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N-Glycan-calnexin interactions in human factor VII secretion and deficiency

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ABSTRACT

Factor VII (FVII) is a key serine protease in blood coagulation. N-glycosylation in FVII has been shown to be critical for protein secretion. To date, however, the underlying biochemical mechanism remains unclear. Recently, we found that *N*-glycans in the transmembrane serine protease corin are critical for calnexin-assisted protein folding and extracellular expression. In this study, we tested the hypothesis that *N*-glycans in the FVII protease domain mediate calnexin-assisted protein folding and that naturally occurring *F7* mutations abolishing *N*-glycosylation impair FVII secretion. We expressed human FVII wild-type (WT) and mutant proteins lacking one or both *N*-glycosylation sites in HEK293 and HepG2 cells in the presence or absence of a glucosidase inhibitor. FVII expression, secretion and binding to endoplasmic reticulum chaperones were examined by immune staining, co-immunoprecipitation, Western blotting, and ELISA. We found that *N*-glycosylation at N360 in the protease domain, but not N183 in the pro-peptide domain, of human FVII is required for protein secretion. Elimination of *N*-glycosylation at N360 impaired calnexin-assisted FVII folding and secretion. Similar results were observed in WT FVII when *N*-glycan-calnexin interaction was blocked by glucosidase inhibition. Naturally occurring *F7* mutations abolishing *N*-glycosylation at N360 reduced FVII secretion in HEK293 and HepG2 cells. These results indicate that *N*-glycans in the FVII protease domain mediate calnexin-assisted protein folding and subsequent extracellular expression. Naturally occurring *F7* mutations abolishing *N*-glycosylation in FVII may impair this mechanism, thereby reducing FVII levels in patients.

1. Introduction

Factor VII (FVII) is a serine protease of the trypsin fold essential for normal hemostasis (D'Alessandro et al., 2018; Gailani and Broze, 1991; Mackman et al., 2007). Upon binding to tissue factor at vessel injury sites, FVII is converted to an active enzyme, *i.e.* FVIIa, which initiates blood coagulation, leading to fibrin clot formation. FVII deficiency impairs blood coagulation, thereby causing bleeding (Mandhyan et al., 2010; Mariani and Bernardi, 2009; Perry, 2002). Conversely, increased FVII activity promotes blood coagulation and hemostasis. To date, recombinant FVIIa has been used as a therapeutic agent to treat FVII deficiency and other bleeding disorders, including hemophilia, factor XI deficiency and Glanzmann's thrombasthenia (Hedner, 2015; Hoffman, 2018; O'Connell et al., 2008; Sumner et al., 2007).

N-glycosylation is a post-translational modification that regulates protein expression and function (Eklund and Freeze, 2005; Varki, 1993). Based on bioinformatic analysis, *N*-glycosylation sites are present in approximately two thirds of predicted proteins encoded by the human genome (Apweiler et al., 1999). Similar to many trypsin-like serine proteases, human FVII is a glycoprotein, containing two *N*-glycosylation sites: one in the pro-peptide domain and the other in the protease domain (Hagen et al., 1986; Thim et al., 1988). In Chinese hamster ovary (CHO) cells and African green monkey kidney COS-7 cells, *N*-glycosylation was found to be required for the intracellular stability and secretion of recombinant human FVII (Bolt et al., 2005, 2007). To date, the biochemical mechanism underlying the *N*-glycan function in FVII expression and secretion has not been elucidated. It has been shown that other post-translational modifications such as gamma-carboxylation in the endoplasmic reticulum (ER) and trafficking through the distal portion of the Golgi network are also important for FVII secretion (Bolt et al., 2008).

Corin is a transmembrane serine protease that regulates salt-water balance and cardiovascular homeostasis (Chen et al., 2015; Cui et al., 2012; Dong et al., 2013; Li et al., 2017; Yan et al., 2000). We and others have shown that *N*-glycosylation is important for the cell surface expression of corin (Gladysheva et al., 2008; Liao et al., 2007; Wang et al.,

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2015). Most recently, we reported that *N*-glycans in the corin protease domain interacted with calnexin in assisting glycoprotein folding and ER exiting (Wang et al., 2018). If the *N*-glycosylation site in the protease domain was eliminated, corin was retained in the ER. This *N*-glycan-calnexin interaction appears to participate in the folding and extracellular expression of other trypsin-like serine proteases. Mutations abolishing *N*-glycosylation sites in the protease domain of enteropeptidase, a digestive enzyme (Wu, 2003), or prothrombin, a clotting factor (Wu et al., 1991), impaired the extracellular expression of these proteases in cultured cells (Wang et al., 2018).

N-glycans at different sites in a given protein may have distinct roles in regulating protein production and function (Wang et al., 2015). In FVII, *N*-glycosylation site locations differ from those in corin, enteropeptidase and prothrombin. It is unknown if *N*-glycans in FVII, especially those in the protease domain, also interact with calnexin in assisting protein folding and extracellular expression. We expressed mutant FVII proteins lacking one or both of the *N*-glycosylation sites in transfected human embryonic kidney 293 (HEK293) cells and hepatoma HepG2 cells. We analyzed the mutant FVII proteins for their expression levels and interactions with calnexin and other ER chaperones by co-immunoprecipitation and Western blotting. These studies should help us to understand the biochemical and cellular mechanisms underlying the functional importance of *N*-glycosylation in FVII synthesis and secretion.

2. Material and methods

2.1. Plasmids

The full-length human FVII cDNA was amplified from a liver cDNA library and inserted into pcDNA 3.1/V5 plasmid (Thermo Fisher). The resultant plasmid, which encoded wild-type (WT) FVII, was used as a template in site-directed mutagenesis (QuikChange II, Agilent Technologies) to make additional plasmids encoding mutant FVII proteins with N183Q, N360Q, N183Q/N360Q, N360D, and T362 M amino acid substitutions, respectively. The amino acid numbering is based on the full-length human FVII isoform with 444 amino acids including the signal peptide (GenBank: NM_019616). Recombinant FVII proteins encoded by these plasmids contained a C-terminal V5 tag for protein detection, as described previously (Chen et al., 2015). All plasmids used in this study were verified by DNA sequencing.

2.2. Cell culture and transfection

HEK293 cells (ATCC, CRL-1573, authenticated by STR DNA profiling) and HepG2 cells (ATCC, HB-8065, authenticated by STR DNA profiling) were grown in DMEM with 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO_2, as described previously (Wang et al., 2018). The plasmids encoding FVII proteins and a control pcDNA 3.1/V5 plasmid were transfected into the cells at ˜70–80% confluence using Fugene reagents (Promega). After 24 h, the conditioned medium was collected and the cells were lysed with a solution containing 50 mM Tris − HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40 (v/v), and a protease inhibitor mixture (1:100, Sigma) (Chen et al., 2010). FVII proteins in the conditioned medium and the lysates were analyzed in the following experiments.

2.3. Western blotting of FVII proteins

Proteins in cell lysates were denatured in a sample buffer (Bio-Rad) with 2.5% β-mercaptoethanol. Recombinant proteins in the conditioned medium were immunoprecipitated with an anti-V5 antibody (Thermo Fisher, R96025) and protein A-Sepharose beads (Thermo Fisher). The proteins on the beads were eluted with the sample buffer and analyzed by SDS-PAGE and Western blotting using a horseradish peroxidase-conjugated anti-V5 antibody (Thermo Fisher, R96125). Western blots were exposed to X-ray films and protein bands were quantified by densitometry, as described previously (Liao et al., 2007).

2.4. Immunostaining

HEK293 cells on coverslips were transfected with plasmids encoding FVII WT and mutant proteins. Immunostaining was done, as described previously (Dong et al., 2014). Briefly, the cells were fixed with 4% paraformaldehyde, blocked with 5% bovine serum albumin in PBS, and incubated with primary antibodies against V5 (Thermo Fisher, R96025), KDEL (Abcam, ab2898) and TGN46 (Abcam, ab50595), respectively, followed by a secondary antibody conjugated with Alexa Fluor-488 (green) or 594 (red) (Thermo Fisher). After washing, the coverslips were mounted with a solution containing 6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclei staining. The cells were examined and images were taken with a confocal microscope (Olympus).

2.5. ELISA analysis of FVII proteins

Cell lysates and conditioned media containing FVII proteins from transfected HEK293 and HepG2 cells were prepared, as described above. FVII protein levels in these samples were measured by ELISA (Abcam, ab168545), based on the manufacturer's instructions.

2.6. Protein co-immunoprecipitation

Cell lysates from transfected HEK293 cells were immunoprecipitated with an anti-V5 antibody and protein A-Sepharose beads, as described above. Proteins eluted from the Sepharose beads were analyzed by Western blotting. FVII and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins in the original cell lysates were verified by Western blotting. In these experiments, primary antibodies against V5 (Thermo Fisher, R96125), GAPDH (EMD Millipore, MAB374), binding immunoglobulin protein (BiP) (Cell Signaling, 3177 T), calnexin (Cell Signaling, 2679 T), and calreticulin (Cell Signaling, 12238S) were used. Secondary antibodies used were horseradish peroxidase-conjugated (KPL, 474–1806; 474–1516).

2.7. Glucosidase inhibition

HEK293 cells transfected with FVII-encoding plasmids or HepG2 cells were cultured with DMEM containing 1-deoxynojirimycin (DNJ) (2 mM, Alfa Aesar), which inhibits glucosidases I and II (Saunier et al., 1982). After 24–36 h at 37 °C, the conditioned medium was collected and the cells were lysed. FVII proteins in the conditioned medium and cell lysates were analyzed by co-immunoprecipitation and Western blotting, as described above.

2.8. Statistical analysis

Data were analyzed with Prism software (Graphpad). The Student's *t* test was used to compare data from two groups. ANOVA followed by Tukey's *post-hoc* analysis was used to compare data from three or more groups. p -values of < 0.05 were considered to be statistically significant.

3. Results

3.1. N-glycosylation at N360 is important for FVII secretion from HEK293 cells

Human FVII consists of a signal peptide, a gamma-carboxyglutamic acid-rich (GLA) domain, two epidermal growth factor (EGF) domains and a trypsin-like serine protease domain (Fig. 1A). Two *N*-glycosylation sites are at N183 and N360 (Fig. 1A). To examine the importance of

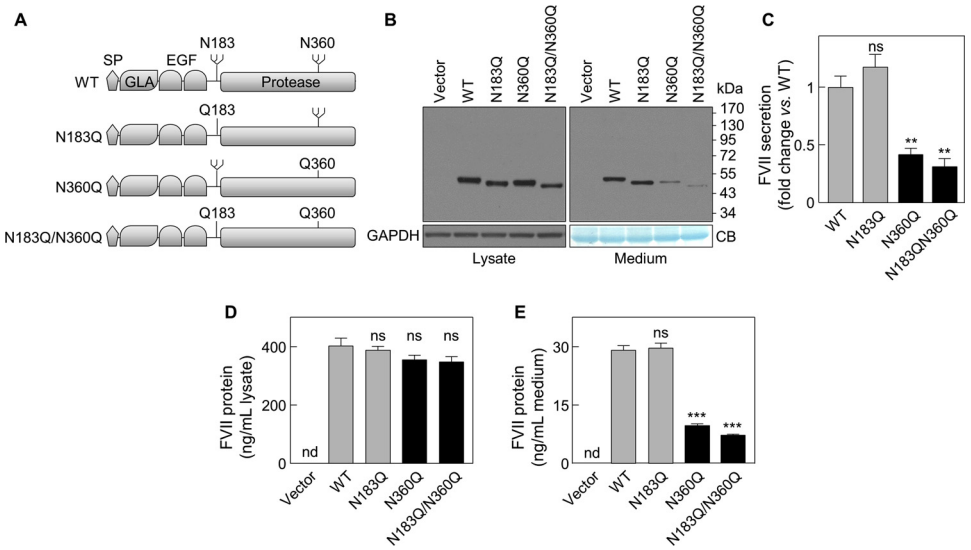

Fig. 1. Importance of *N*-glycosylation in human FVII production and secretion. (A) Illustration of FVII domains and mutant proteins lacking one or both *N*-glycosylation sites. The signal peptide (SP), gamma-carboxyglutamic acid-rich (GLA), epidermal growth factor-like (EGF) and protease domains are indicated. *N*-glycosylation sites at N183 and N360 are shown. (B) Western blotting of FVII proteins in lysates (left) and conditioned media (right) from transfected HEK293 cells. Levels of GAPDH in lysates and a Coomassie blue (CB)-stained non-specific protein band in media were used to assess loaded protein amounts. (C) Levels of FVII secretion were estimated by densitometric analysis of Western blots of the lysates and media. Data are means ± S.E. from five independent experiments. ns: not statistically significant *vs*. WT; $^{**}p < 0.01$ *vs*. WT. (D and E) ELISA analysis of FVII levels in cell lysates (D) and media (E) from transfected HEK293 cells. Data are means ± S.E. from three independent experiments. nd: not detectable; ns: not statistically significant *vs*. WT; $^{***}p < 0.001$ *vs*. WT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

N-glycosylation for FVII expression, we transfected plasmids encoding FVII WT and mutant proteins lacking one (N183Q or N360Q) or both (N183Q/N360Q) of the *N*-glycosylation sites in HEK293 cells (Fig. 1A). In Western blotting, FVII WT and mutant proteins in cell lysates and conditioned media appeared as single bands of ~50–55 kDa (Fig. 1B). N183Q and N360Q bands migrated faster than the WT band, whereas the N183Q/N360Q band migrated faster than N183Q and N360Q bands did (Fig. 1B). The results indicate that both N183 and N360 are *N*-glycosylated and that amino acid replacements at these two sites prevented *N*-glycosylation, thereby reducing the molecular mass of the mutant proteins.

On Western blots, levels of WT and the mutant proteins in cell lysates were similar, whereas levels of N360Q and N183Q/N360Q bands in the conditioned media were lower than those of WT and N183Q (Fig. 1B). Based on densitometric analysis of the blots, ratios of FVII proteins in the conditioned medium *vs*. the cell lysate for N183Q, N360Q and N183Q/N360Q proteins were 118 ± 11% ($p > 0.05$), 42 ± 5% ($p < 0.01$), and 31 ± 5% ($p < 0.01$), respectively, compared to that of WT (Fig. 1C). We verified the results by ELISA. In cell lysates, levels of WT and the mutant proteins were similar (Fig. 1D). In the conditioned medium, levels of WT (29.1 ± 1.2 ng/mL) and N183Q (29.8 ± 0.9 ng/mL) were higher than those of N360Q (9.6 ± 0.6 ng/mL, $p < 0.001$) and N183Q/N360Q (7.0 ± 0.5 ng/mL, $p < 0.001$) (Fig. 1E). These results indicate that *N*-glycosylation at N360, but not N183, is important for FVII secretion from transfected HEK293 cells.

3.2. ER retention of FVII mutant proteins lacking N-glycosylation at N360

To understand how amino acid replacements of N360Q and N183Q/N360Q impair FVII secretion, we did co-immunostaining with markers for the ER (KDEL) and the Golgi (TGN46) in transfected HEK293 cells. We found overlapping staining of KDEL and mutant proteins N360Q and N183Q/N360Q, which was not observed in FVII WT and the N183Q mutant protein (Fig. 2A). In co-staining with TGN46, no overlapping staining was found with FVII WT and the mutant proteins (Fig. 2B). These results suggest that a lack of *N*-glycosylation at N360, but not at N183, may cause FVII retention in the ER.

3.3. N-glycosylation at N360 promotes calnexin-assisted FVII protein folding

Recently, we reported that *N*-glycans in corin, enteropeptidase and prothrombin are required for calnexin-assisted protein folding and ER exiting (Wang et al., 2018). Abolishing *N*-glycosylation sites in the protease domains of these proteins increased the direct binding of poorly folded proteins with calnexin and BiP, another ER chaperone (Behnke et al., 2015). To test if *N*-glycans at N360 in FVII have a similar role in glycoprotein folding, we analyzed FVII interactions with ER chaperones. In co-immunoprecipitation and Western blotting, levels of calnexin and BiP that were co-precipitated with N360Q were higher than those co-immunoprecipitated with WT FVII (124 ± 7% for calnexin, $p < 0.05$; 230 ± 39% for BiP, $p < 0.05$) (Fig. 3A). In contrast, levels of calreticulin, another ER chaperone involved in glycoprotein folding (Caramelo and Parodi, 2008; Hebert et al., 1995), were similar between WT and N360Q in the co-immunoprecipitates (Fig. 3A). As controls, FVII levels in the co-immunoprecipitates and original cell lysates were similar between WT and N360Q (Fig. 3A). These results suggest that lacking *N*-glycans at N360 in FVII blocked the calnexin-*N*-glycan interaction, thereby impairing calnexin-assisted glycoprotein folding. As a result, direct protein-protein interactions between poorly folded N360Q protein and calnexin or BiP were increased in the ER.

To verify this hypothesis, we tested the effect of DNJ, a glucosidase inhibitor (Saunier et al., 1982), which blocks the trimming of triglucosylated to monoglucosylated oligosaccharides that are required for calnexin-assisted glycoprotein folding (Boedeker et al., 1999; Lamriben et al., 2016; Saunier et al., 1982). In DNJ-treated HEK293 cells, calnexin levels co-precipitated with WT FVII increased (129 ± 5% *vs*. WT in untreated cells; $p < 0.01$) and were similar to that of N360Q in DNJ-treated cells (Fig. 3B). Similar results were found for BiP levels in DNJ-treated cells (Fig. 3B). These results support the idea that *N*-glycans at N360 are critical for calnexin-assisted glycoprotein folding and that blocking the interaction between *N*-glycans and calnexin causes the retention of poorly folded FVII proteins in the ER by direct protein-protein binding with calnexin and BiP.

3.4. DNJ inhibition reduces FVII secretion

Increased ER retention is expected to reduce protein secretion. We examined FVII secretion in transfected HEK293 cells cultured with or without DNJ. In Western blotting, similar WT FVII levels were found in lysates from the cells treated with or without DNJ, whereas the FVII level in the conditioned medium from DNJ-treated cells was 58 ± 8% ($p < 0.01$) of that from the untreated cells, an indication of reduced

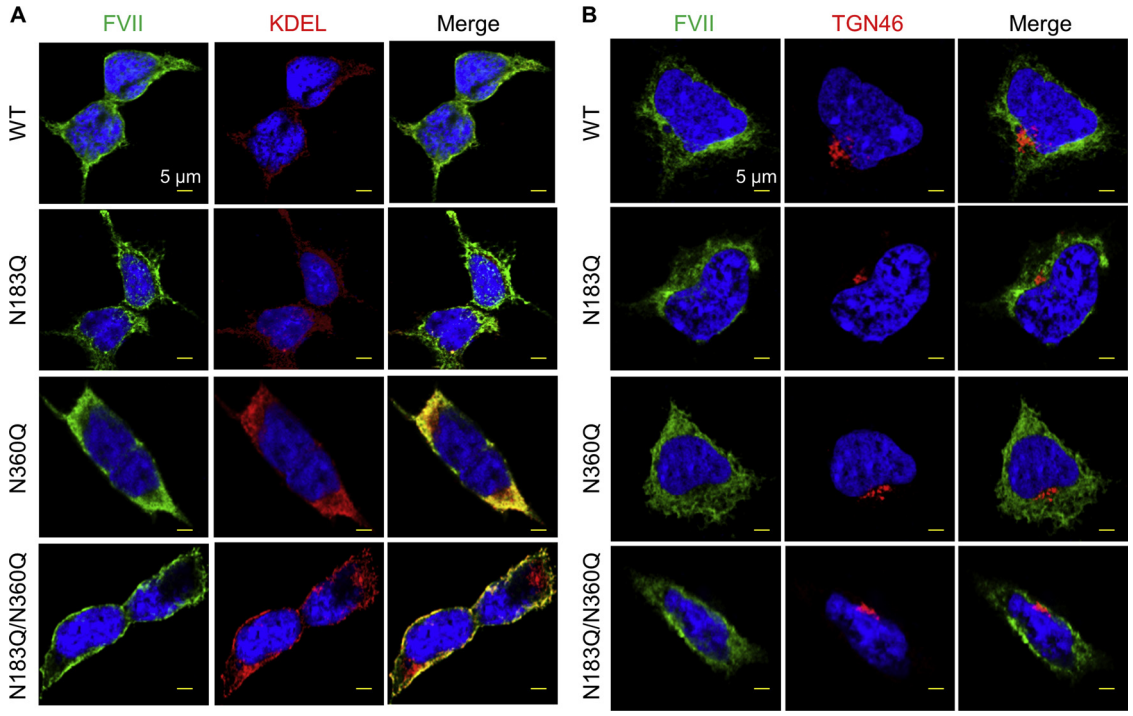

Fig. 2. Intracellular distribution of FVII and mutant proteins in HEK293 cells. Transfected HEK293 cells producing human FVII WT and mutant proteins N183Q, N360Q and N183Q/N360Q were co-immunostained for FVII and KDEL, an ER marker (A) or TGN46, a Golgi marker (B). The images were taken with a confocal microscope. Scale bars are indicated. Data are representative of three independent experiments.

FVII secretion (Fig. 4A). We next examined the effect of DNJ on HepG2 cells producing endogenous FVII. By ELISA, FVII levels were similar in HepG2 cells cultured with or without DNJ, whereas the level in the conditioned medium was reduced by ˜40% in DNJ-treated HepG2 cells (23.2 ± 1.0 *vs.* 38.0 ± 1.9 ng/mL in untreated cells, $p <$ 0.01) (Fig. 4B). These results indicate that blocking *N*-glycan-calnexin interaction impairs calnexin-assisted FVII folding, thereby reducing the secretion of FVII in DNJ-treated cells.

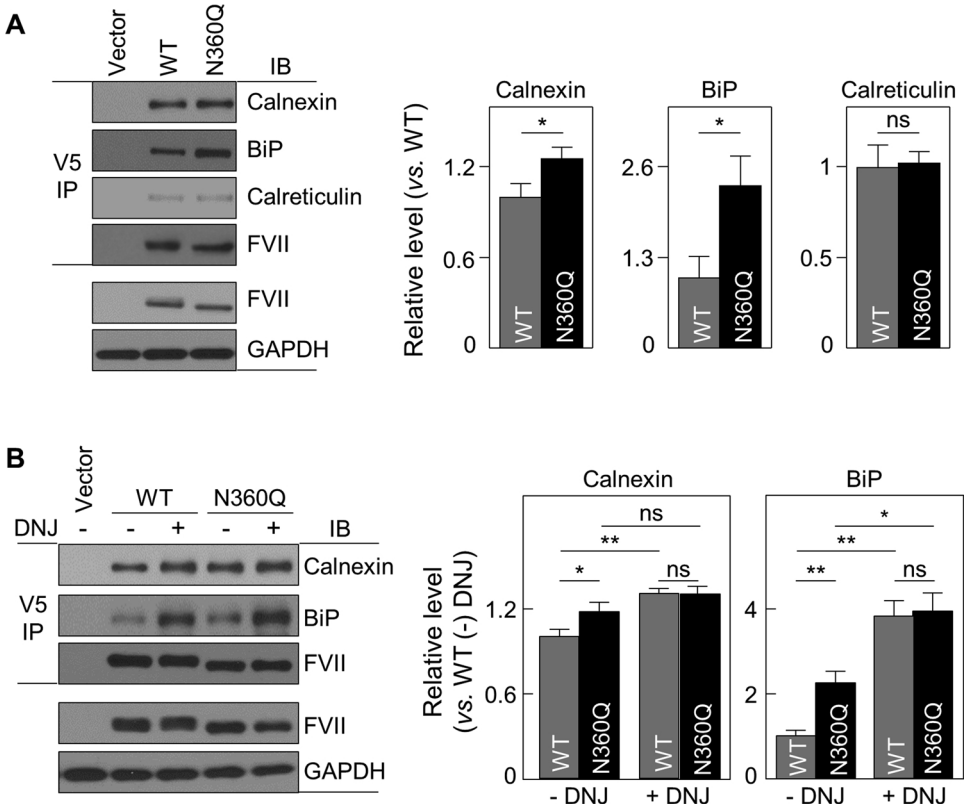

Fig. 3. Interaction between FVII and ER chaperones. (A) FVII WT and the N360Q mutant protein were produced in HEK293 cells. Co-immunoprecipitation (IP) and Western blotting (IB) analysis of calnexin, BiP and calreticulin (top three panels) bound to FVII proteins pulled down with an anti-V5 antibody. FVII proteins in V5 pull-down samples were verified (fourth panel). FVII and GAPDH in the starting cell lysates were also verified (bottom two panels). Relative levels of calnexin, BiP and calreticulin were estimated by densitometric analysis of Western blots. Data are means ± S.E. from ≥ 3 independent experiments. $*p < 0.05$; ns: not significant. (B) Western blotting of calnexin and BiP co-immunoprecipitated with FVII in HEK293 cells producing WT or N360Q without (−) or with (+) DNJ treatment (top two panels). FVII in V5 pull-down samples was verified (third panel). FVII and GAPDH in the starting cell lysates were verified (bottom two panels). Relative levels of calnexin and BiP were estimated by densitometric analysis of Western blots. Data are means ± S.E. from 3 independent experiments. $*p < 0.05$; $**p < 0.01$ *vs.* indicated groups.

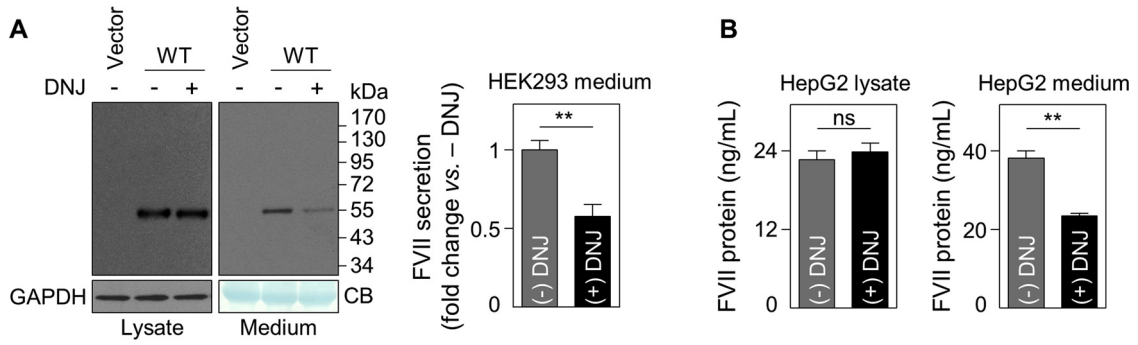

Fig. 4. Effects of DNJ on FVII secretion. (A) Western blotting of WT FVII in lysates and media from transfected HEK293 cells incubated without (−) or with (+) DNJ. GAPDH in lysates and a Coomassie blue (CB)-stained non-specific protein in media were used to assess loaded protein amounts. Levels of FVII secretion were estimated by densitometric analysis of the Western blots of lysates and media. Data are means ± S.E. from 4 independent experiments. $^{**}p < 0.01$. (B) ELISA analysis of FVII protein levels in lysates (left) and conditioned media (right) from HepG2 cells incubated without (-) or with (+) DNJ. Data are means ± S.E. from 4 independent experiments. $^{**}p < 0.01$; ns: not statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.5. Gene variants abolishing N-glycosylation at N360 impair FVII secretion

Previously, an *F7* variant causing T362 M substitution, or T324 M if the numbering is based on the mature FVII without the signal peptide, was reported in patients with FVII deficiency (Giansily-Blaizot et al., 2004; Mota et al., 2009; Salcioglu et al., 2012) (Fig. 5A). This amino acid replacement alters the *N*-glycosylation consensus sequence, Asn360-Ile361-Thr362, and is expected to abolish *N*-glycosylation at N360 (Fig. 5A). To examine the effect of the T362 M replacement on FVII secretion, we expressed and analyzed the T362 M mutant protein in transfected HEK293 cells. On Western blots, the T362 M band migrated faster than that of WT, indicating that T362 M replacement abolished *N*-glycosylation at N360 (Fig. 5B). In cell lysates, levels of WT FVII and T362 M proteins were similar, whereas the level of T362 M in the conditioned medium was 25 ± 11% of WT ($p < 0.001$) (Fig. 5C), indicating that the secretion of the T362 M mutant protein was impaired in the transfected HEK293 cells.

By searching the human single nucleotide polymorphism (SNP) database (www.ncbi.nlm.nih.gov/snp) (Sherry et al., 2001), we identified another *F7* gene variant that causes N360D amino acid substitution and abolishes *N*-glycosylation at N360 (Fig. 6A). We analyzed the mutant protein in similar experiments with transfected HEK293 cells and Western blotting. Compared to WT FVII, the N360D mutant protein migrated faster and had a lower level (25 ± 6% of WT) in the conditioned medium (Fig. 6B and C), a similar phenotype to that of the T362 M mutant protein.

We verified the results by ELISA, which showed similar time-dependent increases in FVII levels in cell lysates from the HEK293 cells producing WT and the mutant proteins (Fig. 7A). In the conditioned medium, levels of N360Q, T362 M and N360D mutant proteins were

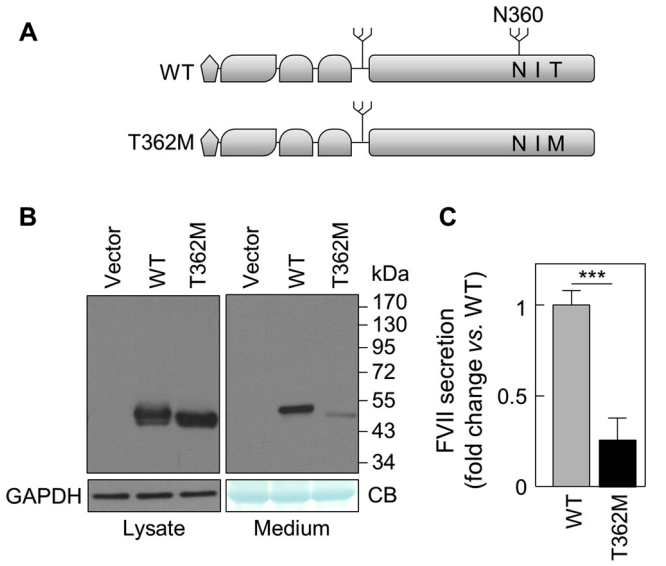

Fig. 5. *F7* mutation causing T362 M replacement reported in patients with FVII deficiency. (A) Illustration of the T362 M replacement abolishing the *N*-glycosylation sequence in the FVII protease domain. (B) Western blotting of FVII WT and the T362 M mutant protein in lysates and conditioned media from transfected HEK293 cells. Levels of GAPDH in lysates and a Coomassie blue (CB)-stained non-specific protein in the media were verified. Levels of FVII secretion were estimated by densitometric analysis of the Western blots. Data are means ± S.E. from 4 independent experiments. $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

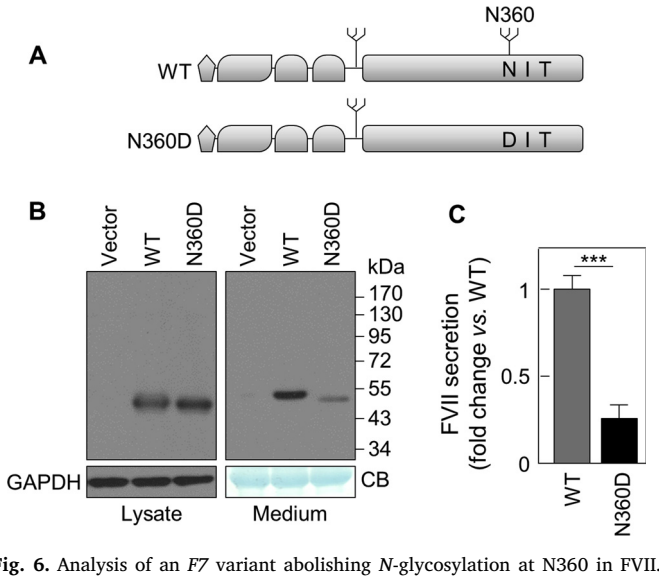

Fig. 6. Analysis of an *F7* variant abolishing *N*-glycosylation at N360 in FVII. The *F7* variant was identified by searching the human SNP database. (Sherry et al., 2001) (A) Illustration of the N360D replacement abolishing the *N*-glycosylation site in the FVII protease domain. (B) Western blotting of FVII WT and the N360D mutant protein in lysates and conditioned media from transfected HEK293 cells. Levels of GAPDH in lysates and a Coomassie blue (CB)-stained non-specific protein in the media were verified. Levels of FVII secretion were estimated by densitometric analysis of the Western blots. Data are means ± S.E. from 5 independent experiments. $^{***}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

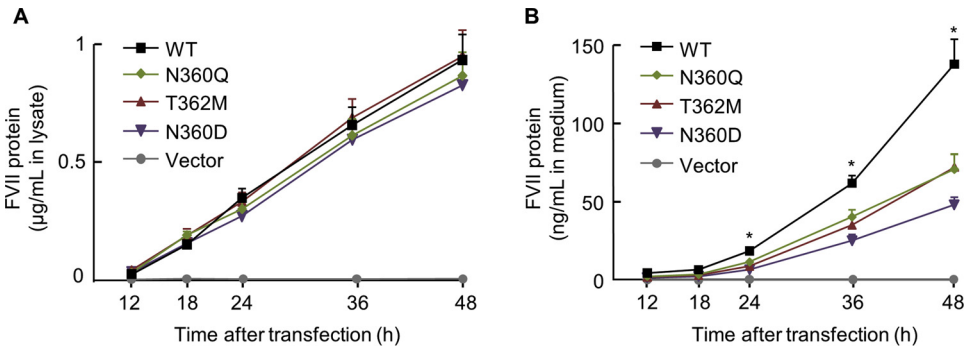

Fig. 7. ELISA analysis of FVII secretion in HEK293 cells. FVII WT and mutant proteins were produced in HEK293 cells. Levels of FVII proteins were measured by ELISA in cell lysates (A) and conditioned media (B) collected at 12, 18, 24, 36 or 48 h after transfection. Data are means ± S.E. from 3 independent experiments. *$p < 0.05$ WT *vs.* mutant proteins at the same time point.

lower than those of WT when measured at 24, 36 and 48 h post-transfection (Fig. 7B), consistent with impaired secretion of these mutant proteins.

3.6. Impaired secretion of mutant FVII proteins in HepG2 cells

We next analyzed the secretion of the mutant FVII proteins in HepG2 cells by transfection and Western blotting. In samples from both cell lysates and conditioned media, N360Q, T362 M and N360D mutant proteins migrated faster than WT on Western blots (Fig. 8A). In the cell lysates, levels of WT and the mutant proteins were similar (Fig. 8A), whereas levels of the mutant proteins in the conditioned media were lower than that of WT (54 ± 3% for N360Q, 36 ± 3% for T362 M, and 39 ± 7% for N360D *vs.* WT; all p values < 0.01) (Fig. 8B), indicating that the amino acid replacements abolishing *N*-glycosylation at N360 impaired FVII secretion in human hepatoma cells.

4. Discussion

N-glycosylation has been shown to be critical for FVII production and secretion in cultured cells (Bolt et al., 2005, 2007). To date, however, the biochemical mechanism underlying the functional importance of *N*-glycans in FVII remains unknown. By site-directed mutagenesis, cell transfection, immune staining, Western blotting, and ELISA, we found that *N*-glycosylation at N360 in the protease domain is most critical for FVII secretion in HEK293 cells. This result is consistent with previous studies by Bolt et al., in which a greater reduction of FVII secretion was detected in transfected COS-7 cells when *N*-glycosylation was abolished at N360, compared with a minor reduction caused by eliminating *N*-glycosylation at N183 (Bolt et al., 2007).

Calnexin is an ER chaperone that assists glycoprotein folding (Caramelo and Parodi, 2008; Helenius and Aebi, 2001). The calnexin function depends on its binding to monoglucosylated oligosaccharides on nascent glycoproteins after triglucosylated *N*-glycans are trimmed by glucosidases I and II. Blocking the conversion of triglucosylated to monoglucosylated oligosaccharides inhibits calnexin-assisted glycoprotein folding (Caramelo and Parodi, 2008; Helenius and Aebi, 2001). As reported recently (Wang et al., 2018), poorly folded serine proteases such as corin, enteropeptidase and prothrombin lacking *N*-glycans in their protease domains were trapped by calnexin and BiP in the ER *via* direct protein-protein interactions. In FVII, *N*-glycosylation site locations differ from those in corin, enteropeptidase and prothrombin. It is unknown if a similar *N*-glycan-calnexin-mediated mechanism occurs in FVII folding in the ER. In this study, we detected ER retention of the N360Q mutant protein by immune staining and increased binding of the mutant protein to calnexin and BiP in co-immunoprecipitation and Western blotting. The results indicate that *N*-glycans at N360 in FVII are critical for calnexin-assisted protein folding. BiP is known for its role in protein folding, quality control and ER exiting (Dudek et al., 2009). Apparently, the poorly folded N360Q mutant protein failed to pass the protein quality control and captured by BiP, resulting in ER retention. In supporting this idea, we found increased binding of WT FVII to calnexin and BiP and reduced FVII secretion in HEK293 and HepG2 cells treated with DNJ, which inhibits the activity of glucosidases I/II and hence the *N*-glycan-calnexin interaction (Saunier et al., 1982). In a previous study Bolt et al. (2008), detected FVII and BiP interactions in transfected CHO cells, supporting a role of BiP in FVII folding and quality control. In that study, however, only WT FVII was studied and there were no findings to indicate a critical role of calnexin in interacting with *N*-glycans on FVII.

In addition to calnexin, calreticulin is another major chaperone involved in glycoprotein folding in the ER (Caramelo and Parodi, 2008; Hebert et al., 1995; Helenius and Aebi, 2001). In our experiments, we did not found increased binding of the N360Q mutant protein to calreticulin. These results are consistent with our previous findings, indicating that calnexin, but not calreticulin, is primarily responsible for the interaction with *N*-glycans in the FVII protease domain, which facilitates FVII folding, ER exiting, and extracellular secretion. Together with our previous findings in corin, enteropeptidase and prothrombin (Wang et al., 2018), our results indicate that *N*-glycans at different locations in the protease domain of various trypsin-like serine proteases play a similar role in calnexin-assisted folding and subsequent extracellular expression.

Mutations in the *F7* gene are common causes of FVII deficiency

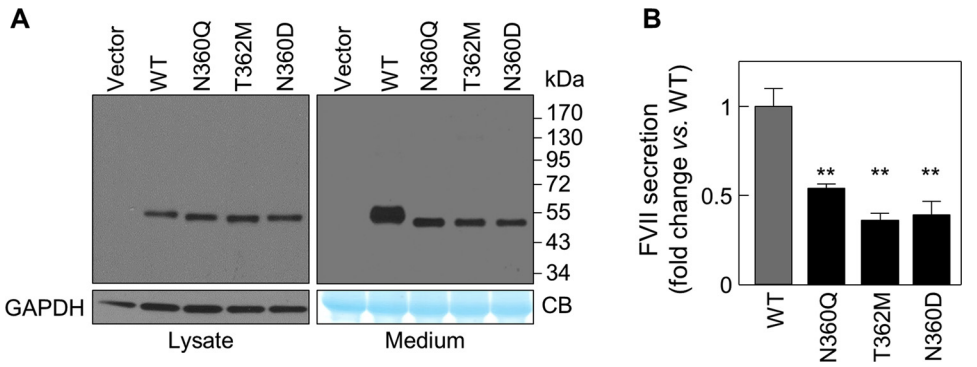

Fig. 8. Production and secretion of FVII mutant proteins in HepG2 cells. (A) Western analysis of FVII WT and mutant proteins in lysates and conditioned media from transfected HepG2 cells. Levels of GAPDH in lysates and a Coomassie blue (CB)-stained non-specific protein in the media were verified. (B) FVII secretion levels were estimated by densitometric analysis of the Western blots. Data are means ± S.E. from 4 independent experiments. **$p < 0.01$ *vs.* WT. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Mandhyan et al., 2010; Mariani and Bernardi, 2009; Perry, 2002). To date, more than 120 *F7* mutations have been reported in patients with FVII deficiency (McVey et al., 2001; Perry, 2002). The consensus sequence for *N*-glycosylation is Asn-X-Thr/Ser, where X can be any amino acid but Pro (Shakin-Eshleman et al., 1996). In principle, genetic mutations altering the *N*-glycosylation sequence in the protease domain of FVII, *i.e.* Asn360-Ile361-Thr362, could impair *N*-glycosylation, thereby reducing FVII secretion *in vivo*. Among the reported *F7* mutations, one mutation, identified in patients with FVII deficiency in Europe and Asia, causes T362 M replacement (Giansily-Blaizot et al., 2004; Mota et al., 2009; Salcioglu et al. 2012). It might not be appreciated that this amino acid replacement alters the *N*-glycosylation site at N360 in FVII, since there was no discussion about such a possibility (Giansily-Blaizot et al., 2004; Mota et al., 2009; Salcioglu et al., 2012). In addition, we found another *F7* variant in the human SNP database (Sherry et al., 2001), which changes Asn360 to Asp, thereby abolishing the *N*-glycosylation site. In this study, we found that T362 M and N360D proteins migrated faster on Western blots and had reduced levels in the conditioned media from transfected HEK293 and HepG2 cells. The results show that naturally occurring *F7* mutations abolishing *N*-glycosylation at N360 impair FVII secretion in cultured cells. Currently, the phenotype of individuals with the mutant *F7* allele causing N360D substitution remains unknown. In individuals with the *F7* mutation causing T362 M substitution, either homozygotes or compound heterozygotes, reduced plasma FVII antigen (~27–70% of pooled normal plasma) and activity (~4–30% of pooled normal plasma) were reported (Giansily-Blaizot et al., 2004; Mota et al., 2009; Salcioglu et al., 2012), which is consistent with our findings of reduced T362 M protein secretion in HEK293 and HepG2 cells. Together, these data indicate that *N*-glycosylation at N360 is important for FVII secretion *in vitro* and *in vivo*.

In summary, FVII is a key clotting protease that undergoes extensive post-translational modifications, including gamma-carboxylation, *N*- and O-glycosylation, and propeptide cleavage (Bolt et al., 2005, 2008; Bolt et al., 2007; Hansson and Stenflo, 2005; Kaufman, 1998). In this study, we studied the biochemical mechanism underlying the functional importance of *N*-glycosylation in FVII expression and secretion. Our results indicate that *N*-glycans at N360 in the protease domain of human FVII interact with calnexin, which is critical for FVII protein folding and subsequent ER exiting. These findings, for the first time, provide biochemical insights into the functional importance of *N*-glycosylation in FVII production and secretion. Moreover, our results help to explain how naturally occurring *F7* mutations abolishing *N*-glycosylation in FVII may impair protein synthesis and secretion, thereby lowering plasma FVII levels in patients. Such a disease mechanism has never been reported in patients with FVII deficiency. Thus, these findings are important for understanding the biology of FVII in health and disease. Currently, recombinant FVIIa is used to treat patients with bleeding disorders (Hedner, 2015; Hoffman, 2018; O'Connell et al., 2008; Sumner et al., 2007). The insights gained from our studies may help to design new strategies to improve the folding and production of recombinant FVII in cell-based systems.

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