RNA Biology

Molecular correction of Duchenne muscular dystrophy by splice modulation and gene editing.

--Manuscript Draft--

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Full Title:	Molecular correction of Duchenne muscular dystrophy by splice modulation and gene editing.
Article Type:	Review
Order of Authors:	Britt Hanson
	Matthew J.A. Wood
	Thomas Roberts
Abstract:	Duchenne muscular dystrophy (DMD) is a currently incurable X-linked neuromuscular disorder, characterised by progressive muscle wasting and premature death, typically as a consequence of cardiac failure. DMD-causing mutations in the dystrophin gene are highly diverse, meaning that the development of a universally-applicable therapy to treat all patients is very challenging. The leading therapeutic strategy for DMD is antisense oligonucleotide-mediated splice modulation, whereby one or more specific exons are excluded from the mature dystrophin mRNA in order to correct the translation reading frame. Indeed, three exon skipping oligonucleotides have received FDA approval for use in DMD patients. Second-generation exon skipping drugs (i.e. peptide-antisense oligonucleotide conjugates) exhibit enhanced potency, and also induce dystrophin restoration in the heart. Similarly, multiple additional antisense oligonucleotide drugs targeting various exons are in clinical development in order to treat a greater proportion of DMD patient mutations. Relatively recent advances in the field of genome engineering (specifically, the development of the CRISPR/Cas system) have provided multiple promising therapeutic approaches for the RNA-directed genetic correction of DMD, including exon excision, exon reframing via the introduction of insertion/deletion mutations, disruption of splice signals to promote exon skipping, and the templated correction of point mutations by seamless homology directed repair or base editing technology. Potential limitations to the clinical translation of the splice modulation and gene editing approaches are discussed, including drug delivery, the importance of uniform dystrophin expression in corrected myofibres, safety issues (e.g. renal toxicity, viral vector immunogenicity, and off-target gene editing), and the high cost of therapy.
Manuscript Classifications:	Neurobiology/Neurological Disease; RNA Binding Proteins; RNA in Disease; Small and Large Non-coding RNAs; Splicing/Pre-mRNA Processing; Therapeutics
Keywords:	Duchenne muscular dystrophy; dystrophin; exon skipping; antisense oligonucleotides; gene editing; CRISPR/Cas9
Response to Reviewers:	Reviewer 1: Hanson et al provide a very well written review on 'Molecular correction of DMD by splice modulation and gene-editing'. Both ASO and CRISPR based approaches are critically described, making this one of the most comprehensive review available on the current state of DMD therapeutics. I only have some minor suggestions- 1.It is desirable to see the full name of the disease in the title. ‘DMD’ changed to ‘Duchenne muscular dystrophy’ as requested. 2.In page 7, arginine, X and B should read R, X and B for consistency. Text changed as requested. 3.It will make more sense to start the discussion with ASOs. We agree with the Reviewer that it would make sense to discuss ASOs first in the discussion, based on the structure of the preceding text. However, the discussion

	section does not deal with the ASO and CRISPR sections separately, but rather considers these two approaches together. Therapy-specific concepts are included in the main body text, in the appropriate section. The focus of the discussion is primarily directed towards CRISPR therapies, as these are more complex and novel. (In contrast, there are a plethora of reviews covering ASO therapies for DMD, so our review has focused only on the key points, approved drugs, and most recent developments). As such, the discussion cannot be re-organised into ASO and CRISPR sections without a substantial re-write. Since there are no specific additional points related to DMD ASO therapies which we wish to add to the revision, we have not altered this section. Reviewer 2: This manuscript represents a comprehensive review of the state of the art in therapeutic correction of the phenotypes in Duchenne muscular dystrophy - a currently incurable and fatal X-linked neuromuscular disease. The review reads very well and is well-structured; it is written in concise and accessible language and overall a pleasure to read. The only suggestion would be to mention whether there are any efforts to correct dystrophin splicing using small molecule drugs rather than oligonucleotides - similar to the use of risdiplam and branaplam in SMA. We have added a paragraph to address this point. The recent approval of risdiplam is a major achievement in the fields of SMA therapeutics and splice modulation. We are not aware of any equivalent compounds which can modulate the splicing of specific DMD exons. Several suggestions on how to improve the abstract: 1. "..the potential to restore dystrophin in the heart" - heart is mentioned here for the first time here, it is better to state at the beginning that heart problems is the main cause of mortality in this disease. The abstract has been modified as requested. 2. It would be helpful to mention in the abstract that a variety of mutations exist that's why it is so challenging to find a blanket approach suitable for all disease subtypes. This key point has been added to the abstract as requested. 3. "Indeed, three exon skipping compounds.." sounds a bit confusing because "compounds" often equate to small molecules, better replace it with oligonucleotides ‘Compounds’ has been changed to ‘oligonucleotides’ as requested.

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To:

04 January 2021

Renee Schroeder, PhD
Editor-in-Chief
RNA Biology

Dear Dr Schroeder,

Re: Submission of revised manuscript to *RNA Biology.*

We would like to submit the accompanying revised manuscript now titled '**Molecular correction of Duchenne muscular dystrophy by splice modulation and gene editing**' (KRNB-2020-0689) for publication as a review article in *RNA Biology* (Special Focus on RNA in neurological development and disease).

We thank-you the Reviewers for their comments and note that they were positive, describing it as 'very well written' and 'a pleasure to read' and that it was 'comprehensive'. The Reviewers comments are reproduced below in bold text, together with our point-by-point responses. Changes to the revised manuscript are indicated with red text. We hope that the changes that we have made will now qualify our manuscript for publication.

With best wishes,

Thomas C. Roberts DPhil

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Title

Molecular correction of Duchenne muscular dystrophy by splice modulation and gene editing.

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Abstract

Duchenne muscular dystrophy (DMD) is a currently incurable X-linked neuromuscular disorder, characterised by progressive muscle wasting and premature death, typically as a consequence of cardiac failure. DMD-causing mutations in the dystrophin gene are highly diverse, meaning that the development of a universally-applicable therapy to treat all patients is very challenging. The leading therapeutic strategy for DMD is antisense oligonucleotide-mediated splice modulation, whereby one or more specific exons are excluded from the mature dystrophin mRNA in order to correct the translation reading frame. Indeed, three exon skipping oligonucleotides have received FDA approval for use in DMD patients. Second-generation exon skipping drugs (i.e. peptide-antisense oligonucleotide conjugates) exhibit enhanced potency, and also induce dystrophin restoration in the heart. Similarly, multiple additional antisense oligonucleotide drugs targeting various exons are in clinical development in order to treat a greater proportion of DMD patient mutations. Relatively recent advances in the field of genome engineering (specifically, the development of the CRISPR/Cas system) have provided multiple promising therapeutic approaches for the RNA-directed genetic correction of DMD, including exon excision, exon reframing via the introduction of insertion/deletion mutations, disruption of splice signals to promote exon skipping, and the templated correction of point mutations by seamless homology directed repair or base editing technology. Potential limitations to the clinical translation of the splice modulation and gene editing approaches are discussed, including drug delivery, the importance of uniform dystrophin expression in corrected myofibres, safety issues (e.g. renal toxicity, viral vector immunogenicity, and off-target gene editing), and the high cost of therapy.

Keywords

Duchenne muscular dystrophy; dystrophin; exon skipping; antisense oligonucleotides; gene editing; CRISPR/Cas9

Introduction

Duchenne muscular dystrophy (DMD, OMIM #300377) is a highly debilitating, and ultimately fatal, hereditary neuromuscular disease. DMD is one of the most severe monogenic skeletal muscle dysfunction disorders, affecting approximately 1 in 3,500-5,000 boys worldwide [1]. An estimated two thirds of patients inherit a deleterious mutation from their mother, often an unknowing carrier, while the remainder of cases are believed to result from *de novo* germline mutations [2]. As such, it is not possible to eradicate DMD by genetic counselling alone, and so treatment and optimal management of this disease remain urgent unmet clinical needs.

DMD is caused by loss-of-function mutations in the dystrophin gene (*DMD*) which encodes dystrophin, a sub-sarcolemmal protein required for myofibre function and integrity [1,3]. The *DMD* gene is located on the X chromosome (Xp21.2-p21.1) and so the disease is X-linked. *DMD* is the largest known human gene, spanning ~2.3 megabases of genomic DNA. The largest dystrophin isoform (and the predominant isoform expressed in skeletal muscle) encodes a 3,685 amino acid, 427 kilodalton rod-shaped protein [3–7]. Dystrophin acts as an organising centre for the multi-protein dystrophin associated glycoprotein complex (DAPC) at the sarcolemma and, as such, interacts with cytoskeletal actin via its N-terminus and connects to the extracellular matrix (ECM) via the DAPC [8]. In addition to this structural role, dystrophin has also been implicated in signalling processes such as the production of nitric oxide mediated by neuronal nitric oxide synthase (NOS1), a DAPC component [9–11].

Loss of dystrophin increases the fragility of the sarcolemma, sensitising it to contractile damage [12]. This leads to chronic cycles of myonecrosis and compensatory regeneration, together with persistent immune cell infiltration. Over time, muscle quality declines as a consequence of extensive fibrosis and fat deposition [13–15]. Affected boys typically present with delays in the

development of certain motor skills (e.g. independent ambulation and standing from a supine position) around the age of three years. DMD patients become wheelchair bound around the age of ten, suffer from diaphragm-related respiratory complications, cardiomyopathy, and ultimately succumb to the disease around the age of thirty [16–18].

To date, the efficacy of approved DMD therapies is very limited and there is currently no cure. The majority of patients are treated with corticosteroids, which have demonstrated some minimal efficacy in terms of prolonging ambulation and delaying disease progression [19]. DMD-causing mutations are highly diverse, which poses a significant challenge for the development of broadly applicable therapies. Exon skipping mediated by antisense oligonucleotides (ASOs) is currently the leading therapeutic strategy for the treatment of DMD, which aims to restore the dystrophin reading frame via RNA-targeted splice correction. Other approaches include classical gene therapy to restore dystrophin using viral vectors expressing compact recombinant mini/micro-dystrophins [20], promoting stop codon read-through (e.g. ataluren) [21,22], upregulation of utrophin (the developmental paralogue of dystrophin) [23], cell therapy [24], and drug repurposing (e.g. tamoxifen) [25]. Importantly, technologies that also involve RNA-based target recognition and engagement (i.e. CRISPR/Cas9, clustered regularly interspaced short palindromic repeat/CRISPR associated protein 9), but which are aimed at permanently correcting disease-causing mutations at the genomic DNA level, are rapidly emerging as novel approaches for treating DMD. These exciting developments have the potential to supersede, or at least complement, the more established ASO strategies.

This review describes the state-of-the-art for RNA-targeted exon skipping, and RNA-guided CRISPR therapeutic approaches to treating DMD, and discusses the obstacles to their successful implementation in patients.

Dystrophin restoration by pre-mRNA-targeted exon skipping

The *DMD* gene is comprised of 79 exons which encode an actin-binding N-terminal domain, a central rod domain, a cysteine-rich domain, and a C-terminal domain [26]. The central rod domain consists of multiple redundant spectrin repeat units which are dispensable for dystrophin protein function. This is exemplified by the related condition Becker muscular dystrophy (BMD, OMIM #300376) which is typically less severe, and presents with later onset, relative to DMD [27,28]. Both DMD and BMD are caused by mutations in the dystrophin gene, however, BMD-causing mutations do not disrupt the translation reading frame, leading to the production of an internally truncated dystrophin protein that retains some functionality [29]. Indeed, in some rare cases BMD patients are effectively asymptomatic [30,31]. This observation inspired the exon skipping approach, whereby alternative splicing is modulated in order that a specific exon, or combination of exons, are selectively excluded (or 'skipped') from the mature mRNA transcript [32]. Exon skipping aims to restore the reading frame of the mutant dystrophin transcript, and thereby convert the DMD phenotype into a BMD-like phenotype [33]. Exon skipping is most commonly achieved through the use of steric block ASOs, which are short (15-35 nt), single-stranded nucleic acid polymers that bind to splicing signals in the pre-mRNA via complementary base pairing, and thereby mask them from the spliceosome [34] (**Fig. 1**). The leading approach in the case of DMD is to target removal of exon 51, which would theoretically be applicable for the treatment of ~13% of patients [33]. For example, patients carrying a deletion in *DMD* exon 52 can be treated by skipping exon 51, as exons 50 and 53 are in-frame with one-another, leading to the generation of slightly truncated dystrophin protein that retains the majority of its functionality.

DMD-causing mutations occur throughout the *DMD* gene body, with the majority being whole exon deletions [35]. However, there are 'hotspots' where mutations tend to cluster, such as at exons 45-55. This region has attracted particular interest, as exon skipping therapy targeting simultaneous removal of these eleven exons would be applicable to ~63% of DMD patients [36]. Furthermore, there are several reports of BMD patients who carry natural deletions of this region [36,37]. Multiple exon skipping in the case of DMD is technically challenging as skipping efficiencies are typically very low, variable between exons, and dependent on the order of intron removal [38]. It is worth noting the extraordinarily long distance that RNA polymerase II must travel between these exons (it has been estimated that it takes 16 hours to transcribe the *DMD* locus [39]) and that the splice modulation events at each exon must therefore be temporally coordinated. Nevertheless, a recent report showed that a cocktail of 10 octaguanidine dendrimer-conjugated PMOs (phosphorodiamidate morpholino oligonucleotides), i.e. vivo-morpholinos, was able to induce exon 45-55 skipping in the *mdx52* mouse model of DMD [40]. Notably, high concentrations of oligonucleotides were required (raising potential toxicity issues), and the level of exon skipping was modest relative to that of single exon skipping [40].

To date, there are three FDA-approved exon skipping drugs for DMD: eteplirsen which targets exon 51, and golodirsen and viltolarsen which both target exon 53. Eteplirsen and golodirsen were developed by Sarepta Therapeutics (Cambridge, MA, USA), whereas viltolarsen was developed by NS-Pharma (Tokyo, Japan). A further Sarepta-developed compound targeting exon 45 (casimersen) is under consideration, with a regulatory decision expected in early 2021 [41]. All of these drugs are comprised of PMO, a non-natural nucleotide analogue with a charge neutral phosphorodiamidate backbone and a six-membered morpholine ring (which substitutes for the arabinose in the equivalent RNA), and are administered via the intravenous route. PMOs have repeatedly been found to be remarkably safe, with very few adverse effects in clinical

trials [42,43], and very high doses (up to 3 g/kg) are tolerated in mice [44]. In contrast, a rival compound, drisapersen (developed by Biomarin Pharmaceutical Inc., San Rafael, CA, USA), was discontinued due to lack of efficacy at Phase III [45,46]. This drug consists of a fully 2′-*O*-methyl substituted phosphorothioate-backbone RNA oligomer, and exhibited dose-limiting renal toxicity, which contributed to its inability to achieve efficacy goals.

Despite their low toxicity, effective delivery of PMOs to muscle (and especially to the heart, an organ that is critical to the lethality of DMD) remains a major obstacle to successful therapy. This is exemplified by the very low efficacy of the FDA-approved exon skipping drugs. Indeed, the approval of eteplirsen in September 2016 proved to be highly controversial [47–50]. The levels of dystrophin protein expression measured in muscle biopsies of eteplirsen clinical trial participants was ~1% of that expressed in healthy muscle [51], and is of debatable therapeutic relevance. Similarly, viltolarsen was recently shown to achieve ~5% dystrophin correction [52]. Importantly, data from animal studies suggest that ~15% of wild-type (WT) dystrophin expression is sufficient to protect against dystrophic muscle pathology [53]. A further challenge for the treatment of DMD is the development of ASOs that target other exons (and therefore mutation types). For example, Sarepta is currently developing therapeutic products targeting exons 43, 44, 45 (i.e. casimersen), 50, 52, and 55 (https://www.sarepta.com/pipeline/exon-skipping-duchenne).

The neutrally-charged character of PMO molecules means that they can be covalently coupled to cationic cell-penetrating peptides in order to generate peptide-PMO (PPMO) conjugates. PPMOs have been extensively investigated for exon skipping applications in DMD mouse models. Early success was observed using the $(RXR)_4$ [54] and B peptides ($(RXRRBR)_2XB$) [55] which are rich in arginine (R), and also incorporate the non-natural amino acids 6-

aminohexanoic acid (X) and β-alanine (B). Further PPMO variants developed by our group (i.e. Pip5 and Pip6 series peptide conjugates) are active at much lower doses than naked PMO, and critically show activity in cardiac muscle [53,56–60]. These peptides constitute iterative improvements on the previous designs and similarly consist of R, X and B residues, with the addition of a core sequence containing several hydrophobic amino acid residues. The leading PPMO drug is SRP-5051 (developed by Sarepta and targeting *DMD* exon 51) which is currently in phase II (NCT04004065) and for which preliminary data is suggestive of enhanced efficacy over eteplirsen [61]. PepGen Ltd (Oxford, UK) also aims to develop PPMOs for use in DMD patients. A potential limitation of PPMO technology is that high doses of arginine rich peptides have in much earlier studies been reported to be associated with lethargy in rats [62], and renal tubule degeneration in cynomolgus monkeys [63]. Notably, multiple other exon skipping chemistries are similarly in the early stages of development, including peptide nucleic acid [64] and tricylcoDNA (tcDNA) [65]. Other enhanced muscle delivery strategies are also being explored, such as the antibody-ASO conjugate approaches, currently being developed by Avidity Biosciences (La Jolla, CA, USA) and Dyne Therapeutics (Waltham, MA, USA).

An alternative to ASOs is the use of expressed exon skipping triggers (i.e. U1 and U7 small nuclear RNA expression systems) which can be delivered using adeno-associated viral (AAV) vectors [66,67]. AAV is a single-stranded *Parvovirus* that infects human cells but does not cause human pathology [68]. AAV vector genomes are typically maintained as episomal chromatin with the potential for long-term, persistent transgene expression [69]. AAVs are attractive gene therapy vectors as multiple serotypes exist with a broad range of tissue tropisms (including for skeletal and cardiac muscle), they are able to transduce both dividing and non-dividing cells, and exhibit low immunogenicity [70,71]. Furthermore the non-integrative lifecycle of AAV reduces

the risk of insertional oncogenesis, a major advantage over other commonly used viral vectors, such as lentivirus [72].

Notably, AAV-mediated dystrophin restoration (either by U7-mediated exon skipping or micro-dystrophin gene therapy) has been augmented by pre-treatment with PPMO [73]. The effects of AAV-mediated gene transfer are dependent on second strand DNA synthesis of the viral genome, meaning that there is a ~7-10 day delay between injection of the virus and the manifestation of therapeutic effects [74,75]. Importantly, the turnover of muscle fibres associated with dystrophic pathology means that viral genomes are progressively lost over time, including during this lag period [72,76]. In contrast, the correction of dystrophin transcripts mediated by ASOs, such as PPMO conjugates, begins immediately after cellular entry of the oligonucleotides. As such, transient dystrophin correction mediated by PPMO re-treatment has been shown to stabilise muscle turnover and thereby enhance the effects of subsequently administered AAV therapies [73].

Targeted alternative splicing of specific exons has also been achieved using small molecules in other contexts. The orally-available drug risdiplam (developed by PTC Therapeutics, South Plainfield, NJ, USA) received FDA-approval for the treatment of spinal muscular atrophy (SMA) in August [77]. Risdiplam treatment restores expression of the survival motor neuron protein by promoting inclusion of *SMN2* exon 7. Whether similar small molecule therapeutics could be identified which promote similar specific alternative splicing events in the context of the *DMD* gene remains to be demonstrated, and to date, PTC Therapeutics has not disclosed such a therapeutic splicing program for DMD.

While ASOs are highly promising for the treatment of DMD, this approach necessitates lifelong repeat administration, constituting an enormous cost to healthcare systems and patients. The development of technologies that can directly manipulate genomic DNA sequences, such as CRISPR/Cas9, have revealed new possibilities for single-intervention treatments and/or cures for a plethora of genetic diseases [78,79]. Multiple CRISPR-based strategies have been investigated as RNA-targeted approaches for genetic correction of DMD.

RNA-programmed nuclease technology

Until recently, genome engineering was achieved using ZFNs (zinc finger nucleases) and TALENs (transcription activator-like effector nucleases). While these are both highly effective tools for manipulating the human genome, they are cumbersome to design and expensive to produce, requiring that a unique synthetic protein be engineered for each target DNA site in order to induce the desired change [80,81]. In contrast, CRISPR/Cas9 is a simple and versatile technology that can be easily directed to recognise a specific genomic DNA site through careful design of a non-protein-coding guide RNA sequence, which programmes the complex [82–84].

CRISPR/Cas9 is derived from a prokaryotic natural defence system found in ~40% of bacteria and ~90% of archaea, which protects against foreign invading nucleic acid (e.g. from bacteriophages) in a sequence-specific manner [85–87]. This natural system has since been engineered for applications in mammalian systems [82,83]. CRISPR/Cas9-mediated gene editing is facilitated by a Cas9 endonuclease protein in complex with a single guide RNA (sgRNA) which is comprised of a ~20 nt RNA targeting component (referred to as the spacer) fused to an invariant, Cas9 orthologue-specific ~80 nt scaffold RNA sequence [84]. The scaffold structure is required for recognition of the sgRNA by the Cas9, resulting in ribonucleoprotein (RNP) complex formation and Cas9 priming. Target recognition requires the presence of a short

protospacer adjacent motif (PAM) sequence directly adjacent to the sgRNA spacer cognate DNA sequence. Cas9 helicase activity results in DNA unwinding and allows for scanning of the genome for the desired target site which is recognised by the sgRNA. Heteroduplex formation of the DNA and spacer activates the HNH and RuvC catalytic domains of the Cas9 endonuclease to cleave the complementary and non-complementary DNA strands respectively. The resulting double strand break (DSB) is introduced within the target recognition sequence, three nucleotides in from the PAM-proximal end [88]. This lesion is resolved by one or more DNA repair pathways, including error-prone non-homologous end joining (NHEJ) and high-fidelity homology directed repair (HDR) [84,89,90]. CRISPR/Cas9 has the potential to either disrupt/modify existing genes, or to introduce recombinant DNA.

The most widely adopted CRISPR/Cas9 systems are those derived from *Streptococcus pyogenes* (SpCas9) and *Staphylococcus aureus* (SaCas9), as these exhibit the most efficient *in vivo* cleavage activity reported to date [82,83,91]. SpCas9 recognises a 5′-NGG trinucleotide PAM, a motif that occurs frequently throughout the human genome and thus enables broad targeting[82,83]. However, the common occurrence of these canonical PAM sites, the potential for non-canonical 5′-NAG PAM recognition, and a high level of tolerance for mismatches between the spacer and similar off-target genomic sequences increase the risk of SpCas9 cleavage promiscuity [84,92,93].

In contrast, the SaCas9 endonuclease has a longer PAM recognition requirement (5′-NNGRRT, where R is either A or G), which significantly reduces the number of potential cleavage sites within the human genome relative to SpCas9, thereby minimising unwanted off-target gene editing activity [91]. SaCas9 also has the advantage of occupying a smaller DNA footprint than SpCas9 (3.3 kb compared with 4.1 kb) and is thus more easily packaged within the limited

capacity of the AAV genome (~4.7 kb) [94]. SaCas9 is currently the orthologue-of-choice for majority of *in vivo* gene editing studies owing to its improved safety profile and compact nature [91]. Nevertheless, SaCas9 and its cognate sgRNA(s) have often still been co-administered separately, using two AAV vectors, for *in vivo* DMD CRISPR applications [95–98].

Dual cut-mediated exon excision

One of the leading CRISPR-based strategies for *DMD* correction is an extension of the RNA targeting ASO-mediated exon skipping approach (described above). Multiplex CRISPR/Cas9-mediated gene editing [82,83] can be used to excise a genomic region containing an exon, thereby achieving permanent 'exon skipping' at the DNA level. To this end, two sgRNAs are used that target introns flanking the target exon(s) of interest. In some cases, the intervening sequence will be excised, and the cleaved genomic ends of the flanking regions are joined by the host cell NHEJ DNA repair pathway. Subsequently, the edited genomic DNA now lacks the excised exon, such that when the locus is transcribed, the upstream and downstream exons will be joined by the spliceosome. Every new *DMD* transcript generated from this locus will therefore lack the targeted exon, leading to correction of the translation reading frame, and production of a functional, albeit truncated, protein [43,95–100] (**Fig. 2**). Repair by the NHEJ pathway typically results in indel (insertion/deletion) formation at the resolved DSB site. These indels are not expected to have a detrimental effect on the *DMD* open reading frame as they occur within the non-coding recombinant intron, provided that a cryptic splice site is not introduced.

Multiple studies have demonstrated the utility of the exon excision strategy *in vivo* by dual sgRNA-mediated targeting of the introns flanking the *mdx* mouse *Dmd* exon 23 (which harbours a nonsense mutation leading to premature termination of dystrophin translation) [95–98,100]. One of the first studies employing dual sgRNA targeting for *Dmd* exon 23 excision in

mdx mice (**Fig. 2A**) demonstrated low levels of editing and dystrophin restoration (~2.2% of WT levels in the tibialis anterior (TA) muscle, and ~0.9% in the heart) three-weeks post systemic administration of SaCas9 and dual sgRNAs delivered as a pair of AAV vectors [97]. A separate study published in the same issue of Science magazine reported that dystrophin protein levels accumulate over time (reaching ~5% in the TA, and ~10% in the heart measured up to three-months post treatment) [100]. Subsequent studies have since confirmed these findings, reporting progressive increases in dystrophin protein levels at 6- [95], 12- [96], and 18-months [98] post treatment, with up to ~20% restoration in the heart at the latest time point [98]. Interestingly, sgRNA levels were found to be a limiting factor for long-term systemic DMD correction *in vivo* [98], an important technological consideration which has also been reported in several subsequent studies (discussed below) [101,102]. This limitation could be attributed to the preferential depletion of sgRNA-AAV genomes relative to Cas9-AAV, and was overcome by increasing the ratio of sgRNA-AAV:Cas9-AAV from 1:1 to 1:3 [98].

Early treatment has the potential to maximise therapy through the induction of immune tolerance to therapy-associated antigens [96]. This is exemplified by the systemic treatment of neonatal *mdx* mice, which were found to have no detectable anti-SaCas9 IgG antibodies in serum one-year post treatment. In contrast, adult *mdx* mice treated by intramuscular injection of the TA displayed high levels of antibodies 6-months post treatment [96]. Additionally, levels of corrected mRNA transcripts were maintained, or increased, in all tissues of the neonatal treated mice, whereas for mice treated at the adult stage, corrected mRNA transcripts were reduced by 5-fold 6-months post treatment [96]. Furthermore, the authors reported that the levels of SaCas9-AAV and sgRNA-AAV genomes were relatively stable in the neonatal treatment group, while levels decreased over time for the mice treated as adults.

The dose of vectors carrying the CRISPR machinery is a crucial factor in the success of gene editing therapy. This was demonstrated in a study that assessed co-excision of 'hotspot' exons 52 and 53 in the *mdx*^{4cv} mouse (which harbours a nonsense mutation within exon 53, **Fig. 2B**) [99]. Dystrophin restoration was achieved in the heart at both low and high doses of the AAV vectors (low dose: 1×10^{12} vg of each AAV, or high dose: 1×10^{13} vg of SpCas9-AAV and 4×10^{12} of sgRNA-AAV), although widespread skeletal muscle restoration was only observed at the higher dose [99]. These findings suggest that while a low dose may be sufficient to extend patient lifespan through alleviation of the cardiac pathology, a higher dose will likely be required in order to improve patient quality of life via dystrophin correction in peripheral skeletal muscle.

One major limitation of the dual sgRNA targeting exon excision CRISPR strategy is that it relies on simultaneous cleavage at both target sites. However, asymmetric cleavage and indel formation at either one of the targets would render the site inaccessible to the CRISPR machinery and preclude the possibility for exon excision.

Both human and mouse dystrophin genes share a high level of sequence similarity and are conserved with respect to their intron-exon boundaries and splicing patterns [103]. It is therefore possible to extrapolate that editing outcomes in mice will be similar in humans. To this end, the dual sgRNA-targeting exon excision strategy has also been applied in human cells [23,104,105]. Induced pluripotent stem cells (iPSCs) derived from human patients offer a useful model to assess the efficacy of DMD gene therapies, and to monitor the function of the modified dystrophin protein within the context of a human DMD genetic background [23,104–110]. Excision of the DMD 'hotspot' region, spanning up to 725 kb of genomic DNA, has successfully been demonstrated in hiPSCs [104,105]. Several studies have also demonstrated DMD pathology

alleviation following transplantation of gene edited murine [111] or human [97,104,105] iPSCs *in vivo*. Cell therapy by the transplantation of genetically engineered haematopoietic stem cells has shown promising results in the clinic for treating non-small cell lung cancer [112], and is reviewed elsewhere in the context of DMD therapy [24].

Single cut-mediated splice signal disruption and exon reframing

An alternative to excising whole exons is the use of a single cut strategy to disrupt splice signal sequences, such as 3′ splice acceptors (SAs) and exonic splicing enhancers (ESEs) (**Fig. 3**). Cas9 targeting of a PAM site adjacent to an SA or ESE site, results in indel formation via the NHEJ DNA repair pathway. These indels can ablate the neighbouring splice signal sequence, such that it is no longer recognised by the spliceosome, leading to permanent exon skipping. Alternatively, indel formation occurring within a mutation-containing exon has the potential for exon reframing and open reading frame restoration, depending on the size of the indel. In instances where the DSB is repaired in a 'scarless' manner (with no indel), the guide recognition site is reconstituted, and the CRISPR complex is able to induce cleavage at the same site again. However, a disadvantage of this approach is that where an indel does not restore the reading frame, the sgRNA target site will be lost and the *DMD* gene edited in a non-productive manner.

The single cut strategy for splice signal disruption and exon reframing has been assessed for targeting of a putative ESE in exon 51 in ΔExon50 mice [113] (**Fig. 3A**). Approximately half of all indels introduced at the target site consisted of a single adenosine insertion (which would accomplish productive reading frame restoration) [113]. The remainder of the indels consisted of deletions with a range of sizes spanning the putative ESE site and, in some cases, also encompassing the proximal SA site of exon 51, resulting in the further promotion of productive

exon 51 skipping. Widespread dystrophin expression was observed in the heart, diaphragm and peripheral skeletal muscles after treatment, which increased between 4- and 8-weeks post injection [113].

Expanding on these promising murine studies, the single cut system was further tested in a ΔExon50 canine model of DMD (**Fig. 3A**), with only a single nucleotide difference in the sgRNA target site relative to the mouse model [114]. SpCas9-AAV and sgRNA-AAV delivered systemically at two doses (low dose: 2×10^{13} vg/kg of each virus, or high dose: 1×10^{14} vg/kg of each virus) resulted in widespread dystrophin restoration in multiple muscle tissues at the higher dose, including the heart and the diaphragm. While these results are highly promising, only a single animal was injected per treatment group. Future studies will be required to evaluate the longevity of dystrophin restoration beyond 8-weeks, the stability of the AAV genomes over time, persistence of Cas9/sgRNA transgene expression, and whether the treatment induces unwanted immune responses.

Notably, the Olson group has made several iterative improvements to the single cut site CRISPR approach [101,102]. The ratio of Cas9-AAV:sgRNA-AAV was found to be critical, with a 1:10 ratio being most effective (in the context of targeting a putative ESE in exon 44 in ΔExon44 mice, **Fig. 3B**) [101]. Furthermore, the authors also investigated the potential for delivery of the sgRNA component using a self-complementary AAV (scAAV) vector, instead of the conventional single-stranded AAV [102]. scAAVs form dsDNA hairpin structures, thereby bypassing the rate-limiting second strand synthesis step and accelerating transgene expression [115,116]. The authors observed up to a 70-fold improvement in editing efficiency with sgRNAs expressed from scAAV relative to conventional AAV at the same dose [102].

Homology directed repair

An alternative DMD therapeutic approach involves CRISPR/Cas9-mediated targeting adjacent to an exonic point mutation, enabling seamless correction via the HDR pathway [99,110,117] (**Fig. 4**). HDR requires that a single-stranded oligodeoxynucleotide (ssODN) repair template, with ~90 nt of sequence homology on either side of the cut site, be provided together with the Cas9 and sgRNA components. HDR offers the potential to make precise, templated corrections to small genetic mutations. However, this repair pathway is only active during the late S and G2 phases of the cell cycle, whereas NHEJ occurs in all cells, regardless of cell cycle status [118–120]. Given that the relevant cell populations for therapeutic targeting in skeletal muscle are myofibres (which are post-mitotic) and satellite cells (resident muscle stem cells responsible for regeneration upon myofibre damage, which remain quiescent until activated), the utility of HDR approaches for treating DMD patients is likely to be very limited.

HDR has been tested for correction of the nonsense mutation within exon 53 in *mdx*^{4cv} mice, although this approach was designed in such a way that alternative repair pathways could also restore dystrophin expression should HDR be unsuccessful [99] (**Fig. 4A**). Dual sgRNAs flanking the nonsense mutation, and in close proximity to one another, enabled a partial in-frame deletion of the intervening exonic region. Targeted deep sequencing revealed that a total of 2.3% of target sites analysed were edited, and of these only 0.18% were seamlessly corrected via HDR [99]. As such, the vast majority of edits were a result of NHEJ repair, with most being non-productive indels. The overall editing efficiency was up to 4-fold lower than that achieved by the full exon 52 and 53 dual sgRNA excision approach (discussed above) [99]. This highlights the limited utility of HDR-mediated editing in post-mitotic cells.

Zhang and colleagues sought to circumvent the limitation of the minimal HDR activity in post-mitotic tissue by treating *mdx* zygotes [110]. The authors employed an alternative CRISPR system called Cpf1 (CRISPR from *Prevotella* and *Francisella*) derived from *Lachnospiraceae bacterium* (LbCpf1) [121–124] to asses a single cut-mediated HDR strategy, with the added potential for exon reframing (in the context of the *Dmd* exon 23 mutation, **Fig. 4B**) [110]. LbCpf1 exhibits robust DNA cleavage activity in mammalian cells and in mice, and recognises a T-rich PAM sequence (5′-TTTV, where V is A, C or G). This is an attractive choice of CRISPR system as SA regions typically exist within a pyrimidine-rich genetic context. Furthermore, LbCpf1 is smaller than SpCas9 (i.e. 3.7 kb), and is thus more suitable for packaging in AAV vectors. Unlike SpCas9 and SaCas9 which generate blunt incisions, LbCpf1 generates a staggered cut which presents new possibilities for gene editing through the activation of alternative DNA repair pathways to NHEJ and HDR. Five of the twenty-four resulting offspring derived from the zygotes treated with the LbCpf1 CRISPR components carried corrected alleles, with levels of dystrophin restoration ranging from 2-100%. This mosaicism suggests a high level of variability in CRISPR editing efficiency, even when CRISPR-mediated gene editing was performed at the zygote stage [110].

Base editing

Base editing is a more recent addition to the CRISPR toolbox, providing a means for genetic correction of DMD point mutations in a manner that is independent of cell cycle stage. The CRISPR base editor complex facilitates nucleotide conversion at a desired genomic DNA through the fusion of a catalytically impaired Cas9 variant (a ‘nickase’ Cas9, nCas9) and a deaminase enzyme. The first generation of base editors to be developed employed a cytidine deaminase (initially derived from rat APOBEC1, although there are now various versions) which facilitates the targeted conversion of specific cytidine bases to uridine within a defined

target window (i.e. ~14-17 nt upstream of the PAM site) [125,126]. The uridine lesion can then be replaced with thymidine by the DNA replication machinery, or repaired via the base excision or mismatch repair pathways, in either case resulting in a C:G to T:A substitution. Subsequently, a second base editing system was developed: ABE (adenosine deaminase base editor), an RNA-programmable deaminase (derived from *Escherichia coli* TadA) fused to nCas9 [127]. The adenosine on the opposite strand to the 'nick' is converted to inosine which is either read as guanosine by the transcription machinery, or is replaced with guanosine by the base excision repair pathway, enabling an A:T to G:C base change. Several further advantages of base editing systems over the more conventional DSB-inducing CRISPR systems described above, are that they do not require a donor template, and only a single stranded 'nick' is introduced into the target DNA. Genomic 'nicks' are repaired via the high-fidelity base excision repair pathway [128]. This is advantageous over CRISPR systems that introduce DSBs where cleavage at unwanted off-target sequences has the potential for indel formation by NHEJ, in addition to the possibility of major chromosomal rearrangements [105,129].

Ryu and colleagues employed the current generation of the ABE system (ABE7.10) to convert a nonsense mutation (from TAG to CAG) in exon 20 of a murine model of DMD (**Fig. 5**) [130]. In order to package the ABE and cognate sgRNA sequences (which are >6 kb in total) for viral delivery, the authors utilised a trans-splicing AAV (tsAAV) vector strategy that enables delivery of the ABE across two separate AAV vectors (the ABE is split into N- and C-terminal fragments and the targeting complex is reconstituted following transduction) [131,132]. The authors observed base editing activity of the target cytidine with an efficiency of ~3.3%, which resulted in ~17% dystrophin restoration as determined by immunohistochemistry (IHC) staining 8-weeks post treatment. This level of correction is less than that achieved by the other NHEJ-based strategies described above, which could be a result of either the low efficiency of

the tsAAV trans-splicing system and/or the low activity of the ABE itself. A further limitation of this approach is that, in some cases, there may be no appropriate PAM sites within the optimal base editing window. Ryu and colleagues tested longer sgRNAs (~20-23 nt) which were found to expand the editing window by 3-4 nt (i.e. to 12-19 nt upstream of the PAM site). However, this expanded base editing window also increases the risk of unintended bystander editing events in close proximity to the desired target.

Discussion

Recent high-profile exon skipping drug approvals [34], together with the potential for CRISPR-mediated gene correction [96,98,114], mean that this is an exciting time for DMD therapeutics. Commercial development of CRISPR therapies for DMD is currently in the early stages at a number of companies, including Editas Medicine (Cambridge, MA, USA), Vertex Pharmaceuticals (Boston, MA, USA), CRISPR Therapeutics (Zug, Switzerland), and Eli Lilly (Indianapolis, IN, USA). Of the various CRISPR strategies for DMD therapy development discussed, the single cut splice signal sequence disruption and exon reframing approach appears to be the most promising, despite the fact that not all editing outcomes will result in open reading frame restoration. Conversely, the dual sgRNA exon excision approach requires temporally synchronised cleavage followed by appropriate DNA repair, and HDR activity appears to be negligible in post-mitotic tissue. Nevertheless, not all DMD mutations will be treatable using the single cut approach, for example, in instances where multiple exons need to be 'skipped' or removed (e.g. for the correction of a multi-exon duplication), or in the case of splice signals lacking an appropriate adjacent PAM site. Similarly, the single cut approach does not allow for development of a broadly-applicable therapy spanning multiple patient-relevant mutations, such as excision of multiple 'hotspot' exons.

Newer versions of SpCas9 that have altered PAM recognition requirements, such as the xCas9 and SpCas9-NG variants, could aide in expanding the number of potential target sites in the genome for multiple CRISPR approaches [133]. For example, an xCas9-ABE base editing system has been developed to increase the availability of PAM sites and, by association, the position of the base editing window [134]. Unlike the approach of lengthening the sgRNA spacer to achieve this end [130], the xCas9-ABE system does pose a greater risk of bystander base substitution events [134]. Furthermore, 'near-PAMless' CRISPR/Cas9 variants have been developed which could offer a simple solution to PAM location constraints, and permit editing at virtually any desired locus within the human genome [135].

Considering the immense hype surrounding the CRISPR/Cas9 system, it is tempting to suggest that gene editing will supersede ASO-mediated exon skipping approaches for the treatment of DMD and other muscle diseases [136]. Indeed, the possibility for permanent therapeutic correction following a single treatment is particularly appealing. However, there are several reasons to be cautious of this notion. The CRISPR/Cas9 system presents new challenges which must be addressed before its therapeutic potential can be fully realised.

The levels of dystrophin restoration in dystrophic animal models after CRISPR-mediated gene correction are typically lower than those observed following ASO-mediated exon skipping. Furthermore, many of the CRISPR studies have utilised IHC for assessing dystrophin expression, which tends to overestimate dystrophin levels relative to more sensitive measures such as Western blot [101,114]. This highlights a technological issue which should be taken into consideration when considering the therapeutic efficacy of DMD CRISPR applications. The relatively low efficacy of CRISPR strategies may be mitigated by the progressive accumulation of dystrophin protein over time [95,98]. Edited myonuclei will continue to generate dystrophin

protein throughout the life of their respective myofibre. Furthermore, newly dystrophin-positive myofibres will likely experience a selection advantage and are therefore expected to accumulate over time. Similarly, successful editing of satellite cells will enable the addition of further dystrophin-producing myonuclei following muscle growth and regeneration. The Wagers group has reported a low level of editing (~2%) in satellite cells [97], although it has been described elsewhere that these cells are intractable to AAV transduction [137]. Similar CRISPR applications for DMD therapy development have employed the muscle-specific CK8 promoter to circumvent expression of CRISPR machinery in non-target cells and avoid off-target mutagenesis. However, this promoter does not appear to be active in satellite cells [138,139]. Further studies are necessary to elucidate the potential for genetic correction in this critical pool of muscle stem cells.

While ASO-mediated exon skipping has shown impressive results in animal models [53–60], the efficacy of this approach in human patients has so far proved to be very limited [51,52]. It is reasonable to expect that similar issues may affect gene editing therapies when they are eventually tested in patients. Sub-optimal gene editing will likely generate the situation whereby treated myofibres contain a mixture of edited (dystrophin-expressing) and non-edited (non-dystrophin-expressing) myonuclei. We have recently modelled such a situation using the *mdx-Xist*$^{\Delta hs}$ mouse, in which dystrophin is expressed in a mosaic manner as a consequence of skewed X-chromosome inactivation [140]. Importantly, *mdx-Xist*$^{\Delta hs}$ mice exhibit a within-fibre, patchy pattern of dystrophin expression, which is insufficient to stabilise myofibre turnover [140]. Additionally, these findings suggest that dystrophin protein is not free to diffuse throughout the sarcolemma. CRISPR-mediated gene editing may result in a similar patchy pattern of sarcolemmal dystrophin if the efficiency of gene correction falls below a certain threshold [140].

This highlights an important potential advantage of exon skipping, which typically results in a uniform pattern of dystrophin distribution, relative to CRISPR-mediated gene editing.

Importantly, almost all CRISPR therapies for DMD are predicated on the assumption that AAV vectors will be safe and effective vehicles for drug delivery. AAV is truly the workhorse of the gene therapy field, and as such has been applied for the treatment of many diverse indications, including for DMD and other muscle diseases [141–143]. The AAV6, AAV8, and AAV9 serotypes have all been shown to exhibit tropism for muscle tissue, and particularly for the heart which is an important therapeutic target for improving the survival of DMD patients [144–146]. However, the majority of patients are expected to be immunised to therapy-associated antigens following treatment, including those associated with the AAV capsid, Cas9 or other CRISPR endonuclease, and the resultant corrected dystrophin protein itself [147–149]. AAV therapies are thus likely to be only effective as a single intervention strategy, and patients will need to be screened for pre-existing immunity to such antigens where possible [150]. Early therapeutic intervention will be important for delaying, or even preventing, the onset of irreversible fibro/fatty muscle degeneration, but is also associated with improved gene editing efficacy. Treatment of mice at the neonatal stage can lead to the acquisition of immune tolerance to therapy-associated antigens [96], although it is currently unknown whether such effects will also be observed in humans. While it would be ideal to treat patients as early as possible after birth, it will also be necessary to treat older patients in which pathology is already established. Furthermore, important safety concerns have recently resurfaced, with the deaths of children participants in a now halted AAV clinical trial for X-linked myotubular myopathy (NCT03199469) [151], and another child participant in a separate AAV trial for mucopolysaccharidosis type IIIA (NCT03612869) [152].

A ‘priming’ strategy could involve pre-treatment of patients with ASOs in order to transiently stabilise dystrophic muscle turnover, and thereby enhance the efficacy of an AAV-CRISPR therapy injected a few weeks later by minimising vector genome loss [73]. Alternatively, should AAV initial treatment prove to be insufficient to alleviate disease pathology, it may be necessary to initiate treatment with ASOs later in life. Such a ‘top up’ style treatment has been proposed in the case of spinal muscular atrophy, whereby initial treatment with zolgensma (FDA-approved classical gene therapy with an AAV vector) may be supplemented with nusinersen (FDA-approved splice-correcting ASO).

All CRISPR-based therapies that result in DNA lesions or base changes at the desired genomic locus have the potential to introduce permanent deleterious mutations at off-target edit sites, which must be carefully monitored. AAV-mediated delivery of the CRISPR gene editing machinery presents a further challenge. Specifically, that AAV genomic DNA has been reported to integrate at Cas9-induced DSBs [96,101,153]. Other gene editing tools that do not rely on DSB formation may be more attractive towards overcoming this hurdle. These include base editing strategies which rely on nicking the DNA and repair by the high fidelity base excision repair pathway [125,127,130], and CRISPR/Cas13 systems which carry out transient editing at the mRNA level [154]. Alternatively, non-viral gene therapy delivery vehicles are currently being developed and have the potential to avoid the risk of viral genome integration into a CRISPR-induced DSB [155]. Such technologies include delivery of Cas9 protein:sgRNA ribonucleoprotein (RNP) complexes facilitated by lipid- or gold-nanoparticles [156], cationic lipids [157,158], or cell-penetrating peptides [159]. Active recruitment of Cas9 into extracellular vesicles has also been demonstrated by a novel and innovative technological approach, coined NanoMEDIC, for CRISPR-mediated DMD correction in patient-derived iPSCs [160].

A further issue for consideration is the cost of any prospective treatments. For example, zolgensma (which consists of a single AAV vector) is estimated to cost $2.1 million dollars per patient, making it the most expensive pharmaceutical product to date. CRISPR therapies will likely be comprised of two AAV vectors, which further compounds this cost issue. It is logical to assume that administration of the CRISPR components within a single AAV genome would be more efficient than a co-delivery approach with the endonuclease and sgRNA being encoded by different AAVs, as both are guaranteed to be delivered to the same target cell. However, improved levels of gene editing were observed when SpCas9 and its cognate sgRNAs were delivered separately as a pair of AAV vectors [97]. Additionally, several studies have demonstrated that the sgRNA component is limiting for CRISPR-mediated gene editing [98,101,102], and so the ability to tailor the ratio of Cas9:sgRNA through the use of separate vectors outweighs the minimal risk of failure to deliver both components to the same target cell. The use of scAAVs for delivery of the sgRNA component enables the optimisation of the Cas9:sgRNA ratio while administering lower doses, which will not only reduce manufacturing costs, but also will likely improve the safety profile of the therapy.

In conclusion, both the RNA-targeted ASO and RNA-directed CRISPR therapeutic strategies present highly promising and exciting avenues for the development of the next generation of effective DMD therapies.

Conflicts of Interest

M.J.A.W. is a founder of, and shareholder in, of Evox Therapeutics and PepGen Ltd, companies dedicated to the commercialisation of extracellular vesicle therapeutics and peptide-enhanced therapeutic oligonucleotide delivery, respectively. The other authors declare no competing financial interests.

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Figure 1.

Antisense oligonucleotide-mediated exon skipping for dystrophin correction.

DMD genomic DNA (gDNA) with an exon 52 deletion results in the prodcution of an out-of-frame mutant transcript. The reading frame can be restored by treatment with a steric block antisense oligonucleotide (ASO) which binds to a splice signal (exonic splice enhancer) located within exon 51, thereby masking this site from the spliceosome and promoting exon 51 skipping. In the skipped transcript, the translation reading frame is restored. Out-of-frame exons are indicated in grey.

Figure 2.

CRISPR/Cas9-mediated *Dmd* exon excision by dual sgRNA targeting.

The CRISPR system introduces double strand breaks (DSBs) at the cut sites and the intervening region is removed. The lesion is thereafter repaired by the error-prone non-homologous end joining (NHEJ) pathway, resulting in the introduction of insertion/deletion mutations (indels) at the repair site. Every transcript derived from the edited locus will lack the excised exon, and the reading frame of the dystrophin mature messenger RNA (mRNA) is thereby restored. This approach has been applied to murine models of DMD: (A) exon 23 in the *mdx* mouse[95–98,100], and (B) both exons 52 and 53 in the *mdx*^{4cv} mice [99]. Out-of-frame exons are indicated in grey, and DMD-causing exons in red.

Figure 3.

CRISPR/Cas9-mediated *Dmd* exon reframing or splice signal disruption by single gRNA targeting.

(A) CRISPR/Cas9-mediated cleavage by a single gRNA targeted to the 5′ end of *Dmd* exon 51 (adjacent to the putative exonic splice enhancer (ESE) and splice acceptor (SA) sites) results in the introduction of indels by the NHEJ DNA repair pathway in ΔExon50 mice [113] and dogs [114]. (B) CRISPR/Cas9-mediated cleavage by a single gRNA at the 5′ end of *Dmd* exon 45 (adjacent to the putative ESE and SA sites) results in the introduction of indels by the NHEJ DNA repair pathway in *ΔExon44* mice [101,102]. This single gRNA targeting strategy has two possible productive editing outcomes for dystrophin reading frame restoration. The indels can either reframe the exon, resulting in the production of a full-length transcript, or disrupt the ESE, resulting in exon skipping. Larger deletions have the propensity to also disrupt the SA site in addition to the ESE, which would further promote productive exon skipping. Out-of-frame exons are indicated in grey, and DMD-causing exons in red.

Figure 4.

CRISPR-mediated homology directed repair of *Dmd* nonsense mutations.

(A) CRISPR-mediated targeting by dual sgRNAs flanking a nonsense mutation within *Dmd* exon 53, and the provision of a single-stranded oligodeoxynucleotide (ssODN) repair template consisting of the desired edited sequence, facilitates seamless homology directed repair (HDR) in *mdx*^{4cv} mice [99]. (B) CRISPR-mediated targeting by a single gRNA adjacent to the nonsense mutation within *Dmd* exon 23, and the provision of a ssODN repair template, facilitates seamless HDR in *mdx* mice [110,117]. The HDR-corrected loci will encode a full-length wild-type *Dmd* transcript, thereby restoring the dystrophin reading frame. Should HDR be unsuccessful, an alternative productive editing outcome is possible whereby indels introduced at the CRISPR cut site by NHEJ DNA repair can either delete the nonsense codon, or reframe the exon, resulting in the production of a full-length transcript containing an in-frame edit within the target exon. Out-of-frame exons are indicated in grey, and DMD-causing exons in red.

Figure 5.

CRISPR-mediated base editing of a *Dmd* nonsense mutation.

A CRISPR base editing system comprised of a catalytically impaired Cas9 that 'nicks' one strand of the target DNA (nCas9), fused to an adenosine deaminase enzyme, is directed to introduce a single-stranded break (SSB) adjacent to a nonsense mutation in *Dmd* exon 20 [130]. The adenosine on the opposite strand to the 'nick' is converted to inosine by the deaminase component, and is either read as guanosine by the transcription machinery, or replaced with guanosine by the base excision repair pathway. This results in conversion of the TAG nonsense codon to a CAG missense codon, leading to dystrophin reading frame restoration. Out-of-frame exons are indicated in grey, and DMD-causing exons in red.

Figure 1

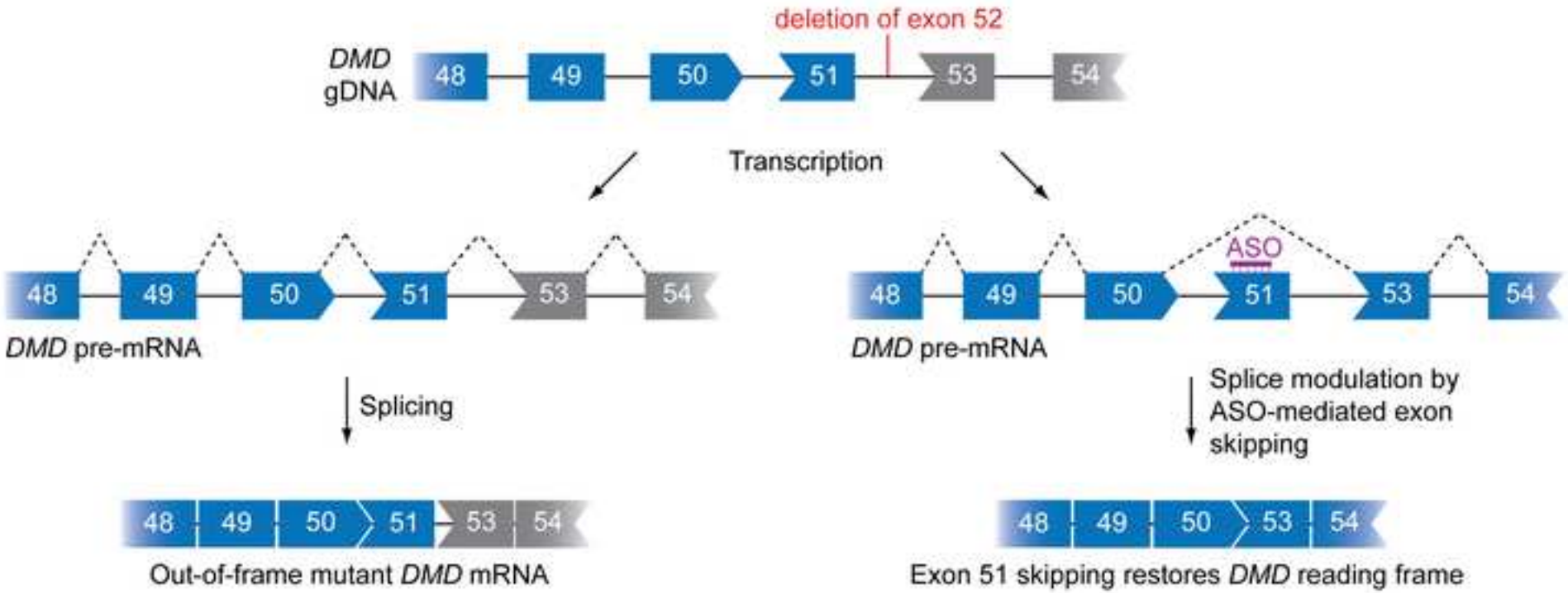

deletion of exon 52
DMD gDNA
48
49
50
51
53
54
Transcription
48
49
50
51
53
54
DMD pre-mRNA
Splicing
48
49
50
51
53
54
Out-of-frame mutant DMD mRNA
ASO
48
49
50
51
53
54
DMD pre-mRNA
Splice modulation by ASO-mediated exon skipping
48
49
50
53
54
Exon 51 skipping restores DMD reading frame

Figure 2

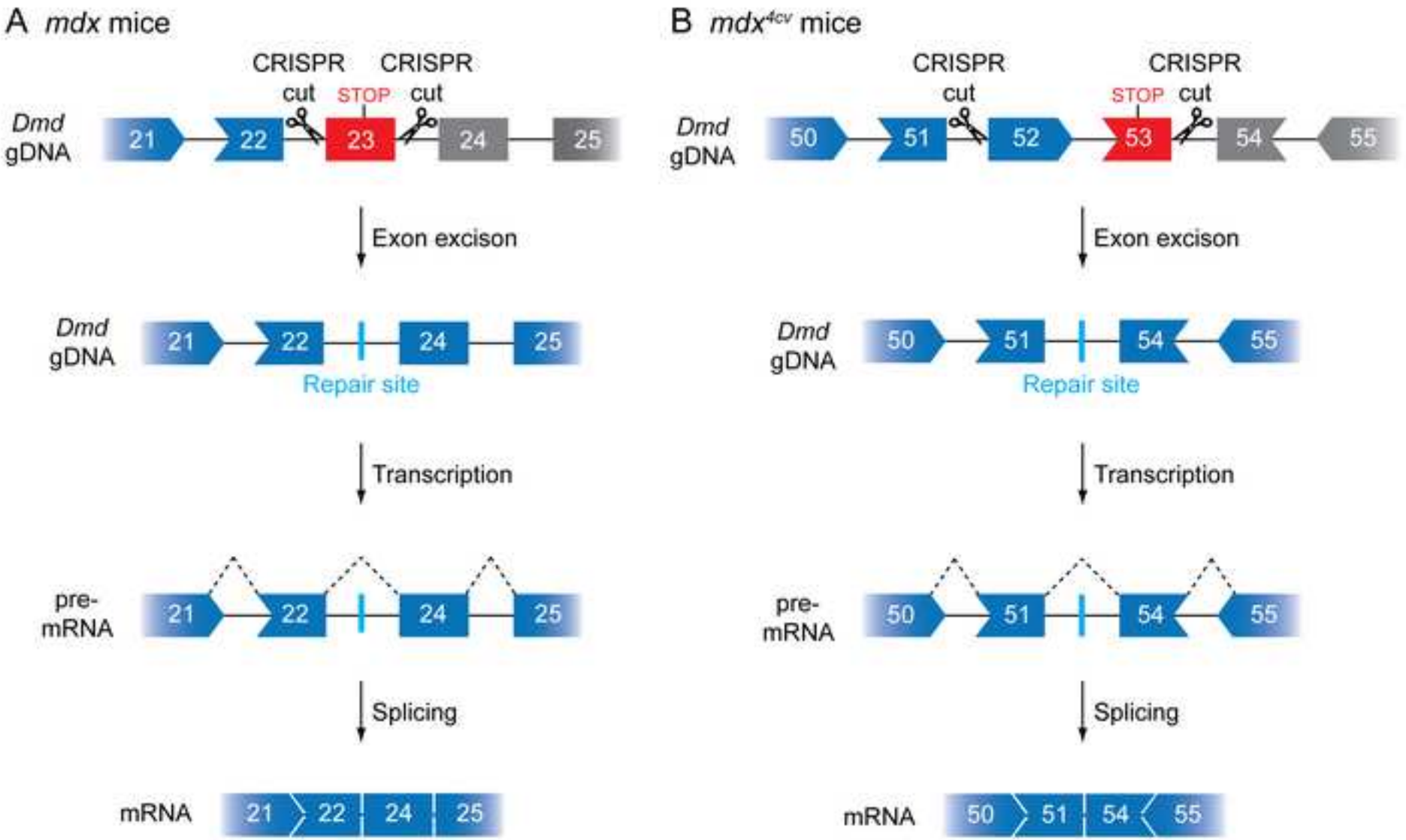

A mdx mice
CRISPR cut
STOP
CRISPR cut
Dmd gDNA
21
22
23
24
25
Exon excison
Dmd gDNA
21
22
24
25
Repair site
Transcription
pre-mRNA
21
22
24
25
Splicing
mRNA
21
22
24
25
B mdx4cv mice
CRISPR cut
STOP
CRISPR cut
Dmd gDNA
50
51
52
53
54
55
Exon excison
Dmd gDNA
50
51
54
55
Repair site
Transcription
pre-mRNA
50
51
54
55
Splicing
mRNA
50
51
54
55

Figure 3

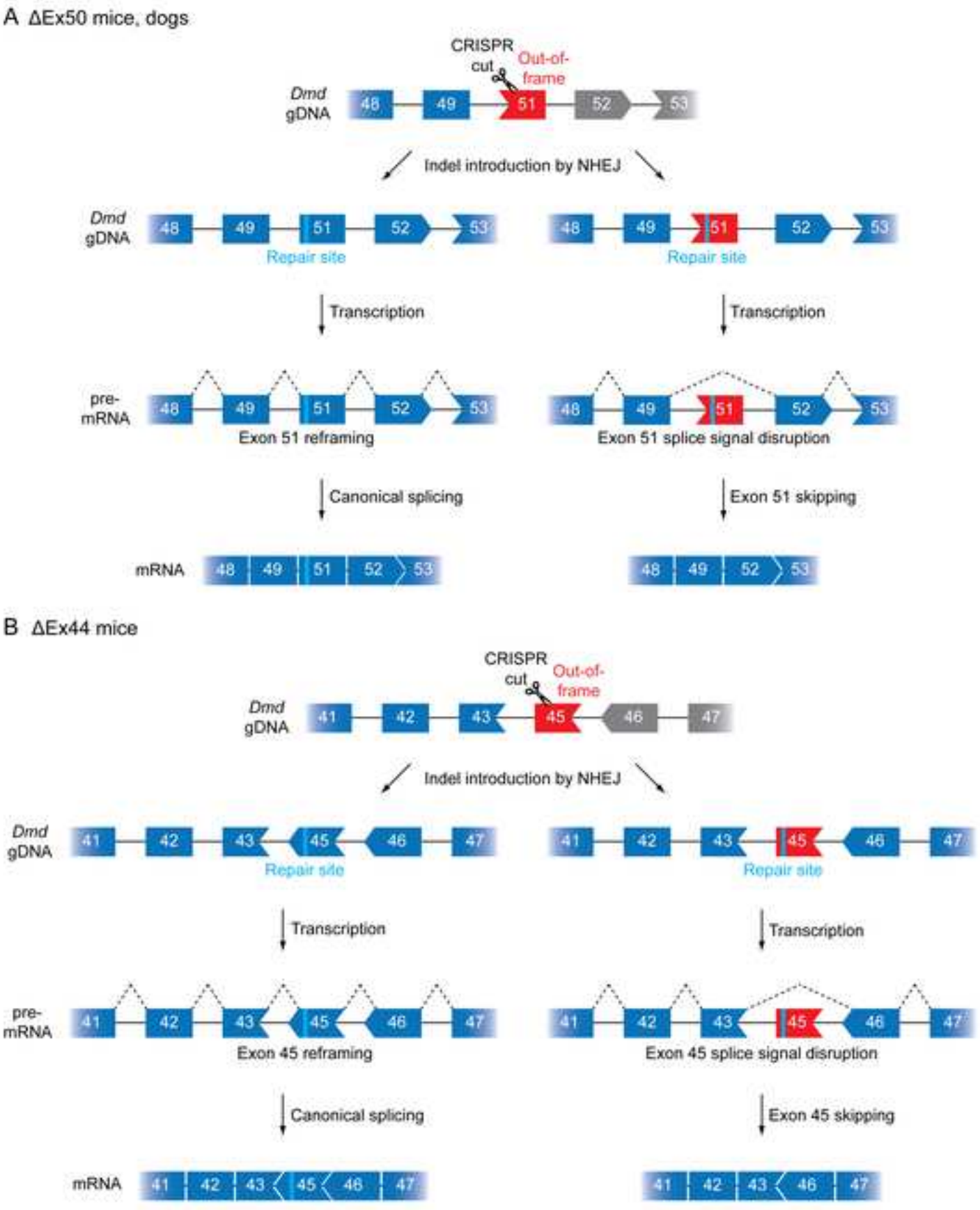

A ΔEx50 mice, dogs
CRISPR cut
Out-of-frame
Dmd gDNA
48
49
51
52
53
Indel introduction by NHEJ
Repair site
Transcription
pre-mRNA
Exon 51 reframing
Exon 51 splice signal disruption
Canonical splicing
Exon 51 skipping
mRNA
B ΔEx44 mice
41
42
43
45
46
47
Exon 45 reframing
Exon 45 splice signal disruption
Exon 45 skipping

Figure 4

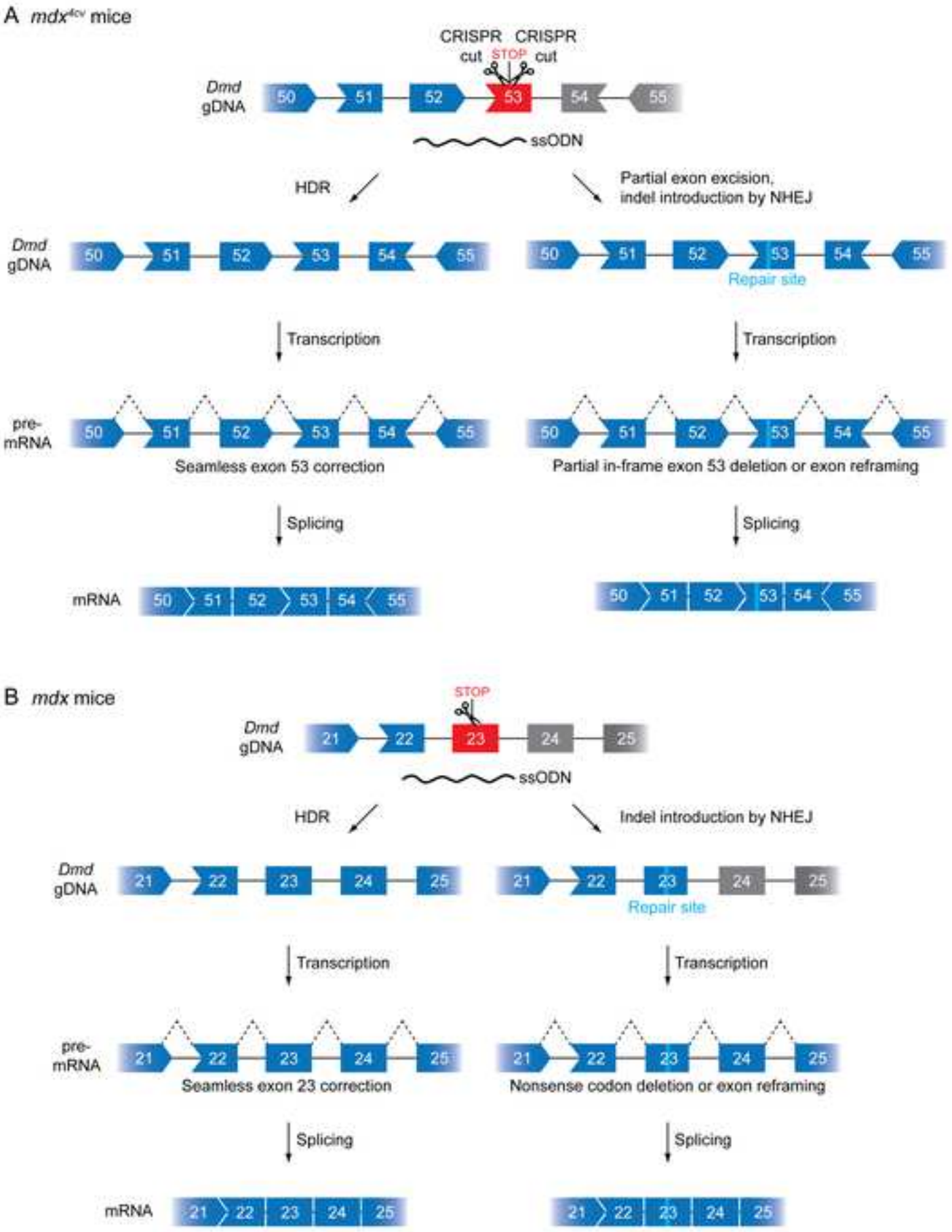
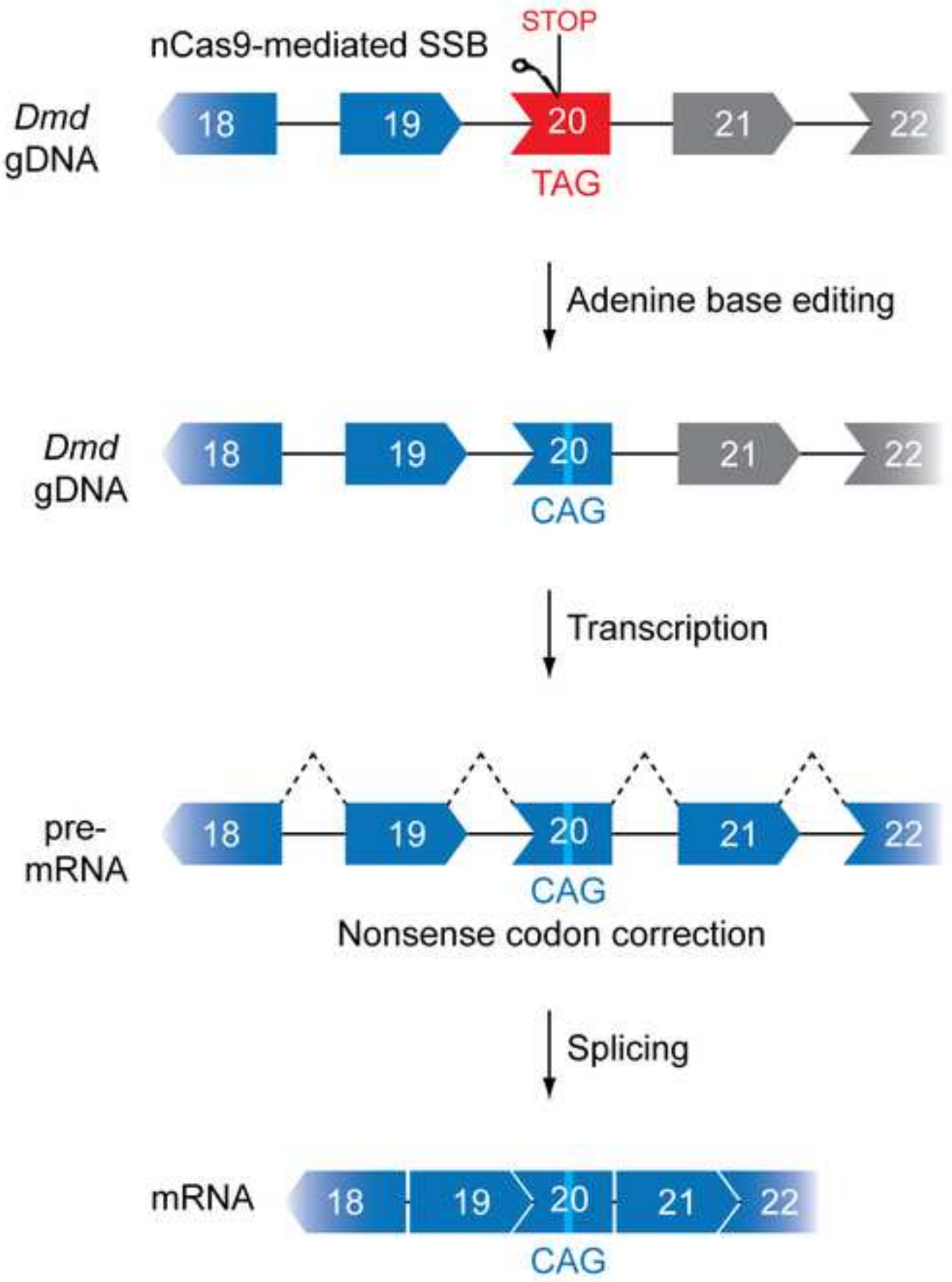

A mdx4cv mice
CRISPR cut
STOP
CRISPR cut
Dmd gDNA
50
51
52
53
54
55
ssODN
HDR
Partial exon excision, indel introduction by NHEJ
Repair site
Transcription
pre-mRNA
Seamless exon 53 correction
Partial in-frame exon 53 deletion or exon reframing
Splicing
mRNA
B mdx mice
STOP
21
22
23
24
25
Indel introduction by NHEJ
Seamless exon 23 correction
Nonsense codon deletion or exon reframing

Figure 5


nCas9-mediated SSB
STOP
Dmd gDNA
18
19
20
21
22
TAG
Adenine base editing
Dmd gDNA
18
19
20
21
22
CAG
Transcription
pre-mRNA
18
19
20
21
22
CAG
Nonsense codon correction
Splicing
mRNA
18
19
20
21
22
CAG