Background: Loss of dystrophin protein causes Duchenne muscular dystrophy (DMD), characterized by progressive degeneration of cardiac and skeletal muscles, and mortality in adolescence or young adulthood. Although cardiac failure has risen as the leading cause of mortality in patients with DMD, effective therapeutic interventions remain underdeveloped, in part, because of the lack of a suitable preclinical model.

Methods: We analyzed a novel murine model of DMD created by introducing a 4-bp deletion into exon 4, one of the exons encoding the actin-binding domain 1 of dystrophin (referred to as DmdE4* mice). Echocardiography, microcomputed tomography, muscle force measurement, and histological analysis were performed to determine cardiac and skeletal muscle defects in these mice. Using this model, we examined the feasibility of using a cytidine base editor to install exon skipping and rescue dystrophic cardiomyopathy in vivo. AAV9-based CRISPR/Cas9-AID (eTAM) together with AAV9-sgRNA was injected into neonatal DmdE4* mice, which were analyzed 2 or 12 months after treatment to evaluate the extent of exon skipping, dystrophin restoration, and phenotypic improvements of cardiac and skeletal muscles.

Results: DmdE4* mice recapitulated many aspects of human DMD, including shortened life span (by ≈50%), progressive cardiomyopathy, kyphosis, profound loss of muscle strength, and myocyte degeneration. A single-dose administration of AAV9-eTAM instituted >50% targeted exon skipping in the Dmd transcripts and restored up to 90% dystrophin in the heart. As a result, early ventricular remodeling was prevented and cardiac and skeletal muscle functions were improved, leading to an increased life span of the DmdE4* mice. Despite gradual decline of AAV vector and base editor expression, dystrophin restoration and pathophysiologic rescue of muscular dystrophy were long lasted for at least 1 year.

Conclusions: Our study demonstrates the feasibility and efficacy to institute exon skipping through an enhanced TAM (eTAM) for therapeutic application(s).

Key Words: cardiomyopathy ◼ CRISPR-associated protein 9 ◼ cytidine deaminase ◼ dependovirus ◼ genetic therapy ◼ muscular dystrophy, Duchenne ◼ RNA splicing

Duchenne muscular dystrophy (DMD) is the most severe form of muscular dystrophy affecting 1 in 3500 to 5000 boys at birth globally. Patients experience a progressive wasting of both skeletal and cardiac muscles attributable to the loss of dystrophin protein encoded by the DMD gene on chromosome Xp21. With the improvement of respiratory support for patients with DMD, heart failure has become the leading cause of morbidity and mortality.1 As early as at 6 to 10 years of age, cardiac involvement is manifested as electrocardiographic abnormalities in patients with DMD, which often progress into dilated cardiomyopathy and cardiac failure.
Clinical Perspective

What Is New?

- A novel murine model of Duchenne muscular dystrophy was generated, which recapitulated many cardiac defects observed in human Duchenne muscular dystrophy, including dilated cardiomyopathy, reduced left ventricular functions, and extensive cardiac fibrosis.
- Built on CRISPR/Cas9-AID (targeted activation-induced cytidine deaminase–induced mutagenesis [TAM]), enhanced TAM (eTAM) improved the editing efficiency by almost 10-fold.
- A single-dose systemic delivery of the AAV9-eTAM together with AAV9-single guide RNA enabled efficient exon skipping, dystrophin protein restoration, and extension of life span.

What Are the Clinical Implications?

- Dystrophin restoration and pathophysiological rescue of muscular dystrophy were durable for at least 1 year after the AAV-eTAM treatment.
- A N-terminal truncated dystrophin protein was functional in vivo, thus expanding the exon-skipping strategy to a broader community of patients with Duchenne muscular dystrophy.
- AAV-eTAM has the potential to be applied to monogenic human diseases to modulate exon skipping/inclusion.

Nonstandard Abbreviations and Acronyms

| AAV | adeno-associated virus |
| AID | activation-induced cytidine deaminase |
| CK  | creatine kinase |
| CRISPR | clustered regularly interspaced short palindromic repeats |
| DMD | Duchenne muscular dystrophy |
| HTS | high-throughput sequencing |
| IVS | interventricular septum |
| LVEF | left ventricle ejection fraction |
| LVFS | left ventricle fraction shortening |
| RT-PCR | reverse transcription polymerase chain reaction |
| TAM | targeted AID-induced mutagenesis |

in their twenties. At present, palliative treatments are provided to delay the progression of cardiac symptoms while no curative intervention is available.

Most mutations identified in the DMD gene disrupt its reading frame, resulting in a complete loss of dystrophin protein that is essential to maintain muscle cell integrity. Meanwhile, early observation that in-frame mutations of the DMD gene cause a less severe Becker muscular dystrophy inspires the therapeutic approaches to restore the open reading frame through exon skipping, which can be achieved by antisense oligonucleotides or clustered regularly interspaced short palindromic repeats (CRISPR)-based technologies. The massive dystrophin protein is composed of an N-terminal actin-binding domain that anchors the protein to the cytoskeleton, central rod domains, a cysteine-rich domain that binds β-dystroglycan, and a C-terminal domain associated with sarcolemma membrane. In DMD animal models that carry various mutations in the central rod domains, applications of the aforementioned technologies have demonstrated that truncated dystrophin proteins missing part of rod domains are functional to rescue the skeletal muscle defects in vivo.

However, it remains largely undetermined whether such reframing strategies can ameliorate the fatal cardiomyopathy associated with DMD, because, to date, no murine models can recapitulate both the genetics and the cardiac pathophysiology observed in patients. The most commonly used animal model of DMD (mdx mouse) harboring a nonsense point mutation in exon 23 only develops minor cardiac phenotypes near the end of a mouse life span. In conjugation with other genetic defects, mdx mice manifest more severe cardiac dysfunctions, which, however, is not concordant with the genetics of human Duchenne (with only DMD mutations).

We and others have recently developed a gene-editing approach to efficiently institute exon skipping without introducing DNA double-strand breaks. Harnessing a fusion of a nuclease-defective Cas9 protein and a cytidine deaminase (Targeted AID induced Mutagenesis [TAM] or Base Editor 3 [BE3]), our approaches precisely edit conserved guanines at splice sites, thus abrogating exon recognition resulting in programmable skipping of the targeted exons. Here, we aimed to develop a TAM-based exon-skipping strategy to restore the open reading frame of the mutant Dmd gene in vivo.

To test the feasibility and efficacy of TAM-base therapy, we analyzed a new preclinical model of DMD in which a frameshift deletion in exon 4 of the Dmd gene was generated, referred to as Dmd<sup>E4*</sup> mice. In contrast with the broadly used mdx model, the Dmd<sup>E4*</sup> model resembled the progression of cardiac pathology observed in the human DMD. We then developed a TAM-based in vivo exon-skipping strategy delivered through AAV9. A single-dose treatment in neonatal mice was able to restore dystrophin protein, prevent cardiac function deterioration, and extend the life span of the Dmd<sup>E4*</sup> mice. Our study thus demonstrates the feasibility and efficacy of using CRISPR-guided cytidine deaminases as a therapeutic strategy to install beneficial splicing changes to treat human diseases, such as DMD.

Methods

Detailed materials and methods used in this study are included in the Supplemental Methods. All data and materials in this...
study are available on reasonable request from the corresponding author.

**Mice**

To generate C57BL/10-\textit{Dmd}^{E4*} mice, Cas9 mRNA and a corresponding single guide RNA (sgRNA; Table S1) were injected into pronucleus of 1-cell zygotes (National Resource Center of Model Mice, China). C57BL/10-\textit{mdx} mice were obtained from the Jackson laboratory. Both \textit{Dmd}^{E4*} and \textit{mdx} mice were back-crossed to C57BL/6 background for at least 8 generations. All the mice were housed in specific pathogen-free conditions, and only male mice were used in the study. All animal studies were performed in compliance with the guide for the care and use of laboratory animals and were approved by the institutional biomedical research ethics committee of Westlake University.

**Statistics**

Different statistical analysis was performed at specific situations, including 2-tailed Student \( t \) tests, unpaired \( t \) test with Welch’s correction, multiple comparison ANOVA tests, 2-way ANOVA tests, or Mantel-Cox text in GraphPad Prism. The \( F \) test was used to compare variances before conducting the \( t \) test. Unless specially described, error bars stand for the standard deviation. *\( P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

**RESULTS**

**N-Terminal Frameshift Mutation in Dystrophin Protein Results in Dystrophic Cardiomyopathy**

It has been hypothesized that mutations in specific domains of the dystrophin protein are associated with varying severity of cardiac or skeletal muscle symptoms in DMD or Becker muscular dystrophy.\(^1\) For example, early-onset dystrophic cardiomyopathies have been associated with mutations at the N terminus of dystrophin,\(^2\) which account for 15% of exon deletions and \( \approx \)50% of exon duplications in DMD.\(^3\) To directly test this possibility, we adopted a mouse model bearing mutation(s) at exons encoding the N terminus of dystrophin.\(^4\) A founder with a 4-bp deletion at exon 4 was chosen for further study and referred to as \textit{Dmd}^{E4*} mice thereafter. In principle, this indel leads to a frameshift mutation and a downstream premature stop codon (Figure 1A).

In the \textit{Dmd}^{E4*} mice, we first confirmed the 4-bp deletion at exon 4 by reverse transcription polymerase chain reaction (RT-PCR) followed by Sanger sequencing (Figure 1B). Using immunoblotting and immunostaining, in 2-month-old \textit{Dmd}^{E4*} mice, a complete loss of dystrophin protein was confirmed in the heart, diaphragm, tibialis anterior, and rectus femoris muscles (Figure 1C and 1D). \( \alpha \)-Sarcoglycan and neuronal nitric oxide synthase (nNOS), components of dystrophin-associated glycoprotein complex, were greatly abolished in the \textit{Dmd}^{E4*} mice (Figure S1D–S1G). It is notable that the magnitude of \( \alpha \)-Sarcoglycan reduction in the hearts of \textit{Dmd}^{E4*} mice was greater than that in the hearts of \textit{mdx} mice (Figure S1E and S1F). Serum levels of creatine kinase (CK), an indicator of muscle damage, were increased \( >10 \) -fold in the 2-month-old \textit{Dmd}^{E4*} mice compared with serum levels of CK in the WT littermates (Figure 1E). Similar to \textit{mdx} mice,\(^2\) at 2 months of age, \textit{Dmd}^{E4*} mice developed muscular dystrophy, manifested histologically as necrotic fibers, inflammatory infiltration, and regenerative muscle fibers with centralized nuclei (Figure 1F). Functionally, the \textit{Dmd}^{E4*} mice demonstrated accelerated muscle fatigue following repetitive measurement of muscle strength compared with the WT littermates (Figure 1G).

In contrast with the \textit{mdx} mice, \textit{Dmd}^{E4*} mice developed an early-onset cardiac dysfunction. As early as 2 months of age, \textit{Dmd}^{E4*} mice exhibited an increase in heart size and weight compared with the WT littermates after normalization to tibia length (Figure 2A and 2B). An increase of the surface areas of cardiomyocytes was also observed in the \textit{Dmd}^{E4*} mice in comparison with the cells in WT mice determined by immunofluorescence staining of laminin (Figure S1N and S1O). Early inflammatory infiltration (hematoxylin and eosin staining; Figure 2C, Figure S2A) and fibrosis (Masson staining; Figure 2C and 2D, Figure S2B) were revealed by the histological and morphometric analysis, such that a 20-fold increase of fibrotic area was observed in \textit{Dmd}^{E4*} mice relative to WT littermates. We further examined cardiac function in the 2-month-old \textit{Dmd}^{E4*} mice versus WT littermates by echocardiography (Table S2). An increase of interventricular septum (IVS) thickness (systolic, 1.85\( \pm \)0.09 mm versus 1.37\( \pm \)0.17 mm; diastolic, 1.03\( \pm \)0.05 mm versus 0.87\( \pm \)0.14 mm) and an accompanying decrease of left ventricle chamber size (systolic, 1.43\( \pm \)0.10 mm versus 2.03\( \pm \)0.33 mm; diastolic, 3.31\( \pm \)0.13 mm versus 3.43\( \pm \)0.25 mm) were observed in the \textit{Dmd}^{E4*} mice relative to controls (Figure 2E and 2F), whereas left ventricular posterior wall thickness was not significantly altered (Figure 2F). In addition, left ventricle ejection fraction (LVEF%), which measures the percentage of blood that pumped out of a filled left ventricle with each contraction, was increased in the \textit{Dmd}^{E4*} mice (87.85\( \pm \)2.00\% versus 72.55\( \pm \)6.44\%; Figure 2F). Left ventricle fraction shortening (LVFS%), the percent change in left ventricle cavity dimensions at the base with systolic contraction, was also elevated in \textit{Dmd}^{E4*} mice (56.85\( \pm \)2.84\% versus 41.11\( \pm \)5.63\%; Figure 2F). Together, these data suggested a condition that mimics interventricular septum hypertrophy, a potential compensatory attempt of the heart to enhance its performance, which is observed in some young patients with DMD.\(^1,2,3\) By 8 months of age, cardiac dysfunctions in the \textit{Dmd}^{E4*} mice progressed into dilated cardiomyopathy with decreased left ventricular contraction, which are often observed in the later stage of human Duchenne.
Figure 1. Generation of Dmd<sup>E4*</sup> mice with a frameshift mutation in exon 4 of the Dmd gene.

A, Schematic showing the generation of the Dmd<sup>E4*</sup> mouse model containing a 4-bp deletion within exon 4 of the Dmd gene. A sequence alignment to compare the WT (Top) and the mutant (Bottom) sequence containing a 4-bp deletion in the exon 4. B, Reverse transcription polymerase chain reaction products from the heart of the Dmd<sup>E4*</sup> mice were analyzed with Sanger sequencing. An amplicon was amplified from cDNA with indicated primers. The boxed sequence represents the 4-bp deletion in the Dmd<sup>E4*</sup> mice. Dystrophin was determined by immunoblotting (C) and immunofluorescence staining (D) in the heart, diaphragm, tibialis anterior (T.A.), and rectus femoris (R.F.) from 2-month-old WT or Dmd<sup>E4*</sup> mice. C, Vinculin (VCL) was included as a loading control and a molecular weight standard was marked on the left. D, Scale bars represent 100 μm. Data are representative of 3 independent experiments (n=2 in each experiment). E, Serum creatine kinase (CK) was determined by an International Federation of Clinical Chemistry reference method (Wako) in 2-month-old Dmd<sup>E4*</sup> mice and littermate controls. Data are a summary of 3 independent experiments. Each dot represents 1 individual mouse (n=6). F, Hematoxylin and eosin staining of various muscle tissues from Dmd<sup>E4*</sup> and littermate controls at 2 months of age. T.A. indicates tibialis anterior; R.F., rectus femoris. Data are representative of 3 independent experiments. G, Decline of grip strength under the fatigue-inducing condition. Ten grip tests of the combined forelimb and hindlimb strength were conducted with a short interval (10 seconds) between each test. The reduction in strength was calculated by normalization to the maximal grip strength. Data are summarized from 2 independent experiments (n=3 in each experiment). Error bars stand for the standard deviation. ****P<0.0001 in unpaired 2-tailed Student t test (E) or 2-way repeated-measures ANOVA test (G). DAPI indicates 4',6-diamidino-2-phenylindole; and WT, wild type.
Figure 2. *Dmd* E4* mice develop progressive cardiac dysfunction.

A and B. The hearts of *Dmd* E4* mice compared with age-matched wild-type mice. A, Representative images. B, Heart weights (HW) normalized by tibia length (TL) are summarized from 2-month-old or 8-month-old wild-type (WT) and *Dmd* E4* mice. C, Hematoxylin and eosin (H&E; Left) and Masson (Right) staining of the heart from the *Dmd* E4*, mdx, and littermate control mice at the indicated age. Data are representative of 3 independent experiments. Scale bars: 100 μm. D. Summary of the fibrotic area observed in the heart based on Masson staining from 4 independent experiments (n = 4). Each dot represents a data point of the whole-mount staining of a heart. E, Representative echocardiographic images of 2- or 8-month-old *Dmd* E4* mice that manifested early interventricular septum hypertrophy (2-month-old) and later cardiac dilation (8-month-old). Age-matched wild-type mice were included as controls. IVS indicates interventricular septum; LVID, left ventricular internal dimension; and LVPW, left ventricular posterior wall. (F, G) Echocardiographic measurements at 2-month-old (F) and 8-month-old mice (G). The thickness of interventricular septum (IVS), left ventricular posterior wall (LVPW), and diameters of the left ventricular (Diam.) at systole or diastole were compared between *Dmd* E4* and control mice at the indicated ages. IVS was determined as shown in E. (Continued)
cardiomyopathy. Histologically, collagen deposition was increased to up to 3% of the myocardial surface in DmdE4* mice (Figure 2C and 2D, Figure S2C and S2D). Echocardiographic analysis indicated the characteristic manifestations of dilated cardiomyopathy. Left ventricular internal diameters were increased in DmdE4* mice relative to the littermate controls (systolic, 2.92±0.25 mm versus 2.09±0.22 mm; diastolic, 4.09±0.20 mm versus 3.74±0.15 mm). LVEF% and LVFS% exhibited significant reduction in DmdE4* mice compared with the controls (LVEF%: 55.38±5.73% versus 75.80±2.21%; LVFS%: 28.60±3.72% versus 44.01±2.12%; Figure 2E and 2G, Table S3). Similar findings of compromised left ventricular functionality were observed in the DmdE4* mice without sedation (Figure S1M). The normalized heart weight was slightly reduced in 8-month-old DmdE4* mice compared with the WT mice (Figure 2B). These findings were further substantiated by microcomputed tomography, revealing enlarged ventricles along with the decreased thickness of ventricular chamber walls in 12-month-old DmdE4* mice relative to littermate controls (Figure S1H and S1I).

The life span of the DmdE4* mice was significantly reduced compared with their littermates. The earliest mortality occurred at 4 weeks of age in DmdE4* mice and the median life span is 65 weeks (Figure 2H and 2I), representing a ≈50% reduction of that in C57BL/6 mice. Furthermore, in 3 DmdE4* mice deceased between 48 and 50 weeks, microcomputed tomography scans of the hearts revealed a significant loss of myocardium and pronounced ventricular dilatation of both left and right ventricles, consistent with congestive heart failure, indicating these mice had likely died of heart failure resulting from dilated cardiomyopathy (Figure S1J ). In addition, in 3-month-old DmdE4* mice, we observed kyphosis (Figure S1K and S1L), an excessive outward curvature of the spine attributable to uneven weakening of trunk muscles and substitution with connective tissue and fat, another classical clinical manifestation of DMD.

As a comparison, the aforementioned phenotypes were not observed in the age-matched mdx mice, including a significantly shorter life span, progressive cardiomyopathy, and kyphosis (Figure S1K and S1L, Tables S2 and S3). Although the level of utrophin, a structural and functional paralog of dystrophin, was increased by ≈3-fold in the heart of mdx mice (Figure 2J and 2K), such an increase was not observed in the DmdE4* mice, suggesting a lack of compensatory upregulation of utrophin that may, in part, account for the more pronounced cardiomyopathy observed in these mice. Similarly, the elevation of utrophin protein was more pronounced in the skeletal muscle of the mdx mice than in the DmdE4* mice (Figure S2E and S2F), and utrophin expression in 12-month-old DmdE4* mice remained lower than that of the mdx mice (Figure S2G and S2H). Meanwhile, mRNA levels of utrophin were comparable among WT, mdx, and DmdE4* mice (Figure S2I), suggesting that the elevation of utrophin protein in the mdx mice may be associated with protein translation or stability.

Furthermore, transcriptome analysis revealed more prominent enrichment of genes associated with inflammatory responses in the cardiac tissue of the 2-month-old DmdE4* mice in comparison with the mdx mice (Figure S3A–S3C). It is notable that proinflammatory genes (eg, Il6) and biomarkers associated with heart failure (eg, atrial natriuretic peptide, Nppa) were only elevated in the heart of DmdE4* mice but not in the mdx mice relative to WT controls, whereas levels of Ifg1 (integrin β1), Ifg7 (integrin α7), and MyoD1 were not altered (Figure S3D and S3E).

Taken together, the progressive cardiac dysfunction developed in DmdE4* mice resembled many aspects of cardiomyopathy observed in patients with DMD, which gradually deteriorates into dilated cardiomyopathy and ultimately heart failure. Thus, the DmdE4* mice could serve as a novel preclinical model that recapitulates both the genetics and many pathophysiological manifestations of human DMD, providing new opportunities to develop efficacious therapies targeting both the heart and the skeletal muscles.

**Optimization of an eTAM-Based Exon-Skipping Strategy for Exon 4 in the Dmd Gene**

In the DmdE4* mice, restoration of the Dmd reading frame can be achieved by removing the indel-containing exon (exon 4), which would generate a truncated dystrophin protein missing a segment of the N-terminal actin-binding domain 1 (Figure 3A). It is notable that patients with in-frame deletion of exon 3 and exon 4 manifested syndromes classified as Becker muscular dystrophy, suggesting that such a truncated dystrophin could maintain most of its functions. To identify an exon-skipping strategy with CRISPR-guided cytidine deaminase in vivo, we characterized a series of TAM configurations to optimize the editing efficiency while minimizing its size to be compatible with the adeno-associated virus (AAV) genome.
Figure 3. Optimization of a TAM-based strategy to induce exon 4 skipping in the Dmd gene.

A. Schematic of TAM-based gene-editing strategy to induce splice site mutation and skipping of exon 4 in the Dmd gene. * indicates the 4-bp deletion in the exon 4. B and C. Editing efficiencies of various configurations of TAM. An sgRNA targeting 5' SS of Dmd Exon 4 (5′SS-E4-Dmd) was transfected into C2C12 cells together with AID (P182X)-nCas9 (KKH)-Ugi (TAM), AID (P182X, K10E, T82I, E156G)-nCas9 (KKH)-Ugi (mTAM), or AID (P182X, K10E, T82I, E156G)-nCas9 (KKH, codon-optimized)-Ugi (eTAM), respectively. Locations of the sgRNA and the corresponding protospacer adjacent motif (PAM) sequence are shown on the top of the intron-exon junction sequence. Editing efficiency was determined by high-throughput sequencing of an amplicon spanning the intron-exon junction of exon 4 generated from the gDNA. The targeted splicing site and the invariant G are highlighted with a red box and a gray shade, respectively. In addition to the targeted G at the splice site, one extra G in the exon 4 was edited. Frequencies of G>A conversions for each G with a detectable mutation (Continued)
Figure 3 Continued. (conversion frequency >0.2%) are highlighted with green bars. The dashed line marks the intron/exon boundary. Data are representative (B) or summary (C) of 3 independent experiments. B, * indicates AID (P182X, K10E, T82I, E156G) variant, D, eTAM mediates efficient skipping of exon 4 in the Dmd gene. cDNA amplicon was generated with indicated primer set (arrows) in the myotubes derived from control and eTAM-edited C2C12 cells. Data are representative of 3 independent experiments. * indicates the 4-bp deletion in the exon 4. E, As shown in D, the smaller polymerase chain reaction product was analyzed by Sanger sequencing. The dashed line marks the boundary between exon 3 and exon 5. F, As in D, an amplicon spanning splicing junctions was amplified and analyzed by high-throughput sequencing. Sashimi plots showed junction coverage and junction reads with >1% of total reads. Data are representative of 2 independent experiments. G, As in D, percentages of the isoform skipping exon 4 were determined by capillary gel electrophoresis. Data are a summary of 3 independent experiments. H, As in B, indels were identified in amplicon spanning the targeted 5′ splice site from genomic DNA of the TAM(s) and control edited C2C12 cells. C, G, and H, Error bars stand for the standard deviation (C, G) or standard error (H). *P<0.05, ** P<0.01, **** P<0.0001 in unpaired, 2-tailed Student t test or Welch t test or multiple comparison test in ANOVA. Ctrl indicates control; sgRNA, single guide RNA; and TAM, targeted AID-induced mutagenesis.

Because Staphylococcus aureus Cas9 (saCas9) is 936 bp smaller than Streptococcus pyogenes Cas9 (3165 bp versus 4101 bp), we fused saCas9 KKH variant recognizing NNNRRT protospacer adjacent motifs with AID (activation-induced cytidine deaminase) P182X and Ugi (uracil glycosylase inhibitor) to generate AID P182X-nsaCas9 (KKH)-Ugi (referred to hereafter as TAM), which can be packaged into AAV vectors. 

Because Staphylococcus aureus Cas9 (saCas9) is 936 bp smaller than Streptococcus pyogenes Cas9 (3165 bp versus 4101 bp), we fused saCas9 KKH variant recognizing NNNRRT protospacer adjacent motifs with AID (activation-induced cytidine deaminase) P182X and Ugi (uracil glycosylase inhibitor) to generate AID P182X-nsaCas9 (KKH)-Ugi (referred to hereafter as TAM), which can be packaged into AAV vectors.27 A sgRNA (5′-SS-E4-Dmd) targeting the 5′-splice site of exon 4 in the Dmd gene was identified with NNNRRT protospacer adjacent motifs (Figure 3B). We observed an on-target nucleotide conversion rate of 5.9±0.1% (Dmd/IVS4+1 g>a) by high-throughput sequencing (HTS) of genomic DNA (gDNA) in murine C2C12 myoblasts, following cotransfection of TAM and the sgRNA (Figure 3B). To further improve the performance, we introduced 3 gain-of-function mutations into human AID P182X (K10E, T82I, E156G),28 which is referred to hereafter as mTAM, leading to an editing efficiency of 30.8±0.4% of the on-target G. Furthermore, codon optimization of the saCas9 (KKH) protein together with an additional nuclear-localization sequence at the N terminus of the fusion protein29 (referred to as eTAM) further improved the editing efficiencies to 50.7±0.5% (Figure 3B and 3C), resulting in a total 9-fold improvement compared with the original configuration.

To assess RNA splicing in the Dmd gene, myotubes were derived from edited C2C12 cells in comparison with parental cells. Using primers that amplify exons 3 to 5 of Dmd, we identified a smaller RT-PCR product only in the edited cells (Figure 3D), which represented a direct joining of exon 3 and exon 5 (Figure 3E). HTS indicated skipping of exon 4 in 62.2±1.3% of reads through eTAM (Figure 3F), a 4-fold improvement compared with that mediated by TAM (Figure 3G). Although TAM introduced extra nucleotide editing (Figure 3B), these mutations were eliminated in the mRNA, because exon 4 was targeted for skipping, which yielded a reframed open reading frame without undesired mutations. eTAM resulted in limited DNA double-strand breaks, demonstrated by <2.5% of indels in contrast to the ~50% of editing rate (Figure 3H). Together, these results established that eTAM was efficient to induce skipping of exon 4 in the Dmd mRNA while introducing minimal DNA double-strand breaks.

Restoration of Dystrophin Through eTAM-Based Exon Skipping in the Dmd<sup>E4*</sup> Mice

Although exon skipping has been exploited in multiple animal models to reframe the Dmd gene, most efforts have been deployed to skip the exons coding the rod domains.4–230 It is unknown whether the reexpression of dystrophin with a truncated N-terminal domain will be sufficient to restore its function in vivo. Previously, adenosine base editors have been used in vivo to correct a nonsense mutation within the Dmd gene in tibialis anterior or muscle after intramuscular delivery of a trans-splicing AAV vector.31 Yet, base-editing–mediated exon skipping has not been attempted in vivo. Here, we postulated that the eTAM can be delivered through AAV9 in vivo, which displays tropism to both skeletal muscles and the heart, to rescue dystrophic phenotypes in the Dmd<sup>E4*</sup> mice.

We initially packaged both nucleotide modulator(s) and sgRNA into a single AAV plasmid. When delivered at 1.1×10<sup>12</sup> virus particles per neonatal mouse at postnatal day 2 (P2), this one-vector strategy resulted in <5% skipping of exon 4 in the heart, in comparison with 9.9±7.6% skipping when the 2 modules were delivered in separate AAV particles at the same total dosage (Figure S4A and S4B). The lower efficiencies were likely attributable to the large genome size (5.2 kb) exceeding the packaging limit of ≤5kb for AAVs. Similar to the observation in C2C12 cells, replacing mTAM with eTAM in the 2-vector scheme further improved the exon-skipping efficiencies and dystrophin expression as detailed next.

We thus packaged eTAM (codon-optimized eAID-x-nCas9(KKH)-Ugi) and the sgRNA into 2 separate AAV9 particles. To achieve muscle-specific delivery, a synthetic muscle-specific promoter, Syn100,32 was used to drive eTAM. In a separate AAV9 vector, 3 copies of the sgRNA (5′-SS-E4-Dmd) were expressed from hU6, h7SK, or hH1 promoter, respectively (Figure 4A). Single dosages of 1.1×10<sup>12</sup> vg per mouse, at a 1:1 vg ratio between the 2 AAV9 particles, were delivered intraperitoneally into neonatal Dmd<sup>E4*</sup> mice (P2 or P3), whereas Dmd<sup>E4*</sup> mice injected with the same amount (vg) of AAV9-eGFP virus or PBS were served as controls (mock-treated; Figure 4B).

Two months after the AAV administration, we collected the heart and various skeletal muscle tissues for analysis.
Figure 4. Systemic delivery of eTAM/sgRNA through AAV9 induces efficient and precise exon skipping in vivo.

A. Schematic of the AAV vectors. eTAM and the sgRNA (5′-SS-E4-Dmd) were packaged into 2 separated AAV9 particles. A muscle-specific promoter Syn100 was used to drive eTAM in the AAV-eTAM vector, and 3 copies of sgRNA were driven by hU6, h7SK, and hH1 promoters, respectively. The size of packaging DNA between ITRs was indicated on the right.

B. Schematic of systemic administration of AAV particles. AAV-eTAM and AAV-sgRNA particles were injected intraperitoneally (IP) into postnatal day 2 (P2) or P3 DmdE4* mice with a total dose of 1.1×10^{12} vg per mouse at a 1:1 vector genome ratio. DmdE4* mice that were injected with the same amount of AAV9-eGFP virus were served as mock-treated controls. Echocardiography (Echo, indicated by green arrows), or tissue collection (red arrows) was performed at indicated time points. * indicates the 4-bp deletion in the exon 4.

C. In vivo skipping of the exon 4 in the Dmd gene. Two months after the AAV delivery, cDNA amplicon was generated with the indicated primers from the heart of the DmdE4* mice subjected to either eTAM/sgRNA (eTAM) or mock treatment. Age-matched WT mice were included as controls. Data are representative of 3 independent experiments. * indicates the 4-bp deletion in the exon 4. (Continued)
In vivo gene editing and exon skipping were first analyzed by RT-PCR covering exon 3 to exon 5 of the *Dmd* gene (Figure 4C). A smaller PCR product was identified in the *Dmd*<sup>E4</sup> mice receiving the AAV-eTAM treatment, which corresponded to the amplicon size without exon 4 (78 bp) and was confirmed by Sanger sequencing (Figure S5A). Next, HTS of the RT-PCR products revealed that the exon 4 skipping rates were 59.98±4.74% in the *Dmd*<sup>E4</sup> mice, which remained unaltered in response to the AAV-eTAM treatment. mRNA sequencing and standard deviations were calculated based on 5 AAV-eTAM/AAV-sgRNA–treated mice. Hence, the mean editing efficiencies were 1.78±0.57% in the mock-treated animals (Figure 4D to 4E). Other than the on-target skipping of exon 4, we did not observe other splicing changes, including usage of alternative 5′-splice site or 3′-splice site and intron retention (Figure 4F). Consistent with the efficient skipping of exon 4, 28.2±7.8% nucleotide conversion rates were identified at the on-target splice site (IVS4+1g>a) in the *Dmd* premRNA from the heart of the *Dmd*<sup>E4</sup> mice treated with AAV-eTAM (Figure 4G).

The efficiencies of exon skipping (≈60%) were much higher than the editing efficiencies in premRNA (≈28%). This is likely caused by nonsense-mediated decay of the mutant (unedited) *Dmd* mRNA, leading to selective enrichment of the Exon4-skipped (reframed) transcripts after the eTAM treatment. In support of this possibility, the *Dmd* mRNA in the *Dmd*<sup>E4</sup> mice was 4-fold lower than that in the WT mice but was partially rescued following the AAV-eTAM treatment (Figure S5E).

Because *Dmd* is only expressed in the cardiomyocytes of the heart, the high exon skipping rates in the *Dmd* gene reflected the nucleotide editing efficiency in the cardiomyocytes that were targeted through a muscle-specific delivery of the gene-editing module. In comparison with the premRNAs, lower nucleotide conversion rates (3.9±1.3%) were observed in the total gDNA extracted from the whole heart (Figure S5B). This discrepancy has been reported in previous studies and is likely because cardiomyocytes only constitute ≈30% of the total cells in the murine heart. At the genomic level, HTS of targeted amplicons revealed 0.83±0.59% of indels and 0.018±0.02% of inverted terminal repeat integration (Figure S5C and S5D). Furthermore, we have bioinformatically identified the top 10 off-target sites of the sgRNA, most of which fall into deep intronic regions of the genome. With the use of HTS of gDNA amplicons, no mutations or indels were observed in the corresponding sites above the background levels (Figure S5F).

As a result of exon 4 skipping, we observed a restoration of dystrophin protein expression to 86.3±10.2% of the WT levels in the heart of the *Dmd*<sup>E4</sup> mice 2 months after the treatment (Figure 5A and 5B). Immunofluorescence staining demonstrated that >86.9±10.1% of the cardiomyocytes in the treated *Dmd*<sup>E4</sup> mice regained dystrophin that was located at the proximity of the plasma membrane (Figure 5C and 5D). Concomitantly, expression of α-Sarcoglycan was recovered at the plasma membrane (Figure 5C), suggesting a potential reorganization of functional dystrophin-associated glycoprotein complexes. It is notable that, since many murine cardiomyocytes are multinucleated (25%–40%), even 1 nucleus in a cardiomyocyte was edited, the whole multinucleated cell would regain dystrophin protein, rendering a higher number of dystrophin<sup>+</sup> cells relative to the bulk percentage of exon skipping.

A similar restoration of dystrophin protein was observed in the various skeletal muscles, including the diaphragm, rectus femoris, and tibialis anterior of the *Dmd*<sup>E4</sup> mice (Figure S6A–S6D). The less efficient dystrophin restoration was likely attributable to the inefficient AAV transduction of the skeletal muscle as determined by the quantification of AAV vector expression (Figure S6E) or AAV copy numbers (Figure S6F).
Correction of Cardiac Function in DmdE4* Mice Through a Single-Dose Delivery of eTAM and sgRNA

On the basis of the extensive restoration of dystrophin protein in vivo, we went on to evaluate the phenotypic changes in the DmdE4* mice after the treatment. Two months after the AAV-eTAM delivery, serum CK in the treated DmdE4* mice was reduced to levels comparable to the WT mice (Figure 6A). Despite the less efficient nucleotide conversion rate in skeletal muscles, histopathologic hallmarks of muscular dystrophy, including necrotic myofibers and regenerating myofibers with centralized nuclei, were largely corrected in the diaphragm, rectus femoris, and tibialis anterior muscles (Figure S7A and S7B). Functionally, DmdE4* mice receiving the treatment...
Figure 6. Systemic delivery of eTAM/sgRNA through AAV9 rescues cardiac and skeletal muscle functions in the DmdE4+ mice.

A. Serum creatine kinase (CK) levels were measured in wild-type (WT), DmdE4+mock, and DmdE4+ mice treated with AAV-eTAM/AAV-sgRNA at 2 months of age. Data are a summary of 3 independent experiments (n=6 in each group).

B. Grip strength was measured in WT, DmdE4+mock, and DmdE4+ mice treated with AAV-eTAM/AAV-sgRNA at 2 months of age. Data are summarized from 3 independent experiments of 6 mice in each group.

C. Hematoxylin and eosin (H&E; Upper) and Masson staining (Lower) of the hearts from WT, DmdE4+mock, and DmdE4+ AAV-eTAM. (Continued)
were largely protected from contraction-induced fatigue, such that the reduction of muscle force following repeated gripping tasks was flattened to a similar curve as observed in the WT mice (Figure 6B). The treated DmdE4* mice were also rescued from kyphosis as revealed by the microcomputed tomography analysis (Figure S7C).

It is important to note that the pronounced cardiomyopathy observed in the DmdE4* mice was largely obviated by a single-dose treatment of AAV-eTAM. At the histological level, inflammatory infiltration and collagen deposition were almost absent in the heart of the treated 2-month-old DmdE4* mice, in comparison with the control DmdE4* mice (Figure 6C and 6D). To assess cardiac function, we performed echocardiography for the WT mice and the DmdE4* mice with mock or eTAM treatment. At 2 months of age, mock-treated DmdE4* mice had shown increases in the thickness of IVS, LVEF%, and LVFS%, along with decreases in left ventricle diameters (Diam) and left ventricle volume, all of which were corrected in the DmdE4* mice treated with AAV-eTAM and AAV-sgRNA to levels comparable to the WT mice (Figure 6E and Table S4).

**Neonatal Treatment Results in Long-Term Dystrophin Restoration and Pathophysiological Improvement**

We then evaluated whether the neonatal administration of AAV-eTAM could confer prolonged dystrophin expression and the pathophysiological improvement. Twelve months after the initial treatment, RT-PCR (Figure 7A and 7B) and RNA-sequencing analysis of amplicons spanning the exon 4 (Figure S8A) demonstrated 60.27±4.80% skipping of the exon 4 in the hearts of the treated mice. Nucleotide conversion rates of the off-target nucleotide in the premRNA (36.6±7.9%) and the gDNA (4.6±1.2%) were also slightly elevated compared with the 2-month-old mice with the same treatment (Figure S8B and S8C). In contrast, indels (Figure S8D), AAV integration to the Dmd locus (Figure S8E), and the off-target mutagenesis at the predicted off-target sites (Figure S8F) remained low or undetectable. Immunoblotting (Figure 7C and 7D) and immunofluorescence staining (Figure 7E) confirmed that dystrophin protein remained elevated in the heart of the DmdE4* mice 12 months after the initial treatment (84.0±6.3% of the WT level). A similar finding was observed in the skeletal muscles of the treated DmdE4* mice (Figure S9A–S9C).

Thus, a single-dose neonatal administration of AAV-eTAM/sgRNA resulted in a long-lasting restoration of dystrophin protein in vivo.

Furthermore, the pathophysiological improvement in the treated DmdE4* mice was preserved for at least 12 months. Serum CK in the AAV-treated DmdE4* mice remained as low as the levels in the WT mice 12 months after the treatment (Figure S9D). Hematoxylin and eosin and Masson staining demonstrated the lack of inflammatory infiltration and collagen deposition in the heart of the DmdE4* mice (Figure S9E and S9F). Necrotic myofibers and regenerating myofibers with centralized nuclei remained lower in the skeletal muscle of the treated mice than the mock-treated mice (Figure S9G and S9H). By 8 months of age, dystrophic cardiomyopathy in the DmdE4* mice had already progressed into dilated cardiomyopathy with reduced LVEF% and LVFS% (Figures 2G and 7F). However, in the treated DmdE4* mice, 8 months after the treatment, no evidence of dilated cardiac dysfunction was observed, such that their LVEF% and LVFS% were maintained at levels similar to those observed in age-matched WT mice (Figure 7F and Table S5). Most prominent, the life spans of the treated DmdE4* mice were significantly extended following a single dose of neonatal AAV-eTAM/sgRNA treatment (Figure 7G).

The expression of the eTAM or the sgRNA, on the other hand, gradually diminished over the 12-month period following the initial treatment. Quantification of AAV copy numbers in the heart revealed a 2-fold reduction of the AAV-eTAM vector 12 months after the treatment (Figure S10A). The expression levels of the eTAM and the sgRNA were also declined (Figure S10B–S10D). These data together suggest that the dystrophin protein restoration instituted by eTAM-mediated exon skipping was sustainable despite the decline of the modulators.

Taken together, our findings demonstrated that a single dose of the systemic delivery of eTAM through AAV9 in the neonatal DmdE4* mouse efficiently installed exon skipping, restored dystrophin protein, and prevented DMD-associated cardiac dysfunction and muscular dystrophy, as well. This treatment afforded enduring dystrophin protein restoration and pathophysiological improvement for at least a year. The substantial phenotypic rescue following exon 4 skipping indicated that the N-terminally truncated dystrophin was fully functional, thus expanding this therapeutic strategy to a broader community of patients with DMD.

**Figure 6 Continued.** DmdE4*–mock, and DmdE4* mice treated with AAV-eTAM/AAV-sgRNA. Data are representative of 5 sets of mice from 2 independent experiments. D. Quantitation of fibrotic tissues was determined by Masson staining in the hearts of WT, DmdE4*–mock, and DmdE4* mice treated with AAV-eTAM/AAV-sgRNA. E. Echocardiographic analysis was performed in WT, DmdE4*–mock, and DmdE4* mice treated with AAV-eTAM/AAV-sgRNA at 2 months of age. Interventricular septum (IVS), diameters of the left ventricle (Diam.), left ventricle ejection fraction (LVEF), and left ventricle fractional shortening (LVFS) were calculated based on the measurements collected from 3 consecutive cardiac cycles in the M-mode. Data are a summary of 3 independent experiments (total n=6 or 7 in each group). Error bars stand for the standard deviation. A, D, E. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, n.s. not statistically significant in multiple comparison test in ANOVA. B. ***P<0.0001, n.s. not statistically significant in 2-way repeated measures ANOVA test. AAV indicates adeno-associated virus; and eTAM, enhanced TAM.
Figure 7. Neonatal treatment of eTAM/sgRNA results in enduring dystrophin restoration and pathophysiological improvement in the DmdE4* mice.

Twelve months after the neonatal AAV treatment, the mice were analyzed for the targeted exon skipping (A, B), dystrophin expression (C–E), and pathophysiological improvement (F, G). A and B, Efficient exon skipping was maintained 12 months after the AAV treatment. cDNA amplicon was generated with the indicated primers from the heart of the DmdE* mice subjected to the eTAM/sgRNA (eTAM) or mock treatment. Age-matched wild-type (WT) mice were included as controls. C and D, Sustained dystrophin (Dys) protein expression was observed 12 months after the AAV treatment. Expression of dystrophin was determined by immunoblotting in the hearts of indicated animals. Vinculin (VCL) was included as a loading control. Quantitation was shown in D. Each dot represents 1 animal. E, Dystrophin and α-sarcoglycan expression in the heart were determined by immunofluorescence staining. F, Echocardiographic analysis was performed in WT, DmdE*–mock, and DmdE* mice treated with AAV-eTAM/AAV-sgRNA at 8 months of age after the neonatal treatment. Interventricular septum (IVS), diameters of the left ventricle (Diam.), (left ventricle ejection fraction (LVEF), and (left ventricle fractional shortening (LVFS) were calculated on the basis of the measurements collected from 3 consecutive cardiac cycles in the M-mode. Data are a summary of 3 independent experiments (total n=5–7 in each group). G, A single dose of AAV-eTAM/AAV-sgRNA treatment rescued the DmdE* mice from early lethality. A Kaplan-Meier survival curve was plotted to compare the probability of survival for the DmdE* mice (untreated, same one plotted in Figure 2H) and DmdE* mice receiving the AAV-eTAM treatment. **, P<0.01 based on Mantel-Cox test. Error bars stand for the standard deviation. *P<0.05, **P<0.01, ****P<0.0001, n.s. not statistically significant in unpaired, 2-tailed Student t test or multiple comparison test in ANOVA. AAV indicates adeno-associated virus; eTAM, enhanced TAM; DAPI, 4′,6-diamidino-2-phenylindole; and WT, wild type.
DISCUSSION

In this study, we have demonstrated that a CRISPR-guided cytidine deaminase (eTAM) is an efficient strategy to modulate exon skipping in vivo. As a result, a functional dystrophin protein was reconstituted, which prevented the onset of dystrophic pathology in a new murine DMD model (DmdE4*). A single-dose systemic delivery of eTAM along with the sgRNA through AAV9 successfully restored dystrophin expression that alleviated the progression of muscular dystrophy for at least a year. Our study thus established a new animal model and an effective therapeutic platform to advance the treatments for patients with DMD.

Significant progress has been made to develop various therapies for DMD. However, the lack of appropriate animal models has dampened the opportunities to further promote and evaluate these therapies. Unlike patients with DMD, the mdx mice have a slow progression of muscle pathology (except for the diaphragm) with normal mobility and display only a 20% reduction in life span. DMD models with more severe phenotype and pathology have been generated with additional genetic alterations. In contrast to these compound genetic murine models, DmdE4* mice contain a single mutation within the Dmd gene and represent most of the key hallmarks of human DMD, including a significantly shorter life span (by ≈50%), progressive cardiomyopathy, kyphosis, profound loss of muscle force, and myocyte degeneration.

The more severe muscular dystrophy in the DmdE4* mice, in part, is likely because of the lack of compensatory elevation of the utrophin protein, which is observed in the mdx mice. It is also possible, through internal translational start sites, that de novo dystrophin fragments can be produced in the DmdE4* mice, which may compete against utrophin and exaggerate cardiac defects. The difference in the nature of mutations between the DmdE4* (4-bp deletion) and mdx (substitution) mice may also associate with differential genetic compensation responses, as recently demonstrated in zebrafish models. Further elucidation of potential disease-modifying factors may uncover new opportunities to mitigate dystrophic cardiomyopathy.

It is worthwhile to note that DmdE4* mice did not recapitulate all the clinical details observed in patients with DMD, in particular, in the early stage of cardiomyopathy. For example, young patients with DMD tend to have an accelerated heart rate, which was not observed in DmdE4* mice. On the other hand, interventricular septum hypertrophy, which was observed in DmdE4* mice, has only been reported in a fraction of patients with DMD. We speculate that the adaptive or compensatory mechanism is likely to be different in mice versus humans because of their disparate anatomy and physiology. For example, the heart rate of mice is 5 times faster than in humans but can only be accelerated by ≈20% even during exercise, whereas humans can ramp up their heart rate by 80% to 100%, demonstrating superior adaptivity. Further investigation is warranted, in particular, in large-animal models, such as golden retriever dogs or non-human primates that may represent the clinical observation more faithfully and provide more opportunities to understand the molecular and pathological progress of DMD.

Our study highlights base editors (eg, TAM) as a new therapeutic platform to institute exon skipping in vivo without causing double-stranded DNA breaks. Exon skipping has been achieved by antisense oligonucleotides or CRISPR nucleases. However, antisense oligonucleotides require repeated administrations and were less efficient in cardiomyocytes, whereas CRISPR nucleases can evoke p53-dependent DNA damage response as a result of double-stranded DNA breaks. In particular, it has been shown that the dystrophic mice with lower p53 activities are susceptible to embryonal rhabdomyosarcoma. DNA breaks mediated by Cas9 nucleases can lead to substantial aberrant genome and transcript alterations in mdx mice, along with the integration of AAV vectors at Cas9-cut sites. In comparison, TAM-based gene editing largely curbs the genomic and transcriptomic toxicity, whereas it installs permanent correction with comparable high efficiencies, especially in the heart.

Although the restoration of dystrophin protein in the skeletal muscles was moderate in this study, it was sufficient to rescue the DmdE4* mice from skeletal muscle dystrophy, consistent with previous estimates that restoration of <15% of functional dystrophin protein could be able to prevent muscular dystrophy in vivo. Further optimization of the ratios between AAV-eTAM and AAV-sgRNA may improve gene-editing efficiency in skeletal muscles, as reported previously.

Although it is challenging to systematically identify off-target genomic edits in vivo, mitigation of such an effect is critical in clinical application. To reduce in vivo off-target effects, we have used a muscle-specific promoter (ie, Syn100) to restrict nucleotide modulator (eTAM) within the muscle, minimizing potential off-target toxicity in non-muscular tissues. Nonetheless, therapeutic applications of TAM will be benefited from future characterization and development of more precise base editors.

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Received March 9, 2021; accepted September 27, 2021.

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Acknowledgments

The authors thank Drs. H. Ushio, K. Takahashi, K. Nakamura, J. Sato, K. Nakamura, M. Tsuchiya, and H. Katsumata for their valuable contributions to this work. They also acknowledge the support of the Japan Science and Technology Agency (JST) CREST program and the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Number 15H05115.

Disclosures

Dr. H. Ushio is a consultant for Pfizer, Inc. The other authors declare no competing interests.

Supplemental Material

Supplemental Methods

Figures S1–S10

Tables S1–S5

References S2–60

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