Level of RUNX1 activity is critical for leukemic predisposition but not for thrombocytopenia

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Conflict of interest: The authors have declared that no conflict of interest exists.

Condensed title: RUNX1 and leukemic predisposition
KEY POINTS

1. A half loss of RUNX1 activity leads to defects in primitive erythropoiesis, megakaryopoiesis and proplatelet formation.

2. An almost complete loss of RUNX1 activity leads to the amplification of granulomonocytic compartment with increased genomic instability.
ABSTRACT

To explore how RUNX1 mutations predispose to leukemia we generated iPSC from two pedigrees with germline RUNX1 mutations. The first, carrying a missense R174Q mutation, which acts as a dominant-negative mutant, is associated with thrombocytopenia and leukemia while the second, carrying a monoallelic gene deletion inducing an haploinsufficiency, presents only a thrombocytopenia. Hematopoietic differentiation of these iPSC clones demonstrated profound defect in erythropoiesis and megakaryopoiesis and deregulated expression of RUNX1 targets. iPSC clones from patients with the R174Q mutation specifically generated an increase amount of granulo-monocytes, a phenotype reproduced by a 80% RUNX1 knock-down in the H9 hESC, and a genomic instability. This phenotype – only found with lower dosage of RUNX1 - may account for leukemic development in patients. Altogether, RUNX1 dosage could explain the differential phenotype according to RUNX1 mutations, with a haploinsufficiency leading in a majority of cases to thrombocytopenia alone while a more complete gene deletion rather predisposes to leukemia.
**Introduction**

RUNX1 protein is the α-subunit of the core-binding factor (CBF) transcriptional complex. The protein contains a N-terminal Runt homology domain (RHD) that binds to DNA and to CBFβ, the β subunit of CBF, and a C-terminal transactivation domain. Germline alterations in the **RUNX1** gene are responsible for the familial platelet disorder with a predisposition to acute myeloid leukemia (FPD/AML, OMIM 601399) \(^1\), a rare constitutive disorder that associates a moderate thrombocytopenia with a variable propensity to develop acute leukemia. Whereas all the germline **RUNX1** alterations found in FPD/AML lead to thrombocytopenia, evolution to leukemia depends on the type of mutations, *i.e.* mutations maintaining CBFβ binding properties to generate dominant-negative (DN) proteins favor leukemic evolution, whereas mutations inducing haploinsufficiency only rarely display leukemic development \(^2\). **RUNX1** alterations that predispose to leukemia, mainly DN-like mutants \(^3\), deregulate critical hematopoietic stem cell and progenitor (HSC/P) target genes such as **NR4A3** \(^4\), leading to the amplification of a pool of cells susceptible to acquire additional somatic mutations, sometimes affecting the second **RUNX1** allele \(^5\).

FPD/AML is a suitable model to study the defects in megakaryopoiesis leading to thrombocytopenia. This disease can also be used to explore the initial events in leukemogenesis, as somatic alterations in the **RUNX1** gene are involved in sporadic myeloid malignancies. Chromosomal translocations that involve the **RUNX1** gene are commonly observed in acute myeloid leukemia (AML) \(^6\), whereas **RUNX1** gene mutations are identified in 6-32% of AML \(^7\)-\(^9\). Mutations in **RUNX1** are also detected in 8-15% of chronic myelomonocytic leukemias \(^10\)-\(^11\) and 9% of early stage myelodysplastic syndromes (MDS) \(^12\). The fact that in MDS, **RUNX1** mutations are detected at an early stage, before acute leukemia occurrence, argues for an early event in leukemic transformation \(^13\).
Analysis of mouse models indicated that RUNX1 is a key regulator of definitive hematopoiesis, including HSC emergence \(^{14}\). In adult murine hematopoietic compartments, RUNX1 is dispensable for HSC maintenance, but negatively regulates myeloid progenitors while promoting lymphopoiesis and megakaryocytopoiesis \(^{15,16}\). Regarding primitive hematopoiesis, an active yolk sac-derived erythropoiesis \(^{14}\) and a normal number of primitive erythroid progenitors were observed in Runx-1 knock-out (KO) mice, but primitive erythrocytes had an abnormal morphology and a reduced expression of Ter119, Klf1 and Gata1 \(^{17}\). Conditional Runx1 KO led to the development of a myeloproliferative syndrome, but failed to reproduce the leukemic development observed in 35% of FPD/AML patients \(^{15}\).

Induced pluripotent stem cells (iPSC) \(^{18}\) offer a new opportunity to model inherited human diseases \textit{in vitro} and allow investigating initial pathogenic events that may occur during embryogenesis. Here, we generated iPSC from FPD/AML patients of two different pedigrees: one harbored the DN-like mutation \textit{R174Q} \(^{4}\) and the second a monoallelic \textit{RUNX1} deletion producing a true haploinsufficiency \(^{2}\). We first observed that RUNX1 played a crucial role in regulating the first wave of human primitive hematopoiesis, giving rise to erythroid and megakaryocytic cells. We noticed also that the phenotype induced by the R174Q mutant was similar to almost complete \textit{RUNX1} knock-down in human embryonic stem cells indicating that the \textit{R174Q RUNX1} mutation should affect the binding of the remaining wild type (WT) RUNX1 protein to CBF\(\beta\) to induce an almost complete loss of function \(^{3}\). Most importantly, we noticed that increased genomic instability of granulomonocytic population depended on the dosage of functional RUNX1, which was associated with decreased expression of \textit{GADD45A} \(^{19}\) as well as other p53 targets. Overall, these results show that the residual activity of wild-type RUNX1 protein would impact the risk for leukemia development in FPD/AML patients.
Material and methods

Cell culture

Human dermal fibroblasts (HDF) were derived from skin biopsies of patients after informed consent in accordance with the declaration of Helsinki. The study was approved by the Ethic Committee of INSERM RBM 01-14 for the project “Network on the inherited diseases of platelet function and platelet production”.

They were cultured in F10 Glutamax medium (Invitrogen, Cergy Pontoise, France) with 20% fetal bovine serum (FBS) (Invitrogen, Cergy Pontoise, France). H9 human embryonic stem cell lines (hESC) (NIH code WA09) were obtained from WiCell Research Institute. iPSC and hESC were maintained at undifferentiated state on irradiated mouse embryonic fibroblasts (MEF) in hESC medium, as previously described 20. The use of hESC was approved by Agence of Biomedicine, N° R04-0020 and N° C04-0019. OP9 cells (Riken Institute, Japan) were maintained on gelatinized dishes.

IPSC generation

HDF transduction with the STEMCCA lentivirus was performed at passage 4 or 5. HDF were transduced twice at 12 hrs intervals. Then, HDF (5x10^5) were plated on MEF and gelatin; 24 hrs later, the medium was replaced by hESC medium and changed every day. The ES like-colonies were picked up separately between day 30 and day 45 and cultivated as for hESC culture 21. One iPSC control cell line (C1/CHOPWT2.2) was already described 22.
Hematopoietic differentiation on OP9 cells

To differentiate iPSC in hematopoietic cells, previously described protocols using co-culture on OP9 and cytokines (VEGF 20 ng/mL (Miltenyi Biotech) added at day 0 (D0), SCF 25ng/mL (Biovitrum AB, Stockholm, Sweden), IL-3 10ng/mL (Miltenyi Biotech), EPO 2U/mL (Amgen Thousand Oaks, CA), TPO 20ng/mL (Kirin Pharma Company, Tokyo, Japan) added at D7 of culture were used.

Clonogenic progenitor assays

HSC/P were sorted on the expression of CD34, CD43 at D11 or D14 and plated in triplicate at a density of 1.5x10^3 cells/mL in human methylcellulose medium H4434 (StemCell Technologies, Vancouver, Canada) to quantify erythroid and CFU-G/M progenitors and at a density of 3x10^3 cells/mL in serum-free fibrin clots to quantify MK progenitors (MK-P). MK-P were stained with an anti-CD41a monoclonal antibody (BD Biosciences, Le Pont de Claix, France), as previously described. Images were obtained with AxioVision 4.6 Software. The same software was used to measure the CFU-G/M colony size.

Gene expression analysis by conventional and quantitative real-time PCR

Genomic DNAs and RNAs were extracted using QIAmp DNA Mini Kit or RNeasy Micro Kit (Qiagen, Courtaboeuf, France). Reverse transcription of RNA was performed with SuperScript Vilo cDNA Synthesis Kit (Invitrogen, Cergy Pontoise, France). Q-RT-PCRs and Q-PCRs were performed on a 7500 Real-Time PCR using SYBR-Green PCR Master Mix (Applied Biosystems, Saint-Aubin, France). Expression levels of genes were normalized to housekeeping genes PPIA and L32. Primers used are listed in ST1.

Statistical analyses
Data are presented as means (±SD) or with 95% Confidence Interval (CI) of the mean. Statistical significance was determined by Student's $t$ test. A $P$ value < 0.05 was considered as statistically significant. For transcriptome analysis, an ANOVA test was performed with a $P$-value threshold $p<10^{-3}$.

For other methods see supplemental data.
Results

**RUNX1 alterations induce a defect in erythroid and megakaryocyte output.**

To compare the consequences of two germline RUNX1 alterations, one leading to an increase in leukemia predisposition (R174Q mutation, pedigree A) and the other to thrombocytopenia alone (monoallelic deletion, pedigree D), four iPSC lines were derived from FPD/AML patient skin fibroblasts. The two pedigrees have been described previously. The strategy of iPSC derivation and their characterization are reported in SF1-SF3. We selected one iPSC clone derived from each of two distinct members of pedigree A (AII_1, AII_2) and 2 iPSC clones derived from pedigree D (D(a) and D(b)). Three independent control iPSC lines (C1, C2 and C3) were used as reference for all experiments. All iPSC lines were passaged 15-20 times to remove memory of origin, which may interfere with differentiation to downstream lineages. Deep sequencing analysis identified 109 de novo mutations for AII_1, 82 for AII_2, and 9 for D(a). The mutations of genes that could play a role in hematopoiesis were confirmed by Sanger sequencing (ST2). However, the results showing that the same gene was never mutated in more than one patient sample suggest that the common phenotype observed in all FPD/AML patients studied here is exclusively linked to the germline RUNX1 alteration and not to the randomly acquired mutations. The protocols of hematopoietic differentiation are described in SF4. Hence we studied the impact of RUNX1 alterations on progenitors generated by culturing CD34⁺CD43⁺ cells obtained on D11, focusing on erythroid/megakaryocytic (E/MK) cells, and those occurring at D14, focusing on granulo/monocytic (G/M) cells. When assessing the colony-forming potential of CD34⁺CD43⁺ cells sorted at D11, we observed a decrease in the number of erythroid progenitors (Ery-P) generated from the RUNX1 R174Q mutated as well as RUNX1 haploinsufficient iPSC clones (Figure 1A, B). We also detected a 3-fold decrease in MK progenitor number for both pedigrees (Figure 1C, D). CD34⁺CD43⁺ cells sorted at D11 were
also cultured on OP9 stromal cells in liquid medium (SF4A), demonstrating a decrease in the percentage of erythroid (GPA⁺) and MK (CD41⁺CD42⁺) populations at D18 in cells derived from the two pedigrees (Figure 1E, F). The absolute numbers of erythroid (GPA⁺) and of MK cells generated from 1×10² CD34⁺CD43⁺ progenitors were decreased for both the RUNX1 R174Q mutation and RUNX1 deletion iPSC clones (Figure 1G, H). Strikingly, these results demonstrate that, in human, RUNX1 plays an important role in the first wave of hematopoiesis yielding primitive erythroid and MK cells.

**Megakaryocytes derived from FPD/AML iPSC display a profound defect in proplatelet formation**

When CD41⁺CD42⁺ MKs were sorted after 14 days of co-culture and analyzed five days later (Figure 2A), the percentage of proplatelet-forming MKs was decreased at least 10-fold in patient iPSC- compared to control iPSC-derived MKs (Figure 2B, C). Since RUNX1 is a positive or negative regulator of cytoskeleton components involved in proplatelet formation (MYL9, MYH9) 25 or in ploidization (MYH10) 26, we examined the expression of these genes. As observed in MKs derived from patient progenitors 25, MYL9 expression level was decreased while MYH10 expression was increased in MKs obtained from patient iPSCs, whatever the RUNX1 alteration (Figure 2D), demonstrating that the defect in megakaryopoiesis is independent of the type of RUNX1 alteration.

**RUNX1 R174Q mutation specifically affects the output of granulo-monocytes**

When we assessed the G/M colony-forming potential of CD34⁺CD43⁺ cells sorted at D14 (SF4A), we observed a significant 2-fold increase in the number of CFU-G/M in the two patients with R174Q mutation compared to the 3 controls (Figure 3A), but not in the patient with a monoallelic RUNX1 deletion (Figure 3B). The size of G/M colonies generated from
R174Q iPSCs was also significantly increased (Figure 3C; median sizes 389420 µm² for AII_2 and 388953 µm² for AII_1 compared to 225078 µm² for C2). This size increase was not seen for iPSCs carrying the RUNX1 deletion (Figure 3D), showing that RUNX1 haploinsufficiency did not affect the proliferation rate of G/M lineage. Accordingly, the generation of mature monocytes/macrophages (CD14<sup>high</sup>CD15<sup>low</sup>) and granulocytes (CD15<sup>high</sup>) in liquid medium (Figure 3E, F) revealed an increase in the proportion of G/M populations carrying the R174Q mutation, but not the RUNX1 deletion. A significant 2-fold increase in the monocyte/macrophage output and a slight increase in granulocyte generation were detected for both patient lines with the R174Q mutation, but not for the patient lines with a RUNX1 monoallelic deletion (Figure 3G, H). In accordance with our previous results<sup>4</sup>, the expression of NR4A3 gene was decreased in CD34<sup>+</sup>CD43<sup>+</sup> progenitors sorted at D14 from the two R174Q mutated iPSC clones, while no significant difference was observed between control and RUNX1 monoallelic deleted cells (Figure 3I). These results indicate that, only the R174Q RUNX1 mutation leads to deregulation of the granulo-monocytic compartment, which correlates with the down regulation of NR4A3.

**RUNX1 transgene targeting corrects hematopoietic defects independently of the type of mutation.**

To exclude a role of iPSC line heterogeneity in the above-reported observation, we reintroduced a WT copy of HA-tagged RUNX1 gene into one iPSC line of each pedigree and used zinc-finger nucleases to place this gene under control of CD43 gene promoter into the “safe harbor” locus AAVS1 (Figure 4A).<sup>27</sup> Clones with homozygous integration were selected and the overexpression of HA-RUNX1 transcripts in CD34<sup>+</sup>CD43<sup>+</sup> progenitors was checked (Figure 4B). In R174Q mutant cells, overexpression of RUNX1 was further verified by western blotting (Figure 4C) and by measuring the expression of two direct RUNX1
targets, p19\textsuperscript{INK4d} and MPL (Figure 4D). Reintroduction of WT RUNX1 induced a 3-fold increase in the number of MK progenitors for the deletion and a 2-fold increase for the R174Q mutant in comparison to the parent iPSC lines (Figure 4E). The gene correction also increased the number of erythroid progenitors produced by the FPD/AML iPSC lines by 2-fold for the deletion and by more than 5-fold for the R174Q mutation, compared to the parent iPSC lines (Figure 4F). Interestingly, the number of G/M progenitors decreased to control levels in the transgene targeted \textit{R174Q} iPSC line, demonstrating reversal of the phenotype (Figure 4G).

**Down-regulation of RUNX1 in H9 ES cell line reproduced the dominant negative phenotype**

To determine whether the R174Q mutant has acquired a new function or leads to a loss-of-function, we transduced undifferentiated H9 ESCs with RUNX1 shRNA or control shRNA constructs. The use of shRUNX1\textsubscript{1} almost completely abolished the expression of RUNX1 at protein level in mesodermal population (Figure 5A). First we confirmed the central role of RUNX1 in hematopoiesis induction by showing a decrease in the generation of hematopoietic clones after RUNX1 knockdown \textsuperscript{28} (SF5). Then, we assessed the colony-forming potential of CD34\textsuperscript{+}CD43\textsuperscript{+} cells sorted at D11, which demonstrated a more than 10-fold decrease in the number of Ery-P (Figure 5B) and a 3-fold decrease in MK-P after RUNX1 knock-down (Figure 5C). In liquid medium, RUNX1 down regulation led to a decreased production of GPA\textsuperscript{+} erythroid cells and CD41\textsuperscript{+}CD42\textsuperscript{+} MKs at D18 (Figure 5D, E). Next, we performed the same experiments with CD34\textsuperscript{+}CD43\textsuperscript{+} cells sorted at D14 and observed an increased number of CFU-G/M after RUNX1 knock-down (Figure 5F) and an increased number of CD14\textsuperscript{high}CD15\textsuperscript{low} mature monocyte/macrophage and CD15\textsuperscript{high} granulocyte populations at D21 in liquid medium (Figure 5G). These results were validated by using a second shRNA,
shRUNX1_2 (SF6). To further confirm that a decrease in RUNX1 activity is crucial for the amplification of G/M progenitors, we transduced the iPSC clone D(b) carrying a monoallelic RUNX1 deletion with the shRUNX1_1. The additional decrease in RUNX1 level led to an increased number of CFU-G/M (Fig 5H).

Altogether, these results suggest that the differences between R174Q mutation and a monoallelic RUNX1 deletion can be explained by RUNX1 dosage. An almost complete functional inhibition or deletion, but not a haploinsufficiency, leads to the deregulation of the G/M compartment, which may contribute to leukemic development. Gene expression was then explored on hematopoietic CD34+CD43+ progenitors derived from shRUNX1_1 and shControl transduced hESC and sorted at D14 of culture. On the 44,000 tested probe sets, 412 probe sets were deregulated at least 1.5-fold in shRUNX1_1 hESC with 327 probe sets decreased and 85 probe sets increased. A focus on hematopoietic genes (Figure 5I) identified 22 erythroid and 11 megakaryocytic genes deregulated. In the G/M compartment, we highlighted a global increase of the G/M signature with an increase of 11 genes. These results confirmed that RUNX1 plays an important role in the commitment between E/MK and G/M lineages.

**R174Q RUNX1 mutation but not haploinsufficiency leads to an increase in genomic instability**

Gene expression analysis also demonstrated a slight decrease in four p53-dependent genes involved in DNA-damage response (Figure 5J). To explore the role of RUNX1 in genomic instability, we first sorted the CD43+ hematopoietic cells at D10 of culture from two FPD/AML iPSC lines with R174Q mutation and control iPSC lines. The accumulation of DNA double strain breaks (DSBs) was investigated using an antibody recognizing the phospho-histone H2AX (P-H2AX). As shown in Figure 6A, B, an increase in the number of
P-H2AX positive cells was observed in CD43+ cells harboring \textit{R174Q} mutation compared to control cells, (from 50% for controls to about 70% for FPD/AML cells) as well as in the number of foci per cell. As replication can induce P-H2AX foci formation, more particularly in highly proliferative erythroid cells that represent the majority of CD43+ cells, we used P53BP1 as a marker of DSBs. The number of cells presenting P53BP1 foci in CD34+CD43+ progenitors was increased in the population of R174Q mutant cells (from 50% in control C3 and C2 cells to 80% in R174Q mutant cells) (\textbf{Figure 6C and D}) while no increase was detected for cells with \textit{RUNX1} deletion (\textbf{Figure 6D}). Then we evaluated the p53-dependent DNA damage response pathway. In the entire CD34+CD43+ cell population, we did not detect any decrease in \textit{p21}, \textit{NOXA} and \textit{BAX} expression, whatever the \textit{RUNX1} alteration (\textbf{Figure 6E}). As the R174Q mutant cell line is characterized by an expansion of the G/M lineage, we focused on this population. As shown in \textbf{Figure 6F} and \textbf{G}, an increase in the number of CD14+CD15+ cells positive for P53BP1 foci (from 26% for control cells to 53%) and in the number of foci per cell was detected for R174Q mutant cells. These results were confirmed by Q-RT-PCR, showing a strong decrease of \textit{p21}, \textit{GADD45A} and in a lesser extent \textit{NOXA} and \textit{BAX} gene expression in CD14+CD15+ cell population (\textbf{Figure 6H}). Importantly, the number of cells showing P53BP1 foci was decreased to control level and the expression of all four genes was restored in FPD/AML iPSCs transduced with WT \textit{RUNX1} (\textbf{Figure 6G, H}). Altogether, our results demonstrate that \textit{R174Q} \textit{RUNX1} mutation could predispose to leukemic transformation at least partly through NR4A3 down-regulation and increased genomic instability in the G/M compartment, which is not observed with cells harboring a \textit{RUNX1} haploinsufficiency. Moreover, the additional decrease of \textit{RUNX1} activity in haploinsufficient iPS cells by the shRUNX1_1 led not only to an increase in CFU-G/M progenitor number but also to a decrease in \textit{p21} and \textit{GADD45A} expression in the
CD14^+CD15^+ cell population (SF7), attesting of the importance of RUNX1 level in this phenotype.
Discussion

The current approaches used to explore FPD/AML pathogenesis remain limited, as access to patient primary cells is difficult, due to the rarity of the disease. In addition, mouse models of Runx1 alterations do not completely reflect the phenotype observed in FPD/AML patients, e.g. adult mice with a conditional Runx1 KO develop a myeloproliferative/myelodysplastic syndrome rather than an acute leukemia 15. Thus, to further explore the mechanisms of dysmegakaryopoiesis and sensitivity to leukemic transformation related to diverse RUNX1 alterations, we generated iPSC lines from two previously studied pedigrees: one with a DN-like mutation R174Q and one with a RUNX1 monoallelic deletion. The two pedigrees are associated with thrombocytopenia, whereas only the first one is associated with AML.

The generation of hematopoietic progenitors during embryonic life occurs initially in large blood vessels by the formation of clusters derived from endothelial cells, a process that strikingly relies on the presence of RUNX1 28,29. The number of human hematopoietic progenitors generated from FPD/AML iPSC depends on the type of RUNX1 alteration 30. Here, we show that the knock-down of RUNX1 in embryonic stem cells limits the commitment to hematopoietic lineage at the clonal level.

The hematopoietic potential of different ESC 31 and iPSC lines can be very heterogeneous 32, therefore, the analysis of hematopoietic lineages obtained from iPSC were normalized to the number of initial hematopoietic progenitors and not of iPSC. To isolate myeloid hematopoietic progenitors, the marker CD34 was used in combination with CD43 (leukosialin), which is an early and specific hematopoietic marker appearing before CD45 during development 33.

In accordance with previously reported data 34, we detected two discrete waves of primitive hematopoiesis with an increased capacity of the first CD34+CD43+ cells to generate
primitive erythroid and megakaryocytic progenitors compared to the CD34+CD43+ cells occurring later that demonstrate a granulo-monocytic potential. By analyzing the clonogenic potential of hematopoietic CD34+CD43+ progenitors derived from FPD/AML iPSC clones, we observed a profound defect in the first wave giving rise to primitive erythroid and MK cells as well as in proplatelet formation from mature MKs, which is consistent with the dysmegakaryopoiesis observed in FPD/AML patients. In addition, the same deregulation of myosin expression was found in MKs derived from both iPSC and adult CD34+ cells confirming the validity of the iPSC model. These defects were independent on the type of RUNX1 alteration (DN-like mutant or haploinsufficiency) and were partially restored after RUNX1 overexpression driven by the CD43 promoter in FPD/AML iPSC lines. In line with our results, defects in MK differentiation from iPSC lines derived from FPD/AML patients with Y260X, G172E, G143W and N233fsX283 RUNX1 mutations were also reported.

Regarding the second wave, DN-like R174Q RUNX1 mutation increased the proliferation rate and clonogenic potential of G/M progenitors, which correlated with a decrease in NRA4A3 expression. Since a similar phenotype was also observed after almost complete RUNX1 knock-down in hESC, we can conclude that R174Q mutant is close to a complete loss-of function. Conversely, the half decrease in RUNX1 dosage due to RUNX1 deletion did not affect the G/M lineages and NR4A3 expression, and further attest that DN-like R174Q mutant could contribute to the induction of a preleukemic state by increasing the G/M progenitor compartment, the main cell target for AML. Even if our differentiation protocol allows us to study only primitive hematopoiesis, these results are in agreement with observations made in adult progenitors from FPD/AML patients harboring the R174Q mutation, where the increase in the G/M compartment has been associated to NR4A3 downregulation by RUNX1. Furthermore, the importance in NR4A3 dosage was already highlighted in induction of myeloproliferative neoplasms and AML in NR4A3 KO mouse
models 36-38. Contrary to our results showing the amplification of G/M lineage after almost complete knock-down of RUNXI or with DN-like R174Q mutation, this amplification was not observed with N233fsX283 mutation 30, which is highly associated with AML. However, this last mutation induces a deep defect in the generation of hematopoietic progenitors, which could be in some way also a preleukemic state as some aplastic anemia.

To our knowledge this is the first study that underlies the differences in granulomonocytic lineage between a haploinsufficiency alone and the DN-like R174Q RUNX1 mutant. These differences are clearly dependent on the protein dosage. The R174Q mutant behaving as a DN, may preclude the formation of active core-binding complexes. This hypothesis becomes even more plausible when we take into account 1) the report that certain RUNX1 mutants including R174Q bind CBFβ more strongly than the WT 3 and mouse models of CBFβ deficiency recapitulate the phenotype observed with this RUNX1 mutant 39; 2) the results obtained with the two shRUNX1 in hESC where abrogation of RUNX1 leads to a phenotype close to R174Q and 3) the rescue of DN phenotype by overexpression of WT RUNX1 probably by changing the equilibrium of WT versus mutant binding to CBFβ. A recent study highlighted the complexity of RUNX1 dosage by reporting that a minimal dosage is necessary for MLL fusion leukemia 40. Here, we show that a very low level of RUNX1 increases the proliferative rate and genomic instability of G/M progenitors that could be at the origin of the preleukemic state in patients whereas a half-decrease in RUNX1 level rather leads to thrombocytopenia alone. However, it should be noted, that leukemia development was described in one pedigree with hemizygous RUNXI deletion 41, attesting that the simple dichotomy of DN versus haploinsufficient mutant for AML development is not so clear and that other genetic events could also contribute to leukemic development. However, our results are consistent with the importance of the transcription factor level in malignant disorder. This
is underscored by the example of PU.1, which needs to be decreased to less than 80% for inducing AML\textsuperscript{42} whereas an haploinsufficiency has no effect\textsuperscript{43,44}.

Finally, we demonstrate that a RUNX1 loss of function leads to an increase in genomic instability linked to a decreased expression of p53-dependent genes and to an increase in the number of P53BP1 foci. Moreover, in the presence of the R174Q mutant, 10-fold more mutations occurred during the reprogramming process as compared to monoallelic \textit{RUNX1} deletion and control cell lines\textsuperscript{45}. In line with these results, a novel role for RUNX1 during DNA damage response has been recently identified. Indeed, it cooperates with p53 after DNA damage and is recruited onto p53 target genes promoters\textsuperscript{46}. In addition, a decreased expression of the direct RUNX1 target \textit{GADD45A}, encoding for a sensor of DNA stress, was observed in bone marrow cells from MDS/AML patients harboring a \textit{RUNX1} mutation in C-terminal domain\textsuperscript{19}. \textit{GADD45A} was shown to be activated synergistically by RUNX1 and p53. Interestingly, Michaud et al reported previously a global genomic instability linked to RUNX1 impairment\textsuperscript{47} and the incidence of \textit{RUNX1} mutations in therapy-related myelodysplastic syndrome and acute myeloid leukemia is particularly high, revealing again a potential role of RUNX1 in genomic instability\textsuperscript{48}.

In conclusion, whereas analysis of mouse models failed to identify any defect in primitive hematopoiesis, we show for the first time that \textit{RUNX1} germline alterations affect human embryonic hematopoiesis, particularly the generation of primitive erythroid and MK cells. As already described by others\textsuperscript{30,35}, defects in the generation of mature megakaryocytes do not depend on the type of \textit{RUNX1} variants. Conversely, a dominant negative R174Q mutant of RUNX1 increases the proliferative rate, and induces a genomic instability of G/M progenitors, thereby contributing to a preleukemic state development.
Acknowledgments

This work was supported by French grants from the Agence Nationale de la Recherche (ANR-physiopathology, ANR-jeunes chercheurs (HR)), the Centre de Référence des pathologies plaquettaires, the ARC (projet libre, IP 2012; projet libre, WV 2009). The CGH array was funded by the apprentice ship tax (TA2011). Labex GR-Ex (IP, WV) is funded by the program “Investissements d’avenir”. IAD was supported by a doctoral fellowship from ARC. ND and WV are recipients of a research fellowship from CHU Bordeaux (ND) and from IGR-INSERM (WV).

We thank P. Rameau and Y. Lécluse for flow cytometry cell sorting and analysis (IRCIV, Gustave Roussy, Villejuif, France). We thank Dr. G. Meurice for the CGH analysis (Plateforme de Bioinformatique, Gustave Roussy, Villejuif, France), Dr. P. Opolon for the teratomas analysis (Plateforme d’ histopathologie, Gustave Roussy, Villejuif, France). We are grateful to Mr. P. Nusbaum and A. Hubas from patient fibroblast culture (Banque de Cellules directed by Pr. J. Chelly, Hôpital Cochin, Paris, France) and to Pr. Jonveaux (CHU de Nancy, France) for his advise. We thank to Dr. A. Foudi and Dr. G. Mostoslavsky (Boston University and Boston Medical Center, Boston, MA, USA) for providing of lentiviral vector STEMCCA and to Dr. A. Galy and Dr. S. Charrier for providing of lentiviral vector pRRLcpptPGKGF-P-WPRE-Alb (Genethon, Evry, France). We thank the patients for participation in this study.
Author’s contribution statement

IAD, VTM, WV, HR designed experiments; IAD, VTM, NB, DB, CT, CL, TL, OB, LT, JAM: performed experiments; IAD, VTM, LT, GT, HR: analyzed data, BL, RF: clinical and biological follow up of patients; ND, IP, ES, RF, WV: discussed the results; HR supervised the work, IAD, VTM, WV, HR: wrote the paper.

IAD and VTM have equally contributed.

The authors declare no competing financial interest.
References


Legends

Figure 1. Deep decrease in erythroid and megakaryocyte potential of progenitors derived from FPD/AML iPSC lines.

AII_1, AII_2: iPSC clones of two patients from pedigree A; D(a) and D(b): two clones of one patient from pedigree D. C1, C2 and C3: control iPS cell lines. Cell numbers were normalized in all experiments to those produced by the C2 clone, which was reported in each experiment as a fix value of 100. (A-D) CD34⁺CD43⁺ cells sorted at D11 and tested for their colony forming potential. (A, B) Methylcellulose assay. (A) The histograms present the numbers of erythroid progenitors (Ery-P) in one representative experiment of three (n=3) each in triplicate. (B) Pictures of primitive erythroid colonies (Ery-P). (C, D) Fibrin clot culture. (C) The histograms present the numbers of MK progenitors (MK-P) in one representative experiment of four (n=4) each in triplicate. (D) Pictures of primitive MK colonies (MK-P) after CD41 immunostaining. (A, C) Error bars represent ±SD of triplicate. (B, D) Scale bar is equal to 50 μm. (E-H) Analysis of hematopoietic populations at D18 from progenitors sorted at D11. (E) May-Grünwald Giemsa staining of erythroblasts (GPA⁺) and megakaryocytes (CD41⁺CD42⁺) derived from iPSC lines. Scale bar is equal to 10 μm. (F) FACS analysis of erythroid cells and MKs from FPD/AML iPS cell lines in one representative experiment. (G) Absolute number of generated erythroid cells normalized to 1x10² plated progenitors. The histograms show average of the cell number obtained in five independent experiments for pedigree A (n=5) and three for pedigree D (n=3). (H) Absolute number of generated MKs normalized to 1x10² plated progenitors. The histograms show average of the cell number obtained in three independent experiments for both pedigrees (n=3). (G, H) Error bars represent ±SD of the average. *P<0.05, **P<0.01, ***P<0.001, Student’s t test.
Figure 2. Defect in proplatelet formation by megakaryocytes derived from FPD/AML iPSC lines

(A) Schema of protocol used for MK differentiation. iPSC lines were seeded on OP9 cells with VEGF and TPO at D0. After 7 days, SCF was added to the culture medium. MKs (CD41+CD42+) cells were sorted at D14 and further cultured in presence of TPO and SCF. Analysis of proplatelet formation and Q-RT-PCR were performed at D19. (B) Representative microscopic images representing controls and patients proplatelet-forming MKs. (C) The percentage of proplatelet-forming MKs was estimated by counting MKs exhibiting one or more cytoplasmic processes with areas of constriction. A total of 200 cells per well was counted. The histograms show one representative experiment of five, each in triplicate (n=5). Error bars represent ±SD of triplicate. (D) Q-RT-PCR analysis of MYL9 and MYH10 expression level in MKs. The histograms show one representative experiment of four for MYL9 (n=4) and five for MYH10 (n=5), each in triplicate. Data are normalized to PPIA and L32 transcript level and expression is compared to control C3. Similar results were obtained, only results normalized to PPIA are shown. Error bars represent ±SD of triplicate. **P<0.01, ***P<0.001, Student’s t test.

Figure 3. RUNX1 R174Q mutation but not RUNXI deletion affects the output of granulo-monocytes

AII_1, AII_2: iPSC clones of two patients from pedigree A; D(a) and D(b): two clones of one patient from pedigree D. C1, C2 and C3: control iPS cell lines. Cell numbers were normalized in all experiments to those produced by the C2 clone. (A-D) CD34+CD43+ cells were sorted at D14 and tested for their colony forming potential in methylcellulose assay. (A, B) The histograms show one representative experiment of three for pedigree A (A; n=3) and of seven for pedigree D (B; n=7), each in triplicate. Error bars represent ±SD of triplicate. (C) Pictures
of granulo-monocytic colonies (CFU-GM). Scale bar is equal to 100 μm. (D) Area of more than 30 CFU-GM colonies obtained from each iPS cell line was analyzed by AxioVisio 4.6 software. (E-H) Analysis of hematopoietic populations generating at D21 from progenitors sorted at D14. (E) May-Grunwald-Giemsa staining of monocytes/macrophages (CD14$^{\text{high}}$CD15$^{\text{low}}$) and granulocytes (CD15$^{\text{high}}$) derived from iPSC. Scale bar is equal to 10 μm. (F) FACS analysis of the G/M cells in one representative experiment. (G, H) Absolute numbers of generated monocytes/macrophages (CD14$^{\text{high}}$CD15$^{\text{low}}$) and granulocytes (CD15$^{\text{high}}$) normalized to 1x10$^2$ plated progenitors. (G) The histograms show average of the cell number obtained in five independent experiments for monocytes/macrophages (n=5) and in three for granulocytes (n=3) for pedigree A. (H) The histograms show average of the cell number obtained in four independent experiments for pedigree D (n=4). (G, H) Error bars represent ±SD of the average. (I) Q-RT-PCR analysis of NR4A3 expression level in CD34$^+$CD43$^+$ cells in both A (left; n=4) and D (right; n=4) pedigrees. Data are normalized to PPIA transcript and expression is compared to control C3. Error bars represent ±SD of triplicate.

*P<0.05, **P<0.01, ***P<0.001, Student’s t test, ns= non significant

**Figure 4. Genetic rescue by RUNX1 WT overexpression in FPD/AML iPS lines**

AII_1,: iPS clones of one patient from pedigree A; D(b): one clone of one patient from pedigree D. AII_1_RUNX1 and D(b)_RUNX1: the iPS clones overexpressing WT RUNX1. C1, C2 and C3: control iPSCs. (A) Gene correction strategy. The constitutively active AAVS1 “safe harbor” locus is shown on the top line and the targeting construct is shown below. cDNA expression cassette driving expression of WT RUNX1 cDNA under the leukosialin promoter (pCD43) was inserted by zinc finger-mediated homologous recombination into intron 1 of AAVS1. HA: homologous arms left (L) and right (R); SA-2A-Puro-PA: puromycin
drug resistance cassette. (B) Overexpression of HA-RUNX1 measured by Q-RT-PCR using forward primer in HA epitope and reverse primer in RUNX1 cDNA. Error bars represent 95% CI. (C) Western blot analysis of total (endogenous and overexpressed) RUNX1. (D) Q-RT-PCR analysis of MPL and P19 INK4D expression level in MKs (CD41⁺CD42⁺) sorted at D14. Data are normalized to PPIA transcript level and expression is compared to control C3 (n=3). (E-G) Cell numbers were normalized in all experiments to those produced by the C2 clone. (E, F) CD34⁺CD43⁺ cells were sorted at D11 and tested for their colony forming potential. (E) Assessment of MK progenitors (MK-P) in fibrin clot cultures (n=3) (F) Assessment of erythroid progenitors (Ery-P) in methylcellulose culture (n=3) (G) CD34⁺CD43⁺ cells of pedigree A were sorted at D14 and tested for their CFU-G/M potential in methylcellulose assay (n=3). (D-G) Error bars represent ±SD of triplicate. *P<0.05, **P<0.01, ***P<0.001, Student’s t test.

Figure 5. RUNX1 knock-down in H9 ESC line and in a FPD/AML iPSC line with monoallelic RUNX1 deletion leads to the same phenotype than FPD/AML-DN iPSC line

(A-G) RUNX1 knock-down in H9 ESC line. (A) Analysis of RUNX1 protein expression in mesodermal population from control and shRUNX1_1 ES lines. (B, C) CD34⁺CD43⁺ cells were sorted at D11 and tested for their colony forming potential in methylcellulose assay and fibrin clot culture. B. Assessment of erythroid progenitors (Ery-P) in methylcellulose culture. C. Assessment of MK progenitors (MK-P) in fibrin clot cultures. (B, C) The histograms show one representative experiment of four for Ery-P (n=4) and three for MK-P (n=3), each in triplicate. Error bars represent ±SD of triplicate. (D, E) Analysis of hematopoietic populations generating at D18 from progenitors sorted at D11. (D) Absolute number of generated erythroid cells (GPA⁺) normalized to 1x10^5 plated progenitors (n=4). E. Absolute number of generated MKs (CD41⁺CD42⁺) normalized to 1x10^5 plated progenitors (n=4). (D, E) The
histograms show the average of four independent experiments. Error bars represent ±SD of average. F. CD34+CD43+ cells were sorted at D14 and tested for their CFU-GM potential in methylcellulose assay. The histograms show one representative experiment of four, each in triplicate (n=4). Error bars represent ±SD of triplicate. G. Analysis of hematopoietic populations generating at D21 from progenitors sorted at D14. Absolute numbers of generated monocytes/macrophages (CD14^{high}CD15^{low}; n=4) and granulocytes (CD15^{high}; n=4) normalized to 1x10^2 plated progenitors. The histograms show the average of four experiments. Error bars represent ±SD of average. (H) RUNX1 knock-down in D(a) iPS cell line with shRUNX1_1. CD34+CD43+ cells were sorted at D14 and tested for their CFU-GM potential in methylcellulose assay. The histograms show one representative experiment of three, each in triplicate (n=3). Error bars represent ±SD of triplicate. (B-H) *P<0.05, **P<0.01, ***P<0.001, Student’s t test. (I, J) Transcriptome analysis of CD34+CD43+ progenitors derived from shControl and shRUNX1_1 transduced hESC line sorted at day 14 of culture. FC: fold change between cells transduced with shRUNX1_1 and shControl lentiviruses. Only genes with expression variation +/-1.5 and P<0.001 (Anova test) are listed. E: erythroid, MK: megakaryocyte, I: immunity, IN: inflammation, G/M: granulo-monocyte. (J) Variation in expression of genes involved in p53-dependent DNA damage response, P<0.001 (Anova test).

Figure 6. RUNX1 DN-like mutant induces increased genomic instability compared to RUNX1 haploinsufficiency

AII_1, AII_2: iPSC clones of two patients from pedigree A; D(a): one clone of one patient from pedigree D; AII_1_RUNX1: the iPSC clone AII_1 overexpressing WT RUNX1; D(a)_shRUNX1_1: the iPSC clone D(a) after RUNX1 knock down by shRUNX1_1; C2 and C3: control iPS cell lines. (A-E) CD43+ cells (A, B) and CD34+CD43+ cells (C-E) were
sorted at day 14 of culture. A, C. Representative pictures are shown, nucleus is stained with DAPI (blue) and double strand breaks (DSB) are stained with P-H2AX (red) (A) or with p53-BP1 (red) (C). (B, D). The number of P-H2AX (B) and P53-BP1 (D) foci per 50 counted cells. One representative experiment of four is shown (n=4, p<0.0001 for the increase in P-H2AX positive cells, p=0.0164 for the increase in P53BP1 positive cells in pedigree A). (E) Q-RT-PCR analysis of different p53-dependent genes in CD34⁺CD43⁺ cells. Data are normalized to PPIA transcript level and expression is compared to control C2. The histograms show one representative experiment of three, each in triplicate (n=3). Error bars represent 95% CI.

(F-H) CD14⁺CD15⁺ cells were sorted at day 15 of culture. (F) Representative pictures are shown, nucleus is stained with DAPI (blue) and DSB are stained with P53BP1 (red). (G) The number of P53BP1 foci per 100 counted cells. One representative experiment of three is shown (n=3, p=0.0017 for the increase in P53BP1 positive cells in AII_1). (H) Q-RT-PCR analysis of different p53-dependent genes in CD14⁺CD15⁺ cells. Data are normalized to PPIA transcript level and expression is compared to control C3. The histograms show one representative experiment of three, each in triplicate (n=3). Error bars represent 95% CI.

*P<0.05, **P<0.01, ***P<0.001, Student’s t test.
Level of RUNX1 activity is critical for leukemic predisposition but not for thrombocytopenia