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Supplementary Information for

Inhibition of *DUX4* expression with antisense LNA gapmers as a therapy for facioscapulohumeral muscular dystrophy

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SI Materials and Methods

Animals. For visualizing LNA uptake into muscles, two male hemizygous seven-week-old *FLExDUX4* mice were used. For testing the therapeutic efficacy of LNAs, fifteen male hemizygous adult *FLExDUX4* mice were used. Animals were anesthetized with isoflurane inhalation to perform intramuscular injections, and were euthanized following CO_2 inhalation by cervical dislocation according to standard protocols.

Antisense oligonucleotides. Seven different LNA gapmers against *DUX4* were designed **(Table 1)** and synthesized by Exiqon and Eurogentec for *in vitro* and *in vivo* studies, respectively. Their approximate target sites along the *DUX4* mRNA are illustrated in **Fig. 1a**. One LNA gapmer (LNA3) was designed to target the *DUX4* open reading frame in exon 1, while the remaining ones targeted the 3'-UTR region in exon 3; none of the gapmers targeted the PAS sequence. Gapmers were designed to have 14-16 nucleotides in length, with the first and last three bases having the LNA chemistry and the backbone fully phosphorothioated. Target site GC content was also taken into account. Lab-designed mock and scrambled LNA gapmers, synthesized by Exiqon and Eurogentec respectively for *in vitro* and *in vivo* experiments, were also used for experiments.

Cell culture. Immortalized FSHD patient-derived myoblasts were kindly provided by the Wellstone Program of the University of Massachusetts Medical School, MA, USA. Specifically, two cell lines were used: WS229 (FSHD-affected) and WS234 (FSHD-unaffected). Both originated from biceps-sourced primary myoblast cultures that were immortalized through the stable integration of a *CDK4/hTERT* cassette (1). The individuals from whom the WS229 (male, age 66 years at biopsy, early adult onset of FSHD) and WS234 (female, age 60 at biopsy) lines were sourced are from the same family and are siblings. EcoRI/BlnI allele sizes and 4q haplotypes for WS229 and WS234 are >112kb(4qB)/28kb(4qA) and >145kb(4qB)/107kb(4qB), respectively.

Cells were grown in medium containing 15% fetal bovine serum (FBS) (Sigma), 2.5 ng/ml recombinant human hepatocyte growth factor (EMD Millipore), 10 ng/ml recombinant human fibroblast growth factor (BioPioneer), and 0.055 µg/ml dexamethasone in basal medium (BM). The BM consisted of 20% Medium 199 (Life Technologies), 0.03 µg/ml $ZnSO_4$, and 1.4 µg/ml Vitamin B12 in DMEM (Life Technologies) with 2.5% penicillin-streptomycin (Life Technologies). For differentiation, the growth medium was replaced with the following once a confluence of 80-90% was reached: BM supplemented with 15% KnockOut Serum Replacement (KOSR) (Life Technologies), 10 µg/ml insulin (Sigma), and 100 µg/ml human apo-transferrin (R&D Systems). Cells were cultured at 37°C and 5% CO_2 for the entire study.

AO transfection. For transfection, 4×10^5 WS229 cells were seeded onto each well of a gelatin-coated 6-well plate, grown, and then differentiated as described in the previous section **(Fig. 1b)**. *DUX4*-targeting LNA gapmers were transfected into myotubes 13 days post-differentiation with Lipofectamine® RNAiMAX (Life Technologies) according to manufacturer's instructions, except for the following modifications: 2% of RNAiMAX in OptiMEM (Life Technologies) was used for the initial dilution of the reagent, and differentiation medium was added at the last step such that it comprised 80% of the final transfection medium. LNA gapmers were transfected at a final concentration of either 1, 10, or 100 nM. To serve as negative controls, WS229 cells were either transfected with a mock LNA gapmer at the respective dose or subjected to the transfection procedure but with no AO added. Non-transfected WS234 cells were also prepared as an additional negative control. Cells were harvested the following day for RNA collection.

RNA extraction from cells and qPCR. Total RNA was extracted from cells using the RNeasy® Mini Kit (Qiagen), following the manufacturer's instructions with on-column DNase treatment (RNase-free DNase set, Qiagen). From this, 1400 ng was used for cDNA synthesis with SuperScript IV Reverse Transcriptase (Life Technologies) as directed by the manufacturer, using 0.5 µg of oligo$(dT)_{12-18}$ (Life Technologies) as primer and having a final reaction volume of 20 µl.

The synthesized cDNA was then used as a template for qPCR using the QuantStudio 3 Real-Time PCR System (Applied Biosystems). For *DUX4* and *GAPDH*, the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-rad) was used, with forward and reverse primers added

to achieve final concentrations of 0.4 μM each. Primer sequences are listed in **Table S4**. Gene expression was normalized against *GAPDH*.

Pre-designed TaqMan™ Gene Expression assays (Thermo Fisher) were used for qPCR-based detection of the *DUX4* downstream target genes *ZSCAN4* (Hs00537549_m1), *TRIM43* (Hs00299174_m1, and *MBD3L2* (Hs00544743_m1). Reactions were prepared using these assays, the TaqMan™ Fast Advanced Master Mix (Thermo Fisher), and the synthesized cDNA using amounts recommended by the manufacturer. Expression values of all downstream target genes were normalized against *GAPDH* as well. For both SYBR and TaqMan reactions, the default "Fast" cycling program of the qPCR machine was used, 1) 95°C, 20 s, 2) 40 cycles of 95°C, 1 s then 60°C, 20 s; for SYBR reactions, there was an additional step for melt curve construction.

RNA sequencing. Healthy FSHD-unaffected control, non-treated and LNA4-treated immortalized FSHD patient-derived cells were grown and differentiated as described in the *Cell culture* section, following the timeline indicated in **Fig. 1b**. In the case of the LNA4-treated cells, transfection was done as described in the *AO transfection* section, with 100 nM of LNA4 provided. Total RNA was extracted via the RNeasy® Mini Kit (Qiagen), using the manufacturer's instructions with on-column DNase treatment (RNase-free DNase set, Qiagen). RNA samples were processed at the New York Genome Center (New York, NY) for high output RNA sequencing using the HiSeq 2500 system (Illumina).

Reads were aligned with STAR (version 2.4.0c) (2), and genes annotated in Gencode v18 were quantified with featureCounts (v1.4.3-p1) (3). Normalization and differential expression were done with the Bioconductor package DESeq2 (4). Downstream analysis and visualization of data were conducted using R (version 3.5.0). RNA sequencing data is publicly available from the SRA portal of NCBI under accession number PRJNA606474.

Myogenic fusion index and hypotrophy phenotype analysis. WS229 and WS234 cells were seeded onto gelatin-coated 24-well plates (2.5×10^4 cells/well), grown, and differentiated as described above. Once the cells reached 4 days post-differentiation, LNA gapmers were transfected overnight with Lipofectamine® RNAiMAX as described, at a final dose of 10 nM. Three days after transfection, cells were used for immunocytochemistry. Following aspiration of the culture medium, cells were fixed for 5 min with 4% paraformaldehyde, washed briefly with phosphate-buffered saline (PBS), and then incubated for 5 min in PBS with 0.5% Triton X-100. Cells were then blocked with 20% FBS (Sigma) in PBS for 1 hr, after which they were incubated in 1:200 rabbit polyclonal anti-desmin antibody (Abcam), diluted in the blocking solution, for 1 hr for visualization of muscle cells. After this, cells were subjected to three 5-min PBS washes and subsequently incubated in 1:100 Alexa594 goat anti-rabbit IgG (H+L) secondary antibody (Life Technologies) for 1 hr. Cells were once again subjected to three 5-min PBS washes and finally mounted with SlowFade Gold Antifade Mountant with DAPI (Life Technologies). All steps were conducted at room temperature; stained cells were kept at 4°C until analysis. Visualization was done using a Nikon Eclipse TE 2000-U fluorescence microscope.

The myogenic fusion index (MFI) was calculated by dividing the number of nuclei in myotubes, considered as having at least two nuclei sharing the same cytoplasm, by the total number of nuclei in an image and multiplying by 100 to arrive at a percentage value. Nuclei were counted by personnel blinded to the experimental conditions. Around 966 nuclei on average were counted (range: 548 – 1,400) for each replicate, per condition using Image J (NIH). The average MFI from three random fields of view were used for fusion index calculation for each replicate. Cell diameters were obtained using the measurement tool of Image J (NIH), by taking the average of three measured diameters across each myotube, and performing the measurement for at least 7-15 myotubes in a given field of view. Diameters were measured at the widest points across myotubes and away from branches or areas of overlap with other myotubes. At most 15 randomly selected myotubes were used for quantification; if less than 15 myotubes were present in a given field of view, then all myotubes were considered for quantification. Around 40 myotubes on average were measured (range: 25 – 45) for each replicate, per condition.

Apoptosis assessment by flow cytometry. WS229 and WS234 cells were seeded onto gelatin-coated 24-well plates (5×10^4 cells/well), grown, and differentiated as described above. At 13 days post-differentiation, LNA gapmers were transfected with Lipofectamine® RNAiMAX as previously described, at a final dose of 10 nM. The next day, cells were harvested by trypsinization, washed twice with PBS, and prepared for apoptosis evaluation via flow cytometry using the eBioscience™ Annexin V Apoptosis Detection Kit FITC (Thermo Fisher). Briefly, cells were washed once with 1x Binding Buffer and incubated in a 100 µl solution containing 5 µl each of fluorochrome-conjugated Annexin V and propidium iodide (PI) in 1x Binding Buffer. The mixture was incubated for 15 min at room temperature, and then cells were analyzed using Attune NxT (Life Technologies). Early apoptotic cells are Annexin-(+) and PI-(-), late apoptotic cells are Annexin-(+) and PI-(+).

Off-target effect evaluation. Potential off-targets were found using GGGenome (https://gggenome.dbcls.jp/), which works similarly to BLAST except it is more optimized for searching databases with short sequence inputs (5). Using the sequences of LNA1, LNA4, and LNA6, complementary targets with at least 1 mismatch were searched in the RefSeq human RNA release 90 (Sep 2018) database. Top hits were compiled in **Table S3**. No results were found with 1 mismatch for LNA6. Three potential off-target genes, *RASA4*, *PLEKHH3*, and *MGAT4B*, were chosen for further analysis as the others were not expressed at detectable levels in muscle. Expression levels of *RASA4*, *PLEKHH3*, and *MGAT4B* were evaluated by qPCR, using SYBR as outlined in the *RNA extraction from cells and qPCR* section, with primers in **Table S4**.

In vivo delivery of LNA gapmers. To visualize fluorescein-tagged gapmers, a 20-µl injection containing 20 µg of LNA4 in PBS was delivered intramuscularly (i.m.) to the tibialis anterior (TA) muscle in one of the legs of *FLExDUX4* mice (n = 2). Twenty-four hours after the injection, TAs were collected and snap frozen in isopentane cooled in liquid nitrogen. The frozen tissues were kept at -80°C until further processing. For visualization of fluorescein-tagged gapmers, 8 µm TA sections were prepared using a cryotome and visualized under a fluorescence microscope (Olympus BX61, 20X).

To determine the *in vivo* efficacy of LNA gapmer treatment, *FLExDUX4* mice were randomly assigned to either the treatment or mock groups. For the treatment group, the *FLExDUX4* mice (n = 5) received a 20-µl injection containing 20 µg of LNA4 in PBS via i.m. injections in the TA muscles of one of the legs. The injections were given every other day for a total of three i.m. injections. The TA muscles of the other legs received PBS as the control. Another five *FLExDUX4* mice received mock LNA in one TA muscle and PBS in the contralateral TA muscle. Tissues were harvested from mice 24 hours or 7 days after the final injection. RNAs were isolated from both TA muscles and cDNA was prepared and used for SYBR-based qRT-PCR detection of *DUX4* as described previously (24). Expression was normalized to *Gapdh* (6).

RNA secondary structure analysis. The Mfold RNA Folding web server (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form) (7) hosted by The RNA Institute, College of Arts and Sciences, State University of New York at Albany was used to determine the predicted secondary structure of *DUX4* exon 3. The *DUX4* exon 3 sequence with 50 bases of upstream (intron 2) and downstream sequences was used as input, and the pre-mRNA was folded using default parameters. The predicted folded structure with the most negative ΔG value was used for this study.

Statistical analysis. All statistical tests were conducted using GraphPad Prism 7 (GraphPad Software). For *in vitro* work, one-way ANOVA with post-hoc Tukey's or Dunnett's multiple comparisons test, or an unpaired two-tailed *t*-test was conducted as appropriate. For *in vivo* work, a paired *t*-test was conducted to determine the statistical significance of *DUX4* knockdown.

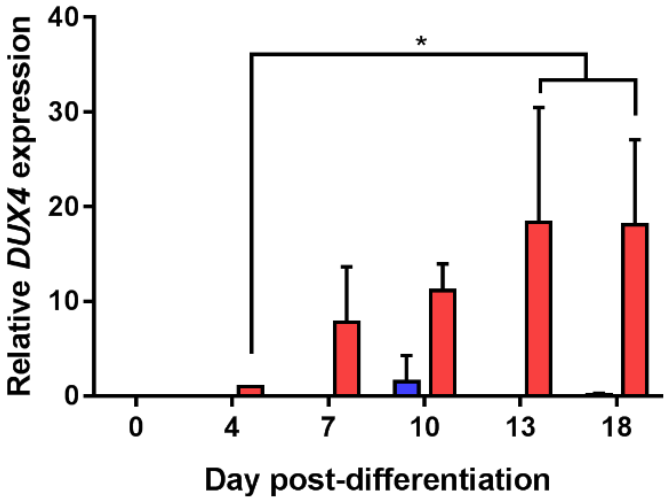

Figure S1. *DUX4* mRNA expression levels in the course of muscle differentiation *in vitro*. *DUX4* expression was evaluated at various times post-differentiation of healthy FSHD-unaffected (blue) or FSHD patient-derived (red) immortalized muscle cells. Error bars: S.D. (n = 3). *$p<0.05$, one-way ANOVA with Tukey's post hoc test.

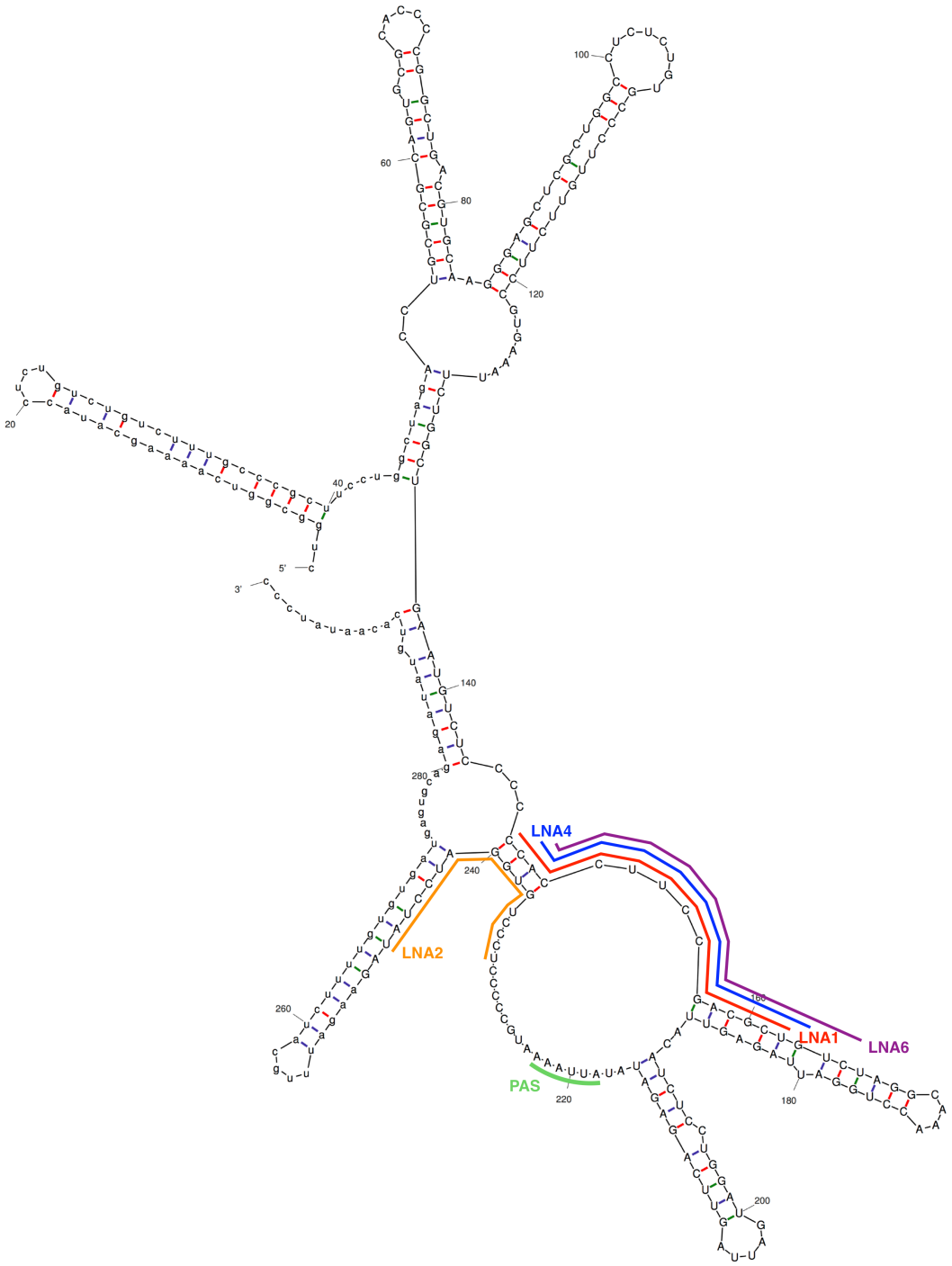

Figure S2. Predicted *DUX4* exon 3 pre-mRNA secondary structure. The locations of four LNA gapmers (LNA1, LNA2, LNA4, and LNA6) are mapped on the folded structure shown for *DUX4* exon 3. The PAS sequence is indicated as well.

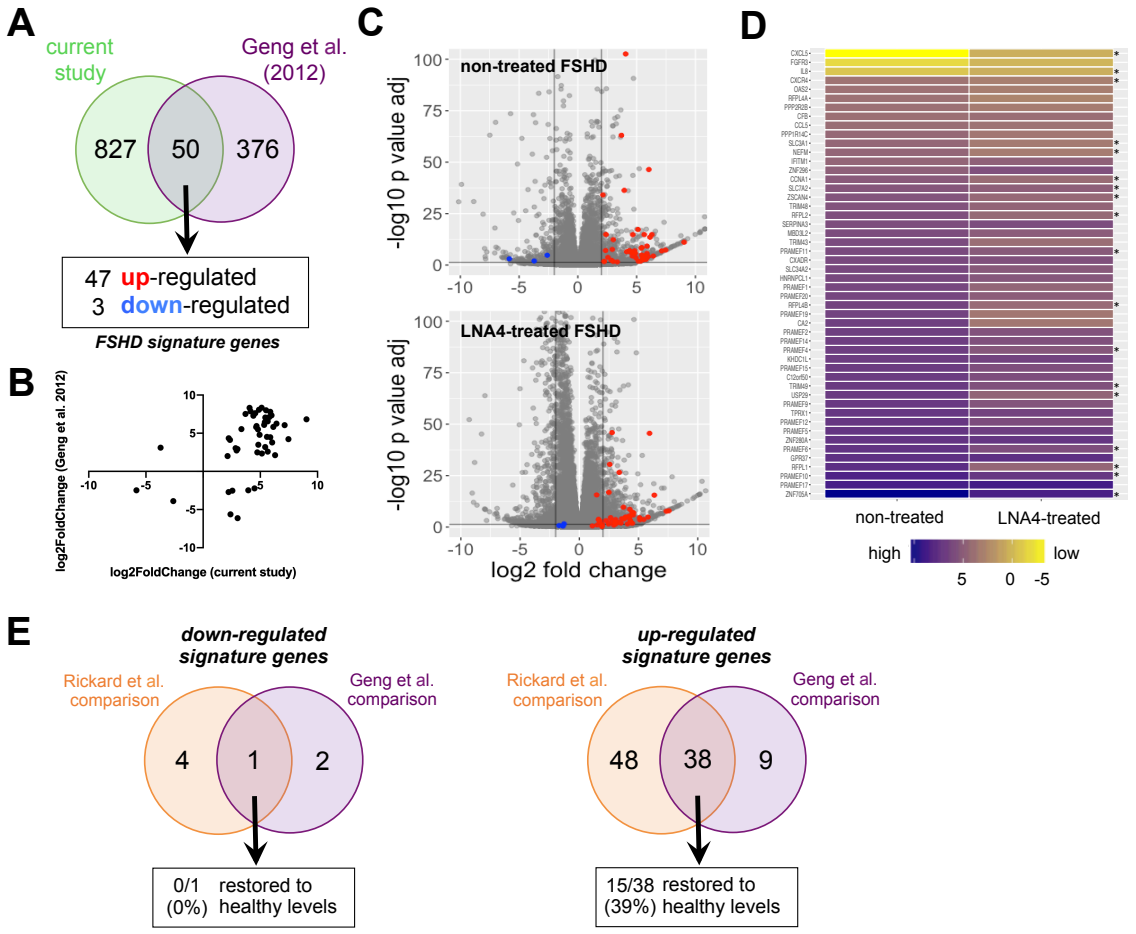

Figure S3. Alternative comparison for RNA sequencing analysis of LNA gapmer therapeutic efficacy. (A) FSHD signature genes were similarly determined following comparison with the dataset of Geng et al. (2012). (B) 2D plot of the $\log_2$(Fold Change) values between the present study and Geng et al. (2012), with a $\log_2$(FoldChange) cut-off of +2/-2 for the significantly affected genes of both datasets. (C) Volcano plots of RNA sequencing data from non-treated and LNA4-treated immortalized FSHD patient-derived muscle fibers, with healthy FSHD-unaffected control muscle fibers as reference. The 50 FSHD signature genes are marked (red: up-regulated, blue: down-regulated in FSHD); gray vertical lines indicate two-fold log2fold change values in either direction. (D) Heat map displaying expression changes for the 50 FSHD signature genes after treatment with LNA4, with the healthy FSHD-unaffected control as reference. High expression is indicated by a more purple shade, and genes with expression values significantly restored to healthy levels are indicated by an asterisk (n = 3). (E) Overlaps in the down- and up-regulated FSHD signature genes obtained from the two comparisons are shown. The numbers of overlapping genes whose expression values were significantly restored to healthy levels after LNA4 treatment are indicated.

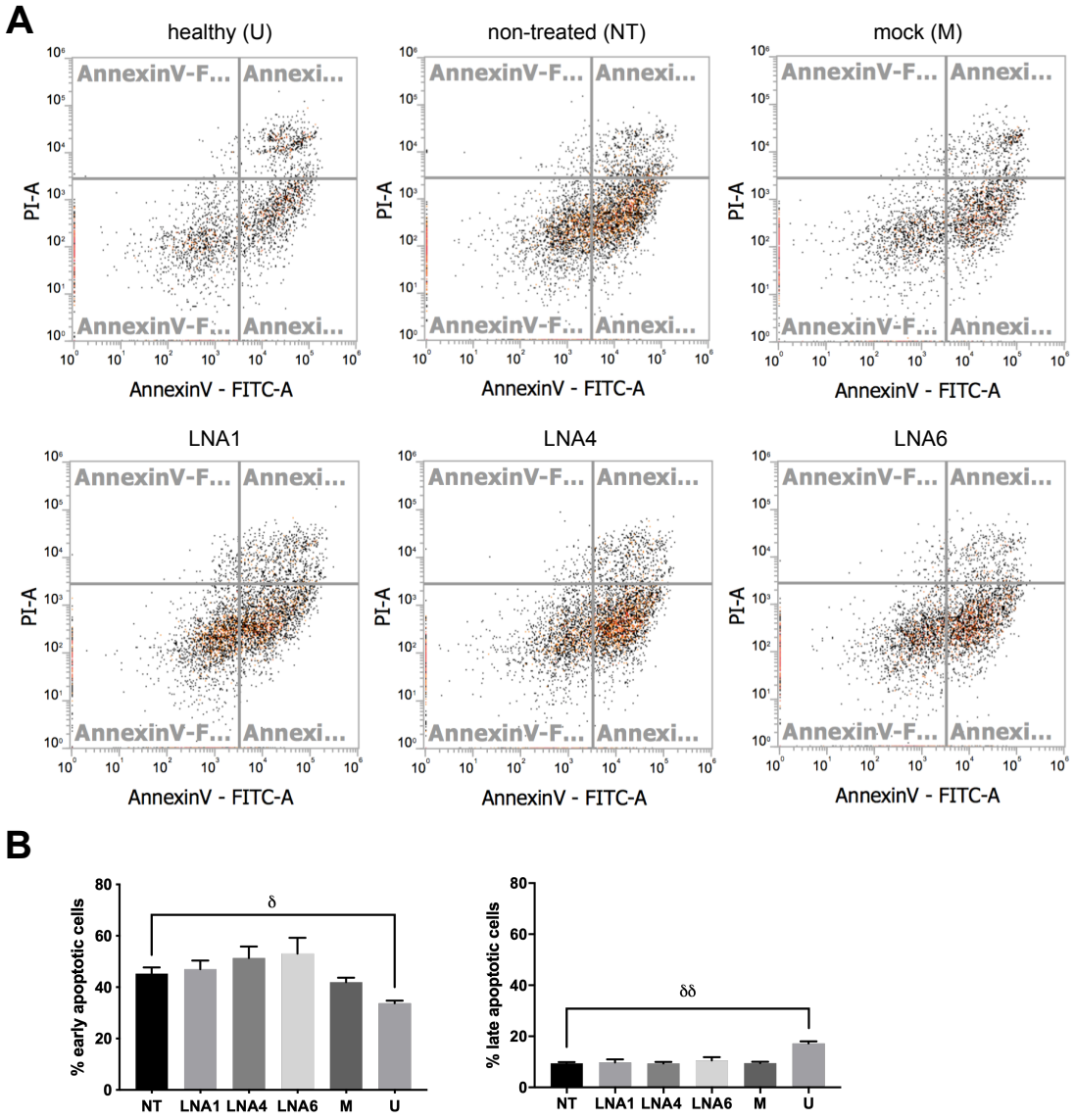

Figure S4. Flow cytometry evaluation of *in vitro* apoptosis. Healthy FSHD-unaffected control immortalized myotubes (U), non-treated (NT), LNA gapmer-treated, and mock-treated (M) immortalized FSHD patient-derived myotubes were harvested and stained with fluorochrome-conjugated Annexin V and propidium iodide to assess apoptotic condition by flow cytometry. (A) Representative plots are shown for when the cells were transfected with gapmers at 13 days post-differentiation and then evaluated for apoptosis the following day. The top-left quadrant represents late apoptotic cells, the bottom-right represents early apoptotic cells, and the bottom-left represents live cells. (B) Percentage of early and late apoptotic cells from immortalized healthy control and non-treated (NT) or gapmer-treated (transfected at 13 days post-differentiation) FSHD patient-derived cells at 18 days post-differentiation. Error bars: S.E.M. (n = 3). δp<0.05, δδp<0.005, unpaired two-tailed t-test.

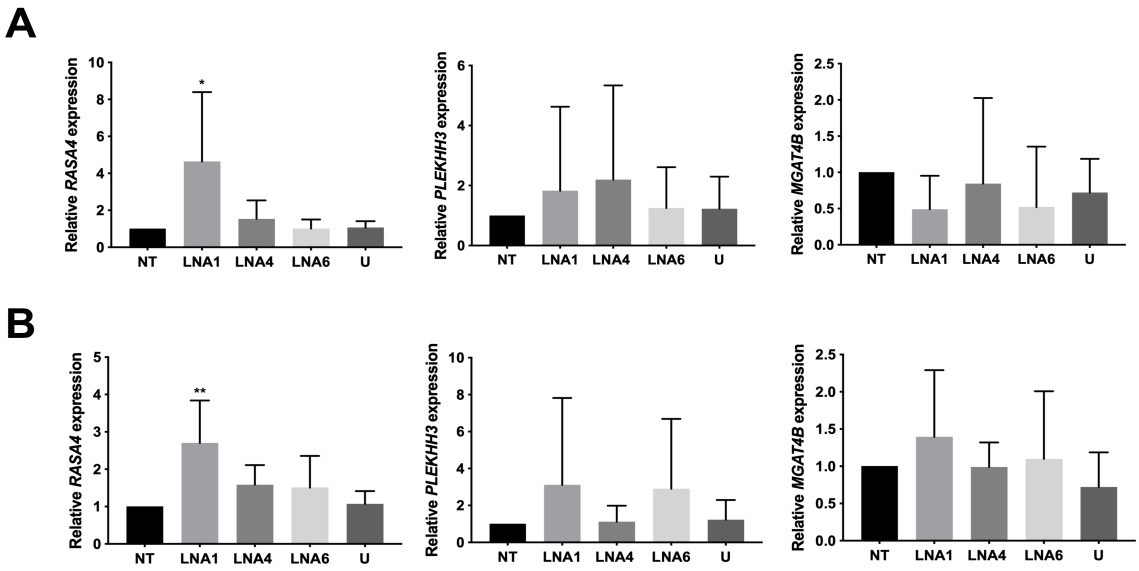

Figure S5. Evaluation of potential LNA gapmer off-target effect. LNA gapmer treatment effect on the expression of potential off-target genes *RASA4*, *PLEKHH3*, and *MGAT4B* was evaluated by qPCR. Expression of these genes was examined at the (A) 100 nM and (B) 10 nM transfected LNA gapmer dose. Error bars: S.D. (n = 5). **p*<0.05, ***p*<0.005, one-way ANOVA with Dunnett's test versus non-treated (NT). U = unaffected/healthy control.

Table S1. Significantly up-/down-regulated genes* comprising the FSHD signature.

Gene name	log_2 Fold Change	Adjusted p-value
FAM151A	10.387	2.20E-16
ALPPL2	9.07	1.68E-12
ZNF705A	9.038	7.96E-12
DPPA3	8.794	1.00E-11
TRIM51	8.737	4.42E-11
TRIM64B	7.648	2.09E-08
PRAMEF17	7.461	5.29E-08
PRAMEF10	7.13	1.39E-07
ALPP	6.779	3.89E-06
LEUTX	6.749	4.43E-11
PRAMEF22	6.643	7.61E-06
RFPL1	6.416	4.08E-05
GPR37	6.299	1.60E-15
TFAP2C	6.219	0.000682
UBTFL1	6.209	2.63E-05
DUXA	6.149	7.13E-07
PRAMEF6	6.137	3.11E-14
ZNF280A	6.036	3.76E-47
PRAMEF5	5.98	7.38E-05
ZSCAN5B	5.941	4.90E-07
TRIM51HP	5.891	0.00355
PRAMEF16	5.887	0.000427
PRAMEF12	5.874	6.28E-10
TPRX1	5.874	2.37E-06
PRAMEF9	5.797	0.00164
TRIM49B	5.687	2.75E-07
USP29	5.653	3.63E-05
ZIM3	5.646	2.63E-06
TRIM49	5.645	1.51E-15
ZNF679	5.59	7.37E-06
C12orf50	5.587	0.00264
KHDC1L	5.51	0.000149
PRAMEF4	5.434	3.14E-09
PRAMEF2	5.399	6.40E-09
CA2	5.342	0.0122
ACOT12	5.338	0.0192
PRAMEF19	5.144	0.0133
ZNF728	5.143	0.00696
CFP	5.13	0.0137
RFPL4B	5.114	5.08E-18
MBD3L3	5.005	1.83E-07
TRIM60	4.962	0.000174
PRAMEF20	4.943	0.00034
PRAMEF1	4.888	1.99E-06
ZNF705D	4.875	0.0476
SLC34A2	4.79	1.29E-06
AMOT	4.766	7.33E-28
CXADR	4.736	9.92E-07
TRIM53AP	4.722	3.75E-13
TRIM43B	4.673	2.25E-06
PRAMEF11	4.672	1.71E-15
TRIM43	4.641	3.92E-05
TRIM49C	4.623	6.41E-09
RFPL4AL1	4.584	0.0198
MBD3L2	4.557	6.31E-07
CCL20	4.475	0.0416
TC2N	4.423	4.96E-08
RFPL2	4.389	8.36E-08
FOXR1	4.204	0.0383
KDM4E	4.202	4.80E-10
TRIM48	4.152	3.66E-07
KLF17	4.135	1.39E-17
ZSCAN4	4.065	2.64E-103
RBP7	3.841	7.75E-05
TRPC5OS	3.734	1.57E-05
CCNA1	3.704	9.22E-64
GRIK1	3.4	3.65E-05
ZNF296	3.354	0.0285
MIAT	3.349	4.44E-28
OLFM1	3.265	3.18E-13
F2RL1	3.232	3.20E-12
SNAI1	3.21	1.92E-20
GRAMD1C	3.117	6.27E-05
NEFM	3.008	4.95E-13
FSTL4	2.959	0.000387
SLC3A1	2.926	2.56E-08
PPP1R14C	2.838	0.00432
ADAMTSL3	2.725	0.00788
ID4	2.648	0.00383
ADCY10	2.522	1.92E-07
PPP2R2B	2.355	8.57E-08
RFPL4A	2.242	0.0221
CXCR4	2.136	8.18E-35
PNMA2	2.134	7.21E-27
CDC42EP5	2.086	0.00018
RIPK4	2.075	4.80E-07
THSD7A	-2.369	0.0181
PLCXD3	-3.521	0.00723
MAP7D2	-3.636	2.17E-41
FGFR3	-3.715	0.0103
DNER	-3.873	1.25E-54

*versus the healthy FSHD-unaffected control

Table S2. FSHD signature genes with expression values significantly restored to healthy levels after LNA4 treatment *in vitro*.

Gene name	Up/Down-regulated in FSHD?	log_2 Fold Change*	Adjusted p-value*	Rickard et al. comparison**	Geng et al. comparison**
TC2N	up	-2.784	6.61E-06	O	X
TRIM49C	up	-1.692	0.00225	O	X
TRIM53AP	up	-1.686	0.000234	O	X
ZNF679	up	-1.584	0.0125	O	X
TRIM51HP	up	-5.838	0.00289	O	X
ZSCAN5B	up	-1.134	0.0164	O	X
DUXA	up	-1.739	0.00466	O	X
ALPP	up	-1.697	0.0436	O	X
TRIM64B	up	-1.567	0.00712	O	X
TRIM51	up	-2.182	5.20E-06	O	X
ALPPL2	up	-1.836	8.39E-08	O	X
FAM151A	up	-0.79	0.0069	O	X
CXCR4	up	-0.67	2.30E-05	O	O
SLC3A1	up	-1.177	0.0148	O	O
NEFM	up	-1.38	0.000635	O	O
CCNA1	up	-1.134	2.60E-07	O	O
ZSCAN4	up	-1.301	7.11E-18	O	O
RFPL2	up	-1.646	0.0235	O	O
PRAMEF11	up	-0.941	0.039	O	O
RFPL4B	up	-2.472	5.65E-09	O	O
PRAMEF4	up	-1.522	0.00625	O	O
TRIM49	up	-1.379	0.000363	O	O
USP29	up	-2.592	0.00295	O	O
PRAMEF6	up	-1.691	1.18E-07	O	O
RFPL1	up	-3.502	0.00305	O	O
PRAMEF10	up	-1.326	0.00883	O	O
ZNF705A	up	-1.688	0.000274	O	O
SLC7A2	up	-0.575	0.0212	X	O
CXCL5	down	4.455	0.0146	X	O
IL8	down	1.319	0.0474	X	O

*versus the non-treated FSHD-affected control

**"O" denotes inclusion in the gene set obtained from the comparison of our RNA sequencing data with the indicated study (Rickard et al. or Geng et al.); "X" denotes that the gene is not part of this comparison set.

Table S3. Potential off-targets of LNA gapmers 1, 4, and 6 as identified by GGGenome.

Gene	Transcript variant/s	Sequence showing mismatch*	# mismatches with LNA gapmer		
			LNA1	LNA4	LNA6
DUX4	n/a	CCACCTTCCGACGCTGTC	0	0	0
GALNT14	4	CCACCTTCGGACGCTGAC	1	1	2
RASA4/4B	1, 2	CCACCTTCC-ACGCTGTG	1	1	2
CHD5	n/a	CCACCTTCCGAAGCTCCT	1	2	4
PLEKHH3	1, 2, 3	GCACCTTCCGAC-CTGGG	2	1	3
MGAT4B	1	GCACCTTCCTGACGCTGCT	2	1	3
LINC01561	n/a	ACACCTTCCGACACTGGG	2	1	3
POLR2J4	n/a	CCACCTTCC-ACGCTGTG	1	1	2

*Red indicates mispairing, green indicates additional/missing base compared to reference *DUX4*

Table S4. Primer sequences used in this study.

Gene	Forward (F) and reverse (R) primers, 5' to 3'
DUX4	F: CCCAGGTACCAGCAGACC R: TCCAGGAGATGTAACTCTAATCCA
GAPDH	F: GCAAATTCCATGGCACCGT R: AGGGATCTCGCTCCTGGAA
RASA4	F: CCCATCATCAACAAGGTGTTTG R: GGAGCACCCTACATCCTTAAC
PLEKHH3	F: AGAGCTGGGAGGAGACTT R: GTACAGCCAACCTTTCACAAC
MGAT4B	F: GAGTCAGGTGGAGGACCAAA R: CGTAGTAGATGCCTTTGGACTG

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