SUPPLEMENTARY APPENDIX

Coated platelets function in platelet-dependent fibrin formation via integrin $\alpha_{IIb}\beta_3$ and transglutaminase factor XIII

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SUPPLEMENTAL MATERIALS AND METHODS

Materials. Horm type-I collagen was purchased from Nycomed. Factor XIII deficient plasma and the glycoprotein VI agonist convulxin came from Stago BNL. Human α-thrombin was from Kordia; human factor XIII (Fibrogammin®) was from CSL Behring GmbH, and PAR1-activating hexapeptide SFLLRN from Bachem. Annexin A5 labeled with Alexa Fluor-647 (AF647) or fluorescein isothiocyanate (FITC) came from Invitrogen; FITC-labeled thrombospondin from Abcam and HiLyte-488 (HL488)-labeled fibronectin from Tebu-bio. Active-site OG488-labeled human factor Xa and prothrombin as well as AF488-labeled bovine factor V(a) were prepared, as described.[1] FITC-labeled PAC1 mAb was from Becton Dickinson; FITC-labeled anti-integrin β_3 mAb from BD BioScience; FITC-labeled WAK mAb against platelet-bound fibrinogen from WAK Chemie; fibrin-specific mAb (anti-fibrin II β chain, clone T2G1) from Accurate Chemical & Scientific Corporation.

The 14-amino acid transglutaminase peptide substrate, GNQEQVSPLTLLK(C-rhodamine)W (Rhod-A14), derived from the *N*-terminal peptide sequence of α_2-antiplasmin, was synthetized as described.[2] The substrate contains the 14 amino acids of α_2-antiplasmin, implicated in its conjugation to fibrin.[3] The tissue-type transglutaminase specific inhibitor, *tert*-butyloxycarbonyl-(6-diazo-5-oxonorleucinyl)-QIV-OMe (Boc-DON) and the general transglutaminase inhibitor, Ac-LGPG-DON-SLVIG-NH_2 (K9-DON) came from Zedira. Other chemicals were from sources described before.[4]

Blood Collection and Platelet Preparation. Experiments were approved by the local Medical Ethics Committees. Blood was taken from healthy volunteers and from a patient with Glanzmann's thrombasthenia, with established deficiencies in integrin $\alpha_{IIb}\beta_3$[5], after informed consent and in accordance with the Declaration of Helsinki. For coagulation experiments, venous blood was collected into 1/10 volume of 129 mM trisodium citrate. For flow cytometry experiments, washed platelets were prepared from acid-citrate-dextrose anticoagulated blood, as described before.[6] Washed platelets were suspended into Hepes buffer A (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5 mg/ml glucose, 1 mg/ml BSA, pH 7.45). Final concentration of platelets in buffer medium was 5×10^7/ml, unless indicated otherwise. Platelet-rich plasma (PRP) was obtained by centrifuging citrate-anticoagulated blood at 240 g for 15 minutes and platelet-free plasma (PFP) by centrifuging twice at 2630 g for 10 minutes. Platelet count in PRP was determined with a Thrombocounter (Coulter Electronics), and normalized with autologous PFP. For flow cytometric experiments,

citrate-anticoagulated human PFP was defibrinated by 30 minutes treatment with ancrod protease (1.3 units/ml)[6] after which formed fibrin clots were removed from the plasma by centrifugation.

Animal studies were approved by the local Animal Experimental Committees. Mice deficient in factor XIII A1 subunit (*F13a1*^{tm1Gdi}, abbreviated as *F13a1*$^{-/-}$)[7] were bred on a mixed 129Sv/CBA background and were compared to *F13a1*$^{+/+}$ mice of the same backgrounds (Harlan Laboratories). Murine blood was taken on trisodium citrate for whole-blood flow experiments; other blood samples were taken on acid-citrate-dextrose anticoagulant to isolate washed platelets, as described.[4] Isolated mouse platelets were resuspended in modified Hepes buffer B (5 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 5 mg/ml glucose, 1 mg/ml BSA, pH 7.45).[8]

Flow Cytometric Platelet Analyses. Washed human or mouse platelets (5×10^7/ml) were preincubated with indicated inhibitors or Me_2SO vehicle for 10 minutes, and stimulated with thrombin (4 nM) and/or convulxin (100 ng/ml) in the presence of 2 mM $CaCl_2$. In the activations, 0.2 mM Gly-Pro-Arg-Pro (GPRP) was added to prevent formation of large fibrin fibers.[6] Subsamples were taken at indicated time intervals to determine incorporation of the transglutaminase-dependent probe Rhod-A14 (10 μg/ml). Platelet sub-populations were distinguished by probing with AF647-annexin A5 (1:200), AF488-factor V (20 nM), OG488-factor Xa (100 nM), AF647-fibrinogen (100 μg/ml) and FITC-PAC-1 (1.25 μg/ml). After staining for 5 minutes, samples were analyzed with a FACScan flow cytometer (BD Accuri Cytometers).[8] AF647-annexin A5 and AF647-fibrinogen were measured in the FL-4 channel, Rhod-A14 was measured in the FL-2 channel, while other fluorescent probes were measured in the FL-1 channel.

In a separate set of experiments, reconstituted PRP was activated with tissue factor (2 pM) and $CaCl_2$ (16.7 mM) in the presence of GPRP (2 mM) at 37 °C, after which samples were taken for fluorescent labeling. Analysis was by flow cytometry as above.

Thrombin Generation. Thrombin generation was measured in citrate-anticoagulated human PRP.[9] The PRP was diluted with autologous PFP to a count of 1.5×10^8 platelets/ml. Samples were activated with convulxin (100 ng/ml) plus thrombin (4 nM) or vehicle for 15 minutes. Aliquots (4 volumes) were transferred to a polystyrene 96-wells plate (Immulon 2HB, Dynex Technologies), already containing 1 volume of buffer C (20 mM Hepes, 140 mM NaCl, 5

mg/ml BSA and 6 pM tissue factor). Coagulation was started by adding 1 volume of buffer D (2.5 mM Z-GGR-AMC, 20 mM Hepes, 140 mM NaCl, 100 mM $CaCl_2$ and 60 mg/ml BSA). First-derivative curves were converted into curves of nanomolar thrombin concentrations using a calibrator for human α-thrombin.[10] All analyses were in triplicate.

Thrombus Formation on Collagen under Flow. Whole blood thrombus formation on collagen was assayed in the absence or presence of coagulation, as described before.[11] In brief, if no coagulation was required, citrate-anticoagulated mouse blood was recalcified in the presence of hirudin (3 μM) and PPACK (40 μM). The blood was then perfused over a coverslip coated with collagen type-I in a transparent parallel-plate perfusion chamber, at a wall-shear rate of 1000 s^{-1} for 4 minutes.[12] If desired, coagulation was introduced by co-perfusion of mouse blood with 0.5 volume of Ca-Hepes buffer (15 mM $CaCl_2$, 7.5 mM $MgCl_2$ and 0.42 mM NaH_2PO_4, pH 7.45) at 1000 s^{-1}. Coagulation of human blood was introduced by co-perfusion with 0.1 volume of adapted Ca-Hepes buffer (75 mM $CaCl_2$, 37.5 mM $MgCl_2$, 136 mM NaCl, 5 mM Hepes, 2.7 mM KCl, 1 mg/ml BSA, pH 7.45) at 1000 s^{-1}. Potential leukocyte contamination was excluded by CD45 and CD15 staining. Thrombi formed on collagen were post-stained with Rhod-A14 and AF647-annexin A5 in Hepes buffer A or B, supplemented with $CaCl_2$ (2 mM) and heparin (1 unit/ml). Phase-contrast and fluorescence images were captured and analyzed with Metamorph software version 7.5.0.0 (MDS Analytical Technologies), as detailed elsewhere.[12] Statistical difference of quantitative colocalization of two-color confocal images was determined using the ZEN software 2010 B SP1.

Microscopic measurement of platelet-dependent star-like fibrin formation under flow. Platelet-dependent star-like fibrin formation was measured as described before.[13] In brief, uncoated glass coverslips were mounted in a flow chamber and incubated for 10-15 min under stasis with a suspension of washed platelets (100×10^9/L). Remaining, non-bound platelets were removed by 5-min perfusion with Hepes buffer pH 7.45 containing 1% BSA. Adhered platelets were superfused with citrate-anticoagulated normal pool plasma or FXIII-deficient plasma at a calculated total shear rate of 250 s^{-1}. During plasma perfusion, coagulation was induced by co-perfusing 10 vol of plasma with 1 vol of 100 mM Hepes, 75 mM $CaCl_2$ and 37.5 mM $MgCl_2$ (pH 7.45). Phase contrast images were recorded in time using a 100×/1.3 NA oil immersion objective and a sensitive EM-CCD camera system (Hamamatsu, Japan). To quantify the orientation of the formed fibrin fibers a circle containing eight equal regions (region 1=0-45°, 2=45-90°, 3=90-135°, 4=135-180°, 5=180-

225°, 6=225-270°, 7=270-315°, 8=315-360°) was superimposed on >30 individual platelets per run, after which the number of fibrin fibers was calculated per region.

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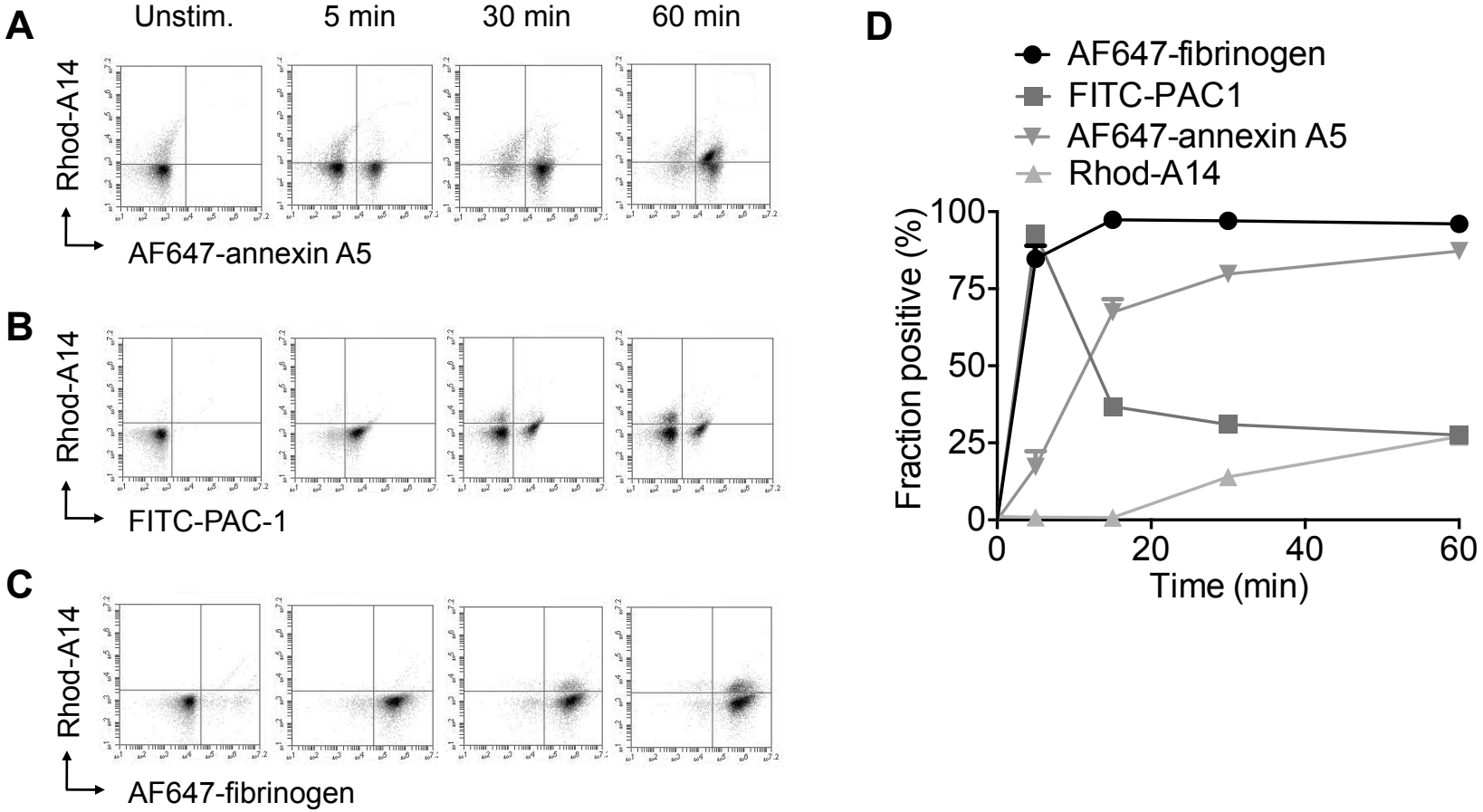
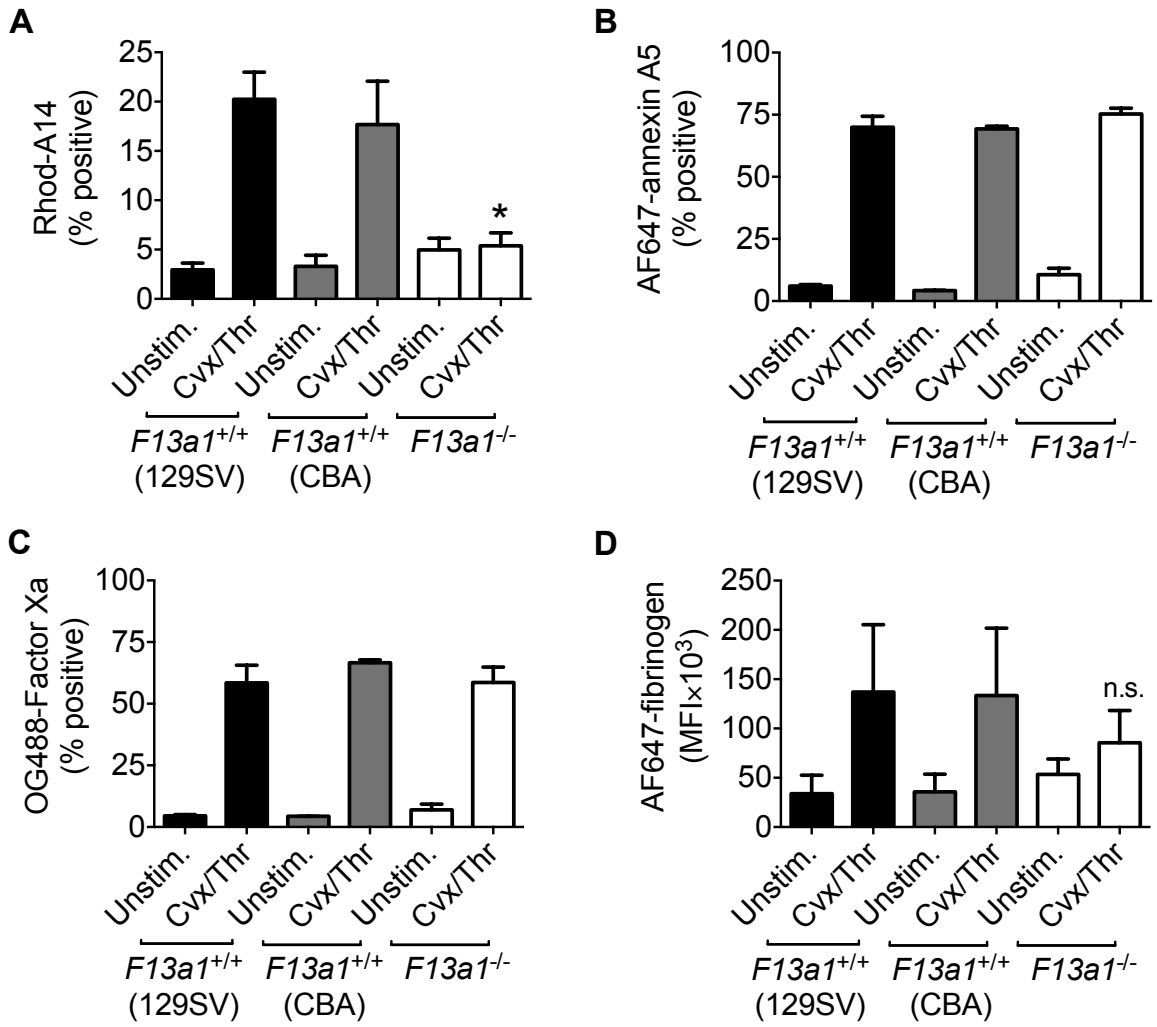
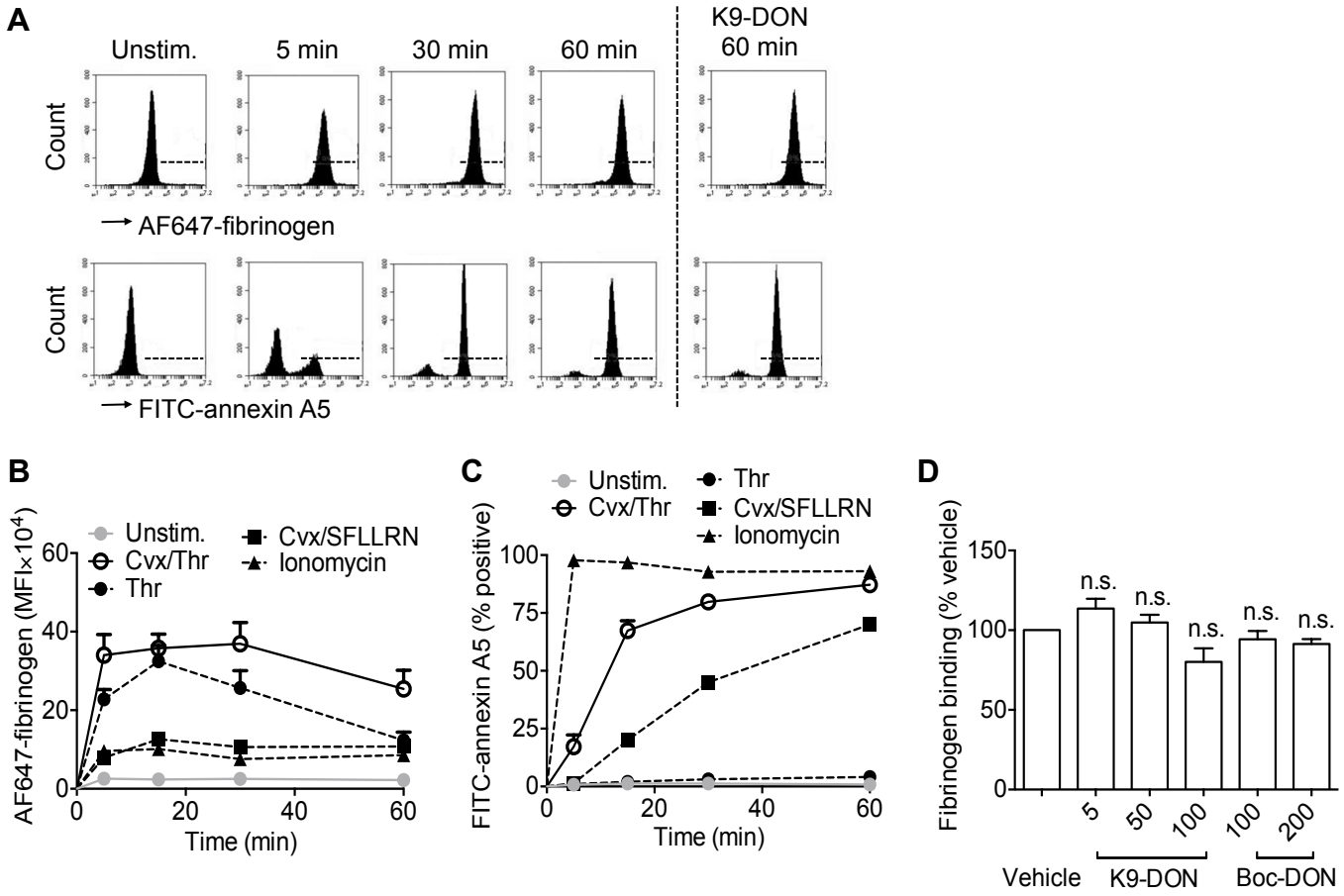
SUPPLEMENTAL FIGURES



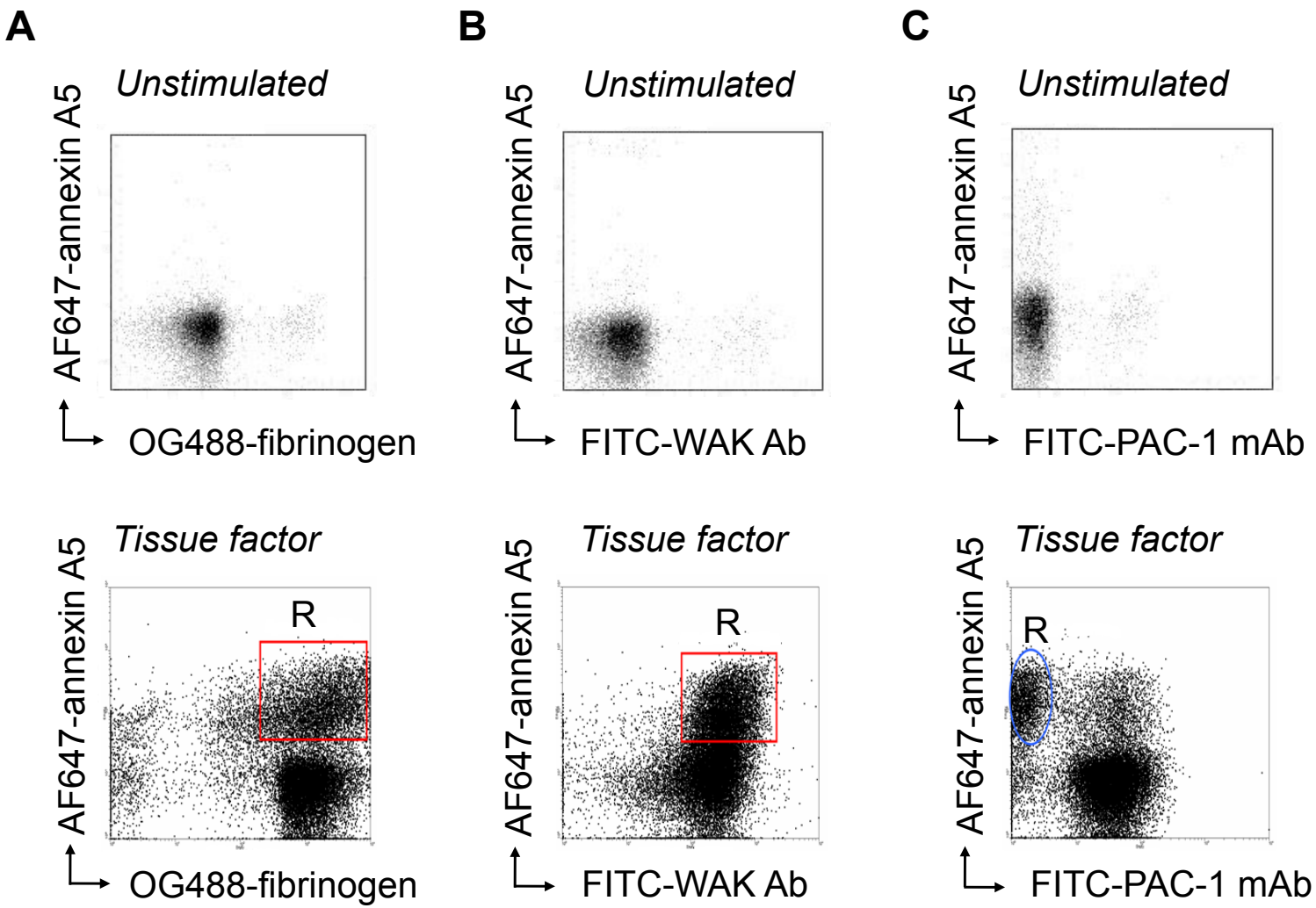
Suppl. Figure 1. Transglutaminase-positive platelets with inactive $\alpha_{IIb}\beta_3$ integrin appear after high fibrinogen binding. (**A**) Washed platelets were stimulated with convulxin and thrombin, as indicated for Figure 1. At given time points, samples were labeled for 5 minutes with Rhod-A14 in combination with AF647-annexin A5, FITC-PAC-1 mAb or AF647-fibrinogen. (**A, B, C,** respectively) Representative dot plots at indicated time points. (**D**) Time courses of fractions of FITC-PAC-1 (■), AF647-fibrinogen (●), AF647-annexin A5 (▼) and Rhod-A14 (▲) positive platelets. Mean ± SEM (n=3).



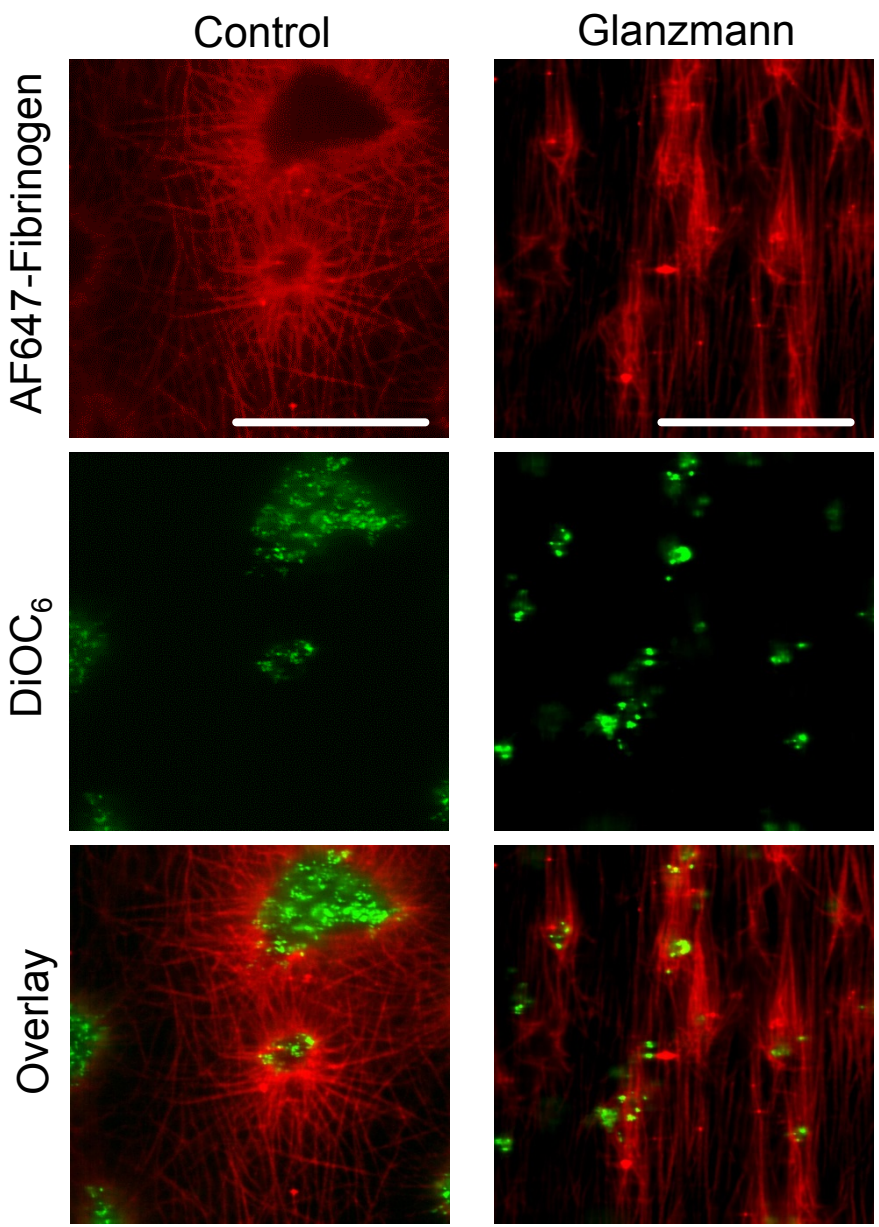
Suppl. Figure 2. Impaired transglutaminase activity and fibrinogen binding of factor XIII-A deficient platelets. Washed platelets from wild-type 129Ola or CBACa mice or *F13a1*$^{-/-}$ mice of mixed 129SV/CBA background were stimulated with convulxin (100 ng/ml) and thrombin (4 nM). After 60 minutes, samples were taken and labeled with Rhod-A14 (**A**), AF647-annexin A5 (**B**), OG488-factor Xa (**C**) or AF647-fibrinogen **(D)**. Shown are fractions of platelets binding the indicated probes. Mean ± SEM (n=3), *p<0.05.



Suppl. Figure 3. High fibrinogen binding precedes PS exposure independently of transglutaminase activity. (**A**) Washed platelets were stimulated with convulxin (Cvx) plus thrombin (Thr), as indicated for Figure 1. At given time points, samples were taken and stained with AF647-fibrinogen and FITC-annexin A5. Shown are representative histograms obtained by flow cytometry. (**B-C**) Staining patterns with AF647-fibrinogen (**B**) and FITC-annexin A5 (**C**) after platelet stimulation with Cvx/Thr (100 ng/ml, 4 nM) (○), Cvx/SFLLRN (100 ng/ml, 15 µM) (■), Thr alone (4 nM) (●) or ionomycin (10 µM) (▲). Solid grey line, unstimulated platelets. (**D**) Platelets were activated with Cvx/Thr, and pretreated with K9-DON (50 µM) or Boc-DON (200 µM), as indicated. Mean ± SEM (n=3-6), *p<0.05.



Suppl. Figure 4. Determinants of high fibrinogen binding of platelets in tissue factor-activated plasma. Defibrinated PPP was reconstituted with platelets and activated with tissue factor (2 pM) and $CaCl_2$ (16.7 mM). Samples were taken for fluorescent labeling after 0 and 60 minutes. Shown are representative dot plots obtained by flow cytometry. Labeling with AF646-annexin A5 plus either OG488-fibrinogen (**A**), FITC-WAK Ab (**B**) or FITC-PAC-1 mAb (**C**). N=3-4.



Suppl. Figure 5. Absence of functional $\alpha_{IIb}\beta_3$ alters thrombus-dependent fibrin formation. Human whole blood from a healthy volunteer (control) and a patient with Glanzmann's thrombasthenia was prelabelled with AF647-fibrinogen (16.5 µg/ml) and the membrane probe $DiOC_6$ (0.5 µg/ml), and then perfused over collagen under coagulating conditions at 1000 s^{-1}. Shown are representative confocal images of AF647-fibrinogen and $DiOC_6$ fluorescence taken one minute after start of fibrin formation (bars, 25 µm).