Studies of the epigenetic disease mechanism in FSHD

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1 Introduction

Facioscapulohumeral muscular dystrophy (FSHD [OMIM 158900]), an inherited myopathy that is predominantly characterized by progressive, often asymmetric, weakness and wasting of the facial, shoulder and upper arm muscles [1], does not seem to be caused by structural mutations within a specific disease gene. Instead, increasing evidence suggests a significant role for a complex epigenetic mechanism, resulting in the perturbation of transcriptional control over multiple disease genes. This introduction aims to discuss the epigenetic changes observed in the FSHD locus and the possible epigenetic disease mechanism that may be associated with and contribute to FSHD pathogenesis.

2 Genetic changes associated with FSHD

FSHD is inherited in an autosomal dominant fashion. The majority of FSHD cases show linkage to the subtelomere of chromosome 4q which harbors the macrosatellite repeat D4Z4 (4q-linked FSHD or FSHD1) [2]. In the general population, this polymorphic repeat array varies between 11 and 100 units of 3.3 kb each. In patients with FSHD1, the D4Z4 repeat array is contracted to 1–10 units on one allele [3, 4]. The smallest residual repeat sizes are correlated with the more severe phenotypes, although a clear linear inverse relationship between residual repeat size and clinical severity has not been observed [5-7]. As monosomy of 4qter is not associated with FSHD, a critical role for the D4Z4 repeat array and flanking sequences in FSHD pathogenesis is to be expected [8]. Interestingly, in ~1% of patients presenting with a classic FSHD phenotype, a partial D4Z4 deletion extending in the proximal direction has been identified [9-11]. In these cases, an inverted D4Z4 repeat unit that is present 42 kb upstream of the D4Z4 repeat array and the candidate gene *FRG2* (FSHD region gene 2) can be deleted [11]. Thus far, the role of this inverted repeat in FSHD is unknown. The role of *FRG2* in FSHD pathogenesis will be discussed below. D4Z4-like repeat arrays are not restricted to chromosome 4qter. Sequences homologous to D4Z4 have been identified on many chromosomes, especially on the acrocentric chromosomes [12]. In addition, as a result of an ancient duplication, the subtelomere of chromosome 10q contains a repeat array that is highly homologous to D4Z4 [13, 14] (Figure 1). In ~10% of the population, subtelomeric exchanges between the D4Z4 repeats on 4qter and 10qter have been observed [15]. These rearrangements can result in the formation of hybrid alleles containing a mixture of 4-type and 10-type repeat units [9]. Translocated repeat arrays on chromosome 10q

are more homogeneous than translocated repeat arrays on chromosome 4q, the latter being almost always comprised of both 4- and 10-derived repeat units [16]. Importantly, FSHD is uniquely linked to chromosome 4q. Although ~10% of chromosomes 10q have been identified with a repeat array <11 repeat units, no contractions on 10qter have been reported to result in FSHD [17, 18]. Contraction of a translocated 4-type allele on chromosome 10q does not result in disease either [9, 15, 16]. Some years ago, two allelic variants of the 4q subtelomere, termed "4qA" and "4qB", were identified [19] (Figure 1).

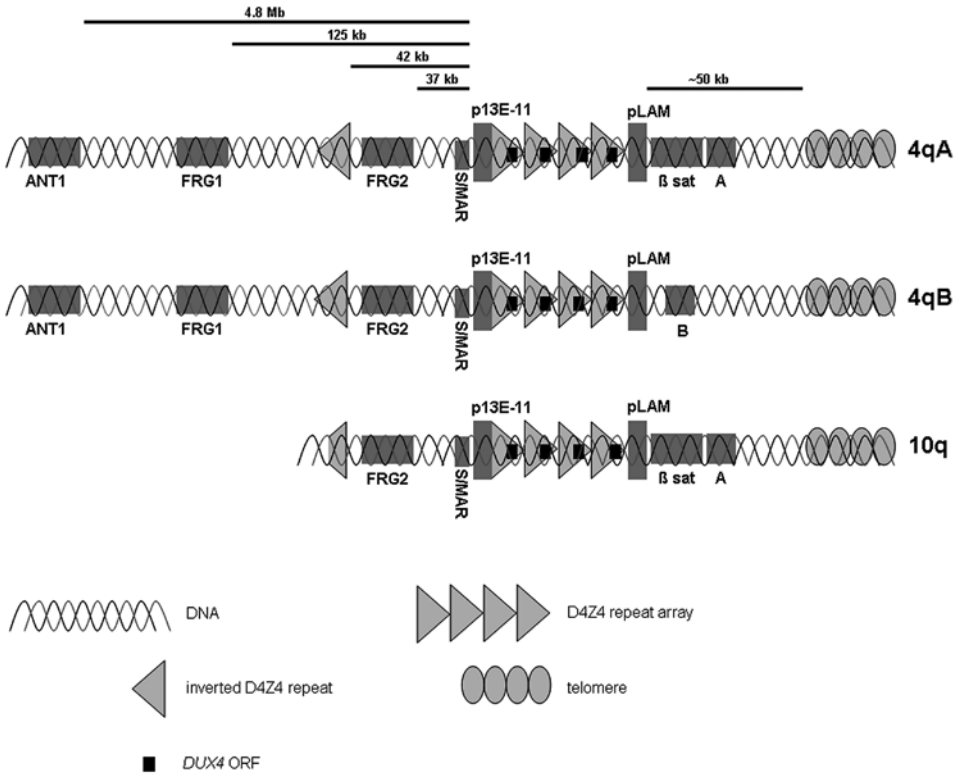

Figure 1

Schematic map of 4qA, 4qB and 10q

The subtelomere of chromosome 10q contains a repeat array that is highly homologous to D4Z4 on 4qter. The homology extends both in proximal (~40 kb upstream) and distal direction. In addition, two allelic variants of the 4q subtelomere have been identified. The presence of beta satellite DNA distal to D4Z4 on 4qA-type alleles is the most prominent difference between these allelic variants.

Although both variants are equally common in the population, FSHD is exclusively associated with a shortened D4Z4 repeat on a 4qA type allele [20]. A FSHD-sized repeat array on a 4qB-type allele does not cause FSHD [21]. The most prominent difference between these two allelic variants is the presence of 6.2 kb beta satellite DNA distal to D4Z4 on 4qA-type alleles [19]. An additional rare 4qter subtype was identified in two FSHD cases [22]. Recently, with the identification of 9 different haplotypes of chromosome 4q on basis of sequence variations in the FSHD locus, the picture became even more complex. Thus far, only contraction in one of these haplotypes, termed "4qA161", was found to cause FSHD, while contractions in other common 4q haplotypes such as "4qA166" and "4qB163" are non-pathogenic [23]. Currently, it is unclear what determines the difference in pathogeneticity between the different haplotypes. Haplotype-specific single nucleotide polymorphisms (SNPs) can be identified in the FSHD locus and are speculated to have an effect on the transcriptional activity of FSHD candidate genes or on the binding of proteins to the D4Z4 repeat array. Finally, a small percentage of FSHD cases (<5%), referred to as patients with phenotypic FSHD or FSHD2, shows no contraction of D4Z4 on one of their chromosomes 4q [24]. Currently, no disease locus has been identified for this heterogeneous patient group. Genes encoding the components of the D4Z4 repressor complex (see below) and *MYOD1* have been excluded as disease genes for this group of patients [25].

3 Epigenetic changes associated with FSHD

Over the years, because of the lack of evidence for transcription emanating from D4Z4 (see below), FSHD studies shifted towards understanding the chromatin structure of D4Z4. 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 [26]. Moreover, D4Z4 is overall very GC rich and has characteristics of a CpG island. Therefore, it has been hypothesized that repeat contraction-induced changes in chromatin conformation leading to inappropriate regulation of FSHD candidate genes, thus an epigenetic mechanism, may underlie FSHD pathogenesis. Major epigenetic mechanisms accounting for and contributing to human disease are changes in DNA methylation and histone modifications. An overview of studies on changes in DNA methylation and histone modifications at the D4Z4 repeat array in FSHD is given below and is summarized in Figure 2.

3.1 DNA methylation in FSHD

In mammalian DNA, the cytosine of CpG dinucleotides can be methylated by DNA methyltransferases like DNMT1, DNMT3A and DNMT3B. Generally, the presence of methyl groups on DNA is associated with increased chromatin condensation and gene silencing. When a promoter region is methylated, transcription factors with CpG dinucleotides in their DNA recognition sequence cannot bind. Reports on the methylation-sensitive binding of proteins, including E2F, CTCF (CCCTC-binding factor) and YY1 (Ying Yang 1), are numerous [27-29]. On the other hand, the methyl binding domain (MBD) proteins bind specifically to methylated DNA. Subsequently, these proteins can recruit histone deacetylases and histone methyltransferases, resulting in increased chromatin condensation and recruitment of the chromatin silencer heterochromatin protein 1 (HP1), respectively [30, 31]. An initial study on DNA methylation in the D4Z4 repeat array did not show a change in this epigenetic marker in FSHD since high methylation levels, consistent with heterochromatin, were observed at several CpG dinucleotides in both normal and FSHD cell lines and somatic tissues. However, the methylation level of both chromosome 4q and 10q repeat arrays was analyzed simultaneously [32]. A few years later, studying two different CpG dinucleotides and discriminating between chromosomes 4q and 10q, significant hypomethylation of the contracted allele was observed in patients with FSHD compared to controls and individuals with non-FSHD muscular dystrophies. Although this study was predominantly performed on lymphoblast DNA, a similar level of hypomethylation was identified in a small group of DNA samples isolated from FSHD muscle. Importantly, low D4Z4 methylation levels were observed at both chromosome 4q alleles in FSHD2 patients who are clinically indistinguishable from FSHD1 patients but who show no D4Z4 contraction [33]. Interestingly, part of the proximal D4Z4 repeat unit seems to be resistant to DNA methylation, as was observed in cancer tissues presenting with high DNA methylation throughout the D4Z4 repeat array. In addition, this 2 kb region showed differential DNaseI accessibility compared to the remainder of the repeat array. These results may suggest the presence of a boundary element at the junction of D4Z4 and the proximal AT-rich p13E-11 region [34]. Such a boundary element can be essential in physically separating active and inactive genomic regions [35]. Further, a subregion within each D4Z4 repeat unit, 1.4 kb from the single *Kpn*I site within D4Z4, also showed resistance to cancer-linked hypermethylation. This subregion contains stretches of G residues that are hypothesized to form stable G-quadruplexes that can play an

important role in D4Z4 chromatin organization [34]. Intriguingly, homodimers of the myogenic regulatory factor MyoD may specifically recognize these G-quadruplexes [36]. These results on cancer-linked hypermethylation were only confirmed at a lower intensity in somatic control DNA samples and not in FSHD DNA samples [34]. Currently, the precise role of D4Z4 hypomethylation in FSHD pathogenesis remains to be established. Altogether, FSHD alleles are hypomethylated compared to controls, but methylation levels can vary substantially between individuals. Generally, patients with residual repeat sizes between 10 and 20 kb are severely affected and show very low DNA methylation levels, while patients with repeat sizes between 20 and 31 kb show large interindividual variation in both clinical severity and D4Z4 hypomethylation [37]. In addition, asymptomatic gene carriers show the same reduction in D4Z4 methylation as FSHD1 patients and strong D4Z4 hypomethylation is also reported in patients with immunodeficiency, centromeric instability and facial anomalies syndrome (ICF syndrome [OMIM 242860]) [33, 38]. Patients with ICF syndrome present with severe immunodeficiency, resulting in recurrent respiratory and gastrointestinal infections, and non-myopathic facial anomalies. In ~60% of patients with ICF, mutations in the DNA methyltransferase gene *DNMT3B* have been identified [39]. As these mutations reduce the methyltransferase activity of DNMT3B, hypomethylation of several repeat arrays, including satellite 2 (Sat2), satellite 3 (Sat3), the NBL2 repeat and the D4Z4 repeat, is observed in patients with ICF [38-40]. Other (epigenetic) factors that differ between FSHD and ICF may contribute to the development of FSHD.

3.2 Histone modifications in FSHD

Chromatin is the assembly of DNA, histone proteins and other chromosomal proteins. A major function of chromatin is to accommodate the packaging of the DNA in the nucleus. The smallest structural unit of packaging is the nucleosome that consists of ~146 bp of DNA wrapped around eight core histone proteins. Histone proteins may undergo several posttranslational modifications, such as acetylation, methylation, phosphorylation and ubiquitination [41]. Currently, two models explaining the function of these histone modifications prevail. Histone modifications may directly affect chromatin structure by preventing transcription factor binding, altering the interactions between nucleosomes or changing the interactions of the histone tails with the DNA in the nucleosome [42]. On the other hand, histone modifications may serve as a site for recruitment of chromatin-associating proteins that recognize a specific histone code.

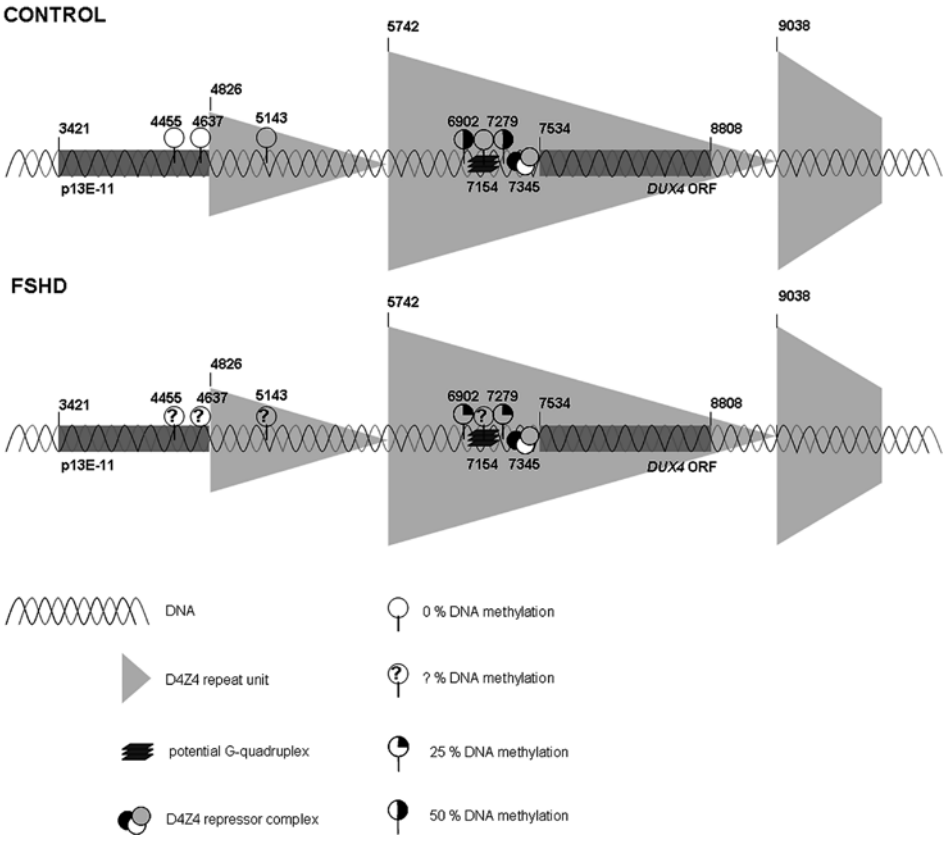

Figure 2

Epigenetic modifications at the D4Z4 repeat array in controls and patients with FSHD

In control individuals methylation levels of 50% are identified at two CpG dinucleotides while in patients with FSHD significant hypomethylation is found at these sites (~25% methylation). Interestingly, part of the proximal repeat unit seems to be resistant to DNA methylation (0% methylation) and is more accessible to DNaseI in cancer tissues, suggesting the presence of a boundary element at the junction of D4Z4 and p13E-11. Finally, a subregion within each D4Z4 repeat unit that may form a G-quadruplex also shows resistance to cancer-linked hypermethylation (0% methylation). In somatic control DNA samples similar results were obtained, although at a lower intensity. FSHD DNA samples have not yet been tested (?% DNA methylation).The presented nucleotide positions are based on AF117653 (GenBank).

As a consequence, downstream events generating a particular chromatin state may occur [43]. Specific histone modifications seem to be associated with either transcriptional activation or transcriptional repression. Methylation at lysine residues 4, 36 and 79 of histone H3 has been correlated with transcriptional activation. Acetylation of arginine residues of histone H3 and H4 is also characteristic for euchromatin and gene activation [44]. In contrast, methylation at lysine residues 9 and 27 of histone H3 and at lysine residue 20 of histone H4 are typically linked to heterochromatin and gene repression [45, 46]. Using chromatin immunoprecipitation (ChIP) assays, the hypothesized heterochromatic nature of the D4Z4 repeat array was studied. The level of histone H4 acetylation of D4Z4 in chromosome 4-containing somatic cell hybrids was higher than expected for a heterochromatic structure. Further, histone H4 acetylation levels at the p13E-11 region immediately proximal to D4Z4 were similar to those observed in the 5' regions of the FSHD candidate genes *FRG1* (FSHD region gene 1) and *ANT1* (adenine nucleotide translocator 1) and did not differ significantly between control and FSHD lymphoid cells. In conclusion, these results suggested that the nature of D4Z4 chromatin is that of unexpressed euchromatin rather than that of constitutive heterochromatin [47]. In a second study, other heterochromatin marks were studied using immunofluorescence in situ hybridization (immuno-FISH) methods. The FSHD locus at 4qter did not colocalize in control and FSHD myoblasts with DAPI-intense loci, not with heterochromatic foci in interphase nuclei and not with chromatin regions enriched in HP1 or histone H3 methylated at lysine 9. In addition, no late replication in S-phase, characteristic for constitutive heterochromatin, was observed. On the other hand, histone H3 methylation at lysine 4 and histone H4 acetylation at lysine 8, both characteristics for highly expressed gene regions, was observed in FSHD and control myoblasts [48]. Again these results indicated a more euchromatic or facultative heterochromatic structure at the D4Z4 repeat.

4 Epigenetic disease mechanism of FSHD

The exact pathogenetic mechanism causing FSHD is still unknown. Over the years, several disease mechanisms for FSHD have been postulated, implying either a direct (protein coding) or an indirect (non-protein coding) role for D4Z4 in the development of FSHD. A number of observations need to be considered when proposing a disease mechanism for FSHD. First, a critical number of D4Z4 repeat units is associated with FSHD pathogenesis. In general, patients with FSHD carry a D4Z4 repeat array

that is contracted to 1–10 repeat units [3, 4] while monosomy of 4qter does not cause FSHD [8]. Second, despite the high homology between D4Z4 repeat arrays derived from chromosomes 4qter and 10qter, only contraction in one of the 4qter haplotypes, termed 4qA161, results in FSHD [20, 23]. FSHD-sized repeat arrays on chromosome 10q or on 4qA166 and 4qB163 alleles do not cause FSHD [21, 23]. Third, a specific change in chromatin structure is observed, namely D4Z4 hypomethylation [33]. At present, it is unknown whether these changes in chromatin structure are causative for FSHD or arise as a consequence of the primary genetic defect. Therefore, it is also unclear what the contribution of these chromatin changes is to the FSHD phenotype. However, a small group of patients presents with a FSHD phenotype but does not show a D4Z4 contraction. Importantly, these patients show both D4Z4 hypomethylation [33]. Thus, it will be imperative to study the functional consequences of this chromatin change.

4.1 Role of D4Z4 transcription in FSHD pathogenesis

Initially, a putative promoter and the putative double homeodomain gene *DUX4* were identified within each D4Z4 repeat unit. As D4Z4 was considered to be of heterochromatic nature, it was hypothesized that partial deletion of the D4Z4 repeat array resulted in destabilization of the D4Z4 heterochromatin and in the inappropriate upregulation of *DUX4* [26, 49]. *DUX4* overexpression may induce cell death by apoptosis, induce caspase 3/7 activation and alter emerin distribution at the nuclear envelope [50]. In addition, *DUX4* overexpression may activate *PITX1* (paired-like homeodomain transcription factor 1), as was determined for both a reporter gene fused to the Pitx1 promoter and the endogenous *Pitx1* gene. Interestingly, upregulation of the PITX1 protein was also observed in muscle biopsies of patients with FSHD [50, 51]. Nevertheless, for a long time, the functionality of the *DUX4* gene was questioned, because of lack of introns and polyadenylation signals and absence of evidence for in vivo transcription [26, 49, 52-54]. Recently however, D4Z4 homologues have been identified in several mammalian species and it was established that the *DUX4* open reading frame (ORF) shows evolutionary conservation, disputing the non-functionality of DUX4 and suggesting a coding role, possibly during development. Interestingly, not only the ORF of *DUX4*, but also their organization in an array is evolutionary conserved. Importantly, this study provided evidence for bidirectional transcription of the mouse Dux array [55]. Next, expression of two different *DUX4* transcripts in cells transfected with D4Z4 elements and in FSHD myoblasts was reported. The first transcript lacks introns and

is transcribed from internal D4Z4 repeat units, while the second transcript has two introns and is transcribed from the most distal D4Z4 repeat unit. Interestingly, the pLAM sequence distal to the second transcript may provide a polyadenylation signal [50, 51]. Thus far, *DUX4* expression seems to be restricted to FSHD myoblasts [50, 51]. As most homeodomain proteins have a function as transcriptional regulators in developmental processes, *DUX4* expression may normally be restricted to embryogenesis [56]. In fact, the DUX4 homeodomain shares high homology with the homeodomain of the proteins Pax3 and Pax7, which are involved in the development of skeletal muscle [57]. As FSHD is specifically linked to the 4qA161 haplotype [23], sequence variations residing within or close to the D4Z4 repeat array may play a role in the regulation of *DUX4* transcription. Therefore, it is very interesting that differences between 4qA and 4qB alleles are observed in the pLAM region, possibly affecting the polyadenylation signal [20, 51]. However, these data need to be extended. At the same time, lower DNA methylation levels at D4Z4 may also influence the regulatory process of *DUX4*, explaining the occurrence of FSHD in FSHD2 patients without a D4Z4 contraction.

4.2 Role of gene deregulation in cis in FSHD pathogenesis

Other models have predicted an indirect role for the D4Z4 contraction in FSHD pathogenesis. Chromatin structure alterations at D4Z4, like D4Z4 hypomethylation, may cause loss of transcriptional control over the expression of candidate genes *in cis*. The identification of a DNA-binding complex, consisting of YY1, HMGB2 (high-mobility group box 2) and nucleolin and acting as a transcriptional repressor, supported the cis-model of gene deregulation. In controls, the presence of a threshold number of D4Z4 repeats may repress 4q35 genes, while in FSHD patients, because of a strong reduction in the number of bound YY1-HMGB2-nucleolin complexes, the transcriptional repression is abrogated, resulting in inappropriate overexpression [58]. A second line of evidence for deregulation *in cis* was recently provided by the identification of a nuclear matrix attachment site (S/MAR) associating with the nuclear matrix immediately upstream of D4Z4 [59]. S/MAR sequences are important for the organization of DNA into loop domains as part of a higher order chromatin structure [60]. In normal cells, the S/MAR is located between the upstream FSHD candidate genes *FRG1* and *FRG2* and the D4Z4 repeat array, thus separating them into two distinct DNA loop domains. In myoblasts from patients with FSHD, dissociation of the S/MAR from the nuclear matrix seems to occur, what may result in the presence of the *FRG1* and *FRG2* genes

in the same loop as the D4Z4 repeat array [59]. Since the 5' end of the D4Z4 repeat array was shown to contain a strong transcriptional enhancer, as a consequence *FRG1* and *FRG2* expression may be upregulated in patients with FSHD [61]. Although initial testing showed that *FRG1*, *FRG2* and *ANT1* were indeed transcriptionally upregulated in FSHD muscle [58], several follow-up studies could not reproduce these findings [47, 52, 53, 62]. The use of different techniques and different sources of RNA may partly explain this lack of reproducibility. The highly conserved nuclear protein FRG1 is a component of the human spliceosome and may have a role in pre-messenger RNA splicing [63-65]. Importantly, mice that overexpress *FRG1* 25- or 40-fold in skeletal muscle develop a muscular dystrophy phenotype. In addition, missplicing of muscle-specific mRNAs was observed in skeletal muscle of these transgenic mice, in *FRG1*-expressing C2C12 cells and in FSHD myoblasts [66]. Although an independent follow-up study could not confirm a splicing defect in FSHD muscle [53], a potential role for *FRG1* in FSHD pathogenesis has to be considered. *FRG2*, mapping 37 kb proximal to D4Z4 and specifically upregulated in differentiating myoblasts of patients with FSHD, is a less attractive FSHD candidate gene, as it is absent in some FSHD patients with a proximally extended deletion [9-11, 67]. Also, mice that overexpress *FRG2* do not present with muscular dystrophy. The same holds for mice overexpressing *ANT1*; these mice do not seem to develop a muscular dystrophy phenotype [66]. Interestingly, ANT1 protein levels were shown to be increased in both unaffected and affected FSHD muscles compared to muscles from controls and patients with Duchenne muscular dystrophy (DMD). An increased expression of *ANT1* may sensitize muscle cells to oxidative stress and apoptosis [68]. Thus, *ANT1* remains an attractive candidate gene and further studies addressing the role of *ANT1* in FSHD pathogenesis are warranted.

4.3 Role of gene deregulation in trans in FSHD pathogenesis

Several studies support an important *trans*-sensing effect in FSHD. An initial study on global gene expression profiles of FSHD muscle suggested a FSHD-specific defect in myogenic differentiation [52]. Since then, both gene and protein expression follow-up studies have been performed, presenting new interesting affected pathways, such as an impairment of slow-to-fast fiber differentiation, increased sensitivity to oxidative stress and a possible link with retinal vasculopathy [53, 62]. As the somatic pairing frequency between the 4q subtelomere and the 10q subtelomere was observed to be slightly but significantly increased in patients with FSHD, a *trans*-sensing effect of the D4Z4 contraction on gene

regulation on 10qter is expected [69]. Evidence supporting this hypothesis is the observation of a distinct level of *FRG2* expression on chromosome 10q in differentiating myoblasts of patients with FSHD [67] and a significant trans effect on myotube formation when D4Z4 repeats were transfected in C2C12 myoblasts [70]. However, a recent study employing chromatin conformation capture (3C) to investigate the three-dimensional structure of the 4q subtelomeric region showed that the majority of interactions at the 4q35 locus occur *in cis* and not *in trans* between chromosomes 4q and 10q [71].

As discussed above, each D4Z4 unit contains a 27 bp D4Z4 binding element (DBE) which binds a multi-protein complex consisting of YY1, HMGB2 and nucleolin [58]. Loss of this repressor complex at the disease allele in patients with FSHD may not only have an effect on transcriptional regulation of 4q35 genes. Genome-wide effects can be expected as well as a result of a local unbalance of D4Z4 binding of these proteins and subsequent interaction with different proteins at the disease allele. HMGB2 is a chromatin-associated DNA binding protein and a member of the high-mobility group (HMG) proteins [72]. Binding of HMGB2 to DNA may have a profound effect on the maintenance of heterochromatic regions, as HMGB2 interacts with SP100B which in turn binds to HP1, which has a function in the establishment and maintenance of higher order chromatin structures [73, 74]. Nucleolin, a nucleolar RNA-binding protein involved in several steps of ribosome biogenesis [75], may have the opposite effect on heterochromatin maintenance. Nucleolin has been shown to interact with histone H1 which may result in chromatin decondensation by displacement of histone H1 from linker DNA [76]. Finally, YY1 may also effect the chromatin structure at D4Z4, since it is the homologue of the Drosophila PcG protein pleiohomeotic (PHO). PcG multiprotein complexes control chromatin accessibility and maintain transcriptional repression during embryogenesis [77]. Depending on its relative concentration, the presence of coactivators or corepressors and the promoter context, YY1 can act as a transcriptional activator or repressor. A local unbalance in YY1 binding at D4Z4 may have multiple consequences. First, YY1 binding to regulatory regions of transcriptionally inactive muscle-specific genes seems to be required for recruitment of the histone lysine methyltransferase Ezh2 in proliferating mouse myoblasts. During myoblast differentiation, the YY1-Ezh2 complex disassociates from the DNA and consequently the transcription factor MyoD, having a key role in the differentiation of all skeletal muscle lineages, is recruited [78]. Thus, an unbalance in YY1 binding at D4Z4 in patients with FSHD may affect muscle differentiation, especially during embryonic development when Ezh2 is expressed [79]. Second, an unbalance in

YY1 binding may influence chromatin structure at or around its target site by recruiting the histone H4-specific methyltransferase PRMT1, resulting in methylation of arginine residue 3 of histone H4 and gene activation [80]. Third, an unbalance in YY1 binding may influence the interaction with the protein CTCF, a chromatin insulator that seems to be essential for homologous X-chromosome pairing [81]. Possibly, YY1-CTCF may have a similar function in pairing between the 4q subtelomere and the 10q subtelomere.

4.4 Role of nuclear organization in FSHD pathogenesis

Appropriate nuclear organization is essential for normal gene expression. Chromosomes are compartmentalized into discrete nuclear territories. The location of a gene within such a nuclear territory determines the availability of regulatory proteins and the accessibility of the DNA to the transcriptional apparatus [82]. The nuclear envelope (NE), consisting of an inner (INM) and outer nuclear membrane (ONM), forms the boundary of the nucleus [83]. The INM is covered with a protein meshwork, the nuclear lamina, which maintains the shape of the nucleus and provides mechanical strength to the nucleus. Besides, it has a role in many nuclear activities, including DNA replication, RNA transcription, nuclear and chromatin organization, cell cycle regulation, cell development and differentiation, nuclear migration and apoptosis [84]. A large group of inherited human diseases, collectively termed the "laminopathies", is caused by mutations in components of the nuclear lamina. Most commonly, adipose tissue, bone and connective tissue, heart and importantly skeletal muscle are affected by these mutations [85]. The 4q subtelomere is preferentially localized in the outer nuclear rim, both in controls and in patients with FSHD. Other subtelomeric regions, including 10qter, localize more to the interior of the nucleus [86, 87]. This peripheral localization of 4qter seems to be caused by an intrinsic property of 4qter as the X chromosome showed a more peripheral localization in a cell line with a X;4 translocation containing the distal 4 Mb of 4qter [87]. A region proximal to D4Z4 seems to be primarily responsible for the perinuclear localization [86, 87]. These results may explain the different nuclear localization of 10q subtelomeres, since the homology between 4qter and 10qter is restricted to the 40 kb proximal to D4Z4. A major role for a correct integrity of the nuclear lamina in the peripheral organization of 4qter is to be expected as the peripheral localization of 4qter is lost in fibroblasts lacking lamin A/C, a protein of the nuclear lamina [86]. Although no change in the localization of disease chromosomes compared to healthy chromosomes was observed, the interaction between 4qter and the nuclear envelope

may be disturbed in FSHD because of alterations in chromatin structure at D4Z4 and the consequent loss of binding of specific proteins that may interact with the nuclear lamina. A possible defective pathway could be via HP1γ and its interacting partner the lamin B receptor (LBR) of the INM [88]. Interestingly, other neuromuscular disorders, like X-linked and autosomal dominant Emery–Dreifuss muscular dystrophies (EDMD), are caused by mutations in emerin and lamin A/C, respectively [85]. Moreover, six nuclear envelope transmembrane proteins (NETs) were identified that are predicted to have an important function in myoblast differentiation and/or muscle maintenance [89]. Finally, transcriptome studies showed that FSHD and EDMD are highly related [90] and DUX4 overexpression may redistribute emerin at the nuclear envelope [50]. In conclusion, it is hypothesized that FSHD may arise from improper chromatin interactions at the nuclear envelope.

5 Thesis outline

As discussed above, an epigenetic change is observed in the FSHD locus and a possible epigenetic disease mechanism may be associated with and contribute to FSHD pathogenesis. However, D4Z4 hypomethylation is also observed in patients with the ICF syndrome [33, 38]. ICF patients present with non-myopathic symptoms including facial anomalies, like a flat nasal bridge and hypertelorism, and severe immunodeficiency leading to recurrent infections mainly affecting the respiratory and gastrointestinal systems [91]. To determine whether the epigenetic similarities between patients with FSHD and patients with ICF are restricted to D4Z4 hypomethylation and to determine whether FSHD2 may be caused by a defect in a similar pathway as the ICF syndrome, in ***Chapter 2*** a study is presented that searched for commonalities and differences between both disorders. First, the DNA methylation of non-D4Z4 repeat arrays (satellite 2, satellite 3, α-satellite and NBL2) is determined in patients with FSHD2. In patients with ICF hypomethylation of these repeat arrays is observed [38-40]. Second, lymphocytes of FSHD2 patients are treated with phytohaemagglutinin (PHA) and studied for pericentromeric abnormalities. PHA-stimulated lymphocytes of patients with ICF tend to form chromosomal abnormalities, including the formation of radial chromosomes, mainly involving chromosomes 1, 9 and 16 [92]. Third, the levels of the immunoglobulins IgA, IgG and IgM are determined in patients with FSHD2, as the levels of these immunoglobulins may be extremely low in patients with ICF [91].

Next, in ***Chapter 3*** a study is presented that searched for additional epigenetic factors discriminating between patients with FSHD and patients with ICF. Therefore, the histone modification status of the D4Z4 repeat array on chromosomes 4q and 10q is examined by ChIP. Trimethylation of lysine 9 and 27 of histone H3 (H3K9me3 and H3K27me3), dimethylation of lysine 4 of histone H3 (H3K4me2) and acetylation of histone H3 (H3Ac) is studied in lymphoblasts, fibroblasts and myoblasts of control individuals, patients with FSHD1, patients with FSHD2, patients with ICF and patients with non-FSHD muscular dystrophies. In addition, the binding of the proteins HP1γ and cohesin to the D4Z4 repeat array on chromosomes 4q and 10q is studied by ChIP in these cell lines. To further scrutinize the role of D4Z4 hypomethylation in FSHD pathogenesis, in ***Chapter 4*** a detailed DNA methylation analysis of the D4Z4 repeat arrays on chromosomes 4q and 10q is presented. Unlike before, not only the methylation status of the proximal D4Z4 repeat unit is determined by methylation-sensitive Southern blot analysis, but also the methylation status of internal D4Z4 repeat units is studied, both on chromosome 4q and on chromosome 10q. Importantly, DNA material of control individuals carrying a short D4Z4 repeat array on a non-pathogenic 4q haplotype or on chromosome 10q is included in this study. Finally, in ***Chapter 5*** a pilot study is presented that focuses on the reclosing of the D4Z4 chromatin structure in patients with FSHD1 and patients with FSHD2 by folic acid and methione supplementation for three months. Outcome measures for this study include clinical severity score, D4Z4 methylation, genome-wide DNA methylation, serum folate levels, serum vitamin B12 levels and plasma homocysteine levels.

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