Supplementary Data

Creation of a novel humanized dystrophic mouse model of Duchenne muscular dystrophy and application of a CRISPR/Cas9 gene editing therapy

Courtney S. Young[a,b,c], Ekaterina Mokhonova[b,d], Marbella Quinonez[b,e], April D. Pyle[b,c,f], Melissa J. Spencer[a,b,c,d *]

[a] Molecular Biology Interdepartmental Program, [b] Center for Duchenne Muscular Dystrophy at UCLA, [c] Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, [d] Department of Neurology, [e] Department of Physiology, [f] Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine, University of California, Los Angeles, USA

Materials and Methods

Mice genotyping

An hDMD genotyping protocol was adapted from Jackson Laboratories for use with GeneMate Taq (BioExpress). Mdx genotyping was performed using ARMS PCR and confirmed by sequencing. Genotyping for the *Ltbp4* allele was done using primers flanking the deletion with GeneMate Taq. The *Anxa6* mutation was checked by sequencing. Genotyping for hDMD del45 mice was performed using GeneMate Taq. Per 12.5µl reaction, 1.25µl NH4 buffer, 1µl 50mM $MgCl_2$, 0.5µl DMSO, 0.6µl hDMD 45 del2 F primer, 0.6µl hDMD 45 del2 R primer, 0.1µl 25mM dNTP, 0.3µl Taq, 7.15µl H_2O, and 1µl tail DNA was mixed. The PCR was run at 95°C 2min, then 35 cycles of 95°C 30s, 56.5°C 30s, 72°C 1:30min, then 72°C 10min. PCR products were run on a 2% gel. No deletion produced a 1254bp band and an exon 45 deletion produced a 550-715bp band. Primer sequences in Supplemental Table 1. Sanger sequencing of PCR products was performed by Laragen Inc.

Supplemental Table 1: Primer sequences for PCR

Primer name, *purpose*	**Sequence**
Ltbp4 21991 F, *genotyping for LTBP4 allele*	AACCGCTACCCAAACCTTCA
Ltbp4 22379 R, *genotyping for LTBP4 allele*	AGGCTTTCTGCCTACTCGTC
hDMD45del2 F, *genotyping for hDMD del45 mice*	TGGCTCAAGTTCCCCTTCAA
hDMD45del2 R, *genotyping for hDMD del45 mice*	TGGGATGCTCCTGAAAGCAA
hdmdseq44F, *Surveyor for I44 and for 293FT deletion PCR*	AGGTGGGCAAAGACAACTGA
hdmdseq44R, *Surveyor for I44*	TGGGAAGCCTGAATCTGCG
hdmdseq45F, *Surveyor for I45*	TCTGACAACAGTTTGCCGC
hdmdseq45R, *Surveyor for I45 and for 293FT deletion PCR*	TGACATAAGGATTTGAGTCATTCAG
44_F, *PCR for exon 45-55 deletion* [11]	CTGGACGGAGCTGGTTTATCT
55surv2_R, *PCR for exon 45-55 deletion* [11]	CCCTTTTCTTGGCGTATTGCC
55undelF, *PCR for undeleted exon 45-55 allele* [11]	GCCTGGGTCTCTGCTATCAA
55undelR, *PCR for undeleted exon 45-55 allele* [11]	GCCACTTTGTACTCCGCACT

CRISPR/Cas9 gRNA generation and screening

gRNAs targeting *DMD* introns 44 and 45 were designed using crispr.mit.edu and cloned into px330 (Addgene 42230, Feng Zhang [15]) as previously described [11]. Sequences in Supplemental Table 2. Each gRNA was screened individually or in pairs in human embryonic kidney (HEK) 293FT cells using TransIT 293 (Mirus Bio) according to the manufacturer's instructions and as described [11]. Surveyor assay (IDT) was performed to assess single gRNA cutting ability as described [11]. The exon 45 deletion was assessed by Accuprime Taq HiFi (Thermo Fisher Scientific) PCR using primers flanking the deleted region (primer sequences in Supplemental Table 1) where a 2037bp undeleted or 1329-1521bp deleted product was produced.

gRNAs for the exon 45-55 deletion (44C4, 55C3) from [11] were cloned into px333 (Addgene 64073, Andrea Ventura [13]) in tandem using BbsI and BsaI.

Supplemental Table 2: gRNA sequences and oligos for cloning

Name	**Sequence**	**Sense oligo**	**Antisense oligo**
44C1	ATATACTTGTGGCTAGTTAG**TGG**	CACCGATATACTTGTGGCTAGTTAG	AAACCTAACTAGCCACAAGTATATC

44C2	ATTCACCTTGAAGCAATCAT**GGG**	CACCGATTCACCTTGAAGCAATCAT	AAACATGATTGCTTCAAGGTGAATC
44C4	TCTTTACTGCTGTTGATTAA**TGG**	CACCGTCTTTACTGCTGTTGATTAA	AAACTTAATCAACAGCAGTAAAGAC
45C1	AAAAACTGGAGCTAACCGAG**AGG**	CACCGAAAAACTGGAGCTAACCGAG	AAACCTCGGTTAGCTCCAGTTTTTC
45C2	TGTCCTACAAATCAATTAGT**TGG**	CACCGTGTCCTACAAATCAATTAGT	AAACACTAATTGATTTGTAGGACAC
45C3	CAGGGAAAAAAGCACCCTCT**CGG**	CACCGCAGGGAAAAAAGCACCCTCT	AAACAGAGGGTGCTTTTTTCCCTGC

Green is NGG PAM

Immunostaining and H&E staining

Harvested muscles were flash frozen in isopentane and cryosectioned at 10µm thickness. Blocking and staining was done as previously described [11]. Primary antibodies consisting of anti-human dystrophin (1:5, MANDYS106, MDA Monoclonal Antibody Resource, [16]) and anti-laminin (1:200, Sigma-Aldrich), were applied overnight at 4°C. The following day secondary antibodies were incubated for 1hr and the slides were mounted with VECTASHIELD containing DAPI (Vector Laboratories) and imaged on the Axio Observer Z1 microscope (Zeiss).

Muscle sections were also stained with hematoxylin and eosin and imaged on an Axio Imager M1 microscope (Zeiss).

Muscle sections from the electroporation experiment were fixed in cold acetone for 1 min then blocked with PBS containing 0.05% Tween-20 and 5% horse serum for 1-1.5hrs. The M.O.M. blocking kit (Vector Laboratories) was applied according to the manufacturer's instructions then primary antibodies of anti-laminin, human dystrophin (1:55, MANDYS106, Millipore-Sigma), human dystrophin E48/50 (1:10, MANEX4850, MDA Monoclonal Antibody Resource, [16]) were added in PBS with 0.05% Tween-20, 5% FBS at 4°C overnight. The rest of the procedure was the same as described above. Control and treated slides were imaged at the same exposure and the contrast adjusted the same.

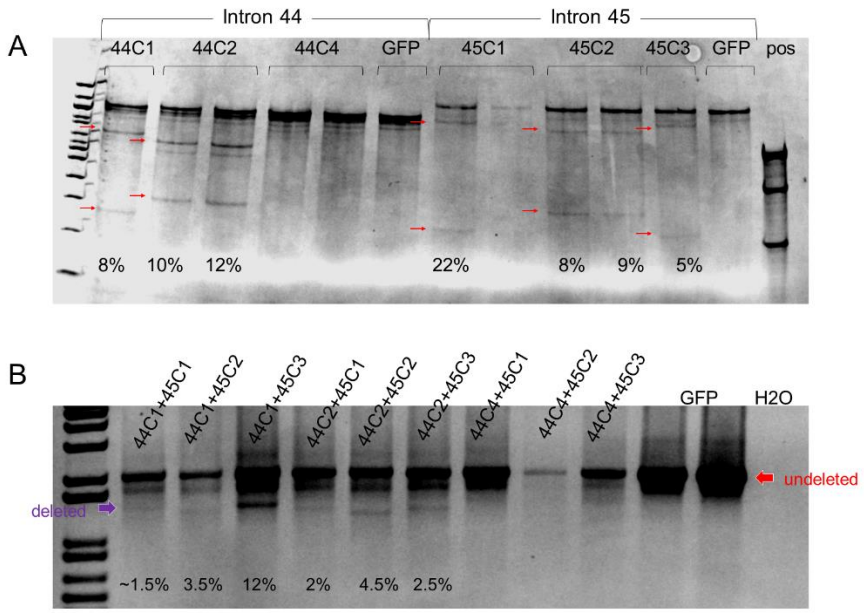
Western blotting

100µg of frozen tissues were solubilized in 50mM tris-HCl (pH 7.4), 7M urea, 2M thiourea, 4% CHAPS, 2% SDS, 50mM β-mercaptoethanol. Lysates were incubated at 4°C for

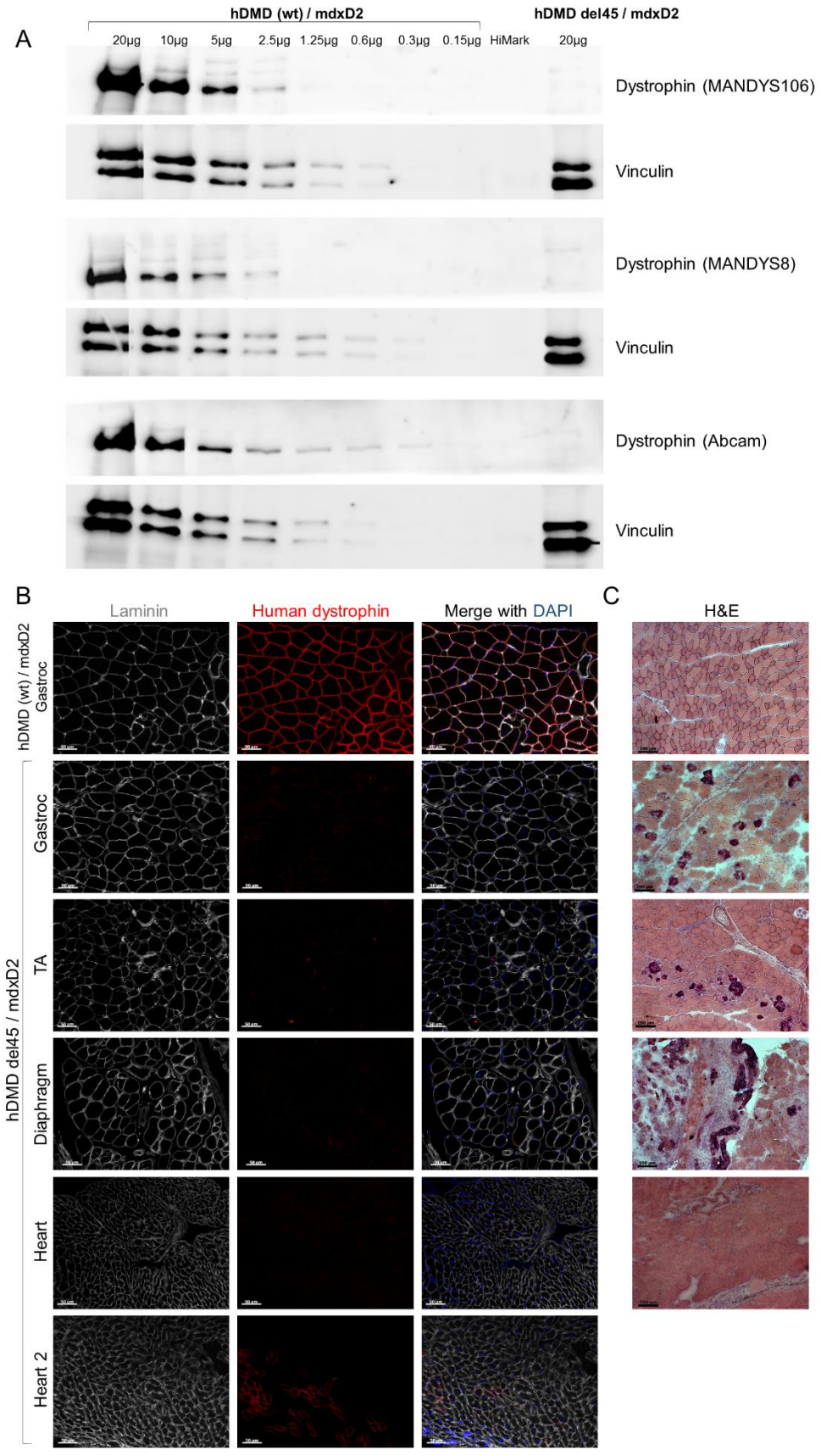
60 min with gentle rotation, and centrifuged for 5 min at 13,000*g.* Clarified lysates were transferred into new tubes, aliquoted and stored at -80°C until use. Protein concentration was determined using 2-D Quant Kit (GE Healthcare Life Sciences). 20µg of hDMD del45 mdxD2 or hDMD del45 mdx gastrocnemius, TA, diaphragm and heart muscle lysates, 20µg of mdxD2 gastrocnemius lysate and 5-20µg of hDMD (wt) mdxD2 or hDMD (wt) mdx gastrocnemius muscle lysate were subjected to electrophoresis in a 6% polyacrylamide gel (PAAG), transferred to a nitrocellulose membrane and blotted with anti-human dystrophin antibody MANDYS106 (1:100 in PBSAT, Millipore Sigma) and stained with Ponceau S (Sigma-Aldrich).

For titration blots, lysates were prepared as described [17]. Briefly, muscle tissue was homogenized for 1min in 1mL of ice-cold Mito buffer [0.2mM EDTA, 0.25mM sucrose, 10mM tris-HCl (pH 7.4)] with protease/phosphatase inhibitor cocktail (Pierce) and deoxyribonuclease/ribonuclease and subjected to low-speed (1500*g*) centrifugation for 10 min at 4°C. The supernatant was centrifuged at 100,000*g* for 30min for isolation of membrane fraction. Isolated membranes and pellet after low-speed centrifugation were combined and resuspended in 300µl extraction buffer [50mM tris-HCl (pH 7.4), 7M urea, 2M thiourea, 4% CHAPS, 2% SDS, 50mM β-mercaptoethanol] followed by centrifugation for 5min at 13,000*g*. Various amounts of hDMD (wt) mdxD2 and hDMD del45 mdxD2 gastrocnemius lysates were subjected to 6% SDS-PAAG electrophoresis, blotted onto nitrocellulose membranes and probed with anti-human dystrophin antibody MANDYS106, anti-dystrophin MANDYS8 (1:400, Sigma-Aldrich), anti-dystrophin (1:300, Abcam Ab15277) or anti-vinculin (1:5000, Sigma-Aldrich) overnight at 4°C.

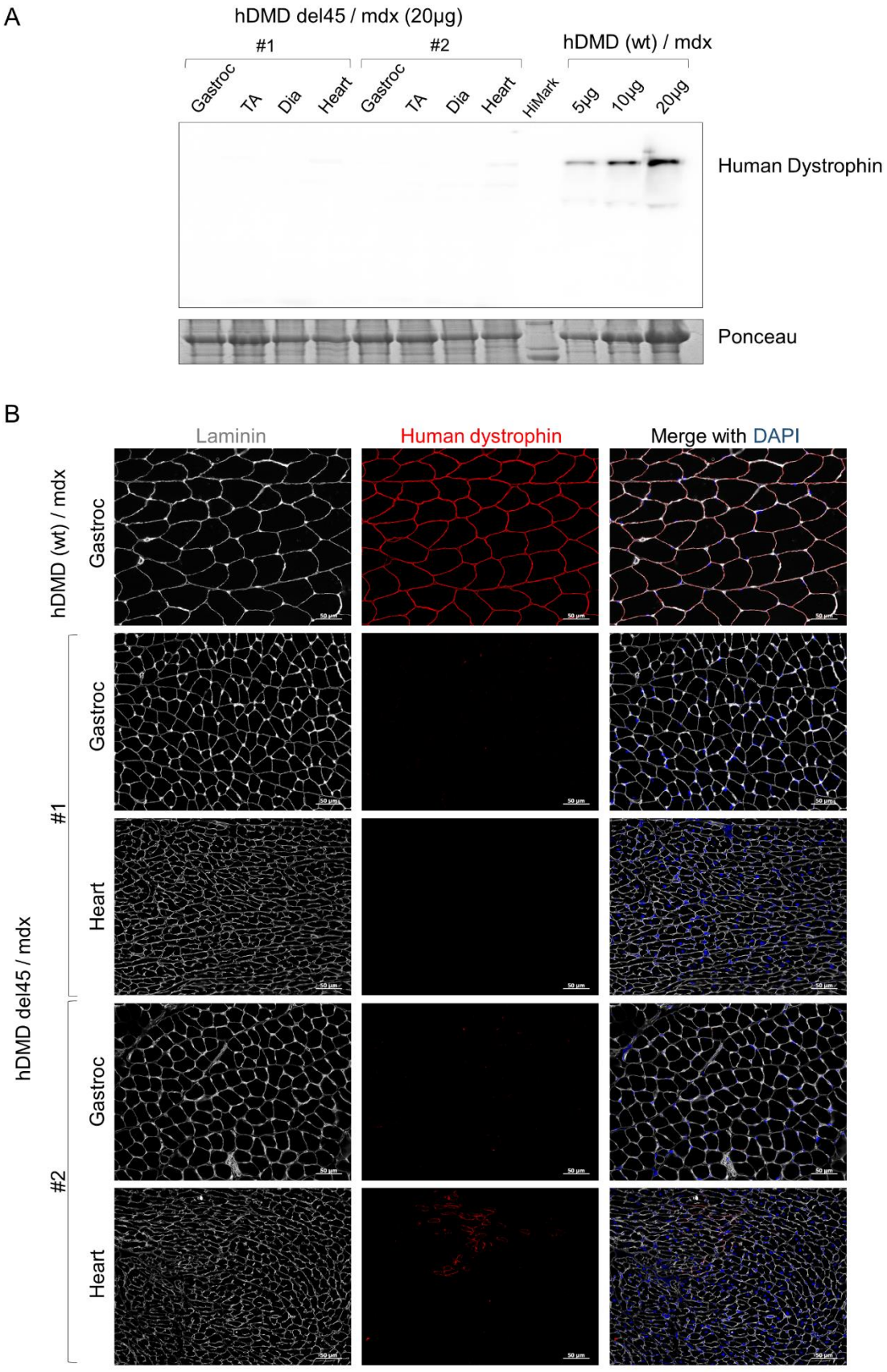
Secondary antibodies used were anti-mouse and anti-rabbit peroxidase conjugates (1:10000 in 5% milk/PBST, Sigma-Aldrich). Blots were developed using ChemiGlow West chemiluminescent detection kit (Protein Simple). Signals were registered by the Azure C300 (Azure Biosystems).



Supplemental Fig. 1. Testing of gRNAs for exon 45 deletion. A) Surveyor assay of individual gRNA screening in HEK293FT cells. Three gRNAs for each intron 44 and intron 45 were designed and their cutting ability assessed in 293FT cells. Expected Surveyor assay cleavage products are shown with the red arrows (sizes in Supplemental Table 3) and the estimated percent cutting is shown below. GFP plasmid was used as a negative control. A 100bp ladder was used. B) Genomic DNA PCR for the exon 45 deletion after paired gRNAs were transfected in HEK293FT cells. The red arrow shows the 2037bp undeleted band and the purple arrow shows the 1329-1521bp deleted band. The estimated percent deletion is shown below. A 1kb ladder was used.


A
hDMD (wt) / mdxD2
hDMD del45 / mdxD2
20µg
10µg
5µg
2.5µg
1.25µg
0.6µg
0.3µg
0.15µg
HiMark
20µg
Dystrophin (MANDYS106)
Vinculin
Dystrophin (MANDYS8)
Vinculin
Dystrophin (Abcam)
Vinculin
B
Laminin
Human dystrophin
Merge with DAPI
hDMD (wt) / mdxD2
Gastroc
hDMD del45 / mdxD2
Gastroc
TA
Diaphragm
Heart
Heart 2
C
H&E

Supplemental Fig. 2. Characterization of hDMD del45 mdxD2 mice. A) Western blotting titration of dystrophin from the gastrocnemius muscle of an hDMD (wt) / mdxD2 mouse (third backcross, 6.5wks old) and lack of dystrophin in the gastrocnemius muscle of an hDMD del45 / mdxD2 mouse (first cross, 4.5wks old). Three different dystrophin antibodies were used. Vinculin is shown as a loading control. B) Immunohistochemistry of muscle sections stained with laminin (white) and human dystrophin (red). Laminin was used to delineate muscle fibers. hDMD del45 / mdxD2 muscles (first cross, 4.5wks old) show a lack of dystrophin staining. A few revertant fibers were seen in a second area of the heart. Scale bar 50µm. C) Hematoxylin and eosin staining of muscle sections show dystrophic pathology in muscles of hDMD del45 / mdxD2 mice (first cross, 4.5wks old). Scale bar 100µm.


A
hDMD del45 / mdx (20µg)
#1
#2
hDMD (wt) / mdx
Gastroc
TA
Dia
Heart
Gastroc
TA
Dia
Heart
HiMark
5µg
10µg
20µg
Human Dystrophin
Ponceau
B
Laminin
Human dystrophin
Merge with DAPI
hDMD (wt) / mdx
Gastroc
hDMD del45 / mdx
#1
Gastroc
Heart
#2
Gastroc
Heart

Supplemental Fig. 3. Characterization of hDMD del45 mdx mice. A) Western blot of whole muscle extracts probed with human dystrophin. Ponceau stain is shown to demonstrate loading. The gastrocnemius (gastroc), tibialis anterior (TA), diaphragm (dia), and heart from two hDMD del45 / mdx mice (#1 and #2, first cross, 2.5wks old) show lack of dystrophin compared to an hDMD (wt) / mdx mouse (third backcross, 8.5wks old) gastroc muscle. B) Immunohistochemistry of muscle sections stained with anti-laminin (white) and human dystrophin (red). Laminin was used to delineate muscle fibers. hDMD del45 / mdx muscles from two mice (first cross, 2.5wks old) show a lack of dystrophin staining with a few revertant fibers seen in the heart of mouse #2. Scale bar 50µm.

Supplemental Table 3: Expected cleavage product sizes from Surveyor assay.

gRNA	Expected sizes (bp)
44C1	257, 700
44C2	309, 648
44C4	190, 767
45C1	257, 750
45C2	326, 681
45C3	253, 754