# Separate roles of LMAN1 and MCFD2 in ER-to-Golgi trafficking of FV and FVIII

Yuan Zhang, Zhigang Liu, and Bin Zhang

Genomic Medicine Institute, Lerner Research Institute of Cleveland Clinic, Cleveland, OH

### **Key Points**

- Efficient ER-to-Golgi transport of FV and FVIII requires the LMAN1-MCFD2 cargo receptor complex.
- MCFD2 likely functions as a primary interacting partner of FV/FVIII cargo, and LMAN1 primarily serves as a shuttling carrier of MCFD2.

Mutations in lectin, mannose-binding 1 (LMAN1) and multiple coagulation factor deficiency protein 2 (MCFD2) cause the combined deficiency of factor V (FV) and FVIII (F5F8D). LMAN1 and MCFD2 form a protein complex that transports FV and FVIII from the endoplasmic reticulum (ER) to the Golgi. Although both proteins are required for the cargo receptor function, little is known about the specific roles of LMAN1 and MCFD2 in transporting FV/FVIII. We used different LMAN1 and MCFD2 deficient cell lines to investigate the LMAN1/MCFD2-dependent FV/FVIII secretion pathway. LMAN1 deficiency led to more profound decreases in FV/FVIII secretion in HEK293T and HepG2 cells than in HCT116 cells, suggesting that regulation of cargo transport by the LMAN1/MCFD2 pathway varies in different cell types. Using these cell lines, we developed functional assays to accurately assess the pathogenicity of recently reported potential LMAN1 and MCFD2 missense mutations. LMAN1 with mutations abolishing carbohydrate binding can still partially rescue FV/FVIII secretion, suggesting that N-glycan binding is not essential for FV/FVIII transport. Surprisingly, overexpression of either wild-type or mutant MCFD2 is sufficient to rescue FV/FVIII secretion defects in LMAN1 deficient cells. These results suggest that cargo binding and transport are carried out by MCFD2 and that LMAN1 primarily serves as a shuttling carrier of MCFD2. Finally, overexpression of both LMAN1 and MCFD2 does not further increase FV/FVIII secretion, suggesting that the amount of the LMAN1-MCFD2 receptor complex is not a rate-limiting factor in ER-Golgi transport of FV/FVIII. This study provides new insight into the molecular mechanism of F5F8D and the intracellular trafficking of FV and FVIII.

# Introduction

Combined deficiency of coagulation factor V (FV) and FVIII (F5F8D) is characterized by the simultaneous decreases in FV and FVIII antigen and activity levels in plasma from 5% to 30% of normal.<sup>1,2</sup> As a rare autosomal recessive disorder, F5F8D is often associated with consanguineous marriages, with the highest estimated occurrence at 1:100 000 among Middle Eastern Jews and non-Jewish Iranians.<sup>3-5</sup> Patients with F5F8D exhibit mild-to-moderate bleeding symptoms. Approximately 70% of F5F8D cases are attributable to lectin, mannose-binding 1 (*LMAN1*) mutations<sup>6</sup> and 30% to multiple coagulation factor deficiency protein 2 (*MCFD2*) mutations.<sup>1,7,8</sup> LMAN1, also called ERGIC-53, is a 53 kDa homo-hexameric transmembrane protein and belongs to the family of L-type animal lectins.<sup>9</sup> MCFD2 is a

Submitted 19 August 2022; accepted 3 December 2022; prepublished online on *Blood Advances* First Edition 9 December 2022; final version published online 31 March 2023. https://doi.org/10.1182/bloodadvances.2022008788.

The full-text version of this article contains a data supplement.

Data are available on request from the corresponding author, Bin Zhang (zhangb@ccf. org).

<sup>© 2023</sup> by The American Society of Hematology. Licensed under Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0), permitting only noncommercial, nonderivative use with attribution. All other rights reserved.

16 kDa soluble monomeric protein with 2 Ca<sup>2+</sup>-binding motifs known as EF-hand domains. LMAN1 and MCFD2 form a Ca<sup>2+</sup>-dependent complex with 1:1 stoichiometry and cycles between the endoplasmic reticulum (ER) and the ER-Golgi intermediate compartment.<sup>7,10</sup> The LMAN1-MCFD2 complex serves as a cargo receptor for FV and FVIII and facilitates their transportation from the ER to the Golgi.<sup>7,10-12</sup> Besides FV and FVIII, potential cargo for this receptor complex include  $\alpha$ 1-antitrypsin (AAT) and other proteins.<sup>11-19</sup>

The mechanism of how the LMAN1-MCFD2 complex transports its cargo is not clear. LMAN1 has all the features of a cargo receptor: it is a type 1 transmembrane protein that has a carbohydraterecognition domain (CRD) in the ER lumen and a short cytoplasmic domain that contains ER exit and retrieval motifs.7,10,20 However, LMAN1 cannot function as a transport receptor for FV, FVIII, AAT, and perhaps other cargo in the absence of MCFD2, because MCFD2 deficiency leads to the same or more severe symptoms as LMAN1 deficiency.<sup>1,12</sup> The requirement of a soluble cofactor MCFD2 in the LMAN1-MCFD2 cargo receptor complex has been an enigma, suggesting a more complex trafficking mechanism than that of previously characterized cargo receptors in yeast. The CRD of LMAN1 contains separable binding sites for MCFD2 and mannose.<sup>20</sup> Both LMAN1 and MCFD2 were reported to interact with cargo.<sup>10,20,21</sup> The mannose-binding activity of LMAN1 is presumably important for cargo selection, but it is not directly demonstrated. A LMAN1-binding deficient MCFD2 missense mutant can still bind to FVIII, suggesting that MCFD2 interaction with FVIII is independent of LMAN1.<sup>10</sup> The EF-hand domains of MCFD2 not only bind to LMAN1, but also interact with FV and FVIII.<sup>21</sup> LMAN1 was recently shown to interact with AAT and this interaction is independent of MCFD2.<sup>22</sup>

LMAN1 and MCFD2 deficiencies in mice also lead to decreased plasma FV, FVIII, and AAT levels.<sup>11,12</sup> Mouse studies also revealed a strain-specific partial lethal phenotype in LMAN1-deficient mice, but not in MCFD2-deficient mice, suggesting distinct functions of the 2 proteins other than transporting FV/FVIII.<sup>11,12</sup> To further understand the molecular mechanism of F5F8D and the regulation of ER-Golgi trafficking of FV and FVIII, we developed a complementation assay in LMAN1 and MCFD2 knockout (KO) cells to rapidly test functions of LMAN1 and MCFD2 variants, as well as features of FV/FVIII required for receptor-mediated secretion. We demonstrated that the reduction of FV/FVIII secretion varied greatly among different KO cells, suggesting that regulation of cargo transport by the LMAN1/MCFD2 pathway varies in different cell types. We provide evidence that carbohydrate binding is not essential for the FV/FVIII transport function of LMAN1. Surprisingly, overexpression of MCFD2 in LMAN1 KO cells is sufficient to rescue FV/FVIII secretion defects. These results suggest that cargo binding and transport are carried out by MCFD2 and that LMAN1 primarily serves as a carrier of MCFD2.

### **Methods**

### Cells

HepG2 cells were grown in American Type Culture Collectionformulated Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, 100 international unit (IU)/mL penicillin, and 100 IU/mL streptomycin at 37°C and in 5% CO<sub>2</sub>. Human embryonic kidney 293T cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin at 37 °C and in 5%  $CO_2$ . HCT116 cells were grown in McCoy's 5A Medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin at 37°C and in 5%  $CO_2$ . Cells were split into 24-well plates the day before transfection. FVIII plasmids (300 ng per well) were transfected into 293T cells using FuGENE 6 (Promega, Madison, WI) and into HepG2 and HCT116 cells using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA). FV plasmids (150 ng per well) were transfected into 293T cells using FuGENE 6 and into HCT116 cells using Lipofectamine 3000. Generation of cell lines stably expressing LMAN1 and MCFD2 was carried out as previously described.<sup>22</sup>

### **Plasmid construction**

Construction of the mutant constructs N156A, H178A, W67S. and  $\Delta\beta1$  of LMAN1, as well as D129E and Y135N of MCFD2 were described previously.7,10,20 Missense mutations (V147I and V100D) were introduced into the plasmid pED-FLAG-LMAN1 and pcDNA-MCFD2-Myc separately using the QuickChange II Sitedirected mutagenesis XL kit (Agilent, Santa Clara, CA). FVIII mutant constructs  $\Delta 807$  to 816 (deletion of amino acids 807-816 of FVIII) and mut807 to 816 (replacement of all amino acids between 807-816 of FVIII with alanine) were prepared by polymerase chain reaction using the QuickChange II Site-directed mutagenesis II XL kit. The pMT2-FVIII wild-type (WT) construct encoding full-length FVIII<sup>23</sup> was used as the template for polymerase chain reaction. Plasmid pED-FV encoding full-length FV was a gift from Rodney M. Camire (Children's Hospital of Pennsylvania). All mutant constructs were confirmed by Sanger sequencing for the presence of desired mutations and the absence of unintended mutations.

### Reagents

Rabbit polyclonal antibody and mouse monoclonal antibody against FLAG were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal anti-Myc antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-AAT antibody was purchased from Proteintech (Rosemont, IL). Rabbit monoclonal anti-LMAN1 antibody was purchased from Abcam (Cambridge, MA). Protein A/G PLUS-Agarose beads were purchased from Santa Cruz Biotechnology. D-mannose agarose was purchased from Sigma-Aldrich. Human AAT enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN).

### Establishment of LMAN1-deficient HCT116 cells

The HCT116 cell line was derived from colorectal cancer with microsatellite instability. The exon 8 of *LMAN1* contains a microsatellite site with a string of 9 adenosines (9A). HCT116 cells consist of mostly heterozygous population with alleles of 8A and 9A (8A/9A) at this site.<sup>24</sup> We discovered that a small number of HCT116 cells had homozygous 8A alleles (8A/8A). Colonies derived from single cells were genotyped to identify cells with homozygous 8A/8A and heterozygous 8A/9A alleles. The lack of LMAN1 expression in homozygous 8A/8A KO cells was confirmed by immunoblotting.

#### FVIII activity and FVIII/FV antigen analysis

FVIII activity was measured by a chromogenic assay using the Coatest SP4 FVIII kit (DiaPharma, West Chester, OH). FVIII antigen was quantified by ELISA using the VisuLize FVIII antigen kit (Affinity Biologicals, Ancaster, ON, Canada). FVIII antigen levels were calculated assuming a concentration of 200 ng/mL in FVIII standard (1 IU/mL). FV antigen was quantified by ELISA using a matched-pair antibody set for human FV antigen from the Affinity Biologicals. FV antigen levels were calculated assuming a concentration of 10 μg/mL in the normal human plasma standard. All assays were performed according to manufacturers' instructions.

#### Immunoprecipitation and mannose-binding assay

Immunoprecipitation of LMAN1 and MCFD2 was performed as previously described.<sup>10</sup> Mannose-binding assay was performed as previously described,<sup>20</sup> with some modifications. Briefly, 293T cells were harvested on ice in homogenate buffer 48 hours after transfection with LMAN1 expression constructs. Cells were homogenized and cleared by centrifugation at 500*g* for 10 minutes. Membrane fractions from postnuclear supernatants were pelleted at 100 000*g* for 1 hour. The pellet was solubilized for 1 hour in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> containing 1% Triton X-100, and protease inhibitors),

followed by centrifugation at 100 000*g* for 1 hour and dialysis of the supernatant overnight against the binding buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 0.15% Triton X-100). The dialysate was incubated with D-mannose agarose beads overnight, and bound LMAN1 was eluted using 0.2 M D-mannose in binding buffer. Eluted LMAN1 was detected by immunoblotting analysis using a rabbit anti-FLAG antibody.

#### Statistical analysis

FVIII activity, FVIII antigen, and FV antigen assay results were analyzed using the Student *t* test for comparison between 2 groups and by 1-way analysis of variance for comparisons of >2 groups. P-values < .05 were considered significant for all assays.

### Results

# Extent of decreases in FV and FVIII secretion varies in different *LMAN1* and *MCFD2* KO cells

Using the CRISPR-Cas9 technology, we previously established 293T and HepG2 cells with *LMAN1* or *MCFD2* KO and found reduced rates of ER-Golgi transport of AAT in these cells.<sup>22</sup> We also now have established LMAN1-deficient HCT116 (HCT116<sup>-/-</sup>) cells with homozygous frameshift mutations in the exon 8 of *LMAN1* 



**Figure 1. Secretion levels of FVIII and FV in different cell lines.** (A) LMAN1 and MCFD2 levels in WT, HepG2<sup>LMAN1-</sup>, HepG2<sup>MCFD2-</sup>, 293T<sup>LMAN1-</sup>, and 293T<sup>MCFD2-</sup> cells, as well as HCT116<sup>+/-</sup> and HCT116<sup>-/-</sup> cells. (B) Conditioned media were collected from HepG2 cell lines 48 hours after a fresh medium change. A FV expression construct was transfected into the indicated 293T and HCT116 cell lines and conditioned media were collected 48 hours after transfection. FV levels were measured by ELISA. (C) A FVIII expression construct was transfected into the indicated cell lines and conditioned media were collected 48 hours after transfection. FV levels were measured by ELISA. (C) A FVIII expression construct was transfected into the indicated cell lines and conditioned media were collected 48 hours after transfection. FVIII activity and antigen levels in conditioned media were measured. (D) An AAT expression construct was transfected into HCT116<sup>+/-</sup> and HCT116<sup>-/-</sup> cells, and conditioned media were collected 48 hours after transfection. FVIII activity and antigen levels in conditioned media were measured. (D) An AAT expression construct was transfected into HCT116<sup>+/-</sup> and HCT116<sup>-/-</sup> cells, and conditioned media were collected 48 hours after transfection. AAT concentrations in conditioned media were measured by ELISA. All data presented are means of 3 independent experiments, and the error bars represent standard deviations. \**P* < .05; \*\**P* < .01; \*\*\**P* < .001.

(supplemental Figure 1).<sup>24</sup> LMAN1 and MCFD2 are expressed in HepG2 cells at much higher levels than in 293T and HCT116 cells (Figure 1A). As expected, *LMAN1* KO cells had a marked reduction in MCFD2 levels owing to MCFD2's reliance on LMAN1 for intracellular retention, whereas MCFD2 KO had no effects on LMAN1 levels (Figure 1A). Secretion of endogenous FV was analyzed by measuring FV antigen levels in conditioned media of WT and KO HepG2 cells. Secretion of FV was also analyzed in WT and KO 293T or HCT116 cells by measuring FV antigen levels in conditioned media of cells transfected with a FV expression plasmid. Results showed that FV secretion levels were reduced to ~50% of the WT level in both LMAN1 and MCFD2 KO 293T and HepG2 cell lines (Figure 1B). However, FV secretion in HCT116<sup>-/-</sup> cells only decreased mildly to ~80% of the HCT116<sup>+/-</sup> level (Figure 1B). To measure secretion of FVIII, a FVIII expression plasmid was transfected into WT and KO cells, and FVIII activity and antigen levels in conditioned media were detected 48 hours after transfection. FVIII activity and antigen levels decreased in all 293T and HepG2 KO cell (293T<sup>LMAN1-</sup>, 293T<sup>MCFD2-</sup>, HepG2<sup>LMAN1-</sup>, lines and HepG2<sup>MCFD2-</sup>) to <10% level of WT cells (Figure 1C). However, reduction of FVIII in conditioned media of HCT116<sup>-/-</sup> cells was less profound, to only ~50% level of HCT116<sup>+/-</sup> cells (Figure 1C). Moderate decrease in FVIII secretion in HCT116<sup>-/-</sup> cells is consistent with a previous report.<sup>25</sup> AAT is another client cargo protein of the LMAN1-MCFD2 complex. Our previous study showed that AAT secretion decreased by ~50% in LMAN1 and MCFD2 KO 293T and HepG2 cells.<sup>22</sup> In this study, we measured the secretion of AAT transfected into HCT116 cells. The amount of AAT secreted into conditioned media of HCT116<sup>-/-</sup> cells decreased to  $\sim$ 70% of the HCT116<sup>+/-</sup> level, similar to FV results (Figure 1D).

# The SDLLMLLRQS sequence in the FVIII B domain is not required for LMAN1/MCFD2-dependent secretion

A recent study reported a putative MCFD2-binding segment from the B domain of FVIII (SDLLMLLRQS at residues 807-816).<sup>25</sup> To test the role of this sequence in the LMAN1-MCFD2 secretion pathway of FVIII, we constructed FVIII with either the deletion of these residues  $[\Delta(807-816)]$  or the alanine replacement of all residues in this segment [mut(807-816)]. Secretion of WT and mutant FVIII was compared in WT, LMAN1 KO, and MCFD2 KO cell lines. Deletion or mutation of this segment did not cause a reduction of activity and antigen levels of FVIII secreted in WT 293T, HepG2, and HCT116 cell lines (supplemental Figure 2). FVIII activity and antigen levels of both  $\Delta(807-816)$  and mut(807-816) mutants were reduced in conditioned media of both LMAN1 KO and MCFD2 KO 293T and HepG2 cells, as well as in LMAN1-deficient HCT116<sup>-/-</sup> cells, to levels identical to that of WT FVIII (supplemental Figure 2). These results suggest that the SDLLMLLRQS sequence in the B domain is not required for LMAN1/MCFD2-dependent secretion of FVIII.

# LMAN1 with mutations abolishing carbohydrate binding can still partially rescue FVIII secretion

To evaluate whether re-expression of LMAN1 in *LMAN1* KO cells can rescue the FVIII secretion defects, we cotransfected FVIII and different LMAN1 expression constructs into 293T<sup>LMAN1-</sup> cells (Figure 2A). Only functional LMAN1 or MCFD2 molecules are expected to rescue FVIII secretion defects in KO cells. As expected, cotransfection of WT LMAN1 could fully restore active

FVIII secretion (Figure 2B-C). In contrast, no rescue of FVIII secretion occurred with cotransfection of the W67S and Δβ1 variants (Figure 2B-C). W67S is a patient mutation that abolishes both MCFD2 and mannose binding.<sup>20</sup> Δβ1 mutant has a deletion of the first β-sheet in the CRD of LMAN1, which is directly involved in MCFD2 binding, thus abolishing MCFD2 binding without affecting mannose binding.<sup>11</sup> V147I is a recently reported missense variant in LMAN1 found in a patient from China with F5F8D.<sup>26</sup> However, cotransfection of the V147I variant resulted in FVIII secretion at a level comparable with the cotransfection of WT LMAN1 (Figure 2A-C), indicating that this variant did not affect the LMAN1 function in FVIII secretion.

Using this LMAN1 complementation assay, we found that LMAN1 variants with point mutations in the mannose-binding site (N156A and H178A) could still rescue FVIII secretion to ~60% level of WT LMAN1 (Figure 2B-C) in 293T cells. To rule out the possibility that the surprising rescue of FVIII secretion by N156A and H178A mutants was an artifact of overexpression, we decreased LMAN1 expression to levels comparable or lower than that in WT cells. Under these conditions, N156A and H178A mutants could still rescue most of the FVIII secretion (supplemental Figure 3). Next, we established HepG2<sup>LMAN1-</sup> cell lines that stably expressed WT, N156A, H178A, and W67S variants of LMAN1 using retroviral expression vectors (Figure 2D). In this system, transfected FVIII secretion was also partially rescued in HepG2<sup>LMAN1-</sup> cell lines stably expressing N156A and H178A variants (Figure 2E-F). In addition, WT LMAN1 rescued endogenous FV secretion in  $HepG2^{\text{LMAN1-}}$  cells to ~90% of WT cells, whereas N156A and H178A variants rescued FV secretion to ~70% level of WT cells (Figure 2G). In all experiments, secreted FVIII activity levels correlated with antigen levels, indicating no reduction in the specific activity.

The N156A mutation changed a critical amino acid in the carbohydrate-binding pocket and was shown to abolish mannose binding without affecting MCFD2 binding.<sup>9</sup> We have previously shown by isothermal titration calorimetry that the H178A mutation abolished D-mannose-binding without affecting Ca<sup>2+</sup> binding.<sup>2</sup> Here, we directly confirmed mannose-binding deficiency of both N156A and H178A mutants in a mannose-binding assav (Figure 3A). This assay also showed that the V147I variant retains mannose-binding activity (Figure 3A). To test the LMAN1-MCFD2 interaction, we performed a coimmunoprecipitation (co-IP) assay. We included WT as a positive control and W67S and  $\Delta\beta$ 1 as negative controls. Results showed that the V147I, N156A, and H178A variants could still coimmunoprecipitate with MCFD2 (Figure 3B), suggesting that the LMAN1-MCFD2 interaction was not disrupted by these mutations. Taken together, these results suggest that carbohydrate binding is not essential for the cargo receptor function of LMAN1, and that V147I is not a deleterious mutation.

# The V100D variant of MCFD2 is a hypomorphic mutation

To test MCFD2 mutant functions, we created HepG2 and 293T cell lines that stably expressed WT and different mutant MCFD2 by transducing cells with retroviruses carrying MCFD2 expression constructs into HepG2<sup>LMAN1-</sup> and 293T<sup>LMAN1-</sup> cells (Figure 4A,E). These cells were then transfected with a FVIII expression construct

2024



**Figure 2. Rescue of FV and FVIII secretion in LMAN1 KO cells by LMAN1 variants.** (A) WT 293T cells were transfected with WT LMAN1 or the vector. 293T<sup>LMAN1-</sup> cells were transfected with WT LMAN1, the indicated LMAN1 variants or the vector. Forty-eight hours after transfection, conditioned media were collected and cell lysates were subjected to immunoblotting analyses with anti-LMAN1, anti-MCFD2, and anti- $\beta$ -actin antibodies. FVIII activity (B) and antigen (C) levels in conditioned media were measured. (D) WT LMAN1 and the indicated LMAN1 variants were stably expressed into HepG2<sup>LMAN1-</sup> cells and LMAN1 expression levels were compared with vector-transduced WT HepG2 and HepG2<sup>LMAN1-</sup> cells by immunoblotting. (E-F) A FVIII expression construct was transfected into HepG2<sup>LMAN1-</sup> cells stably expressing indicated LMAN1 variants and conditioned media were collected 48 hours after transfection. FVIII activity and antigen levels in conditioned media were measured. (G) Conditioned media were collected 48 hours after transfection. FVIII activity and antigen levels in conditioned media were measured. (G) Conditioned media were collected 48 hours after transfection. FVIII activity and antigen levels in conditioned media were measured. (G) Conditioned media were collected 48 hours after transfection. FVIII activity and antigen levels in conditioned media were measured. (G) Conditioned media were collected 48 hours after a fresh medium change. FV antigen levels were measured by ELISA. FV and FVIII level data presented in this figure are means of 3 independent experiments, and the error bars represent standard deviations. \*\*P < .01; \*\*\*P < .001. ns, not significant.

to assess the secretion of FVIII. WT MCFD2 restored FVIII secretion in both HepG2<sup>MCFD2-</sup> (Figure 4B-C) and 293T<sup>MCFD2-</sup> cells (Figure 4F-G) to ~80% of WT cells. Expression of WT MCFD2 restored endogenous FV secretion in HepG2<sup>MCFD2-</sup> cells and

transfected FV secretion in 293T<sup>MCFD2-</sup> cells from ~70 to 80% of WT cells (Figure 4D,H). D129E and Y135N are patient mutations localized to the second EF-hand domain and cause the disease owing to disruption of the LMAN1-MCFD2 complex.<sup>21</sup> We



Figure 3. Interactions of LMAN1 variants with mannose

and MCFD2. (A) Mannose-binding assay. 293T cells were transfected with the FLAG-tagged WT and the indicated LMAN1 variants. Cell lysates were loaded onto a mannose agarose column. The bound LMAN1 was eluted from the column and detected by immunoblotting. (B) Co-IP of LMAN1 variants and MCFD2. 293T cells were cotransfected with FLAG-tagged WT and LMAN1 mutants and a Myc-tagged MCFD2. Cell lysates were immunoprecipitated with anti-Myc for MCFD2 and anti-FLAG for LMAN1 and were detected by immunoblotting.



**Figure 4. Rescue of FV and FVIII secretion in** *MCFD2* **KO cells by MCFD2 variants. (A) WT MCFD2 and the indicated MCFD2 variants were stably expressed in HepG2<sup>MCFD2-</sup> cells and MCFD2 expression levels were compared with vector-transduced WT HepG2 and HepG2<sup>MCFD2-</sup> cells by immunoblotting. A FVIII expression construct was transfected into these cells and conditioned media were collected 48 hours after transfection. FVIII activity (B) and antigen (C) levels in conditioned media were measured. (D) Conditioned media were collected 48 hours after a fresh medium change. FV antigen levels were measured by ELISA. (E) WT MCFD2 and the indicated MCFD2 variants were stably expressed in 293T<sup>MCFD2-</sup> cells and MCFD2 expression levels were compared with vector-transduced WT 293T and 293T<sup>MCFD2-</sup> cells by immunoblotting. A FVIII expression construct or a FV expression construct was transfected into these cells and conditioned media were collected 48 hours after transfection. FVIII activity (F), FVIII antigen (G), and FV antigen (H) levels in conditioned media were measured. FV and FVIII level data presented in this figure are means of 3 independent experiments, and the error bars represent standard deviations. \****P* **< .05; \*\****P* **< .001.** 

observed small but significantly increased FVIII secretion in both HepG2<sup>MCFD2-</sup> and 293T<sup>MCFD2-</sup> cells that expressed D129E and Y135N mutants compared with the vector control (Figure 4B-C,F-G). Expression of D129E and Y135N mutants did not significantly increase FV secretion (Figure 4D,H), likely because of higher basal levels of FV secretion in MCFD2 KO cells. Using this MCFD2 complementation assay, we tested the function of the V100D variant of MCFD2. V100D is a variant reported in a Tunisian patient and is localized at the helix 2 of the first EF-hand domain.<sup>28</sup> Results showed that the V100D variant was also able to partially rescue FVIII secretion to ~50% level of WT cells in HepG2<sup>MCFD2-</sup> cells (Figure 4B,C) and >80% in 293T<sup>MCFD2-</sup> cells (Figure 4F,G). Similarly, this variant also partially rescued the endogenous FV secretion in both cell lines (Figure 4D,H). The V100D variant could also coimmunoprecipitate with LMAN1, indicating that it can form a complex with LMAN1 (supplemental Figure 4). These results suggest that the V100D mutation is hypomorphic and likely not a disease-causing mutation.

# Overexpression of MCFD2 in *LMAN1* KO 293T cells restores FVIII secretion

As noted above, 293T<sup>MCFD2-</sup> and HepG2<sup>MCFD2-</sup> cells stably expressing D129E or Y135N mutants had FVIII secretion levels approximately twofold higher than the vector control (Figure 4B-C,F-G).

One possible reason for this could be that these mutants may retain residual LMAN1-binding activities,<sup>8</sup> and a trace amount of the LMAN1-MCFD2 complex is responsible for the increased FVIII secretion. To exclude this possibility, we established stable cell lines that expressed WT MCFD2 and different MCFD2 variants in 293T<sup>LMAN1-</sup> cells. Steady-state levels of WT and variant MCFD2 were similar in cell lysates (supplemental Figure 5). Interestingly, FVIII secretion still increased by twofold in cells expressing either WT or MCFD2 variants compared with 293T<sup>LMAN1-</sup> cells with vector control after transfection of a FVIII expression construct (supplemental Figure 5), suggesting that the increased FVIII secretion was owing to the expression of MCFD2 or its variants, not the formation of a trace amount of the LMAN1-MCFD2 complex.

To test whether overexpression of MCFD2 could further rescue FVIII secretion, we cotransfected MCFD2 and FVIII expression constructs into 293T<sup>MCFD2-</sup> and 293T<sup>LMAN1-</sup> cells and measured antigen and activity levels of secreted FVIII. MCFD2 was transfected in 2 doses to control the amounts of protein expression. Protein levels of transfected MCFD2 in both KO cells were five- to sixfold higher than the endogenous MCFD2 level in WT cells with 50 ng plasmid DNA and two- to threefold higher than WT cells with 20 ng plasmid DNA (Figure 5B,E). In both cell lines, all MCFD2 variants (WT, V100D, D129E, and Y135N) rescued FVIII secretion. Higher MCFD2 expression rescued FVIII

secretion to ~90% levels of WT MCFD2, whereas lower MCFD2 expression led to 50% to 70% rescue (Figure 5A,D). In contrast, overexpression of LMAN1 in  $293T^{MCFD2-}$  cells did not affect FVIII secretion (supplemental Figure 6). Overexpression of MCFD2 variants also led to near-complete rescue of FV secretion in both cell lines

(Figure 5C,F). In addition, overexpression of MCFD2 in 293T<sup>MCFD2-</sup> and 293T<sup>LMAN1-</sup> cells also rescued the FVIII  $\Delta$ (807-816) secretion (supplemental Figure 7), providing further evidence that this B domain sequence is not important in LMAN1/MCFD2-dependent secretion of FVIII.



Figure 5. Overexpression of MCFD2 rescues FVIII secretion in LMAN1 KO and MCFD2 KO cells. (A) A FV or FVIII expression construct and vectors expressing the indicated MCFD2 variants were cotransfected into  $293T^{\text{MCFD2-}}$  cells in 24-well plates in 2 doses (50 ng and 20 ng). MCFD2 expression levels in cell lysates were compared with vector-transfected WT 293T and 293T<sup>MCFD2-</sup> cells by immunoblotting. (B) FV antigen levels in conditioned media were measured 48 hours after transfection. (C) FVIII activity and antigen levels in conditioned media were measured 48 hours after transfection. (D) A FV or FVIII expression construct and vectors expressing the indicated MCFD2 variants were cotransfected into 293T<sup>LMAN1-</sup> cells in 24-well plates in 2 doses (50 ng and 20 ng). MCFD2 expression levels in cell lysates were compared with vector-transfected WT 293T and 293T<sup>MCFD2-</sup> cells by immunoblotting. MCFD2 expression levels in cell lysates were compared with vector-transfected WT 293T and 293T<sup>LMAN1-</sup> cells by immunoblotting. (E) FV antigen levels in conditioned media were measured 48 hours after transfection. (F) FVIII activity and antigen levels in conditioned media were measured 48 hours after transfection. FVIII activity and antigen data presented are means of 3 independent experiments, and the error bars represent standard deviations. \*\*P < .01; \*\*\**P* < .001.



**Figure 6. Overexpression of the LMAN1-MCFD2 complex does not further increase FV and FVIII secretion.** (A) WT HepG2 and HepG2<sup>LMAN1-</sup> cells with (+) or without (-) stably expressing LMAN1 were transfected with a MCFD2 expressing construct (+) or with an empty vector (-). Cell lysates were subjected to immunoblotting 48 hours after transfection with the indicated antibodies. (B) FV antigen levels were measured in conditioned media 48 hours after transfection. (C) WT 293T cells with (+) or without (-) stably overexpressing MCFD2 were cotransfected with a FVIII expression construct and a LMAN1 expressing construct (+) or an empty vector (-) as indicated. Cell lysates were subjected to immunoblotting 48 hours after transfection. FV and FVIII level data presented are means of 3 independent experiments, and the error bars represent standard deviations.

# Overexpression of the LMAN1-MCFD2 complex does not further increase FV and FVIII secretion

Previous results showed that LMAN1 and MCFD2 form a complex with 1:1 stoichiometry.<sup>10</sup> However, overexpression of LMAN1 did not increase endogenous MCFD2 beyond the WT cell level (Figure 2). Overexpression of MCFD2 also did not increase the endogenous LMAN1 level.<sup>10</sup> These results suggest that levels of LMAN1 and MCFD2 are independently regulated. To increase LMAN1 and MCFD2 levels simultaneously in HepG2 cells, we transfected MCFD2 into HepG2<sup>LMAN1-</sup> cells stably overexpressing LMAN1. The resulting cells express both LMAN1 and MCFD2 to two- to threefold levels of WT HepG2 cells (Figure 6A, lane 2). However, secreted endogenous FV levels in conditioned media of these cells were not significantly different from WT cells and cells overexpressing MCFD2 or LMAN1 alone, and were higher than LMAN1 KO cells (Figure 6B). To assess whether increasing both LMAN1 and MCFD2 could increase FVIII secretion, we cotransfected LMAN1 and FVIII into 293T cells stably overexpressing WT MCFD2, which led to overexpression of both LMAN1 and MCFD2 (Figure 6C, lane 2). FVIII levels in conditioned media of this cell line were unchanged compared with WT 293T cells or 293T cells overexpressing LMAN1 or MCFD2 alone (Figure 6D). These results indicate that increasing the amount of the LMAN1-MCFD2 complex does not lead to increase in FV and FVIII secretion.

# **Discussion**

LMAN1 deficiency in all 3 tested cell lines led to decreased FV and FVIII secretion. MCFD2 deficiency in 293T and HepG2 cells also led to similar decrease in FV and FVIII secretion. These results suggest that these cell lines can be used to model F5F8D in vitro. However, the extent of decrease in secretion varies greatly in different cells, suggesting that regulation of cargo transport by the LMAN1/MCFD2 pathway varies in different cell types. In particular, FV/FVIII secretion in HCT116 cells appears less dependent on the LMAN1-MCFD2 complex suggesting either the existence of an alternative pathway for ER-Golgi transport of FV/FVIII or other cargo receptors with functional overlap. FVIII is primarily expressed in endothelial cells.<sup>29,30</sup> Human FV is expressed in hepatocytes and taken up by megakaryocytes through endocytosis.<sup>31</sup> Although none of the cell lines naturally synthesize FVIII, HepG2 cells have endogenous FV expression and much higher expression levels of LMAN1 and MCFD2. Our study has limitations. FVIII expression in transient transfection experiments is low and variable. Owing to the sensitivity limitations of the ELISA, low FVIII expression levels could complicate the interpretation of the data. Curiously, the extent of decrease in FV secretion is much less than the decrease in FVIII secretion in the same KO cells. This is in contrast to both patients with F5F8D<sup>1</sup> and LMAN1/MCFD2-deficient mice,<sup>12</sup> which have moderate correlations in plasma FV and FVIII levels. The reason for this discrepancy is unclear but may be because of intrinsic differences between FV and FVIII expressed in vitro, or the use of nonnative cells to express FVIII. FV has much higher messenger RNA expression levels than FVIII in transfected cells, and its translation product is fivefold more efficiently secreted into conditioned media.<sup>32</sup> FVIII is more prone to misfolding and aggregation than FV.<sup>33</sup> ER chaperones BiP and calnexin are required for FVIII folding but not for FV folding.<sup>34,35</sup> Intracellular adenosine triphosphate is required for FVIII secretion but not for FV secretion.<sup>35</sup> In addition, AAT is also expressed at a higher level and is less prone to misfolding. Its secretion is decreased to a similar extent as of FV in KO cells.

Signals in FVIII required for the interaction with the LMAN1-MCFD2 complex have not been identified, although it is thought that N-glycans in cargo proteins play a major role in LMAN1 interaction. To our surprise, we found that the N156A and H178A mutations that abolish sugar binding were still able to rescue most of the FVIII secretion defects in LMAN1 KO cells. This result may also explain why no missense mutations have been identified in the carbohydrate-binding region of LMAN1.<sup>1</sup> Any LMAN1 missense mutation would have to severely affect either the protein expression or function, as a hypomorphic mutation that reduces murine Lman1 messenger RNA expression to 6% to 8% of WT level leads to intermediate plasma FV and FVIII reductions.  $^{\rm 36}$  There are only 2 LMAN1 missense mutations reported to date. The W67S mutation causes a MCFD2 binding defect in addition to the mannosebinding defect.<sup>20,37</sup> The C558R mutation leads to an unstable protein.<sup>8</sup> More missense mutations were identified in MCFD2, all of which abolish LMAN1 binding. These results suggest that LMAN1-MCFD2 complex formation, but not N-glycan binding of LMAN1, is required for the cargo receptor function. A possible explanation is that LMAN1 can interact with FVIII through a lectin-independent, direct protein-protein interaction. Our previous studies showed that cross-linking of FVIII with LMAN1 could still be observed in cells treated with N-glycosylation inhibitor, tunicamycin.<sup>10</sup> Targeting of procathepsin Z appears to require both an oligosaccharide chain and a surface-exposed peptide β-hairpin loop.<sup>38</sup> Matrix metallopeptidase 9 N-glycosylation mutants also strongly co-IP with LMAN1.<sup>19</sup> Crystal structures of the LMAN1-CRD suggested potential protein-binding sites for cargo proteins.<sup>39,40</sup>

We also tested a recently reported MCFD2-binding motif from the FVIII B domain. It was reported that deletion of this putative motif caused reduced secretion of FVIII from HCT116 cells.<sup>25</sup> However, we could not duplicate this result in HCT116 cells. We noted that Yagi et al<sup>25</sup> used a codon-optimized version of FVIII, which could explain some differences from our results. No secretion defects of motif-deleted or -mutated FVIII were observed in 293T and HepG2 cells either. Moreover, secretion of both WT FVIII and motif-deleted or -mutated FVIII was reduced to the same extent in all 3 *LMAN1* KO cell lines, as well as in 293T<sup>MCFD2-</sup> and HepG2<sup>MCFD2-</sup> cells. These results strongly suggest that this motif is neither a signal nor sufficient by itself to serve as a signal for LMAN1-MCFD2- dependent secretion of FVIII. Further studies are needed to identify signals recognized by the LMAN1-MCFD2 cargo receptor complex.

*LMAN1* and *MCFD2* KO cell lines provide convenient functional assays to accurately assess the pathogenicity of LMAN1 and MCFD2 mutations. The V147I variant is located at a highly conserved site and in silico analysis predicted that this is a

pathogenic mutation.<sup>26</sup> Our results showed that it can fully rescue FVIII secretion defects in LMAN1 KO cells and has no detected defects in MCFD2 and mannose binding. Therefore, it is likely not a disease-causing mutation. Reduction of FV and FVIII levels in patients may result from a mutation in either LMAN1 or MCFD2 that was missed by DNA sequencing. There were previous reports of patients who were deficient in LMAN1 with no mutations identified in the exons and exon-intron junctions.<sup>1,8</sup> V100D is carried together in a heterozygous state with another missense mutation D81H in a homozygous state.<sup>28</sup> Although MCFD2 proteins with D81H and D81H/V100D mutations have been extensively studied, the impact of the V100D variant on MCFD2 structure is still inconclusive.<sup>41-43</sup> Circular dichroism analysis of the recombinant proteins indicated that the D81H single mutant and the D81H-V100D double mutant had nearly identical circular dichroism spectra similar to the disordered apo state of native MCFD2.<sup>41</sup> Our results indicate that this variant can partially rescue FV and FVIII secretion in MCFD2 KO cells and co-IP with LMAN1; therefore, it is unlikely to cause F5F8D even if inherited in a homozygous or compound heterozygous state.

Overexpression of both LMAN1 and MCFD2 rescued FV/FVIII secretion to levels comparable to, but not exceeding WT cells, suggests that the LMAN1-MCFD2 cargo receptor level is not a rate-limiting factor in ER-Golgi transport of FV/FVIII. All MCFD2 missense mutations identified to date are localized to the EF-hand domains<sup>21</sup> which are important for LMAN1 binding.<sup>39,40</sup> Here, we present a surprising finding that overexpression of MCFD2 alone could rescue FV/FVIII secretion defects in 293T<sup>LMAN1-</sup> cells. Our previous studies showed that MCFD2 interacts with FV and FVIII independent of LMAN1, suggesting that MCFD2 contains distinct FV/FVIII and LMAN1 binding sites.<sup>21</sup> Patients with LMAN1 deficiency have only trace amounts of intracellular MCFD2 because of the requirement of LMAN1 for intracellular retention.<sup>7,10</sup> This trace amount of MCFD2 is insufficient to compensate for the loss of LMAN1 but could explain the statistically higher FV/FVIII levels in patients with LMAN1 mutations than in patients with MCFD2 mutations.<sup>1</sup> Our data suggest that LMAN1 and MCFD2 have distinct functions in cargo transport. MCFD2 is the "cargo capture module" that brings cargo proteins to the LMAN1-MCFD2 complex in the ER for packaging into COPII vesicles. The major function of LMAN1 in cargo transport is to serve as an ER-Golgi shuttling carrier for MCFD2. The mannose-binding site of LMAN1 may further stabilize the tertiary complex with FV/FVIII. Although LMAN1 could co-IP with AAT independent of MCFD2, this interaction is apparently not sufficient to form a stable transport-competent complex, as AAT secretion is also decreased in MCFD2 KO cells.<sup>22</sup>

When overexpressed in *LMAN1* KO cells, the flux of MCFD2 protein may be sufficient to overcome the lack of a LMAN1 carrier. This function of MCFD2 is independent of LMAN1-binding as mutant MCFD2 that cannot bind LMAN1 can still rescue FVIII secretion in *LMAN1* KO cells. How does MCFD2 transport FV/FVIII without LMAN1? Either it could serve as a chaperonin protein that stabilizes cargo in the ER or the MCFD2-cargo complex formation is sufficient to facilitate the ER exit of cargo by bulk flow.

In conclusion, our results demonstrate that LMAN1- and MCFD2deficient cell lines provide valuable tools to study cargo receptormediated secretion of FV/FVIII and other proteins. Using these cells, we functionally characterized LMAN1 and MCFD2 variants from patients with F5F8D and showed that regulation of cargo transport by the LMAN1/MCFD2 pathway varies in different cell types. We present evidence supporting a model in which MCFD2 functions as a primary interacting partner of FV/FVIII cargo and LMAN1 primarily serves as a vehicle that shuttles MCFD2 between the ER and the Golgi.

### **Acknowledgments**

This study was supported by the National Heart, Lung, and Blood Institute (R01 HL094505 and R03 CA202131) (B.Z.) and the Alpha-1 Foundation. Y.Z. is a recipient of the Judith Graham Pool Postdoctoral Research Fellowship from the National Hemophilia Foundation.

### Authorship

Contribution: Y.Z. and B.Z. designed the study and wrote the manuscript; Z.L. provided critical comments; Y.Z. and Z.L. performed research; and all authors analyzed the data.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Bin Zhang, Genomic Medicine Institute, Cleveland Clinic Lerner Research Institute, 9500 Euclid Ave, Cleveland, OH 44195; email: <a href="mailto:zhangb@ccf.org">zhangb@ccf.org</a>.

### References

- 1. Zhang B, Spreafico M, Yang A, et al. Genotype-phenotype correlation in combined deficiency of factor V and factor VIII. *Blood.* 2008;111(12): 5592-5600.
- 2. Zheng C, Zhang B. Combined deficiency of coagulation factors V and VIII: an update. Semin Thromb Hemost. 2013;39(6):613-620.
- 3. Seligsohn U, Zivelin A, Zwang E. Combined factor V and factor VIII deficiency among non-Ashkenazi Jews. N Engl J Med. 1982;307(19):1191-1195.
- 4. Zhang B. Recent developments in the understanding of the combined deficiency of FV and FVIII. Br J Haematol. 2009;145(1):15-23.
- 5. Peyvandi F, Tuddenham EG, Akhtari AM, Lak M, Mannucci PM. Bleeding symptoms in 27 Iranian patients with the combined deficiency of factor V and factor VIII. *Br J Haematol.* 1998;100(4):773-776.
- 6. Nichols WC, Seligsohn U, Zivelin A, et al. Mutations in the ER-Golgi intermediate compartment protein ERGIC-53 cause combined deficiency of coagulation factors V and VIII. *Cell.* 1998;93(1):61-70.
- Zhang B, Cunningham MA, Nichols WC, et al. Bleeding due to disruption of a cargo-specific ER-to-Golgi transport complex. Nat Genet. 2003;34(2): 220-225.
- 8. Zhang B, McGee B, Yamaoka JS, et al. Combined deficiency of factor V and factor VIII is due to mutations in either LMAN1 or MCFD2. *Blood.* 2006; 107(5):1903-1907.
- Itin C, Roche AC, Monsigny M, Hauri HP. ERGIC-53 is a functional mannose-selective and calcium-dependent human homologue of leguminous lectins. Mol Biol Cell. 1996;7(3):483-493.
- 10. Zhang B, Kaufman RJ, Ginsburg D. LMAN1 and MCFD2 form a cargo receptor complex and interact with coagulation factor VIII in the early secretory pathway. J Biol Chem. 2005;280(27):25881-25886.
- 11. Zhang B, Zheng C, Zhu M, et al. Mice deficient in LMAN1 exhibit FV and FVIII deficiencies and liver accumulation of alpha1-antitrypsin. *Blood*. 2011; 118(12):3384-3391.
- 12. Zhu M, Zheng C, Wei W, Everett L, Ginsburg D, Zhang B. Analysis of MCFD2- and LMAN1-deficient mice demonstrates distinct functions in vivo. *Blood Adv.* 2018;2(9):1014-1021.
- 13. Nyfeler B, Zhang B, Ginsburg D, Kaufman RJ, Hauri HP. Cargo selectivity of the ERGIC-53/MCFD2 transport receptor complex. *Traffic*. 2006;7(11): 1473-1481.
- 14. Nyfeler B, Reiterer V, Wendeler MW, et al. Identification of ERGIC-53 as an intracellular transport receptor of a1-antitrypsin. J Cell Biol. 2008;180(4): 705-712.
- Chen Y, Hojo S, Matsumoto N, Yamamoto K. Regulation of Mac-2BP secretion is mediated by its N-glycan binding to ERGIC-53. *Glycobiology*. 2013; 23(7):904-916.
- Vollenweider F, Kappeler F, Itin C, Hauri HP. Mistargeting of the lectin ERGIC-53 to the endoplasmic reticulum of HeLa cells impairs the secretion of a lysosomal enzyme. J Cell Biol. 1998;142(2):377-389.
- 17. Appenzeller C, Andersson H, Kappeler F, Hauri HP. The lectin ERGIC-53 is a cargo transport receptor for glycoproteins. *Nat Cell Biol.* 1999;1(6): 330-334.
- 18. Fu YL, Zhang B, Mu TW. LMAN1 (ERGIC-53) promotes trafficking of neuroreceptors. Biochem Biophys Res Commun. 2019;511(2):356-362.
- 19. Duellman T, Burnett J, Shin A, Yang J. LMAN1 (ERGIC-53) is a potential carrier protein for matrix metalloproteinase-9 glycoprotein secretion. *Biochem Biophys Res Commun.* 2015;464(3):685-691.
- 20. Zheng C, Liu HH, Yuan S, Zhou J, Zhang B. Molecular basis of LMAN1 in coordinating LMAN1-MCFD2 cargo receptor formation and ER-to-Golgi transport of FV/FVIII. *Blood.* 2010;116(25):5698-5706.

- 21. Zheng C, Liu HH, Zhou J, Zhang B. EF hand domains of MCFD2 mediate interactions with both LMAN1 and coagulation factor V or VIII. *Blood.* 2010; 115(5):1081-1087.
- 22. Zhang Y, Zhu M, Zheng C, Wei W, Emmer BT, Zhang B. LMAN1-MCFD2 complex is a cargo receptor for the ER-Golgi transport of alpha1-antitrypsin. *Biochem J.* 2022;479(7):839-855.
- 23. Pittman DD, Millenson M, Marquette K, Bauer K, Kaufman RJ. A2 domain of human recombinant-derived factor VIII is required for procoagulant activity but not for thrombin cleavage. *Blood.* 1992;79(2):389-397.
- 24. Roeckel N, Woerner SM, Kloor M, et al. High frequency of LMAN1 abnormalities in colorectal tumors with microsatellite instability. *Cancer Res.* 2009; 69(1):292-299.
- 25. Yagi H, Yagi-Utsumi M, Honda R, et al. Improved secretion of glycoproteins using an N-glycan-restricted passport sequence tag recognized by cargo receptor. *Nat Commun.* 2020;11(1):1368.
- Shao Y, Wu W, Xu G, Wang X, Ding Q. Low factor V level ameliorates bleeding diathesis in patients with combined deficiency of factor V and factor VIII. Blood. 2019;134(20):1745-1754.
- 27. Zheng C, Page RC, Das V, et al. Structural characterization of carbohydrate binding by LMAN1 protein provides new insight into the endoplasmic reticulum export of factors V (FV) and VIII (FVIII). J Biol Chem. 2013;288(28):20499-20509.
- Abdallah HE, Gouider E, Amor MB, Jlizi A, Meddeb B, Elgaaied A. Molecular analysis in two Tunisian families with combined factor V and factor VIII deficiency. *Haemophilia*. 2010;16(5):801-804.
- 29. Fahs SA, Hille MT, Shi Q, Weiler H, Montgomery RR. A conditional knockout mouse model reveals endothelial cells as the principal and possibly exclusive source of plasma factor VIII. *Blood.* 2014;123(24):3706-3713.
- 30. Everett LA, Cleuren AC, Khoriaty RN, Ginsburg D. Murine coagulation factor VIII is synthesized in endothelial cells. Blood. 2014;123(24):3697-3705.
- Gould WR, Simioni P, Silveira JR, Tormene D, Kalafatis M, Tracy PB. Megakaryocytes endocytose and subsequently modify human factor V in vivo to form the entire pool of a unique platelet-derived cofactor. J Thromb Haemost. 2005;3(3):450-456.
- 32. Pittman DD, Marquette KA, Kaufman RJ. Role of the B domain for factor VIII and factor V expression and function. Blood. 1994;84(12):4214-4225.
- Poothong J, Pottekat A, Siirin M, et al. Factor VIII exhibits chaperone-dependent and glucose-regulated reversible amyloid formation in the endoplasmic reticulum. *Blood*. 2020;135(21):1899-1911.
- Pipe SW, Morris JA, Shah J, Kaufman RJ. Differential interaction of coagulation factor VIII and factor V with protein chaperones calnexin and calreticulin. J Biol Chem. 1998;273(14):8537-8544.
- Pittman DD, Tomkinson KN, Kaufman RJ. Post-translational requirements for functional factor V and factor VIII secretion in mammalian cells. J Biol Chem. 1994;269(25):17329-17337.
- Everett LA, Khoriaty RN, Zhang B, Ginsburg D. Altered phenotype in LMAN1-deficient mice with low levels of residual LMAN1 expression. Blood Adv. 2020;4(22):5635-5643.
- Yamada T, Fujimori Y, Suzuki A, et al. A novel missense mutation causing abnormal LMAN1 in a Japanese patient with combined deficiency of factor V and factor VIII. Am J Hematol. 2009;84(11):738-742.
- Appenzeller-Herzog C, Nyfeler B, Burkhard P, Santamaria I, Lopez-Otin C, Hauri HP. Carbohydrate- and conformation-dependent cargo capture for ERexit. Mol Biol Cell. 2005;16(3):1258-1267.
- 39. Velloso LM, Svensson K, Schneider G, Pettersson RF, Lindqvist Y. Crystal structure of the carbohydrate recognition domain of p58/ERGIC-53, a protein involved in glycoprotein export from the endoplasmic reticulum. *J Biol Chem.* 2002;277(18):15979-15984.
- 40. Satoh T, Nishio M, Suzuki K, et al. Crystallographic snapshots of the EF-hand protein MCFD2 complexed with the intracellular lectin ERGIC-53 involved in glycoprotein transport. Acta Crystallogr F Struct Biol Commun. 2020;76(Pt 5):216-221.
- Elmahmoudi H, Wigren E, Laatiri A, et al. Analysis of newly detected mutations in the MCFD2 gene giving rise to combined deficiency of coagulation factors V and VIII. *Haemophilia*. 2011;17(5):e923-e927.
- Abdallah HE, Gouider E, Stambouli N, et al. Structural analysis of two novel mutations in MCFD2 gene causing combined coagulation factors V and VIII deficiency. Blood Cells Mol Dis. 2010;44(2):120-123.
- 43. Hamza A, Wei NN, Johnson-Scalise T, Naftolin F, Cho H, Zhan CG. Unveiling the unfolding pathway of F5F8D disorder-associated D81H/V100D mutant of MCFD2 via multiple molecular dynamics simulations. J Biomol Struct Dyn. 2012;29(4):699-714.