Studies of the epigenetic disease mechanism in FSHD

Greef, J.C. de

6

General Discussion

Parts of this chapter are published inMutat Res. 2008 Dec 1;647(1-2):94-102.

1 INTRODUCTION

More than fifteen years ago, facioscapulohumeral muscular dystrophy (FSHD) was linked to D4Z4 repeat array contractions in the subtelomere of chromosome 4q [1-3]. Since then, several putative candidate genes in the 4q35 region have been identified, including *ANT1, FRG1, FRG2* and *DUX4* [4-8]. Because of lack of consistent evidence of transcriptional deregulation of these candidate genes in patients with FSHD [9-13], other disease mechanisms have been postulated. One of these includes an epigenetic disease mechanism, which was supported by observation of D4Z4 hypomethylation at two CpG dinucleotides within the D4Z4 repeat array [14]. However, the existence of D4Z4 hypomethylation in patients with an unrelated syndrome, namely the immunodeficiency, centromeric instability and facial anomalies (ICF) syndrome [15], and without muscle pathology, challenged a causal role for D4Z4 hypomethylation in FSHD [14].
In this thesis, the epigenetic disease mechanism was further scrutinized. Additional epigenetic factors, some of which potentially discriminating between FSHD and ICF, were identified (Chapters 2 and 3). Next, the necessity of a more open chromatin structure at D4Z4, although alone insufficient to develop disease, was underscored (Chapter 4). Finally, a small intervention pilot study focusing on closing the chromatin structure at D4Z4 by folic acid and methionine supplementation was performed (Chapter 5).

2 HYPOMETHYLATION IS RESTRICTED TO THE D4Z4 REPEAT ARRAY IN FSHD2

To understand the epigenetic similarities between two unrelated disorders, i.e. the muscular dystrophy FSHD and the primary immunodeficiency ICF, and to further determine whether D4Z4 hypomethylation is causally related to development of FSHD, in Chapter 2 a study is presented that searched for commonalities and differences between the two disorders. The DNA methylation level of non-D4Z4 repeat arrays [15, 16], the tendency of mitogen-stimulated lymphocytes to present with pericentromeric abnormalities [17] and the levels of the immunoglobulins IgA, IgG and IgM [17], all features of ICF syndrome, were analyzed in several FSHD2 cases without D4Z4 contraction. As FSHD2 patients, unlike FSHD1 patients but similar to ICF patients, show D4Z4 hypomethylation at both chromosome 4q alleles [14], a defect in a pathway similar to that of ICF syndrome was predicted. However, no commonalities were identified between FSHD2 patients and ICF patients other than the earlier reported D4Z4 hypomethylation

[14]. While several repeat arrays including D4Z4 are hypomethylated in ICF patients [15, 16, 18], in FSHD2 lower DNA methylation levels seem to be restricted to D4Z4. In addition, no ICF-like symptoms such as low immunoglobulin levels and radial chromosome formation upon PHA-stimulation of PBLs [17] were identified in FSHD2 patients.

3 Specific loss of histone H3 lysine 9 trimethylation and HP1γ/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD)

In Chapter 3, loss or gain of other epigenetic factors in patients with FSHD are described. The presence of several histone modifications at the D4Z4 repeat array was studied by chromatin immunoprecipitation (ChIP). Four of the six histone modifications six studied could be identified at D4Z4; trimethylation of histone H3 at lysine 9 (H3K9me3) and lysine 27 (H3K27me3), both repressive chromatin marks [19], and dimethylation of histone H3 at lysine 4 (H3K4me2) and acetylation of histone H3 (H3Ac), both associated with a more permissive chromatin structure [20]. One of these histone modifications, H3K9me3, was specifically lost at D4Z4 in patients with FSHD, both in FSHD1 and FSHD2 patients. Importantly, loss of H3K9me3 was not observed to the same extent in ICF patients. In addition, control cells treated with 5-azacytidine, a demethylating agent, also showed high levels of H3K9me3 at D4Z4, suggesting that H3K9me3 may be upstream of DNA methylation at D4Z4.

Also in Chapter 3, evidence is presented that heterochromatin protein 1 γ (HP1γ) and cohesin binding to the D4Z4 repeat array is lost in patients with FSHD. Most importantly, no HP1γ/cohesin binding to D4Z4 was observed in lymphoblastoid cell lines; neither in FSHD patients nor in control individuals who have high H3K9me3 levels at D4Z4. These results cells suggest that a possible tissue-specific component is involved in the binding of HP1γ/cohesin to D4Z4, as these two proteins do bind to D4Z4 in fibroblasts and myoblasts.

4 Common epigenetic changes of D4Z4 in contraction-dependent and contraction-independent FSHD

In FSHD1 patients, loss of DNA methylation seems to be restricted to the contracted allele. In FSHD2 patients, D4Z4 hypomethylation seems much more pronounced,

which can be explained by loss of DNA methylation on both chromosome 4q alleles [14]. We therefore hypothesized that a gene defect responsible for the occurrence of the D4Z4 hypomethylation is causally underlying FSHD2. In FSHD1, the contraction of the D4Z4 repeat array may precede the D4Z4 hypomethyation. Interestingly, patients with FSHD1 always carry a contracted repeat array in association with the 4qA161 haplotype. Haplotype-specific sequence variations may therefore determine why only a contracted repeat on this specific haplotype results in disease [21]. Alternatively, there may be a different epigenetic response to D4Z4 contractions in the different haplotypes. Therefore, an important question to answer was whether D4Z4 hypomethylation of contracted repeats is restricted to the 4qA161 haplotype. Also other important questions on the role of D4Z4 hypomethylation in FSHD pathogenesis had remained unanswered for some years, including the methylation levels of D4Z4 on chromosome 10q and the exact relationship between D4Z4 repeat length and D4Z4 methylation.

In Chapter 4, the results of an extensive DNA methylation study, both on chromosomes 4q and 10q, is presented. The results clearly show that D4Z4 hypomethylation is not 4qA161-specific or FSHD-specific. Individuals carrying a contracted repeat on a non-pathogenic 4qA166, 4qB163 or 10qA166 haplotype also presented with a significant loss of DNA methylation at D4Z4. These results suggest that D4Z4 hypomethylation is necessary, but not sufficient, to develop FSHD; other epigenetic factors, like H3K9me3, may be important. Another possibility, not necessarily mutually exclusive, is that sequence variations determine the development of FSHD, while a change in chromatin structure, as evidenced by D4Z4 hypomethylation, facilitates the function of these sequence variations. Next, we reported in Chapter 4 that the methylation defect in FSHD2 patients extends to chromosome 10q; a chromatin modifier responsible for D4Z4 methylation on chromosomes 4q and 10q may therefore be defective in these patients. Importantly, all FSHD2 patients carry a 4qA161-type D4Z4 repeat array, unifying FSHD1 and FSHD2 since all FSHD1 patients carry a contracted D4Z4 repeat array in association with the 4qA161 haplotype [21]. Further, no linear relationship between D4Z4 repeat length and D4Z4 methylation was detected; neither on chromosome 4q nor on chromosome 10q. However, a threshold effect was observed that may explain the abrupt transition from pathogenic to non-pathogenic repeat arrays; below 40 kb a significant drop in D4Z4 methylation was observed. Finally, we showed that D4Z4 hypomethylation is restricted to this repeat array, as similar methylation levels were observed between controls and patients with FSHD at the proximal *Sma*I restriction site.

5 No effect of folic acid and methione supplementation on D4Z4 methylation in patients with FSHD

The chromatin structure of the D4Z4 repeat array is changed in FSHD patients; loss of DNA methylation and H3K9me3 followed by loss of HP1γ/cohesin binding is observed. Therefore, we aimed at closing the chromatin structure of the D4Z4 repeat array. A rather simple method to affect chromatin structure of D4Z4 is through elevation of the DNA methylation levels, as DNA methylation is a reversible process [22]. DNA methylation levels may be influenced by supplementation with folic acid, which is important for the synthesis of S-adenosylmethionine (SAM), the common methyl donor needed for DNA methylation maintenance [23].

In Chapter 5, data of a pilot study performed in a small group of FSHD patients, both FSHD1 and FSHD2, and in unaffected family members is presented. Supplementation for three months with folic acid (5 mg, daily) and methionine (1 g, three times a day) was performed. Outcome measures of this study were clinical severity score determined by the Ricci score [24], DNA methylation at the D4Z4 repeat array and total DNA methylation levels, serum folate levels, serum vitamin B12 levels and plasma homocysteine levels. Although serum folate levels rose significantly and genome-wide DNA methylation levels increased in most subjects, no significant change in DNA methylation at D4Z4 was observed, neither in controls nor in FSHD patients.

6 Recent advances in epigenetic FSHD research

Several other groups have also focused on a possible epigenetic role in the pathogenesis of FSHD. I will now discuss some of the recent advances that were made in this field.

6.1 A nuclear matrix attachment site proximal to the D4Z4 repeat array

Besides DNA methylation and histone modifications, the organization of DNA into loop domains may contribute to the chromatin structure of a region and thus may have an effect on transcriptional regulation [25]. These loop domains are anchored to the nuclear matrix via specific sequences, the scaffold/matrix-associated regions (S/MAR regions) [26]. Recently, Petrov *et al* reported the presence of three S/MAR sites in the vicinity of D4Z4. One of these sites was immediately upstream of D4Z4, between the FSHD candidate genes *FRG1* and *FRG2*; the other two sites were located proximal to *FRG1* and distal to D4Z4. It was hypothesized that the former S/MAR site separates the FSHD

candidate genes from the D4Z4 repeat array into two distinct DNA loop domains. Interestingly, in myoblasts from FSHD patients, dissociation of this S/MAR from the nuclear matrix seems to occur. As a consequence, the upstream candidate genes and the D4Z4 repeat array are residing in a single loop domain in patients with FSHD [27]. Since the 5' end of the D4Z4 repeat array was reported to contain a strong transcriptional enhancer, this may affect the transcriptional regulation of *FRG1* and *FRG2* in patients with FSHD [28]. However, there is still debate on the transcriptional deregulation of *FRG1* and *FRG2* in FSHD [9-13]. Furthermore, part of the D4Z4 repeat array seems to function as a transcriptional repressor; the D4Z4 binding element (DBE) that binds the transcriptional repressor complex YY1-HMGB2-nucleolin [9]. In addition, when myoblasts were transfected with an increasing number of D4Z4 repeat units preceding the FRG2 promoter in a luciferase reporter assay, luciferase activity initially increased with one D4Z4 repeat unit, but subsequently decreased with the addition of extra D4Z4 repeat units. This suggests that D4Z4 may act as a transcriptional activator or repressor depending on the context [7]. It is thus unclear what the exact effect on transcription will be if *FRG1* and *FRG2* reside in the same DNA loop. Finally, as changes in DNA methylation seem restricted to the D4Z4 repeat array in FSHD (no hypomethylation was observed on the proximal *Sma*I restriction site which is located in the S/MAR sequence in FSHD patients (Chapter 4)), it is yet unclear why the loop domain organization in patients with FSHD is lost. The methyl-binding protein MeCP2 has been implicated in loop domain organization [29]. Although no changes in DNA methylation were observed between controls and FSHD patients at the S/MAR site, it will be interesting to study binding of MeCP2 and other proteins that mediate specific association of DNA with the nuclear matrix in myoblasts of controls and patients with FSHD.

6.2 DNA methylation and chromatin DNaseI sensitivity; a role for the insulator protein CTCF at D4Z4

We have described that D4Z4 contraction results in significant D4Z4 hypomethylation. This hypomethylation occurs irrespective of the chromosomal background of the contracted repeat array and the hypomethylation is restricted to the D4Z4 repeat array. Recently, Tsumagari *et al* also performed a thorough DNA methylation analysis of the D4Z4 repeat array using cancer tissues that normally present with high DNA methylation throughout the D4Z4 repeat array. Additionally, they performed chromatin DNaseI sensitivity assays in and adjacent to the D4Z4 repeat array. They identified a 2

kb region in the proximal D4Z4 repeat unit that seems resistant to DNA methylation and shows differential DNaseI accessibility compared to the remainder of the repeat array. Therefore, they hypothesized the presence of a boundary element at the junction of D4Z4 and the AT-rich p13E-11 region [30]. This boundary element may represent the recently identified CTCF binding site at the proximal end of the D4Z4 repeat array. CTCF may function as an insulator at this site and prevent spreading of hypomethylation in a proximal direction. Interestingly, increased CTCF binding at this site was observed in patients with FSHD (G. Filippova, personal communication). Our recent results on the methylation level of the *BstB*I restriction site in the p13E-11 region, located ~200 bp from the proximal D4Z4 repeat unit, support these findings. We observed high methylation levels at the *BstB*I restriction site and, unlike the significant hypomethylation in the proximal D4Z4 repeat unit in FSHD patients, no differences in DNA methylation were observed between controls and FSHD patients (unpublished results). Thus, the enriched binding of CTCF in patients with FSHD may indeed prevent the spreading of the D4Z4 hypomethylation towards the p13E-11 region.

6.3 The three-dimensional structure of the 4q subtelomeric region

To determine the spatial proximity of genes of the 4q subtelomeric region and the D4Z4 repeat array and thus to further explore the higher order chromatin structure of the region, Pirozhkova *et al* performed chromatin conformation capture (3C) assays. This technique evaluates the spatial proximity of two genomic fragments based upon their tendency to become crosslinked *in vivo* [31, 32]. Using this technique it was shown that in normal myoblasts the distal part of the *FRG1* and *FRG2* promoters interact; this interaction was lost in myoblasts derived from a FSHD patient. Furthermore, they showed that in normal myoblasts the inverted D4Z4 repeat *DUX4c* interacts strongly with the distal part of the *FRG1* promoter and that there is also an interaction, albeit to a lower extent, with the *FRG2* promoter and with the subtelomeric region proximal to the 4qA/4qB marker. Interestingly, in FSHD myoblasts novel interactions between both the proximal and the distal part of the *FRG1* promoter and the 4qA/4qB marker and between the *ANT1* promoter and the 4qA/4qB marker were identified. Finally, they showed that in normal myoblasts the D4Z4 repeat array interacts with the region proximal to *DUX4c*.

In conclusion, the 4qA/4qB marker seems to have a very important role in FSHD, as multiple novel interactions with this region were identified in myoblasts derived

from an FSHD patient. This hypothesis is further supported by the observation that the 4qA allele contains a transcriptional enhancer [33]. However, recently several additional 4q haplotypes, both of the 4qA-type and the 4qB-type, were identified [21]. It will be important to test whether non-pathogenic 4qA haplotypes and non-pathogenic 4qB haplotypes do not act as transcriptional enhancers. Furthermore, additional 3C assays studying myoblasts from control individuals carrying a short D4Z4 repeat array on either a non-pathogenic 4q haplotype or on chromosome 10 will be necessary. The experiments described in this thesis suggest that changes in chromatin structure are not restricted to patients with FSHD; D4Z4 hypomethylation was also observed in controls with a short repeat on a non-pathogenic haplotype (Chapter 4).

7 An integrative model for FSHD pathogenesis

Although the D4Z4 repeat contraction in patients with FSHD was discovered more than 15 years ago, the exact molecular mechanism causing FSHD still remains elusive. It seems unlikely that a single candidate gene is responsible for the development of FSHD. Probably, a complex epigenetic disease mechanism involving the deregulation of multiple genes, both *in cis* and *in trans*, underlies its pathogenesis. One of the major challenges for future FSHD research will be to integrate all thus far reported disease mechanisms into a unifying model and to obtain consistent evidence supporting this model.

I hypothesize that the D4Z4 repeat array and its chromatin structure will be central in such a model, because every patient with FSHD shows genetic and/or epigenetic changes at this repeat array. Most importantly, all FSHD patients carry a hypomethylated 4qA161-type repeat array. I propose that in control individuals the D4Z4 repeat array is packaged in a relatively closed chromatin structure, probably as facultative heterochromatin. In patients with FSHD, this chromatin structure is more open. As a consequence, proteins may bind to D4Z4, influencing the regulation of candidate genes and the interaction with the nuclear envelope (Figure 1). Sequence variations residing within or close to the D4Z4 repeat array may be important for binding of such proteins and thus determine why FSHD is specifically linked to the 4qA161 haplotype [21]. Alternatively, the open chromatin structure facilitates only steady state expression levels of D4Z4 transcripts in the presence of critical SNPs in the 4qA161 haplotype.

In FSHD1, the open chromatin structure is only reached at a certain threshold. With

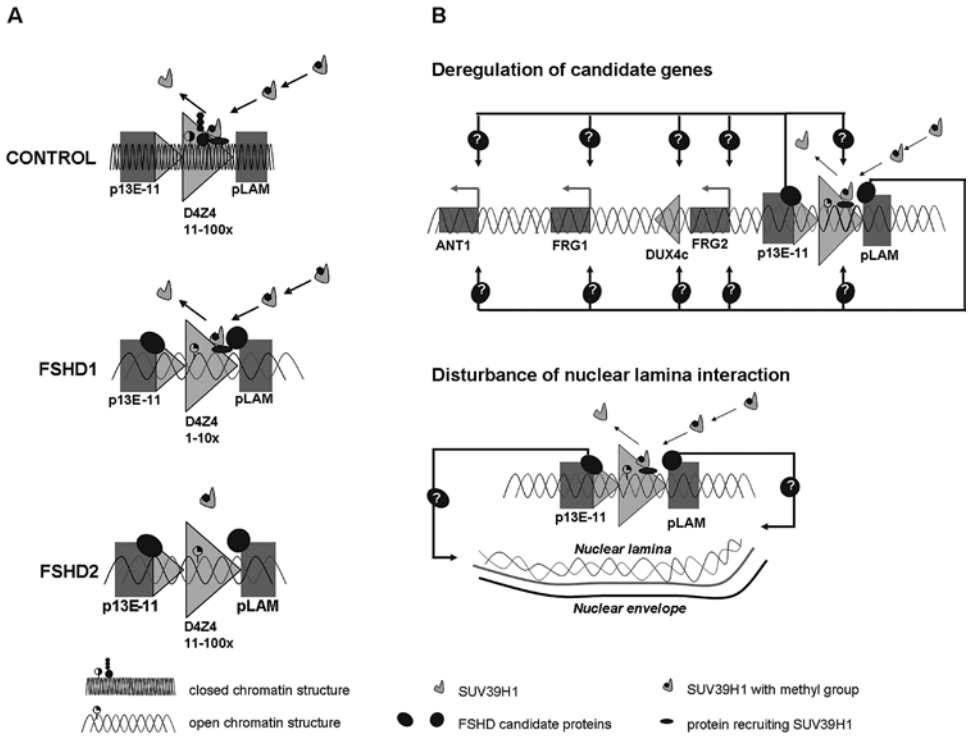

Figure 1

An integrative model for FSHD pathogenesis.

A. *In control individuals the D4Z4 repeat array is organized as facultative heterochromatin. In patients with FSHD1 only below 11 D4Z4 repeat units, a more open chromatin structure is induced. Below this threshold insufficient levels of a protein (complex) responsible for H3K9me3 and D4Z4 methylation or a presently unidentified chromatin modifier (complex) may be present at D4Z4. In patients with FSHD2, changes in chromatin structure may occur because of a yet unidentified gene defect responsible for the epigeneticmodifications in D4Z4.Therefore, a more open chromatin structure is present in these patients even above the critical threshold of 10 D4Z4 repeat units. Because of the more open chromatin structure in patients with FSHD, binding of protein(s) to D4Z4 that normally do not bind may occur.This will most likely occur either proximal or distal to D4Z4 or to a critical D4Z4 element, since the amount of repeat units with an open chromatin structure differs significantly between FSHD1 and FSHD2 patients, while their phenotypes are highly similar. In addition, specific SNPs discriminating between the different haplotypes may be important for the binding of these proteins.*

B.*When the chromatin structure is in a more open conformation, candidate genes may be deregulated in cis (upper panel) and the interaction with the nuclear envelope may be disturbed (lower panel).*

more than 10 D4Z4 repeat units still present the chromatin structure is kept in a closed state, for example because of a sufficient level of binding of proteins that attract SUV39H1, the histone methyltransferase responsible for H3K9me3 and possibly DNA methylation at D4Z4, or the binding of yet unidentified chromatin modifiers. When less than 11 D4Z4 repeat units are left, the critical threshold is reached, resulting in loss of H3K9me3 and loss of DNA methylation at D4Z4.

In FSHD2, a presently unidentified gene defect may disturb the recruitment of SUV39H1 or other chromatin modifiers to D4Z4 with consequent loss of H3K9me3 and DNA methylation, thus also resulting in a more open chromatin structure at D4Z4. Since the number of D4Z4 repeat units with an open chromatin structure differs significantly between FSHD1 and FSHD2 patients (1–10 repeats versus >10 repeats), it seems unlikely that a protein that binds to each D4Z4 repeat unit, e.g. the D4Z4 repressor complex [9], plays an important role in FSHD development. If this were the case, differences in phenotype between FSHD1 and FSHD2 were to be expected. More likely, binding of a protein just upstream or downstream of D4Z4 or binding to a specific D4Z4 element (e.g. the proximal or distal unit) seems to be critical. Interestingly, the chromatin structure of the p13E-11 region just upstream of D4Z4 is different from the remainder of the D4Z4 repeat array [10, 30] and this open chromatin configuration extends into the proximal D4Z4 repeat unit just distal to p13E-11 in both controls and patients with FSHD [30]. Also, this region harbors haplotype-specific SNPs that may be critical to disease development. Apparently, there is an unusual small transition zone from a very open to a compact chromatin structure. Changes in this transition zone may uncover presently unidentified D4Z4 elements essential for the pathogenesis of FSHD. It is therefore imperative to investigate whether such a transition zone also exists at the distal end of the repeat. In addition, a DUX4 transcript from the distal D4Z4 repeat unit was reported recently [34], which is provided with a polyadenylation signal by the distally located pLAM sequence. Interestingly, the pLAM sequence is only present on 4qA alleles [34, 35].

Currently, I cannot explain the large clinical intra- and interfamilial variability observed in FSHD, varying from gene carriers without symptoms to patients that eventually become wheelchair-bound [36]. This intrafamilial variability also applies for FSHD2 families, in which a non-affected family member with significant D4Z4 hypomethylation and a D4Z4 repeat array on a 4qA161 haplotype was identified. However, the 4qA161-type repeat array in this family member is relatively large in size, while most FSHD2

patients carry a 4qA161-type repeat <100 kb. Further analysis of D4Z4 chromatin structure in gene carriers will be essential. Second, because of the lack of the FSHD phenotype when D4Z4 is contracted on 10q alleles, 4qB alleles and 4qA166 alleles, it will be important to study chromatin changes other than DNA methylation in individuals carrying such a short repeat array [21, 37-39]. In Chapter 4 it is already described that D4Z4 hypomethylation occurs upon repeat contraction irrespective of the haplotype the contracted repeat array is residing in. It will also be important to study H3K9me3 and HP1γ/cohesin binding in control individuals with a contracted repeat on non-pathogenic haplotypes. Finally, the differences between all recently identified haplotypes need to be studied in detail as SNPs in the D4Z4 repeat array may affect binding of proteins or affect steady state expression levels of D4Z4-derived transcripts and thus may explain the lack of FSHD development on non-4qA161 alleles [21].

8 Future perspectives

8.1 FSHD2

In <5% of patients with FSHD, no D4Z4 contraction is observed by pulsed field gel electrophoresis (PFGE). Nevertheless, these patients do show loss of similar repressive chromatin marks as FSHD1 patients; D4Z4 hypomethylation on chromosomes 4q (Chapter 2) [14] and loss of H3K9me3 with subsequent disturbed HP1γ/cohesin binding to D4Z4 (Chapter 3). Furthermore, these patients show additional D4Z4 hypomethylation on chromosomes 10q (Chapter 4). Because of the epigenetic overlap between FSHD1 and FSHD2, the presence of a more open chromatin structure at the D4Z4 repeat array seems essential for FSHD pathogenesis.

At present, most FSHD2 patients identified are single cases within a family. In addition, all FSHD2 cases with pronounced D4Z4 hypomethylation carry at least one allele of the 4qA161 haplotype, similar to all FSHD1 patients. This suggests that also the presence of a 4qA161 allele is indispensable for the development of FSHD in addition to the changes in chromatin structure at D4Z4.

8.1.1 Genotype-phenotype study in FSHD2

The phenotype of FSHD1 patients and FSHD2 patients seems identical, although the epigenetic defect is not completely overlapping (additional D4Z4 hypomethylation is observed on chromosome 10q alleles in patients with FSHD2 (Chapter 4)). There is also

a large difference in amount of D4Z4 repeat units present with an open chromatin structure, both on chromosomes 4q and 10q, in patients with FSHD2. Hence, it will be important to perform a thorough genotype-phenotype study with the objectives to compare clinically FSHD1 and FSHD2 patients and to determine whether the more pronounced and more widespread hypomethylation observed in FSHD2 has additional effects on the phenotype. The FSHD phenotype is not restricted to the muscles; epilepsy, mental retardation, sensorineural hearing loss and retinal vasculopathy have been reported [40-42]. When performing such a genotype-phenotype study, these non-muscular symptoms also have to be included in the examination.

8.1.2 Genome-wide SNP studies in FSHD2

I anticipate that a mutation in a gene responsible for the repressive chromatin marks at the D4Z4 repeat array underlies the pathogenesis of FSHD2. Already several candidate genes, mainly genes involved in the establishment and/or maintenance of chromatin structure, have been tested for FSHD2, but thus far no mutations were identified (Chapter 2) [43]. A reasonable alternative is to perform genome-wide SNP studies to identify the disease locus in this small group of FSHD patients. First, it will be crucial to determine the inheritance pattern of FSDH2. With multiple affected siblings and unaffected parents in one family (Family 8, Chapter 4) and an affected mother-daughter pair (Family 2, Chapter 4), it is yet difficult to rule out either a dominant or a recessive inheritance pattern. Importantly, some unaffected family members also show pronounced hypomethylation at the D4Z4 repeat array (Chapter 2), suggesting that the gene defect in FSHD2 patients is not fully penetrant. Another explanation for this observation is that these unaffected family members with D4Z4 hypomethylation are gene carriers of the D4Z4 methylation defect but do not have a 4qA161-type repeat array. However, this last explanation does not explain all unaffected family members with D4Z4 hypomethylation (Individual 1.4, Chapter 2). Thus, it will be very important to rule out the diagnosis of FSHD in these individuals.

8. 2 ICF syndrome

The two unrelated disorders FSHD and ICF share an epigenetic hallmark, namely D4Z4 hypomethylation [14, 44]. In patients with FSHD, the loss of DNA methylation at D4Z4 seems to occur as a consequence of loss of H3K9me3 at D4Z4 (Chapter 3). In most ICF cases, the loss of DNA methylation at specific genomic loci, including D4Z4, can

be explained by the presence of mutations in the DNA methyltransferase 3B *(DNMT3B)* gene (ICF1) [45, 46].

To date, it is still unknown how mutations in *DNMT3B* cause ICF syndrome. It seems unlikely that loss of DNA methylation at D4Z4 plays an important role in ICF pathogenesis, since patients with FSHD show no symptoms of a primary immunodeficiency as observed in ICF patients (Chapter 2). It also seems unlikely that hypomethylation of α-satellite repeat DNA is important for ICF pathogenesis, as loss of DNA methylation in this repetitive DNA is only observed in patients with ICF2 syndrome, thus in patients in whom no mutation in the *DNMT3B* gene can be detected [16]. ICF2 patients cannot be discriminated from ICF1 patients based on their phenotype [47]. More likely, a yet to be identified mechanism involving hypomethylation of a specific repeat array or another locus may cause the ICF phenotype in both patient groups. Thus far, it has been speculated that hypomethylation of the Sat2 repeat array is crucial for pathogenesis, but evidence for this is lacking [48].

Similar as suggested above for FSHD2, it will be important to perform genome-wide SNP studies in patients with ICF2. As it is anticipated that ICF2, like ICF1, is inherited in an autosomal recessive fashion, consanguineous families will be instrumental in such studies. Using the method of homozygosity mapping, large regions of homozygosity that may contain the disease gene can be readily identified in a small set of patients [49]. As the number of ICF2 patients is limited (thus far ~50 ICF cases, both ICF1 and ICF2, have been described in literature [47]), combining homozygosity mapping with genome-wide expression studies in patients with ICF2 may facilitate the identification of the ICF2 disease locus considerably. I expect that genes operating in a similar pathway are affected in both types of ICF syndrome, as no prominent differences in phenotype are observed [47].

8.3 Epigenetic causes and consequences in FSHD

FSHD can be considered an epigenetic disorder, since loss of H3K9me3 and DNA methylation are observed in the D4Z4 repeat array of both FSHD1 and FSHD2 patients (Chapter 3) [14]. However, although necessary for FSHD development, the causes and consequences of the loss of these repressive chromatin marks are unknown. I will now discuss some possible future experiments that can be performed to obtain a more comprehensive understanding of these causes and consequences.

8.3.1 Additional histone modification analyses

We were unable to develop primers that specifically amplify the contracted allele in FSHD patients for our histone modification analyses (Chapter 3). As a result, histone modification levels at D4Z4 were determined on both chromosomes 4 and in addition on the homologous D4Z4 repeat array on both chromosomes 10. It was thus surprising that we observed such a pronounced loss of H3K9me3 in FSHD1, comparable with the loss observed in FSHD2 where the loss of DNA methylation is much more extensive (Chapter 4) [14]. Therefore, we speculated that loss of H3K9me3 may also occur on the other D4Z4 repeat arrays in addition to the contracted repeat array. This is in contrast with the loss of DNA methylation, which only occurs on the contracted allele in FSHD1 (Chapter 4) [14]. It will be crucial to develop primers that are 10q-specific to investigate in more detail the loss of H3K9me3. Besides, also 4qA161-specific primers should be developed which may be used to study the contracted allele specifically in patients in which the normal allele is of the 4qB-type and determine whether a relationship between H3K9me3 levels and clinical severity score (CSS) exists [24]. Furthermore, other histone modifications may also be changed in patients with FSHD, but they may not have been tested yet or they may have been missed if the changes are restricted to the contracted allele.

Another unanswered question is whether loss of DNA methylation is downstream of H3K9me3 loss or whether it occurs independently. The relationship between histone modifications and DNA methylation seems different for other genomic loci. DNA methylation may recruit histone deacetylases and histone methyltransferases via the methyl-binding protein MeCP2 [50]. On the other hand, specific histone modifications may recruit DNA methyltransferases resulting in methylation at CpG dinucleotides [51]. At D4Z4, I speculate that H3K9me3 is independent or upstream of DNA methylation, as no loss of H3K9me3 was observed in ICF patients with D4Z4 hypomethylation and this histone modification was also still present in control cells treated with the demethylating agent 5-azacytidine. However, the occurrence of D4Z4 hypomethylation without loss of H3K9me3 in the unaffected mother of two FSHD2 cases argues against this hypothesis (Chapter 3). To study this process in more detail, ChIP experiments can be performed using antibodies against SUV39H1 and DNMT3B, the histone methyltransferase and DNA methyltransferase responsible for the repressive chromatin marks at D4Z4, in combination with siRNA knockdown experiments. If H3K9me3 is indeed upstream of DNA methylation at D4Z4, no DNMT3B binding to D4Z4 and no

D4Z4 methylation is expected after knockdown of SUV39H1. In addition, when cells are treated with siRNA against DNMT3B, it is expected that SUV39H1 can still bind to D4Z4. However, it will be first necessary to determine where DNMT3B binds to D4Z4, as DNMT3B binding to DNA may be restricted to specific DNA sequences [52]. Finally, it will be essential to study the presence of histone modifications and DNA methylation at those D4Z4 regions that are implicated in transcription [34, 53], e.g. the distal D4Z4 repeat unit and the adjacent pLAM sequence, which may contain a polyadenylation signal [34]. Thus far, only a small part of the D4Z4 repeat array has been studied for chromatin changes [14, 30, 44]. Interestingly, although the maximum level of DNA methylation differs between different CpG dinucleotides tested in our lab, the percentage of reduction in DNA methylation between controls and FSDH1 patients is ~35% on each site investigated (Chapter 4). Furthermore, loss of H3K9me3 was only studied in one region of approximately 300 bp. A limited set of data on the border of the p13E-11 region and the first partial D4Z4 repeat unit suggests that no H3K9me3 is present at this region, neither in FSHD patients nor in controls (Chapter 3).

8.3.2 HP1γ/cohesin binding to D4Z4

D4Z4 contraction, D4Z4 hypomethylation and loss of H3K9me3 at D4Z4 can be observed in different cell types. Thus far, lymphoblastoid cells, fibroblasts and myoblasts were tested. The observation that HP1γ/cohesin binding may be tissue-specific, as it could not be detected at D4Z4 in lymphoblastoid cells of control and FSHD individuals (Chapter 3), is very intriguing and may perhaps partly explain why FSHD patients mainly present with muscular symptoms. However, binding of these two proteins in additional primary cell lines has to be tested. Especially since lymphoblastoid cell lines are obtained by infection of PBLs with Epstein-Barr virus (EBV). Such treatment may have an effect on mechanisms like histone modifications and DNA methylation. If indeed no HP1γ/cohesin binding to D4Z4 is observed in several additional cell types, it will be crucial to determine the function and consequences of HP1γ/cohesin binding to D4Z4. For some years, it has been speculated that FSHD may be regarded as a nuclear envelopathy. The observation that the subtelomere of chromosome 4q localizes to the nuclear periphery [54, 55], but also the overlap in expression profiles between patients with FSHD and Emery-Dreifuss muscular dystrophy (EDMD) [56], have fueled this hypothesis. Our data deliver additional evidence for a possible link between FSHD pathogenesis and the nuclear envelope, because HP1 is known for its interaction with

the lamin B receptor (LBR) in the nuclear lamina [57]. As a result of loss of HP1γ binding to D4Z4 in patients with FSHD, this interaction with the nuclear lamina may be lost. An initial experiment to study this hypothesis is to perform a ChIP study using an antibody against LBR in cells of controls and FSHD patients followed by (quantitative) PCR with D4Z4-specific primers. Another method is to knock down HP1γ by siRNA and determine the binding of LBR to D4Z4 by ChIP. If indeed this interaction with the nuclear lamina is lost, other interactions, for example via the DNA binding protein barrier to autointegration factor (BAF), may also be lost. All these interactions can be studied in more detail using ChIP and treating control cells with siRNA against different proteins in the nuclear lamina.

Another important experiment will be to determine whether the nuclear peripheral organization of the subtelomere of chromosome 4q is lost in FSHD, e.g. using 3D-FISH. Previously, two studies could not observe a significant change in peripheral organization in FSHD patient cells, although one of these studies observed a small non-significant detachment from the nuclear periphery [54, 55]. This indicates that the changes may be very subtle; thus, larger groups of patients and controls have to be studied. Treatment of control cells with siRNA against HP1γ may increase the differences in peripheral organization. Finally, as HP1γ binding to D4Z4 may be tissue-specific, it may be interesting to study peripheral organization of 4qter in different cell types. Thus far, the peripheral localization of the subtelomere of chromosome 4q was studied in a limited amount of lymphoblastoid, fibroblast and myoblast cell lines. In these studies, D4Z4 was located in the nuclear periphery in all tested cell types [54, 55]. Therefore, it seems unlikely that HP1γ has a major role in D4Z4 tethering to the nuclear periphery, but still HP1γ loss may greatly influence the interactions between D4Z4 and the nuclear lamina.

The protein cohesin is mainly known for its role during mitosis where it holds the sister chromatids together [58]. Recently, an additional function of cohesin together with the insulator protein CTCF was reported [59]. Importantly, four different CTCF binding sites were identified in the D4Z4 repeat array. CTCF binding to these sites is enriched in patients with FSHD (G. Filippova, personal communication) [60]. As CTCF and cohesin proteins may compete for similar binding sites [61], the loss of cohesin binding to D4Z4 in FSHD patients may explain the increased binding of CTCF. To study this in more detail, a cohesin ChIP using primers that specifically amplify the four CTCF binding sites may be performed.

8.4 Haplotype-specific sequence variations; the effect on transcription and protein binding

We observed that D4Z4 contraction results in a more open chromatin structure irrespective of the haplotype of the contracted allele (Chapter 4). Therefore, it seems crucial to study the effect of haplotype-specific sequence changes and particularly 4qA161-specific, thus FSHD-specific, sequence variations. First, transcription of FSHD candidate genes, like *DUX4, FRG1* and *FRG2*, may be affected. Second, binding of certain proteins may vary between the different haplotypes because of sequence variations.

To study the first option, transfection studies with D4Z4 elements representing the different haplotypes and/or haplotype-specific SNPs may be informative. Both RNA and protein levels of the *DUX4* candidate gene could be determined. Using a similar system, *DUX4* expression was measured for the first time two years ago. Both an intronless transcript from each D4Z4 repeat unit [53] and a transcript with two introns which is transcribed from the most distal D4Z4 repeat unit [34] were identified. In addition, several new sense and antisense transcripts originating from D4Z4 were recognized very recently. All identified transcripts seem to be generated from the DUX4 transcript from the distal D4Z4 repeat unit and not only novel mRNAs but also mi/siRNA-sized RNA fragments were identified [62]. Another possibility is to use luciferase reporter assay that determine the effect of specific SNPs and/or haplotypes on the promoter activity of the FSHD candidate genes. Previously, this method was successfully applied to determine the effect of one or multiple D4Z4 repeat units on *FRG2* promoter activity [7]. However, it is still unclear which transcript(s) is(are) responsible for FSHD pathogenesis; it has been hypothesized that a combinatorial action of candidate genes may underlie this disease. Therefore, a third possibility is to quantitatively compare RNA isolated from cells transfected with the different haplotypes on a genome-wide scale using either expression arrays covering the whole genome or performing genome-wide deep sequencing of (mi)RNA transcripts. This way, not only the expression of thus far identified FSHD candidate genes is determined. Instead, genome-wide expression differences between the different haplotypes of chromosomes 4q and 10q are determined. Preferably, all studies should be performed in human muscle cells.

Second, the haplotype-specific SNPs may affect the binding of certain proteins to the D4Z4 region. Examples of proteins that bind to the D4Z4 repeat array are YY1 and indirectly HMGB2 and nucleolin [9]. Thus far, no difference in binding of this repressor complex in FSHD patients and control individuals has been observed. However, FSHD

research is complicated by the presence of homologous D4Z4-like sequences on many chromosomes, including the highly homologous repeat array on chromosome 10q [63, 64]. Therefore, it will be very important to develop haplotype-specific primers that can be used in ChIP experiments to specifically study the binding of proteins to the different haplotypes. Alternatively, one could focus on the identification of additional proteins that bind to the D4Z4 repeat array. Using the sequences of the different haplotypes and software programs that specifically identify protein binding sites, proteins that bind haplotype-specific may be identified. Next, these protein binding sites may be confirmed by electrophoretic mobility shift assay (EMSA) and ChIP; techniques that study protein binding to DNA *in vitro* or *in vivo*, respectively. Also the recently developed PICh (proteomics of isolated chromatin segments) technique that identifies the proteins bound to a specific genomic region by combining ChIP and mass spectrometric analysis may be suitable for this type of experiments [65].

8.5 Treatment of patients with FSHD

As described in Chapter 5, supplementation with folic acid and methionine did not significantly increase the methylation level at the D4Z4 repeat array, neither in FSHD patients nor in control individuals. It is unclear whether the intervention was sufficient to remethylate D4Z4 and whether the D4Z4 locus is susceptible to DNA remethylation. Therefore, different conditions can be tested by *in vitro* treatment of myoblasts of FSHD patients with different concentrations of folic acid for different time periods. Further, the addition of vitamin B12 as a supplement may increase the remethylation of D4Z4 significantly, since vitamin B12 is an important co-regulatory enzyme in the folate cycle [23].

A more specific method to remethylate D4Z4 may be by the use of short methylated oligonucleotides which induce DNA methylation at specific loci. When such oligonucleotides were applied to the proximal promoter region of the human oncogene Bcl-2, gene silencing of the gene occurred [66]. However, *Bcl-2* is a single-copy gene, while D4Z4-like sequences can be found on many chromosomes [63, 64, 67]. In addition, to apply this method in FSHD patients more information on transcription from the D4Z4 region is needed, as the oligonucleotides have to be directed towards the regions responsible for FSHD pathogenesis. Finally, we observed that DNA methylation may be downstream of H3K9me3 at D4Z4 (Chapter 3); thus, the lack of results in the initial pilot study with folic acid and methionine may partly be explained by this. The addition

of the two supplements may not have resulted in higher methylation levels at D4Z4 because H3K9me3 levels were still low. Probably, higher levels of H3K9me3 have to be present at D4Z4 to recruit DNMT3B and thus to remethylate D4Z4. However, since DNMT3B is considered a *de novo* DNA methyltransferase that mainly functions during embryogenesis [68], this treatment may not be effective.

A better idea might be to perform an experiment to determine whether addition of the histone methyltransferase SUV39H1 has an effect on H3K9me3 levels at D4Z4 and consequently on D4Z4 methylation levels. An initial experiment studying the effect of transfection of FSHD myoblasts with a construct containing the SUV39H1 sequence may be performed. Such a SUV39H1 construct has already been successfully used to transfect human cells [69]. Readouts of a SUV39H1 overexpression experiment will be the determination of H3K9me3 levels at D4Z4 by ChIP, the D4Z4 methylation levels by methylation-sensitive Southern blot analysis and the expression levels of FSHD candidate genes by RT-PCR. Also, control cells may be transfected with the construct to determine the side-effects of the treatment. A disadvantage of this method is that SUV39H1 not only targets the D4Z4 repeat array but also additional genomic regions. However, SUV39H1 mainly localizes to pericentromeric heterochromatin and telomeres, because of its main function in heterochromatic gene silencing [70]. Probably, most histone H3 proteins associated with these genomic regions will already be completely methylated. Nevertheless, the risk exists that regions that are normally unmethylated become methylated and a consequence is that genes that are normally expressed may be silenced. This is especially dangerous when SUV39H1 overexpression affects tumor suppressor genes. Therefore, it will be better to design a construct that is only recruited to the genomic region that has to be treated. The design of such a construct will be hindered by the presence of D4Z4 repeats not only on chromosomes 4q and 10q, but also on all acrocentric chromosomes [63, 64, 67].

9 References

[1] Wijmenga C, Frants RR, Brouwer OF, Moerer P, Weber JL, Padberg GW. Location of facioscapulohumeral muscular dystrophy gene on chromosome 4., Lancet 336 (1990) 651-653.

[2] Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG, Gruter AM, Hofker MH, Moerer P, Williamson R. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy., Nat. Genet. 2 (1992) 26-30.

[3] van Deutekom JC, Wijmenga C, van Tienhoven EA, Gruter AM, Hewitt JE, Padberg GW, van Ommen GJ, Hofker MH, Frants RR. FSHD associated DNA rearrangements are due to deletions of integral copies of a 3.2 kb tandemly repeated unit., Hum. Mol. Genet. 2 (1993) 2037-2042.

[4] Haraguchi Y, Chung AB, Torroni A, Stepien G, Shoffner JM, Wasmuth JJ, Costigan DA, Polak M, Altherr MR, Winokur ST. Genetic mapping of human heart-skeletal muscle adenine nucleotide translocator and its relationship to the facioscapulohumeral muscular dystrophy locus, Genomics 16 (1993) 479-485.

[5] Wijmenga C, Winokur ST, Padberg GW, Skraastad MI, Altherr MR, Wasmuth JJ, Murray JC, Hofker MH, Frants RR. The human skeletal muscle adenine nucleotide translocator gene maps to chromosome 4q35 in the region of the facioscapulohumeral muscular dystrophy locus, Hum. Genet. 92 (1993) 198-203.

[6] van Deutekom JC, Lemmers RJ, Grewal PK, van Geel M, Romberg S, Dauwerse HG, Wright TJ, Padberg GW, Hofker MH, Hewitt JE, Frants RR. Identification of the first gene (FRG1) from the FSHD region on human chromosome 4q35, Hum. Mol. Genet. 5 (1996) 581-590.

[7] Rijkers T, Deidda G, van Koningsbruggen S, van Geel M, Lemmers RJ, van Deutekom JC, Figlewicz D, Hewitt JE, Padberg GW, Frants RR, van der Maarel SM. FRG2, an FSHD candidate gene, is transcriptionally upregulated in differentiating primary myoblast cultures of FSHD patients., J. Med. Genet. 41 (2004) 826-836.

[8] Gabriels J, Beckers MC, Ding H, De Vriese A, Plaisance S, van der Maarel SM, Padberg GW, Frants RR, Hewitt JE, Collen D, Belayew A. Nucleotide sequence of the partially deleted D4Z4 locus in a patient with FSHD identifies a putative gene within each 3.3 kb element., Gene 236 (1999) 25-32.

[9] Gabellini D, Green MR, Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle, Cell 110 (2002) 339-348.

[10] Jiang G, Yang F, Van Overveld PG, Vedanarayanan V, van der Maarel S, Ehrlich M. Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q., Hum. Mol. Genet. 12 (2003) 2909-2921.

[11] Winokur ST, Chen YW, Masny PS, Martin JH, Ehmsen JT, Tapscott SJ, van der Maarel SM, Hayashi Y, Flanigan KM. Expression profiling of FSHD muscle supports a defect in specific stages of myogenic differentiation, Hum. Mol. Genet. 12 (2003) 2895-2907.

[12] Celegato B, Capitanio D, Pescatori M, Romualdi C, Pacchioni B, Cagnin S, Vigano A, Colantoni L, Begum S, Ricci E, Wait R, Lanfranchi G, Gelfi C. Parallel protein and transcript profiles of FSHD patient muscles correlate to the D4Z4 arrangement and reveal a common impairment of slow to fast fibre differentiation and a general deregulation of MyoD-dependent genes, Proteomics. 6 (2006) 5303-5321.

[13] Osborne RJ, Welle S, Venance SL, Thornton CA, Tawil R. Expression profile of FSHD supports a link between retinal vasculopathy and muscular dystrophy, Neurology 68 (2007) 569-577.

[14] Van Overveld PG, Lemmers RJ, Sandkuijl LA, Enthoven L, Winokur ST, Bakels F, Padberg GW, van Ommen GJ, Frants RR, van der Maarel SM. Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy., Nat. Genet. 35 (2003) 315-317.

[15] Kondo T, Bobek MP, Kuick R, Lamb B, Zhu X, Narayan A, Bourc'his D, Viegas-Pequignot E, Ehrlich M, Hanash SM. Whole-genome methylation scan in ICF syndrome: hypomethylation of non-satellite DNA repeats D4Z4 and NBL2., Hum. Mol. Genet. 9 (2000) 597-604.

[16] Jiang YL, Rigolet M, Bourc'his D, Nigon F, Bokesoy I, Fryns JP, Hulten M, Jonveaux P, Maraschio P, Megarbane A, Moncla A, Viegas-Pequignot E. DNMT3B mutations and DNA methylation defect define two types of ICF syndrome, Hum. Mutat. 25 (2005) 56-63.

[17] Maraschio P, Zuffardi O, Dalla Fior T, Tiepolo L. Immunodeficiency, centromeric heterochromatin instability of chromosomes 1, 9, and 16, and facial anomalies: the ICF syndrome, J. Med. Genet. 25 (1988) 173-180.

[18] Jeanpierre M, Turleau C, Aurias A, Prieur M, Ledeist F, Fischer A, Viegas-Pequignot E. An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome, Hum. Mol. Genet. 2 (1993) 731-735.

[19] Ebert A, Lein S, Schotta G, Reuter G. Histone modification and the control of heterochromatic gene silencing in Drosophila, Chromosome. Res. 14 (2006) 377-392.

[20] Bernstein BE, Humphrey EL, Erlich RL, Schneider R, Bouman P, Liu JS, Kouzarides T, Schreiber SL. Methylation of histone H3 Lys 4 in coding regions of active genes, Proc. Natl. Acad. Sci. U. S. A 99 (2002) 8695-8700.

[21] Lemmers RJ, Wohlgemuth M, van der Gaag KJ, van der Vliet P, van Teijlingen CM, de Knijff P, Padberg GW, Frants RR, van der Maarel SM. Specific sequence variations within the 4q35 region are associated with facioscapulohumeral muscular dystrophy., Am. J. Hum. Genet. 81 (2007) 884-894.

[22] Robertson KD, Wolffe AP. DNA methylation in health and disease, Nat. Rev. Genet. 1 (2000) 11-19.

[23] Fenech M. The role of folic acid and Vitamin B12 in genomic stability of human cells, Mutat. Res. 475 (2001) 57-67.

[24] Ricci E, Galluzzi G, Deidda G, Cacurri S, Colantoni L, Merico B, Piazzo N, Servidei S, Vigneti E, Pasceri V, Silvestri G, Mirabella M, Mangiola F, Tonali P, Felicetti L. Progress in the molecular diagnosis of facioscapulohumeral muscular dystrophy and correlation between the number of KpnI repeats at the 4q35 locus and clinical phenotype., Ann. Neurol. 45 (1999) 751-757.

[25] Razin SV, Iarovaia OV, Sjakste N, Sjakste T, Bagdoniene L, Rynditch AV, Eivazova ER, Lipinski M, Vassetzky YS. Chromatin domains and regulation of transcription, J. Mol. Biol. 369 (2007) 597-607.

[26] Mirkovitch J, Mirault ME, Laemmli UK. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold, Cell 39 (1984) 223-232.

[27] Petrov A, Pirozhkova I, Carnac G, Laoudj D, Lipinski M, Vassetzky YS. Chromatin loop domain organization within the 4q35 locus in facioscapulohumeral dystrophy patients versus normal human myoblasts, Proc. Natl. Acad. Sci. U. S. A 103 (2006) 6982-6987.

[28] Petrov A, Allinne J, Pirozhkova I, Laoudj D, Lipinski M, Vassetzky YS. A nuclear matrix attachment site in the 4q35 locus has an enhancer-blocking activity in vivo: implications for the facio-scapulo-humeral dystrophy, Genome Res. 18 (2008) 39-45.
[29] Stratling WH, Yu F. Origin and roles of nuclear matrix proteins. Specific functions of the MAR-binding protein MeCP2/ARBP, Crit Rev. Eukaryot. Gene Expr. 9 (1999) 311-318.
[30] Tsumagari K, Qi L, Jackson K, Shao C, Lacey M, Sowden J, Tawil R, Vedanarayanan V, Ehrlich M. Epigenetics of a tandem DNA repeat: chromatin DNaseI sensitivity and opposite methylation changes in cancers, Nucleic Acids Res. (2008).
[31] Dekker J, Rippe K, Dekker M, Kleckner N. Capturing chromosome conformation, Science 295 (2002) 1306-1311.
[32] Tolhuis B, Palstra RJ, Splinter E, Grosveld F, de Laat W. Looping and interaction between hypersensitive sites in the active beta-globin locus, Mol. Cell 10 (2002) 1453-1465.
[33] Pirozhkova I, Petrov A, Dmitriev P, Laoudj D, Lipinski M, Vassetzky Y. A functional role for 4qA/B in the structural rearrangement of the 4q35 region and in the regulation of FRG1 and ANT1 in facioscapulohumeral dystrophy, PLoS. ONE. 3 (2008) e3389.
[34] Dixit M, Ansseau E, Tassin A, Winokur S, Shi R, Qian H, Sauvage S, Matteotti C, van Acker AM, Leo O, Figlewicz D, Barro M, Laoudj-Chenivesse D, Belayew A, Coppee F, Chen YW. DUX4, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PITX1, Proc. Natl. Acad. Sci. U. S. A 104 (2007) 18157-18162.
[35] Lemmers RJ, de Kievit P, Sandkuijl L, Padberg GW, van Ommen GJ, Frants RR, van der Maarel SM. Facioscapulohumeral muscular dystrophy is uniquely associated with one of the two variants of the 4q subtelomere., Nat. Genet. 32 (2002) 235-236.
[36] Padberg GW, Lunt PW, Koch M, Fardeau M. Diagnostic criteria for facioscapulohumeral muscular dystrophy., Neuromuscul. Disord. 1 (1991) 231-234.
[37] Lemmers RJL, de Kievit P, van Geel M, van der Wielen MJ, Bakker E, Padberg GW, Frants RR, van der Maarel SM. Complete allele information in the diagnosis of facioscapulohumeral muscular dystrophy by triple DNA analysis, Ann. Neurol. 50 (2001) 816-819.
[38] Zhang Y, Forner J, Fournet S, Jeanpierre M. Improved characterization of FSHD mutations, Ann. Genet. 44 (2001) 105-110.
[39] Lemmers RJ, Wohlgemuth M, Frants RR, Padberg GW, Morava E, van der Maarel SM. Contractions of D4Z4 on 4qB subtelomeres do not cause facioscapulohumeral muscular dystrophy., Am. J. Hum. Genet. 75 (2004) 1124-1130.
[40] Funakoshi M, Goto K, Arahata K. Epilepsy and mental retardation in a subset of early onset 4q35-facioscapulohumeral muscular dystrophy, Neurology 50 (1998) 1791-1794.
[41] Brouwer OF, Padberg GW, Ruys CJ, Brand R, de Laat JA, Grote JJ. Hearing loss in facioscapulohumeral muscular dystrophy, Neurology 41 (1991) 1878-1881.
[42] Padberg GW, Brouwer OF, de Keizer RJ, Dijkman G, Wijmenga C, Grote JJ, Frants RR. On the significance of retinal vascular disease and hearing loss in facioscapulohumeral muscular dystrophy., Muscle Nerve 2 (1995) S73-S80.
[43] Bastress KL, Stajich JM, Speer MC, Gilbert JR. The genes encoding for D4Z4 binding proteins HMGB2, YY1, NCL, and MYOD1 are excluded as candidate genes for FSHD1B, Neuromuscul. Disord. 15 (2005) 316-320.
[44] Tsien F, Sun B, Hopkins NE, Vedanarayanan V, Figlewicz D, Winokur S, Ehrlich M. Methylation of the FSHD syndrome-linked subtelomeric repeat in normal and FSHD cell cultures and tissues., Mol. Genet. Metab 74 (2001) 322-331.

[45] Hansen RS, Wijmenga C, Luo P, Stanek AM, Canfield TK, Weemaes CM, Gartler SM. The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome, Proc. Natl. Acad. Sci. U. S. A 96 (1999) 14412-14417.

[46] Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X, Russo JJ, Viegas-Pequignot E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene, Nature 402 (1999) 187-191.

[47] Hagleitner MM, Lankester A, Maraschio P, Hulten M, Fryns JP, Schuetz C, Gimelli G, Davies EG, Gennery A, Belohradsky BH, de Groot R, Gerritsen EJ, Mattina T, Howard PJ, Fasth A, Reisli I, Furthner D, Slatter MA, Cant AJ, Cazzola G, van Dijken PJ, van Deuren M, de Greef JC, van der Maarel SM, Weemaes CM. Clinical spectrum of immunodeficiency, centromeric instability and facial dysmorphism (ICF syndrome)., J. Med. Genet. 45 (2008) 93-99.

[48] Ehrlich M, Sanchez C, Shao C, Nishiyama R, Kehrl J, Kuick R, Kubota T, Hanash SM. ICF, an immunodeficiency syndrome: DNA methyltransferase 3B involvement, chromosome anomalies, and gene dysregulation, Autoimmunity 41 (2008) 253-271.

[49] Lander ES, Botstein D. Homozygosity mapping: a way to map human recessive traits with the DNA of inbred children, Science 236 (1987) 1567-1570.

[50] Fuks F, Hurd PJ, Wolf D, Nan X, Bird AP, Kouzarides T. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation, J. Biol. Chem. 278 (2003) 4035-4040.

[51] Lehnertz B, Ueda Y, Derijck AA, Braunschweig U, Perez-Burgos L, Kubicek S, Chen T, Li E, Jenuwein T, Peters AH. Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin, Curr. Biol. 13 (2003) 1192-1200.

[52] Handa V, Jeltsch A. Profound flanking sequence preference of Dnmt3a and Dnmt3b mammalian DNA methyltransferases shape the human epigenome, J. Mol. Biol. 348 (2005) 1103-1112.

[53] Kowaljow V, Marcowycz A, Ansseau E, Conde CB, Sauvage S, Matteotti C, Arias C, Corona ED, Nunez NG, Leo O, Wattiez R, Figlewicz D, Laoudj-Chenivesse D, Belayew A, Coppee F, Rosa AL. The DUX4 gene at the FSHD1A locus encodes a pro-apoptotic protein., Neuromuscul. Disord. 17 (2007) 611-623.

[54] Masny PS, Bengtsson U, Chung SA, Martin JH, van Engelen B, van der Maarel SM, Winokur ST. Localization of 4q35.2 to the nuclear periphery: is FSHD a nuclear envelope disease?, Hum. Mol. Genet. 13 (2004) 1857-1871.

[55] Tam R, Smith KP, Lawrence JB. The 4q subtelomere harboring the FSHD locus is specifically anchored with peripheral heterochromatin unlike most human telomeres, J. Cell Biol. 167 (2004) 269-279.

[56] Bakay M, Wang Z, Melcon G, Schiltz L, Xuan J, Zhao P, Sartorelli V, Seo J, Pegoraro E, Angelini C, Shneiderman B, Escolar D, Chen YW, Winokur ST, Pachman LM, Fan C, Mandler R, Nevo Y, Gordon E, Zhu Y, Dong Y, Wang Y, Hoffman EP. Nuclear envelope dystrophies show a transcriptional fingerprint suggesting disruption of Rb-MyoD pathways in muscle regeneration, Brain 129 (2006) 996-1013.

[57] Ye Q, Worman HJ. Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to Drosophila HP1, J. Biol. Chem. 271 (1996) 14653-14656.

[58] Losada A, Hirano M, Hirano T. Identification of Xenopus SMC protein complexes required for sister chromatid cohesion, Genes Dev. 12 (1998) 1986-1997.

[59] Wendt KS, Yoshida K, Itoh T, Bando M, Koch B, Schirghuber E, Tsutsumi S, Nagae G, Ishihara K, Mishiro T, Yahata K, Imamoto F, Aburatani H, Nakao M, Imamoto N, Maeshima K, Shirahige K, Peters JM. Cohesin mediates transcriptional insulation by CCCTC-binding factor, Nature 451 (2008) 796-801.

[60] Ottaviani A, Rival-Gervier S, Boussouar A, Foerster AM, Rondier D, Sacconi S, Desnuelle C, Gilson E, Magdinier F. The D4Z4 macrosatellite repeat acts as a CTCF and A-type lamins-dependent insulator in facio-scapulo-humeral dystrophy, PLoS. Genet. 5 (2009) e1000394.

[61] Parelho V, Hadjur S, Spivakov M, Leleu M, Sauer S, Gregson HC, Jarmuz A, Canzonetta C, Webster Z, Nesterova T, Cobb BS, Yokomori K, Dillon N, Aragon L, Fisher AG, Merkenschlager M. Cohesins functionally associate with CTCF on mammalian chromosome arms, Cell 132 (2008) 422-433.

[62] Snider L, Asawachaicharn A, Tyler AE, Geng LN, Petek LM, Maves L, Miller DG, Lemmers RJ, Winokur ST, Tawil R, van der Maarel SM, Filippova GN, Tapscott SJ. RNA transcripts, mRNA-sized fragments and proteins produced from D4Z4 units: new candidates for the pathophysiology of facioscapulohumeral dystrophy, Hum Mol Genet 18 (2009) 2414-2430.

[63] Bakker E, Wijmenga C, Vossen RH, Padberg GW, Hewitt J, van der Wielen M, Rasmussen K, Frants RR. The FSHD-linked locus D4F104S1 (p13E-11) on 4q35 has a homologue on 10qter., Muscle Nerve 2 (1995) S39-S44.

[64] Deidda G, Cacurri S, Grisanti P, Vigneti E, Piazzo N, Felicetti L. Physical mapping evidence for a duplicated region on chromosome 10qter showing high homology with the facioscapulohumeral muscular dystrophy locus on chromosome 4qter., Eur. J. Hum. Genet. 3 (1995) 155-167.

[65] Dejardin J, Kingston RE. Purification of proteins associated with specific genomic Loci, Cell 136 (2009) 175-186.

[66] Hoffman AR, Hu JF. Directing DNA methylation to inhibit gene expression, Cell Mol. Neurobiol. 26 (2006) 425-438.

[67] Lyle R, Wright TJ, Clark LN, Hewitt JE. The FSHD-associated repeat, D4Z4, is a member of a dispersed family of homeobox-containing repeats, subsets of which are clustered on the short arms of the acrocentric chromosomes, Genomics 28 (1995) 389-397.

[68] Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development, Cell 99 (1999) 247-257.

[69] Krouwels IM, Wiesmeijer K, Abraham TE, Molenaar C, Verwoerd NP, Tanke HJ, Dirks RW. A glue for heterochromatin maintenance: stable SUV39H1 binding to heterochromatin is reinforced by the SET domain, J. Cell Biol. 170 (2005) 537-549.

[70] Schotta G, Ebert A, Reuter G. SU(VAR)3-9 is a conserved key function in heterochromatic gene silencing, Genetica 117 (2003) 149-158.