SUPPLEMENTARY APPENDIX

Mechanisms of thrombocytopenia in platelet-type von Willebrand disease

Loredana Bury,[1] Alessandro Malara,[2,3] Stefania Momi,[1] Eleonora Petito,[1] Alessandra Balduini[2,3] and Paolo Gresele[1]

[1]Department of Medicine, Section of Internal and Cardiovascular Medicine, University of Perugia; [2]Department of Molecular Medicine, University of Pavia and [3]Biotechnology Research Laboratories, IRCCS San Matteo Foundation, Pavia, Italy

 doi:10.3324/haematol.2018.200378

Received: June 22, 2018.

Accepted: January 11, 2019.

Pre-published: January 17, 2019.

Correspondence: *PAOLO GRESELE* - paolo.gresele@unipg.it

SUPPLEMENTARY METHODS

Human and murine megakaryocyte cultures

CD45+ or, alternatively, CD34+ cells were separated from peripheral blood of a PT-VWD patient (studied in 8 different occasions) and of 15 healthy controls and were induced to differentiate into megakaryocytes, as previously described[5-7,21,22]. The *in vitro* studies on CD34+ derived megakaryocytes were performed at University of Perugia while in University of Pavia studies were carried out on CD45+ derived megakaryocytes. All subjects gave their informed consent and all studies were carried out in conformity with the declaration of Helsinki. Comparable results were obtained when human megakaryocytes were retrieved from either CD45+ or CD34+ cells. The percentage of CD41+ cells was measured by flow cytometry using the CD41-FITC clone P2 mAb (Immunotech, Beckman Coulter, Milan, Italy). Megakaryocyte differentiation stage was evaluated at day 14 of culture by fluorescence microscopy, staining cells ($1x10^5$ cells *per* well) with the CD41-FITC clone P2 mAb (Immunotech) and nuclei with Hoechst (Sigma-Aldrich, Milan, Italy), as previously described[5-7,21,22]. Proplatelet tips were counted and diameter measured using the AxioVision4 Software (Carl Zeiss Inc., Oberkochen, Germany)[21,22].

To obtain murine megakaryocytes, bone marrow cells were flushed from mouse femurs, filtered through a 40 μm filter and washed in PBS. They were then cultured for 4 days in DMEM (Life Technologies Italia, Monza, Italy) supplemented with 1% of penicillin/streptomycin, 20 mM of L-glutamine, 10% of fetal bovine serum (Life Technologies Italia) and 10 ng/ml of recombinant mouse TPO (Peprotech, UK), and then megakaryocytes were purified using a 1.5/3% BSA gradient [22].

In none of the cultures VWF was exogenously added to the medium, in human megakaryocyte culture medium (Stem Span SFEM) VWF was absent (VWF:Ag=0.34±0.07%) in murine megakaryocytes culture medium bovine VWF was present in the 10% fraction of FBS (VWF:Ag=39.93±0.83%).

Megakaryocyte spreading and proplatelet formation

To evaluate spreading and proplatelet formation onto adhesive substrates, human and murine megakaryocytes were separated on a BSA gradient (3-4%), at day 14 or 4 of culture respectively, plated onto glass coverslips coated with 100 μg/ml fibrinogen (Sigma-Aldrich) or 10 μg/ml VWF (Sigma-Aldrich) or 25 μg/ml type I collagen (Sigma-Aldrich) in 24-well plates ($1x10^5$ cells *per* well), and allowed to adhere for 4h to evaluate spreading and 16 h to evaluate proplatelet formation at 37°C and 5% CO_2. In selected experiments control human megakaryocytes were incubated with 1.5 mg/ml ristocetin (Mascia Brunelli, Milan, Italy) and plated onto glass coverslips coated with 25 μg/ml type

I collagen. To assess whether GPIbα/VWF complexes may mediate interaction of megakaryocytes with fibrinogen independently from $\alpha_{IIb}\beta_3$ PT-VWD megakaryocytes were pre-incubated with the $\alpha_{IIb}\beta_3$ antagonist monoclonal antibody abciximab (1 μg/ml) and then plated for 4 hours on 100 μg/ml of fibrinogen. To evaluate proplatelet formation in suspension, megakaryocytes were placed in 24-well plates coated with 1% BSA and incubated for 16 h at 37°C and 5% CO_2. After incubation with an anti-β_1-tubulin antibody (kindly given by Dr. J.E. Italiano, Harvard Medical School, Boston, MA) to stain microtubules, TRITC-phalloidin (Life Technologies, Italy) to stain actin and Hoechst to stain nuclei, as previously described[5-7,21,22], samples were analyzed by immunofluorescence using a Carl Zeiss Axio Observer.A1 fluorescence microscope (Carl Zeiss Inc, Oberkochen, Germany) with a 63x/1.4 Plan-Apochromat oil-immersion objective. Image acquisition was obtained using the AxioVision software (Carl Zeiss Inc). All polynucleated cells extending protrusions with terminal tips were considered as proplatelet-forming megakaryocytes, while those displaying a flattened shape with actin organized in focal adhesion points and fibers as spreading megakaryocytes. At least 100 megakaryocytes from 5 different replicates were analyzed.

VWF binding to megakaryocytes

VWF binding was assessed by confocal microscopy[5] and by flow cytometry[20,23]. For confocal microscopy, human megakaryocytes at day 7, 10 and 14 and murine megakaryocytes at day 4 of differentiation were cytospun on poly-L-lysine-coated glass coverslips and stained with a rabbit anti-VWF Ab (Dako, Cernusco sul Naviglio, Milan, Italy), a mouse anti-human CD42b mAb (Beckman Coulter) and the appropriate Alexa-Fluor®-conjugated secondary antibodies, as previously described[5]. Nuclear counterstaining was performed with Hoechst. Confocal microscopy was performed using a TCS SPII confocal laser scanning system equipped with a DM IRBE inverted microscope with a 40x oil NA objective (Leica, Bensheim, Germany). Cellular localization of VWF was assessed by analyzing the graphics reporting the intensity of the fluorescence signal along the x axis for each fluorochrome on the optical section, using the LAS-X software (Leica) and the ImageJ software (NIH, USA)

For flow cytometry, human and murine megakaryocytes were incubated with increasing doses of ristocetin (0, 1, 1.2, 1.5, 2 mg/ml) for 3 min at 37°C, fixed with 2% PFA and the binding of VWF was evaluated using a mouse anti-human VWF antibody, clone 4f9, that cross-reacts also with murine VWF (Immunotech, Marseille, France), and a FITC-conjugated goat anti-mouse IgG (Beckman Coulter, Miami, FL, USA)[23]. Samples were analyzed in a Cytomics FC500 flow cytometer (Coulter

Corporation, Miami, Florida, USA), equipped with an argon laser operating at 488 nm.

Megakaryocyte intracellular signaling triggered by type I collagen

Megakaryocytes were plated for 16 hours in 12-well plates pre-coated with 25μg/ml of type I collagen or 1% BSA and then lysed in HEPES-glycerol lysis buffer (HEPES 50 mM, 10% glycerol, 1% Triton X-100, $MgCl_2$1.5 mM, EGTA 1 mM, 1% protease inhibitors). RhoA activity (Rho-GTP) was assessed on 500 μg of proteins using an active Rho pull-down and detection kit (Pierce biotechnology, Rockford, IL, USA). Immunoprecipitates were analyzed by immunoblotting, using an anti-RhoA mAb and an appropriate peroxidase-conjugated secondary antibody, as described[24]. As loading control, 30 μg of proteins from total lysates were analyzed using the same anti-RhoA mAb.

To assess the phosphorylation of MLC2 and Src-family kinases (SFKs), 30 μg of proteins were analyzed by immunoblotting using an anti-pMLC2 (Ser19) mAb, or an anti-pSrc (Tyr416) mAb (Cell Signaling Technology, Beverly, MA, USA) reacting with all members of the SFK, and an appropriate peroxidase-conjugated secondary antibody. As loading control 30 μg of proteins from total lysates were analyzed using an anti-MLC2 mAb or an anti β-actin mAb (Cell Signaling). To assess Lyn phosphorylation, Lyn was immunoprecipitated from 100 μg of proteins using an anti-Lyn mAb (Thermo Scientific, Life Technologies, Italy) and Protein G Sepharose beads (Life Technologies, Italy). Immuno-precipitates were analyzed by immunoblotting using the anti-pSrc (Tyr416) mAb and an appropriate peroxidase-conjugated secondary antibody. As loading control, 10 μg of proteins from total lysates were analyzed using an anti β-actin mAb.

Western blots were developed with ECL chemiluminescence (Amersham, GE Healthcare, Fairfield, CT, USA). Band intensities were quantified by densitometry from at least four different experiments using the ImageJ software (NIH, USA).

To assess whether Lyn phosphorylation was triggered by VWF binding to GPIbα control megakaryocytes were incubated with ristocetin (1.5 mg/ml) and Lyn phosphorylation was assessed as above described.

Megakaryocyte migration assay

Megakaryocyte migration assay was performed as described[25] in transwell migration chambers (8 μm, Millipore) coated or not with 25μg/ml type I collagen. Human megakaryocytes (25×10^3 in 100 μl of

StemSpan SFEM medium [Stem Cell Technologies] containing 10 ng/ml of TPO [PeproTech]) were seeded in the upper well, while the lower wells contained the same medium supplemented with 100 ng/ml SDF-1α (PeproTech), and chambers were incubated at 37 °C and 5% CO_2. After 16 h, megakaryocytes that had passed to the lower wells were recovered and counted by flow cytometry after staining with a FITC-conjugated mouse anti-human CD41-MoAb and the addition of Flow-Count™ Fluorosferes (Beckman Coulter)[26]. Samples were then analyzed by flow cytometry for CD41 and results expressed as chemotaxis index (CI), that is the number of cells that have passed through the filter in response to SDF-1α divided by the number of cells that have passed in the absence of SDF-1α[27,28].

Bone marrow histology

10 sections of human bone marrow from 1 patient with PT-VWD, 3 patients with ITP (they were included because we would like to compare PT-VWD patient bone marrow, that showed an increased number of megakaryocytes, with bone marrow specimens deriving from patients with an increased number of megakaryocytes due to an acquired condition) and 3 controls (i.e. subjects who underwent bone marrow biopsy for a suspected hematologic disorder but who then revealed not to have any bone marrow anomaly) and 10 sections of murine bone marrow from femurs and tibiae of 3 Tg^{WT} and 3 Tg^{G233V} mice were fixed in phosphate buffered saline containing 4% formaldehyde, and paraffin-embedded 5 μm sections were made. These were rehydrated using xylene and a graded alcohol series and stained with hematoxylin and eosin (Sigma-Aldrich) to assess cell morphology. Immunostaining was carried out using a primary rabbit-anti-human β_1-tubulin antibody for human samples, or a rabbit-anti-mouse CD41 antibody (Abcam, Cambridge, UK) for murine samples and a secondary biotinylated Goat Anti-Rabbit IgG (Vector Laboratories, Burlingame, CA)[27]. Pictures were analyzed by two different unaware operators using the Image J software and platelets present in bone marrow were expressed as number *per* 1000 μm^2 of cell-covered section.

Measurement of platelet life span in mice

Mice were injected intravenously with 0.5 μg/g body weight of an anti-GPIX mAb (Emfret Analytics, Eibelstadt, Germany) conjugated with DyLight 488 (Life Technologies Italia). 50 μl of blood was collected 10 min after injection from tail cuts in tubes containing 4% sodium citrate and thereafter each day for 5 days and the percentage of residual fluorescent platelets relative to the total CD41/61-positive platelet population was calculated by flow cytometry as described[4,26]. One hour after the injection of DyLight 488–conjugated anti-GPIX mAb around 80% of circulating platelets showed

fluorescent staining.

The same experiments were repeated by administering to mice desmopressin (DDAVP) (0.3 □g/kg) by sub-cutaneous injection immediately after intravenous injection of the DyLight 488–conjugated anti-GPIX mAb. 50 µl of blood was collected before DDAVP administration and after 30 and 60 minutes, platelet count was performed by flow cytometry after staining with a PE-conjugated anti-mouse CD41/61-mAb and the addition of Flow-Count™ Fluorosferes (Beckman Coulter)[26] and platelet life span was assessed as above described.

Platelet exposure of annexin V was assessed by flow-cytometry using FITC–annexin V (Beckman Coulter) after PRP stimulation for 10 min at room temperature with TRAP-6 (10µM) and convulxin (500ng/ml) the reaction was stopped with 500 µl of binding buffer.

VWF-bound circulating platelets

Blood smears were prepared from EDTA-anticoagulated human or mouse blood from cardiac puncture, fixed with 4% PFA, immediately blocked with 1% BSA and stained with a rabbit anti-VWF Ab (Dako, Cernusco sul Naviglio, Milan, Italy), a mouse anti-human CD42b mAb (Beckman Coulter) and the appropriate Alexa-Fluor®-conjugated secondary antibodies and analyzed by fluorescence microscopy as previously described[21,22]. The percentage of platelets carrying VWF bound at their surface was measured by evaluating at least 10 fields from 5 different specimens.

Electron microscopy

Megakaryocytes were fixed for 4 h at 4°C, using cacodylate buffer containing 4% wt/vol of glutaraldehyde. The samples were 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 10000xg for 30 sec. Ultrathin sections of the pellets were stained with uranyl acetate and lead citrate and observed with a Philips Electron Optics EM208 transmission electron microscope at 80 kv.

Mouse platelet count in bone marrow

Bone marrow cells or whole blood were incubated with a FITC-conjugated rat-anti-mouse CD41/61 mAb (Emfret Analytics) to stain platelets. Platelets were counted after the addition of Flow-Count™ Fluorosferes (Beckman Coulter), as described[26].

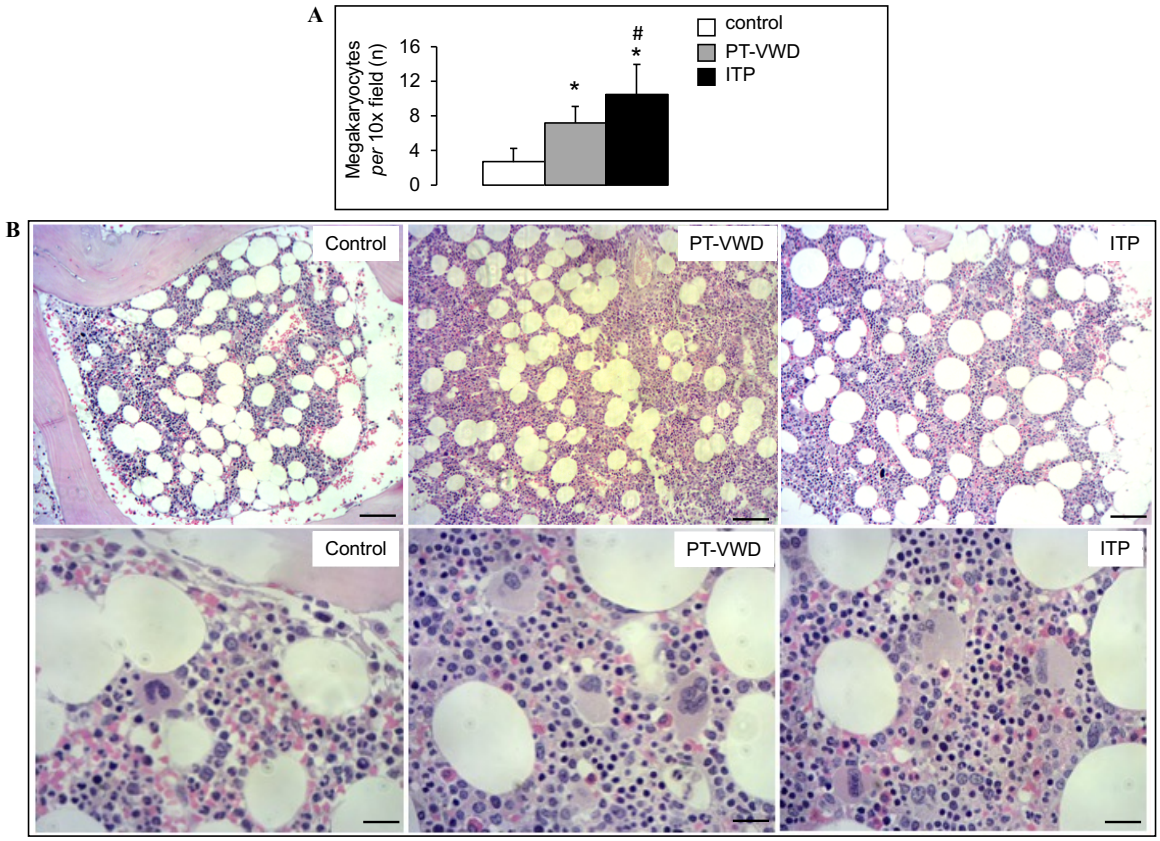
Supplementary Table 1. Surface expression of $\alpha_2\beta_1$ measured by flow cytometry and common genetic variants in *GP6* and *ITGA2*

Antigen	PT-VWD (Mean fluorescence intensity)	Normal values (Mean fluorescence intensity)
CD49 (α_2)	4.8	4.8±0.8

Common variant (SNP)	Patient genotype
GP6 rs1613662 (c.655C>T)	C/C
GP6 rs1654416 (c.709G>A)	G/A
GP6 rs2304167 (c.691G>A)	A/A
GP6 rs1654413 (c.954T>A)	T/T
GP6 rs1671152 (c.968A>C)	A/A
ITGA2 rs1126643 (c.759C>T)	C/C
ITGA2 rs10471371 (c.1600G>A)	G/G

SUPPLEMENTARY FIGURES

Supplementary Figure 1

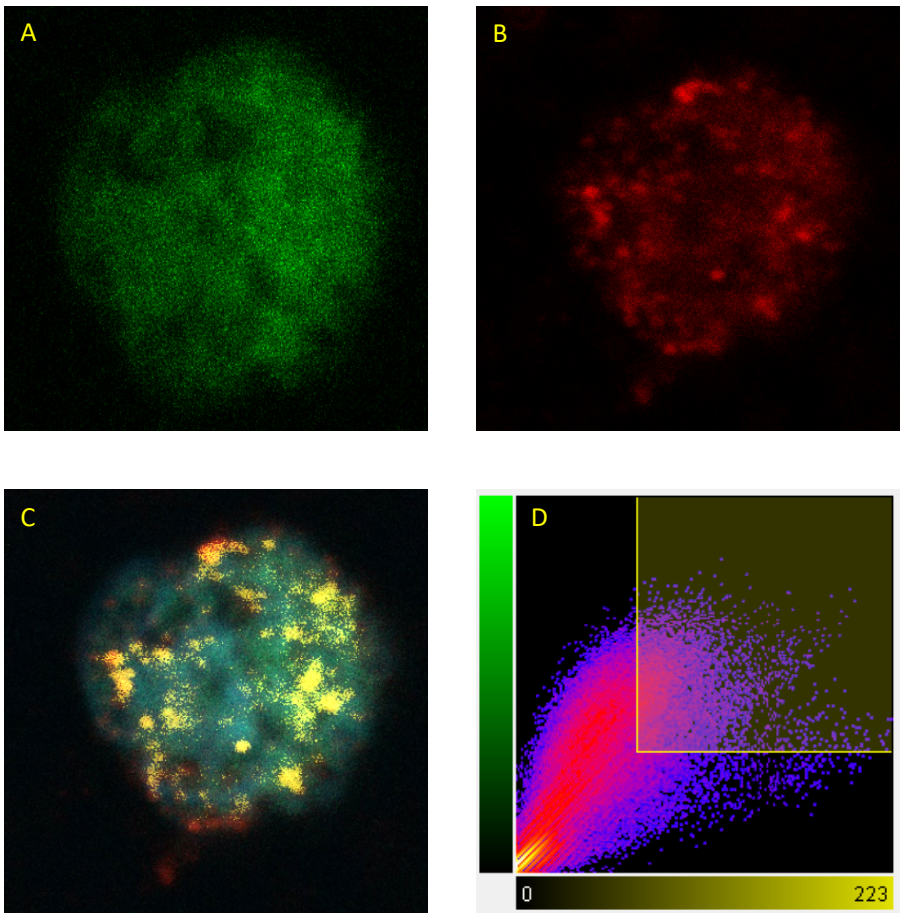


A) Number of megakaryocytes in bone marrow biopsies in 10x fields of 3 controls, one PT-VWD patient and 3 patients with immune thrombocytopenia. At least 15 fields for each sample were evaluated. Data represent means ± SD; *p<0.05 vs controls; # p<0.05 vs PT-VWD. 10 different sections of human bone marrow were analyzed from the PT-VWD patient, from 3 patients with ITP and from 3 controls.

B) Representative images of bone marrow biopsies stained with hematoxylin–eosin of a healthy control, one PT-VWD patient and one patient with immune thrombocytopenia (ITP) (top=10x fields, bottom=40x fields; scale bars 80 μm (top), 40 μm (bottom).

The increased number of megakaryocytes in PT-VWD bone marrow was expected because probably, as in immune thrombocytopenia, the bone marrow attempts to compensate the enhanced platelet clearance by increasing megakaryocyte number.

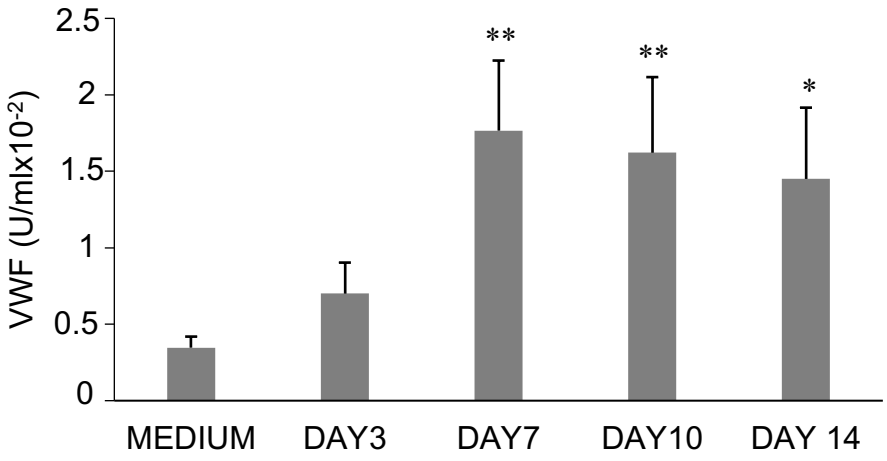
Supplementary Figure 2



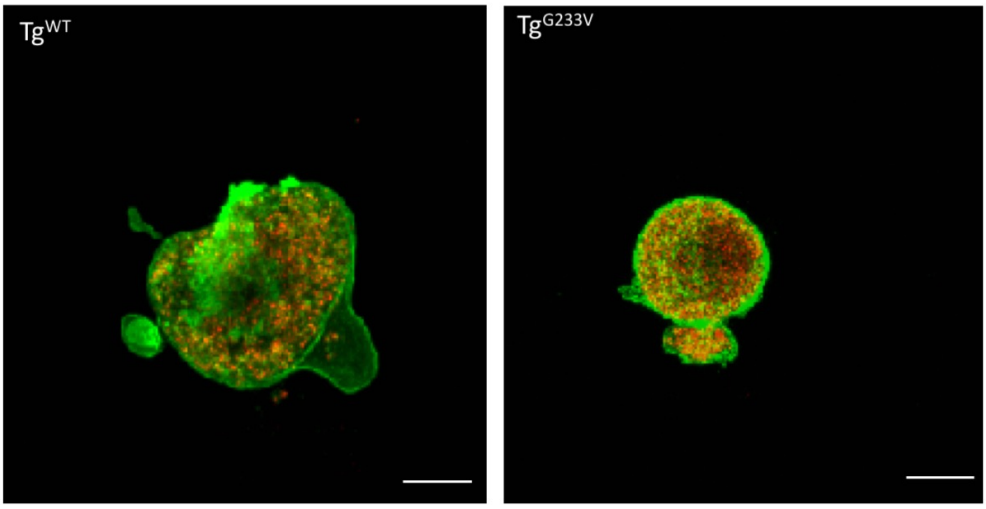
Representative image of a GP1bα – VWF co-localization analysis. To assess the intracellular colocalization of VWF and GPIbα, 16 confocal images (acquired at 1µm distance from the contact surface, z-stack thickness 0.34µm) were analyzed. Mean % of colocalization was 11.8% (S.D. 6.7%). A) GP1bα (green); B) vWF (red); C) Merging of A+B, white=colocalized signals; D) relative correlation diagram and the selection limits of colocalized signals. The images are representative for 3 repeated measures for control megakaryocytes and 3 different cultures for PT-VWD megakaryocytes.

Supplementary Figure 3

A



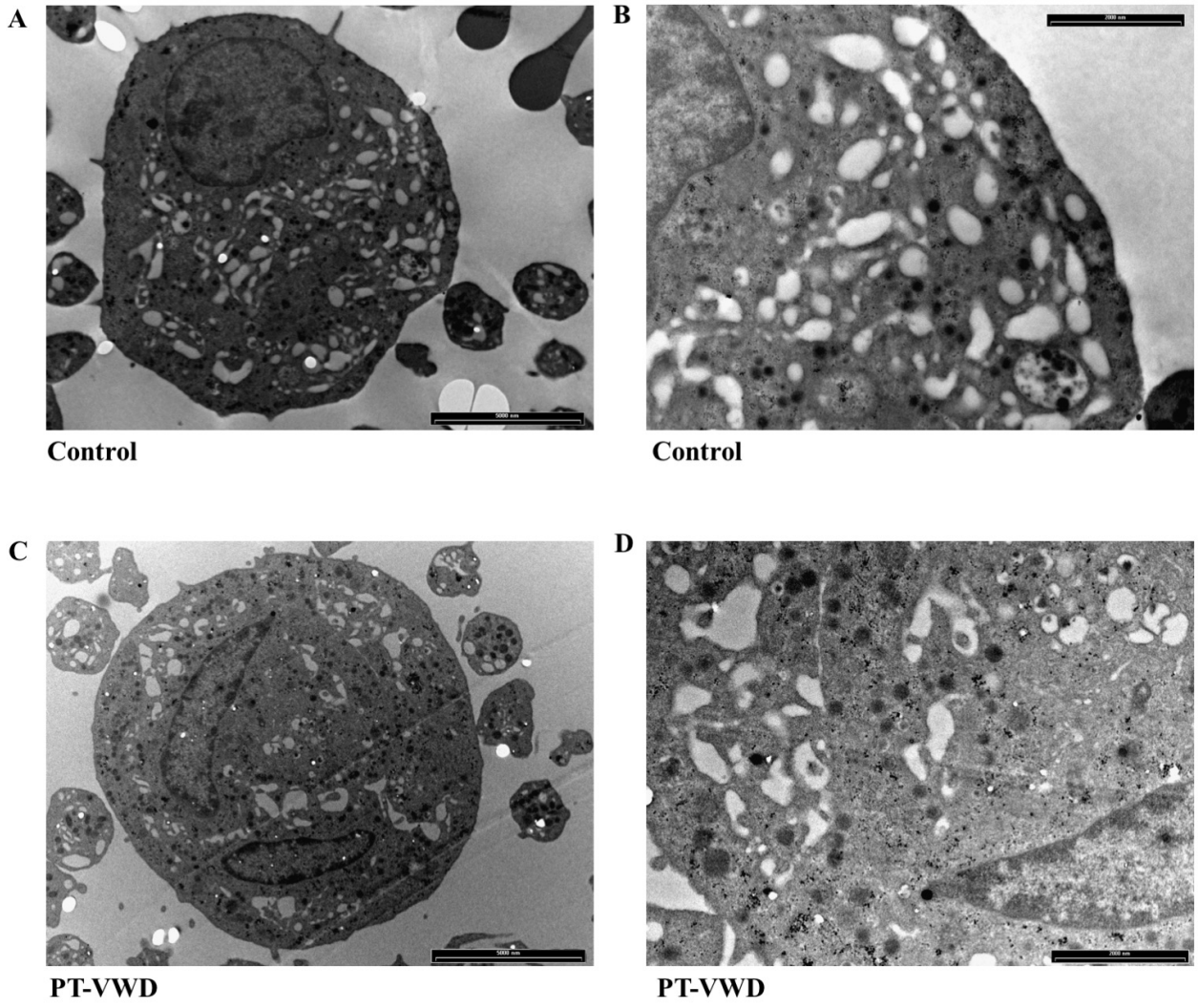
B



A) Levels of VWF in the culture medium of 5 different megakaryocyte cultures at different days of cell differentiation expressed as % of VWF antigen (Ag). **= $p<0.01$ vs medium; *=$p<0.05$ vs medium

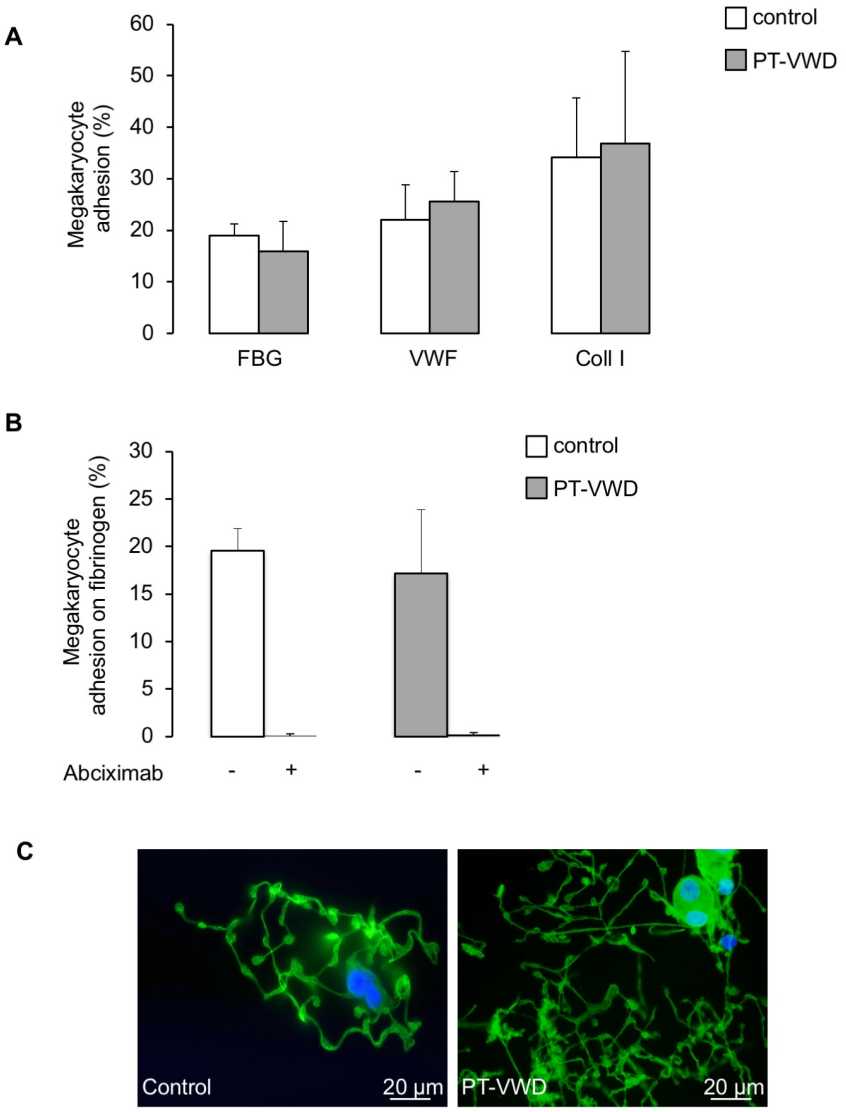
B) Representative images of VWF binding to murine megakaryocytes analyzed by confocal microscopy. VWF is stained in green (Alexa Fluor® 488 Goat Anti-Rabbit IgG), CD42b is stained in red (Alexa Fluor® 568 goat Anti-mouse IgG). Yellow shows merging. Samples were mounted with the ProLong Antifade medium (Molecular Probes) and observed at room temperature using a TCS SPII confocal laser system equipped with a DM IRBE inverted microscope and a 40x OIL NA objective (Leica, Bensheim, Germany). Scale bars= 10 μm. The images are representative for 3 repeated measures for Tg^{WT} and 3 repeated measures for Tg^{G233V} megakaryocytes. VWF was not detected on the surface of PT-VWD megakaryocytes also when experiments were carried out in a serum-free medium or immediately fixing cells after isolation from bone marrow (data not shown).

Supplementary Figure 4



A-D) Representative images of the ultrastructure of control and PT-VWD megakaryocytes. Figures B and D are particulars at higher magnification of A and C. Scale bars for A and C are 5000 nm, for B and D 1000 nm. The images are representative for 3 repeated measures for control megakaryocytes and 3 different cultures for PT-VWD megakaryocytes.

Supplementary Figure 5

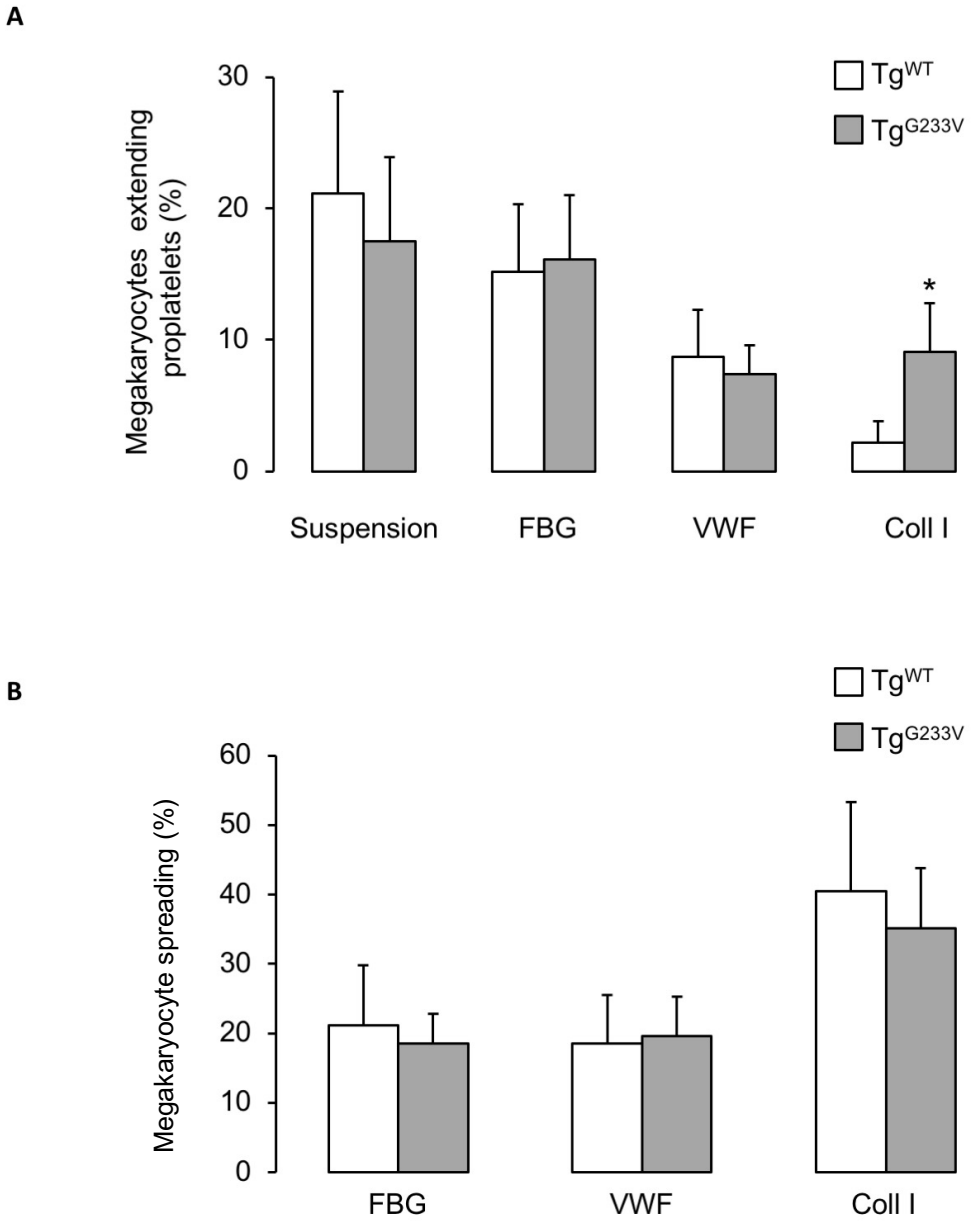


A) Megakaryocyte spreading on glass coverslips coated with fibrinogen (FBG), VWF or type I collagen (Coll I). 5 repeated measures were carried out for control megakaryocytes and 5 different cultures for PT-VWD megakaryocytes.

B) Spreading on glass coverslips coated with fibrinogen of megakaryocytes pre-incubated with Abciximab (1 µg/ml) or its veichle. 3 repeated measures were carried out for control megakaryocytes and 3 different cultures for PT-VWD megakaryocytes.

C) Representative images of control and PT-VWD megakaryocytes plated on fibrinogen. Scale bars =10 µm.

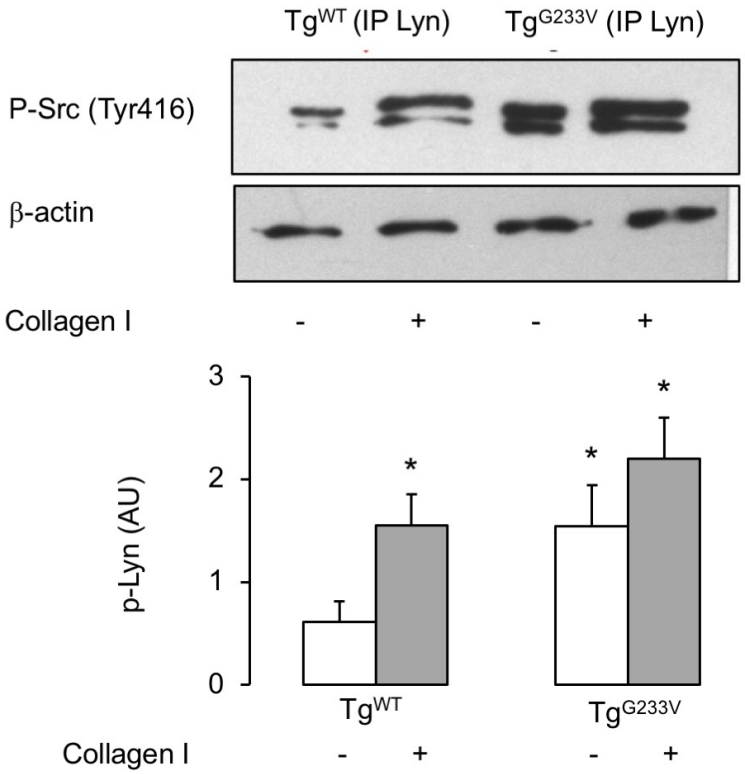
Supplementary Figure 6



A) Percentage of murine megakaryocytes-extending proplatelets in suspension, or onto glass coverslips coated with fibrinogen (FBG), VWF or type I collagen (Coll I). Data represent mean ± SD of 5 different experiments (*$p<0.05$ vs controls).

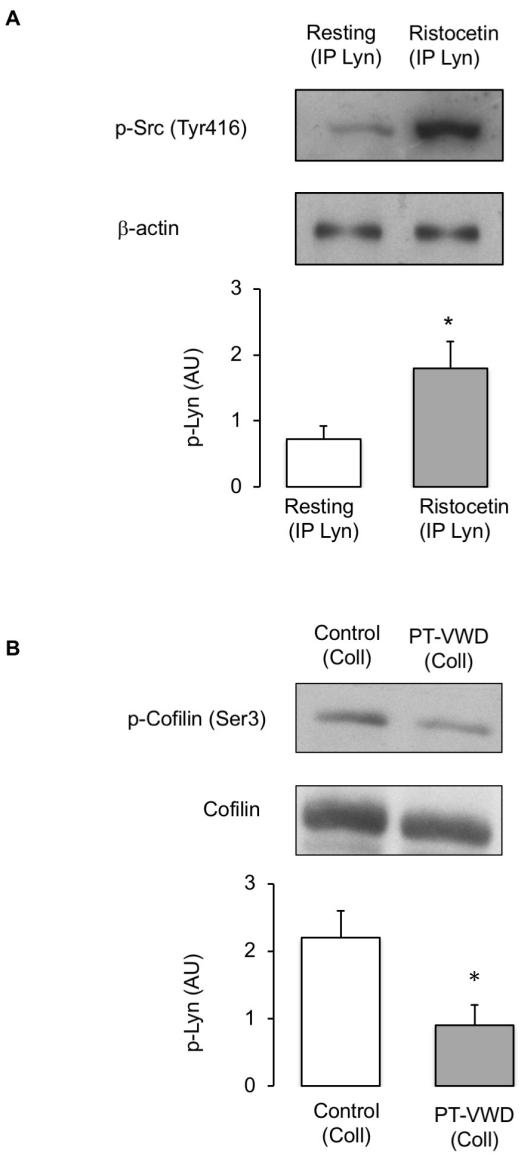
B) Megakaryocyte spreading on glass coverslips coated with fibrinogen (FBG), VWF or type I collagen (Coll I). Data represent mean ± SD of 5 different experiments.

Supplementary Figure 7



Lyn phosphorylation (P-Lyn) of murine megakaryocytes in suspension (-) or after 16 hours of adhesion to type I collagen (+). Densitometric analysis was performed using the Image J software. Quantification of P-Lyn is relative to that of β–actin in total cell lysates and is expressed in arbitrary units (AU) (*n=5; $p<0.05$ vs Tg^{WT}-).

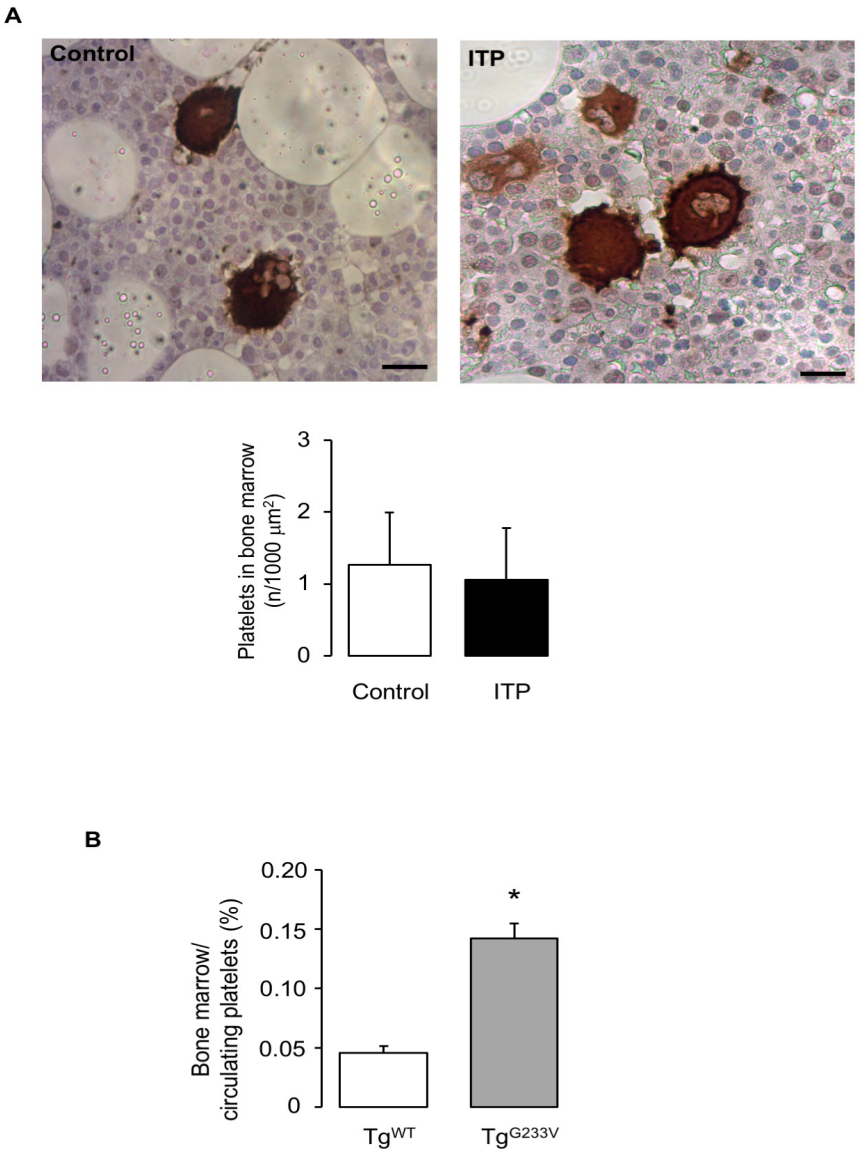
Supplementary Figure 8



A) Lyn phosphorylation (p-Lyn) of human control megakaryocytes under resting conditions or after incubation with ristocetin (1.5 mg/ml). Densitometric analysis was performed using the Image J software. Quantification of P-Lyn is relative to that of β–actin in total cell lysates and is expressed in arbitrary units (AU) (*n=3; $p<0.05$ vs resting).

B) Cofilin phosphorylation (p-Cofilin) of human control and PT-VWD megakaryocytes platelet on type I collagen. Densitometric analysis was performed using the Image J software. Quantification of p-Cofilin is relative to that of Cofilin in total cell lysates and is expressed in arbitrary units (AU) (*n=3; $p<0.05$ vs control).

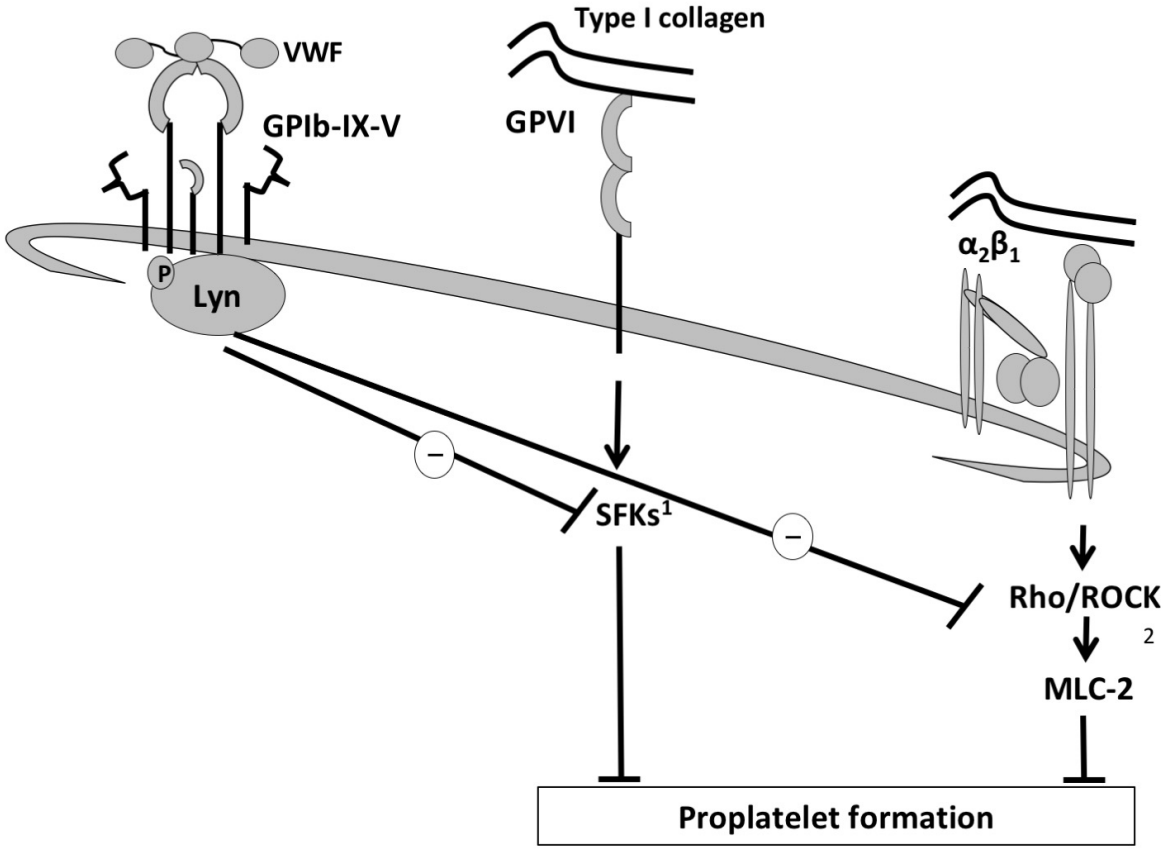
Supplementary Figure 9



A) Megakaryocytes and platelets were stained on human bone marrow biopsies using a primary rabbit-anti-human β_1-tubulin antibody and a secondary biotinylated goat anti-rabbit IgG; specimens were counterstained with hematoxylin–eosin. Scale bars=40 µm. Pictures were analyzed using the Image J software and bone marrow platelet number was expressed as the number of platelets *per* 1000 μm^2 of cells. 10 different sections of human bone marrow were analyzed from 3 patients with ITP and 3 controls.

B) Platelet count in bone marrow of Tg^{WT} and Tg^{G233V} mice performed by flow cytometry. Platelets were stained with a FITC-conjugated rat-anti-mouse CD41/61 mAb and counted after the addition of Flow-Count™ Fluorosferes (Beckman Coulter). Data are expressed as the ratio between platelet count in the bone marrow specimens and in venous blood specimens (n=4, *=$p<0.05$ vs Tg^{WT}).

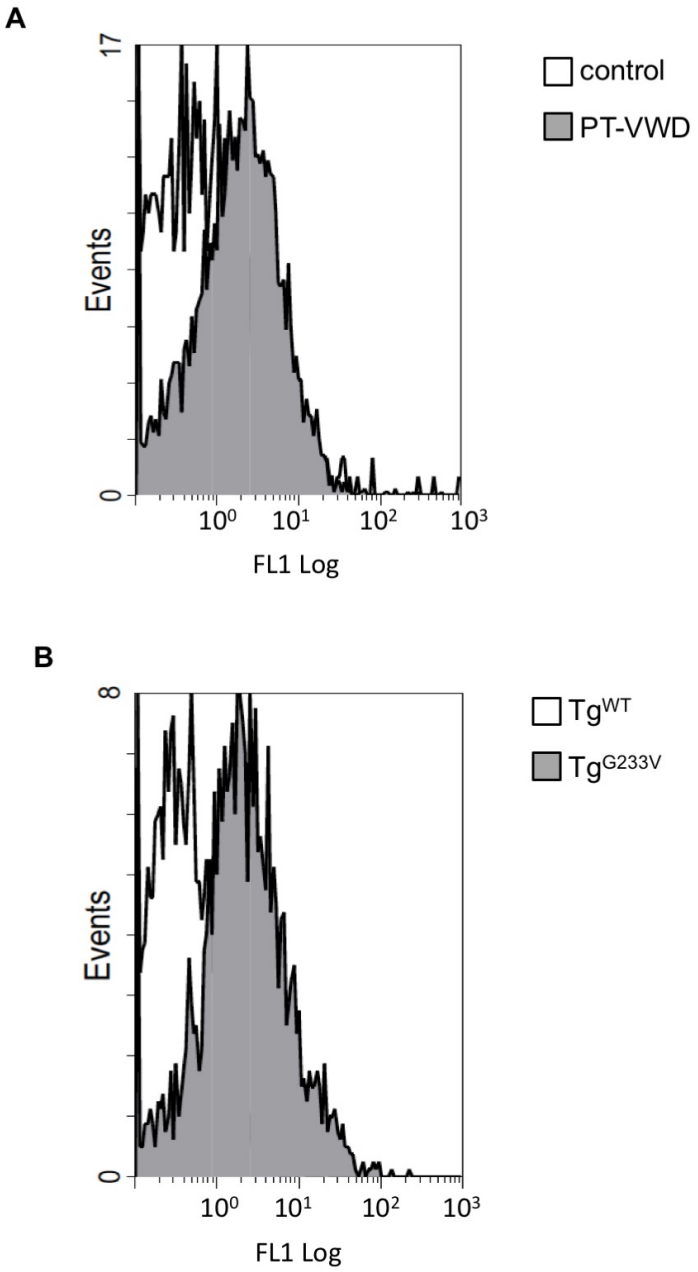
Supplementary Figure 10



[1]Semeniak D et al. J Cell Sci. 2016; 129:3473-84.
[2]Chang Y et al. Blood 2007;109: 4229-4236.

Schematic diagram of the effect of Lyn phosphorylation upon binding of VWF to GPIbα on the signaling involved in the inhibition of PPF on type I collagen.

Supplementary Figure 11



A) Raw FACS data relative to Figure 1B: VWF binding to control and PT-VWD human megakaryocytes at day 10 of differentiation under resting conditions

B) Raw FACS data relative to Figure 1C: VWF binding to Tg^{WT} and Tg^{G233V} murine megakaryocytes at day 4 of differentiation after incubation with ristocetin (1.2 mg/ml)