Deregulation of the Protocadherin Gene FAT1 Alters Muscle Shapes: Implications for the Pathogenesis of Facioscapulohumeral Dystrophy

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Abstract

Generation of skeletal muscles with forms adapted to their function is essential for normal movement. Muscle shape is patterned by the coordinated polarity of collectively migrating myoblasts. Constitutive inactivation of the protocadherin gene Fat1 uncoupled individual myoblast polarity within chains, altering the shape of selective groups of muscles in the shoulder and face. These shape abnormalities were followed by early onset regionalised muscle defects in adult Fat1-deficient mice. Tissue-specific ablation of Fat1 driven by Pax3-cre reproduced muscle shape defects in limb but not face muscles, indicating a cell-autonomous contribution of Fat1 in migrating muscle precursors. Strikingly, the topography of muscle abnormalities caused by Fat1 loss-of-function resembles that of human patients with facioscapulohumeral dystrophy (FSHD). Fat1 lies near the critical locus involved in causing FSHD, and Fat1 mutant mice also show retinal vasculopathy, mimicking another symptom of FSHD, and showed abnormal inner ear patterning, predictive of deafness, reminiscent of another burden of FSHD. Muscle-specific reduction of Fat1 expression and promoter silencing was observed in foetal FSHD1 cases. CGH array-based studies identified deletion polymorphisms within a putative regulatory enhancer of Fat1, predictive of tissue-specific depletion of Fat1 expression, which preferentially segregate with FSHD. Our study identifies Fat1 as a critical determinant of muscle form, misregulation of which associates with FSHD.

Introduction

Developmental genetics has provided considerable insight into the regulatory networks controlling overall skeletal muscle development. Perturbation of these common mechanisms is associated with congenital abnormalities of the muscle lineage as well as with later-onset muscle pathologies [1]. In contrast, less is known about the mechanisms of functional diversification within the muscle lineage. Such diversification may be either metabolic - fast versus slow fibres, for example - or morphological, such as the position and shape of individual muscles. Genes controlling diversification too are likely to be of clinical significance [2–4], since several human muscular dystrophies do not affect all muscles evenly, but specifically target regionalized groups [5]. This is true for limb girdle muscular dystrophy (LGMD), oculopharyngeal muscular dystrophy (OPMD), myotonic dystrophies with oculomotor involvement, distal myopathies, scapulopeloneal dystrophy, and facioscapulohumeral dystrophy (FSHD) [5–6]. In no case, however, is the rationale for this geographic specificity currently understood.

One characteristic example of focal myopathies is FSHD, which affects subsets of muscles in the facial and shoulder areas [6]. The main form of FSHD - FSHD1 - is an autosomal dominant disorder associated with the contraction of an array of 3.3 Kb macrosatellite repeats (D4Z4), located at the subtelomeric 4q35 locus [6]. The mechanism by which the D4Z4 contraction triggers the
Deregulated FAT1 Expression as a Model of FSHD

**Author Summary**

Facioscapulohumeral muscular dystrophy (FSHD) is a hereditary human myopathy affecting groups of skeletal muscles in the face and shoulders. Despite recent advances on the molecular cascade initiated by its main genetic cause, with identification of DUX4 as the main pathogenic agent, how this leads to the specific clinical picture is still poorly understood. Here, we investigated the role of the FAT1 protocadherin gene, located near the FSHD locus, which was repressed by DUX4 in human muscle cells. Disruption of the mouse Fat1 gene causes muscular and non-muscular phenotypes highly reminiscent of FSHD symptoms. We show that Fat1 is required in migrating muscle precursors, and that the altered muscle shapes caused by fat1 mutations are predictive of early onset defects in muscle integrity in adult mutants, with a topography matching the map of muscles affected in FSHD. In humans, we observed Fat1 lowering in muscle but not brain of foetal cases with canonical FSHD1, and identified deletions of conserved elements in the FAT1 locus predictive of changes in FAT1 expression, that were enriched among FSHD patients. Thus, deregulating Fat1 in FSHD-related tissues provides a unique means to mimic FSHD symptoms in mice and learn about pathogenesis of this complex disease.

Deregulated FAT1 array has been suggested to act as an insulator between telomeres and subtelomeric genes [7–8], such that its contraction might result in regulatory changes in neighbouring genes that could in turn alter muscle physiology [6,9–11]. Despite intense focus on deregulated 4q35 genes, including one of the close neighbours, FRG1 [12], and despite numerous large-scale investigations aimed at uncovering additional relevant candidates, none of the genes reported accounts for all aspects of FSHD, and additional players are still actively sought [6,9,13]. An emerging model is that the pathogenic effect of D4Z4 contraction in FSHD1 is mediated in part by DUX4, a retrogene present within D4Z4 repeats themselves encoding a homeobox containing transcription factor that is normally silent in muscle [14–15]. In FSHD1 patients, the contraction of the D4Z4 repeat array leads to a change in chromatin structure that facilitates DUX4 expression [16]. Furthermore, the pathogenicity of the D4Z4 contraction requires polymorphisms distal to the last D4Z4 repeat, that create a polyadenylation signal and thereby stabilize DUX4 mRNA [17]. This stabilized RNA thus leads to increased expression levels in FSHD muscles of a pathogenic isoform of DUX4, which activity is thought to be toxic for muscles through transcriptional activation of various target genes including Pax1 and p53 [18–21]. Another less frequent form of FSHD, clinically identical to FSHD1, is observed in absence of D4Z4 contraction. These cases, referred to as contraction-independent FSHD, include cases called FSHD2, that were shown to exhibit hypomethylated D4Z4 repeats, recently shown to be caused by mutations in the SMCHD1 gene [22]. FSHD2 is caused by the combination of such SMCHD1 mutations with a DUX4 permissive (polyA) context, and also leads to DUX4 overexpression [22]. While FSHD2 cases represent so far the majority of contraction-independent cases, rare cases of contraction-independent FSHD with typical symptoms may also occur without hypomethylation, and be caused by yet unidentified pathogenic contexts. Neither the specificity of SMCHD1 or of DUX4 expression nor of its target genes identified so far [18–21,23–24], provide sufficient account for the specificity of the muscle map and the non-muscular symptoms that characterize FSHD.

The regional specificity in the map of muscles affected in FSHD suggests that the causal abnormality interferes with a muscle subtype-specific developmental process. A gene involved in functional diversification during muscle development would thus provide a logical candidate to fill this gap. We focused on the cell adhesion molecule Fat1 because Fat-like protocadherins are known modulators of the planar cell polarity (PCP) pathway [25–27], a genetic cascade involved in coordinating tissue polarity, morphogenetic movements, and polarized cell flow [28–30]. Fat1 has been reported to be expressed in developing muscles and tendons [31] and to be regulated by muscle developmental genes such as Pax3, Lbx1, or MyoD [32–34]. Thus, Fat1 may control muscle shape through PCP-like mechanisms analogous to those involved in polarized migration of vascular endothelial smooth muscle cells [35].

Here, we report the unexpected finding that Fat1-deficient mice reproduce the highly selective muscular and non-muscular aspects of the clinical picture of FSHD. We show that Fat1 is required during development to shape specific groups of shoulder and facial muscles by modulating the polarity of myoblast migration. While constitutive inactivation of Fat1 leads to neonatal lethality due to defects in kidney development [36], Fat1 hypomorphic mice exhibit defects of muscle integrity with a topography prefiguring the map of muscles affected in FSHD. Furthermore, conditional mutagenesis suggests that a cell-autonomous function of Fat1 in migrating muscle cells may account for a significant part of its muscle shaping function. The human Fat1 gene is located only 3.6 Mb from the critical FSHD genomic region at 4q35, and emerges as a potential transcriptional target of DUX4 or p53 [18,37–38]. We present evidence of altered Fat1 levels in some foetal FSHD1 cases, in muscle, but not brain, accompanied with epigenetic modifications characteristic of silenced chromatin. Finally, we identified genetic variants deleting variable lengths of a putative cis-regulatory enhancer in the FAT1 locus, which segregate with FSHD. Thus, either in presence or absence of D4Z4 contractions, mechanisms leading to tissue-specific deregulation of FAT1 expression are associated with FSHD and may contribute to causing regional-specific muscle shape abnormalities that prefigure muscle degeneration in the adult.

**Results**

*Fat1* regulates myoblast polarity during planar migration

In search of mechanisms that control muscle position and form, we studied Fat1 expression at stages of muscle morphogenesis. We chose first to study a muscle with a characteristic fan-shaped form, the subcutaneous muscle cutaneous maximus (CM). During embryogenesis, following delamination from the dermomyotomal lip at forelimb levels, CM precursors, identified through their specific expression of GDNF, reach the base of the limb, turn, and spread under the skin in a radial manner [39–40] (Figure 1A). This migration pattern reflects collective and polarized cell migration, visible owing to expression of the MLC3F<sup>ΔC</sup> reporter line or of the muscle fate marker MyoD, through the formation of chains of myoblasts aligned in radial directions (Figure 1B and 1E top right panel). At the stages of CM migration, whole mount X-gal staining in embryos carrying a LacZ<sup>ΔC</sup> reporter gene-trap insertion in the mouse Fat1 gene revealed a hot-spot of Fat1 expression highlighting the migration area (Figure 1C, Figure S1). We found that CM myoblasts express Fat1 RNA and appear to be positioned in a subcutaneous layer which itself expresses Fat1 RNA, this surrounding subcutaneous tissue displaying a rostrocaudal gradient of
Figure 1. Fat1 controls the shape of subsets of scapular muscle by modulating myoblast polarity during planar migration. 

(A–C) Reporter gene expression in the forelimb and flank of mouse embryos between E11.5 and E13.5. (A) Gdnf-lacZ staining labels myoblasts of the latissimus dorsi (LD) and cutaneous maximus (CM). CM myoblasts migrate away from the brachial plexus to form a subcutaneous muscle sheath, composed of radially-oriented chains of myoblasts. (B) At E13.5, MLC3f-lacZ staining reveals the characteristic fan-shaped form of the CM (dotted white purple line) as compared to other limb muscles. (C) Fat1 expression detected using the lacZ gene trap allele KST249 (Fat1LacZ) is selectively localized within the CM and in surrounding tissue (pink arrow). 

(D) CM myoblasts express Fat1 and migrate towards an increasing gradient of Fat1 expression. Alternate vibratome cross-sections of a wild type E12.5 embryo were hybridized with Fat1 (left column) and MyoD (purple, right column) RNA probes. Photographs of adjacent sections were superimposed (photoshop) after conversion of Fat1 staining color in pink (right column; Fat1 in pink, MyoD in purple). MyoD expression is used as a marker of the muscle lineage. Superimposition was meant to compare the relative levels of Fat1 expression within and around the cutaneous maximus (CM) muscle (indicated with purple arrows), at three consecutive antero-posterior positions, respectively within the CM (top row), at the posterior end (middle row), and posterior to the caudal extremity of the CM at that stage. CM myoblasts, migrating from anterior to posterior, express lower levels of Fat1 RNA than the surrounding subcutaneous cell layer (pink arrows). Intensity of Fat1 staining in this subcutaneous layer increases gradually in caudal sections. 

(E–H) Orientation of CM myoblast migration in whole-mounts of E12.5 Fat1LacZ/LacZ and control embryos detected using MyoD in situ hybridization. In all panels anterior is to the left, dorsal is to the top. (E) The CM muscle
Expression of this transgene (MLC3F-2E:LacZ) is detected in myocytes and sarcomere assembly [43], hence it allows visualising a domain (Figure 2A,B). Analysis of myogenic differentiation by in situ hybridization with a myoD probe indicated that Fat1TM/ATM mutant myoblasts. The angle between the longest diameter of each myoblast nucleus and the axis of the closest myoblast chain was measured on flat-mounted CM muscles. The bar graph presents mean (+ s.e.m.) percentages of myoD+ nuclei displaying a given angle (by angle ranges of 10°) for wild type (gray) and Fat1<sub>ΔN2ΔLacZ</sub> (black) embryos. (G, H) High magnification images of MyoD-expressing myoblasts in equivalent positions – within the chains (G) or at the leading edge (migration front, H) – in the CM of mutants and controls. Scale bars: (A–C), 0.8 mm; (D) 300 μm; (E), left: 0.5 mm; (E), right: 100 μm; (G, H) 10 μm.

doi:10.1371/journal.pgen.1003550.g001

Regulation of myoblast polarity requires FAT1 transmembrane domain

Further genetic evidence of such a function of FAT1 in control of muscle shape was obtained with another targeted allele of the Fat1 locus, which we engineered by flanking two exons, 24 and 25, the latter containing the transmembrane domain, with LoxP sites (Figure S3A, targeted allele referred to as Fat1<sub>ΔN2ΔLacZ</sub>). Crossing of mice carrying the conditional Fat1<sub>ΔN</sub> allele with a ubiquitous CRE-expressing mouse line produced, by germline excision of the floxed exons, a constitutively recombined allele, Fat1<sub>ΔN</sub>, which encodes FAT1 protein isoforms lacking the corresponding transmembrane domain (Figure 2A,B). Analysis of myogenic differentiation by in situ hybridization with a myoD probe indicated that Fat1<sub>ΔN/ΔN</sub> embryos exhibited phenotypes identical to those seen in Fat1<sub>ΔN2ΔLacZ</sub> embryos (data not shown). This new allele also allowed studying later steps of muscle differentiation by crossing Fat1<sub>ΔN</sub> mice with a transgenic line in which nls-LacZ reporter activity is driven by an enhancer from the m<sub>ymi3</sub> gene (MLC3F-2E) [43]. Expression of this transgene (MLC3F-2E:LacZ) is detected slightly later than myoD expression as it reflects differentiation in myocytes and sarcomere assembly [43], hence it allows visualising muscle shapes, but not migrating myoblasts. MLC3F-2E expression in Fat1<sub>ΔN/ΔN</sub> embryos revealed again the altered morphology of the CM muscle, with misoriented chains of myocytes in the ventral/pectoral half of the CM and shoulder belt muscles (Figure 2D, and Figure S3B). Furthermore, Fat1<sub>ΔN/ΔN</sub> embryos were found to exhibit an extra muscle ectopically located in the shoulder area (Figure 2D). Finally, we also visualized multinucleated myofibres owing to the nuclear β-galactosidase staining at late gestation stages, and confirmed the persistence of misoriented myofibres in the mature CM muscle of Fat1<sub>ΔN/ΔN</sub> E18.5/P0 embryos (Figure 2D). Taken together, our data show that Fat1 is required to control the shape and position of subsets of migratory muscles in the developing embryo, by controlling coordinated polarity of collectively migrating myoblasts.

The developmental map of Fat1-dependent muscles

We next wished to extend our description of the map of Fat1-dependent muscles by exploiting the phenotypes exhibited by Fat1<sub>ΔN/ΔN</sub> embryos carrying the MLC3F-2E transgene at later developmental stages (E14.5 and E15.5), when migration has been completed and muscle shapes are determined. In the scapulohumeral area of all Fat1<sub>ΔN/ΔN</sub>MLC3F-2E embryos examined, we consistently observed an extra muscle in a stereotyped ectopic position, systematically attached between the spinodeltoid muscle and the triceps brachii muscles (Figure 3A,B). Just dorsal to the spinodeltoid, we found a subcutaneous portion of the spinotrapezius muscle (SpTS) to be drastically reduced in Fat1<sub>ΔN/ΔN</sub>MLC3F-2E embryos (Figure 3A, orange arrows). Observation from a dorsal point of view reveals that midline junction of the CM muscle and of Rhomboid muscles (Rh) is delayed, so that a large gap is seen in the back of an E14.5 Fat1<sub>ΔN/ΔN</sub> embryo (Figure 3B, orange line). Numerous mispositioned myofibres create ectopic bridges between the acromiotorpezius and spinotrapezius muscles in Fat1<sub>ΔN/ΔN</sub>MLC3F-2E embryos (Figure 3B; read arrows in top and middle picture). Analysis of muscles in the face at E14.5, E15.5, and at P0, reveals abnormalities in shape, myofibre orientation, and density in several subcutaneous muscles in the facial skin (Figure 3C, red arrows) that occupy positions reminiscent of the position of human muscles of facial expression. The flat structure of these subcutaneous muscles is analogous to that of the CM muscle, and the alterations observed in Fat1<sub>ΔN/ΔN</sub> neonates also include random orientation of multinucleated myofibres (Figure 3C). In contrast, deeper muscles such as the masseters display normal shape in Fat1<sub>ΔN/ΔN</sub> mutants (see Figure 3C and data not shown). Of notice, although muscle shape defects were found in stereotyped places, their severity was variable, and Fat1<sub>ΔN/ΔN</sub> embryos were frequently asymmetrically affected (Figure S4, see also Figure S12A). As previously observed in Fat1<sub>ΔN2ΔLacZ</sub> embryos, examination of muscle development at E14.5 and E15.5 in Fat1<sub>ΔN/ΔN</sub> embryos confirmed that Fat1 loss of function selectively affects muscles of the facial and scapulohumeral area, and that Fat1 is not required to shape other migratory muscles such as the diaphragm or hindlimb muscles, which were identical between wild type and Fat1<sub>ΔN/ΔN</sub> embryos (Figure S4 and data not shown). Overall, in addition to the abnormal shape of the cutaneous maximus muscle, we found that Fat1 was required to shape selective and stereotyped groups of muscles in the scapulohumeral interface, as well as subcutaneous muscles of the face.

Mice with reduced Fat1 expression develop early regionalized muscle wasting restricted to mis-shaped muscles

We next asked what the consequences of these muscle shape abnormalities were at postnatal stages. Constitutive deletion of Fat1 was initially shown to lead to neonatal lethality most likely due to defects in kidney filtration [36,42]. Likewise, constitutive deletion of the transmembrane domain ( Fat1\textsuperscript{DTM} /\textsuperscript{DTM} mice) also leads to more than 50% lethality at birth, with only a small proportion of mutants surviving to adulthood (Figure S3C). We chose to examine adult Fat1\textsubscript{LacZ}/\textsubscript{LacZ} mutants, since the hypomorphic Fat1\textsubscript{LacZ} allele, which results from an insertion of a gene-trap construct in an intron, not deleting any functional domain, allows expression of variable amounts of residual Fat1 RNA and FAT1 protein in Fat1\textsubscript{LacZ}/\textsubscript{LacZ} mutants (Figure 2E, Figures S5, and S13). This hypomorphic allele, in the genetic background we used, allowed bypassing the neonatal lethality in Fat1\textsubscript{LacZ}/\textsubscript{LacZ} mutants, with more than half the mutant mice surviving after 3 months (Figure 4C), and enabled us to study the postnatal consequences of reduced Fat1 levels. The variable amounts of residual Fat1 correlate with the variability in the severity of phenotypes and in the age of death of Fat1\textsubscript{LacZ}/\textsubscript{LacZ} mice. A fraction of these adult phenotypes, in particular the lethality, is likely to result from systemic consequences of kidney phenotype. Indeed, analysis of kidney morphology in the subset of Fat1\textsubscript{LacZ}/\textsubscript{LacZ} mice that exhibited severe weight loss revealed features characteristic of polycystic kidneys, such as cysts formed of enlarged tubules in the cortical renal area (data not shown). Therefore, to score with an objective criterion the progression through adult phenotype stages,
body weight was measured for each individual and compared to its own maximal weight \cite{44}. We arbitrarily set the moment a Fat1LacZ/LacZ mutant mouse has lost 10% of its weight as the visible onset of symptoms associated with kidney malfunction or with other phenotypes likely to have systemic consequences. Mutant mice showing more than 10% loss at the stage of analysis were defined as “symptomatic” (related to generalized symptoms, and not to muscles only), and the degree of severity was recorded as percentage weight loss, while Fat1LacZ/LacZ mutant mice that did not exhibit any weight loss yet were defined as presymptomatic.

**Figure 3. Fat1 loss of function alters shapes of selective facial and scapulohumeral muscles.** Skeletal muscle groups were visualized in E14.5, E15.5, and E18.5 wild type and Fat1\textsuperscript{TM/\textsuperscript{TM}} embryos carrying the MLC3f-2E (LacZ) transgene, by X-gal staining. (A) overview of the face and forelimb musculature at E14.5. Overall, constitutive ablation of Fat1 causes developmental abnormalities of muscle shape, affecting selective subcutaneous muscles in the face (Zyg. Min and Zyg maj, muscles, Occip. F, orbic. Or. and temporalis Muscles) and selective muscles in the scapulohumeral region. Muscle names are indicated. Muscles which are reduced or show an altered shape have their name underlined in Fat1\textsuperscript{TM/\textsuperscript{TM}} mutant pictures. Ectopic muscles are indicated with red arrows. (B) Muscles of the scapulohumeral area at E14.5 and E15.5, visualized with dorsal views of the scapular muscles at E14.5, and side views of the forelimb at E15.5. Dorsal views reveal the reduced extent of the CM and Rhomboid muscles, and the abnormal connections between the upper and lower parts of the trapezius (amT and spT, respectively). A large additional ectopic muscle (red dotted line, bottom picture) is observed in Fat1\textsuperscript{TM/\textsuperscript{TM}} embryo, that appears ectopically inserted between the spindelteid and Triceps brachii (LoTb and LaTb) muscles. (C) Analysis of muscles in the face at E14.5 (A), E15.5 (C), and at P0 (C, bottom), reveals abnormalities in shape, myofibre orientation and density in several subcutaneous muscles (red arrows) that occupy positions equivalent to that of human muscles of facial expression, while deeper muscles such as the masseters (see Figure 6D and data not shown) display normal shape. Overall the topography of muscles affected in Fat1 mutant mice resembles the map of muscles affected in human FSHD muscle in early phases of the disease. Muscle names abbreviations: amT: acromiotrapezius; amd: acromiodeltoid; Bra: brachialis; CM: cutaneous maximus; Ecu: Extensor carpi ulnaris; Ecr: Extensor carpi radialis; Edc: Extensor digitorum communis; Edl: extensor digitorum longus; Fr: Frontalis; LaTb: lateral Triceps Brachii; LoTb: Longitudinal Triceps Brachii; Occ: occipitalis; Orbic. Oc: orbicularis oculi; Orbic. Or: Orbicularis Oris; Risor: Risorius (position equivalent to that of Risorius in human); SpD: spindelteid; SpT: spinitrapezius; SpTS: Subcutaneous part of the Spinitrapezius muscle; Temp: Temporo-parietal muscle; Zyg: Zygomaticus (position inferred from equivalent position in human). doi:10.1371/journal.pgen.1003550.g003
Although this threshold of 10% weight loss was defined arbitrarily, and even though we cannot exclude that kidney phenotypes also have systemic consequences earlier than this limit, it is difficult, during symptomatic phase, to attribute a primary cause to the symptoms observed. We therefore focused on the presymptomatic phase for most of our studies of adult muscle, and also chose to exclude from our adult studies mutant mice with an impaired growth curve. While Fat1<sup>TM/ATM</sup> mice at symptomatic stages (with 20–30% body weight loss) displayed generalized muscle mass reduction (Figures S6B–C, presymptomatic mutant mice showed scapular winging, whereas lumbar posture and hindlimb function appeared unaffected (Figure 4A). Postural abnormalities affecting the shoulder area, indicating weakness of the muscles involved in scapular movements, can be seen when presymptomatic mice move on a cage grid, especially in situations in which they challenge the shoulder girdle muscles by transferring bodyweight rostrally on their forelimbs. These postural abnormalities were accompanied by functional motor defects evidenced in rotarod assays at presymptomatic stages (Figure 4E). Early symptomatic mice (around the 10% threshold) also showed kyphosis, a curvature of the spine known as a hallmark of muscle wasting in the shoulder girdle (Figure 4D, F), without displaying skeletal abnormalities (Figure 4B, X-ray). Similar observations were made in the small proportion of Fat1<sup>TM/ATM</sup> mice that survived to adult stages.

We next investigated the pathological basis for the selective postural abnormality of the scapulae at presymptomatic stages. Dissection of individual muscles in presymptomatic Fat1<sup>TM/ATM</sup> mice revealed a significant mass reduction for both rhomboid muscles when compared to controls (Figure 4D). As expected from the embryonic defect, a severe reduction in thickness of the CM muscle was also observed, although its subcutaneous location made accurate dissection and therefore mass measurement unfeasible. Defects in myofibre orientation similar to those observed at late embryonic stages were confirmed in CM (Figure S6D and data not shown) and in rhomboid muscles (Figure 4G) at all stages examined. In contrast, masses of muscles with unaltered shape when examined during development (i.e hindlimb muscles such as gastrocnemius or soleus) were also not significantly reduced at presymptomatic stages (Figure 4D, Figure S6B, S7). This argues that persistence in mature muscles of misoriented myofibres resulting from fusion of depolarized myoblasts contributes to the shoulder muscle phenotype in presymptomatic mice, although it does not rule out an additional direct function of Fat1 in muscle, whose loss may also cause muscle degeneration. Lastly, another consequence of developmental dysgenesis that is likely to contribute to focal muscle wasting is the persistence of ectopic muscles (Figure S7). Such ectopic muscles were found to share tendon attachment sites with existing muscles (typically two ipsilateral muscles) including shoulder belt muscles (trapezius, LD, pectoral muscles), and the humeral muscle triceps brachii (Figure S7). This association correlated with a unilateral reduction of the corresponding muscle mass, reduction that nevertheless did not result significant until early symptomatic stages (Figure 4D and data not shown).

The phenotypes resulting from developmental dysgenesis were not restricted to muscle shape and mass. Histological analyses revealed that a significant reduction in fibre diameter was detectable already at early symptomatic stages in those muscles in which we detected developmental defects, including the CM, Rhomboids (Figure 4G, superior and profundus), and Trapezius muscle (Figure 5C, pooled analysis). This was also true for Fat1<sup>TM/ATM</sup> mice analysed at presymptomatic stages (Figure S8). In contrast, at presymptomatic stages, analysis of myofiber diameters in muscles whose shape was unaffected at development stages (such as gastrocnemius or soleus, and also diaphragm) revealed no significant abnormality as compared to control mice (Figure 4D, Figure S6B, and data not shown). In affected muscles (trapezius, rhomboid, Pectoralis Major, LD, and CM), we observed a range of additional abnormalities including inflammatory infiltrations between myofibres, most frequently perivascular, in both presymptomatic Fat1<sup>LacZ/LacZ</sup> and Fat1<sup>TM/ATM</sup> mice (Figure S6D and Figure S7). Fibre necrosis was also observed at more advanced symptomatic stages (beyond 10% weight loss, Figure S7L and data not shown), but as mentioned earlier, it is impossible to distinguish whether any abnormality at symptomatic stage is strictly related to muscle defects, or reflects systemic consequences of unrelated phenotypes. Finally, observation of myofibre structure in affected muscles (trapezius, rhomboid, Pectoralis Major, LD, and CM) revealed progressive disruption of higher level organization, with appearance at presymptomatic stages of multiple faults disrupting the regular alignment of sarcomeric structures (Figure 5A, D), and the detachment of the sarcolemma from the contractile apparatus (Figure 5D). Overall, alterations of muscle integrity at pre-symptomatic stages were only detected in those muscles in which we reported fully penetrant myoblast or myofibre orientation defects (CM, Rhomboids, and Trapezius). Analysis of neuromuscular junctions in affected shoulder muscles also revealed a proportion of junctions showing fragmentation (Figure 5B), denervation, and atrophy (Figure S9). Such defects did not reflect a primary failure of NMJ innervations, as all neuromuscular junctions observed at early postnatal stages (P3) were indistinguishable from wild type (data not shown). Nevertheless, although the muscles that were spared during development and at presymptomatic stages (e.g gastrocnemius, soleus, masseters) were seen to harbour histological signs of muscle atrophy (evenly reduced myofiber diameter) at advanced symptomatic stages (Figure S6B), we did not observe muscle degeneration, inflammation, necrosis, or fragmentation of the contractile apparatus (data not shown). These results are consistent with the possibility that the developmental abnormalities of muscle shape constitute a topographic frame in which muscles might be predisposed to undergo early onset muscle wasting, prior to the appearance of systemic consequences of non-muscle phenotypes and the concomitant generalization of muscle wasting. These findings do not exclude however the possibility that Fat1 may play additional roles during muscle biology other than controlling shape during development.

Ablation of Fat1 functions in premigratory myoblasts with Pax3<sup>-cre</sup> is sufficient to alter muscle shape

We next asked if the function of Fat1 in shaping facioscapulo-humeral muscles was exerted cell-autonomously in migrating muscle precursors. In order to perform tissue-specific ablation of Fat1 in muscles at a stage compatible with migration, we reasoned that transgenic lines in which CRE expression would reproduce that of genes of the muscle differentiation cascade, such as myoD or Myf5, would occur too late to have an impact on the migration itself. Therefore, to ablate Fat1 exons 24 and 25 in premigratory myoblasts, we took advantage of the Pax3<sup>-cre</sup> knock-in line [45] (Figure S10). Our conditional allele of Fat1 (Fat1<sup>fl/fl</sup>) initially includes the neo cassette that was used to engineer the mouse model. Although presence the neo cassette caused mild lowering of Fat1 expression levels (Figure S11), this only resulted in subtle, although statistically significant, morphological defects in Fat1<sup>fl/fl</sup> embryos/mice compared to controls (Figure 6 and Figure S12). This allowed using the Fat1<sup>fl/fl</sup> mutants for conditional studies with tissue-specific CRE lines, without
Figure 4. Presymptomatic adult Fat1 mutant mice show selective defects in scapular muscles. (A) Adult Fat1<sup>LacZ/LacZ</sup> mice show visible scapular winging (orange arrow) at stages prior to detectable weight loss (defined as presymptomatic). Pictures (extracted from movies) show a posture in which the mice challenge their shoulder girdle muscles by extending their head as far rostral as possible. At 7 weeks, wasting of the rhomboid muscles can already be detected in presymptomatic Fat1<sup>LacZ/LacZ</sup> mice as they move on a cage grid. Note the large gap (orange arrow) between scapulas (where rhomboids normally maintain scapulas attached to the dorsal spine), not visible in the corresponding position in the wild type littermate. (B) At advanced symptomatic stages (30% weight loss, anesthetized mice), there is marked curvature of the spine in the upper back and shoulder area, also visible through X-ray post-mortem imaging. (C) Kaplan-Meier plot showing survival of wild type, Fat1<sup>LacZ/+</sup>, and Fat1<sup>LacZ/LacZ</sup> mice. Most Fat1<sup>LacZ/LacZ</sup> mice die between 2 and 4 months, with a median survival of 3 months, while a small group survives beyond 6 months. (D) Masses of dissected muscles of Fat1<sup>LacZ/LacZ</sup> mice at presymptomatic disease stage (0% weight loss, n = 3) relative to age-matched controls (n = 6; average wild type weight defined as 100%). (E) Motor performance defects in presymptomatic adult Fat1<sup>LacZ/LacZ</sup> mice. Rotarod analysis shows that the latency to fall off from the rod was significantly shorter in presymptomatic adult Fat1<sup>LacZ/LacZ</sup> mice. In this set of experiments, additional Fat1<sup>LacZ/LacZ</sup> mice that were symptomatic at the stage when training started had died by the time the test was performed and are therefore not included in the graph. (F) Scapular muscle dissection in adult wild type and Fat1<sup>LacZ/LacZ</sup> mice reveals a pronounced reduction in volume and thickness of the rhomboid superficialis (Rh. Sup.) and rhomboid profundus (Rb. P.). This likely underlies the scapular winging phenotype. In the top pictures, the trapezius cervicalis (Trap) has been removed on the right side of each mouse to uncover the other scapular muscles (rhomboids: Rho; levator scapula: LS). Yellow dotted lines indicate the extent of the scapula, red and orange dotted lines that of the two rhomboid muscles. The intermediate magnification highlights the respective shapes of the rhomboid superficialis (orange dotted line) and rhomboid profundus (purple dotted line). (G) Phalloidin staining of flat-mounted rhomboid superficialis muscles of wild type and Fat1<sup>LacZ/LacZ</sup> mice at presymptomatic (middle panel) or advanced disease (20% weight loss; bottom panel) stages shows that early defects of myofiber orientation precede reduction of myofibre diameter. Scale bars: (F) 2 mm; (G) 300 μm.
doi:10.1371/journal.pgen.1003550.g004
requiring Flp/FRT recombination to further ablate the neo cassette. We therefore compared muscle development in Fat1Flx/Flx; Pax3cre/+ and Fat1Flx/Flx embryos, taking advantage of the MLC3F-2E transgene 1) to visualize the shape of every muscle and 2) to quantify the number of muscle cells dispersed in ectopic areas. We followed muscles belonging to Pax3-derived territories in the scapulohumeral area, where ablation of Fat1 leads to measurable phenotypes in Fat1Flx/Flx;MLC3F-2E embryos (Figure 6A). First, we found significantly higher numbers of dispersed myocytes in the forelimb of Fat1Flx/Flx;Pax3cre/+ embryos than in Fat1Flx/Flx embryos (Figure 6A, B). Second, an ectopic muscle similar to the one found in Fat1Flx/Flx;MLC3F-2E embryos, and its surface was significantly larger than in Fat1Flx/Flx embryos (Figure 6A, C). At later developmental stages, in addition to confirming the persistence and position of this ectopic muscle in Fat1Flx/Flx;MLC3F-2E embryos, as in Fat1Flx/Flx;MLC3F-2E embryos, we also detected a reduced density of myofibers in the CM muscle and in

Figure 5. Abnormally shaped shoulder muscles of Fat1-deficient mice develop phenotypes involving reduced muscle fibres diameter and structural abnormalities. (A) Muscle architecture visualized on transverse (A1,2) or longitudinal (A3,4) sections of rhomboid muscles from wild type and Fat1LacZ/LacZ mice (20% weight loss), using antibodies against laminin and α-actinin, or toluidine blue staining. (B) NMJs were visualized by immunolabeling nerve endings with anti-neurofilaments antibodies (NF, red) and AchR clusters with α-bungarotoxin (green). (C) Plot of muscle fiber diameter in scapular muscles (rhomboid, trapezius, latissimus dorsi, and cutaneous maximus) of adult Fat1LacZ/LacZ mice at early symptomatic (n=5, red bars) and advance stages (n=7, green bars), compared to wild type littermates (n=12, blue bars). (D) Electron micrographs at three different magnifications in rhomboid muscle fibres from Fat1LacZ/LacZ adult mice at early symptomatic stages (6-15% weight loss) show fragmentation of the myofibre architecture and loss of t-tubule integrity. In wild type myofibres, t-tubules (purple arrows) are visible between myofibrils, precisely aligned on either side of each Z-band, at a position coinciding with the end of the myosin filaments. By contrast, in dystrophic fibres from Fat1LacZ/LacZ mice, the general disorganization correlated with missing (stars), mis-oriented, mis-aligned (orange arrows), or fragmented (red arrows) triads. An increased distance (indicated as blue double arrowed bar) between the sarcolemma and contractile apparatus is observed in Fat1LacZ/LacZ muscles, compared to wild types, indicating a loss of the tight association between the contractile apparatus and the sarcolemma. Scale bars: (A1–2) 50 μm; (A3–6) 20 μm; (B) 15 μm; (D1,2) 5 μm; (D3,4) 0.5 μm; (D5,6) 0.2 μm.

doi:10.1371/journal.pgen.1003550.g005
the subcutaneous part of the spinotrapezoid muscle (Figure S12). As the Pax3cre/+ line is a CRE knock-in, but also a knock-out of the endogenous Pax3 locus, the resulting loss of one copy of Pax3 may be in itself sufficient to enhance FAT1-dependent phenotypes. To rule this out, we have evaluated the effect of combining a Pax3cre/+ context to the recombined Fat1^D^TM allele, and found no enhanced phenotype in either Fat1^D^TM/+Pax3^cre/+ or Fat1^D^TM/ATM;Pax3^cre/+ embryos compared to Fat1^ATM/+ or Fat1^ATM/ATM embryos, respectively (data not shown). Finally, Fat1^Fln/Fln;Pax3^cre/+ embryos did not display significantly more abnormalities in the subcutaneous facial muscles or in the spinotrapezius muscle than the mild phenotypes observed in Fat1^Fln/Fln embryos (Figure S12), consistent with the fact that facial muscles do not belong to the Pax3-CRE lineage [46]. Furthermore, if ablation in facial neural crest cells, driven by Pax3-CRE activity, had been responsible for altering muscle shape, it would have done so as efficiently in facial muscles as in trunk muscles. The lack of enhancement of facial muscle phenotypes in Fat1^Fln/Fln;Pax3cre/+ compared to Fat1^Fln/Fln embryos thereby also excludes a contributing role of Fat1 expression in neural crest-derived cells. Thus ablating Fat1 in Pax3-derived cells is

Figure 6. Ablation of Fat1 in premigratory myoblasts using Pax3-cre partially reproduces the muscle migration/shape abnormalities of the constitutive knockout. (A) Skeletal muscle cells were visualized at E12.5 in WT, Fat1^D^TM/ATM, Fat1^Fln/Fln, and Fat1^Fln/Fln;Pax3^cre/+ embryos, owing to the MLC3F-2E transgene by performing X-gal staining, after clearing in 100% glycerol. The upper panels show micrographs of the forelimb area, and indicate the positions at which higher magnification pictures shown in the two lower panels were taken. (B, C) The phenotype was quantified in WT, Fat1^ATM/ATM, Fat1^Fln/Fln, and Fat1^Fln/Fln;Pax3^cre/+ as well as in the control genotypes in Fat1^ATM/, Fat1^Fln/+, and Fat1^Fln/+, Pax3^cre/+ and Pax3^cre/+ in two different manners: (B) by counting the number of dispersed myocytes found in the elbow area (orange dotted lines in the lower panels in (A)), (C) by measuring the area occupied by the ectopically positioned muscle (or myocyte cluster) that appears inserted between (red dotted line in middle panels). All data from a given genotype are plotted on a vertical line. Overlapping dots were arbitrarily moved away from the vertical lines to allow showing all results distinctly. In both cases, the Fat1^ATM/ATM, Fat1^Fln/Fln, and Fat1^Fln/Fln;Pax3^cre/+ groups were each significantly different from the control genotypes (WT, Fat1^Fln/+, and Fat1^Fln/+, Pax3^cre/+ respectively, t-test, p values indicated), and were significantly different from each other (Fat1^ATM/ATM from Fat1^Fln/Fln, and from Fat1^Fln/Fln;Pax3^cre/+), but also Fat1^Fln/Fln from Fat1^Fln/Fln;Pax3^cre/+ (t-test, p values indicated).

doi:10.1371/journal.pgen.1003550.g006
sufficient to partially reproduce the defects observed in scapulohumeral muscles of the constitutive Fat1 mutants, indicating that Fat1 is required cell-autonomously in migrating myoblasts to control the polarity of their migration.

Later FAT1 expression in differentiated muscles

As we asked whether in addition to the control of muscle migration, Fat1 may play additional roles in mature muscle, we noticed that in mouse, Fat1 is also expressed in differentiated muscle fibres after migration stages. This expression can be detected through the pattern of β-galactosidase expression in Fat1<sup>lacZ/+</sup> embryos, and by in situ hybridization (Figure 7A). Furthermore antibodies against FAT1 C-terminal cytoplasmic tail detected a protein localized in stripes within muscle fibres (Figure 7B–D), on either side of alpha-actinin-positive sarcomere boundaries (so called Z-bands, Figure 7B). In adult mouse muscle, the stripes of FAT1 protein are closely juxtaposed with DHPR, a calcium channel present in transverse (t)-tubules [47] (Figure 7B). Such localization is consistent with Fat1 also playing a direct role in muscle biology, distinct from its early function in orienting myoblast polarity. Consistent with previous reports showing that cytoplasmic variants in FAT1 proteins exhibit distinct subcellular localisation [48], and that the cytoplasmic domain can translocate in the nucleus [49], another antibody directed against the cytoplasmic domain (FAT1-1465 antibody) also detected FAT1 protein in significant proportion of nuclei in adult mouse muscle fibres (data not shown). Western blot analyses indicated that a full length FAT1 protein is only detected in whole embryo extracts (at E12.5, Figure 2B) or in isolated brain tissue, but not in muscle tissue, where the most abundant bands detected with anti-FAT1-ICD antibodies were smaller molecular weight proteins (Figure S13), which production is spared by the genetic alterations in both Fat1<sup>lacZ/lacZ</sup> and Fat1<sup>ATM/ATM</sup> mutants (Figure 7C,D, Figure S5, S11, S13 and data not shown). While some of these smaller isoforms might be cleavage products of full length FAT1 [50–52], additional short isoforms are also consistent with gene products resulting from transcript initiation at alternative downstream promoters, as proposed by genome browsers (Ensembl, UCSC; Figure S5A, with EST-based genes referenced in NCBIM37 mouse genome and in GRCh37 human genome assemblies).

Neither the gene trap insertion after the first exon (this study), nor the removal of the entire first exon (in the published knockout allele [36]), suppress such gene products. Deletion of the transmembrane domain in Fat1<sup>ATM/ATM</sup> mutants also allowed expression of protein products with unchanged size (Figure S13), although it nevertheless led to a more severe phenotype with drastic neonatal lethality (compare Figure S3C and Figure 4C).

Quantitative RT-PCR confirmed the presence of significant amounts of Fat1 RNA containing the last exons (26 to 28) in Fat1<sup>ATM/ATM</sup> mutants, albeit at reduced levels when compared to wild types (Figure S11). Thus, in the case of all mutant alleles, the remaining smaller isoforms might still carry out Fat1 functions at least partially, resulting in hypomorphic phenotypes with variable

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**Figure 7. Fat1 expression at late stages of muscle differentiation.** (A) Fat1 expression was visualized in E13.5 embryos or in neonate (P0) muscle by β-galactosidase staining or by in situ hybridization with a Fat1 3' UTR RNA probe. (B–D) Immunolocalization of FAT1 (anti-FAT1-ICD from [35], green) was performed in E12.5 mouse embryo (C), and on adult (B, D) muscle fibers on longitudinal muscle cysections from wild type (B, C1–3, D1–4), from Fat1<sup>12/12tm1<sub>C4</sub></sup> embryos (C4–6), and from Fat1<sup>12/12tm1<sub>D2</sub></sup> (D2–3, D5–6) mice, combined with either antibodies against alpha-actinin (red, B3), DHPR (Cacna1s) (red, B2, B4), or RyR (red, B5), or with Phalloidin (red, C, D). In D, Green channel images (FAT1) were first captured with either identical exposure time between wild type and mutants (D1–4 and D2–3, 421 ms), or with longer exposure time (D3–6, 2222 ms). This indicates that the epitope detected by the anti-FAT1-ICD antibody (from ref [35]) is present in reduced but detectable amounts in Fat1<sup>12/12tm1<sub>C4</sub></sup> muscles. This observation was made when Fat1<sup>12/12tm1<sub>C4</sub></sup> mice (n = 2 at P0; and n = 3 at adult stages) displayed severe muscle defects at the stage of dissection, indicating that levels of FAT1 protein inversely correlate with phenotype severity. Scale bars: (B–D) 4 μm, (C) 6 μm.

doi:10.1371/journal.pgen.1003550.g007
Figure 8. Selective changes in Fat1 mutant mice recapitulate the clinical picture of FSHD. (A) Schematic representation of the human 4q35.2 region, including 5 Mb upstream of the FSHD-associated D4Z4 repeat array. (B–C) Retinal defects and exudative vasculopathy in adult Fat1LacZ/LacZ retinas. Fat1 mutant mice have an opaque appearance, in contrast to wild-type eyes (B—yellow arrow). Removal of the cornea reveals absence of opening of the pigmented retina (aniridia), which therefore covers the lens and prevents light from entering the eye. (C) Retinal vasculature visualized using isoelectinB4 (GS-IB4) staining of flat-mounted adult retinas from wild type and Fat1LacZ/LacZ mice. The retina of Fat1LacZ/LacZ mice displayed zones in which the normal vasculature of secondary and tertiary vessels was replaced by disorganized vasculature, revealing numerous intra-retinal microvascular abnormalities, including IB4-binding microaneurysms (orange arrows). Insert: Example of severe retinal detachment (red arrows) observed in Fat1LacZ/LacZ eyes, visible even through the lens prior to its removal during dissection. (D) The shape of the inner ear was visualized at E12.5 in WT and Fat1TM/ATM embryos owing to the MLC5F-2E transgene, which is expressed in the developing inner ear in addition to differentiating muscles. Micrographs show an area of the face around the ear. This area shows: left: the masseter muscles (unaffected); bottom: a stream of muscle fibres of Fat1 mice; residual FAT1 staining is also observed in Fat1LacZ/LacZ embryos at E12.5 (7 affected sides out of 12); this shortening being frequently asymmetric (Figure 8D, E). These phenotypes are expected to influence audition. Thus, in addition to the similarity of muscle abnormalities, adult Fat1LacZ/LacZ mice also show non-muscular defects reminiscent of clinical symptoms of FSHD. Nevertheless, the severity scale of these phenotypes includes phenotypes more dramatic than those seen in FSHD, and Fat1-deficiency also leads to phenotypes such as the previously reported kidney abnormalities, that have no equivalent in FSHD.

Deregulated FAT1 expression in human FSHD1 foetal muscles

Considering the gene location and the provocative similarities between Fat1-deficiency in mouse and FSHD, we therefore asked whether alterations in Fat1 expression might be an essential step in the molecular mechanism leading to FSHD pathology in human. As in spite of the essential role of Fat1 in kidney development, FSHD is not known to be associated with kidney abnormalities, if a mechanism linking FSHD to Fat1 exists, it is expected to involve partial functional alterations only, such as tissue-specific deregulation of FAT1 during development. We thus first asked whether in addition to the previously reported gene expression changes [9–11,60], any deregulation of FAT1 expression levels could be detected in the classical context of FSHD1, in which the pathology is due to the presence of a contracted D4Z4 array on a permissive/pathogenic DUX4-activating context (4q4 haplotype) [17]. This possibility was reinforced by the finding that FAT1 appears to be downregulated by DUX4-β, but not by DUX4-β in human myoblasts [18]. This result was further validated by qPCR, after lentiviral infection of human myoblasts with DUX4-β as compared with GFP control (Figure S1D), indicating that DUX4 overexpression is capable of lowering FAT1 expression in cultured muscle cells. As our results in mice point to the crucial severity. Consistently, in immunohistochemistry experiments on muscle sections, residual FAT1 staining is also observed in myofibres of Fat1TM/ATM mutants and Fat1LacZ/LacZ mice, and staining intensity in Fat1LacZ/LacZ mice that survived to adulthood inversely correlated with phenotype severity at the level of individual myofibers (Figure 7C,D and data not shown). Presence of unchanged smaller Fat1 isoforms in muscles of Fat1TM/ATM mutants precludes using this mouse line to investigate their function. However, it indicates that the phenotype of muscle migration is not the consequence of their deletion, but results from ablation (constitutive or driven by Pax3-cre) of the transmembrane domain in full length FAT1 proteins that are abundant at developmental stages (Figure 2B).
role of FAT1 deregulation during development, we aimed to analyse FAT1 expression in rare cases of biopsies from foetuses with a prenatal diagnosis of FSHD, in spite of the fact that stages of myoblast migration were not accessible to experimentation in this context. Nevertheless, the observation that FAT1 protein is a component of differentiated muscle fibres, enriched in the t-tubule system, is consistent with additional later functions of FAT1 necessary for muscle integrity.

Possible alterations of FAT1 expression were therefore assessed in muscle biopsies of human FSHD cases at foetal stages through a series of independent approaches. Human FAT1 protein was detected by immunohistochemistry in human muscle biopsies from control foetuses of various stages with antibodies against FAT1 C-terminal cytoplasmic tail, with a striped pattern similar to that seen in mice (Figure 9A, Figure S15). We thus first studied FAT1 expression levels in tissues from an FSHD human foetus carrying a pathogenic 4qA allele harbouring 1.5 D4Z4 copies, expected from previous family history to lead to severe infantile FSHD (Figure S14). Immunocytochemistry with anti-FAT1 antibodies on sections from the quadriceps muscle revealed an overall decrease in FAT1 protein levels compared to quadriceps biopsies from control foetuses (Figure 9A), with an irregularly striped pattern of FAT1 in myofibres that otherwise show a normal distribution of other muscle proteins, such as DHPR. To assess this FAT1 lowering quantitatively, mRNA expression levels were then followed by qRT-PCR in muscle biopsies from 4 FSHD human foetuses carrying pathogenic 4qA alleles harbouring 1.5, 4.3, and 7 D4Z4 copies (referred to as F1 to F4, respectively; Figure S14A). In F1 foetus, FAT1 levels were reduced 5-fold in the deltoid (a muscle belonging to the FSHD map) and 3-fold in the quadriceps muscles (a muscle traditionally affected only at late stages in the human disease; Figure 9B). This was also confirmed by Western Blot with anti-FAT1-ICD antibodies (Figure S15A). Additional regulatory changes were detected (Figure S15B), such as an increased level of MURF1 or dysferlin RNAs, while RNA of other muscle components, such as DHPR or c-Sarcoglycan, were unchanged, ruling out secondary effects of loss of muscle integrity at this stage or quality of the biopsy. In contrast, no significant quantitative changes were observed in brain when comparing FSHD and control samples from the same foetuses (Figure 9B). Reduction of FAT1 mRNA levels, albeit to a lesser extent (25% reduction; Figure 9B), and aberrant protein localisation (Figure S15C) were observed in the quadriceps of a second FSHD foetus harbouring 4.3 D4Z4 repeats (F2), from an independent family with previous FSHD history (Figure S14). Finally, no significant quantitative changes were observed in

Figure 9. FAT1 protein and RNA levels are mis-regulated in human foetal FSHD tissues. (A) Immunolocalization of FAT1 (Rb-1465 anti FAT1-ICD, green) and DHPR (Cacna1s, magenta) in longitudinal sections from human quadriceps biopsies from a control (top) or and FSHD (F1, bottom) foetus with 1.5 D4Z4 repeats. (B) qPCR analysis of FAT1 mRNA levels in quadriceps (3 left graphs) and deltoid muscles (middle graph) and in brain (right graph), comparing respectively with age-matched control foetuses (blue bars), a 26 weeks old FSHD1 foetus (F1) harbouring 1.5 D4Z4 repeats in the 4q35 region (dark red bars), a 16 weeks old FSHD1 foetus harbouring 4.3 D4Z4 repeats at 4q35 region (F2), and twin FSHD1 foetuses aged 28 weeks, with 7 D4Z4 repeats. (C) Analysis of the regulatory status of the promoter region by Chromatin immunoprecipitation. The respective level of the following histone marks: H3K27me3 (silenced chromatin; C-left), and H3K4m3 (promoter active; C-right), in muscle extracts from four age matched controls (ct1 to 4) or four FSHD1 foetuses (F1 to F4) are shown. Relative quantities were normalized with the level of histone marks at the promoter of the GUSB gene as internal control, and expressed as % of control 1 (ct1). Scale bars: (A) 50 µm.

doi:10.1371/journal.pgen.1003550.g009
Figure 10. Identification of contraction-independent FSHD patients carrying deletions of an intronic FAT1 enhancer. (A) View of the Human genomic FAT1 locus focusing on an area including FAT1 exons 17-18-19. The lower image is a USCC browser based screen-copy image showing a track displaying ENCODE enhancer and promoter associated histone mark (H3K4me1) on 8 cell lines. (B) Positions of copy number variants identified in 5 FSHD patients by CGH and positioned on the genome by CGHweb analysis. Patients are identified with a specific number, and their characteristics are available in the Table S1. The deletion span varies from deletions restricted to intron 17 to deletions spanning over intron 17, exon 17 and intron 16, including a ENCODE-putative enhancer visible through genomic browsers. (C) Copy number validation of the deletion by qPCR. The three graphs show the relative amounts of PCR fragments obtained using primers couples 1–2 (exon 17), 2–3 (enhancer intron 16) and 4–5 (exon 16), in a group of 40 healthy controls (blue area), a group of 10 FSHD1 patients (red area), and a group of 19 contraction-independent patients (c.i.FSHD). All data were normalized using an unrelated genomic fragment (Adora) as internal control, and one of the control DNAs (number 21) was used as the reference DNA (where all values are set to 1). A cut-off of 0.75 has been set. Individuals in which the relative value is lower than the cut-off are considered as having lowered copy numbers (indicated as loss). Information on each patient (regarding clinical and genetic diagnostic) are available in the Table S1. (D) The distribution of CNVs corresponding to loss CNV (seen as red) is shown in controls and in FSHD groups (all together, or FSHD1 and c.i.FSHD separately) for each of the three spots considered individually (top three graphs) or considered together (bottom plot, where loss represents the number of cases having a loss for at least one of the three spots). The cases where a significant link (as measured by X² or Fischer tests) are indicated with one or two stars (* for p<0.05; ** for p<0.001; p-values indicated in the result section). (E) Analysis by Chromatin
immunoprecipitation of the relative enrichment of the chromatin marks H3K27me3 (silenced chromatin), H3K4m3 (promoter active), and H3K4me1 (enhancer active), at the level of the intronic enhancer located between exons 17 and 18 of FAT1, in muscle extracts from two age matched controls and FSHD1 foetus F1. (F) Schematic summary of the finding, showing a conformation switch of the chromatin to repressed state in FSHD1 foetal muscle (in cases with severe expected outcome with <5 D4Z4 repeats), at the level of both the promoter and the intron 17/18 enhancer of FAT1 exons (middle). (bottom) Deletions of part of all the enhancer at introns 16/17 are predicted to interfere with tissue-specific regulation of FAT1 expression, and. represent (when carried on one allele) a polymorphism that segregates with FSHD (FSHD1 and c.i.FSHD).

doi:10.1371/journal.pgen.1003550.g010

muscle biopsies of twin FSHD foetuses with 7 D4Z4 repeats (Figure 9B), although accumulation of FAT1 protein could be observed in some myofibre nuclei (data not shown), a localization never observed in age matched control biopsies, but reminiscent of adult mouse muscles. In contrast to foetal stages, analysis of FAT1 mRNA levels in a series of adult FSHD1 biopsies or FSHD-derived myoblasts did not reveal any significant change compared to control biopsies or myoblasts (data not shown), a result consistent with published data [10,60], or with data available on GEO NCBI. Overall, these results indicate that 1) a reduction of FAT1 levels in differentiated muscles can be observed is some FSHD1 cases but is not common to all FSHD1 cases at the stages examined; 2) the observed changes in FAT1 expression levels in FSHD1 occur only during development.

We next asked whether the changes we observed were accompanied with alterations in chromatin state around regulatory sequences of the FAT1 locus. We thus performed chromatin immunoprecipitations (ChIP) on muscle biopsies derived from these same FSHD1 and control foetuses (Figure 9C), looking for potential changes in the levels of two widely studied chromatin marks: H3K4me3 (trimethylation of histone H3 on lysine 4), a mark of active promoters, and H3K27me3 (trimethylation of histone H3 on lysine 27), which marks transcriptionally silent chromatin [61–62]. Consistent with RT-PCR data, we observed a significant decrease in the level of H3K4me3 decorating the FAT1 promoter region in the two FSHDs foetuses with less than 5 repeats, but not in the foetuses with 7 repeats, as compared to 4 control muscle biopsies of similar age range (Figure 9C right). However, all 4 FSHD1 foetuses nevertheless showed a significant increase in H3K27me3 levels (Figure 9C left). These data are consistent with a switch in chromatin conformation towards the silenced state in the same FSHD1 samples in which RNA levels were reduced, a switch that has the potential to account for a large part of the observed decrease in FAT1 levels.

CGH-based identification of contraction-independent FSHD cases carrying deletions of an intronic regulatory element of FAT1

FAT1 deregulation is not the only gene expression change reported to be associated with the D4Z4 contraction causing FSHD1. As we also wished to determine to what extent the changes we found were relevant to the specific clinical phenotype, rather than a silent consequence of the D4Z4 contraction, we therefore extended our investigation to contraction-independent FSHD cases. Such patients have typical FSHD symptoms, but are not genetically associated to a pathogenic contraction of the D4Z4 array on chromosome 4. A large fraction of these contraction-independent FSHD cases is now known as FSHD2, in which hypomethylated D4Z4 repeats are combined with with a normal sized D4Z4 array on chromosome 4 permisive for DUX4 expression [22,63–64]. Besides, other rare cases of contraction-independent FSHD cases remains unexplained, and represent interesting candidates to test whether alterations of the FAT1 locus might be directly associated with FSHD. To identify such alterations of the FAT1 locus, we performed an array-based comparative genomic hybridization screen (CGH [65]), a method used to uncover copy number variants. The custom-designed CGH array we employed covered the whole FAT1 genomic region, including non-coding sequences. In our CGH survey of 29 FSHD cases, including 10 FSHD1 cases and 19 contraction-independent cases (5 of which at least not showing D4Z4 hypomethylation, see Table S1 for clinical and genetic characterization of patients), we detected 5 cases exhibiting loss of portions of the intron 17 (between exons 17 and 18), or intron 16 of the FAT1 gene (Figure 10A,B, Figure S16). Besides the overlap with exon 17, we noticed that these deletions mapped near or within a hot spot of H3K4me1 methylation, a hallmark of cis-regulatory enhancers [61], spanning across intron 16 and part of intron 17 (Figure 10A, and Encode high throughput data, available on the UCSC browser [66]). According to the ENCODE ChIP seq data set [67], this element appears labeled as having strong enhancer activity in a human skeletal muscle myoblast line (HSMM) but not in 8 other non-muscle cell lines (Figure S16B). Examining the chromatin status at this locus by ChIP experiments, we consistently found that in control foetal muscle biopsies, intron 16 but also intron 17 were decorated by high levels of the enhancer signature H3K4me1 and negligible amounts of H3K4me3 (promoter signature) (Figure 10D, blue lanes, and data not shown), providing further in vivo support to the possibility that this sequence might indeed act as regulatory element in vivo.

To determine whether loss of functional portions of the putative enhancer were associated with FSHD, we analyzed copy number variants (CNVs) in a set of 40 healthy controls, 19 contraction-independent FSHD cases, and 10 FSHD1 cases. As the sensitivity of the CGH method might not allow detecting all cases with accurate precision, we applied a more precise qPCR method, and evaluated relative copy numbers by comparing 5 positions within and around the putative enhancer to a control spot on another chromosome (Figure 10A, C; 3 additional positions shown in Figure S16). Having set the threshold for considering a genome as carrying reduced copy numbers (loss) to 75% of the value in a healthy control used as reference genome, we found some healthy controls that exhibited reduced copy numbers of genomic regions at the core of the H3K4me1 hotspot in intron 16 (5% of controls) or in either surrounding exons (10% of control cases in both cases). This finding is consistent with a study, available through public databases, that identified cases with loss of similar genomic segments at this locus in a group of 90 healthy individuals [68]. Thus, such deletions/copy number reductions are not sufficient on their own to cause FSHD symptoms, when occurring on only one allele of FAT1. However, in all three positions, the proportion of FSHD cases (all cases included) who exhibited loss was significantly higher than the proportion of healthy controls carrying reduced copy numbers at the same spot (Figure 10C,D; X^2 test, p values<0.016; <0.00075; and <0.00041, for exon 17; enhancer; and exon 16, respectively). Cases with a deletion spanning the whole region were also significantly more frequent in the FSHD group than among controls. When considering only contraction-independent FSHD cases, as much as 47% carried the CNV including the putative enhancer, as compared to 5% of controls, and up to 68% carried a CNV encompassing at least one of the three considered positions, as opposed to 20% of the controls.
(Figure 10C,D, Fischer test, p<0.0004 and p<0.0001 for enhancer and exon 16, respectively). Conversely, when considering the distribution of cases with increased copy numbers (gain, above a threshold of 1.25× over the average control value) we found that there were significantly less FSHD cases with gain-CNVs than among the control group (X² test, p<0.017 and p<0.014 when considering all FSHD cases or contraction-independent cases only, respectively). Finally, we also analyzed the methylation status at DAZ4 repeats on chromosome 4 on a subset of our group of contraction-independent FSHD patients (5 out of 19), and found no indication of hypomethylation at the CpG site, Table S1) on the proximal DAZ4 unit [64]. This does not exclude that others patients in our c.i-FSHD group would be diagnosed as FSHD2, but indicates that FSHD can occur in non-contracted patients independently of the hypomethylation, known FSHD2 hallmark [22,64]. Together, these results indicate that partial or complete deletions of FAT1 intron 16/17 putative enhancer represent a polymorphism not sufficient to cause FSHD by itself when present on one allele only of chromosome 4, but which segregates with FSHD. Therefore, this CNV can be combined with pathogenic or sub-pathogenic contexts, and may act as a novel disease modifier in FSHD.

Discussion

FAT-like cadherins play various roles in tissue morphogenesis, by modulating cell polarity, adhesion and tissue growth. Here we show that during development, FAT1 controls the shape of subsets of muscles in the facial and scapulohumeral regions, and does so by modulating the polarity of collective myoblast migration, a function in accordance with the emerging link between planar cell polarity and collective directional migration events [29–30,69]. These muscle shape abnormalities are predictive of early onset muscle wasting, as observed in Fat1-deficient mice that bypassed neonatal lethality. Using Pax3-cre for conditional ablation of Fat1 functions in premigratory myoblasts, we show that a cell autonomous requirement for Fat1 function in the migrating myoblasts accounts for a significant component of this role in shaping muscles. Taken together, the location of the human FAT1 gene next to the critical FSHD locus at 4q35, the similarity between the Fat1-dependent muscles and those affected in FSHD, and the appearance in Fat1 mutants of non-muscle features of FSHD, suggest a possible role of FAT1 in the pathophysiology of this disease. In our human studies, we found two ways by which altered FAT1 regulation underlies a link with FSHD: 1- we observed muscle-specific lowering in foetal FSHD1 biopsies; 2- we identified a polymorphism deleting a putative cis-regulatory enhancer in the FAT1 locus, which significantly segregated with FSHD. Together, these results strongly support the idea that tissue-specific de-regulation of FAT1 expression/function might play a critical role in FSHD pathophysiology.

Fat1 is required in migrating myoblasts to shape selective muscles in the face and shoulder

The altered myoblast migration polarity caused by loss of Fat1 functions leads to selective developmental dysgenesis of scapulohumeral and subsets of subcutaneous muscles of the face. Understanding how Fat1 controls muscle shape required first determining which part of its expression domain accounts for this function. In addition to the muscles, Fat1 is expressed in several of the cell types that interact with migrating muscle cells. The highest expression was seen in non-muscle cells, such as the subcutaneous layer towards which CM myoblasts migrate (Figure 1). This muscle-skin interface is analogous to the bone-muscle interfaces (tendons, joints) of skeletal muscles, where Fat1 also accumulates at later stages (Figure 7A). Here, however, we show that ablating the floxed transmembrane domain of FAT1 with a Pax3-cre knock-in line leads to efficient excision in premigratory muscles of the limb but not the face, and reproduces at least partially the migration phenotype observed in constitutive Fat1 knockouts in the scapulohumeral region. Pax3-cre excision does not occur in motor neurons, hence ablation in this cell type does not contribute to the phenotype observed in Fat1+/floxFat1−/− embryos. No significant muscle shape defects were caused by Pax3-cre-mediated Fat1 ablation in subcutaneous muscles of the face. This is not surprising, as muscles in the face do not derive from Pax3expressing precursors but were previously shown to derive from a subset of islet1-expressing pharyngeal mesoderm cells [46,70]. In addition to trunk migrating myoblasts, Pax3-cre-mediated excision occurs in dorsal neural tube and neural crest. Although Fat1 expression is detected in Schwann cells (neural crest-derived) along the nerves at P0, we did not detect such an expression at the stage of muscle migration (E12.5, see Figure S11C), making it unlikely to be required to control migration polarity by acting in neural crest derivatives. Furthermore, as Pax3-cre-derived neural crest amply colonizes the developing face, the lack of enhanced muscle phenotype in the face of Fat1+/floxFat1+/− embryos disqualifies the neural crest component of Fat1 expression from playing a major contribution in muscle shaping, and strongly suggests that Fat1 is required cell-autonomously in migrating myoblasts to control the polarity of their migration. As however, the muscle phenotype of Fat1+/floxFat1−/− embryos is significantly weaker than the phenotype of constitutive mutants, it leaves the possibility that other component of Fat1 expression domain may also contribute to its function in muscle patterning.

The rationale for why such a selective group of muscles is affected by Fat1 loss of function is still unclear. This group of muscle includes subsets of migratory muscles of the face and shoulder area. In the face, defects are restricted to branchiомeric muscles derived from the second brachial arch (subcutaneous muscles of the skin, Figure 3), while first brachial arch derived muscles (masseters and temporalis), as well as extraocular muscles, are unaffected (Figure 5 and data not shown) [70–72]. The scapulohumeral region can be divided in two components: 1) the CM, as well as humeral muscles (triceps, deltoid, or muscles which pattern is affected by the supernumerary muscle) derive from somitic Pax3-driven hypaxial migratory precursors (Figure S10); 2) In contrast, some of the shoulder muscles such as the acromioclavicular or spinotrapezius, or the rhomboids, belong to the cucullaris group and were previously shown to derive from non-somatic, occipital lateral plate mesoderm [46,72–73]. Such specificity is consistent with the broader expression domain of Fat1 in muscles as observed at E12.5 and later (Figure 1, 7, and S1), although clear differences in expression levels between muscles can be distinguished (Figure 7A). Given that distinct regulatory programs govern the development of these muscle groups [2,74], the selective impact of Fat1 on muscle shapes could be determined by its interaction with some of the selective myogenic regulators.

Does altered muscle shape predispose to muscle wasting?

Advanced symptomatic stages in Fat1-deficient mice are likely systemic consequences of such non-muscle phenotypes. Nevertheless, the muscle wasting and dystrophic features measured at presymptomatic stages were detectable selectively in those muscles that exhibited myofiber orientation defects, even in cases with no other detectable phenotypes. Despite the important variability in postnatal phenotype strengths observed with the Fat1+/− allele,
myofibre orientation defects and dystrophic features in the CM and shoulder muscles (Rhomboids, Trapeze) were observed in all mutant cases examined, not only of embryos, but also at adult stages, even in cases of Fat1<sup>−/−/LacZ</sup> mice surviving to old ages with no other detectable phenotype. This specificity argues against the idea that restricted topography of muscle defects would be a consequence of renal problems or of other non-muscular defects.

Furthermore, the observed match between the topography of the developmental phenotype and the specific map of muscles that undergo wasting at presymptomatic stages in adult Fat1<sup>−/−/LacZ</sup> mice supports the idea that the selective muscle degeneration might occur as a consequence of the altered muscle shape. Future experiments will be necessary to determine whether the limited defects observed in Pax3-cre/Fat1 embryos are sufficient to predispose muscles to early onset degeneration, and whether additional triggers might be required for degeneration to occur in adult life. Among phenotypes observed in adult Fat1-deficient muscles, it will also be interesting to distinguish secondary consequence of the altered muscle shapes, from phenotypes reflecting additional, independent functions of Fat1, whether exerted in muscles too or in other cell types.

**Tissue-specific de-regulation of FAT1 as a potential mechanism in FSHD pathogenesis**

The spatial distribution of muscles mis-shaped as a result of Fat1 loss of function as seen at E14.5/E15.5 (Figure 3) appears to overlap very closely with, and thus to predict, the map of muscles affected at early stages in FSHD. Furthermore, the observation of non-muscle phenotypes such as defects in retinal vascularisation or inner ear patterning also bears some similarities with symptoms observed in FSHD patients. Despite this strong concordance between the phenotype of Fat1-deficient mice and FSHD symptoms, the selectivity of the shared phenotypes raises a paradox. Fat1 expression during development is not restricted to FSHD-relevant tissues, and constitutive deletion of Fat1 leads to pronounced renal defects and neonatal lethality. Even the Fat1 hypomorphic phenotypes presented above cannot be considered as an exact phenocopy of FSHD. Overall this mouse model is also more severe than FSHD, and 50% of the mice die within 3 months, likely of milder versions of the kidney phenotype (such as polycystic kidney). In contrast, FSHD is not known as a lethal disease, and has no reported association with kidney problems. Absence of renal dysfunction in FSHD is a strong indication that FSHD cannot simply be considered a “Fat1 knockout”. Thus, cases of patients with severe Fat1 loss of functions and kidney failure might be fatal before onset of muscle dystrophy and might thus fail to be classified as FSHD. In support of this hypothesis, a rare case of a 5-year-old girl carrying a duplication of the D4Z4 array and showing vescicourethryopathy and sensorineural deafness was also reported to have focal glomerulosclerosis of the kidney [75]. Instead, lack of association between FSHD and renal dysfunction indicates that any FSHD mechanism involving Fat1 alterations must necessarily preserve Fat1 expression/function in kidney (at least). Our results with mice suggest that such selective alterations of Fat1 function/expression may matter during development, in muscle precursors, at a stage when their migration occurs, for which FSHD human material was not available so far - and can ethnically not be sought. Fat1 levels may not be changed to an equal extent in all tissues and times, consistent with our observation that Fat1 levels were reduced in disease-relevant muscles but not in brain, and at foetal but not adult stages. Thus, an engineered mouse model in which Fat1 functions are specifically ablated in muscles and preserved in the renal system, even though lacking effects of other DUX4 target genes, may represent a more suitable tool to study consequences of the muscle abnormalities in adult, and a better model reflecting the tissue-specific Fat1 depletion that we propose might be occurring in FSHD.

**Loss of a putative FAT1 enhancer as a novel disease modifier in FSHD**

The finding that human cases of contraction-independent FSHD, with such a characteristic and restricted set of clinical symptoms, segregate with the deletion of a putative regulatory genomic element in the Fat1 locus instead of the traditional D4Z4 contraction, strongly supports the idea that altered Fat1 regulation plays a key role in the pathology. The putative cis-regulatory enhancer reported in this study, which deletion segregates with FSHD in contraction-independent cases is likely to carry tissue-specific information driving Fat1 expression in FSHD-relevant cell types, and future experiments are required to demonstrate such activity. The finding that healthy controls can exhibit heterozygous loss of this fragment of the Fat1 locus, containing two exons and an enhancer, is consistent with the observation that heterozygous loss of Fat1 functions in mice does not have major consequence of life span, health, and muscle integrity. However, we did observe a significant degree of haploinsufficiency in Fat1<sup>tm1/s</sup> embryos, evidenced by the presence of subtle muscle shape defects (Figure 6B,C), suggesting that muscles in the shoulder area are highly sensitive to Fat1 dosage. While copy number variants outside of the putative enhancer might occur without causing any regulation change, we reasoned that the further such deletions would extend into the ENCODE predicted enhancer, the more functional transcription factor binding sites they may remove, hence increasingly interfering with Fat1 regulation on the deleted allele, thereby sensitizing the locus to additional contexts that may additionally impact on Fat1 expression.

Interestingly, two of the FSHD1 cases presented here were monozygotic twins, both carrying a contracted 4q35 allele with 3 D4Z4 units, one of the twins being asymptomatic while the other twin had been diagnosed with a classical FSHD. We found that the twin with FSHD symptoms displayed reduced copy numbers throughout the length of the studied area, encompassing both exons 16 and 17 and the intron 16 putative enhancer, while the asymptomatic twin exhibited reduced copy numbers only at the distal-most region towards exon 16, this difference possibly representing a de novo somatic mutation (Figure 10 and Table S1). Although this correlation does not constitute a demonstration of causality, it provides support to the hypothesis that this lowered copy numbers (heterozygous) of Fat1 exons 17/16 and of portions of the putative Fat1 enhancer portions have the potential to worsen FSHD symptoms when combined to a pathogenic context. However, obtaining a formal demonstration of this hypothesis will require studying phenotypes/genotype correlations on a large cohort of patients, and knowing in each case if the FSHD-causing genetic context is FSHD1, FSHD2, or other un-identified contraction-independent contexts. Overall, deregulation of the Fat1 gene is associated with FSHD, either as a consequence of DUX4 overexpression, and/or epigenetically encoded in FSHD1 and FSHD2, or through the deletion of a putative enhancer that segregates with contraction-independent FSHD patients.
An additional function for \( FAT1 \) in muscle differentiation and/or physiology?

Among possible products of the \( Fat1 \)-gene, our results in mice indicate that the control of migration polarity and muscle shape requires a \( Fat1 \) RNA containing a transmembrane domain encoded by the floxed exons and deleted in the \( Fat1^{TM} \) allele. In contrast, other functions can be executed by incomplete \( Fat1 \) isoforms. Residual RNAs containing 3′ \( Fat1 \) exons can rescue (to an extent correlating with RNA levels) kidney defects and their consequences, but not muscle dysgenesis. Interestingly, however, both mouse models retain the capacity to produce \( FAT1 \) protein isoforms containing an intracellular domain, albeit at reduced levels quantified by qPCRs (Figure S10B,C), ruling out a major contribution of these isoforms to the muscle shape phenotypes observed in both mouse models. In muscle fibres, \( FAT1 \) is a novel component of t-tubules. Does \( Fat1 \) expression in differentiating and mature muscle reflect additional functions in muscle biology? The presence of \( FAT1 \) protein in close association with the contractile apparatus, as soon as differentiation starts, may reflect a role in sarcomere assembly. These \( FAT1 \)-enriched stripes are maintained in mature muscle fibres, tightly juxtaposed with the t-tubule system (Figure 7B). This may indicate a further involvement in excitation-contraction coupling, an essential process required throughout adult life for muscle function and maintenance. However, this striped pattern is established as early as the contractile apparatus assembles (Figure 7C), before the alignment and docking of T-tubules to the contractile apparatus takes place, the latter phenomenon occurring postnatally in mice [76]. This indicates that in muscle, \( FAT1 \) isoforms are not inserted in the t-tubule compartment itself, but may be located at an interface juxtaposing t-tubules and the contractile units, possibly reflecting a new function for \( Fat1 \) for example during assembly of the t-tubule network. As myoblast migration precedes differentiation and sarcomere assembly, the accumulation of these \( FAT1 \) protein isoforms in the contractile apparatus occurs too late to be accountable of the function in migration polarity.

\( FAT1 \)-like proteins were previously reported to be subject to various cleavage events by Furin convertase or by \( \alpha \) - or \( \gamma \)-secretases [50–52]. Furthermore, alternative splicing events in the cytoplasmic exons were reported to influence subcellular targeting of \( FAT1 \) proteins [48]. Our work in mice unexpectedly indicated that in addition to producing a large transmembrane protein and its cleavage products, the \( Fat1 \) gene also produces small molecular weight protein products which appear not to contain a transmembrane domain, and synthesis of which is largely preserved in both \( Fat1 \)-deficient mouse models, although at reduced levels. Bioinformatic scans and existing ESTs reported on all genomic browsers are indeed consistent with the possibility that short isoforms may result from transcript initiation at alternative downstream promoters, and may code for protein products devoid of leader peptide and transmembrane domain and potentially produced in the cytosol (lacking a leader sequence). Thus, understanding the roles played by the isoforms of \( FAT1 \) produced in muscles will require first characterizing the exact exon and domain composition of the \( Fat1 \) RNA and protein isoforms produced in muscle (wild type and \( Fat1^{TM/ATM} \)), and second designing novel strategies to ablate them independently of the transmembrane domain containing isoforms.

Interestingly, residual expression of such muscle-specific isoforms is genetic background dependent and its levels in \( Fat1^{TM/ATM} \) mice inversely correlated with phenotype severity. Furthermore, reduced expression levels and abnormal sub-cellular localization were observed in muscle of human foetal cases with expected severe and early onset FSHD1 (as predicted by the degree of \( D4Z4 \) contraction and family history), while no significant changes in RNA levels were detected in adult FSHD1 muscles compared to controls. These observations are consistent with the idea that deregulated \( FAT1 \) expression in differentiated muscle may be predictive of early (infantile) onset and severe dystrophy. These data suggest that the causes of the early phase, common to all FSHD patients and restricted to muscles of the face and shoulder, might be uncoupled from the causes of later phases of the disease - which spreads to other muscles, a condition that occurs in a subset of FSHD patients with childhood onset, the latter ending up wheel-chair bound [6].

Link with the known mechanisms in FSHD1

Recent studies have brought to light several possible molecular pathways by which the \( D4Z4 \) contraction on a 4qA allele may exert its pathological effect in FSHD1. Among those, stabilization of \( DUX4 \)-mRNAs by polyA-creating polymorphisms was shown to enable expression of a toxic form of \( DUX4 \), the latter causing muscle dystrophy through altered regulation of numerous target genes, including \( Ptx1 \), \( p53 \), and other germline-specific genes or myogenic regulators [17–21,23–24]. Another mechanism involves production by the contracted region of \( DBE-T \), a chromatin-associated long-non-coding RNA that causes de-repression of several 4q35 genes [77], including \( FRG1 \), whose overexpression was previously proposed to contribute to causing muscle degeneration too [11–12]. Other mechanisms also influencing 4q35 gene expression include a telomeric position effect, according to which propagation across 4q35 of changes in methylation or chromatin conformation might be due to the loss of the CTCF barrier function of the \( D4Z4 \) array [8,13,78]. The relative contribution of \( DUX4 \)-mediated gene regulation and of mechanisms leading to altered 4q35 gene expression is controversial [9,17] and may reflect an underestimated diversity in the clinical expression of FSHD1 [79–80]. Understanding which of these mechanisms, or what combination, contributes to modifying tissue-specific distribution of \( FAT1 \) will require developing cellular or animal models adequately reproducing FSHD mechanisms and mimicking in vitro key steps of muscle shape development. This will also allow defining whether there are differences in the sensitivity to a contracted allele between developmental stages and adult muscle, but also between \( FAT1 \) isoforms. \( DUX4 \) can repress \( FAT1 \) expression in human myoblasts ([18] and Figure S15D). Such regulatory influence could involve some \( DUX4 \) target genes such as \( p53 \) [37–38], or myogenic transcription factors. Our data suggest that irrespective of whether \( FAT1 \) is regulated by \( DUX4 \), by \( DBE-T \), or by anyone of their respective downstream or upstream targets, this regulation must occur primarily during development, in the cell type in which \( FAT1 \) is required to control migration polarity. This model does not exclude the possibility that the pathogenic 4q5 allele may further contribute to directly triggering muscular dystrophy in adult muscle, through additional mechanisms independent of \( FAT1 \) de-regulation.

Can deregulated \( FAT1 \) in FSHD lead to altered PCP/ Frizzled signaling?

A number of clinical features of FSHD, including non-muscular symptoms such as hearing loss and retinal vasculopathy [81–82], carry the signature of defects in the Wnt/PCP pathway [26], a cascade of tissue polarity regulating genes, involving non-canonical Wnt/Frizzled signalling (core PCP genes) and modulated by the protocadherins \( FAT \) and \( Dachsous \) [25,27]. Sensory hair cell polarity in the cochlea is the best mammalian PCP paradigm, and...
deafness has become a traditional hallmark of altered PCP signalling [26,57–58]. Even through the anatomical nature of auditory abnormalities in FSHD is not known, it will be relevant to explore whether it carries further characteristics in common with altered PCP. Furthermore, vascular abnormalities in the retina, also known as Coats disease, are phenotypically similar to familial exudative vitreoretinopathy (FEVR), recently linked to mutations in the Wnt receptor Frizzled4 (FZD4) and its ligand Norrin [83–85]. Moreover, the Wnt/PCP pathway is also known to play key roles in muscle biology. PCP-activating Wnts, such as Wnt11 or Wnt7a act as instructive signals for myofibre orientation during muscle morphogenesis [86], for muscle satellite cell expansion through symmetric division [87], and for neuromuscular synapse development [88]. Thus, altered regulation of FAT1 may in turn deregulate the function or expression of its genetic partners, such as other components of the planar cell polarity cascade but also of the Hippo pathway. Mutations in other components of these genetic cascades may also play a causal role in a subset of the FSHD patients lacking the D4Z4 contraction. Overall, by linking FSHD to FAT1, our work opens new avenues for the exploration and treatment of this and other neuromuscular disorders.

Methods

Ethics statement

Animals were maintained and sacrificed in accordance with institutional guidelines. Adult mice were either sacrificed for experiments through anaesthesia, or euthanized by cervical dislocation. Efforts were made to minimize the number of adult Fat1-deficient mutant mice examined after more than 25% weight loss.

Human DNAs were obtained from FSHD and control cases at La Timone Hospital (Marseille, France). The protocol for their collection was approved by the Université de la Méditerranée (Marseille, France) Committee on Human Research and an agreement of informed consent authorizing scientific experiments was signed by each individual patients. Human Tissues samples were obtained from abortus cases at La Timone Hospital (Marseille, France) and at AP-HP (Assistance Publique-Hôpitaux de Paris, France). The protocol for their collection was approved by the Université de la Méditerranée (Marseille, France) Committee on Human Research and an agreement authorizing scientific experiments was signed by the parents. Termination of pregnancy (performed at the stages corresponding to individual cases) was decided after late prenatal diagnosis.

Mouse lines

Characterization and genotyping of the Fat1<sup>LacZ</sup> allele. Fat<sup>LacZ</sup> mice, previously generated using the genetrap ES line KST249 (see detailed characterization below), were obtained from Marc Tessier-Lavigne. Initial characterization of the transgene insertion site on Fat1 transcript was performed by 5′RACE PCR (as documented on the international genetrap consortium databases [http://www.genetrap.org/cgi-bin/ annotation.py?cellline = KST249]) indicated insertion downstream of the first exon. Genome walking experiments (LAM-PCR, GATC Biotech; plasmid rescue, restriction analysis by Southern blot) consistently indicated that multiple copies of the transgene were inserted in tandem, thus preventing so far identification of genomic sequences flanking the transgene. Genotyping was performed on genomic DNA using the following PCR to detect the transgene [primer sets OF47: 5′ GGA ACT TCT CAG ATC TGG GGG CTG 3′; and OF48: 5′-TCT CAT CTT GGG TGA GGT GGG TCCC-3′; or OF49: 5′-GGA ACT TCT GGA TCT GGC ATC TGG CCA ACT ATG-3′ and OF57: 5′ CCC CAA ACA CTG CCA ACT ATG-3′]. To recognize heterozygotes (one mutant allele) from homozygotes (two mutant alleles) at postnatal and adult stages, we performed dot blot hybridization experiments (using a beta-gene probe made with OF47-OF48 PCR product, dig-labelled by random priming) and discriminated difference in staining intensity, or qPCR analysis, using OF47 and OF48 primers for the transgene, and the following primers as reference (met primers we: FM20: 5′-AAG CTT GTG GTT ATG CTG ATC TGT CAG -3′; Met-610: 5′- AGG ATT GAT CAT TGG TGC GGT C-3′). At embryonic stages, we also performed X-gal staining on yolk sacs (or any dissected fragment of embryo), the intensity of staining being a reliable indicator of the genotype until E16.5.

To follow progression through adult phenotype, each mouse’s weight was measured weekly, and the weight at a given stage is compared to its maximal measured weight. Mice with less than 10% weight loss are considered presymptomatic (with respect to systemic consequences of phenotypes such as kidney filtration defects).

Transgenic mouse lines. <i>Gadfi-lacZ</i> mice were used with permission of Genentech, and genotyped as previously described [40]. Mice 2E2 transgenic mice were kindly provided by Robert Kelly, and genotyped as previously described [43]. Pax3<sup>cre</sup> knock-in (Pax3<sup>tm1<sup>cre</sup></sup>) mice were used with permission of Jonathan Epstein and genotyped with the following generic CRE-specific primers: MSP4: 5′-ATC CGA AAA GAA ACG GTT GA-3′; MSP5 5′-ATC CAG GTT ACG GAT ATAG T-3′. Rosa26-YFP mice (Gt(Rosa)26Sor<sup>tm1<sup>YFP</sup></sup>Cos line, [90]) were kindly provided by Teddy Fauquier and obtained from the Jackson laboratory (mouse strain 006148), and were genotyped following Jasmime instructions.

Generation of Fat1 conditional and constitutive mutants. Mice carrying a conditional Fat1<sup>ATM</sup> allele were constructed in the research facility of iTL (ingenious Targeting laboratories, genetargeting.com). Construction of mice with the conditional Fat1<sup>ATM</sup> allele began by isolation of the 129SvEv BAC clone RP22: 41E14, containing the murine sequence of the Fat1 locus, including the exons 24 and 25, which we aimed to flox, exon 25 containing the transmembrane domain. An 11 kb region used to construct the targeting vector was first subcloned from the BAC using a homologous recombination-based technique. The region was designed such that the short homology arm (SA) extends about 2.3 kb to the 5′-end of the LoxP/FRT-flanked Neo cassette. The long homology arm (LA) extends 6.22 kb to the 3′-end of the single Lox P site. The single Lox P site is inserted upstream of exon 24 in intron 23–24, and the LoxP/FRT-flanked Neo cassette is inserted downstream of exon 25 in intron 25–26. The target region is ~2.6 kb containing exons 24–25. The BAC was sub cloned into a ~2.4 kb pSP72 (Promega) backbone vector containing an ampicillin selection cassette for retransformation of the construct prior to electroporation. A pGK-gb2 LoxP/FRT-flanked Neomycin cassette was inserted into the gene as described in Figure S4. The targeting construct can be linearized using NotI prior to electroporation into ES cells. Ten micrograms of the targeting vector was linearized by NotI, and transfected by electroporation of iTL1 129/SvEv embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for PCR analysis to identify recombinant ES clones. Screening primers A1 and A2 were designed downstream of the short homology arm (SA) outside the 3′ region used to generate the targeting construct. PCR reactions using A1 or A2 with the F3 primer (located within the Neo cassette) amplify 2.42 or 2.51 kb fragments, respectively. The control PCR reaction was performed...
using the internal targeting vector primers AT1 and AT2, which are located at the 3’ and 5’ ends, respectively, of the SA. This amplifies a product 2.05 kb in size. Primers for PCR Screening: A1: 5’-AAG CCT CTT TCC GT TCC ACT AAG G -3’; A2: 5’-ACG TGT AGT TTA ACT GGG TAG AC-3’; AT1: 5’-AGG TTC TGA ACA GGC AAG TAA AGC -3’; AT2: 5’-TCT GTT GAG CAT ATG TGC AGA -3’; OUT1: 5’-GGC TGC TAG TCT TCA GGC G -3’; F3: 5’-GCA TAA GCT TGG ATC GTG TCT TCG GAC -3’. Individual clones from positive pooled samples were screened using A1 and F3 primers. Positive recombaint clones were identified by a 2.42 kb PCR fragment. A PCR was performed on SA positive clones to detect presence of the third LoxP site. The PCR conditions used for ES screening (A1 described in Figure S4A) from pups with agouti coat color. generate F1 heterozygous offspring. Tail DNA was analyzed as correctly targeted and used for injection in blastocysts. Targeted alleles can be recognized from each other using specific PCR products (For Fat1, the combination of ON17 and Uni primer yields a 406 bp product; for Fat/Flox, the combination of ON17 and ON30 yields a 345 bp PCR product). In the current study, all the conditional experiments were done using the Fat1 allele. To perform the conditional ablation, of Fat1 in Pax3-cre-derived tissues, we mated Fat1Pax3cre/+ mice with Pax3cre/+ mice. Because of our observation of female germline activity of the Pax3-cre line, we exclusively selected Fat1Pax3cre/+; Pax3cre/+ males to perform crosses with Fat1Pax3cre/+ or Fat1Pax3cre/+ females (also carrying the MLC3F-2E transgene) to produce conditional embryos.

Human tissue collection

Human Tissues samples were obtained from abortus cases (see ethics statement) after termination of pregnancy, decided after late prenatal diagnosis of FSHD or non muscular medical symptoms for control cases. The cases used, and their respective stages are described in Figure S8A. Four cases of foetuses diagnosed with FSHD were used (Figure S8A) referred to as F1, F2, F3 and F4, respectively. Family history included in the F1 case early-onset and severe FSHD phenotypes in a sibship carrying the same haplotype (family tree shown in Figure S8D). In the she second (F2) and third (F3 and F4, twin foetuses) cases one parent had FSHD. FSHD diagnosis was characterized by standard procedures involving southern blotting using a combination of restriction enzymes and probes, to characterize contraction status, 10 versus 4 chromosome, and haplotype. The p13E-11 probe was used on genomic DNA digested with EcoRI alone or with EcoRI and BlnI, then determined D4Z4 array length and distinguishing 4q contractions from 10q contractions [93]. Molecular combing is then performed with a combination of probes (including those for D4Z4, the p13E-11, q14 and qB-specific probes, and 10q versus 4q specific) allowing to distinguish simultaneously 10q from 4q as well as qA from qB haplotypes and the degree of contraction [94] (see also simplified probe set in Figure S14B-E). Control biopsies (Figure S14A) were also obtained from abortus cases, for which termination of pregnancy was performed on the basis of medical diagnosis different from FSHD or other muscle related diseases. Detailed information on clinical and genetic diagnostic for the patients used for CGH and qPCR studies is provided in the Table S1.

X-gal staining, immunohistochemistry, antibodies

X-gal staining was performed using classical procedures on embryos or postnatal tissues previously fixed in paraformaldehyde (PFA) 4% (time depending on strength of lacZ expression), rinsed in PBS, and incubated in X-gal in combination with potassium ferri- and ferro-cyanide (FeCN). Staining was terminated by rinsing in PBS, and post-fixing in PFA4%. Embryos were transferred in 100% glycerol for imaging and counting dispersed myoblasts.

For adult murine tissues, anesthetized mice were perfused with PFA 4% in phosphate buffer saline (PBS) prior to dissection. Shoulder and hindlimb muscles were carefully dissected under a stereomicroscope, rinsed in PBS, shortly incubated with fluorescent alpha-Bungarotoxin to visualize neuromuscular junctions. When necessary, observation under fluorescence was used to visualise and sub-dissect zones enriched in neuromuscular junctions. Samples were cryoprotected in
25% sucrose (in PBS), embedded in a mix with 7.5% gelatine and 15% sucrose in PBS, and frozen for cryostat sections.

Immunofluorescence was performed using primary antibodies to neurofilament (NF-M, Ab1789, Chemicon), tau (AbCam), laminin (Sigma), alpha-actinin (Clone EA-53, Sigma), Ryamodine Receptor Ryr1 (MA3-923, Thermo scientific), Dhydropyridine Receptor alpha 1S (MA3-920, Thermo scientific), and rabbit anti-FAT1 (invitrogen). Antibodies against FAT1 were the following: two rabbit polyclonal antibodies raised against human FAT1, HPA001869, HPA023882 from Sigma (epitopes described in the human protein Atlas [http://www.proteinatlas.org]) recognized two regions of the extracellular domain of FAT1 indicated FAT1-1869 and FAT1-23882, respectively, in Figure 2A, B. Two antibodies against the intracellular domain of mouse FAT1 were used: Fat1-ICD from ref [35], and an additional anti-Fat1 rabbit antiserum (Rh1465) we raised against a GST-fusion protein encompassing the intracellular domain of mouse FAT1 (see complete procedure below). Secondary antibodies used were Cy3- or Cy5-conjugated (Jackson Immuonoresearch) or conjugated with Alexa-488 or Alexa-555 (Invitrogen). NMJs were visualised with Alexa-488 conjugated alpha-Bungarotoxin (1/5000) and Alexa-488-conjugated GS-IB4 (Invitrogen), as described [83], and F-actin with alexa-594 or Alexa-647-conjugated-Phalloidin (Invitrogen). Retinal vasculature was visualised with Alexa-488-conjugated GS-IB4 (Invitrogen), as described [83], including CaCl2 1 mM and MgCl2 1 mM in all incubating solutions. Image acquisition was performed with a Zeiss Axioplan equipped with Apotome.

Ultrastucture studies

For electron microscopy analysis, muscles were dissected from mice previously perfused in 4% PFA, and postfixed in 2% PFA, 2.5% glutaraldehyde, 50 mM CaCl2 in 0.1 M cacodylate buffer (pH 7.4). Muscles were additionally postfixed with 1% OsO4, 2.5% glutaraldehyde, 50 mM CaCl2 in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C and dehydrated in a graded series of ethanol, with a 2h incubation step with 2% uranyl acetate in 70% ethanol at 4°C. Samples were further dehydrated and embedded in epon resin. Thin (70-nm) sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscope (Zeiss EM 912). Images were acquired with a digital camera Gatan BioScan 792, using the Digital Micrograph software.

In situ hybridizations and measurement of myoblast orientation

Embryos were collected in PBS and fixed in 4% PFA. In situ hybridizations were performed with a MyoD RNA probe on whole mount E12.5 embryos, according to previously published procedures [95]. In order to assess the orientation of myoblasts, the CM muscle sheet was dissected and flat mounted, after completion of the ISH procedure, for high magnification imaging with a Zeiss Axioplan. For each muscle, three areas in stereotyped positions of the CM (3 positions in which the main chain direction made a 10°, 45°, and 70° angle with the DV axis, respectively) were imaged at 63X resolution. Scoring myoblast direction was done using AxioVision image software (Zeiss Imaging). For each picture, three to four chains were outlined. For each cell, an angle between the closest outlined chain and the nucleus direction was measured. Every cell for which the nucleus was visible was assigned such an angle. The distribution of angles was thus determined for each embryo side (two CM muscles per embryo), by defining angle ranges of 10°, and determining the percentage of cells showing an angle in the given angle range. This distribution was averaged between 3 wild types embryos sides (n = 3), and 5 Fat1+/−;Zfa−/− embryo sides (n = 5).

Western blot analysis

Tissue extracts were prepared in EBM buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton, 5 mM EDTA, 5 mM EGTA, 10% glycerol) supplemented with protease inhibitors [95]. To enrich the lysates in membrane associated proteins, lysates were lectin-purified by incubation with Lectin-sepharose beads. For immunoblotting, 50 μg of protein extracts were separated by SDS-PAGE using 3–8% gradient gels (Invitrogen), blotted onto nitrocellulose membrane and detected with specific antibodies. Immunoblots were revealed by ECL (Amersham).

Anti-FAT1 antisera production and purification

We first constructed a GST-FAT1 fusion protein, containing the C-terminal part of the intracellular domain of mouse FAT1 (from aa 4451 to 4588; an epitope entirely contained by exon 28, and comprising approximately one third of the cytoplasmic domain), in PGEX2 vector. Serum was collected from two rabbits immunized with the GST-FAT1 fusion protein (Rb 1463 and 1464). Antibodies were affinity purified from the two antisera, using the same GST-FAT1 (GST-Fat1(aa4451-4588)) fusion protein, loaded on Affi-gel 15 support in poly-prep chromatography columns, following the manufacturer’s instruction (Biorad).

RNA extraction and Real-Time quantitative Polymerase Chain Reaction (RT-PCR)

In both mouse and human samples, total RNA was isolated using Trizol reagent (Gibco, BRL). RNA was resuspended in 100 μL DEPC-treated H2O and quantified by spectrophotometry; samples used for RT-PCRs had a 260/280 absorbance ratio greater than 1.8. cDNA was synthesized from 1 μg of total RNA using Superscript III (Invitrogen) or the First Strand cDNA Synthesis Kit (Fermentas RevertAid: K1622) and random oligonucleotides.

In mouse RNA samples, expression levels of Fat1, Creatine kinase B (CKB) or HPRT were determined by semi-quantitative and/or quantitative RT-PCRs using real-time sybgreen PCR assay (life technologies), using the following primer sets. Fat1 Primer set exons 20–21 (product size: 511 or 525 bp), 5’ CCA CGC GGT TGT CAT GTA CG 3’ (exon 20-Fw), and 5’ TCC AGT AGG CGA GGG ATT GC 3’ (exon 21-rev). Fat1 Primer set exon 6–8 (product size: 545); 5’ AAG CCC CCTT GAT GCA GAA CA 3’ (exon6-Fw); 5’ TCA GGG TTC CTC CCT TTG TC 3’ (exon6-rev). Fat1 Primer set exons 24–25 (product size 142 bp) 5’ TGC TGT CTG TCA GTG TGA CTC AGG C 3’ (exon 24-Fw); 5’ GAG AGG CAT CCT CAC AGT GCT TCC C 3’ (exon 25-rev); Fat1 Primer set exons 26–28 (product size varies according to splice variants expressed; 3 products are observed:268 bp, 304 bp; and 330 bp) 5’ CGC TTA GCT TCC ATG AGG CTG AGT CC 3’ (exon 26-Fw); 5’ GGG TGG GTG TAT GGA CTC G 3’ (exon 24-Fw); 5’ GAG CAT CCT CAC AGT GCT TCC C 3’ (exon 25-rev); HPRT primer set exons 24–25 (product size 142 bp) 5’ TGC TGT CTG TCA GTG TGA CTC AGG C 3’ (exon 24-Fw); 5’ GGG TGG GTG TAT GGA CTC G 3’ (exon 28-Rev); HPRT Primer set: HPRT-Fw: 5’ CAC AGG ACT AGA ACA CCT GC 3’; HPRT-rev: 5’ GCT GGT GAA AAG GAC CTC T 3’. Creatine kinase B-type (CKB); CKB-Fw: 5’ AGG ACC ACT TCC TCT TCG ATA A 3’; CKB-rev: 5’ TTT TCA GTG TCA ACA ACA GTA C 3’. For qPCR experiments, the HPRT gene was used as endogenous reference gene to normalize the data across all samples. For each gene examined, primers were chosen at the junction between two exons, to distinguish by size the RTPCR products from the genomic DNA PCR products. For Fat1 primer sets, sizes expected from genomic PCR amplicons, in case of genomic DNA contamination, have been indicated on Figures S4 and Figures S5).

Expression of the human FAT1 gene was monitored by a real time quantitative RT-PCR method using TaqMan gene expression
assay reference number Hs00170627_m1 targeting the 5' part of the FAT1 sequence (Applied biosystem), or using real-time sybr-green PCR assay (Roche) (see primers below). The ubiquitous beta-glucuronidase (GUS), was used as endogenous reference gene to normalize the data across all samples. FAT1 primers were chosen at the exon2-3 junction: forward primer: 5'-CAT TAG AGA TGG CTC TGG GC-3'; reverse primer: 5'-ATG GGA GGT CCA TTC ACG-3'). (Fw GUS: 5'-CTC ATT TGG AAT TTT GCC GAT T-3'; Rev GUS: 5'-CCG AGT GAA GAT CCA CCT TTT A-3'). Primers used for other muscle genes: DHPR-Fw: 5'-CGG AAC TAC GTC GGT GCC AGC-3'; DHPR-Rev: 5'-GCC CCA TCC TCC AGC ACG GC-3'; MURF1-Fw: 5'-CTT GCG TAC TGC CAA GCA ACT CA-3'; MURF1-Rev: 5'-CGG AAC AGC CCT GCT CTT TCT TC-3'; DYSF-Fw: 5'-GAA GCC GCC AAG GTG CCA CTC CGA C-3'; DYSF-Rev: 5'-CAG GCA GCC GTC TG TGT AGG ACA-3'; Calp3-Fw: 5'-TCT CTT CAC CAT TGG CTT CGC-3'; Calp3-Rev: 5'-TGCG TGC TGG TCC CCT CGG TGC-3'; B2M-Fw: 5'-CTC TTC TCT TTC TGG CCA GG-3'; B2M-Rev: 5'-TGG TGG ATG ACG TGA GTA AAC C-3'; gSAR-Chip: 5'-CCA CCC GTT TCA AGG CCT TA-3'; gSAR-Rev: 5'-CCT CAA TTT TCC CAC GCT GA-3'. Similar results were obtained with two other normalizing genes (β-2-microglobulin (B2M) or the human acidic ribosomal phosphoprotein (PO)).

Each experiment was performed in triplicate and repeated at least three times against age matched unaffected foetuses used as controls (see Figure S6A). For quantitative RT-PCR experiments, relative quantities of RNA expression were calculated using the comparative cycle threshold (ΔΔCt) method [96] and were normalized with GUS RNA levels as endogenous reference gene. Briefly, the fold change of RNA expression levels was calculated by the equation 2-ΔΔCt, where Ct is the cycle threshold. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. ΔCt was calculated by subtracting the Ct values of the endogenous control (GUS) from the Ctx values of the RNA of interest (FAT1 or control muscle genes, such as DHPR, MURF1, DYSF, Calp3, γ-Sarco/ylgan). ΔΔCt was then calculated by subtracting ΔCt of the sample used as control from the ΔCt of FDHS biopsies.

Repression of FAT1 by DUX4-f1

HUMAN primary myoblasts unaffected by muscle disease were infected with lentivirus carrying either DUX4-f1 or GFP as control for 24 hr. RNA was extracted with Qiagen RNeasy kit, DNAsed with Ambion Turbo DNase and reverse transcribed with Invitrogen SuperScript III according to manufacturers' instructions. Real time quantitative PCR was performed with the following primers: FAT1-1F 5' – GGA AAG CCT GCT TGA AGT GC-3'; FAT1-1R 5' – TGT ATG TCC GCC AGA AGA AC-3'; RPL13a-Fw 5' – AAC CTC CTC CTT TTT GCA GAC-3'; RPL13a-R 5' – GGA GTA CCT GTT TAG CCA CGA-3'; FAT1 values were normalized to the internal standard RPL13a and expressed as percent relative to control condition.

Chromatin immunoprecipitation assay

ChIP assays were performed on chromatin from fetal muscle biopsies (tissue samples obtained as described above) using the Magna A ChIP kit (Millipore/Upstate). For chromatin preparation, muscle samples (∼50 mg) were weighed, cut in small pieces, and cross-linked with 1.5% paraformaldehyde for 10 minutes, the cross-linking reaction being stopped by addition of glycin. Nuclei were extracted from the tissue samples by using a 2 ml dounce tissue grinder and the kid’s cell lysis buffer. Chromatin was then sheared by sonication and quantified after DNA extraction. Immunoprecipitations were performed on 5 μg of Chromatin, with 3 μg of the following antibodies: anti-H3K4me3 (17-614, Millipore), anti-H3K27me3 (07-449, Millipore), and anti-H3K1me1 (ab8095, Abcam), following the ChIP kit instructions, using _proteinA-conjugated marreferredbeads. Immunoprecipitated material was then washed, cross linking was reversed with protease K at 56° for 2 h, and DNA was extracted. The presence of individual regulatory regions in immunoprecipitated chromatin was analyzed by qPCR using sybr green (Invitrogen) on a Biorad CFX96 apparatus. Relative quantities of each chromatin bound fragment expression were calculated using the comparative cycle threshold (ΔΔCt) method again [96] and were normalized either relative to the amount of input DNA (in the same amount of chromatin before immunoprecipitation, quantified with the same PCR), or with levels of the promoter region of a normalizing genes GUSB.

Oligonucleotides for FAT1 Promoter are: FAT1_P1_Fw: 5’ GGT AAG GGT CCT GCG AGC AC-3’; FAT1_P1_Rev: 5’ AAA GTC CCT GCC AGC AGC ATC C-3’; FAT1_P2_Rev: 5’ AAA GTC CCT GCC AGC AGC ATC C-3’. Oligonucleotides for the 17/18 intronic enhancer were: FAT1_inton17_Fw: 5’ gga gta ggg agg agg gag gag tgg g 3’; FAT1_inton17_Rev: 5’ ctt cct tgc ttc ttc tat cag c-3’. Primers for the normalizing GUSB promoter were: GUSB_E/P_Fw: 5’ AGA GGA TGT GCC AGA GCC AAA AGC C-3’; GUSB_E/P_Rev: 5’ TAG AGG ACA GGA CAT GAC ATG C-3’. All sequences were selected based on the Encode ChIP tracks on the UCSC browser (available with the base genome Human Mar. 2006 (NCBI36/hg18)).

Genomic DNA array screen for microdeletions in FSHD patients

DNA was hybridized on a Nimblegen HD2.1 genomic array consisting of 135,000 probes targeting the 4q35.2 genomic region. Probes were designed with a spacing of 10 bp between consecutive probes in the exon/intronic regions and 100 bp in the intergenic regions. Labeling of DNA and hybridization was based on the Nimblegen protocols. Arrays were scanned with the MS200 scanner (Roche) and the acquired paired files were analyzed using the CGHweb algorithms [97]. To visualize the deletion/duplication events, coordinates were formatted as a bed file and added in the custom tracks of the genome browser (genome.ucsc.edu). Patients that had CNVs in the intron 16–17 area are referred to with the number corresponding to their number in the patient summary table (Table S1).

A first PCR validation was performed using primers flanking the deleted area. This approach is expected to yield a PCR product of approximately 1500 bp from a control locus, and a smaller size product in the case of the deleted allele, the deletions being approximately 1 kb long. Primer sequences are: Del-Fw: 5’- CCT TCA CCT GCA GTG AG-3’; Del-Rev: 5’- CTA GGA TTC CTA AGA GC-3’. This approach led to validate the presence of both the 1500 band and the smaller band in the three independent patients (numbers 11, 12 and 25), plus patient 13 (sibling of 12), as carrying a deleted allele as well. Moreover, 20 unaffected controls were tested with this method and only yielded the control 1500 bp band, indicating the absence of deleted alleles.

Further validation of the deletion was performed by quantitative PCR, comparing the relative amount of PCR products scanning the deleted area, using as reference a PCR product outside the considered zone, a method used to quantify copy number variations [96,98]. All DNA samples, whether from healthy controls or from FSHD patients, were normalized with the ADORA Reference PCR, against one healthy control DNA used as standard DNA (set to 100%). The reference primers are as follows: chr17p12 : ADORA2B2-2F: 5’-GTC ACT CTT TTT
CAG CCA GC-3’ ; ADORAB2-2R.5’-AAG TCT CGG TTC CGG TAA GC – 3’. The primers corresponding to the deleted area were as follows: qPCR-primer-1: 5’ – GCA ACA GAG GCC AAT GGA AA – 3’; qPCR-primer-2: 5’ – CTG AAA AGA TTG CAG TGT ACA CCC T – 3’; qPCR-primer-3: 5’ – TTC GGT AAG ATG GGA GCA GCC TCC TTC G – 3’; qPCR-primer-4: 5’ – GGT CCT GAC AAG ATT CTT CTG AAG A – 3’; qPCR-primer-5: 5’ – GGA GTG TGG TGT GTT CTG TAT GGG – 3’; qPCR-primer-6: 5’ – AGC AGA CAA GAG GAC AAG GCA TTC C – 3’; qPCR-primer-7: 5’ – GGA ACA CAG CAG CAA ATT CTA TGG – 3’; qPCR-primer-8: 5’ – TCT TCC TCC TCA GAC TCC TCT CTG – 3’; qPCR-primer-9: 5’ – CCT GGG CAA TGA GTG TAA CTG C – 3’; qPCR-primer-10: 5’ – CCA ACC TCC TCC CTA CTC CAC TT – 3’; qPCR-primer-11: 5’ – CCA GTG GCA GCA GCA GTG CTG ATT ACG C – 3’; qPCR-primer-12: 5’ – GGG AAA CTT ATT AAG GAA GTC GC – 3’ (primers numbered as in Figure 1A and Figure S1A).

Assay of hypomethylation status at D4Z4 repeats

Among contraction-independent cases, a large proportion, referred to as FSHD2, harbours hypomethylated D4Z4 [63–64], while others do not and are expected to carry unrelated causative abnormalities. Hypomethylation was assessed for 8 patients as indicated in the patient summary table, by digesting genomic DNA, with BlnI, CpoI and EcoRI, and hybridizing the southern blot with the p13E-11 probe, as described [64]. Numbers indicated in the patient summary table, by digesting genomic DNA, with BlnI, CpoI and EcoRI, and hybridizing the southern blot with the p13E-11 probe, as described [64].

Statistical analysis

Results were expressed as the mean ± s.e.m. Statistically significant differences were assessed by unpaired t-Student test, or Mann-whitney test for non-Normally distributed data, X² test or Fischer tests (for linkage studies), calculated with the StatEL add-in program to excel. The Kaplan-Meier plot was made with the StatEL add-in program to excel, and P value was calculated with the logrank test. * indicates P value<0.05; ** indicates P value<0.001.

Supporting Information

Figure S1 Fat1-LacZ expression. (A) Fat1Lox-/+ E10.5; E11.5 and E12.5 embryos stained with X-Gal to reveal β-galactosidase activity. The dotted areas are magnified in Figure 1C. The hotspot of expression in/around the Cutaneous Maximus (CM) is indicated with a red arrow. (B) LacZ expression in Fat1Lox-/+ embryos faithfully reproduces Fat1 expression as seen by in situ hybridization on transverse sections of E12.5 embryos in equivalent positions (upper thoracic). Positions of the CM, Latissimus Dorsi (LD) and Trapezius. (C) X-Gal staining of an E12.5 embryo carrying the MLC3F-2E (LacZ) transgene, showing the pattern of muscle differentiation. (TIF)

Figure S2 Myoblast orientation phenotypes in non-CM scapular belt muscles of Fat1Lox-/+LacZ embryos. Whole mount in situ hybridization with a MyoD RNA probe on wild type (A, C, E) or Fat1Lox-/+LacZ (B, D, F) E12.5 embryo. (A, B) Low power magnification micrographs showing a side view of shoulder area. Anterior is to the left, dorsal is to the top. (C, D) Higher power magnification micrographs showing an enlargement of the corresponding boxed areas in (A and B), respectively. Fat1Lox-/+ LacZ embryos present numerous dispersed myoblasts in ectopic positions in the shoulder area, either as individual cells (red arrows), or clustered and forming ectopic muscles (orange arrows). (E, F) Higher (x63) magnification views of the corresponding boxed areas within the Trapezius muscles in (C and D) showing misoriented myoblasts in Fat1Lox-/+LacZ embryos. Scale bars: (A–B) 0.5 mm; (C, D) 50 µm; (E, F) 10 µm. (TIF)

Figure S3 Targeted conditional deletion of FAT1 transmembrane domain. (A) Strategy used to generate the conditional allele. 1- Top: genomic organization of the Fat1 locus around the targeted area. 2- Targeted Fat1 locus, in which exons 24 and 25 (the latter containing the transmembrane domain) were flanked by LoxP sites (Fat1tm allele). The locus also contains a pgk-neo selection cassette, itself flanked by LoxP sites (yellow triangles) and by FRT sites (blue triangles, for later removal of the pgk-neo cassette only). An external probe (red bar) was used to identify recombinant ES clones by Southern blotting. The sizes of the Neol restriction fragments are indicated. 3- No cassette removal is permitted by FRT-mediated excision of the neo cassette, which is flanked with both FRT (blue) and LoxP (yellow) sites. This generates a Fat1tm allele, in which exons 24–25 are flanked on the 5’ side with one LoxP site, and on the 3’ side with one leftover FRT site, followed with a LoxP site. 4- CRE-Recombined Fat1 locus: Genomic organisation of the targeted Fat1 locus after cre-mediated excision of the entire fragment comprised between LoxP sites, including exons 24–25 and the neo cassette. This new recombined allele is referred to as Fat1tm allele. Primers indicated (1 to 7) are the ones used for genotyping by PCR. ES screening primers are given in the method section. (B) Abnormal shape of the Cutaneous Maximus in Fat1tm/+/tm embryos. Flat mounted preparations of dissected skeletal muscle groups from E13.5 and E18.5 control and Fat1tm/+/tm embryos, carrying the MLC3F-2E transgene, in which differentialed skeletal muscle cells are revealed by X-gal staining. Analysis of skeletal muscles confirms the reduced and misshaped CM at E13.5. The MLC3F transgene also reveals the presence of disoriented muscle cells in the forming CM at higher magnification. The shape of CM from E13.5 embryos is shown on the right. (C) Kaplan-Meier plot showing the probability of survival of Fat1tm/+/tm mice. Most (70%) Fat1tm/+/tm mice die between postnatal days P0 and P1, and less than 15% of Fat1tm/+/tm mice survive beyond 3 months after birth. (TIF)

Figure S4 Selective and asymmetric muscle shape abnormalities in E14.5 Fat1tm/+/tm embryos. Skeletal muscle groups were visualized in E14.5, wild type and Fat1tm/+/tm embryos carrying the MLC3F-2E (LacZ) transgene, by X-gal staining. (A) Low magnification images showing the entire embryos. The drastically reduced length and density of the CM muscle is also visible at that stage. (B) High magnification views of hindlimb shank musculature, showing no obvious shape differences, in particular in the tibialis anterior muscles, at that stage. Muscle name abbreviations: lb: biceps femoris; CM: cutaneous maximus; edl: extensor digitorum longus; pd: peroneus digitorum; pl: peroneus longus; ta: tibialis anterior. (C) Illustration of asymmetry of muscle shape abnormalities observed in two Fat1tm/+/tm embryos, by showing their respective left and right sides. All red arrows point to shape abnormalities that are different between the two sides. (TIF)

Figure S5 Residual expression of Fat1 RNAs in the Fat1Lox-/+LacZ hypomorphic allele. In the Fat1Lox-/+ insertion allele (ES line name Fat1EsT/j), multiple copies of a secretory-trap vector [41–42] were inserted in tandem downstream of the first exon of the Fat1 gene (Figure S3A). As a result, the main product of the Fat1 locus is a
fusion protein of 291 kDa, including the first exon of Fat1 (the first β-cadherin domains), in frame with the exogenous transmembrane and beta-geo fusion protein (Figure S2A). (A, top) Schematic representation of the gene trap vector and its insertion point in the mouse Fat1 locus. Precise content of the gene-trap vector has been described previously [41–42]. With its splice acceptor site, the depicted cassette behaves as an exon. Following (in blue), is an element encoding a transmembrane domain, and a beta-geo fusion reporter (in frame fusion of the β-galactosidase and the neomycin resistance gene). The ES selection procedure ensures that this reporter cassette is in frame with the preceding exon (exon 1 of mouse FAT1). The resulting FAT1-β-gal fusion is a transmembrane protein depicted in Figure 2A. This protein is recognized by antibodies raised against an epitope of FAT1 mapping in exon 1 (Fat1-1869; Figure 2B), but not by an antibody raised against a downstream epitope in the extracellular domain (Fat1-23882; Figure 2B). (A, bottom) Representation of several possible RNA products of the Fat1 gene in mouse as they appear proposed by Ensembl as Ensembl-Havana and as EST-based gene products, respectively. At least three alternative sites of transcription initiation were identified, two of which located downstream of KST249 integration site. (B) RT-PCR analysis of Fat1 transcripts in wild type and Fat1LacZ/LacZ mice, using primers matching exons 6–8 of mouse Fat1, and HPRT as control RNA. RT-PCRs were performed on RNA extracted from kidneys. mRNA containing Fat1 exons 6–8 in kidneys are absent in adult Fat1LacZ/LacZ mice compared to control. (C) RT-PCR analysis of Fat1 transcripts in wild type and Fat1LacZ/LacZ mice, using primers matching exon 20–21 junction of mouse Fat1, Creatine kinase b (CKB), and HPRT as control RNA. RT-PCRs were performed on RNA extracted from rhomboid and GM muscles. Residual Fat1 mRNA levels containing exons 21–22 in Fat1LacZ/LacZ mice inversely correlate with phenotype severity. In all cases, primers were chosen in two consecutive exons, so that the size of the amplicon resulting from cDNA and from any potential genomic DNA contamination would result different (sizes indicated). The band sizes shown are those resulting from cDNA amplification, and cannot be genomic DNA contamination. Animals used for RNA extractions were 10–12 week old littermates, a wild type mouse of 23 g, and 2 cases of Fat1LacZ/LacZ mice: one presymptomatic (with only slightly reduced weight as compared to wild type 20.4 g), and one with strongly impaired growth and with 15% weight loss (11 g when sacrificed). Levels of residual Fat1 RNA differ between Fat1LacZ/LacZ mice, but also between muscles in a given mouse. The lowest levels of Fat1 correlate with the most severe phenotype, as assessed by body weight, or by levels of CKB, known to be elevated in dystrophic muscles. (D) In situ hybridization with an RNA probe corresponding to the 3’UTR of Fat1 on whole mount spinal cords from E12.5 Fat1LacZ/LacZ (left) and Fat1LacZ/LacZ (right) embryos. Results show that Fat1 RNA levels are reduced in most but not all cells within the Fat1-expression domain. (TIF)

**Figure S6** Specific muscle wasting in presymptomatic Fat1LacZ/LacZ mice turns to generalized muscle wasting in symptomatic Fat1LacZ/LacZ mice. (A) Kaplan-Meier plot showing the relationship between disease span and age of onset, as evidenced through the comparison of the probability of onset (blue curve: probability of being presymptomatic) with the survival curve (probability of survival), on the same set of 49 Fat1LacZ/LacZ mice. Fat1LacZ/LacZ mice with early onset show short disease span, while the disease span can be long for mice with late onset. (B) Extent of muscle wasting in adult wild type (B1,4) and Fat1LacZ/LacZ mice at presymptomatic (B2,3) or advanced symptomatic (B5,6) stages. (C) Mice with advanced symptomatic stage (B5, 20% weight loss), using antibodies against laminin. (C) Histological section of Cutaneous mass muscles from adult wild type and Fat1LacZ/LacZ mice at presymptomatic stage, lightly stained with Toluidin Blue, showing reduced thickness (top), as well as reduced myofibre density, reduced myofibre diameter, and infiltration with connective tissue (bottom, higher magnification). Scale bars: (B1,4) 4 mm; (B5,6) 50 μm. (TIF)

**Figure S7** Reduced myofiber diameter and cellular infiltrations in affected muscles from adult Fat1LacZ/LacZ mice. (A) Cryosections of the Trapetzus Thorius, Pectoralis Major, Tibialis anterior, and Solus muscles from adult wild type and presymptomatic Fat1LacZ/LacZ mice (4 months old) were stained with phalloidin-Alexa47 (purple), anti-Laminin (green), Collagen I (red), and DAPI (blue). The selected areas illustrate places in the trapetzus and Pectoralis Major muscles with cellular infiltrations (yellow arrows) between myofibres, with two examples (right pictures) of perivascular infiltrations. In tibialis Anterior, the selected area contains nuclei of infiltrated cells surrounded with Collagen I-positive deposit (yellow arrow). (B) Average myofiber diameters were quantified in the analysed muscles, and presented as %age of the area of the corresponding Wild type muscle. Myofiber diameter is significantly smaller in Trapetzus (** p<0.001), in Pectoralis Major (** p<0.001), and in Tibialis anterior (p<0.01), but not in solus. (TIF)

**Figure S8** Ectopic muscles variably connecting shoulder or humeral muscles, which are subject to loss of integrity at adult stages. Ectopic muscles (Ect) were identified during dissection of shoulder or limb musculature from P3 Fat1LacZ/LacZ pups (A–C) and several adult Fat1LacZ/LacZ mice at presymptomatic (D–I) or advanced (J–L) stages of disease progression. Each ectopic muscle was first photographed in its original context (except rhomboids), where the dissection procedure makes it impossible, to visualise original attachment sites with other muscles, and at higher magnification, after dissection pinned on sylgard, without disconnecting the ectopic muscles from the limb or shoulder muscles they were connected to. (C, L) Toluidin counterstained seminifi sections of the ectopic muscles seen in (A and B) and (J and K), respectively. Images show that myofibers properly assemble the contractile apparatus (C, P3) but display severe alterations of their histology at adult stages (L, LD: lattissimus dorsi; Trp: trapetzus; Tri: triceps brachii; s. sc: subcapularis muscle. Scale bars: (A) 2 mm; (B) 0.5 mm; (D) 6 mm; (E) 1 mm; (F, G) 20 μm. (TIF)

**Figure S9** Examples of NMJ denervation and atrophy in Fat1LacZ/LacZ mice. NMJs were visualized in wild type (A1–A3) and Fat1LacZ/LacZ (B1–B3) rhomboid muscles by immunolabelling.
The two magnified areas show the limb and spinal cord region. In the Spinal cord, Pax3-cre-derived lineage includes dorsal neural precursors and their derivatives mostly confined dorsally. Motor neurons in the motor columns do not express YFP, dorsal neural precursors and their derivatives mostly confined dorsally. Motor neurons in the motor columns do not express YFP, except for cells in the spinodeltoid, the Triceps brachii muscles. (TIF)

Figure S10 Tissue-Specificity of Pax3-cre recombinase activity. (A, B) Pax3-cre activity was assayed by performing anti-YFP immunohistochemistry (rabbit anti-GFP antibody, Invitrogen) on cryosections of an E12.5 Pax3stop; Rosa26Lox-STOP-Lox-YFP/+ embryo. (A) Section at Forelimb level, showing muscle masses in the limbs. The two magnified areas show the limb and spinal cord region. In the limb, besides its activity in muscles, Pax3-cre also leads to excision in neural crest derivatives including Schwann cells along the nerves. In the Spinal cord, Pax3-cre-derived lineage includes dorsal neural precursors and their derivatives mostly confined dorsally. Motor neurons in the motor columns do not express YFP, hence are not part of the Pax3-cre lineage. (B) A slightly posterior section shows efficiency of Pax3-cre expression in the Cutaneous maxunmus muscle. (C) Section of an E12.5 Fat1Lox-24/25; Rosa26Lox-STOP-Lox-YFP/+ embryo stained with X-gal at a forelimb level comparable to that shown in (A), illustrating Fat1 expression in the same muscle mass as that expressing YFP in the magnified area in (A), as well as in a cervical motor neuron pool, throughout the ventricular zone, and in multiple non muscle sites, including the vertebral bodies. (D) In situ hybridization was performed with antisense probes for myoD (top) and for Fat1-exon24/25 (the floxed exons) on alternate cryostat sections of a Fat1Lox-24/25 and a Fat1Lox-24/25; Pax3stop/+ E12.5 embryos, at a level slightly posterior to that shown in (A). In contrast to Fat1 expression in the ventral neuroepithelium (∅NE) or in the lung, which is preserved because it does these tissue-types do not derive from Pax3-expressing precursors, Fat1 expression is reduced or abolished in Pax3-derived cell types, such as the dorsal neuroepithelium (dNE) or muscles (CM, LD and spinotrapezius are indicated). (TIF)

Figure S11 Fat1 RNA levels are mildly affected by the conditional strategy. (A) Scheme of the murine Fat1 genomic locus, showing exon/intron structure, highlighting the area that has been floxed in the conditional allele, as well as the positions of primers that have been used for quantitative RT-PCR studies on mouse tissues. (B,C) RT-PCR studies were performed on RNA preparations from embryos of the indicated genotypes, to evaluate the impact of Fat1 targeting on expression of Fat1 mRNAs containing either the floxed exons (24–25), or the last C-terminal exons (26 to 28). (B) All PCRs were performed on cDNA preparations with or without reverse-transcriptases (+ or − RT), and were loaded on agarose gels to validate that the observed amplicons were specifically obtained from cDNA. In all cases, since primers were chosen in two different exons, and the sizes expected from amplicons from cDNA and genomic DNA are indicated. (C) Quantitative PCRs were performed on cDNAs from wild type, Fat1ATM/ATM; Fat1ATM/ATM; Fat1ATM/ATM and Fat1ATM/ATM embryos, to measure the relative amount of RNA containing the floxed exons (24–25, top graph, blue), or the last C-terminal exons (26 to 28, bottom graph, red). Data for each genotype were averaged from 3 embryos, and HPRT was used as normalizing gene. (Top graph): As expected, expression of mRNAs containing exons 24–25 is abrogated in Fat1ATM/ATM embryos, and reduced by 50% Fat1ATM/ATM embryos. The presence of a neo cassette in the conditional Fat1A5 allele exerts a mild effect on Fat1 expression, visible through a 50% reduction of the exon 24–25 signal in Fat1A5/ATM embryos. (Bottom graph): Expression of mRNAs containing the last exons is not abrogated by the constitutive deletion of exons 24–25, and represents less than 50% in Fat1ATM/ATM embryos compared to wild-type. In contrast to upstream exons 24–25, expression of RNAs containing exons 26–28 is only moderately influenced by the neo cassette in Fat1A5/ATM embryos. This suggests that the lowering observed in Fat1ATM/ATM embryos is a consequence of loss of Fat1 function and indicates an autoregulation mechanism. (TIF)

Figure S12 Fat1 ablation in trunk premigratory muscle precursors under Pax3-cre reproduces the scapulohumeral muscle shape phenotypes of the constitutive mutants. Muscle anatomy was visualized at E14.5 (A, B) and E15.5 (C), by X-Gal staining in Fat1A5/ATM; MLC3F-2E and Fat1A5/ATM; Pax3cre/-; MLC3F-2E embryos. Mild phenotypes can be detected in Fat1A5/ATM embryos in the face (reduced occip. Frontalis muscle, and zygomaticus), and through appearance of misplaced muscle fibres between Trapezius Ceravodis and Trapezius ThomeS, frequently unilateral or asymmetric (red arrow). While Pax3-cre driven recombination in Fat1A5/ATM; Pax3cre/+-; MLC3F-2E embryos does not cause any worsening in muscle shape and size in the face, abnormalities can be seen in the scapulohumeral region, such as the appearance of an additional muscle, in an ectopic position reminiscent of that seen in Fat1ATM/ATM embryos, without insertion of its extremity between the spinodeltoid and the Triceps brachii muscles. (TIF)

Figure S13 Residual Fat1 protein isoforms are produced in Fat1Lox-24/25 and Fat1ATM/ATM mice. Residual FAT1 protein levels in Fat1Lox-24/25 mice inversely correlate with phenotype severity. (A) Western blot analysis on Lectin-purified muscle protein extracts from 9 days old pups, comparing a wild type and two Fat1Lox-24/25 cases with different phenotype severity, was performed using a previously characterized anti-FAT1-1CD antibody from ref [35]. (B, C) Western blot analysis was performed with: (B) total brain protein lysates from the same cases shown in (A), or with (C) brain and muscle protein lysates from wild type and Fat1ATM/ATM P0 pups. Membranes with were blotted with anti-FAT1 antibodies (Rb-1465). EROs protein levels were used as loading controls (lower panels in A and B). Mutant Fat1Lox-24/25 and Fat1Lox-24/25 mice survive postnatally with variable phenotype severity (see Figure 3C). In the two examples shown here, both Fat1Lox-24/25 and Fat1Lox-24/25 mutants showed strong phenotypes since birth including impaired growth, with a milder case weighing 3.6 g and a severe case weighing 2.1 g (where reduced weight reflects phenotype) compared to their wild type littermates weighing 6.5 g in average. (TIF)

Figure S14 Characteristics of human foetal FSHD1 and control cases. (A) Tables listing the FSHD1 foetuses (top) and control fetuses (bottom) from which biopsies were used for the present study. These tables indicate for each case, the ID symbol, the stage at which termination of pregnancy was performed (in weeks of amenorrhea), their sex, and for FSHD1 cases, the number of D4Z4 microsatellite repeats. (B) Scheme representing the design of DNA probes and fluorophores used for genotyping FSHD patients by molecular combing, in combed genomic DNA as described in ref [94]. (C) Molecular combing genotyping results showing 3 different alleles of 4q35 were detected in the genome of one male.
Figure S15 qRT-PCR and immunohistochemistry data from foetal FSHD muscle. (A) Western blot analysis of Fat1 levels in muscle protein extracts from the 26 weeks old FSHD Foetus with 1.5 D4Z4 repeats (F1) and 2 age-matched control foetuses (C1 and C2) with the anti-FAT1-ICD antibody from ref [35]. (B) qPCR analysis of mRNA levels of several genes other than FAT1 (shown in Figure 7) involved in muscle biology (DHRP, γ-sarcoglycan (γ-SARC), MURF1, D13B) in quadriceps muscles of a 26 weeks old FSHD1 foetus (F1) harbouring 1.5 D4Z4 repeats in the 4q35 region (dark red bars), and a 16 weeks old FSHD1 foetus harbouring 4.3 D4Z4 repeats at 4q35 region (F2), respectively compared with age-matched control foetuses (blue bars). (C) Immunolocalization of FAT1 (Rb-1465 anti FAT1-ICD, green) and α-actin (α-act, red) in longitudinal sections from human quadriceps biopsies from a control (top) and or the FSHD1 (F2, bottom) foetus with 4.3 D4Z4 repeats. (D) Quantitative PCR shows that DUX4-β downregulates expression of FAT1 in human primary myoblasts. DUX4-β or GFP (control) were expressed in unaffected muscle cells by lentiviral delivery. Data were normalized to internal standard RPL13a and represented as mean +/- SD of triplicates with control set at 100%. (TIF)

Figure S16 Custom array CGH analyses of genome copy number changes in the 4q35.2 region. (A) Scheme of the genomic region in which CNVs were identified. The region represented is identical to that shown in Figure 10. The dotted line boxes represent positions of the PCR primers used for qPCR validation of copy number variants. The boxes in grey are those shown in Figure 10, the brown ones represent position of the primers for qPCRs shown in (D). (B) Screen copies of USCC browser lanes matching the position shown in (A), representing ENCODE- derived data (available on http://genome.ucsc.edu). The “layered H3K27Ac track”, shows enrichment of the H3K27Ac histone mark across the genome as determined in 7 cell lines by a ChIP-seq assay (the H3K27Ac histone mark is the acetylation of lysine 27 of the H3 histone protein). The lower tracks represent expanded image showing a chromatin state segmentation [67] for each of 7 of the nine human cell types, computationally integrating ChIP-seq data for nine factors plus input. The intron 16 enhancer appears labelled as exhibiting strong enhancer activity in HSMM cells (human skeletal myoblast muscle cells). (C) Genome copy number variation frequencies are plotted as a function of position in the same region as that shown in (A). Chromosome locations (NCBI36/hg18 build) are indicated by numbers above graph. Negative values (log2ratio<−0.3) indicate frequencies of probes showing copy number decreases between DNA of patient 11 respect to a control DNA. The extent of deletion is of around 1 kilobase. (D) Copy number validation of the deletion by qPCR. The relative amounts of a PCR fragments obtained using primers couples indicated in (A), which amplification corresponds to the control allele (blue bars), were compared between a control patient DNA (with 2 copies of the normal allele), and three C.1.FSHD patients carrying one copy of a deleted allele (green bars); but also three additional c.i.FSHD patients (orange bars) that did not show copy number variations in the CGH experiments. Patients’ numbers refer to the numbers indicated in Table S1. Data were normalized using an unrelated genomic fragment (Adora) as internal control. Patients 12 and 15 were both confirmed, with two Primer sets matching the deletion span (qPCR (1–2) and qPCR (3–4), corresponding primers shown in A), to carry half the amount of the normal allele compared to the control DNA, validating the deletion. (TIF)

Table S1 Characterization of FSHD patients involved in the study. Information for each patient includes genetic and clinical characteristics, as well as raw results for each FSHD patient of qPCR measurement of copy number variants at 6 positions in the FSHD locus, and of analysis of D4Z4 methylation (at the CpoI site, proximal repeat, 4q). STATUS indicates whether the diagnosis was FSHD1 or contraction-independent FSHD (c.i.FSHD), but also which cases were considered as carrying a deletion (Loss at any of the 6 positions considered). Patients are numbered as they appear in Figure 10. (XLSX)

Acknowledgments

We thank V. Girou-David, L. Jullien and S. Corby, for help with mouse husbandry; J.P. Chauvin, A. Aouane, J. Astier, K. Minko, and K Raffaelli for contributions to histology and Electron Microscopy; C. Castro for technical help on the CGH arrays, D. De Petris for help with qPCRs on human DNA, S. Cayre for performing the X-ray imaging; N. Sibinga for providing the anti-Fat1 antisemur; R. Klein and R. Sanchez for the production of additional polyclonal antiserum against mouse Fat1; M. Tessier-Lavigne for providing the Fat1-lacZ (KST249) mouse line; Genetech for Gdnf-lacZ mice; P. Maire for Pax3cre mouse [with permission of J. Epstein], T. Faquvier for Rosal26-FFP/mice; S. Whalen, N. Seta and C. Fernandez for human sample collection; A. Lu (iTL, genetargeting.com) and G. Buttler-Browne for helpful advice and discussions throughout the work. We also thank all subjects and family members for their participation. We deeply thank C. Henderson for many helpful discussions and suggestions during the preparation of the manuscript.

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