

Widespread and subtle: alternative splicing at short-distance tandem sites

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Alternative splicing at donor or acceptor sites located just a few nucleotides apart is widespread in many species. It results in subtle changes in the transcripts and often in the encoded proteins. Several of these tandem splice events contribute to the repertoire of functionally different proteins, whereas many are neutral or deleterious. Remarkably, some of the functional events are differentially spliced in tissues or developmental stages, whereas others exhibit constant splicing ratios, indicating that function is not always associated with differential splicing. Stochastic splice site selection seems to play a major role in these processes. Here, we review recent progress in understanding functional and evolutionary aspects as well as the mechanism of splicing at short-distance tandem sites.

Alternative splicing as a source of proteome complexity

A fundamental difference between prokaryotic and eukaryotic gene structures is that the typical eukaryotic gene contains introns [1]. In the splicing process, these introns are excised from the primary transcript while the remaining sequences (exons) are joined together (Box 1). Alternative splicing enables one gene to produce multiple mature transcripts with different sequences. Large-scale characterization of eukaryotic transcriptomes using expressed sequence tags (ESTs), cDNAs, and splicing sensitive microarrays revealed that alternative splicing is frequent [2], with 75% of the human genes estimated to undergo alternative splicing [3]. Alternative splicing (along with other mechanisms such as alternative transcriptional initiation, alternative polyadenylation, RNA editing) is believed to be a major mechanism to bridge the gap between the surprisingly small gene number and the much higher transcript and protein number in higher eukaryotes [4].

Alternative splice variants can have biological roles by giving rise to functionally different proteins [5], and the regulation of alternative splicing is important for diverse biological processes. For example, the brain-specific splicing factor Nova regulates alternative splicing of several genes with neuronal functions [6]. In *Drosophila*, alternative acceptor use of the *tra* gene is the beginning of a regulatory cascade that leads to male or female development [7]. Furthermore, alternative splicing can regulate gene expression at a posttranscriptional level by producing

transcripts that are subject to the surveillance mechanism nonsense-mediated decay (NMD) that degrades mRNAs with stop codons >50 nucleotides (nt) upstream of the last exon–exon junction [8,9]. Defects in splicing are associated with cancer [10,11] and other human diseases [12].

There are several types of alternative splice events, which vary in frequency. In vertebrates, the inclusion or skipping of entire exons is the most frequent alternative splice event, whereas in plants, the retention of entire introns is most prevalent [13,14]. Intensive research has focused on exon skipping, and progress has been made in understanding the function, evolution, and characteristics of such exons and in distinguishing them from constitutive exons. Another major mode of alternative splicing is the selection of two or sometimes more alternative splice sites. In humans and mice, alternative donors and acceptors each make up >25% of all alternative splice events [15]. In contrast to exon skipping or intron retention, such splice events are roughly equally frequent in vertebrates, invertebrates and plants [14].

In recent years, several studies showed that many alternative donor and acceptor sites are close to each other, thus producing mRNAs that differ by a few nucleotides [2,16–19]. Such splice site pairs are called tandem sites (Figure 1; please note that we use the notation ‘tandem site/donor/acceptor’ for alternatively spliced sites and the notation ‘tandem motif’ for those that currently lack experimental evidence for the use of both splice sites). Here, we discuss recent progress in characterizing the frequency, the splicing mechanism and the functional and evolutionary implications of tandem sites. It should be noted that U12-dependent introns also produce subtle splice events [20], but we focus on U2-dependent introns because they represent the majority of introns. For reviews on other aspects of alternative splicing, please see Refs [21–24].

Widespread subtle alternative splicing

Although alternative splicing at tandem sites was observed only a few years after the discovery of introns, the extent of this phenomenon was only recently appreciated. Computational analysis using large EST databases found that one third of all alternative donor and acceptor pairs are located 2–10 nt apart [2,19]. Such short-distance alternative acceptor events are about twice as frequent as alternative donor events [16,18,19]. Of all alternative acceptors, $\Delta 3$ (nucleotide distance between two splice sites is denoted with Δ nt; Figure 1) sites having a NAGNAG motif (N = A,C,G,T) are by far most frequent in plants [13]

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Box 1. Introns and splicing

Introns possess several sequence signals required for splicing: the 5' or donor splice site, the branch point, the polypyrimidine tract and the 3' or acceptor splice site (Figure 1). We prefer here the notation donor and acceptor because 5' and 3' splice site can be ambiguous if there are alternative sites. Apart from rare exceptions [75,76], the 5' terminal intronic dinucleotide is GT or sometimes GC (<1%) and the 3' terminal dinucleotide is always AG.

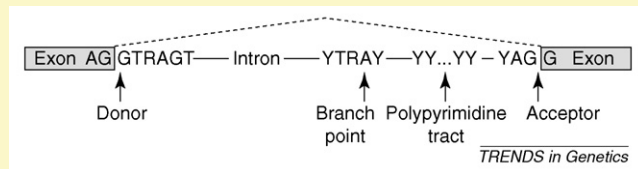


Figure 1. Schematic illustration of basic splicing signals. Splicing is carried out by the spliceosome, which consists of several small nuclear (sn)RNAs (U1, U2, U4, U5 and U6) and numerous proteins. Splicing proceeds in two steps. First, the mRNA is cleaved at the donor splice site and the 5' intron end is attached to the branch point adenosine, forming a lariat structure. Second, the mRNA is cleaved at the acceptor site, both exons are ligated, and the intron is released. During the first step, the U1 snRNA initially binds to the donor site by base pairings to the last three exonic and first six intronic positions and is later displaced by the U6 snRNA [77]. The 3' intron end is recognized by a heterodimer U2AF. The U2AF65 subunit binds to the polypyrimidine tract and the U2AF35 component binds to the YAG (Y = C,T). The U2 snRNA binds to the branch point by base pairings, and this process is aided by protein factors. The recognition of these basic splicing signals is influenced by splicing factors such as serine-arginine-rich proteins and heterogeneous nuclear ribonucleoproteins that bind to sequence motifs in exons and introns [23]. These splicing factors can have enhancing or silencing effects and are essential for alternative and constitutive splicing.

and animals [2,17–19,25], with almost 2000 alternatively spliced acceptors observed in humans [26]. At the donor site, alternative $\Delta 4$ donor events are most frequent [13,19,27]. This is because of the intronic donor consensus sequence GTRAGT (R = A,G) that provides a second GT 4 nt from the donor site in $\sim 40\%$ of introns [27]. Accurate identification of such subtle splice events from noisy EST data requires stringent filtering of the EST-to-genome alignments [2,17]. Experimental validation using RT-PCR succeeded in identifying alternative splicing in 92% of 220 tested NAGNAG acceptors [25], suggesting that alternative splicing at tandem sites can be accurately identified using ESTs.

The frequency of donor and acceptor events decreases with increasing distance between the two splice sites. At short distances, $\Delta 4$ and $\Delta 5$ events outnumber the $\Delta 6$ cases, even though the frameshifted transcripts arising by $\Delta 4$ and $\Delta 5$ events are less likely to be detected, because NMD reduces their abundance [19]. This shows that potential splice sites at a close distance to the dominant site are very prone to alternative splicing [16,19]. At larger distances, the greater fraction of frame-preserving events is likely caused by the suppression of frameshifted transcripts by NMD [19].

Tandem splice events are found in species in which alternative splicing is frequent, including mammals, chicken, zebrafish, *Drosophila* and plants [13,17,28]. The only exception found thus far is the nematode *Caenorhabditis elegans*, where alternative splicing at NAGNAG motifs is very rare. In *C. elegans*, the intron-proximal acceptor is exclusively used in almost all NAGNAG motifs, and only 33 alternative cases (constituting <1% of all

NAGNAG motifs) are found [17,28]. This seems to be caused by a unique acceptor selection mechanism, because the *C. elegans* U2AF dimer binds to a tightly restricted TTTTCAG motif, and deviations from this consensus affect binding [29]. This restricted acceptor motif may prevent recognition of an alternative intron-distal AG, indicating that flexibility in U2AF binding is a crucial step for alternative splicing at tandem acceptors with the potential for regulation. In contrast to NAGNAG sites, alternative splicing at $\Delta 3$ donors was observed in *C. elegans* with a frequency similar to other species [28].

Insights into the tandem splice mechanism

Experimental studies confirmed that alternative donor or acceptor splice events are the cause of the observed subtle splice variants. Disruption of one of the two splice sites by a targeted single nucleotide mutation [25,30,31] or a naturally occurring polymorphism [32–35] leads to the disappearance of the respective splice variant in all investigated cases. This excludes very short exons or highly similar mutually exclusive exons being a frequent cause of subtle alternative transcripts.

The two splice sites in alternative tandems generally resemble normal splice sites in terms of their sequence preference. That is, both alternative donor sites often have a reasonable base pairing potential to the U1 small nuclear RNA (snRNA) and both alternative acceptors mostly have YAG motifs (where Y = C,T), which are preferred U2AF35 binding sites (Box 1) [19,28,36]. For example, one half of all CAGCAG acceptor motifs in humans are known to be alternatively spliced [17]. Furthermore, basically all of the 16 possible NAGNAG motifs allow alternative splicing except for those having a GAG acceptor [17,35], consistent with experimental observations that GAG is a very inefficient acceptor [37]. This indicates that the capability of alternative splicing at tandem sites is an intrinsic property of the spliceosome.

We know from computational studies that the NAG sequence and its local sequence context are the most important features to distinguish alternative from constitutive NAGNAG acceptors [16,38]. Features derived from the local context (e.g. strength of the splice sites, the polypyrimidine tract and the branch point, as well as the distance between them) enable the prediction of alternative splice sites using machine learning methods [38,39]. The relative strength of both splice sites in a tandem is also predictive for the predominantly used site [16,19,27,28,40–42]. However, for NAGNAG acceptors, experiments have shown that the ratio of splicing at the intron-proximal versus the distal site also depends on the intronic sequence between the branch point and the NAGNAG acceptor [35]. Tsai *et al.* [35] switched the intronic context between NAGNAG acceptors that are predominantly spliced at the proximal and at the distal acceptor. The recombinant NAGNAG tandem with the heterologous intron context resembled the splicing ratio of the NAGNAG tandem from which the intron sequence was taken. However, neither the sequence motifs responsible for a specific ratio nor the splicing factors involved are known. At larger distances, the location and strength of the polypyrimidine tract is important for alternative acceptors [38]. This is

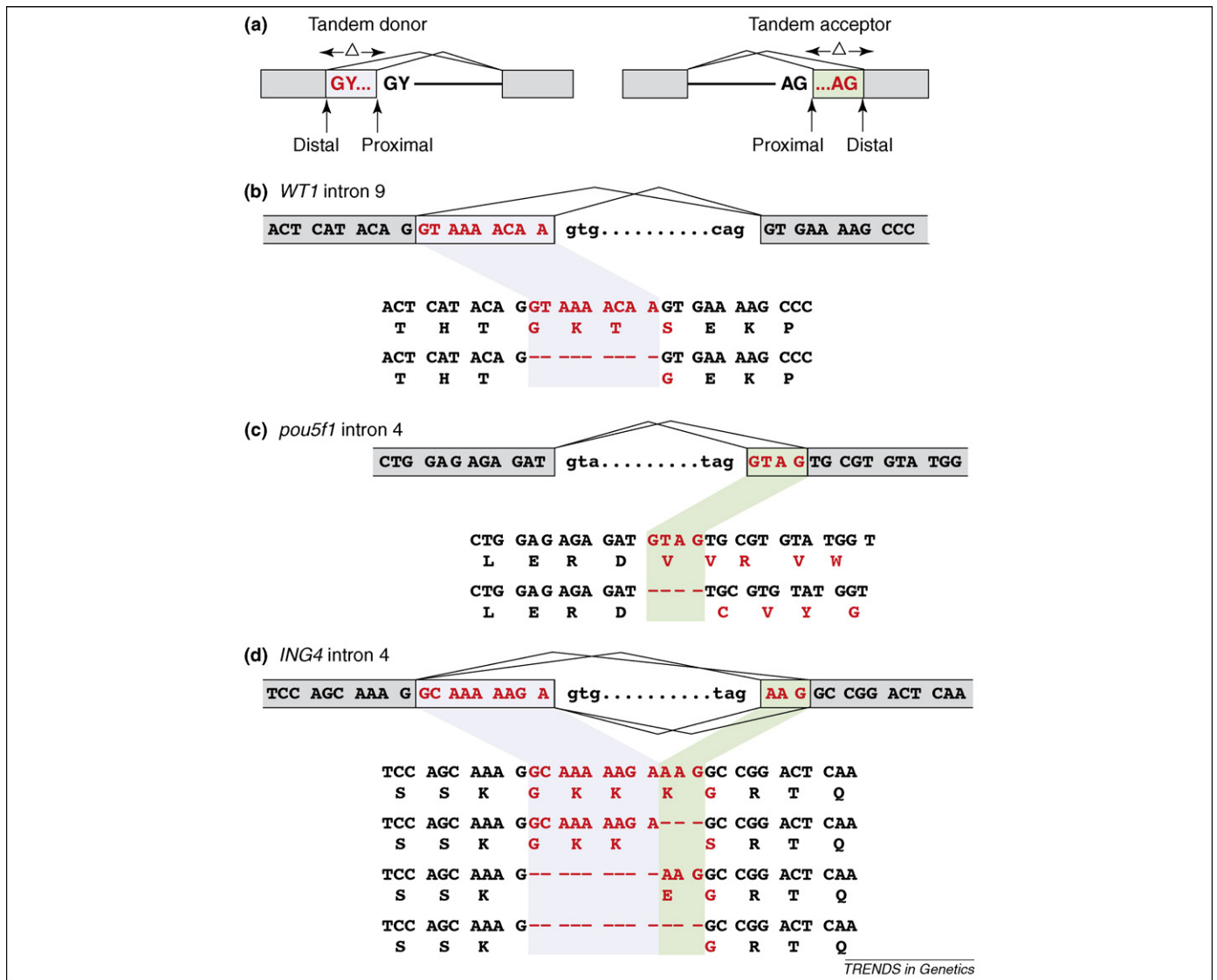


Figure 1. Examples of alternative splicing at short-distance tandem sites. (a) Schematic illustration of tandem donors and acceptors. (b) A $\Delta 9$ donor in the last intron of human *WT1* results in the indel of three amino acids (KTS). (c) A $\Delta 4$ acceptor in the last intron of zebrafish *pou5f1* leads to a frameshift and a different C terminus of the protein. (d) Intron 4 in the human *ING4* gene exhibits a $\Delta 9$ donor and a $\Delta 3$ acceptor [31,34]. The combination of both tandem sites leads to four different transcript and protein variants. The partial exon–intron structure is shown in boxes; missing residues in the transcript sequences are indicated by dashes.

consistent with the finding that at distances of >8 nt, the sequence between the two AGs resembles a second polypyrimidine tract [19]. Thus, at shorter distances, only one polypyrimidine tract seems to be sufficient, whereas at larger distances, two polypyrimidine tracts are needed.

Tandem splicing ratios: constant and variable

Alternative splicing is a highly regulated process with the potential to express a splice variant only under specific spatio-temporal conditions. For example, alternative acceptor use of the *Drosophila tra* gene is controlled in a sex-specific manner [7]. Experimental studies showed that the ratio between two splice variants at short-distance tandem sites may differ between tissues or cell types [17,25,43,44], between developmental stages [45–47] or with respect to external stimuli [47]. Splicing factors such as serine-arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoprotein (hnRNPs) regulate alternative splicing [23], and these proteins are candidates to regulate

tandem splicing by promoting intron–proximal or distal splice sites, respectively (Box 2). Although the short distance between two tandem sites largely excludes the possibility that splicing regulatory motifs are placed between them [48], the up- and downstream regions can contain potential splicing factor binding sites, as shown for $\Delta 3$ tandem sites [28,36]. In case of a $\Delta 3$ acceptor involving a noncanonical TG dinucleotide in the GNAS complex locus gene, SF2/ASF and hnRNP A1 were shown to affect the ratio between the proximal and distal site [49]. It was also suggested that alternative donor/acceptor use is associated with splicing motifs that are distinct from those that regulate exon skipping [42]. Because the position of mRNA cleavage at the acceptor site is ultimately determined in the second splicing step (Box 2), variations in the concentration of second-step splicing factors can lead to variations in the splicing ratio, as shown for hSlu7 [50,51]. However, the details of the underlying mechanism(s) for these variable splicing ratios are currently unclear.

Box 2. Mechanisms of alternative donor and acceptor selection

Donor selection

A widely accepted mechanism for alternative donor splicing is the differential binding of the U1 snRNA to one of the potential donor sites. According to this splice site competition model, alternative splicing happens when one donor is sufficiently good to compete with the other donor for U1 binding. Constitutive splicing at a tandem motif (exclusive selection of only one donor) occurs when either donor is much stronger and consequently outcompetes the other.

Apart from the intrinsic strength of donor sites, splicing factors such as serine-arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) affect splice site selection. SF2/ASF and other SR proteins promote splicing at the intron-proximal donor site, whereas hnRNP A1 promotes the distal site [78]. The relative concentration of SR proteins and hnRNPs affects donor selection, and tissue-specific variations in this ratio might lead to tissue-specific splicing patterns [78].

U1 snRNA binding is almost always required for splicing. One exception is an alternative donor site in *FGFR1*, where the U6 snRNA is responsible for splicing at a noncanonical GA donor site [79]. Also, *in vitro* experiments have shown that splicing can proceed without U1 when the donor site allows a sufficiently stable U6 snRNA binding [77]. However, these cases seem to be rare because computational studies found no evidence that U6 snRNA activates alternative donor sites [19].

Acceptor selection

In vitro experiments found evidence for different modes of acceptor AG selection that depend on the distance of the AG to the branch point. If the branch point is more than ~20–35 nucleotides (nt) away from the AG, the AG selection occurs by a scanning mechanism that starts from the branch point and usually selects the intron-proximal AG [37,80]. The proximal AG can be bypassed if it is too close to the branch point or if it is in competition with a more distal AG. This competition can lead to alternative splicing and depends on (i) the distance between the AGs (shorter distances lead to a higher competition), (ii) the nucleotide upstream of the AGs (C and T are preferred over A and especially over G) and (iii) the sequence between both AGs [19,37,80,81]. Scanning does not occur if the distance to the branch point is short (<20 nt) [80]. In these cases, a distal AG can efficiently compete with a proximal AG given the distance between both AGs <6 nt [81].

However, initial selection of one AG does not ultimately determine the site of exon ligation. It was shown that a proximal AG can be necessary for mRNA cleavage at a distal AG in the second splicing step [81] and vice versa [82]. Splicing step II is subject to proofreading mechanisms, and several proteins are involved in selecting the ultimate AG. For example, changing the concentration of hSlu7 affects the splicing ratio of two acceptors [50,51]. The second step splicing factor SPF45 promotes the use of a proximal AG despite the presence of a polypyrimidine tract and an AG located downstream, which are bound by the U2AF heterodimer [82].

Similar to donor selection, SR proteins were shown to promote proximal acceptor splice sites, whereas hnRNP A1 promotes distal sites [83]. Thus, although alternative splicing is often regulated at the early splicing step, alternative acceptor selection can be regulated at the early and the late step [19,82].

In other experimentally well-documented cases, the tandem splicing ratio is rather constant in different tissues [25,31,34,52–54], developmental stages [25,30,34,55–57], as a response to external stimuli [31,53], and in different disease states [34,55] or with respect to other factors such as sex and ethnic background [55]. For example, the four splice variants of the *ING4* (inhibitor of growth family, member 4) gene that arise by a $\Delta 9$ donor and a $\Delta 3$ acceptor (Figure 1d) have a similar ratio in different tissues and cell

lines, which is unaffected by serum starvation, DNA damage or overexpression of SF2/ASF [31,34]. Likewise, the ratio of the $\Delta 9$ tandem donor variants of the human and mouse *WT1* (Wilms tumor 1) gene (Figure 1b) are constant in tissues, cell lines, and during development [30,58].

The mechanism behind such constant splicing ratios is also not well understood. The splice site competition model (Box 2) suggests that two donors compete for U1 snRNA binding and two acceptors for U2AF35 binding, which leads to interference between the overlapping sites. If U1 snRNA and U2AF are critical factors that enable tandem splicing, this might explain constant splicing ratios because both factors are ubiquitously expressed. This competition model is supported by the finding that the splice site with the higher affinity (inferred from the splice site sequence) is generally the predominantly used site [16,19,27,28,40–42]. For NAGNAG tandems, the splice site strength is predictive for alternative or constitutive splicing and also correlated with the preferred acceptor [16]. Further support for this competition model comes from the observed independence of splicing at NAGNAG acceptors in two adjacent introns in the *MLLT4* (myeloid/lymphoid or mixed-lineage leukemia) gene [33] or at tandem donor and acceptor sites within the same intron of *ING4* (Figure 1d) [31]. Thus, stochastic splice site selection based on spliceosomal core components might represent a simple mechanism for producing constant splicing ratios.

However, this competition model has some limitations because it does not explain observed tissue- and/or developmental-specific variations in the splicing ratio or cases where a weaker splice site is predominantly used. It is conceivable that additional factors (Box 2) bias the outcome of an underlying stochastic mechanism of splice site selection under specific conditions.

Functional implications of short-distance tandem sites

The impact of tandem sites affecting the protein coding sequence depends on whether they are frameshifting ($\Delta 2$, 4, 5, 7, 8, etc.) or frame-preserving ($\Delta 3$, 6, 9, etc.). Frame-shifting tandems have severe effects for protein function because they result in proteins with a different C terminus or mRNA degradation through the NMD pathway [16,19,27] (Figure 2). By contrast, the protein effects of frame-preserving tandems are mostly subtle but highly diverse. Most of them lead to the insertion or deletion (indel) of a few amino acids, but the exchange of an oligopeptide for an unrelated residue is also observed (Figure 2). This increases the proteome diversity by producing highly similar protein isoforms of many genes [17]. Further diversity emerges from the independent usage of a tandem donor and acceptor in one intron [28], as described for *ING4* [31,34], or the existence of more than one tandem site in one gene [17]. Apart from subtle effects, a few frame-preserving tandems have a drastic effect by creating a stop codon [17,28].

Several experimental studies have shown that subtle splice events can yield functionally different proteins. The functional implications range from differences in protein, DNA or RNA binding, modifying subcellular localization to modulating ion channel and receptor properties (Table 1).

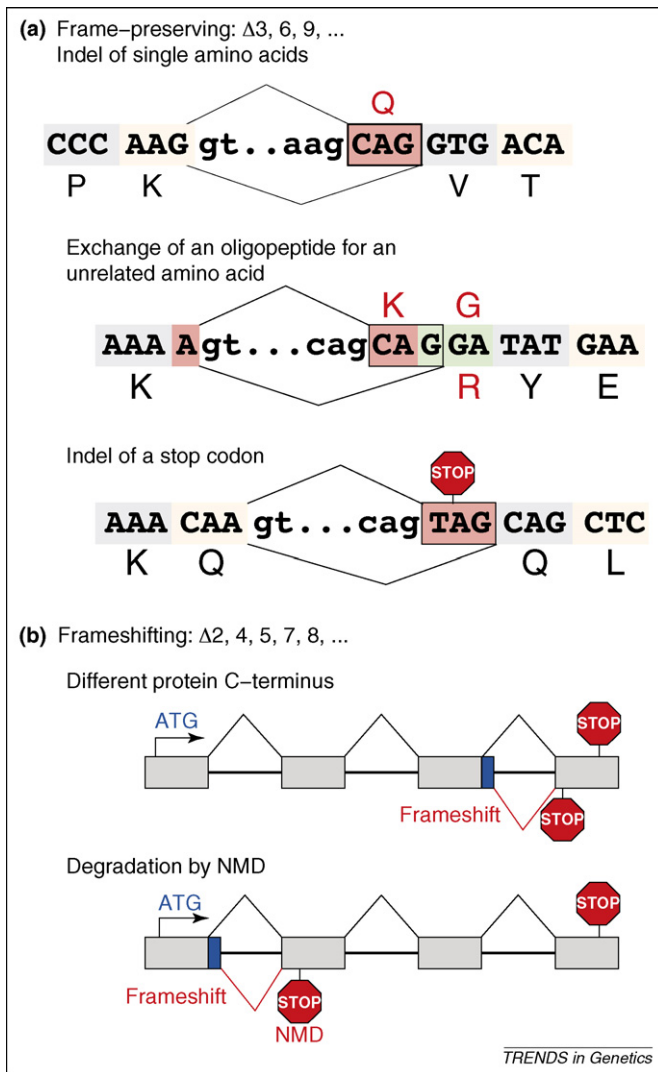


Figure 2. Effects of alternative tandem splicing on proteins. Depending on the distance between the two splice sites, one can distinguish between (a) frame-preserving and (b) frameshifting tandem sites. The protein effects of frame-preserving tandems is illustrated for NAGNAG acceptors and make up the indel of a single amino acid [shown for the insulin-like growth factor 1 receptor (*IGF1R*) gene, intron 13], the exchange of an oligopeptide for another amino acid [paired box 3 (*PAX3*) gene, intron 2] and the indel of a stop codon [protein phosphatase, EF-hand calcium binding domain 2 (*PPEF2*) gene, intron 5]. Frameshifting tandems can lead to a protein with a different C terminus or mRNA degradation by nonsense-mediated decay if the premature stop codon occurs upstream of the last exon-exon junction. It should be mentioned that the original reading frame can be rescued by a second frameshifting splice event, but such events are rare.

For example, the four different protein variants of *ING4* differ in several functional aspects such as protein binding, transcriptional activation and the ability to suppress cell growth, cell spreading and migration [31]. Furthermore, these splice events affect a nuclear localization signal and alternative splicing results in a switch from nuclear to cytoplasmic protein localization. The biological role of these alternative splice events seems to be conserved because the tandem donor and acceptor splice variants exist in mouse, rat and pig [31].

Another striking example is a $\Delta 9$ tandem donor in the transcription factor *WT1* that leads to the indel of three amino acids, Lys-Thr-Ser (KTS). This tandem donor together with its flanking regions is almost perfectly conserved in vertebrates, with only a single mutation found in

fugu [30]. Both protein variants differ in their nuclear localization pattern and their affinity to bind DNA, RNA and proteins, which has consequences for transcriptional regulation [58]. Experiments with hetero- or homozygous knockout mouse mutants, where either donor site was destroyed, demonstrated distinct functions for both splice variants in kidney and gonad development, as well as male sex determination [30]. Homozygous mouse mutants died within 1 d after birth.

The human ectodysplasin A (*EDA*) gene provides an example where the indel of two amino acids caused by splicing at a conserved $\Delta 6$ donor results in distinct receptor binding specificity [46]. These two amino acids lead to a remodeled receptor-binding site, such that the longer protein binds only to the ectodysplasin A receptor, whereas the shorter variant binds exclusively to the ectodysplasin A2 receptor. This affinity switch is of interest because both receptors have distinct functional domains and diverge in their signaling pathways. Apart from frame-preserving tandems, a function for a frameshifting $\Delta 4$ acceptor was proposed for the zebrafish transcription factor *pou5f1* [45] (Figure 1c). Furthermore, tandem splice sites in the 5' untranslated region can have a function by affecting the translational efficiency [59]. As for other alternative splice events, many details about the biological role of splicing at short-distance tandems in cells and organisms remain to be determined.

By contrast, for several tandem splice variants, functional assays revealed no differences [47,53,60]. This shows that small variations can leave protein function largely unaffected, making it likely that such events are evolutionary neutral and thus simply tolerated by the cell.

Subtle splice events can also be associated with diseases. In the *WT1* gene, human mutations that disrupt the proximal donor site lead to a deviation in the defined +KTS/–KTS variant ratio and are associated with Frasier syndrome causing glomerulosclerosis, abnormal gonad development and XY sex reversal [61]. Knockout mice where the proximal donor site is destroyed resemble the human phenotype of Frasier syndrome [30]. This shows that deviations in the +KTS/–KTS ratio are highly deleterious. In another case, a mutation in the *ABCA4* gene that changes the acceptor sequence from TAGGAG to TAGCAG has much higher frequency in patients with macular degeneration (Stargardt disease 1) [62]. The effect of this mutation is twofold: (i) it changes a Gly to an Ala and (ii) it activates the distal acceptor resulting in the lack of the CAG in ~50% of the patient's transcripts. Together with other more severe *ABCA4* mutations, this mutation may cause Stargardt disease [62]. Finally, it is noteworthy that the frequency of alternative donor and acceptor events is increased in cancer tissues, whereas the frequency of exon skipping events is reduced [11].

Functional tandem splice events with constant ratios

Differential regulation of alternative splice events is often considered to be an indication for function. In this respect, it is striking that several of the tandem sites with clear functional implications exhibit remarkably constant splicing ratios (Table 1) [25,31,52,54–57]. In fact, for some splice events, a deviation in the splicing ratio is deleterious

Table 1. Examples of short-distance tandem splice sites with functional implications

Species	Gene ^a	Splice site distance (Δ)	Splice site pattern (= intron–exon boundary)	Functional consequences of alternative splicing	Variation in splicing ratio observed	Refs
Tandem donor						
Human	<i>NR3C1</i>	3	GTA GTG	Transcriptional activation	No	[54]
Mouse	<i>ALDH18A1</i>	6	GTAAAT GTG	Sensitivity versus insensitivity to ornithine inhibition	Yes	[43]
Human	<i>EDA</i>	6	GTAGAA GTG	EDAR versus XEDAR receptor binding specificity	Yes	[46]
Mouse	<i>FGFR1</i>	6	GTAACA GAA ^b	Signaling through the Ras/MAPK pathway by modifying the binding of adaptor protein FRS2	No	[52]
Human, mouse	<i>WT1</i>	9	GTAAAACAA GTG	Localization within the nucleus; binding to U2AF65, RNA, DNA; transcriptional regulation of <i>SRY</i> , <i>NR5A1</i> , <i>BCL2</i> , <i>NROB1</i> , <i>AMH</i> ; kidney and gonad development and male sex determination	No	[58] ^e
Tandem acceptor						
Human	<i>ATN1</i>	3	CAG CAG	Nuclear versus cytoplasmic localization	No	[25]
Human	<i>IGF1R</i>	3	CAG CAG	Signaling activity and the receptor internalization rate	no	[84]
Mouse	<i>GGT1</i>	3	CAG CAG	Translational efficiency	Unknown	[59]
	<i>AT2G4337</i>	3	TAG CAG	Binding to SR proteins and U11 snRNA	Unknown	[85]
<i>Arabidopsis</i>						
	0					
Mouse	<i>PAX3</i>	3	AAG CAG	DNA binding and transcriptional activation	No	[57]
Human	<i>SCN5A</i>	3	CAG CAG	Current density and recovery time of the sodium channel	No	[55]
Human	<i>NUDT4</i>	3	AAG CAG	Catalytic activity toward diphosphoinositol and diadenosine polyphosphates	Unknown	[86]
Rat	<i>CACNA1A</i>	3	TTG TTG ^c	Inactivation kinetics of calcium channel	Yes	[87]
Zebrafish	<i>pou5f1</i>	4	TAG GTAG	Protein reading frame which leads to loss of DNA binding caused by a truncated homeo domain and developmental arrest in overexpression experiments	Yes	[45]
<i>Drosophila</i>	<i>acj6</i> (CG9151)	6	AAG AAAAAG	DNA binding, binding to Cf1-a, switch between transcriptional activator and inhibitor	No	[56]
Mouse	<i>IRF2</i>	6	AAG TTGTAG	Transcriptional regulation	Yes	[44]
Tandem donor...acceptor^d						
Human	<i>ING4</i>	9...3	GCAAAAAGT GTG...TAG AAG	Nuclear versus cytoplasmic localization; binding to Liprin α 1 and G3BP2; transcriptional activation of the p21 ^{WAF1} promoter; cell growth, spreading and migration	No	[31]

^aFull names for the genes are as follows: *NR3C1*, nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor); *ALDH18A1*, aldehyde dehydrogenase 18 family, member A1; *EDA*, ectodysplasin A; *FGFR1*, fibroblast growth factor receptor 1; *WT1*, Wilms tumor 1; *ATN1*, atrophin 1; *IGF1R*, insulin-like growth factor 1 receptor; *GGT1*, gamma-glutamyltransferase 1; *AT2G4337*, U1 small nuclear ribonucleoprotein 70 kDa; *PAX3*, paired box 3; *SCN5A*, sodium channel, voltage-gated, type V, α subunit; *NUDT*, nudix (nucleoside diphosphate linked moiety X)-type motif 4; *CACNA1A*, calcium channel, voltage-dependent, P/Q type, α 1A subunit; *pou5f1*, POU domain, class 5, transcription factor 1; *acj6*, abnormal chemosensory jump 6; *IRF2*, interferon regulatory factor 2; *ING4*, inhibitor of growth family, member 4.

^bUse of a noncanonical GA donor.

^cUse of noncanonical TG acceptors.

^dTandem donor and acceptor in the same intron.

^eAnd references therein.

and leads to disease [12], exemplified by the *WT1* Δ 9 donor (see above) [30]. These constant splicing ratios can be advantageous in situations where alternative splicing simply increases the functional diversity of proteins and where both functional protein isoforms are required ubiquitously. As mentioned above, it is tempting to speculate that expression of two functionally important splice variants in a constant ratio might be achieved just by a stochastic mechanism in which the two splice sites compete for binding to spliceosomal core components. If so, these cases would represent further examples of ‘cultivated noise’ in biology [63]. In this context, it is noteworthy that stochastic splicing of 48 mutually exclusive exons in *Drosophila DSCAM* is needed for proper axon guidance [64], and stochasticity in gene expression is involved in cell fate divergence and cell individualization [63].

Furthermore, some studies found no function for alternative splice events that are produced in variable ratios. For example, assays showed no functional difference between Δ 9 acceptor variants in *Arabidopsis PIMT2*, despite changes in the splicing ratio during development

and under hormone treatment [47]. Similarly, tissue- and development-specific skipping of a NAGNAG acceptor containing exon in mouse *Psap* has no detectable consequences in a knockout mouse lacking this alternative exon [65]. Although it remains possible that these events have a yet undetected function, these findings suggest that variable splicing ratios do not always imply functional importance. In analogy, the tissue-specific expression of a gene does not always imply a tissue-specific function [66].

Selection for and against tandem sites in evolution

Several computational studies investigated conservation of tandem sites in other species because conservation implies purifying (negative) selection and thus function [16–18,27,28,36]. For NAGNAG acceptors, initial studies reached conflicting conclusions [16,17,67] that are most likely caused by an unbalanced distribution of NAGNAG motifs together with different conservation levels for specific motifs [68]. Controlling for these biases, a fraction of tandem sites was found to be under purifying selection, even for large evolutionary distances such as between human and

chicken or fish. Remarkably, both frame-preserving and frameshifting tandems are under selection [68]. A hallmark of conserved alternative exons is that their upstream and downstream intron flanks are more conserved than the intron flanks around constitutive exons. Similarly, for exons with a tandem site, only the intron flank that is associated with the alternative splice event exhibits a higher conservation [40]. With respect to short-distance tandems, this finding is currently controversial because some studies found a higher intronic conservation for alternatively spliced tandems in comparison to constitutively spliced sites [28,36,68], whereas others did not [40]. However, differences in datasets and methods do not enable a direct comparison of these results. In summary, although it is mostly unknown which tandem sites are functionally important, the conservation of tandem splice events between distant species (as observed for *EDA*, *WT1* or *PAX3*; Table 1) and/or strong conservation of the intron flank (such as in *WT1*) seem to be reasonable criteria to select promising candidates for further experimental efforts.

Although many tandem splice events might have little or no effect on the function of the transcript and protein and are probably tolerated, other events can be deleterious and therefore expected to be eliminated by natural selection. Consistent with the latter assumption, $\Delta 3$ tandem sites are significantly underrepresented in protein coding regions compared with untranslated regions [16,69]. This might be caused by selection against mutations that create a

NAGNAG motif that allows alternative splicing. Indeed, $\geq 60\%$ of these mutations are thought to have been eliminated in coding regions [69], suggesting that even the indel of a single triplet into the coding sequence can be deleterious to protein function.

Furthermore, the strength of selection in the coding sequence is not uniform, because protein regions that fold into a fixed three-dimensional structure show a stronger avoidance of frame-preserving tandem acceptor events [69]. This agrees with observations that tandem acceptors are preferentially located at the protein surface and avoided within protein secondary structure elements such as α -helices or β -sheets. Similar results were also reported for other alternative splice events [70,71]. A preferred location of tandem acceptors at the protein surface is also in line with the enrichment of polar amino acids in their flanking protein regions [17,69]. Moreover, the protein effects of $\Delta 3$ tandems are enriched in single amino acid indels [17,25,28], indicating that such events are often more compatible with protein function than events that affect dipeptides (Figure 2).

Emergence of tandem sites in evolution

One can basically distinguish two main mechanisms for the emergence of alternative splicing at a tandem site. The first mechanism involves the emergence of a novel splice site around a constitutive exon [40,42]. For example, a single nucleotide exchange can result in a NAGNAG

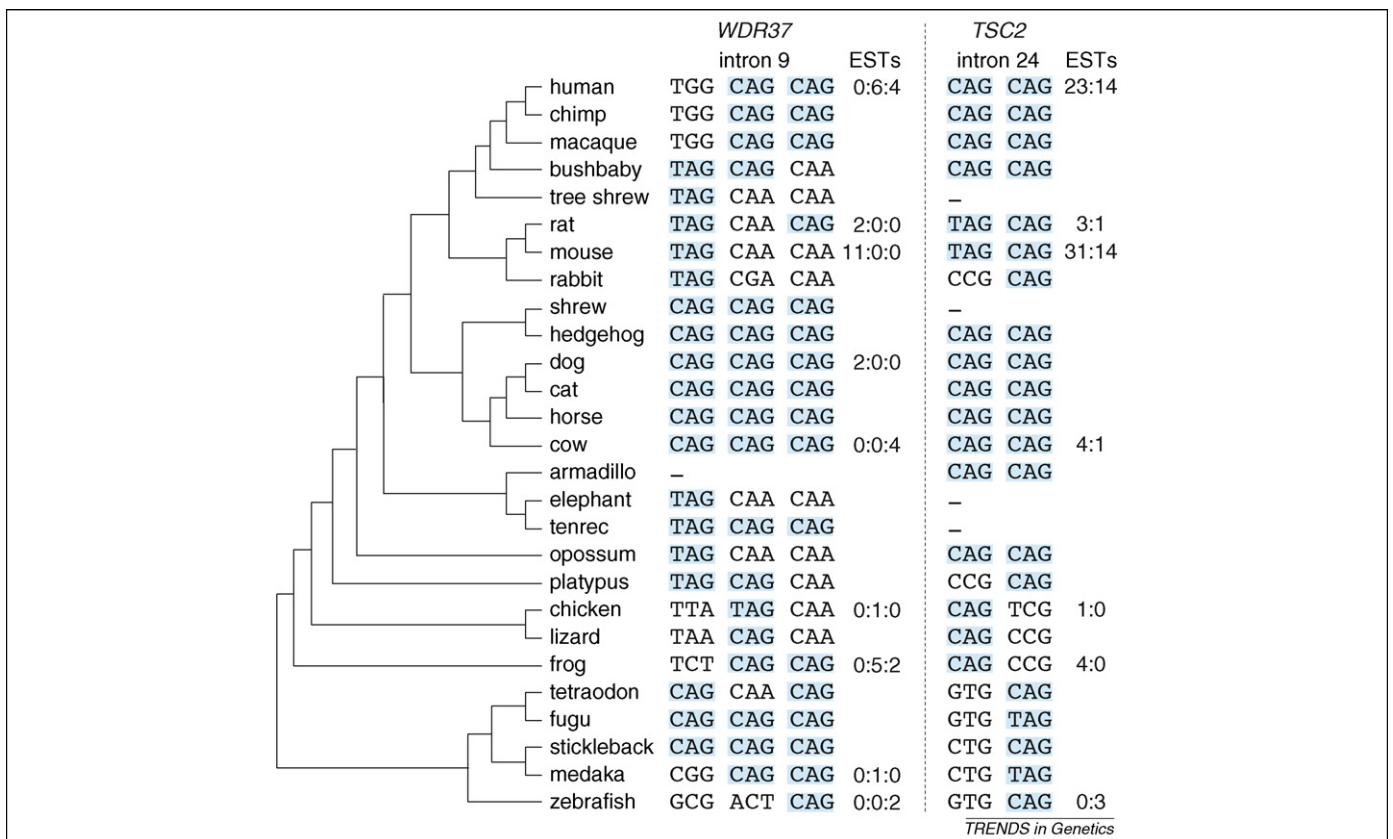


Figure 3. Multiple and independent gains or losses of short-distance tandem acceptor NAG motifs in evolution. The phylogenetic vertebrate tree shows that the acceptor AGs were created or lost several times in independent evolutionary lineages [such as in mouse and opossum for *WDR37* (WD repeat domain 37) or in rabbit, platypus and fish for *TSC2* (tuberous sclerosis 2)]. These acceptor variations result in a change of the exon length of 3 nucleotides (nt) (*TSC2*) or up to 6 nt (*WDR37*) as indicated by available expressed sequence tag counts. In case of *WDR37*, all eight possible combinations of the three acceptor NAG motifs are observed with alternative splicing observed in human and frog. NAG triplets that may function as an acceptor site are highlighted in blue. Branch lengths are not drawn to scale.

acceptor that is alternatively spliced [32]. Often, the ancestral splice site remains the preferred site, presumably because of purifying selection maintaining the ancestral transcript and protein isoform and because the sequence context of the novel site is initially not optimized for efficient splice site selection [40]. If the new splice site is located in the intron, the region that extends the exon shows rapid evolution of the protein coding sequence [40], consistent with the notion that alternative splicing enables a rather neutral or even directional evolution, allowing this region to potentially acquire a new function [72].

A second mechanism does not directly create new splice site motifs. Here, existing donor or acceptor motifs can function as splice sites if the strength of an adjacent constitutive splice site is reduced by a mutation. In the wild-type context, these so-called cryptic sites are never used, indicating that they are only competitive to the weakened but not to the original splice site [73]. This agrees with the finding that both splice sites in a tandem are weaker than the splice sites of alternative and constitutive exons [40,73]. It is also conceivable that mutations in splicing regulatory sequences enable alternative tandem splicing, for example, by destroying silencer sequences that inhibit the use of intron-proximal splice sites [48].

Most orthologous mammalian exons have identical lengths (90% for human and mouse). By contrast, some NAGNAG acceptors show an exceptional flexibility in that one of the two acceptors is destroyed or created multiple times in independent lineages in evolution (Figure 3). It is conceivable that such tandem sites represent intermediate stages during an evolutionary process, leading to shorter or longer exon lengths. Because this mechanism requires as little as a single mutation to change the exon length, it might be more frequent than exon length evolution through genomic indels. Furthermore, this mechanism might counteract the slow sequence evolution at exon boundaries that is caused by constraints on splicing enhancer motifs and the protein coding sequence [74].

Concluding remarks

Splice events at short-distance tandem sites are widespread and contribute to transcriptome and proteome complexity. From the viewpoint of evolution, alternative splice events can be roughly divided into being deleterious (maybe causing a disease), neutral (being tolerated), or advantageous (having functional implications). This classification also holds for short-distance tandem splice events because experimental and computational studies found evidence for all three aspects. A remarkable feature of tandem sites is that events with functional implications can have constant splicing ratios. This suggests that the lack of detectable variation in alternative splicing does not rule out a function for the event. Moreover, stochastic splice site selection is likely important for both variable and constant splicing ratios and functional and nonfunctional events.

Further studies are needed to provide more insights into the tandem splicing mechanism, for example by investigating which splicing factors are involved, which motifs they bind and in which splicing steps they act. This will increase our understanding of how variable and constant

splicing ratios at tandem sites arise and how these processes are regulated. Detailed characterization of further tandem splice events should reveal more examples of events with functional consequences, contributing to the question of what fraction of the tandem site events is deleterious, neutral and advantageous. Finally, mutations that create or destroy a tandem site or that change its splicing pattern might be candidates to investigate their potential involvement in dysfunction and disease.

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