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Review

A complex interplay of genetic and epigenetic events leads to abnormal expression of the *DUX4* gene in facioscapulohumeral muscular dystrophy

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Abstract

Facioscapulohumeral muscular dystrophy (FSHD), a prevalent inherited human myopathy, develops following a complex interplay of genetic and epigenetic events. FSHD1, the more frequent genetic form, is associated with: (1) deletion of an integral number of 3.3 Kb (D4Z4) repeated elements at the chromosomal region 4q35, (2) a specific 4q35 subtelomeric haplotype denominated 4qA, and (3) decreased methylation of cytosines at the 4q35-linked D4Z4 units. FSHD2 is most often caused by mutations at the *SMCHD1* (Structural Maintenance of Chromosomes Hinge Domain 1) gene, on chromosome 18p11.32. FSHD2 individuals also carry the 4qA haplotype and decreased methylation of D4Z4 cytosines. Each D4Z4 unit contains a copy of the retrotransposed gene *DUX4* (double homeobox containing protein 4). *DUX4* gene functionality was questioned in the past because of its pseudogene-like structure, its location on repetitive telomeric DNA sequences (i.e. *junk* DNA), and the elusive nature of both the *DUX4* transcript and the encoded protein, DUX4. It is now known that DUX4 is a nuclear-located transcription factor, which is normally expressed in germinal tissues. Aberrant DUX4 expression triggers a deregulation cascade inhibiting muscle differentiation, sensitizing cells to oxidative stress, and inducing muscle atrophy. A unifying pathogenic model for FSHD emerged with the recognition that the FSHD-permissive 4qA haplotype corresponds to a polyadenylation signal that stabilizes the *DUX4* mRNA, allowing the toxic protein DUX4 to be expressed. This working hypothesis for FSHD pathogenesis highlights the intrinsic epigenetic nature of the molecular mechanism underlying FSHD as well as the pathogenic pathway connecting FSHD1 and FSHD2. Pharmacological control of either *DUX4* gene expression or the activity of the DUX4 protein constitutes current potential rational therapeutic approaches to treat FSHD.

Keywords: Facioscapulohumeral muscular dystrophy; FSHD1; FSHD2; DUX4; *SMCHD1*

1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most prevalent inherited neuromuscular disorders [1,2], having significant intra- and interfamilial variability in disease presentation, progression and age of onset, with most individuals becoming symptomatic in their second decade [1–4]. Patients with childhood onset (i.e. infantile FSHD) have a more severe clinical presentation [5,6]. Juvenile or adult onset male patients are more severely affected than females [7,8]. The disease is characterized by progressive weakness and atrophy of muscles of the face and shoulder girdle, further extending to the proximal arms and legs [9]. FSHD is also characterized by a marked right/left asymmetry of muscle involvement [10]. Non-muscular manifestations of the disease include sensorineural deafness and retinal vasculopathy [11] as well as central nervous system alterations in some severely affected children [12]. The original detailed phenotypic description of FSHD by Landouzy and Dejerine [13] has both historical and clinical relevance.

2. The genetics and epigenetics underlying FSHD

FSHD1 displays an autosomal dominant mode of inheritance with reduced penetrance and a high frequency of sporadic cases [5]. Genetic linkage analyses localized FSHD1 to the subtelomeric region of the long arm of chromosome 4

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(4q35) [14]. This region contains a macrosatellite repeat consisting of head-to-tail tandem repeat units of approximately 3.3 kb in size designated D4Z4 [15]. In healthy individuals the number of D4Z4 repeats at the array is highly polymorphic and varies from 8 to 100 units [16,17]. FSHD1 individuals carry a shortened version of this D4Z4 tandem repeat (i.e. 1–10 D4Z4 units) on one of the two chromosomes 4q [14,15]. FSHD-sized alleles with 8–10 D4Z4 units show incomplete penetrance probably dependent on the epigenetic status of the D4Z4-repeat array [[18]; see below]. An inverse relationship has been established between the residual number of 4q35-linked D4Z4 units and the severity of the disease [19]. Patients with a low number of D4Z4 units (i.e. 1–3) are generally affected in early childhood, while patients with pathologically large alleles have adult onset and are mildly affected [20]. Complete loss of the D4Z4 tandem array does not result in FSHD, suggesting that one or more residual D4Z4 units are required for FSHD to develop [21]. A D4Z4-like repeat is present at the subtelomeric region of chromosome 10 (i.e. 10q26), and complex chromosome rearrangements involving 4q35 and 10q26 (i.e. D4Z4 and D4Z4-like) sequences have been described [22]. Only shortened D4Z4 arrays at 4q35, however, are linked to FSHD1 [23]. Moreover, specific 4q subtelomeric DNA sequences, categorized as permissive haplotypes, are required for FSHD to develop when D4Z4 is contracted (Fig. 1). These permissive haplotypes, located immediately distal to the D4Z4 repeat array, have been designated 4qA (with α-satellite repeat) [24,25]. Whereas chromosomes carrying 4qA and 4qB haplotypes are almost equally distributed in the human population, FSHD chromosomes seemed to be exclusively associated to the 4qA type [25]. Further studies have identified over 18 subtelomeric DNA sequence variants on chromosome 4q, but only three 4qA variants resulted permissive for FSHD: the common variant 4qA161 and the rare variants 4qA159 and 4qA168 [26].

D4Z4 DNA sequences are highly methylated in somatic tissues of healthy subjects (see Fig. 1) [27]. In FSHD patients (i.e. FSHD1 and FSHD2), however, a marked demethylation of D4Z4 cytosines is observed (Fig. 1) [27,28]. In addition to cytosine demethylation, these D4Z4 sequences show a remarkable loss of the repressive patterns of histone modifications [29]. The combination of decreased DNA methylation and loss of histone heterochromatin markers is recognized at open chromatin structures which allow for gene expression [30].

FSHD2-affected individuals (i.e. about 5% of the FSHD patients) are clinically identical to FSHD1 [31]. FSHD2 patients carry smaller but normally sized D4Z4 repeat arrays (i.e. 8–20 units) [31]. Distinctive genetic and epigenetic molecular signatures associated to FSHD1 are also present in FSHD2: the permissive 4qA haplotype and decreased cytosine methylation at D4Z4, respectively [31]. FSHD2 is most often caused by mutations at the *SMCHD1* (Structural maintenance of chromosomes flexible hinge domain-containing 1) gene [31,32], on chromosome 18, thus showing a digenic inheritance: haploinsufficiency of SMCHD1 and the 4qA haplotype [18,32]. *SMCHD1* belongs to the ubiquitous SMC gene superfamily contributing to the control of the repressed status of eukaryotic chromatin [33–35]. Individuals carrying moderately contracted D4Z4 alleles and mutations at the *SMCHD1* gene are considered FSHD1 + 2 patients [36]. These individuals are much more severely affected than expected from their D4Z4 copy number, indicating that SMCHD1 is a modifier of disease severity in FSHD1 [18,36,37]. Mutations in the *DNMT3B* (DNA methyltransferase 3B) gene have recently been found in FSHD2 families [38]. *DNMT3B* mutations also result

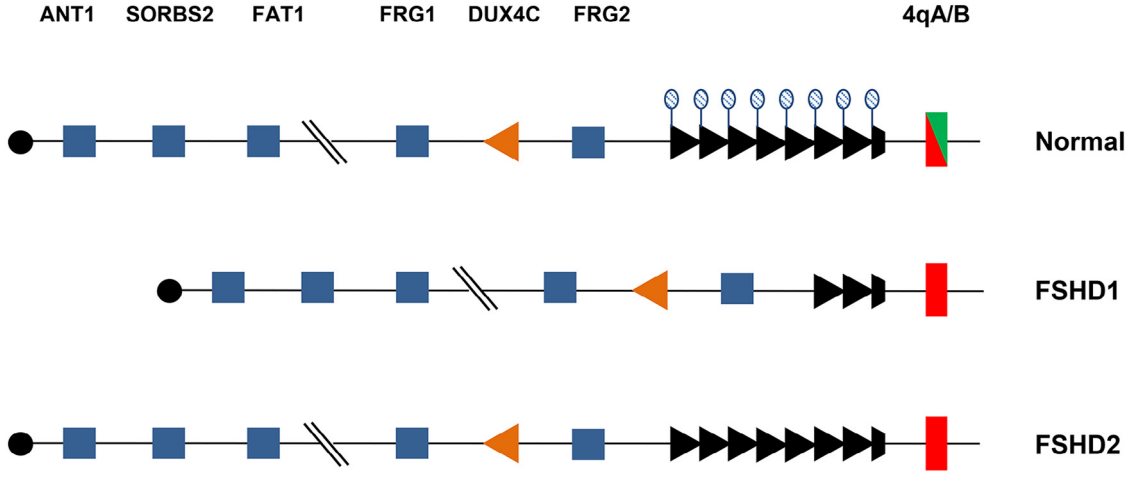

Fig. 1. Human chromosome region 4q35. Healthy (*Normal*) individuals carry a D4Z4 *tandem* (**black** *arrow heads*) with methylated cytosines (*pins*) and the haplotypes 4qA or 4qB (i.e. *red* or *green*, respectively). *FSHD1* patients carry a shortened D4Z4 *tandem*, demethylated cytosines and the haplotype 4qA. *FSHD2* patients carry a normal-sized D4Z4 tandem repeat, cytosine demethylation, haplotype 4qA and mutations at *SMCHD1* (not illustrated). Linked genes in the region are *DUX4c (brown arrow head)*, *FRG2*, *FRG1*, *FAT1*, *SORBS2* and *ANT1* (*blue boxes*) which have a more centromeric *(black circle)* location. See text for further details.

in increased penetrance of FSHD [38], highlighting the contribution of epigenetic phenomena on FSHD severity and progression [18,39,40].

3. Epigenetic control of *DUX4*

Following the recognition of the association between FSHD and 4q35-linked D4Z4-contracted alleles, early models of pathogenesis suggested an epigenetic nature of the disease [18]. The original hypotheses considered that contraction of the D4Z4 tandem-repeat exerts an influence on the transcriptional control of genes in the direct vicinity of D4Z4, a mechanism known as position effect variegation (PEV) [41]. Expression of several candidate genes proximal to 4q35 potentially contributing to the FSHD pathogenic pathway were studied. Among them, the FSHD region gene 1 (*FRG1*), FSHD region gene 2 (*FRG2*) and adenine nucleotide translocator 1 (*ANT1*) were the most relevant candidates (Fig. 1). Each of the aforementioned genes were initially found to be up-regulated in FSHD muscle [42]. *FRG2* is the only gene reproducibly shown to be induced in primary FSHD-derived muscle cells [27,43–45]. Interestingly, *FRG2* is a target of DUX4 (see below), offering an alternative explanation to the PEV for its upregulation in FSHD cells [45]. The 4q35-linked gene *FAT1* has also been recognized as a candidate gene for FSHD [46–48] and expression of the *SORBS2* (*sorbin and SH3 domain-containing protein 2*) gene, also located at 4q35 (Fig. 1), has only been observed in FSHD myoblasts carrying short telomeres [49]. The existence of enhancers and insulators at the D4Z4 region, contributing to the aberrant expression of DUX4 and/or other(s) potential 4q35-liked FSHD-causative gene(s), has been recognized [50–53]. The remarkable intra- and inter-familial clinical heterogeneity in FSHD [1,4], the finding of asymptomatic or minimally affected carriers of contracted 4q alleles [8,16,17,54], as well as the recognition of the various genetic and epigenetic contributors to the FSHD phenotype [see [18]], highlight the complexity on the molecular mechanism underlying FSHD pathogenesis as well as the resemblance of FSHD with multigenic diseases.

D4Z4 units were initially considered non-coding DNA regions, having a regulatory role on the formation and/or maintenance of 4q telomeric heterochromatin [55,56]. D4Z4 units have a high content of highly methylated GC-dinucleotides, as well as two dispersed repeat elements (LSau and hhspm3), characteristic of heterochromatic, non-expressed regions of the human genome [57]. The possibility that this region contains a functional gene was largely discounted because *DUX4*, the only recognizable ORF at each D4Z4 unit, had no evident introns or polyadenylation signals, suggesting that it represented a pseudogene [41]. D4Z4 was, in fact, considered *junk* DNA [58]. In addition, initial attempts to demonstrate *DUX4* gene expression in normal or pathologic tissue were unsuccessful [41,44,59–63].

A major breakthrough connecting *DUX4* to FSHD pathogenesis came from the demonstration that the *DUX4* mRNA and the DUX4 protein were endogenously expressed in cultured myoblasts from FSHD patients [64]. In these studies, it was first recognized that DUX4 is a toxic protein [64]. Detailed

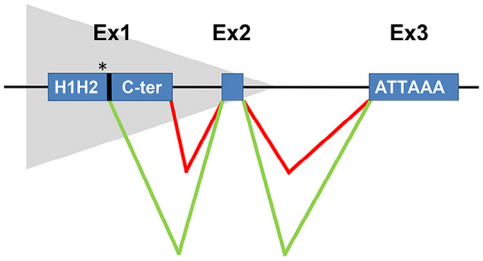

Fig. 2. *DUX4* gene expression. Two main *DUX4* mRNAs, *DUX4-fl* and *DUX4-s*, are generated from alternative splicing. The *DUX4-fl mRNA* (pattern of splicing in *red*) preserves the C-terminal region (*C-ter*) of DUX4 and the resulting DUX4 protein is toxic. The DUX4-s *mRNA* (pattern of splicing in *green*) eliminates the C-terminal region of DUX4 and the resulting DUX4 protein is non-toxic. Splicing generating *DUX4-s* utilizes a cryptic splice donor site (*asterisk*). The shadow-gray arrow represents the more telomeric, distal D4Z4 unit at the D4Z4 tandem. Exons (*Ex*) *1*, *2* and *3*, and the 4qA haplotype, consisting of the consensus polyadenylation sequence (*ATTAAA*), are indicated. See text for further details.

additional analyses of DUX4 toxicity, on cultured myoblasts carrying an inducible *DUX4* transgene, confirmed and extended these original observations [65]. A recent study validates the fidelity and FSHD relevance of myoblast models expressing a *DUX4* transgene [66]. A second key contribution to the current model of FSHD pathogenesis came from the analysis of the 4q subtelomeric haplotypes 4qA/4qB (Fig. 1). Elegant genetic and molecular studies [67,68] demonstrated that the permissive 4qA haplotype provides an intron and a polyadenylation signal (i.e. ATTAAA) stabilizing the distal DUX4 transcripts (see Figs. 1 and 2). This finding suggested a unifying model for the molecular basis of FSHD [68]. In this model, D4Z4 chromatin relaxation associated with D4Z4 shortening (FSHD1) and/or D4Z4 cytosine demethylation (FSHD1 and FSHD2) allows expression of the *DUX4* gene. In turn, the 4qA permissive haplotype provides a polyadenylation signal that stabilizes the *DUX4* mRNA allowing expression of the pathogenic protein DUX4 (Fig. 2), thereby causing FSHD [[68]; see [18]].

4. *DUX4* gene expression

Bidirectional transcription at the D4Z4 array generates sense and antisense RNA transcripts as well as small si/mi/pi-like RNA fragments [69]. These transcripts might recruit the Heterochromatin Protein 1 (HP1) as well as other chromatin repressive factors [70], which may help to preserve the heterochromatic nature of the D4Z4 repeat array [69]. The epigenetic nature of this repressive control is highlighted by the fact that *DUX4* is transcriptionally activated when the SMCHD1 transcripts and protein are suppressed to <50% of normal levels [32].

Sense *DUX4* transcripts are generated from alternative splicing as two major products: a full-length transcript (DUX4-fl) that originated from the last D4Z4 repeat at the D4Z4 tandem that spans the entire DUX4 ORF, and a shorter transcript (DUX4-s) that utilized a cryptic splice donor site and retains the double-homeobox domains but loses the carboxy terminal end (Fig. 2). It has been suggested that usage of the non-canonical splice donor site that produces DUX4-s may be regulated by

relatively higher levels of repressive chromatin modifications of the D4Z4 region [71]. An alternative, longer *DUX4* mRNA, encoding a DUX4 protein isoform of 58 kDa, has recently been recognized in cells committed to osteogenic differentiation [72].

The DUX4-fl is normally expressed in human testes and pluripotent stem cells and is suppressed in most somatic tissues [71]. Low levels of DUX4-fl are present in FSHD muscle and are mostly absent in muscle from healthy individuals. DUX4-s, on the other hand, is present at low levels in muscle from both FSHD patients and control individuals [71]. The low abundance of the DUX4-fl observed in mRNA preparations from muscle cells could reflect either a homogeneous low rate of transcription at every cell, or a heterogeneous expression in different cells, with some cells exhibiting a very high level of expression. This last hypothesis has been supported by RT-PCR amplification of DUX4 mRNA from small pools of differentiated FSHD muscle cells, where some "expressing" pools contain a high abundance of DUX4-fl transcripts [71]. The frequency of positive expressing pools suggested that approximately 1 in 1000 FSHD muscle cell nuclei was expressing a high amount of *DUX4* mRNA [71]. Immunostaining of DUX4 in cultured FSHD muscle cells supported the RT-PCR small pool studies and showed that approximately 0.1% of cells have intense nuclear staining for the DUX4 protein [71]. Interestingly, certain genetically unaffected first-degree relatives of FSHD patients express both DUX4-fl and DUX4, although at significantly low levels, in the absence of clinical symptoms. This observation suggests that potential modifiers of DUX4 expression and/or activity may be involved in FSHD pathology [73].

Stadler et al. [74] have demonstrated that *DUX4* gene expression is up-regulated by telomere shortening in cells from FSHD subjects, suggesting that this mechanism may play a role in age-related issues during FSHD pathogenesis. These authors suggest that telomere shortening associated with myoblast proliferation increases DUX4 expression and DUX4 toxicity in a positive feedback loop that contributes to disease progression. Additionally, variations in telomere length may contribute to the variable penetrance of FSHD [74].

Recently, Feng et al. [75] reported that DUX4-triggered proteolytic degradation of the factor UPF1, a central component of the nonsense-mediated decay (NMD) machinery, is associated with profound inhibition of NMD. DUX4 expression also resulted in the increased abundance of many coding RNA isoforms created by alternative splicing containing premature translation termination codons upstream of splice junctions [69]. These isoforms, which are predicted substrates for degradation by NMD, are present at very low levels in control myoblasts [75]. The accumulation of stabilized NMD substrates in cells expressing DUX4 may cause direct or indirect toxic effects in muscle cells due to intrinsic toxicity of abnormal RNAs, or a stress response to the production of abnormal proteins with antigenic potential, contributing to an immune response [76]. DUX4 and the NMD pathway may participate in a feedback loop where DUX4-mediated inhibition of NMD results in increased perdurance of the DUX4 mRNA as a possible mechanism of positive autoregulation causing FSHD pathogenesis. The connection between DUX4 and RNA metabolism has also been observed by Rickard et al. [77], using an elegant DUX4-activated reporter system. These authors found that DUX4 activation disrupts RNA metabolism (i.e. RNA splicing, surveillance and transport) as well as cell signaling, polarity and migration pathways. Interestingly, some of these DUX4-mediated toxic effects [77] are similar to the cellular phenotypes observed in cells carrying FAT1 mutations. Variants in the *FAT1* gene, as well as low expression of *FAT1* in early affected muscle of FSHD patients, have been found [44,47,48]. The normal mechanism underlying repression at D4Z4 may also involve an Argonaute (AGO)-dependent siRNA pathway [78]. Other potential mechanisms underlying the subtle control of *DUX4* gene expression, DUX4 protein activity and/or DUX4-associated toxicity remain to be addressed and recognized.

5. The DUX4 protein

DUX4, a 52 kDa double homeodomain nuclear-located protein, belongs to a large family of proteins (i.e. DUX1, DUX2, DUX3 and DUX5) encoded by 3.3-kb repeated elements dispersed in the human genome, homologous to D4Z4 [56]. While some members of the DUX family have multiple introns, DUX4 appears to be originated from retrotransposition of a processed transcript, most likely the DUXc-mRNA which is widely present in mammals [79,80]. In a pioneering study, Gabriëls et al. [60] demonstrated that the DUX4 open reading frame (ORF) is preceded by an active promoter element potentially driving endogenous *DUX4* gene expression. The DUX4 ORF and its organization at the D4Z4 array has been evolutionarily conserved for >100 million years, suggesting that DUX4 has a relevant (although still unknown) physiological role [79]. The DUX4 homeodomains H1 and H2 (Fig. 2) have remarkable sequence similarity to the *paired* (*prd*) and *orthodenticle* (*otx*) homeodomain classes [81,82] present in the Pax and Otx families of vertebrate proteins, respectively. Pax3 and Pax7 participate in the development of skeletal muscle [83], whereas Otx1 and Otx2 specify the anterior territory of the central nervous system [84]. Thus, considering the identity of its H1 and H2 homeodomains, DUX4 constitutes an attractive candidate causative protein in FSHD [83].

The carboxy-terminal domain of DUX4 confers to this protein strong transcription factor activity [85]. This activity has also been observed in tumor cells carrying a chromosomal rearrangement producing a CIC–DUX4 fusion protein [86]. In a pioneering work, Dixit et al. [67] identified that PITX1 (i.e. paired-like homeodomain transcription factor 1), a member of a paired family of homeoproteins involved in specification of hind limb identity [87], was up-regulated in muscle biopsies of FSHD patients. Using transient co-transfection with a Pitx1-LUC reporter and DUX expression vectors, this group showed that DUX4 activate transcription of PITX1 [67]. Recent detailed studies on DNA-binding sequence specificity [88] show that DUX4 does not interact physically or functionally with the *Pitx1* promoter sequence.

DUX4c, a 47-kDa protein highly homologous to DUX4, is encoded by the gene *DUX4c* located 42-kb proximal to the D4Z4 array on an incomplete inverted truncated copy of a D4Z4-repeat (Fig. 1) [85]. DUX4c also has transcription factor properties [67]. Over-expression of DUX4c in cultured human primary myoblasts activates proliferation and inhibits cell differentiation, suggesting a role in muscle regeneration [89]. The amino acid sequences of DUX4 and DUX4c only differ at their C-terminal domains [90]. Extensive mutagenic analyses of relevant DUX4 protein domains showed that the toxic effect of DUX4 is associated with its homeodomains and its C-terminal region [91]. These studies highlighted the existence of redundant mechanisms for DUX4 nuclear location, including at least three nuclear localization signals (NLS), and a particular domain at the C-terminal region [91]. DUX4 NLS1 and NLS2 sequences overlap with the N-terminal portion of H1 and H2 [92]. NLS1/NLS2 double mutants are still localized to the nucleus but have a very low cell toxic effect, suggesting that H1/H2 activity is required for DUX4 mediated cell-toxicity [91].

The original finding indicating that DUX4 is a toxic protein [64] was confirmed and extended using several cellular systems and living organisms (for a recent review see [93]). DUX4 toxicity occurs at least partly through a pro-apoptotic mechanism, indicated by caspase-3 activation [64] and DUX4 DNA binding and activation of p53 [94]. In addition to stimulating apoptosis, DUX4 may negatively regulate myogenesis [65,69,95]. Although DUX4 is expressed at low levels in FSHD-affected muscle (see above) [64,67,71], expression of high levels of DUX4 has been used to identify its target genes and participating pathways. DUX4 overexpression in mouse C2C12 cells [65] recapitulated key features of the FSHD molecular phenotype, including repression of MyoD, upregulation of p21 and increased susceptibility to oxidative stress leading to differentiation defects [96,97]. Overexpression of DUX4 in human primary myotubes induced Atrogin1 (MAFbx) and MuRF1 activation, two genes specific to the muscle atrophy pathway [98], showing a similar atrophic phenotype to the phenotype previously described in FSHD myotubes [99]. Block et al. [95] demonstrated that the activation of the Wnt/b-catenin pathway in FSHD myotubes results in reduced DUX4 expression levels and prevents DUX4-dependent myotube apoptosis, suggesting that DUX4 transcription is under active Wnt-mediated suppression. Wnt/b-catenin signaling plays important roles in muscle development [100] and postnatal muscle repair by facilitating myoblast differentiation and myotube fusion [101]. In addition, individuals with mutations of the Wnt ligand and receptors have specific peripheral retinal vascular pathology similar to some FSHD patients [102]. These last findings may provide a framework for understanding the non-muscular features of FSHD.

It has been found that DUX4 binds transposable elements forming alternative promoters for human protein-coding genes, long-noncoding (lnc) RNAs, and antisense transcripts [103]. These DUX4-activated repeats belong to the MaLR and ERV subfamily of LTR elements, and their transcripts are present in FSHD muscle and in the testis of unaffected individuals. In addition, DUX4 also binds and activates transcription of the pericentromeric satellite HSATII. These repeat-initiated transcripts could also contribute to FSHD pathogenesis. The *HEY1* gene induced by DUX4-mediated activation of a MaLR element can inhibit myogenesis [104]. Some ERV-encoded proteins contain a peptide with immunosuppressive properties [105], perhaps contributing to the suppression of innate immunity observed upon DUX4 over-expression in myoblasts [106]. On the other hand, ERV-encoded protein fragments could be antigenic, and might elicit an immune response and some of the inflammation seen in FSHD muscle [107]. In this sense, DUX4 induces the expression of human beta-defensin3 in FSHD cultured muscle cells, being able to block the innate immune response and to inhibit muscle differentiation [106].

The role of DUX4 in spermatogonia and primary spermatocytes (i.e. the only normal human cells expressing DUX4 [71]) is still unknown. Interestingly, when expressed in human muscle cells, DUX4 is able to activate a germline transcriptional program and genes involved in early stem cell development [106]. Eventual expression of these developmentally regulated genes in FSHD might be incompatible with the post-mitotic status of skeletal muscle cells, leading to tissue dysfunction.

6. DUX4 protein synthesis and cytoplasmic diffusion

Tassin et al. [108] have proposed a dynamic model for DUX4 protein expression in FSHD myotubes. These authors suggest that DUX4 gene transcription occurred in pulses in rare nuclei followed by a diffusion of the expressed protein to additional nuclei, a mechanism previously described for muscle nuclear proteins [109]. In this model DUX4 transcription factor further activates a transcriptional deregulation cascade in every nucleus to which it has diffused. The transcription factors expressed from some of its target genes, such as PITX1 [[67]; see also [88]], would similarly diffuse to additional nuclei, thus further extending the transcriptional deregulation cascade leading to fiber atrophy or death and causing the pathological FSHD phenotype. Because DUX4 is a potent transcriptional activator [67,86], small amounts of DUX4 could be sufficient to initiate this deregulation cascade. Interestingly, nuclei from FSHD myoblasts expressing the endogenous DUX4 protein exhibit DNA fragmentation [108], which may reflect an apoptotic process initiated by a pulse of DUX4 expression, followed by DUX4 protein degradation. The cellular stability of DUX4 in cells appears to be highly regulated, as expected from its transcription factor activity and likely role in early development [71]. The protein is degraded by the ubiquitin-proteasome pathway, likely targeting its carboxyl-terminal domain [108]. In affected muscles, the DUX4 signal is often at the limit of detection and even missing in biopsy of severely affected muscles, suggesting that DUX4 expression is an early event in FSHD. In this sense, Tassin et al. [108] detected a weak DUX4 signal in control primary myoblasts, but only upon differentiation. The number of DUX4-positive nuclei was significantly lower than in FSHD myotubes, but the possibility that DUX4 might be expressed normally, in a very limited

window, during the myoblast differentiation process cannot be excluded. The relatively higher presence of DUX4 in myotubes versus proliferating myoblasts may suggest that DUX4 transcription is induced upon differentiation [108].

7. Conclusions

Deregulation of the expression of the *DUX4* gene appears to be central to FSHD1 and FSHD2. It is proposed that D4Z4 chromatin relaxation on FSHD-permissive chromosome 4 haplotypes leads to activation of DUX4 pathogenic molecular networks. This DUX4-model for FSHD pathogenesis requires a deep understanding of the various potential biological roles of DUX4, the relevant protein motifs involved in its various functions, as well as the recognition of its relevant targets downstream. The various potential molecular mechanisms underlying FSHD pathogenesis have rapidly evolved. There is, however, an important gap in our knowledge between the basic molecular findings related to FSHD pathogenesis and the observed muscle pathology. Perhaps, significant questions remain about the potential pathological role of the various recognized *DUX4* RNAs, the relevance and function of DUX4 evolutionarily conserved motifs and the central role(s) of DUX4 in human cells and tissues. Concerning DUX4, other many basic questions also remain unanswered: Are there any roles for DUX4 during normal muscle development? In this case, when and where is DUX4 expressed in muscle cells and what regulates *DUX4* gene expression and DUX4 protein activity? What is the role of DUX4 in gonadal tissues? Why are gonadal cells protected from the toxic effect of DUX4? The understanding of the normal role of DUX4 would shed light on the pathological consequences of DUX4 misexpression as well as on the eventual benefits of therapeutic strategies based on the control of *DUX4* gene expression or DUX4 activity. Model organisms (i.e. Drosophila, zebrafish and mouse) [110–113] and cultured myoblasts [see [66]] constitute relevant experimental systems to study the pathways affected in FSHD as well as in pharmacological studies [114–118] directed to control *DUX4* gene expression and/or DUX4 activity.

In addition to DUX4, the recognition of additional genes causing FSHD and additional genetic-epigenetic modifiers of the FSHD-phenotype would contribute to the better understanding of the disease as well as potential factors that modulate DUX4 activity and disease penetrance.

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