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Supplementary Materials for

Single-cut genome editing restores dystrophin expression in a new mouse model of muscular dystrophy

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This PDF file includes:

MATERIALS AND METHODS

PCR and T7E1 analysis of genomic regions

These methods were performed as previously described *(16)*. PCR products were subcloned into pCRII-TOPO vector (Invitrogen) according to the manufacturer's instructions. Individual clones were picked and the DNA was sequenced.

Targeted deep DNA sequencing

PCR of genomic DNA and cDNA from TA and cardiac muscles was performed using primers designed against the respective target region and off-target sites (**Table S1**). A second round of PCR was used to add Illumina flowcell binding sequences and experiment-specific barcodes on the 5' end of the primer sequence (**Table S1**). Before sequencing, DNA libraries were analyzed using a Bioanalyzer High Sensitivity DNA Analysis Kit (Agilent). Library concentration was then determined by qPCR using a KAPA Library Quantification Kit for Illumina platforms. The resulting PCR products were pooled and sequenced with 300 bp paired-end reads on an Illumina MiSeq instrument. Samples were demultiplexed according to assigned barcode sequences. FASTQ format data was analyzed using the CRISPResso software package version 1.0.8. *(47)*.

Histological analysis of muscles and morphometric analysis

Histological analysis of muscles was performed as described previously*(16)*. Morphometric analyses of dystrophin-positive and total myofibers were carried out on replicates of whole step-sections of TA muscles and hearts. Scanned images were analyzed using Image J software. Dystrophin-positive myofibers were individually counted using Image J software, while number of total myofibers was estimated from

cell-counts per field area made from the mean of eight 10x objective images and extrapolated to the whole scanned section area.

Western blot analysis

Western blot was performed as described previously*(48)*. Antibodies to dystrophin (1:1000, D8168, Sigma-Aldrich), vinculin (1:1000, V9131, Sigma-Aldrich), goat anti-mouse and goat-anti rabbit HRP-conjugated secondary antibodies (1:3000, Bio-Rad) were used for the described experiments.

Grip strength test

Muscle strength was assessed by a grip strength behavior task performed by the Neuro-Models Core Facility at UT Southwestern Medical Center. These measurements were performed as previously described *(16)* in a blinded way.

Serum creatine kinase (CK) measurement

Mouse serum CK was measured by the Metabolic Phenotyping Core at UT Southwestern Medical Center. Serum CK level was measured using the VITROS 250 Chemistry System in a blinded way.

SUPPLEMENTAL MATERIALS

Supplementary Figures

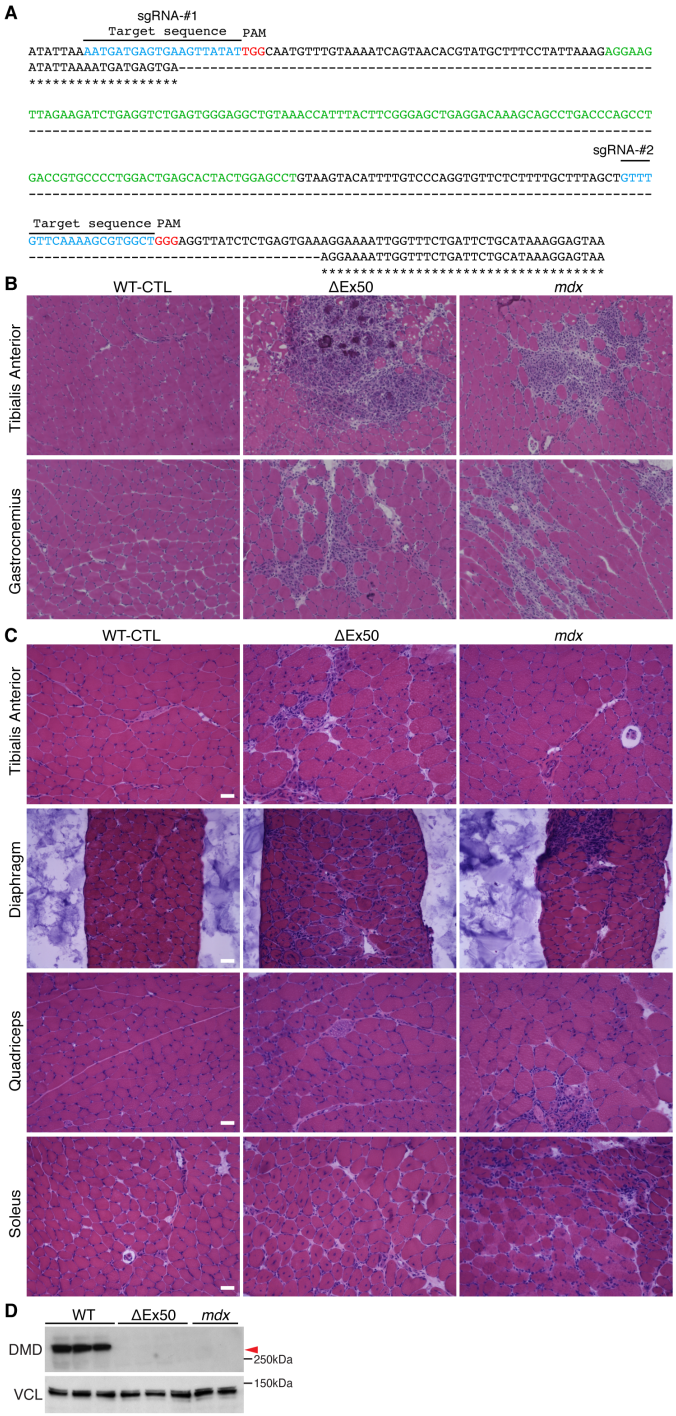


Fig S1. ΔEx50 mouse model analyses. (**A**) Genomic sequence of targeted locus (top line) and ΔEx50 founder (bottom line) with a 245 base pair deletion that eliminated exon 50 (indicated in green). sgRNA-#1 and #2 are indicated in blue. (**B**) Hematoxylin and eosin (H&E) staining of tibialis anterior and gastrocnemius muscles of 3 week old WT, ΔEx50 and *mdx* mice. (**C**) Hematoxylin and eosin (H&E) staining of tibialis anterior, diaphragm, quadriceps and soleus muscles of 1-month old WT, ΔEx50 and *mdx* mice. (**D**) Western blot analysis of dystrophin (DMD) and vinculin (VCL) expression in skeletal muscle of each group. n=5. Scale bar: 50μm.

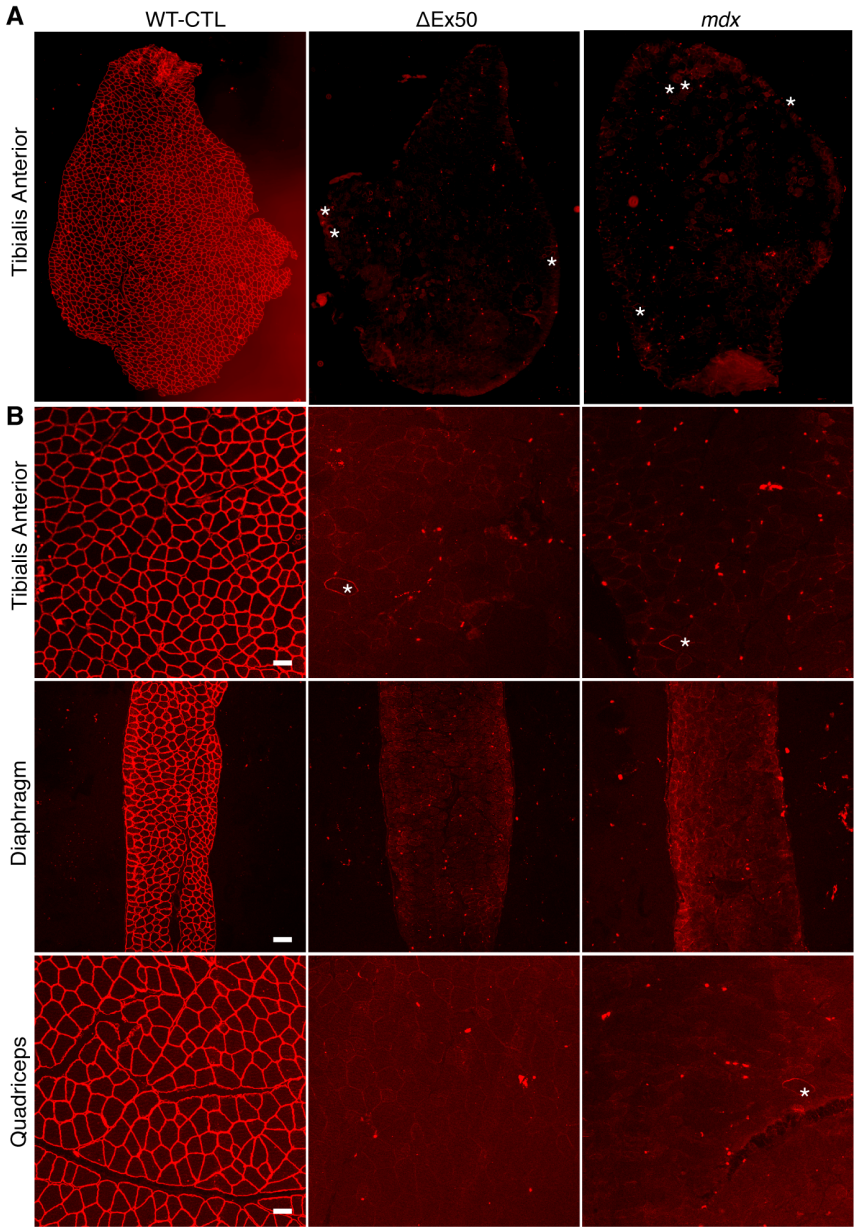


Fig S2. Comparison of dystrophin staining in ΔEx50 and *mdx* mice at one month of age. (A) Dystrophin immunohistochemistry of entire tibialis anterior of 1-month old WT, ΔEx50 and *mdx* mice. (**B**) Dystrophin immunohistochemistry of tibialis anterior, diaphragm and quadriceps muscles of 1-month old WT, ΔEx50 and *mdx* mice. Asterisk indicates revertant fibers. n=5. Scale bar: 50μm.

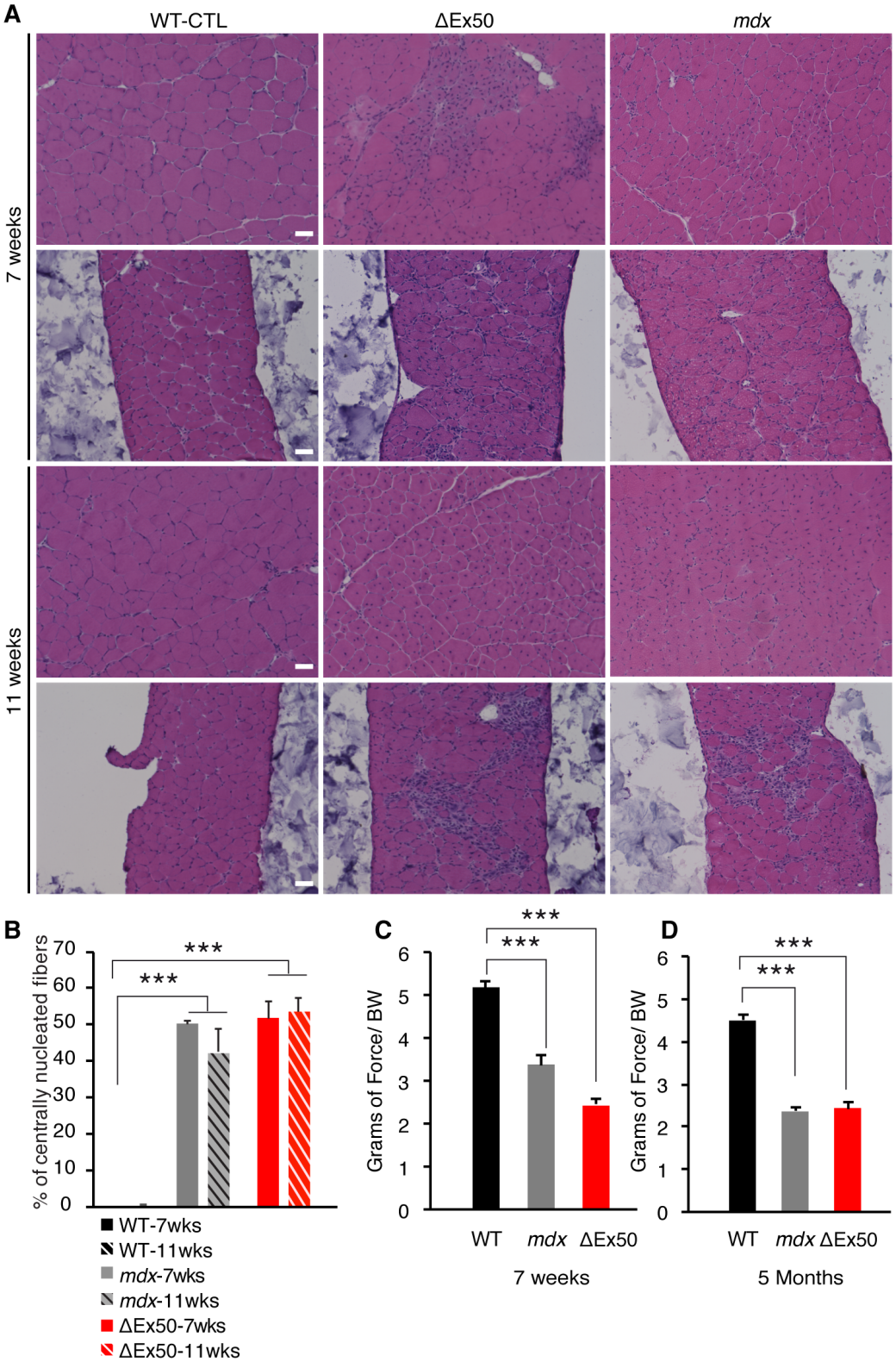

A
WT-CTL
ΔEx50
mdx
7 weeks
11 weeks
B
% of centrally nucleated fibers
0
10
20
30
40
50
60
70
***
***
WT-7wks
WT-11wks
mdx-7wks
mdx-11wks
ΔEx50-7wks
ΔEx50-11wks
C
Grams of Force/ BW
0
1
2
3
4
5
6
***
***
WT
mdx
ΔEx50
7 weeks
D
Grams of Force/ BW
0
1
2
3
4
5
6
***
***
WT
mdx
ΔEx50
5 Months

Fig S3. Characterization of ΔEx50 mice at different ages. (**A**) Hematoxylin and eosin (H&E) staining of tibialis anterior and diaphragm muscle of 7-week-old and 11-week-old WT, ΔEx50 and *mdx* mice. (**B**) Percentage of centrally nucleated fibers in tibialis anterior muscle of 7- and 11-week old WT, ΔEx50 and *mdx* mice. (**C**) WT, *mdx* and ΔEx50 mice were subjected to grip strength testing to measure muscle performance that was normalized by body weight (BW) (grams of force/BW) at 7-weeks and (**D**) 5-months of age. n=5. Scale bar: 50μm.

A

Mouse Exon 51:

CTGCCAGTCAGACTGTTACTCTAGTGACACAATCTGTGGTTACTAAGGAAACTGTCATCTCCAAACTAGAAATGCCATCTTCT
TTGCTGTTGGAGGTACCTGCACTGGCAGACTTCAACCGAGCTTGGACAGAACTTACAGACTGGCTGTCTCTGCTTGATCGAGT
TATAAAATCACAGAGAGTGATGGTGGGTGATCTGGAAGACATCAATGAAATGATCATCAAACAGAAG

B

SRSF1 threshold: 1.956			SRSF1 (IgM-BRCA1) threshold: 1.867			SRSF2 threshold: 2.383		
Position	**Site**	**Score**	**Position**	**Site**	**Score**	**Position**	**Site**	**Score**
9	CAGACTG	3.49605	1	CTGCCAG	1.96756	11	GACTGTTA	2.78337
26	GACACAA	2.43404	9	CAGACTG	3.04097	38	GGTTACTA	4.72957
43	CTAAGGA	2.55243	34	CTGTGGT	2.49220	95	GGTACCTG	3.18459
62	CAAACTA	2.15904	43	CTAAGGA	2.99670	111	GACTTCAA	3.62772
91	TGGAGGT	1.98779	62	CAAACTA	2.09258	127	GGACAGAA	2.58025
109	CAGACTT	3.86959	103	CACTGGC	2.10803	148	TGTCTCTG	3.07384

C

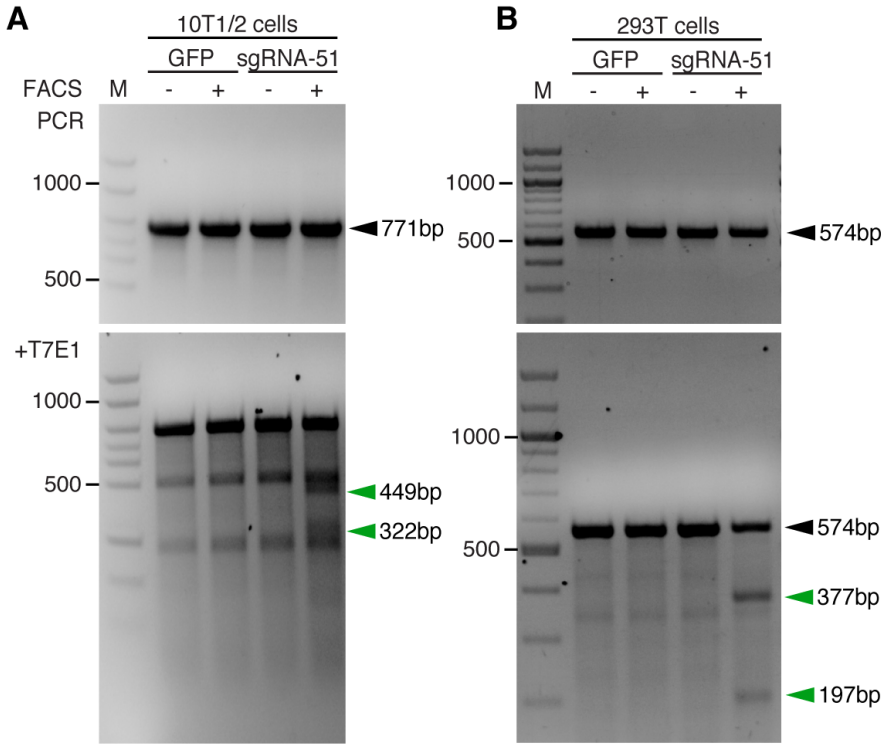
Human Exon 51:

CTCCTACTCAGACTGTTACTCTGGTGACACAACCTGTGGTTACTAAGGAAACTGCCATCTCCAAACTAGAAATGCCATCTTCC
TTGATGTTGGAGGTACCTGCTCTGGCAGATTTCAACCGGGCTTGGACAGAACTTACCGACTGGCTTTCTCTGCTTGATCAAGT
TATAAAATCACAGAGGGTGATGGTGGGTGACCTTGAGGATATCAACGAGATGATCATCAAGCAGAAG

D

SRSF1 threshold: 1.956			SRSF1 (IgM-BRCA1) threshold: 1.867			SRSF2 threshold: 2.383		
Position	**Site**	**Score**	**Position**	**Site**	**Score**	**Position**	**Site**	**Score**
9	CAGACTG	3.49605	9	CAGACTG	3.04097	11	GACTGTTA	2.78337
19	CTCTGGT	2.05834	19	CTCTGGT	2.87347	31	AACCTGTG	2.66418
26	GACACAA	2.43404	34	CTGTGGT	2.49220	38	GGTTACTA	4.72957
43	CTAAGGA	2.55243	43	CTAAGGA	2.99670	95	GGTACCTG	3.18459
62	CAAACTA	2.15904	52	CTGCCAT	2.17523	111	GACTTCAA	3.62772
91	TGGAGGT	1.98779	62	CAAACTA	2.09258	127	GGACAGAA	2.58025

Fig S4. Exon splicing enhancers (ESEs) of exon 51. (**A**) Mouse Exon 51 sequence with the predicted exon splicing enhancers (ESEs) located at the site of sgRNA is indicated in red. (**B**) Mouse ESE sites of exon 51 predicted using ESEfinder3. (**C**) Human Exon 51 sequence with the predicted exon splicing enhancers (ESEs) located at the site of sgRNA is indicated in red. (**D**) Human ESE sites of exon 51 predicted using ESEfinder3.



C

Target Sequence

Mouse-sgRNA : GTCAGACTGTTACTCTAGTG

Human-sgRNA : CTCAGACTGTTACTCTGGTG

D

	PAM ▼	%Reads
WT	ttttagCTCCTACTCAGACTGTTACTCTGGTGACACAACCTGTG	64.3079
(+1)	ttttagCTCCTACTCAAGACTGTTACTCTGGTGACACAACCTGTG	21.5385
(+1)	ttttagCTCCTACTCCAGACTGTTACTCTGGTGACACAACCTGTG	5.3930
(-2)	ttttagCTCCTA--CAGACTGTTACTCTGGTGACACAACCTGTG	0.7932
(-1)	ttttagCTCCTACT-AGACTGTTACTCTGGTGACACAACCTGTG	0.3759
(-2)	ttttagCTCCTACT--GACTGTTACTCTGGTGACACAACCTGTG	0.1999
(-4)	ttttagCTCCTA----AGACTGTTACTCTGGTGACACAACCTGTG	0.0030
(-5)	ttttagCTCC-----CAGACTGTTACTCTGGTGACACAACCTGTG	0.0018
(-3)	ttttagCTCCTAC---AGACTGTTACTCTGGTGACACAACCTGTG	0.0018
(-13)	----------CTCAGACTGTTACTCTGGTGACACAACCTGTG	0.0006

Fig S5. Validation of sgRNAs in mouse 10T1/2 and human 293T cells. (**A**) Cas9 was expressed in the presence or absence of mouse sgRNA-51 in 10T1/2 cells and gene editing was monitored by T7E1 assay in fluorescence-based cell sorted (FACS) (+) and non-sorted cells (-). GFP was used as a control. Guide RNA-51 is defined in

Figure 2. Undigested PCR products (upper panel) and T7E1 digestion (lower panel) are shown on a 2% agarose gel. Black arrowhead indicates the undigested 771bp PCR band. Green arrowheads in the lower panel indicate the cut bands by T7E1 assay. M denotes size marker lane. bp indicates the length of the marker bands. (**B**) Cas9 was expressed in the presence or absence of human sgRNA-51 in 293T cells and gene editing was monitored by T7E1 assay in fluorescence-based cell sorted (FACS) (+) and non-sorted cells (-). GFP was used as a control. Undigested PCR products (upper panel) and T7E1 digestion (lower panel) are shown on a 2% agarose gel. Black arrowhead indicates the undigested 574bp PCR band. Green arrowheads in the lower panel indicate the cut bands by T7E1 assay. M denotes size marker lane. bp indicates the length of the marker bands. (**C**) Alignment of mouse and human target sequence conservation. Highlighted in red are nucleotide difference in target sequence. (**D**) Sequence of representative indels aligned with sgRNA sequence (indicated in blue) revealing deletions and insertions. Black arrowhead indicates the cleavage site.

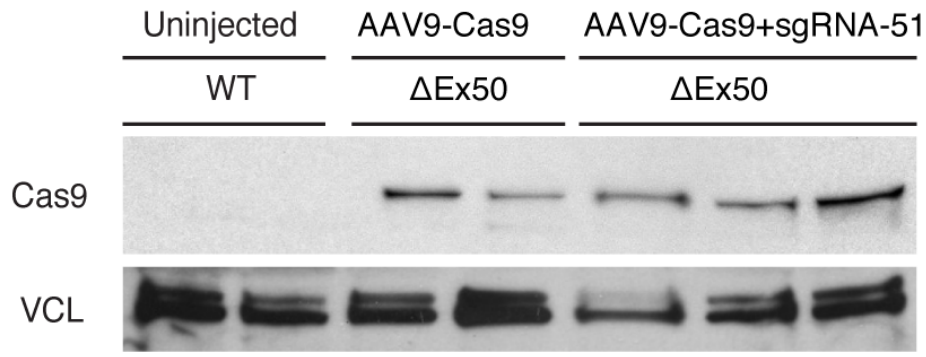
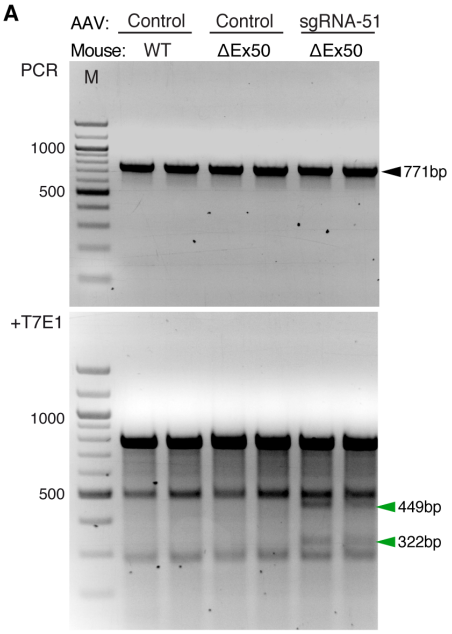


Fig S6. Cas9 expression in injected muscles. Western blot analysis of Cas9 and vinculin (VCL) expression in tibialis anterior muscles 3 weeks after intramuscular injection.

A



B **ΔEx50-AAV9-sgRNA-51-TA-#1**

	PAM ▼	%Reads	#Reads
(NE)	acactagCTGCCAGTCAGACTGTTACTCTAGTGACA	67.17	36253
(+1)	acactagCTGCCAGTCAAGACTGTTACTCTAGTGACA	18.24	9846
(S1)	acactagCTGCCAGTCATACTGTTACTCTAGTGACA	1.17	631
(+2)	acactagCTGCCAGTCATAGACTGTTACTCTAGTGACA	0.64	345
(S1)	acactagCTGCCAGTCAGACTTTTACTCTAGTGACA	0.57	309
(+2)	acactagCTGCCAGTCAAAGACTGTTACTCTAGTGACA	0.49	263
(-2)	acactagCTGCCAGTC--ACTGTTACTCTAGTGACA	0.36	194
(+2)	acactagCTGCCAGTCACAGACTGTTACTCTAGTGACA	0.30	164
(-1)	acactagCTGCCAGTC-GACTGTTACTCTAGTGACA	0.17	92
(-4)	acactagCTGCCAGT----CTGTTACTCTAGTGACA	0.09	51
(-6)	acactagCTG------AGACTGTTACTCTAGTGACA	0.09	51
(-4)	acactagCTGCCAGTCA----GTTACTCTAGTGACA	0.09	50

ΔEx50-AAV9-sgRNA-51-TA-#2

	PAM ▼	%Reads	#Reads
(NE)	acactagCTGCCAGTCAGACTGTTACTCTAGTGACA	72.79	39665
(+1)	acactagCTGCCAGTCAAGACTGTTACTCTAGTGACA	15.43	8407
(S1)	acactagCTGCCAGTCATACTGTTACTCTAGTGACA	1.02	556
(-2)	acactagCTGCCAGTC--ACTGTTACTCTAGTGACA	0.34	183
(+2)	acactagCTGCCAGTCACAGACTGTTACTCTAGTGACA	0.31	171
(+2)	acactagCTGCCAGTCATAGACTGTTACTCTAGTGACA	0.30	162
(+2)	acactagCTGCCAGTCAAAGACTGTTACTCTAGTGACA	0.25	135
(S1)	acactagCTGCCAGTCAAGACTTTTACTCTAGTGACA	0.27	147
(S1)	acactagCTGCCAGTCAGCCTGTTACTCTAGTGACA	0.21	113
(-1)	acactagCTGCCAGTC-GACTGTTACTCTAGTGACA	0.10	54
(-1)	acactagCTGCCAGTCA-TGTCACTAGAGTAACAGT	0.04	21
(-4)	acactagCTGCCAGTCA----TGTTACTCTAGTGACA	0.04	20

ΔEx50-AAV9-sgRNA-51-TA-#3

	PAM ▼	%Reads	#Reads
(NE)	acactagCTGCCAGTCAGACTGTTACTCTAGTGACA	76.19	40992
(+1)	acactagCTGCCAGTCAAGACTGTTACTCTAGTGACA	12.38	6659
(S1)	acactagCTGCCAGTCATACTGTTACTCTAGTGACA	1.12	603
(+2)	acactagCTGCCAGTCAAAGACTGTTACTCTAGTGACA	0.33	175
(+1S1)	acactagCTGCCAGTCAAGACTTTTACTCTAGTGACA	0.24	131
(-1)	acactagCTGCCAGTCA-TGTCACTAGAGTAACAGT	0.18	96
(S1)	acactagGCTGCCAGTCAAACTGTTACTCTAGTGACA	0.15	79
(-2)	acactagCTGCCAGTC--TGTCACTAGAGTAACAGT	0.12	67
(S1)	acactagCTGCCAGTCCGACTGTTACTCTAGTGACA	0.11	58
(S1)	acactagCTGCCAGTTAGACTGTTACTCTAGTGACA	0.08	45
(-4)	acactagCTGCCAGT----TGTCACTAGAGTAACAG	0.05	25
(-6)	acactagCTGCCAGTCA------TGTCACTAGAGTA	0.04	22

Fig S7. In vivo *Dmd* gene editing. (**A**) Undigested PCR products (upper panel) and T7E1 digestion (lower panel) are shown on a 2% agarose gel of tibialis anterior muscle samples from WT and ΔEx50 mice 3 weeks after intramuscular injection with AAV9-sgRNA-51 and AAV9-Cas9 expression vectors. Controls were injected with only AAV9-Cas9 not AAV9-sgRNA-51. Black arrowhead in the upper panel indicates the 771bp PCR band. Green arrowheads in the lower panel indicate the cut bands by T7E1 assay. M denotes size marker lane. bp indicates the length of the marker bands. n=4. (**B**) Genomic deep sequencing analysis of PCR amplicons generated across the exon 51 target site in ΔEx50 mice injected with AAV9-sgRNA-51 and AAV9-Cas9. Sequence of representative indels aligned with sgRNA sequence (indicated in blue) revealing insertions (highlighted in green) and deletions (highlighted in red). Black arrowheads indicate the cleavage site. n=3.

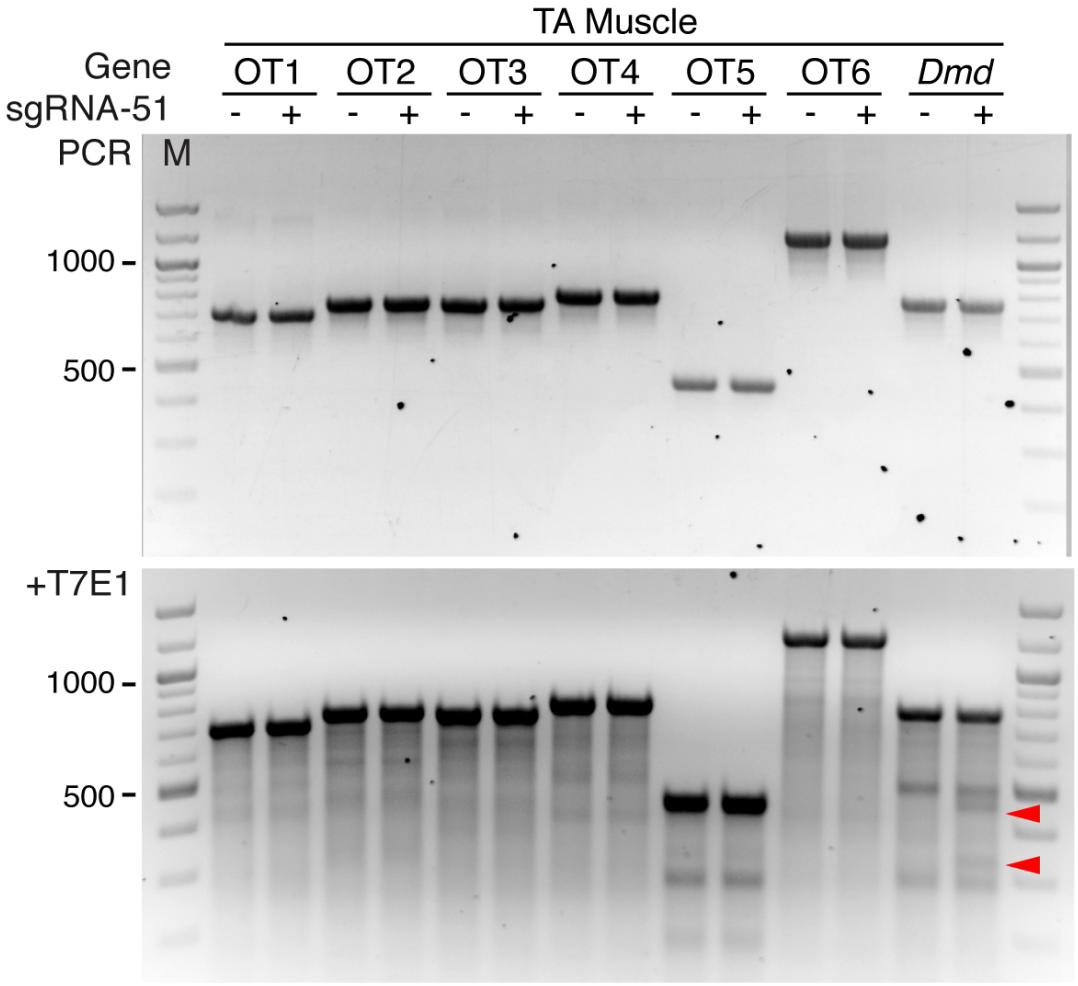


Fig S8. Off-target analyses for sgRNA-51. Undigested PCR products and T7E1 digestion from T7E1 assay on *Dmd* (Target) site and genome-wide theoretical exonic off-target sites (OT1 to OT6) in tibialis anterior (TA) muscle. AAV9-Cas9 was injected into TA muscle in the presence (+) or absence (-) of AAV9-sgRNA-51 and gene editing was monitored by T7E1 assay. Undigested PCR products (upper panel) and T7E1 digestion (lower panel) are shown on a 2% agarose gel. Red arrowheads in the lower panel indicate the cut bands by the T7E1 assay. M denotes size marker lane in base pairs.

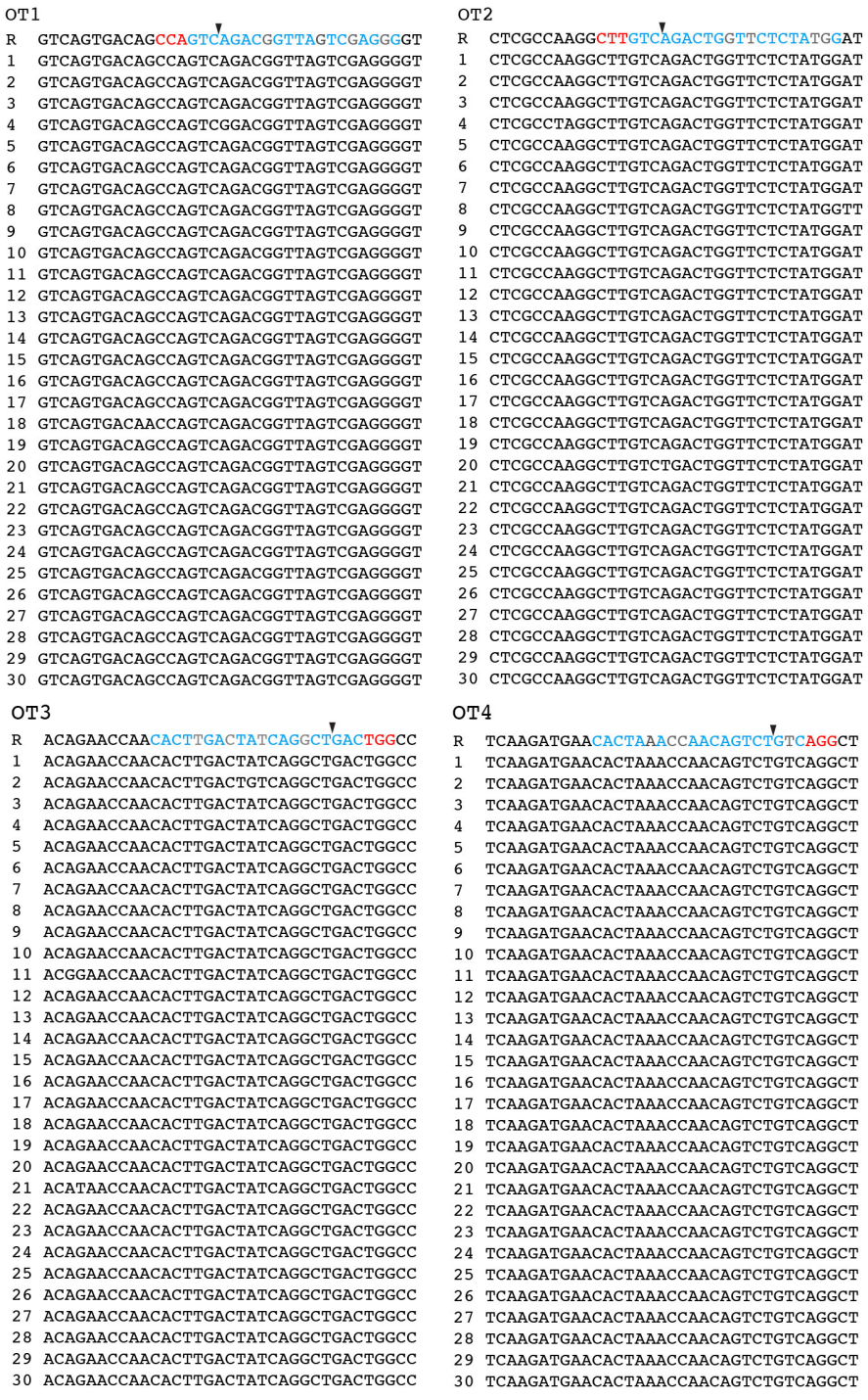
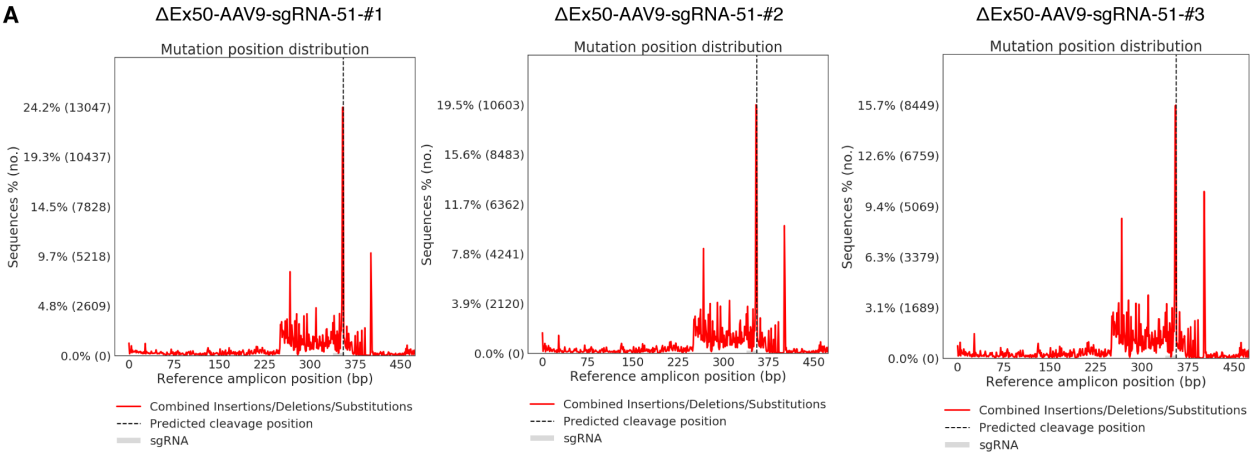


Fig S9. Off-target sequence analyses for sgRNA-51. DNA sequence analysis of the genomic PCR amplicons of exonic off-target sites (OT1 to OT4) in tibialis anterior muscle. Black arrowheads indicate the cleavage site.

A



B

Sample	PCR	sgRNA used for analysis	sgRNA sequence used for analysis	Chr.	Total Reads	Modified Reads	NHEJ (%)	Average NHEJ (%)
ΔEx50-AAV9-sgRNA-51-#1	Ex51	ex51-SA	GTCAGACTGTTACTCTAGTG	chr X	53969	17716	32.826	27.949±1.876
ΔEx50-AAV9-sgRNA-51-#2				chr X	54494	14829	27.212	
ΔEx50-AAV9-sgRNA-51-#3				chr X	53803	12811	23.810	
ΔEx50-AAV9-sgRNA-51-#1	Ex51	NS internal control	ATGGATAAAACAATAATATT	chr X	53969	245	0.453	0.443±0.015
ΔEx50-AAV9-sgRNA-51-#2				chr X	54494	220	0.403	
ΔEx50-AAV9-sgRNA-51-#3				chr X	53803	255	0.473	
ΔEx50-AAV9-sgRNA-51-#1	OT#1	predicted OT ex51	GTCAGACGGTTAGTCGAGGG	chr5	52641	184	0.349	0.334±0.005
ΔEx50-AAV9-sgRNA-51-#2				chr5	47708	158	0.331	
ΔEx50-AAV9-sgRNA-51-#3				chr5	46582	151	0.324	
ΔEx50-AAV9-sgRNA-51-#1	OT#1	NS internal control	TAACAGGGCTGGCAAGCCGA	chr5	51321	124	0.241	0.230±0.009
ΔEx50-AAV9-sgRNA-51-#2				chr5	46500	113	0.243	
ΔEx50-AAV9-sgRNA-51-#3				chr5	45467	94	0.206	
ΔEx50-AAV9-sgRNA-51-#1	OT#2	predicted OT ex51	GTCAGACTGGTTCTCTATGG	chr5	49202	33	0.067	0.062±0.005
ΔEx50-AAV9-sgRNA-51-#2				chr5	58293	28	0.048	
ΔEx50-AAV9-sgRNA-51-#3				chr5	53767	28	0.0706	
ΔEx50-AAV9-sgRNA-51-#1	OT#2	NS internal control	GAACATCCAAAAATTTCATC	chr5	43331	218	0.503	0.365±0.059
ΔEx50-AAV9-sgRNA-51-#2				chr5	58293	223	0.382	
ΔEx50-AAV9-sgRNA-51-#3				chr5	55804	118	0.211	
ΔEx50-AAV9-sgRNA-51-#1	OT#3	predicted OT ex51	GTCAGCCTGATAGTCAAGTG	chr2	66188	119	0.179	0.155±0.012
ΔEx50-AAV9-sgRNA-51-#2				chr2	56941	93	0.163	
ΔEx50-AAV9-sgRNA-51-#3				chr2	59680	73	0.122	
ΔEx50-AAV9-sgRNA-51-#1	OT#3	NS internal control	GACAATGGACCATCTTCCTA	chr2	66188	78	0.117	0.125±0.005
ΔEx50-AAV9-sgRNA-51-#2				chr2	56941	79	0.138	
ΔEx50-AAV9-sgRNA-51-#3				chr2	59680	72	0.120	

Fig S10. Amplicon PCR deep sequencing analyses for sgRNA-51. (**A**) Representative graph generated by the CRISPResso version 1.0.8 software pipeline during genomic deep sequencing analysis of PCR amplicons generated across the exon 51 target. The left panel y-axis represents % sequence and mutation distribution. The average insertion size (center panel) and average deletion size (right panel) at each nucleotide position across the PCR amplicons are indicated. Dotted lines represent predicted Cas9 cleavage sites. (**B**) Genomic deep sequencing analysis of PCR amplicons generated across the exon 51 target and exonic off-target sites in tibialis anterior muscle. Mismatches in the target sequence are highlighted in red. Non-specific internal sgRNA sequence, highlighted in grey, were used for analysis to determine the background of the sequencing analysis. n=3.

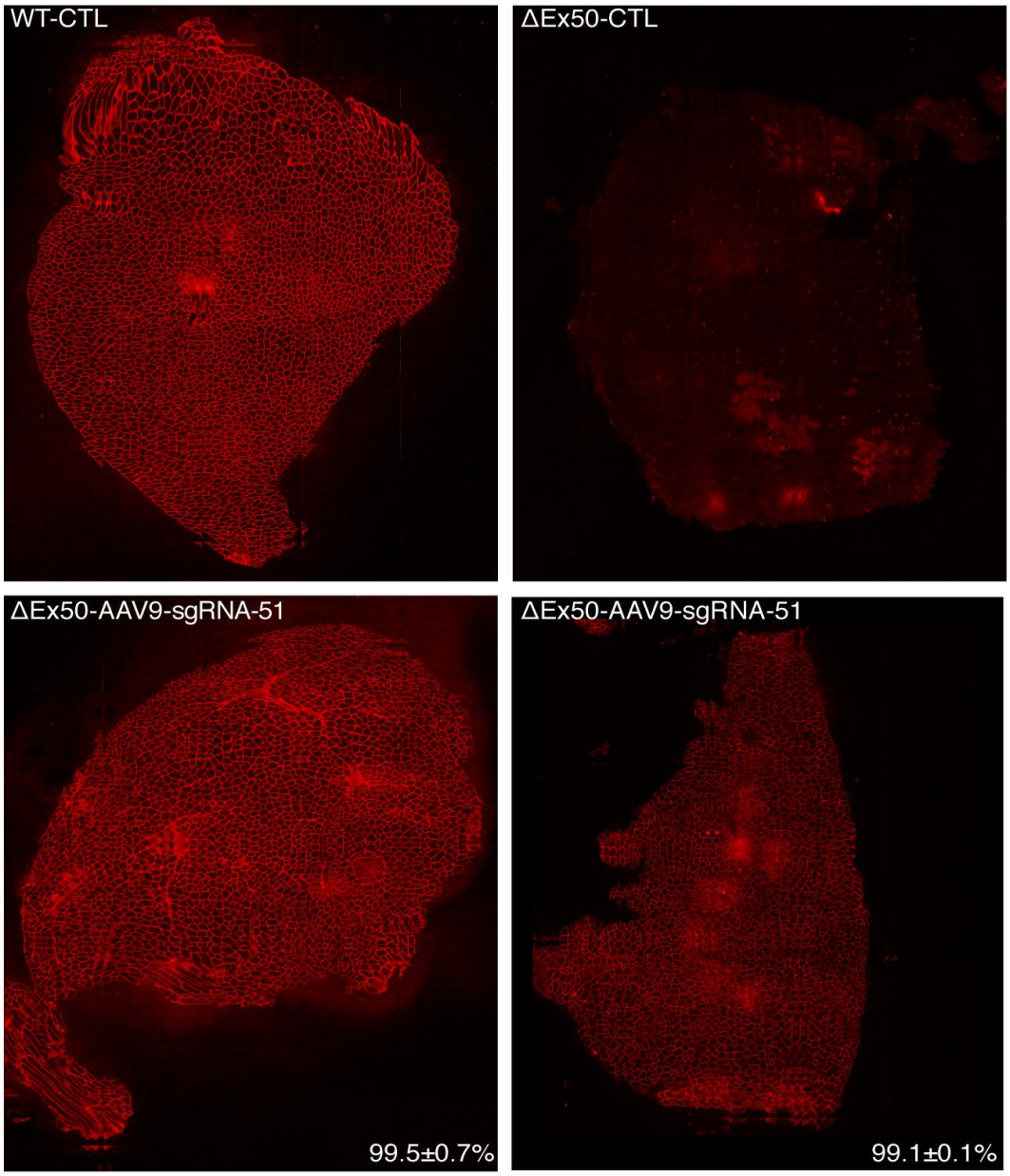


Fig S11. Rescue of dystrophin expression following intramuscular injections of AAV9-Cas9 and AAV9-sgRNA-51 in ΔEx50 mouse model. Dystrophin immunohistochemistry of entire tibialis anterior muscle. CTL mice were injected with AAV9-Cas9 alone without AAV9-sgRNA-51. n=5.

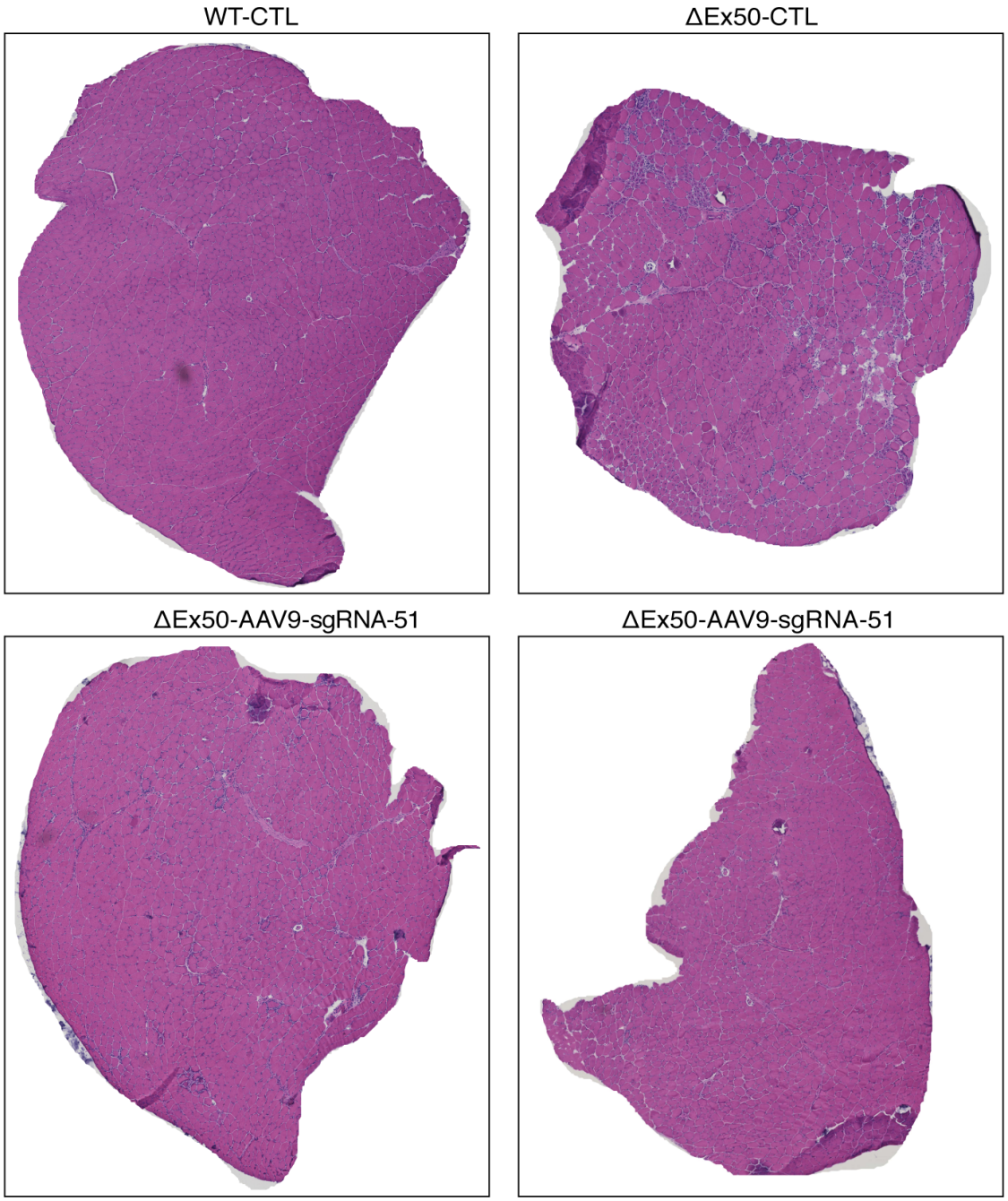


Fig S12. AAV9-Cas9 and AAV9- sgRNA-51-injected muscle show histological improvement after 3 weeks. Hematoxylin and eosin (H&E) staining of entire tibialis anterior muscle. CTL mice were injected with AAV9-Cas9 alone without AAV9-sgRNA-51. n=5

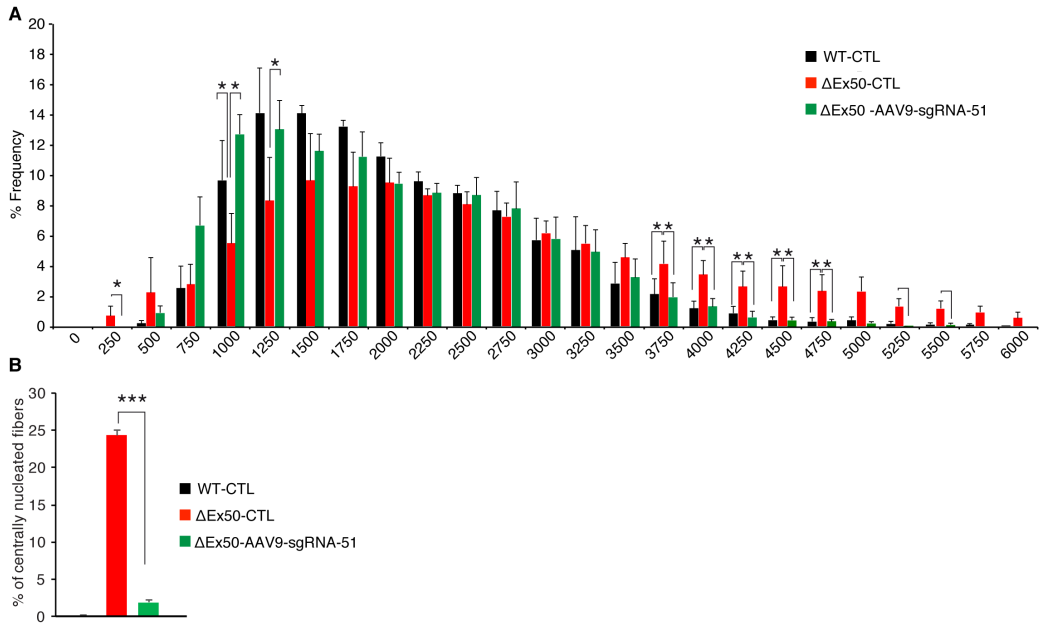


Fig S13. Quantification of histological improvement of AAV9-Cas9 and AAV9-sgRNA-51-injected muscle from ΔEx50 mice after 3 weeks. (**A**)Transverse muscle sections were analyzed for fiber area. Fiber size is grouped into 250 μm^2 intervals, and represented as the percentage of total fibers in each group. n=3. (**B**) Percentage of centrally nucleated fibers in tibialis anterior muscle of WT, ΔEx50 and ΔEx50-AAV-sgRNA-51 mice. CTL mice were injected with AAV9-Cas9 alone without AAV9-sgRNA-51. n=4. Data are represented as mean ± SEM. (*P<0.05, ***P<0.0005)

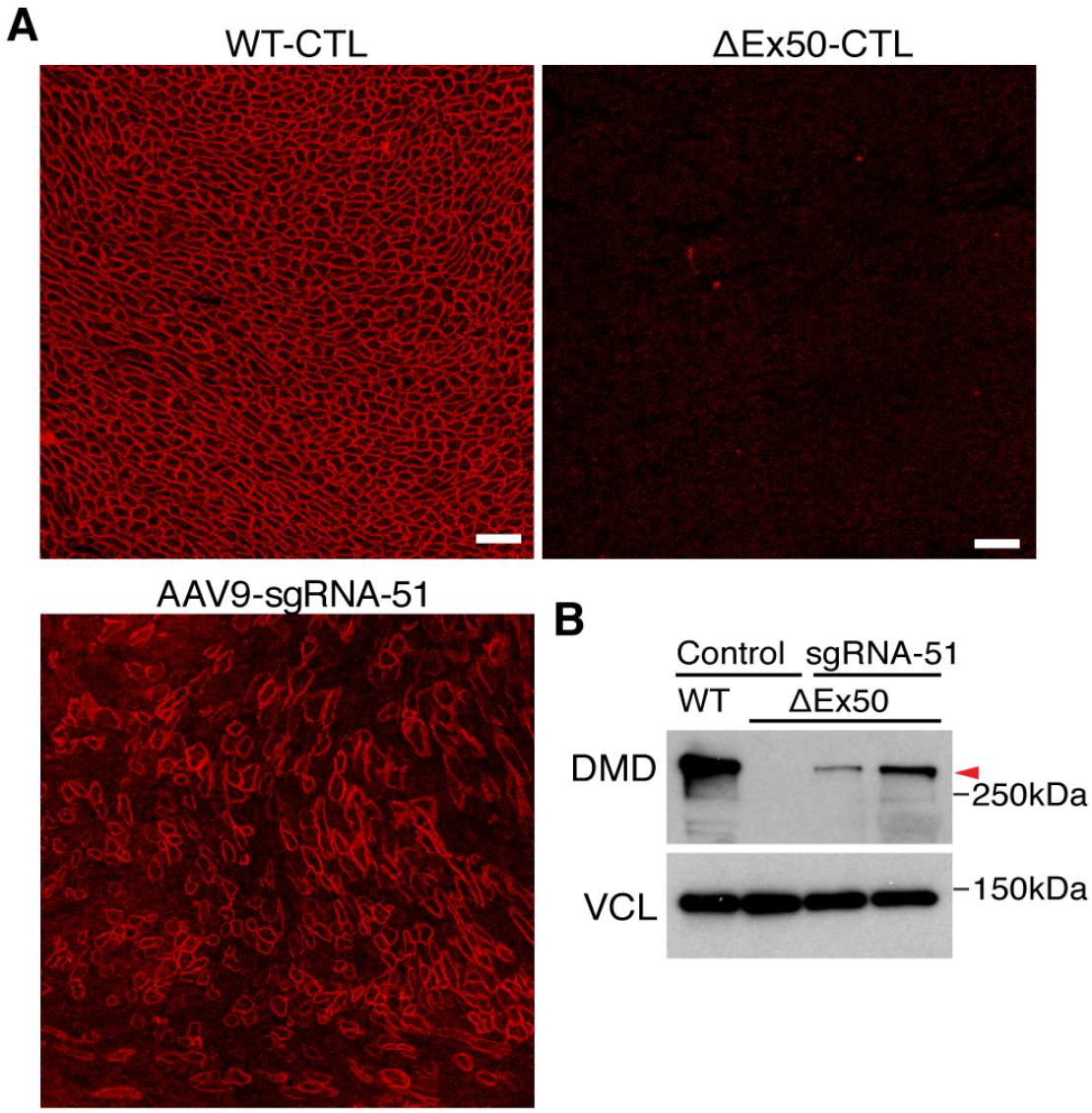


Fig S14. Intramuscular injections of AAV9-Cas9 and AAV9-sgRNA-51 in ΔEx50 mice corrects dystrophin expression in heart. (**A**) Dystrophin immunohistochemistry of cardiac muscle. CTL were injected with AAV9-Cas9 alone without AAV9-sgRNA-51. (**B**) Representative western blot analysis of dystrophin (DMD) and vinculin (VCL) expression in cardiac muscle of two mice for each group. n=5. Scale bar: 50μm.

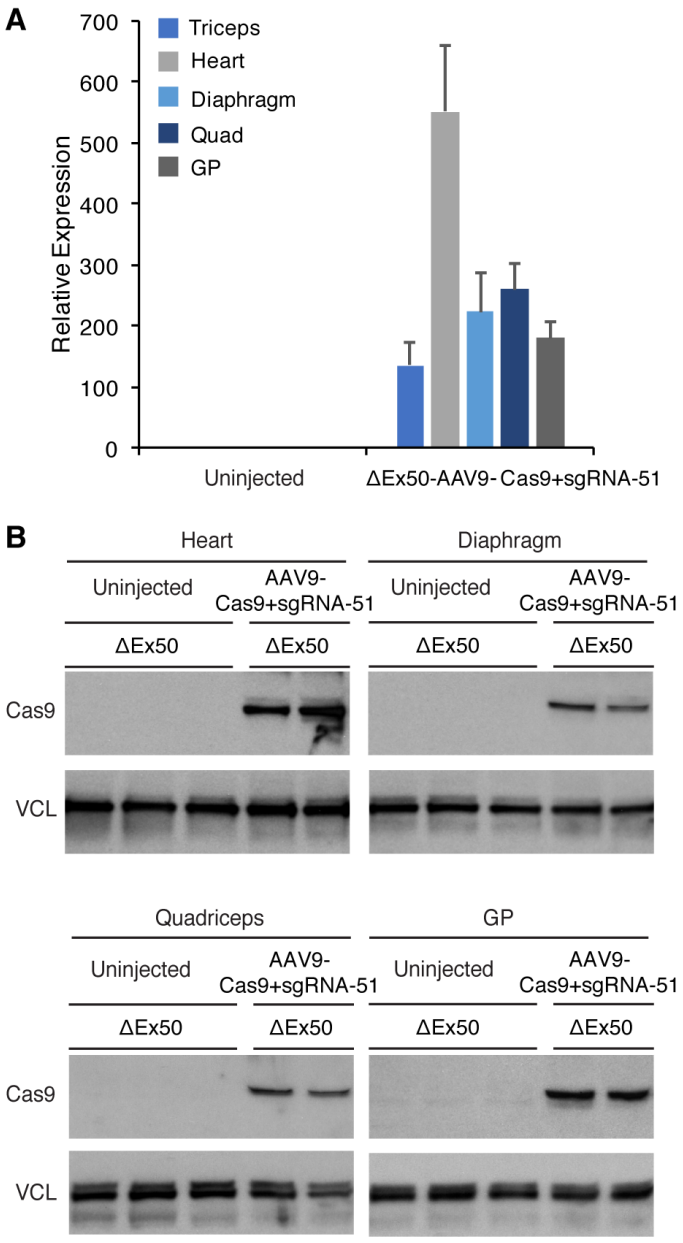
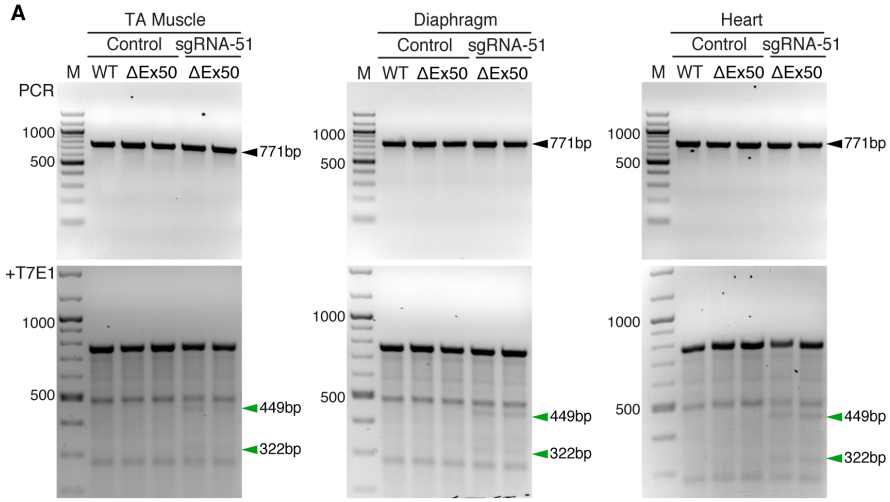


Fig S15. AAV9-Cas9 expression after systemic delivery in mice. (**A**) qPCR analysis of Cas9 expression in triceps, heart, diaphragm, quadriceps and gastrocnemius-plantaris (GP) tissues. n=5. (**B**) Western blot analysis of Cas9 and vinculin (VCL) expression in heart, diaphragm, quadriceps (Quad) and gastrocnemius-plantaris (GP) tissues 4 weeks after systemic injection.



B **Heart-DNA-ΔEx50-AAV9-sgRNA-51-#1**

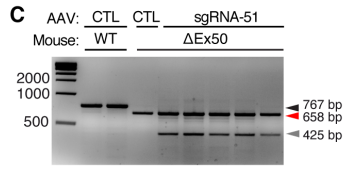
		%Reads	#Reads
	PAM ▼		
(NE)	acactagCTGCCAGTCAGACTGTTACTCTAGTGACA	76.46	37332
(+1)	acactagCTGCCAGTCAAGACTGTTACTCTAGTGACA	10.35	5052
(+1S1)	acactagCTGCCAGTCAAGACTTTTACTCTAGTGACA	0.61	300
(+2)	acactagCTGCCAGTCATAGACTGTTACTCTAGTGACA	0.30	148
(-4)	acactagCTGCCAGT----CTGTTACTCTAGTGACA	0.18	86
(S1)	acactagCTGCCAGTCATACTGTTACTCTAGTGACA	0.16	78
(+2)	acactagCTGCCAGTCACAGACTGTTACTCTAGTGACA	0.16	76
(S1)	acactagCTGCCATTCAGACTGTTACTCTAGTGACA	0.15	75
(-22)	ac----------ACTCTAGTGACA	0.11	54
(+13)	acactagCTGCCAGTCTAGTGTTTTTTTTAGACTGTTACTCTAGTGACA	0.10	47
(-10)	acactagCTGCCAGTCA----------CTAGTGACA	0.08	40
(-4)	acactagCTGC----CAGACTGTTACTCTAGTGACA	0.08	40

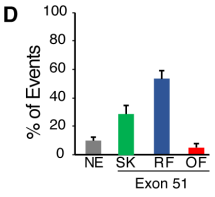
Heart-DNA-ΔEx50-AAV9-sgRNA-51-#2

		%Reads	#Reads
	PAM ▼		
(NE)	acactagCTGCCAGTCAGACTGTTACTCTAGTGACA	82.92	46216
(+1)	acactagCTGCCAGTCAAGACTGTTACTCTAGTGACA	5.36	2985
(S1)	acactagCTGCCAGTCAGACTTTTACTCTAGTGACA	0.75	420
(+2)	acactagCTGCCAGTCAAAGACTGTTACTCTAGTGACA	0.39	220
(S1)	acactagCTGCCAGTCAAACTGTTACTCTAGTGACA	0.19	104
(S1)	acactagCTGCCAGTCATACTGTTACTCTAGTGACA	0.18	99
(S2)	acactagCTGCCAGTCATAGACTGTTACTCTAGTGACA	0.13	73
(-4)	acactagCTGC----CAGACTGTTACTCTAGTGACA	0.11	60
(-4)	acactagCTGCCAGT----CTGTTACTCTAGTGACA	0.08	42
(-2)	acactagCTGCCAGTC--ACTGTTACTCTAGTGACA	0.07	39
(-12)	acactagCTGCCAG----------TCTAGTGACA	0.06	33
(-8)	acactagCTGCCA--------GTTACTCTAGTGACA	0.05	28

Heart-DNA-ΔEx50-AAV9-sgRNA-51-#3

		%Reads	#Reads
	PAM ▼		
(NE)	acactagCTGCCAGTCAGACTGTTACTCTAGTGACA	77.54	36255
(+1)	acactagCTGCCAGTCAAGACTGTTACTCTAGTGACA	8.05	3763
(+2)	acactagCTGCCAGTCAAAGACTGTTACTCTAGTGACA	0.95	444
(+2)	acactagCTGCCAGTCATAGACTGTTACTCTAGTGACA	0.72	337
(S1)	acactagCTGCCAGTCAGACTTTTACTCTAGTGACA	0.69	321
(+1S1)	acactagCTGCCAGTCAAGACTTTTACTCTAGTGACA	0.49	228
(-1)	acactagCTGCCAGT-AGACTGTTACTCTAGTGACA	0.28	129
(S1)	acactagCTGCCAGTAAGACTGTTACTCTAGTGACA	0.26	120
(+9)	acactagCTGCCAGTTACTCTGTTAGACTGTTACTCTAGTGACA	0.24	113
(+2)	acactagCTGCCAGTCACAGACTGTTACTCTAGTGACA	0.22	102
(-4)	acactagCTGC----CAGACTGTTACTCTAGTGACA	0.20	94
(+12)	acactagCTGCCAGTTACTGTTACTCTAGACTGTTACTCTAGTGACA	0.11	53





E **Heart-cDNA upper band**

		Average %reads
49 51	(+1) AAGCTGCCAGTCAAGACTGTTACTCTAGTGACACAATCTGTGGTTA	66.50±3.05
49 51	(NE) AAGCTGCCAGTCAGACTGTTACTCTAGTGACACAATCTGTGGTTA	24.89±7.53

Fig S16. *Dmd* gene editing 4 weeks after systemic delivery of AAV9-Cas9 and AAV9- sgRNA-51 in mice. (**A**) Undigested PCR products (upper panel) and T7E1 digestion (lower panel) are shown on a 2% agarose gel of tibialis anterior (TA), diaphragm and heart muscle samples from WT and ΔEx50 mice 4 weeks after systemic injection with AAV9-sgRNA-51 expression vectors and AAV-Cas9. CTL were injected with AAV9-Cas9 alone without AAV9-sgRNA-51. Black arrowheads in the upper panels indicate the 771bp PCR bands. Green arrowheads in the lower panels indicate the cut bands by T7E1 assay. M denotes size marker lane. bp indicates the length of the marker bands. n=4. (**B**) Genomic deep sequencing analysis of PCR amplicons generated across the exon 51 target site in heart DNA samples of ΔEx50 mice injected with AAV9-sgRNA-51 and AAV9-Cas9. Sequence of representative indels aligned with sgRNA sequence (indicated in blue) revealing insertions (highlighted in green) and deletions (highlighted in red). Black arrowheads indicate the cleavage site. (**C**) RT-PCR of RNA from heart samples of WT and ΔEx50 mice 4 weeks after systemic injection with the AAV9-sgRNA and Cas9 expression vectors. Lower bands indicate deletion of exon 51. Primer positions in exons 48 and 53 are indicated (Fw, Rv). (**D**) Percentage of events detected at exon 51 after AAV9-sgRNA-51 treatment using RT-PCR sequence analysis of TOPO-TA generated clones. For each of 4 different samples, we generated 40 clones. RT-PCR products we divided in 4 groups not-edited (NE), exon51-skipped (SK), reframed (RF) and out of frame (OF). (**E**) Deep sequencing analysis of RT-PCR products from the upper band containing ΔEx50 not-edited (NE) and ΔEx50-RF. Sequence of RT-PCR products revealing insertions (highlighted in green) and deletions (highlighted in red). n=4.

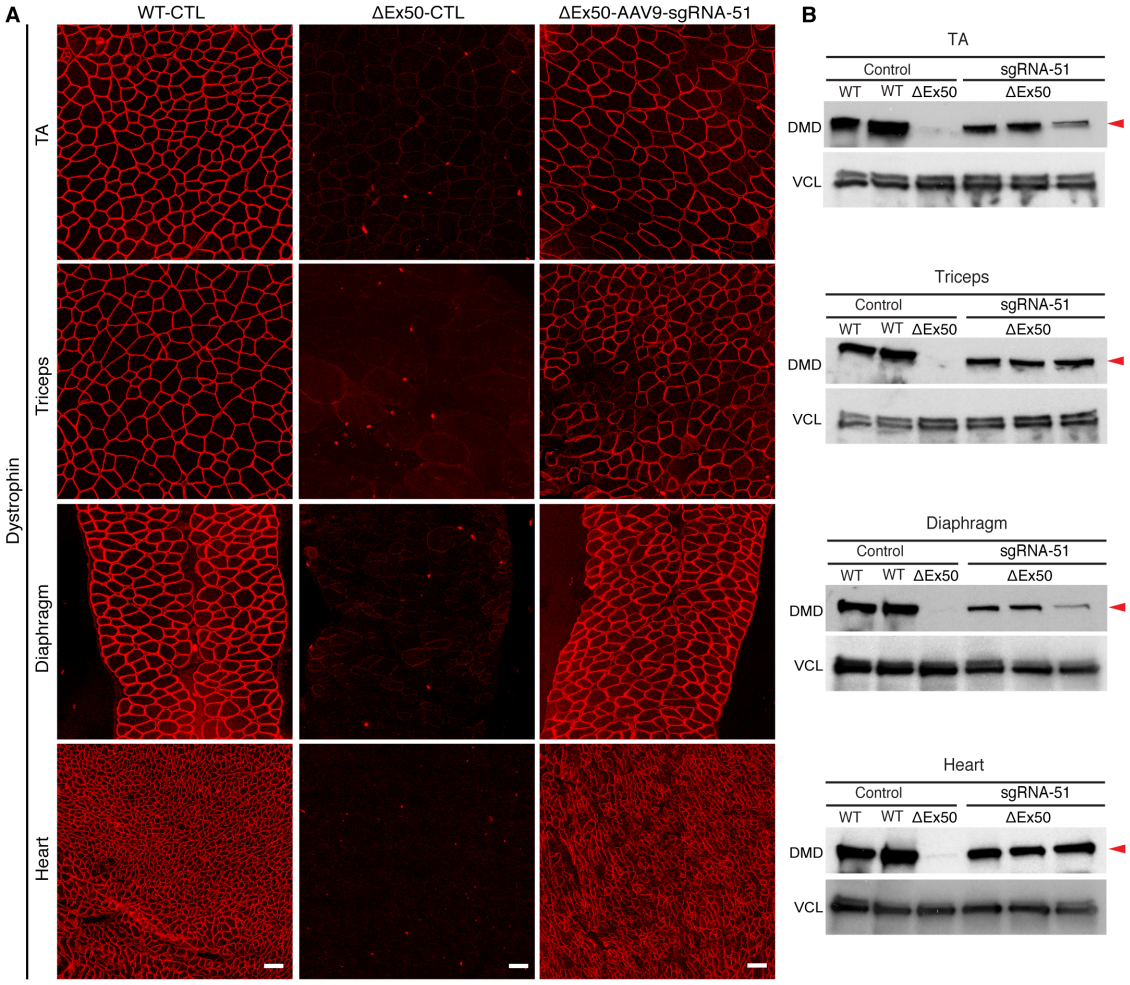


Fig S17. Rescue of dystrophin expression 8 weeks after systemic delivery of AAV9-Cas9 and AAV9-sgRNA-51 in ΔEx50 mice. (**A**) Dystrophin immunostaining of tibialis anterior (TA), triceps, diaphragm and cardiac muscles 8 weeks after systemic injection of AAV9-sgRNA-51. (**B**) Western blot analysis of dystrophin (DMD) and vinculin (VCL) expression in TA, triceps, diaphragm muscles and heart. n=5 for each group. Scale bar: 50μm.

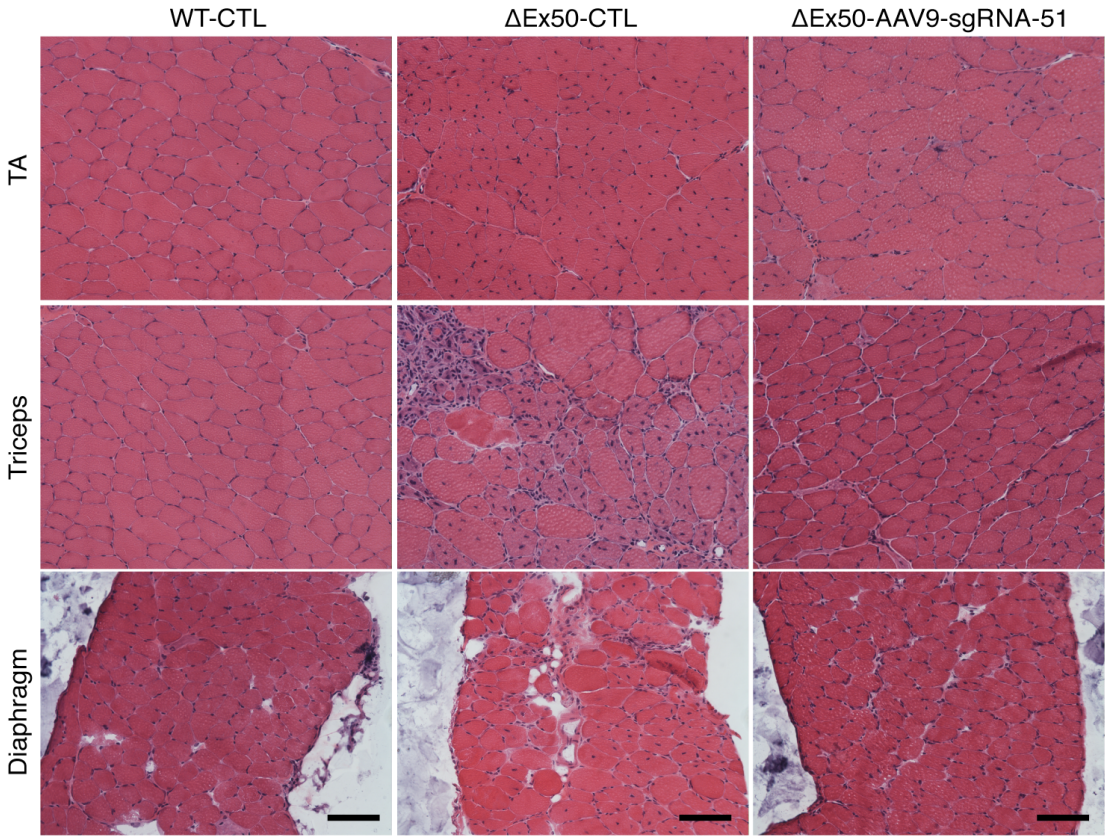


Fig S18. Histological analysis of dystrophin correction 8 weeks after systemic delivery of AAV9-Cas9 and AAV9-sgRNA-51 in ΔEx50 mice. Histochemistry of tibialis anterior (TA), triceps and diaphragm muscle by H&E staining. n=3. Scale bar: 50μm.

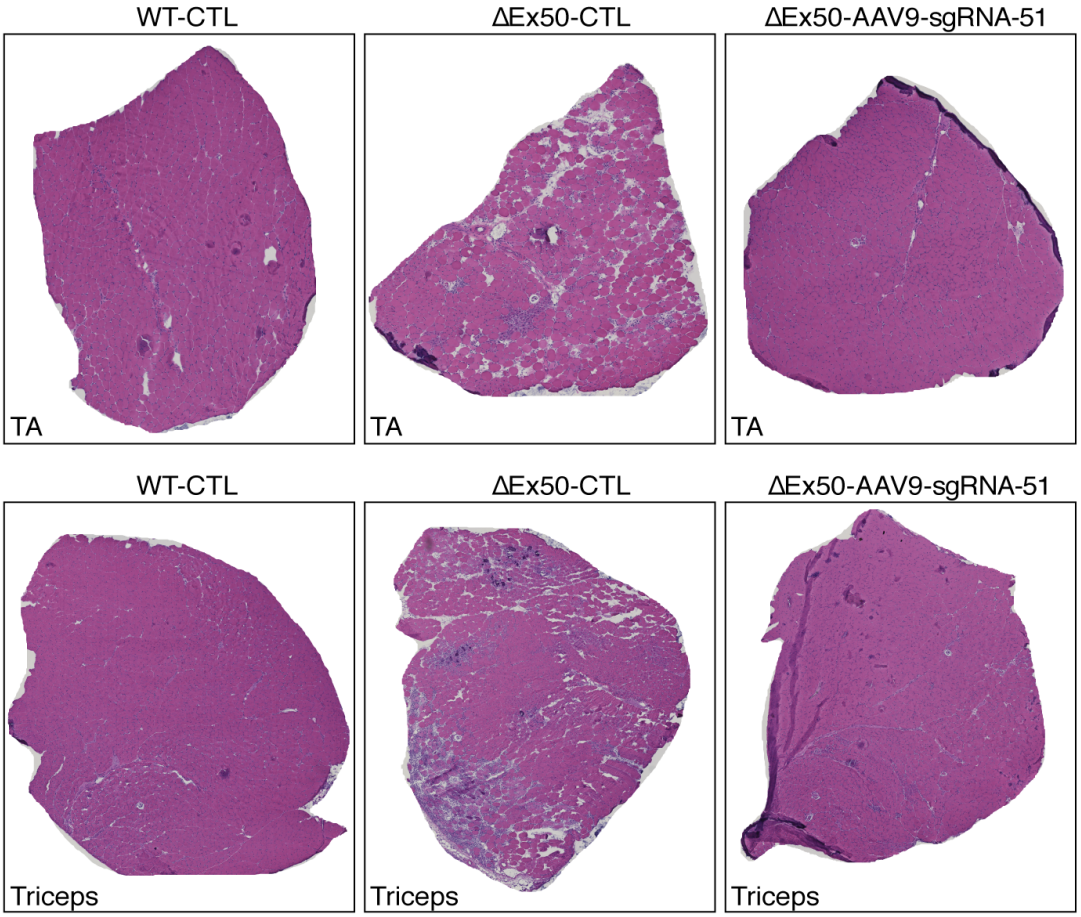


Fig S19. Histological improvement of ΔEx50 mice 4 weeks after systemic injection of AAV9-Cas9 and AAV9-sgRNA-51. Hematoxylin and eosin (H&E) staining of entire tibialis anterior (TA) and triceps muscles. CTL were injected with AAV9-Cas9 alone without sgRNA. n=5

Supplementary Tables

Table S1. Sequences of potential exonic off-target (OT) sites in the mouse genome.

	All exonic off target sites sequence	Score	Mismatches	UCSC gene	Locus
1	CCCTCGACTAACCGTCTGACTGG	0.6	4MMs [2:5:8:13]	NM_016926	chr5:+114194829
2	CCATAGAGAACCAGTCTGACAAG	0.5	4MMs [2:3:9:11]	NM_172880	chr5:+87174896
3	CACTTGACTATCAGGCTGACTGG	0.2	4MMs [5:8:11:15]	NM_028809	chr2:+38870861
4	CACTAAACCAACAGTCTGTCAGG	0.2	4MMs [6:8:9:19]	NR_040363	chr8:-113667574
5	CAATAGGGTATCAATCTGACCAG	0.1	4MMs [3:7:11:14]	NM_172951	chr12:-30859804
6	CACTAGAGGAACAGAGTCACTGG	0	4MMs [9:15:16:18]	NM_009502	chr14:+21851627

Mismatches in the target sequence are highlighted in red.

Table S2. Sequences of top 45 off-target (OT) sites in the mouse genome.

#	Sequence	Score	Mismatches	UCSC gene	Locus
1	GACTTTAGTAACAGTCTGACAGG	1.5	3MMs [1:5:6]		chr5:+12910014
2	AACAGGTGTAACAGTCTGACGGG	0.9	4MMs [1:4:5:7]		chr6:-39148523
3	CACGAGAGCACCAGTCTGACTGG	0.9	3MMs [4:9:11]		chr4:-139770221
4	ACCTAGAAGAACAGTCTGACTAG	0.9	4MMs [1:2:8:9]		chr4:+31992687
5	GAAAAGAGAAACAGTCTGACAGG	0.8	4MMs [1:3:4:9]		chr6:+93420151
6	GACTTGAGCCACAGTCTGACCGG	0.8	4MMs [1:5:9:10]		chr19:+58410134
7	CTCCACAGTTACAGTCTGACCAG	0.8	4MMs [2:4:6:10]		chr4:-132987789
8	CACAGGAGAGACAGTCTGACAGG	0.8	4MMs [4:5:9:10]		chr14:+47694929
9	CACAAGACTCAAAGTCTGACAAG	0.6	4MMs [4:8:10:12]		chr3:-19123041
10	CCCCAGGGAAACAGTCTGACAGG	0.6	4MMs [2:4:7:9]		chr11:-66106190
11	CCCTCGACTAACCGTCTGACTGG	0.6	4MMs [2:5:8:13]	NM_016926	chr5:+114194829
12	AATTAGAGTGACAGTCAGACCAG	0.6	4MMs [1:3:10:17]		chr2:+134267774
13	GGCTAGAGTTACGGTCTGACTGG	0.5	4MMs [1:2:10:13]		chr1:+137500176
14	CTCAAGATTAACAGTCTGGCAGG	0.5	4MMs [2:4:8:19]		chr8:+89915056
15	TAGTAGAGAAGCAGTCTGACTAG	0.5	4MMs [1:3:9:11]		chr11:-51429578
16	AGCTAGGGTAACAGTCTGAATAG	0.5	4MMs [1:2:7:20]		chrX:+123650835
17	TCCTAGGGTAACAGTCTGAACAG	0.5	4MMs [1:2:7:20]		chr1:-195484703
18	CCATAGAGAACCAGTCTGACAAG	0.5	4MMs [2:3:9:11]	NM_172880	chr5:+87174896
19	AACTACAGTGTCAGTCTGACAGG	0.4	4MMs [1:6:10:11]		chr12:-41095983
20	AACTGAAGTAACAGTCTGAATGG	0.4	4MMs [1:5:6:20]		chr10:+67936212
21	CACAAAAGTGGCAGTCTGACAGG	0.4	4MMs [4:6:10:11]		chr13:+113568808
22	GCCTAGATTAACAGGCTGACGGG	0.4	4MMs [1:2:8:15]		chr11:+8453241
23	CACTCGTGTCACAGTCTGAATAG	0.4	4MMs [5:7:10:20]		chr15:+3352089
24	GACTATAGTCATAGTCTGACTGG	0.4	4MMs [1:6:10:12]		chr14:+123509659
25	TACTACAGTTAAAGTCTGACCAG	0.4	4MMs [1:6:10:12]		chr1:-39197660
26	CCATAGTGTAACTGTCTGACTAG	0.4	4MMs [2:3:7:13]		chr12:+87163814
27	AAGTAGAGTGACAGGCTGACAGG	0.4	4MMs [1:3:10:15]		chr3:-70665468
28	CCCCAGAGAAACAGTCAGACAGG	0.4	4MMs [2:4:9:17]		chr14:-17330765
29	CACTGAAGTTACAGTCTGATCGG	0.4	4MMs [5:6:10:20]		chr10:+117084662
30	CACAAGAAAAACTGTCTGACAAG	0.3	4MMs [4:8:9:13]		chr18:+74824438
31	CAATACAGTCACAGTCAGACAAG	0.3	4MMs [3:6:10:17]		chr17:-38068956
32	CAGTAGAGTCAAAGTCTGAGGAG	0.3	4MMs [3:10:12:20]		chr9:-100294992
33	AAGTAGAGTAAGAGTCAGACAAG	0.3	4MMs [1:3:12:17]		chr1:+170850566
34	CACTGGAGTCAGAGTCTGATCGG	0.3	4MMs [5:10:12:20]		chr18:-39033098
35	GACTAGAGCCACAGTCTGTCCGG	0.3	4MMs [1:9:10:19]		chr12:-8868558
36	CACTGTAGTTACAGTCTGGCTAG	0.3	4MMs [5:6:10:19]		chr6:+14422702
37	CACTAGGCTAACATTCTGACAAG	0.3	3MMs [7:8:14]		chr8:+65459205
38	CCCTAGACTAAGAGTCTGGCAAG	0.3	4MMs [2:8:12:19]		chr9:+7157367
39	CCCTAGACTAAGAGTCTGGCAAG	0.3	4MMs [2:8:12:19]		chr9:+7157311
40	AACTAGAGTGACTGTCTGATTAG	0.3	4MMs [1:10:13:20]		chr19:-4529462
41	CACAAGAGTAATAGTGTGACAGG	0.3	3MMs [4:12:16]		chr8:+20011535
42	CACAAGAGTAATAGTGTGACAGG	0.3	3MMs [4:12:16]		chr8:-19793456
43	CACAAGAGTAATAGTGTGACAGG	0.3	3MMs [4:12:16]		chr8:+19884248
44	CTCCACAGTAACAGGCTGACGAG	0.2	4MMs [2:4:6:15]		chr19:+23293758
45	CAGTGGAGGAACAGACTGACGAG	0.2	4MMs [3:5:9:15]		chr7:-73086063

Table S3. Primer sequences.

Experiment	Primer Name	Primer sequence
sgRNA for exon 50 deletion	*Dmd* ex50-g1-Top	5'-CACCGAAATGATGAGTGAAGTTATAT-3'
	Dmd ex50-g1-Bottom	5'-AAACATATAACTTCACTCATCATTTC-3'
	Dmd ex50-g2-Top	5' CACCGGTTTGTTCAAAAGCGTGGCT-3'
	Dmd ex50-g2-Bottom	5'- AAACAGCCACGCTTTTGAACAAAC-3'
T7 in vitro transcription primers	*Dmd* ex50-T7-F1	5'-GAATTGTAATACGACTCACTATAGGAATGATGAGTGAAGTTATAT-3'
	Dmd ex50-T7-F2	5'-GAATTGTAATACGACTCACTATAGGGTTTGTTCAAAAGCGTGGCT-3'
	Dmd ex50-T7-Rv	5'-AAAAGCACCGACTCGGTGCCAC-3'
Genotyping of Δ Ex50 mice	Geno-ex50-F	5'-GGATTGACTGAAATGATGGCCAAGG-3'
	Geno-ex50-R	5'-CTGCCACGATTACTCTGCTTCCAG-3'
sgRNA for mouse exon 51	*Dmd* M-ex51-Top	5'-CACCGCACTAGAGTAACAGTCTGAC-3'
	Dmd M-ex51-Bottom	5'-AAACCCAGTCAGACTGTTACTCTC-3'
sgRNA for human exon 51	*Dmd* H-ex51-Top	5'-CACCGCACCAGAGTAACAGTCTGAG-3'
	Dmd H-ex51-Bottom	5'-AAACCTCAGACTGTTACTCTGGTGC-3'
Primers for T7E1 mouse exon51	*Dmd* M-ex51-T7E1-F	5'-GATAAATCCTGAAAATTCCCCAATA -3'
	Dmd M-ex51-T7E1-R	5'-TTTCACCCTAACTTTTCATCCCTT-3'
Primers for T7E1 human exon51	*Dmd* H-ex51-T7E1-F	5'-TTCCCTGGCAAGGTCTGA -3'
	Dmd H-ex51-T7E1-R	5'-ATCCTCAAGGTCACCCACC-3'
RT-PCR primer flanking exon 48 and exon 53	*Dmd*-ex48-F	5'- AGGTTCACTTAAAAGATTTTAGGCAG-3'
	Dmd-ex52-R	5'-TAACATTTCATTCAACTGTTGTCTCC-3'
Primers for exonic off-targets sites of sgRNA-SA	OT1-T7E1-F	5'-GAGAAACACAAACTCTTCATCTCTG -3'
	OT1-T7E1-R	5'-CTGACTCTGAGTCTCAGCAATG-3'
	OT2-T7E1-F	5'-TTCCAACACAGGCTTATAGTTAATTC -3'
	OT2-T7E1-R	5'-TACATCACTGTCTCCTTTACATTTTG-3'
	OT3-T7E1-F	5'-AGGCTCCATTATAAGAGTTCTTACAG -3'
	OT3-T7E1-R	5'-GAAAAATAAACACTCAGCAAAAGGTC-3'
	OT4-T7E1-F	5'-TTAAAGCCAAACAGAAGAAACTCTG -3'
	OT4-T7E1-R	5'-CCTCAGTCGCTGTAATAATTAAAGTC-3'
	OT5-T7E1-F	5'-TCTTAAGAAACTAATCTTTCCTGCAC -3'
	OT5-T7E1-R	5'-ATTCAAATTCCCTCTTCACTCTATTC-3'
	OT6-T7E1-F	5'-TCTCTCTATAAACCCTGTACTTTCAG -3'
	OT6-T7E1-R	5'-ACTTTTACACTAACTTTGCCCAATAG-3'
Primers for Mi-Seq Genomic DNA Amplicon Deep Sequencing	*Dmd* M-ex51-Mi-seq-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAAATTTTACCTCAAACTGTTGCTTC-3'
	Dmd M-ex51-Mi-seq-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATGAATAGTGTGTTCACAGAGAAAAG-3'
	OT1-Mi-seq-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTCAGGCTGGTCACTAACAG-3'
	OT1-Mi-seq-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTGATGACATTCTCTCTGATGGTC-3'
	OT2-Mi-seq-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGAGGCTGAGAGGCATAACTC-3'
	OT2-Mi-seq-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAACAGTCAGATATAGATCCTTGAGTG-3'
	OT3-Mi-seq-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGTTACCTTGAGAAGAATTTTGGATG-3'
	OT3-Mi-seq-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGTCTGTACAAACTCTCAGGTTTTTC-3'
	OT5-Mi-seq-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCTTAAGAAACTAATCTTTCCTGCAC-3'
	OT5-Mi-seq-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTCAAATTCCCTCTTCACTCTATTC-3'
	Dmd H-ex51-Mi-seq-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTTAACTTAAGTTACTTGTCCAGGC-3'
	Dmd H-ex51-Mi-seq-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTTATAACTTGATCAAGCAGAGAAAGC-3'
Primers for Mi-Seq cDNA Amplicon Deep Sequencing	*Dmd* M-ex51-Mi-seq-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAAGTGGAAATTTATAACCAACCAAGTCAG-3'
	Dmd M-ex51-Mi-seq-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCGATCAGTAATGATTGTTCTAGCTTC-3'
Primers for Mi-Seq for PCR with Bar Codes	Univ-F	5'-AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTC-3'
	BC1-R	5'-CAAGCAGAAGACGGCATACGAGATACATCGGTCTCGTGGGCTCGG-3'
	BC2-R	5'-CAAGCAGAAGACGGCATACGAGATTGGTCAGTCTCGTGGGCTCGG-3'
	BC3-R	5'-CAAGCAGAAGACGGCATACGAGATCACTGTGTCTCGTGGGCTCGG-3'
	BC4-R	5'-CAAGCAGAAGACGGCATACGAGATATTGGCGTCTCGTGGGCTCGG -3'
	BC5-R	5'-CAAGCAGAAGACGGCATACGAGATGATCTGGTCTCGTGGGCTCGG-3'
	BC6-R	5'-CAAGCAGAAGACGGCATACGAGATTACAAGGTCTCGTGGGCTCGG-3'
	BC7-R	5'-CAAGCAGAAGACGGCATACGAGATCGTGATGTCTCGTGGGCTCGG-3'
	BC8-R	5'-CAAGCAGAAGACGGCATACGAGATGCCTAAGTCTCGTGGGCTCGG-3'
	BC9-R	5'-CAAGCAGAAGACGGCATACGAGATTCAAGTGTCTCGTGGGCTCGG-3'
	BC10-R	5'-CAAGCAGAAGACGGCATACGAGATAGCTAGGTCTCGTGGGCTCGG-3'
	BC11-R	5'-CAAGCAGAAGACGGCATACGAGATGTCGTCGTCTCGTGGGCTCGG-3'
	BC12-R	5'-CAAGCAGAAGACGGCATACGAGATCGATTAGTCTCGTGGGCTCGG-3'
	BC13-R	5'-CAAGCAGAAGACGGCATACGAGATGAATGAGTCTCGTGGGCTCGG-3'
	BC14-R	5'-CAAGCAGAAGACGGCATACGAGATCTTCGAGTCTCGTGGGCTCGG-3'
	BC15-R	5'-CAAGCAGAAGACGGCATACGAGATCTCTACGTCTCGTGGGCTCGG-3''
	BC16-R	5'-CAAGCAGAAGACGGCATACGAGATAGGAATGTCTCGTGGGCTCGG-3'