Functional consequences of developmentally regulated alternative splicing

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Abstract | Genome-wide analyses of metazoan transcriptomes have revealed an unexpected level of mRNA diversity that is generated by alternative splicing. Recently, regulatory networks have been identified through which splicing promotes dynamic remodelling of the transcriptome to promote physiological changes, which involve robust and coordinated alternative splicing transitions. The regulation of splicing in yeast, worms, flies and vertebrates affects a variety of biological processes. The functional classes of genes that are regulated by alternative splicing include both those with widespread homeostatic activities and those with cell-type-specific functions. Alternative splicing can drive determinative physiological change or can have a permissive role by providing mRNA variability that is used by other regulatory mechanisms.

Transcriptomes
The transcriptome technically refers to all of the RNA in a cell; however, the term is often used to describe the polyadenylated RNAs transcribed by RNA polymerase II, which are selected for analysis by oligo(dT).

RNA sequencing
(RNA-seq). High-throughput shotgun sequencing of cDNA to obtain the sequence of the transcriptome.

One of several recent advances towards understanding regulated gene expression is the discovery of the high level of mRNA complexity that is generated by alternative splicing within metazoan transcriptomes. Recent estimates based on RNA sequencing (RNA-seq) are that 90%, 60% and 25% of genes in humans, *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively, undergo alternative splicing[1–5]. Alternative splicing is the most prominent of several mechanisms generating mRNA structural complexity; others include alternative transcription initiation, RNA editing and alternative poly(A) site selection[6,7]. The predicted outcomes of this complexity are: extensive proteome diversity; introduction of premature termination codons (PTCs), which causes mRNA downregulation by nonsense-mediated decay (NMD); and variability in mRNA UTRs, which affects *cis*-acting elements that mediate regulation of mRNA translation efficiency, stability and localization[6,8,9].

The prevalence of alternative splicing raises questions about its biological importance. What fraction of the multiple mRNA isoforms expressed from each of the ~19,000 alternatively spliced human genes has a functional impact? Much of the mRNA diversity observed includes low-abundance transcripts that arise from alternative splicing events that are not conserved, suggesting a high level of stochastic noise[10,11]. Although splicing events that undergo transitions during a physiological change are suggestive of functional consequences, most splicing transitions do not completely switch mRNA isoforms but rather produce a change in the ratios of the isoforms expressed. Often the changes are rather small, and it is difficult to discern whether there are functional consequences. Even for robust transitions, detailed experimental analysis of individual isoforms is required to ascertain whether the transition is a determinative event or a 'fine-tuning' event. However, it is clear that alternative splicing produces determinative biological effects, as seen in the long-standing examples of determination in *D. melanogaster*[12,13], production of functionally distinct peptide hormones in mammals[14] and the meiotic developmental program in budding yeast[15]. The fraction of alternative splicing that has a biological impact is currently difficult to estimate. It is likely that a large fraction of mRNA diversity has no detectable function within an organism, although some of it provides fodder for the derivation of functional splice variants on an evolutionary timescale[16,17]. However, the new awareness of alternative splicing prevalence has resulted in increased investigation and identification of a rapidly growing number of physiologically important splicing events.

Here we discuss regulated alternative splicing networks that are operative during development, differentiation or in response to cell stress in a variety of organisms. The mechanisms of splicing regulation have been covered in several excellent reviews[6,8,18–22].

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Our focus is on the physiological outcomes of alternative splicing transitions. We also address broad questions relating to alternative splicing regulation: what are the crucial splicing transitions that are relevant to a developmental program or physiological response? Which RNA-binding proteins are determinative for splicing transitions? How are the activities of the regulators modulated to mediate the transition (for example, change in protein abundance, intrinsic activity or intracellular localization)? What are the 'upstream' signalling pathways that control the activities of these splicing regulators? How are the networks integrated with parallel transcriptional and post-transcriptional regulatory programs? And what are the most important 'downstream' functions performed by the regulated splicing transitions? We discuss specific examples that illustrate the range of scenarios in which splicing transitions have a potential impact and the principles of the regulatory systems that control these transitions.

Global control of alternative splicing

Alternative splicing is primarily regulated by RNA-binding proteins that bind precursor mRNAs near variably used splice sites and modulate the efficiency of their recognition by the basal splicing machinery (the spliceosome). Large-scale quantification of alternative splicing combined with genome-wide identification of *in vivo* binding sites of splicing regulators provides an unprecedented global view of splicing regulatory networks[23] (BOX 1). The large-scale analysis of alternative splicing has recently progressed from comparisons of generally static cell populations to transitions in mRNA complexity that are associated with physiological change. The results reveal that mRNA structural complexity is not only extensive but is also highly dynamic. For example, recently generated transcriptome data sets for 27 stages of *D. melanogaster* development[1] and 17 growth stages and conditions in *C. elegans*[4,5] revealed that a large fraction of alternative splicing (>60% and >30%, respectively) undergoes developmental changes, which often occurs in coordinated sets that are suggestive of co-regulated networks. Studies that used transgenes expressing fluorescent proteins to indicate different splicing outcomes in *C. elegans* have revealed developmental transitions in real time and provide a genetic approach for identifying the regulators[24,25]. Large-scale analyses of alternative splicing in mammals have identified coordinated splicing within genes enriched for specific functions in different tissues (TABLE 1).

There are several emerging themes regarding large-scale alternative splicing transitions during periods of physiological change. First, any given physiological change that is associated with a transcriptional transition is likely to have a co-integrated post-transcriptional response, including coordinated alternative splicing transitions. Second, as noted above, subsets of splicing transitions undergo distinct temporally coordinated transitions that are suggestive of co-regulation by different sets of splicing factors. Third, a large fraction of the splicing transitions that are associated with a physiological change are conserved. For example, more than 40% of splicing transitions observed during heart development or skeletal muscle differentiation in mice were conserved in birds, not only in terms of the alternatively spliced region but also with regard to splicing pattern, the direction of the change and timing of the transitions[26,27]. For most of these events, the coding potential is also conserved, such that the homologous protein isoforms undergo transitions with the same timing relative to birth or hatching, strongly suggesting functional importance. These results are in stark contrast to genome-wide comparisons in which less than 20% of alternative splicing events were conserved between humans and mice[28,29], emphasizing the role for splicing in physiological transitions. Fourth, genome-wide studies in which transitions in both alternative splicing and mRNA expression levels were simultaneously analysed identified two separate gene sets[26,27,30,31]. One gene set showed changes in mRNA levels without a difference in the mRNA splice variants expressed. The second set showed a change in the mRNA splice variants without a change in total expression. Although splicing and transcriptional regulation are linked[18], the results indicate that different genes can be primarily regulated either at the level of splicing or at the level of expression. This has expanded the view of regulated gene expression to include transitions in the complexity of the mRNA and protein isoforms that are expressed from individual genes, as well as changes in overall gene output.

Alternative splicing in cell division

Meiotic splicing regulatory programs. Meiosis in the budding yeast *Saccharomyces cerevisiae* is driven by a well-characterized transcriptional program that was recently found to be intricately linked to multiple splicing regulatory programs. Only 5% of genes in budding yeast contain introns (290 of 6,000 genes). However, introns are enriched in highly expressed genes and more than one-quarter of the ~38,000 RNAs transcribed per hour during vegetative growth are spliced[32]. With a few exceptions[33], alternative splicing in yeast is limited to splice versus do not splice decisions that relate to intron retention in single-intron genes. Large-scale analyses that used tiling- and splicing-sensitive microarrays have determined that 45 intron-containing genes are inefficiently spliced during vegetative growth[34,35]. These include 13 of the 20 intron-containing meiotic genes that specifically undergo efficient splicing during the meiotic cycle[34,35]. Meiosis-specific alternative splicing in budding yeast has been known for two decades[15], but recent investigations have revealed at least three separate but overlapping meiotic splicing regulatory programs[35,36]. The most well-characterized meiotic splicing regulatory program is regulated by *MER1*, which encodes an RNA-binding protein (Mer1) that is transcriptionally induced during initiation of the meiotic cycle[37]. The primary function of Mer1 is to activate splicing of four single-intron genes, thereby inducing their expression during meiosis[35] (FIG. 1). The Mer1 target genes perform diverse functions, including chromosome pairing, recombination and cell cycle control. Inefficient splicing of each gene during vegetative growth is due to suboptimal

mRNA structural complexity
The number and ratio of different transcripts produced from each gene. It is one component of mRNA complexity, along with the number of genes that produce transcripts and the abundance of the transcripts from each gene.

Nonsense-mediated decay
(NMD). An mRNA surveillance mechanism that degrades mRNAs containing nonsense mutations to prevent the expression of truncated or erroneous proteins.

Spliceosome
The complex and conserved nuclear machinery that removes introns. The spliceosome contains five small uridylate-rich small nuclear RNAs (UsnRNAs) and ~150 proteins.

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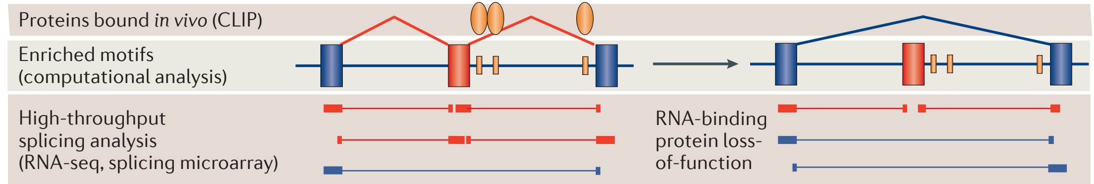
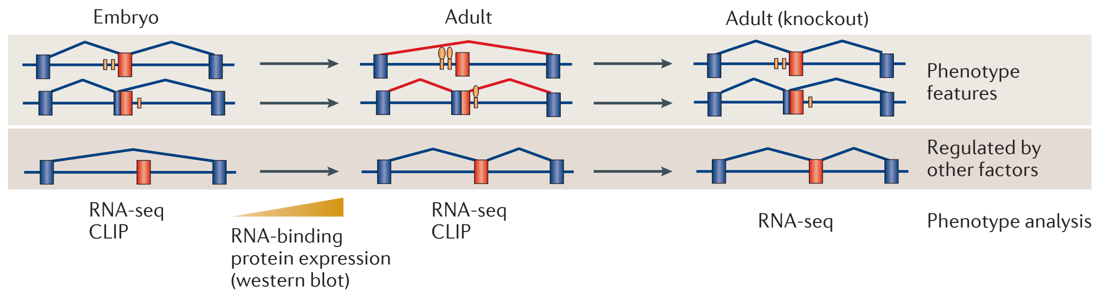
Box 1 | Identification of splicing regulatory networks

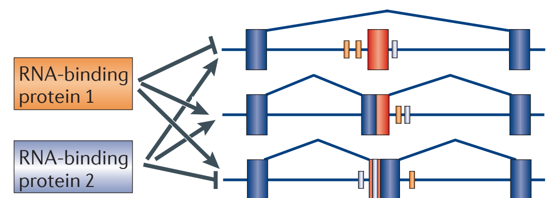
a Identify alternative splicing events that are responsive to a specific RNA-binding protein



b High-throughput approaches identify developmental alternative splicing transitions directly regulated by specific RNA-binding proteins



c Combined results from multiple RNA-binding proteins



A splicing regulatory network can be defined as the set of alternative splicing events that are directly regulated by an individual RNA-binding protein. RNA-binding proteins recognize preferred 5- to 8-nucleotide sequence motifs located either in the regulated exon or in the flanking introns, commonly within 300 nucleotides of the regulated exon or the upstream or downstream constitutive exons[144] (panel **a** of the figure; motifs shown as orange bars, proteins shown as orange ovals). Approximately 50 mammalian RNA-binding proteins that act as auxiliary splicing regulators separately from the basal splicing machinery have been characterized and have been shown to regulate splicing directly by binding to precursor mRNAs[8,145]. Splicing events that are sensitive to the loss- or gain-of-function of the splicing regulator (by RNAi, genetic knockout or overexpression) are identified using large-scale analyses such as RNA sequencing (RNA-seq; depicted in the figure) or splicing-sensitive microarrays[146]. Hundreds of splicing events can be sensitive to changes in the level of a single splicing regulator. A portion of the responsive events are directly regulated by protein–RNA binding and others change because of secondary effects. To identify direct targets, bound protein is covalently linked to the RNA *in vivo* by ultraviolet crosslinking; this is followed by immunoprecipitation and then specific binding sites are identified by high-throughput sequencing[147]. Although crosslinking followed by immunoprecipitation (CLIP) produces false negatives and positives[148], it is a highly effective screen for target identification. The massive data sets from these assays are managed and extensively analysed computationally for genome-wide identification of splicing events that are both sensitive to the splicing regulator levels and are associated with local *in vivo* binding sites[149].

High-throughput splicing analyses applied to normal physiological transitions have demonstrated roles for alternative splicing networks in a variety of cellular responses. In mammals, the combined results from RNA-seq or splicing microarrays, CLIP, genetic knockouts and computational analysis have identified networks of tissue specific as well as developmental alternative splicing programs that are regulated by specific RNA-binding proteins (for example, REFS 26,109,150). Despite the large number of splicing events that are dependent on individual RNA-binding proteins, careful analysis can reveal specific features of a complex knockout phenotype that are due to loss of individual alternative splicing events[107,108] (panel **b**).

Individual splicing events are regulated by cooperative as well as antagonistic effects of multiple RNA-binding proteins. The multiple inputs integrate the effects from diverse external cues to promote an appropriate splicing response (panel **c**). Combining experimental data from multiple splicing regulators with computational analyses of large numbers of features has been used to define splicing codes with predictive capabilities for either cell-specific splicing or responsiveness to a specific splicing regulator[102,144].

splice sites at the intron–exon boundaries. Mer1 binds to an enhancer element within the intron of each of the four genes and promotes spliceosome assembly by direct interactions with Nam8, a spliceosomal component[38,39].

The *MER1* splicing network serves a crucial role in the transition from early meiotic genes, which are transcriptionally regulated by *UME6*, to the middle meiotic genes, which are regulated by *NDT80* (REF. 35) (FIG. 1).

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Table 1 | **Functional enrichment in genes undergoing alternative splicing or transcriptional changes during physiological transitions***

Tissue type, cell type or process	Gene ontology terms enriched in genes regulated through alternative splicing	Gene ontology terms enriched in genes regulated through transcript levels	Refs
Brain or neural tissue	GTPase-based signalling, cell–cell signalling, cytoskeletal organization and biogenesis, vesicular-mediated transport, transmission of nerve impulse and neurophysiologic processes	Synaptic function, nerve impulse and transmission, nervous system development, cytoskeletal organization and biogenesis and secretory pathways	163
Heart development	Cell structure and motility, cytoskeletal remodelling, cell signalling, RNA splicing, muscle specification, excitation–contraction coupling and cell cycle control	Signal transduction and oxidative (lipid and steroid) metabolism, cell adhesion, cytoskeletal organization and biogenesis, nucleic acid metabolism and cell signalling	26
Skeletal muscle differentiation	Cytoskeletal organization, actin binding, cell junction and nucleotide kinase, integrin signalling pathway, nucleic acid metabolism and RNA splicing	Muscle contraction, muscle development, cytoskeletal organization, cell signalling, cell cycle, transcription, nucleic acid and protein metabolism, cell adhesion and ion transport	27, 164
Epithelial-to-mesenchymal transition	Cytoskeleton structure, cell adhesion, polarity, cell migration and RNA splicing	Cell cycle inhibition, apoptotic inhibition, cytoskeletal organization and biogenesis, cell structure and motility and cell adhesion	77, 165
T cell activation	Interphase of mitotic cell cycle (affected early); cell division (affected late)	Cell adhesion, immune defence response, cytoskeletal protein binding (affected early); cell cycle (affected late)	30
Ca^{2+}-induced cell excitation	Cell signalling (affected early); RNA splicing, transcription, cell cycle, apoptosis, lipid and carbohydrate metabolism (affected late); Ca^{2+} binding, cell adhesion, plasma membrane, and extracellular matrix (affected throughout the time course)	Lipid and carbohydrate metabolism (affected late); transcription, Ca^{2+} binding and retrograde vesicle-mediated transport from the Golgi to the ER (affected throughout the time course)	31

*Several studies probing alternative splicing and steady-state mRNA level changes during physiological transitions have found that genes undergoing alternative splicing and/or transcript level changes are enriched in an overlapping yet distinct set of gene ontology annotation terms.

SR proteins
A highly conserved family of RNA-binding proteins that contain arginine/serine-rich domains. They function in constitutive as well as alternative splicing and are primarily splicing activators.

CLIP
A biochemical technique that uses ultraviolet crosslinking of protein and RNA *in vivo* followed by immunoprecipitation to identify direct protein–RNA interaction sites in living cells.

Analysis of *NAM8*-null strains identified intron retention of the four *MER1*-dependent genes — a finding that is consistent with the mechanism of Mer1 activation — as well as two genes that are not dependent on *MER1* for meiosis-specific splicing[35,40]. Another study found two genes for which meiosis-specific splicing requires trimethylguanosine synthase 1 (*TGS1*), which synthesizes the specialized 2,2,7-trimethylguanosine cap at the 5′ end of small nuclear RNAs (snRNAs) — the snRNAs are essential components of the spliceosome[41]. Splicing for one *TGS1*-dependent gene is also *NAM8*-dependent, indicating the existence of multiple overlapping networks (FIG. 1). Unlike the situation for *MER1*, expression levels of *NAM8* and *TGS1* do not substantially change during meiosis, suggesting that, although they are required, these genes are not the primary determinants of meiosis-specific splicing. It is also unclear what regulates meiosis-specific splicing of the six remaining genes that are not dependent on *MER1*, *NAM8* or *TGS1*. Even the moderately straightforward *MER1* splicing regulatory network contains multiple splicing subnetworks and is interlinked with the meiotic transcriptional program. As such, it is an instructive paradigm for metazoan splicing regulatory programs.

Cell cycle control and apoptosis. A high fraction of mammalian apoptotic regulators — including death receptors, adaptors, caspases and caspase targets — are alternatively spliced to produce dramatic biological outcomes[42]. In particular, splicing of the BCL-2 family of apoptosis regulators (including BCL-2, BCL-X and MCL1) yields long and short isoforms to provide anti-apoptotic versus pro-apoptotic functions, respectively[42]. A recent genome-wide RNAi screen using BCL-X- and MCL1-splicing reporters established coordinated alternative splicing as an integral component of cell cycle control. The study found that knockdown of 52 genes induced pro-apoptotic splicing of both reporters. The list of genes included a network of factors linked to the cell cycle regulator, aurora kinase A, a central regulator of mitosis[43]. Loss of aurora kinase A promoted post-translational degradation of serine/arginine-rich splicing factor 1 (SRSF1), a member of the SR protein family of splicing regulators[44]. Results from CLIP analysis (BOX 1) showed that SRSF1 directly binds *BCLX* and *MCL1* pre-mRNAs, as well as revealing additional endogenous apoptotic splicing events that shifted towards pro-apoptotic splicing upon cell cycle inhibition. Cell cycle inhibitors were previously known to induce pro-apoptotic splicing; however, an important new finding from this study was that the splicing response preceded mitotic arrest, indicating that the splicing change is induced in parallel with, rather than as a secondary response to, mitotic arrest. The indications are that SRSF1, which promotes anti-apoptotic splicing patterns, is integrated in decisions of whether to continue through the cell cycle or undergo apoptosis. SR proteins have an established relevance to cell cycle control and oncogenesis[45–47], including the recent demonstration that an SR-related protein (SON) is required for efficient splicing of cell-cycle-regulated genes[48]. Furthermore, the expression of all SR protein genes is maintained under strict homeostatic control by an ancient mechanism involving alternative splicing coupled with NMD (AS–NMD) (BOX 2).

Splicing in cell fate decisions

Stem cell self-renewal and differentiation. Embryonic stem cells (ESCs) are pluripotent cells that can proliferate

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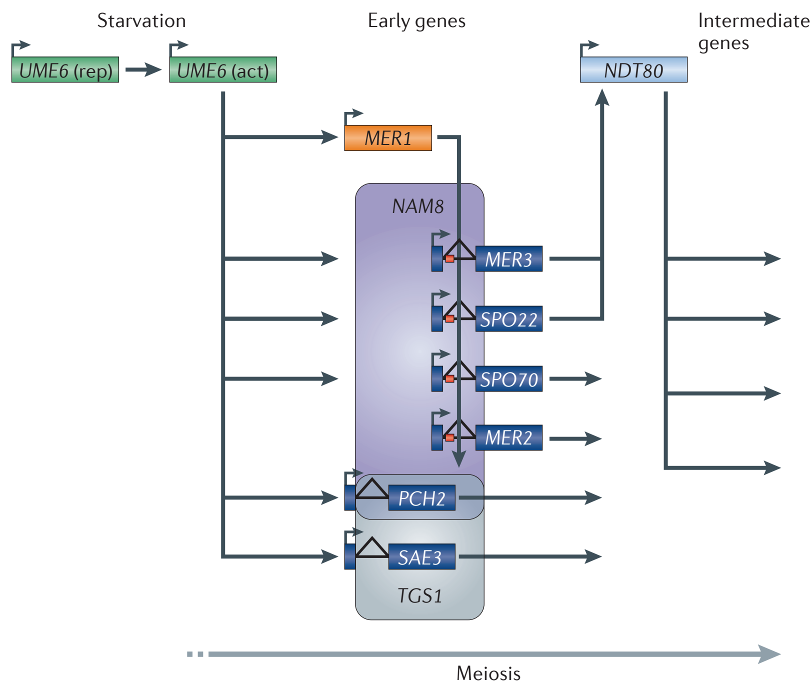

Figure 1 | **Role of splicing regulation during early meiosis in *Saccharomyces cerevisiae*.** Starvation induces a switch in the Ume6 transcription factor from a repressor (*UME6* (rep)) in vegetative cells to an activator (*UME6* (act)) to initiate meiosis during sporulation. Ume6 activates multiple early meiotic genes including *MER1* and 13 intron-containing genes. *MER1* encodes an RNA-binding protein that binds a motif near the 5′ splice site (orange box) and activates splicing of four intron-containing genes specifically during meiosis. Two Mer1 targets, *MER3* and *SPO22*, are required for activation of *NDT80*, the transcriptional regulator of intermediate meiotic genes. Transcriptional regulation of *MER1* and three of its four targets by *UME6* delays expression of Mer1-regulated genes compared to other *UME6* targets (for example, *PCH2* and *SAE3*), creating a lag period prior to *NDT80* activation[35]. Mutation analyses demonstrated separate but overlapping meiotic-dependent splicing requiring *MER1*, *NAM8* (purple shaded area) or *TGS1* (grey shaded area)[41].

Core pluripotency factors
A set of transcription factors (including OCT4, NANOG, SOX2 and TCF3) that form a core transcriptional circuit to maintain the pluripotent state of embryonic stem cells.

MicroRNA
(miRNA). An evolutionarily conserved small non-coding RNA (~22 nucleotides long) that silences gene expression by degrading or inhibiting translation of mRNA transcripts in a sequence-specific manner.

Epithelial-to-mesenchymal transitions
(EMTs). Phenotypic conversions that disrupt the polarity of epithelial cells to establish invasive mesenchymal features through alterations in cytoskeletal organization, cell adhesion and the extracellular matrix.

indefinitely while retaining the capacity to differentiate into the three germ layers. A number of genome-wide studies have found specific transcriptome changes during differentiation of ESCs into distinct lineages, including the contributions of alternative splicing to cell-fate decisions and pluripotency[49–54]. Splice isoform diversity is high in undifferentiated ESCs, decreases upon their differentiation[49,50] and is enriched in cell cycle, pluripotency, signalling and general metabolic pathways[49,51,52,55,56] (TABLE 1).

Alternative splicing has a central impact in stem cell biology by affecting the core pluripotency factors, which produce functionally diverse splice isoforms that have determinative roles on cell state. The pluripotent state of ESCs is maintained by a core set of transcription factors — OCT4 (also known as POU5F1), NANOG, SOX2 and transcription factor E2α (TCFE2α; also known as TCF3) — that induce and crossregulate a network of target genes that are involved in self-renewal and pluripotency[57]. The *OCT4* gene encodes a POU domain transcription factor and produces functionally distinct protein isoforms at specific stages of ESC differentiation[58,59]. Three OCT4 isoforms have been identified: OCT4A, OCT4B and OCT4B1 (REF. 58). OCT4A contains amino- and caboxy-terminal transcription transactivation domains and a POU domain. OCT4B has a different N-terminal transactivation domain from that of OCT4A and a shortened POU domain. Whereas OCT4A target genes are responsible for stemness[57,59], OCT4B cannot sustain ESC self-renewal and instead targets genes that are responsive to cell stress[58,60]. The expression and function of OCT4B1 remains to be determined[58]. The *OCT4* paralogue *OCT2* is also alternatively spliced to generate isoforms that can either activate or repress neuronal differentiation. The OCT2.4 isoform lacks the C-terminal transcription transactivation domain and suppresses neuronal differentiation[61]. During differentiation, multiple splice variants containing the C-terminal transactivation domain are expressed, and one of these isoforms in particular, OCT2.2, induces neuronal differentiation[61].

Alternative splicing of several other genes in addition to the core pluripotency factors is linked to stem cell self-renewal and lineage specification[62–66], but little is known about the regulatory factors that dictate these splicing outcomes. A group of recent studies provides important insights into how splicing factors may influence neural differentiation from ESCs or neural progenitors. Exons that are alternatively spliced in human ESCs[52] are associated with conserved binding sites of the RNA-binding protein fox (RBFOX) family of splicing regulators. This result is consistent with findings that RBFOX2 is highly expressed, regulates a large splicing network and is essential for viability of human ESCs, but not of differentiated stem cells or transformed cell lines[64]. Another splicing factor, polypyrimidine tract-binding protein (PTB), prevents differentiation of a proliferative neural cell line by repressing expression of its neuronal homologue nPTB[67–68]. It does this by directly promoting skipping of nPTB exon 10 in non-neuronal cells, thus introducing a PTC, which results in nPTB mRNA degradation by NMD. Neural-specific expression of nPTB is due to silencing of PTB by a neuronal-enriched microRNA (miRNA), miR-124 (REF. 69), establishing a first example of a regulatory hierarchy from miRNAs to splicing networks. A neuron-specific SR protein, nSR100, participates in this network by promoting the inclusion of nPTB exon 10 to increase nPTB expression, as well as functioning as a co-regulator with nPTB, in a complex regulatory relationship[21,70–71].

The identification of lineage-specific splice variants, their *cis*-acting elements and their *trans*-acting regulators will refine our understanding of how alternative splicing integrates with the transcriptional and post-transcriptional networks of ESCs. It will be particularly interesting to determine whether, like transcription[72], alternative splicing is also reset when somatic cells are reprogrammed to pluripotency.

Epithelial-to-mesenchymal transitions. Phenotypic conversions of cells between epithelial and mesenchymal states, known as epithelial-to-mesenchymal transitions (EMTs) and mesenchymal-to-epithelial transitions (METs), are fundamental to organ morphogenesis and tissue remodelling during embryonic development[73].

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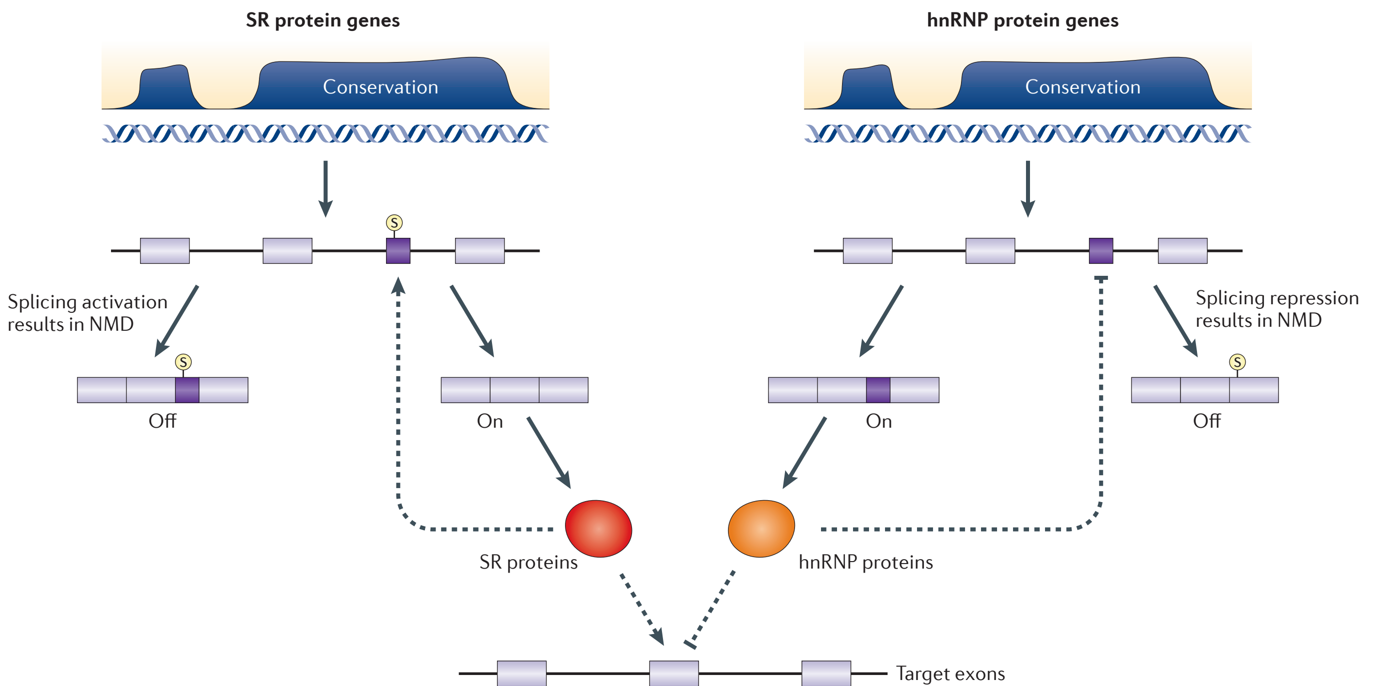
Box 2 | **AS–NMD produces homeostatic control of splicing regulators**



Alternative splicing has a particularly broad physiological impact by maintaining homeostatic levels of splicing regulators as well as spliceosome components through coupling of alternative splicing with nonsense-mediated decay (AS–NMD)[151–154]. NMD is a conserved and multifunctional surveillance mechanism for degrading mRNAs that contain premature termination codons (PTCs)[9]. A general rule for mammalian cells is that spliced mRNAs containing a termination codon located >50 nucleotides upstream of the position of the last intron is degraded by NMD[155]. In AS–NMD, alternative splicing controls an NMD signal by insertion or removal of an mRNA segment that either contains a PTC or introduces a downstream PTC owing to a frameshift. Most splicing regulators affected by AS–NMD autoregulate homeostatic levels by promoting a splicing pattern that results in NMD and downregulation. Splicing regulators also use AS–NMD for cross-regulation, such as the repression of neuron-specific PTB (nPTB) in non-neuronal cells by its paralogue, PTB[68].

The genes encoding the SR protein families and heterogeneous nuclear ribonucleoprotein (hnRNP) families present a striking example of homeostatic control by AS–NMD with pervasive biological and evolutionary implications[153,154]. SR and hnRNP proteins are a long-standing paradigm for antagonistic splicing regulators affecting multiple and diverse alternative splicing events[156]. All SR protein genes and many hnRNP protein genes use AS–NMD for negative autoregulatory feedback. Strikingly, the regions of the genes that are crucial for the AS–NMD 'on–off' switch are within ultraconserved elements. Furthermore, autoregulation of the genes within each family promotes negative feedback that is 'polar' with regard to the effects of each family on the splicing pattern[154] (see the figure). In the genes for SR proteins, which predominantly (although not exclusively) activate splicing, activation of an alternative splicing event results in NMD-mediated downregulation, whereas in genes of hnRNP proteins, which are most often splicing repressors, repression of a splicing event results in NMD-mediated downregulation. The full importance of this ancient regulatory network remains to be elucidated; however, the implication is that maintaining the appropriate levels of both protein families is a crucial component of cellular homeostasis for a broad spectrum of cell types and across metazoan phylogeny. Consistent with this, alterations of each protein family have been associated with disease-causing aberrant splicing[45,157,158]. The figure is reproduced, with permission, from REF. 154 © (2007) Cold Spring Harbor Press.

These *trans*-differentiation events are required for physiological responses to injury and wound healing in adult tissues, and they have roles in pathological responses, such as fibrosis and metastasis[74]. At a cellular level, EMTs are characterized by a loss of epithelial features, including cell adhesion and polarity, and acquisition of mesenchymal features, such as motility and invasiveness[75].

Alternative splicing has a determinative role in EMTs by regulating multiple splicing events and using several regulatory proteins[76–79]. In a high fraction of breast cancer cell lines, SRSF1 upregulation triggers EMTs through alternative splicing of the *RON* (also known as *MST1R*) tyrosine kinase receptor proto-oncogene to produce a constitutively active, pro-invasive isoform, ΔRON[76]. Induction of EMTs by activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 (REF. 80) proceeds, in part, through phosphorylation of its substrate SAM68 (also known as KHDRBS1), which then upregulates SRSF1 by inhibiting AS–NMD-mediated downregulation (FIG. 2a).

Downregulation of the RNA-binding proteins epithelial splicing regulatory protein 1 (ESRP1) and ESRP2 is also determinative for physiological EMT-splicing changes. Splicing-sensitive microarrays were used to identify nearly 100 splicing events that displayed reciprocal changes in epithelial cells that were depleted of ESRP1 and ESRP2 or mesenchymal cells expressing ectopic ESRP1 (REF. 77). A large fraction of these contained an ESRP-binding motif that defined a position-dependent effect on splicing. The ESRP target genes were enriched

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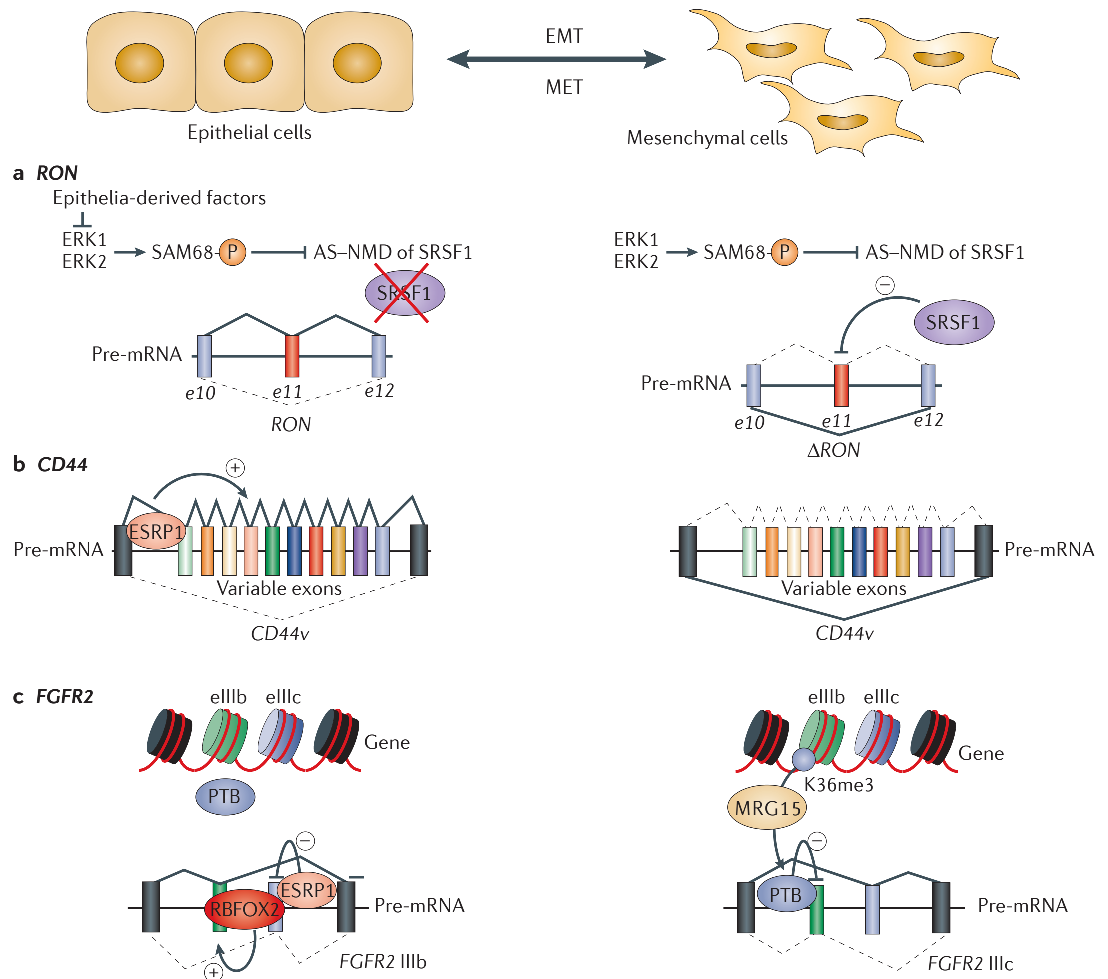

Figure 2 | **Integration of alternative splicing with epithelial-to-mesenchymal transitions.** The scheme at the top represents epithelial and mesenchymal interconversion. Epithelial (left) and mesenchymal (right) splicing that directly influences an epithelial-to-mesenchymal transition (EMT) is depicted in the three panels below. **a** | Serine/arginine-rich splicing factor 1 (SRSF1) triggers EMT by promoting skipping of exon 11 of the *RON* proto-oncogene to produce a constitutively active isoform (ΔRON) that confers an invasive phenotype[76]. SRSF1 levels are dynamically controlled during EMT through alternative splicing coupled with nonsense-mediated decay (AS–NMD) by another splicing factor, SAM68 (REF. 78). Epithelial cell-derived soluble factors repress extracellular signal-regulated kinase (ERK) activity, thereby inhibiting SAM68 phosphorylation, which reduces SRSF1 levels through increased AS–NMD[78]. **b** | Epithelial splicing regulatory proteins (ESRPs) are key regulators of the epithelial cell splicing network[77]. ESRP1 downregulation leads to a switch from the CD44 variant isoform (CD44v) to the CD44 standard isoform (CD44s) that is crucial for EMT[79]. Knockdown of all CD44 isoforms impaired EMT progression, and the phenotype could be rescued by re-expressing the mesenchymal CD44s isoform but not the epithelial CD44v isoform[79]. CD44s and not CD44v potentiates the AKT serine/threonine kinase activation in transforming growth factor-β (TGFβ)-induced EMT assays, providing a functional link between alternative splicing and a key signalling pathway that drives EMT[79]. **c** | Mutually exclusive splicing of fibroblast growth factor receptor 2 (*FGFR2*) exon IIIb (eIIIb) and eIIIc is regulated by multiple splicing factors in communication with chromatin modifications. ESRP proteins inhibit eIIIc, whereas RNA-binding protein fox 2 (RBFOX2) promotes eIIIb inclusion in epithelial cells[160,161]. Polypyrimidine tract-binding protein (PTB) is similarly expressed in mesenchymal and epithelial cells but specifically suppresses eIIIb in mesenchymal cells[82]. The selective suppression is due to mesenchyme-specific enrichment of the histone H3 trimethylation at lysine 9 (H3K36me3) modification on chromatin near eIIIb. Binding of the adaptor protein MRG15 to the H3K36me3 histone mark recruits PTB near eIIIb, resulting in eIIIb skipping[84]. MET, mesenchymal-to-epithelial transition; pre-mRNA, precursor mRNA.

in functions that support EMTs, such as changes in actin cytoskeleton, cell adhesion, cell migration and cell polarity (TABLE 1). Importantly, sustained ESRP1 and ESRP2 knockdown resulted in global epithelial-to-mesenchymal splicing transitions and EMT-like phenotypic changes. Among ESRP targets, the splice isoforms of the CD44 cell adhesion molecule in particular participate in multiple EMT-relevant functions, including proliferation, adhesion and migration[81] (FIG. 2b).

Fibroblast growth factor receptor 2 (FGFR2) contains two mutually exclusive alternative exons, IIIb and IIIc, that produce distinct ligand-binding specificities in

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epithelial and mesenchymal cells[82] to ensure appropriate signalling for different mesenchymal–epithelial interactions during organogenesis[83]. The studies of FGFR2 in EMTs identified crucial interplay between the histone code, adaptor proteins and alternative splicing regulators to generate cell-type-specific splice isoforms[82–84] (FIG. 2c).

Alternative splicing in tissue maturation

Alternative splicing in heart development. The vertebrate heart is the first organ to both form and begin functioning in the embryo. Cardiac morphogenesis is complete by embryonic day 14.5 (E14.5) in mice, after which growth occurs primarily via hypertrophic pathways (cell growth) rather than hyperplastic pathways (cell proliferation)[85,86] (FIG. 3a). The first 4 weeks after birth are characterized by extensive remodelling of the heart in response to the physiological demands of rapid growth and increased activity of the animal.

A large-scale transcriptome analysis using splicing-sensitive microarrays identified alternative splicing transitions during late embryonic and postnatal mouse heart development[26]. A time course revealed three sets of temporally coordinated splicing transitions[26] (FIG. 3b). Remarkably, more than 40% of the splicing transitions are conserved between mammalian and avian species in terms of the pattern, direction, timing and coding potential of splicing changes, and this is indicative of functionally important embryonic-to-adult protein isoform transitions. The genes exhibiting conserved splicing transitions were enriched for functions that were consistent with heart remodelling (TABLE 1). Interestingly, different gene sets were found to be regulated by two distinct mechanisms: changes in mRNA expression levels or isoform switching without changes in mRNA expression levels[26].

Computational analysis identified substantially enriched and conserved pentamer motifs near the developmentally regulated exons. A subset of motifs matched binding sites for known families of splicing regulators, such as CUGBP Elav-like family (CELF), muscleblind-like (MBNL), RBFOX and PTB. In a manner consistent with roles in regulating postnatal splicing transitions, levels of the two CELF paralogues expressed in the heart, CELF1 and CELF2, decrease more than tenfold during postnatal development, whereas MBNL levels increase fourfold[26,87,88]. Importantly, CELF gain-of-function and MBNL loss-of-function in the hearts of adult mice caused over half of the postnatal splicing transitions to revert to the embryonic pattern, a finding that is consistent with a determinative role for these protein families in driving the postnatal splicing transitions. CLIP analysis will be required to identify the direct targets of these splicing regulators.

MicroRNA-mediated regulation of multiple splicing regulators was revealed by deletion of *Dicer1* in adult cardiomyocytes in mice; this resulted in rapid induction of a subset of splicing regulators (CELF, PTB and RBFOX) and re-expression of large numbers of fetal mRNA splice variants[89]. These findings identify a regulatory hierarchy in which miRNAs and alternative splicing act to coordinate the switch from fetal-to-adult gene expression programs (FIG. 3c).

Transgenic overexpression (in the case of CELF1), genetic knockout (for SRSF1, SRSF2 and SRSF10) or overexpression of a dominant negative mutant (in the case of CELFΔ) of splicing regulators in the heart has a severe impact on heart physiology[26,90–94]. Interestingly, heart-specific deletion of either SRSF1 or SRSF2 beginning at around E8.5 had a delayed effect on postnatal heart development in mice: most mice exhibited splicing abnormalities by 2 weeks of age and developed dilated cardiomyopathy within 8 weeks[92,93] (FIG. 3). SRSF1 is essential for cell viability in culture[95], and a constitutive SRSF1 or SRSF2 knockout is lethal in embryos[92,93]. The finding that an early embryonic knockout in cardiomyocytes has no obvious phenotype until the postnatal period presents a paradox with regard to temporal- and cell-specific requirements. This result illustrates the functional versatility of individual SR proteins, as well as the dynamic nature of the postnatal period. Constitutive knockout of a third SR protein, SRSF10, resulted in lethality from mid-gestation until birth owing to several cardiac defects, thereby implicating the protein in multiple critical splicing events[94] (FIG. 3). Loss of each of the three individual SR proteins resulted in misregulation of only a few transcripts that were unique to each protein, demonstrating an unexpected level of target specificity.

Permissive roles of splicing in brain development. In addition to controlling biological outcomes directly, alternative splicing can provide 'biological options' for a determinative biological response by other mechanisms. Two examples, one from *D. melanogaster* and the other from mammals, illustrate how the production of multiple protein isoforms can be used by subsequent mechanisms to produce a biological outcome.

The neuronal circuitry of the brain is bewilderingly complex. Each neuron connects with thousands of other neurons to establish a functioning network. Two features of neuronal architecture that ensure broad coverage of a receptive field are arborization (in which axons and dendrites become widely spaced), and non-overlapping arrangements of adjacent neurons. The *D. melanogaster* Down's syndrome cell adhesion molecule 1 (*Dscam1*) gene encodes a cell adhesion molecule that has a crucial role in both features by using splicing to generate unique cell surface codes. The *Dscam1* gene is an immunoglobulin superfamily member that produces >19,000 extracellular domain variants through alternative splicing, making it unlike the vertebrate gene, which is not alternatively spliced. Initially, the DSCAM1 code was thought to direct circuitry assembly, providing a specific identity to individual neurons to establish specific connections. In fact, the role of DSCAM1 is quite different. Individual neurons express 14–50 DSCAM1 splice variants that are generated stochastically rather than by an invariant signature for specific neurons[96,97]. Homophilic binding of identical DSCAM1 isoforms is highly specific and produces intracellular signalling that results in neurite repulsion. This response prevents intraneural connections, promotes broad arborization and produces extensive non-overlapping receptive fields. Loss of DSCAM1 results in bundling

Histone code
Post-translational modifications of histone proteins that regulate the accessibility of chromatin-bound DNA to the general transcription machinery to provide an instructive code for cell- and tissue-specific gene expression.

Dicer1
A gene encoding the endoribonuclease Dicer that cleaves double-stranded RNAs to produce small interfering RNAs and microRNAs with a two-nucleotide overhang at the 3′ end.

Arborization
A tree-like branching process through which a neuron expands its dendritic coverage in three-dimensional space to integrate multiple synaptic or sensory inputs.

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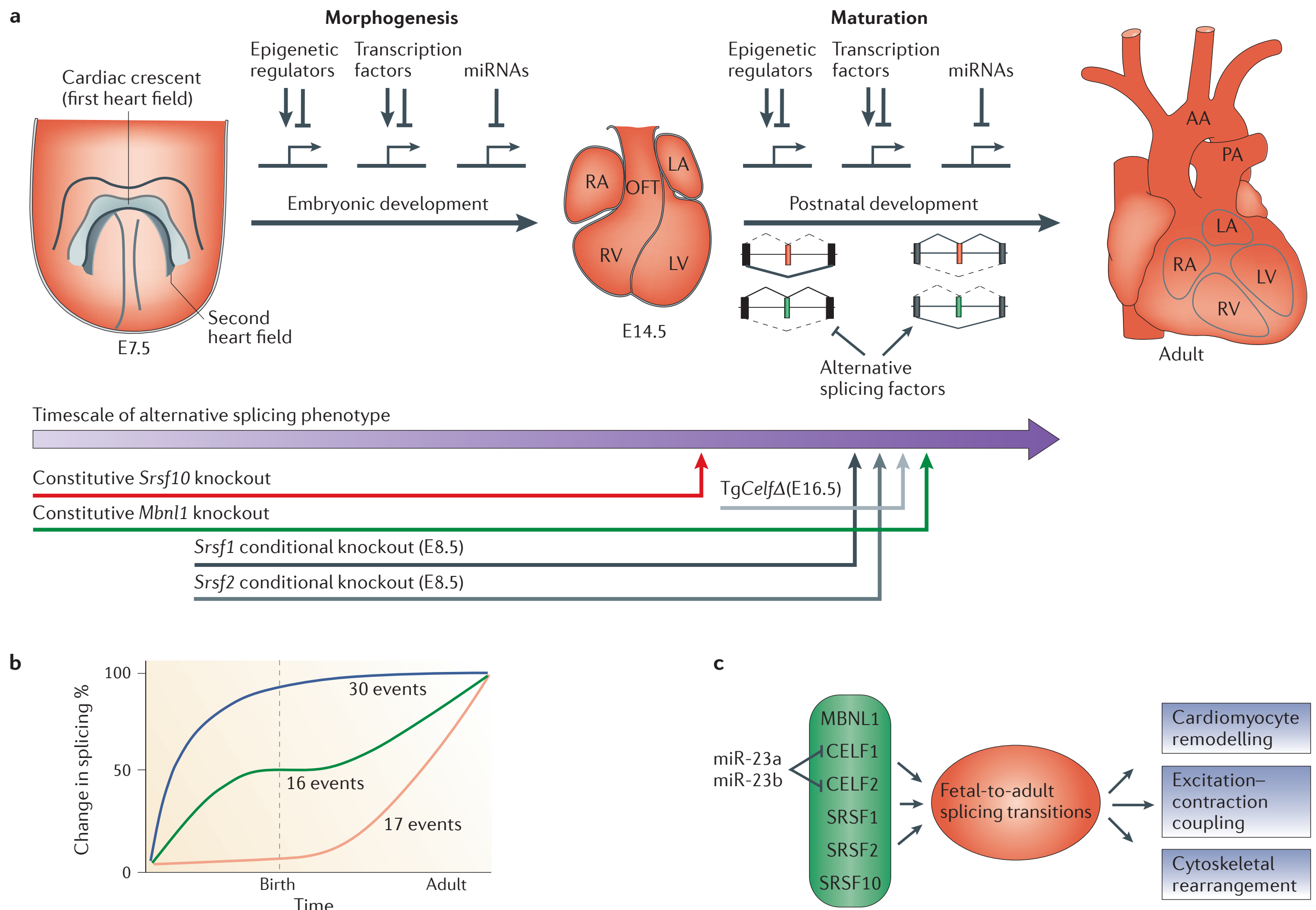

Figure 3 | **Coordinated alternative splicing changes drive fetal-to-adult transitions during postnatal heart development. a** | Two phases of heart development in mice. The morphogenesis phase occurs during early embryonic development; distinct cell populations from the first and second heart field migrate, divide and proliferate to give rise to specific structures of the four-chambered heart. The morphogenesis phase is regulated by complex epigenetic, transcriptional and post-transcriptional networks, which can include microRNA (miRNA)[86]. Growth and maturation in the second phase starts at embryonic day 14.5 (E14.5) and is largely hypertrophic, as cardiomyocytes exit the cell cycle and become post-mitotic. The importance of splicing networks during the second growth phase has become evident from mouse knockouts (namely of muscleblind-like 1 (*Mbnl1*) and serine/arginine-rich splicing factor 1 (*Srsf1*), *Srsf2* and *Srsf10*)[92–94,162] or transgenic (Tg) expression of several splicing regulators in the heart (namely of CUGBP Elav-like family (CELF)Δ)[91]. Such mouse models display late embryonic or postnatal phenotypes, preceded by alternative splicing changes. The bottom part of **a** shows the timing of individual splicing regulator knockouts or transgenic expression (indicated with coloured lines) and also shows when splicing changes appear (indicated by the arrows). **b** | Fetal-to-adult alternative splicing transitions are temporally coordinated to occur at specific times during development[26]. **c** | Members of the CELF, MBNL and SR families of splicing factors are regulators of splicing transitions that directly influence cardiac remodelling, excitation–contraction coupling and cytoskeletal rearrangement. Postnatal upregulation of miRNAs suppresses expression of multiple alternative splicing regulators, which results in a physiological shift in fetal-to-adult splice patterns. In particular, postnatal upregulation of two miRNAs that bind to the same seed sequence (miR-23a or miR-23b) causes the greater than tenfold downregulation of CELF1 and CELF2 proteins[89]. AA, aortic arch; LA, left atrium; LV, left ventricle; OFT, outflow tract; PA, pulmonary artery; RA, right atrium; RV, right ventricle.

and overlapping of neurites due in part, to repulsion failure. Elegant studies using homologous recombination replaced the wild-type *Dscam1* allele with modified genes able to produce from 1 to 4,752 randomly generated DSCAM1 isoforms. These sets of experiments provided several conclusions, including the findings that: a single DSCAM1 isoform is sufficient for homophilic repulsion; the specific isoform expressed is not important, as multiple isoforms tested had the same effect; and more than 1,000 isoforms are required within a neuronal population to produce normal neuronal patterning[98,99]. In contrast to a model in which neuron-specific expression of individual DSCAM1 isoforms directs neuronal connections, the crucial feature is that

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a population of neurons expresses sufficient DSCAM1 diversity to prevent inappropriate interactions.

Another example in which expression of different isoforms is not determinative but is crucial for the biological outcome is the mouse roundabout 3 (*Robo3*) gene[100]. Two ROBO3 protein isoforms that are generated by alternative splicing act as a binary switch to control targeting of growing commissural neurons to ensure that they cross the midline of the spinal cord only once. Whereas the two isoforms are restricted to different sides of the midline, the mRNAs are not. Therefore, splicing produces the two mRNA isoforms bilaterally, but a different regulatory mechanism, presumably translation or protein stability, produces spatially restricted expression of the two protein isoforms.

Determinative roles of splicing in brain development. One of the most well-characterized alternative splicing regulatory networks is controlled by the two RNA-binding protein paralogues, NOVA1 and NOVA2 (hereafter referred to collectively as NOVA). Expression of both genes is neuron-specific, although *Nova1* and *Nova2* are reciprocally expressed in different subregions of the brain[101]. Network analysis that integrated multiple types of data identified approximately 700 splicing events regulated by NOVA in the brains of mice[102]. Interestingly, NOVA uses an activity code that is conserved with the *D. melanogaster* orthologue, Pasilla, with regard to the binding motif (YCAY) and its positive or negative effect on splicing based on the location of the binding site relative to the exon[103]. The biological function, however, has diverged over evolutionary time, acquiring different tissue-specific expression in different metazoan lineages. For example, whereas the regulatory function of NOVA is neuron-specific in chordates, its function is gut-specific in starfish and sea urchins[104]. These analyses suggest that, after it has been established, an RNA-binding code is extremely stable and can be applied to different functional outputs through evolutionary time[103].

Results from NOVA-null mice indicate that NOVA proteins control multiple aspects of brain development, and it is likely that each paralogue controls multiple splicing subnetworks in different neuron subtypes. Another factor that is likely to affect the range of NOVA targets is the expression of co-regulators. For example, 15% of NOVA targets also contain binding sites for the RBFOX family[102], and functional connections that have recently been found to exist between RBFOX and the SR protein family[105] could also influence NOVA activity. Analysis of the genes containing NOVA-regulated splicing events defined functions that are related to synapse development and activity[106]. Additional roles for NOVA have been teased out through detailed analysis of the complex phenotype of NOVA-null mice. For example, abnormal cellular layering within the neocortex of NOVA2-knockout mice revealed a defect in post-mitotic neural migration owing to a failed developmental splicing transition of disabled 1 (*Dab1*), a component of the reelin pathway[107]. NOVA is also required in developing motor neurons for neuron-specific splicing of agrin, which assembles the proper postsynaptic architecture on the skeletal muscle membrane[108]. These studies demonstrate that splicing regulatory networks of individual RNA-binding proteins regulate diverse functions and that, despite the prediction of large numbers of targets (~700 in the case of NOVA), detailed analyses can link specific splicing events with individual components of a complex knockout phenotype.

A splicing-regulatory network has recently been associated with neuronal electrical homeostasis in the brain by knockout of RBFOX1 (REF. 109). RBFOX1 mutations have been linked with epilepsy, mental retardation and autism[110,111], and RBFOX1 expression was found to be altered in brain samples from individuals with autistic spectrum disorder (ASD)[112]. Misregulated splicing of RBFOX targets, many of which are important to synaptic function, was observed in ASD brain samples, a finding that was consistent with altered expression of RBFOX1 (REF. 112).

Alternative splicing response to extracellular stimuli. Alternative splicing is dynamically regulated in response to naturally occurring external stimuli, such as immune cell activation (reviewed in REF. 113) and neuronal depolarization. Depolarization of excitable cells in culture by exposure to elevated potassium chloride levels causes multiple plasma membrane proteins to undergo rapid changes in splicing[31,114,115]. Because blocking L-type calcium channel activity restores these splicing changes, a direct role of calcium signalling has been proposed. For example, in rats, inclusion of the stress-axis-regulated exon (STREX) in the *Slo* (also known as *Kcnma1*) transcript, which encodes a subunit of calcium and voltage-gated potassium channels, is repressed after depolarization[116]. The presence of STREX confers higher calcium sensitivity, slowing the channel deactivation. Repression of STREX upon depolarization is mediated by calcium/calmodulin-dependent protein kinase IV (CaMKIV) through intronic CaMKIV-responsive RNA elements (CaRREs)[116]. Two types of CaRRE motifs, CaRRE1 and CaRRE2, have been identified close to the SLO and many other depolarization-responsive exons[117,118]. Heterogeneous nuclear ribonucleoprotein (hnRNP)L can regulate splicing through CaRRE1 elements[119], whereas the proteins that bind to CaRRE2 elements are unknown. The hnRNPA1 protein also mediates depolarization-dependent splicing repression by binding to a different motif that is located close to regulated exons[120]. Remarkably, depolarization inhibits inclusion of a cassette exon in *Rbfox1* to produce an isoform with enhanced nuclear localization, which, in turn, leads to enhanced splicing activity that promotes the inclusion of RBFOX1 target exons that were repressed owing to depolarization[121]. Thus, separate mechanisms may execute splicing changes as an adaptive feedback response to hyperstimulation.

Stress-responsive splicing programs

In addition to the moderately slow transitions in alternative splicing described above, splicing is used as a component of acute responses to stresses such as DNA damage and hypoxia, as well as oxidative, osmotic,

Commissural neurons
Neurons that cross the midline of the brain to connect the right and left brain hemispheres.

Heterogeneous nuclear ribonucleoprotein
A conserved family of RNA-binding proteins, many of which are highly abundant, that tend to repress splicing.

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thermal or nutrient stress. In yeast, for example, splicing of ribosomal protein-encoding genes is inhibited within minutes of amino acid starvation[122]. The most well-characterized examples are during heat shock or during genotoxic stress due to ultraviolet irradiation.

Thermal stress. Heat shock in mammalian cells results in splicing inhibition, which can be recapitulated in cell-free splicing reactions using nuclear extracts prepared from heat-shocked cells[123]. The majority of heat-shock protein (HSP) genes lack introns; HSP genes that contain introns escape the splicing inhibition of thermal shock by an unknown mechanism[124]. For *Hsp47*, alternative splicing is activated by heat shock to include an additional 169 nucleotides within the 5′ UTR, producing an mRNA isoform that is more efficiently translated[125].

There are two proposed mechanisms for splicing inhibition in response to thermal stress. First, SRSF10 — a splicing factor that regulates both constitutive and alternative splicing — is rapidly dephosphorylated by heat shock. Dephosphorylation increases SRSF10 interaction with U1 small nuclear ribonucleoprotein (U1snRNP), preventing association with other SR proteins[126] and producing splicing inhibition. SRSF10 is rephosphorylated within an hour of recovery; this is similar to the splicing restoration seen for a model precursor mRNA (pre-mRNA) substrate (β-globin). Whether SRSF10 dephosphorylation affects splicing of select substrates or produces a general splicing defect is currently unclear. However, poor heat-shock recovery of SRSF10-deficient cells suggests that it affects crucial genes[126]. SR-protein-specific kinase 1 (SRPK1) dynamically interacts with heat-shock proteins HSP70 and HSP90 in mammalian cells[127]. Stress signals, such as osmotic shock, disrupt these interactions and promote cytoplasmic-to-nuclear translocation of SRPK1, and this results in differential phosphorylation of SR proteins and splicing alterations[127].

In a second mechanism, heat shock and chemical and osmotic stress lead to the formation of nuclear stress bodies; these bodies sequester a subset of alternative splicing factors and affect their splicing functions (reviewed in REFS 113,128). The kinetics of these two mechanisms are quite different. Whereas SRSF10 dephosphorylation occurs rapidly and is completely reversed within an hour of recovery[126], recruitment of splicing factors to nuclear stress bodies peaks at 3 hours and is reversed over 10–12 hours[128]. How the different mechanisms are integrated on different timescales to promote recovery remains to be determined. Large-scale studies could be used for the following applications: identifying genes that are splicing-inhibited versus those that are spared from inhibition in response to thermal stress; determining whether spared genes are enriched for recovery functions; and defining commonalities among inhibited versus spared genes to investigate the different regulatory mechanisms.

Genotoxic stress. Rapid, reversible and coordinated skipping of multiple exons from the MDM2 and MDM4 transcripts following ultraviolet irradiation provided the first indication that alternative splicing synchronizes a rapid response to genotoxic stress[129]. MDM2 is an E3 ligase that is responsible for targeting the tumour suppressor protein p53 for ubiquitin-dependent degradation[130]. Skipping of MDM2 exons deletes the p53 binding region in MDM2 (REF. 129), allowing activation of p53 during stress and its rapid shut-off on stress removal. Furthermore, MDM2 is a transcriptional target of p53 that creates a negative feedback loop to control p53 activity in response to stress.

In addition to ultraviolet irradiation, DNA damage induced by common anticancer agents, such as inhibitors of topoisomerase I and cyclin-dependent kinase, can alter splicing patterns of a large number of genes[131,132]. Intriguingly, these splicing changes are not dependent on general DNA damage response signals, such as p53 or the signalling kinases ATM and ATR. Two different co-transcriptional mechanisms provoke splicing changes in response to genotoxicity. One set of exons undergoes increased skipping after DNA damage largely owing to impaired communication between the transcription and splicing machineries that is normally mediated by EWS, an RNA polymerase II (RNAPII)-associated factor, and YB1, a spliceosome-associated factor[131]. Normally, EWS binds co-transcriptionally to its target RNAs, but ultraviolet irradiation reduces this association owing to a transient relocation of EWS to the nucleoli[133]. Reduced association of EWS with its target RNA affects splicing among genes that are preferentially involved in DNA repair and genotoxic stress signalling[133], linking EWS-mediated splicing regulation and DNA damage response. Conversely, another set of exons exhibits increased inclusion in response to genotoxicity owing to slowing of RNAPII elongation rate[132]. In particular, ultraviolet irradiation increases the phosphorylation of the C-terminal repeat domain of RNAPII, and this slows RNAPII elongation. Alternative exons are typically flanked by inefficiently recognized splice sites, and the slowing of RNAPII allows time for alternative exons to commit to splicing[18]. Future work will provide a broader understanding of the functional consequences associated with both observations.

Conclusions and perspectives

RNA-binding proteins are at the centre of regulatory networks in which hundreds of splicing events are associated with binding sites. It is possible that most binding sites serve to dampen the effects of the RNA-binding protein and that only a minority of binding sites are associated with physiological splicing events (BOX 3). However, as highlighted in this Review, loss-of-function analyses have been successfully used in yeast, flies and mice to identify individual splicing targets of RNA-binding proteins and specific functions of individual splicing events. To understand the full biological context of the splicing network, a next step is to determine whether a natural change in the activity of the RNA-binding protein is used to modulate the network in response to physiological need.

In addition to auxiliary RNA-binding proteins, which are the focus of this Review, the basal splicing machinery makes important contributions to cell-type-specific

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Box 3 | **Binding of a splicing regulator to weakly regulated sites could buffer functional splicing events**

Computational and biochemical genome-wide analyses for individual RNA-binding proteins often identify hundreds of putative target exons that are associated with multiple binding sites. Genetic loss-of-function analyses often identify a much smaller number of splicing events, that exhibit a robust response to the loss-of-function. There are several potential explanations for such discrepancies. First, there may be redundancy with related splicing regulators, as the multifactorial nature of splicing regulation means that individual factors contribute to large numbers of events but are determinative for a limited number of events. Second, the specific cell type exhibiting the robust response might not have been assayed. Third, under certain circumstances, splicing transitions do not have to be large to have physiological consequences. Finally, it might be the case that a large fraction of binding sites do not direct a splicing response that has functional consequences but rather are used as 'sinks' that sequester the RNA-binding protein and dampen its physiological impact.

The hypothesis that natural genome-wide sinks regulate biological function has been proposed to explain a similar set of paradoxes for microRNAs (miRNAs), for which there are large numbers of predicted targets, but only a small fraction of these targets are conserved and an even smaller fraction have a measurable physiological impact[159]. This regulatory mechanism was recently proposed for RNA-binding proteins that regulate alternative splicing (H. Seitz, personal communication). In this hypothesis, most binding sites serve to regulate the activity of the protein negatively by sequestration from the active pool, such that a large number of 'molecular targets' titrate the levels of the regulator against a few 'physiological targets'. Criteria for distinguishing physiological from molecular targets could include: conserved binding sites associated with the regulated exon; a robust change in response to genetic loss- and gain-of-function; and regulation during periods of physiological change in which the RNA-binding protein undergoes a change in activity that is conserved in different species.

splicing (for example, see REFS 134,135). Splicing regulation can be independent from the activity of auxiliary splicing factors[136]. Knockdown of spliceosomal components results in gene-specific splicing effects in yeast, flies and mammals, indicating a large potential for regulation through modulation of spliceosomal components[135,137–139]. Furthermore, specific cell types, such as neurons, are exquisitely sensitive to hypomorphic mutations within core spliceosomal components[140], revealing dramatic cell specificity of what was once considered to be a ubiquitous part of the basal machinery that was constant among cell types. It is likely that cell-type-specific differences in the basal splicing machinery not only produce cell-specific splicing patterns but also have an impact on the function of auxiliary splicing regulators.

Identification of splicing transitions that function during a physiological change requires knowledge of the cell population sampled. For example, less than 20% of the cells in the heart are cardiomyocytes: most are cardiac fibroblasts, vascular smooth muscle cells and endothelial cells[141]. Therefore, it is often unclear which cell type undergoes the splicing transitions that are measured in whole tissue. Another consideration is whether the detected splicing transitions represent regulation within a constant cell population or a change of cell populations. This is relevant to developmental changes that involve cell migrations as well as pathological samples, in which there can be substantial loss and replacement of parenchymal cells by fibroblasts or cell gain by inflammatory infiltration. In the not too distant future, the complication of cell heterogeneity will be circumvented by transcriptome analysis in single cells[142], which will reveal the variability among cells of the same type and enable the analysis of different cell types within a population.

The remarkable complexity of gene regulation becomes increasingly apparent in proportion to the improving resolution of the available assays. Splicing is one component of an interacting continuum of epigenetic, transcriptional and post-transcriptional control[18,143]. Regulation of individual alternative splicing events has multiple inputs into the decision of whether to use splice sites, with different factors acting antagonistically or as negative or positive co-regulators. Splicing factors autoregulate themselves and crossregulate each other, thus generating network-wide influences on splicing. Rapidly developing high-throughput approaches are leading to the delineation of regulatory networks for large numbers of RNA-binding proteins. The combined data sets will aid in identifying how splicing regulatory networks are integrated in different cell types and, ultimately, how they produce diverse physiological responses.

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Competing interests statement
The authors declare no competing financial interests.

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