Recessive grey platelet-like syndrome with unaffected erythropoiesis in the absence of the splice isoform GFI1B-p37

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Histomorphology and Immunofluorescence

Blood smears were fixed with ice cold acetone (0°C; 2 - 5 min) and covered with rabbit antibodies against CD34 (Clone QBEnd/10, Immunologic, Duiven, Netherlands), von Willebrand factor (VWF) (Clone A0082, DAKO, Glostrup, Denmark), or P-selectin (CD62P) (AK4, BD, Franklin Lakes, USA). Targets were visualized using a secondary fluorescence-labelled antibody. The bone marrow biopsy was performed according to a modified Hammersmith protocol[1]. The primary antibodies were directed against CD34 (BD, Franklin Lakes, USA), CD36 and CD41 (Proteintech, Rosemont, USA), CD42b, or CD61 (LabVision Corporation Fremont, USA) and GFI1B (Abcam Plc, Cambridge, UK). Immunoblot analyses were performed with corresponding secondary antibodies on a PATH FLX automated staining machine (Biocare Medical-intelli Automated Slide Staining System, USA).

Targeted panel-based next-generation sequencing

Initial mutation analysis was performed by targeted, panel-based next-generation sequencing (NGS) comprising exons of 59 genes known to have a role in platelet biogenesis or function (list available upon request) including selected regulatory sequences. Target enrichment was performed using Nextera Rapid Capture Enrichment, and sequencing was performed on the MiSeq platform (Illumina, San Diego, CA, USA). 98% of the target sequences had a 20-fold coverage. The generated bam files were analyzed using GensearchNGS software (PhenoSystems SA, Wallonia, Belgium). Filtering criteria included coverage >20 reads, variant frequency >20 %, minor allele frequency <0.01 and selected for mutation types such

as missense, nonsense, or splice variants. The segregation of the detected variant was confirmed by Sanger sequencing.

RNA isolation and quantitative PCR

Platelet-rich plasma (PRP) was obtained from citrated whole blood by centrifugation at room temperature (100 x g, 10 min). The supernatant was re-centrifuged three times (100 x g, 10 min) for depletion of red blood cells. White blood cell depletion was achieved using CD45 magnetic beads (DynaBeads, Invitrogen, Carlsbad, CA; 25 µl/ml PRP). After solvent removal, beads were incubated with PRP under rotation for 20 min at room temperature. Beads were removed from the PRP with a magnet, and platelets were pelleted by centrifugation (1500 x g, 10 min). Platelets were placed on ice and disrupted in RLT buffer using repetitive ultrasonic bursts (6 x 15 s). RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantitative (q)PCR was performed following cDNA synthesis using the High Capacity cDNA reverse transcription kit (ThermoFisher Scientific, Waltham, MA, USA), conducted according to manufacturer's recommendations. For real-time qPCR, the Power SYBR® Green PCR Master Mix (ThermoFisher Scientific) was used following the standard protocol on a 7500 Fast Real-Time PCR System (ThermoFisher Scientific). QPCR-Primer sequences are as follows: GAPDH: fw 5'-AAG GTG AAG GTC GGA GTC AA-3'; rev 5'-AAT GAA GGG GTC ATT GAT GG-3'; GFI1B: fw 5'-CAG CAC TGA GCC CGC CTT GGA CTT-3', p32/37_rev 5'-GTG GGT GGA CAG CGT GGA CGA GCG-3'; p37_rev 5'-CGC ACA TGC ACT TCG AGC CCG TGA; p32_rev 5'-TCG AAG CTG CGC TCC TTG TTG CAC-3'. Three different healthy controls were analyzed in parallel while three independent measurements were made of the index patient.

Measurement of platelet aggregation and secretion

Light transmission aggregometry (LTA) was performed on a PAP4 analyzer (Biodata Corp., Horsham, PA). Aggregation profiles were only obtained if the platelet counts in platelet-rich plasma were higher than 100 x 10^9/L. PRP with giant platelets was obtained after incubation of blood samples for one hour, resulting in auto-sedimentation of red blood cells. Autologous platelet-poor plasma was prepared as a transmission reference by centrifugation of whole blood at room temperature (1500 x g for 15 min). Aggregation was triggered by addition of one of the following agonists in final concentrations: ADP (10 µM), arachidonic acid (0.5 mM), collagen (2 µg/ml), or TRAP-6 (10 µM). Flow cytometric analyses of platelet function were performed by examining mepacrine uptake and secretion as described previously[2].

Supplementary References

1. Naresh KN, Lampert I, Hasserjian R, et al. Optimal processing of bone marrow trephine biopsy: the Hammersmith Protocol. *J Clin Pathol*. 2006;59(9):903-911.

2. Wall JE, Buijs-Wilts M, Arnold JT, et al. A flow cytometric assay using mepacrine for study of uptake and release of platelet dense granule contents. *Br J Haematol*. 1995;89(2):380-385.

Supplementary Table 1. Laboratory blood cell analyses in a GFI1B-p37-deficient family.

(age and gender-specific normal ranges), pathologic values in bold	Index patient (homozygous) II.3	Daughter (homozygous) III.4	Son (homozygous) III.5	Husband (heterozygous) II.4	Nephew (heterozygous) III.1	Nephew (heterozygous) III.2
Complete blood counts						
WBC G/L	9.76 (4.4-11.3)	6.88 (4.5-12.0)	10.85 (4.5-12.0)	5.1 (4.4-11.3)	6.77 (4.4-11.3)	4.39 (4.4-11.3)
RBC T/L	4.59 (4.1-5.1)	4.73 (4.0-5.3)	4.40 (4.0-5.3)	4.57 (4.5-5.9)	5.34 (4.5-5.9)	4.89 (4.5-5.9)
Hb g/dl	13.5 (12.0-15.3)	13.8 (11.8-15.5)	13.0 (11.8-15.5)	13.5 (13.0-17.5)	14.4 (13.0-17.5)	13.8 (13.0-17.5)
Hct %	41.6 (35-45)	40.0 (37-49)	37.1 (37-49)	39.0 (40-50)	42.5 (40-50)	**39.4** (40-50)
MCV fl	90.6 (80-98)	84.6 (77-93)	84.3 (77-93)	85.3 (80-98)	**79.6** (80-98)	80.6 (80-98)
MCH pg	29.4 (28-33)	29.3 (28-33)	29.5 (28-33)	29.5 (28-33)	**27.0** (28-33)	28.2 (28-33)
MCHC g/dl	32.5 (33-36)	34.5 (30-36)	35.0 (30-36)	34.6 (33-36)	33.9 (30-36)	35.0 (30-36)
Platelets 10^9/L	**119**$^{\$}$ (140-440)	**44** (140-440)	**23** (140-440)	186 (140-440)	270 (140-440)	195 (140-440)
MPV fl	Error (7-13)*	Error (7-13)*	Error (7-13)*	11.8 (7-13)	11.8 (7-13)	11.2 (7-13)
IPF % (<6)	**35.1**	**7.6**	**6.1**	n.d.	n.d.	n.d.
Reti abs T/L (0.027-0.095)	**0.258**	0.044	0.022	0.034	0.067	**0.097**

(age and gender-specific normal ranges), pathologic values in bold	Index patient (homozygous) II.3	Daughter (homozygous) III.4	Son (homozygous) III.5	Husband (heterozygous) II.4	Nephew (heterozygous) III.1	Nephew (heterozygous) III.2
RDW % (11-16)	**20.8**	15.8	**16.3**	12.7	13.2	12.4
Differential WBC (microscopic)						
Rods % (-6)	0	0	0	0	0	0
Neutr % (50-75)	**39**	**39**	63	48	39	42
Eo % (-5)	**10**	1	**7**	**6**	4	4
Baso % (-1)	0	0	0	2	0	1
Mono % (2-12)	7	5	5	6	8	9
Lymph % (20-40)	**44**	**55**	25	38	**50**	**44**
Cytomorphology (-, absent; +, mildly present; ++, moderately present; +++, extensively present)						
Anisocytosis	**+**	-	-	-	-	-
Poikilocytosis	**++**	+/-	+/-	-	-	-
Howell-Jolly B.	**++**	-	-	-	-	-
Polychromasy	-	-	-	-	-	-
***Giant platelets**	**+++**	**+++**	**+++**	-	-	-

(age and gender-specific normal ranges), pathologic values in bold	Index patient (homozygous) II.3	Daughter (homozygous) III.4	Son (homozygous) III.5	Husband (heterozygous) II.4	Nephew (heterozygous) III.1	Nephew (heterozygous) III.2
Coagulation						
PT % (70-120)	87	76	88	106	93	79
aPTT s (26-36)	33.6	34.6	28.5	26.2	31.2	33.0
Fbg mg/dl (210-400)	253	232	272	212	291	**204**
DDI mg/L (-0.5)	**0.66**	**0.64**	**0.52**	0.41	0.42	<0.17
Immune phenotyping						
WBC n/µL						
CD19+	**234** (300-500)	**256** (300-500)	**155** (700-1300)	**211** (300-500)	**180** (300-500)	**121** (300-500)
CD19+/CD10+	79	47	141	12	11	5
CD10+/CD34+	18	7	26	5	3	3
CD19+/CD34+	35	13	62	5	8	4
CD3+	1591 (1400-2000)	**1236** (1400-2000)	**858** (1800-3000)	**1311** (1400-2000)	1517 (1400-2000)	**1166** (1400-2000)
CD56+/CD3-	**191**	**623**	231	**191**	**303**	213

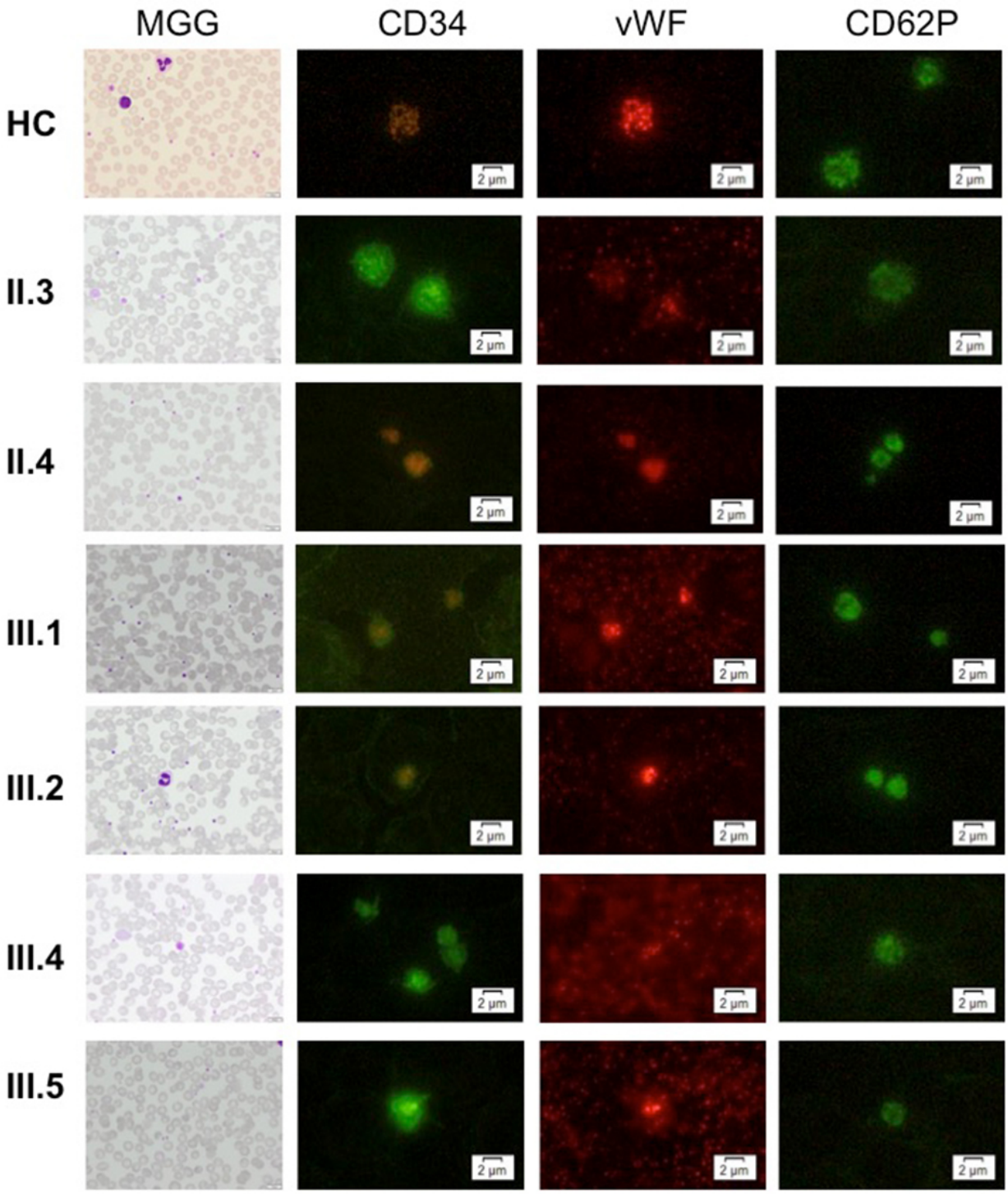
(age and gender-specific normal ranges), pathologic values in bold	Index patient (homozygous) II.3	Daughter (homozygous) III.4	Son (homozygous) III.5	Husband (heterozygous) II.4	Nephew (heterozygous) III.1	Nephew (heterozygous) III.2
	(200-300)	(200-300)	(200-600)	(200-300)	(200-300)	(200-300)
CD34+ # (<5-20)	**295 #**	**50 #**	**64 #**	20	15	5
CD117+	14	17	17	12	6	7
CD33+CD34+#	**157 #**	**45 #**	**49 #**	10	12	11
CD30+CD34+#	**15 #**	**42 #**	**57 #**	4	6	3
Platelet surface receptor expression [% MFI of healthy control]						
Platelets [%]						
GPIIb (CD41) (>80)	94	94	97	99	99	98
GPIIIa (CD61) (>80)	99	99	99	99	99	99
GPIX (CD42a) (>80)	99	98	96	99	99	99
GPIbα (CD42b) (>80)	99	99	99	99	99	99
GPIV (CD36) (>80)	**51**	**53**	**40**	98	98	**77**
GPVI (>80)	**70**	**55**	**41**	95	94	95

(age and gender-specific normal ranges), pathologic values in bold	Index patient (homozygous) II.3	Daughter (homozygous) III.4	Son (homozygous) III.5	Husband (heterozygous) II.4	Nephew (heterozygous) III.1	Nephew (heterozygous) III.2
Platelet activation (CD62P-expression)[§] [%MFI of healthy control]						
Unstimulated (<7)	<1	<1	<1	**10**	4	5
TRAP-6 (>63)	**27**	**31**	**17**	96	97	92
ADP (>42)	**1**	**2**	**3**	**31**	**20**	**24**
Arachidonic acid (>42)	**2**	**2**	**5**	**22**	**15**	**17**
CRP collagen (>28)	**<1**	**1**	**3**	67	48	57
EPI (>18)	**<1**	**1**	**7**	**16**	**9**	**11**
Platelet GPIIb/IIIa activation (PAC-1 binding)[§] [%MFI of healthy control]						
Unstimulated (<7)	<1	<1	<1	2	1	1
TRAP-6 (>23)	**3**	**7**	**8**	38	32	**22**
ADP (>57)	**9**	**8**	**12**	58	**31**	**37**
Arachidonic acid (>55)	**11**	**4**	**7**	**37**	**8**	**14**
CRP collagen (>28)	**1**	**1**	**4**	68	31	46
EPI (>25)	**5**	**2**	**8**	40	**13**	**13**

(age and gender-specific normal ranges), pathologic values in bold	Index patient (homozygous) II.3	Daughter (homozygous) III.4	Son (homozygous) III.5	Husband (heterozygous) II.4	Nephew (heterozygous) III.1	Nephew (heterozygous) III.2
Mepacrine uptake and secretion						
Mepacrine uptake into alpha granula (>25%)	**17**% (reduced)	**12**% (reduced)	35% (normal)	26% (normal)	32% (normal)	34% (normal)
Mepacrine after activation (<20%)	**14**% (no secretion)	**11**% (no secretion)	**33**% (no secretion)	6% (normal)	4% (normal)	5% (normal)

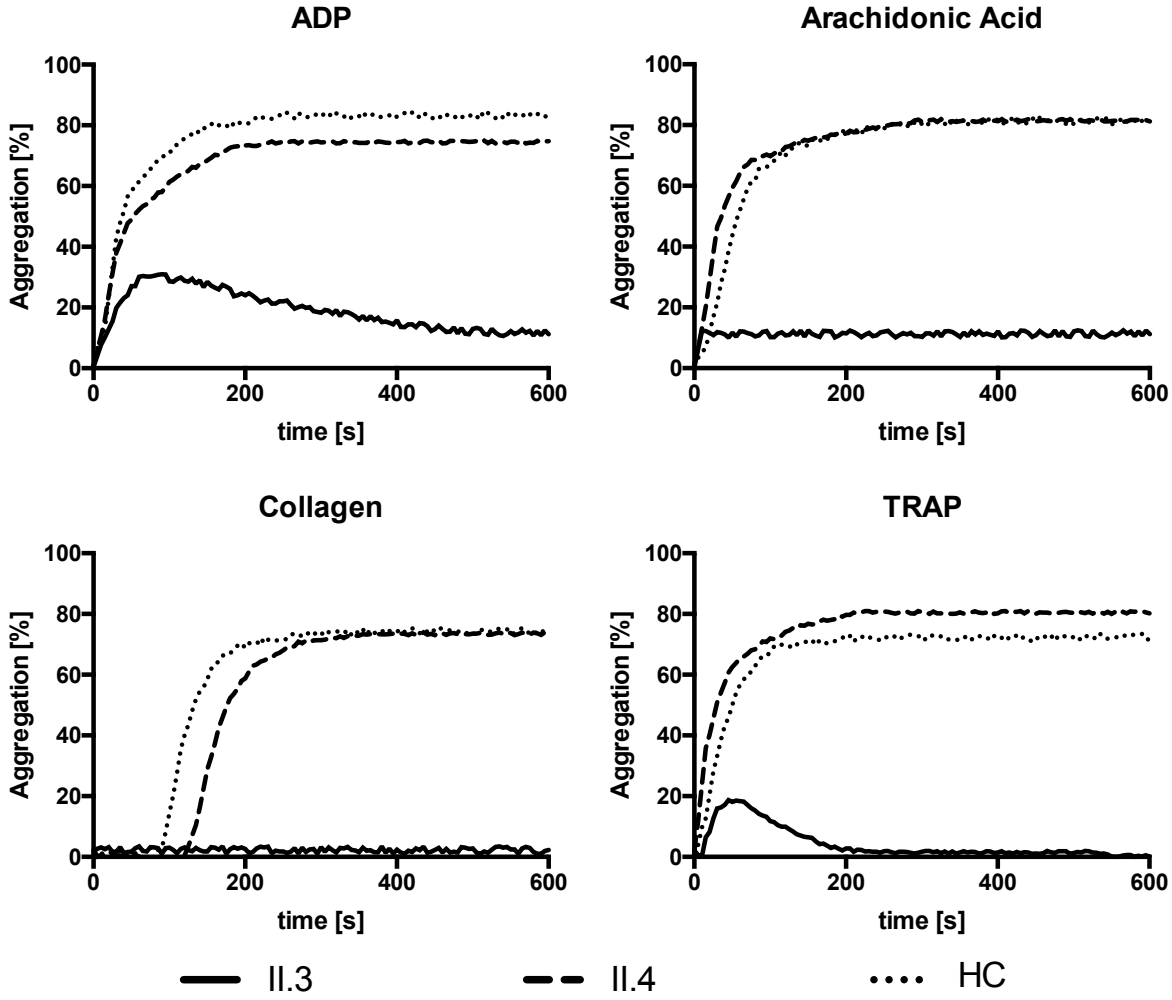
Age and gender-specific normal ranges in brackets, pathologic values in bold.
[$], platelet count of individual II.3 at a time point post-splenectomy; platelet counts were 17-38 G/L before splenectomy on more than 10 occasions between 2010 and 2013;
*, the volume of giant platelets was above the range of automated detection.
[#], a contamination with CD34+ platelets cannot be excluded, although CD34+ cell counts and CD34+ subpopulations were performed after extensive washing to eliminate platelets and gated for CD41-negative cells; no clonal expansion was detectable in any individual.
[§], agonist concentrations for platelet activation: TRAP-6: 14.25 µM, ADP: 1 µM, Arachidonic acid: 80 µM, CRP-collagen: 0.04 µg/ml, EPI: 10 µM.
MFI, mean fluorescence intensity.

Supplementary Figure 1



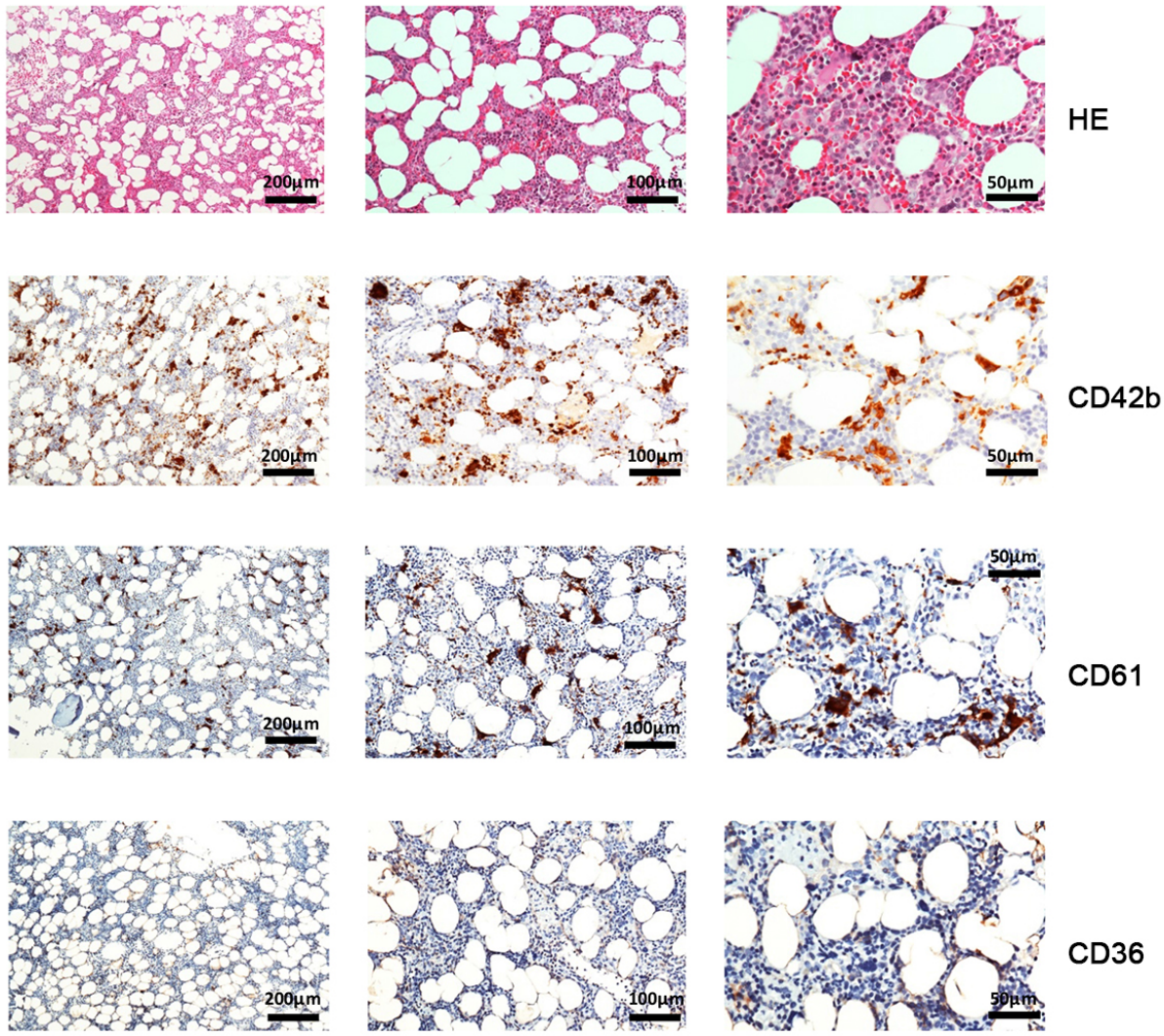
Supplementary Figure 1: Light and immunofluorescence microscopy of platelets: Blood smears were air dried and stained with May-Grünwald-Giemsa (MGG) for light microscopy (left panels) or fixed with ice cold acetone (0°C; 2 - 5 min) and then covered with rabbit antibodies against P-selectisn (CD62P), von Willebrand factor (VWF), or CD34. Appropriate fluorophore-labeled secondary antibodies were added to visualize the target protein. The slides of homozygous patients (II.3, III.4, and III.5) or heterozygous patients (II.4, III.1 and III.2) were compared to those from a healthy control (HC).

Supplementary Figure 2



Supplementary Figure 2. Light transmission aggregometry (LTA) traces induced by various agonists as indicated for patient II.3 (index patient, solid line), subject II.4 (husband, dashed line), and healthy control (HC, dotted line). Data from LTA is depicted in % of platelet poor plasma transmission.

Supplementary Figure 3



Supplementary Figure 3. Histology and immune histochemistry of bone marrow from a GFI1B-p37-deficient patient II.3. Hematoxylin-eosin (HE) staining of bone marrow reveals dysplastic, small to normal-sized and diffusely dispersed megakaryocytes with mostly hypolobulated nuclei and dense chromatin, but otherwise unremarkable hematopoiesis (*upper row*). Normal megakaryocyte staining with typical markers is shown for CD42b (GPIbα; *second row*) and CD61 (integrin β3; *third row*). Expression of CD36 (thrombospondin receptor, *bottom row*) is absent in the patient's megakaryocytes. Magnifications are 100x, left column; 200x, central column; 400x, right column; bars indicate 200 µm, 100 µm and 50 µm scales, respectively.