Supplemental Material

Equine type I collagen was obtained from Kordia (Leiden, The Netherlands). Human purified von Willebrand Factor (VWF) was a kind gift of the Laboratoire Français de Fractionnement et Biotechnologies (LFB, Courtaboeuf, France). Fibrinogen was obtained from HYPHEN BioMed SAS (Andresy, France). Convulxin and the monoclonal antibody directed against GPVI were produced as previously described. The thrombin receptor-derived peptide (SFLRN-NH2: PAR1-AP) was purchased from Bachem (Weil am Rhein, Germany). ADP was obtained from Kordia. D-Phe-Pro-Arg chlormethylketone dihydrochloride (PPACK) was from Calbiochem-VWR (Fontenay-sous-Bois, France). Thrombin, leupeptin, aprotinin, apyrase grade VII, prostaglandin E1 and rhodamine-6G were from Sigma-Aldrich (Saint-Quentin Fallavier, France). The monoclonal antibody directed against Syk polyclonal antibodies directed against the phosphorylated form of linker for activation of T cells (LAT) and against the Fc Receptor (FcR) γ-chain were purchased from Millipore (Billerica, MA). The monoclonal antibody directed against the phosphorylated forms of Syk, was obtained from Cell Signaling Technology (Danvers, MA). The polyclonal antibody specific for C-terminal FLNa was obtained from Abcam (Cambridge, UK). Peroxydase-conjugated AffiniPure secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Supplemental Methods

Platelet preparation
Samples from patients and control random donors were always processed in parallel. The quantification of FLNa levels in controls showed that variability of FLNa content between random controls did not exceed 6%. Venous blood from patients and control was collected in 10% (vol/vol) ACD-A buffer (75 mM trisodium citrate, 44 mM citric acid, 136 mM glucose, pH 4) for experiments with washed platelets or in PPACK (80 μM) for flow experiments using whole blood. Platelet-rich plasma was obtained by centrifugation (100g for 15 minutes at 20°C) and platelets were isolated by differential centrifugations as previously described. The platelet pellet was resuspended in Tyrod’s buffer (5 mM HEPES, 137 mM NaCl, 2 mM KCl, 12 mM NaHCO3, 0.3 mM NaH2PO4, 1 mM MgCl2, 2 mM CaCl2 and 55 mM glucose, pH 7.4).

Platelet aggregation
Platelet aggregation of washed platelets was monitored by measuring light transmission through the stirred suspension of platelets (2.5x10^8 platelets/mL) during 3 minutes using a Chronolog aggregometer (Coultronics, Margency, France). Platelet aggregation was triggered by adding fibrillar equine type I collagen, convulxin (Cvx), thrombin, PAR1-AP or ADP as described in Results. Platelet aggregation was assessed as the percentage change of light transmission with respect to the blank (buffer without platelets) set at 100%.

Platelet dense granule secretion
Dense granule secretion was quantified by measuring ATP release during platelet aggregation. After 3 minutes, platelet aggregation was stopped by adding cold ethylenediaminetraacetic acid (EDTA) (16 mM) followed by centrifugation (12 000g, 1 minute). ATP release was quantified by an ATP determination kit using luciferase and its
substrate D-luciferin according to the manufacturer’s instructions. Light emission was assessed using a luminometer (Fluostar Optima; BMG Labtech). Dense granule secretion was expressed as pmoles of ATP released.

**Platelet spreading**

Glass coverslips were pre-coated with type I collagen (50 µg/mL), VWF (10 µg/mL) or fibrinogen (100 µg/mL) overnight at 4°C. Then washed platelets (10⁷ platelets/mL; 150 µL) from patients or control donors were allowed to adhere at room temperature. After 30 minutes, unbound platelets were removed and adherent platelets were fixed with 4% paraformaldehyde in cytoskeleton buffer (0.1 M PIPES, 2 M glycerol, 1 mM EGTA, 1 mM MgCl₂ pH 6.9) for 15 minutes, then permeabilized in the same buffer containing 0.2% Triton X-100 for 5 minutes. Platelets were stained with Alexa Fluor488-labeled phalloidin (1/500) and anti-C-terminal FLNa and then visualized under an epifluorescence microscope (Nikon, Eclipse 600). Cell surfaces were analyzed using the Image J software (rsb.info.nih.gov/ij).

**Immunoblotting**

Washed platelets (2.5x10⁸/mL; 300 µL) were stimulated with Cvx (800 pM) in the absence of stirring. After 3 minutes, the platelets were lysed in SDS denaturing buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 100 µM phenylarsine oxide, 1% SDS, 5 µg/mL leupeptin, 10 µg/mL aprotinin, pH 7.4). The proteins were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with various primary antibodies (see Results Section). Immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (Pierce, Rockford, IL). Images of the chemiluminescent signal were captured using G:BOX Chemi XT16 Image Systems and quantified using Gene Tools version 4.0.0.0 (Syngene, Cambridge, UK).

**Thrombus formation under flow**

Blood perfusion experiments were performed in a parallel plate perfusion chamber. PPACK (80 µM) anticoagulated blood from patients or controls was incubated with rhodamine 6G (10 µg/mL) for 5 minutes at 37°C, then perfused on glass coverslips pre-coated overnight at 4°C with fibrillar equine type I collagen (50 µg/mL) or human VWF (50 µg/mL) at various shear rates (300 s⁻¹ to 5000 s⁻¹) with a syringe pump (Fisher Scientific, Illkirch, France) as previously described.⁴ Thrombus formation was recorded with an inverted epifluorescence microscope (Nikon Eclipse TE2000-U) coupled to the Metamorph 7.0rl software (Universal Imaging Corporation) and was quantitated by assessment of the mean percentage of the total area covered by thrombi.

Supplemental Figures

Figure I: Results of FLNA mutation searches in Patient 4

Custom array-CGH analysis was performed. Both hybridization experiments with fluorochrome swapping show the same deletion (153531400-153590150, minimum size: 58.75 kb; 153530306-153592216, maximum size: 61.91 kb) including the 3’ terminal part of FLNA from exon 20 to exon 48 (black arrow), the intergenic region and TKTL1 (exon 2 to 13). Genomic coordinates are indicated on the left (according to the human reference sequence GRCh37/hg19). FLNA is shown as a box on the left, and Log2 values of the ratio of fluorescence patient/reference are indicated above the plot.
Figure II: Quantification of GPVI and Fc Receptor (FCR) γ-chain in platelets
Western blotting with antibody against GPVI and Fc Receptor (FCR) γ-chain in platelets was assessed. These results are representative of at least three independent experiments.

Figure III: Platelet aggregation and secretion induced by other agonists.
Platelet aggregation and secretion were initiated by adding various concentrations of thrombin (0.1 or 0.2 U/mL), PAR1-AP (5 or 10 µM) or ADP (5 or 20 µM) during 3 minutes. Aggregations of P1, P2 and P4 were expressed as the percentage change in light transmission, with the value of the blank (buffer without platelets) set at 100%. Traces are representative of at least two experiments. Dense granule secretions of P1, P2 and P4 were evaluated by assessment of ATP release after aggregation. Results are expressed as the amount of the ATP released (pmoles).

**Figure IV : Role of ADP on thrombus formation**

Apyrase (5 U/ml) was added to blood during 5 minutes and then perfused on collagen matrix (50 µg/mL) at 300 s⁻¹. After 5 minutes (300 s⁻¹) thrombi were observed under an epifluorescence microscope (original magnification x20). Total area covered by platelets was expressed as the mean ± SEM of three independent experiments. * p<0.05 (unpaired Student t test)

**Table S1: Clinical and functional parameters of the FLNa patients**

<table>
<thead>
<tr>
<th>Patients</th>
<th>FLNA Mutations</th>
<th>Putative FLNa protein</th>
<th>X-inactivation in lymphocytes</th>
<th>PNH</th>
<th>Platelet count x 10⁹/L</th>
<th>Aggregation Collagen/Cvx</th>
<th>Spreading VWF FG</th>
<th>Spreading collagen</th>
<th>VWF adhesion 5000s⁻¹</th>
<th>Remaining wt FLNa (%)</th>
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</thead>
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<tr>
<td>P1</td>
<td>Frame-shift c.4573_4614delTA, p.Tyr1526X</td>
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<td>terminus exon 29 junction IgG14-15 182KD</td>
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PNH = Periventricular nodular heterotopia