Studies of the epigenetic disease mechanism in FSHD

Greef, J.C. de

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

Jessica C. de Greef[1]
Richard J.L.F. Lemmers[1]
Baziel G.M. van Engelen[2]
Sabrina Sacconi[3]
Shannon L. Venance[4]
Rune R. Frants[1]
Rabi Tawil[5]
Silvère M. van der Maarel[1]

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1 Department of Human Genetics, Leiden University Medical Center (LUMC), Leiden, The Netherlands

2 Department of Neurology, University Medical Center Nijmegen, Nijmegen, The Netherlands

3 Centre de référence des Maladies Neuromusculaires, Nice Hospital and CNRS UMR 6543, University of Nice, Nice, France

4 Department of Clinical Neurological Sciences, London Health Sciences Centre, London, ON, Canada

5 Neuromuscular Disease Center, University of Rochester Medical Center, Rochester, USA

Abstract

Facioscapulohumeral muscular dystrophy (FSHD), caused by partial deletion of the D4Z4 macrosatellite repeat on chromosome 4q, has a complex genetic and epigenetic etiology. To develop FSHD, D4Z4 contraction needs to occur on a specific genetic background. Only contractions associated with the 4qA161 haplotype cause FSHD. In addition, contraction of the D4Z4 repeat in FSHD patients is associated with significant D4Z4 hypomethylation. To date however, the methylation status of contracted repeats on non-pathogenic haplotypes has not been studied. We have performed a detailed methylation study of the D4Z4 repeat on chromosome 4q and on a highly homologous repeat on chromosome 10q. We show that patients with a D4Z4 deletion (FSHD1) have D4Z4-restricted hypomethylation. Importantly, controls with a D4Z4 contraction on a non-pathogenic chromosome 4q haplotype or on chromosome 10q also demonstrate hypomethylation. In fifteen FSHD families without D4Z4 contractions but with at least one 4qA161 haplotype (FSHD2), we observed D4Z4-restricted hypomethylation on chromosomes 4q and 10q. This finding implies that a genetic defect resulting in D4Z4 hypomethylation underlies FSHD2. In conclusion, we describe two ways to develop FSHD; (1) *contraction-dependent* or (2) *contraction-independent* D4Z4 hypomethylation on the 4qA161 subtelomere.

Introduction

Macrosatellite DNA is composed of large and highly homologous repeat units that are arranged in tandem over regions that typically exceed 50-100 kb. Thus far, several classes of macrosatellite repeat arrays have been identified in the human genome, most of which are located at centromeres. One example is satellite DNA which has an important role in the formation and maintenance of the centromeric chromatin structure by specific interactions with DNA-binding proteins and by establishing heterochromatin formation through an RNAi mechanism [1]. Macrosatellite repeats that are non-centromerically located have also been identified. Examples include the RNU2, RS447, DXZ4 and D4Z4 repeats. All these repeats are highly polymorphic; they usually vary between few and >100 units [2-5].

Currently, the only disease-associated macrosatellite repeat is the D4Z4 repeat array. Contraction of D4Z4 in the subtelomere of chromosome 4q is associated with autosomal dominant facioscapulohumeral muscular dystrophy (FSHD1 [OMIM 158900]) [6]. The

D4Z4 repeat array consists of 3.3 kb repeat units, normally varying in numbers between 11 and 100 copies (>40 kb). In the majority of patients with FSHD, a contraction of the repeat array to 1-10 repeat units (<40 kb) is observed [7, 8]. Monosomy of 4q does not cause disease suggesting a critical role for the D4Z4 repeat unit in the etiology of FSHD [9]. Sequences homologous to D4Z4 have been identified on many, mainly acrocentric, chromosomes [10]. Due to an ancient duplication event, the subtelomere of chromosome 10q also contains a D4Z4-like repeat [11, 12] and in ~20% of individuals subtelomeric exchanges between repeats on 4q and 10q can be observed [13]. However, repeat contractions on 10q do not cause FSHD, although ~25% of chromosomes 10q carry a repeat array of 10 units or less [14, 15].

With the initial identification of two allelic variants of the 4q subtelomere, 4qA and 4qB [16] and the recent further specification into 9 different haplotypes of chromosome 4q [17], it has become evident that D4Z4 contraction alone is not sufficient to cause FSHD. Instead, it needs to occur on a specific genetic background, the 4qA161 haplotype. The 4qA161 haplotype is the most prevalent A haplotype in the Caucasian population and can be observed in ~39% of control individuals [17]. Thus far, only contractions in the 4qA161 haplotype have been shown to cause FSHD, while contractions in other 4q haplotypes such as 4qA166 and 4qB163 are non-pathogenic [17-19]). Therefore, it is hypothesized that haplotype-specific sequence polymorphisms are mechanistically linked to FSHD pathogenesis [17].

Some non-centromeric macrosatellite repeat arrays, including D4Z4, are extremely GC-rich, making them attractive candidates for DNA methylation. DNA methylation is a common modification of mammalian DNA which occurs at cytosine residues of CpG dinucleotides, a process executed by DNA methyltransferases. Often, DNA methylation is associated with increased chromatin condensation and gene silencing [20]. The D4Z4 repeat array is frequently considered heterochromatic because of the presence of the low-copy-repeats hhspm3 and LSau, repetitive sequences that are mainly found in heterochromatic regions of the human genome [21]. However, several studies have indicated that D4Z4 has both euchromatic and heterochromatic features [22, 23]. Interestingly, DNA methylation of the proximal D4Z4 repeat unit on the disease allele was significantly reduced in patients with FSHD1, both in DNA isolated from peripheral blood lymphocytes (PBLs) and from muscle [24]. Also in a small group of FSHD2 patients without D4Z4 contraction but with clinical symptoms indistinguishable from FSHD1 patients, significant D4Z4 hypomethylation at both chromosome 4q alleles was observed [24, 25].

Thus far, a limited number of CpG dinucleotides of the ~290 CpGs in each D4Z4 repeat unit have been tested for DNA methylation. Because of technical limitations, only in a small group of patients could the methylation level of the disease allele be studied, while in all other cases, the observed methylation levels were confounded by the simultaneous analysis of D4Z4 repeats on the normal chromosome 4q carrying a normal-sized D4Z4 repeat array [24, 26]. These limitations have left many questions unanswered about the nature and extent of D4Z4 hypomethylation, including: (1) What is the methylation level of internal D4Z4 repeat units compared to the proximal D4Z4 repeat unit?, (2) What is the methylation level of a contracted D4Z4 repeat on a non-pathogenic 4q haplotype?, (3) What is the methylation level of a contracted D4Z4 repeat on chromosome 10q?, (4) Is there a relationship between D4Z4 repeat size and D4Z4 methylation? and (5) What is the methylation level of the D4Z4 repeats on chromosome 10q in FSHD2 patients?

To address these questions, we performed detailed methylation analyses of the D4Z4 repeat array using the methylation-sensitive restriction enzyme *Cpo*I, which enabled us to interrogate D4Z4 methylation on proximal and internal repeat units on chromosomes 4q and 10q separately. Importantly, we also examined the DNA of several unique control individuals, including individuals carrying contracted D4Z4 repeats on non-pathogenic haplotypes. Our results demonstrate that a generic mechanism at the D4Z4 repeats results in significant hypomethylation upon contraction of the repeat array below a certain threshold, and that this change is observed irrespective of the haplotype on which the D4Z4 contraction occurs. Furthermore, FSHD2 patients show D4Z4 hypomethylation not only on both chromosomes 4q, but also on both chromosomes 10q, which implies that in these individuals a genetic defect responsible for methylation of D4Z4 may exist. Importantly, all FSHD2 patients carry at least one copy of the pathogenic 4qA161 haplotype. In conclusion, we show that epigenetic changes in D4Z4 at the pathogenic 4qA161 haplotype underlie and unify both FSHD1 and FSHD2.

Materials and Methods

Most protocols used in this paper and other protocols used by researchers of the Fields Center in FSHD and neuromuscular research are described in detail on the Fields Center website http://www.urmc.rochester.edu/fields-center. The Fields Center for FSHD and Neuromuscular Research is an international collaboration between the University of Rochester Medical Center (USA) and the Leiden University Medical Center (The Netherlands) that performs clinical and genetic research to find treatments for patients with FSHD.

Patients and controls

All individuals included in this study were analyzed previously for their allele size, constitution of repeats arrays on chromosomes 4q and 10q and haplotypes after informed consent was obtained [17, 18]. In total, DNA of 70 control individuals, both family members and unrelated individuals (16 monosomic, 40 disomic and 14 trisomic), and DNA of 54 FSHD1 patients with a D4Z4 contraction (10 monosomic, 29 disomic and 15 trisomic) were analyzed. For a detailed explanation on monosomic, disomic and trisomic individuals see the Results section of this article. DNA of 18 FSHD2 patients without a D4Z4 contraction was collected from fifteen families. These FSHD2 patients are mainly single cases, but also two sibling pairs and an affected mother and daughter are present within the group of FSHD2 patients. FSHD2 patients present with a phenotype indistinguishable from FSHD1 patients with a contraction of D4Z4 on the 4qA161 haplotype.

DNA isolation and cell lines

Genomic DNA was isolated from PBLs or from myoblasts and myotubes in culture. DNA was extracted using a standard salt extraction protocol [27]. Myoblasts were cultured in DMEM F-10 medium (Invitrogen) with heat-inactivated fetal calf serum (Invitrogen), penicillin/streptomycin (Invitrogen), basic human recombinant fibroblast growth factor (Promega) and dexametazone (Sigma Aldrich). When grown until 80% confluency, DNA was isolated from part of the myoblast culture. The remaining myoblasts were induced to differentiate into myotubes using DMEM medium containing glucose (Sigma Aldrich), L-glutamine (Invitrogen) and sodium pyruvate (Invitrogen) with heat-inactivated horse serum (Invitrogen).

*Cpo**I** methylation analysis*

For methylation analysis of the proximal D4Z4 repeat unit on either chromosome 4q or chromosome 10q, we applied a method illustrated in Figure 1A and 1C and modified from methods described previously [24]. For methylation analysis of internal D4Z4 repeats units on either chromosome 4q or 10q, we used the same Southern blot membranes being used for methylation analysis of the proximal unit, but the membrane was hybridized with the D4Z4 probe [28] instead of the p13E-11 (D4F104S1) probe [7] (Figure 1B and 1D). Briefly, 5 µg of genomic DNA was first digested with the restriction enzymes *Eco*91I (MBI Fermentas; isoschizomer of *Bst*EII) and either *Bln*I (Takara Bio Inc) or *Xap*I (MBI Fermentas) for methylation analysis of chromosomes 4q or 10q, respectively. Next, the methylation-

sensitive restriction enzyme *Cpo*I (MBI Fermentas) was used, both for chromosome 4q and for chromosome 10q methylation analysis. All digestion reactions were performed according to the manufacturer's instructions. After digestion, DNA was separated by linear gel electrophoresis on a 0.8% agarose gel (Invitrogen) followed by Southern blotting of the DNA on a hybond-XL membrane (GE Healthcare) and hybridization of the membrane first with the radioactive labeled probe p13E-11 for methylation determination of the proximal D4Z4 repeat unit [7] and subsequently with the D4Z4 probe for methylation analysis of internal D4Z4 repeat units [28]. Hybridizations for p13E-11 were performed for a minimum of 16 hours at 65 °C in a buffer containing 0.125 M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 1 mM EDTA, 7% SDS and 100 µg/ml denatured fish sperm DNA (Roche). Hybridizations for D4Z4 were performed for at least 16 hours at 62 °C in a buffer containing 0.125M Na_2HPO_4 (pH 7.2), 0.25 M NaCl, 7% SDS, 50% formamide and 100 µg/ml denatured fish sperm DNA (Roche). After hybridization, membranes labeled with p13E-11 were washed with 2xSSC/0.1%SDS, followed by washing with 1xSSC/0.1%SDS and a final wash step with 0.3xSSC/0.1%SDS. D4Z4-labeled membranes were washed first with 2xSSC/0.05%SDS and second with 0.1xSSC/0.1%SDS. Finally, the membranes were exposed to a phosphor-imager screen and signal intensities were analyzed and quantified with ImageQuant software (Molecular Dynamics).

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Figure 1

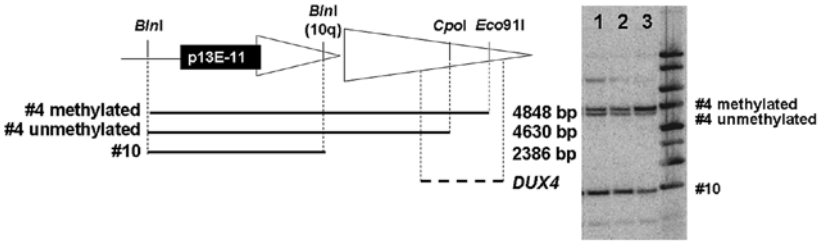
Schematic overview of the CpoI methylation analyses on chromosomes 4q and 10q.

Genomic DNA was digested with either Eco91I/BlnI (A, B) or Eco91I/XapI (C, D) to separate D4Z4 repeat units on chromosome 4q from those on chromosome 10q. Subsequently, DNA was digested with the methylation-sensitive restriction enzyme CpoI. Lines represent the different fragment sizes obtained after digestion, Southern blotting and visualization by hybridization with the p13E-11 probe for the proximal D4Z4 repeat unit (A, C) or with the D4Z4 probe for internal D4Z4 repeat units (B, D). Probe recognition sequences are indicated with black boxes. In the repeat unit the location of the DUX4 open reading frame (ORF) is indicated.

Besides the schematic overview of each methylation analysis a final gel picture showing three DNA samples as examples is depicted.

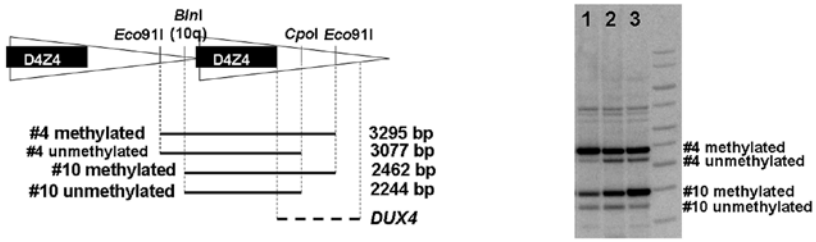
A

CHROMOSOME 4 – PROXIMAL UNIT (*Cpo*I site)



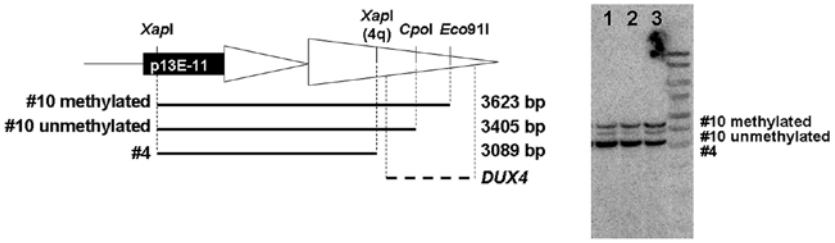
B

CHROMOSOME 4 – INTERNAL UNITS (*Cpo*I site)



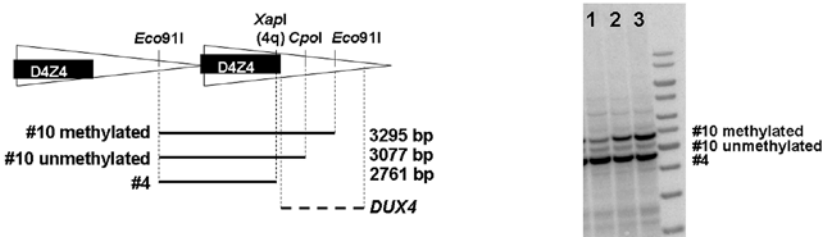
C

CHROMOSOME 10 – PROXIMAL UNIT (*Cpo*I site)



D

CHROMOSOME 10 – INTERNAL UNITS (*Cpo*I site)



*Sma*I *methylation analysis*

For methylation analysis of the region proximal to the p13E-11 region and the D4Z4 repeat array on either chromosome 4q or chromosome 10q, we applied a method illustrated in Supplemental Figure S1 and modified from methods described previously [24]. 5 µg of genomic DNA was first digested with the restriction enzyme *BseM*I (MBI Fermentas). Next, the methylation-sensitive restriction enzyme *Sma*I (MBI Fermentas) was used. All digestion reactions were performed according to the manufacturer's instructions. After digestion, DNA was separated by linear gel electrophoresis followed by Southern blotting of the DNA as described above for the *Cpo*I methylation analysis. Next, the membranes were hybridized with the p13E-11 (D4F104S1) probe [7]. Hybridization, washing conditions and quantification of signal intensities were as described above for the *Cpo*I methylation analysis.

Statistical analyses

Samples showing an incorrect ratio of signal intensities with the p13E-11 hybridization were excluded from the analyses; appropriate 4:10 ratios can only be obtained upon full digestion. In case of a disomic individual with two 4-type D4Z4 repeats and two 10-type D4Z4 repeats the ratio of signal intensities between all bands representing chromosome 4q and all bands representing chromosome 10q should be 1:1. In monosomic and trisomic individuals with respectively one 4-type D4Z4 repeat and three 10-type D4Z4 repeats or three 4-type D4Z4 repeats and one 10-type D4Z4 repeat, the correct signal intensities are 1:3 and 3:1. In addition, at random samples were spiked with an equimolar amount of plasmid DNA containing one D4Z4 repeat to test for complete digestion by hybridization with an empty vector probe (Supplemental Figure S2).
Statistical evaluation of the methylation levels between the different groups was done by either Mann-Whitney U tests or independent Student's T-tests. The mean of a data set is given as mean ± standard deviation. To determine whether the occurrence of at least one repeat on the pathogenic 4qA161 haplotype in FSHD2 patients was not due to chance, we compared the frequency of nine possible 4q haplotypes in the 18 FSHD2 patients with the frequency of the same nine 4q haplotypes in 222 control individuals using Pearson Chi-Square test.

Results

For all experiments described in this paper, muscle cell and lymphocyte DNA of control individuals and FSHD patients was used. Not all individuals carried a standard allele constitution of two 4-type (*Bln*I-resistant) D4Z4 repeat arrays on chromosome 4q and two 10-type (*Xap*I-resistant) D4Z4 repeat arrays on chromosome 10q (Supplemental Figure S3A; disomic). Instead, large groups of individuals with either a translocated 10-type repeat on one chromosome 4q (Supplemental Figure S3B; monosomic) or a translocated 4-type repeat on chromosome 10q (Supplemental Figure S3C; trisomic) were studied. An important advantage of using DNA material from these individuals is that by judicious use of specific restriction enzymes, methylation levels can be determined on a single allele (i.e. a single 4-type D4Z4 repeat array on chromosome 4q in case of monosomic individuals or a single 10-type D4Z4 repeat array on chromosome 10q in case of trisomic individuals) without the interference of the methylation level of the second allele. We used the restriction enzyme *Bln*I to digest 10-type repeat units; for digestion of 4-type repeat units the restriction enzyme *Xap*I was added. In monosomic individuals with a single 4-type repeat on chromosome 4q, the translocated repeat on chromosome 4q is composed of both 4- and 10-type repeat units (Supplemental Figure S3B) [29]. In trisomic individuals with a single 10-type repeat on chromosome 10q, the translocated repeat on chromosome 10q is homogenous, thus consists solely of 4-type repeat units (Supplemental Figure S3C) [29-30].

D4Z4 hypomethylation on chromosome 4q in FSHD1 patients

To confirm previous observations of D4Z4 hypomethylation in patients with FSHD1, we analyzed a large group of controls and FSHD1 patients at the *Cpo*I site in the proximal D4Z4 repeat unit on chromosome 4q (Figure 1A). For most analyses DNA isolated from PBLs was used. As with two other tested previously tested restriction sites [24], FSHD1 patients displayed significantly lower methylation levels compared with controls (Figure 2A; $P \leq 0.010$). The hypomethylation was most pronounced in monosomic FSHD1 patients where the methylation status was determined specifically on the contracted allele ($P < 0.001$). As FSHD primarily affects skeletal muscle, we also measured methylation levels in muscle cell lines, which also showed D4Z4 hypomethylation in FSHD1 patients. In addition, differentiation of myoblasts to myotubes did not change D4Z4 methylation (Supplemental Figure S4A).

Next, using the same membranes that were used to determine the *Cpo*I methylation level at the proximal D4Z4 unit, but instead performing a hybridization with the D4Z4 probe [28], we determined the average methylation level of internal D4Z4 repeat units on chromosome 4q (Figure 1B). We also observed significant hypomethylation of the *Cpo*I site in internal D4Z4 units of disomic FSHD1 patients (Figure 2A; $P=0.013$). However, methylation levels on internal units were 20-25% higher than on the proximal unit, which may be explained by the numerical overrepresentation of *Cpo*I sites with a high methylation level on the normal-sized allele in FSHD1 patients. Also the results for internal units in monosomic FSHD1 patients were much higher than expected (Figure 2A), but translocated 10-type repeats on chromosome 4q are mixtures of both 4q- and 10q-derived units [29] and therefore obscure the analysis of internal repeat units in the disease allele.

D4Z4 hypomethylation on chromosome 4q in controls

Since only the 4qA161 haplotype is associated with FSHD [17], we hypothesized that D4Z4 hypomethylation may be restricted to this haplotype. The group of controls described above included one individual with a 24 kb D4Z4 repeat array on the non-pathogenic haplotype 4qA166, thus not resulting in FSHD [17]. The methylation level at the proximal *Cpo*I site in this individual was very low (33%), similar to the levels on disease alleles of FSHD1 patients (Figure 2A). As we were unable to test additional controls carrying a single contracted 4-type repeat array on a non-pathogenic haplotype, we included disomic FSHD1 patients with two contracted D4Z4 repeats, one on the pathogenic 4qA161 allele and one on a non-pathogenic haplotype. The methylation levels on proximal and internal units in this group of FSHD1 patients were significantly reduced compared to the methylation levels in the group of patients with a single contracted allele (Figure 2B; $P \leq 0.033$). Thus, we conclude that D4Z4 hypomethylation is not restricted to the disease haplotype on chromosome 4q, but also occurs in contracted repeats of other 4q haplotypes.

D4Z4 hypomethylation on chromosome 10q

Next, we investigated whether D4Z4 contraction-dependent hypomethylation is chromosome-specific and studied *Cpo*I methylation in proximal and internal units on chromosome 10q. As before, we applied methylation-sensitive Southern blotting, but instead of adding the restriction enzyme *Bln*I to remove 10-type repeats from our assay,

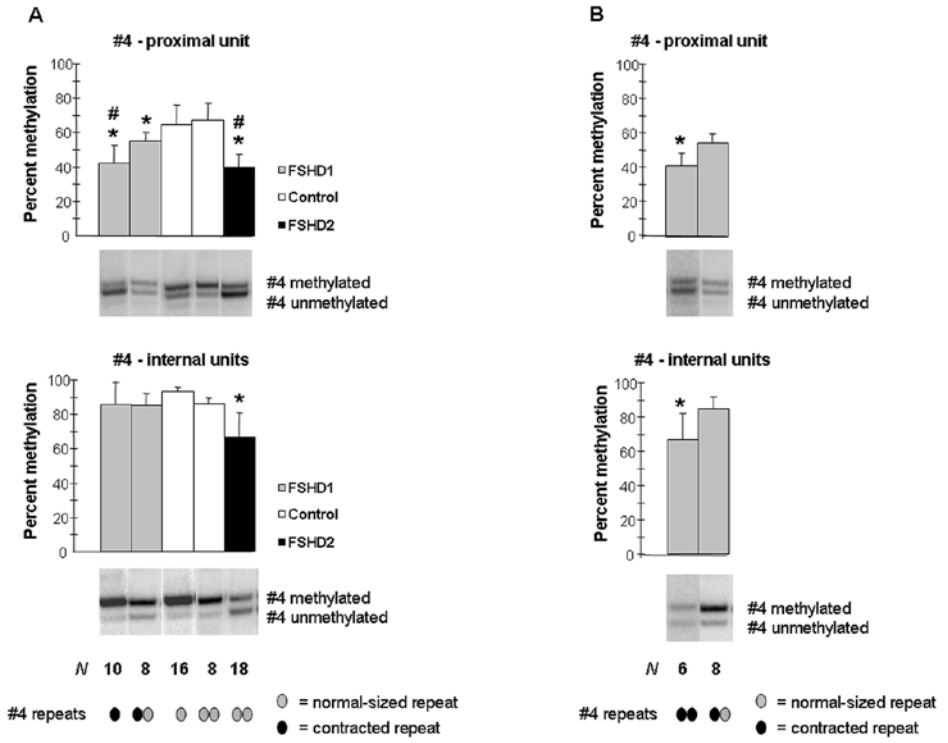


Figure 2

Bar diagrams of CpoI methylation analyses in proximal and internal D4Z4 repeat units on chromosome 4q.

Below each bar an example of a representative gel picture is shown. Below the graphs the number of tested individuals per group and the allele constitution of each group are indicated. In monosomic individuals the methylation level of a single repeat on chromosome 4q was determined (indicated by a single button). In disomic individuals the methylation level of both repeats on chromosome 4q is measured simultaneously (indicated by two buttons). A grey button represents a normal-sized (>40 kb) repeat, a black button denotes a contracted (<40 kb) repeat.

A. *CpoI methylation on chromosome 4q in PBL DNA from FSHD1 patients, control individuals and FSHD2 patients (upper panel: methylation level of proximal D4Z4 repeat unit; lower panel: methylation level of internal D4Z4 repeat units). *P<0.05 versus controls with a single 4-type repeat (monosomic) and P<0.05 versus controls with two 4-type repeats (disomic). #P<0.05 versus FSHD1 patients with two 4-type repeats (disomic). Data are presented as mean ± s.d.*

B. *CpoI methylation on chromosome 4q in PBL DNA from FSHD1 patients with two contracted repeats or from FSHD1 patients with one contracted repeat and one normal-sized repeat (upper panel: methylation level of proximal D4Z4 repeat unit; lower panel: methylation level of internal D4Z4 repeat units). *P<0.05. Data are presented as mean ± s.d.*

we added the restriction enzyme *Xap*I which specifically removes 4-type repeats. Next, we hybridized each membrane with the p13E-11 probe [6] to determine methylation on the proximal repeat unit (Figure 1C) and subsequently with the D4Z4 probe [28] to establish methylation at internal repeat units (Figure 1D). We analyzed individuals with a standard allele constitution (Supplemental Figure S3A) or with a single 10-type repeat on chromosome 10q (Supplemental Figure S3C).

First, we compared *Cpo*I methylation levels between controls and FSHD1 patients. No significant differences were observed; not in the proximal unit and not in internal units (Figure 3A). Also, methylation levels in muscle cell lines of controls and FSHD1 patients were comparable (Supplemental Figure S4B). Second, to mimic the situation on chromosome 4q, we looked at *Cpo*I methylation on chromosome 10q by dividing all individuals into four groups; two groups containing individuals, both controls and FSHD1 patients, with a repeat array smaller than 40 kb and two groups containing individuals, both controls and FSHD1 patients, with a repeat array larger than 40 kb. Similar to the results on chromosome 4q, a D4Z4 repeat array <40 kb on chromosome 10q resulted in a significantly lower methylation level at the *Cpo*I site, both in proximal and internal units (Figure 3B; $P \leq 0.032$). We conclude that D4Z4 methylation is repeat size-dependent but does not depend on either the haplotype or the chromosome in which it resides, as the presence of a contracted repeat on chromosome 10q also results in D4Z4 hypomethylation.

D4Z4 hypomethylation on chromosomes 4q and 10q: a threshold effect

Previously, we reported that in the proximal D4Z4 repeat unit FSHD1 patients with 1-3 residual repeat units show pronounced hypomethylation; FSHD1 patients with 4-8 residual repeat units show large interindividual variation in hypomethylation. In controls, no clear linear relationship between D4Z4 methylation and the repeat length was established [31]. However, for these studies only a small group of monosomic FSHD1 patients with a single 4-type repeat array was available. As disomic FSHD1 patients with two contracted repeats on chromosome 4q showed significantly lower D4Z4 methylation levels than disomic FSHD1 patients with a single contracted repeat (Figure 2B), we aimed to study disomic individuals grouped for comparable repeat sizes on chromosomes 4q or 10q in a more extensive correlation study.

We determined *Cpo*I methylation in disomic individuals with two repeats of approximately 30 kb (7 D4Z4 repeat units), 55 kb (14 D4Z4 repeat units), 80 kb

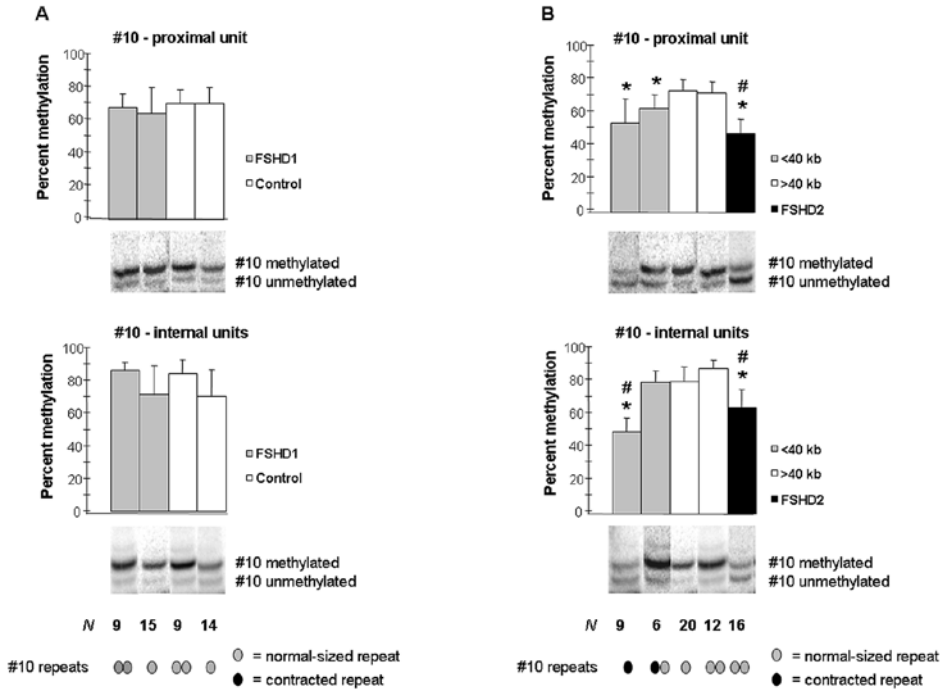


Figure 3
Bar diagrams of CpoI methylation analyses in proximal and internal D4Z4 repeat units on chromosome 10q.

Below each bar an example of a representative gel picture is shown. Below the graphs the number of tested individuals per group and the allele constitution of each group are indicated. In disomic individuals the methylation level of both repeats on chromosome 10q is measured simultaneously (indicated by two buttons). In trisomic individuals the methylation level of a single repeat on chromosome 10q was determined (indicated by a single button). A grey button represents a normal-sized (>40 kb) repeat, a black button denotes a contracted (<40 kb) repeat.

A. CpoI methylation on chromosome 10q in PBL DNA from FSHD1 patients and control individuals (upper panel: methylation level of proximal D4Z4 repeat unit; lower panel: methylation level of internal D4Z4 repeat units). Data are presented as mean ± s.d.
*B. CpoI methylation on chromosome 10q in PBL DNA from individuals with one repeat <40 kb (1-10 units) and one repeat >40 kb (≥11 units), in individuals with two repeats >40 kb (≥11 units) and in FSHD2 patients (upper panel: methylation level of proximal D4Z4 repeat unit; lower panel: methylation level of internal D4Z4 repeat units). *P<0.05 versus individuals with a single 10-type repeat >40 kb (trisomic) and P<0.05 versus individuals with two 10-type repeats >40 kb (disomic). #P<0.05 versus individuals with one 10-type repeat <40 kb and one 10-type repeat >40 kb (disomic). Data are presented as mean ± s.d.*

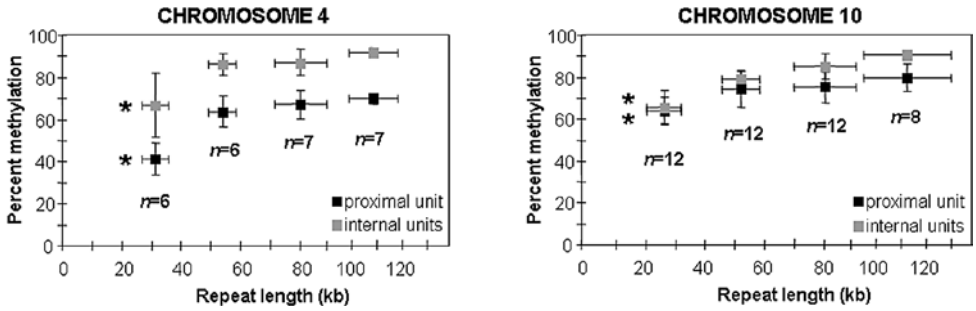


FIGURE 4

A threshold effect for DNA methylation at the D4Z4 repeat array.

*CpoI methylation levels on chromosomes 4q and 10q in PBL DNA from individuals with homozygous 30 kb (7 units), 55 kb (14 units), 80 kb (22 units) or 105 kb (30 units) D4Z4 repeats. *P<0.05 versus all groups. Data are presented as mean ± s.d.*

(22 D4Z4 repeat units) or 105 kb (30 D4Z4 repeat units) on chromosomes 4q or 10q (Figure 4). Both on proximal and internal units on chromosomes 4q and 10q, methylation was significantly lower in the 30 kb group ($P \leq 0.024$), while in the 55 kb group methylation levels reached levels comparable to those observed in the 80 kb and 105 kb groups. This absence of a linear relationship between repeat size and DNA methylation was not an artifact of DNA saturation in the Southern blot procedure, as in serial dilutions of four DNA samples we found similar methylation levels in the range of 1.25 to 5 µg of genomic DNA (data not shown). Thus, our results suggest a clear reduction in D4Z4 methylation below a certain threshold of repeat units, while above this threshold methylation levels are overall not strongly influenced by repeat size.

HYPOMETHYLATION IS RESTRICTED TO THE D4Z4 REPEAT ON CHROMOSOMES 4q AND 10q

In a subset of control individuals and FSHD1 patients we also tested the methylation-sensitive *Sma*I restriction site immediately proximal to D4Z4 (Supplemental Figure S1). No differences in DNA methylation were observed between FSHD1 patients and controls, not on chromosome 4q and not on chromosome 10q (Figure 5). Thus, hypomethylation of the FSHD locus seems to be restricted to the D4Z4 repeat.

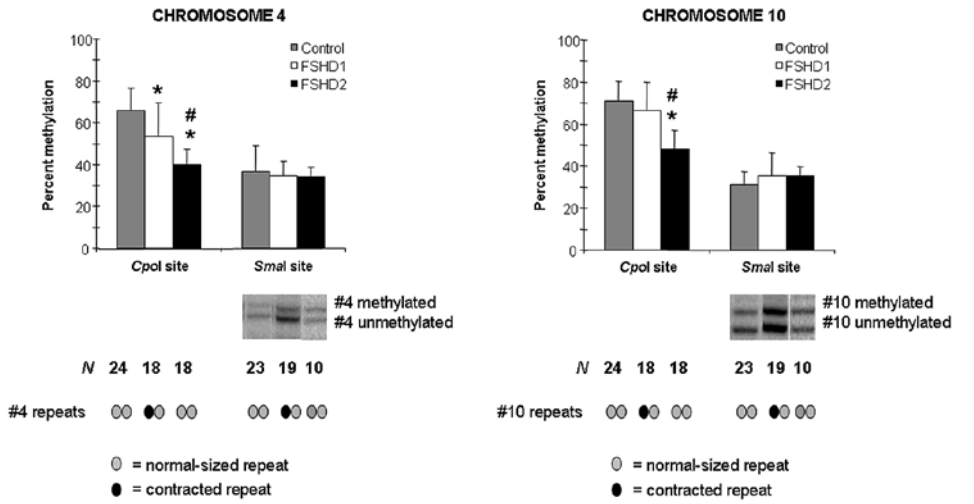


Figure 5

Hypomethylation is restricted to the D4Z4 repeat array in FSHD.

*Bar diagrams of methylation levels on chromosomes 4q and 10q in the proximal D4Z4 repeat unit (CpoI site) and in the region immediately proximal to D4Z4 (SmaI site). Methylation levels were determined in PBL DNA from control individuals, FSHD1 patients and FSHD2 patients. Below each bar of the SmaI site an example of a representative gel picture is shown. Below the graphs the number of tested individuals per group and the allele constitution of each group are indicated. *P<0.05 versus control. #P<0.05 versus FSHD1. Data are presented as mean ± s.d.*

D4Z4 hypomethylation on chromosomes 4q and 10q in FSHD2 patients

In addition to FSHD1, a subset of patients with FSHD does not have D4Z4 contractions (FSHD2). Currently, we have collected fifteen of those FSHD2 families totaling 19 patients (Table 1). These patients were assigned as FSHD2 patients when (1) a phenotype consistent with FSHD1 was observed, (2) no D4Z4 contraction was identified by pulsed field gel electrophoresis (PFGE) and (3) a methylation level of ≤20% was measured at the *Fse*I restriction site. The *Fse*I site was previously used to study D4Z4 methylation. Methylation levels ≤20% were only detected in a small cohort of FSHD2 patients while FSHD1 patients showed on average 33% methylation at the *Fse*I restriction site [24]. Some FSHD2 cases have been described before [24, 25], but most of them were collected recently. In all of them, we observed significant *Cpo*I hypomethylation in proximal and internal units on chromosomes 4q and 10q (Figure 2A, 3A and 3B; $P \leq 0.002$). Like in FSHD1 patients, the proximal *Sma*I restriction site showed normal methylation levels

in patients with FSHD2 (Figure 5). Importantly, we noticed that all FSHD2 patients that met the three criteria stated above carried at least one repeat on the pathogenic 4qA161 haplotype (Table 1; P=0.005). Only in two potential FSHD2 patients of a large family (Table 1; 2.3 & 2.5) no 4qA161 allele was identified. However, in these patients also no D4Z4 hypomethylation was observed, suggestive of the possible presence of an additional muscular dystrophy in this family. In addition, in one of these two patients there was insufficient clinical certainty of FSHD [25].

Discussion

Previously, we reported that an epigenetic mechanism, D4Z4 hypomethylation, might play a role in the pathogenesis of FSHD. At that time, two CpGs in the proximal D4Z4 repeat unit on chromosome 4q were investigated and PFGE analysis suggested that the methylation level at the proximal repeat unit was representative for the entire repeat array [24]. In the present study, we investigated the methylation level at the proximal and internal D4Z4 repeat units on chromosomes 4q and 10q in controls and patients with FSHD using methylation-sensitive Southern blot analysis. Southern blot-based methylation analysis can be applied to a large number of individuals and all additional CpGs we tested using this method with the use of other methylation-sensitive restriction enzymes showed a similar degree in loss of DNA methylation in FSHD1 patients (Figure 6A). This proves that Southern blot-based methylation analysis of D4Z4 is a reliable and robust method to study DNA methylation changes in this repeat. In contrast, bisulphite conversion-based methylation analysis of D4Z4 is very challenging because of the dispersion of numerous D4Z4 homologous sequences in the human genome. This method would necessitate separation of D4Z4 alleles prior to bisulphite conversion precluding the analysis of large cohorts of individuals.

Contraction-dependent hypomethylation at D4Z4 is a generic mechanism

We demonstrated that contraction-associated hypomethylation is not restricted to FSHD1 patients. On the contrary, both on chromosomes 4q and 10q, a repeat <40 kb is associated with significant D4Z4 hypomethylation at the *Cpo*I site, irrespective of the haplotype of the repeat (Figure 2 and 3). We also observed D4Z4 hypomethylation in controls carrying a repeat <40 kb on 4qA166, 4qB163 and 10qA166 haplotypes. This suggests that hypomethylation as a consequence of repeat contraction is a common

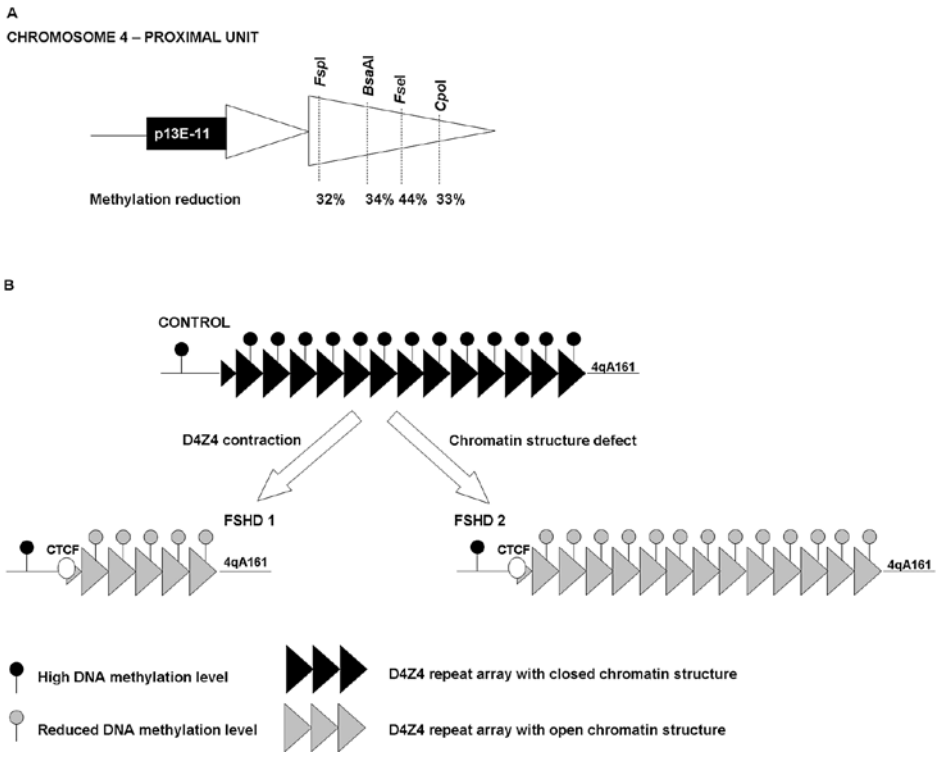


Figure 6

The combination of an epigenetic change in D4Z4 on a 4qA161 haplotype unifies FSHD1 and FSHD2.

A. *Schematic overview of four methylation-sensitive restriction sites in the D4Z4 repeat array. The methylation levels on the BsaAI and FseI restriction sites were reported earlier [24]. The methylation levels on the CpoI site are presented in this paper.The methylation levels on the FspI site are unpublished results. Compared to control individuals the D4Z4 methylation level on these four sites is on average 32-44% reduced in FSHD1 patients.*

B. *D4Z4 contraction-induced chromatin changes are the cause for FSHD1 while a yet unidentified factor that affects the D4Z4 chromatin structure causes FSHD2. Importantly, this phenomenon needs to occur on the 4qA161 haplotype. Binding of CTCF to the proximal end of the D4Z4 repeat may prevent spreading of hypomethylation proximally in patients with FSHD.*

mechanism. A similar mechanism may apply to other macrosatellite repeat arrays with a high GC content, like RNU2 [3, 32] and DXZ4 [2, 33]. It will be interesting to determine the relationship between repeat length and DNA methylation in these two macrosatellite repeats.

D4Z4 repeat size and chromosomal context determine D4Z4 methylation

At the *Cpo*I site, the D4Z4 repeats on chromosomes 4q and 10q behaved mostly in a similar fashion, but some discrete differences were noted (Figure 4). First, on chromosome 10q the proximal unit was almost similarly methylated as in internal units. In contrast on chromosome 4q the proximal unit was clearly less methylated compared to internal sites. Second, the sudden decrease in D4Z4 methylation below the threshold of 40 kb (11 D4Z4 repeat units) is more prominent on chromosome 4q. Third, while methylation levels remained constant above 40 kb on chromosome 4q, a small increase in methylation levels, especially when all repeat units were measured simultaneously, with increasing D4Z4 repeat length was observed on chromosome 10q. Therefore, we hypothesize that not only repeat size, but also chromosomal context, is important for D4Z4 methylation. We expect that a contracted repeat on chromosome 4q, irrespective of its composition, will show lower methylation levels than a contracted repeat on chromosome 10q. This idea is supported by data in a single control individual with a contracted 4qA166 repeat. In this individual a very low methylation level was detected, comparable to methylation levels in FSHD1 patients with a contracted repeat on the pathogenic 4qA161 haplotype and much lower than methylation levels in individuals with a contracted 10qA166 repeat. Interestingly, the sequence of the 4qA166 haplotype is most similar to the sequence of the 10qA166 haplotype on chromosome 10q (unpublished results) [17].

D4Z4 hypomethylation is necessary but not sufficient for FSHD development

We observed a sudden decrease in D4Z4 methylation below a certain threshold on chromosomes 4q and 10q (Figure 4). This steep increase in D4Z4 methylation between repeats of 7 units (30 kb) and 14 units (55 kb) may explain the relatively abrupt transition from pathogenic D4Z4 repeat sizes (1-10 units) to non-pathogenic D4Z4 repeat sizes (≥11 units). However, as D4Z4 hypomethylation was also observed in controls with a contracted repeat on a non-pathogenic haplotype, we hypothesize that D4Z4 hypomethylation is necessary but not sufficient to develop FSHD. Other epigenetic

factors or haplotype-specific sequence polymorphisms may eventually determine the development of the FSHD phenotype.

D4Z4 hypomethylation is restricted to the D4Z4 repeat array

The observed D4Z4 hypomethylation in FSHD1 and FSHD2 patients is restricted to the D4Z4 repeat array, as no differences in DNA methylation were observed between FSHD patients and controls at the *Sma*I site immediately proximal to D4Z4 (Figure 5). This argues against a *cis* spreading effect from D4Z4 in proximal direction. Also analysis of histone modifications in 4qter argues against this disease model for FSHD [22]. We propose that the recently identified CTCF binding site at the proximal end of the D4Z4 repeat (G. Filippova, personal communication) may prevent spreading of hypomethylation in a proximal direction. Also another CTCF binding site in the insulator portion of D4Z4 was recently reported on [34]. It will be interesting to study the methylation level at these sites in patients with FSHD and control individuals, since CTCF binding may be methylation-dependent and CTCF binding prevents propagation of DNA methylation [35].

Proximal to the D4Z4 repeat array several genes are located of which FSHD region gene 1 *(FRG1)*, FSHD region gene 2 *(FRG2)* and adenine nucleotide translocator 1 *(ANT1)* have been proposed to be causally involved based on their transcriptional deregulation in FSHD [36]. Interestingly, muscle-specific overexpression of *FRG1* in transgenic mice leads to a muscular dystrophy phenotype with missplicing of muscle-specific mRNAs [37]. Increased expression of *ANT1* seems to sensitize muscle cells to oxidate stress and apoptosis [38]. The absence of *FRG2* on the disease alleles in some FSHD patients with a proximal deletion makes this gene a less attractive candidate gene [39]. However, transcriptional deregulation of these candidate genes in patients with FSHD is still under debate, as several studies showed contradictory results [22, 40-43]. Based on our data showing that hypomethylation is restricted to the D4Z4 repeat and not spreading in a proximal direction in addition to previous data showing no change in the chromatin structure of proximal sequences in FSHD [22], we do not support a *cis*-spreading mechanism emanating from D4Z4 in FSHD.

FSHD2 patients showed D4Z4 hypomethylation not only on both chromosomes 4 but also on both chromosomes 10 (Figure 2 and 3). This argues that FSHD2 is not caused by a *cis* effect on chromosome 4q but that a genetic defect responsible for DNA methylation of D4Z4 underlies this condition. In addition, as judged from our methylation studies immediately proximal to D4Z4 also in these patients, hypomethylation does no spread beyond the D4Z4 repeat, arguing that a similar yet contraction-independent disease mechanism is operating in these patients.

Previously, we have sequenced several candidate genes involved in chromatin structure, including *DNMT1, DNMT3A, DNMT3B, DNMT3L, MTHFS, LMNA, CBX2, CBX5* and *SUV39H1,* in a subset of FSHD2 patients. However, no disease-specific SNPs or mutations were identified [25]. Furthermore, the hypomethylation in FSHD2 patients seems restricted to D4Z4, as no hypomethylation of other repeats, including satellite 2 and 3 DNA, α-satellite DNA and the NBL2 repeat, was observed in these patients [25].

As judged from the fact that all FSHD2 patients in our collection carry at least one pathogenic 4qA161 allele (Table 1), we hypothesize that to develop FSHD a change in the chromatin structure of D4Z4 and a 4qA161 allele are required, as was already noted in FSHD1 patients, where the D4Z4 contraction is always associated with the 4qA161 haplotype [17]. Our data are further supported by a study of histone modifications in the D4Z4 repeat array. A loss of the histone modification histone H3 lysine 9 trimethylation followed by a secondary loss of the heterochromatin protein 1γ and the cohesin complex to the D4Z4 repeat array was observed in both FSHD1 and FSHD2 patients [44]. It will be crucial to identify 4qA161-specific sequence polymorphisms and show their effect on the binding of proteins to the D4Z4 repeat or on the production of transcripts from the D4Z4 repeat. Expression of two different transcripts from D4Z4 was reported previously [45, 46]. While the first transcript is transcribed from internal repeat units, the second transcript is transcribed from the distal D4Z4 repeat unit. Importantly, the pLAM sequence distal to the D4Z4 repeat may provide a polyadenylation signal for the second transcript and this sequence is only present on 4qA alleles [17, 45]. Recently, new evidence was presented on the complex transcriptional activity of the D4Z4 repeat [47]. We hypothesize that the observed changes in DNA methylation in both FSHD1 and FSHD2 patients may affect the transcriptional activity on the 4qA161 haplotype.

In conclusion, in this study we showed that D4Z4 hypomethylation is not restricted to FSHD1 patients. A contracted repeat (<40 kb) on a non-pathogenic haplotype

	Status	4q repeat (1)	4q repeat (2)	*Fse*I methylation
1.1	Control	96 kb 4qA161	173 kb 4qB163	68%
1.2	FSHD2	96 kb 4qA161	170 kb 4qA161	12%
1.3	FSHD2	96 kb 4qA161	170 kb 4qA161	19%
1.4	Control	75 kb 4qB163	170 kb 4qA161	16%
2.1	FSHD2	50 kb 4qA161	90 kb 4qB163	5%
2.2	FSHD2	50 kb 4qA161	135 kb 4qB163	4%
2.3	FSHD?	48 kb 4qB163	90 kb 4qB163	41%
2.4	Control	90 kb 4qB163	128 kb 4qB168	53%
2.5	FSHD?	90 kb 4qB163	130 kb 4qB168	63%
3.1	FSHD2	65 kb 4qA161	110 kb 4qA161	17%
3.2	Control	65 kb 4qA161	75 kb 4qB163	47%
4.1	Control	75 kb 4qA161	160 kb 4qB163	56%
4.2	FSHD2	50 kb 4qA161	70 kb 4qA161	15%
4.3	Control	90 kb 4qB163	160 kb 4qB163	55%
5.1	FSHD2	48 kb 4qA161	120 kb 4qB168	13%
6.1	FSHD2	55 kb 4qA161	70 kb 4qA161	13%
6.2	FSHD2	55 kb 4qA161	60 kb 4qB163	17%
7.1	FSHD2	42 kb 4qA161	65 kb 4qB163	2%
8.1	Control	80 kb 4qA161	95 kb 4qB162	36%
8.2	FSHD2	75 kb 4qA161	80 kb 4qA161	14%
8.3	Control	48 kb 4qA161	80 kb 4qA161	64%
8.4	FSHD2	48 kb 4qA161	95 kb 4qB162	9%
8.5	Control	48 kb 4qA161	75 kb 4qA161	49%
9.1	FSHD2	48 kb 4qA161	205 kb 4qA161	7%
10.1	FSHD2	48 kb 4qA161	100 kb 4qA166	12%
11.1	FSHD2	46 kb 4qA161	128 kb 4qA161	10%
12.1	FSHD2	65 kb 4qA161	160 kb 4qA161	13%
12.2	Control	65 kb 4qA161	160 kb 4qA161	50%
12.3	Control	131 kb 4qA161	160 kb 4qB168	45%
12.4	Control	145 kb 4qB163	160 kb 4qB168	11%
13.1	FSHD2	40 kb 4qA161	155 kb 4qA161	16%
14.1	FSHD2	40 kb 4qA161	100 kb 4qB163	17%
15.1	FSHD2	50 kb 4qA161	147 kb 4qB168	20%

Table 1

FSHD2 patients have at least one hypomethylated 4qA161 repeat.

Overview of fifteen FSHD2 families consisting of 19 FSHD2 patients with pronounced hypomethylation in the proximal D4Z4 repeat unit at the FseI restriction site [24]. Only the combination of a 4qA161 allele and D4Z4 hypomethylation results in FSHD. Family 1, 2, 3, 4 and 5 were described previously [25].

also results in significantly lower methylation levels. Thus, our results suggest that a general mechanism of DNA hypomethylation occurs upon D4Z4 repeat contraction. Furthermore, the methylation defect in FSHD2 patients also includes hypomethylation on chromosome 10q. A genetic defect in D4Z4 methylation may underlie the disease in these patients. Finally, we noted that all FSHD2 patients carried at least one repeat on the pathogenic 4qA161 haplotype. Therefore, we conclude that there are two different conditions predisposing to the development of FSHD: *contraction-dependent* and *contraction-independent* epigenetic changes in D4Z4. In both forms of FSHD, the resultant D4Z4 hypomethylation needs to occur on the 4qA161 haplotype (Figure 6B). This commonality will facilitate and guide the identification of the molecular basis for FSHD.

Acknowledgements

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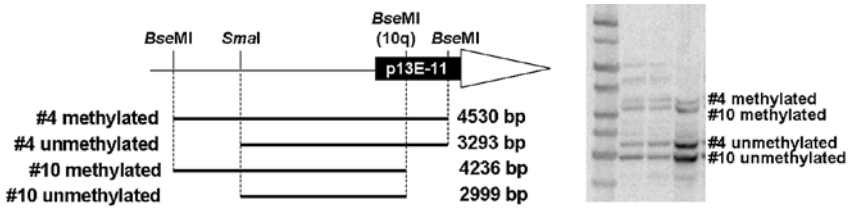
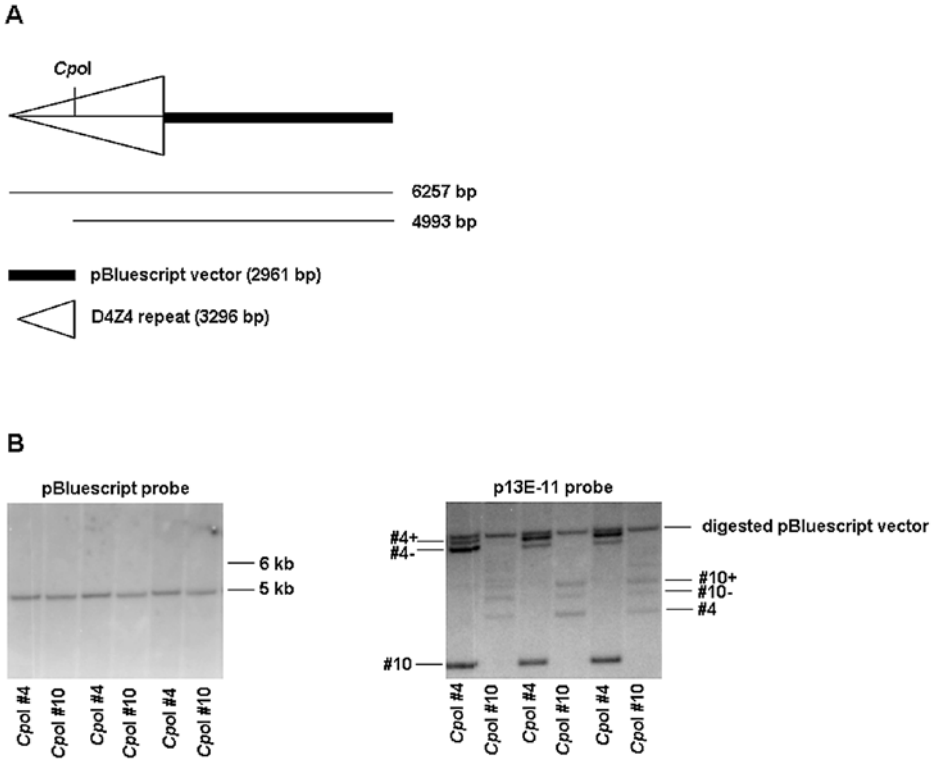
Supplemental information



Supplemental Figure S1

Schematic overview of the SmaI methylation analysis on chromosomes 4q and 10q.

Genomic DNA was digested with BseMI to separate D4Z4 repeat units on chromosome 4q from those on chromosome 10q. Subsequently, DNA was digested with the methylation-sensitive restriction enzyme SmaI. Lines represent the different fragment sizes obtained after digestion, Southern blotting and visualization by hybridization with the p13E-11 probe (indicated with black box). Besides the schematic overview a final gel picture showing three DNA samples as examples is depicted.

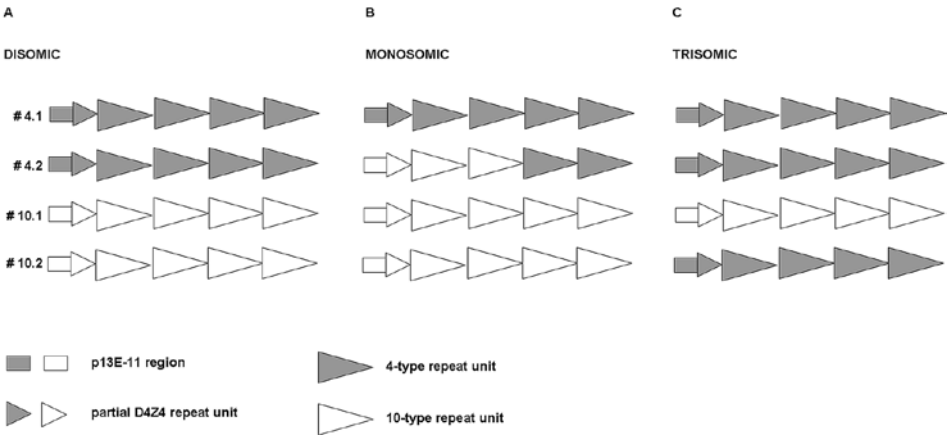


Supplemental Figure S2

Control experiment to confirm complete digestion by the methylation-sensitive restriction enzyme CpoI.

A. *At random, samples were spiked with an equimolar amount of plasmid DNA (pBluescript vector containing a single D4Z4 KpnI repeat unit) to test for complete digestion by CpoI. Complete digestion of the plasmid DNA gives a single ~5 kb band after hybridization with the empty pBluescript vector, while a second band of ~6 kb appears in case of incomplete digestion.*

B. *An example of the control experiment to confirm complete digestion. First, complete digestion was verified by hybridization using the empty pBluescript vector as a probe (left panel). Second, the methylation levels at the CpoI site were determined after hybridization of the same membrane with the p13E-11 probe (right panel). In the right panel, the band obtained after hybridization with the empty pBluescript vector is still visible.*



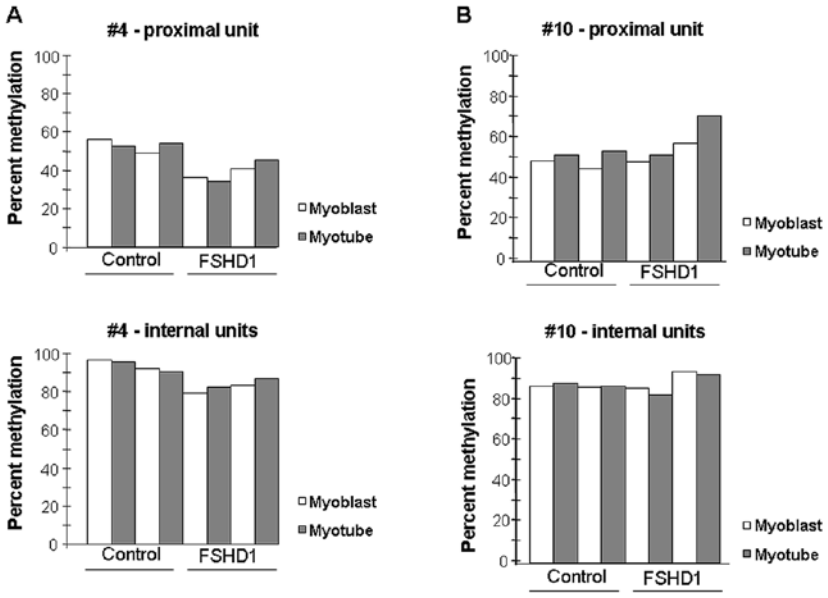
Supplemental Figure S3

Schematic overview of the allele constitution on chromosomes 4q and 10q in DNA of all individuals tested.

A. *A disomic individual has a standard allele constitution; two 4-type repeat arrays on chromosome 4q and two 10-type repeat arrays on chromosome 10q.*

B. *A monosomic individual has a single 4-type repeat on chromosome 4q. The most proximal units of the second chromosome 4q allele are 10-derived repeat units. In monosomic individuals the methylation level of a single repeat on chromosome 4q can be measured.*

C. *A trisomic individual has a single 10-type repeat on chromosome 10q. The second chromosome 10q allele is composed of only 4-type repeat units. In trisomic individuals the methylation status of a single repeat on chromosome 10q can be determined.*



Supplemental Figure S4

Bar diagrams of* CpoI *methylation analysis in DNA isolated from muscle cell lines.

A. CpoI *methylation levels on chromosome 4q in myoblasts and myotubes from two controls and two FSHD1 patients on proximal (upper panel) and internal (lower panel) D4Z4 repeat units. Methylation data of individual cell lines are shown.*

B. CpoI *methylation levels on chromosome 10q in myoblasts and myotubes from two controls and two FSHD1 patients on proximal (upper panel) and internal (lower panel) D4Z4 repeat units. Methylation data of individual cell lines are shown.*

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