SUPPLEMENTAL MATERIALS for "Activated platelets retain and protect most of their factor XIII-A cargo from proteolytic activation and degradation"

Yaqiu Sang, Robert H. Lee, Annie Luong, Éva Katona, Claire S. Whyte, Nicholas L. Smith, Alan E. Mast, Matthew J. Flick, Nicola J. Mutch, Wolfgang Bergmeier, Alisa S. Wolberg

METHODS

Materials. Prostaglandin I_2 (PGI_2, catalog number [cat]: 548925), apyrase (cat: A6535), Gly-Pro-Arg-Pro (cat: G5779), A23187 (cat: C7522), cycloheximide (cat: C1988), colchicine (cat: C9754) and rhosin (cat: 555460) were from Sigma (Burlington, Massachusetts). Recombinant human FXIII-A_2 (rhFXIII-A_2, Tretten®) was from Novo Nordisk. Human α-thrombin (cat: HT1002a), human FXa (cat: HFXa1011), mouse FXIII (cat: MFXIII), and sheep anti-human FXIII-A polyclonal antibody (cat: SAF13A-AP) were from Enzyme Research Laboratory (South Bend, Indiana). Rabbit anti-human FXIII-A polyclonal antibody was a generous gift from Dr. László Muszbek (University of Debrecen, Debrecen, Hungary). Convulxin (cat: 19082) was from Cayman Chemical (Ann Arbor, Michigan). Mouse anti-human CD41/61 monoclonal antibody (cat: MA1-19303, clone: IVA30), polyvinylidene difluoride membrane (cat: 88518), beaded-polyacrylamide resin (UltraLink™ Biosupport, cat: 53110), Alexa Fluor™ 647-labeled goat anti-mouse (cat: A-21235) and DyLight™ 488-labeled rabbit anti-sheep IgG (H+L) cross-adsorbed secondary antibody (cat: SA5-10054) were from Thermo Fisher Scientific (Waltham, Massachusetts). Radioimmunoprecipitation assay buffer (R26200) was from Research Products International (Mt Prospect, Illinois). Protease/Phosphatase inhibitor cocktail (100X) was from Cell Signaling Technology (cat: 5872). Recombinant hirudin (cat: RE120A) was from Aniara (West Chester, Ohio). Calpeptin (cat: 0448) was from Tocris Bioscience (Minneapolis, Minnesota). Anti-human tissue factor pathway inhibitor (TFPI) polyclonal antibody (TFPI-160) was made in-house in rabbit. Rabbit anti-human von Willebrand factor (vWF) polyclonal antibody (cat: ab6994) and Alexa Fluor® 555-labeled donkey anti-rabbit were from Abcam (cat: ab150062). IRDye® 800CW goat anti-rabbit IgG secondary antibody (cat: 92632211) and blocking buffer (cat: 92760003) were from Li-Cor Biotechnology (Lincoln, Nebraska). Transfer kit (cat:1704270) and 10% precast polyacrylamide gels (cat: 4568036) were from Bio-Rad Laboratories (Hercules, California). Vectashield Vibrance® antifade mounting medium (cat: H-1700) was from Vector Laboratories (Newark, California).

Preparation of human washed platelets. Fresh blood was drawn from consenting heathy donors into acid-citrate-dextrose anticoagulant buffer (ACD, 85 mM trisodium citrate dehydrate, 66.6 mM citric acid monohydrate, 111 mM D-glucose, 6:1 vol:vol).[1] All platelet preparation steps were performed at room temperature. Platelet-rich plasma (PRP) was prepared from whole blood by centrifugation (150×g, 15 minutes). Prostaglandin I_2 (PGI_2,1 μg/mL final) and apyrase (0.1 U/mL final) were added to PRP. PRP was centrifuged (100×g, 5 minutes) to pellet red blood cells. Platelets were pelleted from PRP by centrifugation (870×g, 10 minutes). The platelet pellet was washed in Tyrode's buffer (137 mM NaCl, 0.3 mM Na_2HPO_4, 12 mM $NaHCO_3$, 2 mM KCl, 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5 mM D-glucose, 0.175% bovine serum albumin, pH 7.4) containing PGI_2 and apyrase, and centrifuged (870×g, 10 minutes). The platelet pellet was resuspended in Tyrode's buffer containing PGI_2 and apyrase and rested at 37°C for 30 minutes before use. Platelets were counted on a BD Accuri™ C6 flow cytometer.

Preparation of mouse washed platelets. *F13a1*$^{-/-}$, *Fga*$^{-/-}$, and *Plg*$^{-/-}$ mice were backcrossed six generations on a C57BL/6J background.[2-4] *Stim1*$^{fl/fl}$ mice[5] were crossed with *Pf4-Cre* mice to generate *Stim1*$^{fl/fl}$ *Pf4-Cre*$^{+}$ mice, and maintained on a C57BL/6J background. Blood from anesthetized mice was

collected from the inferior vena cava into ACD (6:1 vol:vol) or from the retro-orbital plexus into heparinized tubes and then transferred into ACD. Blood was centrifuged (150×g, 5 minutes) to prepare PRP. PGI_2 (1 μg/mL final) and apyrase (0.1 U/mL final) were added to PRP, which was re-centrifuged (100×g, 5 minutes to remove residual red blood cells, followed by 700×g, 5 minutes to pellet the platelets). The platelet pellet was washed in Tyrode's buffer containing PGI_2 and apyrase, centrifuged (700×g, 5 minutes), resuspended in Tyrode's buffer containing PGI_2 and apyrase, and rested at 37°C for 30 minutes before use. Platelets were counted on a BD Accuri™ C6 flow cytometer.

Preparation of activated platelet fractions. Washed human (2×10^8 platelets/mL) or mouse (3×10^8 platelets/mL) platelets were stimulated with 5 U/mL thrombin, 100 ng/mL convulxin+5 U/mL thrombin, or 10 μM A23187 for the indicated times in the presence of 2 mM $CaCl_2$ at 37°C. Reactions were quenched with EDTA (20 mM final). For reactions with thrombin, 100 U/mL hirudin was added to inactivate thrombin. The reaction mixture was centrifuged (1500×g, 15 minutes) to separate the platelet pellet and releasate. The platelet pellet and releasate were resuspended in equal volumes of Tyrode's buffer. For absolute quantification, human platelet pellets were further diluted 5 times after resuspension. Inhibitors of transglutaminase (T101, 1, 3, 4, 5-Tetramethyl-2-[(2-oxopropyl)thio] imidazolium chloride, 50 μM final), protein synthesis (cycloheximide, 50 μg/mL final), microtubule polymerization (colchicine, 1 mg/mL final), calpain (calpeptin, 80 μg/mL final), or RhoA (rhosin, 40 μg/mL final) were preincubated with platelets for 5 minutes at 37°C before activation. In selected experiments, plasmin (50 nM final) was added at the same time as the agonists.

To characterize the effect of platelets on exogenous FXIII, 20 μg/mL mouse FXIII-A_2B_2 (mFXIII-A_2B_2) or 10 μg/mL recombinant human FXIII-A_2 (rhFXIII-A_2) was added to FXIII-A-deficient mouse platelets and then platelets were stimulated with 100 ng/mL convulxin+5 U/mL thrombin for 30 minutes. Reactions were quenched with 20 mM EDTA and 100 U/mL hirudin, as above. The reaction mixture was centrifuged (1500×g, 15 minutes) to separate the pellet and releasate and remove unbound mouse or human FXIII(a).

Immunoblotting. For FXIII-A and vWF quantification, the pellet and releasate were boiled for 5 minutes in reducing SDS sample buffer. For TFPI quantification, pellets were lysed and centrifuged (5000×g, 10 minutes) to remove cell debris. TFPI in the supernatant of the pellet lysate and the original releasate (centrifuged at 1500×g) was precipitated with FXa-agarose beads (2 hours, room temperature) as described.[6] FXa-agarose beads were washed 3 times with phosphate-buffered saline (PBS)/0.5% Tween-20 and once with PBS, and boiled 5 minutes in reducing SDS sample buffer. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes for immunoblotting. FXIII-A was detected with sheep anti-human FXIII-A polyclonal antibody (1:1000 dilution, 2 μg/mL final) and DyLight 488-labled rabbit anti-sheep secondary antibody (1:5000 dilution, 0.1 μg/mL final). TFPI was detected with rabbit anti-human TFPI polyclonal antibody (1:1000 dilution, 1.4 μg/mL final) and IRDye® 800CW goat anti-rabbit IgG secondary antibody (1:10,000 dilution, 1 μg/mL final). vWF was detected with rabbit anti-human vWF polyclonal antibody (1:1000 dilution, 1 μg/mL final) and IRDye® 800CW goat anti-rabbit IgG secondary antibody (1:10,000 dilution, 1 μg/mL final). Proteins were quantified by densitometry.

Flow cytometry. Washed human platelets (2×10^7/mL) or mouse platelets (3×10^7/mL) were stimulated with 5 U/mL thrombin, 100 ng/mL convulxin+5 U/mL thrombin, or 10 μM A23187 for the indicated times in the presence of 2 mM $CaCl_2$ at 37°C. For reactions with thrombin, 5 mM Gly-Pro-Arg-Pro was included to inhibit fibrin polymerization. Platelets were analyzed on a BD Accuri™ C6 flow cytometer equipped with BD Accuri™ C6 software, with 5000 events collected per sample. Platelets were gated by forward *versus* side scatter.

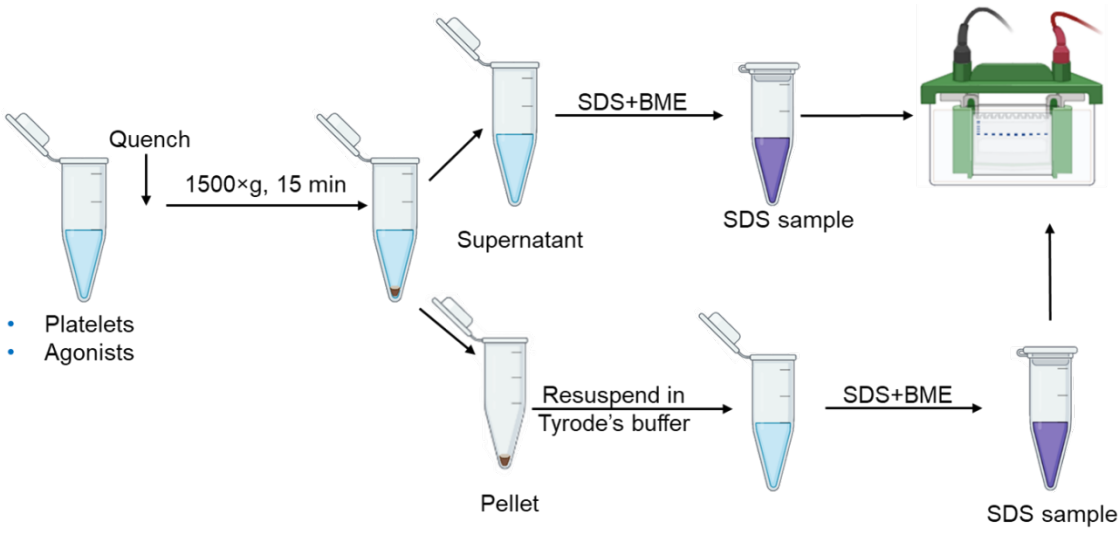
Nanoparticle tracking analysis and measurement of FXIII-A in extracellular vesicles (EVs). Washed human platelets (2×10^8 platelets/mL) were stimulated with 5 U/mL thrombin, 100 ng/mL convulxin+5 U/mL thrombin, or 10 μM A23187 for 30 minutes in the presence of 2 mM CaCl2 at 37°C. For select experiments, platelets were preincubated with calpain inhibitor (calpeptin, 80 μg/mL final) for 5 minutes at 37°C before activation. Reactions were quenched with EDTA (20 mM final). For reactions with thrombin, 100 U/mL hirudin was added to inactivate thrombin. The reaction mixture was centrifuged (1500×g, 15 minutes) to separate the platelet pellet and releasate. The releasate was centrifuged to separate large EVs (pellet of 20,000×g, 15 minutes), small EVs (pellet of 100,000×g, 70 minutes), and soluble proteins (supernatant of 100,000×g, 70 minutes), as described.[7,8] Aliquots of large EVs, small EVs, and soluble proteins were boiled for 5 minutes in reducing SDS sample buffer for FXIII-A quantification. Large and small EVs were washed once and suspended in 20 nm-filtered PBS and diluted to optimal concentration (1×10^7-1.5×10^8 particles/mL) for analysis by nanoparticle tracking analysis (UNC Nanomedicines Characterization Core). Videos (60 seconds each) were recorded of 11 positions using ZetaView software (version 8.05.12 SP2). The particle concentrations were corrected for the dilution necessary for nanoparticle tracking analysis.

Immunofluorescence. Washed human platelets (2×10^8 platelets/mL) were stimulated with 100 ng/mL convulxin+5 U/mL thrombin for 30 minutes in the presence of 2 mM $CaCl_2$ at 37°C. Reactions were quenched with 100 U/mL hirudin and 20 mM EDTA. Samples were diluted 10 times with PBS. For nonpermeabilized platelets, an aliquot of each sample was incubated with rabbit anti-human FXIII-A polyclonal (1:200 dilution, 19 μg/mL final), and mouse anti-human CD41/61 monoclonal (1:200 dilution, 5 μg/mL final) antibodies for 1 hour with rocking. Platelets were then immobilized onto glass slides ($1\text{-}2\times10^6$ cells/slide) by Cytospin centrifuge (Thermo Fisher Scientific (Waltham, Massachusetts) at 750 rpm for 5 minutes. Slides were air-dried and labeled with Alexa Fluor® 555-labeled donkey anti-rabbit (1:200, 10 μg/mL final), and Alexa Fluor™ 647-labeled goat anti-mouse (1:200, 10 μg/mL final) secondary antibody for 1 hour. For permeabilized platelets, platelets were directly immobilized onto glass slides, fixed by 4% paraformaldehyde for 10 minutes, permeabilized by 0.5% Triton-100 for 5 minutes, and incubated with primary and then secondary antibodies for 1 hour each. After staining, slides were washed and mounted with Vectashield Vibrance® antifade mounting medium.

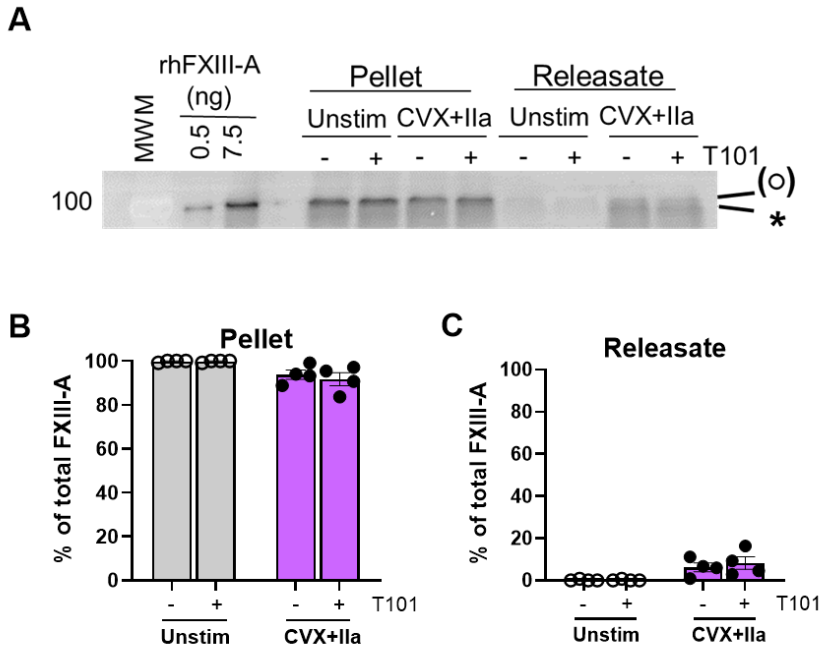
Platelets were imaged on a Zeiss LSM 900 confocal microscope (UNC Microscopy Services Laboratory) through a Plan-Apochromat 63X/1.40 oil immersion objective. The laser excitation wavelengths were 640 nm and 561 nm for the Alexa Fluor 647 and Alexa Fluor 555 channels, with the detection wavelength of 656-700 nm and 560-640 nm, respectively. The detector was a GaAsP photomultiplier tube. The pinhole diameter was set to 57 μm, corresponding to 1 and 1.13 airy units for 640 and 561 nm, respectively.

Platelets from 3 healthy donors were analyzed. For each donor, 10 images from each treatment group (101.4x101.4 μm, 1192×1192 pixels) were captured randomly; 3 images (50.7x50.7 μm, 596×596 pixels) containing ≥3 platelets in the view field from each treatment group were cropped from the larger images and processed on ImageJ (Version 1.54f). These images were then cropped further to show selected single platelets (17.0x17.0 μm, 200×200 pixels). The brightness/contrast of color images of each treatment was adjusted separately for optimal display. Grayscale images with equivalent brightness/contrast settings are shown in **Supplemental Figure 8**.

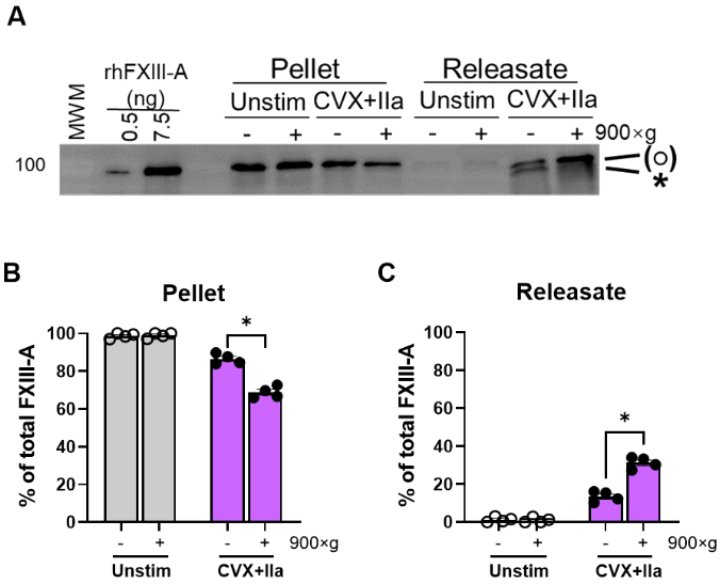
FIGURES



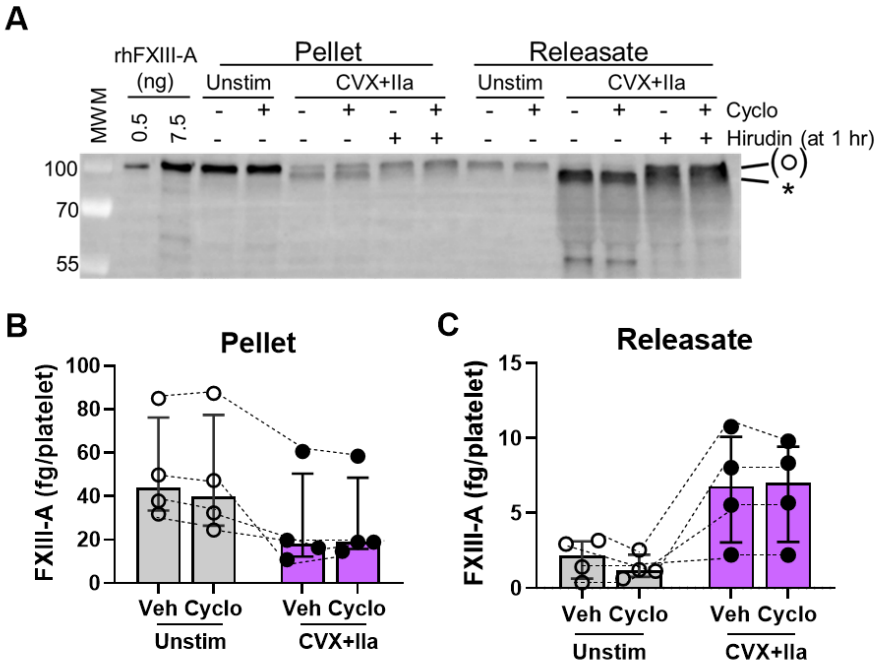
Supplemental Figure 1. Schematic of platelet FXIII-A quantification by immunoblotting. The quantification of platelet FXIII-A in Figure 1A-B was taken as an example. Washed human platelets (2×10^8 platelets/mL, 100 µL) were unstimulated or stimulated with 10 µL solution of convulxin (CVX)+thrombin (IIa) in $CaCl_2$ (2 mM final) for 30 minutes at 37°C. A solution of hirudin/EDTA (12 µL) was added to quench the reaction. The platelet pellet and releasate were separated by centrifugation (1500×g, 15 minutes). The supernatant (81 µL) was collected and boiled for 5 minutes with 19 µL 6X reducing SDS sample buffer. The pellet was resuspended in 610 µL Tyrode's buffer (5×original volume of suspension) and then 81 µL of resuspended pellet was collected and boiled for 5 minutes with 19 µL 6X reducing SDS sample buffer. Four or eight µL of each sample (containing ~0.5 or 1×10^6 platelets and 1 or 2×10^5 platelets for releasate and pellet, respectively) was subjected to 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes for immunoblotting. Recombinant human FXIII-A (rhFXIII-A) (1.5 and 6.5 ng) were loaded to generate a standard curve to convert densitometric measurements to FXIII-A concentrations.



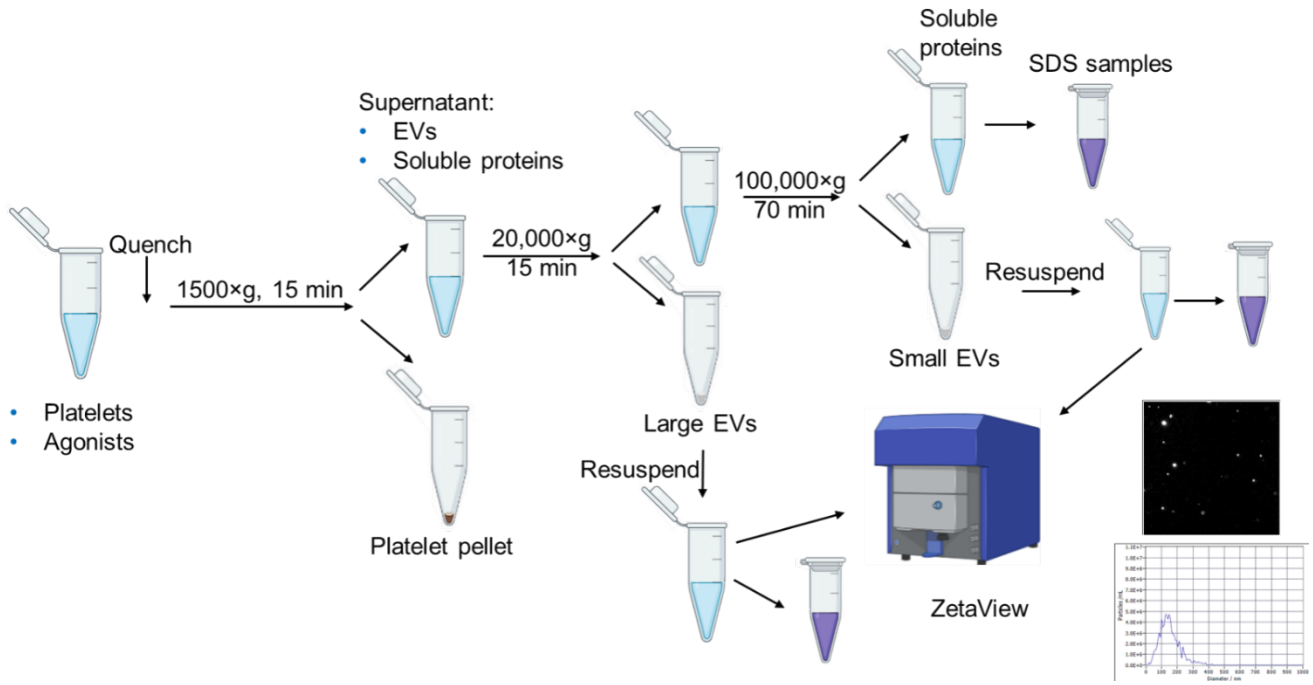
Supplemental Figure 2. Most platelet FXIII-A is retained by platelets even in the presence of a transglutaminase inhibitor. Washed human platelets were unstimulated (Unstim) or stimulated with convulxin (CVX)+thrombin (IIa) for 30 minutes in the absence or presence of T101. The platelet pellet and releasate were separated by centrifugation. FXIII-A content in each fraction was visualized by immunoblotting and quantified by densitometry. (**A**) Representative immunoblot for FXIII-A (rhFXIII-A, recombinant human FXIII-A loading control). (**B-C**) Quantification of FXIII-A in the (**B**) pellet and (**C**) releasate. The data show mean±SEM of 4 separate donors; FXIII-A release in the absence and presence of T101 were not significantly different.



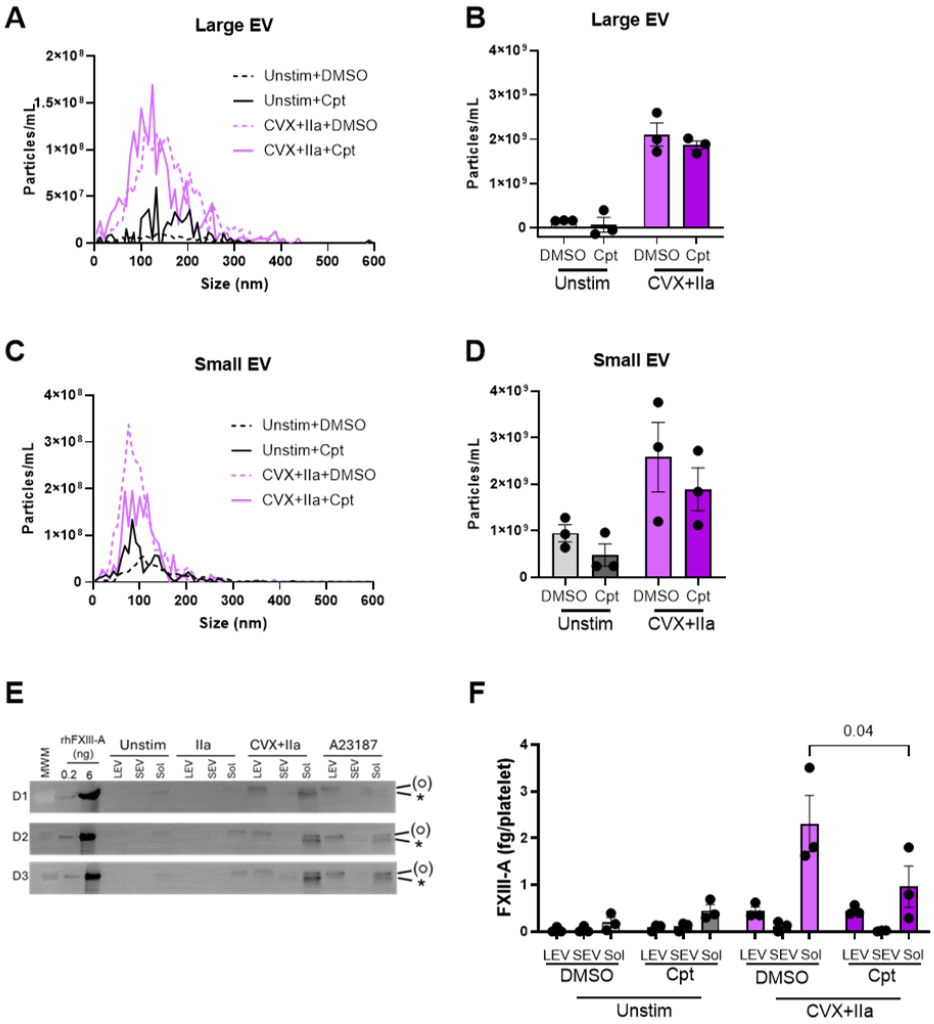
Supplemental Figure 3. Platelet FXIII-A release is slightly increased by platelet agglutination. Washed human platelets were unstimulated (Unstim) or stimulated with convulxin (CVX)+thrombin (IIa) for 30 minutes during which time the platelets were centrifuged at 900×g (or not). The agonists were then quenched, and the platelet pellet and releasate were separated by centrifugation. FXIII-A content in each fraction was visualized by immunoblotting and quantified by densitometry. (**A**) Representative immunoblot for FXIII-A (rhFXIII-A, recombinant human FXIII-A loading control). (**B-C**) Quantification of FXIII-A in the (**B**) pellet and (**C**) releasate. The data show mean±SEM of 4 separate donors. **P<0.05.*



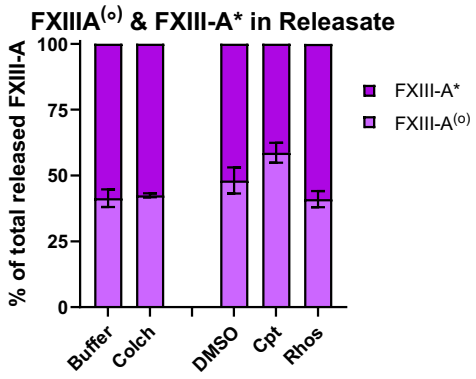
Supplemental Figure 4. Platelet pellet-associated FXIII-A is not newly synthesized even after 7 hours. Washed human platelets were unstimulated (Unstim) or stimulated with convulxin (CVX)+thrombin (IIa) for 7 hours in the absence (Veh, vehicle [ethanol]) or presence of cycloheximide (Cyclo). The platelet pellet and releasate were separated by centrifugation. FXIII-A content in each fraction was visualized by immunoblotting and quantified by densitometry. (**A**) Representative immunoblot for FXIII-A (rhFXIII-A, recombinant human FXIII-A loading control). (**B-C**) Quantification of FXIII-A in the (**B**) pellet and (**C**) releasate. The data show median±interquartile range of 4 separate donors. The increased variability seen at this long time point reflected individual donors, which is illustrated by dashed lines connecting individual donors.



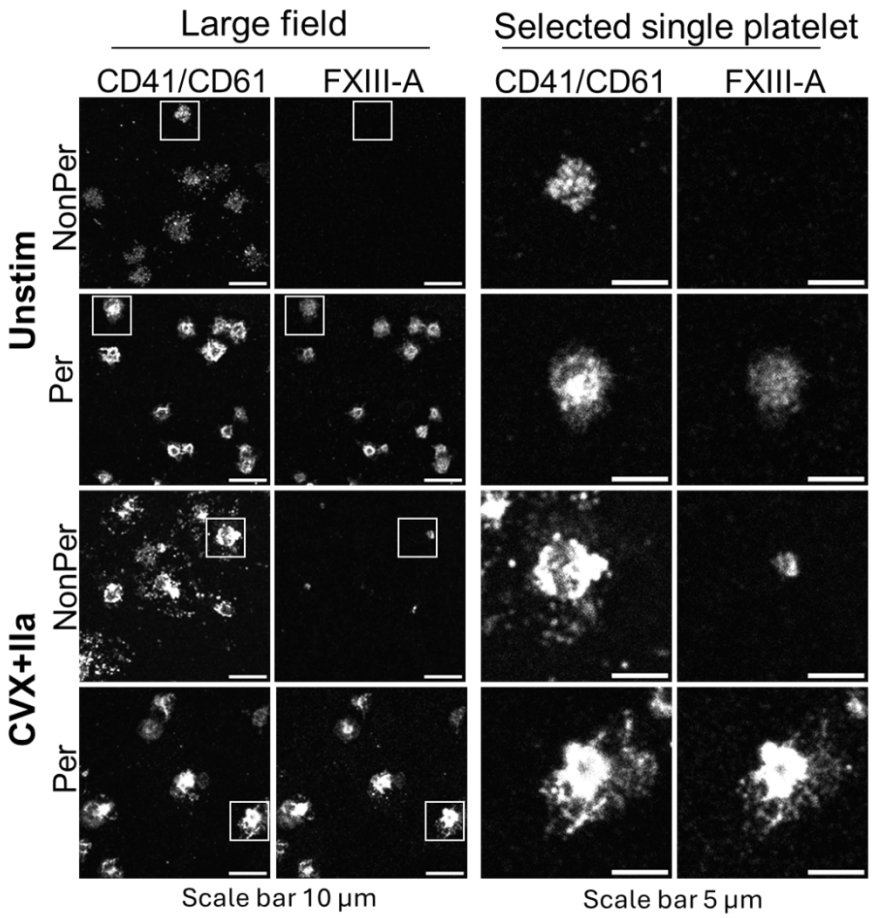
Supplemental Figure 5. Schematic of platelet-derived extracellular vesicles (EVs) isolation and quantification, and quantification of FXIII-A in EVs and soluble proteins. Washed human platelets were unstimulated or stimulated with thrombin, convulxin+thrombin, or A23187 for 30 minutes at 37°C. Reactions were quenched with EDTA±hirudin. The reaction mixture was centrifuged (1500×g, 15 minutes) to separate the platelet pellet and releasate. The releasate was centrifuged to separate large EVs (pellet of 20,000×g, 15 minutes), small EVs (pellet of 100,000×g, 70 minutes) and soluble proteins (supernatant of 100,000×g, 70 minutes), as described.[7,8] An aliquot of large and small EVs and soluble proteins were boiled for 5 minutes in reducing SDS sample buffer for FXIII-A quantification. Large and small EVs were washed once and suspended in 20 nm-filtered PBS and diluted to optimal concentration (1×10^7-1.5×10^8 particles/mL) for analysis by nanoparticle tracking analysis. Videos were recorded for 60 seconds each, with a measurement of 11 positions using ZetaView software (version 8.05.12 SP2). The particle concentrations were corrected for the dilution necessary for NTA analysis.



Supplemental Figure 6. Calpeptin partially reduces EV formation and FXIII-A release. Washed human platelets were unstimulated (Unstim) or stimulated with convulxin (CVX)+thrombin (IIa) for 30 minutes in the absence (buffer, DMSO [vehicle]) or presence of calpeptin (Cpt). Reactions were quenched with EDTA±hirudin. The reaction mixture was centrifuged serially to separate the platelet pellet and releasate (large EVs, small EVs, soluble proteins), as described in the Methods and illustrated in Supplemental Figure 5. (**A-B**) Nanoparticle tracking analysis of size distribution and enumeration of (**A-B**) large EVs and (**C-D**) small EVs. (**E**) Immunoblots of FXIII-A in large EVs, small EVs, and soluble proteins; rhFXIII-A, recombinant human FXIII-A loading control. (**F**) Quantification of FXIII-A in large EVs (LEV), small EVs (SEV), and soluble proteins (Sol). The data show mean±SEM of 3 separate donors (D).



Supplemental Figure 7. Calpeptin, rhosin, and colchicine do not alter FXIII-A* formation in the releasate. Washed human platelets were stimulated with convulxin+thrombin for 30 minutes in the absence (buffer, DMSO [vehicle]) or presence of calpeptin (Cpt), rhosin (Rhos), or colchicine (Colch). The platelet pellet and releasate were separated by centrifugation. FXIII-A was visualized by immunoblotting and quantified by densitometry. Relative quantification of FXIII-A$^{(\circ)}$ and FXIII-A* in the releasate, the data show mean±SEM of 4 separate donors.



Supplemental Figure 8. Activated platelets retain most of their FXIII-A within the platelet body. Grayscale images from the 4 groups shown in Figure 7 using the same brightness/contrast settings.

References

1. Cazenave JP, Ohlmann P, Cassel D, Eckly A, Hechler B, Gachet C. Preparation of washed platelet suspensions from human and rodent blood. *Methods Mol Biol*. 2004;272:13-28.
2. Souri M, Koseki-Kuno S, Takeda N, Degen JL, Ichinose A. Administration of factor XIII B subunit increased plasma factor XIII A subunit levels in factor XIII B subunit knock-out mice. *Int J Hematol*. 2008;87(1):60-68.
3. Suh TT, Holmback K, Jensen NJ, et al. Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. *Genes Dev*. 1995;9(16):2020-2033.
4. Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes Dev*. 1995;9(7):794-807.
5. Oh-Hora M, Yamashita M, Hogan PG, et al. Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nat Immunol*. 2008;9(4):432-443.
6. Maroney SA, Haberichter SL, Friese P, et al. Active tissue factor pathway inhibitor is expressed on the surface of coated platelets. *Blood*. 2007;109(5):1931-1937.
7. Sachetto ATA, Archibald SJ, Hisada Y, et al. Tissue factor activity of small and large extracellular vesicles in different diseases. *Res Pract Thromb Haemost*. 2023;7(3):100124.
8. Krishnamachary B, Cook C, Kumar A, Spikes L, Chalise P, Dhillon NK. Extracellular vesicle-mediated endothelial apoptosis and EV-associated proteins correlate with COVID-19 disease severity. *J Extracell Vesicles*. 2021;10(9):e12117.