

Platelet dysfunction in platelet-type von Willebrand disease due to the constitutive triggering of the Lyn-PECAM1 inhibitory pathway

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SUPPLEMENTARY METHODS

The terms control and PT-VWD along this manuscript refer to human platelets while the respective terms used for murine platelets are Tg^{WT} and Tg^{G233V}. All human and animal studies were approved by the responsible Institutional review boards (human: CEAS Umbria, approval number 2663/15; animal: Italian Ministry of Public Health authorization number 561/2015-PR).

Human studies and blood sampling

The PT-VWD patient carrying the M239V *GP1BA* variant (to our knowledge the only patient diagnosed in Italy so far) was previously reported and presented the typical PT-VWD phenotype with mild macrothrombocytopenia, enhanced ristocetin induced platelet agglutination (RIPA), increased VWF-binding to platelets^{9,10}, and mucocutaneous bleeding with an ISTH-BAT score of 11¹¹. Each experiment was repeated from 3 to 5 times from blood samples collected on different days (in the text indicated as independent experiments, 8 in total). Age- and sex- matched healthy controls were studied in parallel. Given that the degree of thrombocytopenia typically varies in time in PT-VWD patients², platelet counts and mean platelet volume (MPV) at the various blood samplings are reported under Supplementary Table 1 and samples used in each experiment are indicated under figure legends.

To minimize platelet activation, blood was taken without tourniquet using a 21 gauge needle after discarding the first 2 ml in trisodium citrate (3.2%, 0.109M, 1/10 v/v) tubes except for platelet counting, MPV and electron microscopy for which it was taken in EDTA tubes.

Mouse strains and blood sampling

The generation of PT-VWD mice expressing the human *GP1BA* transgene carrying the p.G233V variant (Tg^{G233V}) and of control mice expressing a wild-type human *GP1BA* transgene (Tg^{WT}) was previously described^{4,12}. These animals express either normal or mutant human GPIIb/IIIa (Tg^{WT} and Tg^{G233V}) and no mouse GPIIb/IIIa, and have been consistently backcrossed with C57BL/6J animals. Mice expressing the G233V *GP1BA* variant have macrothrombocytopenia, prolonged tail bleeding time and increased VWF-binding to platelets (Supplementary Figure 1).^{5,10,12} Mice were bred and housed in the animal facility of the University of Perugia, Perugia, Italy, and all experiments were performed with 3 to 6 months-old mice. The number of males and females and platelet counts are reported under each figure legend.

To minimize platelet activation blood was collected by cardiac puncture using a 21 gauge needle in a syringe containing trisodium citrate (4%, 0.136M, 1:10 v/v) from mice anesthetized with xylazine (5 mg/kg) and ketamine (50 mg/kg) injected intraperitoneally¹³.

Light transmission aggregometry (LTA)

Platelet-rich plasma (PRP) was obtained by centrifugation of citrated human blood at 160g for 10 minutes. PRP was stimulated with ADP 10 μ M, epinephrine 100 μ M, TRAP-6 20 μ M, arachidonic acid 1mM, collagen 1 mg/ml, convulxin (CVX) 5 μ g/ml and aggregation was monitored by LTA using a four-channel ATRACT4 aggregometer (Helena Biosciences, UK).¹⁴ For the study of shape change, PRP was pretreated with the $\alpha_{IIb}\beta_3$ blocker Arg-Gly-Asp-Ser peptide (RGDS) (120 μ g/ml) in order to prevent platelet aggregation and then stimulated with ADP 10 μ M, TRAP-6 2 μ M or collagen 1 mg/ml and shape change was monitored by LTA by magnifying the length of the y and

x axes through the ATRACT4 software. The shape change reaction speed was measured by the slope of the tangent to the steepest part of the curve in mm *per* minute.^{15,16}

$\alpha_{IIb}\beta_3$ activation

Five μ l of human or murine citrated blood were diluted with 100 μ l of PBS and incubated with a stimulus for 15 minutes and with saturating concentrations of antibodies recognizing the active conformation of $\alpha_{IIb}\beta_3$: FITC-conjugated mouse PAC-1 MoAb (BD Bioscience, Franklin Lakes, NJ, USA) for human platelets and PE-conjugated JON/A MoAb (Emfret Analytics, Eibelstadt, Germany) for murine platelets. A range of agonists were used as stimuli including ADP 10 μ M, TRAP6 20 μ M or CVX 20 ng/ml, rhodocytin 10 nM for human platelets and thrombin 0.05U/ml + the coagulation-blocking peptide Gly-Pro-Arg-Pro (GPRP) 2 mM or CVX 25 ng/ml for murine platelets. ADP was not used to stimulate murine platelets because it provides a weak and unpredictable aggregation response.¹⁷ Antibodies were added to the samples together with the agonist. PE-conjugated anti-CD42b (Beckman Coulter Italia, Milan, Italy) and FITC-conjugated anti-GPIX (Emfret Analytics) were used to gate for human and murine platelets, respectively.

To define negative staining samples were incubated with an isotype control that was a FITC-labelled mouse IgM antibody.

Samples were analyzed in a CytoFLEX or in a Cytomics FC500 flow cytometer (Coulter Corporation, Miami, Florida, USA) equipped with an argon laser operating at 488 nm.^{14,18}

Granule content and secretion

Platelet granule content was assessed by electron microscopy. Platelets were fixed for 4 h at 4°C using cacodylate buffer containing 4% wt/vol of glutaraldehyde, then washed and kept in cacodylate buffer for 4 h. The buffer was then replaced with 1% osmic acid and the samples were pelleted by centrifugation at 10,000xg for 30 sec. Ultrathin sections of the pellets were stained with uranyl acetate and lead citrate and observed under a Philips Electron Optics EM208 transmission electron microscope at 80 kv^{10,14}. At least 50 platelets were analyzed.

Alpha-granules secretion was measured as P-selectin expression¹⁹, by incubating 5 μ l of human or murine citrated whole blood diluted with 100 μ l of PBS with different agonists and saturating concentrations of a FITC-conjugated anti P-selectin antibody (anti-CD62P from Beckman Coulter for human and from Emfret Analytics for murine platelets) for 15 minutes in the dark. Agonists used were ADP 10 μ M, TRAP6 20 μ M or CVX 20 ng/ml, rhodocytin 10 nM for human platelets and thrombin 0.05 U/ml + GPRP 2mM or CVX 20 ng/ml for murine platelets.^{20,21} PE-conjugated anti-CD41 antibodies (Beckman Coulter for human and Emfret Analytics for murine platelets) were used to gate for platelets. To define negative staining samples were incubated with an isotype control that was a FITC-labelled mouse IgG1 antibody.

To assess dense granule content and release, the green fluorescent dye mepacrine, which accumulates specifically in dense granules, was used.²² Five μ l of blood were added to 100 μ l of PBS in the presence of a PE-labeled anti CD41 MoAb to gate for platelets (Beckman Coulter for human and Emfret Analytics for murine platelets) and of mepacrine 5 μ M (Sigma, Darmstadt, Germany) in polypropylene tubes. To define negative staining samples were incubated with a PBS-CD41 solution.

After 30 min of incubation in the dark, samples were diluted in PBS, thrombin 0.05 U/ml was added and after 5 min samples were analyzed by flow cytometry in a CytoFLEX or in a Cytomics

FC500 flow cytometer (Coulter Corporation).²⁰ Mepacrine release was calculated by the following formula: 1-(residual mepacrine content after thrombin stimulation with 0.05 U/ml / total mepacrine incorporated) x100.

Platelet washing

Human PRP or murine PRP, the latter obtained by centrifugation of citrated murine blood for 10 minutes at 120xg, were washed according to the Mustard method²³, as described.²⁴

Spreading assay

Washed platelets (20×10^6 /ml) resuspended in Tyrode's buffer were layered onto glass slides coated with 100 μ g/ml of human fibrinogen or 25 μ g/ml of human type I collagen for 30 minutes at room temperature. After one washing with PBS, attached platelets were fixed with 3.7% PFA, permeabilized with 0.1% Triton-X100, stained with FITC-conjugated phalloidin (Life Technologies) and analyzed by fluorescence microscopy using a Carl Zeiss Axio Observer.A1 fluorescence microscope (Carl Zeiss Inc., Oberkochen, Germany) with a 100x/1.4 Plan-Apochromat oil-immersion objective.²⁵

Spreading was expressed as the percentage of the total surface area covered by platelets in 20 microscopic fields measured by the ImageJ software (NIH, USA).²⁵

Platelet adhesion under flow conditions

Citrated human or murine blood was passed through a rectangular parallel plate perfusion chamber over a glass coverslip coated with acid-insoluble fibrillar type I collagen from equine tendon (Mascia-Brunelli, Milan, Italy) ($30 \mu\text{g}/\text{cm}^2$), at a wall shear rate of 250 s^{-1} , 1500 s^{-1} and 3000 s^{-1} .^{21,26 21,26 21,26} The chamber was then perfused with 0.1% bovine serum albumin in PBS to remove all residual blood and the coverslip was harvested, gently washed with 10 mM HEPES, and fixed with 0.25% glutaric-dialdehyde. Attached platelets were stained with May-Grünwald/Giemsa and observed under an optical microscope. The area covered by platelets was measured by the ImageJ software (NIH, USA).^{21,26}

Measurement of intracellular calcium (Ca^{2+})

Platelets were loaded with the Ca^{2+} -sensitive dye FLUO 3-acetoxymethyl ester (FLUO 3-AM; Molecular Probes) by incubating PRP with FLUO 3-AM 8 μ M for 20 minutes at 37°C, and Ca^{2+} mobilization induced by various agonists (TRAP-6 10 μ M, ADP 5 μ M and CVX 50 ng/ml) was assessed by flow cytometry in a CytoFLEX flow cytometer (Coulter Corporation). Baseline fluorescence was measured, then agonists were added and changes in green fluorescence were recorded for 300 seconds^{18,27}.

Rap-1b pull-down assay

Unstirred washed platelets were stimulated for 30 seconds at room temperature with ADP 10 μ M, thrombin 0.1 U/ml or their vehicle. Platelets were centrifuged at 10,000g for 30 seconds and lysed in protein extraction buffer (Tris 40mM, NaCl 0.3M, EDTA 1mM, Na_3VO_4 , NaF, pH 7.4, additioned with 1% NP-40 and 0.1% protease inhibitors) for 30 minutes in ice, centrifuged for 10 minutes at 10,000g and protein concentration was assayed. Rap-1b activity (Rap-1b-GTP) was

assessed using an active Rap-1b pull-down and detection kit (Pierce biotechnology, Rockford, IL, USA). Precipitates were analyzed by immunoblotting, using an anti-Rap-1b mAb and an appropriate peroxidase-conjugated secondary antibody.²⁸ As loading control, 30 µg of proteins from total lysates were analyzed using the same anti-Rap-1b mAb.

Phosphorylation of signaling proteins

Human washed platelets ($300 \times 10^9/L$) were stimulated for 30 seconds under continuous stirring with ADP 2 µM, CVX 60 ng/ml, ristocetin 0.3 mg/ml or their vehicle. To exclude $\alpha_{IIb}\beta_3$ -mediated outside-in signaling as a cause of signaling protein phosphorylation, platelets were treated with the $\alpha_{IIb}\beta_3$ inhibitor tirofiban 0.4 µM for 10 minutes before stimulation. Platelets were then centrifuged at 10,000g for 30 seconds and proteins were extracted as above described. 30 µg of proteins were analyzed by immunoblotting using an anti-pSrc (Tyr416, active kinase) mAb, which reacts with all members of the SFK, an anti pLyn Tyr397 (active kinase), an anti- pLyn Tyr507 (inactive kinase), an anti-pPLC β_3 (Ser537, active kinase) mAb, an anti-pAkt (Ser473, active kinase) mAb, an anti-pSyk (Tyr525/526, active kinase) and an anti-p(Ser) PKC substrate Ab (all generated in rabbits and all diluted 1:1000; Cell Signaling Technology, Beverly, MA, USA) or the affinity-purified rabbit polyclonal anti-PECAM1 pY686 and anti-PECAM1 pS702 (both a final concentration of 2 µg/ml) antibodies recognizing PECAM1 phosphorylated on the tyrosine residue at Tyr686 or at Ser702, both detecting PECAM1 activation (kindly given by Prof. Debra K. Newman)²⁹, and an appropriate peroxidase-conjugated goat-anti-rabbit secondary antibody. Anti-Src, anti-Lyn, anti-PECAM1 and anti- β actin antibodies were used as loading controls (all generated in mice and all diluted 1:1000; Cell Signaling Technology, Beverly, MA, USA).¹⁰ Western blots were developed with ECL chemiluminescence (Bio-Rad, Segrate, MI, Italy) and band intensities quantified by densitometry, from at least three independent experiments, using the ImageJ software (NIH, USA).

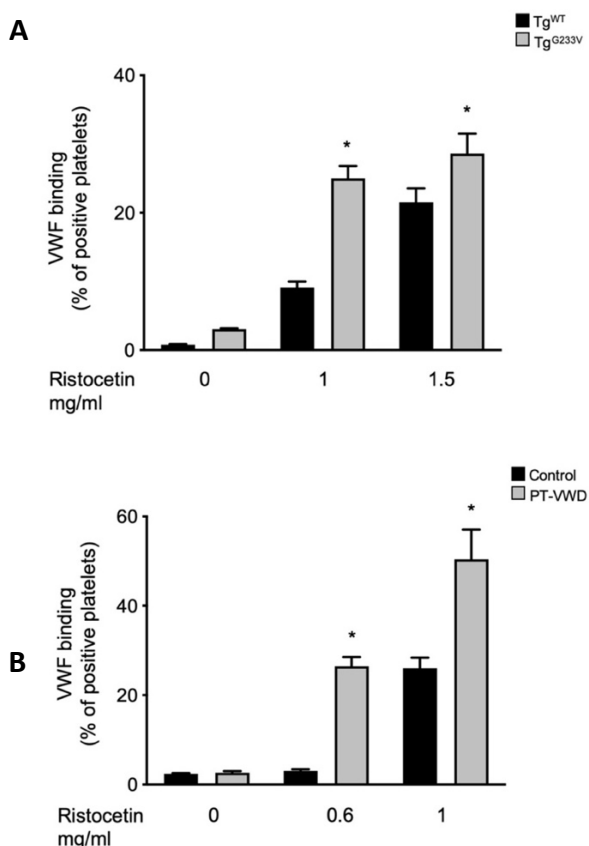
SUPPLEMENTARY TABLE S1

Platelet count and mean platelet volume of patient's samples at the different independent experiments

Patient sample	Platelet count (n x 10⁹/L)	Mean platelet volume (fl)
A	110	12.4
B	104	10.9
C	173	11.8
D	108	12.3
E	130	12.7
F	110	13.7
G	93	13.5
H	109	10.5
Healthy control sample	Platelet count (n x 10⁹/L)	Mean platelet volume (fl)
A	174	7.5
B	229	8.7
C	209	8.2
D	212	7.9
E	250	7.4
F	210	8.8
G	283	9.0
H	286	9.7

SUPPLEMENTARY FIGURES

Supplementary Figure S1

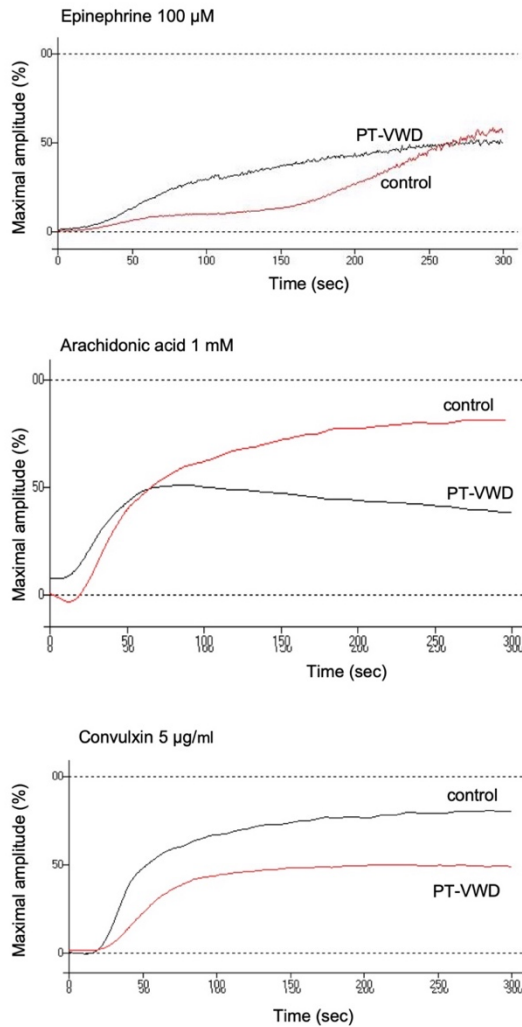


Supplementary Figure S1: VWF binding to Tg^{G233V} platelets is increased

Binding of VWF to PRP from Tg^{WT} and Tg^{G233V} mice (A) or from controls and the PT-VWD patient (B) induced by increasing doses of ristocetin, as assessed by flow cytometry. Platelet VWF-binding by flow cytometry

The binding of VWF to platelets induced by ristocetin was evaluated in PRP by flow cytometry using a mouse anti-human VWF antibody, clone 4f9 (Immunotech, Marseille, France) and a FITC-conjugated goat anti-mouse IgG (Beckman Coulter, Miami, FL, USA), as previously described (Giannini S, Mezzasoma AM, Leone M, Gresele P. Laboratory diagnosis and monitoring of desmopressin treatment of von Willebrand's disease by flow cytometry. *Haematologica*. 2007;92:1647-1654). Samples were analyzed in a Cytomics FC500 flow cytometer (Beckman Coulter). To allow the measurement of ristocetin-mediated VWF binding to murine platelets, purified human VWF (8 µg/ml) was added to murine PRP before the addition of ristocetin. Data are means ± SEM from 8 independent experiments (samples from A to H, **Supplementary table 1**) or from n=5 mice (Tg^{WT}: 2 females and 3 males, mean platelet count: 669650± 54800/µl; Tg^{G233V}: 2 females and 3 males; mean platelet count: 357250± 126218,5604/µl). * = p < 0.05 vs 0 mg/ml, Two-way ANOVA.

Supplementary Figure S2

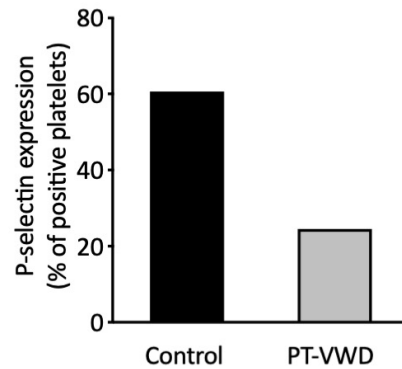


Supplementary Figure S2: Platelet aggregation in response to epinephrine, arachidonic acid and convulxin is defective in PT-VWD

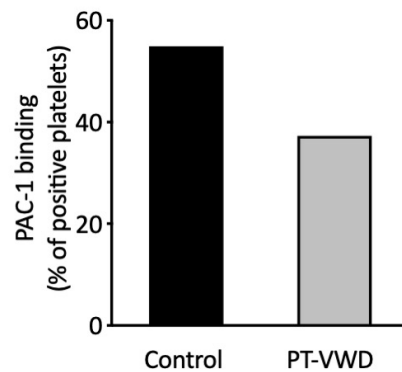
Aggregation in response to arachidonic acid 1mM was: controls $69.2 \pm 11.1\%$, PT-VWD $50.1 \pm 6.5\%$ ($p < 0.05$ vs control, unpaired Student's t test), in response to epinephrine 100 μ M was: controls $56.9 \pm 12.8\%$, PT-VWD $55.1 \pm 10.2\%$ ($p = ns$) but second wave was absent, while in response to CVX was: controls $75.1 \pm 9.8\%$, PT-VWD $35.4 \pm 10.3\%$ ($p < 0.05$ vs control, unpaired Student's t test). Traces are representative of 3 independent experiments (samples A, C, D Supplementary table 1).

Supplementary Figure S3

A



B



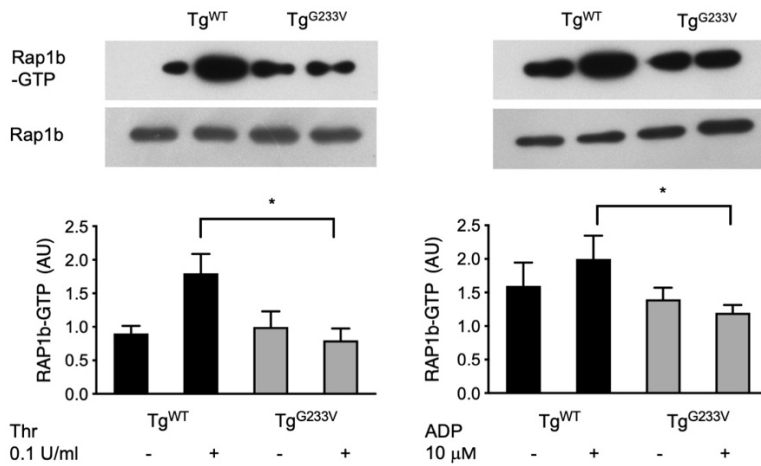
Supplementary Figure S3. α -granule secretion and $\alpha_{IIb}\beta_3$ activation in response to rhodocytin is impaired in PT-VWD.

A) α -granule secretion in human platelets as assessed by the measurement of P-selectin expression by flow cytometry in response to rhodocytin 10 nM in human whole blood from the PT-VWD patient and a parallel healthy control. P-selectin expression is reported as % of positive platelets.

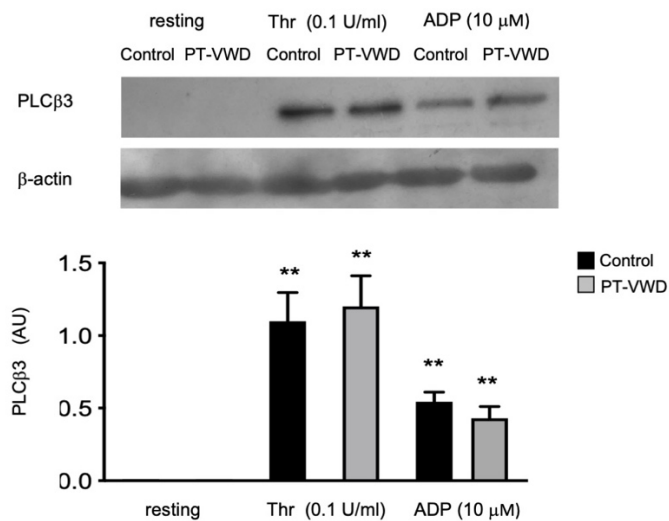
B) Integrin $\alpha_{IIb}\beta_3$ activation (PAC-1 binding) as assessed by flow cytometry in response to rhodocytin 10 nM in human whole blood from the PT-VWD patient and a parallel healthy control. PAC-1 binding is reported as % of positive platelets.

Supplementary Figure S4

A



B

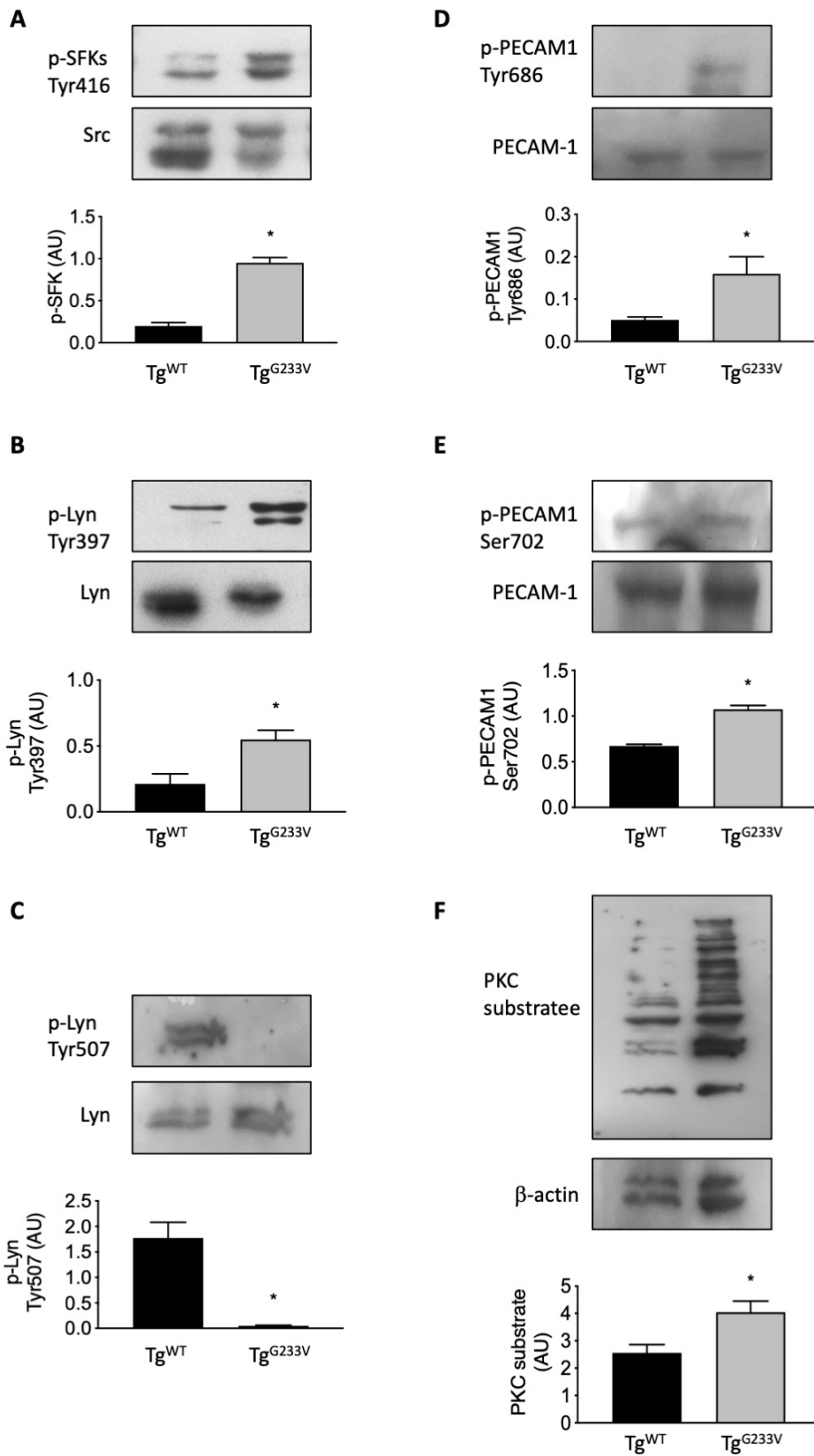


Supplementary Figure S4: Rap-1b activation is defective in Tg^{G233V} platelets while PLCβ₃ activation is not affected

A) Rap1b activation (Rap1b-GTP) of murine Tg^{WT} and Tg^{G233V} platelets after stimulation with thrombin (0.1 U/ml) or ADP (10 μM). Densitometric analysis was performed using the Image J software. Quantification of Rap1b-GTP is relative to total RAP1b expression and is expressed in arbitrary units (AU). Data are means ± SEM n=3 (Tg^{WT}: 1 female and 2 males, mean platelet count: 623650±51406/μl; Tg^{G233V}: 1 female and 2 males; mean platelet count: 466200±139158/μl). *p<0.05; Two-way ANOVA.

B) PLC-β₃ phosphorylation (p-PLCβ₃) of PT-VWD and control platelets after stimulation with thrombin (0.1 U/ml) or ADP (10 μM). Densitometric analysis was performed using the Image J software. Quantification of p-PLCβ₃ is relative to β-actin expression and is expressed in arbitrary units (AU). Data are means ± SEM from 3 independent experiments (samples F, G, H Supplementary table 1) **p<0.01 vs resting; Two-way ANOVA.

Supplementary Figure S5



Supplementary Figure S5: SFKs, Lyn and PECAM-1 are constitutively phosphorylated in Tg^{G233V} platelets

A) SFKs phosphorylation at Tyr416 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Densitometric analysis was performed using the Image J software. Quantification of p-SFKs is relative to total SFK expression and is expressed in arbitrary units (AU). Data are means \pm SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550 \pm 11950/ μ l; Tg^{G233V}: 3 males; mean platelet count: 453850 \pm 156624/ μ l) (*p<0.05 vs Tg^{WT}; **p<0.01; Two-way ANOVA).

B) Lyn phosphorylation at Tyr397 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-Lyn is relative to total Lyn expression and is expressed in arbitrary units (AU). Data are means \pm SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550 \pm 11950/ μ l; Tg^{G233V}: 3 males; mean platelet count: 453850 \pm 156624/ μ l) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

C) Lyn phosphorylation at Tyr507 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-Lyn is relative to total Lyn expression and is expressed in arbitrary units (AU). Data are means \pm SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550 \pm 11950/ μ l; Tg^{G233V}: 3 males; mean platelet count: 453850 \pm 156624/ μ l) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

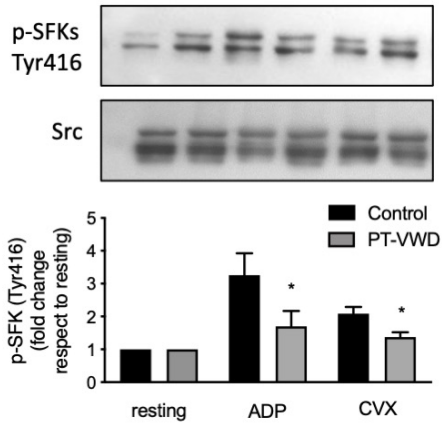
D) PECAM1 phosphorylation at Tyr686 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-PECAM1 is relative to total PECAM1 expression and is expressed in arbitrary units (AU). Data are means \pm SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550 \pm 11950/ μ l; Tg^{G233V}: 3 males; mean platelet count: 453850 \pm 156624/ μ l) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

E) PECAM1 phosphorylation at Ser702 in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of p-PECAM1 is relative to total PECAM1 expression and is expressed in arbitrary units (AU). Data are means \pm SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550 \pm 11950/ μ l; Tg^{G233V}: 3 males; mean platelet count: 453850 \pm 156624/ μ l) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

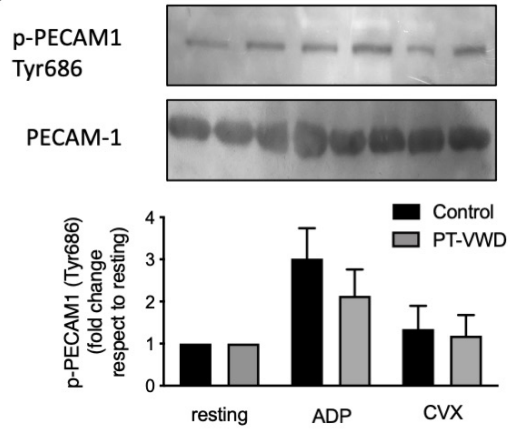
F) PKC substrates phosphorylation in murine Tg^{WT} and Tg^{G233V} washed resting platelets. Quantification of PKC substrate phosphorylation is relative to actin expression and is expressed in arbitrary units (AU). Data are means \pm SEM from n=3 mice (Tg^{WT}: 3 males, mean platelet count: 651550 \pm 11950/ μ l; Tg^{G233V}: 3 males; mean platelet count: 453850 \pm 156624/ μ l) (*p<0.05 vs Tg^{WT}; Two-way ANOVA).

Supplementary Figure S6

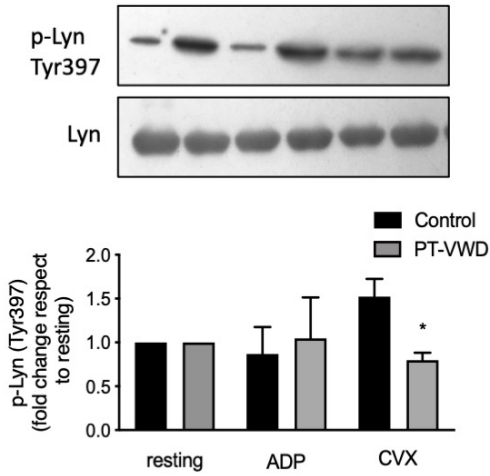
A



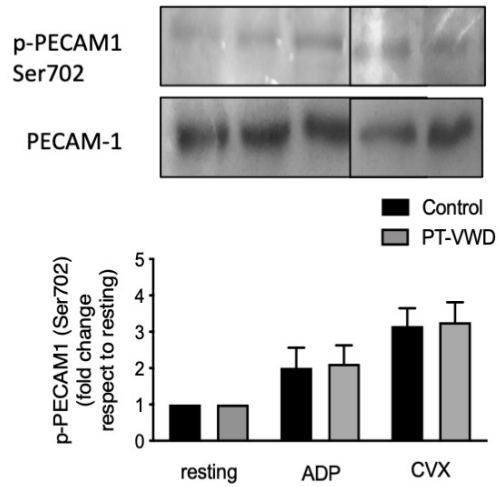
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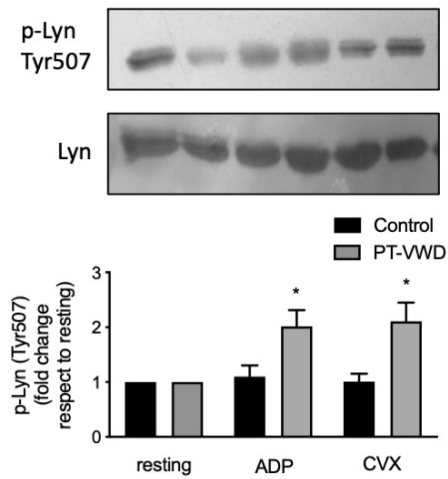
B



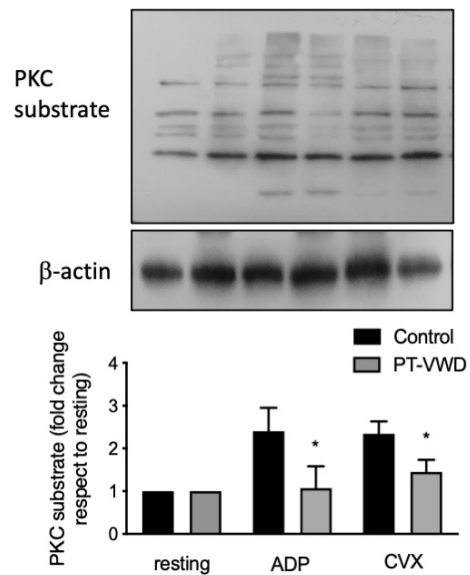
E



C



F



Supplementary Figure S6: activation of SFKs, Lyn and PKC is impaired in stimulated PT-VWD platelets

A) SFKs phosphorylation at Tyr416 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pSFK/total SFK ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (* p <0.05 vs control; Two-way ANOVA).

B) Lyn phosphorylation at Tyr397 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pLyn/total Lyn ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (* p <0.05 vs control; Two-way ANOVA).

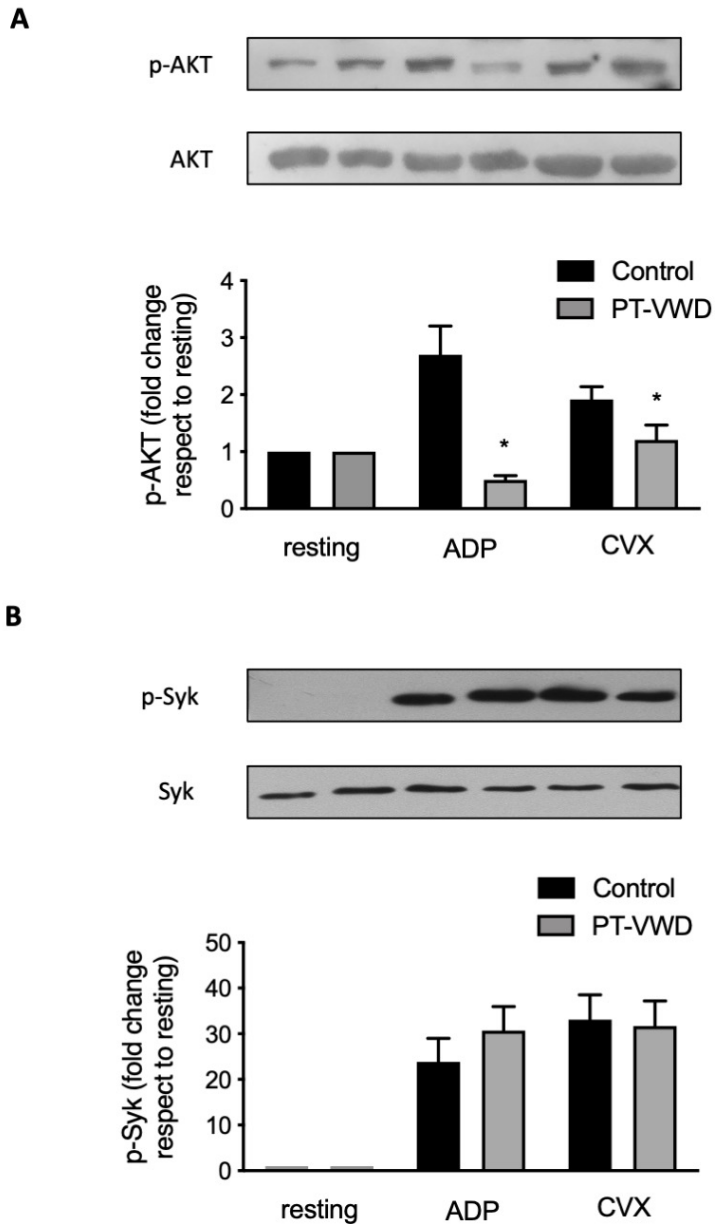
C) Lyn phosphorylation at Tyr507 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pLyn/total Lyn ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (* p <0.05 vs control; Two-way ANOVA).

D) PECAM1 phosphorylation at Tyr686 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pPECAM1/total PECAM1 ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (p =ns vs control; Two-way ANOVA).

E) PECAM1 phosphorylation at Ser702 in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pPECAM1/total PECAM1 ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (p =ns vs control; Two-way ANOVA).

F) PKC substrate phosphorylation in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the PKC substrate/actin ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (* p <0.05 vs control; Two-way ANOVA).

Supplementary Figure S7



Supplementary Figure S7: activation of AKT but not of SYK is impaired in stimulated PT-VWD platelets

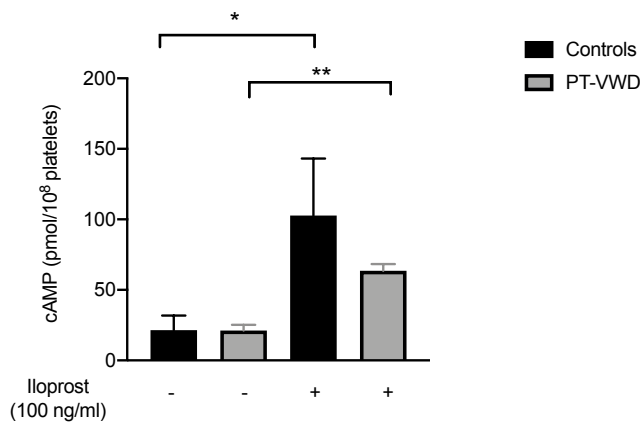
A) Akt phosphorylation at Tyr in human control and patient (PT-VWD) washed platelets under stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pAKT/total AKT ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (* p <0.05 vs control; Two-way ANOVA).

B) Syk phosphorylation at Tyr in human control and patient (PT-VWD) washed platelets under

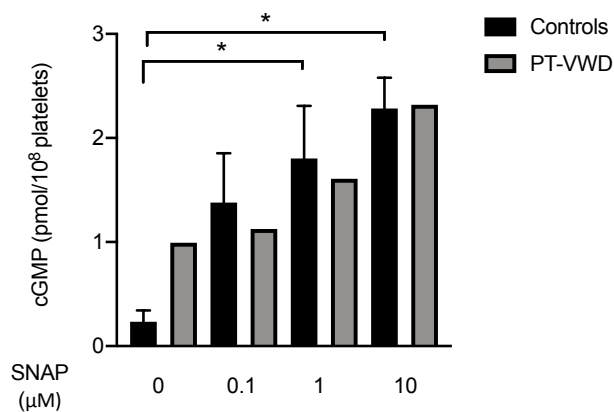
stirring conditions, stimulated with ADP (2 μ M) or CVX (60 ng/ml) for 30 sec. Densitometric analysis of the pSyk/total Syk ratio was performed using the Image J software and is expressed in arbitrary units (AU). Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1) (p=ns vs control; Two-way ANOVA).

Supplementary Figure S8

A



B

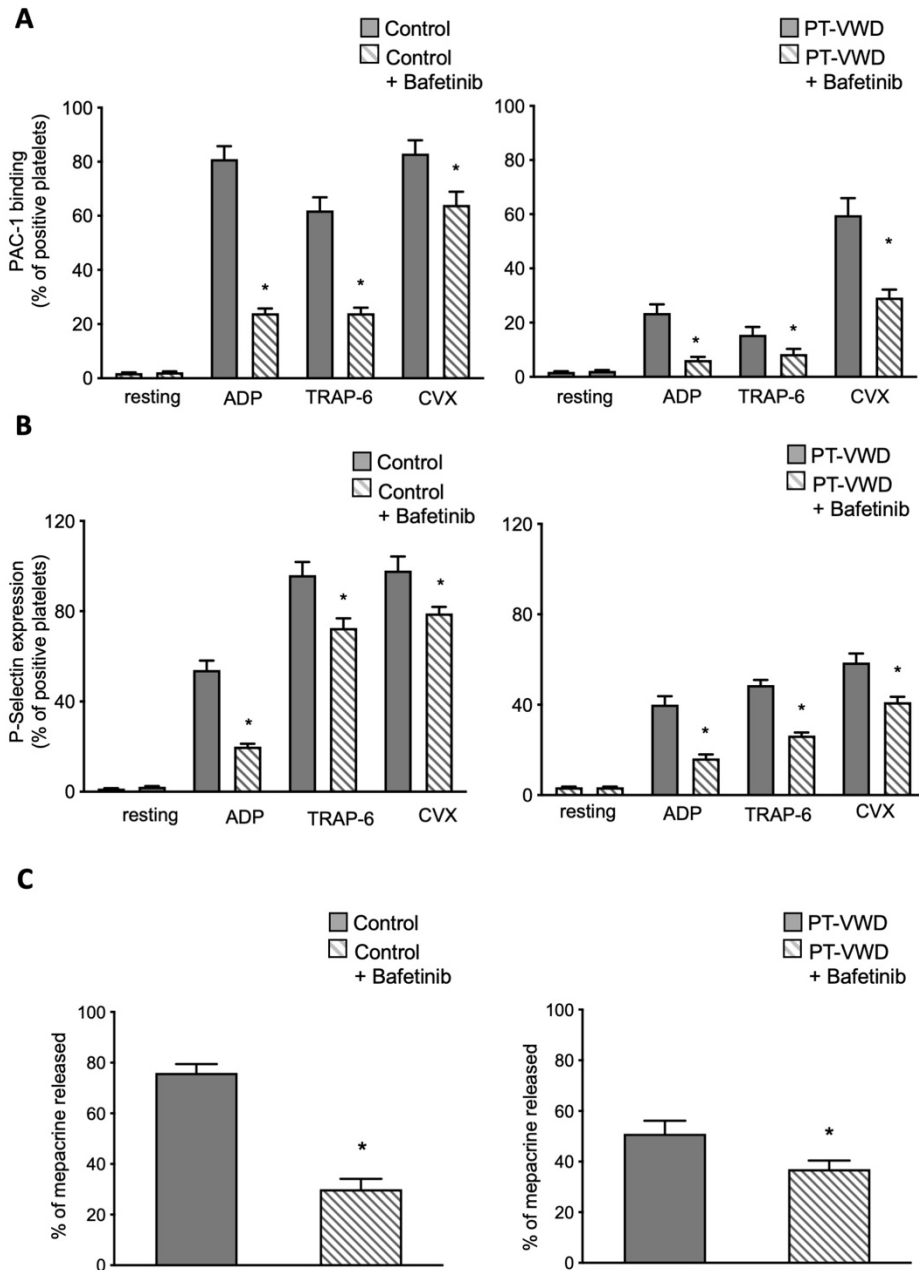


Supplementary Figure S8: cAMP and cGMP production is normal in PT-VWD platelets.

A) Platelets were incubated with Iloprost (100 ng/ml) or its vehicle at 37°C for 5 minutes under stirring. Samples were centrifuged and cAMP production from platelet lysates was assessed using the cAMP Enzymeimmunoassay Biotrak (EIA) System dual range (Amersham, GE Healthcare, Milan, Italy).

B) Platelets were incubated with the nitric oxide (NO)-donor s-nitroso-n-acetyl penicillamine (SNAP, 0.1-1-10-50 μM) or its vehicle at 37°C for 5 minutes under stirring. Samples were centrifuged and cGMP production from platelet lysates was assessed using the cGMP Enzymeimmunoassay Biotrak (EIA) System dual range (Amersham, GE Healthcare, Milan, Italy). Data are means ± SEM from 3 independent replicates. p=ns One-way ANOVA.

Supplementary Figure S9



Supplementary Figure S9: Blockade of Lyn by Bafetinib inhibits $\alpha_{IIb}\beta_3$ activation and granule secretion

Platelets were incubated with Bafetinib 50 μ M for 30 minutes and then $\alpha_{IIb}\beta_3$ activation and granule secretion were assessed by flow cytometry as described under methods. Data are means \pm SEM from 3 independent experiments (samples F, G, H Supplementary table 1). * $p < 0.05$; Two-way ANOVA (A and B) or unpaired T-test (C).