Downregulation of TREM-like transcript-1 and collagen receptor α2 subunit, two novel RUNX1-targets, contributes to platelet dysfunction in familial platelet disorder with predisposition to acute myelogenous leukemia

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Supplementary appendix

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Supplementary Methods

Gene expression analysis

Transcriptome analysis was performed using the Agilent Whole Human Genome Microarray 4x44K microarrays (AMADID 14850). The dual-color analysis method in which probes from specimens and from the reference are differentially labelled with Cyanine 5 and Cyanine 3 was used. A set of 4 dye swap experiments was performed to compare patient versus control MKs and 3 dye swap experiments, to compare shRUNX1- versus shSCR- transduced MKs. From each of the 4 combined experiments for patient MKs and from each of the 3 combined experiments for shRUNX1-transduced MKs, signatures were obtained by selecting genes with a *P*. value, obtained from a moderated t-test (LIMMA package), lower than $5x10^{-2}$ and with hybridization intensity more than 100 in at least one of two samples hybridized in dye swap for each experiment. List of significantly deregulated genes is provided in Online Supplementary Table S3. Microarray data and protocols have been submitted to ArrayExpress at the European Bioinformatics Institute under the number E-MTAB-5598. Venn's diagrams were obtained by using Venny 2.1.0. (http://bioinfogp.cnb.csic.es/tools/venny/index.html).[1] The DEGs (differentially expressed genes) common for patient MKs and shRUNX1-transduced MKs were analyzed using Cytoscape 3.5.1 and ClueGO plug-in (version 2.3.5; http://apps.cytoscape.org/apps/cluego) to decipher functionally grouped gene ontology and pathway annotation networks.[2] Benjamini-corrected Term and Group values of $P < 0.05$ were considered statistically significant. The settings were GO level 5-18, Kappa Score = 0.4, H. sapiens [9606], genes in GO_ImmuneSystemProcess-EBI-QuickGO-GOA, GO_BiologicalProcess-EBI-QuickGO-GOA. Additionally, the genes from Gene Product Association Data for Platelet Activation GO:0030168 were subsequently uploaded into ClueGO, and the node group "GO_Platelet" was created. Taking into account the supporting experimental evidence regarding the *TREML1* gene, the P value of 0.59 was included for this analysis (file:///C:/Users/Proteomics/Downloads/functions_pathways_pval_whitepaper_85.pdf).

Chromatin Immunoprecipitation (ChIP) and promoter activity assays

ChIP assays were performed on 4 x 10^6 sorted ($CD41^+CD42^+$) MKs using the ChIP assay kit (17-295, Merck Millipore), with the anti-RUNX1 antibody (ab23980, Abcam, Paris, France), nonspecific rabbit IgG (ab171870, Abcam, Paris, France) and anti-H3K27ac (pAb 39133, Active Motif, Paris, France). 1% sonicated chromatin was used as input for qPCR analysis. Enriched DNA from ChIP and Input DNA fragments were end-repaired, extended with an 'A' base on the 3′ end, ligated with indexed paired-end adaptors (NEXTflex, BiooScientific) using the Bravo Platform (Agilent), size-selected after 4 cycles of PCR withAMPure XP beads (Beckman Coulter) and amplified by PCR for 10 cycles more. Fifty-cycle single-end sequencings were performed using Illumina HiSeq 2000 (Illumina, San Diego, CA). Reads were aligned *in toto* to human genome hg19 with BWA aln (v0.7.5a) and peak calling assessed using MACS 2.0 with a q-value cut-off of 10^{-4} for RUNX1, and 10^{-3} for H3K27ac mark. Integrative Genomics Viewer (IGV 2.3.69) was used for representation. The *ITGA2* and *TREML1* regulatory regions (RR) were identified by the overlap between H3K27ac mark and RUNX1 peak (Figure 2A, B) in intragenic regions for both *ITGA2* and *TREML1*. ChIP-qPCR data are presented as percent input using the formula 2^(Ct (Input) - Ct (ChIP). Primer sequences are listed below. To assess promoter activity, *ITG2A* and *TREML1* promoter regions were cloned upstream of mCherry, which was inserted into a sinPRRL-PGK-GFP lentiviral vector. The *ITGA2* and *TREML1* regulatory regions (RR) were cloned upstream of the promoter regions, using the In-Fusion HD Cloning Kit (Ozyme, France). Putative RUNX1 binding sites in RR regions were identified using the ChipMapper online software and these sites were deleted using the In-Fusion HD Cloning Kit (Ozyme, Montigny-le-Bretonneux, France). For genome position, see Figure 2C and Online Supplementary Figure S2. Primer sequences are listed below. $CD34^+$ cells isolated from leukapheresis samples were grown as described above and, on days 6 and 7 of culture, cells were transduced with lentiviral particles. On day 10 of culture, cells were stained with APC-conjugated anti-CD41 antibody for 30 minutes at 4°C, and the $CD41^+GFP^+$ gate was analyzed for mCherry expression dependent on promoter/RR activity on the LSRFortessa analyzer (BD, Mountain View, CA, USA).

Primers used for ChIP	Forward	Reverse
ITGA2_A	AAGCATGACCCTCTTCATCC	TTGTGGTGTCATGGCAACTT
ITGA2_B	CATCCTTTCTGGCTCCTCTG	TGGCAGGAGTTCGAGAAGTT
ITGA2_C	CTGTCCCACACTGGCCTTAT	GCAAGAGACAGTGGCATGAA
ITGA2_D	CTTCCGTCAACTGGTGACAA	TTCTCTTGTGGGGAATCTGC
ITGA2_NC	GAGCAGAGAATGGGCATCTT	GCCCCTCAAAACAACTCATC
TREML1_A	GCTCCAGGATGTCAAAGCTC	TCCTGCAGGGTAACCATTTC
TREML1_B	TTCTCATCTCAAGCCTGTGG	GAATGAGGAGGGCAAAGGAT
TREML1_NC	GATAGTGCCACTGCACTCCA	AGCATTTTCCCTTCCCTTGT

Primers used for cloning promoter and regulatory regions of *ITGA2* and *TREML1*	
cherry-cloning-F	ctcgagccaccatggtgagcaag
cherry-cloning-R	TATcAAGcTTcTcGATTATCTAGATCCGGTTGATC
Cherry-infusion_MLU1_F	TCCTAGGCCTACGCGgccaccatggtgagcaag
Cherry-infusion_MLU1_R	GCATGCCGGCACGCGttatctagatccggttgatc
TREML1pr_pRRL_infusion_F	gAgACTAgCCTCgAgCTCTTCACACAACACGCCT
TREML1pr_pRRL_infusion_R	ccatggtggctcgagGGGCTTGCCTGGGCACTG
TREML1RR_F	TCCCAGGGAGTCTCATCTTG
TREML1RR_R	AAGCTCCATTCTGGTGCAGT
TREML1RR_infusion_F2	TCgACggTATCggTTAAGCTCCATTCTGGTGCAGT
TREML1RR_infusion_R2	TTTcTTTTAAAAGTTTCCCAGGGAGTCTCATCTTG
TREML1RR_RUNXdel_F	TCgACggTATCggTTTGCCGGTTCTTGCCGGAG
TREML1RR_RUNX1del_R	TTTcTTTTAAAAGTTGGCTTGAGATGAGAAGGATT
ITGA2pr_pRRL_infusion_F	gAgACTAgCCTCgAgTCCTTCACAAAAGTAGTCACTG
ITGA2pr_pRRL_infusion_R	ccatggtggctcgagCGAGGGATACGAGAACCTG
ITGA2RR_infusion_F	TCgACggTATCggTTGTTTCCAGCTCCATCCATGT
ITGA2RR_infusion_R	TTTcTTTTAAAAGTTTTCTCTTGTGGGGAATCTGC
ITGA2RRdel1_R	TGGGGGATGAAGAGGGTCATGC
ITGA2RRdel1_infusion_R	atgaggtttaaggtgTGGGGGATGAAGAGGGTCATGC
ITGA2RRdel2_F	CACCTTAAACCTCATCCTTTCTGG
ITGA2RRdel2_infusion_F	cctcttcatcccccaCACCTTAAACCTCATCCTTTCTGG
ITGA2RRdel2_R	CATGGCAACTTGGTTAGGAATAG
ITGA2RRdel2_infusion_R	gcaggagttcgagaaCATGGCAACTTGGTTAGGAATAG
ITGA2RRdel3_F	TTCTCGAACTCCTGCCAAATTC
ITGA2RRdel3_infusion_F	aaccaagttgccatgTTCTCGAACTCCTGCCAAATTC
ITGA2RRdel3_R2	CAGATTGAATTTGGTAAGTGAGCC
ITGA2RRdel3_infusion_R2	tggtagggaaaagaataaggcCAGATTGAATTTGGTAAGTGAGCC
ITGA2RRdel4_F2	GCCTTATTCTTTTCCCTACCA
ITGA2RRdel4_infusion_F2	ggctcacttaccaaattcaatctgGCCTTATTCTTTTCCCTACCA
ITGA2RRdel4_R	TCTGCTTTTGAGACATTTGGG
ITGA2RRdel4_infusion_R	tttCttttaaaagttTCTGCTTTTGAGACATTTGGG

Real-time analysis of *RUNX1, ITGA2, ITGB1,* and *TREML1* transcripts

RNA was isolated from 2x 10^9 washed, leukocyte-depleted platelets and lysed with Trizol (Gibco-BRL. Grand Island, NY, USA), as described,[3] or from MKs using the RNeasy Micro Kit (Qiagen, France). Expression levels of *RUNX1*, *ITGA2*, *ITGB1*, and *TREML1* were measured in triplicate by real-time PCR relative to *GAPDH*, *PPIA* or *HPRT* using SYBR® Green in an iCycler (Bio-Rad, Life Science) or a 7500 Real-Time PCR (Applied Biosystems). Primer sequences are detailed below.

Primers used for qPCR	Forward	Reverse
RUNX1	TGCAAGATTTAATGACCTCAGGTTT	TGAAGACAGTGATGGTCAGAGTGA
ITGA2	TTGCGTGTGGACATCAGTCTGG	GCTGGTATTTGTCGGACATCTAG
TREML1	CCCCAGATTTTGCACAGAGT	AATGCGTTCTCAGCCAGACT
ITGB1	TGGGTGGTGCACAAATTCAAC	TCTTTGGAGCCTCTGGGATTT
PPIA	GTCAACCCCACCGTGTTCTT	CTGCTGTCTTTGGGACCTTGT
GAPDH	ATCTTCCAGGAGCGAGATCC	CTGCAAATGAGCCCCAGCCT
HPRT	GGCAGTATAATCCAAAGATGGTCAA	TCAAATCCAACAAAGTCTGGCTTATAT

Rescue experiments in shRUNX1- MKs transduced with RUNX1 cDNA

Wild type RUNX1 cDNA was cloned into the lentivirus pRRL_EF1a_MCS/PGK-Cherry and mutation in 4 nucleotides, keeping the same aminoacids, were introduced to avoid the recognition of cDNA by shRUNX1_2 ($RUNX1^{mut}$). $CD34^+$ cells were isolated from leukapheresis, cultured in presence of TPO and SCF and transduced at day 6 and 7 of culture with lentivirus expressing shRUNX1_2 and Cherry and lentivirus expressing $RUNX1^{mut}$ and GFP. $Cherry^+GFP^+CD41^+$ cells were sorted at day 10 of culture. Transcript levels of RUNX1, *TREML1* and *ITGA2* normalized to *HPRT* were assessed by qPCR.

Western blot analysis of platelet TLT-1 and integrin subunit α2

Platelet-rich plasma was obtained by centrifugation at 200 *g* at room temperature for 10 minutes. After leukocyte depletion (Purecell PL; Pall Biomedical Products, East Hills, NY, USA) and red blood cell lysis, platelets were washed and lysed, as described,[3] followed by SDS-PAGE. For immunoblotting, membranes were probed with rabbit anti-human TLT-1 (kindly provided by Paquita Nurden, Pessac, France) or anti-integrin α2 (Abcam, Cambridge, MA) antibodies, followed by HRP-conjugated secondary antibodies and enhanced chemiluminescence detection. Membranes were reprobed with mouse anti-β-actin

(SigmaAldrich, St. Louis, MO, USA) to assess protein loading and a ratio between TLT-1 or integrin subunit α2 and β-actin was calculated by densitometry.

Effect of anti-TLT-1 antibody in thrombin-induced platelet aggregation

Platelets were washed twice in modified Tyrode´s buffer (140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgCl_2$, 22mM sodium citrate, 0,55 mM glucose, 0,35% BSA, pH=6.5) after addition of 1µM PgE1 and resuspended at 300 $x10^9$/L in Tyrode´s buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 5 mM glucose, pH 7.4) supplemented with bovine serum albumin (BSA) 0.35%. Washed platelets were incubated with a mouse anti-human TLT-1 monoclonal antibody (provided by Valance Washington, University of Puerto Rico) 3.4 mg/mL, 1:20 dilution, under stirring during 3 minutes at 37ºC in a lumiaggregometer (ChronoLog, Havertown, PA, USA) and then challenged with suboptimal concentrations of thrombin (Biopool, Bray, Ireland), ranging from 0.01 to 0.035 U/mL. As patients had reduced response to thrombin, thrombin concentration was titrated until a response was achieved, ranging between 0.045 to 0.1 U/mL.

Potentiation of thrombin-induced fibrinogen binding by soluble TLT-1

Washed platelets (50 x 10^9/L) were incubated with 325 µg/mL recombinant soluble TLT-1 (provided by Valance Washington, University of Puerto Rico) at 37ºC during 15 minutes in the presence 10 µg/mL Alexa Fluor 488 human fibrinogen (Molecular Probes, Eugene, OR, USA) and stimulated with 0.05, 0.1 and 0.5 U/mL thrombin for 15 min. After stimulation, platelets were fixed and analysed on a flow cytometer. Mean fluorescence intensity was recorded and normalized to values obtained in simultaneously assayed control platelets stimulated with 0.05 U/mL thrombin, set as 1.

Platelet integrin subunit α2 and glycoprotein levels by flow cytometry

To assess integrin subunit α2 (GPIa), platelet-rich plasma was adjusted to 50$x10^9$/L, labelled with fluorescein isothiocyanate (FITC)-conjugated CD49b (BD Biosciences, San Jose, CA, USA) during 30 minutes at room temperature and analyzed on a flow cytometer. PE-conjugated anti-CD41 was used to identify the platelet population. Relative

fluorescence intensity (RFI) was calculated as the ratio between mean fluorescence intensity obtained with the CD49b antibody and the corresponding isotypic control. For comparison between assays performed on different days, results were normalized according to variations in RFI measurements of a sample belonging to the same healthy individual, which was run on every assay and set as a calibrator. All assays were performed in duplicate in one or two different blood samples. To assess levels of other glycoproteins (GP), platelets were labelled with FITC or PE-conjugated antibodies against integrin β1 (GPIIa) (CD29), integrin α5 (CD49e) (Biolegend, San Diego, CA, USA), GPVI, GPIIb (CD41a), GPIIIa (CD61), GPIb (CD42b) and GPIX (CD42a) and integrin α5 (CD49f) (BD Biosciences). In separate experiments, 200 x 10^3 platelets in PBS-diluted whole blood were stained, fixed and analysed as described above.

Platelet adhesion

Platelets were diluted in HEPES buffer containing 2 mM Cl_2Mg, 0.1% bovine serum albumin (BSA) and 5.5 mM glucose, and 35 x 10^9/L platelets were seeded on coverslips previously coated with 50 µg/mL fibrillar Horm collagen I (Nycomed, Munich, Germany), monomeric collagen I (kindly provided by Alessandra Balduini, Maria Enrica Tira and Cristian Gruppi, Pavia, Italy), 100 µg/mL human fibrinogen (Sigma-Aldrich) and 10 ug/mL convulxin (kindly provided by Martine Jandrot-Perrus, INSERM UMR 1148, CHU Bichat, Paris, France) on 24-well plates, and incubated during 30 minutes at room temperature. After removing non-adherent platelets and gentle washing, the adherent platelets were fixed, blocked with 1% BSA, labeled with Phalloidin-FITC or TRITC (Sigma Aldrich) and analyzed by fluorescence microscopy (Carl Zeiss GmbH, Germany). Results were expressed as the number of adherent platelets per field in images obtained with a 100x objective. At least 20 non-overlapping fields were analyzed for each sample. Assays were performed in duplicate or triplicate.

Flow chamber assays

Chamber slides were precoated with 100 ug/mL type I fibrillar collagen and placed in a flow chamber (GlycoTech, Gaithersburg, MD, USA). Platelets were pelleted from PRP, resuspended at 300 x10^9/L in RPMI 1640 supplemented with 1mM Cl_2Ca, 1mM Cl_2Mg and

BSA (1%), prewarmed at 37ºC and perfused through the flow chamber at 1 dyn/cm^2 for 10 min. After gentle washing, slides were fixed, labeled with Phalloidin-TRITC and analysed under fluorescence microscopy with a 40x objective. Surface area coverage was calculated using the Image J software after conversion of digital images to grayscale images. Experiments were performed in duplicate or triplicate.

Megakaryocyte α2 integrin expression and adhesion to type I collagen and fibrinogen

Megakaryocytes were cultured from patient and control peripheral blood CD34$^+$ cells, as detailed in Methods, harvested on day 12, stained with APC-CD41, PE-CD42 and FITC-CD49b or the corresponding isotypic controls and analyzed by flow cytometry. To evaluate adhesion, cells were used on day 12 of culture or, in separate experiments, CD41$^+$CD42$^+$ cells were sorted on day 10 by flow cytometry. On day 12, 0.5-1 x 10^5 cells were seeded on coverslips previously coated with 25 µg/mL fibrillar type I collagen (kindly provided by Alessandra Balduini, Maria Enrica Tira and Cristian Gruppi, Pavia, Italy) or Horm collagen (Takeda Austria GmbH, Linz, Austria) or 100 µg/ml fibrinogen (Merck-Millipore, Milan, Italy or Sigma-Aldrich, Saint-Quentin Fallavier, France) and placed on 24-well plates. Following 4- or 16-hour incubation at 37°C in 5% CO2, non-adherent cells were removed and, after gentle washing, adherent cells were fixed, stained with FITC-conjugated anti-CD61 or Phalloidin-FITC and Hoechst 33258 (Sigma-Aldrich) or Vectashield with DAPI (Molecular Probes, Saint Aubin, France) and analyzed under a fluorescence microscope (Carl Zeiss GmbH) with a 40x objective or under Leica TCS SP8 inverted laser scanning confocal microscope (Leica, Microsystem, France) with a 40x/1.15 numeric aperture (NA) oil objective. The number of adherent cells was counted in 10-20 non-overlapping fields and results were expressed as percentage of simultaneously assayed control cells. Experiments were performed in duplicate or triplicate.

Immunofluorescence for TLT-1 in megakaryocytes

In separate experiments, patient and control MKs were sorted as described above, cultured for 2 additional days and immunofluorescence analysis was performed using rabbit anti-human TLT-1 (kindly provided by Paquita Nurden, Pessac, France), mouse anti-vWF (kindly provided by Dominique Meyer, French Academy of Sciences, France), Phallodin

coupled to Alexa 633. Anti-rabbit Alexa-488 and anti-mouse Alexa-546 were used as secondary antibodies. Slides were mounted using Vectashield with DAPI (Molecular Probes) and visualized by confocal microscopy. Images were acquired under a Leica DMI 4000 SPE laser-scanning microscope, with a 63x/1.4 numeric aperture oil objective (Leica Microsystem) and image analysis was performed with the LASX software.

Effect of a blocking anti-TLT-1 antibody on normal megakaryocytes

Cord blood $CD34^+$ cells were cultured as described[4] and incubated at day 4 of culture with a mouse anti-human TLT-1 monoclonal IgG2a (provided by Valance Washington, University of Puerto Rico) 3.4 mg/mL, 1:100 dilution, or the same concentration of a control mouse IgG2a (R&D, Minneapolis, MN, USA). At day 11 of culture, MK output (number of $CD61^+$ cells), percentage of mature MKs ($CD61^+CD42^+$ cells) and cell viability, determined by trypan blue exclusion, were assessed. In separate experiments, leukapheresis- or cord blood- derived $CD34^+$ cells were incubated at day 9 or 11, respectively, of culture with increasing concentrations of anti-TLT-1 antibody or control IgG. After 24-72hs, the percentage of MKs forming proplatelets was counted, as described,[4,5] under an inverted microscope (Axiovert 25, Carl Zeiss GmbH, Göttingen, Germany).

Supplementary Tables

Table S1. Coagulation factors and VWF levels in FPD/AML patients

	APTT	PTs	I	II	V	VII	X	VIIIC	IXC	XIC	XII	XIII	VWF:Ag	VWF:RCo	α2-antiplasmin
	sec	sec	g/L	%	%	%	%	%	%	%	%	%	%	%	%
BIII-1	43	14.4	2.73	83	93	84	76	78	95	110	45	132	73	70	100
BII-2	38	13	3.06	91	105	113	104	140	101	121	75	100	139	142	96
DII-1	34.5	10.8	2.61	98	110	102	104	121	109	nd	nd	nd	95	87	nd
DIII-1	31	10.8	2.96	110	108	103	102	102	89	nd	nd	nd	142	160	nd
DIII-3	36.2	10.8	2.88	105	103	112	99	117	109	nd	nd	nd	71	65	nd
Ref. range	26-41	12.2-15.2	2.0-4.0	60-140	60-140	60-140	60-140	50-150	50-150	70-130	60-150	50-150	50-150	50-150	80-130

Table S2. List of experiments performed for each patient

Patient	**AII-1**	**AII-2**	**BII-2**	**BIII-1**	**DII-1**	**DIII-1**	**DIII-3**
Platelet aggregation	x	x	x	x	x	x	x
Expression of platelet receptors by flow cytometry	x	x	x	x	x	x	x
Coagulation factors	nd	nd	x	x	x	x	x
Transcriptome analysis of mature megakaryocytes	x	x	x	x	nd	nd	nd
Gene expression levels in platelets by qPCR	nd	nd	nd	nd	x	x	x
Western blot for α2 integrin subunit and TLT-1	nd	nd	nd	nd	x	x	x
Inmunofluorescence for TLT-1 in megakaryocytes	nd	nd	nd	nd	x	nd	x
Platelet aggregation with a blocking anti-human TLT-1 antibody	nd	nd	nd	nd	x	x	x
Soluble TLT-1-induced potentiation of fibrinogen binding	nd	nd	nd	nd	x	nd	x
Platelet Adhesion	nd	nd	nd	nd	x	x	x
Megakaryocyte Adhesion	nd	nd	nd	x	x	x	x

nd means not done.

Table S3. List of genes significantly up- or down-regulated, respectively, in (A) and (B) FPD/AML vs, control megakaryocytes; (C) and (D) shRUNX1 vs. shSCR-transduced megakaryocytes; (E) and (F) both patient and shRUNX1-transduced megakaryocytes.

Previously reported RUNX1-targets are highlighted in red colour.

Table S4. GO terms associated to (A) up- and (B) down-regulated genes

Table S5. Whole blood flow cytometry of platelet glycoproteins

Patient	Relative fluorescence intensity (patient/control ratio)						
	GPIa (α2)	GPIIa (β1)	GPVI	GPIIb	GPIIIa	GPIb	GPIX
DII-1	0.36	0.47	0.77	0.71	0.71	1.37	1.24
DIII-1	0.61	0.84	0.80	0.65	0.73	1.14	1.23
DIII-3	0.45	0.53	0.97	0.70	0.91	1.03	1.09

Relative fluorescence intensity (RFI) was calculated as the ratio between the corresponding antibody and the isotypic control. Results are expressed as the ratio between RIF in each patient and a simultaneously assayed control sample.

Table S6. Platelet adhesion to type I collagen and fibrinogen-coated surfaces

	Monomeric collagen	Fibrillar collagen	Convulxin	Fibrinogen
DII-1	43.9	66.9	122	90.1
DIII-1	35.3	50.6	105	81.2
DIII-3	31.5	82.8	81	95.9

Results are expressed as percentage of a simultaneously assayed control sample, set as 100%.

Supplementary Figures

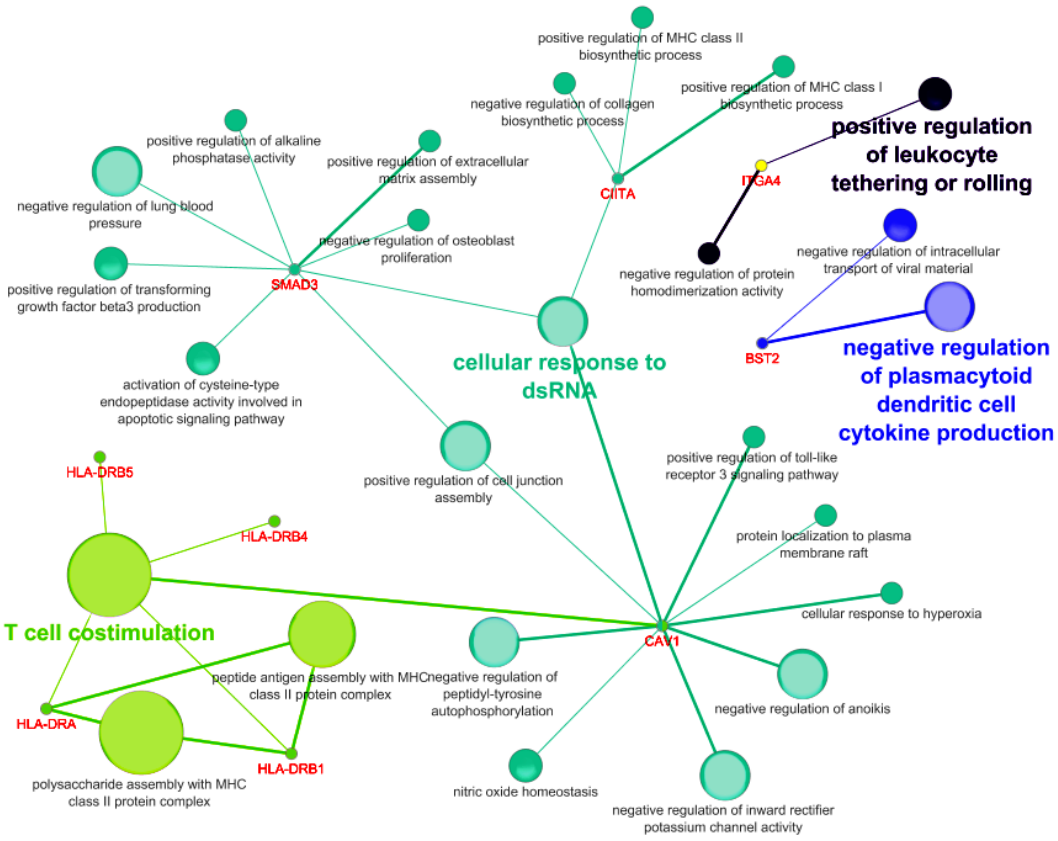
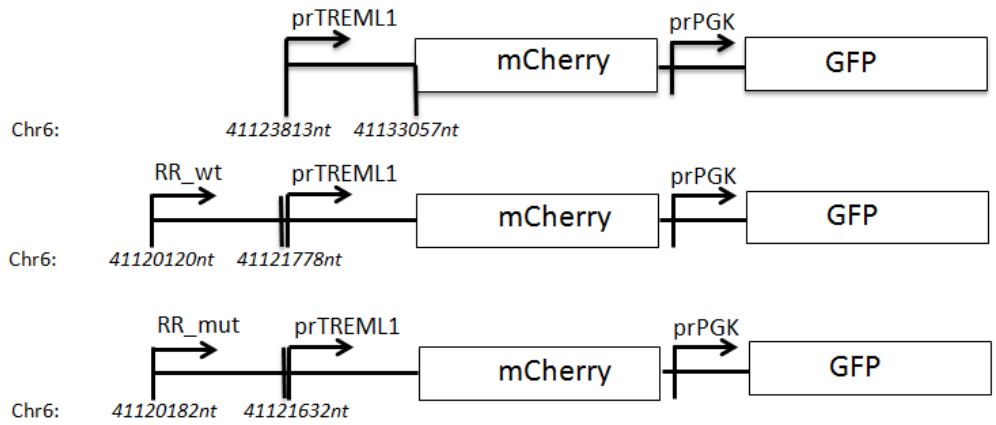


Figure S1. Differentially up-regulated genes and functions associated with different biological processes. GO terms and differentially up-regulated genes are represented as nodes based on their kappa score more than 0.4. The node size represents the GO terms enrichment significance.

A.



B.

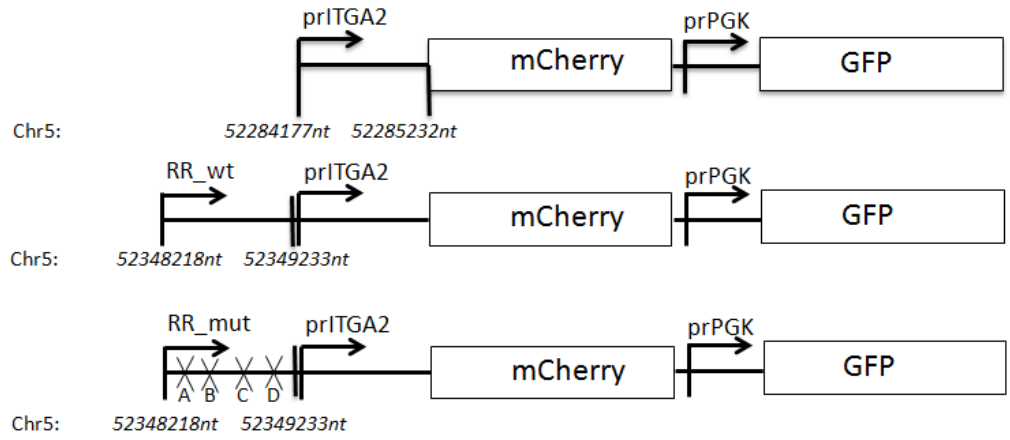


Figure S2. Schematic representation of lentiviral vectors used for regulation of *TREML1* and *ITGA2* transcription. mCherry was cloned upstream of a PGK-GFP cassette into the sinPRRL-PGK-GFP lentiviral vector. 1761nt *TREML1* and 1055nt *ITGA2* promoters were cloned upstream of mCherry cDNA. Regulatory regions identified by ChIP-seq containing RUNX1 binding sites were cloned upstream of their respective promoters. A. In the *TREML1* construct, RUNX1 binding sites are located at both extremities of the regulatory region and both sites were entirely deleted (RR_mut). B. For *ITGA2*, A-D indicate RUNX1 binding sites, their precise position is indicated in Figure 2C. Localization in the genome is indicated according to the UCSC database (CGCh37/hg19).

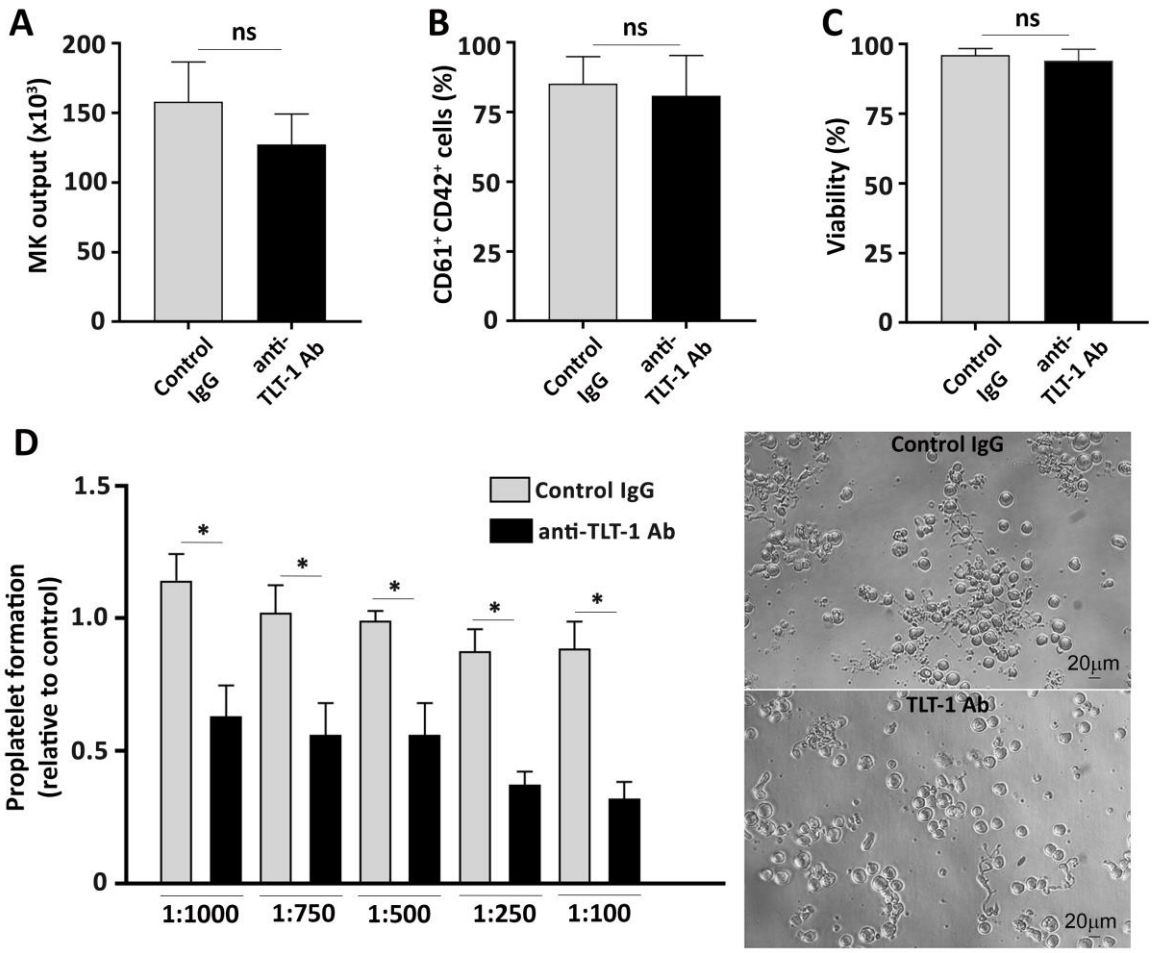


Figure S3. Effect of anti-TLT-1 antibody on normal megakaryocytes. A. Cord blood-derived MKs were incubated with a blocking anti-TLT-1 antibody (Ab), 1:100 dilution, or control IgG at the same concentration since day 4 of culture. Megakaryocyte ($CD61^+$ cells) output (A), percentage of mature MKs ($CD61^+$ $CD42^+$ cells) (B) and cell viability (C) were measured at day 11 of culture, n=4 independent experiments. Bars represent mean and mean error; ns, not significant. D. Cord blood- or leukapheresis-derived MKs were incubated at day 11 or day 9 of culture, respectively, with increasing concentrations of a blocking anti-TLT-1 Ab (black bars) or a control IgG (gray bars), used at the same concentration. After 24 or 48-hour incubation in 96-well plates, MKs forming proplatelets were scored by enumeration of no less than 200 cells per well using an inverted microscope (Axiovert 25, Carl Zeiss GmbH, Göttingen, Germany) and expressed relative to control cells cutured without any antibody. Bars represent mean and standard mean error of 6 independent experiments (n=5 for cord blood-derived cells and n=1 for leukapheresis-

derived cells). * *P*<0.05. Representative images of MKs incubated with control IgG or anti-TLT-1 antibody obtained at 200x magnification are shown.

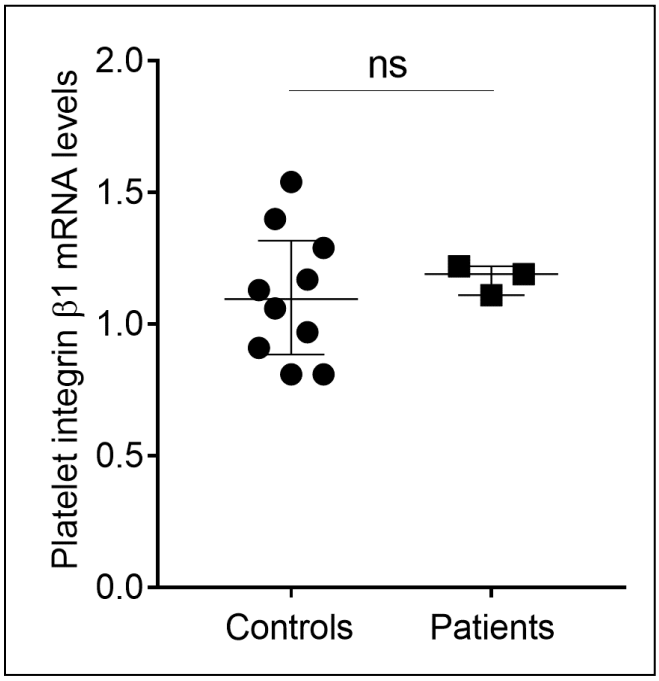


Figure S4. Platelet integrin subunit β1 mRNA levels. A. Real-time PCR analysis of *ITGB1* transcript levels normalized to *GAPDH* in platelets from patients of pedigree D harboring the T219Rfs*8 mutation (n=3) and healthy subjects (n=10); ns, not significant.

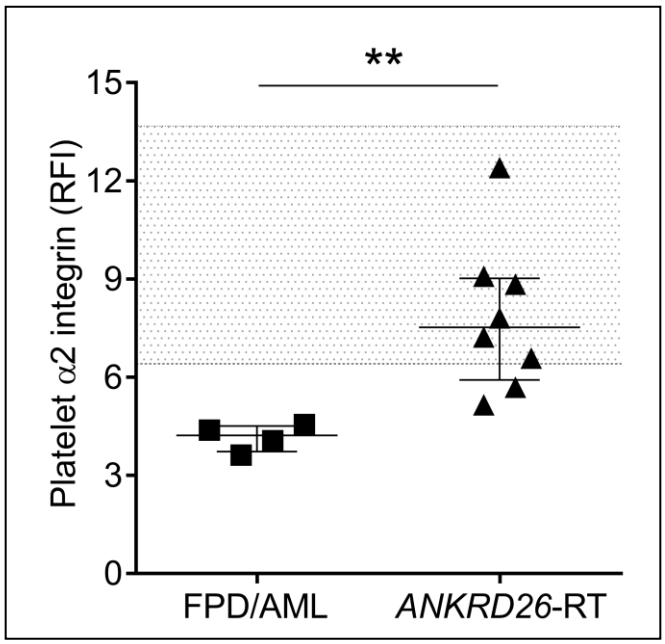


Figure S5. **Comparison of platelet surface integrin subunit α2 expression between patients with FPD/AML and *ANKRD26*-related thrombocytopenia.** Platelet surface expression of integrin subunit α2 in FPD/AML (n=4) vs. *ANKRD26*- related thrombocytopenia (RT) (n=8, from 3 different pedigrees) by flow cytometry. Results are expressed as the ratio between fluorescence intensity obtained with anti-CD49b antibody and the corresponding isotype control (RFI). Median values and interquartile range are depicted. ***P*<0.01. Filled space between the horizontal dashed lines represents upper and lower limits of the reference range, established by the mean ± two standard deviation of healthy subjects.

Supplementary references

1. Oliveros, J.C. (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. http://bioinfogp.cnb.csices/tools/venny/index.html.

2. Bindea G, Mlecnik B, Hackl H et al. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics. 2009;25(8):1091-1093.

3. Glembotsky AC, Bluteau D, Espasandin YR et al. Mechanisms underlying platelet function defect in a pedigree with familial platelet disorder with a predisposition to acute myelogenous leukemia: potential role for candidate RUNX1 targets. J Thromb Haemost. 2014;12(5):761-772.

4. Espasandin YR, Glembotsky AC, Grodzielski M et al. Anagrelide platelet-lowering effect is due to inhibition of both megakaryocyte maturation and proplatelet formation: insight into potential mechanisms. J Thromb Haemost. 2015;13(4):631-642.

5. Bluteau D, Glembotsky AC, Raimbault A et al. Dysmegakaryopoiesis of FPD/AML pedigrees with constitutional RUNX1 mutations is linked to myosin II deregulated expression. Blood. 2012;120(13):2708-2718.