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Alternative splicing: decoding an expansive regulatory layer

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Alternative splicing (AS) is the process by which splice sites in precursor (pre)-mRNA are differentially selected to produce multiple mRNA and protein isoforms. During the past few years the application of genome-wide profiling technologies coupled with bioinformatic approaches has transformed our understanding of AS complexity and regulation. These studies are further driving research directed at elucidating the functions of networks of regulated AS events in the context of normal physiology and disease. Major strides have also been made in understanding how AS is functionally integrated with- and coupled to- gene regulation at the level of chromatin and transcription. Particularly intriguing is the discovery of new AS 'switches' that control transcriptional networks required for animal development and behavior.

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Introduction

Precursor (pre)-mRNA splicing is executed by the spliceosome, one of the most complex cellular machines. The spliceosome comprises five small nuclear RNAs (snRNAs) and on the order of 200 protein components [1]. Decades of research have established that spliceosome assembly occurs in a step-wise fashion. Recently, multi-wavelength fluorescence microscopy has been used to study the assembly process of single spliceosomes. This work has provided evidence that each step in the assembly pathway contributes to the formation of a fully-committed splicing complex, but is reversible [2••]. Therefore, potentially any step during spliceosome formation might be subject to regulation.

Although examples of alternative splicing (AS) regulation at later steps in spliceosome assembly have been reported [3], most regulation is thought to occur at the earliest stages of the assembly pathway, via the interplay between *cis*-acting and *trans*-acting factors that either promote or repress the recognition of core splicing signals (5′ splice site, branch site, and polypyrimidine tract-3′ splice site) (Figure 1). Although interesting examples of secondary structures controlling the use of alternative splice sites have been discovered [4], most characterized regulatory mechanisms involve the recognition of short, degenerate RNA motifs in exons and introns by RNA binding proteins and associated factors [5] (Figure 1).

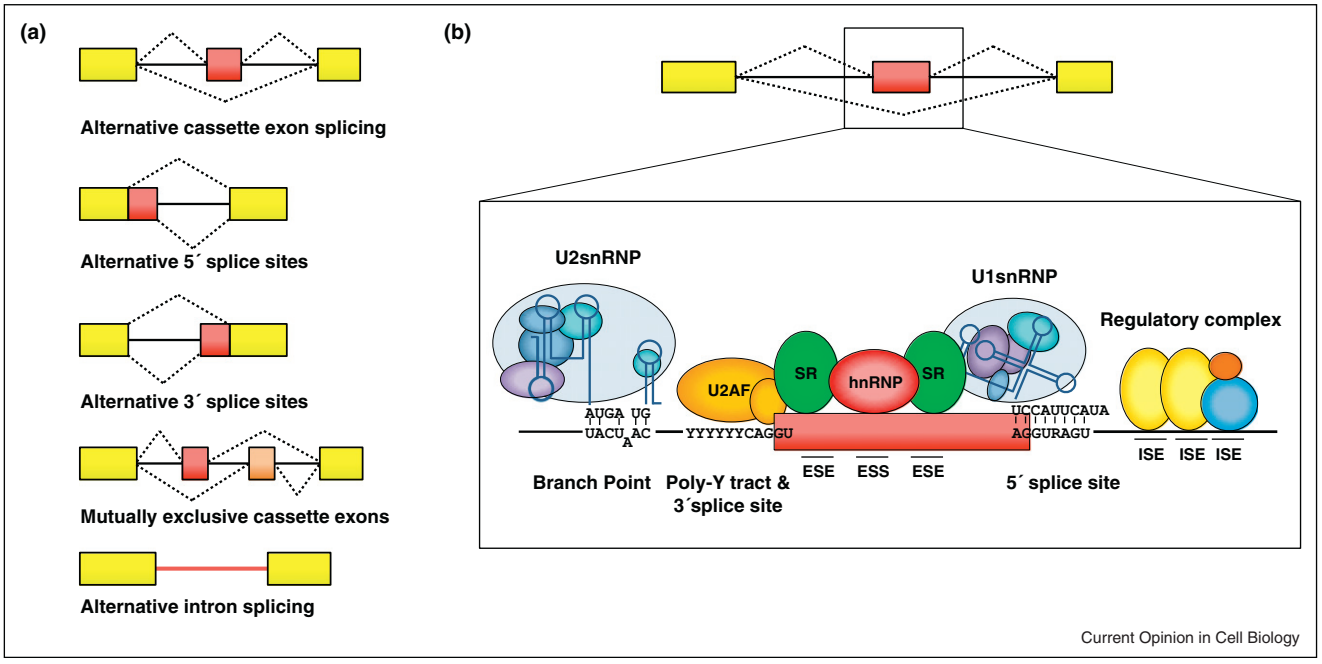
A major challenge in the AS field for many years has been to determine the complex combinations of *cis*-elements in pre-mRNA, collectively referred to as the 'splicing code', that discriminate correct from incorrect splice sites, and that direct constitutive and AS patterns. A related challenge has been to understand how AS is integrated with other layers of gene regulation, as well as how physically-linked complexes, such as those involved in chromatin remodeling and transcription, impact AS regulation.

In this brief review, we will highlight recent advances that relate to these questions, focusing in particular on studies that have successfully integrated transcriptome profiling, focused experimental approaches, and computational methods, to provide new and timely insights into AS regulation and important roles for AS in normal physiology and disease (Figure 2). Since this review cannot be a comprehensive survey of progress in the field of AS in recent years, we refer the reader to several excellent reviews that cover specific subtopics in greater detail [5–16].

Exon networks, RNA binding protein maps and the splicing code

The advent of splicing-sensitive microarrays and the more recent implementation of high-throughput RNA sequencing (RNA-Seq) technologies have transformed how we view and analyze AS. Datasets generated by these methods, which are on an exponential rise, have facilitated the identification of thousands of regulated AS events (as well as other types of transcriptomic changes) in diverse biological contexts. These include various models of cell differentiation and fully differentiated tissues (e.g. [17–20], reviewed in [21]), developmental time series in model organisms [22••,23–25], the circadian cycle [26••], normal-versus-disease states (e.g. [27•,28–31]), and sets of AS events that respond to physiologically-normal and disease-associated changes, such as genotoxic stress [32••,33•,34••,35•]. These datasets have further revealed AS events affected by specific experimental conditions, such as siRNA-mediated knockdown or genetic knockout of individual splicing factors (e.g.

Figure 1



(a) Major types of alternative splicing events. **(b)** Schematic representation of core spliceosomal components that bind to the canonical splicing signals (5′ splice site, branch point, polypyrimidine tract, and 3′ splice site). Additional *cis*-acting elements in exons and introns that control splice site recognition are also shown. Although the diagram depicts positive and negative acting roles for SR and hnRNP proteins, respectively, depending on the location of the binding sites of these factors, they can also act in the opposite manner. Similarly, various tissue-dependent splicing factors can either promote or repress splice site selection depending on the location of their binding sites with respect to splicing signals. ISE, intronic splicing enhancer; ISS, intronic splicing silencer; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; SR, Ser/Arg-repeat containing protein; hnRNP, heterogeneous ribonucleoprotein (hnRNP); and U2AF, U2 snRNP auxiliary factor.
Adapted from Ref. [99].

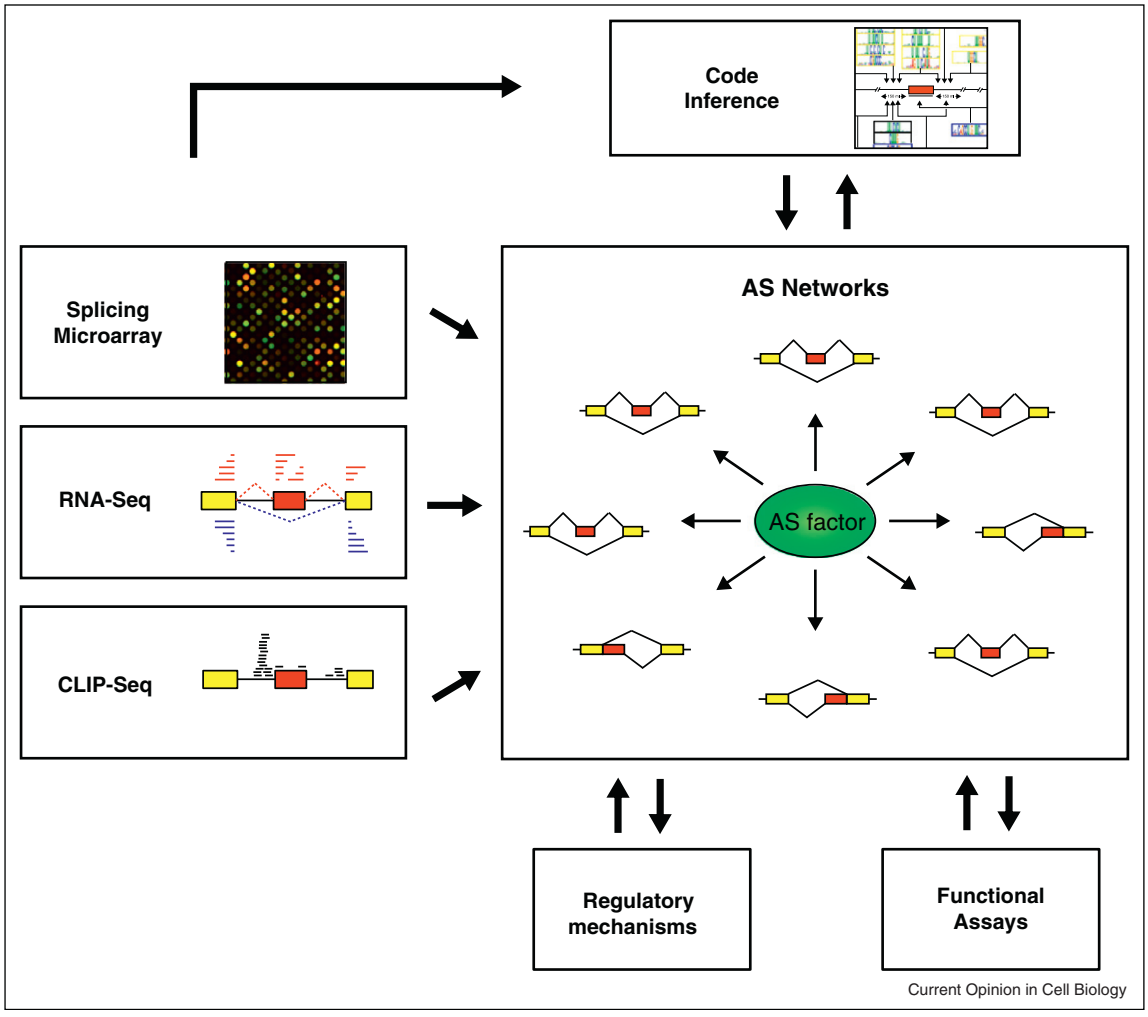
[36,37,38••,39–44]), or perturbations to coupled processes, including transcription and chromatin components that impact AS via coupling mechanisms (see below).

The parallel development of methods enabling the transcriptome-wide mapping of RNA binding protein interactions *in vivo* has greatly complemented AS profiling studies [7,45••,46••]. Increasingly used to date are methods in which RNA fragments cross-linked *in vivo* to a target protein are co-immunoprecipitated using a specific antibody and subjected to RNA-Seq. Mapping of the resulting RNA-Seq reads to genomic sequence provides a snapshot of where the protein of interest binds in transcripts. First referred to as CLIP-Seq or HITS-CLIP [47••], recent variations of this method such as PAR-CLIP incorporate into nascent pre-mRNA a photoactivatable ribonucleoside that preferentially cross-links to bound protein upon exposure to a specific wavelength of light [46••]. The incorporation of the modified nucleoside into RNA creates a base change during reverse transcription allowing more precise mapping of the contact between immunoprecipitated protein and bound RNA [46••].

Originally applied to the neural-specific AS regulator Nova [47••], CLIP-Seq and related methods have since been used to generate binding maps for dozens of RNA binding proteins (RBPs) [45••,46••,48–52]. A recurring theme is that RBPs tend to bind to discrete 'zones' within an alternative exon and/or flanking intronic sequences that correlate with increased exon skipping or inclusion of the exon, as defined by parallel AS profiling [7,8]. In most cases, when the protein binds upstream or within the alternative sequence, exon inclusion is inhibited, whereas binding to downstream regions often enhances inclusion [8]. Interestingly, while the binding specificity and topology of the binding map for Nova and its *Drosophila* ortholog Pasilla have been highly conserved, the sets of exons and genes regulated by these proteins have not [40,53,54].

Another recurring theme is that RBPs involved in AS regulation appear to often 'moonlight' by controlling other post-transcriptional processes such as alternative polyadenylation [47••]. Likewise, RBPs originally implicated in processes such as mRNA stability appear to also function as AS regulators [55••,56]. The widespread multi-tasking nature of RBPs revealed by recent

Figure 2



Scheme illustrating the integration of technologies and resulting datasets for the large-scale analysis of AS. Data generated from high-throughput profiling (using microarrays, RNA-Seq, and CLIP-Seq) of different tissues, cell types, or conditions, facilitates the identification of networks of co-regulated exons (AS networks). This information is used to infer splicing codes, regulatory mechanisms, and serves as a basis for directing the functional analysis of AS events in genes that operate in common processes and pathways.

high-throughput approaches echoes earlier findings demonstrating that splicing factors from the Ser/Arg (SR)-repeat and hnRNP families have diverse RNA-related functions in the cell (e.g. [57–60]). Interestingly, the multi-functional nature of RBPs could facilitate coordination between different regulatory layers (see below), while maximizing the repertoire of regulatory interactions within a finite set of *trans*-acting factors.

The combination of transcriptome-wide data and advanced machine learning algorithms is beginning to have a major impact on studies directed at elucidating the splicing code. For example, the integration within a Bayesian network of information from Nova HITS-CLIP experiments, microarray profiling, and computational searches of Nova binding motifs, enabled the identification of ~700 high-confidence Nova-dependent AS events [61••]. Assembly of this expanded set of AS events led to the discovery that motifs corresponding to binding sites for Rbfox (Fox) proteins are often located in intron sequences flanking Nova-dependent exons, which led to the demonstration that Nova and Fox proteins can synergize in the regulation of a subset of Nova-dependent exons [61••].

The ultimate goal in deciphering a splicing code is to be able to infer from genomic sequence alone comprehensive sets of *cis*-elements that impart AS patterns in the widest possible range of cell types and conditions, as well as to reliably predict the consequence of genomic variation and mutations on splicing. While the field is some way off this lofty goal, progress has been made. A machine learning algorithm has been developed that is capable of

automatically extracting combinations of *cis*-elements (from a compendium of over 1000 splicing-associated *cis*-elements), that are maximally predictive of brain-specific, muscle-specific, digestive-organ specific, and embryo versus adult-specific AS patterns [62••]. Using the resulting splicing code, genome-wide predictions for the different classes of tissue-dependent AS events were generated and validated at a high rate. This work demonstrates that it is feasible to computationally infer complex splicing codes for diverse types of regulated splicing events, and therefore has provided useful groundwork for future development in this area.

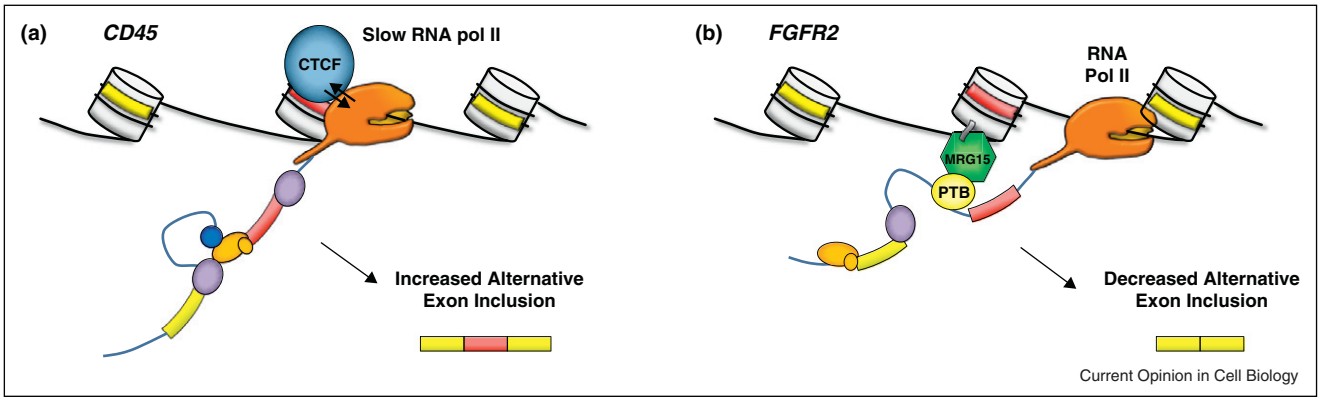
In related work [63••], a computational method was developed for predicting splicing disrupting mutations by exploiting the principle that the preferred binding location of a splicing factor with respect to splice sites is directly correlated with its positive-acting function, whereas a mutation that creates a binding site for the splicing factor in the 'wrong' location is expected to disrupt splicing. Using this principle as predictive strategy on a genome-wide scale, the authors were able to correctly predict splicing alterations for previously uncharacterized disease mutations. The results from this study further reinforce previous estimates that one third or more of human disease mutations affect splicing.

Regulatory communication between chromatin, transcription and splicing

A major development in the past few years was the discovery that nucleosomes, together with associated specific modifications such as trimethylation of histone 3 at lysine 36 (H3K36me3), are preferentially enriched over exons relative to introns, and further display characteristic density profiles surrounding the 5′ and 3′ splice sites [9,10,64]. These observations immediately suggested that local differences in chromatin modification and compaction might play an important role in exon definition and AS regulation. In light of previous models for coupling of chromatin and transcription to splicing [16,65], it was proposed that modified histones might affect AS regulation by facilitating the recruitment of AS regulators to nascent RNA, or by altering RNA polymerase II (pol II) elongation rate (Figure 3).

Recent work has provided evidence for both models. Cell type-dependent increases in H3K36me3 levels over specific exonic regions appear to influence AS of the corresponding exons in nascent RNA, via a mechanism involving an H3K36me3 binding 'adapter' protein (MRG15), which in turn facilitates the recruitment of the splicing repressor PTBP1 to the regulated exons [66••]. In another study, elevated levels of histone 3 trimethylated at lysine 9 (H3K9me3), which are associated with an accumulation of the transcriptional repressor HP1γ, were correlated with reduced pol II elongation and a change in AS of CD44 transcripts [67]. Conversely, splicing activity was shown to be important for establishing H3K36me3 levels, apparently by influencing the recruitment of the H3K36 methyltransferase SETD2 to nascent RNA pol II [68••,69]. Moreover, binding of Hu family proteins to RNA can induce local histone hyperacetylation, leading to increased pol II elongation rates and changes in exon inclusion levels [55••]. Collectively, these studies emphasize the importance of bi-directional communication between chromatin and nascent splicing complexes in not only controlling AS, but also in establishing or perhaps reinforcing epigenetic and other chromatin modification patterns.

Figure 3



Models for coupling between chromatin and AS. **(a)** Specific histone modifications and/or protein factors alter local RNA polymerase (pol) II elongation rate, which affects the accessibility of *cis*-competing splice sites and leads to a change in AS levels. A recent example is the creation of a pol II pause site by binding of the transcription/chromatin factor CTCF in the *CD45* gene. This results in the increased inclusion of exon 5 of this gene during B cell maturation [70••]. **(b)** Chromatin modifications recruit splicing regulators that directly control AS. In the example, increased levels of H3K36me3 (histone H3 modified by trimethylation of lysine 36) in an alternatively spliced region of *FGFR2* facilitates the recruitment of the splicing factor PTBP1 (PTB; yellow) to nascent RNA via the H3K36me3 binding 'adaptor' protein MRG15 (green) [66••].

While it is well established that perturbations to RNA pol II elongation can impact AS [16,65], only recently has the extent and functional significance of such regulation been investigated. UV-induced DNA damage affects the AS levels of many genes associated with the cell cycle and apoptosis, and these effects were linked to altered elongation rates arising from changes in phosphorylation status of the pol II carboxyl-terminal domain [32••]. It was subsequently demonstrated that a variety of conditions that inhibit pol II elongation, including cell stress, preferentially affect AS levels of genes that function in RNA binding and processing [33•]. A disproportionately high number of the affected exons were shown to introduce premature termination codons that elicit nonsense-mediated decay (NMD), thereby additionally contributing to decreased transcript levels. Pol II elongation-coupled regulation of AS may therefore serve as a rapid response mechanism to coordinate the levels of RNA processing factors with mRNA levels in response to cell stress and other changes in cell growth conditions [33•].

In principle, any chromatin component or transcription factor that impacts pol II elongation has the potential to affect the regulation of proximal AS events in nascent RNA. A recent and interesting example involves the CCCTC-binding factor (CTCF), which controls transcription through effects on nucleosome organization and insulator activity. It was shown that CTCF controls AS of CD45 transcripts during B cell maturation by creating a local site of pol II pausing proximal to the alternatively spliced exon 5 in this gene [70••]. Since CTCF binding is controlled by CpG methylation, its effects on AS may in turn be controlled by differential methylation patterns [70••]. In this regard, several reports have shown that CpG methylation levels are elevated over exons relative to introns, and that different densities of CpG methylation over specific exonic regions may be related to differential AS (e.g. [71•,72,73]). An important future goal will be to determine the extent to which epigenetic marks such as DNA methylation, as well as various histone modifications, impact AS levels in functionally important ways.

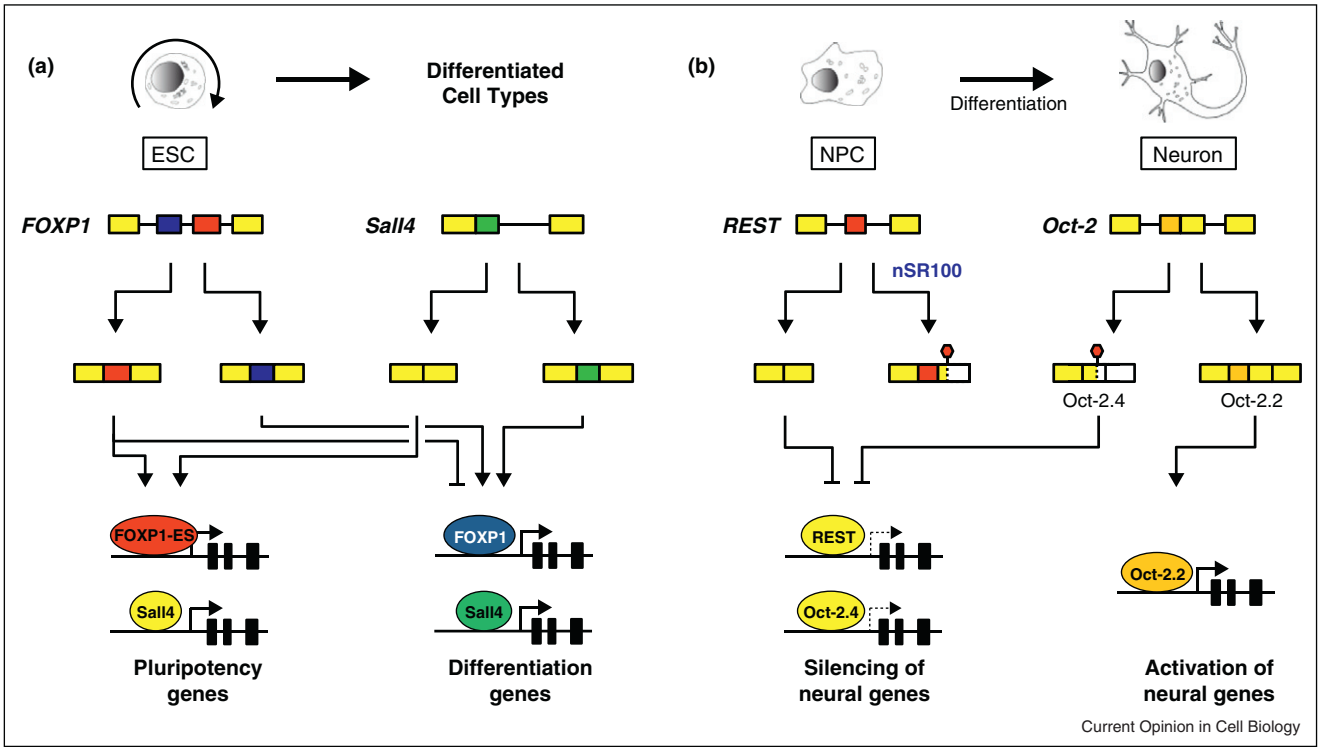
New roles for AS in development and disease

Alternative splicing is a rapidly evolving layer of regulation, and in comparisons of species separated by >70 million years of evolution (such as human and mouse), most AS events are found to be species-specific [12]. Interesting cases of functional roles for species-specific AS events are emerging. For example, the vampire bat expresses a trigeminal ganglion-specific splice isoform of the transient receptor potential cation channel V1 (TRPV1) gene. In contrast to the othologous receptor in its fruit-feeding bat relatives, this isoform has a lower temperature threshold, enabling infrared sensing of warm-blooded animals [74••]. By contrast, large-scale profiling studies have revealed sets of AS events associated with changes in cell differentiation and development that are often evolutionarily conserved. These sets of co-regulated exons (referred to as exon or AS networks) are typically enriched in functionally-related genes, which generally do not overlap with genes that are differentially regulated in the same biological contexts at the transcriptional level (reviewed in [11,14,75]).

Profiling of AS during differentiation provided evidence that isoform complexity may be more extensive in embryonic stem cells (ESCs) and becomes restricted or 'specialized' as ESCs differentiate [19]. Intriguingly, a few AS events specifically detected in ESCs have been implicated in the control of transcription and signaling factors that have key roles in pluripotency and other aspects of ESC biology [76–78,79••]. A particularly striking example is an ESC-specific AS switch that alters the DNA binding specificity of the FOXP1 transcription factor, such that it stimulates the expression of key pluripotency transcription factors including *OCT4* and *NANOG* in ESCs, while simultaneously repressing many genes involved in differentiation [79••] (Figure 4). The ESC-specific isoform of FOXP1 was further shown to promote pluripotency and to be required for the efficient reprogramming of somatic cells to induced pluripotent stem cells [79••]. Examples of other AS events impacting the activity of transcriptional regulators that have key roles in the control of neurogenesis, other aspects of development, and animal behavior, have also been reported recently (e.g. [80•,81–83]) (Figure 4). These AS switches are reminiscent of classic examples of pivotal AS events that control transcriptional programs required for sex determination and courtship behavior in *Drosophila* [84].

High-throughput sequencing and other profiling technologies are greatly accelerating our understanding of the roles of altered splicing in the predisposition, onset and progression of human diseases. The combination of genomic DNA sequencing and RNA-Seq analysis of disease versus normal tissue is revealing disease-driving mutations, as well as the direct and indirect consequences of these mutations on AS and other layers of transcriptome regulation (e.g. [85,86]). Whole exome sequencing of tumor and control DNA from patients with myelodysplasia has revealed frequent mutations in components of the splicing machinery required for the recognition and regulation of 3′ splice site selection [87•]. Other exome sequencing studies have identified mutations linked to poor prognosis in chronic lymphocytic leukemia that target the splicing factor SF3B1 [88,89]. Transcriptome profiling of brain tissue has revealed frequent AS changes linked to the misregulation of RBFOX1 in patients with autism spectrum disorder [27•]. Other profiling studies are uncovering widely occurring AS changes in animal models of human diseases, as well as in patients with neurodegenerative disorders including Alzheimer's disease, amyotrophic

Figure 4



Alternative splicing switches control the activity of key transcription factors during cell differentiation. **(a)** Embryonic stem cell (ESC)-specific isoforms of the transcription factors FOXP1 (red) and Sall4 (yellow) induce the expression of sets of pluripotency genes while concomitantly repressing genes required for differentiation, whereas alternative isoforms (inclusion of dark blue and green sequences, respectively) of these factors lack these activities and contribute to differentiation [76,79••]. **(b)** During neural differentiation, an nSR100/SRRM4-dependent AS switch in transcriptional silencer of neurogenesis genes REST, results in a truncated isoform that lacks the capacity to repress gene expression [81]. In the case of AS of *Oct-2*, isoforms with differing C-termini have opposing roles: inclusion of an alternative sequence (light green) produces an isoform containing a transactivation domain that induces the expression of neural genes, whereas the exclusion of this sequence produces a shorter protein that lacks the transactivation domain and represses neuronal differentiation [83].

lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), as well as cancers (e.g. [28–31]).

Finally, AS is being implicated in almost every aspect of tumor biology, including promotion of Warburg type metabolism, apoptosis, cell cycle control, metastasis, and angiogenesis (reviewed in [15]). In a recent report, Paronetto *et al.* investigated the role of Ewing sarcoma (EWS) protein in the regulation of AS in response to DNA damage [34••]. Upon UV irradiation, EWS transiently localizes to the nucleoli, and thus no longer can bind to its pre-mRNA targets. This affects the regulation of many AS events in genes that function in DNA repair and genotoxic stress signaling, including an AS event that is required for the expression of c-ABL protein. Depletion of EWS protein reduced cell viability and proliferation upon UV irradiation, but these effects could be reversed by restoring c-ABL expression. This and other recent studies highlight important cancer-relevant roles for misregulated AS events as a consequence of altered expression of individual splicing regulators.

Future directions

From reviewing recent progress in the field of AS, it is clear that we are in the middle of a 'transcriptomics revolution', in which increasingly powerful technologies and the datasets they produce are facilitating major advances in our understanding of the complexity, regulation, integration, and function of AS. Combined computational and experimental approaches are further expected to ultimately yield a 'complete' regulatory code, that is, a full map of *cis*-acting elements operating at the levels of DNA (i.e. in chromatin) and RNA that dictate all classes of transcriptomic events, including AS. Identifying and systematically determining the functions of biologically-relevant AS events remains a major challenge for the field. A first step will be to comprehensively determine which of the myriad of splice variants are actually translated. Increasingly sensitive mass-spectrometry methods coupled to cellular fractionation, as well as 'ribosome profiling' promise to advance knowledge in this area [90,91]. Likewise, systematic functional studies will be greatly accelerated by the development of automated assays for interrogating process and

pathway-specific activities of networks of individual splice variants [92].

A more comprehensive understanding of the mechanisms of AS regulation will emerge not only from the combination of genome-wide and focused experimental and computational approaches, but also from ongoing important studies investigating roles for upstream signaling pathways that impact AS by altering the stability and post-translational modification status of regulatory factors [93••]. In this regard, we can anticipate the discovery of many new AS regulators in the years to come, in particular with the recent identification of thousands of new ncRNAs and antisense transcripts [94,95] that as yet lack known functions. In addition, the discovery that AS of UTRs can regulate miRNA targeting (e.g. [77]) further highlights the importance of understanding how different gene regulatory layers are integrated.

Finally, with ongoing major strides being made in linking specific AS events and RNA regulators to the onset and progression of human diseases [13], the future holds tremendous promise for the development of a new generation of therapeutics designed to specifically correct splicing defects and reprogram AS in medically beneficial ways [96••,97,98].

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UV-induced DNA damage is shown to affect the AS of many genes linked to cell cycle control and apoptosis. These effects, which occur via a p53-independent pathway, are linked to reduced rates of pol II elongation through a mechanism that is associated with the hyperphosphorylation of pol II.

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Diverse conditions that inhibit pol II elongation, including cell stress, are shown to concomitantly affect the AS and steady-state transcript levels of many genes encoding RNA processing factors. Many of the regulated AS events trigger nonsense-mediated mRNA decay, thereby leading to further reductions in transcript levels. The results provide evidence that elongation-coupled regulation of AS functions to coordinate the levels of RNA processing factors with transcript levels in response to changes in growth conditions including cell stress.

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Depletion of the Ewing sarcoma protein (EWS), which binds to DNA and RNA, results in changes in AS of genes involved in DNA repair and genotoxic stress signaling. Similar AS changes are observed following irradiation of cells with UV light, which causes EWS to transiently localize in nucleoli. One of the EWS-regulated AS changes leads to reduced c-ABL protein expression. Supporting an important role for the EWS-regulated AS network, the authors demonstrate that its depletion reduces cell viability and proliferation upon UV irradiation, but that these effects can be attenuated by restoring c-ABL expression.

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Genotoxic stress inducers are shown to inhibit an interaction between EWS and the splicing associated protein YB-1. This results in the cotranscriptional skipping of exons of many genes, including exons in the *MDM2* gene, a p53 ubiquitin ligase. This leads to reduced *MDM2* expression and an accumulation of p53 during stress exposure. Collectively, the results in Refs. [32••,33•,34••] and this study highlight the importance of communication between the transcriptional and splicing machineries in the control of AS networks linked to genotoxic stress response.

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