Inherited missense variants that affect GFI1B function do not necessarily cause bleeding diatheses

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Supplemental Methods

Patient recruitment and ethics

The NIHR BioResource (NBR) – Rare Disease Study is a multi-centre whole-exome and whole-genome sequencing study including approximately 10,000 patients. The NBR–Rare Diseases study was approved by the East of England Cambridge South national research ethics committee (REC) under reference number: 13/EE/0325. The inclusion and exclusion criteria were as described before.[1] In short, the inclusion criteria for enrolment are: (i) positive history of bleeding, (ii) abnormal platelets (abnormal count, volume, aggregation, morphology). In addition, patients were only included when their disease was highly likely of genetic etiology (e.g. early onset, informative pedigrees, absence of acquired cause). Variant classification was performed according to the ACMG criteria[19] and using Sapienta™ software (Congenica).

Clinical evaluation and laboratory tests

The clinical and laboratory phenotypes including electron microscopy and CD34 expression were determined as previously described.[1, 2] Patients from the pedigrees with the H181Y, R184P, and R190W variants were recalled for this study.

Expansion of GFI1B variant transduced MEG-01 cells

MEG-01 cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% heat inactivated FCS and retrovirally transduced with pMIGR1-GFI1B variant-flag-IRES-GFP constructs. The GFP% was measured on the Coulter FC500 flow cytometer (Beckman Coulter) for 26 days with 2-3 day intervals. GFP percentages were normalized to the FACS measurement of day 5 using the following formula: (GFP% day X/(100 - GFP% day X))/(GFP% day 5/(100 - GFP% day 5)). On day 23, GFP^+ cells were sorted using the BD FACSAria (BD Bioscience) to determine total and endogenous *GFI1B* expression using quantitative RT-PCR. *GFI1B* exon 1-2 primers and probe are as follows: forward 5'-CCCGTGTGCAGGAAGATGA, reverse 5'-CAGGCACTGGTTTGGGAATAGA, probe 5'-FAM-TTACCCCGGTGCCCAGA-MGB. 5'UTR *GFI1B* expression was determined using the TaqMan gene expression assay Hs01062474_m1. *GAPDH* expression was determined using Human GAPDH mix Hs99999905_m1 (FAM™ Dye/MGB Probe) (Applied biosystems).

GFI1B variant reporter assays

To determine GFI1B transcriptional activity, we performed Dual-Luciferase Reporter Assays (Promega) in 293FT cells. 293FT cells were maintained in DMEM (GIBCO) supplemented with 10% non-heat inactivated fetal calf serum (FCS), 1% glutamine, 1% non-essential amino acids, 1% pyruvate, and 1% penicillin/streptomycin (MP Biomedical). 293FT cells were transfected using Lipofectamine 2000 (Invitrogen). A total of 2µg DNA was used for transfection consisting of 0.5µg pcDNA3.1 flag-tagged wild type or variant GFI1B, 0.5µg pcDNA3.1-empty vector, 0.8µg pGL3-basic Firefly Luciferase vector harboring the *Gfi1* promoter, and 0.2µg pGL3-basic Renilla Luciferase vector. Forty-eight hours after transfection, cells were washed with PBS and lysed in 100µl of passive lysis buffer (Dual-Luciferase Reporter Assay System, Promega) for 1 hour. Luciferase signal from 1.5µl lysate was detected using 10µl LAR II and 10µl Stop&Glo (Dual-Luciferase Reporter Assay System, Promega) on the Fluostar Optima (BMG LABTECH). Experiments were performed at least three times, in duplicate. Firefly Luciferase activity was normalized to Renilla activity and each condition was normalized to empty vector.

Statistics

Two-tailed paired *t*-tests or one-way Anova testing was performed with Graphpad Prism version 5.03 to determine statistically significant differences.

GFI1B-Q89fs-flag expression after MG132 treatment

293FT cells were transfected in duplo with 20µg pcDNA3.1 (empty vector), pcDNA3.1-GFI1B-Q89fs-flag, or pcDNA3.1-GFI1B-Q287*-flag using calcium phosphate. Sixteen hours after transfection, the medium was refreshed, and 24 hours after transfection 5µM MG132 or 1µl DMSO was added to the cells. After 16 hours of MG132 proteasome inhibitor treatment cells were lysed in passive lysis buffer (Promega) and loaded on a SDS-PAGE gel. The proteins were transferred to a PVDF membrane which was stained with mouse α-flag (SIGMA-ALDRICH, Merck) and mouse α-GAPDH (Abcam) followed by probing with goat α-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Luminescence signal was visualized using a ChemiDox XRSþ (Bio-Rad).

Table S1. Clinical characteristics GFI1B variants

Identifier used by clinician	BRIDGE identifier	*Patient identifier (1) Gender*	*GFI1B variant*	*GnomAD (heterozyous-homozygous/total alleles)*	*Bleeding*	*Platelets (10^9/L) (normal range: 150-450)*	*Mean Platelet Volum (fL) (normal range: 8-12)*	*CD34 expression by FACS (2)*	*Blinded blood smear analysis (3)*	*Electron Microscopy (4): platelet area (µm2) (normal range: 6.2 ± 2.15)*	*Electron Microscopy: Number of α-granules (normal range: 15.7 ± 7.45)*	*Platelet aggregation and other functional assays*	*Other variant in a known BPD gene*	*Classification of GFI1B variant using ACMG criteria (6)*
RFH000073X	B200037	P1 (female)	D23N	401 - 5/274546	Yes	408	ND	ND	ND	ND	ND	Abnormal for Epinephrine only.	No	BS1: Benign
ipd85	B200217	P2 (female)	Q89fs	Absent	Yes	119	11.5	ND	ND	7.98 ± 3.37	7.2 ± 3.93*	Abnormal for ADP, Collagen, Aracidonic acid and Ristocetin.	No	PM2, PM4: VUS
TRS13	B200721	P3 female)	G139S	29 - 0/277110	Yes	81	9.7	ND	ND	ND	ND	ND	No	BS1, BS3: Benign
RFH000098F	B200597 (Asian)	P4 (female)	C168F homozygous	122 - 1/243904 (Asian: 121 - 1/30762)	Yes	100	ND	ND	ND	ND	ND	Abnormal for ADP and Epinephrine.	No	PP5: VUS + partial contribution to the phenotype (previously reported as linked to thrombocytopenia but not bleeding)
	sibling	P5.1 (female)	H181Y	Absent	Yes ISTH BAT score: 10	152	11.5	Yes	Mild thrombocytopenia with platelet anisocytosis. Few large "grey" (agranular) platelets and others are hypogranular. The majority are however normal in size and granulation.	5.67 ± 2.27	4.5 ± 2.88**	Abnormal for ADP, Epinephrine & ristocetin. Nucleotide assay: low ADP and abnormal ATP:ADP ratio.	No WGS data	PM2, PS3-P, PP3, PP1: VUS
5H10000348	B200645	P5.2 (male)	H181Y		Yes ISTH BAT score: 11	184	12.7	Yes	Mild platelet anisocytosis and variable granulation.	5.75 ± 1.59	5.3 ±3.30***	Abnormal for ADP, Collagen, Aracidonic acid and Ristocetin.	No	
	sibling	P5.3 (male)	Normal		No ISTH BAT score: -1	237	10.4	ND	ND	ND	ND	ND		
	sibling	P5.4 (female)	H181Y		Yes ISTH BAT score: 4	178	12.4	Yes	Thrombocytopenia with large hypogranular platelets.	ND	ND	Not done but PFA prolonged in both cartridges.	No WGS data	
	niece	P5.5 (female)	H181Y		Yes ISTH BAT score: 8	257	10.8	ND	ND	ND	ND	Abnormal for ADP, Epinephrine, Aracidonic acid (low dose) & Collagen. Decreased ATP release in response to ADP.	No WGS data	
	mother	P6.1 (female)	Normal	Absent	No	242	9.1	Normal	Normal	ND	ND	Normal		PM2, PS3-P, PP3: VUS
	father	P6.2 (male)	R184P		No ISTH BAT score: 0	124	12.6	Yes	Mild thrombocytopenia with platelet anisocytosis. Some large hypogranular and a few agranular platelets.	ND	ND	ND	No WGS data	
	B200600	P6.3 (female)	R184P		Yes ISTH BAT score: 7	196	10.2	Yes	Mild platelet anisocytosis with some hypogranular and a few large agranular platelets.	ND (sample issue)	ND	Normal	No	
BRI000015D	sibling	P6.4 (female)	Normal		No	200	11.1	Normal	Normal	ND	ND	Normal		
IPD11	B200239	P7 (male)	R190W	33 - 0/246058	Yes	44	8.3	ND	ND	ND	ND	ND	Confirmed WAS defect: p.Arg364Ter	BS1, BP5: Benign
ADD000021H	B200077	P8.1 (female)	R190W	33 - 0/246058	No	167	12.9	Yes - weak	Normal count, large platelets.	7.11 ± 4.01	11.2 ± 6.68	ND	No	BS1: Benign
	relative	P8.2 (male)	R190W		Yes	103	12.1	Yes - weak	Macrothrombocytopenia	7.43 ± 2.50	10.2 ± 5.05	ND	No	
TRS28	B200735	P9 (female)	G198S	40 - 1/276984	No	52	12	ND	ND	ND	ND	Abnormal for ADP.	Confirmed ACTN1 defect: p.R46Q (5)	BS1, BS3, BP5: Benign

Values outside the normal range for number of platelets, mean platelet volume, platelet area and number of α-granules are underlined.
One way Anova test number of α-granules *p=0.0069, **p=0.0002 and ***p=0.0005
(1) Pedigrees of P5 and P6 shown in Figure 4
(2) Flow cytometry for CD34 expression shown in Figure S3
(3) Representative images of blood smears in Figure S4
(4) Representative images of electron microscopy in Figure S5
(5) ACTN1 variant: Westbury et al, Genome Medicine 2015,
(6) ACMG criteria: Richards S et al, Genet Med 2015, 17(5), 405-24
ND= not determined; VUS= Variant of Unknown Significance
ISTH BAT= ISTH bleeding assesment tool, Normal range for the ISTH BAT score is <4 in adult males, <6 in adult females and <3 in children.
Several laboratory and clinical findings have previously been published in Supplementary Table 15 of Chen et al.[2]

BS1: allele frequency in control population higher then expected, strong evidence for benign
BS3: functional studies, strong evidence benign
BP5: alternate locus observation, supporting evidence for benign
PM2: absent or low frequency in control population, moderate evidence for pathogenic
PM4: protein length changes due to in-frame deletions/insertions and stop losses, moderate evidence for pathogenic
PP1: cosegregation with disease in multiple affected family members, supporting evidence for pathogenic
PP3: computational (in silico) data, supporting evidence for pathogenic
PP5: reputable source, supporting evidence for pathogenic
PS3-P: functional studies, strong evidence pathogenic

Supplemental Figures

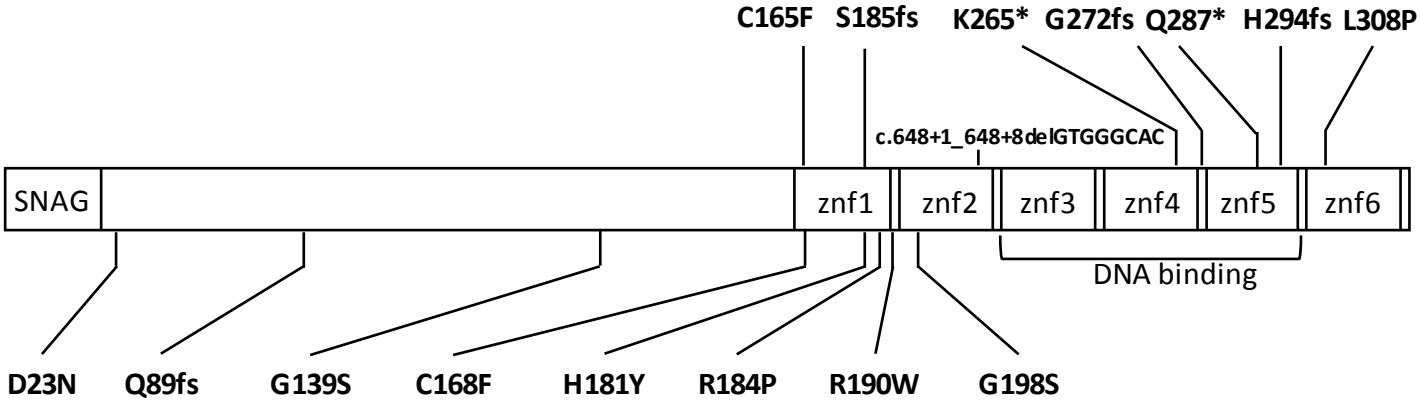

Figure S1. Overview of reported GFI1B variants. Schematic overview of GFI1B protein structure with variants identified in inherited bleeding and platelet disorders. The variants displayed below are analyzed in this study (C168F also identified in another study[1]). The upper variants have been published and studied before: C165F,[4] S185fs (homozygous, GFI1B-p37 transcript is mostly degraded and the short GFI1B-p32 isoform, lacking intact zinc finger (znf) 1 and 2, is unaffected),[5] c.648+1_648+8delGTGGGCAC7 (NM_004188.6; splice variant resulting in coding exon 4 skipping and expression of GFI1B-p32),[3] K265*,[6] G272fs,[7] Q287*,[8] H294fs,[9] and L308P.[6] GFI1B contains an N-terminal SNAG domain and six C-terminal znfs, of which znf 3-5 are involved in DNA binding.

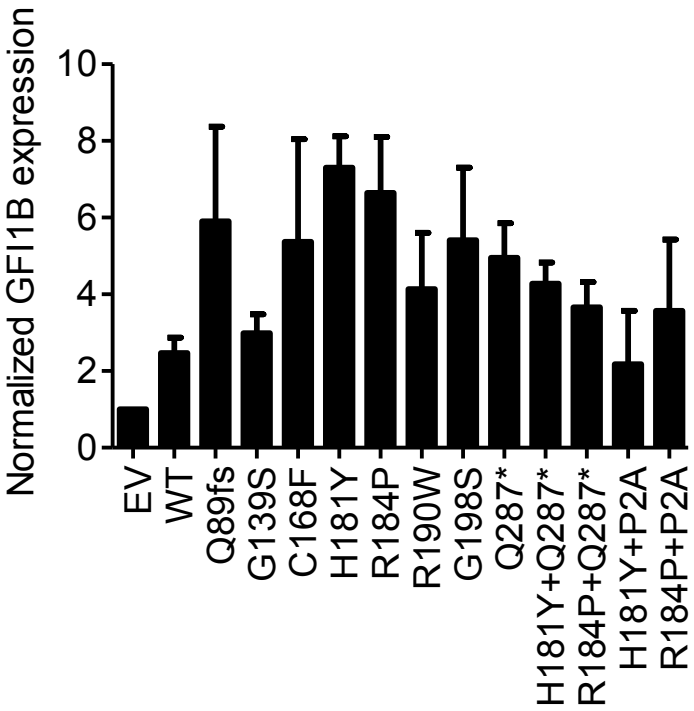

Figure S2. Expression of GFI1B in MEG-01 expansion cultures. GFI1B expression in FACS-sorted GFP positive MEG-01 cells transduced with empty vector (EV) or GFI1B variant-flag at day 23 following transduction. These data correspond to the cultures in Figures 1 and 2. GFI1B expression is normalized to GAPDH and endogenous GFI1B expression in the empty vector (EV) condition. Error bars represent mean ± standard deviation of at least three experiments.

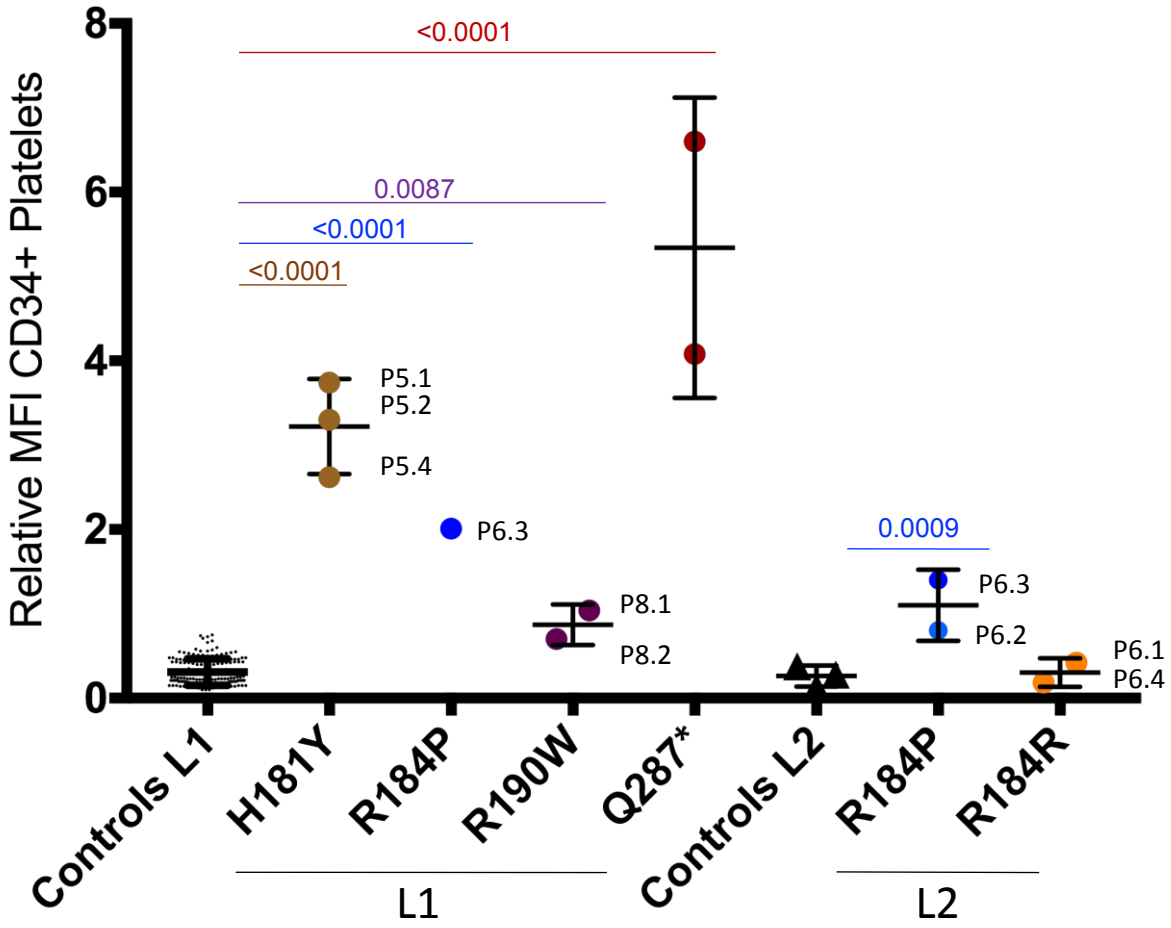

Figure S3. CD34 expression on platelets. Flow cytometry of CD34 expression on patients' platelets carrying GFI1B H181Y, R184P, or R190W variants. Measurements were performed in two laboratories (L1 and L2) using 169 (L1) and three (L2) unrelated healthy individuals as controls (see Table 1). Subjects P6.1 and P6.4 are wild type for GFI1B (R184R) and do not have bleeding symptoms or platelet defects. Error bars represent mean ± standard deviation. P values were determined by one-way ANOVA (Tukey's multiple comparisons test).

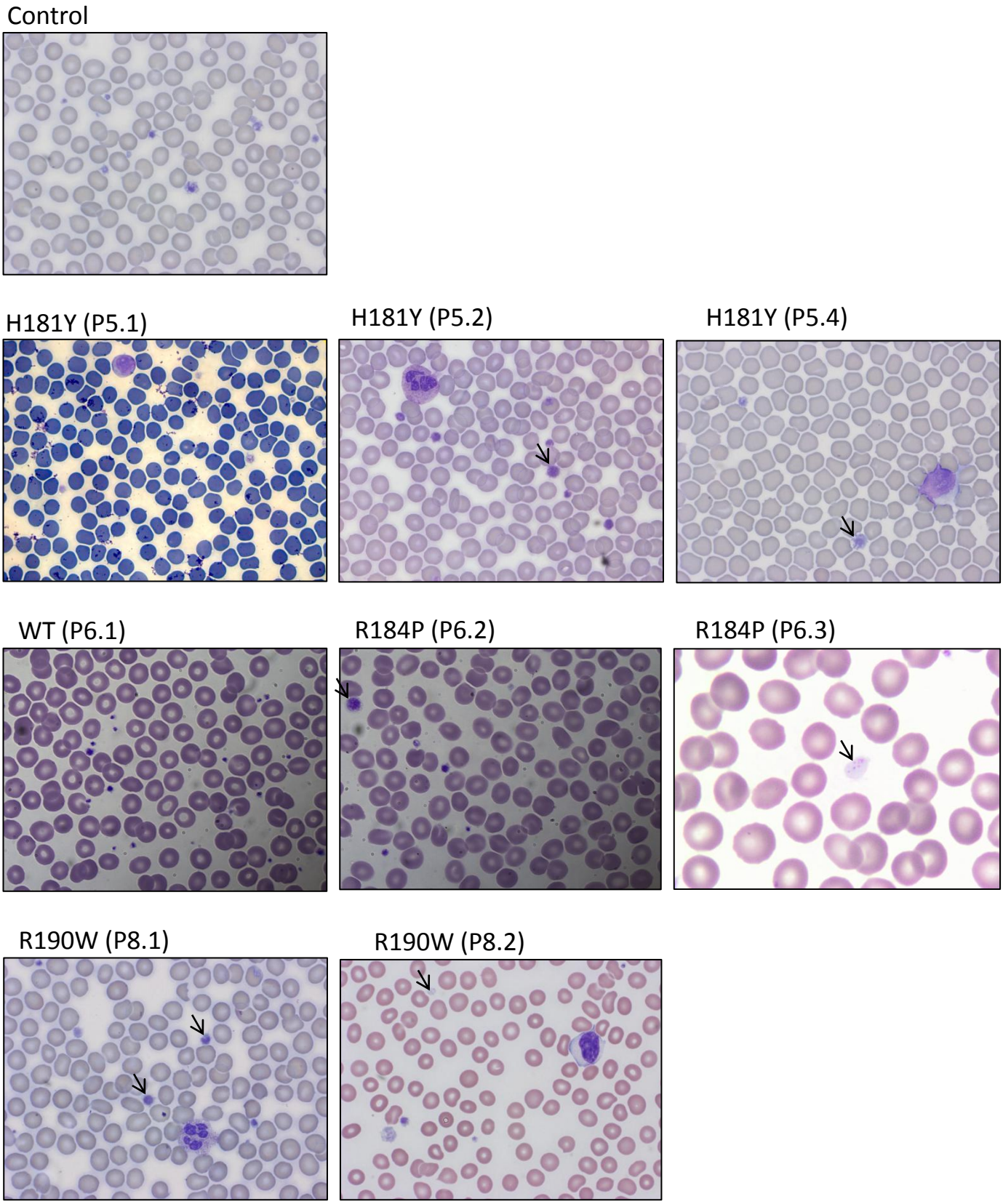

Figure S4. May-Grünwald-Giemsa stained peripheral blood smears of control and patients with the H181Y, R184P, or R190W variant. Overall analysis of the blood smears is presented in Table 1. Representative photos are depicted. Several macrothrombocytes and/or hypogranular platelets are indicated by arrows. Photo P6.3 is a 100x magnification. The remaining photos are a 40x magnification.

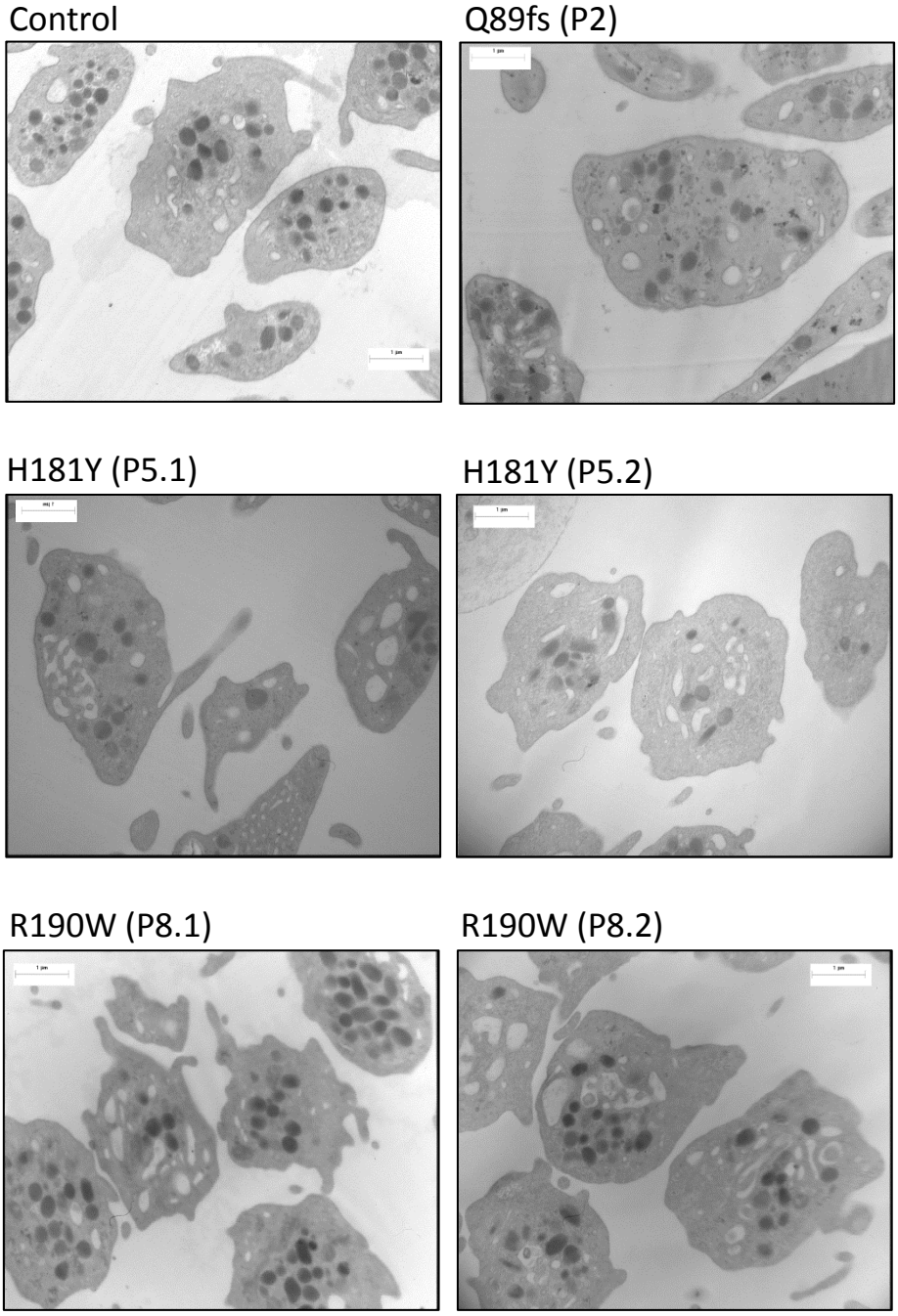

Figure S5. Platelet electron microscopy for control and patients with Q89fs, H181Y, and R190W variants. Platelet area and α- granule number was quantified and reported in Table 1. Representative photos are depicted. α-granule numbers were reduced in P2 (Q89fs), P5.1 (H181Y) and P5.2 (H181Y). Platelet area was in the normal range for all presented cases.

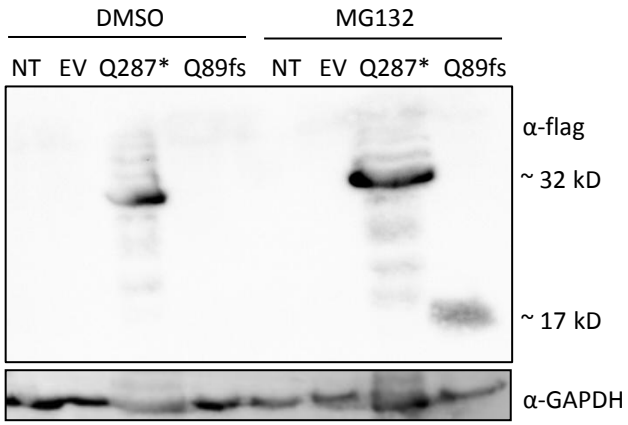

Figure S6. GFI1B Q89fs is only detected after proteasome inhibition treatment. Protein expression of transfected GFI1B-Q287*-flag (~32kD) and GFI1B-Q89fs-flag (~17kD) in HEK293FT cells 24 hour after treatment with 5µM MG132 or DMSO (solvent control). PVDF membrane was stained with α-flag and α-GAPDH antibodies.

References

1. Chen L, Kostadima M, Martens JH, et al. Transcriptional diversity during lineage commitment of human blood progenitors. Science. 2014;345(6204):1251033.
2. Di Michele M, Thys C, Waelkens E, et al. An integrated proteomics and genomics analysis to unravel a heterogeneous platelet secretion defect. J Proteomics. 2011;74(6):902-913.
3. Rabbolini DJ, Morel-Kopp MC, Chen Q, et al. Thrombocytopenia and CD34 expression is decoupled from alpha-granule deficiency with mutation of the first growth factor-independent 1B zinc finger. J Thromb Haemost. 2017;15(11):2245-2258.
4. Uchiyama Y, Ogawa Y, Kunishima S, et al. A novel GFI1B mutation at the first zinc finger domain causes congenital macrothrombocytopenia. British journal of haematology. 2017;
5. Schulze H, Schlagenhauf A, Manukjan G, et al. Recessive grey platelet-like syndrome with unaffected erythropoiesis in the absence of the Splice Isoform GFI1B-p37. Haematologica. 2017;102(9):102(109):e375-e378.
6. Ferreira CR, Chen D, Abraham SM, et al. Combined alpha-delta platelet storage pool deficiency is associated with mutations in GFI1B. Molecular genetics and metabolism. 2017;120(3):288-294.
7. Kitamura K, Okuno Y, Yoshida K, et al. Functional characterization of a novel GFI1B mutation causing congenital macrothrombocytopenia. J Thromb Haemost. 2016;14(7):1462-1469.
8. Monteferrario D, Bolar NA, Marneth AE, et al. A dominant-negative GFI1B mutation in the gray platelet syndrome. The New England journal of medicine. 2014;370(3):245-253.
9. Stevenson WS, Morel-Kopp MC, Chen Q, et al. GFI1B mutation causes a bleeding disorder with abnormal platelet function. J Thromb Haemost. 2013;11(11):2039-2047.