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Journal

Wiley Interdisciplinary Reviews - RNA, 8(2)

ISSN

1757-7004

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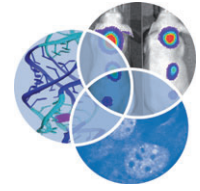
Publication Date

2017-03-01

DOI

10.1002/wrna.1398

Peer reviewed



Developmental regulation of RNA processing by Rbfox proteins

John G. Conboy*

The Rbfox genes encode an ancient family of sequence-specific RNA binding proteins (RBPs) that are critical developmental regulators in multiple tissues including skeletal muscle, cardiac muscle, and brain. The hallmark of Rbfox proteins is a single high-affinity RRM domain, highly conserved from insects to humans, that binds preferentially to UGCAUG motifs at diverse regulatory sites in pre-mRNA introns, mRNA 3'UTRs, and pre-miRNAs hairpin structures. Versatile regulatory circuits operate on Rbfox pre-mRNA and mRNA to ensure proper expression of Rbfox1 protein isoforms, which then act on the broader transcriptome to regulate alternative splicing networks, mRNA stability and translation, and microRNA processing. Complex Rbfox expression is encoded in large genes encompassing multiple promoters and alternative splicing options that govern spatiotemporal expression of structurally distinct and tissue-specific protein isoforms with different classes of RNA targets. Nuclear Rbfox1 is a candidate master regulator that binds intronic UGCAUG elements to impact splicing efficiency of target alternative exons, many in transcripts for other splicing regulators. Tissue-specificity of Rbfox-mediated alternative splicing is executed by combinatorial regulation through the integrated activity of Rbfox proteins and synergistic or antagonistic splicing factors. Studies in animal models show that Rbfox1-related genes are critical for diverse developmental processes including germ cell differentiation and memory in *Drosophila*, neuronal migration and function in mouse brain, myoblast fusion and skeletal muscle function, and normal heart function. Finally, genetic and biochemical evidence suggest that aberrations in Rbfox-regulated circuitry are risk factors for multiple human disorders, especially neurodevelopmental disorders including epilepsy and autism, and cardiac hypertrophy. © 2016 Wiley Periodicals, Inc.

How to cite this article:

WIREs RNA 2017, 8:e1398. doi: 10.1002/wrna.1398

INTRODUCTION

Tissue-specific regulation of alternative pre-mRNA splicing diversifies a gene's functional output by facilitating expression of distinct protein isoforms in cell type- and developmental-specific patterns. Transcriptome characterization via RNA-seq analysis has revealed an enormous capacity for cells to alter splicing patterns as they specialize through normal

differentiation or respond to signals in the environment. Much has been learned about the trans-acting RNA binding proteins (RBPs) and the cis-acting regulatory motifs that govern RNA splicing events, leading to improved models of the splicing regulatory code.^{1,2} Based on these foundational studies of basic RBP mechanisms, current efforts focus on exploring the physiological function of RBP-regulated splicing networks during normal differentiation and development. Correlating specific physiological deficits that occur upon RBP knockdown or knockout, with specific alternative splicing defects induced in the relevant tissue, provides new insights into normal developmental processes. Moreover, since aberrant splicing networks are increasingly appreciated as major contributors to

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Conflict of interest: The author has declared no conflicts of interest for this article.

human disease, mechanistic studies may ultimately provide therapeutic avenues for new classes of disease.

The Rbfox protein family represents an ancient group of RBPs that exert a powerful impact on a variety of RNA metabolic processes. Rbfox1 encodes highly versatile RBPs that play integral roles in RNA metabolism during development of organisms from nematodes and insects to mammals. In humans, Rbfox1 and the closely related Rbfox2 and Rbfox3 genes specify proteins that bind with high affinity and specificity to (U)GCAUG motifs located at strategic regulatory points in the transcriptome: introns that flank tissue-specific alternative exons, 3' untranslated region (UTR) sequences in mRNAs, pre-microRNA hairpins, and likely others. Moreover, their function can be expanded by participation in a larger assemblage of splicing regulators (LASR) that can bind at additional sites.³ Versatility is encoded in the Rbfox gene architecture: via alternative promoter choice and alternative pre-mRNA splicing, the genes encode multiple protein isoforms with differential subcellular localization and differential interaction with regulatory co-factors. New data support the hypothesis that Rbfox1 functions in a complex cellular circuitry that (1) integrates inputs from multiple physiological and developmental cues to modulate the output of structurally and functionally distinct protein Rbfox1 isoforms in the appropriate spatio-temporal patterns; and (2) utilizes this complement of Rbfox1 isoforms to execute powerful controls on RNA processing events that specify the cell-type specific proteome. The latter includes not only regulation of alternative splicing, its most well studied function, but also mRNA stability and translation efficiency, and microRNA processing. This review will focus on the complex expression of Rbfox genes themselves, and summarize recent studies investigating the importance of Rbfox-regulated networks to normal development. These principles will be examined mainly in regard to Rbfox1 and Rbfox2 function in brain and muscle, due to increasing evidence for their developmental functions in these tissues in animal models and human disease states. Functions of Rbfox2 in other tissues, and functions of Rbfox3 in brain, are considered briefly where relevant; future studies will surely provide new insights into these functions for reporting at a later date.

RBFOX1 GENES AND PROTEINS

Understanding the range of Rbfox1 developmental functions requires appreciation of its complex gene structure and regulation. Rbfox1 is encoded by an

extremely large gene of ~1.7 mb having multiple transcription start sites at alternative first exons spanning ~1.3 mb⁴ (Figure 1(a)). Even this representation may understate the transcription potential, because it does not include a few 'singleton' genome browser annotations representing additional candidate start sites. This long expanse of sequence is often described as the '5'UTR'; however, it contains exons of 63, 48, and 42 nt that are highly conserved from reptiles to man, and which maintain an open reading frame that can in principle be translated to generate Rbfox1 proteins with alternative N-terminal domains (transcript classes Ia and II). In the downstream coding region of the gene, alternative splicing produces diversity by modulating inclusion of exons that generate active vs inactive RRM (RNA recognition motif) domains, exons that encode brain- and muscle-specific protein isoforms that may have different target specificities,⁵ and exons that change the C-terminal reading frame to control expression of a nuclear localization signal.⁸ Finally, a little-noticed alternative 3' end, also highly conserved among mammals, can encode two additional C-termini. Notably, Rbfox gene structure in *Drosophila* already possessed two important features of the mammalian genes: an RRM coding domain, the core of which is contained within two exons identical in size and similar in sequence to human Rbfox genes; and the capability to generate cytoplasmic and nuclear isoforms via alternative splicing of 3' exons.⁹

Together these transcriptional and post-transcriptional processes specify a family of Rbfox1 protein isoforms summarized in Figure 1(a). The single RRM has been highly conserved through evolution, being identical in human Rbfox1 and Rbfox2, almost identical (97%) in Rbfox3 and only slightly diverged from the evolutionarily distant *Drosophila* (94% identity) and *Caenorhabditis elegans* (77% identity) proteins. In binary binding assays Rbfox proteins exhibit remarkable binding specificity and high affinity for the (U)GCAUG motif,¹⁰⁻¹³ properties conferred by extensive RNA-protein contacts evident in the solution structure of UGCAUGU-bound human Rbfox1.¹⁴ Even *Drosophila* and *C. elegans* Rbfox1 orthologs associate with UGCAUG elements.^{9,15} Regarding other domains, at least some of the alternative N- and C-terminal domains are required for splicing activity,^{5,10,16} and may facilitate interactions with other RBPs^{16,17} and assembly into the LASR complex.³

Analogous to Rbfox1, the Rbfox2 and Rbfox3 genes exhibit features that facilitate expression of diverse isoforms (Figure 1(b)), including: multiple transcriptional promoters distributed over hundreds

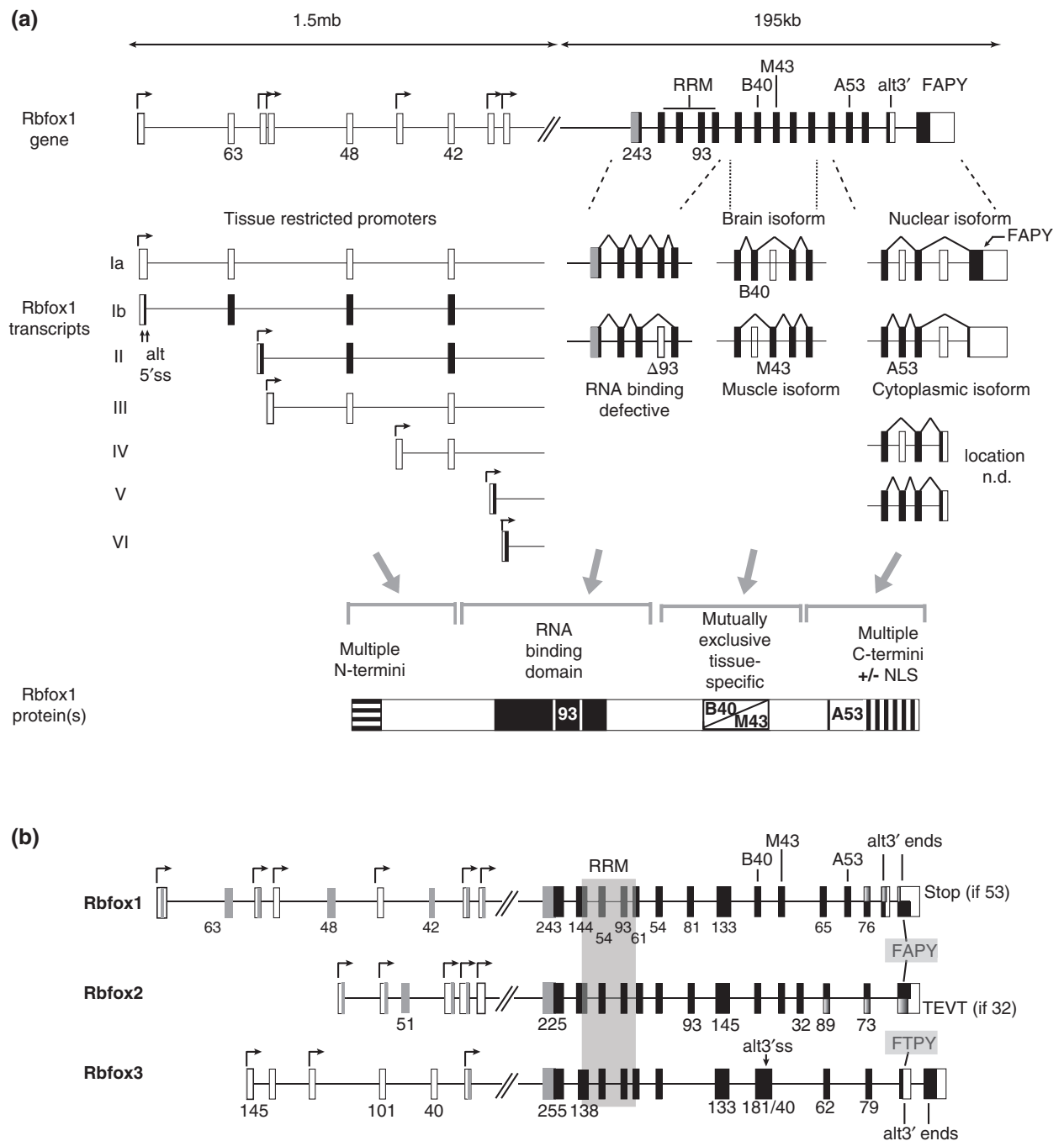


FIGURE 1 | Rbfox1 gene architecture encodes diverse protein isoforms. (a) In the long 5' region, multiple promoters operating at distinct first exons provide potential for independent regulation by various physiological signals. In the RRM region, exons of 54 and 93 nucleotide are conserved from *Drosophila* to humans; the 93 nt exon is deleted in some isoforms to generate isoforms with non-functional RRM. Mutually exclusive exons B40 (brain-expressed 40 nt exon) and M43 (muscle-expressed 43 nt exon) are differentially spliced to generate tissue-specific protein isoforms.⁵ In the 3' region, inclusion of exon A53 (alternative 53 nt exon),⁵ also designated as exon 19,⁶ shifts the translational reading frame of the downstream exons and results in loss of the NLS. White box, untranslated sequence; black boxes, translated sequence; gray boxes, alternative N-terminal sequences predicted by reading frame analysis. Alternative C-terminal reading frames are generated by inclusion or exclusion of A53 (Rbfox1) or 32 nt exon (Rbfox2). n.d., not determined. (b) Organization of Rbfox family genes. Rbfox2 and Rbfox3 are also encoded by large complex genes with many of the same features as Rbfox1.^{4,7} Excluding genome browser annotations only represented once in the database, Rbfox2 and Rbfox3 have 5 and 3 promoters, respectively, spanning ~200–300 kb of 5' sequence. In the core coding region between alternative N- and C-terminal domains, paralogous exons of the three genes are aligned (intron lengths not to scale). Highly conserved RRM domains and RF(A/T)PY nuclear localization signals are indicated by shaded regions.

of kilobases of 5' sequence; a highly conserved RRM domain that can be spliced to generate active or inactive forms through alternative splicing of a 93 nt exon; and alternative splicing events that generate alternative C-terminal domains. All three genes express isoforms that either contain or lack the RF(A/T)PY nuclear localization signal.⁸ All of these features likely contribute to the increasingly wide array of RNA metabolism functions associated with various Rbfox proteins.

REGULATION OF RBFOX EXPRESSION

Precise control of splicing factor expression is critical for normal cell identity, and conversely, aberrant expression is a frequent cause of disease including cancer. Increasing evidence suggests that proper spatiotemporal expression of Rbfox proteins is dictated by continuous integration of inputs from various developmental and environmental signals that control Rbfox transcription, alternative splicing, and mRNA stability.

Rbfox1 transcription is regulated during development and differentiation of skeletal and cardiac muscle, as well as being differentially regulated in various neuronal regions. Some of the promoters are tissue-specific,^{4,5} but little is known concerning their regulation. Presumably the long 5' region is also populated with multiple enhancers sensitive to developmental and environmental input.

Alternative splicing decisions within Rbfox pre-mRNAs are in some cases subject to auto-regulation and/or cross-regulation. In Rbfox1 and Rbfox2, a 93 nt coding exon in the RRM domain possesses UGCAUG motifs in the immediate flanking introns, two of which are conserved to *Drosophila*.^{4,5,18} All three mammalian Rbfox paralogs can bind to these sites¹⁹ and induce exon skipping, which leads to synthesis of a dominant negative isoform that cannot bind RNA but antagonizes splicing enhancer activity of the full length protein.^{4,5,18} In the Rbfox2 gene, inclusion of two poison exons that induce nonsense mediated decay (NMD)²⁰ can be regulated via Rbfox binding to highly conserved UGCAUG motifs in the flanking introns. Finally, the alternative 3' terminal exon in Rbfox1 possesses a highly conserved upstream UGCAUG and thus is a candidate for auto-regulation.

Interestingly, an environmentally-responsive splicing switch involving the A53 exon can modulate the relative output of nuclear vs cytoplasmic isoforms of Rbfox1 to alter splicing outcomes.⁶ Neuronal depolarization induces numerous splicing switches, among which is repression of Rbfox1 exon A53

splicing. Rbfox1 transcripts lacking A53 encode nuclear isoforms of the protein, effectively increasing nuclear Rbfox1 and rescuing some depolarization-induced splicing changes as part of an adaptive response.

Rbfox1 mRNA stability and/or translation represent additional 'post-splicing' steps at which the cellular content of available Rbfox protein can be modulated, e.g., by interaction with other RBPs²¹ or with microRNAs.²² In humans, FRG1 is an RBP that binds Rbfox1 mRNA, decreasing its half-life, reducing expression of Rbfox1 protein, and disturbing Rbfox1 splicing networks.²¹ In *Drosophila*, A2bp1/Rbfox1 acts as a memory-promoting gene of potential relevance to human patients with autism. Behavioral, molecular, cellular, and genetic data suggest that miR-980 represses Rbfox1 expression via interaction with 3'UTR motifs, and may trigger downstream changes in Rbfox1-regulated splicing networks to impact specific memory phenotypes.²²

Additional regulatory effects on Rbfox1 mRNA stability and translation, on protein activity or availability, almost certainly remain to be discovered. Unexplained discordance between changes in Rbfox1 mRNA levels and protein levels have been observed during skeletal muscle differentiation *in vitro*,²³ suggesting the possibility of translational regulation. Finally, the availability of Rbfox proteins for splicing regulation may also be regulated by expression of nuclear lncRNAs that harbor multiple Rbfox binding motifs and are proposed to function as molecular sinks for Rbfox proteins.²⁴

Distribution of Rbfox Binding Sites in the Genome

Genome-wide surveys of conserved UGCAUG motifs and unbiased CLIP-seq analyses of actual Rbfox binding sites indicate that major sites for Rbfox proteins include introns in pre-mRNA, 3'UTRs in mRNA, and intergenic sites (Figure 2). In mouse brain most of the Rbfox CLIP tags (~70%) reside in introns, with ~15% located in 3'UTRs^{19,26} and ~13% in intergenic regions;¹⁹ fewer sites map to coding exons and 5'UTRs. Subcellular fractionation studies show that chromatin-associated nascent transcripts are enriched for intronic CLIP tags, while cytoplasmic RNA primarily binds Rbfox at 3'UTRs.²⁷ Rbfox binding has also been documented in hairpins of pre-miRNAs,^{28,29} in a subset of sno-lncRNAs,²⁴ and at promoter-proximal sequences in nascent RNAs that can influence transcription.²⁵ Rbfox binding can positively or negatively impact RNA processing activities as indicated in the figure. The obvious implication is that Rbfox plays major

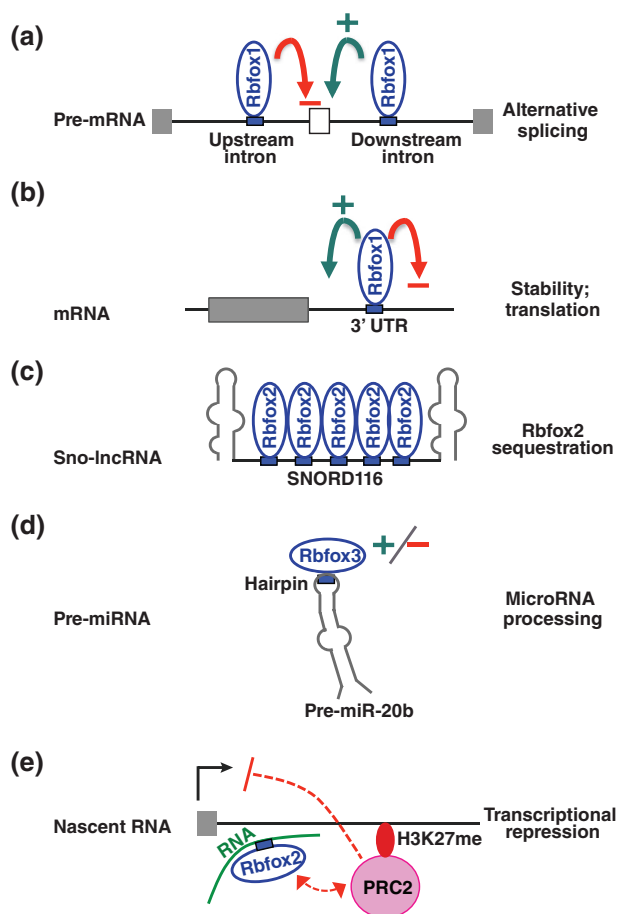


FIGURE 2 | Sites of Rbfox function in the transcriptome.

(a) Binding of Rbfox proteins upstream inhibits, while binding downstream enhances, inclusion of the adjacent alternative exon. (b) Binding of Rbfox proteins to 3'UTR elements can enhance or inhibit RNA stability and/or translation. (c) Binding to a specific subset of sno-lncRNAs can reduce the amount of Rbfox2 protein available for other nuclear functions. (d) Binding of Rbfox3 to the hairpin loop of pre-miRNAs can promote or repress processing. (e) Binding of Rbfox2 to nascent promoter-proximal transcripts can recruit PRC2 complexes that repress transcription.²⁵ Black arrow indicates transcription start site; curved green arrow indicates enhancer activity; curved red arrow represents silencer activity. (Adapted from Ref. 25).

regulatory roles in pre-mRNA alternative splicing, potentially alternative polyadenylation, mRNA stability and translation, miRNA processing, and even transcription.

Many Rbfox CLIP-seq tags lack the prototypical UGCAUG motif, due in part to direct binding with reduced affinity at related motifs such as GCACG,¹² or by virtue of RNA secondary structure that juxtaposes distal UGCAUG motifs with other sequences.²⁶ Additionally, Rbfox proteins can reside in a bigger complex termed LASR (large assembly of splicing regulators).³ In this complex Rbfox can be recruited to RNA

indirectly, e.g., through the binding of other components including hnRNP M protein, allowing it to cross-link to RNA and exhibit splicing enhancer activity in the absence of UGCAUG motifs.³

ALTERNATIVE SPLICING REGULATION BY RBFOX1

UGCAUG motifs are greatly enriched and evolutionarily conserved in proximal introns within 200–300 nt of tissue-specific alternative exons, but are less common and less conserved near non-tissue-specific alternative exons³⁰ and constitutive exons. The degree of exon inclusion correlates with the number of UGCAUG motifs, at least for muscle-specific exons.³¹ UGCAUG motifs are enriched adjacent to tissue-specific alternative exons in brain, muscle, selected epithelial and mesenchymal cells, and certain breast cancers;^{31–38} microexons in brain;^{39,40} near alternative ‘poison’ exons in mouse embryonic stem cells that contain premature termination codons;⁴¹ and are even enriched and conserved in more distal intronic regions >500 nt from neighboring alternative exons.^{26,42,43} Genome-wide mapping of Rbfox binding sites in the brain transcriptome have revealed ~1059 direct Rbfox target alternative splicing events.¹⁹ Over half of these targets show dynamic changes during brain development, suggesting a major role for the Rbfox splicing program.

Rbfox motifs in the downstream intron generally function to enhance splicing, while upstream motifs tend to inhibit splicing, of a neighboring alternative exon. This conclusion is supported by bioinformatic^{1,19,26,32,33,44,45} and experimental analyses (Ref 10 and others) that correlate splicing behavior and motif location. The molecular mechanisms for position-dependent effects on exon splicing are not well understood. In some cases, Rbfox binding may inhibit splicing by competing with constitutive splicing machinery at the branchpoint and/or 3' splice site.^{46,47} Alternatively, Rbfox binding might positively influence splicing by recruiting U1 snRNP to the 5' splice site via interaction with the U1-C polypeptide.^{48,49}

Cooperative Regulation of Splicing Networks by Rbfox1 and Other RBPs

Given that 10^5 to 10^6 UGCAUG hexamers likely reside in the human transcriptome, what distinguishes functional vs non-functional sites, and what determines tissue-specificity of functional sites? Functional sites tend to reside in highly conserved regions^{26,30,32} having specific sequence³ and

secondary structure biases,⁵⁰ indicating that the neighborhood matters. Presumably adjacent sequences are relatively deficient in splicing silencer elements, except when required for regulated antagonism, and enriched in motifs that favor binding of co-regulatory factors that confer tissue-specificity.

A well documented example of coordinate Rbfox splicing regulation occurs in the fibroblast growth factor receptor pre-mRNA in *C. elegans* muscle.¹⁵ Genetic and biochemical evidence show that SUP-12 and the Rbfox-related protein ASD-1 bind cooperatively and stably to adjacent motifs upstream of exon 5B to inhibit its splicing and allow activation of the downstream exon 5A in muscle. Loss of either interaction results in weaker binding *in vitro* and weaker repression of exon 5B *in vivo*. This study concluded that co-regulation of tissue-specific splicing, through cooperative binding of multiple factors having partially overlapping expression patterns, provides greater regulatory flexibility than would a simple binary interaction of Rbfox with its motifs.¹⁵

In fact, several co-regulatory networks involving Rbfox proteins have been at least partially characterized in mammalian tissues, likely providing considerable flexibility in regulating sub-networks of tissue-restricted Rbfox target exons (Figure 3). In mouse brain, about 15% of the exons in the NOVA-regulated splicing network have UGCAUG motifs in the flanking intron,⁵² and experimental analysis confirmed that NOVA and Rbfox synergistically regulate *Gabgr2* exon 9. In the human embryonic muscle cell line HFN, a co-regulatory network of splicing events under combinatorial control by Rbfox1 and Mbnl1 was demonstrated.⁵¹ In embryonic stem cells, Rbfox2 and MBNL1 cooperate in regulating exons important for pluripotent stem cell differentiation,⁵³ and similar cooperation between Rbfox2 and Mbnl1 can occur in mesenchymal cells.⁵⁴ In epithelial cells coordinated networks of Rbfox2/ESRP-regulated exons have been reported.^{55,56} Finally, co-regulation of splicing events has also been reported between Rbfox and PTBP proteins^{38,39} and between Rbfox and HNRNPH

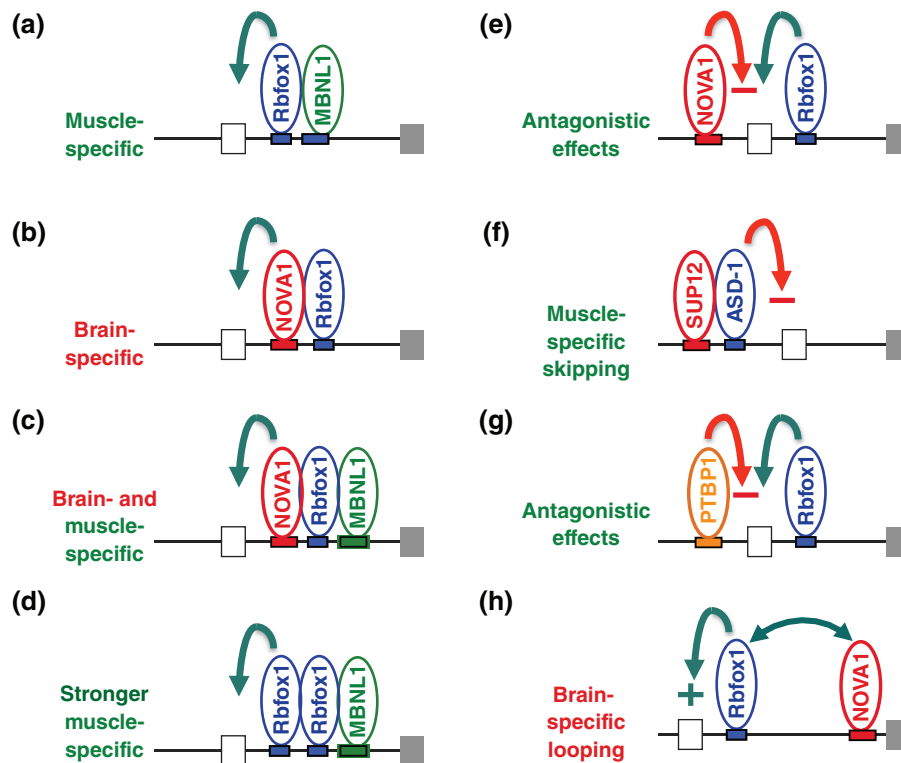


FIGURE 3 | Coordinate determination of lineage-specific alternative splicing by Rbfox and other RBPs. (a) Model based on co-regulation by Rbfox1 and Mbnl1 in a muscle cell line.⁵¹ (b) Model based on the common overlap between Rbfox- and Nova-regulated exons in brain.⁵² (c) Model of dual specificity by intersection of muscle- and brain-specific regulatory patterns. (d) Model based on co-regulation by Rbfox1 and Mbnl1 in a muscle cell line⁵¹ and correlation of exon inclusion with number of UGCAUG motifs.³¹ (e) Demonstrated antagonistic effects on splicing for an alternative exon in *Arhgef12*.³² (f) Coordinated repression by SUP12 and the Rbfox-related gene ASD-1 of exon 5B in the *egl-15* gene in *C. elegans*.¹⁵ (g) Antagonistic effects on splicing of brain microexons H. Brain-specific enhancer activity by looping across the intron, proposed for *Gabgr2* exon 9.⁵² Green arrows indicate enhancer activity; red arrows indicate silencer activity.

proteins,^{16,17} as well as Rbfox3 and PSF.⁵⁷ For most of these examples, it appears that Rbfox can either synergize with or antagonize activity of the co-regulators, providing considerable flexibility to the networks.

Regulating Alternative Splicing of Noncoding Exons Down-regulates Gene Expression via AS-NMD

Rbfox proteins can indirectly effect changes in gene expression, when they mediate alternative splicing coupled to nonsense-mediated decay (AS-NMD), due to the introduction of premature termination codons in specific transcript isoforms. This mechanism was demonstrated for Rbfox2-mediated regulation of numerous AS-NMD events in embryonic stem cells,⁴¹ but similar outcomes likely could be effected by Rbfox1 or Rbfox3 in other tissues. Changes in expression of Rbfox that increase or decrease relative expression of exons containing premature termination codons will modulate the proportion of transcripts subject to nonsense-mediated decay, thus altering gene expression. Some of the affected transcripts encode splicing factors, and could provide a mechanism for escaping the self-limiting window of expression enforced by negative auto-regulation of such transcripts.⁴¹

NON-SPLICING FUNCTIONS OF RBFOX1

Pre-mRNA splicing is but one of many RNA metabolic processes that are regulated by RBPs in the nucleus, and in the cytoplasm, of higher eukaryotes. This section briefly reviews evidence of Rbfox regulation of microRNA processing in the nucleus, and of mRNA stability and translation efficiency in the cytoplasm. The discovery of cytoplasmic Rbfox1 isoforms,^{5,20} and the strong enrichment of UGCAUG motifs in 3'UTRs,^{13,32} clearly indicate that this versatile RBP must have non-splicing functions. Interestingly, both negative and positive effects on mRNA abundance have been demonstrated experimentally in contexts as diverse such as mouse hippocampal neurons²⁷ and *Drosophila* germline,⁹ suggesting that the direction and degree of cytoplasmic Rbfox-dependent gene regulation might depend on combinatorial interactions with other regulators.

Rbfox Regulation of microRNA Processing

Two groups have reported that Rbfox proteins can regulate processing of microRNA precursors.^{28,29}

One study used a CLIP-seq approach to identify pri-miRNAs that bind Rbfox3 both in neuronally-differentiated P19 cells and in the mouse central nervous system (CNS). Rbfox3 was shown to positively regulate processing of individual pri-miRNAs to pre-miRNAs (e.g., pre-miR-15a), while negatively regulating other pre-miRNA processing events (e.g., pre-miR-485).²⁸ Selected isoforms of Rbfox1 and Rbfox2 exhibited similar regulation of these two representative pre-miRNAs when exogenously expressed in P19 cells, albeit with weaker activity than Rbfox3. Surprisingly, most of the miRNAs regulated by Rbfox3 in this system do not contain the typical (U)GCAUG sequence motif, suggesting that Rbfox functions in combination with other factors to mediate recruitment of the microprocessor complex to pri-miRNAs. The second study focused on two miRNAs (miR-20b and miR-107) that do contain essential GCAUG motifs in the terminal loops of their primary transcripts.²⁹ Binding of these transcripts to Rbfox1 and Rbfox2 inhibits processing of the pri-microRNAs to pre-microRNAs, reduces expression of the mature microRNAs, and increases expression of targets normally downregulated by these microRNAs. Together these studies show that Rbfox family members can exert physiological effects in yet another aspect of RNA metabolism, i.e., regulation of miRNA biogenesis.

Cytoplasmic Rbfox1 Regulatory Networks Impact mRNA Stability of Developmentally Important Genes from *Drosophila* to Man

The *Drosophila* A2BP1/Rbfox1 gene encodes a cytoplasmic isoform that affects development through its effects on mRNA stability and translation during germ cell differentiation.⁹ In this context, cytoplasmic Rbfox1 represses expression of target transcript(s). Isoform-specific knockdown and rescue experiments show that mRNAs with 3'UTR GCAUG motifs, including transcripts encoding the RBP Pumilio, are targets of repression by cytoplasmic but not nuclear Rbfox1 protein. Here Rbfox1 functions in circuitry that titrates Pumilio as needed for germ line maintenance: knockdown of cytoplasmic Rbfox increases Pumilio expression and inhibits germ cell differentiation, while over-expression represses Pumilio to cause precocious germ cell differentiation. Of note, the authors point out that Rbfox1, Rbfox2, and Rbfox3 physically associate with *Pumilio1* and *Pumilio2* mRNA in the mouse nervous system,⁹ raising the possibility that critical functions for cytoplasmic Rbfox have been conserved to man.

In fact, functional studies of individual Rbfox1 isoforms in mouse hippocampal neurons had already implicated cytoplasmic Rbfox1 in regulating expression of synaptic and autism-related genes through effects on mRNA abundance.²⁷ Primary hippocampal neurons express abundant cytoplasmic Rbfox that can be depleted by knockdown of Rbfox1 and Rbfox3 transcripts, the two paralogs known to encode cytoplasmic isoforms. Transcriptome analyses of these cells, along with binding assays and genetic rescue experiments, all support the hypothesis that cytoplasmic Rbfox binds to, and positively regulates abundance of, cytoplasmic mRNAs naturally tagged with the UGCAUG motif. Cytoplasmic Rbfox1-regulated transcripts are enriched for genes involved in cortical development and autism, implying that cytoplasmic Rbfox impacts an essential component of the neural development program with disease relevance.²⁷ In the same cells, Rbfox knockdown elicited changes in alternative splicing that could be rescued by expression of nuclear Rbfox1, independent of the abundance effects of cytoplasmic Rbfox.

That Rbfox1-mediated effects on mRNA stability are relevant to human disease is further suggested by bioinformatics analyses that correlate 3'UTR motifs with RBP expression.¹³ Abundance of transcripts with UGCAUG motifs in the 3'UTR correlates positively with Rbfox1 expression across many tissues, and knockdown of Rbfox1 decreases their abundance, supporting the hypothesis that Rbfox1 enhances mRNA stability. As predicted by this model, Rbfox1-deficient autistic brains exhibit reduced abundance of mRNAs having 3' Rbfox motifs.¹³ Since these transcripts are enriched for voltage-gated ion channels, Rbfox1-mediated regulation of transcript stability may affect nervous-system-specific processes.

Rbfox2 Interaction with PRC2 can Impact Transcription Networks

Finally, Rbfox2 can impact transcript abundance via a novel mechanism involving interaction with Polycomb repressive complex 2 (PRC2),²⁵ which methylates H3K27 and is associated with gene silencing activity. Rbfox2 was shown to interact with chromatin-associated RNA, often near gene promoters, and also to bind directly to purified PRC2 complex. An array of bioinformatics and biochemical studies was employed to develop an intriguing model that Rbfox2 interacts with nascent RNA and recruits PRC2 to target genes so as to repress expression. Functional interplay between Rbfox2 and PRC2 may regulate homeostatic gene expression in a nascent

RNA abundance-dependent manner to increase or decrease PRC2 recruitment.²⁵ This activity might be restricted to Rbfox2, since it depends on a unique C-terminal domain not found in other Rbfox paralogs.

DEVELOPMENTAL FUNCTIONS OF RBFOX PROTEINS IN ANIMAL MODELS

Rbox Function in Brain

A brain function for human Rbfox1 was initially proposed on the basis of its interaction with ataxin2, the gene affected in patients with spinocerebellar ataxia type 2,⁵⁸ and on the abundance of UGCAUG motifs near brain-specific alternative exons. Subsequent exploration of Rbfox protein expression and function in the brain has focused on the mouse cerebellum utilizing *Rbfox1^{loxP/loxP}* and *Rbfox2^{loxP/loxP}* strains generated in the Black lab to engineer conditional knockouts.^{59,60} Mice with CNS knockout of either Rbfox1 or Rbfox2 have distinct neurological phenotypes, consistent with their different pattern of expression in the normal cerebellum.^{59,60} Rbfox1 knockout mice experience spontaneous seizures and greatly exaggerated responses to systemic administration of kainic acid, a neuroexcitatory agent used in epilepsy studies. Rbfox2 knockout mice have small cerebellums, exhibit progressive difficulty in movement, abnormalities in Purkinje cell function, and not infrequently develop hydrocephalus at a young age. In the search for Rbfox-mediated splicing switches that might explain these phenotypes, normal *Rbfox1^{-/-}* and *Rbfox2^{-/-}* brains were compared using Affymetrix exon-junction (MJAY) microarrays. Modest numbers of significant splicing differences were detected for alternative exons in the knockout brains, most of which had adjacent intronic UGCAUG motifs and are likely to be direct targets. Correlating specific splicing changes with specific defects in these pleiotropic phenotypes is challenging, probably due to compensatory changes in Rbfox expression when one of the genes is inactivated. Nevertheless, splicing changes in the Rbfox1 knockout were observed in a few ion channels and neurotransmitters that are involved in synaptic transmission and membrane excitation, and might contribute to the seizure phenotype. The authors cite previous reports showing that two genes with disturbed splicing previously have been implicated in patients with epilepsy (*Gabrg2a*), or have been shown to alter seizure susceptibility in mice (*Grin1*).

Knockout of Rbfox gene(s) specifically in postnatal Purkinje cells resulted in aberrant alternative

splicing in the *Scn8a* gene for sodium channel $\text{Na}_v1.6$, likely relevant to the observed electrophysiological abnormalities in firing of pacemaking action potentials.⁶⁰ Importantly, Purkinje cell-specific knockout of the same sodium channel gene was shown by others to generate a pacemaking defect similar to the Rbfox deficiency.

Together these studies have begun to reveal critical functions for both Rbfox1 and Rbfox2 not only during brain development, but also for maintenance of functioning circuits in the adult brain. However, there are inherent difficulties in trying to deconvolute relevant splicing targets and physiological functions across complex brain regions for individual Rbfox proteins, especially in the face of compensatory regulation by other Rbfox paralogs when one is knocked out. This might explain why the number of observed splicing changes is much fewer than expected given the large number of targets predicted by CLIP-seq experiments,¹⁹ and why changes in gene expression expected due to loss of cytoplasmic Rbfox1²⁷ were not observed.

Interestingly, Hamada et al. employed *in utero* electroporation of RNAi vectors to knock down expression of cytoplasmic⁶¹ or nuclear⁶² isoforms of Rbfox1 in ventricular zone progenitor cells of D14.5 mouse embryos. Using a co-transfected GFP expression vector to facilitate imaging of newly generated excitatory neurons during corticogenesis, they demonstrated similar but not identical phenotypes, with defects in neuronal migration and axon growth being more severe under conditions of nuclear Rbfox1 deficiency. Importantly, developmental defects could be rescued by expression of the relevant RNAi-resistant Rbfox1 isoforms. These studies concluded that both cytoplasmic and nuclear isoforms of Rbfox1 are critical for establishing the architecture of the developing cerebral cortex, but detailed transcriptome analyses to identify alterations in splicing or transcript stability have not yet been reported.

Rbfox Function in Muscle

An important role for Rbfox proteins in vertebrate muscle was suggested by the abundance of intronic UGCAUG motifs flanking muscle-specific alternative exons,^{31,32,34} especially those undergoing developmentally-regulated splicing transitions during maturation of embryonic to adult heart.⁶³ Mutation of the *fox-1* and *asd-1* paralogs in *C. elegans* caused aberrant splicing in body wall muscles,⁶⁴ and knockdown of two muscle-expressed Rbfox genes in zebrafish resulted in abnormal splicing of muscle-specific alternative exons having intronic UGCAUG motifs.⁶⁵

Rbfox-deficient zebrafish exhibit severe functional defects in both cardiac and skeletal muscle, including reduced heart rate and disorganized skeletal muscle myofibrils leading to loss of embryo motility. Because muscle-specific splicing events are often highly conserved from zebrafish and chickens to mammals,^{34,63,65} it is not surprising that Rbfox knockdowns described in the next section were shown to disturb myoblast differentiation, mature skeletal muscle function, and cardiac function in mice. Importantly, the latter have potential relevance to dilated cardiomyopathy (DCM) in humans.

Conditional Knockout in Skeletal Muscle

In the C2C12 mouse myoblast differentiation model, siRNA-mediated Rbfox1 knockdown had only modest effects, which was unexpected because substantial upregulation of Rbfox1 accompanies induction of differentiation in these cells. Instead, it was knockdown of Rbfox2 that strongly impaired myoblast fusion.⁶⁶ Two Rbfox2-regulated alternative exons were identified as critical splicing targets: one that generates an alternative isoform of Mef2d transcription factor, and another that ultimately reduces ROCK2 kinase expression by introducing a premature stop codon that induces NMD. Remarkably, the fusion defect in Rbfox2-deficient myoblasts could be substantially rescued by forced expression of the Rbfox-dependent Mef2d isoform and knockdown of ROCK2 expression.

In normally-developed mature skeletal muscle, Rbfox1-deficiency was engineered in adult mice by doxycycline-induced expression of muscle actin promoter-driven Cre recombinase²³ using the *Rbfox1*^{loxP/loxP} model. Phenotypic effects included progressive loss of performance in force generation assays, structural irregularities in sarcomeres, and functionally aberrant calcium handling. RNA-seq analysis detected many changes in alternative splicing, enriched in genes functioning in calcium signaling, cytoskeleton organization, and myofibrillar structure. The authors concluded that the phenotype of Rbfox1-deficient muscle probably results from combinatorial effects on splicing of multiple genes necessary for maintenance of muscle structural and functional integrity.

These studies show that Rbfox1 and Rbfox2 regulate distinct sets of splicing events in proliferating myoblasts and differentiated myotubes. To what extent Rbfox effects on mRNA stability and translation, or on miRNA processing might contribute to the phenotype is unknown. Future studies will be needed to integrate the Rbfox-dependent component

of muscle differentiation with the overall splicing program, since differentiating muscle expresses many Rbfox-independent splicing transitions.

Conditional Knockouts in Heart

Rbfox1 protein levels increase post-natally while Rbfox2 expression is higher in the neonatal heart but reduced in the mature adult mouse heart,^{63,67} suggesting both overlapping and unique functions during cardiac maturation. Many splicing transitions are dynamically regulated during normal heart development, and the strong enrichment of UGCAUG flanking many of the switched exons further supports a critical role for Rbfox splicing networks.⁶³

Two recent papers explored the biomedical importance of cardiac Rbfox regulation, using mechanical and genetic models of heart failure in the mouse.^{67,68} Both Rbfox1 and Rbfox2 were specifically down-regulated in response to pressure-overload induced heart failure, in contrast to most other splicing factors, leading to reversion of many developmentally regulated Rbfox target exons to a more fetal-like splicing pattern. Importantly, re-expression of an Rbfox1 transgene not only ameliorates the pathological hypertrophy in these animals, but also rescues some of the aberrant splicing events. In the genetic model, mice with conditional knockout of cardiac Rbfox1 were engineered using the *Rbfox1*^{loxP/loxP} system. These mice exhibit decreased cardiac function at 6 months, greatly accelerated adverse reactions to pressure overload induced by transaortic constriction, and substantial overlap in aberrant splicing events compared with mechanical heart failure.

An important Rbfox1 regulatory target that likely contributes to the aberrant phenotype in failing hearts is the MEF2 family of transcription factors. Splicing of a key Rbfox- and UGCAUG-regulated alternative exon in MEF2a is perturbed in both heart failure models, but rescued by expression of transgenic Rbfox1 in mice, or by expression of the correct MEF2a isoform in zebrafish. Remarkably, relevance of the Rbfox1/Mef2 regulatory circuit to human disease is supported by the finding of decreased Rbfox1 expression and the same aberrant splicing event in DCM human heart samples.⁶⁷

Mice with conditional knockout of cardiac Rbfox2 at the start of cardiogenesis are born normally, with hearts of grossly normal morphology, but they experience progressive heart failure involving a progressive DCM phenotype with severe contraction defects.⁶⁸ Precise comparison with the Rbfox1 knockout is challenging because the two models were generated using different transcriptional

promoters to drive expression of Cre recombinase, but both models clearly generate serious cardiac defects, and both exhibit aberrant splicing in genes implicated in heart function and disease.

DISRUPTION OF RBFOX FUNCTION IS ASSOCIATED WITH HUMAN DISEASE

Based on the multi-faceted functions of Rbfox proteins in regulating gene expression, and given the phenotypes observed in animal models, one might expect defects in Rbfox networks to be associated with human disease. Indeed, genome-wide studies have repeatedly identified large deletions and translocations in the Rbfox1 gene for small percentages of patients with neurodevelopmental diseases such as autism and epilepsy (summarized in Ref 69). However, evidence implicating Rbfox networks in human disease continues to expand, as defects in Rbfox1 are associated with heart disease and other disorders, and Rbfox2 mutations have now been associated with heart disease.⁷⁰ In a few cases, reduced Rbfox1 mRNA expression has been confirmed in autistic brains, where Rbfox1 behaves as a 'hub' in the ASD gene transcriptome network.⁷¹ Reduced Rbfox1 expression has also been reported in failing hearts of patients with DCM.⁶⁷

So far, mechanistic relationships between Rbfox1 gene deletions, aberrant Rbfox1 protein expression, and neurodevelopmental disorders are not well understood. Nor has the incomplete penetrance of neurodevelopmental disorders sometimes observed in families with Rbfox1 deletions been explained.⁷² A further confounding factor is that Rbfox1 splicing networks can be perturbed not only by direct mutation, but also by defects in Rbfox1-regulatory factors that secondarily disturb splicing.^{21,24}

Mutations in the Rbfox1 Gene

Rbfox1 gene aberrations associated with neurodevelopmental disorders were discovered by virtue of substantial translocations or intragenic deletions mapping to the extraordinarily long 5' region of the gene. A few of the informative deletions reported in the literature^{73–78} are shown in Figure 4. Most deletions remove a first exon (shaded green) or potentially coding internal exons (shaded blue), but a few deletions (depicted in red) apparently encompass only intronic sequence. Not shown here, the farthest upstream promoter region is also disrupted in at least two translocations.^{80,81} Mechanistically, these deletions might disrupt Rbfox1 expression by

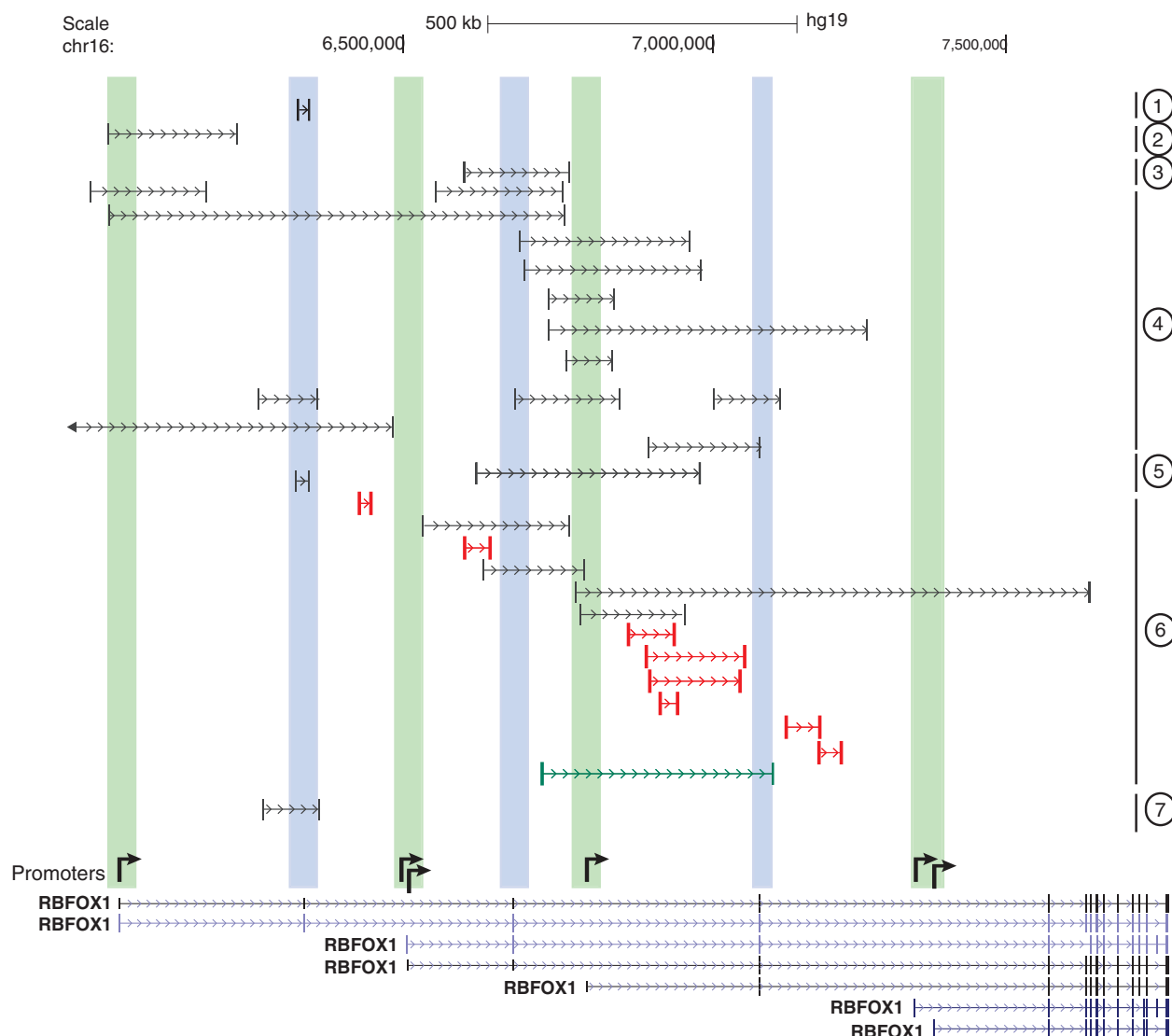


FIGURE 4 | Rbfox1 deletions associated with neurodevelopmental disorders in humans. Below are Rbfox1 annotations with arrows depicting presumed promoter locations as described in Figure 1. Green bars, deletions that span promoters; blue bars, deletions that span internal exons likely translated in some contexts as shown in Figure 1. Red lines indicate deletions apparently in introns only. Other Rbfox1 deletions have also been reported.^{73,75} Numbers at the right margin indicate the relevant citations for deletions as follows: group 1,⁷⁸ group 2,⁷⁴ group 3,⁷³ group 4,⁷⁵ group 5,⁷² group 6,⁷⁶ group 7.⁷⁷

eliminating promoters, disrupting transcriptional enhancers, or affecting mRNA stability and translation efficiency. Alternatively, given the likelihood that the internal 5' exons are translated in some brain regions in a promoter-dependent manner (Figure 1), it is possible that functionally important Rbfox1 domains are lost in selected neurons that express extended N-termini.

Taken together, other recent observations indicate that the contribution of Rbfox1 (and Rbfox2) mutations to human disease could be far greater than currently appreciated. Intragenic deletions of Rbfox1 have been found in patients with heart disease,⁷⁰

visual refractive errors,^{82,83} and pediatric food allergy.⁸⁴ Moreover, small mutations within the traditional downstream Rbfox1 coding exons have been reported,^{72,85} and exome sequencing studies have suggested that a small proportion of patients with congenital heart disease (CHD) have damaging Rbfox2 mutations.⁸⁶ In particular, hypoplastic left heart syndrome (HLHS) patients with a truncating nonsense mutation express a shorter Rbfox2 protein that is associated with reduced nuclear localization, reduced expression of transcripts with 3'UTR Rbfox2 binding sites, and reduced activity in splicing.⁸⁷

Defects in Rbfox Expression Secondary to Aberrations in Other Metabolic Pathways

Besides direct mutations in the Rbfox1 gene, disturbance in homeostatic mechanisms that maintain normal activity levels of Rbfox1 protein can also cause human disease. Facioscapulohumeral muscular dystrophy (FSHD) is a common neuromuscular disorder associated with myogenic defects and progressive wasting of specific skeletal muscles. Contributing to the pathogenesis in FSHD patients is reduced Rbfox1 mRNA expression that appears to be secondary to over-expression of FRG1, an RBP that binds Rbfox1 mRNA and reduces its half-life.²¹ Over-expression of FRG1 in mouse muscle and in C2C12 cells reduces Rbfox1 mRNA and induces aberrant splicing of exons that do not interact with FRG1 directly, but rather are Rbfox target exons. Interestingly, mental retardation and autism sometimes occur in severely affected FSHD infants.²¹

A second candidate Rbfox1-related disease is Prader-Willi Syndrome (PWS), a neurogenetic disorder typically associated with deletion of a 5–6 Mb region on the paternally derived chromosome 15. This region encodes a class of nuclear sno-lncRNAs (SNORD116) that harbor multiple Rbfox binding motifs and can indirectly modulate splicing of Rbfox2 target exons by functioning as molecular sinks for Rbfox proteins.²⁴ By inference, Rbfox1 and/or Rbfox3 could be partially sequestered by a similar mechanism in the brain, but this has not been demonstrated directly.

Finally, aberrant expression of Rbfox2 or Rbfox1 has been described in diverse disease contexts including diabetic heart and Parkinson’s disease (PD) neurons, respectively.^{88,89} In the diabetic heart, upregulation of Rbfox2 has been proposed to autoregulate its own splicing, promoting an exon skipping event that generates a dominant negative protein isoform with an aberrant RRM having reduced

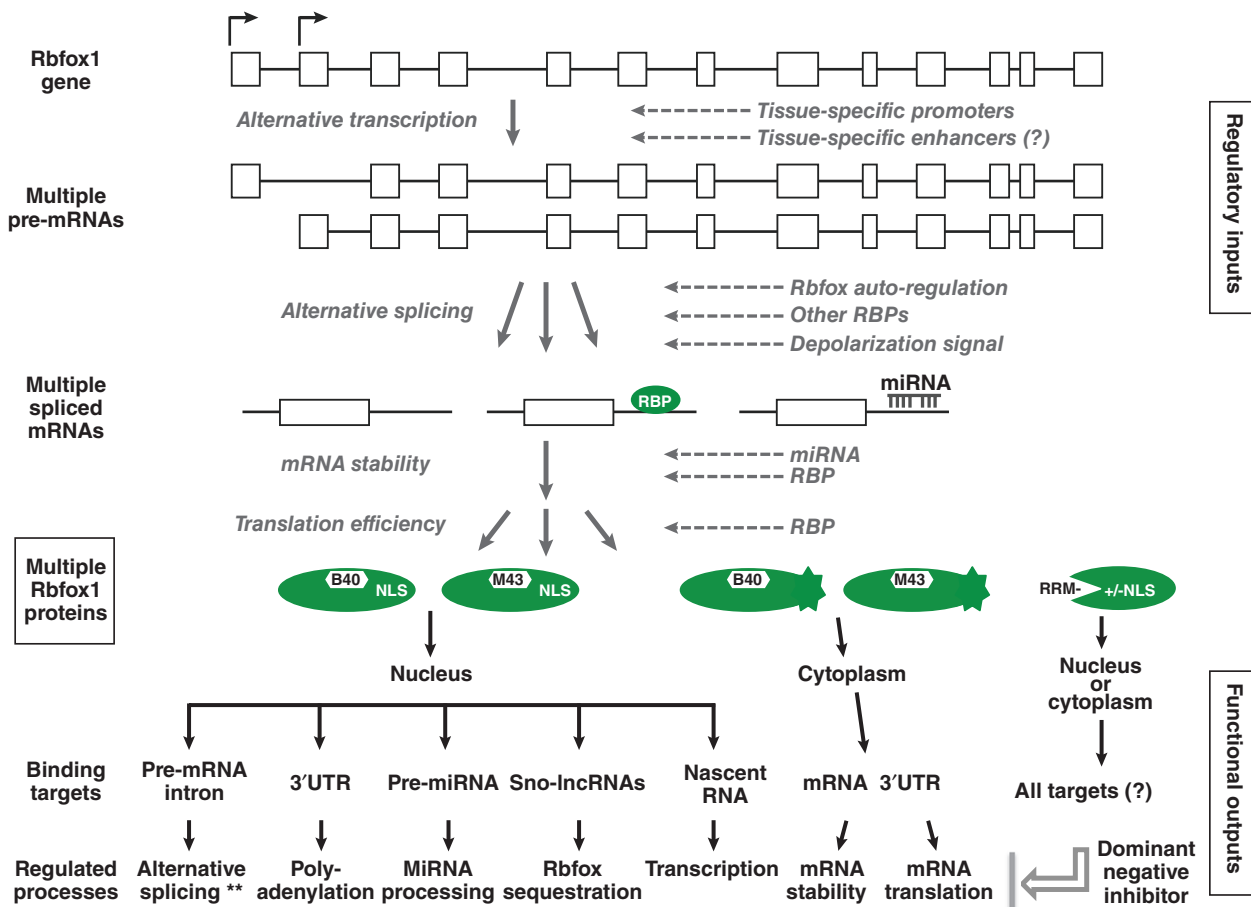


FIGURE 5 | Rbfox1 regulatory circuitry. The top half of the figure indicates multiple steps of gene expression at which the expression of Rbfox1 proteins is regulated, while the lower half depicts some of the functional outputs manifested by these proteins. ** Alternative splicing regulates coding exons in many target transcripts, but also noncoding exons in others that can induce NMD and reduced transcript levels.

Rbfox2 activity.⁸⁸ In dopaminergic neurons differentiated from iPS cells of PD patients, Rbfox1 transcripts are substantially over-expressed in conjunction with aberrant splicing of Rbfox-regulated exons. Whether this effect also involves dominant negative Rbfox isoforms was not reported. Notably, elevated neuronal Rbfox1 has been seen in patients with primary mutations in three different genes (*LRKK2*, *SNCA*, and *PARKIN*).⁸⁹ It will be interesting and important to elucidate the metabolic pathways by which Rbfox1 upregulation in PD neurons and Rbfox2 upregulation in diabetic hearts is mediated.

CONCLUSIONS

Rbfox proteins represent a versatile, multifunctional group of RBPs encoded by large and complex genes. Differentiating and mature cells of muscle and neuronal lineages each express a particular complement of Rbfox isoforms that can be regulated by numerous inputs at major transcriptional and post-transcriptional steps (shown for Rbfox1 in Figure 5). The resulting pools of nuclear and cytoplasmic Rbfox1 proteins, often co-expressed in the same cells, then bind to specific target sequences at key regulatory sites in the transcriptome to control downstream gene networks.

There are many unanswered questions on both sides of the ledger, regarding inputs that regulate

Rbfox isoform production, and functional outputs executed by these isoforms. Very little is known about transcriptional controls that activate expression developmentally, at different promoter sites and in different populations of skeletal and cardiac muscle cells, and especially in various neuronal populations in the brain. The functions of N-terminal variants predicted to be encoded by some of the alternative first exons (Figure 1) is unknown. Presumably, tissue- and development-specific transcriptional enhancers play important roles in determining spatio-temporal expression patterns, and these might even be the affected elements in some patients with Rbfox1 gene deletions.

Exciting advances have been made in characterizing developmental processes that are strongly influenced by Rbfox-regulated networks in both cytoplasm and nucleus and, in a few cases, identifying key transcript targets. However, complete Rbfox knockouts in complex tissues remain difficult to interpret. Better understanding of Rbfox regulation might enable more surgical strikes to knock down selected Rbfox isoforms or to limit knock-down to specific neuronal regions relevant to human disorders. Together, hopefully, new studies will provide new insights into normal development, that will also inform therapeutic efforts to treat human disorders that arise from perturbations in Rbfox1 networks.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health [DK094699] and Director, Office of Science and Office of Biological & Environmental Research of the US Department of Energy [DE-AC02-05CH1123].

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