

# Combined deficiency of factor V and factor VIII is due to mutations in either *LMAN1* or *MCFD2*

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**Mutations in *LMAN1* (*ERGIC-53*) or *MCFD2* cause combined deficiency of factor V and factor VIII (F5F8D). *LMAN1* and *MCFD2* form a protein complex that functions as a cargo receptor ferrying FV and FVIII from the endoplasmic reticulum to the Golgi. In this study, we analyzed 10 previously reported and 10 new F5F8D families. Mutations in the *LMAN1* or *MCFD2* genes accounted for 15 of these families, including 3 alleles resulting in**

**no *LMAN1* mRNA accumulation. Combined with our previous reports, we have identified *LMAN1* or *MCFD2* mutations as the causes of F5F8D in 71 of 76 families. Among the 5 families in which no mutations were identified, 3 were due to misdiagnosis, with the remaining 2 likely carrying *LMAN1* or *MCFD2* mutations that were missed by direct sequencing. Our results suggest that mutations in *LMAN1* and *MCFD2* may account for all cases of**

**F5F8D. Immunoprecipitation and Western blot analysis detected a low level of *LMAN1*-*MCFD2* complex in lymphoblasts derived from patients with missense mutations in *LMAN1* (C475R) or *MCFD2* (I136T), suggesting that complete loss of the complex may not be required for clinically significant reduction in FV and FVIII. (Blood. 2006;107:1903-1907)**

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## Introduction

Combined deficiency of factor V and factor VIII (F5F8D) was first described in a family by Oeri et al<sup>1</sup> in 1954. Most patients have plasma levels of factor V (FV) and factor VIII (FVIII) (both antigen and activity) in the range of 5 to 30 U/dL and exhibit mild to moderate bleeding manifestations.<sup>2</sup> The disease is inherited as an autosomal recessive disorder. Positional cloning efforts initially demonstrated that F5F8D results from null mutations in the gene encoding the endoplasmic reticulum (ER)–Golgi intermediate compartment (ERGIC) marker protein ERGIC-53 (now called *LMAN1*).<sup>3</sup> However, a significant subset (30%) of affected individuals exhibited evidence for mutations in at least one additional gene, and cells from these affected individuals had normal levels of *LMAN1*.<sup>4,5</sup> We recently identified a second gene (*MCFD2*) that accounted for most of the remaining families without mutations in *LMAN1*.<sup>6</sup> *LMAN1* and *MCFD2* form a stable, calcium-dependent complex that likely serves as a cargo receptor for efficient ER-to-Golgi transport of FV and FVIII. Cargo receptors selectively package secreted proteins into COPII-coated vesicles budding from the ER for trafficking to the Golgi compartment.<sup>7-9</sup> Consistent with this idea, FVIII was shown to bind to both *LMAN1* and *MCFD2* through calcium-dependent protein-protein interactions

in cultured cells.<sup>10,11</sup> The B domain of FVIII appears to be the primary determinant of binding to the *LMAN1*-*MCFD2* complex.<sup>6,12</sup> In vitro data suggest that the other cargo proteins including the lysosomal proteins cathepsin Z<sup>13</sup> and cathepsin C<sup>14</sup> may also depend on *LMAN1*/*MCFD2* function, though evidence for deficiency of these or other proteins in F5F8D patients has not been reported.

A total of 19 different mutations in the *LMAN1* gene have been reported to date in 49 families,<sup>2,15,16</sup> all of which appear to be null mutations that abolish protein expression. Seven *MCFD2* mutations have been identified in 10 families.<sup>6,16</sup> Five of the latter mutations disrupt the open reading frame, with the remaining 2 mutations resulting in single-amino acid substitutions in the second putative EF-hand domain (D129E and I136T). Both missense mutations were shown to abolish the interaction of *MCFD2* with *LMAN1*.<sup>6</sup> Mutant *MCFD2* expressed in COS1 cells failed to coimmunoprecipitate with *LMAN1* but appears to still retain the ability to bind FVIII.<sup>11</sup>

We now report the analysis of 20 F5F8D families, including 10 families previously reported to lack *LMAN1* mutations.<sup>4,5</sup> Novel mutations in *LMAN1* and *MCFD2* were identified, including

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**Table 1. *LMAN1* and *MCFD2* mutation analyses, geographic origins, and FV/FVIII levels in F5F8D patients**

Patient	Origin	FV/FVIII levels, U/dL	<i>LMAN1</i> mutation	<i>MCFD2</i> mutation	Reference
B2	Japan*	11/19	None	None	3 in Nichols et al <sup>5</sup>
B3	Italy*	2.3/6.3	None	149+5G>A	5 in Nichols et al <sup>5</sup>
B4-1	Italy	7.5/6.0	None	149+5G>A	6 in Nichols et al <sup>5</sup>
B4-2	Italy	10/10	None	149+5G>A	6 in Nichols et al <sup>5</sup>
B5	Italy	11/23	None	149+5G>A	8 in Nichols et al <sup>5</sup>
B6	Iran*	10/6	None	-6-IG>C	A14 in Neerman-Arbez et al <sup>4</sup>
B7	Italy	8/24	None	None	A22 in Neerman-Arbez et al <sup>4</sup>
B8	Italy	VWD	None	N/A	A33 in Neerman-Arbez et al <sup>4</sup>
B9	Kosovo	4.5/1.5	N/A	407T>C (I136T)	This study
B10	Serbia	7/7	N/A	149+5G>A	This study
B11	Italy	13/20	2T>C (M1T)	None	This study
B12-1	Austria	15/12	780delT	None	This study
B12-2	Austria	24/13	780delT	None	This study
B13	Iraq	14/25	G961>T (E321X)	None	This study
B14	Poland*	16/23	839-841delA	None	This study
B15	Belgium	11/35	822+1G>A	None	This study
B16	USA	23/24	822+33insGGTT†‡	None	This study
B17	Argentina	18/23	1423T>C (C475R)‡	None	This study
B18	Greece	7/5	None	266A>C (D89A)	This study

None indicates no mutations detected; VWD, von Willebrand disease; and N/A, not analyzed.

Nucleotide numberings are relative to the initiation codon of GenBank sequences NM\_005570 for *LMAN1* and NM\_139279 for *MCFD2*.

\*Known consanguinity.

†Patient is heterozygous for this mutation with no mutation detected on the other allele.

‡Cause-effect relationship not established for this intronic mutation.

evidence for regulatory mutations that abolish *LMAN1* mRNA expression. In addition, we studied the interaction of *LMAN1* and *MCFD2* in lymphoblasts derived from a patient with the I136T mutation in *MCFD2* and a patient with a novel missense mutation in *LMAN1* (C475R).

## Patients, materials, and methods

### Patient samples

Peripheral blood (10-15 mL) was collected from the affected individuals, with informed consent from all individuals, after diagnosis of F5F8D. The study protocol was approved by the institutional review board at the University of Michigan. The levels of FV and FVIII activities are reported in Table 1. Epstein-Barr virus (EBV)-immortalized lymphoblast lines were derived from 3 affected individuals (family 9, Zhang et al<sup>6</sup>; B16; B17) as previously described.<sup>3</sup>

### Sequencing of the *LMAN1* and *MCFD2* genes in F5F8D patients

All coding exons and intron-exon junctions were amplified from genomic DNA prepared from peripheral blood as previously reported. Polymerase chain reactions (PCRs) were performed as previously described<sup>3,6</sup> and DNA sequencing was completed in the University of Michigan DNA Sequencing Core. Total RNA was prepared from patient-derived lymphoblasts using the Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcriptase-PCRs (RT-PCRs) were performed as previously described,<sup>6</sup> using primers listed in Table 2. Heterozygous mutations were confirmed by cloning PCR products and sequencing individual clones.

### Immunoprecipitation and Western blot analysis

Patient-derived lymphoblasts were lysed and immunoprecipitated with rabbit anti-*MCFD2*<sup>6</sup> or monoclonal anti-*LMAN1* antibodies (a gift from H.-P. Hauri, University of Basel, Switzerland) as previously described.<sup>6</sup> The immunoprecipitates were further analyzed by Western blot with monoclonal anti-*MCFD2*<sup>6</sup> or rabbit anti-P58 (a gift from R. F. Pettersson, Karolinska Institute, Stockholm, Sweden).

## Results

### Further analysis of previously reported F5F8D patients

Previous *LMAN1* gene sequence analysis failed to identify mutations in 17 of 54 F5F8D families.<sup>4,5</sup> Ten of these families were studied in our recent report, which identified *MCFD2* mutations in 7 families.<sup>6</sup> No *LMAN1* or *MCFD2* mutations were identified in the remaining 3 families, raising the possibility of additional locus heterogeneity and the involvement of a third F5F8D gene. One of the 3 families (family 2, Zhang et al<sup>6</sup>) exhibited evidence for linkage to the *MCFD2* locus,<sup>6</sup> suggesting a mutation in a regulatory region of the gene that was missed by direct sequence analysis. The other 2 families (families 3 and 8, Zhang et al<sup>6</sup>) showed genetic evidence against linkage to either *LMAN1* or *MCFD2*. Reanalysis of the FV and FVIII levels in probands from these 2 families now suggests errors in the initial F5F8D diagnosis. Reevaluation of the family 3 proband demonstrated a normal level of FVIII, with a FV level at 1% of normal, consistent with a diagnosis of simple FV

**Table 2. Primers used in RT-PCR and genotyping analyses**

Primer	Sequence 5' → 3'
a	GAATTGGAGCTGATGGCCTA
b	CGCAACAAACCCTATCCTGT
c	ATCTGCTGCAACTGGAGGTC
d	GACCTCCAGTTGCAGCAGAT
e	TGAAATCAAGCAGCTGAACC
f	AGGCATTCCTGCTCCTCTTT
g	TGCAGGTGCTCTTTGATGTC
MCFD2F	GACCATGAGATCCCTGCTCAGA
MCFD2R	CTGCAGTGATTTTGCAAAATTCAG
BZ1F	CCAGCAGCCAAAGAGAAAAC
BZ1R	GCTTGTCCCAGAAAAAGAG
BZ20F	CATGTGCCACTGACAACAAC
BZ20R	TCTCTGACACCCTGAGGAC

**Table 3. Haplotype analysis for 6 families with the 149+5G>A mutation and 2 families with the 407T>C (I136T) mutation**

Family	Haplotype	Mutation
10 in Zhang et al <sup>6</sup>	N/D-C-C-C-N/D-17	149+5G>A
12 in Zhang et al <sup>6</sup>	N/D-C-C-C-N/D-17	149+5G>A
B3	N/D-A-C-C-N/D-19	149+5G>A
B4	N/D-A-C-C-N/D-18	149+5G>A
B5	N/D-A-C-C-N/D-19	149+5G>A
B10	N/D-C-T-G-N/D-19	149+5G>A
9 in Zhang et al <sup>6</sup>	19/22-C-C-C-19-20	I136T
B9	19-C-C-C-18/19-18/19	I136T

Three single-nucleotide polymorphisms located at the nucleotides 2895, 3099, and 3116 relative to the initiation codon of *LMAN1* mRNA, as well as 2 CA repeat markers (BZ1 and *D2S2227*), were genotyped. Haplotypes are shown in the format BZ1 (CA repeat numbers)-C2895A-C3099T-C3116G-*D2S2227* (CA repeat number)-BZ20 (GAAA repeat number).

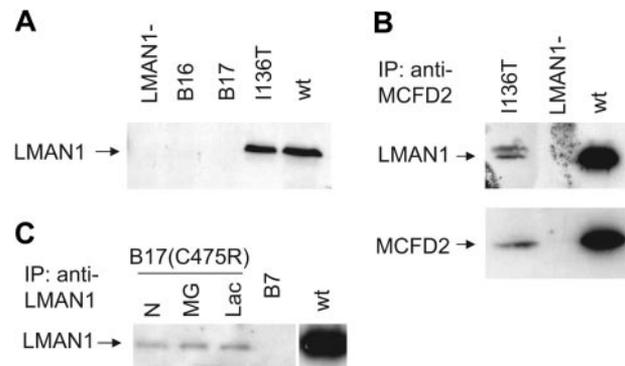
N/D indicates genotyping not done for this marker.

deficiency. Indeed, linkage data from this family are consistent with a mutation on chromosome 1 in the vicinity of the *F5* gene. Reanalysis of both male and female probands from family 8 (Zhang et al<sup>6</sup>) identified a FVIII level of approximately 10%, with a FV level of 40% to 50%. This family may have von Willebrand disease combined with a *F5* null allele or a coincidental FV level at the lower end of normal.

We sequenced the *MCFD2* gene of the probands from the remaining 7 of the 17 previously reported F5F8D families<sup>4,5</sup> (renumbered as B2-B8; Table 1) and identified mutations in 4 families. A novel homozygous mutation (-6-1G>C) that abolishes the consensus splice acceptor site of *MCFD2* exon 2 was identified in family B6. Homozygosity for a previously identified splice donor site mutation (149+5G>A) was identified in 3 Italian families (B3-B5). No *LMAN1* or *MCFD2* mutations were identified in families B2, B7, and B8. Genotyping revealed that the proband of family B2, whose parents are first cousins, is homozygous at the *LMAN1* locus and heterozygous at the *MCFD2* locus (Table 3), suggesting an unidentified *LMAN1* mutation. Although no mutation was identified in B7, Western blot analysis of the proband detected no *LMAN1* protein.<sup>4</sup> Immunoprecipitation of cell lysates from  $5 \times 10^6$  lymphoblasts derived from the B7 proband also failed to detect *LMAN1* (Figure 1C), suggesting a regulatory mutation in the *LMAN1* gene that was missed by direct sequence analysis. Indeed, RT-PCR detected only a trace amount of *LMAN1* mRNA and revealed an aberrantly spliced species of *LMAN1* mRNA that skipped exon 4 (Figure 2). The low level of mRNA in this patient may be the result of nonsense-mediated decay. Reevaluation of the B8 proband revealed reduced von Willebrand factor and FVIII levels and a normal level of FV, consistent with a diagnosis of von Willebrand disease.

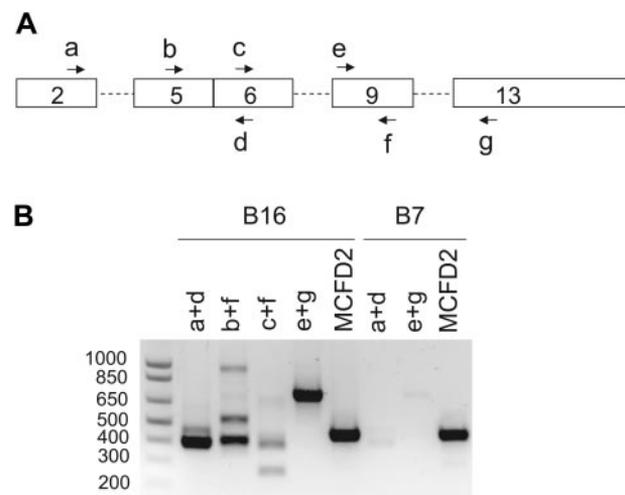
#### Mutational analysis of additional F5F8D patients

We analyzed the *LMAN1* and *MCFD2* genes of 11 F5F8D patients from 10 previously unreported families (B9-B18; Table 1). These families included the first reported cases from the Balkans, Austria, Poland, Belgium, Argentina, and Greece. Homozygosity for *LMAN1* null mutations were identified in 5 families, including 4 novel mutations (780delT in B12, 839-841delA in B14, E321X in B13, and 822+1G>A in B15) and 1 previously reported mutation (2T>C in B11). We also identified homozygous *MCFD2* mutations in 2 families from the former Yugoslavia (B9-B10) and a family from Greece (B18). B9 carries a missense mutation (I136T) that has previously been reported in a Venezuelan family.<sup>6</sup> B10 carries the 149+5G>A splicing mutation identified here in 3 Italian



**Figure 1. Immunoprecipitation and Western blot analysis of patient lymphoblasts.** (A) Western blot analysis of *LMAN1* in lymphoblasts from probands in families B16 and B17 compared with cells carrying the *MCFD2* I136T mutation and wild-type (wt) cells. (B) A small amount of *LMAN1*-*MCFD2* complex is detected in lymphoblasts with the *MCFD2* I136T mutation. Lysates from  $5 \times 10^6$  cells were immunoprecipitated (IP) with rabbit anti-*MCFD2* followed by Western blot analysis with monoclonal anti-*LMAN1* (top panel) or anti-*MCFD2* antibodies (bottom panel). (C) Lymphoblasts from the B17 proband were untreated (N) or treated with the proteasome inhibitors MG132 (MG) or lactacystin (Lac) for 6 hours. Lysates from  $5 \times 10^6$  cells of B17 and B7 lymphoblasts were immunoprecipitated with monoclonal anti-*LMAN1* followed by Western blot analysis with polyclonal rabbit anti-*LMAN1*.

families and previously in US, Swiss, and Indian families.<sup>6,16</sup> To examine the genetic origin of this mutation, we constructed haplotypes using 3 single-nucleotide polymorphisms in the 3' untranslated region (UTR) of *MCFD2* and 3 microsatellite markers (BZ1, located ~570 kb upstream of *MCFD2*; *D2S2227*, located ~123 kb downstream of *MCFD2*; and BZ20, located ~1900 kb downstream of *MCFD2*). Among 6 families with the 149+5G>A mutation, 4 different haplotypes were identified, indicating at least 4 independent origins for this mutation (Table 3). These data are consistent with distinct geographic origins for many of these families and suggest a recurring mutation, perhaps due to a mutation hotspot at a CpG dinucleotide.<sup>17</sup> The haplotypes of the 2



**Figure 2. RT-PCR analysis of *LMAN1* mRNA from probands of families B7 and B16.** (A) Schematics of the *LMAN1* cDNA with locations of PCR primers (Table 2, a-g) indicated. (B) Agarose gel electrophoresis of the RT-PCR products after amplification of *LMAN1* mRNA with the various primer combinations indicated. The bottom bands in B16/b+f and B16/c+f lanes represent alternatively spliced species with exon 8 skipping, whereas the top bands represent the normally spliced products. The 900-bp band in the B16/b+f lane was a nonspecific PCR product according to sequence analysis. Two specific PCR products were observed in the B7/a+d lane. Sequencing of the latter PCR products further amplified by nested PCR revealed that the top band represents the normal spliced product, whereas the bottom band represents an alternatively spliced species with exon 4 skipping. Amplification of the coding region of *MCFD2* mRNA served as a control for RNA quality.

families (B9 and 9II-5, Zhang et al<sup>6</sup>) with the I136T missense mutation in MCFD2 diverge at both the BZ1 and D2S2227 markers (Table 3), suggesting that this mutation also arose independently. Lastly, B18 carries a novel homozygous missense mutation (D89A) that changes an amino acid at a position in the first EF-hand domain that is invariant in all MCFD2 orthologs.<sup>6</sup> Although cells from family B18 are not available for study, we expect the D89A mutation to abolish binding of the mutant MCFD2 to LMAN1, similar to previously described D129E and I136T mutations.<sup>6</sup>

Western blot analysis of lymphoblasts derived from the proband of family B16 detected no LMAN1 (Figure 1A), indicating that loss of LMAN1 protein expression is the cause of F5F8D in this patient. Sequencing of the genomic DNA detected no mutations within the exons or splice junctions of the *LMAN1* and *MCFD2* genes. We isolated mRNA from lymphoblasts and performed RT-PCR analysis, identifying an alternatively spliced species of *LMAN1* mRNA (Figure 2). Sequencing confirmed that the lower band corresponds to splicing of exon 7 to exon 9, with skipping of exon 8. The upper band corresponds to the wild-type LMAN1 product (exons 7-8-9). Sequencing of intron 7 demonstrated a heterozygous 4-nucleotide (GGTT) insertion 33 bp downstream of exon 7. The 4-nucleotide (nt) insertion may be responsible for this aberrant splicing, although an effect of another more distant mutation cannot be excluded. The wild-type product could represent residual normal splicing for the same allele. Alternatively, the second allele may carry an unidentified mutation that markedly reduces transcription or mRNA processing.

#### Missense mutations disrupt LMAN1-MCFD2 complex formation

We identified a heterozygous missense LMAN1 mutation (C475R) in the proband of an Argentinean family of Italian origin (B17). No other mutations were found in this patient. RT-PCR analysis of lymphoblast RNA revealed that only the missense allele is expressed at the mRNA level, indicating that the second *LMAN1* allele in this patient carries an unidentified mutation that results in a *cis*-defect in transcription or mRNA stability. Cysteine 475 plays an important role in forming disulfide-linked homodimers and homohexamers of LMAN1. Substitution of C475 with alanine has previously been reported to abolish homohexamer formation without affecting homodimer formation.<sup>18,19</sup> LMAN1<sup>C475A</sup> was shown to exhibit impaired transport out of the ER<sup>18</sup> but retained a normal intracellular localization pattern.<sup>19</sup> We analyzed cell lysates of lymphoblasts derived from the proband of B17. No endogenous LMAN1 was detectable by Western blot analysis (Figure 1A), though upon immunoprecipitation of lysates from  $5 \times 10^6$  cells, a trace amount of LMAN1 was detected (Figure 1B). This is in contrast to the reported C475A substitution, which exhibited a turnover comparable to the wild-type protein.<sup>18</sup> In addition to disrupting a disulfide bond that is critical for homohexamer formation, the C475R mutation presumably further compromises LMAN1 folding, leading to ER-associated protein degradation. However, treatment of cells with the potent proteasome inhibitors MG132 and lactacystin only slightly increased the amount of LMAN1 accumulation (Figure 1C), suggesting that degradation of LMAN1<sup>C475R</sup> is mediated largely through a proteasome-independent pathway.

We previously showed that the I136T mutation in MCFD2 identified in B9 and a Venezuelan family (family 9, Zhang et al<sup>6</sup>) abolished binding to LMAN1 in an *in vitro* transfection assay.<sup>6</sup> To further study the endogenous behavior of this mutant, we performed coimmunoprecipitation analysis in lymphoblasts derived from a Venezuelan patient homozygous for the I136T mutation.

Cell lysates were immunoprecipitated with anti-MCFD2 and analyzed by Western blotting with anti-MCFD2 or anti-LMAN1. A small amount of MCFD2<sup>I136T</sup> was detected at a level higher than that observed in *LMAN1* mutant lymphoblasts (Figure 1B). A trace amount of LMAN1 also coimmunoprecipitated with MCFD2<sup>I136T</sup> (Figure 1B). Taken together, these data suggest that LMAN1 and MCFD2<sup>I136T</sup> retain a weak interaction. However, the residual amount of LMAN1-MCFD2 complex is apparently either functionally deficient or of insufficient quantity to support FV and FVIII secretion.

## Discussion

We report the analysis of *LMAN1* and *MCFD2* mutations in 10 previously reported and 10 new F5F8D families. Homozygous *MCFD2* and *LMAN1* mutations were identified in 12 families. Heterozygous mutations were identified in only one *LMAN1* allele in 3 families, all of which exhibit markedly reduced LMAN1 protein expression, indicating the existence of unidentified mutations in the second *LMAN1* allele. Of the remaining 5 families, 3 can be explained by misdiagnosis of a similar bleeding disorder. Unidentified mutations in *LMAN1* or *MCFD2* are likely to explain F5F8D in the remaining 2 families that exhibit linkage to 1 of the 2 loci. Presumably regulatory or splicing mutations in unsequenced portions of the corresponding gene are responsible, though patient-derived cells were not available for further study. Thus, the locus heterogeneity identified in F5F8D appears to be limited to the *LMAN1* and *MCFD2* genes. These data are consistent with the failure of previous biochemical studies to identify additional components of the LMAN1-MCFD2 receptor complex or this receptor-mediated secretory pathway.<sup>11</sup> Alternatively, mutations in other components of this pathway may exhibit a different phenotype distinct from F5F8D.

Combined with previous reports, at least 25 *LMAN1* and 9 *MCFD2* mutations have now been identified. Among these, 8 *LMAN1* mutations and 3 *MCFD2* mutations were identified in multiple families. In addition, 3 families carry unidentified regulatory mutations. No clear genotype/phenotype relationship can be identified to account for variations in FV and FVIII levels among F5F8D patients (Table 1). For example, even for patients homozygous for the same 149 + 5G>A mutation, levels of FV/FVIII varied from 2.3/1.2 to 11/23 (Table 1).<sup>16</sup> Although variability in FV and FVIII assays could contribute, the range of observed values more likely reflects the combined effects of modifier genes for FV/FVIII and environmental factors. Although most reported F5F8D patients are from the Mediterranean region,<sup>3-6,20-22</sup> an increasing number of cases have been identified in other geographic regions.<sup>4,5,15,16,23,24</sup> A high percentage of F5F8D patients are associated with known consanguinity and/or are homozygous for a single mutation, suggesting that the geographic distribution of this disease is due at least in part to the varying prevalence of consanguineous marriages rather than specific positive selection for heterozygous carriers.

All previously reported *LMAN1* mutations were predicted to be null, leading to the speculation that even a trace amount of functional LMAN1 expression may be sufficient to support normal FV and FVIII trafficking. However, we detected a low level of LMAN1-MCFD2 complex in patient lymphoblasts carrying either the *LMAN1* C475R mutation (data not shown) or the MCFD2

I136T mutation (Figure 1). The *LMAN1*-*MCFD2*<sup>I136T</sup> complex may retain residual FVIII-binding activity, since *MCFD2* carrying the similar D129E mutation was shown to bind FVIII.<sup>11</sup> The latter observation suggests that a threshold level of *LMAN1*-*MCFD2* activity above that observed for the *LMAN1*-*MCFD2*<sup>I136T</sup> complex may be required for the intracellular trafficking of FV and FVIII,

though we cannot rule out the possibility that the *LMAN1*-*MCFD2*<sup>I136T</sup> interaction may be further weakened in more relevant cells such as hepatocytes *in vivo*. These results also suggest that targeting the *LMAN1*-*MCFD2* complex as a therapeutic approach to anticoagulation is likely to require marked reduction of one or both components of this protein complex.

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