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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 remodeling 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 genes with cell-type-specific functions. Alternative splicing can drive determinative physiological change or can have a permissive role by providing mRNA variability that is utilized by other regulatory mechanisms.

Keywords

Developmental Biology; Gene Expression; RNA

INTRODUCTION

One of several recent advances toward 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-Seq are that 90%, 60%, and 25% of genes in humans, *Drosophila melanogaster*, and *C. elegans*, respectively, undergo alternative splicing¹⁻⁵. Alternative splicing is the most prominent of several mechanisms generating mRNA structural complexity that also include alternative transcription initiation, editing, and alternative polyA site selection⁶⁻⁷. The predicted outcomes of this complexity are: extensive proteome diversity; introduction of premature termination codons (PTCs), which causes mRNA down-regulation due to nonsense mediated decay (NMD); and variability in mRNA untranslated regions (UTRs), which affects *cis*-acting elements that mediate regulation of mRNA translation efficiency, stability and localization^{6,8-9}.

Competing interests statement

The authors declare no competing financial interests.

ONLINE LINKS

Tom Cooper lab homepage <http://www.bcm.edu/pathimmuno/cooper/?PMID=14453>

Flybase <http://flybase.org/>

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The prevalence of alternative splicing raises questions about its biological significance. What fraction of the multiple mRNA isoforms expressed from each of ~19,000 alternatively spliced human genes has a functional impact? Much of the mRNA diversity that is observed includes low abundance transcripts that arise from alternative splicing events that are not conserved, suggesting a high level of stochastic noise¹⁰⁻¹¹. While 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. On the other hand, it is clear that alternative splicing produces determinative biological effects exemplified by longstanding examples such as sex determination in *D. melanogaster*¹²⁻¹³, production of functionally distinct peptide hormones in mammals¹⁴, and the meiotic developmental program in budding yeast¹⁵. 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 time scale¹⁶⁻¹⁷. 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 review networks of regulated alternative splicing 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}. Our focus is on the physiological outcomes of alternative splicing transitions. We also address broad questions relating to alternative splicing regulation: what are the critical 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 (e.g., change in protein abundance, intrinsic activity, or intracellular localization)? What are the “upstream” signaling 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 critical “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 premRNAs near variably used splice sites and modulate the efficiency of their recognition by the basal splicing machinery (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 (Box 1)²³. The large-scale analysis of alternative splicing has recently progressed from comparisons of relatively 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 datasets for 27 stages of *D. melanogaster* development¹ and 17 growth stages and conditions in *C. elegans*⁴⁻⁵ revealed that a large fraction of alternative splicing (>60% and 30%, respectively) undergo developmental changes, often in coordinated sets that are suggestive of co-regulated networks. Studies using transgenes expressing fluorescent proteins to indicate different splicing outcomes in *C. elegans* have revealed developmental transitions in real

time and provide a genetic approach to identify the regulators²⁴⁻²⁵ 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 associated with an 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 associated with a physiological change are conserved. For example, more than 40% of splicing transitions observed during mouse heart development or skeletal muscle differentiation were conserved in birds, not only in terms of the alternatively spliced region but also with regard to splicing pattern, the direction of the changes, and timing of the transitions²⁶⁻²⁷. 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 significance. These results are in stark contrast to genome-wide comparisons in which fewer than 20% of alternative splicing events were conserved between human and mouse²⁸⁻²⁹, emphasizing the role for splicing in physiological transitions. Fourth, genome-wide studies in which transitions in both alternative splicing and mRNA expression levels were analyzed simultaneously 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. While splicing and transcriptional regulation are linked¹⁸, the results indicate that different genes can be regulated primarily 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 recently found to be intricately linked with multiple splicing-regulatory programs. Only 5% of genes in budding yeast contain introns (290 of 6000 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 spliced³². With a few exceptions³³, alternative splicing in yeast is limited to ‘splice versus don’t splice’ decisions that relate to intron retention in single intron genes. Large-scale analyses using tiling and splicing sensitive microarrays have determined that 45 intron-containing genes are inefficiently spliced during vegetative growth³⁴⁻³⁵. These include 13 of the 20 intron-containing meiotic genes that undergo efficient splicing specifically during the meiotic cycle³⁴⁻³⁵. Meiosis-specific alternative splicing in budding yeast has been known for two decades¹⁵ but recent investigations have revealed at least three separate but overlapping meiotic splicing regulatory programs³⁵⁻³⁶. The best-characterized is regulated by *MER1* which encodes an RNA binding protein that is transcriptionally induced during the initiation of the meiotic cycle³⁷. The primary function of Mer1p is to activate splicing of four single-intron genes and induce their expression during meiosis³⁵ (Figure 1). The Mer1p 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 splice sites at the intron-exon boundaries. Mer1p binds to an enhancer element within the intron of each of the four genes and promotes spliceosome assembly by direct interactions with Nam8p, a spliceosomal

component³⁸⁻³⁹. The *MER1* splicing network serves a critical role in the transition from early meiotic genes, transcriptionally regulated by *UME6*, to the middle meiotic genes, which are regulated by *NDT80*³⁵ (Figure 1).

Analysis of *NAM8* null strains identified intron retention of the four *MER1*-dependent genes, consistent with the mechanism of Mer1p activation, as well as two genes not dependent on *MER1* for meiosis-specific splicing^{35,40}. Another study found two genes for which meiosis-specific splicing requires *TGS1* which synthesizes the specialized 2,2,7-trimethylguanosine cap at the 5' end of small nuclear RNAs (snRNAs), essential components of the spliceosome⁴¹. Splicing for one *TGS1*-dependent gene is also *NAM8*-dependent, indicative of multiple overlapping networks (Figure 1). Unlike *MER1*, *NAM8* and *TGS1* expression levels do not substantially change during meiosis, suggesting that while 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 relatively straightforward *MER1* splicing regulatory network contains multiple splicing sub-networks and is interlinked to 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, adapters, caspases and caspase targets, are alternatively spliced to produce dramatic different biological outcomes⁴². In particular, splicing of the Bcl2 family of apoptosis regulators (Bcl2, Bcl-x, and Mcl1) yields long (L) and short (S) isoforms to provide anti-apoptotic versus pro-apoptotic functions, respectively⁴². 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 mitosis⁴³. Loss of aurora kinase A promoted posttranslational degradation of (serine-arginine splicing factor 1) SRSF1, a member of the SR protein family of splicing regulators⁴⁴. Results from CLIP analysis (Box 1) showed that SRSF1 directly binds Bcl-x and Mcl1 RNAs, as well as revealing additional endogenous apoptotic splicing events that shifted toward pro-apoptotic splicing upon cell cycle inhibition. Cell cycle inhibitors were previously known to induce pro-apoptotic splicing; however, a critical 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 oncogenesis⁴⁵⁻⁴⁷, including the recent demonstration that an SR-related protein (SON) is required for efficient splicing of cell cycle regulated genes⁴⁸. 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 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 pluripotency⁴⁹⁻⁵⁴. Splice isoform diversity is high in undifferentiated ESCs, decreases upon their differentiation⁴⁹⁻⁵⁰

and is enriched in cell-cycle, pluripotency, signaling 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, Nanog, Sox2, and Tcf3 — that induce and cross-regulate a network of target genes involved in self-renewal and pluripotency⁵⁷. 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 Oct4B1⁵⁸. Oct4A contains N- and C-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. While Oct4A target genes are responsible for stemness^{57,59}, Oct4B cannot sustain ESC self-renewal and instead targets genes responsive to cell stress^{58,60}. The expression and function of Oct4B1 remains to be determined⁵⁸. 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 differentiation⁶¹. During differentiation, multiple splice variants containing the C-terminal transactivation domain are expressed and, one of these isoforms in particular, Oct2.2 induces neuronal differentiation⁶¹.

Alternative splicing of several other genes in addition to the core pluripotency factors is linked to stem cell self-renewal and lineage specification⁶²⁻⁶⁶ yet little is known about the regulatory factors that dictate these splicing outcomes. A group of recent studies provide important insights into how splicing factors may influence neural differentiation from ESCs or neural progenitors. Exons that are alternatively spliced in human ESCs⁵² are associated with conserved binding sites of the RNA binding protein Fox1 homologue (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 differentiated stem cells or transformed cell lines⁶⁴. Another splicing factor, polypyrimidine track binding protein (PTB), prevents differentiation of a proliferative neural cell line by repressing expression of its neuronal homologue nPTB⁶⁷⁻⁶⁸. It does this by directly promoting skipping of neuronal PTB (nPTB) exon 10 in non-neuronal cells, which introduces a PTC resulting in nPTB mRNA degradation by NMD. Neural-specific expression of nPTB is due to silencing of PTB by a neuronal-enriched miRNA, miR-124⁶⁹, 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 in addition to transcription⁷², alternative splicing is also re-set when somatic cells are reprogrammed to pluripotency.

Epithelial to Mesenchymal Transitions—Phenotypic conversions of cells between epithelial and mesenchymal states, known as epithelial-mesenchymal (EMT) and mesenchymalepithelial (MET) transitions, are fundamental to organ morphogenesis and tissue remodeling during embryonic development⁷³. These *trans*-differentiation events are required for physiological responses to injury and wound healing in adult tissues and play roles in pathological responses such as fibrosis and metastasis⁷⁴. At a cellular level, EMT is

characterized by a loss of epithelial features, including cell adhesion and polarity, and acquisition of mesenchymal features, such as motility and invasiveness⁷⁵.

Alternative splicing plays a determinative role in EMT through regulation of multiple splicing events and utilizing several regulatory proteins⁷⁶⁻⁷⁹. In a high fraction of breast cancer cell lines, SRSF1 up-regulation triggers EMT through alternative splicing of the Ron tyrosine kinase receptor proto-oncogene to produce a constitutively active, pro-invasive isoform, Ron Δ ⁷⁶. Induction of EMT via ERK1/2 activation⁸⁰ proceeds in part through phosphorylation of its substrate SAM68, which then upregulates SRSF1 by inhibiting AS-NMD-mediated downregulation (Figure 2a).

Downregulation of the RNA binding proteins, Epithelial Splicing Regulatory Proteins 1 and 2 (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 depleted of ESRP1/ESRP2 or mesenchymal cells expressing ectopic ESRP1⁷⁷. A large fraction of these contained an ESRP-binding motif that defined a position-dependent effect on splicing. The ESRP target genes were enriched in functions that support EMT, such as actin cytoskeleton, cell adhesion, cell migration, and cell polarity (Table 1). Importantly, sustained ESRP1/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 (Figure 2b)⁸¹.

The fibroblast growth factor plasma membrane receptor 2 (FGFR2) contains two mutually exclusive alternative exons, IIIb and IIIc, which produce distinct ligand binding specificities in epithelial and mesenchymal cells⁸² to ensure appropriate signaling for different mesenchymal-epithelial interactions during organogenesis⁸³. The studies of FGFR2 in EMT identified critical interplay between the histone code, adapter proteins, and alternative splicing regulators to generate cell-type specific splice isoforms (Figure 2c)⁸²⁻⁸⁴.

Alternative splicing in tissue maturation

Alternative splicing in heart development—The vertebrate heart is the first organ to both form and function in the embryo. Cardiac morphogenesis is complete by embryonic day 14.5 (E14.5) in mice, after which growth occurs primarily via hypertrophic (cell growth) rather than hyperplastic (cell proliferation) pathways (Figure 3a)⁸⁵⁻⁸⁶. The first four weeks after birth are characterized by extensive remodeling 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 development²⁶. A timecourse revealed three sets of temporally coordinated splicing transitions²⁶ (Figure 3b). Remarkably, greater 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, indicative of functionally important embryonic to adult protein isoform transitions. The genes exhibiting conserved splicing transitions were enriched for functions consistent with heart remodeling (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 levels²⁶.

Computational analysis identified significantly 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 CELF, MBNL, Rbfox and PTB. Consistent with roles

in regulating postnatal splicing transitions, levels of the two CELF paralogues expressed in heart, CELF1 and CELF2, decrease more than 10-fold during postnatal development while MBNL increases 4-fold^{26,87-88}. Importantly, CELF gain-of-function and MBNL loss of function in adult mouse heart caused over half of the postnatal splicing transitions to revert to the embryonic pattern, 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.

MiRNA-mediated regulation of multiple splicing regulators was revealed by deletion of *Dicer* in adult cardiomyocytes in mice, which resulted in rapid induction of a subset of splicing regulators (CELF, PTB and Rbfox) and re-expression of large numbers of fetal mRNA splice variants⁸⁹. These findings identify a regulatory hierarchy in which miRNAs and alternative splicing act to coordinate the switch from fetal-to-adult gene expression programs (Figure 3c).

Transgenic overexpression (CELF1), genetic knockout (SRSF1, SRSF2, SRSF10) or overexpression of a dominant negative mutant (CELFΔ) of splicing regulators in heart has a severe impact on heart physiology^{26,90-94}. Interestingly, heart-specific deletion of either SRSF1 or SRSF2 beginning around E8.5, had a delayed effect on mouse postnatal heart development with most animals exhibiting splicing abnormalities by two weeks of age and developing dilated cardiomyopathy within eight weeks (Figure 3)⁹²⁻⁹³. SRSF1 is essential for cell viability in culture⁹⁵ and a constitutive SRSF1 or SRSF2 knockout is embryonic lethal⁹²⁻⁹³. 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 due to several cardiac defects implicating the protein in multiple critical splicing events (Figure 3)⁹⁴. 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 directly controlling biological outcomes, alternative splicing can also 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 widely separated neurites (axons and dendrites) (arborization) and non-overlapping arrangements of adjacent neurons. The *D. melanogaster* Down syndrome cell adhesion molecule 1 (*Dscam1*) gene encodes a cell adhesion molecule that plays a critical role in both features using, splicing to generate unique cell surface codes. The *Dscam1* gene is an immunoglobulin (Ig) superfamily member that produces >19,000 extracellular domain variants through alternative splicing, 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 neurons⁹⁶⁻⁹⁷. Homophilic binding of identical *Dscam1* isoforms is highly specific and produces intracellular signaling 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

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 4752 randomly generated *Dscam1* isoforms. These sets of experiments provided several conclusions including that: (i) a single *Dscam1* isoform is sufficient for homophilic repulsion, (ii) the specific isoform expressed is not important since multiple isoforms tested had the same effect, and (iii) more than 1000 isoforms are required within a neuronal population to produce normal neuronal patterning⁹⁸⁻⁹⁹. In contrast to a model in which neuron-specific expression of individual *Dscam1* isoforms directs neuronal connections, the critical feature is that 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 critical for the biological outcome is the mouse Roundabout 3 (*Robo3*) gene¹⁰⁰. Two *Robo3* protein isoforms generated by alternative splicing act as a binary switch to control targeting of growing commissural neurons to ensure that they cross the mid-line of the spinal cord only once. While the two isoforms are restricted to different sides of the mid-line, 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 best characterized alternative splicing regulatory networks is controlled by the two RNA binding protein paralogues, Nova1 and Nova2. Expression of both genes is neuron-specific although Nova1 and Nova2 are reciprocally expressed in different subregions of the brain¹⁰¹. Network analysis that integrated multiple types of data identified approximately 700 splicing events regulated by Nova in mouse brain¹⁰². Interestingly, Nova utilizes an activity code that is conserved with its *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 exon¹⁰³. The biological function, however, has diverged over evolutionary time acquiring different tissue-specific expression in different metazoan lineages. For example, while Nova's regulatory function is neuron-specific in chordates, its function is gut-specific in star fish and sea urchins¹⁰⁴. These analyses suggest that once established, an RNA binding code is extremely stable and can be applied to different functional outputs through evolutionary time¹⁰³.

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 sub-networks in different neuron subtypes. Another factor likely to affect the range of Nova targets is expression of co-regulators. For example, 15% of Nova targets also contain binding sites for the Rbfox family¹⁰² and functional connections that have recently been found to exist between Rbfox and the SR protein family¹⁰⁵ could also influence Nova activity. Analysis of the genes containing Nova-regulated splicing events defined functions related to synapse development and activity¹⁰⁶. 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 due to a failed developmental splicing transition of Disabled1 (*Dab1*), a component of the reelin pathway¹⁰⁷. Nova is also required in developing motor neurons for neuron-specific splicing of agrin which assembles the proper postsynaptic architecture on the skeletal muscle membrane¹⁰⁸. 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 been recently associated with neuronal electrical homeostasis in the brain by a knockout of *Rbfox1*¹⁰⁹. *Rbfox1* mutations have been linked with epilepsy, mental retardation, and autism¹¹⁰⁻¹¹¹ and *Rbfox1* expression was found to be altered in brain samples from individuals with autistic spectrum disorder (ASD)¹¹². Mis-regulated splicing of *Rbfox* targets, many of which are important to synaptic function, was observed in ASD brain samples, consistent with altered expression of *Rbfox1*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 in¹¹³) and neuronal depolarization. Depolarization of excitable cells in culture by exposure to elevated potassium chloride 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 signaling has been proposed. For example, inclusion of the stress-axis-regulated (STREX) exon in the SLO transcript, which encodes a subunit of calcium and voltage-gated potassium channels, is repressed after depolarization¹¹⁶. The presence of the STREX exon confers higher calcium sensitivity, slowing the channel deactivation. Repression of the STREX exon upon depolarization is mediated by calcium/calmodulin-dependent protein kinase (CaMK)IV 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 exons¹¹⁷⁻¹¹⁸. HnRNP L can regulate splicing via CaRRE1 elements¹¹⁹ while the proteins that bind to CaRRE2 elements are unknown. HnRNPA1 also mediates depolarization-dependent splicing repression by binding to a different motif located close to regulated exons¹²⁰. 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 due to depolarization¹²¹. Thus, separate mechanisms may execute splicing changes as an adaptive feedback response to hyperstimulation.

Stress-responsive splicing programs

In addition to the relatively slow transitions in alternative splicing described above, splicing is also utilized as a component of acute responses to stresses such as DNA damage and hypoxia, as well as oxidative, osmotic, thermal or nutrient stress. In yeast, for example, splicing of ribosomal protein-encoding genes is inhibited within minutes of amino acid starvation¹²². The best characterized examples are during heat shock or 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 cells¹²³. 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 mechanism¹²⁴. For HSP47, alternative splicing is activated by heat shock to include an additional 169 nucleotides within the untranslated region, producing an mRNA isoform that is more efficiently translated¹²⁵.

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 snRNP, which prevents association with other SR proteins¹²⁶, producing splicing inhibition. SRSF10 is re-phosphorylated within an hour of recovery that parallels splicing restoration of a model pre-mRNA substrate (β -globin). Whether SRSF10 dephosphorylation affects splicing of select substrates or produces a general splicing defect is

presently unclear. However, poor heat shock recovery of SRSF10-deficient cells suggests that it affects critical genes¹²⁶. SR protein-specific kinase, SRPK1, dynamically interacts with heat shock proteins Hsp70 and Hsp90 in mammalian cells¹²⁷. Stress signals such as osmotic shock disrupt these interactions and promote cytoplasmic-to-nuclear translocation of SRPK1 which results in differential phosphorylation of SR proteins and splicing alterations¹²⁷.

In a second mechanism, heat shock as well as chemical and osmotic stress lead to formation of nuclear stress bodies that sequester a subset of alternative splicing factors affecting their splicing functions (reviewed in^{113,128}). The kinetics of these two mechanisms are quite different. While SRSF10 dephosphorylation occurs rapidly and is completely reversed within an hour of recovery¹²⁶, recruitment of splicing factors to nuclear stress bodies peaks at three-hours and is reversed over ten-to-twelve hours¹²⁸. How the different mechanisms are integrated on different time scales to promote recovery remains to be determined. Large scale studies could be used to identify genes that are splicing inhibited versus spared in response to thermal stress, determine whether spared genes are enriched for recovery functions, and define 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 (UV) irradiation provided the first indication that alternative splicing synchronizes a rapid response to genotoxic stress¹²⁹. MDM2 is an E3 ligase responsible for targeting the tumor suppressor protein p53 for ubiquitin-dependent degradation¹³⁰. Skipping of MDM2 exons deletes the p53 binding region in MDM2 protein¹²⁹, which allows p53 activation during stress and its rapid shut-off upon stress removal. Furthermore, MDM2 is a transcriptional target of p53 which creates a negative feedback loop to control p53 activity in response to stress.

In addition to UV irradiation, DNA damage induced by common anti-cancer agents such as inhibitors of topoisomerase I and cyclin dependent kinase can alter splicing patterns of a large number of genes¹³¹⁻¹³². Intriguingly, these splicing changes are not dependent on general DNA damage response signals such as p53 or signaling kinases ATM and ATR. Two different cotranscriptional mechanisms provoke splicing changes in response to genotoxicity. One set of exons undergoes increased skipping after DNA damage, largely due to impaired communication between the transcription and splicing machineries that is normally mediated by EWS, a RNA Polymerase II (RNAPII)-associated factor, and YB-1, a spliceosome-associated factor¹³¹. Normally, EWS binds co-transcriptionally to its target RNAs, but UV irradiation reduces this association due to a transient relocation of EWS to the nucleoli¹³³. Reduced association of EWS with its target RNA affects splicing among genes preferentially involved in DNA repair and genotoxic stress signaling¹³³ linking EWS mediated splicing regulation and DNA damage response. Conversely, another set of exons exhibit increased inclusion in response to genotoxicity due to slowing of RNAPII elongation rate¹³². In particular, UV irradiation increases the phosphorylation of the carboxy-terminal repeat domain of RNAPII, which slows RNAPII elongation. Alternative exons are typically flanked by inefficiently recognized splice sites and slowing of RNAPII allows time for alternative exons to commitment to splicing¹⁸. Future work will provide a broad understanding of the functional consequences associated with both observations.

CONCLUSIONS AND PERSPECTIVES

RNA binding proteins are at the center 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 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 utilized 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 critical contributions to cell type-specific splicing (for example, see ¹³⁴⁻¹³⁵). Splicing regulation can be independent from the activity of auxiliary splicing factors¹³⁶. 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 components¹⁴⁰ revealing dramatic cell specificity of what was once considered a ubiquitous basal machinery 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 impact the function of auxiliary splicing regulators.

Identification of splicing transitions that function during a physiological change requires knowledge of the cell population sampled. As one example, less than 20% of the cells in heart are cardiomyocytes, the majority being cardiac fibroblasts, vascular smooth muscle cells and endothelial cells¹⁴¹. 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 cells¹⁴² that will reveal the variability among cells of the same type and analysis of different cell types within a population.

The remarkable complexity of gene regulation becomes increasing apparent in proportion to the improving resolution of the available assays. Splicing is one component of an interacting continuum of epigenetic, transcriptional and posttranscriptional control^{18,143}. Regulation of individual alternative splicing events has multiple inputs into the decision of whether or not to use splice site(s), with different factors acting antagonistically or as negative or positive co-regulators. Splicing factors autoregulate themselves and cross-regulate 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 datasets 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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GLOSSARY DEFINITIONS

Transcriptome	technically refers to all of the RNA in a cell; however, often the term is used to describe the polyadenylated RNAs transcribed by RNA polymerase II, which are selected for analysis by oligo dT.
RNA-Seq	high throughput shot gun sequencing of cDNA to obtain the sequence of the transcriptome
mRNA structural diversity	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.
Spliceosome	the complex and conserved nuclear machinery that removes introns. The spliceosome contains 5 small uridylyate-rich small nuclear RNAs (U snRNAs) and ~150 proteins.
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.
HnRNP proteins	a conserved family of RNA binding proteins, many of which are highly abundant, that tend to repress splicing.
Nonsense mediated decay	mRNA surveillance mechanism that degrades mRNAs containing nonsense mutations to prevent the expression of truncated or erroneous proteins.
CLIP	a biochemical technique that utilizes UV crosslinking of protein and RNA <i>in vivo</i> followed by immunoprecipitation to identify direct protein-RNA interaction sites in living cells.
Core Pluripotency factors	A set of transcription factors (Oct4, Nanog, Sox2 and Tcf3) that form a core transcriptional circuit to maintain the pluripotent state of embryonic stem cells.

EMT	Phenotypic conversion that disrupts the polarity of epithelial cells to establish invasive mesenchymal features through alterations in cytoskeletal organization, cell adhesion and the extracellular matrix.
Micro (mi)RNAs	evolutionarily conserved small noncoding RNAs (~ 22 nucleotides long) that silence gene expression by degrading or inhibiting translation of mRNA transcripts in a sequence-specific manner.
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.
Dicer	An endoribonuclease that cleaves double-stranded RNAs to produce small interfering RNAs (siRNAs) and microRNAs with a two-nucleotide overhang at the 3' end.
Dendritic 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.
Commissural neurons	Neurons that cross the midline of the brain to connect the right and left brain hemispheres.

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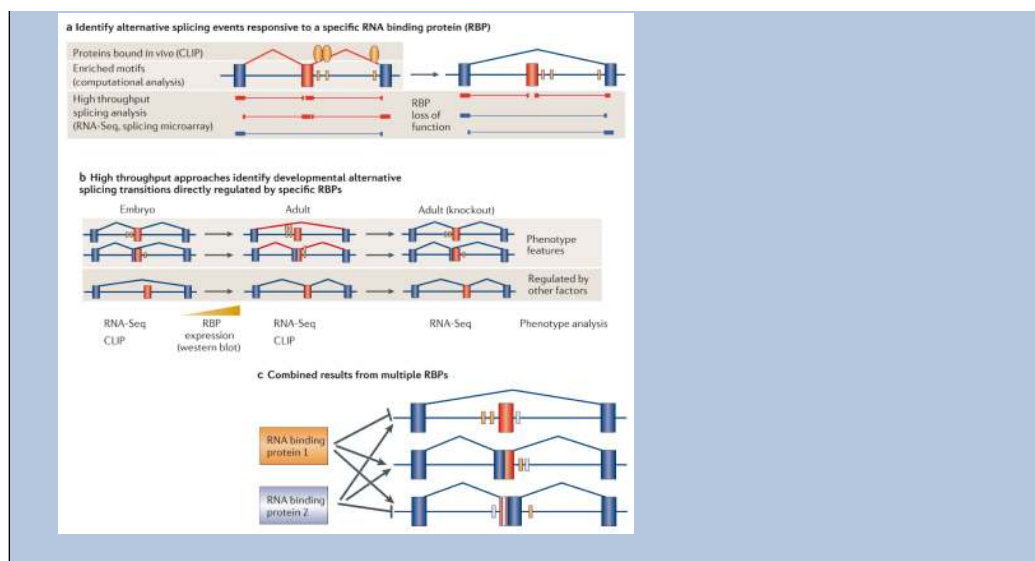
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BOX 1: Identification of splicing regulatory networks

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 5-8 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 exons¹⁴⁷ (**panel a**). Approximately fifty mammalian RNA binding proteins that act as auxiliary splicing regulators, separate from the basal splicing machinery, have been characterized and shown to directly regulate splicing by binding to pre-mRNAs^{8,148}. Splicing events that are sensitive to the loss-or gain-of-function of the splicing regulator (via RNAi, genetic knockout, or overexpression) are identified using large scale analyses such as RNA-Seq (represented in the diagram) or splicing sensitive microarrays¹⁴⁹. 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 due to secondary effects. To identify direct targets, bound protein is covalently linked to the RNA *in vivo* by UV crosslinking followed by immunoprecipitation (CLIP) and identification of specific binding sites by high throughput sequencing¹⁵⁰. While CLIP produces false negatives and positives¹⁵¹, it is a highly effective screen for target identification. The massive data sets from these assays are managed and extensively analyzed 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 sites¹⁵².

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/splicing microarrays, CLIP, genetic knockouts, and computational analysis have identified networks of tissue specific as well as developmental alternative splicing programs regulated by specific RNA binding proteins (for example, ^{26,109,153}). Despite the large number of splicing events 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 events¹⁰⁷⁻¹⁰⁸ (**panel b**).

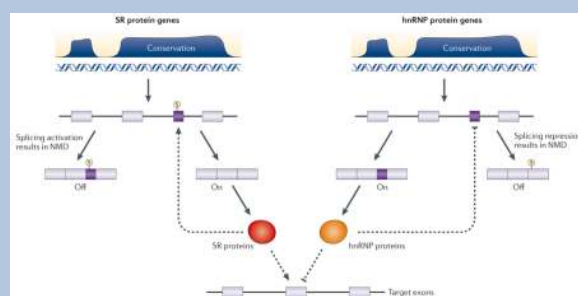
Individual splicing events are regulated by cooperative as well as antagonistic effects of multiple RNA binding proteins. The multiple inputs integrate the effects from diverse external cues to promote an appropriate splicing response (**panel c**). Combining experimental data from multiple splicing 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^{102,147}.



BOX 2: Alternative splicing linked with nonsense mediated decay (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)¹⁵⁴⁻¹⁵⁷. NMD is a conserved and multifunctional surveillance mechanism to degrade mRNAs containing 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 NMD¹⁵⁸. In AS-NMD, alternative splicing controls an NMD signal by insertion or removal of an mRNA segment that contains either a PTC or introduces a downstream PTC due to a frameshift. The majority of splicing regulators affected by AS-NMD autoregulate homeostatic levels by promoting a splicing pattern that results in NMD and down-regulation. Splicing regulators also use AS-NMD for cross regulation such as the repression of neuron-specific nPTB in non-neuronal cells by its paralogue, PTB⁶⁸.

The genes encoding the SR and hnRNP protein families present a striking example of homeostatic control by AS-NMD with pervasive biological and evolutionary implications¹⁵⁶⁻¹⁵⁷. SR and hnRNP proteins are a long-standing paradigm for antagonistic splicing regulators affecting multiple and diverse alternative splicing events¹⁵⁹. All SR protein genes and many hnRNP protein genes utilize AS-NMD for negative autoregulatory feedback. Strikingly, the regions of the genes that are critical 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 splicing pattern¹⁵⁷ (**Figure**): in the genes for SR proteins, which predominantly (though not exclusively) activate splicing, activation of an alternative splicing event results in NMD-mediated down-regulation while in genes of hnRNP proteins, which are most often splicing repressors, repression of a splicing event results in NMD-mediated down-regulation. The full significance of this ancient regulatory network remains to be elucidated; however, the implication is that maintaining the appropriate levels of both protein families is a critical 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^{45,160-161}. The figure is reproduced from reference¹⁵⁷.



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 associated with multiple binding sites. Genetic loss-of-function analyses often identify a much smaller number of splicing events exhibiting a robust response. 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. 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 utilized as “sinks” that sequester the RNA binding protein and dampen its physiological impact.

The hypothesis of natural genome-wide sinks to regulate biological function has been proposed to explain a similar set of paradoxes for 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 impact¹⁶². 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 negatively regulate the activity of the protein 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 to distinguish 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 conserved in different species.

Online at a glance summary

- A large fraction of genes in worms, flies and vertebrates express multiple mRNAs via alternative splicing. This produces extensive mRNA structural diversity that ultimately affects protein coding potential as well as mRNA *cis*-acting elements that are determinative for translation, mRNA stability, and mRNA intracellular localization.
- Global analyses of alternative splicing regulation during periods of biological transition, such as during development, have revealed coordinated and conserved networks of alternative splicing.
- Several splicing regulatory networks controlled by individual RNA binding proteins have been identified by combining recent advances in genome wide analyses of alternative splicing with the identification of RNA binding sites *in vivo*.
- A high proportion of RNA binding proteins that regulate alternative splicing are themselves regulated by alternative splicing and are subject to auto- and cross-regulatory feedback. This type of regulation includes alternative splicing linked with nonsense-mediated decay (NMD), which results in mRNA downregulation.
- Diverse physiological processes are regulated in a determinative fashion by alternative splicing patterns, including meiosis in budding yeast, neuronal arborization in the *D. melanogaster* brain, and stem cell determination in vertebrates.
- The regulation of gene expression by alternative splicing is intricately linked with transcription, the epigenetic state of chromatin, and subsequent RNA processing events, such as 3' end formation, mRNA export, and mRNA translation efficiency.

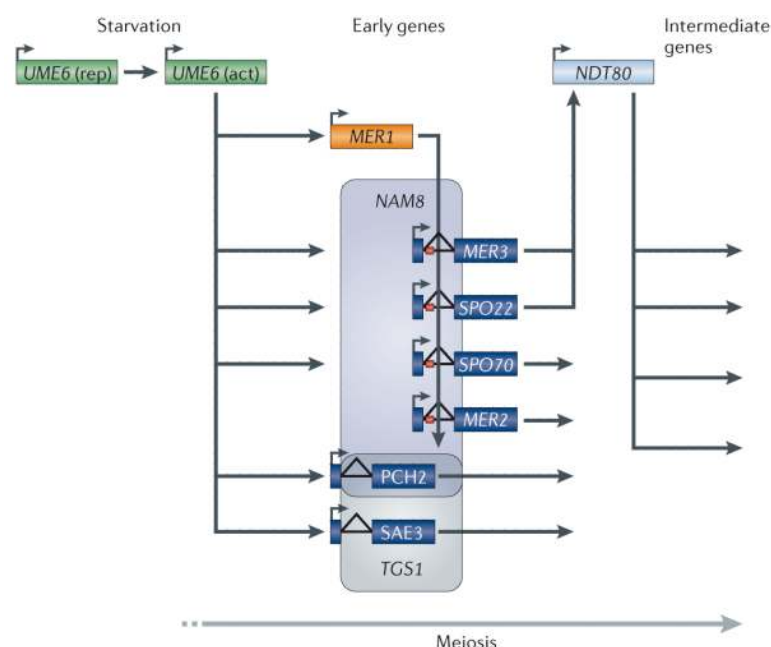


Figure 1. Role of splicing regulation during early meiosis in *Saccharomyces cerevisiae*
 Starvation induces a switch in the Ume6p transcription factor from a repressor [UME6 (rep)] in vegetative cells to an activator [UME6 (act)] to initiate meiosis during sporulation. Ume6p activates multiple early 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 (orange arrow). Two Mer1p 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 Mer1p-regulated genes compared to other *UME6* targets creating a lag period prior to *NDT80* activation³⁵. Mutation analyses demonstrated separate but overlapping meiotic-dependent splicing requiring *MER1*, *NAM8* (blue shaded area), or *TGS1* (grey shaded area)⁴¹.

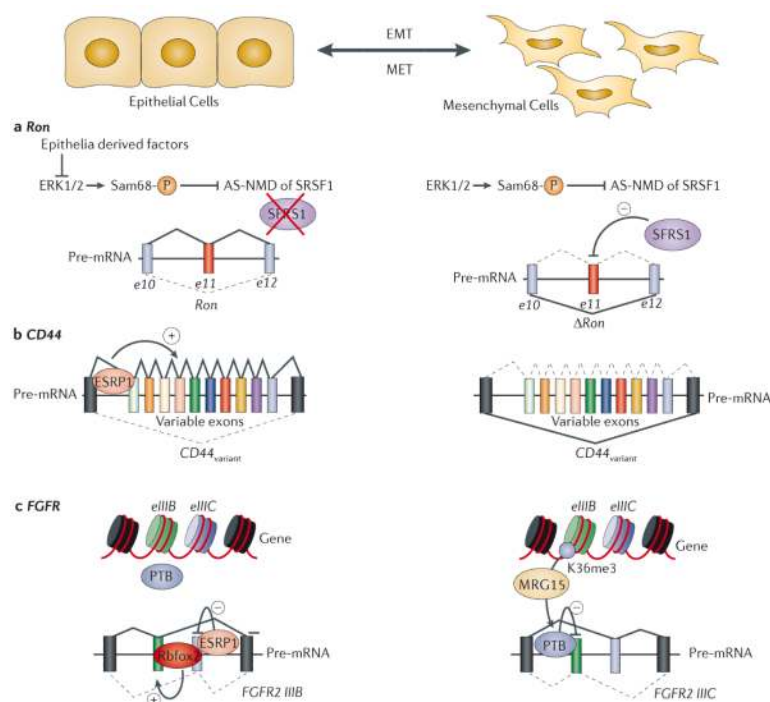


Figure 2. Integration of alternative splicing with epithelial-to-mesenchymal transitions

The scheme at the top represents epithelial and mesenchymal inter-conversion. Epithelial (left) and mesenchymal (right) splicing that directly influence EMT are depicted in the three panels below. (a) SRSF1 triggers EMT by promoting skipping of exon 11 of the Ron proto-oncogene to produce a constitutively active isoform (Δ Ron) that confers an invasive phenotype⁷⁶. SRSF1 levels are dynamically controlled during EMT through AS-NMD by another splicing factor, Sam68⁷⁸. Epithelial cell-derived soluble factors repress ERK activity, thereby inhibiting Sam68 phosphorylation, which reduces SRSF1 levels through increased AS-NMD⁷⁸. (b) ESRP proteins are key regulators of the epithelial cell splicing network⁷⁷. ESRP1 downregulation leads to a switch from CD44(v)ariant to CD44(s)tandard isoforms that is crucial for EMT⁷⁹. 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 isoform⁷⁹. CD44s and not CD44v potentiates Akt activation in TGF β -induced EMT assays, providing a functional link between alternative splicing and a key signaling pathway that drives EMT⁷⁹. (c) Mutually exclusive splicing of fibroblast growth factor receptor 2 (FGFR2) exons IIIb and IIIc is regulated by multiple splicing factors in communication with chromatin modifications. ESRP proteins inhibit exon IIIc while Rbfox2 promotes exon IIIb inclusion in epithelial cells¹⁴⁴⁻¹⁴⁵. PTB is expressed similarly in mesenchymal and epithelial cells but suppresses exon IIIb specifically in mesenchymal cells⁸². The selective suppression is due to mesenchyme-specific enrichment of the H3K36me3 histone modification on chromatin near the IIIb exon. Binding of the adapter protein MRG15 to the H3K36me3 histone mark recruits PTB near exon IIIb resulting in IIIb skipping⁸⁴.

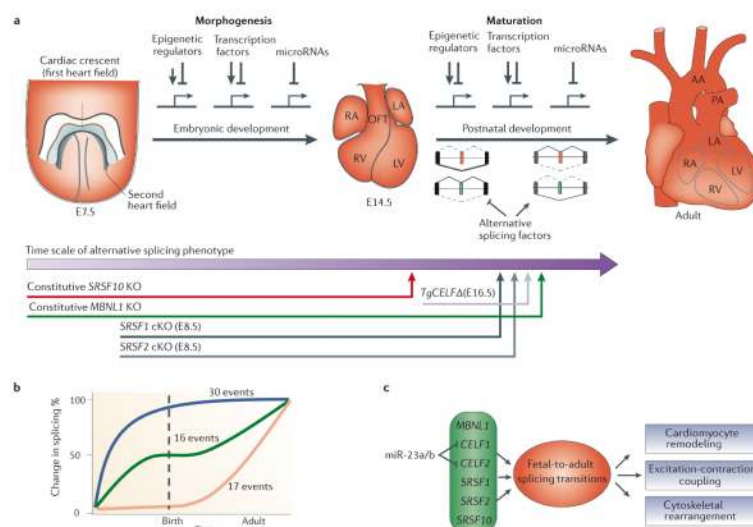


Figure 3. Coordinated alternative splicing changes drive fetal-to-adult transitions during postnatal heart development

(a) Two phases of mouse heart development. 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 including miRNAs⁸⁶. Growth and maturation in the second phase starts at 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 (MBNL1 and SRSF1, 2, 10)^{92-94,146} or transgenic expression (TgCELFΔ)⁹¹ of several splicing regulators in heart, which display late embryonic or a postnatal phenotypes, preceded by alternative splicing changes. The bottom shows timing of individual splicing regulator knockouts/transgenic expression indicated with colored lines and when splicing changes appear indicated by arrows. LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium; OFT, outflow tract; AA, aortic arch; PA, pulmonary artery. (b) Fetal-to-adult alternative splicing transitions are temporally coordinated to occur at specific times during development²⁶. (c) Members of CELF, MBNL, and SR family of splicing factors are regulators of splicing transitions that directly influence cardiac remodeling, EC coupling and cytoskeletal rearrangement. Postnatal up regulation of miRNAs suppress expression of multiple alternative splicing regulators which results in a physiological shift in fetal-to-adult splice patterns. In particular, postnatal up-regulation of two miRNAs that bind to the same seed sequence (miR-23a/b) causes the >10-fold down-regulation of CELF1 and CELF2 proteins⁸⁹.

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

Tissue type, cell type or process	GO terms enriched in genes regulated through Alternative splicing	GO terms enriched in genes regulated through transcript levels	Refs
Brain or Neural Tissue	GTPase-based signaling, cell-cell signaling, cytoskeletal organization and biogenesis, vesicular mediated transport, transmission of nerve impulse, and neurophysiologic process	Synaptic function, nerve impulse and transmission, nervous system development, cytoskeletal organization and biogenesis, and secretory pathways	163
Heart development	Cell structure and motility, cytoskeletal remodeling, cell signaling, 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 signaling	26
Skeletal muscle differentiation	Cytoskeletal organization, actin binding, cell junction and nucleotide kinase, integrin signaling pathway, nucleic acid metabolism, and RNA splicing	Muscle contraction, muscle development, cytoskeletal organization, cell signaling, 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, 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 defense response, cytoskeletal protein binding (affected early); cell cycle (affected late)	30
Ca ²⁺ induced cell excitation	Cell signaling (affected early); RNA splicing, transcription, cell cycle, apoptosis, lipid and carbohydrate metabolism (affected late); Ca ²⁺ ion-binding, cell adhesion, plasma membrane, and extracellular matrix (affected throughout the time course).	Lipid and carbohydrate metabolism (affected late); transcription, Ca ²⁺ ion-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 (GO) annotation terms.