Molecular Therapy
Review

The road toward AAV-mediated gene therapy of Duchenne muscular dystrophy

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Forty years after the dystrophin gene was cloned, significant progress has been made in developing gene therapy approaches for Duchenne muscular dystrophy (DMD). The disorder has presented numerous challenges, including the enormous size of the gene (2.2 MB), the need to target muscles body wide, and immunogenic issues against both vectors and dystrophin. Among human genetic disorders, DMD is relatively common, and the genetics are complicated since one-third of all cases arise from a spontaneous new mutation, resulting in thousands of independent lesions throughout the locus. Many approaches have been pursued in the goal of finding an effective therapy, including exon skipping, nonsense codon suppression, upregulation of surrogate genes, gene replacement, and gene editing. Here, we focus specifically on methods using AAV vectors, as these approaches have been tested in numerous clinical trials and are able to target muscles systemically. We discuss early advances to understand the structure of dystrophin, which are crucial for the design of effective DMD gene therapies. Included is a summary of efforts to deliver micro-, mini-, and full-length dystrophins to muscles. Finally, we review current approaches to adapt gene editing to the enormous *DMD* gene with prospects for improved therapies using all these methods.

INTRODUCTION

Development of gene therapy for Duchenne muscular dystrophy (DMD) has been ongoing almost since the identification of the defective dystrophin gene, *DMD*.[1–3] DMD is a severe neuromuscular disorder characterized primarily by progressive muscle wasting that leads to loss of ambulation and later respiratory and/or cardiac failure.[4] The disease has been of great interest in the genetics and medical fields as it represents a common inherited disorder, and the dystrophin gene displays one of the highest known rates of spontaneous new mutation. DMD is inherited in an X-linked recessive pattern, which, combined with the high incidence and few treatment options, makes this condition an attractive but challenging candidate for genetic therapies.[5]

The initial cloning of dystrophin cDNAs led to early studies of causative mutations and efforts to understand the gene organization. Several unique features of the locus soon became apparent. First, the *DMD* gene was found to be enormous, more than 2.2 MB in length.[6] Second, almost two-thirds of all cases were caused by deletions that remove portions of the gene or, rarely, the entire gene.[7] The remaining mutations were mostly partial gene duplications or small lesions such as nonsense and splice site alterations.[8] Analyses of deletion mutations in particular provided significant insights into the structure and function of the gene and protein and were instrumental in guiding early development of gene therapy strategies. For example, while most deletions were genetic null alleles that caused DMD, some were found in patients with the milder and more slowly progressing Becker MD (BMD). These different phenotypes were explained by the effects of mutations on the mRNA open reading frame (ORF).[9] Deletions that preserved an ORF were associated with milder BMD, while those that disrupted the normal ORF led to severe DMD, although some exceptions were noted that reflect effects on protein stability, the removal of critical functional domains, and cryptic pre-mRNA splicing (see below).[10–13] These observations led to the realization that large parts of the dystrophin protein were not essential for many functional properties. In particular, several very mildly affected BMD patients were identified who carried enormous deletions within the gene, including some greater than 1 MB. Two such deletions, of exons 13–48 and 17–48, generated mRNAs about half the normal size, ~6 kb, instead of the full 11.2 kb coding sequence (13.9 kb with untranslated regions).[14–17] From a gene therapy perspective, these phenotypes suggested the possibility of delivering cDNAs in the size range of 6 kb rather than having to manipulate the full 2.2-MB gene.

EARLY STUDIES TOWARD GENE THERAPY

Efforts to understand the structure and function of dystrophin were hampered by the enormous size of the locus. Partial cDNAs from

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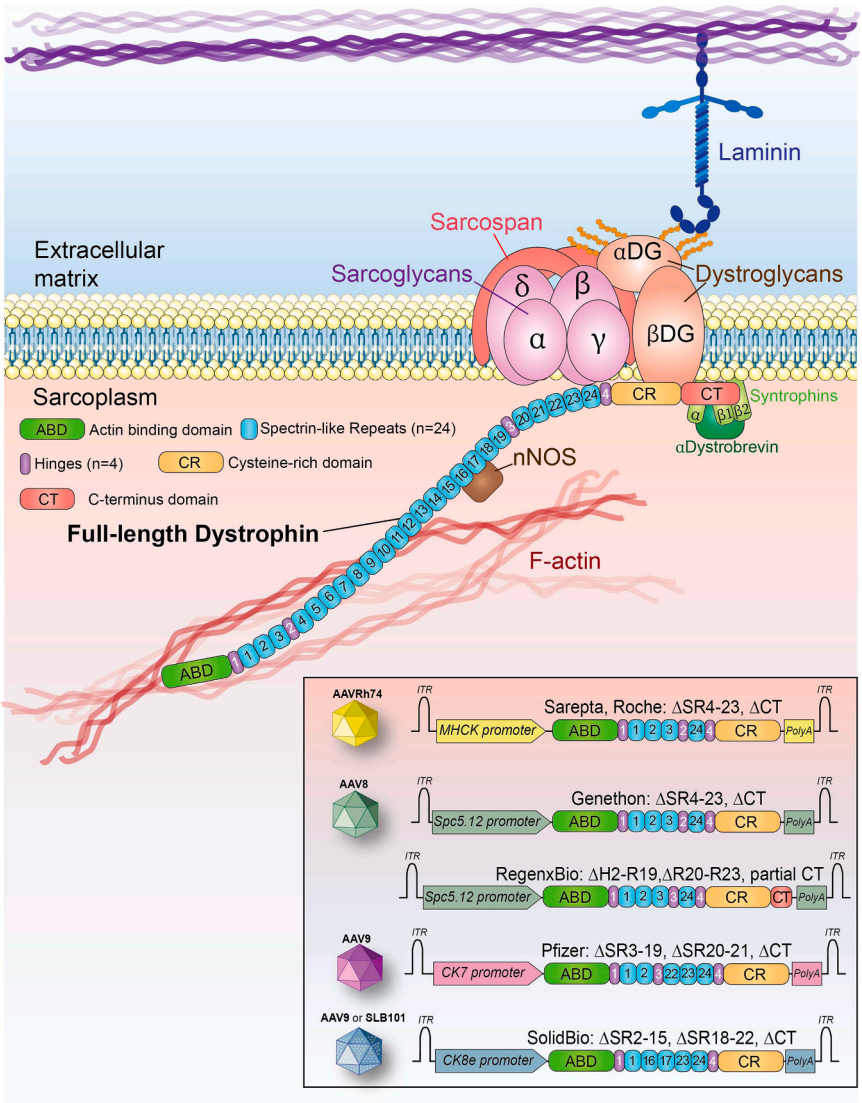

Figure 1. Structure of the dystrophin-glycoprotein complex and the AAV-μDys vectors being used in the clinic

(Top) Dystrophin is a rod-like protein that links at the N-terminal domain and in parts of the central rod domain to the subsarcolemmal cytoskeleton. The hinge 4 and the cysteine-rich (CR) domain bind β-dystroglycan, dystrobrevin, and possibly some of the sarcoglycans. The C-terminal (CT) domain has binding sites for various isoforms of syntrophin and dystrobrevin. β-Dystroglycan spans the sarcolemma, where it links to α-dystroglycan, the major laminin-binding protein in muscle. This linkage is reinforced by the sarcoglycan complex and sarcospan. The dystrophin-glycoprotein complex helps protect muscles from contraction-induced injury while also localizing some signaling molecules such as nNOS inside the sarcolemma, while also organizing several ECM proteins such as perlecan. (Inset) Diagram of the various μDys vectors. Shown are the different AAV serotypes, the organization of the various μDys proteins being expressed, and the companies directing each trial. To date, only the Sarepta vector has received approval by the US FDA.

therapy targets of their own.[32] Elucidation of the DGC structure began to reveal the role of dystrophin and the DGC, which are viewed as critical for maintaining the mechanical integrity of muscle cells by linking the subsarcolemmal actin cytoskeleton to the extracellular matrix (ECM) (Figure 1).[33,34] This complex facilitates lateral transmission of forces from within muscle cells into the stronger ECM, allowing uniform force development and distribution among neighboring myofibers, protecting muscle cells from contraction-induced injury, while also helping organize ECM proteins and localizing several intracellular signaling proteins, including neuronal nitric oxide synthase (nNOS).[35–38]

the initial gene cloning enabled great advances in prenatal diagnosis and carrier detection of the disorder, initially by Southern analysis[18] and later by the first application of multiplex PCR.[19–21] These cDNAs also allowed the identification of several mouse models for DMD, known as *mdx* (MD, X-linked) mice.[22–26] Antibodies raised against dystrophin revealed it to be a peripheral membrane protein localized to the inner face of the sarcolemma in all muscle types, and that its expression was absent, reduced, or of abnormal size in DMD and BMD patients.[27–29] Comparisons between wild-type and *mdx* mice led to the identification of a large complex of integral and peripheral membrane proteins later called the dystrophin-glycoprotein complex (DGC).[30,31] Discovery of the DGC led to the cloning of more than 20 genes encoding proteins that are either part of the DGC or are needed for assembly or function of the complex. In turn, many of these other genes have been found to be defective in other types of MD and have become gene

It was nearly 6 years after the dystrophin gene was identified before intact, full-length dystrophin cDNAs were cloned (13.9 kb cDNA, including the untranslated regions),[39] and several laboratories soon began generating transgenic animals expressing various dystrophin cDNAs to study the numerous protein domains. One early study used a muscle-specific expression cassette (MSEC) derived from the muscle creatine kinase (MCK) gene promoter and enhancers[40] (MCK-6.5) to express full-length dystrophin in *mdx* mice, which led to complete prevention of disease onset in muscles.[41] This approach not only demonstrated the feasibility of gene therapy for DMD but also showed that expression of a single dystrophin isoform only in striated muscles could eliminate essentially all muscle pathology.[41] While dystrophin has several upstream and internal promoters, is alternatively spliced, and has numerous non-muscle isoforms (e.g., motor neurons, CNS) this result greatly simplified further development of gene therapy for DMD, showing that an

effective therapy for loss of ambulation and premature death could result from the expression of a single cDNA in muscle.

Generating transgenic mice is not a clinically useful gene therapy, however, and numerous labs began searching for methods to deliver dystrophin expression cassettes to muscles of neonatal, adolescent, and adult mice. These early efforts were initially met with enormous excitement, followed by disappointment due to observations of inefficiency (of expression and/or biodistribution) or transient expression (due to immune responses against the delivery vector).[42] Nonetheless, such efforts added to the growing proof-of-principle that DMD could be treated by gene therapy and allowed elucidation of many of the critical requirements for successful gene therapy. The first such example came from the laboratory of Jon Wolff, who made the remarkable observation that intramuscular injection of naked plasmid DNA could lead to long-term gene expression.[43] Initially this approach was used to identify portions of dystrophin required to bind the sarcolemma[44] but later formed the basis for the first human DMD gene therapy trial.[45,46] While that trial demonstrated safety, only trace amounts of dystrophin were produced, which did not diffuse significant distances beyond the site of intramuscular plasmid injection. In the early 1990s enormous interest grew around the use of vectors derived from adenoviruses (Ads). Ad vectors lacking several viral genes led to high levels of dystrophin expression when injected directly into muscles.[47–49] However, these early Ad vectors could only carry so-called mini-dystrophins, based on the 6-kb cDNA derived from deletions in one of the aforementioned mild BMD cases.[14] Unfortunately, gene expression was found to be transient due to cellular immune responses that were elicited by residual, low-level Ad protein expression.[50] Later studies using such Ad vectors resulted in the tragic death of a patient in a liver gene therapy trial for ornithine transcarbamylase deficiency, which ground down the progression of the gene therapy field to a crawl for several years.[51] Improved Ad vectors lacking all viral genes and which could carry full-length dystrophins were developed (so-called gutted or helper-dependent Ad vectors).[52–54] However, such vectors were largely limited to intramuscular injection protocols, precluding widespread distribution, unless administered to entire limbs under high pressure and/or in the presence of vascular permeability or vasodilating agents.[55,56] Similar limitations tempered the enthusiasm for using oncoretroviral and lentiviral vectors,[57–60] although a new lentiviral vector pseudotyped to enable efficient targeting of activated satellite stem cells has recently generated newfound enthusiasm for delivering dystrophin cDNAs in the 6- to 8-kb range to skeletal muscles.[61]

A major breakthrough for muscle gene delivery came in 1996, when several groups reported that vectors derived from adeno-associated virus (AAV) displayed minimal immunogenicity when intramuscularly injected into mice.[62,63] This seeming lack of an immune response enabled long-term gene expression in muscles of adult mice for at least 1 year. Thus, for the first time a realistic scenario began to emerge for achieving long-term gene expression in skeletal muscles. However, numerous obstacles remained. First, AAV vectors have a carrying capacity of only ~4.7 kb, which includes the required 300 bp of viral inverted terminal repeat (ITR) sequences, thus allowing only ~4.4 kb for an expression cassette (promoter, cDNA, and polyadenylation site).[64] This created some difficulties for delivering a 13.9-kb cDNA. Second, intramuscular injection of a vector did not allow for cardiac muscle targeting, nor was there an obvious way to enable gene transfer to the more than 650 skeletal muscles in the human body. Even the 6-kb cDNAs derived from mildly affected BMD patients were too large for AAV. However, leading up to this time, several groups had been generating dozens of transgenic animal lines to develop an understanding of the function of different dystrophin structural domains. These studies showed that modest dystrophin function was compatible with deletions within the actin-binding domains but that such truncated proteins were often unstable.[65,66] In contrast, some, not most, deletions within the central rod domain, composed of 24 spectrin-like repeats (SRs), were compatible with high functional activity.[67–70] Importantly, half of all exon-intron boundaries in the rod domain do not align with the boundaries of the individual SRs. Consequently, the stability of many Becker-like dystrophins resulting from deletions in the rod domain is reduced due to the retention of partial SRs in the protein.[69] Inclusion of these domain fragments affects not only mini-dystrophin design but also exon-skipping strategies. By synthetically generating dystrophin cDNAs encoding precise, or properly phased SR domains, mini-dystrophins can be produced that are often more stable and functional than so-called natural BMD-proteins derived from multi-exon deletions (e.g., exon 17–48 or 13–48 deletions).[14,16,17,67,69] Surprisingly, deletion of the entire dystrophin C-terminal domain was not associated with any obvious functional deficits in *mdx* mice,[71,72] despite this region carrying binding sites for various syntrophin and dystrobrevin isoforms (Figure 1). Interestingly, the only region of dystrophin that when removed led to a total loss of function was the so-called cysteine-rich domain, now known to be the binding site for β-dystroglycan (the key transmembrane linker of the DGC).[73] Consequently, in 1997, our group proposed to the Muscular Dystrophy Association (MDA) and NIH (J.S. Chamberlain, personal communication) that therapeutically relevant dystrophins could be generated by deletion of most of the dystrophin rod-domain coupled with removal of the C-terminal domain. We also proposed that such micro-dystrophins (μDys) could be carried by AAV vectors together with MSECs, such as those being developed from the MCK locus.[69,74] Indeed, several such AAV/μDys expression cassettes were subsequently shown to be highly functional when injected into skeletal muscles of dystrophic *mdx* mice.[69,75,76] Importantly, not all μDys are equally functional, due to differences in their overall organization and choice of different functional domains that influence protein folding, stability, and binding to components of the DGC.[69,77–81] Consequently, efforts have continued for many years to optimize new μDys for enhanced DGC assembly and function.[80,81]

Despite the development of AAV/μDys vectors, there was still no obvious way to target muscles body-wide, including the heart. A clue to a possible way to do this came from the aforementioned studies by the group of Hansell Stedman, who demonstrated that Ad and AAV vectors could be delivered to isolated limb muscles

under conditions that included elevated pressure and/or co-delivery with vascular permeabilizing agents and vasodilators.[55] Consequently, we began testing intravascular delivery of AAV vectors together with similar cofactors. Our earliest studies enabled vector delivery only to the liver; however, in the presence of vascular endothelial growth factor (VEGF), which has vascular permeabilizing activity, we observed that intravascular administration of relatively high doses of AAV6 ($\sim 10^{13}$ vector genomes [vg]/kg) was associated with gene transfer into cardiac muscles, and to a lesser extent, skeletal muscle. While such levels of gene transfer are sub-therapeutic, we realized that further increases of the vector dose, at levels in the 10^{14} vg/kg range, enabled high-level, body-wide gene transfer into muscles even in the absence of VEGF.[82] These studies also suggested that high intravascular concentrations of the AAV capsid were sufficient to enable systemic gene delivery to striated muscles, suggesting a vascular permeabilizing activity of certain capsid serotypes. Consequently, this combination of μDys, coupled with an MCK-based MSEC, when delivered via a subset of AAV serotypes, enabled systemic delivery of dystrophin cassettes to muscles, paving the way for clinical trials.[82–84] Muscle-restricted gene expression was important for this approach, as delivery of some bacterial proteins, such as *Escherichia coli* β-galactosidase (β-gal) expressed from the ubiquitously active cytomegalovirus (CMV) enhancer/promoter, led to the rapid death of all systemically injected mice via a cytotoxic T cell-mediated immune response against β-gal. In contrast, swapping CMV for MCK expression cassettes led to lifelong, body-wide expression of β-gal (and μDys) with no adverse events.[82,85]

AAV CLINICAL TRIALS: PROS AND CONS

The relatively low-immunogenic nature of AAV vectors coupled with a method for systemic delivery spurred the onset of clinical trials for many types of MD, including DMD. The initial trials began cautiously and used intramuscular injections of therapeutic constructs into a single muscle.[86–90] Several important lessons were learned from these early trials. While the intramuscular injections were unable to produce functional benefits, they gave a readout on gene expression levels and immune responses. In particular, one DMD trial led to minimal gene expression, which was found to be due to a cellular immune response against several epitopes in μDys, and possibly in the AAV vector used.[89,90] Future studies with systemic delivery using muscle-restricted gene regulatory cassettes (RCs) avoided most of these immune-mediated events and enabled widespread μDys expression, albeit in a mosaic pattern.[91–93]

Six different biotech groups have entered the AAV-μDys clinical gene therapy space (Figure 1).[94,95] These groups include Sarepta (partnering with Roche in Europe), Solid Biosciences, Pfizer, Genethon, and RegenexBio. Sarepta has published several papers on their results (see above), while results from the other groups have largely come from press releases and conference presentations. While all involved intravascular delivery of AAV vectors carrying μDys cDNAs driven from MSECs, the composition of each company's vectors is unique. Sarepta and Roche have been using a rhesus monkey-derived serotype known as rh74, Solid and Pfizer have been using AAV9 (see below), while Genethon and RegenexBio use AAV8. Sarepta, Roche, and Genethon each use a cDNA that is similar, if not identical, to the first μDys developed (μDysH2) (J.S. Chamberlain, personal communication).[69] Pfizer used a somewhat similar μDys designed by the Xiao lab.[75] RegenexBio uses a μDys designed by George Dickson that carries a portion of the dystrophin C-terminal domain,[96] while Solid Biosciences uses the most recent μDys design, which is the only one that enables localization of nNOS to the DGC.[81] Different MSECs are used in the various trials, although all are muscle specific (Sarepta and Roche, MHCK7[97]; Genethon and RegenexBio, Spc5.12[98]; Pfizer, CK7[97]; and Solid Biosciences, CK8e[81]). Of these various trials, those by Sarepta and Roche had the most consistent outcomes with few serious adverse events (SAEs). Consequently, Sarepta obtained US Food and Drug Administration (FDA) approval for their drug in 2024, which is now able to be prescribed to all boys with DMD ages 4 and older.

The overall results of these various trials can best be described as mixed, with modest clinical benefit, although long-term outcomes remain unclear. None have resulted in a clear increase in strength, yet there often appears to be a slowing of disease progression. Neither Sarepta nor Pfizer were able to reach their primary clinical endpoints, although Sarepta did meet several secondary outcomes. Consequently, while Sarepta obtained FDA approval, Pfizer decided to abandon further development of their drug. Solid Biosciences, Pfizer, and Genethon each encountered some SAEs early on that slowed development. In those trials, some patients developed concerning, albeit transient issues, including thrombocytopenia and thrombotic microangiopathy (TMA). These issues appear to be related to innate immunity against the AAV vectors and may be dose, and possibly serotype, dependent. Almost all the trials have encountered issues with transient elevation of serum transaminases, which have largely been controlled via corticosteroids such as prednisone, although Sarepta announced in March 2025 that one of their patients died of liver failure within 2 months of vector infusion. Improved manufacturing methods, lower doses, and, in the case of Solid Biosciences, switching serotypes, appear to have largely eliminated many of these AEs. Thus, while initial trials administered doses in the range of $1–3 \times 10^{14}$ vg/kg, most of the ongoing trials are at the lower part of that range. Solid Biosciences recently reported early results from a new trial using one of a class of novel myotropic vectors that incorporate an integrin-binding ligand into the AAV9 capsid. Their latest results, reported in an early 2025 press release and at the 2025 MDA Clinical & Scientific Conference, showed no SAEs and the highest expression levels reported to date. Recent results from RegenexBio and Genethon are also reporting no SAEs and dystrophin positivity in the majority of patient myofibers (also presented at the 2025 MDA Clinical & Scientific Conference). μDys expression has been seen in all these studies in the range of ∼20%–80% dystrophin-positive myofibers with levels, via western blot, in the range of ∼20%–100% of endogenous dystrophin levels from healthy controls. It is important to note that surveys of μDys expression come from analysis of a single biopsy taken from a single muscle, although usually at two to three time points, including

baseline levels. Thus, it remains unclear how well these expression levels reflect what is occurring body-wide, and none of the groups have been able to assess what expression, if any, is occurring in the heart. Cardiac muscle expression is a critical consideration, as it has been shown that restoration of dystrophin in skeletal muscles but not cardiac muscle will exacerbate cardiomyopathy, sometimes with serious consequences.[99–101]

The SAEs observed to date have generally been transient, largely subsiding within 4–12 weeks of vector administration. However, several exceptions have occurred that have led to serious consequences and in at least two cases, a fatal outcome. One patient treated with the Pfizer vector died within 1 week of vector administration, likely due to a severe innate immune response against the AAV9 capsid that resulted in systemic inflammation and myocarditis. No autopsy was performed, however, preventing a full assessment of the cause of death. A somewhat similar rapid and fatal event resulting from acute respiratory distress syndrome was also seen in an older patient treated with an AAV9 vector designed to upregulate an upstream, alternate dystrophin promoter.[102] Also, a second Pfizer-treated patient died more than 1 year after treatment, in 2024, although the relationship to the gene therapy treatment remains unclear. As noted above, one Sarepta patient recently died of liver failure. The other SAEs of note have been found in 5 DMD patients treated with AAV-μDys in the Sarepta, Roche, and Genethon trials. In these cases, a very serious immune complication resulted from a cytotoxic T cell-mediated response against epitopes within the μDys coding sequence. From a joint analysis and highly collaborative investigation between the companies involved and outside experts, it became clear that the epitopes recognized were encoded in the region of dystrophin exons 8–11, and possibly just the 8–9 region.[103,104] Consequently, all ongoing AAV-μDys administration is excluding patients with deletions including exons 8–11, and in some cases additional regions encoded by company-specific μDys cDNAs. This is in addition to exclusion from all trials of any patient displaying elevated serum levels of neutralizing antibodies against the AAV vector, which can block muscle transduction and may be linked to instances of TMA.[103,105]

Together, the ongoing studies of AAV-μDys therapy suggest that the current approaches are having a modest impact on patient outcomes, but they also identify several issues that can be addressed to improve efficacy. At the same time, it is important to note that DMD is slowly progressive, and μDys-mediated improvements in muscle function may manifest further and increase over time. Still, AAV-μDys therapies have consistently been far more impressive and efficacious in animal models than in the clinic, partly because similar AAV doses lead to much higher and more uniform expression of μDys. So, what aspects of these therapies may be limiting, and what might be done to improve patients' responses? Some have already been mentioned above, including using lower doses and addressing immunological problems. Lower doses by themselves will not improve efficacy since they result in reduced gene transfer, which already appears significantly lower than in animal models.[78,82,106–110] However, the use of improved AAV vectors that are able to target muscle at lower doses, such as used in the recent Solid Bioscience clinical trial could dramatically increase expression at lower doses.[111–113] Such vectors could reduce, if not eliminate, TMA, thrombocytopenia, and liver toxicity. Improved MSECs could lead to higher and possibly more uniform μDys expression in different striated muscle types and could further blunt immune responses. Avoiding immune responses against dystrophin might be achieved by delivery of μUtrophin, a dystrophin paralog, as it would be less likely to be recognized as a foreign antigen in DMD patients. However, utrophin cannot replace all of the functions of dystrophins.[78,114–116] An alternate approach might be to use protein design software to redesign and deimmunize problematic epitopes in the dystrophin sequence, for example, with analyses via AlphaFold or RoseTTaFold.[117–120] Finally, strong evidence from analyses of patient deletions, transgenic mouse studies, and AAV delivery indicate that the ability to express dystrophins larger than μDys could lead to a significant improvement in muscle function.[69,110,121] Examples of such approaches are explored in the following sections.

DEVELOPMENT OF MYOTROPIC AAVs

A critical consideration in DMD gene replacement therapy is the need to target a sufficient number of myofibers. Ideally, therapeutic dystrophins should be expressed across the entire myofiber to efficiently protect it from the mechanical stress generated during muscle contraction. To this end, several groups have engineered a variety of new AAV capsid variants with high tropism to striated muscles (Table 1). Using ligand insertion, computational and rational design, *in vivo*-directed evolution, and capsid shuffling, a new capsid family has been developed, called myotropic AAVs, with high targeting to striated muscles at low doses (10- to 50-fold lower versus natural capsids).[111–113,122,123] Intriguingly, this novel class exhibits very low liver uptake (except for AAVcc47), making them more attractive to human application since administration of natural serotypes at high doses has resulted in acute liver damage.[124] Even though the efficacy of these new AAVs has been demonstrated in different mouse models of muscular dystrophies and congenital myopathies, as well as large animal models such as pigs and monkeys,[112,113,122,123] there are few data available about their efficacity in humans. As noted above, Solid Biosciences issued a press release in early 2025 showing promising data using a new myotropic capsid in their clinical trial. The data showed a high transduction efficacy with 70%–80% of μDys-positive myofibers, with protein expression detected at up to 135% of normal dystrophin levels. Importantly, these results support the safety of this new class of engineered AAV vectors, with 3 DMD participants being administered a dose of 1×10^{14} vg/kg. Additional studies are needed to confirm these promising data with a larger cohort of patients.

NOVEL TOOLS TO CIRCUMVENT THE AAV PACKAGING LIMITATION AND EXPRESS LARGE CONSTRUCTS

Several innovative approaches have been explored to deliver and express large dystrophins using dual or triple AAV vectors. The general

Table 1. Examples of myotropic vectors developed using different strategies

Capsid backbone	Myotropic AAV ID	Modification	Reference
AAV9	AAV9 AAV-MYO1	insertion of GQSGRGDLGL (P1ligand) at position 585–586	Weinmann et al. (2020)[111]
	AAV-MYO2	capsid shuffling (serotypes 1, 6, 8) + insertion of GQSGRGDLGL (P1ligand) at position 585–586	El Andari et al. (2022)[122]
	AAV-MYO3		
	MyoAAV2a	insertion of GPGRGDQTTL at position 585–589	Tabebordbar et al. (2021)[112]
	MyoAAV3a	insertion of RGDYVGL at position 588–589	
	MyoAAV4a	insertion of NSRGDYNSL at position 586–589	
	MyoAAV4c	insertion of QERRGDYTSM at position 585–589	
	MyoAAV4e	insertion of ENRRGDFNNT at position 585–589	
	AAV.cc47	mutagenesis of residues 452–458 (GVSLGGG)	Gonzalez et al. (2022)[113]
Hybrid capsid Cap9-Rh74	AAV-LICA1	insertion of TDGRGDLGRLGP at position 452–459	Hong et al. (2024)[123]

idea is to break the dystrophin coding sequence into pieces that fit into an AAV particle. Subsequently, upon simultaneous delivery, the fragmented dystrophin construct is restored through biomolecular processes involving DNA, RNA, or protein intermolecular interactions (Figure 2; Table 2).

AAV DNA *trans*-splicing

During the 1990s, different groups showed high levels and persistent transgene expression using AAV in muscle cells, neurons, and other nondividing cells.[62,137–139] This long-term expression was partly associated with the assembly of multiple AAV genomes and the formation of large circular concatemers via intermolecular and complementary joining of the palindromic ITRs.[137,140,141] These observations revealed unique biological properties of AAVs that have expanded the usefulness of this vector system. Early attempts have utilized the AAV genome concatemerization and the *trans*-splicing mechanism mediated by donor and acceptor sequences included in the 5′ and 3′ vectors, respectively, to express large transgenes.[142–146] For DMD, this method was first tested to express the mini-dystrophin ΔH2-R19, where the authors evaluated the splicing efficiency of four native *DMD* exon/intron junctions.[125] The study identified two sequences with high splicing activity. However, *in vivo* testing revealed higher protein expression with one combination, which led to encouraging improvements in muscle morphology and function in young *mdx* mice. This proof-of-concept study was followed by a series of dual-vector combinations to express a variety of mini-dystrophins (Table 2).[126,129,147] In these follow-up studies, the Duan lab examined the efficacy of this AAV *trans*-splicing approach to produce mid-size dystrophin constructs and the therapeutic effects in murine models of DMD via intramuscular injections. Despite encouraging data demonstrating successful formation of a large ORF, low levels of mini-dystrophins were detected from the co-delivery of the two vectors. A similar approach was evaluated to express full-length dystrophin (Dp427) using a triple vector delivery.[127,131] Two junctions with high consensus splicing value were designed to maximize the splicing activity. Nonetheless, simultaneous delivery of triple vectors into *mdx* muscles resulted in low efficiency, with few myofibers expressing detectable full-length dystrophin. Despite the tremendous effort from the different groups, this approach demonstrated limited protein expression with poor correction of the dystrophic phenotype.

Homologous recombination of AAV genomes

In parallel to the *trans*-splicing method, other studies explored another approach to express a variety of mini-dystrophin constructs. An overlapping and highly recombinogenic sequence was employed to reconstitute a single and large ORF. For DMD, early work demonstrated successful and broad expression of mini-dystrophin (ΔH2-R19) following systemic co-delivery of two AAV vectors.[128] A similar triple AAV approach from this same group to generate full-length dystrophin via homologous recombination resulted in only low levels of the protein (Guy Odom and J.S.C., unpublished observations). Other studies showed that the selection of highly recombinogenic sequences and hybrid ITRs, as well as codon optimization of the transgene cassette and the use of newly designed AAV vectors, are critical factors in the success of this approach.[130,131,148] Given the promising data obtained with dual AAV delivery to express various mini-dystrophin constructs, this strategy was evaluated to deliver the full-length dystrophin by administering triple AAV vectors. Due to the inability to identify a potent recombinogenic sequence to join the 5′ to the middle vector, Lostal et al. developed a hybrid approach where both DNA *trans*-splicing and overlapping homologous recombination approaches were tested to co-join the three DNA elements.[131] Although detectable reconstitution of the dystrophin ORF was achieved, expression was limited to only a few myofibers following local injections.

mRNA *trans*-ligation mediated by ribozyme

A recent study harnessed ribozymes to actively join two RNA fragments delivered by AAV vectors.[132] Several ribozyme sequences were designed, optimized, and tested to catalyze a seamless ligation of two RNA fragments into one mRNA, which was

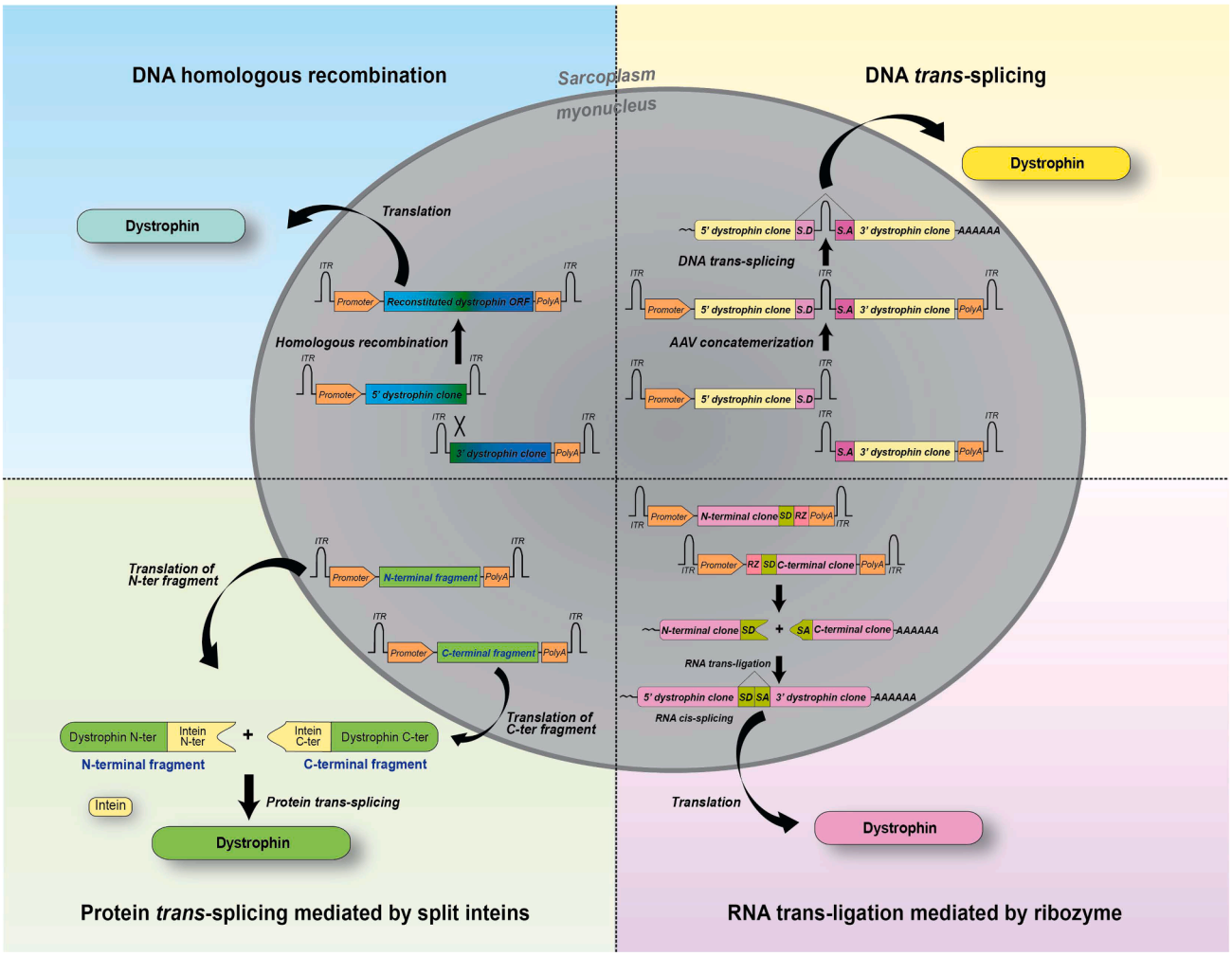

Figure 2. Different approaches developed to bypass the limited cargo capacity of AAVs and express large proteins from dual vectors

then translated to a functional protein. This novel method was validated to express various constructs such as reporter genes, gene-editing Cas9, full-length dysferlin, and a mini-dystrophin. For DMD, high levels of mini-dystrophin were detected across different muscles of young D2-*mdx* mice, resulting in a correction of markers of muscle damage, such as circulating creatine kinase and centrally nucleated myofibers. This novel technology has been successfully validated using a dual vector strategy and offers a new perspective on developing large therapeutic dystrophin constructs. It will be valuable to test its efficacy and specificity to express large proteins, such as full-length dystrophin, requiring delivery via three (or more) AAVs.

Joining dystrophin polypeptides with split inteins

Another strategy to express large dystrophins from smaller polypeptides involves protein *trans*-splicing (PTS) mediated by split inteins. PTS is a unique post-translational process described in unicellular organisms where two protein halves are joined seamlessly into a functional unit via the fusion and the extraction of inteins. Using first-generation Ssp6803 split inteins, early work attempted to express a mini-dystrophin based on genomic deletions in mildly affected patients with BMD.[133] Successful expression of this mini-dystrophin was confirmed in at least one intramuscularly injected muscle. Our group developed this approach further by comparing the PTS activity of an extensive split intein library.[110] We identified several potent candidates with high orthogonality. These candidates were successfully validated using dystrophin sequences in a dual vector approach where we demonstrated robust expression of one of the largest mid-sized dystrophins *in vivo* using young (mildly affected) and very old (very affected) *mdx*^{4cv} mice. By combining optimized split inteins, a potent MSEC (CK8e), and the novel AAV-MYO, we showed broad expression of midi-dystrophin in hindlimb, diaphragm, and heart muscles with significant improvement of the dystrophic phenotype. In the same study, as a proof of concept, we adapted this approach to express the full-length muscle isoform (Dp427) by selecting two highly specific split inteins to join three

Table 2. Summary of the approaches developed to express mid-size or full-length dystrophins from dual or triple AAV vectors

Approach	Dystrophin construct	DMD model	AAV serotype	Route (total dose)	Promoter	Reference
DNA splicing	mini-dystrophin ΔH2-SR19	*mdx* mice	AAV6	intramuscular ($1–4 \times 10^{10}$ vg)	CMV	Lai et al. (2005)[125]
	mini-dystrophin ΔSR2-15, ΔSR18-19	*mdx*^{4cv} mice	AAV6	intramuscular (2.4×10^{10} vg)	CMV	Zhang et al. (2012)[126]
	full-length dystrophin (Dp427)	*mdx* mice	AAV5	intramuscular (2×10^{10} vg)	CAG	Koo et al. (2014)[127]
DNA overlapping homologous recombination	mini-dystrophin ΔH2-SR19	*mdx*^{4cv} mice	AAV6	intramuscular (2×10^{10} vg); systemic, tail vein (2×10^{12} vg)	CMV	Odom et al. (2011)[128]
	mini-dystrophin ΔSR2-15, ΔSR18-19	*mdx*^{4cv} mice	AAV6	intramuscular (2.4×10^{10} vg)	CMV	Zhang et al. (2012)[126]
	mini-dystrophin ΔH2-SR19	DMD dog with point mutation in *DMD* intron 6	AAV9	intramuscular (4×10^{13} vg)	CMV	Kodippili et al. (2018)[129]
	quasi-dystrophin ΔSR4-7, ΔSR10-17, ΔSR18-19	*DBA2 mdx* mice	myotropic AAV (AAV9 based)	systemic, retro-orbital (8×10^{13} vg/kg)	Sp5-12	Albini et al. (2023)[130]
	full-length dystrophin (Dp427)	*mdx*^{4cv} mice	AAV9	intramuscular ($1.4\text{-}3.6 \times 10^{12}$ vg)	CMV	Lostal et al. (2014)[131]
mRNA *trans*-ligation mediated by ribozyme	mini-dystrophin ΔH2-SR15	*DBA2 mdx* mice	AAV9	systemic, intraperitoneal (3×10^{12} vg)	CK8e	Lindley et al. (2024)[132]
Protein *trans*-splicing mediated by split inteins	mini-dystrophin ΔH2-SR15	*mdx* mice	AAV1	intramuscular (2×10^{11} vg)	CMV	Li et al. (2008)[133]
	midi-dystrophin ΔSR5-15	*mdx*^{4cv} mice	AAV6, AAVMYO1	intramuscular (5×10^{10} vg); systemic, tail vein (2×10^{14} vg for AAV6, 2×10^{13} for AAVMYO1	CK8e	Tasfaout et al. (2024)[110]
	full-length dystrophin (Dp427)	*mdx*^{4cv} mice	AAV6, AAVMYO1	intramuscular (5×10^{10} vg); systemic, tail vein (2×10^{14} vg for AAV6, $4\text{-}8 \times 10^{13}$ for AAVMYO1	CK8e	Tasfaout et al. (2024, 2025)[110,134]
	full-length dystrophin (Dp427)	*mdx*^{4cv} mice	MyoAAV4A	systemic, retro-orbital ($8 \times 10^{13}–2 \times 10^{14}$ vg/kg)	Sp5-12	Zhou et al. (2024)[135]
	midi-dystrophins: ΔSR4-7, ΔSR14-15, ΔSR18-23; ΔSR2-3, ΔSR4-7, SR13-15, SR18-19; ΔSR4-7, Δ10-15, Δ18-19	*DBA2 mdx* mice	AAV9	systemic, retro-orbital (4×10^{13} vg/kg)	CK8e	Palmieri et al. (2024)[136]

CAG. chicken β-actin; CK8e, creatine kinase 8; CMV, cytomegalovirus.

protein fragments delivered by AAV vectors. We observed variable expression in different muscles analyzed using the natural serotype AAV6.[110] However, in a follow-up study, we found that using triple myotropic vectors improves the delivery to striated muscles, which alleviates or restores functional skeletal and cardiac defects.[134] Using the same split intein sequences, another study showed that the stoichiometry of the three vectors is critical to driving high expression of the full-length dystrophin.[135] More recently, Palmieri et al. utilized artificial intelligence tools to predict the stability of the protein fragments and simulate the interaction between split intein/dystrophin moieties.[136] These *in silico* tools will certainly guide future studies in designing novel constructs with improved and safer profiles.

THE CASE FOR GENOME EDITING

Advances in delivery and expression of larger dystrophins will undoubtedly increase the therapeutic potential of gene therapies for DMD. However, significant improvements, including long-term (and potentially lifelong) production of large dystrophins, may be achieved through genome editing approaches.

Early studies utilizing CRISPR-Cas9 to excise mutation-containing exons and reframe functional dystrophin mRNAs hinted at the power of genome editing for treating DMD.[149–151] Building on these studies, the path toward therapeutic application was seemingly made clear with seminal reports showing that *in vivo* delivery of CRISPR-Cas9 using AAV vectors could restore widespread dystrophin expression and ameliorate associated pathophysiology in mouse models of DMD.[152–156] Further methodological refinements restricted *in vivo* editing activity to striated muscles with improved efficiency[157,158] and expanded the scope of studies to preclinical large animal models.[159–161] While initial excitement over this new technology has been somewhat tempered recently, as several preclinical

studies have highlighted exciting possibilities but also major hurdles, its immense promise remains undeniable. One major advantage is that correcting DMD-causative mutations via genome editing restores native dystrophin expression from the endogenously regulated locus, substituting constitutive expression of dystrophin from episomal AAV vectors using MSECs. Although MSECs have proven incredibly effective for expressing AAV-delivered dystrophins,[81,109,110] their transcriptional activities are several log-fold higher than the endogenous dystrophin gene and offer little adaptive control over when and how much dystrophin is made. AAV-mediated dystrophin gene editing paradoxically often relies on the very same MSECs to express CRISPR-Cas,[155,157,158] and while muscle-specific gene expression precludes expression in immune effector cells, constitutive expression of bacterially derived editing components in muscle still presents a significant immunological challenge. Indeed, previous studies suggest that although Cas-like proteins may not be immunogenic in neonatal mice, they can be in older mammals.[162–165] Encouragingly, unlike AAV-delivered dystrophins, gene editing components theoretically only need to be expressed briefly to permanently correct dystrophin expression. Remodeling of skeletal muscles due to postnatal growth, exercise, or insufficiently corrected disease pathology presents a significant challenge to the stability of restored dystrophin expression, but stable correction is certainly possible in the heart, where cardiomyocytes are rarely replaced.[166] Encouragingly, long-term dystrophin expression may also be achieved in skeletal muscles upon editing of muscle stem cells (satellite cells[167] [SCs]).[154,168–170]

Compared to the broad patient applicability of dystrophin replacement methods, editing strategies will require some level of bespoke design to address the wide range of identified DMD genotypes. This entails screening and selecting highly efficient guide RNAs (gRNAs) that specifically target genomic regions comprising individual or clusters of closely grouped causative mutations. Additionally, patient mutation types (e.g., duplications, inversions, deletions, substitutions) will inform which editing strategy to employ for optimal results. More widely applicable approaches may aim to skip or excise one or more exons that are common between various patient mutations,[152–154,157,158,165,171] resulting in the production of truncated but partially functional mini-dystrophins, depending on the number of exons that need to be excised and/or the size of the deletion mutation that is being targeted. A somewhat related strategy has been employed using AAV vectors to deliver antisense oligonucleotides expressed from pol III promoters such as U7 to induce exon skipping.[172–176] Such designs could accelerate therapy development and help reduce treatment costs for groups of amenable patient mutations. However, precise editing strategies that restore expression of the full-length native dystrophin protein would also be tremendously valuable to maximize therapeutic potential. One such approach to restoring native dystrophin expression is to target duplication mutations. By inducing a double-stranded break at one site of a duplicated exon or series of exons, or by inducing skipping of one set of duplicated exons, the entire duplicated region can be excised, leading to the production of an essentially normal transcript.[177,178] This latter strategy has been reported by the Flanigan lab to be remarkably efficient in at least one exon 2 duplication patient, although the results have not yet been published.

Base and prime editing, which eliminate the need for CRISPR-induced double-stranded DNA (dsDNA) breaks,[179–181] have emerged as promising methods to restore dystrophin expression.[182–184] These represent safer and more specific approaches to earlier methods designed to excise or skip affected exons,[152–154,157,158,161,166,171,185] which often produce unwanted and possibly detrimental editing effects.[186] Base editing has shown significant therapeutic potential for inducing exon skipping or making pinpoint corrections of nonsense mutations with relatively high efficiencies.[165,182–184] However, this approach is not easily adaptable for mutations that span multiple exons and is not applicable for addressing deletion mutations without removing additional exons to reframe an even shorter mRNA. Thoughtfully tailored prime-editing techniques can achieve editing of larger genomic regions,[187] but most proof-of-principle studies have been conducted *in vitro*, and it is unclear whether resulting editing frequencies are sufficiently high to yield reliably significant therapeutic benefits *in vivo*.[182,188] Complete correction of deletion mutations by inserting missing genomic regions arguably represents some of the most challenging treatment scenarios from an efficiency perspective. Approaches leveraging homologous DNA repair (HDR) to integrate corrected DNA templates have been described but exhibit limited efficacy due to the low activity of HDR in postmitotic myonuclei.[157] Alternative promising homology-independent or homology-related approaches can improve editing efficiency,[157,189–191] but such methods often rely on the generation of dsDNA breaks and will likely need further efficiency optimizations to yield meaningful therapeutic benefits following systemic administration. Modified prime-based editing systems that initially install genomic landing sites for subsequent and more efficient integration of larger genomic sequences using add-on recombinases present a potential alternative for replacing or repairing large to very large mutations with relatively high efficiencies. However, prospectively useful levels of editing have thus far only been demonstrated *in vitro*.[192,193] Additionally, they require significantly more genetic material to be delivered to muscle via multiple AAV vectors or other effective delivery vehicles, including prime editor components to install the landing sites, the enzyme to catalyze the integration, and the DNA template to be inserted. The administration of additional foreign enzymes that catalyze template integration is also likely to exacerbate prospective immune responses. Overall, the portfolio of prospective editing approaches capable of correcting diverse mutational contexts continues to grow (Figure 3). However, different methods will likely be needed to optimally address divergent classes of DMD-causing mutations, which undoubtedly will increase development times and costs, as each approach may need separate approval to enter the clinic. Efforts are under way to streamline development by installing mechanisms for achieving approval for steps that are commonly shared across drug development platforms for different disease applications. This may entail broader approvals pertaining to the type of vector and editing system used, where only specific gRNA sequences and editing effects on intended target genes would warrant additional in-depth investigation.

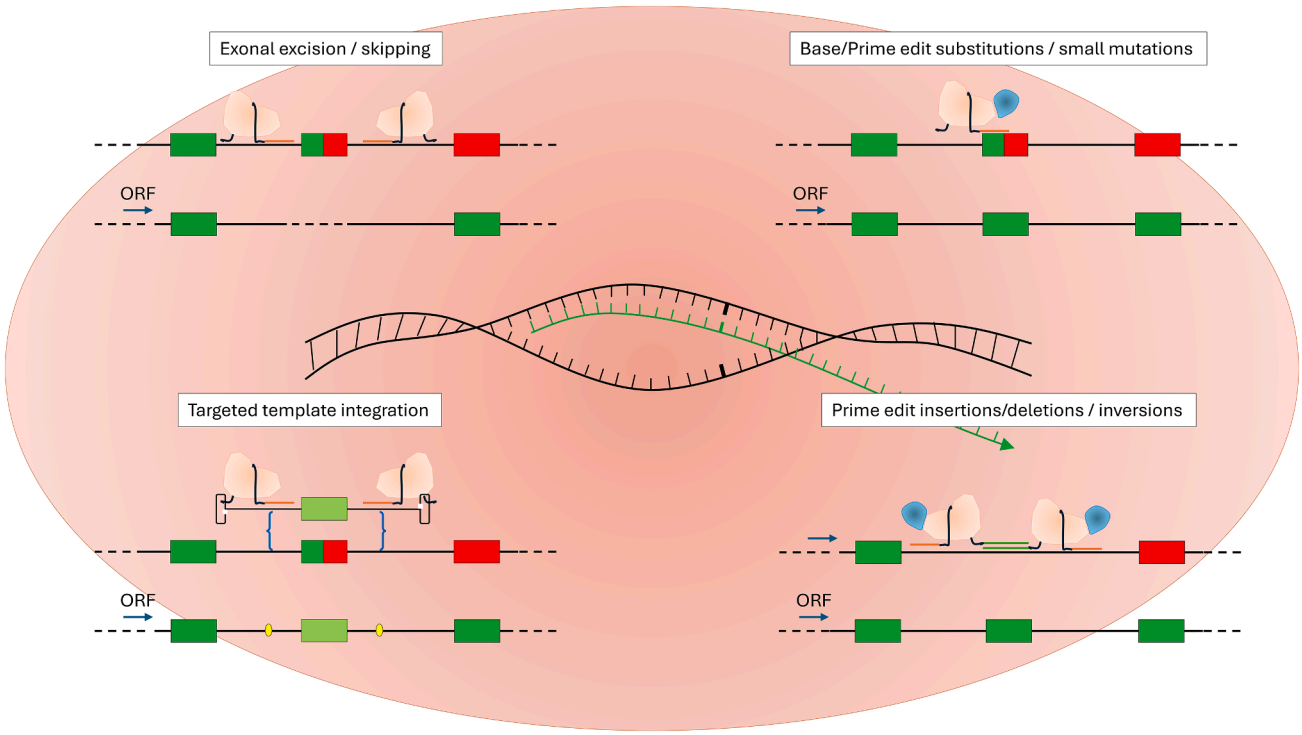

Figure 3. Examples of *in vivo* editing approaches for DMD

(Top left) Exonal excision- or skipping-based approaches to bypass one or more causative exons to restore an ORF encoding a truncated dystrophin. (Top right) Base or prime-based editing approaches to perfectly correct point or small mutations. (Bottom left) Homology dependent or independent integration of corrected template DNA to substitute affected or missing genomic regions. (Bottom right) Prime-based deletion or insertions to correct affected regions. Similar approaches can be used to reverse-transcribe site-specific recombinase landing sites into a genomic position for subsequent high-efficiency recombinase-mediated integration of larger genomic segments.

DELIVERY REMAINS A PRIMARY CHALLENGE TO TREATMENT EFFICACY

To date, the most promising results from efforts to correct dystrophin expression in animal models of DMD have been achieved using AAV vectors, but these studies have also identified significant concerns regarding the combined use of AAV and CRISPR-Cas.[165,186] The chief concerns include the integration of AAV vector sequences at sites of CRISPR-induced dsDNA breaks, large genomic rearrangements, and elicited anti-Cas immune responses.

In lieu of this, alternative methods to deliver editing components in the form of mRNA or mRNA/protein complexes to affected muscles using non-viral vehicles such as lipid nanoparticles have been explored. The advantage of such approaches is that editing components and editing activity would only be present during the time it takes for the mRNA or mRNA/protein components to be degraded. Conversely, these approaches offer little spatial control over editing activity, potentially increasing unintended editing in non-target tissues while elevating the risks of editing components being taken up by immune effector cells. Crucially, while non-viral-mediated gene editing can be used to correct dystrophin mutations using direct intramuscular injections,[194,195] widespread uptake of editing components in all muscle groups affected by DMD via the vasculature substantially lags transduction levels achieved with AAV. This gap has expanded significantly following the development of myotropic AAV capsids.[110] Encouragingly, more effective muscle transduction promises to improve editing efficiency and alleviate limitations associated with suboptimal dystrophin gene editing outcomes.[166] It also facilitates systemic delivery of split-AAV vectors encoding larger base or prime editors that increase therapeutic specificity and safety (Figure 3).[165,186]

SMALL CRISPR SYSTEMS, DRUG-INDUCIBLE VECTORS, AND SC EDITING WILL PLAY KEY ROLES IN DEVELOPING SUCCESSFUL EDITING-BASED TREATMENTS

Significant effort is also being made to identify and adapt smaller Cas systems for mammalian gene editing.[165,196–199] Such systems may allow packaging of base or prime editors in a single AAV vector, potentially improving editing outcomes by eliminating the requirement for target cells to be transduced with two separate AAVs.[200] Intriguingly, miniaturized CRISPR systems may also allow for the incorporation of additional regulatory elements to confer spatiotemporal and drug-inducible control over editing activity.[201] Such elements may be crucial for abolishing constitutive expression of

editing components that otherwise are likely to elicit immune responses or genotoxicity.

As the potential for making permanent genetic corrections arguably represents the biggest promise of genome editing in DMD, effective methods to edit SCs must be developed. Due to the relative rarity and quiescent nature of SCs, establishing optimal methods to deliver and express editing components will be crucial to ensure efficient and safe outcomes. Encouragingly, evidence suggests that AAV vectors may be capable of transducing cells that express SC markers,[112,154,169] although it is unclear to what extent these represent true long-term quiescent SCs,[202] as opposed to activated SCs and myocytes committed to terminal differentiation. Further screening of alternative AAV capsids, as well as other types of viral or non-viral delivery vehicles, will be useful in optimizing gene delivery to SCs. Importantly, effective and safe editing via the use of viral vectors will likely require RCs capable of expressing editing components specifically in SCs, ideally for no longer than what is required to achieve genome correction. As episomal AAV vectors are doomed to be lost in pools of proliferating SC progeny during muscle regeneration, there has been limited interest in such RCs for gene therapy applications prior to the emergence of genome editing. Now, however, their development could play a large role in fulfilling the potential for long-term correction of dystrophin expression in DMD.

Most published reports of virally mediated SC targeting have thus far relied on ubiquitous and constitutively active RCs (e.g., driven by CMV) to demonstrate successful SC targeting.[154] For reasons discussed earlier, such RCs are unlikely to be clinically applicable due to unwarranted risks of unintended editing and elicitation of immune responses.

Developing novel RCs based on gene regulatory elements involved in the expression of transcription factors that govern SC quiescence*versus* activation and differentiation appear to be prime candidates to achieve this goal. Our efforts to develop such RCs are showing great promise,[203] and further optimizations and validation studies are ongoing.

The gene therapy field has made enormous recent strides, and while *in vivo* muscle gene editing must still undergo extensive optimization before it can safely be administered to patients, depending on one's viewpoint, the future is as close as only a day away. Continuous improvements and novel discoveries are made almost daily, bringing hope that effective long-term therapies for DMD will soon be clinically available.

PROSPECTS

Developing gene therapy for DMD has made enormous progress since the dystrophin gene was isolated 40 years ago. The field has advanced from trying to understand the structure and function of dystrophin, exploring possible gene delivery systems, and attempting to overcome immunological barriers that have slowed nearly all attempts at human gene therapy. Alternate approaches to gene therapy are being developed by many labs, but here we chose to focus on those that use AAV vectors as they remain the only system able to demonstrate even modest efficiency for transferring genes to muscles body-wide. Still, new vectors are constantly being developed, and we should not lose sight of approaches that involve small molecules, oligonucleotides, and non-dystrophin approaches that may modify muscle pathology. The recent FDA approval for the first gene therapy of an MD (*elevidys*) was an enormous step forward, but it is only the beginning, as far more effective therapies are needed. Whether these will come from improved or novel vectors, larger transgenes, gene editing methods, or approaches not yet imagined remains unclear. However, progress is coming rapidly, and it is only a matter of time, and critically, continued research support, before the field can feel comfortable with the current state of progress.

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AUTHOR CONTRIBUTIONS

N.E.B., H.T., and J.S.C. planned, wrote, and edited the article. N.E.B. and H.T. created the figures.

DECLARATION OF INTERESTS

The University of Washington holds intellectual property related to μDys, mini-dystrophin, midi-dystrophin, AAV vectors, split-intein vectors, systemic gene delivery, muscle-specific expression cassettes, and gene editing of which the authors are inventors. J.S.C. and H.T. are members of the Scientific Advisory Board of Kinea Bio.

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