Alternative splicing: a pivotal step between eukaryotic transcription and translation

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Abstract | Alternative splicing was discovered simultaneously with splicing over three decades ago. Since then, an enormous body of evidence has demonstrated the prevalence of alternative splicing in multicellular eukaryotes, its key roles in determining tissue- and species-specific differentiation patterns, the multiple post- and co-transcriptional regulatory mechanisms that control it, and its causal role in hereditary disease and cancer. The emerging evidence places alternative splicing in a central position in the flow of eukaryotic genetic information, between transcription and translation, in that it can respond not only to various signalling pathways that target the splicing machinery but also to transcription factors and chromatin structure.

Intron
Gene segment that is present in the primary transcript but absent from the mature RNA as a consequence of splicing.

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Alternative splicing, through which multiple mRNA variants are produced from a single gene, was discovered together with splicing itself[1,2]. Since then, what seemed to be an interesting mechanism of expression control restricted to a few dozen mammalian genes has matured into a fundamental regulatory crossroad between transcription and translation. Alternative splicing affects nearly 95% of mammalian genes[3,4] and multiple regulatory processes, including chromatin modification and signal transduction.

The splicing process is performed by the spliceosome, a ribonucleoprotein megaparticle that assembles around splice sites at each intron (BOX 1). Each splice site consists of a consensus sequence that is recognized by spliceosomal components, although 'strong' splice sites (that is, those that are more adjusted to the consensus sequence) are more efficiently recognized and used than 'weak' splice sites, which are suboptimal. It is the vicinity of competing strong and weak splice sites along a nascent pre-mRNA that leads to alternative splicing.

Transcription of mRNA-coding genes by RNA polymerase II (Pol II) starts at a nucleotide conventionally numbered as +1 and proceeds downstream of the cleavage/polyadenylation site of the pre-mRNA to terminate at positions and sequences that vary from gene to gene[5]. In this way, the 5′ end of the transcript is determined by the +1 nucleotide but its 3′ end is not marked by the transcriptional termination site but by the cleavage/polyadenylation site. The unspliced mRNA is usually known as the primary transcript. However, due to the co-transcriptional nature of splicing (discussed below), for most genes the primary transcript is a virtual entity, and the actual excision of introns is set by the pace at which relevant splicing sites and splicing regulatory sequences emerge during transcription. This is particularly crucial for alternative splicing.

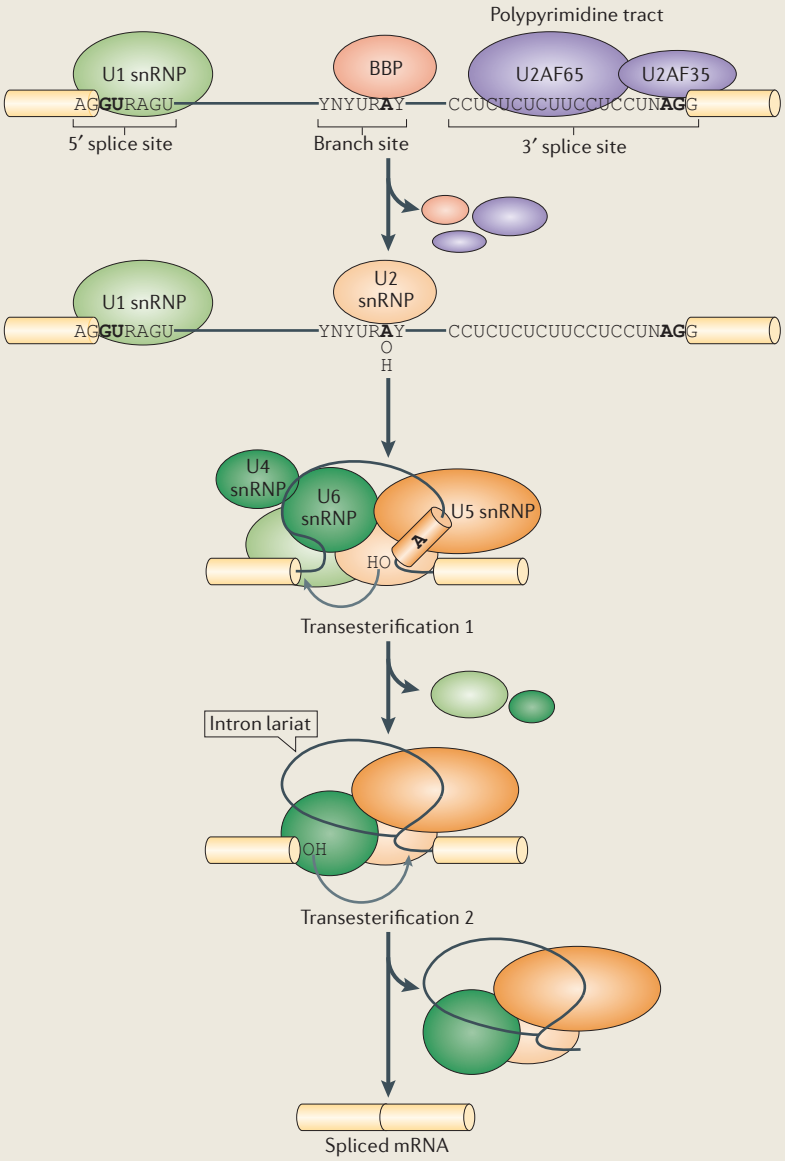
In this Review, we discuss the emerging features of the mechanisms, regulation and biological functions of alternative splicing, a rapidly evolving subject that has been extensively reviewed in the literature[5–8]. We focus on the mechanisms by which both transcription and chromatin structure directly influence splicing events, as well as the effects of signalling pathways, the roles in disease and the development of gene therapies that are based on alternative splicing.

The evolution of alternative splicing

Alternative splicing is more prevalent in multicellular than unicellular eukaryotes; unicellular eukaryotes, including for example trypanosomes, have mostly intron-less genes[9]. In *Saccharomyces cerevisiae,* introns are rather short and only present in a small subset of genes, so although splicing exists, alternative splicing is very rare[10]. Alternative splicing is common in invertebrates, with *Drosophila melanogaster* being a key example that illustrates the biological importance of alternative splicing and the extreme expansion in coding capacity it provides. In flies, alternative splicing is crucial for sex

Box 1 | **The spliceosome mediates a two-step splicing reaction**

During a splicing reaction, the points that will undergo 'cutting and sewing' are located at the 5′ and 3′ splice sites that mark the beginning and end of each intron (see the figure). These sites, together with the branch site that is located near the 3′ splice site, contain consensus sequences that are recognized in the pre-mRNA by the small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5 and U6 and auxiliary factors, including U2AF65 and U2AF35. Together, these factors form the spliceosome, a complex ribonucleoprotein megaparticle that performs the two transesterification reactions that are necessary to excise introns and join together the selected exons. The branch site is initially recognized by the branchpoint-binding protein (BBP). The transition from an inactive to a catalytically active spliceosome implies a series of snRNP and RNA rearrangements, aided by RNA-dependent ATPases and helicases. The first transesterification step consists of the nucleophilic attack by the 2'OH group of a key adenosine in the branch consensus site on the 5′ splice site, resulting in the formation of a branched RNA intermediate known as the intron lariat; a subset of snRNPs are released after this first step. In the second transesterification step, the 3'OH group of the upstream exon attacks the 3′ splice site, and this produces the spliced mRNA and the excised intron lariat, which is subsequently degraded. There is also a minor form of the spliceosome, which works on less than 1% of introns with particular end sequences and is characterized by the use of U12, U11, U4atac and U6atac snRNPs[125] (for a comprehensive review on the composition and functions of the two types of spliceosome, see REF. 126).



determination[11] and *DSCAM* (*Down syndrome cell adhesion molecule*) encodes more than 38,000 mRNA variants through a complex pattern of alternative splicing in four different regions of its pre-mRNA[12]. Remarkably, this single gene produces more protein variants than the number of genes that *D. melanogaster* has. Nonetheless, this is an unusual case, and the number of genes that are alternatively spliced and the average number of variants that are encoded by the whole genome are even higher in vertebrates[13]. The number of protein-coding genes in vertebrates is not radically different from the number in invertebrates (for example, 20,000 human genes versus 19,000 genes in *Caenorhabditis elegans*), so it is reasonable to assume that the prevalence of alternative splicing in vertebrates is important for their higher complexity (see BOX 2 for discussion of alternative splicing in plants).

The split organization of eukaryotic genes into exons and introns and the concomitant evolution of pre-mRNA splicing seem to have had at least two advantages. At the phylogenetic level, non-disruptive recombination events (that is, events that leave the exons intact) at intronic sequences allowed protein-coding exons from different ancestor genes to be placed together to form new genes. Through this mutation process, known as exon shuffling[14], new genes carry the splicing signals of the ancestor genes, and the resulting recombined mRNAs have high chances of encoding novel functional polypeptides that combine, in a single molecule, functional domains previously tested by natural selection. The second evolutionary advantage is that alternative splicing allows a single gene to produce two or more mature mRNA variants that are similar but not identical, greatly expanding the coding capacity of eukaryotic genomes.

What makes splicing alternative?

Enhancers, silencers and regulatory proteins. Splice sites can be strong or weak depending on how far their sequences diverge from the consensus sequence. This effectively determines their affinities for cognate splicing factors (BOX 1). In general, strong splice sites lead to constitutive splicing and full usage of the site. The percentage of usage of weak splice sites varies depending on the cellular context, even at normal saturating levels of spliceosomal components. The relative positions of weak and strong sites give rise to the different modes of alternative splicing including: inclusion of alternative cassette exons; inclusion of alternative mutually exclusive cassette exons; use of alternative 5′ splice sites or alternative 3′ splice sites; and retention of alternative introns. Alternative initiation of transcription at different promoters and alternative mRNA cleavage/polyadenylation should not be considered as alternative splicing modes.

The degree to which weak sites are used is regulated by both *cis*-regulatory sequences and *trans*-acting factors. *Cis*-regulatory sequences include exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs), depending on their locations and on how they affect the usage of a splice site (FIG. 1). *Trans*-acting factors function through binding to splicing enhancers and silencers and include members of well-characterized

Box 2 | **Alternative splicing in plants**

The splicing machinery is mostly conserved between plant and animal cells. RNA sequences that define exon–intron boundaries, spliceosome components and splicing factors characteristic of mammalian cells are also present in plants[132,133]. Nevertheless, in plants, splicing exhibits key differences: plant introns are on average shorter and more U/A-rich than their animal counterparts; intron retention is the most common alternative splicing event found in plants, whereas exon skipping prevails in animals; and the number of genes encoding Ser/Arg-rich proteins is higher in plants (for example, there are 18 genes in *Arabidopsis thaliana* and 22 genes in rice) compared with mammalian cells (for example, there are 12 genes in humans)[132–134]. The extent to which genes are alternatively spliced in *A. thaliana* was known to be smaller than in mammalian cells[135]. Nevertheless, recent estimates have increased the percentage of genes that undergo alternative splicing in *A. thaliana* from 5% to 60% (REF. 134). The realization that alternative splicing occurs at a high frequency in plants was not matched by similar advances in the knowledge of its functional roles and regulatory mechanisms. Some key studies indicate that several biotic and abiotic stresses influence splicing decisions[136–139] and that alternative splicing is important for photosynthesis, defence responses and flowering, among other functions[123]. A new link has also been made to the control of circadian rhythm, as alternative splicing defects produced by a mutation in the gene encoding Arg *N*-methyltransferase 5 (PRMT5) lead to circadian clock defects in *A. thaliana*[140].

Plants are sessile organisms, which forces them to respond and adapt to environmental changes in order to survive. As photoautrophs, they are extremely sensitive to light. Light affects many developmental and physiological responses during their life cycle[141] and regulates approximately 20% of the transcriptome in *A. thaliana* and rice[142]. Therefore, light is a strong candidate for being an environmental cue that controls alternative splicing, and further investigation into this possibility is required.

Ser/Arg-rich and heterogeneous nuclear ribonucleoprotein (hnRNP) protein families, as well as tissue-specific factors such as nPTB and PTB[15], NOVA[16] and FOX[17]. Some of these factors activate, whereas others inhibit the use of splice sites. Many of them can act in both ways depending on the sequence and position of the target site in the pre-mRNA[18].

The multiple roles and mechanisms of action of these *cis*-regulatory sequences and *trans*-acting factors have been extensively studied (for comprehensive reviews, see REFS 19–21). The emerging concept is that, if splicing occurred only post-transcriptionally, the degree of inclusion of an alternative exon would, for example, depend on: the intrinsic weakness of its 3′ splice site and/or 5′ splice site; the presence of different ESEs and ESSs within the alternative exon and of ISEs and ISSs in the surrounding introns; the particular secondary structures that may expose or hide these sequences[22]; and the nuclear concentration and post-translational modifications of the cognate regulatory proteins. However, the real situation is in fact more complex because splicing and alternative splicing are coupled to transcription, and factors that regulate transcription also affect alternative splicing.

Deciphering an alternative splicing code. The functions of most splicing enhancers and silencers were originally characterized through individual gene analyses. However, these sequences are widely dispersed and repeated throughout the genome, which suggests a high degree of redundancy and raises the possibility that only a small subset of them are effectively involved in alternative splicing at any one time. Maps of genome-wide splicing factor binding, combined with genome-wide RNA expression analysis that detects levels of alternatively spliced variants, have allowed the identification of regulatory elements that are specific for alternative splicing. These inferred combinations of *cis*-regulatory features have been assembled into a putative 'splicing code' that is useful in predicting, for example, variations in tissue-specific splicing patterns[4].

To assemble a splicing code, an analysis was first performed of the extent to which more than 3,600 alternative splicing combinations (consisting of cassette exons) occur in four different tissues: the central nervous system; muscle; digestive system; and whole embryos (including embryonic stem (ES) cells)[4]. In parallel, a compendium of ~1,000 RNA features was compiled that included: target sites for known splicing regulators such as FOX, nPTB and PTB, NOVA, Mbl, Ser/Arg-rich splicing factor 2 (SRSF2), SRSF5 and SRSF6, QKL, TIA1 and TIAR; new motifs including 12 clusters of putative and validated exonic and intronic splicing enhancers and silencers; short sequences (5–7 nucleotides long) that are conserved in introns flanking alternative exons; transcript structural features such as exon and intron length and secondary structures; and whether exon inclusion or exclusion introduces premature stop codons, thus triggering nonsense-mediated mRNA decay (NMD). A putative code was then determined by combining features for each tissue group that are associated with exon inclusion, exon skipping or 'no change' in the alternative splicing events. Once assembled, the code was tested by extracting the same RNA features for a particular alternative splicing event.

Another approach, studying changes in exon–intron structure during vertebrate evolution, allowed the identification of *cis*-regulatory sequences that became fixed during the transition from early vertebrates to mammals[23]. Using this information, an algorithm was developed that proved to be robust for predicting whether an exon is constitutive or alternative[23]. Together, these studies show that complex combinations of *cis*-regulatory elements are often required to control individual tissue-dependent alternative splicing events. The splicing code[4] shows good rates of prediction when comparing relative levels of exon inclusion between two different tissues. One limitation of this code analysis is that the predicted levels of exon inclusion or exon skipping were not completely accurate, most likely because other layers of alternative splicing regulation were not

Cleavage/polyadenylation
Endonucleolytic cleavage at the poly(A) site and subsequent addition of a poly(A) tail at the 3′ end of the eukaryotic pre-mRNA. The poly(A) site is defined by the poly(A) signal, which contains the consensus sequence AAUAAA.

Co-transcriptional
Any modification of or addition to the mRNA taking place while it is still being transcribed, that is, before its 3′ end is generated by cleavage/polyadenylation.

Exons
Gene segments that are or can be present in the mature RNA as a consequence of splicing. Because mRNA exons also harbour 5′ and 3′ untranslated regions (UTRs) and genes encoding RNAs other than mRNAs may have introns, exons cannot be simply defined as protein-coding segments.

Nonsense-mediated mRNA decay
(NMD). Mechanism that degrades mRNAs harbouring a premature translational termination codon as a result of gene mutation.

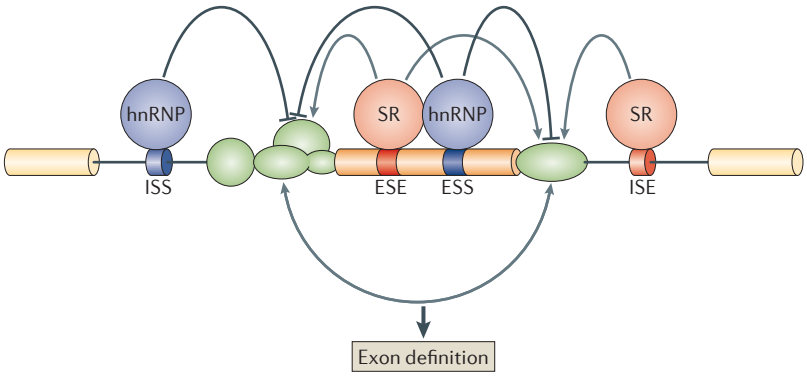


Figure 1 | **Alternative splicing regulatory sequences and factors.** Splicing is governed by *cis*-regulatory sequences in the pre-mRNA (that is, exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs)) and two main families of alternative splicing regulatory proteins, Ser/Arg-rich proteins (SRs) and heterogeneous nuclear ribonucleoproteins (hnRNPs). These regulatory proteins target components of the spliceosome (shown in green) that associate with both the 5′ and the 3′ splice sites flanking the alternative exon and can have either activating or inhibitory effects on the recognition and use of that site. In addition, interactions among components of the spliceosome that are recruited to the 3′ and 5′ splice sites can mediate exon definition, by, for example, ensuring that the recognition of one of these sites by a Ser/Arg-rich protein may indirectly stimulate recognition of the other one.

included, such as the coupling with transcription and the influence of chromatin structure.

Coupling alternative splicing to transcription

Evidence so far indicates that alternative splicing is not only regulated by the relative abundance of splicing factors but also by a more complex process involving the transcription machinery. Indeed, for many years transcription and splicing were thought to be independent events until a series of biochemical, cytological and functional experiments demonstrated that all three processing reactions (that is, capping, splicing and cleavage/polyadenylation) can occur co-transcriptionally and in certain situations be coupled to transcription (FIG. 2).

Co-transcriptional splicing. The first demonstration that splicing can occur co-transcriptionally was achieved using electron microscopy to visualize nascent transcripts containing splicing loops[24]. Deep sequencing analysis of nascent transcripts has provided further support for this[25], as has the detection of spliced mRNA, spliceosomal components and splicing factors in chromatin fractions of actively transcribed genes before their release to the nucleoplasm[26–30]. Coupling to transcription can have a positive effect on splicing efficiency. For example, in *Saccharomyces cerevisiae* in which most genes have a single short intron, Pol II-mediated elongation has been shown to pause on the terminal exon of a subset of intron-containing genes, and this delay in transcription allows for co-transcriptional splicing of the single intron[31]. A minority of budding yeast genes have a single long intron and, interestingly, pausing of Pol II is more abundant in genes that contain short exons than in those containing long exons. This suggests that the presence of a long terminal exon can compensate for faster elongation and help ensure co-transcriptional splicing[31]. Other studies also confirm widespread co-transcriptional splicing in mammalian cells[32]. *In vitro* experiments revealing that Ser/Arg-rich proteins enhance splicing efficiency only when added before transcription also support the idea that co-transcriptional splicing is advantageous; co-transcriptional recruitment of Ser/Arg-rich proteins would prevent binding of inhibitory hnRNPs which would otherwise shuttle pre-mRNA molecules to mRNA degradation complexes[33].

Single molecule imaging analysis indicates that, although catalysis of constitutive splicing is strictly co-transcriptional, this is not always true for alternative splicing[34]. Specifically, mutations that weaken the 3′ splice site lead to an increase in post-transcriptional splicing during alternative splicing. Similarly, alternative splicing events in which exon skipping is induced by inhibitory factors that bind to intronic 3′ regions trigger the same transition from a co-transcriptional to a post-transcriptional event. However, these experiments assess only the timing of splicing catalysis and do not rule out the possibility that these factors controlling alternative splicing might be recruited co-transcriptionally. Consistent with this idea, a striking accumulation of incompletely spliced transcripts has been observed in the chromatin fraction of macrophages, suggesting that transcripts are retained on the chromatin until fully spliced[35].

Coupling is more than just a co-transcriptional event. Similarly to other pre-mRNA processing events, including capping, cleavage/polyadenylation and editing, splicing is coupled to transcription[36,37], in that the two processes influence each other through coordinated mechanisms. Although co-transcriptional splicing is probably necessary for this, it is not sufficient[38]. In this section, we focus on the ways in which transcription can influence coupling during alternative splicing. Because it is not strictly related to alternative splicing, we will not discuss here the increasing evidence indicating that coupling is reciprocal and also occurs via effects of splicing on transcription (for key reports on the subject, see REFS 39–43).

Early evidence that alternative splicing patterns for a single gene differed depending on whether splicing took place *in vivo* (during ongoing RNA synthesis) or *in vitro* (on a pre-made pre-mRNA template) was suggestive of coupling[44]. The subsequent finding that different Pol II promoters placed in front of the same gene elicited different proportions of two splicing isoforms[45,46] provided more conclusive evidence for this. Indeed, promoters[46,47], transcription factors[48,49] and co-activators[50–52], transcriptional enhancers[53], proteins with dual activities such as transcription and splicing factors[54], chromatin remodellers[55] and factors affecting chromatin structure[56–60] can also influence alternative splicing decisions. Two mechanisms, which are not mutually exclusive, have been proposed to explain how coupling works: recruitment coupling and kinetic coupling.

Capping
Addition of 7-methylguanosine nucleotide to the 5′ end of eukaryotic mRNAs.

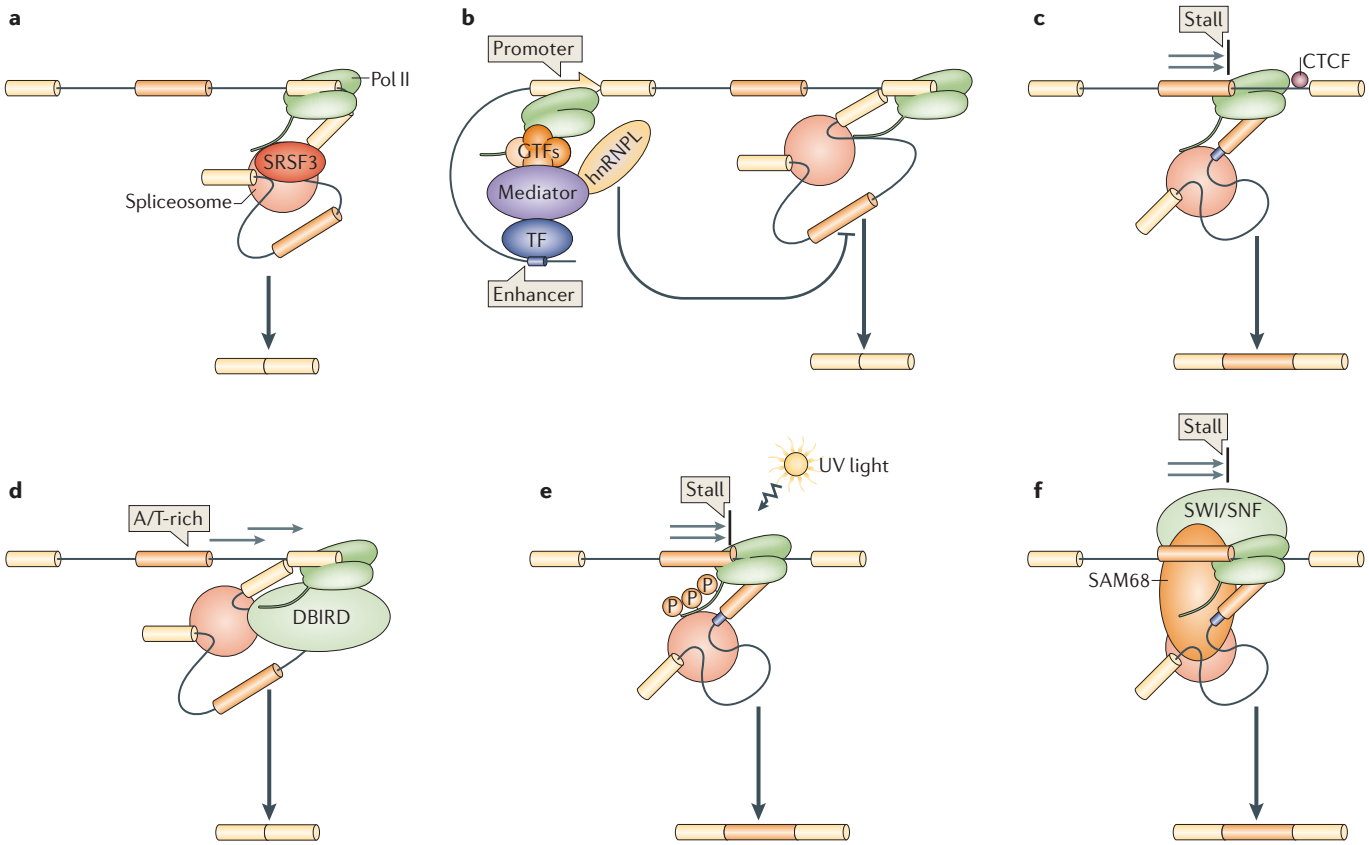


Figure 2 | **Different mechanisms can couple transcription with alternative splicing.** **a** | The carboxy-terminal domain (CTD) of RNA polymerase II (Pol II) is necessary for the recruitment of Ser/Arg-rich splicing factor 3 (SRSF3), and this inhibits inclusion of alternative exons[62]. **b** | The basal transcription complex, known as the Mediator complex, associates with general transcription factors (GTFs) at promoters and specific transcription factors (TFs) that are bound to gene enhancers and recruits the negative splicing factor heterogeneous nuclear ribonucleoprotein L (hnRNPL). This allows hnRNPL to inhibit inclusion of an alternative exon during splicing[64]. **c** | The transcriptional insulating factor CCCTC-binding factor (CTCF) binds to unmethylated CG-rich DNA sequences located downstream of an alternative exon, thus creating a roadblock to Pol II-mediated elongation that results in stalling of transcription and favours exon inclusion[68]. **d** | Through kinetic coupling, the transcription factor complex DBIRD promotes rapid Pol II-mediated elongation of A/T-rich gene sequences, which facilitates skipping of alternative exons[70]. **e** | Another kinetic coupling mechanism occurs following DNA damage that is caused by ultraviolet (UV) light irradiation, which triggers hyperphosphorylation of the Pol II CTD. This subsequently inhibits Pol II-mediated elongation and allows inclusion of alternative exons[82]. **f** | The chromatin remodelling factor SWI/SNF recruits the splicing factor SAM68 during transcription and creates an intragenic roadblock that stalls transcriptional elongation and promotes exon inclusion[55]. In all panels, only the splicing variant that is favoured is shown.

Recruitment coupling. The first mode of coupling involves the recruitment of splicing factors to transcription sites by the transcription machinery. The Pol II CTD (carboxy-terminal domain) has a key role in functionally coupling transcription with factors that affect capping and 3′ processing, and this domain has also been implicated in alternative splicing[61]. Several alternative splicing factors associate with the Pol II CTD[33], although it is not yet clear whether these interactions are direct or mediated by the nascent mRNA. The role of SRSF3 (formerly known as SRp20) in promoting inclusion of cassette exon 33 in the fibronectin mRNA depends on the Pol II CTD, as a mutant polymerase with a truncated CTD abolishes exon 33 inclusion upon SRSF3 knockdown[62] (FIG. 2a). Another classic example of a recruitment mechanism is observed for the thermogenic activator PGC1 (PPARγ co-activator 1), which modulates inclusion of fibronectin exon 25 only if it can bind to the promoter of the gene[63]. More recently, the Mediator complex subunit MED23 has been implicated in alternative splicing[64]. The Mediator complex physically links transcription factors bound to regulatory DNA sequences with general transcription factors at core promoters. A large number of RNA processing factors was found to bind MED23, including the alternative splicing regulator hnRNPL. Moreover, most alternative splicing events regulated by hnRNPL were also shown to be regulated by MED23 (REF. 64) (FIG. 2b).

Kinetic coupling. Another mechanism by which the transcription machinery influences alternative splicing is through kinetic coupling. Here, the rate of Pol II-mediated elongation influences the outcome of splicing by affecting the pace at which splice sites and regulatory sequences

Pol II CTD
(RNA polymerase II carboxy-terminal domain). This domain consists of a repeating consensus heptad amino acid sequence, Tyr-Ser-Pro-Thr-Ser-Pro-Ser (52 repeats in humans and 26 in yeast). The CTD has important roles in pre-mRNA processing.

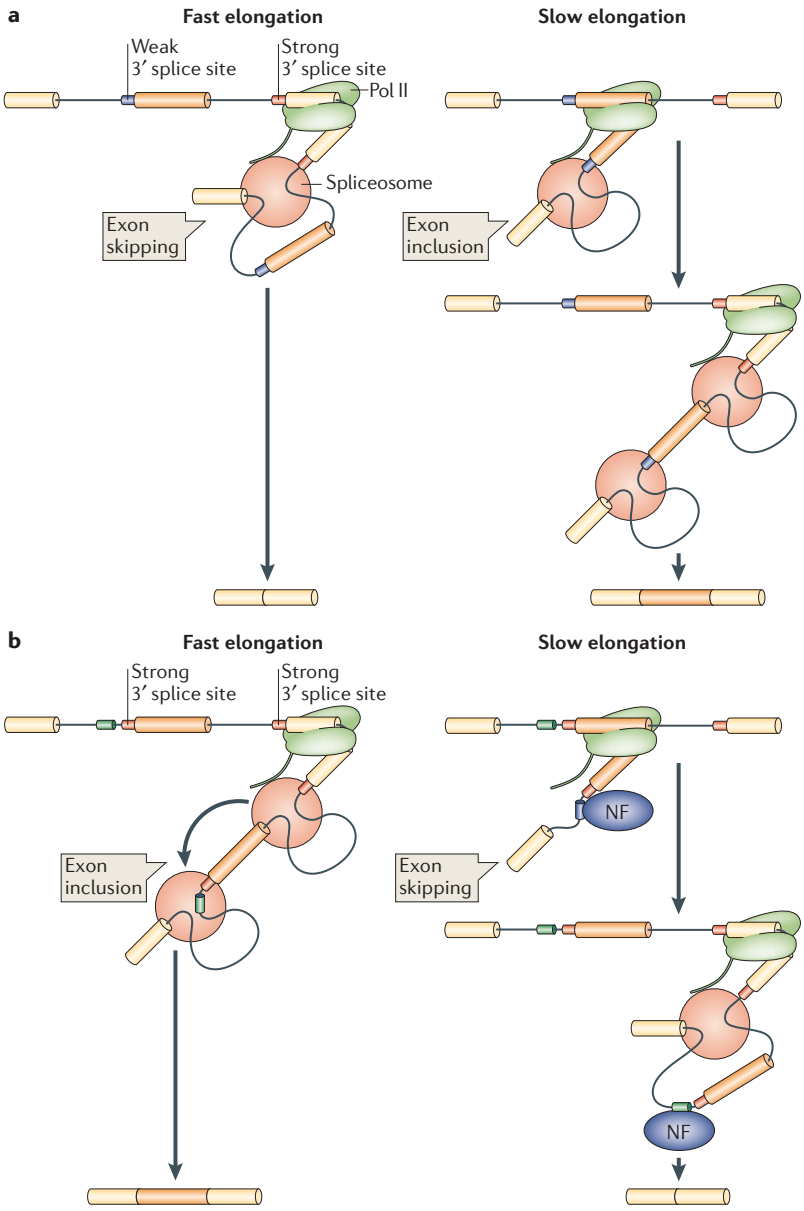


Figure 3 | **Dual effects of transcriptional elongation on alternative splicing.** **a** | During RNA polymerase II (Pol II)-mediated transcription, fast elongation (left) favours the recruitment of the spliceosome to the strong 3′ splice site of a downstream intron instead of the weak 3′ splice site of the upstream intron, which results in exon skipping. By contrast, slow elongation (right) favours the recruitment of spliceosome components to the upstream intron, which results in splicing commitment and exon inclusion. **b** | In conditions in which both 3′ splice sites are equally strong and the upstream intron also has a binding site (green) for a splicing factor that inhibits exon inclusion (negative factor (NF)), fast elongation of Pol II (left) favours the recruitment of spliceosome components to both introns, ensuring exon definition and subsequent exon inclusion. On the contrary, slow elongation (right) provides a time window for the negative splicing factor to be recruited to its target site before spliceosome components can bind to the 5′ splice site of the downstream intron and mediate exon definition. This results in exon skipping.

emerge in the nascent pre-mRNA during transcription (FIG. 3). Sequences that induce Pol II pausing[65], or drugs[66,67] that reduce Pol II-mediated elongation, promote increased inclusion rates of cassette exons into the mature mRNA, whereas drugs that elicit a more open chromatin state[48,54,57] or elongation-promoting transcription factors[49] increase exon skipping. For example, the DNA-binding protein CCCTC-binding factor (CTCF), previously implicated in targeting gene insulators, promotes inclusion of an alternative exon 5 in *CD45* by binding to a target site in its downstream intron, which creates a 'roadblock' to Pol II-induced elongation (FIG. 2c). Interestingly, DNA methylation of this intronic site prevents CTCF binding, releasing Pol II and reverting the effects on exon 5 splicing[68,69].

The effects that factors such as CTCF have on alternative splicing through the induction of pausing during elongation may be counterbalanced by factors that enable Pol II to overcome these blocks. For example, the DBIRD protein complex binds directly to Pol II and promotes exclusion of a subset of exons embedded in A/T-rich sequences, which are sequences that are particularly difficult for Pol II to transcribe (FIG. 2d). Thus, the DBIRD complex may facilitate Pol II-mediated elongation through these A/T-rich tracts[70].

Perhaps the most direct support for kinetic coupling has come from the use of 'slow' Pol II mutants that harbour an amino acid substitution in the catalytic domain of its large subunit and show a reduced elongation rate both *in vitro*[71] and *in vivo*[72]. Transcription by these mutants increases fibronectin exon 33 inclusion[73], affects the choice of alternative 5′ splice sites for *BCL-X* (B cell lymphoma X)[74] in human cells, regulates alternative splicing of *ultrabithorax* (*Ubx*) in *D. melanogaster*[73] and modulates the inclusion of an artificially created alternative exon in yeast[10]. Furthermore, a global analysis showed that dozens of alternative splicing events are affected by treatment of human cells with DRB (D-ribofuranosylbenzimidazole) and camptothecin, two drugs that have been reported to inhibit elongation[67]. Most of the DRB- and camptothecin-sensitive genes also displayed increased levels of alternative exon inclusion when transcribed by a slow Pol II mutant, compared with wild-type Pol II. By contrast, none of the ten DRB- and camptothecin-insensitive genes were affected by the slow Pol II. Thus, these studies support the idea that slowing of Pol II can facilitate inclusion of alternative exons.

'First come, first served' revisited. The influence that elongation rates have on splicing can be interpreted as being consistent with the 'first come, first served' model, first postulated 25 years ago[75]. One interpretation of this model is that slow elongation favours the removal of the intron that lies upstream of an alternative cassette exon. Alternatively, slow elongation might favour the recruitment of splicing factors to the upstream exon before the downstream exon is synthesized. Once commitment to include the exon is achieved, the order of intron removal in fact becomes irrelevant (FIG. 3a). This is supported by evidence demonstrating that the intron downstream of fibronectin exon 33 is excised before the upstream intron has been removed[28,76]. Notably, however, slower elongation induces higher inclusion of exon 33 without affecting the order of intron removal, which suggests that slow elongation favours the commitment to exon inclusion during spliceosome assembly. In light of these findings, the original 'first served' model could be re-interpreted not as 'first excised' but as 'first committed'.

Insulators
Sequences that 'isolate' sets of genes co-regulated by the same DNA *cis*-acting sequences.

In other contexts, slow elongation can also lead to higher exon skipping during alternative splicing by creating a window of opportunity for negative regulatory splicing factors to bind to their target sequences in the upstream intron of the pre-mRNA (FIG. 3b). Several studies have shown that drugs or signals that are known to reduce Pol II-mediated elongation can promote exon skipping events[67,77,78]. Another example is alternative splicing at the *CFTR* (cystic fibrosis transmembrane conductance regulator) locus. Inclusion of the *CFTR* cassette exon 9 is regulated by a U-G-repeat polymorphism, located just upstream of the polypyrimidine tract in the 3′ splice site, that is the target site for the negative factor ETR3 (REF. 79). Under conditions that that slow down Pol II-dependent elongation, including the use of a slow Pol II mutant, ETR3 binding to this U-G-repeat sequence is increased, and it displaces the constitutive auxiliary factor U2AF65 from the polypyrimidine tract. This hinders the recognition of the *CFTR* exon 9 as a proper exon, and results in skipping of this exon (G.D. and A.R.K., unpublished observations). So, the effects that kinetic coupling has on splicing depend on the microenvironment and the particular combination of splicing regulators that are involved.

Modelling kinetic control. Genome-wide studies[67] clearly indicate that not all alternative splicing events are subjected to elongation control. Although it seems plausible that splicing events involving longer introns would be more prone to be affected by elongation rates, so far we do not know in detail the specific sequence and/or structural features that would render a particular splicing event sensitive to elongation. In fact, real-time imaging of co-transcriptional splicing has demonstrated that the observed kinetics of splicing fit better with a mathematical model in which splicing has not one limiting step (that is, assembly or catalysis) but three limiting steps, one of which occurs at the level of transcriptional elongation[80]. This model is consistent with the estimated rates for Pol II-mediated elongation (which is 1.4–4 kb per minute)[72,81] and, most importantly, the model predicts that changes in elongation rates similar to those observed with the slow Pol II mutant (which slows elongation by about twofold) would have a high impact on alternative splicing decisions only if there are three limiting steps that affect kinetic coupling (as opposed to only one limiting step).

How physiologically relevant is kinetic control? Evidence that kinetic coupling is relevant for the physiopathology of mammalian cells is still scarce. Most of the evidence discussed above was obtained from studies in cells that were transfected with reporter minigenes, Pol II mutants or perturbation of endogenous gene splicing events with drugs or overexpression of proteins. Although such studies are highly instrumental for defining regulatory mechanisms, they are somewhat artificial. In an attempt to understand the physiological relevance of kinetic control, the effects of DNA damage caused by ultraviolet (UV) light irradiation on splicing was assessed. Co-transcriptional alternative splicing was shown to be affected in a p53-independent manner through hyperphosphorylation of the Pol II CTD and subsequent inhibition of transcriptional elongation[82] (FIG. 2e). Phosphomimetic Pol II CTD mutants not only displayed slower elongation but also duplicated the effect of UV light irradiation on alternative splicing. Consistent with this, the effect of UV light irradiation on splicing could be prevented by expression of non-phosphorylatable mutants of the Pol II CTD. This regulatory mechanism is physiologically important as it can upregulate the pro-apoptotic splicing isoforms of BCL-X and caspase 9, which would be consistent with the adaptive apoptotic response that follows DNA damage.

Moreover, splicing control via kinetic coupling can act at a global level. For example, during a stress response, this may ensure the coordination between changes in mRNA levels and the expression levels of RNA processing factors. Indeed, alternative splicing events that are sensitive to elongation rate control are significantly increased in genes encoding RNA splicing factors and other RNA processing factors. In addition, a subset of these splicing events can introduce premature termination codons that elicit NMD[67]. Splicing control through kinetic coupling is also conserved in *C. elegans*; in response to amino acid starvation, increased Pol II occupancy is observed proximal to exons that show increased exon inclusion[67].

Chromatin and alternative splicing

There is increasing evidence that chromatin has a key role in alternative splicing through the effects of both histone modifications and nucleosome positioning (for recent reviews, see REFS 83,84).

The idea that changes in chromatin structure could affect alternative splicing decisions was first suggested by experiments in mammalian cells using reporter minigenes. These studies showed that the inclusion of a cassette exon in a mature mRNA was increased in response to replication of the template DNA[48]. It was concluded that the more compact chromatin structure of the replicated reporter plasmid acted as a barrier to Pol II-mediated elongation and resulted in higher exon inclusion owing to kinetic coupling. Consistent with this, the effect on splicing was reverted by trichostatin A, a drug that promotes histone acetylation and subsequent chromatin opening. A seminal study later showed that, in response to an external signal, the chromatin remodeller SWI/SNF binds to a central portion of *CD44* and recruits the splicing factor SAM68 to regulate alternative splicing of a set of exons by creating a roadblock to Pol II-induced elongation[55] (FIG. 2f). SWI/SNF control of alternative splicing was also confirmed to operate on a subset of *D. melanogaster* genes[85].

Histone modifications. Histone post-translational modifications are major regulators of alternative splicing (FIG. 4). In a simplistic way, these modifications may be divided into those associated with active transcription (for example, histone H3 Lys36 trimethylation (H3K36me3), H3K4me2, H3K4me3 and H3K9 acetylation (H3K9ac)) and those associated with transcriptional silencing (for example, H3K9me2, H3K9me3 and H3K27me3). H3K36me3 is generally enriched at exons,

Histone
Highly basic nuclear protein that is a structural component of a nucleosome (core histone families H2A, H2B, H3 and H4) or associates with DNA that links nucleosomes (linker histone families H1 and H5).

Nucleosome
Repeating unit of eukaryotic chromatin that consists of a segment of approximately 147 bp of DNA wound around a histone octamer comprising two copies of each core histone (which are H2A, H2B, H3 and H4).

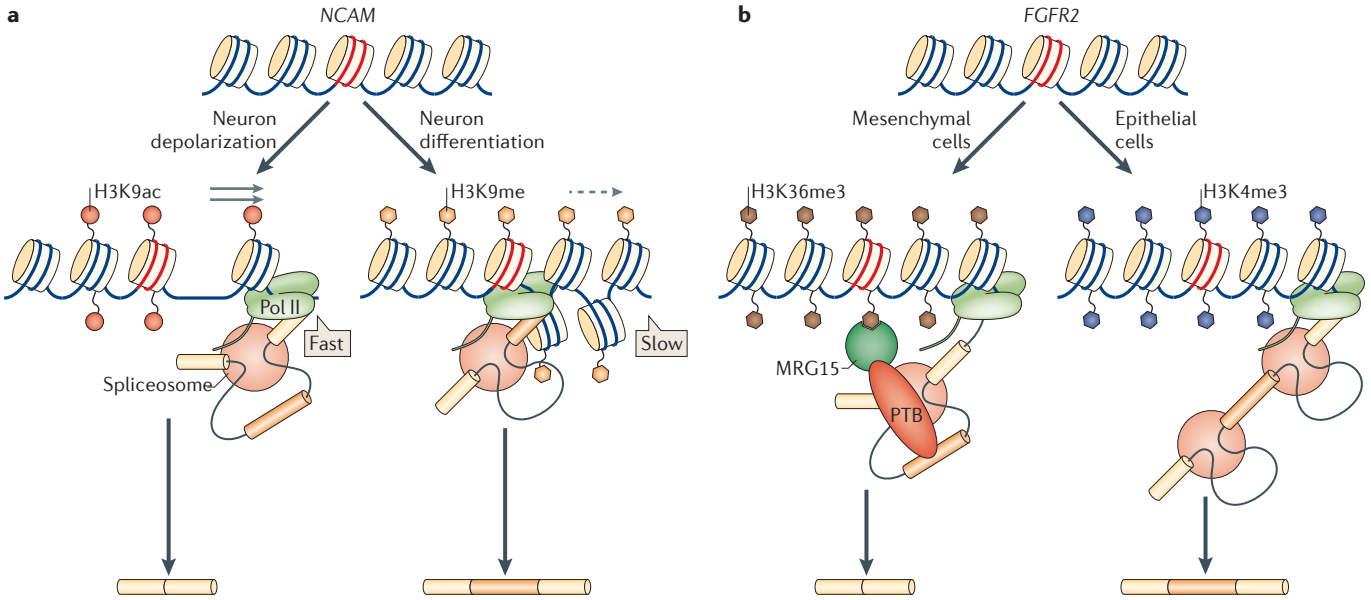


Figure 4 | **Two alternative mechanisms by which chromatin may influence alternative splicing.** Alternative splicing decisions are affected by the nature of histone marks that are deposited on the chromatin around a gene in response to external stimuli or to the differentiation state of the cell. **a** | Example of how histone modifications can affect kinetic coupling between transcription and alternative splicing. Neuron depolarization triggers intragenic acetylation of histone 3 at Lys9 (H3K9ac) and a subsequent increase in RNA polymerase II (Pol II)-mediated elongation; this favours skipping of neural cell adhesion molecule (*NCAM*) exon 18. Conversely, neuron differentiation promotes inclusion of exon 18 in *NCAM* through H3K9 methylation (H3K9me), causing a reduction in Pol II-mediated elongation (REF. 57 and I.E.S. and A.R.K., unpublished observations). **b** | Histone modifications can affect alternative splicing through a recruitment coupling mechanism. In mesenchymal cells, intragenic H3K36 trimethylation (H3K36me3) at the *FGFR2* (fibroblast growth factor receptor 2) locus recruits the negative splicing factor PTB through the adaptor protein MRG15, and this results in exclusion of an alternative exon. Conversely, inclusion of this *FGFR2* exon is increased in epithelial cells in which levels of H3K36me3 are lower compared with H3K4me3, which reduces MRG15 recruitment[59].

but less prominent at alternative exons[86]. This histone mark has been shown to regulate alternative splicing by recruiting the adaptor protein MRG15, as MRG15 association with the splicing factor PTB reduces the inclusion of a subset of alternative exons. Interestingly, when PTB-dependent genes are enriched in the H3K4me3 mark, the MRG15–PTB complex is not recruited, and exon inclusion is favoured[59] (FIG. 4b). The H3K36me3 mark can also regulate alternative splicing through the recruitment of the Ser/Arg-rich protein SRSF1 via another adaptor protein[60].

Membrane depolarization of neuronal cells causes intragenic H3K9ac of *NCAM* (neural cell adhesion molecule), which promotes an open chromatin state that facilitates Pol II-mediated elongation and subsequent skipping of *NCAM* exon 18 (REF. 57) (FIG. 4a). The opposite phenomenon occurs during neuron differentiation; the silencing marks H3K9me2 and H3K27me3 are enriched throughout the body of the *NCAM* gene, and this coincides with reduced elongation and higher inclusion of NCAM exon 18 (I.E.S. and A.R.K., unpublished observations). Together, this demonstrates that external signals and differentiation pathways may control alternative splicing through changes in chromatin.

siRNAs can also influence alternative splicing through effects on chromatin. When targeted to promoter sequences, siRNAs promote the deposition of silencing marks (such as H3K9me2 and H3K27me3) that inhibit transcription. Through a similar mechanism, siRNAs designed to target the intron downstream of an alternative exon generate intragenic roadblocks to elongation and effectively increase exon inclusion via kinetic coupling[56]. This requires the presence of the small RNA-associated protein Argonaute 1 (AGO1) and heterochromatin-binding protein 1α (HP1α). Another member of the HP1 family, HP1γ, also facilitates exon inclusion in *CD44* and other genes, by interacting with intragenic H3K9me3 marks and inhibiting Pol II-mediated elongation[58]. A general role for AGO proteins in the nucleus has been proposed[87] in light of the finding that immunopurified human AGO1 and AGO2 from chromatin-embedded proteins associate with chromatin modifiers and splicing factors. Both AGO1 and AGO2 facilitate spliceosome recruitment and increase histone H3K9 methylation on variant exons to modulate Pol II elongation rate, thereby affecting alternative splicing.

Nucleosome positioning. Through the analysis of genome-wide datasets, several groups reported almost simultaneously the unexpected finding that nucleosomes are preferentially positioned at exons[88–91]. Although introns are not devoid of nucleosomes, intronic nucleosome distribution is rather random. At exons, nucleosomes seem to be more fixed at a ratio

of one nucleosome per exon, which is in agreement with the similar length of the DNA stretch that wraps around a nucleosome and the average length of exons (~150 base pairs). On the basis of these findings, it has been proposed that nucleosome positioning may help the splicing machinery to 'find' exons because the nucleosomes would create transient pauses to Pol II-mediated elongation by 'marking' the beginning of each exon and providing extra time for the recognition of 3′ splice sites by the auxiliary factor U2AF and other factors. Three lines of evidence support this idea. First, nucleosome positioning seems to be stronger on alternative exons than on constitutive exons, which suggests that nucleosomes collaborate for exon definition[92]. Second, single molecule studies of Pol II-dependent transcription on a 'chromatinized' template reveal that elongation rates are not constant and that nucleosomes act as transient 'bumps'[93]. Last, when transcription is monitored at single-nucleotide resolution, nucleosome-induced pausing is observed to constitute a major barrier to transcriptional elongation *in vivo*[94].

The importance of being an alternative exon

Alternative exon, flexible protein. The existence of exons for which inclusion is regulated increases the functional diversity of proteins. A key question is whether the protein domains encoded by alternative exons display distinct functional or structural features compared with domains encoded by constitutive exons. Two recent reports indicate that tissue-specific alternative exons often encode structurally disordered protein regions that are enriched in target sites for post-translational modification and in binding motifs that mediate protein–protein interactions, which increases the functional versatility of the corresponding proteins[95,96]. How these features may contribute to tissue identity is unclear, but these intriguing results greatly strengthen the idea that regulation of alternative splicing is at least as important to cell differentiation as regulation of transcription. In support of this concept, an ES cell-specific alternative splicing isoform of the transcription factor forkhead box P1 (FOXP1) includes a segment encoding a DNA-binding domain that is essential for stimulating the expression of transcription factor genes required for pluripotency[97]. In this way, a cell-specific alternative splicing event in ES cells controls transcriptional programmes that are essential for development.

Species-specific alternative splicing. An important feature of tissue-specific alternative splicing events is that they tend to be associated with specific functions of the tissue in which they are manifested[98]. For instance, brain-specific alternative splicing events are associated with neural-specific functions[99]. Sometimes this association is linked to species-specific characteristics, such as the intriguing case of infrared detection by vampire bats, which are obligate blood feeders that have evolved the ability to detect infrared radiation in order to locate warm-blooded prey. For this purpose, vampire bats 'fine tune' the characteristics of the heat-sensitive transient receptor potential cation channel V1 (TRPV1), which is already present in other non-blood-feeding mammals, by lowering its thermal activation threshold from 43 °C to 30 °C. This is achieved through alternative splicing of *TRPV1* transcripts to produce a channel with a truncated C-terminal cytoplasmic domain. This splicing event is both species-specific and tissue-specific because it occurs exclusively in the trigeminal ganglia of vampire bats and not in their dorsal root ganglia, thereby maintaining the role for TRPV1 as a detector of noxious heat in other regions of the nervous system[100].

High-throughput strategies are now being used to characterize species-specific alternative splicing events and their evolutionary implications. Notably, although chimpanzee and human genes are >99% identical, between 6% and 8% of their alternative exons are spliced differently in equivalent tissues[101]. Comparisons of organ transcriptomes from vertebrate species spanning ~350 million years of evolution has also revealed that alternative splicing patterns evolve rapidly and that, in an evolutionary period as short as 6 million years, the splicing profiles of physiologically equivalent organs diverged so much that they are more related to the identity of a species than to an organ type[102].

Signal transduction and alternative splicing

Many signalling molecules affect alternative splicing by targeting splicing regulators or the kinases that target these regulators, including the same protein phosphatases and kinases that control transcription and translation (for reviews, see REFS 103–105). For example, the MAPK pathway regulates alternative splicing of *CD44* through phosphorylation of the splicing factor SAM68 (REF. 106). During T cell development, the phorbol ester- or cytokine-activated RAS pathway regulates alternative splicing of exon 4 in *CD45* through glycogen synthase kinase 3 (GSK3). Whereas in resting T cells GSK3 phosphorylates and inactivates the *CD45* splicing regulator PSF (PTB-associated splicing factor), this is relieved upon T cell differentiation by downregulation of GSK3 levels[107]. A role for the AKT pathway in the control of alternative splicing through the phosphorylation of Ser/Arg-rich proteins has been reported[108]. Most interestingly, by affecting SRSF1 phosphorylation, activation of AKT was able to simultaneously control alternative splicing and translation of mRNAs that contain target sites (ESEs) for SRSF1 in their exons. The AKT pathway is also the main intermediate in promoting changes in alternative splicing in response to epidermal growth factor (EGF) signalling[109]. AKT activation promotes phosphorylation and activation of Ser/Arg-rich protein kinase 1 (SRPK1), which is highly specific for the phosphorylation of Ser/Arg-rich proteins. Surprisingly, AKT does not seem to act as a kinase of SRPK1 but promotes its autophosphorylation in an allosteric manner, which provokes its subsequent translocation to the nucleus. Interestingly, *SRPK1* gene transcription is repressed by the tumour suppressor Wilms' tumour 1 (WT1), whereas overexpression of SRPK1 contributes to angiogenesis by inducing changes in *VEGF* (vascular endothelial growth factor) alternative splicing that lead to renal failure and Wilms' tumour[110].

Box 3 | **Alternative splicing as a target of gene therapy**

Because the *trans*-acting factors that regulate alternative splicing also participate in constitutive splicing, a strategy of searching for small molecules that target these proteins in order to correct alternative splicing defects without affecting general splicing may have little success. Instead, important efforts are being made to develop nucleic acid-based tools that target the aberrant regulatory sequences of the pre-mRNA. These therapeutic approaches include: antisense oligonucleotides (ASOs) that associate with splicing enhancers to prevent their recognition by cognate factors; siRNAs that specifically knock down aberrant splicing isoforms; modified U1 small nuclear RNAs targeting sequences that are downstream of the 5′ splice site[127]; and the use of a *trans*-splicing strategy known as SMarT (spliceosome-mediated RNA *trans*-splicing[128]) to create chimeric mRNAs in which the aberrantly spliced region is replaced by a 'healthy' segment of spliced mRNA. One of the most fascinating examples that has illustrated the power of the ASO strategy is the gene therapy protocol for spinal muscular atrophy (SMA). This recessive hereditary disease is caused by disruption of *SMN1* (survival of motor neuron 1). Humans have a second SMN encoding gene, *SMN2*, of almost identical sequence except for a nucleotide change in exon 7 that causes its partial skipping. In other words, the essential exon 7 is constitutive in *SMN1* but alternative in SMN2. When *SMN1* is mutated, the expression of SMN2 cannot compensate for the low levels of the SMN protein. This issue has been overcome through the design of 2′-O-(2-methoxyethyl) (MOE) phosphorothioate-modified ASOs[129,130] that base pair with an intronic splicing silencer located in intron 7 of the *SMN2* pre-mRNA. By blocking the binding of splicing repressors to this *cis*-element, the ASO restores inclusion of exon 7. Correction of the splicing defect was confirmed in cell-free splicing assays, in cultured human cells, including fibroblasts from patients, and in the central nervous system of SMA mouse models. Unexpectedly, and for unknown reasons, reversion of the SMA phenotype in mice also required correction of *SMN2* splicing in peripheral tissues such as liver, despite being a neurological disease in which the functionally affected cells are neurons[131]. These experiments suggest that there is promise of a cure for patients suffering from SMA.

Signalling pathways also affect the subcellular localization of splicing regulators. For example, the nuclear distributions of the splicing factors hnRNPA1, SLU7 and TRA2 are controlled by the p38 protein kinase casacade[111], UV light-mediated stress[112] and ischemia, respectively[113]. Global relocalization of both alternative and constitutive splicing factors also occurs upon the perturbation of chromatin structure that results from histone acetylation[114].

These examples suggest that the same complex signalling cascades that regulate transcription operate during alternative splicing. Detailed genome-wide studies therefore seem to be necessary to dissect the relative contributions that the effects on transcription and alternative splicing have towards the resulting phenotype in each case.

Alternative splicing and disease

The link between alternative splicing and disease is well established (for reviews, see REFS 115–120). Mutations in regulatory sequences that affect alternative splicing are a widespread cause of human hereditary disease and cancer. These mutations can disrupt existing splicing enhancers or silencers or create new ones, thereby altering the alternative exons that are included or even converting constitutive exons into alternative exons. When these changes occur in the protein-coding sequences of exons, their effects can be misinterpreted by considering only the putative effects they would have in the encoded protein. For example, a single nucleotide change that does not change the encoded amino acid of a protein (a silent mutation) might be mistaken as a neutral polymorphism when in fact it causes disruption of a crucial ESE and is a disease-causing mutation. Similarly, base changes that would result in premature stop codons (that is, nonsense mutations) or in amino acid changes (that is, missense mutations) might not be acting at the level of the encoded protein but at the pre-mRNA level by affecting the fate of alternative splicing events. Therefore, the protein-coding sequences of exons are subjected to a twofold selective pressure: conservation of the encoded amino acid sequence, and of the exonic elements that regulate alternative splicing of the mRNA. The best-characterized pathologies caused by *cis*-acting mutations are tauopathies such as frontotemporal dementia with parkinsonism, myotonic dystrophy and spinal muscular atrophy (SMA).

Mutations in genes encoding *trans*-acting factors that regulate alternative splicing can also cause disease. Unlike the *cis*-acting mutations that only affect the compromised gene, this second type of mutation can affect large sets of genes. The most studied disorders that arise from this type of mutation are facioscapulohumeral muscular dystrophy[121] and various types of cancers that are related to the overexpression of SRSF1. Experimental overexpression of SRSF1 causes malignant transformation, and SRSF1 expression is also highly upregulated in many human tumours[122]. These observations have led to the conclusion that, similarly to the genes encoding several transcription factors, the gene encoding SRSF1 is a proto-oncogene[122]. One of the well-characterized targets of SRSF1 is the pre-mRNA of the proto-oncogene *RON*. Upon SRSF1 overexpression, exon 11 of *RON* is excluded, giving rise to the *ΔRON* variant that is upregulated in breast cancer and the presence of which increases the metastatic behaviour of different tumour cells[123]. Changes in other splicing factors also cause disease through effects on alternative splicing events. For example, hnRNPA1, hnRNPA2 and PTB affect the proportions of two splicing isoforms of pyruvate kinase, PKM1 and PKM2 (REF. 124). Whereas PKM1 is characteristic of differentiated cells, PKM2 is upregulated in embryonic and cancer cells, which is key for the high levels of glycolysis that are typical of cancer cells (known as the Warburg effect) and allows them to survive in hostile anaerobic conditions.

Warburg effect
Metabolic property of cancer cells characterized by energy production through a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by mitochondrial aerobic respiration as in most healthy cells.

A detailed knowledge of the basic mechanisms operating in those alternative splicing events that are implicated in human disease will be important for the development of potential gene therapies (BOX 3).

Conclusions

Many emerging concepts justify a renewed interest in alternative splicing. More the rule than the exception, alternative splicing not only expands the proteomic landscape of higher eukaryotes but also explains species specific patterns of gene expression as well as the unique features of different cell types. The mechanisms that regulate alternative splicing are multiple and varied; they are not restricted to the interactions of splicing factors with their cognate target sites in pre-mRNA but also involve complex interactions with the transcription and chromatin machineries.

Histone marks and nucleosome positioning have recently emerged as key features that also affect alternative splicing decisions. Signalling pathways triggered by diverse external cues, ranging from growth factors to UV light irradiation, regulate alternative splicing by altering splicing factor activity as well as at the levels of chromatin and transcription.

Despite this panorama of insight, many fundamental questions still remain. How much alternative splicing is 'noise', only detected because of extremely sensitive high-throughput sequencing, and how much is biologically relevant? How much does alternative splicing contribute to cell fate and tissue or organ differentiation compared to differential transcriptional regulation? Do signalling cascades control this contribution? It is also unclear whether alternative splicing has a role in determining species identity. Moreover, the physiological relevance of coupling of alternative splicing with transcription and chromatin remains to be determined. If this is important, how much does it contribute to cell type-specific alternative splicing patterns? Is there an epigenetic *trans*-generational inheritance of alternative splicing patterns? Answers to these questions will require multidisciplinary approaches and combined efforts through genome-wide and individual gene studies. This information will be vital for efforts currently underway to assemble a potential 'splicing code' that would have the ability to predict alternative splicing patterns from a set of objective features and to design gene-therapy protocols that can correct alternative splicing defects.

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Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
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CORRIGENDUM

Alternative splicing: a pivotal step between eukaryotic transcription and translation

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In the above article, the sentence on page 156 should have read: "A minority of budding yeast genes have a single long intron and, interestingly, pausing of Pol II is more abundant in genes that contain short exons than in those containing long exons. This suggests that the presence of a long terminal exon can compensate for faster elongation and help ensure co-transcriptional splicing[31]."

This has been corrected online, and the authors apologize for any confusion caused.