference has also been widely used for mechanistic studies of alternative splicing of endogenous or reporter minigene transcripts. Nevertheless, we are still at the beginning of our understanding of the mechanistic and, in particular, physiological aspects of alternative splicing regulation.
Our understanding of the physiological consequences of alternative splicing still largely relies on genetic approaches. For example, knockout of splicing factors in animals in combination of mRNA isoform comparison can facilitate the identification of their *in vivo* targets and biological functions. Study of disease-related splicing factors can in particular provide insights into pathogenesis of aberrant splicing. Moreover, knock-in or knockout of specific mRNA isoforms can help to unveil their functional consequence(s), which is poorly understood, and may even allow delineation of causal effects (Moroy & Heyd, 2007). However, progress has been relatively slow owing to limitations of genetic techniques in mammalian systems. At present, efficient recombination technologies are being developed to facilitate high-throughput gene knockout in embryonic stem cells (Valenzuela, et al., 2003), which may allow large-scale analysis of biological functions of splicing factors as well as mRNA isoforms. Besides more efficient/convenient genetic tools, high-throughput whole-transcriptome sequencing and extensive bioinformatics tools have proved their advantage. With these techniques, we will begin to establish a more accurate paradigm for mRNA splicing regulatory networks with physiological significance.

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8. References

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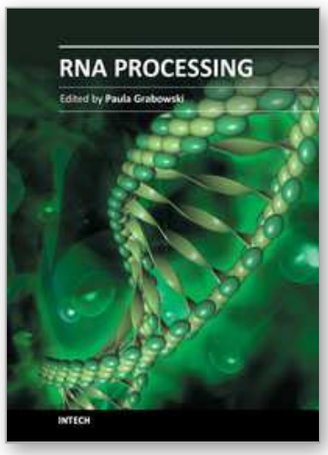

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RNA functions broadly as informational molecule, genome, enzyme and machinery for RNA processing. While these functions reflect ancient activities, they also remain vital components of contemporary biochemical pathways. In eukaryotic cells RNA processing impacts the biogenesis of RNA molecules of essentially every shape and function. The collection of articles in this volume describes the current state of understanding of the broad array of RNA processing events in animal and plant cells, key unanswered questions, and cutting edge approaches available to address these questions. Some questions discussed in this volume include, how viruses subvert the RNA processing machinery of the host cell, how the coordination of co-transcriptional RNA processing is regulated at the level of chromatin, the status of RNA processing in plant organelles, and how micro RNA machinery is biosynthesized and regulated.

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