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Review

To splice or not to splice: pseudoexons in neurological disease and opportunities for intervention

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Accurate exon selection and processing of pre-messenger RNA are crucial for normal gene expression. Mutations that alter splicing disrupt pre-mRNA processing and can have diverse effects on transcript structure, making the consequences of many such mutations difficult to predict. While next-generation sequencing technologies have transformed genetic diagnosis for many patients, deep intronic variants generally evade detection and characterisation. Of all the known types of splicing mutations, the most elusive to predict are those that activate pseudoexons. Because transcripts that contain pseudoexons are otherwise generally intact, exclusion (or 'skipping') of the pseudoexon during processing of the pre-mRNA is likely to generate a normal, functional mRNA. Characterisation of pseudoexon mutations will open opportunities for the development of antisense oligonucleotide strategies to overcome these disease-causing mutations.

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Introduction

The search for causative mutations in human genetic disorders has largely focused on mutations that impact coding regions [1]. Exome analysis can identify obvious candidates — insertions, deletions, splice site mutations, nonsense, and other protein-truncating mutations [2] — but will generally provide an answer for fewer than 60% of patients. Up to 30% of disease-associated genetic variants affect *cis*-acting splicing motifs [3] and may cause aberrant splice site selection or alter exon recognition, summarised in Table 1. The impact of these mutations on splicing is context dependent and influenced by the strength of exon definition [4] and may generate multiple splice variants, further complicating predictions of pathogenicity. Mutations that activate pseudoexons are especially challenging to predict, particularly those that do not create splice sites within the intron, but instead alter splicing regulatory motifs (reviewed in Ref. [5]).

Transcripts carrying pseudoexons generally retain intact, functional coding sequences; therefore, splice-modulating antisense oligonucleotides (ASOs) designed to exclude pseudoexons from the mature transcript are expected to generate a normal, functional mRNA (Figure 1a,b). Accurate characterisation of these deep intronic mutations will therefore lead to opportunities for targeted therapies [6].

Pre-mRNA splicing, splice motif mutations, and disease

Most human genes contain introns, tracts of sequence that intervene the exons. Transcription of intron-bearing genes yields nascent pre-messenger RNA; the introns are removed and through a process known as splicing, the flanking exons are precisely ligated by a multimolecular assembly called the spliceosome. However, not all adjacent exons are spliced in every transcript. Alternative splicing, whereby specific exons or splice sites are differentially selected, produces multiple transcript variants (reviewed in Ref. [7]) and is an essential mechanism that increases the complexity of gene expression and gene regulation. In eukaryotic cells, splicing and other pre-mRNA maturation events are closely linked to transcript elongation; evidence is emerging that splicing of individual introns is extremely plastic and that this functional coupling may exert widespread effects on splicing (reviewed in Ref. [8]).

Splicing at sites other than canonical exon boundaries is referred to as 'cryptic' splicing (reviewed in Ref. [9]). Cryptic splicing is observed in transcripts of many genes and may occur as low-frequency events of unknown

Table 1

Noncanonical exons implicated in genetic disease NMD.

Feature	Location	Characteristics	Mechanism	Outcome	Consequence
Pseudoexon	Usually deep intronic	Latent sequence resembling an exon, activated by a mutation that strengthens a splice site or enhances exon selection	'Exonized' or activated by the mutation	Pseudoexon inclusion in the transcript	Inclusion of aberrant peptide sequence and/or frameshift and premature termination of translation
Cryptic exon	Usually near canonical splice sites	Activated when mutations disrupt normal splicing	Spliceosome selects alternative (cryptic) splice site	Inclusion of intronic sequence in the transcript	Inclusion of aberrant peptide sequence and/or frameshift and premature termination of translation
Poison exon (NMD exon)	Intron	Alternatively spliced exon carrying a premature termination codon	NMD	Transcript downregulation	Endogenous mechanism of transcript regulation; dysregulated poison exon selection can cause disease
Recursive splice sites	Deep intron (large introns)	Specialised, noncanonical splicing	Large introns are removed in segments through multiple splicing reactions	Recursively spliced sequences are normally transient	Mutations or deletions affecting recursive splice sites can lead to aberrant gene expression and genetic disease

NMD, nonsense mediated decay

function, as a regulatory mechanism [6], or as the consequence of mutation [3] (reviewed in Ref. [5]).

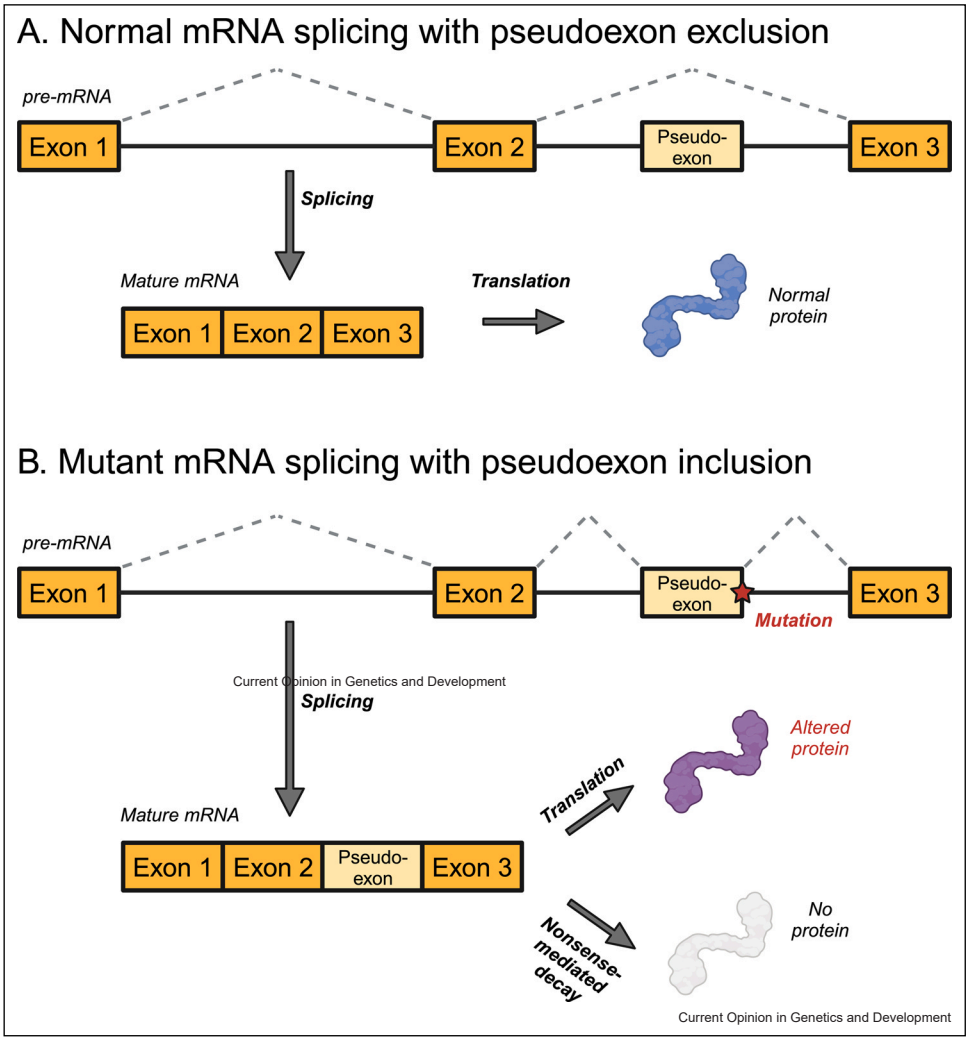
Exon selection and pre-mRNA splicing are highly dependent on *cis*-elements, present throughout the primary transcript sequence, that are recognised and bound by splicing factors, including splicing enhancers and silencers. Consequently, mutations that modify or create a splicing motif (categorised by Wimmer *et al.* [10]) can affect pre-mRNA processing and transcript structure in complex ways and, in some cases, may create a noncanonical exon or cause an existing one to become predominant in the processed transcripts (reviewed in Refs. [9,11]). We refer to a noncanonical exon in a gene as a 'pseudoexon' if its inclusion in transcripts is largely due to mutations in that gene (Figure 1 a,b), and otherwise as a 'cryptic exon', or a 'poison exon' (Figure 1c,d) if it appears to serve a regulatory role (reviewed in Ref. [12]; Table 1). If the reading frame of the gene is altered by inclusion of the noncanonical exon, the functional consequence is usually reduced gene expression due to the introduction of a premature termination codon, leading to degradation of the transcript through the nonsense-mediated decay (NMD) pathway.

Pseudoexons are commonly activated by deep intronic variants of a single nucleotide [10,13–18]. Why evolution tolerates intronic tracts that are one nucleotide away from possessing all elements required for exon definition, despite their potential impact on splicing efficiency remains unclear. Possible answers may lie in transcript regulatory mechanisms such as poison exons that abolish unneeded transcripts by introducing premature termination codons and trigger NMD, or recursive splicing [19]; a conserved mechanism that supports splicing of large introns by stepwise removal of the intron in segments [20] [21]. Recursive splicing likely evolved to more efficiently process very long introns/large transcripts; a proposed mechanism uses intronic 'ratchet points' [22] resembling acceptor site motifs that are spliced successively to remove the entire intron. Single-nucleotide variants that strengthen splice motifs (in particular a *potential* donor splice site or exon splicing enhancer) or weaken intronic silencer motifs within the recursive splicing region can result in selection of the sequence as a pseudoexon in the maturing mRNA [21] (for review, see Ref. [5]).

Pseudoexons and pathogenesis

Pseudoexon activation is challenging to predict and an underreported disease mechanism [23] in part due to the different types of mutations that can enhance splice site selection (Figure 2) [24]. Deep intronic variants are often missed by routine genomic characterisation or classified as variants of unknown significance. In addition, pseudoexon-containing transcripts in many cases

Figure 1



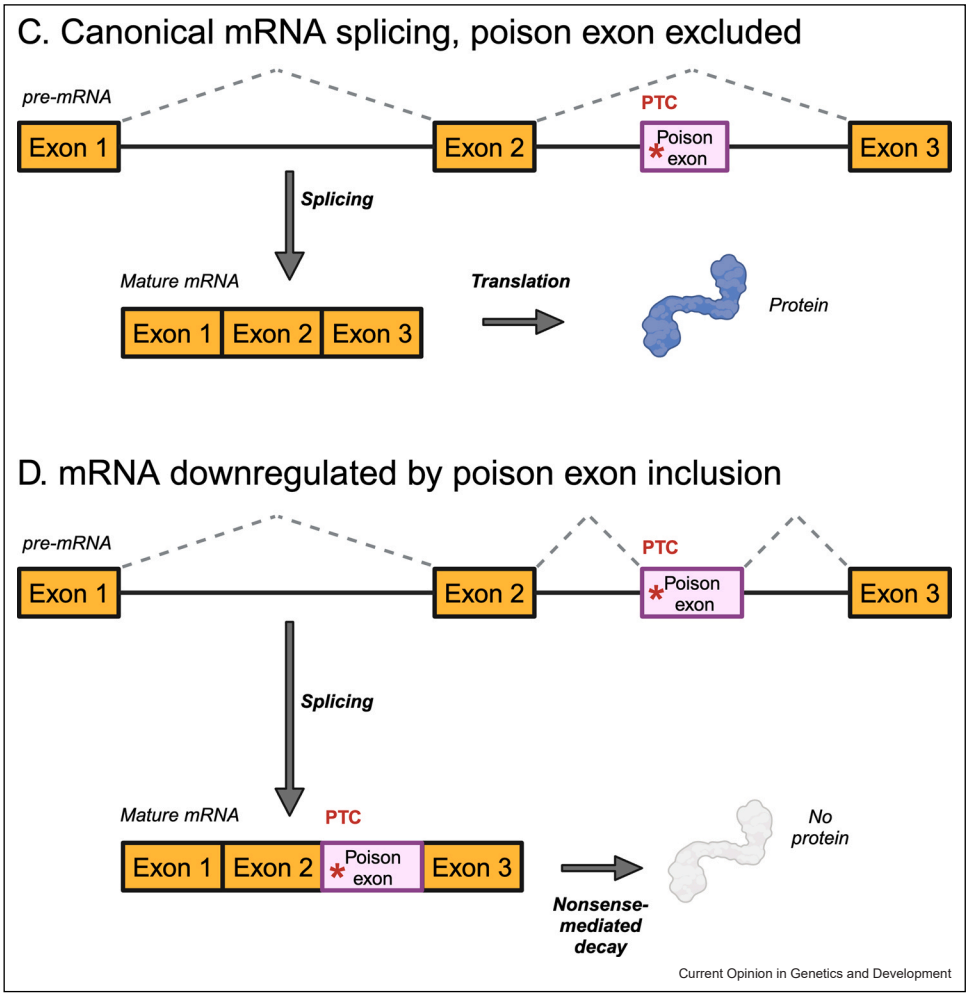
Pseudoexons and poison exons; an overview. **(a)** In this hypothetical three-exon gene, a latent pseudoexon in the second intron is typically excluded from the mature transcript, allowing the canonical exons to be spliced contiguously. **(b)** When a mutation enhances splicing of the pseudoexon (in this case at the donor splice site), inclusion of the pseudoexon in the mature transcripts predominates. **(c)** A poison exon, located between canonical exons 2 and 3, carries and in frame premature termination codon (PTC) but is normally excluded during pre-mRNA splicing. **(d)** When canonical processed transcripts reach a threshold level, the poison exon is selected during splicing, leading to NMD and downregulation of gene expression. Created in BioRender.com.

trigger nonsense-mediated mRNA decay, making them more difficult to detect. Genomic technologies (gene panels, microarrays, and exome sequencing) provide molecular characterisation for a significant proportion (25–35%) of previously undiagnosed diseases [2]; however, others remain unresolved. Paired genome and transcriptome analyses, coupled with novel computational tools [25], use of patient-derived cell models [26] and new insights into the impact of noncoding variations on gene expression are revealing previously unexplored mechanisms of disease [27] (for review, see Ref. [28]). Analysis of genome-wide paired DNA-RNAseq is enabling discovery of deep intronic splice mutation hotspots (SpliPath) [25] and has classified pathogenic and benign variants within deep introns in a neurodegeneration data set [29].

Pseudoexons and opportunities for intervention

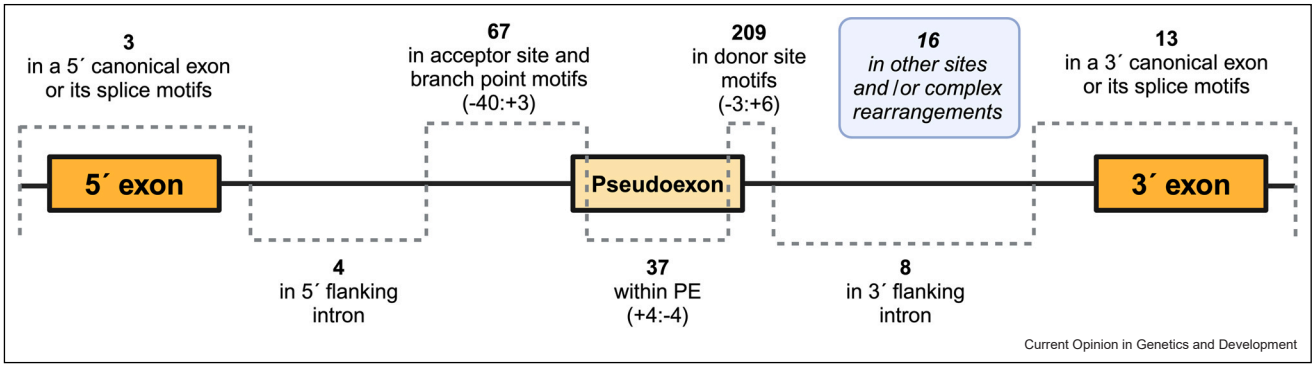
ASOs are short nucleic acid analogues that are designed to target and bind an RNA and modulate gene expression through various mechanisms. Pre-mRNA splicing can be altered by RNA analogue ASOs targeted to splice sites or *cis*-regulatory elements to modulate exon or splice site selection. Despite the potential for splice-modulating interventions to overcome pseudoexon

Figure 1



(*continued*)

Figure 2



Summary of locations of mutations relative to the activated pseudoexons, as reported in Keegan et al., 2022 [20]. Mutations that directly enhance the pseudoexon (PE) donor site are the most frequent, followed by enhancement of the acceptor site/branch point motifs and internal exon splice enhancer motifs. More rarely, mutations in neighbouring introns and exons are sufficient to increase pseudoexon inclusion, presumably due to the loss of distal silencing mechanisms or introduction of distal enhancers. Created in BioRender.com.

mutations, surprisingly few ASO strategies have been developed to address cryptic or pseudoexons associated with neurodevelopmental disorders (Figure 3).

Yamada et al. developed a 2′-O,4′- C-ethylene bridged nucleic acid–modified 24mer 2′-O-methyl ASO to promote skipping of a pathogenic *WDR45* pseudoexon, identified in a female patient with developmental delay and progressive motor impairment [30]. Similarly, a pseudoexon-skipping 2′-O-methyl ASO was developed for a mutation identified in a patient with *PCCA* propionic acidemia, a metabolic disorder with neurodevelopmental consequences. This ASO reduced pseudoexon inclusion and restored the enzyme activity *in vitro* [31]. Although *PCCA* is broadly expressed, studies of propionic acidemia patients who received liver transplants have shown that restoring functional expression in this organ alone is sufficient to halt or resolve most symptoms [32]. This suggests that, if developed into a therapeutic, the ASO may have the additional advantage of not needing to cross the blood–brain barrier (BBB) and being amenable to the enhanced methodology available for delivery of nucleic acid drugs to the liver.

Genomic sequencing and transcript analysis in a patient with tetrahydrobiopterin deficiency, a disorder with usually severe neurological manifestations, identified two novel 6-pyruvoyl-tetrahydropterin synthase (*PTS)* splicing variants [33]. The deep-intronic c.164–672C > T variant creates a potential 5′splice site and inclusion of four overlapping pseudoexons. Two of the identified pseudoexons were also detected at residual levels in healthy cells. The major pseudoexon is near-identical to a disease-associated pseudoexon with the same 3′ and 5′ splice sites, activated in the Factor VIII gene [34]. Splice-switching ASOs excluded the *PTS* pseudoexon and rescued protein expression in cells from the two patients with different pseudoexon-activating variants in the region [33].

In 2023, Shah et al. reported skipping of a pseudoexon in fragile X messenger ribonucleoprotein 1 *(FMR1),* that contributes to Fragile X syndrome pathology in some cases. This pseudoexon is atypical, as it arises due to the CGG trinucleotide repeat expansion in the 5′ UTR of *FMR1* and is a 'terminal' pseudoexon, meaning it induces transcription termination and polyadenylation rather than splicing to downstream canonical exons [35]. An ASO targeting the 5′end of the pseudoexon suppressed splicing and significantly increased levels of FMRP in patient-derived cells. This finding, that blocking splicing of the pseudoexon appears sufficient to prevent recognition of the transcription termination signals it contains, suggests a novel coordination between these two processes.

A strategy to reduce the natural inclusion of cryptic exons that function as 'poison' exons is being pursued by Stoke Therapeutics with their STK-001/Zorevunersen 2′-MOE ASO for the treatment of Dravet syndrome (NCT04740476) [36]. Dravet syndrome is predominantly caused by *SCN1A* haploinsufficiency, with a phenotype of severe epilepsy and neurodevelopmental delay, onset in early infancy and high mortality [37]. STK-001 targets *SCN1A* exon 20N, thereby increasing the levels of *SCN1A* productive transcripts encoded by the wild-type allele, with reduction in seizure frequency in the treated cohorts [38]. The crucial advantage of this approach is that it relies on upregulation of the healthy, functional allele and is therefore mutation agnostic.

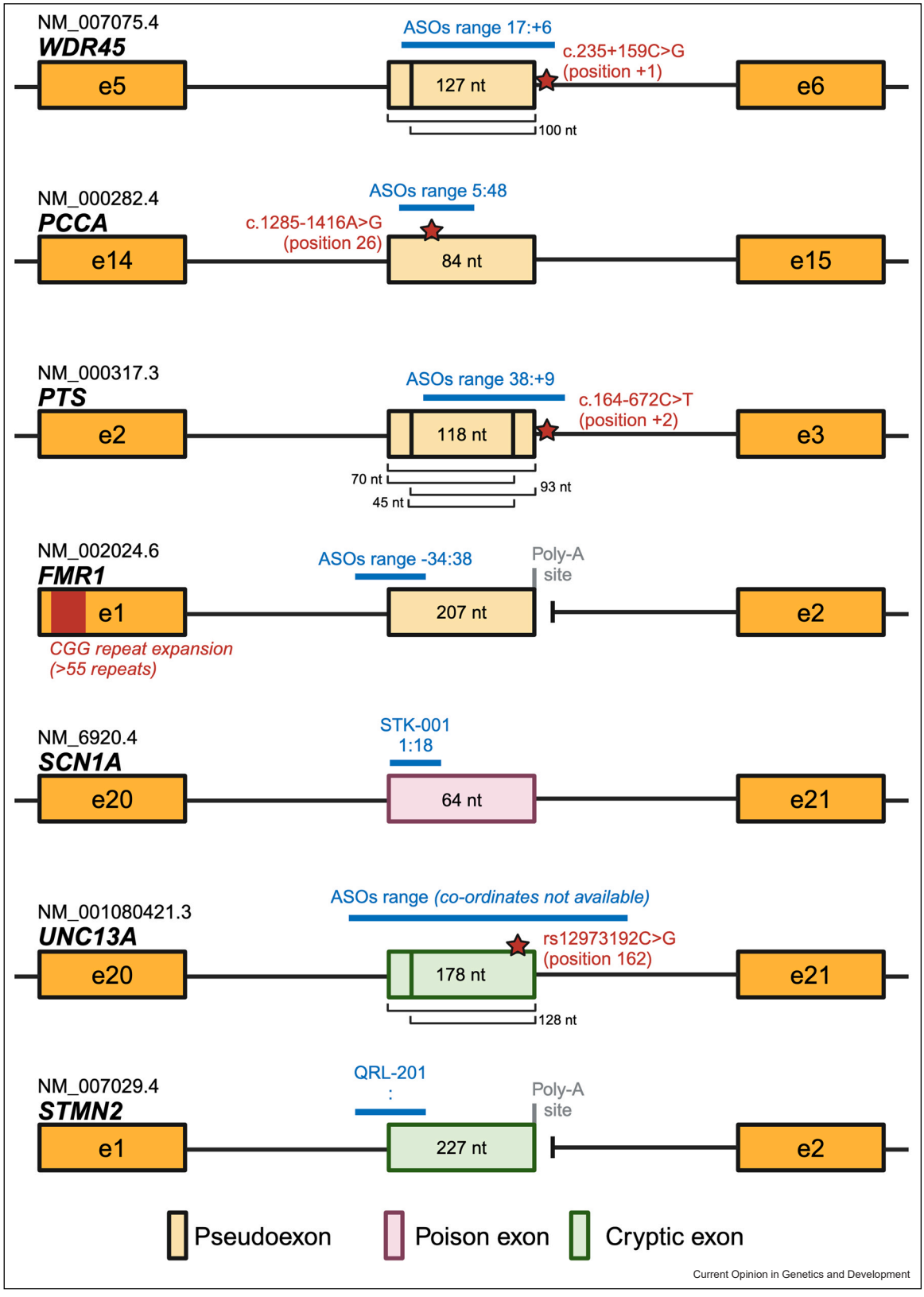
Evidence that cryptic- and pseudo-exons play an important role in the pathology of neurodevelopmental and neurological disorders is increasing. Abnormal mRNAs are a hallmark in neurodegeneration; mutations in *TARDBP*, encoding the splicing factor TDP-43, can result in relaxed splicing stringency and increased inclusion of cryptic exons in transcripts of multiple genes, leading to cell stress and neurodegeneration [39–41]. Splicing of *STMN2,* dysregulated by aberrant TDP-43 expression, is corrected by a cryptic exon-targeting ASO (QRL-201) developed by QurAlis, currently undergoing a phase I clinical trial in amyotrophic lateral sclerosis patients (NCT05633459).

In our 2022 report [24], we discussed how some intronic single-nucleotide polymorphisms (SNPs) confer disease risk through modulating splicing of a nearby cryptic exon. Ma et al. [42] and Brown et al. [43] recently confirmed a novel example of one such SNP (rs12973192) within a cryptic exon in *UNC13A*. Although the risk allele of this SNP alone is not sufficient to cause neurodegeneration, in patients with TDP-43 loss-of-function, it hastens symptom progression [42] and increases accumulation of mis-spliced *UNC13A* transcripts in the brain [43]. ASOs targeting the *UNC13A* cryptic exon rescued UNC13A expression and restored function [44].

Concluding remarks

Pseudoexons as therapeutic targets present unique opportunities and challenges in the field of RNA therapeutics for disorders of the central nervous system (CNS). The brain is characterised by unique gene expression patterns and neuronal-specific alternative splicing events. Long-lived cell types, such as neurons and retinal cells, are especially vulnerable to aberrant gene expression, resulting in neurodevelopmental disorders, neuronal dysfunction, and cumulative effects on cell biology over time. Splice-modifying ASOs are uniquely capable of modulating *only* the target pre-mRNA and for pseudoexon targets, generating a normal, fully functional transcript under endogenous regulation, an outcome not currently possible using small molecule or gene replacement approaches.

Figure 3



(caption on next page)

Seven ASO strategies targeting noncanonical exons (pseudoexon, poison exon, cryptic exon) discussed in this review. In cases where multiple variants of an exon exist, ASO target coordinates are given relative to the largest isoform (*–n* to –1 for nucleotides 5′ of the exon, 1 to *n* for nucleotides within the exon, +1 to +*n* for nucleotides 3′ of the exon). Mutations and SNPs that increase inclusion of a noncanonical exon are shown in red, where applicable. Created in BioRender.com.

Evidence is now emerging of recurrent mutations that activate pseudoexons, warranting the development of splice-correcting therapies that could potentially treat multiple patients. A splice defect in *COL6A1* resulting in inclusion of an in-frame pseudoexon is one of the more frequent mutations causing COL6-related dystrophy; antisense oligomer-mediated exon skipping and CRISPR/Cas9 editing strategies both restored correct splicing of the transcript carrying this +189C > T mutation [45]. Kumar et a. studied two siblings with a severe, progressive neurodevelopmental disorder and identified a deep intronic *TIMMDC1* c.597–1340A > G variant, also reported by others in unrelated children with phenotypic similarities. ASO-mediated splice switching restored *TIMMDC1* transcript and protein expression and mitochondrial function in patient cells [46]. The population frequency of this *TIMMDC1* variant in gnomAD 4.1.0 is 0.0001051, supporting potential application for this ASO strategy.

The BBB presents a significant challenge to the development of nucleic acid therapeutics for CNS disorders. The BBB limits which molecules can cross from the blood to the cerebrospinal fluid and where they can cross. The upper limit of lipid-soluble molecules that can passively diffuse across the BBB is ~600 Da [47] (reviewed in Ref. [48]), which precludes most antisense therapeutics, including the active molecule in Spinraza to treat spinal muscular atrophy, necessitating intrathecal injection as a delivery method. While effective in the case of *Spinraza*, intrathecal delivery brings its own set of challenges and drawbacks, primarily related to the complex and unpredictable ways in which molecules distribute through the CNS from a given entry point [49]. The limited regenerative capacity of CNS neurons [50] also means that any prospective therapeutic must demonstrate very low toxicity, as even minor losses of function may be cumulative and irreversible.

Wider implementation of nucleic acid therapeutics is hindered by inefficient delivery of ASOs to target cells and costs of developing bespoke ASO strategies to address pseudoexons and other private mutations for small cohorts. However, academic, not-for-profit, and special interest groups are pursuing *n*-of-1 studies in rare diseases, whereby a novel therapy may be custom-designed for a single patient or family who has no other treatment options (reviewed in Ref. [51]). Regulatory guidelines specifically for the development of antisense therapies to treat small cohorts and *n*-of-1 patients will remove some of the barriers to advancing ASO drugs for rare diseases [51] and support ongoing development of oligonucleotide chemistries and delivery strategies.

Author contributions

S Fletcher: Conceptualization, Writing – original draft preparation and editing. **IL Pitout**: Writing – review & editing. **NP Keegan**: Conceptualization, Original draft preparation and editing, Figure preparation. **R Mejzini:** Writing – review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

SF is named on intellectual property (licensed to Sarepta Therapeutics) that is unrelated to the topic of this review. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence this review.

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