Mini-dCas13X-mediated RNA editing restores dystrophin expression in a humanized mouse model of Duchenne muscular dystrophy

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Approximately 10% of monogenic diseases are caused by nonsense point mutations that generate premature termination codons (PTCs), resulting in a truncated protein and nonsense-mediated decay of the mutant mRNAs. Here, we demonstrate a mini-dCas13X-mediated RNA adenine base editing (mxABE) strategy to treat nonsense mutation-related monogenic diseases via A-to-G editing in a genetically humanized mouse model of Duchenne muscular dystrophy (DMD). Initially, we identified a nonsense point mutation (c.4174C>T, p.Gln1392*) in the DMD gene of a patient and validated its pathogenicity in humanized mice. In this model, single adeno-associated virus (AAV)-packaged mxABE reached A-to-G editing rates up to 84% in vivo, which is at least 20-fold greater compared to rates reported in previous studies using other RNA-editing modalities. Furthermore, mxABE restored robust expression of dystrophin protein to over 50% of wild-type (WT) levels by enabling PTC read-through in multiple muscle tissues. Importantly, systemic delivery of mxABE by AAV also rescued dystrophin expression to averages of 37%, 6%, and 54% of WT levels in the diaphragm, tibialis anterior, and heart muscle, respectively, as well as rescued muscle function. Our data strongly suggest that mxABE-based strategies may be a viable new treatment modality for DMD and other monogenic diseases.
Title: Mini-dCas13X-mediated RNA editing restores dystrophin expression in a humanized mouse model of Duchenne muscular dystrophy

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Abstract: Approximately 10% of monogenic diseases are caused by nonsense point mutations that generate premature termination codons (PTCs), resulting in a truncated protein and nonsense-mediated decay of the mutant mRNAs. Here, we demonstrate a mini-dCas13X-mediated RNA adenine base editing (mxABE) strategy to treat nonsense mutation-related monogenic diseases via A-to-G editing in a genetically humanized mouse model of Duchenne muscular dystrophy (DMD). Initially, we identified a nonsense point mutation (c.4174C>T, p.Gln1392*) in the DMD gene of a patient and validated its pathogenicity in humanized mice. In this model, single adeno-associated virus (AAV)-packaged mxABE reached A-to-G editing rates up to 84% in vivo, which is at least 20-fold
greater compared to rates reported in previous studies using other RNA-editing modalities. Furthermore, mxABE restored robust expression of dystrophin protein to over 50% of wild-type (WT) levels by enabling PTC read-through in multiple muscle tissues. Importantly, systemic delivery of mxABE by AAV also rescued dystrophin expression to averages of 37%, 6%, and 54% of WT levels in the diaphragm, tibialis anterior, and heart muscle, respectively, as well as rescued muscle function. Our data strongly suggest that mxABE-based strategies may be a viable new treatment modality for DMD and other monogenic diseases.

**Main Text:**

**Introduction**

The majority of monogenic diseases are caused by point mutations(1), among which ~50% can be reversed by A-to-G conversion with adenine base editors (ABEs). Specifically, A-to-G conversion can restore protein expression by converting premature termination codons (PTCs) to non-termination codons, thus enabling PTC read-through. Nonsense mutations that introduce PTCs, UAG, UGA, and UAA, account for ~10% of monogenic diseases(2). Although base editors derived from the nickase Cas9, have been demonstrated to correct some monogenic mutations, these proteins are generally too large for efficient *in vivo* delivery by single AAV vectors(3, 4), which have a maximum cargo size of only 4.7 kb. Given the relatively small size and reversible nature of RNA editing activity, flexible RNA base editing tools have recently been the focus of intensive development efforts, resulting in REPAIR(5), RESTORE(6), LEAPER(7), mxABE(8), and other systems. While currently used exclusively as effective research tools, potential clinical applications of RNA base editing have been proposed as personalized gene therapies for monogenic diseases.

Among monogenic degenerative muscular diseases, Duchenne muscular dystrophy (DMD) represents the second most common hereditary muscular disease, affecting an estimated 1 in 3,500 to 5,000 male newborns(9). DMD still lacks any effective treatment and is often fatal. Statistically, 60–70% of human DMD diseases are caused by exon deletions in the *DMD* gene(10), whereas ~10% of cases result from nonsense point mutations that introduce PTCs in *DMD*, of which 40% are UAG, 39% are UGA, and 21% are UAA PTCs(11). A recent study showed that RNA base editors can indeed restore dystrophin expression in mice with nonsense mutation-induced X-linked muscular dystrophy (mdx)(12). However, in that study, they achieved only limited dystrophin expression, and moreover, no alleviation of damaged muscle function in the treated mice was observed. Nonetheless, these results validated RNA editing modalities for targeting nonsense mutation-induced PTCs and encourages the development of more effective approaches to potentially treat monogenic diseases such as DMD.
In this study, we identified an uncharacterized c.4174C>T mutation that introduced a PTC in the DMD gene of a patient suffering from progressive muscle weakness. We validated that this nonsense mutation caused loss of dystrophin in multiple muscle tissues in a genetically humanized mouse model of DMD. We then applied the mini-dCas13X-based RNA base editor, mxABE, to convert the TAG codon to a TGG codon in DMD mRNA, which efficiently restored dystrophin expression in the skeletal, heart, and diaphragm muscular tissue of humanized DMD mice. In addition, systemic or local administration of mxABE in an AAV vector significantly improved muscular growth and function in humanized DMD mice. Notably, in cultured cells, mxABE also exhibited high editing activity of other pathogenic nonsense mutations that have been documented in patients with DMD, suggesting its versatility for treating different DMD subtypes. Cumulatively, these findings demonstrate that mxABE is an efficient RNA base editor of known PTCs in DMD in vitro and in vivo and prompt further studies toward developing mxABE technology for personalized gene therapy to treat monogenic diseases.

Results

Validation of c.4174C>T as the causative nonsense DMD mutation in a humanized DMD mouse model

An 8-year-old male patient with progressive limb weakness came to our clinic for the first diagnosis. His medical history indicated onset of abnormal walking posture at age 4. Other symptoms included tiptoe walking, inability to climb stairs, and difficulty standing up after squatting, all of which presented with progressive exacerbation. To exclude neurological causes, we performed physical and psychiatric examinations. The patient was sober without obvious mental illness. His proximal and distal muscle strength levels were IV and V, respectively. No abnormalities were found upon cranial nerve examination, and tendon reflexes were normal. No abnormalities that suggested sensory ataxia were observed. Overall, no evident pathological signs were elicited during these examinations; however, the patient was positive for Gower sign. Bilateral calf muscles displayed hypertrophy and stiffness and mild contractures in both Achilles tendons were also observed. His walking speed was 45.5 meters/min and North Star Ambulatory Assessment (NSAA) score was 18 points. Electromyography examination revealed muscle-induced damage and low compound muscle action potential (CMAP) volatility of upper- and lower-limb movements.

To investigate the etiology of his muscle-related dysfunction, we measured blood lactate dehydrogenase (LDH) and creatinine kinase (CK) levels and found that both were extremely elevated (851 U/L and 13,342 U/L, respectively), consistent with DMD-like symptoms. We next performed multiplex ligation-dependent probe amplification (MLPA), which detected no copy number variation in the DMD gene. Therefore, whole-exome deep
sequencing was used to screen for mutations in the protein-coding sequence that could be potentially responsible for muscle weakness in the patient. This analysis identified a nonsense mutation, c.4174C>T, p.Gln1392*, in exon 30 of the *DMD* gene (Figure 1A). *DMD* follows a Mendelian hereditary pattern, and the *DMD* gene is located on the X chromosome. The patient’s mother was heterozygous for the null allele resulting from this nonsense mutation and also carried a wild-type (WT) allele, whereas only the WT allele was present in the paternal genome (Figure 1A). We examined dystrophin expression by histological immunostaining of tissue from a biopsy of the left bicep muscle of the patient. This showed complete loss of dystrophin expression (Figure 1B), supporting a causative role of c.4174C>T in the pathological symptoms observed in the patient.

To further confirm the causal relationship between c.4174C>T and DMD pathology, we designed a genetic humanization strategy to generate a personalized mouse model of the effects of DMD<sup>E30mut</sup> by replacing mouse *Dmd* exon 30 with the corresponding human exon from the patient with the c.4174C>T mutation (Figure 1C). A CRISPR-assisted knock-in method was used to generate several founder mice with the correctly humanized genotype. To ensure that the human exon was correctly spliced with the flanking mouse exons, we performed reverse-transcription PCR (RT-PCR) to check the junction sequence around exon 30, which confirmed that the expected splicing was seamless in the humanized DMD<sup>E30mut</sup> mice (Figure 1D).

Because DMD-related mutations affect males more often than females, histological analysis with Sirius red and HE staining were conducted in male DMD<sup>E30mut</sup> mice. This showed that humanized DMD<sup>E30mut</sup> mice had severe muscle wasting in the heart, diaphragm (DI), and tibialis anterior (TA) (Figure 1E). Also, the muscle fibers of DMD<sup>E30mut</sup> mice exhibited significant variation in size with widened inter-fiber space, inward movement of cell nuclei, and abnormal infiltration of inflammatory cells compared to normal muscle, which are all characteristics of DMD. Immunostaining for dystrophin and spectrin expression in the heart, DI, and TA muscle of WT and DMD<sup>E30mut</sup> mice showed that DMD<sup>E30mut</sup> mice exhibited complete loss of dystrophin expression but maintained normal spectrin expression compared to WT mice (Figure 1F). Western analysis confirmed the absence of dystrophin in DMD<sup>E30mut</sup> mice (Figure 1G). To examine muscle function in DMD<sup>E30mut</sup> mice, we measured the grip strength of 8-week-old mice. These tests indicated that grip strength was significantly reduced in DMD<sup>E30mut</sup> mice compared to WT mice (Figure 1H), consistent with the progressive limb weakness that is characteristic of human patients with DMD. Moreover, CK levels were strikingly higher in DMD<sup>E30mut</sup> mice compared to WT mice (Figure 1I), implying severe muscle damage.

Overall, the humanized DMD<sup>E30mut</sup> mice exhibited molecular and functional
phenotypes that were highly similar to the phenotypes observed in patients with DMD, strongly supporting a causative role for the c.4174C>T mutation in the DMD symptoms of the patient. These findings indicate that DMD^{E30mut} mice accurately recapitulate the symptoms of DMD observed in human patients and validate it as a suitable model for subsequent evaluation of potential intervention strategies.

mxABE can mediate RNA-level correction of c.4174C>T and other common DMD mutations

Similar to our previous work, here we developed the hypercompact RNA base editor, mxAPE, which can be packaged in an AAV vector for in vivo delivery of genetic therapies(8). Importantly, the c.4174C>T mutation fit the criteria for potential correction by mxAPE-mediated A-to-G conversion. To identify the most effective gRNA for targeting the c.4174C>T site, we engineered a dual fluorescence reporter for gRNA screening. Specifically, we integrated the genomic sequence from the patient containing the c.4174C>T mutation between mCherry and GFP reporters and expressed the dual reporter with c.4174C>T mutation (Figure 2A). The stop codon resulting from c.4174C>T induced premature translational termination, thus blocking GFP expression, which could be rescued by mxAPE editing of the c.4174C>T mutation (Figure 2A). A total of 24 gRNAs, all either 30 nt or 50 nt long, were tested in our GFP rescue screen, and all of them resulted in detectable GFP fluorescence compared to an untargeted control gRNA. Among these, the 50-nt g6 gRNA exhibited the highest GFP rescue efficiency, restoring 95.70% of signal intensity, on average (Figure 2B and Supplemental Figure 1A,B). Deep-sequencing of the reporter mRNAs after GFP rescue confirmed that g6 provided the most efficient editing, with a 77.88% A-to-G editing rate (Figure 2C. Supplemental Figure 1C).

To identify the optimal AAV-compatible expression construct for efficient RNA base editing, we assembled 10 mxAPE expression cassettes (Supplemental Figure 2) with different promoters (i.e., EFS, CBh, MHCK7), single or dual direct repeats (DR), different nuclear localization signals (NLS), and with or without a translational regulatory element (WPRE), and we tested them in GFP-rescue assays in HEK293T cells. All 10 mxAPE expression cassettes provided at least 79.5% GFP rescue and 79.08% average A-to-G conversion efficiency (Figure 2D,E). Targeted sequencing of mutant DMD transcripts edited by the different mxAPEs showed low, variable efficiency for A-to-G editing of adjacent A sites (Figure 2F and Supplemental Figure 3), which was consistent with previous studies of bystander editing activity by a truncated RNA base deaminase, ADAR2dd(5-7). Analysis of the bystander A-to-G editing events indicated that no premature stop codons were introduced at other sites in the DMD transcript by mxAPE, thus suggesting this approach should enable PTC read through. To evaluate the transcriptome-wide off-target
effects of mxABE targeting c.4174C>T, we conducted RNA-seq analysis of
HEK29T cells expressing different mxABE cassettes driven by an EFS (E1),
CBh (C3, C4) or MHCK7 (M2) promoter, and we evaluated a non-targeting
mxABE as a control. Our results showed no significant differences in
A-to-G/C-to-U single-nucleotide variant (SNV) counts generated by any of the
different reporter constructs (851.67 ± 36.62) compared to the control (829.17
± 28.09) (Supplemental Figures 4 and 5), suggesting high editing specificity for
mxABE constructs to target c.4174C>T at the transcriptome scale.

To investigate the general efficacy of mxABE to rescue other types of DMD
mutations, we designed mxABE gRNAs that targeted c.2977C>T (p.Gln993*,
exon 23) and c.8009C>T (p.Trp2670*, exon 54), which are among the most
common causative DMD mutations, and we tested these gRNAs using our
RNA editing GFP rescue assay workflow. Among 62 different gRNAs targeting
either c.2977C>T or c.8009C>T, two potent gRNAs were identified that
provided 77.98% and 78.90% editing rates, respectively (Supplemental Figure
6). These results suggest that mxABE-based RNA editing could be employed
for correction of different mutations that introduce PTCs in DMD.

AAV delivery of mxABEs rescues dystrophin expression in mice
Given the high efficiency of c.4174C>T correction by mxABEs in vitro, we next
tested the effectiveness of mxABEs in vivo in our humanized DMDE30mut model.
We generated 10 vector constructs (AAV9-mxABE-T) with their respective
mxABEs driven by different expression cassettes, designated as E1-, E2-, E3-,
C1-, C2-, C3-, C4-, M1-, M2-, or M3-mxABE (Supplemental Figure 2) and
packaged each of them into AAV9 for in vivo transfection into muscle tissue.
Specifically, the right TA of 8-week-old male DMDE30mut mice was injected with
3 × 10^11 vg of AAV9-mxABE viral particles targeting c.4174C>T, while the left
TA of the same mouse was injected with saline as a control (Figure 3A). All
mice were kept under identical conditions and the cohort was divided in half
and euthanized after 3 weeks or 6 weeks for molecular and histological
analyses of muscle tissue (Figure 3A), including editing efficiency, western
analysis, and histological staining for dystrophin expression.

We first evaluated the short-term effects of mxABE treatment after 3 weeks.
Deep sequencing of DNA extracted from mxABE-treated TA tissue from
DMDE30mut mice revealed an average editing rate of ~35% for all 10 constructs,
and editing efficiency reached 70% in TA of mice treated with C1-mxABE or
C3-mxABE (Figure 3B, Supplemental Figure 7A,B). In general, EFS (E1-, E2-,
E3-mxABE) and CBh promoter-driven mxABEs (C1-, C2-, C3-, C4-mxABE)
exhibited slightly higher rates of RNA correction compared to those driven by a
muscle tissue-specific MHCK7 promoter (M1-, M2-, M3-mxABE) (Figure 3B,
Supplemental Figure 7A,B). Also, western analysis of TA tissue indicated that
dystrophin protein levels in DMDE30mut mice treated with CBh- or
MHCK7-mxABEs were similar to levels in WT mice; however, dystrophin protein expression was lower in EFS-mxABE-treated DMD\textsuperscript{E30mut} mice (Figure 3C,D). We also found that base editor expression was higher in CBh- and MHCK7-mxABE-treated compared to EFS-mxABE-treated DMD\textsuperscript{E30mut} mice (Supplemental Figure 7C). Histological examination of TA tissue showed that all mxABE constructs potently rescued dystrophin expression (43.57 ± 21.31% of WT level) (Figure 3E,F and Supplemental Figure 8).

To evaluate the long-term effects of EFS-, CBh- and MHCK7-mxABEs in DMD\textsuperscript{E30mut} mice, RNA editing rates, protein expression, and histological analysis were conducted in TA tissue 6 weeks after treatment. Editing rates were approximately 40% lower (25.69 ± 3.65%) 6 weeks after treatment compared to the 3-week values (Figure 4A and Supplemental Figure 9A,B). Also, we found that base editor mRNA levels were reduced in the mxABE-treated muscles after 6 weeks compared to after 3 weeks (Supplemental Figure 9C), potentially due to dilution of the AAV titer in rapidly growing muscle, which aligns well with results from another study (13).

Intriguingly, dystrophin staining showed that the Dys+ area was similar 6 weeks compared to 3 weeks after mxABE treatment (Figure 4B,C and Supplemental Figure 10), indicating a sustained therapeutic effect. In addition, quantification of western blot bands from TA lysates of mxABE-injected DMD\textsuperscript{E30mut} and WT mice indicated that the M2 vector expressing MHCK7-mxABE induced the highest level of dystrophin protein expression, which was around 50% of WT levels (Figure 4D,E). These results suggest that the mxABE vectors can provide sustained and proliferation-tolerant therapeutic effects, despite the rapid growth of muscle fiber and the reversibility of RNA editing.

In addition, to investigate off-target effects of mxABE \textit{in vivo}, we collected RNA from CBh-mxABE-, MHCK7-mxABE- and saline-injected TA tissue for RNA-seq and SNV analysis. CBh- and MHCK7-mxABE treatment induced a similar number of SNVs compared to the saline control (Supplemental Figures 11 and 12), indicating that these reagents are highly specific for the target sequence and the delivery vector is highly efficient.

**Systemic delivery of AAV-mxABE restores dystrophin expression and muscle function in multiple tissues**

Having demonstrated that local injection of mxABE robustly restored dystrophin expression in TA muscle, we next examined the effects of systemic
delivery of AAV-mxABE in DMD<sup>E30mut</sup> mice. For systemic delivery, we
intrapertitoneally injected 1 × 10<sup>12</sup> vg viral vehicles of all 10 mxABE constructs
or control saline into neonatal DMD<sup>E30mut</sup> mice 3 days after birth (P3) (Figure
5A). We collected TA, DI, and heart tissue for analysis at different time points
(Figure 5A). Three weeks after injection, deep sequencing analysis indicated
that C3-mxABE and M2-mxABE were remarkably efficient RNA base editors in
heart tissue (77% and 61% on average, respectively, after 3 weeks), but their
RNA base editing efficiencies were lower in DI and TA (Figure 5B). Between
the two, C3-mxABE had generally higher or at least comparable base editing
efficiency relative to M2-mxABE in all three tissues (Figure 5B and
Supplemental Figure 13).

To investigate possible reasons underlying the observed differences in
correction efficiency among heart, DI, and TA tissues, we performed qPCR to
quantify mxABE expression in each tissue. We found that, regardless of
whether constitutive or tissue-specific promoters were used, expression was
highest in heart tissue (Supplemental Figure 14A), which suggested that the
higher mxABE activity in heart was due to either stronger mxABE expression
or more efficient vehicle delivery to heart tissue compared to DI and TA tissues.

Subsequent quantitative western analysis showed that dystrophin protein was
most abundant in heart tissue compared to DI and TA (Figure 5C and
Supplemental Figure 16B), which was consistent with the respective base
editing rates in these tissues. Immunohistochemical analysis uncovered an
extensive region of Dys+ muscle fibers in the heart, DI, and TA of
mxABE-treated DMD<sup>E30mut</sup> mice, whereas Dys+ tissue was completely absent
in the saline-treated controls (Figure 5D,E and Supplemental Figure 15).
Furthermore, HE and Sirius red staining showed that histopathologic hallmarks
of muscular dystrophy, such as fibrosis, necrotic myofibers, and regenerated
fibers with central nuclei, were substantially alleviated in the TA, DI, and heart
muscles 3 weeks after AAV9-mxABE delivery compared to saline-treated
control tissues (Supplemental Figure 16). Compared with untreated DMD<sup>E30mut</sup>
mice, serum CK levels in CBh(C3)-mxABE and MHCK7(M2)-mxABE-treated
DMD<sup>E30mut</sup> mice were significantly reduced by 87.29% and 71.25%,
respectively (Figure 5F).

Finally, we examined whether mxABE could restore muscle function by
measuring forelimb grip strength in mxABE-treated and untreated DMD<sup>E30mut</sup>
mice as well as in WT mice. Untreated DMD<sup>E30mut</sup> mice exhibited ~40% less
forelimb grip strength 3 weeks after birth compared to WT mice (Figure 5G).
Notably, 3 weeks after treatment, forelimb grip strength was significantly
restored in DMD<sup>E30mut</sup> mice treated with CBh-mxABE(C3) compared to
saline-treated DMD<sup>E30mut</sup> mice, whereas the improvements in grip strength in
DMD<sup>E30mut</sup> mice treated with the MHCK7-mxABE(M2) were not statistically
significant (Figure 5G). Repetitive forelimb grip tests showed that grip strength
declined rapidly with increasing grip time in mxABE-treated DMD\textsuperscript{E30mut} mice compared to in WT mice (Figure 5H), suggesting that muscle dysfunction was only partially alleviated in mxABE-treated DMD\textsuperscript{E30mut} mice; thus, further optimization of mxABE expression or delivery is necessary to enhance therapeutic effects.

At 6 weeks, we also checked A-to-I base editing efficiency, dystrophin restoration level, and grip strength in mxABE-treated and untreated DMD\textsuperscript{E30mut} mice (Figure 5A). RNA editing rate 6 weeks after systemic mxABE administration was still as high as 85% in the heart, but only 29% in TA and 16% in DI (Figure 6A and Supplemental Figure 17), which might be because of different mxABE expression levels among the three tissues (Supplemental Figure 18A). Both western analysis and immunostaining confirmed that dystrophin expression was sustained in all three muscle tissues after 6 weeks (Figure 6B,C and Supplemental Figure 18B,C and Supplemental Figure 19). C3-mxABE-treated mice had decreased dystrophin levels compared to M2-mxABE-treated mice after 6 weeks, which is consistent with what we observed after 3 weeks (Figure 5 and 6). Forelimb grip strength of both C3- and M2-mxABE-treated DMD\textsuperscript{E30mut} mice was significantly improved (Figure 6D), and both groups of mxABE-treated DMD\textsuperscript{E30mut} mice also had significantly increased running time in the rotarod test compared to untreated mice, suggesting improved muscle function. Finally, similar to results 3 weeks after treatment, 6 weeks after treatment, mxABE only partially restored performance in a repetitive grip test (Figure 6E).

To examine the long-term therapeutic effect of mxABE treatment, DMD\textsuperscript{E30mut} mice with or without mxABE administration were monitored for 6 months and then euthanized to analyze muscle tissue (Figure 5A). RNA base editing rate was up to 80% in heart muscle 6 months after treatment, but it was only 10% in DI and TA muscle (Figure 7A), which is in agreement with our findings in shorter-term studies (Figure 7B). Western analysis confirmed detectable dystrophin expression in heart, TA, and DI 6 months after treatment, but protein levels were dramatically decreased compared to earlier time points (Figure 7C,D). We also quantified Dys+ muscle using immunostaining, which showed that only ~ 20% of muscle area maintained dystrophin expression in heart muscle, and even less Dys+ muscle was observed in DI and TA (Figure 7E,F and Supplemental Figure 20). In addition, collagen staining of heart tissue from treated and untreated DMD\textsuperscript{E30mut} mice as well as WT mice was conducted to detect cardiac fibrosis, and this showed no differences among the three cohorts (Supplemental Figure 21), which is consistent with previous findings showing that most mdx mice have less severe and slower cardiac progression compared to human patients\cite{14-16}. To measure the host immune response to AAV-mxABE treatment, IL2, IL15, and IL18 activation were examined by qPCR at baseline and 3 weeks, 6 weeks, and 6 months after
treatment. Expression of all three cytokines was substantially increased 6 weeks after treatment but returned to basal levels by 6 months after treatment (Supplemental Figure 22A-C). Furthermore, we examined potential toxicity of mxABE administration by analyzing ALT, total protein, and urea. All three serum biomarkers were normal before and after AAV-mxABE treatment (Supplemental Figure 22D-F), indicating that AAV-mxABE administration is relatively safe. Overall, mxABE was effective at restoring dystrophin expression in DMD<sup>E30mut</sup> mice for up to 6 months after systemic administration of mxABE via AAV.

DMD is usually diagnosed in patients when they are around 5 years old. To closely mimic the clinical scenario, we performed systemic injection of AAV-mxABE in 8-week old mice and investigated treatment outcomes. Both intravenous (AIV group) and intraperitoneal (AIP group) injection delivering 1×10<sup>12</sup> vg of AAV particles with mxABE-M2 per mouse were tested (Supplemental Figure 23A). We collected heart, DI, and TA muscle 6 weeks after injection for analysis (Supplemental Figure 23A). In heart muscle, the RNA base editing rate was up to 49%, whereas it was only 6% in DI and TA muscle (Supplemental Figure 23B,C). Western analysis only detected dystrophin expression in heart tissues, but not in DI or TA (Supplemental Figure 23D). However, immunostaining showed that mxABE treatment of adult DMD<sup>E30mut</sup> mice restored Dys+ muscle in heart, DI, and TA to up to 38% of WT levels (Supplemental Figure 23E,F and Supplemental Figure 24). In the forelimb grip strength test, no significant improvement in muscle function was observed 6 weeks after mxABE injection in adult DMD<sup>E30mut</sup> mice (Supplemental Figure 23G), implying that an optimized approach is required to potentially functionally rescue adult DMD<sup>E30mut</sup> models.

Overall, our findings demonstrate that systemic delivery of AAV-mxABE can rescue DMD gene expression and support further exploration of this approach as a potential gene therapy to treat patients with DMD and other monogenic diseases.

Discussion

PTCs resulting from point mutations account for 10% of the lesions leading to DMD(17); thus, strategies that induce PTC read-through for all three stop codons (UAG, UAA, and UGA) hold great potential as a therapeutic strategy to treat DMD caused by nonsense mutations. Here, we demonstrated that the compact mxABE RNA base editor can mediate the correction of several pathogenic nonsense mutations in DMD transcripts with sufficient efficiency to stably restore dystrophin expression in skeletal, DI, and heart muscle in humanized DMD<sup>E30mut</sup> mice harboring the causative genetic lesion detected in an 8-year-old patient with DMD. In particular, our mxABE approach rescued dystrophin expression in DMD<sup>E30mut</sup> mice to up to 50% of WT levels, which
reflects a 16-fold improvement compared to the 3% of dystrophin restoration recently achieved using an MS2-derived RNA base editor delivered by local muscle injection\(^\text{(12)}\). In addition, systemic administration of mxABE in the present study also induced robust restoration of dystrophin expression in multiple tissues, which, so far, has not been demonstrated using other RNA base editors. Therefore, our findings serve as a proof-of-concept for RNA base editing as a strategy for DMD treatment.

Nonsense point mutations have been identified as causative factors in \(\sim 10\%\) of all monogenic diseases\(^\text{(2)}\). Our results suggest that mxABE-mediated PTC read-through may be used to effectively treat other hereditary diseases induced by nonsense point mutations. Moreover, we found that systemic administration of mxABE resulted in greater and more durable rescue of dystrophin expression in heart tissue compared to skeletal muscle in mice, suggesting this approach may be particularly effective to reduce the likelihood of poor outcomes or mortality in patients with DMD or other monogenic diseases that put them at high risk of cardiac failure.

mxABE driven by the muscle-specific MHCK7 promoter produced a slightly lower base editing rate and level of dystrophin restoration compared to expression under the control of a ubiquitous CBh promoter, which might be due to slightly weaker activity of MHCK7 compared to CBh. Intriguingly, dystrophin restoration in skeletal muscle after mxABE treatment progressively decreased approximately 6 weeks after treatment, but this decrease was less pronounced in heart tissue. Because mxABE is correcting RNA rather than DNA, sustainable RNA editing activity and therapeutic effect would rely on the long-lasting expression and editing activity of mxABE \textit{in vivo}. Previous studies have shown low tropism of AAV for satellite cells\(^\text{(13, 18)}\) and preexisting immunity against CRISPR reagents\(^\text{(19, 20)}\), possibly leading to progressive loss of mxABE and decreased dystrophin restoration in skeletal muscles over time. These results suggest that mxABE may be more suitable as a therapeutic intervention for non-dividing tissue such as the eye, neurons, and cardiomyocytes than in rapidly dividing tissue like skeletal muscle.

RNA base editors induce A-to-G or C-to-U conversion in transcripts through deaminase activity of ADAR proteins acting on adenine or cytosine nucleobases near the targeted sequence. This activity may also result in off-target, unintended bystander base editing, and therefore monitoring and controlling for bystander effects is an essential step in the development of effective base editors. Notably, bystander effects have also proven to be a significant limitation of the most efficient previously described RNA base editors\(^\text{(5-7)}\). In this work, the mxABE editor showed A-G editing rates of up to 84% \textit{in vivo}, converting nonsense mutant UAG stop codons to UGG in \textit{DMD} mRNA transcripts. However, bystander A-to-G conversions in sequences...
adjacent to the disease-causing mutation were consistently detected. Although we did not detect introduction of any new PTCs through bystander A-to-G editing, such aggressive editing activity could foreseeably cause other pathogenic amino acid substitutions. To address this long-standing issue in base editing, another recent study described a gRNA design strategy that employs U deletion to minimize bystander editing effects in ADAR proteins (21), which could enhance the specificity of PTC correction for RNA base editors, and hence increase the safety of candidate base editing gene therapies.

In addition to analyzing bystander effects, we also analyzed the transcriptome of mice treated with AAV-mxAEBE, which showed no statistically significant difference in the number of SNVs between treated and untreated mice, suggesting that the off-target effects were limited to sequences in the immediate proximity of the target sequence. As a strategy to potentially reduce off-target effects, especially in non-target tissue, we evaluated the efficiency of tissue-specific promoters to drive mxABE expression. Our results showed that these promoters can rescue dystrophin expression in muscle tissues to levels comparable to ubiquitous promoters.

We validated a pathogenic nonsense mutation identified in a patient with DMD admitted to our clinic and established a humanzined mouse model of DMD to study the efficiency of RNA base editing to rescue DMD-related phenotypes. Using the compact mxABE base editor packaged in AAV, we demonstrated that dystrophin expression can be restored in multiple muscle tissues through local or systemic administration, indicating the potential of this system to treat monogenic diseases resulting from PTCs.

Methods
Plasmid construction
The pU6-BpiI-EFS-Cas13X.1 plasmid encodes a human codon-optimized Cas13X.1 driven by the EFS promoter and a U6-driven crRNAs with a BpiI cloning site. The sgRNAs (including missense mutation in exon 23, 30, 54) were designed using the CHOPCHOP tool, then synthesized as DNA oligonucleotides and cloned into pU6-BpiI-EFS-Cas13X.1 to form the CRISPR targeting plasmids (listed in Supplementary Table 1). A reporter vector was constructed with mCherry and ATG-removed GFP as well as the mutant sequence identified in the patient with DMD (c.4174C>T mutation). The high-efficiency EFS promoter in the sgRNA plasmid for DMD gene exon 30 was replaced with the CBh or the MHCK7 promoter using the Gibson assembly method, and SV40 NLS was also replaced with NP NLS or BP NLS, to construct different AAV9 vectors. All AAV9 vectors used in this study are listed in Supplementary Figure 2.
Cell culture, transfection, and flow cytometry analysis

HEK293T from the American Type Culture Collection (ATCC) were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco, 11965092) supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂ in a humidified incubator. For sgRNA screening, CRISPR targeting plasmids and reporters were co-transfected using polyethylenimine (PEI) transfection reagent. After transfected cells were cultured for 48 hours, we carefully resuspended the cell pellet and then analyzed or sorted cells using a BD FACSaria II. Flow cytometry results were analyzed with FlowJo X (v.10.0.7).

Generation of DMD\textsuperscript{E30mut} mice

Mice were housed in a barrier facility with a 12-hour light/dark cycle and maintained in accordance with the Instructive Notions with Respect to Caring for Laboratory Animals issued by the Ministry of Science and Technology of China. DMDE\textsuperscript{E30mut} mice were generated in the C57BL/6J background using the CRISPR-Cas9 system. In brief, two sgRNAs targeting mouse DMD exon 30 were designed (Supplementary Table 1), and then the T7 promoter sequence was added to the sgRNA template. After PCR product was purified directly using a Omega gel extraction kit (Omega, D2500-02), templates were used for \textit{in vitro} transcription using the MEGAshortscript T7 Kit (Invitrogen, AM1354). sgRNAs were purified using a MEGAclear Kit (Invitrogen, AM1908) and eluted with nuclease-free water. The concentration of target sgRNA was measured using a NanoDrop instrument. For cytoplasmic injection, spCas9 mRNA (100 ng/μl), sgRNA (100 ng/μl), and HMEJ donor (100 ng/μl) were mixed and then injected into fertilized eggs using a FemtoJet microinjector (Eppendorf) with constant flow settings. The injected zygotes were cultured in KSOM medium for 12 hours and surgically transferred to the oviduct of recipient mice 24 hours after estrus was observed. Genomic DNA from the tail tissue of founder (F0) mice was isolated according to manufacturer's instructions for the OMEGA Kit (Omega, D3396-02) for PCR (Supplementary Table 1), followed by gel electrophoresis.

AAV9 production and delivery to DMD\textsuperscript{E30mut} mice

AAVs were produced using PackGene Biotech (Guangzhou, China) and purified by iodixanol density gradient centrifugation. For intramuscular injection, DMDE\textsuperscript{E30mut} mice were anesthetized and TA muscle was injected with 50 μL of AAV9 (3 × 10¹¹ vg) preparations or with the same volume saline solution. For intraperitoneal injection, the P3 DMDE\textsuperscript{E30mut} mice were injected using an ultrafine needle (31 gauge) with 50 μl of AAV9 (1 × 10¹² vg) preparations or with saline solution.
Targeted deep sequencing

To analyze A-to-I base editing efficiency, the RNA of successfully transfected cells or of AAV9-mxABE-treated tissues were isolated with RNA-easy isolation reagent according to the manufacturer’s protocol. cDNA was synthesized using an HiScript II One Step RT-PCR Kit (Vazyme, P611-01) following the manufacturer’s protocol and amplified with Phanta max super-fidelity DNA polymerase (Vazyme, P505-d1) for Sanger or deep sequencing methods. Deep sequencing libraries were used to add Illumina flow cell binding sequences and specific barcodes on the 5’ and 3’ ends of primer sequences. The products were pooled and sequenced with 150 paired-end reads on an Illumina Hiseq instrument. FASTQ format data were analyzed using the Cutadapt (v.2.8)41 according to assigned barcode sequences.

qPCR and RNA-sequencing

Total mRNA was extracted from muscle tissue and cDNA was synthesized using a HiScript II One Step RT-PCR Kit (Vazyme, P611-01) following the manufacturer’s protocol. Then, each 20-µl PCR reaction contained approximately 2 µl cDNA, 0.25 µM of each forward and reverse primer, and 10 µl of AceQ Universal SYBR qPCR Master Mix (Vazyme, Q511-02). Amplification was performed on a Roche 480 II-A using the following program: initial hold for 5 min; 45 cycles of 95 °C for 10 s; 60 °C for 10 s; and 72 °C for 10 s. qPCR results were calculated by normalizing to GAPDH mRNA level.

Western blot analysis

Samples were homogenized with RIPA buffer supplemented with protease inhibitor cocktail. Lysate supernatants were quantified using a Pierce BCA protein assay kit (Thermo Fisher Scientific, 23225) and adjusted to an identical concentration using H2O. Equal amounts of sample were mixed with NuPAGE LDS sample buffer (Invitrogen, NP0007) and 10% β-mercaptoethanol then boiled at 70 °C for 10 min. Ten µg total protein per lane was loaded into 3% to 8% tris-acetate gels (Invitrogen, EA03752BOX) and electrophoresed for 1 hour at 200 V. Protein was transferred onto a PVDF membrane under wet conditions at 350 mA for 3.5 hours. The membrane was blocked in 5% non-fat milk in TBST buffer and then incubated with primary antibody to label the specific protein. After washing three times with TBST, the membrane was incubated with HRP-conjugated secondary antibody specific to the IgG of the
species of primary antibody against dystrophin (Sigma, D8168) or vinculin (CST, 13901S). The target proteins were visualized using Chemiluminescent substrates (Invitrogen, WP20005).

**Histology and Immunofluorescence**

Tissues were collected and put into preconditioned 4% paraformaldehyde. The fixed tissues were dehydrated from low to high concentrations of alcohol. After xylene treatment, tissues were placed in melted paraffin wax, cut into 10 μm slices and attached to slides. Xylene was used to wash the paraffin, then slides were passed through high-concentration to low-concentration alcohol, and finally put it into distilled water. For HE staining, slides were stained with hematoxylin for 3-8 min followed by color separation using acid water and ammonia water. After dehydration using 70% and 90% alcohol for 10 minutes each, tissues were stained in eosin staining solution for 1-3 minutes, and dehydrated in ascending alcohol solutions (50%, 70%, 80%, 95%, 100%). Coverslips were mounted onto the section on glass slides with neutral resin.

For Sirius red staining, slides were stained with picrosirius red for one hour and washed in two changes of acidified water. Physical removal of most of the water from the slides was accomplished by vigorous shaking. Then, slides were dehydrated in three changes of 100% ethanol, cleared in xylene, and finally mounted in neutral resin.

For immunofluorescence, tissues were mounted in optimal cutting temperature (OCT) compound and snap-frozen in liquid nitrogen. Serial frozen cryosections (10 μm) were fixed for two hours at 37 °C followed by permeabilization with PBS + 0.4% Triton-X for 30 min. After washing with PBS, samples were blocked with 10% goat serum for 1 hour at room temperature. Next, the slides were incubated overnight at 4 °C with primary antibodies against dystrophin (Abcam, ab15277) and spectrin (Millipore, MAB1622). The next day, samples were washed extensively with PBS and incubated with compatible secondary antibodies (Alexa Fluor® 488 AffiniPure donkey anti-rabbit IgG (Jackson ImmunoResearch labs, 711-545-152) or Alexa Fluor 647 AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch labs, 715-605-151)) and DAPI for 3 h at room temperature. Samples were washed for 15 min with PBS, and slides were sealed with fluoromount-G mounting medium. All images were visualized using an Olympus FV3000 or Nikon C2. The number of Dys+ muscle fibers is represented as a percentage of total spectrin-positive muscle fibers.

**Forelimb grip strength test and rotarod test**

Muscle strength was assessed at various time points. Briefly, mice were
removed from the cage, weighed and held from the tip of the tail, causing the forelimbs to grasp the pull-bar assembly connected to the 47200-grip strength meter. The mouse was drawn along a straight line leading away from the sensor until the mouse could no longer grasp the gridiron and the peak amount of force in grams was recorded. The assessment was repeated 10 times with 10-second intervals between.

For the rotarod test, the week before the experiment, we performed daily 30-minute training trials. During the test, mice were trained on the rotarod (Ugo Basile Inc.) with accelerating speed from 4 to 40 rpm over 30 second. Four mice were tested simultaneously on the rotarod and the test was repeated five times per animal. Latency was measured as the time from the beginning of the trial (start of the accelerated rod rotation) until the mouse fell off and onto the lever that stops the timer.

**Serum CK**

To measure CK levels, a blood sample was collected in an Eppendorf tube via cardiac puncture under ketamine anesthesia prior to euthanasia. Samples were centrifuged at 3,000 × g for 10 min and then the serum was collected. CK activity was measured with creatine kinase (CK10) reagent (Pointe Scientific, 23-666-208) according to the manufacturer’s instructions.

**Statistical analysis**

The data are presented as mean ± SEM. Differences were assessed using unpaired two-tailed Student’s t test or one-way ANOVA. Differences in means were considered statistically significant when they reached P < 0.05. Significance levels are: * P < 0.05. ** P < 0.01. *** P < 0.001.

**Study approval**

The objectives of the present study were to generate a humanized DMD model and to obtain proof-of-concept for *in vivo* CRISPR-mediated RNA base editing in DMD. Male mice were used in all experiments, such as grip tests, CK analysis and AAV9 injection. The number of independent biological replicates are shown in figure legends. All animal experiments were performed and approved by the Animal Care and Use Committee of the Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China. The patient study was approved by the ethics committee of First Affiliated Hospital of Fujian Medical University. Written informed consent was obtained from each subject to use their DNA for molecular analysis of the *DMD* gene and for further research. For the minor patient, parental consent for muscle biopsy and molecular analysis was obtained. The research was conducted according to the principles of the declaration of Helsinki.
Author contributions: H.Y., G.L., and C.X. jointly conceived the project and designed experiments. H.Y., C.X., and W.C. supervised the project. G.L. and Z.L. generated the humanized mouse model. G.L. and M.J. designed vectors, performed in vitro experiments and conducted confocal imaging. C.X. assisted with construction of plasmids. G.L. and M.J. performed in vivo virus injection, tissue dissection, histological immunostaining and muscle function experiments. Y.L. and Q.X. analyzed RNA-seq data. Q.X., J.L., H.W. and D.Y. assisted with tissue dissection, immunostaining, and animal breeding. G.L., M.J., Z.L., Q.X., J.L., D.Y., and C.X. analyzed the data and organized figures. H.Y., C.X., and W.C. wrote the manuscript with data contributed by all authors who participated in the project.

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References


**Supplementary Materials:**

Figures S1 to S24

Table S1

Supplemental Sequences
**Figure 1**

A. Schematic representation of the Dystrophin Spectrin gene with the indicated mutation (c.4174C>T, p.Gln1392*).

B. Immunohistochemical staining of Sirius Red HE for normal and DMD patients in heart (Heart), diaphragm (DI), and tibialis anterior (TA) muscles.

C. Schematic diagram showing the humanized DMD230mut mice with the donor donor (HAL) and Exon30-HAR, and the natural mutation (c.4174C>T, p.Gln1392*).

D. Schematic diagram of the Dmd gene exon 29 to exon 31, with human and mouse alleles, showing the expression of Vinculin and Dystrophin in different tissues.

E. Western blot analysis of Dystrophin and Spectrin in normal and DMD230mut mice in heart, diaphragm, and tibialis anterior muscles.

F. Bar graphs showing grip strength (g) and CK activity (U/liter) for normal (WT) and DMD230mut mice.
Figure legends

Figure 1. Establishment and characterization of a humanized DMD mouse model.

A. Pedigree of patient with DMD (proband) with a nonsense mutation p.(Gln1392*). Squares represent males, circles represent females. B. Histological analysis of left bicep muscle from normal and proband. Dystrophin (Sigma, D8168) is shown in brown. C. Strategy for generating humanized DMD mouse model. CRISPR-Cas9 editing using two sgRNAs flanking an exon was used to delete mouse Dmd exon 30, and replaced with human DMD exon 30 carrying the nonsense mutation. D. RT-PCR products from muscle of DMD\textsuperscript{E30mut} mice were sequenced to validate the exon 30 mutation. E. Sirius red staining and HE staining of TA, DI, and heart muscle of WT and DMD\textsuperscript{E30mut} mice. F. Dystrophin immunohistochemistry from indicated muscles of WT and DMD\textsuperscript{E30mut} mice. Dystrophin (Abcam, ab15277) and spectrin (Millipore, MAB1622) are shown in green and mangenta, respectively. G. Western blot confirming the absence of dystrophin (Sigma, D8168) in indicated muscle tissues. H. WT and DMD\textsuperscript{E30mut} mice were subjected to forelimb grip strength testing to measure muscle performance (n=6). I. Serum CK, a marker of muscle damage and membrane leakage, was measured in WT and DMD\textsuperscript{E30mut} mice (n=8). All mice were 8 weeks old at the time of the experiment. Data are represented as mean ± SEM. Each dot represents an individual mouse. **P < 0.01 using unpaired two-tailed Student’s t test. Scale bar, 200 μm.
Figure 2

A. Schematic diagram of the CRISPR-Cas9 construct used for gene editing.

B. Bar graph showing the efficiency of gene editing at different positions.

C. Graph indicating the efficiency of A→I conversion at various positions.

D. Graph displaying the efficiency of GFP/mCherry expression at different sgRNA positions.

E. Graph showing the efficiency of A→I conversion at different sgRNA positions.

F. Heatmap illustrating the expression levels of different sgRNA positions.
**Figure 2. mxAPE-mediated correction of mutant DMD RNA**

A. The reporter construct containing the mCherry cassette fused with a 2A peptide, mutant human exon 30 (c.4174C>T) and ATG-removed GFP. Correction of the stop codon within the target sequence allows GFP expression.

B. Flow cytometry analysis of GFP expression in HEK293T cells transfected with 24 gRNAs. C. Deep-sequencing of the reporter RNA transcribed from the reporter vector after GFP rescue experiment. Comparison of the editing efficiencies of different mxAPE vectors by flow cytometry (D) and deep sequencing (E).

F. Measurement of bystander A to I editing rate for multiple adenosines within a 50-nt region of the DMD\textsuperscript{E30mut} target sequence. gRNA g6 was used in the analysis. Adenosines (As) with position number (n) are indicated with A\textsubscript{n} from the 5’ to 3’ end in the 50-nt target sequence. Data are represented as mean ± SEM (n=3). **P < 0.01 using unpaired two-tailed Student’s t test.
Figure 3
Figure 3. mxABE robustly rescues dystrophin expression in TA three weeks after AAV injection.

A. Overview for the in vivo intramuscular (IM) injection of the AAV9-mxABE construct into the TA muscle of the right leg of 8-week-old DMD\textsuperscript{E30mut} mice. Left leg was injected with saline as a control. Black arrows indicate time points for tissue collection after injection. B. The A to I efficiency of different AAV9 vectors was measured (n=4). A cDNA amplicon spanning exon 30 was generated from the TA muscle and analyzed by deep sequencing. C. Western blot analysis of dystrophin (Sigma, D8168) and vinculin (CST, 13901S) expression in TA muscles 3 weeks after injection with AAV9-mxABEs or saline. D. Quantification of dystrophin expression from Western blots after normalization to vinculin expression (n=3). Age-matched WT and saline-treated DMD\textsuperscript{E30mut} mice were included as control. E. Comparison of dystrophin expression among the 10 AAV9-mxABE systems by immunofluorescence. Dystrophin (Abcam, ab15277) is shown in green. Scale bar, 200 μm. F. Quantification of Dys+ fibers in cross sections of TA muscles (n=4). Data are represented as mean ± SEM. Each dot represents an individual mouse. Significance is indicated by asterisk (P < 0.05). NS represents not statistically significant using unpaired two-tailed Student’s t test.
Figure 4

A

Ato i efficiency (%)

WT Control E1 C3 C4 M2

B

WT

Dystrophin

Vinculin

C

Relative intensity

Dystrophin / vinculin (%)

WT Control E1 C3 C4 M2

D

Dys + (%)

WT Control E1 C3 C4 M2

E

WT Control E1 C3 C4 M2

Images and graphs showing the effects of different conditions on Ato i efficiency, relative intensity, and Dys + percentage in various groups (WT, Control, E1, C3, C4, M2).
Figure 4. AAV-mxABE robustly rescues dystrophin expression in TA six weeks after injection

A. Deep-sequencing of in vivo RNA editing 6 weeks after IM injection with AAV9-E1, -C3, -C4 and -M2 constructs (n=4). B. Western blot analysis of dystrophin (Sigma, D8168) protein expression in TA muscles of WT and DMD<sup>E30mut</sup> mice. Intramuscular injection in the DMD<sup>E30mut</sup> mice or saline as control. Vinculin (CST, 13901S) was used as the loading control. C. Quantification of dystrophin expression from Western blots after normalization to vinculin (n=3). D. Immunohistochemistry of dystrophin in TA muscles 6 weeks after IM injection with different AAV9 constructs. Dystrophin (Abcam, ab15277) is indicated in green. Scale bar, 200 μm. E. Quantification of Dys+ fibers in cross sections of TA muscles (n=4). Dots and bars represent biological replicates and are mean ± SEM. Unpaired two-tailed Student’s t test. *P< 0.05, **P< 0.01 versus control.
A B

Intraperitoneal Injection
Treatment: mxAEBEs
T1×10^12 vg/mouse
Control: Saline

C

Heart

D

Grip strength (g)

E

Dystrophin
Vinculin

Dystrophin
Vinculin

Dystrophin
Vinculin

Dystrophin
Vinculin

WT Control C3 M2

Heart DI TA

Control WT

Remaining strength (%)

P0 P3 3 weeks 6 weeks 6 months

Collection Collection Collection

Figure 5
Figure 5. Systemic delivery of AAV-mxAPE rescues dystrophin expression and muscle function in multiple organs after 3 weeks

A. Schematic of systemic administration of AAV particles. AAV9-C3 and AAV9-M2 particles were injected intraperitoneally (IP) into postnatal-day-3 (P3) DMD<sup>E30mut</sup> mice. Some DMD<sup>E30mut</sup> mice were injected with saline as mock-treated controls. Black arrows indicate time points for tissue collection after IP injection. B. Measurement by deep sequencing of dystrophin transcripts of the targeted A to I editing efficiency in TA, DI, and heart after systemic delivery (n=4). C. Western blot analysis shows restoration of dystrophin expression in the TA, DI, and heart of DMD<sup>E30mut</sup> mice 3 weeks after injection. Dilutions of protein extract from WT mice were used to standardize dystrophin expression (5%, 10%, and 50%). Vinculin (CST, 13901S) was used as the loading control. D. Immunohistochemistry for dystrophin in the TA, DI, and heart of DMD<sup>E30mut</sup> mice was performed 3 weeks after systemic injection. Dystrophin (Abcam, ab15277) is shown in green. Scale bar, 200 μm. E. Quantification of Dys+ in cross sections of TA, DI, and heart muscles (n=4). F. CK levels were measured in WT, DMD<sup>E30mut</sup> mock-treated and DMD<sup>E30mut</sup> AAV9-mxAPE-treated mice 3 weeks after injection (n=4). G. Forelimb grip strength was measured in WT, DMD<sup>E30mut</sup> mice, and DMD<sup>E30mut</sup> mice treated with AAV9-C3/M2 particles (n=6). H. The remaining strength was also measured during 10 repetitions at 10-second intervals (n=6). Dots and bars represent biological replicates and are mean ± SEM. Different asterisks represent statistical significance (P< 0.01) in multiple comparison test using ANOVA. NS, not significant.
Figure 6. Restored dystrophin expression and muscle function 6 weeks after systemic mxABE treatment

A. Base editing efficiency in heart, DI, and TA muscle 6 weeks after systemic treatment with mxABE. Unpaired Student’s t-test (n=4). B. Western blot analysis of dystrophin (Sigma, D8168) restoration in heart, DI, and TA. C. Histological immunostaining analysis of Dys+ (Abcam, ab15277) muscle area in heart, DI, and TA after treatment. Scale bar, 200 μm. D. Forelimb grip strength test results for WT, untreated, M3- and C1-treated DMD^{E30mut} mice. Unpaired Student’s t test (n=4). E. Exhausted strength test showed partial rescue of forelimb muscle function in mxABE-treated DMD^{E30mut} mice. Different asterisks represent statistical significance (P< 0.01) in multiple comparison test using ANOVA (n=4). F. Rotarod running test indicated functional muscle improvement in mxABE-treated DMD^{E30mut} mice (n=4). Dots and bars represent biological replicates and are mean ± SEM. Significance is indicated by an asterisk. NS, not significant.
Figure 7

Intraperitoneal injection (6 months)

A
A to I efficiency (%)

Heart
Control
C3
M2

DI
C3
M2

TA
Control
C3
M2

sgRNA position

B
Relative mRNA expression

Heart
Control
C3
M2

DI
C3
M2

TA
Control
C3
M2

Relative intensity

Dystrophin / vinculin (%)

Heart
Control
C3
M2

DI
C3
M2

TA
Control
C3
M2

C
WT
Control
C3
M2

Dystrophin

Vinculin

Heart
Dystrophin

Vinculin

DI
Dystrophin

Vinculin

TA
Dystrophin

Vinculin

D
Relative intensity

Dystrophin / vinculin (%)

Heart
Control
C3
M2

DI
C3
M2

TA
Control
C3
M2

E
WT
Control
C3
M2

Heart

DI

TA

F
Dys + (%)

Heart
Control
C3
M2

DI
C3
M2

TA
Control
C3
M2

ns

ns

ns
Figure 7. Systemic AAV-mxABE delivery sustained dystrophin restoration after 6 months

A. Heatmap of base editing rate in heart, DI, and TA muscle 6 months after treatment with AAV-mxABE (n=4). B. mxABE expression level in heart, DI, and TA 6 months after systemic injection (n=4). C. Western blot analysis of dystrophin (Sigma, D8168) restoration in different muscle tissues 6 months after treatment. D. Quantification of western blot results in (C). E. Histological immunostaining analysis of Dys+ (Abcam, ab15277) muscle area in heart, DI, and TA 6 months after treatment (n=3). Scale bar, 200 μm. F. Quantification of immunostaining results in (E) (n=4). Dots and bars represent biological replicates and are mean ± SEM. Unpaired Student's t-test. Significance is indicated by an asterisk. NS, not significant.
Supplementary materials for

**Mini-dCas13X-mediated RNA editing restores dystrophin expression in a humanized mouse model of Duchenne muscular dystrophy**

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This PDF file includes:

- Figure S1. In vitro studies of c.4174C>T mutation correction using the mxABE system.
- Figure S2. Construction of 10 mxABE expression cassettes.
- Figure S3. Representative Sanger sequencing trace of reporter transcripts.
- Figure S4. Evaluation of SNV counts by transcriptome-wide off-target analysis in vitro.
- Figure S5. Manhattan plots of transcriptome-wide off-target RNA editing analysis for different mxABE constructs.
- Figure S6. mxABE-mediated A to I correction of other mutations in the DMD gene.
- Figure S7. *In vivo* DMD gene editing by IM injection with mxABEs after 3 weeks.
- Figure S8. Rescue of dystrophin expression following IM injection of mxABEs in DMD$^{E30mut}$ mice.
- Figure S9. *In vivo* DMD gene editing by IM injection with mxABEs after 6 weeks.
- Figure S10. Intramuscular AAV9 delivery of gene editing components rescues dystrophin expression.
- Figure S11. Calculation of SNV counts by transcriptome-wide off-target analysis in vivo.
- Figure S12. Manhattan plots of transcriptome-wide off-target RNA editing analysis in vivo.
- Figure S13. Gene editing 3 weeks after systemic delivery of mxABEs in DMD$^{E30mut}$ mice.
- Figure S14. AAV9-mxABE expression and dystrophin protein level after systemic delivery in DMD$^{E30mut}$ mice.
- Figure S15. Histological analysis of dystrophin expression 3 weeks after systemic delivery of AAV9-mxABE in DMD$^{E30mut}$ mice.
- Figure S16. Histological analysis 3 weeks after systemic delivery of AAV9-mxABE.
Figure S17. Base editing efficiency achieved by systemic delivery of mxAEB after 6 weeks.

Figure S18. mxAEB expression and dystrophin restoration level 6 weeks after systemic administration of AAV9-mxAEB.

Figure S19. Immunostaining of dystrophin in heart, TA, and DI tissues 6 weeks after systemic administration of AAV-mxAEB.

Figure S20. Immunostaining of dystrophin in heart, TA, and DI 6 months after systemic administration of AAV-mxAEB.

Figure S21. Collagen staining shows no obvious fibrosis in hearts of treated or untreated DMD\textsuperscript{E30mut} mice 6 months after systemic administration of AAV-mxAEB.

Figure S22. Host immune response to AAV-mxAEB treatment indicates no overt toxicity after 6 months.

Figure S23. Systemic delivery of AAV-mxAEB rescues dystrophin deficiency in adult DMD\textsuperscript{E30mut} mice.

Figure S24. Immunostaining of dystrophin in adult DMD\textsuperscript{E30mut} mice 6 weeks after systemic injection of AAV-mxAEB.

Tables S1. Target sgRNA and primer sequences.

Supplemental Sequences
Figure S1

A  mCherry  GFP

control

30nt-g2

50nt-g6

B

control

30nt-g2

50nt-g6

C

A to I efficiency (%)  

Control

30nt-g2

50nt-g6

sgRNA A position
Figure S1. *In vitro* studies of c.4174C>T mutation correction using mxABE constructs.

A. Fluorescence microscopy images of HEK293T cells transfected with reporter alone, or reporter and mxABE constructs. Scale bar, 200 µm. B. Representative Sanger sequencing trace of reporter transcripts. C. Measurements of the representative gRNA (30 nt-g2 and 50 nt-g6) effect on bystander A to I editing efficiency (n=3).
Vector List

- SV40 NLS
- Bipartile NLS
- Nucleoplasmin NLS
- DR

E1
- hU6
- EFS
- minidCas13 X
- hADAR2 DDV1

E2
- hU6
- EFS
- minidCas13 X
- hADAR2 DDV1

E3
- hU6
- EFS
- minidCas13 X
- hADAR2 DDV1
- WPRE

C1
- hU6
- CBh
- minidCas13 X
- hADAR2 DDV1

C2
- hU6
- CBh
- minidCas13 X
- hADAR2 DDV1
- WPRE

C3
- hU6
- CBh
- minidCas13 X
- hADAR2 DDV1
- WPRE

C4
- hU6
- CBh
- minidCas13 X
- hADAR2 DDV1
- WPRE

M1
- hU6
- MHCK7
- minidCas13 X
- hADAR2 DDV1
- WPRE

M2
- hU6
- MHCK7
- minidCas13 X
- hADAR2 DDV1
- WPRE

M3
- hU6
- MHCK7
- minidCas13 X
- hADAR2 DDV1
- WPRE
Figure S2. Construction of 10 mxABE expression cassettes.

The mxABE expression cassettes contained different promoters (EFS, CBh, MHCK7), direct repeat (DR) numbers (single, dual DR), nuclear localization signals (NLS), and were constructed with or without a translational regulatory element (WPRE).
A

Control

E1

E2

E3

M1

M2

M3

C1

C2

C3

C4

B

Control

E1

E2

E3

E4

C1

C2

C3

C4

M1

M2

M3

200 μm
Figure S3. Representative Sanger sequencing trace of reporter transcripts.

A. Sanger sequencing results showing representative A to I conversion on reporter transcripts by different mxABE editors. B. Fluorescence microscopy images of HEK293T cells transfected with reporter alone or with reporter and mxABE construct. Scale bar, 200 µm. Figure S3B and S1A showed similar results but from different experiments investigating the effect of gRNA length and construct expression elements on mutation correction efficiency respectively.
Figure S4

SNV counts

A to G

Control
E1 C3 C4 M2

C to T

Control
E1 C3 C4 M2

ns

SNV counts
Figure S4. Evaluation of SNV counts \textit{in vitro} using transcriptome-wide off-target analysis.

The transcriptome-wide off-target effect of different mxABE constructs targeting the DMD c.4174C>T mutation were performed \textit{in vitro}. The E1 mxABE construct was driven by an EFS promoter; C3 and C4 were driven by an CBh promoter; and M2 was driven by an MHCK7 promoter. Non-targeting mxABE construct was analyzed as a control. Values are shown as mean ± SEM (n=3). NS, not statistically significant in multiple comparison test using ANOVA (P< 0.01).
Figure S5
Figure S5. Manhattan plots of transcriptome-wide off-target RNA editing analysis for different mxABE constructs.

Non-targeting mxABE construct (control) and E1, C3, C4, and M2 constructs were transfected into HEK293T cells. mCherry-positive cells were sorted and endogenous transcripts were analyzed by deep sequencing. The x and y axes are proportionally enlarged with each Manhattan plot to make the axis legend clear (n=3).
A B

C E

mxABEs
c.2977 C>T, p.Gln993*

CMV
mCherry
E23
GFP

CMV
mCherry
E23
GFP

TAA to TGG

30nt

0

10

20

30

ControlGFP/mcherry efficiency (%)

30nt

50nt

38.22%

GFP/mcherry efficiency (%)

77.98%

TAA to TGG efficiency (%)

70.30%

Control A to I efficiency (%)

D F

E

mxABEs
TAG to TGG

c.8009 G>A, p.Trp2670*

CMV
mCherry
E54
GFP

CMV
mCherry
E54
GFP

Figure S6
Figure S6. mxAEB-mediated A to I correction of other mutations in the 
DMD gene.

Schematic diagram of reporter cassettes containing an mCherry sequence 
fused with 2A peptide, mutant human DMD exon 23 (A) or exon 54 (D) and 
ATG-removed GFP. Correction of the stop codon within the target sequence 
would allow EGFP expression. Flow cytometry analysis of GFP expression in 
HEK293T cells transfected with 28 gRNAs targeting exon 23 (B) and exon 54 
(E). Deep sequencing of the reporter RNA after GFP rescue experiment in 
exon 23 (C) and exon 54 (F). Data are represented as mean ± SEM (n=3).
Figure S7

A

Intramuscular injection (3 weeks)

Control

E1

E2

E3

C1

C2

C3

C4

M1

M2

M3

Interpretation of the figures:

A:

The figures show the results of intramuscular injection experiments with different sgRNA positions. The top panel shows the control group and the treatment groups E1, E2, E3, C1, C2, C3, C4, M1, M2, and M3. The panels display the expression levels of a specific mRNA transcript over time, with peaks indicating higher expression.

B:

The bar graph represents the AtoI efficiency (3-weeks, %) for each sgRNA position. The colors range from red to green, indicating different levels of efficiency. The x-axis shows the sgRNA positions, and the y-axis shows the efficiency percentages.

C:

The graph shows a comparison of the relative mRNA expression of a specific gene over 3 weeks. The horizontal axis represents the time points, while the vertical axis shows the expression levels. Each group (Control, E1, E2, E3, C1, C2, C3, C4, M1, M2, M3) is represented by a color-coded bar, with error bars indicating variability.
Figure S7. *In vivo* DMD gene editing by IM injection with mxABEs at 3 weeks.

A. Representative Sanger sequencing trace of DMD transcripts 3 weeks after IM injection with ten AAV9-mxABE particles. B. Heat map indicates the A>G edits in the vicinity of the target. C. qPCR analysis of mxABE expression in TA muscles 3 weeks after intramuscular injection. Values are shown as mean ± SEM (n=4).
Intramuscular injection (3 weeks)

WT
NT
E1
E2
E3
C1
C2
C3
C4
M1
M2
M3

TA tissue
Figure S8. Rescue of dystrophin expression following IM injection of mxABEs in DMD$^{E30mut}$ mice.

Dystrophin immunohistochemistry of entire TA muscle. Control mice were injected with saline. Dystrophin (Abcam, ab15277) is shown in green. Scale bar, 500 µm.
Figure S9

A

Intramuscular injection (6 weeks)

Control

E1

C3

C4

M2

B

A to I efficiency (6 weeks) (%)

C

Relative mxABE mRNA expression (6 weeks)
Figure S9. In vivo DMD gene editing by IM injection with mxABEs after 6 weeks.

A. Representative Sanger sequencing trace of DMD transcripts 6 weeks after IM injection with four AAV9-mxABE particles. B. Heat map indicates the A>G edits in the vicinity of the target. C. qPCR analysis of mxABE expression in TA muscles 6 weeks after intramuscular injection. Values are shown as mean ± SEM (n=4).
Intramuscular AAV9 delivery of gene editing components rescues dystrophin expression.

Immunohistochemistry of dystrophin in entire TA muscle 6 weeks after intramuscular AAV9 delivery. Control mice were injected with saline. Dystrophin (Abcam, ab15277) is showed in green. Scale bar, 500 µm.
Figure S11
Figure S11. Calculation of SNV counts by transcriptome-wide off-target analysis in vivo.

The transcriptome-wide off-target effect of two AAV9-mxABEs (C3 and M2) were analyzed in vivo. Control mice were injected with saline. Values are shown as mean ± SEM (n=4). NS, not statistically significant using unpaired two-tailed Student’s t tests (P<0.01).
Figure S12
Figure S12. Manhattan plots of transcriptome-wide off-target RNA editing analysis in vivo.

Control mice were injected with saline. The x and y axes are proportionally enlarged with each Manhattan plot to make the axis legend clear. n=4.
Figure S13

A

Heart

Control

A G G G G C T G T T

C3

A A G A G C T G T T

M2

A G G G G C T G T T

DI

Control

A G G G G C T G T T

C3

A A G A G C T G T T

M2

A A G A G C T G T T

TA

Control

A G G G G C T G T T

C3

A A G A G C T G T T

M2

A A G A G C T G T T

B

A to I efficiency (%)

Intraperitoneal injection (3 weeks)

Heart

Control

sgRNA position

Control

C3

M2

DI

Control

C3

M2

TA

Control

C3

M2

Intramuscular injection (3 weeks)
Figure S13. Gene editing 3 weeks after systemic delivery of mxABEs in DMD<sup>E30mut</sup> mice.

A. Representative Sanger sequencing trace of DMD transcripts in TA, DI, and cardiac muscle 3 weeks after intraperitoneal (IP) injection with C3 and M2 particles. B. Heat map indicates the A>G edits in the vicinity of the target in indicated tissues. n=4.
Figure S14

(A) Relative mRNA expression (3 week)

(B) Relative intensity

Heart DI TA

C3 - 3 weeks M2 - 3 weeks

ns ns ns
Figure S14. AAV9-mxABE expression and dystrophin protein levels after systematic delivery in DMD$^{E30mut}$ mice.

A. qPCR analysis of mxABE expression in TA, DI, and heart muscles 3 weeks after systemic delivery (n=4). B. Relative dystrophin (Sigma, D8168) intensity was calibrated against a vinculin (CST, 13901S) internal control before normalizing to the WT control. The results showed restoration of dystrophin in TA, DI, and heart muscles of DMD$^{E30mut}$ mice 3 weeks after systemic delivery of AAV9-packaged C3 and M2 (n=3). Values are shown as mean ± SEM. NS, not statistically significant using unpaired two-tailed Student’s t tests (P<0.01).
Figure S15

Heart

Intraperitoneal injection (3 weeks)

WT | Control | C3 | M2

TA

WT | Control | C3 | M2

DI

WT | Control | C3 | M2

C3 | M2
**Figure S15.** Histological analysis of dystrophin expression 3 weeks after systemic delivery of AAV9-mxABE in DMD<sup>E30mut</sup> mice.

Whole-muscle scanning of TA, DI, and heart of DMD<sup>E30mut</sup> mice 3 weeks after systemic delivery AAV9-C3 and M2 particles. Control mice were injected with saline. Dystrophin (Abcam, ab15277) is showed in green. Scale bar, 500 µm.
Figure S16

A

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Intraperitoneal injection (3 weeks)

Sirius Red

200 μm

B

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<th>C3</th>
<th>M2</th>
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Intraperitoneal injection (3 weeks)

HE

200 μm
Figure S16. Histological analysis 3 weeks after systemic delivery of AAV9-mxABE.

Sirius red staining (A) and HE staining (A) of TA, DI, and heart of WT, untreated DMD<sup>E30mut</sup> mice and DMD<sup>E30mut</sup> mice treated with AAV9-mxABE 3 weeks after systemic injection. n=4 for each group. Scale bars, 200 μm.
Figure S17

A

Heart

Control

C A A G T A T T G G

C3

C A G G T G T T G G

M2

C A A G T G T T G G


DI

Control

C A A G T A T T G G

C3

C A G G T G T T G G

M2

C A A G T G T T G G


TA

Control

C A A G T A T T G G

C3

C A G G T G T T G G

M2

C A A G T G T T G G


B

Intraperitoneal injection (6 weeks)

Heart

Control

C3

M2

Intraperitoneal injection (6 weeks)

DI

Control

C3

M2

Intraperitoneal injection (6 weeks)

TA

Control

C3

M2

A to I efficiency (%)

sgRNA position

10 20 30 40 50

A14 A18 A22 A24 A25 A28 A35 A40 A41

80 70 60 50 40 30 20 10
Figure S17. Base editing efficiency achieved by systemic delivery of mxAPE after 6 weeks.

A. Representative Sanger sequencing trace of DMD transcripts in TA, DI, and heart tissues 6 weeks after intraperitoneal (IP) injection with C3 and M2 particles. B. Heat map shows base editing rates in tissues indicated after IP injection with C3 (n=3) and M2 (n=4).
Figure S18. mxBE expression and dystrophin restoration level after 6-week administration of AAV-mxBE systemically.

A. qPCR analysis of mxBE expression in TA, DI, and heart muscles 6 weeks after systemic delivery (n=4). B. Relative dystrophin (Sigma, D8168) intensity was calibrated against a vinculin (CST, 13901S) internal control before normalizing to the WT control. The results showed restoration of dystrophin in TA, DI, and cardiac muscles of DMD\textsuperscript{E30mut} mice 6 weeks after systemic delivery of C3 and M2 AAV-mxBE (n=3). C. Percentage of Dys+ tissue area in TA, DI, and heart from treated and untreated DMD\textsuperscript{E30mut} mice (n=4). Values are shown as mean ± SEM. NS, not statistically significant using unpaired two-tailed Student’s t test (P<0.01).
Figure S19

Heart

WT
Control
C3
M2

TA

WT
Control
C3
M2

DI

WT
Control
C3
M2

Intraperitoneal injection (6 weeks)
Figure S19. Immunostaining of dystrophin in heart, TA, and DI tissues 6 weeks after systemic administration of AAV-mxABE.

Whole-muscle scanning of TA, DI, and heart muscle of DMD$^{E30mut}$ mice 6 weeks after systemic delivery of AAV9-C3 and M2 particles. Control mice were injected with saline. Dystrophin (Abcam, ab15277) is shown in green. Scale bar, 500 µm. Images shown in both Figure 6C and Figure S19 were obtained from the same tissue at 20x magnification. Figure 6C showed the local region staining image rather than the reconstituted whole-tissue scanning image in Figure S19.
Figure S20. Immunostaining of dystrophin in heart, TA, and DI 6 months after systemic administration of AAV-mxABE.

Whole-muscle scanning of TA, DI, and heart muscle of DMD^{E30mut} mice 6 months after systemic delivery of AAV9-C3 and M2 particles. Control mice were injected with saline. Dystrophin (Abcam, ab15277) is shown in green. Scale bar, 500 µm.
Figure S21
Figure S21. Collagen staining shows no obvious fibrosis in treated and untreated hearts 6 months after systemic administration of AAV-mxABE. Scale bar, 200 µm.
Figure S22

A

B

C

D

E

F
Figure S22. Host immune response to AAV-mxABE treatment indicates no overt toxicity after 6 months. (A-C) IL2, IL15, and IL18 transcript levels at baseline for untreated mice, 3 weeks, 6 weeks, and 6 months for treated mice (n=3). (D-F) No obvious change in ALT, total protein and BUN level after AAV-mxABE treatment (n=3). Significance is indicated by asterisk (P < 0.05). NS represents not statistically significant using unpaired two-tailed Student’s t test.
**Figure S23**

**A**

- Treatment: mxABE-M2 $1 \times 10^{12}$ vg
- Tail vein injection
- Intraperitoneal injection

**B**

- A to I efficiency (%)
- Heart
- Control
- AIV
- AIP
- WT

**C**

- Relative mxABE mRNA expression
- WT
- Control
- AIP
- AIV

**D**

- Western blots
- Heart
- DI
- TA
- Dystrophin
- Vinculin

**E**

- Heart
- DI
- TA

**F**

- Grip strength (g)
- WT
- Control
- AIP
- AIV

- ns
- *
Figure S23. Systemic delivery of AAV-mxABE rescued dystrophin deficiency in adult DMD$^{E30mut}$ mice.

A. Experimental design for mxABE administration and therapeutic analysis in adult DMD$^{E30mut}$ mice. Both intravenous (AIV group) and intraperitoneal (AIP group) injection of AAV-mxABE were evaluated. B. Heatmap shows base editing rate in heart, DI, and TA muscles (n=3). C. mxABE expression level in heart, DI, and TA muscles. D. Dystrophin restoration level analyzed by western blot in heart, DI, and TA muscles of treated and untreated mice. E. Tissue section immunostaining for dystrophin in heart, DI, and TA muscles of treated and untreated mice. Scale bar, 200 µm. F. Quantification of Dys+ immunostaining in (E) (n=3). G. Forelimb grip strength was measured in WT, DMD$^{E30mut}$ mice, and DMD$^{E30mut}$ mice treated with AAV9-M2 (n=4). Significance is indicated by asterisk ($P < 0.05$). NS represents not statistically significant using unpaired two-tailed Student’s t test.
Figure S24

Intraperitoneal injection (6 weeks)

Heart

WT
Control
M2-AIV
M2-AIP

TA

WT
Control
M2-AIV
M2-AIP

DI

WT
Control
M2-AIV
M2-AIP
Figure S24. Immunostaining of dystrophin in adult DMD$^{E30mut}$ mice 6 weeks after systemic injection of AAV-mxABE.

Scale bar, 500 µm.
### Supplementary Table S1: Target sgRNA and primer sequence.

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**RT-PCR primer for Reporter**

- **RT-rep765F**: CCACAACGAGGACTACACCA
- **RT-rep765R**: TCCTTGAAGTCGATGCGCTTT

**qPCR primer**

- **GAPDH-119F**: TCAACGACCCCTCCTATTGACC
- **GAPDH-119R**: TTTCCCGTTGATGACAAGCTTC
- **Cas13X-140F**: GCCATGCAAGAAATAATATCTCGG
- **Cas13X-140R**: CATTCTCCTTCAGCCTAAACC

| 50nt-g1 | CTGAGATAGTATAGGCCACTccATTGCTCTTGCAGAGAACTTTGTAAAGC |
| 50nt-g2 | GCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGTAA |
| 50nt-g3 | TGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g4 | GTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g5 | GTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g6 | TGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g7 | GTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g8 | CACAGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
| 50nt-g9 | TCACAGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
| 50nt-g10 | AGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
| 50nt-g11 | CAGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
| 50nt-g12 | ACAGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
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| 30nt-g2 | ATAGTATAGGCCACTCCATTGCTCTTGAGA |
| 30nt-g3 | GATAGTATAGGCCACTCCATTGCTCTTGAGA |
| 30nt-g4 | AGATAGTATAGGCCACTCCATTGCTCTTGAGA |
| 30nt-g5 | GAGATAGTATAGGCCACTCCATTGCTCTTGAGA |
| 30nt-g6 | TGAGATAGTATAGGCCACTCCATTGCTCTTGAGA |

**RNA base editing**

**sgRNA for exon 23**

| 50nt-g1 | CTGAGATAGTATAGGCCACTccATTGCTCTTGCAGAGAACTTTGTAAAGC |
| 50nt-g2 | GCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGTAA |
| 50nt-g3 | TGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g4 | GTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g5 | GTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g6 | TGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g7 | GTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGCAGAGAACTTTGAA |
| 50nt-g8 | CACAGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
| 50nt-g9 | TCACAGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
| 50nt-g10 | AGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
| 50nt-g11 | CAGTGGTGCTGAGATAGTATAGGCCACTCCATTGCTCTTGAGAGAAG |
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RNA base editing
sgRNA for exon 54

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**Supplemental sequences AAV constructs’ sequences of C1 to C4, E1 to E4 and M1 to M3**

C1 to C4 AAV constructs are CBh-driven mxABE. CBh promoter sequence is highlighted in **red**

E1 to E4 AAV constructs are EFS-driven mxABE. EFS promoter sequence is highlighted in **green**

M1 to M3 AAV constructs are MHCK7-driven mxABE. MHCK7 promoter sequence is highlighted in **blue**

> C1

```
CTCGCGAGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCG
TCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCA
GAGGAGGGAGTGGCAAACCGGCTGGAGCAGCCCCGATTTGTGGGGTG
TTACAGCGTCTGCAATATAAGCTGCAACCACTTGTCAATGAATGTGAGGG
ACTCctGCTGGAGCAGCCCCGATTTGTGGGGTGATTACAGCTTTTTTccg
```

M1 to M3 AAV constructs are MHCK7-driven mxABE. MHCK7 promoter sequence is highlighted in **blue**

```
gagggcttatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

>`E1`

```
TGGAAAGGACGAAACACCGGCTGGAGCAGCCCCGATTTGTGGGGTG
TTACAGCGTCTGCAATATAAGCTGCAACCACTTGTCAATGAATGTGAGGG
ACTCctGCTGGAGCAGCCCCGATTTGTGGGGTGATTACAGCTTTTTTccg
gagggcttatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> E4

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

>`M1`

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> C4

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> E2

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> M3

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> C3

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> M2

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> C2

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> M1

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> E3

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> M4

```
gagggctatatccagatctcctcatatgcatacggataaggcttagagagataattggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaataatttctttgtagtttgcagttt
```

> C1

```
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