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Decrypting the genome's alternative messages

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Alternative splicing of messenger RNA (mRNA) precursors affects the majority of human genes, has a considerable impact on eukaryotic gene function and offers distinct opportunities for regulation. Alterations in alternative splicing can cause or modify the progression of a significant number of pathologies. Recent high-throughput technologies have uncovered a wealth of transcript diversity generated by alternative splicing, as well as examples for how this diversity can be established and become misregulated. A variety of mechanisms modulate splice site choice coordinately with other cellular processes, from transcription and mRNA editing or decay to miRNA-based regulation and telomerase function. Alternative splicing studies can contribute to our understanding of multiple biological processes, including genetic diversity, speciation, cell/stem cell differentiation, nervous system function, neuromuscular disorders and tumour progression.

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Introduction

The 30-year-old discovery of the bizarre syntax of eukaryotic genes, whereby primary transcripts of messenger RNAs are interrupted by intronic sequences, was soon followed by the realization that alternative pathways of intron removal (alternative splicing, AS) can lead to the synthesis of multiple mRNAs from a single gene (Figure 1). Generating distinct combinations of RNA and protein domains by AS affords diversification and regulation of gene function. Important advances in our understanding of the prevalence of AS, its biological and medical significance and the underlying molecular mechanisms of regulation have been made in recent years and have been covered by a comprehensive series of reviews [1••]. The aim of this manuscript is to highlight information published over the past two years that makes an eloquent case for AS as an essential component of gene function (and its alterations) in higher eukaryotes. We will first review examples of AS events with deep impact on fundamental biological processes, from the function of the nervous system to tumour progression to speciation. We will then provide an overview of molecular mechanisms of regulation and their functional coupling with other steps of gene expression. Finally, we will review novel high-throughput approaches used to detect and quantify AS and evaluate their impact on our views for how cells establish cellular programmes of splicing regulation.

Biological and biomedical significance

Instances of AS events with profound implications in gene function proliferate in a diverse range of biological contexts. For example, AS of the N-type calcium channel-coding gene $Ca_v2.2$ modulates signals transmitted by pain receptive neurons to the brain. Inclusion of exon e37a results in a protein isoform with a tyrosine phosphorylation site that increases the sensitivity of the channel to the analgesic effects of endocannabinoids and opiate drugs [2]. In another remarkable example, AS generates isoforms of the receptor Robo3 with opposing functions in the migration of axon growth cones of spinal commisural neurons. Expression of these isoforms is regulated at subsequent steps of the gene expression cascade, switching growth cones from attraction to repulsion and facilitating proper neuronal wiring [3] (Figure 1b).

Evidence accumulates that AS is important in cancer [4]. The gene encoding SF2/ASF, one of the founder members of the SR family of splicing regulators, was demonstrated to be a potent oncogene [5••]. The tumourigenic effects of SF2/ASF overexpression can be explained, at least partly, by changes in AS of tumour suppressors and oncogenic kinases that lead to activation of the mTORC1 pathway [6]. Also consistent with the notion that changes in AS can contribute to tumour biology, AS changes have been identified that represent good markers of tumour grade [7], and aberrant splicing has been correlated with resistance to anti-cancer drugs [8]. One example of how AS can contribute to tumour progression concerns a switch between mutually exclusive exons in the gene encoding the glycolytic enzyme pyruvate kinase (Figure 1a). This switch enables tumour cells to maintain elevated levels of glucose uptake and lactate production under aerobic conditions (the Warburg effect), which facilitates tumour growth [9•].

Other pathologies, including myotonic dystrophy and spinal muscular atrophy, are caused by an imbalance of the activities of splicing regulators or factors involved in

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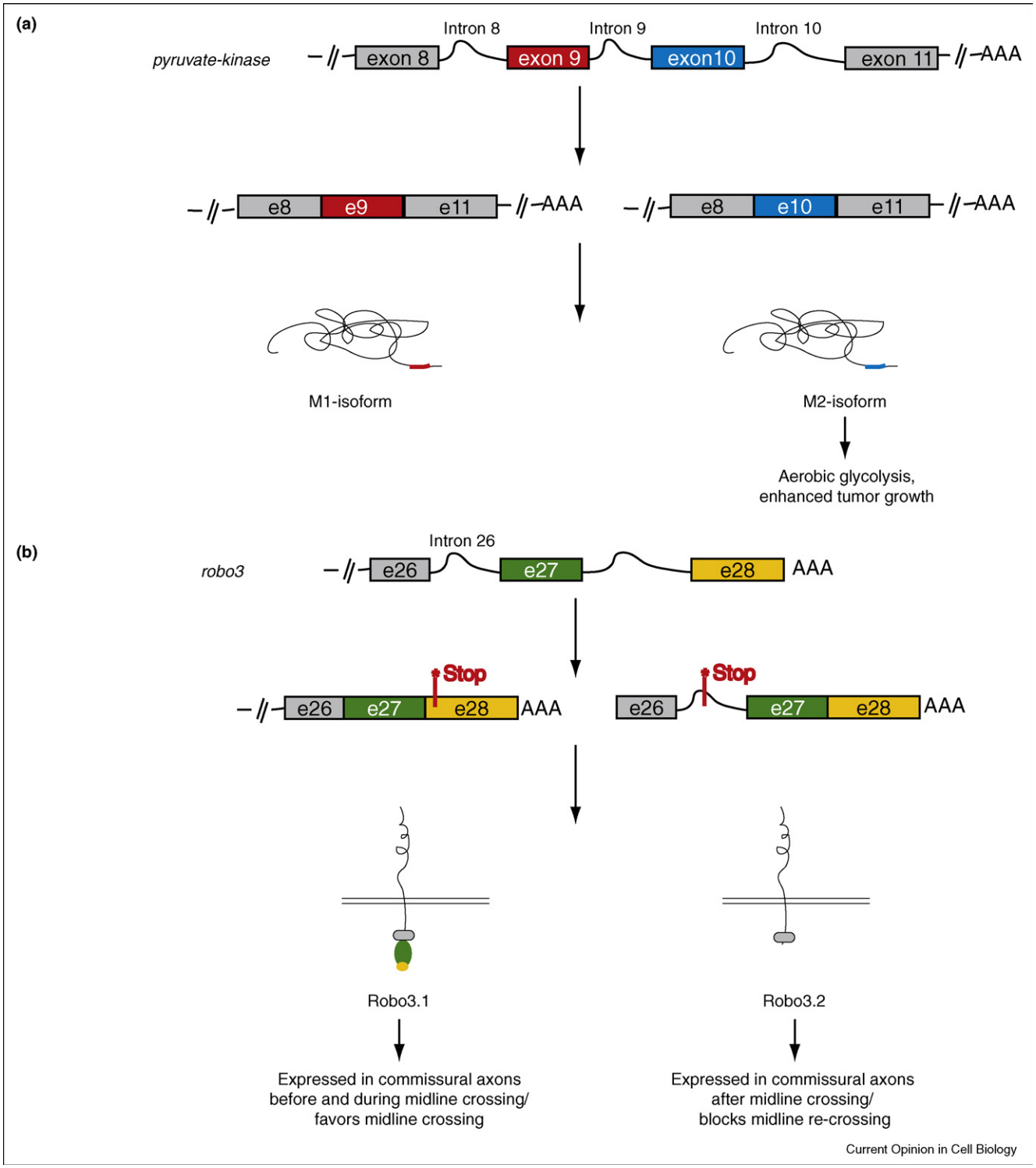
Figure 1



Examples of AS events with important biological consequences. **(a)** AS of *pyruvate kinase*. Exons 9 and 10 of the pre-mRNA are included in a mutually exclusive fashion to generate mRNAs encoding, the M1 and M2 isoforms of the enzyme, respectively. Expression of the M2 isoform confers a metabolic advantage to cancer cells and contributes to tumour progression [9•]. **(b)** AS of *robo 3*. The intron between exons 26 and 27 can be removed or retained in mature mRNAs, encoding proteins with different carboxy-termini that confer distinct repulsive/attractive properties to the axon growth cones of spinal commissural neurons [3].

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assembling splicing complexes. AS changes associated with these conditions have been identified and offer the potential to explain clinical features and tissue-specific effects of these conditions [10,11•,12•]. These findings open the door to novel therapy strategies. For example, isoform-specific drugs could provide higher efficacy and reduce secondary effects of analgesics and anticancer compounds. Modulation of AS by chemicals or oligonucleotide-based reagents is an additional therapeutic possibility that is starting to yield encouraging results [13–15].

While the functional relevance of the majority of AS events remains to be documented, it seems likely that this layer of gene regulation makes general contributions to cell and organism phenotypes. Eight percent of differences in tissue-specific AS of orthologous exons between humans and chimpanzees could be at the basis of species-specific traits, including cognitive functions [16•]. Differences have been also detected between human individuals and among populations [17•,18•,19•], suggesting that transcriptome variation reflects, and possibly contributes to, human diversity.

Regulation at the molecular level

Five small nuclear ribonucleoprotein particles (snRNPs) and more than 200 proteins are involved in the process of intron removal and AS regulation (Figure 2a). *In vitro*, this complex machinery assembles in a stepwise manner on the pre-mRNA. A complex algebra of numerous sequence motifs modulates splice site recognition [20,21] by recruiting protein complexes that antagonize or facilitate initial steps in the assembly of basal splicing components [1••] (Figure 2c). Recent reports are challenging several tenets of these classical models of AS regulation.

First, live imaging studies indicate that at least some regulatory factors interact *in vivo* in the absence of transcription, suggesting the existence of pre-assembled regulatory complexes [22,23]. Second, while these complexes are thought to modulate primarily the thermodynamic stability of the assembly of basal splicing factors on the pre-mRNA, recent results indicate that also kinetic features of the interaction of basal factors with different splice site sequences can determine their relative usage under conditions of competition, a poorly explored aspect of splicing regulation [24•] (Figure 2b). Interestingly, perturbations in the activity of basal spliceosomal components also lead to intron-specific differences in splicing efficiency in budding yeast [25]. Third, while targeting the initial steps of splice site recognition is an efficient mechanism of AS control [1••,26], several reports indicate that regulation can also occur after early complexes are assembled and at the time when specific pairs of 5′ and 3′ splice sites become committed to delineate the boundaries of intron removal [27•,28•,29•,30•,31•,32]. These observations open a wealth of still poorly understood molecular interactions as possible targets for AS regulation.

Two other long-standing concepts have been revisited recently. Most of the known AS regulatory factors, including the large families of hnRNP and SR proteins [1••], are ubiquitously expressed and therefore it has been proposed that tissue-specific splicing relies on variations in their relative expression or activity in different cell types [33,34•]. Interestingly, genetic screens exploiting dual fluorescence AS-based reporters in *C. elegans* and mammalian cells have revealed novel regulators expressed in a tissue-specific manner, suggesting that the catalogue of splicing regulators is incomplete and that cell type-restricted factors can play a major role in tissue-specific splicing [35••,36•]. Although the prevalence of RNA secondary structures as modulators of splice site selection remains unclear, phylogenetic conservation analyses have spurred renewed interest in this topic [37,38]. Of special potential is the observation, reported in fission yeast, that riboswitches (aptamer RNA fragments capable of adopting distinct conformations depending on the binding of a metabolite) induce changes in internal pre-mRNA base pairing that activate or repress particular splice sites [39•].

Finally, knowledge about how signaling pathways regulate the activity of splicing factors and AS remains fragmentary [40]. One interesting example concerns arginine and serine-rich (RS) domains present in SR proteins, which are important for various splicing steps [41] and are targets of phosphorylation by dedicated kinases [42]. Upon heat shock, SRp38 dissociates from inhibitory complexes and becomes dephosphorylated by phosphatase PP1. The versatility of splicing regulators is illustrated by this change in phosphorylation status, which converts this SR protein from a sequence-specific splicing activator to a general repressor that inhibits splicing under heat shock conditions [31•,43].

Collectively, these findings indicate that fundamental principles of the molecular mechanisms of AS regulation remain to be discovered.

Coupling processes, coordinating mechanisms

Intron removal can occur co-transcriptionally and this circumstance influences AS. For example, differences in transcription elongation rate affect the timing at which alternative splice sites become available for competition [1••,44], and factors that influence elongation can thus modulate AS. These include transcription factors [45] and interestingly, also some SR proteins [46•], thus offering a novel mechanism by which they can regulate AS. A second mechanism for coupling between transcription and splicing involves the physical association between RNA polymerase and factors that participate in early

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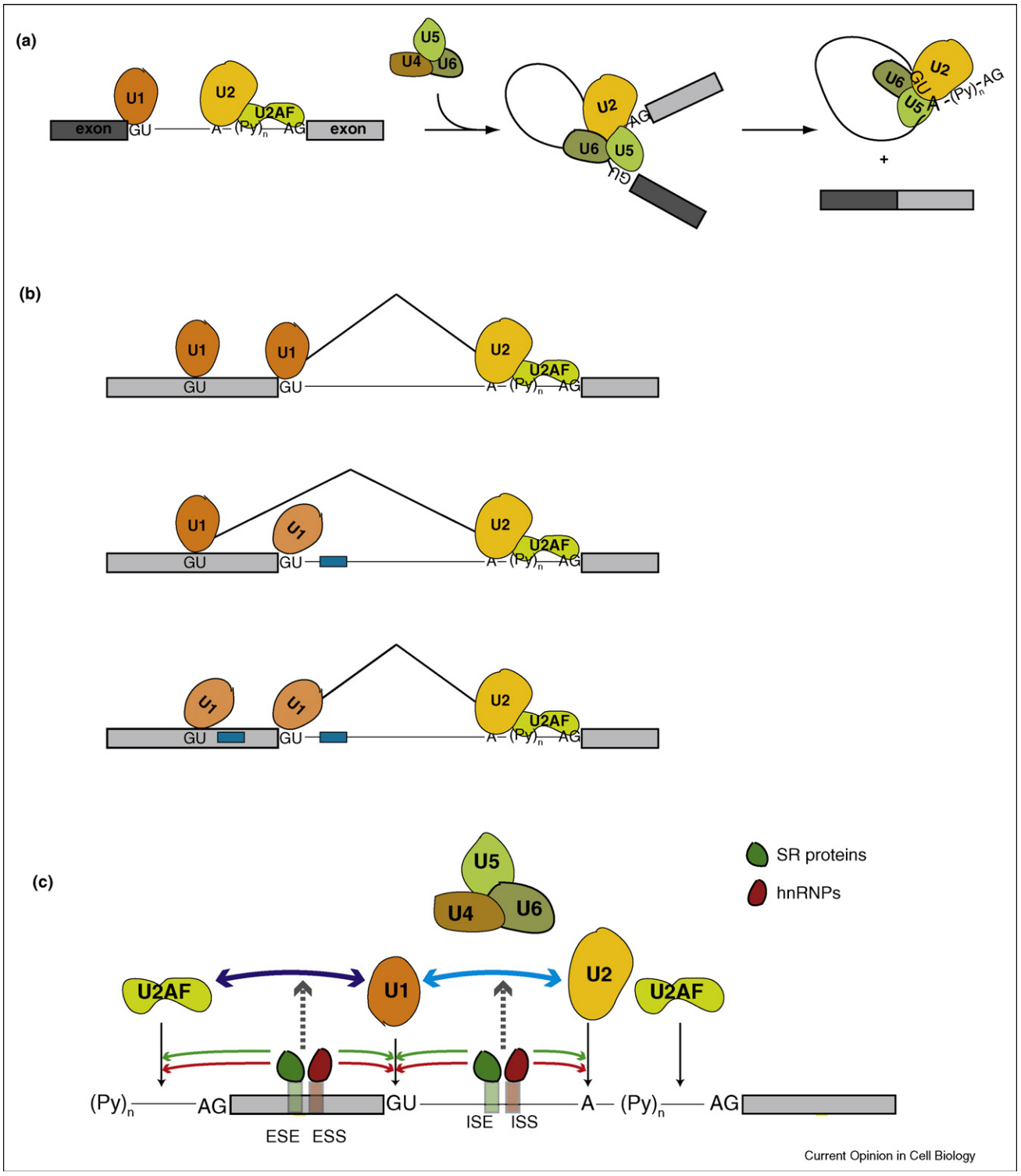
Figure 2



Mechanisms of AS regulation. **(a)** Spliceosome assembly pathway. Sequences at the 5′ and 3′ end of the intron direct assembly of early spliceosomal factors: U1 snRNP recognizes the 5′ splice site (which includes a conserved GU dinucleotide), while the U2AF heterodimer binds to the pyrimidine-rich tract (Py) and the conserved AG dinucleotide at the 3′ splice site. U2AF facilitates assembly of U2 snRNP on an intronic sequence adjacent to the Py-tract, which contains a conserved Adenosine known as the branch site. Incorporation of the U4/5/6 tri-snRNP leads to fully assembled spliceosomes, where the two catalytic steps of the splicing reaction take place, splicing together the exons and releasing the intron in a lariat configuration. **(b)** Kinetic regulation of splice site choice. Different sequence contexts around 5′ splice sites can generate differences in the kinetics with which they are recognized by U1 snRNP, influencing the efficiency of engagement with the downstream 3′ splice site to direct intron removal. Upper diagram: the

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recognition of splice sites, which facilitates loading of splicing factors on the nascent RNA and intron removal [47]. Although SR proteins were thought to be exclusive of organisms which display extensive alternative splice site selection, one budding yeast SR protein-like factor also promotes efficient splicing by enhancing co-transcriptional recruitment of U1 and U2 snRNP to a large set of introns [48]. Other effects of transcriptional coupling on splicing regulation include the influence of promoter architecture, which determines for example meiosis-specific splicing in fission yeast [49••], and the activities of transcription factors, including those leading to modifications of histone tails and chromatin structure [50•].

In addition to generating various protein products, splicing can also influence the posttranscriptional fate of mRNAs. Loading of SR proteins on the mRNA enhances its export to the cytoplasm [51] and even translation [52]. A significant fraction of AS events leads to the synthesis of mRNAs containing premature stop codons that trigger mRNA decay (NMD). In addition to serve as a quality control device for deficient or inaccurate splicing [53], this mechanism can also help to establish the levels of protein expression through auto-regulatory and cross-regulatory circuits [54•,55•]. This is prominent, for example, in splicing factor-coding genes where some of the genomic regions involved correspond to ultraconserved elements, ~200 base pair-long segments which are extremely conserved through long evolutionary distances [56•,57•,58•].

Different untranslated regions generated by AS can determine the susceptibility of an mRNA to other regulatory factors, including miRNAs. For example, proliferating cells tend to generate alternatively spliced 3′ UTRs containing fewer miRNA-binding sites [59•]. Conversely, miRNAs that regulate expression of splicing factors can indirectly control a variety of AS decisions in the genes regulated by the splicing factor, as reported for nPTB [60,61]. The examples above illustrate how different steps in gene expression can be coordinately regulated and how decisions affecting one step can be amplified through their impact in other steps. An interesting extension of this concept is the observation that RNA editing of transcribed transposable Alu elements located in introns is associated with shifted splicing of neighboring exons from constitutive to alternative in human evolution [62].

The splicing process can also contribute to the biosynthesis of other RNA molecules. miRNAs are often contained within introns, and the RNAse III enzyme Drosha carries out the initial endonucleolytic cleavage that leads to the release of miRNA precursors. For a subset of miRNA-containing introns (known as mirtrons), the ends of the miRNA precursors coincide with the intron 5′ and 3′ splice sites, and in these instances the combined activities of the spliceosome and lariat debranching enzyme replace Drosha for the first processing step in the biogenesis of these miRNAs [63•,64]. In fission yeast, the 3′ end of the essential RNA component of telomerase is generated from an intron-containing precursor that undergoes the first, but not the second catalytic step of the splicing reaction, making the (partial) activity of the spliceosome essential to maintain chromosome ends [65•]. These examples expand the classical functions of the spliceosome and illustrate how splicing regulation can indirectly control a variety of cellular processes.

Global views

The advent of high-throughput technologies (Figure 3), including splicing-sensitive microarrays, sequencing of large collections of cDNA/EST libraries and, recently, major advances in our capacity to obtain DNA and RNA sequences, provide a unique momentum for understanding global features of AS [1••,20,66,67]. Combined with elaborate computational tools for sequence analysis [68•] and phylogenetic conservation of regulatory networks [69], these technologies have the potential to provide a comprehensive picture of splicing codes.

Microarray analyses of tens of thousands of AS events in hundreds of biological situations have provided reference catalogues for various transcriptomes [16•,17•,18•,19•,68•,70]. Novel general insights have already emerged from these initial studies, including the realization that genes and functional categories regulated at the level of splicing are largely non-overlapping with those regulated at the level of transcription [16•,66,70]. They have also provided a new perspective to challenge old assumptions. For example, a microarray study in yeast (where splicing regulation was considered anecdotal) demonstrated that splicing of intron-containing genes varies substantially and is differentially regulated under stress conditions [71•].

Deep-sequencing technologies can provide unambiguous, quantitative information about transcript isoform identity and abundance, as well as facilitate the discovery of novel isoforms. While it is unclear whether current state-of-the-art in sequence output and bioinformatic analysis allows

(**Figure 2 Legend Continued**) proximal 5′ splice site is utilized under basal conditions. Central diagram: the presence of particular silencer sequences near the proximal site (blue box) switches splicing to the upstream site because U1 binds in a different, kinetically less favoured conformation. Bottom diagram: these differences become obvious only under conditions of splice site competition, because if the silencer sequence is present at both 5′ splice sites, the proximal site is again favoured. Adapted from reference [24•]. **(c)** Multiple targets of splice site control. Exonic and intronic splicing enhancers and silencers (ESE, ISE, ESS, ISS) bind regulatory factors (e.g. proteins belonging to the SR and hnRNP families) and facilitate (green arrows) or prevent (red arrows) binding of early factors to the splice sites. They can also regulate early factors involved in exon or intron definition as well as target later events in splice site pairing after initial recognition of the splice sites (grey arrows).

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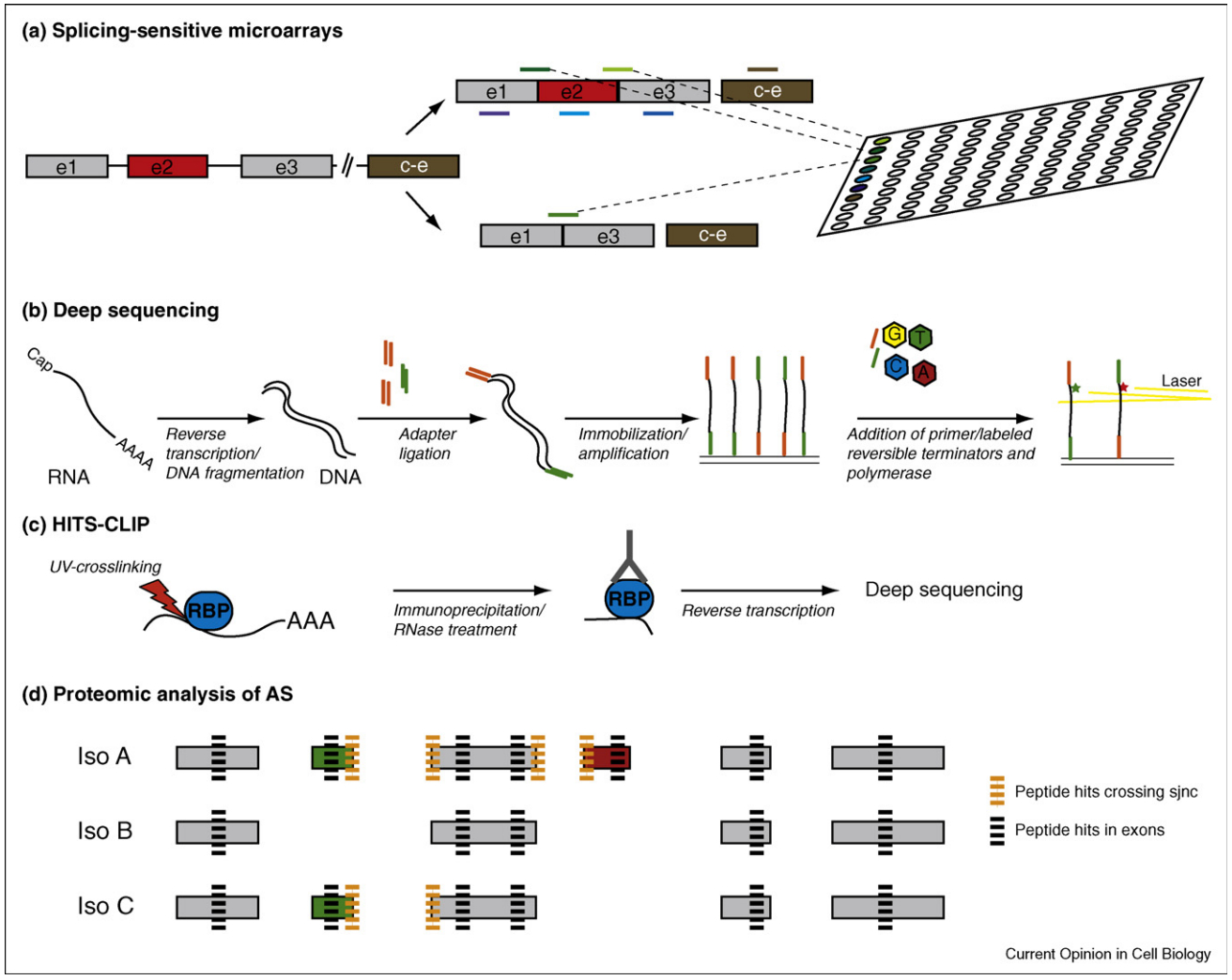
Figure 3



High-throughput technologies for the analysis of AS. **(a)** Splicing-sensitive microarrays. Oligonucleotide probes complementary to constitutive and alternative exons (blue and brown lines), as well as complementary to junction sequences generated upon splicing together constitutive or alternative exons (green lines), are immobilized on a solid surface and hybridized to RNA, typically labeled with fluorescent dyes. The analysis of the relative hybridization signals can be used to infer the expression of AS variants. **(b)** Deep sequencing. Fragments of DNA products of reverse transcription of RNA are ligated to linkers and immobilized on a solid surface, where they are amplified and sequenced using primers complementary to the linker sequences. Immobilization of individual molecules allows parallel sequencing of millions of DNA fragments, which can be used to infer the relative abundance of isoforms generated by AS. **(c)** HITS-CLIP. This technology, utilized to identify RNAs and binding sites for RNA-binding proteins, is based upon capturing RNAs bound to the protein *in vivo* by UV light-mediated crosslinking, partial digestion of the captured RNA molecules and their characterization by reverse transcription and deep sequencing. **(d)** Proteomic analysis of AS. Mass-spectrometry analyses can provide information about protein isoforms by detecting peptides that correspond to alternatively spliced regions of mRNAs (alternative exons or alternative exon junctions).

genome-wide coverage over the full dynamic range of transcript levels present in the cell [72•], third generation sequencers will likely allow the large scale description of full-length mRNAs and therefore complete transcriptome catalogues. The results so far demonstrate that over 90% of the human genes are subject to AS and that our current understanding of transcriptome complexity is far from complete, requiring revised gene models and description of a multitude of non-coding RNAs [73•,74•,75•]; they also indicate that large switches in AS correlate with increased sequence conservation affecting long open reading frames and suggest coordinated regulation of AS and alternative 3′ end processing [74•].

A key question to decipher the logic of splicing control is the identification of RNAs bound by a particular regulatory factor *in vivo*. Crosslinking / immunoprecipitation followed by deep RNA sequencing (HITS-CLIP) can yield this information [76•,77•]. Combining the annotation of binding sites with AS information has generated RNA maps for the splicing regulatory factors NOVA and Fox which are able to predict the outcome of AS in other

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genes [76•,77•,78•]. A reciprocal approach has been to use functional screens to identify factors that regulate a particular AS event. For this purpose, reporters have been generated that generate different fluorescent dyes depending on the pattern of AS used. Screens monitoring fluorescence changes have lead to the identification of splicing regulatory factors whose mutation, deletion or overexpression influences AS events involved in developmental transitions or T cell activation [35••,36•,79,80].

Finally, differential display and sensitive mass-spectrometry techniques able to resolve and quantify protein isoforms are emerging [55•,81]. While the impact of AS changes in protein structure and function remains difficult to assess, these studies confirm the detection of protein isoforms generated by AS in large scale [81] and reveal regulatory circuits relevant to AS [55•].

The emerging picture

Recent technical advances hold the promise to provide reference transcriptomes for organisms, populations and individuals, including quantitative information about isoform abundance in different cell types or biological situations. But decrypting the genome's alternative messages will additionally require cracking the codes of regulatory sequences and activities of regulatory factors that weave cellular programmes of AS—a feat demanding extensive combination of experimental and computational tools-[68•,76•,77•,78•]. The description of AS and its mechanisms of regulation is bound to provide substantial gains in our understanding of many cellular processes. Many exciting questions remain: Will a cocktail of splicing regulatory factors be sufficient to reprogramme a differentiated cell, as is the case for transcription factors? Can drugs be found that regulate biomedically relevant AS events with sufficient specificity? What fraction of AS events contribute to phenotypic differences relevant for evolutionary change? We are still far from mastering the broken syntax of the genome's messages, but its secrets and potential remain increasingly inviting.

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