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In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a devastating disease affecting about 1 out of 5000 male births and caused by mutations in the dystrophin gene. Genome editing has the potential to restore expression of a modified dystrophin gene from the native locus to modulate disease progression. In this study, adeno-associated virus was used to deliver the CRISPR/Cas9 system to the *mdx* mouse model of DMD to remove the mutated exon 23 from the dystrophin gene. This includes local and systemic delivery to adult mice and systemic delivery to neonatal mice. Exon 23 deletion by CRISPR/Cas9 resulted in expression of the modified dystrophin gene, partial recovery of functional dystrophin protein in skeletal myofibers and cardiac muscle, improvement of muscle biochemistry, and significant enhancement of muscle force. This work establishes CRISPR/Cas9-based genome editing as a potential therapy to treat DMD.

Duchenne muscular dystrophy (DMD) is among the most prevalent fatal genetic diseases, occurring in 1 out of 5000 male births (*1*). It results in muscle degeneration, loss of mobility, and premature fatality. DMD mutations are often deletions of one or more exons in the dystrophin gene that disrupt the reading frame of the gene and lead to a complete loss of functional dystrophin expression. In contrast, Becker muscular dystrophy (BMD) is associated with much milder symptoms relative to DMD and is caused by internal, in-frame deletions of the dystrophin gene resulting in expression of a truncated but partially functional dystrophin protein (*2*). Because of the genetic nature of the disease, gene therapy is a promising option to treat DMD. However, the very large size of the dystrophin cDNA presents a challenge to gene delivery. Consequently, some therapeutic strategies aim to generate a BMD-like dystrophin. These approaches include the development of mini/micro-dystrophin genes for delivery by adeno-associated virus (AAV) vectors (*3*–*6*) and oligonucleotide-mediated exon skipping therapies designed to restore the reading frame of the transcript (*7*, *8*). For example, removal of exon 51 can address 13% of DMD patient mutations, and exon skipping strategies could be extended to other regions of the gene to collectively treat 83% of DMD patients (*9*). In contrast, genome editing technologies can be used to directly correct disease-causing genetic mutations (*10*) and may be a preferred approach for a single treatment to restore stable expression of a dystrophin protein that contains most of the normal structure and function and is also under physiologic control of the natural promoter. In particular, the CRISPR/Cas9 genome editing system, which uses the Cas9 nuclease to cleave DNA sequences targeted by a single guide RNA (gRNA) (*11*), has recently created new possibilities for gene therapy by making precise genome modifications possible in cultured cells (*12*–*15*) and in animal studies (*16*–*19*). Analogous to exon-skipping therapies, CRISPR-mediated removal of one or more exons from the genomic DNA could be applied to the treatment of 83% of DMD patients. Moreover, this approach can be easily extended to targeting multiple exons within mutational hotspots, such as the deletion of exons 45-55 that could address 62% of DMD patients with a single gene editing strategy (*20*). We and others have applied these tools to correct dystrophin mutations in cultured human cells from DMD patients (*20*–*25*) and in *mdx* mouse embryos (*26*). A critical remaining challenge is to translate these proof-of-principle results into a clinically relevant approach for genome editing in muscle tissue in vivo. The use of genome editing for exon removal, rather than replacing missing exons to restore a full-length gene, may be desirable for several reasons. Editing by exon removal takes ad-

vantage of the relatively efficient non-homologous end joining pathway that is active in all cell types, in contrast to targeted gene addition by the homology-directed repair pathway that is downregulated in post-mitotic cells such as skeletal myocytes and myofibers. This method also avoids the need to deliver a DNA repair template. Finally, gene editing to delete exons will be more applicable to large patient populations that include a variety of mutations, in contrast to patient-specific editing strategies that restore unique gene deletions.

The *mdx* mouse model of DMD has a nonsense mutation in exon 23, which prematurely terminates protein production (*27*). Removal of exon 23 from the transcript through oligonucleotide-mediated exon skipping restores functional dystrophin expression and improves muscle contractility (*28*, *29*). Here, we have developed an AAV-based strategy for the treatment of DMD in the *mdx* mouse by harnessing the unique multiplexing capacity of CRISPR/Cas9 to excise exon 23 from the dystrophin gene. We hypothesized that CRISPR-mediated removal of exon 23 from the genomic DNA would restore dystrophin expression and improve muscle function (Fig. 1a).

We used AAV serotype 8 (AAV8) as a vector for delivery and expression of the components of the CRISPR/Cas9 system to skeletal and cardiac muscle (*30*). Due to the packaging size restrictions of AAV (~4.7 kb), we utilized the 3.2 kb *Staphylococcus aureus* Cas9 (SaCas9) cDNA that was recently described for in vivo genome editing applications (*19*). A second AAV vector with two guide RNA (gRNA) expression cassettes was also produced to express gRNAs targeted to introns 22 and 23. We expected that simultaneous DNA cleavage in both introns by Cas9/gRNA complexes would remove exon 23 from the genome and result in production of an internally truncated, but highly functional, dystrophin protein. A panel of gRNAs was designed by manual inspection for the SaCas9 PAM (5′-NNGRRT-3′) with close proximity to exon 23 and prioritized according to predicted specificity by minimizing potential off-target sites in the mouse genome. The best set of gRNAs was then selected based on in vitro gene editing efficiency (fig. S1).

The Cas9 and gRNA AAV vectors were premixed in equal amounts and injected into the tibialis anterior muscle of *mdx* mice. Contralateral limbs received saline injection. At eight weeks post-injection, the muscles were harvested and analyzed for deletion of exon 23 from the genomic DNA and mRNA, and expression of dystrophin protein. End-point PCR across the genomic locus revealed the expected ~1,171 bp deletion in all injected limbs (Fig. 1b). Droplet digital PCR (ddPCR) was used to quantify the percent of modified alleles by separately amplifying the unmodified or deleted DNA templates. ddPCR showed that exon 23 was deleted in ~2% of all alleles from the whole muscle lysate (Fig. 1c). Sanger sequencing of gel-extracted bands confirmed the deletion of exon 23 as predicted without any additional indels (Fig. 1b). Deep sequencing of these amplicons indicated a strong preference (~66%) for precise ligation of cut products (fig. S2). Regardless, the distribution of indels in the deletion should not impact transcript production as the indels occur in the intronic region. Deep sequencing of gRNA target sites and the top 10 predicted off-target sites for each gRNA indicated ~3% indel formation at the target sites and low (~1%, gRNA1-OT8) or undetectable off-target gene editing at the predicted off-target sites (tables S2-S3, fig. S3).

RT-PCR of mRNA extracted from muscle lysates showed a fraction of transcripts without exon 23 (Fig. 1d). Quantitative ddPCR of the cDNA also showed significant editing of the mRNA transcript with exon 23 excluded in 59% of transcripts (Fig. 1e). The high frequency of mRNA modification is likely due to protection of the Δ23 transcripts from nonsense-mediated decay. This is supported by an increase in total dystrophin mRNA from 5% of wild-type mRNA levels in non-treated muscles to 12% in Cas9/gRNA-treated muscles (fig. S4).

Western blot of whole muscle lysates showed substantial recovery of dystrophin protein to ~8% of the normal level (Fig. 2a). By immunostaining, ~67% of myofibers expressed dystrophin (Fig. 2b, Fig. 3a). Immunofluorescence staining also confirmed Cas9 expression in myonuclei (fig. S5). Collectively, the molecular analyses of genomic deletion, exon removal from the transcript, and abundant protein expression validated CRISPR-mediated restoration of a near-full length dystrophin protein to levels above the established benchmarks for functional recovery and therapeutic benefit. In particular, it is reported that as little as 4% of normal dystrophin expression level is sufficient to improve muscle function (*31*, *32*), and human natural history studies show that 30% protein expression may be sufficient for a completely asymptomatic phenotype (*33*). Therefore we evaluated therapeutic benefit in CRISPR-treated *mdx* mice. We first examined sarcolemmal neuronal nitric oxide synthase (nNOS) localization. nNOS is absent in the sarcolemma of the *mdx* mouse and DMD patients due to the loss of the nNOS-binding site in spectrin repeats 16 and 17 in the dystrophin protein (*34*, *35*). The mislocalization of nNOS contributes to DMD pathogenesis (*34*, *35*). In CRISPR-treated muscles, nNOS activity was restored at the sarcolemma closely mirroring dystrophin staining in serial sections (Fig. 3a-b) and resembling that of wild-type muscle (fig. S6).

Dystrophin assembles a series of transmembrane and cytosolic proteins into the dystrophin-associated glycoprotein complex (DGC) to link the cytoskeleton and the extracellular matrix (*1*). In *mdx* mice and DMD patients, these proteins are mislocalized. Immunostaining of serial muscle sections showed recovery of DGC proteins in Cas9/gRNA-treated

muscles, but not the contralateral controls (figs. S7-S9). Histological examination showed improved overall morphology of CRISPR/Cas9-treated muscles, including reduced fibrosis (Fig. 3c, fig. S10). The number of infiltrating macrophages and neutrophils was substantially decreased in treated muscle, indicating a reduction of the inflammation typical of dystrophic muscle (fig. S11). Hematoxylin and eosin staining of serial sections showed no obvious response to the vector or transgene in this study (Fig. 3c). However, potential immune responses to the AAV capsid, Cas9, and dystrophin are important subjects for future studies (*6*, *36*–*38*).

Next, we assessed muscle function. The specific twitch (Pt) and tetanic (Po) force were significantly improved in Cas9/gRNA-treated muscle (*p<0.05, Fig. 3d). Treated muscles showed significantly improved resistance to eccentric contraction injury, maintaining 50% of the initial force relative to 37% in untreated muscle (*p<0.05 at marked cycles, Fig. 3d). Further, pathologic hypertrophy was mitigated in Cas9/gRNA-treated muscle (table S4). Collectively these results show that CRISPR/Cas9-mediated dystrophin restoration improved muscle structure and function.

To assess the effects of dystrophin restoration early in life, we performed intraperitoneal injections of the AAV vector into P2 neonatal mice. This led to recovered dystrophin expression in abdominal muscles, diaphragm, and heart at seven weeks post-injection (figs. S12-S13). Importantly, these muscles are responsible for cardiac and pulmonary health, which are severely weakened and responsible for the premature death of DMD patients. Finally, intravenous administration of AAV vectors in six-week-old adult *mdx* showed significant recovery of dystrophin in the cardiac muscle (Fig. 4). Efficient cardiac correction will be a significant end-point to prevent premature death of DMD patients.

In this study we have demonstrated the therapeutic benefit of AAV-mediated CRISPR/Cas9 genome editing in an adult mouse model of DMD. Our results include correction in a single muscle following local delivery and in the heart following intravenous delivery to neonatal and adult mice. As systemic delivery of AAV vectors to skeletal and cardiac tissue is well-established, we expect this approach to confer body-wide therapeutic benefits. The accompanying article from Wagers and colleagues (*39*) uses a similar approach to CRISPR/Cas9-based correction of dystrophic mice using delivery with AAV9, demonstrating generality across muscle-tropic AAV serotypes. Moreover, their demonstration of efficient editing of Pax7-positive muscle satellite cells (*39*) suggests that gene correction may improve as the mature muscle fibers are populated with the progeny of these progenitor cells, as was observed in mosaic mice generated by CRISPR/Cas9 delivery to single cell zygotes (*26*). In fact, we have observed that dystrophin restoration by genome editing is maintained for at least six months post-treatment (fig. S14).

Continued optimization of vector design will be important for potential clinical translation of this approach, including evaluation of various AAV capsids and tissue-specific promoters. Additionally, although dual vector administration has been effective in body-wide correction of animal models of DMD (*40*), optimization to engineer a single vector approach may increase efficacy and translatability. These two studies (*39*) establish a strategy for gene correction by a single gene editing treatment that has the potential to achieve similar effects as seen with weekly administration of exon skipping therapies (*7*, *8*, *28*, *29*). More broadly, this work establishes CRISPR/Cas9-mediated genome editing as an effective tool for gene modification in skeletal and cardiac muscle and as a therapeutic approach to correct protein deficiencies in neuromuscular disorders and potentially many other diseases. The continued developed of this technology to characterize and enhance the safety and efficacy of gene editing will help to realize its promise for treating genetic disease

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/science.aad5143/DC1
Materials and Methods
Figs. S1 to S22
Tables S1 to S4
References (*41*–*45*)

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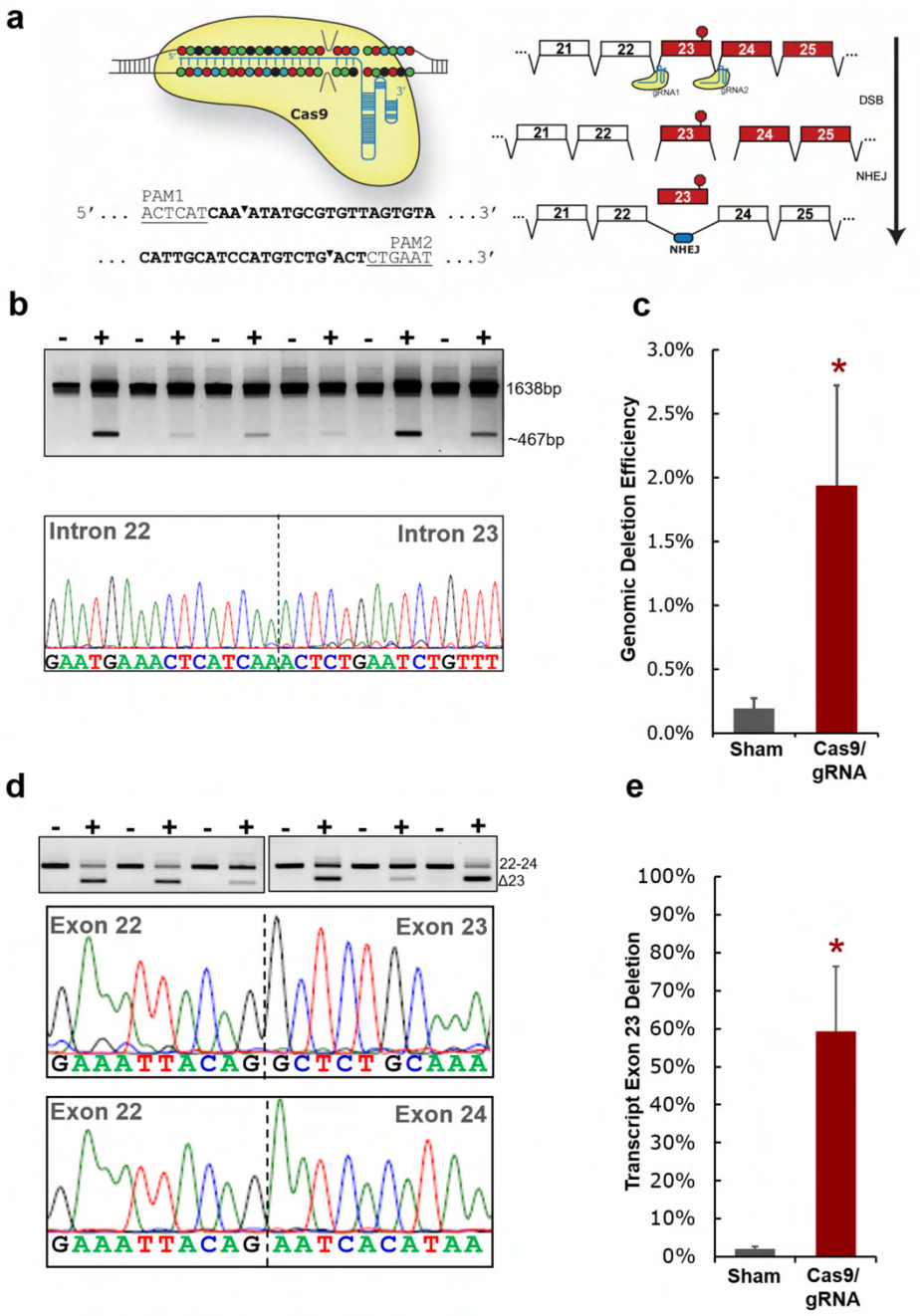

Fig. 1. CRISPR/Cas9-mediated genomic and transcript deletion of exon 23 through intramuscular AAV-CRISPR administration. (**a**) The Cas9 nuclease is targeted to introns 22 and 23 by two gRNAs. Simultaneous generation of double stranded breaks (DSBs) by Cas9 leads to excision of the region surrounding the mutated exon 23. The distal ends are repaired through non-homologous end joining (NHEJ). The reading frame of the dystrophin gene is recovered and protein expression is restored. (**b**) PCR across the genomic deletion region shows the smaller deletion PCR product in treated muscles. Sequencing of the deletion band shows perfect ligation of Cas9 target sites (+, AAV-injected muscles; -, contralateral muscles). (**c**) ddPCR of deletion products shows 2% genome editing efficiency (n=6, mean+s.e.m.). (**d**) RT-PCR across exons 22 and 24 of dystrophin cDNA shows a smaller band that does not include exon 23 in treated muscles. Sanger sequencing confirmed exon 23 deletion. (**e**) ddPCR of intact dystrophin transcripts and Δ23 transcripts shows 59% of transcripts do not have exon 23 (n=6, mean+s.e.m.). bGHpA, bovine growth hormone polyadenylation sequence; ITR, inverted terminal repeat; NLS, nuclear localization signal. Asterisk, significantly different from the sham group ($p<0.05$).

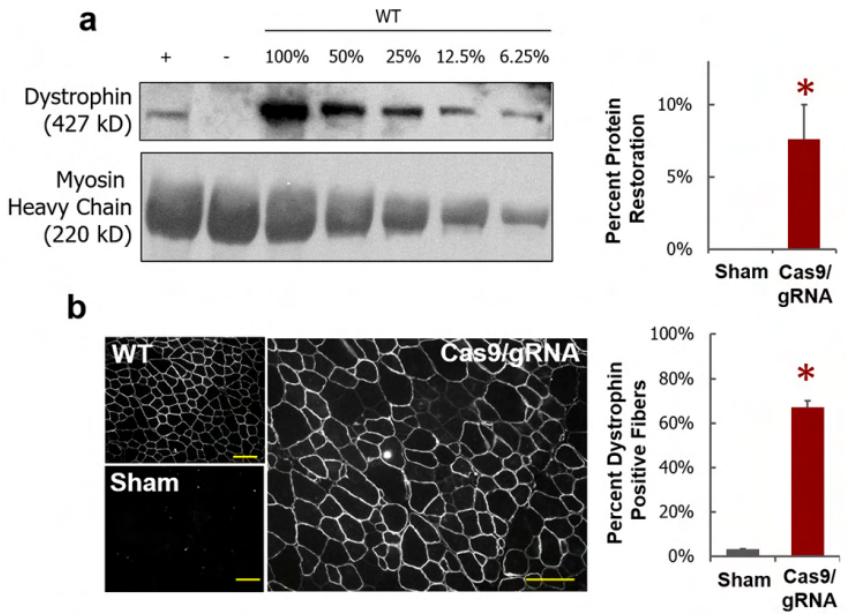

Fig. 2. In vivo genome editing restores dystrophin protein expression. (**a**) Western blot for dystrophin shows recovery of dystrophin expression (+, AAV-injected muscle; -, contralateral muscle). Comparison to protein from wild-type (WT) mice indicates restored dystrophin is ~8% of normal levels (n=6, mean+s.e.m.). (**b**) Dystrophin immunofluorescence staining shows abundant (67%) dystrophin-positive fibers in Cas9/gRNA treated groups (scale bar = 100 μm, n=7, mean+s.e.m.). Asterisk, significantly different from the sham group ($p<0.05$)

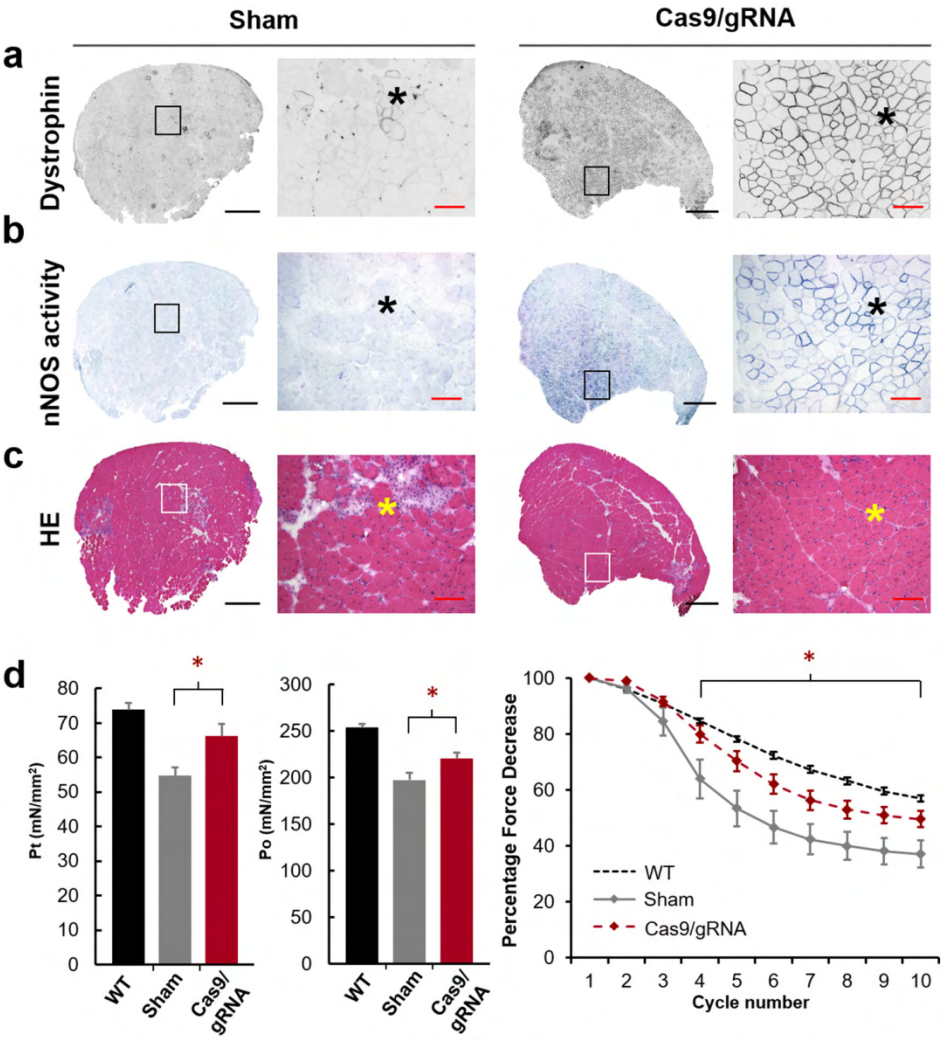

Fig. 3. CRISPR/Cas9 gene editing restores nNOS activity and improves muscle function. (**a**) Whole muscle transverse sections show abundant dystrophin expression throughout the tibialis anterior muscle. (**b**) Staining of serial sections shows recruitment and activity of nNOS in a pattern similar to dystrophin expression. (**c**) H&E staining shows no obvious adverse response to the AAV/Cas9 treatment. Additionally, there is reduction of regions of necrotic fibers. Scale bars = 600 μm in full-view images and 100 μm in high-power images. (**d**) Significant improvement in specific twitch force (Pt) and tetanic force (Po) as measured by an in situ contractility assay in Cas9/gRNA-treated muscles. Treated muscles also showed significantly better resistance to damage caused by repeated cycles of eccentric contraction (n=7, mean+s.e.m). Overall treatment effect by ANOVA ($p<0.05$). Asterisk, significantly different from the sham group ($p<0.05$).

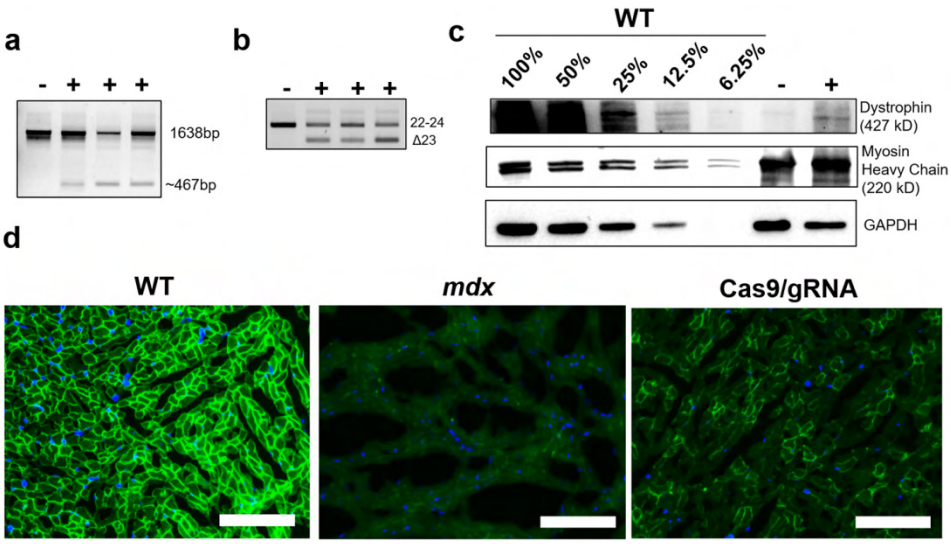

Fig. 4. Systemic delivery of CRISPR/Cas9 by intravenous injection restores dystrophin expression in adult *mdx* mouse cardiac muscle. (**a**) PCR across the deletion region in the genomic DNA from cardiac tissues shows the smaller deletion PCR product in all treated mice. (b) RT-PCR across exons 22 and 24 of dystrophin cDNA from cardiac tissue shows a smaller band that does not included exon 23 in treated mice. (c) Western blot for dystrophin in protein lysates from cardiac tissue shows recovery of dystrophin expression (+, AAV injected mice; -, saline injected controls). (d) Dystrophin immunofluorescence staining shows dystrophin recovery in cardiomyocytes. Scale bar = 100μm