

Facioscapulohumeral muscular dystrophy

Are telomeres the end of the story?

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Facioscapulohumeral muscular dystrophy (FSHD) is a progressive myopathy with a relatively late age of onset (usually in the late teens) compared with Duchenne and many other muscular dystrophies. The current FSHD disease model postulates that contraction of the D4Z4 array at chromosome 4q35 leads to a more open chromatin conformation in that region and allows transcription of the DUX4 gene. DUX4 mRNA is stable only when transcribed from certain haplotypes that contain a polyadenylation signal. DUX4 protein is hypothesized to cause FSHD by mediating cytotoxicity and impairing skeletal muscle differentiation. We recently showed in a cell culture model that DUX4 expression is regulated by telomere length, suggesting that telomere shortening during aging may be partially responsible for the delayed onset and progressive nature of FSHD. We here put our data in the context of other recent findings arguing that progressive telomere shortening may play a critical role in FSHD but is not the whole story and that the current disease model needs additional refinement.

Facioscapulohumeral muscular dystrophy (FSHD, FSHD1A MIM 158900) has been linked to a reduction in size of the D4Z4-array at chromosome 4q (reviewed in¹). Healthy individuals have up to 150 of these macrosatellite repeats (each 3.3 kb in length), whereas in FSHD patients this number is reduced to less than 11. Short D4Z4 arrays lose marks of heterochromatin, such as DNA methylation and

histone H3K9 methylation (reviewed in¹). It has been proposed that D4Z4 repeat contraction is pathogenic only on specific, so called permissive haplotypes, i.e., in the presence of the A-allele (but not B- or C-alleles) distal to D4Z4,² and one of several SSLP (simple sequence length polymorphisms) proximal to D4Z4 (161, 161L, 159, or 168).³ A-type alleles contain the pLAM region harboring a polyadenylation signal for transcripts from the most telomeric D4Z4 unit.^{3,4} Each D4Z4 unit contains an evolutionarily conserved ORF for DUX4,⁵ suggesting that its gene product has an important function. Indeed, DUX4 has been found to be expressed not only in FSHD muscle,^{6,7} where it presumably contributes to pathology, but also in human testes and iPS cells,⁷ where it presumably has beneficial, yet unknown functions. The “DUX4-model” is the latest and most attractive in a long series of candidates thought to explain the molecular pathogenesis of FSHD. Increasing amounts of data support this model, but important aspects of the molecular pathogenesis of FSHD are still missing and several reports contain cases that cannot be explained by the current model. DUX4 expression is found in only ~1:1,000–2,000 nuclei in myotubes from FSHD samples.^{6,7} Although possible, it appears biologically implausible that this isolated expression would cause the observed myopathy. FSHD has the characteristics of a complex, multifactorial disease and the evidence is exceptionally strong that “DUX4 expression from a contracted and permissive 4q allele” is not generally

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sufficient and may not even be necessary to cause FSHD. Instead we envision a two (or more) hit mechanism, where the first hit is a contracted allele (causing misexpression of DUX4 in most cases), and the second the co-occurrence of one of several conditions: contraction of the second D4Z4 allele on 4q⁸ (or the presence of only 1–3 repeats, where DUX4 expression may be unusually high even with monoallelic genetics), an independent myopathy, a family background of high CRYM or other muscle-toxic protein expression,⁹ or an unusually short telomere at the contracted 4q. It is also conceivable that low levels of SMCHD1, which cause the rare contraction-independent form of FSHD (FSHD2, MIM 158901),¹⁰ represents a second hit in some patients with the much more common contraction-dependent FSHD.

We want to emphasize that many candidates for the cause of FSHD were discovered in a small set of samples and, though they may be relevant for particular FSHD cases, these findings often have not generalized to larger populations, becoming non-significant when the sample size increased. In carefully planned cell culture studies it was shown that for several readouts, there were bigger differences between families (each with FSHD and unaffected individuals) than between FSHD and unaffected individuals within families.¹¹ Hence, these family effects may lead to misinterpretation of small data sets.

Testing more than 800 independent and healthy individuals from Italy and Brazil, the previously described permissive allele (contracted D4Z4 on 4qA161 with a polyadenylation site) was found with an unexpectedly high frequency (1.4%), two orders of magnitude higher than the incidence of FSHD.¹² This clearly argues that the permissive signature is not sufficient to cause disease. In addition, these numbers are probably underestimates, as several other haplotypes previously described as “non-permissive” did not prevent disease in ~25% of 223 FSHD patients, including the 4qA166 haplotype (which had been suggested to be nonpathogenic in the Dutch population¹³), but also the B-type-allele that lacks pLAM. Interestingly, DUX4 transcripts from chromosome 4q

with the B allele and chromosome 10 have been detected in human testes,⁷ the latter using a polyadenylation signal ~6.5 kb distal of pLAM. If these transcripts are stable enough to be detected in testes, transcription and translation from “non-permissive” alleles might also be possible in skeletal muscle. Hence, expanding the sample size expanded the number of haplotypes on 4q that are compatible with FSHD, and revealed that only a small percentage of carriers of contracted and permissive alleles develop disease. These observations are still compatible with the notion that inappropriate DUX4 expression in skeletal muscle contributes to FSHD. However, definitive proof that DUX4 is the primary cause of FSHD is still missing. Cytotoxicity has been shown mostly in overexpression studies and is not specific to muscle (e.g., 14-17). In addition, differentiation of myoblasts induces not only endogenous DUX4,¹⁸ but also a mechanism rendering them more resistant to overexpressed DUX4.¹⁴ This is consistent with our observations showing no obvious increase in cell death in differentiating FSHD cells with short telomeres (with higher endogenous DUX4 expression) compared with isogenic cells with long telomeres (and lower DUX4 expression), or cells from unaffected siblings with no detectable DUX4. Apoptosis genes were not upregulated in cells with high endogenous DUX4 expression due to short telomeres, in contrast to data generated after ectopic DUX4 overexpression by others (e.g., BAX, BID, TP53¹⁶). On the molecular level, we also could not detect any inhibition of myogenic differentiation by endogenously high DUX4 levels, as opposed to ectopic overexpression. Similarly, transcriptional profiling of muscle biopsies from FSHD subjects, most of which express DUX4,⁶ show only few and mild differences when compared with unaffected siblings,¹⁹ without major changes in apoptosis genes or skeletal muscle differentiation markers. Therefore it is entirely possible that strong cytotoxicity and inhibition of differentiation was an artifact of ectopic overexpression. Recently, the first indirect evidence that endogenous DUX4 may trigger apoptosis *in vitro* has been reported.²⁰ The authors optimized cellular culture conditions to

achieve higher percentages of DUX4-positive nuclei in differentiated myotubes (more than 10% in some cases). Prolonged differentiation resulted in apoptosis of myotubes from FSHD cell lines, which impressively could be prevented by DUX4 knock down. However, four out of ten FSHD cell lines did not show the apoptotic phenotype, including one with a high percentage of DUX4-positive nuclei. These observations again indicate that heterogeneous pathways are active in FSHD cells from different patients. In contrast, inappropriate activation of some DUX4 target genes²¹ in FSHD muscle has been independently confirmed in a large cohort.¹⁹ To summarize, the functions of endogenous DUX4 and how it may cause or contribute to FSHD are still largely correlative. In addition, expression patterns of DUX4 in muscle biopsies reveal a complex picture, incompatible with the simple model that DUX4 is expressed exclusively in FSHD muscle where it causes disease. First, full length DUX4 mRNA is not exclusively found in FSHD muscle. It is also observed in a significant subset of controls with normal sized D4Z4 repeats and no sign of muscle disease (ref. 6: 3 out of 26). Second, full length DUX4 mRNA is not detected in all muscle biopsies from FSHD subjects [5 out of 10 (ref. 7), and 13 out of 59 (ref. 6) FSHD samples had no DUX4 detected]. Third, muscle weakness in FSHD subjects does not strongly correlate with DUX4 expression: DUX4 was present in several subjects containing contracted D4Z4 on a permissive allele, but without clinical manifestations of the disease.⁶ Finally, DUX4 expression was absent in some FSHD muscles showing clear signs of weakness.⁶ We therefore conclude that the presence of DUX4 in skeletal muscle is not by itself sufficient to cause FSHD in most cases. However, it is possible that transient bursts of DUX4 expression may cause or contribute to FSHD while being undetectable at a certain time or area of biopsy, as speculated before.⁷

Because detectable DUX4 apparently does not cause disease in some individuals, we postulate there often needs to be involvement of at least a second factor in the etiology of FSHD. Theoretically, there might be protective factors preventing

FSHD in individuals expressing DUX4, and/or factors working synergistically with DUX4 to drive disease progression. Different factors and different mechanisms in different individuals/families would help explain the difficulty to detect an FSHD-specific signature in large cohorts. The simplest case would be increasing the dose of DUX4 by adding a second contracted allele, which has been suggested by unusually high numbers of compound heterozygous FSHD patients in several Italian families.⁸ Another way to increase DUX4 dosage would be a short telomere at 4q. We showed in a well-controlled cell culture model that transcription of DUX4 and FRG2 (centromeric of D4Z4) on contracted alleles is strongly upregulated by telomere shortening.¹⁸ Telomere length not only decreases with aging (and could therefore explain delayed onset and progression of FSHD), but is also highly variable between individuals. For example, if the contracted 4q allele is paternal in origin, the length of the 4q telomere in the sperm fertilizing the embryo could be dramatically different between siblings.²² This might partially explain cases in which siblings carrying the same contracted 4q allele nonetheless exhibit dramatically different ages of onset or severity of symptoms.

The mechanism by which telomere length affects gene expression at 4q35 remains to be determined. It is likely that telomere length influences the epigenetic landscape in this region, e.g., that heterochromatin spreading from telomeres decreases during their shortening with increased age. In this scenario, normalized D4Z4 repeats would act as a repressor, so that the subtelomeric genes would be repressed regardless of telomere length. This repressor function would be reduced when < 11 repeats were present (and lost completely with 1–3 repeats). Expression could then increase with telomere shortening as heterochromatin spreading from the telomere diminished as telomeres shortened.

Theoretically, telomere length regulated genes other than DUX4 may contribute to FSHD as well. These might be located on chromosome 4q, such as FRG2, or other chromosomes. FRG2 has been found elevated in FSHD samples in

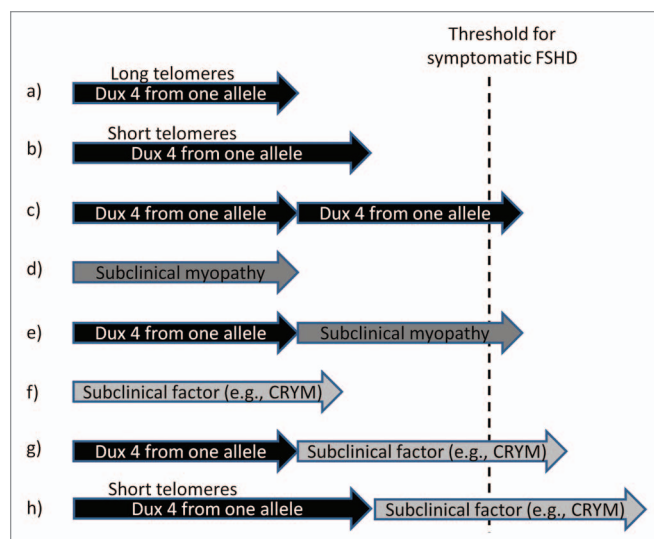


Figure 1. Possible mechanisms in FSHD. By itself, DUX4 expression from a contracted allele might not be sufficient to cause FSHD symptoms (A). Short telomeres would increase expression, but still might not be sufficient (B). A variety of additional factors could cooperate with DUX4 to increase muscle toxicity and produce weakness. A far from exhaustive list could include compound heterozygosity (having two contracted alleles, each increasing the dose of DUX4 expression so the total was sufficient to exceed a threshold, C), and other conditions that compromised muscle function (such as a subclinical myopathy (D, E), or the increased expression of other factors that by themselves produced decreased muscle reserve but not overt symptoms: (F-H). Telomere length could modulate the age of onset and severity of symptoms by influencing DUX4 levels or the level of other genes.

several studies (e.g., 23), but a universal causal involvement in FSHD is ruled out because there are FSHD patients with deleted FRG2 on chromosome 4q, and by the absence of a phenotype when overexpressed *in vitro*¹⁴ or *in vivo*.²³ Although clearly not sufficient to cause FSHD, high expression of FRG2, whose function is still unknown, may synergize with DUX4 or other misregulated factors and contribute to disease in some patients. Other genes on chromosome 4q that may synergize with DUX4 and contribute to FSHD, possibly each only in a subset of patients, include FRG1, DUX4c and FAT1. FRG1 has been found elevated in FSHD cells and muscle by some investigators (e.g., 23), but not by others (e.g., 11,19), and FRG1 has been reported to cause an FSHD-like phenotype when highly overexpressed in mice.²⁴ DUX4c protein is identical to DUX4 (outside of having a different C-terminus), and inhibits differentiation when overexpressed in myoblasts.²⁵ Hypomorphic FAT1 alleles cause FSHD-like symptoms in mice and FAT1 levels are reduced in a subset of fetuses with D4Z4 contraction.²⁶ There is at least one study providing some

evidence that chromosome looping can occur between the subtelomere of 4q and genes ~5 Mb away.²⁷ It is also possible that these interactions might change with telomere length and could influence muscle physiology.

Besides genes at chromosome 4q, other loci may contribute to FSHD, such as CRYM, which has been found to be expressed at high levels in some FSHD families,^{9,11} and has functions putatively misregulated in FSHD. Noteworthy are numerous case reports about the co-occurrence of D4Z4 contraction and an independent myopathy.²⁸⁻³⁴ Figure 1 summarizes some of the many possibilities in which short telomeres and other factors could cooperate in the production of FSHD symptoms.

In conclusion, the molecular mechanism leading to FSHD is complex and not yet adequately understood. Current data and models are insufficient to fully explain this disease. Key assumptions, such as DUX4's exclusive causative role, need to be unequivocally demonstrated. We believe that FSHD will prove to be a multifactorial disease, and that several

disease-promoting factors in addition to D4Z4 contraction will be heterogeneous, such as an unrelated additional myopathy, or short telomeres at chromosome 4q. Multiple factors will likely be discovered, possibly including some that are protective.

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Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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