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Blood coagulation factor VIII D1241E polymorphism leads to a weak malectin interaction and reduction of factor VIII posttranslational modification and secretion

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ABSTRACT

Blood coagulation factor VIII (FVIII) is a key cofactor in regulation of blood coagulation. This study investigated the mechanism by which FVIII is translated and transported into the endoplasmic reticulum (ER) and processed in the Golgi apparatus before secretion using an *in vitro* cell model. HEK-293T cells were transfected with vectors carrying wild-type (WT) FVIII or polymorphic FVIII D1241E for coexpression with ER lectins and treatment with tunicamycin (an N-linked glycosylation inhibitor), 1-deoxynojirimycin (an alpha-glucosidase inhibitor), endoglycosidase H, or MG132 (Cbz-Leu-Leu-leucinal; a proteasome inhibitor). The data showed that the minor allele of FVIII D1241E was able to reduce FVIII secretion into the conditioned medium but maintain a normal level of procoagulation ability, although both FVIII WT and the minor allele of FVIII D1241E showed similar levels of transcription and translation capacities. Functionally, the D1241E polymorphism led to a reduced level of FVIII in the Golgi apparatus because of its reduced association with malectin, which interacts with newly synthesized glycoproteins in the ER for FVIII folding and trafficking, leading to degradation of the minor allele of FVIII D1241E in the cytosol. This study demonstrated that malectin is important for regulation of the FVIII posttranslational process and that the minor allele of FVIII D1241E had a reduced association with malectin but an increased capacity for proteasomal FVIII degradation. These data imply the role of the ER quality control in future recombinant FVIII development.

1. Introduction

Blood coagulation factor VIII (FVIII) is an essential blood-coagulation glycoprotein [1], and a defect in the gene and protein expression of FVIII causes hemophilia A, an inherited disorder resulting in an increase in bleeding [2]. The secreted FVIII, mainly from the liver, will circulate in the bloodstream as a complex with the von Willebrand factor to maintain blood homeostasis in the human body. During the blood coagulation cascade, FVIII is activated by factor IIa and then dissociates from the von Willebrand factor to subsequently activate factor IX; and the latter interacts with factor Va to activate more factor II, which will cleave fibrinogen to fibrin, leading fibrin to polymerize and crosslink by factor XIII into thrombosis [3]. The *FVIII* gene is localized at chromosome Xq28 and covers a 186-kb region with 26 exons to encode the FVIII protein with 2351 amino acids [1]. FVIII mRNA is translated into a single-chain precursor of approximately 280 kD with the domain structure of A1-a1-A2-a2-B-a3-A3-C1-C2 [4] (Fig. 1A). Except for the B domain, each FVIII domain plays a role in normal procoagulant function, while the B domain is cleaved by thrombin (FIIa) upon activation [1] (Fig. 1C). Moreover, the B domain of FVIII contains 19 out of a total of 25 asparagine (N)-linked glycosylation sites in FVIII (Fig. 1A) [5]. N-linked glycosylation plays a critical role in the regulation of glycoprotein folding and posttranslational modifications; thus, it is believed that the B domain plays an important role in FVIII intracellular processing and trafficking [6]. Before it is secreted into the blood, FVIII undergoes cleavage at the B-a3 junction and additional cleavages within the B domain in the Golgi apparatus. These posttranslational modifications generate the heavy chains (A1-a1-A2-a2-B) of FVIII with different molecular weights and an 80-kD light chain (a3-A3-C1-C2) (Fig. 1B) [7].

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Genetically as well as clinically, there is still a debate regarding whether there are quantitative trait loci in the FVIII gene that control FVIII expression and secretion. In 2007, Viel et al. performed a measured-genotype association analysis and demonstrated that the level of FVIII activity (FVIII:C) was significantly associated with the minor allele of FVIII D1241E and that this polymorphism reduced the FVIII:C level by 14.3 IU dL^{-1} [8]. FVIII D1241E is also named as FVIII D1260E according to the Human Genome Variation Society (HGVS) because the 19-amino-acid signal peptides were counted. It is a single-nucleotide substitution polymorphism with the base cytosine at the position of 3951 converting into guanine (Fig. 1A) [9,10]. Scanavini et al. confirmed that this FVIII polymorphism was associated with an 11% reduction of the mean FVIII:C [11], while Nossent et al. showed that the HT1 haplotype of D1241E was associated with a 6% reduction of FVIII:C and reduced the risk of developing venous thrombosis in male individuals [12]. Another *in vitro* study revealed a relative decrease of FVIII:C and FVIII:Ag by up to 22% in FVIII D1241E [13]. However, the precise molecular basis for the effect of the minor allele of D1241E on the FVIII level remains to be defined. Thus, further study of the underlying mechanism by which the minor allele of D1241E regulates FVIII expression and secretion will lead to a better understanding and the future control of FVIII in the clinic.

In this study, we investigated the interaction of wild-type FVIII (FVIII WT) and the minor allele of FVIII D1241E with malectin and ERGIC-53 as a mechanism by which FVIII is trafficked into the endoplasmic reticulum (ER) after translation, transported into the Golgi apparatus, and secreted into the conditional medium using an *in vitro* model of HEK-293T cells. The results of this study will provide useful mechanistic information regarding the role of malectin in FVIII modification and regulation.

2. Materials and methods

2.1. Cell line and culture

Human embryonic kidney-293T (HEK-293T) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH7.4) at 37 °C in a humidified incubator with 5% CO_2.

2.2. Plasmids and transfection

All vectors were purchased from Genechem (Shanghai, China), i.e., the pGV362 vector carrying the full-length FVIII cDNA has the backbone of CMV-MCS-3FLAG-IRES-EGFP-SV40-neomycin so that the user can detect the transfection efficiency by measuring the enhanced green fluorescent protein (EGFP) level. The full length of the FVIII cDNA vector is referred as FVIII WT. The plasmid carrying the minor allele of FVIII D1241E was generated through site-directed mutagenesis using the FVIII WT vector. The pGV366 vector harboring malectin cDNA has the backbone of CMV-MCS-HA-SV40-Neomycin. The pGV168 vector carrying ERGIC-53 cDNA has the backbone of CMV-MYC-MCS-SV40-Ampicillin. The vector-3FLAG and vector-HA were only used as negative controls. Before these vectors were available for purchase, they were amplified *in vitro* and DNA-sequenced for verification.

For transfection, HEK-293T cells in the exponential growth phase were detached from tissue culture dishes using 0.025% trypsin-EDTA and then inoculated into a 25-cm^2 flask with 5 mL of DMEM supplemented with 10% FBS and grown overnight to reach 60–80% confluency. Thereafter, cells were transfected for 48 h with 6 μg of plasmid

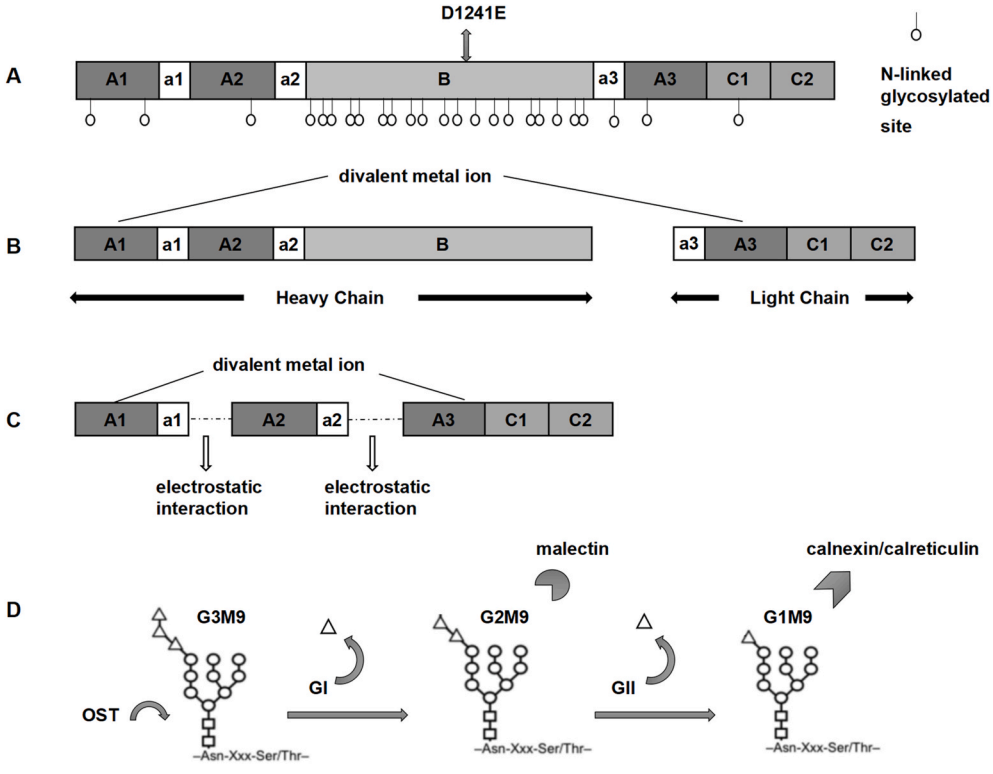

Fig. 1. FVIII structure and oligosaccharide trimming. (A) FVIII gene structure and protein size. The primary FVIII translation product is a multidomain protein of 280 kD, containing a total of 25 asparagine (N)-linked glycosylation sites, 19 of which reside in the B domain. The D1241E polymorphism is localized in the B domain. (B) FVIII processing. FVIII undergoes cleavage at the B-a3 junction and additional cleavages within the B domain in the Golgi apparatus to form heavy chains (HCh) of A1-a1-A2-a2-B and a light chain of (LCh) a3-A3-C1-C2. The A1 and A3 subunits are noncovalently linked via a metal ion-mediated interaction. (C) FVIII activation. Upon activation, the B and A3 domains are trimmed to form FVIIIa, in which the A1 and A3 domains retain the metal ion-mediated interaction, while the A1/A3-C1-C2 dimer is weakly associated with the A2 subunit through electrostatic interactions. (D) N-linked glycosylation. Oligosaccharyltransferase (OST) transfers the G3M9 oligosaccharide to the asparagine residue within the amino acid sequences of Asn-Xxx-Ser/Thr of FVIII. G3M9 consists of two N-acetylglucosamine (squares), nine mannose (circles), and three glucose (triangles) residues, while glucosidase I (GI) can remove the outermost glucose in G3M9 to form G2M9, which has a high affinity to malectin. Glucosidase II (GII) can remove the second glucose from G2M9 to form G1M9, which can interact with calnexin and calreticulin for the initial stages of correct FVIII folding.

DNA per 1×10^6 cells in 5 mL of culture medium using Lipofectamine 2000 (Invitrogen) with a DNA:transfection reagent ratio of 1:2.5. The conditioned cell culture media and transfected cells were harvested at various time points for the experiments. The cotransfection of FVIII-3FLAG and malectin-HA or ERGIC-53-Myc was set for the two plasmids at a ratio of 1:1. Furthermore, to assess the levels of the primary translation products, the FVIII WT-3FLAG or D1241E-3FLAG plasmid was transfected into HEK-293T cells for 24 h and then treated with tunicamycin (5 μg/mL) for 24 h to inhibit N-glycosylation, resulting in the accumulation of the primary translation products in the cells.

2.3. Antibodies and other reagents

The polyclonal anti-FLAG (Cat. #ab1162) antibody was purchased from Abcam (Cambridge, UK); while monoclonal anti-HA (#66006-2-Ig), polyclonal anti-malectin (#26655-1-AP), monoclonal anti-Myc (#60003-2-Ig), polyclonal anti-ERGIC-53 (#13364-1-AP), and polyclonal anti-β-tubulin (#10094-1-AP) antibodies were obtained from Proteintech (Wuhan, China). The monoclonal anti-ubiquitin (P4D1; #sc-8017) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-FVIII (#MA1-10589) antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Tunicamycin (an N-linked glycosylation inhibitor) and 1-deoxynojirimycin (an alpha-glucosidase inhibitor) were purchased from Med Chem Express (Shanghai, China), and endoglycosidase-H (EndoH) was from Promega (Madison, WI, USA). MG132 (Cbz-Leu-Leu-leucinal; a potent, reversible, and cell-permeable proteasome inhibitor) was from Beyotime (Shanghai, China), and the protein A/G Plus-Agarose was purchased from Santa Cruz Biotechnology.

2.4. Enzyme-linked immunosorbent assay (ELISA) and chromogenic assay of FVIII

To detect the secreted FVIII level *in vitro*, the conditioned cell culture medium was collected 72 h after transfection, i.e., the conditioned medium was collected from 5×10^5 HEK-293T cells per well from a 12-well plate and centrifuged at 16,000 *g* and 4 °C for 15 min to obtain the supernatant. The ELISA kit contained 96-well plates previously coated with various anti-FVIII light chain antibodies for the sandwich ELISA reaction from CUSABIO (Wuhan, China). Actually, this ELISA kit utilizes various anti-light chain antibodies to recognize the C1 and C2 domains of FVIII. The minor allele of FVIII D1241E is localized in the heavy chain, some of which might even be removed during B domain processing before secretion. So, the antibodies should recognize the two FVIII variants with similar affinity. The conditioned medium samples were subjected to the ELISA to measure the FVIII antigen level (FVIII:Ag), according to the manufacturer's instructions.

Furthermore, the FVIII:C in the conditioned media was measured by using a chromogenic assay kit (Biophen, France), which utilizes the acetic acid-stopped endpoint method, according to the manufacturer's instructions. After that, the FVIII:Ag and FVIII:C values (as a ratio) in the FVIII D1241E-transfected cells were assessed against those in the FVIII WT-transfected cells.

2.5. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

HEK-293T cells at 48 h after transcription were harvested and isolated for cellular RNA collection using RNAiso Plus reagent (Takara Bio, Dalian, China) and reversely transcribed into cDNA using a PrimerScript RT reagent kit (Takara Bio), according to the manufacturer's instructions. The FVIII mRNA was amplified in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using TB Green Premix Ex *Taq*II (Takara Bio) and qPCR primers (Takara Bio). The primer sequences were as follows: FVIII, 5′-GCATTCGCAGCACTCTTCG-3′ and 5′-GAGGTGAAGTCGAGCTTTTGAA-3′; GAPDH, 5′-TGTGGGCATCAATGGATTTGG-3′ and 5′-ACACCATGTATTCCGGGTCAAT-3′. The qPCR conditions were set as 95 °C for 5 min and then 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The ΔΔCq method was used to quantify the FVIII mRNA level against the GAPDH mRNA level.

2.6. Western blot

The total cellular protein was extracted from cells after transfection using radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL aprotinin, 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10 mM sodium fluoride, and 1 mM sodium orthovanadate on ice. The lysates were then centrifuged at 16,000 *g* and 4 °C for 15 min, and the total protein concentration in the supernatants was quantified using a bicinchoninic acid protein assay kit (CWBIO, Beijing, China). The proteins were denatured in denaturing buffer (1% SDS, 5% glycerol, 0.062 M Tris-HCl, pH 6.8) at 100 °C for 5 min. For western blotting, 20 μg of each protein sample was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) in 6–10% gels at a constant voltage (120 V) for 100 min using a Bio-Rad apparatus (Hercules, CA, USA). The proteins were then transferred onto a 0.45-μm polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) using a Bio-Rad apparatus at a constant current (250 mA) for 60–240 min in transfer buffer containing 10–20% (v/v) methanol. After that, the membranes were blocked in 5% nonfat milk in Tris-based saline-Tween 20 (TBST) buffer at room temperature for 2 h and then incubated with the appropriate primary antibody at a dilution recommended by the manufacturer at 4 °C overnight. On the next day, the membranes were washed with TBST three times, incubated with a horseradish peroxidase-conjugated secondary antibody (Proteintech, Wuhan, China) at room temperature for 1 h, then detected for positive protein bands using the enhanced chemiluminescence system (Millipore), and exposed to x-ray films. The anti-β-tubulin rabbit antibody was used as a loading control. The membranes were quantified by using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.7. Coimmunoprecipitation

HEK-293T cells were grown and transfected with FVIII WT-3FLAG or D1241E-3FLAG together with malectin-HA for 48 h. To assess the interaction between FVIII and ERGIC-53-Myc, we grew HEK-293T cells and transfected them with FVIII WT-3FLAG or D1241E-3FLAG together with ERGIC-53-Myc for 48 h. After that, 1×10^7 cells of each group were lysed in buffer containing 1% Triton X-10, TBS pH7.4, 0.5 mM PMSF, 10 mM iodoacetamide, and Roche complete protease inhibitor for 30 min on ice. The cells were then scraped into Eppendorf tubes and centrifuged at 16,000 *g* and 4 °C for 15 min. Next, an aliquot of the supernatant was used for western blotting, while the remaining sample was precleaned with 30 μL of Protein A/G Plus-agarose (50%) with a gentle rotation at 4 °C for 1 h, followed by centrifugation at 16,000 *g* and 4 °C for 15 min to collect the supernatant. The supernatant was then incubated with 2 μg of the appropriate antibody with a gentle rotation at 4 °C for 4 h, then 40 μL of Protein A/G Plus-agarose was added to the lysate-antibody

mixture, and the mixture was further incubated with a gentle rotation at 4 °C for 1.5 h. After that, the mixture was washed five times with washing buffer containing Tris-HCl, pH 7.5, 0.1% (vol/vol) Triton X-100, 150 mM NaCl, 1 mM PMSF, and 1 μg/mL leupeptin. The immunoprecipitate was eluted by boiling in elution buffer containing 100 mM Tris-HCl, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, and 0.004% (wt/vol) bromophenol blue; the proteins were separated by SDS-PAGE gels and immunoblotted.

To assay the FVIII WT and D1241E ubiquitination in cells, HEK-293T cells were grown and transfected with plasmids carrying FVIII WT-3FLAG or D1241E-3FLAG for 48 h. After that, the cells were treated with 50 μM MG132 (Beyotime) for 2 h, and approximately 1×10^7 cells of each group were lysed using lysis buffer containing 1% Triton X-100, 0.5 mM PMSF, 10 mM iodoacetamide, and Roche complete protease inhibitor in TBS (pH 7.4). The cell lysates were then subjected to immunoprecipitation and Western blot analysis.

2.8. Small interfering RNA (siRNA) and transfection

To knock down malectin expression, we utilized the siRNA technique. The predesigned siRNA products targeting malectin were synthesized by and obtained from Genepharma (China). The specific target sequences of malectin were as follows: 5′-GGAUGAUGUACCAAAGCUUTT-3′ and 5′-AAGCUUUGGUACAUCAUCCTT-3′. The negative control siRNA sequences were 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′. For cell transfection, 70 pmol of siRNA oligonucleotides and 1.5 μL of Lipofectamine 2000 transfection agent (Invitrogen) were added to 5×10^5 HEK-293T cells, and then the cells were incubated for 24 h before being transfected with FVIII WT-3FLAG or D1241E-3FLAG for another 48 h. After that, cells were harvested for Western blot analysis.

2.9. EndoH sensitivity assay

To detect the level of deglycosylated FVIII in FVIII WT and D1241E, we performed the EndoH sensitivity assay using an EndoH kit (Cat. #V4850; Promega). In brief, HEK-293T cells were grown and transfected with plasmids carrying FVIII WT-3FLAG or D1241E-3FLAG for 48 h. Next, approximately 1×10^7 cells of each group were lysed using lysis buffer containing 1% Triton X-100, 0.5 mM PMSF, 10 mM iodoacetamide, and Roche complete protease inhibitor in TBS (pH 7.4), and the cell lysates were then subjected to immunoprecipitation using an anti-FLAG antibody. After that, 1 μL of 10 × denaturing buffer was mixed with 9 μL of immunoprecipitate, and the mixture was incubated at 95 °C for 5 min and allowed to cool down to room temperature for 5 min. The samples were mixed with 2 μL of 10 × EndoH Reaction Buffer and 5 μL of EndoH, the mixture was incubated at 37 °C for 5 h, the reaction was stopped by the addition of SDS-sample buffer, and then the sample was subjected to Western blot analysis.

2.10. Statistical analysis

The proteins on the Western blot membranes were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA). The data were expressed as the mean ± standard deviation of at least duplicated or triplicated experiments. The difference between the two groups was analyzed using the Student's *t*-test with SPSS software, version 18.0 (SPSS, Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. The minor allele of FVIII D1241E downregulated FVIII secretion but maintained normal procoagulation activity

In this study, we first transfected plasmids carrying FVIII WT-3FLAG and D1241E-3FLAG cDNA into HEK-293T cells for 72 h, while the vector-3FLAG only was used as a negative control. We then collected the conditioned cell culture media and determined the FVIII levels (FVIII:Ag) using an ELISA. We found that there was an approximately 25% reduction of the FVIII:Ag in the D1241E-transfected cells compared to that of the FVIII WT-transfected cells (Fig. 2A). Next, we analyzed the FVIII:C using a chromogenic assay and found a 28% reduction of the FVIII:C in FVIII D1241E compared to that of FVIII WT (Fig. 2B). These data suggest that the D1241E polymorphism significantly reduced FVIII secretion into the conditioned medium, but it did not cause a change in the procoagulation activity in terms of the FVIII:C level.

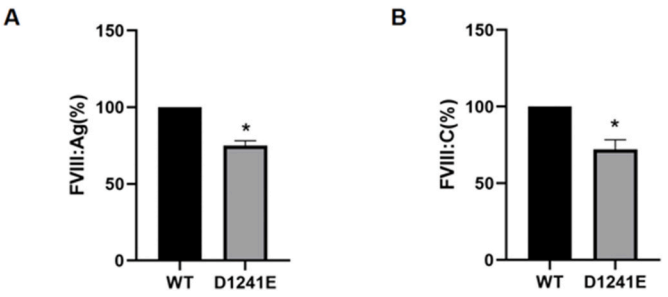

Fig. 2. Effect of FVIII D1241E on the regulation of FVIII secretion and procoagulation activity. Plasmids carrying FVIII WT-3FLAG or D1241E-3FLAG were transfected into HEK-293T cells for 72 h, and the conditioned cell culture media were collected for analysis. (A) ELISA. The conditioned media were subjected to ELISA analysis of the FVIII antigen level (FVIII:Ag). (B) Chromogenic assay. The conditioned media were subjected to the chromogenic assay for FVIII coagulation activity (FVIII:C). The antigen level and activity of FVIII WT was set to 100%. The data represent the mean ± standard deviation of three independent experiments , and the error bars represent the standard deviation. *$p < 0.05$ using the Student's *t*-test.

3.2. Levels of FVIII WT and D1241E transcription and translation were identical in cells

Next, we assessed the level of FVIII mRNA in FVIII WT-FLAG- and D1241E-FLAG-transfected cells using qRT-PCR and found that after 48 h transfection, there was no significant difference in the mRNA level between these two types of cells (Fig. 3A).

It is known that FVIII is translated into a 280-kD protein in cells, then is transported into the ER for N-linked glycosylation, and interacts with lectins and chaperones for folding to the native form [14]. In this study, we determined whether there was any discrepancy in the translation of functional protein between FVIII WT and D1241E. After their 24-h transfection, we treated cells with 5 μg/mL tunicamycin (an N-linked glycosylation inhibitor that causes the accumulation of nonglycosylated protein in the ER lumen [14]) for 24 h. The cell lysates were then subjected to immunoblotting with an anti-FVIII antibody. The data showed that both FVIII WT and D1241E had similar levels of a 280-kD primary translation protein (Fig. 3B). Thus, this finding confirmed that there was no difference in the mRNA transcription and polypeptide translation between FVIII WT and D1241E.

3.3. Level of FVIII D1241E was reduced in the golgi apparatus

As is well known, FVIII protein is processed in the Golgi apparatus before secretion; specifically, FVIII protein undergoes cleavage at the B-a3 junction and additional cleavages within the B domain to generate heavy chains (A1-a1-A2-a2-B domain) and a light chain (a3-A3-C1-C2 domain) [2]. The 80-kD FVIII light chain indicates a successful Golgi apparatus process. Thus, we transfected cells with FVIII WT-3FLAG and D1241E-3FLAG for 48 h and then analyzed the level of the 80-kD FVIII light chain using Western blot. Our data showed that FVIII WT-3FLAG transfection exhibited a higher level of the 80-kD FVIII light chain

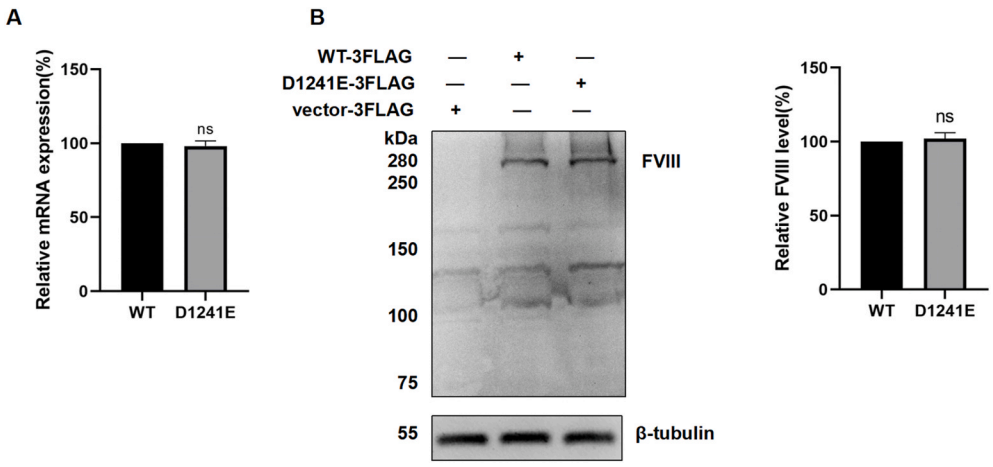

Fig. 3. Effect of FVIII D1241E on the regulation of FVIII mRNA transcription and polypeptide translation. (A) qRT-PCR. Plasmids carrying FVIII WT-3FLAG or D1241E-3FLAG were transfected into HEK-293T cells for 48 h and then subjected to qRT-PCR. (B) Western blot. Plasmids carrying FVIII WT-3FLAG or D1241E-3FLAG were transfected into HEK-293T cells for 24 h and treated with tunicamycin (5 μg/mL) for 24 h and then subjected to protein extraction and Western blot using an anti-FVIII antibody. The data represent the mean ± standard deviation of three independent experiments, and the error bars represent the standard deviation. ns, not significant after the Student's *t*-test.

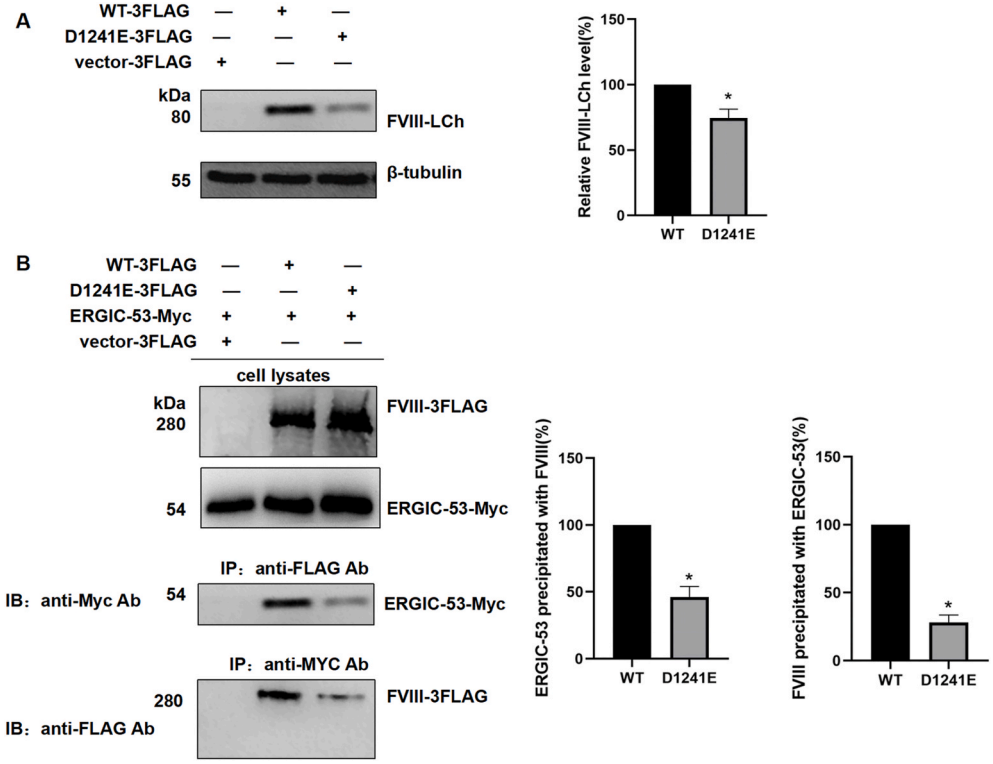

Fig. 4. Effect of FVIII D1241E on the regulation of FVIII polypeptide transport into the Golgi apparatus. (A) Western blot. Plasmids carrying FVIII WT-3FLAG or D1241E-3FLAG were transfected into HEK-293T cells for up to 48 h and subjected to Western blot using an anti-FLAG antibody to detect the 80 kD FVIII light chain (FVIII-LCh). (B) Coimmunoprecipitation–western blot. HEK-293T cells were grown and transfected with FVIII WT-3FLAG plus ERGIC-53-Myc or D1241E-3FLAG plus ERGIC-53-Myc for 48 h and then subjected to coimmunoprecipitation. Cell lysates were immunoprecipitated using an anti-FLAG antibody, followed by Western blot using an anti-Myc antibody; the reciprocal coimmunoprecipitation was conducted using an anti-Myc antibody, followed by Western blot using an anti-FLAG antibody. The data represent the mean ± standard deviation of the three independent experiments, and the error bars represent the standard deviation. *p < 0.05 using Student's *t*-test.

compared to that of FVIII D1241E-3FLAG (Fig. 4A), suggesting that more FVIII WT protein was processed in the Golgi apparatus and a normal level of FVIII WT would be secreted.

Furthermore, FVIII protein is transported from the ER to the Golgi apparatus by the cargo receptor ERGIC-53 [15]. Thus, we cotransfected FVIII WT-3FLAG and ERGIC-53-Myc, or FVIII D1241E-3FLAG and ERGIC-53-Myc into HEK-293T cells for 48 h and then performed coimmunoprecipitation analysis using the anti-FLAG and anti-Myc antibodies (Fig. 4B). We found that when the cell lysates were precipitated with an anti-FLAG antibody, there was a significant proportion of ERGIC-53-Myc that was coprecipitated with FVIII WT-3FLAG (Fig. 4B). However, there was a weak signal corresponding to ERGIC-53-Myc with cells coexpressing FVIII D1241E-3FLAG (Fig. 4B). Similarly, when the cell lysates were precipitated with the anti-Myc antibody, there was more FVIII WT-3FLAG that was coprecipitated with ERGIC-53-Myc compared to that of FVIII D1241E-3FLAG (Fig. 4B). These data demonstrated that there was less FVIII D1241E in the complex and transportation by ERGIC-53, resulting in a reduced level of FVIII light chain formation in the Golgi apparatus.

3.4. FVIII D1241E reduces association with malectin

Before transport into the Golgi apparatus, FVIII interacts with a sequence of lectins and chaperones in the ER to acquire its native form [14]. Indeed, malectin is one of the initial lectins that interacts with newly synthesized glycoproteins in the ER; however, the precise interaction between FVIII and malectin has not been reported. We speculated that malectin could selectively interact with FVIII WT and D1241E to regulate their folding process accordingly. To confirm this hypothesis, we coexpressed FVIII WT-3FLAG plus malectin-HA and FVIII D1241E-3FLAG plus malectin-HA in HEK-293T cells. Next, we treated these cells with 1 mM 1-deoxynojirimycin, an alpha-glucosidase

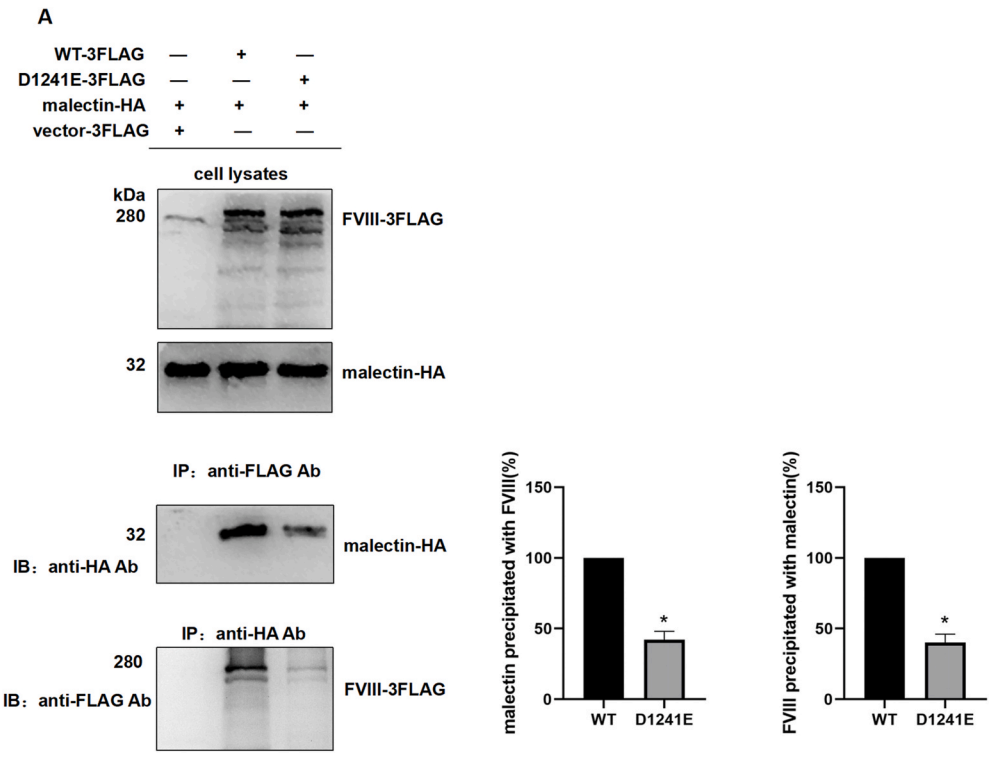

Fig. 5. Association of FVIII D1241E with decreased malectin. Plasmids carrying FVIII WT-3FLAG plus malectin-HA or D1241E-3FLAG plus malectin-HA were transfected into HEK-293T cells for 48 h and treated with 1-deoxynojirimycin (1 mM) for 3 h and then subjected to Western blot using the appropriate antibodies. The remaining cell lysates were subjected to coimmunoprecipitation, immunoprecipitated with an anti-FLAG antibody, followed by Western blot using an anti-HA antibody. The reciprocal coimmunoprecipitation with an equal cell number was performed with an anti-HA antibody and immunoblotted by an anti-FLAG antibody. The data represent the mean ± standard deviation of the three independent experiments, and the error bars represent the standard deviation. *$p < 0.05$ using the Student's *t*-test.

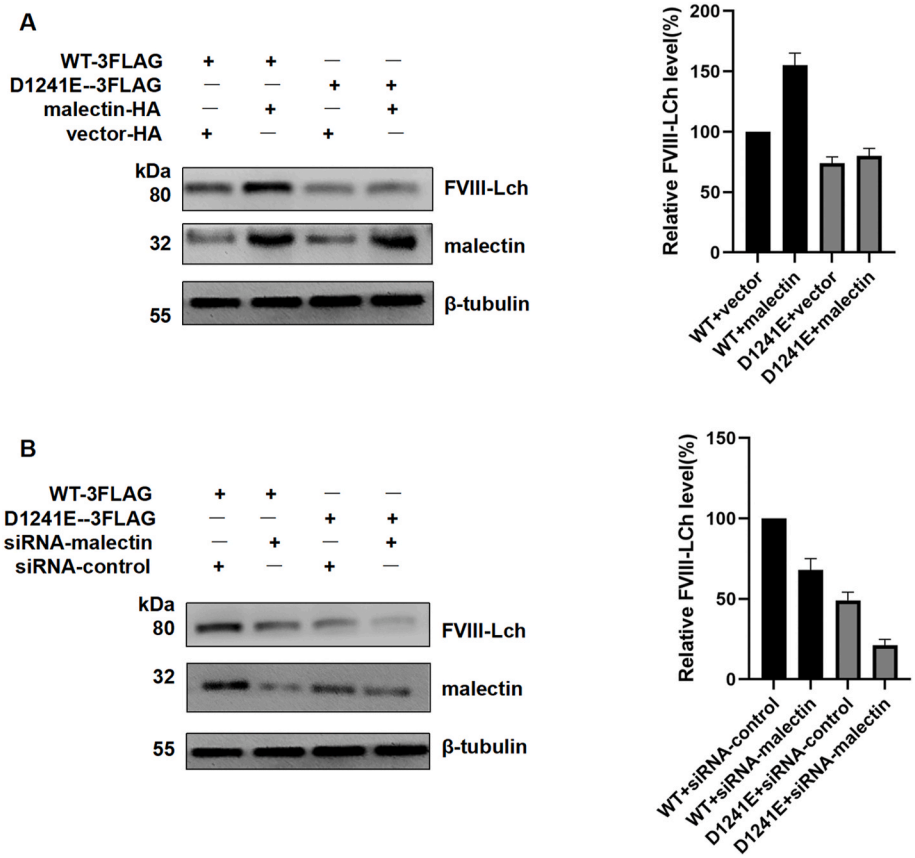

Fig. 6. Malectin regulation of the FVIII trafficking. (A) Western blot. HEK-293T cells were grown and transfected with plasmids carrying malectin-HA or the vector-HA only with FVIII WT-3FLAG or D1241E-3FLAG for 48 h and then subjected to Western blot using an anti-FLAG antibody to detect FVIII light chain. (B) Western blot. HEK-293T cells were grown and transfected with malectin siRNA or control siRNA for 24 h and then with plasmid carrying FVIII WT-3FLAG or D1241E-3FLAG for 48 h and then subjected to Western blot using an anti-FLAG antibody to detect FVIII light chain. The data represent the mean ± standard deviation of the two independent experiments, and the error bars represent the standard deviation.

inhibitor, for 3 h to induce the accumulation of G2M9 glycans before harvesting the cells that had been transfected for 48 h. We then analyzed the expression levels of FVIII WT-3FLAG, D1241E-3FLAG, and malectin-HA using Western blot (Fig. 5), while the remaining cell lysate was immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-HA antibody. As shown in Fig. 5, both FVIII WT-3FLAG and D1241E-3FLAG could precipitate malectin, while more malectin was in the complex with FVIII WT-3FLAG. The reciprocal coimmunoprecipitation with an equal cell number was conducted using an anti-HA antibody and then immunoblotted using an anti-FLAG antibody; the data showed that a higher level of FVIII WT-3FLAG was precipitated with malectin compared to that of FVIII D1241E-3FLAG (Fig. 5). Taken together, these data revealed that malectin was able to interact with FVIII, although FVIII WT and D1241E had different binding capacities with malectin. FVIII D1241E had a reduced association with malectin, resulting in a decrease in FVIII polypeptides undergoing the subsequent protein folding process in the Golgi apparatus as well as secretion.

3.5. Malectin regulates FVIII trafficking

To further explore the role of malectin in FVIII regulation, we coexpressed FVIII WT-3FLAG plus malectin-HA or FVIII D1241E-3FLAG plus malectin-HA in HEK-293T cells for 48 h, while FVIII WT-3FLAG plus vector-HA onlyor FVIII D1241E-3FLAG plus vector-HA only were used as negative controls. The cell lysates were then subjected to Western blot analysis using an anti-FLAG antibody. We found that compared to that of the negative controls, the expression of the 80-kD FVIII light chain was increased in the malectin plus FVIII WT-cotransfected cells (Fig. 6A). This observation suggested that malectin was able to facilitate FVIII transport from the ER to the Golgi apparatus for the protein folding and modification processes.

Next, we knocked down malectin expression using specific siRNA. After that, 24-h malectin siRNA-transfected HEK-293T cells were further transfected with FVIII WT-3FLAG or FVIII D1241E-3FLAG for 48 h. Our Western blot data showed that the level of the 80-kD FVIII light chain was reduced in both the FVIII WT and D1241E groups, compared to that of the siRNA controls (Fig. 6B), suggesting that malectin was essential for FVIII folding and modification in the ER and the Golgi apparatus.

3.6. FVIII D1241E was degraded in the cytosol

It is well known that any polypeptides that fail to undergo the folding and modification processes will enter into the ER-associated protein degradation (ERAD) process, which causes the polypeptides to translocate from the ER into the cytosol; any glycans will be detached, thus reducing their molecular weight, and the polypeptides will be degraded. Therefore, we analyzed the levels of deglycosylated FVIII WT and D1241E using an EndoH sensitivity assay. The transfected cells were immunoprecipitated with an anti-FLAG antibody and treated with the glycosidase EndoH to cleave the high-mannose oligosaccharides; the samples were then subjected to Western blot analysis using an anti-FVIII antibody (Fig. 7A). The Western blot data showed two protein bands, i.e., the slow-migrating band represented the EndoH-resistant portion (EndoH-R), which was still localized in the ER, while the quick-migrating band refers to the EndoH-sensitive portion of FVIII protein, which had been translocated into the cytosol (Fig. 7A). The EndoH sensitivity assay showed that the minor allele of FVIII D1241E led to a higher portion of EndoH-sensitive FVIII compared to that of FVIII WT, indicating that FVIII D1241E transfection resulted in a higher level of nonprocessed FVIII, which was translocated into the cytosol for degradation.

Furthermore, in the protein degradation process, ubiquitin, a small regulatory protein of 8.6 kD, is added to deglycosylated polypeptides for proteasome recognition and degradation. Therefore, we analyzed the ubiquitination level of FVIII WT vs. D1241E in transfected cells after

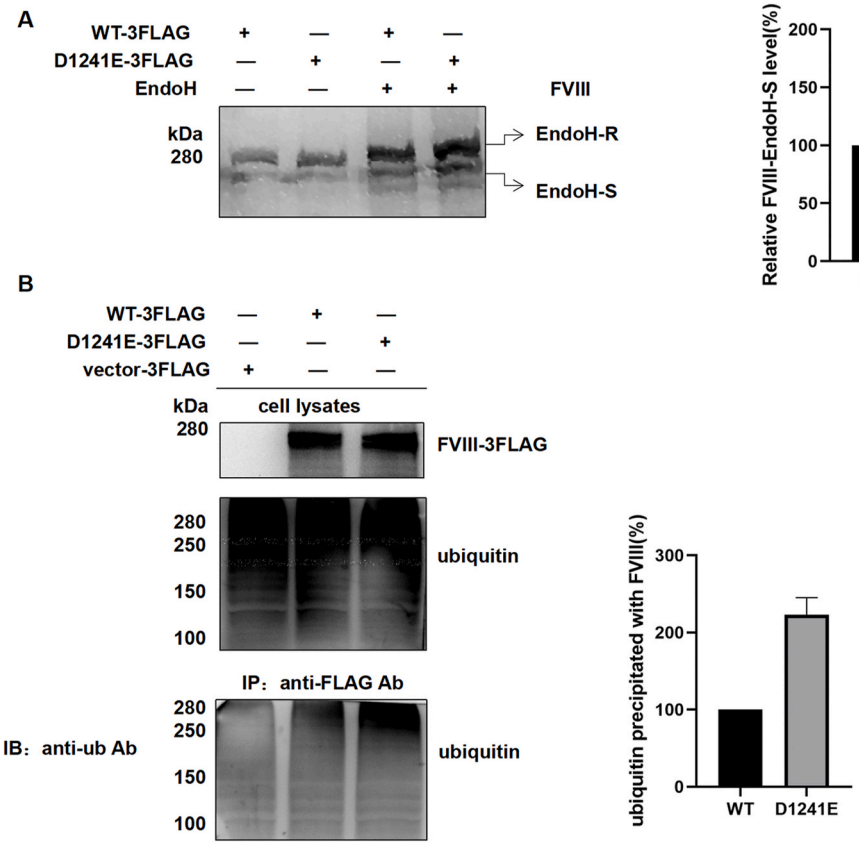

Fig. 7. Promotion of FVIII D1241E into the cytosol for degradation after 48 h transfection. (A) Immunoprecipitation–western blot. HEK-293T cells were grown and transfected with plasmid carrying FVIII WT-3FLAG or D1241E-3FLAG for 48 h, and then subjected to immunoprecipitation. The immunoprecipitates were treated with the glycosidase EndoH (5 μL) for 5 h, then subjected to Western blot (immunoprecipitation with an anti-FLAG antibody, Western blot with an anti-FVIII antibody). (B) Coimmunoprecipitation–western blot. HEK-293T cells were grown and transfected with plasmid carrying FVIII WT-3FLAG or D1241E-3FLAG for 48 h, treated with MG132 (50 μM) for 2 h, and then subjected to immunoprecipitation–western blot (immunoprecipitation with an anti-FLAG antibody, followed by Western blot with an anti-ubiquitin antibody). The data represent the mean ± standard deviation of the two independent experiments, and the error bars represent the standard deviation.

treatment with MG132. The cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody and Western blot analysis with an anti-ubiquitin antibody. We found that the FVIII D1241E-transfected cells had a higher ubiquitination level than that of the FVIII WT-transfected cells (Fig. 7B), suggesting that the D1241E polymorphism led to a higher level of proteasome degradation.

4. Discussion

FVIII is an important blood coagulation glycoprotein, the deficiency of which leads to the development of hemophilia A. Meanwhile, the FVIII levels in the general population can vary by more than fivefold [16]. In addition, an increase in the FVIII level has been associated with an increased risk of developing venous and arterial thrombosis in patients [17,18]. A previous study that searched for the FVIII quantitative trait loci in the *FVIII* gene has revealed that the minor allele of FVIII D1241E [8], localized in the B domain, affected FVIII secretion; however, the underlying mechanism remains to be clarified. Thus, our current study assessed the effect of this minor allele on the regulation of FVIII expression and secretion as well as the underlying mechanism. We found that the minor allele of FVIII D1241E reduced FVIII secretion into the conditioned medium *in vitro*, whereas it maintained the FVIII procoagulation activity in the normal range. Furthermore, the minor allele of FVIII D1241E led to reductions of FVIII in the Golgi apparatus and malectin association. In contrast, the minor allele of FVIII D1241E promoted the translocation of FVIII protein into the cytosol and the ERAD process. In summary, this study demonstrated that malectin has an important role in the regulation of FVIII for posttranslational processes and modification, while the association of FVIII with decreased amounts of malectin could cause the reduced amount of FVIII in individuals carrying the minor allele of FVIII D1241E.

Indeed, FVIII is an N-linked glycosylated protein, and N-glycosylation occurs when a nascent polypeptide enters into the ER, i.e., oligosaccharyltransferase will transfer the $Glc_3Man_9GlcNAc_2$ (G3M9) oligosaccharide from a lipid donor to an asparagine residue residing in the amino acid sequence of Asn-Xxx-Ser/Thr of the polypeptide, which is named as N-linked glycosylation [19] (Fig. 1D). Furthermore, malectin is a novel carbohydrate-binding protein in the ER that plays an important role in the early steps of protein folding [20], i.e., after N-glycosylation starts in the ER, the outermost glucose residues in G3M9 are sequentially removed by the ER-resident glucosidase I, while the remaining $Glc_2Man_9GlcNAc_2$ (G2M9) possesses a high affinity to malectin [20]; thereafter, glucosidase II removes the second glucose to form $Glc_1Man_9GlcNAc_2$ (G1M9), which in turn recruits calnexin and calreticulin [21]. At this stage, the newly synthesized and modified polypeptides will be correctly folded into their native conformation [22] and then transported from the ER to the Golgi apparatus for additional processing [23] (Fig. 1D). The ER-Golgi intermediate compartment 53-kD protein (ERGIC-53; also named as lectin mannose-binding 1) is a transport receptor for FVIII that is transported from the ER to the Golgi apparatus (15, 24, 25). However, a previous study has shown that a fraction of newly translated intracellular FVIII undergoes ERAD by the proteasome [14]. The ERAD substrates are usually misfolded proteins, fully glycosylated proteins, or even partially folded proteins [26], in which the misfolded proteins will be retrotranslocated from the ER into the cytosol for deglycosylation, ubiquitination, and proteasomal proteolysis [27]. Overall, the recently identified malectin in the ER does play an important role in the posttranslational modification and transportation of newly translated protein from the ER to the Golgi apparatus [28,29]. Our current study showed that the minor allele of FVIII D1241E did reduce the level of FVIII in the Golgi apparatus and also reduced its association with malectin. In contrast, this minor allele promoted the translocation of FVIII protein into the cytosol for the ERAD process and degradation. These data further support the normal process of FVIII in cells as well as the importance of malectin in the post-translation and maturation processes of FVIII protein.

Furthermore, newly translated polypeptides in the ER will interact with various lectins sequentially to facilitate glycoprotein folding and sorting [30]. Oligosaccharides recognized by lectins may act as markers to direct glycoprotein folding and trafficking in the secretion pathway [31]; however, before the discovery of malectin, calnexin, an integral membrane chaperone, and its soluble luminal counterpart calreticulin were thought to be two crucial lectins involved in recognition of the G1M9 oligosaccharide and binding to the glycoprotein [32]. In cells, the G1M9 oligosaccharide is synthesized through a two-step reaction, i.e., sequential removal of the glucose residues from G3M9 with glucosidases I and II [33]. However, malectin can recognize the G2M9 oligosaccharide, indicating that malectin is between glucosidases I and II before the glycoprotein binds to calnexin and calreticulin. Indeed, malectin has been shown to stably associate with misfolded AT^{NHK}, a folding-defective human α1-antitrypsin variant, thus preventing the misfolded glycoprotein from secretion [29]. A previous study has demonstrated that FVIII is a binding partner to calnexin and calreticulin [14]. Our current study was the first to assess the association (binding) of FVIII to malectin, i.e., when cells were treated with 1-deoxynojirimycin (an alpha-glucosidase inhibitor) to accumulate G2M9, our coimmunoprecipitation–western blot data showed that both FVIII WT and D1241E were able to interact with malectin, but the latter had a reduced association with malectin, resulting in a lower amount of FVIII polypeptide to be processed thereafter. However, future studies are needed to show their binding at the molecular level.

Another previous study has reported that malectin is responsible for carbohydrate recognition, especially diglucosides [34], while malectin can form a complex with ribophorin I to induce the association with misfolded glycoproteins [35]. In the current study, we revealed the minor allele of FVIII D1241E had a reduced association with malectin, although we did not know how FVIII D1241E lost its affinity toward malectin. Theoretically, FVIII D1241E *per se* does not induce changes to any N-glycosylation sites, so we speculate that it might lead to weak binding to ribophorin I, and, therefore, to a relatively weak binding to malectin. Another possible explanation may be that FVIII D1241E changes the FVIII protein conformation and in turn alters the N-linked glycosylation sites to prevent N-glycosylation and the interaction with malectin. However, future studies are needed to confirm these speculations. Moreover, the accumulation of unfolded or misfolded proteins in the ER can also cause ER stress [36], and the ER can initiate several mechanisms to alleviate such a stress, e.g., inhibition of client protein influx into the ER and an increase in protein folding and/or protein degradation [37,38]. Indeed, a previous study has demonstrated that approximately 60% of intracellular FVIII undergoes ERAD and degradation by the proteasome [14,39]. Furthermore, previous studies have revealed that ERGIC-53 is a cargo transporter that transports FVIII from the ER to the Golgi apparatus [15,24,25]. Our current study of ERGIC-53 coexpression showed that FVIII WT had a strong interaction with ERGIC-53, but the minor allele of FVIII D1241E only associated with rather reduced levels of ERGIC-53, suggesting that FVIII D1241E led to weak FVIII trafficking to the Golgi apparatus, resulting in reduced FVIII D1241E secretion into the conditioned medium *in vitro*. In contrast, the excessive amount of FVIII D1241E in the ER will undergo ERAD by the proteasome. The EndoH sensitivity assay further confirmed these findings. Following FVIII dislocation from the ER, the level of the deglycosylated form of FVIII D1241E in the cytosol became higher than that of FVIII WT. Our ubiquitination analysis showed a higher ubiquitination level in FVIII D1241E, implying that more FVIII D1241E was in the cytosol for degradation than FVIII WT.

Overall, FVIII has the lowest level in the blood among all coagulation factors, but FVIII has an important role in blood coagulation [40], indicating that the human body has developed a precise mechanism to control the blood FVIII level because any increase in the plasma FVIII level might increase the thrombosis potential [41]. For normal homeostasis in the human body, most of the newly synthesized FVIII undergoes ERAD degradation. To date, hemophilia A is primarily treated with

recombinant FVIII (rFVIII). It is not only expensive [39], but the level of rFVIII expression is relatively low [42]. Therefore, insight into the malectin coexpression data could help us to facilitate an increase in FVIII production, especially, the level of FVIII light chain in the Golgi apparatus; thus, modification of the ER lectins, such as malectin, could improve the efficiency of FVIII production using a mammalian expression system, leading to a more cost-efficient rFVIII production system.

However, our current study does have some limitations. For example, we used HEK-293 cells (normal human embryonic kidney cells) instead of a normal liver cell line. The reasons for this choice were that we were unable to obtain a normal liver cell line and that liver cancer cells, like HepG2 cells, may have endogenous FVIII production, which would make it difficult for us to compare the effects of exogenous FVIII vs. D1241E minor allele. Indeed, HEK-293T, CHO, and COS-1 cells are frequently used in *in vitro* studies of FVIII mutations in the literature [13,43,44]. Moreover, our current study is just a proof-of-principle investigation. Future *in vivo* and *ex vivo* studies are needed to confirm our current *in vitro* data.

In conclusion, the current study is the first to provide *in vitro* evidence revealing the interaction between FVIII and malectin and to describe the important role of malectin in FVIII post-transfection regulation. We also demonstrated that the minor allele of FVIII D1241E had a reduced association with malectin, resulting in its degradation in the cytosol and leading to reduction of its secretion. Our current data might help us to improve our understanding of the FVIII trafficking after protein translation and secretion, thus providing a potentially novel strategy for the production of rFVIII for the treatment of hemophilia A.

Consent for publication

Consent to publish has been obtained from the participants.

CRediT authorship contribution statement

Ning Jiang: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Yanfeng Xiao:** Conceptualization, Supervision, Funding acquisition. **Yuesheng Liu:** Software, Data curation. **Weihua Liu:** Software, Data curation. **Shanxi Liu:** Project administration.

Declaration of competing interest

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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