

Riboflavin-Responsive and -Non-responsive Mutations in FAD Synthase Cause Multiple Acyl-CoA Dehydrogenase and Combined Respiratory-Chain Deficiency

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Multiple acyl-CoA dehydrogenase deficiencies (MADDs) are a heterogeneous group of metabolic disorders with combined respiratory-chain deficiency and a neuromuscular phenotype. Despite recent advances in understanding the genetic basis of MADD, a number of cases remain unexplained. Here, we report clinically relevant variants in *FLAD1*, which encodes FAD synthase (FADS), as the cause of MADD and respiratory-chain dysfunction in nine individuals recruited from metabolic centers in six countries. In most individuals, we identified biallelic frameshift variants in the molybdopterin binding (MPTb) domain, located upstream of the FADS domain. Inasmuch as FADS is essential for cellular supply of FAD cofactors, the finding of biallelic frameshift variants was unexpected. Using RNA sequencing analysis combined with protein mass spectrometry, we discovered *FLAD1* isoforms, which only encode the FADS domain. The existence of these isoforms might explain why affected individuals with biallelic *FLAD1* frameshift variants still harbor substantial FADS activity. Another group of individuals with a milder phenotype responsive to riboflavin were shown to have single amino acid changes in the FADS domain. When produced in *E. coli*, these mutant FADS proteins resulted in impaired but detectable FADS activity; for one of the variant proteins, the addition of FAD significantly improved protein stability, arguing for a chaperone-like action similar to what has been reported in other riboflavin-responsive inborn errors of metabolism. In conclusion, our studies identify *FLAD1* variants as a cause of potentially treatable inborn errors of metabolism manifesting with MADD and shed light on the mechanisms by which FADS ensures cellular FAD homeostasis.

Introduction

Riboflavin, or vitamin B2, is the precursor of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN),

which are essential cofactors of numerous dehydrogenases involved in cellular metabolism and a number of other essential cellular pathways, such as antioxidant defense, protein folding, and apoptosis.¹ These flavoenzymes are

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<http://dx.doi.org/10.1016/j.ajhg.2016.04.006>

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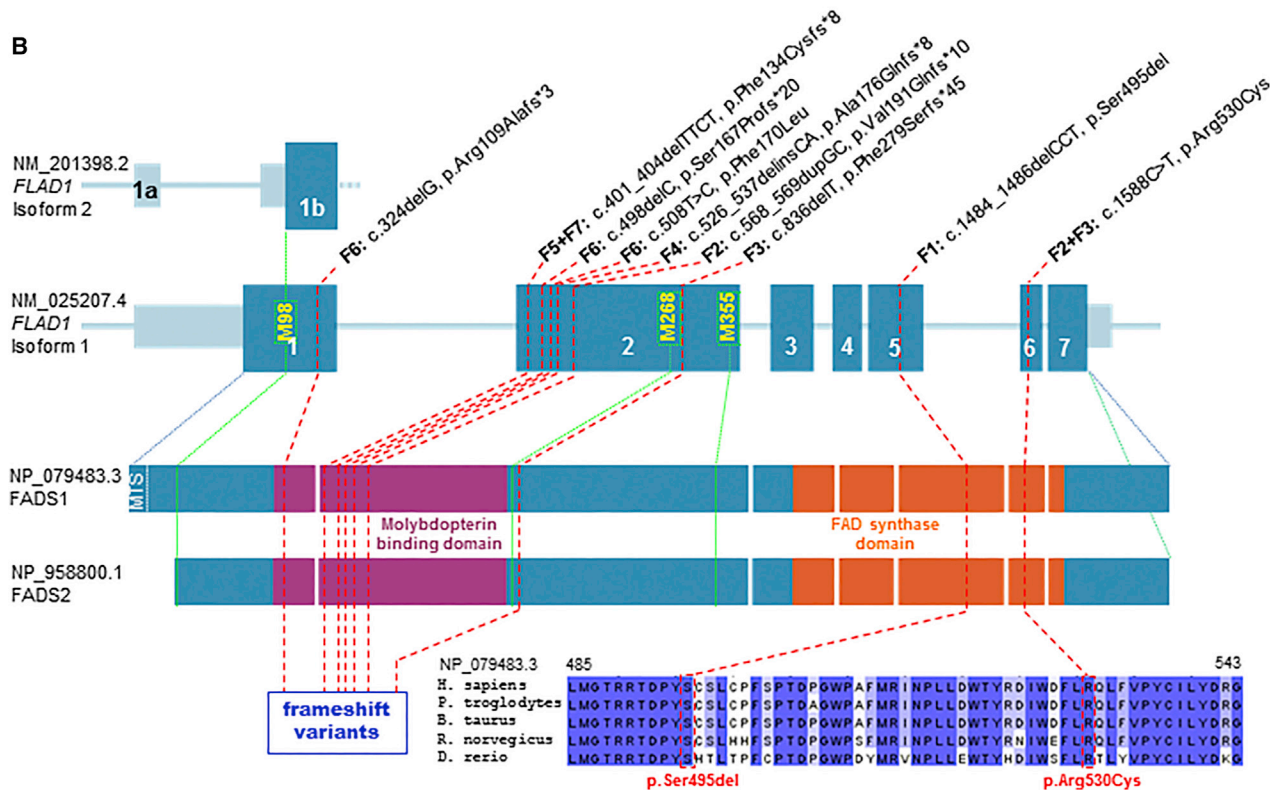
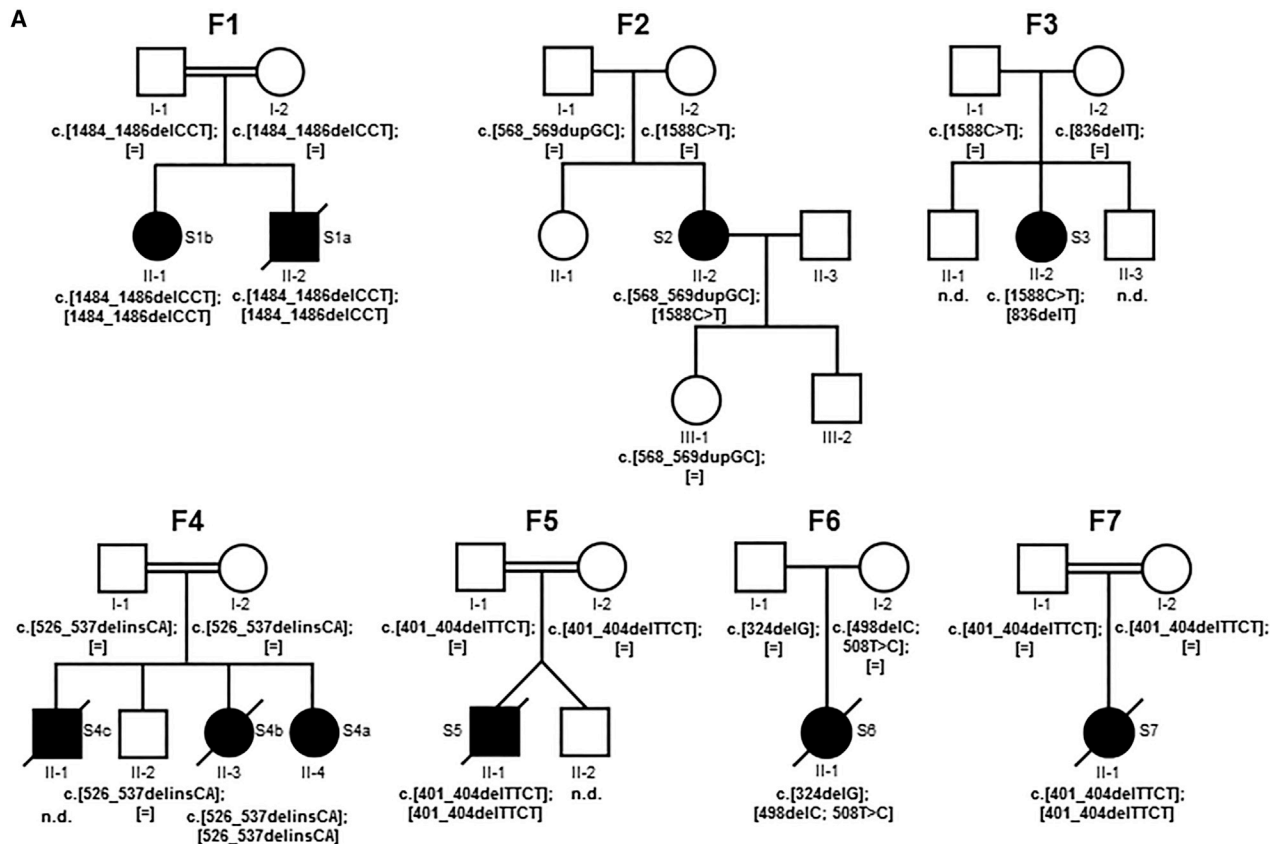


Figure 1. FLAD1 Variants and Gene and Protein Structure

(A) Pedigrees of the investigated families (F1–F7) with recessively inherited *FLAD1* variants. Affected individuals are indicated by closed symbols.

(B) Gene structure with exons and introns shows the localization of the investigated gene variations (homozygous variants are underlined). Met residues located upstream of the FADS domain are presented in yellow with their corresponding protein positions with

(legend continued on next page)

located in the cytosol or compartmented in the cellular organelles, where they ensure the functionality of mitochondrial electron transport, the metabolism of fatty acids, some amino acids, choline, and betaine, the synthesis of protoporphyrin, and the metabolism of vitamins B6, B9, and B12.^{2–6}

Riboflavin cannot be synthesized in humans and must therefore be obtained as a nutrient via intestinal absorption. Riboflavin is transported into the bloodstream and taken up by target cells by the human riboflavin transporters (RFVTs). Three RFVTs (RFVT1, RFVT2, and RFVT3), each differing in tissue-specific presence, have been identified thus far and assigned to a new solute carrier family named SLC52.⁴ Within cells, riboflavin is converted into FMN by riboflavin kinase (RFK), and FMN is then converted into FAD by FAD synthase (FADS), previously known as FAD synthetase or FMN:ATP adenylyltransferase.^{7,8} FADS is the product of *FLAD1* (MIM: 610595), which according to the UCSC Genome Browser encodes two transcripts resulting in proteins with an intact FADS domain. Those two transcripts have been characterized in detail;^{7–12} a longer isoform derives from a large exon 1 and has the potential to encode FADS1 with a predicted mitochondrial targeting sequence (MTS). In a shorter isoform, exon 1 is interrupted by an additional intron. This leads to usage of a downstream ATG start codon at position 98 of the longer transcript, resulting in FADS2.¹³ Both FADS proteins contain an N-terminal molybdopterin binding (MPTb) domain, which has been shown to have FAD hydrolase activity,¹⁰ and a C-terminal 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase domain. This second domain is sufficient to catalyze FAD synthesis.¹¹ Therefore, it is here named the FADS domain. All recombinant FADS produced up to now exhibit the ability to bind FAD, the product of their activity, tightly but not covalently.⁹ FAD release from FADS is likely to be tightly controlled and presumably requires well-defined conditions, including a correct redox state.^{9,12} FAD (or as recently proposed, catalytically active FADS enzyme) binds to apoproteins and chaperones their folding into stable and functional flavoenzymes.^{14–19} Accordingly, several studies have shown that a number of flavoproteins, and in particular mature mitochondrial acyl-CoA dehydrogenases, undergo fast degradation under depletion of riboflavin or flavin cofactors.^{20–23} The biochemistry of riboflavin deficiency, with elevated multiple acylcarnitines and urine organic acids, resembles that seen in individuals who suffer from multiple acyl-CoA dehydrogenase deficiency (MADD [MIM: 231680]) or glutaric aciduria type 2 and have genetic defects in three genes encoding two enzymes, electron transfer flavoprotein (ETF) and electron

transfer flavoprotein dehydrogenase (ETFHD). These two enzymes functionally link the acyl-CoA dehydrogenation reactions to ATP production by delivering their electrons to coenzyme Q10 and further on to complex III in the respiratory chain.²⁴ Riboflavin-responsive forms of MADD leading to improvement in biochemical and clinical symptoms after high doses of riboflavin treatment were first explained by missense variants in *ETFHD* (MIM: 231675)^{25–29} and more recently by variants in the riboflavin transporter genes (*SLC52A1* [MIM: 607883], *SLC52A2* [MIM: 607882], and *SLC52A3* [MIM: 613350]),^{30–32} as well as in the mitochondrial FAD transporter gene (*SLC25A32* [MIM: 610815]).³³

In this study, we describe individuals who are from seven unrelated families and have *FLAD1* variants causing MADD and a disturbed riboflavin metabolism. The affected individuals presented with muscle symptoms and multiple-respiratory-chain deficiency. Depending on the *FLAD1* genotype, some responded to riboflavin treatment.

Subjects and Methods

Subjects

Subjects (S1–S7) from seven unrelated families (F1–F7 in Figure 1A) recruited from medical centers located in Australia, Finland, Italy, Austria, Turkey, and France were enrolled in the current project. All studies were completed according to local approval by institutional review boards. Informed consent for participation in this study was obtained from the parents of all investigated subjects in agreement with the Declaration of Helsinki and approved by the ethical committees of the centers participating in this study, where biological samples were obtained.

Genetic Analyses

For subjects S4a and S5, whole-exome sequencing (WES) was performed in genomic DNA by AROS Applied Biotechnology AS with Illumina's TruSeq DNA Sample Prep Kit and Exome Enrichment Kit on the Illumina HiSeq 2000 platform (for S5)³⁴ or with SureSelect Human All Exon 50Mb V5 Kit (Agilent) on a HiSeq 2500 system (Illumina) (for S4a).^{35,36} The remaining affected individuals were investigated by sequencing of candidate genes associated with fatty-acid oxidation and/or mitochondrial disorders, including *FLAD1* and other genes involved in riboflavin uptake or metabolism. For subjects S1a, S2, and S3, Sanger sequencing of the target genes listed in Table S1 was performed as described previously.^{37,38} For subject S6, a customized gene panel³⁹ (TruSeq Custom Amplicon, Illumina) containing 200 genes associated with mitochondrial disorders, including the genes listed in Table S1, was used for library preparation; then, samples were analyzed by a MiSeq system (Illumina). For subject S7, a panel of 17 genes (Table S1) was analyzed with a targeted resequencing approach

respect to isoform 1 (GenBank: NM_025207.4). Isoforms 1 and 2 are reported in the UCSC Genome Browser as transcripts possessing an intact and active FADS domain. The protein structure highlights the MPTb domain in violet and the FADS domain in orange. Protein consequences of the identified *FLAD1* mutations include the frameshift variants located in the MPTb domain and the two amino acid changes in a region of the FADS domain, which is highly conserved among eukaryotic species. Amino acid residues that are conserved across all species are highlighted in dark blue.

by Ion AmpliSeq technology (Life Technologies) on a PGM analyzer (Life Technologies). All identified variants and their segregation in the affected families were validated by Sanger sequencing.

Skeletal-Muscle and Liver Homogenates and Fibroblast Cultures

Muscle biopsy specimens were obtained from seven subjects, and a post-mortem liver specimen was obtained from individual S5. Homogenization and preparation of mitochondrial fractions were performed according to standard procedures. Primary human dermal fibroblasts from subjects S1a, S2, and S4a and healthy control individuals were cultivated until confluent at 37°C and 5% (v/v) CO₂ in RPMI-1640 media (Lonza) with 10% FCS, 2 mmol/L L-glutamine (Sigma-Aldrich), and 1% penicillin and streptomycin (Sigma-Aldrich).

Histological and Respiratory-Chain Analyses

Histological analyses and measurements of respiratory-chain function were performed via polarographic and spectrophotometric analyses as previously described.^{25,40}

SDS-PAGE and Immunoblotting

Liver tissue was homogenized with a potter-type homogenizer on ice in buffer (250 mmol/L sucrose, 50 mmol/L Tris-HCl [pH 7.4], 5 mmol/L MgCl₂, EDTA-free protease inhibitor cocktail, and 1% Triton X-100), and frozen fibroblast pellets were lysed in a buffer containing 50 mmol/L Tris-HCl (pH 7.8), 5 mmol/L EDTA (pH 8.0), 1 mmol/L DTT, 10 µg/mL Aprotinin (Sigma-Aldrich), 1 mg/mL trypsin inhibitor (Roche) in 10 mL, and 1% Triton X-100. Aliquots of total liver protein (50–100 µg) were loaded on 14% SDS-PAGE gels, and 20 µg fibroblast protein lysate was loaded on Criterion GGX Stain-Free Precast Gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes. Membranes were probed with human anti-FLAD1 antibody (fibroblasts: HPA028563, Sigma-Aldrich; liver: ab95312, Abcam) and anti-GAPDH (sc25778, Santa Cruz). Detected proteins were visualized with the ECL Plus Western Blotting Detection System (GE Healthcare).

Mass Spectrometry to Confirm the Identity of Anti-FADS Gel Bands

80 µg of total protein from control fibroblasts was extracted and separated by SDS-PAGE as described above. The bands of interest (~60, 50, 37, 32, and 27 kDa) were excised and processed by an in-gel trypsin-digestion protocol as previously described.⁴¹ Selected reaction monitoring (SRM) was performed as previously described.⁴² Peptide selection, method development, and data analysis were performed with the software Skyline.⁴³ Five FADS peptides passed selection criteria based on detectability and FADS uniqueness (checked in the whole human proteome, UniProt version 2012_06_19, containing 20,202 reviewed sequences), and these five FADS peptides were analyzed by liquid chromatography coupled with the TSQ Vantage Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific). Four or more transitions were detected for each peptide, and synthetic isotopically labeled tryptic peptides (SpikeTide from JPT Peptide Technologies) were added to each sample for quality assurance. The list of peptides and transitions can be found in Table S2.

Measurements of FAD Synthesis Rate and Flavin Content

For investigating different fractions of EDTA blood from subject S4a, plasma, mononuclear cells, and enriched erythrocytes purified by Ficoll gradient centrifugation, 50 µl of each was mixed with 375 µl of 10% trichloroacetic acid and 75 µl of water. After incubation on ice for 5 min and centrifugation at 13,000 × g for 5 min, the supernatant was extracted with equal amounts of diethyl ether twice, and 100 µl was applied to high-performance liquid chromatography (HPLC) separation on a reversed-phase column (Agilent, Eclipse Plus C18, 5 µm, 4.6 × 150 mm) for measuring riboflavin, FMN, and FAD.

Flavin content in muscle homogenate from subject S5 was measured by HPLC with a reverse-phase C18 column and fluorescence detection (excitation and emission wavelengths of 470 and 530 nm, respectively) according to the previously described method.⁴⁴ 200 µl of muscle homogenate was mixed with 500 µl methanol and centrifuged, and the supernatant was injected for HPLC analysis (mobile phase: 50 mmol/L NaH₂PO₄ and 13% acetonitrile [pH 2.9]; flow rate = 1.2 mL/min).

For measurements in fibroblast cells, the frozen cells were re-suspended in 150 µl lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 1% Triton X-100, 5 mmol/L β-mercaptoethanol [2-ME], 1 mmol/L NaF, 0.1 mmol/L PMSE, and 1× protease-inhibitor cocktail) and passed through a 26G needle. After incubation for 30 min on ice, the cell suspension was centrifuged at 13,000 × g for 10 min at 4°C, and the supernatant was recovered as cell lysate. Riboflavin, FMN, and FAD were measured in neutralized perchloric acid extracts of cell lysates (0.2 mg) by HPLC as previously described.^{45–48} Separation was achieved with a Gilson HPLC system, including a model 306 pump and a model 307 pump equipped with a FP-2020 Plus Jasco Fluorescence Detector and uniPoint software. Quantitative determination of riboflavin, FMN, and FAD was carried out with a calibration curve made in each experiment with standard solutions diluted in the extraction solution. The rate of FAD synthesis was measured at 37°C in 500 µl of 50 mM Tris-HCl (pH 7.5) in the presence of 0.08 mg cell lysate, 1 µmol/L FMN, 5 mmol/L ATP, and 5 mmol/L MgCl₂. At the appropriate time, 100 µl aliquots were taken, extracted with perchloric acid, and neutralized. Riboflavin, FMN, and FAD were analyzed with HPLC (see above).

qRT-PCR and Transcriptome Analysis

Total RNA was isolated according to the TRIzol Reagent protocol (Ambion) or with the RNeasy Plus Mini Kit (QIAGEN). The quality of the RNA was determined with the Agilent 2100 BioAnalyzer (RNA 6000 Nano Kit, Agilent). cDNA was synthesized from subject S2's RNA with the iScript cDNA Synthesis Kit (Bio-Rad), and the c.1588C>T genotype was assessed by sequencing.

qRT-PCR analysis (iQ SYBR Green Supermix, iCycler, Bio-Rad) of subject S4a's fibroblast RNA (TRI Reagent, Molecular Research Center), treated with DNase (TURBO DNase, Thermo Fisher), was performed with oligo-dT-amplified cDNA (Maxima Reverse Transcriptase, Thermo Fisher) with a set of primers spanning all coding regions of *FLAD1* (Figure S1). Threshold cycles (Ct) were normalized (ΔCt) to housekeeping genes *HPRT* (GenBank: NM_000194.2; MIM: 308000) and *RPL27* (GenBank: NM_000988.3; MIM: 607526), and the expression of PCR products was compared to that of control fibroblasts (ΔΔCt).

For transcriptome analysis, quantitative library preparation and enrichment were performed as described in Haack et al.⁴⁹ RNA

libraries were assessed for quality and quantity with the Agilent 2100 BioAnalyzer and the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) and sequenced as 100 bp paired-end runs on an Illumina HiSeq 2500 platform. Sashimi plots were used for quantitative visualization of splice junctions of their alignment data in the Integrative Genomics Viewer.⁵⁰

Lentiviral Gene Expression

The wild-type (WT) sequence of isoform 2 (GenBank: NM_201398.2) was obtained as a plasmid from DNASU (HsCD00039838). Shorter *FLAD1* transcripts coding for FADS proteins starting at methionine 268 (Met268) or methionine 355 (Met355) were amplified from a human control cDNA and cloned into the pLenti6.3/V5-TOPO TA Cloning Kit (Invitrogen) for production in human fibroblasts.³⁶ The following primers were used: 5'-CGCCATGAAGGGACTATTCC-3' (forward Met268), 5'-CGCCA TGCCCAACGCTGTGG-3' (forward Met355), and 5'-TCATGTG CGGGAGTTCGCT-3' (reverse).

Biophysical Studies of Recombinant 6His-p.Ser495del and 6His-p.Arg530Cys FADS2

Site-Directed Mutagenesis

The coding region for human FADS isoform 2 (FADS2) was amplified as previously described.⁷ The Ser495 deletion and the p.Arg530Cys substitution were obtained via overlap-extension PCR¹² with the following primers: 5'-ATCTGGGATTTTCTGTGT CAGCTGTTTGTGTC-3' (forward) and 5'-GACAAACAGCTGACACAG AAAATCCCAGAT-3' (reverse) for p.Arg530Cys and 5'-TGACCC CTACTGTAGCCTCTGCCCTTCAGCCCCACTGAC-3' (forward) and 5'-AGAGGCTACAGTAGGGGTCAGTCCGGCGGGTGCCC-3' (reverse) for the Ser495 deletion.

The PCR products were purified, digested with EcoRI and XhoI, and ligated into the pH6EX3 expression vector as previously described.⁹ The resulting recombinant plasmid carried the extra N-terminal sequence MSPIHHHHHHLVPRGSEASNS.

Production and Purification of Recombinant 6His-Ser495del and 6His-p.Arg530Cys FADS2

Protein production in Rosetta (DE3) cells and purification of WT or mutant FADS2 were carried out as described previously.⁹ Where indicated, the WT and variant proteins were incubated with a 2.5-fold molar excess of FAD at 4°C overnight, and the free cofactor was eventually removed on a PD10-column run in 40 mmol/L HEPES/Na and 5 mmol/L 2-ME (pH 7.4).

Protein Concentration and Measurements of FAD Saturation

Protein concentration was measured by the Bradford method with BSA as a standard. In an alternative procedure, the concentration of the purified FADS2 was estimated by absorbance spectra as previously described⁹ with the use of ϵ_{280} (56,435 $\text{mM}^{-1} \cdot \text{cm}^{-1}$ and 0.998 mg/mL^{-1} for the WT protein; 55,810 $\text{mM}^{-1} \cdot \text{cm}^{-1}$ and 0.997 mg/mL^{-1} for 6His-p.Ser495del FADS2; and 56,435 $\text{mM}^{-1} \cdot \text{cm}^{-1}$ and 0.999 mg/mL^{-1} for 6His-p.Arg530Cys FADS2) as calculated from the protein sequence with the ExPasyProtParam tool. The degree of FAD saturation (i.e. the ratio of FAD to protein monomer) was calculated from the spectra as previously described.⁹

Measurements of FAD Synthesis Rate

The different fluorescence properties of FAD and FMN were exploited for measuring the rate of FAD synthesis. Fluorescence time courses (excitation at 450 nm and emission at 520 nm) were followed in a LS50 Perkin Elmer spectrofluorometer. FAD and FMN fluorescence were calibrated with standard solutions, whose

concentrations were calculated with $\epsilon_{450} = 12.2 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for FMN and $11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for FAD. The rate of FAD synthesis ($\text{nmol FAD} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) was calculated from the rate of decrease in fluorescence, measured as the tangent to the initial part of the experimental curve. For activity measurements, purified protein fractions (10 μg protein as a monomer, unless otherwise indicated) were incubated at 37°C in 2 ml of 50 mol/L Tris-HCl (pH 7.5) containing 5 mmol/L MgCl_2 , 2 $\mu\text{mol/L}$ FMN, 100 $\mu\text{mol/L}$ ATP, and additional reagents as appropriate.

Limited Proteolysis with Trypsin

WT and mutant FADS2 proteins with or without incubation with a molar excess of FAD were used for testing the susceptibility to trypsin digestion. Samples (10 μg) were incubated with or without 0.015 mg/mL trypsin (bovine pancreas trypsin, T8003, Sigma-Aldrich) at 0°C in 40 mmol/L HEPES/Na and 5 mmol/L 2-ME (400 μL). Aliquots of 2.5 μg protein were sampled at different time points, and the reaction was stopped by the addition of 0.3 mg/mL of soybean trypsin inhibitor (T9003, Sigma-Aldrich). As an internal standard for the quantity of loaded protein in the gel, BSA (1 μg) was also added to the loading buffer solution. The products of the proteolysis reaction were separated by 12% SDS-PAGE and electro-transferred onto a nitrocellulose membrane with a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The immobilized proteins were incubated for 3 hr with a 3,000-fold dilution of a polyclonal antiserum against FADS.¹³ The bound antibodies were visualized with the aid of secondary anti-rabbit IgG antibodies conjugated with alkaline phosphatase (1:3,500 dilution). Quantitative evaluation of immuno-reactive protein bands was carried out by densitometric analysis with ImageLab software 5.1 (Bio-Rad).

Statistical Analysis

Statistical analysis was performed with the Poisson test for determining the number of loss-of-function (LOF) variants in *FLAD1* and with the Student's t test for pairwise comparisons of means in Tables 2, 3, and 4. A p value of <0.05 was considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Metabolic research centers from Australia, Austria, Denmark, England, Finland, France, Germany, Italy, and Turkey independently achieved and subsequently shared results from candidate-gene or WES analysis on families and individuals affected by riboflavin-responsive MADD and/or a suspected mitochondrial respiratory-chain disorder (Table 1). As a result of this collaborative effort, from seven unrelated families (F1–F7 in Figure 1A) we identified nine affected individuals harboring recessive mutations in one gene, *FLAD1* (GenBank: NM_025207.4), coding for FADS. Detailed case reports are available in the Supplemental Note.

Genetic Analyses

In family F1, both siblings (S1a and S1b) were found to carry a homozygous mutation resulting in an in-frame deletion of serine 495 (c.1484_1486delCCT [p.Ser495del]) in the FADS domain. Individuals S2 and S3 were found to carry the same missense mutation, c.1588C>T

Table 1. Pre-treatment Genetic, Clinical, Biochemical, and Histopathological Findings in Individuals with FLAD1 Variants

ID	Sex	Consanguineous	FLAD1 Variants (GenBank: NM_025207.4)	FADS1 Variants (GenBank: NP_079483.3)	Affected Domain	Age of Onset	Status	Responsive to Riboflavin	Increased Blood Acylcarnitines ^a	Elevated Organic Acids ^a	Muscle Investigations		
											Respiratory- Chain Activities ^b	Other Mitochondrial Enzyme Activities	Histology
S1a	F	yes	c.[1484_1486delCCCT]; [1484_1486delCCCT]	p.[Ser495del]; [Ser495del]	FADS	32 hr	died at 3 days	NT	C5, C8, C14, C5-DC	ethylmalonic, adipic, suberic, and dehydrosebacic acids, hexanoylglycine	within control range	NR	vacuoles in muscle fibers (EM not done)
S1b	M	yes	c.[1484_1486delCCCT]; [1484_1486delCCCT]	p.[Ser495del]; [Ser495del]	FADS	3 months	alive at 22 years	yes	C4	ethylmalonic and methylsuccinic acids	NR	NR	NR
S2	F	no	c.[568_569dupGC]; [1588C>T]	p.[Val191Glnfs*10]; [Arg530Cys]	MPTb (fs), FADS	20 years	alive at 44 years	yes	C8, C10, C10:1	ethylmalonic and glutaric acids, lactate	CII↓, CIII↓, CIV↓	CS↑	lipid-storage myopathy, faint COX staining
S3	F	no	c.[836delT]; [1588C>T]	p.[Phe279Serfs*45]; [Arg530Cys]	MPTb (fs), FADS	44 years	alive at 56 years	yes	C5, C8, C10, C10:1, C14	ethylmalonic acid, tiglylglycine	CI+III↓, CII+III↓	CS↑	lipid-storage myopathy, muscle beta oxidation (C4: 132%; C8: 15%; C16: 56%)
S4a	F	yes	c.[526_537delinsCA]; [526_537delinsCA]	p.[Ala176Glnfs*8]; [Ala176Glnfs*8]	MPTb (fs)	4 months	alive at 8 years	yes	C4, C6, C8, C10, C14, C14:1, C14:2, C16:1, C18, C18:1, C18:2	adipic, suberic, ethylmalonic, and methylsuccinic acids, ketosis	CI↓, CII↓	PDH↓	lipid-storage myopathy
S4b	F	yes	c.[526_537delinsCA]; [526_537delinsCA]	p.[Ala176Glnfs*8]; [Ala176Glnfs*8]	MPTb (fs)	8 months	died at 16 years, 2 months	discontinued (side effects)	normal or C4-OH, C5, C6, C8, C10, C14:1, C16:1	NR	NR	NR	NR
S5	M	yes	c.[401_404delTTCT]; [401_404delTTCT]	p.[Phe134Cysfs*8]; [Phe134Cysfs*8]	MPTb (fs)	6 months	died at 6 months	NT	C3, C5, C6, C8:1	adipic acid	CI↓, CII+III↓	CS↑	lipid-storage myopathy, absent SDH staining, several NADH- and COX- negative fibers
S6	F	no	c.[324delG]; [498delC;508T>C]	p.[Arg109Alafs*3]; [Ser167Profs*20; Phe170Leu]	MPTb (fs)	birth	died at 9 months	no	NR	NR	CI↓, CII+III↓	CS↑	lipid-storage myopathy, absent SDH staining, several NADH- and COX- negative fibers

(Continued on next page)

Muscle Investigations													
ID	Sex	Consanguineous	FLAD1 Variants (GenBank: NM_025207.4)	FADS1 Variants (GenBank: NP_079483.3)	Affected Domain	Age of Onset	Status	Responsive to Riboflavin	Increased Blood Acylcarnitines ^a	Elevated Organic Acids ^a	Other		
											Respiratory Chain Activities ^b	Mitochondrial Enzyme Activities	Histology
S7	F	yes	c.[401_404delTTCT]; [401_404delTTCT]	p.[Phe134Cysfs*8]; [Phe134Cysfs*8]	MPTb (fs)	2 months	died at 4 months	NT	C4, C6, C8, C10, C10:1, C12, C14:1, C16, C16:1, C18:1	ethylmalonic and glutaric acids	NR	NR	lipid-storage myopathy, faint SDH staining, normal COX

Abbreviations are as follows: ↓, decrease; ↑, increase; C4, butyrylcarnitine; C4-OH, OH-butyrylcarnitine; C5, isovalerylcarnitine; C5-DC, glutarylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C10:1, decenoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C14:1, tetradecenoylcarnitine; C14:2, tetradecenoylcarnitine; C16:1, hexadecanoylcarnitine; C16, hexadecenoylcarnitine; C18, octadecanoylcarnitine; C18:1, octadecenoylcarnitine; C18:2, octadecenoylcarnitine; C18-2, octadecadienoylcarnitine; C1-CIV, complexes I-IV; COX, cytochrome c oxidase; CS, citrate synthase; EM, electron microscopy; F, female; FADS, FAD synthase domain; fs, frameshift; M, male; MPTb, molybdopterin binding domain; NR, not reported; NT, not tested; PDH, pyruvate dehydrogenase; and SDH, succinate dehydrogenase.

^aActual values are reported in Table S3.

^bActual values are reported in Table S5.

ID	Rate of FAD Synthesis (pmol/min/mg)
C1	4.37 ± 0.61
C2	4.36 ± 0.4
C3	3.66 ± 0.38
S1a	2.05 ± 0.59**
S2	1.57 ± 0.7***
S4a	1.04 ± 0.47***

Data represent the mean ± SD of three to four independent cell lysates. **p < 0.01, ***p < 0.001.

(p.Arg530Cys), affecting the FADS domain and a frameshift variant in exon 2 on the other allele. The four additional individuals were all found to carry biallelic frameshift variants in exon 1 or 2. Carrier testing showed that each parent analyzed was heterozygous for one allele (Figure 1A). None of the predicted LOF variants was present in an in-house database with WES data from approximately 6,000 individuals or in >120,000 alleles from the Exome Aggregation Consortium (ExAC) Browser (accessed January 2016). Only variants c.1588C>T (p.Arg530Cys) and c.508T>C (p.Phe170Leu) were recorded in the ExAC Browser; c.1588C>T was recorded as heterozygous three times in 121,410 alleles, and c.508T>C was recorded as heterozygous two times in 121,408 alleles. Of note, the affected individuals can be distinguished by their symptoms and the identified FLAD1 variants (Table 1 and the Supplemental Note). Whereas subjects S1b, S2, and S3 (who showed a milder clinical course and a remarkable response to riboflavin) carried at least one allelic variant that changes or deletes a single amino acid in the FADS domain, subject S4a (who showed only a partial response to riboflavin) and subjects S4b, S5, S6, and S7 (who had an early onset and lethal disease) all carried two predicted LOF variants affecting the MPTb domain.

FADS activity is expected to be essential for human metabolism, and no other protein with analogous function has been reported. Therefore, FLAD1 variants predicted to result in complete LOF were unexpected.

Residual FADS Activity and Flavin Content in FLAD1 Mutant Cells

The mild increase in metabolites derived from deficient multiple acyl-CoA dehydrogenases argues for only a partial defect in FAD synthesis and levels (Table S3). In accordance with this, mild but significant decreases in the rate of FAD synthesis were measured in cultured fibroblasts derived from the affected individuals, whose activity was 50% (S1a), 38% (S2), and 25% (S4a) of that of control individuals (Table 2). Analysis of flavin content in the same cell extracts and in blood from S4a and S4b supported remaining FADS activity in all investigated subjects and revealed FAD, FMN, and riboflavin amounts within the control

Table 3. FAD, FMN, and Riboflavin Quantification in Cellular and Mitochondrial Fibroblast Samples from Control and Affected Individuals

	ID	FAD	FMN	Riboflavin	FAD/FMN Ratio
Cellular flavin content (pmol/mg)	C1	111.7 ± 2.8	13.3 ± 1.4	2.6 ± 0.5	8.5 ± 0.7
	C2	171.2 ± 18.8	12.4 ± 0.3	3.1 ± 0.1	13.8 ± 1.9
	C3	105.8 ± 6.9	13.0 ± 0.0	2.1 ± 0.6	8.2 ± 0.5
	S1a	82.3 ± 8.6	11.7 ± 0.8	2.1 ± 0.8	7.0 ± 0.2
	S2	84.3 ± 3.1	11.5 ± 0.6	2.4 ± 0.9	7.3 ± 0.1
	S4a	111.4 ± 6.6	15.8 ± 0.4	2.5 ± 0.5	7.0 ± 0.6
Mitochondrial flavin content (pmol/mg)	C1	2,093	3.0 ± 0.1	NT	697.7 ± 24.0
	C2	1,940	3.2	NT	606.3
	C3	1,895	3.2	NT	592.2
	S4a	1,310 ± 70**	2.0 ± 0.7	NT	655.0 ± 195.7

For cellular flavin content, data represent the mean ± SD of two independent cell lysates. NT, not tested. **p < 0.01.

range but increased amounts of FMN in erythrocytes (Table 3 and Figure S3). Only when enriched mitochondrial fractions from subject S4a's fibroblasts were investigated were amounts of FAD and FMN found to be slightly but significantly reduced (Table 3). In accordance with decreased availability of mitochondrial FAD, subject S4a's fibroblast samples had reduced amounts of ETFDH and complex II, two mitochondrial dehydrogenases that use FAD as a co-factor (Figure S4). The amount of FAD in a skeletal-muscle biopsy from S5 (who, like S4a, carried biallelic frameshift variants in exon 2) was 145 nmol/L, slightly below the control range (174–471 nmol/L). Whereas reduced FADS activity in S1a and S2 can be explained by missense mutations, the residual FADS activity and the relatively high content of FAD in S4a and S5 suggest alternative mechanisms that allow expression of functional FADS from *FLAD1* variants predicted to result in complete LOF.

Immunoblot Analysis of *FLAD1* Mutant Cells

In order to investigate the molecular consequences of the identified *FLAD1* variants, we performed immunoblot analyses for FADS in cell extracts from cultured fibroblasts (from S1a, S2, and S4a) or from liver tissue (from S5) (Figure 2). Using antibodies recognizing the FADS C terminus, containing the FADS domain, we detected a number of bands, including three high-intensity bands that were expressed in both fibroblast and liver tissue: ~60 and ~50 kDa bands corresponding to the expected mitochondrial (isoform 1 [FADS1]) and cytosolic (isoform 2 [FADS2]) proteins, respectively (Figure 1B), and a ~26 kDa band. Mass spectrometry confirmed only the ~50 and 26 kDa bands to be FADS, and only peptides located downstream of the MPTb domain encoded by exon 2 were detected in the 26 kDa band (Figure 2A and 2C). As predicted by the identified variants, individuals S4a and S5, both homozygous for frameshift variants in exon 2, showed no detectable FADS2. In contrast, subject S1a, homozygous for a single amino acid deletion (p.Ser495del) in the

FADS domain, showed FADS2 amounts within the control range. Notwithstanding, in subject S2, in whom one allele is predicted to cause an amino acid change (p.Arg530Cys) in the FADS domain and a late-onset riboflavin-responsive MADD phenotype, immunoblot analysis also revealed severely decreased amounts of FADS2. The observation that all mutations affect only the FADS2 band argues for unspecific cross-reactive signals at 60 and 26 kDa. Nonetheless, the findings of unique FADS peptides in the 26 kDa band suggest that at least a minor amount of isoform(s) might add to the observed residual FADS activity. A faint 26 kDa band was repeatedly observed in liver tissue from S5, but not in control liver (Figure 2B), suggesting that compensatory increases in FADS isoforms are masked by unspecific signals in fibroblast cells (Figure 2A). However, because of the limited amount of liver tissue, we were not able to verify the identity of the 26 kDa band. Two shorter isoforms (isoform 3 [GenBank: NM_00114891] and isoform 4 [GenBank: NM_00114892]) have been reported in RefSeq (accessed January 2016). It is not likely that the 26 kDa protein corresponds to any of these, because both short isoforms contain sequences specific to the MPTb domain. This argues for alternative mechanisms by which residual FADS activity is produced in *FLAD1* mutant cells.

Alternative *FLAD1* Transcripts and Activation of Downstream ATG Start Sites Might Rescue LOF Variants

Because most of the investigated individuals carried frameshift variants in exon 2, we were interested in the mutation load of *FLAD1*. We therefore divided *FLAD1* into two parts: one 1,117 bp fragment covering exons 1 and 2 and coding for the MPTb domain and a second 647 bp fragment covering exons 3–7 and coding for the FADS domain. Although the ExAC Browser records an equal distribution of missense and synonymous variants among the first (0.178 variants/bp) and second (0.156 variants/bp) parts of the gene, it lists 47 alleles with predicted LOF variants

Table 4. Kinetic Properties of 6His-p.Ser495del, 6His-p.Arg530Cys, and WT FADS2

Forward Reaction	6His-p.Arg530Cys	6His-p.Ser495del	WT
K_m FMN (μ M)	0.39 \pm 0.07	0.98 \pm 0.23***	0.32 \pm 0.10
K_m ATP (μ M)	4.63 \pm 1.00*	8.02 \pm 2.23	8.10 \pm 1.37
V_{max} (nmol·min ⁻¹ ·mg ⁻¹)	7.9 \pm 3.7***	10.2 \pm 1.6**	43.3 \pm 12.2
Mg^{2+}_{50} (mM)	0.04 \pm 0.02*	0.09 \pm 0.01	0.07 \pm 0.01

Data represent the mean \pm SD of three experiments. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

affecting exon 1 or 2 and only two LOF variants affecting exons 3–7. This significant accumulation of LOF variants in exons 1 and 2 (47 LOF variants within 1,117 bp) rather than in exons 3–7 (two LOF variants within 647 bp, $p = 10^{-7}$, Poisson test) suggests alternative mechanisms resulting nonetheless in functional FADS.

It has been shown that the FADS domain alone, without the MPTb domain, is able to mediate FADS activity.¹¹ Because the frameshift variants affect the MPTb domain, we speculated whether additional splice variants or a re-initiation of translation using a downstream ATG codon could encode an active FADS. A prerequisite for re-initiation of translation would be a stable *FLAD1* transcript. qRT-PCR analysis from subject S4a's fibroblast cells with frameshift variants on both alleles revealed stable *FLAD1* transcript levels, which were reduced only by about 50% in comparison to control levels (Figure S1), indicating that the transcripts were only partially degraded by nonsense-mediated decay. Likewise, sequence analysis of *FLAD1* RT-PCR products amplified from individual S2's fibroblasts showed heterozygosity for the c.1588C>T variant, indicating that the C allele containing c.568_569dupGC in exon 2 was stably expressed (data not shown).

The human *FLAD1* transcript contains two in-frame downstream ATG codons located between the frameshift variants and the region encoding the FADS domain (Figures 1B and 3A). Theoretically, these truncated FADS proteins, starting at Met268 or Met355, could be translated from the variant *FLAD1* transcripts and thereby ensure cytosolic FAD synthesis. On the basis of the idea of truncated products with an active FADS domain, three different constructs were expressed in primary fibroblast cells from a control individual: the full-length construct starting at Met98, a second construct starting at Met268, and a third construct at Met355. All of the *FLAD1* constructs were expressed and resulted in stable proteins easily detected by immunoblotting (Figure 3C). When analyzing FADS activity in cell extracts, we found that expression of the Met268 construct resulted in a FAD synthesis rate higher than that of control cells (data not shown). These data support the possibility that re-initiation of translation using an in-frame ATG codon would result in a stable protein containing an active FADS domain. However, the Met268 and Met355 proteins did not migrate with a mass similar to that of any of the FADS bands detected by immunoblot

analysis of case and control fibroblasts, including the 26 kDa band (Figure 3C).

We next investigated the possibility of alternative transcripts by analyzing RNA sequencing data generated from two fibroblast samples (C1 and C3) or a whole-blood RNA sample (C2). Figure 3A summarizes in Sashimi plots all detected RNA splice variants. In both tissues, FADS2 (comprising exons 1a and 1b) represents the most abundant transcript; the next most-abundant transcript is missing exon 1a but still encodes the same cytosolic protein isoform of 54 kDa. In none of the investigated samples did we find evidence of FADS1, which covers all of exon 1 and retains intron 1a, or isoforms 3 (GenBank: NM_00114891) and 4 (GenBank: NM_00114892) reported in RefSeq. These observations are in agreement with the immunoblotting experiments (Figure 2). In control fibroblast C3, 27 out of 28 reads starting with exon 1a continued in exon 1b; however, one paired read detected a transcript variant covering exon 1a, including an ATG codon directly connected in frame to the open reading frame in exon 3 (isoform 5 in Figure 3A). Isoform 5, missing exons 1b and 2, has the potential to encode a 26 kDa functional FADS missing the MPTb domain. In addition, we found a substantial number (11) of mRNA reads directly connecting exons 1a and 2 (whereas 114 reads connected exons 1b and 2). None of them included the ATG codon in exon 1a. This isoform, now isoform 6, is predicted to code for the FADS starting with Met268. In order to validate the presence of isoform 6, we performed an RT-PCR experiment by using primers specific to exons 1a and 2. Unexpectedly, we found substantial amounts of both isoforms 2 and 6 (Figure 3B). None of the identified frameshift variants in the ExAC Browser or the frameshift variants presented here, which are predicted to be pathogenic considering isoforms 1 and 2, would affect the FADS encoded by isoform 6. Immunoblot analysis using case and control fibroblasts failed to detect the predicted FADS proteins starting with Met268 or Met355, but it did identify a 26 kDa band containing two FADS peptides located downstream of the MPTb domain (Figures 2 and 3C). The FADS uniqueness of these peptides to the human proteome, as confirmed by bioinformatics analyses, strongly suggests that the 26 kDa band indeed contains FADS (see Subjects and Methods). We speculate that the 26 kDa band is based on isoform 5 and/or a proteolytically processed version of the Met268 isoform 6 and has an

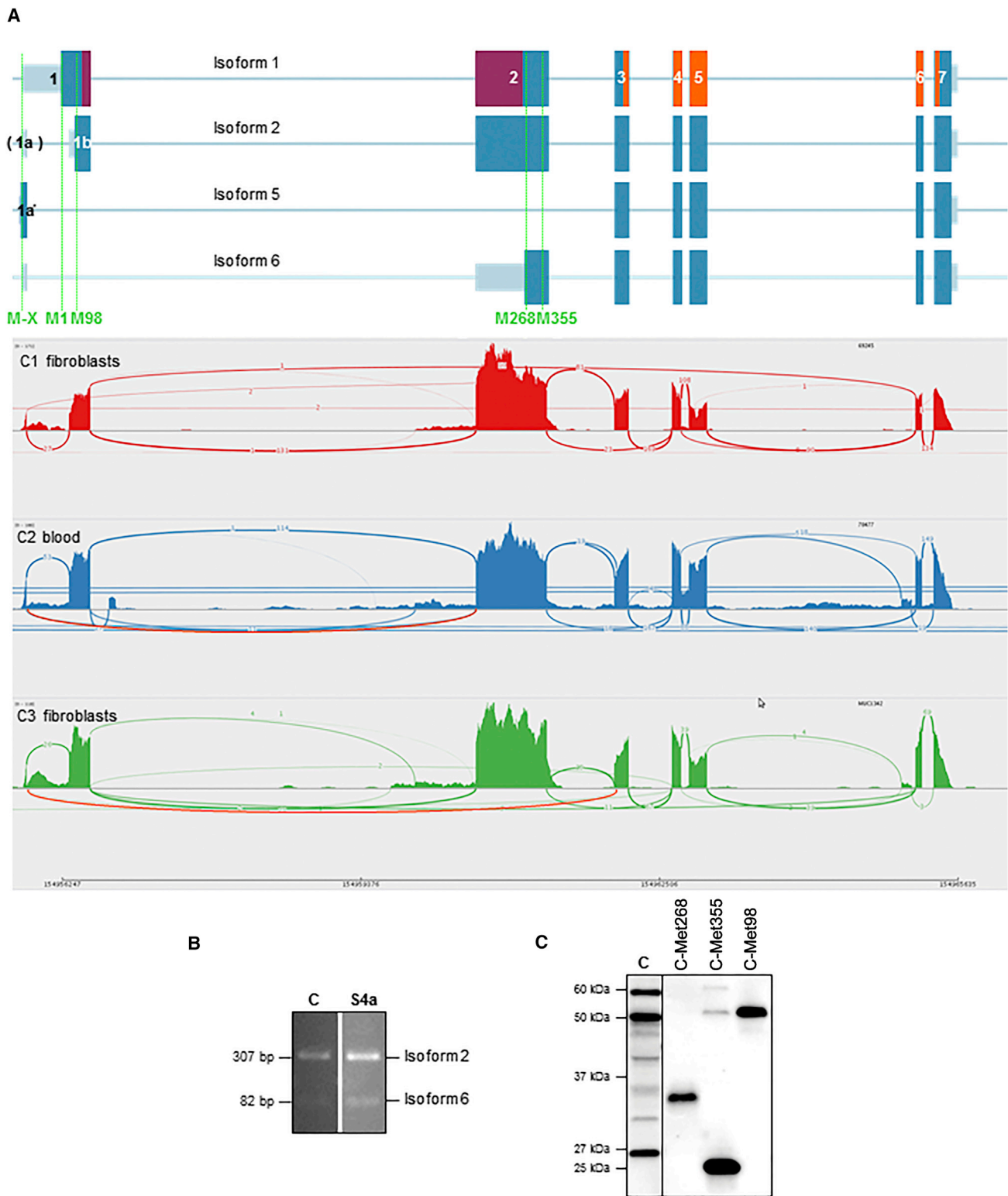


Figure 3. Alternative *FLAD1* Transcripts and Their Predicted Products

(A) Analysis of transcriptome data for *FLAD1* from two unrelated fibroblast control samples (C1 and C3) and one human blood sample (C2) revealed several possible transcripts, summarized as Sashimi plots (bottom). Schematic diagram shows isoform transcript structures with MPTB and FADS domains marked in violet and orange, respectively (top). The most abundant transcript (isoform 2) corresponds to the sequence reported in RefSeq (GenBank: NM_201398.2) but has an alternative intron in exon 1 and lacks the predicted MTS. Isoforms 5 and 6 represent transcripts without exons 1b and/or 2, where the identified *FLAD1* frameshift variants are located, but both are able to express an active FADS domain. Isoform 5 can use the Met marked as M-X in the very beginning of the transcript, whereas isoform 6 utilizes Met268. In RNA from fibroblasts or blood, we did not identify a single transcript resulting in isoforms 1 (GenBank: NM_025207.4), 3 (GenBank: NM_00114891), or 4 (GenBank: NM_001184892) as reported in RefSeq. Isoforms 3 and 4 are not shown.

(legend continued on next page)

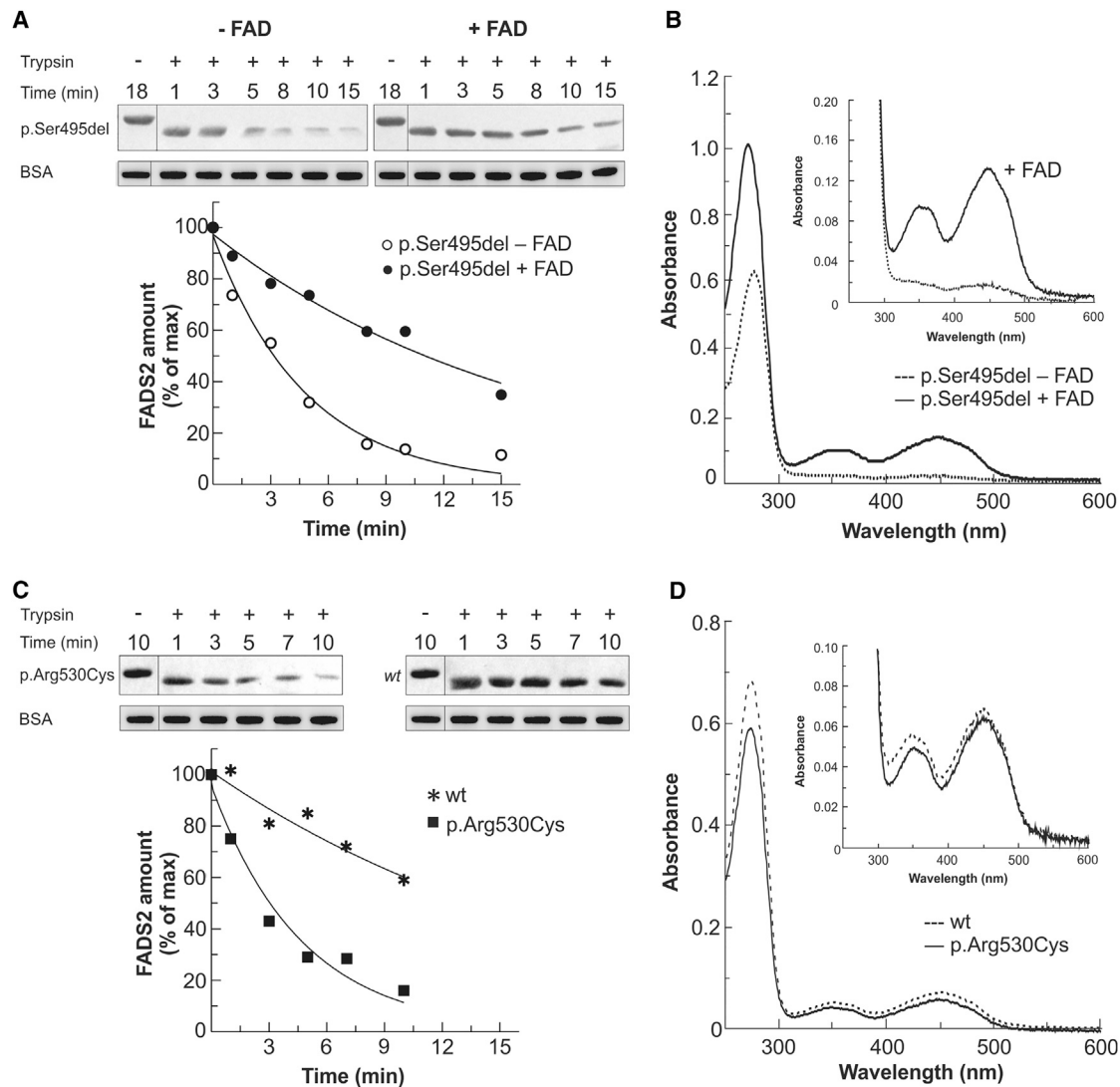


Figure 4. Characterization of Recombinant 6His-p.Arg530Cys and 6His-p.Ser495del FADS2 Proteins

(A and B) The trypsin sensitivity (A) and degree of FAD saturation (B) of 6His-p.Ser495del FADS2 are reported in comparison with those of the same protein after reconstitution with a 2.5-fold molar excess of FAD. In (B), spectra of both 6His-p.Ser495del FADS2 purified in its apoform (0.58 mg/mL protein concentration, dashed line) and the reconstituted holoform (0.64 mg/mL protein concentration, black line) are reported.

(C and D) The trypsin sensitivity (C) and degree of FAD saturation (D) of 6His-p.Arg530Cys FADS2 are reported in comparison with those of WT FADS2. In (D), spectra of both 6His-p.Arg530Cys FADS2 (0.44 mg/mL protein concentration, straight line) and WT protein (0.5 mg/mL protein concentration, dashed line) are reported.

Trypsin sensitivity was analyzed by immunoblotting. The control represents protein treated under the same condition but in the absence of trypsin (-). The slower migration of the control band might reflect that the 6His tag is rapidly removed upon addition of trypsin. BSA was added to the loading buffer as an internal standard. In the graph, the time course of the limited proteolysis is reported as a percentage of the control amount (arbitrarily set to 100%). The degree of FAD saturation was estimated from the UV/Vis absorbance spectra.

progressive neurological signs, involving motor, sensory, or cranial neuropathies that are seen in the more recently identified individuals diagnosed with Brown-Vialetto-Van Laere syndrome (MIM: 211530), involving defects in RFVT-encoding genes.^{32,35,51-53} When investi-

gated, individuals with *FLAD1* variants showed no hearing or visual impairment, and their peripheral-nerve conduction velocity was normal. Typical clinical phenotypes were hypotonia and swallowing and speech difficulties. Scoliosis was reported in three individuals, and many

(B) PCR products of cDNA from control subjects and subject S4a confirmed the presence of non-degraded *FLAD1* mRNA (all details are provided in Figure S1). The primer pair is able to amplify either isoform 2 or isoform 6 of the transcript, resulting in 307 or 84 bp products, respectively.

(C) Immunoblot analysis of the different FADS isoforms (Met98, Met268, and Met355) overproduced in fibroblasts derived from a healthy control individual shows their stability and detectability by anti-FADS antibody. A control fibroblast sample (C) is shown for comparison.

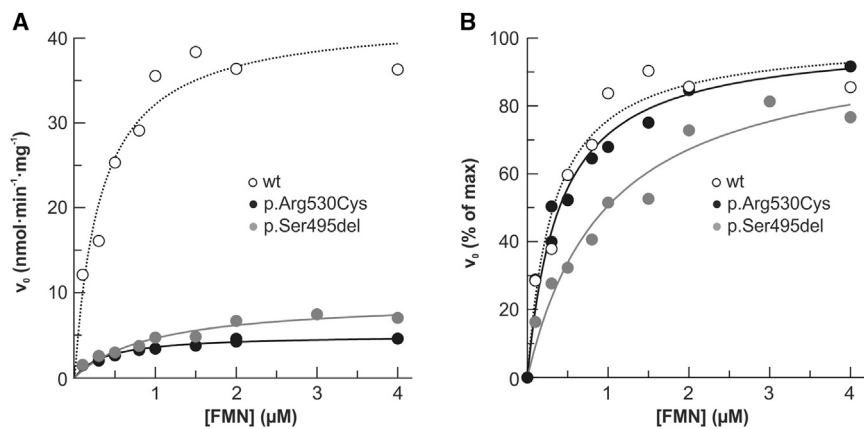


Figure 5. Kinetic Characterization of Recombinant 6His-p.Arg530Cys and 6His-p.Ser495del FADS2 Proteins

The dependence of FMN concentration on the rate of FAD synthesis catalyzed by WT (0.17 nmol), 6His-Arg530Cys (0.17 nmol), or 6His-Ser495del (0.18 nmol) FADS2. The FAD synthesis catalyzed by WT FADS2 is presented as open symbols. Mutant FADS is presented as closed symbols. The rate of FAD synthesis was measured in the presence of 100 $\mu\text{mol/L}$ ATP and 5 mmol/L MgCl_2 . v_0 was measured by the initial rate of fluorescence decrease (excitation at 450 nm and emission at 520 nm) and expressed in $\text{nmol FMN} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ mutant FADS2 (A) and as a percentage of the V_{max} value (set arbitrarily to 100%) (B). Data points are fitted according to the Michaelis-Menten equation.

developed respiratory insufficiencies or arrest. Cardiomyopathy was reported only in a single individual, and two required pacemaker implantation because of tachycardia or sudden cardiac arrest. Most subjects presented with disease in early infancy, and four died within the first year of life or in late childhood. Four subjects are still alive, and three (S1b, S2, and S3, aged 22, 44, and 56 years, respectively) differ from the others because they have gene variants affecting a single amino acid in the FADS domain, share a milder clinical course, and are responsive to treatment with riboflavin.

The clinical heterogeneity is mirrored by an allelic series of variants, including transcript-specific frameshift variants and variants affecting only a single amino acid. FAD is an essential cofactor, and FADS is the only known human enzyme catalyzing the synthesis of FAD from FMN. The unexpected *FLAD1* frameshift variants led us to discover previously undescribed transcript isoforms. *FLAD1* codes for a protein consisting of MPTb and FADS domains. All of the identified frameshift variants affect the MPTb domain. Early in evolution, these two domains appeared as separate proteins, encoded by two different genes, whereas in mammals they appear as one protein containing two domains.¹ However, the FADS domain was shown to function independently from the other domain.¹¹ Indeed, we have discovered *FLAD1* isoforms that code for only the FADS domain (isoforms 5 and 6 in Figure 3). The existence of these isoforms might explain why affected individuals with biallelic *FLAD1* frameshift variants still harbor substantial FADS activity (Table 2). However, despite residual FADS activity, homozygous carriers of frameshift variants are affected, and their disease seems more severe than that observed in individuals harboring single amino acid changes in the FADS domain (Table 1). This could be explained by at least four different mechanisms. (1) the mRNA expression levels of FADS isoforms 5 and 6 were found to be low, and although we did not find conclusive evidence of their protein products in fibroblasts from case and control individuals, protein mass spectrometry of the 26 kDa band supports their exis-

tence (Figures 2 and 3). Determining the exact identity of the 26 kDa band will require further studies. (2) We only studied blood and fibroblast transcriptomes, and we have no information about the isoform expression pattern in the affected (muscle) tissue. (3) Although, there might be sufficient FADS activity, the isoforms lack the uncertain but potentially important activity of the MPTb domain. Indeed, it has been recently suggested that the MPTb domain possess FAD hydrolase activity¹⁰ and might be needed for optimal FAD release to apoproteins.^{9,12,54} Addressing this question will require more experiments. (4) Finally, even though our protein and transcriptome data do not support substantial expression of the mitochondrial *FLAD1* isoform 1, the mitochondrial isoform might be expressed in other tissues or in other experimental conditions where direct interaction between FADS and apoproteins is needed to ensure sufficient or catalytically controlled flavinylation of mitochondrial proteins.^{9,54} Loss of isoforms 1 and 2 could result in disturbed subcellular distribution of FADS. Isoforms 5 and 6 do not harbor a MTS. Although some polypeptides can reach mitochondria without a cleavable MTS,⁵⁵ the frameshift variants in the MPTb domain most likely result in deficiency of mitochondrial FADS. Mitochondrial biogenesis and function in these cells would then depend on supply from cytosolic FAD synthesis. In accordance with this idea, cellular FAD amounts within or slightly below the control range were observed in all investigated subjects. Significant decreases in FAD content were found only in enriched mitochondrial fractions from subject S4. Moreover, the lipid storage and decreased activity of respiratory-chain enzymes in muscle homogenates from several individuals (Tables 1, S4, and S5) points specifically to a mitochondrial defect. In subject S4a, this was accompanied by decreased amounts of ETFDH and the SDHA flavo-component of complex II (Figure S4). Similarly, we would expect amounts of other mitochondrial flavoproteins, such as acyl-CoA dehydrogenases, to be decreased as well.^{20–23} Even though this was not investigated, all reported individuals with variant *FLAD1* genotypes showed

MADD or ethylmalonic and/or adipic aciduria (Tables 1 and S3), which argues for a more general impairment in flavinylation of mitochondrial proteins under these conditions. Decreased activity of complex II combined with MADD biochemistry was also observed in a recently reported subject with riboflavin-responsive exercise intolerance due to a defect in mitochondrial FAD transport.³³

Variants affecting only a single amino acid were detected in four families. The c.508T>C (p.Phe170Leu) missense variant identified in F6 in *cis* with a frameshift variant most likely does not contribute to the FADS deficiency. In families F2 and F3, we discovered a c.1588C>T (p.Arg530Cys) missense variant, and in a family F1, we found a 3 bp deletion (c.1484_1486del [p.Ser495del]); both of these affect the FADS domain. In vitro studies demonstrated that these variants result in impaired but detectable FADS activity (Figure 5). At least for the deletion variants, we could show that incubation with a molar excess of FAD significantly improved protein stability (Figure 4), arguing for a chaperone-like action of FAD, similar to what has been reported in a number of other metabolic disorders caused by gene variations (mainly of the missense type) in proteins using FAD as a cofactor.^{15–19,56,57} Concluding on the effectiveness of riboflavin treatment in individuals with different types of *FLAD1* variants will require further in vitro studies of *FLAD1* genotypes and a larger group of individuals with longer follow up. However, because there are no reported side effects of riboflavin, all *FLAD1*-defective individuals should initially be treated with riboflavin no matter their type of mutation. The fatal outcome of the untreated brother of an older sister (S1a), who responded to riboflavin, stresses the importance of early diagnosis and treatment in these disorders.

In conclusion, our studies have identified *FLAD1* variants as a cause of potentially treatable myopathies associated with MADD. This further establishes the heterogeneous etiology of MADD; some have ETF and/or ETFDH variants, and others have defects in genes responsible for cellular uptake of riboflavin, synthesis of flavin cofactors, or mitochondrial FAD transport. Because there are still unsolved cases of riboflavin-responsive MADD, it can be expected that the list of involved genes will still grow in the future. Besides being important for the pathomechanism of *FLAD1* defects, our findings also stress that care should be taken in the interpretation of predicted LOF variants in large-scale sequencing projects. Sometimes LOF variants affect only specific isoforms, and such findings do not allow conclusions that the particular gene is not essential.

Accession Numbers

The accession numbers for the nine *FLAD1* variants reported in this paper are ClinVar: SCV000266355, SCV000266356, SCV000266357, SCV000266358, SCV000266359, SCV000266360, SCV000266361, SCV000266362, and SCV000266363.

Supplemental Data

Supplemental Data include a Supplemental Note, four figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2016.04.006>.

Acknowledgments

We thank all affected individuals and their families for their participation and for providing important samples for the present research study. We thank Dr. Francesco De Cillis for his participation in the early stages of this work, Dr. James Pitt for biochemical evaluation of individuals S1a and S1b, Dr. Norma Romero for muscle histology analysis of individual S7, and Dr. Maria Teresa Balsimelli, Dr. Carla Cozzolino, Prof. Vincenzo Nigro, and Dr. Filippo Maria Santorelli for their clinical care and biochemical evaluation of individual S6. The study was supported by the Aarhus County Research Initiative, the Danish Council of Independent Medical Research (4004-00548 to R.K.J.O.), the John and Birthe Meyer Foundation (N.G.), the European Commission FP7-PEOPLE-ITN Mitochondrial European Educational Training Project (GA 317433 to R.H., J.A.M., and H.P.), the Neuromuscular Research Association Angela Maria Nieddu (L.V.), the Telethon Genetic BioBank (GTB12001D to E.P.), the EuroBioBank network (E.P.), the Telethon Foundation (grant GGP15041 to D.G.), the Italian Ministry of Health (GR2010–2316392 to D.G.), the Programma Operativo Nazionale “Ricerca e Competitività” 2007–2013 (01_00937 to M.B.), the European Research Council (309548 to R.H.), the UK Medical Research Council (G1000848 to R.H.), the German Bundesministerium für Bildung und Forschung (BMBF) through the German Network for Mitochondrial Disorders (mitoNET; 01GM1113C to T.M. and H.P.), the E-Rare project GENOMIT (01GM1207 to T.M. and H.P.), and the Juniorverbund in der Systemmedizin “mitOmics” (FKZ 01ZX1405C to T.B.H.).

Received: January 22, 2016

Accepted: April 13, 2016

Published: June 2, 2016

Web Resources

ClinVar, <http://www.ncbi.nlm.nih.gov/clinvar/>
ExAC Browser, <http://exac.broadinstitute.org/>
ExPasy ProtParam, <http://web.expasy.org/protparam/>
Integrative Genomics Viewer, <https://www.broadinstitute.org/igv/>
MutationTaster, <http://www.mutationtaster.org/>
OMIM, <http://www.omim.org/>
RefSeq, <http://www.ncbi.nlm.nih.gov/refseq/>
Skyline, <https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/>
UCSC Genome Browser, <http://www.genome.ucsc.edu/>
UniProt, <http://www.uniprot.org/>

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Supplemental Data

Riboflavin-Responsive and -Non-responsive Mutations in FAD Synthase Cause Multiple Acyl-CoA Dehydrogenase and Combined Respiratory-Chain Deficiency

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Supplemental Note: Case Reports

Family 1 (F1): Subject S1a, a boy of Turkish consanguineous parents presented with cardiorespiratory collapse at 32 hours of life and died 3 days later. A metabolic post-mortem analysis, done immediately after his death, showed elevated C5-, C8-, C14-carnitines and slightly elevated C5-DC/glutaryl-carnitine in blood spots. Urine organic acids revealed markedly elevated ethylmalonic acid and adipic acid, elevated suberic and dehydro-sebacic acids and slightly elevated hexanoylglycine levels. ETF and ETFDH activities in cultured fibroblasts were within control range. Muscle and liver respiratory chain complexes activities were within control range. Family history revealed an older sister (individual S1b), who had been treated for cardiomyopathy in the first year of her life. Screening of this girl showed elevated C4-carnitine on a blood acylcarnitine profile, and urine organic acids showed elevation of ethylmalonic acid and trace methylsuccinic acid. After treatment with riboflavin all metabolites normalized. Compliance with treatment was variable; in periods without treatment high urinary ethylmalonic acid was found, accompanied by episodes of supraventricular tachycardia. She was treated with riboflavin again and urinary metabolites decreased to control range. She is currently 22 years old and doing well with a pacing/defibrillator device. There were four miscarriages between individual S1a and his sister, S1b.

Family 2 (F2): Subject S2's developmental milestones were normal. During childhood she was able to run but was slower than her peers and experienced symptoms of illness during long-lasting exercise. First evident muscle weakness was observed at age 20 years as she carried heavy bags. Thereafter, she experienced muscle discomfort and weakness during physical activity, and after strenuous exercise she had occasional muscle pain, vomiting and loss of weight. She had tachycardia and used beta blocking medication. During her pregnancy at age 30, one month before the delivery, her muscle weakness drastically deteriorated with a raise in creatine kinase (CK) activities ranging between 5000-6612 U/l (control range 35-210 U/l). It was difficult for her to control her head. She had swallowing and speech difficulties and a prominent scoliosis. No hearing or visual impairments were found. She was advised to

follow a high carbohydrate, moderate protein and low fat diet (dietary fat max. 20 g/day), which ameliorated her symptoms. Blood analysis revealed increases in C8- and C10-acylcarnitine species, and urine analysis showed increased amount of lactate, mildly increased ethylmalonic acid, an increased 2-hydroxyglutarate combined with a low/normal 2-ketoglutarate excretion that was consistent with MADD. Fibroblast fatty acid oxidation flux studies and western blot analysis of ETF and EFTDH protein were found to be within control range. Multiple respiratory chain deficiency with lipid storage myopathy was detected in a muscle biopsy. Riboflavin therapy (100 mg daily) was introduced, and she continued the dietary therapy. This resulted in drastic amelioration of muscle symptoms, and increase in muscle strength. A more comprehensive description of subject S2 with long-term treatment follow-up studies will be published elsewhere.

Family 3 (F3): Subject S3 is a 56 year-old woman, who was born at term after an uneventful pregnancy from non-consanguineous Italian parents. Early psychomotor development was normal. In the teens a mild scoliosis was diagnosed that did not required surgical or medical correction. At 45 years the affected individual presented with exercise intolerance, gait difficulties, weakness in both arms and muscle hypotrophy prevalent in the shoulder girdle. Her symptoms progressed since then and prompted neurological evaluation at age 46. Neurological examination revealed a mild waddling gait with bilateral foot drop. Standing or walking on heels was impossible. Scapular winging with hypotrophy of upper limbs, calf pseudohypertrophy and scoliosis were noticed. She was not able to lift her arms to the horizontal level, but was able to rise from the floor with Gowers maneuver. Lower limb weakness, graded using the medical research council (MRC) scale, involved iliopsoas and tibialis anterior muscles (4/5 MRC). In the upper limb, deltoid and teres muscles were the weakest (3+/5 MRC), while biceps and triceps were mildly affected (4+/5 MRC). A mild weakness in the orbicularis oris was observed. A needle EMG showed mild myopathic changes in the proximal muscles. CK was mildly elevated at 380 U/L (control range <170 U/L). Acylcarnitine profile showed increased C5-, C8-, C10-, C10:1-, and C14-

carnitines in blood and glutaryl-carnitine in urine. Urine organic acid revealed elevated ethylmalonic acid and presence of tiglylglycine. A muscle biopsy showed a vacuolar myopathy with fiber size variation. No muscle fiber necrosis or regeneration was observed. Oil Red O staining showed lipid storage myopathy. Several cytochrome c oxidase (COX) negative fibers were also observed. Fatty acid oxidation analysis in a fresh muscle biopsy showed a reduced oxidation of C8 and C16 but not of C4. Activity of CI+CIII and CII+CIII complexes were reduced compared to control, whereas citrate synthase activity was augmented. Heart and respiratory evaluations were normal. Lipid storage myopathy was diagnosed and supplementation with riboflavin (100 mg/day) and carnitine (2 g/day) was initiated. At follow up evaluation, 7 months later, the affected individual reported a complete resolution of exercise intolerance and improvement of muscle strength. She was able to walk on her heels, to lift her arm above the horizontal level, and to rise from the floor with minimal support of one hand. Muscle weakness was limited to deltoid, teres, and iliopsoas muscles and graded 4+/5 MRC. Muscle hypotrophy and scapular winging did not improve. An acylcarnitine/free carnitine ratio within control range, and normal respiratory chain and citrate synthase activities were found in a post-treatment muscle biopsy, but with reduced total carnitine (7.6 nM/mg protein; control range: 10.5-29.5 nM/mg protein). Currently, 10 years after initial diagnosis, she is stable and still on riboflavin and carnitine supplementation.

Family 4 (F4): S4a, a girl of consanguineous Turkish parents, presented after term delivery and uneventful neonatal period with increasing muscle hypotonia within the first months of life. Metabolic work up showed moderate elevation of acylcarnitines from C4 - C18:2 without the full pattern of MADD. Urine organic acids revealed variable ketosis and excretion of adipic and suberic acids in early age and moderate ethylmalonic acid and methylsuccinic acid excretion later. Activities of muscle respiratory chain enzymes were reduced, mainly for complexes I, II. Pyruvate dehydrogenase was also reduced. Family history revealed an older sister (affected individual S4b), who also suffered from severe muscle

hypotonia, which was diagnosed in the first year of life after term delivery and uneventful neonatal period. Metabolic follow-up showed either normal acylcarnitines, but also a measurement with an increase of multiple acylcarnitines (C4-OH, C5, C6, C8, C10, C14:1, C16:1). Variable mild ketosis was seen in organic acid evaluation. Both girls presented with severe muscle hypotonia and weakness, and S4b developed massive scoliosis. Tube feeding was necessary to avoid aspiration. Both sisters had reduced speech capacity. S4b was mentally able to finish elementary school training, whereas her sister S4a attended special school due to global developmental delay. Brain MRIs performed at the age of 6 years (S4a) or 16 years (S4b) revealed no abnormalities. Investigations of nerve conduction velocity (NCV) of peripheral nerves (tibial nerve, median nerve) were normal. No hearing or visual impairments were found, however, eyelid ptosis was observed on both eyes. Riboflavin (3x 60 mg daily) was introduced in the treatment of S4a, but could not correct biochemical abnormalities such as acylcarnitine profiles. Clinical improvement of hypotonia was however obvious. The sister was not treated with riboflavin. Unfortunately, S4b died at the age of 16 years after pneumonia and subsequent septicaemia. The family also had one older son, who died at the age of 4 years with similar symptoms and suspicion of mitochondrial disorder as well, but no detailed clinical or biochemical data are available (S4c). There is also one healthy son in this family, who is heterozygous for the *FLAD1* mutation.

Family 5 (F5): Subject S5 is a boy, who was born at 31st week of gestation following a twin pregnancy from Turkish consanguineous parents. He presented with poor sucking and hypotonia in the first few months of life, and was hospitalized due to pulmonary infection at age 6 months. During the hospital course, he developed sudden cardiac arrest few times. Holter monitoring revealed pause and pacemaker was implanted. Multiple respiratory chain deficiency with lipid storage myopathy and faint COX staining was seen in skeletal muscle (Figure S2). Blood analysis revealed increase in C3, C5, C6 and C8:1 acylcarnitine species with a low free carnitine, and urine analysis showed increased amount of adipic acid. He died of multiorgan failure at 7 months of age. Post-mortem examination showed mild

macrovascular steatosis in the liver, acute lung injury, nephrolithiasis and hemorrhagic necrosis of the kidney and spleen. There were no features of cardiomyopathy and COX staining was normal in heart (Figure S2).

Family 6 (F6): Subject S6 is a girl born to Italian non-consanguineous parents at 33 weeks after pregnancy complicated with uterine contractions. The mother had a previous miscarriage at 10 weeks. Soon after birth she presented with hypotonia, poor sucking, frequent vomiting episodes, and gastro-esophageal reflux. She was repeatedly hospitalized because of bronchiolitis and severe respiratory insufficiency. Due to persistent hypotonia with no head control, a suspicion of muscle disease was raised at age 5 months, and the girl underwent muscle biopsy. The spectrophotometric determination of respiratory chain complex activities in the muscle biopsy displayed a severe reduction of complex II and complex II+III activities, with a marked increase of citrate synthase, suggesting mitochondrial proliferation. She was treated with riboflavin, but with bad compliance and without response. She died aged 9 months due to septic shock following pneumonia.

Family 7 (F7): Subject S7, a girl of Turkish consanguineous parents, presented with severe neonatal hypotonia and muscle weakness with elevated plasma CK activity (717 U/L – control range < 240). When she was 2 months old she was hospitalized for swallowing difficulties, requiring nasogastric feeding. Outcome was severe with a sudden respiratory deterioration at 4 months of age, requiring non-invasive ventilation and leading to death. Acylcarnitine profile in plasma revealed increase in C10-, C12-, C14:1-, C16:1-, C16- and C18:1-carnitines and to a lesser extent in C4-, C6-, C8- and C10:1-carnitines. Urine organic acids revealed markedly elevated ethylmalonic and 2-hydroxyglutaric acid. Muscle biopsy revealed major lipid accumulation and a faint SDH staining with normal COX.

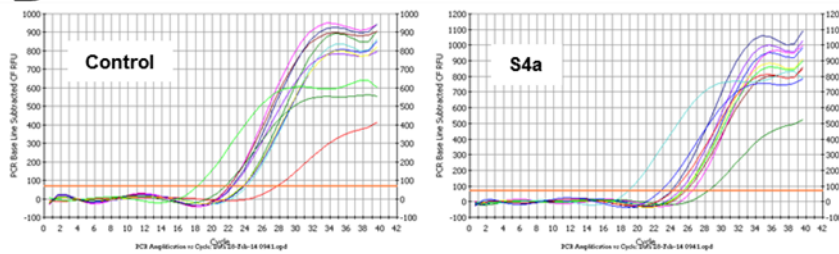
Figure S1. Quantitative Real Time PCR of *FLAD1*

A

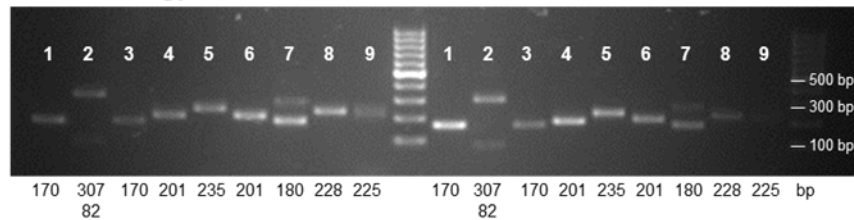
Reaction	Size [bp]	ct control	ct patient	Δ ct control	Δ ct patient	$\Delta\Delta$ ct control-patient
1-FLAD1	170	23,6	25,4	3,8	4,7	-0,9
2-FLAD1	82 (307)	24,0	26,4	4,2	5,7	-1,5
3-FLAD1	170	23,7	25,6	3,9	4,9	-1,0
4-FLAD1	201	22,0	23,9	2,2	3,2	-1,0
5-FLAD1	235	22,0	24,9	2,2	4,2	-2,0
6-FLAD1	201	22,4	25,3	2,6	4,6	-2,0
7-FLAD1	180	22,5	24,1	2,7	3,4	-0,7
8-FLAD1	228	23,6	25,3	3,8	4,6	-0,8
9-FLAD1	225	27,7	28,3	7,9	7,6	0,3
RPL27	179	18,2	18,8			
HPRT	137	21,5	22,7			

mean $\Delta\Delta$ ct
-1,1

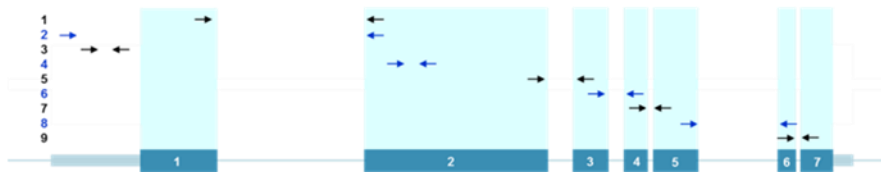
B



C

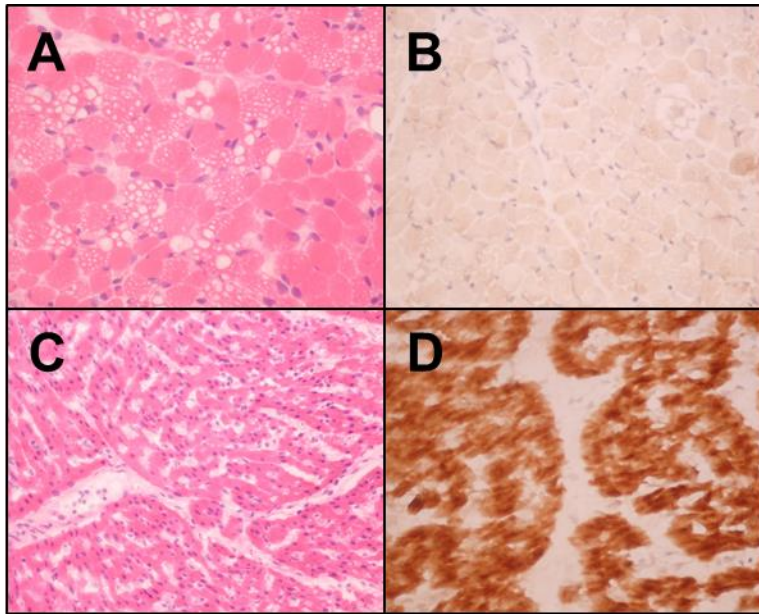


D



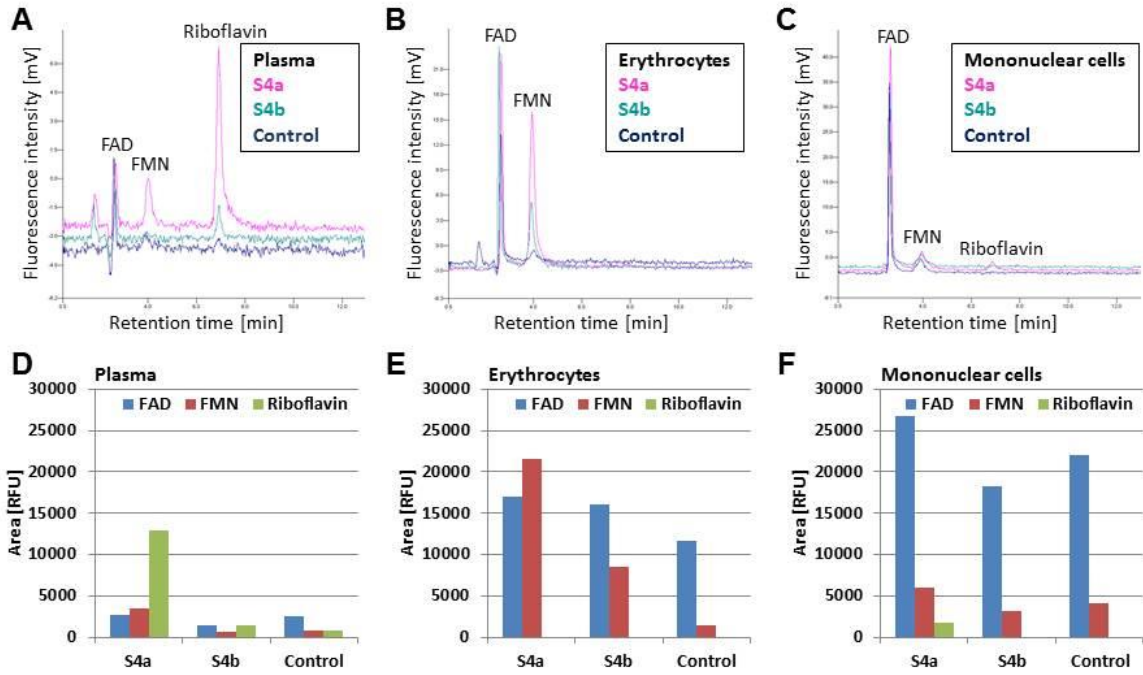
- (A) Quantitative real time PCR (qRT-PCR) of fibroblast cDNA from control and affected individual S4a identified reduction of *FLAD1* mRNA in samples from S4a compared to the control. qRT-PCR was analyzed using the comparative CT method. ct is the PCR cycle at which the PCR fluorescent signal crosses the threshold. Δ ct = ct(*FLAD1*) – ct(mean of reference genes). $\Delta\Delta$ ct = Δ ct(affected individual) - Δ ct(control). RPL27 and HPRT are stably expressed reference genes.
- (B) All single qRT-PCR products were amplified later in S4a in respect to the control.
- (C) qRT-PCR products visualized on the agar gel.
- (D) Schematic representation of primer bindings.

Figure S2. Muscle Histology in S5



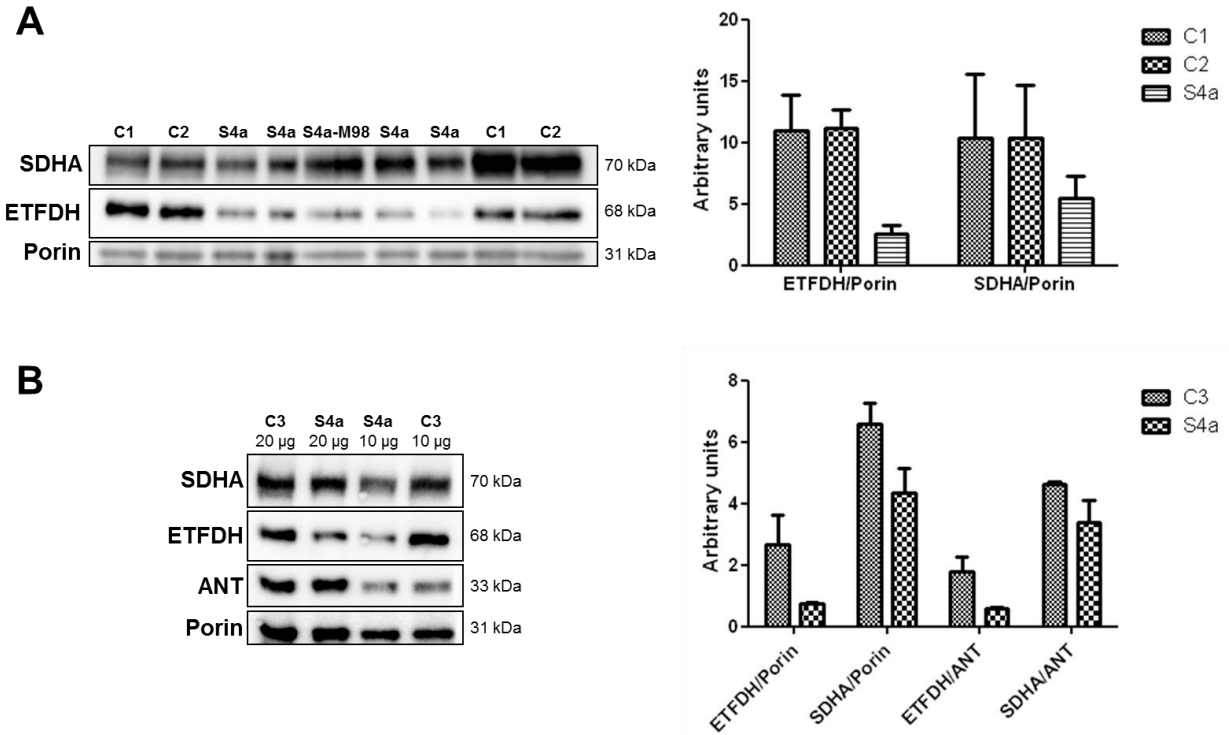
Investigations of muscle histopathology in individual S5. Skeletal muscle biopsy detected vacuolar changes (A) and faint COX staining (B). Heart muscle showed no cardiomyopathy (C) and normal COX staining (D).

Figure S3. Flavin Content in Blood Cells from S4a and S4b



HPLC quantification of riboflavin, FMN and FAD in (A, D) plasma, (B, E) enriched erythrocytes, or (C, F) mononuclear cells from Ficoll gradient centrifugation of EDTA blood derived from a control, subject S4a (under riboflavin treatment) or S4b. Areas in relative fluorescence units (RFU).

Figure S4. Decreased ETFDH and Complex II Protein Levels in Fibroblast Mitochondria from S4a



Two immunoblot experiments, (A) and (B) were performed to measure ETFDH and SDHA protein levels in enriched fibroblast mitochondria from three controls (C1, C2, C3) and four independently grown cells at different passage numbers of subject S4a. Protein extracts (10 µg in A, and 10-20 µg in B) were separated by SDS-PAGE and immunoblotted with antibodies against the proteins indicated to the left of the blots. ETFDH and SDHA protein intensities were quantified relative to porin or adenine nucleotide translocator (ANT). Data are presented as mean of two independent protein extracts (C1 and C2 in A, C3 and S4a in B), or four independent protein extracts (S4a in A). The error bars represent the standard error of the mean. In (A), there are also loaded protein extracts from enriched fibroblast mitochondria of a cell culture of S4a, which was transduced with a lentiviral construct bearing the full length wild-type construct starting at methionine-98 (S4a-M98). However, no normalization of ETFDH and SDHA was seen in these cells, probably due to insufficient *FLAD1* expression or mitochondrial targeting.

Table S1. Sequenced Genes in Subjects S1a, S2, S3, S6 and S7

Gene	Genbank	MIM	S1a	S2	S3	S6	S7
<i>ETF A</i>	NM_000125	608053	+	+	+	+	+
<i>ETFB</i>	NM_001985	130410	+	+	+	+	+
<i>ETFDH</i>	NM_004453	231675	+	+	+	+	+
<i>SLC52A1</i>	NM_017986.3	607883	+	+	-	-	+
<i>SLC52A2</i>	NM_024531	607882	+	+	-	-	+
<i>SLC52A3</i>	NM_033409.3	613350	+	+	-	-	+
<i>RFK</i>	NM_018339.5	613010	+	+	-	-	+
<i>FLAD1</i>	NM_025207.4	610595	+	+	+	+	+
<i>ACADM</i>	NM_000016.5	607008	-	-	-	-	+
<i>ACADS</i>	NM_000017.3	606885	-	-	-	-	+
<i>ACADVL</i>	NM_000018.3	609575	-	-	-	-	+
<i>CPT1a</i>	NM_001876.3	600528	-	-	-	-	+
<i>CPT2</i>	NM_000098.2	600650	-	-	-	-	+
<i>HADHA</i>	NM_000182.4	600890	-	-	-	-	+
<i>HADHB</i>	NM_000183.2	143450	-	-	-	-	+
<i>SLC22A5</i>	NM_001308122.1	603377	-	-	-	-	+
<i>SLC25A20</i>	NM_000387.5	613698	-	-	-	-	+
<i>SLC25A32</i>	NM_0300780	6108156	-	-	-	-	+

Table S2. Mass Spectrometry Detection of Peptides from Human FADS (Uniprot codes Q8NFF5|FAD1_HUMAN)

Peptide Analysis no.	FADS Domain Location	Peptide Amino Acid Sequence	Precursor	Transitions	m/z of Product Ions
P1	MPTb	VSVVPDEVATIAAEVTSFSNR	1096.0657++	A - y10+	0.1877
				A - y9+	0.015
				T - y6+	12.51
				S - y5+	0.057
				P - y17++	903.95
P1-heavy	MPTb	VSVVPDEVATIAAEVTSFSNR-	1101.0698++	A - y10+	1091.54
				A - y9+	1020.5
				T - y6+	721.35
				S - y5+	620.3
				P - y17++	908.95
P2	MPTb	NVYLFPGIPELLR	765.9376++	F - y9+	1041.61
				P - y8+	894.54
				P - y5+	627.38
				Y - b3+	377.18
				I - b8+	904.49
P2-heavy	MPTb	NVYLFPGIPELLR-heavy	770.9417++	F - y9+	1051.62
				P - y8+	904.55
				P - y5+	637.39
				Y - b3+	377.18
				I - b8+	904.49
P3		GLFQNPAVQFHSK	736.8859++	N - y9+	1027.53
				P - y8+	913.49
				Q - y5+	646.33
				F - y4+	518.27
				F - y11++	651.83
P3-heavy		GLFQNPAVQFHSK-heavy	740.8930++	N - y9+	1035.55
				P - y8+	921.5
				Q - y5+	654.34
				F - y4+	526.29
				F - y11++	655.84
P4		LGLGSYPDWGSNYYQVK	973.9678++	P - y11+	1356.62
				W - y9+	1144.54
				G - y8+	958.46
				P - y11++	678.81
				P4-heavy	
W - y9+	1152.56				
G - y8+	966.48				
P - y11++	682.82				
P5	FADS synthase	LPDVPNPLQILYIR	825.9825++		
				P - y8+	1015.63
				P - y13++	769.44
				P - y10++	613.87
				V - b4+	425.24
P5-heavy	FADS synthase	LPDVPNPLQILYIR-heavy	830.9866++	P - y10+	1236.73
				P - y8+	1025.64
				P - y13++	774.44
				P - y10++	618.87
				V - b4+	425.24

Abbreviations are as follows: MPTb, Molybdopterin binding

Table S3. Plasma Acylcarnitines and Urine Organic Acids in Individuals with *FLAD1* Variants

		S1a (-)	S1b (-)	S1b (+)	S2 (-)	S2 (+) ^a	S2 (+) ^b	S3 (-)	S4a (-) ^c	S4b (-) ^c	S5 (-)	S7 (-)
Acylcarnitines μmol/L (control range)	C4	NR	2.2↑ (<1.7)	UD	0.68 (≤0.68)	0.31 (≤0.68)	0.23 (0.14-0.66)	NR	0.72↑ (<0.56)	0.53 (<0.56)	NR	0.85↑ (<0.6)
	C3	NR	NR	NR	NR	NR	NR	NR	NR	NR	3.89↑ (0.28-2.90)	NR
	C5	elevated↑	NR	NR	0.39 (≤0.64)	0.29 (≤0.64)	0.33 (0.12-0.43)	1.45↑ (<0.85)	0.32 (<0.35)	0.41↑ (<0.35)	1.20↑ (≤0.52)	0.28 (<0.3)
	C5-DC	elevated↑	NR	NR	0.06 (≤0.13)	UD	0.05 (0.04-0.46)	NR	0.22 (<0.23)	0.13 (<0.23)	NR	0.20 (<0.2)
	C6	NR	NR	NR	0.26 (≤0.29)	0.16 (≤0.29)	0.11 (0.03-0.20)	NR	0.34↑ (<0.17)	0.33↑ (<0.17)	2.32↑ (≤0.59)	0.50↑ (<0.4)
	C8	elevated↑	NR	NR	0.45↑ (≤0.19)	0.47↑ (≤0.19)	0.40↑ (0.05-0.21)	1.23↑ (<0.30)	0.63↑ (<0.54)	0.79↑ (<0.54)	NR	0.54↑ (<0.2)
	C10:2	NR	NR	NR	NR	NR	NR	NR	0.08↑ (<0.07)	0.03 (<0.07)	NR	0.10 (<0.25)
	C10:1	NR	NR	NR	0.17↑ (≤0.10)	0.26↑ (≤0.10)	0.13 (0.04-0.19)	0.76↑ (<0.17)	0.23 (<0.27)	0.19 (<0.27)	NR	0.38↑ (<0.2)
	C10	NR	NR	NR	0.76↑ (≤0.39)	1.06↑ (≤0.39)	0.69↑ (0.05-0.27)	1.86↑ (<0.30)	0.67 (<0.80)	0.85↑ (<0.80)	NR	1.70↑ (<0.2)
	C12	NR	NR	NR	0.48 (≤0.59)	0.23 (≤0.59)	0.31↑ (0.05-0.19)	NR	0.16 (<0.19)	0.13 (<0.19)	NR	0.89↑ (<0.1)
	C14:2	NR	NR	NR	NR	NR	0.05↑ (0.01-0.03)	NR	0.10↑ (<0.08)	0.05 (<0.08)	NR	0.15 (<0.15)
	C14:1	NR	NR	NR	0.46 (≤0.75)	0.18 (≤0.75)	0.24↑ (0.04-0.12)	NR	0.44↑ (<0.25)	0.35↑ (<0.25)	NR	0.60↑ (<0.10)
	C14	elevated↑	NR	NR	0.47 (≤0.96)	0.14 (≤0.96)	0.20 (0.07-0.23)	1.49↑ (<1.41)	0.16↑ (<0.09)	0.08 (<0.09)	NR	0.44 (<0.10)
	C16:1	NR	NR	NR	0.54 (≤0.54)	0.09 (≤0.54)	0.16 (0.04-0.18)	NR	0.37↑ (<0.07)	0.20↑ (<0.07)	NR	0.47↑ (<0.10)
	C16	NR	NR	NR	1.65 (0.63-5.27)	0.72 (0.63-5.27)	1.08 (0.58-2.08)	NR	0.23 (<0.24)	0.10 (<0.24)	NR	0.35↑ (<0.20)
	C18:2	NR	NR	NR	0.32 (≤0.85)	0.55 (≤0.85)	0.26↑ (0.04-0.13)	NR	0.16↑ (<0.14)	<0.05 (<0.14)	NR	0.23↑ (<0.12)
	C18:1	NR	NR	NR	2.10 (0.54-3.11)	0.89 (0.54-3.11)	1.36 (0.49-1.78)	NR	0.69↑ (<0.42)	0.42 (<0.42)	NR	0.49↑ (<0.30)
C18	NR	NR	NR	0.89 (0.55-1.43)	0.55 (0.55-1.43)	0.68 (0.30-1.04)	NR	0.17↑ (<0.13)	0.1 (<0.13)	NR	0.08 (<0.1)	
Urine Organic Acids mmol/mol Creatinine (control range)	ethylmalonic acid	41↑ (<21)	elevated↑	UD	elevated↑	2 (<10)	UD	32↑ (0,1-14,6)	elevated↑	NR	NR	248 (<15)
	adipic acid	elevated↑	NR	NR	NR	NR	NR	NR	elevated↑	NR	elevated↑	<30 (<30)
	glutaric acid	NR	NR	NR	elevated↑	4 (<20)	UD	NR	NR	NR	NR	109 (<50)
	suberic acids	elevated↑	NR	NR	NR	NR	NR	NR	elevated↑	NR	NR	<20 (<20)
	hexanoylglycine	elevated↑	NR	NR	NR	NR	NR	NR	NR	NR	NR	<5 (<5)
	dehydrosebacic acid	elevated↑	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	methylsuccinic acid	NR	elevated↑	UD	NR	NR	NR	NR	NR	NR	NR	<5 (<5)
	lactate	NR	NR	NR	elevated↑	NR	NR	elevated↑	NR	NR	NR	<150 (<150)
	tiglylglycine	NR	NR	NR	NR	NR	NR	NR	elevated↑	NR	NR	<5 (<5)

Abbreviations are as follows: NR, not reported; UD, undetectable, (-), before riboflavin; (+), after riboflavin; †, elevated; ^a, measurements 1 year after starting treatment; ^b, measurements 11 years after starting treatment; ^c, C3-DC/C4-OH S4a 0.27(<0.28) S4b 0.55(≤0.28); C4, butyrylcarnitine; C4-OH, OH-butyrylcarnitine; C5, isovalerylcarnitine; C5-DC, glutarylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C10:1, decenoylcarnitine; C10:2, decadinoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C14:1, tetradecenoylcarnitine; C14:2, tetradecenoylcarnitine; C16, hexadecanoylcarnitine; C16:1, hexadecenoylcarnitine; C18, octadecanoylcarnitine; C18:1, octadecenoylcarnitine; C18:2, octadecenoylcarnitine.

Table S4. Muscle Respiratory Control for S2 and S4a, Pre-treatment

Substrate	S2 (control range)	S4a (control range) (nmol/h/mg protein)
Pyruvate + malate	27 (54 ±44)	121 (263-900)
Succinate + rotenone	25 (95 ±54)	NR
Ascorbate + TMPD	266 (828 ±318)	NR
Palmitoyl-CoA	NR	NR
Alpha-ketoglutarate	NR	NR
Pyruvate + carnitine	NR	174 (302-856)
Pyruvate + malate + ADP	NR	39 (32-102)
Pyruvate + malate + CCCP	NR	148 (304-889)
Pyruvate + malate + atractyloside	NR	28 (19-90)
Malate + pyruvate + malonate	NR	114 (282-874)
Malate + acetylcarn. + malonate	NR	148 (273-678)
Malate + acylcarn. + arsenite	NR	98 (156-378)
Glutamate + acylcarn.	NR	43 (86-209)

Abbreviations are as follows: NR, not reported

Table S5. Muscle Respiratory Chain Enzyme Activities for S2^a, S3, S4a, S5 and S6

	S2^a (control range) [nmol/minmg]	S4a (control range) [mUnit/mg protein]	S5 (control range) (U/UCS)	S6 (control range) [mmol/min/g]
Citrate synthase	2223 (1316±486)	228 (150-338)	175.5 (45.00–100.00) ^b	47.38 (7.80-10.90)
CI	NR	8 (28-76)	UD (0.17-0.56)	26.61 (27.50-39.50)
CI+III	155 (324 ±180)	32 (49-218)	NR	1.39 (0.65-1.50)
CII	77 (181 ±81)	13 (33-102)	NR	0.51 (1.20-2.00)
CII+III	56 (155 ±111)	22 (65-180)	0.03 (0.08–0.45)	0.17 (0.45-0.90)
CIII	NR	238 (304-896)	NR	NR
CIV	1805 (2177 ±808)	169 (181-593)	0.145 (1.10–5.00)	2.40 (1.80-2.45)
CV	NR	68 (86-257)	NR	NR
PDHC	NR	3.6 (5.3-19.8)	NR	NR

Abbreviations are as follows: NR, not reported; UD, undetectable; UCS, unit citrate synthase. ^ameasured after treatment. ^bmeasured in U/gNCP