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

Charis L. Himeda,1,2 Takako I. Jones,1,2 Ching-Man Virbasius,3,4 Lihua Julie Zhu,1,5 Michael R. Green,3,4 and Peter L. Jones1,2

INTRODUCTION
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.

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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 inhibitor and the Dicer/Argonaute system. 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, 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 an 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. 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 strategies 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. 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. 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. 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. Levels of the muscle structural protein MYH1 and FREG1, 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; 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
relevant functional readout of DUX4 activity. Importantly, DUX4-FL targets thought to have pathogenic consequences 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

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<tr>
<th>Gene</th>
<th>Alias</th>
<th>Full Name</th>
<th>Function</th>
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<td>absent, small, or homeotic-like</td>
<td>H3K4me3, H3K36me2/3 methyltransferase</td>
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<td>splicosome component; B-WICH complex</td>
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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 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.
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). Knockdown of the H3K36 methyltransferase ASH1L led to the expected decrease in levels of H3K9me3 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.
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 repeat 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). 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. 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...
of at least five chromatin-remodeling complexes (CHRAC, RSF, ACF/ WCRF, B-WICH, and NoRC). 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. SMARCA5 is also part of the B-WICH-remodeling complex—which includes two other candidates from our screen, SF3B1 and the splicing factor SF3B1—and part of the NURF complex with BPTF (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 Mutante genetic screen, 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. For example, the highly specific de novo DNA methyltransferase DNMT3B is localized at D4Z4 arrays, and SMCHD1, the chromatin-remodeling protein responsible for FSHD2, has a very restricted genomic distribution, including D4Z4 macrosatelites.

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. 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 knockout represses DUX4-fl levels without major effects on muscle genes, whereas knockout 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), 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 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, would be
in a majority of these cells, indicating a general deregulation of myocytes at any given time, it is epigenetically poised for expression of related genes. Although silencing of 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, 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: the abnormal activation of testis genes normally expressed in the male germ line that encode immunogenic proteins—are epigenetically activated in many types of cancer. Interestingly, many DUX4-FL targets are epigenetically activated and the D4Z4 macrosatellite repeat is critical in so-

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; however, silencing of DUX4 and the DAZ4 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, 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 germ line that encode immunogenic proteins—are epigenetically activated in many types of cancer. Interestingly, many DUX4-FL targets are also cancer/testis antigens. 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. 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 developed by Rene Maehr and Scot Wolfe (Addgene plasmid 50920). 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/rna pública análise 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). 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, and normal human primary myoblasts were obtained from Lonza (HSMM, 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. Lentiviral particles expressing shRNAs were generated using The RNAi Consortium (TRC) shRNA expression plasmids as previously described, 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. For the assessment of fusion index, normal primary myoblasts were switched to differentiation conditions 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...
SYBR green qPCR assays were performed as described.29 Oligonucleotide primer sequences are provided in Table S7.

ChIP
ChIP assays were performed with lentiviral–infected 17Abic differentiated myocytes using the Fast ChIP method31 as described.32 Chromatin was immunoprecipitated using 2 μg specific antibodies. SYBR green qPCR assays were performed as described.32 Oligonucleotide primer sequences are provided in Table S7.

Bisulfite Sequencing
Bisulfite sequencing (BSS) was performed on genomic DNAs isolated from lentiviral–infected 17Abic differentiated myocytes. DNA methylation at the distal pathogenic D4Z4 repeat was analyzed using the 4qA BSS assay as described33,34 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. Levine’s test shows that the assumption of homogeneity of variances is met. Pre-determined contrasts was made between knockout and the corresponding empty gene expression or ChIP enrichment data. Levene’s test shows that the assumption of homogeneity is met. ANOVA of the randomized block design was performed followed by pre-determined 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

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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REFERENCES

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