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Mechanisms underlying platelet function defect in a pedigree with FPD/AML: potential role for candidate RUNX1-targets

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Short title

Platelet function defect in FPD/AML

Summary

Background

FPD/AML is an inherited platelet disorder caused by germline *RUNX1* mutation and characterized by thrombocytopenia, platelet function defect and leukemia predisposition. The mechanisms underlying FPD/AML platelet dysfunction remain incompletely clarified. We aimed to determine the contribution of platelet structural abnormalities and defective activation pathways to the platelet phenotype. In addition, by using a candidate gene approach, we sought to identify potential RUNX1-regulated genes involved in these defects.

Methods

Lumi-aggregometry, α- and dense- granule content and release, platelet ultrastructure and $\alpha_{IIb}\beta_3$ integrin activation and outside-in signaling were assessed in members of one FPD/AML pedigree. Expression levels of candidate genes were measured and luciferase reporter assay and chromatin immunoprecipitation were performed to study NF-E2 regulation by RUNX1.

Results

Severe decrease in platelet aggregation, together with defective $\alpha_{IIb}\beta_3$ integrin activation and combined αδ storage pool deficiency was found. However, while dense

granules were markedly reduced, α-granule content was heterogeneous. A trend towards decreased platelet spreading was found, while β_3 integrin phosphorylation was impaired, reflecting altered outside-in signaling. A decrease in transcription factor p45 NF-E2 was shown in platelet RNA and lysates, and other deregulated genes included RAB27B and MYL9. RUNX1 was shown to bind *NF-E2* promoter in primary megakaryocytes and wild-type RUNX1, but not FPD/AML mutants, was able to activate NF-E2 expression.

Conclusions

FPD/AML platelet function defect represents a complex trait and RUNX1 orchestrates platelet function by regulating diverse aspects of this process. This study highlights RUNX-1-target NF-E2 as part of the molecular network by which RUNX1 regulates platelet biogenesis and function.

Keywords

FPD/AML; RUNX1 protein; platelet function tests; NF-E2 transcription factor; Rab27B protein, human; blood platelet disorder

Introduction

Familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) is an inherited platelet disorder characterized by autosomal dominant thrombocytopenia, platelet function defect and a lifelong risk of development of hematologic neoplasms. Germline heterozygous mutations in transcription factor *RUNX1* underlie this disease [1], which is now being increasingly recognized due to heightened diagnostic awareness and more than forty families have been reported to

date. Thrombocytopenia, characterized by normal-sized platelets, is usually mild to moderate and, although present in most patients, low-normal or even normal platelet counts have been reported in some cases [2]. Severity of bleeding varies among affected individuals and tends to be more severe than expected according to the degree of thrombocytopenia, due to the presence of associated platelet function defect. Although the presence of platelet dysfunction seems to be an almost constant feature of this disorder, it is presently unknown whether all individuals with germline *RUNX1* mutation display this abnormality. FPD/AML represents a clinically heterogeneous condition. While some affected individuals develop leukemia and have mild or no bleeding diathesis [2], on the other hand, other patients may present with a predominant platelet function disorder without history of malignancy, as recently described in patients recruited to the UK genotyping and platelet phenotyping (GAPP) study [3]. Likewise, the spectrum and severity of platelet functional abnormalities vary among reported cases. In some patients, the predominant pattern seems to be a platelet secretion defect, while others show a more complex phenotype. The mechanisms by which *RUNX1* mutations lead to the platelet function defect remain incompletely understood. Although several studies have revealed the presence of δ-storage pool disease [4-7], it is unclear whether this abnormality fully accounts for the platelet phenotype. In this regard, impaired $\alpha_{IIb}\beta_3$ integrin activation has been shown in one FPD/AML patient, providing an alternative explanation for defective platelet function [8].

RUNX1 is a master hematopoietic transcription factor which dimerizes with CBFβ and is crucial for the establishment of definitive hematopoiesis during embryonic development. Although dispensable for the maintenance of adult hematopoietic stem cells, it plays an essential role in the lymphoid and megakaryocytic lineages [9], where it is involved in megakaryocyte development, platelet formation and function. FPD/AML represents a useful model to unravel the role of RUNX1 in platelet and megakaryocyte

biology. By using a complementary approach including the study of patient samples and shRNA-induced RUNX1 knockdown in primary megakaryocytes, Bluteau et al have recently shown that FPD/AML-related thrombocytopenia is due to decreased megakaryocyte maturation and polyploidization, as well as a block in proplatelet formation [10]. RUNX1-targets involved in decreased platelet production include the *MPL* receptor [11], myosin IIA (*MYH9*) and IIB (*MYH10*) and myosin regulatory light chain (*MYL9*) [10]. Besides *MYL9*, other RUNX1-target genes with a role in platelet function have been found to be decreased in one FPD/AML patient by platelet expression profiling, including platelet 12-lipoxygenase (*ALOX12*) and protein kinase C-θ (*PKCθ*) [12-16].

Considering that the mechanisms underlying FPD/AML platelet function defect and RUNX1-targets involved remain incompletely characterized, in this study, we aimed to determine the contribution of platelet structural abnormalities and defective activation pathways to the platelet phenotype. In addition, by using a candidate gene approach, we sought to further identify potential RUNX1-regulated genes involved in the platelet functional abnormalities.

Patients and Methods

Patients

Four members from a previously described pedigree harbouring the p.Thr219Argfs*8 *RUNX1* mutation, previously reported as p.Pro218fsX225 and renamed according to current nomenclature (www.hgvs.org/mutnomen) [11], were included in this study. Patient features are described in Table 1. During follow-up, patient (P) II.2 developed myelodysplastic syndrome and died after AML transformation. Platelet studies for this

patient were performed before transformation to MDS. The study was approved by the Institutional Ethics Committee and subjects gave written informed consent.

Light transmission aggregometry and ATP release

Platelet-rich plasma (PRP) was prepared by centrifugation at 200g during 10 min. and platelet aggregation was evaluated using a lumi-aggregometer (Chrono Log Corp, Havertown, PA, USA) after stimulation with 1mM arachidonic acid, 4ug/mL collagen, 1,5mg/mL ristocetin, 2µM ADP and 1uM epinephrine (Biopool, Bray, Ireland). ATP release was determined concurrently using luciferin-luciferase (Chrono-Lume, Chrono Log Corp).

Flow cytometry assays

To evaluate dense granule content, PRP ($5x10^7$/mL) was labelled with 2mM mepacrine during 30 min. at room temperature (RT) followed by flow cytometry analysis and a ratio between mean fluorescence intensity (MFI) obtained with and without mepacrine was calculated. For PAC-1 and P-selectin analysis, PRP ($1x10^8$/mL) was diluted 1:10 in modified Tyrode's buffer (134mM NaCl,12mM $NaHCO_3$, 2.9mM KCl, 0.34mM Na_2HPO_4, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM Hepes, 5mM glucose, pH=7.4), preincubated with fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody or anti-P-selectin (BD Biosciences, San José, CA, USA) and stimulated with 10µM ADP during 5 min. at RT. To measure fibrinogen binding, $1x10^8$/mL washed platelets in modified Tyrode's buffer were preincubated with 10µg/mL Alexa Fluor 488 human fibrinogen (Molecular Probes, Eugene, OR, USA) and stimulated with 0.25 U/mL thrombin (Biopool) during 5 min. at RT. After stimulation, platelets were fixed and analyzed. The platelet population was identified with phycoerythrin-conjugated CD42b.

Measurements were performed in duplicate. To determine PAC-1 or fibrinogen binding, MFI obtained after binding to unstimulated platelets was substracted from MFI obtained in activated platelets, whereas P-selectin was expressed as percentage of platelets positive for this marker. For comparison between measurements conducted on different days, MFI was normalized according to variation in control samples and expressed as mean fluorescence units (MFU).

Electron microscopy of platelets

To evaluate platelet ultrastructure, PRP was fixed with 5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, rinsed, postfixed in 1% osmium tetroxide in the same buffer and embedded in Araldite (Sigma-Aldrich, St Louis, MO, USA). Thin sections were stained with uranyl acetate (Riedel-d Haen, AG, Germany) and lead citrate (Sigma-Aldrich) and examined in a transmission electron microscope (Zeiss, EM 900, Germany).

Immunofluorescence analysis for thrombospondin-1

Platelet thrombospondin-1 (TSP1) content was assessed on blood smears by immunostaining with mouse anti-TSP1 antibody (Sigma-Aldrich) followed by FITC-conjugated goat anti-mouse antibody. Platelets were identified by F-actin labeling with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich) and at least 200 platelets were examined under a fluorescence microscope (Zeiss Axiostar Plus, Göttingen, Germany) by two independent observers. For analysis, platelets were classified according to the number of TSP1-positive granules into platelets displaying >5 granules and those with ≤5 granules, as described [18,19].

Platelet spreading

To assess platelet spreading, $5x10^7$/mL platelets were plated on glass coverslips coated with 100 ug/mL human fibrinogen (Sigma-Aldrich) during 30 min. at RT. Adherent platelets were fixed, permeabilized and stained with FITC-labelled phalloidin (Sigma-Aldrich). At least 200 platelets were analyzed by two different examiners under a fluorescence microscope and classified as round, filopodia-bearing or platelets with lamellipodia extension.

Tyrosine phosphorylation of β_3 integrin in platelets

To study β3 integrin phosphorylation, 6-well polystyrene plates were coated with 100 ug/mL human fibrinogen and blocked with 1% bovine serum albumin (BSA). Washed platelets ($3x10^8$/mL) resuspended in modified Tyrode's buffer containing 1mM $CaCl_2$ were added and incubated for 60 min. at RT. Nonadherent cells were removed and adherent cells were lysed in RIPA buffer (50mM Tris-HCl, pH=8, 150mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate). Lysates of non-adherent cells were prepared after plating on 3% BSA-coated plates. After SDS-PAGE, membranes were incubated with rabbit polyclonal anti-phospho integrin β3 (Tyr773) antibody (Abcam, Cambridge, UK) followed by horseradish-peroxidase (HRP)-conjugated anti-rabbit antibody and enhanced chemiluminescence (ECL) detection. Protein loading was assessed on duplicate membranes with rabbit anti-β3 integrin (Santa Cruz, CA, USA).

Gene expression analysis in platelets

To prepare platelet RNA, PRP was filtered through a leukocyte reduction filter (Purecell PL, Pall Biomedical Products Co, NY), as described [20], red blood cells were lysed

with 8.6 gr/L ammonium chloride and $1x10^9$ platelets were lysed in Trizol (Life Technologies, NY, USA). Expression levels of target genes were assessed in triplicate by real-time PCR relative to *GAPDH* in an iCycler (Bio Rad Life Science, CA, USA) using SYBR® Green (Life Technologies), primer sequences are listed in Supplementary Table. Serial cDNA dilutions were assessed to ensure similar amplification efficiency of the target and internal control genes.

Western blot analysis of platelet NF-E2 content

Platelet-rich plasma was subjected to leukocyte depletion and red blood cell lysis, as for preparation of platelet RNA, and washed platelets (2×10^9/mL) were lysed, as described [11], followed by SDS-PAGE. Membranes were probed with anti-NF-E2 rabbit IgG (Santa Cruz Biotechnologies), followed by HRP-conjugated secondary antibody and ECL detection and then reprobed with mouse anti-β-actin (Sigma Aldrich). A ratio between NF-E2 and β-actin calculated by densitometry was expressed as percentage of mean ratio of normal controls assayed on the same blot, set as 100%. Mean values from two different experiments were calculated.

Chromatin Immunoprecipitation (ChIP) and promoter activity assays

ChIP assays were performed with a ChIP assay kit (Millipore Upstate Biotechnology, Billerica, MA, USA) using anti–RUNX1 antibody (ab23980, Abcam). Assays were performed using chromatin prepared from human megakaryocytes, as previously described [21]. Immunoprecipitated DNA was analyzed on a PRISM® 7700 sequence detection system using SYBR® Green (Applied Biosystems, Saint-Aubin, France) in duplicate. Two independent experiments were performed. Promoter activity assays were performed in HEL cells, as previously described [21]. Primer sequences are listed in Supplementary Table.

Statistical analysis

Comparison between patients and controls was performed using Student´s *t*-test or Mann-Whitney-Wilcoxon test, as appropriate. Variables were analyzed for normality and equality of variances using Shapiro-Wilks and F-test, respectively. To account for variability in experimental conditions among flow cytometry measurements performed on different days, control and patient samples handled in parallel were analyzed as pairs using paired *t*-test. *P* values < 0.05 were considered significant. Analysis was performed with InfoStat (Universidad Nacional de Córdoba, Argentina) and GraphPad Prism 5 (La Jolla, CA, USA) software.

Results

Light transmission aggregometry and dense granule content and release

Platelet aggregation tests showed marked abnormalities in response to standard concentrations of ADP, epinephrine, collagen and arachidonic acid (Fig. 1), while response to ristocetin was largely preserved. Tests were performed on two to three occasions for each patient and mean values are depicted in Table 2. As shown, response to epinephrine and collagen was always severely impaired or absent, while aggregation traces in response to ADP and arachidonic acid were more variable among affected family members or during sequential evaluation in the same patient, ranging from severe decrease in primary wave to preserved primary response with lack of secondary aggregation. ATP release triggered by 2μM ADP was severely decreased or absent (Table 2), while ATP secretion in response to 1mM arachidonic acid was absent in PII.2 and PIII.3 and decreased in PII.1, who also showed absent ATP release in response to 1μM epinephrine (data not shown). Mepacrine uptake was reduced in patients (n=4) compared to controls (n=4), 0.61±0.21 vs. 1.00±0.26 MFU, *P*= 0.028, paired *t*-test, indicating that, besides impaired dense granule secretion, there is a

reduction in dense granule number and/or content. For each patient, the measurement was performed twice, with similar results.

Assessment of $\alpha_{IIb}\beta_3$ integrin activation

Agonist binding to their platelet receptors triggers inside-out signaling events that lead to $\alpha_{IIb}\beta_3$ integrin conformational change and exposure of binding sites for fibrinogen. To assess the contribution of impaired $\alpha_{IIb}\beta_3$ integrin activation to defective platelet aggregation, both PAC-1 and fibrinogen binding were evaluated. PAC-1 binding to platelets stimulated with 10μM ADP was decreased in patients compared to controls, 0.58±0.1 vs. 1.00±0.17 MFU, *P*= 0.01, paired *t*-test (Fig. 2A and C and Table 2), despite normal expression of αIIbβ3 on the platelet surface (data not shown). For each patient, the measurement was performed at least twice, with similar results. In two patients, response to 5μM and 20 μM ADP was found to be similarly impaired (data not shown). Accordingly, fibrinogen binding induced by 0.25 U/mL thrombin was decreased in patients compared to controls, 0.55±0.10 vs. 1.00±0.19 MFU, *P*= 0.04, paired *t*-test (Fig. 2B and D and Table 2), further indicating impaired $\alpha_{IIb}\beta_3$ integrin activation.

Electron microscopy of platelets

To evaluate whether *RUNX1* germline mutation leads to platelet structural abnormalities, electron microscopy was performed for two patients revealing heterogeneity in platelet size and shape, disorganized submembrane microfilaments and distended open canalicular system with vacuolated appearance. Marked decrease in the number of dense granules was found, while α-granule content was heterogeneous, as some platelets were completely devoid of granules, while others showed normal or near normal α-granule content (Fig. 3).

Immunofluorescence for α-granule marker Thrombospondin-1 and P-selectin expression

In order to quantify α-granules, immunofluorescence labeling for TSP1 was performed. Five or less than 5 α granules per platelet was used as a stringent cut-off value for α-granule deficiency, as reported [18, 19, 22]. A mild but significant increase in the percentage of platelets with severe α-granule reduction was found in patients compared to controls, 15±2.3% vs. 3.1±1%, p=0.03, Mann-Whitney-Wilcoxon test (Fig. 4A and B). Platelet activation leads to redistribution of P-selectin from α-granule membranes to the platelet surface, representing a marker for α-granule degranulation. P-selectin expression induced by 20μM ADP was decreased in patients (n=4) compared to controls (n=4), 38.7±16.8 vs. 68.5±16%, *P*= 0.008, paired *t*-test.

Outside-in signaling responses

Next, we explored whether abnormal outside-in mechanisms contribute to defective hemostasis in these patients. Fibrinogen binding to $\alpha_{IIb}\beta_3$ triggers downstream signaling cascades that stabilize the platelet aggregate and trigger cytoskeleton reorganization leading to cell spreading, filopodia and lamellipodia formation. Morphologic analysis of platelets adherent to fibrinogen revealed an increased percentage of platelets remaining round or displaying only filopodia, together with a decrease in platelets showing lamellipodia in patients compared to controls, 17.5±11.5 vs. 0.9±0.5, 29.4±10.2 vs. 12.4±3 and 53±16.3 vs. 86.6±1.95%, respectively, although the difference for each parameter was not statistically significant (Fig. 5A and B). Fibrinogen ligation leads to tyrosine phosphorylation of β3 subunit cytoplasmatic tail and downstream signaling substrates. Phosphorylation of β_3 integrin was impaired in patient platelets adherent to immobilized fibrinogen compared to control platelets (Fig. 5C).

Expression of candidate RUNX1-targets in platelets

To identify candidate RUNX1-targets with a possible role in the platelet functional defects, we first assessed gene expression levels of transcription factor p45 NF-E2, which plays a key role in platelet function [23], being essential for $\alpha_{IIb}\beta_3$ inside-out signaling [24] and for development of normal granule content [25]. Both NF-E2 1A and 1F transcripts were measured, which lead to the same protein but are regulated by alternative promoters [26]. We found a reduction in both isoforms, which, although potentially biologically relevant, did not reach statistical significance (Fig. 6A). To determine if reduced gene expression of NF-E2 led to decreased protein levels, we assessed NF-E2 content by Western blot in platelet lysates. A consistent reduction in NF-E2 was found in three evaluated FPD/AML patients, ranging from 33 to 62% of control levels (Fig. 6B), confirming that NF-E2 is downregulated in FPD/AML. Next, we studied downstream NF-E2 effectors with a possible role in the platelet phenotype. Concerning NF-E2 target genes likely to be involved in defective granule biogenesis, we found a trend towards reduced levels of RAB27B, which regulates intracellular vesicle trafficking and dense granule packaging [27,28], Fig. 6A. With regards to NF-E2-regulated genes implicated in inside-out $\alpha_{IIb}\beta_3$ activation, levels of Rap1 guanine nucleotide exchange factor CALDAG-GEFI, which represents a critical step in agonist-induced integrin activation [29, 30], were normal (Fig.6A), while platelet expression of alternative NF-E2 candidate targets with a role in $\alpha_{IIb}\beta_3$ activation, including caspase 12 [31] and thiol isomerase ERP5 [23, 32], was below the detection limit of our assay (data not shown). Besides, we confirmed a profound reduction in RUNX1-target MYL9 in platelets, (Fig. 6A), as already described in FPD/AML samples [10,12].

Chromatin Immunoprecipitation (ChIP) and promoter activity assays

To address whether NF-E2 is a direct transcriptional target of RUNX1, we performed

an *in silico* conserved sequence analysis [33] of its 2Kb 1A upstream promoter region, which is the main NF-E2 promoter activated in megakaryocytes, and identified potential RUNX1 binding sites (numbered 1 and 2 in Fig. 7A). Chromatin immunoprecipitation assays were performed in human primary megakaryocytes and showed that RUNX1 binds to site 2 localized in the F PCR amplified region (Fig. 7B). To test its functional relevance, we cloned this promoter region upstream of the *luciferase* gene and performed gene reporter assays. Over-expression of wild-type RUNX1 with its cofactor CBFβ increased luciferase activity in transient transfection assays in HEL cells, while, in contrast, R174Q or R139X RUNX1 mutants, which are not able to bind DNA [10], did not exhibit this transactivation property (Fig. 7C). Furthermore, it has been previously shown that RUNX1 binds the NF-E2 promoter *in vitro* and *in vivo* in granulocytes from polycythemia vera patients and HEL cells in a different region of the NF-E2 1A promoter, i.e. within the segment at bp –3376, –3235, and –3085 from exon 1A transcription start site (numbered 4, 5 and 6 in Fig. 7A) [34]. In primary megakaryocytes, we tested by ChIP these sites and showed that RUNX1 also binds sites 3, 4 and 5 localized in the A and B PCR amplified regions, but with a lower efficiency than it binds to site 2 (Fig. 7B).

Discussion

Inherited platelet function disorders provide useful tools to dissect the mechanisms involved in platelet activation and aggregation, as well as to clarify the function of genes involved in this process. In the present study, we have studied the effects of a germline *RUNX1* mutation in several members of a FPD/AML pedigree with severe platelet function defect [11]. We have shown abnormalities in platelet morphology and activation that may contribute, together with mild to moderate thrombocytopenia, to this bleeding disorder.

Regarding mechanisms underlying platelet dysfunction, we found decreased dense granule content by both mepacrine staining and electron microscopy, confirming previous findings in other FPD/AML pedigrees [4-7]. In addition, analysis of platelet ultrastructure revealed heterogeneous α-granule content and immunofluorescence for α-granule marker TSP1 confirmed the partial reduction in α-granules. Therefore, although combined α and δ granule deficiency was found, while δ granules were markedly and uniformly decreased, the reduction in α granules was variable, as described for patients with combined αδ storage pool deficiency (SPD). In agreement with our findings, partial α granule plus dense granule deficiency has been previously reported in another FPD/AML pedigree [35,36]. Study of additional patients will be required to establish the significance of α-granule deficiency in FPD/AML and its potential contribution to the defect in hemostasis. In addition, defective αIIbβ3 integrin activation was shown, indicating that impaired inside-out signaling represents an additional mechanism involved in platelet dysfunction. Abnormal αIIbβ3 activation has been previously reported for another FPD/AML patient, although in this case, dense granule content was normal and platelet dysfunction was proposed to rely solely on decreased αIIbβ3 function [8]. The finding that both SPD and impaired αIIbβ3 activation contribute to this phenotype highlights the complexity of FPD/AML platelet function defect.

To search for RUNX1-targets potentially involved in these specific abnormalities, i.e. SPD and impaired αIIbβ3 activation, we focused in transcription factor p45 NF-E2, which besides regulating terminal megakaryocyte maturation and proplatelet formation, plays a key role in both granule biogenesis and inside-out signaling. NF-E2 knockout mice display profound thrombocytopenia and megakaryocytes show a late arrest in maturation together with a striking granule deficiency [25] and failure to bind fibrinogen in response to agonists [24]. However, the virtual absence of platelets precludes the study of platelet function in this model. More recently, the essential role of NF-E2 in

platelet function was revealed in mice expressing a hypomorphic NF-E2 mutation, which show mild thrombocytopenia and reduced thrombin-induced aggregation and P-selectin expression [23]. In the current study, we show that *RUNX1* germline mutation leads to NF-E2 downregulation in FPD/AML patient samples, strongly suggesting that the decrease in this transcription factor could contribute to the platelet function phenotype. In addition, decreased NF-E2 could also participate in altered platelet biogenesis in this disorder. RUNX1 binding to the NF-E2 1A promoter was previously demonstrated in polycythemia vera granulocytes and HEL cells and NF-E2 expression has been shown to be positively regulated by RUNX1 [34]. More recently, NF-E2 was identified as a RUNX1-target in mouse fetal liver megakaryocytes by ChIP-seq analysis and reduced NF-E2 expression was found in conditional RUNX-1 deficient mice [37]. In this work, we confirm NF-E2 regulation by wild-type RUNX1 and show that two FPD/AML RUNX1 mutants [10], lose the ability to mediate NF-E2 promoter activation. In addition, we show that RUNX1 binding to the NF-E2 proximal 1A promoter occurs also in primary human megakaryocytes, which may represent a more relevant physiological setting, and describe a new RUNX1 binding site on this promoter. Altogether, these data point to NF-E2 as a key mediator of the effects of RUNX1 on the megakaryocyte lineage.

Regarding NF-E2 downstream effectors with a possible role in these specific platelet abnormalities, we were able to identify RAB27B decrease as a plausible explanation for FPD/AML-associated δ-SPD. This small GTPase regulates δ-granule number and platelet biogenesis [28], possibly coordinating granule transport and proplatelet formation. Besides being regulated by NF-E2, RUNX1 occupancy at the RAB27B promoter has been described in CMK cells [38], raising the possibility that RAB27B could also be directly regulated by RUNX1. Concerning candidate NF-E2-targets with a

role in αIIbβ3 activation, the potential implication of CALDAG-GEFI in defective inside-out signaling was ruled out, while the involvement of caspase 12 [31] and ERP5 [23,32] could not be ascertained because of their low platelet expression. In this scenario, gene expression analysis in megakaryocytes might be more rewarding. Alternatively, other, not yet identified, NF-E2-targets or genes directly regulated by RUNX1 could contribute to defective inside-out signaling in this setting. In this regard, and in contrast to findings by Sun et al [13], we did not find decreased platelet levels of RUNX1-target PKCθ, by real-time PCR (data not shown), which has been implicated in inside-out signaling triggered by GPVI and PAR agonists [39]. Considering that RUNX1 has been shown to be a master regulator of megakaryocyte gene expression program, it is plausible that reduction in more than one RUNX1-regulated gene may be responsible for the observed phenotypes. Further study of potential RUNX-1 targets may help dissect the gene expression program regulated by RUNX1 in the megakaryocyte.

Dysregulation of genes involved in cytoskeleton rearrangement has been previously demonstrated in FPD/AML. Among genes found to be dysregulated, a profound reduction in RUNX1-target MYL9 has been previously shown in megakaryocytes [10] and platelets [12] and confirmed in platelet samples from this pedigree. Besides RUNX1, recently, NF-E2 has been identified as a direct regulator of *MYL9* expression [23]. The profound decrease in MYL9 found in FPD/AML may thus be ascribed, not only to *RUNX1* mutation, but also, to reduced NF-E2 levels found in this study, which may contribute to this defect. Besides its role in platelet biogenesis, MYL9 plays a key role in platelet function [40], through regulation of myosin motor activity. Myosin is involved in several platelet contractile functions, such as platelet shape change, stress fiber formation and outside-in signaling, as demonstrated in mice with megakaryocyte-

restricted *myh9* inactivation, which display abnormal stress fiber formation, impaired outside-in signaling and decreased thrombus growth under flow conditions [41]. In addition, decreased MYL9 levels secondary to RUNX1 knock-down in HEL cells induces impaired cell spreading on fibrinogen [15]. We therefore assessed whether MYL9 deficiency is associated with a similar phenotype in FPD/AML patients and found an abnormal pattern of spreading for platelets adherent to fibrinogen, with increased round or filopodia-bearing platelets and decreased lamellipodia extension. Furthermore, adhesion to a fibrinogen matrix triggered integrin β3 phosphorylation in control platelets but this response was impaired in patient samples, indicating abnormal outside-in signaling events in FPD/AML. Together with abnormal inside-out signaling, this defect points to the key involvement of αIIbβ3 integrin, the major receptor involved in platelet aggregation and spreading, in FPD/AML pathogenesis.

In conclusion, the finding that both platelet structural abnormalities and defects in activation pathways underlie FPD/AML platelet function defect reveals that multiple abnormalities contribute to this complex trait and that RUNX1 orchestrates platelet function by regulating diverse aspects of this process. In addition, this study highlights RUNX1-target NF-E2 as part of the molecular network by which RUNX1 exerts its effects in the megakaryocyte lineage and regulates platelet formation and function. These data may contribute to current understanding of the molecular basis of platelet function.

Addendum

A. C. Glembotsky designed and performed experiments, analyzed data, discussed results and wrote the paper; D. Bluteau: designed and performed luciferase assay and ChIP experiments, analyzed data and discussed results; Y. R. Espasandin, N.P. Goette, R.F. Marta, C.P. Marin Oyarzun, L. Korin and P. R. Lev performed experiments

and discussed results; R. P. Laguens performed electron microscopy and interpreted results; F.C. Molinas provided patient samples and clinical data and discussed results; H. Raslova contributed to study design, designed experiments, analyzed data and wrote the paper; P. G. Heller designed the study, performed and supervised experiments, analyzed data and wrote the paper. All authors contributed to manuscript editing and final approval.

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Table 1. Patient clinical and laboratory features

	Age (years)	Gender (F/M)	Family member	Platelet count ($x10^9$/L)	MPV (fL)	Bleeding (WHO score)	Disease status at follow-up
PII.1	45	F	sister of II.2	145	8	3	stable
PII.2	37	F	propositus	72	9.2	3	MDS/AML
PIII.1	25	M	son of II.1	137	6.6	2	stable
PIII.3	22	F	daughter of II.2	98	7.5	2	stable

F, female; M, male; MPV, mean platelet volume; WHO, World Health Organization.

Reference values for MPV, 6.2-10.4 fL

Bleeding was assessed according to WHO bleeding scale [17]: grade 0 indicates no bleeding; 1, petechiae; 2, 3 and 4, mild, gross and debilitating blood loss, respectively.

Table 2. Platelet aggregation tests, ATP release and $\alpha_{IIb}\beta_3$ integrin activation in FPD/AML patients

	Platelet aggregation (%)					ATP release (ηmole)	PAC-1 (% of control)	FBG-binding (% of control)
	ADP	Epinephrine	Collagen	AA	Ristocetin			
PII.1	31 ± 16	14 ± 6.4	20 ± 0	28.5 ± 40.3	66 ± 0	0.11	64.8	50.2
PII.2	19.3 ± 5.5	0 ± 0	13 ± 4	8 ± 11.3	52.3 ± 9.2	0	44	ND
PIII.1	30 ± 3	15 ± 0	8 ± 7	4 ± 5.7	50 ± 0	ND	51.8	46.7
PIII.3	34.3 ± 8.7	18.7 ± 1.5	21.3 ± 5.5	33.7 ± 40.8	59.7 ± 23.3	0	58	69.2

AA, arachidonic acid; ND, not done; FBG, fibrinogen.

Mean±SD platelet aggregation, expressed as percentage maximal light transmission, in response to 2μM ADP, 1μM epinephrine, 4μg/mL collagen, 1mM arachidonic acid and 1.5 mg/mL ristocetin is shown. Mean±SD values (%) in healthy controls (n=60) were 75±9, 78±9.5, 77±11, 79±9 and 82±8.6, respectively.

ATP release values normalized to platelet count (1 x 10^8/mL) in response to 2 μM ADP are shown. Mean±SD values in healthy subjects (n=40) were 0.72 ± 0.40 ηmole ATP/ 1 x 10^8/mL platelets.

PAC-1 and fibrinogen binding were expressed as percentage of a simultaneously assayed control sample.

Figure legends

Figure 1. Platelet aggregation by light transmission aggregometry in FPD/AML patients. Representative traces in response to different agonists, as indicated, show severe decrease in platelet aggregation for patients (PII.1, PII.2, PIII.1, PIII.3), as compared to a control (C) sample.

Figure 2. Flow cytometry analysis of integrin $\alpha_{IIb}\beta_3$ activation. (A) ADP-induced-FITC-conjugated PAC-1 binding to PRP (n=4 for patients and controls) and (B) thrombin-induced Alexa Fluor 488-conjugated fibrinogen binding to washed platelets (n=3 for patients and controls) show decreased binding in patients compared to controls.

Binding to unstimulated platelets was substracted from values obtained after agonist stimulation and data are expressed as mean fluorescence units (MFU) (mean ± SEM), * $P < 0.05$. Representative (C) PAC-1 and (D) fibrinogen binding analysis. Agonist-induced binding to platelets from a FPD/AML patient (right panel) and a simultaneously studied healthy control (left panel) is depicted by the open black histogram, while the solid gray trace represents binding to unstimulated platelets.

Figure 3. Electron microscopy of blood platelets. (A) Representative image from a patient sample showing round platelets, enlargement of the open canalicular system with a vacuolated appearance, decrease in alpha granules and absence of dense granules (B) Representative image from a healthy control showing a platelet with normal granule content. Scale bar: 1.1µm.

Figure 4. Immunofluorescence labeling for α-granule marker Thrombospondin-1 (TSP1). Blood smears were fixed, permeabilized, and stained with TSP1 primary antibody followed by FITC-conjugated secondary antibody (green fluorescence) and platelets were identified by TRITC-conjugated phalloidin (red fluorescence). (A) The percentage of platelets harbouring ≤5 TSP1-positive granules, which represents a stringent cut-off value for α-granule deficiency, was increased in patients (n=4) compared to controls (n=10). Data are expressed as mean ± SEM, * $P < 0.05$. (B) Representative image of platelets from a healthy individual (left panel) display a packed granular pattern, while the staining pattern is heterogeneous in the patient (right panel), with some platelets displaying normal TSP1 content while another (arrow) shows a single TSP1-positive granule. Scale bar: 5 µm.

Figure 5. Outside-in signaling events in FPD/AML platelets. (A) Platelets were plated on fibrinogen-coated coverslips and actin fibers were labelled with FITC-phalloidin. Patients (n=3) show a trend for higher percentage of platelets remaining round or displaying only filopodia, while lamellipodia extension is decreased compared to

controls (n=3). Data are expressed as mean ± SEM, $P > 0.05$. (B) Representative image showing morphology of platelets adherent to fibrinogen in a control (left panel) and patient (right panel) sample. Scale bar= 5 μm (C) Western blot analysis of β3 integrin phosphorylation is shown in panel (i). Washed platelets were layered over fibrinogen-coated plates for 60 min. After removal of non-adherent cells, reaction was stopped with lysis buffer and lysates were subjected to SDS-PAGE and probed with anti-phospho (Tyr773) β3 integrin antibody. Protein loading was assessed with anti–β3 integrin antibody. Control platelets show enhanced β3 integrin phosphorylation upon adherence to immobilized fibrinogen compared to non-adherent platelets, while the response is blunted in patient platelets. Densitometric analysis of band intensity is shown in (ii).

Figure 6. Analysis of candidate RUNX1-targets in platelets. (A) Gene expression analysis. Expression levels of target genes relative to GAPDH were assessed in triplicate by real-time PCR in FPD/AML patients (n=3) compared to controls (n=3). Patients show decreased expression of transcription factor p45 NF-E2, isoforms 1A and 1F. Among NF-E2 targets, a trend towards reduced RAB27B was found (n=4 for patients and controls), while CalDAG-GEFI expression was preserved. In addition, a profound reduction of RUNX1-target MYL9 is shown. Data are depicted by mean ± SEM; ** $P < 0.01$ for MYL9; $P > 0.05$ for all other genes. (B) Western blot analysis of NF-E2 content. Platelet lysates were subjected to SDS-PAGE and membranes were probed with anti-NF-E2 antibody followed by HRP-conjugated secondary antibody and ECL detection. To assess protein loading, membranes were reprobed with anti-β-actin. Representative blot of two different experiments shows decreased NF-E2 levels in patients (PII.1, PIII.1, PIII.3) compared to controls (C1, C2, C3).

Figure 7. NF-E2 regulation by RUNX1. (A) Schematic representation of the NF-E2 human promoter region. The arrow represents the putative transcription start site. *Italic numbers* designate genomic positions in UCSC hg18 assembly

(http://genome.ucsc.edu/). Gray boxes numbered from 1 to 6 designate *in silico* putative RUNX1 binding sites (http://bio.chip.org/mapper/). Black bars (A-F) designate genomic positions of amplicons used in ChIP analysis. C amplicon does not contain putative RUNX1 binding site and was used as a negative control. (B) Chromatin immunoprecipitation assay. ChIP analysis in mature $CD41^{+}CD42^{+}$ megakaryocytes was performed with primer sets (NF-E2_A to _F) directed toward RUNX1–binding sites predicted *in silico*. The RUNX1 binding site N°2 is localized inside amplicon F (between 52 982 728 – 52 982 733 nt, hg18). Sonication after ChIP experiment generates about 200bp DNA fragments and both E and F primer sets are able to amplify this immunoprecipitated region. The histogram represents the percentage of input after immunoprecipitation using anti-RUNX1 or IgG. Error bars represent SD of Q-PCR triplicate. * $P < 0.05$. (C) NFE2-promoter luciferase assay with wild-type promoter (NFE2-Luc) containing RUNX1 binding site N°2. Luciferase assay was performed by transient cotransfection of HEL cells with 500 ng of MPI vector containing RUNX1 wild type (WT), RUNX1 R174Q or RUNX1 R139X cDNAs and pEF6/V5-His-TOPO vector containing CBFβ. Luciferase levels are shown as fold change relative to cells transfected with the promoter construct NF-E2 Luc alone. The total amount of transfected DNA was kept constant by transfection with an empty MPI vector. The histograms show one representative experiment of two, each in triplicate. Error bars represent SD of the two experiments, each in triplicate. * $P < 0.05$.