Institutional Repository - Research Portal
Dépôt Institutionnel - Portail de la Recherche

researchportal.unamur.be

UNIVERSITÉ DE NAMUR
University of Namur

RESEARCH OUTPUTS / RÉSULTATS DE RECHERCHE

Inactivation of human coagulation factor X by a protease of the pathogen Capnocytophaga canimorsus

Hack, K; Renzi, F; Hess, E; Lauber, F; Douxfils, J; Dogné, Jean-Michel; Cornelis, G R

Published in:
Journal of Thrombosis and Haemostasis

DOI:
10.1111/jth.13605

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

Link to publication

Citation for pulished version (HARVARD):
Hack, K, Renzi, F, Hess, E, Lauber, F, Douxfils, J, Dogné, J-M & Cornelis, GR 2016, 'Inactivation of human coagulation factor X by a protease of the pathogen Capnocytophaga canimorsus', *Journal of Thrombosis and Haemostasis*. https://doi.org/10.1111/jth.13605

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 02. May. 2026

Accepted Article

Received Date : 09-Jun-2016
Revised Date : 05-Nov-2016
Accepted Date : 08-Dec-2016
Article type : Original Article - Coagulation

Inactivation of human coagulation factor X by a protease of the pathogen *Capnocytophaga canimorsus*

K. Hack[1], F. Renzi[1], E. Hess[1], F. Lauber[1], J. Douxfils[2], J. M. Dogné[2] and G. R. Cornelis[1#]

Departments of Biology [1] and Pharmacy[2], University of Namur, Belgium

Corresponding author:

Guy Richard Cornelis, URBM, Rue de Bruxelles 61, B-5000 Namur

mail: guy.cornelis@unamur.be

Phone: +32 (0)81 724 401

Fax: +32 (0)81 724 420

Running Title: FX inactivation by *C. canimorsus*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jth.13605

This article is protected by copyright. All rights reserved.

Accepted Article

Essentials:

- *C. canimorsus* causes severe dog bite related blood stream infections.
- We investigated if *C. canimorsus* contributes to bleeding abnormalities during infection.
- The *C. canimorsus* protease *Cc*DPP7 causes factor X dysfunction by N-terminal cleavage.
- *Cc*DPP7 inhibits coagulation *in vivo,* which could promote immune evasion and trigger hemorrhage.

Abstract

Background: *Capnocytophaga canimorsus* is a Gram-negative bacterium, which belongs to the oral flora of dogs and causes fulminant sepsis in humans who have been bitten, licked or scratched. In patients, bleeding abnormalities, such as petechiae, purpura fulminans or disseminated intravascular coagulation (DIC) occur frequently.

Objective: We investigated whether *C. canimorsus* could actively contribute to these bleeding abnormalities.

Methods: Calibrated automated thrombogram and clotting time assays were performed to assess the anticoagulant activity of *C. canimorsus* 5 (*Cc*5), a strain isolated from a fatal human infection. Clotting factor activities were measured with factor deficient plasma. Factor X (FX) cleavage was monitored using the radiolabeled zymogen and western blot. Mutagenesis of *Cc*5 genes encoding putative serine proteases was performed to identify the protease cleaving FX. Protein purification was done by affinity chromatography. Edman-degradation allowed detection of N-terminal cleavage of FX. Tail bleeding times were measured in mice.

This article is protected by copyright. All rights reserved.

Accepted Article

Results: We found that *Cc*5 inhibited thrombin generation and increased the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) of human plasma via FX cleavage. A mutant, unable to synthesize a type 7 dipeptidyl peptidase (DPP7) of the S46 serine protease family, failed to proteolyse FX. The purified protease (*Cc*DPP7) cleaved FX heavy and light chains from the N-terminus and was active *in vivo* after i.v. injection.

Conclusions: This is to our knowledge the first study demonstrating a detailed mechanism for FX inactivation by a bacterial protease and it is the first functional study associating DPP7 proteases with a potentially pathogenic outcome.

Key words:

Sepsis

Bacterial infection

Factor X

Coagulation

Protease

Introduction

Capnocytophaga canimorsus is a Gram-negative bacterium belonging to the family of *Flavobacteriaceae* in the *Bacteroidetes* phylum [1]. It is part of the oral flora of dogs [1, 2] and causes rare but severe dog bite related blood stream infections in humans with a mortality as high as 30% [3] (for reviews see [3-5]). *C. canimorsus* sepsis can be accompanied by bleeding disorders, such as disseminated intravascular coagulation (DIC)

This article is protected by copyright. All rights reserved.

(13-35 %*), that often results in gangrene (8-13%*). Patients frequently show skin manifestations, including petechiae and purpura (11-46%*) [3-6]. Furthermore, one third of autopsies, performed on deceased individuals, revealed hemorrhage of adrenal glands [3].

The presence of pathogens or their products in circulation provokes several pro-coagulant processes, such as tissue factor (TF) expression by monocytes and endothelial cells, fostering entrapment of bacteria inside fibrin clots [7]. The importance of coagulation in innate immunity is illustrated by *in vivo* data. For instance, infection of fibrin deficient or coumadin treated mice with *Yersinia enterocolitica* leads to an elevated pathogen burden and reduced survival [8]. Some bacteria interact with coagulation factors, thus modulating this first step of immune defense [9-11]. *Yersinia pestis,* for example, suppresses fibrin generation via the plasminogen activator Pla thereby reducing recruitment of leukocytes to the infection site [12].

In view of these facts and the frequent occurrence of bleeding abnormalities during *C. canimorsus* infection we investigated the impact of *C. canimorsus* on hemostasis. We demonstrate that the blood culture isolate, *C. canimorsus* 5 (*Cc*5), blocks coagulation via the bacterial dipeptidyl peptidase *Cc*DPP7, which inactivates coagulation factor X (FX) by proteolytic cleavage. We hypothesize that this could contribute to immune evasion of the bacteria and to the hemorrhage observed in patients.

---

* of all cases

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Methods

Bacteria and growth conditions

Bacterial strains are listed in table S5. *Cc*5 was grown on blood agar plates (heart infusion agar (Difco)), 5% defibrinated sheep blood (Oxoid), gentamycin). *E. coli* strains were routinely cultured in LB medium (Invitrogen). Concentrations of antibiotics used: 10 µg/ml erythromycin (Em), 10 µg/ml cefoxitin (Cf), 20 µg/ml gentamicin (Gm), 50 µg/ml kanamycin (Km) and 100 µg/ml ampicillin (Ap), nalidixic acid (35 µg/ml).

Preparation of NPP

Human plasma was obtained from 16 healthy volunteers. Blood was withdrawn into vacutainer tubes (venosafe, TERUMO) containing 0.109 mol/l buffered sodium citrate and centrifuged twice at 2500 x g for 15 min at RT. NPP was snap frozen and stored at -80°C. The study was approved by the ethics committee of Mont-Godinne, Belgium (Protocol number B039201316262) and performed according to the declaration of Helsinki and the Belgian law on experiments in humans from March 7th, 2004. Participants were informed about the aim of the study and risks of blood withdrawal and gave written consent.

Calibrated automated thrombogram (CAT) assay

All bacteria were grown on blood agar, incubated with NPP and cell-free supernatants were added to MP- or PPP-Reagent (Thrombinoscope BV). Control NPP was mixed with a Thrombin Calibrator (Thrombinoscope BV). Thrombin generation was monitored on a Fluoroskan Ascent (Thermo Scientific) after FluCa (Thrombinoscope BV) injection. Calculations were performed with the Thrombinoscope software (Thrombinoscope BV).

This article is protected by copyright. All rights reserved.

Clotting Time measurements

PT, aPTT, thrombin and reptilase time were assessed with STA-Neoplastin R (Stago), HemosIL SynthASil (Instrumentation Laboratory), STA-Thrombin (Stago) or STA-Reptilase (Stago), repectively.

Coagulation factor activity assay

Activities were determined with deficient plasma (Stago): STA-Immunodef FII (00740), STA-Deficient FV (00744), STA-Deficient FVII (00734), STA-Immunodef FIX, STA-Deficient FX (00738), STA-Deficient FXI (00723), and STA-Immunodef FXII (00315). Standard curve measurements were performed according to the manufacturer's instructions. In brief a series of NPP dilutions in Owren-Koller buffer (Stago) was mixed 1:1 with deficient plasma. A 1:10 dilution was assigned as 100 % factor activity. PT was measured to determine FII, FV, FX and FVII activity or the aPTT for FIX, FXI and FXII. Clotting times were plotted against factor dilution. Plasma supernatants were diluted 1:10 and mixed 1:1 with factor deficient plasma. Clotting was triggered immediately.

Experiments with radiolabeled FX

Radiolabeling of 50 µg FX (Haematologic Technologies) with $Na^{125}I$ (1 mCi) (Perkin Elmer) was performed using pre-coated iodination tube (cat. no.: 28601, Pierce). Iodine incorporation constituted 3.7% of total iodine. Bacteria were incubated with ^{125}I-FX in NPP for 1h at 37°C. AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride) treatment was performed with $2x10^9$ bacteria/ml at RT. Cell free supernatants were run on a 12 % Nupage Novex Bis-Tris gel (lifetechnologies) with MOPS buffer (lifetechnologies). The dried gel was exposed to a Phosphor Screen (multisensitive, PerkinElmer) which was scanned with a Cyclone Phosphor Imager (PerkinElmer).

This article is protected by copyright. All rights reserved.

Accepted Article

Generation of knock-out mutants

Primers are listed in table S6. Site directed mutagenesis was performed as described by Mally *et al.* [13] using primers 1.1 and 1.2 to amplify the upstream flanking region (fragment 1) and primers 2.1 and 2.2 to amplify the downstream flanking region (fragment 2) of the gene to be knocked out. Primers 3.1 and 3.2 were used to amplify the *ermF* resistance cassette (fragment 3). In contrast to Mally *et al.* we first fused fragment 1 with fragment 3 in a separate PCR reaction before fusing this to fragment 2.

Construction of complementation and expression plasmids

Plasmids and primers are listed in table S7 and S6, respectively. The *dpp7* gene was amplified with primers 7982 and 7983 and inserted in pMM47 [13] generating the complementation plasmid pKH28. *Dpp7* was amplified with primers 7982 and 7989 introducing a C-terminal Strep-His tag, and inserted into pPM5 [14] to generate plasmid pKH32. Plasmids were purified from One Shot® TOP10 Electrocomp™ *E. coli* (Thermo) and transformed into *Cc*5 Δ *dpp7* bacteria. Positive clones were isolated on cefoxitin blood agar plates. Primers 5451 and 4730 or 7809 and 4730 were used for sequencing of inserts in pMM47 or pPM5, respectively.

Construction of the catalytic *Cc*DPP7 mutant

We introduced a point mutation in the *dpp7* gene replacing the active site serine residue of *Cc*DPP7 by an alanine (S649A) and added a C-terminal Strep-His tag for purification. *Dpp7* was amplified in two fragments using primers 7982 and 7999 or 7998 and 7989, which were then fused with primers 7982 and 7989 **(Table S6)**. The product was inserted into pPM5 generating pKH33. We then proceeded as described above.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Protein purification

*Cc*5 Δ*dpp7* (pKH32) and *Cc*5 Δ*dpp7* (pKH33) bacteria were disrupted with a French press. Cleared lysates were purified with a HisTALON resin (Clontech Laboratories, Inc.) and a Streptactin resin (Iba) according to the manufacturers' instructions. Purity was assessed by silver staining as described [15] **(Fig. S1)**.

Immunoblotting

The following primary antibodies were used: rabbit antiserum raised against purified *Cc*DPP7-Strep-His (1:5000, this work), mouse Mab HFX-LC and Mab HFX-HC (1:1000 and 1:1500 respectively, Enzyme Research Laboratories), rabbit anti-F10A cleaved Ala41 (1:1000, AssaybioTech). The following secondary antibodies were used: swine anti-rabbit HRP (1:5000, Dako), goat anti-mouse HRP (1:5000, Dako). Lumiglo Reserve Chemoluminiscent Substrate (KPL) was used for development according to the manufacture's instructions.

Quantification of *Cc*DPP7 release

160 µl of supernatants of NPP or HIP treated with 10^9 *Cc*5 wt, Δ*dpp7 or* with PBS were added to 40 µl of Met-Leu-AMC (L-Methionyl-L-Leucyl-4-methylcoumaryl-7-amide, Peptanova, final concentration of 250 ng/ml) and incubated for 8min at 37°C. Fluorescence was read on a Fluoroskan Ascent (excitation/emission 355nm/460nm). A dilution series of a *Cc*DPP7 standard was added for quantification purposes.

This article is protected by copyright. All rights reserved.

N-terminal sequencing

25 µg of FX in PBS were incubated with or without *Cc*DPP7 for 30min at 37°C. Concentrated samples (Amicon Ultra-0.5 10K MWCO filters, Millipore) were applied to a Nupage Novex Bis Tris gel (lifetechnologies). Proteins were transferred on a PVDF membrane (Amersham) and visualized with Coomassie R250 brilliant blue. Sequencing was carried out at the laboratory for Protein Biochemistry and Biomolecular Engineering at the University of Gent, Belgium.

Effect of *Cc*DPP7 on clotting factors

Purified clotting factors (Haematologic technologies) were incubated with *Cc*DPP7 or *Cc*DPP7$_{S649A}$ in PBS. FIX and FX were diluted 1:5 in factor deficient plasma to 5 µg/ml and 10 µg/ml, respectively. 0.5 µg/ml FVII and 10 µg/ml FII were mixed 1:1 with factor deficient plasma.

Evaluation of tail bleeding time

Balb/c mice were obtained from the University of Namur and Harlan Laboratories (Netherlands) and housed at the animal facility of the University of Namur. Experiments were performed in accordance with institutional guidelines and its animal bioethics consent (approval no. UN LE 13/205). Mice were injected with 200 µl of 1 mg/kg of *Cc*DPP7 or with 200 µl of the vehicle PBS. Warfarin was administered for 3 days with the drinking water. After anaesthesia with ketamin/domitor (82.5 mg/kg / 0.55 mg/kg) a 5 mm long piece of the tail tip was removed and the tail was submerged in 37 °C warm PBS. The total bleeding time represents all bleeding periods that occurred within 10 min.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Results

*Cc*5 inhibits thrombin generation

To determine if *C. canimorsus* interferes with coagulation we performed a CAT assay, in which thrombin generation was monitored with a fluorogenic thrombin substrate. We incubated normal pooled plasma (NPP) with different amounts of *Cc*5 bacteria and induced thrombin generation via the intrinsic (MP-Reagent), or the extrinsic pathway (PPP-Reagent) at different time points. Both pathways were affected at concentrations from 10^7 bacteria/ml and completely blocked from 10^8 bacteria/ml. The inhibitory effect already occurred after 15min **(Fig. 1A; Table S1)** and increased slightly when the incubation time was prolonged to 1h **(Fig. 1B, Table S2)**. A slight, but insignificant acceleration of thrombin generation occurred with MP-Reagent which might result from the activation of the intrinsic pathway on the surface of *Cc*5 as shown for other bacteria [16]. Two other Gram-negative control strains, *E. coli* MG1655 and *Y. enterocolitica* E40, did not inhibit thrombin generation and paraformaldehyde (PFA) fixed *Cc*5 only slightly reduced the amount of generated thrombin **(Fig. 1C, Table S3),** suggesting that the inhibitory effect was specific to *Cc*5 and mediated by an active mechanism.

*Cc*5 prolongs PT and aPTT

We next measured the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) of *Cc*5 treated NPP, which allow the detection of deficiencies in the extrinsic and the common pathway or in the intrinsic and the common pathway, respectively [17, 18]. Since this method is less sensitive than the CAT assay, 10^9 bacteria/ml were used and incubated for 1h. NPP treated with *Cc*5 showed a 10-fold increased PT **(Fig. 1D)** and a 2- to 3-fold increased aPTT **(Fig. 1E)**, thus confirming that *Cc*5 exerts an inhibitory effect on the coagulation cascade and that all pathways are impaired. Testing the activity of different coagulation factors, we found that four vitamin K dependent (VKD) clotting factors were

This article is protected by copyright. All rights reserved.

affected. FX and FIX activity was strongly decreased to 1 % of normal and FII and FVII activity to over 50 %, hinting either towards a dysfunction or a depletion of these factors by the bacteria **(Table 1A)**. There was no effect on fibrin(ogen) as confirmed by a thrombin and a reptilase time assay both of which allow the detection of fibrin(ogen) deficiencies, **(Table 1B).**

FX is cleaved by a bacterial serine protease

We carried on investigating the fate of FX, one of the most affected VKD factors. Incubating *Cc*5 bacteria with NPP spiked with radiolabeled FX we observed protein modifications, with a slight shift of FX heavy chain (HC) and light chain (LC). These no longer occurred upon prior treatment of the bacteria with the irreversible serine protease inhibitor AEBSF **(Fig. 2A)** suggesting cleavage by a bacterial serine protease. In addition, there was *Cc*5 mediated generation of two faint bands below the HC, which were still visible after AEBSF treatment of the bacteria, indicating that another bacterial factor, potentially another protease, or a deglycosylase also affected FX. As pre-incubation of *Cc*5 with AEBSF completely abolished the increased clotting times **(Fig. 2B)**, serine protease mediated FX LC and HC degradation seems to be the main cause of the increase of aPTT and PT and will be the focus of this paper.

Identification of the bacterial serine protease affecting FX activity

According to the MEROPS protease database, the *Cc*5 genome [19] encodes 27 potential serine proteases **(Table S4)** which we individually deleted by gene directed mutagenesis. All mutants behaved like wt bacteria (data not shown), except one, Δ*Ccan_08540,* which no longer led to an increased PT **(Fig. 3A).** There was still a small increase of the aPTT **(Fig. 3B)** which might result from the hypothetical second factor modifying FX HC or from an

This article is protected by copyright. All rights reserved.

interaction of *Cc*5 with molecules in the aPTT reagent. The normalization of PT and aPTT in Δ*Ccan_08540* bacteria treated NPP coincided with intact FX function **(Fig. 3C)** and the disappearance of the HC and LC band shift of FX **(Fig. 3D)**. A complemented strain of Δ*Ccan_08540*, Δ*Ccan_08540* (pKH28), behaved similarly to wt *Cc*5, meaning that the mutation of *Ccan_08540* was indeed responsible for the phenotype **(Fig. 3A-C)**. These findings indicate that *Ccan_08540* encodes a serine protease, which is responsible for FX cleavage and inhibition of coagulation.

_Ccan_08540_ encodes a dipeptidyl peptidase 7 (DPP7) homolog.

In MEROPS the protein encoded by *Ccan_08540* is annotated as a dipeptidyl peptidase 11 (DPP11) like protease, a member of the S46 family of serine proteases. Aligning the amino acid sequence of the Ccan_08540 protease with that of the DPP11 homolog of *Porphyromonas gingivalis* (*Pg*DPP11) [20] **(Fig. S2)** we found that Ccan_08540 has a glycine residue instead of an arginine at P667. Since this arginine is a hallmark of DPP11 proteases [21], its substitution by a glycine as in *Pg*DPP7, another *P. gingivalis* dipeptidyl peptidase, indicates that Ccan_08540 is rather a member of the DPP7 subgroup of S46 proteases [21]. We therefore called it *Cc*DPP7. The *Ccan_08540* gene, which we named *dpp7,* could be identified in all 26 *C. canimorsus* strains, that we have sequenced so far, with a gene identity of over 99% **(Fig. S3A)**. *Cc*5 also possesses a DPP11 homolog, which is encoded by *Ccan_11580*, but it is not implicated in the anticoagulant effect (data not shown).

*Cc*DPP7 homologs are present in the genome of other *Capnocytophaga* species, such as *Capnocytophaga cynodegmi* and *Capnocytophaga canis,* that only cause wound infections, but no sepsis **(Fig. S3A)** [1, 22]. 12 further *C. canimorsus* and 3 *C. canis* strains, which we tested, increased PT and aPTT in a similar manner. 3 tested *C. cynodegmi* strains were less efficient in affecting the PT, but prolonged the aPTT like *C. canimorsus* **(Fig. S3B)**.

This article is protected by copyright. All rights reserved.

*Cc*DPP7 is released into plasma via bacterial lysis

Being devoid of a signal peptide *Cc*DPP7 is likely to be cytoplasmic. Since the *Cc*5 genome contains no evidence of a machinery allowing active secretion, except for the gliding motility system [19], and since *C. canimorsus* is killed by complement at high plasma concentrations [23], we speculated that complement-mediated lysis could induce *Cc*DPP7 release. Incubating 10^9 bacteria/ml in NPP, we observed that 60% of the bacteria had died within 15min **(Fig. 4A)**. No more killing occurred up to 1h. When complement was inactivated by heat treatment of plasma (HIP) there was no killing and even slight growth. Using a fluorogenic dipeptide substrate cleaved by *Cc*DPP7**,** we observed a strong time-dependent increase of fluorescence in the supernatants of NPP, but not in HIP incubated with *Cc*5 wt bacteria (**Fig. 4B)** suggesting the liberation of *Cc*DPP7 by bacterial lysis. In the same conditions, Δ*dpp7* bacteria led to low fluorescence confirming that substrate cleavage was mainly caused by *Cc*DPP7. Using serial dilutions of purified *Cc*DPP7 **(Fig. S1)** we calculated that 10^9 wt *Cc*5/ml of NPP release an average 942 ng of *Cc*DPP7 **(Fig 4C)**. This might be slightly overestimated given the low activity of other *Cc*5 proteases on the substrate. However, if we assume 60% lysis **(Fig. 4A)**, the value is in the same range as that obtained by a quantitative western blot (WB) which showed that 10^9 bacteria contain on average 1.49 µg of *Cc*DPP7 (**Fig. 4D)**. To conclude, *Cc*DPP7 is rapidly released into plasma via bacterial lysis.

*Cc*DPP7 cleaves the N-terminus of FX LC and HC

We next characterized the cleavage of FX by purified *Cc*DPP7. We first confirmed the anticoagulant action of the purified protease. Indeed NPP incubated with *Cc*DPP7 showed a dose dependent increase of PT and aPTT **(Fig. 5A)**, reduced VKD factor activity **(Fig. 5B)** and altered molecular weight of FX **(Fig. 5C,D)**. A catalytic mutant of *Cc*DPP7, *Cc*DPP7$_{S649A}$, where the active serine had been replaced by an alanine, was inactive. To test the efficiency

This article is protected by copyright. All rights reserved.

Accepted Article

of *Cc*DPP7 in cleaving FX we performed a PT assay with FX deficient plasma, which we supplemented with FX that was pre-treated with *Cc*DPP7 at different concentrations. We observed a significant increase of the PT starting from an enzyme to substrate ratio of 1:50 (by weight) compared to the control **(Fig. 6A)**, which corresponds to the ratio needed to significantly increase the PT in NPP **(Fig. 5A),** assuming a plasma FX concentration of 10 µg/ml. The PT maximum was reached at ratios of 1:10 to 1:4. To exclude any interference of *Cc*DPP7 with FX deficient plasma at the moment of induction, we added a control incubating FX and *Cc*DPP7 separately and only mixing them upon addition to FX deficient plasma when clotting was induced. The PT was normal in this condition meaning that the effect was only triggered by the interaction of *Cc*DPP7 with FX.

We additionally monitored FX degradation by SDS-PAGE followed by silver staining and found that a *Cc*DPP7 to FX ratio of 1:1000 was already sufficient to initiate a clearly visible band shift of FX HC **(Fig. 6B)**. The LC shift was more obvious from 1:250 indicating that *Cc*DPP7 could have a higher specificity for the HC. The discrepancy between this test and the PT assay may result from the high sensitivity of the silver staining, which already detected partial degradation, and the low sensitivity of the Neoplastin reagent, which contains high amounts of TF.

The DPP7 homolog of *P. gingivalis* is an aminopeptidase [24]. Exploring whether *Cc*DPP7 degrades the FX N-terminus we incubated FX with *Cc*DPP7 at different ratios and analyzed FX LC by WB using an antibody recognizing the first 20 amino acids (aa). There was reduced antibody binding from a 1:250 ratio indicating N-terminal cleavage **(Fig. 6C)**. CcDPP7$_{S649A}$ did not induce amino-terminal cleavage of the LC **(Fig. S4)**. N-terminal sequencing was performed to confirm the result and to investigate the fate of the HC. Since at a ratio of 1:4 no more binding of the LC antibody occurred and the FX HC and LC were shifted to a maximum, suggesting that all FX molecules had been cleaved **(Fig. 6B),** we decided to use this condition for sequencing. We found that mainly 10 N-terminal aa of the

This article is protected by copyright. All rights reserved.

HC and 2 residues of the LC were missing **(Fig. 6D)** confirming the aminopeptidase nature of *Cc*DPP7.

Apart from FX, *Cc*DPP7 also acted on FIX **(Fig. 5B,S5C)** and FVII, but the latter action was weaker and less specific **(Fig. 5B,S5B)**. It is likely that the N-termini of FIX and FVII undergo limited cleavage, but so far we have no evidence for this. *Cc*DPP7 reduced FII activity in NPP **(Fig. 5B),** but did not reduce the activity of purified FII **(Fig. S5A)**. This raises the question whether the impact of *Cc*DPP7 on FII observed in NPP was overestimated due to an effect on multiple coagulation factors or if our assay with purified FII was not sensitive enough.

Cc*DPP7 is active *in vivo

In order to confirm the anticoagulant activity of *Cc*DPP7 *in vivo* we administered the enzyme to Balb/c mice by intravenous injection. 1h after *Cc*DPP7 administration the median tail bleeding time was 2-fold higher than the control **(Fig. 7)**. Mice of the positive control group, which had received warfarin, showed a 3-fold increase in the median tail bleeding time compared to the MOCK treated control group.

Discussion

Although coagulation disorders classically accompany generalized infections, the bleeding and coagulation abnormalities are particularly severe in the case of *C. canimorsus* infection, often leading to therapeutic amputations [25-29]. Investigating the possible interaction between *C. canimorsus* and hemostasis we found that *Cc*5 inhibited coagulation of human plasma via a bacterial serine protease of the DPP7 type (*Cc*DPP7) which caused cleavage of the N-terminus of FX LC and HC. Removal of all, but three aa of the FX activation peptide hampers the activation by the intrinsic Xase [30]. The HC cleaved by *Cc*DPP7 lacks only 10

This article is protected by copyright. All rights reserved.

Accepted Article

aa, but this could be sufficient to decrease the rate of FX activation. *Cc*DPP7 predominantly cleaves 2 aa from the N-terminus of the LC, which comprises a γ-carboxyglutamic acid (Gla) domain. Already the absence of the very first alanine (A1) residue can affect FX activity by preventing the formation of hydrogen bonds with Gla16, Gla20, Thr21 and Gla21, which normally mediate conformational stability of the Gla-domain [31]. In addition, A1 contributes to the generation of a Ω-loop, which stretches until glycine 11 [31] and is necessary for the interaction of the Gla-domain with membrane surfaces [32]. This suggests that, although *Cc*DPP7 only cleaves few residues from the FX HC and LC N-terminus it could be sufficient for FX inactivation.

It would be of great interest to identify the mechanism of interaction of *Cc*DPP7 with FX, which should help to clarify its preference for certain factors, and to investigate why *Cc*DPP7 stops cleaving after a limited number of aa from HC and LC. Even with very high enzyme concentrations no further cleavage occurs **(Fig. 6B)**. A possible cause could be posttranslational modifications presenting steric hindrance, such as glycans or a complex secondary structure making further aa residues less accessible.

Due to its intracellular location *Cc*DPP7 might essentially be a housekeeping protease, which could nevertheless play a role in cleaving clotting factors in plasma, because it is strongly released by the complement mediated lysis of *Cc*5 bacteria. Since CcDPP7 is also expressed by strains not causing invasive infections it is clear that the possession of *Cc*DPP7 alone does not suffice to make *C. canimorsus* a pathogenic bacterium. Other factors, such as the weakly endotoxic LPS [33], the resistance to phagocytosis [34], the capacity to capture iron [14] and the N-glycoproteins deglycosylation [35] cooperate to determine pathogenicity. Unfortunately the investigation of virulence factors has been hampered by the lack of animal model permitting to investigate the pathogenesis *in vivo*.

This article is protected by copyright. All rights reserved.

Accepted Article

Rabbits [36], mice and hamsters (unpublished data) clear bacteria completely. *In vitro,* using human plasma, *Cc*DPP7 was effective in the nanogram range and 10^7 bacteria/ml sufficed to affect thrombin generation. In the mouse, approx. 17 µg of pure *Cc*DPP7/ml blood were required to increase the tail bleeding time. We have no obvious explanation for this discrepancy, but protein stability, the presence of inhibitors or other unknown targets in murine blood could play a role.

Some clinical reports mention the presence of extracellular bacteria in peripheral blood smears [37, 38] indicating a high bacterial load. However, in most cases, *C. canimorsus* was only detected by culture suggesting lower bacterial numbers. This raises the question whether *Cc*DPP7 could have a systemic activity.

A generalized anticoagulant effect would probably only occur at advanced stages of sepsis when bacteremia is high and many bacteria have lysed releasing their intracellular content. *Cc*DPP7 levels are probably not sufficient to prevent DIC, there could be at most aggravation of DIC induced hemorrhage.

C. canimorsus adheres to various cell types including endothelial cells (unpublished data) and vegetations have been found within the heart [39]. A local inhibition of coagulation by agglomerates of adhering bacteria in tissues, organs or blood vessels releasing *Cc*DPP7 seems to be more probable. Brenner *et al.* mention a *C. canimorsus* isolate derived from a petechial lesion [1] pointing to the presence of *C. canimorsus* in the skin, which is interesting with respect to the skin hemorrhage observed in patients. It is tempting to speculate that *C. canimorsus* could directly induce skin manifestations like *Neisseria meningitidis* [40, 41] or that it could reduce the inflammatory response and clearance like *Y. pestis* [12] by locally blocking hemostasis.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

To conclude, we demonstrate a novel mechanism for the inactivation of FX by a DPP7 type protease. This effect on FX could contribute to local inhibition of coagulation or maybe to an aggravation of DIC induced bleeding. It could equally be a mechanism by which *C. canimorsus* evades detection and killing by the immune system.

Acknowledgements

We are grateful to M. Dol and D. Koudad for their technical assistance. We also thank Dr P. Gillion for taking blood, I. Hamer and M. Boonen for their help with the radioactive labelling of FX and our colleagues from the URBM. This work was supported by grant ERC 2011-ADG 20110310 from the European Research Council to G.R.C. FR is a post-doctoral fellow "chargé de recherche" of the Belgian National Fund for Research (FNRS).

Addendum

Contribution: K. Hack performed the experiments; K. Hack, J. M. Douxfils and G. R. Cornelis wrote the paper; K. Hack, J. M. Douxfils, G. R. Cornelis, E. Hess, F. Renzi, F. Lauber and J. Douxfils contributed to the design of experiments and to the interpretation of data.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

This article is protected by copyright. All rights reserved.

References

1 Brenner DJ, Hollis DG, Fanning GR, Weaver RE. Capnocytophaga canimorsus sp. nov. (formerly CDC group DF-2), a cause of septicemia following dog bite, and C. cynodegmi sp. nov., a cause of localized wound infection following dog bite. *J Clin Microbiol*. 1989; **27**: 231-5.

2 Bobo RA, Newton EJ. A previously undescribed gram-negative bacillus causing septicemia and meningitis. *Am J Clin Pathol*. 1976; **65**: 564-9.

3 Butler T. Capnocytophaga canimorsus: an emerging cause of sepsis, meningitis, and post-splenectomy infection after dog bites. *Eur J Clin Microbiol Infect Dis*. 2015; **34**: 1271-80.

4 Lion C, Escande F, Burdin JC. Capnocytophaga canimorsus infections in human: review of the literature and cases report. *Eur J Epidemiol*. 1996; **12**: 521-33.

5 Pers C, Gahrn-Hansen B, Frederiksen W. Capnocytophaga canimorsus septicemia in Denmark, 1982-1995: review of 39 cases. *Clin Infect Dis*. 1996; **23**: 71-5.

6 Herbst JS, Raffanti S, Pathy A, Zaiac MN. Dysgonic fermenter type 2 septicemia with purpura fulminans. Dermatologic features of a zoonosis acquired from household pets. *Arch Dermatol*. 1989; **125**: 1380-2.

7 Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol*. 2013; **13**: 34-45.

8 Luo D, Szaba FM, Kummer LW, Plow EF, Mackman N, Gailani D, Smiley ST. Protective roles for fibrin, tissue factor, plasminogen activator inhibitor-1, and thrombin activatable fibrinolysis inhibitor, but not factor XI, during defense against the gram-negative bacterium Yersinia enterocolitica. *J Immunol*. 2011; **187**: 1866-76.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

9 Berge A, Sjobring U. PAM, a novel plasminogen-binding protein from Streptococcus pyogenes. *J Biol Chem*. 1993; **268**: 25417-24.

10 Herwald H, Morgelin M, Dahlback B, Bjorck L. Interactions between surface proteins of Streptococcus pyogenes and coagulation factors modulate clotting of human plasma. *J Thromb Haemost*. 2003; **1**: 284-91.

11 Herwald H, Morgelin M, Olsen A, Rhen M, Dahlback B, Muller-Esterl W, Bjorck L. Activation of the contact-phase system on bacterial surfaces--a clue to serious complications in infectious diseases. *Nat Med*. 1998; **4**: 298-302.

12 Degen JL, Bugge TH, Goguen JD. Fibrin and fibrinolysis in infection and host defense. *J Thromb Haemost*. 2007; **5 Suppl 1**: 24-31.

13 Mally M, Cornelis GR. Genetic tools for studying Capnocytophaga canimorsus. *Appl Environ Microbiol*. 2008; **74**: 6369-77.

14 Manfredi P, Lauber F, Renzi F, Hack K, Hess E, Cornelis GR. New iron acquisition system in Bacteroidetes. *Infect Immun*. 2014; **83**: 300-10.

15 Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*. 1987; **8**: 93-9.

16 Frick IM, Bjorck L, Herwald H. The dual role of the contact system in bacterial infectious disease. *Thromb Haemost*. 2007; **98**: 497-502.

17 McCraw A, Hillarp A, Echenagucia M. Considerations in the laboratory assessment of haemostasis. *Haemophilia*. 2010; **16 Suppl 5**: 74-8.

18 Florell SR, Rodgers GM, 3rd. The PT and the pendulum. *Am J Clin Pathol*. 1996; **106**: 699-700.

This article is protected by copyright. All rights reserved.

19 Manfredi P, Pagni M, Cornelis GR. Complete genome sequence of the dog commensal and human pathogen Capnocytophaga canimorsus strain 5. *J Bacteriol*. 2011; **193**: 5558-9.

20 Ohara-Nemoto Y, Shimoyama Y, Kimura S, Kon A, Haraga H, Ono T, Nemoto TK. Asp- and Glu-specific novel dipeptidyl peptidase 11 of Porphyromonas gingivalis ensures utilization of proteinaceous energy sources. *J Biol Chem*. 2011; **286**: 38115-27.

21 Rouf SM, Ohara-Nemoto Y, Hoshino T, Fujiwara T, Ono T, Nemoto TK. Discrimination based on Gly and Arg/Ser at position 673 between dipeptidyl-peptidase (DPP) 7 and DPP11, widely distributed DPPs in pathogenic and environmental gram-negative bacteria. *Biochimie*. 2013; **95**: 824-32.

22 Zangenah S, Abbasi N, Andersson AF, Bergman P. Whole genome sequencing identifies a novel species of the genus Capnocytophaga isolated from dog and cat bite wounds in humans. *Sci Rep*. 2016; **6**: 22919.

23 Zangenah S, Bergman P. Rapid killing of Capnocytophaga canimorsus and Capnocytophaga cynodegmi by human whole blood and serum is mediated via the complement system. *Springerplus*. 2015; **4**: 517.

24 Rouf SM, Ohara-Nemoto Y, Ono T, Shimoyama Y, Kimura S, Nemoto TK. Phenylalanine 664 of dipeptidyl peptidase (DPP) 7 and Phenylalanine 671 of DPP11 mediate preference for P2-position hydrophobic residues of a substrate. *FEBS Open Bio*. 2013; **3**: 177-83.

25 Delanaye P, Dubois C, Mendes P, Bertholet M, Lambermont B. [Dog bite in a splenectomized patient]. *Rev Med Liege*. 2002; **57**: 40-4.

26 Low SC, Greenwood JE. Capnocytophaga canimorsus: infection, septicaemia, recovery and reconstruction. *J Med Microbiol*. 2008; **57**: 901-3.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

27 Kullberg BJ, Westendorp RG, van 't Wout JW, Meinders AE. Purpura fulminans and symmetrical peripheral gangrene caused by Capnocytophaga canimorsus (formerly DF-2) septicemia--a complication of dog bite. *Medicine (Baltimore)*. 1991; **70**: 287-92.

28 Waters MJ, Stern L, Andrew JH. Dysgonic fermenter-2 septicemia. *Pathology*. 1986; **18**: 162-4.

29 van de Ven AR, van Vliet AC, Maraha B, Ponssen HH. Fibrinolytic therapy in Capnocytophaga canimorsus sepsis after dog bite. *Intensive Care Med*. 2004; **30**: 1980.

30 Duffy EJ, Lollar P. Intrinsic pathway activation of factor X and its activation peptide-deficient derivative, factor Xdes-143-191. *J Biol Chem*. 1992; **267**: 7821-7.

31 Venkateswarlu D, Perera L, Darden T, Pedersen LG. Structure and dynamics of zymogen human blood coagulation factor X. *Biophys J*. 2002; **82**: 1190-206.

32 Welsch DJ, Nelsestuen GL. Amino-terminal alanine functions in a calcium-specific process essential for membrane binding by prothrombin fragment 1. *Biochemistry*. 1988; **27**: 4939-45.

33 Ittig S, Lindner B, Stenta M, Manfredi P, Zdorovenko E, Knirel YA, dal Peraro M, Cornelis GR, Zahringer U. The lipopolysaccharide from Capnocytophaga canimorsus reveals an unexpected role of the core-oligosaccharide in MD-2 binding. *PLoS Pathog*. 2012; **8**: e1002667.

34 Meyer S, Shin H, Cornelis GR. Capnocytophaga canimorsus resists phagocytosis by macrophages and blocks the ability of macrophages to kill other bacteria. *Immunobiology*. 2008; **213**: 805-14.

This article is protected by copyright. All rights reserved.

35 Renzi F, Manfredi P, Mally M, Moes S, Jeno P, Cornelis GR. The N-glycan glycoprotein deglycosylation complex (Gpd) from Capnocytophaga canimorsus deglycosylates human IgG. *PLoS Pathog*. 2011; **7**: e1002118.

36 Butler T, Johnston KH, Gutierrez Y, Aikawa M, Cardaman R. Enhancement of experimental bacteremia and endocarditis caused by dysgonic fermenter (DF-2) bacterium after treatment with methylprednisolone and after splenectomy. *Infect Immun*. 1985; **47**: 294-300.

37 Bryson MS, Neilly I, Rodger S, Soutar RL. Purpura fulminans associated with Capnocytophaga canimorsus infection. *Br J Haematol*. 2003; **121**: 1.

38 Tan V, Schwartz JC. Renal failure due to Capnocytophaga canimorsus generalized Shwartzman reaction from a dog bite (DF-2 nephropathy). *Proc (Bayl Univ Med Cent)*. 2014; **27**: 139-40.

39 Sandoe JA. Capnocytophaga canimorsus endocarditis. *J Med Microbiol*. 2004; **53**: 245-8.

40 Melican K, Dumenil G. Vascular colonization by Neisseria meningitidis. *Curr Opin Microbiol*. 2012; **15**: 50-6.

41 Join-Lambert O, Lecuyer H, Miller F, Lelievre L, Jamet A, Furio L, Schmitt A, Pelissier P, Fraitag S, Coureuil M, Nassif X. Meningococcal interaction to microvasculature triggers the tissular lesions of purpura fulminans. *J Infect Dis*. 2013; **208**: 1590-7.

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

Tables

Table 1. (A) Activity of selected coagulation factors and **(B)** thrombin and reptilase times of NPP incubated for 1h at 37°C with 10^9 *Cc*5 bacteria/ml or PBS as control.

A

	Factor activities [%][1] ± SD	
	NPP + PBS	**NPP + *Cc*5**
FII	92 ± 7	44 ± 4 (p=0.0006)[2]
FV	95 ± 17	96 ± 14
FVII	100 ± 4	45 ± 1 (p<0.0001)
FIX	105 ± 0	1 ± 0 (p<0.0001)
FX	81 ± 6	1 ± 0 (p<0.0001)
FXI	64 ± 5	71 ± 6
FXII	98 ± 13	122 ± 17

B

	Clotting Times [s]	
	NPP + PBS	**NPP + *Cc*5**
Thrombin time	17.3 ± 0.1	20.1 ± 1.8
Reptilase time	17.5 ± 0.5	18.3 ± 1.2

[1] Factor activities were determined with factor deficient plasma. Sample plasma was diluted 1:10 in Owren Koller buffer, mixed 1:1 with factor deficient plasma and PT (FII, FV, FVII, FX) or aPTT (FIX, FXI, FXII) were measured. The activity was calculated by means of a standard curve which was obtained by mixing different dilutions of a calibrator NPP, starting from 1:10, with equal volumes of the appropriate factor deficient plasma. The 1:10 dilution of standard NPP was arbitrarily assigned to a factor activity of 100%.

[2] For statistical analysis One-way ANOVA followed by a Bonferroni test was carried out. P-values represent statistical significance compared to the control (NPP + PBS)

SD = standard deviation, NPP = normal pooled plasma, PBS = phosphate buffered saline

This article is protected by copyright. All rights reserved.

Accepted Article

Figure Legends

Figure 1. *Cc*5 inhibits thrombin generation and leads to increased clotting times. Thrombin generation profiles of NPP after incubation with different concentrations of *Cc*5 for 15 min (A) and 1 h (B). PBS only (ctrl) or bacterial suspensions in PBS were diluted 1:10 in NPP to make the final dilutions of 10^6 - 10^9 *Cc*5 bacteria/ml and incubated at 37 °C. (C) Thrombin generation profiles of NPP after incubation for 15 min at 37 °C with PBS (ctrl) or *E. coli* MG1655, *Y. enterocolitica* E40, live *Cc*5, PFA fixed *Cc*5, all at 10^9/ml. Thrombin generation was monitored with a Fluoroskan Ascent after addition of MP-Reagent (4 µM Phospholipids) or PPP-Reagent (5 pM TF, 4 µM Phospholipids) and FluCa (fluorogenic thrombin substrate + $CaCl_2$). Shown are representative results from three independent experiments performed in triplicate. (D, E) *Cc*5 bacteria (10^9/ml) or PBS (ctrl) were incubated at 37°C with NPP for 1 h. For PT measurements coagulation was induced with STA-Neoplastin R (D), for aPTT measurements with HemosIL SynthASil (E). Shown are average clotting times of at least three independent experiments performed in duplicate. Error bars represent the standard deviation. ***, P≤0.001 compared to control determined with a Student's t-test.

Figure 2. A serine protease mediates FX cleavage and causes increased clotting times. (A) Autoradiography of I^{125}-FX. 3 µg/ml iodinated FX were added to NPP and incubated either with PBS (ctrl) or wt bacteria in PBS pre-incubated for 30 min in the presence or absence of 1 mM AEBSF. The dashed line helps to visualize the size shift. Shown is a representative image of three independent experiments. (B) PT and aPTT measurements of NPP treated with *Cc*5 wt bacteria in PBS pre-incubated for 30min in the presence or absence of 1 mM AEBSF. For the control (ctrl) either PBS or 1 mM AEBSF in PBS were added to NPP. Shown are mean values (±SD) of three independent experiments each performed in triplicate (ns, not significant; ***, p≤0.001 compared to control). 10^9

This article is protected by copyright. All rights reserved.

bacteria/ml were used for all conditions, the incubation was always performed for 1h at 37 °C. One-way-ANOVA with a Bonferroni post-test was used for statistical analysis.

Figure 3. Deletion of the gene *Ccan_08540* restores normal PT and aPTT values, FX function and FX protein integrity. Complementation of Δ *Ccan_08540* with the *Ccan_08540* expression plasmid pKH28 leads again to increased clotting times and impaired FX activity. (A, B) PT and aPTT values of NPP incubated with PBS (ctrl) or 10^9 bacteria/ml of *Cc*5 wt, Δ*Ccan_08540* (Δ*08540*) or the complemented strain Δ*Ccan_08540* (pKH28) (Δ*08540* (pKH28)). Shown are mean values (±SD) of at least four independent experiments each performed in duplicate. (C) FX activity of NPP incubated in the absence or presence of 10^9 bacteria/ml was measured with factor deficient plasma. Shown are mean values (±SD) of three independent experiments each done in duplicate. Statistical analysis was performed comparing with the control (***, p≤0.001). (D) Representative autoradiography of I^{125}-FX in NPP incubated with PBS (ctrl) or 10^9 bacteria/ml. The dashed line helps to visualize the size shift. All experiments were performed for 1 h at 37 °C. One-way-ANOVA with a Bonferroni post-test was used for statistical analysis.

Figure 4. *Cc*DPP7 release into NPP is mediated by lysis of *Cc*5. (A) *Cc*5 is killed in NPP. 10^9 *Cc*5 wt bacteria/ml were incubated for the indicated time points with NPP or HIP and cfu counts were quantified by plating. Shown is the percentage of surviving/growing bacteria relative to the inoculum. ***, p≤0.001 (B) *Cc*DPP7 release into plasma is triggered by bacterial lysis. 10^9 bacteria/ml of *Cc*5 wt or Δ*dpp7* were incubated with NPP or HIP for the indicated time-points. PBS only was used as control. *Cc*DPP7 release was monitored using a fluorogenic Met-Leu-AMC substrate. Depicted is the average fluorescence (±SD) of 3 independent experiments. ***, p≤0.001; ns, not significant versus NPP/HIP incubated with Δ*dpp7* bacteria. (C) The average *Cc*DPP7 concentration (±SD) in NPP and HIP was

This article is protected by copyright. All rights reserved.

calculated based on the fluorescence generated in each of the 3 experiments using purified *Cc*DPP7 as a standard. (D) Quantification of *Cc*DPP7 expression by *Cc*5. Shown is an anti-*Cc*DPP7 western blot. 10 µl of purified *Cc*DPP7 or *Cc*5 wt bacteria at the indicated concentrations in sample buffer were loaded onto an SDS-PAGE gel. Shown is a representative blot and standard curve of 3 independent experiments as well as the average *Cc*DPP7 amount (±SD) expressed by 10^9 *Cc*5 wt bacteria. The band intensities were quantified with the Amersham Imager 600 software and the average amount of *Cc*DPP7 expressed by 10^9 *Cc*5 wt bacteria was calculated from 3 independent experiments.

Figure 5. (A, B) NPP treated with purified *Cc*DPP7 shows prolonged PT and aPTT and reduced VKD factor activity. (A) *Cc*DPP7, *Cc*DPP7$_{S649A}$ or PBS were diluted 1:10 in NPP to the indicated concentrations, incubated at 37 °C for 1 h and PT and aPTT were measured. One-way ANOVA with a Bonferroni post-test was performed for statistical analysis comparing to the untreated control, *** $p \leq 0.001$. (B) The activity of FII, FVII, FIX and FX in NPP treated for 1h at 37°C with PBS, 200 ng/ml of *Cc*DPP7 or *Cc*DPP7$_{S649A}$ were measured with factor deficient plasma. Shown are the results of three independent experiments (±SD) each done in duplicate. For statistical analysis one-way ANOVA with a Bonferroni post-test was performed comparing to NPP + PBS. (C, D) *Cc*DPP7 causes FX LC and HC cleavage in NPP. Shown are cropped images of anti-FX LC (C) or HC (D) western blots of NPP incubated for 1h at 37°C in the presence or absence of 200 ng/ml of *Cc*DPP7 or *Cc*DPP7$_{S649A}$. 10 µl of plasma samples diluted 1:20 were loaded. 10 µl of 1:20 diluted FX deficient plasma served as negative control. Dashed lines were drawn to visualize the band shift. Shown is a representative result of 3 independent experiments.

This article is protected by copyright. All rights reserved.

Accepted Article

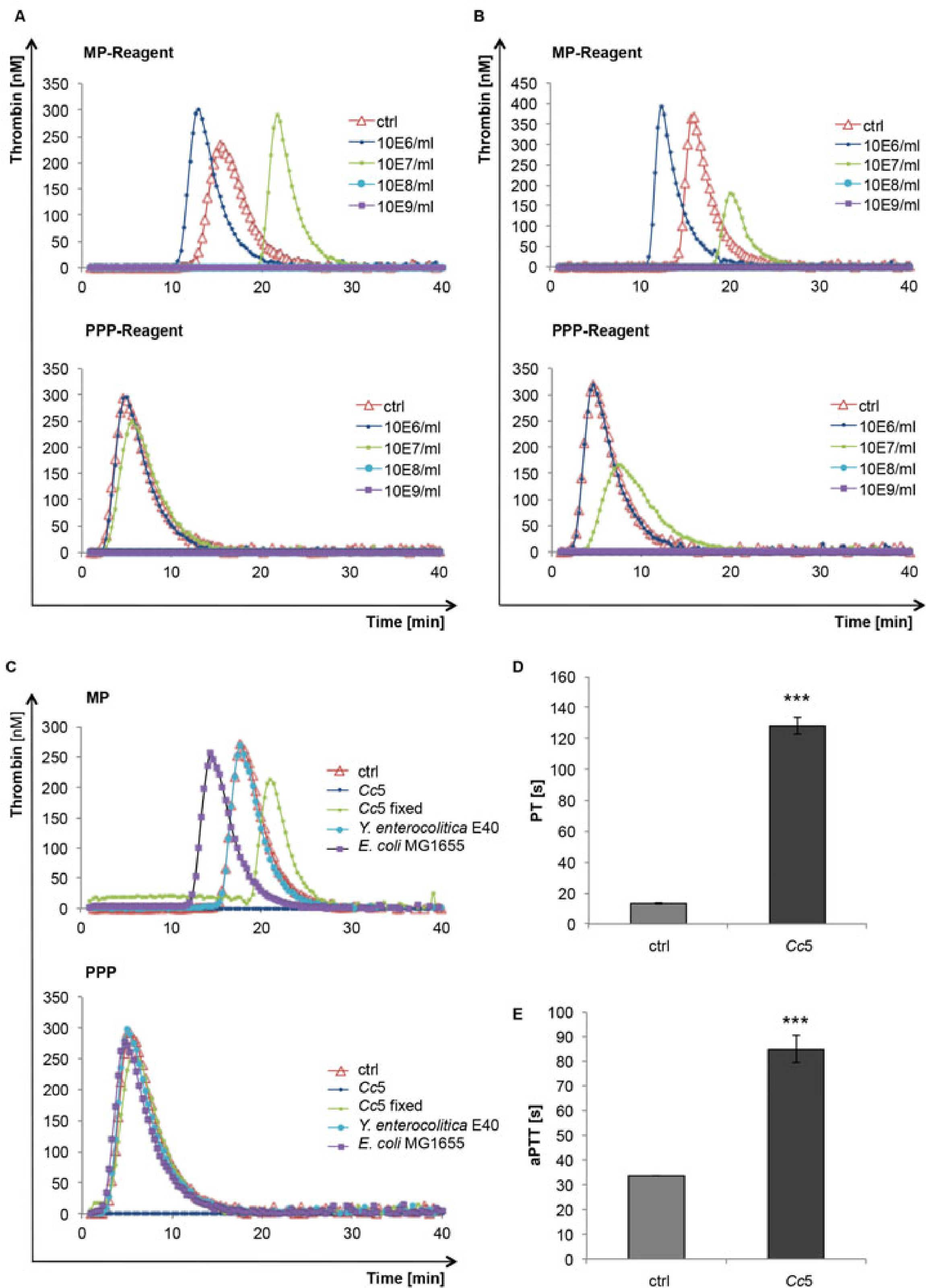
Figure 6. FX is degraded by N-terminal cleavage. (A) *Cc*DPP7 affects FX function in a dose dependent manner. 50 µg/ml of FX were incubated for 30min at 37°C in the presence or absence of *Cc*DPP7 or *Cc*DPP7$_{S649A}$ at the indicated enzyme to substrate ratios (by weight) and subsequently added to FX deficient plasma at a concentration of 10 µg/ml. To exclude any interaction of *Cc*DPP7 with FX deficient plasma at the moment of induction, a control was included for which FX and *Cc*DPP7 were first incubated separately and only mixed upon addition to FX deficient plasma when the inducer was added (n.c. = no coincubation). Shown are mean values and standard deviations of three independent experiments each performed in triplicate. ***, $p \leq 0.001$ versus control. One-way-ANOVA with a Bonferroni post-test was used for statistical analysis. (B) Degradation of pure human FX by *Cc*DPP7 happens in a dose dependent manner, but reaches a limit at high doses. 50 µg/ml of FX in PBS were incubated with *Cc*DPP7 at the indicated ratios (by weight) for 30min at 37°C. 10 µl of each sample in sample buffer were loaded. Shown is a representative silver staining of 3 independent experiments. (C, D) FX is degraded by *Cc*DPP7 via N-terminal cleavage. (C) Western blot for amino acids 1-20 of FX LC. 50 µg/ml of purified human FX in PBS were incubated with the indicated concentrations of *Cc*DPP7 or *Cc*DPP7$_{S649A}$ for 30min at 37°C. 10 µl of each sample in sample buffer were loaded. Shown are representative results of three independent experiments. (D) 50 µg/ml of purified human FX in PBS was incubated in the presence or absence of 12.5 µg/ml of *Cc*DPP7. Automated N-terminal Edman degradation was performed on LC and HC. Amino acid sequences from two independent experiments are shown. Sequences in bold were the most highly abundant in both experiments.

Figure 7. *Cc*DPP7 prolongs the bleeding time *in vivo*. 1 mg/kg *Cc*DPP7 or PBS as control were injected into the tail vein of male Balb/c mice. Bleeding times were measured 1 h after injection. The positive control group received 5 mg/l of warfarin in the drinking water

Accepted Article

This article is protected by copyright. All rights reserved.

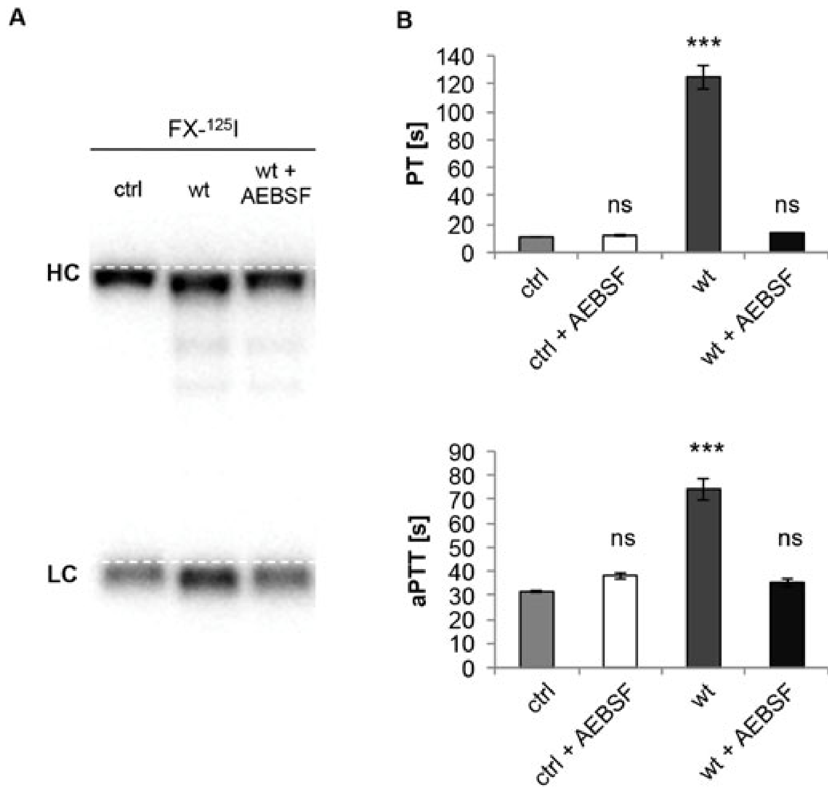
for 3 days. *, p≤0.05; ***, p≤0.001 versus vehicle treated control. One-way-ANOVA with a Bonferroni post-test was used for statistical analysis.



Accepted Article

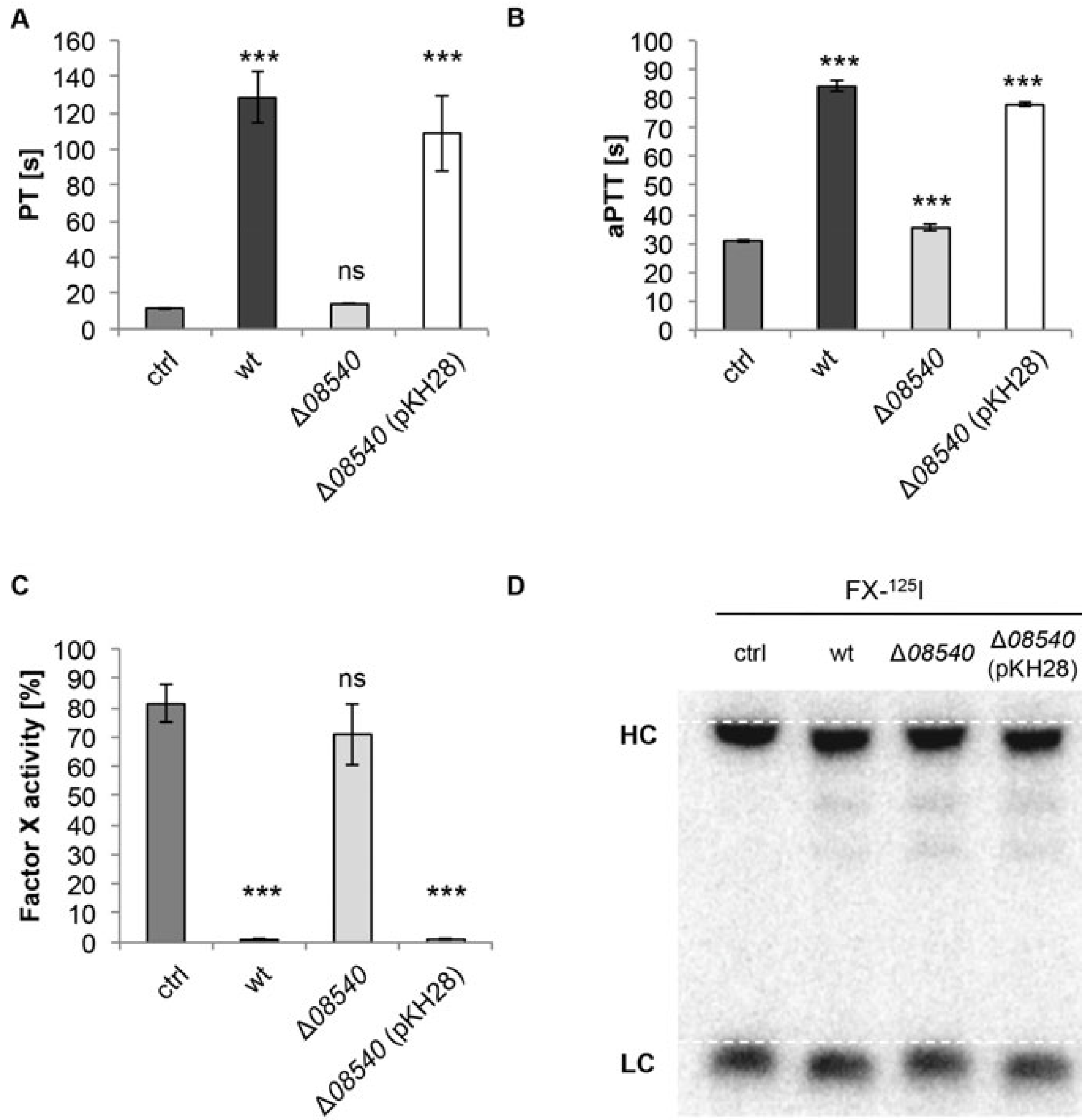
This article is protected by copyright. All rights reserved.

Accepted Article



This article is protected by copyright. All rights reserved.

Accepted Article



This article is protected by copyright. All rights reserved.

Accepted Article

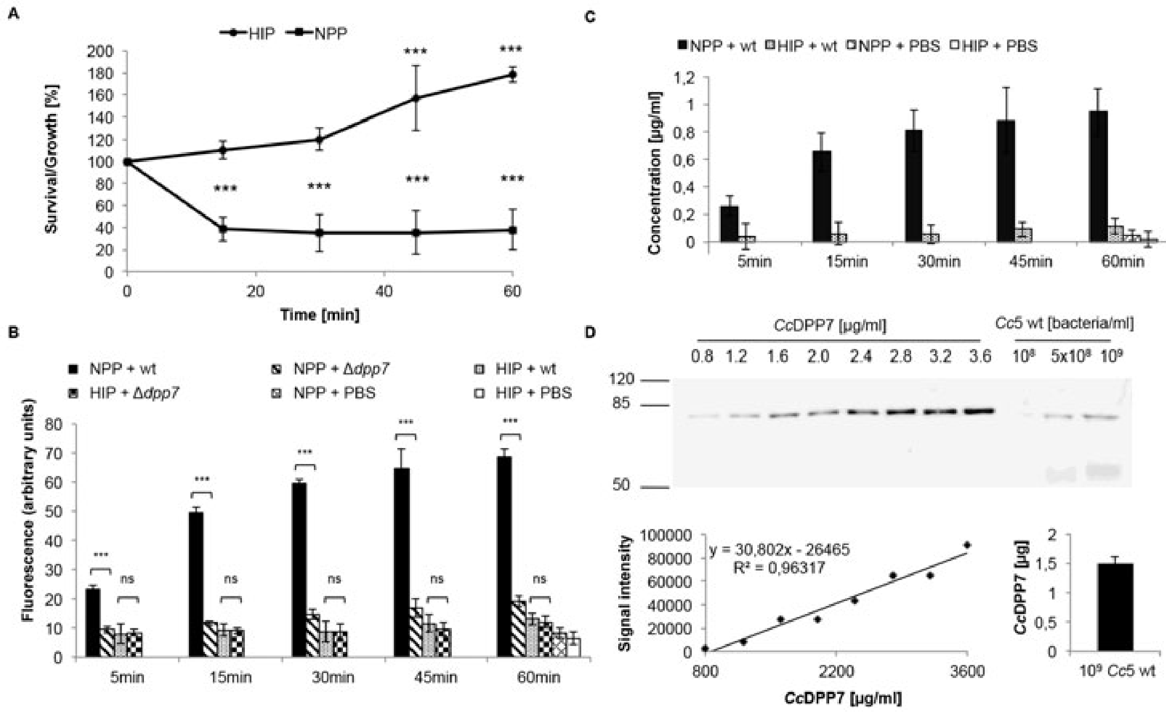
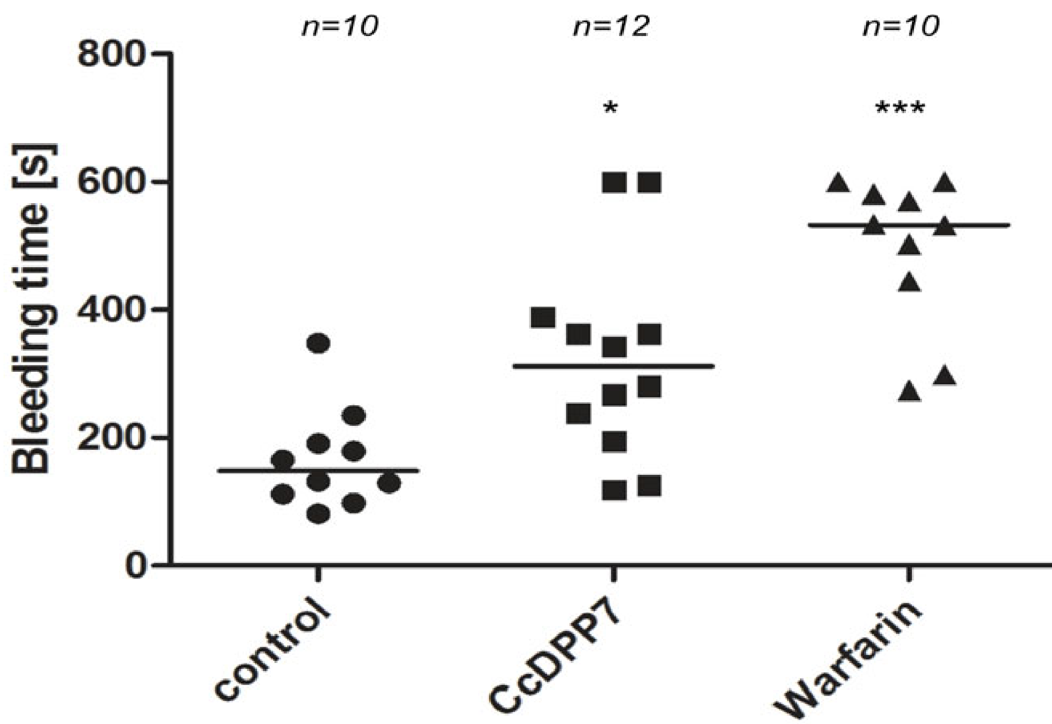
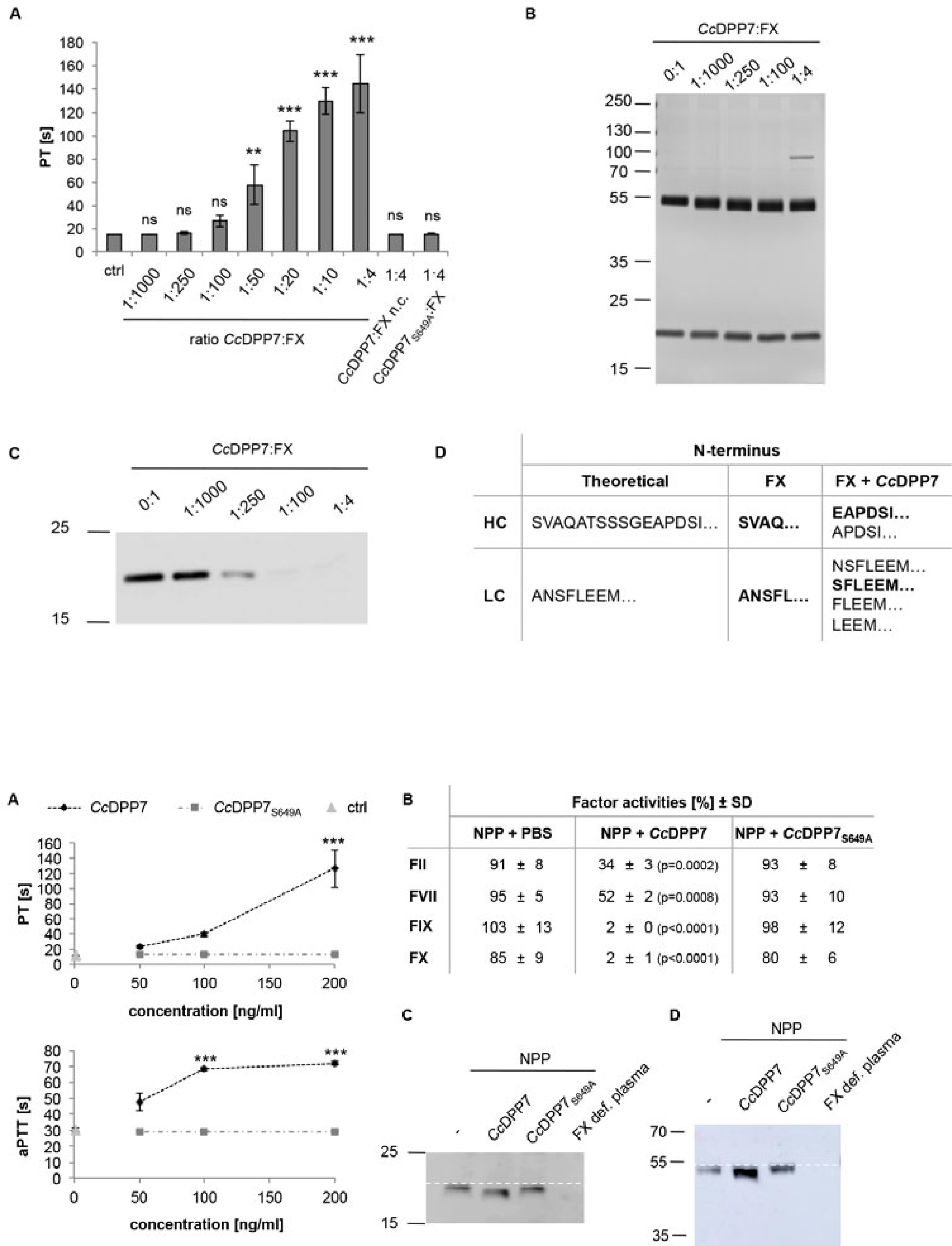


Figure 5



This article is protected by copyright. All rights reserved.

Accepted Article



This article is protected by copyright. All rights reserved.