SUPPLEMENTARY APPENDIX

Cytoskeletal perturbation leads to platelet dysfunction and thrombocytopenia in variant forms of Glanzmann thrombasthenia

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Supplemental methods

Construction of the expression vectors and mutagenesis

Full-length α_{IIb} and β_3 cDNAs were amplified by Pfu DNA Polymerase. α_{IIb} was cloned into the HindIII/NotI sites of pcDNA3.1/Hygro(+) (Invitrogen, Life Technologies, Monza, Italy) to obtain the pcDNA3.1/α_{IIb} expression vector and β_3 was cloned into the HindIII/XhoI sites of pcDNA3 (Invitrogen) to obtain the pcDNA3/β_3 expression vector. β_3 cDNA was amplified by PCR from patient platelets, digested with KpnI and EcoRI and exchanged for the wild-type sequence in the pcDNA3/β_3 construct. β_3 S527F, L743P and D748H mutations were introduced into the pcDNA3/β_3 vector by site-directed mutagenesis[21].

CHO cells-culture and transfection

CHO cells (from European Collection of Cell Cultures, Salisbury, UK) were grown in IMDM supplemented with 10% FCS, 2mM L-Glutamine, 100U/ml penicillin and 100µg/ml streptomycin.
Cells were transiently transfected with equimolar amounts of pcDNA3.1/α_{IIb} and pcDNA3/β_3, pcDNA3/$\beta_3$del647-686, pcDNA3/β_3S527F, pcDNA3/β_3L743P or pcDNA3/β_3D748H using Turbofect (Fermentas, Glen Burnier, MD, USA). Selected experiments were carried out using CHO cells transfected with pcDNA3.1/α_{IIb}, pcDNA3/β_3 and pcDNA3/$\beta_3$del647-686 together, to obtain a heterozygous model, mimicking the patients' genotype. Mock cells were obtained by transfecting equimolar amounts of pcDNA3 and pcDNA3.1/Hygro(+) plasmids.

CHO cells $\alpha_{IIb}\beta_3$ activation

Cells were incubated for 30 minutes with the CD41-FITC clone P2 mAb (Immunotech, Beckman Coulter, Milan, Italy) to assess $\alpha_{IIb}\beta_3$ surface expression or the PAC-1 FITC mAb (BD Biosciences, Milan, Italy) to measure $\alpha_{IIb}\beta_3$ activation. To measure fibrinogen binding cells were incubated with an anti-human-fibrinogen FITC mAb (Immunsystem AB, Uppsala, Sweden) in the presence of 4 µg/ml of human fibrinogen.
Samples were analyzed in a Cytomics FC500 flow cytometer (Coulter Corporation, Miami, Florida, USA), equipped with an argon laser operating at 488 nm[22].

Surface biotinylation, β_3 immunoprecipitation and Western Blotting

CHO cells expressing normal, mutant or heterozygous $\alpha_{IIb}\beta_3$ and mock-transfected cells were detached, washed and resuspended in PBS. Patient PRP was centrifuged at 1000 x g for 10 min, platelets were washed 3 times with PBS containing 10mM EDTA and resuspended in the same

buffer. Samples were incubated with 1 mg/ml of Sulfo-NHS-LC-Biotin reagent (Pierce, Rockford, IL, USA) for 30 minutes at room temperature, washed in PBS containing 100mM glycine and lysed with lysis buffer (40 mM Tris- HCl, 0.3 M NaCl, 1 mM EDTA, 1mM NaF, 1 mM Na_3VO_4, 10 µl NP-40, 10 µg/ml leupeptin/pepstatin).

Lysates were pre-cleared and β_3 was immunoprecipitated with 10 µg of a mouse anti human-β_3 MoAb (Calbiochem, Merck, Darmstadt, Germany) and Protein G Sepharose beads (Invitrogen, Life Technologies). Immuno-precipitates were then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. Biotinylated proteins were identified using HRP-conjugated avidin (Sigma Aldrich, Milan, Italy) and ECL chemiluminescence (Amersham, GE Healthcare, Fairfield, CT, USA). Not-biotinylated CHO cell and platelet lysates were used to measure total β_3 expression, by probing membranes with a mouse anti human-β_3 MoAb (Calbiochem) and an appropriate HRP-conjugated secondary antibody. Densitometric analysis was performed on three different experiments using the ImageJ software (NIH, USA).

$\alpha_{IIb}\beta_3$ and $\alpha V\beta_3$ expression

$\alpha_{IIb}\beta_3$ and $\alpha V\beta_3$ expression were assessed by flow cytometry using the CD41-FITC clone P2 mAb and the CD51-FITC Clone AMF7 mAb (Beckman Coulter, Milan, Italy).

For analysis of $\alpha_{IIb}\beta_3$ expression upon activation platelets were stimulated with 10µM ADP or 20 µM TRAP-6 and then incubated with the CD41-FITC mAb and analyzed by flow cytometry as described above. Alternatively, membrane proteins were biotinylated, β_3 was immunoprecipitated and analyzed by SDS-PAGE as described above.

$\alpha_{IIb}\beta_3$ and fibrinogen internalization

Internalization of $\alpha_{IIb}\beta_3$ and fibrinogen was assessed by flow cytometry. Briefly, washed platelets, wild type and mutant $\alpha_{IIb}\beta_3$-expressing CHO cells were incubated for 60 minutes at 37°C with the CD41-FITC clone P2 mAb and then treated with 20µM ADP (platelets), 10mM DTT (CHO cells) or PBS for 20 minutes at 37°C, and finally analyzed by flow cytometry. After the acquisition of the surface mean fluorescence intensity a saturating concentration of an anti-fluorescein rabbit polyclonal IgG (Molecular Probes) was added to quench the fluorescence of surface-bound antibodies, and samples were immediately re-analyzed for residual CD41-FITC fluorescence. The percentage of internalized $\alpha_{IIb}\beta_3$ was calculated as described[24].

For fibrinogen internalization CHO cells were treated with 10mM DTT or PBS for 20 minutes at 37°C, incubated for 60 minutes at 37°C with the anti-human-fibrinogen FITC mAb in the presence

of 4 μg/ml of human fibrinogen and then fibrinogen internalization was analyzed as above described.

Adhesion assay

CHO cells expressing mutant, wild type or heterozygous $\alpha_{IIb}\beta_3$ ($3x10^6$/ml) were resuspended in IMDM and layered onto glass coverslips coated with 100 μg/ml of human fibrinogen (American Diagnostica, Stamford CT, USA). May-Grunwald-Giemsa staining was performed and samples were analyzed by optical microscopy.

Washed platelets ($20x10^6$/ml) were layered onto glass slides coated with 100 μg/ml of human fibrinogen or with 10 μg/ml of human Von Willebrand Factor (VWF) (Haemate P, CSL Behring, Marburg, Germany). To induce spreading on VWF, which requires activation of $\alpha_{IIb}\beta_3$, platelets were stimulated with 0.1 U/ml of human α-thrombin. Adherent platelets were fixed, permeabilized, stained with FITC-conjugated phalloidin and analyzed by fluorescence microscopy.

Spreading was assessed at 10, 30 and 60 minutes and the mean percentage of the total surface covered was calculated by using the ImageJ software (NIH, USA).

Protein phosphorylation

CHO cells stably expressing normal or mutant $\alpha_{IIb}\beta_3$, mock-transfected CHO cells or washed platelets were resuspended in IMDM and plated for 1 h at 37° C in 6-well plates pre-coated with 100 μg/ml of purified human fibrinogen or with 1% BSA. Cells were then washed twice with PBS and lysed with lysis buffer.

Lysates were recovered, clarified and 20 μg of proteins were analyzed by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with a rabbit anti-phospho-FAK MoAb (Cell Signalling Technology, Danvers, MA), a rabbit anti-integrin β_3 phosphoY773 and a rabbit anti-integrin β_3 phosphoY785 (AbCam, Cambridge, UK) and immunoreactive bands were detected using a peroxidase-conjugated anti-rabbit IgG antibody by chemiluminescence and measured by densitometric analysis using the ImageJ software (NIH, USA).

Clot retraction

CHO cells expressing mutant or wild type $\alpha_{IIb}\beta_3$ and mock-transfected cells, resuspended in 800 μl of IMDM plus 200 μl of human platelet-poor plasma, or alternatively 200 μl of PRP plus 800 μl of IMDM supplemented with 5mM $CaCl_2$, were placed in tubes pretreated with SigmaCote (Sigma Aldrich, Milan, Italy). Human α-thrombin (2 U/ml) was then added, tubes were briefly stirred and

incubated in a water-bath at 37° C for 30 and 60 min and were photographed at various times. The two-dimensional sizes of retracted clots on photographs were quantified using Image J software and clot size was measured. Clot retraction was calculated by the formula: [1-(Vc/Vt)] x100, where Vt is the total cell suspension volume and Vc the volume occupied by the clot.

Actin polymerization

Actin polymerization was assessed by flow cytometry. Briefly, 20 µl of platelet-rich plasma (PRP) or of a suspension of wild type or mutant $\alpha_{IIb}\beta_3$-expressing CHO cells were stimulated with ADP (20µM for platelets) or DTT (10mM for CHO cells) for 15 min at 37°C, fixed with 4% PFA, permeabilized with 0.01% Triton-X and stained with FITC-conjugated Phalloidin (Molecular Probes, Life Technologies, Monza, Italy).

Extraction of platelet cytoskeleton and analysis of cytoskeletal proteins

Cytoskeleton was extracted from washed platelets, either resting or stimulated with thrombin (0.5 U/ml) for 5 min at 37°C under constant stirring, by the addition of an equal volume of cytoskeleton extraction buffer 2x[26]. Samples were carefully mixed and placed on ice for 10 min. Triton X-100-insoluble material was recovered by centrifugation at 12,000 x g for 5 min at 4°C, washed twice with cytoskeletal buffer without Triton X-100, and resuspended in 4% SDS. An equal volume of sample buffer (5% β-mercaptoethanol, 20% glycerol and 0.02% bromophenol blue) was then added, samples were boiled for 5 min, and cytoskeletal proteins (20 µg) were separated on a 10% acrylamide gel and stained with Comassie blue[27]. Quantitative analysis of cytoskeleton-associated proteins was performed by densitometric scanning of stained gels by using the ImageJ software (NIH, USA).

Mass Spectrometry

Protein digestion was performed as previously reported[27]. Briefly, after Coomassie staining, selected bands were sliced from the gel, destained in 300 µL of a 100 mM NH_4HCO_3 in 50% ACN solution at 37°C for 30 min, and with 25 mM NH_4HCO_3 in 50% ACN (3x30 min). Destained gel bands were reduced with a solution of dithiothreitol (DTT) 10 mM in 25 mM of NH_4HCO_3 for 1h at 56°C and alkylated with a solution of 55 mM iodoacetamide (IAA) in 25 mM NH_4HCO_3 for 45 min at room temperature in the dark. Gel bands were then dried in a Speedvac, rehydrated with a 50 mM NH_4HCO_3 solution containing 400 ng of proteomic grade trypsin (Promega) and covered with 50 mM NH_4HCO_3 solution (pH 8) and incubated overnight at 37°C. After centrifugation, oligopeptide-containing supernatants were recovered and concentrated to dryness. Peptides were redissolved in

110 µL of an aqueous solution containing 5% ACN and 0.1% formic acid and analyzed with a LCQ Deca-XP Plus ion-trap mass spectrometer operating in data-dependent acquisition mode, as previously reported[27].

Database searching was performed using the MASCOT software version 2.2 and MyriMatch software v. 2.1.87[28]. Search parameters were as follows: protein sequence database UniProt release 2014_11 (66,922 sequences); enzyme trypsin; fixed modification carbamidomethylation; variable modification oxidation; two missed cleavages allowed; peptide tolerance ±1.2 Da; MS/MS tolerance 0.6 Da, peptide charge +1, +2 and +3. Protein assembly for MyriMatch analysis was made using IDPicker v 3.0[29] using an FDR both at protein and peptide levels of 1%. Spectral counts were used for semiquantitative comparison between controls subjects and patients.

Effect of the perturbation of actin polymerization on platelet function

To induce actin polymerization in control platelets we used Jasplakinolide (Vinci Biochem, Vinci, Italy), a natural cyclic peptide that induces actin polymerization and stabilizes actin filaments. To prove actin polymerization induced by Jasplakinolide, we incubated control platelets with 5µM Jasplakinolide, the cytoskeleton was extracted as above described, and the cytoskeletal and Triton-soluble fractions were analyzed by Western Blotting using an anti-human actin antibody (Sigma Aldrich), as above described.

Jasplakinolide (5µM) or its vehicle were incubated for 10 minutes with PRP and then $\alpha_{IIb}\beta_3$ surface expression and ADP (10 µM)-induced $\alpha_{IIb}\beta_3$ activation were measured by flow-cytometry, as above described.

Moreover, gel filtered platelets, suspended in Hepes-Tyrode buffer at $2x10^5$/µl, were treated for 10 minutes with jasplakinolide 5 µM or its vehicle, supplemented with 0.5 mM $CaCl_2$ and 200 µg/ml of human fibrinogen, and stimulated with ADP (10 µM)[31] and aggregation was followed for 10 min by light transmission aggregometry (APACT4, Helena Bioscences, UK)[32].

Finally, clot retraction and spreading on fibrinogen were evaluated as described above after pre-treatment for 10 minutes with increasing concentrations of jasplakinolide (1µM, 3µM or 5µM) or its vehicle. In this case platelets were stained with CD41-FITC clone P2 mAb because jasplakinolide inhibits the binding of phalloidin to F-actin[33].

Construction of retroviral vectors and retrovirus production

Given that the in-frame fusion of α_{IIb} and β_3 subunits with fluorescent proteins interferes with their function[34] or surface expression (unpublished observations), we decided to use bicistronic vectors

that allowed the simultaneous expression of α_{IIb} with the GFP protein and of β_3 with the Ds-Red protein separately.

Full-length α_{IIb} cDNA was amplified by Pfu DNA Polymerase and cloned into the EcoRI/SnaBI sites of pMYs-IRES-GFP Bicistronic Retroviral Vector (CellBioLabs, San Diego CA, USA) to obtain a retroviral vector expressing independently α_{IIb} and GFP.

The full-length wild-type and del647-686 β_3 cDNA was amplified by Pfu DNA Polymerase and cloned into the BglII/ClaI sites of pRetroX-IRES-DsRedExpress Bicistronic Retroviral Vector (Clontech, Mountain View, CA, USA) to obtain two retroviral vectors expressing independently wild-type or mutant β_3 and the Ds-Red protein. Retroviruses were produced by transfecting vectors with Fugene6 (Roche Applied Science, Mannheim, Germany) in the HEK 293 Phoenix cell line grown in DMEM supplemented with 10% FCS, 2mM L-Glutamine and 100U/ml penicillin/streptomycin. After 72h, retroviral supernatants were obtained and supplemented with 6 μg/ml polybrene (Sigma Aldrich, Milan, Italy).

Megakaryocyte infection

Mouse fetal liver cells were harvested from day 13.5 fetuses and cultured in DMEM supplemented with 10% FCS, 2mM L-Glutamine, 100U/ml penicillin/streptomycin and 0.1 μg/mL mouse thrombopoietin (obtained from the GP+E-86 TPO-secreting cell line) for 4 days; at day 4 of differentiation, cultures were enriched by a single-step gradient (1.5-3% BSA), resuspended in fresh medium and cultured for an additional 24 h[33].

Mouse megakaryocyte cultures at day 3 of differentiation were double-infected with the pMYs-IRES-GFP-α_{IIb} and the pRetroX-IRES-DsRed-wt or -mutant β_3 retroviruses by spin-infection. Transduction efficiency was assessed by flow cytometry measuring the fluorescence emitted by the GFP and Ds-Red proteins.

Megakaryocyte spreading, proplatelet formation and morphology

Megakaryocyte cultures at day 5 were layered on a single-step gradient and allowed to lay down for 30 min to resolve intermediate stages in proplatelet maturation, as previously described[35]. To evaluate spreading, megakaryocytes were plated onto glass coverslips coated with 100 μg/ml of human fibrinogen, incubated for 4 h at 37°C in a 5% CO_2 atmosphere and analyzed by immunofluorescence microscopy, as previously described[3]. To evaluate proplatelet formation megakaryocytes were cytospun on poly-L-lysine coated coverslips, all polynucleated cells extending protrusions with terminal tips were identified as proplatelet-forming megakaryocytes and cells showing both green and red fluorescence (i.e. expressing both α_{IIb} and β_3) were analyzed for

proplatelet formation. At least 20 megakaryocytes from 5 different samples were analyzed. Tips were counted and measured using the AxioVision4 Software (Carl Zeiss Inc., Oberkochen, Germany).

For the assessment of proplatelet morphology in circulating human blood, patient or control PRP was cytospun on poly-L-lysine-coated coverslips and β1-tubulin distribution was analyzed by immunofluorescence, as described[3,36]. At least 20 barbell-proplatelets from 5 different samples were analyzed. Tips were measured using the AxioVision4 Software. Barbell-proplatelet symmetry was assessed by measuring the difference in the diameter between the two tips.

CHO cells immunofluorescence and confocal microscopy

CHO cells expressing normal or mutant $\alpha_{IIb}\beta_3$ were cultured overnight on poly-L-lysine coated coverslips, fixed with 3.7% PFA, permeabilized with 0.01% Triton-X, and blocked with 1% BSA.

For fluorescence microscopy β_3 integrin was stained with the CD41 clone P2 mAb followed by labeling with the Alexa Fluor® 488 Goat Anti-Mouse IgG (Molecular Probes). Samples were the incubated with either Tetramethylrhodamine Conjugate Concanavalin A (ER marker) or Texas Red®-X Conjugate Wheat Germ Agglutinin (WGA, Golgi marker) (Molecular Probes). Nuclei were stained blue with Hoecst. Specimens were mounted with the ProLong Antifade medium (Molecular Probes) and analyzed at room temperature by a Carl Zeiss Axio Observer.A1 fluorescence microscope (Carl Zeiss Inc, Oberkochen, Germany) using a 63x/1.4 Plan-Apochromat oil-immersion objective and acquired using the AxioVision software (Carl Zeiss Inc).

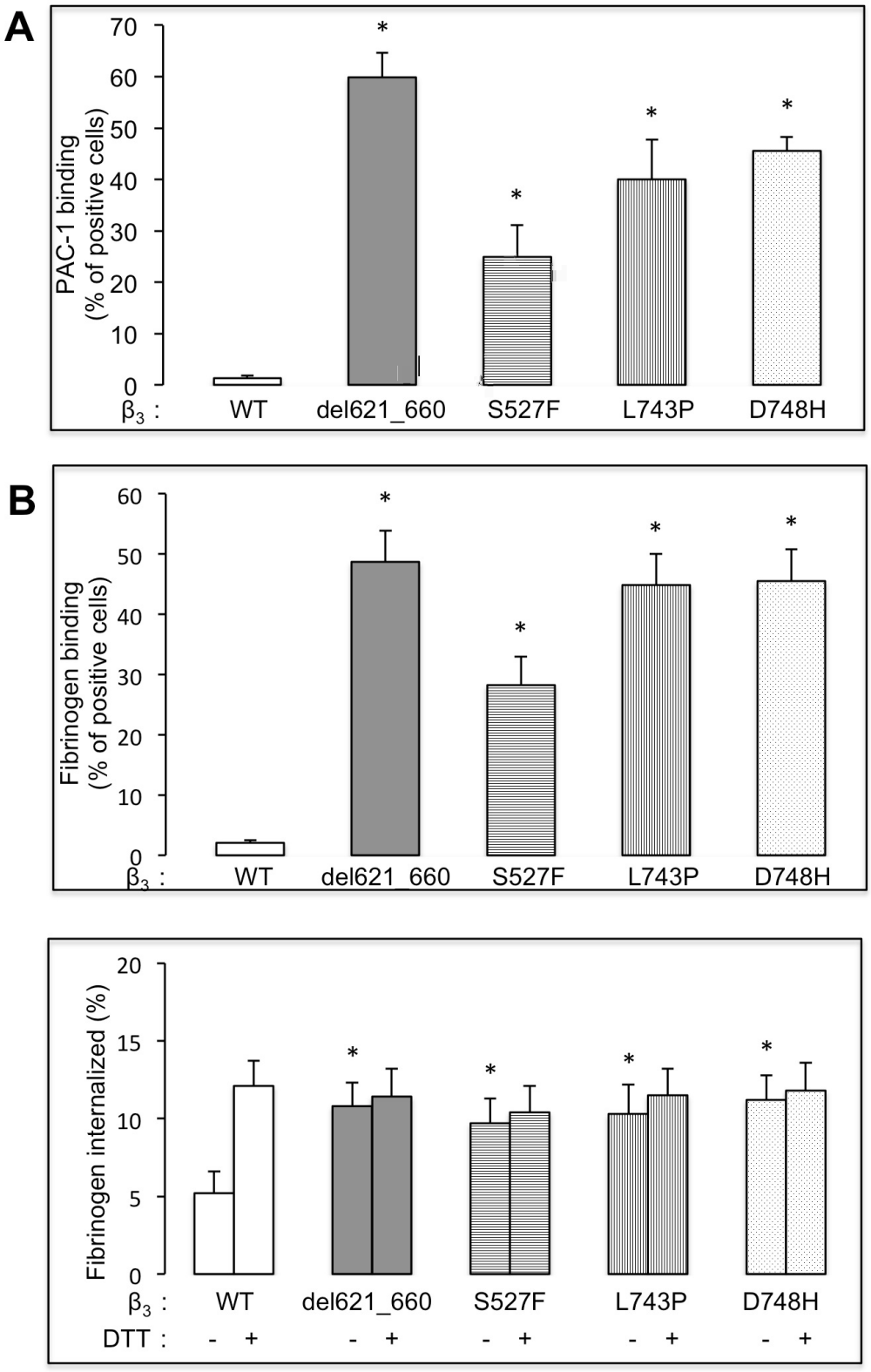
For confocal microscopy β_3 integrin was stained with the CD41 clone P2 mAb followed by labeling with the Alexa Fluor® 488 Goat Anti-Mouse IgG. Specimens were mounted with the ProLong Antifade medium. Confocal analysis was performed at room temperature with a confocal microscope (Bio-Rad MRC 1024) using an Ar/Kr laser. Medial focal planes are shown. Images were elaborated on a SGI Octane work station (SGI, Mountain View, CA) with the Imaris software (Bitplane, Zurich, CH).

Confocal microscopy for β_3 localization in platelets

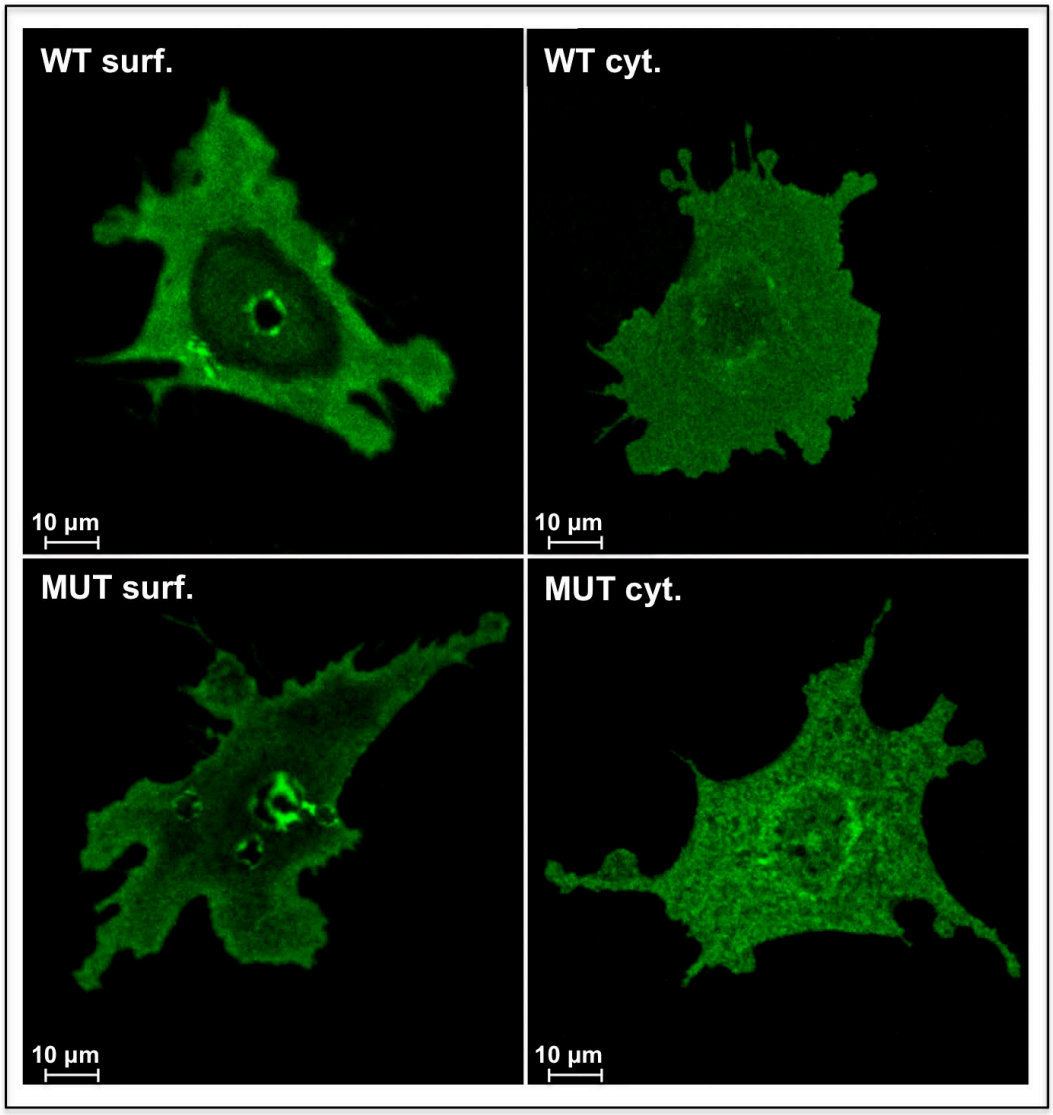
Washed platelets were cytospun on poly-L-lysine coated coverslips, fixed with 3.7% PFA, permeabilized with 0.01% Triton-X, and blocked with 1% BSA. For confocal microscopy β_3 integrin was stained with a rabbit anti-human β_3 antibody (Cell Signaling) followed by labeling with the Alexa Fluor® 594 goat anti-rabbit IgG and P-selectin was stained with the mouse anti-human CD42P mAb (Beckman Coulter) followed by labeling with the Alexa Fluor® 488 goat anti-mouse IgG. Confocal analysis was performed using a TCS SPII confocal laser scanning microscopy

system equipped with a DM IRBE inverted microscope with a 40x OIL NA objective (Leica, Bensheim, Germany). Co-localization of and P-selectin 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).

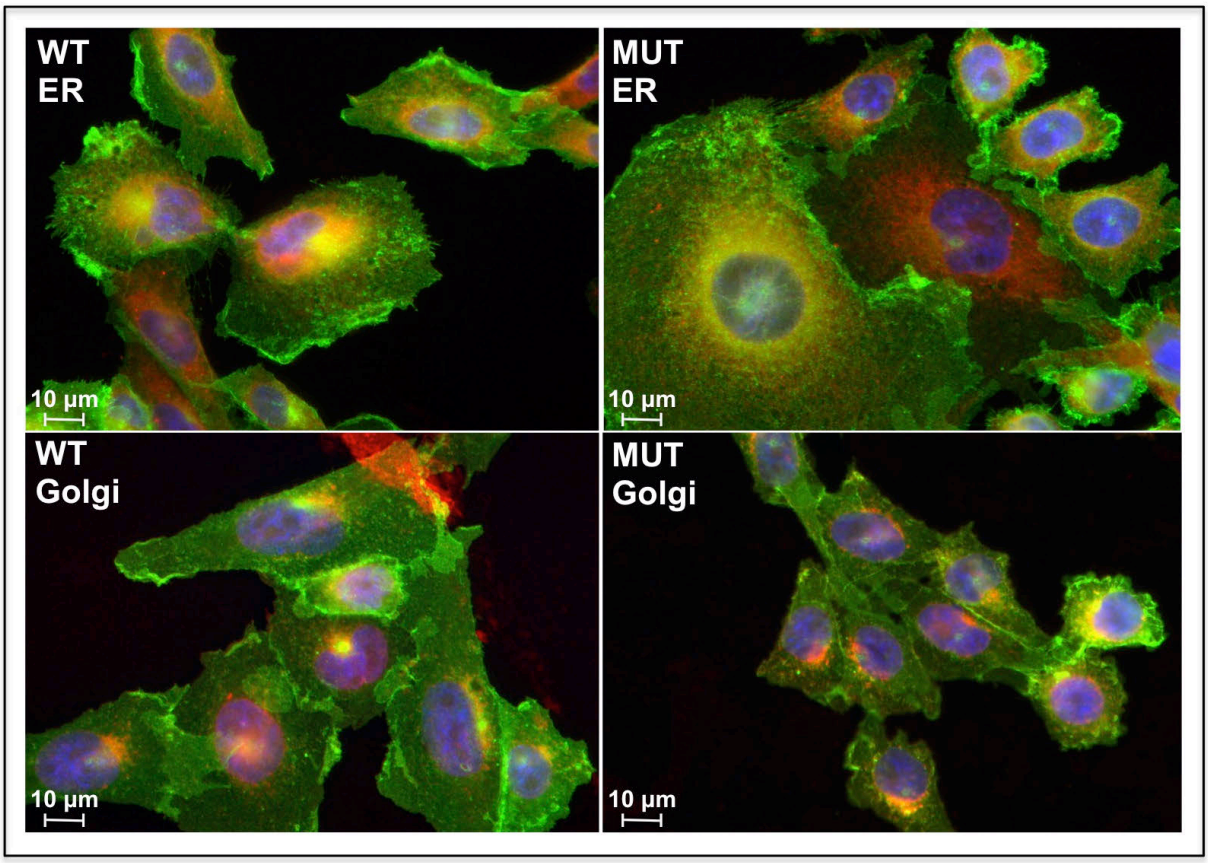
Supplementary figure 1



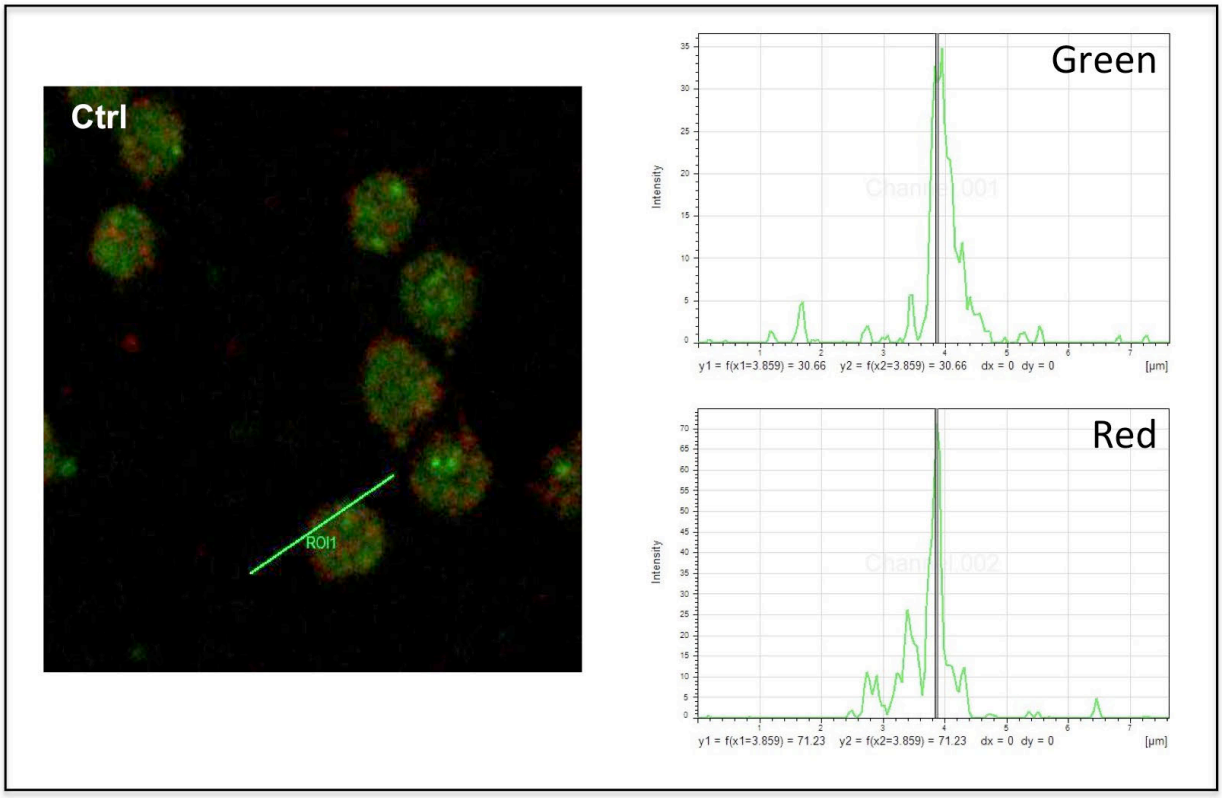
Supplementary figure 2

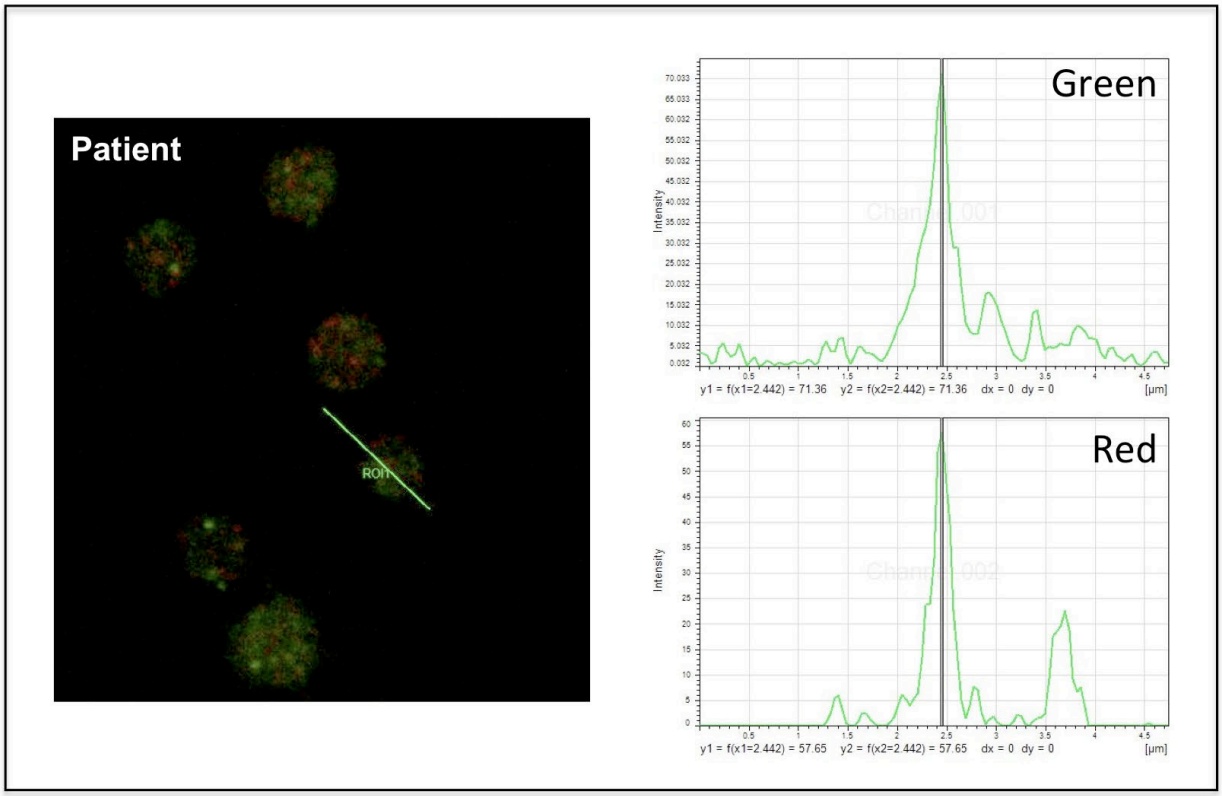


Supplementary figure 3

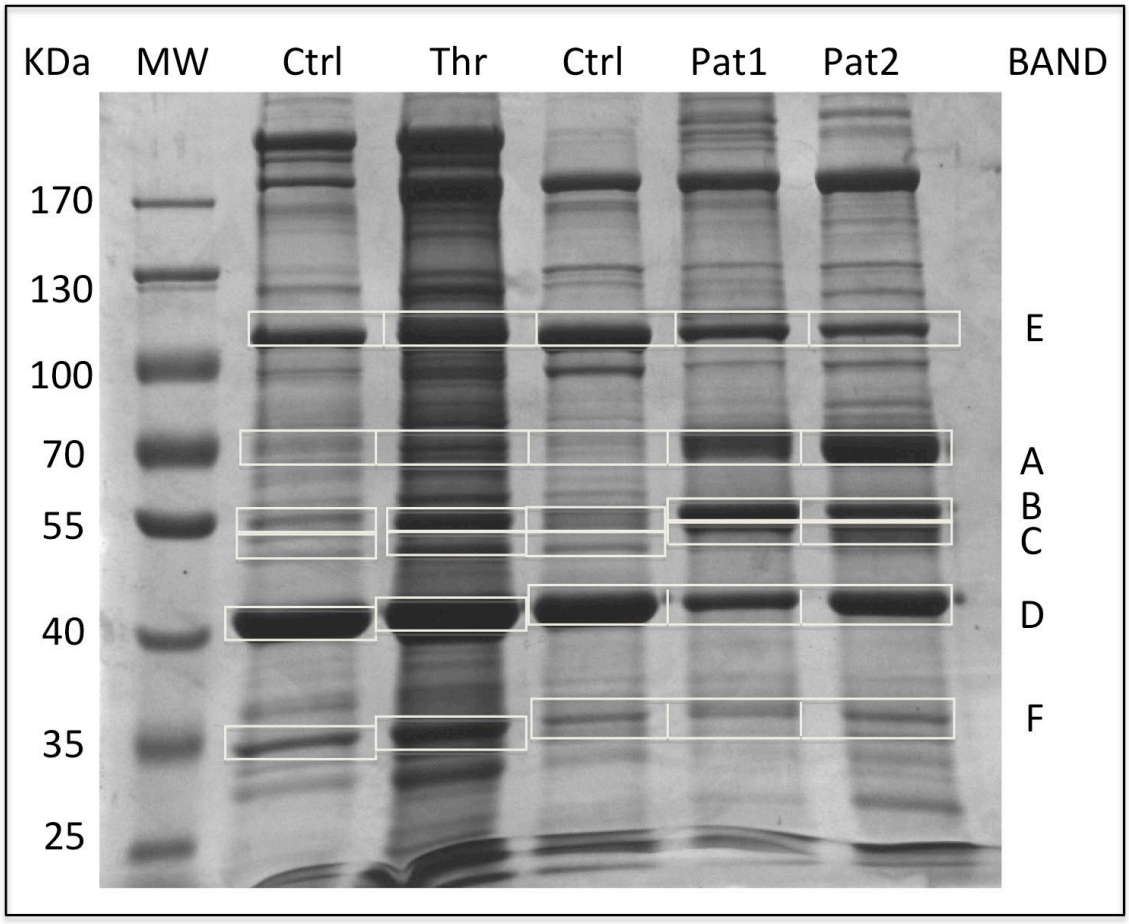


Supplementary figure 4

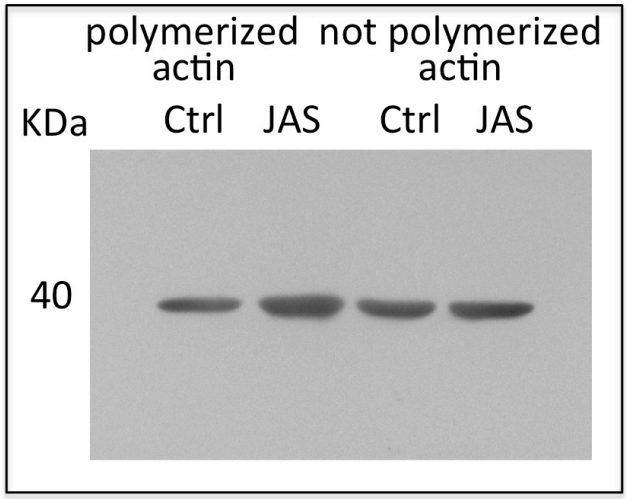




Supplementary figure 5



Supplementary figure 6



Supplementary Figures Legends

Supplementary Figure 1: CHO cells expressing the different *ITGB3* mutations constitutively bind PAC-1 and fibrinogen

A) PAC-1 binding measured by flow-cytometry in CHO cells expressing different *ITGB3* mutations.

B) Fibrinogen binding measured by flow-cytometry in CHO cells expressing different *ITGB3* mutations (*p<0.05 vs WT).

C) Internalized fibrinogen measured by flow cytometry in CHO cells expressing different ITGB3 mutations (*p<0.05 vs WT resting).

Supplementary Figure 2: **the mutant β_3 integrin preferentially localizes in the cell cytoplasm**

Immunolocalization of the wild type (WT) or the mutant (MUT) $\alpha_{IIb}\beta_3$ receptor in CHO cells. To analyze surface expression (surf.) cells were not permeabilized, while to analyze the receptor retained in the cytoplasm (cyt.) they were permeabilized with 0.1% Triton-X. Integrin β_3 was stained with the CD41 clone P2 mAb followed by labeling with the Alexa Fluor® 488 Goat Anti-Mouse IgG. Specimens were mounted with the ProLong Antifade medium. Confocal analysis was performed at room temperature with a confocal microscope (Bio-Rad MRC 1024). Images were elaborated on a SGI Octane work station (SGI, Mountain View, CA) with the Imaris software (Bitplane, Zurich, CH).

Supplementary Figure 3: mutant β_3 integrin is correctly synthesized and undergoes maturation.

Mutant β_3 integrin (green signal) co-localizes with the Endoplasmic Reticulum (ER, red signal) and the Golgi Apparatus (Golgi, red signal) as well as wild-type β_3. Co-localization is detectable as yellow. Integrin β_3 was stained with the CD41 clone P2 mAb followed by labeling with the Alexa Fluor® 488 Goat Anti-Mouse IgG. Samples were the incubated with either Tetramethylrhodamine Conjugate Concanavalin A (ER marker) or Texas Red®-X Conjugate Wheat Germ Agglutinin (WGA, Golgi marker). Nuclei were stained with Hoecst. Specimens were mounted with the ProLong Antifade medium, analyzed at room temperature by a Carl Zeiss Axio Observer.A1 fluorescence microscope using a 63x/1.4 Plan-Apochromat oil-immersion objective and acquired using the AxioVision software.

Supplementary Figure 4: Colocalization of the β_3 integrin with α-granules

Immunolocalization of the β_3 integrin and P-selectin in control and patient platelets. β_3 integrin was stained with a rabbit anti-human β_3 antibody (Cell Signaling) followed by labeling with the Alexa Fluor® 594 goat anti-rabbit IgG and P-selectin was stained with the mouse anti-human CD42P mAb (Beckman Coulter) followed by labeling with the Alexa Fluor® 488 goat anti-mouse IgG. The graphics on the right report the intensity of the fluorescence signal along the x axis for each fluorochrome on the optical section and were obtained using the LAS-X software (Leica).
Of note, also single green signals (α-granules) and single red signals (β_3 integrin) are detectable, indicating that β_3 integrin localizes both inside and outside α-granules.

Supplementary Figure 5: protein bands selected for mass spectrometry analysis.
Selected protein bands associated with the platelet cytoskeleton from resting control platelets (Ctrl1, Ctrl2), control platelets stimulated with thrombin (Thr) and platelets from two different patients (Pat1, Pat2). The molecular weight of the bands is reported on the left, the name of the band on the left. Supplementary Table 1 lists the identity of the bands.

Supplementary Figure 6: treatment of platelets with Jasplakinolide induces actin polymerization
Western blotting of actin linked to the platelet cytoskeleton (polymerized actin) in platelets treated with the vehicle (CTRL) or Jasplakinolide (JAS) and actin present in the Triton-soluble fraction (not polymerized actin). Platelet cytoskeleton was extracted as described under "Material and Methods", proteins were separated on a 10% acrylamide gel and blotted onto nitrocellulose membranes. Membranes were probed with a mouse anti-human actin mAb (Sigma Aldrich), and immunoreactive bands were detected using a peroxidase-conjugated anti-rabbit IgG antibody by chemiluminescence.

Supplementary Table 1: Mass spectrometry identification of selected protein bands

Gel band	Experimental MW (KDa)	Protein Name	Uniprot ID	Mascot Score (maximum)	n° identified peptides	Theoretical MW (kDa)
A*	70	Serum albumin	ALBU_HUMAN	292	25	71.317
		Fibrinogen alpha chain	FIBA_HUMAN	249	18	95.656
		Fermitin family homolog 3	URP2_HUMAN	129	9	76.475
		WD repeat-containing protein 1	WDR1_HUMAN	81	2	66.836
		Heat shock cognate 71 kDa protein	HSP7C_HUMAN	70	2	71.082
		Serum deprivation-response protein	SDPR_HUMAN	58	3	47.202
B*	55	Fibrinogen beta chain	FIBB_HUMAN	270	11	56.577
		Serum albumin	ALBU_HUMAN	51	2	71.317
		Tubulin alpha-3C/D chain	TBA3C_HUMAN	46	4	50.612
		Tubulin alpha-4A chain	TBA4A_HUMAN	83	5	50.634
C*	55	Tubulin beta-1 chain	TBB1_HUMAN	337	19	50.865
		Tubulin beta chain	TBB5_HUMAN	289	15	50.095
		Tubulin beta-4B chain	TBB4B_HUMAN	277	14	50.255
		Tubulin beta-3 chain	TBB3_HUMAN	194	8	50.856
		Fibrinogen beta chain	FIBB_HUMAN	160	7	56.577
		Fibrinogen alpha chain	FIBA_HUMAN	140	7	95.656
		Tubulin beta-1 chain	TBB1_HUMAN	126	4	50.865
		Fibrinogen gamma chain	FIBG_HUMAN	116	6	52.106
		Protein disulfide-isomerase A6	PDIA6_HUMAN	113	2	48.49
		ATP synthase subunit beta	ATPB_HUMAN	96	6	56.525
		Serum albumin	ALBU_HUMAN	89	2	71.317
D*	40	Actin, cytoplasmic 1	ACTB_HUMAN	441	28	42.052
		POTE ankyrin domain family member E	POTEE_HUMAN	343	10	122.882
E*	105	Fibrinogen gamma chain	FIBG_HUMAN	111	8	52.106
		Alpha-actinin-1	ACTN1_HUMAN	45	1	103.563
F*	35	Tropomyosin alpha-4 chain	TPM4_HUMAN	89	3	28.619
		Tropomyosin alpha-3 chain	TPM4_HUMAN	79	2	32.987

*The letters correspond to the different gel bands shown in Supplementary Figure 4.

Supplementary Table 2: Semiquantitative comparison of fibrinogen subunits in patients and controls by spectral counts

Uniprot ID	Protein name	FC Patients vs controls	CTRL1	CTRL2	PAT1	PAT2
FIBA_HUMAN	Fibrinogen alpha chain	4.5	1	0	2	1
FIBB_HUMAN	Fibrinogen beta chain	12.3	3	1	13	36
FIBG_HUMAN	Fibrinogen gamma chain	2.4	0	2	1	8

Fibrinogen chains (alpha, beta, gamma) were enriched in patients carrying the β_3 del647-686 mutation of ITGB3. Spectral counts relative to the whole lane of the different samples were used in the comparison.