Trends in Genetics

CellPress
REVIEWS

Review

RNA Splicing and Disease: Animal Models to Therapies

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Alternative splicing of pre-mRNA increases genetic diversity, and recent studies estimate that most human multiexon genes are alternatively spliced. If this process is not highly regulated and accurate, it leads to mis-splicing events, which may result in proteins with altered function. A growing body of work has implicated mis-splicing events in a range of diseases, including cancer, neurodegenerative diseases, and muscular dystrophies. Understanding the mechanisms that cause aberrant splicing events and how this leads to disease is vital for designing effective therapeutic strategies. In this review, we focus on advances in therapies targeting splicing, and highlight the animal models developed to recapitulate disease phenotypes as a model for testing these therapies.

Highlights

Recent studies have shown that approximately one-third of all disease-causing mutations are related to RNA splicing.

Research on new and/or improved animal models that can better replicate diseases caused by aberrant splicing has helped not only to increase the understanding of these diseases, but also the design of new therapies to combat them.

The appearance of new antisense oligonucleotides and small molecules, together with novel techniques, such as spliceosome-mediated RNA trans-splicing (SMaRT) or exon-specific U1 small nuclear RNA (ExSpeU1), gives hope in the fight against splicing-related diseases because they present as promising therapy options.

The development of new technologies in molecular biology, such as next-generation sequencing or the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 genome-editing technology, has opened the door for research on RNA mis-splicing events and how they relate to disease.

Disrupted Splicing in Disease

Our knowledge of pre-mRNA maturation has greatly expanded since the discovery of 'split' genes by Richard Roberts and Phillip Sharp in 1977, for which they were jointly awarded the Nobel Prize in 1993 for their independent discoveries [1,2]. We now know that eukaryotic genes contain a series of exons and introns, the latter of which have to be removed during RNA maturation for the mature mRNA to be translated into protein (Box 1). Splicing is a precise process by necessity because mistakes in splicing can also lead to unintended effects causing coding or frameshift mutations. To direct splicing effectively with high fidelity, the process relies on signals within the transcript itself, such as the splice sites and associated sequences, as well as factors that recognize them to facilitate molecular reorganization, recruitment of cofactors, and catalysis of the reaction. Hence, genetic mutations that lead to sequence alterations within the transcript can create new splice sites or enhancer sequences that lead to the recognition of new exons, also known as cryptic exons, or can disrupt splicing sequences required for the recognition of exons that lead to the exclusion of constitutive exons from the transcript. These types of splicing mistake create aberrant transcripts and contribute to disease. Similarly, defects in splicing machinery can lead to aberrant splicing of multiple transcripts and also contribute to the disease state.

Further adding to the complexity of splicing is the fact that genes are not always spliced in the same manner; alternative splicing produces different proteins from a single gene (Box 2). It is currently estimated that >95% of human genes are alternatively spliced [3,4]. While alternative splicing expands the genetic diversity of an organism and presents the opportunity for specific discreet transcripts to be expressed in developmentally or tissue-specific ways, there has been a flourish of research defining how inappropriately controlled-alternative splicing events can contribute to disease onset and severity. Likewise, there has been new research investigating how alternative splicing events can be targeted for therapeutic development.

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 https://doi.org/10.1016/j.tig.2018.10.002

In this review, we highlight the role of animal models mimicking splicing dysregulation in both understanding the disease etiology and providing a platform for developing and testing novel treatment strategies that target the deleterious splicing event.

Splicing Regulation

As alluded to earlier, differential splicing choices are regulated through the coordinated interactions between *cis* elements [splicing regulatory elements (SREs)] on the pre-mRNA and trans-binding protein factors. The trans-factors are RNA-binding proteins that form multi-protein complexes and facilitate recruitment (for positive regulation) or inhibition (for negative regulation) of the spliceosome components. Although there are numerous types of RNA-binding protein that interface with splicing regulation, the splicing regulatory proteins that are rich in serine/arginine (SR) residues, also known as SR proteins, and the heterogeneous ribonucleoprotein (hRNPs) are two of the most common. Some splicing regulatory proteins are tissue-specific and help to guide and delineate the tissue-specific profiles of the transcripts. The combinatorial binding of these proteins is dynamic and, consequently, can give rise to various permutations of exons and introns being included or excluded (Box 2). Given the complex regulatory patterns of alternative splicing regulation, there are many functional links between disrupted alternative splicing and disease (see Table S1 in the supplemental information online; reviewed in [5]). From mutations in *cis* SREs on the pre-mRNA, to overexpression or mutation of splicing factors, there are myriad diseases that result from perturbed alternative splicing. In fact, of ~80 000 mutations reported in the human gene mutation database, 10% affect splice sites [6,7]. The number of mutations affecting splicing indirectly by their association with SREs, *cis* elements, or their associated trans factors has not been tracked and, therefore, the number of mutations affecting splice choices is greater than quantitating splice site mutations alone predicts.

Laboratory Animals to Model and Study Splicing Defects

Scientists first began to associate defects in RNA splicing with disease during the early 1980s with the discovery of a retained intron in patients with β-thalassemia [8] (Box 3). Since then, hundreds of different splicing mutations have been identified as the underlying cause of diseases [9,10]. With this growing list of diseases related to splicing defects, it is essential to investigate the factors that contribute to mis-splicing events, to study how they lead to disease, and to devise ways to correct the splicing for therapy. Cellular and animal models have been instrumental in augmenting our understanding of splicing in disease, as well as serving as important tools for preclinical therapy testing. From models in *Drosophila* and zebrafish to mammalian systems, such as mice and dogs, many laboratory models have been designed or fortuitously characterized for the purpose of understanding and treating splicing defects (Table 1, Key Table).

Most animal models were designed and generated by engineering mouse embryonic cells to harbor the splice-affecting mutations via homologous recombination techniques. These models allow the splicing defects to be verified in the affected tissues of the animal. Furthermore, the models enable researchers to prove the causative nature of the defect for the designated disease by characterizing the pathology in the mouse and to provide an appropriate genetic background for preclinical trials in animals.

Additionally, a few models were identified that occur naturally in nature. For example, the widely used *mdx* mouse model of Duchenne muscular dystrophy (DMD) is derived from a mouse line with a naturally occurring nonsense mutation in exon 23 of the mouse dystrophin gene. While there is no splicing defect in the *mdx* mouse, it is a useful backdrop in which to investigate

Glossary

Adeno-associated virus (AAV): gene therapy technology used for the delivery of genetic material. In this case, infection with a harmless virus is performed; the virus can persist as an episome inside the cells and, thus, is not integrated into the host genome. For example, this technology can be used to deliver components of the CRISPR-Cas9 gene-editing system, as discussed within this review.

Cassette exon: a complete exon that may be excluded or included in alternative splicing events.

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 System: gene-editing technique based on bacterial adaptive immunity, where a guide RNA (gRNA) is used to target the gene of interest to be cleaved by the bacterially derived nuclease, Cas9. This can be used to induce mutations taking advantage of the cellular repair machinery.

Cryptic splice site: splice sites that are not normally used in wild-type pre-mRNA splicing, but that are activated as a result of mutations and can be used instead of the actual splice sites, hence affecting the normal splicing pattern of a transcript.

Exon-specific U1 snRNA (ExSpeU1): technology that takes advantage of the degenerate nature of the U1 snRNA base complementarity with the 5′ splice site of a target pre-mRNA. Due to the presence of three to four mismatches in this binding, improving the base complementarity with an artificial exon-specific U1 snRNA can target the splice site more specifically and may be used to correct aberrant splicing (see Figure 2Figure IBiv in Box 2).

Minor spliceosome: a U12-dependent ribonucleoprotein responsible for catalyzing removal of U12-type introns, present in most eukaryotes. U12-type introns are defined by the strong conservation of the 5′ splice site and branch point sequences. In contrast to the major spliceosome, the 5′ splice site and branch point are cooperatively recognized by the U11 and U12 snRNPs, respectively, and the U4atac, U5, and U6atac tri-snRNP

therapies that can induce removal of the mutated exon by interfering with its splicing (Table 1 and Box 4). Similarly, multiple dog breeds with a Duchenne-like phenotype have been identified that carry a variety of *DMD* mutations [11]. Of these, the affected Golden Retriever DMD model carries an A-to-G nucleotide base change in intron 6 of the canine *DMD* gene and is now referred to as the 'GRDMD' model. The intronic mutation in the GRDMD model causes the exclusion of exon 7 from the DMD transcript and an out-of-frame protein. Colonies of this dog model have been established to allow disease characterization and therapeutic testing in a mammalian system other than mouse. In fact, this model has been utilized to show the efficacy of **splice switching oligonucleotides** (**SSOs**; see Glossary) for systemic multiexon-skipping therapy ([11] and see later).

Controlling Splicing for Therapeutic Benefit

One of the challenges to designing therapies that target alternative splicing is ensuring target specificity. There are tens of thousands of mRNA messages in the cell, and modulating the splicing behavior of one particular gene, perhaps in one specific tissue, without undesirable off-target effects is challenging. To this end, targeting specific transcripts and affecting their RNA processing is emerging as a viable option to ensure target specificity and to minimize potentially deleterious off-target effects (Figure IB in Box 2). In diseases that arise from a splicing defect, a common therapeutic strategy is to use SSOs to restore the normal splice variant (reviewed in [12]). One type of SSO is a single-stranded RNA molecule that binds to complementary SRE targets to occlude binding of a specific RNA-binding factor, thereby impacting the levels of splicing. If the SSO is targeting a splice site or enhancer signal, the resultant mature RNA will exclude the exon targeted. By contrast, if the SSO is targeting a splicing silencer, the strategy can be used to force the inclusion of an exon and restore full-length protein expression.

Small-molecule compounds that target splicing factors are another means of changing alternative splicing that have been gaining popularity as treatment for diseases arising from splice defects. Frequently, libraries of compounds are used to screen splicing reporter constructs to discover synthetic and natural compounds that alter splicing of a particular exon. Additionally, some small molecules have shown efficacy in inhibiting splicing in general. Given that mutations in general splicing factors have recently been identified (discussed in more detail later), small molecules affecting the core spliceosome are a viable option for disease treatment. Specifically, Spliceostatin A and pladienolide bind the SF3B spliceosomal protein, which is an essential component of the U2 small nuclear ribonucleoprotein (snRNP) [13]. While these compounds do not target splicing of a particular gene, they are useful in disrupting splicing networks and cellular homeostasis in cells that are metabolically active in diseases such as cancer. AIDS, a systemic disease caused by a viral infection, has shown some benefit from small-molecule splicing inhibitors. IDC16, a small indole derivative, was similarly identified in an *in vitro* screen. IDC16 interferes with exonic splicing enhancers for **serine/arginine-rich splicing factor 1** (**SRSF1)** and suppresses the expression of key viral proteins, inhibiting the replication of several strains, including those that are resistant to viral protease and reverse transcriptase inhibitors [14]. This is an important example highlighting the relevance of splicing inhibition to both infectious and genetic diseases. Finally, another avenue of investigation in correcting splicing mutations is genome editing, where specific mutations can be corrected or circumvented (discussed comprehensively later).

There are multiple instances in which splicing therapies have been designed, tested in animal models, undergone clinical trials, and are now US Food and Drug Administration (FDA)-approved drugs. The evolution of two of these therapies for the treatment of spinal muscular atrophy (SMA) and DMD has been chronicled and discussed thoroughly elsewhere [12,15]. For

complex is utilized for complex B formation.

Peptide nucleic acid (PNA): synthetic polymers based on modified nucleic acids, where the sugar-phosphate backbone is replaced by repeating units of *N*-(2-aminoethyl)-glycine linked through peptide bonds. The lack of charged phosphate groups results in PNA having strong binding affinities. These can be used in antisense technology to modulate gene expression and splicing.

Phase separation: physicochemical phenomenon occurring inside eukaryotic cells in dynamic structures called membrane-less organelles, formed by proteins and nucleic acids. Some examples of these organelles are nucleoli, nuclear speckles, and stress granules. They are thought to be involved in aspects such as gene regulation, mRNA metabolism, and processing.

Pladienolide analog E7107: a derivative of pladienolide B with improved stability that binds to the SF3B component of U2 snRNP to interrupt spliceosome assembly.

Serine/arginine-rich splicing factor 1 (SRSF1) (previously known as ASF/SF2): a splicing factor that contains an arginine- and serine-rich domain and two RNA recognition motifs and is required for 5′ splice site selection and cleavage; it is also reported to be a proto-oncogene.

Serine/arginine-rich splicing factor 2 (SRSF2) (previously known as SC35): similar to SRSF1, this is important for splice site selection and spliceosome assembly. Mutation of SRSF2 is implicated in MDS and AML.

SF3B1: a heptameric protein complex in the U2 snRNP (in humans: SF3b155, SF3b130, SF3b145, SF3b49, SF3b14b, p14/SF3b14a, and SF3b10), in which mutations have been shown to lead to alternative branch point selection and has been linked to cancer.

Small nucleolar RNAs (snoRNA): generally exist in two classes and primarily serve as guides for chemical modification of ribosomal RNA, including methylation (Box C/D class) and pseudouridylation (Box H/ACA).

Splice switching oligonucleotide (SSO): synthetic polymers based on

both of these diseases, SSOs are used to modify splicing, for exon inclusion in the case of SMA, and for exon exclusion for the case of DMD. These two success stories highlight the power of splice-altering therapies and the importance of testing them in animal models for clinical use. While these and additional therapeutics targeting splicing defects are summarized in Table 1, we also detail the use of animal models in the evolution and testing of some of these therapies in the remainder of the review. In three vignettes described here, we discuss how developing technologies and animal models have impacted the RNA-splicing field in the recent past and provide new insights into disease and the therapies to treat them.

Mutations in Splicing Core Components as the Source of Disease

Given the prevalence of splicing alterations in disease, it is not surprising that recent studies point towards components of the spicing machinery as the basis of numerous diseases. This has been reported in myelodysplastic syndromes (MDS), a group of rare clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, which can progress into acute myeloid leukemia (AML), as well as in retinitis pigmentosa, a breakdown and loss retinal cells that can lead to blindness (see Table S1 in the supplemental information online, Table 1, and [16,17]).

In MDS and AML, several mutations in splicing factors have been identified, with **serine/arginine-rich splicing factor 2 (SRSF2)**, **U2 auxiliary factor (U2AF)**, and **SF3B1** being the most common [18]. SF3B1 is mutated in approximately a quarter of patients with MDS and is component of the U2 snRNP [19]. Therefore, mutations in the SF3B1-splicing factor affect binding of the U2 snRNP to the branch-point of pre-mRNAs and, consequently, leads to alterations in the splicing of several genes [20]. The A-to-G transition in exon 15 of *SF3B1*, one of the most common mutations in MDS, leads to the K700E substitution and also affects 3′ splice site selection fidelity. A novel mouse model containing this mutation was recently developed. This mouse develops progressive macrocytic anemia and myelodysplasia, and induces increased alternative 3′ splice site selection in several genes. Notably, a subset of the splicing changes seen in this mouse model are also affected in the human condition [21]. Other mutations from patients with MDS have likewise been modeled in mice. For example, mice with the P95H mutation in splicing factor SRSF2 also replicate the MDS phenotype [22]. Similarly, a mouse model expressing a U2AF S34F mutant splicing factor that showed increased alternative 3′ splice site usage with an increased frequency of myeloid progenitors in both spleen and bone marrow was generated [23]. Each of these models reinforces how important pre-mRNA splicing and its core components are for the development of pathogenesis.

The generation of novel animal models that accurately reflect the molecular mechanisms and phenotype of the disease is a critical step for the development of treatments. For example, in MDS clinical trials, treatment with a **pladienolide analog E7107** in patient-derived xenografts (PDX) decreased leukemic burden in mice with spliceosomal gene mutations [24]. This drug was also tested *in vivo* in a mouse model designed by Obeng and collaborators, where administration of E7107 reduced chimerism after bone marrow transplant of a mixture of *Sf3b1* mutant and wild-type (WT) cells [21]. Moreover, this drug entered Phase I clinical trials, although these were stopped shortly after due to two reported cases of vision loss [25]. H3B-8000, another modulator of the SF3b complex, recently entered clinical trials after showing antitumor effects in PDX mouse models with tumor cells from patients harboring *SF3B1* or *SRSF2* mutations [26]. Therefore, splice modulators emerge as a clear alternative in the treatment of diseases arising from mutations in the core splicing components, with animal models harboring conserved disease-causing mutations being key to early testing in the pipeline to the clinic.

modified nucleic acids, normally designed as single-stranded nucleic acids that are complementary (antisense) to a specific mRNA molecule (also called antisense oligonucleotides, ASOs or AONs). They can be used to modify gene expression by promoting mRNA degradation or altering RNA splicing.

Spliceosome-mediated RNA *trans*-splicing (SMaRT): technology that takes advantage of the uncommon splicing between two separate pre-mRNA molecules or trans splicing. Using an artificial pre-mRNA trans-splicing molecule (PTM), containing a binding domain, splicing domain and coding domain; it is possible to trigger a trans-splicing event between this PTM and a target pre-mRNA. The resultant artificially spliced transcript is able to correct an error in the target pre-mRNA by replacing the mutated exon and all downstream exons (encoded by the PTM) via the trans-splicing event (see also Figure 2Figure IBiii in Box 2).

Stable intronic sequence RNAs (sisRNAs): introns are generally degraded upon splicing; however, sisRNAs have been suggested to have other roles in the cell, including regulation of gene expression, acting as a molecular sponge, and regulating protein translation.

Stress granule: dense aggregates that form in eukaryotic cells under stress conditions such as heat-shock; they comprise RNA and pre-mRNA processing factors, such as splicing-related proteins. They are thought to be a cellular mechanism of rapid reprograming of gene expression.

U2 auxiliary factor (U2AF): a heterodimer comprising a large and a small subunit, U2AF65 and U2AF35, respectively. The complex is required for definition of 3′ splice sites. Mutations in U2AF have been implicated in blood diseases and cancers.

Phase Separation and Alternative Splicing in Neurodegenerative Diseases: New Players in an Old Game

Eukaryotic cells are compartmentalized into specific membrane-bound compartments called organelles. However, membrane-less organelles have emerged as important functional compartments, the cellular organization of which is less well understood. Moreover, these membrane-less organelles comprise a mix of proteins and nucleic acids. It became clear recently that these membrane-less organelles are brought together and maintained as a result of **phase separation** within the cells. The nucleolus, Cajal bodies, nuclear speckles, and nuclear **stress granules** are all examples of membrane-less organelles, where several splicing factors and ribonucleoprotein complexes and/or miRNAs reside. Hence, it is not surprising that RNA metabolism is intricately influenced by phase separation and the formation, reorganization, or disruption of these membrane-less organelles [27].

Phase separation is facilitated in part by proteins with low-complexity domains. When these proteins are mutated, it can hinder their ability to coalesce and disrupts the normal physiological liquid–liquid phase separation. When physiological phase separation is disrupted, pathogenic protein aggregates may occur and, in some cases, lead to neurodegenerative disease. Recent studies have shown that some of these aggregating and disease-causing proteins are in fact splicing factors due to low-complexity regions commonly found in RNA-binding proteins [28,29]. Simply put, specific mutations in some splicing factors alter the biophysical properties of the protein to contribute to pathological aggregation and potentially lead to their sequestration and functional inhibition. One such example is trans-activating response region DNA-binding protein 43 (TDP-43) encoded by *TARDBP*. More than 40 different mutations have been identified in the C-terminal domain that can cause TDP43 to form toxic aggregates by phase separation [30,31]. These aggregates can also alter normal splicing of the cells by promoting

Box 1. Mechanisms of RNA Splicing

Pre-mRNA splicing involves the removal of regions of the transcript called introns and joining together the exons that will comprise the mature RNA transcript. While most of the mature transcript is responsible for coding the resultant protein, some exons are also regulatory in nature, comprising the untranslated region (UTR) of the mRNA. Splicing is a highly ordered process performed by a macromolecular machine called the spliceosome, which rivals the ribosome in its complexity and sheer number of proteins and RNAs involved [64,65]. The spliceosomal small nuclear (sn)RNAs and proteins or small nuclear ribonucleoprotein (snRNP) complexes are essential for the recognition of splicing sites and the catalysis of the splicing reaction (Figure I). The location of splice sites at both the 5′ and 3′ ends of the intron are demarcated by degenerate canonical sequences, which are recognized by core elements of the spliceosome. The composition of the 5′ splice site, or donor splice site, includes a nearly invariant 'GU' dinucleotide sequence along with less conserved residues downstream. In rare cases, a 'GC' dinucleotide has been documented to serve as the 5′ splice site when the remaining nucleotides match the 'consensus' sequence [66]. The 3′ splice site, or acceptor splice site, comprises three conserved elements: a branch point, polypyrimidine or Py tract, and an 'AG' at the 3′ end. The branch point is typically an adenosine located approximately 18–40 nucleotides from the 3′ splice site, and this is followed by a track of 15–20 polypyrimidine residues, particularly uracil. Atypical branch points also exist that can be more distant from the 3′ splice site as well as those that utilize a nonadenosine base [67].

The splicing process is accomplished by a two-step transesterification reaction, which is carried out by either the major spliceosome, for ~99% of transcripts, (Figure IB left, labeled in bold red) or, less frequently, by the minor spliceosome (Figure IB, labeled in gray). The first step is the lariat formation via a nucleophilic attack of the 5′ splice site phosphate group by the 3′ hydroxyl group of the branch point adenosine. In the second step, the free hydroxyl of the detached exon attacks the 3′ splice site, leading to the generation of two ligated exons and a lariat intron [68]. In the first step of spliceosome assembly, the U1 snRNP associates in a pairwise manner with the 5′ splice site, and weakly with the U2 snRNP, forming Complex E [69]. When the U2 snRNP tightly binds the branch point by base-pairing, the complex A is said to be assembled; this is then followed by the recruitment of the tri-snRNP U4/U5/U6, to generate the precatalytic complex B. After several conformational rearrangements facilitated by RNA helicases, the complex B adopts a conformation that catalyzes the first of the transesterification reactions. This process generates complex C, which results in the free 5′ exon and lariat intron. Complex C undergoes rearrangement to perform the second step of splicing that allows for the ligation of adjacent exons and release of the lariat intron. At this point, the snRNPs are released in an ATP-dependent manner to catalyze additional rounds of splicing [69,70].

The minor spliceosome works in much the same way; however, the intron boundaries are characterized by divergent 'AU' and 'AC' dinucleotide sequences at the 5′ and 3′ termini, respectively. These sequence variants require U11 and U12 snRNPs for the recognition of splice sites and the subsequent catalysis. Additionally, U4atac and U6atac replace U4 and U6, with U5 as the only snRNP component in common between the two spliceosomes.

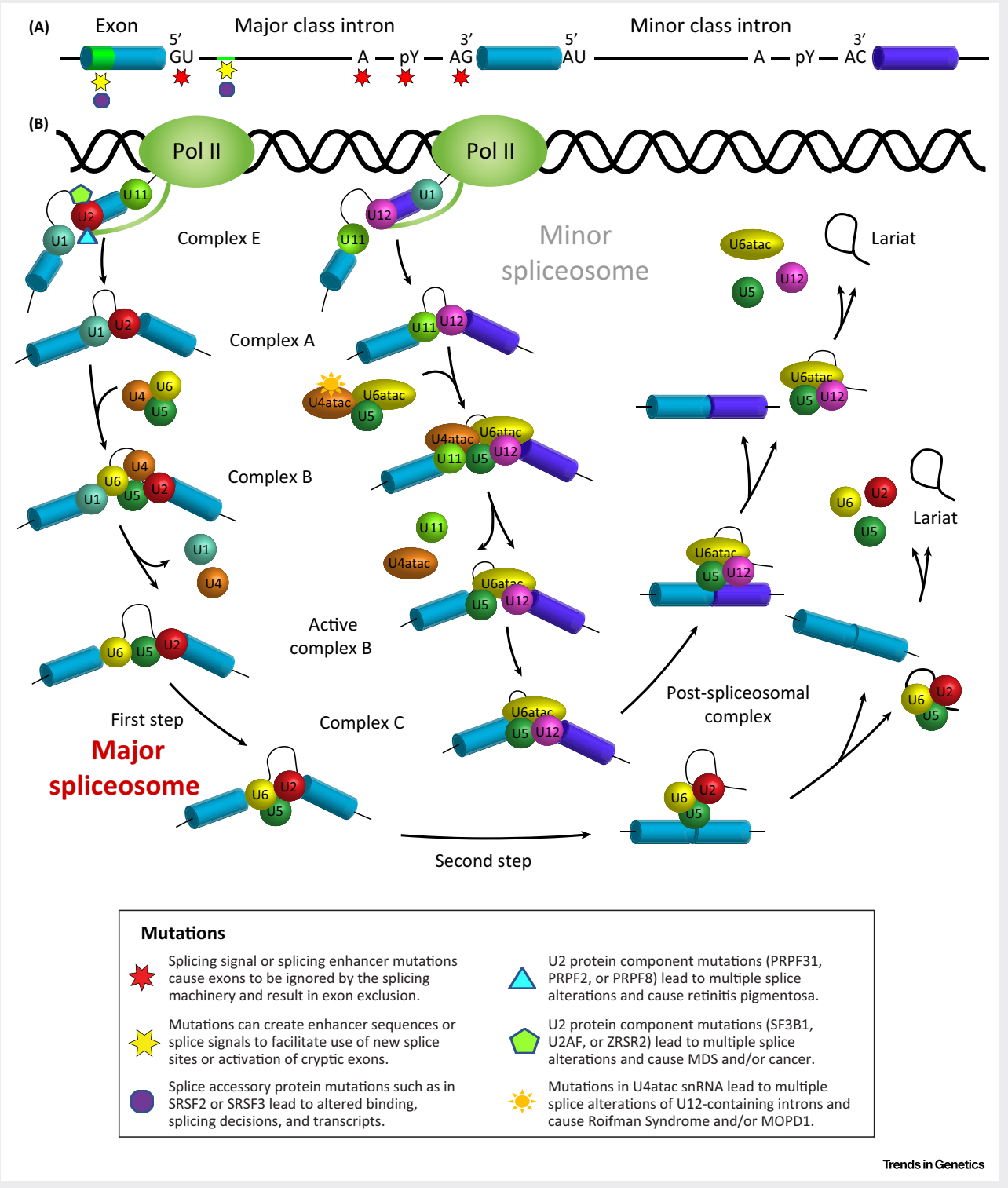

Figure I. Disease Causing Mutations in the pre-mRNA and Major and Minor Spliceosomes. (A) Mutations in the RNA transcript itself can lead to splicing alterations. Similar mutations are also possible in the sequences controlling splicing of the minor introns, although are not depicted here. (B) Similarly, mutations in core spliceosome machinery of both the major and minor spliceosome have been shown to lead to altered splicing networks. Both of these scenarios can manifest in a disease phenotype. Abbreviations: MDS, myelodysplastic syndromes; MOPD1, microcephalic osteodysplastic primordial dwarfism type 1; SRSF, serine/arginine-rich splicing factor.

the inclusion of **cassette exons** or modifying the usage of mutually exclusive exons [32,33]. Moreover, this protein has been shown to affect the splicing of another splicing factor, hnRNP A1, by increasing the inclusion of exon 7B, causing it to also form aggregates [34]. Hence, it is still a matter of debate whether the aggregate accumulation in the cytoplasm is toxic itself, or whether the altered splicing is responsible for the disease phenotype [32,33,35].

Animal models carrying mutant forms of TDP-43 have been generated that replicate some of the abnormal alternative splicing seen in ALS. For example, mice harboring a mutant Q331K TDP-43 transgene led to a loss-of-function in direct splicing targets, such as *Kcnip2* or *Atp2b1*, as well as age-dependent motor neuron loss [36]. Additionally, *Tardbp* was modified to produce a truncated protein by the change of a glutamine to a stop codon in position 110 in another mouse model. These mice exhibited a loss of splicing fidelity in Tdp-43 target genes in the brain as well as functional defects manifesting as impaired hindlimb strength when measured by a classical clasping test and abnormal body tone [37]. These animal models reinforce TDP-43 as a key splicing factor involved in ALS pathogenesis.

The protein fused in sarcoma (FUS) is another example of a splicing factor affected in ALS. Mutations in the SYGQ low-complexity domain situated in the N-terminal end of the protein,

Box 2. Alternative Splicing

In addition to constitutive splicing, most eukaryotic cells are able to perform alternative splicing; the process wherein different coding regions of the pre-mRNA are included in the mature mRNA (Figure IA). Some exons of a gene may be included or excluded from the final transcript. Alternative splicing is one of the leading drivers of proteomic diversity, because one gene can generate many different mRNA messages that translate into several proteins with divergent sizes and/or functions. One of the contributing factors to alternative splicing is the length of introns in higher vertebrates, because long introns have a high likelihood of undergoing alternative splicing [71]. Alternative splicing can occur cotranscriptionally with many of the protein factors that participate in regulated alternative splicing binding to the Carboxyl-terminal domain (CTD) of RNA polymerase II [72].

Alternative splicing is a highly regulated process and happens in response to developmental cues and external stimuli, including stress. Moreover, alternative splicing can result in one of several outcomes in splice site selection: cassette exons (the preferential exclusion of an exon under certain conditions); alternative 3′ and 5′ splice site selection (the selection of a distal splice site instead of the normally utilized splice site); mutually exclusive exons (sets of exons that are never included in the same transcript); and intron retention (a normally excluded intron is retained in the final mRNA transcript).

Alternative splicing is regulated by transcriptional elongation, core spliceosomal proteins, RNA–RNA base pairing, chromatin structure, and a host of RNA-binding proteins that interact with splicing elements on the pre-mRNA. Aside from the canonical splicing signals, 5′ splice site, branch point, and 3′ splice site, there are interactions between *cis* elements on the pre-mRNA and trans-acting protein factors. These *cis*-acting elements are known as SREs, and are generally classified as either being enhancers, which promote the recognition of an exon, or silencers, the proteins of which facilitate the masking of splice signals to result in exon exclusion events. SREs can occur in both introns and exons and have been characterized in many organisms, from flies to humans. SREs can be classified as exonic splicing enhancers (ESE), intronic splicing enhancers (ISE), exonic splicing silencers (ESS), or intronic splicing silencers (ISS).

Numerous avenues have been pursued to accomplish splice alterations for therapeutic benefit (Figure IB). Examples of splice-altering therapies are: antisense oligonucleotides (ASOs) targeting an exon and/or intron boundary to induce exon exclusion (Figure IBi); ASOs targeting a splicing silencer to promote exon inclusion (Figure IBii); trans splicing to an exogenous construct to increase full-length splicing (Figure IBiii); exonic-specific U1 to improve the recognition of a mutant splice site (Figure IBiv); and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9-genome editing to delete a mutant cryptic spice site and restore constitutive splicing (Figure IBv). In Figure IB, exons are depicted graphically by boxes, introns by horizontal lines, and splice choices by angled broken lines. Reported splicing enhancer elements are depicted by a green box within the exon, while splicing silencer elements are depicted by a red line (intronic) or a red box (exonic). Splice choices in the absence of therapeutic intervention are depicted by broken angled lines above each transcript and the therapeutic intervention below. The dark angled lines are the preferred splicing choice, while the light broken lines occur less frequently.

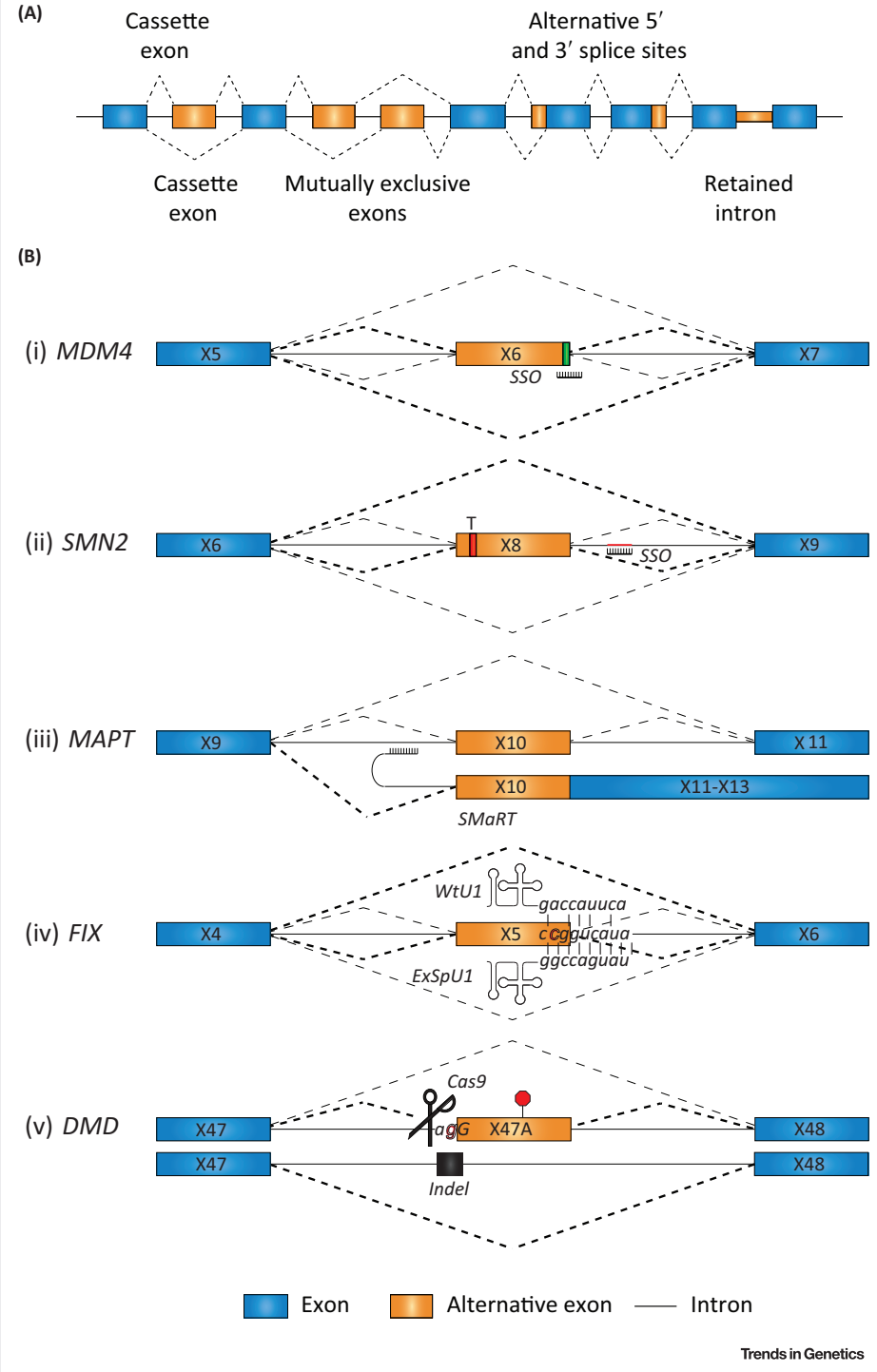

Figure I. Alternative Splicing and Therapeutic Strategies.

Box 3. Evolution of Therapies for Splicing Targets: β-Thalassemia

β-thalassemias are a group of diseases affecting hemoglobin synthesis. There are over 200 identified mutations leading to these genetic disorders, with splicing mutations among the most common [73,74]. Many of the mutations are clear examples of how single nucleotide changes can lead to the activation of what are known as **cryptic splice sites**, or novel 5′-donor or 3′-acceptor sequences that generate aberrantly-spliced transcripts. IVS2-654 β-thalassemia is one of the most common of these splicing mutations, causing a C-to-T change in position 654 of the IVS2 intron in the gene encoding β-hemoglobin (*HBB*) to generate an aberrant 5′-donor splice site at position 652 and to activate a cryptic 3′-acceptor splice site at position 579. This allows for the aberrant inclusion of an intron and the translation of a nonfunctional β-globin protein [75]. Expression of this nonfunctional protein leads to low oxygen levels and a shortage of red blood cells, which can lead to life-threatening anemia.

Lewis and colleagues were the first to successfully generate a humanized mouse model of β-thalassemia that expresses an aberrant splice variant by carrying common IVSII-654 β-thalassemia splicing mutation [76]. More recently, subsequent models were generated harboring additional splice-disrupting mutations [77,78]. Importantly, these models were useful not only for gaining further understanding of the molecular mechanisms involved with the disease, but also as a platform for testing splice-switching oligonucleotides (SSO) [79]. Furthermore, these studies have led to new advances for SSO delivery, including the lentiviral vectors that stably express U7 snRNAs carrying splice-switching SSO sequences targeted to the cryptic 5′ splice site recently tested in erythroid cells from patients [80].

Genome-editing techniques are a new avenue of therapy likewise being applied to the correction of splicing defects in β-thalassemias. Using CRISPR-Cas9 and a piggyBac transposon, Yuet Wai Kan's laboratory was able to correct *HBB* mutations in induced pluripotent stem cells (iSPCs) derived from a patient with β-thalassemia. These corrected clones were differentiated into hematopoietic progenitors expressing a 16-fold increase in HBB [81]. Remarkably, the genome-editing therapy yielded no detectable off-target effects. Thus, from its earliest association as a splice-dependent disease, to the development of animal models, and to the testing of splice-correcting therapies, the β-thalassemia field has paved the way for understanding splicing defects in disease and to finding therapies to correct them.

such as G156E, can induce the disruption of the normal liquid–liquid phase separation in favor of protein aggregates and RNP granules [38,39]. Furthermore, FUS has been shown to interact with the U11 snRNP, a component of the **minor spliceosome**, thus regulating the splicing of minor intron-containing mRNAs. In the case of neurodegenerative diseases such as ALS, cytoplasmic aggregates of FUS sequester and trap U11 and U12 snRNAs, affecting minor intron splicing [40]. A mouse model harboring the mutant R521C FUS protein was able to recapitulate the molecular mechanisms affected in ALS related to alternative splicing defects. In these transgenic mice, several genes had increased intron retention compared with wild-type mice, especially in genes related to the extracellular matrix, such as those encoding collagens and cadherins, which are key in neurite outgrowth and synapsis specificity [41].

Alzheimer's disease (AD) is another neurodegenerative pathology in which abnormal liquid–liquid phase separation is involved; in this case, the microtubule-associated protein Tau forms aggregates. While Tau is not a splicing factor itself, in AD (and other neurodegenerative diseases categorized as tauopathies) the abnormal alternative splicing of the gene encoding microtubule-associated protein tau (*MAPT*) causes an imbalance between Tau splice isoforms, leading to protein aggregates [42]. A total of 14 different mutations have been identified that affect different splicing enhancers or silencers in exon 10 and intron 11, thus altering the inclusion or exclusion of exon 10 of *MAPT* [43,44]. The resultant isoforms, 4R-Tau and 3R-Tau, contain different numbers of the imperfect repeat sequences and are normally represented in the adult brain at a ratio of 1:1. However, the splicing changes affecting exon 10 disrupt this normal ratio and are crucial for the pathogenic accumulation of Tau. Furthermore, the altered ratio of these two isoforms has different clinical manifestations leading to different tauopathies. For example, in Pick's disease, also known as frontotemporal dementia (FTD), there is relative change in the Tau isoform ratio to more 3R-Tau than 4R-Tau [45]. This example, once again, reinforces the importance of alternative splicing changes leading to alterations in cellular phase

Key Table

Table 1. Commonly Used Animals to Model Splicing Changes and Test Splice-Altering Therapies in Disease

Disease	Animal model	Therapies	Outcome	Refs
Alzheimer's disease and other tauopathies	Mouse model expressing N296H mutant Tau isoform	**Peptide nucleic acid (PNA)**, SMaRT	PNA treatment induced *MAPT* exon 10 skipping *in vitro*; transfection of a *MAPT* trans-splicing donor plasmid carrying exons 10–13 induced exon 10 inclusion *in vivo*	[86–89]
	Knock-in mouse model expressing *Apoer2* gene with deleted exon 19	SSO	SSO treatment increased inclusion of *ApoER2* exon 19 *in vitro* and *in vivo*; treatment improved hippocampal basal synaptic transmission, learning, and memory in AD mice	
Amyotrophic lateral sclerosis	Mouse model expressing Q331K mutant TDP-43	SSO	SSO treatment induced *FUS* exon 7 skipping *in vitro*	[32,37,41,90]
	Mouse expressing R521C mutant FUS			
	Mouse model expressing a Q101X mutation in *Tardbp* gene			
Autism spectrum disorder	Mouse model expressing nSR100/Srrm4 isoform with deleted exons 7 and 8	N/A[a]	N/A	[91]
Autoimmune diseases	Knock-in mouse model overexpressing *1/4 Ctla4* splice variant	SSO	Treatment with SSO induced skipping of *CTLA4* exon 2 *in vitro* and *in vivo* increasing expression of liCTLA-4 isoform; induction of splice isoform delayed onset of diabetes and decreased its incidence in NOD mice	[92–94]
Breast cancer	Knock-in mouse model expressing human *Δ16HER2*	Small molecule (dacomitinib)	Dacomitinib treatment completely suppressed Δ16HER2-driven carcinogenesis *in vitro* and *in vivo*; in Δ16HER2 mice, treatment completely abolished autochthonous mammary tumor formation	[95–98]
	Knock-in mice expressing *ERBB4* isoforms *CYT-1* and *CYT-2*	SSO	SSO treatment induced skipping of *ERBB4* exon 26, thus increasing CYT-2 isoform expression *in vitro*	
Cystic fibrosis	Transgenic mouse model carrying YAC with a mutated *CFTR* gene in exon 9 ($TG_{12}T_5$ insertion)	SSO, **exon-specific U1 snRNA (ExSpeU1)**, CRISPR/Cas9	Treatment with SSO or ExSpeU1 induced correct inclusion of *CFTR* exon 16 *in vitro*; treatment with CRISPR/Cas9 induced skipping of cryptic exons in *CFTR* introns 12, 21, and 22 *in vitro*	[99–102]
Deafness	Mouse model expressing nSR100/Srrm4 isoform with deletion on intron 12 and exon 13	N/A	N/A	[103]
Duchenne muscular dystrophy	Multiple models as reviewed in [11]; including a dog model carrying an A-to-G mutation in intron 6 of canine *DMD* gene	SSO, SMaRT, CRISPR-Cas9	Transfection of a trans-splicing AAV vector containing *Dmd* intron 22 and exon 23 induced expression of wild-type protein *in vivo* and *in vitro*; SSO treatment induced skipping of *Dmd*	[11,12,15,84,104–106]

Table 1. (continued)

Disease	Animal model	Therapies	Outcome	Refs
	Mouse models carrying a C-to-T mutation in exon 23 of *Dmd* gene or a deletion of exon 52 Mouse model with *Dmd* exon 50 deleted		exon 51 producing in-frame mRNA *in vivo*; CRISPR/Cas9 induced skipping of exon 51 in *Dmd* gene *in vivo*; treatments and restoration of wild-type protein induced muscle normalization both structurally and functionally; in patients SSO treatment lowered incidence of loss of ambulation	
Epilepsy	Knockout mouse model with deletion of exon E8a in *Kdm1a/Lsd1* gene (neuroLSD1 null)	N/A	N/A	[107]
Familial dysautonomia	Knock-in mouse model carrying mutated human *IKBKAP* gene in intron 20 (T to C) Mouse model with *Ikbkap* knockout	Small molecule (kinetin)	Kinetin treatment induced *IKBKAP* exon 20 inclusion in patients	[108,109]
Fukuyama muscular dystrophy	Knock-in mouse model carrying mutant *Fktn* with exon 10 replaced by SINE-VNTR-*Alu*-containing human exon 10	SSO	SSO treatment abolished inclusion of *fukutin* aberrant exon 11 *in vivo;* treatment restored normal function of Fukutin	[110,111]
Hemophilia B	Mouse model expressing human Factor 9 with mutant exon 5	ExSpeU1	Treatment with ExSpeU1 induced *F9* exon 5 inclusion *in vitro*	[101,112,113]
Hereditary myopathy with lactic acidosis	Mouse model carrying a G-to-C mutation in intron 4 of *ISCU* gene	SSO	SSO treatment induced skipping of cryptic *ISCU* exon 4a *in vitro*	[114,115]
Hereditary tyrosinemia type I	Mouse model carrying G-to-A mutation in exon 8 of *Fah* gene	CRISPR/Cas9	CRISPR/Cas9 treatment induced inclusion of exon 8 in *Fah in vivo*; treatment ameliorated liver damage in $Fah^{mut/mut}$ mice	[116]
Huntington's disease	Knock-in mouse model carrying insertion of 150 CAG repeats into exon 1 of *HTT* gene	SMaRT	Transfection of *HTT* exon 1 trans-splicing donor plasmid with 21 CAG repeats abolished exon 1 skipping *in vitro*	[117,118]
Hutchinson–Gilford progeria syndrome	Knock-in mouse model carrying a C-to-T mutation in exon 11 of *Lmna* gene	SSO	Treatment with SSO induced correct inclusion of *Lmna* exon 11 *in vitro*	[119,120]
Hypertrophic cardiomyopathy	Knock-in mouse model carrying a G-to-A mutation in exon 6 of *Mybpc3* gene	SMaRT	Transfection of a trans-splicing AAV vector containing *Mybpc3* exons 1–6 induced inclusion of exon 6 *in vivo*; although treatment repaired cMyBP-C protein, it was not enough to ameliorate cardiac phenotype in knock-in mice	[121,122]
Infantile hypophosphatasia	Mouse model carrying T-to-G mutation in intron 9 of *Alpl* gene	N/A	N/A	[123]
Leber's congenital amaurosis	Knock-in mouse model carrying a A-to-G mutation in intron 26 of *CEP290* gene	SSO, CRISPR/Cas9	SSO and CRISPR/Cas9 treatment induced skipping of cryptic exon in *CEP290* intron 26 *in vitro*	[124,125]
Melanoma	Knockout mouse expressing *Mdm4-S* (exon 6 deleted)	SSO	Treatment with SSO induced skipping of *Mdm4* exon 6 *in vitro* and *in vivo*; in	[126,127]

Table 1. (continued)

Disease	Animal model	Therapies	Outcome	Refs
			patient-derived mouse xenographs, treatment decreased tumor growth and sensitized them to BRAFV600E inhibition	
Muscular dystrophy type 1A	Mouse model carrying G-to-A mutation in intron 2 of *Lama2* gene	CRISPR/Cas9	Treatment with AAV containing the CRISPR/Cas9 components induced inclusion of exon 8 in *Lama2* gene *in vivo*; genome editing of dy^{2J}/dy^{2J} mice showed improved muscle architecture and decreased fibrosis with concomitant improvement on paralysis of hind limbs and mobility	[52]
Myelodysplastic syndromes and acute myeloid leukemia	Knock-in mouse model expressing K700E mutant Sf3b1 Knock-in mouse model expressing P95H mutant Srsf2 Heterozygous knockout mouse model with *Hnrnpk* gene deleted Mouse model expressing S34F mutant U2AF1 isoform	Small molecules (E7107, H3B-8800)	Treatment with E7107 resulted in widespread intron retention and reduced leukemic burden in *Srsf2* mutant mice; treatment with H3B-8800 produced splicing modulation and inhibited tumor growth in mouse xenografts	[21–24,128,129]
Myotonic dystrophy type 1	Knock-in mouse model expressing mutant *HSA* gene with 250 CTG repeats in its 3′-UTR	Small molecule (manumycin A)	Treatment with manumycin A induced inclusion of *Clcn1* exon 7a *in vivo*	[130,131]
Niemann–Pick type C disease	Mouse model carrying G-to-A mutation in intron 9 of *Npc1* gene	SSO	Treatment with SSO inhibited inclusion of cryptic exon in *Npc1* intron 9 in patient cells	[132,133]
Pompe disease	Knockout mouse models with disrupted/deleted exon 6 on *Gaa* gene	SSO	Treatment with SSO inhibited inclusion of cryptic exon in *Gaa* intron 15 in patient cells	[134]
Prader–Willi syndrome	Knockout mouse model with *Snord115* gene deleted	SSO	Treatment with SSO induced *HTR2C* exon 5b inclusion *in vitro* and *in vivo;* in both fasted mice and genetically altered, hyperphagic mice, treatment reduced food intake	[135,136]
Prostate cancer	Knock-in mouse expressing human *AR3/AR-V7*	Small molecule (niclosamide)	Niclosamide treatment induced AR-V7 proteasomal degradation both *in vitro* and *in vivo*; in mouse xenographs, treatment decreased tumor growth of enzalutamide-resistant prostate cancer	[137,138]
Retinitis pigmentosa	Zebra fish and mouse models expressing mutant *Prpf31* Knock-in mice expressing mutant *Prpf3* or *Prfpf8*	SMaRT	Transfection of trans-splicing AAV vector containing *Rho* exons 2–5 and artificial intron 2 induced expression of wild-type protein *in vivo*; nevertheless, in severely affected transgenic mice, treatment was not sufficient to delay or stop photoreceptor degeneration	[139–142]
Rett syndrome	Knockout mouse model with *Mecp2* gene deleted	N/A	N/A	[143]
Rhabdomyosarcoma		SSO	SSO treatment induced inclusion of *MDM2* exons 4–11, thus increasing	[144,145]

Table 1. (continued)

Disease	Animal model	Therapies	Outcome	Refs
	Transgenic mouse model expressing human *MDM2-ALT1*		MDM2 full-length protein expression *in vitro*	
Spinal muscular atrophy	Multiple models as reviewed in [146]; including a knock-in mouse model carrying a mutated human *Smn* gene in exon 7 (C to T)	SSO, small molecules (7-piperazinylcoumarins, RG7800), ExSpeU1	Treatment with SSO, small molecules or ExSpeU1 induced retention of *SMN2* exon 7 *in vivo* and patient cells; SSO treatment in mouse studies showed marked improvement in muscle morphology and motor function as well as extended life expectancy; treatment with SSO RG7800 has entered clinical trials	[12,147–152]
	Heterozygous *Smn* exon 2 knockout mice			
	Knock-in mouse model carrying a mutated human Smn gene in exon 7 (TTT insertion)			
Usher syndrome	Knock-in mouse model carrying G-to-A mutation in exon 3 of *Ush1c* gene	SSO	Treatment with SSO induced correct inclusion of *Ush1c* exon 3 *in vitro* and *in vivo*; transgenic 216AA mice treated with SSO showed therapeutic correction of deafness	[153,154]
X-linked agammaglobulinemia	Knock-in mouse model carrying mutated human *BTK* gene in intron 4 (A to T)	SSO	Treatment with SSO induced skipping of cryptic exon in *BTK* intron 3 in patient cells	[155,156]
	Mouse model with *BTK* knockout			
β-thalassemias	Knock-in mouse models carrying either a mutated human *HBB* gene in intron 2 (C to T), in intron 1 (G to A), or (T to C)	PNA, SSO	Treatment with PNA induced skipping of cryptic exon in *HBB* intron 2 *in vitro*; treatment with ASO induced skipping of cryptic exon in *HBB* intron 2 *in vivo*; in IVS2-654 mice, treatment improved red blood cell morphology and hemoglobin levels	[157,158]

[a]N/A, not available.

separation, and further highlights how harnessing these processes could lead to the therapeutic avenues for multiple diseases.

In an effort to modulate alternative splicing as a means of therapy for tauopathies and AD, a hTau transgenic mouse model that expresses all six splice isoforms simultaneously from the human *MAPT* gene in a mouse *Mapt* null background was generated. Interestingly, this model showed *Tau* altered splicing where exon 10 exclusion is increased, thus producing more 3R-Tau isoform and modeling what is observed in Pick's disease [46]. The hTau transgenic mouse model was additionally used to test the therapeutic capabilities of **spliceosome-mediated RNA trans-splicing** (SMaRT) technology (Figure IBiii in Box 2). hTau mice infected with a lentivirus carrying the pre-trans-splicing RNA molecule (PTM) that includes the 3′ end of a *Mapt* cDNA including exon 10 and its associated 5′ splice site and splicing signals showed increased exon 10 inclusion in the brain [47]. In another instance, hTau mice were treated with SSOs that targeted regulatory elements within intron 12 of *MAPT*. The SSO treatment successfully shifted the ratio of *Tau* isoforms by increasing the inclusion of exon 10. The resultant changes

Box 4. Splicing Induction as Therapy in Nonsplice-Mediated Diseases

DMD is a genetic disorder characterized by progressive muscle wasting and weakness caused by the loss of a functional dystrophin protein. The disease is caused by hundreds of different known mutations in the *DMD* gene. These mutations encompass a variety of genetic alterations, from exon deletions or duplications and even frameshift mutations, with just one thing in common: they all lead to a nonfunctional dystrophin protein.

One of the ways that researchers have approached the therapeutic design against this disease is to take advantage of alternative splicing and use it to produce a partially functional dystrophin protein. The use of antisense oligonucleotides has been one strategy to do this (see Figure IB in Box 2), by inducing skipping both of a single mutated exon [82] and of multiple exons using a cocktail of SSOs [83]. The goal of these therapies is to delete the exons carrying the deleterious mutations and restore the reading frame of the DMD protein. Although the DMD protein is lacking the sequence encoded by the exon that is induced to be skipped, its function is conserved sufficiently to restore muscle physiology and to alleviate disease symptoms. However, using splice-switching oligomers in the clinic has therapeutic limitations related to delivery and molecule stability.

In an effort to design new therapies for DMD, researchers have explored using novel genome-editing CRISPR-Cas9 technology to correct *DMD* functionality by inducing splicing changes to delete 'bad' exons. A new mouse model of DMD was created by using CRISPR-Cas9 to delete exon 50 of the mouse *Dmd* gene (ΔEx50), which caused a deleterious frameshift in the gene and resulted in mice with severe muscle dysfunction [84]. The DMD phenotype could be rescued by systemic delivery of adeno-associated virus encoding the CRISPR/Cas9 genome-editing components to create reframing mutations and allowed skipping of exon 51 [84]. Armed with this powerful proof of principle, the authors went on to target 12 exons of the human *DMD* gene, for which reframing mutations and exon skipping could result in therapeutic benefit. They were able to correct multiple different mutations in human engineered heart muscle cells using CRISPR-Cas9 [85].

produced higher levels of the isoform *4R-Tau* [48]. Surprisingly, this shift in Tau isoforms also caused increased protein aggregate formation and increased seizures in mouse models, reflecting the importance of the titration of isoform products for alternative splicing in disease manifestation and therapy.

As summarized earlier, it is well known that, in neurodegenerative diseases such as the tauopathies, AD and ALS, different protein aggregates are an underlying cause. Moreover, recent research has shown that some splicing factors can, in fact, also form aggregates via phase separation [28,29]. These events lead to altered pre-mRNA splicing, which can be detected in the different pathologies. Therefore, the generation of novel and improved animal models, which can replicate not only the outcome of the disease, but also the alterations in splicing is crucial. Animal models such as these allow for the design of splicing-modulating therapies to combat the effects of pathological aggregation and splicing defects in neurodegenerative diseases.

Using New Technologies as Therapeutic Modulators of Splicing: Targeted Genome Editing with CRISPR-Cas9

To ensure target specificity of RNA therapeutics, there is a growing interest in developing new and improved ways to regulate alternative splicing or better delivery methods for existing ones. The increase in new technologies in molecular biology and genetics emerges as a fundamental answer to these problems. Examples include next-generation sequencing (NGS), nanoparticles, single-molecule studies, and targeted genome editing with the **clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9)** technology. This powerful system, which is part of the innate immune response in bacteria, has been coopted by molecular biologists to induce precise genome editing in several organisms. For instance, CRISPR-Cas9 has been used to generate genetic knockouts, tag endogenous proteins, induce disease-causing mutations, and also therapeutically correct toxic gene products in *in vitro* systems and animal models [49,50]. In recent years, the use of CRISPR-Cas9 has grown exponentially and its application to diseases related to alternative splicing is not the exception.

Genome-editing technologies have been harnessed for the study of new treatment modalities for multiple diseases arising from mutations that disrupt splicing fidelity, such as the β-thalassemia group of diseases (Box 3). Other studies using this technology have likewise been pursued, as highlighted by recent studies of hereditary tyrosinemia type I (HTI), a fatal genetic disease caused by mutation of the gene encoding fumarylacetoacetate hydrolase (*FAH*). This disease has been modeled in mice by inducing the same mutation that is observed in humans: a homozygous G-to-A point mutation that causes exclusion of exon 8 during splicing and creates a truncated, unstable FAH protein. Using CRISPR-Cas9, researchers have been able to correct the FAH mutation in the livers of mice, generating full-length protein sufficient to restore the weight loss of a mouse model of HTI [51].

Another example of the CRISPR-Cas9 technology being used in a splicing-related disease is work published in congenital muscular dystrophy type 1A (MDC1A). This disease is characterized by severe muscle wasting, as is seen in many other neuromuscular diseases (NMD). The dy^{2j} mouse model harbors a 5′ splice site mutation in intron 2 of the *LAMA2* gene that causes aberrant skipping of exon 2, leading to murine dystrophia muscularis-2J. The authors were able to restore the production of the full-length protein in the dy^{2j}/dy^{2j} mouse model by using CRISPR-Cas9 in conjunction with an **adeno-associated virus (AAV)** for delivery into the mouse. In this model, the authors used genome editing to delete the mutation in intron 2 and, therefore, promote exon 2 inclusion by creating a new functional donor splice site [52].

Similarly, genome editing has been utilized to create new mouse models and treatment options for DMD (Box 4). While therapies aimed at splicing in DMD are not correcting mis-splicing in the disease, splicing changes are being utilized as a powerful way to manipulate the *DMD* gene to remove disease-causing frameshift mutations (see Figure IBv in Box 2).

While the genome-editing technologies we have discussed thus far target the DNA and, hence, the genetic code, new systems have been developed to redirect the CRISPR/Cas 9 toolbox to target RNA directly. Microsatellite repeat expansions in the genome can give rise to pathogenic RNA molecules that cause several diseases, such as myotonic dystrophy, Huntington's disease, or amyotrophic lateral sclerosis (ALS) (when associated with the accumulation of the *C9orf72* mRNA). These diseases lend themselves well to the application of the new RNA-targeting Cas9 enzyme to eliminate toxic RNAs. In a proof-of-principle experiment, the RNA-targeting CRISPR-Cas9 system was used to successfully eradicate repeat-containing RNAs in cells from patients with myotonic dystrophy. Encouragingly, decreasing the amount of toxic RNA also correlated with the correction of some of the validated disease-specific splicing defects [53].

The successes described earlier are examples of what the future holds in the design of new therapies against diseases related to mis-splicing. Nevertheless, several barriers will have to be overcome before these technologies can be used in the clinic. For the CRISPR-Cas9 system specifically, there are potential problems with possible off-target effects that will need to be carefully addressed, because the correction of a specific mutation may lead to other genome-editing events that could have deleterious effects. Additionally, a long discussion about the ethical implications of using this technology is just starting and regulations need to be in order before this emerging technology can be used to treat human diseases.

Concluding Remarks and Future Perspectives

Even though research has taken important steps into understanding how the processes of post-transcriptional regulation are involved in the generation of diseases, there remain many open opportunities (see Outstanding Questions). For example, recent studies have shown that

Outstanding Questions

Will RNA modifications affect the splicing of a subset of RNAs? Does misregulation of these modifications lead to disease and, thus, new therapeutic opportunities?

Is there overlap in chromatin-remodeling drugs and splice-altering therapy? Since transcription and splicing are intricately linked, is it possible to repurpose drugs targeting transcription for RNA-splicing therapy? By contrast, could drugs targeting chromatin also cause unintended deleterious consequences on global RNA-splicing profiles?

As with all therapies, delivery and dosing will be important for therapies targeting the RNA-splicing pathway. How will cellular uptake be optimized? Will it be possible to limit delivery to specific tissues and/or cell types?

Given the success of SSO therapies, can RNA molecules be designed and delivered to make up a 'designer' spliceosome? This is especially appealing for treatment of diseases caused by mutations in core splicing components.

splicing factors in fact act as oncogenes or tumor suppressors in several cancers (Table 1; extensively reviewed in [54]). Consequently, current efforts are focused on developing new cancer therapies that target splice regulatory factors, or even alter their post-translational regulation, as is the case for the SRPK inhibitors that target the kinases that phosphorylate many of the SR proteins [55].

New regulatory methods that impact splicing will also drive the future of therapeutic design. Recent published work indicates that N^6-methyl-adenosine, or m^6A RNA methylation, directly impacts pre-mRNA splicing and may be regulated to impact splicing upon certain types of stress [56]. Although this topic is hotly debated and there is strong evidence that this method of regulation is not broadly applicable to most RNA transcripts, the role of m^6A in RNA metabolism and regulation of stability is broadly accepted [57–59]. Similarly, there have been reports of long noncoding RNAs, **stable intronic sequence (sisRNAs)**, and **small nucleolar RNAs (snoRNAs)** regulating splicing and, in some cases, impacting disease [60–62]. Thus, there is still much to learn about the regulatory intricacies of RNA and its splicing via new modifications, RNA–RNA interactions, and RNA–protein complexes, all paving the way for novel therapeutic targets.

The implementation of new technologies as well as the generation of new knowledge will move us one step closer to the development of new and improved therapies to fight disease. As has been done in DMD, splicing therapy can also be used more broadly to repair a damaged or mutated gene by inducing splicing changes to exclude a bad exon. Conversely, splicing may be harnessed to cause a nonfunctional transcript to be generated for any identified disease-causing gene by deleting an essential exon to induce a deleterious deletions or frameshift. Thus, therapeutics targeting the splicing process may be applicable to a multitude of genetic diseases with gain-of-function mutations or inappropriately expressed proteins, as was accomplished with SSOs targeting the amyloid beta precursor protein as a therapeutic target for AD [63].

Acknowledgments

We apologize that space limitations did not allow us to cite all the important studies that have contributed to this field. We also thank the funding sources for this work: grants from the Elsa U. Pardee Foundation, the Wolfe Foundation, the Sunbeam Foundation, Pelotonia, and the National Institutes of Health NIH CA195324 and NS084187 to D.S.C. as well as NS077984 to B.L.S. (trainee).

Supplemental Information

Supplemental information associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tig.2018.10.002.

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