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Supplemental Information

Targeted epigenetic repression by CRISPR/dSaCas9 suppresses pathogenic *DUX4-fl* expression in FSHD

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Supplemental Figures

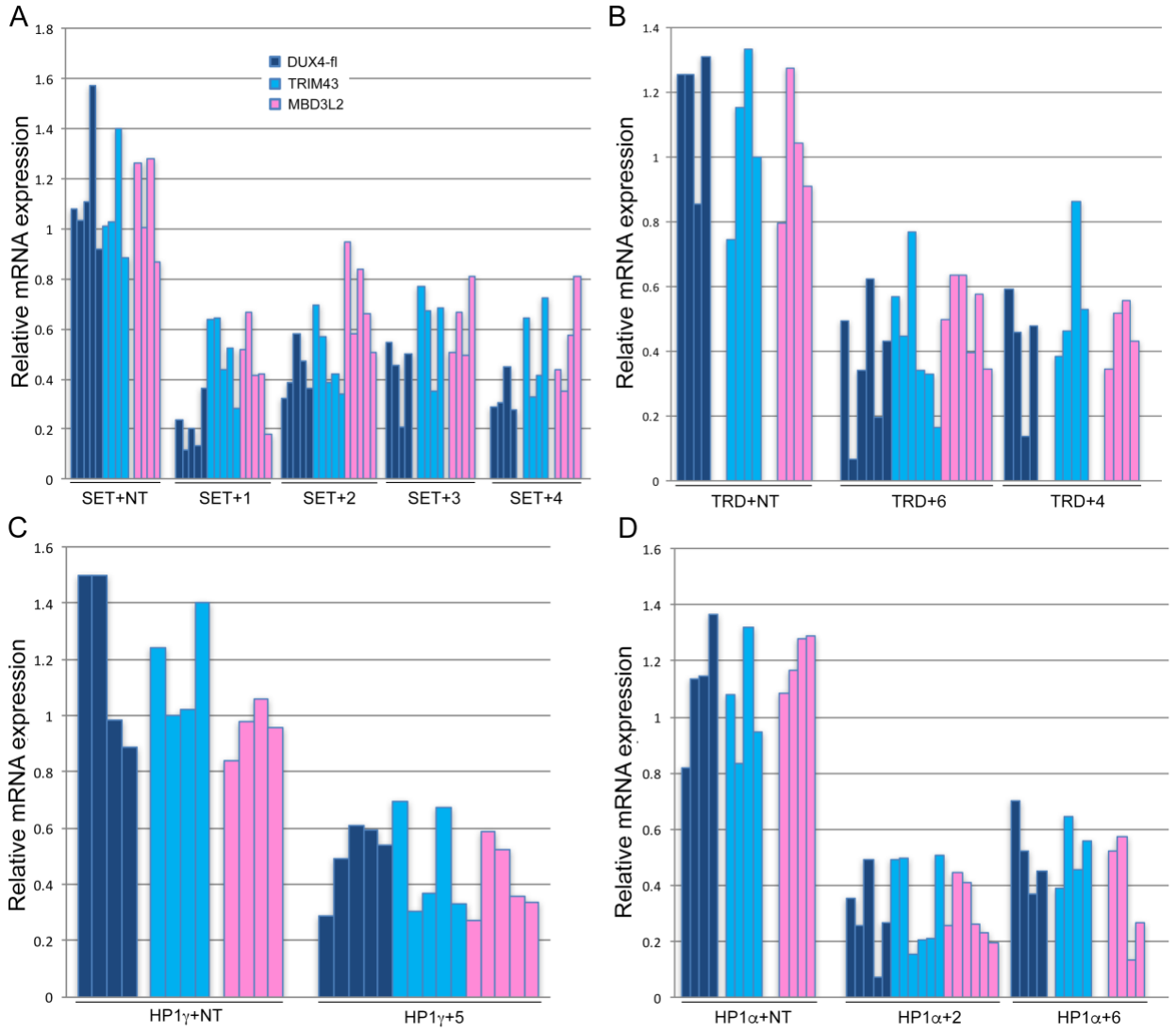

Figure S1. dSaCas9-mediated recruitment of epigenetic repressors to the *DUX4* promoter or exon 1 represses *DUX4-fl* and DUX4-FL targets in FSHD myocytes. A-D) FSHD myocytes were transduced with dSaCas9 fused to either: **A)** the SUV39H1 pre-SET, SET, and post-SET domains (SET), **B)** the MeCP2 TRD, **C)** HP1γ, or **D)** HP1α, with or without sgRNAs targeting *DUX4* (#1-6) or non-targeting sgRNAs (NT). Expression levels of *DUX4-fl* and DUX4-FL target genes *TRIM43* and *MBD3L2* were assessed by qRT-PCR. In all panels, each bar represents relative mRNA expression for a single biological replicate normalized to expression in cells expressing each dCas9-epigenetic regulator alone.

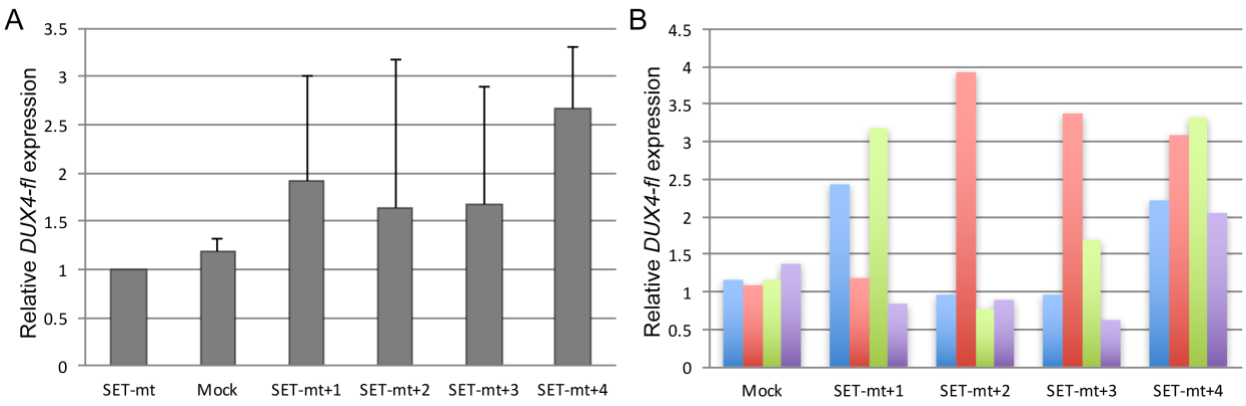

Figure S2. Enzymatic activity of the SET domain is required for *DUX4-fl* repression. FSHD myocytes were transduced with dSaCas9-SET containing a mutation (C326A) within the SET domain that abolishes enzymatic activity (SET-mt)[1] with or without sgRNAs targeting *DUX4* (#1-4). Expression levels of *DUX4-fl* were assessed by qRT-PCR. **A)** Data are plotted as the mean + SD value of four independent experiments, with relative mRNA expression for cells expressing dCas9-SET-mt alone set to 1. **B)** Each bar represents relative mRNA expression for a single biological replicate normalized to expression in cells expressing dCas9-SET-mt alone.

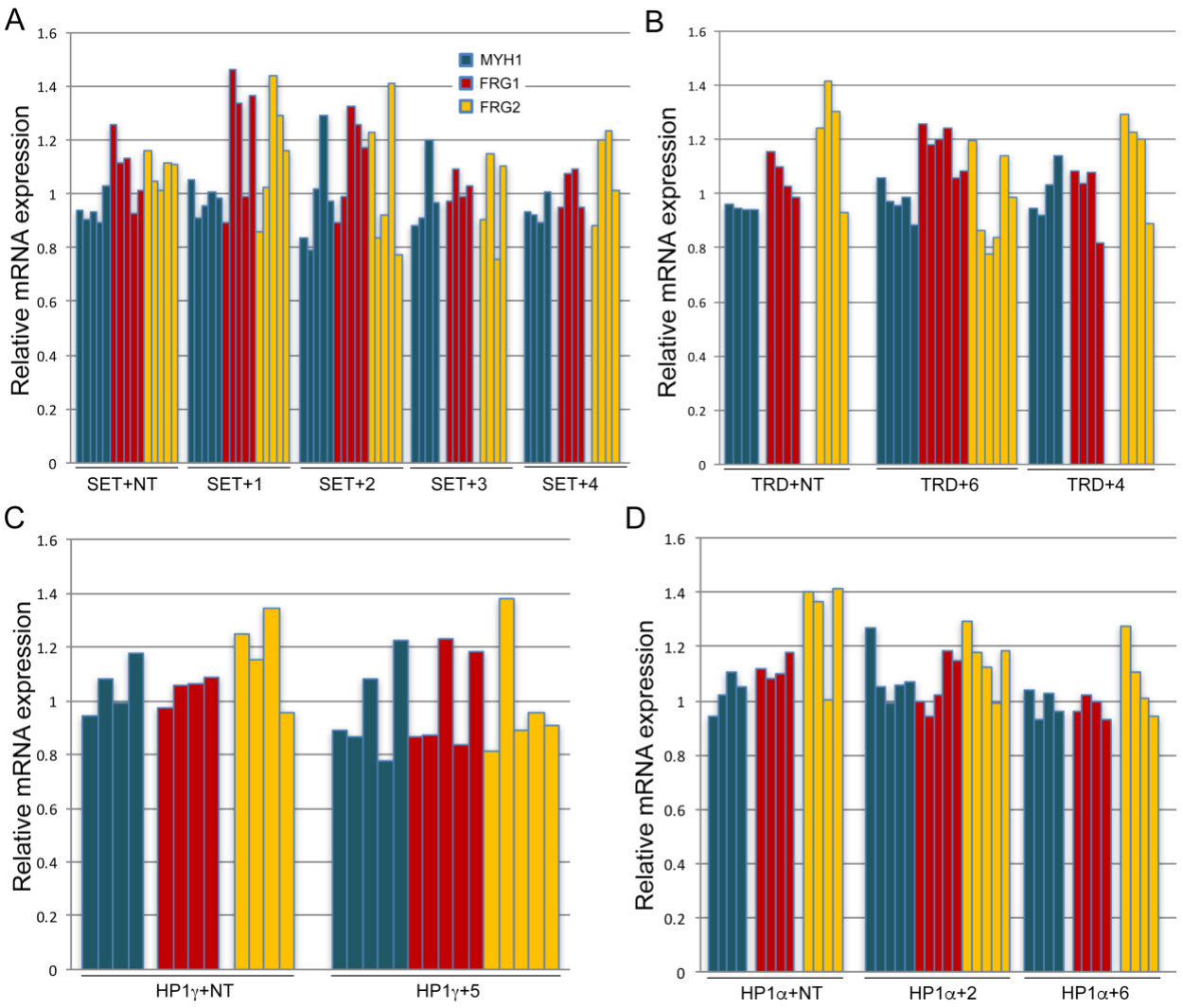

Figure S3. Targeting dSaCas9-repressors to *DUX4* has no effect on *MYH1* or D4Z4 proximal genes. A-D) Expression levels of the terminal muscle differentiation marker *Myosin heavy chain 1 (MYH1)* and the D4Z4 proximal genes *FRG1* and *FRG2* were assessed by qRT-PCR in the FSHD myocyte cultures described in Figure 2. In all panels, each bar represents relative mRNA expression for a single biological replicate normalized to expression in cells expressing each dCas9-epigenetic regulator alone.

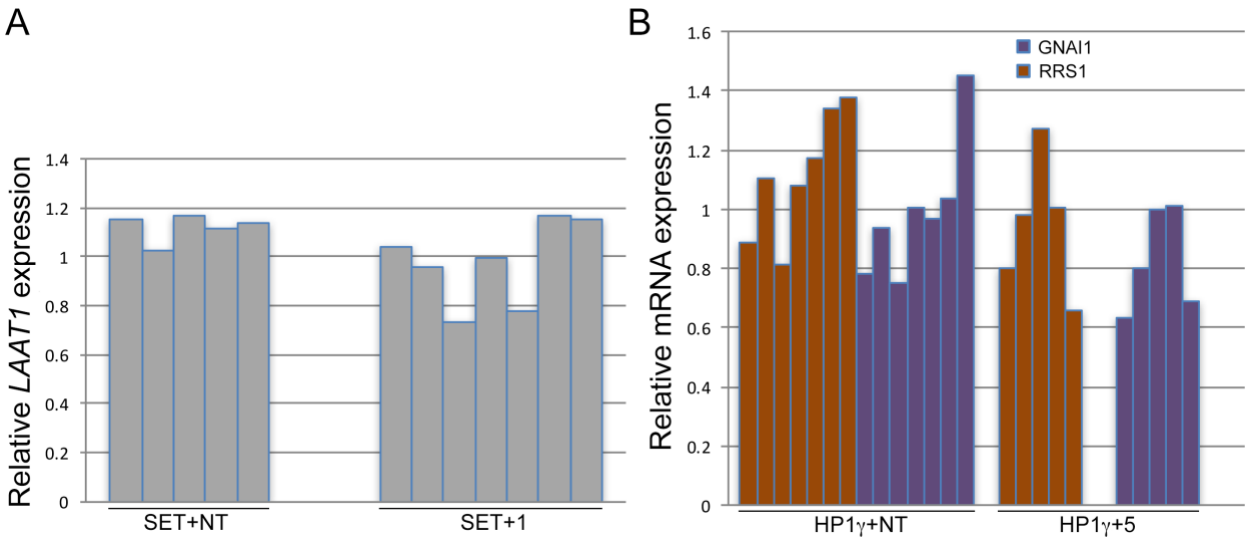

Figure S4. Targeting dSaCas9-repressors to *DUX4* has no effect on closest-match off-target (OT) genes expressed in skeletal muscle. Levels of **A)** *Lysosomal amino acid transporter 1 homolog* (*LAAT1*), **B)** *Ribosome biogenesis regulatory protein homolog* (*RRS1*), or *Guanine nucleotide-binding protein G(i) subunit alpha-1 isoform 1* (*GNAI1*) were assessed by qRT-PCR in the relevant FSHD myocyte cultures described in Figure 2. Intron 1 of *LAAT1* contains a potential OT match to sgRNA #1. The single exon of *RRS1* and the downstream flanking sequence of *GNAI1* contain potential OT matches to sgRNA #5. In all panels, each bar represents relative mRNA expression for a single biological replicate normalized to expression in cells expressing each dCas9-epigenetic regulator alone.

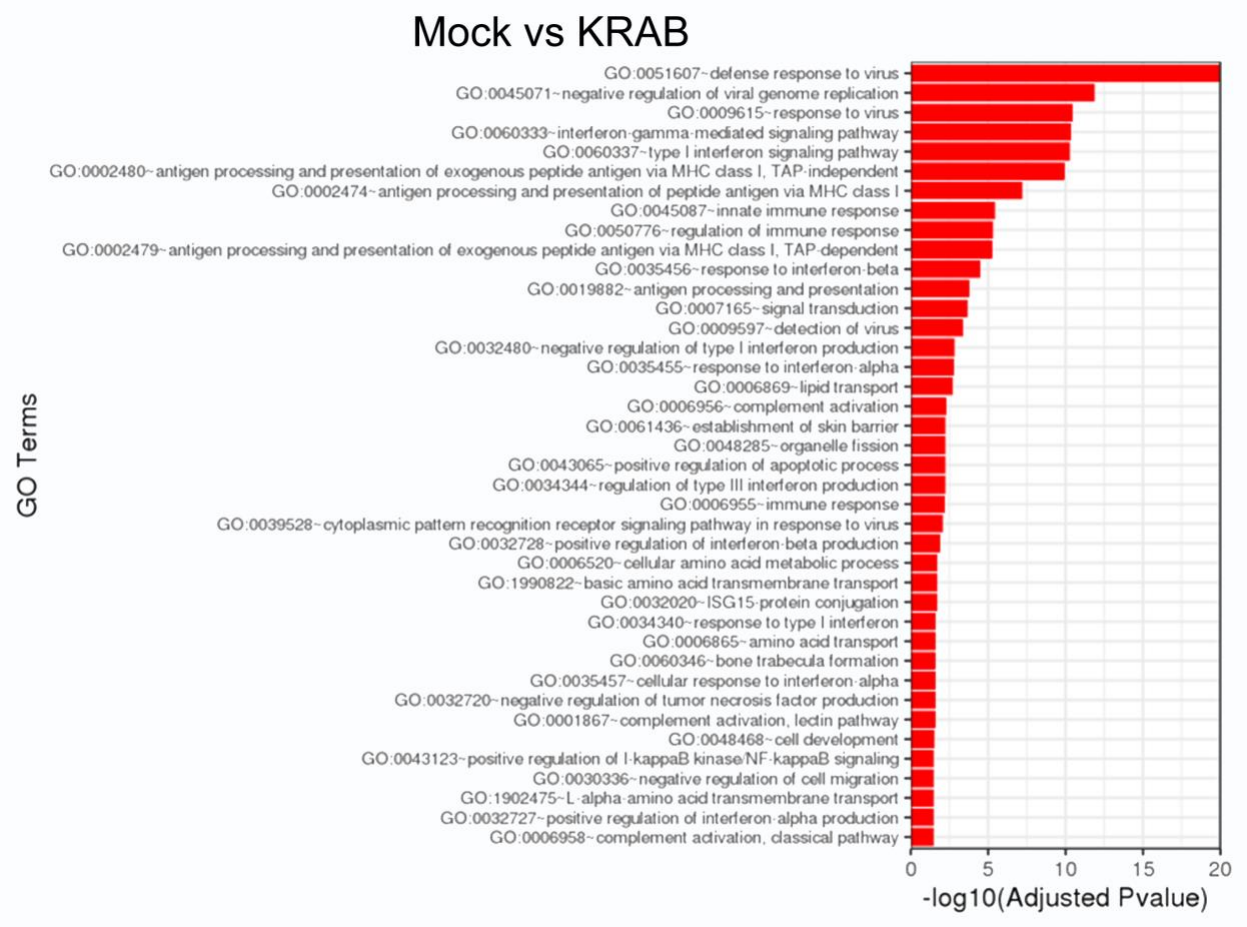

Figure S5. Gene Ontology analysis of DEGs following targeting of dSaCas9-KRAB to *DUX4*. FSHD myocytes were transduced with dSaCas9-KRAB + sgRNA #6. RNA-seq analysis was performed using the Illumina HiSeq 2 x 100bp platform. Significantly differentially expressed genes were clustered by their gene ontology (GO) and the enrichment of GO terms was tested using Fisher exact test (GeneSCF v1.1-p2). Shown are GO terms that are significantly enriched with an adjusted P-value <0.05 in the differentially expressed gene sets.

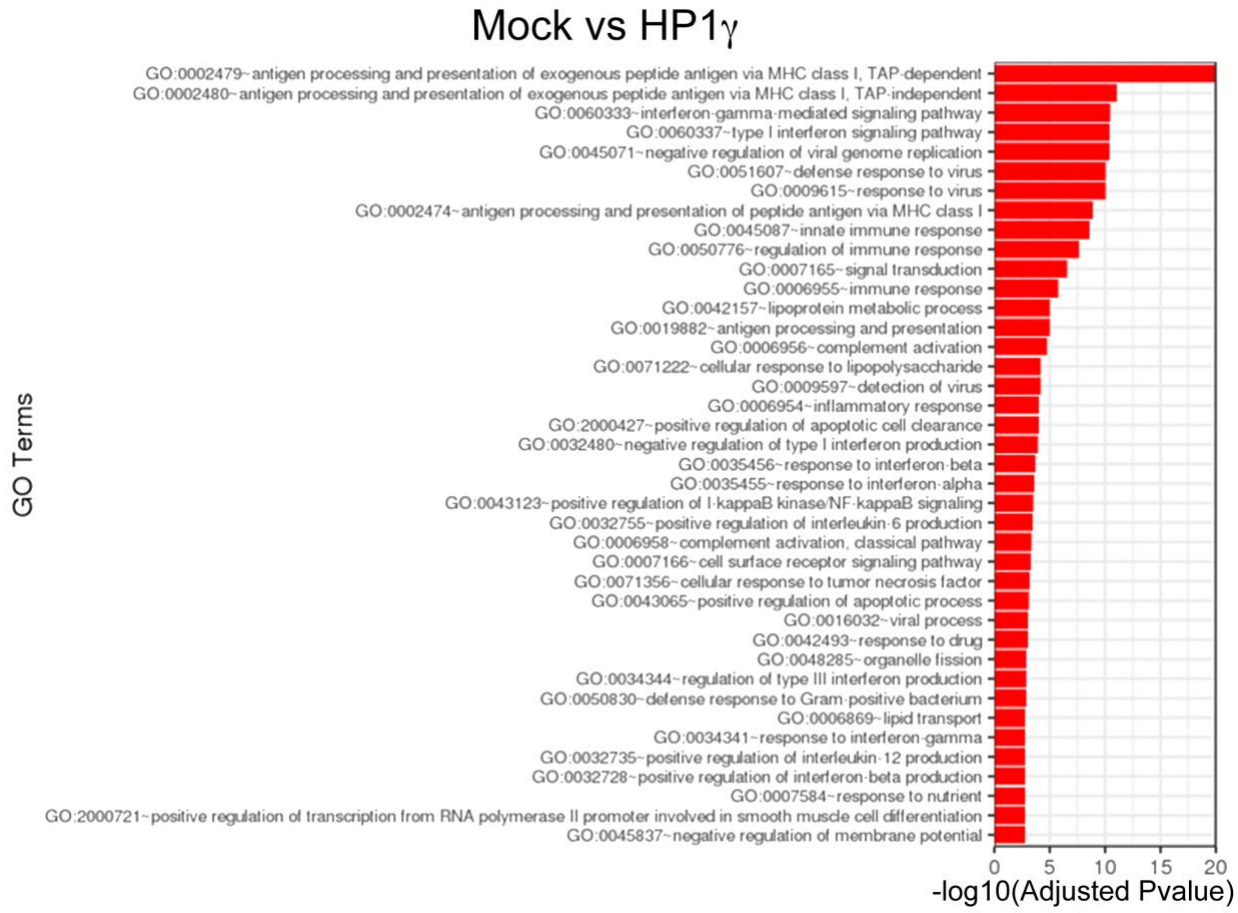

Figure S6. Gene Ontology analysis of DEGs following targeting of dSaCas9-HP1γ to *DUX4*. FSHD myocytes were transduced with dSaCas9-HP1γ + sgRNA #5. RNA-seq analysis was performed using the Illumina HiSeq 2 x 100bp platform. Significantly differentially expressed genes were clustered by their gene ontology (GO) and the enrichment of GO terms was tested using Fisher exact test (GeneSCF v1.1-p2). Shown are GO terms that are significantly enriched with an adjusted P-value <0.05 in the differentially expressed gene sets.

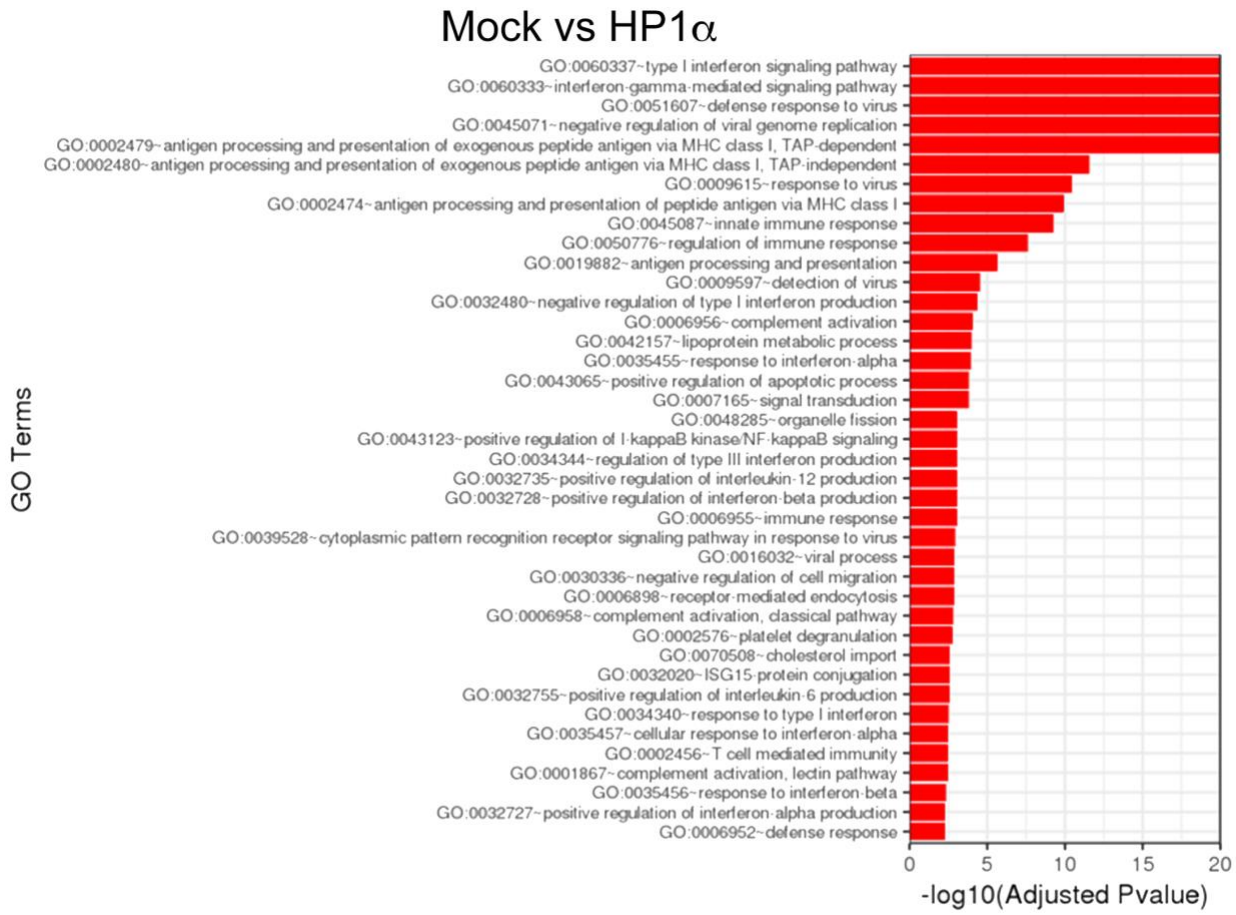

Figure S7. Gene Ontology analysis of DEGs following targeting of dSaCas9-HP1α to *DUX4*. FSHD myocytes were transduced with dSaCas9-HP1α + sgRNA #2. RNA-seq analysis was performed using the Illumina HiSeq 2 x 100bp platform. Significantly differentially expressed genes were clustered by their gene ontology (GO) and the enrichment of GO terms was tested using Fisher exact test (GeneSCF v1.1-p2). Shown are GO terms that are significantly enriched with an adjusted P-value <0.05 in the differentially expressed gene sets.

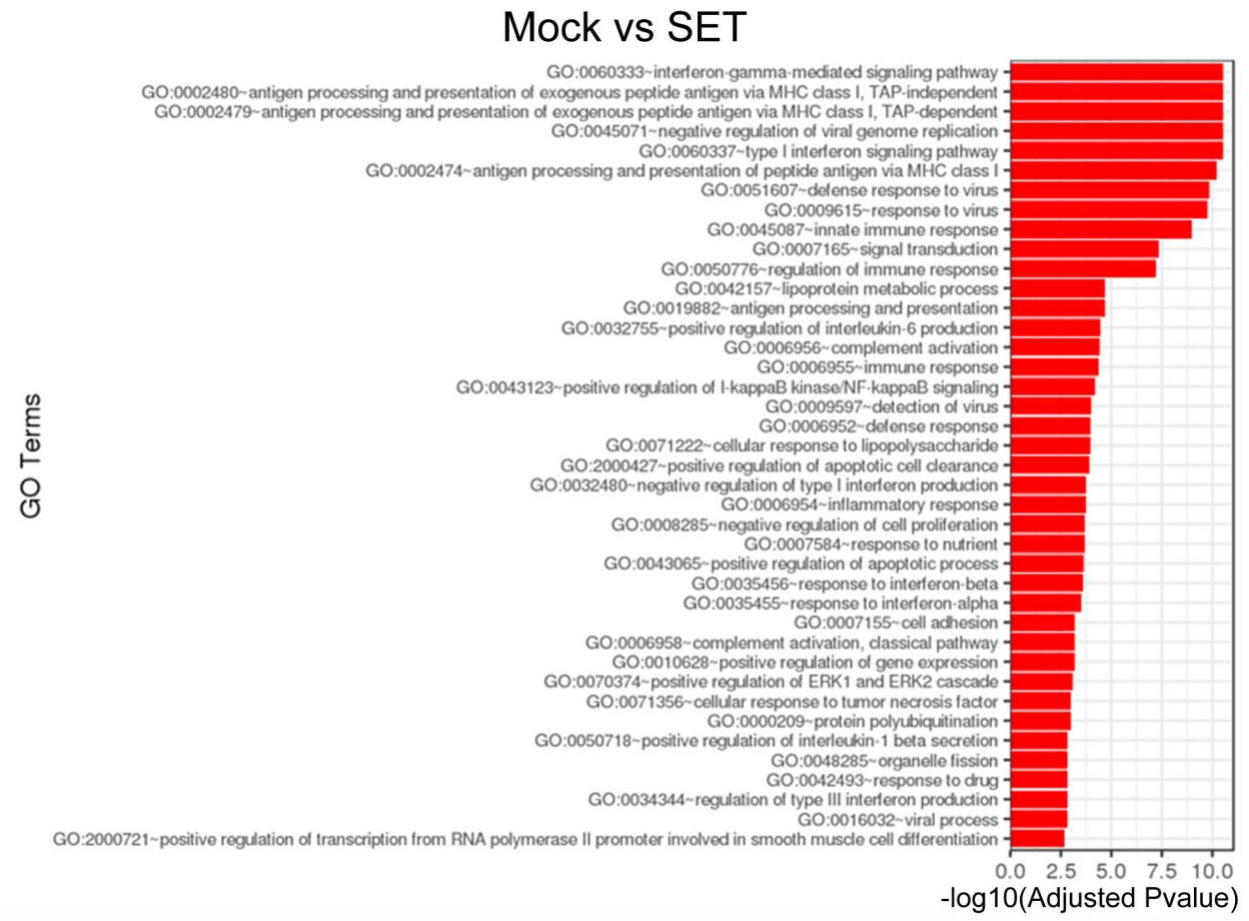

Figure S8. Gene Ontology analysis of DEGs following targeting of dSaCas9-SET to *DUX4*. FSHD myocytes were transduced with dSaCas9-SET + sgRNA #1. RNA-seq analysis was performed using the Illumina HiSeq 2 x 100bp platform. Significantly differentially expressed genes were clustered by their gene ontology (GO) and the enrichment of GO terms was tested using Fisher exact test (GeneSCF v1.1-p2). Shown are GO terms that are significantly enriched with an adjusted P-value <0.05 in the differentially expressed gene sets.

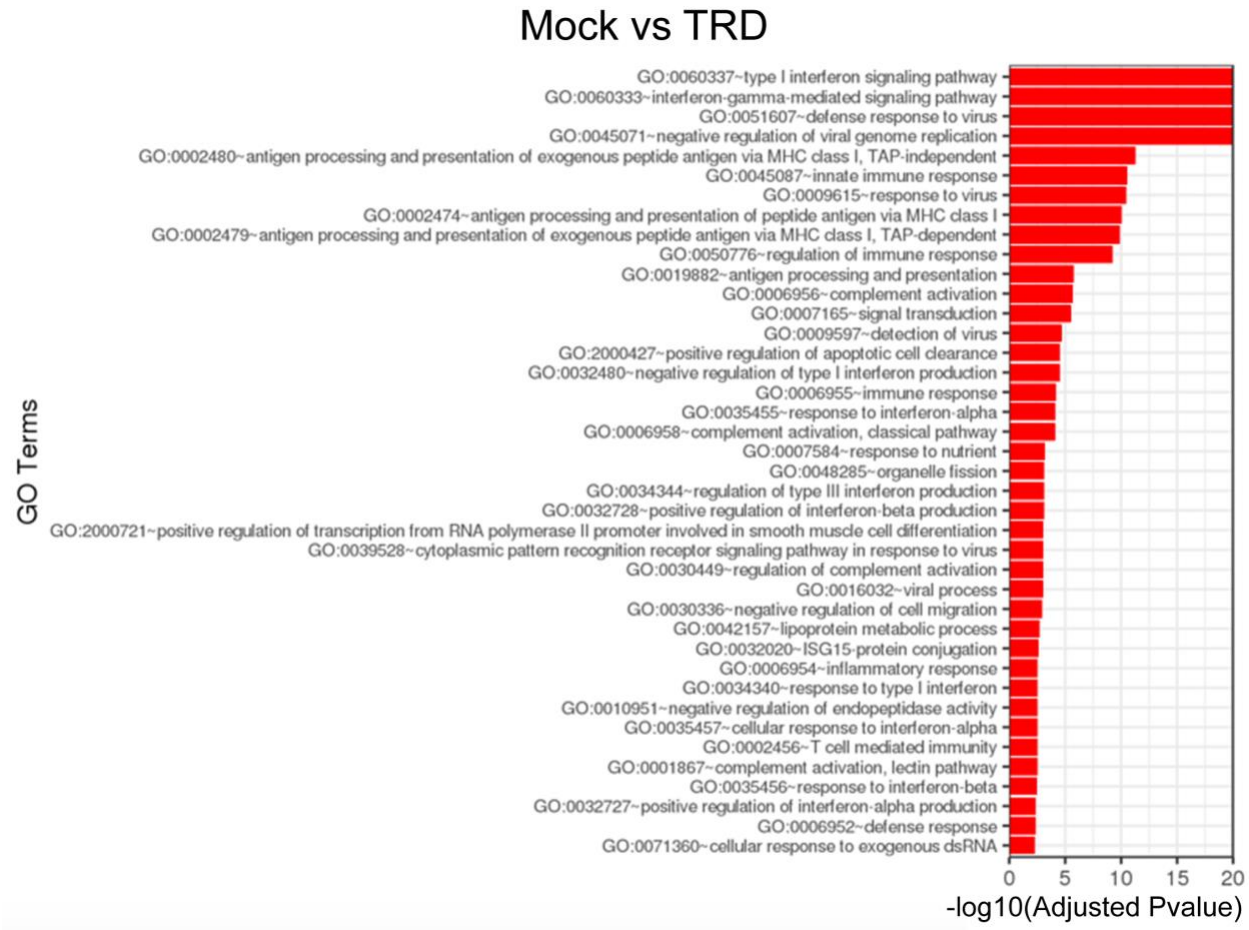

Figure S9. Gene Ontology analysis of DEGs following targeting of dSaCas9-TRD to *DUX4*. FSHD myocytes were transduced with dSaCas9-TRD + sgRNA #6. RNA-seq analysis was performed using the Illumina HiSeq 2 x 100bp platform. Significantly differentially expressed genes were clustered by their gene ontology (GO) and the enrichment of GO terms was tested using Fisher exact test (GeneSCF v1.1-p2). Shown are GO terms that are significantly enriched with an adjusted P-value <0.05 in the differentially expressed gene sets.

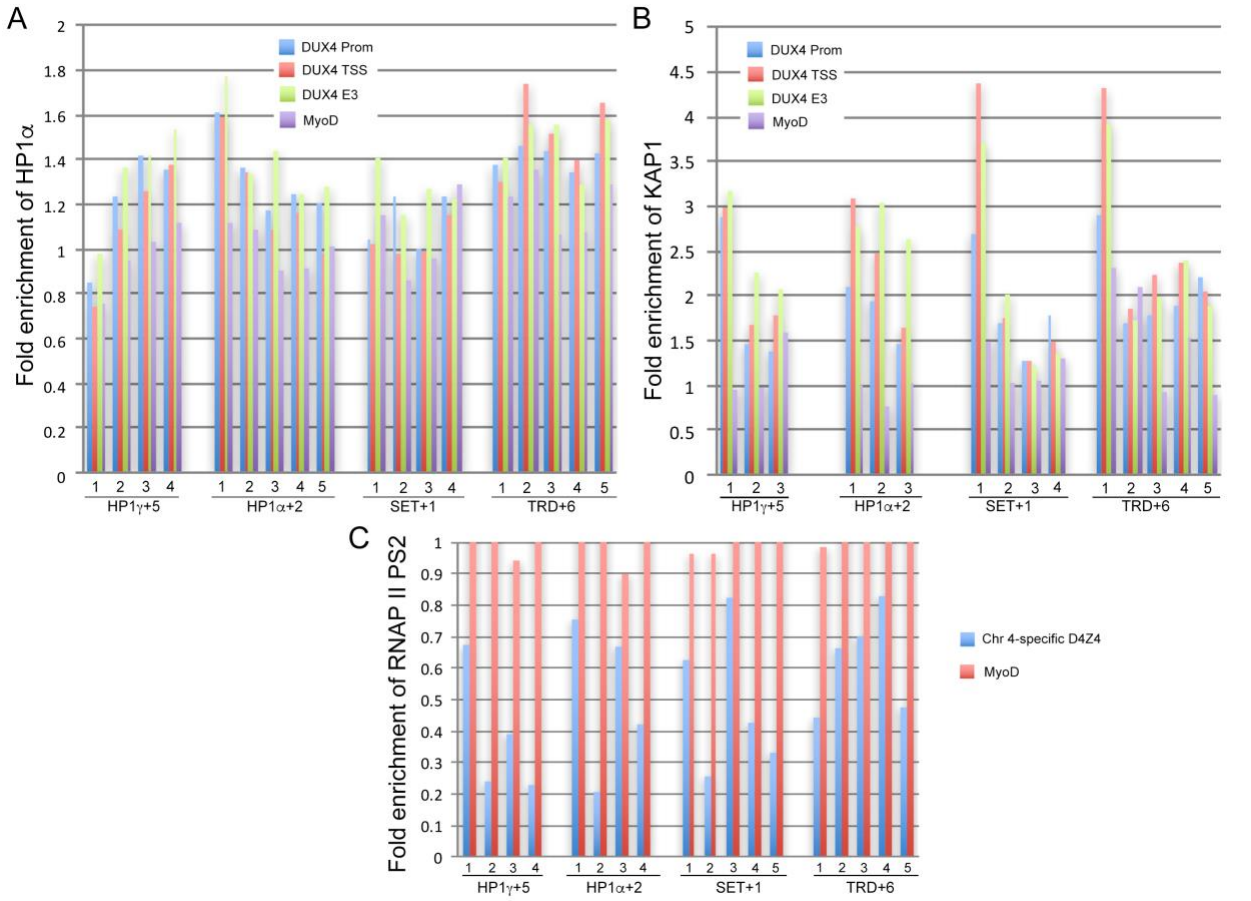

Figure S10. Targeting dSaCas9-repressors to *DUX4* increases chromatin repression at the locus. ChIP assays were performed using FSHD myocytes transduced with each dSaCas9-epigenetic regulator + sgRNA targeting the *DUX4* promoter or exon 1. Chromatin was immunoprecipitated using antibodies specific for **A)** HP1α or **B)** KAP1 and analyzed by qPCR using primers to the promoter, TSS, or exon 3 of *DUX4* or to *MyoD*, or **C)** the elongating form of RNA-Pol II (phospho-serine 2) and analyzed by qPCR using primers specific to *DUX4* exon1/intron1 on chromosome 4 or to *MyoD*. Locations of primers are shown in Figure 1C. Data are presented as fold enrichment of the target region by each specific antibody normalized to α-histone H3, with enrichment for mock-infected cells set to 1. In all panels, each bar represents a single biological replicate.

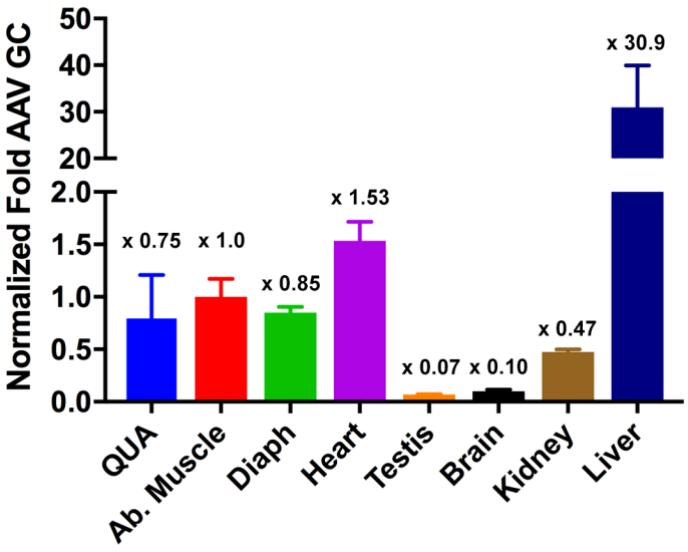

Figure S11. Tissue transduction of AAV9. The presence of AAV genomes in various mCherry expressing and non-expressing tissues was assessed by qPCR using primers against AAV9 and normalizing to the single copy *Rosa26* gene. This confirms that tissues such as heart, kidney, and liver, which did not express any detectable mCherry, were highly transduced, supporting the tissue specificity of the FSHD-optimized expression cassette.

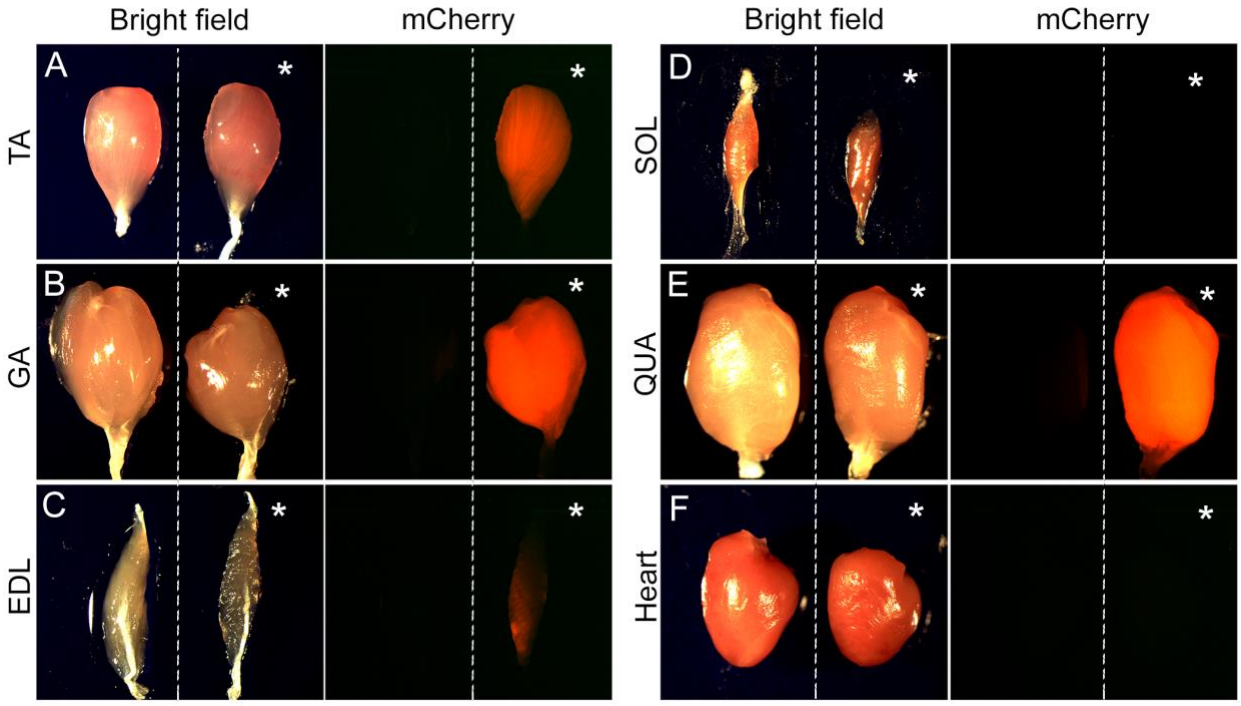

Figure S12. The FSHD-optimized regulatory cassette is active in skeletal muscles, but not in cardiac muscle. Muscles from Figure 6 are shown at 1.0 s exposure. *In vivo* AAV9-mediated mCherry expression under control of the FSHD-optimized regulatory cassette was visualized in the indicated muscles at 12 wk post-injection with tissues from uninjected mice on the left and injected tissues on the right (indicated by an asterisk). Expression of mCherry was detected in skeletal muscles (tibialis anterior TA, gastrocnemius GA, extensor digitorum longus EDL, and quadriceps QUA), and was undetectable in soleus (SOL) and heart.

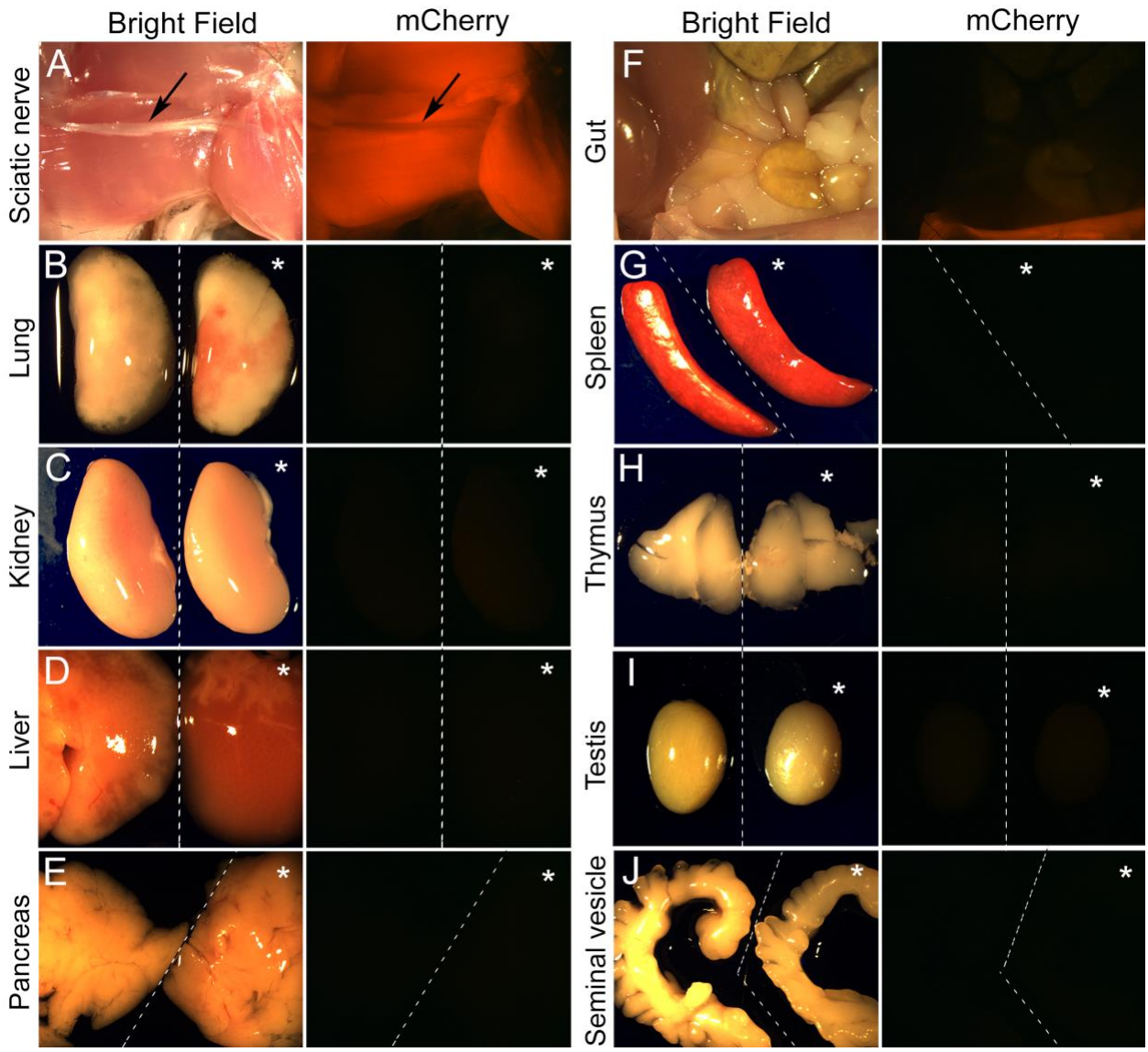

Figure S13. The FSHD-optimized regulatory cassette is not active in non-muscle tissues. Non-muscle tissues from the AAV9 injected wild-type mice assayed in Figure 6 were similarly assayed for mCherry expression and shown at 1.0 s exposure. Panels A and F only show tissues from AAV injected mice; the remaining panels show tissues from uninjected mice (left) and injected mice (right and indicated by an asterisk). **A)** The skeletal muscle from a hindlimb, including the posterior biceps femoris, quadriceps, and gastrocnemius, express mCherry, while the sciatic nerve, indicated by a black arrow, does not express mCherry. **F)** The dorsal view of a dissected abdomen, including liver and the large and small intestines, which do not express mCherry, compared with the mCherry expressing abdominal muscle in view at the bottom of the frame.

Supplemental Tables

Table S1. Specificity of dSaCas9-compatible sgRNAs targeting the FSHD locus

sgRNA	target	21-nt sequence + PAM (NNGRRT)	mismatches	wobbles
1	*DUX4* prom	CGGCCCCAGGCCTCGACGCCCTGGGGT		
OT	*LAAT1*	aGGCCCCAGG-CTCGcCGCCCCAGGAT	2	1
5	*DUX4* exon 1	CTGTGCAGCGCGGCCCCCGGCGGGGGT		
OT	*RRS1*	CTGT--AGCtCGGCCtCCGGCGTGGGT	2	2
OT	*GNAI1*	C--TGCgGCGCGGCCaCCGGCGGGAGT	2	2

Two dSaCas9-compatible sgRNAs used in this study had potential off-target (OT) matches (http://www.rgenome.net/cas-offinder/) in or near genes expressed in skeletal muscle, as indicated. Intron 1 of *Lysosomal amino acid transporter 1 homolog* (LAAT1) contains a potential OT match to sgRNA #1. The single exon of *Ribosome biogenesis regulatory protein homolog* (*RRS1*) and the downstream flanking sequence of *Guanine nucleotide-binding protein G(i) subunit alpha-1 isoform 1* (*GNAI1*) contain potential OT matches to sgRNA #5.

Table S2. Significant DEGs following targeting of dSaCas9-repressors to *DUX4*

See separate Excel file

Table S3. Comparison of DEGs following targeting of dSaCas9-repressors to *DUX4*

See separate Excel file

Table S4. Changes in expression among developmental and myogenic DEGs following targeting of dSaCas9-repressors to *DUX4*

See separate Excel file

Table S5. Oligonucleotide primers to human genes (5' → 3')

qRT-PCR:
DUX4-fl-F: GCTCTGCTGGAGGAGCTTTAGGA
DUX4-fl-R: CGCACTGCTCGCAGGTCTGCWGGT
DUX4-fl-nested-F: AGCTTTAGGACGCGGGGTTGGGAC
DUX4-fl-nested-R: GCAGGTCTGCWGGTACCTGG
TRIM43-F:[2] ACCCATCACTGGACTGGTGT
TRIM43-R:[2] CACATCCTCAAAGAGCCTGA
MBD3L2-F:[2] GCGTTCACCTCTTTTCCAAG
MBD3L2-R:[2] GCCATGTGGATTTCTCGTTT
MYH1-F: ACAGAAGCGCAATGTTGAAG
MYH1-R: CACCTTTGCTTGCAGTTTGT
FRG1-F:[3] TCTACAGAGACGTAGGCTGTCA
FRG1-R:[3] CTTGAGCACGAGCTTGGTAG
FRG2-F:[4] GGGAAAACTGCAGGAAAA
FRG2-R:[4] CTGGACAGTTCCCTGCTGTGT
LAAT1-F: TCTGCTTTGCTGCATCTACC
LAAT1-R: AGTACAGCGTCAGCATCACC
RRS1-F: CACAACCGAGACTTTGGAGA
RRS1-R: TCCCGCTCTGATACACAAAC
GNAI1-F: CATCCCGACTCAACAAGATG
GNAI1-R: TGCATTCGGTTCATTTCTTC
Wfdc3-F:[5] CTTCCATGTCAGGAGCTGTG
Wfdc3-R:[5] ACCAGGATTCTGGGACATTG
Slc34a2-F: TTCTACATGCTCATCTCTGCC
Slc34a2-R: CCCATGTTGCTCTTCCAATTG
Rpl37-F: CATCCTTTGGTAAGCGTCGCA
Rpl37-R: TGGCACTCCAGTTATACTTCCT

ChIP:
DUX4 prom-F:[6] CCTGTTGCTCACGTCTCTCC
DUX4 prom-R:[6] GTGGGGAGTCTGCAGTGTG
DUX4 TSS-F:[6] GACACCCTCGGACAGCAC
DUX4 TSS-R:[6] GTACGGGTTCCGCTCAAAG
DUX4 exon3-F:[2] CTGACGTGCAAGGGAGCT
DUX4 exon3-R:[2] CAGGTTTGCCTAGACAGCG
4-spec D4Z4-F:[7] TCTGCTGGAGGAGCTTTAG
4-spec D4Z4-R:[7] GAATGGCAGTTCTCCGCG[a]
MyoD-F:[3] CGCCAGGATATGGAGCTACT
MyoD-R:[3] CGGGTCGTCATAGAAGTCGT

CRISPRi:[b]
sgRNA-1: CGGCCCCAGGCCTCGACGCCC
sgRNA-2: TCGACGCCCTGGGGTCCCTTC
sgRNA-3: TCCGCGGGGAGGGTGCTGTCC
sgRNA-4: GCCAGCTGAGGCAGCACCGGC
sgRNA-5: CTGTGCAGCGCGGCCCCCGGC
sgRNA-6: TCATCCAGCAGCAGGCCGCAG

[a]G at this position is specific to chromosome 4 (G) vs chromosome 10 (T)

[b]Each sgRNA is a 21-bp sequence preceded by a G for most effective targeting.[8]

AAV qPCR:
bGH-F: TCTAGTTGCCAG CCATCTGTTGT
bGH-R: TGGGAGTGGCACCTTCCA
Rosa26-F: CAATACCTTTCTGGGAGTTCTCTGCTGC
Rosa26-R: TGCAGGACAACGCCCACACACC

Supplemental References

1. Rea, S, Eisenhaber, F, O'Carroll, D, Strahl, BD, Sun, ZW, Schmid, M, Opravil, S, Mechtler, K, Ponting, CP, Allis, CD, *et al.* (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**: 593-599.

2. Himeda, CL, Jones, TI, and Jones, PL (2016). CRISPR/dCas9-mediated Transcriptional Inhibition Ameliorates the Epigenetic Dysregulation at D4Z4 and Represses DUX4-fl in FSH Muscular Dystrophy. *Mol Ther* **24**: 527-535.

3. Bodega, B, Ramirez, GD, Grasser, F, Cheli, S, Brunelli, S, Mora, M, Meneveri, R, Marozzi, A, Mueller, S, Battaglioli, E, *et al.* (2009). Remodeling of the chromatin structure of the facioscapulohumeral muscular dystrophy (FSHD) locus and upregulation of FSHD-related gene 1 (FRG1) expression during human myogenic differentiation. *BMC Biol* **7**: 41.

4. Klooster, R, Straasheijm, K, Shah, B, Sowden, J, Frants, R, Thornton, C, Tawil, R, and van der Maarel, S (2009). Comprehensive expression analysis of FSHD candidate genes at the mRNA and protein level. *Eur J Hum Genet* **17**: 1615-1624.

5. Krom, YD, Thijssen, PE, Young, JM, den Hamer, B, Balog, J, Yao, Z, Maves, L, Snider, L, Knopp, P, Zammit, PS, *et al.* (2013). Intrinsic Epigenetic Regulation of the D4Z4 Macrosatellite Repeat in a Transgenic Mouse Model for FSHD. *PLoS Genet* **9**: e1003415.

6. Himeda, CL, Debarnot, C, Homma, S, Beermann, ML, Miller, JB, Jones, PL, and Jones, TI (2014). Myogenic enhancers regulate expression of the facioscapulohumeral muscular dystrophy associated DUX4 gene. *Mol Cell Biol* **34**: 1942-1955.

7. Himeda, CL, Jones, TI, Virbasius, CM, Zhu, LJ, Green, MR, and Jones, PL (2018). Identification of Epigenetic Regulators of DUX4-fl for Targeted Therapy of Facioscapulohumeral Muscular Dystrophy. *Mol Ther* **26**: 1797-1807.

8. Ran, FA, Cong, L, Yan, WX, Scott, DA, Gootenberg, JS, Kriz, AJ, Zetsche, B, Shalem, O, Wu, X, Makarova, KS, *et al.* (2015). In vivo genome editing using Staphylococcus aureus Cas9. *Nature* **520**: 186-191.