Supplementary Material for Ye *et al.*

STXBP5/Tomosyn-1 is Critical for Platelet Secretion and Hemostasis

SUPPLEMENTARY METHODS:

Washed Human Platelet Preparation: Banked platelets were obtained as anonymized units from the Kentucky Blood Center (Lexington, KY). Platelet-rich plasma (PRP) was isolated in the presence of 0.2 U/ml apyrase and 10 ng/ml PGI_2 by centrifugation at 150 × *g* for 10 min at room temperature. PRP was centrifuged at 700 × *g* for 10 min and pelleted platelets were resuspended in HEPES/Tyrode buffer (10 mM HEPES/NaOH, pH 6.5, 5.56 mM glucose, 137 mM NaCl, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.36 mM KH_2PO_4, 1 mM $MgCl_2$) with apyrase and PGI_2. Washed platelets were prepared by centrifugation at 700 × *g* for 7 min followed by resuspension in HEPES/Tyrode buffer (pH 7.4).

Generation of Anti-STXBP5 Antibody: The rat STXBP5/tomosyn-1 expression vector was a generous gift from Dr. Uri Ashery (Tel Aviv University, Tel Aviv, Israel) (1). His-STXBP5 (1-390 aa) was purified under denaturing conditions (6 M urea) and used as an antigen after dialysis. Polyclonal anti-STXBP5/tomosyn-1 antibodies were prepared by immunizing rabbits and then affinity purified from sera using the same antigen.

Washed Mouse Platelet Preparation: Mice were euthanized by CO_2 inhalation. After the heart was exposed, a 1 ml syringe with ~100 µl of 3.8% sodium citrate and attached to a 26 G needle was used to collect blood from the right ventricle. The citrated blood was then mixed with an equal volume of PBS and adjusted to 0.38% sodium citrate final concentration. Platelet-rich plasma was isolated in the presence of 0.2 U/ml apyrase and 10 ng/ml PGI_2 by centrifugation at 237 × *g* for 10 min at room temperature. PRP was centrifuged at 800 × *g* for 10 min and pelleted platelets were resuspended in HEPES/Tyrode buffer (pH 6.5) with apyrase and PGI_2. Washed platelets were prepared by centrifugation at 800 × *g* for 7 min followed by resuspension in HEPES/Tyrode buffer (pH 7.4).

Mouse Platelet Counting: Mice were euthanized by CO_2 inhalation and blood was collected by heart puncture. Whole blood from *Stxbp5* KO and WT littermates, without dilution, was analyzed using a Hemvet (DREW Scientific Inc.).

Measurement of Secretion from Intact Murine Platelets*:* The secretion assay was performed as described previously (2). Washed platelets were labeled with 2 μCi/ml [^{3}H]-Serotonin (Perkin-Elmer Cetus Life Sciences) for 45 min at 30°C. After washing twice with HEPES/Tyrode buffer (pH 7.4) in the presence of 0.2 U/ml apyrase and 10 ng/ml PGI_2, the platelets were resuspended with HEPES/Tyrode buffer (pH 7.4). Platelet concentration was determined by Z2 Coulter particle counter and size analyzer (Beckman Coulter) and adjusted to 2.5 × 10^5 platelets/μl. A final concentration of 0.7 mM $CaCl_2$ was added to the platelet suspension before stimulation. For thrombin titrations, a concentration series of thrombin (Chrono-Log) was added to stimulate platelets (50 μl of suspension) at room temperature. Reactions were stopped by adding a two-fold excess of hirudin (Sigma) and then placed on ice. For the time course experiments, 0.1 U/ml of thrombin was added for the indicated time periods, and 0.2 U/ml of hirudin was added to stop the reactions. When all reactions were finished, the samples were subjected to centrifugation at 13,000 × *g* for 1 min. The supernatant was recovered to another tube. The pellet was lysed with an equal volume of lysis buffer (PBS, pH 7.4, 1% Triton X-100) for 1 h on ice. Both supernatant and pellet fractions were assayed for the three granule cargo markers: [^{3}H]-5-HT for dense granules, PF4 for α-granules, and β-hexosaminidase for lysosomes, as described previously (3). For the dense granule release assay, 25 μl of the supernatant or the pellet was added to 3 ml of scintillation cocktail solution. The samples were analyzed using a Tri-Carb 2100TR liquid scintillation analyzer (Beckman). For α-granule secretion, a sandwich ELISA method was used to detect the specific α-granule marker, PF4. This ELISA assay was performed according to the manufacturer's instruction (R&D Systems). To assay lysosomal secretion, the enzymatic activity of β-hexosaminidase was measured. Six microliters of supernatant and 3 μl of the pellet samples was added to 100 μl of citrate-phosphate buffer (53.4 mM citric acid, 93.2 mM Na_2HPO_4, pH 4.5) containing 10 mM *p*-nitrophenyl-N-acetyl-β-D-glucosaminide. The reactions were incubated at 37°C in a sealed, 96 well plate for 18 h and stopped with 100 μl of 0.08 N NaOH. The optical density of each well was measured at 405 nm using a Biotek Elx808 plate reader (BioTek Instruments Inc.). The percent release for each

marker at each data point (triplicates) was calculated by using the supernatant fraction divided by the supernatant plus the pellet fraction (total).

Measurement of Secretion from Permeabilized Platelets*:* Streptolysin-*O* (SLO)-Permeablized Platelet Secretion Assay: The SLO-permeabilized platelet secretion assay was described previously (4). Banked human platelets (platelet-rich plasma, PRP) were obtained from Kentucky Blood Center (Lexington, KY) and were labeled with 0.4 μCi/mL [^{3}H]-Serotonin at 37°C for 45 min. After washing twice with HEPES/Tyrode buffer (pH 7.4) in the presence of 3 μg/mL apyrase, the platelets were resuspended in Buffer A (120 mM sodium glutamate, 5 mM potassium glutamate, 20 mM HEPES, pH 7.4, 2.5 mM EDTA, 2.5 mM EGTA, 3.15 mM $MgCl_2$, 1 mM DTT). After adjusting the platelet concentration to 5 × 10^8 platelets/mL, 50 μL was mixed with 50 μL of Buffer A containing 8 mM ATP, 1.6 U/mL SLO (Corgenix) and the purified anti-STXBP5/tomosyn-1 polyclonal antibody at RT for 10 min. The reaction was further incubated on ice for 30 min. The samples were then warmed to 25°C for 5 min and stimulated with calcium for 5 min. The reactions were stopped by centrifugation at 13,800 × g for 1 min to separate the supernatant from the pellet. The pellet samples were lysed with 100 μL Lysis Buffer for 1 h on ice. Assays for granule cargo are described in Methods.

Measurement of Platelet Serotonin: Total platelet serotonin levels were measured with a fluorometric assay as described previously (5). Briefly, washed platelets (5 × 10^5 platelets/μL) in HEPES/Tyrode buffer (pH 7.4) from single WT or *Stxbp5* KO mouse were isolated. Each sample (600 μl) was mixed with 120 μl 6M TCA, followed by centrifugation at 12,000 × *g* for 2 min at room temperature. To 500 μl of this TCA extract, 2.0 ml of *o*-phthalaldehyde reagent was added and the reaction was placed in boiling water bath for 10 min. After cooling, the reactions were extracted with chloroform and read on a spectrofluorometer with excitation and emission wavelengths of 360 nm and 475 nm, respectively.

Ultrastructural Analysis by Electron Microscopy: Washed mouse platelets were warmed in a 37°C metal block for 5 min. Platelets were either kept resting or stimulated with 0.1 U/ml of thrombin for 5 min. The platelets were then processed for electron microscopy as described previously (2). An equal

volume of 0.1% glutaraldehyde in White's saline (120 mM NaCl, 5 mM KCl, 2.3 mM $MgSO_4$, 3.2 mM $Ca(NO_3)_2$, 6.5 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 0.19 mM KH_2PO_4, 0.0005% phenol red) was added to the platelet suspension for 15 min at 37°C. The platelets were centrifuged at 3,000 × *g* for 2 min then resuspended and incubated in ice-cold 3% glutaraldehyde in White's saline at 4°C for 1 h. After three washes with White's saline, the platelets were osmicated in 1% OsO_4 in White's solution. This modification is known to generate a stable fixation of granule contents and provides a better identification of dense granules. Osmicated platelets were washed twice with 0.1 M Sorenson's buffer (16.2 mg/ml KH_2PO_4, 3.76 mg/ml Na_2HPO_4, pH 8.0) and dehydrated in a serial ethanol washes (50%, 70%, 80%, 90%, 100%, and previously unopened absolute ethanol) for 5 min. The platelets were rinsed twice with propylene oxide and infiltrated overnight in a 1:1 mixture of propylene oxide and Spurr's resin (10 g vinyl cyclohexane dioxide (VCD), 6 g DER epoxy resin, 26 g nonenyl succine anhydride (NSA) with final addition of 0.4 g dimethylaminoethanol (DMAE)). After one wash in Spurr's resin for 1 h, samples were embedded in 200 µl Spurr's resin and polymerized in a 60°C incubator for 48 h. Polymerized blocks were sectioned and mounted on copper grids. Following counterstaining with uranyl acetate, samples were examined using a Philips Tecnai 12 transmission EM and images were obtained with Gatan's Digital Micrograph software.

Static Platelet Adhesion to Fibrinogen: The adhesion assay was performed as described previously (6) with slight modification. Briefly, human fibrinogen (Enzyme Research Laboratories) diluted in Tris buffer (50 mM Tris, 100 mM NaCl, pH 7.4) was incubated in wells (50 µg/ml) of a black polystyrene 96-well plate (Nunc). Washed platelets were incubated with 7 µM calcein-AM for 30 min at 37°C. After washing, 100 µl of calcein-labeled WT or *Stxbp5* KO platelets (2×10^5 platelets/µl) in HEPES/Tyrode buffer (pH 7.4) containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ were loaded into fibrinogen-coated wells and allowed to adhere for 1 h at 37°C. The nonadherent platelets were removed by washing with HEPES/Tyrode buffer (pH 7.4), the number of adherent platelets was determined by measuring fluorescence (excitation and emission wavelengths of 490 nm and 519 nm, respectively) and referenced to a standard curve using fixed numbers of WT or KO platelets.

Analysis of Platelet Spreading on Fibrinogen: Washed mouse platelets from WT and KO mice were freshly prepared in HEPES/Tyrode buffer (pH 7.4) containing 1 mM $CaCl_2$ and adjusted to 2.5 X 10^4 platelets/µl. The platelets (100 µl) were seeded onto human fibrinogen- (50 µg/ml; Enzyme Research Laboratories) coated Lab-Tek II 16 well chamber slides (Nunc), followed by incubation at 37°C for 2 h. The samples were washed twice with PBS and then fixed with 4% paraformaldehyde O/N at 4°C. Platelets were permeabilized with buffer (0.1 M Tris, 0.1% Triton X-100, 0.15 M NaCl, 0.01 M EGTA, 5 mM, $MgCl_2$, pH 7.4) for 30 min at room temperature and stained with 0.1 µM TRITC-phalloidin (Sigma) for 30 min at room temperature. After 3 washes with PBS, platelets were mounted with VectaShield (Vector Laboratories) on a Lab-Tek coverglass. The coverglass was sealed with nail polish and samples were visualized using a Nikon Eclipse E600 microscope (Nikon) with a 100X/1.40 NA DIC H oil objective lens (Nikon). Images were taken using Zeiss camera (AxioCam MR). Images were processed with Zen 2011 (blue edition, Zeiss) and analyzed with image J (V1.47, NIH).

Statistical Analysis: Statistical analyses for adhesion and spreading assays were done with a one-tailed student's t-test using SigmaPlot12.0 (Systat Software Inc.) and the *p* values were indicated.

Study Approval: Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Human samples were from anonymized units and thus no IRB approval is required.

SUPPLEMENTARY REFERENCES:

1. Yizhar O, Matti U, Melamed R, Hagalili Y, Bruns D, Rettig J, and Ashery U. Tomosyn inhibits priming of large dense-core vesicles in a calcium-dependent manner. *Proc Natl Acad Sci U S A.* 2004;101(8):2578-83.

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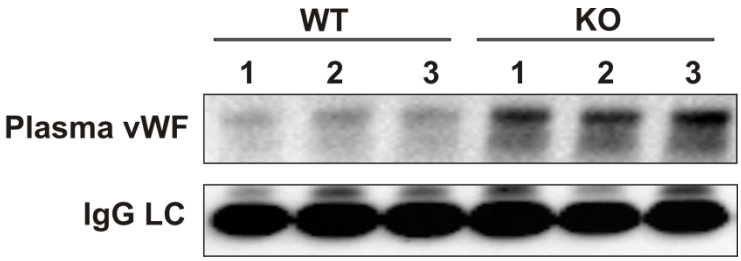
6. Fulkerson Z, Wu T, Sunkara M, Kooi CV, Morris AJ, and Smyth SS. Binding of autotaxin to integrins localizes lysophosphatidic acid production to platelets and mammalian cells. *J Biol Chem.* 2011;286 (40):34654-63.

SUPPLEMENTARY DATA:

Supplementary Table 1: Syntaxin-Containing Complex Interacting Proteins

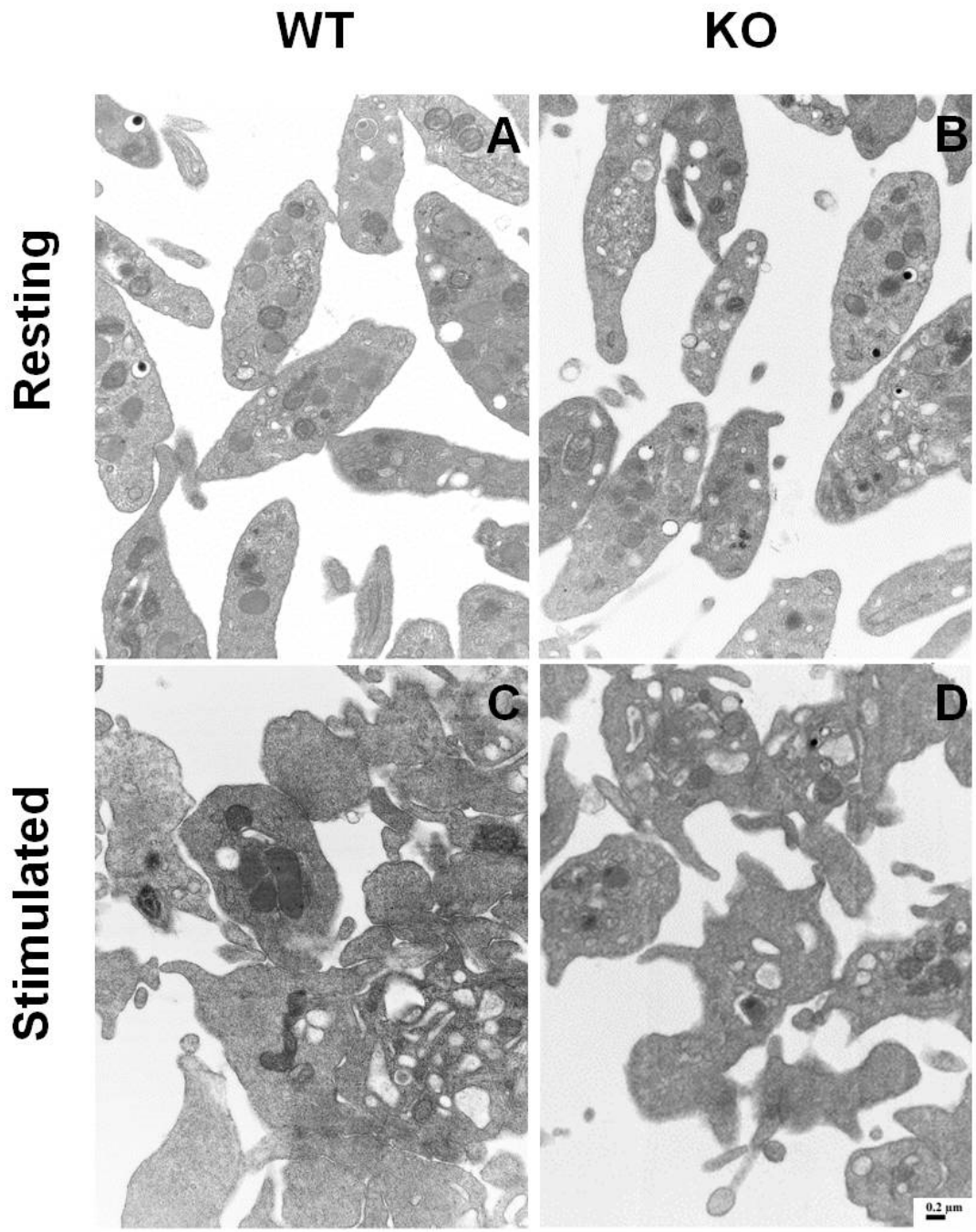
Sample	MOWSE Score	Protein
T1	144	STXBP5/Tomosyn-1
T2	66	STXBP5/Tomosyn-1
T3	150	Phosphofructokinase C
T4	325	Munc18b
T5	259	Granuphilin/SLP4

Supplementary Figure 1



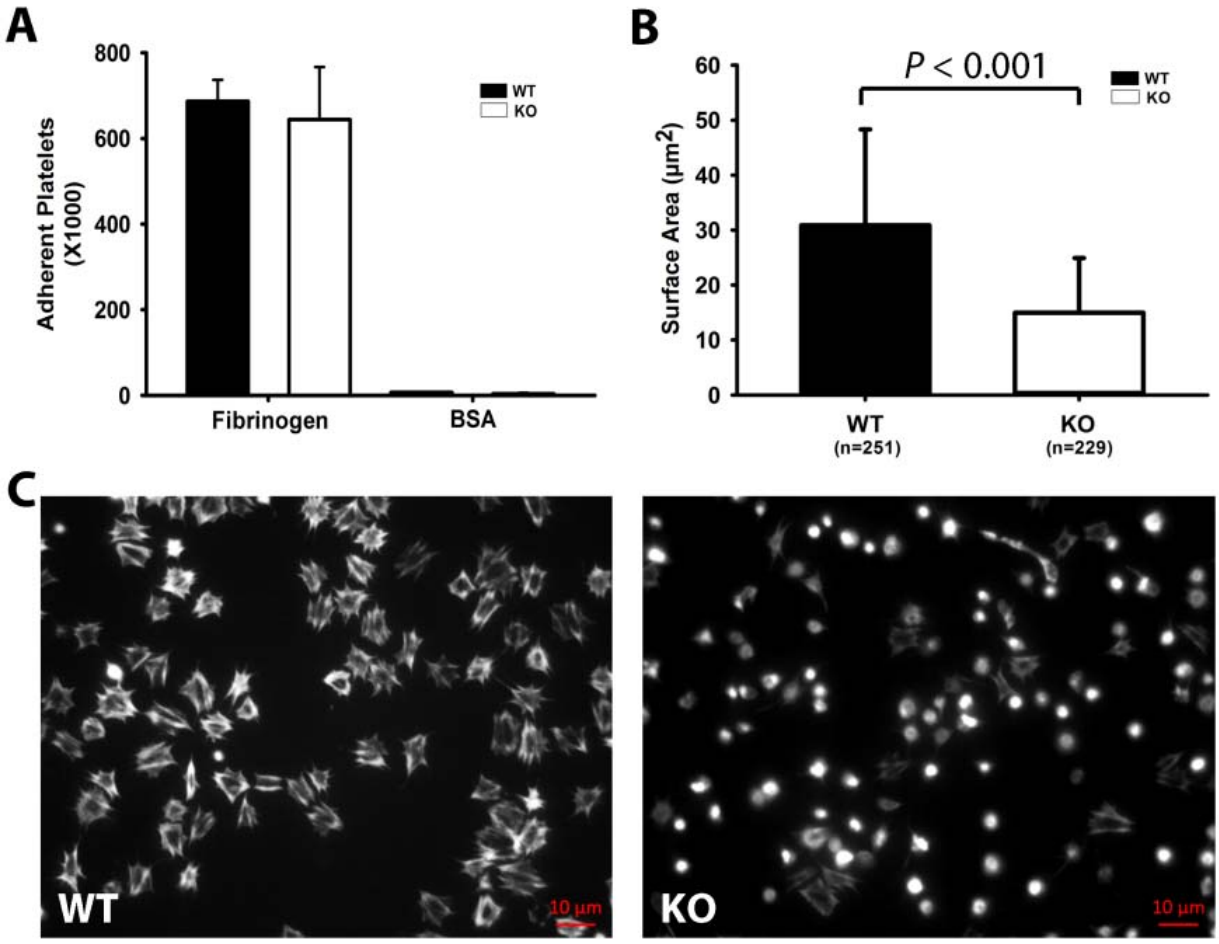
Supplementary Figure 1: STXBP5/tomosyn-1-deficient mice have elevated plasma vWF levels. Plasma samples (2.5 µl) were prepared from three individual *Stxbp5* KO (KO) or WT mice and analyzed by IB with anti-vWF antibody and anti-mouse IgG antibody.

Supplementary Figure 2



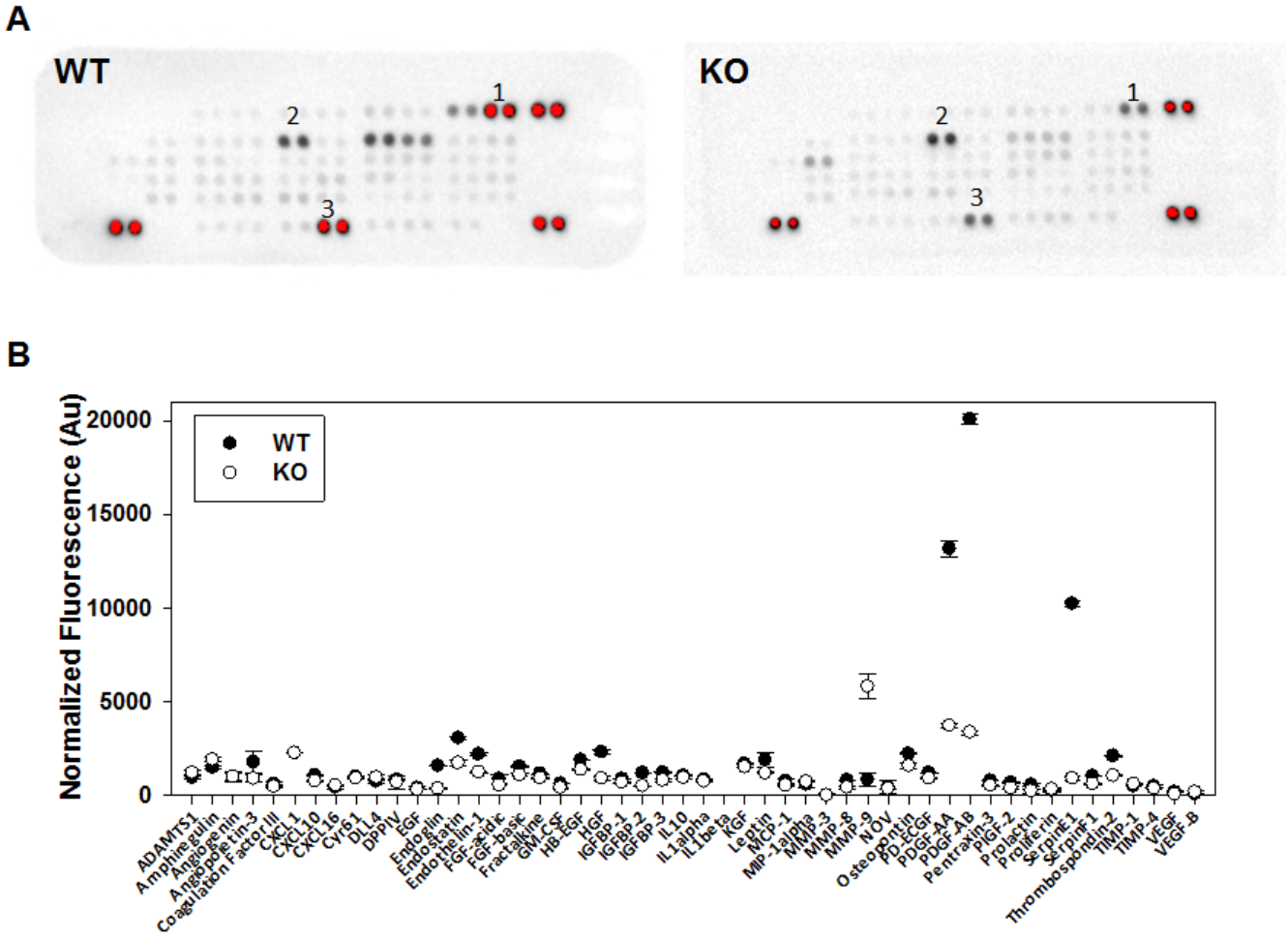
Supplementary Figure 2: Depletion of STXBP5/tomosyn-1 does not affect platelet morphology or cytoskeleton rearrangement: Washed platelets from *Stxbp5* KO (KO) and WT littermates were kept resting with 1 ng/ml PGI_2 (**A-B)** or stimulated with 0.1 U/ml thrombin (**C-D**) for 5 min. The platelets were fixed and processed for EM analysis as described in the Methods. The samples were analyzed by transmission EM and images were obtained using Gatan software. The scale is indicated.

Supplementary Figure 3

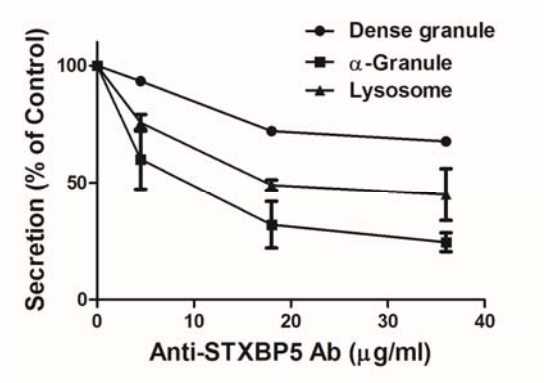


Supplementary Figure 3: Depletion of STXBP5/tomosyn-1 affects platelet spreading on fibrinogen surface but not adhesion. A) Calcein-labeled washed platelets (2 × 10^5 platelets/μl) were isolated from WT (filled bar) or KO (open bar) mice and allow to adhere to microtiter wells coated with fibrinogen. Results are presented as the number of adherent platelets with mean and SD from triplicate determinations. No significant differences were detected. **B)** Quantification of platelet surface area of cells spread on fibrinogen-coated surface. Washed WT and KO platelets (2.5 × 10^4 platelets/μl) were seeded onto fibrinogen-coated surface for 2 h. After washing, the adherent platelets were fixed with 4% paraformaldehyde, then permeabilized and stained for F-actin with TRITC-phalloidin. Images were taken using a Nikon E600 epifluorescence microscope. The surface area was measured using image J. Statistical analysis was done using SigmaPlot12.0. **C)** Representative images of WT and KO platelets spread on fibrinogen-coated surfaces. The scale is indicated.

Supplementary Figure 4



Supplementary Figure 4: Protein profiler array analysis of WT and *Stxbp5* KO platelet extracts. A) Representative image of developed array analysis of WT and *Stxbp5* KO platelet extracts. Platelet extracts were prepared and analyzed on Proteome Profiler Mouse Angiogenesis Antibody Arrays (ARY 015, R &D Systems) according to manufacturer's instructions. The signals for each array spot were detected with Supersignal ELISA Femto Maximum Sensitivity Substrate using a ChemiDoc MP System. Pairs of fiduciary marks are in three of the four corners. **B)** Semi-quantitative analysis of profiles of 50 molecules assessed from arrays. Shown is the representative analysis of a single set of arrays. The fluorescence intensities for each spot were measured, and background was subtracted. The mean fluorescence of the two different array spots is represented with the range of values indicated (closed, WT; open, *Stxbp5* KO). SDF-1 (spots # 1), PF4 (spots # 2), and angiopoietin 1 (spots # 3) were excluded from analysis due to saturation of their respective spots on the array membranes.



Supplementary Figure 5: Introduction of anti-STXBP5 Ab into permeabilized platelets inhibits platelet secretion. The permeabilized platelet exocytosis assay was performed in the presence of increasing amounts of anti–STXBP5 rabbit polyclonal Ab. After stimulation with Ca^{2+} (10 μM), [^{3}H]-Serotonin release for dense granules, PF4 release for α-granules, and β-hexosaminidase release for lysosomes were measured and normalized as a percentage of control (no antibody added) release. Each point was repeated in triplicate and the means with STD are presented.