Chromosome 10q-linked FSHD identifies DUX4 as principal disease gene

Lemmers, R.J.L.F.; Vliet, P.J. van der; Blatnik, A.; Balog, J.; Zidar, J.; Henderson, D.; ... ; Maarel, S.M. van der

Original research

Chromosome 10q-linked FSHD identifies *DUX4* as principal disease gene

Richard J L F Lemmers ,[1] Patrick J van der Vliet,[1] Ana Blatnik,[2] Judit Balog,[1] Janez Zidar,[3] Don Henderson,[4] Rianne Goselink,[5] Stephen J Tapscott,[6] Nicol C Voermans,[5] Rabi Tawil,[4] George W A M Padberg,[5] Baziel GM van Engelen,[5] Silvère M van der Maarel [1]

► Additional material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/jmedgenet-2020-107041).

[1]Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
[2]Cancer Genetics Clinic, Institute of Oncology, Ljubljana, Slovenia
[3]Division of Neurology, Institute of Clinical Neurophysiology, University Medical Centre Ljubljana, Ljubljana, Slovenia
[4]Department of Neurology, University of Rochester Medical Center, Rochester, New York, USA
[5]Department of Neurology, Donders Institute for Brain, Cognition and Behaviour, Radboud University, Nijmegen, The Netherlands
[6]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

Correspondence to
Dr Richard J L F Lemmers, Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden 2300 RC, The Netherlands; r.j.l.f.lemmers@lumc.nl

Received 28 May 2020
Revised 5 October 2020
Accepted 14 November 2020
Published Online First 12 January 2021

To cite: Lemmers RJLF, van der Vliet PJ, Blatnik A, *et al*. *J Med Genet* 2022;**59**:180–188.

ABSTRACT

Background Facioscapulohumeral dystrophy (FSHD) is an inherited muscular dystrophy clinically characterised by muscle weakness starting with the facial and upper extremity muscles. A disease model has been developed that postulates that failure in somatic repression of the transcription factor DUX4 embedded in the D4Z4 repeat on chromosome 4q causes FSHD. However, due to the position of the D4Z4 repeat close to the telomere and the complex genetic and epigenetic aetiology of FSHD, there is ongoing debate about the transcriptional deregulation of closely linked genes and their involvement in FSHD.

Method Detailed genetic characterisation and gene expression analysis of patients with clinically confirmed FSHD and control individuals.

Results Identification of two FSHD families in which the disease is caused by repeat contraction and *DUX4* expression from chromosome 10 due to a de novo D4Z4 repeat exchange between chromosomes 4 and 10. We show that the genetic lesion causal to FSHD in these families is physically separated from other candidate genes on chromosome 4. We demonstrate that muscle cell cultures from affected family members exhibit the characteristic molecular features of FSHD, including *DUX4* and DUX4 target gene expression, without showing evidence for transcriptional deregulation of other chromosome 4-specific candidate genes.

Conclusion This study shows that in rare situations, FSHD can occur on chromosome 10 due to an interchromosomal rearrangement with the FSHD locus on chromosome 4q. These findings provide further evidence that *DUX4* derepression is the dominant disease pathway for FSHD. Hence, therapeutic strategies should focus on DUX4 as the primary target.

INTRODUCTION

Facioscapulohumeral dystrophy (FSHD; MIM 158900) is a progressive muscular dystrophy which affects approximately one in 8500 individuals.[1] FSHD is clinically characterised by muscle weakness and wasting which generally starts in the facial muscles and then progresses to the shoulder girdle and upper arm muscles. At later stages, the lower leg muscles may also become involved. The disease progression and severity is highly variable and many patients have an asymmetric muscle involvement.[2 3] There is no cure for FSHD.

Many studies have suggested that FSHD is caused by somatic derepression of the double homeobox 4 (*DUX4*) gene in skeletal muscle.[4–7] *DUX4* encodes for a transcription factor that is normally expressed in the testis and in cleavage stage embryos, where it is involved in zygotic genome activation.[8–10] Inappropriate *DUX4* expression in skeletal muscle tissue induces, among others, stem cell and germline specific processes eventually resulting in apoptosis.[5 11] Therapeutic strategies targeting DUX4 toxicity in skeletal muscle are currently being pursued.[12]

DUX4 derepression in FSHD is the result of a partial chromatin relaxation of the FSHD locus, the D4Z4 macrosatellite repeat on chromosome 4q, which harbours the *DUX4* gene (figure 1).[13 14] The polymorphic D4Z4 repeat consists of 3.3 kb long repeat units and ranges in non-affected individuals generally between 8 and 100 units.[15] In most autosomal-dominant FSHD cases, D4Z4 chromatin relaxation is caused by a contraction of the D4Z4 repeat to a size between 1 and 10 units (FSHD1).[16] D4Z4 chromatin relaxation in somatic FSHD tissues is marked by a partial loss of repressive chromatin marks, including reduced CpG methylation, and gain of active chromatin marks.[17–20] In rare cases (FSHD2; <5%), variants in the D4Z4-associated chromatin modifiers structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1), DNA methyltransferase 3B (DNMT3B) or ligand dependent nuclear receptor interacting factor 1 (LRIF1) are underlying D4Z4 chromatin relaxation, but these mutations only cause FSHD when combined with a medium-sized (8–20 units) D4Z4 repeat.[21–23] In contrast to FSHD1, where D4Z4 chromatin relaxation is limited to the contracted allele, in FSHD2 the chromatin relaxation occurs on all chromosome 4-derived and 10-derived D4Z4 repeats, which can be detected by an overall reduced CpG methylation at D4Z4.[24–28]

Apart from D4Z4 chromatin relaxation, the chromosomal background is critically important for disease development. Two equally common 4qter variants exist: 4qA and 4qB. D4Z4 chromatin relaxation on most 4qA chromosomes causes FSHD, while D4Z4 chromatin relaxation on the 4qB chromosome is not associated with disease. This is because the *DUX4* gene on 4qB is incomplete. It lacks the 3' end encoding for its polyadenylation signal (PAS) in skeletal muscle (figure 1).[14 29] An

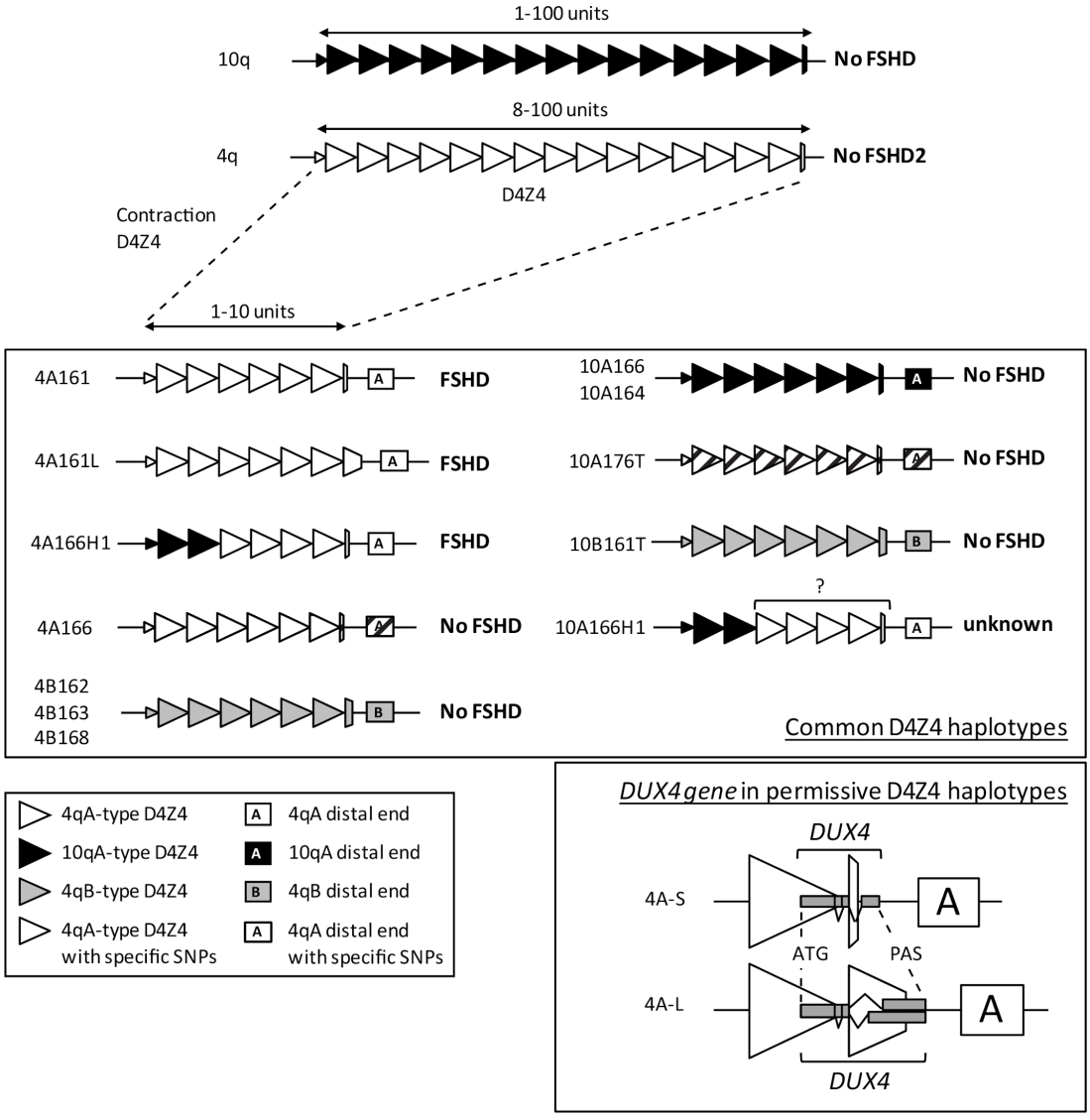

Figure 1 Genetic mechanism for FSHD1 and the most common D4Z4 haplotypes on chromosomes 4 and 10. The D4Z4 repeat array size in unaffected individuals varies between 8–100 units on chromosome 4 and 1–100 units on chromosome 10 (no FSHD, top panel). In patients with FSHD1, the D4Z4 repeat is contracted to a size that varies between 1 and 10 units, but only on specific D4Z4 haplotypes on chromosome 4qA. The most common haplotypes that are permissive to FSHD are shown in the panel below and are annotated 'FSHD' (4A161, 4A161L and 4A166H1). Also indicated are the most common haplotypes that are not associated with FSHD when D4Z4 is contracted to 1–10 units (4B162, 4B163, 4B168, 10A166, 10A164, 10A176T and 10B161BT). The permissivity of the hybrid haplotype 10A166H1 is explored in this study. The legend for the different haplotypes is shown in the left bottom panel. In the right bottom panel, the complete and partial most distal D4Z4 units and the gene structure of DUX4 in permissive 4A-S (4A161 and 4A166H1) and 4A-L (4A161L) chromosomes are shown. DUX4 exons are indicated as grey squares.

equally copy number variable D4Z4 repeat-like structure of the 4qA-type is present on chromosome 10 (10qA) showing 98.5% sequence homology with chromosome 4.[30 31] On chromosome 10, stable somatic expression of the *DUX4* paralog is also failing when the D4Z4 chromatin structure is relaxed because of chromosome 10-specific sequence variants that distort the *DUX4* PAS (online supplemental figure 1). Thus, only D4Z4 chromatin relaxation on most 4qA chromosomes permits the stable yet sporadic expression of DUX4 protein in FSHD skeletal muscle (figure 1).[14] Small sequence variations between chromosome 4 and 10 also render differential sensitivity for the restriction endonucleases BlnI and XapI (4-type D4Z4 units are BlnI resistant and XapI sensitive, while 10-type D4Z4 units are BlnI sensitive and XapI resistant), which facilitate their discrimination by Southern blotting, still the most common genetic diagnosis method for FSHD.[32]

Based on polymorphisms proximal and distal to the D4Z4 repeat, a number 4qA haplotypes have been described with haplotype 4A161 being the most frequent representing 80% of all 4qA haplotypes in the European population.[32] While all 4qA haplotypes have a *DUX4* PAS and can therefore stably express DUX4 in skeletal muscle, one haplotype (4A166) seems to be non-permissive for the disease for reasons that are not well known.[33] A subclass of the common FSHD 4A161 haplotype, the European-specific 4A161L (4A-L) haplotype, encodes for an extended *DUX4* 3'UTR (DUX4L), but an identical DUX4 protein (figure 1).[34] Another infrequent 4qA haplotype is characterised by mixed arrays of BlnI-sensitive and XapI-sensitive

D4Z4 units. This so-called hybrid haplotype (4A166H1) is detected in 4% of the European and Asian control population and is permissive to *DUX4* expression.[32] Previously, we have studied the sequence of several hybrid FSHD alleles and identified for all a 4qA-type most distal D4Z4 unit with a *DUX4* PAS (online supplemental figure 1). In contrast, the rare 10A176T haplotype representing 5% of chromosome 10 alleles consists of a homogeneous 4q-type repeat and is not permissive due to an interrupting sequence variant in the *DUX4* PAS (online supplemental figure 1).[32]

Although the mechanism in which FSHD is caused by inappropriate expression of *DUX4* is generally well accepted, there is ongoing debate about alternative mechanisms. Specifically, considering the proximity of the D4Z4 repeat to the telomere, and based on several studies that have addressed the 4qter 3D chromatin architecture, a critical involvement of other 4qter genes has been repeatedly suggested as a consequence of their transcriptional deregulation through telomere position effects, or spreading and looping effects originating from the D4Z4 repeat.[35–37]

One way of addressing the importance of 4qter genes besides *DUX4* is by making use of the power of genetics. Although there is a strong linkage of FSHD to chromosome 4q, we previously reported one family in which FSHD appears to be associated with a D4Z4 contraction on chromosome 10, thereby separating the epigenetic insult to *DUX4* from other chromosome 4-specific candidate genes.[14] However, formal proof of *DUX4* expression and FSHD molecular pathology was missing from this family. In the current study, we sought to find further evidence that FSHD1-sized repeats on chromosome 10, when combined with a chromosome 4qA-derived *DUX4* PAS, are associated with *DUX4* expression in skeletal muscle and disease presentation.

MATERIALS AND METHODS

Subjects

Clinical evaluation for all members of families Rf25 and Rf1253 was performed by an experienced neurologist after informed consent. For the clinical severity, we used the age corrected severity score (ACSS), based on the 10-scale Ricci score (ACSS=(Ricci score/age at examination)×1000).[38 39]

Primary myocyte cultures

For immunofluorescence and DUX4 transcription analysis, we generated primary myocyte cultures from the proband of each family according to previously described methods (detailed protocols at the Fields Center website (www.urmc.rochester.edu/fields-center/). As controls, we used primary myocyte cultures from patients with genetically confirmed FSHD1 and control individuals, which originated from the University of Rochester Medical Center bio repository. All cultures have at least 80% myocytes, except for that of patient Rf1253.201, where the fraction of myocytes is lower. Detailed genotype and methylation information for all cells can be found in online supplemental table 1).

Genetic analysis of the D4Z4 repeats

These studies were done as described previously (online supplemental methods).

Myocyte cultures and immunofluorescence staining

These studies were done as described previously(online supplemental methods).

Determination of the 4;10 breakpoint in Rf1253.201

Based on the Southern blot analysis, the 4;10 breakpoint was roughly mapped to the most distal BlnI-XapI restriction fragment in the hybrid disease allele. Therefore, genomic DNA of Rf1252.201 was digested using restriction enzymes XapI and XmaJI (isoschizomer for BlnI) and two additional enzymes NcoI and AatII that digest outside the D4Z4 repeat and serve to reduce genomic background. After pulsed field gel electrophoresis (PFGE), an agarose slice around 5.7 kb was isolated from the gel and DNA was extracted. Subsequently, the breakpoint region was PCR-amplified using the following primers F 5'-GCA CAG TCC GGC TGA GGT-3' and R 5'- CCC AGG AAA GAA TGG CAG T-3'. The PCR was performed using Kapa2G robust kit with enhancer (Merck) in a total volume of 20 µL. The PCR conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min and a final extension of 5 min.

Determination of the distal D4Z4 sequence in the 10A166 and the 10A166H1 haplotype

D4Z4-pLAM sequences for the 10A166H1 haplotypes were obtained in individuals that carry two 4qB chromosomes and at least one 10A166H1 chromosome. We used forward primer 9406LRF 5'-AGC GTT CCA GGC GGG AGG GAA G-3' and reverse primer BsatLRR2A(10q) 5'- GAA ACA CAT CTG CAC TGA TCA CCG AAG TTA TGT AAA TC-3'. These PCR reactions were performed on 125–140 ng of genomic DNA, in a solution containing 3.5 µM of each primer, 0.2 mM dATP, 0.4 mM dCTP, 0.2 mM dTTP, 0.2 mM dGTP and 0.2 mM 7-deaza-dGTP, 2.5 U of LA-Taq DNA polymerase and supplemented with 2xGC buffer I (TAKARA), in a total volume of 20 µL. The PCR conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s and extension at 72 °C for 3 min and 30 s. The final extension time was 10 min at 72 °C.

Transcription analysis

Total RNA was extracted using the Qiagen RNAeasy isolation kit with DnaseI treatment. The RNA concentration was determined on a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). cDNA was synthesised using oligo-dT primers (Fermentas RevertAid Transcripase kit) from 2 µg of total RNA. The reverse transcription buffer was added after 5 min at 70°C, followed by 1 hour incubation at 42°C, 10 min at 70°C and then placed on ice. After the reaction, cDNA was treated with RNAseH and 75 µL water was added to an end volume of 100 µL.

Expression analysis for *DUX4, GUSB,* DUX4 target genes, genes proximal to D4Z4 on chromosome 4q and myogenic differentiation genes in the myotubes from Rf25.201, Rf1253.201 and chromosome 4q-linked FSHD1 or control samples was performed in triplicate using the primer pairs shown in online supplemental table 2. For the RT-qPCR analysis, we used in a 15 µL reaction 5 µL cDNA, 1× Sybrgreen PCR mix (buffer and dNTP and Polymerase) and 0.33 µM of each primer. The PCR conditions consisted of an initial denaturation step at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension for 30 s at 60°C. To analyse the sequence of the 4A161L-like DUX4 transcript in both patients, we used three overlapping amplicons (online supplemental table 2). We used the following conditions: input cDNA 2 µL cDNA, 1× Accuprime buffer II (Invitrogen), supplemented with 0.2 mM 7-Deaza-2'-deoxyguanosine, 0.2 µM of each primer and 0.5 µL AccuPrime Taq Polymerase in a 25 µL PCR

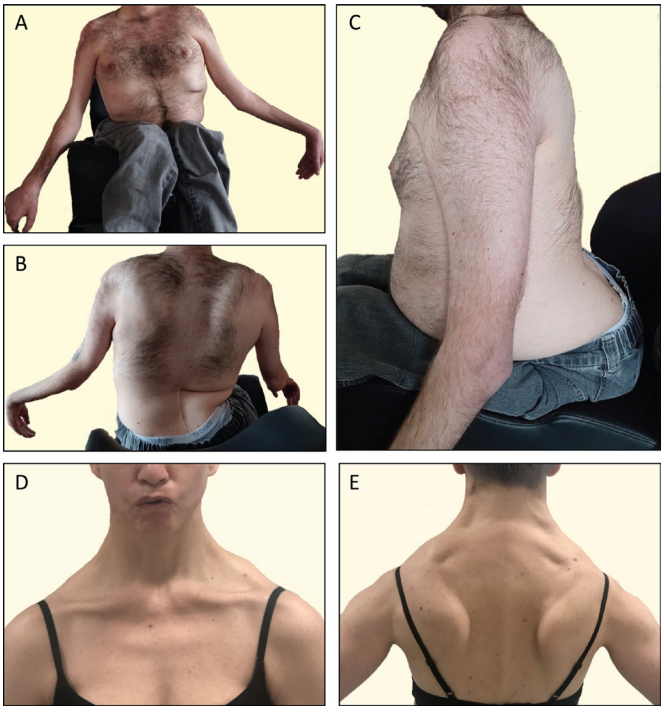

Figure 2 (A)–(C) Proband of family Rf25 at the age of 34. He is not able to stand independently. (A)/(B) Maximal shoulder abduction showing severe weakness and atrophy of the shoulder girdle muscles, upper arm and forearm muscles and bilateral winging of scapula. He underwent a scoliosis surgery at the age of 15. (C) Resting position, lateral view, showing atrophy of deltoid muscle and biceps more than triceps muscle and increased lumbar lordosis. (D,E) Proband of family Rf1253 at the age of 44. (D) Bilateral pectoralis major atrophy and asymmetry of the mouth when pursing the lips due to weakness of the orbicularis oris. (E) Bilateral scapular winging on maximal arm abduction.

reaction. The PCR programme consisted of an initial denaturation step at 95°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 68°C for 2 min and 30 s. The final extension time was 10 min at 72°C. Primers for RT-qPCR and full length DUX4 RT-PCR were designed using Primer 3 software.

RESULTS

Clinical and genetic analysis of FSHD family Rf25

The proband of our previously reported family Rf25 presented with facial weakness and dysarthria at age 2. At age 5, severe facial weakness, scapular winging as well as abdominal, quadriceps and hamstring weakness was noted. He became wheelchair users at the age of 7 years.[40] At the age of 31, he has a severe muscle phenotype and is unable to walk or make transfers. He has mild bilateral hearing loss, a normal vision with severe retinal telangiectasis, a severe lumbar hyperlordosis and a decreased respiratory function without ventilatory assistance (figure 2A–C). He lives semi-independently with assisted care and works on a voluntary basis. The father of the proband has right facial weakness since childhood and examination at age 37 showed facial weakness, right scapular winging with shoulder girdle weakness, asymmetric pectoralis major atrophy and abdominal muscle weakness.

D4Z4 genotyping of family Rf25 has been described before.[14] Briefly, the proband inherited a 2 unit hybrid D4Z4 repeat on chromosome 10 from his father as determined by PFGE and fluorescence in situ hybridisation on stretched DNA (fibre FISH). The father is gonosomal mosaic for this hybrid chromosome 10 repeat with approximately 50% of his blood mononuclear cells carrying the FSHD-sized hybrid repeat explaining the milder phenotype.[41] The mosaicism observed in the father's blood is unusual as we observed mosaicism for the hybrid chromosome 10 and for the 4A161L chromosome with a similar ratio (figure 3A and online supplemental figure 2 and table 1), suggesting that both rearrangements co-occurred. In this study, by using the chromosome-specific endonucleases XapI and BlnI, we determined that the chromosome 10;4 breakpoint in the hybrid D4Z4 repeat is in the *DUX4* promoter in the most distal complete D4Z4 unit (figure 3B and online supplemental figure 2). Further genetic analysis and Sanger sequencing showed that the hybrid allele has a 4A161L partial distal D4Z4 unit (online supplemental figure 1). Molecular combing analysis[42] in the proband confirmed the fiber-FISH analysis[14] and showed that the FSHD-sized D4Z4 repeat is localised on chromosome 10 (figure 3C). Based on normal D4Z4 CpG methylation, we excluded FSHD2 in the proband and his father (online supplemental table 1),[43] which was corroborated by the absence of pathogenic variants in the FSHD2 genes *SMCHD1*, *DNMT3B* and *LRIF1* (online supplemental table 3). Collectively, the genetic analysis suggests that the 2 unit hybrid repeat on chromosome 10 is causal to FSHD in this family.

Clinical and genetic analysis of FSHD family Rf1253

Next, we identified a second family showing FSHD linkage to chromosome 10. The proband in this family (Rf1253) first noticed signs of slight right shoulder girdle weakness and right shoulder pain since the age of 25. The symptoms became more pronounced in the 2 years prior to her first neurological examination at age 36. Neurological examination showed (predominantly lower) asymmetrical facial weakness, trapezius and rhomboid muscle atrophy with severe scapular winging, horizontal clavicles and bilateral pectoralis atrophy (figure 2D,E). There was abdominal muscle weakness with a positive Beevor's sign and increased lumbar lordosis. Muscle strength was normal in both her upper and lower limbs. EMG of the trapezius muscle showed myopathic changes. The proband's parents were reported to be healthy.

D4Z4 genotyping in family Rf1253 revealed that the proband has a mosaic de novo 3 units hybrid repeat on chromosome 10 (online supplemental figure 3). The patient inherited an uncommon 23 D4Z4 units long 4B162 allele from her father and a 16 units 4A161L allele from her mother. Both chromosome 10 alleles appear to have a different repeat sizes than the parental alleles, and one of them is mosaic and hybrid containing either 3 (80% of cells) or 16 (20% of cells) repeat units (figure 3D and online supplemental figure 3 and table 1). All chromosome 10 alleles in both parents are of the most common 10A166 haplotype. The most distal partial D4Z4 unit of the de novo hybrid allele in the patient is of the 4qA-L type, like in Rf25. Based on the Southern blot fragments on different hybridisations, we estimated the position of the chromosome 10;4 breakpoint and showed on targeted PCR and sequencing that this was close to the *DUX4* stop codon (figure 3E, online supplemental figure 4). This implies that the DUX4 protein sequence is chromosome 10-like, as was previously found in a patient with a hybrid FSHD allele on chromosome 4 (patient 4A166H1-F3 in online supplemental figure 1).[14] Molecular combing analysis confirmed that the FSHD1-sized repeat of 3 D4Z4 units is on chromosome 10 (figure 3F). The patient showed normal D4Z4 CpG methylation, which excludes FSHD2 (online supplemental table 1),

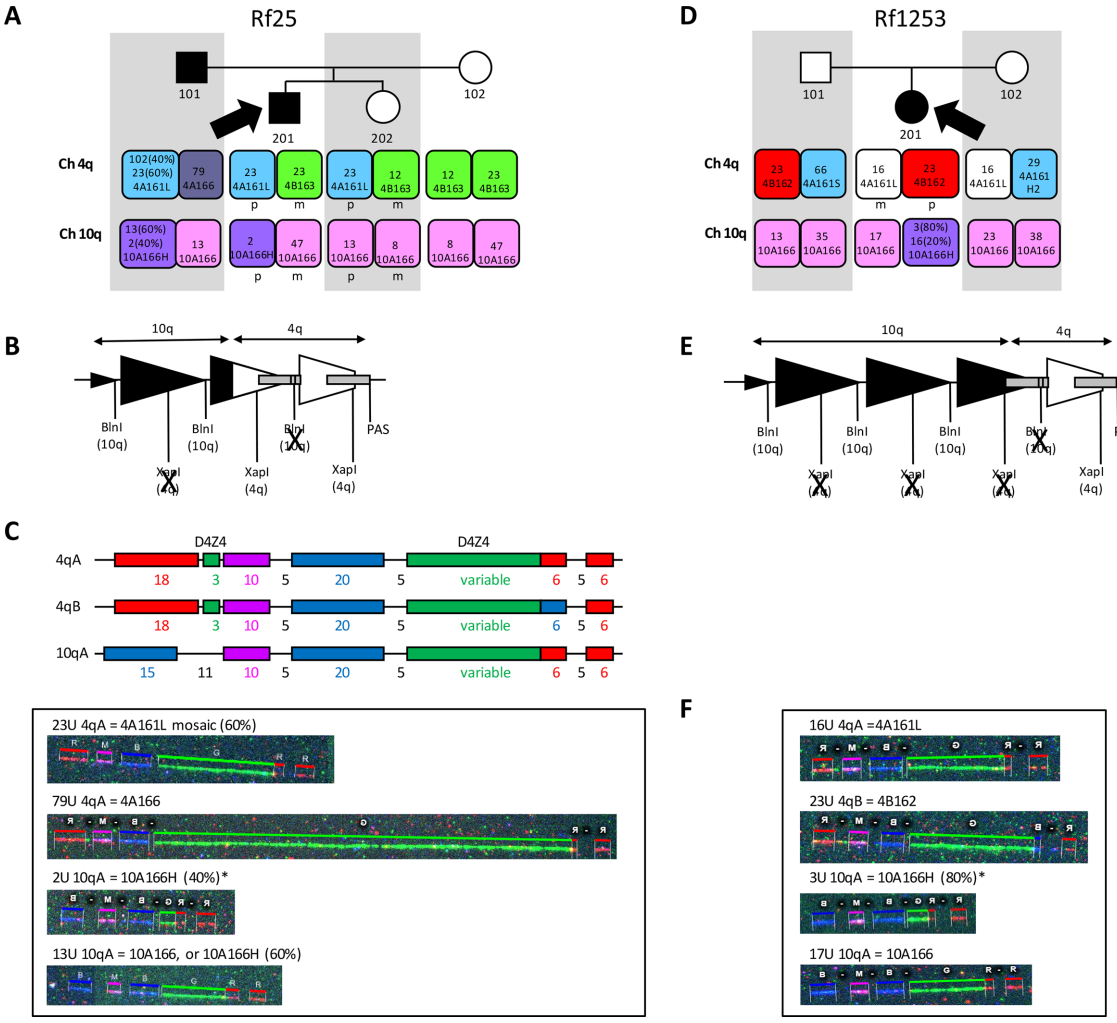

Figure 3 Genetic analysis of FSHD families Rf25 and Rf1253. (A) Pedigree of family Rf25 in which both the index patient (indicated with arrow) and his father carry the 2 unit hybrid D4Z4 repeat on chromosome 10. Below each individual in the coloured boxes, the D4Z4 haplotype and repeat size of all chromosomes 4 and 10 alleles are shown. Identical haplotypes are indicated with the same colour (eg, pink for all 10A166 alleles). The parental origin of the alleles is also indicated (p for paternal and m for maternal). (B) Schematic representation of the hybrid FSHD-sized D4Z4 repeat on chromosome 10. The position of the chromosome-specific endonucleases sites *Xap*I and *Bln*I are indicated and the chromosome 4-derived and 10-derived D4Z4 sequences (triangles) are depicted in white and black, respectively. The distal *DUX4* gene is indicated in grey. (C) Top: Schematic overview of the position and colour of the fluorescence molecular combing probes for chromosomes 4qA, 4qB and 10qA. The D4Z4 repeat and the highly homologous inverted unit on chromosome 4 are indicated in green. Bottom: Molecular combing on Rf25.101 confirms the genetic background for all alleles identified by Southern blotting and shows that the contracted 2 unit D4Z4 repeat (marked with asterisk) is on chromosome 10. (D) Pedigree of family Rf1253, in which the sporadic patient inherited two normal-sized chromosome 4 alleles from both parents. The patient acquired two rearranged alleles on chromosome 10; one has a de novo hybrid D4Z4 repeat and one a de novo rearranged D4Z4 repeat. The hybrid allele is mosaic and approximately 80% of the her cells have a 3 unit repeat due to the rearrangement. (E) Composition of the FSHD-sized hybrid allele on chromosome 10, which ends with a 4A161L-like D4Z4 unit. (F) Molecular combing confirms that the contracted 3 unit D4Z4 repeat in the patient (marked with asterisk) is on chromosome 10.

which was again corroborated by the absence of possible pathogenic variants in the FSHD2 genes (online supplemental table 3).

Expression analysis of DUX4 and its target genes

Since the presence of DUX4 in myotube cultures is considered a molecular hallmark of FSHD, to further study the pathogenicity of the FSHD-sized repeats on chromosome 10 in both families, we tested patient-derived myogenic cell cultures for the presence of DUX4 and its target genes.[4 11] Muscle biopsy-derived myotube cultures from individuals from each family, Rf25.201 and Rf1253.201, were compared with myotube cultures from genetically and patients with clinically confirmed chromosome 4q-linked FSHD1 and control individuals (online supplemental table 1) using immunofluorescence microscopy and RT-qPCR. Immunofluorescence analysis for DUX4 and myosin as differentiation marker in primary myotube cultures showed multiple, often clustered, DUX4-positive nuclei in Rf25.201 and Rf1253.201 typical for FSHD (figure 4).

We showed, as expected, DUX4 expression in Rf25.201 and Rf1253.201 (figure 5A). Sequence analysis of the full length transcript showed that it was identical to the transcript derived from the standard 4A161L FSHD alleles (data not shown). MYOG and embryonic MHC (MYH3) muscle differentiation marker expression analysis showed that all cells were adequately differentiated. We also analysed the expression of three DUX4 target genes:[11] *MBD3L2*, *ZSCAN4* and *TRIM43* and found robust expression in myotube cultures in both patients.

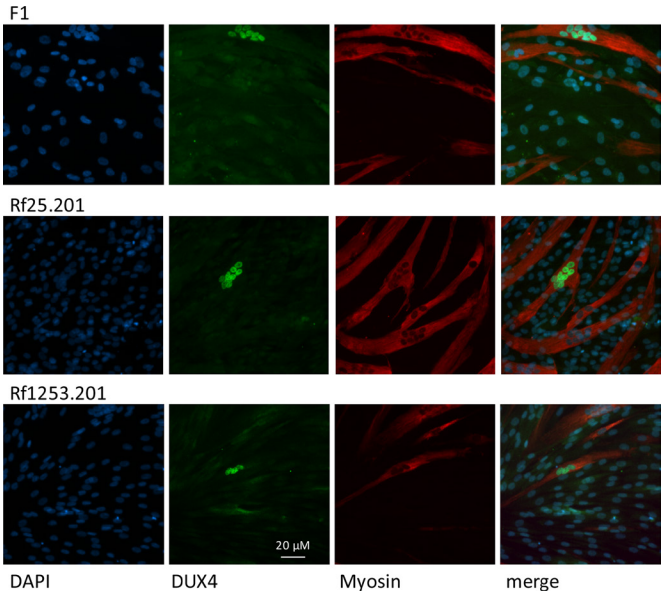

Figure 4 Immunofluorescence staining of myotubes derived from the probands of the two families described. Genetically confirmed chromosome 4q-linked FSHD1 samples F1 served as a positive control. All samples show clustered DUX4 positive nuclei. DAPI staining is in blue, myosin in red and DUX4 in green. Scale bar is indicated.

Expression analysis of 4qter genes

To address the possibility that D4Z4 chromatin relaxation may indirectly affect the expression of other genes on chromosome 4qter that have been implicated in FSHD pathogenesis, we studied the expression of 4qter genes (*FRG2*, *FRG1*, *FAM149A*, *SORBS2*, *SLC25A4 (FAT1)* and *WWC2*) within a 7 Mb window from D4Z4 (online supplemental figure 5). These genes were previously shown to be transcriptionally dysregulated in FSHD muscle cells or biopsies.[36 37 44] *SORBS2* and *WWC2* were suggested to have a direct DNA interaction with the D4Z4 repeat in control cells, which is lost on D4Z4 contraction.[37] *FAT1* is located 4 Mb from D4Z4 and was reported to be transcriptionally downregulated in FSHD muscle biopsies[45] and disrupted FAT1 functions cause a FSHD-like phenotype in mice.[46] We found all genes to be expressed and observed a slight but significant upregulation for *FAT1, SORBS2* and *WWC2* in chromosome 4q-linked, but not 10q-linked, FSHD1 samples compared with controls. *FRG2*, a known DUX4 target gene,[47] was also slightly but significantly upregulated in all chromosome 4q-linked and 10q-linked FSHD samples (figure 5B).

Consequences for the genetic confirmation of FSHD

In both families, the hybrid FSHD allele on chromosome 10 contains, respectively, 2 and 3 units. The hybrid composition of the repeat can be determined by Southern blotting, but not when using molecular combing or single-molecule optical mapping (SMOM),[48] both emerging technologies for the genetic diagnosis of FSHD. To determine the risk for missing FSHD-causing alleles on chromosome 10 using these novel diagnostic methods, we analysed the repeat size on chromosome 10qA (haplotypes 10A164, 10A166, 10A176T and 10A180T) in 724 unrelated European control individuals, which have been described previously.[34] We identified 10 (0.7%) chromosome 10qA alleles with a repeat size between 1 and 4 units (like in families Rf25 and Rf1253), indicating that these short repeat sizes on chromosome 10 are rare (online supplemental figure 6). We determined the sequence of the most distal D4Z4 sequence and flanking region for all 10A166 alleles with a 1–4 units D4Z4 repeat in the control population and observed standard chromosome 10A166 sequence lacking the *DUX4* PAS (data not shown).

Determination of the permissivity of a prevalent chromosome 10 hybrid D4Z4 repeat

As discussed before, hybrid D4Z4 repeats on chromosomes 4 and 10 are frequently observed in the population and those ending with 4-type repeats are potentially a yet underappreciated risk factor for FSHD.[14 32] This especially holds true for the hybrid D4Z4 repeat on chromosome 10 (10A166H1) which might be, like the 4A166H1 hybrid repeat, permissive when having a *DUX4* PAS, which has not been explored thus far. Because of the high frequency of this haplotype in Asia[32] and thus the potential disease risk, we sequenced the distal D4Z4 region for four Asian 10A166H1 alleles and found that their sequence was very similar to the common 10A166 haplotypes and also lacking the *DUX4* PAS (online supplemental figure 1). Therefore, this haplotype is likely non-permissive.

DISCUSSION

Identification of two chromosome 10q-linked FSHD families

In this study, we describe two families in which FSHD is caused by a D4Z4 rearrangement on chromosome 10. The chromosomal position of the pathogenic D4Z4 repeat was verified by multiple independent technologies. Detailed genetic analysis of these alleles showed that in both cases, it has an unusual hybrid composition and, most probably, arose by a de novo exchange between the D4Z4 repeat of chromosomes 4 (4A161L) and 10. In family Rf25, the hybrid repeat on chromosome 10 was identified in the gonosomal mosaic father and his son. Gonosomal mosaicism for the D4Z4 contraction is common in de novo FSHD. It typically arises by a D4Z4 rearrangement between sister chromatids, thus involving a single allele.[49 50] In the father of the proband, however, mosaic D4Z4 repeats on chromosome 10 (13 units and 2 units) and on chromosome 4 (102 units and 23 units) were observed with similar ratio's (40% and 60%), which suggests that two chromosomes were involved in an interchromosomal rearrangement event between chromosome 4 and 10 repeats (online supplemental figure 2). As previously reported for mosaic carriers of an FSHD allele, the mosaic father is less severely affected than his non-mosaic son. In family Rf1253, we actually could show the de novo exchange between chromosomes 4 and 10 as both parents carry only homogeneous D4Z4 repeats on 4q and 10q. This rearrangement probably occurred immediately after fertilisation as the D4Z4 repeat size of the paternal and maternal 10q allele in the patient differ from her parents, while the chromosome 4 alleles showed normal parental inheritance. Furthermore, the de novo hybrid allele on chromosome 10 shows a mosaic D4Z4 contraction; in one cell population (20% of blood cells), the D4Z4 repeat is 16 units long and in the other (80% of the cells) 3 units. Both parents are unaffected, while the proband showed a classical FSHD phenotype.

Because of the different positions of the chromosome 4;10 breakpoints, the sequence in the DUX4 protein coding exon 1 is chromosome 4-derived in Rf25 and chromosome 10-derived in Rf1253. The chromosome 4-derived predicted DUX4 protein has an alanine-serine duplication at amino acid positions 340–343, which is absent in its chromosome 10 paralog. The functional consequence of this sequence variation is unknown.

Previously, two myogenic enhancers for *DUX4* have been described on chromosome 4, which are located 7 kb (DME1)

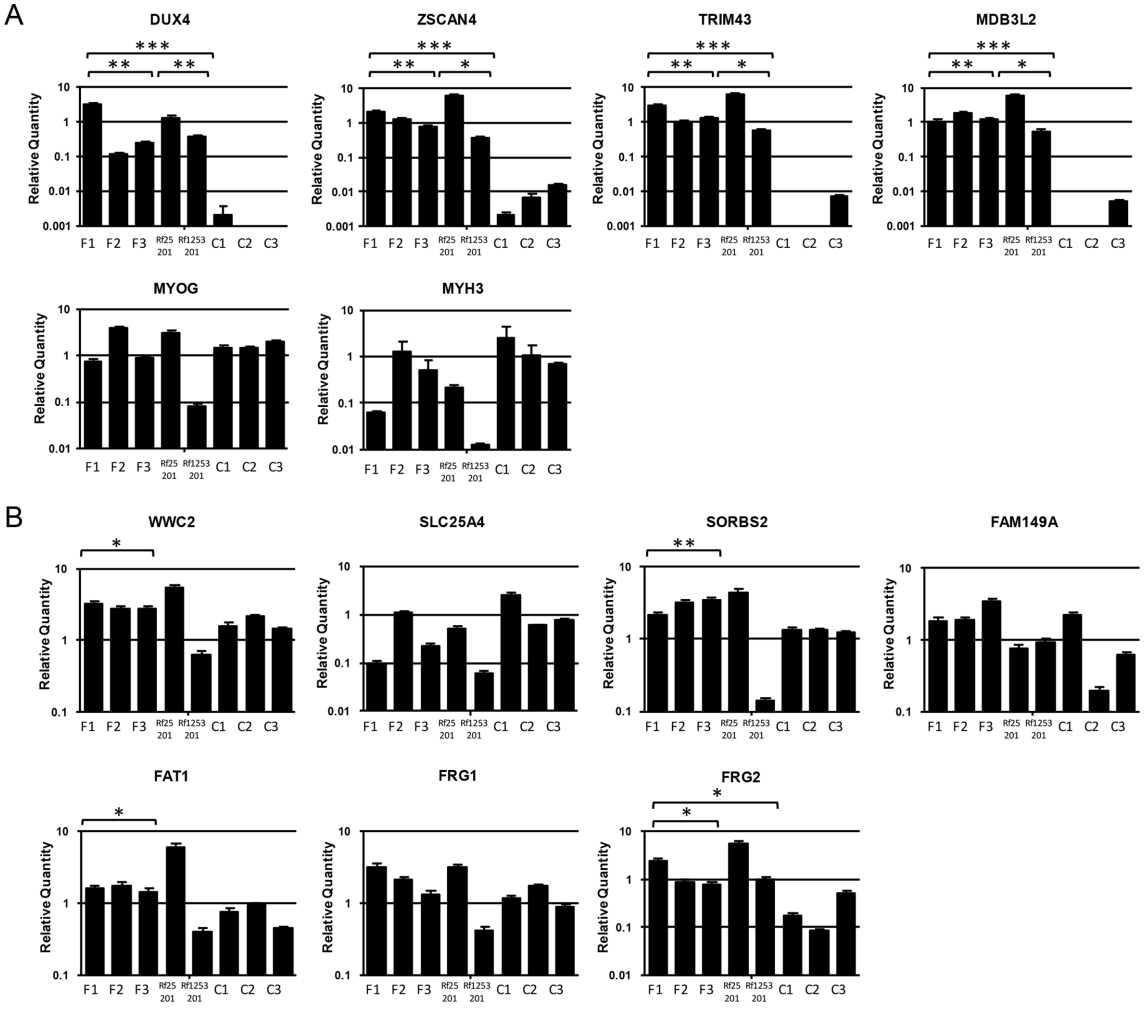

Figure 5 (A) Quantitative RT-PCR on myotubes from the probands of the two chromosome 10q-linked FSHD families described, three patients with genetically confirmed chromosome 4q-linked FSHD (F1, F2 and F3) and two control individuals (C1 and C2). Expression levels of *DUX4* relative to *GUS* are shown in the top panel and that of the DUX4 target genes *ZSCAN4*, *TRIM43* and *MBD3L2* in the middle panel. The expression levels of the early and late myogenic differentiation markers (*MYOG* and *MYH3*, respectively) are shown in the bottom panel. Myotubes Rf25.201 and Rf1253.201 showed DUX4 and target expression levels comparable to the positive controls. (B) RT-qPCR on the same myotubes of the chromosome 4q *cis* genes *FRG2*, *FRG1*, *FAT1*, *FAM149A*, *SORBS2* and *WWC2*. A low but significant dysregulation was observed in patients with chromosome 4q-linked FSHD1 compared with controls for *WWC2*, *SORBS2* and *FAT1*. *FRG2* is significantly upregulated in patients with chromosome 4q-linked and 10q-linked FSHD compared with controls, which corroborates previous findings showing that *FRG2* is a DUX4 target gene. Analyses were done in triplicate and the graph represents mean±SEM. Comparisons between two groups were performed using unpaired two-tailed t-tests. Significant differences between chromosome 4-linked FSHD (4F1), chromosome 10-linked FSHD (10F1) and both groups of patients with FSHD (4F1+10F1) compared with controls are indicated by an asterisk on top of the bracket assembling the specific FSHD groups (where $p<0.05$ is indicated by *, $p<0.005$ by ** and $p<0.0005$ by ***).

and 20 kb (DME2) upstream of the DUX4 MAL start codon, respectively (online supplemental figure 5).[51] The permissive *DUX4* gene in Rf25 and Rf1253 has a chromosome 10-type sequence proximal to the D4Z4 repeat and therefore could lack both enhancer sequences. However, DME1 and DME2 are in a region with 99% sequence homology (99.4% DME1; 98.8% DME2) between chromosomes 4 and 10 and therefore likely present on both chromosomes. However, the region encompassing these enhancers is deleted from FSHD1 alleles in which the partial deletion of the D4Z4 repeat extends into proximal sequences.[52 53] The frequency of proximally extended deletions in FSHD1 is approximately 2% and therefore the relevance of these enhancers for FSHD is unclear.

Consequences for the genetic confirmation of FSHD

The hybrid nature of FSHD-sized alleles on chromosome 10 can be identified by PFGE but not by molecular combing or SMOM. Because of this limitation, some FSHD cases might be missed by this technique. Therefore, we analysed the repeat size distribution of all chromosome 10qA haplotype and observed that 1–4 unit D4Z4 repeats on chromosome 10 are rare in the population. When using molecular combing or SMOM as first tier genetic test, it seems worthwhile to analyse the composition of the D4Z4 repeat by Southern blot analysis or by sequencing when such a short repeat is found on chromosome 10. Generally, these individuals might turn out to have a standard non-permissive 10qA allele lacking a canonical PAS sequence, as we have shown for 10 control individuals. Yet, a recent molecular combing analysis on a large cohort of FSHD families identified two families in which a 1–3 units repeat on chromosome 10 was the only FSHD-sized fragment segregating with the disease without providing evidence that this allele is disease-causing.[42] In these cases, testing for DUX4 expression in differentiated muscle cells, as we have done in this study, may provide additional diagnostic evidence for FSHD.

Determination of the permissivity of a prevalent chromosome 10 hybrid D4Z4 repeat

Previously, we showed that hybrid D4Z4 repeats on chromosome 10 can be found in 0.5% of the European control population, 3% of the African control population and almost 12% of the Asian control population.[32] However, these repeats were identified in the haplotype 10A166H1 and did not arise by a de novo rearrangement like in the families we describe here. Phylogenetic analysis previously suggested that the 10A166H1 haplotype likely originates from the hybrid chromosome 4 haplotype 4A166H1 which is permissive to *DUX4* expression, and therefore this haplotype might also be permissive. Here, we sequenced several 10A166H1 alleles and identified a sequence that is closest to the common 10A166 haplotype with the 10qA SNP in the *DUX4* PAS, which renders it non-permissive to FSHD.

The sporadic presence of DUX4 protein in myonuclei, the molecular hallmark of FSHD, was confirmed in myotube cultures of Rf25.201 and Rf1253.201. We also analysed DUX4 mRNA expression and DUX4 target gene expression in myotube cultures. Using targeted RT-qPCR, we verified FSHD by showing expression of DUX4 and its targets in both cultures comparable to chromosome 4q-linked FSHD1 samples.

Expression analysis of DUX4 and of 4qter genes

Several studies have addressed the 3D architecture of the D4Z4 repeat on chromosome 4 and tested the hypothesis that D4Z4 contractions and chromatin changes may indirectly deregulate the expression of D4Z4 adjacent genes as part of FSHD pathology. Indeed, although sometimes inconsistently, transcriptional deregulation of 4qter genes has been reported in FSHD.[36 37 45] Our targeted RT-qPCR studies confirmed a mild transcriptional dysregulation of three chromosome 4 genes in the proximity of D4Z4 (*WWC2*, *SORBS2* and *FAT1*) in chromosome 4q-linked FSHD samples. However, the chromosome 10 translocated disease alleles as reported in these two families displaying a classical FSHD phenotype, argue against an important role of these genes in FSHD pathology, as in these myotube cultures *WWC2*, *SORBS2* and *FAT1* were not differentially expressed, likely because the epigenetic insult to D4Z4 is physically separated from these FSHD candidate genes on chromosome 4. Therefore, the two chromosome 10q-linked families reported here show that *DUX4* derepression is the dominant disease pathway for FSHD and that therapeutic strategies should focus on DUX4 as the primary target.

Acknowledgements We thank all FSHD families for participating in our studies. We thank professor J J Goeman from the LUMC the Netherlands for statistical advice. Several authors are members of the European Reference Network for Rare Neuromuscular Diseases (ERN EURO-NMD) and/or members of the Netherlands Neuromuscular Centre (NL-NMD).

Contributors RJLFL and SMvdM contributed to the study design and conception of study. RJLFL, PJvdV, AB, JB, JZ, DH, RJMG, NV, RT, GP, BvE contributed to the data collection and assembly. RJLFL and PJvdV contributed to the data analysis and interpretation. RJLFL and SMvdM contributed to the writing of manuscript. RJLFL, PJvdV, JB, SJT, NV, GP and SMvdM critical reviewed and revised the manuscript. RJLFL submitted the manuscript.

Funding This work was supported by funds from the National Institute of Neurological Disorders and Stroke grant number P01NS069539, the Prinses Beatrix Spierfonds grant numbers W.OP14-01 and W.OB17-01 and Spieren voor Spieren.

Competing interests None declared.

Patient consent for publication Obtained.

Ethics approval This study protocol has been approved by the Medical Review Ethics Committee region Arnhem-Nijmegen (NL53213.091.15) and was conducted according to the criteria set by the declaration of Helsinki and its later amendments.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

ORCID iDs
Richard J L F Lemmers http://orcid.org/0000-0002-7102-710X
Silvère M van der Maarel http://orcid.org/0000-0001-8103-711X

REFERENCES

1 Deenen JCW, Arnts H, van der Maarel SM, Padberg GW, Verschuuren JJGM, Bakker E, Weinreich SS, Verbeek ALM, van Engelen BGM. Population-Based incidence and prevalence of facioscapulohumeral dystrophy. *Neurology* 2014;83:1056–9.
2 Mul K, Lassche S, Voermans NC, Padberg GW, Horlings CG, van Engelen BG. What's in a name? the clinical features of facioscapulohumeral muscular dystrophy. *Pract Neurol* 2016;16:201–7.
3 Padberg GW. *Facioscapulohumeral disease. PhD thesis*. Leiden University, 1982.
4 Balog J, Thijssen PE, Shadle S, Straasheijm KR, van der Vliet PJ, Krom YD, van den Boogaard ML, de Jong A, F Lemmers RJL, Tawil R, Tapscott SJ, van der Maarel SM. Increased DUX4 expression during muscle differentiation correlates with decreased Smchd1 protein levels at D4Z4. *Epigenetics* 2015;10:1133–42.
5 Rickard AM, Petek LM, Miller DG. Endogenous DUX4 expression in FSHD myotubes is sufficient to cause cell death and disrupts RNA splicing and cell migration pathways. *Hum Mol Genet* 2015;24:5901–14.
6 Snider L, Geng LN, Lemmers RJLF, Kyba M, Ware CB, Nelson AM, Tawil R, Filippova GN, van der Maarel SM, Tapscott SJ, Miller DG. Facioscapulohumeral dystrophy: incomplete suppression of a retrotransposed gene. *PLoS Genet* 2010;6:e1001181.
7 Wang LH, Friedman SD, Shaw D, Snider L, Wong C-J, Budech CB, Poliachik SL, Gove NE, Lewis LM, Campbell AE, Lemmers RJFL, Maarel SM, Tapscott SJ, Tawil RN. MRI-informed muscle biopsies correlate MRI with pathology and DUX4 target gene expression in FSHD. *Hum Mol Genet* 2019;28:476–86.
8 De Iaco A, Planet E, Coluccio A, Verp S, Duc J, Trono D. DUX-family transcription factors regulate zygotic genome activation in placental mammals. *Nat Genet* 2017;49:941–5.
9 Hendrickson PG, Doráis JA, Grow EJ, Whiddon JL, Lim J-W, Wike CL, Weaver BD, Pflueger C, Emery BR, Wilcox AL, Nix DA, Peterson CM, Tapscott SJ, Carrell DT, Cairns BR. Conserved roles of mouse DUX and human DUX4 in activating cleavage-stage genes and MERVL/HERVL retrotransposons. *Nat Genet* 2017;49:925–34.
10 Whiddon JL, Langford AT, Wong C-J, Zhong JW, Tapscott SJ. Conservation and innovation in the DUX4-family gene network. *Nat Genet* 2017;49:935–40.
11 Geng LN, Yao Z, Snider L, Fong AP, Cech JN, Young JM, van der Maarel SM, Ruzzo WL, Gentleman RC, Tawil R, Tapscott SJ. DUX4 activates germline genes, retroelements, and immune mediators: implications for facioscapulohumeral dystrophy. *Dev Cell* 2012;22:38–51.
12 Bouwman LF, van der Maarel SM, de Greef JC. The prospects of targeting DUX4 in facioscapulohumeral muscular dystrophy. *Curr Opin Neurol* 2020;33:635–40.
13 Dixit M, Ansseau E, Tassin A, Winokur S, Shi R, Qian H, Sauvage S, Mattéotti C, van Acker AM, Leo O, Figlewicz D, Barro M, Laoudj-Chenivesse D, Belayew A, Coppée F, Chen Y-W. DUX4, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PITX1. *Proc Natl Acad Sci U S A* 2007;104:18157–62.
14 Lemmers RJLF, van der Vliet PJ, Klooster R, Sacconi S, Camaño P, Dauwerse JG, Snider L, Straasheijm KR, van Ommen GJ, Padberg GW, Miller DG, Tapscott SJ, Tawil R, Frants RR, van der Maarel SM. A unifying genetic model for facioscapulohumeral muscular dystrophy. *Science* 2010;329:1650–3.
15 Scionti I, Fabbri G, Fiorillo C, Ricci G, Greco F, D'Amico R, Termanini A, Vercelli L, Tomelleri G, Cao M, Santoro L, Percesepe A, Tupler R. Facioscapulohumeral muscular dystrophy: new insights from compound heterozygotes and implication for prenatal genetic counselling. *J Med Genet* 2012;49:171–8.
16 Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG, Gruter AM, Hofker MH, Moerer P, Williamson R, van Ommen GJ, Padberg GW, Frants RR. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. *Nat Genet* 1992;2:26–30.
17 Balog J, Thijssen PE, de Greef JC, Shah B, van Engelen BGM, Yokomori K, Tapscott SJ, Tawil R, van der Maarel SM. Correlation analysis of clinical parameters with epigenetic modifications in the DUX4 promoter in FSHD. *Epigenetics* 2012;7:579–84.
18 Haynes P, Bomsztyk K, Miller DG. Sporadic DUX4 expression in FSHD myocytes is associated with incomplete repression by the PRC2 complex and gain of H3K9 acetylation on the contracted D4Z4 allele. *Epigenetics Chromatin* 2018;11:47.

19 van Overveld PGM, Lemmers RJFL, Sandkuijl LA, Enthoven L, Winokur ST, Bakels F, Padberg GW, van Ommen G-JB, Frants RR, van der Maarel SM. Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy. *Nat Genet* 2003;35:315–7.
20 Zeng W, de Greef JC, Chen Y-Y, Chien R, Kong X, Gregson HC, Winokur ST, Pyle A, Robertson KD, Schmiesing JA, Kimonis VE, Balog J, Frants RR, Ball AR, Lock LF, Donovan PJ, van der Maarel SM, Yokomori K. Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). *PLoS Genet* 2009;5:e1000559.
21 Hamanaka K, Šikrová D, Mitsuhashi S, Masuda H, Sekiguchi Y, Sugiyama A, Shibuya K, Lemmers RJLF, Goossens R, Ogawa M, Nagao K, Obuse C, Noguchi S, Hayashi YK, Kuwabara S, Balog J, Nishino I, van der Maarel SM. Homozygous nonsense variant in *LRIF1* associated with facioscapulohumeral muscular dystrophy. *Neurology* 2020;94:e2441–7.
22 Lemmers RJLF, Tawil R, Petek LM, Balog J, Block GJ, Santen GWE, Amell AM, van der Vliet PJ, Almomani R, Straasheijm KR, Krom YD, Klooster R, Sun Y, den Dunnen JT, Helmer Q, Donlin-Smith CM, Padberg GW, van Engelen BGM, de Greef JC, Aartsma-Rus AM, Frants RR, de Visser M, Desnuelle C, Sacconi S, Filippova GN, Bakker B, Bamshad MJ, Tapscott SJ, Miller DG, van der Maarel SM. Digenic inheritance of an SMCHD1 mutation and an FSHD-permissive D4Z4 allele causes facioscapulohumeral muscular dystrophy type 2. *Nat Genet* 2012;44:1370–4.
23 van den Boogaard ML, Lemmers RJLF, Balog J, Wohlgemuth M, Auranen M, Mitsuhashi S, van der Vliet PJ, Straasheijm KR, van den Akker RFP, Kriek M, Laurense-Bik MEY, Raz V, van Ostaijen-Ten Dam MM, Hansson KBM, van der Kooi EL, Kiuru-Enari S, Udd B, van Tol MJD, Nishino I, Tawil R, Tapscott SJ, van Engelen BGM, van der Maarel SM. Mutations in Dnmt3b modify epigenetic repression of the D4Z4 repeat and the penetrance of facioscapulohumeral dystrophy. *Am J Hum Genet* 2016;98:1020–9.
24 Calandra P, Cascino I, Lemmers RJLF, Galluzzi G, Teveroni E, Monforte M, Tasca G, Ricci E, Moretti F, van der Maarel SM, Deidda G. Allele-specific DNA hypomethylation characterises FSHD1 and FSHD2. *J Med Genet* 2016;53:348–55.
25 Hamanaka K, Goto K, Arai M, Nagao K, Obuse C, Noguchi S, Hayashi YK, Mitsuhashi S, Nishino I. Clinical, muscle pathological, and genetic features of Japanese facioscapulohumeral muscular dystrophy 2 (FSHD2) patients with SMCHD1 mutations. *Neuromuscul Disord* 2016;26:300–8.
26 Jones TI, Yan C, Sapp PC, McKenna-Yasek D, Kang PB, Quinn C, Salameh JS, King OD, Jones PL. Identifying diagnostic DNA methylation profiles for facioscapulohumeral muscular dystrophy in blood and saliva using bisulfite sequencing. *Clin Epigenetics* 2014;6:23.
27 Larsen M, Rost S, El Hajj N, Ferbert A, Deschauer M, Walter MC, Schoser B, Tacik P, Kress W, Müller CR. Diagnostic approach for FSHD revisited: SMCHD1 mutations cause FSHD2 and act as modifiers of disease severity in FSHD1. *Eur J Hum Genet* 2015;23:808–16.
28 Lemmers RJLF, van der Stoep N, Vliet PJvander, Moore SA, San Leon Granado D, Johnson K, Topf A, Straub V, Evangelista T, Mozaffar T, Kimonis V, Shaw ND, Selvatici R, Ferlini A, Voermans N, van Engelen B, Sacconi S, Tawil R, Lamers M, van der Maarel SM. SMCHD1 mutation spectrum for facioscapulohumeral muscular dystrophy type 2 (FSHD2) and Bosma arhinia microphthalmia syndrome (BAMS) reveals disease-specific localisation of variants in the ATPase domain. *J Med Genet* 2019;56:693–700.
29 Lemmers RJLF, de Kievit P, Sandkuijl L, Padberg GW, van Ommen G-JB, Frants RR, van der Maarel SM. Facioscapulohumeral muscular dystrophy is uniquely associated with one of the two variants of the 4q subtelomere. *Nat Genet* 2002;32:235–6.
30 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* 1995;2:39–44.
31 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* 1995;3:155–67.
32 Lemmers RJLF, van der Vliet PJ, van der Gaag KJ, Zuniga S, Frants RR, de Knijff P, van der Maarel SM. Worldwide population analysis of the 4q and 10q subtelomeres identifies only four discrete interchromosomal sequence transfers in human evolution. *Am J Hum Genet* 2010;86:364–77.
33 Lemmers RJLF, Wohlgemuth M, van der Gaag KJ, van der Vliet PJ, van Teijlingen CMM, 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* 2007;81:884–94.
34 Lemmers RJ, van der Vliet PJ, Balog J, Goeman JJ, Arindrarto W, Krom YD, Straasheijm KR, Debipersad RD, Özel G, Sowden J, Snider L, Mul K, Sacconi S, van Engelen B, Tapscott SJ, Tawil R, van der Maarel SM. Deep characterization of a common D4Z4 variant identifies biallelic DUX4 expression as a modifier for disease penetrance in FSHD2. *Eur J Hum Genet* 2018;26:94–106.
35 Cortesi A, Pesant M, Sinha S, Marasca F, Sala E, Gregoretti F, Antonelli L, Oliva G, Chiereghin C, Soldà G, Bodega B. 4q-D4Z4 chromatin architecture regulates the transcription of muscle atrophic genes in facioscapulohumeral muscular dystrophy. *Genome Res* 2019;29:883–95.
36 Gabellini D, Green MR, Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle. *Cell* 2002;110:339–248.
37 Gaillard M-C, Broucqsault N, Morere J, Laberthonnière C, Dion C, Badja C, Roche S, Nguyen K, Magdinier F, Robin JD. Analysis of the 4q35 chromatin organization reveals distinct long-range interactions in patients affected with facio-scapulo-humeral dystrophy. *Sci Rep* 2019;9:10327.
38 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* 1999;45:751–7.
39 van Overveld PGM, Enthoven L, Ricci E, Rossi M, Felicetti L, Jeanpierre M, Winokur ST, Frants RR, Padberg GW, van der Maarel SM. Variable hypomethylation of D4Z4 in facioscapulohumeral muscular dystrophy. *Ann Neurol* 2005;58:569–76.
40 Goselink RJM, Mul K, van Kernebeek CR, Lemmers RJLF, van der Maarel SM, Schreuder THA, Erasmus CE, Padberg GW, Statland JM, Voermans NC, van Engelen BGM. Early onset as a marker for disease severity in facioscapulohumeral muscular dystrophy. *Neurology* 2019;92:e378–85.
41 Lemmers RJLF, van der Wielen MJR, Bakker E, Padberg GW, Frants RR, van der Maarel SM. Somatic mosaicism in FSHD often goes undetected. *Ann Neurol* 2004;55:845–50.
42 Nguyen K, Broucqsault N, Chaix C, Roche S, Robin JD, Vovan C, Gerard L, Mégarbané A, Urtizberea JA, Bellance R, Barnérias C, David A, Eymard B, Fradin M, Manel V, Sacconi S, Tiffreau V, Zagnoli F, Cuisset J-M, Salort-Campana E, Attarian S, Bernard R, Lévy N, Magdinier F. Deciphering the complexity of the 4q and 10q subtelomeres by molecular combing in healthy individuals and patients with facioscapulohumeral dystrophy. *J Med Genet* 2019;56:590–601.
43 Lemmers RJLF, Goeman JJ, van der Vliet PJ, van Nieuwenhuizen MP, Balog J, Vos-Versteeg M, Camano P, Ramos Arroyo MA, Jerico I, Rogers MT, Miller DG, Upadhyaya M, Verschuuren JJGM, Lopez de Munain Arregui A, van Engelen BGM, Padberg GW, Sacconi S, Tawil R, Tapscott SJ, Bakker B, van der Maarel SM. Inter-individual differences in CpG methylation at D4Z4 correlate with clinical variability in FSHD1 and FSHD2. *Hum Mol Genet* 2015;24:659–69.
44 Rijkers T, Deidda G, van Koningsbruggen S, van Geel M, Lemmers RJLF, van Deutekom JCT, 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* 2004;41:826–36.
45 Mariot V, Roche S, Hourdé C, Portilho D, Sacconi S, Puppo F, Duguez S, Rameau P, Caruso N, Delezoide A-L, Desnuelle C, Bessières B, Collardeau S, Feasson L, Maisonobe T, Magdinier F, Helmbacher F, Butler-Browne G, Mouly V, Dumonceaux J. Correlation between low FAT1 expression and early affected muscle in facioscapulohumeral muscular dystrophy. *Ann Neurol* 2015;78:387–400.
46 Caruso N, Herberth B, Bartoli M, Puppo F, Dumonceaux J, Zimmermann A, Denadai S, Lebossé M, Roche S, Geng L, Magdinier F, Attarian S, Bernard R, Maina F, Levy N, Helmbacher F. Deregulation of the protocadherin gene FAT1 alters muscle shapes: implications for the pathogenesis of facioscapulohumeral dystrophy. *PLoS Genet* 2013;9:e1003550.
47 Thijssen PE, Balog J, Yao Z, Pham TP, Tawil R, Tapscott SJ, Van der Maarel SM. DUX4 promotes transcription of FRG2 by directly activating its promoter in facioscapulohumeral muscular dystrophy. *Skelet Muscle* 2014;4:19.
48 Zhang Q, Xu X, Ding L, Li H, Xu C, Gong Y, Liu Y, Mu T, Leigh D, Cram DS, Tang S. Clinical application of single-molecule optical mapping to a multigeneration FSHD1 pedigree. *Mol Genet Genomic Med* 2019;7:e565.
49 Lemmers RJLF, Van Overveld PGM, Sandkuijl LA, Vrieling H, Padberg GW, Frants RR, van der Maarel SM. Mechanism and timing of mitotic rearrangements in the subtelomeric D4Z4 repeat involved in facioscapulohumeral muscular dystrophy. *Am J Hum Genet* 2004;75:44–53.
50 Qiu L, Ye Z, Lin L, Wang L, Lin X, He J, Lin F, Xu G, Cai N, Jin M, Chen H, Lin M, Wang N, Wang Z. Clinical and genetic features of somatic mosaicism in facioscapulohumeral dystrophy. *J Med Genet* 2020;57:777–85.
51 Himeda CL, Debarnot C, Homma S, Beermann ML, Miller JB, Jones PL, Jones TI. Myogenic enhancers regulate expression of the facioscapulohumeral muscular dystrophy-associated DUX4 gene. *Mol Cell Biol* 2014;34:1942–55.
52 Deak KL, Lemmers RJLF, Stajich JM, Klooster R, Tawil R, Frants RR, Speer MC, van der Maarel SM, Gilbert JR. Genotype-phenotype study in an FSHD family with a proximal deletion encompassing p13E-11 and D4Z4. *Neurology* 2007;68:578–82.
53 Lemmers RJLF, Osborn M, Haaf T, Rogers M, Frants RR, Padberg GW, Cooper DN, van der Maarel SM, Upadhyaya M. D4F104S1 deletion in facioscapulohumeral muscular dystrophy: phenotype, size, and detection. *Neurology* 2003;61:178–83.