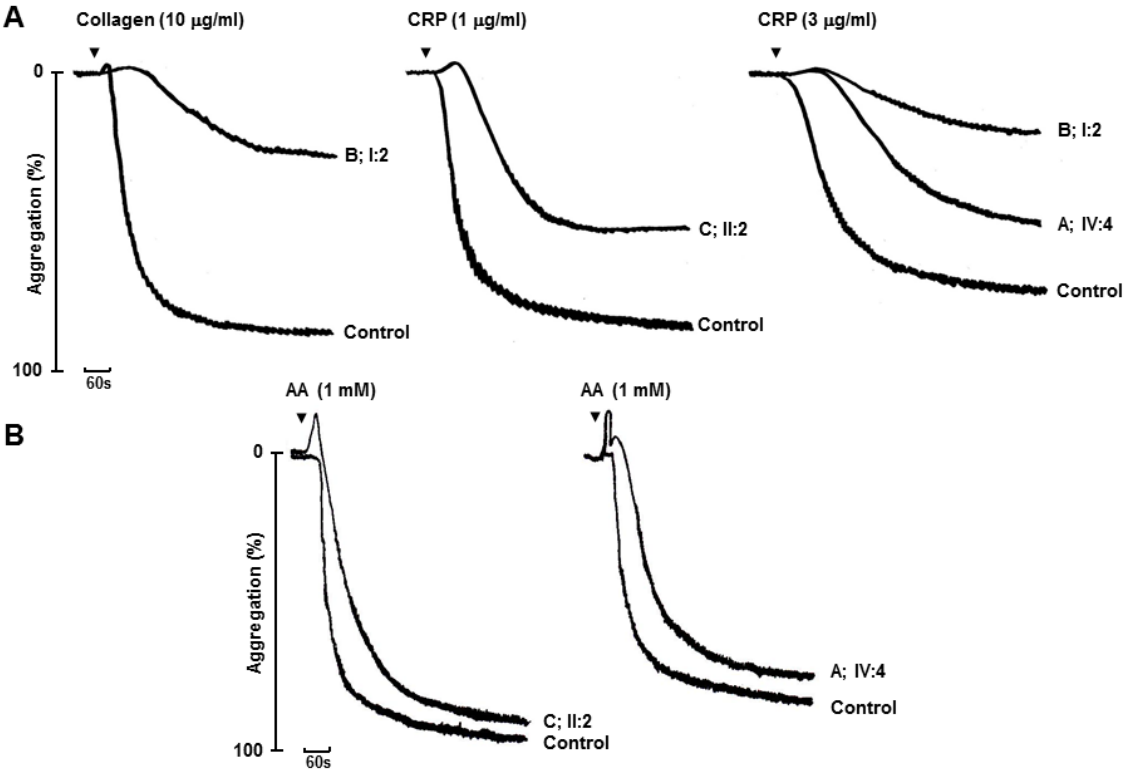
Supplementary data

Supplementary Table 1

	Patient IV:2	**Patient IV:4**	**Patient III:3**
Total variants	22,867	23,334	23,153
Novel or Rare variants	124	137	128
Overlapping variants	8		
minus synonymous variants	4		
Segregation analysis (5 patients and 1 unaffected)	2		

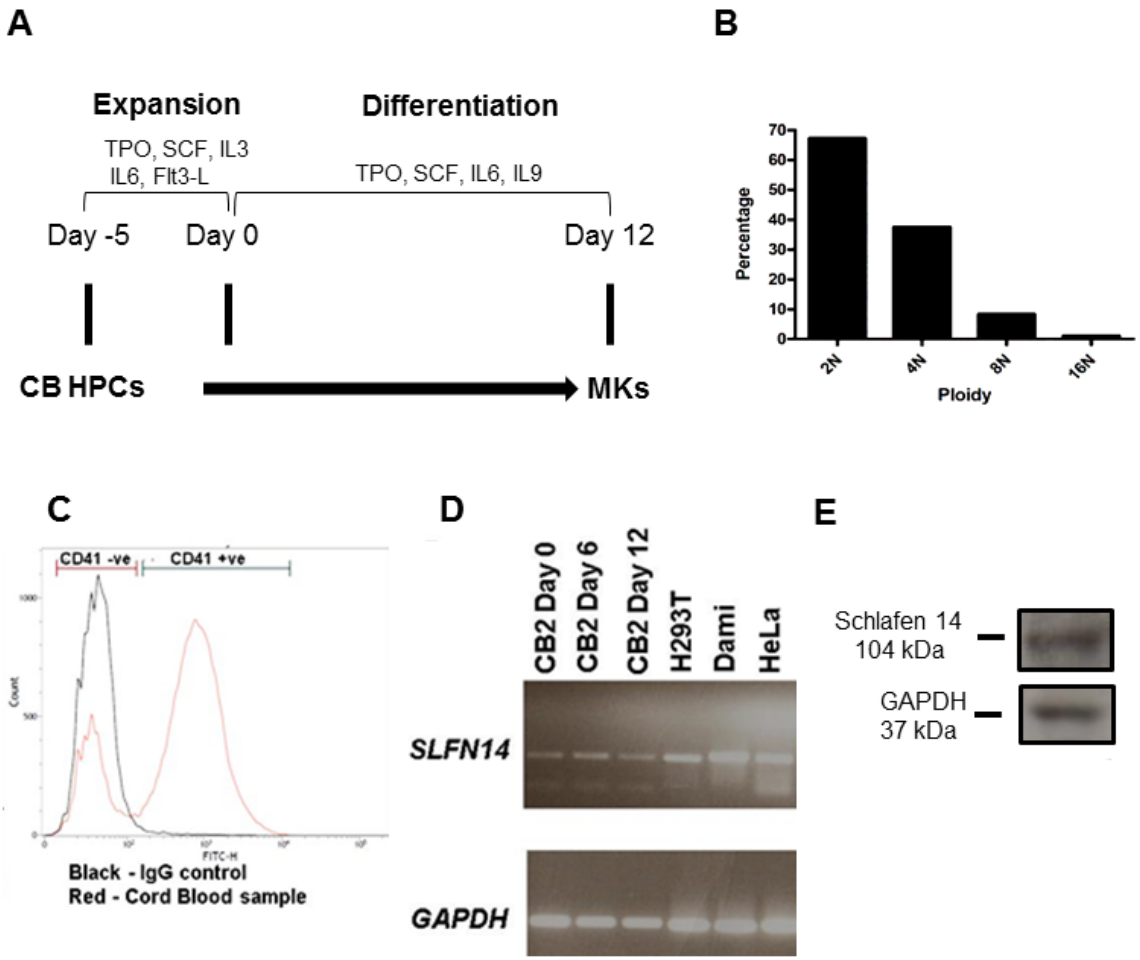
Whole exome sequencing analysis in 3 affected members of Family A showing the number of sequence variants indicated following each filtering step. Novel or rare variants are selected based on filtering out from variants in dbSNP build 135, the 1000 Genomes project database and in-house database (composed of >600 exomes).

Supplementary figure 1



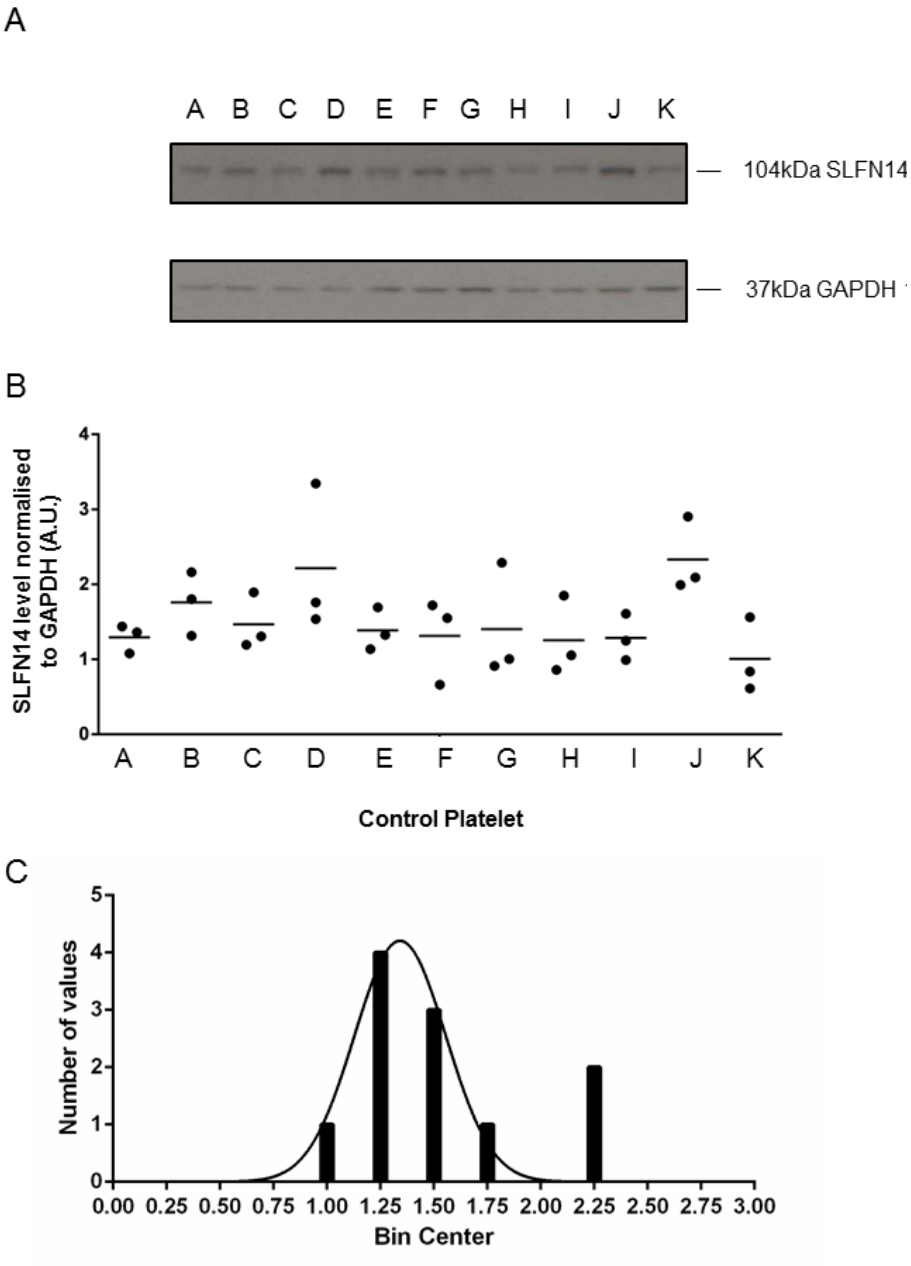
Supplementary figure 1. Platelet phenotyping of patients from the 3 families with *SLFN14* mutations. (A) Impaired aggregation in PRP from patients represented from of each of the 3 families (Family A; IV:4, Family B; III:3 and Family C; II:2) following lumiaggregometry performed on native undiluted PRP to assess percentage aggregation after stimulation with collagen (10μg/ml), CRP (1 and 3 μg/ml 100μM). (B) Normal responses in patients (Family A; IV:4 and Family C; II:2) after stimulation with arachidonic acid (AA) (1 mM). All assays were performed once on platelet samples from affected patient/healthy volunteer platelets.

Supplementary figure 2



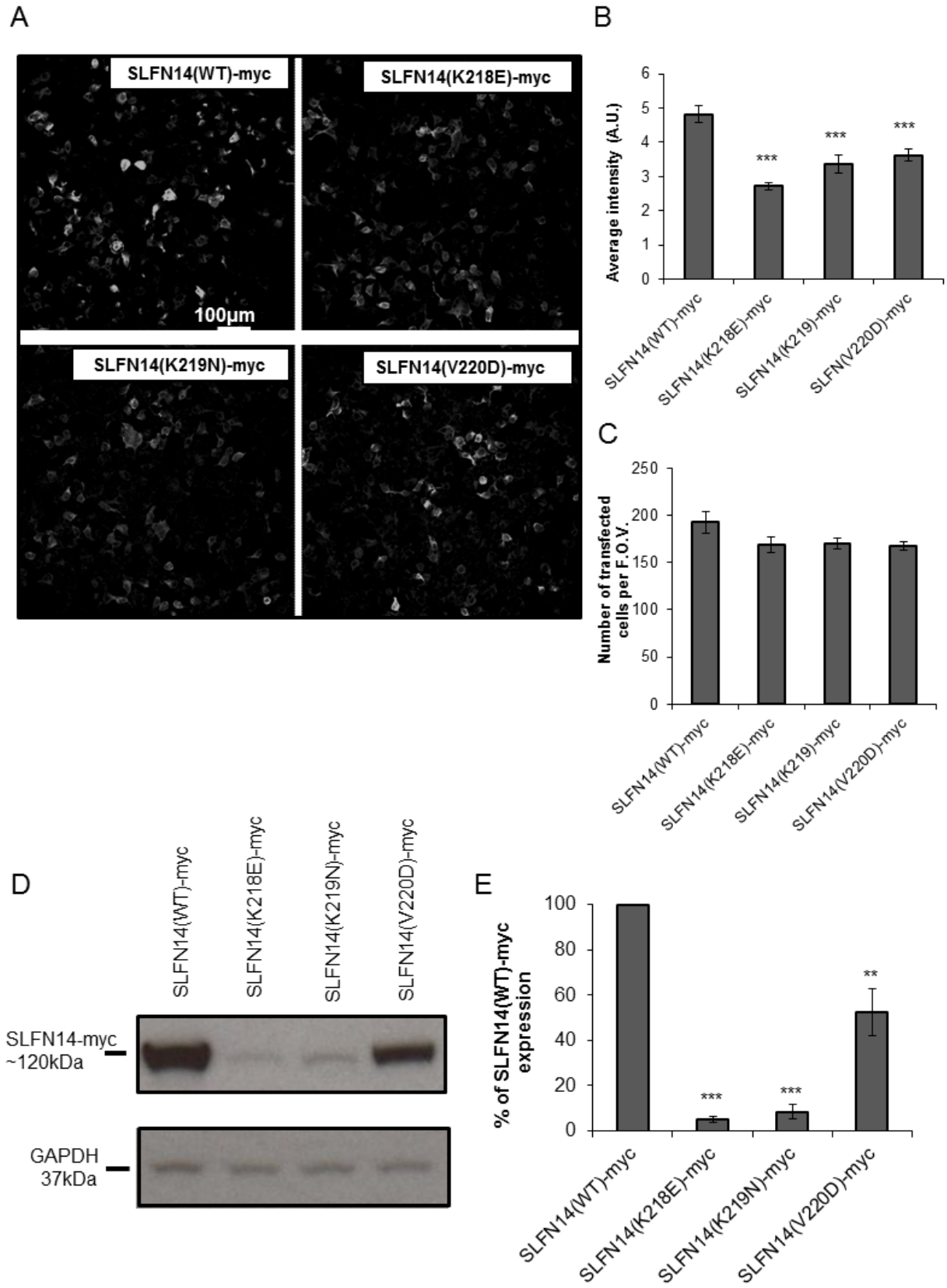
Supplementary figure 2. *SLFN14* mRNA expression in megakaryocytes derived from CD34+ hematopoietic progenitor cells (A) Workflow and time course of stages in isolating and differentiating CD34+ cell into megakaryocytes from cord blood. (B) Ploidy analysis of isolated mature megakaryocytes. (C) CD41 expression of mature megakaryocytes using flow cytometry (D) RT-PCR analysis of *SLFN14* and *GAPDH* in primary megakaryocytes at different stages of development and cell line expression. (E) SLFN14 protein expression in primary megakaryocytes 12 days post differentiation. All results from one cord blood sample from one experiment.

Supplementary figure 3



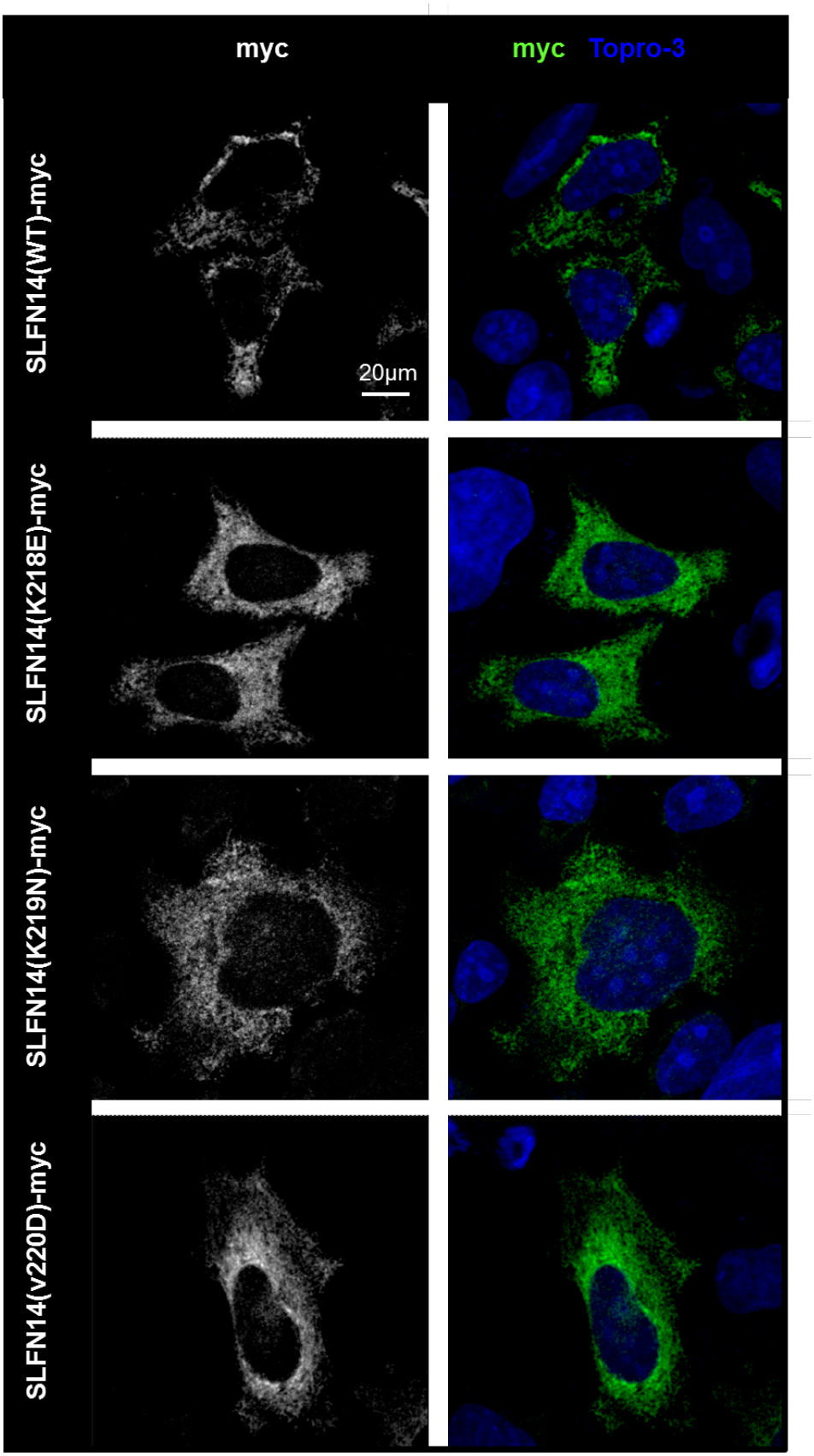
Supplementary figure 3. Healthy control platelet SLFN14 protein levels. (A) Representative Western blot analysis showing SLFN14 protein levels in platelets from 11 healthy volunteers. (B) Densitometry quantification of 3 independent Western blots (A) performed using a single platelet sample obtained from each healthy volunteer. (C) Frequency histogram demonstrating a Gaussian distribution of SLFN14 protein levels in control platelets (mean 1.5, 95% CI 1.2 to 1.8).

Supplementary figure 4



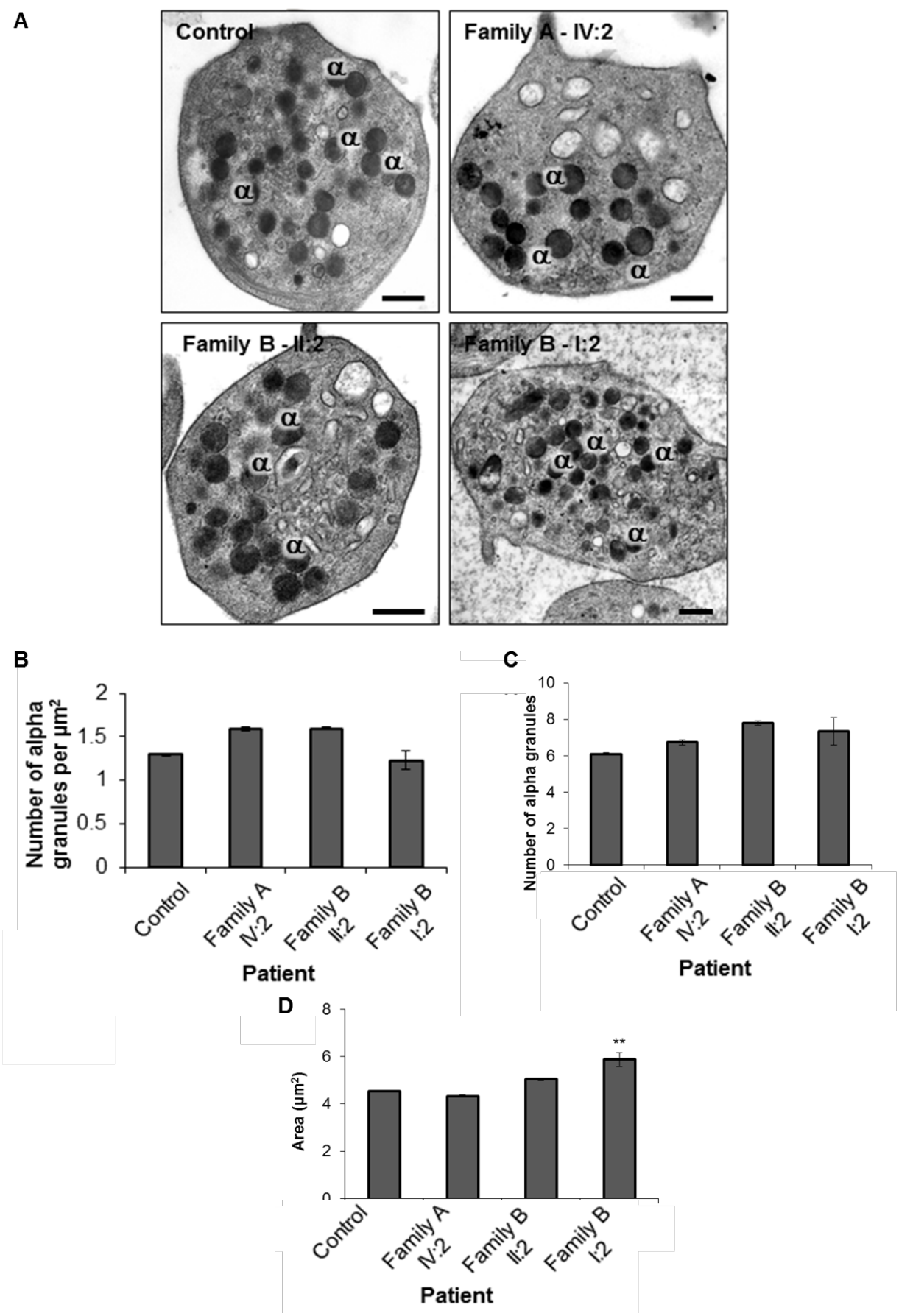
Supplementary figure 4. Overexpression of mutant SLFN14 in HEK293T cells. (A) Representative greyscale microscopy image following transfection of HEK293T cells with SLFN14(WT)-myc, SLFN14(K218E)-myc, SLFN14(K219N)-myc and SLFN14(V220D)-myc expression constructs. Immunocytochemistry was performed probing with mouse anti-myc followed by anti-mouse AlexaFluor 488. (B) Average intensity quantification of field of views for SLFN14(WT/mut)-myc (C) Quantification of transfection, number of transfected cells per field of view (F.O.V.). (B and E) Values mean ± SE with 15 fields of view analysed per expression condition, from three independant experiments. (D) Western blot image showing levels of SLFN14(WT/mut)-myc protein in HEK293T cells transiently expressing the above constructs. The blot was probed with anti-myc primary followed by incubation with anti-mouse HRP. (E) Quantification of SLFN14(WT/mut)-myc protein expression from Western blot analysis of n=3 lysate samples per condition from 3 independent experiments. In all figures Student's T-test provided values of $^{**}P \leq 0.005$ and $^{***}P \leq 0.001$, all values are mean ± SE.

Supplementary figure 5



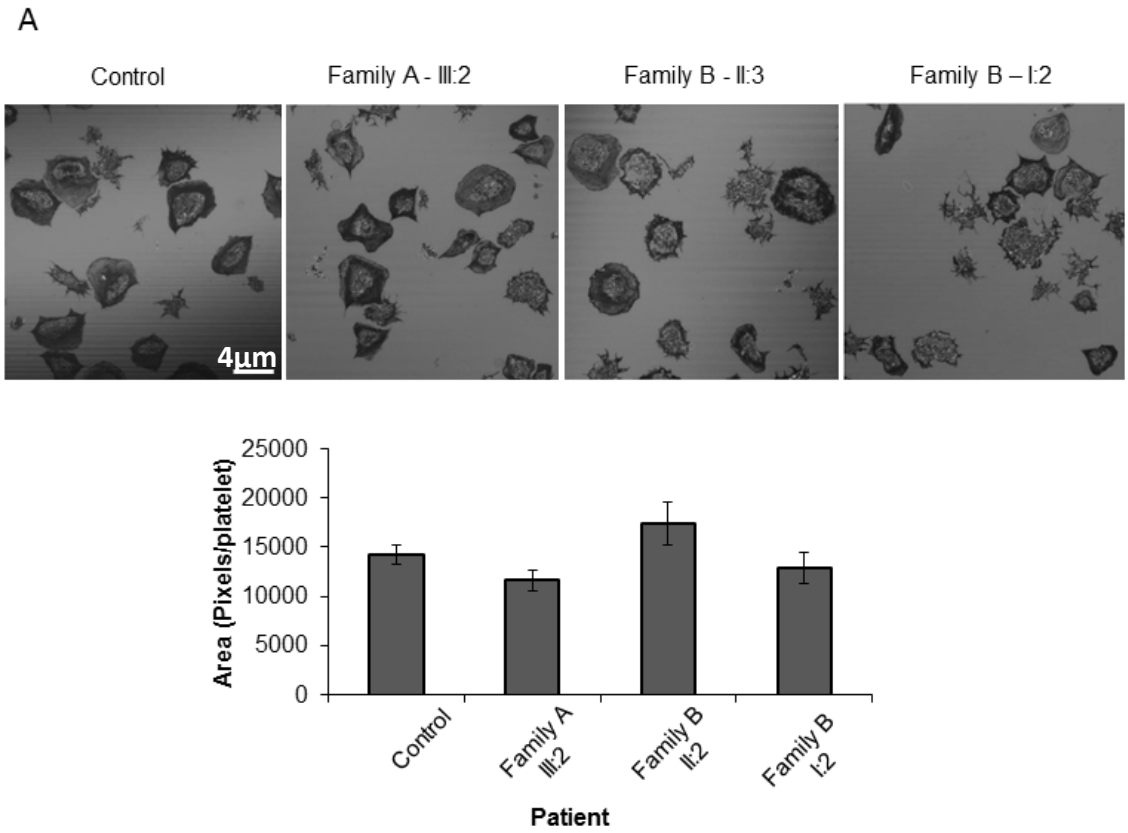
Supplementary Figure 5. Localisation of overexpressed SLFN14(WT/mut)-myc in HEK293T cells. Transiently transfected HEK293T expressing SLFN14(WT/mut)-myc for 24 hours were probed with anti-myc primary antibodies followed by incubation with anti-mouse AlexaFluor488 secondary antibodies and Topro-3 to label the nucleus. Represenative image from 2 independent experiments.

Supplementary figure 6



Supplementary figure 6. Ultrastructural characterisation of SLFN14 patient platelets. (A) Transmission electron micrographs of patient platelets showing normal morphology in comparison to healthy control platelets. (B) Quantification of (A) demonstrating no significant difference in alpha granule number per μm2 between patient and heathy control platelets. (C) Analysis showing the number of alpha granules per platelet section in healthy volunteer and patient platelets. (B) Analysis demonstrating the area (μm^2) of healthy volunteer and patient platelets. 50 platelets analysed per patient/healthy control. The experiment was repeated once from a single platelet sample from an affected patient/healthy control. In all experiments Student's T-test provides values of $^{**}P \leq 0.001$. All values are mean ± SE.

Supplementary figure 7



Supplementary figure 7. Patient platelets demonstrate no significant difference in spreading on fibrinogen in comparison to healthy control platelets. (A) Platelet spreading on 100µg/ml fibrinogen coated coverslips for 45 minutes. n=1. (B) Analysis demonstrating no significant difference in spreading of healthy control and patient platelets. 30 platelets analysed per patient/healthy control. The experiment was repeated once from a single platelet sample from an affected patient/healthy control. A Student's T-test was used to ascertain significance.

Supplementary Methods

All reagents were supplied by Sigma unless otherwise stated.

Whole exome sequencing

Following enrichment of coding regions and intron/exon boundaries with the SureSelect human AllExon 50Mb kit (Agilent Technologies) WES was carried out on the HiSeq 2500 (Illumina) with 100 bp paired-end reads. Bioinformatic analysis was carried out as in previous studies (1) with the exception of filtering of variants for novelty by comparison to dbSNP135, 1000 Genomes dB and variants identified in >600 control exomes analysed by the same method described above. To verify candidate mutations Sanger sequencing was performed using standard methods on an ABI 3730 automated sequencer. We designed primer pairs using exon-primer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html) to amplify the candidate variants (sequences are available on request).

Platelet Preparation and Lumiaggregometry

Fifty millilitres of blood was collected in 10% by volume 3.8% trisodium citrate for patients and healthy controls. Platelet rich plasma (PRP) was prepared by centrifuging at 200g for twenty minutes. A PRP platelet count and mean platelet volume was then measured. Lumiaggregometry was performed on native undiluted PRP. Secretion of ATP from dense granules was assessed using the luciferase assay. Agonists and concentrations tested for aggregometry were ADP (3, 10, 30 and 100 μM), adrenaline (10 and 30 μM), arachidonic acid (0.5, 1 and 1.5 mM), PAR-1 peptide (30 and 100 μM), PAR-4 peptide (100, 250 and 500 μM), collagen (1, 3 and 10 μg/ml), CRP (1 and 3μg/ml) and ristocetin (1 and 1.25mg/ml) (8).

Washed platelets were prepared as described by Hughes et al., 2010 (2). To isolate platelet proteins for Western blotting, platelets were used at a density of 5 × 10^8/ml, while in platelet spreading assays platelets were seeded at a density of 2 x 10^7/ml.

Flow Cytometry

We assessed platelet function in samples from affected individuals by flow cytometry using an assay that was validated with platelet rich plasma (PRP) from healthy volunteers. PRP was stimulated with ADP (3 and 30 μM), collagen-related peptide (CRP) (0.3 and 3μg/ml) and PAR-1 peptide (10 and 100 μM) (PAR-1 specific thrombin receptor activating peptide with sequence SFLLRN). Membrane expression of P-selectin (FITC conjugated mouse anti-human CD62P antibody, BD Pharmingen, Clone AK-4 Catalogue #555523), a marker of platelet alpha granule release, was assessed by flow cytometry on an Accuri C6 flow cytometer. Incubation took place at 37ºC for 2 minutes and was terminated by adding a fivefold excess of ice cold PBS.

Cell culture, plating and transfection

HEK293T cells were cultured in DMEM plus L-glutamine (Invitrogen) (plus 10% foetal calf serum, 1% pen/strep (both from Gibco)). Cells were plated into 6 well plates with/without sterilised 23mm glass coverslips at a density of $5x10^5$ cells/ml. Cells were transfected 24 hours post-plating with 5.5μl (1mg/ml pH 7.4 PEI stock), 1.2μg DNA and 140μl Optimem (Invitrogen) per well of a 6 well plate and used in studies 24 hours post-transfection.

Immunoblot analysis and densitometry

Platelet stored at -80ºC at a density of 5×10^8/ml, were lysed in 100 µl lysis buffer (1% Triton-X100, 2mM Na_3VO_4, 1mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 10µg/ml leupeptin, 10µg aprotinin, 1µg pepstatin) on ice for 45 minutes. Membranes were pelleted by centrifugation in an Eppendorf 5417R centrifuge at 12000g at 4°C for 20 minutes.

Protein levels were then quantified using a DCTM Protein Assay (Bio-Rad) performed as per the manufacturer's instructions and measured using a VERSA max tunable plate reader (Molecular Devices) running SoftMax Pro Version 6.3 Software. Samples were standardised to contain 0.5mg protein/ml in 3x Sample buffer. Protein samples were separated on 1.5mm, 15 well, pre-poured NuPAGE® Novex® 10% Bis-Tris Protein Gradient Gels (Invitrogen) in MOPS 20x electrophoresis buffer diluted to 1x in dH_2O (Invitrogen). Proteins were transferred onto PVDF membrane (BioRad) in a Mini-PROTEAN Trans-Blot Cell (BioRad) in transfer buffer (dH_2O plus 20% Methanol, 14.4mg/ml glycine, 3mg/ml Tris). Membranes were blocked in TBST (200mM Tris, 1.37mM NaCl, plus 0.1% TWEEN) containing 5% non-fat milk powder (Applichem), probed with rabbit anti-GAPDH (Abcam clone AB9485) or rabbit anti-SLFN14 (Abcam clone AB106406), washed in TBST, and then incubated with donkey anti-rabbit IgG (catalogue # NA934-1ML) conjugated to horseradish peroxidase (GE healthcare) and developed using a Compact X4 Xograph machine (Scientific Laboratory Supplies) onto Amersham Hyperfilm (GE Healthcare).

Western blot band intensity was quantified in NIS Elements version 3.16 as follows: the ROI selection tool was used to draw around the largest band and the average intensity was measured. This box was used to measure the average band intensity of other bands. Background intensity was measured using the same ROI box moved to 4x non-band region in the same lane as the band measured. These values were

logged to Excel, for both SLFN14 and GAPDH the average band value was then subtracted from the average background value. To correct for minor differences in protein levels seen in the GAPDH protein control, the band value for SLFN14 was divided by the average band value for GAPDH. For the healthy volunteer control platelet analysis a one-way ANOVA was performed using Prism 6 Graphpad (Graphpad Software, INC). The values were obtained from 3 separate experiments. For the patient platelets and corresponding healthy control platelets, control values were averaged and classified as 100%, SLFN14 protein levels in patients were expressed as a percentage of the average control SLFN14 level. The values were obtained from 3 separate experiments.

In overexpression analysis studies HEK293T cells were plated as described above and transfected as for I.C. Cells were lysed and protein levels established as for platelet protein preparation. Western blotting was performed as stated previously probing with mouse anti-myc primary antibody (Cell Signalling clone 9B11) followed by incubation with sheep anti-mouse IgG-HRP (catalogue # RPN4201) (GE Healthcare). SLFN14-myc wild type/mutant protein expression level was quantified as described above. In order to examine SLFN14 levels in mature megakaryocytes, CD34+ cells were subcultured as described below. 12 days post differentiation, megakaryocytes were sorted in a 3% and 5% BSA gradient, the lower half of the gradient centrifuged at 300g for 10 minutes, pelleted and the cells were lysed as described above. Lysates were prepared for Western blotting, and Western blotting was performed as described above.

Platelet spreading

13mm coverslips were washed in 100% ethanol, air dried, then subsequently washed in sterile PBS (all PBS -Mg2+ and -Ca2+) (Invitrogen) and placed in a 24 well plate. Coverslips were coated overnight in 100µg/ml fibrinogen (Enzyme Research) in sterile PBS at 4ºC. The coverslips were blocked in denatured, sterile filtered 5mg/ml BSA (First Link (UK) Ltd) in PBS. 300µl of $2x10^7$/ml platelets were added to each well and allowed to spread for 45 minutes at 37ºC at 5% CO_2. Spread platelets were washed in PBS and fixed at room temperature in 4% PFA for 5 minutes.

Plasmid constructs

A wild type expression plasmid containing the full coding region of human *SLFN14* was purchased from Origene (SLFN14(WT)-myc). The *SLFN14* mutations p.K218E, p.K219N and p.V220D were created using the QuikChange site-directed mutagenesis kit (Agilent) with primers:

K218E_F (5-AAAAGGTTCACCACCGAAAAAGTCATACCTC-3')
K218E_R (5'-GAGGTATGACTTTTTCGGTGGTGAACCTTTT-3')
K219N_F (5'-GTTCACCACCAAAAATGTCATACCTCGGATT-3')
K219N_R (5'-AATCCGAGGTATGACATTTTTGGTGGTGAAC-3')
V220D_F (5'-TCACCACCAAAAAAGACATACCTCGGATTAA-3')
V220D_R (5'-TTAATCCGAGGTATGTCTTTTTTGGTGGTGA-3')

All plasmid constructs were verified by sequencing.

Immunocytochemistry (I.C.)

HEK293T (American Type Culture Collection) cells were plated onto glass coverslips and transfected with SLFN14(WT)-myc, SLFN14(K218E)-myc, SLFN14(K219N)-myc or SLFN14(V220D)-myc as described above. Cells were fixed in 4% PFA for 5

minutes and permeabilised in 0.1% Triton-X-100 in PBS for 5 minutes. Cells were then blocked in block buffer (PBS (Invitrogen) plus 10% goat serum (Gibco) plus 5% BSA) for 1 hour. Cells were then incubated for 1 hour in primary mouse anti-myc (Cell Signalling Technology clone 9B11) diluted in block. Cells were washed then incubated for 1 hour in secondary goat anti-mouse AlexaFluor488 (Invitrogen catalogue # A-10667) plus Topro-3 (Invitrogen) diluted 1:1000 in block. Cells were mounted on glass slides using Hydromount mounting media (National Diagnostics). In overexpression quantification studies five regions from each coverslip were imaged and the numbers of transfected cells was counted.

Immunofluorescence microscopy

All images were taken using a DM IRE2 Leica inverted microscope, SP2 confocal system running Leica Confocal Software Version 2.61 Build 1537. For platelet spreading and SLFN14(WT/mut)-myc localisation assays reflectance and confocal imaging was performed using the 488nm line of an Argon-Ion laser 457-514nM (to image AlexaFluor 488 labelled constructs) and the 633 line of a HeNe 688nm laser (to image TOPRO-3) with an HCX Plan Apo Ibd.BL 63x NA 1.4, Olympus objective. HEK293T cells expressing SLFN14(WT/mut)-myc to examine protein expression were imaged using the 488nm line of an Argon-Ion laser 457-514nM and the HC Plan FLUOTAR 40x NA 0.5 Olympus objective.

Electron Microscopy

For transmission electron microscopy (TEM) platelet-rich plasma (PRP) was prepared by centrifugation (Harrier 18/80 (Sanyo)) at 200g for 10 minutes and fixed with an equal volume of 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH

7.4) at room temperature for 15 minutes. The platelets were post-fixed with 3% glutaraldehyde in sodium cacodylate buffer at 4°C for 30 minutes. They were then washed three times in 0.1 M sodium cacodylate buffer and postfixed with 1% OsO_4 for 1 hour at 4°C. Cells were rinsed in distilled water, dehydrated in a graded series of ethanol and embedded in Agar 100 (Electron Technology, Cambridge, UK). Ultra-thin sections (70 nm) were stained with uranyl acetate and lead citrate, and examined using a JEOL 1200EX transmission electron microscope (Hertfordshire, UK).

Platelet alpha-granules were counted and the area of each platelet was measured. The number of alpha-granules per μm^2 was calculated for at least 40 platelets from each patient/control. Platelet whole-mount electron microscopy was performed by placing 10 mL of PRP onto carbon-coated formvar copper grids (Elektron Technology, Cambridge, UK) for 5 minutes. Grids were rinsed with distilled water and excess liquid was removed with a filter paper. All imaging was done in using a JEOL 1200EX transmission electron microscope (Hertfordshire, UK).

Platelet dense-granules were counted for 40 platelets from each patient/control.

In vitro culture of megakaryocytes from $CD34^+$ cells

Umbilical cord blood was obtained from the Human Biomaterials Resource Centre, University of Birmingham under project application 12-098. $CD34^+$ cells were isolated by Lymphoprep (Axis-Shield) density gradient centrifugation followed by magnetic cell sorting according to the manufacturer's instructions (MiniMacs CD34 MicroBead kit, Myltenyi Biotec). The purity of the isolated population, based on CD34 expression (anti-CD34 antibody, Myltenyi Biotec), was determined by flow cytometry and always exceeded 90%. $CD34^+$ cells were cultured for 5 days in an expansion

cocktail, StemPro-34 medium (Life Technologies) supplemented with 20 ng/ml human TPO, 50 ng/ml human SCF, 10 ng/ml IL-3, 10 ng/ml IL-6 and 50 ng/ml Flt3-L (all from Myltenyi Biotec). To induce megakaryocyte development, the differentiation cocktail 1 ng/mL SCF, 30 ng/ml TPO, 7.5 ng/ml IL-6 and 13.5 ng/ml IL-9 was added to the culture media on day 5 of subculture and incubated for an additional 12 days.

Supplemental References

1. Stockley J, et al. Enrichment of *FLI1* and *RUNX1* mutations in families with excessive bleeding and platelet dense granule secretion defects. *Blood.* 2013.

2. Hughes CE, et al. CLEC-2 activates Syk through dimerization. *Blood.* 2010;115(14):2947-55.

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Dr Neil Morgan has authorized authorship on behalf of the UK Genotyping and Phenotyping of Platelets Study Group.