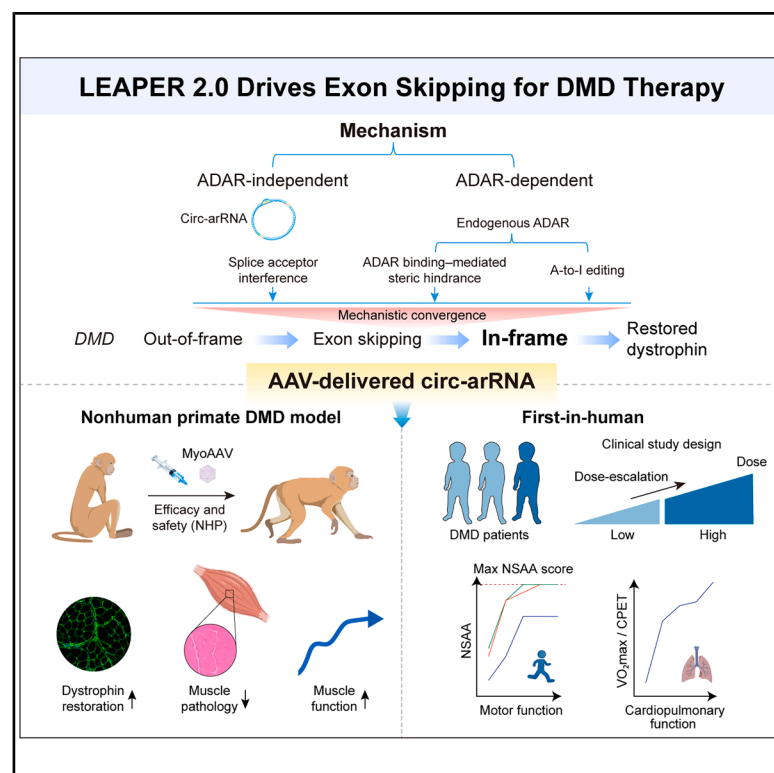


Long-term reversal of Duchenne muscular dystrophy via circular arRNA-guided exon skipping in monkeys and humans

Graphical abstract



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In brief

Circular ADAR-recruiting RNAs enable programmable exon skipping to bypass out-of-frame mutations in Duchenne muscular dystrophy and restore dystrophin expression. LEAPER 2.0 shows sustained efficacy in nonhuman primates and early clinical promise in patients, supporting circ-arRNA-mediated RNA editing as a translatable therapeutic strategy.

Highlights

- LEAPER 2.0 harnesses circ-arRNAs to drive exon skipping via endogenous ADAR
- circ-arRNAs restore dystrophin through both ADAR-dependent and -independent mechanisms
- A single administration confers durable benefit in DMD NHPs without anti-dystrophin immunity
- First-in-human dosing shows safe, dose-dependent exon skipping with early functional gain

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Article

Long-term reversal of Duchenne muscular dystrophy via circular arRNA-guided exon skipping in monkeys and humans

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SUMMARY

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular disorder caused by mutations in the *DMD* gene, leading to progressive muscle degeneration, loss of mobility, and premature death. Here, we applied leverage endogenous adenosine deaminase acting on RNA (ADAR) for programmable editing of RNA (LEAPER) 2.0, an RNA editing platform, to achieve exon skipping by harnessing endogenous ADAR through circular ADAR-recruiting RNAs (circ-arRNAs). By engaging key splicing elements, circ-arRNAs bypass out-of-frame *DMD* mutations and restore dystrophin expression through ADAR-dependent and ADAR-independent mechanisms. In DMD nonhuman primates (NHPs) carrying hotspot mutations, a single administration achieved durable dystrophin restoration and sustained motor improvement for at least 1.5 years without eliciting anti-dystrophin immune responses. In a first-in-human study, a single dose of adeno-associated virus (AAV)-delivered circ-arRNA produced safe, dose-dependent exon skipping in three patients, accompanied by measurable gains in motor and cardiopulmonary function. Together, consistent dystrophin restoration across DMD NHP models, patient-derived cardiomyocytes, and treated patients highlights the translational potential of circ-arRNA-mediated exon skipping as a therapeutic strategy for DMD.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a prevalent and devastating genetic disorder, impacting approximately 1 in every

3,500–5,000 male births.^{1–3} It stems from mutations in the *DMD* gene on the X chromosome, leading to the absence or malfunction of dystrophin in muscle fibers.⁴ This deficiency causes progressive muscle degeneration, loss of mobility, and premature



mortality. Most mutations involve deletions of one or more exons in *DMD*, destroying the gene's reading frame and preventing the production of functional dystrophin.^{5,6} In contrast, patients with Becker muscular dystrophy (BMD) have internally deleted but partially functional dystrophin.^{7,8} The challenges of gene therapy for DMD are mainly related to delivery, primarily due to the large size of the dystrophin cDNA.^{9,10} As a result, therapeutic strategies that focus on generating a dystrophin similar to that in BMD provide a promising avenue for treating DMD.^{7,8}

Oligonucleotide-mediated exon-skipping therapies transiently restore the open reading frame of dystrophin but require repeated administration due to rapid RNA turnover.¹¹ It is noteworthy that exon skipping could potentially benefit up to 80% of DMD patients.^{8,11} In principle, targeted adenosine-to-inosine (A-to-I) editing at splice regulatory elements can also induce exon skipping, as inosine is interpreted as guanosine during RNA processing.¹² We previously developed LEAPER (leverage endogenous adenosine deaminase acting on RNA [ADAR] for programmable editing of RNA),¹³ an RNA editing platform now advanced to LEAPER 2.0.¹⁴ This upgraded version is more efficient and durable, leveraging a circular RNA form (circular ADAR-recruiting RNA [circ-arRNA]) to recruit endogenous ADAR proteins for A-to-I editing. Delivered via adeno-associated virus (AAV), LEAPER 2.0 mediates long-lasting RNA editing in both mice and nonhuman primates (NHPs).¹⁵

In this study, we employed circ-arRNA to disrupt splicing of *DMD* through both ADAR-dependent and ADAR-independent pathways, enabling exon 5 skipping (to correct mutations within exon 5) and exon 51 skipping (to address mutations in exon 50). Notably, targeting exon 51 could potentially benefit approximately 13% of DMD patients.¹⁶ When delivered via muscle-tropic AAV (MyoAAV)¹⁷ in NHP *DMD* models, circ-arRNA efficiently restored functional dystrophin expression and reversed disease phenotypes with durable safety. Moreover, clinical application of AAV-delivered circ-arRNA demonstrated dose-dependent exon skipping and improved motor and cardiac function in DMD patients, underscoring its translational promise as a durable and safe therapeutic strategy.

RESULTS

circ-arRNA enables efficient exon skipping at multiple endogenous loci through both ADAR-dependent and ADAR-independent mechanisms

Unlike DNA editing, which acts on a single genomic copy, RNA editing can modify multiple RNA transcripts within each cell; therefore, increasing the abundance of RNA-editing components can enhance overall editing efficiency. Circular RNAs, lacking both 5' and 3' ends, are resistant to exonuclease degradation and therefore exhibit greater stability and persistence than linear RNAs.¹⁸ Theoretically, the LEAPER platform enables adenosine targeting for A-to-I RNA editing,^{13,14} including those located at splice acceptor (SA) sites, intron branch points (BPs), exonic splicing enhancers, and premature termination codons (PTCs). To evaluate this concept, we designed a 151-nt circ-arRNA targeting an adenosine at a canonical SA site, with 75 nucleotides flanking each side to encompass the intron-exon boundary (Figure 1A). Urea-PAGE confirmed robust

expression and circularization of arRNA in HEK293T cells (Figure S1A). We reasoned that by directing circ-arRNA to critical splicing elements, exon skipping could be triggered through both ADAR-dependent RNA editing and ADAR-independent steric interference, particularly involving nuclear ADAR1 p110.¹⁹ To dissect the contribution of ADAR activity, we compared exon-skipping efficiencies in wild-type, *ADAR1*-knockout, and *ADAR1*-rescued HEK293T cells.

Three key observations emerged. First, loss of ADAR1 or expression of a catalytically inactive ADAR1 abolished A-to-I editing, whereas reintroduction of catalytically active ADAR1 restored efficient editing in the CG2 reporter (Figure 1B). CG2 is a convenient dual-fluorescent reporter (mCherry-TAG-green fluorescent protein [GFP]) in which arRNA targets the intervening UAG codon, and endogenous ADAR-mediated editing converts UAG to UGG, thereby restoring GFP expression as a direct readout of functional ADAR activity.¹⁴ Second, circ-arRNA alone was sufficient to induce exon skipping even in *ADAR1*^{-/-} cells (Figure 1C), suggesting that steric hindrance by highly expressed circ-arRNA (Figures 1D and S1A) impedes spliceosome access to the SA and BP sites, functionally mimicking antisense oligonucleotides (ASOs). Third, the presence of active ADAR1 further augmented exon skipping, highlighting a synergistic effect between ADAR1 recruitment and RNA editing (Figures 1B and 1C).

Mechanistically, ADAR binds double-stranded RNA (dsRNA) through its dsRNA-binding domains.²⁰ The formation of a dsRNA duplex between circ-arRNA and pre-mRNA may hinder spliceosome recognition and promote exon exclusion (Figure 1E). Previous studies suggest that ADAR occupancy can negatively correlate with other RNA-modifying proteins on transcripts.²¹ Consistent with this model, catalytically inactive ADAR1, despite lacking editing activity, enhanced exon skipping in *ADAR1*^{-/-} cells (Figures 1B, 1C, S1B, and S1C), implying that ADAR1 binding alone disrupts exon definition.^{22,23} Conversely, catalytically active ADAR1 further promoted exon skipping by introducing multiple A-to-I edits that destabilize splice motifs (Figures 1D–1F).

Applying LEAPER 2.0, we achieved up to 60% exon skipping of *RELA* exon 7 (Figure 1G). circ-arRNA targeting four *PPIB* introns exhibited variable skipping efficiencies (Figure 1H) and differential editing rates across intron-exon boundaries (Figures S1D–S1H). Notably, steric hindrance contributed more prominently to exon skipping in *RELA*, whereas RNA editing dominated in *PPIB* exon 3 (Figure 1I). These differences likely reflect transcript-specific determinants such as sequence context, RNA secondary structure, accessibility, and local RNA-binding protein (RBP) occupancy.^{24–26}

Together, these findings demonstrate that circ-arRNA-mediated exon skipping arises from a coordinated interplay between ADAR recruitment, A-to-I editing of splice elements, and steric blockade of spliceosome assembly. This dual-mode mechanism, combining editing-dependent and editing-independent pathways, confers upon LEAPER 2.0 a unique advantage over conventional ASO-based exon-skipping approaches (Figures 1D–1F).

Optimization of tandem circ-arRNA design enhances exon skipping in DMD

To investigate the exon-skipping potential of circ-arRNA in *DMD*, we designed constructs based on rhesus monkey sequences

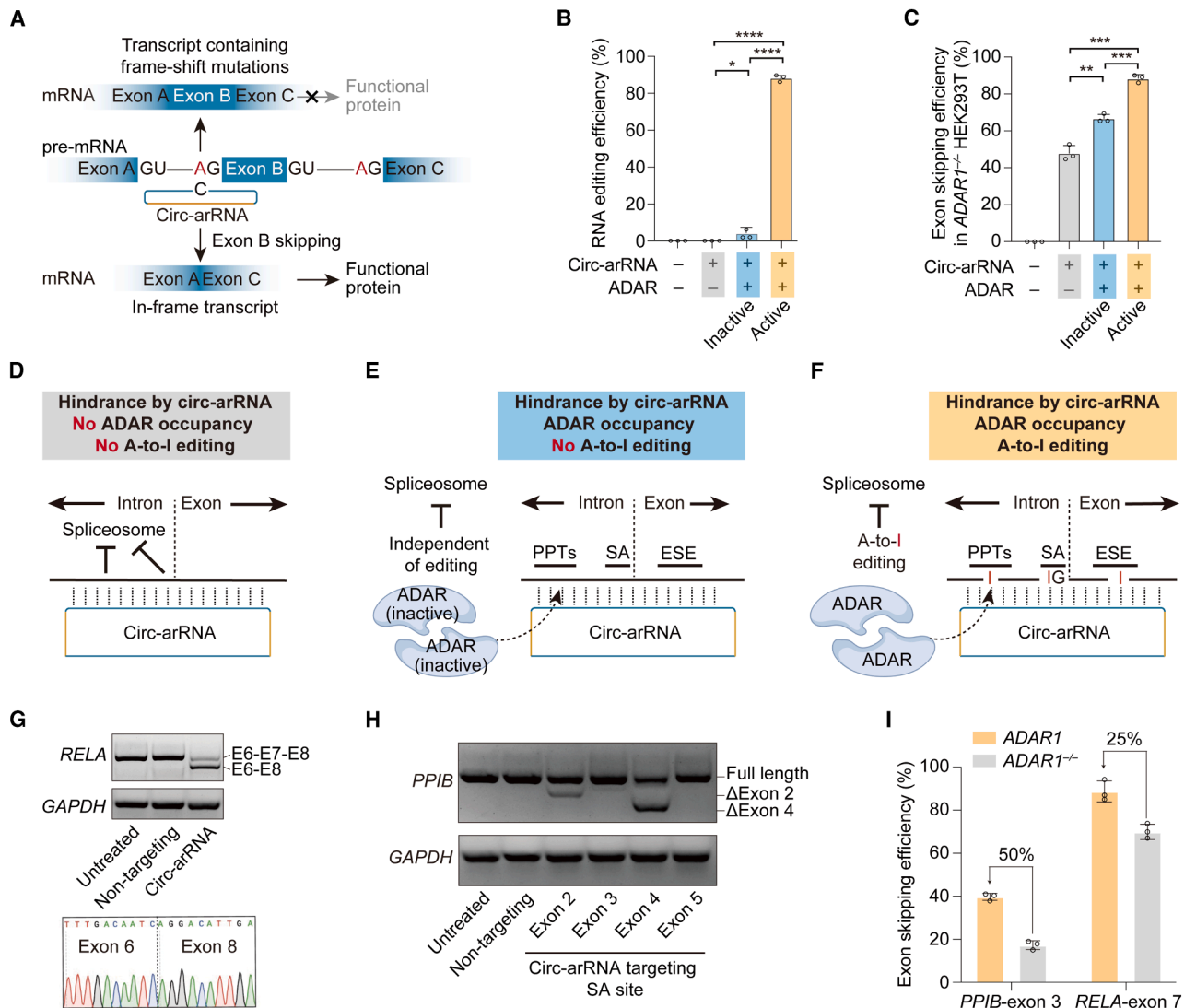


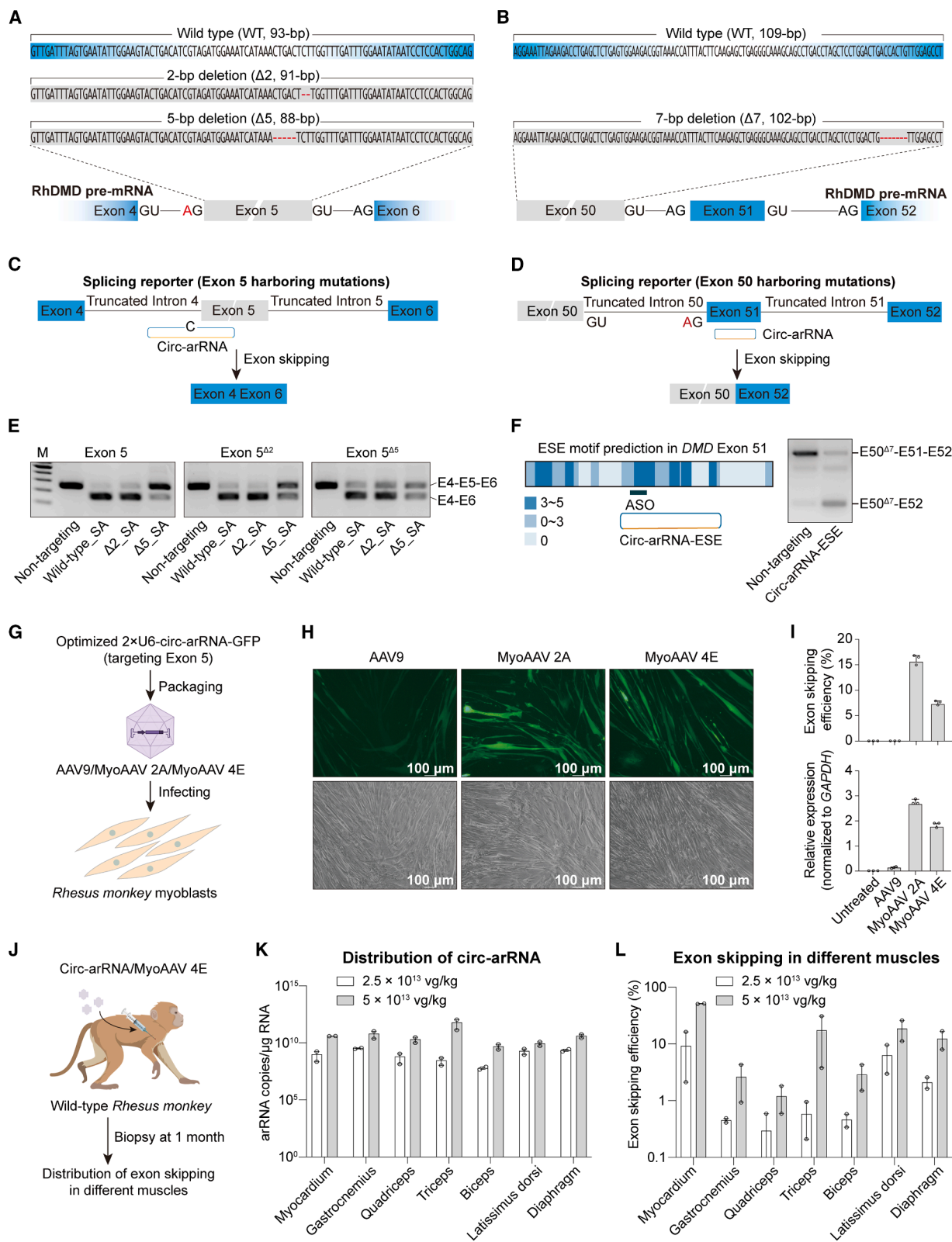
Figure 1. circ-arRNA facilitates effective exon skipping through ADAR-dependent and -independent mechanisms

(A) Illustration s how circ-arRNA mediated exon skipping restores expression of in-frame transcripts.
 (B) RNA editing efficiency was quantified by the CG2 reporter¹⁴ in HEK293T *ADAR1*^{-/-} cells with overexpression of different forms of ADAR1. HAE in ADAR1 represents the catalytically active form (editing by ADAR, yellow), whereas QAA or HAG represents inactive forms (blue). $n = 3$, mean $\pm$ SD. * $p = 0.0127$, **** $p = 2.6410 \times 10^{-8}$ (gray vs. yellow), **** $p = 3.0240 \times 10^{-10}$ (blue vs. yellow).
 (C) Exon 5 skipping efficiency of the splicing reporter (exon 4-intron 4-exon 5-intron 5-exon 6) derived from the rhesus monkey *DMD* sequence in HEK293T *ADAR1*^{-/-} cells with overexpression of different forms of ADAR1 as in (B). $n = 3$, mean $\pm$ SD. ** $p = 0.0018$, *** $p = 0.0001$ (gray vs. yellow), *** $p = 0.0003$ (blue vs. yellow).
 (D–F) Mechanism of circ-arRNA-mediated exon skipping.
 (G) Exon-skipping efficiency in HEK293T cells transfected with circ-arRNA₁₅₁ targeting the splice acceptor (SA) site of intron 7 in *RELA* (top), and Sanger sequencing of exon junctions after exon skipping (bottom).
 (H) Exon-skipping efficiencies of individual *PPIB* exons in HEK293T cells transfected with circ-arRNA₁₅₁ targeting SA sites in introns 1–4 of *PPIB*.
 (I) Exon-skipping efficiency of circ-arRNA₁₅₁ targeting the SA site at intron 6 of *RELA* and intron 3 of *PPIB* with or without ADAR in HEK293T *ADAR1*^{-/-} cells. $n = 3$, mean $\pm$ SD.
 See also Figure S1.

(Figures 2A and 2B). Circular arRNAs displayed markedly higher editing efficiency and broader editing scope compared with their linear counterparts (Figures S2A–S2C).

To further enhance editing and splicing modulation, we engineered a series of tandem circ-arRNA expression cassettes by

linking two U6 promoters via an extended linker sequence, following previously optimized architectures.^{27–29} The resulting variants, designated 2×U6-circ-arRNA₁₅₁-v1–3, were evaluated for their ability to induce exon 5 skipping (Figures S2A–S2C). Among them, 2×U6-circ-arRNA₁₅₁-v3 exhibited the strongest



(legend on next page)

activity, achieving the highest expression level and editing rate at the SA site and adjacent splicing elements (Figures S2C and S2D). The degree of exon skipping correlated closely with SA-site editing efficiency across variants (Figure S2B). Quantitative analyses further demonstrated that 2×U6-circ-arRNA₁₅₁-v3 maintained robust expression even under low transfection conditions (Figures S2E and S2F). However, the mechanistic basis for the superior performance of v3 remains unclear, as all linker variants uniformly increased circ-arRNA abundance (Figure S2G). One possibility is that linker length or sequence composition modulates local cassette spacing or transcriptional interference between adjacent U6 promoters, effects that become more evident under conditions of low vector copy number (VCN). Overall, this optimized construct achieved up to 80% exon 5 skipping, attributed to enhanced circ-arRNA expression and editing activity at splice sites. Based on these superior properties, 2×U6-circ-arRNA₁₅₁-v3 was used for all subsequent studies.

circ-arRNA efficiently induces exon 5 and exon 51 skipping in DMD models

Our previously generated DMD NHP models harboring 2- or 5-bp deletions in exon 5 and a 7-bp deletion in exon 50 recapitulate the key pathological features of DMD, including dystrophin loss and progressive muscle weakness.^{30,31} Using these pathogenic contexts, we generated reporter systems carrying Δ2 or Δ5 deletions in exon 5 and Δ7 deletions in exon 50 to evaluate exon-skipping efficiency (Figures 2C and 2D). In splicing reporter assays to evaluate exon 5 skipping, we designed circ-arRNAs complementary to either the wild-type or mutant sequences to restore the reading frame.

Strikingly, circ-arRNAs targeting the wild-type sequence consistently outperformed those designed against mutant sequences across all exon 5 reporters (Figure 2E), suggesting that the wild-type configuration offers a more accessible or stable binding interface. This enhanced activity correlated with higher circ-arRNA expression levels (Figure S2H), underscoring the importance of transcript abundance in determining functional efficacy. However, circ-arRNAs fully complementary to Exon 5^{Δ5} or Exon 5^{Δ2} exhibited reduced exon-skipping efficiency, likely due to changes in sequence or structural context

that impair binding stability and/or circ-arRNA abundance, although the exact mechanism remains to be defined.

Because skipping exon 51 could rescue the most frequent mutation hotspots in human *DMD*, we next examined whether circ-arRNA could promote exon 51 skipping to correct such mutations. Targeting exon splicing enhancers (ESEs) significantly increased skipping efficiency (Figures S2I and S2J). In particular, a circ-arRNA directed against an ESE motif within the central region of exon 51 achieved the most potent exon-skipping activity (Figures S2K and S2L). Length-dependent analyses further revealed that longer circ-arRNAs correlated with higher exon-skipping efficiencies (Figures S2M and S2N). Using a reporter harboring a 7-bp deletion in exon 50 (exon 50^{Δ7}), circ-arRNA-mediated exon 51 skipping successfully restored the open reading frame (Figure 2F).

Collectively, these results demonstrate that optimized tandem circ-arRNA designs enable efficient and programmable exon skipping in *DMD*, offering a versatile strategy to correct diverse mutations by skipping exon 5 or exon 51.

Multiple adenosine edits synergistically drive exon skipping

We next examined whether multiple adenosine edits within intronic and exonic regions synergistically promote exon skipping. We hypothesized that A-to-I editing occurring in the dsRNA formed between circ-arRNA and its target transcript could further hinder spliceosome recognition and thereby enhance exon exclusion. To identify which edits most effectively promote splicing modulation, we introduced a series of A-to-G substitutions at specific adenosines near the *DMD* exon 5 region and assessed their impact on exon skipping using splicing reporter assays (Figure S3).

To our surprise, individual A-to-G substitutions at either the SA or BP sites modestly increased exon 5 skipping (Figures S3A and S3B). Combining multiple substitutions at SA and BP produced stronger effects, though exon skipping was still incomplete (Figure S3C). Strikingly, when all intronic adenosines were converted to inosines, exon skipping reached 100% efficiency (Figure S3D). Reverse-transcription PCR (RT-PCR) confirmed precise exon 5 exclusion, producing transcripts that joined exons 4 and 6 (Figure S3B). Similarly, complete conversion of

Figure 2. circ-arRNA effectively induces exon skipping of *DMD* and achieves high muscle transduction efficiency via MyoAAV

(A and B) Diagrams illustrating the mutations in exon 5 and exon 50 of *DMD* in DMD monkey models.

(C and D) Splicing reporters for analyzing exon skipping of *DMD* mutant sequences (rhesus monkey) in HEK293T cells. RT-PCR results illustrating the exon-skipping efficiency achieved by 2×U6-circ-arRNA₁₅₁ targeting the SA site (wild-type, Δ2, and Δ5) in exon 5 with different sequences (exon 5, exon 5^{Δ2}, and exon 5^{Δ5}) and 2×U6-circ-arRNA₁₅₁ targeting the ESE (exon 50^{Δ7}).

(E) RT-PCR results of exon skipping in different sequences of exon 5. Wild-type_SA, Δ2_SA, and Δ5_SA represent circ-arRNAs designed for different sequences of rhesus monkey exon 5 in splicing reporter (C).

(F) RT-PCR results of skipping rhesus monkey exon 51 in splicing reporter (D). The targeted position of arRNA is shown on the left.

(G) A schematic diagram of AAV-delivered circ-arRNAs infecting rhesus monkey myoblasts.

(H) Visual inspection of AAV-delivered enhanced green fluorescent protein (EGFP) expression in rhesus monkey myoblasts after injection on day 10. Scale bar, 100 μm.

(I) (Top) The percentages of exon skipping in exon 5 achieved by AAV-delivered 2×U6-circ-arRNA₁₅₁-v3 targeting the wild-type SA site. (Bottom) Relative expression levels of arRNAs in rhesus monkey myoblasts injected with different capsids of AAV, normalized to *GAPDH*; *n* = 3, mean ± SD.

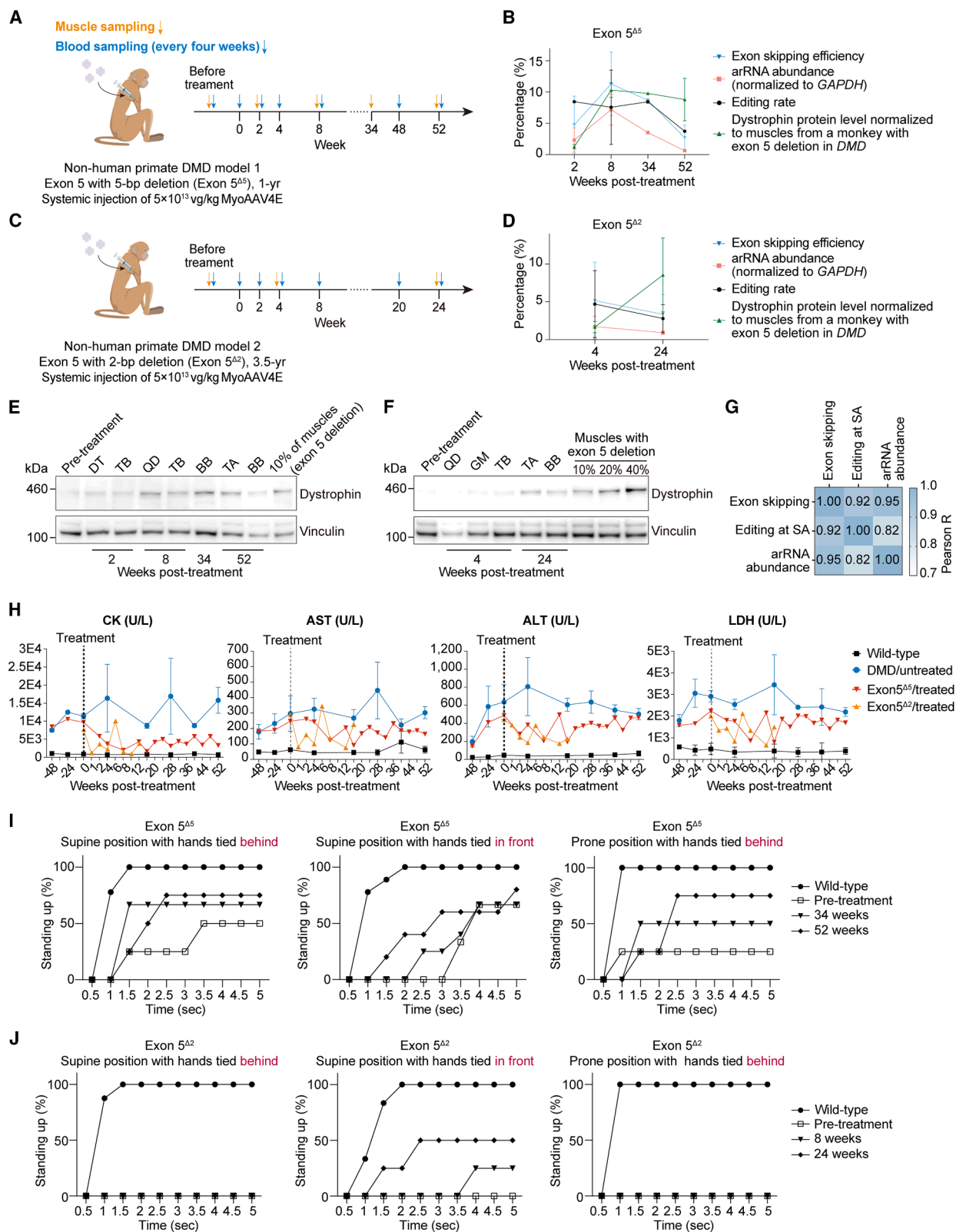
(J) 5 × 10¹³ vg/kg MyoAAV 4E injected systemically into wild-type monkeys carrying circ-arRNA₁₅₁ targeting exon 51 of *DMD* (rhesus monkey).

(K) Expression levels of arRNAs in monkeys administered different doses of MyoAAV4E across various muscle tissues.

(L) Exon-skipping efficiency of exon 51 in rhesus monkey *DMD* in different muscle tissues.

For data in (K) and (L), *n* = 2 for treated groups at two doses, mean ± SEM. *n* represents the number of monkeys.

See also Figures S2 and S3.



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adenosines within the exon and SA site fully abolished exon inclusion (Figure S3D).

We next focused on five adenosines identified as key determinants of exon definition (Figure S3E). Next-generation sequencing (NGS) revealed activation of cryptic splice sites in these contexts (Figure S3F), suggesting that extensive editing destabilizes canonical splicing signals (Figure S3G). These data indicate that robust exon skipping results from the cumulative disruption of multiple adenosines rather than a single key edit.

In our previous work, we showed that removing uridines paired with non-target adenosines can reduce bystander editing in coding regions.¹⁴ However, in the context of exon skipping, deletion of these uridines suppressed editing at critical positions and markedly reduced exon-skipping efficiency (Figure S3H). Thus, simultaneous editing of multiple adenosines within the circ-arRNA-targeted region yields superior exon skipping compared with editing only the SA (0) site. Although bystander editing poses concerns in coding regions due to possible amino acid changes, this is less problematic in splicing modulation, where the objective is to disrupt exon recognition rather than alter coding potential. Moreover, rational circ-arRNA design, guided by splicing regulatory databases, can mitigate risks of unintentional splicing perturbations or bystander edits when targeting mRNA rather than pre-mRNA.

Collectively, these findings demonstrate that ADAR-mediated multi-site editing enhances exon skipping by globally impairing spliceosome recognition of targeted regions. This mechanism distinguishes circ-arRNA-mediated exon skipping from ASOs (which rely solely on steric blockade¹¹) and from DNA base editors (which operate over limited ranges constrained by short single-guide RNA [sgRNA] targeting³²).

circ-arRNAs delivered via MyoAAV achieve efficient muscle transduction and exon skipping *in vivo*

To enable *in vivo* delivery, 2×U6-circ-arRNA_{151-v3} targeting the wild-type exon 5 was packaged into AAV9, MyoAAV 2A, or MyoAAV 4E vectors and tested in rhesus monkey myoblasts

(Figure 2G).¹⁷ Both MyoAAV 2A and MyoAAV 4E significantly outperformed AAV9 in driving exon skipping (Figures 2H and 2I). Taking into account that MyoAAV 4E demonstrated the highest transduction efficiency, particularly in cardiomyocytes—a key therapeutic target in DMD due to the high prevalence of cardiac involvement—it was selected for subsequent NHP studies. Although MyoAAV 4E showed lower exon-skipping efficiency than MyoAAV 2A in certain *in vitro* assays (Figure 2I), its favorable tissue tropism and overall *in vivo* performance, characterized by stronger cardiac transduction and reduced liver targeting relative to AAVrh74, were considered more relevant for advancement in monkeys.¹⁷

To validate systemic delivery, we first administered MyoAAV 4E-circ-arRNA in wild-type monkeys and evaluated exon 51 skipping (Figure 2J). MyoAAV transduced a broad spectrum of muscle tissues, including skeletal and cardiac muscle, with high efficiency (Figure 2K). Four weeks post-injection, exon skipping was readily detected, especially in the myocardium (Figure 2L), notable given the major contribution of cardiomyopathy to DMD morbidity and mortality.³³

MyoAAV-delivered circ-arRNA restores functional dystrophin in two DMD monkey models

To assess therapeutic potential, MyoAAV 4E carrying the 2×U6-circ-arRNA_{151-v3} targeting exon 5 was systemically administered (5×10^{13} vg/kg) to two DMD rhesus monkey models: a 1-year-old harboring a 5-bp deletion in exon 5 (exon 5^{Δ5}) and a 3.5-year-old with a 2-bp deletion (exon 5^{Δ2}). Because the two animals differed in age, disease status, and biopsy feasibility, the sampling schedules were deliberately tailored. Exon 5^{Δ5} underwent both early and late biopsies (2, 8, 34, and 52 weeks) to assess onset and durability, whereas exon 5^{Δ2} was sampled at selected post-treatment time points (4 and 24 weeks) to minimize repeated invasive procedures. In both animals, blood samples were collected longitudinally at regular intervals (Figures 3A and 3C). The dose of AAV-delivered circ-arRNA was selected based on prior safety data in NHPs.¹⁵ Immunosuppressive therapy was initiated 3 days before injection and continued for

Figure 3. Efficient exon skipping and dystrophin restoration in two DMD monkey models harboring mutations in exon 5

- (A) Schematic illustrating the injection of MyoAAV-delivered circ-arRNA in a DMD rhesus monkey with a 5-bp deletion in exon 5 (exon 5^{Δ5}).
- (B) Persistence of circ-arRNAs (RT-qPCR results showing arRNA abundance), resulting in corresponding RNA editing rates (targeted amplicon NGS of the SA site) and exon-skipping ratios (RT-PCR) at different time points after systemic injection. $n = 3$ (DT, TB, and QD) at 2 and 8 weeks, mean $\pm$ SD. $n = 1$ (BB) at 34 weeks. $n = 2$ (TA and BB) at 52 weeks, mean $\pm$ SD. n represents the sampling number of muscles.
- (C) Schematic illustrating the injection of MyoAAV-delivered circ-arRNA in a DMD rhesus monkey with a 2-bp deletion in exon 5 (exon 5^{Δ2}). Blue arrows denote the time points for blood sample collection, while orange arrows indicate the time points for muscle sampling in (A) and (C).
- (D) Persistence of circ-arRNAs (RT-qPCR results showing arRNA abundance), resulting in corresponding RNA editing rates (targeted amplicon NGS of the SA site) and exon-skipping ratios (RT-PCR) at different time points after systemic injection; $n = 3$ (GM, TB, and QD) at 4 weeks, $n = 2$ (TA and BB) at 24 weeks, mean $\pm$ SD. n represents the sampling number of muscles.
- (E and F) Western blot results of muscle samples from DMD monkeys before and after treatment in the Exon 5^{Δ5} (E) and Exon 5^{Δ2} (F) models. Control refers to muscle from a monkey with 100% exon 5 deletion. DT, deltoid; TB, triceps brachii; QD, quadriceps; BB, biceps brachii; TA, tibialis anterior; GM, gastrocnemius.
- (G) Correlation coefficient (R) among exon-skipping efficiency, circ-arRNA abundance, and editing rates of A at the SA site. $R = 0.95$, **** $p = 9.1150 \times 10^{-6}$; $R = 0.92$, **** $p = 5.4950 \times 10^{-5}$; $R = 0.82$, ** $p = 0.0020$. For data analysis in (G), $n = 11$.
- (H) Serum creatine kinase (CK), aspartate transaminase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) levels in the exon 5^{Δ5} and exon 5^{Δ2} models before and after circ-arRNA treatment. For the wild-type group, $n = 3$; for the untreated DMD group, $n = 2$. Mean $\pm$ SD. n represents the number of monkeys. Age-matched DMD and wild-type monkeys were used as controls, with DMD monkeys sharing mutations in exon 5 matching those of the treated group.
- (I and J) Probability of exon 5^{Δ5} (I) and exon 5^{Δ2} (J) monkeys standing up at different time points when their hands were tied behind them while in a supine position, tied in front while in a supine position, or tied behind while in a prone position.

See also Figure S4.

35 days. The exon 5^{Δ5} model was monitored for 52 weeks and the exon 5^{Δ2} model for 24 weeks, with serial muscle biopsies and comprehensive mobility assessments. To enable intuitive visualization of treatment dynamics in individual monkeys, Figures 3B and 3D depict longitudinal changes in editing efficiency, circ-arRNA abundance, and dystrophin expression, with lines representing the mean values across all muscle samples collected at each time point.

In the exon 5^{Δ5} model, exon 5 skipping was detected as early as 2 weeks post-treatment in the triceps brachii (TB) and expanded to all examined muscles (deltoid [DT]; quadriceps [QD]; TB) by week 8, with TB exhibiting the highest skipping efficiency (15%) corresponding to peak circ-arRNA expression (Figure 3B). Exon-skipping efficiency was detectable through week 52 but showed a gradual decline, decreasing from ~10% at week 34 to ~4% at week 52 (Figure 3B). In contrast, in the exon 5^{Δ2} model, exon skipping increased progressively from weeks 4 to 24 (Figure 3D). The corresponding RT-PCR raw data are provided in Table S1.

In the NHP models with complete exon 5 deletion, dystrophin expression was restored to approximately 10% of wild-type levels, comparable to that observed in BMD (Figures 3E, 3F, and S4A). This dystrophin restoration remained stable throughout the observation period in both models (Figures 3E, 3F, S4C, and S4D). In the TB muscle, dystrophin levels at 8 weeks post-treatment reached roughly 8% when normalized to the exon 5-deleted model, reflecting corresponding efficiency of exon skipping (Figures 3B, 3E, and S4C). The variation in exon 5 skipping among different muscles likely stems from differences in circ-arRNA expression (Figures 3B and S4E), which are influenced by variable infection efficiencies. A strong positive correlation ($R = 0.95$) between circ-arRNA abundance and exon-skipping efficiency across samples underscores the dose-dependent nature of the therapeutic effect (Figure 3G). Consistent with this, expression of ADAR1 p110 in muscles (QD, TB, and BB) and detection of both intronic and exonic edits confirmed active ADAR recruitment and *in vivo* RNA editing (Figures S4B and S4E). Editing rates shown in Figures 3B and 3D were determined based on A-to-I editing at the SA site, which strongly correlates with overall A-to-I editing efficiency (Figure S4E). Furthermore, serum biomarkers mirrored these therapeutic benefits: creatine kinase (CK) levels, a hallmark of muscle injury, were markedly reduced after treatment in both monkeys (Figure 3H), accompanied by decreases in aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), enzymes typically elevated in DMD,³⁴ demonstrating attenuated muscle pathology (Figure 3H).

To evaluate the *in vivo* transcriptome-wide safety of circ-arRNA, we performed RNA-seq on native skeletal muscle from three NHPs before and after treatment. Acknowledging the substantial biological variability inherent to NHP muscle, arising from tissue heterogeneity, inter-individual variability, and ongoing regenerative processes, we applied stringent filtering criteria (false discovery rate [FDR] < 0.01, average inclusion level difference > 0.1) to identify potential off-target splicing or exon-skipping events. Outside the DMD target locus, only a minimal number of differential splicing events (DSEs) were observed; these events showed no enrichment in muscle-structural or DMD-

associated pathways (Figure S4F). The infrequency of these events falls within the expected baseline biological variation in primates and is substantially lower than the perturbations typically induced by splicing regulators. Although post-treatment analysis revealed differential expression of *MOB3C*,³⁵ a gene potentially involved in muscle repair, its precise regulatory role requires further investigation. Collectively, these findings indicate high splicing fidelity and suggest that circ-arRNA does not trigger systematic off-target splicing *in vivo*.

Together, these results demonstrate that systemic MyoAAV 4E-mediated delivery of circ-arRNA induces efficient exon skipping, restores dystrophin expression, improves muscle pathology, and maintains safety and long-term efficacy in DMD primate models, validating LEAPER 2.0 as a potent RNA-based therapeutic platform for muscular dystrophy.

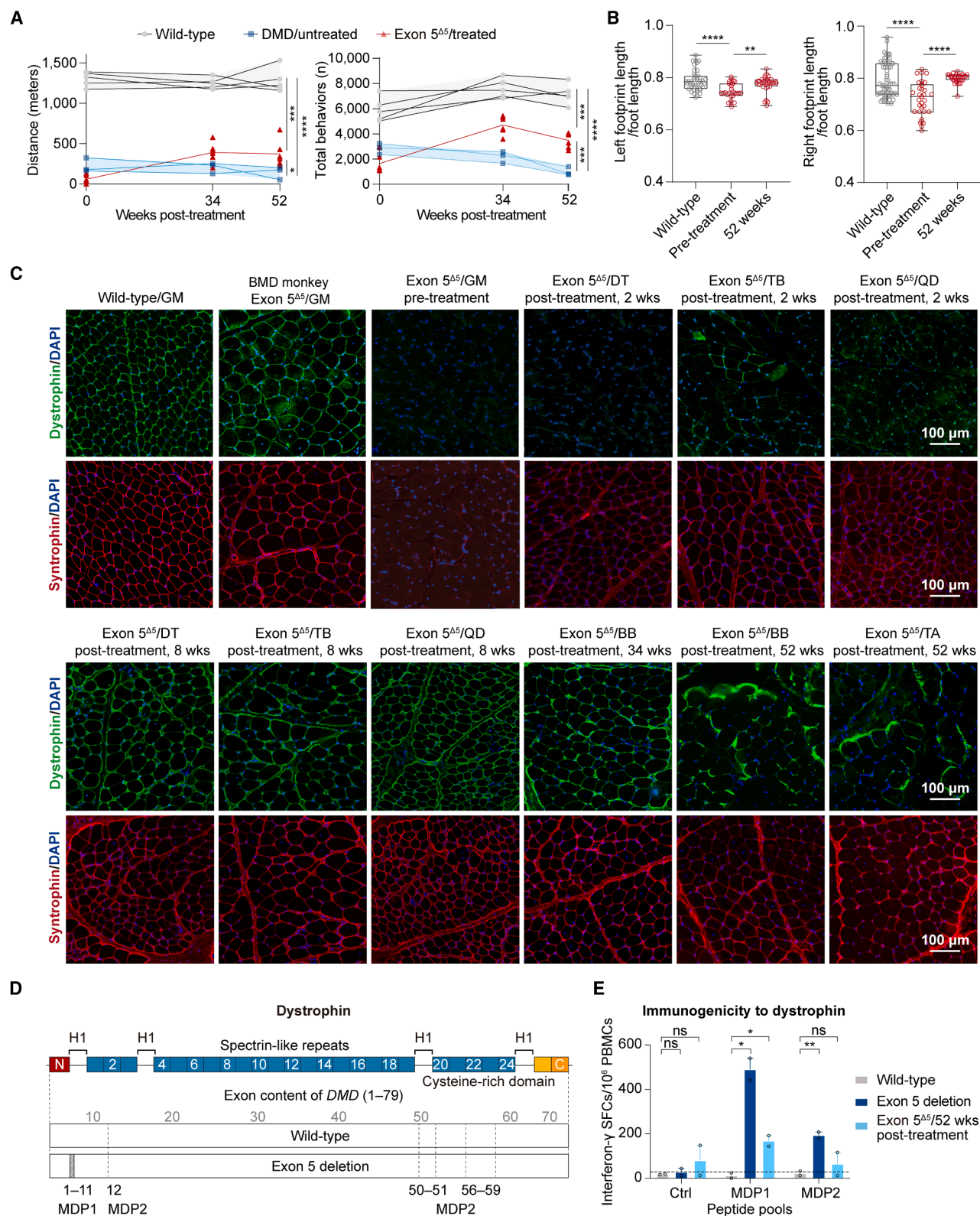
The importance of early intervention in achieving phenotypic improvement in DMD NHP models, in line with clinical observations in patients

NHP models recapitulate human neuromuscular physiology and behavior more faithfully than existing rodent models of DMD. To evaluate mobility, we continuously monitored spontaneous movement for 1 h, quantifying walking distance and behavioral activity. Muscle strength was further assessed by measuring the time required for the monkeys to rise from prone or supine positions with their hands restrained either behind or in front of the body.

Following treatment, the exon 5^{Δ5} model showed a marked functional improvement, with significantly shorter times required to stand up, indicative of improved muscle strength (Figure 3I; Video S1). Histological analyses using H&E and Sirius Red staining demonstrated pronounced amelioration of DMD-associated pathology, including fewer internal nuclei, less necrosis and fibrosis, and improved myofiber organization (Figure S5). These improvements were evident by 8 weeks post-treatment, further enhanced at 34 weeks, and persisted for up to 1 year in the exon 5^{Δ5} model (Figure S5).

Although the exon 5^{Δ5} model exhibited pronounced post-treatment improvements in both muscle pathology and motor function (Figures 3I and S5), the exon 5^{Δ2} model, comparable to a 14-year-old human treated at approximately 3.5 years of age, showed minimal recovery in standing performance (Figure 3J). This limited functional gain occurred despite robust exon skipping and restoration of dystrophin expression (Figures 3D, 3F, and S4D). Consistent with the exon 5^{Δ5} model, dystrophin localization along the sarcolemma and histopathological improvements were confirmed in the exon 5^{Δ2} model at 8 and 24 weeks post-treatment (Figure S5). These findings are consistent with clinical data from Sarepta Therapeutics, which indicate that DMD patients achieve maximal therapeutic benefit when treated between 4 and 5 years of age. In contrast, interventions initiated after this window, especially with ASO-based exon-skipping therapies, show reduced efficacy, reflecting the progressive and largely irreversible nature of muscle degeneration in DMD and highlighting the critical importance of early intervention.³⁶

Collectively, these findings underscore the critical importance of early therapeutic intervention in achieving durable phenotypic



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improvement in DMD and highlight the translational concordance between our NHP models and clinical outcomes.

Sustained motor improvement and dystrophin immunogenicity in the Exon 5^{Δ5} model

Before treatment, DMD monkeys displayed significantly reduced walking distances and overall behavioral activities compared with age-matched wild-type controls (Figure 4A). By 34 weeks after MyoAAV-circ-arRNA administration, the exon 5^{Δ5} model exhibited substantial gains in walking distance and overall activity, which persisted for a full year. In contrast, age-matched untreated DMD monkeys showed progressive motor decline over the same period (Figure 4A).

DMD patients often develop a characteristic “duck-like” gait and toe-walking due to proximal muscle weakness. Analogously, pre-treatment gait analyses revealed shortened stride lengths and absent heel imprints in the monkeys. One year post-treatment, footprint analysis demonstrated elongated step length and improved heel contact, consistent with enhanced lower-limb strength and coordination (Figure 4B). At 1 year (52 weeks) post-treatment, footprint analyses revealed longer step lengths relative to foot size, indicating improved gait and muscle strength. Raw behavioral data across all longitudinal time points are presented in Table S1.

Serial muscle biopsies from the exon 5^{Δ5} model (collected before treatment and at 2, 8, 34, and 52 weeks post-treatment) showed that dystrophin was nearly undetectable prior to treatment, as confirmed by western blot and immunofluorescence (Figures 3E and 4C). Robust dystrophin restoration was observed at 8 and 34 weeks, coinciding with high editing efficiency, and immunostaining confirmed dystrophin localization along muscle fiber membranes (Figure 3B and 4C). However, by 1 year post-treatment, both immunofluorescence and western blot analyses revealed a modest decline in dystrophin levels (Figures 4C and S4C).

To elucidate the cause of this decline, we examined multiple potential factors, including vector persistence, promoter methylation, and immune responses. A gradual 26% reduction in AAV vector genome copies was detected at 52 weeks compared with 2 weeks post-injection (Figure S6A). C-phosphate-G (CpG)

methylation analyses showed minimal changes across this period, suggesting that methylation was not the principal contributor to reduced exon skipping or dystrophin expression (Figure S6B). Nonetheless, partial methylation of the U6 promoter was detected in NHPs, highlighting an opportunity for optimization through promoter redesign or co-administration with FDA-approved DNA methyltransferase inhibitors (DNMTis).^{37,38}

We further hypothesized that the decline in dystrophin could be associated with an immune response post-treatment. A recent study reported that DMD patients lacking exons 8–11 mounted a cytotoxic T cell response to AAV-delivered micro-dystrophin containing these exons within 3 months of treatment.³⁹ Similarly, the monkeys in our study, which lacked exons 8–11 prior to therapy, regained expression of these regions following exon 5 skipping, potentially eliciting an immune response (Figure 4D). Consistent with this notion, chronic inflammatory features were observed in both the BMD monkey with an exon 5 deletion and the exon 5^{Δ5} model after exon skipping (Figure 4E). This immune response likely underlies the gradual decline in dystrophin expression, distinguishing it from the more stable expression observed with micro-dystrophin transgene delivery.³⁹ However, even though complete exon 5 skipping yielded a maximal dystrophin restoration of only ~10% of wild-type levels, the exon 5^{Δ5} model maintained stable phenotypic recovery for an entire year.

DMD monkeys with hotspot mutations exhibit durable dystrophin restoration without immunogenicity

Given that a recent *New England Journal of Medicine* study reported immunogenicity triggered by the introduction of non-self dystrophin epitopes,³⁹ whereas patients lacking exons 45–52 typically do not elicit such immune responses, we next examined a rhesus monkey DMD model harboring a 7-bp deletion in exon 50 (exon 50^{Δ7}).

Systemic delivery of circ-arRNA via MyoAAV 4E (5×10^{13} vg/kg) was initiated at 1 year of age (Figure 5A). Immunosuppressants were administered starting 3 days before AAV injection and continued for 35 days. Eight weeks post-treatment, muscle biopsies from the QD and TB showed exon-skipping efficiencies of 31% and 62%, respectively, with dystrophin restoration

Figure 4. Sustained mobility improvements and immunogenicity to dystrophin in the Exon 5^{Δ5} model following treatment

(A) Walking distance and total behavioral numbers of the exon 5^{Δ5} model before and after circ-arRNA treatment. Age-matched DMD ($n = 3$) and wild-type monkeys ($n = 5$) were used as controls, with DMD monkeys sharing mutations in exon 5 matching those of the treated group. For treated DMD, each point represents a technical replicate. See also Table S1. On the left, $^*p = 0.0461$, $^{***}p = 0.0001$, $^{****}p = 1.1000 \times 10^{-5}$. On the right, $^{***}p = 0.0002$ (wild-type vs. Exon 5^{Δ5}/treated), $^{***}p = 0.0003$ (DMD/untreated vs. exon 5^{Δ5}/treated), $^{****}p = 1.7000 \times 10^{-5}$.

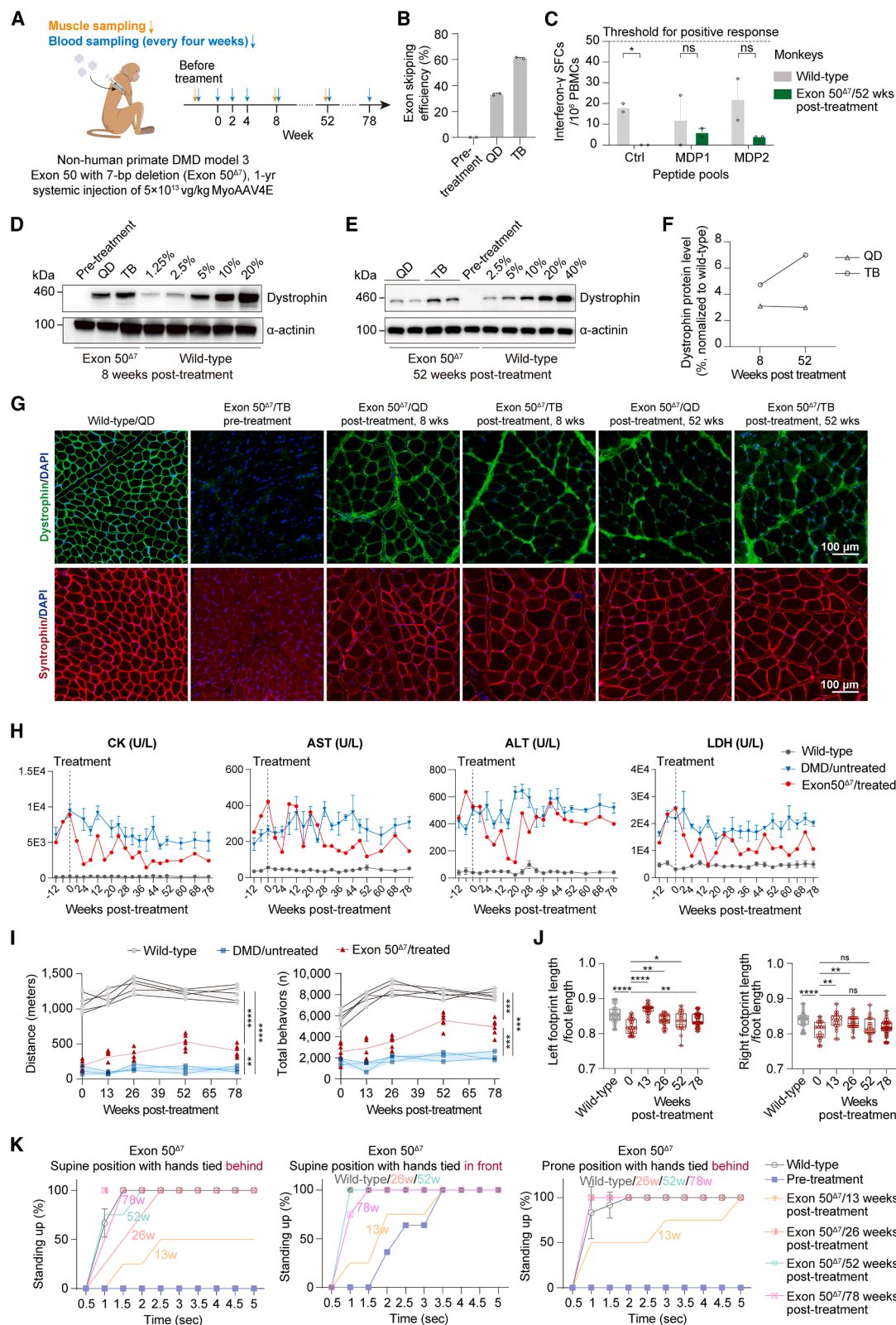
(B) Ratio of footprint length to actual foot length in the exon 5^{Δ5} model before and after circ-arRNA treatment. One-way ANOVA followed by Tukey's post hoc test was used for data analysis. Mean $\pm$ SD. On the left, $^{**}p = 0.0051$, $^{****}p = 1.3000 \times 10^{-5}$. On the right, $^{***}p = 7.7922 \times 10^{-8}$ (pre-treatment vs. wild-type), $^{****}p = 9.4962 \times 10^{-7}$ (pre-treatment vs. 52 weeks).

(C) Immunofluorescence staining of the exon 5^{Δ5} model before and after circ-arRNA treatment. GM, DT, TB, QD, BB, and TA represent the muscle tissue source of biopsy. GM, gastrocnemius; DT, deltoid; TB, triceps brachii; QD, quadriceps; BB, biceps brachii; and TA, tibialis anterior. Scale bar, 100 μ m.

(D) Illustration of dystrophin (top), exon content of DMD (middle), and area covered by peptide pools (MDP1 and MDP2).

(E) Dystrophin-specific immune response. Peripheral-blood mononuclear cells (PBMCs) from monkeys of wild-type, exon 5 deletion, and exon 5^{Δ5} 1-year post-treatment were cultured with two pools of synthetic peptides (MDP1 and MDP2) spanning the 5' and middle domain of dystrophin (D). Interferon- γ production was assessed 48 h later with the use of an interferon- γ enzyme-linked immunosorbent spot (ELISpot) assay. Data are presented as interferon- γ spot-forming cells (SFCs) per 1 million PBMCs. The dashed lines represent the threshold for a positive assay response (50 SFCs per 1 million PBMCs). $n = 2$, mean $\pm$ SEM. Significance was analyzed using a two-tailed unpaired Student's t test. In the control group (ctrl), $^{ns}p = 0.5984$ (wild-type vs. exon 5 deletion), $^{ns}p = 0.4583$ (wild-type vs. exon 5^{Δ5}/52 weeks post-treatment). In the MDP1 group, $^*p = 0.0114$ (wild-type vs. exon 5 deletion), $^*p = 0.0283$ (wild-type vs. exon 5^{Δ5}/52 weeks post-treatment). In the MDP2 group, $^{**}p = 0.0099$, $^{ns}p = 0.5108$.

See also Figures S5 and S6.



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reaching 3.4% and 4.7% of wild-type levels (Figures 5B, 5D, and 5E). As anticipated, no T cell-mediated immune response was detected in the exon 50^{Δ7} model 1 year after treatment (Figure 5C). At this time point, arRNA expression remained stable (Figure S6C), and western blot analysis revealed persistent dystrophin levels of ~3% in QD and up to 7% in TB (Figure 5F). Immunostaining further confirmed dystrophin localization along the sarcolemma (Figure 5G).

Consistent with other DMD NHP models, serum biomarkers of muscle injury, including CK, AST, ALT, and LDH, were elevated compared with wild-type before treatment but declined progressively after treatment (Figure 5H). Histological analyses by H&E and Sirius Red staining demonstrated marked improvement in muscle pathology, with less internal nucleation, necrosis, and fibrosis (Figure S5).

Functional assessments demonstrated marked improvements in locomotor performance, including increased walking distance and activity counts (Figure 5I), greater step length relative to foot size—particularly in the left limb (Figure 5J), and reduced times required to rise from both prone and supine positions (Figure 5K; Video S1). Compared with the exon 5^{Δ5} model, the exon 50^{Δ7} monkey exhibited stronger muscle function and higher dystrophin expression at 1 year, with these benefits of functional improvement remaining stable through 1.5 years (Figures 3E, 3I, 4A, 4B, 5D–5F, and 5I–5K). These improvements underscore the superior therapeutic potential of circ-arRNA at hotspot loci, where exon skipping minimally affects expression of dystrophin. Raw behavioral data over the longitudinal time course are presented in Table S1. Collectively, these findings demonstrate that a single systemic administration of AAV-circ-arRNA can induce durable dystrophin restoration and functional recovery without eliciting dystrophin-specific immune responses (Figures S6E and S6F).

circ-arRNA restores dystrophin in cardiomyocytes derived from DMD patients

To further validate the translational potential of circ-arRNA therapy in human contexts, we used induced pluripotent stem cells (iPSCs) derived from two DMD patients harboring a deletion of exons 49–50 (Δexon 49 + 50) or exon 52 (Δexon 52), mutations located near the exon 50 hotspot (Figure 6A). These mutations generate aberrant transcripts joining exon 48 to 51 or exon 51 to 53, both resulting in frameshifts in the *DMD* gene. Skipping exon 51 is predicted to restore the reading frame by connecting exon 48 to 52 or exon 50 to 53, potentially rescuing dystrophin expression even though the targeted exon does not contain the causal mutation (Figure S2I).

Upon differentiation of these iPSCs into cardiomyocytes, AAV-delivered circ-arRNAs targeting exon 51 efficiently promoted exon skipping in a dose-dependent manner (Figure 6A). Increasing the multiplicity of infection (MOI) led to a proportional rise in editing efficiency and dystrophin restoration. circ-arRNA achieved up to 80% exon skipping (Figure 6B) and restored dystrophin protein levels to approximately 60% of those in healthy controls (Figures 6C–6E).

Together, these findings highlight the versatility and efficacy of circ-arRNA-mediated exon skipping across species and genetic contexts, establishing a robust foundation for its clinical translation as a durable, immunologically compatible therapeutic strategy for DMD.

Treatment in DMD patients

Building on the consistent efficacy induced by circ-arRNA observed in NHP models, we next examined whether these therapeutic benefits could be translated to human patients. To this end, we designed LE051, a splice-modulating therapy mediated by circ-arRNA and delivered via AAV that targets human

Figure 5. Long-term dystrophin restoration and disease phenotype amelioration in the exon 50^{Δ7} model

(A) Schematic illustrating the injection of MyoAAV-delivered circ-arRNA in a DMD rhesus monkey with a 7-bp deletion in exon 50 (exon 50^{Δ7}). Blue arrows denote the time points for blood sample collection, while orange arrows indicate the time points for muscle sampling.

(B) Exon-skipping ratios by droplet digital PCR (ddPCR) at 8 weeks after systemic injection; $n = 2$, mean $\pm$ SD.

(C) Dystrophin-specific immune response. Peripheral-blood mononuclear cells (PBMCs) from monkeys of wild-type and exon 50^{Δ7} 1-year post-treatment were cultured with two pools of synthetic peptides (MDP1 and MDP2) spanning the 5' and middle domain of dystrophin. Interferon- γ production was assessed 48 h later with the use of an interferon- γ enzyme-linked immunosorbent spot (ELISpot) assay. Data are presented as interferon- γ spot-forming cells (SFCs) per 1 million PBMCs. The dashed lines represent the threshold for a positive assay response (50 SFCs per 1 million PBMCs). $n = 2$, mean $\pm$ SEM. Significance was analyzed using a two-tailed unpaired Student's t test. In the control group (ctrl), $^*p = 0.0121$. In the MDP1 group, $^{ns}p = 0.6707$. In the MDP2 group, $^{ns}p = 0.2137$.

(D–F) Western blot results of muscle samples from DMD monkeys before and after treatment at 8 and 52 weeks.

(G) Immunofluorescence staining of the exon 50^{Δ7} model before and after circ-arRNA treatment. TB and QD represent the muscle tissue source of biopsy. TB, triceps brachii; QD, quadriceps. Scale bar, 100 μ m.

(H) Serum creatine kinase (CK), aspartate transaminase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) levels in the exon 50^{Δ7} model before and after circ-arRNA treatment. For the wild-type group, $n = 3$; for the untreated DMD group, $n = 3$. Mean $\pm$ SD. n represents the number of monkeys. Age-matched untreated DMD and wild-type monkeys were used as controls, with DMD monkeys sharing mutations in exon 50 matching those of the treated group. (I) Walking distance and total behavioral activity numbers of the exon 50^{Δ7} model before and after circ-arRNA treatment. Age-matched DMD ($n = 3$) and wild-type monkeys ($n = 5$) were used as controls, with DMD monkeys sharing mutations in exon 50 matching those of the treated group. For treated DMD, each point represents a technical replicate. See also Table S1. On the left, $^{**}p = 0.0016$, $^{****}p = 4.000 \times 10^{-6}$ (wild-type vs. DMD/untreated), $^{****}p = 9.000 \times 10^{-6}$ (wild-type vs. exon 50^{Δ7}/treated). On the right, $^{***}p = 0.0001$ (wild-type vs. DMD/untreated), $^{***}p = 0.0001$ (wild-type vs. exon 50^{Δ7}/treated), $^{***}p = 0.0007$ (DMD/untreated vs. exon 50^{Δ7}/treated).

(J) Ratio of footprint length to actual foot length in the exon 50^{Δ7} model before and after circ-arRNA treatment. One-way ANOVA followed by Tukey's post hoc test was used for data analysis. On the left, $^*p = 0.0176$, $^{**}p = 0.0048$ (0 vs. 26 weeks), $^{**}p = 0.0024$ (0 vs. 78 weeks), $^{****}p = 5.4542 \times 10^{-9}$ (0 vs. wild-type), $^{****}p = 9.7589 \times 10^{-14}$ (0 vs. 13 weeks). On the right, $^{**}p = 0.0014$ (0 vs. 13 weeks), $^{**}p = 0.0026$ (0 vs. 26 weeks), $^{****}p = 2.3 \times 10^{-5}$, $^{ns}p = 0.4917$ (0 vs. 52 weeks), $^{ns}p = 0.6391$ (0 vs. 78 weeks).

(K) Probability of the exon 50^{Δ7} monkey standing up at different time points when their hands were tied behind them while in a supine position, tied in front while in a prone position, or tied behind while in a prone position. Age-matched wild-type monkeys ($n = 3$) were used as controls.

See also Figures S5 and S6.

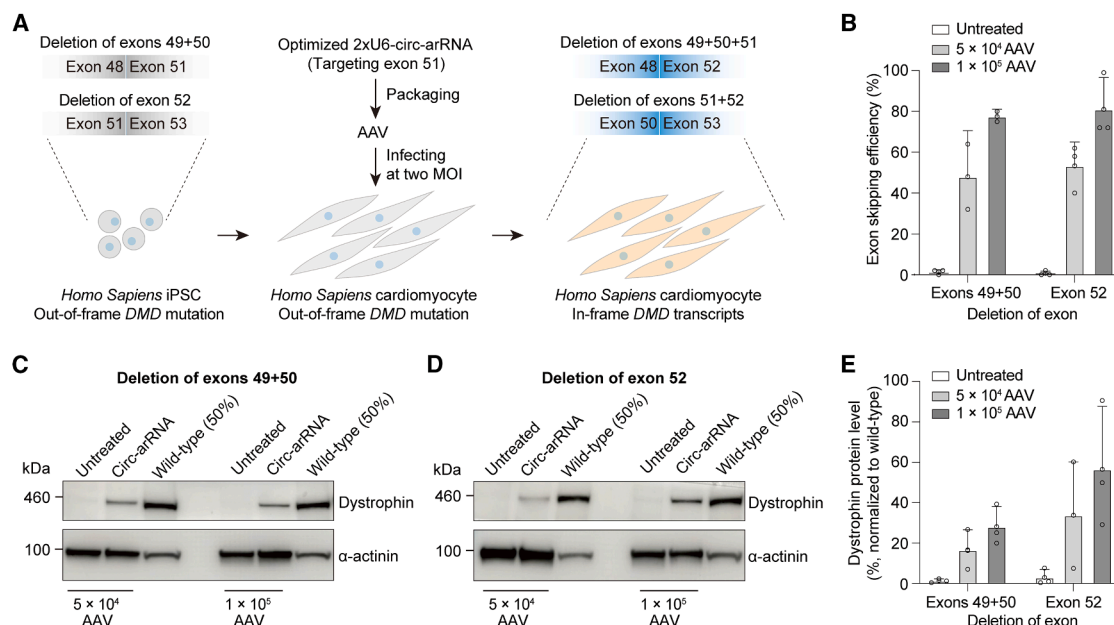


Figure 6. Effective restoration of dystrophin expression in patient-derived cardiomyocytes

(A) Illustration of the induction of cardiomyocytes carrying hotspot mutations in the *DMD* gene from iPSCs and their correction using AAV-delivered circ-arRNA targeting exon 51.

(B) Skipping efficiency of exon 51 before and after circ-arRNA treatment. $n = 3$, mean $\pm$ SD.

(C and D) Western blot results of cardiomyocytes from exon (49 + 50) deletion and exon 52 deletion before and after circ-arRNA treatment.

(E) Quantitative western blot results of cardiomyocytes before and after circ-arRNA treatment. $n = 3$, mean $\pm$ SD.

dystrophin exon 51, inducing exon 51 skipping in individuals with DMD who are amenable to this approach. In light of the progressive nature of DMD and the benefits of early intervention demonstrated in our NHP studies, compassionate-use treatment was approved and administered to three DMD patients under 7 years of age, in accordance with ethics committee approvals (SCMCIRB-K2024186-1). Preliminary in-human data demonstrated the safety and efficacy of our RNA-editing AAV therapy in individuals with DMD (Figure 7). By promoting exon 51 skipping, LE051 has the potential to benefit approximately 13% of DMD patients (Figure 7A). Among three patients (Figure 7B), two administered a low dose of 2×10^{13} vg/kg and one receiving a higher dose of 5×10^{13} vg/kg, we observed encouraging signs of efficacy and an overall favorable safety profile, reflecting the robustness of the therapeutic design and adherence to rigorous ethical and safety standards.

Harnessing endogenous protein recruitment for exon skipping safeguards the LEAPER system

The safety of LE051 was assessed according to the Common Terminology Criteria for Adverse Events (CTCAE). Across all three participants, only mild treatment-emergent adverse events (TEAEs) were reported. These included transient headache, nausea, and vomiting in patient 001, and transient abdominal pain with occasional vomiting in patient 003, which occurred within the first week post-infusion and resolved spontaneously. In patient 002, an upper respiratory tract infection was reported on day 24, accompanied by an asymptomatic rise in serum CK to 43,813 U/L ($3.6 \times$ baseline) and hemoglobin positivity (2+) on uri-

analysis. CK levels declined to 7,551 U/L by day 42 with nebulization therapy, and no muscle tenderness, weakness, or pain was observed. No biomarker or clinical evidence of liver injury was detected in any patient. A complete summary of adverse events is provided in Table S2.

Monitoring for treatment-related serious adverse events (TR-SAEs) is essential for evaluating the risk profile of emerging therapeutics. Previous AAV-based micro-dystrophin studies, such as those by Sarepta Therapeutics, reported TR-SAEs in 11.1% of cases, including hepatotoxicity, myocarditis, and rhabdomyolysis.⁴⁰ Similarly, severe TR-SAEs occurred in two patients receiving 40 mg/kg of DYNE-251, a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO), though dose reduction improved tolerability.⁴¹ However, concerns remain regarding the cumulative toxicity of repeated PPMO administration.

In contrast, no TR-SAEs or dose-limiting toxicities (DLTs) were observed following LE051 treatment, supporting its favorable safety and tolerability. Importantly, LE051 avoids the immune complications associated with pre-existing immunity to exogenous proteins such as Cas9 or Cas13, which have been reported in other gene-editing systems.^{42–47} By leveraging endogenous ADAR recruitment, the LEAPER 2.0 platform mitigates these immunological risks, providing a safer and more sustainable RNA-based therapeutic modality. Collectively, these early clinical findings highlight the strong translational potential of LE051 and reinforce the emphasis on patient safety and ethical integrity in its ongoing development.

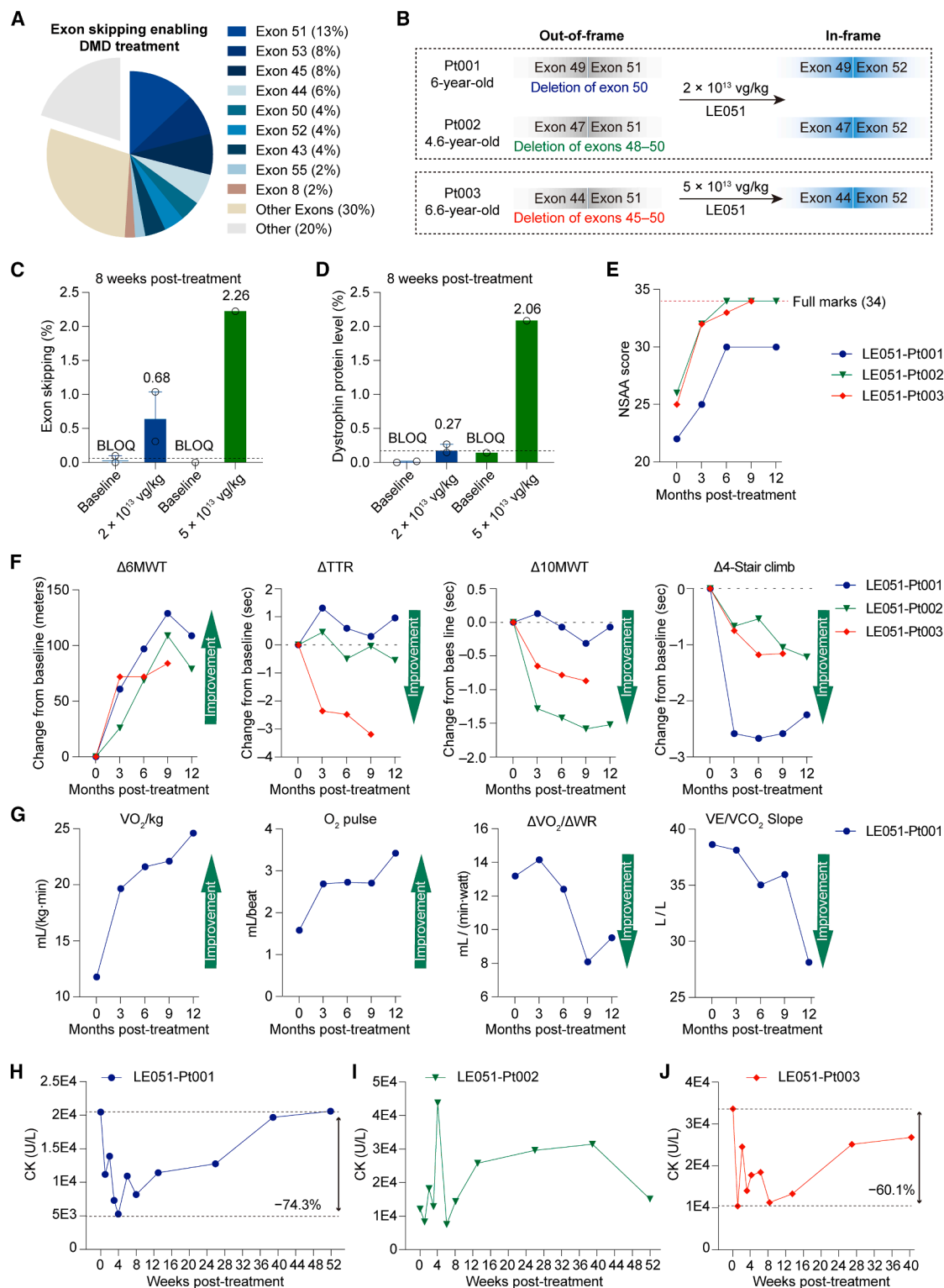


Figure 7. Efficacy and tolerability of LE051 assessed by functional improvement in motor and cardiopulmonary function

(A) A statistical analysis was conducted using ClinVar to identify which exon skipping events could restore the expression of dystrophin in DMD patients.

(B) Basic information of three DMD patients, including age, mutations in *DMD*, and administered dosage during treatment.

(C and D) ddPCR (C) and western blot (D) results of patient samples at 8 weeks post-treatment. For 2×10^{13} vg/kg group, $n = 2$ (Pt001 and Pt002), mean $\pm$ SEM. For 5×10^{13} vg/kg group, $n = 1$ (Pt003). BLOQ represents below the limits of quantification.

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Improvement in motor and cardiopulmonary function in DMD patients following treatment

A clear dose-dependent response was observed, with the patient receiving 5×10^{13} vg/kg showing greater exon skipping and dystrophin restoration than those treated with 2×10^{13} vg/kg (Figures 7C and 7D). Dystrophin protein levels in the high-dose patient reached 2.06% of healthy control levels at 8 weeks post-treatment, as determined by western blot (Figures 7D and S7A), comparable to levels achieved following six 10 mg/kg injections of DYNE-251, a peptide-conjugated PPMO.⁴¹

Functional outcomes were evaluated using the North Star Ambulatory Assessment (NSAA), a validated 17-item scale (0–34 points) for assessing ambulatory function in DMD. The two patients treated with the lower dose showed preliminary improvements of +3 to +6 points at 3 months and +8 points by 6 months, with sustained gains lasting at 12 months post-treatment. Notably, patient 002 achieved the maximum score of 34 at 6 months, which was then maintained for a full year (Figure 7E). The high-dose patient showed an improvement of +7 points at 3 months, which increased to +8 points at 6 months, reaching the maximum score by 9 months (Figure 7E). Quantitative analysis of NSAA subtests revealed enhanced performance in the stand-from-lying (time-to-rise [TTR]) and 10-m walk/run test (10MWT) assessments, reflecting faster movement and improved muscular strength (Figures 7F and S7B). Endurance measures, including the 6-min walk test (6MWT) and 4-stair climb, also demonstrated substantial preliminary increases in distance and speed (Figure 7F). Following treatment, patients exhibited functional improvements consistent with meaningful gains in muscle strength (Video S2).

To evaluate potential effects on cardiac and respiratory performance, key determinants of survival in DMD, cardiopulmonary exercise testing (CPET) was conducted. CPET offers quantitative insights into cardiac, ventilatory, and muscular function under maximal exertion.^{48,49} In patient 001, peak VO_2/kg increased from 11.78 to 24.61 mL/(kg·min) (Figure 7G), indicating a preliminary improvement in aerobic capacity and functional fitness.⁵⁰ O_2 pulse rose from 1.58 to 3.42 mL/beat, suggesting enhanced cardiac output and oxygen delivery per heartbeat. Additionally, an elevation in $\Delta\text{VO}_2/\Delta\text{WR}$ (work rate) reflected improved muscular efficiency and oxygen utilization, while the VE (minute ventilation)/ VCO_2 slope decreased toward healthy control values (26.6 L/L), reflecting preliminary gains in ventilatory efficiency and pulmonary gas exchange.⁵¹ Together, these findings point to stabilization and functional recovery of myocardial performance following LE051 administration, supporting the potential for improved cardiac output and exercise tolerance. A comprehensive CPET dataset is presented in Table S3.

Serum biomarkers of muscle injury exhibited favorable trends, and comprehensive hepatic monitoring (AST, ALT, LDH, GGT, ALP, and GR) demonstrated no evidence of progressive or clin-

ically significant liver injury during follow-up (Figure S7C). In patient 001, CK levels decreased by ~70% post-treatment (Figure 7H). Although CK levels partially rebounded by 9 months post-treatment due to the lower dose (2×10^{13} vg/kg), motor function remained stable (Figure 7E). Patient 002 exhibited a transient rise in CK 1 month post-treatment, likely associated with an upper respiratory tract infection (Figures 7I and S7C), with levels thereafter remaining elevated, consistent with the lower dose administered. In contrast, high-dose patient 003 showed a prolonged reduction in CK levels, reaching a maximum reduction of 60%, further supporting the dose-dependent therapeutic benefit of LE051 (Figure 7J), although transient fluctuations were noted, consistent with prior observations.

To assess systemic vector dynamics, we performed longitudinal monitoring of VCN in peripheral blood. VCN levels peaked shortly after infusion and declined over time, a trend consistent with expected systemic clearance kinetics (Figure S7D). Viral shedding was further evaluated by measuring DNA in urine, feces, and oral mucosal swabs. Although transient vector DNA was detectable in the early post-dosing phase, it became nearly undetectable across all compartments by day 42 post-infusion (Figure S7E). These findings indicate efficient vector clearance and minimal risk of long-term environmental shedding.

Based on published natural history studies of DMD, NSAA scores in patients aged approximately 5–6 years generally exhibit minimal year-to-year fluctuation.⁵² The NSAA improvements observed in LE051-treated patients exceed what would be expected from age-related natural variability alone. However, these findings are exploratory, derived from a small cohort with only 1 year of follow-up. Although the magnitude of improvement is notable, contributions from developmental variability, motivational effects, or other non-TR factors cannot be completely excluded.

Collectively, LE051 showed dose-responsive trends toward improvement in protein restoration, motor function, cardiopulmonary performance, and blood biomarkers, accompanied by an overall favorable safety profile. These preliminary clinical findings highlight the therapeutic potential of LE051 as a safe and effective RNA-based treatment therapy for DMD.

DISCUSSION

DMD remains one of the most challenging genetic disorders to treat, primarily due to the need for durable, muscle-wide dystrophin restoration. Although several ASO therapies have been approved by the FDA in recent years, they require repeated high-dose administration and face limitations in endosomal escape, nuclear delivery, and potential dose-related toxicities.^{53–56} In contrast, AAV vectors offer superior programmability, long-term expression, and broad tissue tropism across multiple serotypes.^{57–59}

(E) Changes in NSAA scores in three DMD patients before and post-treatment.

(F) Quantitative analysis of changes in four NSAA items: stand-from-lying (time-to-rise [TTR]), 10-meter walk/run test (10MWT), 6-min walk test (6MWT), and 4-stair climb.

(G) Cardiopulmonary exercise testing (CPET) of VO_2 , O_2 pulse, $\Delta\text{VO}_2/\Delta\text{WR}$, and VE/ VCO_2 slope in Pt001 during 12-month post-treatment.

(H–J) Serum creatine kinase (CK) levels of the three patients measured post-treatment.

See also Figure S7.

To leverage these advantages, our RNA editing platform (LEAPER 2.0)-based strategy combines three cooperative mechanisms, splicing element editing, ADAR occupancy, and circ-arRNA-mediated RNA interaction, that are distinct from conventional ASO-based exon-skipping approaches (Figures 1D–1F). The ADAR1 p110 isoform, which is constitutively nuclear and differentially expressed across muscle types, is essential for circ-arRNA-mediated splicing modulation and facilitates circ-arRNA-guided exon skipping *in vivo* (Figure S4B).¹⁹ Although these mechanisms were mainly characterized in cell-based systems, the consistent editing observed in NHP muscle suggests their relevance *in vivo*. The exact quantitative contribution of each pathway in mature tissues has yet to be determined. Overall, AAV-delivered tandem circ-arRNA demonstrates comparable or superior efficacy relative to high-dose ASO treatments, reflecting a major advantage in both delivery efficiency and tissue distribution in NHPs (Table S4).

In NHP models of DMD carrying mutations in exon 5 or exon 50, this strategy induced robust dystrophin restoration and improved motor function lasting up to 1 year (Figures 3, 4, and 5). Observations in the two age-differentiated exon 5 models provided valuable translational insights into clinical outcomes and underscore the importance of early intervention for phenotypic improvement (Figures 3I and 3J). Notably, in the exon 5^{Δ5} model, immune reactivity to dystrophin, also observed in patients,³⁹ contributed to a gradual decline in efficacy over time, highlighting the importance of immune modulation in durable efficacy. To address this, we developed a third NHP model carrying a patient-relevant hotspot mutation (exon 50^{Δ7}), which exhibited stable dystrophin expression for 1 year without eliciting dystrophin-specific immune responses and sustained behavioral recovery for 1.5 years (Figure 5). These findings provide mechanistic insight into the interplay between disease progression, genetic background, and immune tolerance in determining the durability of therapeutic efficacy.

Building on these preclinical findings, administration of LE051, the optimized clinical candidate from this platform, in three DMD patients led to measurable dystrophin restoration and preliminary functional improvement with a favorable safety profile. Although the cohort remains small and long-term follow-up is ongoing, these early data suggest that LE051 may deliver meaningful molecular and functional benefits at substantially lower doses compared with existing ASO- or micro-dystrophin-based therapies in similarly aged patients (Table S4).

Notably, the myocardium represents a critical therapeutic target, as cardiopulmonary complications account for ~80% of DMD-related mortality, with a median survival of 29.9 years.^{60,61} Dystrophin loss promotes myocardial fibrosis and left ventricular dysfunction, increasing the risk of premature death.^{62,63} Although extended functional monitoring in NHPs precluded comprehensive postmortem cardiac analysis, a parallel study in wild-type monkeys confirmed efficient cardiac transduction 4 weeks after AAV administration. Moreover, improvements in CPET observed in patient 001 provide preliminary evidence of enhanced cardiac performance following LE051 treatment.

Collectively, these findings define a seamless translational trajectory from mechanistic validation in NHPs to preliminary clinical outcomes in patients, highlighting the potential of AAV-deliv-

ered RNA editing as a safe, durable, and scalable therapeutic strategy for DMD.

Limitations of the study

This study has several notable limitations. In the NHP model, observed dystrophin restoration levels (1%–5% by bulk tissue analysis) reflect partial molecular correction rather than full normalization, and bulk measurements may underestimate fiber-level heterogeneity, which could disproportionately contribute to functional improvement. Consistent with prior preclinical and clinical studies, low-level dystrophin restoration can be biologically meaningful but does not represent full normalization, highlighting the need for further optimization to maximize therapeutic benefit. Although AAV-delivered circ-arRNA achieved sustained exon skipping, dystrophin expression, with functional improvement for up to 1.5 years in DMD NHPs and 1 year in patients, the long-term durability of these effects beyond the current follow-up remains unknown. Consequently, repeat AAV administration could be considered in the future if durability declines over time. The number of DMD monkeys was limited by the high cost and long generation time of NHP models, and behavioral assessments included only a small cohort due to inherent individual variability; nevertheless, monitoring was conducted under strictly controlled conditions with age, body size, and mutation type matched across subjects. By comparison, referenced clinical trials enrolled substantially larger patient cohorts, which limits direct quantitative comparison, but the consistency of outcomes across individual monkeys and patients supports the overall robustness of the results.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Wensheng Wei (wswei@pku.edu.cn).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

RNA-seq data have been deposited in the National Genomics Data Center (NGDC) database and are publicly accessible as of the publication date. The accession number NGDC: PRJCA058659 is provided in the [key resources table](#). This study does not report original code. Additional information necessary to reanalyze the data is available from the [lead contact](#) upon reasonable request.

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AUTHOR CONTRIBUTIONS

W.W., Y.C., W.J., and J.W. conceived the project. W.W., Y.C., W.J., and J.W. supervised the project. H.T., W.G., and P.Y. designed the experiments, performed the research, and analyzed data. H.T., W.G., and W.W. wrote the manuscript, with contributions from all participating authors. H.T., Zongyi Yi, X.C., L.Q., and Y.Y. performed the preliminary exploration of LEAPER 2.0 in exon skipping and performed the analysis of muscle samples from DMD NHPs. J.R. and W.T. analyzed the bioinformatics results. W.G., T.Z., S.R., W.S., and J.L. designed and performed the experiments in nonhuman primates. P.Y., Z.L., Zexuan Yi, and Y. Zhao designed the vector for patients. J.H., C.W., and Xinyan Sun drafted the study plan for patients. F.H. and Y.X. provided support for the interpretation of patient data. G.T., W.Z., H.L., Xueqing Sun, H.S., Y. Zhang, C.Z., Y.W., and L.Y. performed the analysis of patient samples. All authors discussed the results and contributed to the final manuscript.

DECLARATION OF INTERESTS

A patent application has been filed by Peking University (with W.W., Zongyi Yi, and H.T. as inventors). P.Y., Z.L., Zexuan Yi, Y. Zhao, J.H., G.T., W.Z., H.L., Xueqing Sun, H.S., Yongjian Zhang, C.Z., Y.W., and L.Y. are employees of Leaper Bio Inc. W.W. is a founder of and scientific advisor to EdiGene and Theroma.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Dystrophin antibody	Abcam	Cat#ab15277; RRID: AB_301813
Anti-Vinculin antibody	Abcam	Cat#ab129002; RRID: AB_11144129
Anti- α -actinin antibody	Abcam	Cat#ab68167; RRID: AB_11157538
Peroxidase-AffiniPure Goat Anti-Mouse IgG (H + L)	Jackson ImmunoResearch	Cat#115-035-003; RRID: AB_10015289
Anti-ADAR1 antibody	Santa Cruz Biotechnology	Cat#sc-271854; RRID: AB_10708553
Anti- β -tubulin antibody	CWBiotech	Cat#CW0098; RRID: AB_2814800
Anti-Syntrophin antibody	Abcam	Cat#ab11425; RRID: AB_298030
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher Scientific	Cat#A-11008; RRID: AB_143165
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568	Thermo Fisher Scientific	Cat#A10037; RRID: AB_11180865
Anti-Laminin antibody	Abcam	Cat#ab11575; RRID: AB_298179
Anti-rabbit IgG HRP-linked Antibody	Cell Signaling Technology	Cat#7074S; RRID: AB_2099233
Bacterial and virus strains		
Trans1-T1 Phage Resistant Chemically Competent Cell Trans1-T1	Transgene	Cat#CD501-03
AAV9, MyoAAV2A, MyoAAV4E for exon 5 skipping	Packgene Biotech	N/A
AAV for exon 51 skipping	Ubrigene Co., Ltd	N/A
Biological samples		
Primary monkey skeletal muscle myoblasts	This paper	N/A
iPSC (healthy control)	Shanghai Gempile Biotech Co.Ltd	Cat#hiPSC-12
iPSC (Deletion of exons 49+50)	This paper	N/A
iPSC (Deletion of exon 52)	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Fetal Bovine Serum	VivaCell BIOSCIENCES	Cat#C04001-500
Dulbecco's modified Eagle's medium	Gibco	Cat#C11995500BT
X-tremeGENE HP DNA transfection reagent	Roche	Cat#6366546001
PEI	Proteintech	Cat#B600070
Matrigel	Gibco	Cat#354277
mTeSRTM 1 media	StemCell	Cat#85850
ddPCR supermix for probes (No dUTP)*	Bio-rad	Cat#1863024
NuPAGE™ Tris-Acetate Mini Protein Gels, 3 to 8%, 1.0–1.5 mm	Thermo Fisher Scientific	Cat#EA0378BOX
NuPAGE™ Tris-Acetate Midi Protein Gels, 3 to 8%, 1.0 mm	Thermo Fisher Scientific	Cat#WG1603BOX
EASY Dilution	TAKARA	Cat#9160
TB Green Premix Ex TagII (Tli RNaseH Plus)	TAKARA	Cat#RR820A
FastKing gDNA Dispelling RT SuperMix	TIANGEN	Cat#KR118
TransScript® II First-Strand cDNA Synthesis SuperMix	TransGen Biotech	Cat#AH301

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TRIzol reagent	Thermo Fisher Scientific	Cat#15596026
MDP1	GenScript	N/A
MDP2	GenScript	N/A
Ficoll-Paque™ PREMIUM sterile solution	Cytiva	Cat#17544202
Critical commercial assays		
Atrial Cardiomyocyte Differentiation Kit	StemCell	Cat#100-0215
STEMdiff™ Cardiomyocyte Dissociation Kit	StemCell	Cat#05025
STEMdiff Cardiomyocyte Maintenance Kit	StemCell	Cat#05020
Direct-zol RNA Microprep Kits	Zymo	Cat#R2062
Quick-RNA Miniprep Kit	Zymo	Cat#R1055
ProtoScript II First Strand cDNA Synthesis Kit	NEB	Cat#E6560L
Hematoxylin and Eosin staining kit	Solarbio	Cat#G1120
Sirius Red staining kit	Solarbio	Cat#G1472
Interferon- γ enzyme-linked immunosorbent spot (ELISPOT) assay	MABTECH	Cat#3421M-4HPW-2
Deposited data		
Next generation sequencing data	https://ngdc.cncb.ac.cn/	PRJCA058659
Experimental models: Cell lines		
HEK293	C. Zhang (Peking University)	N/A
HEK293T ADAR1 ^{-/-}	This paper	N/A
COS-7	ATCC	Cat#CRL-1651
Experimental models: Organisms/strains		
Rhesus monkey	The Laboratory Animal Center of Kunming University of Science and Technology	N/A
DMD ^{Ex5} and DMD ^{Ex50} rhesus monkey	This paper	N/A
Oligonucleotides		
Primers for cloning	See Table S5 for a list of cloning sequences	N/A
Primers for NGS, exon skipping and qPCR	See Table S5	N/A
synthetic RNA standard, L-TC-RNA	GenScript	N/A
Recombinant DNA		
pcDNA3.1	Addgene	Cat#V790-20
pcg 2.0-BFP-circ-arRNA backbone	Addgene	Cat#213965
AAV backbone	This paper	N/A
Software and algorithms		
Prism 9	GraphPad	https://www.graphpad.com/
PrimateScan Version 1.0	CleverSys Inc	https://cleversysinc.com/CleverSysInc/csi_products/primatescan/
OlyVIA Viewer	Olympus	https://www.olympus-lifescience.com/
Image Lab	Bio-rad	https://www.bio-rad.com/zh-cn/product/image-lab-software?ID=KRE6P5E8Z
ImageJ	NIH	https://imagej.net/
FastQC (v0.11.9)	Babraham Institute	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
STAR (v2.2.1)	Cold Spring Harbor Laboratory	https://github.com/alexdobin/STAR
Samtools (v1.7)	Sanger Institute	https://www.htslib.org/
Mosdepth (v0.3.12)	University of Utah	https://github.com/brentp/mosdepth

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
rMATS (v4.2.0)	University of Pennsylvania / Children's Hospital of Philadelphia	https://github.com/Xinglab/rmats-turbo
Fastp (v0.12.4)	OpenGene	https://github.com/OpenGene/fastp
Other		
QX200™ system	Bio-Rad	N/A
SLIDEVIEW VS200 scanner	Olympus	N/A
Vacuum vessel	BD	Cat#367955
Kinect 2.0 camera	N/A	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**Animals**

The nonhuman primates (NHPs) used in this study were male rhesus monkeys (*Macaca mulatta*). Male animals were specifically selected to reflect the male-predominant clinical manifestation of DMD, and sex was incorporated into both the experimental design and interpretation of results. Two DMD model monkeys carried exon 5 mutations, designated Exon 5^{Δ5} (1 year old) and Exon 5^{Δ2} (3.5 years old), while one monkey carried an exon 50 mutation, designated Exon 50^{Δ7} (1 year old). Age-matched wild-type (WT) male monkeys served as controls.

All rhesus monkeys used in this study were kept in a controlled environment. The temperature was maintained at $22 \pm 1^\circ\text{C}$, and the humidity was kept at $50\% \pm 5\%$ RH. They were exposed to a consistent 12-h light/dark cycle, with the lights being turned on at 08:00 a.m. The monkeys were given commercial diets twice a day, and fruits and vegetables were provided once a day as a supplement. All care and experimental procedures for the rhesus monkeys were approved by the Laboratory Animal Center of Kunming University of Science and Technology (LPBR201803002, LPBR202003003, and LPBR202101031).

Human participants

This study involved 3 Asian male DMD patients (6-, 4.6- and 6.6-year-old). Prior to receiving our treatment, all three patients had undergone corticosteroid therapy with minimal functional improvement and notable adverse effects, including weight gain and muscle inflammation. They were enrolled in a compassionate use program approved by the ethics committee (SCMCIRB-K2024186-1), with written informed consent obtained in accordance with the Declaration of Helsinki. Comprehensive clinical and genetic evaluations were performed prior to treatment.

Prior to treatment, the patients underwent a comprehensive diagnostic workup for DMD, including clinical, serological, and imaging evaluations. Eligible participants were ambulatory male patients aged 4–8 years with genetically confirmed Duchenne muscular dystrophy amenable to exon 51 skipping, stable on corticosteroids for ≥ 3 months, and a 6-min walk distance ≥ 150 m. Key exclusions included significant cardiac (LVEF $< 40\%$), respiratory (FVC $< 50\%$), or systemic disease, high anti-AAV neutralizing antibody titers ($> 1:100$), active viral infection, recent major surgery or trial participation, or prior use of exon-skipping drugs other than corticosteroids.

Cell lines

HEK293T cells were obtained from C. Zhang's laboratory (Peking University). COS-7 (ATCC, CRL-1651) cells were maintained in our laboratory at Peking University. Rhesus monkey myoblasts were induced from monkey myogenic precursor. These mammalian cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, C11995500BT) with 10% fetal bovine serum (FBS) supplemented with 1% penicillin–streptomycin under 5% CO₂ at 37°C.

Plasmids were transfected into cells with X-tremeGENE HP DNA transfection reagent (Roche, 06366546001) or PEI (Proteintech, B600070) according to the manufacturer's instructions.

The HEK293T ADAR1^{−/−} cell lines were generated according to a previously reported method.⁶⁴ ADAR1-targeting single-guide RNA and PCR-amplified donor DNA containing the CMV-driven puromycin resistance gene were co-transfected into HEK293T cells. Then, the cells were treated with puromycin 7 days after transfection. Single clones were isolated from puromycin-resistant cells and verified through sequencing and western blot analysis.

Circ-arRNAs were packaged in AAV9, MyoAAV 2A, MyoAAV 4E by PackGene Biotech and then infect myoblasts at MOI of 1×10^5 .

Primary cell cultures

These cells were cultured in 6-well plates coated with Matrigel (Gibco, 354277) and maintained in mTeSR™ 1 media (StemCell, 85850). The culture medium was refreshed daily. To differentiate the iPSCs into functional cardiomyocytes, a differentiation protocol

provided by the company was followed, using the Atrial Cardiomyocyte Differentiation Kit (StemCell, 100-0215). In brief, 4.5×10^5 human iPSCs were seeded in 12-well plates per well. The patient-derived iPSCs were subjected to the differentiation process using the Atrial Cardiomyocyte Differentiation Kit (StemCell, 100-0215) for a period of 15 days. Following differentiation, cells were digested using the STEMdiff™ Cardiomyocyte Dissociation Kit (StemCell, 05025), and the cardiomyocytes were then replated onto Matrigel-coated 24-well plates with the STEMdiff Cardiomyocyte Maintenance Kit (StemCell, 05020). After a 48-hr period, the cells were infected with MyoAAV 4E at varying MOIs, specifically 1×10^5 or 5×10^4 . Four days post-infection, the cells were harvested, and total RNA was extracted (Zymo, R2062) and subsequently reverse-transcribed into cDNA (NEB, E6560L).

METHOD DETAILS

Plasmid construction

For genetically encoded circ-arRNA-expressing constructs, we first constructed a cloning vector based on a pLenti-sgRNA-lib 2.0 vector that included a Twister P3 U2A, 5' ligation sequence, a 3' ligation sequence and Twister P1. Then, the sequences of arRNAs or random sequence non-targeting control RNAs were synthesized and Golden Gate-cloned into the autocatalytic circular RNA expression vector (Addgene, 213965). Different mutation versions of ADAR 1 p110 were constructed into pcDNA3.1 backbone (Addgene, V790-20). Sequence of ADAR1 p110 mutants were listed in [Table S5](#).

To assess RNA editing in the sequence flanking splicing acceptor (SA) in intron 4 of *DMD*, reporter contained intron 4-exon 5-intron 5 was construct into pcDNA3.1 backbone. A truncated version of intron was adopted because of its long size.

To increase expression of circ-arRNA, tandem forms was designed by hU6-circ-arRNA-linker-mU6-circ-arRNA. Corresponding arRNAs sequence targeting SA of wild-type or 2/5 bp deletions were cloned into the genetically encoded circ-arRNA-expressing vector.

To create reporter for testing exon skipping efficiency in disease model sequence of exon 5, reporter contained exon 4-intron 4-exon 5 (with 2- or 5-bp deletions)-intron 5-exon 6 of *DMD* (rhesus monkey) were construct into pcDNA3.1 backbone.

To create splicing reporter for testing importance of multiple As in intron 4 and exon 5 about exon skipping efficiency, splicing reporter contained exon 4-intron 4-exon 5-intron 5-exon 6 of *DMD* (rhesus monkey) with A-to-G transitions to mimic 100% RNA editing were construct into pcDNA3.1 backbone.

Sequence of arRNA and reporter are listed in Tables S5, respectively.

RNA editing of target site

HEK293T cells were initially seeded in 12-well plates at a density of 3×10^5 cells per well. After 24 h, the cells underwent transfection with 2 μ g of circ-arRNA and 500 ng of reporter plasmids. After 48 h post-transfection, the cells were harvested, and RNA was extracted (Zymo, R1055) and then subjected to reverse-transcription to produce cDNA (TIANGEN, KR118). The targeted locus was subsequently PCR-amplified with the appropriate primers (listed in [Table S5](#)). The resulting PCR products were further purified and used for Sanger sequencing (when verifying exon junction after exon skipping) or subjected to NGS (Illumina HiSeq X Ten) analysis (targeted amplicon next-generation sequencing).

Exon skipping efficiency

COS-7 cells were initially seeded in 6-well plates (2×10^5 cells per well). After 24 h, the cells were transfected with 4 μ g of circ-arRNA. After 48 h post-transfection, the cells were sorted and collected using FACS to enrich the transfection-marker⁺ cells. Subsequently, RNA was isolated (Zymo, R1055) from these cells, and reverse-transcribed into cDNA (Tiagen, KR118).

To test exon skipping efficiency in the disease model sequence, cDNA was obtained from cells that were transfected as above in HEK293T cells and iPSCs.

To generate myogenic precursor cells, 5×10^3 cells were seeded per well in 24-well plates. Monkey myogenic precursor cells were induced by 2% horse serum for a period of 3-7 days. Once myotubes had formed, the cells were infected with AAV at an MOI of 1×10^6 (AAV9, MyoAAV 2A, MyoAAV 4E). The cells were collected on day 10 post-infection, and total RNA was isolated (Zymo, R1055), followed by reverse-transcription into cDNA (TransGen Biotech, AH301).

RNA samples from monkey muscles, taken at different time points, were subjected to extraction using the Trizol-chloroform method, following the manufacturer's instructions. The extracted RNA was then reverse-transcribed into cDNA (Tiagen, KR118).

In all experimental setups, exon skipping efficiency was assayed by both RT-PCR (skipping exon 5) and digital PCR (ddPCR for exon 51 skipping) after acquiring cDNA. The quantitative analysis of RT-PCR was performed using Image Lab software. The primer sequences are listed in [Table S5](#). Uncropped and unprocessed scans of RT-PCR were provided in [Table S1](#).

ddPCR reaction was performed using the QX200™ system (Bio-Rad) with the following components: ddPCR Supermix for Probes (No dUTP, Bio-Rad), custom primers and dual-labeled probes (FAM-labeled probe: detects wild-type transcript containing exons 50–51–52; HEX-labeled probe: detects exon 51-skipped transcript containing exons 50–52). Droplets were generated using the QX200™ Droplet Generator. PCR amplification was performed under the following conditions: 95°C for 10 min;

40 cycles of: 94°C for 30 s, 58°C for 30 s, 72°C for 90s; 98°C for 10 min and 4°C hold. Droplets were read using the QX200™ Droplet Reader and analyzed with QuantaSoft software. Acceptance criteria: Total droplets >10,000 per well; NTC copies ≤10 or <1% of the lowest sample; CV ≤30% for replicate wells; Control recovery rate: 50–150%. Exon skipping percentage was calculated as: Exon Skipping (%) = $(1 - \text{HEX}/\text{FAM}) \times 100\%$.

RNA editing analysis of samples from monkeys

For targeted amplicon next-generation sequencing (NGS) analysis, we first constructed a reference index comprising sequences spanning the targeted sites, including 20 nucleotides upstream and downstream of the arRNA-covered regions. Subsequently, we aligned and quantified the reads using BWA (v.0.7.10-r789). The resulting BAM alignment files were sorted with SAMtools (v1.1), and the analysis of RNA editing sites was conducted using REDitools (v.1.0.4). The specified parameters used in the analysis were as follows: -t 8 -U [AG] -n 0.0 -T 6-6 -e -d -u. Any A-to-G conversions within the arRNA-targeted regions that were statistically significant, as determined by Fisher's exact test (P value < 0.05), were categorized as edits attributed to the arRNAs. Conversions occurring at sites other than the targeted adenosines were considered off-target edits. Mutations that appeared simultaneously in both the control and experimental groups were attributed to single nucleotide polymorphisms.

Bulk RNA-seq analysis for potential off-target splicing events

Raw sequencing reads were evaluated for quality using FastQC (v0.11.9). Adapter trimming and quality filtering were performed with fastp (v0.12.4). Filtered reads were aligned to the T2T-MMU8v2.0 (GCF_049350105.2) reference genome using STAR (v2.2.1), with BestRefSeq annotation as the transcript reference. The resulting alignments were processed using samtools (v1.7) for format conversion and indexing. Genome-wide average sequencing depth was calculated with mosdepth (v0.3.12).

Alternative splicing analysis was performed using rMATS (v4.2.0), which quantifies splice junction usage based on inclusion junction counts (IJC) and skipping junction counts (SJC). To ensure robust detection, only splice junctions with a combined read count (IJC + SJC) exceeding 40 in each sample were retained for downstream analysis.

Immunosuppressant administration and intravenous AAV injection in DMD monkeys

The immunosuppressant treatment regimen involved a combination of Prednisolone and Tacrolimus (FK506). Prednisolone was administered orally at a dosage of 4 mg/kg once a day for 3 days prior to AAV injection. It was then continued for an additional 7 days following the AAV injection. Subsequently, the prednisolone dosage was adjusted to 2 mg/kg once a day for 7 days, then to 1 mg/kg once a day for 7 days, followed by 0.5 mg/kg once a day for 7 days, and finally 0.5 mg/kg every other day for an additional 7 days. Tacrolimus (FK506) was administered orally at a dosage of 0.2 mg/kg once a day for 3 days before AAV injection and was continued for 4 weeks following the AAV injection.

Two monkeys, identified as Exon 5^{Δ5} and Exon 5^{Δ2}, and aged 1 year old (Exon 5^{Δ5}) and 3.5 year old (Exon 5^{Δ2}), respectively, received a dose of 5×10^{13} vg/kg of AAV (packaged by PackGene Biotech.). Exon 50^{Δ7} aged 1 year old received a dose of 5×10^{13} vg/kg of AAV (packaged by Ubrigene Co., Ltd.). Systemic AAV injection was administered through the saphenous vein in the right lower limb.

Western blot analysis

For dystrophin, we used monoclonal primary antibodies directed against dystrophin (abcam, ab15277), anti-vinculin antibody (abcam, ab129002) and anti-actinin antibody (abcam, ab68167). For secondary antibody binding, we employed an HRP-conjugated goat anti-mouse IgG (H + L, 115-035-003) purchased from Jackson ImmunoResearch. A total of 60 μg of muscle lysate protein was loaded onto NuPAGE gels (Thermo, EA0378BOX and WG1603BOX). Subsequently, the protein samples were transferred to polyvinylidene difluoride membranes at 30V for 16 hrs (Bio-Rad Laboratories). These PVDF membranes were subjected to immunoblotting with the respective primary antibodies (anti-dystrophin, 1:1,000; anti-vinculin, 1:3,000; anti-actinin, 1:20,000), followed by incubation with a secondary antibody (1:10,000), and subsequent exposure. For ADAR1, we used monoclonal primary antibodies directed against ADAR1 (Santa Cruz, sc-271854) and β-tubulin (CWBio, CW0098). Muscle tissues were lysed and an equal amount of protein was loaded for SDS-PAGE. Then, sample proteins were transferred onto polyvinylidene difluoride membrane (Bio-Rad Laboratories) and immunoblotted with primary antibodies against one of the ADAR enzymes (anti-ADAR1, 1:500; anti-β-tubulin, 1:1000), followed by secondary antibody incubation (1:10,000) and exposure.

The experimental procedure was repeated three times, and semi-quantitative analysis was performed using Image Lab software. Uncropped and unprocessed scans were provided in [Table S1](#).

Immunofluorescence staining

Fresh muscle tissues were dissected and mounted on pieces of cork using tragacanth gum. The tissues were then rapidly frozen in liquid nitrogen-cooled isopentane and cryosectioned at 8 μm thickness. Followed by fixation with 4% paraformaldehyde, the sections were blocked in a blocking buffer consisting of PBS with 5% fetal bovine serum, 3% bovine serum albumin, and 0.3% Triton X-100 for 1 h at room temperature (RT). Subsequently, they were incubated with primary anti-dystrophin (Abcam, ab15277, 1:500 dilution), or anti-syntrophin (Abcam, ab11425, 1:500 dilution) antibodies at 4°C overnight. After primary antibody incubation, the

sections were washed three times for 15 min each in PBS and then incubated with secondary Goat anti-Rabbit IgG (H+L), Alexa Fluor™ 488, or Donkey anti-Mouse IgG (H+L), Alexa Fluor™ 568 antibody for 2 h at RT. The slides were washed three times for 15 min each in PBS and mounted with a mounting medium containing DAPI.

Histological staining

Hematoxylin and Eosin (H&E) staining and Sirius Red staining were performed using the Hematoxylin and Eosin staining kit (Solarbio, G1120) and Sirius Red staining kit (Solarbio, G1472), respectively. All experimental procedures followed the instructions provided in the staining kits, and the staining time was optimized based on the staining results. Images were captured using a SLIDEVIEW VS200 scanner (Olympus, Germany).

Quantification of myofiber morphology and pathology

Myofiber morphology and pathological features were quantified on transverse sections co-stained for laminin (to delineate myofiber boundaries) and DAPI (to visualize nuclei), as well as on H&E-stained sections to assess necrosis. For each animal $\times$ muscle $\times$ time point, two non-adjacent sections (separated by 80–150 μ m to prevent resampling of the same fibers) were analyzed. Within each section, five non-overlapping fields were selected using a systematic random sampling approach. Briefly, sections were first surveyed at low magnification; areas containing artifacts (e.g., folding or tearing) or non-muscle structures (e.g., large vessels or tendon) were excluded, and fields were then sampled at predefined positions across the remaining tissue to minimize regional bias.

Percentage of internal nucleated fibers

Internal nucleated fibers (INFs) were quantified on laminin/DAPI-stained sections. A fiber was classified as internal nucleated when one or more DAPI-positive nuclei were located within the interior of the laminin-defined boundary rather than in a subsarcolemmal position. INF% was calculated as (internal nucleated fibers / total fibers scored) $\times$ 100, with $\geq 1,000$ fibers scored per animal per muscle per time point.

Myofiber cross-sectional area

Myofiber cross-sectional area (CSA) was measured from laminin-stained images using Fiji (ImageJ) by segmenting laminin-defined boundaries, with manual correction as needed. Fibers exhibiting incomplete boundaries, oblique sectioning, or edge truncation were excluded. A total of ≥ 800 –1,500 fibers per animal per muscle per time point were quantified.

Necrotic fiber density

Necrotic fibers were assessed on H&E-stained sections and reported as necrotic fiber percentage (%). Necrosis was identified based on established morphological criteria, including fiber fragmentation, pale sarcoplasm, disruption of normal architecture, and frequent inflammatory infiltration. Necrotic fiber% was calculated as (necrotic fibers / total fibers scored) $\times$ 100.

All analyses were conducted by investigators blinded to group identity. The animal was defined as the biological replicate, where sections, fields, and individual fibers were treated as within-animal sampling units to estimate distributions and enhance measurement robustness.

Serum enzyme measurement

Monkey blood was collected from the submandibular vein by a vacuum vessel (BD, 367955). The blood samples were centrifuged at 5,000 rpm for 10 min at 4°C to obtain serum. The levels of creatinine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were measured by Kunming Biomed International.

Activity measurement

During the experiment, the monkeys were kept in a clean and transparent video cage. To ensure no interference from other monkeys, the cage was placed in a separate room. The monkeys had free access to water and could move around the cage without any restrictions. To avoid any interference, no one was present in the room after the recording began. The monkey activity was continuously recorded for 60 min using a Kinect 2.0 camera and analyzed with Primate Scan 1.0 software (Clever Sys Inc, VA). Total behavior was quantified as the cumulative frequency of predefined behavioral events recorded during the observation period, including bouncing, climbing, descending, hanging (cuddled or vertically), jumping, vertical landing, moving left to right, pacing, sitting, standing up, swinging, and turning. Raw behavioral data across all longitudinal time points are presented in [Table S1](#).

Gait analysis

Patients with DMD often exhibit a duck-like gait. To investigate the hind limb walking characteristics in DMD monkeys, we conducted a footprint analysis. The monkeys were trained to walk on a treadmill with white paper attached to it, and their hind feet were dipped in edible ink. The monkeys were then allowed to walk at a predetermined speed of 0.8 km/h on the treadmill. We collected the footprints and measured the left and right footprint lengths to compare them with the actual foot lengths of the monkeys. A minimum of 20 steps was collected for each monkey. Each experiment was repeated for at least three times.

Lie-to-stand assay

Monkeys were placed in supine or prone position with hands tied behind or in front. The standing up time required for each posture was recorded to reflect the muscle function. Each posture was repeated for at least 4 times with adequate rest in between.

Blinded behavioral analysis

Motor function assessments, including activity measurement, gait analysis, and the lie-to-stand assay, were conducted and quantified by two independent investigators blinded to treatment allocation. Video recordings were anonymized and assigned randomized identification codes before analysis. Behavioral parameters were evaluated according to predefined criteria, and group identities were disclosed only after completion of the data analysis.

ELISPOT assay

Interferon- γ enzyme-linked immunosorbent spot (ELISPOT) assay was performed according to the kit from (MABTECH, 3421M-4HPW-2) to evaluate the immune response of PBMC from monkeys. Briefly, PBMC isolated from DMD monkeys isolated using Ficoll-Hypaque density gradients were cultured with synthetic peptides (20 amino acids in length, overlapping by 10 residues) spanning the rhesus monkey dystrophin protein. The peptides were grouped into two pools, designated MDP-1 and MDP-2. MDP-1 encompassed the mini-dystrophin actin-binding domain, H1, and R1 (exons E1–E11/E12), whereas MDP-2 included R2, R22, R23, and H3 (exons E12, E50–51, and E56–59). Sequence of MDP peptides are listed in [Table S5](#).

Fewer than 10 SFCs per well were detected in response to peptides from the control pool (CP) derived from a wild-type monkey. Responses were defined as positive when SFC counts exceeded 50 per 10^6 PBMC in duplicate wells.

Quantification of circ-arRNA

The expression levels of circ-arRNAs both *in vitro* and *in vivo* were quantified using qPCR (TAKARA, RR820A). The primer sequences are listed in [Table S5](#).

Quantification of arRNA copies/ μ g RNA and VCN copies/nucleus

For normalization of arRNA copies/ μ g RNA, a synthetic RNA standard (L-TC-RNA, GenScript) was serially diluted from 5.0×10^3 to 5.0×10^7 copies/ μ l to generate a standard curve. Sample arRNA concentrations were calculated based on the standard curve and normalized to total RNA input (copies/ μ g total RNA). For normalization of VCN copies/nucleus, a linearized plasmid standard containing the target sequence was used. The plasmid was digested with XbaI, purified, and serially diluted in EASY Dilution (TAKARA, 9160) to generate a standard curve ranging from 1.0×10^2 to 1.0×10^7 copies/ μ l.

Administration of LE051

The case was approved by the institutional review board of Shanghai Children's Medical Center Affiliated to Shanghai Jiao Tong University School of Medicine (Ethics Approval Number: SCMCIRB-K2024186-1). Written informed consent was obtained from the patient in accordance with the Declaration of Helsinki prior to enrollment. Prior to infusion, patients underwent adjustment of systemic corticosteroid therapy as follows: starting three days before LE051 infusion (Day –3) and continuing thereafter, participants received an increased dose of prednisolone at 1.75 mg/kg/day (maximum 60 mg/day). For patients intolerant to prednisolone, an equivalent dose of an alternative corticosteroid (e.g., deflazacort) was permitted. This intensified corticosteroid regimen was maintained for at least 30 days post-infusion, unless dose tapering was deemed clinically necessary at the investigator's discretion based on individual tolerability or adverse event profiles. The study comprised two dose cohorts, 2.0×10^{13} vector genomes per kilogram (vg/kg) and 5.0×10^{13} vg/kg. Enrolled participants were allocated to the corresponding cohort and administered LE051 via intravenous infusion at a weight-adjusted dose consistent with the assigned level.

Safety monitoring

- (1) Patient monitoring: The patient's temperature and vital signs were continuously monitored and recorded daily during the first month of hospitalization.
- (2) Routine laboratory testing: Regular follow-up assessments included complete blood counts, cellular immunologic response, and biochemical evaluations to monitor liver, kidney, and cardiac function. These analyses were performed by certified personnel at the Medical Laboratory Center of Shanghai Children's Medical Center Affiliated to Shanghai Jiao Tong University School of Medicine, strictly following the protocols detailed in the test kit manuals.
- (3) Cardiac safety monitoring and assessment: Cardiac monitoring included 24-h continuous electrocardiographic (ECG) surveillance on the day of treatment, a standard 12-lead ECG on the following day, and transthoracic echocardiography at 14 days post-treatment.
- (4) Dose-Limiting Toxicity (DLT) Assessment: Continuous monitoring for DLTs was performed following LE051 administration. DLTs were defined as any adverse event (AE) considered related to LE051 occurring within 28 days after infusion that met predefined severity criteria. In accordance with NCI-CTCAE version 5.0 grading standards, DLTs included: Severe ($\geq$ Grade 3) AEs, excluding elevated alanine aminotransferase (ALT) or aspartate aminotransferase (AST) commonly observed in DMD; Clinically significant hepatotoxicity, defined by marked ALT/AST elevations ($\geq 3 \times$ baseline or $\geq 3 \times$ ULN with bilirubin $\geq 2 \times$ ULN) or disproportionate transaminase increases relative to alkaline phosphatase (ALT/AST:ULN to ALP:ULN ratio ≥ 5); Any LE051-related AE of any grade that, in the investigator's judgment and in consultation with study collaborators, warranted early study withdrawal.

Efficacy evaluation

The major clinical improvement was evaluated using North Star Ambulatory Assessment (NSAA) Scores, 6-min walk distance, supine-to-stand time, 4-stair climb time and 10-meter walk/run time. Moreover, dystrophin protein expression levels in muscle tissue or percentage of dystrophin-positive muscle fibers compared to baseline are also indicators for recovery. In addition, exploratory analyses encompassed changes from baseline in cardiopulmonary exercise testing (CPET), post-treatment variations in blood biomarkers (including CK, CK-MB, LDH, AST, GGT, ALP and GR) and longitudinal monitoring of pulmonary function.

QUANTIFICATION AND STATISTICAL ANALYSIS

n represents the number of independent experiments performed in parallel, unless otherwise stated. For comparisons between two groups, unpaired two-tailed Student's *t*-test was used. One-way ANOVA followed by Tukey's multiple comparisons test was applied for comparisons involving more than two groups, as indicated in the figure legends. Two-way ANOVA with repeated measures was used for analyses of distance and total behaviors. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001, and ^{ns}*P* indicates no statistically significant difference.

Supplemental figures

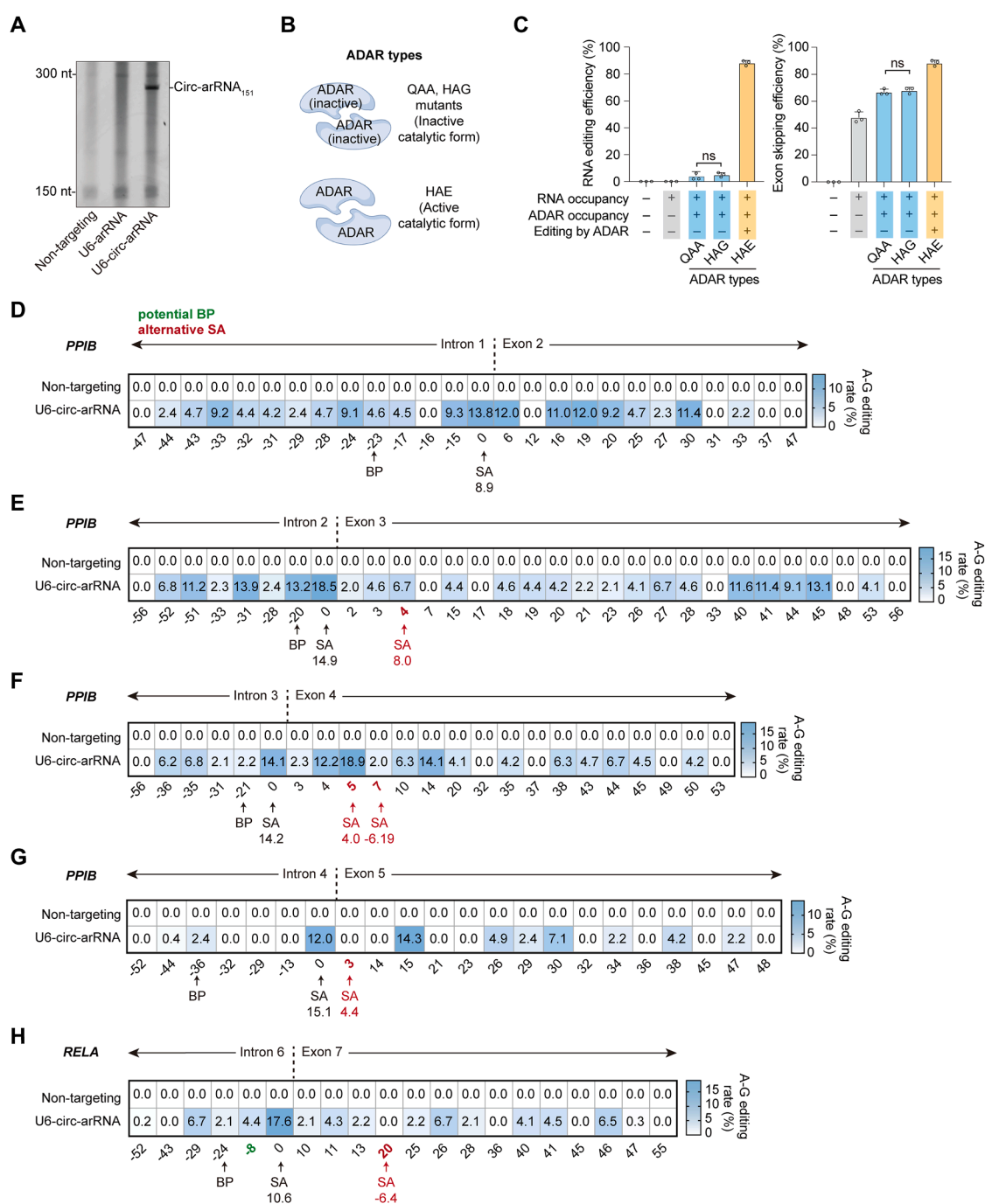


Figure S1. Circ-arRNA achieves exon skipping in multiple endogenous sites, related to Figure 1

(A) Urea PAGE gel analysis of cellular total RNA in HEK293T cells transfected by Nontargeting, U6-arRNA₁₅₁ and U6-circ-arRNA₁₅₁.

(B) Illustration of ADAR inactive and active catalytic form.

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(C) RNA editing efficiency (left, quantified by CG2 reporter¹⁴) and exon skipping efficiency (right, splicing reporter of exon 5 in monkey *DMD* sequence) in HEK293T *ADAR1*^{-/-} cells with overexpression of different forms of ADAR1, HAE in ADAR1 represents catalytic active form (editing by ADAR, yellow) while QAA or HAG represents inactive forms (blue). Significance was analyzed using a two-tailed unpaired Student's *t* test; ns, not significant. ^{ns}*P* = 0.5185 (editing), ^{ns}*P* = 0.5893 (exon skipping).

(D–H) Heat maps show editing rates at intron-exon boundaries targeted by circ-arRNA₁₅₁. Black and red SA indicate original and potential cryptic splicing site predicted by using weighted matrix method (WMM).

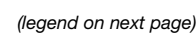


Figure S2. Optimized 2×U6-circ-arRNA₁₅₁-v3 targeting wild-type SA and ESE induced more effective exon skipping, related to Figure 2

(A) Illustration of arRNA in tandem designs.

(B) Next-generation sequencing (NGS) results revealing the rates of targeted adenosine editing in a reporter (illustrated in D) harboring sequences flanking the splicing acceptor site (SA) in intron 4 by six variations of circ-arRNA₁₅₁ in HEK293T cells; $n = 3$, mean $\pm$ SD. Significance was analyzed using a two-tailed unpaired Student's t test, **** $P = 0.0001$ (U6-arRNA vs. U6-circ-arRNA), **** $P = 1.6520 \times 10^{-5}$ (U6-circ-arRNA vs. 2×U6-circ-arRNA-v3), *** $P = 0.0002$, * $P = 0.0123$.

(C) RT-PCR results showing the ratios of exon 5 skipping at endogenous rhesus monkey *DMD* achieved by six variations of circ-arRNA₁₅₁ in COS-7 cells; $n = 3$, mean $\pm$ SD. Significance was analyzed using a two-tailed unpaired Student's t test, **** $P = 0.0001$ (U6-arRNA vs. U6-circ-arRNA), **** $P = 4.3810 \times 10^{-9}$ (U6-circ-arRNA vs. 2×U6-circ-arRNA-v3), *** $P = 0.0007$, * $P = 0.0108$.

(D) Heat map of the rates of editing in dsRNA region covered by six variations of circ-arRNA₁₅₁ in a reporter harboring sequences flanking the splicing acceptor site (SA) in intron 4 (of rhesus monkey *DMD*) by six variations of circ-arRNA₁₅₁ in HEK293T cell.

(E) Schematic illustration of low-dose arRNA transfection; the cell population highlighted in red was gated and collected as the low-dose group.

(F) Collection of low transfection marker⁺ cells by FACS in COS-7 cells transfected with a low mass of 2×U6-circ-arRNA₁₅₁-v1 and -v3. RT-PCR and qPCR results showing exon 5 skipping efficiency at endogenous rhesus monkey *DMD* and circ-arRNA abundance correspondingly. For results in qPCR, $n = 3$, mean $\pm$ SD.

(G) qPCR results displaying the expression levels of circ-arRNAs in COS-7 cells transfected with different arRNA versions. $n = 3$, mean $\pm$ SD.

(H) qPCR results illustrating arRNA abundance in circ-arRNA₁₅₁ targeting the SA (wild-type, $\Delta 2$ and $\Delta 5$), normalized to *GAPDH*; $n = 3$, mean $\pm$ SD.

(I) Illustration about how circ-arRNA mediated exon skipping restore expression of in-frame transcripts when the skipped exon and the mutated exon are not the same.

(J) RT-PCR analysis showing exon 51 skipping of a splicing reporter carrying the *dmd* sequence in HEK293T cells by the constructs in (I).

(K) Illustration of ESE motifs in exon 51 of *dmd* and designs of circ-arRNA respectively.

(L) RT-PCR shows exon skipping efficiency of a splicing reporter (same as the reporter in Figure 2D) carrying the *dmd* sequence in HEK293T cells by constructs in (K).

(M) Detailed designs of circ-arRNA length from 151 to 31 and corresponding location.

(N) RT-PCR shows exon skipping efficiency of a splicing reporter (same as the reporter in Figures 2J and 2L) carrying the *dmd* disease sequence in HEK293T cells by constructs in (M).

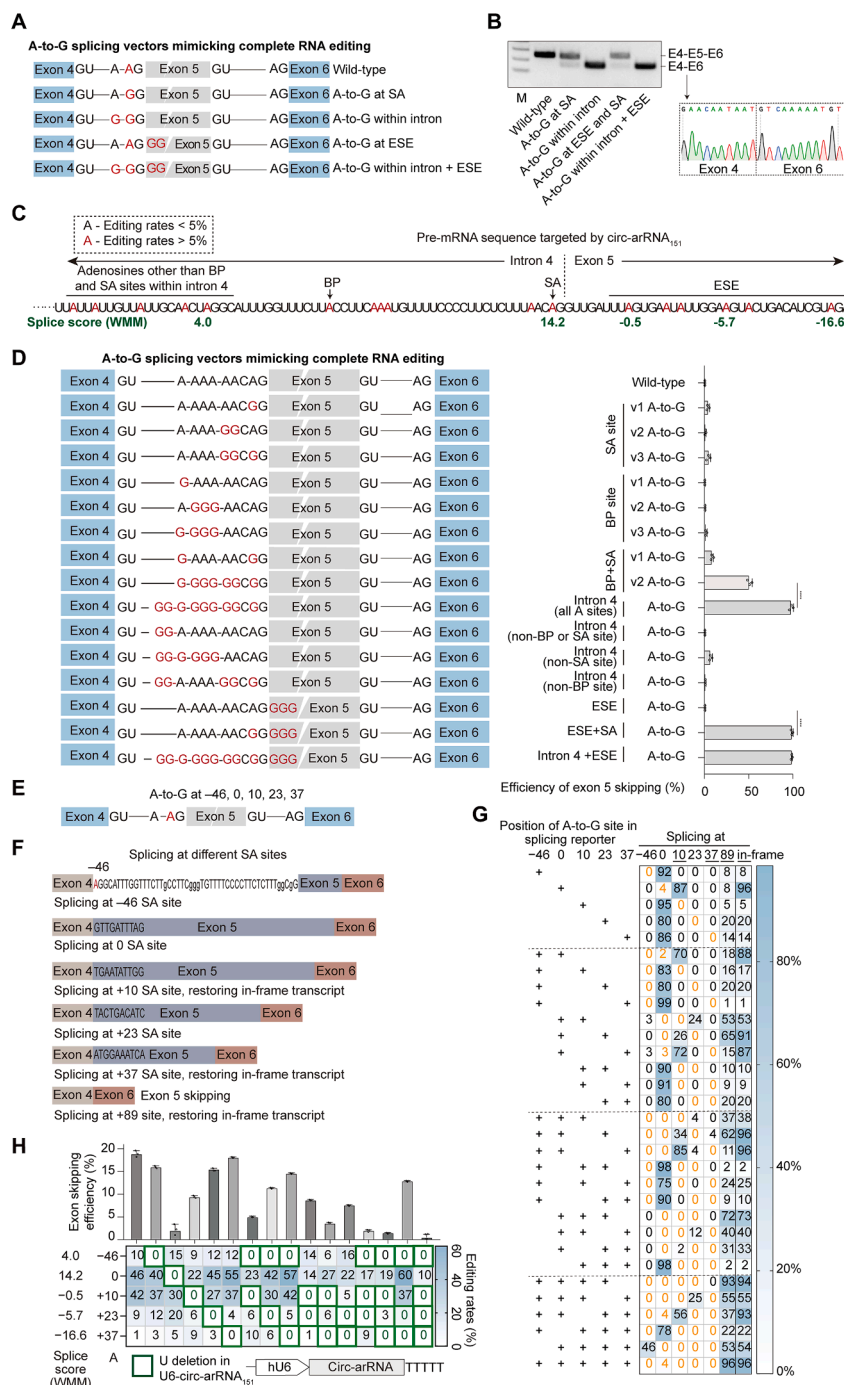


Figure S3. Synergistic RNA editing by circ-arRNA at splicing elements enhances exon skipping, related to Figure 2

(A) Illustration of splicing reporters with A-to-G transitions in intron 4 or exon 5 of rhesus monkey *DMD*.
 (B) RT-PCR analysis of exon 4-exon 5-exon 6 (E4-E5-E6) splicing in HEK293T cells transfected with the reporter constructs shown in (A). Sanger sequencing of exon junction after exon skipping (Exon 4-Exon 6 of endogenous rhesus monkey *DMD*) in COS-7 cells.
 (C) Diagram of the pre-mRNA sequence targeted by circ-arRNA₁₅₁. Splicing score is calculated using Weighted Matrix Method (WMM).
 (D) Construction of splicing reporter plasmids with A-to-G transitions in intron 4 or exon 5 of rhesus monkey *DMD*, mimicking maximum RNA editing by circ-arRNA, along with corresponding exon skipping efficiencies (%) determined by RT-PCR ($n = 3$, mean $\pm$ SD). Statistical significance was assessed using a two-tailed unpaired Student's *t* test; **** $P = 1.2000 \times 10^{-7}$ (BP+SA v2 vs. Intron 4 all A sites), **** $P = 5.1200 \times 10^{-10}$ (ESE+SA vs. ESE).
 (E) Illustration of A-to-G transition sites in splicing reporter plasmids.
 (F) Illustration of splicing pattern at different cryptic SA sites.

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(G) Heat map indicates the percentages of splicing at different SA site of splicing reporter plasmids with A-to-G transitions in intron 4 or exon 5 of rhesus monkey *DMD*. Splicing pattern was detected by NGS analysis, corresponding to illustrations in **CC**. "+" in the left indicates A converted to G, mimicking maximum RNA editing.

(H) Design of circ-arRNA with U deletion with exon skipping efficiency (bar) and editing rates (heatmap) at -46, 0, +10, +23, +37 sites. $n = 3$, mean $\pm$ SD.

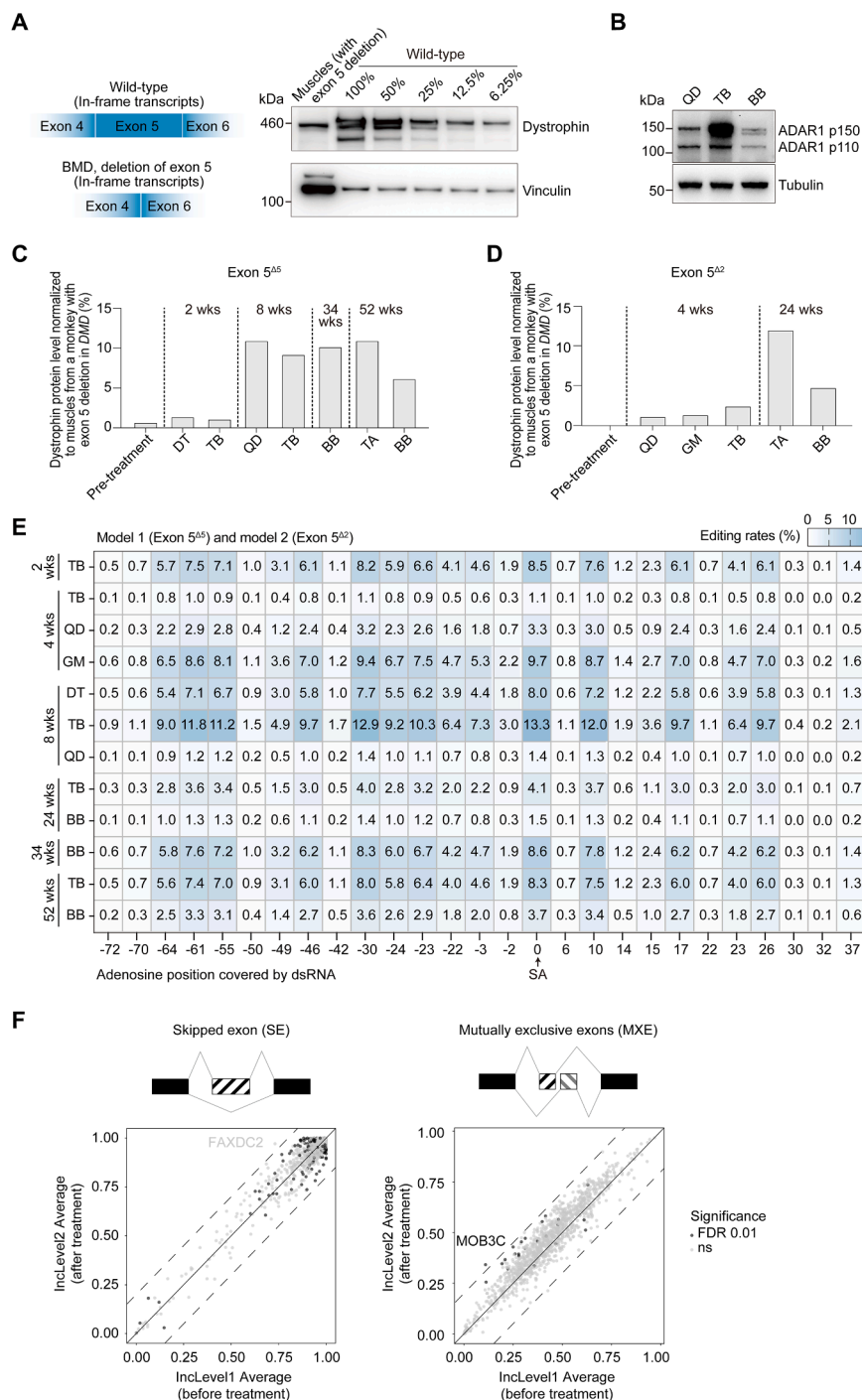


Figure S4. Dystrophin restoration and RNA editing analysis in NHP models, related to Figure 3

(A) Western blot results of muscle samples from BMD monkey with 100% exon 5 deletion.

(B) Western blot results of ADAR distribution in different muscle samples. TB, triceps brachii; QD, quadriceps; BB, biceps brachii.

(C and D) Semiquantitative evaluation of western blot results from muscle samples in DMD monkeys before and after treatment in Exon 5^{Δ5} (Figure 4E) and Exon 5^{Δ2} (Figure 4F) model. Control refers to muscle from monkey with 100% exon 5 deletion.

(E) Heatmap of editing rates covered by dsRNA in 2 DMD models. Data of 2 weeks, 8 weeks, 34 weeks and 52 weeks are from Exon 5^{Δ5} model; data of 4 weeks and 24 weeks are from Exon 5^{Δ2} model.

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(F) Scatter plots showing average inclusion level (IncLevel) averages of alternative splicing events before (x axis) and after treatment (y axis). Two major types of splicing events are presented: skipped exons (SE, left) and mutually exclusive exons (MXE, right). Each dot represents one splicing event. Dashed lines indicate the threshold for differential splicing. Events with a false discovery rate (FDR) < 0.01 (black) were considered statistically significant, while *ns* (gray) indicates non-significant events. Representative genes exhibiting significant changes in splicing patterns are labeled in the plots.

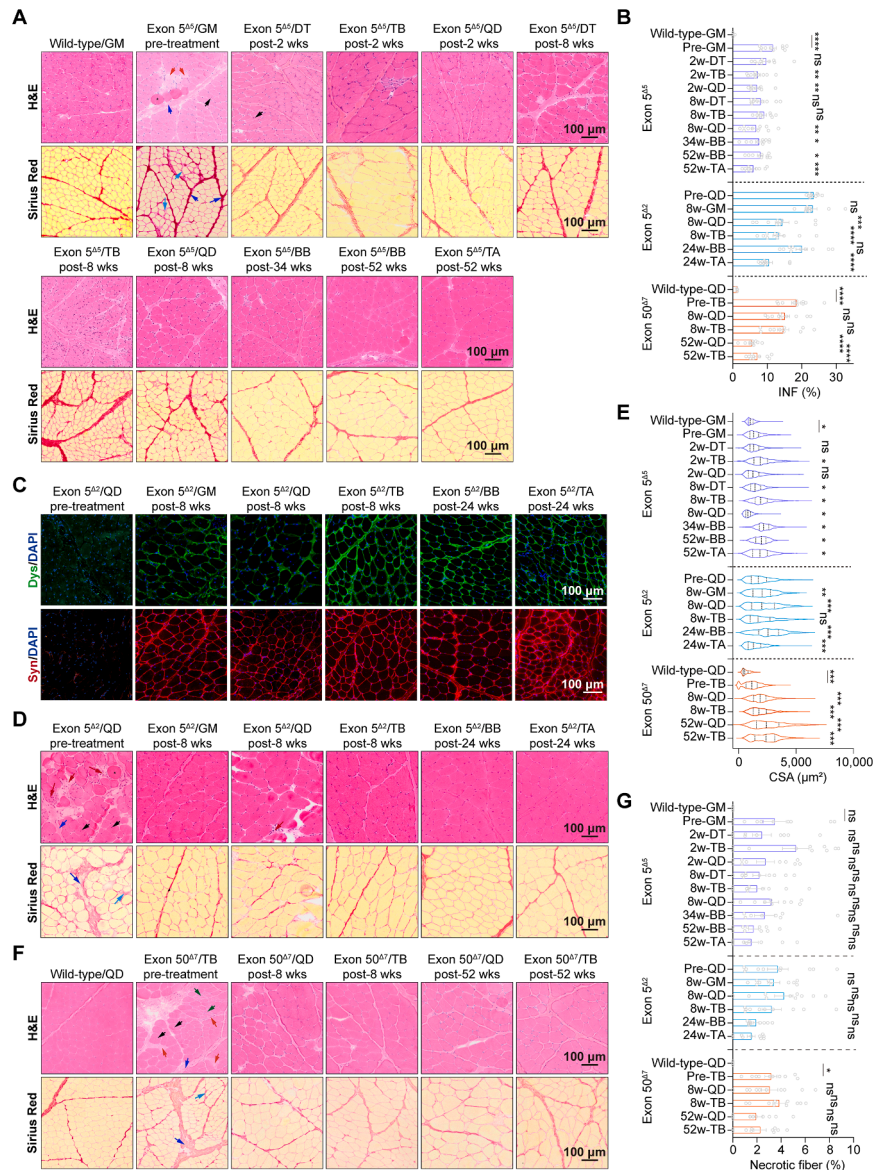


Figure S5. Histopathological biopsy analysis of DMD monkeys before and after circ-arRNA treatment, related to Figures 4 and 5

(A, D, F) H&E and Sirius red staining of muscle tissue biopsies from the Exon 5^{Δ5} (A), Exon 5^{Δ2} (D), or Exon 50^{Δ7} (F) model before and after circ-arRNA treatment. GM, DT, TB, QD, BB, and TA represent the muscle tissue source of biopsy. GM, gastrocnemius; DT, deltoid; TB, triceps brachii; QD, quadriceps; BB, biceps brachii; TA, tibialis anterior. Scale bar = 100 μ m. Red, blue, black, and green arrows indicate necrotic fibers, perimysial fibrosis, internal nucleated, and regenerating fibers, respectively; asterisk (*) marks hypercontracted, deeply eosinophilic fibers in H&E staining. Light blue and dark blue arrows indicate endomysial fibrosis and perimysial fibrosis in Sirius red staining.

(B) Quantification of internal nucleated fibers (INF%) in NHPs. Data are presented as mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA with post-hoc testing. For Exon 5^{Δ5}, * P = 0.0172 (pre-GM vs. 34w-BB), * P = 0.0432 (pre-GM vs. 52w-BB), ** P = 0.0075 (pre-GM vs. 2w-TB), ** P = 0.0039 (pre-GM vs. 2w-QD), ** P = 0.0022 (pre-GM vs. 8w-QD), ^{ns} P = 0.5640 (pre-GM vs. 2w-DT), ^{ns} P = 0.05277 (pre-GM vs. 8w-DT), ^{ns} P = 0.2732 (pre-GM vs. 8w-TB), *** P = 0.0002 (pre-GM vs. 52w-TA), **** P = 3.2707×10^{-7} . For Exon 5^{Δ2}, *** P = 0.0002, **** P = 3.800×10^{-5} (pre-QD vs. 8w-TB), **** P = 2.3790×10^{-7} (pre-QD vs. 24w-TA), ^{ns} P = 0.9999 (pre-QD vs. 8w-GM), ^{ns} P = 0.342126 (pre-QD vs. 24w-BB). For Exon 50^{Δ7}, **** P = 7.3085×10^{-8} (pre-TB vs. wild-type-QD), **** P = 6.2783×10^{-9} (pre-TB vs. 52w-QD), **** P = 1.5632×10^{-7} (pre-TB vs. 52w-TB), ^{ns} P = 0.1986 (pre-TB vs. 8w-QD), ^{ns} P = 0.1220 (pre-TB vs. 8w-TB).

(C) Immunofluorescence staining for dystrophin (Dys) and syntrophin (Syn) in muscle sections from Exon 5^{Δ2} model before and after treatment. Dystrophin expression is shown in green and syntrophin in red, with DAPI staining for nuclei (blue). Scale bar = 100 μ m.

(E) Quantification of cross-sectional area (CSA) of muscle fibers. Data are presented as violin plot. The thick line inside each violin represents the median of the data, while the thin lines at the top and bottom represent the 25th and 75th percentiles. Statistical significance was assessed using one-way ANOVA with post-hoc testing. For Exon 5^{Δ5}, * P = 0.0149 (pre-GM vs. wild-type-GM), * P = 0.0149 (pre-GM vs. 2w-TB), * P = 0.0432 (pre-GM vs. 8w-DT), * P = 0.0432 (pre-GM vs. 8w-TB), * P = 0.0432 (pre-GM vs. 8w-QD), * P = 0.0174 (pre-GM vs. 34w-BB), * P = 0.0149 (pre-GM vs. 52w-BB), * P = 0.0149 (pre-GM vs. 52w-TA), ^{ns} P = 0.0149 (pre-GM vs. 2w-DT), ^{ns} P = 0.0149 (pre-GM vs. 2w-QD). For Exon 5^{Δ2}, ** P = 0.0018, *** P = 0.0002 (pre-QD vs. 8w-QD), *** P = 0.0002 (pre-QD vs. 24w-BB), **** P = 0.0002 (pre-

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QD vs. 24w-TA), $^{ns}P = 0.1745$. For Exon 50 $^{\Delta 7}$, $^{***}P = 0.0002$ (pre-TB vs. wild-type-QD), $^{***}P = 0.0002$ (pre-TB vs. 8-TB), $^{***}P = 0.0002$ (pre-TB vs. 8w-QD), $^{***}P = 0.0002$ (pre-TB vs. 52-TB), $^{***}P = 0.0002$ (pre-TB vs. 52-QD).

(G) Quantification of necrotic fibers as a percentage of total fibers. Data are presented as mean $\pm$ SEM. Statistical significance was assessed using one-way ANOVA with post-hoc testing. For Exon 5 $^{\Delta 5}$, $^{ns}P = 0.1772$ (pre-GM vs. wild-type-GM), $^{ns}P = 0.9282$ (pre-GM vs. 2w-DT), $^{ns}P = 0.4875$ (pre-GM vs. 2w-TB), $^{ns}P = 0.9910$ (pre-GM vs. 2w-QD), $^{ns}P = 0.8268$ (pre-GM vs. 8w-DT), $^{ns}P = 0.6912$ (pre-GM vs. 8w-TB), $^{ns}P = 0.9999$ (pre-GM vs. 8w-QD), $^{ns}P = 0.9823$ (pre-GM vs. 34w-BB), $^{ns}P = 0.4946$ (pre-GM vs. 52w-BB), $^{ns}P = 0.3690$ (pre-GM vs. 52w-TA). For Exon 5 $^{\Delta 2}$, $^{ns}P = 0.9950$ (pre-QD vs. 8w-GM), $^{ns}P = 0.9731$ (pre-QD vs. 8w-QD), $^{ns}P = 0.9697$ (pre-QD vs. 8w-TB), $^{ns}P = 0.1736$ (pre-QD vs. 24w-BB), $^{ns}P = 0.0673$ (pre-QD vs. 24w-TA). For Exon 50 $^{\Delta 7}$, $^{*}P = 0.0323$, $^{ns}P = 0.9999$ (pre-TB vs. 8w-QD), $^{ns}P = 0.8535$ (pre-TB vs. 8w-TB), $^{ns}P = 0.3734$ (pre-TB vs. 52w-QD), $^{ns}P = 0.6812$ (pre-TB vs. 52w-TB).

For data in (B), (E), and (G), the P values are arranged in order of significance.

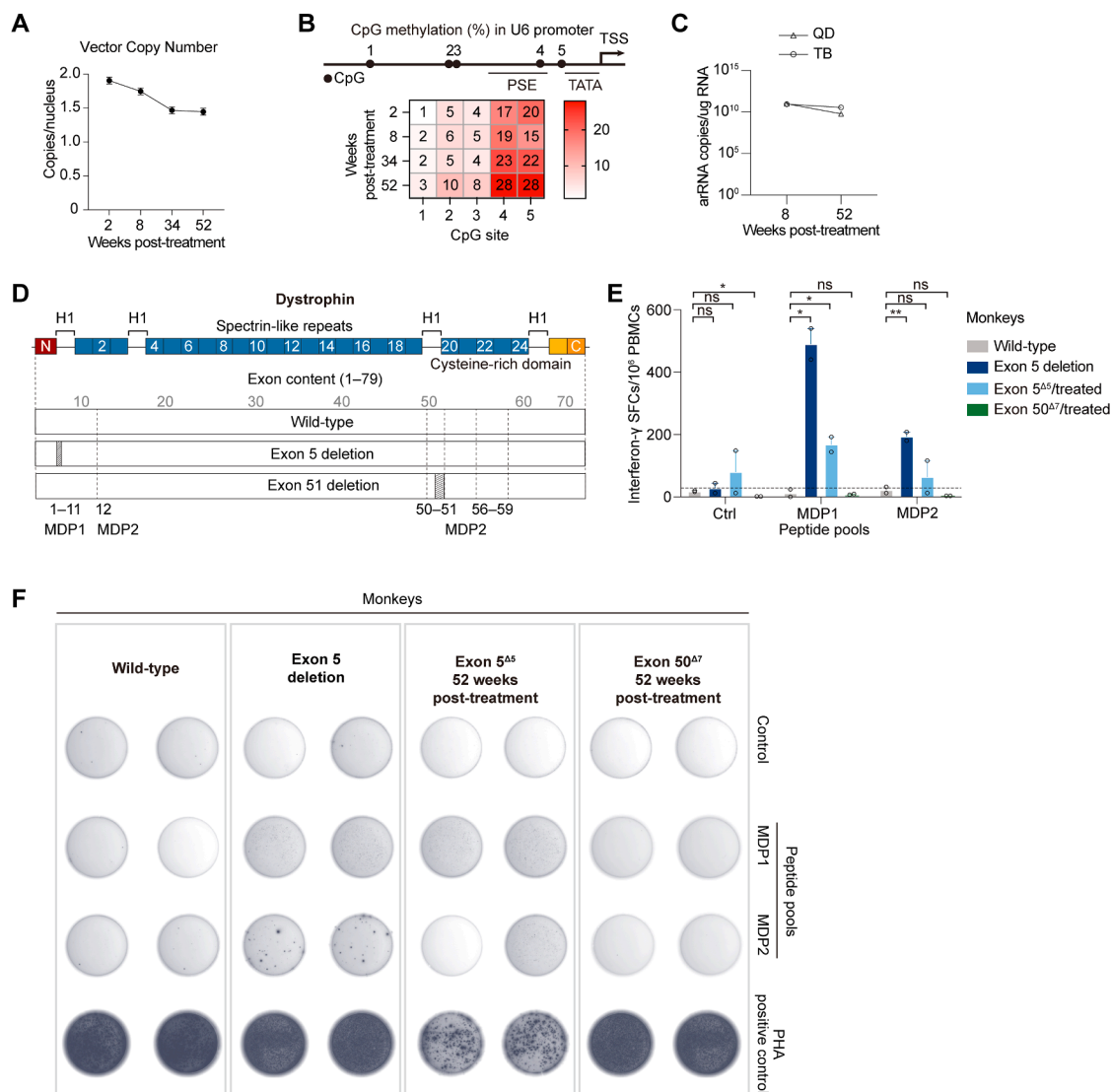


Figure S6. Monitoring AAV genome copy, AAV epigenetic feature, and dystrophin immunogenicity in DMD models, related to Figures 4 and 5

(A) Vector copy number (VCN) in muscle samples derived from the exon 5^{Δ5} model.

(B) Methylation of DNA at cytosine sites by bisulfite sequencing in muscle samples derived from exon 5^{Δ5} model.

(C) Persistence of circ-arRNAs in exon 50^{Δ7} model by qPCR at different time points after systemic injection.

(D) Illustration of dystrophin (up), exon content of *DMD* (middle) and area covered by peptide pools (MDP1 and MDP2).

(E) Dystrophin-Specific immune response. Peripheral-blood mononuclear cells (PBMCs) from monkeys of wild-type, exon 5 deletion, one-year post-treatment of exon 5^{Δ5} and exon 50^{Δ7} were cultured with two pools of synthetic peptides (MDP1, MDP2) spanning 5' and middle domain of dystrophin. Interferon-γ production was assessed 48 h later with the use of an interferon-γ enzyme-linked immunosorbent spot (ELISpot) assay. Data are presented as interferon-γ spot-forming cells (SFCs) per 1 million PBMCs. The dashed lines represent the threshold for a positive assay response (50 SFCs per 1 million PBMCs). $n = 2$, mean $\pm$ SEM. Significance was analyzed using a two-tailed unpaired Student's t test. In the control group (ctrl), $^{ns}P = 0.5984$ (wild-type vs. exon 5 deletion), $^{ns}P = 0.4583$ (wild-type vs. exon 5^{Δ5}/52 weeks post-treatment), $^{*}P = 0.0121$ (wild-type vs. exon 50^{Δ7}/52 weeks post-treatment). In the MDP1 group, $^{*}P = 0.0114$ (wild-type vs. exon 5 deletion), $^{*}P = 0.0283$ (wild-type vs. exon 5^{Δ5}/52 weeks post-treatment), $^{ns}P = 0.6707$ (wild-type vs. exon 50^{Δ7}/52 weeks post-treatment). In the MDP2 group, $^{**}P = 0.0099$ (wild-type vs. exon 5 deletion), $^{ns}P = 0.5108$ (wild-type vs. exon 5^{Δ5}/52 weeks post-treatment), $^{ns}P = 0.2137$ (wild-type vs. exon 50^{Δ7}/52 weeks post-treatment).

(F) Source data of interferon-γ enzyme-linked immunosorbent spot (ELISpot) assay to evaluate dystrophin-specific immune response.

