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

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Several types of *GFI1B* variants have been identified in patients with inherited bleeding and platelet disorders. This includes dominant-negative truncating variants affecting DNA binding,[1-4] missense variants of which the molecular mechanism is unclear,[5-7] and variants changing the amount and ratio of GFI1B isoforms (Figure S1).[7, 8] The severity of the bleeding disorder may differ depending on the type of variant, but frequent abnormalities include macrothrombocytopenia, a reduction in α-granule numbers, and platelet CD34 expression. In this study we performed a molecular and/or clinicopathological characterization of eight GFI1B variants in non-DNA binding domains (Figure S1). These variants were previously identified by the NIHR BioResource rare disease study in cases with an assumed inherited bleeding or platelet disorder.[9] Molecular characterization was not performed for D23N, since the minor allele frequency in the gnomAD database deemed too high for a causal variant. From the characterization of the other variants we can conclude that although some have a clear effect on GFI1B function, they are not necessarily sufficient to cause bleedings on their own.

Previously, we used the megakaryoblast cell line MEG-01 to study the effect of GFI1B and the proven pathogenic GFI1B-Q287* variant on cell expansion. In expansion-competition cultures containing transduced and non-transduced cells, MEG-01 cells ectopically expressing GFI1B were overgrown by non-transduced cells, while the opposite was observed following expression of GFI1B-Q287* (Figure 1; manuscript resubmitted September 2018). Thus, forced GFI1B expression inhibits MEG-01 cell expansion whereas dominant-negative GFI1B-Q287* results in enhanced expansion. The latter is in line with elevated megakaryocyte numbers observed in a bone marrow specimen of a GFI1B p.Q287* affected individual.[1] To investigate the (functional) effect of GFI1B variants, we retrovirally expressed them in MEG-01 cells and performed the expansion-competition culture described above. GFI1B and GFI1B-Q287* were taken along as references. Two variants, one in the intermediate domain (G139S) and one in zinc finger (znf) 2 (G198S), did not affect the inhibitory function of wild type GFI1B on MEG-01 proliferation (Figure 1A, Figure 1B). The R190W variant, located between znf1 and znf2, rendered the protein less effective at inhibiting MEG-01 proliferation (Figure 1C), whilst both the znf1 variant C168F and the truncated variant Q89fs rendered the protein completely inactive (Figure 1D, Figure 1E). Interestingly, expression of znf1 H181Y and R184P variants resulted in increased MEG-01 cell proliferation, although to a lesser extent than cells expressing GFI1B-Q287* (Figure 1F, Figure 1G). To further study H181Y and R184P, we introduced these variants separately in GFI1B-Q287*. This led to partial inhibition of the growth stimulating effect of GFI1B-Q287* (Figure 2A, Figure 2B), indicating that amino acids H181 and R184 are important for the effect of GFI1B-Q287* on MEG-01 proliferation. These findings clearly demonstrate that different variants have qualitatively distinct effects on the function of GFI1B, and that znf1 is important in regulating MEG-01 proliferation.

The increased MEG-01 expansion caused by GFI1B-H181Y and GFI1B-R184P suggests that these variants, like GFI1B-Q287*, act in a dominant-negative manner. However, the molecular mechanism might be different, because these variants are not located in the DNA binding domain like GFI1B-Q287*. GFI1B is a repressive transcription factor that inhibits its own transcription and that of its paralogue *GFI1*.[10, 11] GFI1B-Q287* has lost this repressive function.[1] To study if the variants affect the repressive function of GFI1B, we performed gene reporter assays using the *Gfi1* promoter. Remarkably, all tested GFI1B missense variants, including GFI1B-H181Y and GFI1B-R184P, repressed the *Gfi1* promoter to a similar extent as wild type GFI1B (Figure 2C). However, results obtained in transient gene repression assays may not reflect effects on endogenous target genes. We therefore analyzed the effects of GFI1B-H181Y and GFI1B-R184P on endogenous *GFI1B* expression. Wild type GFI1B, GFI1B-Q287*, GFI1B-H181Y, and GFI1B-R184P were expressed in MEG-01 cells, followed by

endogenous *GFI1B* mRNA expression analysis. In line with earlier reports, wild type GFI1B inhibited endogenous *GFI1B* expression.[12] In contrast, GFI1B-Q287*, as well as GFI1B-H181Y and GFI1B-R184P did not repress endogenous *GFI1B* expression to the same extent as wild type GFI1B (Figure 2D). This indicates that not only the DNA binding znfs, but also amino acids H181 and R184 are required for efficient repression of endogenous *GFI1B*.

The LSD1-RCOR1-HDAC co-repressor complex is one of the main epigenetic regulatory complexes recruited by GFI1B to induce transcriptional repression. To study whether GFI1B-H181Y- and GFI1B-R184P-induced MEG-01 expansion depends on an interaction with this complex, we co-introduced a P2A mutation in the GFI1B-H181Y or GFI1B-R184P variants. The P2A mutation in the N-terminal SNAG domain of GFI1B abrogates its interaction with LSD1,[13] and nullifies the inhibitory effect of wild type GFI1B and stimulatory effect of GFI1B-Q287* on MEG-01 proliferation (manuscript resubmitted September 2018). Expression of the P2A-H181Y and P2A-R184P double mutants resulted in expansion rates similar to empty vector transduced cells (Figure 2E). This strongly suggests that H181Y and R184P variants require the LSD1 interaction to exert their effect on MEG-01 expansion.

The functional data were subsequently correlated with clinical and laboratory features of patient samples to improve classification of the *GFI1B* variants according to ACMG guidelines[14] (Table S1). A minimal set of genetic, clinical and laboratory features have already been published in Chen *et al.*, supplementary table ST15.[9] For this study, we expanded clinical and laboratory phenotype studies for the H181Y and R184P variants, because these GFI1B variants had similar functional effects in the MEG-01 cell models as the proven pathogenic GFI1B-Q287* variant. In addition, we performed clinical and laboratory phenotype studies for R190W variant carriers.

The variants G139S and G198S were classified as 'Benign' as they showed similar inhibition of MEG-01 expansion as wild type GFI1B, and have a relatively high minor allele frequency in gnomAD. Further, the thrombocytopenia in patient P9 with G198S was explained by a pathogenic ACTN1 variant (p.R46Q)[15]. Variants R190W, C168F and Q89fs did not inhibit MEG-01 expansion to the same extent as wild type GFI1B (loss of function effect). R190W platelets were weakly CD34-positive, but R190W in patients P8.1 and P8.2 did not co-segregate with bleeding or result in abnormal α-granules (Table S1; Figures S3-5). Moreover, patient P7 with the same R190W variant was explained by a pathogenic variant in WAS (p.R364*), resulting in a 'Benign' classification for R190W. Patient P4 with a homozygous C168F variant suffered from clinical bleeding symptoms with thrombocytopenia and platelet aggregation dysfunction. Unlike P4, heterozygous C168F patients studied by Rabbolini and colleagues only displayed macrothrombocytopenia with platelet CD34 expression (partial effect on the phenotype).[7] C168F is predicted to disrupt znf1 structure and thereby GFI1B function.[9] This was confirmed in functional experiments performed here (Figure 1D) and by Rabbolini *et al*. showing that C168F disrupts the repressive function of GFI1B gene expression.[7] C168F was classified as a 'variant of unknown significance' (VUS); further studies in the affected patient or of family members was not possible. A 90-year old woman (deceased) carrying the Q89fs variant and without affected siblings had mild thrombocytopenia with bleeding, platelet dysfunction and significantly reduced α-granule numbers; a phenotype very similar to previously described *GFI1B* pathogenic variants (Table S1; Figure S5).[1, 2, 4] The Q89fs variant does not repress the *Gfi1* promoter to the same degree as wild type GFI1B and the missense variants. However, it must be noted that we could only detect the truncated protein after proteasome inhibition, suggesting it is instable (Figure S6). If this is also the case in patient cells, the Q89fs variant would lead to haploinsufficiency. This variant was classified as VUS.

The R184P and H181Y variants stimulated MEG-01 proliferation and failed to repress endogenous *GFI1B* expression in a similar way as the pathogenic Q287* variant. These missense variants were absent from the gnomAD database and co-segregation studies were performed (Figure 3). Both the propositus (P6.1) and her father (P6.2), who are carriers of R184P, showed a small number of hypogranular platelets and platelet CD34 expression (Table S1; Figures S3-4). P6.1 had a normal platelet count whereas her father (P6.2) had mild thrombocytopenia. Importantly, neither parent had clinical bleeding symptoms or platelet dysfunction (Table S1; Figure 3A). Following ACMG criteria, the R184P variant was classified as VUS. For the propositus (P5.1) with the H181Y variant, three affected relatives (P5.2, P5.4-5) and one non-affected (P5.3) relative were screened and the variant co-segregated with clinical bleeding symptoms, platelet dysfunction and CD34-positive platelets (Table S1; Figure S3; Figure 3B). Affected individuals P5.1 and P5.2 had normal platelet counts with few large platelets and a significant reduction of α-granules (Table S1; Figures S4-5). The functional and segregation data suggest that the H181Y variant is causal of bleeding and platelet dysfunction but does not result in thrombocytopenia. Following ACMG guidelines, H181Y was classified as a VUS (Table S1).

We conclude Q89fs, C168F, H181Y, and R184P affect GFI1B function, but are not necessarily sufficient to cause bleedings on their own. Still, their identification and documentation, even when classified as VUS, will help to distinguish pathological from non-pathological *GFI1B* variants and increase our understanding of GFI1B functional domains. The identification of additional patients with similar variants will be essential to clarify their exact role for platelet phenotypes and bleeding.

Authorship

Contribution: R.O., A.E.M., S.M.B., and M.G.J.M.B performed *in vitro* experiments; K.F., W.N.E., H.M. and K.D. performed EM, blood smears and CD34 expression measurements; Patients were followed by K.P., C.L., C.M.M., S.K.W., R.F., and W.H.O.; E.T. and W.H.O. analyzed and coordinated the genetic studies; R.O., A.E.M., and K.F. analyzed results and made the figures; R.O., A.E.M., B.A.R., J.H.J., and K.F. designed the research and wrote the paper; All authors have read and agreed to the contents of the paper.

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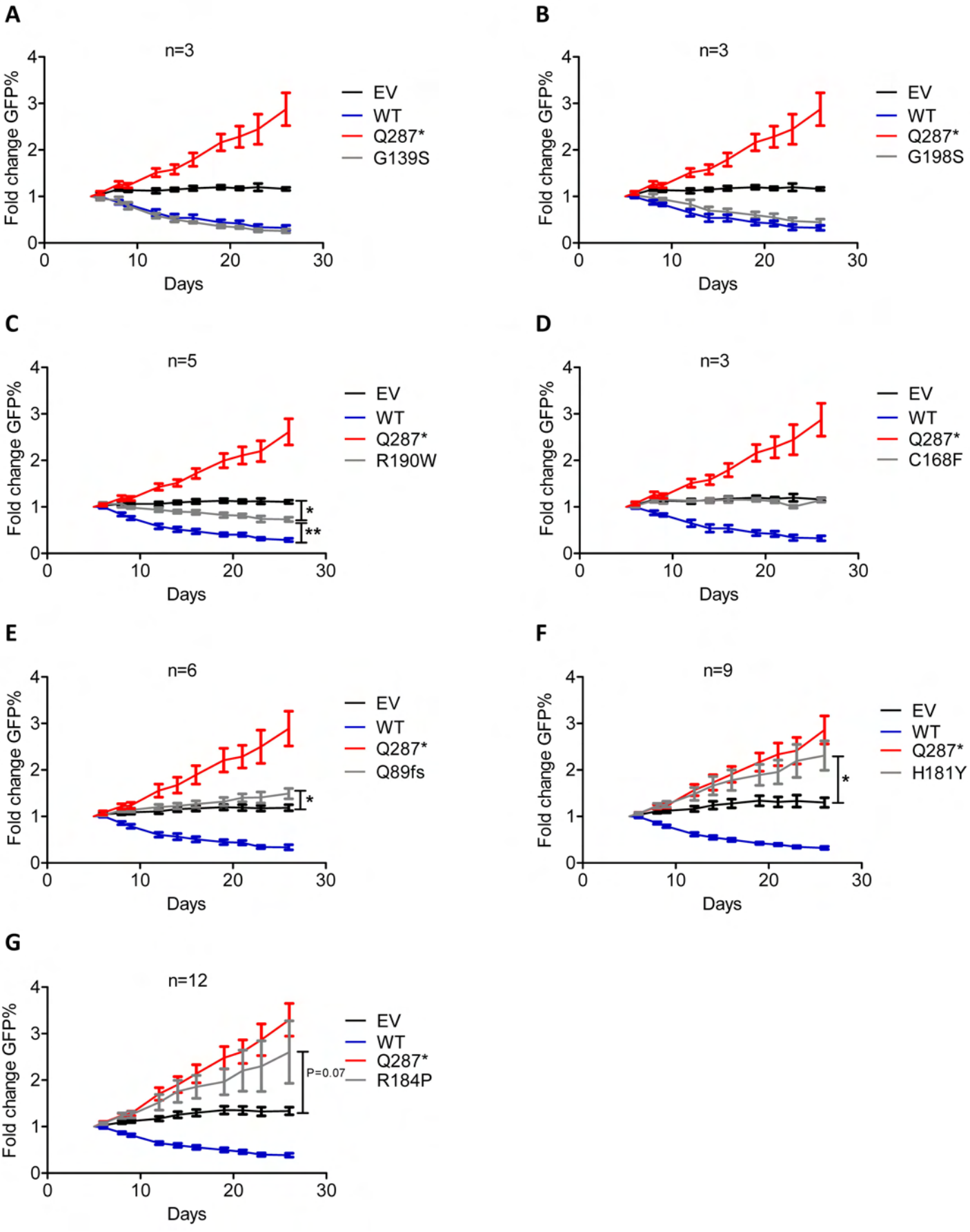
Figure legends

Figure 1. GFI1B variants have different effects on GFI1B function. Expansion competition cultures of MEG-01 cells transduced with flag-tagged GFI1B variants (A) G139S (B) G198S (C) R190W (D) C168F (E) Q89fs (F) H181Y (G) R184P. GFI1B-Q287*-flag, GFI1B-p37-flag wild type (WT), and empty vector (EV) were taken along as controls. Fold change of GFP% to GFP% at day 5 (first GFP measurement) is presented on the y-axis. Results show mean ± standard error of the mean, and two-tailed paired t-tests were performed on day 26 to determine statistical significance *P<0.05, **P <0.01. Of note, all MEG-01 transduced cells showed increased GFI1B mRNA expression indicating expression of the retroviral vector (Figure S2).

Figure 2. Functional effect of GFI1B variants H181Y and R184P. (A-B) Expansion competition cultures of MEG-01 cells transduced with flag-tagged GFI1B-H181Y+Q287* (A), or GFI1B-R184P-Q287* (B). Empty vector (EV) and GFI1B-Q287*-flag transduced cells taken along as reference. (C) Dual luciferase reporter assays in HEK293FT cells transfected with Renilla luciferase construct, *Gfi1* promoter Firefly luciferase construct, and empty vector (EV), wild type GFI1B-p37-flag (WT-p37), wild type GFI1B-p32-flag (WT-p32, lacking coding exon 4 and therefore amino acids 171-216 corresponding to zinc finger 1 and 2), or GFI1B-flag variants. Firefly/Renilla luciferase ratios are normalized to EV transfected cells. Results show mean ± standard deviation, and two-tailed paired t-tests were performed to determine statistical significance between WT-p37 and the other conditions. Corresponding Western blots showing expression of the flag-tagged GFI1B proteins and the GAPDH loading control are depicted below the graph. (D) 5'UTR *GFI1B* expression in GFP positive cells from MEG-01 expansion competition cultures, FACS-sorted 23 days after transduction. *GFI1B* expression is normalized to *GAPDH* expression. Results show mean ± standard deviation, and two-tailed paired t-tests were performed to determine statistical significance. (E) Expansion competition cultures of MEG-01 cells transduced with flag-tagged GFI1B-H181Y+P2A, or GFI1B-R184P-P2A. EV transduced cells taken along as reference. *P<0.05, **P <0.01, ***P <0.001

Figure 3. Pedigrees for families harboring GFI1B variants H181Y (A) and R184P (B). The propositus (arrow) and the family members with signs of pathological bleeding are indicated with a black filled symbol. GFI1B variant status, patient identifier, platelet count (PLT), platelet CD34 expression, and the ISTH bleeding assessment tool (BAT) score are indicated for each patient. Normal range for the ISTH BAT score is <4 in adult males, <6 in adult females and <3 in children. P5.4 has less haemostatic challenges than the other siblings. ND= not determined. Additional clinical and laboratory data obtained in patient samples can be found in Table S1.

Figure 1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WT
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Days
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G198S
C
n=5
Fold change GFP%
Days
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WT
Q287*
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*
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Q287*
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E
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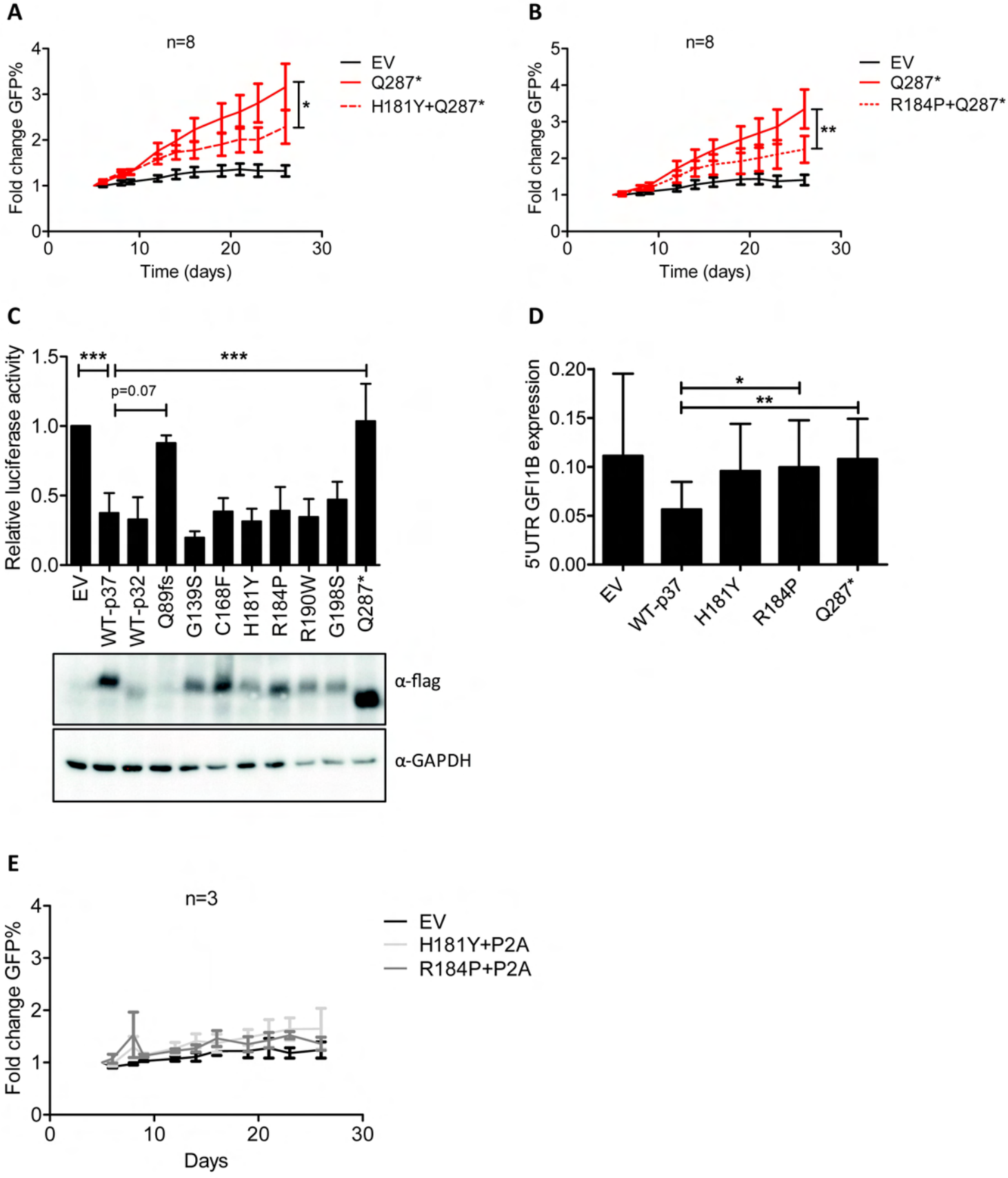
Figure 2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Fold change GFP%
Time (days)
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Q287*
R184P+Q287*
**
C
Relative luciferase activity
***
***
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WT-p32
Q89fs
G139S
C168F
H181Y
R184P
R190W
G198S
Q287*
α-flag
α-GAPDH
D
5'UTR GFI1B expression
*
**
EV
WT-p37
H181Y
R184P
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E
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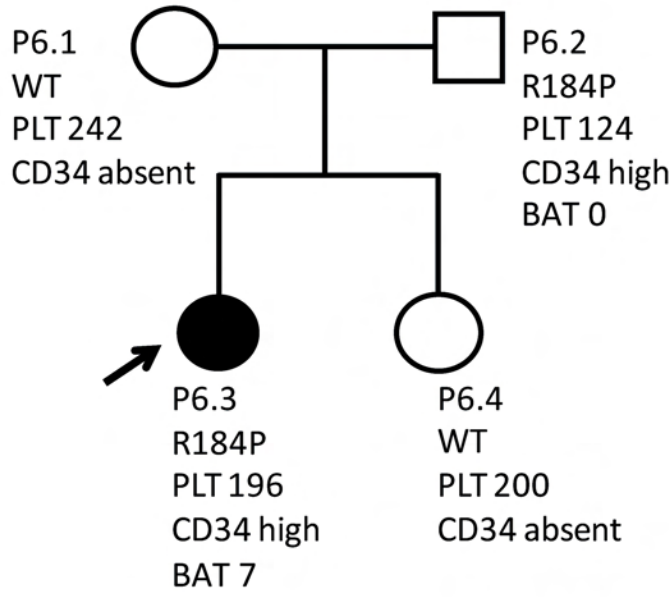
Fold change GFP%
Days
EV
H181Y+P2A
R184P+P2A

Figure 3

A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P6.2
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PLT 124
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BAT 0
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BAT 7
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WT
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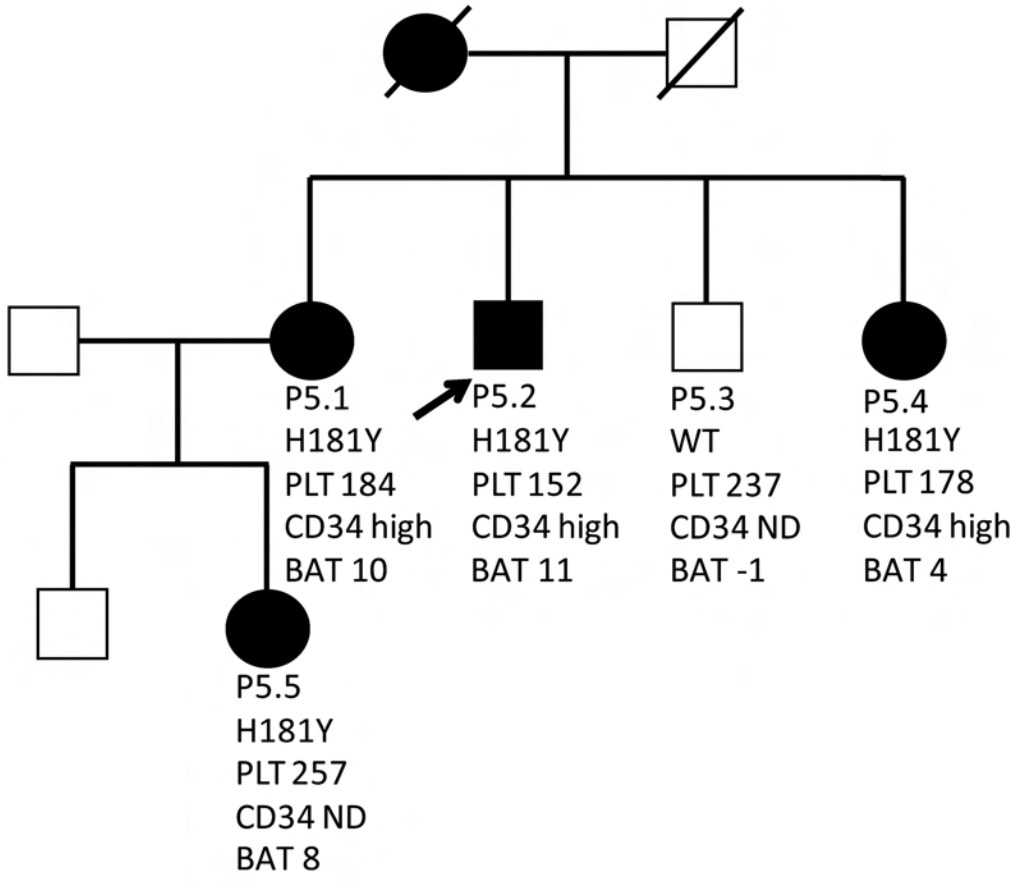
CD34 absent

B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CD34 high
BAT 11
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CD34 ND
BAT -1
P5.4
H181Y
PLT 178
CD34 high
BAT 4
P5.5
H181Y
PLT 257
CD34 ND
BAT 8

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

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)*
B200037	P1 (female)	D23N	401 - 5/274546	Yes	408	ND	ND	ND	ND	ND	Abnormal for Epinephrine only.	No	BS1: Benign
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
B200721	P3 female)	G139S	29 - 0/277110	Yes	81	9.7	ND	ND	ND	ND	ND	No	BS1, BS3: Benign
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
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	
sibling	P6.4 (female)	Normal		No	200	11.1	Normal	Normal	ND	ND	Normal		
B200239	P7 (male)	R190W	33 - 0/246058	Yes	44	8.3	ND	ND	ND	ND	ND	Confirmed WAS defect: p.Arg364Ter	BS1, BP5: Benign
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	
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 3
(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.

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

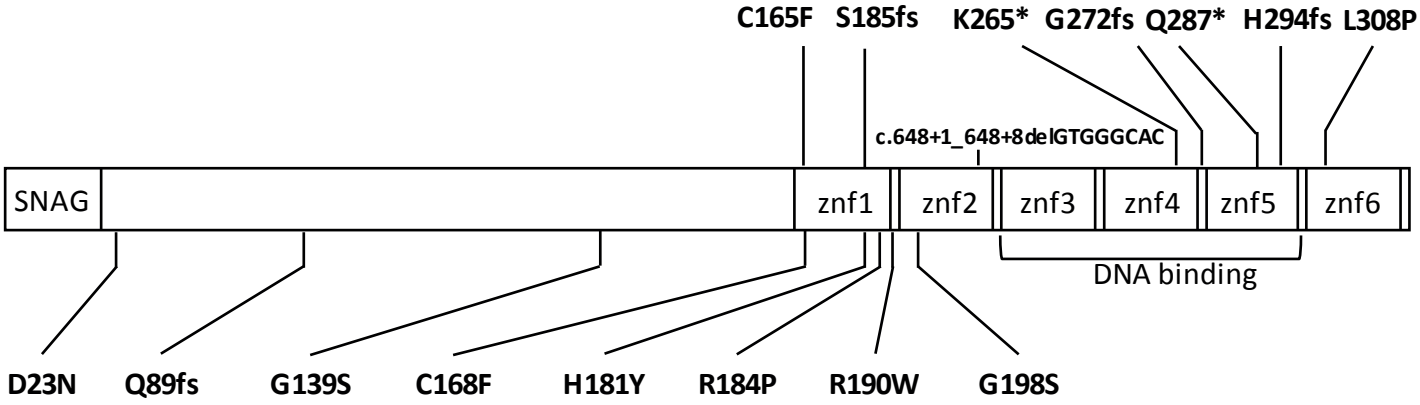

Figure S1. Overview of reported GFI1B variants. Schematic overview of GFI1B protein structure with variants identified in inherited bleeding and platelet disorders. The variants displayed below are analyzed in this study (C168F also identified in another study[1]). The upper variants have been published 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.

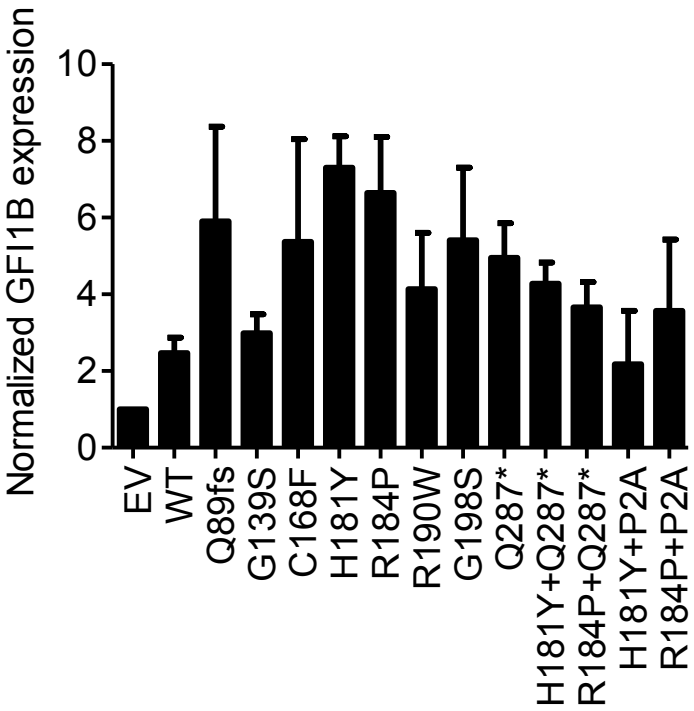

Figure S2. Expression of GFI1B in MEG-01 expansion cultures. GFI1B expression in FACS-sorted GFP positive MEG-01 cells transduced with empty vector (EV) or GFI1B variant-flag at day 23 following transduction. These data correspond to the cultures in Figures 1 and 2. GFI1B expression is normalized to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

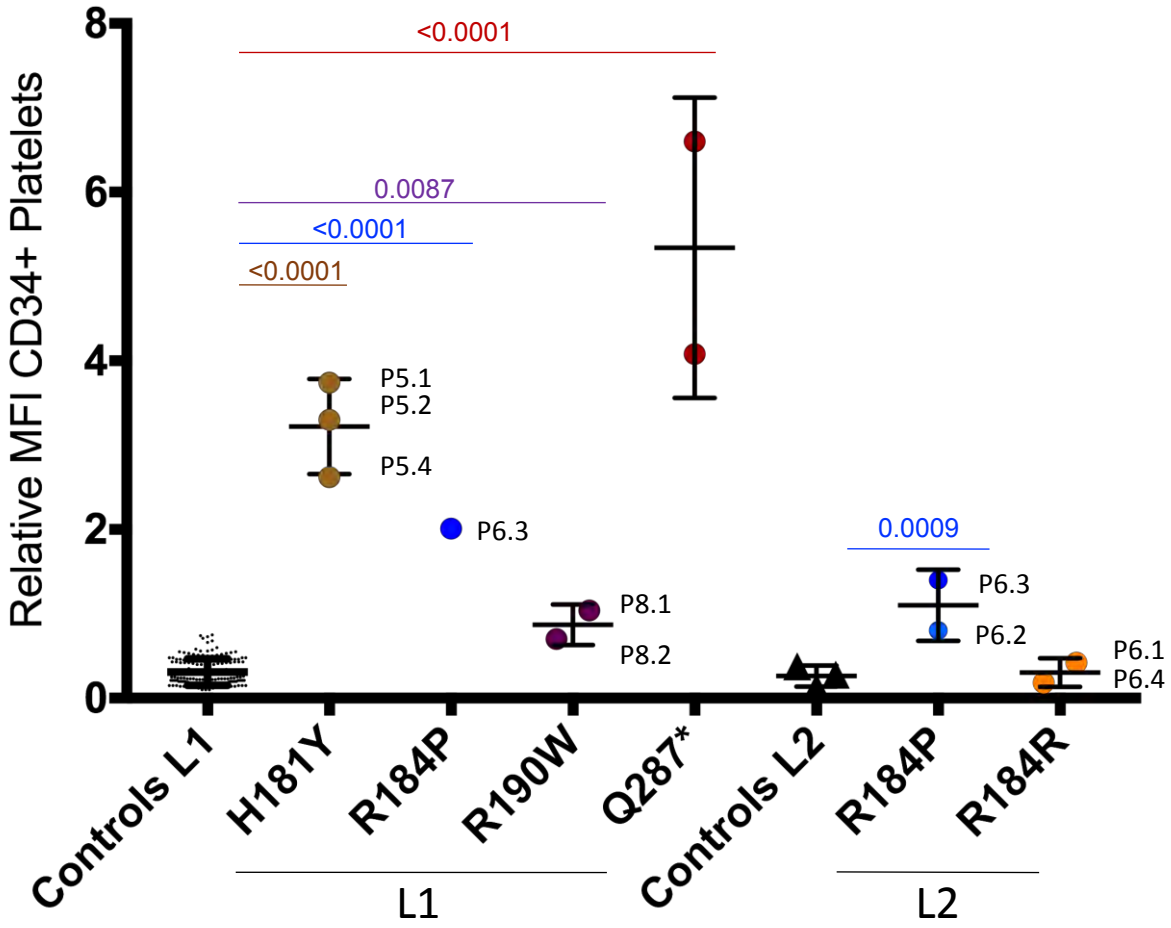

Figure S3. CD34 expression on platelets. Flow cytometry of CD34 expression on patients' platelets carrying GFI1B H181Y, R184P, or R190W variants. Measurements were performed in two laboratories (L1 and L2) using 169 (L1) and three (L2) unrelated healthy individuals as controls (see Table 1). Subjects 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).

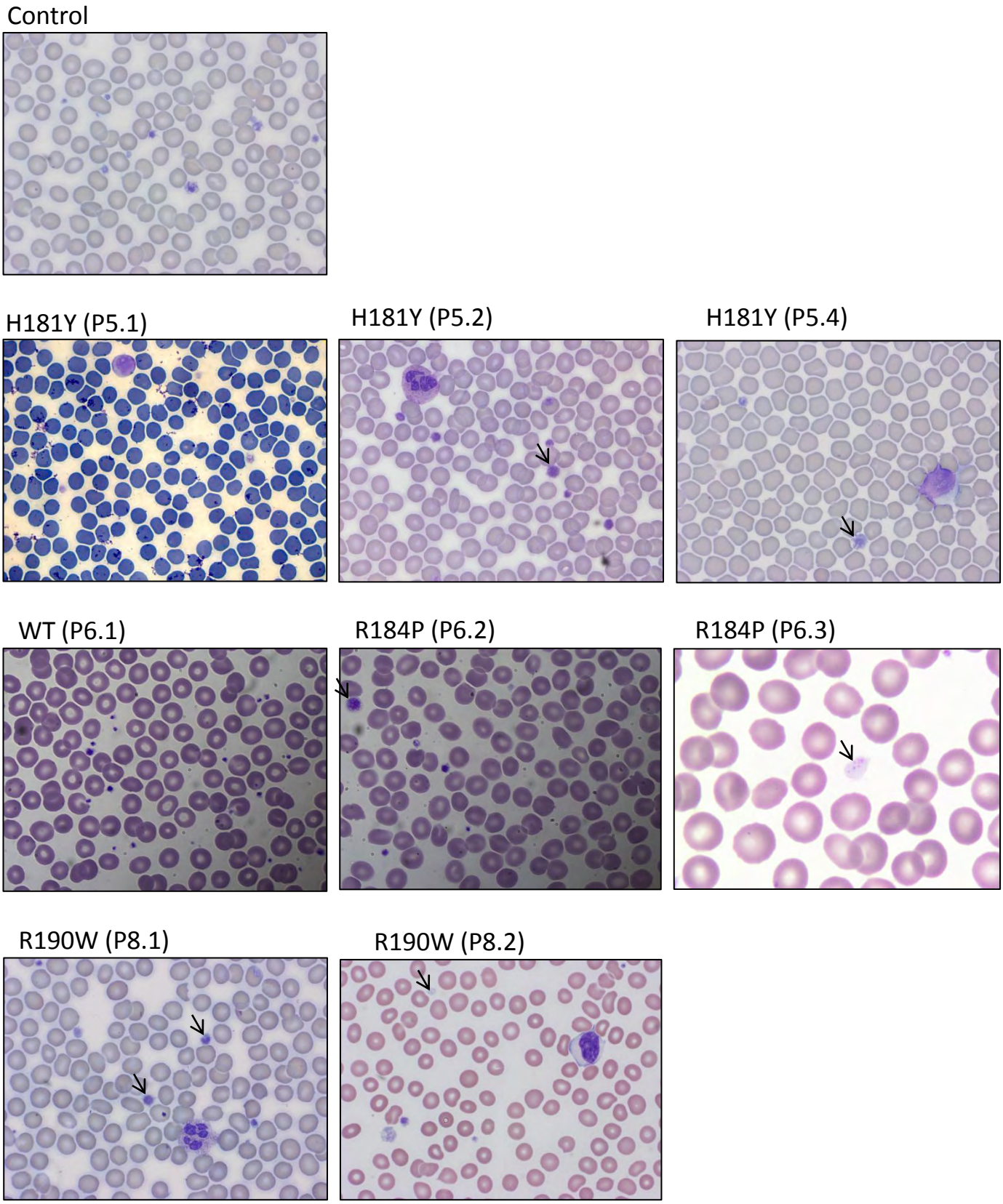

Figure S4. May-Grünwald-Giemsa stained peripheral blood smears of control and patients with the H181Y, R184P, or R190W variant. Overall analysis of the blood smears is presented in Table 1. Representative photos are depicted. Several macrothrombocytes and/or hypogranular platelets are indicated by arrows.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

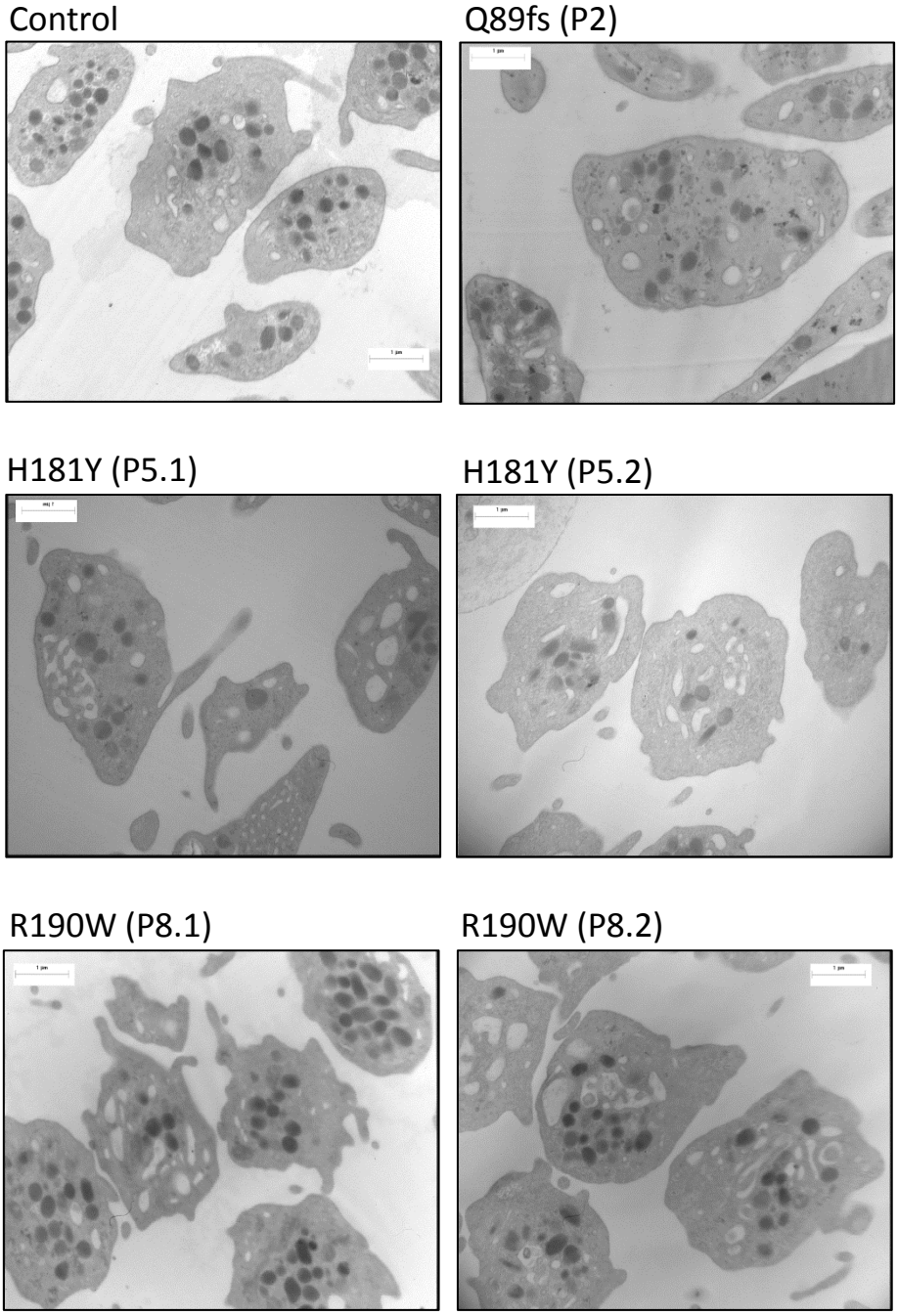

Figure S5. Platelet electron microscopy for control and patients with Q89fs, H181Y, and R190W variants. Platelet area and α- granule number was quantified and reported in Table 1. Representative photos are depicted. α-granule numbers were reduced in P2 (Q89fs), P5.1 (H181Y) and P5.2 (H181Y). Platelet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

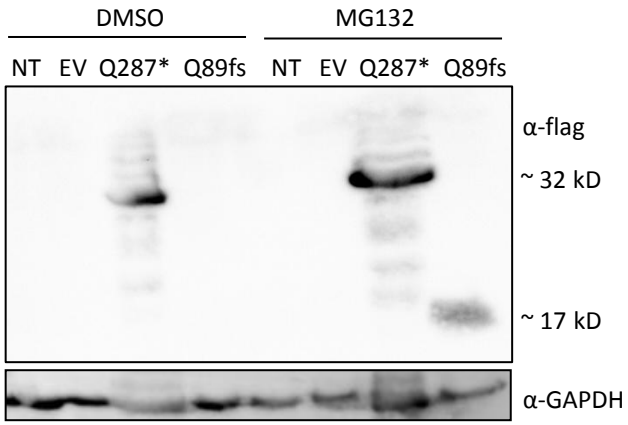

Figure S6. GFI1B Q89fs is only detected after proteasome inhibition treatment. Protein expression of transfected GFI1B-Q287*-flag (~32kD) and GFI1B-Q89fs-flag (~17kD) in HEK293FT cells 24 hour after treatment with 5µM MG132 or DMSO (solvent control). PVDF membrane was stained with α-flag and α-GAPDH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

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