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

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Facioscapulohumeral muscular dystrophy (FSHD) is caused by incomplete silencing of the disease locus, leading to pathogenic misexpression of DUX4 in skeletal muscle. Previously, we showed that CRISPR inhibition could successfully target and repress DUX4 in FSHD myocytes. However, an effective therapy will require both efficient delivery of therapeutic components to skeletal muscles and long-term repression of the disease locus. Thus, we re-engineered our platform to allow in vivo delivery of more potent epigenetic repressors. We designed an FSHD-optimized regulatory cassette to drive skeletal muscle-specific expression of dCas9 from Staphylococcus aureus fused to HP1α, HP1γ, the MeCP2 transcriptional repression domain, or the SUV39H1 SET domain. Targeting each regulator to the DUX4 promoter/exon 1 increased chromatin repression at the locus, specifically suppressing DUX4 and its target genes in FSHD myocytes and in a mouse model of the disease. Importantly, minimizing the regulatory cassette and using the smaller Cas9 ortholog allowed our therapeutic cassettes to be effectively packaged into adeno-associated virus (AAV) vectors for in vivo delivery. By engineering a muscle-specific epigenetic CRISPR platform compatible with AAV vectors for gene therapy, we have laid the groundwork for clinical use of dCas9-based chromatin effectors in skeletal muscle disorders.

INTRODUCTION
Facioscapulohumeral muscular dystrophy (FSHD) (MIM: 158900 and 158901) is the third most common muscular dystrophy,1–3 characterized by progressive weakness and atrophy of specific muscle groups. Both forms of the disease are caused by epigenetic dysregulation of the D4Z4 macrosatellite repeat unit at chromosome 4q35.4–6 FSHD1, the most common form of the disease, is linked to contractions at this array,4–7 resulting in relaxation of chromatin that is normally repressed. FSHD2 is caused by mutations in proteins that maintain epigenetic silencing of the D4Z4 array, leading to a similar chromatin relaxation.8,9 In both forms of the disease, loss of epigenetic repression leads to the aberrant expression of the DUX4 retrogene in skeletal muscle.10 The DUX4 protein, in turn, activates a host of genes normally expressed in early development, which cause pathology when misexpressed in adult skeletal muscle.10,11,12 Although an intact DUX4 open reading frame resides in every D4Z4 repeat unit in the macrosatellite array, only the full-length DUX4 mRNA (DUX4-fl) encoded by the distal-most repeat is stably expressed and translated due to the polyadenylation signal residing in an exon distal to the array in disease-permissive alleles.10,13 Thus, FSHD is a toxic gain-of-function disease.

There are no cures or ameliorative treatments for FSHD, so an effective therapy is critically needed. Since the discovery that FSHD pathogenesis is caused by aberrant expression of DUX4 in skeletal muscles,10,13,14 numerous therapeutic approaches targeting DUX4 and its downstream pathways are being developed.15–21 The most direct path to an FSHD therapy is eliminating expression of DUX4 mRNA. While the amount of DUX4 inhibition required for effective therapy is unknown, data from clinically affected and asymptomatic FSHD subjects support the idea that any reduction in DUX4 expression will have therapeutic benefit.22–24 Small molecules targeting DUX4 expression, independently identified from highly similar indirect expression screens, are promising; however, their discovery is limited by the chemical libraries screened, dosing, and modes of action. Despite the clear overlap in libraries, two published screens with similar approaches identified different molecules, targets, and pathways for DUX4 inhibition, even to the exclusion of other targets,25,26 which is cause for concern. Long-term, harmful side effects are also a concern, as many potential FSHD drug targets play key roles within the target tissue27 (e.g., the p38 inhibitor losmapimod, currently being tested in a clinical trial for FSHD23–27).

With these issues in mind, correcting the fundamental epigenetic dysregulation that leads to FSHD may prove to be the most efficacious and cost-effective avenue of therapy.28 As with many repetitive elements in the human genome, the D4Z4 macrosatellite array that encodes DUX4 is normally under strong epigenetic repression in adult somatic cells. The loss of repression seen in FSHD patients is marked by reduced enrichment of the repressive H3K9me3 mark, HP1γ, and

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cohesin at D4Z4 arrays and DNA hypomethylation at the 4q D4Z4 array. Thus, returning the chromatin at D4Z4 to its normal state of repression in skeletal myocytes is a viable and attractive therapeutic avenue. CRISPR technology provides a means for effectively modifying specific regions of the human genome and, thus, the ability to target the root cause of a disease. Therefore, we are developing CRISPR-based approaches to correct the epigenetic dysregulation in FSHD and therapeutically reduce pathogenic DUX4 expression.

CRISPR gene targeting consists of two components working together: (1) the Cas9 nuclease and (2) a single guide RNA (sgRNA), which directs Cas9 to a specific site in the genome. There are essentially two approaches one could take toward CRISPR-based silencing of DUX4: editing (CRISPRe), which utilizes a functional Cas9 to alter the genomic sequence, and inhibition (CRISPRi), which uses an enzymatically inactive “dead” Cas9 (dCas9) fused to a transcriptional or chromatin repressor. CRISPRi platforms can effectively modulate endogenous gene expression in mammalian cells and for an approach to FSHD with a path to the clinic, CRISPRi has significant advantages over CRISPRe. (1) It does not damage the genome. (2) Any CRISPR approach targeting DUX4 will have unavoidable specific “off targets,” because D4Z4/DUX4 is present in many copies on the non-contracted 4q and both 10q chromosomes, in addition to polymorphic D4Z4s at other loci. However, as these regions are normally heterochromatic, they would simply remain repressed by CRISPRi—not cut, which could result in numerous double-strand breaks and likely apoptosis. (3) CRISPRi, using an appropriate epigenetic regulator, can potentially switch the epigenetic state of a region to make the effect heritable and essentially permanent.

Previously, we demonstrated that CRISPRi using dCas9 from Streptococcus pyogenes (dSpCas9) fused to the KRAB transcriptional repression domain (TRD) can successfully target the pathogenic D4Z4 repeat array to repress DUX4-fl expression in FSHD myocytes. However, repression mediated by dCas9-KRAB requires its continuous expression, which is not ideal for therapeutic applications. Here we extend the utility of CRISPRi to epigenetic regulators capable of mediating stable repression for long-term silencing, yet small enough to be packaged in adeno-associated viruses (AAVs). Key to this advance was minimizing the gene regulatory cassette while maintaining high activity in skeletal muscle, creating more space for delivery of larger epigenetic regulators fused to the smaller dCas9 from Staphylococcus aureus (dSaCas9). We demonstrate that targeting the DUX4 promoter or exon 1 with dSaCas9 fused to any one of four epigenetic repressors (the SUV39H1 SET domain, the MeCP2 TRD, HP1α, or HP1γ) increases chromatin repression at the disease locus, leading to suppression of DUX4 and its target genes in FSHD myocytes and in a DUX4-based, FSHD-like transgenic mouse model. Targeting each dSaCas9-repressor to DUX4 has no significant effects on the closest-matching off-target genes expressed in skeletal muscle and minimal effects on global gene expression. Importantly, by reducing the size of the regulatory cassette, our therapeutic cassettes can be effectively packaged into AAV vectors for in vivo delivery.

RESULTS

Design of an FSHD-specific CRISPRi platform for epigenetic repression of DUX4

While we have demonstrated proof of principle that CRISPRi for FSHD is feasible, an effective therapy will require both efficient delivery of therapeutic components to skeletal muscles and long-term repression of the disease locus. To address these needs, we re-engineered our CRISPRi platform. Our initial study used dSpCas9 fused to the KRAB TRD (Figure 1A), which was sufficient for short-term inhibition in cultured cells but not ideal for long-term silencing. Stable silencing might be directly accomplished by targeting DNA methyltransferases (DNMTs) to DUX4; however, the catalytic domains of these enzymes are much too large to fit the packaging constraints (~4.4 kb) of current AAV vectors needed for in vivo delivery. Thus, we turned our attention to smaller epigenetic regulators and repressive domains that can also effect stable silencing.

Of these, HP1 proteins are key mediators of heterochromatin formation. HP1α predominantly localizes to heterochromatin and HP1γ is enriched at the D4Z4 macrosatellite array in healthy myocytes and lost in FSHD. SUV39H1 is a histone methyltransferase that establishes constitutive heterochromatin at pericentric and telomeric regions. The SET domain of SUV39H1 participates in stable binding to heterochromatin and mediates H3K9 trimethylation, a repressive mark that recruits HP1. Although the SET domain contains the active site of enzymatic activity, both pre-SET and post-SET domains are required for methyltransferase activity and are included in our SET cassette. The methyl-CpG-binding protein MeCP2 plays diverse roles in chromatin regulation, but its TRD is all that is required for binding repressive histone marks and co-repressor complexes to enable efficient gene silencing.

To accommodate dCas9 fused to even these relatively small repressors and repressive domains in AAV vectors required minimizing current regulatory cassettes. Building on key work from the Hauschka lab and taking into account that cardiac muscle is not involved in FSHD, we designed a minimized skeletal muscle regulatory cassette to allow larger therapeutic components to be delivered in vivo. Starting with the CK8 cassette, which is a modified version of three muscle creatine kinase (CKM) enhancers upstream of the CKM promoter, we removed additional space between elements and deleted the CarG and AP2 sites, which are dispensable for expression in skeletal muscle. This reduced the size of the regulatory cassette to 378 bp, allowing us to build constructs containing the smaller dSaCas9 ortholog fused to epigenetic repressors that were previously too large to fit into AAV vectors. Thus, our optimized CRISPRi platform consists of: (1) dSaCas9 fused to one of four epigenetic repressors (HP1α, HP1γ, the MeCP2 TRD, or the SUV39H1 pre-SET, SET, and post-SET domains) under control of our FSHD-optimized regulatory cassette, and (2) an
sgRNA targeting the DUX4 locus under control of the U6 promoter (Figure 1B). These components were expressed in lentiviral (LV) vectors for transduction of cultured myocytes and in AAV vectors for in vivo use.

dSaCas9-mediated recruitment of epigenetic repressors to the DUX4 promoter or exon 1 represses DUX4-fl and DUX4-FL targets in FSHD myocytes

To target our dSaCas9-repressors, we designed sgRNAs compatible with the Sa protospacer adjacent motif (PAM) (NNGRRT) across the DUX4 locus (Materials and methods; Figure 1C). For all experiments, we performed four serial coinfections of FSHD myogenic cultures, as described.15 Cells were transduced with combinations of LV supernatants expressing either dSaCas9 fused to each epigenetic regulator or individual sgRNAs. Cells were harvested 3 days following the final round of infection for analysis of gene expression by quantitative reverse-transcriptase PCR (qRT-PCR).

Although targeting DUX4 exon 3 or the D4Z4 upstream enhancers54 had no effect, targeting each dSaCas9-repressor to the DUX4 promoter or exon 1 significantly reduced levels of DUX4-fl mRNA to ~30%–50% of endogenous levels (Figure 2; Figure S1). As levels of DUX4-FL protein are low and difficult to assess in FSHD myocytes, we routinely assess DUX4-FL target gene expression as the more reliable assay and relevant functional readout of DUX4 activity. Importantly, expression levels of DUX4-FL targets thought to have pathogenic consequences55 are significantly decreased in parallel with the reduction in DUX4-fl mRNA (Figure 2; Figure S1).

To verify that enzymatic activity of the SET domain is required for the effect on DUX4-fl, we targeted dSaCas9-SET containing a mutation (C326A) within the SET domain that abolishes enzymatic activity56 to the DUX4 promoter/exon 1. Although the effect was highly variable, the inactive SET domain did not significantly affect levels of DUX4-fl (Figure S2), indicating that enzymatic activity of this region is required for DUX4-fl repression.

Targeting dSaCas9-repressors to DUX4 has no effect on Myosin heavy chain 1 or D4Z4 proximal genes

To rule out a nonspecific effect of dSaCas9-repressors on muscle differentiation, we assessed levels of Myosin heavy chain 1 (MYH1), a marker of terminal muscle differentiation, by qRT-PCR in the cells described above. Importantly, MYH1 levels were equivalent in all cultures (Figures 3A–3D; Figure S3), indicating that lower levels of DUX4-fl are not due to impairment of differentiation. We also measured expression of FRG1 and FRG2, which lie proximal to the D4Z4 macrosatellite. Recruitment of each dSaCas9-repressor to the DUX4 promoter/exon 1 did not reduce expression of these D4Z4 proximal genes (Figures 3A–3D; Figure S3).

Targeting dSaCas9-repressors to DUX4 has minimal off-target effects in FSHD myocytes

A major concern of CRISPR-based approaches for gene therapy is the prospect of deleterious off-target effects. While many versions of Cas9 are being engineered for increased specificity,57–60 for preclinical studies it is always necessary to determine the off-target effects of each potential treatment. For the sgRNAs that worked best in combination with each dSaCas9-repressor, we used the

![Figure 1. CRISPRi constructs for epigenetic repression of DUX4](image)
publicly available Cas-OFFinder tool (http://www.rgenome.net/cas-offinder/) to search for the closest-matching off-target sequences in the human genome. Only sgRNAs #1 and #5 had close-matching predicted off-targets in or near genes expressed in skeletal muscle (Table S1). Intron 1 of Lysosomal amino acid transporter 1 homolog (LAAT1) contains a potential off-target match to sgRNA #1. The single exon of Ribosomal biogenesis regulatory protein homolog (RBS1) and the sequence 283 bp downstream of Guanine nucleotide-binding protein G(i) subunit alpha-1 isoform 1 (GNAI1) contain potential off-target matches to sgRNA #5. However, in contrast to the striking reduction in DUX4-fl, targeting dSaCas9-SET with sgRNA #1 had no effect on LAAT1 expression (Figure 3E; Figure S4). Similarly, targeting dSaCas9-HP1γ with sgRNA #5 had no effect on levels of RBS1 or GNAI1 (Figure 3F; Figure S4).

Since an analysis of off-target DNA binding (by chromatin immunoprecipitation sequencing [ChIP-seq]) sheds no light on the more critical off-target gene expression profiles, we performed RNA sequencing (RNA-seq) to assess the global effects of targeting each dSaCas9-repressor to DUX4 with the most effective sgRNAs. Primary FSHD myocytes were transduced with each combination of vectors (described in Figure 4) or with dSaCas9-KRAB + sgRNA #6 for comparison. Gene Ontology (GO) analysis shows that the majority of misregulated cellular responses are likely due to the LV transduction or possibly dCas9 expression and not due to...
off-target repression mediated by the dCas9 effectors (Figures S5–S9). The fact that targeting with four different sgRNAs yields very similar profiles of differentially expressed genes (DEGs), consistent with an innate immune response, strongly supports this conclusion (Tables S2–S4), although some immune-related DEGs may represent a correction of DUX4-mediated dysregulation, since DUX4 targets include immune mediators. After removing DEGs consistent with a response to virus, the vast majority remaining are part of embryonic programs or developmental pathways that are deregulated by DUX4 misexpression (Table 1; Table S4). Many of these genes are common to multiple treatments, and their differential expression represents a return to a more normal pattern of gene expression. For example, DUX4 expression reduces levels of TRIM14, KREMEN2, LY6E, and PARP14 in multiple independent studies (compared in Jagannathan et al.); consistent with these studies, we found that all four dSaCas9-epigenetic repressor treatments led to an increase in expression of these genes (Table 1; Table S4). Conversely, TM6SF1 and ITGA8, which are upregulated following DUX4 overexpression, were both decreased following treatment with every dSaCas9-epigenetic repressor (Table 1; Table S4).

Expression levels of myogenic genes that were assessed by qRT-PCR (MYOD1, MYOG, and MYH1) also showed no changes by RNA-seq analysis (Table S4). The only muscle genes with differential expression are CKM, which is increased ~2-fold following
treatment with dSaCas9-SET, -HP1γ, or -TRD, an antisense transcript of MEF2C, and MYBPC2, which are increased 2-fold following treatment with dSaCas9-SET (Table S4). Since DUX4 expression is reported to inhibit myogenesis, these changes also likely represent a beneficial correction of DUX4-mediated transcriptional dysregulation.

Importantly, the number of detectable off-target responses to each treatment was extremely low. Treatment with dSaCas9-TRD or -HP1γ yielded no significant unique DEGs, while treatment with dSaCas9-SET and -HP1α yielded only 7 and 8 unique DEGs, respectively (Figure 4; Table S3). As predicted by the in silico search of sgRNA targets, our system for CRISPRi is highly specific in human myocytes. In contrast, treatment with dSaCas9-KRAB, which was targeted using the same sgRNA (#6) as dSaCas9-TRD, yielded 37 unique DEGs (versus 0 for dSaCas9-TRD). This result was surprising, considering the high specificity reported for dSpCas9-KRAB; however, it suggests that, at least in myocytes, the KRAB repressor is recruited to genomic locations independent of sgRNA targeting and is a more promiscuous repressor than the MeCP2 TRD.

Targeting dSaCas9-repressors to DUX4 increases chromatin repression at the locus

Since targeting each epigenetic repressor to the DUX4 promoter/exon 1 reduces levels of DUX4-fl, we expected that each would mediate direct changes in chromatin structure at the locus. Using ChIP assays, we analyzed several marks of repressive chromatin across D4Z4 following CRISPRi treatment in FSHD myocytes. Increases in repressive marks are difficult to assess across the FSHD1 DUX4 locus, because three of the four 4q/10q alleles, which are comprised of many more copies of D4Z4, are already in a compacted, heterochromatic state. Thus, any attempt to assess an increase in repression at the contracted allele is dampened by the presence of the other three. Unsurprisingly, changes in overall levels of the repressive H3K9me3 histone mark were undetectable across the D4Z4 repeats; however, other repressive marks were detectably and significantly elevated, overcoming the high background. Recruitment of HP1α to DUX4 led to a 30%–40% enrichment of this factor across the locus, as well as increased occupancy of the KAP1 co-repressor (Figures 5A and 5B; Figures S10A and S10B). Recruitment of HP1γ led to an increase in both HP1α and KAP1 at DUX4 exon 3, and recruitment of the MeCP2 TRD led...
to an increase in HP1α across the locus (Figures 5A and 5B; Figures S10A and S10B). Recruitment of each of the four factors also led to a ~40%–60% decrease in the elongating form of RNA Pol II (phospho-serine 2) at the pathogenic repeat (Figure 5G; Figure S10C), consistent with the lower levels of DUX4-fl transcript observed (Figure 2). Taken together, these results indicate that treatment with dSaCas9-repressors returns the chromatin at the disease locus to a more normal state of repression.

The FSHD-optimized regulatory cassette is only active in skeletal muscles

Following development of our optimized cassette, it was important to confirm that our smaller CKM-based regulatory cassette retains high activity in skeletal muscles with low to no activity in other tissues. Thus, in vivo expression of the FSHD-optimized regulatory cassette was analyzed using AAV9-mediated transgene delivery to wild-type mice. Viral particles were delivered by systemic retro-orbital injection (2.8 × 10^{14} genome copy [GC]/kg body weight), and the mCherry reporter signal was visualized at 12 weeks post-injection. As previously reported, AAV9 strongly transduced skeletal muscles, cardiac muscle, and liver (Figure S11). Nonetheless, mCherry expression was detected only in skeletal muscles and was undetectable in the heart (Figure 6), even at a higher exposure (Figure S12), and in non-muscle tissues (Figure S13), indicating that our FSHD-optimized regulatory cassette is only active in the critical target tissue. The lack of tropism/activity in testis is particularly important, because DUX4 is normally expressed in this tissue in healthy individuals.

**In vivo targeting of dSaCas9-repressors to DUX4 exon 1 represses DUX4-fl and DUX4-FL targets in ACTA1-MCM;FLEXDUX4 bi-transgenic mice**

To test the ability of our CRISPRi platform to repress DUX4-fl in vivo, we utilized our lab’s ACTA1-MCM;FLEXDUX4 (FLEXD) FSHD-like bi-transgenic mouse model, which can be induced to express DUX4-fl and develop a moderate pathology in response to a low dose of tamoxifen.69,70 These mice carry one human D4Z4 repeat from which DUX4-fl is expressed and can be targeted by our sgRNAs to exon 1. Mice were injected intramuscularly with AAV vectors encoding dSaCas9-TRD or -KRAB and sgRNAs targeting DUX4 exon 1 at different ratios, followed 3.5 weeks later by intraperitoneal injection of tamoxifen. Although transcript levels of the DUX4-fl transgene are difficult to assess in this model,69,70 targeting either dSaCas9-TRD or -KRAB to DUX4 exon 1 led to a ~30% decrease in expression of DUX4-fl at the higher ratio of sgRNA to effector (Figure 7). Transcript levels of DUX4-FL targets Wtfdc3 and Slc34a2 were also reduced, although the reduction was only significant for dSaCas9-TRD at the lower ratio of sgRNA to effector (Figure 7). Although these effects are modest, they provide proof of principle that our epigenetic CRISPRi platform is a viable strategy for ongoing preclinical development.

**DISCUSSION**

CRISPR/Cas9 technology has been used extensively to target and modify specific genomic regions, offering the potential for permanent correction of many diseases.15,71–73 While the dangers associated with standard CRISPR editing are a concern for any locus, they are of particular concern in a highly repetitive region such as the FSHD locus. However, the use of CRISPR to repress gene expression is ideally suited to FSHD. Unfortunately, CRISPRi platforms for human gene therapy are limited by the large size of Cas9 targeting proteins, which take up most of the available space in AAV vectors, leaving little room for effectors. Not surprisingly, most proof-of-principle studies have utilized dSpCas9 in LV vectors, which have a larger genome capacity and are convenient for expression in cultured cells but not useful for clinical gene delivery. The smaller dSaCas9 ortholog has been shown to work well with a fused effector,74 but its coding sequence is still over 3 kb, leaving little room for a chromatin modulator and regulatory sequences within the 4.4 kb packaging capacity of AAVs. It is worth emphasizing that the packaging limitation of AAV vectors continues to be a major hurdle for gene therapy of FSHD and many other diseases. To bring a CRISPRi platform for FSHD to the clinic, it was imperative to find stable repressors small enough to be included in dCas9 therapeutic cassettes and to reduce the size of current muscle-specific regulatory cassettes.
Studies from many labs, including ours, have utilized dCas9-KRAB to repress target genes; however, repression mediated by this effector requires its continuous expression. While dCas9-effectors may be continuously expressed from stable episomal AAV vectors, this is not guaranteed. From a clinical standpoint, it seems far more desirable to achieve stable repression that does not rely on continuous, lifelong expression of the transgene. Thus, we minimized the widely used CK8 cassette while maintaining high activity and specificity for skeletal muscles and used this FSHD-optimized cassette to drive expression of dSaCas9 fused to each of four small epigenetic repressors capable of mediating stable silencing. Here we demonstrate that dSaCas9-mediated targeting of these repressors returns the chromatin at the FSHD locus to a more normal state of repression and reduces expression of DUX4-fl and its targets in FSHD myocytes and in a DUX4-based transgenic mouse model, with minimal effects on the muscle transcriptome.

We observed more robust repression of DUX4-fl and its targets in primary FSHD myocytes than in ACTA1-MCM;PLExD bi-transgenic mice, likely due to limitations of the mouse model, which contains only a single D4Z4 repeat that may not be enough for efficient epigenetic silencing. Thus, ongoing studies will also test our CRISPRi platform in a human xenograft model containing mature FSHD myofibers. These mice are immunocompromised and, thus, not useful for assessing the effects of CRISPRi on DUX4-mediated immune pathologies. However, because they contain a full D4Z4 array from an FSHD patient, a xenograft model may be ideal for assessing long-term epigenetic changes at the disease locus. Determining the stability of DUX4 repression mediated by CRISPRi is a critical goal, since current AAV vectors for gene therapy can only be administered once.

A major concern of Cas9 editing is the potential for off-target cutting leading to deleterious mutations, something that was not considered to be a problem for dCas9 effectors. However, it was recently demonstrated in yeast that R-loops formed by dCas9 binding to DNA can cause mutagenesis at both on- and off-target sites, although the frequency is several orders of magnitude lower than that induced by Cas9. Consistent with this very low rate, mutations induced by dCas9 have not been detected in mammalian cells. Additionally, this concern is ameliorated when targeting the D4Z4 region, which is normally silent. Fortunately, for CRISPRi of FSHD, both the nature of the targeted region and the type of modulation employed tend to mitigate the general concerns related to CRISPR platforms.

As CRISPR and other gene-targeting systems continue to evolve, it is important that the results of this study can be adapted to a changing platform. The identification of sgRNAs that successfully target the DUX4 locus with minimal off-target effects should prove useful with engineered Cas9 variants and dCas9 fused to other effectors. In addition, we have identified the DUX4 promoter and exon 1 as targets for epigenetic modulation, and these regions contain numerous sgRNA targets compatible with different orthologs of Cas9. Once these orthologs are better characterized, smaller and less immunogenic versions should become available, rendering fusions with larger epigenetic regulators more amenable to in vivo delivery. We are currently making further improvements in vector and cassette design that allow all therapeutic components to be included on single AAV vectors, a clinically important milestone for improving efficiency, lowering the high cost of therapy, and reducing the immunotoxicity associated with high viral doses.

Here we demonstrate the successful use of dCas9-mediated epigenetic repression in a muscle disorder, thus laying the groundwork for...
Figure 6. The FSHD-optimized regulatory cassette is active in skeletal muscles

In vivo AAV9-mediated mCherry expression under control of the FSHD-optimized regulatory cassette (A) was visualized in the indicated tissues at 12 weeks post-injection with the same exposure time (0.5 s). (B–H and L) For two-tissue panels, tissues from uninjected mice are shown on the left. (I’–K) Single-tissue panels are AAV injected, and (I) is uninjected. Expression of mCherry was detected in skeletal muscles (tibialis anterior [TA], gastrocnemius [GA], extensor digitorum longus [EDL], and quadriceps [QUA], as well as pectoral, abdominal, and facial muscles) and was undetectable in soleus (SOL), heart, and testis.
subsequent, ongoing studies to assess the functional efficacy and stability of this approach in vivo. Ultimately, we are attempting to correct the underlying pathogenic mechanism in FSHD using a therapeutically relevant platform. In addition, our successful use of dCas9-based chromatin effectors should be applicable to other diseases of aberrant gene regulation.

MATERIALS AND METHODS

Study design

The primary goal of this study was to design and test a CRISPRi platform that will ultimately allow therapeutic delivery of epigenetic repressors capable of mediating stable repression of DUX4 in FSHD patients. Thus, we designed our therapeutic cassettes (minimized skeletal muscle-specific regulatory sequences driving dSaCas9 fused to different epigenetic repressors) for use in AAV gene therapy vectors. Two experimental systems were used: primary FSHD myocytes, which contain endogenous patient D4Z4 arrays, and ACTA1-MCM;FLExD bi-transgenic mice,69,70 which can be induced to express DUX4-FL at controllable levels in skeletal muscles and exhibit pathologies consistent with FSHD. For experiments in primary cell culture, therapeutic cassettes were delivered via LV, but all in vivo experiments utilized therapeutic cassettes in AAV9 for efficient delivery to skeletal muscles. For in vivo experiments, we tested increasing ratios of sgRNA to dSaCas9-repressor, since dual-vector CRISPR editing has been shown to be more effective using a higher ratio of sgRNA to Cas9.7 Since our AAV-sgRNA contains three sgRNAs in one vector, we delivered AAV-dSaCas9-repressor:AAV-sgRNA at ratios of 1:0, 1:1, and 1:4, which is equivalent to dSaCas9:sgRNA copy number ratios of 1:0, 1:3, and 1:12. All experiments (biological replicates) in primary cells were performed at least four times (for gene expression analysis by qRT-PCR), at least three times (for ChIP analysis), and five times (for global transcriptome analysis by RNA-seq). AAV transductions of dSaCas9-TRD or -KRAB + sgRNAs utilized nine mice for each dSaCas9-repressor.

Antibodies and plasmids

The ChIP-grade antibodies used in this study, α-KAP1 (ab3831), α-H1P1α (ab77256), α-RNA Pol II CTD repeat (phospho S2) (ab5095), and α-histone H3 (ab1791) were purchased from Abcam (Cambridge, MA, USA). dSaCas9 constructs were designed with a skeletal muscle-specific regulatory cassette consisting of three modified CKM enhancers in tandem, upstream of a modified CKM promoter. Enhancer modifications are as follows: (1) Left E-box mutated to Right E-box,80 (2) enhancer CArG and AP2 sites removed, (3) 63 bp between Right E-box and MEF2 site removed,39 (4) sequence between TF binding motifs minimized, (5) +1 to +50 promoter sequence used,39 and (6) consensus Inr site added.39 The sequence is available upon request. This regulatory cassette was designed upstream of the SV40 bipartite nuclear

Figure 7. In vivo targeting of dSaCas9-repressors to DUX4 exon 1 represses DUX4-fl and DUX4-FL targets in ACTA1-MCM;FLExD bi-transgenic mice (A–F) dSaCas9-TRD or -KRAB ± sgRNAs were delivered intramuscularly using AAV9 to the ACTA1-MCM;FLExD moderate pathology FSHD-like transgenic mouse model, which carries one human D4Z4 repeat.70 Expression of DUX4-fl and DUX4-FL downstream markers Wfdc3 and Slc34a2 were assessed by qRT-PCR and normalized to levels of Rpl37. Copy-number ratios of dSaCas9-TRD or -KRAB to sgRNA are indicated. *p < 0.05, **p < 0.01 are from comparing to dSaCas9-TRD or -KRAB control.
localization signal flanking dSaCas9, which was fused in-frame to one of four epigenetic repressors (the SUV39H1 pre-SET, SET, and post-SET domains, the MeCP2 TRD, HP1α, or HP1γ) and HA tag, followed by the SV40 late pA signal. sgRNA constructs were designed with the U6 promoter upstream of BsuAI cloning sites, followed by an SaCas9-optimized scaffold and cPPT/CTS. Constructs were synthesized in pUC57 and sequenced by GenScript Biotech, then cloned into a pRRLSIN LV vector for transduction of primary FSHD myocytes. pRRLSIN.cPPT.PGK-GFP.WPRE was a gift from Didier Trono (Addgene plasmid #12252; http://addgene.org/12252; RRID:Addgene_12252). For evaluation of activity in vivo, the FSHD-optimized regulatory cassette driving mCherry (Figure 6, top) was cloned between the AAV2 ITRs (using MluI and RsrII) of the pAAV-CA plasmid,81 a gift from Naoshige Uchida (Addgene plasmid #69616; http://addgene.org/69616; RRID:Addgene_69616). All AAV9 vectors used this study were produced by Vector Biolabs (Malvern, PA, USA).

sgRNA design and plasmid construction

We used the publicly available sgRNA design tool from the Broad Institute (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design) to design dSaCas9-compatible sgRNAs targeting across the DUX4 locus. sgRNAs were cloned individually into BsuAI sites in the parent construct and sequence verified. We used the publicly available Cas-OFFinder tool (http://www. genome.net/cas-offinder/) to search for the closest-matching off-target sequences in genes expressed in skeletal muscle. Refer to Table S1 for additional details.

Cell culture, transient transfection, and LV transduction

Myogenic cells derived from biceps muscle of an FSHD1 patient (17Abic) were obtained from the Wellstone FSHD cell repository,22,23 and grown as described.15 LV particles were generated using 293T packaging cells, as described.15 At ~70%–80% confluence, 17Abic myoblasts were subjected to four serial infections, as described,15 and allowed to self-differentiate. Cells were harvested ~72 h following the last round of infection.

qRT-PCR

Total RNAs were extracted using TRIzol (Invitrogen) and purified using the RNeasy Mini kit (QIAGEN) after on-column DNase I digestion. From cultured myocytes, total RNA (2 μg) was used for cDNA synthesis using oligo dT and Superscript III Reverse Transcriptase (Invitrogen); DUX4-fl was amplified by qPCR using 200 ng of cDNA, and other genes were amplified using 10–20 ng of cDNA, as described.15 From mouse TAs, DUX4-fl was amplified using 100 ng of cDNA, and downstream targets were amplified using 5–20 ng of iScript cDNA. Oligonucleotide primer sequences are provided in Table S5.15,16,54,82–86

RNA-seq

FSHD myocytes (17Abic) were subjected to four serial co-infections with LV supernatants expressing dSaCas9 fused to either: (1) the SUV39H1 pre-SET, SET, and post-SET domains (SET); (2) the MeCP2 TRD; (3) HP1γ; (4) HP1α; or (5) the KRAB TRD, each in combination with LV expressing an sgRNA targeting DUX4. Cells were harvested ~72 h following the last round of infection. For all treatments, five separate experiments were performed, and reduction of DUX4-fl and DUX4-FL targets was confirmed by qRT-PCR prior to submitting samples for sequencing. RNA-seq analysis was performed by GeneWiz using the Illumina HiSeq 2 × 100 bp platform, which is ideal for identifying gene expression levels, splice variant expression, and the de novo transcriptome assembly (including un-annotated sequences). The rRNA depletion, library construction, sequencing, and initial analysis (mapping all sequence reads to the human genome, reading hit count measurements, and differential gene expression comparisons) were performed by GeneWiz. Sequence reads were trimmed to remove possible adaptor sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Homo sapiens GRCh38 reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences. Unique gene hit counts were calculated by using featureCounts from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted. Since a strand-specific library preparation was performed, the reads were strand-specifically counted. Comparisons of gene expression between groups of samples were performed using DESeq2, described below. A GO analysis was performed on the statistically significant set of genes by implementing the software GeneSCF v.1.1-p2. The goa_human GO list was used to cluster the set of genes based on their biological processes and determine their statistical significance. To estimate the expression levels of alternatively spliced transcripts, the splice variant hit counts were extracted from the RNA-seq reads mapped to the genome. Differentially spliced genes were identified for groups with more than one sample by testing for significant differences in read counts on exons (and junctions) of the genes using DESeq. Volcano plots of differentially expressed genes were generated using Prism 7 (GraphPad).

ChIP

ChIP assays were performed with LV-transduced 17Abic differentiated myocytes using the Fast ChIP method97 as described.15 Chromatin was immunoprecipitated using 2 μg of specific antibodies. SYBR green quantitative PCR assays were performed as described.15 Oligonucleotide primer sequences are provided in Table S5.

In vivo characterization of FSHD-optimized regulatory cassette

All animal experiments were approved by the institutional Animal Care and Use Committee of the University of Nevada, Reno. All mice were anesthetized prior to injection. Systemic AAV9-mediated delivery of the FSHD-optimized regulatory cassette driving mCherry (100 μL of 3.2 × 1011 GC/g body weight) was performed via retro-orbital injection (ROI) to wild-type C57BL/6J mice (2 males and 2 females) at 3.5 weeks of age with an average dose of 2.8 × 1011 GC/g body weight. At 12 weeks post-ROI, mCherry signals in all tissues were captured by fluorescent imaging system and Leica LAS X software, using the same exposure unless indicated. Images were assembled with Adobe Photoshop CS6, and exposures were adjusted equally. For assessment of systemic AAV9-mediated
infection, genomic DNA was isolated from individual tissues, and viral genomes were quantified by qPCR (50 ng genomic DNA) using primers to the bovine growth hormone polyadenylation signal (PAS) and normalized to the endogenous single copy Rosa26 gene. Oligonucleotide primer sequences are provided in Table S5.

**AAV transduction of dSaCas9-TRD or -KRAB in ACTA1-MCM;FLExD bi-transgenic mice**

Tibialis anterior (TA) muscles of 4-week-old male ACTA1-MCM;FLExD bi-transgenic animals were injected with various ratios of AAV9-dSaCas9-TRD or -KRAB and AAV9-sgrRNAs. AAV-dSaCas9-TRD or -KRAB was injected at 5 × 10⁵ GC/TA for all experiments. At 3.5 weeks post-AAV injection, mice were subjected to intraperitoneal injections of 5 mg/kg of tamoxifen (TMX) to induce DUX4-fl expression in skeletal muscles. TA muscles were sampled at 14 days post-TMX injection for gene expression analysis.

**Statistical analysis**

Experiments in primary cells were performed using at least four biological replicates (for qRT-PCR analysis) and at least three biological replicates (for ChIP analysis), and data were analyzed using an unpaired, two-tailed Student’s t test (*p < 0.05, **p < 0.01, ***p < 0.001). RNA-seq analysis was performed by GeneWiz using five biological replicates, and comparisons of gene expression between groups of samples were performed using DESeq2. The Wald test was used to generate p values and log2 fold changes. Genes with a p value < 0.05 and absolute log2 fold change > 1 were called as DEGs for each comparison. Enrichment of GO terms was tested using Fisher exact test (GeneSCF v1.1-p2). Significantly enriched GO terms had an adjusted p value <0.05 in the differentially expressed gene sets. For AAV transductions in mice, gene expression was analyzed using an unpaired, two-tailed Student’s t test.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.12.001.

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**AUTHOR CONTRIBUTIONS**

C.L.H., T.I.J., and P.L.J. designed the study, C.L.H. and P.L.J. wrote the manuscript. C.L.H. designed therapeutic cassettes, performed all experiments using FSHD myocytes, and analyzed data. T.I.J. performed all experiments using mice and analyzed data. All authors edited and approved the final version of the manuscript.

**DECLARATION OF INTERESTS**

C.L.H., P.I.J., and T.I.J. are listed as inventors on two US patent applications (nos. 62/398801 and 63/011476) pertaining to the use of CRISPR inhibition for FSHD.

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