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Supplementary Materials for

Enhanced CRISPR-Cas9 correction of Duchenne muscular dystrophy in mice by a self-complementary AAV delivery system

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Supplementary Materials

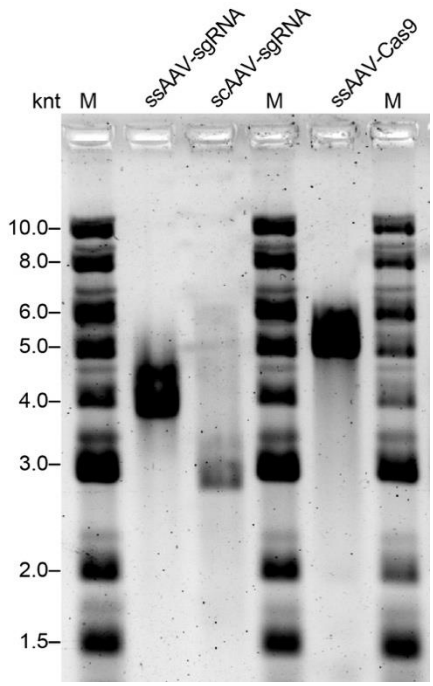

Fig. S1. Alkaline denaturing gel electrophoresis confirms integrity of AAV vectors. The viral genomes of the Cas9 vector and sgRNA vectors were analyzed by gel electrophoresis under alkaline denaturing conditions. The size of ssAAV-sgRNA and ssAAV-Cas9 is 3.9 and 5.1 knt, respectively, and remains unchanged after alkaline gel electrophoresis. The size of scAAV-sgRNA is 1.4 kilo-base pair and is doubled to 2.8 knts under denaturing conditions, indicating its double-stranded viral genome. M, marker; knt, kilo-nucleotides.

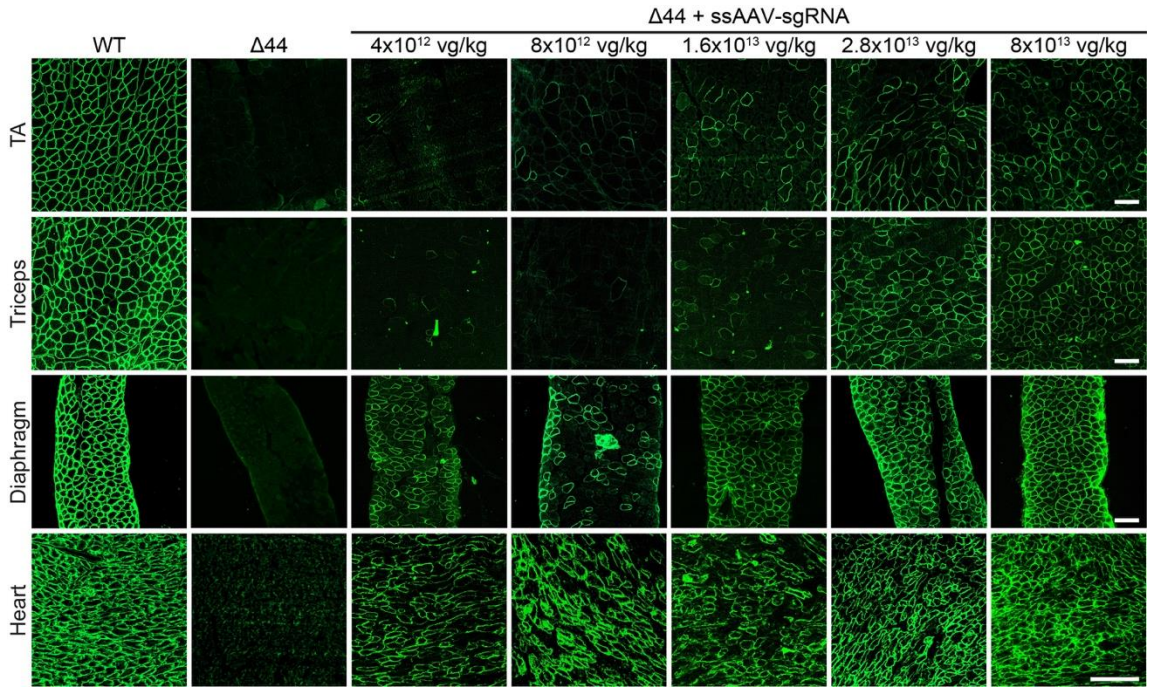

Fig. S2. Systemic delivery of CRISPR-Cas9 genome editing components by single-stranded AAV vector to ΔEx44 mice rescues dystrophin expression. Immunohistochemistry shows restoration of dystrophin in tibialis anterior (TA), triceps, diaphragm, and heart of ΔEx44 mice 4 weeks after systemic delivery of ssAAV-packaged *Sp*Cas9 and ssAAV-packaged sgRNA. *Sp*Cas9 vector was kept at constant dose of 8×10^{13} vg/kg. The dose of sgRNA vector was shown in the figure. Dystrophin is shown in green. n = 5 for each muscle group. Scale bar, 100 μm.

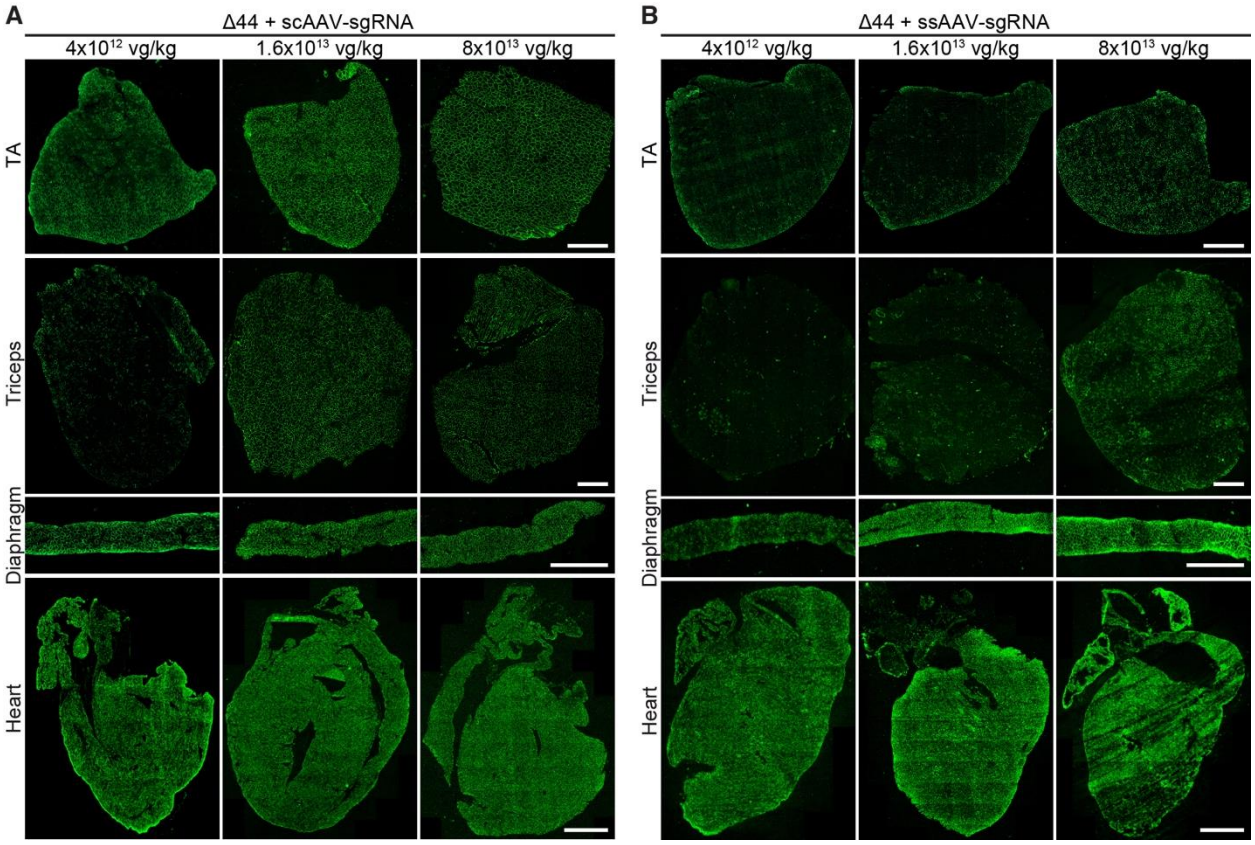

Fig. S3. Whole muscle scanning of immunohistochemistry of TA, triceps, diaphragm, and heart of CRISPR-Cas9–corrected ΔEx44 mice. (**A** and **B**) Whole muscle scanning of TA, triceps, diaphragm, and heart of ΔEx44 mice 4 weeks after systemic delivery of ssAAV-packaged *Sp*Cas9 and scAAV-packaged sgRNA (A) or ssAAV-packaged sgRNA (B). *Sp*Cas9 vector was kept at constant dose of 8×10^{13} vg/kg. The dose of sgRNA vector was shown in the figure. Dystrophin is shown in green. n = 5 for each muscle group. Scale bar in TA, triceps, diaphragm is 500 μm, in heart is 1.5mm.

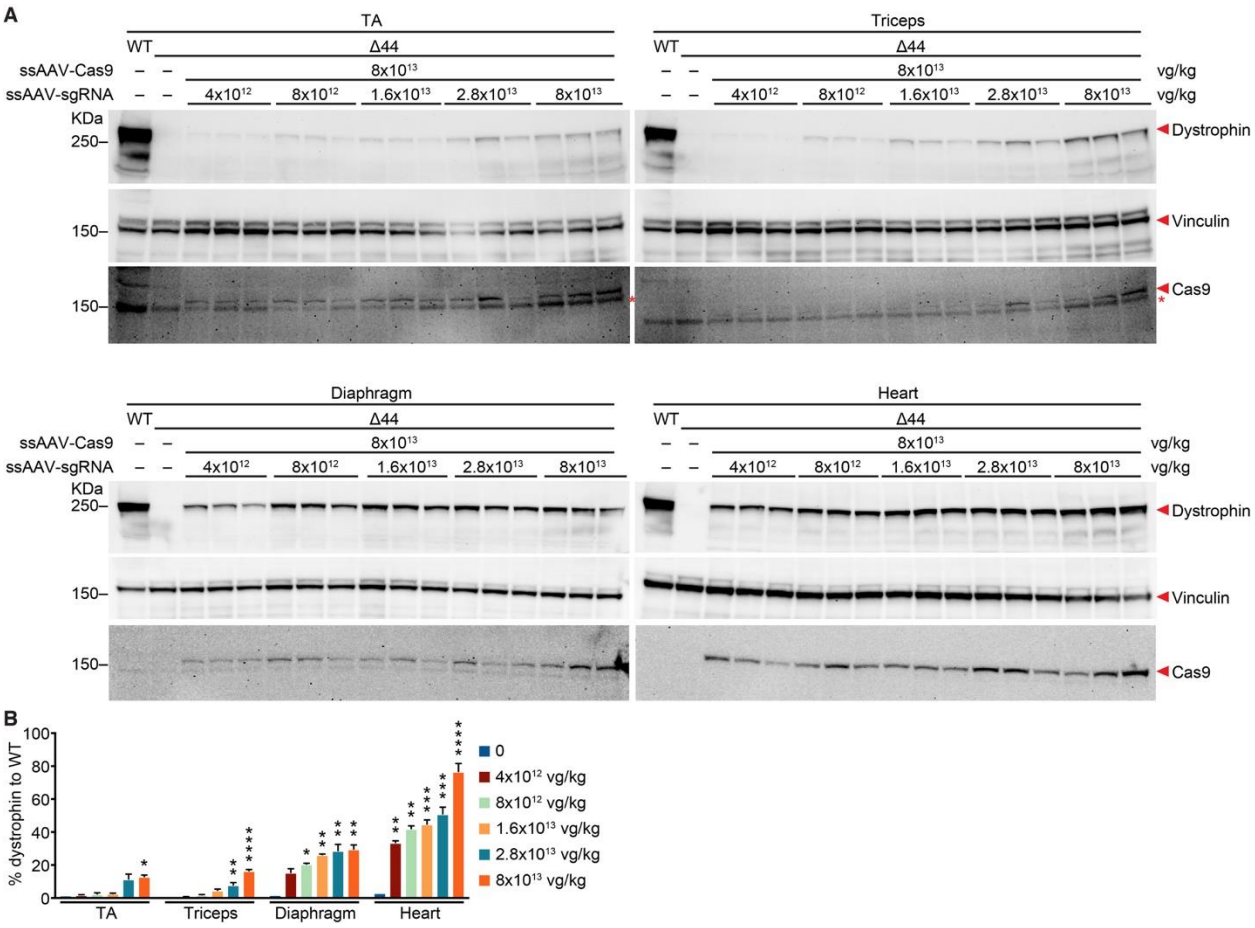

Fig. S4. Western blot analysis of skeletal muscles and heart of ΔEx44 mice treated with ssAAV-packaged CRISPR-Cas9 genome editing components. (**A**) Western blot analysis shows restoration of dystrophin expression in the TA, triceps, diaphragm, and heart of ΔEx44 mice 4 weeks after systemic delivery of ssAAV-packaged *Sp*Cas9 and ssAAV-packaged sgRNA. *Sp*Cas9 vector was kept at constant dose of 8×10^{13} vg/kg. The dose of sgRNA vector was shown in the figure. Vinculin was used as the loading control (n = 3). Red asterisks indicates non-specific band. (**B**) Quantification of dystrophin expression in TA, triceps, diaphragm, and heart. Relative dystrophin intensity was calibrated with vinculin internal control before normalizing to the WT control. Data are represented as mean ± SEM. One-way ANOVA was performed with post-hoc Tukey's multiple comparisons test. *P<0.05, **P<0.005, ***P<0.001, ****P<0.0001 (n=3).

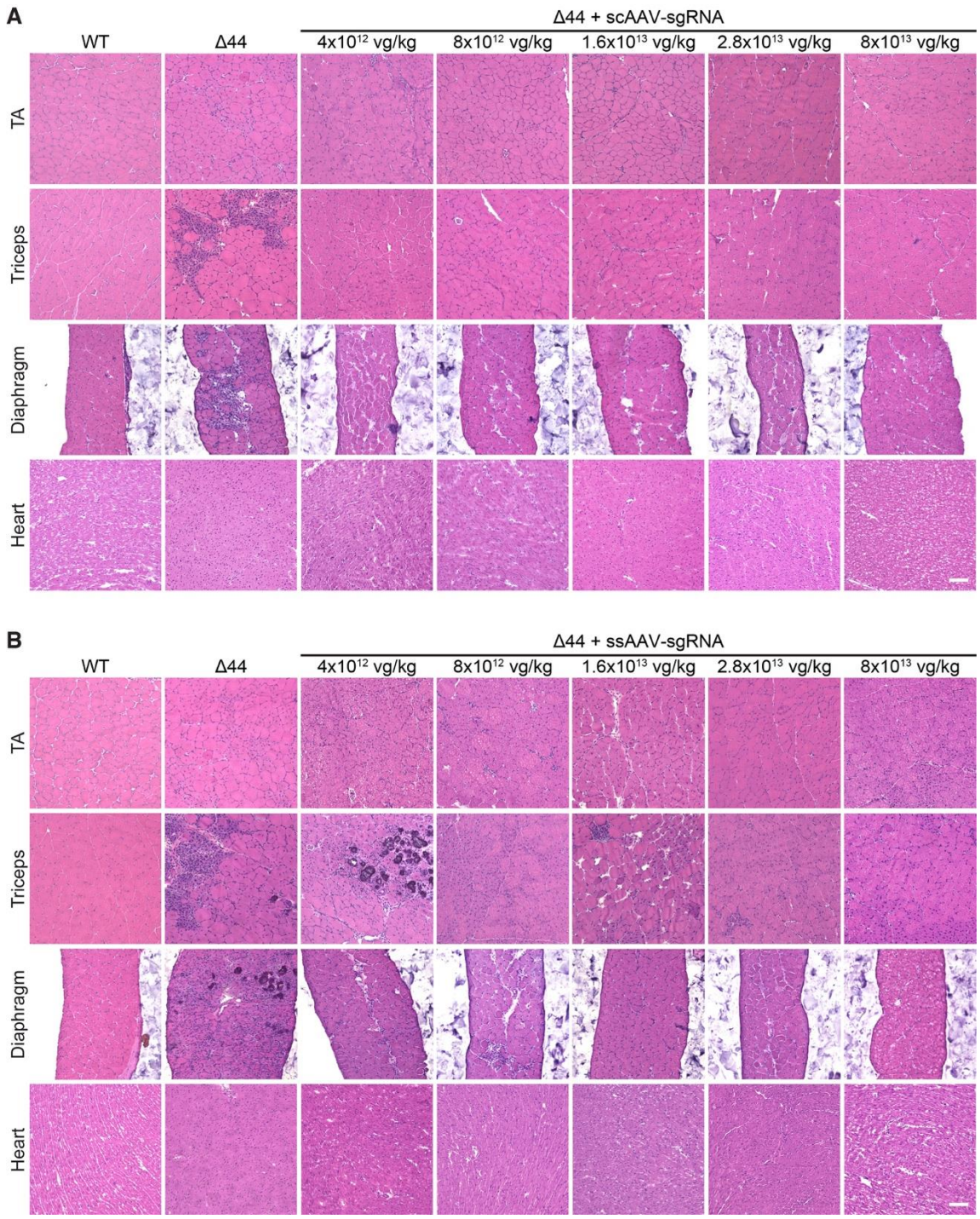

Fig. S5. Muscle histology of ΔEx44 mice after systemic delivery of AAV expressing CRISPR-Cas9 genome editing components. (**A** and **B**) H&E staining of TA, triceps, diaphragm, and heart of ΔEx44 mice 4 weeks after systemic delivery of ssAAV-packaged *Sp*Cas9 and scAAV-packaged sgRNA (A) or ssAAV-packaged sgRNA (B). *Sp*Cas9 vector was

kept at constant dose of 8×10^{13} vg/kg. The dose of sgRNA vector was shown in the figure. n = 5 for each muscle group. Scale bar, 100 μm.

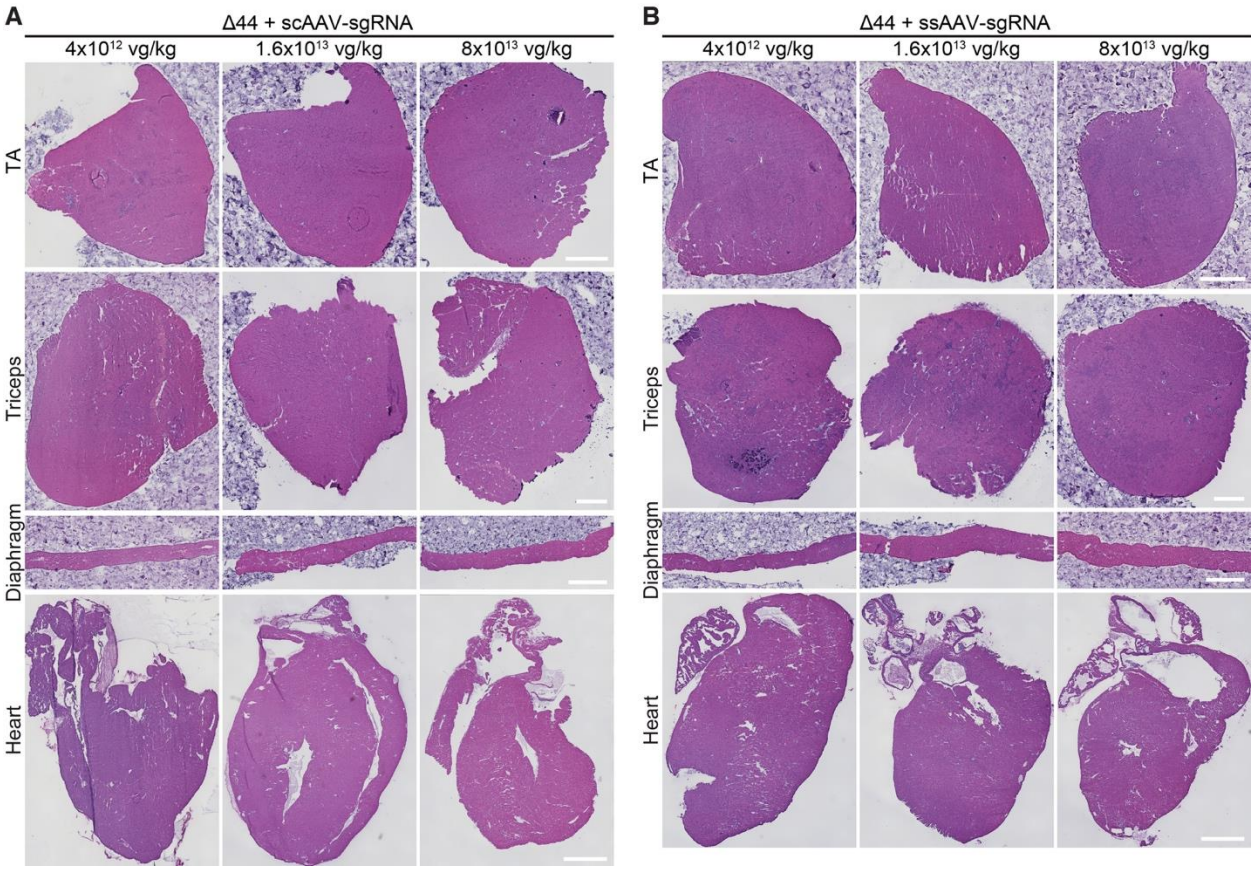

Fig. S6. Whole-muscle scanning of H&E staining of TA, triceps, diaphragm, and heart of CRISPR-Cas9–corrected ΔEx44 mice. (**A** and **B**) Whole muscle scanning of H&E staining of TA, triceps, diaphragm, and heart of ΔEx44 mice 4 weeks after systemic delivery of ssAAV-packaged *Sp*Cas9 and scAAV-packaged sgRNA (A) or ssAAV-packaged sgRNA (B). *Sp*Cas9 vector was kept at constant dose of 8×10^{13} vg/kg. The dose of sgRNA vector was shown in the figure. n = 5 for each muscle group. Scale bar in TA, triceps, diaphragm is 500 μm, in heart is 1.5mm.

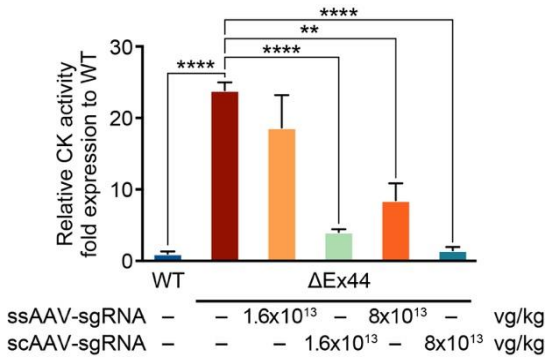

Fig. S7. Serum CK analysis of CRISPR-Cas9–corrected ΔEx44 mice. Serum CK was measured in WT, ΔEx44 mice untreated, and ΔEx44 mice 4 weeks after treatment with ssAAV-packaged *Sp*Cas9 and scAAV or ssAAV-packaged sgRNA. *Sp*Cas9 vector was kept at constant dose of 8×10^{13} vg/kg. The dose of sgRNA vector was shown in the figure. Serum CK was normalized to WT mice and shown as fold expression. Data are represented as mean ± SEM. One-way ANOVA was performed with post-hoc Tukey's multiple comparisons test. **P<0.005, ****P<0.0001 (n=5).

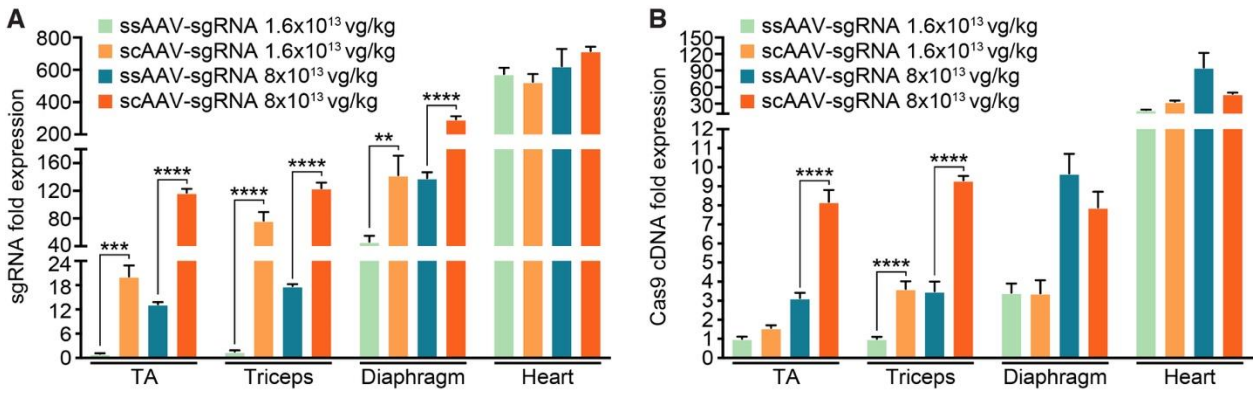

Fig. S8. ΔEx44 mice express more Cas9 and sgRNA transcripts after systemic delivery of scAAV-packaged sgRNA. (**A** and **B**) sgRNA cDNA transcripts (A) and Cas9 cDNA transcripts (B) were detected by quantitative RT-PCR from skeletal muscles and heart of ΔEx44 mice 4 weeks after systemic delivery of ssAAV-packaged *Sp*Cas9 and scAAV or ssAAV-packaged sgRNA. *Sp*Cas9 vector was kept at constant dose of 8×10^{13} vg/kg. The dose of sgRNA vector was shown in the figure. Both sgRNA (A) and Cas9 (B) cDNA transcripts were calibrated with 18s internal control before normalizing to the muscle group with lowest sgRNA transcripts. In both analyses, TA muscle treated with a low dose of ssAAV-packaged sgRNA (1.6×10^{13} vg/kg) had the lowest sgRNA and Cas9 cDNA transcripts compared to other muscle groups. Data are represented as mean ± SEM. One-way ANOVA was performed with post-hoc Tukey's multiple comparisons test. **P<0.005, ***P<0.001, ****P<0.0001 (n=3).

Table S1. Deep sequencing analysis of CRISPR-Cas9–corrected ΔEx44 mice.

Dose (vg/kg)	Treatment	Analyzed Muscle	Samples	Barcode	Total Reads	Non-edited %	Total NHEJ %	NHEJ (+1 nt) %	ITR Integration %
1.60E+13	ssAAV-sgRNA	TA	BC2-ss58	TGGTCA	265,313	94.59	5.41	0.85	<0.2
			BC3-ss59	CACTGT	430,500	94.77	5.23	0.69	<0.2
			BC4-ss60	ATTGGC	343,805	95.69	4.31	0.34	<0.2
		Triceps	BC10-ss58	AGCTAG	336,778	94.97	5.03	0.25	<0.2
			BC11-ss59	GTCGTC	339,549	94.39	5.61	1.18	<0.2
			BC12-ss60	CGATTA	322,347	95.02	4.98	0.32	<0.2
		Diaphragm	BC18-ss58	ATCAGT	423,848	86.51	13.49	5.64	<0.2
			BC19-ss59	TATACT	508,066	89.32	10.68	3.65	<0.2
			BC20-ss60	CAACAA	297,096	90.79	9.21	2.77	<0.2
		Heart	BC26-ss58	AATGGT	414,968	81.29	18.71	7.84	<0.2
			BC27-ss59	GGCGGT	314,206	83.56	16.44	6.36	<0.2
			BC28-ss60	ATCACG	444,263	82.25	17.75	7.99	<0.2
	scAAV-sgRNA	TA	BC2-sc65	TGGTCA	351,570	87.11	12.89	8.29	<0.2
			BC3-sc66	CACTGT	397,695	89.08	10.92	5.47	<0.2
			BC4-sc67	ATTGGC	347,505	87.22	12.78	6.71	0.28
		Triceps	BC10-sc65	AGCTAG	445,116	73.92	26.08	18.96	0.47
			BC11-sc66	GTCGTC	391,352	82.71	17.29	11.15	0.39
			BC12-sc67	CGATTA	413,467	69.96	30.04	18.21	<0.2
		Diaphragm	BC18-sc65	ATCAGT	445,539	86.61	13.39	6.32	<0.2
			BC19-sc66	TATACT	404,070	82.33	17.67	10.3	<0.2
			BC20-sc67	CAACAA	331,964	84.09	15.91	8.41	<0.2
		Heart	BC26-sc65	AATGGT	471,201	79.04	20.96	12.3	0.35
			BC27-sc66	GGCGGT	328,191	86.48	13.52	7.75	<0.2
			BC28-sc67	ATCACG	435,717	85.92	14.08	7.77	0.48
8.00E+13	ssAAV-sgRNA	TA	BC5-ss83	GATCTG	418,020	91.34	8.66	2.67	0.24
			BC6-ss84	TACAAG	337,358	87.34	12.66	5.99	0.23
			BC7-ss85	CGTGAT	291,510	90.57	9.43	3.16	<0.2
		Triceps	BC13-ss83	GAATGA	348,423	90.21	9.79	3.53	<0.2
			BC14-ss84	CTTCGA	394,987	86.25	13.75	5.32	<0.2
			BC15-ss85	CTCTAC	245,385	89.91	10.09	2.55	<0.2
		Diaphragm	BC21-ss83	GTTGTT	339,005	84.47	15.53	6.11	<0.2
			BC22-ss84	TCGGTT	335,216	80.78	19.22	8.98	<0.2
			BC23-ss85	AGTATT	405,971	81.09	18.91	8.51	<0.2
		Heart	BC29-ss83	CGATGT	349,844	81.33	18.67	8.33	<0.2
			BC30-ss84	TTAGGC	329,313	80.97	19.03	8.32	0.23
			BC31-ss85	TGACCA	472,649	74.32	25.68	13.36	<0.2
	scAAV-sgRNA	TA	BC5-sc14	GATCTG	294,064	70.91	29.09	19.76	<0.2
			BC6-sc15	TACAAG	356,949	74.01	25.99	16.8	0.24
			BC7-sc16	CGTGAT	368,727	70.49	29.51	18.87	<0.2
		Triceps	BC13-sc14	GAATGA	380,792	71.12	28.88	16.95	0.24
			BC14-sc15	CTTCGA	339,794	70.4	29.6	17.8	0.7
			BC15-sc16	CTCTAC	365,034	67.38	32.62	20.08	<0.2
		Diaphragm	BC21-sc14	GTTGTT	407,519	77.15	22.85	13.67	<0.2
			BC22-sc15	TCGGTT	393,010	78.78	21.22	11.34	<0.2
			BC23-sc16	AGTATT	443,769	81.34	18.66	9.12	<0.2
		Heart	BC29-sc14	CGATGT	420,871	85.18	14.82	7.7	<0.2
			BC30-sc15	TTAGGC	415,579	84.14	15.86	7.04	0.64
			BC31-sc16	TGACCA	411,603	86.73	13.27	7.93	0.97

Table S2. Primers used in this study.

Primer function	Primer name	Primer sequence
ssAAV-sgRNA cloning primers	IF-SpeI-PacI-U6-F	ATGTATACCTTCGTCACTAGTTTAATTAAATCAGCGTTTGAGTAAGAGCCCG
	IF-XbaI-KpnI-GFP-R	GCTGGCGCGCCTTTTTCTAGAGGTACCCATGGACGAGCTGTACAAGTAAAGGC
scAAV-sgRNA cloning primers	IF-SpeI-KpnI-U6-F	ATGTATACCTTCGTCACTAGTGGTACCATCAGCGTTTGAGTAAGAGCCCG
	IF-XbaI-MluI-GFP-R	GCTGGCGCGCCTTTTTCTAGAACGCGTCATGGACGAGCTGTACAAGTAAAGG
AAV titer ddPCR primers	AAV-Cas9-ddPCR-F	GGACTCCCGGATGAACACTAAG
	AAV-Cas9-ddPCR-R	GTTGTTGATCTCGCGCACTTT
	AAV-Cas9-ddPCR-probe	TGGTGTCCGATTTCCGGA
	AAV-sgRNA-ddPCR-F	AGGGCCTATTTCCCATGATT
	AAV-sgRNA-ddPCR-R	AAACTGCAAACTACCCAAGAAA
	AAV-sgRNA-ddPCR-probe	TGCATATACGATACAAGGCTGTTAGAGAGA
Genomic PCR for TIDE analysis	T7E1-mE45-g4-F	CCCTGAGCTGAAGTGAGAGG
	T7E1-mE45-g4-R	ACCTCTTTCTCCTTTCTGCCAG
AAV vector genome copy number quantification	AAV-Cas9-copy-F	TGAAAGAGGACTACTTCAAGAAAATC
	AAV-Cas9-copy-R	TTGTCCTTGATAATTTTCAGCAGATC
	AAV-sgRNA-copy-F	CCGGAAATCAAGTCCGTTTATC
	AAV-sgRNA-copy-R	GAGCTTCAGCAGGAAATTTAACTA
cDNA RT-PCR quantification	mE43-qPCR-F	AGGTGAAAGTACAGGAAGCCGT
	mE46-qPCR-R	CTGCTGCTCATCTCCAAGTGGA
	Cas9-qPCR-F	TGAAAGAGGACTACTTCAAGAAAATC
	Cas9-qPCR-R	TTGTCCTTGATAATTTTCAGCAGATC
	sgRNA-qPCR-F	GGCTTACAGGAACTCCAGGA
	sgRNA-qPCR-R	CGACTCGGTGCCACTTTTTC
	18s-qPCR-F	GTAACCCGTTGAACCCCATT
	18s-qPCR-R	CCATCCAATCGGTAGTAGCG
On-target deep sequencing primers	On-target-F-Nextera	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCTATTGGATGTGTACATGTCAGGTTCA
	On-target-R-Nextera	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTCTGCTAAAAAGTCTCTGTCACC
	miSeq-Univ-F	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTC
	miSeq-Barcode-R	CAAGCAGAAGACGGCATACGAGAT**XXXXXX**GTCTCGTGGGCTCGG

Green: Nextera adaptor sequence

Red: Transposon end sequence

Blue: On-target primers to *Dmd* exon 45

xxxxxx: barcode sequence see table S2