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INVITED REVIEW

Consequences of epigenetic derepression in facioscapulohumeral muscular dystrophy

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Abstract

Facioscapulohumeral muscular dystrophy (FSHD), a common hereditary myopathy, is caused either by the contraction of the D4Z4 macrosatellite repeat at the distal end of chromosome 4q to a size of 1 to 10 repeat units (FSHD1) or by mutations in D4Z4 chromatin modifiers such as *Structural Maintenance of Chromosomes Hinge Domain Containing 1* (FSHD2). These two genotypes share a phenotype characterized by progressive and often asymmetric muscle weakening and atrophy, and common epigenetic alterations of the D4Z4 repeat. All together, these epigenetic changes converge the two genetic forms into one disease and explain the derepression of the *DUX4* gene, which is otherwise kept epigenetically silent in skeletal muscle. *DUX4* is consistently transcriptionally upregulated in FSHD1 and FSHD2 skeletal muscle cells where it is believed to exercise a toxic effect. Here we provide a review of the recent literature describing the progress in understanding the complex genetic and epigenetic architecture of FSHD, with a focus on one of the consequences that these epigenetic changes inflict, the *DUX4*-induced immune deregulation cascade. Moreover, we review the latest therapeutic strategies, with particular attention to the potential of epigenetic correction of the FSHD locus.

KEYWORDS

DUX4, Epigenetics, facioscapulohumeral, genetic diseases, genetics, immune deregulation, inborn, muscular dystrophy, SMCHD1

1 | INTRODUCTION

Facioscapulohumeral dystrophy (FSHD) is a prevalent inherited myopathy¹ characterized by slowly progressive, often asymmetric, dysfunction of facial, upper and lower extremity muscles.² Extramuscular manifestations occur mostly in early onset FSHD³ and include high-frequency hearing loss and retinal vascular tortuosity which can progress into a

treatable symptomatic condition known as Coats syndrome.⁴ Disease onset is typically in the second decade of life, but can occur at any age from infancy to adulthood. The clinical phenotype varies among mutation carriers, ranging from asymptomatic to wheelchair dependent.⁵

Considerable progress has been made in our understanding of the complex (epi)genetic architecture of the FSHD locus on chromosome 4.^{6,7} Consensus has been reached with respect to a plausible disease mechanism involving the loss of epigenetic control over the subtelomeric D4Z4 macrosatellite repeat, situated at chromosome 4q35

Anna Greco and Remko Goossens contributed equally to this study.

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in arrays of up to 100 units.^{8,9} Chromatin relaxation of the D4Z4 repeat occurs as a consequence of repeat contraction to 1 to 10 repeats (FSHD1) or because of mutations in epigenetic modifiers of the locus (FSHD2).¹⁰⁻¹² This results in the aberrant expression of the retrogene encoding the transcription factor Double Homeobox 4 (DUX4) in skeletal muscle.¹³

DUX4 is expressed in testes and cleavage stage embryos, and epigenetically repressed in most somatic tissues,¹⁴ possibly through a repeat-mediated epigenetic silencing pathway.⁷ Incomplete D4Z4 chromatin repression in FSHD muscle results in high levels of DUX4 expression in a small number (between 1:200 and 1:1000) of myonuclei.^{15,16} Ectopic DUX4 expression in muscle cells activates various molecular pathways, which potentially result in cell death by apoptosis.¹⁷ However, it remains enigmatic what initiates these bursts of DUX4 expression and how they might drive the pathophysiology.¹⁸

Many studies have investigated the events that occur downstream of DUX4 activation. Induced DUX4 expression in cultured myoblasts initiates an abnormal transcriptional cascade, including dysregulation of MyoD/MYOD1 and downstream targets, resulting into defects in myogenic differentiation.^{19,20} DUX4 also represses glutathione redox pathways resulting in increased oxidative stress,²¹ induces muscle atrophy,²² and activates germline and immune transcriptional programs.²³ This raises the question whether the DUX4-induced expression of these genes in FSHD muscle induces an immune response and whether this is the basis of the inflammatory infiltrates associated with FSHD pathology.²⁴⁻²⁶

First, we describe the genetic and epigenetic changes leading to DUX4 expression in FSHD muscle. Then, downstream effects of DUX4 expression are discussed. Finally, we review the different therapeutic strategies that have been explored thus far.

2 | FSHD PHENOTYPE AND GENOTYPE

2.1 | Clinical presentation of FSHD

The classical FSHD phenotype is hallmarked by progressive, often asymmetric weakness and wasting of muscles of the face, shoulder and upper arms. With disease progression and increasing severity, abdominal, axial, foot-extensor and pelvic-girdle muscles can become affected. Generally, the disease manifests in the second decade of life, but onset can be highly variable.²⁷ Facial weakness can be demonstrated in patients by attempts to puff out the cheeks or to whistle, as FSHD often involves wasting of the periorbital and perioral muscles. Scapular winging and inability to raise the arms above shoulder height are also signs of FSHD.²⁸ Disease penetrance is incomplete, with roughly one-third of FSHD mutation carriers remaining asymptomatic throughout their life; although careful clinical examination can often identify FSHD-related symptoms.²⁹ Conversely, ~20% of patients exhibit a severe phenotype and will eventually become wheelchair dependent.²⁸ The prevalence of FSHD was originally estimated to be 1:21.000, but due to advances in diagnostics and awareness, the most recent estimates lie between 1:15.000 and 1:8.500 in Europe.^{1,30,31}

FSHD is considered a slowly progressive muscle disorder, with the rate of muscle weakening thought to occur in bursts after longer periods of no apparent functional decline.³¹ Prognosis is variable, but roughly correlates with age at onset and D4Z4 repeat size (see genetics of FSHD). As involvement of cardiac and respiratory muscles is rare, general life expectancy is not reduced for FSHD patients.³¹ Clinical anticipation has been suggested, but not undisputedly proven.^{32,33} Inheritance from parents who are mosaic for the FSHD mutation has been postulated to explain, at least in part, the suggestion of anticipation.^{31,34}

FSHD affects males more severely and frequently than females.³⁵ Males generally tend to have a higher mean Ricci score, a 10-grade scale used to assess clinical severity,³⁶ and to develop motor impairment approximately 7 years before females do.³⁶⁻³⁸ Female mosaic carriers of an FSHD mutation are more often the unaffected parent of an affected child who inherited the mutation, while mosaic males are more often affected.³⁹ The biological cause underlying the gender difference is not clear, but recent studies suggest that estrogen can influence the intracellular activity and localization of DUX4 in cultured FSHD myoblasts. This study also indicated that female patients that had rapidly diminishing estrogen levels because of early menopause or due to anti-estrogenic treatment experienced an increase in the severity of clinical symptoms.⁴⁰ However, a clinical study on estrogen exposure during the lifetime of female patients did not find conclusive evidence for either benefit or harm of estrogen levels on disease progression.⁴¹ The estrogen levels that could exhibit protective properties for muscle tissue *in vitro* are possibly not of physiological proportions to be of benefit to patients. It is also important to note that estrogen differences between male and female patients would be much greater than between females.⁴¹ Moreover, while 12% to 24% of female FSHD patients experienced worsening of their symptoms following pregnancy, this percentage is relatively low when compared to other neuromuscular disorders.^{42,43}

High-frequency hearing loss is reported in 15% to 32% of FSHD patients and partly depends on the D4Z4 repeat size (see Section 2.2). Retinal vasculopathy is observed in 25% of examined individuals with clinical or genetic evidence for FSHD.^{5,44} High-frequency hearing loss severity is variable, but it usually starts with failure to perceive high tones and can progress to involve all frequencies.⁴⁵ While occasionally observed and postulated to be part of FSHD pathogenesis, cardiac involvement, ptosis, extraocular muscle weakness and extensive contractures are not considered to be FSHD specific.^{30,45}

2.2 | The genetics of FSHD

Linkage studies mapped the FSHD locus to chromosome 4q, which subsequently led to the discovery that FSHD is associated with partial deletions of the D4Z4 repeat.²⁷ The D4Z4 repeat consists of units of 3.3 kb each, ordered head-to-tail, with the number of units varying from 8 to 100 in the European population (Figure 1A). In FSHD1 patients, the repeat is reduced to a size of 1 to 10 units on one of the chromosomes 4. At least one unit is required to develop the disease,

emphasizing the critical role for D4Z4 in FSHD.^{37,46-51} Each D4Z4 unit contains a copy of the *DUX4* retrogene that contains the full open reading frame.⁵² However, only *DUX4* from the most distal repeat unit can be stably expressed in FSHD muscle due to genetic elements downstream to the repeat that are important for mRNA processing.^{14,53}

FSHD1 is inherited in an autosomal dominant fashion with incomplete penetrance,²⁷ with 10% to 30% of cases being the result of de novo mutations.^{30,34,54} De novo mutations are often mitotic in origin, leading to somatic mosaicism. Depending on D4Z4 repeat size and proportion of affected cells, mosaicism can be found in either the clinically unaffected parent or in the proband.^{34,39} These rearrangements seem to occur during early zygotic cell divisions through gene conversions with or without crossover.⁵⁵

Two major allelic forms of chromosome 4q exist, 4qA and 4qB, and while being equally common in the European population, only the 4qA allele is associated with FSHD.⁵⁶ The 4qA sequence contains a 9 kb beta-satellite repeat region immediately distal to the D4Z4 repeat, which is absent from 4qB (Figure 1A).⁵⁷ This distal portion of the FSHD-permissive 4qA allele, called pLAM, contains a unique 3' untranslated region with non-canonical polyadenylation signal (PAS) for *DUX4*.⁷ While this PAS is essential for stable expression of *DUX4* in muscle, it is possible that other elements in the 4qA sequence also contribute to *DUX4* mRNA expression, processing and stabilization.^{58,59} D4Z4 repeat contractions <10 units on a non-permissive 4qB allele do not cause FSHD, as this allele lacks the pLAM region in its entirety.^{7,56} The 4q haplotypes are further classified based on the size of a simple sequence length polymorphism (SSLP) located 3.5 kb proximal to the D4Z4 repeat.⁶⁰ 4q Haplotypes are therefore defined by the chromosomal origin, the size of the SSLP, and the distal polymorphism, for example, the most prevalent FSHD-permissive haplotype 4A161 contains a SSLP of 161 nucleotides on a 4qA chromosome.⁶⁰ The 4A161 haplotype can be further divided into two major subtypes: 4A161S and 4A161L.⁶¹ These two subtypes differ in the size of the distal D4Z4 unit, which is truncated (Figure 1B). Despite this size difference both 4A161 variants produce the same *DUX4* ORF.^{7,61} Although at least 17 unique 4q haplotypes have been identified, only 4A161S, 4A161L, 4A159 and 4A168 have been reported to be associated with FSHD.⁵¹ It is currently unknown why contractions in 4A166 do not cause FSHD, as this haplotype also contains a *DUX4* PAS. The different haplotypes are not equally distributed over the different world populations, which might account for the perceived differences in FSHD prevalence around the world.⁵¹

A highly homologous D4Z4 repeat exists on chromosome 10q26, but this repeat is generally not associated with FSHD as this chromosome has a damaging SNP in the *DUX4* PAS.^{56,62,63} However, individuals with translocations between chromosomes 4 and 10 have been reported. In these individuals, the distal end of the repeat on chromosome 10, including pLAM sequence, is 4qA-derived. When contracted, these hybrid repeats likely give rise to *DUX4* expression in muscle from the 4q related unit on chromosome 10, resulting in disease presentation.^{51,64} These hybrid repeats were initially observed by Southern blot analysis, but recent advances in diagnostic techniques allow to visualize complex D4Z4 rearrangements by use of molecular combing.^{64,65}

FSHD1 patients account for >95% of patients diagnosed with FSHD. The remaining patients are classified as FSHD2, and are often carrying a mutation in the *Structural Maintenance of Chromosomes Hinge Domain Containing 1* (*SMCHD1*) gene (>80% of FSHD2), or rarely in the *De Novo Methyltransferase 3B* (*DNMT3B*) gene (both described in more detail below).^{66,67} Inheritance of FSHD2 occurs in a digenic manner, requiring the transmission of both a mutant *SMCHD1* or *DNMT3B* allele, together with a permissive 4qA allele.⁶⁶ Recently, an FSHD patient without mutations in *SMCHD1* or *DNMT3B* was described to be a carrier of a homozygous mutation in *Ligand-Dependent Nuclear Receptor Interacting Factor 1* (*LRIF1* [also known as *HBIX1*]).⁶⁸ This mutation causes the absence of one *LRIF1* isoform resulting in D4Z4 chromatin relaxation. *LRIF1* and *SMCHD1* protein are known to interact with each other,⁶⁹ and reduced *LRIF1* and *SMCHD1* binding to the D4Z4 repeat was observed in this patient. A small subset of patients do not carry mutations in either *SMCHD1*, *DNMT3B* or *LRIF1*, suggesting that other disease genes are yet to be identified.⁶⁷

For more information on FSHD diagnostic techniques, we would like to refer to the 2019 review by Zampatti et al.⁷⁰

3 | D4Z4 CHROMATIN STRUCTURE AND THE ROLE OF SMCHD1

In somatic cells, the D4Z4 repeat is decorated with a plethora of chromatin marks indicative for a repressive chromatin state, such as the histone modification H3K9me3, and CpG methylation (hypermethylation).⁷¹⁻⁷³

Several studies have demonstrated that D4Z4 methylation levels correlate with the size of the D4Z4 repeat and that the D4Z4 methylation level is reduced in somatic cells of FSHD individuals (hypomethylation).^{12,71,74,75} To account for the repeat size dependency of D4Z4 methylation levels, the delta1 correction model was introduced. This model calculates the corrected D4Z4 methylation value defined by the observed methylation minus the predicted methylation based on repeat size information. The mean of this value is zero, and varies between 10% and -10% in controls and FSHD1 patients, while it is below -21% in FSHD2 patients. The delta1 value facilitates (epi)genotype-phenotype studies of clinical variability resulting from inter-individual differences in D4Z4 methylation, which were originally deemed not to be correlated.^{71,76} In FSHD2, these differences are mainly explained by the type of the *SMCHD1* mutation.⁷¹

While in FSHD1, the contracted D4Z4 allele is hypomethylated,⁷³ in FSHD2, the D4Z4 repeats on chromosomes 4 and 10 are hypomethylated.¹² The loss of methylation in FSHD is restricted to the D4Z4 repeat, as no hypomethylation is observed in the region proximal to the repeat.^{12,77} Methylation facilitates repression of *DUX4*, as treatment of cells with 5'aza-2'deoxyctidine (AZA), a demethylating agent, causes an increase of *DUX4* expression.^{78,79} Use of monochromosomal cell hybrids indicated that D4Z4 is hypoacetylated, and that histone deacetylases (HDACs) are actively recruited to the D4Z4 repeat, similar to the DNMT

proteins.⁷⁸ Furthermore, treatment of cells with trichostatin A (TSA; an inhibitor of HDACs) leads to increased *DUX4* expression, an effect which is amplified by combined treatment of cells with AZA and TSA.^{78,79}

D4Z4 chromatin relaxation is associated with *DUX4* expression in FSHD skeletal muscle. Chromatin relaxation is also observed in other patient derived tissues, indicating that it is not specific for muscle.^{73,80} The apparent tissue-wide D4Z4 chromatin relaxation raised the question which mechanisms confine *DUX4* expression to skeletal muscle. The tissue-restricted expression might be, at least in part, attributed to two myogenic enhancers (called *DUX4* myogenic enhancer 1 and 2). Chromatin confirmation capture (3C) studies showed that these two enhancers located proximally to D4Z4 can associate with the *DUX4* promoter in vivo in myocytes but not fibroblasts, and drive the expression of *DUX4*.⁵⁹ An alternative explanation is that upon myogenic differentiation, SMCHD1 protein levels decline as does SMCHD1 binding to D4Z4, which coincides with increased *DUX4* expression.⁸¹

Apart from *DUX4* mRNA, several other D4Z4 transcripts can be detected, some specific for FSHD while others occur in control and FSHD muscle cells.⁸² Of these, the long noncoding RNA (lncRNA) *DBE-T* was reported to be specifically associated with the D4Z4 chromatin in FSHD. *DBE-T* recruits the H3K36me2 methyltransferase Ash1L, contributing to derepression of genes in the 4q35 region.^{79,83} This same study identified an enrichment of Polycomb components along the FSHD locus, which are necessary for repression of the locus.⁷⁹ The presence of the Polycomb repressive complex 2 (PRC2) and its accompanying histone mark H3K27me3 on the D4Z4 repeat was observed in multiple studies,⁸⁴⁻⁸⁶ and seems to be important for the stability of D4Z4-bound heterochromatin protein 1 alpha HP1 α .⁸⁵ Specifically in FSHD2 myotubes, the loss of SMCHD1 protein at D4Z4 is partially compensated by H3K27me3 deposition in a PRC2-dependent manner.⁸¹ This effect was also observed in control myotubes upon SMCHD1 knockdown, while SMCHD1 overexpression in FSHD1 and FSHD2 myotubes suppresses *DUX4*.⁸¹ Simultaneous presence of the repressive histone marks H3K9me3 and H3K27me3 at D4Z4 was demonstrated by ChIP, while marks for transcriptional permissive chromatin H3K4me2 and H3 acetylation (H3Ac) were also identified.⁷² A specific loss of H3K9me3 was observed in FSHD1 cells, while H3K27me3 and H3K4me2 levels remained relatively unaltered.⁷²

For creating a heterochromatic environment, D4Z4 hosts a range of repressor complexes such as YY1, Nucleosome Remodeling Deacetylase and Chromatin Assembly Factor 1^{87,88} (Figure 2A). SUV39H1-dependent H3K9me3 on D4Z4, which is partially lost in FSHD, was found to recruit HP1 γ and cohesin.⁷² The presence of acetylated histone H4 (H4ac) indicates that D4Z4 is configured similar to unexpressed euchromatin, rather than constitutive heterochromatin, which is hypoacetylated. Euchromatic and heterochromatic D4Z4 units might exist simultaneously within a repeat.^{78,89} The H3K4me2: H3K9me3 ratio represents the chromatin compaction score, which is significantly reduced in FSHD patients.⁸ Separating *DUX4* expressing muscle cells from non-expressing cells by use of a *DUX4*-sensitive reporter showed that the D4Z4 repeat of non-expressing cells is enriched for PRC2 and H3K27me3, while these cells were depleted

for H3K9Ac. No CpG methylation differences could be detected between the two cell pools, indicating the loss of CpG methylation alone is not sufficient to trigger *DUX4* expression.⁸⁶ As indicated, thus far three FSHD2 genes have been identified: *SMCHD1*, *DNMT3B* and *LRIF1*. The *SMCHD1* locus on chromosome 18p contains 48 exons. It encodes a 2005 amino acid (2007 aa in mice) protein consisting of an N-terminal GHKL (DNA Gyrase, HSP90, Histidine Kinase, MutL) type ATPase domain and a C-terminal SMC hinge domain which orchestrates SMCHD1 homodimerization and chromatin binding. The protein is conserved among vertebrates.⁹⁰⁻⁹³ Due to its SMC hinge domain, SMCHD1 is often classified as an atypical member of the SMC protein family, which contains members forming the cohesin and condensin protein complexes. However, while condensin/cohesin ATPases are of the Walker A/B type, the GHKL-type ATPase domain is more similar to what is present in the microorchidia (MORC) family of nuclear proteins. Hence, SMCHD1 can be considered to be a distant MORC-family member.⁹⁴⁻⁹⁶ X-ray crystallography studies of the N-terminus of SMCHD1 identified a unique ubiquitin-like fold N-terminal of the ATPase domain, which potentially aides in homodimerization of the ATPase domain in an ATP-dependent conformational change.⁹⁷ Furthermore, SMCHD1 contains coiled-coil domains both N- and C-terminally of the hinge domain, which possibly mediate protein-protein interactions or assist in SMCHD1 homodimerization, a C-terminal nuclear localization signal and a putative Bromo Associated Motif/Homology (BAM) domain of unknown function are located C-terminal of the ATPase domain.^{83,92,98}

When SMCHD1 mutations were first described in FSHD2, it became clear that SMCHD1 activity is required for *DUX4* repression in somatic tissues.⁶⁶ Similar to the inactive X chromosome in *Smchd1* mutant mice, the D4Z4 repeats are hypomethylated upon loss of SMCHD1 function.^{66,90}

Heterozygous missense mutations in the *DNMT3B* gene on chromosome 20q were identified in a few FSHD2 patients that do not have a mutation in *SMCHD1*. *DNMT3B* mutation carriers have hypomethylated D4Z4 repeats, but only develop FSHD when the *DNMT3B* mutation co-segregates with a relatively short (9 and 13 units) D4Z4 repeat on a 4qA chromosome.⁶⁷ *DNMT3B* mutations have previously been shown to cause immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome.⁹⁹ ICF is a rare autosomal recessive disorder, marked by hypomethylation of CpGs in pericentromeric satellite regions as well as hypomethylation of the D4Z4 repeats and other large repeat structures.^{77,100-102} Although ICF patients show hypomethylated D4Z4 repeats, no ICF patients presenting with muscular dystrophy have so far been reported.⁷⁷ Likewise, no immune phenotype has been reported in FSHD patients with *DNMT3B* mutations, consistent with the absence of an immune phenotype in heterozygous *DNMT3B* mutation carriers in ICF families.

3.1 | Genotype-phenotype relationships

For FSHD1 individuals with a repeat length of 1 to 6 units, the clinical severity depends to some degree on the size of the D4Z4 repeat. In

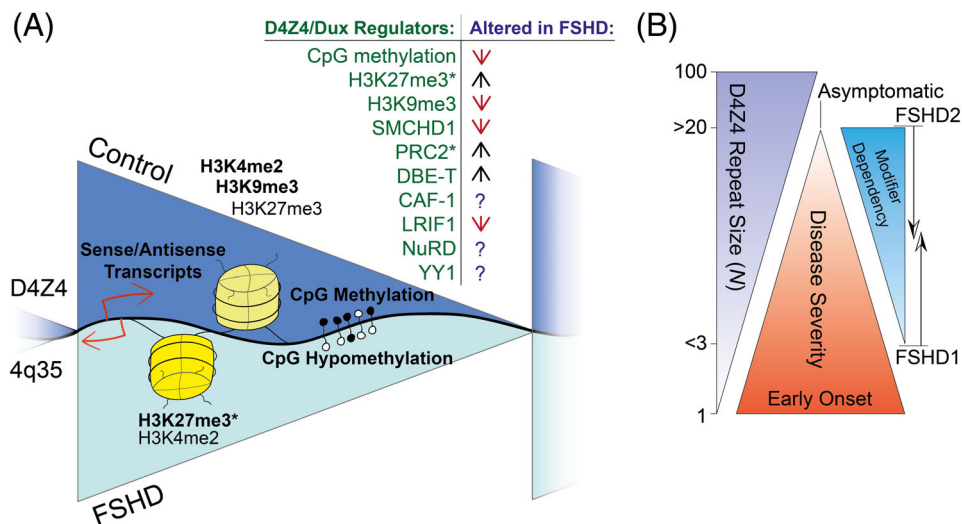


FIGURE 2 D4Z4 chromatin components and the facioscapulohumeral muscular dystrophy (FSHD) disease continuum. A, D4Z4 is host to a plethora of repressor complexes which keep the repeat silenced in healthy controls by direct binding or deposition of repressive chromatin marks (top of (A)). In FSHD, these protein complexes and the chromatin state are altered (bottom of (A)). See main body of text for further information of illustrated complexes. The listed alterations in FSHD do not have to occur simultaneously in a single patient, although some combinations can increase disease severity. (B) Simplified representation of the FSHD disease spectrum. As the D4Z4 repeat size changes from 100 (asymptomatic) to 1 unit, chromatin modifiers (eg, SMCHD1) have less additional effect on the repression of *DUX4* expression, therefore mutations are seldom seen together with shorter repeats. Rare cases of patients with relatively long repeats, or carriers with short alleles are not accounted for

patients with 7 to 10 units, chromatin modifiers acting on D4Z4 play an increasingly prominent role in the susceptibility to D4Z4 chromatin relaxation, *DUX4* expression and disease presentation. The nature of these factors is currently only partly understood.⁷¹ Families harboring a 7 to 10 unit D4Z4 allele show more clinical variability among family members with apparent identical FSHD genotypes.¹⁰³ This includes non-penetrant disease allele carrying siblings of symptomatic FSHD patients.^{37,38,54} The cause of this variability seems to be heritable to some extent, as first degree relatives are almost twice as likely to exhibit motor-impairment when compared to second through fifth degree relatives, which are more frequently asymptomatic.³⁷ Interestingly, carriers of a repeat of 7-10 D4Z4 units on an FSHD-permissive allele have a reduced delta1 value indicative of a lower CpG methylation level than would be expected based on their D4Z4 repeat size. This negative delta1 value can be fully attributed to disease presenting carriers of a 7 to 10 unit repeat, as non-penetrant carriers with a similar repeat size have normal delta1 values.^{71,104,105} In this size range, the disease course is typically milder and non-penetrance is more frequent.¹⁰⁴ Furthermore, comparison of methylation levels of 4qA D4Z4 in myocytes and blood derived from FSHD1 patients and their non-manifesting relatives shows lower methylation levels in affected patients specifically.¹⁰⁶ Although this suggests that individuals with upper-sized FSHD1 repeats are more frequently unaffected, unaffected carriers with permissive D4Z4 repeats of 1 to 3 units have also been observed.¹⁰⁷

Patients who are diagnosed with a severe form of FSHD at a young age, called early onset FSHD, usually have a D4Z4 repeat of 1 to 3 units.¹⁰⁸ The definition of early onset FSHD is: symptoms of facial weakness before the age of 5 and/or signs of scapulohumeral

weakness before the age of 10.³⁰ A recent study of cohort patients between the age of 0 to 17 years and a 22-year follow-up study of another small cohort of early onset FSHD patients revealed that even among early onset patients, there is a wide variety in severity of the disease. Severity spanned the entire FSHD spectrum, that is, some patients were wheelchair dependent, while others could still walk unaided.^{109,110} This indicates that the phenotype and severity of early onset FSHD patients is still not uniformly defined.

Altogether, these studies indicate that the epigenetic state and transcriptional activity of the D4Z4 repeat is not perfectly related to the number of units on a permissive allele, but that other epigenetic modifiers play a role in the degree of D4Z4 chromatin relaxation. An example of such a modifier is SMCHD1, which has been shown to influence expression of *DUX4*.^{66,81} When an FSHD1-sized D4Z4 repeat is inherited together with a mutation in *SMCHD1* (FSHD2) a more severe FSHD phenotype (FSHD1 + 2) is observed¹¹¹ (Figure 2B). Similarly, *DNMT3B* mutations can act as a disease modifier in FSHD1 families.⁶⁷ Although FSHD2 is often referred to as the contraction-independent form of FSHD, analysis of a large number of unrelated controls and FSHD2 patients reveals a repeat size dependency in these patients as well. While the median number of D4Z4 units in controls is 33.7 units, in FSHD2 this is significantly lower with a median of 16.8 units.^{71,112} Sacconi et al provided further evidence for the hypothesis that FSHD1 and FSHD2 form a disease continuum.¹⁰³ This was based on the analysis of the combined effect of D4Z4 repeat size and *SMCHD1* mutation status on the methylation levels at D4Z4 (Diagnostic Region 1 [DR1]) in a group of FSHD patients. This study showed that methylation levels in FSHD1 + 2 (9 or 10 units combined with an *SMCHD1* mutation) and FSHD2

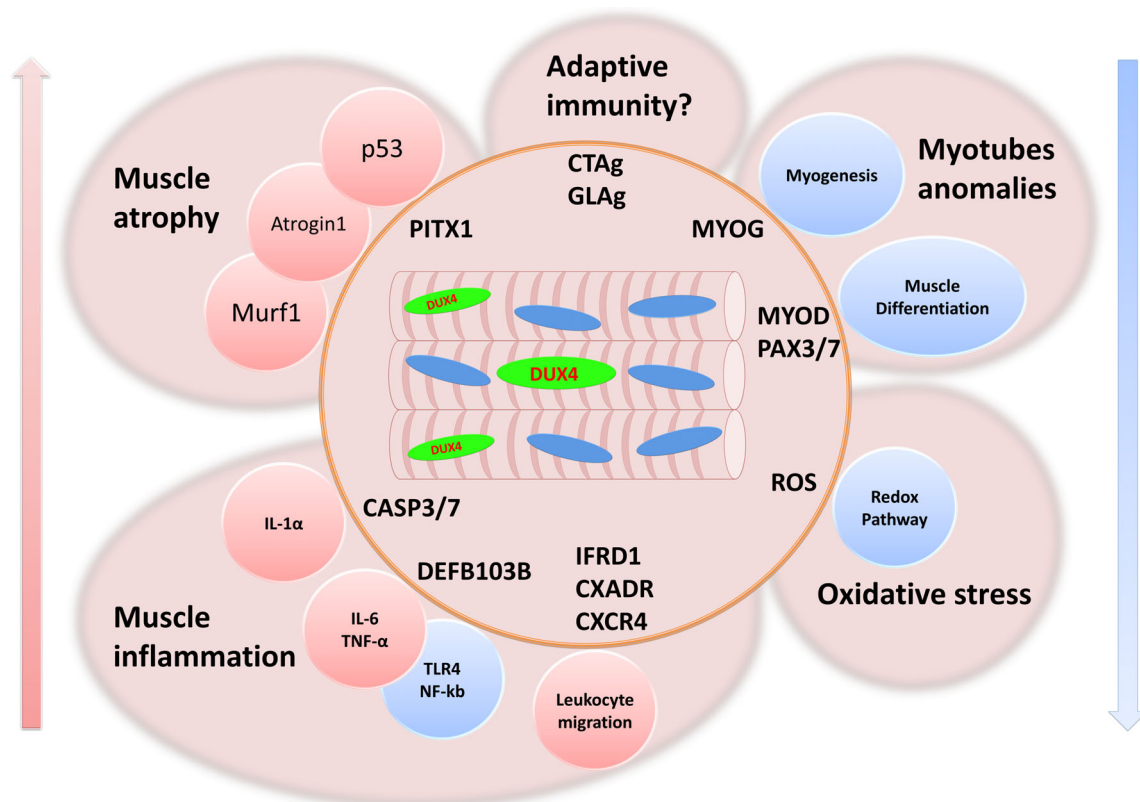


FIGURE 3 Schematic representation of the DUX4-induced transcriptional cascade in skeletal muscle cells. DUX4-induced misexpression of Cancer testis antigens (CTAg) and germline antigens (GLAg) in facioscapulohumeral muscular dystrophy (FSHD) skeletal muscle cells would induce an immune response which could explain the inflammatory infiltrates associated with FSHD histology. PITX1, another DUX4 target gene, is a transcription factor able to activate p53 (cell cycle arrest mediator), Atrogin 1 and Murf1 (proteasome family members) eventually leading to muscle atrophy. DUX4-induced upregulation of caspase 3/7 activity (CASP3/7) would lead to muscle cell death which is a further stimulus for interleukin 1 α (IL-1 α) secretion, a potent inflammatory cytokine; DUX4 also upregulates a group of genes belonging to the innate immunity defense like DEF103B, IFRD1, CXADR, CBARA1 and CXCR4. These findings could be responsible for the presence of muscle inflammation. Genes belonging to the glutathione-redox pathway appear to be downregulated resulting in an elevated reactive oxygen species (ROS) production and therefore in an increased susceptibility to oxidative stress. Finally, DUX4 could also compromise muscle differentiation (by MYOD and PAX3/7 downregulation) and myogenesis (by MYOG downregulation) with consequent myotubes anomalies

(>11 units with an *SMCHD1* mutation) form a continuous scale together with FSHD1. Importantly, in this study *SMCHD1* mutations were exclusively found in FSHD1 patients with a 9 to 10 unit D4Z4 repeat, and never in combination with a 4 to 8 unit D4Z4 repeat. These lower levels of DR1 methylation are associated with higher age-corrected disease severity and faster disease progression.¹⁰³ Additionally, certain unique cases of FSHD2 which were originally thought to have unusually long 4qA alleles (>20 units) can be explained by the presence of D4Z4 duplication events. These cases present as FSHD2 in which a long D4Z4 repeat on a 4qA allele is followed by, or preceded by, a duplication of the D4Z4 repeat, which is of an FSHD2-compatible size (ie, <20 units).^{65,112} Therefore, it is tempting to speculate that there is a repeat size threshold for any type of FSHD.

In FSHD2 patients with a mutation in *SMCHD1*, the disease severity is influenced by the type of mutation. In general, missense mutations in the protein coding sequence lead to a more severe

phenotype than those causing haploinsufficiency. As *SMCHD1* normally forms homodimers, the missense mutations most likely lead to the formation of dysfunctional heterodimers with dominant-negative consequences.^{71,93} *SMCHD1* loss-of-function mutations such as mutations causing frameshifts and premature stop codons or aberrant splicing are well-described causes of FSHD2.^{71,113} Recent studies have also highlighted that the loss of one copy of the *SMCHD1* gene can occur through chromosome 18p microdeletions, or the complete loss of the short arm of chromosome 18 in 18p deletion syndrome (18p-).^{114,115} These 18p- patients with *SMCHD1* among the deleted genes were found to have reduced D4Z4 repressive chromatin marks and express *DUX4* in myonuclei when a permissive 4qA allele is present.¹¹⁴ Although these patients present a wide range of unrelated symptoms, FSHD clinical features were also detected in a few cases, demonstrating that the loss of one copy of *SMCHD1* can cause FSHD2.^{114,116} Furthermore, when FSHD2 patients have more than one permissive 4qA allele of appropriate size (ie, 1-8 units in FSHD1,

<20 in FSHD2), biallelic expression of *DUX4* can occur, which can result in a higher susceptibility to disease presentation and could potentially cause a more severe FSHD phenotype.⁶¹

3.2 | SMCHD1 mutations in BAMS and FSHD2

Recently, several reports showed that heterozygous mutations in *SMCHD1* are also causal to Bosma Arhinia Microphthalmia Syndrome (BAMS). BAMS is a rare developmental disorder in which the nose (arhinia) and olfactory structures are partially or completely absent due to defects in early nasal development. Many BAMS patients show other craniofacial anomalies and ocular defects such as anophthalmia or microphthalmia (absence of the eyes or smaller eyes, respectively) and patients may demonstrate hypogonadotropic hypogonadism.^{117,118}

Mutations causative for FSHD2 cover the entire *SMCHD1* locus, and can be classified as indels, splice site mutations, nonsense or missense mutations.⁷¹ Close to 200 FSHD2 mutations have currently been identified (See the Leiden Open Variant Database).^{119,120} In contrast, only missense mutations have been described in BAMS, and they are exclusively located in the extended ATPase domain.^{117,118,120} In FSHD2, the extended ATPase domain is also enriched for missense mutations, and three-dimensional modeling of FSHD2 and BAMS missense mutations suggests that although mutations occur in the same region, the affected amino acids are largely located in different functional regions of the ATPase domain for either disease.^{97,120} At least one BAMS patient with FSHD symptoms has been reported having a moderately sized D4Z4 repeat on a 4qA allele. This suggests that although the phenotypes are very different, the disorders are not mutually exclusive.¹¹⁸ Intriguingly, two mutations (G137E and L107P) have been reported in both FSHD2 patients and unrelated BAMS patients.^{71,118,121} The FSHD2 patients harboring the L107P mutation do not have BAMS-like features.¹²¹ Based on questionnaires, neither did any of the other FSHD2 patients with a missense mutation other than the L107P mutation in the extended ATPase domain of *SMCHD1*.¹²¹

To investigate whether BAMS and FSHD2 mutations have different functional outcomes, *in vitro* ATPase assays have been employed with a recombinant N-terminal fragment of murine or human *SMCHD1*.^{97,117,122} Whether the ATPase function is differentially affected by FSHD2 or BAMS mutations is still topic of debate. Some data suggest that BAMS mutations exhibit increased capacity to hydrolyze ATP to ADP,^{117,122} while others show no difference.⁹⁷ Interestingly, although the aforementioned G137E mutation also causes FSHD2, this mutant was observed to have increased ATPase activity,^{97,122} while D4Z4 methylation status available for the FSHD2 G137E patient indicates hypomethylation (indicative of FSHD2).⁷¹ This implies that BAMS and FSHD2 mutations cannot be fully functionally distinguished on their ATPase activity alone, and that hypermorphic variants might cause FSHD2 just like hypomorphic variants could potentially cause BAMS. Modeling of BAMS mutations in *Xenopus laevis* indicates a developmental defect leading to smaller

eyes in the tadpole.^{117,122} Downregulation of *smchd1* in the early larvae of zebrafish by either morpholinos or clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) mediated genome editing resulted in smaller eye size as well, indicating that in different organisms either loss (FSHD2) or potential gain (BAMS) of *SMCHD1* function can confer similar phenotypic effects.¹¹⁸ Collectively, the data obtained in these studies show that great care must be taken when interpreting the functional outcome of *SMCHD1* mutations. The functional effect of either BAMS or FSHD2 on full length *SMCHD1* protein is not known, neither is the effect of heterodimerization of mutant and wild-type *SMCHD1* protein *in vivo*.

4 | CONSEQUENCE OF EPIGENETIC DEREPRESSION: THE DUX4 IMMUNE DEREGULATION CASCADE

There is general consensus that D4Z4 chromatin structure reorganization in the context of a specific genetic background results in inappropriate activation of *DUX4* in skeletal muscle.^{7,52,123,124} *DUX4* is expressed in the testis and cleavage stage embryos, and epigenetically silenced in most somatic tissues. In cleavage stage embryos, *DUX4* acts as a transcription factor that is involved in zygotic genome activation.^{14,15,18,125} Among the several candidate genes for FSHD, *DUX4* is currently the strongest candidate since its expression is repeatedly found in both FSHD1 and FSHD2 while absent in control cells^{126,127} thereby connecting two genotypes with a single phenotype.^{7,14,52,126,128-130} Several studies have thus proposed *DUX4* as the initiator of a transcriptional deregulation cascade with ultimately myopathic effects.^{23,131}

DUX4, once epigenetically de-repressed, activates germline genes in skeletal muscle.^{23,132,133} Therefore, it is plausible that *DUX4*-induced misexpression of these genes induces an immune response which can drive the progression of the disease. However, the nature of such immune response is largely unclear. In this section, we recapitulate *DUX4* candidate mechanisms disturbing muscle homeostasis in general (Figure 3).

DUX4 mRNA is only detected in low quantities in FSHD muscle biopsies and primary muscle cell cultures.^{18,134} Based on RT-PCR and immunofluorescent studies this low expression is explained by the presence of a small number of myonuclei expressing relatively high levels of *DUX4*, rather than a uniform low expression level in all nuclei.^{14,135}

Overexpression of *DUX4* in muscle cells induces upregulation of caspase 3/7 activity (a prominent mediator of apoptosis), altered emerin distribution in the nuclear envelope, and cell death.¹³⁶ *DUX4C*, a variant of *DUX4* that lacks the transactivation domain, is located in a single inverted and truncated D4Z4 unit, which is positioned proximally to the D4Z4 repeat. Forced expression of *DUX4C* does not lead to muscle cell degeneration.¹³⁷ Cell death is known to drive a subtype of inflammation defined as “sterile inflammation,”¹³⁸⁻¹⁴² mainly through the release of the IL-1 family cytokines (IL-1 α , IL-1 β , IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ and IL-37).¹⁴³ Once activated, all members

of this family are able to recruit inflammatory cells (such as neutrophils and macrophages) to the site of injury as well as tissue repair factors such as TGF β , which will promote the healing of the inflammation by fibrosis.^{138,143} The effect of IL-1 on skeletal muscle cells has been studied in the early 1980s.¹⁴⁴ Incubation of rat muscles with IL-1 causes increased muscle proteolysis as well as increased secretion of prostaglandin E2 which can further stimulate protein degradation.¹⁴⁴ Therefore, muscle cell death in FSHD initiated by DUX4 might be mediated through the IL-1 pathway. Wallace and colleagues demonstrated that the caspase 3/7 activity is upregulated upon injection of DUX4 protein in the muscles of wild type but not p53 knockout mice.¹⁴⁵ This suggested that DUX4 induced apoptosis is p53 dependent. However, recent findings challenge this model. Bosnakovski et al argued that inhibition and/or deficiency of p53 in murine derived myoblasts and tissues does not suppress cytotoxicity mediated by DUX4 expression, a result which was also observed by Shadle et al in human myoblast deficient for *TP53*.^{146,147} The latter authors propose that DUX4 activates the MYC-mediated apoptosis together with the double-stranded RNA response pathway instead, which can function in a P53-independent manner.¹⁴⁷ Further research is needed to clarify the exact mechanism of DUX4-mediated apoptosis.

Among DUX4 downstream target genes is PITX1, a member of the paired homeodomain family. PITX1 is involved in the early development of the lower limbs,¹⁴⁸ and is upregulated in patients with FSHD.⁵³ PITX1 regulates the expression of the IFN gene family involved in the activation of the innate immune response against viral infection and is a suppressor of both RAS and tumorigenicity.¹⁴⁹ Furthermore, PITX1 is also known to activate components of the p53 pathway causing cell cycle arrest and apoptosis,¹⁵⁰ and to induce MURF1 and ATROGIN1.²² These two proteins are components of the proteasome, which is involved in the degradation of muscle proteins.²² These findings make PITX1 an interesting DUX4 target which abnormal activation could help explain muscle atrophy and inflammatory features in FSHD.

DUX4 upregulates a group of genes belonging to the innate immunity defense like *DEFB103B*, *IFRD1*, *CXADR*, *CBARA1* and *CXCR4*.²³ *DEFB103B* is a member of the defensin family with an anti-inflammatory activity through inhibition of NF- κ B signaling and Toll-like receptor 4, thereby suppressing the release of pro-inflammatory cytokines.¹⁵¹ It has also been suggested that *DEFB103B* inhibits muscle differentiation. Therefore, it has been proposed that DUX4 might suppress the innate immune system and impair muscle differentiation by upregulation of *DEFB103B*.^{13,15,23} *IFRD1* encodes a protein related to interferon gamma and represses transcriptional activity of NF- κ B, contributing to explain the DUX4 immunosuppressive action.^{23,152} On the other hand, DUX4 upregulates *CXADR* and *CXCR4*, which are receptors involved in the migration of leukocytes from the blood into inflamed tissues.^{153,154} Among the innate immunity pathways, several membrane attack complex-related genes were also found highly expressed in normal appearing FSHD muscle fibers.¹⁵⁵ Therefore, complement activation may be an early event in FSHD pathogenesis. It has recently been shown that *DUX4* expression in cancer cells leads to a block of interferon gamma mediated MHC class I expression.¹⁵⁶

As blocking MHC class I antigen presentation lowers the inflammatory response against the *DUX4* expressing cells, it is not yet clear how this finding correlates with FSHD muscle showing an increased inflammatory response.

Another pathway likely disrupted by DUX4 is myogenesis,¹³³ a finely regulated process responsible for normal muscle development.¹⁵⁷ Defects in the myogenic program may perturbate muscle homeostasis contributing to the pathogenesis of muscle disorders.^{158,159} PAX3 and PAX7 are two key regulators of myogenesis¹⁶⁰ that share a high degree of homeodomain homology with the DUX4 DNA binding domain.^{161,162} Therefore, Bosnakovski et al suggested that DUX4 might interfere with myogenesis by competition with PAX3 and PAX7 after induced co-overexpression in mouse C2C12 myoblasts.^{131,161} However, a possible competitive inhibition by DUX4 and PAX3/7 needs further validation and translation to FSHD patient studies¹⁶³ as co-expression of these proteins was not observed in cell cultures.¹⁶⁴ In addition to the hypothesized PAX3/PAX7 inhibition theory, DUX4 would impair myogenesis and muscle differentiation decreasing the expression of the myogenic precursors MyoG,^{133,165} MyoD and of its downstream target genes as confirmed by different laboratories.^{18,130,166,167} This defective myogenic program causes myoblasts to differentiate into abnormal myotubes, as shown in *in vitro* cultures.^{168,169}

DUX4 could also affect muscle differentiation by not only affecting the upstream regulators of myogenesis but also through the induction of oxidative stress. Indeed, *in vitro* cultured FSHD myoblasts are particularly sensitive to oxidative stress.¹⁷⁰ In fact, several oxidative stress related genes have been found to be altered in FSHD muscle cells.^{130,165,167,170-172} Presence of constitutive oxidative stress disturbs muscle homeostasis and reduces the ability of myoblasts to correctly differentiate into myotubes.^{173,174} Dmitriev et al described the presence of high levels of DNA damage lesions, increased reactive oxygen species production, and upregulation of DNA damage repair related genes in cultured FSHD myoblasts.¹⁷⁵

Altogether, the activation of DUX4 in FSHD might trigger a cascade of events which can activate hundreds of genes, ultimately leading to muscle inflammation, muscle atrophy, oxidative stress, and disrupted myogenesis.

5 | NOVEL POTENTIAL THERAPEUTIC TARGETS

Despite the progress in understanding the pathophysiology of the disease, there is no cure for FSHD.

Currently, patients can benefit from symptomatic treatment that can improve muscle function and strength such as physical therapy,² moderate aerobic exercise,¹⁷⁶⁻¹⁷⁹ scapular fixation (a surgical procedure that ameliorates the arm functionality)⁵ and the use of orthotic devices like corsets, back supports, and shoes plus orthoses that can compensate the weakening muscles.¹⁸⁰

Over the last three decades, different clinical trials attempted to improve muscle function and strength in FSHD patients. Antioxidants

like vitamin C and E, zinc gluconate, and selenomethionine have been tested in FSHD patients in the context of a double-blind randomized trial.¹⁸¹ The rationale of this trial stems from the increased susceptibility of FSHD muscle cells to oxidative stress.^{21,175,182} Unfortunately, patients receiving antioxidants did not report a significant improvement in muscle performance compared to the placebo group.

Apart from physical activity, another attempt to improve muscle mass and function in FSHD is represented by use of anti-myostatin therapies. Myostatin, also known as growth differentiation factor 8, belongs to the TGF- β superfamily, a group of proteins with profibrotic activity.¹⁸³ Myostatin is produced by skeletal muscle cells and acts as a negative muscle growth regulator.¹⁸⁴ Animal studies have demonstrated that myostatin deficient mice have a strong increase in muscle mass compared to the wild-type mice.¹⁸⁵ These findings sparked the interest of pharmaceutical companies in designing antibodies against myostatin which have been tested in several neuromuscular diseases.¹⁸⁶ However, despite high expectations, results have been unsatisfying.^{187,188} In 2008, a 9-month multicenter double-blind randomized clinical trial tested the myostatin inhibitor MYO-29. The study tested three different doses in three patient groups, among which 42 patients with FSHD. Although MYO-29 was generally well tolerated, there was no significant improvement in muscle strength and function in any of the groups.¹⁸⁸

The presence of inflammatory features in FSHD muscle provided a rationale for an open-label trial of prednisone.^{29,189} Also, this study did not find significant differences in muscle strength and muscle mass between the treated and the placebo arm. Furthermore, case-reports of FSHD patients receiving corticosteroid therapy have failed to show function improvements.^{190,191} In 2015, the immune involvement in FSHD also provided rationale for a Phase 1b/2 open-label trial of ATYR1940 in patients with early onset FSHD. ATYR1940 is a physiocrine-based protein and a modulator of immune responses in skeletal muscle.¹⁹² Eight genetically confirmed FSHD patients were included and received one placebo dose followed by 12 escalating doses of ATYR1940. The drug was well tolerated up to the highest dose, but there was no clinical improvement in terms of muscle strength and function, or on muscle MRI evaluation.¹⁹³

More recently, research groups are focusing on the identification of specific disease targets to develop a causal treatment. Taking into account the complexity of DUX4 toxicity, a major focus is on (epigenetic) regulators of DUX4 activity, as this would also block all its downstream targets and effects. Different laboratories explored whether it is possible to revert the chromatin structure of the FSHD locus into a repressed state. Snider et al demonstrated that small RNA transcripts consistent with siRNAs and miRNAs (small RNA molecules involved in RNA silencing) are produced by D4Z4, suggesting their role in RNA-mediated epigenetic silencing of the repeat.⁸² Some years later, Lim and coworkers investigated whether these small RNA molecules might contribute to the epigenetic silencing of the D4Z4 repeat. To test this hypothesis, the authors transfected siRNAs identical to the siRNAs endogenously transcribed from D4Z4 into FSHD muscle and observed strongly reduced DUX4 mRNA levels. This silencing

correlated with increased H3K9me2 and with AGO2 recruitment to the D4Z4 repeats. Together these studies suggest that an RNA-mediated silencing pathway is normally involved to prevent DUX4 transcription, making this pathway an interesting potential therapeutic target. Himeda et al¹⁹⁴ demonstrated the benefits of the use of CRISPR and dCas9 (catalytically dead Cas9) protein system (CRISPR-dCas9) to reverse the epigenetic status of the FSHD locus.¹⁹⁵ Targeting the transcriptional inhibitor KRAB to the DUX4 promoter through fusion with dCas9 repressed DUX4 and its downstream target genes in FSHD muscle cell cultures. Additionally, the presence of the KRAB-repressor leads to a slight increase in the levels of repressive proteins, for example, HP1 α and KAP1, at D4Z4, although no increase of H3K9me3 and H3K27me3 could be observed, potentially due to the large amount of non-targeted D4Z4 repeats in the genome.¹⁹⁴ Recently, the same group identified epigenetic pathways that activate DUX4 by knock down of 36 candidate DUX4 activators in FSHD1 myocytes and monitoring the effect on DUX4 expression and other genes involved in muscle homeostasis.⁸³ Selected candidates belong to several functional categories: chromatin modifiers, transcription regulators, as well as several classes of histone modifiers. The screening yielded four validated candidates: ASH1L, BRD2, KDM4C, and SMARCA5. In addition, slight increases in SMCHD1 by ectopic expression or repairing the SMCHD1 gene defect in patient cells efficiently silences DUX4 in muscle cell cultures.^{81,196} Besides that these candidates are potentially druggable targets, the results confirm that multiple epigenetic pathways shape the D4Z4 chromatin structure.

Attempts to improve muscle functionality in FSHD have also been undertaken with salbutamol, a β 2 adrenergic receptor (β 2AR) agonist,¹⁹⁷⁻¹⁹⁹ since β 2 agonists were proven to favor muscle cell regeneration in animal studies, and to prevent muscle proteolysis.²⁰⁰ However, in none of the trials salbutamol proved to benefit the physical performance of the patient group in comparison to the control group. Nevertheless, a recent study using β 2AR agonists salbutamol and formoterol in FSHD myotube cultures showed that both drugs were able to reduce the expression of well-known DUX4 target genes ZSCAN4, TRIM43, MBD3L2, and LEUTX, and to induce the production of cAMP.²⁰¹ cAMP, an ATP derivative, is a second messenger crucial for many biological process such as transport of hormones, ion channel regulation and protein kinase activation like the protein kinase A (PKA).²⁰² Therefore, the authors treated FSHD myotubes with a cAMP analogue which was also able to reduce DUX4 target gene expression levels through a PKA-dependent mechanism.

Campbell et al further investigated the potential of β 2AR agonists and bromodomain and extra-terminal (BET) inhibitors as possible FSHD drugs candidates.²⁰³ BET proteins belong to the BRD protein family including four members: BRD2, BRD3, BRD4 and BRDT. These proteins normally bind to acetylated histones thereby promoting gene transcription.²⁰⁴ They reported a significant suppression of DUX4 and DUX4 target gene levels in both FSHD1 and FSHD2 primary muscle cells treated either with β 2AR agonists through cAMP increase, or with BET inhibitors through BRD4 inhibition.²⁰³ Further research by these authors into the signaling pathway behind the effect of β 2AR agonists identified p38 mitogen-activated protein kinase (p38-MAPK)

as a regulator of *DUX4* expression.²⁰⁵ Clinically approved p38 inhibitors lead to potent suppression of *DUX4* expression in both FSHD myoblasts and a mouse FSHD xenograft model.²⁰⁵ A phase 2 clinical trial using p38 inhibitor Losmapimod in FSHD is currently (early 2020) ongoing.

6 | CONCLUSION

As research on FSHD continues, it is becoming apparent that the genetic underpinnings of FSHD are complex with many modifiers contributing to a wide clinical spectrum: from asymptomatic to wheelchair dependent, from features restricted to muscle to extramuscular involvement such as hearing loss and vision impairment. The involvement of partially known modifiers implies that FSHD should be considered a complex disorder rather than a monogenic or digenic disease, with classical FSHD1 and FSHD2 forming a disease continuum. In this complex and variegating scenario, with two seemingly distinct but converging genotypes, common epigenetic changes explain the cytotoxic gain of function of the otherwise silenced *DUX4* gene. *DUX4* is activated at the wrong time (with unpredictable bursts of expression) and in the wrong place (skeletal muscle). Once activated, it triggers a cascade of events that, among others, elicit a response from our immune system, leading to the presence of inflammatory infiltrates in affected muscles. However, it is not yet clear which means of defense our immune system undertakes and whether this represents a double-edged sword, explaining the progressive replacement of skeletal muscle with fatty fibrosis. Numerous strategies have been undertaken to treat the muscle weakness in FSHD, but unfortunately, apart from increasing physical activity, none of them resulted in functional benefit. However, the field has matured to an exciting new era in which we can expect trials with new and existing drugs that target *DUX4* itself, its regulation, or its damaging effects in skeletal muscle.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Data sharing not applicable—no new data generated.

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