Partial rescue of naturally occurring active site factor X variants through decreased inhibition by tissue factor pathway inhibitor and antithrombin

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ESSENTIALS

- Factor (F)X Nottingham and Taunton are two naturally occuring variants associated with bleeding
- Recombinant variants were expressed and functionally characterised for activity and inhibition
- Both variants exhibited reduced proteolytic activity against a peptide as well as prothrombin
- They can be rescued by decreased inhibition by tissue factor pathway inhibitor and antithrombin

ABSTRACT

Background: Activated coagulation factor X (FXa) is the serine protease component of prothrombinase, the physiological activator of prothrombin. FX Nottingham (A404T) and Taunton (R405G) are two naturally occurring mutations, identified in families with a bleeding phenotype.

Objective: To functionally characterise these FX variants.

Methods: The activity and inhibition of recombinant FX variants was quantified in plasma based and pure component assays.

Results: The prothrombin times in FX-depleted plasma supplemented with FX Nottingham and Taunton were greatly increased compared to wild-type (WT) FX. Kinetic investigations of activated variants in the prothrombinase complex showed k_{cat}/K_m, reduced ~50-fold and ~5-fold, respectively, explaining the prolonged PT times. The substituted residues are located in the protease domain Na^+-binding loop, important for the activity of FXa, as well as its inhibition. Both FXa Nottingham and Taunton showed reduced affinity for Na^+. Plasma-based thrombin generation assays triggered with 1pM tissue factor (TF) demonstrated only small differences in activities compared to WT FX, but large reductions at 10pM TF. Severely reduced inhibition of both FXa Nottingham and Taunton by tissue factor pathway inhibitor (TFPI) and antithrombin (AT), was shown in pure-component FXa inhibition assays. FXa Nottingham and Taunton produced higher amounts of thrombin than WT FXa in pure-component prothrombinase assays in the presence of TFPI and AT, explaining the results from the plasma-based assay.

Conclusions: FX Nottingham and Taunton both display decreased proteolytic activity. However, their reduced activity in plasma triggered by low TF can be rescued by decreased inhibition by the natural FXa inhibitors, TFPI and AT.

INTRODUCTION

Factor (F)X is a vitamin K dependent serine protease which plays a central role in coagulation. The activated form of FX (FXa), is the serine protease component of the prothrombinase complex, the only known physiological activator of prothrombin. FXa function is therefore essential for the formation of a stabilised clot.

FX is activated through proteolytic cleavage of the Arg194-Ile195 scissile-bond (c16-c17, in the chymotrypsin numbering system) by the tissue factor (TF)-FVIIa complex in the extrinsic pathway and the FIXa-FVIIIa complex in the intrinsic pathway. During activation the new N-terminus, Ile195(c16), forms a salt bridge with Asp378(c194), leading to conformational changes of the serine protease domain. This molecular transition changes the folding of the S1-specificity pocket which controls substrate recognition and binding [1, 2] and enables prothrombin cleavage by the active site catalytic triad of His236(c57), Asp282(c102) and Ser379(c195). Together with its cofactor FVa, FXa binds to negatively charged membranes, forming the prothrombinase complex [3, 4]. The prothrombinase complex rapidly activates prothrombin to thrombin, more than 5 orders of magnitude more efficiently than FXa alone [5, 6]. Like all other coagulation proteases, the activity of FXa is strictly regulated. In addition to binding prothrombin, the FX active centre also binds natural FXa inhibitors, including tissue factor pathway inhibitor (TFPI) [7, 8] and antithrombin [9], both of which are essential for regulation of FXa activity and coagulation.

In 2001 Deam and colleagues reported two novel mutations in the FX gene found in families with clinically manifest bleeding [10]. Single base pair mutations resulted in amino acid substitutions, A404(c221)T or R405(c222)G, the variants termed FX Nottingham and Taunton, respectively. The mutation in FX segregated with a bleeding tendency in these families. While the substitutions did not influence the FX antigen levels, the FX coagulant activity was reduced in heterozygous individuals to ~40-60%, which would usually be regarded as sufficient for normal haemostasis. Only one homozygous individual, carrying the FX Nottingham mutation, has been found and it was shown that its substitution essentially abolished any FX activity (< 3%). Interestingly, FX residues Ala404(c221) and Arg405(c222) are both part of one of the loop segments that contribute to a Na^+-binding site in the FXa serine protease domain [11]. Na^+-binding is essential for correct folding around the S1 pocket of the FXa serine protease domain and has been shown to enhance the catalytic activity of FXa [11-13].

In this study we have prepared the two FX variants, FX Nottingham and FX Taunton, using a mammalian expression system and assessed their functional activities. We have confirmed that both variants have severely reduced catalytic efficiency for prothrombin activation. However, they retain unexpectedly high procoagulant activity in plasma-based thombin generation assays triggered with

low tissue factor (TF). This is due to decreased inhibition of the variants by the natural inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin (AT).

METHODS

Generation and expression of recombinant FX

The FX mutations, A404T and R405G, were generated through site directed mutagenesis (KOD Hot Start DNA polymerase kit, Novagen) using the wild-type (WT) FX cDNA in the pCMV4 vector [14] (a kind gift from Prof. Rodney Camire, University of Pennsylvania) as template and mutagenic oligonucleotide primers (Life Technologies). All mutations were verified by DNA sequencing.

The FX vectors were stably transfected into HEK293 cells (ATCC) according to the supplier's instructions. Cotransfection with a plasmid containing neomycin resistance gene (pdDNA3.1) was performed at a 1:4 molar ratio (pCMV-4-ss-pro-II-FX/pcDNA3.1) and selected using G418. The FX variants were expressed in OptiMEM I (GIBCO, Invitrogen) supplemented with 10 µg/ml vitamin K (Roche Products Ltd.) for 2-3 days before harvesting and purification.

Purification of FX(a)

To specifically purify fully γ-carboxylated FX, FX was initially purified by barium citrate precipitation [15] followed by anion-exchange chromatography. The partially purified protein was applied on a HiTrap DEAE fast flow column (5 ml, GE healthcare) equilibrated with 20mM Tris pH 7.5, 150mM NaCl (TBS). After washing with the same buffer, FX was eluted with a 35 ml linear $CaCl_2$ gradient (0-30mM). Fractions were analysed using SDS-PAGE and those containing pure FX were pooled and concentrated. Test experiments showed activity of WT FX similar to plasma derived FX (Enzyme research laboratories) in plasma-based assays (data not shown), confirming that the purification protocol results in fully γ-carboxylated FX.

In a reaction volume of 800 µl, 10µM FX was incubated with 50nM FX activating enzyme (RVV-X; Abcam) in TBS for one hour at room temperature. As previously described [14, 16, 17], even at longer incubation times full activation was not accomplished (data not shown). The FXa was separated from the unactivated fraction by size exclusion chromatography, essentially as described previously [16]. Briefly, the FX/FXa was loaded on a Sephacryl S100 16/60 column (GE Healthcare) and eluted using TBS. Fractions were screened for FXa activity using S-2765 (Chromogenix). Fractions containing FXa were tested for purity using heavily loaded SDS-PAGE and only the fractions where no FX was detected were pooled and concentrated.

The FX(a) concentration was determined by absorption at 280nm using extinction coefficient (E1%, 1cm) of 11.6 [18]. The concentration of WT FXa was confirmed by active site titration with TFPI (data not shown).

Prothrombin and activated partial thromboplastin time

The prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined using Neoplastine-R and Cephascreen (Stago), respectively, using a STA R Max2 analyser, according to the manufacturer's instructions.

Phospholipid vesicle preparation

Phospholipids (Avanti Polar Lipids) 1,2 -Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-Dioleoyl-sn-glycero-3-phosphoserine (DOPS), and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were mixed in required molar ratio and extruded as previously described [19].

Activation of FX by TF/FVIIa

TF (2pM) was incubated with 1nM FVIIa (NovoSeven) in the presence of 15µM phospholipids (DOPS/DOPC/DOPE, 20:60:20) at 37°C in TBS supplemented with 0.5% BSA and 3mM $CaCl_2$. FXa generation was started by addition of 5nM FX. After different time intervals (0-210 min), aliquots were taken from the reaction mixture and diluted 3-fold in ice-cold TBS supplemented with 0.5% BSA and 20mM EDTA. Generated FXa was detected using the chromogenic substrate S-2765 (200µM; Chromogenix). The kinetic efficiency (k_{cat}/K_m) of the reaction was derived by nonlinear fitting using pseudofirst-order kinetics: $Y = Y_{max} (1 - e^{-k*t})$, where *t* is the reaction time, *Y* is the amount of FXa generated at any given time point, and *k* is the observed rate of proteolysis (k_{cat}/K_M), multiplied by the enzyme concentration (E_0). Full activation of FX to FXa (Y_{max}) was assumed after 210 min and the FXa activity detected at each time point was normalised to the activity achieved after 210 min reaction time. The equation can therefore be rewritten as $Y = 1 - e^{-kcat/Km*E0*t}$.

Determination of Kinetic Parameters for Peptidyl Substrate Hydrolysis

All kinetic measurements were performed in TBS supplemented with 0.1% PEG-6000, 2mM $CaCl_2$ and 0.5% BSA. The kinetic parameters of peptidyl substrate hydrolysis (S-2765) were measured at RT using increasing concentrations of substrate (0-1000µM for WT FXa and 0-5000µM for the mutants) and initiated with FXa (1nM WT FXa, 5nM FXa Nottingham or 3nM FXa Taunton). Alternatively, the reaction was initiated using FXa assembled into prothrombinase (3nM WT FXa/5nM FXa Nottingham/3nM FXa

Taunton, 30nM FVa (Haematologic Technologies Inc.), and 50µM phospholipids (DOPS/DOPC/DOPE, 20:60:20)) which was added to S-2765 (0-2000µM for WT FXa and 0-5000µM for the variants).

Influence of Na^+ on activities

Cleavage of the substrate S-2765 at RT was measured using 1nM WT FXa [17], 5nM FXa Taunton or 50nM FXa Nottingham in the presence of increasing (0-450mM) concentrations of Na^+. LiCl was used as the compensating salt to adjust for the changes in ionic strength.

Kinetics of prothrombin activation

The kinetic parameters of prothrombinase-catalysed prothrombin activation were determined at 37°C in TBS supplemented with 2mM $CaCl_2$ and 0.05% ovalbumin by measuring the initial rate of thrombin formation at increasing concentrations of prothrombin [6]. Assay mixtures contained 50µM phospholipids (DOPS/DOPC, 10:90), FXa (0.1nM WT FXa or 0.4nM mutant FXa) and FVa (20nM or 0-40nM). The reaction was initiated with prothrombin (0-2µM or 2µM; Enzyme Research Laboratories). Aliquots of the reaction mixture were quenched during the initial period of the reaction (at 0, 30, 60, 90 and 120 s).

Prothrombin activation was also assessed in the presence or absence of inhibitors. Assay mixtures contained 50µM phospholipids (DOPS/DOPC/DOPE, 20:60:20), FXa (0.1nM) and FVa (20nM) in the presence or absence of 0.25nM TFPI-100nM protein S and/or 20nM AT-20µg/ml fondaparinux. The reaction was initiated with prothrombin (2µM). Aliquots of the reaction mixture were quenched at time points up to 8 minutes.

The amount of thrombin generated in each sample was determined using the chromogenic substrate S-2238 and compared to a standard curve generated from known amounts of thrombin.

Thrombin generation assay

The procoagulant activities of the FX variants and their inhibition by TFPI or antithrombin in plasma were assessed by calibrated automated thrombography (CAT) using a Fluoroscan Ascent FL plate reader (Thermo Labsystem) and Thrombinoscope software (Synapse BV) [19, 20]. Thrombin generation was initiated in FX-depleted plasma (Affinity Biologicals) supplemented with physiological concentrations of FX (136nM) by 1, 4 or 10pM TF, 4µM phospholipid vesicles (DOPS/DOPC/DOPE, 20:60:20) and 16.6mM $CaCl_2$. To test the ability of the FX variants to be inhibited, inhibitory antibodies against TFPI (a mix of anti-TFPI Kunitz domain 1, anti-TFPI Kunitz domain 2 and anti-TFPI C-terminus; 120nM of each; Sanquin) or anti-antithrombin (16.7µM; Enzyme Research Laboratories) were added.

To inhibit contact activation, 65 μg/ml of plasma corn trypsin inhibitor was added. Thrombin generation was monitored using 0.42mM of the fluorogenic substrate Z-GlyArg-AMC-HCl (Bachem).

TFPI and antithrombin-mediated inhibition of FXa

For inhibition by TFPI, TFPI (0, 4 or 8nM) was mixed with 20μM phospholipids (DOPS/DOPC/DOPE, 20:60:20), 5mM CaCl$_2$ and 400μM S-2765 in the presence or absence of 100nM protein S at RT. Protein S was expressed and purified as previously described [19]. For inhibition by antithrombin, antithrombin (0-200nM; Enzyme Research Laboratories) was mixed with 20μg/ml fondaparinux (determined to be saturating), 5mM CaCl$_2$ and 400μM S-2765. The reactions were initiated by the addition of FXa (1nM) and the FXa activity was measured by the cleavage of S-2765 at 405nm for 30-120 minutes.

For FXa inhibition by antithrombin, the first order constant (k$_{obs}$) of inhibition was determined at various antithrombin concentrations as previously described by Griffith [21]. The pseudo first and second order rate constants, k' and k'', were derived from k$_{obs}$ according to:

$$k' = k'' \times [AT] = (1 + \frac{[S]}{K_m}) \times k_{obs}$$

RESULTS

FX Nottingham and Taunton variants and clotting times, PT and APTT

Plasma from patients carrying the FX Nottingham and FX Taunton variants have previously been shown to display decreased FX activity in assays using both the extrinsic and intrinsic pathway [10]. To confirm these findings, FX-depleted plasma was supplemented with physiological concentrations of recombinant WT FX, FX Nottingham or FX Taunton and the prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured. As expected, the PT and APTT were both prolonged in plasma supplemented with the two FX mutants compared to that supplemented with WT FX. The PT was increased to 109±4.71 and 61.3±6.74 seconds for the Nottingham and Taunton variants, respectively, compared to 14.1±0.49 seconds for WT FX (Figure 1A). Similarly, the APTT was increased to 78.9±8.44 and 52.2±4.86 seconds for FX Nottingham and Taunton, respectively, compared to 40.0±0.24 seconds for WT FX (Figure 1B).

Activation of FX Nottingham and Taunton by TF/FVIIa

Following the results from the PT and APTT assays, their procoagulant activities were studied in pure-component assays. The activation of the two FX variants by TF-FVIIa was first examined. A fixed amount of TF-FVIIa was used to activate the preparations over 210 minutes. Sub-samples were taken

at various time points and the amount of FXa generated was measured using a chromogenic substrate. The FXa activities detected at all time points were normalised to those measured in the samples stopped after 210 minutes (complete) activation (Figure S1). One advantage of this approach is that the efficiency of activation could be quantified independently of potential differences in proteolytic activities of the FXa variants. The catalytic efficiencies of FX activation by TF-FVIIa were similar for all three recombinant proteins, with only a small significant decrease detected for FX Taunton compared to WT FX; 137 ± 10.3 compared to 169 ± 31.9 x10^6M^{-1}s^{-1}. The k_{cat}/K_m for activation of FX Nottingham was similar to WT FX at 176 ± 72.8 x10^6M^{-1}s^{-1}.

Quantification of peptidolytic activities of the FXa preparations

While little differences were detected in the activation of FX Nottingham and Taunton by TF-FVIIa, these experiments showed apparent decreased peptidolytic activities (Figure S1). The initial rate of peptidolytic substrate cleavage by FXa was measured at increasing substrate concentrations (Figure S2A-B and Table 1). While the peptidolytic activity of WT FXa was comparable to that of plasma derived FXa and to results described previously [17, 22], the activities determined for FXa Nottingham and Taunton were severely to moderately reduced with overall 140- and 12-fold decreases in k_{cat}/K_m of FXa Nottingham and Taunton, respectively, compared to WT FXa (Table 1). Several recombinant FXa variants with reduced activities caused by mutations in the FXa serine protease domain have been shown to be rescued by incorporation into the prothrombinase complex [12, 17, 23]. The peptidolytic activities were therefore also estimated in the presence of phospholipid membranes and FVa. The catalytic efficiency of WT FXa was, as expected, half of that quantified in the absence of FVa and phospholipids [17, 22]. The activities detected for FXa Nottingham and Taunton were unaffected by the presence of phospholipids and FVa, and remained severely or moderately reduced, respectively, compared to WT FXa (Figure S2C-D, Table 1). These results confirm loss of overall protease activity. They also demonstrated that the loss of activity was not recovered by the presence of the cofactor, FVa.

Interaction with Na$^+$

Ala404(c221) and Arg405(c222) are both part of one of the loop segments (c221-225), involved in Na$^+$-binding within the serine protease domain. It has previously been established that Na$^+$-binding is essential for activity of FXa [24]. It was considered possible that impaired protease activities of FXa Nottingham and Taunton were caused by disruption of Na$^+$-binding within the protease domain. To determine the functional affinities of FXa Nottingham and FXa Taunton for Na$^+$, the initial rate of peptide proteolysis at different Na$^+$ (in the presence of CaCl$_2$) were determined. It was estimated that

WT FXa bound to Na$^+$ with a K_d of ~60mM (Figure 1C). This is in the range of previously estimated affinities [13, 17, 23]. In contrast, and as expected, FXa Taunton and Nottingham showed much lower affinities. Even at Na$^+$ concentrations as high as 450mM neither of the variants exhibited saturation for functional Na$^+$-binding (Figure 1C-D). These results strongly suggest that the lack of peptidolytic activity of the variants is associated with lack of Na$^+$-binding at physiological concentrations (~140nM).

Kinetics of prothrombin activation

The initial velocity of prothrombin cleavage to thrombin when the FXa was incorporated in the prothrombinase complex were next determined. Consistent with the reduced peptidolytic activities, both FXa Nottingham and FXa Taunton showed reduced proteolytic activities against prothrombin, with the largest influence being the decrease in turn-over rate (k_{cat}) for both variants (Table 1 and Figure 1E). The k_{cat} was reduced by ~19-fold for FXa Nottingham and ~4-fold for FXa Taunton, compared to WT FXa. In contrast, the effect of the mutations upon the variants ability to bind prothrombin was relatively mild. FXa Nottingham bound prothrombin with a lower affinity (~3-fold) compared to WT FXa whereas no change was observed for FXa Taunton. Combined, the k_{cat}/K_m was reduced to 4.16 and 30.1 x10^{6}M^{-1}s^{-1} for FXa Nottingham and Taunton, respectively, compared to 181 x10^{6}M^{-1}s^{-1} for WT FXa.

Next, we investigated the prothrombin activation of the FXa variants at increasing FVa concentrations, to assess whether FXa Nottingham and Taunton are incorporated into a prothrombinase complex (Figure 1F). While the affinity of WT FXa for FVa was estimated to be 4.66±1.81nM, which is in similar range to those previously described [12], the affinities of FXa Nottingham and Taunton were reduced. The affinity of FXa Taunton was ~3-fold reduced with a K_d of 13.8±3.28nM while the affinity of FXa Nottingham for FVa was too low to determine. This suggests that not all FXa Nottingham and Taunton were incorporated in a prothrombinase complex at 20nM FVa, explaining further their reduced activities (Figure 1E and Table 1).

Global evaluation of procoagulant activities of FX Nottingham and Taunton determined with CAT

The three different FX variants were added to FX-depleted plasma at physiological concentrations and thrombin generation initiated using increasing concentrations of TF (1-10pM) (Figure 2). No thrombin generation was observed in the absence of FX, confirming complete depletion of the plasma (Figure S3). While the peak heights generated in the plasma supplemented with FX Nottingham and Taunton were significantly lower than in that supplemented with WT FX after initiation with 4 or 10pM TF, the differences were far less pronounced than the differences observed in the PT assays (Figure 1A). However, most striking, when the coagulation was initiated with low TF (1pM) the peak heights

generated in plasma supplemented with FX Nottingham and Taunton were higher or the same as those generated in the presence of WT FX (Figure 2). Also the endogenous thrombin potential (ETP) was similar in plasma supplemented with FX Taunton to those observed in the presence of WT FX. Only plasma supplemented with FX Nottingham showed mildly reduced ETP when thrombin generation was initiated by 4 or 10pM TF (Figure 2F). However, the lag times were prolonged in plasma supplemented with both variants at high (4 and 10pM) TF stimulation which is in agreement with the prolonged clotting times observed in the PT and APTT assays and a reduced procoagulant FXa activity (Figure 2E vs Figure 1A-B).

To confirm that the high thrombin generation in plasma supplemented with FX Nottingham and Taunton, compared to that of WT FX, was independent on activation by TF-FVIIa, thrombin generation was also initiated by addition of 0.1nM of respective FXa variant (Figure S4). In these experiments the rescued activity of FX Nottingham and Taunton was even more apparent with a significantly increased peak height and ETP, compared to WT FX.

Inhibition of FX Nottingham and Taunton by antithrombin and TFPI

One important difference between the PT/APTT assays compared to the CAT assay is that the former are designed to be initiated by high concentrations of TF or contact activators such as ellagic acid or kaolin, respectively. Additionally, the CAT assays, in particular when initiated at low TF, are sensitive to the natural inhibitors of FXa, while the PT and APTT assays are relatively insensitive. Na^+-binding is not only essential for substrate recognition, but also for recognition by TFPI and antithrombin [12, 13]. It was therefore of interest to investigate the abilities of TFPI and antithrombin to inhibit the FX variants. This was initially done using CAT where thrombin generation was initiated using 1pM TF. Inhibitory antibodies against TFPI and antithrombin were added to FX depleted plasma, supplemented with physiological concentrations of WT FX, FX Nottingham or FX Taunton (Figure 3). It is worth noting that while the antibodies directed against TFPI have been shown to cause complete inhibition [20], due to the high concentrations of AT, full inhibition by AT cannot be assumed. In the absence of antibodies, similar, or moderately increased levels of thrombin generation were observed in the presence of FX Nottingham and Taunton, compared to WT FX. Inhibition of TFPI and antithrombin dramatically increased the peak height observed in the plasma supplemented with WT FX (Figure 3A). The peak height increased also in the plasma supplemented with FX Nottingham and Taunton, however, the effect was relatively moderate compared to WT FX (Figure 3B-C). In fact, both TFPI and antithrombin had to be inhibited for the increase in peak height to be significant in the plasma supplemented with FX Taunton. In contrast, a clear increase in ETP was observed for all FX variants, after addition of inhibitor antibodies, while little difference was observed between the variants in each

condition. This is of particular importance since blocking AT influences the inhibition of FIXa and thrombin activities as well as that of FXa, suggesting that the effect upon FXa inhibition by AT may be larger than visually observed in these experiments.

To investigate these findings further, direct inhibition of WT FXa, FXa Nottingham and FXa Taunton by TFPI was next evaluated by pure-component FXa inhibition assays. WT FXa was efficiently inhibited by TFPI, as expected. The inhibition was further enhanced by the TFPI cofactor, protein S (Figure 4A). In contrast, very little inhibition of FXa Taunton or FXa Nottingham was observed, even with prolonged incubation (Figure 4B-C). Similar experiments were also performed to assess inhibition of the FXa variants by antithrombin. The advantage of using a pure component assay was that it enabled us to investigate the inhibition of FXa by AT without any confounding effects by other AT targets. For this, the pseudo first-order (k'; Figure 5) and second order (k'') rate constants of inhibition were determined. The k'' for AT inhibition of FXa Nottingham and Taunton were both reduced at 0.39 and 2.04 x10^6M^{-1}min^{-1}, respectively, compared to 21.1 x10^6M^{-1}min^{-1} for WT FXa.

To assess whether the reduced inhibition could rescue the activity of FXa Nottingham and Taunton we performed prothrombinase assays in the presence and absence of TFPI and AT. While prothrombin activation by WT FXa was reduced in the presence of 0.25nM TFPI and 100nM protein S, it was almost abolished in the presence of 20nM AT and 20µg/ml fondaparinux (Figure 6A). In contrast, the inhibition of FXa Taunton was moderately to severely reduced whereas essentially no inhibition of FXa Nottingham could be detected (Figure 6B-C). Importantly, in the presence of both TFPI-protein S and AT-fondaparinux, both variants generated more thrombin than WT FXa (Figure 6D).

DISCUSSION

Mutations causing FX deficiency often contribute to bleeding observed within the presenting families [25-29]. In this study we have investigated two naturally occurring FX variants, FX Nottingham and FX Taunton, both of which arise from a single amino acid substitution in the serine protease domain (Figure 7A). In contrast to many FX mutations, these do not affect the expression of FX, and all affected individuals have FX antigen levels that are normal [10]. We have aimed to characterise these variants and to determine the molecular mechanisms that might be implicated in clinical presentation.

We have generated recombinant WT FX, FX Nottingham and FX Taunton and compared their activation, activities and inhibition. As expected from the results obtained from examination of the patient plasmas [10], we found that the substitutions caused increases in PT and APTT (Figure 1A-B). Since FX Nottingham and FX Taunton both were activated by TF-FVIIa as efficiently as WT FX (Figure S1), the prolonged PT and APTT therefore appeared to be due to impaired activities of the generated

FXa preparations. This was confirmed in pure component assays where both variants showed a severe reduction in activity towards a peptide substrate as well as towards the physiological substrate of FX, prothrombin (Figures S2 and 1E-F).

Many crystal structures of FX(a) have been generated [30-32]. These have led to development of FXa inhibitors which have proven to be efficient treatments against deep venous thrombosis [33, 34]. The crystal structures of the FX serine protease domain have also helped define the Na^+ binding site, which is known to be important for its activity, and overall substrate recognition by FX [12, 13, 23]. Importantly, the amino acids which are substituted in FX Nottingham and Taunton are both in very close proximity to the bound Na^+ [11, 32]. Indeed, Arg405(c222), which is substituted in FX Taunton, plays a direct role in Na^+ coordination (Figure 7A) [11]. While recombinant FX variants, such as FX Y409(c225)P, have been generated to study the functional impact of disruption of the Na^+-binding [12], the present study is the first of natural variants, illustrating the physiological importance of coordination of Na^+. It was clear from our assessment of functional Na^+-binding (Figure 1C-D) that both FX Nottingham and Taunton have severely reduced affinity for Na^+. The decreased affinity for Na^+ of FX Taunton is expected, due to the direct role of Arg405(c222) in its coordination. Decreased affinity of FX Nottingham may arise because of its adjacent position. Proximity of the residue change and the substitution of alanine to the more bulky threonine might be expected to alter conformation and thereby disrupt Na^+-coordination. However, while the peptidolytic activity of FXa Y408(c225)P was partially rescued by FVa [12], no such rescue was observed for FXa Nottingham and Taunton (Table 1). This could potentially be due to their reduced affinities for FVa (Figure 1F). The substitution of Tyr408(c225) into a proline is predicted to shift the backbone so that the oxygen atom of Lys408(c224) points away from the Na^+ and so that it no longer can take part in its coordination [11, 35, 36]. It is possible that the positioning of Ala404(c221) and Arg405(c222) leads to a more critical structural change which cannot be restored by FVa binding. This may also explain the reduced activities observed for FXa Nottingham and Taunton in the complete absence of Na^+ (Figures 1 C and D). While it is unlikely that the Na^+-levels would fluctuate sufficiently *in vivo* for the binding of Na^+ to cause any functional regulation of FX, the loss of cation coordination in these two variants highlights the functional importance of Na^+ binding for FXa protease activity.

It is noteworthy that the individual who is homozygous for the FX Nottingham mutation showed a dramatic reduction of FX activity: she was diagnosed after suffering from menorrhagia. Severe FX deficiency is commonly associated with a severe bleeding phenotype, often apparent during early childhood [25]. The results from the present study could potentially explain this late and relatively mild presentation of bleeding. Whilst FX Nottingham and Taunton showed minimal activity in PT and APTT assays, they still retained almost normal activity when added to FX-depleted plasma in CAT

assays stimulated with low TF (1pM; Figure 2). These observations clearly highlight the difference in read outs from these three different assays. The PT and APTT assays give an accurate and unimpeded measurement of the FX protease functional deficit in plasma. However, they give an incomplete global picture as they are insensitive to the activity of the natural FX inhibitors, TFPI and antithrombin, which strongly regulate FXa activity. The conditions of the CAT assay can be adapted to reflect integrated haemostasis. When initiated at low TF, the assay is highly sensitive to the inhibitory pathways, and may better reflect the FXa protease activity and its inhibition. When thrombin generation was initiated with high TF concentrations, the results corresponded well to the results obtained from the PT and APTT assays, with significantly reduced peak heights and prolonged lag times of both variants (Figure 2D-E). In contrast, when initiated at low TF concentrations, the activities of FX Nottingham or Taunton were similar, or even higher, than those of WT FX (Figure 2). To clarify the role of plasma inhibitors, TFPI and antithrombin were inhibited using inhibitory antibodies in FX-depleted plasma supplemented with physiological concentrations of WT FX, FX Nottingham or FX Taunton (Figure 3). While the thrombin generation peak heights increased dramatically in plasma supplemented with WT FX when TFPI and antithrombin were inhibited, the increases observed in plasma supplemented with the variants were relatively moderate. The results suggested a potentially diminished role of these natural inhibitors against FX Nottingham and Taunton, compared with WT. However, it should be appreciated that blocking AT influences the inhibition of FIXa and thrombin activities as well as that of FXa. TFPI and antithrombin-mediated inhibition of FXa were therefore studied in further detail in pure-component FXa inhibition assays. The inhibition of FXa Taunton by both inhibitors was severely reduced and the inhibition of FXa Nottingham was essentially abolished (Figures 4 and 5). Pure-component prothrombinase assays in the presence of inhibitors resulted in higher amounts of thrombin generation by FXa Nottingham and Taunton than by WT FXa (Figure 6). These results can explain the relatively high thrombin generation in plasma at low TF (Figure 2), where efficient inhibition of WT FXa is expected. Similarly, the lack of inhibition of FXa Nottingham and Taunton is likely to strongly enhance their activities during the initiation phase of coagulation where TFPI and AT both play major roles [37]. A model of TFPI Kunitz domain 2 docked onto FXa, Figure 7B, and the crystal structure of the antithrombin-FXa complex, Figure 7 C, illustrates their close relationship to the inhibitor docking sites. Protein Z-dependent protease inhibitor (ZPI), together with its cofactor, protein Z (PZ), is also a natural FXa inhibitor [38]. It is not known whether binding of ZPI to FXa is influenced by Na$^+$ coordination within the FXa serine protease domain. However, it is possible that reduced inhibition of FXa Nottingham and Taunton by ZPI-PZ further restores the activity of these FX variants at low procoagulant stimuli.

In contrast to the relatively mild phenotype observed in the individual who is homozygous for the FX Nottingham mutation, the bleeding phenotype in the individuals who are heterozygous for the FX Taunton mutation cannot readily be explained by the present findings. If competition between FX Taunton and the normal FX, either towards prothrombin/FVa or inhibitors, contributes towards a very large reduction in overall FXa activity in plasma [10], this has proven difficult to demonstrate *in vitro* (results not shown). However, there are high levels of TFPI present on the endothelium and the presence of heparan sulphate, which functions as cofactor for antithrombin. These factors, combined with global haemostasis involving flow, whole blood, together with the preference of natural inhibitors for WT FXa, are difficult to replicate *in vitro*. Finally, bleeding may arise from multiple and complex haemostatic abnormalities. Disorders unrelated to FX may also contribute to the clinical phenotype.

This work illustrates the importance of investigating protease activity of coagulation variants in a context that closely relates to that experienced *in vivo*. Assays should be employed that not only detect any altered protease domain function against natural substrates, but also assesses the influence of naturally occurring inhibitors. Partial rescue of active site-mediated defects by impaired inhibitor action against a variant protease demonstrates an additional complexity to clinical management of coagulation protease variants.

AUTHORSHIP CONTRIBUTIONS

Contributions: J.A. designed the research, analysed the results and wrote the paper. J.A., M.G. and J.T. performed the experiments. M.L. and D.A.L. designed the research and revised the manuscript.

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Table 1. Kinetic constants for peptidyl substrate and prothrombin cleavage.

FXa variant	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^6 M^{-1}s^{-1}$)
Peptidolytic activity			
Free FXa			
pFXa	65.5 ± 18.9	200 ± 17.9	3.26 ± 0.96
WT FXa	71.3 ± 11.6	227 ± 25.6	3.27 ± 0.81
FXa Nottingham	1890 ± 182****	43.7 ± 2.47****	0.023 ± 0.002**
FXa Taunton	575 ± 85.1****	156 ± 16.2**	0.270 ± 0.014**
PTase (FXa, FVa, PLs)			
WT FXa	110 ± 8.70	182 ± 21.1	1.67 ± 0.24
FXa Nottingham	1400 ± 156***	43.7 ± 12.9***	0.031 ± 0.010***
FXa Taunton	479 ± 35.6***	165 ± 3.98	0.350 ± 0.018***
Activation of prothrombin by PTase			
WT FXa	0.400 ± 0.065	70.8 ± 5.06	181 ± 21.2
FXa Nottingham	1.41 ± 0.43**	5.49 ± 1.48****	4.16 ± 1.77****
FXa Taunton	0.580 ± 0.198	16.9 ± 4.02****	30.1 ± 3.27****

The results were obtained from 3-4 independent experiments and are expressed as mean ± SD.
pFXa, plasma derived FXa; N.A., not applicable.

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared to WT FXa according to students t-test.

FIGURE LEGENDS

Figure 1. Procoagulant activities of FX variants. (A-B) The procoagulant activities of WT FX, FX Nottingham and Taunton were assessed in FX-depleted plasma using prothrombin time (PT) and activated thromboplastin time (APTT). PT (A) and APTT (B) were determined in FX-depleted plasma supplemented with 136nM FX. The reactions were initiated using Neoplastine-R or Cephascreen for PT and APTT, respectively (n=4). (C-D) To determine the functional affinities of all three preparations towards Na^+, the initial velocity of S-2765 conversion (100µM) by FXa (WT: 1nM; Nottingham: 50nM; Taunton: 5nM) was determined as a function of the NaCl concentration (0-450mM). Data for WT FXa, FXa Nottingham and FXa Taunton are all shown in (C). Data for FX Nottingham are also shown with a shortened y-axis (D). (E-F) Procoagulant activities of the FX variants in pure-component prothrombinase assays. (E) To assess the ability of the FXa mutants to convert prothrombin to thrombin, reaction mixtures containing, WT FXa (0.1nM), 2nM FXa Nottingham or 0.4nM FXa Taunton, phospholipids (50µM) and 20nM FVa were incubated at 37°C. The reaction was initiated through addition of increasing concentrations of prothrombin (0-2µM) and aliquots of the reaction mixture was quenched during the initial rate of the reaction (at 0, 30, 60, 90 and 120 s). The amount of thrombin generated in each sample was determined using the chromogenic substrate S-2238, compared to a standard curve generated from known amounts of thrombin. The initial rates of prothrombin proteolysis were divided by the enzyme concentration. (F) Prothrombin activation was also assessed at increasing concentrations of FVa (0-20nM). These were essentially run as those presented in panel E at a fixed concentration of prothrombin (2µM). (C-F) All results are presented as mean ± SD (n=3).

Figure 2. Procoagulant activities of FX variants in plasma measured by CAT. The procoagulant activities of WT FX, FX Nottingham and Taunton were assessed in FX-depleted plasma using calibrated automated thrombography (CAT). Thrombin generation was measured in FX-depleted plasma supplemented with 136nM recombinant WT FX (A), FX Nottingham (B) or FX Taunton (C). Coagulation was initiated using increasing concentrations of TF (1-10pM) in the presence of 4µM phospholipids. Representative experiments are shown (n=3). (D-F) Peak thrombin (D), lag time (E) and ETP (F) was plotted at the difference TF concentrations. Results are presented as mean ± SD (n=3). *$p<0.05$, **$p<0.01$, ***$p<0.001$ according to students t-tests. The statistically significant differences given are compared to WT FX, initiated with the same TF concentration.

Figure 3. Inhibition of FX variants by TFPI and antithrombin during thrombin generation. Thrombin generation was measured in 60µl FX-depleted plasma supplemented with 136nM recombinant WT FX

(A), FX Nottingham (B) or FX Taunton (C) in the presence or absence of inhibitory antibodies against TFPI or antithrombin. Coagulation was initiated using 1pM in the presence of 4μM phospholipids. Representative experiments are shown (n=3). (D-F) Peak thrombin (D), lag time (E) and ETP (F) was plotted for each condition investigated. Results are presented as mean ± SD (n=3). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ according to students t-tests. The statistically significant differences given are compared to the absence of antibodies, unless indicated otherwise.

Figure 4. Inhibition of FXa variants by TFPI in direct FXa inhibition assays. Conversion of 400μM S-2765 by 1nM FXa in the presence of 20μM phospholipids was monitored by the absorbance at 405nm in the presence or absence of 4 or 8nM TFPI and in the presence or absence of 100nM protein S. The reaction was started upon addition of FXa. Results from a representative experiment is shown (n=3). PS; protein S.

Figure 5. Inhibition of FX variants by antithrombin in direct FXa inhibition assays. Conversion of 400μM S-2765 by 1nM FXa was monitored by the absorbance at 405nm in the presence or absence of 0-200nM AT in the presence of 20μg/ml fondaparinux. The reaction was started upon addition of FXa. The pseudo first order rate constants (k') were calculated according to Griffith[21] and are presented as a function of AT. Results are presented as mean ± SD (n=3).

Figure 6. Prothrombin activation by FX variants in the presence and absence of inhibitors. To assess the ability of the FXa variants to convert prothrombin to thrombin in the presence or absence of TFPI and AT, reaction mixtures containing, FXa (0.1nM), phospholipids (50μM) and 20nM FVa were incubated at 37°C in the presence or absence of 0.25nM TFPI-100nM protein S and 20nM AT-20 μg/ml fondaparinux. The reaction was initiated through addition of prothrombin (2μM) and aliquots of the reaction was quenched at various time points (at 0, 0.5, 1, 2.5, 5 and 8 mins). The amount of thrombin generated in each sample was determined using the chromogenic substrate S-2238, compared to a standard curve generated from known amounts of thrombin. All results are presented as mean ± SD (n=3-4).

Figure 7. Location of Ala404(c221) and Arg405(c222) with respect to the Na⁺-binding loop and molecular interactions with TFPI. FXa is shown in different shades of blue, Ala404(c221) is shown in red, Arg405(c222) is shown in green and the catalytic triad is shown in blue. (A) The positions of Ala404(c221) and Arg405(c222) within the FXa serine protease domain are shown in relation to the Na⁺-binding site in a cartoon model. The FXa serine protease domain/EFG2 structure is taken from Gunaratne *et al.* (pdb 5VOE) [32]. The Na⁺ coordination by Tyr367(c185), Asp368(c185A), Arg405(c222), and Lys408(c224) is shown in more detail in insert. (B) The position of Ala404(c221) and Arg405(c222) are also shown in relation to the interaction with TFPI Kunitz domain 2 (B) and

antithrombin (C), both shown in beige. The FXa/TFPI Kunitz domain 2 structure was created by structural alignment using the crystal structure of FXa by Gunaratne *et al.* [32] on to the crystal structure of Kunitz domain 2 in complex with procine trypsin (pdb 1TFX) using PyMOL. The FXa/antithrombin structure is taken from Johnson *et al.* (pdb 2GD4) [9]. Amino acid residues are referred to by chymotrypsin numbering in this figure.
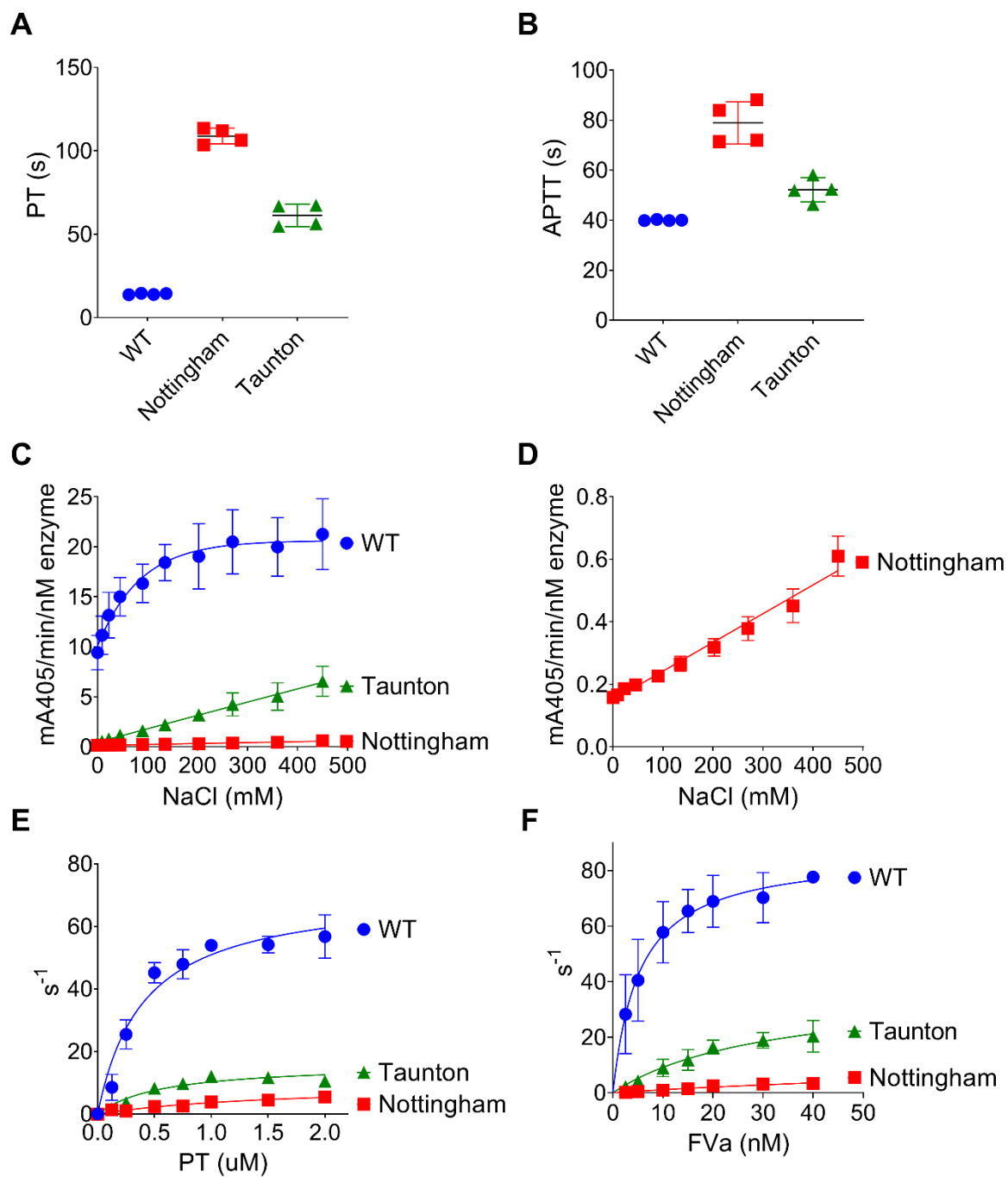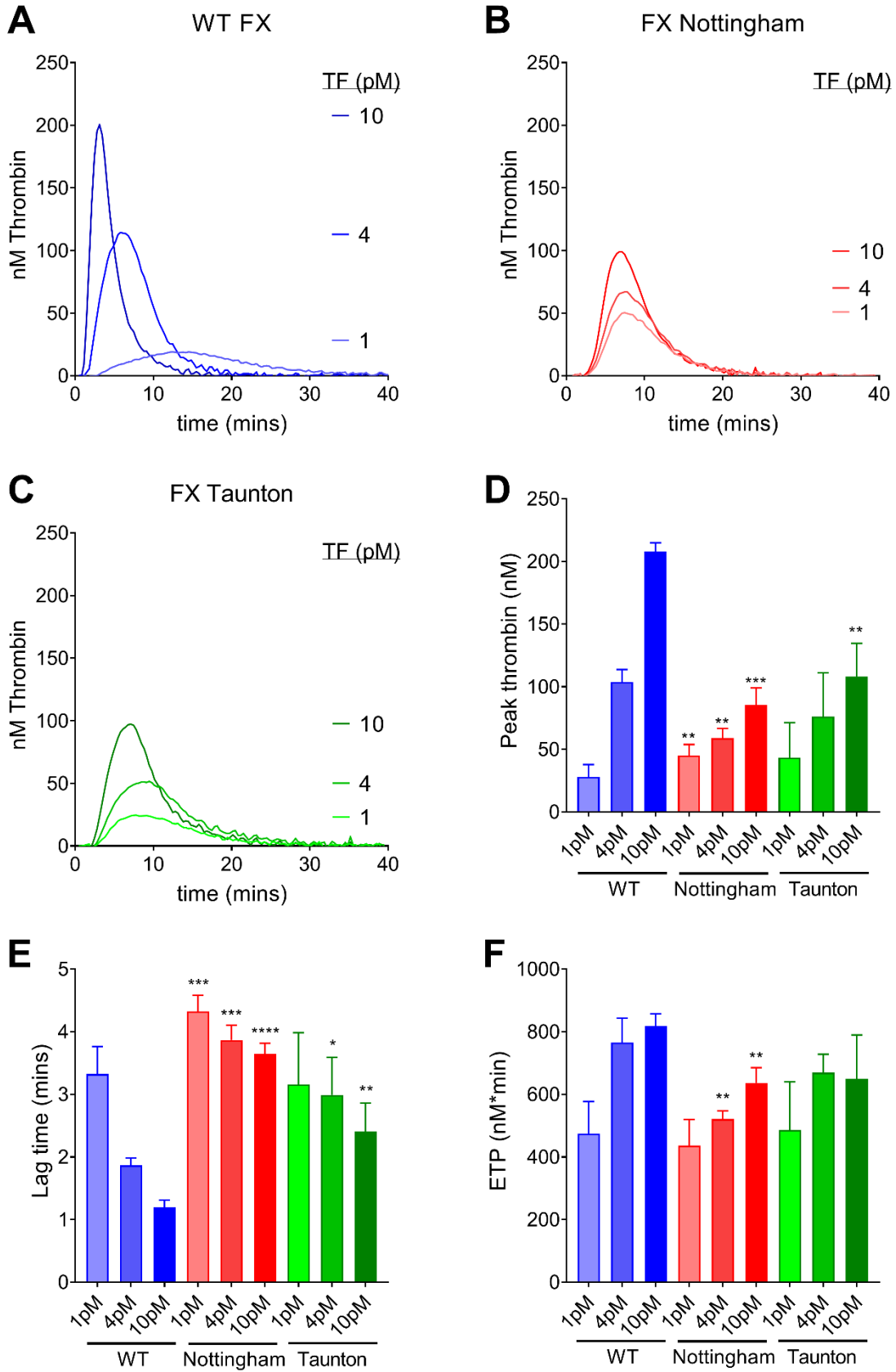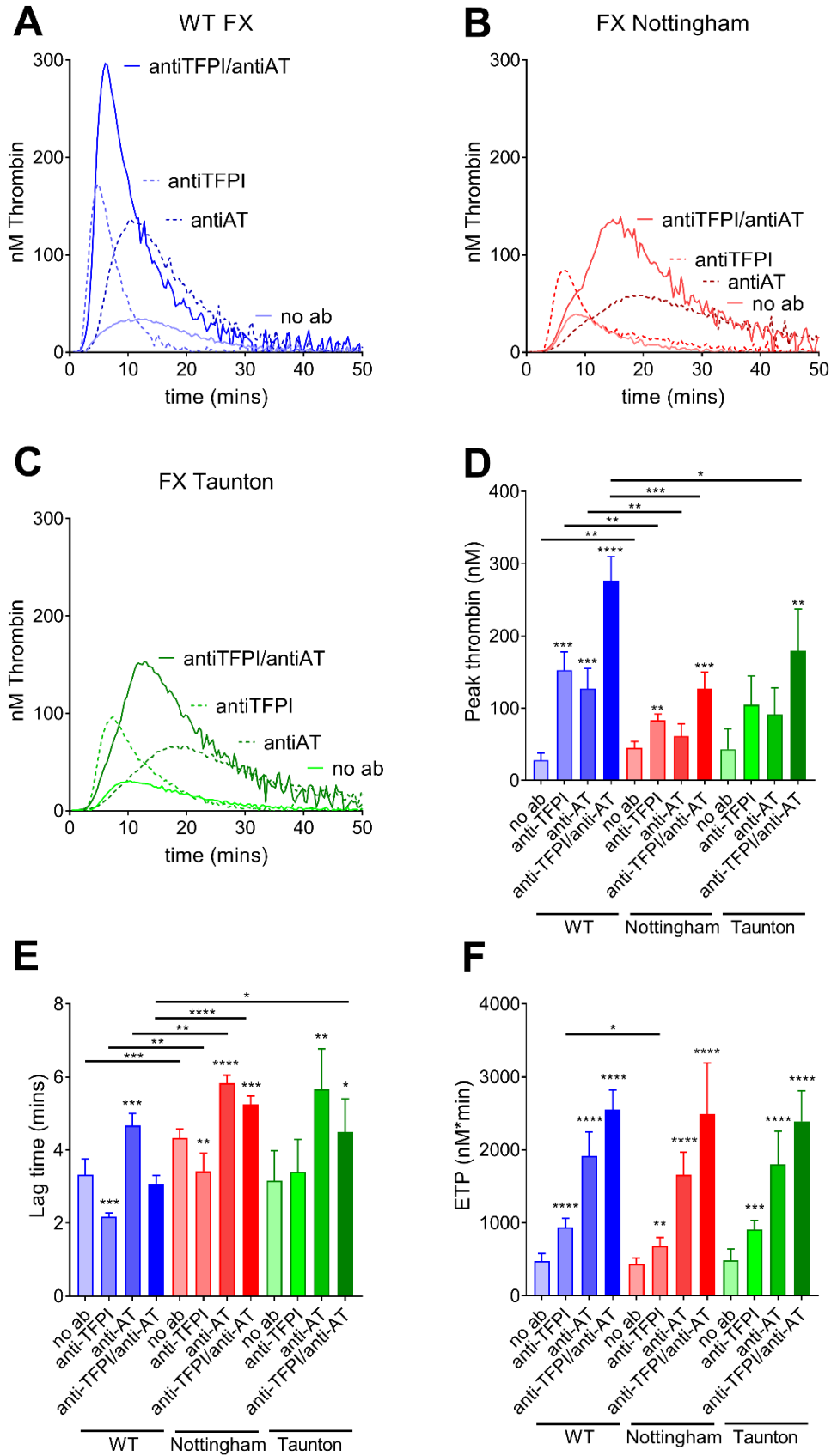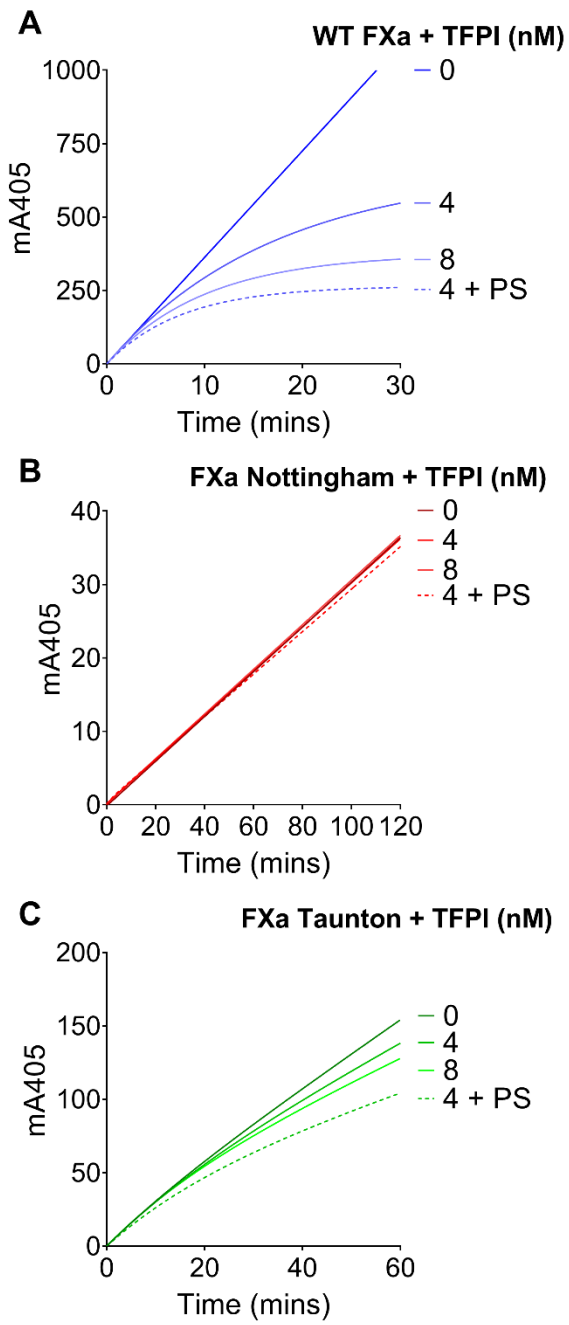
Figure 1.

Figure 2.

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