Supplemental Materials

Materials and Methods

Mice

Mice (C57BL/10ScSn, C57BL10ScSn.utr/mdx and C57BL/10ScSn-*Dmd*^{mdx}/J) were maintained at The Ohio State University Laboratory Animal Resources in accordance with animal use guidelines. All animal studies were authorized by the Animal Care, Use, and Review Committee of the Ohio State University.

Construction of Cas9 and gRNA plasmids

All plasmids for Adenovirus-CRISPR/Cas9 (Ad-CRISPR) vector system were constructed as previously described[1]. For AAV production, a single vector AAV-Cas9 system containing Cas9 from Staphylococcus aureus (SaCas9) and its sgRNA was obtained from Addgene (Plasmid #61591, pX601-AAV-CMV: NLS-SaCas9-NLS-3xHA-bGHpA;U6::BsaI-sgRNA) (Addgene, MA, USA). Two gRNAs (i20 and i23) were designed to target the intron 20 and intron 23, respectively, with the following sequences (i20: GGGCGTTGAAATTCTCATTAC CAGAGT and i23: CACCGATGAGAGGGAAAGGTC CTGAAT; Note: the underlined PAM sequences were not included in the gRNA). The CMV promoter in pX601-i20 and pX601-i23 were replaced with CK7-miniCMV.

Generation of EGFP-2A-cas9, i20-gRNA and i23-gRNA adenoviruses

EGFP-2A-cas9, i20-gRNA and i23-gRNA cassettes were subcloned into pShuttle-CMV vector (Clontech, Mountain View, CA) and recombinant adenovirus genomic DNA were generated using the AdEasy-1 Adenovirus system (Agilent Technologies, La Jolla, CA)

according to the manufacturer's instructions as previously described[1] . The adenoviral particles were packaged and amplified in AD293 cells and purified by cesium chloride gradient ultracentrifugation followed by dialysis in storage buffer (10mM Tris-HCl pH 8.0, 2mM $MgCl_2$, 4% sucrose). The titers of the adenovirus preparations were quantified by measuring the OD_{260}[2].

Generation of adeno-associated virus (AAV)

AAV production was performed at the viral vector core of the Nationwide Children's Hospital. The SaCas9 and gRNA expression cassette was packaged into AAV serotype rh74 capsid using the standard triple transfection protocol[3]. A quantitative PCR-based titration method[4] was used to determine an encapsulated vg titer utilizing a Prism 7500 Fast Taqman detector system (PE Applied Biosystems Grand Island, NY USA). The following primers/probes were used: 5'-CCAGGGACAGGGTTATTTTTAGC-3' AND 5'-CCCGAGATGCCTGGTTATAATT-3'. Titers are expressed as DNase resistant particles per ml (DRP/ml) and rAAV titers used for injection in mice were $9.1X10^{12}$ DRP/ml.

Adenovirus transduction *in vivo*

The *mdx* pups (day 1-3) were injected with ~2.5×10^{10} viral particles systemically into the temporal vein or into the thoracic cavity. Tissues were collected for genomic DNA, RNA, immunoblotting and immunofluorescence staining experiments at 4 weeks after treatment.

AAV transduction *in vivo*

The *mdx/utr*$^{+/-}$ pups (day 3) were injected with ~3 x 10^{11} or 1 x 10^{12} viral particles systemically via a retro-orbital approach or an intraperitoneal approach. Tissues were collected for genomic DNA, RNA, immunoblotting and immunofluorescence experiments at ten weeks after treatment.

Cell culture and transfection

C2C12 cells were cultured in DMEM supplemented with 10% FBS and electroporated with Neon Transfection System (Invitrogen, Carlsbad, CA). Briefly, 1 × 10^5 cells were electroporated with 0.25 μg cas9 and 0.125 μg i20-gRNA and 0.125 μg i23-gRNA plasmids for both SpCas9/gRNA and SaCas9/gRNA systems. The electroporation conditions were 1650 V, 10 ms, 3 pulses. After 48 hours, C2C12 cells were collected for the genomic DNA and RNA analysis.

Extraction of DNA and RNA, and PCR analysis

Total genomic DNA from heart tissues and C2C12 cells were isolated and precipitated by isopropanol. Total RNA was extracted from heart tissues by using Trizol reagent (Life Technologies, Carlsbad, CA). Total RNA was pre-treated with an RNase-free DNase and 5 μg of treated RNA was used as template for first-strand cDNA synthesis by using RevertAid RT Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Aliquots of the RT product were used for RT-PCR analysis of dystrophin expression. *Glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) was used as a reference gene for PCR analysis. The primers used for mouse dystrophin genomic DNA were: 5'-GGCCAAAGCAAACTCTGGTA and 5'-TTTAATCCCACGTCATGCAA. The primers used

for mouse dystrophin mRNA were: 5'-GGCTAGAGTATCAAACCAACATCAT and 5'-TGGAGGCTTACGGTTTTATCC. The primers used for *Gapdh* were: 5'-GGAGTTGCTGTTGAAGTC and 5'-ACCTGCCAAGTATGATGA.

Immunoblotting

Heart tissues from *mdx/Utr*$^{+/-}$ mice treated with or without cas9/gRNA adenovirus or AAV were lysed with cold RIPA buffer supplemented with protease inhibitors and extracted protein samples were separated by SDS-PAGE (BioRad, 4-20%) and transferred onto PVDF membranes (0.45 μm). The rabbit polyclonal anti-dystrophin (E2660, 1:500, Spring Bioscience, Pleasanton, CA), mouse polyclonal HA (sc-7392, 1:500, Santa Cruz Biotechnology, Inc, Dallas, Texas) and mouse monoclonal anti-Gapdh (MAB374, 1:2500, Millipore, Billerica, MA) antibodies were used for immunoblotting analysis. HRP conjugated rabbit anti-mouse (1:5000) and goat anti-rabbit secondary antibodies (1:10000) were obtained from Cell Signaling Technology, Danvers, MA. The membranes were developed using ECL western blotting substrate (Pierce Biotechnology, Rockford, IL) and exposed to film (Kodak, Rochester) or scanned by ChemiDoc MP system (BioRad, Hercules, California). Western blots were quantitated using Image J (U. S. National Institutes of Health, Bethesda, Maryland, USA) as described previously[5].

Illumina next generation sequencing (NGS)

PCR products were purified using a commercial purification kit (Promega, Madison, WI, USA), and electrophoresed on an agarose gel, showing a single sharp peak. Their quality and quantity were assayed using an Agilent Bioanalyzer 2100 (Genomics

Shared Resource, Ohio State University Comprehensive Cancer Center). The purified PCR products were polyadenylated, and a generic Illumina sequencing adaptor was ligated using T4 DNA ligase and amplified by PCR for 2-3 cycles to enrich for ligated adaptors using appropriate primers. Ligation products were cleaned up using Agencourt Ampure beads. Samples were amplified again by PCR for 4-5 cycles to incorporate flanking Agilent SureSelect indices A01, B01 and C04, and again cleaned up using AMpure beads. Quality again was checked using a Bioanalyzer and Qubit, and then sent for sequence clustering using the Illumina cBot. Sequence data were generated for the pooled samples using a MiSeq nano-scale flow cell (Molecular and Cellular Imaging Center, OSU Wooster campus).

Ion Torrent NGS

PCR products were checked for quality. Following instructions for the Ion Torrent Fragment DNA library preparation kit, they were sheared using the Covaris S2 following manufacturer's recommendation to result in 200 nt products. Ends were repaired using T4 DNA polymerase Klenow fragment. Adaptors for Ion Torrent P1 and index were ligated, cleaned up, quality was checked, and products were amplified by PCR for a few cycles, and were cleaned up and sequenced. Beads were templated on the One Touch, then loaded for 316 chip. Outputs were processed using the Ion Torrent server software at default settings to remove adaptor sequences, resulting in FASTQ files.

Informatics analysis of targeted sequencing data

Adapter and primers sequences were removed using standard Illumina MiSeq BCL2FASTQ software. For amplicons A and B, we confirmed where the amplicons started and stopped by aligning the data to the reference genome (hg19). For amplicon C, we generated an artificial template sequence comprised of amplicons A and B, joined by 50 nt buffer sequences to the right and to the left of the amplicons, respectively. We provided these reference template amplicon sequences in using CRISPResso with default parameters[6]. For Ion Torrent sequence data, to confirm the specificity of the target amplicons A, B and C, we aligned each set first to the reference human genome (hg19) using GSNAP[7]. We extracted the ~ 350 nt target region for each amplicon. Then, we used GSNAP to locally realign the sequences to these short reference templates. Variants were visualized using the IGV sequence browser (the Broad Institute).

Immunofluorescence staining and confocal imaging

Heart tissues were collected from the *mdx/Utr*$^{+/-}$ mice injected with cas9/gRNA-expressing adenovirus or AAV. Hearts were mounted in Optimal Cutting Temperature (OCT) and frozen in liquid nitrogen cooled isopentane. The frozen hearts were transversely cryo-sectioned on a Leica CM3050S cryostat (Leica Biosystems, Buffalo Grove, IL, USA) at a thickness of 8 µm. Eight µm frozen sections were fixed with 4% paraformaldehyde for 15 min at room temperature. The samples were washed twice with PBS and incubated with blocking solution (10% goat serum) for 1h prior to overnight incubation at 4º C with primary antibodies. Primary antibodies against dystrophin (ab15277, 1:200, Abcam) and caveolin 3 (sc-5310, 1:500, Santa Cruz Biotechnology, Inc, Dallas, Texas) were used. The slides were then extensively washed with PBS and

incubated with secondary antibodies (Alexa Fluor 555 goat anti-mouse IgG, 1:500, Invitrogen or Alexa Fluor 594 goat anti-rabbit IgG 1:500, Invitrogen) for 1h at room temperature. Finally, the glass slides were mounted using VECTASHIELD® Mounting Medium with DAPI (Vector Laboratories, Inc). Then the slides were imaged with an inverted confocal microscope (Zeiss 780, Germany). Caveolin-3-positive cardiomyocytes and dystrophin-positive cardiomyocytes were counted using Image J Version 1.48 (U. S. National Institutes of Health, Bethesda, Maryland, USA). The amount of dystrophin positive cardiomyocytes is represented as a percentage of total caveolin-3-positive cardiomyocytes.

Hematoxylin & Eosin (H&E) staining

H&E staining was used to visualize the structure of the heart, nuclei and connective tissue. The frozen sections were allowed to thaw at room temperature for 15 minutes prior to immersion in 70% ethanol, followed by distilled water and were subsequently stained with Harris hematoxylin single strength (Leica Biosystems, Buffalo Grove, IL, USA) for one minute. The slides were then immersed in Scott's tap water (distilled water with 8 drops of ammonia per 300mL water) and then 70% ethanol before staining in 1% (w/v) Eosin (Leica Biosystems, Buffalo Grove, IL, USA) for one minute. Slides were then dehydrated in two steps of increasing ethanol concentrations of 70%, 95% and 100%, cleared in two washes of xylene and mounted onto coverslips with Permount (Fisher Scientific, Waltham, MA USA).

Sirius red/fast green staining

Sirius red/fast green staining was performed to visualize the fibrotic area within the cardiac muscles. Slides were washed in PBS prior to being fixed in Kahle fixative (60 ml distilled water, 28 ml 96% ethanol, 10 ml 37% formaldehyde and 2 ml concentrated acetic acid) for 10 minutes at room temperature. Slides were then washed in PBS prior and subsequently stained with dye solution (Chrondex, Redmond, WA, USA) for 30 minutes at room temperature. Slides were rinsed in distilled water and mounted onto coverslips in 50% glycerol: 50% distilled water. The area of the heart was measured using Image J Version 1.48 (U.S. National Institutes of Health, Bethesda, Maryland, USA). Color deconvolution was performed to specifically measure areas of fibrosis as previously described [8]. Fibrosis is represented as a percentage of the total heart area.

Functional measurements of contractility of cardiac papillary muscles

Ten week old mice were administered 0.1mL heparin (10,000u/mL) mixed with 0.1mL 0.9% NaCl via an intraperitoneal injection and allowed to rest for 10 minutes. Mice were then euthanized using cervical dislocation before quickly opening the chest cavity to remove the heart, which was placed directly into Krebs-Henseleit (K-H) solution containing (in mmol/L) 137 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 20 $NaHCO_3$, 0.25 Ca^{2+}, 10 glucose and 20 2,3-butanedione monoxime (BDM) in equilibrium with 95% O_2-5% CO_2. Under a dissection microscope, the right ventricle was carefully opened along the septum and blood was flushed out of the ventricle using a gentle flow of K-H/BDM solution. One or two papillary muscles were then dissected from the right ventricle to include connection with a tricuspid valve leaflet at one end and a chunk of right ventricular or septal tissue at the other. A papillary muscle was then mounted into the experimental muscle bath which contained a continuous flow of K-H solution in equilibrium with 95%

O_2-5% CO_2 (without BDM) heated to 37°C. The muscle was fixed at one end by piercing the valve leaflet over a small hook connected to a linear micromanipulator. The other end of the muscle was held in place via a basket-shaped extension of the force transducer. After mounting, the Ca^{2+} concentration was increased to 1.5 mmol/L and electrical stimulation (at 4 Hz frequency) was provided via a wire connected to the force transducer. The muscle was stabilized for 10-15 minutes at optimal length, comparable to the length at the end of diastole when sarcomere length is approximately 2.2 μm. After the muscle was stabilized, three main mechanisms of cardiac contractile regulation were assessed[9]; length dependent activation, frequency-dependent activation, and beta-adrenergic stimulation were assessed as previously described[10]. Briefly, the length-dependent activation was evaluated by recording the contractile parameters at 85, 90, 95, and 100% of optimal length. Then, the force-frequency response was measured at optimal length by adjusting the stimulation frequency from 4 to 6, 8, 10, 12 and 14 Hz, with 2-3 minute stabilization time between each frequency. Finally, after allowing the muscle to stabilize for 10 minutes at 4 Hz, the β-adrenergic receptor responsiveness was assessed by measuring the contractile parameters with the addition of isoproterenol at semi-log steps between 1 nM and 1 μM. Length, width and thickness of each papillary muscle were recorded to allow force values to be normalized to cross-sectional area.

Statistical analysis

Data are expressed as mean ± standard error (S.E.M.). Statistical differences were determined by unpaired Student's *t*-test for two groups and one-way ANOVA with

Bonferroni's post-tests for multiple group comparisons using Prism 5.02 (Graphpad). A *p* value equal or less than 0.05 was considered to be significant.

Sequences

>Amplicon A range=chrX:83781683-83782025 strand=+

I20 guide RNA sequence underlined site of most frequent recombination highlighted yellow primers I20-F and I20-R italicized and bolded

GACAACTACTGCCGAAAACTTGTTGAAAACCCAGTCTACCACCCTATCAGAGCCAACAGCAATT
AAAAGCCAGTTAAAAATTTGTAAGGTAAGGTTTTCCTCCTTCCACTTTAAGCATGATCAAAAGG
TGCTTCTTGGCAAATTGCAAGAGGGCCAAAGCAAACTCTGGTAATGAGAATTTCAACGCCCAGG
CATTGAGCTCTGTACAGTGAAGATCAAAATTTGTAAAGAATATCAGTTTAAATAAGTACTGAAT
TGATGCGTTAATTCCAGAGTTGATTTATGCAATTTTTACATGGCTACTTGATTTTGTCAGTTTT
AC***AGATGGGTGAGATAGGAAGCA***

>Amplicon B range=chrX:83804421-83804763 strand=+

I23 guide RNA sequence underlined site of recombination highlighted yellow primers I23-F and I23-R italicized and bolded

TTGCATCCATGTCTGACTCTGAATCTGTTTCTGTCTATCCTGCTCCCTAACACTGTACCATCTT
CCTTTTTGAAAAAAAAATATTGAATTATTTTATTTATTTACTTTCCAAAGTTGCTCCTGCCTGT
TCCTCCTTCTCCAAGTTCTTCAGTCCCCCCTGCTCCCCACCGATGAGAGGGAAAGGTCCTGAAT
TCACTGGGCTCCATGGGGGTCCTTTTGCATTTTCTTAACCTTCTTAATAAAATAGGCCTTCTAG
AATTATATCATATACATTGTGATATGACAAATGATAAAGTATATTGTTCAGAGTTTTACCTTGT
TCA***TATTTGCAATGTCCCCCTGT***

>Amplicon C (+50 nt padding 3' of A most frequent recombination site and 50 nt 5' of B recombination site)

GACAACTACTGCCGAAAACTTGTTGAAAACCCAGTCTACCACCCTATCAGAGCCAACAGCAATT
AAAAGCCAGTTAAAAATTTGTAAGGTAAGGTTTTCCTCCTTCCACTTTAAGCATGATCAAAAGG
TGCTTCTTGGCAAATTGCAAGAGGGCCAAAGCAAACTCTGGTAATGAGAATTTCAACGCCCAGG
CATTGAGCTCTGTACAGTGAAGATCAAAACTTCTCCAAGTTCTTCAGTCCCCCCTGCTCCCCAC
CGATGAGAGGGAAAGGTCCTGAATTCACTGGGCTCCATGGGGGTCCTTTTGCATTTTCTTAACC
TTCTTAATAAAATAGGCCTTCTAGAATTATATCATATACATTGTGATATGACAAATGATAAAGT

ATATTGTTCAGAGTTTTACCTTGTTCA***TATTTGCAATGTCCCCCTGT***

>Amplicon C_A range=chrX:83781683-83781903 strand=+

primers I20-F and I23-R italicized and bolded

GACAACTACTGCCGAAAACTTGTTGAAAACCCAGTCTACCACCCTATCAGAGCCAACAGCAATT
AAAAGCCAGTTAAAAATTTGTAAGGTAAGGTTTTCCTCCTTCCACTTTAAGCATGATCAAAAGG
TGCTTCTTGGCAAATTGCAAGAGGGCCAAAGCAAACTCTGGTAATGAGAATTTCAACGCCCAGG
CATTGAGCTCTGTACAGTGAAGATCAAAA

>Amplicon C_B range=chrX:83804554-83804763 strand=+

CTTCTCCAAGTTCTTCAGTCCCCCCTGCTCCCCACCGATGAGAGGGAAAGGTCCTGAATTCACT
GGGCTCCATGGGGGTCCTTTTGCATTTTCTTAACCTTCTTAATAAAATAGGCCTTCTAGAATTA
TATCATATACATTGTGATATGACAAATGATAAAGTATATTGTTCAGAGTTTTACCTTGTTCA***TA***
TTTGCAATGTCCCCCTGT

Supplemental References

1. Xu L, Park KH, Zhao L, Xu J, El Refaey M, Gao Y, Zhu H, Ma J, Han R. Crispr-mediated genome editing restores dystrophin expression and function in mdx mice. *Mol Ther*. 2015;24:564-569
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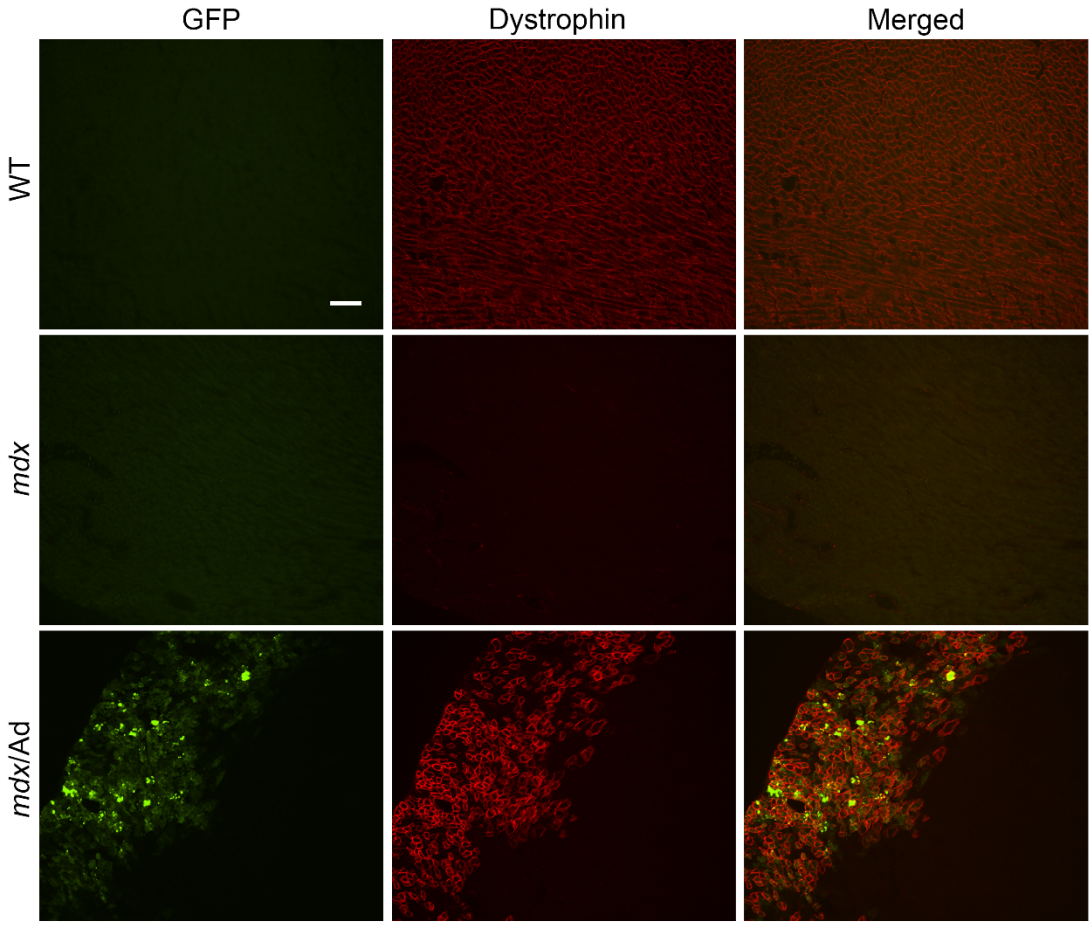
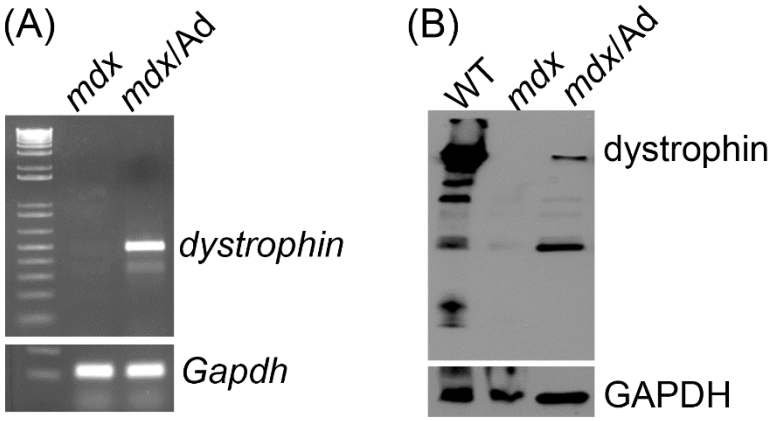
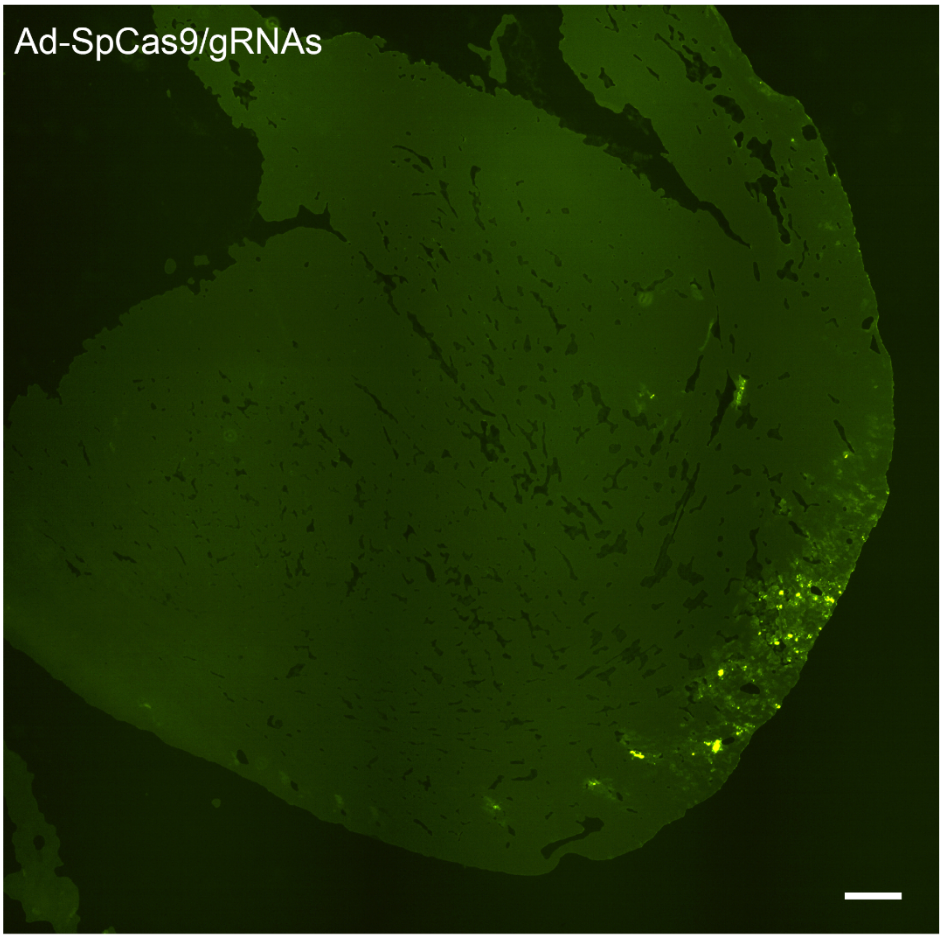


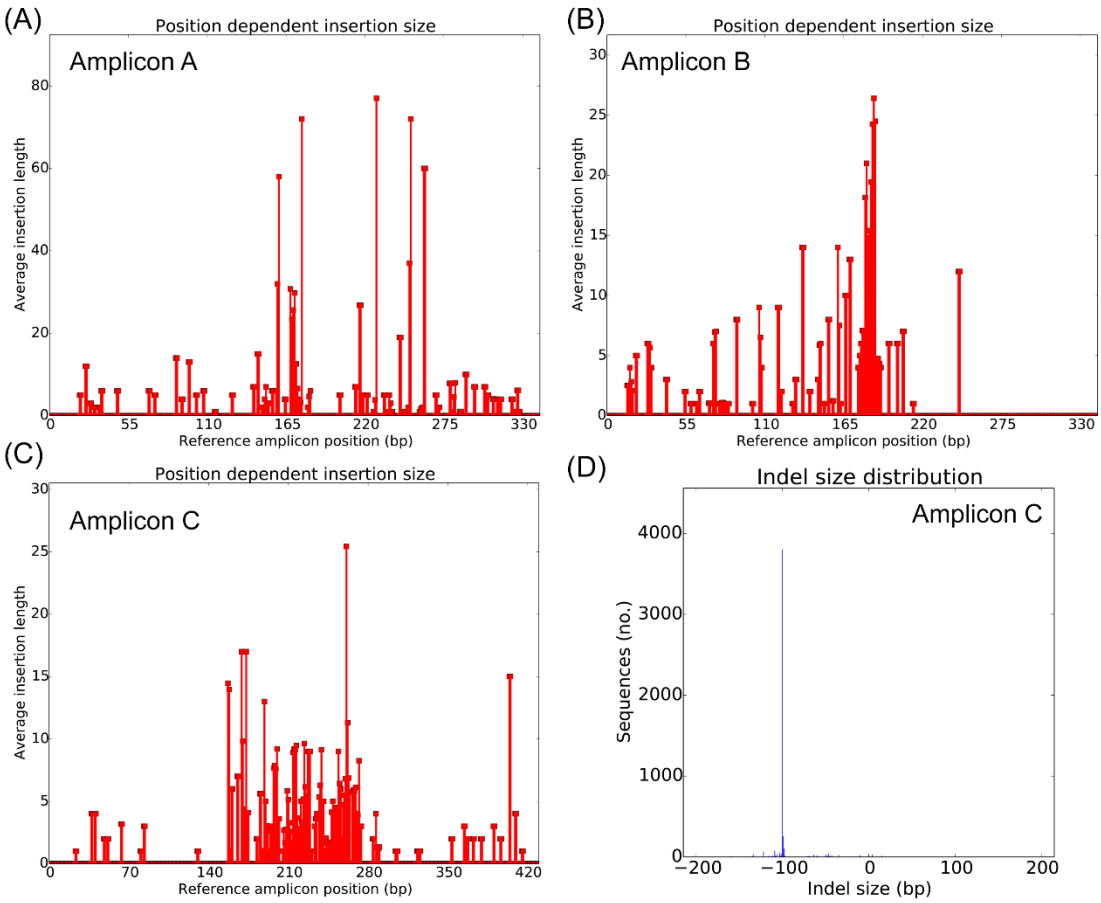
Online Figure I. Restoration of dystrophin protein expression at the sarcolemma of *mdx* cardiomyocytes by *in vivo* genome editing with adenovirus-SpCas9/gRNAs. Immunofluorescence images of dystrophin (red) and green fluorescent protein (GFP, green) in *mdx* heart cryosections treated with or without SpCas9/gRNA adenovirus after systemic injection at 8 weeks. Wild-type hearts (WT) were used as positive controls. Scale bar: 50 μm. All data are representative of a minimum of three experiments.



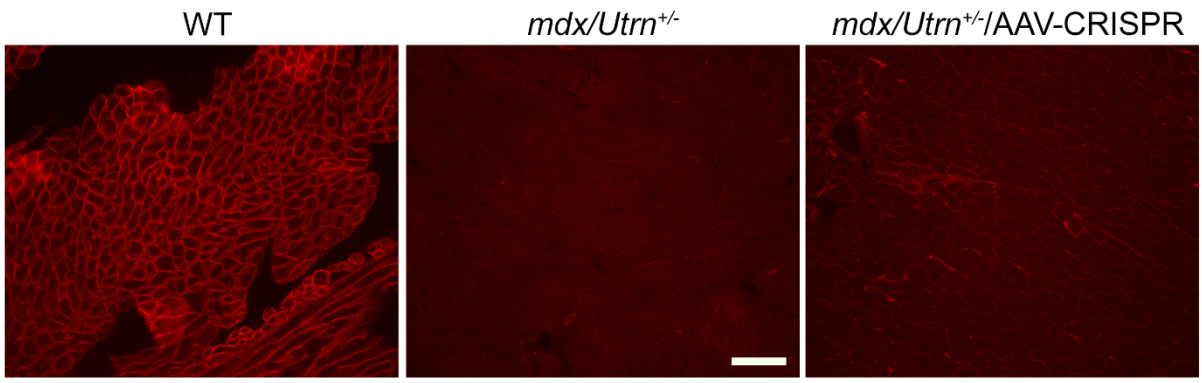
Online Figure II. (A) PCR analysis of genomic DNA extracted from *mdx* hearts transduced with or without SpCas9/gRNAs adenovirus. (B) Western blotting of heart homogenates from WT, *mdx*, and *mdx* treated with adenoviral vectors carrying SpCas9/gRNAs using anti-dystrophin and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies. All data are representative of a minimum of three experiments.



Online Figure III. GFP fluorescence of the entire heart section of an *mdx* mouse showing the focal transduction area in the pericardial myocardium after systemic injection of SpCas9/gRNA adenovirus.



Online Figure IV. Targeted deep sequencing confirms highly efficient deletion of mutant exons mediated by the CRISPR/Cas9 system. (A – C) Histograms display lengths of inserted sequences detected by individual next-generation sequences from (A) left junction spanned by amplicon A at chr. X:83781683-83782025, (B) right junction spanned by amplicon B at chr. X: 83804421-83804763, and (C) end joining junction caused by deletion of large intervening genomic fragment. Each vertical red line represents a single sequence. Maximal numbers of insertions (*y-axis*) were detected at or near the sites of gRNA annealing, in the center of each amplicon (*x-axis*). The histograms were generated using CRISPResso software. (D) A histogram shows the counts of individual next-generation sequences aligned to an artificial reference template (amplicon C; see Supp. Fig. S5), comprised of the junction between amplicons A (*left junction*) and B (*right junction*). For this template (amplicon C), we added a buffer of 50 nt additional genomic DNA sequences to the right of A and 50 nt to the left of B. Most sequences confirmed precise joining between the left and right gRNA annealing sites. As shown in the histogram, a peak at -100 represents the majority of sequence reads which aligned without insertions or deletions to this artificial template, confirming efficient deletion of ~23 kb of intervening genomic sequence. Other smaller peaks represent smaller numbers of sequences showing small insertions or deletions, where the size of the indels is shown by alignment with our artificial sequence template (*x-axis*), centered at -100. This histogram was generated using CRISPResso software.



Online Figure V. Restoration of dystrophin in the cardiomyocytes of adult *mdx/Utr*$^{+/-}$ mice (16 weeks old) treated with AAV-SaCas9/gRNAs (1 x 10^{12} vg) via tail vein injection. Immunofluorescence staining of heart sections in WT, *mdx/Utr*$^{+/-}$, and *mdx/Utr*$^{+/-}$ treated with AAV-SaCas9/gRNAs showed dystrophin-positive myocytes in the treated sample. This is a representative of two mouse experiments. Scale bar: 200 μm.