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Intronic *SMCHD1* variants in FSHD: testing the potential for CRISPR-Cas9 genome editing

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Abstract

Background—Facioscapulohumeral dystrophy (FSHD) is associated with partial chromatin relaxation of the *DUX4* retrogene containing D4Z4 macrosatellite repeats on chromosome 4, and

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transcriptional de-repression of *DUX4* in skeletal muscle. The common form of FSHD, FSHD1, is caused by a D4Z4 repeat array contraction. The less common form, FSHD2, is generally caused by heterozygous variants in *SMCHD1*.

Methods—We employed whole exome sequencing combined with Sanger sequencing to screen uncharacterised FSHD2 patients for extra-exonic *SMCHD1* mutations. We also used CRISPR-Cas9 genome editing to repair a pathogenic intronic *SMCHD1* variant from patient myoblasts.

Results—We identified intronic *SMCHD1* variants in two FSHD families. In the first family, an intronic variant resulted in partial intron retention and inclusion of the distal 14 nucleotides of intron 13 into the transcript. In the second family, a deep intronic variant in intron 34 resulted in exonisation of 53 nucleotides of intron 34. In both families, the aberrant transcripts are predicted to be non-functional. Deleting the pseudo-exon by CRISPR-Cas9 mediated genome editing in primary and immortalised myoblasts from the index case of the second family restored wild-type SMCHD1 expression to a level that resulted in efficient suppression of *DUX4*.

Conclusions—The estimated intronic mutation frequency of almost 2% in FSHD2, as exemplified by the two novel intronic *SMCHD1* variants identified here, emphasises the importance of screening for intronic variants in *SMCHD1*. Furthermore, the efficient suppression of *DUX4* after restoring SMCHD1 levels by genome editing of the mutant allele provides further guidance for therapeutic strategies.

INTRODUCTION

Facioscapulohumeral dystrophy (FSHD [FSHD1; OMIM 158900 and FSHD2; 158901]) is a common muscular dystrophy (prevalence ~1:8500) mainly characterised by progressive weakness and wasting of the facial, shoulder girdle, trunk and upper arm muscles.[1 2] With a disease onset typically in the second decade of life, there is a large variability in onset and progression.[3] Two genetic forms have been identified, FSHD1 and FSHD2, which are clinically indistinguishable[4] and seem to represent a disease continuum.[5 6] Both forms are associated with partial chromatin relaxation of the D4Z4 macrosatellite repeat on chromosome 4 in somatic tissue, characterised by reduced CpG methylation and loss of repressive histone marks, as well as changes in other chromatin factors that result in a more relaxed chromatin structure.[7–10] This chromatin relaxation results in transcriptional de-repression of the D4Z4-encoded *DUX4* [MIM 606009] retrogene in skeletal muscle.[11] The DUX4 transcription factor is normally expressed in the germ line and in cleavage stage embryos, while being suppressed in most somatic tissues.[11–14] DUX4 causes cell death when over-expressed in somatic cell lines or when endogenously expressed in FSHD myotubes.[15 16] D4Z4 chromatin relaxation must occur on a so-called permissive chromosome 4 (4qA haplotype), which contains a polymorphic *DUX4* polyadenylation signal (PAS) distal to the D4Z4 repeat, to cause FSHD.[17 18] This PAS is required for the production of stable *DUX4* mRNA in somatic cells. Consequently, chromatin relaxation of the homologous D4Z4 repeats on non-permissive 4qB or 10q chromosomes does not cause FSHD since these chromosomal backgrounds lack a somatic *DUX4* PAS.[17 19]

FSHD1, accounting for >95% of cases, is caused by contraction of the D4Z4 repeat to 1–10 units on a 4qA chromosome.[20 21] FSHD2 is most often caused by heterozygous variants

in *structural maintenance of chromosomes flexible hinge domain containing 1* (*SMCHD1*) [MIM 614982] in combination with a D4Z4 repeat of 8–20 units on a 4qA chromosome.[5 6 22] *SMCHD1* is an atypical member of the SMC gene superfamily and was originally identified as a regulator of epigenetic silencing.[23 24] SMCHD1 binds to the D4Z4 repeat, thereby repressing DUX4 in somatic cells by yet largely unknown mechanisms.[22] FSHD2 patients with a pathogenic *SMCHD1* variant show reduced SMCHD1 binding to the D4Z4 repeat, resulting in D4Z4 chromatin relaxation and *DUX4* (mis) expression in skeletal muscle.[22] SMCHD1 is also a disease modifier for FSHD1 since pathogenic *SMCHD1* variants have been identified in some severely affected members of FSHD1 families.[25] The *SMCHD1* mutation spectrum in FSHD2 patients includes locus-wide missense, nonsense, and splice site variants, as well as insertions and deletions.[5 22 25–31] Some FSHD2 patients with D4Z4 hypomethylation cannot be explained by (exonic) *SMCHD1* variants. In some of these patients D4Z4 hypomethylation can be explained by *SMCHD1* hemizygosity,[30] or by heterozygous variants in *DNA methyltransferase 3B* (*DNMT3B*) [MIM 602900].[32] Intriguingly, missense variants in the ATPase domain of SMCHD1 can also cause Bosma arhinia microphthalmia syndrome, an unrelated severe developmental disorder.[33 34] There is currently no comprehensive explanation for this discordant clinical outcome of missense variants in the ATPase domain of SMCHD1, although recent biochemical and modelling studies have pointed towards differences in the mutation spectrum and ATPase activity between the two conditions.[35–37]

Currently, >180 FSHD causing *SMCHD1* variants have been described.[37] In this study we describe two independent intronic *SMCHD1* variants which result in aberrant *SMCHD1* transcripts. One variant alters splicing by partial intron retention. The other deep intronic variant leads to exonisation of 53 nucleotides. We designed a genome editing strategy to delete this deep intronic variant with the objective to restore wild-type SMCHD1 expression and *DUX4* repression in myoblasts from a patient carrying this variant.

MATERIALS AND METHODS

All materials and methods are available as online supplementary data.

RESULTS

Clinical and genetic characterisation of Rf744 and Rf1034 individuals

Index case Rf744.1 was suspected of FSHD based on physical examination with a Clinical Severity Score (CSS)[38] of 9 at age 66. Physical examination showed asymmetric scapular winging, right foot drop, asymmetric distribution of facial weakness, symmetric weakness of fixator shoulder girdle muscles, weakness of the pelvic girdle muscles, humeral weakness involving both biceps and triceps brachii, abdominal weakness with positive Beevor's sign and tibialis anterior weakness. Rf744.1 also has a benign myelodysplastic syndrome. D4Z4 repeat size and haplotype analysis showed that the shortest permissive D4Z4 allele of Rf744.1 contains 14 units (figure 1A). D4Z4 methylation analysis in Rf744.1 revealed a FseI methylation level of 19% (Delta1 value −27%), which is well within the FSHD2 range.[5] The unaffected sister of the proband (Rf744.4) also shows D4Z4 hypomethylation, but she does

not have a permissive allele. The daughter of the proband (Rf744.3) does not show D4Z4 hypomethylation and is unaffected (figure 1A).

Index case Rf1034.5 was suspected of FSHD based on physical examination with a CSS of 3 at age 19.[38] Physical examination showed a combination of pectus excavatum, progressive weakness of the right arm, bilateral scapular winging, facial weakness and Beevor's sign. D4Z4 repeat analysis showed that Rf1034.5 has a 7-unit D4Z4 repeat on a permissive chromosome and D4Z4 hypomethylation (FseI: 10%, Delta1 score: −29%), suggestive for both FSHD1 and FSHD2 (figure 1B). The father (Rf1034.1) of the proband carries a disease permissive 7-unit D4Z4 repeat (figure 1B) and has pectus excavatum, which is frequently observed in FSHD.[39] He does not have muscle weakness. The unaffected mother (Rf1034.2) of the proband shows D4Z4 hypomethylation and she has two permissive 4qA alleles of 44 and 74 units. The two sisters (Rf1034.3 and Rf1034.4) both have the 7-unit D4Z4 repeat and D4Z4 hypomethylation, and they are also affected. Physical examination of Rf1034.3 showed a combination of weakness of the scapular stabilisers and weakness of the right arm. Physical examination of Rf1034.4 showed weakness of the facial muscles. This family information strengthened the suggestion that there is a combination of FSHD1 and FSHD2 in this family.

Identification of an intronic variant in *SMCHD1* in Rf744

SMCHD1 variant analysis of coding exons and splice regions identified an intronic *SMCHD1* variant in peripheral blood-derived RNA from patient Rf744.1. This variant (NG_031972.1(SMCHD1):c.1843–15A>G, g.2705677A>G) is located 15 base pairs proximal to exon 14 and various splicing prediction tools suggest that this variant creates a 3′ splice site and has not been reported in public variant databases (figure 2A, online supplementary table S1).

The variant was also identified in Rf744.4, who also shows D4Z4 hypomethylation, but not in Rf744.3, who does not present D4Z4 hypomethylation (online supplementary figure S1A). To investigate whether this variant leads to an altered transcript, an RT-PCR targeting *SMCHD1* exon 12 through exon 14 was performed. Besides the normal PCR product of expected size, a longer PCR product was also detected (figure 2B). Sanger sequencing of individual clones derived from PCR products of the target region shows that they contained the altered transcript sequence from c.1843–14 to c.1843–1 confirming that c.1843–15A>G creates a 3′ splice site (figure 2C and online supplementary figure S1B). The inclusion of these 14 nucleotides is predicted to disrupt the open reading frame and to result in a premature stop codon in exon 14. Sanger sequencing also confirmed the wild-type transcript sequence in some clones, consistent with heterozygous expression. No RNA was available from Rf744.3 and Rf744.4. No further material was available from index case Rf744.1 for additional functional testing of the *SMCHD1* variant, but previous studies support the possibility for the development of FSHD from *SMCHD1* haploinsufficiency.[6 40] This is further supported by the negative Delta1 methylation scores observed exclusively in carriers of the *SMCHD1* variant (Rf744.1 and Rf744.4, figure 1A), which is typical for reduced *SMCHD1* activity at D4Z4.[5]

Identification of a deep intronic variant in *SMCHD1* in Rf1034

SMCHD1 variant analysis of all *SMCHD1* exons and splice regions or whole exome sequencing (WES) in the proband did not identify any putative pathogenic *SMCHD1* variant or pathogenic variants elsewhere in the genome.[5] *SMCHD1* transcript analysis using partially overlapping amplicons identified a fragment of increased size suggestive for aberrant splicing. An RT-PCR targeting *SMCHD1* exon 32 through exon 35 revealed two PCR products for Rf1034.3, that is, a product of expected size and a larger PCR product (figure 3A). This larger PCR product was also identified with an RT-PCR performed on RNA isolated from blood of Rf1034.2 and Rf1034.4 (figure 3A), and RNA from myoblasts of Rf1034.5 (not shown), while it was absent in Rf1034.1 (figure 3A). Sequencing of the larger PCR product revealed the presence of a sequence corresponding to 53 nucleotides of intron 34, from c.−235 to c.−183 proximal to exon 35 (online supplementary figure S2A). These 53 nucleotides are included in the transcript as a pseudo-exon and are predicted to disrupt the open reading frame and to lead to a premature stop codon in exon 35 (online supplementary figure S2A). To identify the variant responsible for this pseudo-exon, we used an intronic PCR followed by Sanger sequencing. A heterozygous deep intronic variant (NG_031972.1(SMCHD1):c.4347–236A>G, g.2760414A>G) in *SMCHD1*, not reported in public databases, was identified in subjects Rf1034.2–5, which was absent in Rf1034.1 (figure 3B, online supplementary figure S2B). Splicing prediction tools suggest that this variant creates a 3′ splice site, while a cryptic 5′ splice site is already predicted in the reference sequence at position c.4347–183 (online supplementary table S1, figure 3C). In this family, the deep intronic *SMCHD1* variant segregates with D4Z4 hypomethylation. We further characterised RNA from primary muscle cell cultures from patient Rf1034.5 using RT-qPCR for *DUX4* and for the wild-type and mutant forms of *SMCHD1* (figure 3D). The inclusion of the pseudo-exon in the mutant mRNA allowed us to use primers targeting this exon for specific amplification of the mutant transcript. This analysis supported the diagnosis of FSHD by the apparent expression of *DUX4*, while also showing that the mutant form of *SMCHD1* is readily detectible on mRNA level (figure 3D). The observed increased expression of *SMCHD1* mRNA following myogenic differentiation is consistent with a previous study.[41] Whether mutant *SMCHD1* mRNA is stable and leads to translation of a truncated SMCHD1 protein is unknown, but ChIP-qPCR analysis of SMCHD1 occupancy on D4Z4 in Rf1034.5 myoblasts compared with controls and unrelated FSHD2 myoblast samples suggests that the inclusion of this pseudo-exon creates SMCHD1 haploinsufficiency with consequent partial decompaction of the D4Z4 chromatin structure in myonuclei (figure 3E). To determine whether the mutant transcript is a target for nonsense mediated decay (NMD), we inhibited NMD using cycloheximide (CHX) in Rf1034.5 myotube cultures (figure 3F). RT-qPCR analysis after CHX treatment showed a modest (~2-fold) increase in *SMCHD1* wild-type mRNA, but a ~10-fold increase in mutant transcript. This response of the mutant *SMCHD1* transcript to inhibition of NMD is similar to the known endogenously produced NMD-sensitive isoform of *SRSF2* (figure 3F, *SRSF2* intron inclusion).[42] This indicates that the *SMCHD1* transcript retaining the pseudo-exon is indeed degraded by NMD, leading to SMCHD1 haploinsufficiency. We compared expression of total *SMCHD1* RNA in Rf1034.5 myotubes to other FSHD and control myotube cultures (online supplementary figure S2C). Due to endogenous variability in *SMCHD1* transcript levels, it is not possible to distinguish between control and FSHD2

samples. Only hemizygous expression of *SMCHD1* significantly alters total *SMCHD1* RNA levels, as reported previously.[40] Attempts to detect a truncated SMCHD1 protein by western blotting using an N-terminal targeting antibody (HPA039441) did not yield any detectable specific signal of lower molecular weight when compared with unrelated samples (data not shown), consistent with a haploinsufficiency situation and NMD-mediated degradation of the transcript for Rf1034.5.

Genome editing designed to remove the pathological intronic variant in Rf1034

In an attempt to suppress *DUX4* in primary muscle cell cultures from individual Rf1034.5 by restoring the wild-type *SMCHD1* open reading frame at the expense of the mutant version, we aimed to delete the *SMCHD1* pseudo-exon from the genome. We performed CRISPR-Cas9-mediated genome editing using two gRNA constructs targeting sequences upstream and downstream of the pseudo-exon. To test whether deletion of the intronic target region would not impair SMCHD1 protein expression from a wild-type allele, we transfected HeLa cells with plasmids encoding the two gRNAs (U/D3) and sp.Cas9–2A-GFP. Monoclonal cultures of GFP-positive HeLa cells were genotyped to screen for clones harbouring a homozygous or heterozygous deletion of the targeted region (online supplementary figure S3, bottom panel). We analysed SMCHD1 protein levels in all clones and compared these with those observed in monoclonal cultures transfected with a plasmid encoding the control gRNA X50 and sp.Cas9–2A-GFP. Although some variation in protein levels can be observed, none of the edited clones showed loss of SMCHD1, even when homozygously edited (online supplementary figure S3, top panels). This indicates that deletion of the intronic region corresponding to the location of the pseudo-exon in Rf1034 by means of CRISPR-Cas9 genome editing does not impair SMCHD1 expression in HeLa cells. Thus, deleting the same region in Rf1034 seems a feasible approach to restore SMCHD1 protein levels.

For genome editing of primary myoblast cultures of Rf1034.5, we first employed an approach in which gRNAs were first delivered by lentiviral transduction, and Cas9 protein was subsequently delivered by induced transduction by osmocytosis and propanebetaine (iTOP). To achieve enrichment of targeted myoblasts, a gRNA targeting *B2M* was co-introduced to allow for FACS sorting of MHC class I-negative cells. In a biological replicate experiment, PCR analysis identified genomic deletions in Rf1034.5 myoblasts treated with gRNAs U/D3, but not with control gRNA X50 (figure 4A). Sanger sequencing of the smaller PCR product of the edited genomic DNA showed that there is a deletion of 407±1 bp confirming that the deep intronic variant is absent in this product (figure 4B). Additional RT-PCR analysis indicated that there are no extra products after treatment with Cas9 and gRNAs U/D3 besides the wild-type and mutant products (online supplementary figure S4A–B). The aforementioned myoblasts were allowed to differentiate to myotubes, and subsequently *SMCHD1* expression levels from the wild-type and mutant alleles was determined by using RT-qPCR. A significantly higher expression of the wild-type *SMCHD1* transcript ($p=0.0286$, Mann-Whitney U test) was observed in the myotube samples treated with the gRNAs flanking the pseudo-exon, although we could not detect a significant change in mutant *SMCHD1* transcript expression ($p=0.8857$, Mann-Whitney U test). The increase of wild-type *SMCHD1* transcript was concomitant with reduced expression of *DUX4* but

not with the DUX4 target genes *KHDC1L* (figure 4C) and *ZSCAN4* (online supplementary figure S4E) (p=0.0286, 0.8857 and 0.1143, respectively (Mann-Whitney U test)) levels suggesting that the gain of *SMCHD1* restores D4Z4 chromatin repression (figure 4C). Expression levels of *MYOG* (p=0.0286, Mann-Whitney U test) but not *MYH3* (p=0.2000, Mann-Whitney U test) were significantly higher in Cas9-treated cells expressing gRNAs U/D3 when compared with those expressing control gRNA X50 (figure 4C). This outcome suggests that the myogenic differentiation process is not strongly impaired due to genomic editing of *SMCHD1*.

While editing of primary Rf1034.5 myoblasts successfully increased *SMCHD1* expression levels and decreased expression of *DUX4*, we could not rule out that incomplete editing of *SMCHD1* in the polyclonal cultures obscured a more robust phenotypical change. Therefore, we immortalised Rf1034.5 myoblasts (Rf1034.5-iMB) to allow generation of monoclonal cultures after genome editing. After iTOP-mediated editing and expansion of U/D3 transduced cells, we confirmed genomic editing on the mutated allele, and thus restoration of the *SMCHD1* open reading frame, in 10 independent myocyte clones, of which one clone (U/D3–4.110) was edited on both alleles (figure 4D, lower panel). For comparison, we isolated material from seven clones transduced with the control gRNA X50 (RNA, DNA and protein analysis shown), as well as 18 unedited U/D3 transduced clones (RNA analysis shown) (collectively called *SMCHD1*-unedited patient clones (n=25)), which still have the pseudo-exon and contain no genomic aberrations at the U/D3 gRNA target sites, as confirmed by PCR and Sanger sequencing (data not shown). Additional RT-PCR analysis of X50 and U/D3 treated samples showed no mis-splicing of the *SMCHD1* mRNA (online supplementary figure S4C–D). SMCHD1 western blot analysis of a representative set of edited (n=10) and X50 *SMCHD1*-unedited myotube clones (n=7) (figure 4D, upper panel) and subsequent quantification of SMCHD1 protein levels normalised to the housekeeping protein Actin showed a significant increase (p=0.0020, Mann-Whitney U test) in cellular SMCHD1 protein after genome editing (figure 4E). Protein quantification data corresponding to the monoclonal myotube cultures were in agreement with the RT-qPCR data for the wild-type and variant *SMCHD1* mRNA forms, in that they were significantly increased (p<0.0001, Mann-Whitney U test) and decreased (p<0.0001, Mann-Whitney U test), respectively (figure 4F). Again, we observed a significant decrease in *DUX4* expression (p=0.0001, Mann-Whitney U test). However, now, the decrease in *DUX4* expression also correlated with decreased levels in the amounts of the DUX4 targets *KHDC1L* (p=0.0342, Mann-Whitney U test), and *ZSCAN4* (p=0.0039, Mann-Whitney U test) (figure 4F and online supplementary figure S4F). These data indicate a more robust phenotypic rescue in edited myotube clones when compared with the primary polyclonal myotube cultures (figure 4C). Expression of myogenic differentiation marker *MYH3* (p=0.0008, Mann-Whitney U test), but not *MYOG* (p=0.7877), was significantly higher in gRNA U/D3 edited cultures, which in combination with typical morphological changes (ie, formation of aligned multinucleated myotubes) observed in differentiating myotube cultures (representative examples online supplementary figure S4G) suggests that restoration of *SMCHD1* by genomic editing to physiological levels does not negatively influence myogenic differentiation *in vitro*. Previously published work has shown that *DUX4* expression in FSHD patient-derived myogenic cultures correlates with high

expression of myogenic markers such as *MYH3*.[41] The negative correlation we observe here (ie, higher *MYH3* with lower *DUX4* expression in *SMCHD1*-edited compared with *SMCHD1*-unedited patient cells) strengthens our conclusion that restoration of SMCHD1 levels supresses expression of *DUX4*.

Loss of SMCHD1 in FSHD2 leads to decreased methylation levels of the DR1 region in D4Z4.[43] We therefore analysed a set of four edited U/D3 clones and four *SMCHD1*-unedited patient clones by bisulphite Sanger sequencing, and found no evidence that increased SMCHD1 levels lead to increased CpG methylation at DR1 in these clones (online supplementary figure S5).

DISCUSSION

In this study, we identified two intronic *SMCHD1* variants: one likely acting as a modifier of disease severity in an FSHD1 family and another likely acting as a disease-causing variant in an FSHD2 family.

In family Rf744, an intronic variant located 15 base pairs proximal to exon 14 creates a 3′ splice site. This variant results in the inclusion of the distal 14 nucleotides of intron 13 into the transcript, which is predicted to disrupt the open reading frame to result in the presence of a premature stop codon in exon 14. The intronic variant and the D4Z4 hypomethylation status were also detected in the unaffected sister of the proband. She carries two non-permissive alleles, which explains why she remained unaffected. The unaffected daughter of the proband does not carry the variant and shows no D4Z4 hypomethylation.

In family Rf1034, no disease-causing variants were identified by exonic *SMCHD1* variant analysis by Sanger sequencing or elsewhere in the exome using WES.[5] However, in this study, a deep intronic variant was identified, which segregates with D4Z4 hypomethylation. This *SMCHD1* variant creates a 3′ splice site in intron 34 resulting in exonisation of 53 nucleotides of intron 34. Inclusion of these 53 nucleotides in the transcript is predicted to disrupt the open reading frame and to result in a premature stop codon in exon 35. In family Rf1034, this *SMCHD1* variant acts as a modifier of disease severity. The proband and his two sisters all carry a permissive D4Z4 repeat of 7 units and the deep intronic variant in *SMCHD1*, and are affected. The proband is more severely affected than his sisters, indicating clinical variability, which is common in FSHD.[5] The mother (Rf1034.2) carries the deep intronic variant in *SMCHD1* and two permissive 4qA alleles of 44 and 74 units, while the median D4Z4 repeat size on chromosome 4 in controls is 23 units. The length of the D4Z4 repeats in the mother is much longer than the median length of the shortest permissive allele in FSHD2 patients, which is only 13 units.[44] Probably, the permissive alleles of the mother contain too many repeat units to become severely de-repressed by SMCHD1 loss associated with this *SMCHD1* variant, explaining her FSHD2-free status. This has also been shown in other FSHD2 families, in which *SMCHD1* variant carriers are typically only affected when they also carry a permissive D4Z4 repeat of 11–20 units.[5] The father carries an FSHD1-sized allele of 7 units and is unaffected. However, the father presented with pectus excavatum, a condition often observed in FSHD.[39] Non-penetrance and mild phenotypes are often seen in carriers of FSHD1-sized alleles of 7–10 units.[45]

Indeed, in 1%–3% of the control population, D4Z4 repeats of 7–10 units on disease permissive chromosomes are found, indicating the reduced penetrance of these alleles.[46] [47] Thus, in Rf1034, it is likely that only the combination of a permissive D4Z4 repeat of 7 units with the deep intronic variant in *SMCHD1* causes FSHD. This modifying role of *SMCHD1* variants has been described in multiple FSHD1 families with upper-sized FSHD1 D4Z4 repeats, which provides an explanation for the clinical variability observed in these families.[6] [25] [26]

In order to restore the *SMCHD1* open reading frame in primary myoblasts of Rf1034, we aimed to remove the splice site created by the deep intronic variant by CRISPR-Cas9-mediated genome editing. The simultaneous treatment with these gRNAs and Cas9 was expected to create a genomic deletion in intron 34 of 407 (±1) base pairs on both mutant and wild-type alleles. Since the deletion would be intronic, it was expected to disrupt inclusion of the pseudo-exon without affecting wild-type splicing. This predicted lack of consequences for the wild-type allele was supported by the experiments in HeLa cells in which we did not observe a loss of SMCHD1 protein after deletion of the target sequence in intron 34.

We performed genome editing experiments using plasmid transfection, lentiviral vector gRNA transduction combined with iTOP delivery of Cas9, or complete delivery of the gRNA–Cas9 complex by iTOP. The iTOP transduction was previously shown to be an efficient strategy to deliver proteins to a variety of primary cell types. [48] In this study, we show that this strategy can also be applied in primary and immortalised myoblasts.

Expression analysis showed a significant increase in levels of wild-type *SMCHD1* transcript after CRISPR-Cas9-mediated pseudo-exon excision. In turn, this allowed us to detect a consistent reduction of *DUX4* expression in the edited primary and immortalised myogenic cells. Several factors might have affected wild-type *SMCHD1* levels including the efficiency of the genome editing procedure at wild-type and variant alleles and dynamic changes in *SMCHD1* and *DUX4* expression during muscle cell differentiation.41 In Rf1034, the mutation is likely to cause *SMCHD1* haploinsufficiency, as supported by the sensitivity of the mutant transcript to NMD, and the reduced binding of SMCHD1 to D4Z4 observed in ChIP-qPCR experiments. Our results suggest that restoring wild-type *SMCHD1* expression levels to near-normal, bi-allelic levels is sufficient to effectively repress *DUX4*. Previously, we have shown that mild SMCHD1 overexpression by lentiviral transduction can also repress *DUX4* in FSHD primary muscle cell cultures.[41] Combined with this study, our data suggest that therapeutic strategies aiming at SMCHD1 upregulation to normal or close-to-normal levels in FSHD2 skeletal muscle cells results in efficient suppression of *DUX4*. This suppression of *DUX4* is not dependent on D4Z4 methylation as we did not observe an increase in D4Z4 methylation in edited clones. This seems consistent with a recent report showing that SMCHD1 is important for *de novo* methylation at the pluripotent stage, but dispensable for methylation maintenance in somatic cells.[49]

The variants identified in this study affect splicing by introducing new 3′ splice sites in SMCHD1 outside the consensus sequence. Previously, an intronic *SMCHD1* variant with a similar effect as the variant in Rf744 was identified in another FSHD2 patient.[5] In total,

we have identified >180 variants in *SMCHD1*, which affect gene function.[5 22 25 29 30 37] This includes three intronic variants outside the splice consensus sequence that introduce a 3′ splice site, of which two cases are further described in the current work, and Rf1352 was published previously.[22] This indicates that intronic variants in *SMCHD1* that introduce a new splice site are present in approximately 2% of the FSHD2 patient population. This type of variants might explain FSHD phenotypes in patients in whom no variant has yet been identified in the exonic regions of the *SMCHD1* gene or its splice site consensus sequences. Since *SMCHD1* is expressed in blood, RNA-seq or targeted *SMCHD1* RNA analysis approaches might be considered to identify these intronic variants, although care must be taken to avoid false-negative results due to potential NMD of the mutant transcript.

In summary, this report expands the *SMCHD1* mutation spectrum in FSHD2 by characterising two additional intronic variants in *SMCHD1*. Both variants lead to aberrant splicing with the altered *SMCHD1* transcripts leading to frameshifts generating premature stop codons. Our study also highlights the importance of, whenever warranted, performing additional variant screening in FSHD2 patients that are negative for exonic *SMCHD1* variants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement

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

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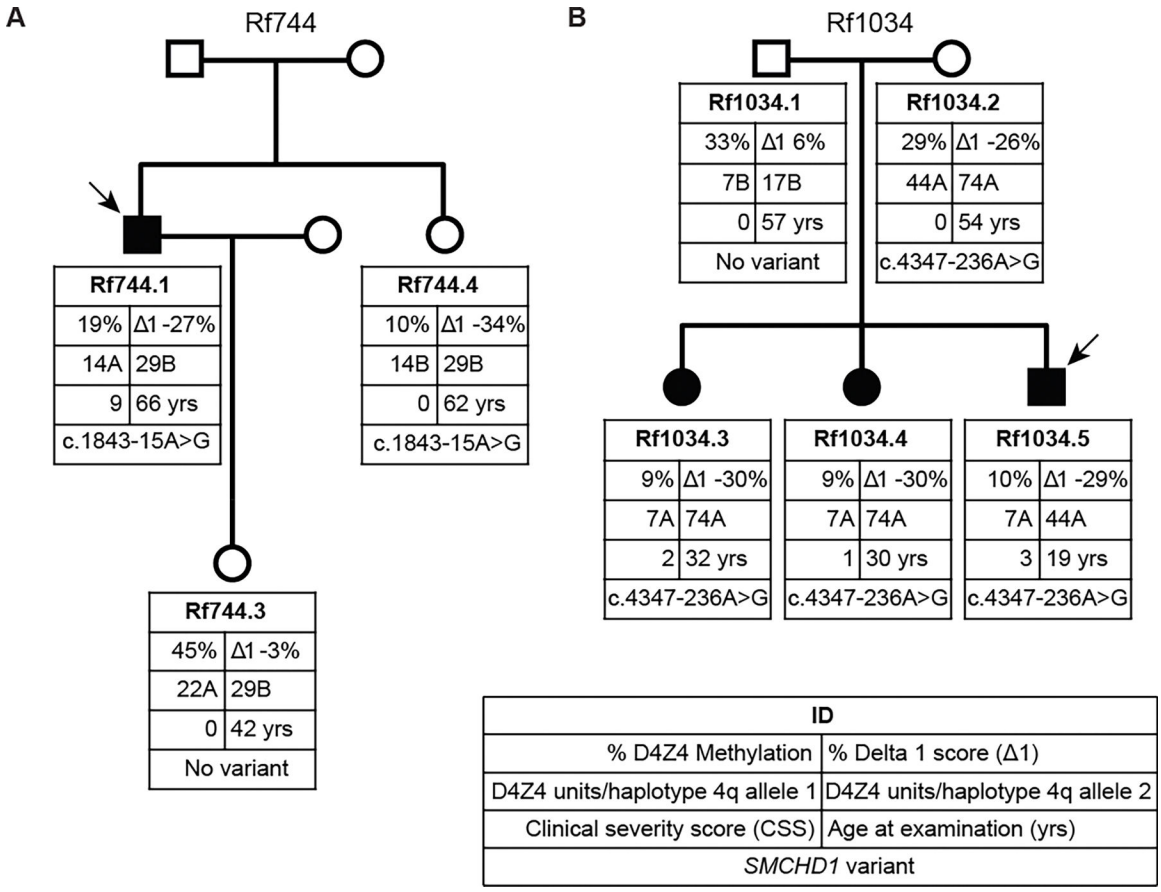

Figure 1.

Pedigrees of families Rf744 (A) and Rf1034 (B). Clinically affected individuals are indicated in black, the index cases are marked by an arrow. The following information is provided: the family identifier, D4Z4 methylation, delta1 score, the size and type (A permissive, B non-permissive) of 4q-linked D4Z4 repeats, the Clinical Severity Score, the age at examination and the *SMCHD1* variant. Key is shown on the bottom right.

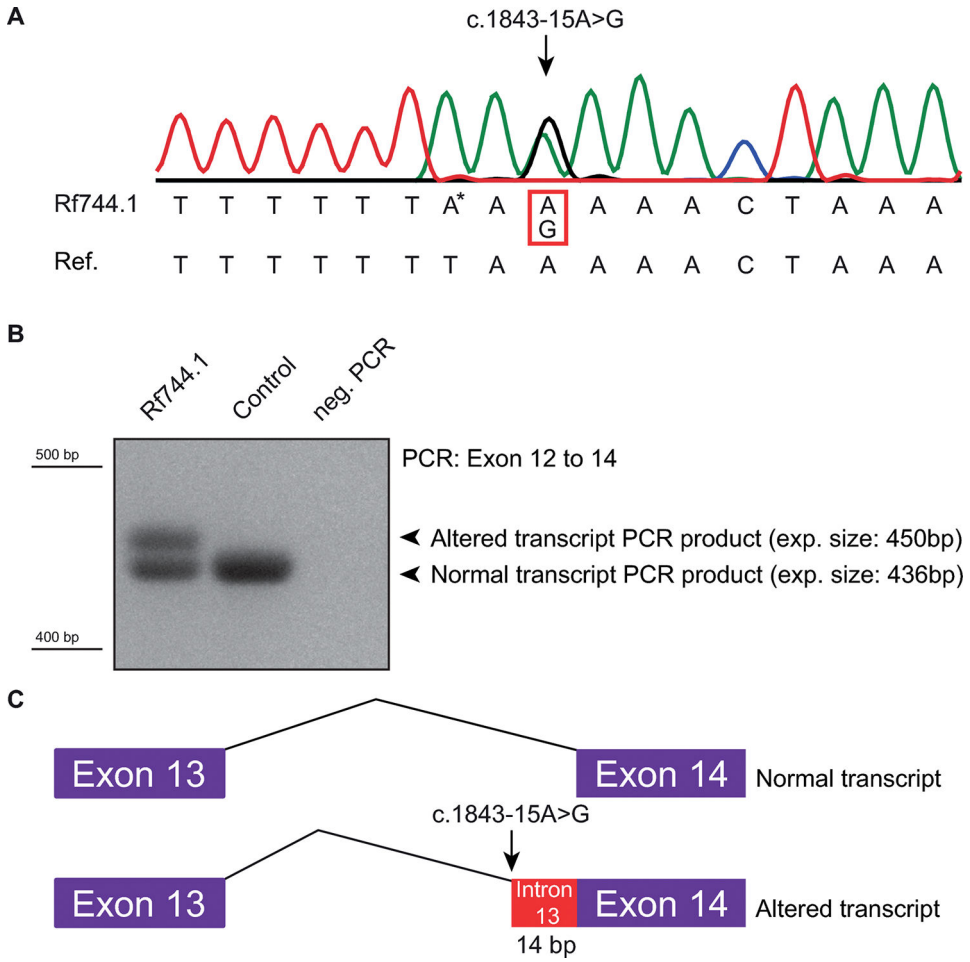

Figure 2.

Identification of an intronic *SMCHD1* variant in Rf744. (A) Sanger sequence track from Rf744.1 showing the intronic variant in *SMCHD1* at position c.1843–15, highlighted with a rectangle. * indicates common SNP rs8090988 (T/A, ancestral T, minor allele frequency 0.33 (A)). (B) RT-PCR analysis of the *SMCHD1* transcript region spanning exons 12 through 14 in Rf744.1. A control sample and a negative control PCR (No DNA) were taken along. (C) Schematic representation of splicing of the normal transcript and the altered transcript containing the intronic variant.

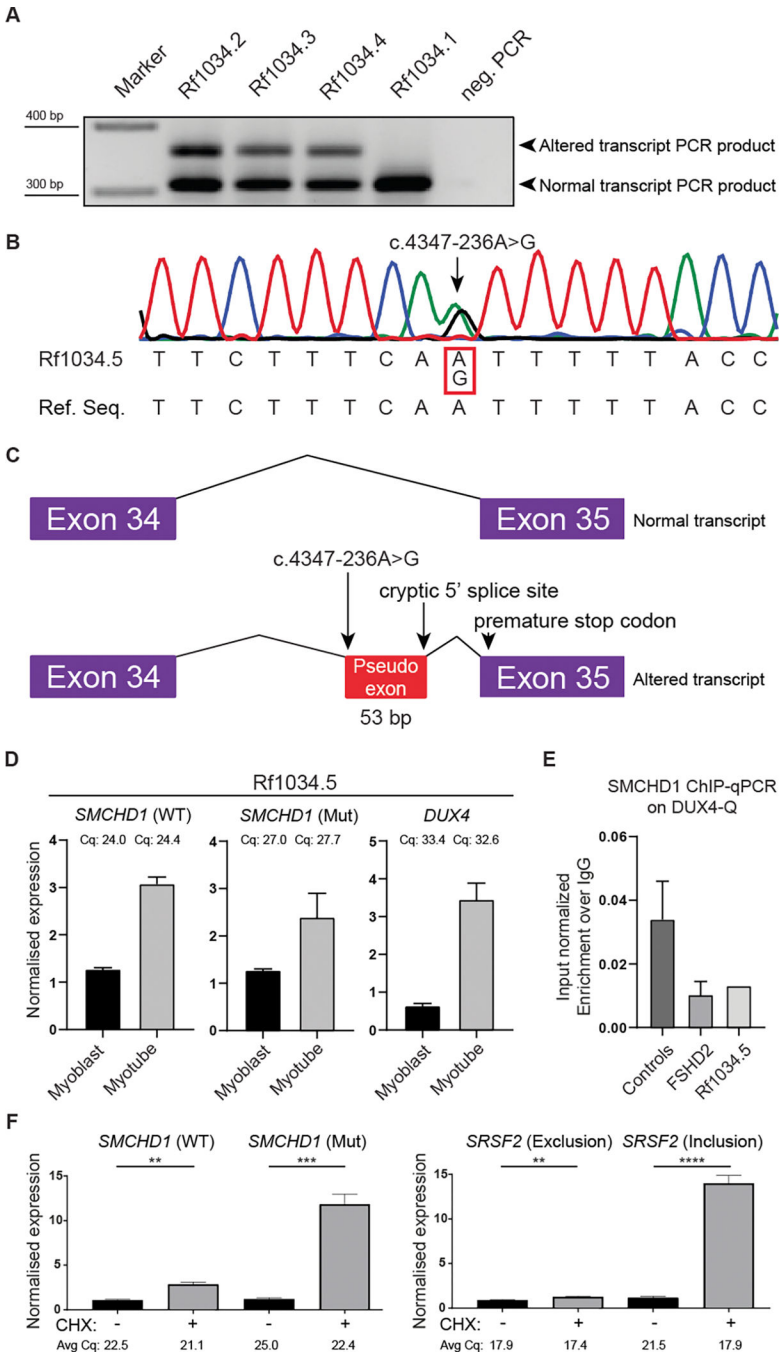

Figure 3.

Identification of a deep intronic *SMCHD1* variant in Rf1034. (A) RT-PCR analysis of *SMCHD1* transcripts spanning exons 32 through 35 in four members of family Rf1034. A negative control PCR (No DNA) was performed in parallel. (B) Sanger sequence track showing the deep intronic variant in *SMCHD1* at position c.4347–236 in Rf1034.5, highlighted with a rectangle. (C) Schematic representation of splicing of the normal transcript and the altered transcript containing the deep intronic variant. The altered transcript shows exonisation of the 53 base pair pseudo-exon. (D) RT-qPCR analysis of

primary Rf1034.5 myoblast and myotube samples. Expression of *SMCHD1* (wild-type and mutant) and *DUX4* are shown, normalised to *GUSB* expression. Error bars indicate SEM. (E) ChIP-qPCR analysis of SMCHD1 occupancy on the DUX4-Q region in D4Z4 on chromosome 4 in myoblasts of three control individuals, three unrelated FSHD2 patients and Rf1034.5. Input normalised enrichment is shown, subtracted for IgG values of the corresponding sample, error bars indicate SD. (F) Inhibition of nonsense mediated decay (NMD) by cycloheximide (CHX) treatment in Rf1034.5 myotubes. RT-qPCR analysis shows that CHX treatment results in a ~10-fold increase of *SMCHD1* mutant transcript, with a smaller (~2-fold) increase in WT transcript. This CHX-mediated increase is similar to a known NMD target, an isoform of *SRSF2* including an intron (*SRSF2* inclusion). This increase of transcript levels is not seen for an *SRSF2* transcript excluding this intron. Expression was normalised to *RPL13*. CHX−: n=3, CHX+: n=4 (**p<0.01, ***p<0.001, ****p<0.0001 — Student's t-test).

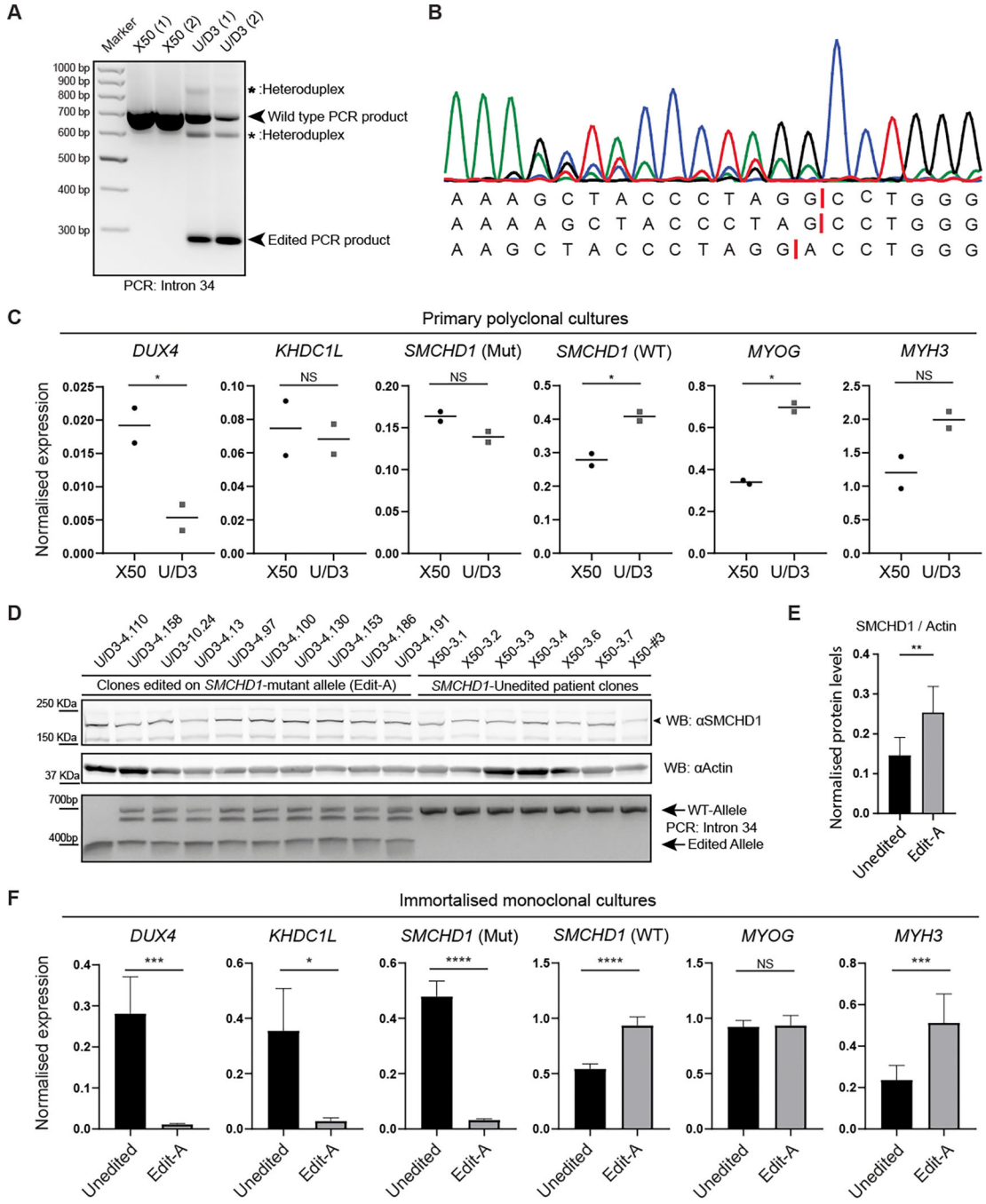

Figure 4.

Genomic deletion of the pseudo-exon in *SMCHD1* in myocytes of Rf1034 by CRISPR-Cas9-based editing. (A) Gel electrophoresis of genomic PCR on primary Rf1034.5 myoblasts treated with Cas9 and control gRNA X50 (targeted against *AAVS1*) or treated with Cas9 and gRNAs U/D3, which cleave upstream and downstream of the pseudo-exon, respectively. Gel electrophoresis image showing the wild-type PCR product, a PCR product with a genomic deletion in intron 34 (edited PCR product), and a heteroduplex formed by hybridisation of the wild-type and edited PCR product. Samples from a biological

replicate experiment are shown. (B) Sanger sequencing track of the PCR product with the deletion in intron 34 (including the deep intronic variant) after genomic editing. The track shows that position chr18:2760070 is mainly repaired to chr18:2760478 (upper line). The other repaired products include position chr18:2760069 to chr18:2760478 (middle line) and position chr18:2760070 to chr18:2760477 (lower line). The vertical lines indicate the breakpoints. (C) Expression of wild-type *SMCHD1*, mutant *SMCHD1* (ie, pseudo-exon containing), *MYOG, MYH3, DUX4* and DUX4 target gene *KHDC1L* by RT-qPCR in Rf1034.5 myotubes treated with Cas9 and gRNA X50 or Cas9 and gRNAs U/D3. Two biological replicates are shown as independent data points, each containing two technical replicate cultures. Expression was normalised to *RPL13* and *GUSB* expression, horizontal bars indicate mean normalised expression. (D) Western blot for SMCHD1 and Actin (top panels) and genomic analysis of intron 35 (bottom panel) of monoclonal Rf1034.5-iMB cultures, edited in intron 34 with the U/D3 gRNA combination (Edit-A) or a representative set of *SMCHD1*-unedited patient clones (X50). (E) Quantification of SMCHD1 levels of the western blot data presented in (D), normalised to Actin. error bars: SD. (F) RT-qPCR analysis of monoclonal *SMCHD1*-edited (n=10) or *SMCHD1*-unedited (n=25) Rf1034.5-iMB myotube clones. Error bars: SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; NS, not significant — Mann-Whitney U test.