Supplementary Material to Marconi et al. “SLFN14-related thrombocytopenia: identification within a large series of patients with inherited thrombocytopenia” (Thromb Haemost 2016; 115.5)

**Suppl. Figure 1.** Multiple species alignment of SLFN14 shows that the Arginine 223 is highly conserved.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. sapiens</td>
<td>THVEFKRFTTKKVIRIKEMLVHVSANFQGGYVILGVDKSKEVGCWKEKVPNPD</td>
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<tr>
<td>M. mulatta</td>
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<tr>
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<td>C. familiaris</td>
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</tr>
</tbody>
</table>

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Suppl. Methods

Patients investigation
Methods of recruitment of patients for exome sequencing are reported in the main text. Each individual gave written informed consent for the study, which was conducted according to the Declaration of Helsinki. The International Society on Thrombosis and Haemostasis (ISTH) bleeding assessment tool (BAT) score was assessed by patients’ interrogation as reported (2,3). Mean platelet diameters were calculated by software-assisted image analysis of peripheral blood smears stained by May-Grünwald-Giemsa as previously described (1). At least 200 platelets were investigated for each patient. Methods of flow cytometry study of platelet glycoprotein complexes have been described in detail previously (4).

Exome sequencing
Whole exome DNA from patient's whole blood was captured using the BGI exome kit (BGI TECH SOLUTIONS, Hong Kong) and sequenced as 100 bp paired-end reads on Illumina HiSeq2000 platform (Illumina Inc., San Diego, CA, USA).

Generated reads were checked with FastQC (http://www.bioinformatics.babraham.ac.uk/publications.html) and aligned with BWA (5) to the reference genome hg19. Aligned reads were treated for realignment and base quality score recalibration with GATK (6) and for duplicate removal with PicardTools (http://picartools.sourceforge.net). Alignment statistics were collected by SAMtools (7) and GATK. Coverage statistics over the targeted regions were calculated with GATK. Variant calling and filtering by quality were performed by GATK and annotated using GEMINI (8). Candidate variants where selected among those having frequency less than 1:1000 in the 1000Genomes database.

Coverage evaluation of SLFN14 in the whole cohort was performed with GATK. Mean coverage of the gene was 285.8±73X (52X-406X). All the sequenced individuals had more than 99% of SLFN14 bases in the target covered >20X.

Validation and segregation were performed using Sanger sequencing following standard protocols.

Immunoblotting of SLFN14
Peripheral blood was collected from patients and three healthy volunteers into acid/citrate/dextrose in a ratio of 9:1. General procedures of immunoblotting analysis of platelet lysates have been previously described in detail (9). Briefly, after preparation of platelet-rich plasma (210g for 10 minutes), platelets were isolated by centrifugation at 730g for 15 minutes in the presence of 0.2 U/ml apyrase grade I and 1 µM PGE_1 (both from Sigma, Milan, Italy) and washed in PIPES buffer (136 mM NaCl, 20mM PIPES, pH 6.5). Whole platelet lysates were prepared and dissociated under reducing conditions as reported (9). Twenty µg of lysates were loaded on the gradient AnyKd gels (Biorad, Hercules, CA, USA) and transferred to
nitrocellulose. After blocking with 5% not-fat milk, membranes were probed with a rabbit polyclonal against SLFN14 (Abcam, Cambridge, UK) or the mouse monoclonal AC-15 against β-actin (Sigma). The appropriate HRP-conjugated secondary antibodies (Ab) (Dako, Glostrup, Denmark) were used for secondary detection. Protein bands were visualized by an enhanced chemiluminescence method (ECL, GE Healthcare, Waukesha, WI, USA). Densitometric analysis was performed by Image J software.

**Megakaryocyte culture**

CD45+ cells from peripheral blood samples of patients and three healthy volunteers were separated by immunomagnetic bead selection (Miltenyi Biotech, Bologna, Italy) and cultured as previously described (10,11). To investigate megakaryocyte differentiation and maturation, 200 x10³ cells were collected, washed in PBS, and stained with the FITC-conjugated mouse monoclonal HIP8 against CD41a (eBioscience, Milan, Italy) and the PE-conjugated mouse monoclonal HIP1 against CD42b (Abcam, Cambridge, UK), at room temperature in the dark for 30 minutes. After incubation, samples were immediately analyzed by a Beckman Coulter Navios flow cytometer. Off-line data analysis was performed using Beckman Coulter Navios software package. Proplatelet formation was investigated as previously reported (10,11). Briefly, megakaryocyte population at day 14 was enriched through a BSA gradient and 1 x10⁵ cells were allowed to adhere on glass coverslips previously coated with 100 µg/ml fibrinogen (Sigma). After 16 hours at 37°C and 5% CO₂, adhering cells were fixed in 4% PFA (Sigma), permeabilized with Triton X-100 (Sigma), and stained with rabbit anti-β1-tubulin Ab (kindly provided by Prof. J. Italiano Jr.). Proplatelet forming megakaryocytes were identified as the β1-tubulin+ cells displaying at least one proplatelet with respect to total number of round megakaryocytes. At least 50 fields per sample were analyzed.
Suppl. References