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Diverse regulation of 3′ splice site usage

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Abstract The regulation of splice site (SS) usage is important for alternative pre-mRNA splicing and thus proper expression of protein isoforms in cells; its disruption causes diseases. In recent years, an increasing number of novel regulatory elements have been found within or nearby the 3′SS in mammalian genes. The diverse elements recruit a repertoire of *trans*-acting factors or form secondary structures to regulate 3′SS usage, mostly at the early steps of spliceosome assembly. Their mechanisms of action mainly include: (1) competition between the factors for RNA elements, (2) steric hindrance between the factors, (3) direct interaction between the factors, (4) competition between two splice sites, or (5) local RNA secondary structures or longer range loops, according to the mode of protein/RNA interactions. Beyond the 3′SS, chromatin remodeling/transcription, posttranslational modifications of *trans*-acting factors and upstream signaling provide further layers of regulation. Evolutionarily, some of the 3′SS elements seem to have emerged in mammalian ancestors. Moreover, other possibilities of regulation such as that by non-coding RNA remain to be explored. It is thus likely that there are more diverse elements/factors and mechanisms that influence the choice of an intron end. The diverse regulation likely contributes to a more complex but refined transcriptome and proteome in mammals.

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Introduction

Alternative pre-mRNA splicing allows the generation of up to thousands of variants from a gene, greatly contributing to the proteomic diversity of metazoans [1, 2]. In humans, about 92–95 % of multi-exon genes undergo alternative splicing [3, 4], with functional consequences ranging from altered mRNA stability and fine-tuning of protein functions to antagonistic effects. Aberrant splicing, resembling alternative splicing but in abnormal conditions, causes diseases [5, 6]. Therefore, it is important to understand how the splice sites (SS) are alternatively or aberrantly used in cell physiology or diseases.

In mammals, the two splice sites at the start and end (5′ and 3′) of introns have distinctly different arrangements of particular motifs. The 5′SS is mostly comprised of a GU dinucleotide within a short consensus sequence. In contrast, the 3′SS consists of tripartite consensus motifs: the branch point (BP), polypyrimidine tract (Py) and 3′AG dinucleotide (Fig. 1, upper diagram). The three motifs are differently conserved, required or spaced among different metazoan species *S. cerevisiae* [7], *S. pombe* [8], *C. elegans* [9–11], and mammals [12], as summarized by Hollins et al. [9]. Particularly, the mammalian tripartite motifs have more permissive in-between spaces and different requirements for the 3′AG [12, 13], likely providing more targets for splicing regulation than the 5′SS, as well as more than the 3′SS in other species.

The splice sites are recognized sequentially by a set of small nuclear ribonucleoproteins (snRNPs) during spliceosome assembly on pre-mRNA introns. This process
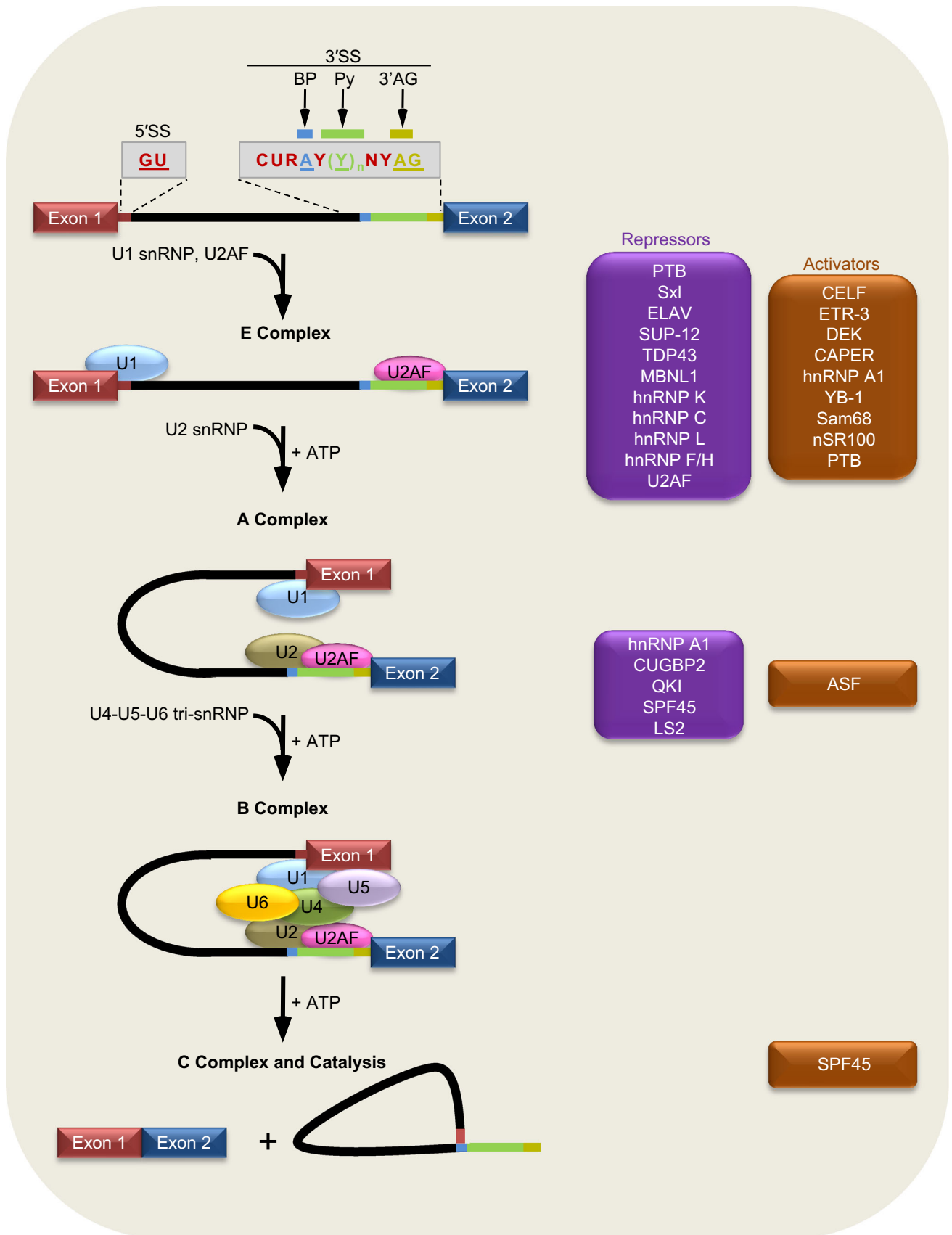
Fig. 1 Diagram of pre-mRNA splicing steps and *trans*-acting 3′SS regulators. Shown at the *top* is splice sites motifs shown in *different colors* with consensus sequence and represented in the intron with respective colors. 5′SS (*maroon line*), BP (*blue line*), Py (*green line*) and 3′AG (*olive line*). Below is the stepwise assembly of constitutive splicing factors (*ovals*) for excision of intron between exon 1 (*red*) and exon 2 (*blue*). *Boxes* on the *right* list *trans*-acting alternative splicing factors that regulate the corresponding steps of spliceosome assembly for exon 2 splicing

has been reviewed recently by Will and Luhrmann [14], to which and references therein readers are referred for more details. Briefly, after the 5′SS is bound by the U1 snRNP, the 3′SS Py and 3′AG are bound by the U2 auxiliary factor (U2AF) heterodimer to form an E (early) complex (Fig. 1). Then the BP is base-paired with the U2 small nuclear ribonucleic acid (snRNA) of the U2 snRNP to form the A complex in an ATP-dependent manner. Subsequent recruitment of U4-U5-U6 tri-snRNP and complex rearrangements form the catalytic C complex where the intron is removed and exons are joined through two transesterification steps. In total, a spliceosome has over 170 proteins that assemble on pre-mRNA introns in in vitro systems using HeLa nuclear extract. It should be noted that, in the above systems relatively short introns have been used, mostly for intron definition and spliceosome formation; for longer introns and certain regulated exons, exon definition has been proposed as well [15–17].

The spliceosome assembly or splicing can be affected by a wide variety of *cis*-acting regulatory RNA elements called splicing enhancers or silencers [18, 19]. Many elements overlap with or are in-between the consensus motifs of splice sites though others are away from the sites. The elements often recruit *trans*-acting splicing factors and/or form RNA secondary structures to regulate splicing [18–20]. In addition to the commonly known regulatory factors, more and more so-called "constitutive splicing factors", which were identified mostly from the HeLa nuclear extract system and model pre-mRNA transcripts, have also been demonstrated to regulate alternative splicing, for example the U2AF65 [21, 22], SmB/B′ [23] or U1C [24]. The list is probably going to extend to more factors in the future. Thus, before a complete survey of these factors is available, here we will still follow the tradition to call them regulatory or constitutive factors respectively.

Recent studies on the regulation of 3′SS usage have uncovered a diverse group of elements and corresponding factors previously unidentified in this region and suggested novel mechanisms beyond the earlier competition model. Here we review the recently characterized 3′SS RNA elements, their binding factors and try to categorize their mechanisms of action into several models according to their common features. Other layers of splicing regulation

including chromatin modification and rate of transcription elongation, as well as posttranslational modification of splicing factors and upstream signaling are also discussed regarding their effects on 3′SS usage. Focus is mainly on progresses in the last decade in mammalian systems.

Regulation of 3′ splice site usage by RNA elements/splicing factors

Table 1 lists representative 3′SS regulatory RNA elements (silencers or enhancers) and *trans*-acting factors in the control of alternative splicing. Their regulatory targets during spliceosome assembly are shown in Fig. 1. Here we describe the elements/factors according their mechanisms of actions.

Repression of 3′ splice site usage

There is a diverse group of silencers and repressors, but they mostly interfere with the early spliceosome factors (Fig. 2).

Inhibition of U2AF binding to the Py

Inhibition of U2AF65 binding was observed upon repressor binding to RNA silencers overlapping with the Py (Fig. 2a). Well-studied early examples include the inhibition of exon 6B of beta-tropomyosin [25, 85], exon 3 of alpha-tropomyosin [27, 86], N1 exon of Rouse sarcoma oncogene (*c-Src*) [16, 29, 30], SM exon of alpha-actinin [32], and gamma amino-butyric acid (*GABA*) receptor, Clathrin light chain B and *N*-methyl-D-aspartate receptor subunit NR1 (*NMDAR1*) exons [33], by polypyrimidine tract-binding protein (PTB) binding to elements such as CUCU to compete with U2AF65 binding to the Py in mammalian systems. A more recent example of PTB-mediated repression of U2AF65 binding is its inhibition of neuron-enriched exons in non-neuronal cells [34]. A similar mechanism represses the male-specific alternative 3′SS of drosophila transformer (*Tra*) exon 2 by sex lethal (sxl) [35, 36].

Other elements include the U-rich and UG repeat elements that are bound by embryonic lethal abnormal visual system protein (ELAV) and SUP-12, respectively, to repress splicing of the *neuroglian* 3′ terminal exon and *ADF/Cofilin* exon 2A [37, 38], or UG motifs by TAR DNA-binding protein 43 (TDP43) to repress cystic fibrosis transmembrane conductance regulator (*CFTR*) exon 9 [39, 40]. These probably target U2AF65 as well. A similar YGCU(U/G)Y motif also inhibits U2AF65 when bound by muscleblind-like 1 (MBNL1), to repress the splicing of cardiac troponin T (*cTNT*) exon 5 [41, 42]. In this case, it

Table 1 List of 3′SS RNA elements and protein factors that regulate 3′SS usage, grouped according to their target constitutive factors

| Target constitutive factor	Location	Regulatory element	Trans-acting factor	Regulated exon	Gene	Mechanism of regulation	References
Silencers/repressors							
U2AF	Py	UCUU and UCUCU	PTB	6B	β-tropomyosin	i	[25]
		UCUU	PTB	3	α-tropomyosin	i	[26–28]
		CUCUCU	PTB	N1	c-Src	i	[16, 29, 30]
		UCUU	PTB	SM	α-actinin	i	[31, 32]
		CUCU like	PTB	24 nt exon, EN and 5	GABA receptor, Clathrin light chain B and NMDAR1	i	[33]
		C/U-rich motifs	PTB	Group[b]	Group[b]	i[a]	[34]
		UUUUUGUUGUUUUUUUU	Sxl	2	Tra	i	[35, 36]
		8/10 U	ELAV	Terminal	Neuroglian	i[a]	[37]
		UG repeats	SUP-12	2A	ADF/Cofilin	i[a]	[38]
		UG	TDP43	9	CFTR	i[a]	[39, 40]
		YGCU(U/G)Y	MBNL1	5	cTNT	i	[41, 42]
		UCCCU	hnRNP K	5a, 6	Snap25, Runx1	i	[43]
		UUUs	hnRNP C	10	CD55	i	[44]
	Between Py and 3′ AG	CA repeats	hnRNP L	V10	CD44	ii	[45]
		CaRRE	hnRNP L	STREX	Slo gene	ii	[46–48]
		CA repeats	hnRNPL	20	TJP1	ii	[49]
		GGGUGGGGG	hnRNP F/H	3	PRMT5 (group)[b]	ii	[50, 51]
U2	Near BP	UAGGG(A/U)	hnRNP A1	3	Tat (HIV)	ii	[52]
		UGUGU and GU	CUGBP2	N1	NMDA R1	ii	[53]
		UGU motifs	CUGBP2		CUGBP2	ii	[53]
		AUUAAC	QKI	12	NUMB	i	[54]
		ULM of SF1	SPF45	6	Fas	ii[a]	[55]
		G rich	LS2	Minigene	Ftc and PEP	ii[a]	[56]
U2AF of weaker 3′SS[a]	Upstream of 3′SS	UUUCUU, UUUUUC, UUUUCU	U2AF	Group[b]	Group[b]	iii	[22]
Constitutive factors e.g. U1, U2AF	Py	ISS in IVS9	SF2/ASF, SRp40	9	CFTR	iii	[57]
		Stem loop	MBNL1	5	cTNT	iv	[41, 42]
		U runs	Sxl	3	Sxl	iv	[58–60]
		CUCUCU	PTB	N1	c-Src	iv	[16, 29, 30]
		UUUs	hnRNP C	10	CD55 (group)[b]	iv[a]	[61]

Table 1 continued

Target constitutive factor	Location	Regulatory element	*Trans*-acting factor	Regulated exon	Gene	Mechanism of regulation	References
Enhancers/activators							
U2AF, U2 snRNA	Py	CUG and UG motifs	CELF	SM, NM	*Actinin*	I	[62]
		U/G motifs	ETR-3	5	*cTNT*	I	[63]
		U2AF interaction (Py)	DEK	Minigene	*IgM-AdML*	II	[64]
		Py	CAPER	6	*VEGF*	II[a]	[65, 66]
		Py with 3′ CG or AG	hnRNP A1	Group[b]	Group[b]	II	[67]
		CAUC	YB-1	V5	*CD44*	II	[68]
	3′AG	3′AG	Urp	Minigene	*AdML*	II	[69]
	BP	AUUAAA	Sam68	V5	*CD44*	II	[70]
		Purine-rich ESE and BP	ASF/RS domain	Minigene	*Actin, IgM*	II	[71, 72]
	Between Py and 3′AG	UGC	nSR100	Group[b]	Group[b]	II	[34]
U2AF of competing 3′SS[a]	Py	CU rich	PTB	Group[b]	Group[b]	III	[73]
Constitutive factors e.g. U1, U2AF	Near 3′SS	Docking/selector	hrp36[a]	Minigene	*Dscam*	IV	[74–76]
		SR binding	U1, U2AF35	Minigene	*dsx*	IV	[77–80]
		Artificial binding sites	hnRNP A/B or hnRNP F/H	Minigene	*hnRNPA1*	IV	[81–84]

For mechanisms of silencing/repression, i: competition, ii: steric hindrance, iii: competing 3′SS, or iv: local RNA secondary structure/looping out. For enhancement/activation, I: competition with a repressor for the same or overlapping RNA element, II: interaction with U2AF or U2 snRNA, III: competing 3′SS, or IV: local RNA secondary structure or longer range RNA loops

[a] Predicted

[b] Group of regulated exons as detailed in the text
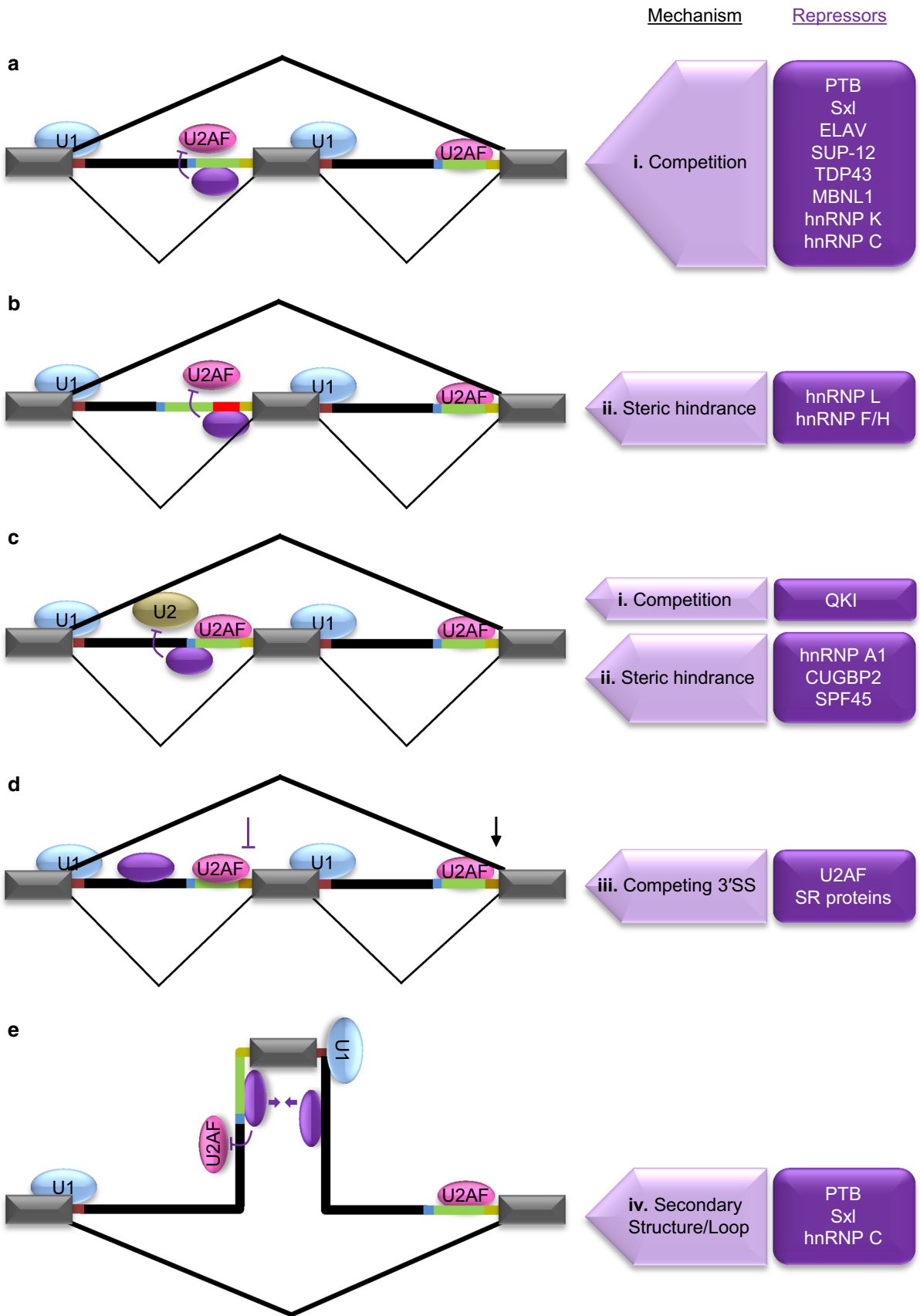
◄**Fig. 2** Mechanisms of splicing repression at 3′SS. The regulated exon shown in the *middle* is flanked by introns and exons. Splicing patterns in *bold lines* indicate the outcome of regulation. *Purple oval* represents one of the repressors listed in *purple boxes* on the *right side* that are involved in the regulation through respective mechanisms indicated on the *left side*. **a** Splicing repression through *cis*-acting elements within Py. The location of a major class of silencers within 3′SS overlaps with Py. These elements recruit *trans*-acting factors to inhibit U2AF binding to Py through competition (i). **b** Splicing repression through *cis*-acting element insertions between Py and 3′AG. A group of 3′SS silencers is uniquely positioned between Py and 3′AG (*red line* in intron). Such elements also inhibit U2AF mainly through steric hindrance (ii) caused by specific binding of repressors. **c** Splicing repression through *cis*-acting elements near BP. Some 3′SS silencers are located near or in overlap with BP. The binding of *trans*-acting factors to these elements results in inhibition of U2 snRNP interaction with BP either through competition (i) or steric hindrance (ii). **d** Inhibition of weakened 3′SS by competition of downstream stronger 3′SS. The weakening of upstream 3′SS by intronic binding of U2AF (*purple oval*) results in inhibition by competing 3′SS (iii). **e** Splicing repression through 'RNA secondary structures or loops'. In some cases Py binding *trans*-acting alternative splicing factors interact cooperatively with another RNA-binding protein bound to downstream intron (*purple ovals* and *arrows*). This interaction results in looping out (iv) of the regulated exon and interferes with U1 and U2AF binding to its splice sites leading to inhibition of exon definition. RNA secondary structures also trap 3′SS motifs in a similar way but without proteins to repress splicing

also involves a mutually exclusive secondary structure encompassing the Py, to which U2AF65 binding is competed by MBNL1 [42].

A recently reported regulatory element within the Py is the UCCCU motif that is bound by heterogeneous ribonucleoprotein particle K (hnRNP K) to repress the exon 5a of neural SNARE complex component synaptosomal-associated protein 25 (*Snap25*) and exon 6 of runt-related transcription factor 1 (*Runx1*) [43]. Mutation of the hnRNP K consensus motifs abolished its binding and addition of hnRNP K reduces U2AF65 binding to the 3′SS in a dosage-dependent way in UV-crosslinking assays. The hnRNP K target motif appears to be enriched in the 3′SS of a group of neuronal alternative exons, implying a wider impact of this regulation [43].

Another recent study has found that hnRNP C binds a U-rich element within Py, to compete with U2AF65 causing complement decay-accelerating factor 55 (*CD55*) exon 10 skipping [44, 61]. The binding of U2AF65 to Py and exon inclusion increased upon hnRNP C knockdown as shown in individual-nucleotide resolution UV-crosslinking and immunoprecipitation (iCLIP) assays and RNA sequencing analysis. Direct competition of U2AF65 binding by hnRNP C was observed in UV cross-linking assays using U$_{10}$ RNA oligonucleotides. This hnRNP C competition with U2AF65 similarly inhibits cryptic 3′SS to avoid the exonization of Alu elements [44].

Besides the Py, a second location of 3′SS elements and their binding factors that interfere with U2AF65 binding is

between the Py and 3′AG (Fig. 2b). We have called these elements REPA (regulatory elements between the Py and 3′AG) [45–49, 51]. These include CA repeats and purine-rich elements particularly G-tracts.

CA repeat elements at this location are bound by hnRNP L to inhibit U2AF65 binding to upstream Py and splicing of a group of exons [45–49, 87]. The binding of hnRNP L and inhibition of U2AF65 were demonstrated in UV cross-linking/immunoprecipitation assays [45, 46, 49, 87]. Moreover, hnRNP L loss of function/rescue assays confirmed its role in the regulation of stress hormonal axis-regulated exon (STREX) [46].

One may argue that the boundary of the CA repeats actually could not be clearly distinguished from the Py, but the recently identified G-tracts between the Py and 3′AG in a group of genes should have defined the REPA elements without any doubt [50, 51]. G-tracts are recognized by the quasi RNA recognition motifs (RRMs) of hnRNP F/H [88–90]. A 'GGGTGGGGG' REPA element is identified in the upstream 3′SS of exon 3 of the protein arginine methyltransferase 5 (*PRMT5*) gene. It is bound by hnRNP F/H to inhibit U2AF65 binding to the Py in UV cross-linking and immunoprecipitation assays. Depleting hnRNP F/H enhanced U2AF65 binding to the Py and adding-back hnRNP H decreased it in a dosage-dependent manner. Moreover, hnRNP F/H knockdown increased the endogenous exon inclusion [50]. Therefore, unlike the enhancer effect of G-tracts at other intronic locations [91–93], the uniquely positioned REPA G-tracts between the Py and 3′AG are splicing silencers. The presence of similarly positioned G-tract elements in over a thousand of human 3′SS suggests a wider impact of these elements in diversifying the protein products and functions [51].

The 3′SS inhibition by the REPA elements could be better explained by steric hindrance between the *trans*-acting repressor and U2AF65/35 heterodimer, which could be co-purified stably from nuclear extracts [94]. The inhibition could be due to the space constraint imposed by the element 'insertion' between the Py and 3′AG, which are often bound by the heterodimer for splicing of AG-dependent 3′SS [95–98]. If so, the 'insertion' represents an opposite way to cause steric hindrance from that by the shortened distances between splice site motifs such as BP and 3′AG or 5′SS in repressing the 3′SS of the (mutually exclusive) SM exon of alpha-actinin [28, 32, 99].

It should be noted that a REPA might not necessarily always inhibit splicing. In the case of a UGC motif at the REPA location [34], it is bound by nSR100 (neural-specific Ser/Arg repeat-related protein of 100 kDa) in neurons to directly interact with U2AF65 and enhance its binding, which will be detailed in the enhancement section. Therefore, whether a REPA causes steric hindrance between the splicing regulators might depend on the

particular factor. Moreover, since such *trans*-acting factors can also receive upstream signaling [46], the effect by even the same factor could be changed depending on the cell environment.

Taken together, these recent cases demonstrate that: (1) the py-rich elements and bound factors could be diverse, and (2) location of the elements can also be out of the Py: "inserted" between the Py and 3'AG (REPA) to recruit more *trans*-acting factors, to interfere with U2AF65 binding.

Inhibition of U2 snRNP interaction with the BP

The interaction of U2 snRNP with the BP is a critical step in spliceosome assembly. It is regulated by a number of *trans*-acting repressors binding to RNA motifs near or directly to the BP (Fig. 2c). An earlier example showed that the U2 snRNP could be interfered sterically by the arginine/serine-rich SR protein ASF/SF2 (SRSF1) binding several nucleotides upstream of the BP [100]. In the splicing repression of HIV Tat exon 3, hnRNP A1 binding to the −26nt does not interfere with U2AF binding but inhibits the subsequent U2 snRNP interaction with the BP [52]. The N1 exon of NMDA receptor subunit *NMDAR1* and exon 6 of CELF RNA-binding protein family member CUG triplet repeat RNA-binding protein 2 (*CUGBP2*) are also regulated in this manner [53, 101–103]. *NMDAR1* pre-mRNA contains UGUGU and GU motifs around the predicted BP of N1 exon upstream 3'SS and UGU motifs are similarly located for *CUGBP2* exon 6 [53]. Such UG-rich motifs are one of the two groups of sequences recognized by CUGBP2 to regulate alternative splicing, the other group being CUG triplet repeats [102–111]. CUGBP2 bound to NMDAR1 and its own 3'SS motifs in chemical modification footprinting assays [53], and its inhibition of lariat formation was confirmed by using recombinant CUGBP2 or mutagenesis of its binding sites under in vitro splicing conditions [53]. Nuclear extract complementation and mutagenesis analyses revealed that CUGBP2 binding interferes with U2 snRNP association [53]. These studies support the model of branch site-perimeter-binding and interference with U2 snRNP for exon skipping.

A more recent example is QKI (quaking homolog, KH domain) competition with the BP binding constitutive factor SF1 (splicing factor 1) to repress the exon 12 of *NUMB* [54], a Notch pathway regulator [112–115]. QKI is a member of STAR (signal transduction and activation of RNA) family of proteins and binds ACUAAY motif [116, 117], which is similar to the consensus ACUNAC of SF1 [118]. Both QKI and SF1 bind the AUUAAC motif immediately upstream of Py in *NUMB* and QKI competes with SF1 in UV-crosslinking and mutagenesis assays [54].

In case of *FAS* (TNF receptor superfamily member 6) exon 6, the splicing repression requires the interactions

between U2AF-homology domain (UHM) of SPF45 (splicing factor 45) with UHM-ligand motifs (ULMs) of constitutive splicing factors including SF1 and U2AF65 [55]. SPF45 crosslinked to RNA in a U2AF dependent manner [119]. The interactions of SPF45 with SF1 and U2AF likely compete with UHM-ULM interactions required for *FAS* exon 6 splicing, possibly by inhibiting the interaction of U2AF65 with SF1 and subsequent U2 snRNP binding to BP [55].

There are also G rich motifs that are enriched in the upstream 60nt of 3'SS to repress the exon usage of some testis-specific genes in Drosophila [56]. These motifs are bound by the LS2 protein; a homologue of the U2AF larger subunit emerged through gene duplication but has diverged in binding specificity for G-rich motifs, as determined by systematic evolution of ligands by exponential enrichment (SELEX) and electrophoretic mobility shift assays (EMSA) [56]. This change in binding specificity switched the protein function to a repressor. The motifs appear to be around the usual location of BP but whether it represses U2 snRNP remains unknown.

Together, the current evidence for the splicing repression of a number of these exons indicates that regulatory motifs overlapping or near BP can specifically inhibit the interaction of SF1 or U2 snRNP with the essential 3'SS motif. The observations in the above examples strongly support this model, particularly *NUMB* exon 12 which is repressed by a direct competition between QKI and SF1 to inhibit BP recognition.

Competition between 3' splice sites

Competition between 3'SSs of two consecutive exons in splicing repression has been described recently [22]. In this scenario, the competitiveness of a downstream 3'SS is enhanced upon 'weakening' of the upstream 3'SS due to U2AF65 binding to an upstream intronic sequence. This mechanism has been proposed for a group of exons for which U2AF binding events in the intronic regions upstream of 3'SS were identified by CLIP-seq experiments. U2AF binding to these sites inhibits the usage of the 3'SS of these introns and promotes the usage of competing 3'SSs (Fig. 2d).

3'SS scanning and competition have been proposed for more than two decades but on the basis of a common BP shared by two competing 3'AGs [99]. The above-mentioned cases do not share the same BP between the two sites; therefore they are likely regulated differently. In this regard, a very interesting study is the biochemical characterization of the regulation of *c-Src* N1 exon by PTB [16, 17]. Here intron-bound PTB blocks the upstream 3'SS of N1 exon [91], prevents definition of the downstream intron but allows usage of the downstream 3'SS with

the constitutive 5′SS further upstream of the N1 exon [17]. According to these effects, splicing of a weak middle exon like N1 is repressed from both sides by splicing repressors; therefore, it could not splice efficiently to its downstream 3′SS. However, the latter could still be spliced efficiently with the upstream constitutive 5′SS, thereby promoting skipping of the middle exon. This provides molecular details for the dynamic transition of spliceosome recognition from alternative to constitutive splice sites.

Another way that could be considered as competition between 3′SSs involves the SR proteins activating decoy 3′SS. The role of SR proteins in the regulation of alternative splicing has been largely based on their binding to exonic splicing enhancers (ESEs) but their binding sites have also been found within introns as well as intron–exon and exon–exon junctions [120–123]. Interestingly, the intronic binding of SR proteins is more frequently associated with splicing repression [57, 121, 124, 125]. Particularly, in regulation of CFTR exon 9, the SR proteins SF2/ASF and SRp40 interact with an intronic splicing silencer (ISS) to inhibit splicing by favoring a 'decoy' sequence through an alternative 3′SS usage [57]. A similar effect of SR proteins has been observed in the preferential usage of proximal or distal 3′SS in β-globin, hnRNP A1 and Adenovirus pre-mRNA substrates [100, 125, 126]. Besides the splicing repression through an ISS, the SR proteins in some cases antagonize the effect of each other to modulate the choice of splice sites [127, 128].

Together, these provide examples for the inhibition of 3′SS by enhancing the relative strength of competing 3′SSs.

Inhibition of 3′ splice site usage by local RNA secondary structure or longer range loops

Another possible mechanism of splicing repression is trapping a splicing motif inside the double stranded region of a stem loop or 'looping out' an entire exon through interaction between complementary regions of pre-mRNA, RNA-binding proteins or different domains of a protein bound to the flanking introns (Fig. 2e).

The effect of pre-mRNA secondary structure on splicing was first observed three decades ago followed by more examples later on [129–132]. Large-scale analyses indicate that stable secondary structures based on free energy prediction are enriched at alternative 3′ or 5′ splice sites and are conserved across species [133, 134]. Complementary mutagenesis analyses of particular sites indicate that RNA stem loops can trap motifs including the Py or 3′AG, or loop out the target exon, thereby inhibiting 3′SS usage [42, 130, 135, 136]. Regulation of cTNT exon 5 is a clear example of the formation of a splicing inhibitory stem loop in 3′SS [42].

For those secondary structures/loops bridged by RNA-binding proteins, two well-studied examples involving 3′SS elements are in the repression of Drosophila Sxl exon 3 and c-Src exon N1 [16, 58–60]. In the case of Sxl exon 3, an autoregulation through binding of SXL in the upstream 3′SS and downstream intron mediates the skipping. SXL exists as a monomer in solution but it can form higher order complexes through RNA-binding-dependent multimerization [58]. In regulation of Sxl exon 3, it binds to the two sites and interacts cooperatively through N terminus to ensure the splicing repression [58–60]. The amino terminus of SXL is essential for cooperative protein–protein interaction and plays a vital role in splicing regulation. Therefore, the multimerization of SXL suggests the formation of protein–protein bridges in the looping out model. For c-Src exon N1, PTB binds to both CU elements within the upstream 3′SS and in the downstream intron to repress exon inclusion, likely by looping out the N1 exon [30]. Whether PTB binds as a monomer or multimer remains unknown though PTB is a monomer in experimental solutions [137]. A model with different RRM domains of PTB contacting different target sites within the pre-mRNA has also been proposed [138]. However, they do self-interact in yeast two hybrid assays [139]. Therefore, it remains undetermined how the loop is formed if it does.

A more recent study proposed the looping out mechanism for the regulation of CD55 exon 10 by hnRNP C binding to upstream Py as described above [44], and downstream intron [61]. The binding of hnRNP C at these positions with 165 nucleotides in-between was determined by iCLIP. The spacer sequence between the two binding sites of hnRNP C flanking CD55 alternative exon is proposed to allow the cooperative particle formation and splicing repression through a looping out mechanism.

Although observations in the above cases suggest the protein-RNA or protein–protein interactions between the splicing factors bound to the flanking introns but direct physical evidence for the "looping out" of an exon in the pre-mRNA remains to be seen. Moreover, for the secondary structures formed by complementary RNA regions, how the structures are regulated in cells to determine the usage or skipping of a 3′SS is unclear. It could be imagined that trans-acting factors (protein or RNA) might be involved to receive the upstream signals.

From the above examples, it is clear that the elements and factors now appear to be quite diverse in sequence and location in the repression of 3′SS usage. Their mechanisms of action mainly involve RNA–RNA, RNA–protein or protein–protein interactions. The binding of trans-acting factors inhibits the recruitment of constitutive splicing factors (Fig. 2; Table 1). Such inhibition could possibly be caused by protein competition (i), steric hindrance (ii), 3′SS competition (iii) or RNA secondary structure/looping

out (iv), for which the first one is the simplest and best characterized.

Enhancement of 3′ splice site usage

The enhancement of 3′SS usage mainly involves the inhibition of repressor binding, binding of *trans*-acting activator to enhancers or direct interaction with U2AF, to promote the early steps of spliceosome assembly (Figs. 1, 3; Table 1).

Inhibition of repressor binding

The binding of a *trans*-acting factor to enhancer element within Py to interfere with the binding of a *trans*-acting repressor is a well-known mechanism of splicing activation at 3′SS (Fig. 3a). The CUG and UG enhancer motifs in the upstream 3′SS of *actinin* exons SM, NM and *cTNT* exon 5 are such examples [62, 63]. In this regulation, these motifs are bound by CELF proteins leading to competitive displacement of splicing repressor PTB that binds to adjacent sites, thereby enhancing the usage of the downstream exon. A recent example involves the displacement of hnRNP L by hnRNP LL due to a genetic mutation in the *CHRNA1* (cholinergic receptor, nicotinic, alpha polypeptide 1) gene [140]. The latter, unlike hnRNP L, does not interact with PTB, thereby allowing U2AF65 interaction with a cryptic 3′SS and inclusion of an intronic sequence.

Interaction with U2AF or U2 snRNA by activators

Other regulatory elements at different positions of 3′SS are recognized by RNA-binding proteins that interact with constitutive splicing factors for splicing activation (Fig. 3b–d). A well-known example of such regulation is the splicing activation of minigene constructs of IgM and AdML pre-mRNAs through interaction of U2AF subunits with DEK (DEK proto-oncogene) [64]. The minigene activation is achieved by the interaction of a chromatin- and RNA-associating protein DEK with U2AF35 (Fig. 3b). However, the binding of DEK to the pre-mRNA is not demonstrated. Moreover, a similar mechanism is likely involved in the splicing activation of vascular endothelial growth factor (*VEGF*) exons 6 and 7 by CAPER proteins that share sequence homology with U2AF65 [65, 66, 141]. Although the molecular details for this mechanism are not known, it was postulated that interaction of CAPER with EWS/FLI-1, which interacts with some 3′SS constitutive factors, is involved [66].

A more recent example of similar regulation is the interaction between RNA-bound hnRNP A1 with U2AF to proofread the splice site and promote its recognition [67]. HnRNP A1 is often observed as a splicing repressor but this study explained a novel role in proofreading of 3′SS. The

pre-mRNAs with pyrimidine rich sequences followed by AG or CG were utilized to study the ability of constitutive splicing factor U2AF to discriminate between the two dinucleotides. HnRNP A1 formed a complex with U2AF heterodimer facilitating it to discriminate between these 3′SS (Fig. 3b). Moreover, upon knockdown of hnRNP A1 in cells, the binding of U2AF to U-rich sequences of an intronless gene *c-Jun* as well as to 5′UTRs (5′ untranslated region) of three other transcripts increased, which suggests a role of hnRNP A1 for preventing the binding of U2AF to U-rich sequences beyond 3′SS in cells. However, whether hnRNPA1 has an enhancing effect on U2AF65 binding to the 3′SS of an endogenous exon in cells is not known.

A similar mechanism may also contribute to the activation of *CD44* (CD44 molecule) exon V5 splicing [68], in addition to a previously known model [70]. A recent study reported CAUC splicing enhancer motifs within the weak Py of V5 upstream 3′SS that are bound by the Y box-binding protein-1 (YB-1) to recruit U2AF65 and U2AF35 through protein–protein interaction [68] (Fig. 3b). The YB-1 interacts through its C-terminal domain with the arginine-serine-rich (RS) domain of the U2AFs and enhances the recruitment of U2AF65 to Py, to which the U2AF65 alone does not bind [68]. Another RNA motif (AUAAA) immediately upstream of Py is bound by Sam68 (Src-associated in mitosis, 68 kDa protein) and enhances the 3′SS usage by facilitating U2AF65 binding to Py [70] (Fig. 3c).

The recently identified UGC motifs between Py and 3′AG also promote the inclusion of a group of exons specifically in neuronal cells [34]. nSR100 binds to these motifs and interacts with early spliceosome components including U2AF65 to promote exon inclusion (Fig. 3d). The nSR100 binding motifs were identified in the upstream introns of a group of regulated neural exons. The in vivo binding of nSR100 to these motifs was determined by photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP). Mutagenesis of minigenes in splicing reporter assays confirmed their critical role in splicing activation. Furthermore, immunoaffinity purification coupled to mass spectrometry analysis showed that nSR100 interacts with a number of constitutive splicing factors including U2AF subunits that bind in a close proximity to nSR100 binding motifs. Moreover, higher nSR100 expression was able to restore exon inclusion after U2AF65 knockdown, which suggests a compensatory effect when U2AF65 is rate limiting. This provides an interesting example of tissue specific splicing activation through the binding of a *trans*-acting factor to the motif uniquely positioned between Py and 3′AG, where locates the REPA elements.

Several examples involve the interaction of activators with the 3′AG, which is important for introns with weak Py tracts. In activating the *c-Src* N1 exon, ASF/SF2 binds to an
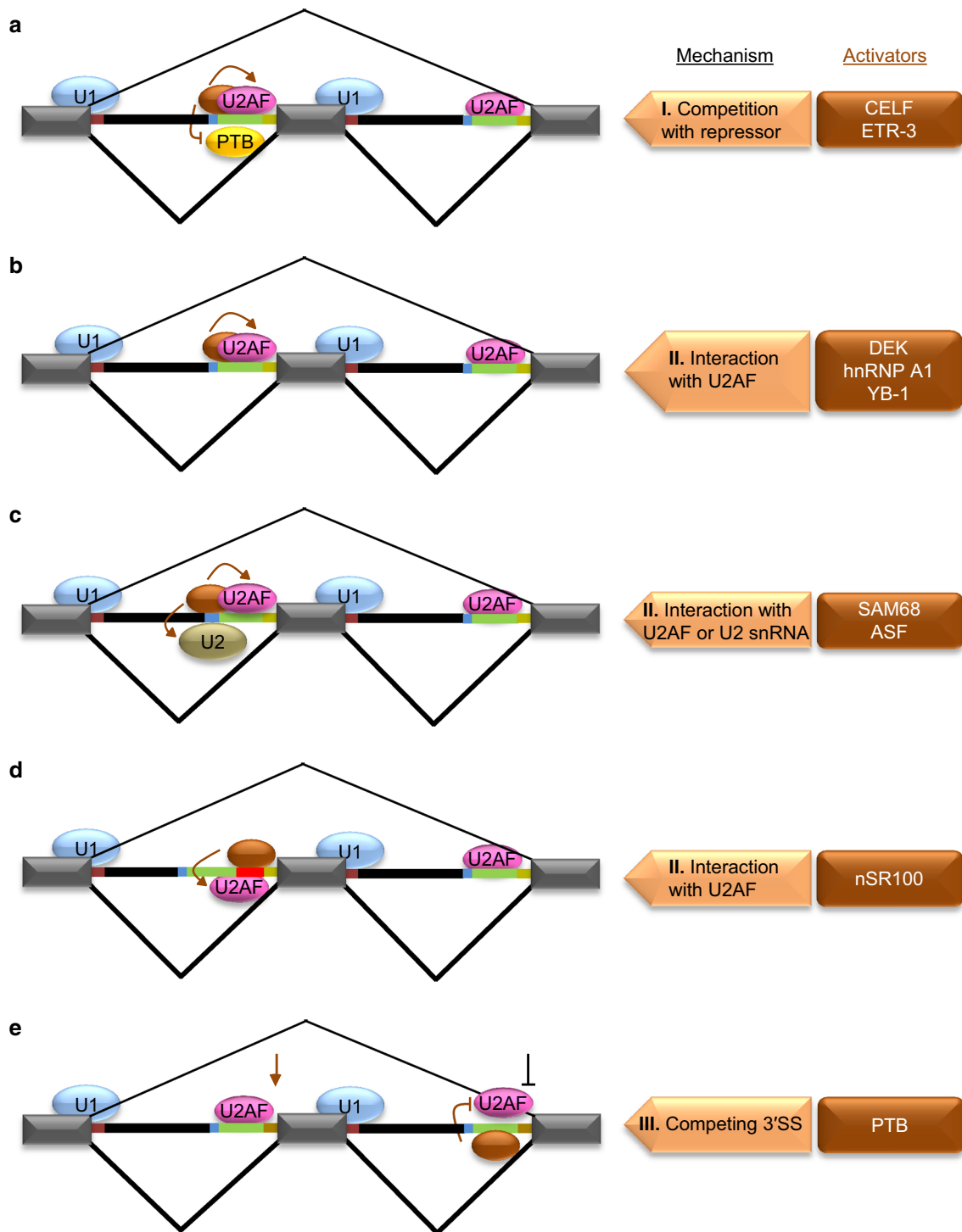
Fig. 3 Mechanisms of splicing activation at 3′SS. Individual activators are shown as *brown ovals* and they are listed on the *right side* in *brown boxes* along with respective mechanisms for the regulation shown on the *left side*. **a** Splicing activation through inhibition of a repressor by enhancer motifs in Py. The binding of a *trans*-acting factor to an enhancer motif within Py results in competition with a repressor (*yellow oval*/PTB) binding (I) and facilitates U2AF binding to Py to promote 3′SS usage. **b–d** Splicing activation through *cis*-acting elements located at different positions within 3′SS. The locations of these elements are within Py (**b**), near BP (**c**) or between Py and 3′AG (*red line*) (**d**). A common feature of 3′SS regulation by these elements is their recognition by respective *trans*-acting factors for interaction with U2AF or U2 snRNA (II) to facilitate their RNA-binding leading to activation of splicing. **e** Activation of 3′SS by repression of downstream 3′SS. The repression of a downstream 3′SS through binding of PTB to Py inhibiting U2AF results in activation of a competing 3′SS (III)

ESE in the 5′ half of the exon but has two nucleotides overlapping with the 3′AG [142]. In regulating the *Sxl* exon 3, SPF45 also binds 3′AG to enhance its usage by enhancing the second catalytic step of splicing [119]. A U2AF35-related protein Urp contacts the 3′AG (or AC of the minor U12 type introns) and interacts with U2AF65 to promote splicing [69, 143], particularly the second step for U2 introns [69]. Another U2AF35-related protein U2AF26 also interacts with U2AF65 and promotes splicing [144]. The detailed molecular mechanisms of the splicing activation by ASF/SF2, SPF45, Urp or U2AF26 in these cases remain unclear. Interestingly, both ASF and Urp contain RS domains. SPF45 also contains RS dipeptides that can be phosphorylated by a SR protein kinase Clk1 in its regulation of splicing [145]. It was shown that ASF binding to or phosphorylated RS domain tethered to an ESE contacts the doubled stranded U2 snRNA-BP region to promote early spliceosome assembly and splicing [71, 72].

Taken together, these examples indicate that interaction with U2AF or U2 snRNA is another way for activators to enhance 3′SS usage.

Competition between 3′ splice sites

In this case, weakening of a downstream site activates an upstream site (Fig. 3e). This was observed in the control of alternative splicing by the commonly known repressor PTB [73]. This mechanism was inferred from the genome-wide PTB binding map based on CLIP-seq, which allowed the identification of a group of regulated exons in the context of PTB binding. Similar competitive activation of upstream 3′SSs has also been described for U2AF65, which binds an intronic site to weaken the downstream 3′SS [22]. A group of exons are regulated through this mechanism but the molecular details of this competition remain unknown.

Enhancement of weaker 3′ splice site by local RNA secondary structure or longer range looping

Besides the direct involvement of 3′SS elements and *trans*-acting factors in its regulation, another mechanism is the enhancement of an otherwise weaker 3′SS by RNA secondary structures or looping, to bring motifs or splice sites closer, particularly in splicing of long introns [84].

The usage of a distal 3′SS could be facilitated by a hairpin structure that brings it closer to the BP in an E1A intron [131], and more examples have been found recently in yeast introns [146]. A similar hairpin structure also blocks cryptic 3′AG to facilitate correct 3′AG usage [147]. Besides the 3′SS motifs, the 5′ and 3′ splice sites can also be brought closer by intra-molecular base-pairing, for instance between two complementary regions near the 5′SS

and BP to aid the early spliceosome assembly in the yeast rp5l B pre-mRNA [148], as supported by both in vitro and in vivo evidence [148].

Studies on drosophila *Dscam* pre-mRNAs suggest a similar model of interaction between two intronic regions to bring splice sites closer in selecting a downstream 3′SS of mutually exclusive exons [75, 76] (Fig. 4a). The selective enhancement of 3′SS usage in the exon 6 cluster of *Dscam* requires base-pairing interaction between one of the selector sequences near 3′SS and a docking site [75, 76] (Fig. 4a). However, how a specific 3′SS among that of the other mutually exclusive exons is chosen in a particular cell or cell state remains unknown. It likely involves *trans*-acting factors such as hrp36, an hnRNP A1 homologue [74].

Besides RNA intramolecular base-pairing, protein–protein interaction may also bring the 5′ and 3′ splice sites closer (Fig. 4b). Proteins such as SRSF1 (ASF/SF2) and SRSF2 (SC35) specifically interact with both the U170K, a component of the 5′SS-binding U1 snRNP, and the 3′AG-binding U2AF35 [77, 78]. Moreover, SC35 interacts with Tra and Tra2 which also shows interaction with U2AF35 and suggested to facilitate interaction between splice sites in regulation of *dsx* sex specific splicing [77–80]. Studies with hnRNP A/B and F/H binding sites near introns ends indicate that their protein-RNA interactions could bring the splice sites closer as well [81–84] (Fig. 4c). Insertion of the binding sites near intron ends significantly increased the splicing of long introns suggesting looping out of a portion of pre-mRNA to bring two splice sites in close proximity. This model was further explained in vivo by hybridizing the ends of introns with oligonucleotides containing hnRNP A/B binding sites within their non-hybridizing tails. Computational analysis of hnRNP A/B and F/H binding sites at the end of introns supports this model as well [84].

Taken together, the current evidence on 3′SS activation by different elements/factors suggests that the mechanisms mainly involve the inhibition of a repressor (Fig. 3a), protein–protein interactions between an RNA-binding protein and U2AF (Fig. 3b–d), activation of a 3′SS by weakening of competing 3′SS (Fig. 3e), or local RNA secondary structures, longer range RNA loops through protein–protein bridge or RNA base-pairing to bring closer two motifs or splice sites to promote early spliceosome assembly (Fig. 4). However, many questions still remain, for instance, how does the activator protein help 3′SS recognition by interacting with the U2AF? Does it need U2AF65/35 binding to the RNA in such a constrained space or simply serve to bring the U2AFs close, like that by YB-1 [68], to help SF1 binding to the BP? Moreover, how do two splice sites compete and is it limited only to nearby 3′SSs?

Overall, according to the mode of protein/RNA interaction in the above examples, the mechanisms of 3′SS regulation (repression or activation) can be divided into: (1)
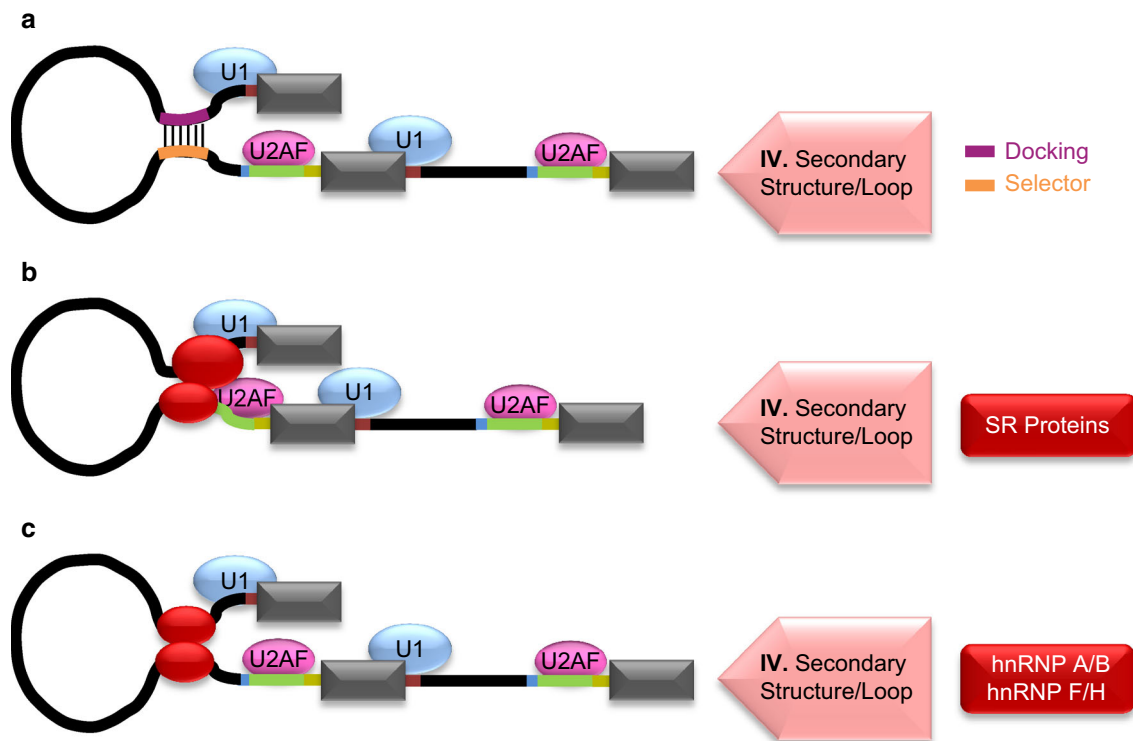
Fig. 4 Mechanisms of weaker 3′SS activation through RNA secondary structures/loops. **a** Base-pairing interaction between intronic RNA motifs. The activation of a 3′SS through looping of pre-mRNA can also result from base-pairing of an intronic motif (*purple line*) with a second motif located near regulated splice site (*orange line*). These motifs are referred to as docking or selector sites as indicated on the *right side*. The formation of such secondary structures or loops (IV) promotes 3′SS usage. **b** Interaction between a complex of splicing factors and 3′SS spliceosome components. In this mechanism a protein complex (*red oval*) binds to an intron upstream of regulated exon and interacts with spliceosome proteins of 3′SS to promote its usage. SR proteins shown in *red box* on the *right side* form such known complexes. **c** Cooperative interaction of a splicing factor. This mechanism of pre-mRNA looping involves the binding of a *trans*-acting splicing factor (*red oval*) near intron ends. Examples of such factors are shown in *red box* on the *right side*. The cooperative interaction between the factors bound near intron ends creates a loop of spanning intronic sequence that brings its splice sites along with their spliceosomal components in close proximity to activate 3′SS

protein competition for a common RNA element/sequence (repression i; enhancement I), (2) steric hindrance between protein factors (repression ii), (3) direct interaction between activators and U2AF or U2 snRNA (enhancement II), (4) competition between splice sites (repression iii, enhancement III), and (5) local RNA secondary structures or RNA loops (repression iv; enhancement IV).

Regulation of 3′ splice site usage by chromatin configuration/transcription rate

Chromatin-remodeling and the rate of transcription elongation also modulate exon usage with enhancing or repressing effect depending on the target exon [149–154]. However, the positional role of chromatin/nucleosome modifications and remodeling with respect to the precise splice sites remains largely unknown. Studies based on computational models revealed that the nucleosome occupancy at approximately 25 bp upstream of 3′AG is lowest as compared to other intronic or exonic regions [155], a position generally corresponding to BP [155, 156].

SWI/SNF is a chromatin-remodeling complex that uses ATP to facilitate conformational changes for transcriptional regulation [157–159]. The ATPase activity lies in the Brahma (Brm) or Brahma-related protein (Brg1). Overexpression and knockdown assays of Brm strongly suggest its role in splicing regulation [160]. Its most profound specific effect was observed for *CD44* exon V5. However, the chromatin-remodeling activity of Brm does not seem to be required for this regulation as mutations or deletions of the ATPase domain did not substantially change splicing whereas deletion of its chromatin binding C-terminal part resulted in decreased exon inclusion. RNA immunoprecipitation assays of Brm revealed that it interacts with U1 and under conditions that favor the inclusion of *CD44* exon V5 it also interacts with U5 snRNA [160].

The splicing of *CD44* exon V5 is enhanced by Sam68 binding in the exon [161], as well as near BP as described above [70]. Co-immunoprecipitation assays revealed that

Brm also associates with Sam68 and they cooperatively enhance splicing of this exon [160]. Moreover, ChIP (chromatin immunoprecipitation) walking using antibodies against phospho-CTD (C-terminal domain) of PolII (RNA polymerase II) revealed higher occupancy in the CD44 variant region including V5, which suggests a slower transcription rate for enhanced exon usage of CD44 V5 [160]. These data support a role for a factor of the chromatin-remodeling complex in the regulation of splicing, likely involving its interaction with those of the 3′SS.

Role of posttranslational modifications and upstream signaling in 3′ splice site regulation

Besides the *cis*-acting RNA elements, *trans*-acting factors and chromatin/transcriptional machinery, posttranslational modifications of *trans*-acting splicing factors by cellular signaling pathways add another layer of regulation. Table 2 lists the posttranslational modifications of 3′SS *trans*-acting factors that influence splicing regulation. The effects on splicing regulation through these factors are described below.

Hydroxylation

U2AF65 undergoes posttranslational hydroxylation by the Fe(II) and 2-oxoglutarate–dependent dioxygenase jumonji domain-6 protein (JMJD6) in the presence of oxygen [162]. The hydroxylation of Lys15 and Lys276 of the endogenous U2AF65 was detected by liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis of HeLa cells. U2AF65 co-immunoprecipitates with JMJD6 in an RNAse-sensitive manner. Knockdown of JMJD6 resulted in increased exon 19 skipping of endogenous *MGEA6* pre-mRNA and a similar effect was observed for a α-

tropomyosin minigene construct comprised of exons 1, 3 and 4. This indirectly shows the role of U2AF65 hydroxylation in splicing activation of these exons as JMJD6 catalyzes it. The effect of hydroxylation on *MGEA6* splicing is further supported by treatment of cells with oxygenase inhibitor desferrioxamine (DFO), which showed a similar effect as JMJD6 knockdown. These data demonstrate a unique posttranslational modification of a splicing factor in the presence of oxygen, likely related to splicing regulation under hypoxia conditions. However, a direct effect of U2AF65 hydroxylation on splicing, the underlying molecular mechanism and the hydroxylation status of other splicing factors under similar conditions remain unknown.

Phosphorylation

Phosphorylation of 3′SS *trans*-acting factors such as SF1 also contributes to alternative splicing regulation [163–165]. The protein–protein interaction of SF1 and U2AF65 and their cooperative binding to 3′SS motifs is a critical step for further spliceosome assembly. In vitro kinase assays followed by mass spectrometry analysis revealed the phosphorylation of SF1 at serines 80 and 82 [165]. In vivo metabolic labeling of overexpressed SF1 or its mutants confirmed this phosphorylation. The phosphorylation of SF1 facilitates its ternary complex formation with U2AF65 and slightly enhances the RNA-binding in EMSA [163, 165], hence likely to promote the spliceosome assembly and splicing. Contrary to the activating effect of Ser80 and Ser82 phosphorylation, previous studies of SF1 Ser20 phosphorylation by protein kinase G (PKG) suggest a negative effect on protein–protein interaction as well as spliceosome assembly [164, 166]. Thus, phosphorylation of different residues of SF1 may have different effects on spliceosome assembly and interaction of SF1 with U2AF.

Table 2 Posttranslational modifications of 3′SS factors

Splicing factor	Modification	Residue	Effect on splicing	References
U2AF65	Hydroxylation	Lys-15, Lys-276	Activating	[162]
SF1	Phosphorylation	Ser-80, Ser-82, Ser-20	Activating/repressing	[163–166]
hnRNP K	Phosphorylation	Ser/Thr (predicted)	Repressing	[43]
hnRNP L	Phosphorylation	Ser-513	Repressing	[46]
DEK	Phosphorylation	Ser-19, Ser-32	Activating	[64]
Sm proteins	Methylation	Arg rich motifs	Activating	[167]
SRSF2	Acetylation	Lys-52	Activating	[168, 169]
U2AF35	Ubiquitination	Within residues 43–146	Repressing	[170]
Prp3	Ubiquitination	Two Lys residues	Repressing	[171]
SF3b[a]	Spliceostatin binding	NA	Repressing	[172]

NA not applicable

[a] Non-covalent binding

Two other examples of splicing factor phosphorylation are of hnRNPs K and L [43, 46]. The hnRNP K is phosphorylated in the PKA-regulated splicing of SNAP25 exon 5a. Its binding to the 3′SS Py is abrogated by pre-treatment with lambda protein phosphatase, suggesting an essential role of its phosphorylation in its regulation of the 3′SS. The serines 284 and 353 of hnRNP K are phosphorylated by the MAP/ERK pathway [173], but their role in the splicing regulation is unknown. In case of hnRNP L, depolarization activated CaMKIV (Calcium/calmodulin-dependent kinase IV) phosphorylates serine 513 to regulate splicing of STREX [46–48, 174]. The phosphorylation enhances its binding to the CaRRE1 (Calcium/calmodulin-dependent kinase IV-responsive RNA element) pre-mRNA and inhibition of U2AF65 [46].

The phosphorylation of DEK is required for intron removal [64]. This protein interacts with U2AF35 to form a complex that is disrupted upon phosphatase treatment. Mutagenesis analysis revealed that the complex formation is dependent on the phosphorylation of serines 19 and 32.

Other regulatory factors acting on the 3′SS are also phosphorylated but the phosphorylation effect on 3′SS usage has not been reported. For instance, hnRNP A1 is phosphorylated upon activation of the p38 MAP kinase pathway by osmotic or UV stress, which caused its redistribution from nucleus to the cytoplasm and accompanied enhanced proximal 5′SS usage of E1A reporter minigenes [175]. The redistribution requires phosphorylation of a number of serines within a C-terminal peptide that interacts with transportin 1 [176]. PTBP1 is phosphorylated by protein kinase A upon forskolin stimulation [177]. The target Ser16 is essential for its redistribution from the nucleus to the cytoplasm and neurites [177, 178].

The phosphorylation of SR proteins, which also influence 3′SS selection [57, 100, 126], regulates their subcellular redistribution, RNA-binding and effect on splicing [179–183]. Their specific kinase yeast Sky1 is implicated in 3′AG recognition [184]. Importantly, both the hypo- and hyperphosphorylation of SR proteins can inhibit splicing suggesting the role of their phosphorylation levels in alternative splicing [185–187].

Methylation

Many constitutive and alternative splicing factors are methylated including the spliceosome core Sm proteins [167, 188]. The reduction in methylation of Sm proteins leads to aberrant splicing of specific pre-mRNAs. For instance, the splicing of Mdm4 (MDM4, p53 regulator) exon 7 is repressed in the absence of PRMT5 (protein arginine methyltransferase 5) that methylates Sm proteins [167].

Acetylation

Acetylation inhibitors also affect various steps of spliceosome assembly, suggesting that acetylation of multiple components/steps of the spliceosome assembly are involved in this process [169]. SRSF2 acetylation on lysine 52 in its RRM destabilizes the protein through proteosomal degradation in the regulation of the cell growth/apoptosis variants of caspase-8 gene [168]. A large number of splicing regulator proteins including most of hnRNPs mentioned above are also acetylated in cells [150]. Particularly lysines 87, 98 and 224 of hnRNP F that are potential targets of acetylation and ubiquitination are critical for increased protein stability by a deacetylase inhibitor trichostatin A (TSA) [189]. Their acetylation effect on 3′SS usage remains to be examined.

Ubiquitination

Ubiquitination of U2AF35 contributes to splicing regulation. One of the Shigella factors IpaH9.8 specifically interacts with U2AF35 to interfere with splicing of IgM pre-mRNA [190]. IpaH9.8 catalyzes U2AF35 ubiquitination in vitro [170].

U4 snRNP component Prp3 is modified by Prp19 complex with nonproteolytic ubiquitin chains [171]. This ubiquitination facilitates the interaction of Prp3 with U5 snRNP and therefore promotes the stability of U4-U5-U6 tri-snRNP. Usp4 deubiquitinates Prp3 leading to disruption of the U4-U5-U6 tri-snRNP in HeLa splicing extracts, which inhibits in vitro splicing of a substrate ftz pre-mRNA. However, knockdown of Usp4 decreased the levels of correctly spliced products of a group of pre-mRNAs [171]. This suggests the role of Prp3 ubiquitination in maintenance of the U4-U5-U6 tri-snRNP and its reversal by Usp4 in spliceosomal rearrangements, particularly release of Prp3 and other U4 proteins, required for U6 to participate in active spliceosome [171, 191]. Moreover, a component of U5 snRNP called Prp8p is also ubiquitinated and this modification has been suggested to be involved in the maintenance of U4-U5-U6 tri-snRNP assembly [192, 193]. Therefore, more than one spliceosomal factors are ubiquitinated during splicing.

Spliceostatin binding to SF3b

Spliceostatin is a methylated derivative of a natural product FR901464 [172, 194]. Originally this product was identified for its anti-cancer effect through enhanced transcription activity of SV40, which promotes cell cycle arrest [195]. Later on it was discovered that spliceostatin binds to SF3b [172], an important component of U2 snRNP critical for its interaction with U2AF [196–199]. The

binding of spliceostatin to SF3b is not a posttranslational modification. However, this binding results in inhibition of U2 snRNP recognition of the BP, in complex formation and in vitro splicing assays [172, 200, 201]. Spliceostatin treatment of HeLa cells leads to nonsense-mediated mRNA decay (NMD) of splice variants and downregulation of genes important for cell division, such as cyclin A2 and Aurora A kinase [200].

Taken together, current evidence has demonstrated that various modifications of *trans*-acting factors regulate protein level, protein–protein or protein-RNA interactions to control the 3′SS usage. However, the modifications of many more 3′SS splicing factors remain unknown.

Evolutionary emergence of regulatory elements and factors

Global insights into alternative exon creation revealed that over the course of mammalian evolution the abundance of alternative splicing significantly increased as compared to lower vertebrates [202]. This is further consolidated by independent alternative splicing data sets from different species [203, 204]. However, the evolutionary changes that contributed to the splicing regulatory mechanisms involved in the emergence of alternative exons remain largely unknown.

Evolutionary mechanisms for the de novo creation of alternative exons include tandem exon duplication, exonization of transposable elements and transition of a constitutive to alternative exon [205–209]. The transitions are achieved through accumulation of mutations at splice sites or splicing regulatory elements [209–212]. The splice sites of lower vertebrates such as fish shows remarkably low prevalence of regulatory elements such as GGG [213]. This implies that the 3′SS regulatory elements perhaps are largely characteristic of mammals.

Sequence alignment of the upstream 3′SS of *Snap25* exon 5a from different species showed that the hnRNP K binding motifs are mostly conserved in vertebrates and expanded into multiple copies in mammals whereas they are absent in invertebrates [43]. Similar sequence alignment of STREX upstream 3′SS of different species indicated that the CA contents of CaRRE1 increased from lower to higher vertebrates [46, 214]. For the *PRMT5* G-tract element [51], it clearly emerged in mammals over the course of evolution [215]. Human and several other mammals contain two copies of G-tract that are reduced to one copy in lower mammals and marsupials; in lower vertebrates including fish and amphibians this element is absent. The skipping of exon 3 is significantly increased in human *PRMT5* transcripts. Moreover, the critical role of the G-tracts in splicing repression is confirmed by

mutagenesis and splicing assays [51]. The evolutionary emergence of this splicing regulatory element provides a clear example of the transition of a mainly constitutive to an alternative exon. Together, these evolved regulatory elements may have contributed to the proteomic diversity in mammals [214].

Further considerations

Of the regulation mechanisms, the competition model is perhaps by far the simplest and most well supported for the 3′SS regulation. However, many more examples have been accumulating that cannot be simply explained by this model. Therefore, other mechanisms such as steric hindrance, splice site competition and local RNA secondary structure or looping have also been proposed. Yet, more direct evidence and molecular details are still needed for some of the latter models. Moreover, how the inhibition or activation happens after recruitment of the *trans*-acting factors remains unknown in most cases.

Despite the above progresses, further study is necessary for insights into the regulatory mechanisms. The regulation of splicing takes elaborate interplay of multiple factors to determine the fate of an exon. The examples discussed in this review mainly focus on the role of a particular element/factor within 3′SS. How they collaborate with other elements/factors or splice sites to determine the choice of a particular splice site among the many other sites remains largely unknown. For example in the regulation of STREX splicing, hnRNP L is a repressor through binding to CaRRE1 [46–48], but its interaction with other factors such as PTBP1, hnRNP K and LL in the regulation remains unknown [46, 216]. Moreover, the regulation of *trans*-acting factors by upstream signals has just started to be unveiled; more effort is needed to fully understand the impact of this regulation on 3′SS choice and splicing in general. A number of other modifications of splicing factors such as acetylation and methylation in the regulation of alternative splicing have just emerged recently [149, 150, 167–169, 189, 217, 218]. Furthermore, chromatin modification and the transcriptional machinery also have important impact on splice site choice; however, their detailed mechanisms of action remain to be answered, though models based on transcription rate and splice site strength have been proposed.

The functions of novel so-called non-coding RNAs (ncRNA) have just started to be revealed as well but already shown to impact many steps of gene expression [219]. Their role in splicing regulation particularly through 3′SS remains unexplored. One study showed that snoRNA HBII-52 regulates the splicing of serotonin receptor 5HT$_{2C}$ by base-pairing with a silencer element in its exon to

influence the splicing of Vb through alternative usage of a 5′SS [220]. In another study, a natural anti-sense transcript (NAT) that overlaps with 5′SS is associated with intron retention in the 5′ UTR of *Zeb2* pre-mRNA [221]. This intron retention preserves the IRES hence allowing the translation of Zeb2 protein. Several other studies also suggest a link between anti-sense RNAs and posttranscriptional processing particularly splicing [222–224]. Such mechanisms for 3′SS regulation remain to be explored.

Beyond the expected regulatory elements/factors, how do the constitutive (or even "core") spliceosome factors regulate alternative splicing? Besides differences in the splice site sequences and strengths of target transcripts, the role of the homologous factors such as PUF60 and CAPER to U2AF65 [141, 225], U2AF26 to U2AF35 [144] in this process remains to be intensively explored. Moreover, many splicing factors themselves are alternatively spliced. Though some of their variant transcripts including those of the core spliceosome factors are subject to NMD [226], in other cases variant protein products of splicing regulators do have differential effects on splicing [227]. Furthermore, pseudogenes have also come into play with gene expression [228]. As analysis of the genome/transcriptome accelerates, more such homologous factors, splice variants, pseudogene or even non-homologous factors but with overlapping functions might be identified beyond the previously known constitutive or "core" spliceosome factors.

In addition to studying the details of molecular mechanisms regulating individual exons, genome/transcriptome-wide analyses among different species have identified conserved splicing regulatory elements within/nearby splice sites and reveal their evolutionary emergence [213, 229]. A human genome search identified several potential splicing regulatory elements including evolutionarily emerged splicing silencer G-tract element between Py and 3′AG [51]. Application of these approaches to more elements/factors will help to reveal the diverse regulation of 3′SS choice and evolutionary changes that contributed to the increased proteomic diversity and organismal complexity in mammals. The knowledge of large-scale analysis of splicing regulation will also help us understand the pathological conditions that arise from aberrant splicing due to genetic mutations. For instance, the mutations of several 3′SS spliceosome factors are associated with different clinical outcomes [230–232], suggesting their cooperation with other genetic lesions [233], or different target exon profiles.

In conclusion, the diversity of characterized 3′SS regulation has been increasing rapidly in the past decade. With more possibilities of regulation remaining to be explored, this diversity is likely going to continue to increase. The diverse regulation likely contributes to the higher transcriptome and proteome diversity in mammals than lower vertebrates. Further studies will help us understand not only this important step of gene regulation in normal cell physiology but also the pathophysiology and development of therapeutic approaches for aberrant splicing-caused diseases.

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