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Review article

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# Regulation of pre-mRNA splicing: roles in physiology and disease, and therapeutic prospects

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## Abstract

The removal of introns from mRNA precursors and its regulation by alternative splicing are key for eukaryotic gene expression and cellular function, as evidenced by the numerous pathologies induced or modified by splicing alterations. Major recent advances have been made in understanding the structures and functions of the splicing machinery, in the description and classification of physiological and pathological isoforms and in the development of the first therapies for genetic diseases based on modulation of splicing. Here, we review this progress and discuss important remaining challenges, including predicting splice sites from genomic sequences, understanding the variety of molecular mechanisms and logic of splicing regulation, and harnessing this knowledge for probing gene function and disease aetiology and for the design of novel therapeutic approaches.

## Sections



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## Introduction

Most primary RNA transcripts produced by eukaryotic RNA polymerase II (RNA Pol II) contain introns that need to be removed by the process of splicing (Box 1, see the figure), to generate functional mRNAs and long non-coding RNAs. Although many aspects of the origin and function of introns remain uncertain (Box 1), they likely originated from autocatalytic RNAs that spread through the genomes of primitive eukaryotes as retrotranscribed DNA transposable elements[1]. During evolution, the strict sequence and 3D structure required for self-removal of intronic RNAs were progressively relaxed as their excision became dependent upon an increasingly complex cellular machinery known as the spliceosome (Fig. 1; see Supplementary Tables 1–3). The function of the spliceosome depends on the recognition of intronic boundaries by small nuclear ribonucleoprotein (snRNP) complexes, followed by a series of conformational transitions that involve remodelling of numerous RNA–RNA, RNA–protein and protein–protein interactions to enable protein-assisted, RNA-based catalysis of intron removal[2–5]. A general principle of spliceosome assembly is that intron boundaries are recognized multiple times, ensuring accuracy in the splicing process[4,6]. Two classes of introns and spliceosomes coexist in complex organisms, the minor class being spliced with slower kinetics but being nevertheless essential for the expression of genes involved in multiple processes, including early development[7].

Variations in the patterns of intron removal (known as alternative splicing) occur in the majority of genes in multicellular organisms[8–10], contributing to proteome diversification as well as to the regulation of gene expression by the degradation of transcripts containing premature termination codons (Box 2). There are numerous examples of alternative splicing events that are important for cell identity, pluripotency and organismal physiology, or that contribute to various pathologies (reviewed in refs. 11–15). How widespread is the functional relevance of alternative splicing, however, remains an open question (Box 2).

Owing to recent developments in the methods, software and data sets available to study alternative splicing (Supplementary Box 1), now is a particularly exciting time for studies of RNA splicing and its regulation. For the first time, detailed cryogenic electron microscopy structures of the spliceosome at various steps of its assembly and catalysis have been determined at unprecedented resolution (Fig. 1; see Supplementary Table 4), providing a structural framework to interpret decades of previous biochemical and genetic studies[2–5]. There is also an unprecedented wealth of transcriptome data showing the large diversity of transcript isoforms, with profound implications for understanding basic biology and disease outcomes. In parallel, strong evidence has accumulated indicating that mutations in factors involved in post-transcriptional regulation contribute to cancer and neurodegeneration[16]. Finally, the success of splicing-modulating therapies for the treatment of spinal muscular atrophy[17], as well as unexpected applications of splicing inhibitors as cancer therapies or for the maintenance of cell totipotency[18,19], bring hope that our increasing understanding of splicing mechanisms will provide a new generation of therapeutics. Alternatively spliced isoforms should also be given careful consideration in the design of mRNA-based therapeutics[20].

This Review focuses on the most recent advances in our understanding of the molecular mechanisms that help distinguish between introns and exons to enable accurate splicing and to regulate alternative splicing. These include the interplay between components of the splicing machinery that recognize the 5′ splice site (5′ss) and 3′ splice site (3′ss), their coupling with the process of transcription, the role of RNA structures and the contribution of RNA modifications. In higher eukaryotes, accurate splicing is a formidable challenge because, whereas intron removal requires single-nucleotide precision to preserve coding information in exons, the sequences delineating exon–intron boundaries are remarkably diverse, often resembling other sequences that are not splice sites[21] (Figs. 2 and 3). Testifying to the high level of specificity required, mutations in pre-mRNAs or in splicing factors lead to alterations in the splicing process that are associated with human pathologies ranging from genetic diseases to neurodegeneration or cancer[13,14,18]. Here, we provide examples of the clinical impact of splicing alterations, and discuss how a deeper understanding of the regulatory mechanisms of splicing can help design therapies that counteract these detrimental effects of splicing alterations or even exacerbate them to facilitate immune responses against tumour cells[22].

## 5′ splice site recognition

Initial recognition of the 5′ss of an intron is carried out by the U1 snRNP complex of the spliceosome through base-pairing interactions involving the 5′ end of U1 small nuclear RNA (snRNA) (Figs. 1 and 2). Given the variability of 5′ss sequences, base pairing is key to defining the efficiency (or 'strength') with which a particular 5′ss is used[23]. 5′ss mutations that weaken base pairing with U1 snRNA can cause defective splice site recognition and disease. For example, a single mutation at position +6 of intron 20 of the *IKBKAP* gene causes exon skipping, introducing a premature termination codon that reduces expression of functional protein[24] (Fig. 4a). This leads to the autosomal recessive condition familial dysautonomia, a neurodegenerative disorder that often causes premature death owing to cardio-respiratory arrest[25]. Conversely, mutations in the 5′ end sequence of one of the multiple gene copies of U1 snRNA, which are observed in patients with chronic lymphocytic leukaemia and sonic hedgehog medulloblastoma, can result in the recognition of novel 5′ss that have base-pairing complementarity with the mutated U1 snRNA, and more generally alter patterns of splicing of multiple genes. These genes include known cancer drivers – for example, resulting in inactivation of tumour suppressor genes or activation of proto-oncogenes – and correlate with worse disease prognosis[26,27].

However, it is striking that most 5′ss that are efficiently recognized do not form perfect base-pairing interactions with the 5′ end of U1 snRNA, even after considering non-canonical base-pairing schemes (such as bulged or other unpaired nucleotides[28–30]). It is also striking that mutations of the 5′ss that are not predicted to significantly disrupt base pairing with U1 snRNA nevertheless alter 5′ss recognition, being associated with diseases such as Fanconi anaemia, haemophilia, neurofibromatosis and phenylketonuria[29]. Massively parallel splicing assays assessing all of the 32,768 possible 5′ss sequences – NNN/GYNNNN (N = any nucleotide, maintaining at position +1 the G required for catalysis; Y = pyrimidine at position +2, present in 99.6% of 5′ss) – in three different minigene contexts confirmed the relevance of base pairing between U1 snRNA and the 5′ss for splicing efficiency but also revealed marked context-related differences[31]. This suggests that additional nearby sequences, their cognate factors and interactions between these and U1 snRNP components can aid the efficient use of 5′ss that have suboptimal base pairing with U1 snRNA[32–34]; for example, such additional sequences and factors have been shown to have a role in alternative splicing that regulates the physiological shift in energy metabolism from glycolysis to oxidative phosphorylation[35]. Systematic approaches (such as those described in ref. 31) can help predict the pathogenic effects of 5′ss mutations or of natural sequence variation. For example, they verified that nearly 90% of the 5′ss mutations found in *BRCA2* in breast cancer samples do affect splicing, potentially facilitating genetic screening[31].

### Therapeutic targeting

Blocking the inhibitory effect of an intronic splicing silencer (ISS) on the recognition of a 5′ss using an antisense oligonucleotide known as nusinersen (approved for clinical use in 2016) has provided a major therapeutic breakthrough for patients with spinal muscular atrophy, which is a leading genetic cause of infant mortality[17,36–38] (Fig. 4a). Spinal muscular atrophy is caused by mutations that inactivate *SMN1*, which encodes a protein important for snRNP assembly. For reasons that remain unclear, loss of SMN1 function mainly affects the function of motor neurons, leading to progressive muscle weakness and in the most severe cases to death within the first 2 years of life[38]. Quarterly intrathecal injection of nusinersen increases the levels of protein generated from a second gene, *SMN2*, which under normal circumstances fails to produce functional protein owing to limited inclusion of exon 7 (refs. 17,39).

Facilitated by the early success of nusinersen, an orally available, small-molecule, pyrido-pyrimidinone drug known as risdiplam that has similar effects on *SMN2* exon 7 inclusion obtained US Food and Drug Administration (FDA) approval in 2020 and has shown promising clinical results[40,41]. One mode of action proposed for risdiplam analogues is to 'repair' the bulge formed by the lack of base pairing between U1 snRNA and the last nucleotide of *SMN2* exon 7, thus stabilizing U1 snRNP recruitment[42] (Figs. 2b and 4a). This is achieved, at least in part, by facilitating an interaction between the zinc finger of the U1 snRNP protein U1C and the minor groove of the U1–5′ss helix[42], although other mechanisms may also be involved[43]. It is quite remarkable that a compound that modulates structural features of particular U1–5′ss configurations has therapeutic properties, paving the way to a new generation of compounds targeting 5′ss recognition.

Interestingly, engineering U1 snRNA such that its 5′ end can base pair to intronic sequences downstream of a 5′ss activates the use of the bona fide upstream 5′ss (refs. 44,45) (Fig. 4a). This suggests that an increase in the local concentration of and/or cooperativity between U1 snRNP complexes can enhance 5′ss recognition, perhaps by propagating complexes that change the physical behaviour of the exon. This approach has been used in vitro to correct exon skipping events associated with various pathologies including spinal muscular atrophy, cystic fibrosis or neurological disorders such as CDKL5-deficiency disorder[46–48] (Fig. 4a). However, the oncogenic properties of U1 snRNA mutations mentioned above[26,27] bring a note of caution when considering the general applicability of this approach.

Although not directly related to 5′ss recognition, another major function of the U1 snRNP complex is to bind to 3′ untranslated regions of mRNAs and inhibit the use of proximal 3′ end formation sites, which are often used in actively proliferating cells, including cancer cells[49,50]. Consistent with this, inhibition of U1 snRNP using antisense morpholino oligonucleotides increases cancer cell migration and invasion, whereas increased levels of U1 snRNP inhibit these phenotypes[51].

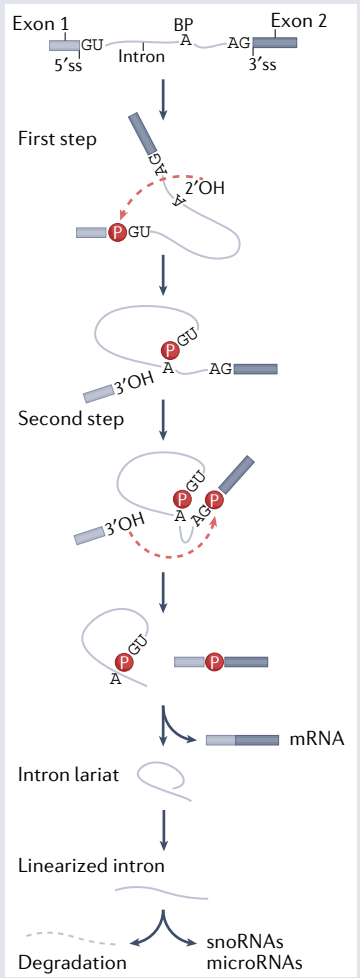
Similar approaches have been proposed for the therapeutic correction of 5′ss recognition in familial dysautonomia (Fig. 4a). These include antisense oligonucleotides targeting ISSs downstream from the 5′ss of IKBKAP exon 20 (refs. 52,53), modified U1 snRNAs[54] and small molecules such as kinetin, a plant cytokinin that enhances the recognition of 5′ss flanked by a particular sequence motif[55,56], or RECTAS, which enhances the phosphorylation of SRSF6, a splicing regulatory factor that functions through an intronic splicing enhancer (ISE) located in intron 20 (refs. 57,58).

Other examples of 5′ss recognition that have significant therapeutic potential for modulation include the induction of a pro-apoptotic

## Box 1

# The function of introns

Introns are internal sequences that are removed from precursor mRNA transcripts by a two-step splicing process (see the figure). The first step involves cleavage of the phosphodiester bond between the upstream exon (exon 1) and the intron, and concomitant formation of a 2′–5′ phosphodiester bond between the 5′ guanosine and an internal adenosine (the branch point (BP)), generating a lariat intermediate. The second step involves cleavage of the phosphodiester bond between the 3′ end of the intron and the downstream exon (exon 2), concomitant with ligation of the two exons and release of the intron in a lariat configuration.




Despite great progress in understanding the splicing process and its regulation, important fundamental questions remain. One such question is whether intronic sequences can have functions of their own or are simply by-products of a process designed to eliminate ancient transposon insertions. Recent work has shown that particular introns in the yeast *Saccharomyces cerevisiae* accumulate as linear RNA species under various stress conditions, with their accumulation contributing to stress responses through a regulatory network that involves the target of rapamycin complex (TORC), a key integrator of growth signalling[295]. Indeed, systematic deletion of introns in *S. cerevisiae* led to an impaired response to starvation, which was linked to the function of intronic sequences as repressors of ribosomal protein synthesis[296]. These observations are consistent with the long-standing proposal that introns might provide, via autonomous functions, an additional layer of genetic information to that provided by mature mRNAs and long non-coding RNAs[297]. It remains unclear, however, whether the functions documented above for certain yeast introns, as well as other examples of non-coding RNAs located within introns (such as small nucleolar RNAs (snoRNAs)[298] and microRNAs), are important exceptions to a general lack of function for introns.

3′ss, 3′ splice site; 5′ss, 5′ splice site.

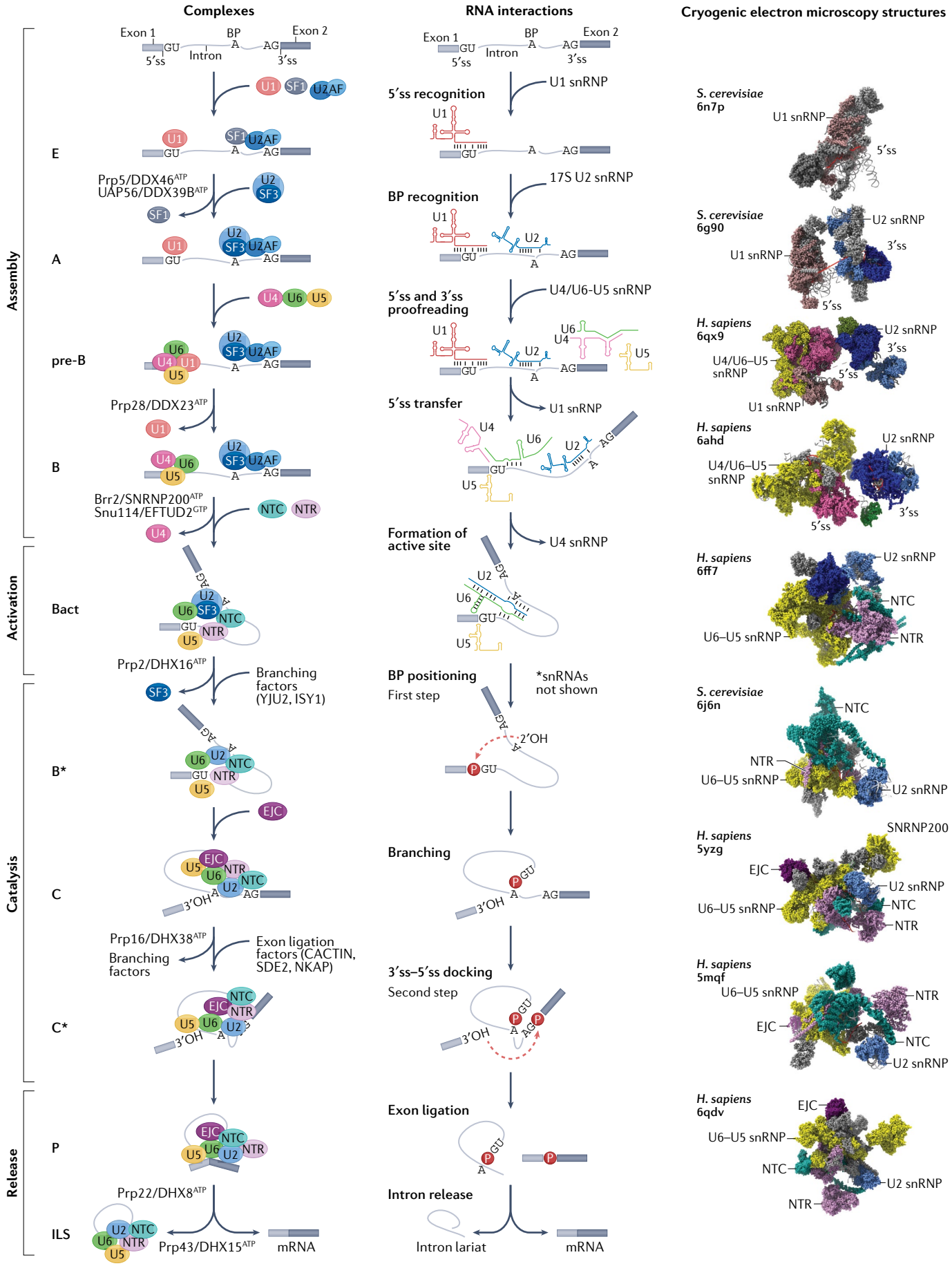

Complexes
RNA interactions
Cryogenic electron microscopy structures
Assembly
Activation
Catalysis
Release
Exon 1
BP
Exon 2
GU
Intron
AG
5′ss
3′ss
E
A
pre-B
B
Bact
B*
C
C*
P
ILS
U1
SF1
U2AF
U2
SF3
U4
U6
U5
NTC
NTR
EJC
Prp5/DDX46ATP
UAP56/DDX39BATP
Prp28/DDX23ATP
Brr2/SNRNP200ATP
Snu114/EFTUD2GTP
Prp2/DHX16ATP
Branching factors (YJU2, ISY1)
Prp16/DHX38ATP
Branching factors
Exon ligation factors (CACTIN, SDE2, NKAP)
Prp22/DHX8ATP
Prp43/DHX15ATP
mRNA
5′ss recognition
U1 snRNP
BP recognition
17S U2 snRNP
5′ss and 3′ss proofreading
U4/U6-U5 snRNP
5′ss transfer
Formation of active site
U4 snRNP
BP positioning
First step
*snRNAs not shown
2′OH
Branching
3′–5′ss docking
Second step
Exon ligation
Intron release
Intron lariat
S. cerevisiae 6n7p
S. cerevisiae 6g90
H. sapiens 6qx9
H. sapiens 6ahd
H. sapiens 6ff7
S. cerevisiae 6j6n
H. sapiens 5yzg
H. sapiens 5mqf
H. sapiens 6qdv
U4/U6–U5 snRNP
U6–U5 snRNP
SNRNP200

**Fig. 1 | The splicing mechanism and the spliceosome.** Pre-mRNA splicing involves the identification of intron–exon boundaries (splice sites) and two successive transesterification reactions (catalytic steps) (see Box 1 for details). The spliceosome comprises 5 small nuclear ribonucleoprotein (snRNP) complexes (U1, U2, U4, U5 and U6) and more than 150 additional proteins, which together recognize the splice sites, bring them together and catalyse intron removal[4,6]. Left-hand column: the dynamics of spliceosome assembly and the exchanges of snRNPs and other factors, which are driven by ATP-consuming RNA helicases — (yeast/human) Prp5/DDX46, Uap56/DDX39B, Prp28/DDX23, Brr2/SNRNP200, Snu114/EFTUD2, Prp2/DHX16, Prp16/DHX38, Prp22/DHX8 and Prp22/DHX15 — that can resolve kinetic traps along the pathway to spliceosome activation, catalysis and product release. Central column: key RNA–RNA interactions that occur during the process. Right-hand column: snapshot cryogenic electron microscopy structures of the different complexes so far available in different organisms[2–4,6,34,95,261–267]. The structures with the highest resolution available are shown (Protein Data Bank (PDB) codes indicated at the top left of each). Structures from *Saccharomyces cerevisiae* are shown for complexes that have not yet been determined for *Homo sapiens* (for example, the E and A complex structures). For simplicity and owing to space constraints, a recently described pre-Bact complex has not been included[268] and additional snapshots are likely to emerge from future work, including those corresponding to spliceosome proofreading mechanisms. See Supplementary Tables 1–3 for a full list of spliceosome components in *H. sapiens*, *S. cerevisiae* and *Schizosaccharomyces pombe*, and see Supplementary Table 4 for details of all published cryogenic electron microscopy structures of spliceosome complexes. BP, branch point; EJC, exon junction complex; NTC, Prp19 (NineTeen) complex; NTR, ntc-related complex; SF1, splicing factor 1; snRNA, small nuclear RNA; 3′ss, 3′ splice site; 5′ss, 5′ splice site; ILS, intron lariat spliceosome.

isoform of BCL-X in cancer cells[59] or the repression of a cryptic 5′ss in lamin A, which becomes activated in Hutchinson–Gilford's progeria[60].

## 3′ splice site recognition

Recognition of the 3′ end of introns in higher eukaryotes is initiated by the cooperative binding of three interacting proteins — splicing factor 1 (SF1; also known as branch point-binding protein (BBP)) and the U2AF heterodimer (U2AF1–U2AF2) — to three adjacent sequence motifs, namely the branch point (BP), polypyrimidine tract (PPT) and 3′ss (Figs. 1 and 2). Although distance constraints determine the use of a particular 3′ss upon recognition of the BP, a given 3′ss is frequently associated with more than one functional BP[61]. Mutations in U2AF1 that have been identified in various types of cancer, including myeloid malignancies and lung adenocarcinomas, alter the specificity of 3′ss recognition such that different mutations enhance binding to and selection of 3′ss that have specific nucleotides flanking the conserved 3′ss AG[62–64]. For example, whereas S34F/Y mutants of U2AF1 favour the inclusion of exons harbouring CAG 3′ss and disfavour the inclusion of exons harbouring UAG 3′ss, Q157P/R mutants of U2AF1 favour the inclusion of exons with AG/G 3′ss and promote skipping of exons with AG/A 3′ss[63]. One study found that U2AF1 mutations directly affect stress granule components and responses[65]. Mutations in U2AF2 have also been found in cancer samples and correlate with reduced binding to PPTs[66]. These results illustrate how modulating the binding of core splicing factors, which are generally required for splicing of most introns, can be rate-limiting for splice site selection.

SF1 is subsequently replaced by U2 snRNP at the BP (Fig. 1) and the U2 snRNP proteins SF3B1 and PHF5A have a key role in BP recognition (Fig. 2a). A major rearrangement of SF3B1, from an open to a closed conformation, is triggered by recognition of the pre-mRNA, with the HEAT repeats domain of SF3B1 establishing specific contact with the adenosine at the BP sequence, which is sandwiched between SF3B1 and PHF5A (refs. 67–69) (Fig. 2b). As occurs for multiple other transitions in the spliceosome cycle, an RNA-dependent helicase (PRP5; associated with U2 snRNP) provides a mechanism for proofreading, ensuring that proper recognition of the BP has been achieved within the closed conformation of SF3B1 (ref. 70).

Mutations in SF3B1 are common in various types of tumour[62,71]. They occur in 81% of patients with a class of myelodysplastic syndrome having perinuclear iron accumulations known as ring sideroblasts[72]; the SF3B1 K700E mutation is among the most common single mutations detected in any gene in patients with chronic lymphocytic leukaemia[73,74]; and SF3B1 mutations are detected in 15–36% of eye melanomas[75,76]. SF3B1 mutations are associated with changes in alternative splicing of numerous genes, and involve a characteristic pattern of activation of cryptic 3′ss 10–30 nucleotides upstream of canonical 3′ss (at least in some cases associated with the use of an alternative BP[77]), intron retention and, intriguingly, enhanced splicing of certain partially retained introns[78]. The activation of cryptic 3′ss has been linked to a reduced interaction of mutant SF3B1 with the splicing factor SUGP1 (ref. 79).

One key question relates to how transcriptome changes induced by mutations in factors that recognize 3′ss can influence tumour progression, particularly considering that the same mutation, for example K700E in SF3B1, correlates with worse prognosis in chronic lymphocytic leukaemia but with better prognosis in myelodysplastic syndrome[72–74], or with shorter or longer overall survival depending on the melanoma class[75,76]. Pan-cancer splicing analysis and positive-enrichment CRISPR screening showed that the effects of various SF3B1 mutations converge on repression of BRD9, a core component of the non-canonical BAF chromatin remodelling complex that is a potent tumour suppressor for uveal melanoma, through activation of a 'poison exon' that introduces a premature termination codon and leads to degradation of *BRD9* mRNA[80] (Fig. 4b). Other splicing alterations associated with SF3B1 mutations are also likely to contribute, including for example increased expression of telomerase RNA (and telomerase activity) or decreased expression of the MAP3K7 kinase (which is related to increased NF-κB signalling) or of the haem transporter ABCB7 (which is relevant to sideroblastic anaemia)[81–83].

## Therapeutic targeting

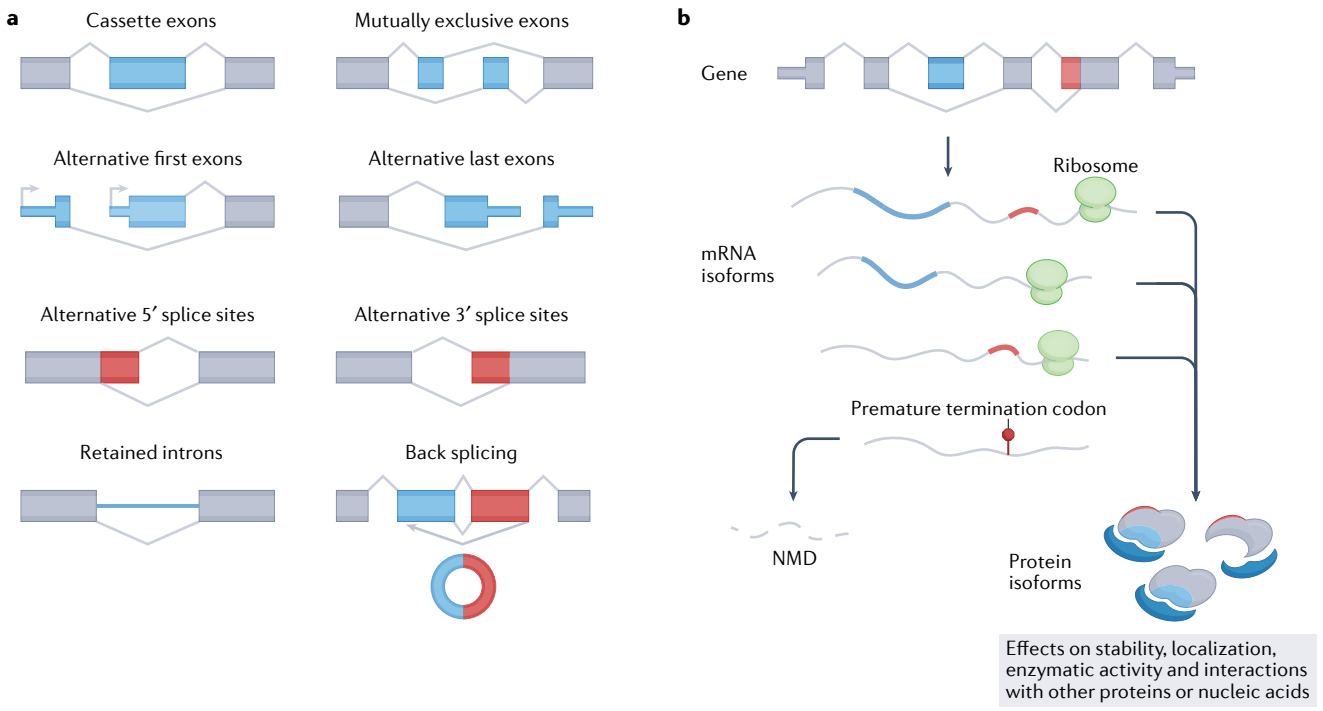
Surprisingly, various families of small molecules that inhibit the conformational change of SF3B1 during BP recognition at the 3′ end of introns have anti-proliferative effects in vitro and inhibit tumour growth in various mouse cancer models[18,67–69,84]. One of these (H3B-8800) is currently in clinical trials for myelodysplastic syndrome[71] (Fig. 4b). Different BP sequences have differential sensitivity to these compounds, with BP sequences that have more extensive base pairing with U2 snRNA being more resistant to their effects. As a result, these compounds can induce changes in splice site selection, rather than general splicing inhibition, at concentrations that have cytostatic rather than cytotoxic effects[85–88]. Treatment with one such compound, pladienolide B, reprogrammes mouse pluripotent cells into totipotent blastomere-like cells that can be cultured stably in vitro[19], suggesting that SF3B1 inhibitors can exert specific effects on various biologically relevant programmes of post-transcriptional regulation. Different structural variants of these small molecules induce alternative splicing changes that are only partially overlapping[89], which suggests that slight modifications to their chemical structures might generate drugs of improved specificity.

Cancer cells, particularly those with mutations in SF3B1, seem to be more sensitive to compounds that inhibit SF3B1 than are non-cancer cells[71]. These observations led to the concept that although cancer cells can tolerate marked alterations in their transcriptomes (for example, induced by mutations in splicing factors) that contribute to tumour progression, as a result they become more susceptible to further perturbations of the splicing process. A similar synergistic effect (known

## Box 2

# Functions of alternative splicing

Different combinations of binary splice site choices produce various classes of alternative splicing events, which are observed at different frequencies in the human transcriptome. These include cassette exons or mutually exclusive exons that can be included or skipped, the use of alternative splice sites associated with the use of alternative promoters or polyadenylation sites (giving rise to alternative first or last exons, respectively), the use of alternative 5′ or 3′ splice sites (5′ss or 3′ss) within exons, the retention of certain introns and reverse splicing reactions (back splicing) that generate circular RNA molecules (see the figure, part **a**). However, the extent to which alternative splicing affects protein and/or cell function or, rather, represents a by-product of transcriptome noise is unclear. There are many examples of alternative splicing generating protein isoforms that are relevant for cellular or organismal phenotypes, disease progression or the ecology of organisms, ranging from apoptotic switches to opioid analgesia, from neural function to sexual behaviour or seed edibility (reviewed in refs. 299,300). The question is whether these functional examples are the norm or, rather, are exceptions among the hundreds of thousands of alternatively spliced transcripts that exist. Whereas some proteomic studies detect mainly a single isoform expressed at the protein level in most tissues[301], or a limited number of tissue-specific isoforms in specific protein families[302], other studies suggest that a major fraction of alternatively spliced mRNAs is translated to different protein isoforms[303] (see the figure, part **b**). Systematic analyses of the effects of alternative splicing on protein–protein interaction networks are also more compatible with alternative splicing having widespread effects on protein and cellular function[304–309]. Alternative splicing tends to affect disordered protein domains, which are often involved in functionally important protein–protein interactions[304,310,311]. Protein isoforms can also have differences in stability, localization, enzymatic activity and protein–nucleic acid interactions. Also of relevance to the functions of alternative splicing, 30% of alternative splicing events introduce premature termination codons that can trigger nonsense-mediated decay (NMD) and other mechanisms of RNA degradation and, therefore, alternative splicing often functions to control mRNA abundance[312–315]. The exon junction complex (EJC), which is deposited on the mRNA on completion of splicing, has a role in translation-coupled nonsense-mediated decay as well as impairing cryptic splice site usage[316]. A pooled CRISPR–Cas9 screen assessing the relevance of such ‘poison exons’ that cause premature termination of translation showed that, for 50% of the tested exons, deletion had effects on cancer cell viability and xenograft growth, with a subset of these exons having tumour suppressor activity[317]. These results suggest the widespread functionality of splicing regulation, even in cases where alternative splicing does not generate alternative protein products.



as synthetic lethality) applies to the cell cycle inhibitor indisulam and related compounds, which enhance degradation of the U2AF2-related factor RBM39, resulting in alternative splicing changes that have cytotoxic effects in haematological malignancies, particularly in those that already have mutations in other splicing factors such as SF3B1 or U2AF (refs. 90,91) (Fig. 4b).

Recent results suggest an interesting additional use of splicing factor inhibitors such as pladienolide B or indisulam in oncology. Previous work has shown that splicing alterations in cancer can lead to the production of tumour-associated neoantigens, for example through the activation of cryptic splice sites that introduce in-frame or out-of-frame novel amino acid sequences in protein-coding genes[92,93]. This, in turn, can enhance the

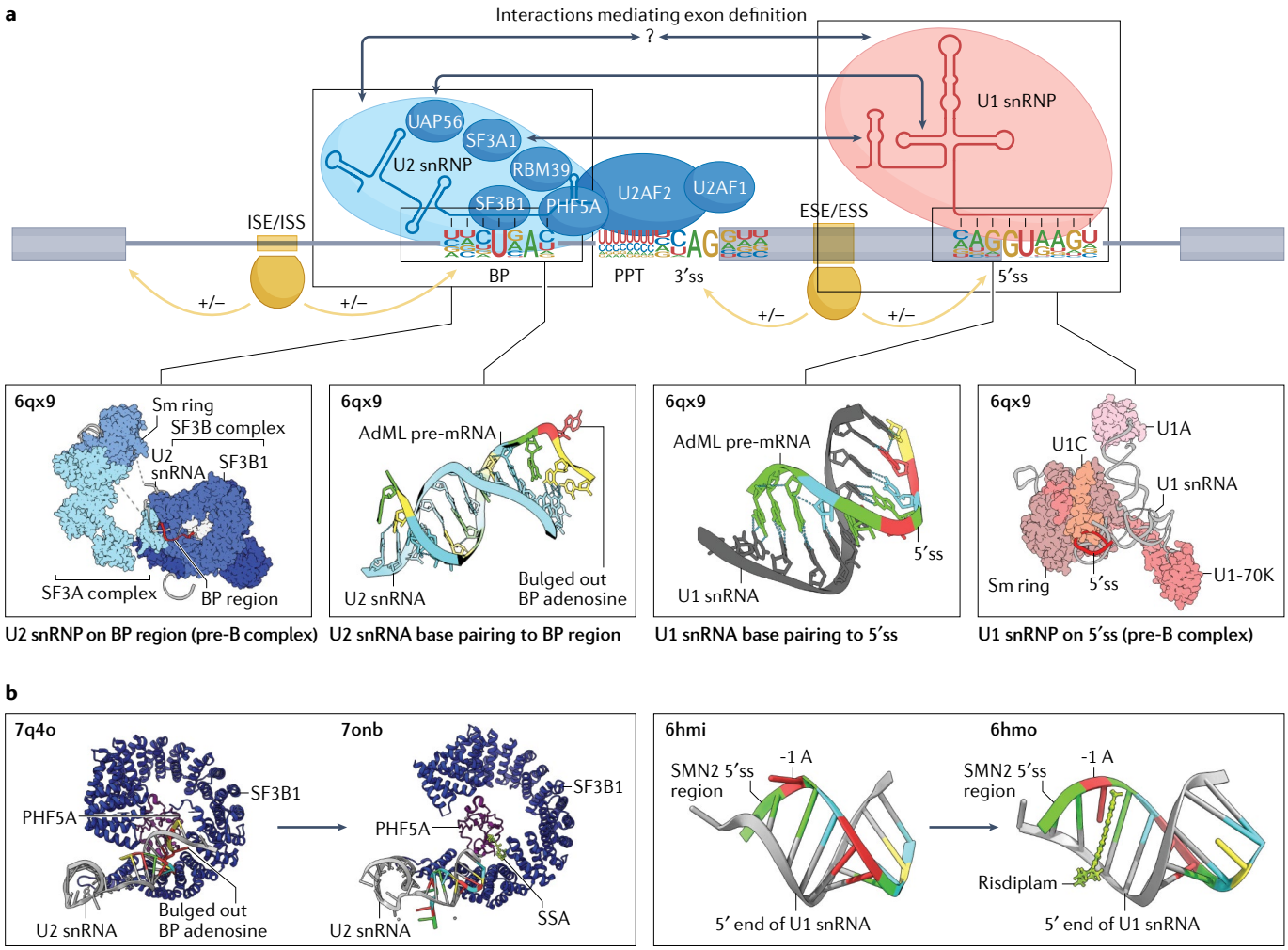
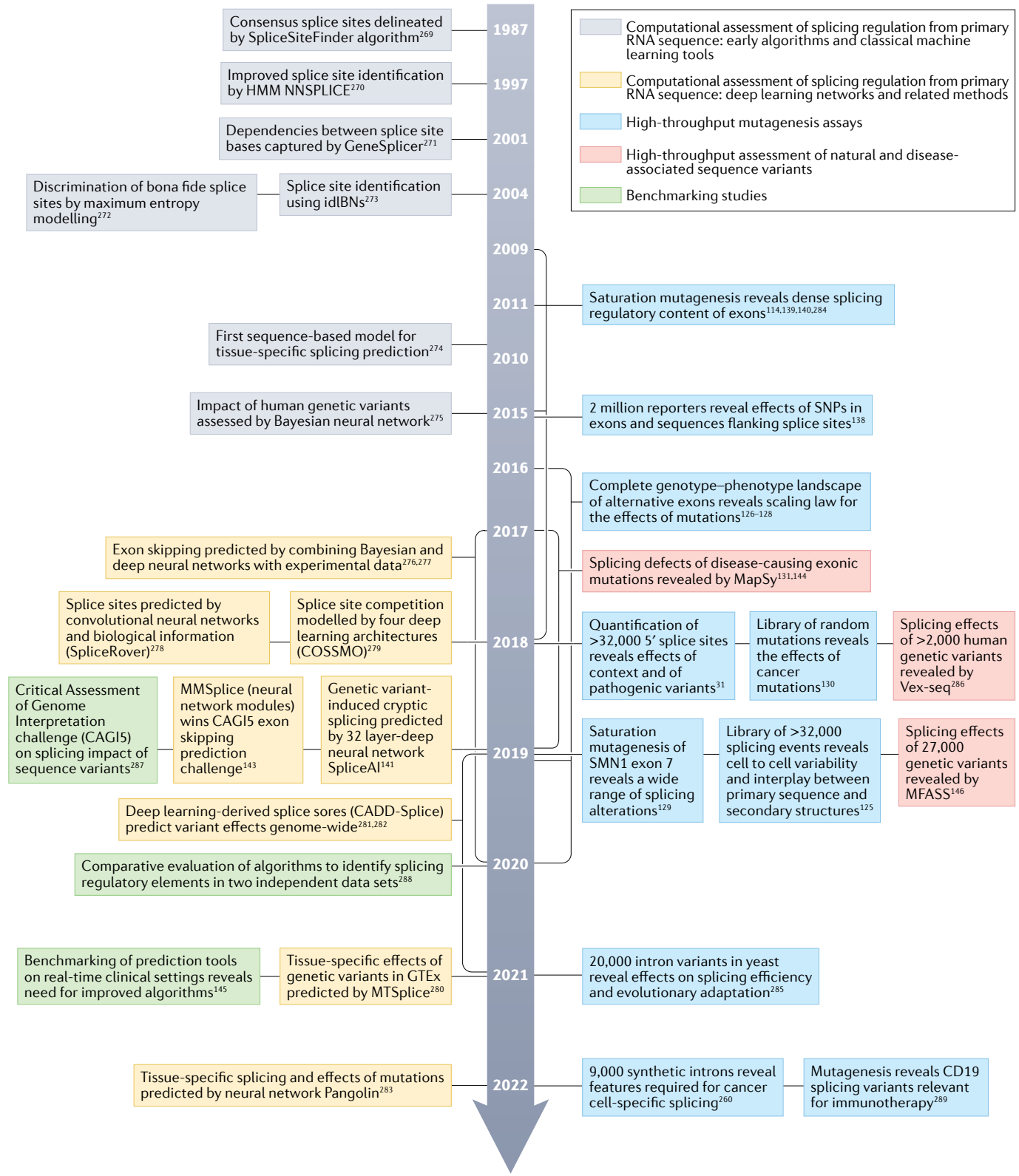



**Fig. 2 | Early splice site recognition and exon definition. a**, Base-pairing interactions between pre-mRNA sequences (the branch point (BP) and 5′ splice site (5′ss)) and small nuclear RNA (snRNA) components of small nuclear ribonucleoprotein (snRNP) complexes (U2 and U1, respectively) are crucial for the definition of intron–exon boundaries. These interactions are assisted by proteins of the snRNP complexes and other auxiliary factors — such as U2AF2, which binds to the polypyrimidine tract (PPT), and U2AF1, which binds to the 3′ splice site (3′ss) adenosine–guanosine (AG)[4,6]. Various sequences can function as splice sites (represented by sequence logos), their strength generally correlating with their potential to base pair with U2 or U1 snRNAs and with the length and uridine-richness of the PPT. Intronic and exonic splicing enhancers (ISEs and ESEs) and intronic and exonic splicing silencers (ISSs and ESSs) are recognized by regulatory factors (shown in orange), such as SR proteins, heterogenous nuclear ribonucleoproteins (hnRNPs), RBM or CELF proteins, that enhance or inhibit the association of U1 and U2 snRNPs with the splice sites. Interactions between U1 and U2 snRNP complex components have been proposed to mediate intron (and possibly also exon) definition and splice site pairing (see main text for details); additional stabilizing interactions across exons and introns are likely. The four structural snapshots illustrate the principles of 3′ss and 5′ss recognition by U2 and U1 snRNPs, respectively, and the role of RNA–RNA interactions in these processes. **b**, Structural snapshots showing the changes in conformation of U2 and U1 snRNP complex components upon binding to spliceostatin A (SSA) and a risdiplam analogue. SSA belongs to a family of splicing inhibitors with antitumour properties that prevent the transition of the SF3B1–PHF5A complex that mediates BP recognition from an open to a closed conformation. Risdiplam is a small molecule that stabilizes the interaction between U1 snRNA and the 5′ss of *SMN2* exon 7, facilitating exon inclusion and the production of functional SMN2 protein as a therapy for spinal muscular atrophy. Protein Data Bank (PDB) codes for each structure are indicated at the top left of each box.




immune surveillance of cancer cells. Thus, small-molecule splicing modulators can enhance the production of tumour neoepitopes that trigger effective antitumour immune responses. Pharmacological modulation of splicing can therefore be combined with therapies that prevent the inhibition of T cell-mediated immune responses – 'checkpoint blockers' such as antibodies to PD1 – to enhance their effects[22] (Fig. 4b).

**Fig. 3 | A timeline of key events in cracking the 'splicing code'.** How does a cell distinguish between exons and introns? How does one cell type decide that a particular sequence should be included in the mature mRNA whereas another cell type decides to skip it? How is this achieved given that the sequences at intron boundaries (5′ splice site (5′ss) and 3′ splice site (3′ss)) are highly diverse in multicellular organisms — with the exception of GU/C at the 5′ end of the intron and adenosine–guanosine (AG) at the 3′ end of the intron (Fig. 2)? In the past four decades, several computational and high-throughput experimental approaches have been developed to crack the 'splicing code' — in other words, to identify from genomic sequences alone bona fide splice sites and their differential use[31,114,125–131,138–141,143–146,260,269–289]. See Supplementary Table 5 for further details of these studies. GTEx, Genotype-Tissue Expression database; idlBNs, inclusion-driven learned Bayesian networks; SNP, single nucleotide polymorphism.

Similarly to the regulation of 5′ss recognition, antisense oligonucleotides targeting 3′ss also have potential therapeutic applications. For example, blocking activation of the poison exon in *BRD9* that is activated by SF3B1 mutations suppresses tumour growth[80], and targeting a 3′ss in the oncogene *ERG* inhibits the proliferation of prostate cancer cells[94] (Fig. 4b).

## Splice site communication

5′ss and 3′ss necessarily need to pair ('commit') to each other for the splicing process to occur. Assembly of the U4/U6–U5 tri-snRNP on pre-mRNAs at positions where 5′ss and 3′ss are recognized by U1 and U2 snRNPs, respectively, establishes such pairing through multiple RNA–RNA, RNA–protein and protein–protein interactions with the pre-mRNA and/or early splicing factors[6,95] (Fig. 1). Connections between 5′ss and 3′ss can, however, also occur before tri-snRNP assembly and such interactions are thought (although not yet fully proven) to stabilize complexes formed on the splice sites across the intron. Examples of such connections involve interactions between U1 snRNP and the 3′ and 5′ domains of U2 snRNP[34]; the interaction of stem–loop IV of U1 snRNA (which is essential for splicing) with a non-canonical RNA binding domain in the U2 snRNP protein SF3A1 (refs. 96,97); the interaction of stem–loop III of U1 snRNA with the U2AF-associated RNA helicase UAP56 (ref. 98); and the interaction of SF1 with the U1 snRNP-associated protein Prp40p in yeast[99,100] (Fig. 2). It is currently unclear whether these are the main contacts for early splice site communication or whether multiple other molecular bridges can be formed on different introns.

An important complementary concept posits that some of these interactions might occur not only between splice sites across introns but also across internal exons through a process known as exon definition[101,102] (Fig. 2). The mutual stabilization of splice site recognition complexes across internal exons can explain various long-standing observations, including the upper (250 nucleotides) and lower (50 nucleotides) length constraints of these exons (which define the boundaries for optimal exon definition interactions) and the fact that genetic mutations in splice sites can induce exon skipping (as a consequence of the failure of exon definition) instead of intron retention (which would be the consequence of a failure of intron definition)[103,104]. It might also explain the results of saturation mutagenesis of 5′ss, whereby the strength of the upstream 3′ss was found to influence the effects of 5′ss mutations[31].

An important exception to the length constraints of internal exons are microexons, which are ~3–27 nucleotides in length but have important regulatory effects on protein functions in nervous system development, synaptic transmission and autism spectrum disorder[105–107]. Microexons have evolved specific mechanisms of recognition coordinated by the neuron-specific regulatory protein SRRM4, which functions as a master regulator for this programme[108–110]. Master regulatory factors have been described to coordinate other programmes of splicing regulation, for example for sex determination in fruit flies, shaping synapses or coordinating epithelial–mesenchymal transition in vertebrates[111–113].

## Role of regulatory sequences

Exon sequences themselves can also contribute to splice site recognition and exon definition through the function of exonic splicing enhancers (ESEs) and exonic splicing silencers (ESSs) (Fig. 2). Together with ISEs and ISSs, these regulatory sequences are thought to nucleate the assembly of complexes of regulatory factors that promote or inhibit splice site recognition by the core splicing machinery[108,114,115] (Fig. 2). This can be achieved through various mechanisms, including the recruitment of core splicing factors through direct interactions with these regulatory complexes[116,117], the establishment of exclusion zones through cooperative coating of the RNA by RNA binding proteins[118,119], interference with specific interactions mediating exon and/or intron definition[120] or the formation of higher-order assemblies (possibly even experiencing local phase transitions) involving tyrosine-rich intrinsically disordered protein domains that are themselves regulated by alternative splicing[121,122]. Classical examples include proteins of the arginine–serine-rich (SR) family, which have positive effects on splicing from exonic enhancers, and proteins of the heterogenous nuclear ribonucleoprotein (hnRNP) family, which inhibit spliceosome assembly from intronic silencers[108,115]. However, often the same sequence motif and cognate factors can have positional effects, for example promoting exon skipping when bound upstream of an alternative exon but promoting exon inclusion when bound downstream[108,115,123–125].

Not unexpectedly, exon mutations have broad effects on alternative splicing. Recent high-throughput, saturation mutagenesis studies have shown that two thirds of all possible mutations in an alternative exon can affect its inclusion, whereas this was not the case for constitutive exons[126–130]. This has obvious implications for the joint evolution of splicing and protein codes[131,132] and for understanding the effects of synonymous mutations in natural genetic variation associated with genetic diseases[133–135] as well as in cancer[136,137].

Several recent studies have aimed to systematically assess the effects of exonic or proximal intronic sequence motifs on splice site selection using high-throughput read-outs (Fig. 3; see Supplementary Table 5). For example, one study assessing and modelling the effects of random libraries of 25 nucleotides flanking 5′ss or 3′ss (involving more than 2 million synthetic minigenes) showed that the vast majority of possible hexamer sequence motifs influence splice site selection, having similar effects in 5′ss or 3′ss competition assays[138]. This large variability of sequence motifs is consistent with results from other reports, although these studies found differential positional effects of ESSs but not ESEs[139,140]. Other studies have also highlighted the importance of genomic context and of starting levels of exon inclusion on the effects of mutations and in the generation of transcriptome complexity during evolution[127,141,142], as well as the contribution of various inputs to splice site selection in most native contexts[125] (Fig. 3; see Supplementary Table 5).

These efforts have obvious relevance for predicting the effects of potentially pathogenic mutations, and various strategies have been envisioned to assist in genetic counselling by modelling and/or

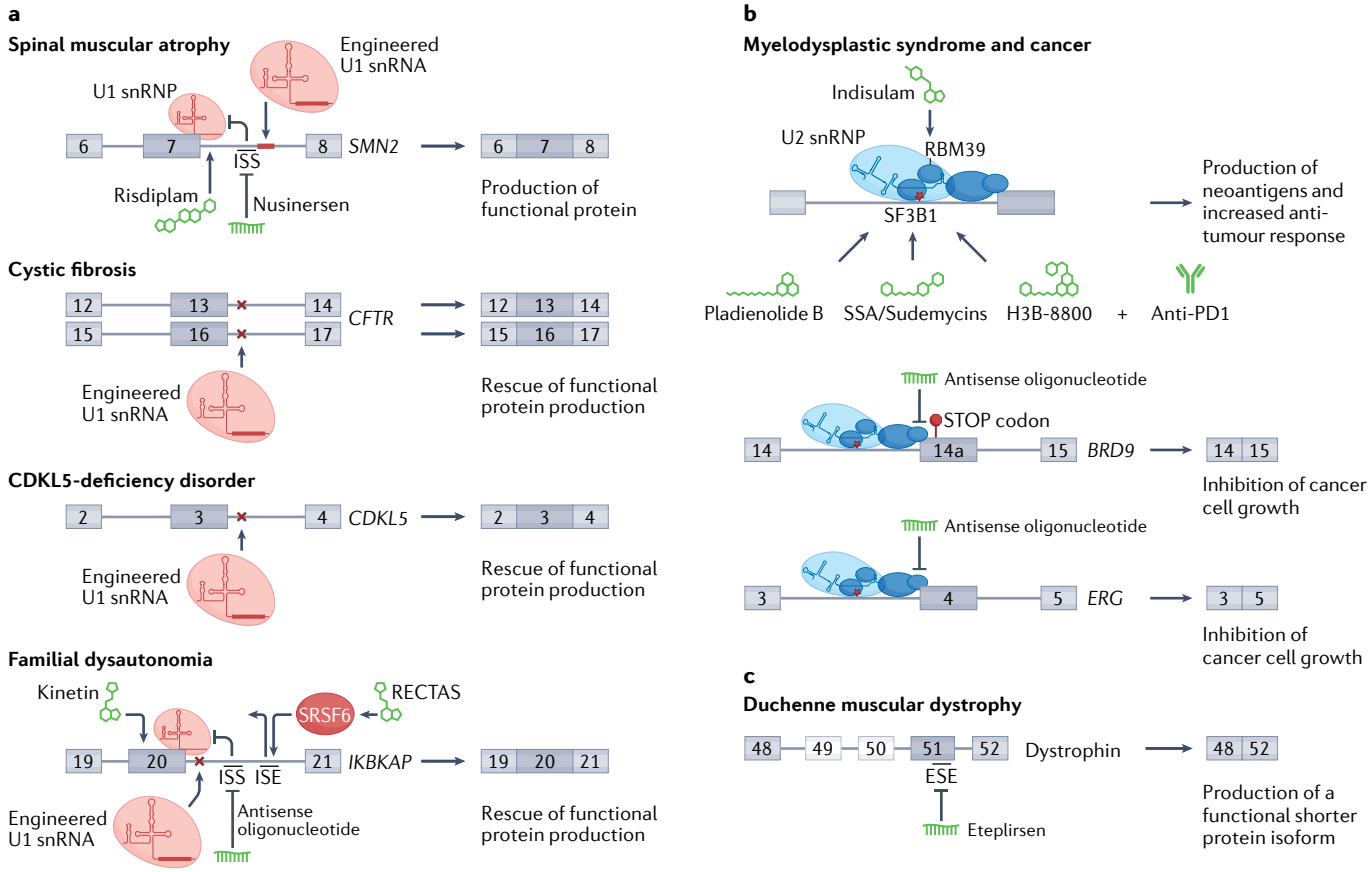



**Fig. 4 | Therapeutic targeting of splice site recognition and exon definition.** Examples of how small-molecule drugs, antisense oligonucleotides or engineered U1 small nuclear ribonucleoprotein (snRNP) complexes can be used as potential or current therapies for genetic diseases or cancer. **a**, Targeting 5′ splice site (5′ss) recognition. Examples are provided of four diseases in which promoting 5′ss recognition can enhance the inclusion of exons and lead to the synthesis of functional proteins whose production was disrupted in the indicated pathologies. This is achieved by using engineered U1 snRNP (in which the 5′ end sequence of U1 small nuclear RNA (snRNA) has been modified to target the complex to specific locations within a transcript), antisense oligonucleotides (including nusinersen) targeting intronic silencers or small molecules (see text for details). **b**, Targeting 3′ splice site (3′ss) recognition. Examples of therapeutic interventions relevant in oncology include the production of neoantigens by inducing inhibition of 3′ss recognition using the indicated SF3B1-targeting or RBM39-targeting drugs; neoantigens in combination with immune checkpoint blockade (such as anti-PD1 therapy) can elicit immunotherapy responses. Other examples include blocking 3′ss recognition by antisense oligonucleotides, leading to exon skipping events that inhibit cancer cell growth. **c**, Targeting regulatory sequences. Antisense oligonucleotides (including eteplirsen) targeting exonic enhancers induce skipping of exons containing inactivating mutations in the Dystrophin gene that cause Duchenne muscular dystrophy, leading to in-frame deletion and production of a shorter protein isoform that rescues function. ESE, exonic splicing enhancer; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; SSA, spliceostatin A.

experimentally assessing splicing perturbations in genes of interest[141,143–145]. For example, a high-throughput system designed to test the effects of more than 27,000 variants annotated in the Genome Aggregation Database found that very rare variants had large effects on splicing, mostly located outside the splice sites themselves[146].

### Therapeutic targeting

The antisense oligonucleotide eteplirsen, which targets ESE sequences, has been approved as a therapy for Duchenne muscular dystrophy. Eteplirsen promotes skipping of an exon in the dystrophin gene that harbours inactivating mutations, leading to in-frame production of a shorter, but still functional, protein that restores muscle function[147] (Fig. 4c). More generally, approaches targeting exon sequences may be used to generate truncated protein variants lacking specific domains, for interrogating gene and protein function without requiring complex genome engineering.

## Co-transcriptional regulation

Long-standing evidence ranging from electron microscopy[148] to transcriptome analyses of RNAs physically associated with transcribing RNA Pol II[149] indicates that the removal of introns from pre-mRNAs can occur co-transcriptionally, even almost immediately after the 3′ss exits from the polymerase tunnel (reviewed in refs. 150,151) (Fig. 5). Although in vitro-transcribed model pre-mRNAs can be spliced in nuclear extracts or upon transfection or injection (for example, in the nucleus of *Xenopus* oocytes)[152], which indicates that splicing can be uncoupled from transcription, co-transcriptional splicing has important mechanistic implications, as functional connections between the transcription and

splicing machineries can enhance splicing efficiency and influence splice site choice[153,154]. Transcription elongation rates determine the time window during which alternative splice sites enter into competition (reviewed in refs. 153,155,156), which can influence splice site choice via a kinetic model that has been shown to operate both in vitro and in vivo, in animals and in plants[157–159] (Fig. 5a). Conversely, splice site recognition can influence promoter choice, transcription elongation and proper 3′ end formation[154,160–162].

However, more recently, the extent to which co-transcriptional splicing occurs and/or contributes to the majority of splicing events has been questioned[163]. Results from long-read sequencing of nascent pre-mRNA transcripts indicate that splicing in human and *Drosophila* cells typically occurs after RNA Pol II has transcribed several kilobases of pre-mRNA, with the order of intron removal not following strictly the order of transcription[164]. Another study combining similar technologies with precision run-on sequencing found that, during mouse erythropoiesis, although introns are often spliced during the time of transcription of the downstream intron, nascent transcripts with a high proportion of unspliced introns are also detected[162]. Along the same lines, a three-pronged methodology to characterize nascent RNAs

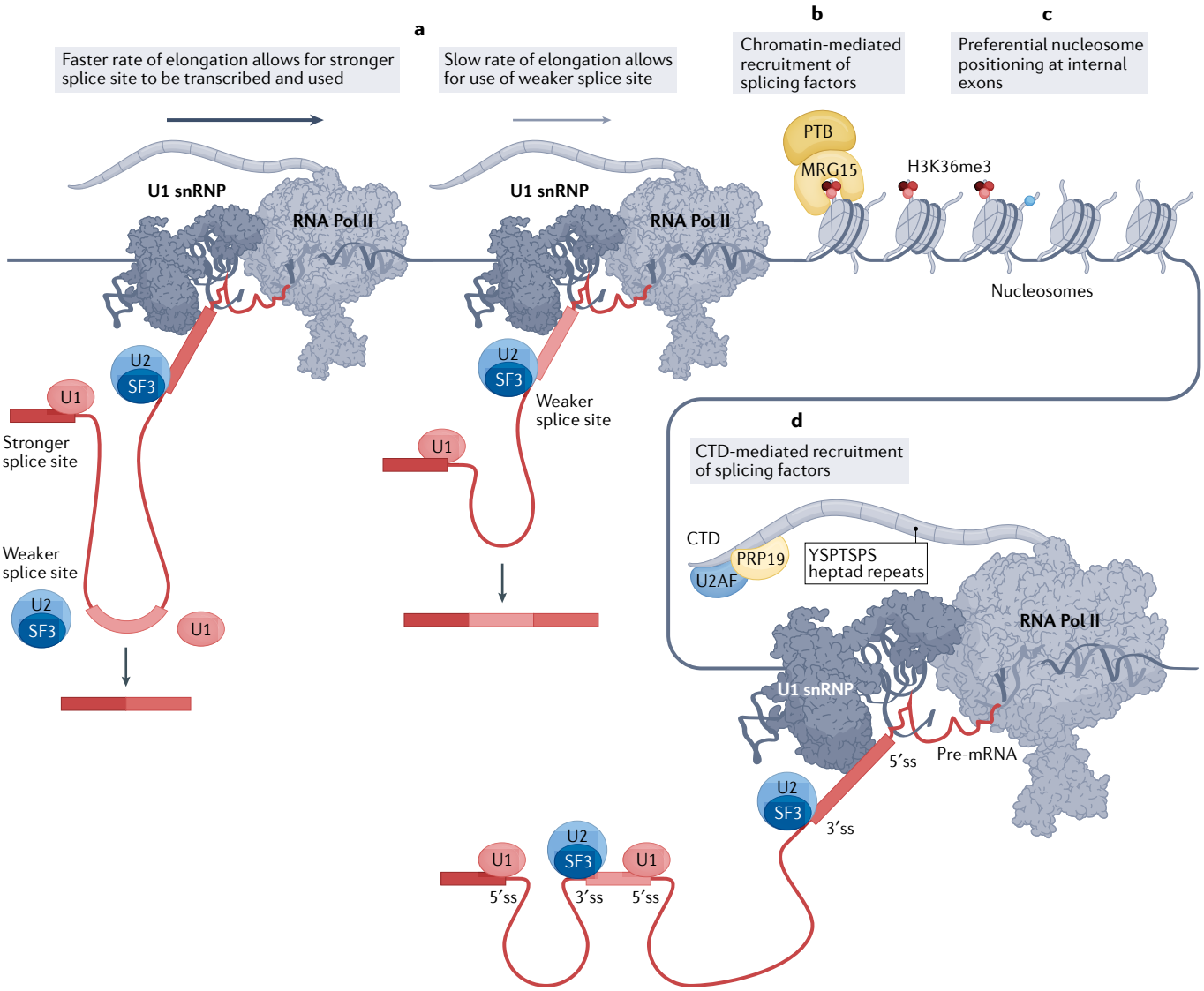



**Fig. 5 | Co-transcriptional splicing.** Intron removal can occur on nascent mRNA transcripts during transcription by RNA polymerase II (RNA Pol II), which has important mechanistic implications for splice site selection. **a**, The elongation rates of RNA Pol II can affect the competition between alternative splice sites. For example, slow rates of elongation facilitate the use of weaker splice sites before stronger splice sites are transcribed[155]. **b**, Chromatin can recruit splicing regulators (such as PTB) through proteins that recognize epigenetic marks, such as MRG15, which binds both PTB and the trimethylation of histone H3 on lysine 36 (H3K36me3)[186,290]. **c**, Preferential nucleosome positioning at internal exons may favour exon definition[291–293]. **d**, There is evidence to suggest that splicing factors (such as U2AF–PRP19)[294] may be recruited through direct interactions with RNA Pol II, including its carboxy-terminal domain (CTD). The CTD is composed of multiple repeats of the heptad amino acid sequence YSPTSPS harbouring potential phosphorylation sites that are linked to different elongation states of the enzyme[156] and to a switch between transcriptional and splicing condensates[178]. Importantly, the interaction between U1 small nuclear ribonucleoprotein (snRNP) and RNA Pol II requires a 5′ splice site (5′ss) being present in the nascent transcript[253]. 3′ss, 3′ splice site.

also documented examples of co-transcriptional splicing occurring immediately after 3′ss transcription, as well as other examples in which splicing was delayed until RNA Pol II had transcribed sequences much further downstream[165]. Nevertheless, clear examples of post-transcriptional splicing do exist. These include examples of coordinately regulated distant alternative splicing events within the same transcript in different cell types[166,167], and the recently reported category of detained introns, excision of which can be induced by signalling cues during development or meiosis, or in certain cancers[168–171]. The otherwise fully processed polyadenylated transcripts containing detained introns are retained at the gene locus until splicing of the detained intron allows for mRNA export to the cytoplasm and translation, thus allowing for rapid changes in protein expression in response to external cues[169–172].

A long-standing issue related to co-transcriptional splicing is whether splicing is subject to some form of compartmentalization in the nucleus. For example, splicing has been proposed to occur in the vicinity of nuclear speckles, regions of the nucleus that have an accumulation of splicing factors, contain active spliceosomes and have phase transition properties (reviewed in refs. 168,173–177). This is particularly relevant considering that different phosphorylation states of the intrinsically disordered carboxy-terminal domain (CTD) of RNA Pol II can drive an exchange from condensates involved in transcription initiation to condensates in nuclear speckles[178]. Two recent studies reported two distinct areas of the nucleus, one associated with nuclear speckles and the other with peripheral nuclear lamina, that had characteristic patterns of splicing regulation[179,180]. Such patterns correlate with differences in the levels of splicing factors and/or genomic guanosine–cytosine (GC) content in these areas, the latter being associated with distinct genomic architectures that have been linked to exon versus intron definition[179–181].

Chromatin organization, including nucleosome positioning and epigenetic marks such as histone modifications and DNA methylation, has also been associated with splice site recognition and regulation (reviewed in refs. 182,183) (Fig. 5b,c). Proposed mechanistic models involve proteins that recognize methylated DNA and slow down RNA Pol II, indirectly affecting splice site selection[184], or adaptor proteins such as MRG15 that recognize both histone tail modifications and splicing factors, thus increasing the local concentration of splicing regulators such as PTB[185] (Fig. 5b). Proving causality, a recent study used genome editing tools to introduce histone modifications that are observed during epithelial–mesenchymal transition at specific genomic locations, which induced corresponding splicing modifications and concomitant biological effects[186].

It is clear that splicing regulation can be influenced by chromatin spatial organization and epigenetic modification, as further illustrated by the recent report of enhanced effects of combining the splicing modulatory drug nusinersen with a histone deacetylase inhibitor[187]. Future work will establish how general and diverse are co-transcriptional mechanisms influencing the splicing code.

## Epitranscriptomic regulation

More than 70 chemical modifications of RNA molecules have been described in eukaryotes and, for some of these, dedicated protein factors involved in their deployment, reading or erasing are known[188]. Such epitranscriptomic modifications might establish a regulatory code on RNA[189], resembling the epigenetic code on DNA and histones that functions to recruit or inhibit enzymatic complexes that modulate transcription, replication or DNA repair[190]. For example, in *Caenorhabditis elegans*, $N^6$-methyladenosine ($m^6A$) modification at the adenosine–guanosine (AG) dinucleotide of 3′ss inhibits its recognition by U2AF1, leading to the retention of an intron in the pre-mRNA encoding *S*-adenosylmethionine (SAM) synthetase[191]. This results in the downregulation of SAM synthetase expression as part of a negative feedback loop by which an excess of methionine triggers accumulation of methylated SAM, which itself functions as the methyl donor for the enzymatic deposition of $m^6A$ on pre-mRNA. A similar mechanism operates in human cells[192]. In this case, under conditions of abundant SAM, the $N^6$-adenosyl-methyl transferase METTL16 methylates a loop structure at the 3′ untranslated region of SAM synthetase pre-mRNA. Under conditions of low levels of SAM, METTL16 binds to the unmethylated loop and enhances splicing of SAM synthetase pre-mRNA, facilitating expression of the enzyme[192]. Thus, in both *C. elegans* and humans, $m^6A$ functions as a sensor of the availability of metabolites to switch off the expression of SAM synthetase via regulation of splicing.

Another mechanism by which $m^6A$ can regulate splicing is via proteins that recognize this modification. For example, hnRNPG (also known as RBMX) recognizes $m^6A$ modifications at exonic positions close to regulated splice sites and, through interactions with RNA polymerase, modulates alternative splicing[193]. Another example is the nuclear $m^6A$ reader YTHDC1, which recruits splicing regulatory factors of the SR protein family (such as SRSF3), but antagonizes the binding of other factors such as SRSF10, with the result of promoting inclusion of alternative exons[194]. The effects of YTHDC1 on splicing and polyadenylation may underlie its essential function in germline development[195]. $m^6A$-mediated regulation of the splicing kinetics of multiple introns, associated with positional effects of $m^6A$ deposition on nascent transcripts[196], is an attractive proposed mechanism for the coordinated control of developmental programmes. However, the extent to which $m^6A$ modifications generally regulate splicing programmes remains unclear, with one study arguing that their major functional effect is on cytoplasmic mRNA stability[197].

Recent work highlights the regulatory potential of pseudouridine modifications at alternatively spliced regions of pre-mRNAs and their regulatory sequences, with direct effects on splicing efficiencies[198]. The tissue-specific expression of pre-mRNA pseudouridine synthases thus offers another potential mechanism for the control of alternative splicing and 3′ end formation[198].

Functionally important RNA modifications also occur in snRNAs, which have 2′-*O*-methyl and pseudouridylated residues at phylogenetically conserved positions, in addition to characteristic cap structures at their 5′ ends (2,2,7-trimethyl-guanosine for U1, U2, U4 and U5 snRNAs and γm-guanosine for U6 snRNAs) (reviewed in ref. 199). Some of these modifications have been shown to be important for snRNP biogenesis and/or for efficient splice site recognition, and an additional $m^6A$ modification in U2 snRNA has been proposed to modulate 3′ss choice[199,200].

## Regulation by RNA structure

One difficulty in assessing the functional effects of RNA structure on alternative splicing is that RNAs exist, almost invariably from birth, as RNP complexes in which the associated proteins strongly influence the conformation(s) that RNAs adopt during or after folding. Although methods for the high-throughput analysis of higher-order transcriptome structure in living cells (reviewed in ref. 201) remain to be fully exploited in investigating splicing regulation, recent studies argue that introns may be more highly structured than exons and that distinct RNA folding around alternative exons, depending on RNA Pol II elongation rates, influences splicing outcomes[202,203]. Additional

## Glossary

**Alternative splicing**
The process by which intron and/or exon sequences are differentially recognized in different cell types or biological conditions to generate distinct mRNAs and long non-coding RNAs from the same primary transcript.

**Back splicing**
The process by which a 5′ splice site is spliced to a 3′ splice site located upstream in the same pre-mRNA molecule, leading to the generation of a circular RNA, typically spanning one or a few exons.

**Branch point**
(BP). An intronic adenosine nucleotide, typically located 15–45 nucleotides 5′ of the 3′ end of introns, which engages in formation of a 2′–5′ phosphodiester bond with the 5′ end of the intron after the first catalytic step of the splicing reaction.

**Exon definition**
A model for the mutual stabilization of splicing factors recognizing splice sites flanking internal exons in multicellular organisms.

**Intron definition**
A model for the mutual stabilization of splicing factors recognizing the splice sites across an intron, which likely has a major role in the efficient co-transcriptional splicing of many introns.

**Introns**
Internal sequences within primary transcripts produced by eukaryotic RNA polymerase II (RNA Pol II) that are removed through the process of pre-mRNA splicing, allowing their flanking sequences (exons) to be spliced together and thus generate functional mRNAs and long non-coding RNAs.

**MicroRNAs**
A class of small regulatory RNAs whose function is to induce the degradation or repress the translation of mRNAs with which they have full or partial complementarity, respectively. They are often transcribed as part of intronic sequences, from which they are released to be assembled with specific proteins on microRNA-induced silencing complexes.

**Premature termination codons**
Translation termination codons in mRNA arising from single-nucleotide mutations or from alternative splicing events that disrupt an open reading frame, often leading to mRNA degradation by the process of nonsense-mediated decay.

**Recursive splicing**
The sequential excision of shorter pieces of a long intron, each piece being separated from the next by a zero-length exon.

**Small nucleolar RNAs**
(snoRNAs). A class of small regulatory RNAs whose function is to guide the addition of chemical modifications at specific residues in other RNAs, including ribosomal, transfer or small nuclear RNAs (snRNAs). They are often transcribed as part of intronic sequences, from which they are released to be assembled with specific proteins on small nucleolar RNP complexes.

**Spliceosome**
The molecular machinery involved in intron removal, composed of 5 small nuclear ribonucleoprotein (snRNP) complexes (U1, U2, U4, U5 and U6) and more than 150 accessory proteins.

evidence has accumulated supporting roles for secondary RNA structures in splice site recognition (reviewed in refs. 204,205), explaining for example temperature-sensitive splice site selection[206], or bringing together distant splice sites through long-range base pairing[207]. In mammalian cells, stem–loop structures involving splice sites or regulatory sequences, chaperoned by various RNA binding proteins, modulate alternative splicing decisions during development and are of relevance for potential therapeutic approaches in spinal muscular atrophy and tauopathies (neurodegenerative disorders characterized by the deposition of abnormal Tau protein in neurons)[208–211]. Links have also been found between the function of RNPs containing structured RNAs and tumour biology, including G-quadruplexes recognized by hnRNPF that enhance exon inclusion events[212] and are relevant for cancer progression[213], and a promestastatic splicing programme that is regulated by interactions between the protein SNRPA1 and structured splicing enhancers[214].

Base-pairing interactions involving sequences flanking internal exons have been shown to contribute to the production of circular RNAs by facilitating back splicing between the 3′ss and 5′ss associated with the looped-out exon[215–220]. Such sequences are often associated with repetitive DNA elements and their limited conservation has been viewed as evidence against the general functionality of at least some families of circular RNAs[221–223]. There is, however, evidence for the functional relevance of specific circular RNAs, for example as 'sponges' for proteins or microRNAs[224–226], and a functional CRISPR screen showed that a group of circular RNAs are important for cell growth, mostly in a cell type-specific manner, or for the preimplantation development of mouse embryos[227]. Furthermore, some circular RNAs can direct the synthesis of peptides or proteins, the general functional relevance of which is under intense debate[228–232].

## Conclusions and future perspectives

The progress reviewed above provides a valuable framework to understand how the spliceosome has evolved various mechanisms to regulate splice site selection. These range from the control of RNA structure or RNA modifications to the spatial organization of genes in the nucleus, from tight coupling between transcription, chromatin and RNA processing to the complex interactions of regulatory sequences and factors that modulate exon and intron definition. However, important challenges to our understanding of pre-mRNA splicing remain.

A comprehensive and quantitative assessment of the isoform structure of individual full transcript molecules using long-read sequencing in single cells remains challenging[233], but this will be necessary to reconstruct the spatial regulation of alternative splicing (spatial transcriptomics) and to understand precisely the contributions of alternative splicing to tissue development and homeostasis. Linked to this is the need for high-resolution methods to reliably assess cell to cell variability in splice site selection and in the levels and/or activity of regulatory factors[234–237] (Supplementary Box 1). If individual cells of the same type diverge markedly in their alternative splicing decisions[125,238–244], this would call for major revision of our understanding of the molecular mechanisms of splicing regulation, which are currently mostly based upon the study of cell populations. For example, a recent study showed that the regulation of intron excision in certain yeast ribosomal protein genes can be used to induce phenotypic heterogeneity that facilitates population adaptation to starvation or high

levels of sugar availability[245]. Similarly important will be to assess the levels, origins, proofreading mechanisms and potential biological functions of 'noise' in the splicing process, for example by observing synthesis and processing kinetics of single nascent RNA molecules in real time[246,247]. In this regard, recursive splicing[246,248,249] and variations in the order of intron removal[164,250] can have important roles in the kinetics of RNA processing and in splice site selection.

Our knowledge of rate-limiting, regulatable steps in spliceosome assembly and catalysis remains mostly based upon (painstaking) efforts to understand the biochemical process as it occurs in a limited number of pre-mRNA substrates that are detectably spliced in cell or nuclear extracts, which is uncoupled from transcription and chromatin, and neither recapitulates the complexity of splicing decisions nor reflects cell type-specific variations in splicing, epigenetic or epitranscriptomic factors. It is conceivable that cell type-specific or even substrate-specific spliceosomes exist, characterized by different composition, stoichiometry and/or modifications of their components, in addition to the modulation of their function by master regulatory factors that respond to environmental cues to shape tissue-specific transcriptomes during development[251]. Recent efforts to develop cryogenic electron microscopy methods to visualize the complexes involved in coupling between transcription and splicing[252,253], in early steps of splice site communication[34,254] and in the function of higher-order supraspliceosomes[255], as well as tomography-based visualization of spliceosomes in situ, should pave the way to a better understanding of the molecular basis of cell type-specific splicing regulation. Such efforts can be complemented by genetic analyses aimed at reconstructing networks of splicing regulation[91,109,256] and by detailed characterization of pathogenic variants in pre-mRNAs and splicing factors. Other important open questions concern the extent to and mechanisms by which long non-coding RNAs can contribute to the regulation of alternative splicing[257] and how alternative splicing of long non-coding RNAs — including the combination of exons across classical transcriptional units — can contribute to generating a large repertoire of RNA modules with possible functions in gene regulation[258].

Ultimately, integrating structural and functional information to predict patterns of alternative splicing is likely to benefit from artificial intelligence methods that can generate models of splice site selection[259] (Fig. 3). Such approaches will have applications for understanding the effects of genetic variation or pathogenic mutations, as well as for the design of novel therapies to correct splicing alterations or to eliminate cells, such as cancer cells, that have pathogenic splicing phenotypes[260].

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## Author contributions
All authors contributed to all aspects of the article.

## Competing interests
The authors declare no competing interests.

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