Germline variants in *ETV6* underlie reduced platelet formation, platelet dysfunction and increased levels of circulating CD34+ progenitors

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Received: April 18, 2016.

Accepted: September 22, 2016.

Pre-published: September 23, 2016.

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Supplemental methods

Exome sequencing and sequencing validation in the F1 family

Whole-exome sequencing was performed on genomic DNA, as previously described[1], from 6 members of F1: the index case (F1-IV3), 2 unaffected members (F1-II1 and F1-IV2) and 3 affected members (F1-II2, F1-III3 and F1-III8). Direct Sanger sequencing confirmed the genotype among all family members (Big Dye Terminator kit V3; Life Technologies). Sequences were subsequently analyzed (Chromas X software) and aligned (Multalign: http://multalin.toulouse.inra.fr/multalin/).

Antibodies

Immunoblots were performed using goat polyclonal anti-ETV6 (N19, #sc8546, Santa Cruz), mouse monoclonal anti-SMRT (1212, #sc32298, Santa Cruz), rabbit polyclonal anti-RhoA (119, #sc179, Santa Cruz), rabbit polyclonal anti-MYH10 (#3404, Cell Signaling Technology), mouse monoclonal anti-Cdc42 (#05-542, Millipore), mouse monoclonal anti-Rac1 (23A8, #05-389, Millipore) and mouse monoclonal anti-GAPDH (6C5, MAB374, Millipore) antibodies.

Real-Time PCR

cDNA was synthesized using MMLV-reverse transcriptase from 10 ng of total RNA. Real-time PCR were performed using a LightCycler 480 (Roche) and Eva Green MasterMix (Euromedex). Relative levels of mRNA were measured using the comparative CT method. Primer sequences are available upon request.

Quantification of circulating CD34^{+} hematopoietic progenitors in the blood

Before labeling, 2 mL of each blood sample was subjected to red blood cell lysis. Equivalent numbers of cells were incubated for 15 minutes at 20°C with the appropriate monoclonal antibody cocktails: CD133-PhycoErythrin (BD Biosciences), CD19-FITC, CD34-AlloPhyco Cyanin/Alexa Fluor750, CD3-Pacific Blue, CD45-Krome Orange, CD33-PE/Cyanin7, CD38-AlloPhyco Cyanin, CD41-PE/Cyanin5, CD42b-PE/Cyanin5, CD61-FITC and CD123-PE (Beckman Coulter). Progenitors were characterized by CD34/CD133/CD33/CD38 co-expression; cells were gated on the CD34^{+} and SS^{low}/CD45^{low} population and excluded co-expression of lymphoid markers (Navios, Beckman Coulter).

Glycoprotein surface expression on platelets

PRPs were incubated in the presence or absence of platelet agonist ADP (10 µM) or TRAP (50 µM) with antibodies against $\alpha_{IIb}\beta_3$ (clone P2), the active form of $\alpha_{IIb}\beta_3$ (clone PAC-1; BD), glycoprotein (GP) Ibα, Ia, IV, CD63 (clone CLB-grad12; Beckman Coulter) and CD62P (clone CLB-Thromb/6) for 30 min at 20°C. Scatter signals and fluorescence intensity were analyzed using a FC500 flow cytometer (Beckman Coulter**).**

Platelet survival assay

The platelet survival assay was based on the method of Thakur *et al.*[2]. Autologous platelets were washed and incubated with 3 MBq of ^{111}In-oxine for 10 min at 37° C. The platelets were then washed

and suspended in autologous platelet-poor plasma. An aliquot was withdrawn for platelet count and measurement of labeling efficiency. The remainder of the labeled platelets was used for intravenous administration. Successive blood samples were collected at 15 minutes as well as 2, 24, 48, 72, 96, 120 and 144 hours post-injection for platelet count and radioactivity quantification. An exponential model was used to calculate platelet survival time according to the International Committee for Standardization in Hematology (ICSH).[3] The platelet recovery and platelet production rates were calculated based on platelet survival time, initial platelet recovery and platelet count.

Platelet spreading analysis

Non-stimulated or ADP-stimulated (10 µM) washed platelets (10^7 platelets/ml) were allowed to adhere (45 min, 37°C) to fibronectin-coated coverslips (20 µg/ml; Sigma-Aldrich). Fixed platelets were stained with Alexa 488-phalloidin (F-actin) and Alexa 594-DNAse I (G-actin). Images were recorded (Axio-Imager M1 microscope with an Axiocam MRm camera; Carl Zeiss) and analyzed (ImageJ software). Filopodia and lamellipodia were manually quantified in five different view fields. The G-actin/F-actin ratio was evaluated on 20 different platelets. For each pixel, the G-actin fluorescence intensity was divided by the corresponding F-actin signal. A 5-ramps look-up table was applied to the ratio image. The surface of high G-actin/F-actin ratio corresponded to the platelet area with a signal above the LUT threshold, defined as $\geq$3. Measurements were made on unprocessed images obtained using the same staining conditions, microscope objective and settings, and camera exposure time.

Clot retraction

PRPs were diluted in Tyrode's buffer with red blood cells. Coagulation was triggered using thrombin (1.25 U/mL), and clots were allowed to retract (1h, 37°C). Images were recorded using a CoolSNAP CCD-camera and analyzed to evaluate the reduction (%) of the initial clot surface (ImageJ software).

Co-immunoprecipitation assay

Whole cell extracts were prepared in 20 mM Tris, 140 mM NaCl, 1 mM EDTA and 0.05% Nonidet P-40, with EDTA-free protease cocktail inhibitor. The cell lysates were pre-cleared with protein A/G magnetic beads (Millipore) for 2 hours at 4ºC. The immunoprecipitation was carried out overnight at 4°C via incubation of cell extracts (500 µg protein) with anti-ETV6-coated beads. Immunoprecipitates were washed five times with lysis buffer, suspended in SDS sample buffer and boiled for 5 min at 95 ºC. The bound proteins were assessed for SMRT expression (Santa Cruz) via western blotting.

Cell transfection and luciferase assays

Transcriptional regulatory properties of wt*ETV6* and mut*ETV6* were analyzed using p(E74)$_3$tk80Luc plasmid containing the luciferase gene driven by an enhancer/promoter cassette composed of three tandem copies of the Ets Binding Site (E74-binding sites AACCGGAAGTA, found in the Drosophila E74 gene promoter) inserted 5′ of the herpes simplex virus thymidine kinase promoter. This plasmid was a gift from J Ghysdael[4]. GripTite™ 293 MSR Cells were transfected with the indicated reporter gene constructs (166 ng), the expression plasmid (333 ng) and pGL473-hRLuc

(50 ng) to normalize transfection efficiency. Luciferase activity was assayed 48 hours after transfection (Dual-Luciferase® Reporter Assay system, Promega).

Mammalian two-hybrid experiments. GripTite™ 293 MSR cells were co-transfected with GAL4(UAS)$_5$-TkLUC reporter plasmid (166 ng); GAL4(DBD)-N-CoR, GAL4(DBD)-SMRT, or GAL4(DBD)-mSin3A expression vector (or an empty vector, pGALO) (166 ng); expression vector pVP16-wtETV6 or pVP16-mutETV6 (166 ng) and pGL4-hRLuc (50 ng) to normalize transfection efficiency[5]. Luciferase activity was assayed 48 hours after transfection.

Ploidy analysis

At culture day 10, Hoechst 33342 (10 µg/mL; Sigma-Aldrich) was added to the medium of cultured MKs for 1 hour at 37°C. The cells were then stained with directly coupled monoclonal antibodies: anti–CD41-allophycocyanin and anti-CD42a-phycoerythrin (BD Pharmingen) for 10 minutes at 4°C. Ploidy was measured on the CD41$^+$CD42$^+$ cell population by means of a flow cytometer (Navios BD Biosciences) and calculated as previously described.[6]

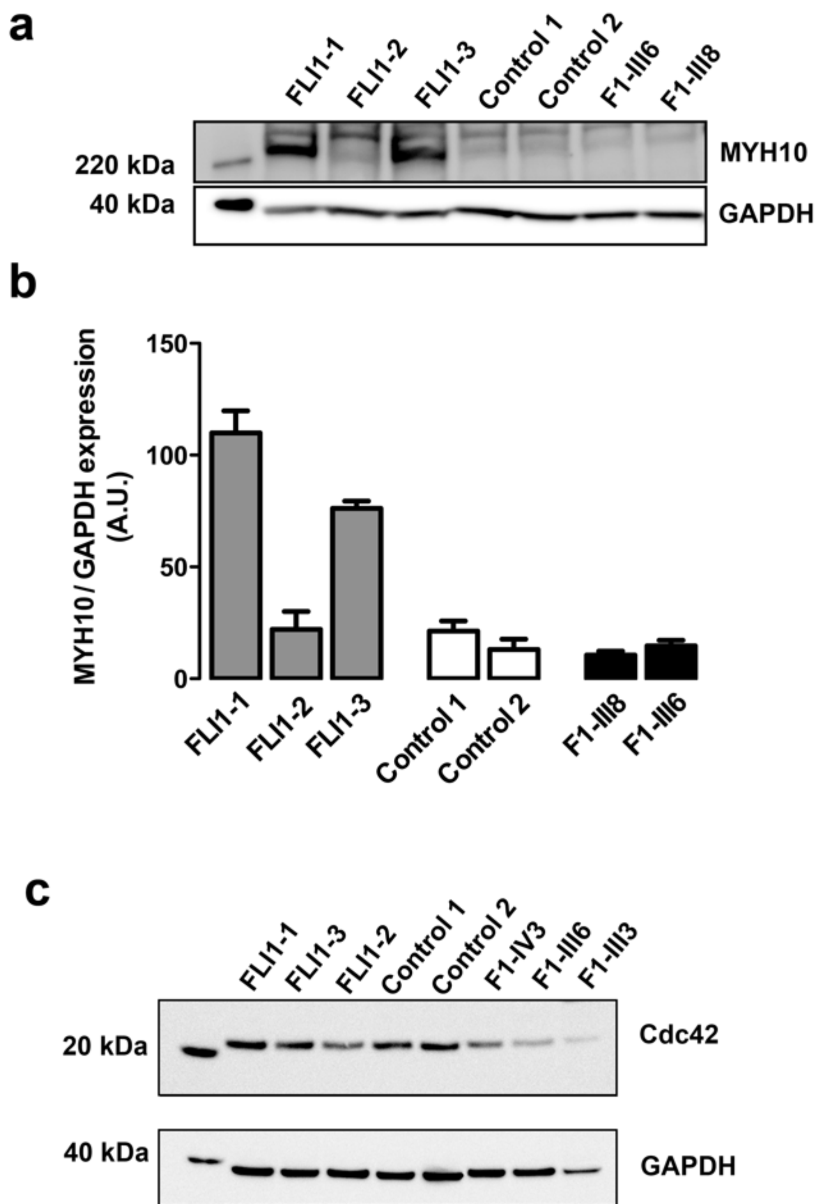
Quantification of proplatelet-bearing MKs

PPT-forming MKs were quantified on 300-500 total cells between days 11 and 15 of culture. PPT-bearing MKs exhibited cytoplasmic extensions with constriction areas (3 separate culture wells for each individual and condition). Microtubule and F-actin organization was determined on MKs (12 days) adhering to fibrinogen with fluorescently labeled anti-tubulin antibody (Sigma-Aldrich) and phalloidin (Life Technologies).

Lentiviral particle production and CD34+ cell transduction

Wild-type (wt) and p.P214L ETV6 DNA were subcloned into a third generation of HIV-derived lentiviral vector pRRLsin-PGK-IRES2-eGFP-WPRE (Genethon), and lentiviral stocks were prepared as previously described.[7, 8] CDC42 DNA was subcloned into pRRLsin-PGK-IRES-ZsGreenGFP-WPRE, and lentiviral particles were produced by Vect'UB (Plateforme de Vectorologie, Bordeaux, France). CD34$^+$ cells (3.5 to 5x10^4) were infected twice with lentiviral particles. After 8 hours, the cells were washed and cultured in serum-free medium supplemented with TPO (100 ng/ml) and SCF (250 ng/ml).

Supplemental figure 1



Supplementary Figure 1: Effect of ETV6 mutations on MYH10 levels.

Western blot analysis **(a)** and quantification of MYH10 **(b)** and Cdc42 (**c**) expression in platelets of affected members (F1-III3, F1-III6, F1-III8, F1-IV3), 2 healthy controls and members of family affected by a FLI1 mutation: 2 carriers of the mutation (FLI1-1, FLI1-3) and 1 non-carrier (FLI1-2). GAPDH was used as a protein loading control. First lane corresponds to the protein ladder. The results are expressed as mean ± SEM, n=2.

Supplemental Table 1: Summarized results of platelet aggregation, ATP secretion and dense granule defects by electron microscopy or a mepacrine uptake assay. Human Phenotype Ontology (HPO) phenotyping was used as described (PMID: 25949529) to present the functional platelet laboratory results. As the different laboratories have used different type of aggregometers, concentrations of agonists and dense granule studies, the HPO methodology allows standard platelet function phenotyping of laboratory data. As expected, platelet aggregation performed with platelet rich plasma containing < 120 x 10^3 platelets/µL plasma is highly variable and was not included in the analysis.

	Impaired ADP-induced platelet aggregation	Impaired epinephrine-induced platelet aggregation	Impaired collagen-induced platelet aggregation	Impaired arachidonic acid-induced platelet aggregation	Impaired ristocetin-induced platelet aggregation	Impaired thrombin-induced platelet aggregation	Impaired thromboxan A2 analog-induced platelet aggregation	Abnormal platelet ATP dense granule secretion	Abnormal dense granule
F1-III3	Yes	No	No	Yes	Yes	*ND*	*ND*	*ND*	No
F2-II3	No	No	No	Yes	No	No	No	No	No
F3-II4	No	No	No	Yes	No	No	Yes	No	No
F4-I2	No	Yes	No	Yes	No	No	No	No	No
F4-II2	No	Yes	No	Yes	*ND*	No	No	No	No
F4-II3	No	Yes	No	Yes	*ND*	No	No	No	No
F5-I2	No	No	No	Yes	No	*ND*	No	No	No
F5-II1	No	No	No	No	No	*ND*	*ND*	No	No
F6-I1 (WT)	No	No	No	No	*ND*	No	No	Yes	Yes
F6-II1	Yes	Yes	Yes	Yes	*ND*	*ND*	No	No	Yes

ND: not done

Supplemental Table 2: Flow Cytometric Analysis of Platelet-Membrane Glycoproteins

Baseline	αIIbβ3 (P2)	αIIbβ3 (Pac1)	GPIb	GPIa	GPIV	CD63	CD62P
MFI Normal range	23-40	2.8-6.9	21-40	1.8-6.9	6.0-16	0.3-0.9	1.1-4.3
F1-II2	29	2.1	23	2.2	5.9	0.2	2.0
F1-III3	37	3.3	35	3.4	5.4	1.6	1.9
F1-III5	30	2.9	18	3.0	5.1	0.4	2.2
F1-IV3	22	2.3	21	2.8	7.0	0.6	2.9

ADP (10 μM)	αIIbβ3 (P2)	αIIbβ3 (Pac1)	GPIb	GPIa	GPIV	GP53	CD62P
MFI Normal range	23-45	5.4-16	5-21	2.2-6.7	6.7-19	0.4-1.2	3.2-16
F1-II2	32	3.1	9.8	ND	ND	0.3	3.2
F1-III3	45	20	16	ND	ND	2.1	8.5
F1-III5	22	17	4.8	ND	ND	0.6	6.8
F1-IV3	24	12	8.3	ND	ND	0.7	7.9

TRAP (50 μM)	αIIbβ3 (P2)	αIIbβ3 (Pac1)	GPIb	GPIa	GPIV	GP53	CD62P
MFI Normal range	29-55	3.5-9	10-27	2.0-6.5	7.2-18	0.5-3.6	5-12
F1-II2	37	2.4	10	ND	ND	0.5	5.1
F1-III3	47	9.4	18	ND	ND	3.4	12
F1-III5	30	5.5	12	ND	ND	1.1	9.7
F1-IV3	33	3.4	13	ND	ND	1.3	8.7

ND: not done

Supplemental Table 3: Platelet kinetic parameters for patient F3-II4

	Parameter value	Reference value
Platelet survival time (days)	4.6	8 - 10
Platelet recovery (% per day)	15	10 - 15
Platelet production rate	0.8 N	NA
Platelet sequestration	Absence	NA

The platelet count at the day of analysis was 128 x 10^9 /L. The injected dose was 2.2 MBq. N: reference value of the laboratory.

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