Designed U7 snRNAs inhibit DUX4 expression and improve FSHD-associated outcomes in DUX4 overexpressing cells and FSHD patient myotubes

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INTRODUCTION
Facioscapulohumeral muscular dystrophy (FSHD) arises from epigenetic changes that de-repress the DUX4 gene in muscle. The full-length DUX4 protein causes cell death and muscle toxicity, and therefore we hypothesize that FSHD therapies should center on inhibiting full-length DUX4 expression. In this study, we developed a strategy to accomplish DUX4 inhibition using U7-small nuclear RNA (snRNA) antisense expression cassettes (called U7-asDUX4). These non-coding RNAs were designed to inhibit production or maturation of the full-length DUX4 pre-mRNA by masking the DUX4 start codon, splice sites, or polyadenylation signal. In so doing, U7-asDUX4 snRNAs operate similarly to antisense oligonucleotides. However, in contrast to oligonucleotides, which are limited by poor uptake in muscle and a requirement for lifelong repeated dosing, U7-asDUX4 snRNAs can be packaged within myotrophic gene therapy vectors and may require only a single administration when delivered to post-mitotic cells in vivo. We tested several U7-asDUX4s that reduced DUX4 expression in vitro and improved DUX4-associated outcomes. Inhibition of DUX4 expression via U7-snRNAs could be a new prospective gene therapy approach for FSHD or be used in combination with other strategies, like RNAi therapy, to maximize DUX4 silencing in individuals with FSHD.

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to deliver artificial microRNAs (miRNAs) engineered to knock down the DUX4 mRNA via RNA interference (RNAi) in vitro and in vivo. Other groups demonstrated that antisense oligonucleotides (ASOs), designed to mask splice sites and/or the poly(A) signal, could interfere with DUX4 mRNA production and suppress full-length DUX4 expression in FSHD cells. The system we describe here has some similarity to these ASO studies because we utilize antisense sequences in our designs. However, our approach is distinct because it uses a recombinant U7 small nuclear RNA (U7-snRNA) expression system to produce antisense sequences, overcoming some major limitations of ASOs, such as inefficient delivery to muscle and a requirement for lifelong administration.

The U7-snRNA is a component of the small nuclear ribonucleoprotein complex (U7-snRNP), involved in 3' end processing of histone pre-mRNAs in the nucleus. The wild-type U7 snRNA contains an antisense RNA sequence at its 5' end, which normally base pairs with a histone pre-mRNA, as well as binding sites to seed the formation of 7 core Sm proteins. At the 3' end is a hairpin structure that provides stability to the RNA. Importantly, the antisense sequence of U7-snRNA can be changed to retarget the RNP to other mRNAs, such as DUX4. When embedded into an snRNP complex, the U7-snRNA is protected from cellular enzymatic digestion, allowing it to remain in the nucleus for a longer time compared with ASOs and extending the effects of masking RNA sequences like splice sites, start codons, or poly(A) signals. Furthermore, U7-snRNA expression cassettes can be packaged into adeno-associated virus (AAV) particles to achieve widespread delivery of therapeutic antisense sequences at high efficiency into animal and human target tissues, including muscle. Thus, although the ASO approach for DUX4 suppression would require chronic, lifelong administration of chemically synthesized sequences, AAV-delivered U7-snRNAs are produced in target cells using endogenous transcriptional machinery, offering a chance to provide long-term DUX4 inhibition with one administration. In this study, we designed several U7-snRNAs containing antisense regions with sequence complementarity to the DUX4 start codon, splice sites and splice enhancers, or regulatory elements (3' UTR and poly(A)) and investigated their efficacy for DUX4 silencing in HEK293 cells overexpressing DUX4 and in myotubes from individuals with FSHD expressing endogenous levels of DUX4. We show that the U7-snRNA system can reduce DUX4 and DUX4-associated outcomes in these human cell models, supporting further translation of the U7-snRNA system for gene therapy of FSHD.

RESULTS

DUX4-targeting U7-snRNAs reduce apoptosis and increase the viability of co-transfected HEK293 cells

Recombinant U7-snRNAs were developed previously to induce skipping of mutated exons as potential treatment for Duchenne muscular dystrophy and β-thalassaemia. In these studies, U7-snRNAs were used to restore the expression of frameshifted genes by skipping entire exons. In contrast, our goal here was to develop a novel gene silencing strategy by using U7-snRNAs to interfere with DUX4 pre-mRNA maturation or inhibit translational initiation. To do this, we developed recombinant U7-snRNAs targeting splice donor (SD), splice acceptor (SA), and splice enhancer (SE) sequences or the polyadenylation signal (PAS) in DUX4 exon 3 (Figures 1A and 1B). In addition, we generated two constructs (9 and 10) designed to sit atop the full-length DUX4 start codon and potentially interfere with translation. The structure of our DUX4-targeting U7-snRNAs (called U7-antisense [as]DUX4) is shown in Figure 1A, where the key feature for specificity is an antisense sequence modified to base-pair with various regions of the DUX4 pre-mRNA (Figure 1A). To choose effective sequences for interfering with correct splicing, we used the Human Splicing Finder tool (Figure S1) to predict potential SD, SA, and SE sites for all three DUX4 exons and within introns 1 and 2. We then designed U7-asDUX4s to target the highest-scoring sites (Figure 1B). For those U7-asDUX4s targeting the poly(A) signal or start codon, we ensured that the antisense sequences provided complete coverage of the cognate sites on the DUX4 mRNA. All U7-asDUX4 sequences and their important features are summarized in Table S1.

HEK293 cells do not normally express DUX4 protein or polyadenylated DUX4 mRNA but are susceptible to DUX4-induced cell death following transfection with a cytomegalovirus (CMV).DUX4 expression plasmid. We therefore initially assessed the efficacy of U7-asDUX4 expression plasmids by measuring apoptotic cell death using caspase-3/7 and cell viability assays as outcome measures in co-transfected HEK293 cells. We designed and tested 18 U7-asDUX4 sequences and found that 13 significantly reduced cell death (>50%) and increased viability (>50%) of co-transfected HEK293 cells (Figures 1C and 1D). Combining the two parameters, the most effective U7-asDUX4s were constructs 4, 7, and 8, targeting the exon 1-intron 1 junction or the PAS. Cells transfected with these constructs showed reduced relative caspase-3/7 activity (U7-asDUX4-4, 75% ± 7% reduction; U7-asDUX4-7, 60% ± 9% reduction; U7-asDUX4-8, 50% ± 8% reduction) and significantly increased viability (U7-asDUX4-4, 79% ± 8%; U7-asDUX4-7, 95% ± 5%; U7-asDUX4-8, 85% ± 4%) compared with only DUX4 (19% ± 2%) or DUX4 with nontargeting U7-snRNA (23% ± 0.5%) (Figures 1C and 1D). Because of their superior protective properties, we selected U7-asDUX4 constructs 4, 7, and 8 as our lead candidate sequences.

U7-asDUX4s significantly decrease DUX4 expression in transfected HEK293 cells

The reduction in DUX4-related cell death outcomes in samples treated with U7-asDUX4 plasmids suggested that these sequences operated to inhibit full-length DUX4 gene expression. To investigate the specificity of our lead U7-asDUX4 sequences to target and reduce overexpressed DUX4 mRNA in HEK293 cells, we first used the RNA-scope in situ hybridization assay to detect DUX4 mRNA in co-transfected cells. Fixed cells were incubated with probes targeting DUX4, control transcripts, or negative control reagents and then treated with a dianimobenzidine (DAB) reagent that stains hybridized target mRNAs brown. As we reported previously, cells transfected with DUX4 expression plasmid alone showed abundant, spider-like brown signals when incubated with DUX4 probes as well as relatively low cell...
density consistent with death (Figure 2A). In contrast, the DUX4 probe signal was reduced significantly in cells co-transfected with DUX4 and our three lead U7-asDUX4 (Figures 2B–2D). Specifically, in U7-asDUX4-treated wells, there were significantly fewer DUX4-positive cells and/or reduced intensity of DUX4 staining in cells that still showed DUX4 signal (Figures 2B–2D). Positive and negative controls behaved as expected; we found little to no DUX4 signal in untransfected HEK293 cells (Figure 2E), whereas abundant signal was evident in cells stained with probes to the peptidylprolyl isomerase B (PPIB) gene (Figure 2F), a positive control for the RNAscope assay. Consistent with DUX4 knockdown, which provided some protection from cell death (Figure 1D), wells transfected with U7-asDUX4 plasmids had greater cell density compared with “DUX4-only”-transfected samples.

U7-asDUX4 snRNAs reduce full-length DUX4 protein in transfected HEK293 cells

To detect DUX4 protein, we then stained cells with fluorescence-labeled antibodies to the V5 tag (CMV.DUX4.V5-full-length [FL]) (Figure 3A). To additionally confirm DUX4 protein knockdown by our lead U7-snRNAs, we performed similar co-transfection experiments in HEK293 cells but used western blots as an outcome measure. In addition, because the C-terminal V5 tag disrupted the U7-asDUX4-4 binding site, we utilized a different DUX4 expression construct in this set of experiments. Specifically, we generated a CMV.DUX4 expression plasmid containing an amino-terminal, in-frame Myc epitope tag (Figure 3C). We also reasoned that this construct would allow us to determine whether the U7-asDUX4 sequences designed to mask the FL DUX4 mRNA SD/SA near the 3’ end of exon 1 would bias splicing to produce a truncated and non-toxic DUX4-s protein isoform (Figure 3C). We performed western blotting using protein lead U7-asDUX4 constructs or a non-targeting control along with a CMV.DUX4 expression plasmid. To facilitate protein detection, we used a full-length DUX4 construct containing an in-frame COOH-terminal fusion of the V5 epitope tag (CMV.DUX4.V5-full-length [FL]) (Figure 3A).
However, we did not note a strong correlation between DUX4 levels and the abundance of that upper band if it is DUX4 s transcript using 3' rapid amplification of cDNA ends (RACE) RT-PCR (Figure S3). We also noted that the non-specific DUX4 protein that migrated at the size of endogenous c-Myc protein (52 kDa) in all transfected cells, and all samples contained a larger non-DUX4 protein that was detected in all HEK293 cells and served as a positive control for the assay. (G) The bacterial gene dapB probe was used a negative control for the RNAscope assay. (H) RNAscope quantification showed a significantly reduced DUX4-positive signal in DUX4-transfected cells co-expressing U7-asDUX4 snRNAs 4, 7, and 8. 40× objective. Scale bar, 50 μm. Quantification was performed as described previously.50 Two representative microscopic fields were counted from 3 independent experiments; each point represents quantification of one field. *p < 0.01, ANOVA.

Figure 2. U7-asDUX4 snRNAs significantly reduced overexpressed DUX4 mRNA in transfected HEK293 cells.

extracts from HEK293 cells co-transfected with plasmids expressing Myc.DUX4 and five different U7-asDUX4 constructs, including our 3 leads and sequences 5 and 6, which were designed to base-pair near the exon 1-intron 1 junction. Using a Myc epitope antibody, we detected the FL Myc-DUX4 protein band (52 kDa) in all transfected cells, and all samples contained a larger non-DUX4 protein that migrated at the size of endogenous c-Myc protein (~60 kDa). Consistent with our previous experiments, our lead constructs, U7-asDUX4 sequences 4, 7, and 8, significantly reduced DUX4 protein by 87% ± 10%, 66% ± 15%, and 85% ± 14% (n = 3 independent experiments; Figures 3D and 3E; Figure S2). We did not detect evidence of DUX4 s production by western blot (predicted size, 22 kDa), suggesting that the reduction of FL DUX4 gene expression by U7-asDUX4 sequences designed to mask DUX4 splice sites (4, 5, 6, and 7) did not operate by shifting splicing patterns to favor the DUX4 s isoform. Similarly, we found no evidence of a shorter DUX4 s transcript using 3' rapid amplification of cDNA ends (RACE) RT-PCR (Figure S3). We also noted that the non-specific upper band on these western blots showed variable expression. Because DUX4 has been shown to activate Myc, it is possible that changing DUX4 levels could affect the abundance of that upper band if it is Myc.15 However, we did not note a strong correlation between residual DUX4 and Myc abundance in these experiments.

U7-asDUX4 snRNAs decrease DUX4-activated biomarker expression in FSHD myotubes

Our results in HEK293 cells suggested that several U7-asDUX4 snRNAs could reduce FL DUX4 expression and offer protection from cell death in an overexpression model. Next we assessed the ability of lead U7-asDUX4 snRNAs (4, 7, and 8) to decrease endogenous DUX4 mRNA in myotubes from individuals with FSHD using RNAscope in situ hybridization. We previously used RNAscope to detect DUX4 in FSHD myotubes.50 Consistent with prior reports, we found that DUX4 staining was only present in a small percentage of cells at any given time, but importantly, we were also able to quantify DUX4 knockdown following delivery of an artificial DUX4-targeted miRNA (mi405).40,41 We therefore used RNAscope to determine whether U7-asDUX4 snRNAs could reduce endogenous DUX4 signal in myotubes derived from individuals with FSHD, supporting the potential translatability of this approach. To do this, we used electroporation to transfect FSHD muscle cells, which typically yields ~50%–70% transfection efficiency (Figure S4). Consistent with our previous results, untransfected 15A FSHD myotubes showed brown DUX4 signals, whereas those transfected with U7-asDUX4 sequences 4, 7, and 8 had significantly reduced RNAscope signals (Figures 4A–4H). This supported that these three U7-asDUX4s lead to destabilization and degradation of endogenous DUX4 mRNA in FSHD muscle cells.

U7-asDUX4 snRNAs decrease DUX4-activated biomarker expression in FSHD myotubes

With the emergence of prospective FSHD therapies came a need in the FSHD field to develop clinical outcome measures and biomarkers that could be used to establish therapeutic efficacy.41-44 Although DUX4 expression is the most direct measure of target engagement by a prospective drug or gene therapy, it is difficult to detect and relatively scarce in FSHD muscle biopsies. Thus, DUX4 expression in human muscle biopsies is currently not a reliable outcome measure for FSHD clinical trials, and several groups have now turned to examining DUX4-activated biomarkers as an indirect measure of DUX4.
expression. At least 67 different genes contain regulatory regions with DUX4 binding sites and are consistently activated upon DUX4 expression. However, recent studies suggest that only a few biomarkers are needed to represent the entire set.\(^{17,19,20,45-48}\) We selected four biomarkers in this study (ZSCAN4, PRAMEF12, TRIM43, and MBD3L2) because they are established DUX4 target genes and FSHD disease biomarkers and consistently show differential expression between FSHD and healthy control cells in our experiments.\(^{19,20,24,26,30}\) We therefore tested the ability of our lead U7-as-DUX4 snRNAs to suppress these DUX4-activated biomarkers in cells from individuals with FSHD. To do this, we transfected 15A myoblasts from individuals with FSHD with U7-asDUX4-4, -7, and -8 as well as a non-targeting control. We then differentiated cells into myotubes for 7 days and performed quantitative RT-PCR to measure expression of the DUX4-activated human biomarkers TRIM43, MBD3L2, PRAMEF12, and ZSCAN4. All four biomarkers were present in untreated 15A myotubes and reduced significantly in U7-asDUX4-treated 15A cells (Figure 4I).
DISCUSSION

The FSHD field has made great strides in the last decade or so by identifying DUX4 as a primary target for therapeutic intervention, generating numerous cell and animal models, producing several prospective therapeutic strategies, and working to define outcome measures for clinical trials. Despite this progress, there are still no approved treatments for FSHD, and therapeutic development remains a critical need in the field. We propose that FSHD therapies should focus on inhibiting DUX4, and several strategies that target the gene, mRNA, and/or protein could be utilized.24–29,31,49 Our lab has been primarily focused on attacking the DUX4 mRNA, and we previously demonstrated the safety and efficacy of DUX4 silencing using RNAi-based gene therapy delivered by AAV vectors in pre-clinical studies.31 This strategy is now being translated. However, because even very small amounts of DUX4 protein may be toxic in muscle cells, we believe that it is beneficial to develop additional DUX4 silencing strategies employing alternative mechanisms, which could be used alone or in combinatorial therapies, to help maximize DUX4 silencing in muscles of individuals with FSHD. Here we describe a new method to accomplish DUX4 silencing using designed U7-asDUX4 snRNAs.

The U7-asDUX4 constructs we developed have some parallels to ASOs, which have been tested previously in FSHD models.18,23–25,29 A significant amount of knowledge about ASOs for treatment of muscle disease comes from their use in preclinical and clinical studies for treating Duchenne muscular dystrophy (DMD) and myotonic dystrophy (DM1).50–54 In the case of DMD, ASO uptake is facilitated by porous cell membranes associated with loss of the dystrophin glycoprotein complex.55–57 In contrast, intact muscle membranes in DM1 patients serve as a barrier to efficient ASO delivery, suggesting that improved delivery mechanisms are needed when treating muscle diseases without membrane defects.58 Similar to DM1, membrane damage is not a feature of muscles of...
individuals with FSHD; thus, delivering ASOs to FSHD muscles could be challenging. In addition, DUX4 is rare and not present in most FSHD myonuclei at any given time, but when it turns on at sufficiently toxic levels, it could rapidly kill cells expressing it. Thus, it is likely that therapies designed to inhibit DUX4 will need to be present within target muscles prior to DUX4 activation. Accomplishing this with ASOs will require repetitive, lifelong systemic dosing to produce optimal therapeutic efficacy. In contrast to ASOs, U7-snRNAs can be packaged as DNA expression cassettes within AAV vectors, many of which have natural myotropism. Thus, AAV-delivered U7-asDUX4 snRNAs should express the therapeutic antisense RNAs as long as the vector is present within a target muscle cell and the promoter is active. In stable, post-mitotic muscle, this could theoretically provide lifelong protection following a single administration, although the persistence of AAV transduction in human muscle still requires more study. In addition, U7-snRNAs are produced in the host nucleus, facilitating access to DUX4 pre-miRNA, and their association with the U7-snRNPs increases their resistance to degradation. These properties increase their effectiveness and reduce the necessity for repeated administration, especially for diseases that require lifelong treatment, such as FSHD. In the next step of our study, it will be important to assess the long-term safety and efficacy of our lead U7-asDUX4 sequences, or combinations thereof, using AAV vectors in our FSHD mouse models. Part of this will include investigation of sequence-specific off-target effects. Nucleotide BLAST results using U7-asDUX4-4 and -7 against the human transcriptome yielded no significant hits, whereas sequence 8 has partial sequence complementarity to some human transcripts, including ZNF91 (80%), NAA16 (60%), CTB5A (53%), CDH19 (53%), and STEAP2 (50%) and 46% sequence complementarity with TCAIM LIPK, FAM177A1, MAN1A1, XCR1, and the FREM2 and ZDHHC3 non-coding RNAs. These predicted changes could be confirmed experimentally using RNA sequencing (RNA-seq) and/or western blotting to detect protein changes.

U7 snRNAs are transcribed in the nucleus and then exported to the cytoplasm, where they assemble with Sm and Lsm proteins. The assembled U7-snRNP can remain in the cytoplasm or be imported back into the nucleus. In the nucleus, they are associated with splicing machinery, whereas in the cytoplasm, they associate with P bodies, which normally function in mRNA turnover. Similarly, miRNAs can be detected in the nucleus and the cytoplasm because they are transcribed and matured in the nucleus and then transported to the cytoplasm for translation. The sequences we identified as our lead constructs target the exon 1-intron1 junction (U7-asDUX4-4 and -7) or the DUX4 poly(A) signal (U7-asDUX4-8). Targeting the splice junction is a new approach, but using antisense sequences to bind the DUX4 PAS has been done previously using chemically synthesized ASOs, which have been shown to reduce DUX4 and DUX4-activated biomarkers in vitro and in vivo. Polyadenylation is an important process required for stabilizing nascent miRNAs and coordinating mRNA transit through nuclear pores to the cytoplasm for translation. Chemically synthesized DNA-based ASOs may operate by forming DNA:RNA hybrids and activating RNase H against the target transcript, but it is also possible that published ASO sequences designed to base-pair with the DUX4 poly(A) signal could operate by masking the signal and preventing polyadenylation, leading to DUX4 mRNA destabilization. Because the antisense portion of our PAS-targeting U7-asDUX4-8 was composed of RNA, it should not be able to reduce DUX4 expression via RNase H, which requires base-pairing of DNA:RNA molecules. It is therefore more likely that U7-asDUX4-8 operates by recruiting snRNP proteins to the DUX4 transcript and sterically hinders poly(A) machinery, destabilizing DUX4 mRNA. Thus, sequence 8 might work in the nucleus, whereas sequences 4 and 7, which are located near the exon 1 splice junction, could operate in the nucleus or the cytoplasm.

In conclusion, we identified three lead U7-asDUX4 constructs that significantly reduced DUX4 and DUX4-associated outcomes in cotransfected cells and myotubes derived from individuals with FSHD. Our findings provide a proof of concept for DUX4 silencing using recombinant U7-asDUX4 as a treatment for FSHD. Translating this approach will require evaluating efficacy and safety in FSHD mouse models following AAV-mediated delivery, which is now ongoing in our laboratory.

MATERIALS AND METHODS

Designing DUX4 targeting U7-snRNAs

The Human Splicing Finder version 3.1 program from Marseille University (http://umd.be/Redirect.html) was used to predict potential SA, SD, and SE sites at the end of DUX4 exon 1 (coding sequence) and within the untranslated exons 1 and 2. For designing U7-snRNAs against DUX4 (called U7-asDUX4s), we selected 18 high-scoring target sites with the fewest number of CpGs and one non-targeting region (Table S1). Predicted off-target matches were determined by BLAST, using each sequence against the human genome database (http://blast.ncbi.nlm.nih.gov/blast.cgi). The expression cassettes of all U7-asDUX4s, containing a mouse U7 promoter, were synthesized and cloned into the pUCIDT plasmid (Integrated DNA Technologies). Sequences were also designed to bind the DUX4 start codon and poly(A) signal via reverse complementary base-pairing (Table S1). The non-targeting control snRNA antisense sequence is 5’-GTCATATCGTGCCCGGTGCTGCAGACACGTCGG-3’.

Cell culture

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin at 37°C in 5% CO2. Affected and unaffected immortalized human myoblasts derived from an individual with FSHD and an unaffected relative (15Abic and 15Vbic) were expanded in DMEM supplemented with 16% medium 199, 20% fetal bovine serum, 1% penicillin/streptomycin, 30 ng/mL zinc sulfate, 1.4 mg/mL vitamin B12, 55 ng/mL dexamethasone, 2.5 ng/mL human growth factor, 10 ng/mL fibroblast growth factor, and 20 mM HEPES. Cells were maintained as myoblasts and differentiated prior to measuring DUX4 mRNA and DUX4-activated biomarkers by qRT-PCR and RNAscope. To differentiate myoblasts into myotubes, transfected
myoblasts were switched to differentiation medium composed of a 4:1 ratio of DMEM:medium 199 supplemented with 15% KnockOut Serum (Thermo Fisher Scientific), 2 mM L-glutamine, and 1% antibiotics/antimycotics for up to 7 days before harvesting.

Viability assay
HEK293 cells (250,000 cells/well) were co-transfected (Lipofectamine 2000, Invitrogen) with an expression plasmid from which FL DUX4 pre-mRNA (DUX4-FL) was transcribed from the CMV promoter (CMV.DUX4-FL) along with plasmids expressing U7-asDUX4 snRNAs or the non-targeting U7-snRNA at a 1:6 ratio using the protocol. The cells were trypsinized 48 h after transfection and collected in 1 mL of growth medium. Automated cell counting was performed using Countess cell counting chamber slides. The results were confirmed with traditional cell counting using a hemocytometer and trypan blue staining. Three independent experiments were performed, and data were reported as a mean of total cell number ± SEM per group.

Cell death assay
HEK293 cells (42,000 cells/well) were plated on a 96-well plate 16 h prior to transfection. The next morning, cells were co-transfected (Lipofectamine 2000, Invitrogen) with CMV.DUX4-FL and U7-asDUX4 snRNAs or a non-targeting U7-snRNA expression plasmid at a 1:6 molar ratio. Cell death was measured using the Apo-ONE homogeneous caspase-3/7 assay (Promega, Madison, WI) 48 h after transfection using a fluorescent plate reader (Spectra Max M2, Molecular Devices, Sunnyvale, CA). Three individual assays were performed in triplicate, and data were averaged per experiment and reported as mean caspase activity ± SEM relative to our control assay, which was transfected with CMV.DUX4-FL only.

Western blot assay
For this experiment, DUX4 expression plasmids were used with and without epitope tags (CMV.Myc-DUX4-FL, which contained a Myc epitope tag fused to the DUX4 N terminus, or CMV.DUX4-FL). HEK293 cells were co-transfected at a 1:6 ratio of DUX4:U7asDUX4 expression plasmids. Twenty hours after transfection, cells were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100) supplemented with a cocktail containing protease inhibitors. Protein concentration was determined using the Lowry protein assay kit (Bio-Rad). 25 μg of each protein sample was run on a 12% SDS-polyacrylamide gel. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes via a semi-dry transfer method, blocked in 5% non-fat milk, and incubated with primary monoclonal mouse anti-DUX4 (1:500; P4H2, Novus Biologicals), mouse anti-Myc (R95125, Invitrogen), or rabbit polyclonal anti-α-tubulin antibodies (1:1,000; ab15246, Abcam, Cambridge, MA) overnight at 4°C. The next day, following multiple washes, blots were probed with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1:100,000; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Protein bands were developed on X-ray films after short incubation in Immobilon chemiluminescent HRP substrate (Millipore, Billerica, MA). Protein quantification was assessed by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA; https://imagej.nih.gov/ij/).

Immunofluorescence staining
V5 epitope-tagged DUX4 protein in treated and untreated cells was visualized using V5 immunofluorescence staining. 14 HEK293 cells were transfected with a plasmid carrying a FL DUX4 sequence consisting of the coding and 3′ UTR sequences but engineered to express DUX4 protein with an in-frame carboxy-terminal V5 epitope fusion. Twenty hours after transfection, cells were fixed in 4% paraformaldehyde (PFA) for 20 min, and nonspecific antigens were blocked with 5% BSA in PBS supplemented with 0.2% Triton X-100. The cells were incubated at 4°C, overnight in rabbit polyclonal anti-V5 primary antibody (1:2,500, Abcam, ab9116). The following day, cells were washed with PBS, incubated with goat anti-rabbit Alexa 594 secondary antibodies (1:2,500, Invitrogen), and mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA).

RNAseq assay and quantification
Detecting overexpressed DUX4 in HEK293 cells
We used RNAseq in situ hybridization assay to measure DUX4 mRNA levels following co-transfection of CMV.DUX4-FL and U7.asDUX4 expression plasmids in HEK293 cells (1:6 ratio). Specifically, HEK293 cells were seeded in triplicate on glass coverslips in 24-well plates at a density of 120,000 cells per well 16 h prior to transfection. The next morning, upon reaching 70% confluence, cells were co-transfected with 250 ng of CMV.DUX4-FL expression plasmid (Lipofectamine 2000, Thermo Fisher Scientific), according to the manufacturer’s instructions. Sixteen hours after transfection, cells were fixed with 4% PFA, and RNAseq staining was performed following the manufacturer’s instructions (ACDBio; detailed below).

Detecting endogenous DUX4 in human FSHD myotubes
To determine the specificity of U7-asDUX4 snRNAs for targeting endogenous DUX4 mRNA, 15Abic FSHD myoblasts (15A, 500,000 cells/reaction) were transfected with U7-asDUX4 expression plasmids via electroporation (Lonza, VVPD-1001) and then differentiated into myotubes for up to 7 days. RNAseq staining was performed as described previously. 15 The cells were fixed in 4% PFA and dehydrated/rehydrated with ethanol gradients. Endogenous peroxidase activity was blocked by hydrogen peroxide treatment. Protease III was added to increase the permeability of fixed cells for RNAseq probes. The cells were treated with a DUX4-specific RNAseq probe (ACDBio, catalog number 498541) or probes to detect the positive control housekeeping gene peptidylprolyl isomerase B (PPIB) and negative control bacterial gene dihydrodipicolinate reductase (dapB). Following probe incubation, cells were treated with several signal amplification steps using RNAseq 2.5 HD Assay Brown according to the manufacturer’s protocol (ACDBio). The cells were counterstained with 50% Gill’s hematoxylin I (catalog number HXGHE1LT, American Master Tech Scientific) for 2 min at room temperature, followed by several washes. After mounting, images were captured using an Olympus DP71 microscope. DUX4
RNAsecope signals were quantified using ImageJ-Fiji software as described previously.\textsuperscript{30}

**Quantitative real-time PCR analysis of DUX4 biomarkers**
15A FSHD myoblasts were transfected as described in RNAsecope assay and quantification above and differentiated into myotubes. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol, and yield was measured by Nanodrop. Isolated RNA was then DNase treated (DNA-Free, Ambion, TX), and cDNA was generated with a high-capacity cDNA reverse transcription kit (Applied Biosystems) using random hexamer primers. Subsequent cDNA samples were then used as a template for the TaqMan assay using pre-designed TRIM43, MBD3L2, PRAMEF12, and ZSCAN4 (biomarkers of DUX4 activity) and human RPL13A control primer/probe sets (Applied Biosystems). All data were normalized to the non-targeting U7-snRNA-transfected cells. Data were generated from two independent experiments performed in triplicate for each biomarker.

**Statistical analysis**
All statistical analyses (caspase-3/7 assay, cell viability assay, RNAsecope, western blot, and qRT-PCR) were performed in GraphPad Prism 5 (GraphPad, La Jolla, CA) using the indicated statistical tests.

**SUPPLEMENTAL INFORMATION**
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.12.004.

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**AUTHOR CONTRIBUTIONS**
A.R. designed U7-asDUX4s, performed or directed all experiments, created figures and legends, and wrote the paper. G.A.-C. performed the RNAsecope assay and quantified the corresponding results. N.K.T cloned the Myc-DUX4 plasmid, contributed to western blots, and assisted with manuscript writing and editing. N.W. contributed to construct design. S.Q.H. conceptualized and obtained funding for the project, designed U7-asDUX4s, and edited the manuscript.

**DECLARATION OF INTERESTS**
The sequences and methods described here were included in a provisional patent application filed on December 1, 2020 (USPTO serial no. 63/120,190). S.Q.H., A.R., and N.W. are listed as inventors.

**REFERENCES**
1. Deenen, J.C., Arnts, H., van der Maarel, S.M., Pijdberg, G.W., Verschuuren, J.J., Bakker, E., Weinreich, S.S., Verbeek, A.L., and van Engelen, B.G. (2014). Population-based incidence and prevalence of facioscapulohumeral dystrophy. Neurology 83, 1056–1059.
2. Pastorino, E., Cao, M., and Trevisan, C.P. (2012). Atypical onset in a series of 122 cases with FaciOcapulOcular Muscular Dystrophy. Clin. Neurol. Neurosurg. 114, 230–234.
3. Klinge, L., Eagle, M., Haggerty, L.D., Roberts, C.E., Straub, V., and Bushby, K.M. (2006). Severe phenotype in infantile facioscapulohumeral muscular dystrophy. Neuromuscul. Disord. 16, 553–558.
4. Statland, J.M., and Tawil, R. (2016). Facioscapulohumeral Muscular Dystrophy. Continuum (Minneap. Minn.) 22, 1916–1931.
5. Lemmers, R.J., van der Vlist, P.J., Klooster, R., Sacconi, S., Camacho, P., D’Aswerve, J.G., Snider, L., Straussheim, K.R., van Ommen, G.J., Padberg, G.W., et al. (2010). A unifying genetic model for facioscapulohumeral muscular dystrophy. Science 329, 1650–1653.
6. Hendrickson, P.G., Dorais, J.A., Grow, E.J., Whiddon, J.L., Lim, J.W., Pike, C.L., Weaver, B.D., Pflueger, C., Emer, B.R., Wilcox, A.L., et al. (2017).Conserved roles of mouse DUX and human DUX4 in activating cleavage-stage genes and MERVL/HERV L retrotransposons. Nat. Genet. 49, 925–934.
7. Whiddon, J.L., Langford, A.T., Wong, C.J., Zhong, J.W., and Tapscott, S.J. (2017). Conservation and innovation in the DUX4-family gene network. Nat. Genet. 49, 935–940.
8. Bosnakovski, D., Xu, Z., Gang, E.J., Fallico, C.L., Liu, M., Simsek, T., Garner, H.R., Agba-Mohammadi, S., Tassin, A., Coppée, F., et al. (2008). An isogenic myoblast expression screen identifies DUX4-mediated FSHD-associated muscular pathologies. EMBO J. 27, 2766–2779.
9. Drait, M., Anseau, E., Tassin, A., Winokur, S., Shi, R., Qian, H., Sauvage, S., Mattetti, C., van Acker, A.M., Leo, O., et al. (2007). DUX4, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PTX1. Proc. Natl. Acad. Sci. USA 104, 18157–18162.
10. Wallace, L.M., Garwick, S.E., Mei, W., Belayew, A., Coppee, F., Ladner, K.J., Otto, J., and Harper, S.Q. (2011). DUX4, a candidate gene for facioscapulohumeral muscular dystrophy, causes p53-dependent myopathy in vivo. Ann. Neurol. 69, 540–552.
11. Giesege, C.R., Wallace, L.M., Heller, K.N., Eidahl, J.O., Saad, N.Y., Fowler, A.M., Pyne, N.K., Al-Kharsan, M., Rashnongejad, A., Chermahini, G.A., et al. (2018). AAV-mediated follistatin gene therapy improves functional outcomes in the TIC–DUX4 mouse model of FSHD. JCI Insight 3, e123538.
12. Kowaljow, V., Marcowycz, A., Anseau, E., Conde, C.B., Sauvage, S., Mattetti, C., Arias, C., Corona, E.D., Nuñez, N.G., Leo, O., et al. (2007). The DUX4 gene at the FSHD1A locus encodes a pro-apoptotic protein. Neuromuscul. Disord. 17, 611–623.
13. Snider, L., Aswachaicharn, A., Tyler, A.E., Geng, L.N., Petek, L.M., Maves, L., Miller, D.G., Lemmers, R.J., Winokur, S.T., Rawl, R., et al. (2009). RNA transcripts, miRNA-sized fragments and proteins produced from D4Z4 units: new candidates for the pathophysiology of facioscapulohumeral dystrophy. Hum. Mol. Genet. 18, 2414–2430.
14. Snider, L., Geng, L.N., Lemmers, R.J., Kyba, M., Ware, C.B., Nelson, A.M., Tawil, R., Filippova, G.N., van der Maarel, S.M., Tapscott, S.J., and Miller, D.G. (2010). Facioscapulohumeral dystrophy: incomplete suppression of a retrotransposon gene. PLoS Genet. 6, e1001181.
15. Shadle, S.C., Zhong, J.W., Campbell, A.E., Conery, M.L., Jagannathan, S., Wong, C.J., Morello, T.D., van der Maarel, S.M., and Tapscott, S.J. (2017). DUX4-induced dsRNA and MYC mRNA stabilization activate apoptotic pathways in human cell models of facioscapulohumeral dystrophy. PLoS Genet. 13, e1006658.
16. Dmitriev, P., Bou Saada, Y., Dib, C., Ansseau, E., Barat, A., Hamade, A., Dessen, P., Robert, T., Lazar, V., Louzada, R.A.N., et al. (2016). DUX4-induced constitutive DNA damage and oxidative stress contribute to aberrant differentiation of myoblasts from FSHD patients. Free Radic. Biol. Med. 99, 244–258.

17. Rickard, A.M., Petek, L.M., and Miller, D.G. (2015). Endogenous DUX4 expression in FSHD myotubes is sufficient to cause cell death and disrupts RNA splicing and cell migration pathways. Hum. Mol. Genet. 24, 5901–5914.

18. Vanderplank, C., Ansseau, E., Charron, S., Stricwant, N., Tassin, A., Laoudy-Chenvesse, D., Wilton, S.D., Coppée, F., and Belayev, A. (2011). The FSHD atrophic myotube phenotype is caused by DUX4 expression. PLoS ONE 6, e26280.

19. Yao, Z., Snider, L., Balog, J., Lemmers, R.J., Van Der Maarel, S.M., Tawil, R., and Tapscott, S.J. (2014). DUX4-induced gene expression is the major molecular signature in FSHD skeletal muscle. Hum. Mol. Genet. 23, 5342–5352.

20. Eisdahl, J.O., Giesige, C.R., Domire, J.S., Wallace, L.M., Fowler, A.M., Guckes, S.M., Garwick-Coppens, S.E., Labhart, P., and Harper, S.Q. (2016). Mouse Dux is myotytic and shares partial functional homology with its human paralog DUX4. Hum. Mol. Genet. 25, 4577–4589.

21. Lyle, R., Wright, T.J., Clark, L.N., and Hewitt, J.E. (1995). The FSHD-associated repeat, D4Z4, is a member of a dispersed family of homeobox-containing repeats, subsets of which are clustered on the short arms of the acrocentric chromosomes. Genomics 28, 389–397.

22. Gabriëls, J., Beckers, M.C., Ding, H., De Vriese, A., Plaisance, S., van der Maarel, S.M., Ruzzo, W.L., Gentleman, R.C., and Tapscott, S.J. (2012). DUX4 activates germline genes, retroelements, and immune mediators: implications for facioscapulohumeral muscular dystrophy. BMC Biol. 10, 443–458.

23. Ansseau, E., Vanderplank, C., Wauters, A., Harper, S.Q., Coppée, F., and Belayev, A. (2017). Antisense Oligonucleotides Used to Target the DUX4 mRNA as Therapeutic Approaches in Facioscapulohumeral Muscular Dystrophy (FSHD). Genes (Basel) 8, 94.

24. Lyle, R., Wright, T.J., Clark, L.N., and Hewitt, J.E. (1995). The FSHD-associated repeat, D4Z4, is a member of a dispersed family of homeobox-containing repeats, subsets of which are clustered on the short arms of the acrocentric chromosomes. Genomics 28, 389–397.

25. Gabriëls, J., Beckers, M.C., Ding, H., De Vriese, A., Plaisance, S., van der Maarel, S.M., Ruzzo, W.L., Gentleman, R.C., and Tapscott, S.J. (2012). DUX4 activates germline genes, retroelements, and immune mediators: implications for facioscapulohumeral muscular dystrophy. BMC Biol. 10, 443–458.

26. Lek, A., Zhang, Y., Woodman, K.G., Huang, S., DeSimone, A.M., Cohen, J., Ho, V., Conner, J., Mead, L., Kodani, A., et al. (2020). Applying genome-wide CRISPR-Cas9 screens for therapeutic discovery in facioscapulohumeral muscular dystrophy. Sci. Transl. Med. 12, eaay0271.

27. Himeda, C.L., Jones, T.I., and Jones, P.L. (2016). CRISPR/dCas9-mediated transcriptional Inhibition Ameliorates the Epigenetic Dysregulation at D4Z4 and Represses DUX4 mRNA expression in vitro. RNA 22, 1211–1217.

28. Wallace, L.M., Saad, N.Y., Pyne, N.K., Fowler, A.M., Eisdahl, J.O., Domire, J.S., Griffin, D.A., Herman, A.C., Sahenk, Z., Rodino-Klapac, L.R., and Harper, S.Q. (2017). Preclinical Safety and Off-Target Studies to Support Translation of AAV-Mediated RNAi Therapy for FSHD. Mol. Ther. Methods Clin. Dev. 8, 121–130.

29. Verma, A. (2018). Recent Advances in Antisense Oligonucleotide Therapy in Genetic Neuromuscular Diseases. Ann. Indian Acad. Neurol. 21, 3–8.

30. Ideue, T., Adachi, S., Nagatomo, T., Tanigawa, A., Natsume, T., and Hirose, T. (2012). U7 small nuclear ribonucleoprotein represses histone gene transcription in cell cycle-arrested cells. Proc. Natl. Acad. Sci. USA 109, 5693–5698.

31. Schümerl, D., and Pillai, R.S. (2004). The special Sm core structure of the U7 snRNP: far-reaching significance of a small nuclear ribonucleoprotein. Cell. Mol. Life Sci. 61, 2560–2570.

32. Goyenvalle, A., Babbs, A., van Ommen, G.J., Garcia, L., and Davies, K.E. (2009). Enhanced exon-skipping induced by U7 snRNA carrying a splicing silencer sequence: Promising tool for DMD therapy. Mol. Ther. 17, 1234–1240.

33. Goyenvalle, A., Wright, J., Babbs, A., Wilkins, V., Garcia, L., and Davies, K.E. (2012). Engineering multiple U7snRNA constructs to induce single and multixon-skipping for Duchenne muscular dystrophy. Mol. Ther. 20, 1212–1221.

34. Schümerl, D., and Pillai, R.S. (2004). The special Sm core structure of the U7 snRNP: far-reaching significance of a small nuclear ribonucleoprotein. Cell. Mol. Life Sci. 61, 2560–2570.

35. Goyenvalle, A., Babbs, A., van Ommen, G.J., Garcia, L., and Davies, K.E. (2009). Enhanced exon-skipping induced by U7 snRNA carrying a splicing silencer sequence: Promising tool for DMD therapy. Mol. Ther. 17, 1234–1240.

36. Goyenvalle, A., Wright, J., Babbs, A., Wilkins, V., Garcia, L., and Davies, K.E. (2012). Engineering multiple U7snRNA constructs to induce single and multixon-skipping for Duchenne muscular dystrophy. Mol. Ther. 20, 1212–1221.

37. Schümerl, D., and Pillai, R.S. (2004). The special Sm core structure of the U7 snRNP: far-reaching significance of a small nuclear ribonucleoprotein. Cell. Mol. Life Sci. 61, 2560–2570.

38. Goyenvalle, A., Babbs, A., van Ommen, G.J., Garcia, L., and Davies, K.E. (2009). Enhanced exon-skipping induced by U7 snRNA carrying a splicing silencer sequence: Promising tool for DMD therapy. Mol. Ther. 17, 1234–1240.

39. Goyenvalle, A., Wright, J., Babbs, A., Wilkins, V., Garcia, L., and Davies, K.E. (2012). Engineering multiple U7snRNA constructs to induce single and multixon-skipping for Duchenne muscular dystrophy. Mol. Ther. 20, 1212–1221.

40. Schümerl, D., and Pillai, R.S. (2004). The special Sm core structure of the U7 snRNP: far-reaching significance of a small nuclear ribonucleoprotein. Cell. Mol. Life Sci. 61, 2560–2570.
51. Voit, T., Topaloglu, H., Straub, V., Muntoni, F., Deconinck, N., Campion, G., De Kimpe, S.J., Eagle, M., Guglieri, M., Hood, S., et al. (2014). Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled phase 2 study. Lancet Neurol. 13, 987–996.

52. Kinali, M., Arechavala-Gomeza, V., Feng, L., Cirak, S., Hunt, D., Adkin, C., Guglieri, M., Ashton, E., Abbi, S., Nihoyannopoulos, P., et al. (2009). Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. Lancet Neurol. 8, 918–928.

53. Mendell, J.R., Goemans, N., Lowes, L.P., Alfano, L.N., Berry, K., Shao, J., Kaye, E.M., and Mercuri, E.; Eteplirsen Study Group and Telethon Foundation DMD Italian Network (2016). Longitudinal effect of eteplirsen versus historical control on ambulation in Duchenne muscular dystrophy. Ann. Neurol. 79, 257–271.

54. Carrell, S.T., Carrell, E.M., Auerbach, D., Pandey, S.K., Bennett, C.F., Dirksen, R.T., and Thornton, C.A. (2016). Dmpk gene deletion or antisense knockdown does not compromise cardiac or skeletal muscle function in mice. Hum. Mol. Genet. 25, 4328–4338.

55. Aoki, Y., Nagata, T., Yokota, T., Nakamura, A., Wood, M.J., Partridge, T., and Takeda, S. (2013). Highly efficient in vivo delivery of PMO into regenerating myotubes and rescue in laminin-a2 chain-null congenital muscular dystrophy mice. Hum. Mol. Genet. 22, 4914–4928.

56. Heemskerk, H., de Winter, C., van Kuik, P., Heuvelmans, N., Sabatelli, P., Rimessi, P., Braghetta, P., van Ommen, G.J., de Kimpe, S., Ferlini, A., et al. (2010). Preclinical PK and PD studies on 2’-O-methyl-phosphorothioate RNA antisense oligonucleotides in the mdx mouse model. Mol. Ther. 18, 1210–1217.

57. Shimizu-Motohashi, Y., Komaki, H., Motohashi, N., Takeda, S., Yokota, T., and Aoki, Y. (2019). Restoring Dystrophin Expression in Duchenne Muscular Dystrophy: Current Status of Therapeutic Approaches. J. Pers. Med. 9, 1.

58. González-Barriga, A., Kranzen, J., Croes, H.J., Bijl, S., van den Broek, W.J., van Kessel, I.D., van Engelen, B.G., van Deutekom, J.C., Wieringa, B., Mulders, S.A., and Wansink, D.G. (2015). Cell membrane integrity in dystrophic muscle type 1: implications for therapy. PLoS ONE 10, e0121556.

59. Brun, C., Suter, D., Pauli, C., Dunant, P., Lochmüller, H., Burgunder, J.M., Schümerli, D., and Weis, J. (2003). U7 snRNAs induce correction of mutated dystrophin pre-mRNA by exon skipping. Cell. Mol. Life Sci. 60, 557–566.

60. Liu, J.J., and Gall, J.G. (2007). U bodies are cytoplasmic structures that contain uridine-rich small nuclear ribonucleoproteins and associate with P bodies. Proc. Natl. Acad. Sci. USA 104, 11655–11659.

61. Stadler, G., Rahimov, F., King, O.D., Chen, J.C., Robin, J.D., Wagner, K.R., Shay, J.W., Emerson, C.P., Jr., and Wright, W.E. (2013). Telomere position effect regulates DUX4 in human facioscapulohumeral muscular dystrophy. Nat. Struct. Mol. Biol. 20, 671–678.