

# Increased DUX4 expression during muscle differentiation correlates with decreased SMCHD1 protein levels at D4Z4

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Facioscapulohumeral muscular dystrophy is caused by incomplete epigenetic repression of the transcription factor DUX4 in skeletal muscle. A copy of *DUX4* is located within each unit of the D4Z4 macrosatellite repeat array and its derepression in somatic cells is caused by either repeat array contraction (FSHD1) or by mutations in the chromatin repressor SMCHD1 (FSHD2). While DUX4 expression has thus far only been detected in FSHD muscle and muscle cell cultures, and increases with *in vitro* myogenic differentiation, the D4Z4 chromatin structure has only been studied in proliferating myoblasts or non-myogenic cells. We here show that SMCHD1 protein levels at D4Z4 decline during muscle cell differentiation and correlate with DUX4 derepression. In FSHD2, but not FSHD1, the loss of SMCHD1 repressor activity is partially compensated by increased Polycomb Repressive Complex 2 (PRC2)-mediated H3K27 trimethylation at D4Z4, a situation that can be mimicked by SMCHD1 knockdown in control myotubes. In contrast, moderate overexpression of SMCHD1 results in DUX4 silencing in FSHD1 and FSHD2 myotubes demonstrating that DUX4 derepression in FSHD is reversible. Together, we show that in FSHD1 and FSHD2 the decline in SMCHD1 protein levels during muscle cell differentiation renders skeletal muscle sensitive to DUX4.

## Introduction

Facioscapulohumeral dystrophy (FSHD, OMIM 158900/158901) is characterized by progressive and often asymmetrical weakness of facial and upper extremity muscles typically starting in the second decade of life.<sup>1</sup> FSHD is one of the most common myopathies with a recently reported prevalence of 12:100,000 in the Dutch population and is inherited in an autosomal dominant fashion in most families.<sup>2</sup> FSHD is genetically linked to the polymorphic D4Z4 macrosatellite repeat array at 4q35, which in healthy subjects consists of 11–100 units, each of them being 3.3 kb in size and containing a copy of the *DUX4* retrogene.<sup>3–5</sup>

The prevailing FSHD disease model postulates that *DUX4* is normally silenced in somatic cells by repeat mediated epigenetic repression and that failure of this mechanism leads to partial decompaction of the D4Z4 chromatin structure and the expression of DUX4 in skeletal muscle of FSHD individuals.<sup>6</sup> Stable

*DUX4* expression requires a specific genetic background of chromosome 4 that contains a polymorphic *DUX4* polyadenylation signal (PAS) immediately distal to the D4Z4 repeat array.<sup>7</sup> Partial D4Z4 chromatin decompaction on a *DUX4*-PAS containing chromosome leads to the FSHD-specific pattern of DUX4 expression in myotube cultures with few myonuclei expressing relatively abundant amounts of DUX4 protein.<sup>8</sup> Expression of the germline double homeobox transcription factor DUX4 is harmful to skeletal muscle since it activates germline and early stem cell programs, and modulates inflammatory pathways eventually resulting in increased cell death.<sup>9–11</sup>

In >95% of FSHD individuals (FSHD1) incomplete repression of *DUX4* can be attributed to a contraction of the D4Z4 repeat array to a size of 1–10 units, whereas in the remainder of individuals with FSHD (FSHD2) the D4Z4 repeat arrays are in the normal size range.<sup>5,12,13</sup> The majority of FSHD2 individuals carry heterozygous mutations in the structural maintenance of

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chromosomes hinge domain 1 (*SMCHD1*) gene on chromosome 18.<sup>14</sup> *SMCHD1* is a chromatin repressor that binds to D4Z4 and germline mutations in *SMCHD1* result in reduced D4Z4 chromatin compaction and *DUX4* expression.<sup>14</sup> Moreover, *SMCHD1* was shown to act as a modifier of disease severity in FSHD1<sup>15</sup> and the functional consequences of *SMCHD1* mutations, in combination with D4Z4 repeat size, determines the disease penetrance.<sup>16</sup>

Several lines of evidence indicate that epigenetic derepression of the D4Z4 macrosatellite repeat array in FSHD results in *DUX4* expression in skeletal muscle cells.<sup>17,18</sup> In somatic cells originating from healthy individuals, such as myoblasts, the D4Z4 repeat array is characterized by high, but inhomogeneous levels of CpG methylation and histone 3 lysine 9 trimethylation (H3K9me3).<sup>18-22</sup> In proliferating FSHD somatic cell cultures, a reduction of both CpG methylation and H3K9me3 at D4Z4 has been reported together with reduced cohesin and heterochromatin protein 1  $\gamma$  (HP1 $\gamma$ ) levels, presumably facilitating sporadic activation of *DUX4*.<sup>18</sup> In addition, members of the Polycomb Repressive Complex 2 (PRC2) and the histone modification deposited by the PRC2 complex, H3K27me3, were identified at D4Z4 in control and FSHD1 proliferating myoblasts.<sup>17,18,23</sup> The mechanism of PRC2 recruitment remains however to be determined. D4Z4 non-coding RNAs may have a role, since the endogenous RNAi pathway was recently demonstrated to facilitate DICER/AGO-dependent epigenetic silencing of the D4Z4 repeat arrays in muscle cells.<sup>24,25, 26</sup>

Although *DUX4* expression has repeatedly been shown to be increased in FSHD muscle cells upon *in vitro* differentiation,<sup>8,27-29</sup> data on the epigenetic regulation of the D4Z4 repeat array during this process is scarce. Nevertheless, extensive changes in the genome wide chromatin structure have been reported upon myogenic differentiation in mouse muscle cells. In particular, the Polycomb complexes were shown to be pivotal for the regulation of lineage commitment and differentiation specific genes.<sup>30</sup> Moreover, multi-nucleated myotubes more closely resemble the *in vivo* structural composition of muscle than proliferating myoblasts. Taken together, differentiated myotubes are likely a more relevant *in vitro* model to study the epigenetic requirements for *DUX4* expression than proliferating myoblasts.

This prompted us to examine whether we could identify changes in D4Z4 chromatin regulation specific to differentiated muscle cells that might account for the susceptibility of this tissue to express *DUX4* at higher levels than in myoblasts or many other tissues. Our study highlights the importance of *SMCHD1* in regulating D4Z4 chromatin structure and *DUX4* expression in muscle cells of both FSHD1 and FSHD2. Whereas a decline in *SMCHD1* protein levels, and specifically its reduction at D4Z4, during skeletal muscle differentiation leads to *DUX4* expression, ectopic expression of *SMCHD1* in either FSHD1 or FSHD2 muscle cell cultures efficiently suppresses *DUX4* expression. Moreover, in FSHD2 but not in FSHD1, the loss of *SMCHD1* is accompanied by a gain in PRC2-mediated H3K27 trimethylation, likely as a compensatory but incomplete mechanism to silence *DUX4* in skeletal muscle.

## Results

### DUX4 expression is induced upon myogenic differentiation and coincides with reduced *SMCHD1* levels

Although *DUX4* expression has been reported repeatedly in FSHD myotubes,<sup>8,27,29</sup> comprehensive data on the regulation of *DUX4* expression in FSHD1 and in particular on FSHD2 muscle cells is scarce. Therefore, our first aim was to firmly establish that *DUX4* expression increases with differentiation of FSHD1 and FSHD2 muscle cells. We cultured a large set of primary myoblasts derived from control, FSHD1 and FSHD2 individuals and harvested RNA from both proliferating myoblasts and differentiating myotubes (further referred to as myoblasts and myotubes). The differentiation status of the cultures was confirmed by myotube formation, as visualized by immunofluorescent labeling of myosin, and MYOG transcript levels (Fig. 1A, Fig. S1 and S2). Multinucleated myofibers were observed in all conditions and differentiation was confirmed by the increase in MYOG levels in control, FSHD1, and FSHD2 muscle cell cultures.

mRNA quantification by qRT-PCR confirmed the activation of *DUX4* in FSHD1 and FSHD2 myotubes, whereas we could not reproducibly detect *DUX4* transcripts in control samples (Fig. 1B). While the increase of *DUX4* expression during myotube formation in FSHD1 cells was statistically significant, FSHD2 cells were more variable. In FSHD2 cells, an increase of *DUX4* during myogenesis can be observed; however, FSHD2 muscle cell cultures already show variable levels of *DUX4* transcripts before differentiation, which may explain the non-significant effect (Fig. 1B). Immunofluorescence microscopy on a subset of myotube cultures established the variegated pattern of *DUX4* protein expression in both forms of the disease (Fig. 1A, Fig. S2). We also quantified expression levels of the previously established *DUX4* target gene *ZSCAN4* and observed FSHD-specific induction during myoblast differentiation (Fig. 1C).<sup>9</sup>

Since we previously demonstrated that *SMCHD1* is a D4Z4 chromatin repressor,<sup>14</sup> we examined *SMCHD1* protein levels. Western blot analysis showed a reduction in *SMCHD1* protein levels after control myoblast differentiation (Fig. 1D), a phenomenon that could be reproduced by forced myogenic differentiation of control fibroblasts transduced with the myogenic transcription factor MyoD (Fig. 1E). Upon forced myogenesis by MyoD transduction in 2 FSHD1 and 2 FSHD2 fibroblast cell lines we observed induction of *DUX4*, whereas *DUX4* remained undetectable in 2 control samples treated with MyoD (Fig. 1F and Fig. S3). Employing ChIP-qPCR in control fibroblast, myoblast and myotube cultures from the same donor we observed higher density of *SMCHD1* at qD4Z4, located around the transcriptional start site of *DUX4* (Fig. S4),<sup>18</sup> in fibroblasts as compared to myoblasts, with a further decrease in myotubes (Fig. 1G). Analysis of 3 additional independent pairs of control myoblast and myotube cultures confirmed the significant decrease in *SMCHD1* levels at D4Z4 after myoblast differentiation (Fig. 1H). Together, we confirm the FSHD-specific induction of *DUX4* expression upon myogenic differentiation in a large set of FSHD1 and FSHD2 primary myotube cultures and upon MyoD-induced differentiation in FSHD fibroblasts.

Moreover, we demonstrate that DUX4 expression negatively correlates with SMCHD1 levels at D4Z4 in myogenic cell cultures, specifically in differentiated myotubes.

#### SMCHD1 levels control DUX4 expression in somatic cells

To establish a causal relationship between SMCHD1 protein levels and DUX4 expression in myotubes and SMCHD1 protein levels, we conducted lentiviral shRNA-mediated depletion experiments in differentiating primary control myoblast cultures (Controls 2 and 8; Table S1) carrying an FSHD-permissive *DUX4*-PAS containing 4A161 allele with D4Z4 repeat sizes varying between 20 and 32 units. Depletion of SMCHD1 resulted in robust activation of DUX4 expression (Fig. 2A, C, S5A), which is in agreement with our earlier observations.<sup>14</sup> This phenomenon of DUX4 activation seems to be rather specific for SMCHD1 since knocking down other known chromatin repressors of D4Z4 in parallel experiments using the same control myoblast cultures did not lead to DUX4 expression. Upon knockdown of SUV39H1, a histone methyltransferase previously shown to be involved in establishing the H3K9me3 modification at D4Z4<sup>18</sup> we did not observe a reduction in relative abundance of H3K9me3 at D4Z4 (Fig. 2B, Fig. S5, S6B), nor the transcriptional activation of *DUX4*, in 2 independent control myotube cultures (Fig. 2C). Similarly, independent depletion of cohesin proteins SMC3 or RAD21 by a similar strategy did not cause a consistent activation of DUX4 either under these conditions (Figs. 2D-F). In support, we did not see significant changes in H3K9me3 levels or RAD21 levels between control and FSHD muscle cell cultures, in myoblasts or myotubes (Fig. S7 and S8).

Since SMCHD1 levels decline at D4Z4 during myoblast differentiation, we next tested whether ectopic expression of SMCHD1 in FSHD myotubes can rescue the repression of DUX4. To this end, we transduced FSHD1 and FSHD2 myoblast cultures with SMCHD1 expressing lentiviruses and allowed the cells to form myotubes. In both FSHD1 and FSHD2 myotubes, 2-3-fold overexpression of SMCHD1, as determined by western blot analyses, resulted in a 70-90% reduction in DUX4 mRNA levels (Fig. 2G-H). To confirm the silencing of *DUX4*, we analyzed the mRNA expression levels of 3 known downstream target genes of

DUX4 protein. ZSCAN4, RFPL2, and TRIM43 expression levels decreased 70-90% upon overexpression of SMCHD1 (Fig. 2I). To rule out that the lower DUX4 transcript levels were a consequence of reduced muscle cell differentiation, we measured MYOG transcript levels. MYOG increased similarly between the GFP expressing control and SMCHD1 expressing FSHD myotubes, with the exception of one FSHD2 myotube culture that showed substantially higher MYOG levels in the SMCHD1 expressing compared to control GFP expressing conditions (Fig. S9). This demonstrates that modest upregulation of SMCHD1 has no inhibiting effect on myogenic differentiation. In conclusion, DUX4 expression levels are strongly decreased upon moderate overexpression of SMCHD1, emphasizing its role as D4Z4 repressor in both genetic forms of the disease.

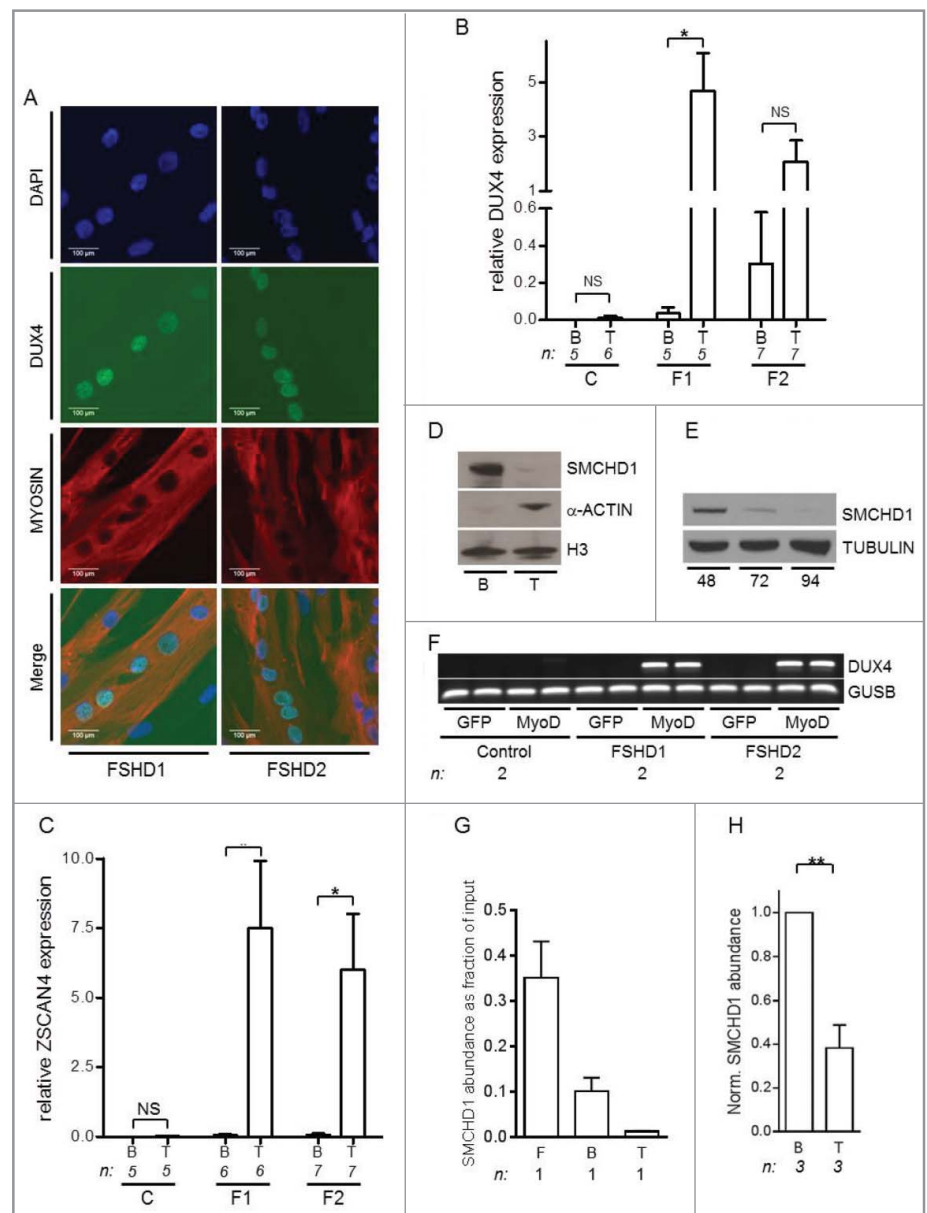


Figure 1. For figure legend, see page 1136.

### SMCHD1 reduction leads to PRC2 enrichment at D4Z4 in FSHD2 but not FSHD1

Since SMCHD1 was recently shown to associate with the repressive H3K27me3 modification at the inactive X chromosome,<sup>31</sup> we further determined the levels of H3K27me3 and the PRC2 complex at D4Z4 upon depletion of SMCHD1 in 2 independent control myotube cultures. SMCHD1 knockdown resulted in a 2-fold decrease in SMCHD1 levels at D4Z4 using 2 different SMCHD1 shRNA vectors (Fig. 3A) and increased H3K27me3 levels at D4Z4 (Fig. 3B). Because of the increase in H3K27me3 at D4Z4 upon SMCHD1 depletion, we further analyzed the involvement of the PRC2 complex and observed a significant increase of the PRC2 protein SUZ12 at D4Z4 (Fig. 3C).

To validate these data derived from knock down experiments in control myotube cultures, we investigated the levels of PRC2 complex in primary control and FSHD myotubes. Previously, H3K27me3 levels at D4Z4 were reported to be similar in control and FSHD1 proliferating myoblast cultures at the DBE locus just proximal to qD4Z4 (Fig. S4).<sup>24</sup> We measured H3K27me3 levels at qD4Z4 and observed significantly higher levels of H3K27me3 in FSHD2 myotubes (Fig. 3D), consistent with the increase we observed following knockdown of SMCHD1 (Fig. 3B). Increased H3K27me3 levels were also observed in FSHD2 myoblasts (Fig. S10). Next, we quantified the levels of the PRC2 protein SUZ12 at D4Z4 and detected higher levels of SUZ12 at D4Z4 in FSHD2 samples (Fig. 3E), all consistent with an increase in H3K27me3 through PRC2 recruitment at D4Z4.

The decreased SMCHD1 activity in FSHD2 myotubes correlates with increased PRC2 levels at D4Z4. To test the repressive role of PRC2 at D4Z4, we inhibited PRC2 activity by treating a control, FSHD1, and FSHD2 muscle cell cultures with increasing concentrations of the EZH2 inhibitor GSK126.<sup>32</sup> After differentiation, total H3K27me3 levels were decreased in all cell cultures as shown by western blot analysis. DUX4 transcript levels increased significantly in FSHD2 samples treated at 2  $\mu$ M GSK126, (Figs. 4A and 4B) suggesting that PRC2 is involved in the repression of D4Z4 in FSHD2, but not in FSHD1.

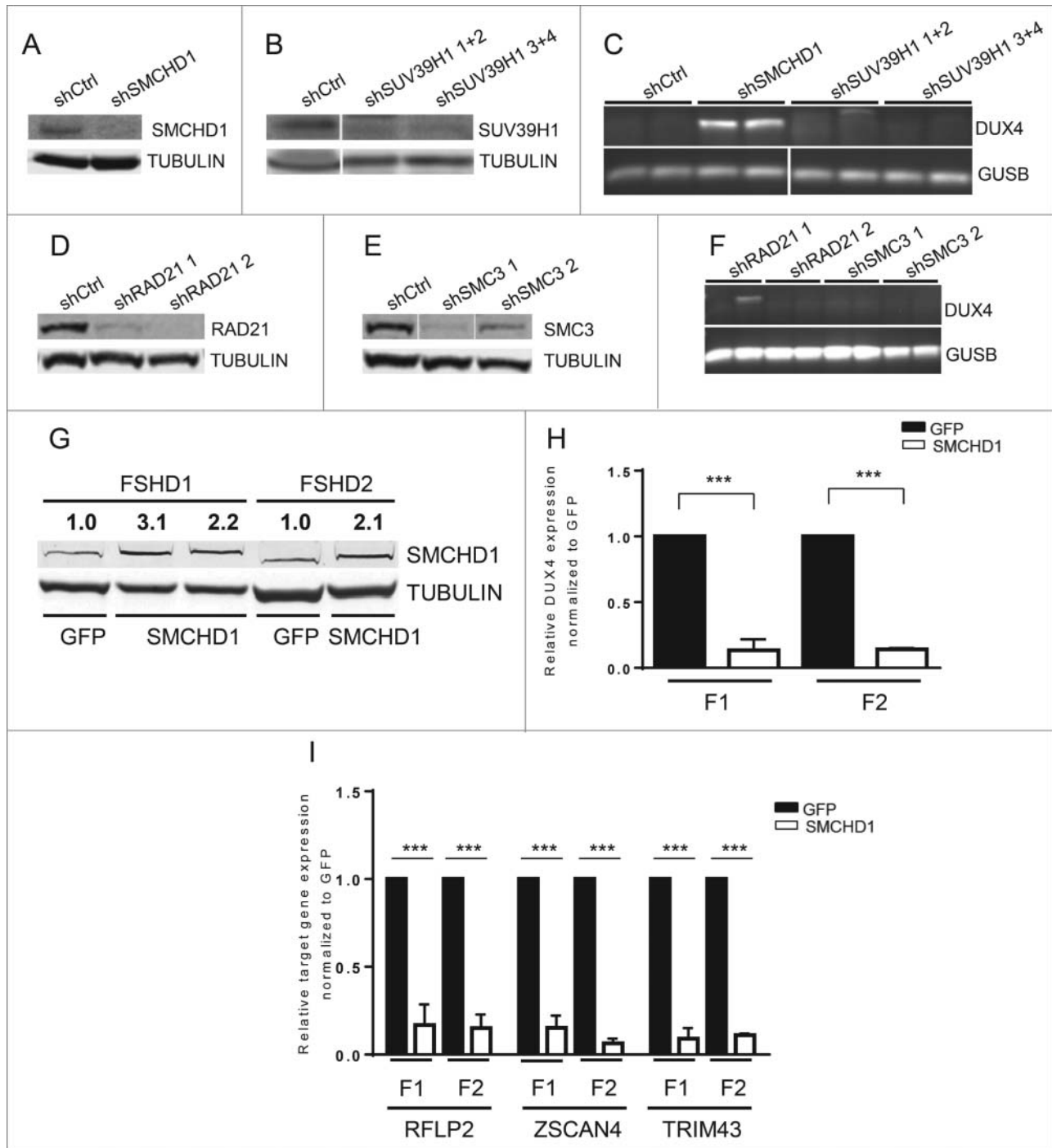
Despite the recent advances in our understanding of common molecular disease mechanism in FSHD1 and FSHD2 and the consensus model of incomplete epigenetic repression of DUX4 in somatic cells, the key epigenetic changes at D4Z4 in FSHD1 and FSHD2, and the selective tissue involvement is only partly understood. FSHD is mainly a disease of skeletal muscle, suggesting that differentiated skeletal muscle might have an epigenetic state more permissive for DUX4 expression. *In vitro*, it was observed that DUX4 expression in FSHD muscle cells specifically increases upon myogenic differentiation. To date, however, most D4Z4 chromatin studies have focused on myoblast and non-myogenic cell cultures. The dynamics of the different epigenetic mechanisms involved in D4Z4 chromatin organization during muscle cell differentiation, and their causality to DUX4 expression, have not been studied in detail. Here we show that SMCHD1 protein levels decrease during muscle cell differentiation and correlate with DUX4 expression, possibly making differentiated muscle cells particularly prone to incomplete D4Z4 repression. Moreover we have identified the involvement of PRC2-mediated H3K27 trimethylation of D4Z4 selectively in FSHD2.

Thus far, most studies have been performed in FSHD1 muscle cell cultures, but we here firmly establish that in FSHD1 and FSHD2 DUX4 expression is strongly upregulated during muscle cell differentiation, emphasizing the importance of studying D4Z4 chromatin structure in differentiated muscle cells. Our observation of the decline in SMCHD1 levels during muscle cell differentiation may provide an explanation toward the susceptibility of muscle in expressing DUX4 and prompted us to further explore the role of SMCHD1 in somatic DUX4 repression. Depletion of SMCHD1 in control myotubes resulted in robust DUX4 expression. Conversely, 1.5-3-fold increases in SMCHD1 protein levels by ectopic expression in FSHD1 and FSHD2 myotubes led to a significant decrease in DUX4 levels and that of its target genes. This demonstrates that the derepression of DUX4 in FSHD muscle cells is a reversible process that can be rescued by increasing SMCHD1 levels.

**Figure 1.**(See previous page) DUX4 activation during myogenic differentiation coincides with SMCHD1 reduction. (A) Immunofluorescence microscopy analysis confirmed the typical DUX4 protein expression pattern in myosin positive, multinucleated myotubes derived from FSHD1 and FSHD2 individuals. Images were taken using a 200x magnification, scale bars are displayed. (B) Quantitative mRNA analysis in control (C), FSHD1 (F1), and FSHD2 (F2) primary myoblasts (B) and myotubes (T) showing increased levels of DUX4 expression upon differentiation. *GAPDH* and *GUSB* were used as reference genes, *n* indicates number of samples, error bars display SD, and significance was calculated using a 2-tailed Student t-test. (C) Quantitative mRNA analysis showed robust *ZSCAN4* activation upon myogenic differentiation in FSHD myotubes. *GAPDH* and *GUSB* were used as reference genes, *n* indicates number of samples, error bars display SD, and significance was calculated using a 2-tailed Student t-test. (D) Western blot analysis showing reduced levels of SMCHD1 upon muscle cell differentiation in a primary muscle cell culture derived from a control individual. Myoblasts (B) were differentiated into myotubes (T) for 48 h. H3 serves as a control for equal protein loading,  $\alpha$ -ACTIN serves as a control for the induction of myogenic differentiation. (E) Western blot analysis of a control derived primary fibroblast undergoing forced myogenesis by ectopic MyoD expression for 48, 72, or 94 h showed a decrease in SMCHD1 protein expression. TUBULIN serves as a loading control. (F) Duplicate RT-PCR analysis of DUX4 expression upon ectopic MyoD expression in a Control 1, FSHD1 1 or FSHD2 1 fibroblast (Table S2) revealed DUX4 activation in patient cells exclusively. Ectopic GFP expression was used as a control and *GUSB* serves as an internal PCR control. (G, H) Normalized ChIP qPCR analysis of SMCHD1 binding at D4Z4 in isogenic fibroblasts (F), myoblasts (B), and myotubes (T) derived from the same control individual (panel G) and 3 independent control derived myoblast – myotube pairs showed the highest SMCHD1 abundance in myoblasts with a further decrease during myogenesis. Error bars display SD in panel G and H and significance was calculated using a 2-tailed Student's test. NS = not significant; \* =  $P < 0.05$ ; \*\* =  $P < 0.005$ .

SMCHD1 was recently shown to associate with H3K27me3 at the inactive X chromosome and we observed that loss of SMCHD1 at D4Z4 leads to an increase in PRC2 and H3K27me3 levels at D4Z4 in FSHD2. This strongly suggests that there is aberrant PRC2 regulation in FSHD2, but not FSHD1, myoblasts and myotubes. Since SMCHD1 has a role in the establishment and/or

maintenance of CpG methylation, this observation consistent with recent studies showing an inverse relationship between CpG methylation and PRC2-mediated H3K27 methylation.<sup>33</sup> While the overall levels of some PRC2 complex proteins decline during murine myogenic differentiation,<sup>34</sup> our data suggest a local enrichment of PRC2 at D4Z4 in FSHD2 cells during this process.



**Figure 2.** For figure legend, see page 1138.

Previous studies did not observe a difference in PRC2 and H3K27me3 at D4Z4 in FSHD. However, these studies were mostly restricted to FSHD1 myoblast cultures,<sup>17,24</sup> used primers that were not specific to chromosome 4,<sup>17</sup> or analyzed a single cell line<sup>35</sup> which may explain this difference. Indeed, a recent study by Zeng et al. showed that although D4Z4 units are dispersed throughout the genome, epigenetically the D4Z4 repeat arrays on chromosomes 4 and 10 are distinctly regulated from their homologs.<sup>35</sup>

Our data also identifies a difference between FSHD1 and FSHD2 in the epigenetic regulation of D4Z4. Depletion of SMCHD1 in control cells, mimicking FSHD2, leads to increased abundance of PRC2. The involvement of PRC2 in FSHD2 is further substantiated by the observation that GSK126 specifically increases DUX4 transcript levels in FSHD2, but not in FSHD1. Despite this epigenetic difference, SMCHD1 depletion and overexpression respectively leads to activation and repression of DUX4 in both FSHD1 and FSHD2 derived cells. It is unlikely that a selective involvement of a single contracted D4Z4 repeat array in FSHD1 creates a technical limitation in our ChIP approach (interrogating all 4 D4Z4 repeat arrays simultaneously) and explains the FSHD2-specificity of the involvement of PRC2 at D4Z4. Therefore, we conclude that although in both conditions DUX4 is derepressed, there are some unique epigenetic responses to repeat contraction or SMCHD1 malfunctioning in FSHD1 and FSHD2, respectively.

Increased PRC2 recruitment and H3K27me3 levels are generally associated with transcriptional repression.<sup>36</sup> However, in FSHD2 they are associated with the transcriptional derepression of the *DUX4* locus. PRC2 recruitment to the derepressed D4Z4 region may reflect a rather effective fail-safe mechanism to compensate for the loss of other repressive mechanisms. Indeed a compensatory repressive mechanism by PRC2 is supported by our observation of increased DUX4 transcript levels in FSHD2 myotubes in the presence of the EZH2 inhibitor GSK126. The specific nature of the trigger leading to the sporadic escape of repression typical for DUX4 remains unclear at this point and requires experimental approaches, which can dissect differences between single nuclei.

We could not confirm a role for SUV39H1 or the cohesin complex in DUX4 repression in a myogenic context: we did not observe different H3K9me3 or SMC3 levels in myotubes and knockdown of SUV39H1 and cohesin in myotubes also did not activate *DUX4*. Knockdown of SUV39H1 did not decrease H3K9me3 at D4Z4 in myotubes either. Previously, siRNA mediated knockdown of SUV39H1 led to reduced levels of H3K9me3 at D4Z4 in HeLa cells.<sup>18</sup> Similarly, reducing SUV39H1 activity in proliferating immortalized myoblasts by knock down or treatment of primary and immortalized myoblasts with Chaetocin decreased H3K9me3 levels and resulted in transcriptional derepression of *DUX4*.<sup>35</sup> The specificity of Chaetocin was recently challenged, however, as it might also inhibit the activity of other histone lysine methyltransferases.<sup>37</sup> Our data thus suggest that the regulation of this modification at D4Z4 is different between primary and immortalized myoblasts, and between proliferating and differentiating myoblasts, perhaps by involvement of additional methyltransferases such as G9a.<sup>35</sup> A recent report suggested a role for the telomere in the epigenetic regulation of D4Z4.<sup>38</sup> Since myoblasts are immortalized by telomerase expression, it will be important to comprehensively compare the epigenetic regulation of the D4Z4 repeat array between primary and immortalized myoblasts.

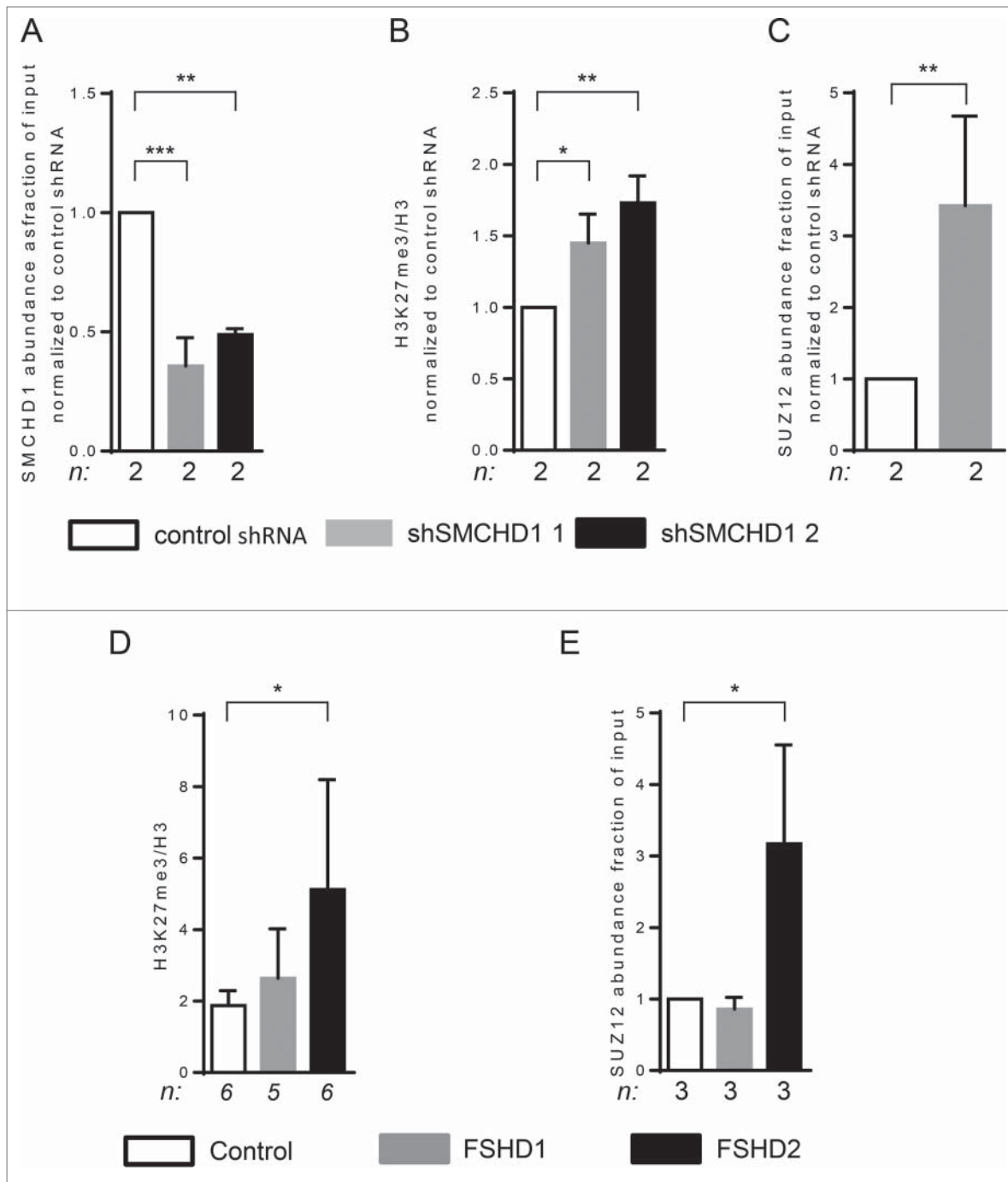
Together with earlier observations that SMCHD1 is a modifier of disease severity in FSHD1, our study suggests that D4Z4 derepression in FSHD1 and FSHD2 converges at SMCHD1. Contraction of D4Z4 in FSHD1 may lead to specific loss of SMCHD1 at the contracted allele, analogous to its interaction with telomeric repeats, which shows a non-linear positive correlation to telomere length.<sup>39</sup> All together, this emphasizes the crucial role of SMCHD1 in suppressing DUX4 activity in somatic cells and its decline during muscle cell differentiation provides a plausible explanation for the increased expression of DUX4 in skeletal muscle cells.

## Materials and Methods

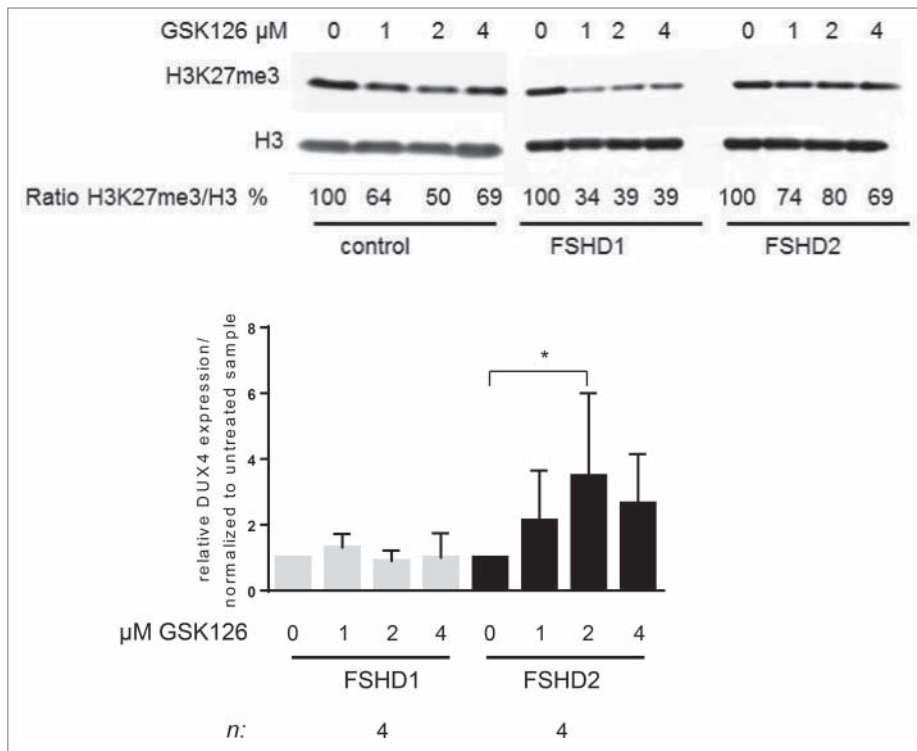
### Culturing of human primary myoblast cell lines

Human primary myoblast cell lines were originating from the University of Rochester bio repository (<http://www.urmc>).

**Figure 2. (See previous page) SMCHD1, but not SUV39H1 and Cohesin, regulates DUX4 expression.** Western blot confirmation of (A) SMCHD1, (B) SUV39H1, (D) RAD21, and (E) SMC3 knockdown in control myotubes expressing the indicated lentiviral transduced shRNAs. Tubulin was used as a loading control. Representative blots of at least duplicate experiments are shown. (C, F) Standard gel electrophoresis analysis of DUX4 expression upon lentiviral knockdown of SMCHD1, SUV39H1, RAD21 and SMC3 in control myotubes. Only depletion of SMCHD1 resulted in reproducible activation of DUX4 transcription. Representative gel photos are shown of at least duplicate experiments. The PCR fragment visible in one RAD21 knockdown was sequenced and shown to be the product of an a-specific amplification. (G) Western blot analysis confirms a 2-3 fold increase of SMCHD1 expression upon its lentiviral transduction in 2 FSHD1 and 1 FSHD2 myotube cultures. GFP transduced myotubes served as a negative control. Numbers indicate normalized relative expression levels of SMCHD1 using Tubulin as a loading control followed by setting normalized SMCHD1 levels of GFP transduced samples (only expressing endogenous SMCHD1) to 1. (H) Expression levels of *DUX4* were significantly reduced upon ectopic expression of SMCHD1 in 2 FSHD1 and 2 FSHD2 myotube cultures. Relative DUX4 expression was calculated for each sample by normalization to *GUSB* and *GAPDH* housekeeping genes. Bars show values of each samples adjusted to the expression value of GFP transduced sample as 1. (I) Expression levels of the DUX4 target genes *RFPL2*, *ZSCAN4*, and *TRIM43* showed a significant reduction upon ectopic expression of SMCHD1 in 2 FSHD1 and 2 FSHD2 myotubes, concordant with decreased DUX4 protein expression. Expression levels were normalized as described for panel **Figure 1A**. For panel H, I: Error bars display SD and significance was calculated using a 2-tailed Student's t-test. All  $P < 0.0005$  indicated by \*\*\*.



**Figure 3. SMCHD1 depletion at D4Z4 leads to increased H3K27me3 and PRC2 binding.** (A) ChIP-qPCR analysis showed a statistically significant reduction of SMCHD1 at qD4Z4 upon its lentiviral knockdown in control myotubes with 2 independent shRNA constructs in 2 independent control myotube cultures. (B) ChIP-qPCR analysis of H3K27me3 at qD4Z4 upon SMCHD1 depletion showed a significant increase at qD4Z4. Enrichment values were normalized to H3 enrichment values. (C) Normalized ChIP-qPCR analysis of SUZ12 upon SMCHD1 depletion showed a statistically significant increase at qD4Z4 in 2 independent experiments performed on 2 control myoblast cultures. *n* indicates sample size, error bars indicate SD, and significance was tested with Student t-test. (D) ChIP-qPCR analysis of H3K27me3 at qD4Z4 showed a significant increase in FSHD2 myotubes compared to controls. Enrichment values were normalized to H3 enrichment values. (E) ChIP-qPCR analysis of SUZ12 abundance showed a significant increase in FSHD2 myotubes at qD4Z4. On panel A, B, D, and E, *n* indicates sample size, error bars display SD, and significance was tested using a one way-ANOVA followed by Bonferroni multiple comparison test. \* =  $P < 0.05$  \*\* =  $P < 0.005$  \*\*\* =  $P < 0.0005$ .



**Figure 4. Treatment of FSHD2 myotube cultures with EZH2 inhibitor GSK126 increases DUX4 levels.** (A) Western blot analysis of control, FSHD1, and FSHD2 myotube samples after treatment with 0, 1, 2, and 4  $\mu$ M GSK126. Blots were probed with H3K27me3 antibody and H3 antibody as loading control. Shown are the reduced ratios of H3K27me3:H3 signal intensities in samples treated with GSK126 compared to the untreated sample as a result of EZH2 inhibition. (B) qRT-PCR analysis of DUX4 expression in FSHD1 and FSHD2 myotubes after GSK126 treatment shows that relative DUX4 transcript levels are significantly increased in FSHD2 samples treated with 2  $\mu$ M GSK126 but not in FSHD1 myotubes. Graph shows the results of 4 FSHD1 and 4 FSHD2 cell lines. Results of control cell lines are not shown, DUX4 transcript was not detectable. Error bars show SD, *n* indicates number of independent cell lines, and significance was tested by 2-way ANOVA followed by Bonferroni multiple comparison test. \* = *P* < 0.05.

rochester.edu/fields-center/). Muscle samples were obtained after subjects were consented under a protocol approved by the institutional review board at the University of Rochester. Myoblasts were cultured in DMEM/F-10 media (#31550, Gibco/Life Technologies, Bleiswijk, The Netherlands) supplemented with 20% heat inactivated fetal bovine serum (FBS #10270, Gibco/Life Technologies), 1% penicillin/streptomycin (#15140, Gibco/Life Technologies) and 10ng/ml rhFGF (#G5071, Promega, Leiden, The Netherlands) and 1 $\mu$ M dexamethasone (#D2915, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to the medium. Myoblasts were fused at 80% confluency by culturing them in DMEM/F-12 Glutamax media (#31331, Gibco/Life Technologies) containing 1% penicillin and streptomycin and 2% KnockOut serum replacement formulation (#10828, Gibco/Life Technologies) for 36 h. Human control fibroblast cell lines were maintained in DMEM/F-12, supplemented with 20% FBS, 1% penicillin and streptomycin, 10 mM HEPES (#15630#, Gibco/Life Technologies) and 1mM sodium pyruvate (#11360, Gibco/Life Technologies). Used cell lines, D4Z4 allele information, and experimental use are listed in Table S1.

#### RNA isolation, cDNA synthesis, and qRT-PCR

Myoblast and myotube samples were harvested for RNA isolation by adding QIAzol lysis reagent (#79306, Qiagen N.V., Venlo, The Netherlands). RNA was isolated by miRNeasy Mini Kit (#217004, Qiagen) including DNase treatment according to the manufacturer's instructions. cDNA was synthesized with RevertAid H Minus First strand cDNA Synthesis Kit (#K1632 Thermo Fischer Scientific Inc., Waltham, MA) using 2 $\mu$ g template RNA and poly-dT primers. Gene specific cDNA products were quantified by qPCR in duplicate using SYBR green master mix supplemented with gene specific primers (Table S2) using the CFX96 system (Bio-Rad, Veenendaal, The Netherlands). qRT-PCR data were analyzed by Bio-Rad CFX manager version 3.0 (Bio-Rad) using *GAPDH* and *GUSB* as reference genes for every individual sample.

#### Transduction of primary cell cultures

Full length *SMCHD1* was PCR amplified from cDNA and cloned into the pRRL-CMV lentiviral backbone containing a puromycin selection marker using standard cloning procedures and the obtained construct was verified by Sanger sequencing. shRNA constructs originating from the Mission shRNA library (MISSION shRNA library, TRC1 or TRC2; Sigma Aldrich) and are listed in Table S2. Constructs were used to generate lentiviral particles, and myoblast cell lines were transduced at 50% confluency and 24 h after transduction they were grown in media containing 0.5  $\mu$ g/ml puromycin for selection. Differentiation to myotubes was induced by serum reduction at 80% confluency and cells were harvested after 36 h of differentiation. Transduction of fibroblasts to ectopically express MyoD1 and induce myogenesis was performed as described previously.<sup>40</sup>

#### Chromatin immunoprecipitation

Histone ChIP studies were carried out as described before<sup>19</sup> using antibodies against H3 (ab1791, 2  $\mu$ l/rxn, Abcam, Cambridge, UK), H3K9me3 (39161, 5  $\mu$ l/rxn, Active Motif, Carlsbad, USA), H3K27me3 (#17-622, 5  $\mu$ l/rxn, Merck-Millipore, Amsterdam Zuid-Oost, The Netherlands), and total IgG (5  $\mu$ l/rxn, Merck-Millipore). Non-histone ChIPs were carried out as described before<sup>14</sup> using antibodies against SMCHD1 (ab31865, 5  $\mu$ g/rxn, Abcam, Cambridge, UK) RAD21 (ab992, 5  $\mu$ g/rxn, Abcam), SMC3 (ab9263, 5  $\mu$ g/rxn, Abcam), SUZ12



(D39F6, 5 µg/rxn, Cell Signaling, Leiden, The Netherlands). All antibodies were qualified for ChIP application by the manufacturer. All ChIP-qPCR experiments were performed 2 independent times.

### GSK126 treatment of primary myoblast cultures

Three control, 4 FSHD1, and 4 FSHD2 human primary myoblast cultures were grown to 50–60% confluency and culturing was continued in the presence of 0 µM, 1 µM, 4 µM and 8 µM GSK126 (#1346574-57-9, MedKoo Biosciences, North Carolina, USA) in proliferation media. GSK126 was dissolved in DMSO (#D2650, Sigma-Aldrich, St Louis, USA) and the different concentration of GSK126 was added in equal volumes of DMSO to the cells. Cultures shown as 0 µM GSK126 were treated only with DMSO. After 24 h culturing was continued in fusion media in the presence of the same concentration of GSK126 for another 24 h. RNA and protein samples were harvested as described above.

### Western blot

Cell were directly lysed in NuPAGE LDS Sample Buffer (#NP0008, ThermoFisher Scientific) and loaded on Novex 4–12% Bis-Tris Protein Gels (#WT4121A, ThermoFisher Scientific) according to manufacturer's instructions. Running of the gel and transfer to Hybond nitrocellulose membranes (#10600048, GE Healthcare, Diegem, Belgium) were carried out using the Nupage Novex SDS-page gel system (ThermoFisher Scientific). Blots were blocked in 4% milk in PBS and incubated with antibodies against SMCHD1 (1:250), SUV39H1 (0.1 µg/ml, #07-958, Merck-Millipore), RAD21 (1:500), SMC3 (1:1000) and Tubulin (1:2000, T6199, Sigma-Aldrich). Detection and relative quantification were done using the Odyssey system (V3.0, LI-COR Biosciences, Lincoln, NE, USA), except for blots in **Figure 1**, which were visualized by enhanced chemiluminescence (ECL).

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### Statistical analysis

All statistical analysis was done using GraphPad Prism 6 software and the exact method applied at different experiments is described in the figure legends.

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### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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