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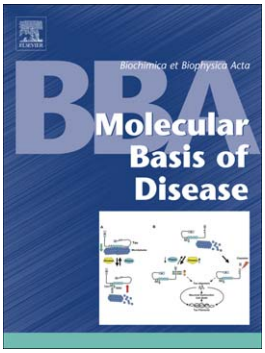

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Alternative splicing and disease

Jamal Tazi[1], Nadia Bakkour [1], and Stefan Stamm[2]*

[1]University of Montpellier II, Institute of Molecular Genetics, Centre Nationale de Recherche Scientifique, 1919 Route de Mende, France
[2]Department of Molecular and Cellular Biochemistry, University of Kentucky, College of Medicine, Lexington, KY 40536, fax: 859-323-1037; E-mail: stefan@stamms-lab.net
*corresponding author

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Abstract

Almost all protein-coding genes are spliced and their majority is alternatively spliced. Alternative splicing is a key element in eukaryotic gene expression that increases the coding capacity of the human genome and an increasing number of examples illustrates that the selection of wrong splice sites causes human disease. A fine-tuned balance of factors regulates splice site selection. Here, we discuss well-studied examples that show how a disturbance of this balance can cause human disease. The rapidly emerging knowledge of splicing regulation now allows the development of treatment options.

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1. General principles of alternative splicing

Alternative pre-mRNA splicing regulates the function of the majority of protein-coding genes

An average human protein-coding gene contains a mean of 8.8 exons with a mean size of 145 nt. The mean intron length is 3365 nt and the 5' and 3' UTR are 770 and 300 nt, respectively; as a result, this "standard" gene spans about 27 kbp. After pre-mRNA processing the average mRNA exported into the cytosol consists of 1340 nt coding sequence, 1070 nt untranslated regions and a poly (A) tail [1]. This shows that more than 90% of the pre-mRNA is removed as introns, and only about 10% of the average pre-mRNA are joined as exonic sequences by pre-mRNA splicing. Almost all protein-coding genes contain introns that are removed in the nucleus by RNA splicing during pre-mRNA processing. Exon usage is often alternative, i.e. the cell decides whether to remove a part of the pre-mRNA as an intron or include this part in the mature mRNA as an alternative exon [2]; (**Figure 1**). Alternative pre-mRNA processing is a key regulator of gene expression as it generates numerous transcripts from a single protein-coding gene, which largely increases the use of genetic information. The process is more widely used than previously thought and was recently estimated to affect more than 88% of human protein-coding genes [3]. An estimated 75% of alternative exons encode protein parts [4] and their alternate use allows to generate multiple proteins from a single gene, which increases the coding potential of the genome. Mapping of alternatively spliced regions on known protein structures suggest that most alternative exons are in coiled or loop regions that are located on the surface [5]. Alternative splicing generates protein isoforms with different biological properties that differ in protein:protein interaction, subcellular localization, or catalytic ability [6]. More than a quarter of alternative exons introduce premature stop codons in their mRNAs. This can result either in the formation of truncated proteins or in the degradation of the mRNA in nonsense-mediated decay. Recent array analyses indicate that although frequently found, alternative exons with premature stop codons are present only in low abundance, which question their role as a general shut-off mechanism for protein production [7, 8].

Changes in alternative splicing can be the cause or consequence of human diseases

There are only a few reports of mutations in core elements of the splicing machinery that result in human diseases. For example, autosomal dominant forms of retinitis pigmentosa is caused by mutation in the splicing factors PRPF31/U4-61k [9, 10] and PRP8 [11]. It is possible that defects in the general splicing machinery are generally not compatible with life, whereas changes in alternative splicing can be tolerated by an organism, although these changes might manifest in a disease. As alternative splicing affects numerous genes, it is not surprising that changes in alternative splicing are frequently associated with human diseases. It is often not clear whether a change in alternative splicing causes a disease or is an indicator for an underlying defect. A better mechanistical understanding of splice site selection has helped in distinguishing these effects. The first demonstration that exon sequences can have an effect on splice site selection was published 20 years ago [12]. Ten years later, the first review about the impact of exonic mutations on splice site selection postulated that silent mutations can interfere with exon usage and explained

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how these mutations that do not change the predicted encoded protein can cause a human disease [13]. Since then, a better understanding of alternative splice site selection contributed to a better understanding of human diseases and *vice versa*. The number of diseases reported to be associated with changes in alterative splicing increased dramatically and has been frequently reviewed [14-17], including in form of a book [18, 19]. To facilitate the access to this fast growing area for colleagues in other fields, we briefly summarize disease-relevant aspects of splice site selection, discuss well-established examples of alternative splicing changes that lead to human disease and point out links between the diseases and aberrant splice site selection.

Alternative exons are regulated by combinatorial control through transient formation of recognition complexes

Since splice sites follow only loose consensus sequences, the key questions in alternative splicing regulation are: How are splice sites recognized in the vast genomic sequence background, and how are they differentially regulated (11)? The mechanism of alternative splice site recognition has been extensively reviewed **[16, 20-23]**. Exon recognition is regulated by the interaction of proteins and ribonuclear proteins (trans-factors), with sequence elements on the pre-mRNA (cis-factors), which is summarized below.

Five small nuclear RNAs form the core of the spliceosome

During processing, the pre-mRNA has extensive, specific interaction via base pairing with five small nuclear RNAs (U1, U2, U4, U5, and U6). Early in spliceosome assembly, U1 forms a base-pairing interaction with the 5'-splice site and U2 similarly base-pairs with the branch-point. Then a tri-snRNP complex containing U4, U5 and U6 associates with the forming spliceosome and U4 is removed from the complex. This allows U6 to replace U1 at the 5' splice site and leads to a U6-U2 interaction that brings the 5'-splice site and the branch point close together, allowing for a transesterification step. By forming non-canonical interactions, U5 brings the two exons into close proximity and allows for the second step of splicing, when the two exons are joint. This shows that small RNAs play a crucial role in splicing and there is increasing evidence that some of the RNAs also play a role in catalysis of the splicing reaction [24, 25].

Protein assembling on the pre-mRNA allow exon recognition by interaction with the core spliceosome

Since the information in splice sites is not sufficient for regulation, ribonuclear-protein complexes (RNPs) forming on the pre-mRNA help in the recognition of exons (Figure 2). The majority of splicing regulatory proteins in these complexes belong to two major classes: hnRNPs and SR-proteins. HnRNPs are operationally defined as proteins binding to RNA and SR-proteins are characterized by serine and arginine-rich protein domain. These proteins contain RNA-binding and protein interaction domains [26]. They bind with low specificity to accessible, mostly single-stranded parts of the pre-mRNA. To overcome the low RNA-binding specificity, splicing regulatory proteins use their protein-interaction domains to bind to each other. Another frequent strategy to overcome the low affinity between splicing factors and their recognition sequence is a repetitive arrangement of regulatory sequences. For example, exon 4 of the doublesex pre-mRNA

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contains six repeats, each 13 nt long, that bind to the SR-proteins tra, tra2 and 9G8 [27]. Once these protein complexes have been formed around an exon, they aid ribonuclear protein components of the core spliceosome in establishing an RNA:RNA interaction at the 5' splice site and at the branchpoint. A well-studied interaction occurs between SR-proteins and the U1 snRNP, which is part of the spliceosome. The RNA component of the U1 snRNP interacts with the 5' splice site, which defines one border of an exon. Since SR-proteins that bind to an exon stabilize the interaction with U1 snRNP and the 5' splice site, they generally promote inclusion of exons to which they bind. The high fidelity of exon recognition is thus achieved by the combination of multiple weak protein:protein, protein:RNA and RNA:RNA interactions, which are schematically shown in Figure 1. Different pre-mRNAs seem to associate with a unique arrangement of proteins, which constitutes the 'splicing- or mRNP code'[28]. By interacting with the spliceosome, the protein complexes forming on the pre-mRNA, i.e. the splicing code, determine which RNA parts will be removed as introns and which parts will be included in the mature mRNA. The low affinity of each interaction is an intrinsic property of pre-mRNA processing and allows the transient formation of a specific protein:RNA complex from several intrinsically weak interactions. This has several advantages: (i) it allows a high sequence flexibility of exonic regulatory sequences that puts no constrains on coding requirements, (ii) the protein interaction can be influenced by small changes in the concentration of regulatory proteins which allows the alternative usage of exons depending on a tissue and/or developmental- specific concentration of regulatory factors, (iii) phosphorylation of regulatory factors that alter protein:protein-interactions can influence splice site selection, (iv) the regulatory proteins can be exchanged with other proteins after the splicing reaction, allowing a dynamic processing of the RNA.
Alternative splice site selection is connected to other processing steps, such as transcription, 5' end capping, 3'end polyadenylation and nuclear export. Splicing regulatory proteins often function in multiple processing events while they remain bound to 'their' pre-mRNA [29].

The secondary structure of pre-mRNA influences splice site selection
Secondary structures of the pre-mRNA can influence splice site selection (reviewed in [30]). For example, the stability of a predicted stem-loop structure at the 5' splice site of tau exon 10 regulates usage of this exon (15) and the alternative exon of the fibronectin gene is influenced by secondary RNA structure [31]. Bioinformatic analysis indicate that the splicing regulatory sequences are preferentially in a single-stranded conformation, which allows these sequences to interact with RNA binding proteins that mostly have a preference for single strand RNA [32].

Small nucleolar RNAs regulate splice site selection
Tiling array data and detailed expression analysis of parts of the human genome in the ENCODE project showed that the expression data and gene structures collected in current databases are largely incomplete [33]. Numerous noncoding regions are transcribed into polyadenylated, stable RNAs, which are named TUF (transcript of unkown function). Within most previously well-characterized protein coding genes, there are large numbers of transcribed fragments (transfrags) that could represent new exons or short RNAs of unknown sequence. The most recent study of human gene expression using tiling arrays

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demonstrates that about 57% of the transfrags are not annotated in Genbank or EnsEMBL databases [34]. Recently, it was discovered that a snoRNA, HBII-52, regulates alternative splicing by binding to an alternative exon of the serotonin receptor. This snoRNA is not expressed in people with the Prader-Willi syndrome and it is likely that the lack of HBII-52 snoRNA expression contributes to the disease [35]. In support of this theory, a child with a 174,584 bp long microdeletion was reported. The deletion encompasses only snoRNAs: HBII-438A, all snoRNAs of the HBII-85 cluster and 23 of the 47 HBII-52 snoRNAs. Since this child shows most clinical features of Prader-Willi syndrome, it is now very clear that the loss of snoRNAs cause the disease [36], which is in agreement with genetic studies and reflected by two recent mouse models [37-39]. A possible link between snoRNPs and splice site selection was mechanistically hard to understand, as snoRNPs reside in the nucleolus and splicing takes place in the nucleoplasma. Recently, it was shown that nuclear export and import factors are directly involved in U8 C/D box snoRNA biogenesis, suggesting that C/D box snoRNPs are transported through the nucleoplasma while being assembled [40]. Furthermore, the 15.5K protein that was originally identified as part of a the C/D box snoRNP complex where it binds to a conserved kink turn, binds also to a similar structure in the U4 snRNA where it interacts with the splicing factor hPrp31 [41]. These findings raise the possibility that 15.5K protein bound to snoRNPs interferes with the U4/U6 rearrangement during splicing by interacting with hPrp31. It is therefore possible that other small RNAs are also involved in splice site selection.

Exon-recognition complexes are targeted by cellular signal transduction pathways

Cells frequently regulate alternative splicing events, often in response to external stiumuli (reviewed in [42-44]). In most cases studied, rapid changes observed in splice site selection are influenced by phosphorylation events that are regulated by known signal transduction pathways. Reversible phosphorylation is a key regulatory step in the formation of protein complexes on pre-mRNA (reviewed in [45]). SR-proteins are phosphorylated by several kinases, including the Clk/Sty kinase family (cdc2 like kinases, Clk1-4), the SRPK family (SRPK1,2), mammalian PRP4, topoisomerase I, CDC2 kinase 2 [46], and GSK3 [47]. A change in phosphorylation of the RS-domain changes its ability to interact with other proteins. For example, phosphorylation of SRp38 decreases its binding to U1 70K snRNP, but increases its binding to tra2-alpha [48]. Phosphorylation of the SR-protein SF2/ASF increased its binding to U1 70K snRNP [49] and decreased its binding to the RNA export factor TAP/NXF1 [50]. Finally, the ability of the RS-domain to bind to RNA depends on its phosphorylation [49, 51].

The phosphorylation is reversed by phosphatases; only about 25 serine/threonine protein phosphatases are known [52]. This is in contrast to the estimated 474-518 protein kinases representing 1.7% of human proteins [53]. The low number of protein phosphatases is the result of a combinatorial control. The protein phosphatase represents only the catalytically active subunit that forms numerous regulatory complexes with other proteins. Splicing regulatory proteins are dephosphorylated by protein phosphatase 1 (PP1), PP2A and protein phosphatase 2Cgamma [54, 55]. Complete blocking of phosphatases inhibits splicing as dephosphorylation is necessary for the transesterification step [54, 56]. However, modulation of phosphatase activity *in vitro*

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[57] and *in vivo* [58, 59] influences alternative exon usage, demonstrating that adjusting phosphatase activity can be used by cells to control alternative splicing.

Alternative exons are generated during evolution and their usage can be changed by point mutations located outside the splice sites

Alternative exons can be generated by three mechanisms (reviewed by [60]): (i) exon shuffling, where an existing exon is duplicated within the same gene and is then alternatively spliced, (ii) exonization of mobile genetic elements, such as Alu elements [61] and (iii) a transformation of formally constitutive exons into alternative ones [62]. Since the approximately one million human Alu elements are primate-specific elements that account for 10% of the human genome [63], their exonisation provides a large reservoir to generate new alternative exons. Numerous studies showed that synonymous mutations in coding regions can influence splice site selection. There is now also emerging evidence that intronic mutations and single-nucleotide polymorphism can alter exon usage [64]. It is thus likely that alternative exon usage is an evolutionary 'substrate' that is subject to a large number of mutations. Due to the complexity of the splicing regulation, the effects of mutations are difficult to predict, but become obvious when they lead to human diseases.

Each of the regulatory principles listed here can be altered to cause a human disease, which is schematically summarized in Figure 3.

2. Examples of diseases caused by alternative splicing

The sequences of the exons mutates in the diseases are listed in Table 1, further access to internet information is given in Table 4.

2.1 Diseases caused by point mutations in regulatory sequences

Spinal muscular atrophy as an example of a recessive disease caused by a point mutation in an exonic regulatory element.

Spinal muscular atrophy (SMA) describes several different diseases that are characterized by degeneration of alpha motoneurons in the brainstem and spinal cord. Autosomal recessive SMA associated with chromosome 5 is molecularly the best understood. It is characterized by progressive paralysis caused by the loss of alpha-motor neurons in the spinal cord. The incidence is 1:6,000 to 1:10,000 for live births and the carrier frequency is 1 in 40 [65]. SMA is the second most common autosomal recessive disorder. Since children suffering from cystic fibrosis now largely survive childhood, it is the most frequent genetic cause of infantile death. SMA is caused by the loss of the SMN1 gene that encodes the SMN protein, which regulates snRNP assembly. It is not clear how the loss of SMN protein causes the disease and leads to a specific death of motoneurons. Mouse studies revealed that the loss of SMN protein causes cell-type specific changes in snRNAs and a generally reduced snRNP assembly capacity. Numerous pre-mRNA splicing events are deregulated in all tissues analyzed. Some of the changes observed reflect a shift in known alternative splicing patterns. However, the majority of the deregulated splicing events are aberrant mRNAs, which are normally not produced. These findings suggest that (i) the selective death of motoneurons could be caused by the cumulative effect of aberrantly splicing mRNAs and (ii) that changes in cells surrounding the motoneurons cause their death [66]. Genetic studies identified six families with eight

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female members that were asymptomatic for SMA, although they inherited the same SMN1 and SMN2 alleles as their affected siblings [67]. Plastin 3, an actin binding protein was identified as a modifier. Overexpression of plastin 3 in SMN knock-out mice partially rescued the short neuronal axon length causes by the absence of SMA protein. These findings argue that that the death of motoneurons could be caused by a mechanism different from a change in splicing. Although it is not understood how the loss of SMA protein causes the disease, it is clear that restoration of SMA protein production would be a therapeutic approach.

Humans posses a gene, SMN2, that is almost identical to SMN1. SMN2 was generated through a recent duplication. Although both genes are almost identical in sequence, due to a translationally silent C->T change at position 6 in exon 7, they have different splicing patterns and exon 7 is predominantly excluded in SMN2 (reviewed in [68]). This exon-skipping event generates a truncated, less stable and probably non-functional protein. Therefore, SMN2 cannot compensate the loss of SMN1. At least one copy of SMN2 is retained in humans with SMA, as lack of both SMN2 and SMN1 is embryonically lethal. Mice have only one SMN gene where exon 7 is constitutively spliced. A homozygous knock out of this gene is lethal. To study the splicing regulation of the human gene in mice, transgenic animals that contain the human gene were developed [69]. Although, in SMA patients the SMN protein is almost completely absent from all cells, for unknown reasons, alpha motoneurons are most severely affected and die, which causes the muscular atrophy. The disease can manifest in four phenotypes (type I to IV) that differ in onset and severity. The phenotypes correlate roughly with the number of SMN2 copies in the genome, most likely because more SMN2 copies produce more SMN protein [70]. Since stimulation of SMN2 exon7 usage would increase SMN protein levels and potentially cure the disease, work has concentrated on understanding the regulation of exon 7. Typical for the combinatorial control of exon regulation, multiple factors determine the regulation, including a suboptimal polypyrimidine tract [71], a central tra2-beta1-dependent enhancer [72] and the sequence around the C->T change at position 6. Recent large scale mutagenesis studies indicate that a composite regulatory exonic element termed EXINCT (extended inhibitory context) is responsible for the regulation of exon 7 inclusion [73, 74]. The exon skipping event is caused by the C->T change at position 6 and currently two models are proposed for its mechanism. In one model, the base exchange destroys the exonic enhancer that normally binds to SF2/ASF [75, 76] and in the other model, the mutation creates an hnRNPA1 binding site that acts as a silencer [77, 78]. Both models can explain the predominant skipping of exon 7. Inclusion of exon 7 depends on a central tra2-beta1 enhancer sequence [72]. Tra2-beta1 is an SR-related protein. Its activity is regulated by dephosphorylation mediated by protein phosphatase 1 and, not surprisingly, exon 7 usage depends on cellular PP1 activity [79]. SMN illustrates several common features of diseases caused by missplicing. Evolutionary changes in the genome, here the recent dublication of genes that facilitate their recombination, can manifest in splicing changes. Alternative exons are regulated by numerous factors and sequence elements and a single mutation can disturb the balance necessary for normal exon recognition. Finally, splicing factors are regulated by reversible phosphorylation controlled by cellular signaling pathways.

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Tauopathies as an example for a disease caused by a change in the ratio of protein isoforms generated by alternative splicing

Tauopathies describe several diseases of the central nervous system that show abnormal intracellular accumulations of abnormal filaments that contain the microtubule associated protein tau. The tau protein is encoded by a single gene (MAPT, (microtubule associated protein tau) located on chromosome 17. The gene undergoes extensive alternative splicing and eight of the sixteen exons are alternatively spliced. In humans, these splicing events are spatially and temporally regulated. For example, exons 2, 3 and 10 are adult specific and show differences in splicing in various brain regions. The tau protein binds to microtubules via microbuble repeat regions. One of these microtubule binding regions is encoded by the alternatively used exon 10. Exon 10 inclusion creates a protein with four microtubule repeats (4R), whereas exon 10 skipping creates an isoform with three repeats (3R). This splicing event is species-specific in the adult. In humans, exon 10 is alternatively spliced in the adult, whereas in mice the exon is constitutively used. In both species, the exon usage is regulated during development.

Genetic studies identified rare dominant mutations in the tau gene that caused frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), which are collected on the world wide web (*http://www.molgen.ua.ac.be/ADMutations/*) and currently list 42 mutations. The majority of the mutations affect the splicing regulation of exon 10 that encodes part of one microtubule binding site. The mutations in tau exon 10 helped dissect its regulatory elements. The exon shows an alternating arrangement of four enhancer and three silencer regions. A mutation that falls into a silencer or enhancer regions either promotes or decreases exon usage, respectively (reviewed in [80]). Mutations in exon 10 alter its normal fraction of inclusion and changes of pre-mRNA encoding 3R and 4R repeat tau isoforms were found associated with FTDP-17 (recently reviewed by [81], [82]). These data clearly suggested that the splicing mutations cause the neuropathology by changing the ratio between the 3R and 4R isoforms. One mechanistically well understood mutation is N279K [83]. This mutation is caused by changing a TAAGAAG into GAAGAAG. The GAAGAAG sequence forms the core of a tra2-beta1 binding site. Similar to the situation in exon 7 of SMN2, this mutated version contains two partially overlapping versions of the GAAG binding site. Biochemical studies showed that the mutation increases affinity to tra2-beta1 *in vitro* [84] and cotransfections experiments showed that tra2-beta1 promoted exon 10 inclusion in reporter gene constructs [85]. *In vitro* studies showed that the asparagine to lysine exchange in the mutation does not alter the binding between tau and tubulin, the tau aggregation or microtubule assembly [86]. These data suggested that mainly the change in the ratio of expressed isoforms is responsible for the disease. Testing this hypothesis in mouse models was difficult, as the mouse tau gene constitutively expresses the 4R isoform in the adult. Therefore, a minigene of human tau, containing the promoter and all exons flanked by shortend intronic regions was expressed in mice. These constructs show alternative splicing resembling the human situation and express human tau protein containing either 3 or 4 microtubule binding domains. When the mutation that promotes exon 10 inclusion (N279K) was introduced into exon 10 of this construct, pre-mRNA splicing was shifted as expected towards exon 10 inclusion. Interestingly, the mice showed similar pathophysiology as humans with the same mutation and also showed

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behavioral changes [87]. These data suggest that a change in the ratio between 3R and 4R tau isoforms is an important underlying cause for FTDP-17.
Abnormal intracellular tau aggregations are also found in other tauopathies, such as Alzheimer's disease [88]. Several studies were performed to investigate whether the 4R to 3R ratio of tau pre-mRNA is changed in Alzheimer's disease. The studies gave conflicting results, which most likely reflects that only post-mortem human tissue can be analysed. Two studies showed no statistical regional differences in the 4R/3R ratio [89, 90], one study showed regional differences but no clear link to Alzheimer pathology [91] and a forth study showed a statistical significant increase of the 4R to3R ratio in temporal cortex. The later study also investigated possible changes in other splicing factors regulating exon 10 and found changes in clk2 and tra2-beta1 splicing, suggesting that multiple changes in alternative splice site selection are either causing or contributing to Alzeheimer's disease [92]. This example shows that a change in the ratio of protein isoforms generated by alternative splicing can cause human diseases. It further illustrates how analysis of genetic mutants helps to understand splicing regulation, which is often complicated due to species-specific splicing differences. Despite these problems, animal models can be generated that reflect the human pathology.

Hutchinson-Gilford progeria syndrome as an example for a disease caused by an intronic mutation that activates a cryptic splice site

Hutchinson-Gilford progeria syndrome (HGPS) is a rare genetic disorder phenotypically characterized by many features of premature aging. It is clinically characterized by postnatal growth retardation, midface hypoplasia, micrognathia, premature atherosclerosis, absence of subcutaneous fat, alopecia and generalized osteodysplasia. At birth, the appearance of patients is generally normal, but by one year of age patients show severe growth retardation, balding and sclerodermatous skin changes. Patients live a median of 13.4 years and die of heart attacks or congestive heart failure. Mutations causing HGPS have been identified in the nuclear lamin A/C (*LMNA*) gene. Lamin proteins are distributed throughout the nucleoplasm and are involved in numerous functions, including DNA replication, transcription, chromatin organization, nuclear positioning and shape, as well as the assembly/disassembly of the nucleus during cell division. Out of 14 mutations affecting lamin A/C, three have been reported to specifically alter lamin A splicing. The changes in splicing lead to the production of truncated protein products (p.G608G, p.T623S and IVS11+1G>A). Most of the typical Hutchinson-Gilford progeria cases are due to a recurrent, *de novo* point mutation in *LMNA* exon 11: c.1824C>T [93]. This mutation occurs in a probable exon splicing enhancer. As a result, a cryptic splice site is activated in transcripts generated from the mutated allele, which is located 5 nucleotides upstream of the mutation. The use of the cryptic splice site leads to the production of a truncated Lamin A protein lacking the last 150 base pairs of exon 11. The truncated protein is called "progerin" and acts in a dominant fashion to generate the HGPS phenotype. This example shows how a mutation can cause activation of a nearby otherwise 'hidden', cryptic splice site.

LDL receptor splicing variants caused by a single nucleotide polymorphism are a sex-specific factor for hypercholesterolemia

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Hypercholesterolemia is a major risk factor for arteriosclerosis. Low-density lipoproteins are removed from the bloodstream by the LDL receptor (LDLR). Mutations in the LDLR are a primary cause for hypercholesterolemia. Recently, a single nucleotide polymorphism was identified in exon 12 of the LDLR that promotes skipping of this exon. The SNP was found to promote exon 12 skipping in the liver of pre-menopausal women. However, the SNP had no effect on men and post-menopausal women. The SNP and the splicing pattern are associated with a higher level of cholesterol in pre-menopausal women, but not in men. Exon 12 skipping generates a truncated form of the receptor that lacks the transmembrane domain necessary for membrane binding and internalization. It is possible that the protein generated by exon 12 skipping prevents, in a dominant negative form, the uptake of LDL. This model explains the interesting finding that exon 12 skipping caused by this SNP is associated with cholesterol levels. The reason for the sex-dependency of the SNP is unclear, but is possible that high estrogen levels influence transcription level of the gene or its alternative splicing [94]. Apo lipoprotein E (ApoE) is a ligand for the LDLR receptor and the apoE allel status is a major risk factor for Alzheimers disease. It was therefore investigated whether the SNP in exon 12 of the LDLR associates with Alzheimer's disease. It was found that the SNP associates with an increased chance to develop Alzheimer's disease in males, but not females [95]. This example nicely illustrates that a SNP can influence alternative splicing, which in turn predisposes to a disease. Reflecting the combinatorial control of alternative exon regulation, the result of a mutation depends on other factors, in this case the sex and age.

Familial dysautonomia as an example for a disease caused by a mutation in the 5' splice site

About 10% of roughly 80,000 mutations reported in the human gene mutation database affect splice sites [96]. Well-studied diseases caused by changes in splice site selection include thalassemias [97] and Familial dysautonomia (FD). FD, (also Riley-Day syndrome, hereditary sensory and autonomic neuropathy type III) is a recessive disease that is caused by loss of function of the i-kappa-B kinase complex associated protein (IKBKAP). In the Ashkenazi jewish population, the incidence is 1/3600 in live birth (carrier frequency 1:30) [98]. Affected children show abnormal development of the nervous system that is associated with demyelination in various regions. This leads to a large clinical spectrum that includes vomiting crises, unsteady gait, and decreased perception of pain. In more than 99.5% of FD patients the 5' splice site of exon 20 is mutated T->C in position 6 of intron 20. This point mutation interrupts base pairing with U1snRNA. U1 snRNA interacts both with the last three nucleotides of the exon and the first six nucleotides of the downstream intron. The majority of 5' splice sites show complementarity to seven base pairs of U1 snRNA. This means there are usually three mismatches between the 5' splice site and U1 snRNA. Bioinformatic analyses indicate that these mismatches are not randomly distributed. They either weaken the exonic portion of the 5' splice site, which is then compensated by strong binding to the intronic portion, or a weak intronic portion is compensated by a strong exonic portion. In exon 20 of the IKBKAP gene, the exonic part of the splice site is weak, due to an A at position –1. The T->C mutation weakens the intronic part of the 5' splice site, which causes exon skipping [99]. Exon 20 usage is susceptible to a weak 5' splice site, as the exon has a

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weak 3' splice site that has an A at the –3 position, and contains several exonic silencer elements [100]. Array analysis indicates that IKBKAP promotes expression of genes involved in oligodendrocyte and myelin formation, which could explain the demyelination phenotype caused by the loss of IKBKAP [101].
The example of FD illustrates the complexity of mutations in splice sites that have to be carefully analyzed within the context of other regulatory elements. It further shows that a missplicing event of a key regulatory gene can have profound impact by affecting other genes, and finally indicates that splicing is influenced by small substances.

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency illustrates how multiple mutations affect exon usage leading to a human disease
Medium-chain acyl-CoA dehydrogenase (MCAD) is a mitochondrial enzyme that participates in the degradation of medium chain length fatty acids. Deficiencies of this enzyme are the most frequently diagnosed defect of mitochondrial beta-oxidation. The patients show metabolic crisis, characterized by hypoglycemia, lethargy and seizures, when first exposed to viral infections or challenged by fasting. About 20% of the infants die. MCAD deficiency results in accumulation of medium-chain acylcarnitines in the urine, which can be analyzed by mass-spectroscopy. A large newborn screen showed an incidence from 1:15,000 in the US population. The major reason for deficiency is a K304E missense mutation leading to a less active protein [102]. The newborn screening project identified a 362C->T missense mutation in exon 5 of the MCAD gene that causes exon skipping and subsequent degradation of the mRNA by nonsense-mediated decay [103]. This mutation disrupts a splicing enhancer that is highly similar to the SF2/ASF enhancer in the SMN2 exon 7. Cotransfection experiments demonstrate that an increase of the SF2/ASF concentration promotes inclusion of the mutated exon, suggesting that the mutation weakens an SF2/ASF-dependent enhancer. Interestingly, a synonymous mutation 351A->C was identified 11 nucleotides upstream in the same exon. This mutation affects an hnRNP A1 dependent silencer. Since the exon is constitutively used in the absence of the 362C->T SF2/ASF enhancer mutation, it has no effect on splicing. However, in the presence of the 362C->T enhancer mutation, it promotes exon inclusion, which antagonized the exon-skipping effect of the 362C->T SF2/ASF enhancer mutation. This example illustrates the fine-tuned balance of positive and negative acting factors that exists in splicing regulation. It also shows that seemingly irrelevant mutations can have an effect on splicing when they are combined with other mutations. Finally, the similarities between the regulation of MCAD exon 5 and SMN2 exon 7 suggest that there are degenerate 'building blocks' or 'regulatory modules' in the splicing code.

Frontotemporal lobar dementias are caused by the loss of the splicing factor TDP43
TDP43 (TAR DNA binding protein 43 kd) was originally identified as a transcriptional repressor that associates with the transcriptional activator DNA region (TAR) in HIV [104] and was later also found associated with the spermatid-specific gene SP-10 promoter [105], reviewed in [106]. TDP43 is a member of the heterogeneous nuclear ribonucleoproteins (hnRNP) family of proteins. The protein was later identified as a factor that binds to 12 UG repeats that cause aberrant skipping of exon 9 of the CFTR gene, leading to cystic fibrosis [107]. It contains two RNA binding domains. The first RNA binding domain is necessary and sufficient for binding to RNA that contains at least

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four UG repeats. In addition, TDP43 shows binding to single stranded TG DNA repeats *in vitro* [108]. TDP43 is a nuclear protein and it was therefore completely surprising when it was detected in ubiquitin-positive, tau and alpha synuclein-negative cytosolic inclusions that are the characteristic of frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS, Lou Gehring's disease) [109]. During the disease, TDP43 is cleaved by caspase-3 and the cleavage fragments accumulate in the cytosol, where they form aggregates. The caspase-3 reaction is inhibited by progranulin, which explains why mutations reducing the progranulin expression cause FTLD [110]. It is not clear whether the disease is caused by a loss of nuclear function of TDP43 or by a possible cytotoxoic accumulation. However, siRNA mediated knock down of TDP43 in HeLa cells followed by RNA microarray analysis demonstrated a strong upregulation of cyclin-dependent kinase 6 (cdk6). The human Cdk6 gene contains numerous intronic TG repeats and interestingly a change in cdk6 expression was not observed for the chicken gene that lacks these repeats. These data strongly suggest that TDP43 represses cdk6 expression by sequestering its RNA by binding to UG repeats. Loss of TDP43 expression leads to abnormal cdk6 activity, resulting in pRB phosphorylation, genomic instability and apoptosis, which could explain the cell death in FTLD [111]. Mutations the gene encoding TDP43 are found in families with amyotrophic lateral sclerosis, as well as in sporadic cases, indicating a direct link between TDP43 and amyotrophic lateral sclerosis [112].

This example illustrates that splicing factors like TDP43 operate in a regulatory network. A loss of their function can have drastic, but indirect effects. HnRNPs typically perform several different functions, which can obscure possible disease mechanisms. Finally, the interaction between UG repeats of the cdk6 gene and TDP43 illustrate how an hnRNP can be recruited to a new function during evolution.

Diseases associated with repeat elements

Short sequence repeats can be detected in numerous exons, where they serve to increase the recognition by a certain splicing factor [27, 113], reviewed in [114]. A change in length of simple repeat sequences can therefore change the splicing pattern of a gene. For example, the endothelial NO synthase gene contains an intronic polymorphic CA-repeat region and the number of repeats correlate with the risk of coronary artery disease and hyperhomocysteinemie in a sex-specific fashion [115]. SELEX experiments and functional studies showed that CA repeats bind to hnRNP L and the action of hnRNP L depends on the CA repeat length. Microarray studies showed that intronic CA-rich repeats can influence alternative splicing decisions, strongly suggesting that other intronic CA-repeat polymorphisms could cause human disease [116]. Another disease-relevant repeat is the UG repeat that can cause aberrant splicing of exon 9 of the CFTR gene, leading to cystic fibrosis [107]. This repeat binds to TDP43, which is discussed below. Myotonic dystrophy (DM) is the most common form of muscular dystrophy in adults. The disease is caused by extensions of two repeats: a CUG repeat in the 3' region of the DMPK gene leads to DM1 and extension of a CCUG repeat in an intron of the ZNF9 gene leads to DM2 [117]. The common CUG element binds to at least two groups of RNA binding proteins, muscleblind-like1 and CUG binding protein. Extension of the repeats leads a sequestration of muscleblind protein in nuclear foci. This sequestration of muscleblind-like 1 protein reduces its cellular concentration and changes alternative

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splicing events that depend on this protein. Surprisingly, the extended CUG repeats lead to an increase of CUG binding protein concentration. This increase is due to a stabilization of CUG-binding protein. CUG RNA repeats induce via an unknown mechanism protein kinase C, which phosphorylates the CUG protein, leading to a more stable protein [118].

Alu elements are the largest group of repetitive elements in the human genome. They represent about 10% of the total genome sequence (reviewed in [63]). Alu elements contain potential splice sites and can evolve into exons [61]. It has been estimated that up to 5% of human alternative exons could be derived from Alu sequences [119]. It is therefore not surprising that mutations in existing Alu elements can cause their abnormal inclusion in mRNA, which leads to human diseases, such as the Alport syndrome [120], congenital cataracts facial dysmorphism neuropathy syndrome [121], and mucopolysaccharidosis type VII [122].

These examples illustrate how repeats can change the balance of alternative splicing regulation, even when they are located in introns or in seemingly unrelated mRNAs. The exonisation of Alu elements shows how new repetitive elements can be used by evolution to aquire new functions, and diseases caused by improper Alu-element exonisations can be viewed as failed evolutionary experiments.

3. Numerous changes in alternative splicing are found in cancer

Numerous reports have shown that alternative splicing patterns are changed in cancer (reviewed in [123]). The expression of alternative or even tumour-specific splice variants significantly affects many cellular events critical for cancer biology such as cell proliferation, motility, and drug response [124]. It is still unclear, however, to what extent alternative splicing functionally contributes to the initiation and progression of cancers. Most altered splicing patterns could be largely symptomatic and attributed to a generalized lack of fidelity of the splicing apparatus in cancer cells. The existence of particular splice variants in cancer could merely be a consequence of the malignant phenotype without contributing to the cancer phenotype. Here we will describe general changes of the splicing machinery in cancer and discuss specific examples illustrating the action on tumor suppressor genes.

3.1 Splicing regulatory factors and cancer

Changes in the concentration, localization, composition, or activity of trans-acting regulatory factors, such as hnRNP and SR proteins, can alter splice site selection. Several studies have demonstrated specific alterations in the expression of splicing factors in cancer. The increased phosphorylation and the elevated mRNA expression levels of Tra2-beta1, YB-1 and classical SR proteins, including SC35 and ASF/SF2, have been observed in human ovarian cancer and in mouse models of breast cancer development [125-128].

One of the best characterized examples of splicing changes observed in cancer tissues is CD44, a type 1 transmembrane glycoprotein involved in cell-cell and cell-matrix interactions. Alternative splicing of 10 of its 20 exons is found in association with several biological processes, such as lymphocyte recruitment, epithelial cell-matrix adhesion, and tumour metastasis. Due to its abundant alternative splicing, it has been correlated with

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changes in splicing factors, for example the concentration of ASF/SF2 or hnRNP A1 in mouse lung tumours [129] and human colon carcinomas [130].

SF2/ASF is a prototypical splicing factor and has been widely used in studying the connection between cancer and alternative splicing. SF2/ASF controls alternative splicing of the oncogene Ron to modulate cell motility, a general property of metastatic cancer cells [131]. An increased expression of SF2/ASF can transform NIH3T3 and Rat1 cells *in vitro*, and NIH3T3 cells that express high levels of ASF/SF2 produce tumours *in vivo*. This increase correlates with a change in splicing of genes involved in the Ras-mitogen-activated protein kinase (MAPK) and the phophatidylinositol-3-kinas (PI3K)-mammalian target of rapamycin (mTOR) pathways, which are often deregulated in cancer. MNK2 and S6K1 kinases involved in signal transduction are alternatively spliced in response to elevated SF2/ASF concentration. A novel SF2/ASF-induced isoform of S6K1 is sufficient to cause a transformation phenotype. Overexpression or knockdown of ASF/SF2 resulted in specific splicing changes in a number of genes involved in these pathways. SF2/ASF overexpression led to cellular transformation resulting in anchorage-independent cell growth in soft-agar and tumour formation in nude mice.[132]. These data argue that SF2/ASF is a proto-oncogene [132]. It is likely that other splicing factors have similar features.

3.2 Mutations of tumour suppressor genes affecting splice site selection can cause cancer

Mutations in splicing regulatory elements that change splice site selection provide a clear link between pre-mRNA processing and cancer development. The mutations ultimately lead to the generation of non-functional tumor suppressor genes, which predisposes to cancer. The identification and understanding of inherited mutations is important for genetic counselling and for follow-up aimed at cancer prevention in affected family members. A list of well-characterized mutations is shown in Table 2.

The most frequent early molecular alteration in the initiation of colon cancer involves mutations in the adenomatous polyposis coli (APC) gene, which occur both in rare familial cancer syndromes and in ~85% of sporadic cancers. The majority of these mutations result in the truncation of the APC gene product. Germline mutations in APC gene result in familial adenomatous polyposis (FAP). Two mutations that disrupt splicing regulatory elements have been found. The first one affects a splicing donor site in intron 4 resulting in skipping of exon 4 and leading to an attenuated form of FAP [133]. This splicing deregulation was found in liver metastases, where the wild-type allele was deleted [134]. The second mutation is a G to A substitution at the splice acceptor site of intron 7 creating a cryptic splice site and causing a single nucleotide deletion at the beginning of exon 8 [134]. The behaviour of these mutations can be complicated by additional APC transcripts which are generated due to alternative splicing of exons 3-4, 9, 10A/X, and 14 [135] [136, 137]

The MMR MLH1 (MIM 120436) and MSH2 (MIM 609309) genes responsible for hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome; MIM 120435), are prone to mutations. Double exon skipping in MLH1 gives rise to hereditary nonpolyposis colorectal cancer [138, 139]. Also in various hereditary nonpolyposis colorectal cancer, different mutations in codon 659 result in skipping of exon 17 of MLH1 gene. This causes an internal deletion of 31 amino acids that abrogates binding to

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the other mismatch repair protein PMS2 [140]. A major difficulty in the diagnosis of the Lynch syndrome, like other Mendelian diseases, is the interpretation of numerous variants of unknown clinical and biological significance. In most cases the pathogenic role of the unclassified variants cannot be established from clinical data and segregation analysis, because of the small number of family members available, the incomplete penetrance of MMR mutations and the possibility of additional undetected mutations in the MMR genes. Recently, using an *ex vivo* functional splicing assay based on a novel splicing reporter minigene to test 87 intronic or exonic variants (20 and 67, respectively) corresponding to 85 alleles found in MLH1 or MSH2 by the French HNPCC consortium, it was possible to show that a high level of mutations alter splicing regulatory elements [141]. However, the biological significance of the six variants is unkown.

Breast cancer gene 1 (BRCA1) is responsible for the majority of hereditary breast and ovarian cancers [142]. Among sporadic cases of breast cancer, expression of BRCA1 is reduced or undetectable in high-grade ductal carcinomas, suggesting the involvement of this gene in the etiology of breast cancer. Germline sequence variations in both splice sites and regulatory elements of BRCA1 gene have been implicated in susceptibility to cancer. For example, an inherited nonsense mutation in exon 18 of the BRCA1 gene disrupts an Exonic Splicing Enhancer. This enhancer binds to the SR protein SF2/ASF, and its mutation causes inappropriate skipping of the constitutive exon 18 [143]. It has also been shown that an AA to AG mutation creates a cryptic 3' splice site that adds 11 nucleotides to BRCA1 mRNA, which encodes a truncated protein in a breast cancer family [144]. In addition, multiple BRCA1 splice variants are present in different tissues with different expression profiles [145].

The neurofibromatosis 1 (NF1) gene that is linked to development of neurofibromas has one of the highest mutation rates described for any human disorder, and a large proportion of NF1 mutations bear consequences for the correct splicing of the RNA. A systematic study of somatic NF1 mutations [146] demonstrated that most point mutations resulted in splicing defects including exon skipping and the usage of alternative 5' and 3' splice sites. Also, a double point mutation near the middle of NF1 exon 7 has been found to cause a high level of in-frame deletion of that exon. These mutations disrupt consensus binding sites for the SR proteins SC35 and ASF/SF2 [147]. Some mutations like a CTAGC to CTAAC in the NF1 gene in the central nervous system, creates a consensus branch point adenosine that activates a cryptic site in the intron leading to an additional exon (exon 5a). Because some normal splicing remains, this mutation is associated with a relatively mild phenotype [148].

These examples illustrate that numerous changes in splicing patterns are characteristic for cancer development and progression. It is currently not clear whether these changes are the cause or the consequence of a malignant phenotype. Gain of function changes in alternative splicing have been observed in several systems and well-characterized examples are summarized in Table 3. These mutations, and the mutations in the tumor supressor genes that cause missplicing and predispose cancer, are strongly suggesting that abarrant processing of some pre-mRNAs is the cause of cancer and could be a therapeutic target.

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4. Treatment options for missplicing events

The examples discussed here clearly show that a misregulation of alternative splicing plays a large role in numerous human diseases. The identification of molecules capable of correcting and or inhibiting pathological splicing events is therefore an important issue for future therapeutic approaches.

4.1 Antisense strategies

The major challenge in treating splicing disorders is to specifically target one splicing event in a certain pre-mRNA. Since there are several thousand other pre-mRNAs in the cell, the selectivity of splice site intervention is a major problem. One way to address this problem is the use of oligonucleotides that will specifically bind to one sequence.
Special chemistries were devised to prevent RNAseH-mediated cleavage of the RNA and to lower toxicity (reviewed in [149, 150]). A major drawback of the therapeutic use of oligonucleotides is their delivery and uptake in the cells. This problem has been addressed by coupling of oligonucleotides to arginine-rich cell penetrating peptides. [151, 152].
Antisense oligonucleotides have been tested for beta thalassemias [153], Duchenne muscular dystrophy [154, 155], cystic fibrosis [156] cyclophilin transcripts [157], Hutchinson Gilford Progeria Syndrome (HGPS) [158] as well as block HIV replication [159] and alter *tau* pre-mRNAs [160].

Antisense oligonucleotides can also be used to block splicing enhancers or silencers, which can be found in introns or exons quite far away from the splice sites [161]. Inhibition of splicing silencers (ESSs or ISSs) is of particular interest since it provides a way to activate otherwise repressed exons. This was achieved for the α exon of fibroblast growth factor receptor-1 (FGFR1) transcripts [162] where an antisense morpholino oligonucleotides that blocks silencer elements in glioblastoma cells in culture promotes the inclusion of the α exon. The antisense approach was further developed in ESSENSE (exon-specific splicing enhancement by small chimeric effectors). ESSENSE uses bifunctional reagents that contain a peptide effector domain and an antisense-targeting domain. The effector domains of these protein-nucleic acids were arginine-serine (RS) repeats that mimic the effect of SR proteins [163]. A second incarnation of bifunctional oligomers uses a 2'-*Ome*-modified binding domain and an effector domain, which is composed of RNA that contains binding sites for known splicing *trans*-acting factors [164] [165]. The recruited factors mediate activation or silencing of the targeted exon. An example of this type of bifunctional oligonucleotide acts as an ESE and promotes inclusion of the SMN2 exon 7 fibroblasts from SMA patients leading to partial restoration of the SMN function [164].

5.2 Substances that change alternative splicing

The use of RNA-binding molecules as antibiotics, such as gentamicin, chloramphenicol, and tetracycline illustrates that drugs can be targeted against RNA and/or RNA binding proteins. High-throughput screens and testing of substances in model systems have now identified more than 30 substances that change splice site selection. The substances fall into several categories, including HDAC inhibitors, kinase and phosphatase inhibitors, as

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well as cAMP antagonist and agonists. The currently known substances are reviewed in [166] and updated on the web.

The usefulness of substances that change splice site selection is evident from potential HIV therapies that could be combined with other antiviral strategies. Using an *in vitro* splicing assay, a chemical screen was performed that identified an indole derivative (IDC16) that interferes with exonic splicing enhancer activity of the SR protein splicing factor SF2/ASF [167]. IDC16 suppresses the production of key viral proteins and inhibits replication of macrophage- and T cell–tropic laboratory strains, clinical isolates, and strains with high-level resistance to inhibitors of viral protease and reverse transcriptase. Thus, human splicing factors represent novel and promising drug targets for the development of antiretroviral therapies, particularly for the inhibition of multidrug-resistant viruses.

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Figures

Figure 1: Alternative splicing modes

Constitutive exons are shown as white boxes, introns as vertical lines. Open arrows indicate the 3' splice site, closed arrows the 5' splice site. A black box indicates a cassette exon, the most frequent form of alternative splicing. Hatched boxes indicate other splicing modes. Alternative polyadenylation sites and alternate promoters are shown as two other mechanisms to increase mRNA diversity. They are mechanistically different from alternative splicing.

Figure 2: Elements involved in alternative splicing of pre-mRNA

Exons are indicated as boxes, introns as thin lines. Splicing regulator elements (enhancers or silencers) are shown as green or red boxes in exons. The 5' splice-site (CAGguaagu) and 3' splice-site $(y)_{10}$ncagG, as well as the branch point (ynyyray), are indicated (y=c or u, n=a, g, c or u). Upper-case letters refer to nucleotides that remain in the mature mRNA. Two major groups of proteins, hnRNPs (green) and SR or SR related proteins (red), bind to splicing regulator elements; the protein:RNA interaction is indicated by shading. The protein complex assembling around the exon stabilizes binding of spliceosomal copmponents, indicated in blue. For example, SR-proteins can interact with U1 snRNA, which helps in the recognition of the 5' splice site, as it allows hybridization (thick black line) of the U1 snRNA (black line) with the 5' splice-site. The formation of the multi-protein:RNA complex allows discrimination between proper splice-sites (bold letters) and cryptic splice-sites (small *gt ag*) that are frequent in pre-mRNA sequences. Factors at the 3' splice-site include U2AF, which recognizes pyrimidine rich regions of the 3' splice-sites, and help stabilize the interaction of U2 snRNP with the adenosine of the branch point.

Figure 3: Disease causing mutations

Exons and introns are indicated as in Figure 2. Arrows indicate repetitive elements.

A: Mutation in silencer or enhancer sequences change the interaction between regulatory proteins and pre-mRNA, leading to changes in exon recognition.
B: Mutations in splice sites change the interaction with core spliceosomal proteins.
C: Due to formation of secondary structures, intronic sequences can be brought close to splice sites, accounting for the effect of some intronic mutations.
D: Secondary pre-mRNA structures contribute to exon recognition and their mutations can cause aberrant splicing.
E. Expanded repeat elements can sequester splicing regulatory proteins, changing splice site selection in other messages.
F. Some snoRNAs regulate splice site selection and their loss can cause disease.

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Table 1: Summary of disease-causing mutations

Exons associated with diseases mentioned in the text are listed. Since there are no unique accession numbers for exons, the NCBI accession number is listed. The mutations having an effect on splicing are listed under features and changed nucleotides are underlined. These nucleotides are underlined in the sequence. Capital letters are exons, small letters are introns.

disease	sequence	Accession number	Features	Ref.
FD	tttaagATGCCAAGGGGAAACTTAGAAGTTGTTCATCA TCGAGCCCTGGTTTTAGCTCAGATTCGGAAGTGGTTGG ACAAgtaagtgccat	AF044195	Change from agtgc to agcgc causes skipping	[168]
FTDP-17	GTGCAGATAATTAATAAGAAGCTGGATCTTAGCAACGT CCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACG TCCCGGGAGGCGGCAGT	J03778	AATAAGA to AAGAAGA causes inclusion	[169]
FTDP-17	GTGCAGATAATTAATAAGAAGCTGGATCTTAGCAACGT CCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACG TCCCGGGAGGCGGCAGT	J03778	AATAAGAAGCT to AATAAGCT causes skipping	[170]
FTDP-17	GTGCAGATAATTAATAAGAAGCTGGATCTTAGCAACGT CCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACG TCCCGGGAGGCGGCAGT	J03778	ATCTTAGCA to ATCTCAGCA causes inclusion	[171]
FTDP-17	GTGCAGATAATTAATAAGAAGCTGGATCTTAGCAACGT CCAGTCCAAGTGTGGCTCAAAGGATAATATCAAACACG TCCCGGGAGGCGGCAGT	J03778	GCAGT to GCAAT causes inclusion	[172]
HGPS	GGCTCCCACTGCAGCAGCTCGGGGGACCCCGCTGAGTA CAACCTGCGCTCGCGCACCGTGCTGTGCGGGACCTGCG GGCAGCCTGCCGACAAGGCATCTGCCAGCGGCTCAGGA GCCCAGGTGGGCGGACCCATCTCCTCTGGCTCTTCTGC CTCCAGTGTCACGGTCACTCGCAGCTACCG	BC014507	GGGCGGA to GGGTGGA or GTCACTCGCA to GTCATTCGCA Activates cryptic splice site	[93]
hypercholesterolemia	ATCTCCTCAGTGGCCGCCTCTACTGGGTTGACTCCAAA CTTCACTCCATCTCAAGCATCGATGTCAACGGGGGCAA CCGGAAGACCATCTTGGAGGATGAAAAGAGGCTGGCCC ACCCCTTCTCCTTGGCCGTCTTTGAG	AY114155	CAACGGG To CAATGGG Causes skipping	[94, 95]
MCAD deficiency	GAGGTCTTGGACTTGGAACTTTTGATGCTTGTTTAATT AGTGAAGAATTGGCTTATGGATGTACAGGGGTTCAGAC TGCTATTGAAGGAAATTCTTTGGGG	M16827	GACTGC To GATTGC causes exon skipping, ACAGGG to ACCGGG promotes inclusion	[103]
SMA	GGTTTTAGACAAAATCAAAAAGAAGGAAGGTGCTCACA TTCCTTAAATTAAGGA	U18423	TTTAGA to TTCAGA promotes exon skipping	[173]

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Table 2: Splicing mutations of tumour suppressor genes

Gene	Mutation	Effect	Reference
APC	Donor splice site mutation in intron 4	Exon 4 skipping → attenuated form of FAP	[133]
APC	G→A substitution at the splice acceptor site of intron 7	Cryptic splice site creation causing a single nucleotide deletion a the beginning of exon 8	[174]
BRCA1	Non sense mutation in exon 18	ASF/SF2 ESE disruption → exon 18 skipping	[143]
BRCA1	Intronic point mutation AA → AG (IVS5-12 G→A)	Cryptic 3' splice site creation → production of a truncated protein	[144]
Estrogen receptor	AT → GT point mutation in intron 5	Cryptic 5' splice site creation in intron 5 → insertion of a 69 nucleotide cryptic exon into the reading frame	[175]
NF1	Double point mutation in exon 7	Disruption of ASF/SF2 and SC35 ESE binding sites → In frame deletion	[147]
NF2	Intronic CTAGC → CTAAC	Consensus branch point adenosine creation → cryptic splice site creation → additional exon 5a insertion	[148]
MLH1	Different codon 659 mutations	Exon 17 skipping → internal deletion of 31 aa that abrogates binding to the other mismatch repair protein PMS2	[140]

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Table 3: Gain of function of proteins promoting tumour development by alternative splicing

Gene	Protein Property/ function	Splice variant	Isoform expression in cancer	Cancer type	Reference
Survivin	Inhibitor of apoptosis	Survivin 2B with pro-apoptotic properties	Down regulated	Breast carcinoma and late stage or metastatic gastric cancer	[176, 177]
VEGF	Role in angiogenesis	Isoforms lacking exon 6	Upregulated	Non-small cell lung cnacer	[178]
Cathepsin B	Role in the development and progression of cancers	Certains isoforms	Overexpressed	Colon cancer	[179]
FHIT	Tumour suppressor	Aberrant transcripts	Aberrant expression	Gastric, cervical, thyroid and testicular germ-cell tumours	[180-185]
Actinin 4	Actin-binding protein	Variant Va, a mutually exclusive splice variant where exon 8 is replaced by a new exon of the same size that exists in intron 8	Aberrant expression of the splice isoform	Small cell lung cancer	[186]
AIB1	Hormone coactivator	Isoform lacking exon 3	Aberrant expression	Breast cancer	[187]
RON	Tyrosine kinase receptor	Ron? 165 Ron? 160 Ron? 155	Overexpressed	Colorectal carcinoma	[188, 189]

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Table 4: Information about diseases on the web

General information about alternative splicing	http://www.eurasnet.info/
Familial dysautonomia	http://www.familialdysautonomia.org/
Frontotemporal lobar dementias/ amyotrophic lateral sclerosis	http://www.alsa.org/
Hutchinson-Gilford progeria syndrome	http://www.progeriaresearch.org/
Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	http://www.fodsupport.org/mcad_fam.htm
Myotonic dystrophy	http://www.myotonic.com http://www.mda.org
Prader-Willi Syndrome	http://www.pwsausa.org/ http://www.fpwr.org/
Spinal muscular atrophy	http://www.fsma.org/ http://www.mda.org
Tauopathies	http://www.molgen.ua.ac.be/ADMutations/
Substances that influence alternative splicing	http://www.stamms-lab.net/cpds.htm

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Figure 1

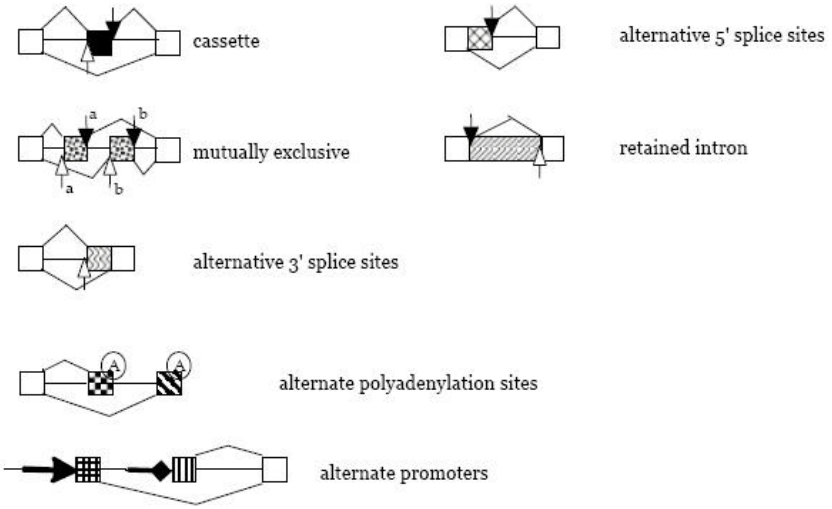

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Figure 2

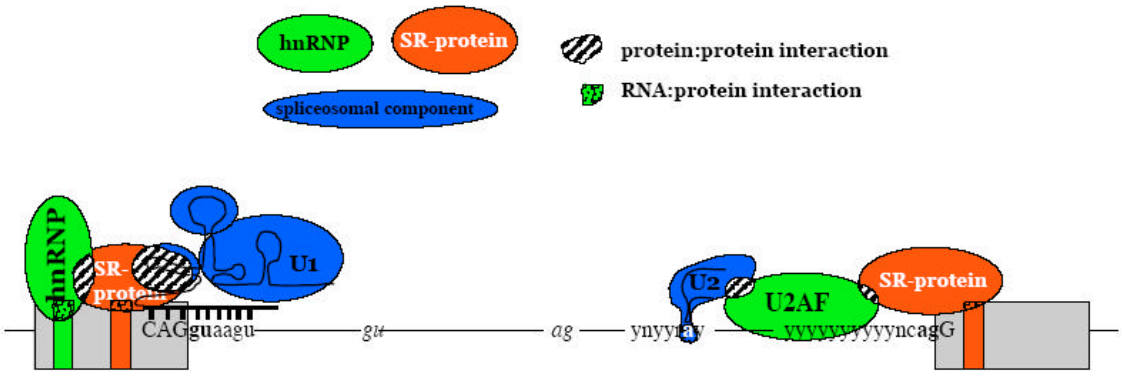

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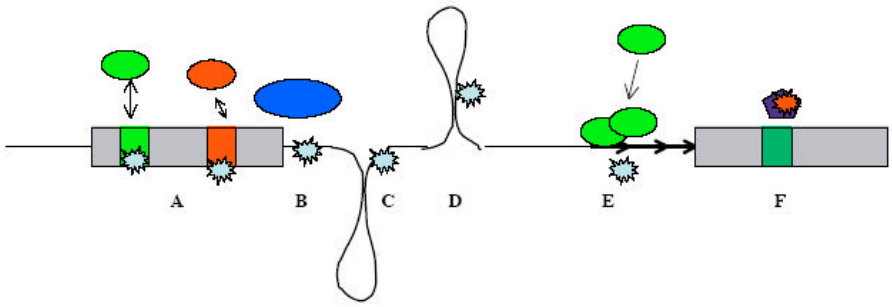
Figure 3



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