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ORIGINAL ARTICLE

Factor XII truncation accelerates activation in solution

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Essentials

- During contact system activation, factor XII is progressively cleaved by plasma kallikrein.
- We investigated the role of factor XII truncation in biochemical studies.
- Factor XII contains naturally occurring truncating cleavage sites for a variety of enzymes.
- Truncation of factor XII primes it for activation in solution through exposure of R353.

Summary. *Background:* The contact activation system and innate immune system are interlinked in inflammatory pathology. Plasma kallikrein (PKa) is held responsible for the stepwise processing of factor XII (FXII). A first cleavage activates FXII (into FXIIa); subsequent cleavages truncate it. This truncation eliminates its surface-binding domains, which negatively regulates surface-dependent coagulation. *Objectives:* To investigate the influence of FXII truncation on its activation and downstream kallikrein-kinin system activation. *Methods:* We study activation of recombinant FXII variants by chromogenic assays, by FXIIa ELISA and western blotting. *Results:* We demonstrate that FXII truncation primes it for activation by PKa in solution. We demonstrate this phenomenon in three settings. (i) Truncation at a naturally occurring PKa-sensitive cleavage site, R334, accelerates FXIIa formation in solution. A site-directed mutant FXII-R334A displays ~50% reduced activity when exposed to PKa. (ii) A pathogenic mutation in FXII that causes hereditary angioedema, introduces an additional plasmin-sensitive cleavage site. Truncation at this site synergistically accelerates FXII activation in solution. (iii) We identify new, naturally occurring cleavage sites in FXII that have so far not been functionally linked to contact system activation. As examples, we show that non-activating truncation of FXII by neutrophil elastase and cathepsin K primes it for activation by PKa in solution. *Conclusions:* FXII truncation, mediated by either pathogenic mutations or naturally occurring cleavage sites, primes FXII for activation in solution. We propose that the surface-binding domains of FXII shield its activating cleavage site, R353. This may help to explain how the contact system contributes to inflammatory pathology.

Keywords: bradykinin; factor XII; hereditary angioedema; neutrophil elastase; plasma kallikrein.

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Introduction

The pro-inflammatory peptide bradykinin is a powerful modulator of vascular permeability. It is a key disease mediator in hereditary angioedema (HAE) [1] and implicated in sepsis [2], anaphylactic shock [3] and rheumatoid arthritis [4]. Bradykinin is produced by the plasma contact system, in which the enzyme factor XII (FXII) plays an initiating role. FXII belongs to the family of trypsin-like serine proteases, which are produced in an inactive form (zymogen). A cleavage event near their catalytic domain causes a conformational change, which mediates activation [5,6]. When plasma contacts anionic surface materials, FXII is activated through successive cleavage events by plasma kallikrein (PKa). It is generally assumed that FXII (an 80 kDa single chain protein) is first cleaved after position R353 to yield activated FXII (αFXIIa; an 80 kDa disulfide-linked two-chain protein). This molecule can act as a clotting factor. Subsequent cleavages, in particular after R334, truncate the molecule (βFXIIa; a 28 kDa disulfide-linked two-chain protein). This eliminates the domains that are essential for binding to prothrombotic surfaces, resulting in a selective activator of plasma prekallikrein (PK) that lacks the capacity to act as a clotting factor [7].

During binding to activating surfaces, the FXII molecule changes conformation, which exposes the R353 cleavage site that is essential for zymogen activation [8]. This conformational change can be recapitulated by the binding of specific monoclonal antibodies that recognize the FXII fibronectin type II or kringle domain [9,10]. This suggests that the activating cleavage site is shielded from PKa when FXII is in a globular form, restricting FXII activation to the activating surface. Similar mechanisms have already been described for prothrombin and plasminogen [11–13].

We recently identified that mutations in FXII that cause HAE (FXII-HAE) introduce additional cleavage sites into the FXII molecule. When plasmin cleaves at these sites, uncontrolled FXII activation occurs and bradykinin production follows [14]. We proposed that this represents a two-stage activation mechanism: first, FXII is cleaved, which truncates the molecule and exposes the activating cleavage site. Subsequently, it is activated through cleavage after R353. We hypothesized that this leads to excessive FXII activation in solution, without a surface as a prerequisite [15].

In this study, we hypothesized that the disease mechanism of FXII-HAE reflects a physiological pathway for FXII activation in solution. We here report that the proline-rich region of FXII contains several enzyme cleavage sites for a variety of enzymes, including neutrophil elastase and monocyte-expressed cathepsin K, two enzymes that are not classically linked to contact system activation. Cleavage by these enzymes removes a large shielding sequence from the FXII molecule and primes it for activating cleavage by PKa. Based on these findings, we propose a general two-step model for FXII activation by cell-derived enzymes, which helps us to understand how bradykinin production may occur in inflammatory conditions.

Methods

Reagents

A list of reagents is provided in Data S1.

Protein expression and purification

Factor XII mutagenesis and production were performed as previously described [14]. Briefly, mutations R334A and R353A were generated via site-directed mutagenesis using Pfu turbo (see Table S1 for primer sequences). FXII constructs were ligated into the pSM2 eukaryotic expression vector via the *HindIII* and *EcoRI* digestion sites. pSM2 contains an N-terminal murine Igκ secretion signal followed by two strep-tags for purification. FXII constructs were transfected into HEK293T cells with Lipofectamine 2000, after which transfected cells were selected by resistance to 5 μg mL^{-1} blasticidin. After 3 weeks of selection, stable transfected cells were expanded and grown in 1L cell factories. The production medium (Dulbecco's modified eagle's medium, 5% (v/v) fetal bovine serum [FBS], 0.5% (v/v) Ultroser G, Pen/Strep) was harvested twice a week, supplemented with benzamidine (0.174 g L^{-1}), Soybean trypsin inhibitor (0.024 g L^{-1}) and polybrene (0.056 g L^{-1}), centrifuged to remove cell debris and stored at −20 °C. Harvested media were thawed at 37 °C, pooled, concentrated and buffer exchanged (against 100 mmol L^{-1} Tris-HCl, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA, pH = 8.0) on a 10 kDa dialysis membrane in a Quixstand benchtop system (GE Life Sciences, Eindhoven, the Netherlands). Recombinant FXII protein was purified using strep-tactin sepharose beads. Purified recombinant FXII was dialyzed against 4 mmol L^{-1} sodium acetate-HCl, 150 mmol L^{-1} NaCl, pH = 5.4, and stored at −80 °C. Protein concentrations were determined by absorption spectroscopy at OD_{280nm}. Absorption coefficients of the FXII mutants were determined based on their mature amino acid sequences using ProtParam [16]. Purity and degradation were assessed via 4–12% Bis Tris-PAGE gel and coomassie blue staining.

Plasminogen purification and activation

Plasminogen was purified from human plasma as previously described [14]. Plasmin was freshly prepared for functional experiments by preactivation of plasminogen with 10 U mL^{-1} streptokinase in 10 mmol L^{-1} HEPES, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} $MgSO_4$, 5 mmol L^{-1} KCl, pH = 7.4 HEPES-buffered saline (HBS), with 0.2% (w/v) bovine serum albumin (BSA) for 15 min at 37 °C, after which it was kept on ice until use.

Contact system activation assays

Purified FXII exposure to single purified enzymes. For chromogenic assays and FXIIa ELISAs, recombinant FXII (375 nmol L^{-1}) in HBS-BSA (0.2% w/v) was incubated with plasmin (555.6 nmol L^{-1}), PKa (11.6 nmol L^{-1}) or vehicle. For western blotting experiments, recombinant FXII (375 nmol L^{-1}) was incubated with plasmin (1.1 μmol L^{-1}), PKa (93 nmol L^{-1}) or vehicle.

Purified FXII exposure to multiple purified enzymes. For chromogenic assays and FXIIa ELISAs, recombinant FXII (375 nmol L^{-1}) in HBS-BSA (0.2% w/v) was incubated with plasmin (66.7 nmol L^{-1}), neutrophil elastase (33.8 nmol L^{-1}), cathepsin K (80 nmol L^{-1}), PK (1.43 nmol L^{-1}), vehicle or combinations thereof, as indicated in the Figures. For western blotting, 375 nmol L^{-1} recombinant FXII in HBS-BSA (0.2% w/v) was incubated with plasmin (66.7 nmol L^{-1}), neutrophil elastase (15.4 nmol L^{-1}), cathepsin K (80 nmol L^{-1}), PKa (23.3 nmol L^{-1}), vehicle or combinations thereof.

Sample preparation. After the above procedures, PKa and plasmin activity were neutralized with 100 KIU mL^{-1} aprotinin. FXIIa activity was determined through conversion of chromogenic substrate H-D-Pro-Phe-Arg-pNA (0.5 mmol L^{-1}) at 405 nm, 37 °C. Alternatively, samples were collected in assay-specific buffers for analysis by western blotting or ELISA.

Plasma experiments. FXII (3.75 μmol L^{-1}) was pretreated with neutrophil elastase (338 nmol L^{-1}) in HBS-BSA (0.2% w/v) for 15 min at 37 °C. Hereafter, samples were diluted 10x in FXII-deficient plasma to neutralize the neutrophil elastase and reconstitute the plasma to a final concentration of 375 nmol L^{-1} FXII. Subsequently, the reconstituted plasma was incubated with PKa (11.6 nmol L^{-1}) for 15 min. Samples were collected in assay-specific buffer for analysis by western blotting or ELISA, as described below.

FXIIa nanobody ELISA

FXIIa ELISA was performed as described previously with minor modifications [17]. Briefly, 5 μg mL^{-1} nanobody B7 against FXIIa was immobilized on Maxisorp plates in phosphate-buffered saline (PBS; 21 mmol L^{-1} Na2HPO4, 2.8 mmol L^{-1} NaH2PO4, 140 mmol L^{-1} NaCl, pH = 7.4) overnight at 4 °C. Nanobody B7 recognizes both αFXIIa and βFXIIa, but not when complexed with C1 esterase inhibitor [17]. Samples from contact system activation assays were collected by 8x dilution in 0.5% (w/v) skimmed milk in HBS (mHBS), containing 200 μmol L^{-1} D-Phe-Pro-Arg-chloromethylketone (PPACK) and 100 KIU mL^{-1} aprotinin. Wells were rinsed with PBS, blocked with 4% (w/v) mHBS, for 1 h at room temperature (RT) while shaking. Hereafter samples were added and incubated for 2 h at RT while shaking. Next, the wells were rinsed with 0.05% (v/v) PBS-Tween-20 (PBST) and incubated with the biotinylated monoclonal anti-FXII 'B2' nanobody (5 μg mL^{-1} in 0.5% (w/v) mHBS) for 1 h at RT, which recognizes all forms of (activated) FXII [17]. After washing with PBST, wells were incubated with a peroxidase-conjugated anti-sheep polyclonal (1 : 8000 in 0.5% (w/v) mHBS) for 1 h at RT while shaking. Finally, wells were rinsed with PBST and developed with 100 μL/well 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate. Substrate conversion was stopped by adding 50 μL/well H_2SO_4 (0.3 mol L^{-1}) and absorbance was read at 450 nm. A βFXIIa standard curve was included on each plate, which was plotted in Prism Graphpad 7.0 (Graphpad Software Inc., La Jolla, CA, USA) using a sigmoidal 4PL fit model to which sample concentrations of FXIIa were related.

C1-inhibitor-enzyme complex ELISA

C1INH-enzyme complex ELISAs were performed as previously described [14]. Capture nanobody 1B12 against cleaved C1INH was immobilized at 5 μg mL^{-1} in HBS on a maxisorp plate overnight at 4 °C. Samples from contact system activation experiments were 32× diluted in stopmix (0.5% (w/v) mHBS, 200 μmol L^{-1} PPACK). The coated plate was rinsed with HBS, after which it was blocked with 1% (w/v) mHBS for 1 h at RT while shaking. Samples were added to the plate and incubated for 2 h at RT while shaking. Thereafter the plate was rinsed (0.1% (v/v) Tween-20 in PBS (PBST)) and FXIIa-C1INH and PKa-C1INH complexes were detected via a biotinylated polyclonal nanobody mix against βFXIIa (5 μg mL^{-1} in mHBS) or via a sheep polyclonal anti-PK IgG (1 : 2000 in mHBS), respectively. After 1 h, the plate was rinsed with PBST and incubated for 1 h at RT with streptavidin-poly HRP (1 : 5000 in mHBS) or a peroxidase-conjugated polyclonal rabbit anti-sheep antibody (1 : 8000 in mHBS). Wells were rinsed with PBST and developed with 50 μL TMB substrate. The reaction was stopped by the addition of 25 μL H_2SO_4 (0.3 mol L^{-1}) and absorbance was measured at 405 nm. As a standard curve, a DXS-activated plasma (30 μg mL^{-1} DXS-500k for 30 min) was included. Sample concentrations were plotted against the standard curve via Prism Graphpad 7.0 (Graphpad Software Inc.) using a sigmoidal 4PL fit.

Western blotting

Samples from contact system activation assays were diluted 20 times in (non-)reducing sample buffer (15.5% (v/v) glycerol, 96.8 mmol L^{-1} Tris-HCL, 3.1% (w/v) SDS, 0.003% (w/v) Bromophenol Blue, with or without 25 mmol L^{-1} dithiotreitol [DTT] for reduction) and boiled for 10 min at 95 °C. Samples were separated on 4–12% Bis-Tris gels at 165 V for 65 min in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer. Proteins were transferred onto Immobilon-FL membranes at 125 V for 60 min in blotting buffer (14.4 g L^{-1} glycine, 3.03 g L^{-1} Tris-HCL, 20% (v/v) ethanol) and blocked with blocking buffer (0.5x Odyssey blocking reagent in TBS) for 1 h at RT. FXII was detected by overnight incubation at 4 °C with an affinity purified polyclonal antibody in blocking buffer (1 : 2000). Blots were washed with TBS-0.05% (v/v) Tween-20 (TBST), and the primary antibody was detected with Alexa Fluor 680 donkey anti-sheep IgG (1 : 7500 in blocking buffer). Blots were extensively washed with TBST, followed by distilled water and analyzed on a near infra-red Odyssey scanner (LI-COR, Leusden, the Netherlands).

In silico *predictions*

Proline-rich regions of mature FXII amino acid sequences (https://www.uniprot.org; Human, P00748; Mice, Q80YC5; Rat, D3ZTE0; Bovine, P98140; Pig, O97507) were analyzed with Peptide Cutter or Prosper software for the presence of putative cleavage sites with probability scores > 50% [16,18].

Statistical analysis

Statistics were performed by one-way ANOVA. $P < 0.05$ was considered significant. All data analyses were performed with Prism Graphpad 7.0 (Graphpad Software Inc.).

Results

The surface-binding domains of activated factor XII limit its activity in solution

Factor XII contains three cleavage sites that are cleavable by PKa (R334, R343 and R353) [19,20]. Cleavage after R343 and R353 will result in a two-chain molecule (αFXIIa), as a result of an intramolecular disulfide bridge (Cys340-Cys467) in FXII. In contrast, cleavage after R334 will result in a large (50 kDa; sequence I1-R334) fragment that contains the surface-binding domains and small fragment (~28 kDa; βFXIIa; sequence N335-S596) containing the catalytic domain. Mutagenesis of R353 into an alanine (Fig. 1A) prevents FXII activation by PKa, as determined by FXIIa ELISA (R353A; Fig. 1B). A minor fraction of FXII is truncated by cleavage after R334, as is visible in western blots under non-reducing conditions (Fig. 1C). Under reducing conditions it becomes clear that PKa predominantly cleaves after R353 in wild-type FXII (FXII-WT; Fig. 1C). Residual cleavage is seen with FXII-R353A as a result of the two alternative

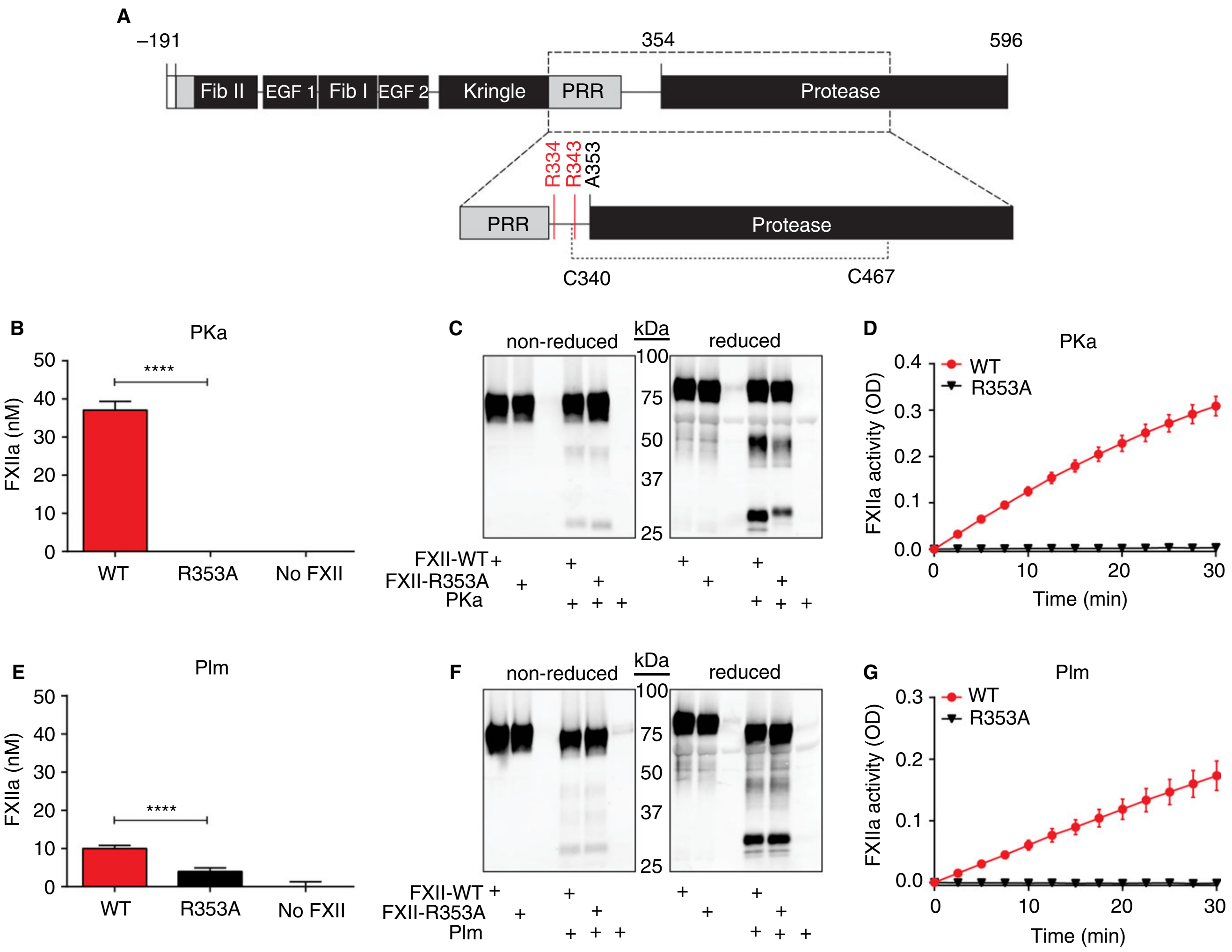

Fig. 1. Cleavage after arginine (R) 353 is critical for factor XII (FXII) activation by plasma kallikrein (PKa) and plasmin (Plm). (A) Schematic overview of PKa cleavage sites in FXII-R353A; available cleavage sites are indicated in red and incapacitated cleavage sites in black. Fib II, Fibronectin type II-domain; EGF 1; Epidermal Growth Factor-like domain 1; Fib I, Fibronectin type I-domain; EGF 2; Epidermal Growth Factor-like domain 2; PRR, Proline-rich region. (B) FXIIa levels determined by ELISA after 15 min of exposure to PKa (11.6 nmol L^{-1}). WT, wild type FXII; R353A, FXII-R353A. (C) Western blots of FXII cleavage by PKa (93 nmol L^{-1}). (D) Chromogenic assay for enzyme activity of FXII-WT or FXII-R353A after 15 min of exposure to PKa (11.6 nmol L^{-1}). (E) FXIIa levels (determined by ELISA) after 15 min of exposure to plasmin (555.6 nmol L^{-1}). (F) Western blots of FXII cleavage by plasmin (1.11 μmol L^{-1}). (G) Chromogenic assay for enzyme activity of FXII-WT or FXII-R353A after 15 min of exposure to plasmin (555.6 nmol L^{-1}). Data represent means ± SD of three separate experiments, all performed in duplicate. Data were analyzed by one-way ANOVA (****$P < 0.0001$).

cleavage possibilities. Cleavage after R353 is essential for the development of enzymatic activity in chromogenic substrate assays (Fig. 1D, Figure S1A) [19]. In a comparable manner, plasmin generates FXIIa from FXII zymogen, albeit much less efficiently (Fig. 1E). Some FXII is truncated into similar non-disulfide-linked products (Fig. 1F; non-reduced). In contrast to PKa-mediated cleavage, plasmin does not show a preference for FXII-WT over FXII-R353A; both form similar amounts of two-chain products (Fig. 1F; reduced). This can be attributed to a plasmin-specific cleavage event at position K346 [21]. Nonetheless, enzymatic activity is critically dependent on R353 cleavage, indicating that K346 cleavage does not independently trigger FXII activation and R353 cleavage is a prerequisite (Fig. 1G, Figure S1A).

To investigate the importance of FXII truncation at position R334, we generated FXII-R334A (Fig. 2A). This mutant generates comparable amounts of FXIIa in response to PKa (Fig. 2B) and plasmin (Fig. 2E). As expected, truncation by both enzymes is abrogated (Fig. 2C,F; non-reduced; for PKa and plasmin, respectively). Correspondingly, FXII-R334A is less fragmented by PKa when analyzed by SDS-PAGE under reducing conditions (Fig. 2C; reduced). As before, fragmentation is similar between FXII-WT and FXII-R334A when exposed to plasmin (Fig. 2F; reduced). Remarkably, we observed a ~50% lower enzymatic activity in FXII-R334A compared with FXII-WT in chromogenic substrate assays after exposure to either PKa or plasmin (Fig. 2D,G, respectively, Figure S1B). These experiments suggest that cleavage after R334 accelerates FXII activation or enhances enzyme activity, possibly because the surface binding domains of FXIIa interfere with substrate conversion (Fig. 2H).

Truncation of pathogenic factor XII mutant T309K primes it for activation in solution

We recently identified that mutations in FXII that cause HAE (FXII-HAE) introduce additional cleavage sites within the proline-rich region of FXII, which are sensitive to plasmin (Fig. 3A, T309K indicated in yellow). Stimulation of plasmin activity in patient plasma provokes uncontrolled bradykinin production [14]. Motivated by our previous experiments on the role of FXII truncation (Fig. 2), we investigated the consequences of plasmin-mediated truncation of this pathogenic mutant in further detail. We therefore exposed FXII-T309K to low levels of plasmin (67 nmol L^{-1}). At these concentrations, there is negligible FXIIa formation (Fig. 3B), despite considerable truncation (Fig. 3C; non-reduced). This indicates that plasmin prefers cleavage after residue K309 in the pathological mutant over R353. In a similar manner, we exposed FXII-T309K to low levels of PKa (1.4 nmol L^{-1}). At these PKa concentrations, negligible FXIIa formation is observed (Fig. 3B) and no truncation takes place (Fig. 3C; non-reduced). However, plasmin and PKa act synergistically on FXII-T309K: when this mutant is simultaneously exposed to low levels of both enzymes, FXIIa formation is strongly amplified (Fig. 3B; prognosed cooperative activity of both enzymes indicated by striped bar). During this reaction, plasmin-truncated FXII is cleaved at position R353 by PKa, yielding a two-chain molecule (Fig. 3C; reduced). As a result, FXII-T309K only develops enzyme activity when simultaneously exposed to both enzymes (Fig. 3D, Figure S1C). This phenomenon is specific to the pathogenic form of FXII. FXII-WT does not form FXIIa in response to the combined presence of low levels of plasmin and PKa (Fig. 3E), is resistant to truncation by plasmin (Fig. 3F) and does not develop enzymatic activity (Fig. 3G, Figure S1C). These findings indicate that site R353, which is critical to FXII activation, is shielded from enzymatic cleavage in solution by the N-terminally positioned surface-binding domains (Fig. 3H). We next considered the possibility that these observations reflect a physiological phenomenon.

Non-canonical cleavage by neutrophil elastase or cathepsin K primes factor XII for activation in solution

Because of its composition, the unique highly unstructured proline-rich region of FXII may act as a hinge to connect the surface-binding domains of FXII to its catalytic domain. Our previous experiments with FXII-T309K indicated that cleavage sites that are present in this region are accessible for cleavage. We subsequently considered the possibility that naturally occurring cleavage sites are present in this region.

For the identification of putative naturally occurring cleavage sites for alternative enzymes in the proline-rich region, we performed an *in silico* analysis. A selection of the predicted enzymes and their cleavage sites across several species is presented in Table 1. These predictions show possible cleavage sites for neutrophil elastase (elastase 2) in the proline-rich region (Fig. 4A). This enzyme is amongst others expressed by neutrophils, basophils and mast cells, which have been previously tied to contact activation [22]. Interestingly, it was previously established that neutrophil elastase cleaves FXII into two fragments of 52 kDa and 28 kDa [23,24]. This suggests a cleavage site in the vicinity of the proline-rich region. Extended exposure of FXII to elastase destroys its procoagulant and enzymatic activity [25]. This may be mediated through a predicted elastase cleavage site after V354, a single amino acid behind the activating cleavage site R353. We explored this in further detail: when FXII is exposed to neutrophil elastase, elevated levels of FXIIa are detected by ELISA (Fig. 4B). This is accompanied by FXII truncation, seen under non-reducing conditions (Fig. 4C). The presence of low, non-activating levels of PKa (1.4 nmol L^{-1}) increases FXIIa levels synergistically (Fig. 4B; prognosed cooperative activity of both enzymes

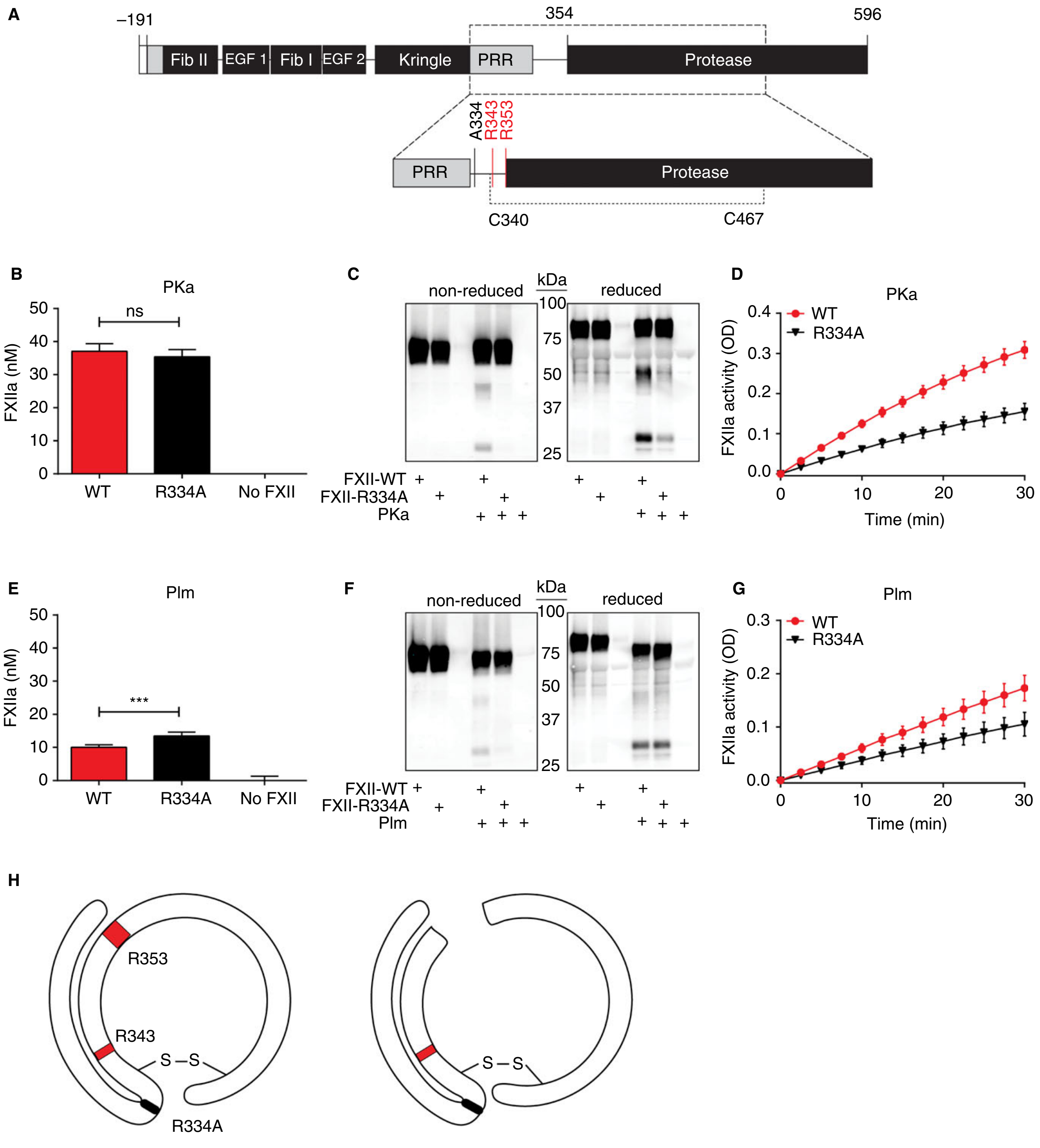

Fig. 2. A plasma kallikrein (PKa)-sensitive shielding sequence blocks the active site of factor XII. (A) Scaled schematic overview of PKa cleavage sites in FXII-R334A; available cleavage sites are indicated in red and incapacitated sites in black. Fib II, Fibronectin type II-domain; EGF 1; Epidermal Growth Factor-like domain 1; Fib I, Fibronectin type I-domain; EGF 2; Epidermal Growth Factor-like domain 2; PRR, Proline-rich region. (B) FXIIa levels (determined by ELISA) after 15 min of exposure to PKa (11.6 nmol L^{-1}). WT, wild type FXII; R334A, FXII-R334A. (C) Western blots of FXII cleavage by PKa (93 nmol L^{-1}). (D) Chromogenic assay for enzyme activity of FXII-WT or FXII-R334A after 15 min of exposure to PKa (11.6 nmol L^{-1}). (E) FXIIa levels (determined by ELISA) after 15 min of exposure to plasmin (Plm; 555.6 nmol L^{-1}). (F) Western blots of FXII cleavage by plasmin (1.11 μmol L^{-1}). (G) Chromogenic assay for enzyme activity of FXII-WT or FXII-R334A after 15 min of exposure to plasmin (555.6 nmol L^{-1}). (H) Model for FXII-R334A activation in solution; available cleavage sites are indicated in red and incapacitated sites in black. Data represent means ± SD of three separate experiments, all performed in duplicate. Data were analyzed by one-way ANOVA (***$P < 0.0005$).

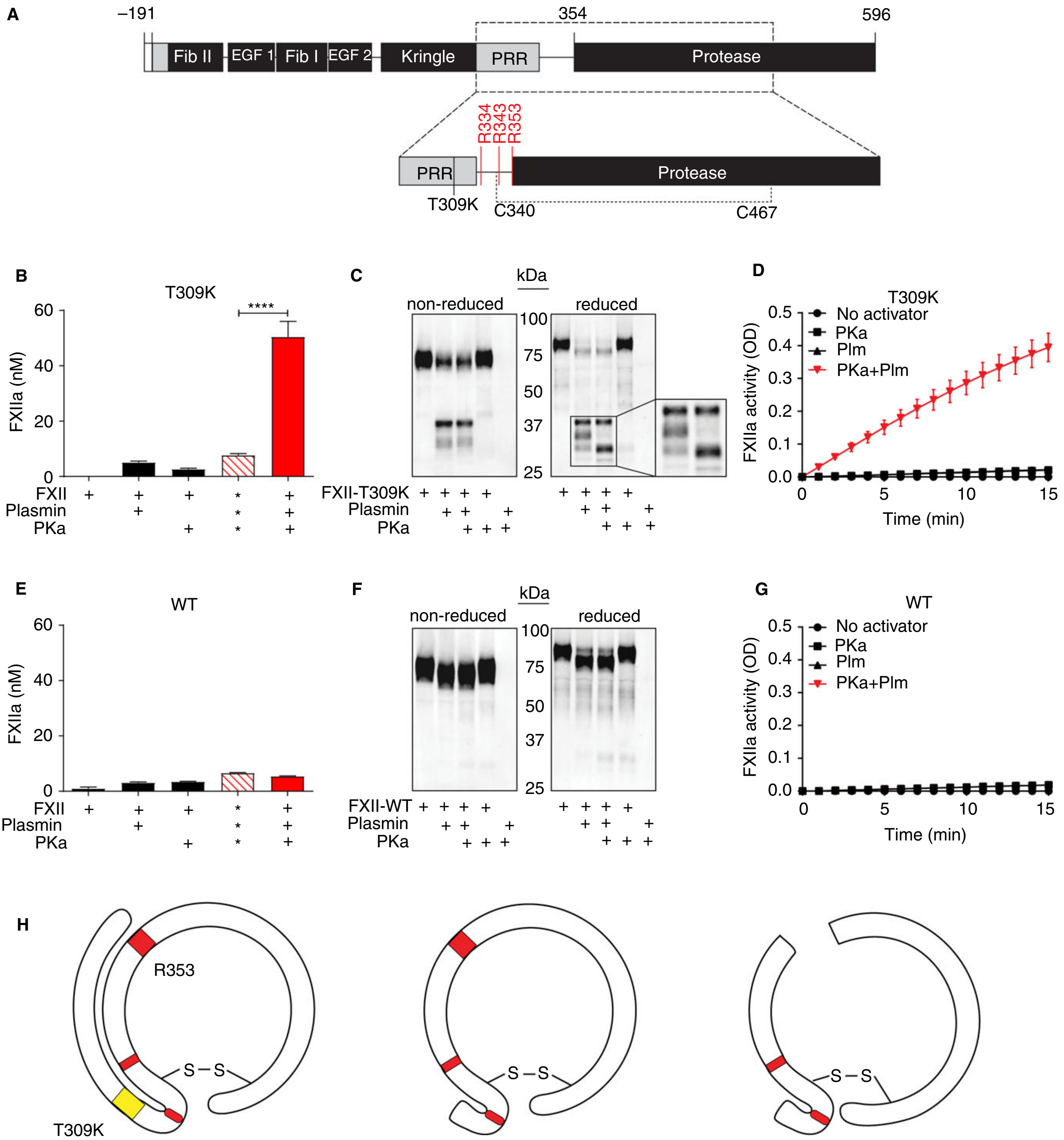

Fig. 3. Plasmin (Plm) cleavage primes factor XII-T309K for activation by plasma kallikrein (PKa). (A) Scaled schematic overview of cleavage sites in FXII-T309K. PKa cleavage sites are indicated in red. Fib II, Fibronectin type II-domain; EGF 1; Epidermal Growth Factor-like domain 1; Fib I, Fibronectin type I-domain; EGF 2; Epidermal Growth Factor-like domain 2; PRR, Proline-rich region. (B) FXIIa levels (determined by ELISA) that FXII-T309K (T309K) develops after 15 min of exposure to 66.7 nmol L^{-1} plasmin and/or 1.43 nmol L^{-1} PKa. The prognosed cooperative activity of both enzymes is indicated by the striped bar. (C) Western blots of FXII cleavage by 66.7 nmol L^{-1} plasmin and/or 23.3 nmol L^{-1} PKa. (D) Chromogenic assay for enzyme activity of FXII-T309K after 15 min of exposure to 66.7 nmol L^{-1} plasmin and/or 1.43 nmol L^{-1} PKa. (E) FXIIa-WT (WT) levels after 15 min of exposure to 66.7 nmol L^{-1} plasmin and/or 1.43 nmol L^{-1} PKa. The prognosed cooperative activity of both enzymes is indicated by the striped bar. (F) FXII-WT cleavage by 66.7 nmol L^{-1} plasmin and/or 23.3 nmol L^{-1} PKa. (G) Enzymatic activity of FXII-WT after 15 min of exposure to 66.7 nmol L^{-1} plasmin and/or 1.43 nmol L^{-1} PKa. (H) Model for synergistic FXII-T309K activation by PKa and plasmin in solution; available cleavage sites are indicated in red and incapacitated sites in black. The pathogenic plasmin-sensitive cleavage site of T309K is indicated in yellow. Data represent means ± SD of three separate experiments, all performed in duplicate. Data were analyzed by one-way ANOVA (**** $P < 0.0001$).

Table 1 *In silico* predictions of cleavage sites in the proline-rich region of factor XII and its activation loop

Enzyme	No. of cleavage sites Human (Q277-S339)	Mice (Q277-G321)	Rat (Q276-V320)	Bovine (Q288-G335)	Pig (Q277-G338)
Trypsin	5	1	3	1	2
Neutrophil elastase	2	3	2	1	1
Cathepsin K	1	-	2	3	3
Matrix metallopeptidase 9	2	1	1	2	1

indicated by the striped bar). Analyses under reducing conditions point out that ~50% of FXIIa forms a two-chain product in the presence of neutrophil elastase (Fig. 4C). This cleavage by elastase does not independently activate FXII in a chromogenic substrate assay (Fig. 4D, Figure S1D), despite recognition of (some of) the cleavage products by ELISA. However, in the presence of low, non-activating levels of PKa (1.4 nmol L^{-1}), all truncated FXII is converted into a two-chain molecule, indicated by a migratory shift seen by SDS-PAGE under reducing conditions (Fig. 4C). This synergistic action strongly promotes enzymatic activity (Fig. 3D). These experiments indicate that two naturally occurring cleavage sites for neutrophil elastase in FXII are able to control its activation in solution. One of these cleavage sites (at a currently unidentified position) mediates truncation by elastase (Fig. 4H). This removes the sequence that shields R353, priming FXII for activation by PKa in solution.

Cathepsin K is expressed by osteoclasts and monocytes [26]. This cysteine protease mediates the breakdown of collagen and elastin and is implicated in osteoporosis and emphysema. Its expression is upregulated by inflammatory cytokines after tissue injury. Our *in silico* analyses predict a cleavage site for cathepsin K in the proline-rich region of FXII (Table 1; Fig. 4A). Similar to our findings with elastase, we found that cathepsin K generates products from FXII that are recognized in FXIIa ELISAs (Fig. 4E). By western blots, we confirmed that cathepsin K cleaves FXII into two fragments (Fig. 4F; non-reduced). In the presence of low, non-activating levels of PKa (1.4 nmol L^{-1}), FXIIa levels increase synergistically (Fig. 4E; prognosed cooperative activity of both enzymes indicated by the striped bar). This corresponds with a migratory shift that is visible by western blotting under reducing conditions, indicating activating cleavage by PKa (Fig. 4F; reduced). As a result, FXII only develops enzymatic activity in the combined presence of both enzymes (Fig. 4G, Figure S1E).

Removal of the shielding sequence by neutrophil elastase primes factor XII for activation in plasma

In a final series of experiments, we reconstituted FXII-depleted plasma with FXII or FXII that had been pre-exposed to neutrophil elastase. We determined the plasma levels of complexes between FXIIa or PKa and C1INH as a measure of contact activation in plasma in the presence or absence of low levels of PKa (11.3 nmol L^{-1}). We found that the combined actions of neutrophil elastase and PKa led to high levels of FXIIa-C1INH complexes, as well as PKa-C1INH complexes (Fig. 5A,B; prognosed cooperative activity of both enzymes indicated by striped bar). Finally, we analyzed the cleavage of high-molecular-weight kininogen (HK) by western blotting. This reflects the liberation of bradykinin from its precursor molecule [27]. Our experiments showed that pre-exposure of FXII to neutrophil elastase (which truncates it) boosts HK cleavage (Fig. 5C). Densitometric analysis of HK cleavage from repeated experiments at low PKa levels (11.6 nmol L^{-1}) shows that cleaved HK levels are ~2-fold higher than can be attributed to the cooperative actions of both enzymes (Fig. 5D; prognosed cooperative activity of both enzymes indicated by the striped bar).

Discussion

Excessive contact system activation has been linked to a variety of inflammatory conditions [4,28–30]. Because the contact system derives its name from surface-dependent enzyme reactions in coagulation assays, it is logical to assume that surfaces are a prerequisite for contact system activation *in vivo*. We here present evidence for a general biochemical mechanism that controls FXII activation in solution.

1. Cleavage after R334 enhances the enzyme activity of FXIIa. A mutant in which this cleavage site is disabled (R334A) has a strongly reduced enzyme activity after exposure to PKa or plasmin (Fig. 2). Our findings suggest that the surface-binding domains of FXIIa shield its active site.
2. Truncation of the pathological mutant FXII-T309K by plasmin primes it for activation by PKa in solution (Fig. 3). These experiments suggest that the activating cleavage site after R353 is shielded from cleavage. These findings indicate a two-step mechanism in which FXII-T309K is first truncated, exposing R353 for subsequent cleavage by PKa, as we previously proposed [15].

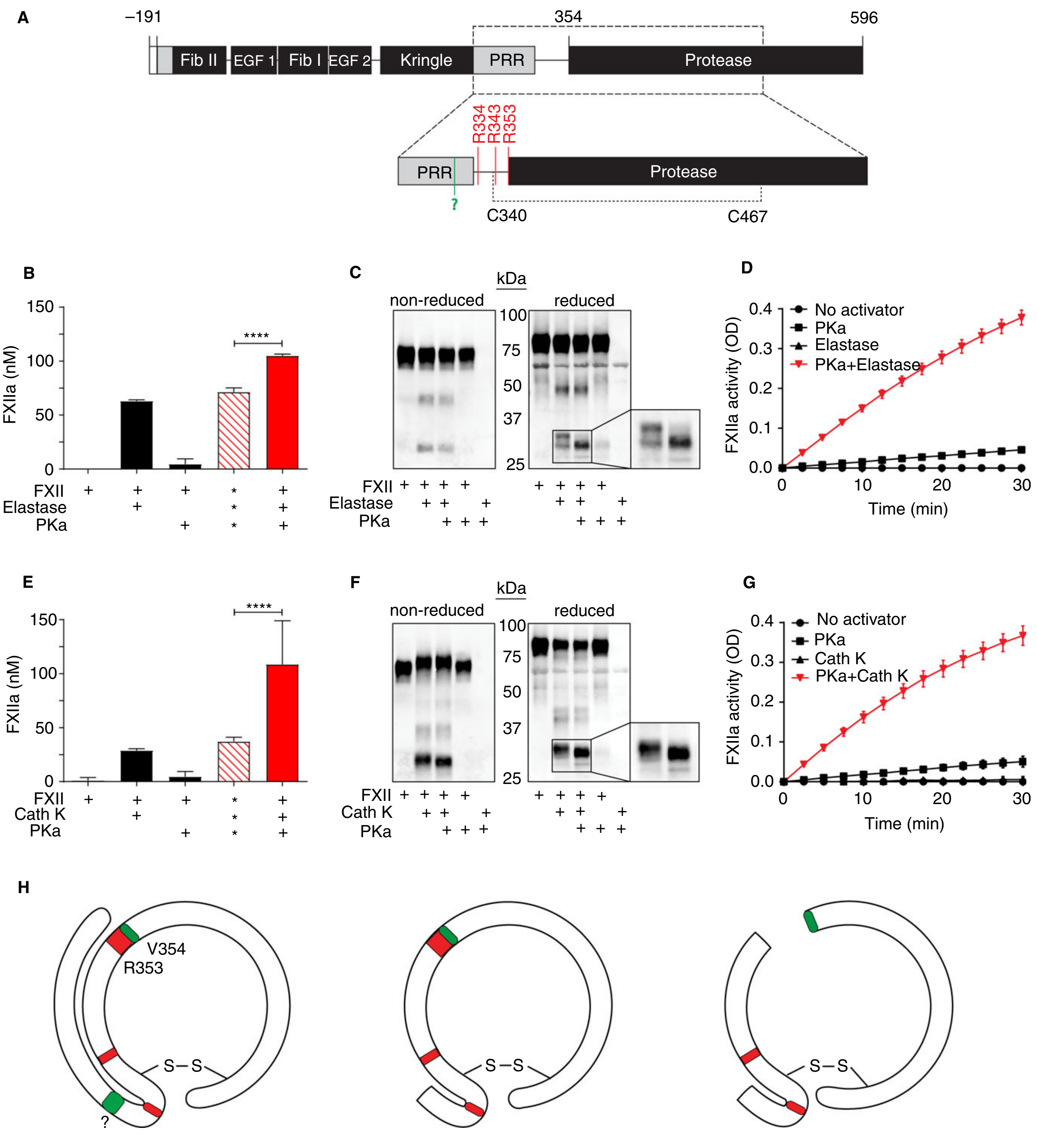

Fig. 4. Neutrophil elastase and monocyte cathepsin K prime factor XII (FXII) for activation by plasma kallikrein (PKa). (A) Scaled schematic overview of cleavage sites in FXII. PKa cleavage sites are indicated in red. Putative neutrophil elastase and cathepsin K cleavage site(s) are indicated in green by a '?'. Fib II, Fibronectin type II-domain; EGF 1; Epidermal Growth Factor-like domain 1; Fib I, Fibronectin type I-domain; EGF 2; Epidermal Growth Factor-like domain 2; PRR, Proline-rich region. (B) FXIIa levels (determined by ELISA) that FXII develops after 15 min of exposure to 33.8 nmol L^{-1} elastase and/or 1.43 nmol L^{-1} PKa. The prognosed cooperative activity of both enzymes is indicated by the striped bar. (C) Western blots of FXII cleavage by 15.4 nmol L^{-1} elastase and/or 23.3 nmol L^{-1} PKa. (D) Chromogenic assay for enzyme activity of FXII after 15 min of exposure to 33.8 nmol L^{-1} elastase and/or 1.43 nmol L^{-1} PKa. (E) FXIIa levels (determined by ELISA) that FXII develops after 15 min of exposure to 80 nmol L^{-1} cathepsin K and/or 1.43 nmol L^{-1} PKa. The prognosed cooperative activity of both enzymes is indicated by the striped bar. (F) Western blots of FXII cleavage by 80 nmol L^{-1} cathepsin K and/or 23.3 nmol L^{-1} PKa. (G) Chromogenic assay for enzyme activity of FXII after 15 min of exposure to 80 nmol L^{-1} cathepsin K and/or 1.43 nmol L^{-1} PKa. (H) Model for synergistic FXII activation in solution activation; available cleavage sites for PKa are indicated in red. The putative cleavage sites for neutrophil elastase or cathepsin K are indicated in green. Data represent means ± SD of three separate experiments, all performed in duplicate. Data were analyzed by one-way ANOVA (****$P < 0.0001$).

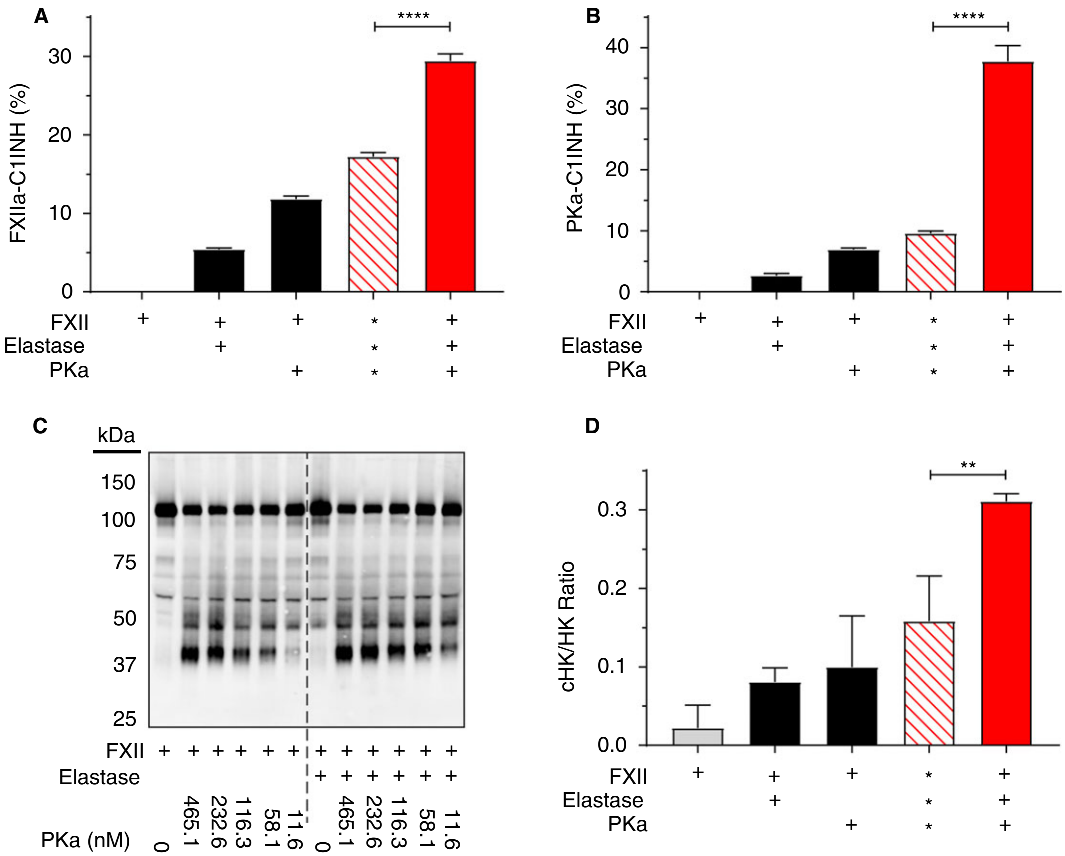

Fig. 5. Primed factor XII (FXII) accelerates contact system activation in plasma. Factor XII was pretreated with neutrophil elastase or vehicle, and afterwards reconstituted in FXII-depleted plasma. FXIIa-C1INH (A) and plasma kallikrein (PKa)-CINH (B) complexes were measured after 15 min in the presence or absence of 11.3 nmol L^{-1} PKa. The prognosed cooperative activity of both enzymes is indicated by the striped bars. (C) High-molecular-weight kininogen (HK) consumption in plasma was determined by western blotting after 15 min exposure to a concentration range of PKa. (D) Densitometric determination of cleaved HK (cHK)/HK ratio after 15 min exposure to 11.3 nmol L^{-1} PKa. The prognosed cooperative activity of both enzymes is indicated by the striped bar. Data represent means ± SD of three separate experiments, all performed in duplicate. Data were analyzed by one-way ANOVA (**$P < 0.005$, ****$P < 0.0001$).

3 The proline-rich region of FXII contains naturally occurring cleavage sites for a variety of enzymes, including neutrophil elastase and cathepsin K. These enzymes are unable to directly activate FXII. However, truncation by these enzymes primes FXII for activating cleavage by PKa (Figs 4 and 5). These findings are strikingly similar to our previous observations with FXII-T309K. However, in this case no pathological mutation is required.

In earlier studies, it was demonstrated that the binding of FXII to the polyanion dextran sulfate [31], or the binding of monoclonal antibodies to the surface binding domains, lowers the threshold for FXII activation by PKa [9,10]. This suggests that the activating cleavage site R353 is shielded, unless FXII binds to activating surfaces.

In the current study, we explore the proline-rich region for the presence of truncating cleavage sites. The proline-rich region is of particular interest given its unique presence in FXII. The sequence from the proline-rich region up to Cys^{340} is not strictly conserved between species. However, prediction models indicate that cleavage sites for neutrophil elastase, matrix metalloprotease 9 (MMP-9) and cathepsin K are generally present within these sequences across species (Table 1). Truncations with similar functionality have been shown to take place outside the proline-rich region: previous studies identified a cleavage site for MMP-12 and MMP-14 after S331 [32]. These studies concluded that this cleavage event degrades FXII, impairing its ability to contribute to coagulation and fibrinolysis. Our current findings suggest otherwise: although elimination of its surface-binding domains should reduce its capacity as a clotting factor, its potential to become activated in solution should increase. Our data suggest that neutrophil elastase is not only a negative regulator of FXII [25]: truncation strongly accelerates FXII activation in solution (Fig. 4). The localized swelling that is generally seen in HAE suggests that bradykinin production is a local process [33]. However, simultaneous swelling at multiple locations occurs in some patients, which suggests a systemic activation process [34].

Despite intensive investigation, a contact surface has not been pinpointed in bradykinin-driven disease. We generally assume that physiological contact activation requires a surface, which may be delivered by platelets during clot formation [35]. By analogy, we proposed that bradykinin production on vascular endothelium is a cell surface-receptor-dependent mechanism [36]. These mechanisms depend on the surface-binding domains of FXII and (c)HK. When FXII is fragmented by PKa, βFXIIa can continue its role as a PK activator (but not as a clotting factor) in a surface-independent manner [7]. Our findings in FXII-HAE suggest that accelerated FXII fragmentation and unregulated PK activation by dissociated βFXIIa form the molecular basis for swelling attacks [14]. Our current findings expand on this principle: we propose that bradykinin production can also be initiated in the absence of a surface when FXII is truncated by enzymes that are not classically linked to the contact system. Future studies are needed to explore whether non-canonical truncation of FXII contributes to the role of the contact system in pathology. This may, for example, be the case in anaphylaxis, where FXII is cleaved in a PK-independent manner [3].

Our studies identify that truncation of FXII primes the molecule for activation in solution. We hypothesize that the proline-rich region acts as a versatile sensor: it can be cleaved by a variety of enzymes that are released by or activated by (inflammatory) cells. This cleavage removes the sequence that shields R353, accelerating FXII activation and, ultimately, bradykinin production.

Addendum

S. de Maat, C.C. Clark, M. Boertien, N. Parr, W. Sanrattana, Z.L.M. Hofman and C. Maas performed experiments. S. de Maat, C.C. Clark, Z.L.M. Hofman and C. Maas were involved in the development of the concept, design and interpretation of data. S. de Maat and C. Maas wrote the manuscript.

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Disclosure of Conflict of Interests

C. Maas and S. de Maat are inventors on a patent application (patent P6064890EP) on enzyme inhibitors for treatment of bradykinin-mediated disease and have a financial interest in SERPINx BV, a spin-off company of the UMC Utrecht. The other authors state that they have no conflict of interest.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Chromogenic enzyme activation experiments.
Table S1. Primers for site-directed mutagenesis.
Data S1. Reagents.

References

1 Nussberger J, Cugno M, Cicardi M. Bradykinin-mediated angioedema. *N Engl J Med* 2002; **347**: 621–2.
2 Nuijens JH, Huijbregts CC, Eerenberg-Belmer AJ, Abbink JJ, Strack van Schijndel RJ, Felt-Bersma RJ, Thijs LG, Hack CE. Quantification of plasma factor XIIa-Cl(-)-inhibitor and kallikrein-Cl(-)-inhibitor complexes in sepsis. *Blood* 1988; **72**: 1841–8.
3 Sala-Cunill A, Björkqvist J, Senter R, Guilarte M, Cardona V, Labrador M, Nickel KF, Butler L, Luengo O, Kumar P, Labberton L, Long A, Di Gennaro A, Kenne E, Jämsä A, Krieger T, Schlüter H, Fuchs T, Flohr S, Hassiepen U, *et al*. Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions. *J Allergy Clin Immunol* 2015; **135**: 1031–43.e6.
4 McLaren M, Alkaabi J, Connacher M, Belch JJF, Valenete E. Activated factor XII in rheumatoid arthritis. *Rheumatol Int* 2002; **22**: 182–4.
5 Bode W, Schwager P. The refined crystal structure of bovine beta-trypsin at 1.8 A resolution. II. Crystallographic refinement, calcium binding site, benzamidine binding site and active site at pH 7.0. *J Mol Biol* 1975; **98**: 693–717.
6 Hedstrom L. Serine protease mechanism and specificity. *Chem Rev* 2002; **102**: 4501–24.
7 Revak SD, Cochrane CG, Bouma BN, Griffin JH. Surface and fluid phase activities of two forms of activated Hageman factor produced during contact activation of plasma. *J Exp Med* 1978; **147**: 719–29.
8 Samuel M, Pixley RA, Villanueva MA, Colman RW, Villanueva GB. Human factor XII (Hageman factor) autoactivation by dextran sulfate. Circular dichroism, fluorescence, and ultraviolet difference spectroscopic studies. *J Biol Chem* 1992; **267**: 19691–7.
9 Citarella F, te Velthuis H, Helmer-Citterich M, Hack CE. Identification of a putative binding site for negatively charged surfaces in the fibronectin type II domain of human factor XII – an immunochemical and homology modeling approach. *Thromb Haemost* 2000; **84**: 1057–65.
10 Ravon DM, Citarella F, Lubbers YT, Pascucci B, Hack CE. Monoclonal antibody F1 binds to the kringle domain of factor XII and induces enhanced susceptibility for cleavage by kallikrein. *Blood* 1995; **86**: 4134–43.
11 Chinnaraj M, Planer W, Pozzi N. Structure of coagulation factor II: molecular mechanism of thrombin generation and development of next-generation anticoagulants. *Front Med* 2018; **5**: 281.
12 Chinnaraj M, Chen Z, Pelc LA, Grese Z, Bystranowska D, Di Cera E, Pozzi N. Structure of prothrombin in the closed form reveals new details on the mechanism of activation. *Sci Rep* 2018; **8**: 2945.
13 Law RHP, Abu-Ssaydeh D, Whisstock JC. New insights into the structure and function of the plasminogen/plasmin system. *Curr Opin Struct Biol* 2013; **23**: 836–41.
14 de Maat S, Björkqvist J, Suffritti C, Wiesenekker CP, Nagtegaal W, Koekman A, van Dooremalen S, Pasterkamp G, de Groot PG, Cicardi M, Renné T, Maas C. Plasmin is a natural trigger for bradykinin production in patients with hereditary

angioedema with factor XII mutations. *J Allergy Clin Immunol* 2016; **138**: 1414–1423.e9.

15 Jukema BN, de Maat S, Maas C. Processing of factor XII during inflammatory reactions. *Front Med* 2016; **3**: 52.

16 Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF. Protein identification and analysis tools in the ExPASy server. *Methods Mol Biol* 1999; **112**: 531–52.

17 de Maat S, van Dooremalen S, de Groot PG, Maas C. A nanobody-based method for tracking factor XII activation in plasma. *Thromb Haemost* 2013; **110**: 458–68.

18 Song J, Tan H, Perry AJ, Akutsu T, Webb GI, Whisstock JC, Pike RN. PROSPER: an integrated feature-based tool for predicting protease substrate cleavage sites. Srinivasan N, editor. *PLoS ONE* 2012; **7**: e50300.

19 Hovinga JK, Schaller J, Stricker H, Wuillemin WA, Furlan M, Lämmle B. Coagulation factor XII Locarno: the functional defect is caused by the amino acid substitution Arg 353–>Pro leading to loss of a kallikrein cleavage site. *Blood* 1994; **84**: 1173–81.

20 Fujikawa K, McMullen BA. Amino acid sequence of human beta-factor XIIa. *J Biol Chem* 1983; **258**: 10924–33.

21 Ewald GA, Eisenberg PR. Plasmin-mediated activation of contact system in response to pharmacological thrombolysis. *Circulation* 1995; **91**: 28–36.

22 Oschatz C, Maas C, Lecher B, Jansen T, Bjorkqvist J, Tradler T, Björkqvist J, Tradler T, Sedlmeier R, Burfeind P, Cichon S, Hammerschmidt S, Müller-Esterl W, Wuillemin WA, Nilsson G, Renné T. Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. *Immunity* 2011; **34**: 258–68.

23 Meier HL, Heck LW, Schulman ES, MacGlashan DW. Purified human mast cells and basophils release human elastase and cathepsin G by an IgE-mediated mechanism. *Int Arch Allergy Appl Immunol* 1985; **77**: 179–83.

24 Meier HL, Schulman ES, Heck LW, MacGlashan D, Newball HH, Kaplan AP. Release of elastase from purified human lung mast cells and basophils. Identification as a Hageman factor cleaving enzyme. *Inflammation* 1989; **13**: 295–308.

25 Meier HL, Flowers B, Silverberg M, Kaplan AP, Newball HH. The IgE-dependent release of a Hageman factor cleaving factor from human lung. *Am J Pathol* 1986; **123**: 146–54.

26 Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, Lee-Rykaczewski E, Coleman L, Rieman D, Barthlow R, Hastings G, Gowen M. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. *J Biol Chem* 1996; **271**: 12511–6.

27 Suffritti C, Zanichelli A, Maggioni L, Bonanni E, Cugno M, Cicardi M. High-molecular-weight kininogen cleavage correlates with disease states in the bradykinin-mediated angioedema due to hereditary C1-inhibitor deficiency. *Clin Exp Allergy* 2014; **44**: 1503–14.

28 Hess R, Wujak L, Hesse C, Sewald K, Jonigk D, Warnecke G, Fieguth H-G, de Maat S, Maas C, Bonella F, Preissner KT, Weiss B, Schaefer L, Kuebler WM, Markart P, Wygrecka M. Coagulation factor XII regulates inflammatory responses in human lungs. *Thromb Haemost* 2017; **117**: 1896–907.

29 Zamolodchikov D, Chen Z-L, Conti BA, Renné T, Strickland S. Activation of the factor XII-driven contact system in Alzheimer's disease patient and mouse model plasma. *Proc Natl Acad Sci USA* 2015; **112**: 4068–73.

30 Pixley RA, De La Cadena R, Page JD, Kaufman N, Wyshock EG, Chang A, Taylor FB, Colman RW. The contact system contributes to hypotension but not disseminated intravascular coagulation in lethal bacteremia. In vivo use of a monoclonal anti-factor XII antibody to block contact activation in baboons. *J Clin Invest* 1993; **91**: 61–8.

31 Citarella F, Wuillemin WA, Lubbers YT, Hack CE. Initiation of contact system activation in plasma is dependent on factor XII autoactivation and not on enhanced susceptibility of factor XII for kallikrein cleavage. *Br J Haematol* 1997; **99**: 197–205.

32 Hiller O, Lichte A, Oberpichler A, Kocourek A, Tschesche H. Matrix metalloproteinases collagenase-2, macrophage elastase, collagenase-3, and membrane type 1-matrix metalloproteinase impair clotting by degradation of fibrinogen and factor XII. *J Biol Chem* 2000; **275**: 33008–13.

33 Nussberger J, Cugno M, Cicardi M, Agostoni A. Local bradykinin generation in hereditary angioedema. *J Allergy Clin Immunol* 1999; **104**: 1321–2.

34 Hofman ZLM, Relan A, Zeerleder S, Drouet C, Zuraw B, Hack CE. Angioedema attacks in patients with hereditary angioedema: local manifestations of a systemic activation process. *J Allergy Clin Immunol* 2016; **138**: 359–66.

35 Verhoef JJF, Barendrecht AD, Nickel KF, Dijkxhoorn K, Kenne E, Labberton L, McCarty OJT, Schiffelers R, Heijnen HF, Hendrickx AP, Schellekens H, Fens MH, de Maat S, Renné T, Maas C. Polyphosphate nanoparticles on the platelet surface trigger contact system activation. *Blood* 2017; **129**: 1707–17.

36 de Maat S, de Groot PG, Maas C. Contact system activation on endothelial cells. *Semin Thromb Hemost* 2014; **40**: 887–94.