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# Human promoter mutations unveil Oct-1 and GATA-1 opposite action on Gfi1b regulation

Aurora Hernández · Ana Villegas · Eduardo Anguita

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**Abstract** Growth factor-independence 1b (Gfi1b) is a zinc finger transcription factor essential for erythroid and megakaryocytic development. To better understand Gfi1b regulation and to know the implication of the level of expression of this gene in human pathology, we have searched for promoter punctual sequence variations in 214 patients with different hematological diseases. We found two previously unknown congenital mutations at evolutionary conserved GATA and octamer-binding (Oct) transcription factor sites. The Oct site mutation was also found in five relatives of the patient. The GATA motif mutation reduced promoter activity by 50% *in vitro*, while homozygous patients with the octamer site mutation showed a four-to-five times increase of Gfi1b RNA in platelets. Electrophoretic mobility shift analyses demonstrated that different protein complexes bind to both sites and that binding is reduced by the mutations. Finally, we found that GATA-1 and Oct-1 are the main components of each complex. This study provides evidences of a new mechanism for Gfi1b repression. This is also the first report of Gfi1b mutations with a functional implication; further investigation and follow-up will clarify the involvement of these mutations in hematological disease.

**Keywords** Gfi1b · Mutation · Gene regulation · Leukemia

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## Introduction

Transcription factor growth factor-independence 1b (Gfi1b) is expressed in hematopoietic stem cells, common myeloid and megakaryocyte/erythroid progenitors, and erythroid and megakaryocytic lineages. Moderate levels of expression are found in immature B cells, a subset of early T cell precursors and peripheral blood granulocytes and monocytes [1, 2]. During erythroid differentiation, Gfi1b is upregulated in early erythroblast stages and decreases with terminal differentiation [1, 3, 4]. Knockout mice have demonstrated that this gene is required for development of both erythroid and megakaryocytic lineages [5]. Additionally, Gfi1b expression has been found to increase in leukemia patients and cell lines [6, 7].

These facts indicate that correct Gfi1b expression is important to achieve normal erythroid and megakaryocytic differentiation. Therefore, it is crucial to understand how Gfi1b is regulated.

Here, we have identified two different congenital Gfi1b promoter mutations. These mutations reveal the presence of different protein complexes, including GATA-1 and Oct-1, involved in Gfi1b regulation, giving clues of Gfi1b repression mechanism. Sequence variations like these ones together with additional abnormalities are likely to have an impact in hematological disease.

## Methods

### Patients

We have studied 128 cases of acute leukemia of all FAB types (107 analyzed at diagnosis and 21 of them in relapse); 69 patients with acute myeloid leukemia (AML): 7 AML-M0, 12 AML-M1, 11 AML-M2, 6 AML-M3, 13 AML-M4, 16 AML-

M5, 2 AML-M6, 2 AML-M7, and 59 cases of acute lymphoblastic leukemia (ALL): 46 B lineage ALL and 13 cases of T-ALL. We also analyzed eight myelodysplastic syndromes; two myelofibrosis; five congenital dyserythropoietic anemia (CDA) type II; one CDA type III; one case of “variant CDA type II” with negative Ham test [8, 9]; one case of CDA with thrombocytopenia; one congenital sideroblastic anemia (the last three patients had normal GATA-1 by exon sequencing; data non shown); 36 patients with thrombocytosis (platelets between 429 and  $1,437 \times 10^3/\mu\text{l}$ ), 15 of them with JAK 2 V617F mutation, detected as described [10], and 31 with polyglobulia, 22 males with hemoglobin (Hb) range 17.7 to 18.6 g/dl and 9 females with Hb between 15.2 and 19.4 g/dl. Only ten individuals with polyglobulia had JAK 2 V617F mutation that confirmed the diagnosis of polycythemia vera; the other cases were studied to investigate the involvement of Gfi1b promoter single nucleotide polymorphisms (SNPs) in polyglobulias that cannot be classified as myeloproliferative disorders.

#### DNA isolation

Samples were isolated from bone marrow (BM) or peripheral blood (in the cases of thrombocytosis, polyglobulia, myelofibrosis, and 24 acute leukemia patients with 60–94% blasts in peripheral blood in which BM was not available) and DNA extracted by phenol/chloroform.

#### Point mutation analysis

The Gfi1b promoter was amplified by polymerase chain reaction (PCR) with AmpliTaq Gold (Applied Biosystems, ABI, Foster City, CA, USA) and Pwo DNA Polymerase (Roche, Basel, Switzerland) with the following nucleotides:

5'-AAAGCGTGCCGCTCCAAGTG-3'  
5'-ACAGTGGCTCAGACGAGGCTACGTC-3'.

The resulting products were denatured and then reannealed to allow heteroduplex formation. Afterward, PCR fragments were analyzed by denaturing high-performance liquid chromatography (DHPLC) in a WAVE 3500HT Fragment Analysis System (Transgenomic, Omaha, NE, USA). When an abnormal WAVE pattern was detected, we performed direct sequencing with the same primers and BigDye terminator sequencing kit (ABI) in an ABI Prism 3100 genetic analyzer.

SNPs were confirmed in at least another PCR, cloned in pGEM-T easy vector system (Promega, Madison, WI, USA), and sequenced.

#### Cell culture

L8057 cells, which have some megakaryocytic characteristics, and K562 and MEL 585 erythroid cells were grown and

differentiated as described [11, 12]. Primary human erythroblasts were obtained as described [13, 14]. Briefly, peripheral blood mononuclear cells were isolated by ficoll gradient and cultured in phase I medium: minimum essential medium (MEM) alpha modification (Sigma, St Louis, MO) with 1.5 mmol/l glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (both from Gibco BRL, Rockville, MD), 10% fetal calf serum (FCS; Biosera, East Sussex, UK), 1  $\mu\text{g}/\text{ml}$  cyclosporine A (Novartis, Basel, Switzerland), and 10% conditioned medium from 5,637 bladder carcinoma cells. After 7 days in culture, nonadherent cells were incubated in phase II medium  $\alpha$ -MEM with 30% FCS with 1 U/ml human erythropoietin (Janssen-Cilag, New Brunswick, NJ, USA),  $10^{-6}\text{M}$  dexamethasone (Merck, Whitehouse Station, NJ, USA), 1% deionized BSA,  $10^{-5}\text{M}$   $\beta$ -mercaptoethanol, 0.3 mg/ml holo-transferrin human, and 10 ng/ml human recombinant stem cell factor (last four from Sigma) for 13 days.

#### Promoter functional study

Alleles with promoter mutation and normal control were amplified with the following primers:

5'-TGCAGATGTTATTCAAAATTTCC-3'  
5'-CTGTCAACTGTGTGACTCTTTGC-3'.

PCR products were cloned in pGL3-Enhancer vector (Promega) and sequenced.

K562 cells were transiently cotransfected in a Bio-Rad Gene Pulser Electroporator (960  $\mu\text{F}$ , 220 mV) with 10  $\mu\text{g}$  of each promoter construct and 1  $\mu\text{g}$  of pEGFP-C2 (Clontech, Mountain View, CA) control.

Luciferase activity was determined with Dual-Luciferase Reporter Assay (Promega). Green fluorescent protein (GFP) expression was analyzed in a Dako (Glostrup, Denmark) flow cytometer.

#### RNA isolation and semiquantitative and quantitative real-time PCR

RNA was extracted with Tri reagent (Sigma) and transcribed with MuLV reverse transcriptase (ABI) and primer random (Roche).

The cDNA was amplified in a reaction containing Taqman Universal PCR Master Mix (ABI) and appropriate primers and FAM-NFQ probe: glycoprotein VI, Hs00212574\_m1; platelet glycoprotein IIb; Hs00166246\_m1; platelet glycoprotein IIIa, Hs00173978\_m1; Gfi1b, Hs00180261\_m1 and transferrin receptor, 4333770;  $\beta$ -actin, 4333762, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 4333764, endogenous controls (Gene Expression Assays, ABI). Real-time PCR expression was calculated relative to GPVI and GAPDH or  $\beta$ -actin using the  $\Delta\text{C}_T$  method. Statistical

**Table 1** Oligonucleotides used in EMSA studies (WT = wild type)

Gfi1b promoter WT	
Sense	5'-GGAAAGTTTTGATAAGCAAATACGGCTGAGCT-3'
Antisense	5'-GGAGCTCAGCCGTATTGCTTATCAAACTTT-3'
Mutant -8099 (A-G)	
Sense	5'-GGAAAGTTTTGATGAGCAAATACGGCTGAGCT-3'
Antisense	5'-GGAGCTCAGCCGTATTGCTCATCAAACTTT-3'
Mutant -8092 (T-C)	
Sense	5'-GGAAAGTTTTGATAAGCAAACACGGCTGAGCT-3'
Antisense	5'-GGAGCTCAGCCGTGTTTGCTTATCAAACTTT-3'

analysis was performed with nonparametric Mann–Whitney test and SPSS 15.0 program. A *P* value <0.05 was considered significant.

#### Electrophoretic mobility shift analyses

DNA binding proteins were extracted from  $10^7$  induced MEL and L8057 cells as described [15]. Electrophoretic mobility shift analysis (EMSA) was performed as described [16]. Consensus GATA, Oct, and Gfi1b binding control oligonucleotides have been previously published [17–19]. The other oligonucleotides used are shown in Table 1.

#### Antibodies

We used anti-Oct-1 (ab15112, Abcam, Cambridge, UK), Oct-2 (C-20 X, sc-233, Santa Cruz Biotechnology, Santa Cruz, CA), Oct-3/4 (H-134 X, sc-9081, Santa Cruz), mouse Gfi1b (D19, sc-8559, Santa Cruz), mouse GATA-1 (N6 X, sc-265, Santa Cruz) antibodies, and rabbit immunoglobulin fraction (normal; X 0903, DAKO, Glostrup, Denmark).

## Results

#### Gfi1b SNP screening

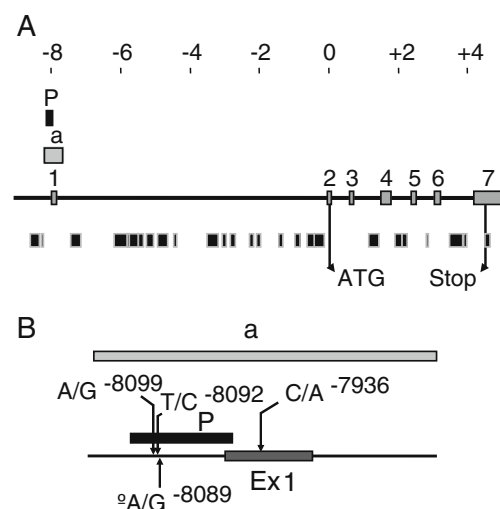
To gain further insight into Gfi1b regulation, we have searched for promoter polymorphisms in 214 patients with hematological abnormalities and 96 controls, blood donors.

We amplified Gfi1b promoter, (MN 004188; Fig. 1a) [20] and searched for SNPs through DHPLC followed by sequencing of cases with an abnormal pattern. We found two unknown SNPs (Figs. 1b and 2a, b). DHPLC and direct sequencing indicated a similar amount of normal and abnormal alleles (Fig. 2a, b). These were not present in the controls.

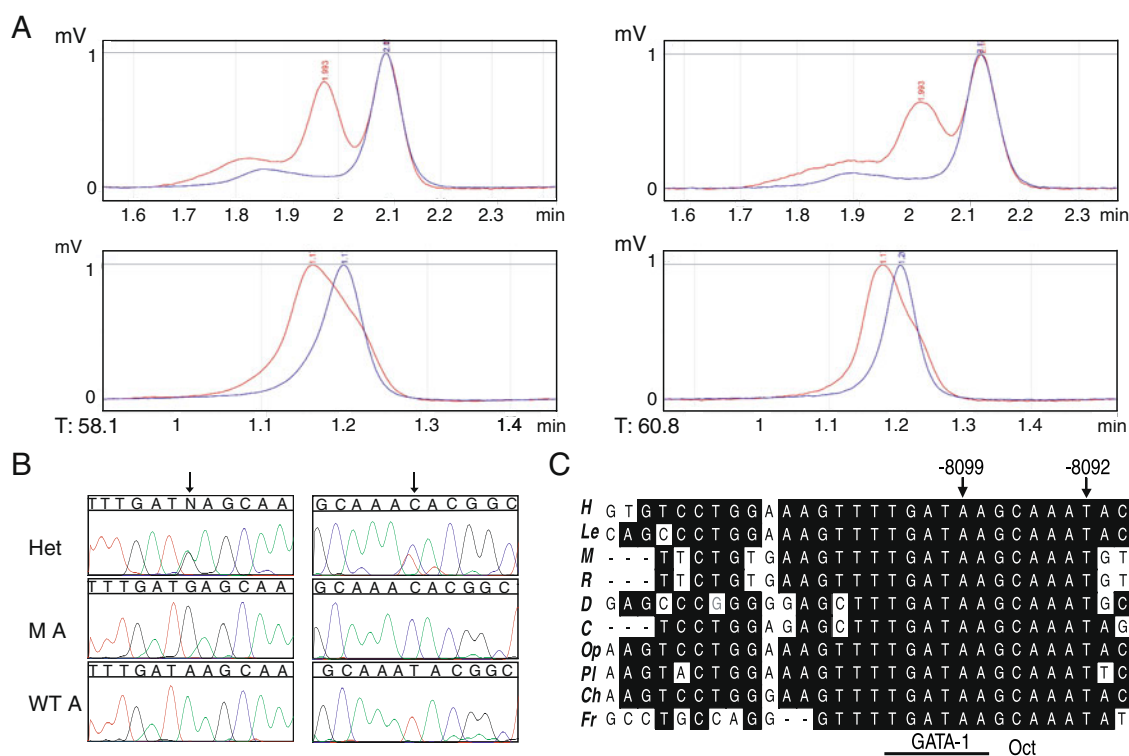
Both changes lied in an area of highly conserved sequence through evolution from frog to human (Fig. 2c). One mutation is located at position -8099 (coordinate in relation to the initiation codon), changing the sequence from TGATAA to TGATGA at a GATA-1 binding site previously validated by gel shift and chromatin immuno-

precipitation [20]. This change was detected in an acute myeloid leukemia M5a patient at diagnosis and relapse, but it was also found in complete remission bone marrow (data not shown). Additional studies could not be performed, as the patient died after relapse and had no relatives.

The other mutation affected an acute lymphoblastic leukemia patient. It is localized at -8092 (T-C; Fig. 2b, c). Unlike the previous case, in which mouth cell sample was not available, this mutation was detected in mouth cell DNA, indicating that it is congenital. Next, we performed a family study, which concluded that both parents and one sister of the proband are heterozygous for this base change, and two sisters are homozygous for it (-8092 C+/+). The family did not report consanguinity, but they are Colombian Caucasians from the same small community in the Andes. Further study of the parents and of both homozygous sisters showed they had normal blood counts (Table 1 of the Electronic Supplementary Material).



**Fig. 1** SNPs are found at the human Gfi1b promoter. **a** Human Gfi1b locus. DNA sequence is represented by a *black horizontal line*. Coordinates are relative to the initiation ATG codon. *Gray boxes* on the sequence are the exons. Gfi1b promoter is shown as a *black box* over the sequence and the promoter amplicon as a *gray box* underneath this (*a*). *Black boxes* below the sequence are repeats. **b** Location of Gfi1b promoter SNPs. The diagram **a**. SNPs are indicated with *arrows* including base changes and the coordinate. ° SNP included in NCBI database but not found in this study. *P* promoter, *Ex* exon



**Fig. 2** Promoter mutations localize at conserved sequence motifs. **a** DHPLC tracing of the promoter amplicon from mutant (red) and normal (blue) cases. The *top diagrams* represent the result obtained after running the samples at 58.1°C, and the *bottom tracings* are the consequence of performing the experiment at 60.8°C. **b** Sequence analysis of patients with abnormal DHPLC study: on the *left* -8099 (A-G) patient and on the *right* -8092 (T-C) heterozygous patient. *Top* direct sequence of PCR products (*Het*). The middle analyses correspond to the mutant allele after subcloning the PCR products (*MA*). *Bottom* wild-

type alleles (*WT A*). The *arrows* indicate the position of the mutations. **c** Sequence alignment of Gfi1b promoter including the mutated nucleotides indicated by *arrows* and *coordinates*. The alignment was performed using CLUSTALW (MacVector, Accelrys, Cary, NC, USA). *H* human (*Homo sapiens*); *M* mouse (*Mus musculus*); *R* rat (*Rattus norvegicus*); *D* dog (*Canis familiaris*); *C* cow (*Bos taurus*); *Op* opossum (*Monodelphis domestica*); *Ch* chicken (*Gallus gallus*). *Fr* frog (*Xenopus tropicalis*). GATA-1 and the potential Oct binding sites are *underlined*

### Mutations -8099 (A-G) and -8092 (T-C) affect different protein complexes

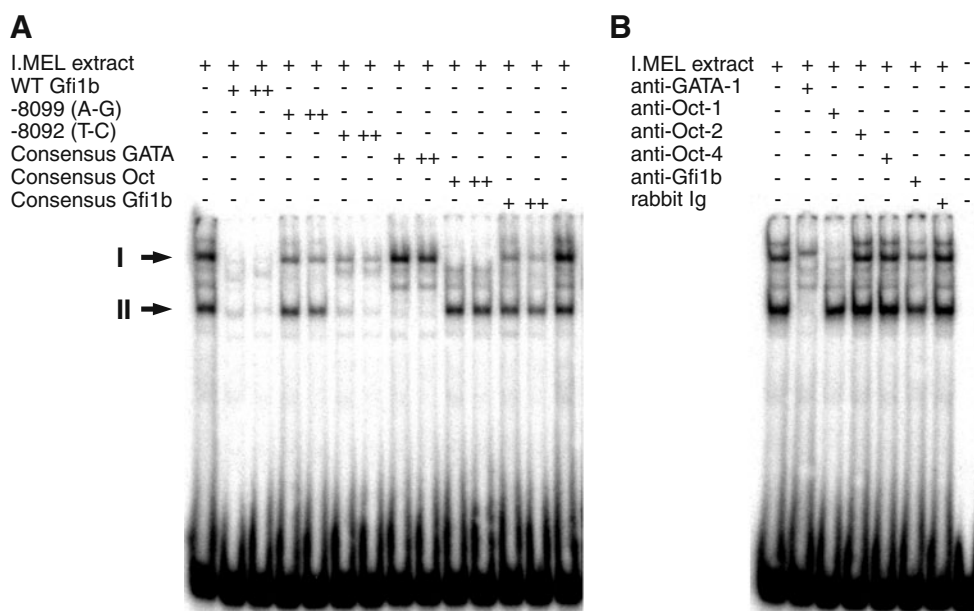
To further understand these mutations, we studied protein binding to the normal and mutated sequences by EMSA. An oligonucleotide containing the normal sequence including positions -8099 and -8092 was radioactively labeled and reacted with nuclear proteins from induced MEL and L8057 cells (Figs. 3a, b and Figure 1 of the Electronic Supplementary Material). Two protein/DNA complexes were obtained in both cell lines; they were completely abolished by an excessive amount of unlabeled oligomer. The -8099 (A-G) oligo did not compete completely with any of both complexes, although competed preferentially for the upper complex (I), while -8092 (T-C) oligo inhibited the bottom complex (II) but did not abolish complex I.

Excess amount of unlabeled consensus GATA oligo blocked complex II binding to the probe (Fig. 3a and Figure 1 of the Electronic Supplementary Material) and GATA-1 antibody-inhibited and shifted complexes I and II (Fig. 3b and Figure 1 of the Electronic Supplementary Material). This

indicates that GATA-1 is present in both complexes, but only complex II directly binds GATA recognition sequence. Mutation -8099 (A-G) decreases this binding, and it also reduces complex I DNA affinity.

Mutation -8092 (T-C) affects a sequence where Oct proteins have been shown to bind that differs for one base to the canonical octamer (ATGCAAAT) [21]. Thus, we used an unlabeled consensus octamer oligomer to compete with normal Gfi1b probe; this destroyed complex I (Fig. 3a and Figure 1 of the Electronic Supplementary Material). Antibody against Oct-1 (POU2F1) abolished complex I, suggesting that Oct-1 is part of this complex (Fig. 3b and Figure 1 of the Electronic Supplementary Material).

Gfi1b can repress its own transcription, binding to Gfi1b promoter [19, 22]. This could be achieved through GATA-1 binding [19]. We incubated the nuclear extracts with an oligonucleotide containing Gfi1b binding sequence or Gfi1b antibody. These consistently produced an attenuation of the protein/DNA complexes (Fig. 3a, b and Figure 1 of the Electronic Supplementary Material).



**Fig. 3** –8099 (A-G) and –8092 (T-C) mutations mainly affect two different DNA/protein complexes. GATA-1 and Oct-1, respectively, bind to DNA at the sites affected by each of these mutations. Gel shift analysis of the –8112/–8081 region of human Gfi1b promoter (coordinates relative to the translation start site). **a** The <sup>32</sup>P-labeled double-stranded oligonucleotide corresponding to the –8112/–8081 sequence of Gfi1b locus was used for the analysis in the presence or

absence of unlabeled competitor in 50 (+) or 100 (++) fold molar excess with nuclear proteins extracted from induced MEL cells. **b** Gel shift analysis performed as before; when indicated, antibodies or rabbit immunoglobulins (*rabbit Ig*) were added. A control with no protein extract is also shown. The *arrows* indicate the location of complexes I and II

In conclusion, –8099 (A-G) and –8092 (T-C) mutations mainly affect two different DNA/protein complexes. GATA-1 and Oct-1, respectively, bind to DNA at the sites affected by each of these mutations. Gfi1b itself can be part of the complexes under certain circumstances.

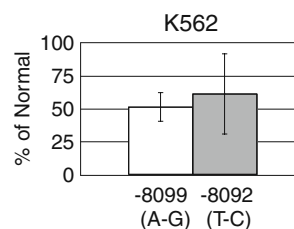
Natural occurring mutation at GATA-1 site decreases promoter activity

To study the impact of these changes in vitro, the abnormal and normal alleles were cloned in pGL3-Enhancer Vector (Promega), which encodes firefly luciferase, and then they were transiently transfected to K562 cells. The vector with GATA site mutation consistently decreased luciferase activity to 50% of the normal (Fig. 4), while the octamer mutation produced some decrease in activity, which cannot be considered significant due to the high interexperimental variability (Fig. 4).

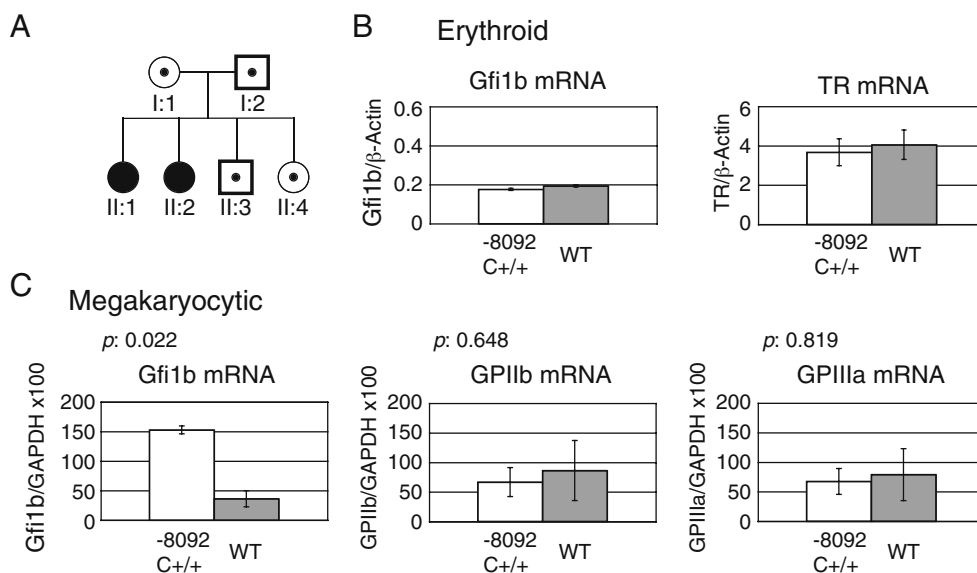
Homozygous Gfi1b promoter mutation –8092 (C+/+) increases Gfi1b expression in megakaryocytes

To further investigate the role of mutation –8092 (T-C), we studied the impact of this mutation in the erythroid and megakaryocytic lineages. First, we differentiated peripheral blood mononuclear cells from a homozygous individual (–8092 C+/+) (patient II:1, Fig. 5a) and a normal control

onto erythroid cells in vitro and studied Gfi1b and transferring receptor expression in late erythroblasts. Patient and control showed no differences in mRNA levels (Fig. 5b). Next, we assessed Gfi1b RNA level in platelets by real-time PCR. We studied three different samples from patient II:1 and one from patient II:2 and compared them with 20 normal individuals (Fig. 5c and Figure 2 of the Electronic Supplementary Material), demonstrating an increase in Gfi1b expression more than fourfold in –8092 C+/+ individuals. Control platelet glycoproteins IIb



**Fig. 4** The –8099 (A-G) mutation causes a reduction in Gfi1b expression in vitro. K562 cells were cotransfected with pGL3-Enhancer reporter plasmid containing –8222/–7925 region of wild-type Gfi1b, –8099 (A-G) or –8092 (T-C) mutant alleles and pEGFP-C2. Luciferase activity was standardized relative to GFP expression, and data of each mutant allele were plotted relative to wild-type Gfi1b promoter result set arbitrarily to 100. Data from –8099 (A-G) mutation are shown in the *white column*; the results from –8092 (T-C) mutation are represented in the *gray column*. *Error bars* represent  $\pm$ standard deviation from the mean of three independent experiments



**Fig. 5** The  $-8092$   $C^{+/+}$  mutation associates with increase Gfi1b mRNA in platelets but not in erythroid cells. **a** Pedigree of the family with  $-8092$  (T-C) mutation. *Black circles* represent  $-8092$   $C^{+/+}$  homozygous females, *circles and squares with black disks* represent  $-8092$  (T-C) heterozygous females and males, respectively. **b** Real-time PCR mRNA analysis of in vitro differentiated erythroid cells. Study on cells after 13 days on second phase culture, when the predominant cells are mainly polychromatophilic and in less proportion orthochromic erythroblasts. *Left* Gfi1b and *right* transferring receptor (*TR*); mRNA level was normalized with  $\beta$ -actin mRNA

expression. Analysis in cells from patient II:1 ( $-8092$   $C^{+/+}$ , *white boxes*) and a wild-type control (*WT*, *gray boxes*) show similar results. The standard deviations correspond to three different studies starting from the cDNA synthesis. **c** Real-time PCR mRNA study in platelets. *Left* Gfi1b expression in three samples extracted in different days of patient II:1 and one of II:2 (*white box*) compared to 20 normal controls (*gray box*), and normalized with GAPDH mRNA level, shows significant differences. *Middle panel* platelet glycoprotein IIb and *right panel* glycoprotein IIIa mRNA, studied and plotted as before, showing similar levels of expression in patients and controls

and IIIa showed similar RNA levels in  $-8092$   $T^{+/+}$  and  $C^{+/+}$  cases (Fig. 5c and Figure 2 of the Electronic Supplementary Material).

## Discussion

Gfi1b promoter mutations indicate that an octamer-binding complex represses Gfi1b in the latest stages of megakaryocyte maturation and confirm that a GATA-1-containing complex activates the gene. We have recently studied Gfi1b regulation through erythropoiesis. As erythroid cells differentiate the general decrease in transcription factor expression, and subsequently of binding to Gfi1b promoter and regulatory elements, correlate with progressive reduction in Gfi1b expression [23]. Nevertheless, in the megakaryocytic lineage, additional unknown factors must be required to achieve Gfi1b repression. This would explain that mutation  $-8092$  ( $C^{+/+}$ ) increases Gfi1b expression in megakaryocytes, but Gfi1b level does not change in late erythroid cells, strongly suggesting that Oct-1 is the missing repressor. Accordingly, Oct-1 has intrinsic silencing activity [18]. The octamer-like sequence where the mutation localizes (GATAAGCAAAT to GATAAGCAAAC) partly overlaps the previously described GATA-1 binding site. Likewise, a GATA/Oct

combined site is present at  $\gamma$ -globin promoter [24]. While this paper was under review, Laurent et al. also described Oct-1 binding to this site together with another position in Gfi1b promoter [25]. These authors observed that Oct-1 increases the activity of Gfi1b promoter linked to a reporter gene when transfected together with GATA-1 and HGMB2 in HeLa cells. In agreement with our data in erythroid cells, they found a no significant decrease in the promoter activity when the Oct-1 site where  $-8092$  (T-C) localizes was mutated. Nevertheless, Gfi1b promoter activity reduced when both Oct-1 sites were destroyed. These in vitro findings may correspond to the assembly of certain protein complexes on Gfi1b promoter that can be relevant in vivo in some cell types or stages of differentiation. However, our in vivo data strongly suggest that a repressive complex forms around  $-8092$  in terminally differentiated megakaryocytes.

This is the first report of Gfi1b mutations with a functional implication. Considering the evidences that point to the importance of Gfi1b hyperexpression in leukemia [6, 7], promoter changes, particularly activating mutations like  $-8092$  (T-C), may be important in blood malignancy. Nevertheless, it is likely that several abnormalities or sequence variations converge to have a pathological impact. Further investigation and follow-up will rule out the involvement of these mutations in hematological disease.

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**Authorship and disclosures** Contribution: EA was the principal investigator, designed the study, performed the laboratory work, and wrote the paper. AV provided information on the patients. AH performed some research. The authors reported no potential conflicts of interest.

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