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Identification of Epigenetic Regulators of *DUX4-fl* for Targeted Therapy of Facioscapulohumeral Muscular Dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is caused by epigenetic de-repression of the disease locus, leading to pathogenic misexpression of the *DUX4* gene in skeletal muscle. While the factors and pathways involved in normal repression of the FSHD locus in healthy cells have been well characterized, very little is known about those responsible for the aberrant activation of *DUX4-fl* in FSHD myocytes. Reasoning that *DUX4-fl* activators might represent useful targets for small molecule inhibition, we performed a highly targeted, candidate-based screen of epigenetic regulators in primary FSHD myocytes. We confirmed several of the strongest and most specific candidates (*ASH1L*, *BRD2*, *KDM4C*, and *SMARCA5*) in skeletal myocytes from two other unrelated FSHD1 patients, and we showed that knockdown led to reduced levels of *DUX4-fl* and *DUX4-FL* target genes, as well as altered chromatin at the *D4Z4* locus. As a second mode of validation, targeting the CRISPR/dCas9-KRAB transcriptional repressor to the promoters of several candidates also led to reduced levels of *DUX4-fl*. Furthermore, these candidates can be repressed by different methods in skeletal myocytes without major effects on certain critical muscle genes. Our results demonstrate that expression of *DUX4-fl* is regulated by multiple epigenetic pathways, and they indicate viable, druggable candidates for therapeutic target development.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common muscular dystrophy,^{1,2} characterized by progressive weakness and atrophy of specific muscle groups. FSHD is also marked by a high variability in disease onset, progression, and severity, which ranges from asymptomatic to clinically severe.^{1,3–5} This high variability within the clinical spectrum suggests the involvement of multiple factors, including epigenetic regulators. Indeed, both forms of the disease are linked by epigenetic dysregulation of the *D4Z4* macrosatellite repeat array at chromosome 4q35. FSHD1, the most common form of the disease, is linked to contractions at this array,^{6–8} resulting in relaxation of chromatin that is normally repressed. FSHD2 is

contraction independent, but mutations in proteins that maintain epigenetic silencing lead to a similar relaxation of chromatin in the region.^{9,10} One critical consequence of this epigenetic alteration is the aberrant expression of the *DUX4* retrogene in skeletal muscle. While *DUX4* 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, due to the presence of a polyadenylation signal in disease-permissive alleles.^{11,12} The *DUX4-FL* protein acts as a transcription factor and is highly cytotoxic when overexpressed. Endogenous *DUX4-FL* activates germline genes, immune mediators, and retroelements,^{13,14} alters RNA and protein metabolism,^{15,16} and leads to accumulated muscle pathology.

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 (reviewed in Himeda et al.¹⁷). FSHD patients exhibit a loss of this repression, displaying chromatin relaxation (reduced enrichment of the repressive H3K9me3 mark, HP1 γ , and cohesin) at *D4Z4* arrays^{18,19} and DNA hypomethylation at the 4q *D4Z4* array.^{20–25} Thus, targeting the epigenetic dysregulation in FSHD is a viable potential therapeutic avenue.¹⁷ In support of this approach, a forward genetic screen to identify regulators of repeat-induced variegation of expression in transgenic mice carrying a metastable repeat epiallele identified numerous chromatin-modifying factors, classified as the *Momme* (Modifier of Murine Metastable Epiallele) genes, involved in either repressing or enhancing the variegated gene expression phenotype.^{26–28}

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Importantly, mice carrying mutations in genes encoding enhancers of gene expression from large DNA repeat arrays were otherwise healthy, suggesting that the levels of certain factors functioning in the regulation of large repeat arrays can be decreased without global epigenetic dysregulation.

Correction of the epigenetic defect in FSHD has been attempted in proof-of-principle studies by our lab and others, using the dCas9-KRAB transcriptional inhibitor²⁹ and the DICER/Argonaute system.³⁰ Here we took a different approach, screening a pre-selected panel of epigenetic regulators, including the human orthologs of several *Momme* genes, and chromatin-modifying enzymes for their role in maintaining *DUX4-fl* mRNA expression in primary human FSHD myocytes. Since we are keenly interested in identifying targets for developing small molecule inhibitors and therapeutics for FSHD, it was vital that our selected targets have a high potential for selective druggability. Using a combination of techniques for validation, we have uncovered several epigenetic regulators as promising targets for FSHD therapeutic development.

RESULTS

Multiple Epigenetic Pathways Regulate the Expression of *DUX4-fl* in FSHD Myocytes

Utilizing our understanding of the epigenetics impacting the FSHD region and the *Momme* enhancer genes,¹⁷ we hypothesized that multiple epigenetic and chromatin-regulatory pathways might be deregulated in FSHD myocytes, with many potential targets for therapeutic manipulation. Since the pathogenic expression of *DUX4-fl* is restricted to differentiated human skeletal muscle, where it is expressed sporadically at very low levels, regulators of *DUX4-fl* expression are not readily amenable to high-throughput screening. Thus, we designed a highly focused, candidate-based screen in a larger format using primary FSHD myocytes. We pre-selected 36 candidate activators of *DUX4-fl* as potential drug targets based on likelihood of functioning at the contracted D4Z4 macrosatellite, activity predicting a role in transcriptional activation, or establishing or maintaining a euchromatic environment, and the presence of druggable protein domains (Table 1). Although very little is known about mechanisms of activation at D4Z4, mechanisms of repression have been well characterized. Thus, we reasoned that epigenetic regulators opposing D4Z4-repressive factors and histone marks would be good candidates for our small-scale, biased screen. These candidates include transcriptional regulators, chromatin remodelers, and histone-modifying enzymes.

For our initial screen, we used primary skeletal myocytes from an FSHD1 patient (05Abic) that express consistent and relatively high levels of *DUX4-fl* when terminally differentiated.³¹ Using lentivirus-encoded small hairpin RNAs (shRNAs), we knocked down each candidate in terminally differentiated cultures, then harvested the cells 4 days later, and assessed the expression of *DUX4-fl* and other genes, including key muscle factors. The lack of a major effect on muscle genes is critically important from the perspective of ultimately developing and administering a chronic therapeutic agent. However, this also represents a potential problem with gene knockdown strate-

gies targeting ubiquitous transcriptional activators or with non-specific epigenetic drugs. Somatic *DUX4-fl* expression is predominantly restricted to differentiated skeletal muscle, due to regulation by two myogenic enhancers that activate *DUX4-fl* in an epigenetically permissive environment.³² Therefore, any manipulation that indirectly decreases *DUX4-fl* expression by affecting myogenic differentiation or the levels of key muscle factors is not a viable therapeutic avenue for FSHD. Thus, the minimal key criteria for viable therapeutic targets emerging from our screen are those regulators whose knockdown results in a reduction of *DUX4-fl* levels without affecting the expression of critical muscle genes.

Interestingly, the results of our targeted knockdown screen revealed that many of these candidates do, in fact, appear to play a role in regulating *DUX4-fl* expression as predicted (Table S1). For example, *ASH1L*, the mammalian homolog of the *Drosophila* Trithorax group protein that counteracts Polycomb-mediated gene silencing, is a histone methyltransferase that has been reported to activate *DUX4-fl* expression in FSHD.^{33–36} *ASH1L* is thought to be recruited proximal to the D4Z4 array by the *DBE-T* long noncoding RNA (lncRNA), resulting in H3K36me2 enrichment and de-repression of the FSHD locus.³⁶ Strikingly, we found that knockdown of *ASH1L* with three different shRNAs reduced *DUX4-fl* expression by ~70%–80% (Figure 1A). Likewise, knockdown of the epigenetic reader *BRD2*, the lysine-specific histone demethylase *KDM4C*, and the chromatin-remodeling factors *BAZ1A* and *SMARCA5* substantially reduced levels of *DUX4-fl* (Figures 1B–1E). Importantly, these knockdowns had minimal effects on expression of the key muscle transcription factors *MYOD1* and *MYOG* (Figure 1), indicating that *DUX4-fl* repression was not caused by reduced levels of myogenic regulatory factors.³² Levels of the muscle structural protein *MYH1* and *FRG1*, an FSHD candidate gene that lies proximal to the D4Z4 array, were also relatively unchanged (Figure 1). Depletion of *BRD2* in HeLa cells has been reported to cause widespread changes in gene expression, including a decrease in *UTRN* levels;³⁷ we observed a similar slight decrease in expression of *UTRN* following *BRD2* knockdown in FSHD myocytes (Figure 1C). We also confirmed that the knockdown of our top candidates does not affect the ability of myoblasts to fuse and form multinucleated myotubes (Figure S1). Thus, although a global analysis of changes in gene expression is beyond the scope of this study, these results suggest that certain epigenetic pathways controlling *DUX4-fl* expression in FSHD can be modulated without major adverse effects on muscle differentiation.

To confirm our top candidates across FSHD patient cohorts, we tested shRNA knockdowns of *ASH1L*, *BRD2*, *KDM4C*, and *SMARCA5* in myocytes from two other unrelated FSHD1 patients (18Abic and 17Abic), with similar results (Table S2). Although knockdown of these candidates was incomplete (~40%–60% reduction in mRNA expression), at least one shRNA for each target reduced levels of *DUX4-fl* mRNA significantly in myocytes from all three patient cohorts (Figure 2; Table S3; Figure S2). As *DUX4-FL* protein levels are low and difficult to assess in FSHD myocytes, we chose to assess *DUX4-FL* target gene expression as the more reliable assay and

Table 1. Candidate Epigenetic Regulators of *DUX4-fl*

| Gene | Alias | Full Name | Function |
|---------|----------------|---|---|
| ASH1L | – | absent, small, or homeotic-like | H3K4me3, H3K36me2/3 methyltransferase |
| BAP1 | – | BRCA1-Associated Protein 1 | histone deubiquitinase; tumor suppressor |
| BAZ1A | ACF1 | Bromodomain Adjacent to Zinc-Finger Domain 1A | ATP-dependent chromatin remodeler |
| BAZ1B | WSTF | Bromodomain Adjacent to Zinc-Finger Domain 1B | ATP-dependent chromatin remodeler |
| BAZ2A | TIP5 | Bromodomain Adjacent to Zinc-Finger Domain 2A | NoRC chromatin-remodeling complex |
| BPTF | NURF301 | Bromodomain PHD Finger Transcription Factor | NURF chromatin-remodeling complex |
| BRD2 | – | Bromodomain containing 2 | epigenetic reader |
| BRD3 | – | Bromodomain containing 3 | epigenetic reader |
| BRD4 | – | Bromodomain containing 4 | epigenetic reader |
| BRDT | BRD6 | Bromodomain testis associated | epigenetic reader |
| BRPF1 | – | Bromodomain and PHD Finger Containing 1 | epigenetic reader; MOZ HAT complex |
| BRPF3 | – | Bromodomain and PHD Finger Containing 3 | epigenetic reader; HBO1 HAT complex |
| CARM1 | PRMT4 | Coactivator Assoc. Arginine Methyltransferase 1 | H3R17 methyltransferase |
| KDM4A | JMJD2A | Lysine (K)-specific demethylase 4A | H3K9me3 & H3K36me3 demethylase |
| KDM4B | JMJD2B | Lysine (K)-specific demethylase 4B | H3K9me3 & H3K36me3 demethylase |
| KDM4C | JMJD2C | Lysine (K)-specific demethylase 4C | H3K9me3 & H3K36me3 demethylase |
| KDM4D | JMJD2D | Lysine (K)-specific demethylase 4D | H3K9me2/3 demethylase |
| KDM6A | UTX | Lysine (K)-specific demethylase 6A | H3K27me2/3 demethylase |
| KDM6B | JMJD3 | Lysine (K)-specific demethylase 6B | H3K27me2/3 demethylase |
| KMT2A | MLL | Lysine (K)-specific methyltransferase 2A | H3K4me1/2 methyltransferase |
| KMT2C | MLL3 | Lysine (K)-specific methyltransferase 2C | H3K4me1 methyltransferase |
| KMT2E | MLL5 | Lysine (K)-specific methyltransferase 2E | (no methyltransferase activity) |
| MYSM1 | – | Myb-like, SWIRM, and MPN Domains 1 | histone (H2A) deubiquitinase |
| NEK6 | – | NIMA-Related Kinase 6 | histone (H1, H3) kinase |
| PHF2 | CENP-35 | PHD-Finger Protein 2 | H3K9me2 demethylase |
| PRMT1 | – | Protein Arginine Methyltransferase 1 | H4R3 methyltransferase |
| SETD1A | KMT2F | SET Domain Containing 1A | H3K4 methyltransferase |
| SETD1B | KMT2G | SET Domain Containing 1B | H3K4 methyltransferase |
| SF3B1 | SAP155 | Splicing Factor 3b Subunit 1 | spliceosome component; B-WICH complex |
| SMARCA5 | SNF2H | SWI/SNF-Related, Matrix-Associated, Actin-Dependent Regulator of Chromatin, Subfamily A, Member 5 | NoRC, B-WICH, NURF, ACF1, RSF, chromatin-remodeling complexes |
| SMARCB1 | SNF5 and BAF47 | SWI/SNF-Related, Matrix-Associated, Actin-Dependent Regulator of Chromatin, Subfamily B, Member 1 | ATP-dependent chromatin remodeling |
| SMYD3 | KMT3E | SET and MYND Domain Containing 3 | H3K4 methyltransferase |
| UFL1 | – | UFM1-Specific Ligase 1 | E3 ligase |
| USP3 | UBP | Ubiquitin-Specific Peptidase 3 | histone (H2A, H2B, and H2AX) deubiquitinase |
| USP7 | TEF1 | Ubiquitin-Specific Peptidase 7 | histone (H2A, H2B) and non-histone deubiquitinase |
| USP16 | UBPM | Ubiquitin-Specific Peptidase 16 | histone (H2A) deubiquitinase |

relevant functional readout of *DUX4* activity. Importantly, *DUX4-FL* targets thought to have pathogenic consequences¹³ are significantly decreased by knockdown of all four candidates in myocytes from all three FSHD patients (Figure 2; Table S3; Figure S2). Thus, reducing individual levels of these four FSHD therapeutic targets significantly decreases expression of *DUX4-fl* and its downstream targets without altering the expression of certain key myogenic genes.

Transcriptional Repression of Epigenetic Regulators by dCas9-KRAB Reduces *DUX4-fl* Expression in FSHD Myocytes

In our initial screen, knockdown of eight candidates by at least two shRNAs resulted in >70% reduction of *DUX4-fl* expression, with minimal effects on other tested genes (Table S1). Based on the potential for selective druggability, we selected five of these candidates, *ASH1L*, *BAZ1A*, *BRD2*, *KDM4C*, and *SMARCA5*, for verification by

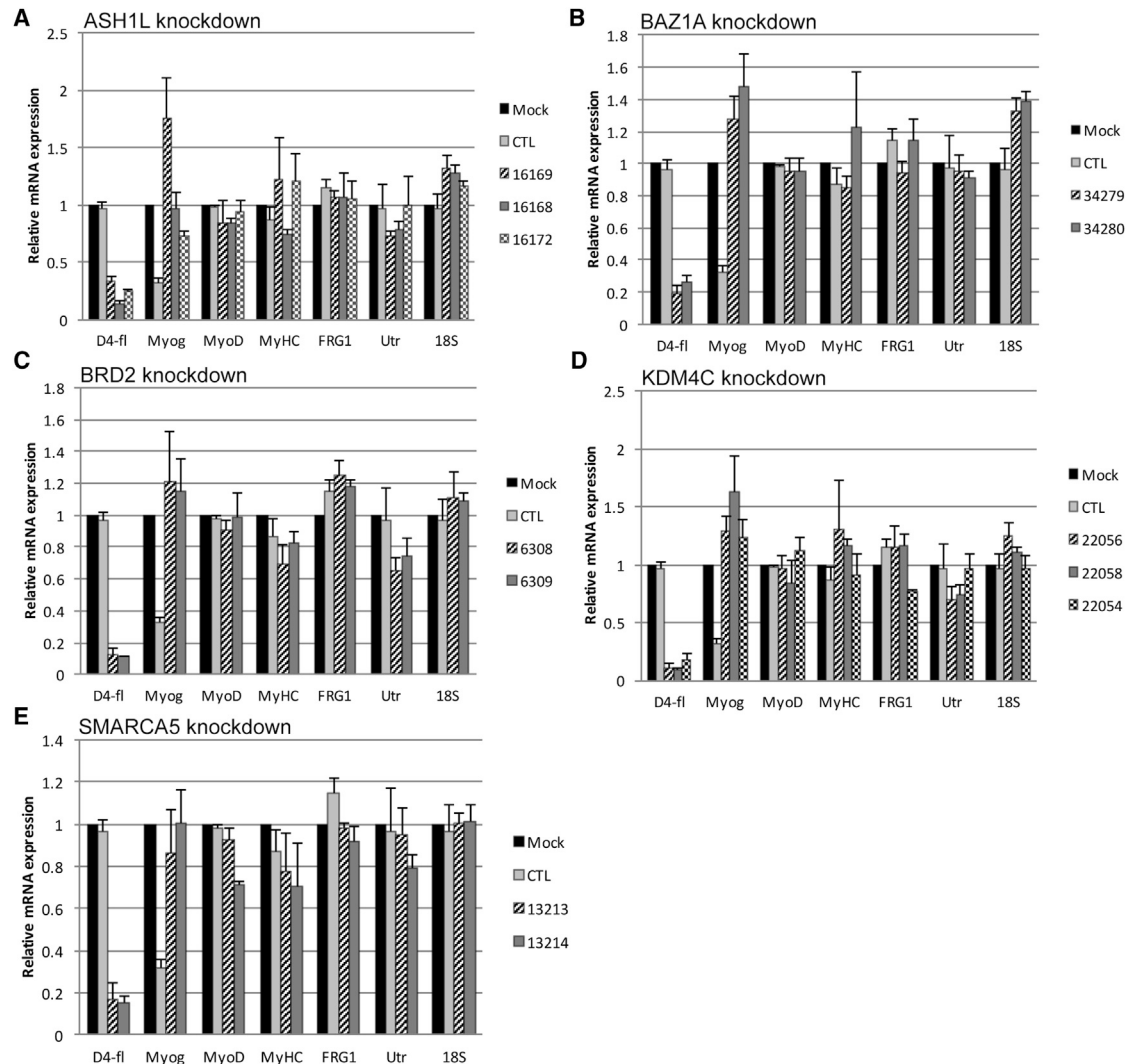


Figure 1. Knockdown of Epigenetic Regulators Reduces Expression of *DUX4-fl* in FSHD Myocytes

(A–E) Differentiated FSHD myocytes were infected in two serial rounds with lentivirus expressing shRNAs indicated in each of the panel keys specific to *ASH1L* (A), *BAZ1A* (B), *BRD2* (C), *KDM4C* (D), *SMARCA5* (E), or a scrambled control. Cells were harvested 4 days later for expression analysis of the full-length *DUX4* isoform (D4-fl), myogenin (Myog), MyoD, myosin heavy chain 1 (MyHC), *FRG1*, utrophin (Utr), and *18S* by qRT-PCR. In all panels, data are plotted as the mean + SD value of three technical replicates, with relative mRNA expression for mock-infected cells set to 1. Refer to [Table S1](#) for results of the full screen.

an independent method. Guided by appropriate single-guide RNA(s) (sgRNA[s]), the enzymatically inactive dCas9 fused to transcriptional effectors (KRAB, LSD1, VP64, and p300) can modulate endogenous target gene expression in mammalian cells.^{38–42} When recruited to regions near the transcription start site (TSS) of active genes (–50 to +250), dCas9-KRAB can be an effective transcriptional repressor,⁴³ and we have used it successfully to repress *DUX4-fl* expression in differentiated FSHD myocytes, which are not readily amenable to Cas9 cutting and selection.²⁹ Thus, for each candidate, we designed 6–8 sgRNAs targeting the promoter or exon 1, and we transduced these with dCas9-KRAB into 17Abic FSHD myocytes using four serial co-infections with centrifugation, as in our previous study.²⁹ Cells were harvested 72 hr later and assayed for changes in gene expression.

While none of the tested sgRNAs targeting *ASH1L* consistently affected expression of this candidate gene, we identified one functional sgRNA for *BRD2* and two independently functional sgRNAs for *BAZ1A*, *KDM4C*, and *SMARCA5* ([Table S4](#)). It is difficult to achieve strong repression of many transcriptional regulators, and although the levels of CRISPR inhibition achieved with these independent sgRNAs were modest (~20%–60%), the results were similar to those reported by other labs.^{39,43} However, as with the shRNA knockdowns, even a small reduction of target gene expression proved sufficient to significantly reduce levels of *DUX4-fl* (by ~40%–60%) ([Figure 3](#); [Figure S3](#); [Table S5](#)), which is ideal from a therapeutic perspective. By contrast, the expression levels of other genes (*MYOD1*, *MYOG*, *MYH1*, *FRG1*, and *18S*) were

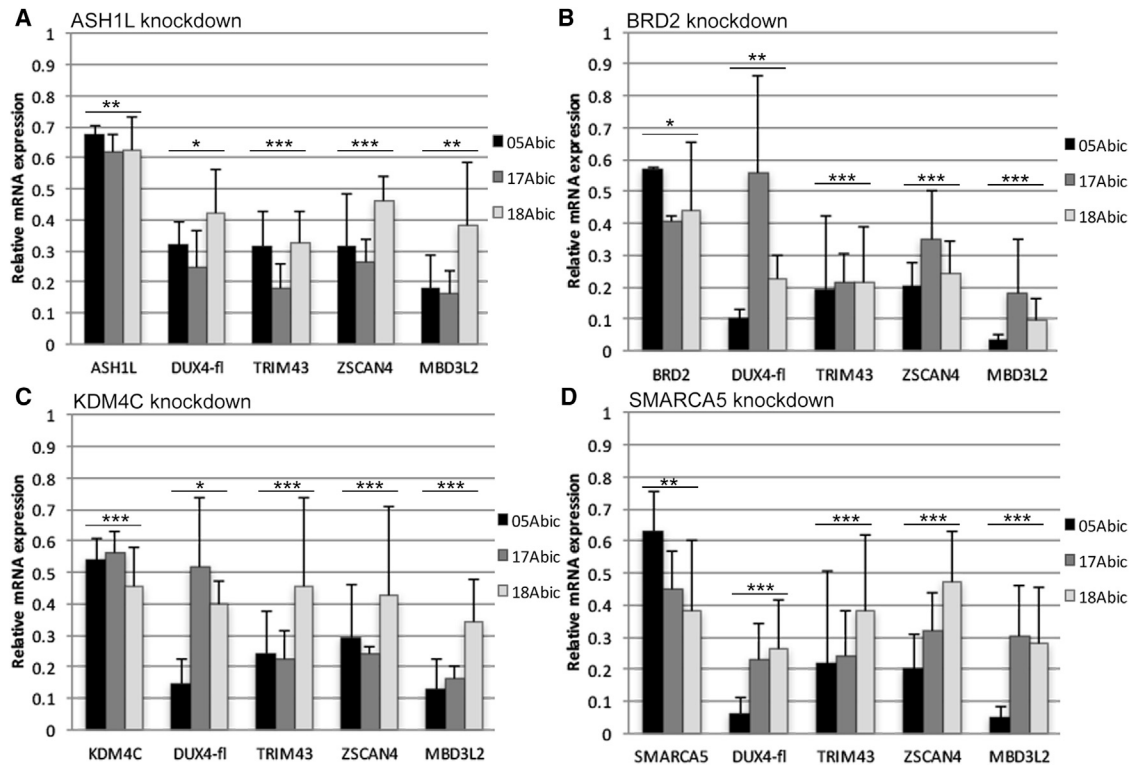


Figure 2. Knockdown of Epigenetic Regulators Reduces *DUX4-fl* and *DUX4-FL* Target Gene Expression across Multiple FSHD Cohorts

(A–D) Differentiated myocytes from three unrelated FSHD patients (05Abic, 17Abic, and 18Abic) were infected in two serial rounds with shRNAs to *ASH1L* (16169) (A), *BRD2* (6308) (B), *KDM4C* (22058) (C), *SMARCA5* (13214) (D), or a scrambled control. Cells were harvested 4 days later for expression analysis of shRNA target genes; *DUX4-fl*; and *DUX4-FL* target genes *TRIM43*, *ZSCAN4*, and *MBD3L2* by qRT-PCR. In all panels, data are plotted as the mean + SD value of three independent experiments, with relative mRNA expression for control-infected cells set to 1. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are from comparing knockout to the corresponding control ($n = 3$ patients). Refer to the [Materials and Methods](#), [Figure S2](#), and [Tables S2](#) and [S3](#) for additional details.

relatively unaffected by reduction of the candidate regulator ([Figure 3](#); [Figure S3](#)). Importantly, *DUX4-fl* was the only tested gene to be significantly reduced by both methods of repression (shRNA knockdown and CRISPR inhibition). For example, knockdown of *SMARCA5* with one shRNA (13214) resulted in a 30% decrease in *MYOD1* ([Figure 1E](#)), but this was not recapitulated by the other shRNA (13213) or by either sgRNA in the CRISPR inhibition experiment, and, thus, it likely represents an off-target effect.

Knockdown of Epigenetic Regulators Increases Chromatin Repression at the D4Z4 Macrosatellite Array

Since knocking down any of several epigenetic regulators in FSHD myocytes led to a substantial decrease in expression of *DUX4-fl*, we wanted to assess changes in chromatin at the pathogenic locus. Although *DUX4* is present in every D4Z4 repeat unit at both 4q and 10q alleles, the chromatin at three of these alleles is already in a compacted, heterochromatic state. Thus, any attempt to assess repression at the contracted allele would be dampened by the presence of the other three alleles. To remove 10q alleles from the analysis, we took advantage of a chromosome 4- versus 10-specific sequence polymorphism in the *DUX4* exon 2 in our primer design.

To assess detectable chromatin changes, we performed chromatin immunoprecipitation (ChIP) for several histone modifications following shRNA knockdown of *ASH1L*, *BRD2*, *KDM4C*, or *SMARCA5* in 17Abic FSHD myocytes. For these experiments, we used shRNAs that gave strong, consistent knockdowns of each target gene across all FSHD cohorts tested. We found that levels of the repressive H3K9me3 mark were increased in *BRD2* and *KDM4C* knockdown cells at the chromosome 4 *DUX4* exon1/intron1 ([Figure 4](#); [Figure S4](#)). Knockdown of the H3K36 methyltransferase *ASH1L* led to the expected decrease in levels of H3K36me3 at *DUX4*; conversely, knockdown of the H3K9/H3K36 demethylase *KDM4C* led to the expected increase in both marks at *DUX4* ([Figure 4](#); [Figure S4](#)). Although changes in enrichment were slight (~45%–85%), these differences were significant ([Table S6](#)) and likely to be an underestimate, as they reflect an increase in repression at the distal de-repressed pathogenic repeat among a background of heterochromatic 4q repeats. With regard to this, patient 17A has ~5 repeat units on the contracted 4A161 allele and ~26 repeat units on the non-contracted 4A-L161 allele. In all cases, there was no significant change in levels of enrichment at the heterochromatic 4p macrosatellite array, indicating that these epigenetic modifiers are not acting broadly at repeat regions across the genome.

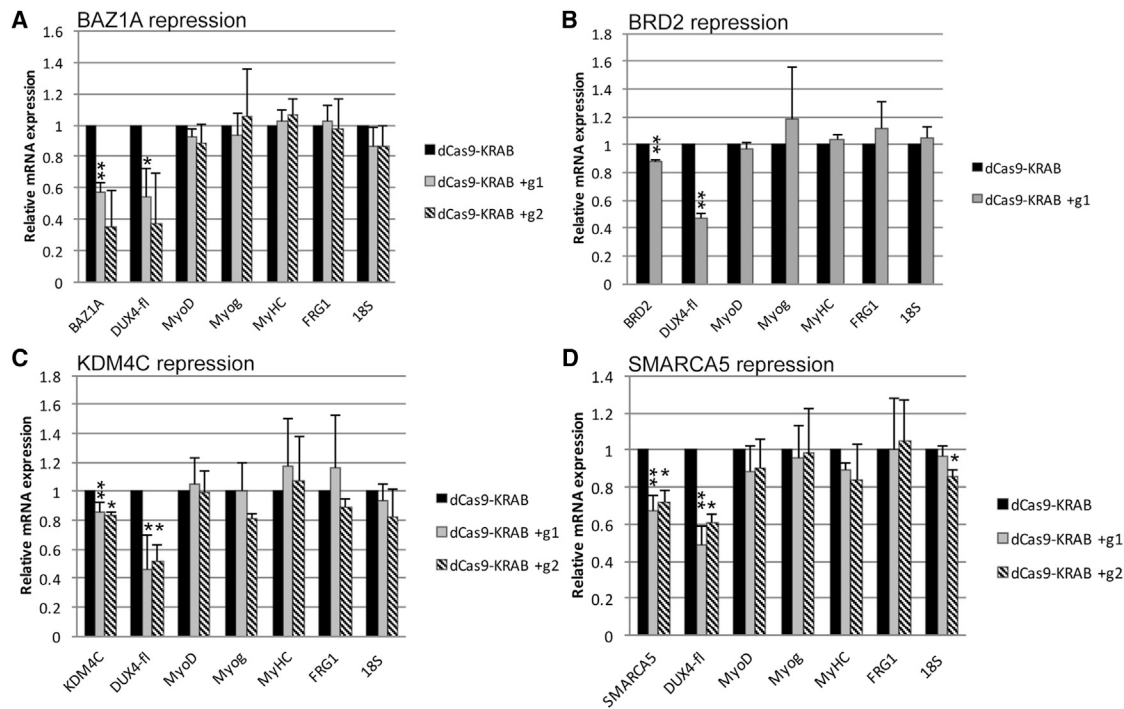


Figure 3. Transcriptional Repression of Epigenetic Regulators by dCas9-KRAB Reduces Expression of *DUX4-fl* in FSHD Myocytes

(A–D) Differentiated FSHD myocytes were subjected to four serial co-infections with combinations of lentiviral supernatants expressing either dCas9-KRAB or individual sgRNAs (g1–2 for each target gene) targeting *BAZ1A* (A), *BRD2* (B), *KDM4C* (C), or *SMARCA5* (D). Cells were harvested ~72 hr later for analysis of gene expression by qRT-PCR (as in Figure 1). Data are plotted as the mean + SD value of three independent experiments, with relative mRNA expression for cells infected with dCas9-KRAB alone set to 1. * $p < 0.05$ and ** $p < 0.01$ are from comparing sgRNAs to dCas9-KRAB alone. Refer to the [Materials and Methods](#), [Figure S3](#), and [Tables S4](#) and [S5](#) for additional details.

As repressive histone modifications can lead to more stable repression in the form of DNA methylation, we performed bisulfite sequencing across the gene body of the distal pathogenic *DUX4* repeat²³ following shRNA knockdown of *ASH1L*, *BRD2*, *KDM4C*, or *SMARCA5* in 17Abic FSHD myocytes. While there were no changes in the pattern of DNA hypomethylation seen across the main gene body of *DUX4* (Figure S5A), we found that, by 4 days post-infection, there was a small increase in DNA methylation at exon 3 in *ASH1L*, *BRD2*, and *SMARCA5* knockdown cells (Figure S5B). Although the functional significance of methylation at exon 3 is unclear, knockdown of these epigenetic regulators serves to drive the methylation pattern at this region toward the higher levels seen in healthy myocytes (Figure S5B). Since primary, terminally differentiated myocytes in culture are not the ideal system in which to assess long-term changes, we expect that any substantial increase in DNA methylation will require assessment in a more physiologically relevant model.

In summary, using several different methods, we have demonstrated that independent knockdown of multiple chromatin regulators results in chromatin repression at *D4Z4* and a substantial decrease in *DUX4-fl* expression in FSHD myocytes. Our results provide proof of principle that even modest inhibition of certain epigenetic pathways can substantially reduce levels of *DUX4-fl*, demonstrating their potential as novel drug targets for FSHD.

DISCUSSION

In a previous study, we successfully corrected the underlying defect in FSHD by using dCas9-KRAB (CRISPR inhibition) to return the aberrantly relaxed chromatin at the disease locus to a more repressed state.²⁹ However, due to limitations in current technology and delivery, our CRISPR inhibition approach is many years away from being clinically applicable. Small molecule therapeutics targeting the regulatory factors modulating *DUX4-fl* expression at the FSHD locus could overcome these limitations. Therefore, here we took a different approach to this correction, identifying the potentially druggable chromatin-regulatory pathways converging on the contracted FSHD1 *D4Z4* macrosatellite repeat, which represent therapeutic targets for designing inhibitory molecules. Using two complementary modalities to knock down specific candidate regulators, we found that many of these pathways serve to regulate *DUX4-fl* expression in FSHD myocytes. Importantly, while we have focused on only several of these factors, the results from our initial screen indicate that there are many viable potential candidates for FSHD drug development, for any investigator who wishes to pursue them.

The identification of multiple candidate regulators of *DUX4-fl* is perhaps not surprising, as many of these factors are part of the same multi-protein complexes or function in the same regulatory pathways (Table 2). For instance, *SMARCA5* is a catalytic component

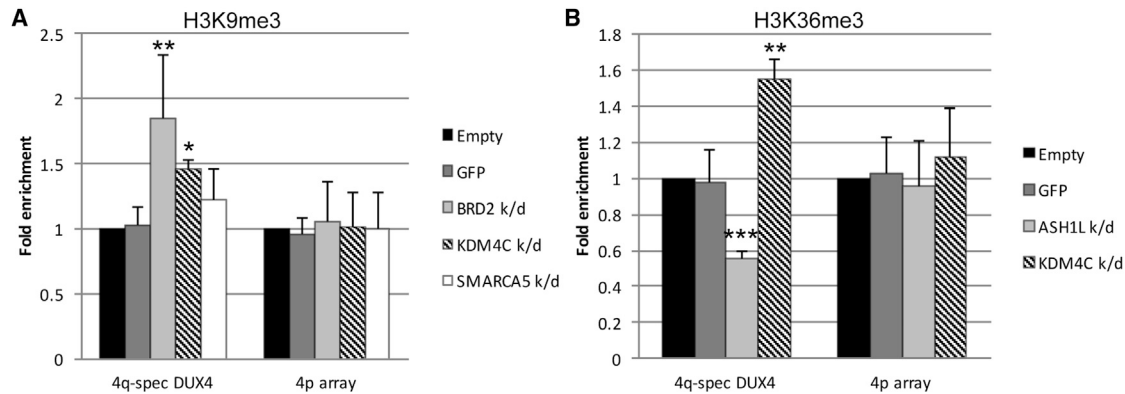


Figure 4. Knockdown of Epigenetic Regulators Alters Chromatin at the D4Z4 Macrosatellite Array

(A and B) Differentiated FSHD myocytes were infected in two serial rounds with shRNAs to *ASH1L*, *BRD2*, *KDM4C*, *SMARCA5*, an empty control, or a GFP control. Cells were harvested 4 days later for ChIP analysis. Chromatin was immunoprecipitated using antibodies specific for H3K9me3 (A), H3K36me3 (B), or H3, and it was analyzed by qPCR using primers specific to *DUX4* exon 1/intron 1 on chromosome 4 (4q-spec DUX4) or the 4p macrosatellite array on chromosome 4. Data are presented as fold enrichment of the target region by α -H3K9me3 or α -H3K36me3 normalized to α -histone H3, with enrichment for the empty control shRNA-infected cells set to 1. Data are plotted as the mean + SD value of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ are from comparing knockout to the corresponding empty control. Refer to the [Materials and Methods](#), [Figure S4](#), and [Table S6](#) for additional details.

of at least five chromatin-remodeling complexes (CHRAC, RSF, ACF/WCRF, B-WICH, and NoRC^{44–48}). SMARCA5 directly associates with BAZ1A and BAZ2A, two other candidates from our screen, in two distinct chromatin-remodeling complexes that enable DNA replication through repressive chromatin and mediate heterochromatin formation at repetitive elements, respectively.^{49,50} SMARCA5 is also part of the B-WICH-remodeling complex—which includes two other candidates from our screen, BAZ1B and the splicing factor SF3B1—and part of the NURF complex with BPTF⁵¹ (Table 2). These complex interaction networks, and the large families these factors belong to, are largely responsible for the toxicity of current epigenetic drugs. However, as with the *Momme* genetic screen,²⁶ our results demonstrate that the reduction of certain epigenetic regulators can have relatively specific effects. While a global gene expression analysis would likely find other changes, it is clear that our candidates are not global regulators and have some specificity for D4Z4 arrays. This specificity is not without precedent, as many repressive epigenetic factors are relatively specific for the chromosome 4q and 10q D4Z4 arrays.¹⁷ For example, the highly specific *de novo* DNA methyltransferase DNMT3B is localized at D4Z4 arrays,^{52,53} and SMCHD1, the chromatin-remodeling protein responsible for FSHD2, has a very restricted genomic distribution, including D4Z4 macrosatellites.^{10,54}

The identification of potential targets from screens such as ours should facilitate the development of more specific small molecule inhibitors. For example, consistent with our results, a recent report indicates that BET bromodomain inhibition decreases *DUX4* expression in FSHD myocytes.⁵⁵ However, the results of our epigenetic screen indicate that pan-BRD inhibition is not necessary or even desirable as a treatment for FSHD. We found that *BRD2* knockdown represses *DUX4-fl* levels without major effects on muscle genes, whereas knockdown of *BRD3* or *BRD4* either led to substantial effects on other genes or was less effective at decreasing *DUX4-fl*. Thus, the

development of drugs targeting specific isoforms as well as specific protein interactions should decrease adverse effects, leading to greater safety and efficacy. This is particularly important, since combination therapies may ultimately prove to be the most effective means of treating FSHD.

These epigenetic regulators have wide-ranging roles (e.g., cell proliferation, differentiation, DNA repair, and apoptosis); thus, it is possible that they are affecting *DUX4-fl* expression indirectly. However, they are most likely reducing *DUX4-fl* expression by modifying the chromatin state at the pathogenic locus. Even a modest reduction in expression of these factors led to enhanced chromatin repression at the chromosome 4q D4Z4 array, detectable even on a background of heterochromatic repeats, and a striking decrease in *DUX4-fl* expression. The ability to modulate levels of *DUX4-fl* without completely abolishing expression of its upstream regulators, all of which play important and diverse cellular roles, is particularly encouraging for the development of targeted therapies. Within FSHD families, asymptomatic individuals still express detectable levels of *DUX4-fl* (lower than those of their manifesting siblings),^{24,31} suggesting that *DUX4* expression doesn't need to be completely silenced, merely reduced, to see a therapeutic effect. The catastrophic effects of small increases in *DUX4-fl* levels in mouse models^{56,57} also suggest that even small decreases in expression will be beneficial to patients. While the establishment of stable, long-term repression will be important to demonstrate, we found that knockdown of several regulators led to an increase in DNA methylation at exon 3 of *DUX4* as early as 4 days post-infection. Unfortunately, primary FSHD myotubes—which are not undergoing replication and are not amenable to gene editing, selection, or long-term culturing—are not the best model in which to test long-term epigenetic changes. Cells such as the newly reported FSHD lymphoblast lines, which express *DUX4-fl* mRNA and mimic patterns of DNA methylation seen in FSHD myocytes,⁵⁸ would be

Table 2. Top Therapeutic Candidates for Targeted Repression of *DUX4-fl*

| Candidate | Target Domains | Function and Complexes | Candidate Interactions |
|---------------|--|--|--------------------------------------|
| ASH1L | SET, ⁶³ Bromo, ⁶⁴ BAH, ⁶⁵ and PHD ⁶⁶ | H3K36me2/3 methyltransferase | – |
| BAZ1A/ACF1 | Bromo, ⁶⁴ PHD, ⁶⁶ and WAC ⁶⁷ | non-catalytic component that enhances and directs function of SMARCA5 in the CHRAC- and ACF-remodeling complexes | SMARCA5 |
| BRD2 | Bromo1 and Bromo2 ⁶⁴ | binds hyperacetylated chromatin; regulates transcription | – |
| KDM4C/JMJD2C | jmjN/jmjC ⁶⁸ and PHD ⁶⁶ | H3K9me3 and H3K36me3 demethylase | PRMT1 |
| SMARCA5/SNF2H | Helicase ATP-binding, Helicase C, ⁶⁹ SANT1, and SANT2 ⁷⁰ | catalytic subunit of ATP-dependent chromatin-remodeling complexes (CHRAC, RSF, ACF, B-WICH, NoRC, and NURF) | BAZ1A, BAZ1B, BAZ2A, SF3B1, and BPTF |

better suited for assessing DNA methylation changes in response to the depletion of *DUX4-fl* regulators. In response to epigenetic drugs, these lymphoblasts show a similar induction of *DUX4-fl* expression to that seen in FSHD myocytes,⁵⁸ suggesting that at least some upstream regulatory pathways are maintained in non-muscle cells.

It is likely that multiple epigenetic pathways have evolved to repress repetitive elements within the human genome. The *DUX4* retrogene appears to have a normal developmental role in the testis;¹² however, silencing of *DUX4* and the D4ZA macrosatellite repeat is critical in somatic tissues. Although *DUX4-fl* is only rarely expressed in FSHD myocytes at any given time, it is epigenetically poised for expression in a majority of these cells,^{24,29} indicating a general deregulation of repressive upstream mechanisms. Our results suggest that, in the absence of normal repression, activating pathways are aberrantly active and even modest perturbations in these pathways may be sufficient to reduce levels of *DUX4-fl*.

How these regulators activate *DUX4* in FSHD myocytes is an important question that may bear on the normal function of *DUX4* during development. *DUX4* is normally expressed in the testis, where epigenetic mechanisms are critically important for regulating spermatogenesis. The abnormal activation of testis genes is not unprecedented: cancer/testis antigens—genes normally expressed in the male germline that encode immunogenic proteins—are epigenetically activated in many types of cancer. Interestingly, many *DUX4-FL* targets are also cancer/testis antigens.¹³ Although the mechanisms controlling *DUX4* expression in germ cells are still uncharacterized, the upstream enhancers driving *DUX4* transcription in FSHD myocytes contain elements for both muscle and testis factors.³² Thus, it seems plausible that, in the absence of normal somatic repression, epigenetic pathways that activate *DUX4* in the testis could be aberrantly activated in skeletal muscle, allowing muscle factors inappropriate access to *DUX4*-regulatory regions. Further characterization of these mechanisms should help to uncover additional targets for therapeutic development.

MATERIALS AND METHODS

Plasmids and Antibodies

The pHAGE EF1-dCas9-KRAB (Addgene plasmid 50919) and pLKO.1-puro U6 sgRNA BfuAI stuffer lentiviral plasmids were devel-

oped by Rene Maehr and Scot Wolfe (Addgene plasmid 50920).⁴⁰ The ChIP-grade antibodies used in this study, α -H3K9me3 (ab8898), α -H3K36me3 (ab9050), and α -histone H3 (ab1791), were purchased from Abcam (Cambridge, MA).

sgRNA Design and Plasmid Construction

We used the publicly available sgRNA design tool from the Broad Institute (<http://www.broadinstitute.org/rnai/public/analysis-tools/sgRNA-design>) to identify candidate sgRNAs targeting the promoter/exon 1 regions of human *BAZ1A*, *BRD2*, *KDM4C*, and *SMARCA5* (Supplemental Information; Table S4). To build in flexibility for experiments beyond the scope of this study, we prioritized sgRNAs that target sequences flanking dual protospacer adjacent motifs (PAMs) recognizable by both SaCas9 and SpCas9. Predicted off-target matches were determined using the CRISPR Design Tool (<http://crispr.mit.edu>).⁵⁹ 6–8 sgRNAs for each target gene were cloned individually into BfuAI sites in the pLKO.1-puro U6 sgRNA BfuAI stuffer plasmid and sequence verified.

Cell Culture, Transient Transfections, and Lentiviral Infections

Myogenic cells were obtained from the Wellstone FSHD cell repository housed at the University of Massachusetts Medical School,^{24,31} and normal human primary myoblasts were obtained from Lonza (HSM, lot 509793). Myogenic cultures derived from biceps muscles of unrelated FSHD1 patients (05Abic, 17Abic, and 18Abic), normal myoblasts, and 293T packaging cells were grown as described.²⁹ Lentiviral particles expressing shRNAs were generated using The RNAi Consortium (TRC) shRNA expression plasmids as previously described,⁶⁰ and they were obtained through the University of Massachusetts Medical School RNAi Core Facility. FSHD1 skeletal myoblasts were grown to confluence, and then allowed to self-differentiate in growth medium for ~48 hr. Cells were subjected to 2 rounds of infection and harvested 4 days later (for shRNA knockdowns and ChIP) or 4 rounds of infection and harvested 3 days later (for CRISPR inhibition experiments), as described.²⁹ For the assessment of fusion index, normal primary myoblasts were switched to differentiation conditions^{24,31} following shRNA knockdown.

qRT-PCR

Total RNAs were extracted using TRIzol (Invitrogen) and purified using the RNeasy Mini kit (QIAGEN) after on-column DNase I

digestion. Total RNA (2 µg) was used for cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen), and 200 ng cDNA was used for qPCR analysis as described.²⁴ Oligonucleotide primer sequences are provided in [Table S7](#).

ChIP

ChIP assays were performed with lentiviral-infected 17A5c differentiated myocytes using the Fast ChIP method⁶¹ as described.²⁹ Chromatin was immunoprecipitated using 2 µg specific antibodies. SYBR green qPCR assays were performed as described.²⁹ Oligonucleotide primer sequences are provided in [Table S7](#).

Bisulfite Sequencing

Bisulfite sequencing (BSS) was performed on genomic DNAs isolated from lentiviral-infected 17A5c differentiated myocytes. DNA methylation at the distal pathogenic D4Z4 repeat was analyzed using the 4qA BSS assay as described^{23,24} or using primers amplifying exon 3 from 4qA ([Table S7](#)).

Statistics

Statistical analysis was performed using R⁶² with log2-transformed gene expression or ChIP enrichment data. Levene's test shows that the assumption of homogeneity of variances is met. Pre-determined contrast was made between knockout and the corresponding empty vector within the framework of ANOVA using randomized block design (patient as block for [Figure 2](#), experiment as block for [Figures 3 and 4](#); n = 3–5). Fusion index ([Figure S1](#)) was first arcsine-transformed to homogenize the variance. Levene's test shows that the assumption of homogeneity of variances is met. ANOVA of the randomized block design was performed followed by predetermined contrasts.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and seven tables and can be found with this article online at <https://doi.org/10.1016/j.ymthe.2018.04.019>.

AUTHOR CONTRIBUTIONS

M.R.G. conceived of the project. C.L.H., T.I.J., M.R.G., and P.L.J. designed research. C.L.H. and T.I.J. performed research. C.-M.V. and M.R.G. contributed new reagents. C.L.H., L.J.Z., M.R.G., and P.L.J. analyzed data. C.L.H., L.J.Z., M.R.G., and P.L.J. wrote the paper.

CONFLICTS OF INTEREST

The results described in this manuscript are part of a patent application that has been licensed by Fulcrum Therapeutics. P.L.J. and M.R.G. are scientific advisors to Fulcrum Therapeutics; C.L.H. is a scientific consultant to Fulcrum Therapeutics.

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