# Making Use of Aberrant and Nonsense: Aberrant Splicing and Nonsense-Mediated Decay as Targets for Personalized Medicine

Jiuyong Xie*

Departments of Physiology, and of Biochemistry & Medical Genetics

Faculty of Medicine, University of Manitoba

745 Bannatyne Ave.,Winnipeg, MB R3E 0J9, Canada

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*Correspondence: E-mail: xiej@cc.umanitoba.ca, Tel: 1-204 975 7774, Fax: 1-204 789 393

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**ABSTRACT**

It is estimated that one-third of disease-causing mutations may induce aberrant splicing of pre-messenger RNA transcripts and a partially overlapping third to premature stop codons (PTC) and nonsense-mediated mRNA decay (NMD). In some diseases, the estimates even go up to 50% and >70% for aberrant splicing and NMD, respectively. These highly prevalent effects of different mutations on mRNA processing have prompted much effort for the identification of compounds towards the therapy of a substantial number of diseases with mutation-specific, personalized medicine. Here I review the widespread occurrence of aberrant splicing, NMD and their association in human genetic diseases, and discuss the rationales underlying the corresponding therapeutic strategies and challenges.

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The ability to sequence and analyze the human genome and transcriptomes of various sources at speeds unimaginable more than 20 years ago has had huge impacts on not only basic biological research but also the development of novel therapeutic strategies for human genetic diseases. Particularly this ability allows the screening of mutations in individuals at a genome/transcriptome scale for the design of different therapeutic strategies for mutation-specific, personalized medicine based on how the mutations take their toll.

For the genetic information-based disease therapy, RNA has also been targeted, besides DNA and protein, with an accelerating speed of research in recent years. This development has mainly benefited from our understanding of different aspects of RNA processing and appreciation of their prevalence, such as the widespread presence of alternative pre-mRNA splicing in human transcriptomes[1, 2], mRNA quality control by nonsense-mediated decay (NMD)[3], as well as microRNA, long noncoding RNA and other non-coding RNAs[4]. Their misregulation due to mutations has been linked to or associated with the development of human genetic diseases. Here I will discuss the therapeutic potential of targeting aberrant splicing and NMD, two widespread and related effects of a large number of genetic mutations that cause human diseases.

## 1. WIDESPREAD OCCURRENCE OF ABERRANT SPLICING OF MUTANT GENES THAT CAUSE HUMAN GENETIC DISEASES:

Pre-messenger RNA splicing is an essential step in the expression of most eukaryotic genes where the expressed regions (exons) are precisely joined together and the intervening sequences (introns) removed to generate mature mRNA transcripts for proteins.

Splicing occurs in the spliceosome, where five small nuclear ribonucleoproteins (snRNPs U1, U2, U4, U5 and U6) and approximately 170 spliceosome-associated factors cooperate to accurately recognize the splice sites at the intron-exon boundaries and catalyze the splicing reaction in a sequential order[5, 6]. Particularly important for recognition are the intronic 5' GT in a consensus of AG|GTRAGT and 3' splice site (branch point consensus CTRAY, polypyrimidine tract and 3' AG in a consensus of NYAG|G) [7]. There is also a small percentage of introns that mostly use AT/AC at their 5' and 3' splice sites, respectively and the corresponding minor spliceosome is comprised of U11, U12, U4atac, U5 and U6atac snRNPs [8-10].

While as some exons tend to be always included (constitutive exons), others can be selectively used in a spatially or temporally dependent way (alternative exons). Alternative splicing occurs in about 95% of human multi-exon protein-coding genes[1, 2], with one-third of the resulting variant transcripts predicted to be degraded through nonsense-mediated decay[11]. Particularly the coupled alternative splicing-NMD controls the homeostasis of a group of splicing regulatory factors[12-19].

The inclusion level of an alternative exon is generally dependent on the balance of the positive and negative effect of *cis*-acting regulatory elements (i.e. exonic splicing enhancers/silencers and intronic splicing enhancers/silencers), *trans*-acting factors and transcriptional/chromatin control in mammalian cells[20] (Fig. 1). Moreover, usage of a splice site can be competed by its nearby sites[21, 22].

Not surprisingly, disruption of either the *cis*-acting elements or *trans*-acting factors can alter constitutive or alternative splicing, causing aberrant splicing and human genetic diseases[23]. The effect of aberrant splicing on mRNA include the aberrant inclusion or exclusion of whole or

partial exonic/intronic sequences (Fig. 2), due to mutations of splice sites, regulatory elements, core spliceosomal snRNA or factors, regulatory splicing factors or microsatellite repeat RNA-induced loss/gain-of-function of splicing factors. For mechanistic details of these splicing defects, readers can find them in recent reviews[24, 25].

The first splice mutation was discovered in the beta-globin gene of a thalassemia patient in 1981[26]. In 1992, 15% (101) of all point mutations causing human diseases (659 in total) were suggested to result in splicing defects[27]. As experimental studies of mutation effects on splicing accumulated, the number increased significantly, up to about 50% (32/62) for the Mutated-in-Ataxia-telangiectasia (*ATM*) gene [28], as well as the Neurofibromatosis type I (*NF1*) gene[29]. The higher occurrence is contributed by point mutations outside of splice sites that cause splicing defects, particularly those in the coding regions that used to be considered only for amino acid changes (missense, nonsense or silent mutations)[30]. Recent computational and biochemical studies estimate that in general about 25% of exonic disease-causing mutations result in splicing defects[31, 32]. Moreover, for most genetic disease genes, intronic mutations outside of splice sites have not been thoroughly examined. Taken together, the conservative estimate is that about one-third of disease-causing mutations may result in aberrant splicing[32].

## 2. WIDESPREAD OCCURRENCE OF NMD OF mRNA TRANSCRIPTS OF MUTANT GENES THAT CAUSE HUMAN GENETIC DISEASES.

NMD is the degradation of mRNA transcripts that harbour premature termination codons[33] (PTC, Fig. 3), which are more than 50-55nt upstream of the last exon-exon junction[34]. The presence of PTC induces the assembly of NDM factors hUPF1-3 and SMG5-7 together with

others onto the exon-exon junction complex (EJC) that deposits at about 20-24nt upstream of the junction during splicing[35-38]. The spliced PTC-containing mRNA transcripts are marked by the NMD factors and recognized for degradation by the endonuclease SMG6 [38].

In cells, NMD plays important roles in mRNA quality control to eliminate transcripts with PTCs or maintain the homeostatic levels of transcripts. Particularly as mentioned above, it keeps the homoestatic levels of a group of splicing regulatory factors through alternative splicing-coupled NMD[12-19]. For example, the control of the switch between the polypyrimidine tract binding proteins 1 and 2 through NMD plays a critical role in neuronal differentiation[17, 18].

In genetic diseases, it has been estimated that a third of disease-causing point mutations lead to NMD of the disease gene transcripts[39]. These mutations either create a PTC directly, or indirectly by frameshift through insertion/deletion or disruption of the splicing of one or more exons/introns (Fig. 3). In the *ATM* gene of AT patients, the percentage of protein truncation mutations in the first 65 of the 66 exons, which supposedly contain PTC and result in NMD, is 72% (56 of 78 unique mutations) [28]. In the *NF1* gene of NF1 patients, the percentages are also close to or more than 70% (14 of 21; 10 of 14; 24 of 29) in three studies [40,41, 42]. Moreover, in an exhaustive analysis, 54 of the 62 mutations (87%) of the *NF1* gene cause protein truncation within the first 42 of the 46 exons examined[43], due to nonsense, missense, insertion, deletion or splice mutations. Overall, these four independent *NF1* studies point to an average of 77% (±8.3) mutations that cause NMD. Thus, the occurrence of NMD is likely far more prevalent than just a third of mutations in at least some diseases.

For aberrant splicing-caused NMD, it could be due to mutations of either the *cis*-acting elements or *trans*-acting splice factors. Particularly the latter could result in the NMD of a group

of transcripts. For example, mutation of the minor spliceosome snRNA *U4ATAC* gene causes the retention of a set of AT/AC introns and decreased mRNA levels in patients with microcephalic osteodysplastic primordial dwarfism type 1 (MOPD 1, or called Taybi-Linder syndrome, TALS) [44, 45].

PTCs that cause NMD may also result in aberrant splicing[46], likely by disrupting or forming splicing regulatory elements with its flanking nucleotides[32]. In a Marfan syndrome patient, a PTC caused not only NMD but also skipping of exon 51 due to the disruption of a SRSF2 (SC35)-dependent splice enhancer[47]. Knocking down the NMD factor Upf2 altered the alternative splicing of about 30% of expressed genes in mouse liver or macrophages suggesting that the NMD factor also controls the alterantive usage of exons in cells[48].

Taken together, the high prevalence of aberrant splicing and NMD among disease gene transcripts and their association with each other provide therapeutic targets for a large number of genetic diseases in a mutation-specific, personalized way.

## 3. POTENTIAL THERAPEUTIC STRATEGIES TARGETING ABERRANT SPLICING OR NMD

For the correction of aberrant splicing or NMD, mainly two categories of compounds have been used: oligonucleotide-based and small molecules (Fig. 4). Each has its advantages and disadvantages.

**Oligonucleotide-Based Compounds Targeting Aberrant Splicing.** Antisense oligonucleotides (ASO) bind the complementary target RNA sequences with relatively high specificity, thereby giving hope for mutation/gene-specific therapy at different steps of mRNA processing. For enhanced stability and/or affinity of oligos in cells or *in vivo*, various

modifications have been made to ASOs, for example, morpholino and 2-O-Methyl RNA oligos for nuclease resistance and specificity [49], and oligos containing locked nucleic acids (LNA) with a methylene bridge between the 2' oxygen and 4' carbon atoms of the sugar ring for high affinity as well as nuclease resistance[50].

ASOs have been used to directly mask the splicing regulatory elements or cryptic splice sites, or conjugated with a chimeric peptide or tagged with a binding motif of a splicing regulatory factor to correct aberrant splicing[51-53] (Fig. 4A). Some of them have been used in animal studies demonstrating promising results. For example, 2-OME or morpholino oligos targeting the intronic ISS-N1 silencer of the *SMN2* gene corrected the aberrant splicing of exon 7 in SMA mice [54, 55].

Besides ASOs, siRNA has been used to knockdown the deleterious microsatellite repeat transcripts that cause aberrant splicing in muscular dystrophy [56].

Although the effects with ASO or siRNA are promising, their efficient delivery into cells/tissues, particularly of the nervous system of patients, had been probably one of the biggest concerns. In this regard, adenovirus-mediated ASO expression[57], or direct injection/infusion of the therapeutic ASOs[58], have appeared to be sufficient to achieve certain levels of therapy. However, it will have to wait for clinical trial results to see the effectiveness and specificity in patients.

**Small Molecules.** Small molecules are another category of compounds that have been screened for their cell/tissue permeability, specificity and efficiency on aberrant splicing or NMD of disease transcripts.

*Small molecules targeting aberrant splicing.* Alternative splicing can be controlled by physiological or synthetic external factors, which act through intracellular signaling pathways or directly bind splicing factors or *cis*-acting RNA structures[59-61]. A list of small molecules with therapeutic potentials can be found in a recent review on "Alternative splicing interference by xenobiotics"[62]. Here I will discuss several representative ones targeting *trans*-acting factors, pathogenic RNA or *cis*-acting elements/structures (Fig. 4A).

Chromatin remodelling compounds such as sodium butyrate and valproic acid (VPA) inhibit deacetylases, which may act on histones as well as splicing factors[63-65]. Sodium butyrate enhanced the inclusion of the *SMN2* exon 7, increased the SMN protein and ameliorated the SMA phenotype in a mouse model of SMA[66]. VPA, likely by increasing the splicing activator TRA2-β1, also increased the SMN protein level in fibroblast cells from SMA patients[67]. However, clinical trials by two groups with VPA failed to see an increase of SMN protein or improved strength or function of SMA children [68-70]. One of the complications in patients could be the low (~15%) efficiency of VPA to cross the blood brain barrier[71], or its side effects on the electrical excitability of motor neurons or gain of weight [68, 69, 72]. Thus, more studies are needed before successful application of the therapeutic compounds to patients.

Digoxin is a cardiotonic steroid that has been in clinical use for decades. In a screen of 1,440 compounds of drugs, enzyme inhibitors and ion channel antagonists for splicing regulators using a tau exon 10 splicing reporter, digoxin and a number of other drugs were

found to enhance or inhibit exon inclusion [73]. Digoxin also regulates the splicing of HIV transcripts[74]. Its closely related form digitoxin down-regulates SRSF3 (SRp20) [75, 76], as well as TRA2β [76] . Interestingly, the suppressive effect of digoxin on SRSF3 increases the exon 20-containing wild type transcripts of the *IkappaB kinase complex-associated protein* (*IKAP*) gene in cells from familial dysautonomia (FD) patients [75]. If its delivery efficiency through the blood brain barrier (BBB) could be enhanced[77], digoxin/digitoxin could bring hope for the therapy of the prevailing mutant alleles (more than 98%) of this debilitating disease [33].

Spliceostatin A is a natural anti-cancer compound isolated from *Pseudomonas*[78]. It inhibits splicing by binding to the SF3B subcomplex of the U2 snRNP [79]. One component SF3b1 and other factors of the 3' splice site are mutated in myelodysplasia [80, 81]. Recently a screen from another bacterial strain *B. thailandensis* MSMB43 isolated compounds named Thailanstatins A, B and C with similar inhibitory activities on splicing and cell proliferation but with greater solution stability[66].

Other small compounds that inhibit *trans*-acting splicing factors include the benzothiazole-4,7-dione BN82685, which blocks the second *trans*-esterification reaction preventing the release of intron lariat and ligation of exons[82], and the biflavonoid isoginkgetin, which prevents the stable recruitment of the tri-snRNP U4/U5/U6 and accumulation of complex A [83].

Besides *trans*-acting factors, *cis*-acting RNA elements/structures could also be targets of small compounds like that in riboswitch. For example, the telomerase inhibitor telomestatin binds G quadruples[84-86], which are formed by multiples of G tracts that are known to control splicing[87-91]. Another chemical LDN-13978 (mitoxantrone) was identified from a library of

110,000 compounds and stabilized the secondary structure around the junction of the tau exon 10/intron 10, giving hope to reduce the exon usage in FTD-17 dementia patients [92-94].

The effect of spliceostatin A and these other small compounds on aberrant splicing and their specificity remains to be tested for genetic disease transcripts in cells or *in vivo*.

There are also compounds binding the hairpin structures of the pathogenic triplet CUG repeats of *DMPK1* transcripts of muscular dystrophy (DM1) [95, 96], one of a group of microsatellite expansion disorders[97]. Particularly interesting is that pentamidine, a drug for pneumonia and other infectious diseases, partially rescued the aberrant splicing of the *Serca1* and *Clc-1* transcripts in a mouse model of DM1 [98].

*Small molecules targeting NMD*. In experimental systems, NMD can be inhibited by global inhibitors of translation elongation such as cycloheximide, puromycin or emetine, which means that they also inhibit the protein levels of disease genes. Therefore, more specific compounds targeting NMD have been sought after for disease therapy (Fig. 4B).

One of the prominent small compounds for the supression of PTC is PTC124 (brand-named Atluren), which was identified from more than ~800,000 small compounds for its effective readthrough of the premature stop codons (most effectively TGA), but not the normal termination codons [99]. PTC124 was used to suppress the PTC of the *DMD* transcripts caused by a PTC UAA in the exon 23 of the *Dmd* gene in the *mdx* mice or a PTC UGA in the exon 28 of the *DMD* gene in a DMD patient[99]. In both cases, the dystrophin protein was significantly increased in myotubes, and in *mdx* mice muscle strength was enhanced [99]. Although the clinical trial result for PTC124 in DMD patients did not bring significant improvements for patients [100], its

clinical trial for cystic fibrosis has shown increased chloride transport of the CFTR protein and improved pulmonary function with generally good tolerability [101, 102].

The molecular basis of PTC124 suppression of PTC remains unclear. However, since it does not change the NMD-reduced mRNA level[99], combining its effect with other compounds that inhibit NMD to increase the transcript level would significantly enhance its therapeutic efficiency.

Besides readthrough of PTCs, targeting NMD factors has also been attempted, for example, by siRNA knockdown of UPF1 or SMG1 to rescue the collagen VI phenotype in Ullrich's disease[103, 104]. A safe and efficient small compound as an inhibitor of NMD factors remains to be identified.

## 4. THERAPEUTIC STRATEGIES TARGETING BOTH ABERRANT SPLICING AND NMD.

*Correction of aberrant splicing that leads to NMD*. In addition to the therapeutic strategies targeting either aberrant splicing or NMD, one can also target the aberrant splicing that cause NMD to eventually inhibit the NMD of disease transcripts using ASOs or small molecules as discussed above (Fig. 5A). For examples, the *NF1* and *NF2* genes with deep intronic mutations cause PTCs and NMD[105, 106]. Antisense morpholino oligos blocking the cryptic splice sites increased the wild type transcripts and the NF1 protein in primary fibroblasts from patients[106]. A similar effect was also demonstrated by the same group for the *NF2* mutations[105]. It'll be interesting to see the *in vivo* effect in animals or patients in the future.

*Inhibition of NMD to increase the splicing regulators that are subject to the homeostatic control by NMD.* Besides the inhibition of NMD through the control of splicing, the regulation of

a group of alternative splicing factors by NMD, as mentioned above, is also a promising target for therapy (Fig. 5B). For example, several of the splice variants of SRSF1 in the control of *SMN2* exon 7 are controlled by NMD for its homeostatic maintenance[19]. This NMD is through the splicing of a normally retained intron within the 3' UTR creating an additional last EJC[19], thus converting the upstream normal stop codon in the major transcript to a PTC in the variant transcript. At least two of the NMD-targeted variants still produce full-length SRSF1 proteins. Application of small compounds that inhibit NMD to increase the SRSF1 variant mRNA and protein level could potentially enhance the *SMN2* exon 7 for SMA therapy (Feng and Xie, unpublished data). For this purpose, the PTC readthrough compound PTC124 or protein synthesis inhibitors do not fit; instead, novel compounds inhibiting the NMD factors UPF1-3 or SMG5-6 without inhibiting protein synthesis need to be identified.

Besides SRSF1, the many other splicing factors that are controlled by NMD could also be enhanced through inhibiting NMD factors, thereby exploited for the correction of aberrant splicing. Since they act in *trans* on splicing, increasing their protein level by safe compounds that inhibit the NMD factors but not protein synthesis will have even wider impact on the correction of aberrant splicing of a large number of disease genes.

## 5. CONCLUDING REMARKS

The highly prevalent occurrences of aberrant splicing and nonsense-mediated decay among the mutant transcripts of human genetic disease genes make them prominent therapeutic targets for mutation-specific, personalized medicine for a large number of patients. Recent advances in the development of therapeutic compounds have shown promising results in

patient cells, animal models of diseases or even some clinical trials. Improved compounds for higher delivery/treatment efficiency and specificity but less side effect and toxicity will bring hope for the personalized therapy of genetic diseases whose mutations are linked to the misregulated steps of RNA processing.

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**Fig. 1. Factors determining the inclusion of an exon.** Shown is part of a transcript containing three exons (blocks) and two introns (horizontal lines). The positive (Green) or negative (Red) effects of *trans*-acting (ovals) factors binding to the respective *cis*-acting pre-mRNA elements on the 3' or 5' splice sites (SS) or splicing pathways are indicated with curves. Competition between the two nearby sites (3' SS here) are indicated with a brown dashed line between the two alternative splicing pathways.

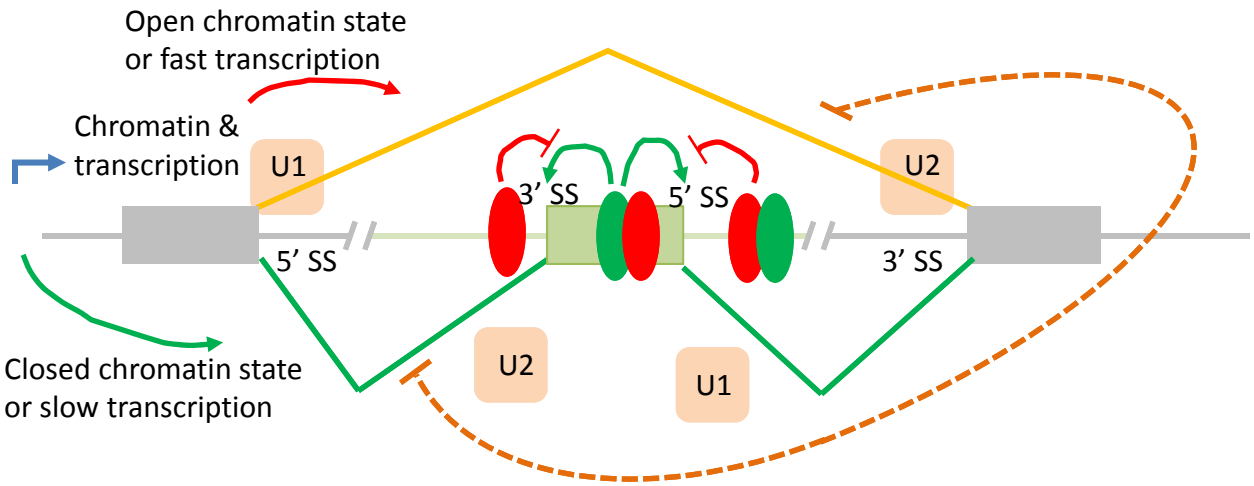
**Fig. 2. Different effects of aberrant splicing on the usage of pre-mRNA sequences due to *cis*- or *trans*-acting mutations.** The normal splicing pathways are in grey and aberrant in red. **A**: branch point. The effects of mutations include aberrant inclusion/exclusion of the whole or part of an exon or intron as shown. The effects of aberrant splicing on mRNA transcripts or proteins are listed in the text box to the right.

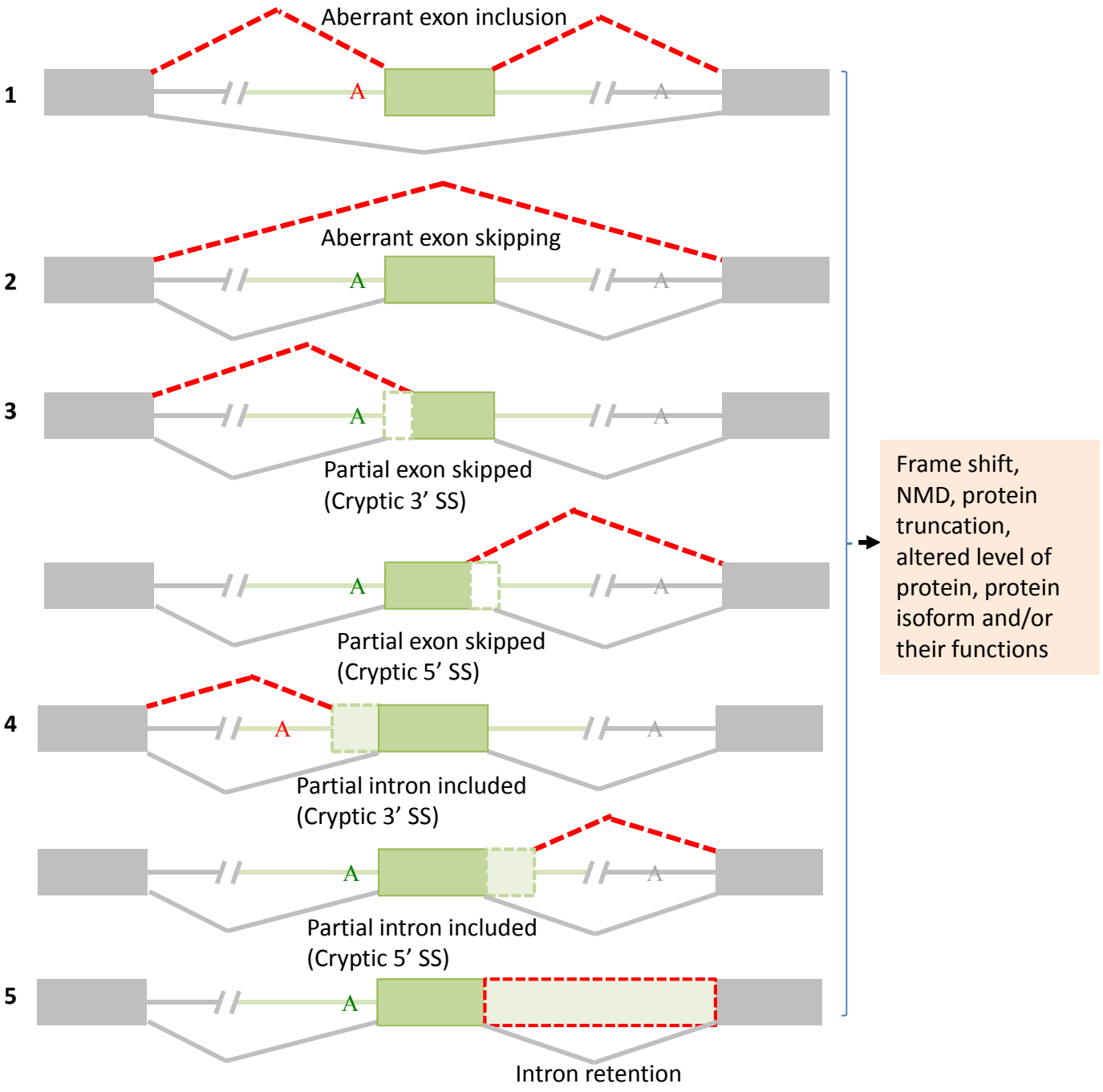
**Fig. 3. Causes of nonsense-mediated decay of mRNA transcripts. A.** NMD by the creation of a PTC (Red STOP) by a nonsense mutation (X) in the exon >55nt upstream of the last exon-exon junction (EJC), or a non-3n insertion/deletion mutation (Red triangles). The normal STOP codon is in yellow. The PTC leads to the marking of the transcript by NMD factors UPF1-3, SMG5-7 and others at the EJC upon splicing and subsequent degradation by SMG6. **B.** NMD caused by aberrant splicing. Aberrant splicing leads to skipping of partial or whole exons or inclusion of intron sequences resulting in frameshift and PTC/NMD/mRNA degradation.

**Fig. 4. Potential therapeutic strategies targeting aberrant splicing or NMD. A.** Strategies against aberrant splicing using ASO (1) or small molecules (2). For an ASO, it could target a regulatory element (here a silencer in red) or tagged with a chimeric peptide or binding site of a regulatory factor as indicated as a small green block (1). It could also block a cryptic splice site (2) to switch the splicing back to the normal pathway. For small molecules (purple), they could target the chromatin/transcriptional machinery, splicing regulators (ovals) or a secondary structure (riboswitch) to correct aberrant splicing (here to restore the inclusion of the middle exon, pathway in green). **B.** Strategies against NMD, by compounds that promote the read-through of the PTC, like PTC124 (1), or inhibition of the NMD factors (2), thereby increasing the level of the disease transcript.

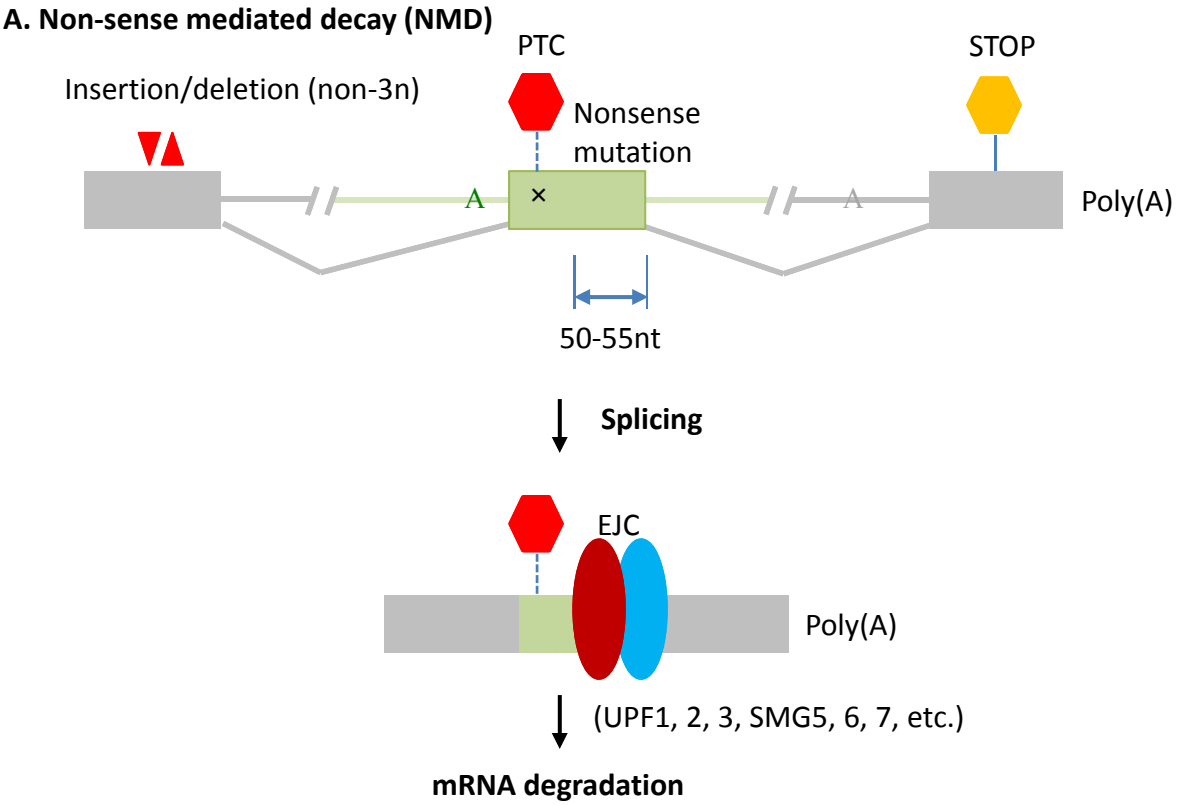
**Fig. 5. Potential therapeutic strategies targeting both aberrant splicing and NMD. A.** A strategy targeting aberrant splicing and to inhibit its resulting NMD. Shown here is inclusion of an intron fragment as an aberrant exon (Red) due to a mutation that created cryptic splice sites. An ASO complementary to the cryptic 5' SS blocks this site leading to switching of splicing to the normal pathway (green), thereby to a normal STOP codon (Yellow), eliminating the PTC (Red). See text and the reference for the *NF1* gene as an example. **B.** A strategy targeting NMD factors and NMD of a splicing regulator to correct aberrant splicing. Here the *SRSF1* gene and its target *SMN2* exon 7 are used as an example. The SRSF1 transcript has a normal STOP codon (Yellow) in the 3'

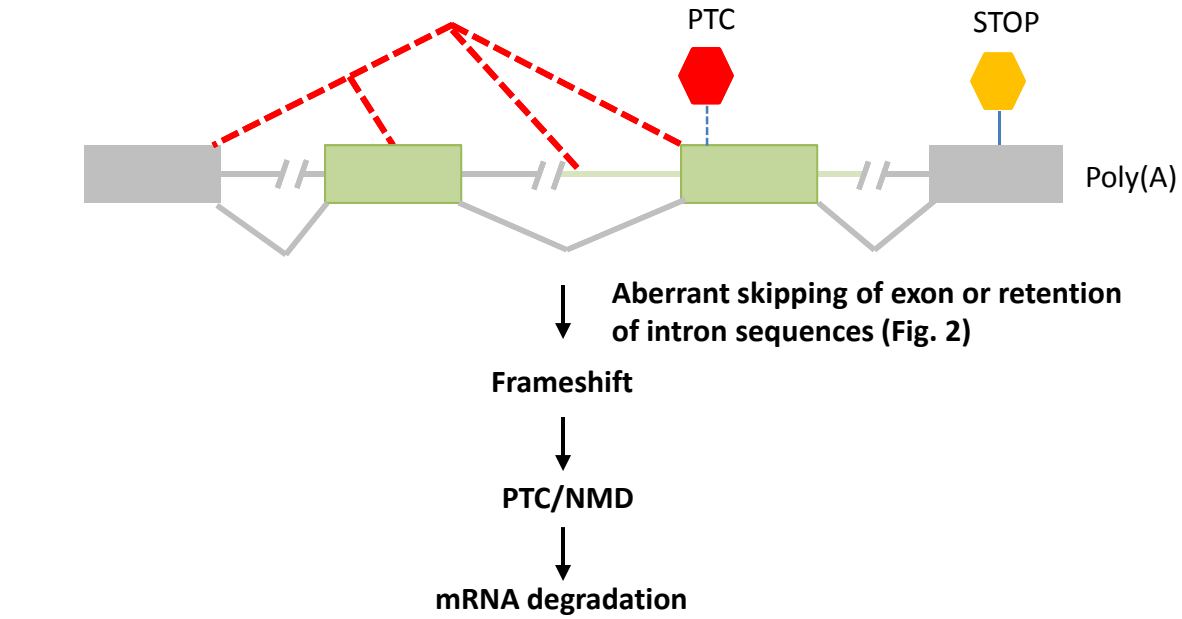
UTR. Upon splicing of an intron (purple) inside the 3’ UTR, the normal stop codon becomes upstream of an EJC and converted to be a PTC (Red outline of the STOP). Compounds that inhibit the NMD factors but not translation (Pink box) will stabilize the SRSF1 NMD-targeted variant transcripts and thereby increasing the SRSF1 protein (green oval) level in cells. Consequently the *SMN2* exon 7 could be included at a higher level to produce the full length SMN protein.


Open chromatin state
or fast transcription
Chromatin &
transcription
U1
5’ SS
3’ SS
5’ SS
U2
3’ SS
Closed chromatin state
or slow transcription
U2
U1




About one-third disease-causing mutations may result in aberrant splicing



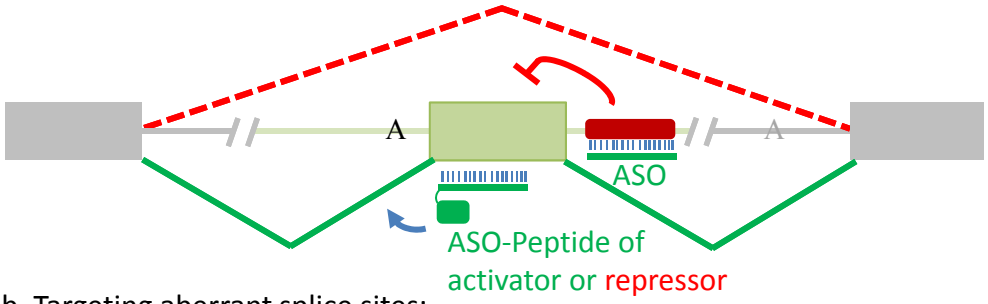




In total (A+B), about 70% PTC/NMD in the *ATM* and *NF1* mutant transcripts examined

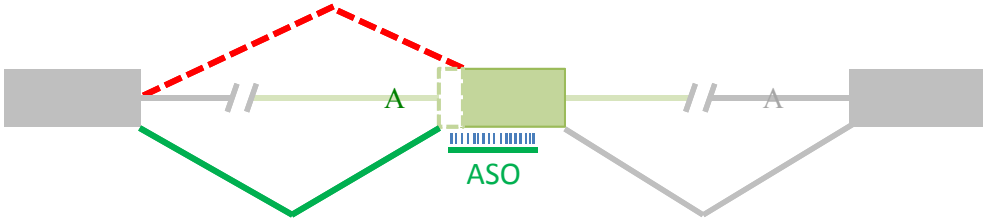
**A. Potential therapeutic strategies targeting aberrant splicing:**

**1. ASO targeting cis-acting sequences:**

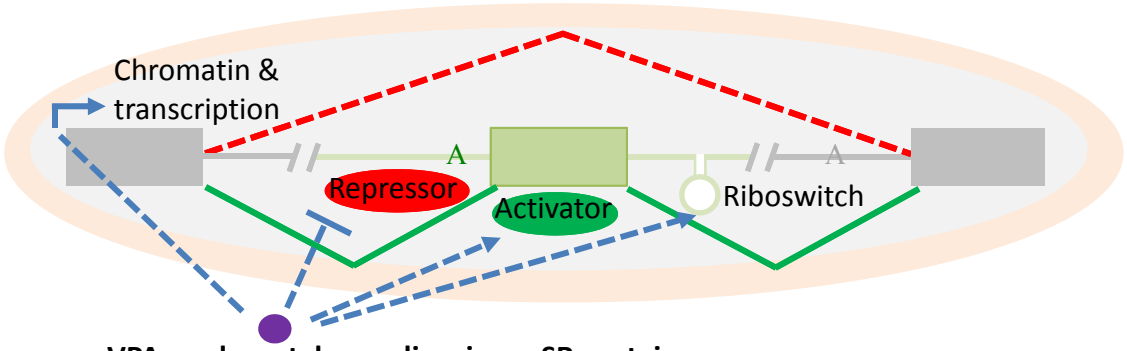
a. Targeting splicing regulatory elements:




b. Targeting aberrant splice sites:

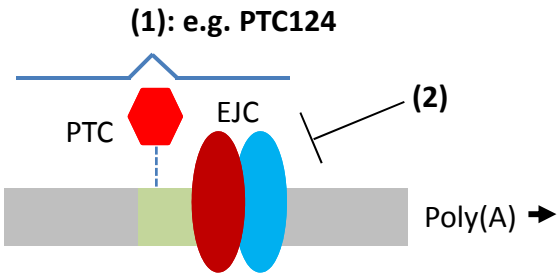



**2. Small molecules:**




**e.g. VPA on deacetylases, digoxin on SR proteins**

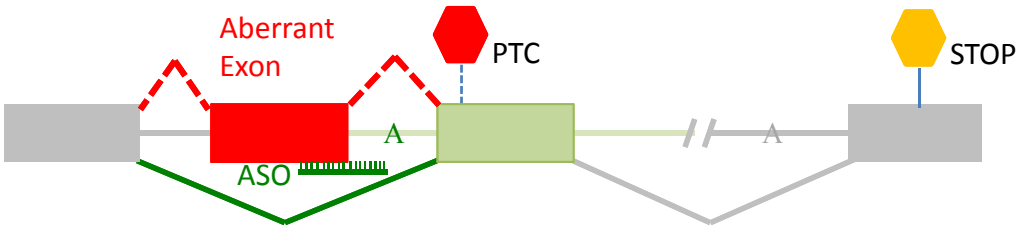
**B. Potential therapeutic strategies targeting NMD:**




1. **Readthrough of PTC**
2. **Inhibition of NMD effectors**

**Increased protein level, e.g. in the correction of DMD in *mdx* mouse or Cystic Fibrosis in clinical trial of CFTR-mutated patients by PTC124.**

**A. Potential therapeutic strategies targeting aberrant splicing to inhibit NMD:**




**B. Potential therapeutic strategies targeting NMD to correct aberrant splicing:**