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A Global Regulatory Mechanism for Activating an Exon Network Required for Neurogenesis

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SUMMARY

The vertebrate and neural-specific Ser/Arg (SR)-related protein nSR100/SRRM4 regulates an extensive program of alternative splicing with critical roles in nervous system development. However, the mechanism by which nSR100 controls its target exons is poorly understood. We demonstrate that nSR100-dependent neural exons are associated with a unique configuration of intronic *cis*-elements that promote rapid switch-like regulation during neurogenesis. A key feature of this configuration is the insertion of specialized intronic enhancers between polypyrimidine tracts and acceptor sites that bind nSR100 to potently activate exon inclusion in neural cells while weakening 3′ splice site recognition and contributing to exon skipping in nonneural cells. nSR100 further operates by forming multiple interactions with early spliceosome components bound proximal to 3′ splice sites. These multifaceted interactions achieve dominance over neural exon silencing mediated by the splicing regulator PTBP1. The results thus illuminate a widespread mechanism by which a critical neural exon network is activated during neurogenesis.

INTRODUCTION

Alternative splicing (AS) is the process by which different pairs of splice sites are selected in pre-mRNA to produce distinct mRNA and protein variants. Transcriptome-wide profiling has uncovered vast repertoires of splice variants in metazoan species, many of which are highly conserved and regulated in a cell- and tissue-specific manner (Irimia and Blencowe, 2012; Licatalosi and Darnell, 2010; Nilsen and Graveley, 2010). Important current challenges are to systematically determine the functions of individual tissue-regulated AS events and establish how they are regulated. These questions are especially important in the context of the vertebrate nervous system, which possesses among the most complex yet highly conserved repertoires of regulated AS events (Barbosa-Morais et al., 2012; Merkin et al., 2012).

Tissue-specific regulation of AS involves the complex interplay of numerous *cis*-acting elements and *trans*-acting factors. Experimental and computational studies have provided evidence that recognition of splice sites flanking neural and other tissue-regulated exons involves tissue-specific AS regulators that, depending on their binding location on pre-mRNA, can function to positively or negatively regulate the assembly of core components of the splicing machinery (spliceosome) at proximal or overlapping splice sites (Witten and Ule, 2011). For example, Nova proteins, which regulate a discrete network of exons enriched in synaptic and axon guidance genes, bind clusters of YCAY motifs concentrated near 5′ splice sites to promote exon inclusion and to YCAY clusters proximal to 3′ splice sites to promote exon skipping (Licatalosi et al., 2008; Ule et al., 2006).

Ptbp1 and its paralog Ptbp2 bind intronic C/U-rich elements upstream of regulated exons to repress the inclusion of neural exons, yet Ptbp1 also promotes exon inclusion when binding to C/U-rich elements downstream of regulated exons (Licatalosi et al., 2012; Llorian et al., 2010; Xue et al., 2009). Ptbp1 and Ptbp2 have mutually exclusive patterns of expression in the developing nervous system. Expression of the microRNA miR-124 silences Ptbp1 in developing neurons (Makeyev et al., 2007). Loss of Ptbp1 facilitates the inclusion of a neural-specific exon (exon 10) in Ptbp2 transcripts that promotes Ptbp2 expression by preventing turnover of its transcripts by the nonsense-mediated mRNA decay pathway (Boutz et al., 2007; Makeyev et al., 2007; Spellman et al., 2007). The relative expression levels of Ptbp1 and Ptbp2 governed by this regulatory circuit contribute to establishing neural-specific AS patterns (Boutz et al., 2007; Li et al., 2014; Licatalosi et al., 2012; Zheng et al., 2012). However, the mechanisms by which Ptbp1/Ptbp2-repressed and other neural-specific exons are activated during neurogenesis are not well understood.

Alternative splicing is additionally regulated by members of a large class of proteins harboring Ser/Arg (SR)-repeat regions (Ankö, 2014). The vertebrate- and neural-specific SR-related protein of 100 kDa (nSR100/SRRM4) regulates a network of brain-specific alternative exons concentrated in genes that function in various aspects of neurogenesis (Calarco et al., 2009; Raj

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et al., 2011). Among this network is exon 10 of Ptbp2, and a neural-specific "switch" exon in REST/NRSF, a transcriptional repressor of neurogenesis genes. Skipping of the switch exon in nonneural cells produces a repressive form of REST, whereas nSR100-dependent inclusion of the exon in differentiating neurons produces a truncated isoform that lacks repressive activity, thereby allowing neurogenesis to proceed (Raj et al., 2011). nSR100 regulates many additional brain-specific exons that are enriched in disordered regions of proteins, which function in modulating protein-protein interactions that contribute to neural-specific functions (Ellis et al., 2012). Recently, a mutation in the Bronx waltzer (bv) mouse, which causes deafness and balance defects, was mapped to the *nSR100/Srrm4* locus and shown to disrupt AS of genes linked to secretion and neurotransmission that are expressed in the inner ear (Nakano et al., 2012). However, the molecular mechanisms that underlie nSR100-dependent neural-specific AS remain poorly understood.

In this study, we employed high-throughput RNA sequencing (RNA-seq) and crosslinking and immunoprecipitation coupled to sequencing (CLIP-seq) to identify an expanded network of neural-specific AS events regulated by nSR100. In addition to revealing nSR100 target exons with important functions in neurogenesis, analyses of these data together with functional studies demonstrate that nSR100 activates neural exon inclusion by binding to intronic UGC-containing motifs proximal to suboptimal 3′ splice sites. The same motifs also serve to weaken 3′ splice site recognition and contribute to skipping of neural exons in nonneural cells. We further provide evidence that this nSR100-dependent regulatory mechanism involves multiple interactions with early spliceosomal assembly components and that it directly outcompetes widespread neural exon repression by PTBP1 during early stages of neurogenesis. These results thus reveal a mechanism underlying the activation of a critical network of neural-specific AS events.

RESULTS

Identification and Characterization of a Conserved Exon Network Regulated by nSR100

To investigate the mechanism by which nSR100 controls neural-specific AS, we sought to computationally identify *cis*-features that mediate its activity in the regulation of conserved target alternative exons in human and mouse cells. RNA-seq profiling was performed following knockdown of nSR100 in mouse neural N2A cells and doxycycline (dox)-induced expression of nSR100 in human 293T cells. Consistent with our previous results from profiling a smaller set of exons using a focused AS microarray (Calarco et al., 2009), knockdown of nSR100 in undifferentiated N2A cells predominantly resulted in skipping of cassette exons that are specifically included in neural tissues (Figure 1A). Of 503 responsive exons (i.e., displaying an absolute *p*ercent *s*pliced *i*n change, $|\Delta PSI| \geq 15\%$), 405 exhibited increased skipping, whereas 98 displayed increased inclusion. Remarkably, although nSR100 is not normally expressed in 293T cells, its dox-induced expression in this cell line resulted in widespread changes in the inclusion levels of exons, many of which are differentially regulated between neural and nonneural tissues (Figure 1B).

To define a high-confidence set of conserved exons that are regulated by nSR100, we focused our analyses on all (157) orthologous exons that undergo skipping upon knockdown of nSR100 in N2A cells and that exhibit increased inclusion in 293T cells upon nSR100 expression (Figures 1A and 1B, red circles, and Figure 1C). This set of 157 orthologous exons represents 53% and 62% of all conserved exons that are dependent on nSR100 for inclusion in 293T and N2A cells, respectively ($p < 0.0001$, chi-square test). Further underscoring the physiological relevance of this set of AS events, the vast majority (95%) correspond to exons that are more included in neural versus nonneural tissues (Figures 1A and 1B; Table S1, available online). In contrast, of the exons that exhibit nSR100-dependent skipping in human and mouse cells, only five overlapped (Figure 1C), one of which is neural enriched (Figures 1A and 1B, black circles), suggesting that many of these changes may be the consequence of indirect effects. RT-PCR assays validated 100% (40/40 events in 20 genes) of the analyzed nSR100-regulated AS events detected by RNA-seq profiling (Figures 1D, 1E, S1A, and S1B; data not shown).

Consistent with previous observations (Calarco et al., 2009; Ellis et al., 2012), the set of 157 conserved nSR100-regulated exons are enriched in genes that function in cytoskeleton remodeling. However, we also observe enrichment in genes with the following terms: GTPase activity, synaptic membrane, neuron projection, establishment of cell polarity, and cell junctions. Additional nSR100-regulated exons are predicted to impact genes that function in transcriptional control of nervous system development, including the myocyte enhancer factor 2 (Mef2) family of transcription activators (Figures 1D and 1E), which are critical for the formation of cortical neuronal layers and synaptic plasticity (Akhtar et al., 2012; Li et al., 2008).

Identification of *cis*-Regulatory Motifs Associated with nSR100-Dependent Exons

PEAKS software (Bellora et al., 2007) was used to identify motifs with significant positional biases relative to the splice sites of the set of 157 conserved nSR100-dependent exons. While significant enrichment of motifs in downstream intronic sequence was not observed, two types of enriched motifs were detected in the upstream introns, with similar sequence and positional biases in human and mouse (Figures 2A, S2A, and S2B). One of these corresponds to C/U-rich hexamers that resemble binding sites of PTBP1/PTBP2, and the other consists of motifs containing one or more UGC triplets.

We compared the cumulative distributions of the first occurrence of a UGC triplet in mouse and human introns upstream of the nSR100-dependent exons versus sets of control alternative exons that are not regulated by nSR100 but have comparable ranges of PSI levels (Figures 2B and S2C). This reveals that 3′ splice sites of nSR100-regulated exons are significantly more often associated with proximal UGC triplets than are control exons ($p < 0.0001$, all comparisons, Mann-Whitney test), but these motifs are not enriched within nSR100-regulated exons relative to control exons (data not shown). Consistent with these findings, UGC motifs were implicated in the regulation of AS events affected in the nSR100 mutant Bronx waltzer mouse strain (Nakano et al., 2012). However, based on the analysis of