

## SHORT COMMUNICATION



# SerpinB2 deficiency in mice reduces bleeding times via dysregulated platelet activation

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## Abstract

SerpinB2, also known as plasminogen activation inhibitor type 2 (PAI-2), is classically viewed as an inhibitor of fibrinolysis. However, we show herein a distinct, hitherto unrecognized role for SerpinB2 in hemostasis. Mice deficient in SerpinB2 expression and mice with an active site mutation in SerpinB2, both showed significant reductions in tail bleeding times. This hemostatic phenotype was associated with platelets, with SerpinB2 and SerpinB2-urokinase complexes clearly present in platelet fractions, and immunohistochemistry of blood clots suggesting SerpinB2 is associated with platelet aggregates. Thromboelastography illustrated faster onset of clot formation in blood from SerpinB2 deficient mice, whereas clotting of platelet-free plasma was unaffected. The results appear consistent with the low circulating SerpinB2 levels and hypercoagulation seen during pre-eclampsia; however, SerpinB2 was not detected in human platelets.

## Keywords

Bleeding times, PAI-2, plasminogen activation inhibitor type 2, platelet, SerpinB2, urokinase

## History

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## Introduction

SerpinB2 (also known as plasminogen activator inhibitor type 2 or PAI-2) is a member of the clade B or ovalbumin-like serine protease inhibitor (ov-serpin) subgroup of the serpin superfamily. SerpinB2 can be expressed by variety of cells including monocytes and macrophages, syncytiotrophoblasts, keratinocytes, fibroblasts, endothelial cells, dendritic cells and cancer cells [1–6]. SerpinB2 lacks a classical secretory signal peptide and is usually localized to the cytoplasm. However, SerpinB2 can reach the extracellular milieu via loss of plasma membrane integrity [5] or microparticle formation, with SerpinB2 expressed on microparticles, potentially via an association with phosphatidylserine and annexins [4,7].

The classical view argues that SerpinB2 is involved in the inhibition of fibrinolysis, via inhibition of plasmin generation by urokinase plasminogen activator (uPA) and, to a lesser extent, tissue plasminogen activator (tPA) [1,8–12]. SerpinB2 inhibits uPA via the

formation of a covalent SerpinB2-uPA complex involving the P1 arginine at position 380 (Arg380) in the reactive site loop of SerpinB2 and the active site serine of uPA. PAI-1 also inhibits fibrinolysis by inhibiting plasmin generation by tPA and uPA, with PAI-1<sup>-/-</sup> mice showing enhanced fibrinolysis and thrombolysis [13–15]. Some in vivo evidence for SerpinB2-mediated inhibition of fibrinolysis was only recently reported, with SerpinB2<sup>-/-</sup> mice showing increased venous thrombus resolution [15], although the observation was complicated by increased uPA and decreased plasminogen activator inhibitor type 1 (PAI-1) expression [16].

Herein we describe a novel function for SerpinB2 in hemostasis using both (i) SerpinB2<sup>-/-</sup> mice (and a littermate control SerpinB2<sup>+/+</sup> mouse line) [17] and (ii) a newly created mouse line where the active site Arg380 was mutated to alanine (SerpinB2<sup>R380A</sup>) using CRISPR technology, which renders the serpin unable to inhibit uPA [2,18]. Both SerpinB2<sup>-/-</sup> and SerpinB2<sup>R380A</sup> mice showed significantly reduced bleed times compared with their respective wild-type controls. This phenotype appears unrelated to fibrinolysis since overt increases in uPA/plasmin-mediated clot dissolution would be expected to increase (rather than decrease) bleed times.

## Materials and Methods

### Ethics Statements and Mice

All mouse work was conducted in accordance with the “Australian code for the care and use of animals for scientific purposes” as defined by the National Health and Medical Research Council of Australia. Mouse work was approved by the QIMR Berghofer Medical Research Institute animal ethics committee. Mice were euthanized using carbon dioxide.

#WAS and TTL should be considered joint first authors.

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## SerpinB2 Deficient Mice

SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice (described previously [17]) were bred in-house at QIMR Berghofer Medical Research Institute. C57BL/6J mice (6–8 weeks) were purchased from Animal Resources Center (Canning Vale, WA, Australia). Heterozygous CRISPR SerpinB2<sup>R380A</sup> mice on a C57BL/6J background were generated by the Australian Genome Research Facility Ltd. (Melbourne, Australia) and a homozygous SerpinB2<sup>R380A</sup> mouse line was generated in-house. The active site (P1) Arg380 [2] (codon AGA) of SerpinB2 was changed to Ala380 (codon GCA); i.e. nucleotides 1222 and 1223 (with reference to accession NM\_011111.4) were changed from AG to GC. Two proximal silent mutations were also introduced (1222-GCAACTGGACATGGTGGCCACAGTTTGTC-1251; mutations underlined) to prevent cutting of the oligonucleotide during genome editing. The genotype was confirmed by tail tipping, extraction of DNA (Extract-N-Amp Tissue PCR Kit, Sigma), PCR (primers Forward 5'-tctgaggtgtccatcaag-3', 5'-Reverse ctacacaatagatcgtgtg-3') and sequencing of the PCR products.

## Tail Bleeding Time Determination

Bleed time was determined in gender and age matched mice by restraining the mouse, removing 1 mm of the tail using a scalpel, placing the tail into normal saline at 37°C and measuring the time until bleeding stopped.

## Plasma Coagulation Time Determination

Mouse blood was collected by heart puncture into citrated blood collection tubes (BD, Franklin Lakes, NJ, USA). For SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice, Thromborel (Dade Behring, Liederbach, Germany) was added to platelet-free plasma according to the manufacturer's recommendation. Coagulation time was measured using a semi-automatic ball coagulometer (Heinrich Amelung GmbH, Germany). For C57BL/6 and SerpinB2<sup>R380A</sup> mice, Thromborel S (Siemens Healthcare Pty Ltd, Bayswater, Australia) was used and clot time was assessed manually.

## Thromboelastography

Citrated mouse blood was treated with recalcified kaolin and clot parameters measured by thromboelastography (TEG 5000, Medicell Ltd., London, UK).

## P-Selectin Staining

Platelet rich plasma was prepared from citrated blood from SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice (n = 4 per strain). Platelets were spun down and resuspended in SGH buffer (120 mM NaCl, 30 mM glucose, 10 mM HEPES pH 7); final plasma concentration 10% v/v. After re-calcification for 20 mins at room temperature, platelets were fixed in paraformaldehyde (1% 15 mins), washed in RPMI 1640, blocked with 10% FCS and stained with FITC-labeled anti-P selectin (BD Biosciences; RB40.34) and analyzed using BD LSRFortessa and data analyzed using BD FACSDiva™ software.

## Immunoblotting of Platelet Fractions

Citrated blood collected by heart puncture was spun at 100 x g for 5 min and the supernatant collected as platelet rich plasma. Platelets were washed once (3000 x g for 30 mins) in CGS buffer (120 mM NaCl, 30 mM glucose, 13 mM trisodium citrate, pH 7) or SGH buffer. Platelet pellets were then lysed with RIPA buffer (0.1% SDS, 1% NP40, 0.1% sodium deoxycholate, 140 mM

NaCl, 1 mM EDTA and Protease Inhibitor Cocktail – Roche) and spun at 2000 x g for 5 min. Supernatants were boiled in SDS-PAGE sample buffer containing 0.1 M dithiothreitol and analyzed by SDS polyacrylamide gel electrophoresis and immunoblotting using an anti-murine SerpinB2 antibody and after stripping (Restore PLUS Western Blot Stripping Buffer; ThermoScientific, Rockford, IL, USA) and reprobed with a rabbit anti-murine uPA antibody (ab20789; Abcam, Cambridge, UK) or anti-murine  $\beta$  actin (13E5, Cell Signaling Technology, Inc., Danvers, MA, USA) as described [5]. The anti-murine SerpinB2 antibody (affinity purified, rabbit polyclonal) was generated by Peptide Specialty Labs GmbH (Heidelberg, Germany). An ovalbumin-coupled CD loop region peptide <sup>62</sup>EIGSYGITTRNPENFSGC<sup>79</sup> was used as the immunogen [5].

## Immunohistochemistry and Histology

Blood clots from tail bleeds were allowed to form in 1.5 ml Eppendorf tubes for 30 mins at room temperature and were then fixed in paraformaldehyde, and processed for immunohistochemistry using the aforementioned anti-SerpinB2 antibody as described previously [5]. Paraffin sections were also stained with a standard Giemsa or Wright-Giemsa (Sigma).

## Results

### Bleed Time Decreases in SerpinB2 Deficient Mice

Bleed times were determined in SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice by tail tipping, with both female (Figure 1A) and male SerpinB2<sup>-/-</sup> mice (Fig. S1) showing significantly lower bleed times compared to the SerpinB2<sup>+/+</sup> control mice. The phenotype was recapitulated in homozygous CRISPR SerpinB2<sup>R380A</sup> mice (on a C57BL/6 background) when compared with wild-type C57BL/6 mice (Figure 1B), illustrating that this bleed time phenotype requires the protease inhibition activity of SerpinB2. The SerpinB2<sup>R380A</sup> mutation did not affect SerpinB2 protein expression (Fig. S2); SerpinB2 activities that involve protease inhibition are thus implicated in this hemostatic phenotype. By extension other activities, such as annexin binding [4] and transglutaminase cross-linking [5] via SerpinB2's CD loop, would thus appear not to be involved.

The coagulation times for platelet-free plasma from (i) SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice and (ii) SerpinB2<sup>R380A</sup> and C57BL/6 mice were not significantly different (Figure 1C), arguing that the phenotype requires the presence of platelets. (The platelet-free plasma retains microparticles, suggesting they are also not involved in the phenotype). The platelet count in SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice was not significantly different (Table S1), suggesting platelet activation rather than platelet numbers were altered in SerpinB2 deficient mice.

Thromboelastography of recalcified citrated mouse whole blood showed that the time to initial clot formation (reaction time, R) was significantly lower (faster) for SerpinB2<sup>-/-</sup> mice when compared to SerpinB2<sup>+/+</sup> mice (Figure 1D). Neither the kinetic time, alpha angle or the maximum amplitude were significantly affected by SerpinB2 deficiency (Fig. S3). Whole blood platelet lumi-aggregometry also showed a tendency for increased and faster platelet ATP release from SerpinB2<sup>-/-</sup> platelets after standard doses of arachidonic acid and collagen, but not thrombin, treatment (Fig. S4).

## P-Selectin Staining

To further examine platelet activation, platelets from SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice were stained with anti-P-selectin. Prior to

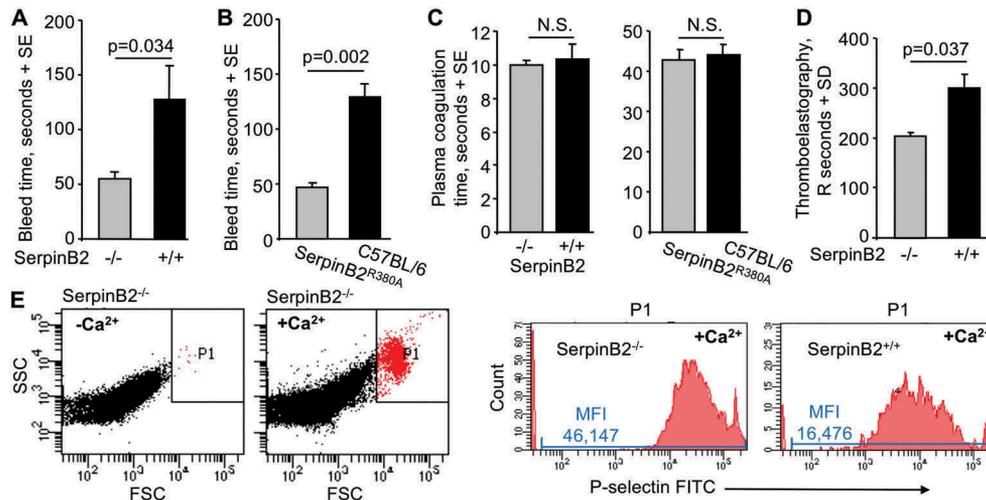


Figure 1. (A) Mean tail bleeding time for SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> female mice. Statistics by Kolmogorov-Smirnov test, n = 12 mice per group. (B) Bleed time for SerpinB2<sup>R380A</sup> and C57BL/6J female mice. Statistics by Kolmogorov-Smirnov test, n = 19–21 mice per group. (C) Coagulation time for platelet-free-plasma. Female SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mouse plasma was assessed using a semi-automatic ball coagulometer (n = 9–10 mice per group). Coagulation of plasma from SerpinB2<sup>R380A</sup> and C57BL/6J female mice was determined manually (n = 8–10). N.S. – not significant. (D) Thromboelastography of recalcified citrated blood from SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> female mice. Mean R values are shown (time to initial clot formation); the remaining parameters are shown in Fig. S3. Statistics by Kolmogorov-Smirnov test, n = 4 mice per group. (E) P-selectin staining by FACS. SSC/FSC plots: platelets from citrated blood (-Ca<sup>2+</sup>) have few events with high side scatter (SSC) and forward scatter (FSC) (P1 gate, red dots). After re-calcification (+Ca<sup>2+</sup>) events with high FSC/SSC (indicative of platelet aggregation) increase (P1 gate, red dots). (Similar FCS/SSC plots for SerpinB2<sup>+/+</sup> mice are shown in Fig. S5A). Histograms; the P-selectin staining for events in the P1 gate for SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice are shown, with mean fluorescent intensity (MFI) indicated. Total events collected was 30,000 for each strain. (Representative of two independent experiments).

re-calcification of citrated platelet rich plasma, P-selectin staining was (as expected) low for platelets from SerpinB2<sup>-/-</sup> and SerpinB2<sup>+/+</sup> mice (Fig. S5, -Ca<sup>2+</sup>). Events with high FSC/SSC (indicative of platelet aggregation) were also relatively low for SerpinB2<sup>-/-</sup> (Figure 1E, -Ca<sup>2+</sup>, P1 red dots) and SerpinB2<sup>+/+</sup> platelets (Fig S5A, -Ca<sup>2+</sup>, P1). After re-calcification events with high FSC/SSC increased for SerpinB2<sup>-/-</sup> (Figure 1E, +Ca<sup>2+</sup>, P1 red dots) and for SerpinB2<sup>+/+</sup> platelets (Fig. S5A, +Ca<sup>2+</sup>, P1 red dots). P-selectin staining of events in the P1 gates (ostensibly aggregated platelets) was higher for SerpinB2<sup>-/-</sup> mice than SerpinB2<sup>+/+</sup> mice (Figure 1E, +Ca<sup>2+</sup>, P1 histogram plots). These results further support the view that platelets from SerpinB2<sup>-/-</sup> mice have a more activated phenotype.

### Platelet Fractions Contain SerpinB2 and uPA

SerpinB2 deficiency thus leads to a bleeding phenotype that appears to be associated with platelets. To determine whether SerpinB2 protein can be found in platelets, platelets were isolated from platelet rich plasma (derived from citrated blood) from SerpinB2<sup>+/+</sup> mice and were analyzed by immunoblotting. Both SerpinB2 and SerpinB2-uPA complexes were clearly identified, although uncomplexed uPA (≈34 kDa) was not detected (Figure 2A, SerpinB2<sup>+/+</sup> mice). The presence of uPA on human [19,20] and mouse [21] platelets has been reported previously, although uPA was not identified in human platelets that had been highly purified [22] nor was it found in platelet alpha-granules from healthy humans [23]. The uPA receptor (uPAR) has been reported to be expressed on mouse platelets [24], with abundant PAI-1 expression in human platelets also reported [22].

To illustrate the specificity of the anti-SerpinB2 antibody, platelets from SerpinB2<sup>-/-</sup> mice were analyzed, with no significant reactivity seen (Figure 2A, SerpinB2<sup>-/-</sup> platelets), consistent with previous reports regarding the high level of specificity of this antibody [5].

### Immunohistochemistry of Blood Clots

Immunohistochemistry (IHC) of clots from C57BL/6 mice illustrated clumps of anti-SerpinB2 antibody staining distributed throughout the clot (Figure 2B, top panels). A similar pattern was observed in clots from SerpinB2<sup>R380A</sup> mice (Fig. S6A). IHC controls are shown in Fig. S6B,C. These clumps correspond to platelet aggregates in Giemsa stained sections of the same blood clot (Figure 2B, bottom panels), although the small clump sizes precludes the ability to match IHC and Giemsa in serial sections. (Wright-Giemsa staining is shown in Fig. S6D). Blood clots from SerpinB2<sup>-/-</sup> mice show a similar pattern of platelet aggregates (Figure 2C). (IHC of blood from a tail bleed dropped straight into fixative is shown in Fig. S6E).

### Discussion

Herein we show that loss of SerpinB2 expression (in SerpinB2<sup>-/-</sup> mice) or loss of SerpinB2 protease-inhibition activity (in SerpinB2<sup>R380A</sup> mice) results in significant reductions in tail bleeding times. This SerpinB2-associated hemostatic phenotype appeared to be associated with platelets. SerpinB2 and SerpinB2-uPA complexes were clearly identified in platelet fractions from wild-type mice, with immunohistochemistry of blood clots supporting the view that SerpinB2 is associated with platelet aggregates. Thromboelastography indicated faster onset of clot formation in blood from SerpinB2-deficient mice, whereas faster clotting was not apparent in platelet-free plasma. To the best of our knowledge, this is the first time SerpinB2 has been associated with a hemostatic phenotype, and the first time SerpinB2 and SerpinB2-uPA complexes have been reported to be associated with platelets.

Reduced bleeding times in the absence of bioactive SerpinB2 is difficult to reconcile with the canonical view that SerpinB2 inhibits plasmin-mediated fibrinolysis, which would predict that loss of SerpinB2 activity would prolong bleeding times. However,

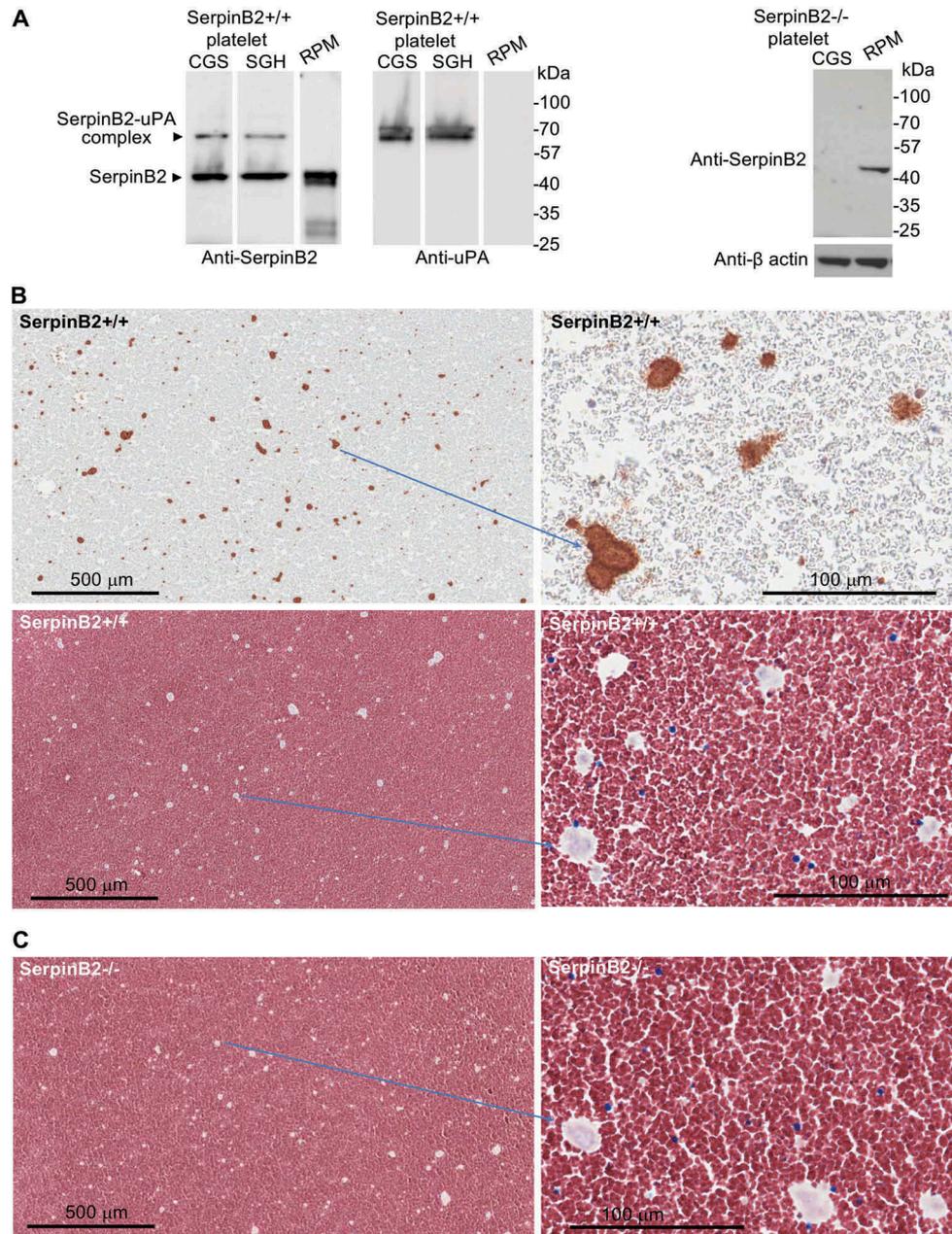


Figure 2. (A) SerpinB2<sup>+/+</sup> platelets; platelets isolated from SerpinB2<sup>+/+</sup> mice in CGS or SGH buffers were analyzed by immunoblotting using anti-murine SerpinB2 antibody and reprobed using anti-uPA antibody. SerpinB2<sup>-/-</sup> platelets; platelets were isolated from SerpinB2<sup>-/-</sup> mice and were analyzed by immunoblotting using anti-murine SerpinB2 antibody and reprobed using anti-murine β actin antibody (loading control). RPM – resident peritoneal macrophages from SerpinB2<sup>+/+</sup> mice; these cells constitutively express high levels of SerpinB2. (B) Blood clots from SerpinB2<sup>+/+</sup> mice were fixed, embedding into paraffin blocks and sections stained with anti-murine SerpinB2 antibody; high and low resolution IHC images are shown (top panels). (IHC controls with no primary antibody and staining of clots from SerpinB2<sup>-/-</sup> mice are shown in Fig. S5B,C). Giemsa staining of a section from the same block is shown in the bottom panels. (Wright-Giemsa staining is shown in Fig. S5D). (C) Example of Giemsa staining of a blood clot from SerpinB2<sup>-/-</sup> mice.

the reduced bleed times in SerpinB2<sup>-/-</sup> and SerpinB2<sup>R380A</sup> mice are consistent with increased bleeding times in uPA<sup>-/-</sup> and plasminogen-deficient mice [25]. (SerpinB2<sup>-/-</sup> and SerpinB2<sup>R380A</sup> mice are likely to have increased uPA/plasmin activation, whereas uPA<sup>-/-</sup> and plasminogen-deficient mice are likely to have reduced uPA/plasmin activation) [25]. Moreover, plasmin not only plays a key role in fibrinolysis, but has also been shown to promote platelet activation [21,26,27] via cleavage of (platelet expressed) protease activated receptor type 4 (PAR4) [28]. Thrombin also cleaves PAR4, with PAR4 considered a promising target for inhibiting thrombosis [29]. Excess exogenous thrombin might thus be expected to override any endogenous plasmin-

mediated activity, consistent with the observations presented in Fig. S4. The presence of SerpinB2 in blood clots (Figure 2B) might support the view that SerpinB2 also has a role in inhibiting fibrinolysis, at least in mice [15,16].

Clear illustrations of SerpinB2-uPA complexes in tissues *ex vivo* have been rare, suggesting that such complexes are usually present in small amounts, are rapidly cleared and/or are only generated in very specific settings/locations [2,4,5]. The immunoblotting results suggest SerpinB2-uPA complexes may form *in vivo*, although we cannot exclude the possibility that SerpinB2-uPA complexes formed in platelet fractions as a result of the isolation procedure. A bewildering array of binding partners and

activities have been attributed to SerpinB2 [2,10,18,30]; however, our data provides rare *ex vivo* evidence supporting the canonical view that SerpinB2 physiological role is inhibition of uPA [1,4–6,15].

How relevant might these observations in mice be to humans? Comprehensive proteomic analysis of human platelets did not detect SerpinB2 [22], consistent with our inability to detect SerpinB2 in human platelets. Quantitative ELISAs confirmed that most of the SerpinB2 in human blood is present in plasma [31], with some (as reported previously [7]) present on microparticles (Fig. S7). Low levels of SerpinB2 were found in platelet fractions from human blood, but this may have been due to the presence of SerpinB2-expressing microparticles in these fractions (Fig. S8), potentially derived from macrophages [4] and/or syncytiotrophoblasts [7]. Another difference is that cleavage of human PAR4 (compared with cleavage of mouse PAR4) requires significantly higher levels of plasmin to stimulate platelet aggregation [32]; although the physiological consequences of this difference remains unclear. Despite these apparent differences between mice and humans, the platelet phenotype seen in SerpinB2<sup>-/-</sup> and SerpinB2<sup>R380A</sup> mice is nominally consistent with observations in pre-eclamptic women. Pre-eclampsia is associated with both reduced levels of circulating SerpinB2 [31,33–35] and a platelet-associated hypercoagulopathy [36–39]. However, PAI-1 levels are often higher during pre-eclampsia, complicating any simple correlation between plasminogen activation and hypercoagulation [31,40].

In conclusion, although SerpinB2 has classically been associated with inhibition of uPA-mediated fibrinolysis, we illustrate herein that SerpinB2 (at least in mice) has an unexpected platelet-associated activity in the regulation clot formation. Further research is required to ascertain how relevant this new role for SerpinB2 might be in human diseases [6,9,41–45].

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## Disclosure Statement

The authors report no declarations of interest.

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## Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

## Supplemental data

Supplemental data for this article can be accessed [here](#)

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