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Facioscapulohumeral Muscular Dystrophy: From Clinical Data to Molecular Genetics and Return

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1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD or Dejerine–Landouzy muscular dystrophy, OMIM #158900) is the third most common hereditary myopathy, with prevalence of 1 in 20,000 (Padberg, 1982; Mostacciuolo et al., 2009). This disease is characterized by the progressive wasting of a highly selective set of muscle groups (Padberg, 1982) and it has been traditionally classified as an autosomal dominant trait (Lunt, 1998; Padberg, 1992). FSHD genetic locus has been mapped on chromosome 4q35 by genetic linkage analysis (Wijmenga et al., 1990). Interestingly, this muscular dystrophy has not yet been related to a classical mutation within a protein-coding gene, but rather the disease has been associated with DNA rearrangements in a polymorphic genomic region consisting of an array of tandemly repeated 3.3 kb segments, named D4Z4 (Wijmenga et al., 1992b). D4Z4 contains an ORF encoding a putative homeobox protein called "DUX4." The existence of native transcripts of DUX4 from D4Z4 single repeats is still controversial (Gabriels et al., 1999; Hewitt et al., 1994; Lyle et al., 1995), although recent data show evidences of the presence of the DUX4 transcript from the last D4Z4 unit in FSHD myoblasts (Lemmers et al., 2010a). The number of D4Z4 repeats varies from 11 to 100 in the general population, whereas less than 11 repeats are usually present in sporadic and familial FSHD patients. A very low copy number of 4q35 D4Z4 repeats (1–3) often correlates with an earlier onset and more severe disease. However no FSHD-linked array has been found to have zero copies of the repeat unit (Tupler et al., 1996; van der Maarel et al., 2007) suggesting that the repeat itself plays a critical role in the disease. Alleles with 4-7 repeats are the most frequent in the FSHD population and are associated with the more common form of FSHD that usually presents in adulthood, whereas alleles with 8-10 repeats typically display milder disease phenotypes with reduced penetrance. Nearly identical and equally polymorphic D4Z4 sequences reside on the subtelomere of chromosome 10q (Bakker et al., 1995; Deidda et al., 1995). The proportion of individuals in the general population carrying 4q or 10q chromosome ends with repeat arrays entirely or partially transferred between both chromosomes, is considerable (van Deutekom et al., 1996b; Lemmers et al., 1998; van Overveld et al., 2000). Rearrangements between 4q and 10q subtelomeres occur in 20% of

subjects and represent an additional complication to FSHD molecular diagnosis. However, only subjects with reduced number of D4Z4 repeats on chromosome 4, but not on chromosome 10, develop FSHD. Thus, molecular diagnosis of FSHD is, at this time, based on the analysis of the D4Z4 polymorphic alleles (Lunt, 1998; Tawil et al., 2010). Despite the identification of the genetic defect associated with FSHD, the pathologic effects of the reduction of D4Z4 elements remain largely unknown. The observation of the unique linkage of the disease with chromosome 4, led to the hypothesis that in *cis* DNA elements must be present on D4Z4 reduced alleles to explain disease onset. An element within D4Z4 has been shown to behave as a silencer that provides a binding site for a transcriptional repressing complex (Gabellini et al., 2002). These results suggest a model in which reduction of D4Z4 leads to the inappropriate transcriptional derepression of proximal chromosome 4-specific genes. Indeed, closely located genes such as FSHD Region Gene 2 (*FRG2*), FSHD Region Gene 1 (*FRG1*), and Adenine Nucleotide Translocator (*ANT1*), with high myopathic potential, were observed to be transcriptionally upregulated in FSHD muscle (Gabellini et al., 2002). However, studies testing this model obtained conflicting results with some showing support (Gabellini et al., 2002; Rijkers et al., 2004; Klooster et al., 2009; Bodega et al., 2009), and others not (Winokur et al., 2003b; Jang et al., 2003; Osborne et al., 2007; Masny et al., 2010). These discrepancies prevented a general consensus on the role of 4q35 genes upregulation in FSHD pathogenesis. Recently it has been proposed that FSHD arises from chromosomes bearing a short D4Z4 repeat array associated with a novel polyadenylation signal capable of stabilizing *DUX4* mRNA from the normally transcriptionally inactive DUX4 gene. However, there is reason to believe that this model does not account for all FSHD cases. For example, several studies described FSHD patients carrying full-length D4Z4 alleles that are clinically indistinguishable from FSHD subjects carrying D4Z4 reduced alleles. Conversely, there is a high percent of normal individuals, unrelated to any FSHD patients, with reduced D4Z4 alleles (Scionti et al., 2012b). Most importantly it has been recently showed that the polymorphism adding the polyadenylation signal to *DUX4* RNA, is common in the general population. Thus, just a portion of subjects carrying this molecular signature develops FSHD (Scionti et al., 2012b). Consequently, the number of D4Z4 repeats at 4q35 by itself or in association with a specific haplotype, does not to fully explain FSHD development. The non linear correlation between the number of D4Z4 elements and FSHD expression raises the question whether or when FSHD can be considered a simple autosomal dominant mendelian disorder, or if a more complex picture should be considered in clinical practice. Indeed, recent findings suggest that epigenetic modification at the disease locus, such as DNA methylation (de Greef et al., 2008), histone modifications (Zeng et al., 2009) or chromosomal architecture (Petrov et al., 2006) can be altered in FSHD patients, adding further levels of complexity. Undeniably, the incomplete knowledge of molecular mechanism(s) leading to FSHD onset has hampered the possibility of developing effective therapeutic strategies. This chapter aims to discuss the recent advances in our understanding of FSHD pathology viewed against its clinical heterogeneity, considering all the factors that can contribute to the complexity of this elusive disease.

2. FSHD Clinical features: Role of D4Z4 repeats reduction

2.1 Clinical features

Facioscapulohumeral muscular dystrophy is considered an autosomal dominant myopathy characterized by progressive atrophy and weakness of a highly selective set of muscle

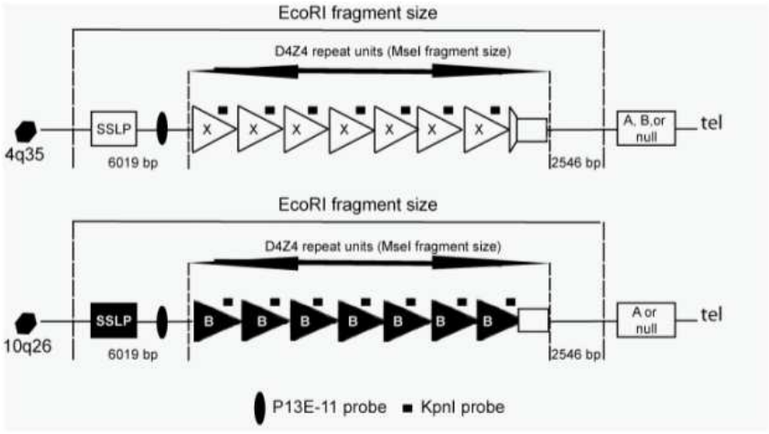
groups (Padberg, 1982). The onset of the disease is in the second/third decade of life and usually involves weakness of facial mimic muscles. The clinical presentation is characterized by an initially restricted distribution of weakness starting with asymptomatic facial weakness followed by scapular fixator, humeral, truncal, and lower extremity weakness. The onset of lower-extremity weakness is typically in the anterior leg compartment, presenting with footdrop. Extraocular and bulbar muscles are typically spared. Although facial weakness is perhaps the most recognizable aspect of FSHD, affected individuals from otherwise clinically typical families may have no or minimal facial weakness (Flanigan et al., 2004). Weak abdominal muscles result in a protuberant abdomen and contribute to the lumbar lordosis. Lower abdominal muscles are weaker than the upper abdominal muscles, causing a strikingly positive Beevor's sign, a physical finding fairly specific for FSHD. In advanced cases, hip girdle may be as affected or more than shoulder girdle muscles, making difficult the clinical distinction between FSHD and limb-girdle muscular dystrophy. A notable distinctive feature of FSHD is that muscle weakness displays an asymmetric distribution, which does not correlate with the handedness of the individual (Brouwer et al., 1992). The chronology of disease progression is unpredictable; for example, long periods of stability can be followed by sudden and dramatic worsening. In addition, there is a wide variability in the spectrum of disease among patients, ranging from subjects with very mild muscle weakness, who are almost unaware of being affected, to those who are wheelchair-dependent. This variability in disease penetrance was exemplified by a set of monozygotic male twins who carried the same genetic mutation but were affected by FSHD to a dramatically different extent (Tupler et al., 1998). Electromyography and histological analysis reveal non-specific myopathic changes associated, in some cases, with neurologic aspects. The creatine kinase (CK) level can be moderately increased or normal. Thus, diagnosis of FSHD is mainly based on the clinical phenotype in combination with a molecular test (Lunt et al 1995; Lunt, 1998). Ancillary features such as sensorineural deafness or retinal vasculopathy have been also reported in FSHD patients, but they are not to be considered decisive criteria for FSHD diagnosis (Fitzsimmons et al., 1987; Padberg et al., 1995; Trevisan et al., 2008).

2.2 Molecular diagnosis

The FSHD genetic defect does not reside in mutation of any protein-coding gene. Instead FSHD has been causally associated with reduction of the number of 3.3 kb DNA elements at the subtelomeric region of chromosome 4q. When digested with the restriction enzyme EcoRI the FSHD allele originates a fragment of 35 kb in size or shorter that can be detected by Southern analysis using the probe p13E-11 (Wijmenga et al., 1992b). The polymorphic genomic region, identified by probe p13E-11, consists of an array of tandem repeat 3.3-kb segments (hereafter referred to as D4Z4 repeats), such that the variation in the size of EcoRI fragments is due to variability in the number of D4Z4 repeats (van Deutekom et al., 1993) (Figure 1). Normal subjects carry p13E−11 EcoRI alleles longer than 50 kb (>10 D4Z4 units) originating from chromosome 4 whereas alleles of 35 kb or shorter (≤ 8 D4Z4 units) are present in the majority of either de novo or familial FSHD patients (Lunt, 1998). *De novo* reduced allele transmitted by an affected parent to the offspring co-segregate with the disorder (Griggs et al., 1993). New mutations account for a surprisingly high percentage of FSHD patients (10%−33%) (Padberg et al., 1995; Zatz et al., 1995). This high incidence can be partly explained by the presence of parental mosaicism for 4q short alleles that has been reported in 19% of *de novo* cases (Upadhyaya et al.; 1995, Köhler et al.; 1996, Lemmers et al.,

2004). The presence of somatic mosaicism for a rearrangement of D4Z4 was found in as much as 3% of the general population (van der Maarel et al., 2000), suggesting that the D4Z4 repeat is highly recombinogenic.

A complication of molecular testing by Southern analysis is represented by the presence of a polymorphic region recognized by probe p13E-11 at the subtelomeric region of chromosome 10q, which shares numerous homologies with the 4q subtelomere (Bakker et al., 1995; Deidda et al., 1996). The repeat element at 10q is 98% identical to D4Z4 at 4q, and the size of 10q EcoRI alleles varies between 11 and 300 kb (1-100 D4Z4 units). Moreover, 10% of these alleles are shorter than 35 kb (8 D4Z4 units) (Bakker et al., 1996; Bakker et al., 1995), overlapping the 4q alleles. Clearly these overlapping features can interfere with the molecular diagnosis of FSHD. Nevertheless the presence of a BlnI restriction site within the 3.3 kb element associated with chromosome 10q allows the discrimination between 4q and 10q alleles (Deidda et al., 1996). As a result, Southern blot hybridization of EcoRI and EcoRI/BlnI digested genomic DNA is used for the molecular diagnosis of FSHD (Lunt, 1998) (Figure 1).



$$\frac{[\text{EcoRI fragment lenght} - (6019 + 2546)]}{3.3\ \text{kb}} = \text{Number of D4Z4 repeats}$$

D4Z4 repeat units	EcoRI fragment approximate size
1U	12-13 kb
2U	14-16 kb
3U	17-20 kb
4U	21-23 kb
5U	24-26 kb
6U	27-30 kb
7U	31-33 kb
8U	34-36 kb
9U	37-39 kb
10U	40-43 kb

Fig. 1. **Schematic representation of Polymorphisms at the 4q and 10q subtelomeres**. Schematic representation of the method used to calculate D4Z4 repeat numbers from *EcoR*I-fragment sizes. Seven and eight D4Z4 repeats (31-36 kb *EcoR*I fragment size) were defined to be the upper diagnostic range for FSHD. D4Z4 repeat units on chromosomes 4 and 10 can be distinguished because all repeats on 10q contain *BlnI* restriction sites (**B**), while all D4Z4 repeats on 4q contain *XapI* restriction sites (**X**).

Through years, additional findings have emerged that need to be considered in the molecular diagnosis of FSHD. First, translocated 4-type repeats residing on chromosome 10q as well as translocated 10-type repeats residing on chromosome 4q are found in 10% of the population (van Deutekom et al., 1996b; van Overveld et al., 2000; Matsumura et al., 2002;). Therefore, FSHD-sized D4Z4 alleles may be attributed incorrectly to chromosome 10 and viceversa. Second, deletions at 4q encompassing the genomic sequence recognized by probe p13E-11 have been detected in FSHD cases. Thus D4Z4 short arrays might not be detected by using the standard diagnostic procedure. The frequency of such extended deletions has been estimated around 3% (Lemmers et al., 2003) and represents a possible caveat of FSHD molecular diagnosis. Third, 5-10% of subjects showing FSHD clinical features do not carry D4Z4 reduced alleles. Possible explanations for such anomalous cases include a different mechanism at 4q35, such as D4Z4 hypomethylation (De Greef et al, 2009) or the presence of other mutations not linked to the FSHD locus at 4q35. At present, no FSHD families linked to other chromosomal loci have been described. Figure 2 summarizes the diagnostic flow chart that should be used to study the 4q35 region in FSHD patients.

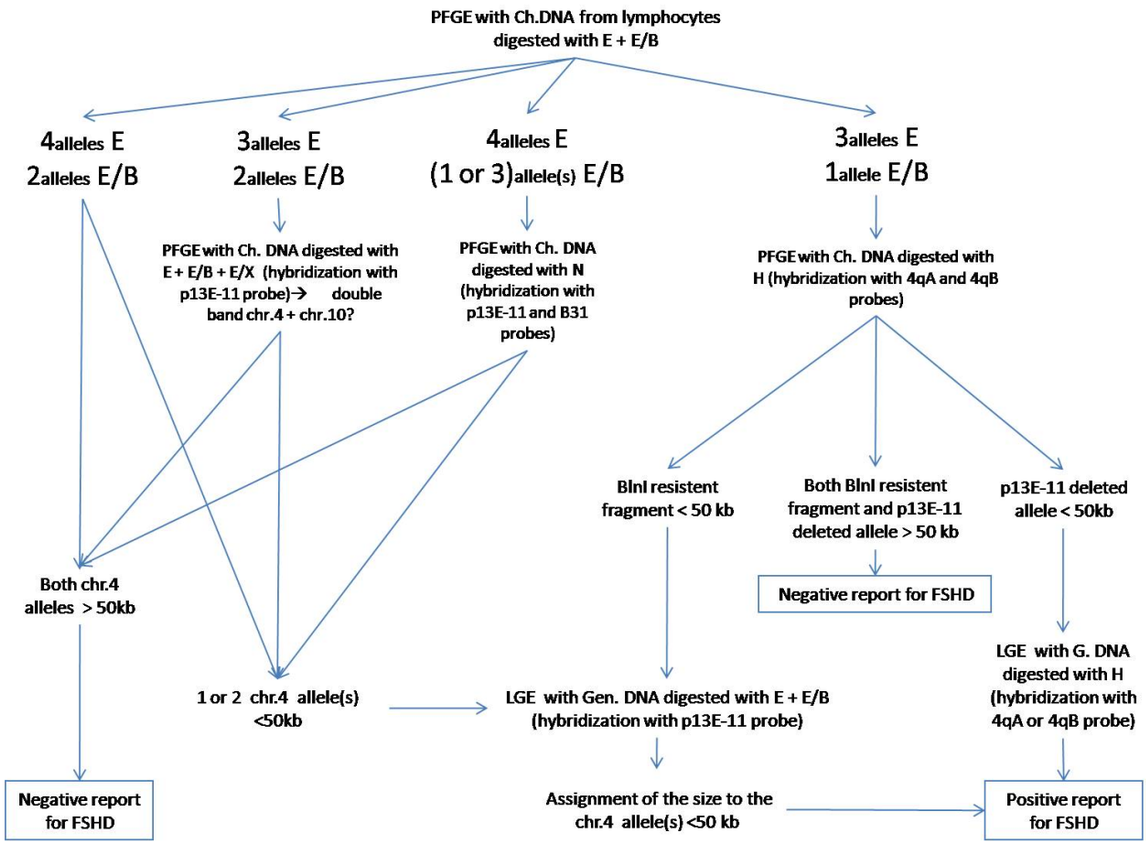


Fig. 2. **Schematic representation of FSHD diagnostic approach at 4q35 locus**.
Abbreviations: **Ch.DNA**: chromosomal DNA embedded in plugs; **Gen.DNA**: genomic DNA in solution; **PFGE**: Pulse Field Gel Electrophoresis; **LGE**: Linear Gel Electrophoresis; **E**: restriction enzyme EcoRI; **B**: restriction enzyme BlnI; **X**: restriction enzyme XapI; **N**: restriction enzyme NotI; **H**: restriction enzyme HindIII.

2.3 FSHD clinical ascertainment and molecular diagnosis: Necessity of standardized clinical examination

As said before, genomic studies conducted on groups of FSHD patients and families revealed the numerous difficulties that can be encountered in the molecular characterization of the 4q35 locus. Through years, the complexity of molecular diagnosis has been paralleled by the emerging complexity of FSHD clinical ascertainment. At present, criteria established in 1991, before the advent of molecular diagnosis (Padberg et al., 1991), and in 1998, following the ENMC workshop on FSHD (Lunt 1998), need probably to be reconsidered in light of the most recent observations.

2.3.1 Penetrance

Non-penetrance in FSHD was estimated to be less than 2% after the age of 50 years and more likely with allele sizes larger than 30 kb (Tawil et al., 1996). However, asymptomatic gene carriers seem to be more prominent in some families, and non-penetrance has even been found in carriers of 25 kb D4Z4 alleles (Ricci et al., 1999). In his work, Ricci et al. detected D4Z4 reduced alleles in several unaffected family members, named non-penetrant carriers, who are capable of transmitting the disease to their offspring. In addition reduced penetrance for D4Z4 reduced alleles was described in families in which patients heterozygous for FSHD alleles on both 4q chromosomes were present (Wohlgemuth et al., 2003; Tonini et al., 2004). Gender differences have been also described in FSHD, with males apparently more affected than females (Tonini et al., 2004). Nowadays correlation between penetrance of FSHD, length of the repeat array, age and sex is unsettled. Thus, the risk of developing the disease in correlation with D4Z4 allele sizes cannot be estimated and no prognostic tools are available. In addition several clinical reports describe patients displaying clinical and genetic features of FSHD associated with other documented muscle disorders including mitochondrial diseases (Chuenkongkaew et al., 2005; Filosto et al., 2008), glycogenosis (Nadaj-Pakleza et al., 2009), dystrophinopathies (Rudnik-Schoneborn et al., 2008). In all these cases the presence of the FSHD molecular defect seems to aggravate the clinical phenotype. Finally, phenotypic features of FSHD can be found in other myopathies (Oya et al., 2001; Saenz et al., 2005) as well as atypical phenotypes can be displayed by subjects carrying the FSHD molecular defect (Figueroa and Chapin, 2010; Tsuji et al., 2009; Zouvelou et al., 2009). All together these observations suggest that the variable penetrance observed in the FSHD population may be the result of the interaction of several factors. Indeed the presence of low-penetrant alleles suggests that susceptibility for FSHD is not only determined by the intrinsic properties of the diseased allele but also by additional factors that can be genetic, epigenetic and/or environmental factors. Identification of factors influencing FSHD clinical outcome remains one of the major challenges of FSHD research.

2.3.2 Severity of the disease and repeats number: Does a linear correlation exist?

An inverse relationship has been established between the D4Z4 repeat size and the severity and progression of the disease (Lunt et al., 1995; Ricci et al., 1999; Tawil et al., 1996; Zatz et al., 1998). In general, individuals with ≥11 repeats are healthy; in contrast, 1-3 D4Z4 repeats is associated with a severe form of disease that presents in childhood, 4-7 repeats with the

most common form of FSHD, and 8-10 repeats with a milder disease and reduced penetrance. Nevertheless great variability of clinical expression has been described among FSHD patients even within the same family. Interestingly it has been suggested that patients harboring D4Z4 alleles of ≥35 kb (≥ 8 repeats) were less likely to present the classic FSHD phenotype as compared with patients with alleles of <35 kb (<8 repeats) (Felice and Whitaker, 2005). Several clinical reports described myopathic patients, carrying alleles of 38 kb (9 repeats) or larger, showing typical and atypical FSHD phenotypes (Vitelli et al., 1999; Felice et al., 2000; Felice and Moore, 2001; Butz et al., 2003; Krasnianski et al., 2003). However D4Z4 repeat arrays of size between 38-45 kb (9-11 repeats) were encountered in 3% of 200 control subjects in a Dutch study (van Overveld et al., 2000). These findings seems to indicate that in a substantial proportion of 38 to 45 kb-sized repeat arrays penetrance may be close to zero, but in some families 38−45 kb alleles are associated with myopathy (Butz et al., 2003). Remarkably D4Z4 repeat array of size between 21-34 kb (4-8 repeats) were found in 3% of 801 Italian and Brazilian samples of normal individuals unrelated to any FSHD patients, indicating that in this size-range, additional factors influence the disease expression (Scionti et al., 2012b). In conclusion the high variability in clinical expression makes difficult to establish a prognostic correlation between the number of the D4Z4 repeats and the severity of the disease. There is the necessity of clinical and molecular studies on large cohorts of FSHD patients and families to obtain significant information on FSHD development and generate useful prognostic information.

2.3.3 A standardized clinical evaluation tool: FSHD score

Based on the need of gaining statistically significant observations through large cohorts studies, a standardized clinical evaluation tool for patients with FSHD was created. The clinical protocol examines muscle groups specifically affected in FSHD. The test uses functional criteria, which allow expression of clinical severity in quantitative terms (Lamperti et al., 2010). The clinical examination results in an evaluation scale, which is divided into six independent sections that assess the strength and the functionality of (I) facial muscles (scored from 0 to 2); (II) scapular girdle muscles (scored from 0 to 3); (III) upper limb muscles (scored from 0 to 2); (IV) distal leg muscles (scored from 0 to 2); (V) pelvic girdle muscles (scored from 0 to 5); and (VI) abdominal muscles (scored from 0 to 1). The evaluation scale allows the functional quantification of muscle weakness in FSHD patients. This examination protocol, which is associated with a questionnaire that collects information on the clinical history of the subject, generates a disability score resulting from the sum of six independent scores of separately evaluated muscle regions, including the facial and abdominal muscles, which are specifically affected by FSHD. The total score can range from 0, when no signs of muscle weakness are present, to 15, when all muscle groups tested are severely impaired. The protocol represents a robust evaluation procedure for FSHD patients that can be performed easily in the medical office and is not influenced by the tester. The robustness of the clinical evaluation protocol provides a tool that can therefore be used by different neurologists in large cooperative clinical studies and allows translating what is called clinical impression of the progressive involvement of specific muscle groups into a number (Lamperti et al., 2010) (The FSHD clinical form and the FSHD evaluation scale form, as well as a visual guide to clinical assessment, are available online at www.fshd.it).

Use of the FSHD score can support studies for defining the natural history of the disease throughout time. Importantly, definition of the clinical involvement of specific muscle groups by a number permits identification and characterization of atypical cases and support the definition of clinical subcategories among FSHD patients.

By assessing the correlation between clinical severity, results of molecular analysis, and anamnestic records, the FSHD score can provide useful information for defining FSHD nosology.

3. Genomic characteristic of the 4q35 region: D4Z4 and role of specific polymorphisms

Since the discovery of the FSHD molecular defect (Wijmenga et al 1992b), many studies suggested the possibility that reduction of D4Z4 repeat units on chromosome 4 alone is not sufficient to FSHD development (Weiffenbach et al 1993; van Overveld et al., 2000; Lemmers et al., 2002, Lemmers et al., 2007). Thus, a detailed genomic characterization of the 4q35 region led to the identification of polymorphic regions flanking the D4Z4 repeat array which could contribute to FSHD onset.

3.1 Genetic variability and haplotypes

D4Z4 is part of a family of 3.3-kb repeats (D4Z4) are dispersed throughout the human genome and are generally found associated with regions of heterochromatin (Lyle et al.,1995). A homologue D4Z4 tandem array is present at the 10q telomere. This homology between the subtelomeric region of 10q and 4q is not confined to D4Z4 repeats but extends proximal 42 kb and distally to include the telomere (van Geel et al., 2002). However, despite the high level of sequence similarity (> 98% nucleotide identity) between the 10q and 4q subtelomeres, FSHD is associated only with the chromosome 4q (Bakker et al., 1995; Deidda et al., 1996). In the attempt of explaining the unique association between D4Z4 reduction of chromosome 4q and FSHD, a bi-allelic polymorphism was identified distal to the repeat array (van Geel et al., 2002). Two distinct polymorphic regions, named 4qA and 4qB, were observed at the distal end of chromosome 4q. Within 4qA there is a polymorphic 8-kb region of 68bp satellite DNA immediately distal to the D4Z4, and adjacent to this is a 1-kb divergent $(TTAGGG)_n$ array. None of these repeats is present within the 4qB sequence. In 4qB polymorphism, the last 3.3-kb repeat contains only the first 570 bp of a complete unit, whereas in 4qA the terminal repeat is a divergent 3.3-kb repeat named pLAM (van Deutekom et al., 1993). Sequence alignment and subsequent phylogenetic analysis of subtelomeric region of 10q showed a close relationship between 4qA sequences and 10q suggesting that the 4q subtelomere has been transferred onto chromosome 10q (van Geel et al., 2002). The distribution of these allelic variants is heterogeneous and depends on the studied population. Lemmers and colleagues (2004), analyzing 80 Dutch control individuals, have observed an almost equal frequencies of 4qA and 4qB alleles (42% and 58%, respectively), whereas in another study, conducted on 66 Italian control individuals, an overrepresentation of 4qA telomeres has been reported (68% for 4qA and 32% for 4qB) (Rossi et al., 2007). Proximal to the D4Z4 repeat array a simple sequence-length polymorphism (SSLP) has been described and those sequences are in a range between 157 bp and 180 bp (Figure 3).

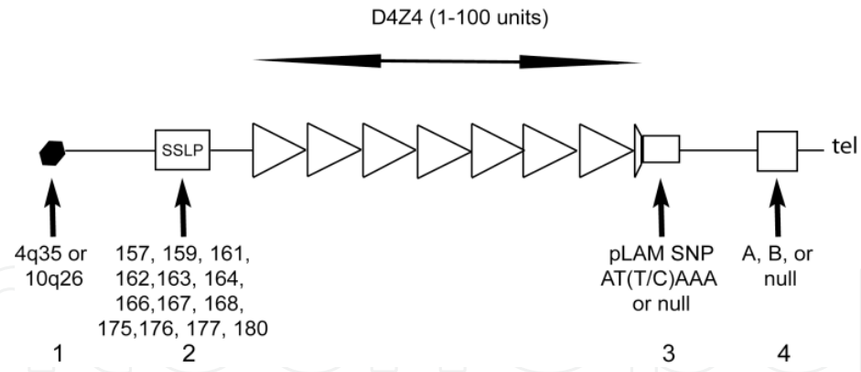


Fig. 3. The D4Z4 repeat array within the subtelomere of chromosomes 4q and 10q varies in size between 1 and 100 D4Z4 units (3.3–330 kb) and it is indicated with triangles. Elements that distinguish subjects include: 1. The chromosomal localization of the D4Z4 repeat, chromosome 4q35 or 10q26. 2. The Simple Sequence Length Polymorphism (SSLP). It is a combination of five Variable Number Tandem Repeats, an 8 bp insertion/deletion, and two SNPs localized 3.5 kb proximal to D4Z4 and vary in length between 157 and 182 bp. 3. Single nucleotide polymorphism AT(T/C)AAA (SNP) in the pLAM region. 4. A large sequence variation (termed 4qA or B) that is distal to D4Z4. In the 4qB variant the terminal 3.3-kb repeat contains only 570 bp of a complete repeat, whereas in the 4qA variant the terminal repeat is a divergent 3.3-kb repeat named pLAM. 4q chromosomes which do not hybridize to probes for A and B are termed "null" and their sequences vary from case to case.

A worldwide population (including African, European and Asian HAPMAP panels) analysis of 4q subtelomeric polymorphisms flanking the D4Z4 array revealed 17 distinct haplotypes on chromosome 4q (Lemmers et al., 2010b). On the basis of sequence similarities, all haplotypes were categorized in two groups: the major group 1 consists mainly of the haplotypes 4A159, 4A161 and 4B163, which are the most common in all three HAPMAP populations. The major group 2 contains other standard and nonstandard 4q haplotypes (4A166 and 4A168). Evolutionary studies showed that haplotypes 4A159 and 4A161 represent the oldest human D4Z4 haplotypes. Similarly, the 4A168 haplotype is most probably the oldest haplotype that belongs to major group 2. It has been hypothesized that all other haplotypes originate from only four discrete sequence-transfer events during human evolution (Lemmers et al., 2010b).

3.2 Permissive and non-permissive genetic background

Analysis of 4qter polymorphisms of 80 unrelated Dutch patients with FSHD revealed that D4Z4-reduced alleles are associated with the 4qA variant (Lemmers et al., 2002). Subsequently, by studying three families in which two D4Z4-reduced alleles segregate, it has been proposed that 4qB chromosomes carrying short D4Z4 repeats do not cause FSHD, since only subjects carrying D4Z4-reduced alleles associated with the 4qA polymorphism had FSHD (Lemmers et al., 2004). The almost exclusive association between FSHD disease-expression and the D4Z4-reduced allele of the 4qA type has been confirmed in a large cohort of 164 unrelated patients with FSHD from Turkey and the UK. Even though that study described FSHD patients lacking the 4qA/4qB end (Thomas et al., 2007). Subsequent studies led to the hypothesis that D4Z4 contraction on 4qA chromosome *per se* is not sufficient to cause disease. Analysis of SSLP proximal to the repeat array in 86 FSHD patients

and 222 healthy controls revealed a unique association of FSHD with the 161 allele and the 4qA sequence. In particular the haplotype 4A166 associated with D4Z4-reduced alleles was detected in multiple unaffected relatives of two independent families and the 4B163 haplotype was associated with 17 FSHD-sized alleles carried in healthy subjects (Lemmers et al., 2007). On this basis it has been hypothesized that FSHD can develop only in a specific "permissive" chromosomal background represented by the haplotype 4A161. Following this hypothesis, proximal and distal sequences of 4A161 chromosome were compared to those of "non-permissive" ones, such as 4B163 and 10A166. This approach led to the identification of a single nucleotide polymorphism (SNP, AT(T/C)AAA) in the adjacent pLAM sequence, immediately distal to D4Z4 array. In particular 4A161 and two other uncommon permissive variants, 4A159 and 4A168 presented the ATTAAA variant, which has been interpreted as a polyadenylation signal able to stabilize the *DUX4* transcript (Figure 4a).

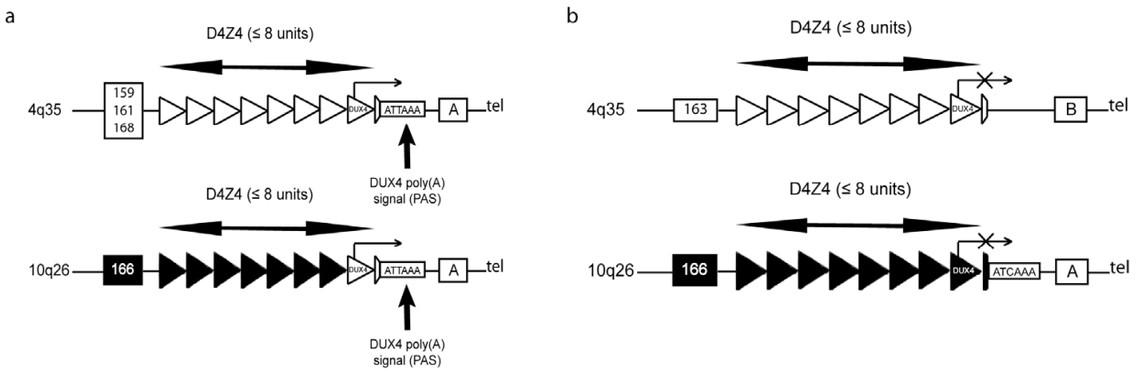


Fig. 4. **Schematic representation of the current view of permissive and not-permissive haplotype. a**. Permissive haplotypes **b.** Non-permissive haplotype. The ATTAAA variant creates a polyadenylation signal (PAS) that stabilizes the *DUX4* transcript and has been postulated to be the critical factor causing FSHD.

By contrast sequences associated with non-permissive chromosome 10A166 and 4B did not allow the expression of DUX4 (Lemmers et al., 2010a). Analysis of more than 300 unrelated FSHD patients and 5 families with one or more FSHD patients carrying D4Z4-reduced allele strongly supported the hypothesis that the last 4qA D4Z4 unit with the directly adjacent pLAM sequence including the ATTAAA is necessary to the FSHD development (Lemmers et al., 2010a). On this basis it has been proposed that FSHD arises through a toxic gain of function attributable to the stabilized distal *DUX4* transcript (Lemmers et al., 2010a) (Figure 4b). Despite the intriguing premise, the notion that FSHD is a fully-penetrant autosomal dominant disorder caused by the reduction of D4Z4 repeat number associated with 4A161PAS haplotype is challenged by recently published data. First a study conducted on 750 unrelated FSHD families from Italy revealed that the frequency of individuals carrying two D4Z4 reduced alleles (compound heterozygotes) is 2,7%, a frequency much higher than expected for a fully penetrant autosomal dominant disorder with prevalence of 1 in 20,000. Interestingly in these families with compound heterozygosity, 25% of relatives carrying D4Z4-reduced alleles and 4A161PAS are healthy (Scionti et al., 2012a). Second, characterization of 253 unrelated FSHD probands from the Italian National Registry for FSHD showed that only 127 of them (50.1%) carry D4Z4 alleles with 1-8 D4Z4 associated with 4A161PAS, whereas the remaining FSHD probands carry different haplotypes or alleles with greater number of D4Z4 repeats (Scionti et al., 2012b). Third, molecular analysis of 801 normal

healthy subjects from Italy and Brazil showed that that 3% of individuals from the general population carry alleles with reduced number (4-8) of D4Z4 repeats on chromosome 4q and one third of these alleles occurs in combination with the 4A161PAS haplotype (Scionti et al., 2012b) All these findings challenge the hypothesis that 4APAS structure is necessary and sufficient for the development of FSHD. This discovery is not incompatible with evidence implicating DUX4 or other factors as important mediators of disease. Nonetheless, it does demonstrate that FSHD pathogenesis is more complex than currently thought.

4. One disease (too) many theories

After the genetic correlation between D4Z4 and FSHD the most difficult task has been to explain the role of D4Z4 in disease development. D4Z4 can directly cause FSHD through DUX4 expression; on the other end D4Z4 reduction might indirectly cause FSHD by exerting long distance effects. None of the proposed models entirely explain the mechanism leading to disease. In this regard the scientific community does not express undisputed consensus.

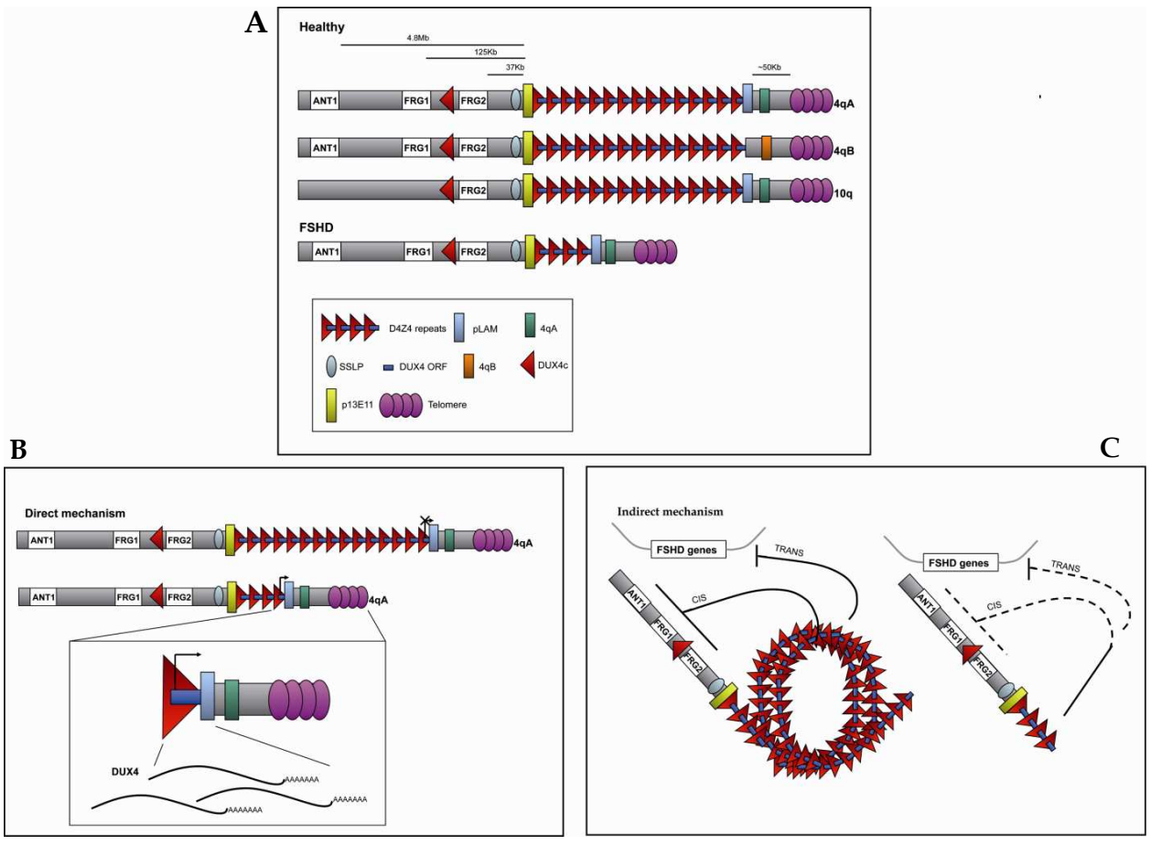


Fig. 5. **Models for the molecular basis of FSHD. A**. Healthy individuals carry 11–150 units of D4Z4, whereas FSHD patients have less than 11 repeats. **B. DIRECT MECHANISM**: reduction of D4Z4 repeat array leads to the synthesis of DUX4 transcript, which is normally not transcribed, through changes in D4Z4 heterochromatin and/or stabilization of DUX4 mRNA. **C. INDIRECT MECHANISM:** the reduction of D4Z4 repeats leads to modifications of the spatial and structural organization of chromatin generating changes of transcriptional control over the expression of candidate genes localized in *cis* or in *trans*.

In examining all the models that have been proposed it is important to remember essential points:

- 80-85% of FSHD patients carry a reduction in D4Z4 whereas loss of the whole array is not associated with FSHD;
- 15-20% of FSHD patients have a normal number of repeats;
- No specific 4q haplotype is associated with FSHD;
- 25% of relatives carrying D4Z4 alleles who are old than 56 years do not have FSHD;
- Healthy individuals bearing allele with reduced number of repeats (4-8 units) are present in 3% of the healthy population;
- Repeat reduction in the highly homologous D4Z4 copy on chromosome 10 is not associated with the FSHD;
- Penetrance of the FSHD is not complete and its severity does not clearly correlate with number of repeats;

Notably in all proposed models, epigenetic changes such as methylation or histone modifications are used as an additional level of complexity that might help interpreting the complex correlation between genotype and phenotype in FSHD. In this paragraph we will shortly describe the main mechanisms that have been proposed. In the following paragraphs all the factors that have been considered important for the disease onset will be explained in detail.

4.1 Direct mechanism: DUX4

The most recent model proposed to explain FSHD pathogenesis is based on the idea that the most distal copy of the *DUX4* gene, whose open reading frame is present in each D4Z4 repeat, is transcribed and the expression of this gene has a direct role in FSHD pathophysiology. At first it has been proposed that partial reduction of the D4Z4 repeat array results in destabilization of the D4Z4 heterochromatin and in the inappropriate upregulation of *DUX4* gene (Gabriels et al., 1999; Hewitt et al., 1994). However this hypothesis has never been proven. This model has been consequently modified introducing the concept of a "permissive" chromosomal background namely a single nucleotide polymorphism in the pLAM sequence that provides a polyadenylation signal (PAS) for the *DUX4* transcript. This should stabilize the *DUX4* transcript from the most distal D4Z4 unit on 4q chromosomes resulting in disease through a toxic gain-of-function mechanism (Lemmers et al., 2010b) (Figure 5A).

4.2 Indirect mechanism: Iindirect overexpression of candidate genes

All the other models proposed to explain the role of D4Z4-reduced alleles in FSHD pathogenesis, predict that D4Z4 reduction is able to generate a modification in the spatial and structural organization of chromatin at 4q35. As a consequence loss of transcriptional control over the expression of candidate genes, localized in *cis* or in *trans,* is generated (Figure 5B).

***Cis*-spreading model: 4q35 genes derepression**

D4Z4 contains heterochromatic DNA elements. It was thus reasoned that D4Z4 and surrounding sequences would be strongly packed as heterochromatin. Based on this idea, it

was hypothesized that loss of D4Z4 repeat would produce a local chromatin relaxation (i.e., loss of heterochromatinization) and, consequently, the transcriptional upregulation of genes nearby D4Z4, possibly in a distance-related manner (Hewitt et al., 1994; Winokur et al., 1994).

The identification of a repressor complex that binds to a specific 27 bp DNA element within D4Z4 (Gabellini et al., 2002) supports the *cis*-spreading model. Consistently, three 4qter genes (*FRG2, FRG1,* and *ANT1)* were found upregulated in muscle of FSHD patients (Gabellini et al., 2002).

Cis-looping model: 4q35 genes derepression

According to this model, D4Z4 is able to interact with target gene(s) by long-distance loops only when the D4Z4 contraction impairs the formation of normal D4Z4 intra-array loops. The hypothesis that normal-sized D4Z4 repeats form intra-array loops is supported by the size distribution of D4Z4 repeats which is multimodal, with equidistant peaks 65 kb apart (van Overveld et al., 2000).

Insulator model

D4Z4 is localized between the distal heterochromatic telomeric sequences and the euchromatic sequences more upstream. It has thus been proposed that it might act as an insulator (van Deutekom, 1996a). Reduction of the repeat arrays would impair the separation between domains, and, consequently, the spreading of heterochromatic would silence proximal genes in *cis*. This model is supported by the finding that D4Z4 itself acts as an insulator, which interferes with enhancer–promoter communication and protects from position effect (Ottaviani et al., 2009). Results obtained with different experimental approaches demonstrated that both, the transcriptional factor CTCF (CCCTC-Binding factor), and the A-type intermediate filament Lamins binding, are necessary for D4Z4 insulator function. In this model, FSHD contracted *D4Z4* array associates with CTCF and A-type Lamins at the nuclear periphery resulting in both *cis* and *trans* insulation of gene(s) physiologically interacting with the 4q35 terminal sequences (Ottaviani et al., 2010). This may lead to the miss regulation of these genes and to the FSHD phenotype.

***Cis* model: Nuclear localization**

The FSHD genomic region at 4q35.2 is consistently and specifically localized at the nuclear envelope (Petrov et al., 2006; Ottaviani et al., 2009) in proliferating myoblasts, fibroblasts, lymphoblasts, and differentiated myotubes. Interestingly it is not the D4Z4 repeat itself that mediates interaction with the nuclear envelope but a chromosome 4 genomic regions just proximal to the D4Z4 repeat (D4S139) (Masny et al., 2004; Ottaviani et al., 2009). Since FSHD region is localized to the nuclear periphery, an alternative model for FSHD pathogenesis has been proposed. In this model improper interaction with transcription factors or chromatin modifiers at the nuclear envelope could induce aberrant expression of genes localized in *cis* or in *trans.* However a differential localization of normal or FSHD alleles to the nuclear periphery has not been observed (Masny et al., 2004).

Trans-effect model: Genome wide effect

It has been also postulated that reduction of D4Z4 might have a more genome-wide effects, affecting other pathways, such the slow-to-fast fiber differentiation pathway (Celegato et al., 2006) and the response to oxidative stress and myogenic differentiation pathway (Winokur

et al., 2003b). Because D4Z4 can be regarded as a docking platform for protein factors, loss of repeats may generate a local imbalance in the availability of D4Z4 proteins in the cell, and/or lead to new interaction with different proteins at the disease allele.

5. Direct role of D4Z4: The double homeobox gene 4 (DUX4)

The D4Z4 unit has been completely sequenced (Hewitt et al., 1994; Winokur et al., 1994 Lee et al., 1995). Each D4Z4 repeat unit contains an ORF with a double homeobox putatively encoding the DUX4 protein. *DUX4* belongs to a family of highly homologous genes scattered throughout the genome. One almost identical copy, named *DUX4c*, is located 42 kb proximal to the repeat array. Based on the presence of the molecular signature 4A-PAS, which should allow the stabilization of the mRNA from the distal copy of the *DUX4* gene; it has been proposed that FSHD arises through a toxic gain-of-function mechanism attributable to the pathological expression of *DUX4* mRNA (Lemmers et al., 2010a). More detailed analysis of *DUX4* expression shows that the *DUX4* pre-mRNA can be alternatively spliced and apparently, the FSHD muscle expresses a different splice form of *DUX4* mRNA compared to control muscle (Snider et al., 2010). It is important to note that *DUX4* is a rare transcript and the amount of *DUX4* has been estimated in one copy per cell. To explain how such low expression level of *DUX4* can cause FSHD, it has been proposed that in FSHD muscle the DUX4 protein may exert its toxic effect in a small subset of nuclei, which express a relatively abundant amount of *DUX4* transcript. The possible toxic effect of DUX4 has been inferred on the basis of in vitro and in vivo studies (Kowaljow et al., 2007; Bosnakovski et al., 2008; Wallace et al., 2011) in which *DUX4* was expressed at very high levels. Thus in order to explain FSHD pathogenesis, it is difficult to reconcile those experimental observations with the very limited amount of *DUX4* detected in human muscle cells.

6. 4q35 genes in FSHD phatogenesis: Role of genes overexpression

Although the exact molecular mechanism responsible for FSHD is unknown, it is a common agreement that reduction of D4Z4 elements might cause up-regulation of gene(s) in *cis*. A few genes have been considered as good candidates for FSHD based on their localization and/or function.

This section will critically discuss function and potential role of 4q35 candidate genes in FSHD development.

6.1 FSHD region gene 2 (FRG2)

FSHD Region Gene 2 (*FRG2*) was originally identified by *in silico* gene prediction (Van Geel et al., 1999) as a region of 3 kb 37 kb proximal to the D4Z4 repeat array. Predicted exons are preceded by a putative muscle specific promoter. *FRG2* gene is composed of four exons and encodes an mRNA of 2084 bp with two alternative polyadenylation sites. The *FRG2* ORF encodes a putative protein of 278 amino acids. The FRG2 protein does not show significant homology to known proteins (Rijkers et al., 2004). Alternative splicing creates an additional alanine codon (Rijkers et al., 2004). Even though *FRG2* has been shown to be nuclear, (Rijkers et al., 2004) its sequence contains not only two potential nuclear localization signals (NLS) but also a peroxisomal targeting signal (PTS1) at the carboxyterminal end of the protein (Swinkels et al., 1992). Copies of *FRG2* are dispersed throughout the genome,

prevalently located in subtelomeric or pericentromeric regions (Winokur et al., 1994; van Geel et al., 1999; van Geel et al., 2002). However, only the *FRG2* copies on chromosomes 4 and 10 show a 98% identity, differing for just five nucleotide mismatches in the ORF (Rijkers et al., 2004). Experiments demonstrated that the *FRG2* promoter is sensitive to the presence of D4Z4 repeat units making *FRG2* an interesting candidate gene for FSHD pathophysiology (Rijkers et al., 2004). Indeed it has been shown that overexpression of *FRG2* is obtained by suppressing the activity of the D4Z4 recognition complex (DRC) (Gabellini et al., 2002). Moreover data suggests that in muscle biopsies from FSHD patients, *FRG2* overexpression inversely correlates with D4Z4 repeat number (Gabellini et al., 2002). However the overexpression of *FRG2* in FSHD is still controversial. If there is a general agreement that mRNA is virtually absent in most of human tissue, there is no consensus regarding the expression of *FRG2* in FSHD patients' samples. *FRG2* overexpression was reported in differentiating, but not proliferating myoblasts of FSHD patients (Rijkers et al., 2004). The overexpression of *FRG2* in FSHD myotubes has not been fully confirmed in other works (Arashiro et al., 2009; Cheli et al., 2011; Masny et al., 2010; Osborne et al., 2007). The different outcomes of expression studies may be explained by the intrinsic difficulties in detecting *FRG2* mRNA due to its low expression level and by the presence in the genome of multiple copies of *FRG2.* Moreover *FRG2* is not represented in the gene arrays currently used for RNA expression studies. Whether *FRG2* is involved in FSHD pathogenesis still remains in discussion. Indeed muscle-specific overexpression of *FRG2* in mice does not result in an aberrant phenotype (Figure 6A) (Gabellini et al., 2006), and FSHD patient with proximal deletion encompassing FRG2 have been found (Lemmers et al., 2003). Nevertheless it is worth mentioning that *FRG2* appears late in the evolution together with D4Z4 repeats and it is not present in the mice genome making the mice model for *FRG2* overexpression not conclusive. The function of this protein is still unknown.

6.2 FSHD region gene 1 (*FRG1*)

In the human genome *FRG1* gene is located 125 kb centromeric to D4Z4 array on chromosome 4. As for many other genes from the 4q subtelomeric region, several copies of FRG1 are present in the human genome (van Deutekom et al., 1996c). The *FRG1* copy on chromosome 4 encodes a 258-amino acid protein. Although the FRG1 protein does not share significant overall homology to any known protein, it contains two nuclear localization signals in the N-terminal region (NLSs, aa 22-25 and 29-32), a bipartite NLS in the C-terminal region (aa 253-261) and a single fascin-like domain (aa 58-176), indicative of an actin-binding protein (Figure 6B), one potential RNA-binding domain (22-35 aa) homologous to several RNA-binding proteins (RBPs). FRG1 protein is highly conserved among invertebrates and vertebrates: human FRG1 shares 42% identity with *C. elegans*, 81% identity with *Xenopus* and 97% identity with mouse protein (Figure 6B). The high level of conservation throughout species suggests that FRG1 might have a very important function that is preserved during the evolution.

Since its discovery, FSHD Region Gene 1 (*FRG1*) has been considered a candidate gene for FSHD (Van Deutekom et al., 1996c). Analysis of its expression level in muscle tissues obtained from FSHD patients and healthy subjects showed that *FRG1* was abnormally up-regulated in FSHD affected muscles. Significantly, in lymphocytes from FSHD patients, its expression was equivalent to that observed in normal tissue, indicating that this over-expression in FSHD is muscle-specific (Gabellini et al., 2002). Consistent with this evidence,

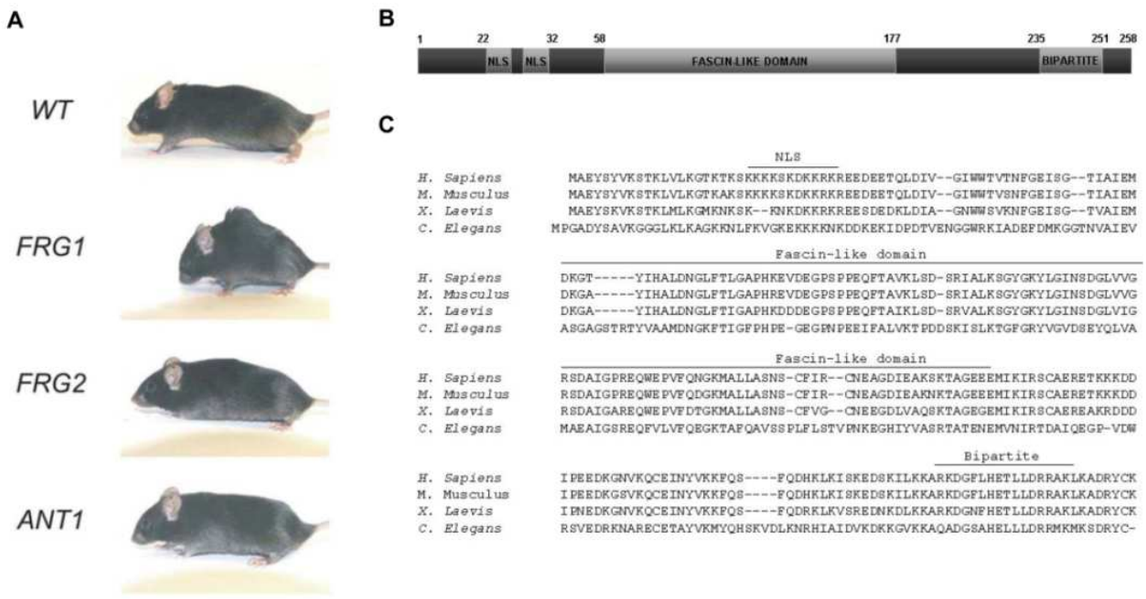


Fig. 6. **FSHD Region Gene 1 (FRG1). A.** WT and FRG1, FRG2 and ANT1 transgenic mice**.** Only FRG1 transgenic mice develop a muscular dystrophy with features of the human disease. **B**. FRG1 protein domains **C.** Alignament of FRG1 homologs: human FRG1 shares 42% identity with *C. elegans*, 81% identity with *Xenopus* and 97% identity with mouse.

transgenic mice over-expressing *FRG1* develop a muscular dystrophy with features of the human disease (Figure 6A). Importantly the myopathic features of these mice are corrected by the use of RNA interference to target and reduce FRG1 level in the affected muscles. Interestingly, the same result was obtained by two groups using two different experimental approaches (Wallace et al., 2011; Bortolanza et al., 2011). Furthermore, in muscles of *FRG1* transgenic mice and FSHD patients, specific pre-mRNAs undergo aberrant alternative splicing. Collectively, these results suggest that FSHD might results from inappropriate over-expression of *FRG1* in skeletal muscle, which leads to abnormal alternative splicing of specific pre-mRNAs (Gabellini et al., 2006).

Recent studies show the crucial role of FRG1 in maintaining proper muscle structure and function (Hanel et al., 2011; Hanel et al., 2009; Liu et al., 2010). In *C. elegans*, frg1 protein localized both in nuclei and in the dense bodies that are homologous to vertebrate Z-disk. Interestingly *frg1* overexpression in this invertebrate model disrupts the body-wall musculature and the muscular organization (Liu et al., 2010). In *Xenopus* both knock down and overexpression of *frg1* resulted in defective growth and morphogenesis of the myotome indicating that precise levels of *frg1* must be maintained for normal muscle morphology (Hanel et al., 2009). Together these results strongly suggest an evolutionary conserved function of *FRG1* in muscular development. Additional evidences support the role of *FRG1* in muscle cell biology. *FRG1* expression increases during myogenic differentiation. Its activation is paralleled by chromatin remodeling at the *FRG1* promoter with loss of the polycomb repressor complex and replacement of the H3K27 trimethylation (H3K27me3) repression marker with the H3K4 trimethylation (H3K4me3) activation marker (Bodega et al., 2009). Interestingly the physical interaction between *FRG1* promoter and D4Z4 array has

been shown (Petrov et al., 2008). In this context replacement of H3K27me3 by H3K4me3 during myoblasts differentiation might indicate that chromatin structure undergoes dynamic changes during myogenic differentiation that lead to the loosening of the FRG1/4q-D4Z4 array loop in myotubes. Consistently, *FRG1* over-expression was detected in the early stages of differentiation in FSHD myoblasts in comparison with control myoblasts (Bodega et al., 2009).

FRG1 molecular function has not been elucidated yet. Several observations suggest that it could be involved in RNA processing. FRG1 is a nuclear protein that localizes in Cajal bodies, in nucleoli and in nuclear speckles, sites where RNA processing takes place (van Koningsbruggen et al., 2004). Its interaction with RNA has been demonstrated *in vitro* and *in vivo* (Sun et al., 2011). Proteomic studies found FRG1 as a component of purified spliceosomes (Rappsilber et al., 2002; Bessonov et al., 2010). Moreover in muscle of *FRG1* over-expressing transgenic mice, specific pre-mRNAs undergo aberrant alternative splicing (Gabellini et al., 2006). Studies showed that FRG1 has nuclear and cytoplasmic localizations. Interestingly in human muscle sections, FRG1 localizes with Z-disc (Hanel et al., 2011) an element of muscle sarcomere. In a muscle cells ribosomes are available at sarcomere for local synthesis of contractile proteins providing a mechanism to quickly respond to changes in the extra-cellular environment. It would be interesting to test wheter FRG1 is involved in Z line targeting and/or translation of specific m-RNAs. Despite the interest in FRG1 as candidate for FSHD pathogenesis has diminished because expression studies failed to detect *FRG1* consistently overexpressed in FSHD biopsies (Gabellini et al., 2002; Jiang et al., 2003; Winokur et al., 2003b; Dixit et al., 2007; Osborne et al., 2007; Arashiro et al., 2009), experimental evidences point at the critical role of *FRG1* in muscle development and indicate the presence of negative regulatory mechanisms on its expression, which is released in a myogenic-specific manner. On this basis *FRG1* remains a very suitable candidate gene for FSHD pathophysiology.

6.3 Adenine Nucleotide Translocator (ANT1)

The Adenine Nucleotide Translocator gene (*ANT1*) is located approximately 4 Mb centromeric to the tandem array and encodes a 298-amino acid protein. This protein is a member of integral membrane transport molecules family that are among the most abundant constituents of the inner mitochondria membrane (IMM), responsible for the transport of adenine nucleotides across the inner mitochondrial membrane, importing ADP for oxidative phosphorylation and exporting ATP to the cytosol (Klingenberg and Aquila, 1982). There are three different ANT genes in humans, *ANT1*, *ANT2*, and *ANT3*. These genes share 88% amino acid sequence identity and are characterized by a distinct tissue specific expression patterns (Levy et al., 2000; Stepien et al., 1992). *ANT1* is the predominant isoform expressed in the mitochondria of heart and skeletal muscle tissue. In addition to regulating adenine nucleotide pools, ANT1 functions as a component of the mitochondrial permeability transition pore (PTP), which is essential for the release of pro-apoptotic proteins during the activation of the intrinsic-apoptosis pathway (Bauer et al., 1999; Sharer et al., 2002). *ANT1* overexpression seems critical in inducing programmed cell death in different eukaryotic cell lines (Bauer et al., 1999). Although mice overexpressing *ANT1* do not show evident dystrophic phenotype (Figure 6A) (Gabellini et al., 2006), an increased amount of ANT1 protein was detected in both unaffected and affected FSHD muscles in

comparison to healthy controls (Laoudj-Chenivesse et al., 2005). Even though both increase of oxidative stress and *ANT1* overexpression are proposed to be early events in the development of FSHD, it remains unclear if these are sequential or parallel processes (Winokur et al., 2003a).

7. Epigenetic and FSHD: Role of methylation and chromatine structure

Several clinical features, such as penetrance variability, gender bias in severity, asymmetric muscle wasting, and discordance in monozygotic twins, suggest that FSHD development involves epigenetic factors that can influence gene expression through local modification of chromatin structure.

7.1 DNA methylation

C5 methylation of cytosine, the most common epigenetic modification of mammalian DNA is known to be involved in development, X-chromosome inactivation, imprinting, and gene silencing (Robertson and Wolffe, 2000). CpG methylation can affect occupancy of specific genomic regions since several transcription factors and chromatin-binding proteins, such as CTCF and Yin Yang 1 (YY1), are sensitive to it (Hark et al., 2000; Kim et al., 2003). Each D4Z4 repeat unit harbors two classes of GC-rich sequences, namely the low copy-repeats hhspm3 and LSau. This type of repetitive DNA is predominantly found in heterochromatic regions of the genome (Hewitt et al., 1994). On this basis, it has been hypothesized that D4Z4 repeat reduction might induce changes in chromatin conformation leading to inappropriate expression of 4q35 genes. The first study of DNA methylation at the D4Z4 repeat array did not show a change in this epigenetic marker in FSHD tissues. D4Z4 was found highly methylated in both normal and FSHD lymphoblasts, as well as in somatic tissues, including skeletal muscle. Nevertheless the study did not discriminate between the methylation status of the repeat array at chromosome 4 or chromosome 10 (Tsien et al., 2001). A subsequent study, revealed a significant hypomethylation of three different CpG dinucleotides of the D4Z4-reduced allele in lymphoblasts and muscle biopsies from FSHD patients (van Overveld et al., 2003). Importantly, low methylation levels at D4Z4 were observed at both chromosome 4 and 10 in the so-called phenotypic FSHD patients. These patients are clinically indistinguishable from 4q-linked FSHD patients but do not carry any D4Z4-reduced (de Greef et al, 2009). However, methylation levels can vary substantially between individuals. Generally, patients with residual repeat sizes between 10 and 19 kb (1-3 D4Z4 units) are severely affected and show very low DNA methylation levels, whereas FSHD patients with repeat sizes between 20 and 31 kb (4-6 D4Z4 units) show inter-individual variation in both clinical severity and D4Z4 hypomethylation (van Overveld et al., 2005). In addition, non-penetrant carriers show the same D4Z4 hypomethylation as their affected relatives and strong D4Z4 hypomethylation is also reported in patients with immunodeficiency, centromeric instability and facial anomalies syndrome (ICF) without any myopatic symptoms (Xu et al., 1999; Kondo et al., 2000; van Overveld et al., 2003). Currently, the role of D4Z4 hypomethylation in FSHD pathogenesis remains elusive.

7.2 Histone modification

Chromatin conformation results from the interaction between DNA and histone proteins and the involvement of other chromosomal proteins. The basic structural unit of chromatin

is the nucleosome that consists of 146 bp of DNA wrapped around a protein octamer of core histone proteins (Kornberg et al., 1974; Finch et al., 1977). Histone proteins may be posttranslational modified, by acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP-ribosylation (Bernstein et al., 2007). Modified histones are likely to control the structure and/or function of the chromatin fiber, with different modifications yielding distinct functional consequences. Furthermore, recruitment of chromatin-associating proteins may depend upon the recognition of a specific histone modification pattern (Strahl and Allis, 2000; Peterson and Laniel, 2004). Extracellular and intracellular stimuli may change these patterns of modification, making the chromatin itself an integrator of various signaling pathways, ultimately affecting basic cellular processes such as transcription or replication (Cheung et al., 2000; Nightingale et al., 2006). *In vivo*, chromatin exists as fibers with differing degrees of compaction. The morphologically distinct classes of chromatin within the nucleus of higher eukaryotes are heterochromatin, which is more compacted and generally transcriptionally inactive, and euchromatin, wich is less compacted and generally transcriptionally active (Frenster et al., 1963). Although D4Z4 unit harbors two classes of repetitive DNA, hhspm3 and LSau, both of which are found predominantly in heterochromatic domains of the genome, FSHD locus at 4qter does not share some of the common properties of heterochromatin. For instance it does not co-localize with DAPI-intense loci or it does not replicate in late S-phase. A recent study on D4Z4 histone modification seems to indicate that the repeat array may be organized in distinct domains, some characterized by transcriptionally repressive heterochromatin and others by transcriptionally permissive euchromatin (Zeng et al., 2009). These results indicate that the D4Z4 locus might display a chromatin structure more similar to euchromatin and favor the hypothesis that this region might be more dynamic than expected. Interestingly loss of marks of unexpressed heterochromatin such as histone H3K9me3 was observed in both FSHD with or without D4Z4 contraction. This phenomenon seems to be strictly associated with FSHD phenotype; in fact it was not found in ICF syndrome, despite its apparent similarity to FSHD with regard to D4Z4 DNA hypomethylation, or in other types of muscular dystrophies tested (Zeng et al., 2009). H3K9 methylation at D4Z4 is specifically mediated by the histone methyltransferase SUV39H1 (Zeng et al., 2009), which interacts with MyoD to suppress MyoD-dependent muscle gene expression (Mal, 2006). Interestingly, the heterochromatin binding protein HP1, which mediates transcriptional silencing (Bannister et al., 2001; Bernard et al., 2001), and the sister chromatid cohesion complex, cohesin, bind to D4Z4 in an H3K9me3-dependent manner and their recruitment is seriously compromised in FSHD (Zeng et al., 2009). These data support the indirect mechanism (Figure 5 C) where loss of repeats generates structural and functional modification, possibly through epigenetic changes in the histone pattern, which in turn might have an effect on transcriptional regulation in *cis* and/or in *trans*. It is reasonable to anticipate that future studies on the possible chromatin organization involving D4Z4 and its changes in FSHD may provide critical insight into the mechanism of FSHD pathogenesis.

7.3 Long distance effect: A repressor complex binding D4Z4

The alteration of 4q35 gene expression observed in FSHD affected muscle (Gabellini et al., 2002) raised the question whether D4Z4 was directly involved in transcriptional control of 4q35 genes. The analysis of the interaction between D4Z4 and nuclear proteins revealed the presence of a 27 bp binding site (DBE, D4Z4 Binding Element) able to recruit a multi-protein

complex in vitro and in vivo comprising of YY1, HMGB2 and nucleolin, termed D4Z4 Recognition Complex (DRC) (Gabellini et al., 2002). The ubiquitous transcription factor Yin Yang 1 (YY1) is a recruiter of polycomb group proteins (PcG), which are responsible for chromatin remodelling and epigenetic silencing in many fundamental biological processes. YY1, exerts its effects on genes involved in normal biologic processes such as embryogenesis, differentiation, replication, and cellular proliferation. Its ability to initiate, activate, or repress transcription depends upon context (Gordon et al., 2006). Furthermore, the activity of YY1 is modulated by histone deacetylases and histone acetyltransferases (Yao et al., 2001). HMGB2 is a member of one of the three families of high mobility group (HMG) proteins (Bustin, 1999; Bianchi and Beltrame, 2000;Agresti and Bianchi, 2003). It has been proposed that HMGB2 might be involved in the organization and/or maintenance of heterochromatic regions through the SP100-mediated interaction with HP1 (Lehming et al., 1998). The third component of the DRC, nucleolin, is an abundant nucleolar protein, which has been implicated in chromatin structure, ribosomal RNA (rRNA) transcription, rRNA maturation, ribosome assembly and nucleo-cytoplasmic transport. To address whether the level of the DRC components influenced transcription of 4q35 genes, antisense experiments to decrease intracellular levels of DRC components were performed. These experiments showed that depletion of YY1, HMGB2 or nucleolin results in overexpression of the 4q35 gene *FRG2*, which is silent in normal cells and tissues (Gabellini et al., 2002). Accumulating evidences indicate that gene regulation can be affected by physical interaction between two distant chromosomal regions in *cis* and in *trans* in mammalian cells (Tolhuis et al., 2002; Horike et al., 2005; Spilianakis et al., 2005; Lomvardas et al., 2006). Thus the DRC might exerts is inhibitory activity either modifying the chromatin structure or acting directly on 4q35 genes promoters through a physical interaction mediated by the formation of a cromatin loop (Gabellini et al., 2002; Pirozhkova et al., 2008). The physical interaction between D4Z4 and FRG1 has been demonstrated (Pirozhkova et al., 2008; Bodega et al., 2009) in normal myoblast by Chromosome conformation capture (3C), which is a technique that identifies long distance intra- and inter-chromosomal interactions (Dekker et al., 2002). Interestingly chromatin seems to undergo remodeling during myogenic differentiation. It has been shown that in normal myoblasts, the *FRG1* gene is repressed and its promoter physically interacts with the D4Z4 array; upon differentiation, *FRG1* gene is expressed and the chromatin loop between FRG1 promoter and D4Z4 is relaxed (Bodega et al.2009). Consistent with the observed mis-regulation of FRG1, a small reduction in the D4Z4–FRG1 promoter interaction was observed in FSHD myoblasts compared with controls (Bodega et al., 2009). Different findings obtained with 3C analysis described the formation of loops between other elements in the FSHD locus (DUX4c and the 4qA/B marker) and the FRG1 promoter (Pirozhkova et al., 2008). These data indicate that the tridimensional structure of the FSHD locus is complex and composite, probably more than one sequence elements (for example, D4Z4, DUX4c,4qA/B) or more than one chromatin modification factor might be required to obtain a fine regulation of *FRG1* gene expression during muscle differentiation (Petrov et al., 2006; Pirozhkova et al., 2008).

7.4 Subnuclear localization of 4q35

The nucleoplasm is a high defined and structured compartment and chromosomes occupy specific and distinct territories. These chromosome territories are related to gene density,

transcriptional activity, replication timing, and chromosome size (Sun HB et al., 2000; Tanabe et al., 2002). The nucleoplasm is separated from the cytoplasm by the nuclear envelope (NE), consisting of an inner (INM) and outer nuclear membrane (ONM), (Gerace et al., 1988). Chromosome 4qter is preferentially localized in the outer nuclear periphery (Masny et al., 2004; Tam et al., 2004) although mammalian telomeres, including 10qter, are usually dispersed in the inner part of the nucleus (Ludérus et al., 1996; Nagele et al., 2001; Amrichová et al., 2003; Weierich et al., 2003). Sequences proximal to D4Z4, and not the repeat array itself, seem to be required to localize the 4q telomere at the periphery (Masny et al., 2004). These sequences are not found at 10qter and this may explain the different nuclear localization of 10qter. Recently, Ottaviani et al. (2009) identified an 80-bp sequence inside the D4Z4 unit that can trigger perinuclear positioning of artificial telomeres in a CTCF- and lamin A–dependent manner. Furthermore in cells lacking the *lamin A* gene, chromosome 4 telomeres are dispersed (Masny et al., 2004). In addition, lamin A is shown to be associated with D4Z4 in vivo by chromatin immunoprecipitation and the perinuclear localization of 4qter is largely lost in fibroblasts lacking lamin A/C (Ottaviani et al., 2009). Although Fluoresece In Situ Hybridization (FISH) analyses showed no change in the chromosome 4 localization, between FSHD and healthy subjects, the peripheral environment of the FSHD 4q35 allele may be altered because of modification in chromatin structure at D4Z4. This nuclear lamina alteration might produce a change in the binding of specific proteins, thereby contribute to the aberrant 4q35 gene expression reported in FSHD (Masny et al., 2004; Tam et al., 2004; Ottaviani et al., 2009).

8. Conclusions

D4Z4 repeat contraction in patients with FSHD was discovered almost 20 years ago, nevertheless the exact molecular mechanism causing the FSHD phenotype has still not been elucidated and the search for a unifying model that can explain all the clinical features that have been observed in time has been frustrated. No histological or biochemical markers are available to independently confirm a specific FSHD diagnosis that remains mainly clinical. The molecular test primarily used for FSHD diagnosis was based on the initial observation that 95% of FSHD patients carry a reduction of integral numbers of D4Z4 repeats at 4q35 with full penetrance (Van Deutekom et al., 2003). However the wide use of this test revealed several exceptions to the original assumption. Through the years the threshold size of D4Z4 alleles has been increased from the original 28 kb (6 D4Z4 repeats) (Wimenga et al., 1999b) to 35 kb (8 D4Z4 repeats) (Van Deutekom et al., 2003), with FSHD cases carrying D4Z4 alleles of 38-41 kb (9-11 D4Z4 repeats) considered borderline alleles (Butz et al., 2003; Vitelli et al., 1999). A further analysis of genotype-phenotype correlation led in time to the identification of subjects carrying D4Z4 reduced alleles with no sign of muscle weakness in FSHD families (Ricci et al., 1999; Tonini et al., 2004) as well as in normal controls (Van Overveld et al., 2000; Weiffenbach et al., 1992). The genotype-phenotype correlation conducted more recently on a large scale using a standardized method of evaluation allowed to estimate that 1) 20% of FSHD patients carry full-length D4Z4 alleles, 2) over 25% of relatives carrying D4Z4 reduced alleles do not have FSHD, 3) 3% of healthy subjects from the general population carry D4Z4 reduced alleles 4) no specific 4q haplotype is uniquely associated with FSHD. Remarkably, these studies established as a general rule rather than an exception that detection of a D4Z4 reduced allele is not sufficient to predict FSHD (Scionti et al., 2012a; Scionti et al., 2012b). Over the years, the molecular etiology of FSHD

has remained enigmatic, and the literature is filled with claims of causes that fail to be confirmed by other groups. Indeed, this might be expected if the clinical manifestation of FSHD symptoms is not only dependent on the structure/haplotype of D4Z4 contractions. This does not exclude an important pathogenic role for DUX4 or other candidate factors, but do establish a complex mechanism beyond current understanding indicating that a profound re-thinking of the genetic disease mechanism and modes of inheritance of FSHD is required.

In-depth examination of disease points to a more complex genetic etiology in which D4Z4 reduction might play a significant role only in association with other determinants, including genetic, epigenetic and environmental factors. Indeed, it is possible that in the heterozygous state a D4Z4 reduction might produce a predisposing condition that requires other epigenetic mechanisms or mutations in additional genes, both in *cis* and in *trans,* to cause overt myopathy. Finally it is also plausible that drugs or toxic agents might contribute to the disease onset and clinical variability. It is likely that, all mechanisms described above may contribute to the diverse phenotypic expression observed in carrier of D4Z4 reduced alleles. One of the major challenges for clinicians and researchers involved in FSHD studies will be to establish the weight that each single factor has in FSHD development. Particular attention should be paid to the relevance of epigenetics in the pathogenesis of FSHD. At the 4q subtelomere chromatin is normally tightly packed, probably as facultative heterochromatin. However this region can be highly dynamic as demonstrated by the fact that in patients with FSHD, this chromatin structure becomes more open. As a consequence, regulation of candidate genes can be influenced by proteins that may bind to or be released from D4Z4. One of the major goals for future FSHD research will be to integrate these disease mechanisms into a single model that can be used to explain the clinical data and to improve the molecular diagnosis; both steps are essential to develop effective therapeutic strategies.

9. References

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