ARTICLE

Mutations of the *FHL1* Gene Cause Emery-Dreifuss Muscular Dystrophy

Lucie Gueneau,[1,2] Anne T. Bertrand,[1,2] Jean-Philippe Jais,[3] Mustafa A. Salih,[4] Tanya Stojkovic,[1,2,5] Manfred Wehnert,[6] Maria Hoeltzenbein,[6] Simone Spuler,[7] Shinji Saitoh,[8] Annie Verschueren,[9] Christine Tranchant,[10] Maud Beuvin,[1,2] Emmanuelle Lacene,[5,11] Norma B. Romero,[1,2,5,11] Simon Heath,[12] Diana Zelenika,[12] Thomas Voit,[1,2,5,11] Bruno Eymard,[5] Rabah Ben Yaou,[1,2,11] and Gisèle Bonne[1,2,13,*]

Emery-Dreifuss muscular dystrophy (EDMD) is a rare disorder characterized by early joint contractures, muscular dystrophy, and cardiac involvement with conduction defects and arrhythmias. So far, only 35% of EDMD cases are genetically elucidated and associated with *EMD* or *LMNA* gene mutations, suggesting the existence of additional major genes. By whole-genome scan, we identified linkage to the Xq26.3 locus containing the *FHL1* gene in three informative families belonging to our *EMD*- and *LMNA*-negative cohort. Analysis of the *FHL1* gene identified seven mutations, in the distal exons of *FHL1* in these families, three additional families, and one isolated case, which differently affect the three FHL1 protein isoforms: two missense mutations affecting highly conserved cysteines, one abolishing the termination codon, and four out-of-frame insertions or deletions. The predominant phenotype was characterized by myopathy with scapulo-peroneal and/or axial distribution, as well as joint contractures, and associated with a peculiar cardiac disease characterized by conduction defects, arrhythmias, and hypertrophic cardiomyopathy in all index cases of the seven families. Heterozygous female carriers were either asymptomatic or had cardiac disease and/or mild myopathy. Interestingly, four of the *FHL1*-mutated male relatives had isolated cardiac disease, and an overt hypertrophic cardiomyopathy was present in two. Expression and functional studies demonstrated that the FHL1 proteins were severely reduced in all tested patients and that this was associated with a severe delay in myotube formation in the two patients for whom myoblasts were available. In conclusion, *FHL1* should be considered as a gene associated with the X-linked EDMD phenotype, as well as with hypertrophic cardiomyopathy.

Introduction

Emery-Dreifuss muscular dystrophy (EDMD [MIM 310300, 181350, 604929]) is a rare hereditary disease characterized by the triad of 1) early joint contractures of Achilles tendons, elbows, and rigid spine, 2) childhood onset of muscle weakness and wasting, usually with a scapulo-peroneal distribution, and 3) adult-onset cardiac disease characterized by conduction defects, arrhythmias, and cardiomyopathy.[1] Cardiac disease usually appears as late as 40 years of age and is the major life-threatening issue in EDMD because it is often responsible for sudden cardiac death.[2–4] The disease can be transmitted in a recessive X-linked manner as a result of mutations in *EMD* (MIM 300384), encoding the emerin protein,[5] or in an autosomal mode with mutations in *LMNA* (MIM 150330), encoding the lamin A and C proteins.[6,7] Emerin and lamins A and C are components of the nuclear envelope and interact with each other. The precise functions of emerin and lamins A and C remain elusive even if they are thought to participate in replication, transcription, regulation of signaling pathways, chromatin organization, and nuclear envelope structure.[8,9]

About 50% of the EDMD patients do not carry mutations in *EMD* and *LMNA* and are orphan of molecular diagnosis,[10–12] thus strongly suggesting the existence of other causative genes that remain to be discovered. Recently, sequence variants in *SYNE1* and *SYNE2* genes (MIM 608441, 608442), encoding nesprin 1 and 2 proteins that bind both emerin and lamins A and C and form a network in muscle linking the nucleoskeleton to the inner and outer nuclear membranes, were identified in patients with the EDMD phenotype without *EMD or LMNA* mutations.[12] These results further suggested the genetic heterogeneity of EDMD. The clinical phenotypes associated with these variants ranged from almost asymptomatic patients with moderately increased creatine kinase to patients manifesting muscular dystrophy combined with severe dilated cardiomyopathy.[12]

In order to identify other genes responsible for the EDMD phenotype, we performed a whole-genome analysis of six informative EDMD families, and we linked the

[1]INSERM, U974, Paris, F-75013, France; [2]Université Pierre et Marie Curie-Paris, UMR-S974, CNRS, UMR-7215, Institut de Myologie, IFR14, Paris, F-75013, France; [3]Université Paris Descartes, EA 4067, Faculté de Médecine, Biostatistique et Informatique Médicale, GH Necker Enfants-Malades, Paris, F-75015, France; [4]Division of Pediatric Neurology, College of Medicine, King Saud University, Riyadh, Saudi Arabia; [5]AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Centre de Référence des Maladies Rares Neuromusculaires, Paris, F-75013, France; [6]Institute of Human Genetics, Greifswald, D-17487, Germany; [7]Muscle Research Unit, Experimental and Clinical Research Center, Charité University Medicine Berlin, D-13125, Germany; [8]Hokkaido University Graduate School of Medicine, Department of Pediatrics, Sapporo, 060-8638, Japan; [9]AP-HM, Hôpital de la Timone, Service de Neurologie et Maladies Neurologiques, Marseille, F-13000, France; [10]Service de Neurologie, Hôpitaux Universitaires, Strasbourg, F-67000, France; [11]Association Institut de Myologie, Unité de Morphologie Neuromusculaire, Groupe Hospitalier Pitié-Salpêtrière, Paris, F-75013, France; [12]Centre National de Génotypage, Evry, F-91000, France; [13]AP-HP, Groupe Hospitalier Pitié-Salpêtrière, U.F. Cardiogénétique et Myogénétique, Service de Biochimie Métabolique, Paris, F-75013, France
*Correspondence: g.bonne@institut-myologie.org
DOI 10.1016/j.ajhg.2009.07.015.

disease to chromosome X in three families with a common locus containing the *FHL1* gene (MIM 300163).

FHL1 is composed of eight exons (Ensembl no. ENSG00000022267, NCBI no. NC_000023.9). The first two are thought to be noncoding, and the others are alternatively spliced and transcribed into three major protein isoforms. FHL1 proteins belong to a protein family containing four and a half LIM domains (Lin-11, Isl-1, Mec3), which are highly conserved sequences constituted by two zinc fingers in tandem, implicated in numerous interactions. Each of the two zinc fingers contains four highly conserved cysteines linking together one zinc ion.[13] The main isoform FHL1A is predominantly expressed in striated muscles.[14,15] The two other isoforms, FHL1B and FHL1C, less abundant, are expressed in striated muscles and also in brain and testis, respectively.[16–18] FHL1A, FHL1B, and FHL1C are, respectively, composed of 4.5, 3.5, and 2.5 LIM domains. Alternative splicing leads to different domains in the C-terminal part of FHL1B and FHL1C, which correspond to nuclear import and export signals in FHL1B and to the RBP-J binding domain in FHL1B and FHL1C.[16–18] FHL1A can be localized to the sarcolemma, sarcomere, and nucleus of muscle cells.[16,18] It has been implicated in sarcomere assembly by interacting with myosin binding protein-C.[19] FHL1C has been shown to modulate transcription factor activities, in particular by inhibiting RBP-J transactivation.[14] Recently, three distinct myopathies have been annotated with mutations in *FHL1*: the X-linked myopathy with postural muscle atrophy and generalized hypertrophy, or X-MPMA[20] (MIM 300696), the X-linked dominant scapuloperoneal myopathy, or X-SM[21] (MIM 300695), and the reducing body myopathy, or RBM[22] (MIM 300717).

We report here seven *FHL1* mutations responsible for the EDMD phenotype in a total of six families and one isolated case, further extending the spectrum of *FHL1*-related diseases. In contrast to all previously described mutations, patients with EDMD were found to carry mutations in distal exons (5 to 8) differently affecting all three FHL1 isoforms. Moreover, four of these mutations are insertion or deletion mutations leading to truncated proteins, which have not been described for *FHL1* so far. Functional analyses showed that FHL1 expression was highly decreased and not detected in sarcomeres in patient muscles. More importantly, there was a substantial delay in muscle differentiation of cultured primary myoblasts from patient skeletal muscles.

Subjects and Methods

This collaborative study was conducted up to April 2008 among more than 2200 patients referred to our institutions for routine diagnostic analysis of *EMD* and *LMNA* genes because they presented clinical features suggesting *EMD*- and/or *LMNA*-related skeletal muscle and/or cardiac disease. Referring physicians were systematically asked to provide detailed clinical data (at least neurological examination, electrocardiography, echocardiography, and respiratory evaluations), as well as the existence of positive family history. Results of creatine kinase (CK) measurement and muscle histology, as well as previous genetic analyses, were also systematically obtained when available. All materials (blood and muscle biopsies) from patients and controls included in this study were taken with the informed consent of the donors and with approval of the local ethical boards. All the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national).

Clinical Assessment: Defining the EDMD Phenotype Inclusion Criteria and Selecting Families for Whole-Genome Scan

We first aimed to define a clinically homogeneous EDMD patient population. According to classical EDMD diagnostic criteria,[1,23,24] the coexistence of the following clinical features was required: slowly progressive muscle wasting and weakness, either in a humero-peroneal, proximal, or diffuse distribution; early joint contractures involving spine (rigid spine and/or stiff neck) and limb joints (elbows, ankles, etc.); and cardiac disease including rhythm and conduction disturbances, with or without cardiomyopathy. The presence of such cardiac disease was not a prerequisite up to 40 years of age. An overt dilated or hypertrophic cardiomyopathy was not mandatory, because EDMD major cardiologic features are classically based on the coexistence of conduction defects and/or arrhythmias.[1] All genetic modes of inheritance were considered. In addition, when clinical data were not conclusive, other genetic defects leading to neuromuscular diseases resembling EDMD were also excluded.[25] Blood sampling for DNA extraction was performed in accordance with standard procedures.

Muscle Biopsy, Histochemistry, and Immunohistochemistry

Prior to this study, skeletal muscle biopsies were performed for diagnostic purpose in the index cases of the seven families, and we retrospectively obtained muscle biopsy records. Of note, patients F11-5 and F1328-4 had two muscle biopsies.

We also had access to muscle sections from patients F8-6, F11-5, F997-8 and the two muscle biopsies from patient F1328-4 and a 30-year-old male control (Table S1, available online). Muscle specimens were frozen in isopentane cooled in liquid nitrogen, and 8 μm and 10 μm cryostat sections were used for histochemical and immunohistochemical techniques. Morphological analysis of muscle was made with the use of routine histological stainings: hematoxylin and eosin (HE), gomori trichrome (GT), and menadione-nitroblue tetrazolium (NBT) staining in the absence of the substrate α-glycerophosphate.

Immunohistochemical studies were performed in patients F8-6, F997-8, and F1328-4 and in the male control. Primary antibodies directed against human desmin (Dako-Desmin, D33, 1:1000; Dako, Carpinteria, CA, USA); human αB-crystallin (NLC-ABCrys, 1:50, Novocastra, Newcastle, UK); human myotilin (NLC-myotilin, 1:50, Novocastra) were used. A mixture of rabbit and mouse secondary antibodies was incubated according to the standard Ventana Nexes System procedure and colored with peroxydase reaction 3′3′-diaminobenzidine. A set of control slides was prepared without primary antibodies. For patients F83-4, F321-3, and F1292-1, no frozen muscle was available for immunohistochemical studies.

Immunofluorescence analysis was performed for FHL1, lamin A and C, and emerin on longitudinal and transversal sections of

patients F997-8 and F1328-4 and one healthy control. After fixation with 2% para-formaldehyde, blocked with 4% BSA in PBS, the muscle sections were stained with rabbit polyclonal anti-FHL1 (1:100, ARP34378_T100; Aviva Systems Biology, San Diego, CA, USA) directed against part of the LIM4 domain of FHL1A, mouse monoclonal anti-lamin A and C, or anti-emerin (1:100, 4A7 and MANEM5, respectively, both kindly provided by Glen Morris, NEWI, UK). Incubation of the primary antibody was performed overnight at 4°C. After 15 min of rinsing in PBS, the sections were incubated with Alexa-fluor 488 goat anti-rabbit or goat anti-mouse IgG (1:500, Invitrogen).

Whole-Genome Scan and Linkage Analysis

Genome-wide analysis was performed on six informative families with the use of 250K SNP *NspI* chips from Affymetrix (Santa Clara, CA, USA). So that a minimal genotyping error rate was ensured, genotype calls were first determined on a collection of more than 300 individuals, with both the BRLMM and Birdseed algorithms implemented in the apt-probeset-genotype procedure (Affymetrix power tools V 1.8.5). For the Birdseed algorithm, the 250K_NSP definition files, available on the Broad Institute website, were used. Genotypes with confidence scores higher than 0.30 for BRLMM or 0.05 for Birdseed or discordance between the two algorithms were flagged as no call. Markers having a minimum allele frequency greater than 0.2 and a call rate superior to 0.98 were selected. These steps retained 63,776 SNPs on autosomes and 1680 on chromosome X. Genome-wide linkage analysis was performed with MERLIN v1.1.2[26] and its specific version for chromosome X (MINX), assuming a recessive mode of inheritance and a complete penetrance for homozygous subjects or hemizygous males on the X chromosome. Allelic frequencies were considered equal in the population, and the position map was estimated by the marker physical position expressed in megabases. Multipoint LOD scores were then computed at each marker position with Merlin. For accommodation of marker-marker linkage disequilibrium[27] due to the SNP map density, analyses were also performed on clusters of consecutive markers for which r^2 was greater than 0.40 (option–rsq 0.40 of Merlin).

Genomic DNA Amplification and Sequencing of *FHL1*

Coding exons and intronic flanking regions of *FHL1* were amplified by touch-down PCR (Taq polymerase GOLD, Applied Biosystems, Courtaboeuf, France) with the use of 80 ng of genomic DNA (primer sequences detailed in Table S2). PCR fragments were purified with the use of multiscreen plates on the Manifold system (Millipore, Saint Quentin en Yvelines, France) and sequenced with the Big Dye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems). Collected sequence data were analyzed with SeqScape v.2.5 (Applied Biosystems) and Sequencher v.4.7. The identified mutations were checked on DNA of 200 healthy unrelated control subjects.

Cell Culture, Differentiation Study, and Immunofluorescence

Primary myoblast cultures were obtained from fresh muscle biopsies from patients F321-3 and F997-5 and from three healthy age-matched male control subjects (Table S1). Primary myoblasts were expanded in proliferation medium containing 80% MYO1 medium (Hyclone, Thermo Scientific, Berbières, France), 20% FBS (GIBCO, Invitrogen, Cergy Pontoise, France), 50 μg/ml gentamycin (Invitrogen, Cergy Pontoise, France), 10 ng/ml human recombinant bFGF (R&D Systems, Lille, France), 10^{-6} M dexamethasone (Merck, Fontenay sous Bois, France) at 37°C, and 5% CO_2. All myoblast cultures from patients and from control subjects were enriched in myoblasts by magnetic cell sorting with the use of anti-CD56 to reach at least 95% of myoblasts. Myoblast differentiation was induced by switching confluent myoblasts to differentiation medium (DMEM containing 4.5g/L glucose, pyruvate, antibiotics without FBS) on 0.5% gelatin-coated dishes. Cells were collected at different times after the addition of differentiation medium. Dried pellets were snap-frozen in liquid nitrogen and stored at −80°C before protein extraction, and differentiated myotubes (D4 and D8 in differentiating media) grown on 0.5% gelatine-coated glass coverslips were fixed for 10 min in 4% PFA before being processed for immunostainings.

PFA-fixed cells were blocked in 5% BSA diluted in PBS and immunostained with the use of subsequent primary antibodies: MF20 (from DSHB, Iowa City, IA, USA), anti-α actinin (Sigma-Aldrich, Saint-Quentin, France), and anti-titin (Novocastra, Newcastle, UK) mouse monoclonal antibodies; anti-FHL1 (ARP34378_T100; AVIVA Systems Biology, San Diego, CA, USA), anti-lamin A and C (Santa Cruz, CA, USA), anti-emerin (generously provided by Glenn Morris, NEWI, UK), and rabbit polyclonal antibodies, followed by secondary Alexa568-conjugated anti-mouse IgG or Alexa488-conjugated anti-rabbit IgG (Invitrogen, Cergy Pontoise, France). Images were collected with a Carl Zeiss Axiophot1 microscope. These different immunostainings were used for fusion index calculation. Fusion index is defined as the number of nuclei in myotubes to total nuclei ratio and was calculated after 4 and 8 days in DM in three independent experiments per individual and per time points.

Protein Analysis

Proteins were extracted either from skeletal muscle fragments (one healthy male control and five male patients: F8-6, F321-3, F1292-1, F1328-4, and F1328-17) or from pelleted myoblasts (patient F997-5). Muscle samples were homogenized with FastPrep in total protein extraction buffer (50 mM Tris-HCl, pH 7.5, 2% SDS, 250 mM sucrose, 75 mM urea, 1 mM DTT) and with protease inhibitor (25 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM 4-[2-aminoethyl]-benzene sulfonylfluoride hydrochloride and 2mM Na_3VO_4). Pelleted cells were extracted with the same buffer. Total protein extracts (50 μg for analysis of FHL1 expression or 20 μg for differentiation kinetic study) were separated on 15% and 10% SDS-PAGE for FHL1 expression and differentiation studies, respectively. Membranes were blocked in 5% skim milk in TBS-tween20 and hybridized with anti-FHL1 (ab58067, directed against the N-terminal domain of FHL1A, FHL1B, and FHL1C), anti-myogenin (ab1835) (both purchased from AbCam, Cambridge, UK), anti-α actinin, or anti-vinculin (both from Sigma-Aldrich, Saint-Quentin, France) mouse monoclonal antibodies and with secondary rabbit anti-mouse IgG HRP-conjugated antibody (Dako, Trappes, France). Immunoblots were visualized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Saint Quentin en Yvelines, France) on a G-Box system with GeneSnap software (Ozyme, Montigny le Bretonneux, France).

Statistical Analysis

One-way analysis of variance was performed on fusion index data with SigmaStat 3.5 software. Statistical significance was assumed at $p < 0.05$.

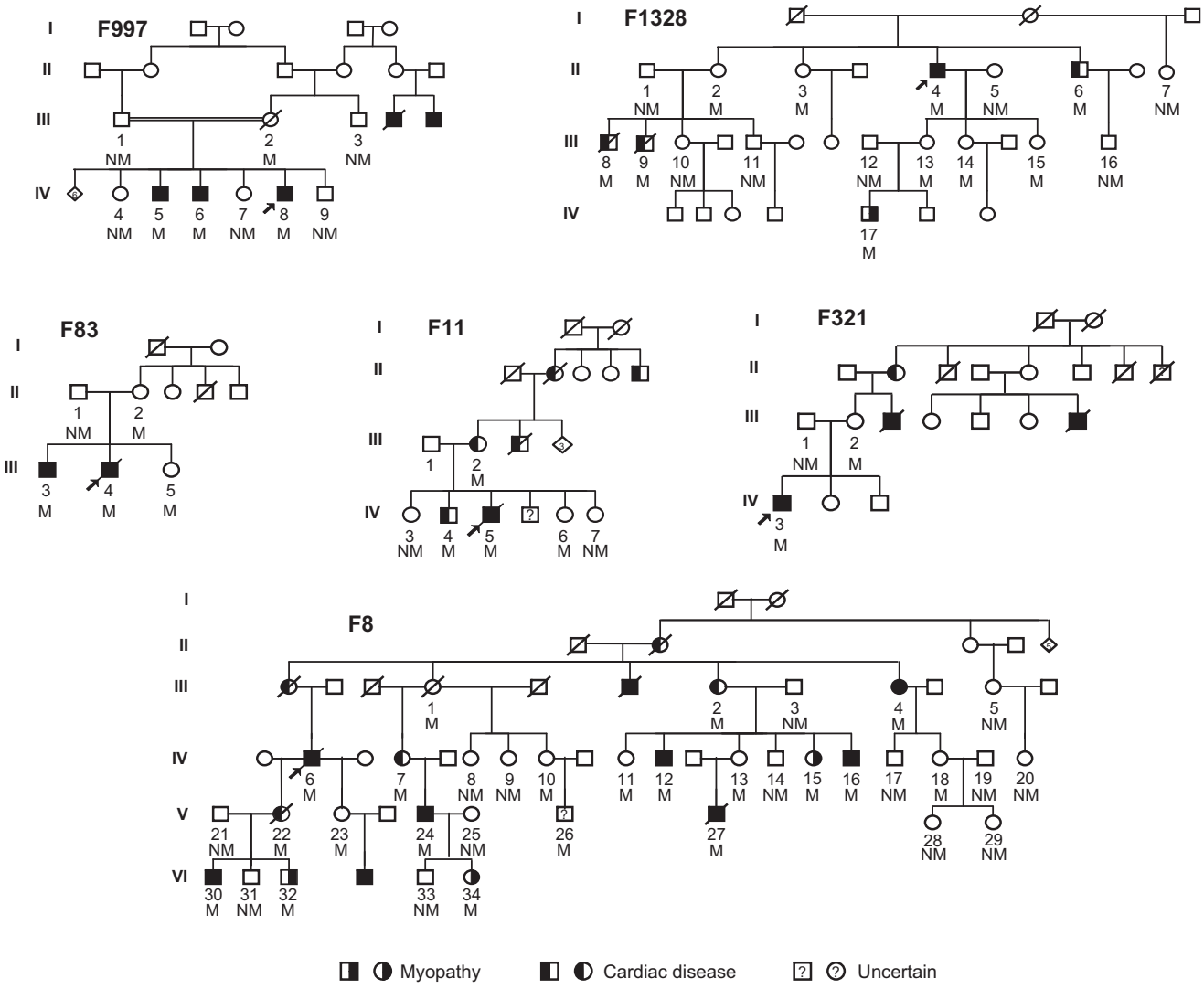

Figure 1. Pedigrees of the X-Linked EDMD Families
Males are depicted by squares, females by circles. Subjects presenting muscle involvement are indicated by right-half-filled symbols. Subjects presenting cardiac disease are indicated by left-half-filled symbols. Question marks indicate uncertain phenotype status. Arrows indicate the family index case. M: mutated; NM: nonmutated. Families F83, F997, and F1328 had whole-genome scan analysis. Family F997 originates from Saudi Arabia and F321 from Japan, whereas others are of European descent.

Results

Identification of the EDMD Cohort

Among the 2200 patients referred to our institutions for *EMD* and *LMNA* gene analysis, a subgroup of 588 index cases corresponding to the inclusion criteria were identified as harboring an EDMD phenotype. *LMNA* or *EMD* genes were found to be mutated in 156 cases (26.5%) and 50 cases (8.5%), respectively. The remaining 382 patients (65%; 280 males and 102 females) were considered as EDMD cases orphan of molecular diagnosis. For these latter patients, a definite family history was found in 90 patients (23.5%), whereas the remaining patients were considered to be isolated cases (170; 44.5%) or to have an uncertain family history (122; 32%).

Whole-Genome Scan Analysis

For six informative families comprising a total of 16 affected and 33 nonaffected members, we performed whole-genome scan analysis. Three of the families (F83, F997 and F1328) were compatible with an X-linked transmission (Figure 1 and Figure S1). Linkage analysis of the consanguineous family F997 with three affected brothers excluded an autosomal-recessive transmission and detected a strong linkage to locus Xq26.3. As for the F83 and F1328 families, linkage analysis detected the same locus but with a smaller size, spanning 16 megabases between SNP rs4281231 and SNP rs4824862 (Figure 2). The cumulative LOD score on the three families was 3.010 (Figure 2). For the three remaining families, the linkage analysis pointed toward other potential loci that are under analysis.

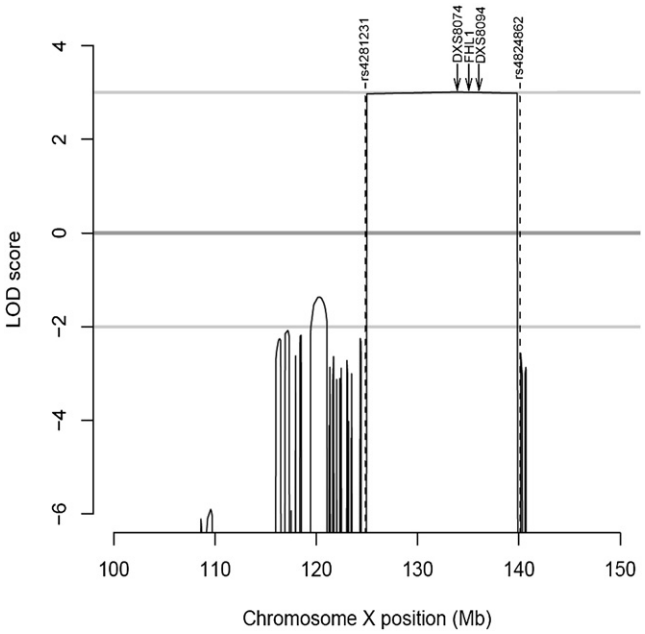

Figure 2. LOD Score Profile of the Multipoint Linkage Performed on the X Chromosome
Markers rs4281231 (position 124.82 Mb) and rs4824862 (position 140.10 Mb) encompass a 16 Mb region including 151 of the 1680 SNPs selected on the X chromosome and three microsatellites (DXS8074 at position 133.91 Mb, intra *FHL1* at position 135 Mb, DXS8094 at position 136.064 Mb). The overall LOD score for the three families (F83, F997, F1328) reaches a maximum of 3.010 near the *FHL1* position. The analysis with cluster option led to the same profile and maximum LOD score.

Table 1. Genomic and Protein Nomenclatures of *FHL1* Mutations According to the Affected Isoforms

***FHL1* Isoform (A, B, C)**	***FHL1* Mutation**	**Protein Nomenclature**
Family F8		
A	c.625T>C	p.Cys209Arg
B	c.625T>C	p.Cys209Arg
C	-	-
Family F11		
A	c.817dup	p.Cys273LeufsX11
B	*c.973+41dup (3′UTR)*	-
C	*c.585+41dup (3′UTR)*	-
Family F83		
A	c.332_688del	p.Gly111_Thr229delinsGly
B	c.332_688del	p.Gly111_Thr229delinsGly
C	c.332_501del	p.Asp112PhefsX51
Family F321		
A	c.469_470delAA	p.Lys157ValfsX36
B	c.469_470delAA	p.Lys157ValfsX36
C	c.469_470delAA	p.Lys157ValfsX62
Family F997		
A	c.841T>G	p.X281Glu
B	*c.973+67T >G (3′ UTR)*	-
C	*c.585+67T>G (3′ UTR)*	-
Family F1292		
A	c.371_372delAA	p.Lys124ArgfsX6
B	c.371_372delAA	p.Lys124ArgfsX6
C	c.371_372delAA	p.Lys124ArgfsX6
Family F1328		
A	c.827G>A	p.Cys276Tyr
B	*c.973+53 (3′ UTR)*	-
C	*c.585+53 (3′ UTR)*	-

Mutations indicated in italics affect the 3′ untranslated region of the *FHL1* gene.

Mutation Screening of *FHL1*

Among the 100 genes with an HGNC name located in the locus identified on chromosome X, the *FHL1* gene was a good candidate because it was highly expressed in skeletal and cardiac muscles and has been implicated in other muscle diseases.[20–22] We therefore directly sequenced the coding regions and flanking splice sites of this gene. In family F997, we identified a T>G transversion on the last codon of the gene in exon 8 (c.841T>G [p.X281E], Table 1) in the DNA of the three affected brothers (Figure 3). This transversion suppresses the FHL1A termination codon, predicting a larger protein with 52 additional amino acids at the C-terminal end of the FHL1A isoform (Figure 4). In family F1328, we detected a missense mutation (c.827G>A [p.C276Y], Figure 3 and Table 1) in exon 8, affecting a highly conserved cysteine of the LIM4 domain present only in the FHL1A isoform. Amplification of exons 5 and 6 on the DNA of affected members of family F83 was impossible. After long-range PCR from exons 4 to 8, we detected a smaller PCR product in the DNA of the two affected brothers that was also present at a heterozygous state in the mother's and sister's DNA, in comparison to control DNA. Sequencing of this shorter PCR fragment revealed a genomic deletion of 1440 bp, encompassing exons 5 and 6, deleting in frame 357 bp of the coding sequence of FHL1A and FHL1B isoforms (c.332_688del [p.G111_T229delinsG]), and deleting out of frame 170 bp of the FHL1C coding sequence (c.332_501del [p.D112FfsX51]; Figure 3 and Table 1). These deletions lead to shorter FHL1A, FHL1B, and FHL1C proteins lacking part of the LIM2 domain and the entire LIM3 domain, together with part of the LIM4 domain for FHL1A. Mutated FHL1A and FHL1C are identical: in FHL1C, the deletion is thought to introduce a frameshift within exon 5, but the junction with exon 8 reintroduces a frame that potentially leads to a pseudo LIM4 domain, like that which occurs in FHL1A (Figures 3 and 4). FHL1B still contains its binding domain to RBP-J, whereas FHL1C loses it. The identification of *FHL1*

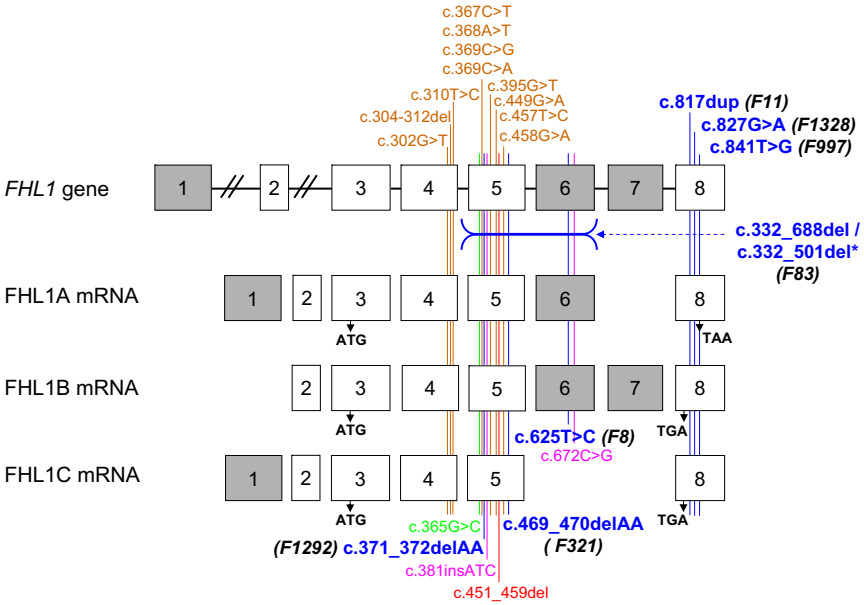

Figure 3. Distribution of the EDMD-Related Mutations along the *FHL1* Gene and Corresponding RNA Isoforms

The *FHL1* gene is composed of eight exons. Exons 1 and 2 are noncoding, whereas exons 3 to 8 are alternatively spliced and give rise to three major transcribed isoforms: FHL1A, FHL1B, and FHL1C. Grey squares represent alternatively spliced exons. Translational start (ATG) and stop (TAA or TGA) codons are indicated for the three FHL1 isoforms. The seven *FHL1* mutations identified in EDMD families are depicted in blue (with corresponding family number in bracket); they affected exons 5, 6, and 8 and are missense or ins and/or del truncating mutations. Mutations related to X-SM[21] (depicted in green), X-MPMA[20] (depicted in pink), RBM[22] (depicted in brown), and RSS[36] (depicted in red) are all missense mutations and essentially affect exons 4 or 5, except one that affects exon 6.

Asterisk indicates the following information: c.332_688del affects FLH1A and FLH1B isoforms, and c.332_501del affects the FHL1C isoform.

mutations in these three families prompted us to directly analyze this gene in additional EDMD patients for whom X-linked inheritance seemed to be the most likely mode of transmission (18 unrelated index cases) or in isolated cases (49 males, nine females). We subsequently found four additional mutations in three families (F8, F11, and F321; Figure 1) and in one isolated male (F1292-1). Family F8 displayed a missense mutation in exon 6 (c.625 T>C [p.C209R], Figure 3 and Table 1), affecting an important cysteine of the LIM3 domain of FHL1A and FHL1B isoforms (Figure 4). We identified in family F11 an insertion of 1 bp in exon 8 (c.817dup [p.C273LfsX11]; Figure 3 and Table 1) introducing a frameshift affecting only the FHL1A isoform that lacks the last two cysteines of the LIM4 domain. Family F321 presented a deletion of 2 bp in exon 5 (c.469_470delAA; Figure 3 and Table 1), predicting a truncated protein with 35 out-of-frame new amino acids in the C terminus of the FHL1A and FHL1B isoforms (p.K157VfsX36) and 61 out-of-frame new amino acids at the C terminus of FHL1C (p.K157VfsX62; Figure 4). Finally, we found a deletion of 2 bp in exon 5 (c.371_372delAA [p.K124RfsX6]; Figure 3 and Table 1) in the isolated patient F1292-1, predicting a truncated protein with five out-of-frame new amino acids in the LIM2 domain of the three FHL1 isoforms. All of these mutations were absent from 200 control DNAs and perfectly cosegregated with the disease (Figure 1).

Clinical Presentation of Patients with *FHL1* Mutations

Clinical evaluation of the seven index cases with mutations, performed by the referring clinicians (A.V., B.E., C.T., M.A.S., M.H., S.Sa., S.Sp., T.S., T.V.), is summarized in Table 2 and Table 3. More detailed clinical features found in families with *FHL1* mutations will be reported elsewhere. Of note, family F8's index case has been previously reported as patient 4 by Voit et al.[28] In all index cases, skeletal muscle symptoms preceded cardiac involvement. The age of onset ranged from 4 to 48 years (14.7 ± 15), with first skeletal muscle symptoms being joint contractures (four cases), muscle weakness (one case) or both (two cases). The disease course was progressive in all cases. All patients were still ambulant after a disease duration ranging from 6 to 30 years (17.7 ± 8.5). Among the seven index cases, the last neurological evaluation, performed at 18 to 54 years (32.4 ± 12.1), revealed lower-limb muscle weakness and/or wasting of pelvic, peroneal, or pelvi-peroneal distribution in all cases and upper-limb involvement in six of the cases, usually in a scapular topography. Additional axial muscle weakness and/or atrophy were observed in four patients, and facial involvement was noticed in two patients. Neck stiffness and rigid spine were observed in six and five patients, respectively. Limb-joint contractures were commonly observed: in ankles (seven patients), requiring surgery in one of the patients; elbows (five patients); knees (three patients); hips (two patients); wrists (one patient); fingers (one patient); and shoulders (one patient). Scoliosis was present in three patients and required arthrodesis in one. In addition, unusual symptoms were observed: swallowing difficulties (one patient), dysphonia characterised by husky voice (three patients), muscle hypertrophy (two patients), and ptosis (one patient). CK levels were either normal or moderately elevated, up to 6-fold times normal. Concerning the three patients showing dysphonic voice, additional laryngeal investigation revealed abduction limitation of either the two vocal cords (two patients) or one of the vocal cords (one patient), with normal electromyographic activities observed. These abnormalities suggested vocal cord palsy.

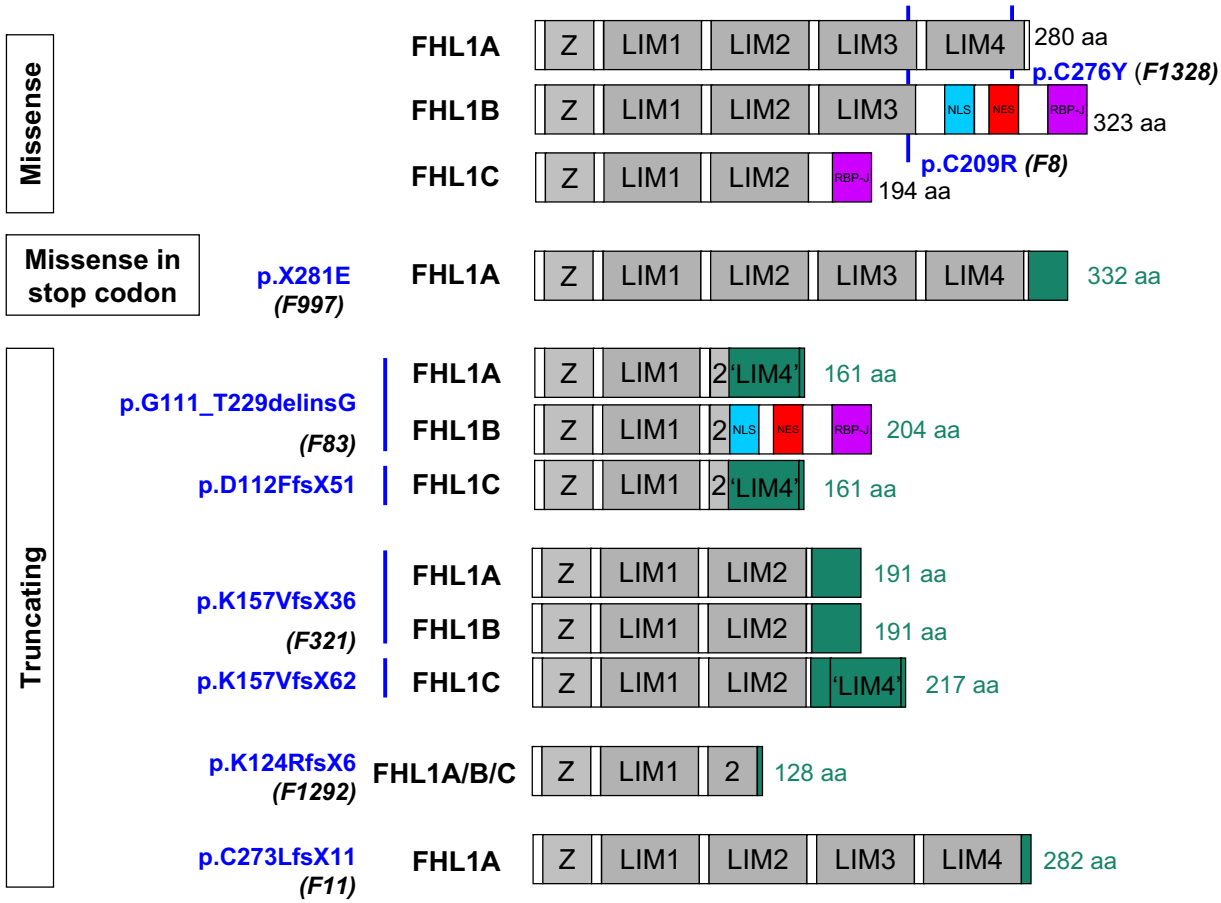

Figure 4. Consequences of the Different EDMD Mutations on the Three FHL1 Isoforms
The three FHL1 protein isoforms are represented at the top of the picture, with indication of the position of the two EDMD missense mutations. Below are presented the putative consequences at protein level of the missense mutation suppressing the FHL1A stop codon and the four truncating mutations. Only FHL1 isoforms affected by the mutations are depicted.

All index cases developed cardiac disease of variable extent at an age ranging from 10 to 48 years (28.2 ± 12) (Table 3). Cardiac symptoms of onset were supraventricular or ventricular arrhythmias (four patients), symptoms of cardiac chamber hypertrophy (three patients), and conduction defects (one patient). Last cardiac evaluation (18 to 54 years, 32.5 ± 12) revealed supraventricular or ventricular arrhythmias in three patients, and conduction defects were present in one patient. However, the most striking finding was the presence of significant cardiac hypertrophy in four patients, biatrial dilation in one patient, and both in one patient. Left ventricular function was decreased in the two oldest patients and normal in the five others. When respiratory evaluation was performed, two patients showed severe impairment requiring noninvasive ventilation. Finally, sudden death occurred in two patients, at 31 and 51 years (one with severe hypertrophic cardiomyopathy and the other with normal documented LV function), and another died at 37 years from respiratory insufficiency. None of the seven index cases had cardiac transplantation.

Among the 68 available relatives, 38 (15 males, 23 females) were found to carry the *FHL1* mutations identified in the seven index cases (Figure 1). Among these male relatives, ten showed EDMD; four had isolated cardiac disease, two presenting overt hypertrophic cardiomyopathy; and one had unknown clinical status. Among them, one died suddenly at 19 years (patient F1328-9) and two died in other circumstances, at 23 and 27 years (patients F1328-8 and F8-27). Regarding female carriers, 16 were asymptomatic (age 26 to 81 years), and four had isolated cardiac disease (age 36 and 74 years), two of them concomitantly suffering from high blood pressure (patients F8-2 and F8-22). The remaining three had either mild muscle involvement, mainly spine rigidity (at 10 and 47 years [patients F8-34 and F8-15]), or onset of motor difficulties with hearth rhythm disturbances at 69 years (patient F8-4).

Histological Study

Prior to this study, muscle biopsies were performed for diagnostic purpose in the seven index cases (Table 2). They showed either unspecific myopathic features (patients F11-5, F83-4, and F321-3) or a dystrophic pattern (patients F8-6, F997-8, F1292-1, and F1328-4). Histological reevaluation in three patients (F11-5, F997-8, and F1328-4) showed fiber size variations and increase of interstitial

Table 2. Neurological Evaluation of EDMD Family Index Cases

		Last Neurological Evaluation											
			Weakness/Wasting		Joint Contractures								
Family Index Case	Onset (Age [Yrs] / Symptoms)	Age [yrs]	Upper and Lower Limbs	Axial/ Facial	RS	SN	Elb.	Ank.	Others	Scoliosis	Other Symptoms	CK[a]	Muscle Biopsy (Age [Yrs] / Muscle / Pattern)
F8-6[b]	11 / stiff neck, unable to roll over	41	UL (scapulo-humeral), LL (pelvic, distal)		Y	Y	Y	Y	small finger joints, knees, hips	Y		x 6	41 / quadriceps / dystrophic
F11-5	6 / elbows and Achilles tendons contractures	30	UL (scapular), LL (pelvic)	Axial	Y	Y	Y	Y	knees		muscle hypertrophy (rectus abdominis, trapezius, biceps brachii, vastus lateralis, right forearm muscles), dysphonia due to vocal cord palsy, swallowing difficulties	x 2.3-3.7	1st: 30 / deltoid / non specific, 2nd: 31 / quadriceps / non specific
F83-4	14 / stiff neck	35	UL (scapular, scapula alata), LL (pelvic, peroneal),	Axial	Y	Y	Y	Y		Y (scoliosis, arthrodesisat 19 yo)		x 4.5	16 / hip muscle / non specific
F321-3	4 / abnormal gait, ankle joint contracture	23	UL (scapular, humeral), LL (pelvic, peroneal)	Axial / Facial	Y	Y		Y	hips		ptosis	x 2	14 / biceps brachii / non specific
F997-8	10 / stiff neck	18	UL (scapular), LL (peroneal)		Y	Y	Y	Y	wrists, knees	Y (scoliosis)	dysphonia due to vocal cord palsy	N	12 / vastus lateralis / dystrophic
F1292-1	10 / tiptoe walking	26	UL (humeral), LL (peroneal)	Facial			Y	Y			dysphonia due to vocal cord palsy	x 2.3	23 / quadriceps / dystrophic
F1328-4	48 / difficulties in climbing stairs and get up from squatting position	54	LL (pelvic)	Axial		Y		Y	shoulders		muscle hypertrophy (paravertebral and shoulders)	x 2.5	1st: 52 / quadriceps / dystrophic, rimmed vacuoles, 2nd: 58 / deltoid / myopathic, mitochondrial infiltration and mononuclear cell infiltrate

Abbreviations are as follows: Ank, ankle; CK, creatine kinase; Elb, elbow; LL, lower limb; N, normal; RS, rigid spine; SN, stiff neck; UL, uppel limb; Y, yes; Yrs, years.

[a] x-fold normal level.

[b] Previously reported (patient 4, Voit et al.[28]).

Table 3. Cardiological and Respiratory Features of EDMD Family Index Cases

	Cardiac Disease							Respiratory Involvement		
		Last Cardiac Assessment								
Family Index Case	Onset (Age [Yrs] / Symptoms)	Age	Conduction Defects	Arrhythmias	Chamber Hypertrophy	Septum Thickness (mm)	LV Dysfunction	FVC at Last Evaluation / Age (Yrs)	Mechanical Ventilation / Age (Yrs)	Circumstance of Death / Age (Yrs)
F8-6	39 / AFl, nocturnal bradycardia, SVES, VES,	42	N	AFb, VES and SVES	Y (LV, RV, septal)	16[a]	Y	UK	N	Sudden death / 51
F11-5	30 / Asthenia, septal hypertrophy	30	N	N	Y (septal)	23	N	40% / 30	NIV / 31	Sudden death / 31
F83-4	27 / complete RBBB, WPW syndrome	35	N	N	N	Normal	N	25% / 31	NIV / 31	Respiratory insufficiency / 37
F321-3	10 / Electric heart hypertrophy	23	N	N	Y (apex)	11.2	N	UK	N	
F997-8	18 / Septal hypertrophy	18	N	N	Y (septal)	20.7	N	UK	N	
F1292-1	26 / AFb, ischemic stroke	26	RBBB	AFb	N (atrial dilation)	Normal	N	69.4% / 26	N	
F1328-4	48 / AFl	54	N	AFb	Y (septal, LV) with atrial dilation	13	Y	UK	N	

Abbreviations are as follows: AFb, atrial fibrillation; AFl, Atrial flutter; FVC, forced vital capacity; LV, left ventricle; N, no; NIV, noninvasive ventilation; RBBB, right bundle branch block; RV, right ventricle; SVES, supraventricular extra systole; UK, unknown; VES: ventricular extra systole; WPW, Wolf-Parkinson-White; Y, yes.
[a] Postmortem analysis: severe left and right ventricular hypertrophic cardiomyopathy.

tissue. Patient F11-5 showed myopathic changes associated with atrophic, angulated fibers. Patient F1328-4 exhibited dissimilarities between two biopsies obtained from different muscles: the quadriceps muscle appeared dystrophic, with large variability in fiber size, internalisation of nuclei, rimmed vacuoles, and significant increase of interstitial tissue, whereas the deltoid muscle showed only minimal unspecific changes, with mitochondrial accumulation in subsarcolemmal position (Table 2 and Figure 5). Menadione-NBT staining showed no reducing bodies (RB) in the three reevaluated patients (Figure 5). Results of immunostaining against myofibrillar proteins αB-crystallin, desmin, and myotilin were normal without the presence of aggregates (data not shown). FHL1 immunofluorescence studies were available for patients F1328-4 (not shown) and F997-8 (Figure 6A). On longitudinal and transversal muscle sections, the immunoreactivity for FHL1 was strongly reduced and the remaining FHL1 protein observed was mainly localized in and/or close to the nucleus. Immunofluorescence analysis of lamin A and C and emerin on muscle sections of patients F1328-4 and F997-8 showed no difference in comparison to that of a healthy control subject (Figure S2).

Protein Expression Study

When available, we used patient muscle biopsies or cultured myoblasts to check the consequences of the different *FHL1* mutations in terms of expression level and presence of truncated proteins. Immunoblot analysis using an antibody detecting an N-terminal epitope common to the three major isoforms showed a strong expression of FHL1A in control muscle biopsy (Figure 6B, upper panel), whereas FHL1B and FHL1C were much less expressed and detected only when membrane was overexposed for 5 min (Figure 6B, middle panel). Protein analysis of patient biopsies or cultured myoblasts clearly showed a moderate (patient F8-6; Figure 6B, upper panel) to strong (patients F321-3, F997-5, F1292-1, F1328-4, and F1328-17) decrease in FHL1 expression level (Figure 6B, middle panel). Truncated or extended mutated FHL1 proteins were observed at expected molecular weight (37.5 kDa and 14.5 kDa for patients F997-5 and F1292-1, respectively). Surprisingly, the band detected for patient F321-3 did not correspond to the truncated FHL1A/B isoform (with expected molecular weight being 13.5 kDa) but, rather, corresponded to the truncated FHL1C (expected molecular weight of 24.5 kDa instead of 22 kDa for full-length FHL1C).

Differentiation and Immunofluorescence Analysis of Primary Myoblasts

To analyze the functional consequences of *FHL1* mutations, we induced primary myoblasts from patients F321-3 and F997-5 to differentiate in culture by switching confluent myoblasts to DM and analyzed myogenin and

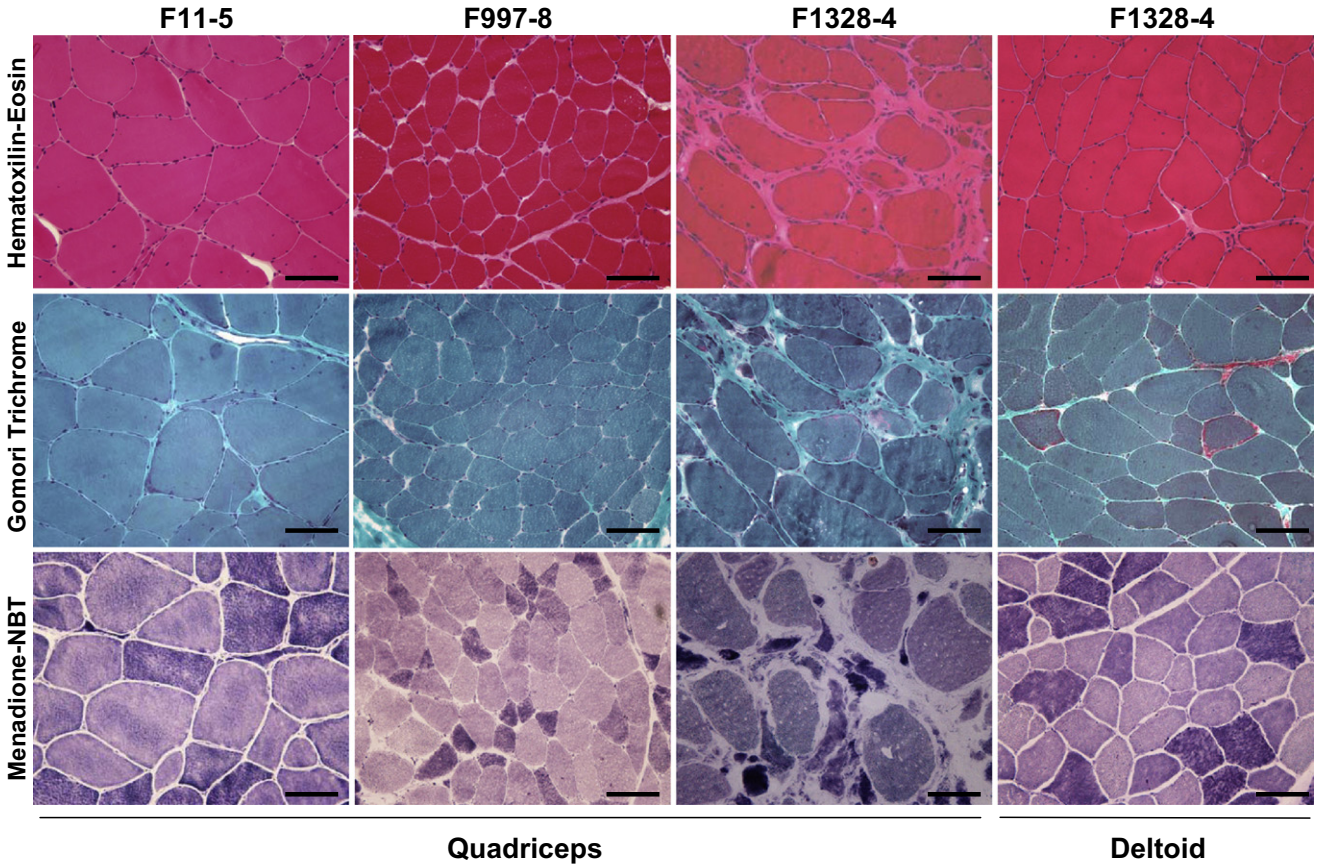

Figure 5. Muscle Histology of Patients
Haematoxylin and eosin (HE), Gomori trichrome (GT), and Menadione-nitroblue tetrazolium (Menadione-NBT) stainings on quadriceps biopsies of patients F11-5, F997-8, and F1328-4 and an additional biopsy of deltoid muscle of patient F1328-4. With Menadione-NBT stain, type II muscle fibers appear as dark purple fibers. Scale bar represents 100 μm.

α-actinin expression at different time points (Figure 7A). The expression of the differentiation factor myogenin, absent in myoblasts, was induced after 24 hr in DM in control cells (Figure 7A). In F321-3 cells, induction of myogenin was low after 48 hr in DM and reached normal expression levels only after 72 hr in DM. In F997-5 cells, we were able to detect a very faint myogenin signal only after 96 hr in DM. Myogenin is a transcription factor, which switches on the myogenic program, resulting in the expression of muscle-specific proteins as well as sarcomeric proteins, such as α-actinin. In control myoblasts and during the first hours in DM, α-actinin expression was very low, but increased by 48 hr in DM (i.e., 24 hr after myogenin expression). In F321-3 cells, the increase in α-actinin expression also appeared 24 hr after myogenin expression had started. The increase in α-actinin expression was not detected in F997-5 cells even after 96 hr in DM. However, at that time, myogenin expression was only faintly expressed in F997-5 cells. Myoblasts fusion takes place after myogenin induction. Considering the delay in myogenin expression, we evaluated myoblast fusion by measuring fusion index after 4 and 8 days in DM (Figure 7B). After 4 days in DM, control cells had 24% (± 3%) to 66% (± 13%) of nuclei belonging to myotubes, whereas F321-3 cells had only 3% (± 3%) of nuclei in myotubes and F997-5 cells have none. After 8 days in DM, fusion index increased in all cultures. Control cells had reached 59% (± 7%) to 73% (± 6%) of nuclei in myotubes, whereas F321-3 cells had reached 43% (± 8%) and fusion of F997-5 cells was still severely delayed, at only 4% (± 1%).

Finally, sarcomeric proteins have to assemble to form the contractile apparatus. Some studies have implicated FHL1 in sarcomere assembly.[19] Hence, we checked the putative impact of *FHL1* mutations on sarcomere formation in control and patient myotubes after 8 days in DM. We studied both thin and thick line formation by staining for α-actinin and titin, respectively (Figure 7C). Despite fewer myotubes in F321-3 and F997-5 cultures, we were able to find normally organized α-actinin and titin proteins. Moreover, we used emerin and lamin A and C immunostaining to look at their localization in *FHL1*-mutated myotubes. No difference was observed in comparison to control myotubes. Therefore, the two *FHL1* mutations identified in families F321 and F997 induced a delay in myogenin activation and, hence, in myoblast fusion, with a more severe impact for the mutation found in family F997. None of the analyzed mutations seemed to

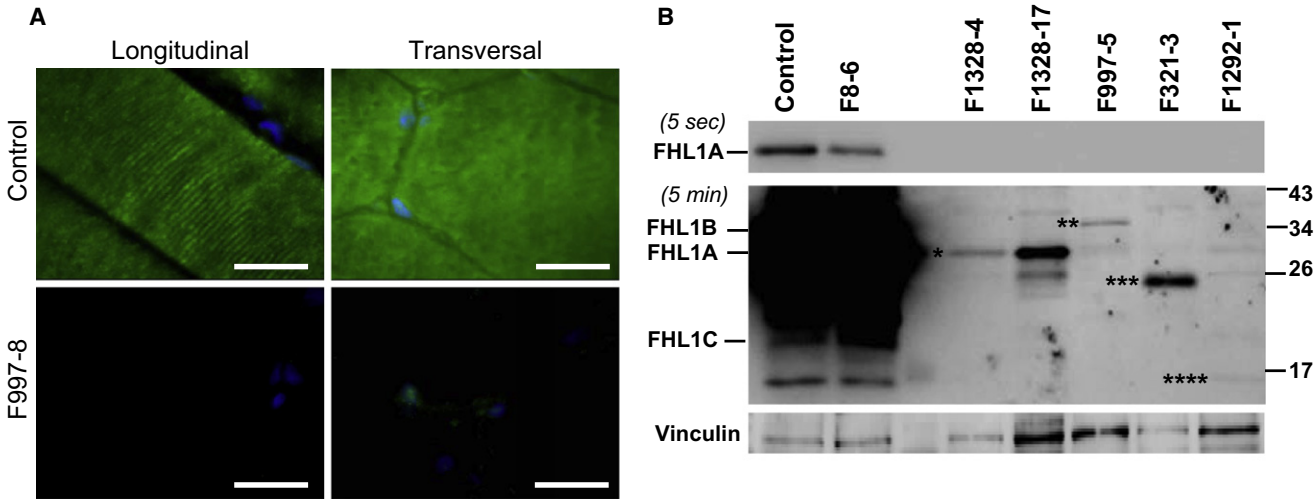

Figure 6. Immunostaining and Immunoblotting for FHL1 Showed a Strong Reduction in FHL1 Protein Expression
(A) Fluorescent immunostaining against FHL1 (green), with the use of the AVIVA antibody directed against the C-terminal domain of FHL1A on longitudinal and transversal muscle sections from patient F997-5 and one healthy control.
(B) Immunoblotting of 50 μg total protein extract from patient biopsies (F8-6, F1328-4, F1328-17, F321-3, and F1292-1) and from cultured primary myoblasts (F997-5), with the use of the FHL1 antibody from Abcam that detected the N-terminal domain common to the three FHL1 isoforms. The membrane was overexposed for 5 min to allow for the detection of wild-type FHL1B and FHL1C isoforms and most of the mutated proteins. Asterisks are used as follows: *, expected size of full-length FHL1A protein: 31.5 kDa; **, expected size of F997 FHL1A protein: 37.5 kDa; ***, expected size of F321 FHL1C protein: 24.5 kDa; ****, expected size of F1292-1 FHL1A, FHL1B, and FHL1C protein: 14.5 kDa. Vinculin was used as a loading control.

have an impact on sarcomere formation or emerin and lamin A/C localization in myotubes.

Discussion

Since the first report of EDMD[29] and its recognition as a separate clinical and genetic entity,[30] the implication of *EMD* and *LMNA* genes[5,6] has allowed precise molecular genetic EDMD diagnosis but does not account for all EDMD cases.[10,11,31] For the identification of other causative genes, a large cohort of *EMD*- and *LMNA*-nonmutated EDMD patients has been selected, and a whole-genome scan allowed us to identify linkage to chromosome Xq26.3 in three families. We report here, for the first time to our knowledge, seven *FHL1* gene mutations associated with EDMD.

FHL1 Mutations Cause X-Linked EDMD

The seven index cases displayed the typical triple muscle-joint-heart involvement of EDMD. Early joint contractures occurred and involved the neck, spine, elbow, and Achilles tendons, concomitantly with a progressive muscle involvement of the four limbs, with a predominant scapulo-humeral and pelvic and/or peroneal topography. Secondarily, cardiac disease, mainly with arrhythmias and cardiac hypertrophy, appeared and was highly life threatening, being responsible for the sudden death of at least two affected males, even despite normal cardiac function.

The EDMD patients reported here are different from other *FHL1*-related disease patients[20–22] and display several specific features. First, histological studies in our patients showed dystrophic or mostly nonspecific myopathic patterns but no RB. Therefore, our patients are different from RBM patients[22] even if a recent report suggested that a subgroup of RBM patients may show EDMD features, as indicated by an RBM patient with early joint contractures, rigid spine, and dilated cardiomyopathy (patient 8 in [32]). In addition, none of our cases had congenital or early childhood onset, as in RBM.[22]

Second, some of our cases displayed heart (5 of 7) and muscle (2 of 7) hypertrophy, features also reported in X-MPMA.[20] Interestingly, predominant EDMD cardiac features are conduction and/or rhythm defects,[1] which in patients with *EMD* and *LMNA* mutations evolve toward dilated cardiomyopathy.[1,33] Therefore, the presence of cardiac hypertrophy in an EDMD patient should prompt clinicians to screen for *FHL1*. As for muscle hypertrophy, it has been observed in some rare *LMNA*-related cases (G.B., unpublished data), but it is clear that X-MPMA is a relevant differential diagnosis of *FHL1*-related EDMD cases.

Third, three of our index cases had a striking vocal cord palsy revealed by dysphonia without a neuropathic or myopathic pattern at electromyographic examination. Speech abnormalities or vocal cord palsy have been reported in myopathies such as myotilinopathies[34] and distal myopathy with vocal cord and pharyngeal weakness[35] but never in EDMD or in other *FHL1*-related diseases. Its association with the EDMD phenotype should therefore also prompt *FHL1* screening. Further investigations have to be performed in order to elucidate the pathomechanism of vocal cord involvement in these patients with *FHL1* mutations.

Of significant importance is that four males and one female among the mutation-bearing relatives had isolated

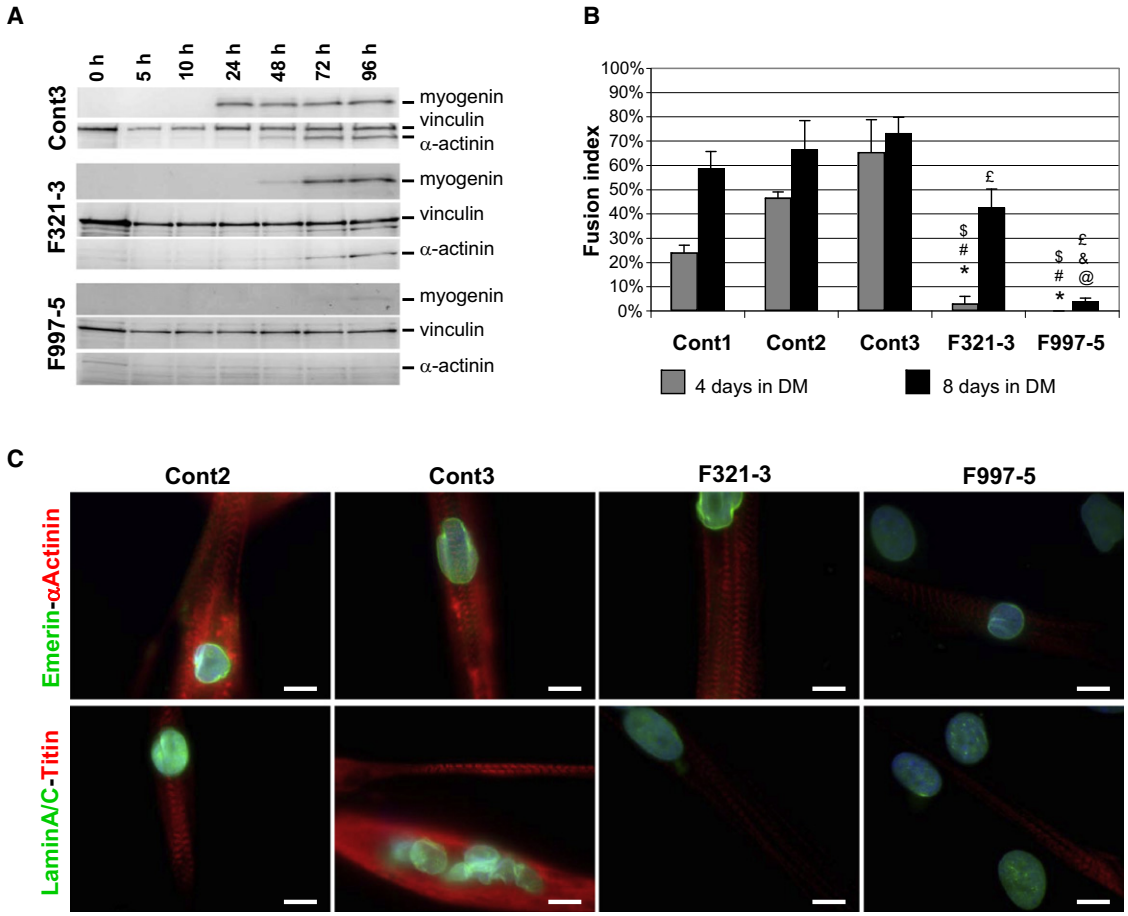

Figure 7. Differentiation Study Showed Major Differentiation Delay without Sarcomere-Formation Defects
(A) Immunoblot from control no. 3, patient F321-3, and patient F997-5 cultured myoblasts induced to differentiate for 0, 5, 10, 24, 48, 72, and 96 hr in differentiation medium (DM). Expression of myogenin was delayed in patients in comparison to healthy controls (only control no. 3 is shown; similar results were obtained with controls no. 1 and no. 2). Expression of α-actinin in patient F321-3 was upregulated 24 hr after myogenin expression had started, as in control cells, whereas for patient F997-5, no upregulation of α-actinin was detected. Vinculin was used as loading control.
(B) Fusion index of F321-3 and F997-5 primary myoblasts compared to three healthy controls after 4 and 8 days in DM. $p < 0.05$. Symbols are used as follows: * and @, patients versus cont1 at J4 and J8, respectively; # and &, patients versus cont2 at J4 and J8, respectively; $ and £, patients versus cont3 at J4 and J8, respectively.
(C) Immunostainings of myotubes of two controls, F321-3 and F997-5, after 8 days in DM. The upper part shows representative pictures of emerin (green) and α-actinin (red) stainings, whereas the lower part shows representative pictures of lamin A and C (green) and titin (red) stainings. Nuclei are stained in blue by DAPI. Scale bar represents 20 μm.

hypertrophic cardiomyopathy (families F11 and F1328), suggesting that *FHL1* mutation may lead to isolated cardiac disease. We therefore propose that *FHL1* screening should be considered in patients displaying isolated cardiac disease, notably hypertrophic cardiomyopathy, even in the absence of any family history of skeletal muscle involvement.

Finally, as previously reported for RBM and X-SM,[21,22] we observed a high frequency of symptomatic female carriers in two of our families (F8 and F11), which may suggest a primary dominantly inherited disease. As for other X-linked diseases, this could be explained by a skewed X chromosome inactivation or a dominant-negative function of mutated FHL1 proteins. On the other hand, the presence of consanguinity (family F997) may suggest an autosomal-recessive mode of inheritance and lead to some diagnostic dilemma. Nevertheless, the absence of male-to-male transmission and/or the exclusive male involvement should suggest an X-linked transmission.

Extension of the *FHL1*-Related Disease Spectrum

The identification of *FHL1* mutations in EDMD extends the spectrum of *FHL1*-related diseases, after the description of X-MPMA,[20] X-SM,[21] and RBM.[22] Interestingly, recent reports clarified the *FHL1*-related disease nosology. Indeed, muscle biopsies from patients of the original X-SM family[21] were found to contain menadione-NBT-positive RB, suggesting that this family, in fact, represents a milder form of RBM.[32] Along the same line, a patient with a prominent rigid spine (RSS) showed RB in his muscle biopsy.[36] Consequently, *FHL1*-related disorders may be categorized into two main subgroups. The first is the "RB subgroup,"

which includes RBM, X-SM, and RSS, in which the hallmark is the typical histopathological finding of RB. Besides this remarkable common histological marker, the RB subgroup patients' clinical spectrum seems to include severe conditions with congenital or early childhood onset, delayed motor milestones, and rapid loss of ambulation, as well as a later-onset and less progressive condition that may not lead to loss of ambulation.[32] Only one patient had an overt cardiac disease consistent with dilated cardiomyopathy at 18 years of age.[32] The second subgroup, with the later-onset phenotype, may comprise EDMD and X-MPMA patients.[20] Interestingly, respiratory insufficiency seems to be a frequent feature in both subgroups (present report and [20,32]).

The EDMD-Related *FHL1* Mutations Exhibit a Specific Pattern

Among the seven *FHL1* gene mutations we report here, two were missense mutations affecting highly conserved cysteines of LIM3 and LIM4 domains, one abolished the FHL1A stop codon, and four were truncating mutations not reported in *FHL1* before. Unlike mutations previously described in other phenotypes, the EDMD mutations were preferentially located in the most distal exons of *FHL1* (exons 5 to 8), whereas previously reported mutations preferentially affected exons 4 and 5 of the gene (Figure 3).[20–22,36] In contrast to other *FHL1*-related diseases in which only the LIM2 domain of the three FHL1 isoforms was affected, the EDMD-related *FHL1* mutations impaired the three FHL1 isoforms to various degrees (Figure 4). They altered either only the FHL1A isoform (F11, F997, F1328) or the three isoforms that were differently modified, resulting in various new truncated proteins (F83, F321, F1292) or carrying a missense mutation affecting FHL1A and FHL1B (F8). To date, *FHL1* gene mutations account for at least 1.2% of our EDMD cohort (7/588 EDMD index cases, but the entire cohort has not yet been completely tested) and do not explain all of our isolated males and likely X-linked patients not carrying a mutation in *EMD*. We have identified other loci that are still under analysis in other informative families (data not shown). Therefore, additional genetic heterogeneity is expected for EDMD.

Regarding the possible genotype-phenotype relations, the type of mutations and their location seem to be crucial. Indeed, mutations associated with the RB subgroup[21,22,36,37] were all missense or short in-frame deletions (deletion of one or three amino acids) affecting highly conserved cysteine and histidine of the LIM2 domain of the three FHL1 isoforms.[13] Histidine 123 and cysteine 153 appeared as hot-spot mutational sites in RBM patients (Figure 3).[22,32,37] To date, all reported mutations affecting these conserved residues in the LIM2 domain of FHL1 have led to the formation of reducing bodies. In contrast, mutations identified in the EDMD and X-MPMA[20] subgroup were either missense or truncating mutations affecting the most distal domains of FHL1 proteins (Figures 3 and 4). Mutations affecting the LIM2 domain in EDMD and X-MPMA were either in-frame or out-of-frame deletions: one short in-frame insertion in X-MPMA[20] and small or large out-of-frame deletions in EDMD (our data). This difference may be at the origin of the absence of RB in EDMD and X-MPMA patients. So far, no hot-spot mutation was observed for this subgroup. The number of published *FHL1* mutations is still limited, and additional reports will help to confirm these genotype-phenotype relations.

EDMD-Related *FHL1* Mutations Lead to Reduction of Protein Expression

As previously reported,[14–18] we also detected strong expression of FHL1A and low expression of FHL1B and FHL1C in skeletal muscle biopsies from control individuals (Figure 6). In all tested patient biopsies or myoblasts, FHL1A expression was severely reduced, except in one case (F8, missense mutation), in which only moderate reduction was observed. However, we always detected the truncated or extended proteins in low amounts in all tested cases, even in out-of-frame deletions (F321 and F1292) or a stop codon mutation (F997). Thus, as for *EMD*-related EDMD diagnosis,[38] immunoblotting can be used for diagnosis before the complete sequencing of the gene.

So far, only FHL1A expression was analyzed in the other *FHL1*-related diseases. It was found to be severely reduced in X-MPMA,[20] whereas it was either only slightly decreased in X-SM[21] and in late-onset RBM cases[37] or increased in early-onset RBM cases.[22] Thus, one can see an inverse relation between duration of the symptoms at the time of the biopsy and expression level of FHL1A, as previously stated for X-SM.[21] In other words, a reduced level of FHL1 expression seems to be less deleterious for skeletal muscle than persistence and accumulation of a mutated FHL1 protein. Missense or small in-frame mutations in the LIM2 domain are identified in disorders with RB (RBM, RSS, X-SM) that only show slight decrease or accumulation of FHL1A, whereas missense or truncating mutations in the distal domains of FHL1, identified in EDMD and X-MPMA, lead to severe reduction of the protein level. In addition, truncated proteins are expressed in EDMD cases. Thus, again, the localization and/or the type of the *FHL1* mutation seems to be important.

Pathophysiological Mechanisms of EDMD-Related *FHL1* Mutations

Two recent papers have pointed out the role of FHL1 in the regulation of the ultra-rapid activating delayed rectifier K^+ current and atrial arrhythmia,[39,40] which may explain the typical arrhythmia and/or conduction defect observed in several EDMD patients with *FHL1* mutations. However, cardiac hypertrophy observed in several EDMD patients was more difficult to understand, considering that *FHL1* knockout mice show blunted response to stress-induced cardiac hypertrophy,[41] whereas EDMD patients with *FHL1* mutations showed reduced FHL1 expression. Several hypotheses may explain this discrepancy. First, we had

access only to skeletal muscle biopsies, and thus FHL1 expression might be different in cardiac muscle. Second, EDMD-related *FHL1* mutations differently affect the three isoforms. The residual expression in the heart of the modified FHL1 isoforms might be sufficient to lead to cardiac arrhythmias and hypertrophy. Third, as we have shown in skeletal muscles, the truncated FHL1 proteins are expressed at low level and may have a dominant-negative effect on still uncharacterized proteins, as has been shown for RBM mutations on the NFAT protein.[42] Finally, before interaction between FHL1 and titin has been identified,[41] a recent report has shown a relationship between the level of titin (N2B region)-FHL2 interaction and the development of dilated (DCM) or hypertrophic (HCM) cardiomyopathy, a decrease of the interaction being related to DCM and an increase in the interaction being related to HCM.[43]

Concerning the skeletal muscles, our in vitro experiments have shown that the reduced FHL1 expression has an impact on myogenin expression and myoblast fusion. These results were obtained only on two different patient primary cells and therefore may be still considered as preliminary. Delay in myogenin expression and myoblast fusion was reported in other muscular disorders.[44,45] However, these observations in *FHL1*-mutated cells are in accordance with the recent study from Cowling et al.,[42] which showed a correlation between FHL1 expression level and myotube size.[42] Further studies are needed to clarify the specificity of these observations. Finally, a role in sarcomere organization has been attributed to FHL1.[19] However, in our in vitro myoblast differentiation assay, performed on *FHL1*-mutated myoblasts, sarcomere formation appeared to be undisturbed.

It is important to note that the different FHL1 isoforms have been described as localizing to separate subcellular compartments of the muscle cell: sarcomere I-band and Z-line, as well as costameres for FHL1A,[19] the nucleus for FHL1C[14] and FHL1B, which is shuttling between the nucleus and the cytoplasm.[16,17] The FHL1 isoforms have specific functions at these different locations. FHL1A has been described as playing an important role in the mechanism of pathological hypertrophy by sensing biomechanical stress responses via activation of the ERK pathway,[41] whereas FHL1C (and probably FHL1B) isoforms that localize to the nucleus are thought to inhibit Notch signaling by the specific interaction with the RBP-J transcription factor.[14] In a context-dependent manner, Notch signals can promote or suppress cell proliferation, cell death, acquisition of specific cell fates, or activation of differentiation programs. This occurs in cells throughout development of the organism and during the maintenance of self-renewing adult tissues, including skeletal muscles (reviewed in [46]). Hence, FHL1 proteins have dual functions in the cell: the maintenance of structural integrity and the regulation of cell signaling. Interestingly, theses functions are shared by emerin and lamin A and C proteins.[8,9,47–49] Moreover, in this study, we have shown that EDMD-related patient myoblasts with an *FHL1* mutation have impaired differentiation, whereas several studies have pointed out the role of emerin and lamin A and C in skeletal muscle differentiation.[50,51] Therefore, beside the similarities in terms of EDMD phenotype, the mutations that we found in *FHL1* and mutations reported in *LMNA* and *EMD* may be associated with similar pathophysiological mechanisms.

Supplemental Data

Supplemental Data include two figures and two tables and can be found with this article online at http://www.cell.com/AJHG.

Acknowledgments

We thank Abdullah S. Al-Jarallah, Khalid H. Al Malki, Denis Duboc, Odile Dubourg, Essam A. Elgamal, Salah A. Elmalik, Yu-ichi Goto, Mohammad M. Kabiraj, Saleh A. Othman, Jean Pouget, Maja Sukalo, Christine S. Wehnert, and Karim Whabi for their precious help in the clinical evaluation of EDMD families. We are also indebted to F. Leturcq and P. Richard, as well as the "Banque de Cellules Cassini," for their support. We thank Myosix SA for allowing us access to the cell culture methodology and to the custom-made MYO1 medium. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Université Paris 06, the Centre National de la Recherche Scientifique (CNRS), the Association Française contre les Myopathies (no. 11057, no. 11034, and financial support for L.G.), the European Union Sixth Framework Programme (Eurolaminopathies no. 018690), the German Network of Muscular Dystrophies (MD-NET, 01GM0302), and the German Ministry of Education and Research (BMBF). M.A.S. was supported by CMRC (project no. 07-581), College of Medicine, King Saud University, Riyadh, Saudi Arabia.

Received: May 29, 2009
Revised: July 15, 2009
Accepted: July 29, 2009
Published online: August 27, 2009

Web Resources

The URLs for data presented herein are as follows:

Birdseed algorithm, http://www.broad.mit.edu/mpg/birdsuite/birdseed.html
Ensembl genome browser, http://www.ensembl.org/index.html
NCBI, http://www.ncbi.nlm.nih.gov/sites/entrez
Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/Omim/
Sequencher v4.7, http://www.genecodes.com

References

1. Emery, A.E.H. (2000). Emery-Dreifuss muscular dystrophy - a 40 year retrospective. Neuromuscul. Disord. *10*, 228–232.
2. Bécane, H.-M., Bonne, G., Varnous, S., Muchir, A., Ortega, V., Hammouda, E.H., Urtizberea, J.-A., Lavergne, T., Fardeau, M., Eymard, B., et al. (2000). High incidence of sudden death with conduction system and myocardial disease due to lamins

A and C gene mutation. Pacing Clin. Electrophysiol. *23*, 1661–1666.
3. van Berlo, J.H., Duboc, D., and Pinto, Y.M. (2004). Often seen but rarely recognised: cardiac complications of lamin A/C mutations. Eur. Heart J. *25*, 812–814.
4. Sakata, K., Shimizu, M., Ino, H., Yamaguchi, M., Terai, H., Fujino, N., Hayashi, K., Kaneda, T., Inoue, M., Oda, Y., et al. (2005). High incidence of sudden cardiac death with conduction disturbances and atrial cardiomyopathy caused by a nonsense mutation in the STA gene. Circulation *111*, 3352–3358.
5. Bione, S., Maestrini, E., Rivella, S., Manchini, M., Regis, S., Romei, G., and Toniolo, D. (1994). Identification of a novel X-linked gene responsible for Emery-Dreifuss muscular dystrophy. Nat. Genet. *8*, 323–327.
6. Bonne, G., Rafaele di Barletta, M., Varnous, S., Becane, H., Hammouda, E.H., Merlini, L., Muntoni, F., Greenberg, C.R., Gary, F., Urtizberea, J.A., et al. (1999). Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. Nat. Genet. *21*, 285–288.
7. Rafaele di Barletta, M., Ricci, E., Galluzzi, G., Tonali, P., Mora, M., Morandi, L., Romorini, A., Voit, T., Orstavik, K.H., Merlini, L., et al. (2000). Different mutations in the LMNA gene cause autosomal dominant and autosomal recessive Emery-Dreifuss muscular dystrophy. Am. J. Hum. Genet. *66*, 1407–1412.
8. Broers, J.L., Ramaekers, F., Bonne, G., Ben Yaou, R., and Hutchison, C. (2006). The nuclear lamins: laminopathies and their role in premature ageing. Physiol. Rev. *86*, 967–1008.
9. Ellis, J.A. (2006). Emery-Dreifuss muscular dystrophy at the nuclear envelope: 10 years on. Cell. Mol. Life Sci. *63*, 2702–2709.
10. Vytopil, M., Benedetti, S., Ricci, E., Galluzzi, G., Dello Russo, A., Merlini, L., Boriani, G., Gallina, M., Morandi, L., Politano, L., et al. (2003). Mutation analysis of the lamin A/C gene (LMNA) among patients with different cardiomuscular phenotypes. J. Med. Genet. *40*, e132.
11. Bethmann, C.I., Wasner, C., and Wehnert, M.S. (2004) Candidate gene testing for Emery–Dreifuss muscular dystrophy. 9th International World Muscle Society Congress. Neuromuscular Disorders, Goteborg, Sweden, 1–4 September, p. 593.
12. Zhang, Q., Bethmann, C., Worth, N.F., Davies, J.D., Wasner, C., Feuer, A., Ragnauth, C.D., Yi, Q., Mellad, J.A., Warren, D.T., et al. (2007). Nesprin-1 and −2 are involved in the pathogenesis of Emery-Dreifuss Muscular Dystrophy and are critical for nuclear envelope integrity. Hum. Mol. Genet. *16*, 2816–2833.
13. Kadrmas, J.L., and Beckerle, M.C. (2004). The LIM domain: from the cytoskeleton to the nucleus. Nat. Rev. Mol. Cell Biol. *5*, 920–931.
14. Taniguchi, Y., Furukawa, T., Tun, T., Han, H., and Honjo, T. (1998). LIM protein KyoT2 negatively regulates transcription by association with the RBP-J DNA-binding protein. Mol. Cell. Biol. *18*, 644–654.
15. Lee, S.M., Tsui, S.K., Chan, K.K., Garcia-Barcelo, M., Waye, M.M., Fung, K.P., Liew, C.C., and Lee, C.Y. (1998). Chromosomal mapping, tissue distribution and cDNA sequence of four-and-a-half LIM domain protein 1 (FHL1). Gene *216*, 163–170.
16. Brown, S., McGrath, M.J., Ooms, L.M., Gurung, R., Maimone, M.M., and Mitchell, C.A. (1999). Characterization of two isoforms of the skeletal muscle LIM protein 1, SLIM1. Localization of SLIM1 at focal adhesions and the isoform slimmer in the nucleus of myoblasts and cytoplasm of myotubes suggests distinct roles in the cytoskeleton and in nuclear-cytoplasmic communication. J. Biol. Chem. *274*, 27083–27091.
17. Lee, S.M., Li, H.Y., Ng, E.K., Or, S.M., Chan, K.K., Kotaka, M., Chim, S.S., Tsui, S.K., Waye, M.M., Fung, K.P., et al. (1999). Characterization of a brain-specific nuclear LIM domain protein (FHL1B) which is an alternatively spliced variant of FHL1. Gene *237*, 253–263.
18. Ng, E.K., Lee, S.M., Li, H.Y., Ngai, S.M., Tsui, S.K., Waye, M.M., Lee, C.Y., and Fung, K.P. (2001). Characterization of tissue-specific LIM domain protein (FHL1C) which is an alternatively spliced isoform of a human LIM-only protein (FHL1). J. Cell. Biochem. *82*, 1–10.
19. McGrath, M.J., Cottle, D.L., Nguyen, M.A., Dyson, J.M., Coghill, I.D., Robinson, P.A., Holdsworth, M., Cowling, B.S., Hardeman, E.C., Mitchell, C.A., et al. (2006). Four and a half LIM protein 1 binds myosin-binding protein C and regulates myosin filament formation and sarcomere assembly. J. Biol. Chem. *281*, 7666–7683.
20. Windpassinger, C., Schoser, B., Straub, V., Hochmeister, S., Noor, A., Lohberger, B., Farra, N., Petek, E., Schwarzbraun, T., Ofner, L., et al. (2008). An X-linked myopathy with postural muscle atrophy and generalized hypertrophy, termed XMPMA, is caused by mutations in FHL1. Am. J. Hum. Genet. *82*, 88–99.
21. Quinzii, C.M., Vu, T.H., Min, K.C., Tanji, K., Barral, S., Grewal, R.P., Kattah, A., Camano, P., Otaegui, D., Kunimatsu, T., et al. (2008). X-linked dominant scapuloperoneal myopathy is due to a mutation in the gene encoding four-and-a-half-LIM protein 1. Am. J. Hum. Genet. *82*, 208–213.
22. Schessl, J., Zou, Y., McGrath, M.J., Cowling, B.S., Maiti, B., Chin, S.S., Sewry, C., Battini, R., Hu, Y., Cottle, D.L., et al. (2008). Proteomic identification of FHL1 as the protein mutated in human reducing body myopathy. J. Clin. Invest. *118*, 904–912.
23. Yates, J.R. (1997). 43rd ENMC International Workshop on Emery-Dreifuss Muscular Dystrophy, 22 June 1996, Naarden, The Netherlands. Neuromuscul. Disord. *7*, 67–69.
24. Bonne, G., Capeau, J., De Visser, M., Duboc, D., Merlini, L., Morris, G.E., Muntoni, F., Recan, D., Sewry, C., Squarzoni, S., et al. (2002). 82nd ENMC international workshop, 5th international Emery-Dreifuss muscular dystrophy (EDMD) workshop, 1st Workshop of the MYO-CLUSTER project EUROMEN (European muscle envelope nucleopathies), 15–16 September 2000, Naarden, The Netherlands. Neuromuscul. Disord. *12*, 187–194.
25. Bonne, G., and Lampe, A. (in press) Muscle diseases with prominent muscle contractures. In Disorders of Voluntary Muscle, Eighth Edition, G. Karpati, D. Hilton-Jones, R.C. Griggs, and K. Bushby, eds. (Cambridge: Cambridge University Press).
26. Abecasis, G.R., Cherny, S.S., Cookson, W.O., and Cardon, L.R. (2002). Merlin–rapid analysis of dense genetic maps using sparse gene flow trees. Nat. Genet. *30*, 97–101.
27. Abecasis, G.R., and Wigginton, J.E. (2005). Handling marker-marker linkage disequilibrium: pedigree analysis with clustered markers. Am. J. Hum. Genet. *77*, 754–767.
28. Voit, T., Krogmann, O., Lenard, H.G., Neuen-Jacob, E., Wechsler, W., Goebel, H.H., Rahlf, G., Lindinger, A., and Nienaber, C. (1988). Emery-Dreifuss muscular dystrophy: disease spectrum and differential diagnosis. Neuropediatrics *19*, 62–71.

29. Emery, A.E.H., and Dreifuss, F.E. (1966). Unusual type of benign X-linked muscular dystrophy. J. Neurol. Neurosurg. Psychiatry *29*, 338–342.
30. Rowland, L.P., Fetell, M., Olarte, M., Hays, A., Singh, N., and Wanat, F.E. (1979). Emery-Dreifuss muscular dystrophy. Ann. Neurol. *5*, 111–117.
31. Bonne, G., Ben Yaou, R., Beroud, C., Boriani, G., Brown, C.A., De Visser, M., Duboc, D., Ellis, J.A., Hausmanowa-Petrusewicz, I., Lattanzi, G., et al. (2003). 108th ENMC International Workshop, 3rd Workshop of the MYO-CLUSTER project: EUROMEN, 7th International Emery-Dreifuss Muscular Dystrophy (EDMD) Workshop, 13–15 September 2002, Naarden, The Netherlands. Neuromuscul. Disord. *13*, 508–515.
32. Schessl, J., Taratuto, A.L., Sewry, C., Battini, R., Chin, S.S., Maiti, B., Dubrovsky, A.L., Erro, M.G., Espada, G., Robertella, M., et al. (2009). Clinical, histological and genetic characterization of reducing body myopathy caused by mutations in FHL1. Brain *132*, 452–464.
33. Bonne, G., Mercuri, E., Muchir, A., Urtiziberea, A., Becane, H.M., Reca, D., Merlini, L., Wehnert, M., Boor, R., Reuner, U., et al. (2000). Clinical and molecular genetic spectrum of autosomal dominant Emery Dreifuss muscular dystrophy due to mutations of the lamin A/C gene. Ann. Neurol. *48*, 170–180.
34. Olive, M., Goldfarb, L.G., Shatunov, A., Fischer, D., and Ferrer, I. (2005). Myotilinopathy: refining the clinical and myopathological phenotype. Brain *128*, 2315–2326.
35. Senderek, J., Garvey, S.M., Krieger, M., Guergueltcheva, V., Urtizberea, A., Roos, A., Elbracht, M., Stendel, C., Tournev, I., Mihailova, V., et al. (2009). Autosomal-dominant distal myopathy associated with a recurrent missense mutation in the gene encoding the nuclear matrix protein, matrin 3. Am. J. Hum. Genet. *84*, 511–518.
36. Shalaby, S., Hayashi, Y.K., Goto, K., Ogawa, M., Nonaka, I., Noguchi, S., and Nishino, I. (2008). Rigid spine syndrome caused by a novel mutation in four-and-a-half LIM domain 1 gene (FHL1). Neuromuscul. Disord. *18*, 959–961.
37. Shalaby, S., Hayashi, Y.K., Nonaka, I., Noguchi, S., and Nishino, I. (2009). Novel FHL1 mutations in fatal and benign reducing body myopathy. Neurology *72*, 375–376.
38. Manilal, S., Sewry, C.A., Man, N.T., Muntoni, F., and Morris, G.E. (1997). Diagnosis of X-linked Emery-Dreifuss muscular dystrophy by protein analysis of leucocytes and skin with monoclonal antibodies. Neuromuscul. Disord. *7*, 63–66.
39. Chen, C.L., Lin, J.L., Lai, L.P., Pan, C.H., Huang, S.K., and Lin, C.S. (2007). Altered expression of FHL1, CARP, TSC-22 and P311 provide insights into complex transcriptional regulation in pacing-induced atrial fibrillation. Biochim. Biophys. Acta *1772*, 317–329.
40. Yang, Z., Browning, C.F., Hallaq, H., Yermalitskaya, L., Esker, J., Hall, M.R., Link, A.J., Ham, A.J., McGrath, M.J., Mitchell, C.A., et al. (2008). Four and a half LIM protein 1: a partner for KCNA5 in human atrium. Cardiovasc. Res. *78*, 449–457.
41. Sheikh, F., Raskin, A., Chu, P.H., Lange, S., Domenighetti, A.A., Zheng, M., Liang, X., Zhang, T., Yajima, T., Gu, Y., et al. (2008). An FHL1-containing complex within the cardiomyocyte sarcomere mediates hypertrophic biomechanical stress responses in mice. J. Clin. Invest. *118*, 3870–3880.
42. Cowling, B.S., McGrath, M.J., Nguyen, M.A., Cottle, D.L., Kee, A.J., Brown, S., Schessl, J., Zou, Y., Joya, J., Bonnemann, C.G., et al. (2008). Identification of FHL1 as a regulator of skeletal muscle mass: implications for human myopathy. J. Cell Biol. *183*, 1033–1048.
43. Matsumoto, Y., Hayashi, T., Inagaki, N., Takahashi, M., Hiroi, S., Nakamura, T., Arimura, T., Nakamura, K., Ashizawa, N., Yasunami, M., et al. (2005). Functional analysis of titin/connectin N2-B mutations found in cardiomyopathy. J. Muscle Res. Cell Motil. *26*, 367–374.
44. Amack, J.D., and Mahadevan, M.S. (2001). The myotonic dystrophy expanded CUG repeat tract is necessary but not sufficient to disrupt C2C12 myoblast differentiation. Hum. Mol. Genet. *10*, 1879–1887.
45. de Luna, N., Gallardo, E., Soriano, M., Dominguez-Perles, R., de la Torre, C., Rojas-Garcia, R., Garcia-Verdugo, J.M., and Illa, I. (2006). Absence of dysferlin alters myogenin expression and delays human muscle differentiation "in vitro". J. Biol. Chem. *281*, 17092–17098.
46. Zammit, P.S. (2008). All muscle satellite cells are equal, but are some more equal than others? J. Cell Sci. *121*, 2975–2982.
47. Worman, H.J., and Bonne, G. (2007). "Laminopathies": a wide spectrum of human diseases. Exp. Cell Res. *313*, 2121–2133.
48. Muchir, A., Pavlidis, P., Decostre, V., Herron, A.J., Arimura, T., Bonne, G., and Worman, H.J. (2007). Activation of MAPK pathways links LMNA mutations to cardiomyopathy in Emery-Dreifuss muscular dystrophy. J. Clin. Invest. *117*, 1282–1293.
49. Muchir, A., Pavlidis, P., Bonne, G., Hayashi, Y., and Worman, H. (2007). Activation of MAP kinase in hearts of Emd null mice: similarities between mouse models of X-linked and autosomal dominant Emery-Dreifuss muscular dystrophy. Hum. Mol. Genet., in press.
50. Frock, R.L., Kudlow, B.A., Evans, A.M., Jameson, S.A., Hauschka, S.D., and Kennedy, B.K. (2006). Lamin A/C and emerin are critical for skeletal muscle satellite cell differentiation. Genes Dev. *20*, 486–500.
51. Melcon, G., Kozlov, S., Cutler, D.A., Sullivan, T., Hernandez, L., Zhao, P., Mitchell, S., Nader, G., Bakay, M., Rottman, J.N., et al. (2006). Loss of emerin at the nuclear envelope disrupts the Rb1/E2F and MyoD pathways during muscle regeneration. Hum. Mol. Genet. *15*, 637–651.