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The neurogenetics of alternative splicing

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Abstract

Alternative precursor-mRNA splicing is a key mechanism for regulating gene expression in mammals and is controlled by specialized RNA-binding proteins. The misregulation of splicing is implicated in multiple neurological disorders. We describe recent mouse genetic studies of alternative splicing that reveal its critical role in both neuronal development and the function of mature neurons. We discuss the challenges in understanding the extensive genetic programmes controlled by proteins that regulate splicing, both during development and in the adult brain.

The precursor-mRNA (pre-mRNA) splicing reaction is a key step in the regulation of eukaryotic gene expression. Nearly all mammalian multi-exon genes produce multiple mRNA isoforms through alterations in the choice of splice sites to produce proteins of different structures and functions, or to alter mRNA localization, translation or decay. In keeping with its cellular and functional complexity, the mammalian nervous system makes extensive use of splicing regulation to generate specialized protein isoforms that affect all aspects of neuronal development and function[1–4]. Splicing defects are being increasingly implicated in neurological and neurodegenerative diseases, which underscores the need to better understand these regulatory processes.

Alternative splicing patterns (BOX 1) are regulated by specialized pre-mRNA binding proteins that alter `spliceosome` assembly at specific splice sites[5–8] (BOX 2). These proteins are structurally diverse and can exert different effects on a target transcript depending on their binding position, their modification by signalling pathways and their interactions with cofactors. Some regulators exhibit tissue-specific expression, whereas others are more ubiquitous, but they all regulate large overlapping programmes of neuronal

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alternative splicing events. Although each regulatory protein can affect many different RNA targets, each transcript is usually targeted by multiple regulators (FIG. 1). These compounded levels of complexity have challenged the characterization of the biological function of splicing regulatory proteins and studies of their mechanisms of action. The biological roles of splicing regulators and the cellular programmes they control are currently being elucidated through mouse genetics.

The neurogenetics of splicing is still in its infancy and can be confounded by several factors. How an alteration in splicing pattern changes the function of an encoded gene product is not usually known. Nevertheless, the mutation of an individual regulator often leads to a phenotype that either exhibits a high degree of pleiotrophy and/or is lethal owing to its extensive target set. In other cases, splicing regulators often have `paralogues`, the partially redundant function of which reduces the phenotypic impact of single gene mutations. Although splicing regulators can be widely expressed throughout the brain, different neuronal cell types often express different combinations of regulators and show a distinct susceptibility to their mutation. Despite this complexity, it is clear that splicing regulatory programmes dramatically affect all aspects of neuronal development and biology, from neurogenesis to mature synaptic function.

In this Review, we survey recent genetic studies of individual splicing regulators and the diverse roles they have in the mammalian nervous system. These studies have been aided by powerful new methods that allow the analysis of changes in splicing across the whole transcriptomes of mutant mice and the genome-wide mapping of regulatory protein binding. These methods have been extensively reviewed elsewhere[9–12], and our main focus is the phenotypic analyses that have begun to define the biological roles of these complex splicing regulatory programmes.

Alternative splicing and neurogenesis

Alternative splicing patterns change dramatically as cells progress along the neuronal lineage. These new splicing events are directed by changes in the expression of particular RNA-binding proteins, such as the `polypyrimidine tract` binding protein 1 (PTBP1) and serine/arginine repetitive matrix protein 4 (SRRM4; also known as nSR100), that affect neuronal fate and early neuronal differentiation.

PTBP1 in neurogenesis

The members of the PTBP family of splicing regulators are similar in structure and RNA-binding properties, but distinct in their cell type expression. PTBP1 (also known as PTB) is broadly expressed, but is largely absent from neurons, muscle cells and certain other mature cells. By contrast, PTBP2 (nPTB or brPTB) is found in neurons, myoblasts and spermatocytes. A third paralogue, PTBP3 (ROD1), expressed in haematopoietic cells and liver cells, is not known to affect splicing in neurons. Each PTBP contains four RNA recognition motif domains that together bind to extended CU-rich sequences[13,14].

PTBP1 is abundant in neural stem cells and progenitors, but on mitotic exit its expression is sharply reduced by the induction of the neuronal microRNA miR-124 (REF. 15). Reduced

PTBP1 expression also enhances miR-124 repression of the REST (repressor element 1-silencing transcription factor) complex[16], a well-known transcriptional repressor of neuronal gene expression[17,18]. Strikingly, the simple depletion of PTBP1 from cultured fibroblasts is sufficient to induce their *trans*-differentiation into neurons[16]. How the many PTBP1 splicing targets contribute to maintaining pluripotency or preventing differentiation is not yet clear. One target exon repressed by PTBP1 is in the transcription factor pre-B cell leukaemia homeobox 1 (*Pbx1*) gene. Precocious expression of the neuronal PBX1 isoform leads to the early induction of neurogenic genes[19]. Another notable PTBP1 target is exon 10 of *Ptbp2*. The repression of exon 10 leads to nonsense-mediated mRNA decay (NMD) of the *Ptbp2* transcript and prevents its expression in PTBP1$^+$ cells[15,20,21]. Induction of the PTBP2 protein has a critical role in neuronal differentiation.

Germline knockout of *Ptbp1* in mice leads to early embryonic mortality[22,23]. Mice with pan-neuronal loss of *Ptbp1* have grossly normal brain morphology at an early age, but show a progressive loss of ependymal cells from the lateral ventricles with hydrocephaly and die by 10 weeks after birth[24]. PTBP1 loss may induce the precocious differentiation of radial glial cells into neurons, thereby depleting the radial glial cell pool that later gives rise to ependymal cells[25] (FIG. 2). The loss of ependymal cells is restricted to the dorsal telencephalon, indicating variable roles for PTBP1 across brain regions.

An interplay between PTBP1 and SRRM4

SRRM4 is similar to the serine/arginine (SR)-rich splicing factor (SRSF) family and SR-related proteins containing serine/arginine repeats[26–28], but is unique for its brain-specific expression[29]. Despite lacking a canonical RNA-binding domain, SRRM4 frequently binds UGC-rich sequences located between the polypyrimidine tract and the `3′ splice site` of target exons[30]. The most enriched motifs surrounding SRRM4-dependent exons are typical PTBP binding elements, suggesting that SRRM4 can antagonize PTBP activity, and the regulatory programmes for these two proteins significantly overlap. SRRM4 promotes splicing of the REST4 isoform, which lacks four of the nine zinc fingers found in the full-length protein[31] and has reduced transcriptional repression activity[32–34]. Conversely, REST inhibits *Srrm4* expression in non-neuronal cells[31]. Through their action on transcription factor pre-mRNA, splicing regulators can indirectly control the transcription of neuronal genes.

Knockdown of *Srrm4* in the developing mouse cortex inhibits neuronal differentiation and leads to the accumulation of Pax6$^+$ progenitor cells in the ventricular zone and the depletion of differentiated cells from the cortical plate[31]. Interestingly, the germline deletion of *Srrm4* results in fewer Pax6$^+$ cells in the ventricular zone and fewer postmitotic NeuN$^+$ neurons[35] (FIG. 2). The different observations of Pax6$^+$ cells may result from different effects of acute versus prolonged loss of SRRM4, or may indicate that SRRM4 has different roles in early versus late neurogenesis. Indeed, *Srrm4*$^{-/-}$ mice have fewer late-born, upper-layer neurons and more early-born, lower-layer neurons, suggesting either depletion of the neural stem/progenitor cell pool or alterations in neuronal subtype specification (FIG. 2).

The seemingly mild neurogenic phenotypes of the *Ptbp1* and *Srrm4* knockout mice compared with the more dramatic results in tissue culture suggest that the *in vivo*

programme of neuronal induction contains multiple fail-safe mechanisms, with PTBP1 and SRRM4 serving opposing roles in reinforcing the robustness of the regulatory network. The interplay of these factors and their interactions with miR-124 and the REST complex constitute an important genetic programme underlying neuronal cell fate commitment.

Regulation of neuronal migration

The alternative splicing of components of the Reelin signalling pathway is important for proper neuronal migration in multiple brain regions. A loss of splicing regulators, such as neuro-oncological ventral antigen 2 (NOVA2) and RNA-binding protein fox-1 homologue 2 (RBFOX2) leads to defects in cortical and cerebellar lamination.

NOVA2 ensures proper migration of late-born cortical neurons

The first NOVA protein was identified as an autoantigen in patients with paraneoplastic opsoclonus-myoclonus ataxia, a human neurological syndrome characterized by motor and cognitive deficits[36]. The two paralogues NOVA1 and NOVA2 each contain three K homology (KH)-type RNA-binding domains and bind clusters of `YCAY elements`. NOVA1 is mainly expressed in the hindbrain and ventral spinal cord, whereas NOVA2 is predominant in the forebrain and dorsal spinal cord, with some overlapping expression in portions of the midbrain and hindbrain[37]. Genetic knockout of *Nova1*, *Nova2*, or both has demonstrated important roles for the two proteins in multiple aspects of brain development[38,39].

Proper cortical lamination requires NOVA2. In *Nova2*-null mice, neurons of cortical layers II/III and IV are mislocalized to lower layers without altering the layer-specific molecular markers[39] (FIG. 2). Progenitor cell proliferation and radial glia morphology are largely unaffected, suggesting a defect in neuronal migration rather than subtype specification. This contrasts with lamination defects in *Srrm4*-null mice, where increased numbers of lower-layer neurons were attributed to the premature commitment of progenitors to neurogenesis or alterations in subtype specification.

The defective migration of *Nova2*$^{-/-}$ upper-layer neurons was attributed to the mis-splicing of disabled 1 (*Dab1*), a component of the Reelin signalling pathway that controls cortical neuronal migration and lamination[40–42]. In wild-type neurons, NOVA2 represses both exon 7b and 7c of the *Dab1* transcript, and the resulting DAB1 protein isoform DAB1Δ7bc is subject to ubiquitylation upon Reelin activation[43–45]. In *Nova2*$^{-/-}$ neurons, the abnormal inclusion of exons 7b and 7c produces a more stable isoform that may antagonize the activity of DAB1Δ7bc[46,47]. The introduction of a Dab1Δ7bc transgene rescues the migration defect for a subset of the layer II–IV *Nova2*$^{-/-}$ neurons. This rescue with a single spliced isoform provides an important method of validating the source for particular aspects of a pleiotropic phenotype. Notably, *Dab1* is mis-spliced in the *Nova2*-null cortex only between E14 and E18. This restricted regulatory window and the limited population of affected cells reflect the complicated landscape of alternative splicing during neuronal development, where overlapping splicing regulatory programmes come into play at specific times and in specific neuronal populations.

RBFOX2 is required for proper Purkinje cell radial migration

Purkinje cell migration in the cerebellum is controlled by RBFOX2, which is a member of the highly conserved RBFOX family of RNA-binding proteins: RBFOX1 (also known as A2BP1), RBFOX2 (also known as RBM9) and RBFOX3 (also known as NeuN). The RBFOX proteins all bind the RNA sequence element (U)GCAUG via a single RNA recognition motif domain. RBFOX binding upstream of, or within, an alternative exon typically inhibits exon inclusion, whereas downstream binding usually promotes splicing[48]. The mechanistic basis for this pattern is not known, but multiple other splicing regulators, such as NOVA[49,50], exhibit the same positional dependence. All three *Rbfox* genes are broadly expressed in the brain, with individual neuronal cell types expressing different combinations at different developmental times[51–55]. For example, Purkinje cells express RBFOX2 early in development, with later onset of RBFOX1 and no expression of RBFOX3. By contrast, cerebellar granule cells switch from expressing RBFOX2 during proliferation and migration to RBFOX1 and RBFOX3 with maturation[55]. It is not known how the different RBFOX proteins differ in activity; however, their complex expression patterns imply that they may serve overlapping but distinct roles.

Pan-neuronal *Rbfox2* knockout mice show increased mortality with frequent hydrocephaly at 1 month of age[55]. The cerebellum is severely affected, with a substantially reduced size and a loss of foliation. Purkinje cells normally migrate outwards from the ventricular zone to be arrayed in a single layer between the external and internal granule layers by embryonic day 18 (REFS 56,57). The *Rbfox2*$^{-/-}$ Purkinje cells show a substantial delay in migration and increased cell death, resulting in a disorganized Purkinje cell layer (FIG. 3). *Rbfox2*$^{-/-}$ brains show altered splicing in transcripts known to control cell migration. In particular, low-density lipoprotein receptor-related protein 8 (LRP8), which normally binds Reelin to control cortical and Purkinje neuron migration, produces higher amounts of a dominant-negative isoform in *Rbfox2*$^{-/-}$ brains[58]. Consistently, *Lrp8*-null mice show ectopic Purkinje cells similar to the *Rbfox2*-null mice[59,60]. It will be interesting to test the roles of LRP8 and other components of the Reelin signalling pathway in *Rbfox2*$^{-/-}$ rescue experiments, similar to those in *Nova2*-null mice.

Multiple components of the Reelin pathway and other signalling pathways affecting neuronal migration are expressed as alternatively spliced isoforms[61,62]. The regulators of these splicing events are largely unknown, and it is likely that other splicing regulator mutants will show migration defects in particular brain structures.

Synaptogenesis and cell survival

After commitment to differentiation and migration to their proper location, neurons undergo a long period of maturation that includes the formation and maturation of synapses. Alternative splicing defines the gene products involved in these processes, and particular splicing regulator mutations have been shown to dramatically affect these developmental steps.

PTBP2 is required for proper neuronal maturation

The downregulation of PTBP1 in neural stem/progenitor cells as they exit mitosis induces the expression of PTBP2, which is required for neuronal development and survival. The role of PTBP2 was revealed in different mutant mice carrying either germline null alleles or pan-neuronal conditional alleles[63,64]. These mice are paralysed and unresponsive to touch at birth and show perinatal lethality with respiratory failure, possibly owing to a loss of innervation to the diaphragm. The brains of these mice appear grossly normal. In one analysis, small ectopic clusters of S and M phase cells were found in reverse orientation from their normal positions[63] (FIG. 2). These cellular abnormalities were possibly mediated by an observed change in the splicing of *Numb*, a known regulator of asymmetrical neural stem cell division[65,66]. Early differentiation defects were not reported in another germline *Ptbp2*-null allele, but these mice were not subjected to the same analyses[64]. This null mutant exhibited a loss of some early developing white matter tracts. Overall, the early lethality of the *Ptbp2*-null alleles and of the pan-neuronal knockout limited analyses of their phenotypes.

The role of PTBP2 in later development was revealed by its depletion from excitatory neurons of the dorsal telencephalon using an *Emx1-Cre* line[64]. At birth, the *Emx1– Ptbp2*$^{-/-}$ brain appears similar to the wild-type brain in morphology, size, neuronal fate commitment and cortical lamination. However, *Emx1–Ptbp2*$^{-/-}$ brains begin to show cortical atrophy as early as P5, and by P15 the cortex shows massive cell death and is almost completely degenerated. Similarly, cultured *Ptbp2*$^{-/-}$ embryonic cortical neurons initially appear normal in plating efficiency and neurite outgrowth, but exhibit progressive cell death beginning in the second week. The cell death is possibly due to a failure of synapse formation or another aspect of maturation, with a resulting lack of activity-dependent survival signals (FIG. 2). Because synaptogenesis occurs later in the forebrain than elsewhere in the central nervous system, similar defects in synaptogenesis with neuronal death may also occur in the lower brain of *Ptbp2*-null mice and lead to the perinatal lethality.

All of the *Ptbp2* mutant mouse models show a precocious expression of many adult mRNA isoforms encoding proteins that affect a variety of cellular functions, including the regulation of transcription, synaptic transmission, synapse organization and endocytosis[63,64]. Crosslinking immunoprecipitation (CLIP) analysis indicates that many of these transcripts are direct PTBP2 targets[63]. In early development, the embryonic splicing pattern of many neuronal genes is maintained during the switch from PTBP1 to PTBP2. Later in maturation, coincident with marked PTBP2 downregulation and synaptogenesis, the adult isoforms become more prevalent[63,64,67]. Thus the high expression of PTBP2 during early development extends the expression of the embryonic splicing programme until late in neuronal maturation. How the premature induction of the adult isoforms contributes to neuronal cell death and other phenotypes of *Ptbp2*$^{-/-}$ neurons is unclear.

One synaptic target of PTBP1 and PTBP2 is exon 18 of postsynaptic density protein 95 (*Psd95*; also known as *Dlg4*), which encodes the major scaffold protein of excitatory synapses. The *Psd95* transcript is expressed in many non-neuronal cells, where skipping exon 18 leads to NMD of the transcript, preventing its productive translation. Overexpression of either PTBP1 or PTBP2 in mature neurons inhibits exon 18 inclusion, PSD95 protein expression and excitatory synapse formation[67]. PTBP1 restricts PSD95

protein expression to neurons, whereas PTBP2 controls the temporal induction of PSD95 late in neuronal maturation. The sequential expression of PTBP1 and PTBP2 thus serves to precisely time the production of this key synaptic protein.

SRRM4 in synaptogenesis and development

Two mutant alleles of *Srrm4* have been analysed and shown to exhibit different phenotypes. The Bronx waltzer (*bv*) mouse arose from a forward genetic screen[68] and produces an unstable truncated or translationally defective protein. *Bv/bv* mutants show deafness, head tossing and circling[69]. A second conditional *Srrm4* allele produces a frame-shifted product lacking the critical RS-rich domain and splicing activity when crossed to a germline Cre strain ($Srrm4^{\Delta 7-8}$)[35]. The phenotypes of homozygous $Srrm4^{\Delta 7-8}$ mice are more severe than those of the *bv/bv* mice, with defects in multiple neurodevelopmental processes. Although *bv/bv* mice live to adulthood, only 15% of $Srrm4^{\Delta 7-8}$ mice survive beyond birth and adult survivors show severe tremors with some balance defects similar to the *bv/bv* mice.

The *bv/bv* mice have defects in the differentiation and/or survival of inner hair cells and vestibular hair cells of the cochlea[70,71]. Both inner hair cells and vestibular hair cells, normally densely innervated by spiral ganglion neurons, progressively degenerate and are completely lost by the first postnatal week. By contrast, the outer hair cells, which normally require only sparse innervation, are unaffected[72]. The *bv/bv* inner ear shows aberrant splicing in genes enriched in neurotransmission and secretion. This is similar to the $Srrm4^{\Delta 7-8}$ brain, which shows aberrant splicing in genes implicated in vesicle trafficking and recycling. Although it remains unclear how these splicing defects lead to decreased cell survival, one possibility is that synaptogenesis and synaptic transmission, which are required for the survival of inner hair cells and vestibular hair cells, are impaired. As seen in the $Ptbp2^{-/-}$ forebrain, the *bv/bv* mouse provides evidence that correct alternative splicing is necessary for proper synaptogenesis and cell survival.

The phenotypes of $Srrm4^{\Delta 7-8}$ mice have similarities to those of the $Ptbp2^{-/-}$ mice. $Srrm4^{\Delta 7-8}$ mice show no gross morphological phenotype during embryonic development, but most die from respiratory failure within a few hours of birth. This appears to result from insufficient phrenic innervation to the diaphragm[35], as secondary branching of motor neuron axons in this region is reduced by twofold. Although the molecular events underlying the phenotypic defects are not yet defined, one-third of *in vivo* SRRM4 targets overlap with PTBP2 targets. Comparisons of these systems will provide interesting insights into how developmental alternative splicing programmes control synaptogenesis.

NOVA control of motor neuron development and survival

Defects in muscle innervation and neuromuscular junction (NMJ) development and function are also seen in *Nova1* and *Nova2* double-knockout (dKO) mice. $Nova1^{-/-}$ *mice* appear normal at birth, but die in the second postnatal week with motor neuron apoptosis, profound motor failure and action-induced tremors[37,38]. *Nova1/2* dKO mice are born paralysed and die from respiratory failure indicative of NMJ defects[73]. Indeed, the dKO mice, but not the single-knockout mice, show fewer acetylcholine receptor (AChR) clusters and a loss of apposition between AChR clusters and phrenic nerve terminals, suggesting redundancy of

the NOVA proteins for controlling NMJ development[38,73]. Although NOVA1 and NOVA2 are detected in the ventral and dorsal spinal cord, respectively[37], the loss of NOVA1 might upregulate NOVA2.

The impaired NMJ synaptogenesis in *Nova1/2* dKO mice is due to aberrant agrin (*Agrn*) splicing. The AGRN protein promotes the clustering of AChRs within the postsynaptic membrane of the innervated muscle. Neuronal-specific *Agrn* isoforms containing the Z exons (32 and 33) are the most potent in promoting AChR aggregation[74–77]. Targeted deletion of the Z exons leads to paralysis and perinatal lethality similar to the phenotype of *Nova1/2* dKO mice[78,79]. Although exon 32 is only slightly affected in single *Nova* knockouts, it is almost completely skipped in dKO mice. Strikingly, restoring Z^+ *Agrn* expression in dKO motor neurons via a transgene rescues AchR clustering, nerve terminal apposition, NMJ morphology and muscle responses to stimuli[73]. The paralysis phenotype and early mortality are unchanged by the transgene, indicating the contributions of additional NOVA targets, possibly in other brain regions. These studies demonstrate how the activity of a particular alternatively spliced isoform can play a crucial part in ensuring proper synaptic development.

Regulation of synaptic function

Alternative splicing contributes to many aspects of synaptic function, including synapse specificity through the action of the KH domain-containing, RNA-binding, signal transduction-associated (KHDRBS) family, regulation of inhibitory synapse function by NOVA2, and splicing of many ion channels and synaptic components by muscleblind-like 2 (MBNL2), the neuronal ELAV-like (nELAVL) proteins, RBFOX1 and RBFOX2, and sodium channel modifier 1 (SCNM1).

KHDRBSs control alternative splicing of neurexin

The KHDRBS family has three members: SRC-associated in mitosis 68 kDa protein (SAM68; also known as KHDRBS1), SLM1 (also known as KHDRBS2) and SLM2 (also known as KHDRBS3)[80]. Each contains a single KH domain for RNA binding and dimerization. SAM68 is found in both the nucleus and the cytosol of many cell types[81] and affects a variety of cellular processes, including splicing in the nucleus[82,83]. SLM1 and SLM2 are more restricted to the nervous system and have distinct expression patterns. For example, SLM1 is found in the dentate gyrus, some cortical neurons and Purkinje cells, whereas SLM2 is in found in the CA1 and CA3 neurons of the hippocampus, most cortical neurons, and sparsely in the granule and molecular layers of the cerebellum[84]. The mutually exclusive expression pattern of SLM1 and SLM2 is enforced by their cross-regulation via alternative splicing coupled with NMD, as seen with other paralogous pairs of RNA-binding proteins[15,20,21,49,53,85,86]. Specifically, SLM2 depletion shifts the splicing of *Slm1* away from an NMD-targeted isoform and towards a productive transcript[87].

One target of the KHDRBS proteins is neurexin (*Nrxn*), encoding presynaptic cell surface proteins that promote synaptogenesis through *trans*-synaptic signalling[88]. Pre-mRNAs from the three *Nrxn* genes undergo extensive alternative splicing to produce more than 3,000 protein isoforms[88,89], presumably to define synapse specificity[90,91]. Regulation of *Nrxn*

exon 20 generates AS4^{+} or AS4^{-} isoforms that show differential binding to cell-type-specific postsynaptic partners[88,92]. NRXNβ AS4^{-} isoforms preferentially bind neurolignin 1 (B) concentrated at glutamatergic synapses, whereas NRXNβ AS4^{+} isoforms preferentially bind NLGN2(A) at GABAergic and glycin-ergic synapses[93] (FIG. 4a). Most interestingly, the inclusion of exon 20 is negatively regulated by KHDRBS proteins.

Although exon 20 is similarly regulated by the three KHDRBS proteins in cell culture[87,94], *Khdrbs* mutant mice show region-specific differential *Nrxn* splicing defects as a result of cell-type-specific expression of the individual KHDRBS proteins. Control of exon 20 by KHDBRS also appears to be modulated by synaptic activity. In cerebellar granule neurons, both KCl depolarization and kainic acid treatment increase SAM68 phosphorylation at Ser20 via CamKIV activation, leading to increased repression of *Nrxn1* exon 20 and changes in trans-synaptic interactions[94] (FIGS 3b,4a). Thus the KHDRBS proteins probably play a key part in modulating synaptic specificity and the plasticity of neural circuits during development and in adults.

Germline *Sam68*-null mice have deficits of bone metabolism, sexual organ development, motor coordination and motor learning[95,96]; recent work indicates possible alterations in long-term depression[97]. As SAM68 is almost ubiquitously expressed throughout the cerebellum, it is a question as to which neuronal subtypes contribute to the motor phenotypes and whether *Nrxn1* is one of the relevant targets.

In contrast to *Sam68*-null mice, *Slm1* and *Slm2* single-null mice are viable with no apparent behavioural or anatomical defects[84,87,98]. *Slm1/2* dKO mice also show no apparent behavioural abnormalities, although possible morphological defects have not been characterized[87]. *Sam68/Slm1* dKO mice were similar to *Sam68* single-null mice, except for additional defects in cerebellar foliation and scattered ectopic Purkinje cells in the molecular layer. SAM68 and SLM1 are both expressed in Purkinje cells and probably have redundant roles in their development. It will be interesting to assess the *Slm2/Sam68* dKO or the triple *Khdrbs* knockout mice. The interplay between the specific effects of particular KHDRBS paralogues and their partial redundancy typifies the complexity of analysing splicing regulator mutants and indicates how a given splicing pattern may be controlled differently across different neuronal subtypes.

NOVA2 and synaptic plasticity

Many NOVA2 target genes, including the $GABA_B$ receptor 2 (*Gabbr2*), glycine receptor α2 (*Glra2*), gephyrin (*Gphn*) and the inward rectifier potassium channel Kir3.2 (*Girk2*), are involved in inhibitory synapse function[99] (FIG. 4a). Mis-splicing of $GABA_B$ receptors and GIRK channels probably leads to deficient long-term potentiation of the slow inhibitory current seen in the *Nova2*-null hippocampus[100]. By contrast, long-term potentiation of excitatory post-synaptic currents is unchanged, as are basal excitatory and inhibitory synaptic transmission. This high degree of phenotypic specificity highlights the variable sensitivity of different forms of synaptic transmission and plasticity to splicing alteration. How the alternative splicing of ion channels and neurotransmitter receptors changes precise physiological functions and how this regulation defines circuit function will be a rich area of investigation going forward.

MBNL2 and neurological symptoms of myotonic dystrophy

The MBNL family of RNA-binding zinc finger proteins has three members in mice and humans. MBNL proteins have been studied extensively in relation to the neuromuscular disorder myotonic dystrophy. In myotonic dystrophy, CTG or CCTG repeat expansions in expressed RNAs sequester MBNL proteins from their normal binding sites, altering MBNL-dependent splicing patterns[101–105]. Although all three MBNLs are expressed in the brain, only *Mbnl2*-null mice exhibit obvious central nervous system phenotypes. Germline deletion of *Mbnl2* results in abnormal sleep patterns, memory loss and learning deficits. *Mbnl2*-null mice are also more susceptible to pentylenetetrazole-induced seizures. Muscle function is, however, unperturbed, probably as a result of abundant MBNL1 expression[106]. Hundreds of exons are mis-spliced in the *Mbnl2*$^{-/-}$ brain and overlap significantly with those known to be mis-spliced in myotonic dystrophy. MBNL2 overall promotes adult-like splicing patterns and its loss leads to continued expression of the fetal isoforms of ion channel-encoding genes such as the calcium-activated potassium channel subunit alpha 1 gene (*Kcnma1*; which encodes BK (also known as Slo1)), the voltage-gated calcium channel subunit alpha 1D gene (*Cacna1d*; which encodes $Ca_v1.3$) and the NMDA receptor subunit gene (*Grin1*; which encodes GluN1) (FIG. 4a). NMDAR-mediated responses and pattern-induced long-term potentiation are impaired in *Mbnl2*-null mice. The observed alterations in synaptic plasticity and perturbations in neuronal excitability may be a result of the continued expression of fetal ion channel isoforms.

It is notable that about half of the MBNL2 CLIP tags are found in three prime untranslated region (3′ UTR) sequences, indicating the non-splicing functions of MBNL2 (REF. 106). Although *Mbnl2*-null brains did not show major changes in transcript levels, recent studies of *Mbnl1/2* dKOs have highlighted MBNL activity in controlling alternative polyadenylation events[105,107,108]. Binding in 3′ UTRs is commonly observed for other splicing regulators and points to the need to distinguish phenotypes driven by splicing changes from those arising from altered mRNA stability, localization and/or translation (FIG. 5).

ELAVL proteins regulate neuronal excitation

The ELAVL (also known as Hu) family of proteins consists of four highly homologous members[109–111] that recognize U- and AU-rich elements[112–118] (FIG. 5b). ELAVL1 (also known as HuR or HuA) is widely expressed in non-neuronal tissues, whereas ELAVL2 (also known as HuB), ELAVL3 (also known as HuC) and ELAVL4 (also known as HuD) show neuronal-specific expression and are called neuronal ELAVLs (nELAVLs)[51,119,120]. Like NOVA, nELAVLs are target antigens in patients with paraneo-plastic neurological disorders[121,122]. The ELAVLs have primarily been studied as regulators of mRNA stability and translation efficiency through their binding to 3′ UTRs[110,111,123] (FIG. 5b), but recent genome-wide profiling analyses have revealed intronic binding of nELAVLs and hundreds of splicing changes in *Elavl3/4* dKO brains. These splicing targets are enriched for proteins involved in microtubule assembly and disassembly at synapses and axons. Interestingly, the biological processes affected at the level of splicing are different from those affected at the level of transcript abundance, suggesting that the regulatory programmes of the nuclear and cytoplasmic nELAVL proteins are distinct[118].

Depletion of *nElavl* in the brain leads to multiple neurological defects[124,125]. Although *Elavl3*-null mice are born grossly normal and fertile, most of the adult animals show poor motor coordination. The specificity of the motor defect may be because ELAVL3 is the only nELAVL protein in Purkinje cells[118]. These mice also show spontaneous cortical hypersynchrony and non-convulsive electrographic seizures. These phenotypes are attributed to aberrant glutamate levels, based on binding of nELAVL to the 3′ UTRs of genes affecting glutamate synthesis. The multiple splicing regulator mutants that show seizure phenotypes may reflect the large number of synaptic and membrane proteins regulated at the level of splicing, with hyperexcitability being a common consequence of their perturbation.

RBFOX1 control of neuronal excitability

Another splicing regulator whose mutation leads to a hyperexcitability phenotype is RBFOX1. Human mutations in *RBFOX1* have been identified in patients with epilepsy[126–128] and autism spectrum disorder[129–131]. Pan-neuronal deletion of *Rbfox1* (*Rbfox1*$^{loxp/loxp}$; *Nestin-Cre*) leads to increased susceptibility to spontaneous seizures and seizures induced by kainic acid, as well as hyperexcitation in the dentate gyrus[53]. Relatively few splicing and expression changes were detected in the *Rbfox1*$^{-/-}$ whole brain, presumably as a result of the redundancy of RBFOX2 and RBFOX3 function[53,132]. However, these splicing changes affect transcripts encoding ion channels, neurotransmitter receptors, structural proteins and Ca^{2+} signalling molecules, many of which are associated with seizure disorders in humans or mice, such as the $GABA_A$ receptor subunit *Gabrg2*, *Grin1*, the voltage-gated sodium channel *Scn8a* (which encodes $Na_V1.6$), and synaptosomal-associated protein 25 (*Snap25*)[133–137] (FIGS 1b,4). Changes in the isoform ratios for these proteins may increase action potential firing or disrupt the excitation/inhibition balance in neuronal circuits. For example, the gene for the SNARE protein SNAP25 uses a pair of mutually exclusive exons (5a and 5b) to produce two alternative isoforms that show differences in the kinetics of synaptic vesicle release[138,139]. The *Rbfox1*$^{-/-}$ brains show decreased 5b and increased 5a inclusion. The *Rbfox1*$^{-/-}$ seizure phenotype is thus consistent with studies showing that mice carrying genetic substitution of exon 5b with exon 5a also exhibit seizures[140].

The *Rbfox1*$^{-/-}$ and *Elavl3*$^{-/-}$ mice present interesting animal models for the study of human epileptogenesis and mechanisms controlling neuronal excitability. It will be interesting to compare their molecular targets and mutant physiology to understand whether they affect a common regulatory programme or perhaps drive different splicing changes that have similar physiological outcomes.

RBFOX proteins control Purkinje cell pace-making

In addition to affecting Purkinje cell migration, RBFOX2, in conjunction with RBFOX1, regulates mature Purkinje cell function. *Rbfox2*$^{-/-}$ Purkinje cells eventually form a proper layer, but show decreased dendritic arborization in the molecular layer, as well as irregular and less frequent spontaneous action potentials. This pace-making defect becomes more severe in *Rbfox1* heterozygous, *Rbfox2*-null brains (*Rbfox1*$^{+/-}$; *Rbfox2*$^{-/-}$)[55]. Specific depletion of *Rbfox1* and *Rbfox2* from mature Purkinje cells after the completion of migration and development, using the *L7*(*Pcp1*)-*Cre* strain (*L7*-DKO), results in a similar

pace-making phenotype and motor defects[55] (FIG. 3). The early and late phenotypes of *Rbfox* mutation demonstrate roles for these proteins in both cerebellar development and mature function.

The pace-making defect in the *L7*-DKO mice is highly reminiscent of mice lacking the voltage-gated sodium channel subunit *Scn8a* (REFS 141–143). $Na_v1.6$ functions, in part, to maintain a resurgent sodium current that enables regular, spontaneous firing. Several alternatively spliced exons in *Scn8a* contribute to the resurgent sodium current[144,145]. Mutually exclusive exons 5A and 5N alter voltage-dependent gating and/or interactions with a blocking subunit[145]. Another pair of mutually exclusive exons, 18A and 18N, determine whether a functional 18A+ *Scn8a* mRNA is produced. Inclusion of 18N introduces a premature termination codon and leads to nonsense-mediated decay, whereas skipping both 18A and 18N produces an isoform that lacks large portions of the third and fourth transmembrane domains[146,147] (FIG. 4b). Although the single *Rbfox1*$^{-/-}$ and *Rbfox2*$^{-/-}$ brains show modest changes in *Scn8a* splicing, the *Rbfox1*$^{+/-}$; *Rbfox2*$^{-/-}$ brains show dramatic splicing changes at both exons 5 and 18, including a twofold decrease in exon18A that decreases the amount of functional $Na_v1.6$ (REF. 55). Thus the Purkinje cell pace-making defect in *Rbfox* mutant mice could largely arise from the loss of $Na_v1.6$. These results further confirm the partial redundancy of the *Rbfox* family members.

SCNM1 enhances non-consensus splicing of Scn8a

Forward genetic studies have identified an *Scn8a* splicing mutation that causes severe neurological defects. This mutation, *med*^{J}, does not alter the coding sequence of *Scn8a* but instead has a four base pair deletion in the `5′ splice site` of intron 3, which causes skipping of both exons 2 and 3 in a majority of *Scn8a* transcripts (FIG. 4b), and produces a severely truncated, non-functional $Na_v1.6$ protein[148]. The *med*^{J} mice show hindlimb paralysis, muscle atrophy and the degeneration of Purkinje cells[149]. Normally, $Na_v1.6$ replaces fetal $Na_v1.2$ at the nodes of Ranvier during the first few weeks of postnatal development[150]. In *med*^{J}/C3H mice, the replacement of fetal $Na_v1.2$ is delayed and the amount of $Na_v1.6$ at the nodes of Ranvier reaches only 10–20% of that seen in wild-type mice. Nerve conduction velocity in *med*^{J} mutants is decreased by half[151], probably as a consequence of insufficient $Na_v1.6$ expression.

The severity of the *med*^{J} phenotype was found to be affected by genetic background and to correlate with the amount of correctly spliced transcript[152]. In the C3H background, *med*^{J} mice live a normal lifespan with dystonia and ataxia, with 10% of *Scn8a* transcripts correctly spliced. The same mutation in a C57Bl/6J background produces only 5% correctly spliced transcript and the mice show progressive paralysis and lethality by 1 month of age[151]. The phenotypic severity of the *Scn8a*^{medJ} hypomorphic allele is determined by a single gene modifier, sodium channel modifier 1 (*Scnm1*). C67Bl/6J mice have a nonsense mutation in *Scnm1* and targeted deletion of *Scnm1* in the C3H strain confirmed that SCNM1 affects both the splicing of the *Scn8a*^{medJ} transcript and the mouse phenotype[152,153]. Finally, a bacterial artificial chromosome transgene expressing wild-type *Scnm1* can rescue the lethality and paralysis of *Scn8a*^{medJ} in C57BL/6J mice[154].

Although the genetic interaction between *Scnm1* and *Scn8a* splicing has been studied in detail, the mechanism of SCNM1 function as a splicing regulator is less clear. The protein has one zinc finger domain, a basic nuclear localization signal and an acidic carboxy terminus. Its overexpression in heterologous cells can enhance the correct splicing of an *Scn8a* mini-gene, possibly via interactions with the spliceosomal proteins U1 small nuclear ribonucleoprotein 70 (U1-70k) and putative RNA-binding protein Luc7-like 2 (LUC7L2)[153]. The studies of *Scnm1* indicate that splicing regulators can be an important class of phenotypic modifiers. Given the many human disease-causing mutations that affect pre-mRNA splicing, polymorphisms in splicing regulatory genes may play a large part in modifying disease severity across individuals.

Splicing regulators and neurodegeneration

Splicing misregulation is increasingly implicated in neurodegenerative disorders. Dysfunction of transactivating response DNA binding protein (TDP43; also known as TARDBP) and fused in sarcoma (FUS; also known as TLS) lead to phenotypes that are characteristic of amyotrophic lateral sclerosis (ALS) and frontal temporal lobar disease (FTLD), whereas mutation of *Rnu2–8*, which encodes part of a core spliceosomal component, the U2 small nuclear RNA (snRNA), leads to specific neurodegeneration in the cerebellum.

TDP43 and FUS in amyotrophic lateral sclerosis

Errors of splicing regulation are increasingly implicated in a variety of neurodegenerative disorders, including ALS and FTLD[155,156]. Familial and sporadic forms of ALS and some cases of FTLD have been associated with mutations in TDP43, FUS[155,156], heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), hnRNPA2B1 (REF. 157) and matrin 3 (REF. 158) — all of which are regulators of splicing. Disruption of TDP43 and FUS function through protein aggregation or mislocalization is a characteristic of ALS and FTLD derived from many different mutations[159–161] and neurogenetic analyses of TDP43 and FUS have been the most extensive.

TDP43 is a major component of cytoplasmic inclusions found in 95% cases of sporadic ALS and FTLD[156]. Widely expressed in many tissues and predominantly nuclear, TDP43 affects multiple steps of RNA metabolism, including transcription, splicing, decay, transport and translation[162] (FIG. 5). One hypothesis for the pathogenic role of TDP43 is that the formation of TDP43 cytoplasmic inclusions leads to its depletion from the nucleus and a loss of splicing function[155,156]. Consistent with this, ALS-like phenotypes are seen in mice with partial depletion of TDP43 by RNA interference, or with targeted deletion of *Tdp43* in motor neurons, whereas germline *Tdp43-null* mice are embryonic lethal[163–165]. Dominant missense mutations are sufficient to cause familial disease in humans[166], and transgenic rodents expressing either wild-type or disease-associated mutants also show neurodegeneration. These phenotypes could be indicative of a toxic gain of function[167–169]; alternatively the mutations and overexpression could somehow both promote cytoplasmic inclusion formation with a loss of function[166,170–173].

Both the ALS-associated mutations and the changes in wild-type TDP43 expression alter a large programme of alternative exons in mutant mice[170,174,175]. However, less than a quarter of affected exons are shared between the transgenic and RNA interference-depletion mouse models. Motor neurons may be particularly sensitive to aberrant splicing changes, or some of the common TDP43 dependent splicing events might be sufficient to cause the phenotype. These many models will allow rich comparisons in identifying potentially causative splicing changes.

Another ALS and FTLD gene, FUS[156], is a widely expressed, predominantly nuclear RNA-binding protein[176,177] with an RNA recognition motif that, like TDP43, has multiple roles in RNA processing, including splicing regulation[162,178]. *Fus*-null mice die soon after birth[179], whereas a transgenic rat model of mutant *Fus* shows various ALS-like phenotypes[180]. Interestingly, TDP43 and FUS regulate distinct groups of alternative exons and target different sets of mRNAs in the cytoplasm[178,181], suggesting different roles for the wild-type proteins. It will be interesting to compare the targets of these two proteins with the targets of other RNA-binding proteins implicated in ALS, including hnRNPA1, hnRN-PA2B1 and matrin 3, and to assess how their mutation might converge on similar disease pathologies.

Rnu2–8 is required for cerebellar granule neuron survival

A forward genetic screen recently uncovered a novel form of neurodegeneration caused by mutation of a core component of the spliceosome. The mouse mutant *NMF291* strain contains a functionally compromised allele of *Rnu2-8* (REF. 182), one of multiple genes encoding the U2 snRNA[183]. U2 snRNA binds to the `branch point` during spliceosome assembly and then forms base pairs with the U6 snRNA to become a key portion of the spliceosome catalytic centre for all `major class introns`[6,8,183,184] (BOX 2); its dysfunction might be expected to be lethal for all cell growth. Mammalian genomes contain multiple clustered copies of the U2 snRNA gene that allow production of the extremely high levels of U2 snRNA found in cells, which were previously thought to be equally expressed across tissues. The highly tissue-specific phenotype of *NMF291* was therefore unanticipated.

NMF291 mice show progressive and severe degeneration of the cerebellum as a result of the loss of cerebellar granule neurons beginning at postnatal week 4 (FIG. 3) and develop tremors at 8 weeks, progressing to truncal ataxia by 12 weeks. Consistent with the phenotype, both wild-type and mutant *Rnu2-8* RNA are selectively expressed in the cerebellum and increase in expression after granule neuron maturation. A transgenic mouse expressing the mutant *Rnu2-8* in the wild-type background displays a similar course of granule neuron loss and ataxia. Conversely, increasing the dose of the wild-type RNA in the *NMF291* mutant decreases neurodegeneration in the granule layer. These data demonstrate that not all U2 genes are the same, but that individual genes within a cluster can show temporal and cell-type-specific patterns of expression. Their mutation can thus lead to a highly specific phenotype.

The *NMF291* mutation is a 5nt deletion that removes the first 2nt of the branch site recognition sequence within the U2 snRNA, as well as a 3nt linker between the branch site recognition sequence and the U2/U6 helix IA. When highly expressed, the mutant U2 snRNA decreases the overall splicing efficiency and affects alternative splicing patterns. In

particular, about 3,000 annotated introns show higher levels of retention in the *NMF291* cerebellum (BOX 1; FIG. 5a). These results are reminiscent of recent data on myelodysplastic syndromes, where mutations in several components of the core splicing apparatus, such as U2 small nuclear ribonucleoprotein auxiliary factor 2 (U2AF65), splicing factor 3B, subunit 1 (SF3B1) and other proteins, were found to cause very specific splicing defects in particular transcripts and to lead to a highly tissue-specific phenotype[185–187]. These studies open the possibility for other neurological disorders being caused by the mutation of general splicing factors.

Conclusions

Alternative splicing as a regulatory process has been studied for many years at the level of individual proteins and target transcripts. The advent of whole genome analyses[10,12,188,189] brought a new appreciation of its pervasiveness and regulatory reach in metazoan organisms[190,191]. However, understanding the biology of splicing regulatory programmes and, in particular, the role of these programmes in the mammalian brain, needs to be addressed through a combination of genetics, neuroanatomy and physiology. The neurogenetics of splicing is still in its infancy. Of the many hundreds of potential splicing regulatory proteins, only a few are beginning to be analysed, and we have focused on those studied through mouse genetics (see Supplementary information S1 (table)). Although not yet studied in the mouse, another family of regulators that will probably have important splicing roles in the brain is the CUGBP, Elav-like family (CELF) proteins[192–194]. The initial targeted genetic studies described here make it clear that changes in the splice site choice have essential roles in nearly all aspects of neuronal development and function.

The analysis of splicing factor mutations is challenged by their highly pleiotropic phenotypes. Mutations are often lethal or lead to developmental abnormalities that obscure additional later functions. Several studies have overcome these obstacles using ever more precise Cre expression to ablate a regulatory gene in particular cell types at particular times. RNA sequencing and `CLIP-seq` now allow the relatively simple identification of mis-spliced targets potentially determining the mutant phenotype. These genome-wide analyses indicate that individual splicing regulators affect coherent sets of transcripts that can be involved in common biological pathways[195,196]. However, the large number of targets makes it difficult to link a phenotype to a particular splicing event. As described above, phenotypes can be connected to splicing events using transgenes expressing single spliced isoforms to rescue particular functions. This strategy will need to be applied to more refined populations of cells and circuits, perhaps through *in utero* electroporation or viral transduction. One challenge will be to match the expression from the rescuing gene to that of an endogenous locus in time and quantity. Developmental phenotypes may be reverted by an overexpressed transgene, but the rescue of physiological defects will probably require the precise control of isoform ratios, perhaps through genome editing of endogenous loci.

The obverse problem to the many targets of splicing regulators is that the regulators frequently occur in highly related gene families. Groups of paralogous regulators that show partially redundant functions can mute the effect of single-gene mutations. In cells where they are co-expressed, double mutation will often lead to new splicing changes that are not

seen in either single mutant and reveal new phenotypes. However, regulators are rarely entirely redundant and usually show differences in their range of expression, as seen with the NOVA, nELAVL and RBFOX proteins.

In this Review, we have focused on splicing regulation. It is important to keep in mind that many RNA-binding proteins controlling splicing choices also affect the choice of poly-A site and can also be found in the cytoplasm, where they control the translation or stability of target transcripts through binding in 3′ UTRs[197] (FIG. 5b). The consequences of their mutation will include the loss of these functions in addition to splicing changes. Cytoplasmic functions can be examined by measuring overall changes in expression by RNAseq, rather than splicing changes, and by identifying 3′ UTR targets in CLIP-seq data sets. The relationships between the nuclear and cytoplasmic regulatory programmes controlled by RNA-binding proteins are only beginning to be examined. It will also be important to define those effects that arise from the direct regulation of a transcript rather than as an indirect consequence of splicing factor loss. Splicing regulators extensively modulate each other's activity, as well as controlling the activity of transcriptional regulators (FIG. 1). Gene expression changes in mutant mice may thus derive in part from extensive secondary effects downstream of the protein being examined.

Recent human genetic studies have clearly implicated splicing regulators in neurodegenerative diseases such as ALS. Other work has connected RNA-binding proteins to mis-splicing in neuropsychiatric disorders, including epilepsy, autism spectrum disorder, inherited ataxias and schizophrenia. Splicing programmes provide a highly interconnected layer of regulation that can alter protein activity without easily discernible changes in the overall expression. Perturbations of these programmes have the potential to alter neuronal connectivity and firing properties in a manner that has dramatic consequences for overall circuit function and behaviour. The mouse mutations described here provide the first glimpses of these regulatory programmes. In the RNA-binding protein knockouts so far analysed, the heterozygous mice develop largely normally, but some splicing targets are still altered by the reduction in regulatory protein dose. It will be particularly interesting to assess these heterozygous mice for behavioural defects. Future work in these genetic systems will provide potential new models for a variety of disorders.

In addition to relating splicing regulation to neurological disease, there are many questions to be addressed. Although many alternative splicing events are conserved across mammalian or vertebrate species, the effect of these splicing changes on protein activity is usually unknown. It will be important to characterize the set of protein isoforms expressed from each gene and understand their different roles in cell biology. This will be a particular challenge for physiology, but such analyses are needed to relate changes in synaptic and membrane protein structure to changes in synaptic activity and firing. Another issue is how the programmes controlled by different regulators interact (FIG. 1a). RNA-binding proteins can antagonize each other or synergize in RNA binding. The complex overlap between their regulatory programmes allows for a high degree of specificity in where and when particular splicing events occur. It will be very interesting to assess how the expression of spliced isoforms contributes to defining specific neuronal subtypes. The future application of mouse

genetics to the characterization of splicing regulatory programmes should allow some of these questions to be answered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Spliceosome	A large macromolecular assembly of small nuclear ribonucleoproteins and proteins that catalyses the excision of introns from precursor mRNAs
Pleiotropy	A condition where one gene can influence multiple phenotypic traits
Paralogues	Related genes within a genome that often arise from gene duplication during evolution
Polypyrimidine tract	A sequence element located between the branch point and the 3′ splice site that is bound by U2AF during normal spliceosome assemblyit is also a binding site for the polypyrimidine tract binding protein that can be found elsewhere in the precursor mRNA.
3′ splice site	The sequence at the 3′ end of the intron that is recognized by the U2AF protein component of the spliceosome. Also called an acceptor site.
YCAY elements	Sequences consisting of a cytosine (C) and an adenine (A) flanked by two pyrimidines (Y)
5′ splice site	The sequence at the 5′ end of the intron that is recognized by the U1 small nuclear ribonucleoprotein component of the spliceosome. Also called a donor site.
Branch point	An intronic sequence element upstream from the polypyrimidine tract of the 3′ splice site where a branched nucleotide at a specific adenosine will be formed by the first transesterification step of splicing. The branch point is recognized by the U2 small nuclear ribonucleoprotein.

Major class introns	Also called GT/AG introns, which are excised by the major spliceosome containing the U1 and U2 small nuclear ribonucleoproteins
CLIP–seq	(Crosslinking immunoprecipita-tion followed by high-throughput sequencing). A method used to identify the binding sites of RNA-binding proteins across the transcriptome

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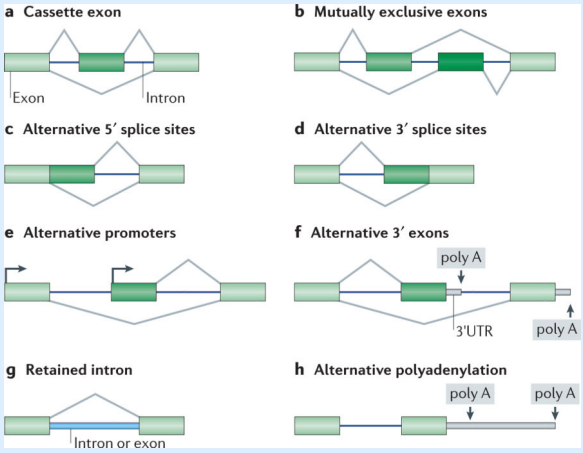
Box 1

Patterns of alternative splicing

The diversity of mRNA isoforms is generated from many different patterns of alternative splicing. Genes are segmented into introns and exons. During the precursor-mRNA splicing process, introns are excised from the precursor mRNA and exons are ligated together to form the mRNA. Special sequences at the intron ends define where the cleavage and ligation reactions occur. The 5′ splice site or donor site is at the 5′ end of the intron. The 3′ splice site or acceptor site is at the 3′ end of the intron. Splicing catalysis by the spliceosome takes place in two cleavage and ligation steps. The 3′ splice site has an associated branchpoint sequence, which is joined to the 5′ splice site after the first cleavage step. This is followed by cleavage at the 3′ splice site and ligation of the two exons. In the figure, the light green boxes indicate exons and the dark green boxes indicate alternative exons. The v-shaped lines show the different ways in which the exons can be joined in a final mRNA. The most common change in splicing pattern is a cassette exon (skipped exon; see part **a** of the figure), the inclusion or skipping of which will insert or delete a sequence from the final mRNA. Mutually exclusive exons (see part **b** of the figure) are a pair of consecutive cassette exons where only one of the exons is included in the mRNA. Alternative 5′ splice sites (see part **c** of the figure) are consecutive ‘donor sites’ that change the length of an exon at its 3′ end. Conversely, alternative 3′ splice sites (see part **d** of the figure) are consecutive acceptor sites that change the 5′ end of the exon. Alternative promoters (see part **e** of the figure) and alternative 3′ exons (see part **f** of the figure) create different first exons and different last exons on the mRNA, respectively. Retained introns (see part **g** of the figure) can be excised as a typical intron or remain in the final mRNA. Alternative polyadenylation (see part **h** of the figure) in the last exon allows for the generation of three prime untranslated regions (3’ UTRs) of varying lengths. A single gene can have multiple positions and patterns of alternative splicing to create a family of many different mRNAs and proteins through the inclusion or skipping of various alternatively spliced RNA segments. Figure adapted from REF. 1, Nature Publishing Group.



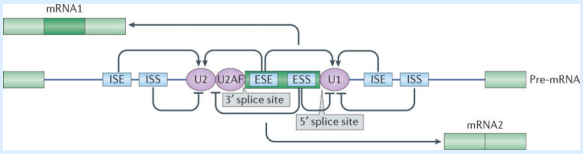
Box 2

Regulation of an alternative exon by RNA-binding proteins

Trans-acting RNA-binding proteins (RBPs) interact with *cis*-sequence elements in the precursor mRNA to facilitate or inhibit the assembly of the spliceosomal machinery at nearby splice sites. The 5′ splice site is initially bound by U1 small nuclear ribonucleoprotein (snRNP, see the figure). The U2 snRNP recognizes the branchpoint and is recruited by the U2AF proteins that are bound to the polypyrimidine tract between the branchpoint and the 3′ splice site. Binding of U1 and U2 allows recognition of an exon in a process called exon definition. These snRNPs are subsequently brought into interaction across an intron to allow further spliceosome assembly and the pairing of splice sites within the catalytic centre of the spliceosome. An alternative splicing event frequently involves multiple competing weak splice sites that are subject to dynamic regulation by neighbouring *cis* elements. These *cis* elements include intronic and exonic splicing enhancers and intronic and exonic splicing silencers that recruit activator or repressor RBPs, respectively. These RBPs, through multiple modes of action that are not yet understood, collectively influence splice site recognition or splice site pairing within the spliceosome. The levels and activity of these *trans*-acting RBPs control the choice of splice sites for many different transcripts.

Activator RBPs binding to enhancer elements are shown as arrows, and repressors binding to silencer elements are shown as inhibitory arrows. Constitutive flanking exons are shown in light green and the alternative exon is shown in dark green.

ESE, exonic splicing enhancer; ESS, exonic splicing silencer; ISE, intronic splicing enhancer; ISS, intronic splicing silencer.



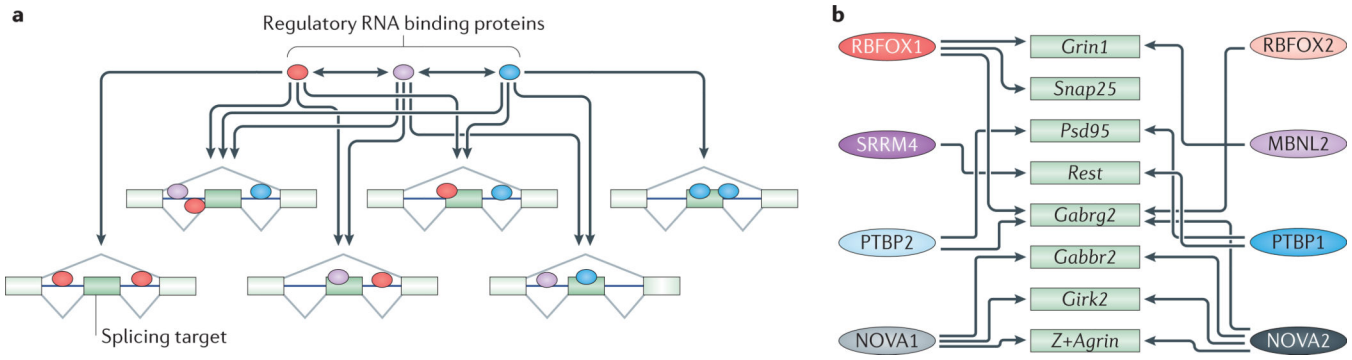


Figure 1. Splicing regulatory networks

a | Splicing regulators control large target exon sets that often overlap with those regulated by other RNA-binding proteins (RBPs). Therefore, splicing of a given alternative exon can be affected by multiple RBPs. RBPs also affect their own splicing and homeostatic expression as well as that of other RBPs. The high degree of cross-regulation (indicated by arrows) between splicing regulators and their target sets creates complex splicing networks where the perturbation of a single RBP can lead to pleiotropic effects. Conversely, the splicing outcome of an exon can result from the combinatorial control of many RBPs. **b** | This figure shows some of the splicing target transcripts discussed in this Review (green boxes) that are cross-regulated (indicated by arrows) by multiple RBPs (coloured ovals on the left and right). *Girk2*, inwardly rectifying potassium channel Kir3.2; *Gabrg2*, $GABA_A$ receptor subunit gamma 2; *Gabbr2*, $GABA_B$ receptor 2; MBNL, muscleblind-like; NOVA, neuro-oncological ventral antigen; *Psd95*, postsynaptic density protein 95; PTBP, polypyrimidine tract binding protein; *Rest*, repressor element 1-silencing transcription factor; RBFOX, RNA-binding protein fox 1 homologue; *Snap25*, synaptosomal-associated protein 25; SRRM4, serine/arginine repetitive matrix protein 4.

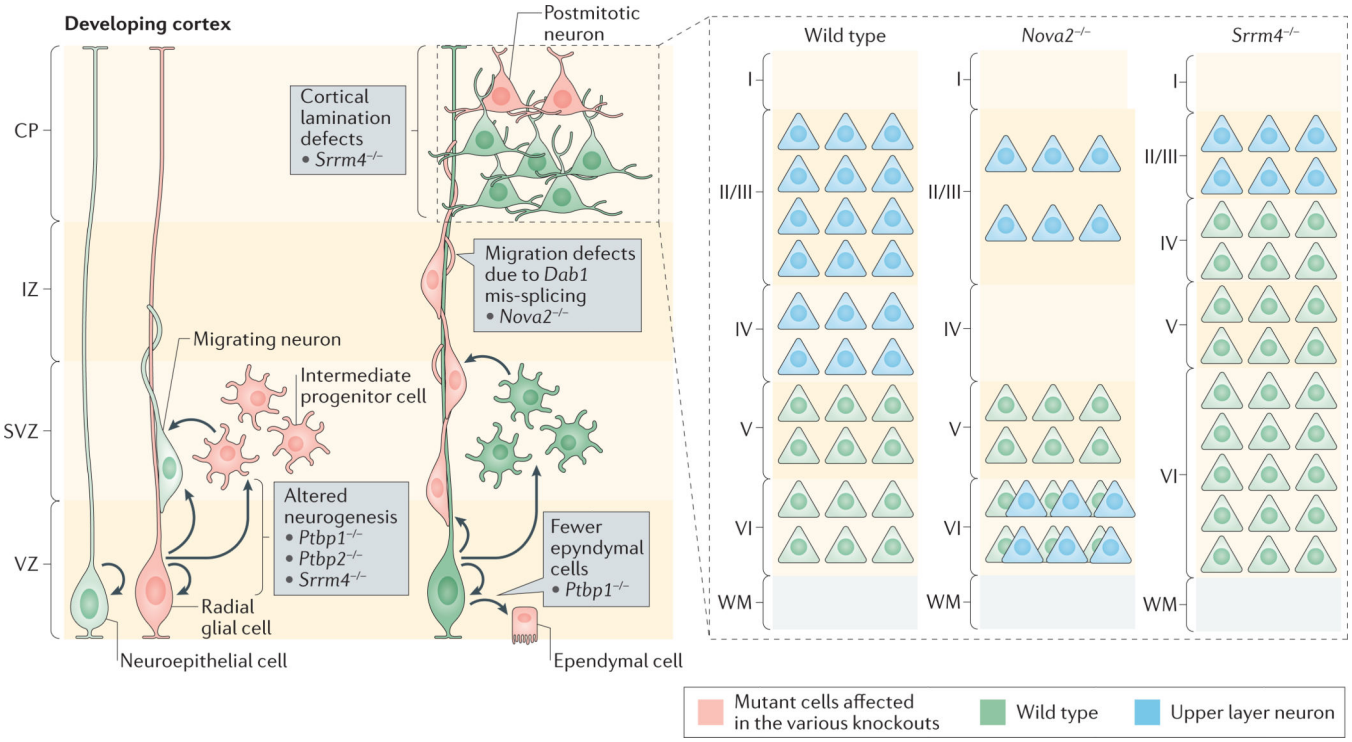


Figure 2. Splicing regulators in cortical development and function

Alternative splicing controls multiple aspects of early neuronal development. Defects in neurogenesis are seen in mouse mutants of a variety of regulatory RNA-binding proteins, including *Ptbp1*$^{-/-}$, *Ptbp2*$^{-/-}$ and *Srrm4*$^{-/-}$. Loss of polypyrimidine tract binding protein 1 (PTBP1) can cause precocious neurogenesis, deplete the neural stem cell pool and lead to fewer ependymal cells arising from radial glia later in development. PTBP2 loss may alter neural stem cell positioning and proliferation. Depletion of serine/arginine repetitive matrix protein 4 (SRRM4) inhibits neurogenesis of upper-layer neurons and causes the accumulation of progenitors or lower-layer neurons, resulting in abnormal cortical lamination (see inset). Defects in cortical lamination are also seen in mice lacking neuro-oncological ventral antigen 2 (NOVA2), where mis-splicing of the Reelin component disabled 1 (*Dab1*) leads to failure of many layer II/III and IV neurons to migrate properly (see inset). C P, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; WM, white matter; VZ, ventricular zone. Figure (left panel) adapted with permission from REF. 199, Elsevier, and from REF. 198: Poduri, A., Evrony, G. D., Cai, X. & Walsh, C. A. Somatic mutation, genomic variation, and neurological disease. *Science* **341**, 1237758–1237758 (2013). Reprinted with permission from AAAS.

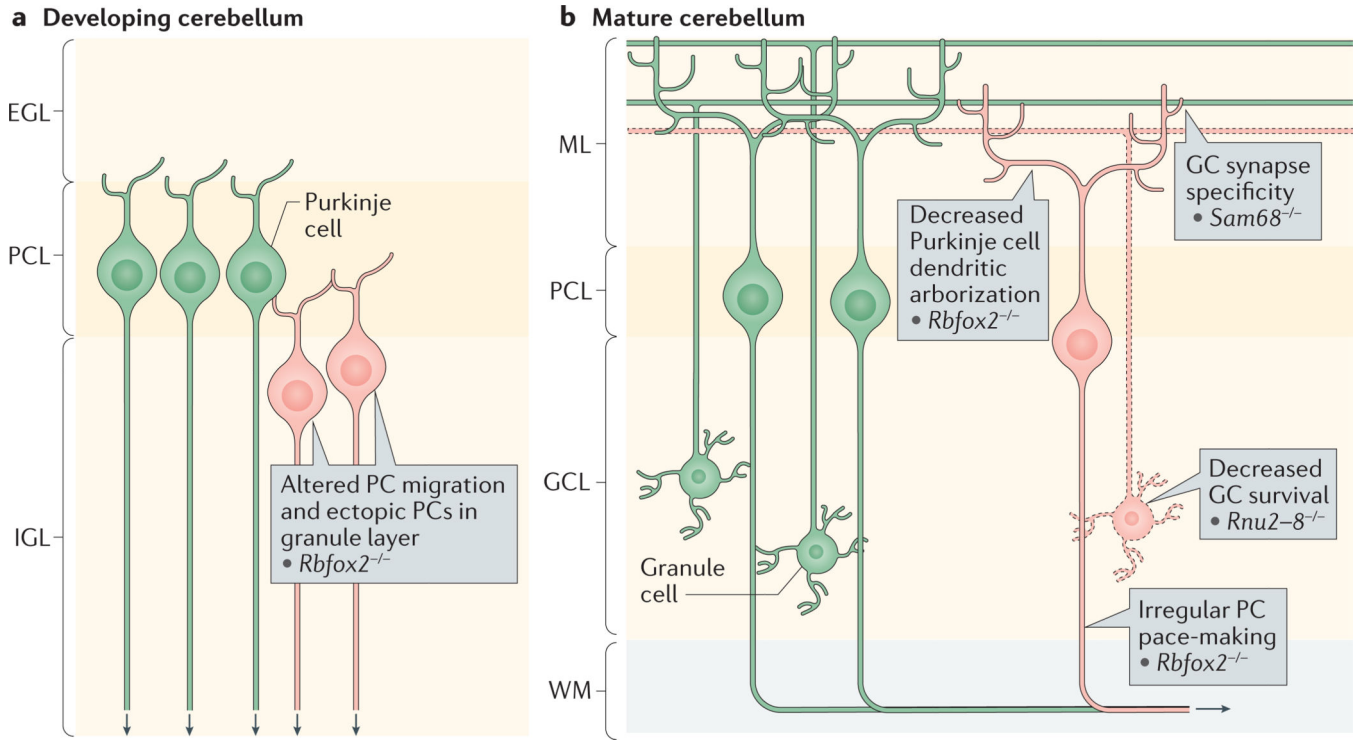


Figure 3. Splicing regulators in cerebellar development and function

a | RNA-binding protein fox1 homologue 2 (RBFOX2) is required for both Purkinje cell (PC) migration and mature function. The cerebellum in *Rbfox2*$^{-/-}$ mouse mutants exhibits a disorganized PC layer (PCL) with ectopic PCs found in the internal granule layer (IGL), as well as reduced PC dendritic arborization later in development. **b** | In mature PCs, RBFOX2 controls the splicing and expression of the sodium channel gene *Scn8a*, which is needed for proper PC pace-making. Splicing regulation is also required for granule neuron survival and proper synaptic specificity. Loss of the U2 small nuclear RNA, a core spliceosomal component that is partially encoded by *Rnu2–8*, leads to increased intron retention and progressive granule neuron death. In granule neurons, SRC-associated in mitosis 68 kDa protein (SAM68) affects trans-synaptic interactions through alternative splicing of *neurexin*. EGL, external granule layer; GC, granule cell; GCL; granule cell layer; ML, molecular layer; WM, white matter.

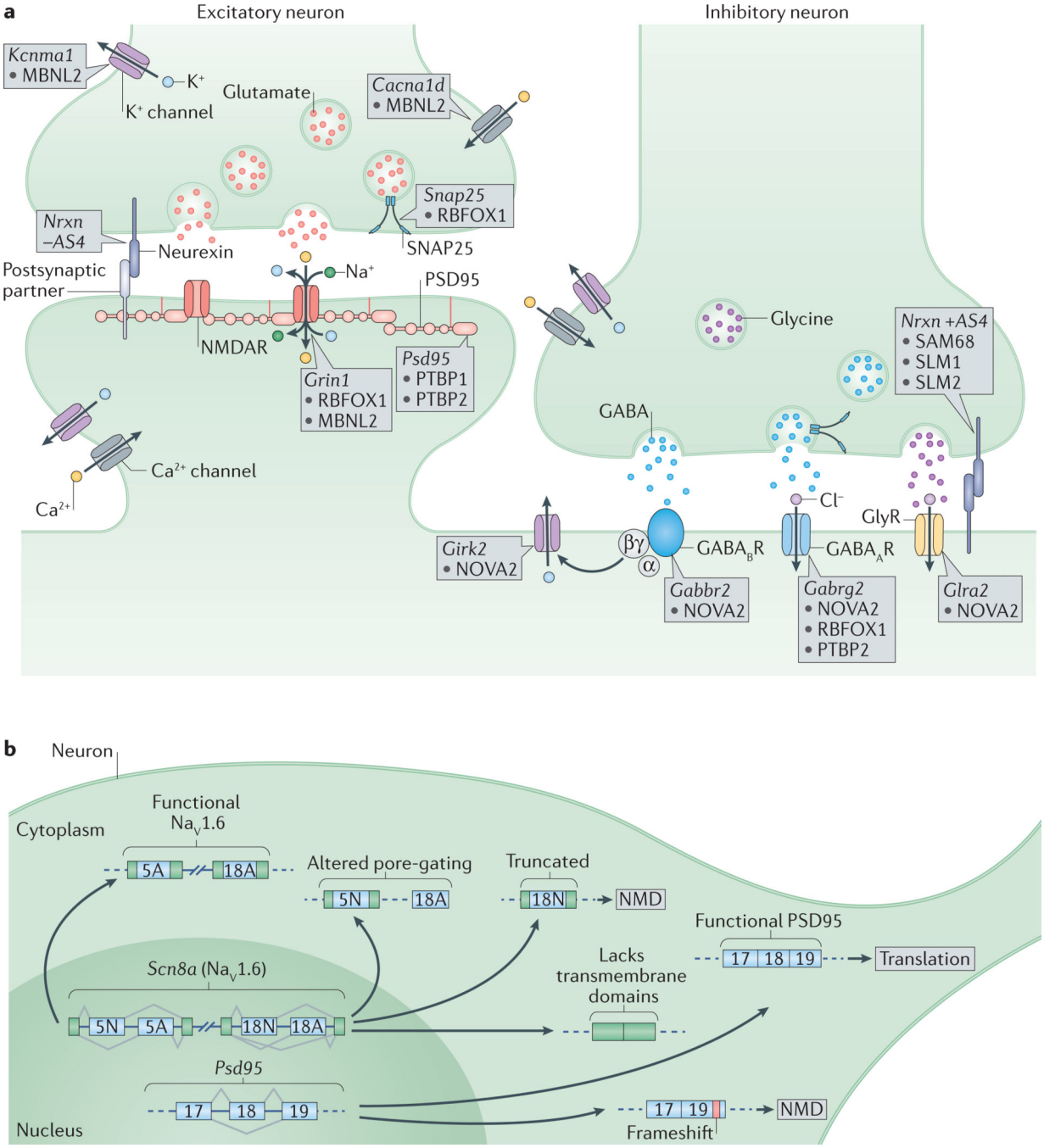


Figure 4. Alternative splicing regulation of synaptogenesis and synaptic function

a |At the presynaptic terminal, alternative splicing of synaptosomal-associated protein 25 (*Snap25*) by RNA-binding protein fox 1 homologue 1 (RBFOX1) and of the calcium-activated potassium channel subunit alpha 1 (*Kcnma1*) by muscleblind-like 2 (MBNL2) are important to control neurotransmitter release. Differential splicing of the presynaptic neurexins (*Nrxns*) at AS4 by KHDRBS proteins (SAM68, SLM1 and SLM2) controls targeting to postsynaptic partners. At excitatory synapses, alternative splicing of the transcript encoding the NMDA receptor subunit GluN1, *Grin1*, is regulated by RBFOX1 and

MBNL2, whereas the polypyrimidine tract binding proteins (PTBPs) control productive splicing of the scaffold protein, postsynaptic density protein 95 (*Psd95*). Splicing of the transcripts encoding L-type voltage-gated calcium channels, such as the pore-forming subunit $Ca_v1.3$ (encoded by *Cacna1d*), by MBNL2 may allow the voltage sensitivity, conductance, or other properties to be tuned as synapses differentiate. At inhibitory synapses, neuro-oncological ventral antigen 2 (NOVA2) mediates alternative splicing of the transcripts encoding many postsynaptic components such as the metabotropic $GABA_B$ receptor (*Gabbr2*), the inwardly rectifying potassium channel Kir3.2 (*Girk2*) and the glycine receptor alpha 2 (*Glra2*). Splicing of the $GABA_A$ receptor subunit transcript (*Gabrg2*) is controlled by multiple splicing regulators including NOVA2, RBFOX1 and PTBP2. **b** | Alternative splicing controls the expression and function of many synaptic components. Expression of PSD95 is repressed by PTBP-controlled exclusion of exon 18 until late in neuronal maturation when it is required for synaptogenesis. The gene encoding the voltage-gated sodium channel $Na_v1.6$, *Scn8a*, has multiple alternative exons (such as 5N, 5A, 18N and 18A as shown in the figure) that can change its gating properties, determine its localization or alter its overall function. $GABA_AR$, $GABA_A$ receptor; $GABA_BR$, $GABA_B$ receptor; GlyR, glycine receptor; NMD, nonsense-mediated decay; NMDAR, NMDA receptor; SAM68, SRC-associated in mitosis 68 kDa protein. Figure adapted from REF. 1, Nature Publishing Group.

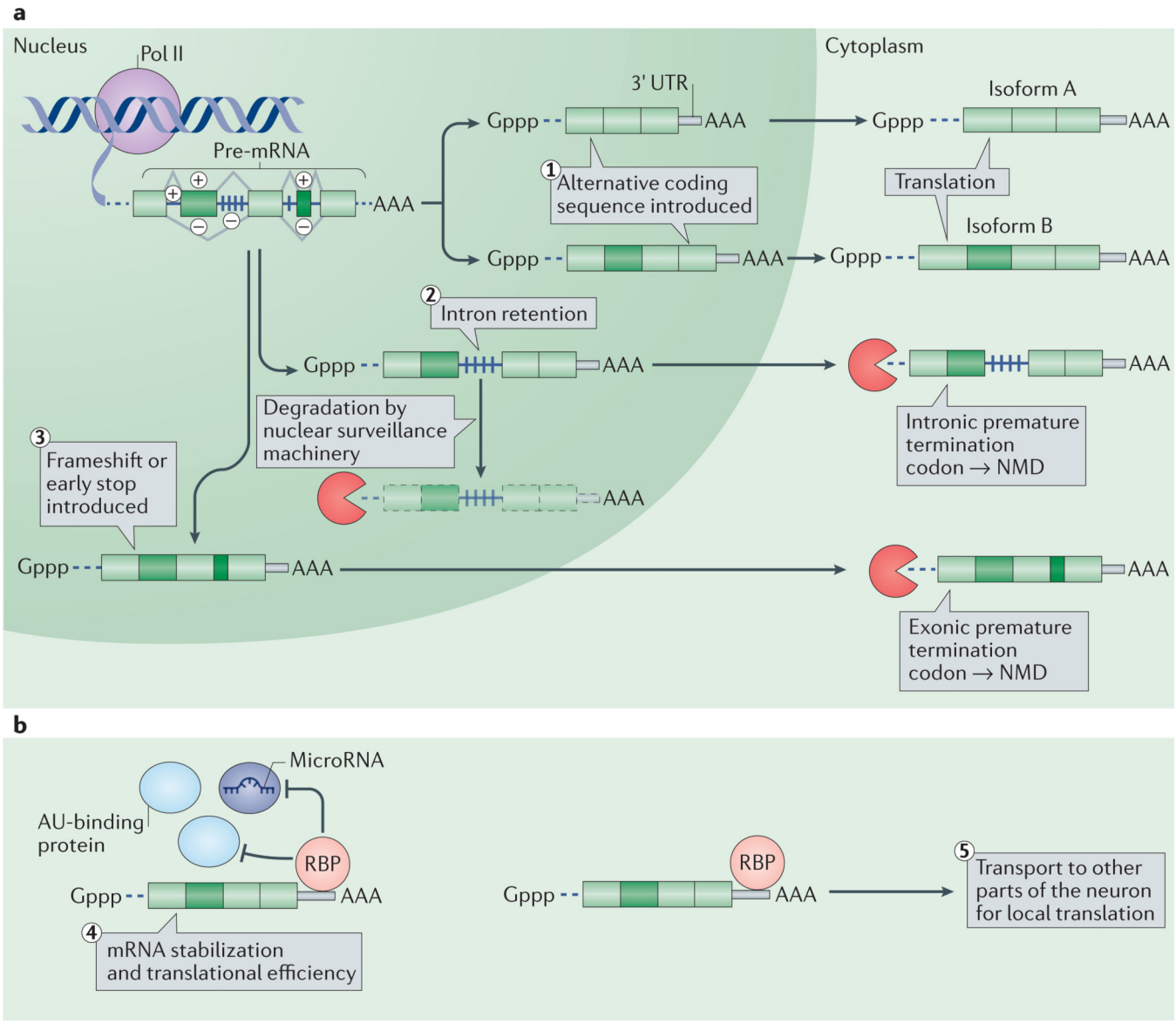


Figure 5. Regulatory outcomes of RNA-binding proteins

RNA-binding proteins (RBPs) regulate transcript splicing, stability, translation and localization, and many RBPs studied as splicing regulators have extended functions in the cytoplasm, affecting every subsequent step of the mRNA life cycle. **a** | Nuclear roles of RBPs. Step 1: RBPs control alternative splicing of precursor mRNA (pre-mRNA) to generate multiple isoforms that differ in functional activity, interactions with cofactors or post-translational modifications. Step 2: regulated intron retention targets transcript isoforms for degradation by nuclear surveillance mechanisms[200,201], or by the introduction of a premature termination codon leading to nonsense-mediated decay (NMD, shown in red; see below). Step 3: by shifting the reading frame or by including a 'poison exon' containing a premature translation termination codon, alternative splicing produces transcript isoforms that are degraded by NMD[202]. Alternative splicing coupled with NMD can control the overall abundance of gene transcripts[203]. **b** | Cytoplasmic roles of RBPs. Step 4: RBPs

compete with AU-binding proteins for binding at AU-rich elements in the 3′ untranslated region (UTR) to stabilize their target transcripts[110,111]. Other RBPs regulate transcript stability in the cytoplasm by either competing with microRNAs for their binding sites or facilitating microRNA binding[131,204,205]. RBP binding in both the 5′ UTRs and 3′ UTRs also affects translational efficiency[206]. Step 5: RBPs regulate the transport and differential localization of mRNA, which are crucial for spatial and temporal control of translation in response to activity-dependent signalling[162,207,208]. The establishment of neuronal polarity and consolidation of synaptic strength through local translation of mRNAs in response to synaptic activity are some well-known examples. AAA, poly-A tail; Gppp, 5′ cap; Pol II, RNA polymerase II.