

## ORIGINAL ARTICLE

# Blocking domain 6 of high molecular weight kininogen to understand intrinsic clotting mechanisms

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**Abstract**

**Background:** The contact system is initiated by factor (F) XII activation and the assembly of high molecular weight kininogen (HK) with either FXI or prekallikrein (PK) on a negatively charged surface. Overactivation of this system contributes to thrombosis and inflammation in numerous diseases. To develop effective therapeutics for contact system disorders, a detailed understanding of this pathway is needed.

**Methods:** We performed coagulation assays in normal human plasma and various factor-deficient plasmas. To evaluate how HK-mediated PK and FXI activation contributes to coagulation, we used an anti-HK antibody to block access to domain 6 of HK, the region required for efficient activation of PK and FXI.

**Results:** FXI's binding to HK and its subsequent activation by activated FXII contributes to coagulation. We found that the 3E8 anti-HK antibody can inhibit the binding of FXI or PK to HK, delaying clot formation in human plasma. Our data show that in the absence of FXI, however, PK can substitute for FXI in this process. Addition of activated FXI (FXIa) or activated PK (PKa) abolished the inhibitory effect of 3E8. Moreover, the requirement of HK in intrinsic coagulation can be largely bypassed by adding FXIa. Like FXIa, exogenous PKa shortened the clotting time in HK-deficient plasma, which was not due to feedback activation of FXII.

**Conclusions:** This study improves our understanding of HK-mediated coagulation and provides an explanation for the absence of bleeding in HK-deficient individuals. 3E8 specifically prevented HK-mediated FXI activation; therefore, it could be used to prevent contact activation-mediated thrombosis without altering hemostasis.

**KEYWORDS**

clotting factors, contact system, intrinsic coagulation, kallikrein, kininogen

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## Essentials

- Domain 6 of high molecular weight kininogen (HK) mediates intrinsic coagulation and disease.
- HK-dependent and -independent coagulation was evaluated in human plasma by blocking HK's domain 6.
- Blocking domain 6, we show that plasma kallikrein can substitute for factor XIa in coagulation.
- Blocking HK's domain 6 could be a safe treatment for thrombosis as it would not alter hemostasis.

## 1 | INTRODUCTION

Blood clotting occurs by activation of the extrinsic or intrinsic coagulation cascade, where fibrin formation is achieved by sequential activation of clotting factors/proteases (Scheme 1).<sup>1,2</sup> The extrinsic pathway requires activation of factor VII (FVII) by tissue factor to activate FX. Activated FX (FXa) cleaves prothrombin into thrombin, which is required for fibrin generation. However, the intrinsic pathway requires activated FIX (FIXa) to activate FX.<sup>2,3</sup> To initiate the intrinsic pathway, coagulation FXII is activated by binding to a negatively charged surface (contact activation). Activated FXII (FXIIa) then activates FXI and prekallikrein (PK).<sup>4</sup> In this simplified scheme, activated FXI (FXIa) activates FIX for downstream activation of FX. FX and its downstream factors constitute the common pathway for both extrinsic and intrinsic clotting.<sup>2,5</sup> Deficiency in common and extrinsic coagulation pathway factors is associated with severe bleeding abnormalities.<sup>3</sup> However, deficiency in FXI shows mild-to-moderate bleeding and mostly only affects organs with high fibrinolytic activity.<sup>4,6</sup> Surprisingly, deficiency in high molecular weight kininogen (HK), PK, or FXII, which are needed for FXI activation, is not associated with bleeding.<sup>2,7</sup> This discrepancy is fairly well explained with evidence that FXI is activated by thrombin.<sup>8</sup>

HK circulates in the plasma complexed with either FXI or PK. This complex is required for the efficient activation of FXI or PK by FXIIa.<sup>9–14</sup> Though FXIIa-mediated activation of FXI is not needed for hemostasis, contribution of HK and FXII in generating FXIa is confirmed in several diseases.<sup>7,15–25</sup> For example, in a mouse model of sickle cell disease, HK contributes to hypercoagulation and increased inflammation, resulting in organ damage and early mortality.<sup>26</sup> Most recently, plasma from COVID-19 patients showed increased levels of cleaved HK<sup>27</sup> and FXII activation<sup>28</sup> as a part of disease pathology. Similarly, blocking contact-mediated activation of FXII or FXI reduces thrombosis<sup>29</sup> and provides protection against bacteria-induced organ damage in baboons.<sup>30,31</sup> Also, reducing contact system activation minimizes extravasation of vascular proteins in the brain parenchyma and improves memory in a mouse model of Alzheimer's disease.<sup>25</sup>

Because HK-mediated contact system activation is associated with pathological conditions, a better understanding of the role of HK in coagulation could be helpful in preventing both PK and FXI overactivation in diseases. Also, it would be important to explore how coagulation occurs in the absence of HK. This information could provide a rationale for why the role of HK is limited to thrombosis and inflammation, whereas hemostasis is not affected by HK deficiency.

To better understand the HK-dependent coagulation and its implication in disease, we used our monoclonal anti-HK antibody (3E8),<sup>32</sup> which recognizes domain 6 of HK,<sup>33</sup> the binding site for both PK and FXI, and studied the coagulation cascade in human plasma.<sup>9–14</sup> Because HK is a nonenzymatic protein and not required for hemostasis, we also analyzed how plasma clots when HK is absent and whether its deficiency can be compensated for by other coagulation factors. Because 3E8 blocks both PK and FXI activation, we used various human factor-deficient plasmas to compare the effect of blocking PK and FXI on coagulation using 3E8. In this study, we analyzed the FXIa-like role of PKa by blocking HK-mediated PK activation in plasma. Also, the efficiency of FXIa and PKa in correcting the coagulation delay in several factor-deficient human plasmas was analyzed and compared. Collectively, this study improves our understanding of HK-dependent and HK-independent coagulation pathways.

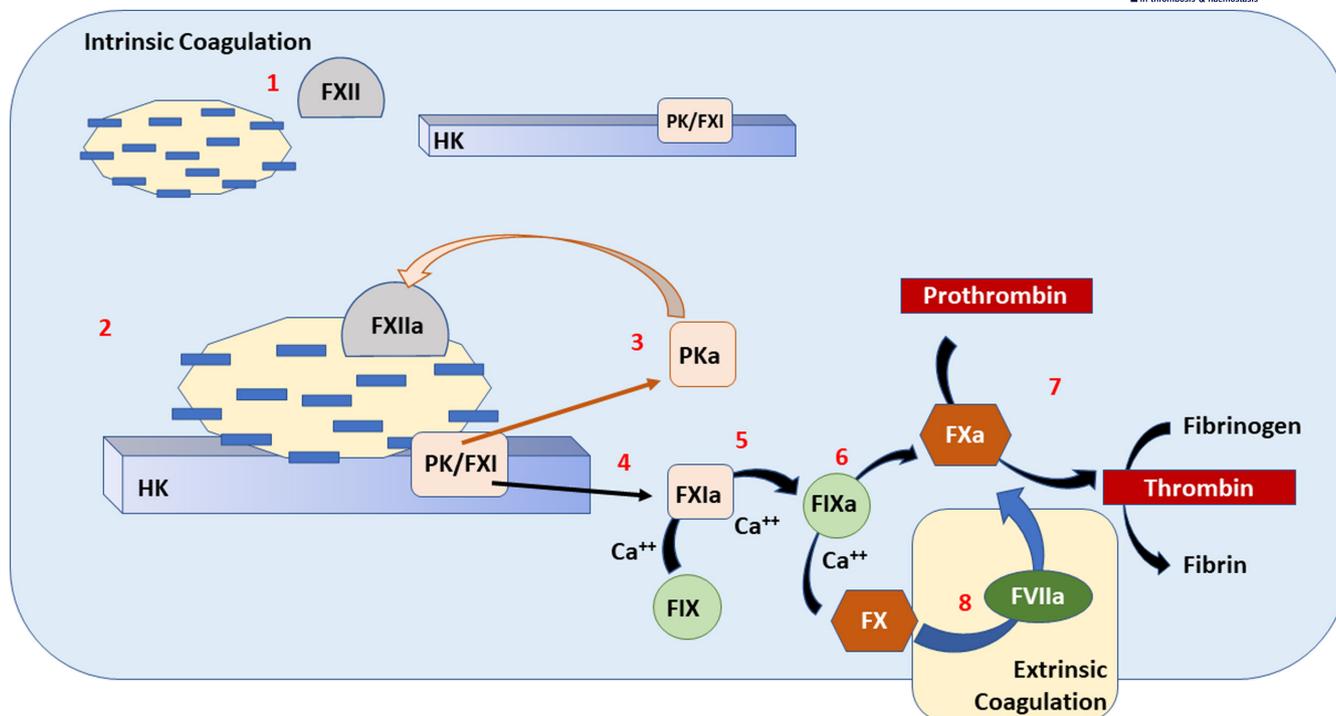
## 2 | MATERIALS AND METHODS

### 2.1 | Human plasma, purified coagulation factors, and reagents

Normal human pooled plasma (NPP) and human coagulation factor-deficient plasmas were obtained from George King Biomedical, Inc., and Technoclone. Purified coagulation factors were obtained from Hematologic Technologies and Enzyme Research Laboratories. We used Thermo Scientific's Pacific Hemostasis ellagic acid-based activated partial thromboplastin time (APTT) reagent (APTT-XL) and tissue factor containing prothrombin time (PT) reagent (Thromboplastin-D). Corn trypsin inhibitor (CTI), human anti-FIX (AHIX5051), and anti-FXIa (AHXI-5061) antibodies were from Hematologic Technologies. Anti-HK antibodies (3E8 and 2B7) were generated in-house as described.<sup>32</sup> Hamster IgG control antibody was from Innovative Research.

### 2.2 | Activated partial thromboplastin time

Briefly, plasma (30  $\mu$ l) was half-diluted in HEPES-buffered saline (HBS; 20mM HEPES, pH 7.4, 140mM NaCl) and incubated in the presence or absence of 3E8, control IgG, or anti-FXI antibodies in a 96-well plate for 10 min at 37°C. After incubation, APTT-XL (30  $\mu$ l) was added to the wells and further incubated for 5 min. Finally, CaCl<sub>2</sub> (30  $\mu$ l, 25 mM) was added, and clotting was monitored at 350nm using a spectrophotometer (Molecular Devices) at 37°C.<sup>34</sup> The antibody concentration in the reaction mixture was 670nM. In the kinetic measurement,



**SCHEME 1** Sequential activation of coagulation proteases in the extrinsic and intrinsic clotting cascades. In the intrinsic pathway, FXIIa formation occurs on a negatively charged surface (1, 2). Also, efficient activation of HK-bound FXI or PK by FXIIa occurs on a negatively charged surface (1, 2). PKa (kallikrein) generated upon PK cleavage by FXIIa also triggers the feedback activation of FXII (3). For simplicity, both PK and FXI are shown to bind on a single HK molecule, and cleaved HK and bradykinin are not shown. FXIIa cleaves FXI into active FXIa (4), which is needed for conversion of FIX to FIXa (5). FIXa subsequently cleaves FX into FXa (6), which is required for prothrombin cleavage into thrombin (7). The presence of calcium ions ( $\text{Ca}^{++}$ ) is required for clotting. FIXa and FXa also require cofactor FVIIIa and cofactor FVa, respectively. In the extrinsic pathway, FVIIa is necessary to generate FXa by cleaving FX (8). FVIIa generation requires tissue factor in extrinsic clotting. The cofactors (FVIIIa, FVa) and tissue factor are not shown in this simplified scheme. "a" denotes the activated form of the coagulation factor.

the time point at which turbidity increased linearly and continuously (at 350nm) was defined as clotting time (seconds). For plotting and analysis, the baseline/initial optical density of plasma in solution was subtracted from the kinetic readings at 350nm.

To evaluate the effect of purified FXIa and PKa on neutralizing the effect of 3E8, these factors were individually added along with APTT-XL solution to the plasma incubated with 3E8. The final concentration of FXIa and PKa in the reaction mixture was 1.5 nM and 60nM, respectively. The concentrations of FXIa and PKa used in the reaction were less than the concentration of their zymogens in human plasma.<sup>7,35</sup> The APTT was also performed in the presence of CTI, an FXIIa blocker.<sup>36</sup> CTI also blocked residual FXIIa contaminant in PKa preparation (Table S1). The final concentration of CTI in the reaction mixture was 2.66  $\mu\text{M}$ . Experiments were repeated at least three times.

### 2.3 | Prothrombin time

The spectrophotometer-based PT test of the plasma incubated with or without 3E8 antibody was also performed in a 96-well plate using a PT reagent (Thromboplastin D with  $\text{CaCl}_2$ ).<sup>34</sup> In brief, plasma (30  $\mu\text{l}$ ) was half-diluted in HBS and incubated in the presence or absence of

3E8 antibody (670 nM) in a 96-well plate for 10 min at 37°C. The PT reagent (60  $\mu\text{l}$ ) was added to initiate the clotting at 37°C and monitored at 350nm using a spectrophotometer (Molecular Devices).

To evaluate the effect of anti-FXI and anti-FIX in PT, NPP and FX-deficient plasma (60  $\mu\text{l}$ ) was incubated with or without anti-FIX or anti-FXI antibody (600 nM) for 10 min at 37°C before adding Thromboplastin-D (120  $\mu\text{l}$ ). Clotting was spectrophotometrically monitored at 37°C. All experiments were repeated at least three times.

### 2.4 | Thrombin time

The thrombin time (TT) test was performed in a 96-well plate by adding purified human thrombin (1 U/ml) to plasma (30  $\mu\text{l}$ ) incubated in the presence or absence of 3E8 antibody (670 nM) in HBS. Clotting was spectrophotometrically monitored at 37°C. Experiments were repeated at least three times.

### 2.5 | Binding experiments

The binding affinity of 3E8, purified human FXI, or purified human PK to purified human HK was determined by ELISA. Briefly, microtiter

plates were coated with 3E8 antibody, FXI, or PK (100ng/well) in binding buffer overnight at 4°C. Wells were washed and blocked (1% bovine serum albumin, 0.1% Tween-20, in phosphate-buffered saline), and then different concentrations of purified HK (in blocking buffer) were added to assess binding. Bound HK was determined using biotinylated 2B7 antibody as described previously.<sup>32</sup> To evaluate how 3E8 interferes with the binding of HK to either FXI or PK, HK was added in the presence or absence of varying concentrations of 3E8, and ELISA was performed. Hamster IgG was used as a control. All the experiments were repeated at least three times.

## 2.6 | Mass spectrometry analysis

Purified coagulation factors were analyzed using mass spectrometry (MS) for their purity. Briefly, commercially purchased coagulation factors (40 µg) were reduced and alkylated with dithiothreitol and iodoacetamide. Proteins were purified by chloroform/water/methanol extraction and digested with LysC and trypsin. Peptides were then purified by reverse phase solid phase extraction. A test sample was injected onto a Q-Exact system to gauge the amounts. Adjusted amounts were injected onto a Fusion Lumos system with two blank injections between every sample. Liquid chromatography–tandem MS data were processed using Proteome Discoverer and Mascot and queried against UniProt human database. The experiments were performed at The Rockefeller University Proteomics Resource Center.

## 2.7 | Statistical analyses

Comparisons between two groups were performed using unpaired two-tailed Student *t*-test and between multiple groups were performed using one-way ANOVA followed by Tukey multiple comparison test (GraphPad Prism).

## 3 | RESULTS

### 3.1 | The 3E8 anti-HK antibody blocks binding of FXI and PK to domain 6 of HK and delays intrinsic coagulation

The 3E8 antibody strongly binds to purified human HK (Figure 1A), and the extent of this binding was determined by its very low dissociation constant ( $K_d = 123.6 \pm 6.2$  pM). HK is required to initiate efficient FXI activation in the presence of a negatively charged surface.<sup>7</sup> To analyze whether blocking plasma HK with 3E8 affects intrinsic clotting, we performed APTT in NPP in the presence or absence of 3E8. The antibody was used at the equivalent physiological concentration of HK (670 nM).<sup>7</sup> The APTT in NPP was significantly delayed in the presence of 3E8 but not control IgG (Figure 1B). The binding of FXI at domain 6 of HK is the

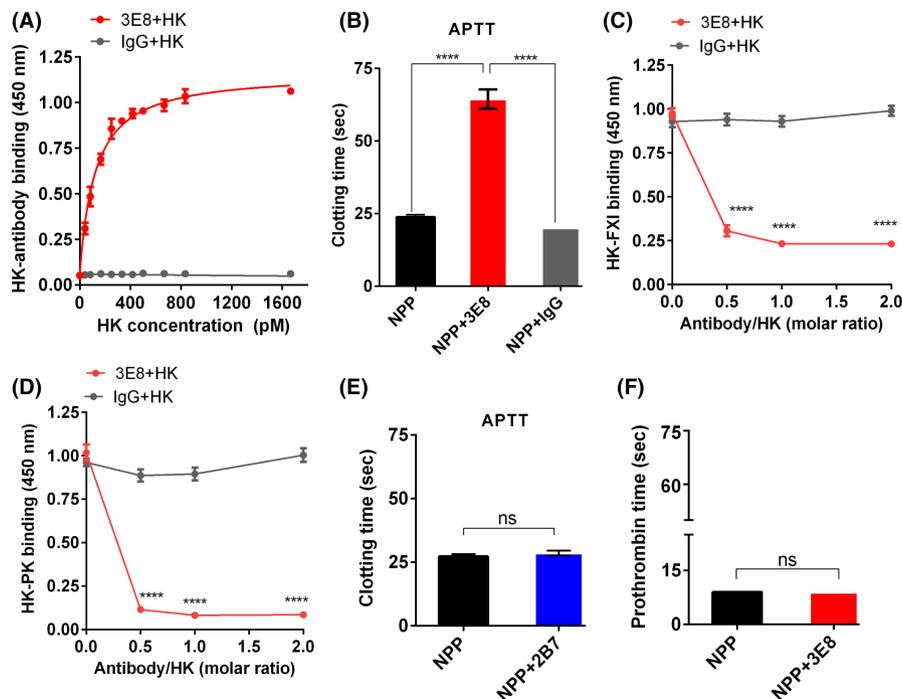
prerequisite for efficient FXI cleavage/activation by FXIIa.<sup>14</sup> The epitope of the 3E8 antibody (IQSDDDWIPDIQIDPNGLSF)<sup>32</sup> overlaps with the binding site of FXI at domain 6 of HK. Therefore, we determined the binding of FXI to HK in the presence and absence of 3E8. We found that FXI/HK binding was dramatically reduced in the presence of 3E8 but was not affected by the presence of IgG control (Figure 1C).

HK-bound PK is cleaved into active kallikrein (PKa) by FXIIa.<sup>7</sup> PKa not only cleaves HK to release proinflammatory bradykinin but also feeds back to continue activation of FXII.<sup>7</sup> Within domain 6 of HK, the binding sites of PK and FXI are overlapped.<sup>14</sup> Therefore, we determined whether 3E8 also affects the PK/HK interaction. By ELISA, we found that 3E8, but not control IgG, dramatically inhibited PK/HK binding (Figure 1D). Therefore, 3E8 could affect the PKa-mediated feedback activation of FXII. A monoclonal anti-HK antibody (2B7)<sup>32</sup> that binds to HK outside of domain 6 did not affect intrinsic clotting in NPP (Figure 1E), suggesting the effect of 3E8 in plasma is due to its ability to interfere with the binding of FXI and PK to domain 6 of HK. The 3E8 antibody also did not affect tissue factor-mediated extrinsic clotting in NPP (Figure 1F).

### 3.2 | In normal human plasma, the effect of 3E8 was modulated by the addition of activated coagulation factors

In plasma, 3E8 blocks the binding of FXI and PK to HK, preventing their efficient activation by FXIIa. Therefore, if 3E8 interferes with FXI activation, the addition of FXIa should abolish 3E8's effect on plasma clotting. Purified FXIa was added to NPP incubated with 3E8, and APTT was performed. Exogenous FXIa (1.5 nM) corrected the 3E8-induced delayed clotting in NPP (Figure 2A, blue). Because the conversion of PK to PKa is required for feedback activation of FXII,<sup>7</sup> we also evaluated the effect of exogenous PKa in the presence of 3E8. We found that the addition of PKa (60 nM) partially corrected the clotting delay caused by 3E8 in NPP (Figure 2A, green). This result suggests that requirement of HK in intrinsic clotting is curtailed in the presence of FXIa or PKa. Exogenous FXIa also corrected the 3E8-induced clotting delay in the presence of CTI, an FXIIa blocker<sup>36</sup> (Figure 2B, blue stripes), indicating that the effect of exogenous FXIa is not via FXIIa feedback activation.

Because PKa contributes to feedback FXIIa generation, the effect of exogenous PKa on correcting the 3E8-induced clotting delay could be also via FXIIa (Figure 2A, green). Consistently, when CTI was added, the effect of exogenous PKa on correcting 3E8-induced clotting delay was minimized (Figure 2B; green stripes). However, when both CTI and 3E8 were added to NPP, intrinsic clotting was severely delayed compared with either CTI or 3E8 alone (Figure S1). When the effect of exogenous PKa was compared with cumulative effect of both CTI and 3E8 (Figure S1), we found that exogenous PKa accelerated NPP clotting (Figure S1). These data indicate that PKa can also induce downstream coagulation in NPP independent of FXIIa, although less effectively.



**FIGURE 1** The 3E8 anti-HK antibody blocks the binding of FXI or PK to HK and delays intrinsic coagulation. HK binding studies were performed using ELISA. The clotting of normal human pooled plasma (NPP) was performed by spectrophotometer-based activated partial thromboplastin time (APTT) or prothrombin time (PT). (A) Representative binding curve shows HK binds to 3E8 (red) but not to the control IgG (gray). (B) The 3E8 antibody (670 nM), but not the control IgG (670 nM), delayed intrinsic clotting of NPP. (C) HK binding to FXI is inhibited in the presence of 3E8 (red) but not control IgG (gray). (D) HK binding to PK is inhibited in the presence of 3E8 (red) but not control IgG (gray). (E) The 2B7 anti-HK antibody (670 nM), which binds to HK outside of domain 6, did not affect the APTT of NPP. (F) 3E8 did not delay the tissue factor-driven extrinsic coagulation as indicated by prothrombin time. The results are presented as mean  $\pm$  SEM.  $n \geq 3$ /group. \*\*\*\* $p < 0.0001$ . ns, not significant ( $p > 0.05$ ). Experiments repeated at least three times.

### 3.3 | 3E8 does not block FXI autoactivation independent of HK and FXII

If the effect of the 3E8 antibody on intrinsic clotting is specifically due to its binding to HK, it should not affect clotting of HK-deficient plasma, and it did not (Figure 2C; red vs. gray). To further evaluate the effect of 3E8, APTT was performed using HK-deficient plasma, where HK deficiency was compensated by exogenous HK. As expected, exogenous HK (670 nM) normalized the delayed clotting in HK-deficient plasma (Figure 2D, gray). However, when purified HK was briefly preincubated with 3E8 (1:1 molar ratio) before adding to plasma, it could not correct the delayed clotting in HK-deficient plasma (Figure 2D, brown). This result demonstrates that when 3E8 is bound to exogenous HK, the endogenous FXI or PK in HK-deficient plasma cannot bind to HK domain 6 for their efficient activation, and therefore, the effect of exogenous HK bound to 3E8 is very minimal (Figure 2D).

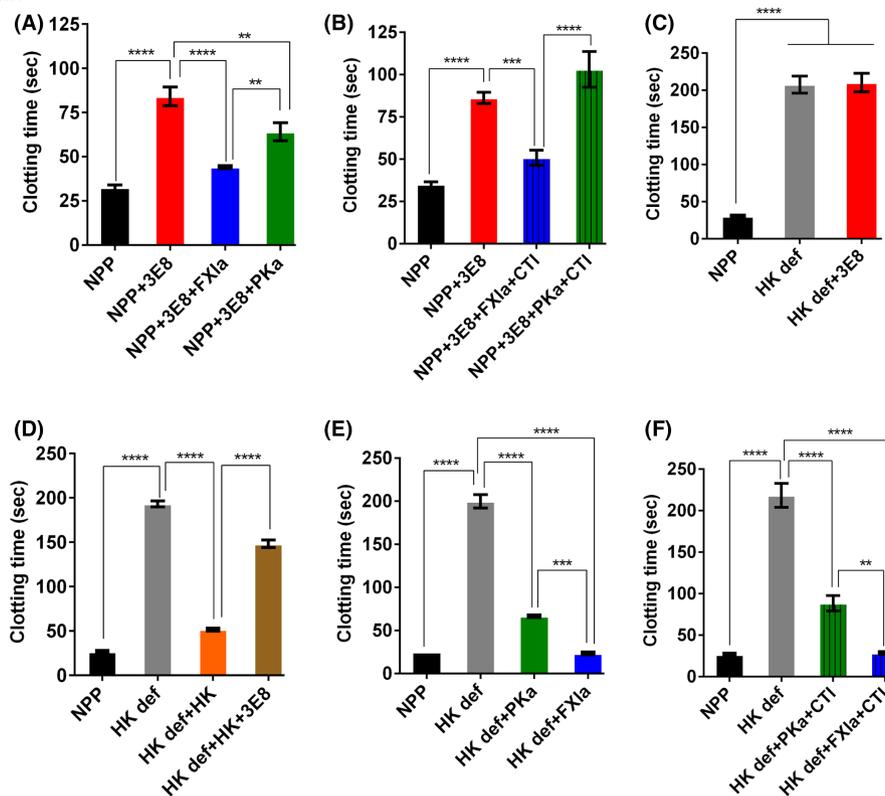
Unlike 3E8, an anti-FXIa antibody further prolonged the intrinsic clotting in HK-deficient plasma, (Figure S2A). However, this antibody did not show any effect in FXI-deficient plasma (Figure S2B), suggesting that in HK-deficient plasma, the anti-FXIa antibody specifically blocks FXIa, which is generated independent of HK. Therefore, in HK-deficient individuals, some FXI activation occurs and contributes to coagulation in an HK-independent manner. However, 3E8, which

specifically blocks HK-mediated FXI activation, did not further delay HK-deficient plasma clotting (Figure 2C).

3E8 did not affect intrinsic clotting of FXII-deficient human plasma (Figure S3), confirming that the activation of HK-bound FXI requires the presence of FXII. Although FXII-deficient plasma's intrinsic clotting is very prolonged (Figure S3), the plasma eventually clots, suggesting the involvement of FXII-independent activation of FXI in this system as previously reported.<sup>37–39</sup> However, 3E8 did not affect FXII-independent or thrombin-induced FXI activation.<sup>33</sup> This result suggests that FXII is involved in 3E8-mediated delayed intrinsic clotting.

### 3.4 | In HK-deficient human plasma, addition of FXIa or PKa compensates for HK

HK is a procoagulant but a nonenzymatic factor because it primarily aids in the activation of FXI and PK by FXIIa.<sup>14</sup> HK also blocks autoactivation of FXI or thrombin-induced FXIa generation.<sup>38,39</sup> Therefore, blocking FXIa generation independent of HK further prolonged clotting in HK-deficient plasma (Figure S2A). Alternatively, when we added exogenous FXIa (1.5 nM), the delayed intrinsic clotting in HK-deficient plasma was normalized (Figure 2E, blue). This effect was still observed when CTI was added to block FXIIa (Figure 2F, blue stripes), indicating that this result was not via FXIIa.



**FIGURE 2** In human plasma, the 3E8-mediated effect is HK-dependent, and 3E8 does not affect FXI autoactivation. Intrinsic clotting of human NPP and HK-deficient (HK def) plasma with and without 3E8 (670 nM) was performed by spectrophotometer-based APTT. Purified factors (PKa, FXIa, and HK) were individually added to plasma as indicated. The final concentrations of these factors were 1.5 nM (FXIa), 60 nM (PKa), and 670 nM (HK). CTI (2.66  $\mu$ M) was used to block FXIIa in reactions where indicated. (A) The intrinsic clotting delay by 3E8 was only partially corrected by addition of PKa (green vs. red) in NPP. However, added FXIa (blue) showed near-complete correction of delayed clotting in NPP (blue vs. red). (B) The effect of exogenous PKa was minimized in the presence of CTI as it blocked FXIIa generated via PKa feedback (green stripes). However, the effect of exogenous FXIa was not affected in the presence of CTI (blue stripes). (C) In the absence of HK, the effect of 3E8 on APTT was not observed (red vs. gray). (D) Addition of purified HK corrected the delayed clotting in HK-def plasma (orange vs. gray). However, when purified HK was briefly preincubated with 3E8, its effect on normalizing the delayed clotting was very minimal (brown vs. orange). (E) In HK def plasma, added FXIa normalized the clotting delay caused by the absence of HK (blue vs. gray). Exogenous PKa also significantly shortened the clotting time, although it was less effective than FXIa. (F) In HK def plasma, the effect of exogenous FXIa and PKa was also observed in the presence of CTI. Results are presented as mean  $\pm$  SEM.  $n \geq 3$ /group. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Experiments repeated at least three times.

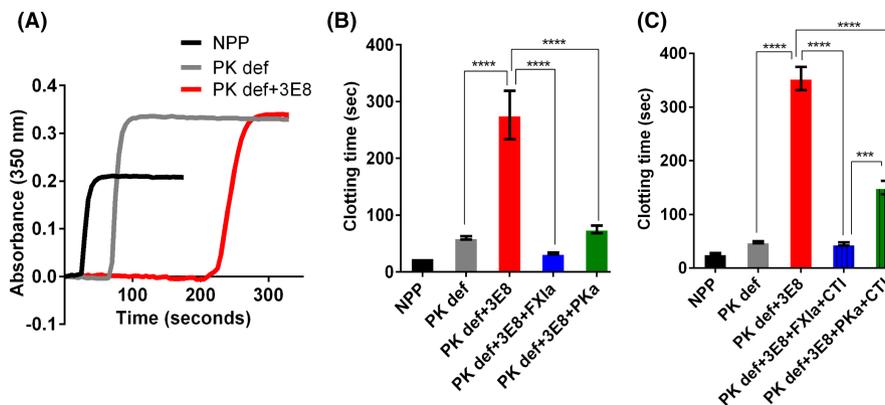
We also evaluated the role of PKa in HK-deficient plasma clotting. When HK-deficient plasma was supplemented with purified PKa (60 nM), the clotting delay was partially normalized (Figure 2E, green). This result further confirms a FXIa-like role of PKa in HK-deficient plasma. However, PKa is also involved in FXII activation via feedback, which could activate FXI. The effect of exogenous PKa on correcting the delayed intrinsic clotting in HK-deficient plasma was still observed when the FXIIa was blocked by CTI (Figure 2F, green stripes). These data indicate that PKa or FXIa can induce downstream coagulation independent of HK, which is also independent of FXII. However, PKa is not as effective as FXIa (Figure 2E and F).

### 3.5 | Effect of blocking HK's domain 6 on intrinsic clotting in the absence of PK and FXI

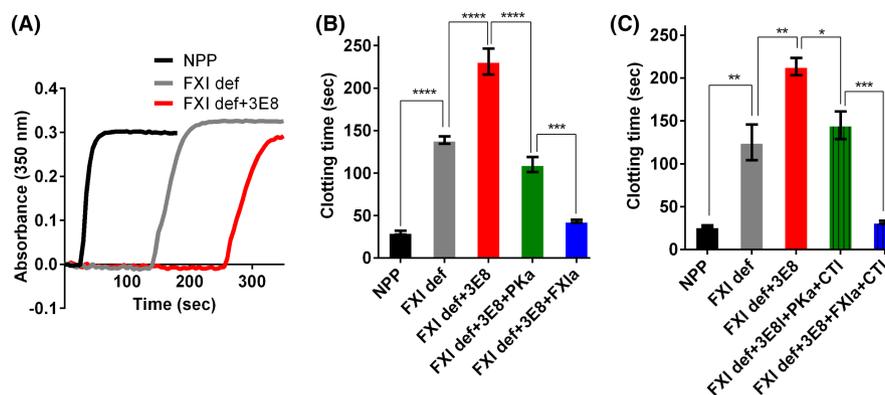
The intrinsic clotting in PK-deficient plasma was delayed in the presence of 3E8 (Figure 3A and B). Consistently, addition of FXIa corrected

the clotting delay in PK-deficient plasma with 3E8 (Figure 3B; blue) or with 3E8 and CTI together (Figure 3C; blue stripes). These results suggest that in the absence of PK, FXIa is a significant contributor to intrinsic clotting, and, therefore, blocking HK-mediated FXI activation with 3E8 severely prolongs APTT (Figure 3A).

Adding PKa to PK-deficient plasma significantly corrected the 3E8-induced clotting delay (Figure 3B; green). The effect of exogenous PKa was still significant when CTI was used to block FXIIa (Figure 3C; green stripes). However, exogenous PKa was less effective in the presence of both 3E8 and CTI (Figure 3C) because the contribution of FXIIa, generated via PKa-mediated feedback, was blocked by CTI. We also directly blocked FXIa in PK-deficient plasma using an anti-FXIa antibody, which led to severely delayed intrinsic clotting (Figure S2B). The effect of directly blocking FXIa in PK-deficient plasma was more profound than blocking HK-mediated FXI activation (Figure 3A and Figure S2B). This result suggests that some HK-independent activation of FXI occurs in PK-deficient plasma.



**FIGURE 3** FXI activation drives intrinsic coagulation in the absence of PK. The APTT of PK-deficient (PK def) plasma with and without 3E8 (670 nM) was performed using a spectrophotometer. CTI (2.66  $\mu$ M) was used to block FXIIa where indicated. (A) Representative clotting curve shows the effect of 3E8 in the APTT in PK def plasma. (B) 3E8 delayed intrinsic clotting time in PK def plasma (red vs. gray; 276.3  $\pm$  42.7 vs. 60.0  $\pm$  2.8 s). Addition of FXIa (1.5 nM) or PKa (60 nM) significantly corrected the effect of 3E8 (blue vs. red and green vs. red, respectively). (C) Effect of exogenous FXIa or PKa in the presence of CTI and 3E8. Exogenous PKa (green stripes) was less effective than FXIa (blue stripes) when CTI (2.66  $\mu$ M) was used to block FXIIa. The results are presented as mean  $\pm$  SEM.  $n \geq 3$ . \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Experiments repeated at least three times.

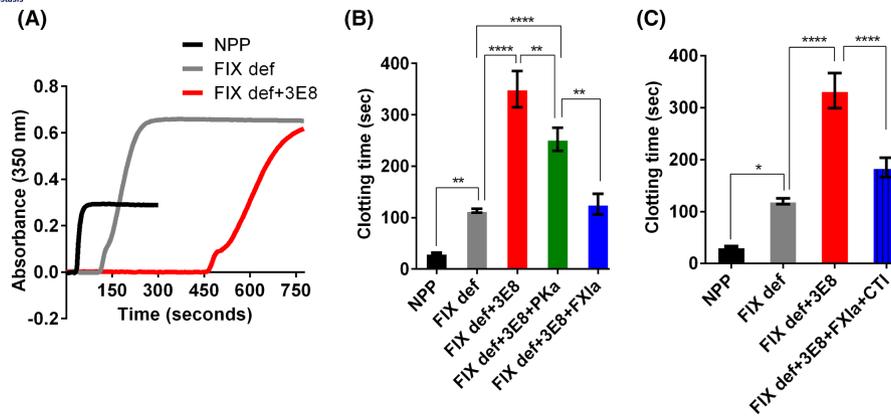


**FIGURE 4** PK activation is required for intrinsic coagulation in the absence of FXI. The APTT of FXI-deficient (FXI def) plasma with and without 3E8 (670 nM) was performed using a spectrophotometer. CTI (2.66  $\mu$ M) was used added to block FXIIa in some reactions. (A) Representative clotting curve shows the effect of 3E8 in the APTT in FXI def plasma. (B) 3E8 delayed the intrinsic clotting time in FXI def plasma (red vs. gray; 231.3  $\pm$  15.3 vs. 138.8  $\pm$  4.2 seconds). Addition of PKa (60 nM) corrected the effect of 3E8 in FXI def plasma (green vs. red). (C) Exogenous PKa significantly corrected the 3E8-induced delayed clotting in the presence of CTI (red vs. green stripes). Exogenous FXIa (1.5 nM) abolished the effect of 3E8 in FXI def plasma in the absence (B; blue vs. red) or presence (C; blue stripes vs. red) of CTI. The results are presented as mean  $\pm$  SEM.  $n \geq 3$ /group. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Experiments repeated at least three times.

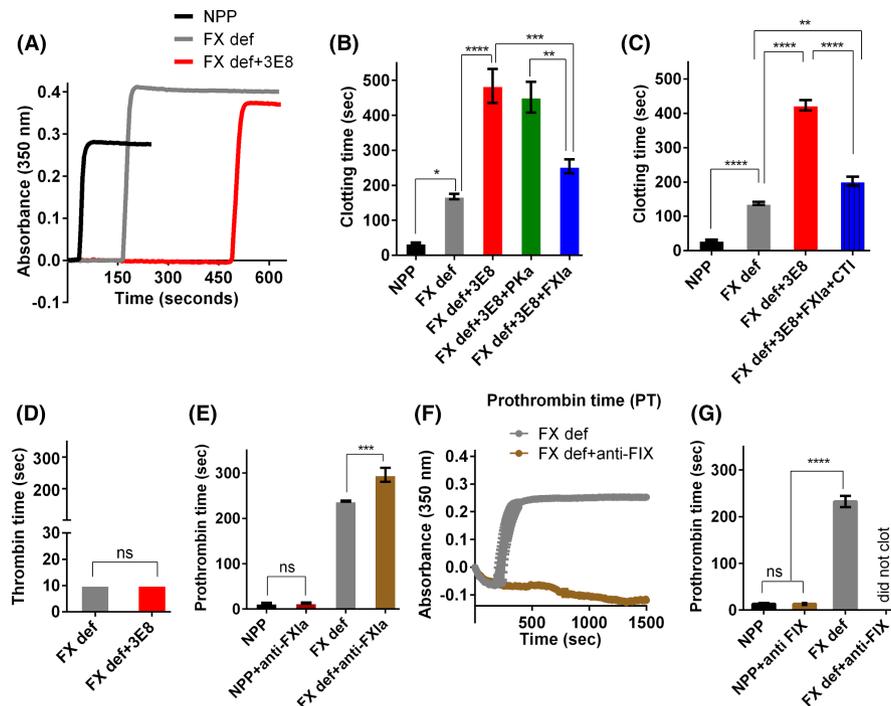
We further explored the role of HK-mediated PK activation in FXI-deficient plasma coagulation. As expected, the anti-FXIa antibody did not show any effect in FXI-deficient plasma (Figure 52B). However, 3E8 caused an additional clotting delay in FXI-deficient plasma because 3E8 prevented PK/HK binding and therefore PK activation (Figure 4A and B). Consistently, when exogenous PKa was added to FXI-deficient plasma with 3E8, plasma clotting time was shortened (Figure 4B; green). This effect was still significant despite CTI blocking FXIIa in FXI-deficient plasma (Figure 4C; green stripes). This result further supports the role of PKa in downstream intrinsic coagulation independent of FXII. As expected, exogenous FXIa completely corrected the clotting delay in FXI-deficient plasma with 3E8 (Figure 4B; blue) or with both 3E8 and CTI (Figure 4C; blue stripes).

### 3.6 | Effect of 3E8 on the common coagulation pathway

Our results are consistent with the findings that FXIa and PKa are both required for downstream activation of FIX.<sup>2,40</sup> We also found that 3E8 significantly prolonged the APTT in FIX-deficient human plasma (Figure 5A and B), suggesting that in the absence of FIX, HK-mediated activation of FXI and/or PK is needed to activate downstream clotting factors other than FIX. This 3E8-induced clotting delay was only partially corrected by exogenous PKa (Figure 5B; green). However, the effect of exogenous FXIa was much more pronounced (Figure 5B; blue). This result indicates a FIX-independent role of PKa and FXIa in coagulation. As



**FIGURE 5** HK-mediated FXI and/or PK activation is necessary for activation of downstream clotting factors other than FIX. The APTT of FIX-deficient plasma (FIX def) with and without 3E8 (670 nM) was performed using a spectrophotometer. (A) Representative clotting curve shows the effect of 3E8 in the APTT in FIX-def plasma. (B) 3E8 delayed the intrinsic clotting time in FIX-def plasma (red vs. gray;  $350.0 \pm 35.1$  vs.  $113.6 \pm 3.7$  seconds). Addition of PKa (60 nM) partially corrected the anticoagulating effect of 3E8 (green vs. red). However, exogenous FXIa (1.5 nM) was much more effective at correcting the clotting time (blue vs. red or green, respectively). (C) FXIa significantly corrected the 3E8-induced delay even in presence of FXIIa inhibitor, CTI (2.66  $\mu$ M; blue stripes vs. red). The results are presented as mean  $\pm$  SEM.  $n \geq 3$ /group.  $**p < 0.01$ ,  $****p < 0.0001$ . Experiments repeated at least three times.



**FIGURE 6** Blocking domain 6 of HK by 3E8 reveals an FX-independent alternate pathway for thrombin generation. Clotting of NPP and FX-deficient (FX def) plasma with and without 3E8 (670 nM) or anti-FIX antibody (200 nM) was performed using a spectrophotometer. CTI (2.66  $\mu$ M) was used to block FXIIa in some reactions. (A) Representative curve shows the effect of 3E8 in the APTT of FX def plasma. (B) 3E8 delayed the intrinsic clotting time in FX def plasma (red vs. gray;  $484.0 \pm 48.5$  vs.  $168.0 \pm 7.5$  seconds). Addition of PKa (60 nM) did not significantly alter the effect of 3E8 (green vs. red). However, addition of FXIa (1.5 nM) significantly normalized the anti-clotting effect of 3E8 (blue vs. red). (C) The effect of FXIa on correcting the 3E8-induced clotting delay was still significant in the presence of CTI (blue stripes vs. red). (D) In FX def plasma, 3E8 did not affect exogenous thrombin-induced conversion of fibrinogen to fibrin as measured by thrombin time test. (E) Directly blocking FXIa prolonged extrinsic clotting, measured by prothrombin time (PT) test, in FX def plasma but not in NPP (gray vs. black). (F) Representative clotting curve shows that blocking FIX using an anti-FIX antibody (200 nM) in FX def plasma dramatically affected PT. Clotting did not begin even by 25 min. (G) Quantification of PT shows that anti-FIX antibody did not affect the PT in NPP (black vs. brown), but it did show an effect in the absence of FX. Results are presented as mean  $\pm$  SEM.  $n \geq 3$ /group.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ ; ns, not significant ( $p > 0.05$ ). Experiments repeated at least three times.

expected, exogenous FXIa also significantly corrected the clotting delay in FIX-deficient plasma with both 3E8 and CTI (Figure 5C; blue stripes). However, the effect of exogenous PKa was minimized when CTI was also added with 3E8 in FIX-deficient plasma (Figure S4). Exogenous PKa accelerated clotting of FIX-deficient plasma with both 3E8 and CTI; however, the clotting was still quite prolonged (Figure S4).

In the downstream coagulation cascade (common pathway), FXa is required for thrombin formation.<sup>41</sup> Surprisingly, in FX-deficient plasma, 3E8 also dramatically prolonged the APTT (Figure 6A and B). The effect of 3E8 in FX-deficient plasma suggests the requirement of PKa and/or FXIa in direct thrombin generation independent of FX. However, addition of PKa did not correct the 3E8-induced clotting delay in FX-deficient plasma (Figure 6B; green). Exogenous FXIa significantly corrected the clotting delays incurred by 3E8 (Figure 6B; blue) or both 3E8 and CTI (Figure 6C; blue stripes) in FX-deficient plasma.

Although FXIa can directly cleave prothrombin, the cleaved product is not able to induce clotting.<sup>42</sup> However, addition of FXIa corrected the 3E8-induced clotting delay in FX-deficient plasma (Figure 6B; blue). MS analysis revealed that the FXIa preparation was extremely pure and devoid of any other coagulation factors (Table S1). Because FXIa does not directly generate functional thrombin,<sup>42</sup> the clotting correction by exogenous FXIa was likely via activation of FIX. However, it is not known whether FIXa can directly cleave prothrombin to generate functional thrombin in the absence of FX. TT was examined to determine if 3E8 also affects thrombin-induced conversion of fibrinogen to fibrin in FX-deficient plasma. Because 3E8 did not affect TT (Figure 6D), the result suggests the involvement of upstream FIX. The FIX gene arose from a duplication of the FX gene, and both have very similar structures.<sup>43</sup> Therefore, we hypothesize that FIX may have FX-like activity, which could be responsible for FIX's role in coagulation in the absence of FX.

In extrinsic clotting, the tissue factor/FVIIa complex directly activates FX for prothrombin cleavage (Scheme 1). Therefore, in the absence of FX, extrinsic clotting measured by tissue factor-mediated prothrombin time (PT) test is severely delayed (Figure 6E). However, if FIXa contributes to extrinsic clotting in FX-deficient plasma, blocking FIX activation should further prolong the PT. Therefore, we decided to block FXIa-mediated FIX activation in FX-deficient plasma. First, we performed PT in FX-deficient plasma in the presence of an anti-FXIa antibody. As expected, the anti-FXIa antibody did not show any effect in extrinsic clotting of NPP (Figure 6E). However, in FX-deficient plasma, it further prolonged the PT (Figure 6E). Blocking FXIa only mildly prolonged extrinsic clotting in FX-deficient plasma (Figure 6E) because the tissue factor used in the assay is also a direct activator of FIX.<sup>44,45</sup>

We therefore directly blocked FIX in FX-deficient plasma using an anti-FIX monoclonal antibody, which binds the FIX heavy chain that contains the catalytic domain.<sup>46</sup> Addition of this anti-FIX antibody severely affected the PT such that the sample was still not clotted by 25 min (Figure 6F and G). However, blocking FIX did not

affect extrinsic clotting of NPP (Figure 6G) because NPP contains FX. Together, these results suggest that a FX-independent coagulation mechanism may exist in FX-deficient patients, where activation of FIX is a critical step for initiating coagulation. However, because FX-deficient individuals suffer from major bleeding problems,<sup>47</sup> this FX-independent pathway may have only very limited protection during bleeding episodes.

## 4 | DISCUSSION

Epidemiological studies suggest that an abnormal APTT, indicative of a defective intrinsic clotting pathway, is a risk factor for thrombosis.<sup>48-50</sup> In intrinsic coagulation, HK-mediated FXI activation by FXIIa is required to initiate downstream thrombin generation (Scheme 1). Depending on FXI levels in plasma, thrombosis or bleeding complications may arise. For example, severe FXI deficiency is associated with mild-to-moderate bleeding in organs with increased fibrinolytic activity.<sup>51</sup> Similarly, an increased FXI level is associated with increased risk of venous thrombosis.<sup>52</sup> Therefore, precise management of FXI activation could be an effective therapeutic strategy to control both bleedings and thrombosis.

Although FXII or HK deficiency is not associated with bleeding in humans, the roles of HK and FXII in FXI and PK activation are evident in many pathological conditions.<sup>24,30,31,53</sup> Also, it is likely that in the individuals with increased FXI levels,<sup>52</sup> elevated FXI activation via HK/FXII could contribute to thrombosis. Therefore, blocking HK's domain 6 could be a therapeutic strategy for thrombotic diseases. This therapy should not affect hemostasis as FXIa for hemostasis is generated by thrombin rather than FXIIa.<sup>8</sup> Furthermore, our 3E8 antibody does not block the thrombin-induced activation of FXI.<sup>33</sup>

To induce its effect, 3E8 replaces bound FXI or PK from HK<sup>33</sup> via ligand substitution, which is possible because of 3E8's very low dissociation constant to HK ( $K_d = 123.6 \pm 6.2$  pM) compared with those of FXI or HK.<sup>13</sup> Consistently, 3E8 did not show any effect in HK-deficient plasma (Figure 2C). However, clotting time was significantly prolonged in HK-deficient plasma treated with an anti-FXIa antibody (Figure S2A). This result further confirms the HK-independent activation of FXI and supports an alternative path for FXIa generation in HK-deficient individuals. Moreover, addition of exogenous FXIa normalized the delayed intrinsic clotting in HK-deficient plasma (Figure 2E and F). This result suggests that any requirement of HK in hemostasis can be largely bypassed if enough FXIa is generated (most likely via thrombin), which could explain why HK-deficient individuals do not show bleeding complications. We also showed that PKa can accelerate clotting in HK-deficient plasma and in a FXII-independent manner, although not as effectively as FXIa (Figure 2F).

HK-mediated PK activation plays a role in FXI-independent coagulation (Figure 4). A FXIa-like role of PKa in downstream coagulation is recently suggested by directly blocking PKa<sup>54</sup> and using purified PKa in plasma.<sup>40</sup> However, in blood, PK circulates bound to HK, and this complex is required for its efficient activation to PKa

(Scheme 1). Therefore, we blocked HK-mediated PK activation to establish the role of both HK and PK in FXI-deficient plasma clotting (Figure 4). In our study, we also compared the effect of exogenous PKa and FXIa in correcting the 3E8-induced clotting delay. Our result clearly demonstrates that PKa can act like FXIa, though it is not as effective as FXIa in downstream coagulation (Figure 4). This result could also partially explain why FXI-deficient individuals show bleeding episodes despite having compensatory PK. Though the role of PK in hemostasis is minimized in the presence of FXI, its role in disease-associated thrombosis should be evaluated. It would also be important to evaluate whether thrombin can directly activate PK as it does to FXI.

In PK-deficient plasma, FXI activation is sufficient (Figure 3), which is likely why overt bleedings do not occur in PK-deficient individuals.<sup>2</sup> Our results suggest that preventing FXIa generation in PK-deficient individuals would increase the risk of bleeding.

FXIa can also cleave prothrombin, although the cleaved product cannot induce clotting.<sup>42</sup> However, we found that exogenous FXIa corrected the 3E8-induced clotting delay in FX-deficient plasma (Figure 6B and C). MS analysis showed that the FXIa preparation was extremely pure (Table S1). Therefore, the effect of FXIa on correcting the delayed clotting is not direct, but via FIX. To the best of our knowledge, there is no prior report that FIX can be substituted for FX in coagulation. This study provides evidence that FIX can induce fibrin formation in the absence of FX. However, because congenital or acquired FX deficiency is associated with severe bleeding episodes<sup>47,55</sup> and mice lacking FX survive only for a few weeks,<sup>56</sup> this FX-independent pathway may have a limited auxiliary role in coagulation and hemostasis. Nevertheless, this pathway must be fully explored in detail to understand its role in human disease.

FIX-deficient plasma does not show prolonged PT, a measure of extrinsic coagulation; however, FIX-deficient individuals show spontaneous bleeding and serious intracranial hemorrhage, which sometimes leads to death.<sup>55</sup> Also, FIX knockout mice bleed to death after tail clipping,<sup>57</sup> indicating the significance of FIX in preventing bleeding. Our results show that blocking FIX activation can severely affect extrinsic coagulation (Figure 6). However, future studies are needed to determine the mechanistic details of this alternative pathway. Even though it is only observed in FX-deficient plasma, our extensive studies suggest a role for FIX in extrinsic coagulation. Therefore, deficiency in FIX or the acquisition of FIX inhibitors could further intensify bleeding complications in FX-deficient individuals. A recent large cohort analysis showed that systemic amyloidosis is associated with significantly increased risk of intracranial hemorrhage (hazard ratio 3.5) and subarachnoid hemorrhage (hazard ratio 6.7)<sup>58</sup> in humans. Acquired FX deficiency is also frequently found in patients with systemic amyloidosis.<sup>59</sup> Interestingly, combined FIX and FX deficiency is also reported in some patients with systemic amyloidosis.<sup>60–62</sup> These patients showed severe to fatal bleeding complications.<sup>60–62</sup> Based on our results, it is possible that combined deficiency of FIX and FX could also significantly increase the hemorrhagic incidences in some of the systemic amyloidosis patients.

Therefore, FIX levels should be carefully monitored in patients with this disease.

Moreover, our study clearly demonstrates the role of HK's domain 6 in coagulation and suggests that blocking this domain could be a safe approach in preventing disease-associated thrombosis without affecting hemostasis.

## AUTHOR CONTRIBUTIONS

P.K.S. designed research studies, conducted experiments, analyzed the data, and wrote the manuscript; Z.L.C. helped design the research studies and analyze the data; K.H. helped conduct the experiments; E.H.N. aided in research study design, data analysis, and manuscript writing. The manuscript has been read and approved for submission by all authors.

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## RELATIONSHIP DISCLOSURE

The 3E8 and 2B7 anti-HK antibodies have been licensed to Millipore Sigma. All authors declare no other conflicts of interest.

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#### SUPPORTING INFORMATION

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

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