

# The transcription factor GATA1 regulates NBEAL2 expression through a long-distance enhancer

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## ABSTRACT

Gray platelet syndrome is named after the gray appearance of platelets due to the absence of  $\alpha$ -granules. It is caused by recessive mutations in *NBEAL2*, resulting in macrothrombocytopenia and myelofibrosis. Though using the term gray platelets for GATA1 deficiency has been debated, a reduced number of  $\alpha$ -granules has been described for macrothrombocytopenia due to *GATA1* mutations. We compared platelet size and number of  $\alpha$ -granules for two *NBEAL2* and two *GATA1*-deficient patients and found reduced numbers of  $\alpha$ -granules for all, with the defect being more pronounced for *NBEAL2* deficiency. We further hypothesized that the granule defect for *GATA1* is due to a defective control of *NBEAL2* expression. Remarkably, platelets from two patients, and *Gata1*-deficient mice, expressed almost no *NBEAL2*. The differentiation of *GATA1* patient-derived CD34<sup>+</sup> stem cells to megakaryocytes showed defective proplatelet and  $\alpha$ -granule formation with strongly reduced *NBEAL2* protein and ribonucleic acid expression. Chromatin immunoprecipitation sequencing revealed 5 GATA binding sites in a regulatory region 31 kb upstream of *NBEAL2* covered by a H3K4Me1 mark indicative of an enhancer locus. Luciferase reporter constructs containing this region confirmed its enhancer activity in K562 cells, and mutagenesis of the GATA1 binding sites resulted in significantly reduced enhancer activity. Moreover, DNA binding studies showed that GATA1 and GATA2 physically interact with this enhancer region. GATA1 depletion using small interfering ribonucleic acid in K562 cells also resulted in reduced *NBEAL2* expression. In conclusion, we herein show a long-distance regulatory region with GATA1 binding sites as being a strong enhancer for *NBEAL2* expression.

## Introduction

Platelets play a critical role in hemostasis and contain secretory granules that are essential to maintain their normal function. Of the three types of granules (dense,  $\alpha$  and lysosomes),  $\alpha$ -granules are the most abundant type that store essential proteins for platelet adhesion and blood coagulation.<sup>1</sup> Platelets are shed into the blood stream after the formation of long cytoplasmic extensions from differentiated megakaryocytes (MKs), a process called proplatelet formation. The  $\alpha$ -granules are formed in the early MKs and are actively transported along microtubules from the MK cell body towards the proplatelets.<sup>1</sup> In circulating platelets, these  $\alpha$ -granules mature further and proteins will be actively or passively taken up from the plasma by receptor-mediated endocytosis, in addition to the proteins loaded in these granules during their biosynthesis in the MK.<sup>2</sup> Recently, it has become clear that

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$\alpha$ -granule proteins play important roles in nonhemostatic events, such as wound healing, cancer, inflammation and innate immunity.<sup>3</sup>

Gray platelet syndrome (GPS, MIM139090) is a rare inherited platelet disorder characterized by mild to moderate bleeding, macrothrombocytopenia and markedly reduced or absent  $\alpha$ -granules that typically results in a grayish appearance of platelets under a light microscope.<sup>4</sup> Using three independent next-generation sequencing approaches, recessive mutations in *NBEAL2* were found to cause GPS.<sup>5-7</sup> *NBEAL2* encodes a 2.754 amino acid polypeptide, neurobeachin-like-2, that contains BEACH (named after Beige and Chediak-Higashi), ARM (Armadillo), Con A-like lectin (Concanavalin A-like lectin domain), PH (Pleckstrin Homology-domain like) and WD40 domains. The exact role of BEACH domain-containing proteins remains largely unknown, but they are generally large proteins that are able to control diverse cellular mechanisms such as vesicular transport, apoptosis, membrane dynamics and receptor signaling.<sup>8</sup> Interestingly, *LYST* and *NBEA*, two other BEACH domain-containing proteins, have been shown to be implicated in platelet dense granule defects.<sup>9-10</sup> Regarding these proteins, their exact contribution in granule formation and trafficking during MK and platelet formation also remains unknown.

In addition, the term GPS has been used for macrothrombocytopenia patients with defects in X-linked *GATA1*<sup>11</sup> (MIM305371), or having an autosomal dominant loss-of-function variant in *GFI1B*<sup>12</sup> (MIM187900). Both genes encode for transcription factors that regulate megakaryopoiesis, and defects result in enlarged platelets with fewer (though not absent)  $\alpha$ -granules, as studies in additional *GATA1* and *GFI1B* patients illustrated.<sup>13-18</sup> Based

on a recent detailed comparison of clinical and laboratory data for *NBEAL2*, *GATA1* and *GFI1B* patients,<sup>3</sup> it was suggested not to use the term GPS for other macrothrombocytopenia disorders that comprise abnormal  $\alpha$ -granule numbers, except for patients with *NBEAL2* defects. Important similarities indeed do exist (e.g., large platelets with paucity of  $\alpha$ -granules, myelofibrosis, platelet dysfunction) but also important differences, such as red cell abnormalities that are only described for *GATA1* and *GFI1B* defects and the presence of CD34-positive platelets for *GFI1B* defects.

In the study herein, we hypothesized that the  $\alpha$ -granule defect found for *GATA1* macrothrombocytopenia might be due to reduced *NBEAL2* expression. We compared platelet morphology and *NBEAL2* protein expression using samples from two *NBEAL2* and two *GATA1*-deficient macrothrombocytopenia patients. *In vitro* megakaryopoiesis from hematopoietic stem cells (HSCs) from the *GATA1* defective patients was studied with a focus on proplatelet formation,  $\alpha$ -granule formation and *NBEAL2* expression. Finally, The Encyclopedia of DNA Elements (ENCODE) and *GATA1/GATA2* Chromatin immunoprecipitation (ChIP) sequencing data were used to predict putative enhancer elements upstream of the *NBEAL2* gene. A long-distance (31 kb upstream) region was identified as a potential enhancer that was characterized using reporter and DNA-binding assays.

## Methods

### Patient studies

Table 1 contains clinical and laboratory data for two previously described *GATA1*-<sup>13,14</sup> and two *NBEAL2*-deficient patients. Genetic screening of *NBEAL2* was performed using a high-

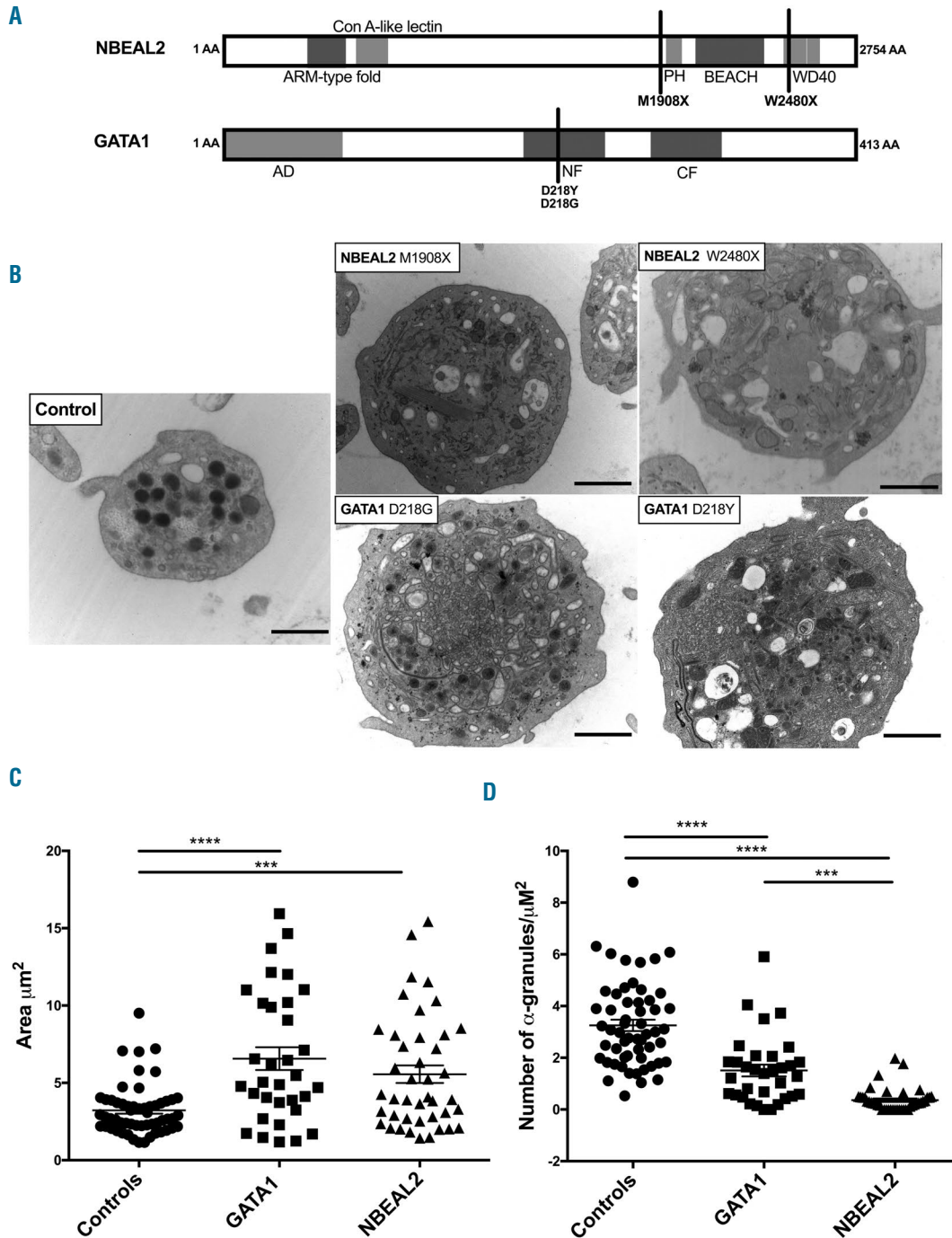
**Table 1.** Comparison of clinical and laboratory data obtained for patients with defects in *NBEAL2* and *GATA1*.

	<i>NBEAL2</i> MIM13909		<i>GATA1</i> MIM300367/314050	
Mode of inheritance	Autosomal recessive		X-linked	
Genetic defect	Homozygous c.7440G>A	Homozygous c.5721-1G>C	c.653A>G	c.652G>T
Protein defect	W2480X	Predicted M1908X	D218G	D218Y
Dyserythropoiesis	No		Yes	Yes + anemia
Myelofibrosis	Yes	No	No	Yes
Macrothrombocytopenia	Yes		Mild TP	Severe TP
$\alpha$ -granule defect as determined by EM	Yes		Yes	
Megakaryocytic emperipoiesis	Yes	ND	Yes	
Plt count, x 10 <sup>9</sup> /L*	40	55	53	8
MPV, fL	> Max value	> Max value	> Max value	> Max value
Bleeding severity	Mild to moderate		Mild	Severe
Defective platelet aggregation	ND	Performed at low plt count in PRP: Impaired ADP, collagen, arachidonic acid, and ristocetin-induced plt aggregation	Performed at low plt count in PRP: Impaired collagen, and ristocetin-induced plt aggregation	ND

Normal platelet count should be between 150-400 x 10<sup>9</sup>/L. ND: not determined; EM: electron microscopy; Plt: platelet; MPV: mean platelet volume; PRP: platelet rich plasma; ADP: adenosine diphosphate; TP: thrombocytopenia.

throughput sequencing test.<sup>19</sup> Platelet testing and electron microscopy (EM) analysis was performed as described.<sup>13</sup> The platelet surface area ( $\mu\text{m}^2$ ) and number of  $\alpha$ -granules/ $\mu\text{m}^2$  were measured with ImageJ software. Patients, or their legal represen-

tatives, signed informed consent to participate in the enhanced clinical and laboratory phenotyping studies. The Ethics Committee of the University Hospitals Leuven approved the study (ref. ML3580).



**Figure 1. Variants in NBEAL2 and GATA1 result in macrothrombocytopenia with a paucity of  $\alpha$ -granules.** (A) Schematic diagram displaying protein domains in the NBEAL2 and GATA1 protein. The NBEAL2 protein contains an ARM-type fold, Con A-like lectin, PH, BEACH and WD40 domain. The NBEAL2 predicted M1908X and NBEAL2 W2480X variants are indicated. The GATA1 protein contains a transactivation domain (TAD), N-terminal finger domain (NF), and C-terminal finger domain (CF). Locations of the GATA1 D218Y and D218G variants are indicated. Protein drawing is not to scale. (B) EM images of platelets for the 4 patients with different variants in comparison to an unrelated control show enlarged platelets that are more round instead of discoid, with only a few  $\alpha$ -granules and more open canalicular system forming vacuoles (Scale bars =  $1\mu\text{m}$ ). (C,D) The platelet area ( $\mu\text{m}^2$ ) and number of  $\alpha$ -granules/ $\mu\text{m}^2$  were quantified. Values are the means  $\pm$  SEM as quantified for two GATA1 patients, two NBEAL2 patients and three unrelated controls. The number of  $\alpha$ -granules/ $\mu\text{m}^2$  showed a significant reduction for GATA1 patients and an even more pronounced reduction for the patients with NBEAL2 variants, which was significant compared to the patients with GATA1 variants. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , one-way analysis of variance (ANOVA) with Bonferroni's multiple test. AA; amino acids; ARM: armadillo.

## Megakaryocyte cultures, proplatelet formation and immunohistochemistry

CD34<sup>+</sup> HSCs were isolated from the peripheral blood of patients with the GATA1 D218Y and D218G variant and unrelated healthy controls. The MK differentiation assays and immunostainings are described in the *Online Supplementary Methods*. Proplatelet-forming MKs were analyzed as described.<sup>20,21</sup>

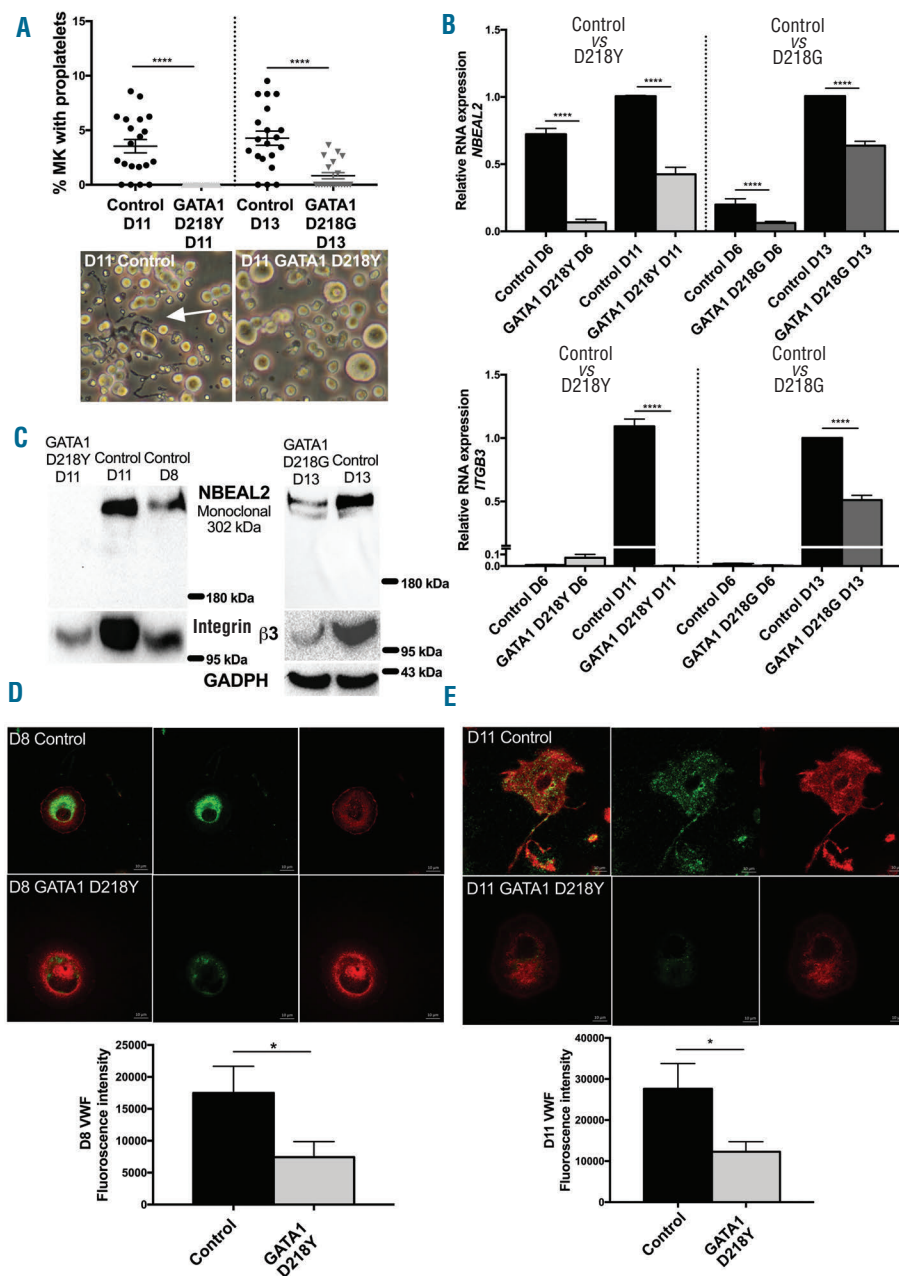
## Immunoblot analysis

Proteins were isolated from platelets and *in vitro* differentiated MKs as described.<sup>13</sup> Platelet extracts were also obtained from C57BL/6/Gata1-deficient mice ( $\Delta$ neo $\Delta$ HS), which have been described previously (Dr. Paresh Vyas, Department of Haematology and MRC Molecular Haematology Unit, Weatherall

Institute of Molecular Medicine).<sup>22</sup> Blots were performed as described in the *Online Supplementary Methods*.

## Luciferase reporter assays to measure NBEAL2 enhancer activity

The NBEAL2 enhancer region is located about 31 kb upstream of the *NBEAL2* gene and within an intron of the *CCDC12* gene (GRCh37/hg19) and was cloned as two fragments, these being Chr3:46988756-46989716 (comprising 3 potential GATA sites BS-1-2-3) and Chr3:46989733-46990332 (comprising 2 potential GATA sites BS-4-5) in the pGL3 promotor (enhancer-less) vector (Promega). Details of cloning, mutagenesis, transfection of K562 cells and luciferase measurements are described in the *Online Supplementary Methods*.<sup>23</sup>



**Figure 2. Effect of GATA1 D218Y and D218G variants on megakaryopoiesis.** MKs were differentiated from peripheral blood-derived CD34<sup>+</sup> HSCs from GATA1 patients D218Y, D218G and unrelated healthy controls. The MK differentiation assay was performed twice with different controls. (A) (Top) Proplatelet formation was quantified at day 11 or day 13 of differentiation in duplicate liquid cultures for the control and the GATA1 patients. \*\*\*\* $P < 0.0001$ , t-test. (Bottom) Proplatelet-forming MKs were present in the control and to a lesser extent in the GATA1 D218G (arrow). Though large MKs were present for GATA1 patient D218Y, proplatelet formation was not seen. (B) PCR data performed on RNA collected at day 6, and day 11 or 13, respectively, from the D218Y and D218G defective MKs. Expression levels of *NBEAL2* and *ITGB3* were normalized to *GADPH* expression. \*\*\*\* $P < 0.0001$ , t-test. (C) Immunoblot analysis for NBEAL2 with a rabbit polyclonal antibody and integrin  $\beta$ 3 were performed (left) using total protein MK lysates at days 8 and 11 for the control and day 11 for GATA1 D218Y (right) using total protein MK lysates at day 13 for the control and GATA1 D218G. Uncropped blots are shown in the *Online Supplementary Figure S3*. (D, E) (Top) Representative immunofluorescence confocal microscopy images of differentiated MKs at days 8 (D) and 11 (E) of culture. The MKs were stained for VWF (green) and actin (red). The control shows VWF present inside the  $\alpha$ -granules at day 8 with a further increase of VWF at day 11. The MKs of GATA1 patient D218Y were only weakly positive for VWF both at day 8 and 11 (Scale bar = 10  $\mu$ m). (Bottom) Quantification of VWF staining showed a significant reduction of VWF in GATA1 D218Y patient at days 8 (D) and 11 (E) of culture in comparison to the control. Values are means  $\pm$  SEM as quantified for 10 randomly selected images. Panel D: day 8, \* $P = 0.0465$ , t-test; panel E: \* $P = 0.0322$ , t-test. RNA: ribonucleic acid; GADPH: glyceraldehyde 3-phosphate dehydrogenase; VWF: von Willebrand factor; MK: megakaryocytes.

### Quantitative (q)RT-PCR to quantify *GATA1*, *GATA2*, *NBEAL2*, and *ITGB3* expression

Total ribonucleic acid (RNA) from K562 cells was extracted with TRIzol (Invitrogen). Expression of *GATA1*, *GATA2*, *NBEAL2* and *ITGB3* were measured with Sybr Green quantitative real-time polymerase chain reaction (qRT-PCR) using the ABI 7000 real-time PCR machine (Life Technologies). Expression was quantified via the  $\Delta\Delta C_t$  method in arbitrary units<sup>24</sup> (see the *Online Supplementary Methods* for primer details).

### DNA binding assay

Biotinylated PCR fragments for BS-1-2-3 and BS-4-5 were bound to Superparamagnetic streptavidin beads (Hyglos) and added to nuclear extracts (NE) isolated from HEK293 cells transfected with *GATA1* or *GATA2*. The cloning, binding reaction, and blots are described in the *Online Supplementary Methods*.

### *GATA1* knockdown in K562 cells using siRNA

*GATA1* depletion in K562 cells was obtained after transfection with SMARTpool *GATA1* small interfering RNA (siRNA)<sup>25</sup> or negative control SMARTpool siRNA (Dharmacon) (see *Online Supplementary Methods*).

### Glutathione S-Transferase-pull down assay with *GATA1*-NF

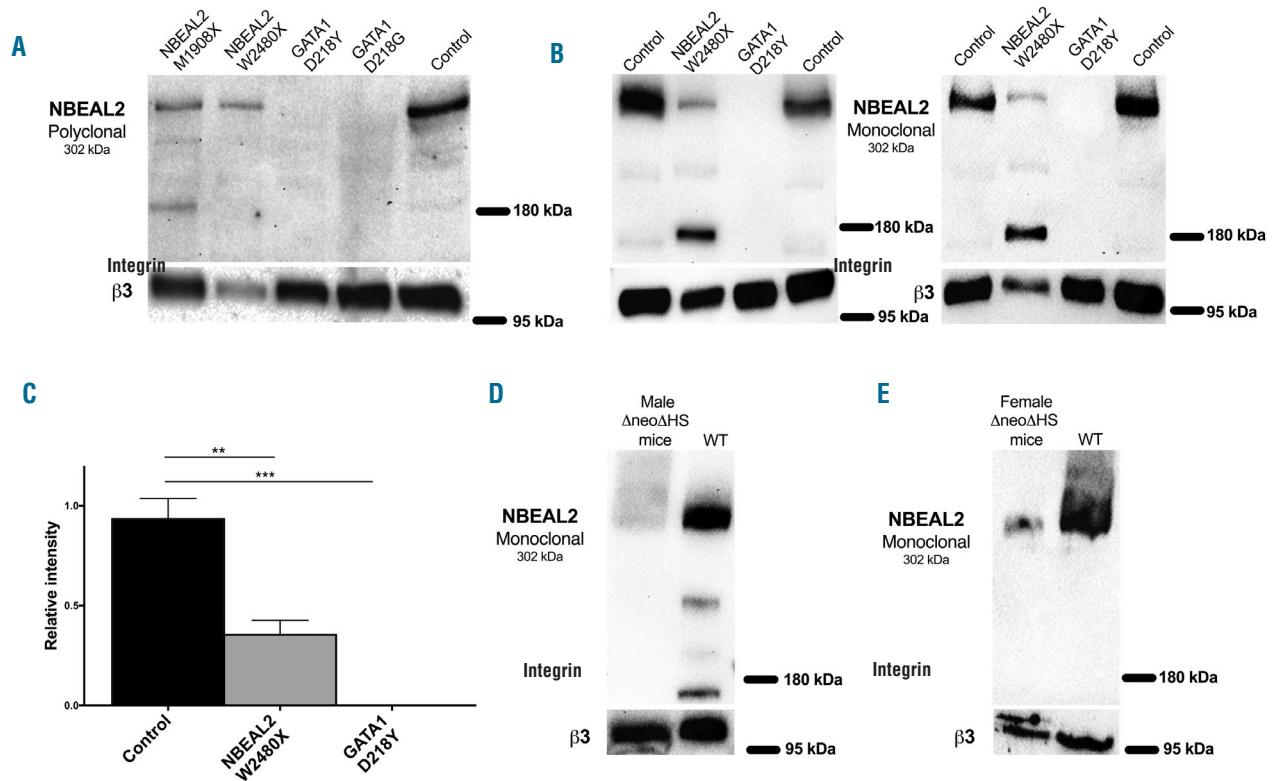
Glutathione S-Transferase (GST)-coupled *GATA1*-NF or GST-

only bound to glutathione sepharose beads (GE Healthcare) was prepared as described.<sup>15</sup> Immunoprecipitation with GF11B<sup>26</sup> was performed as described in the *Online Supplementary Methods*.

## Results

### Clinical description and platelet morphology studies in macrothrombocytopenia patients with *NBEAL2* and *GATA1* variants

Table 1 describes clinical and laboratory phenotypes for two GPS patients with recessive *NBEAL2* variants and two boys with *GATA1* variants. One GPS patient is homozygous for W2480X, that is predicted to result in a non-functional protein without WD40 domain (Figure 1A). The other GPS patient has a homozygous splice variant c.5721-1G>C (Figure 1A), predicted to result in an early stop codon M1908X that would delete the PH, BEACH and WD40 domains. Indeed, a similar splice variant, c.5720+1G>A, in another GPS patient has been demonstrated to result in M1908X, based on expression studies.<sup>27</sup> The macrothrombocytopenia patients with *GATA1* variants D218G<sup>13</sup> and D218Y<sup>6</sup> were previously described (Figure 1A). Both mutations are located in the N-terminal finger domain, which is required for association with the coactivator friend-of-*GATA1* (FOG1) that contributes to

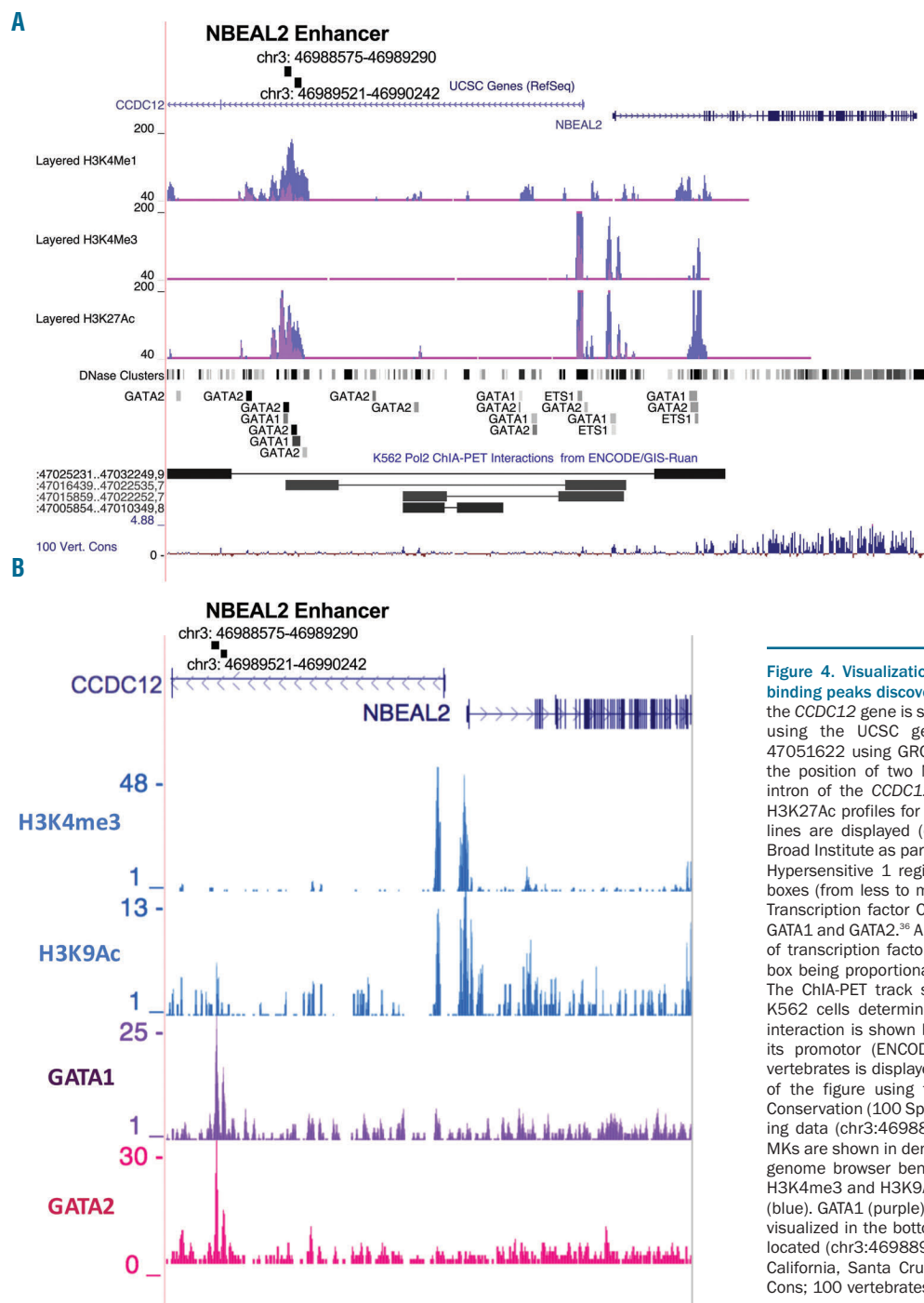


**Figure 3. NBEAL2 expression in *GATA1* and *NBEAL2*-deficient platelets.** (A) Immunoblot analysis for NBEAL2 was performed with a rabbit polyclonal NBEAL2 antibody (epitope against amino acids 1865-1939) for platelet lysates from patients with NBEAL2 M1908X, NBEAL2 W2480X, *GATA1* D218Y and *GATA1* D218G. Integrin  $\beta 3$  was used as loading control. (B) Replicated immunoblots performed with a rabbit monoclonal NBEAL2 antibody (ab187162; epitope against amino acids 1-100) for platelet lysates from patients with NBEAL2 W2480X and *GATA1* D218Y defects. Integrin  $\beta 3$  was used as loading control. (C) Quantification of NBEAL2 expression.  $^{**}P=0.0077$ ,  $^{***}P=0.0006$ , *t*-test. (D, E) Expression of NBEAL2 in platelets from hemizygous male (D) and homozygous female (E) *Gata1*-deficient mice in comparison to sex-matched wild-type mice. For each lane platelets were pooled from 10 mice for *Gata1* and 5 mice for control. Uncropped blots are shown in the *Online Supplementary Figure S4*. WT: wild-type.

the stability of DNA binding to a palindromic GATA recognition sequence.<sup>28</sup> GATA1 D218Y resulted in a severe clinical phenotype with deep macrothrombocytopenia, while patients with GATA1 D218G only have mild macrothrombocytopenia (Table 1). All patients have macrothrombocytopenia, and blood smears and EM studies of their platelets clearly show a gray appearance of large platelets due to a paucity of  $\alpha$ -granules. Myelofibrosis was present in the patient with NBEAL2 W2840X and the patient with GATA1 D218Y. Bone marrow studies observed the presence of megakaryocyte emperipolesis in all patients except the patient with the

NBEAL2 M1908X variant. The platelet aggregation defect for the patient with the NBEAL2 M1908X variant was similar to the defect found for the patient with GATA1 D218G. The major difference between these cases is that the GPS patients had normal red blood cells, while dyserythropoiesis was clearly present in GATA1 D218G<sup>13</sup> and severe anemia with dyserythropoiesis in GATA1 D218Y.<sup>14</sup>

Detailed morphological examination of platelets by EM for all patients was performed and compared. All had enlarged platelets that are more round instead of discoid with fewer  $\alpha$ -granules and a pronounced open canalicular system forming large vacuoles in some platelets (Figure



**Figure 4. Visualization of NBEAL2 enhancer with GATA binding peaks discovered by ChIP sequencing.** (A) Part of the *CCDC12* gene is shown upstream of the *NBEAL2* gene using the UCSC genome browser (chr3:46975201-47051622 using GRCh37/hg19). The upper layer shows the position of two NBEAL2 enhancer peaks within an intron of the *CCDC12* gene. H3K4Me1, H3K4Me3 and H3K27Ac profiles for K562 (blue) and NHEK (purple) cell lines are displayed (data produced by Bernstein lab at Broad Institute as part of the ENCODE database).<sup>34</sup> DNase Hypersensitive 1 regions are displayed as gray to black boxes (from less to more open chromatin conformation). Transcription factor ChIP sequencing data are shown for GATA1 and GATA2.<sup>36</sup> A gray box encloses each peak cluster of transcription factors occupancy (with darkness of the box being proportional to the maximum signal strength). The ChIA-PET track shows the chromatin interaction in K562 cells determined by RNA polymerase II. Physical interaction is shown between the *NBEAL2* enhancer and its promoter (ENCODE/GIS-Ruan). Conservation across vertebrates is displayed as blue histograms at the bottom of the figure using the Vertebrate Multiz Alignment & Conservation (100 Species) UCSC track. (B) ChIP sequencing data (chr3:46988420-47051185) of primary human MKs are shown in density plots and displayed in the UCSC genome browser beneath the tracks for gene structure. H3K4me3 and H3K9Ac are visualized in the upper tracks (blue). GATA1 (purple) and GATA2 (pink) binding peaks are visualized in the bottom tracks. The NBEAL2 enhancer is located (chr3:46988970-46989880). UCSC: University of California, Santa Cruz; DNase: deoxyribonuclease; Vert. Cons: 100 vertebrates conservation.

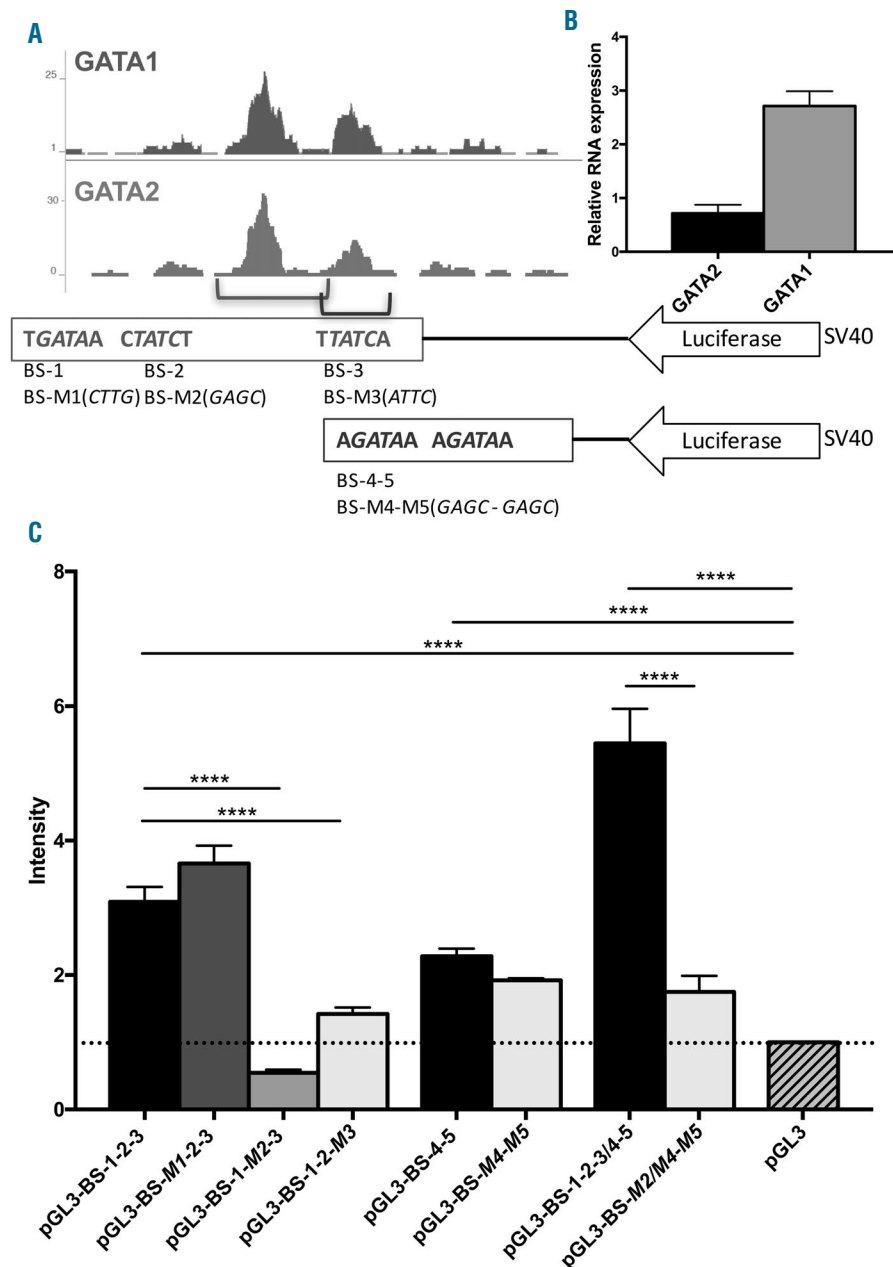
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1B). Quantification of the platelet size for three unrelated controls, two GATA1 and two NBEAL2 patients, showed significantly larger platelets in both patient groups. There was no difference between the patient groups (Figure 1C). The number of  $\alpha$ -granules corrected for platelet size showed a pronounced reduction for GATA1 patients and even more for the NBEAL2 patients, that were now significantly different from GATA1 patients (Figure 1D).

### Abnormal NBEAL2 expression in megakaryocytes from GATA1 patients

Recent *in vitro* megakaryopoiesis studies using HSCs from GPS patients with *NBEAL2* mutations showed normal MK differentiation with defective proplatelet formation and reduced  $\alpha$ -granule proteins such as von Willebrand factor (VWF), thrombospondin and P-

selectin.<sup>29</sup> Peripheral blood-derived HSCs from GATA1 patients D218Y and D218G were differentiated to MK to quantify proplatelet formation during two independent culture experiments with each compared to another unrelated control. Similar as for *NBEAL2* deficiency, large MKs were present, but the MKs for both patients showed a significantly decreased formation of proplatelets that was more pronounced for D218Y (Figure 2A). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using RNA from MKs at differentiation days 6 and 11 (for D218Y) or days 6 and 13 (for D218G) (Figure 2B). *NBEAL2* and *ITGB3* were significantly decreased throughout the MK differentiation for the GATA1 patients, but again the defect was more pronounced for the D218Y patient. We performed immunoblot analysis using total protein lysates from differentiated MKs to quantify



**Figure 5. NBEAL2 enhancer activity using luciferase reporter assays.** (A) The binding peaks for GATA1 (purple) and GATA2 (pink) discovered by ChIP sequencing of primary human MKs are visualized (ch3:46986830-46991970). Schematic overview of the location of the 5 potential GATA binding sites. BS-1-2-3 are located in the first peak and BS-4-5 in the second peak. The two fragments are cloned in a luciferase construct 3' prime to the luciferase reporter gene to generate pGL3-BS-1-2-3 and pGL3-BS-4-5. Binding site mutants are pGL3-BS-M1-2-3, pGL3-BS-1-M2-3, pGL3-BS-1-2-M3 and pGL3-BS-M4-M5 with the mutations indicated. (B) qRT-PCR for RNA from K562 was performed to determine the expression of GATA1 and GATA2 relative to *GADPH*. Expression was quantified via the  $\Delta\Delta Ct$  method in arbitrary units. (C) The luciferase expression after transfection with different constructs as indicated in K562 cells. The pGL3 promoter vector without enhancer sequence was used as control construct. Each plasmid was assayed in triplicate in six separate transfection experiments. \*\*\*\* $P < 0.0001$ , one-way analysis of variance (ANOVA) with Bonferroni's multiple test. RNA: ribonucleic acid; BS: binding site.

NBEAL2 and integrin  $\beta 3$  expression. Remarkably, NBEAL2 expression was absent in GATA1 D218Y compared to control MKs at differentiation day 11 (Figure 2C). As integrin  $\beta 3$  levels were also lower for GATA1 D218Y, we also loaded day 8 differentiated MKs from the control, and these cells expressed comparable integrin  $\beta 3$  levels while NBEAL2 was clearly detectable. NBEAL2 expression was also studied at a later time point in MK differentiation (day 13) for GATA D218G, but its expression remained low compared to control MKs (Figure 2C). To exclude NBEAL2 degradation in GATA1 mutant MKs, the expression of filamin (FLN), a large protein sensitive to calpain-mediated cleavage, was found to be comparable to control MKs (Online Supplementary Figure S1).

MKs were stained at days 8 and 11 for VWF and actin to visualize  $\alpha$ -granule formation (Figure 2D,E). During these final stages of megakaryopoiesis, VWF staining was significantly reduced for GATA1 D218Y MKs. These data could indicate that part of the similar MK and platelet phenotypes between NBEAL2 and GATA1 deficiency are due to the fact that GATA1 is a transcription factor that drives NBEAL2 expression.

### Strongly reduced NBEAL2 expression in GATA1-deficient platelets

Immunoblot analyses were performed to compare NBEAL2 protein expression levels in platelets of patients with variants in GATA1 or NBEAL2 (Figure 3). Complete protein extracts of control platelets showed a large protein that corresponds to full-length NBEAL2 (302 kDa) (Figure 3A,B). Platelets of the patient with the homozygous splice mutation that would result in the predicted M1908X showed a strongly reduced, but not absent expression of full-length NBEAL2. There was also the presence of a band with a lower molecular weight of about 150 kDa, that could represent a cleaved truncated product, though we did not confirm this by mass spectrometry (Figure 3A,B). The homozygous W2480X NBEAL2 variant is predicted to result in a truncated protein of 27 kDa shorter than wild-type NBEAL2, a small difference that is probably not detectable by gel electrophoresis of such a large protein. However, platelets from this patient clearly expressed very low NBEAL2 levels (Figure 3A,B). It was surprising to observe that platelets from the patients with the D218Y or D218G GATA1 variants expressed no NBEAL2 (Figure 3A,B). An antibody for integrin  $\beta 3$  was used as loading control, as we previously found that integrin  $\beta 3$  expression was relatively normal for GATA1-deficient platelets using flow cytometry.<sup>13</sup> NBEAL2 blots were performed with a rabbit polyclonal (Figure 3A; epitope against amino acids 1865-1939) and a rabbit monoclonal (Figure 3B; epitope against amino acids 1-100) NBEAL2 antibody with comparable results. Only for the patients with NBEAL2 W2480X and GATA1 D218Y was sufficient platelet extract available to triplicate the blots and quantify expression (Figure 3C). Quantification showed a marked decrease in NBEAL2 expression for platelets with the NBEAL2 W2480X variant, while NBEAL2 was not detected in GATA1 D218Y deficient platelets. We also performed immunoblot analysis using total platelet extracts from hemizygous male and homozygous female Gata1-deficient mice and compared them to wild-type mice (Figure 3D,E). Equal amounts of integrin  $\beta 3$  positive platelet extracts were loaded. NBEAL2 was again strongly reduced for the Gata1-deficient mice that were previously

described by others to display macrothrombocytopenia, defective megakaryopoiesis and reduced  $\alpha$ -granule proteins in MKs.<sup>22,30,31</sup>

Female carriers of GATA1 variants are asymptomatic, and our earlier studies revealed skewed X inactivation and no mutant *GATA1* RNA in platelets from the D218Y carrier, while the D218G carrier had mild skewing of X inactivation with the presence of the *D218G* mutation in platelet RNA.<sup>14</sup> We also performed immunoblot analysis to compare NBEAL2 protein expression in platelets of female carriers of GATA1 D218G and D218Y. Interestingly, no difference in NBEAL2 expression was found for D218Y, while D218G carriers had slightly lower levels of NBEAL2 (Online Supplementary Figure S2). This decrease in NBEAL2 expression is not enough to cause a phenotype as these carriers have a normal platelet count and volume.<sup>13,14</sup> To support the absence of protein degradation in these samples, blots were performed for FLN and GPIIb $\alpha$  (Online Supplementary Figure S2).

### Identification of a long-distance NBEAL2 enhancer region

GATA1 is an important transcription factor that binds the sequence WGATAR in regulatory elements of many genes important for erythropoiesis and megakaryopoiesis.<sup>32</sup> Our data clearly showed that GATA1 could regulate NBEAL2 expression and therefore, we analyzed the NBEAL2 chromosomal region using ENCODE data.<sup>33</sup> Figure 4A shows potential GATA1 and GATA2 binding sites (BS) that are present in the chromosomal NBEAL2 region. Interestingly, some of these binding sites are clustered in a region that is located 31 kb upstream of the *NBEAL2* gene, within an intronic region of the nearby gene *CCDC12* (Chr3:46988970-46989880). This region is covered by a H3K4Me1 histone mark that is typically associated with an active enhancer. We only subtracted ChIP sequencing data from ENCODE that were determined for the myelogenous leukemia cell line K562 (Figure 4A, in blue), known to express GATA1 and GATA2<sup>34</sup> and epidermal keratinocytes (Normal Human Epidermal Keratinocytes (NHEK), Figure 4A, in purple), included as non-blood cells. A H3K4Me3 mark covers the *NBEAL2* promoter region that contains multiple GATA1 and GATA2 binding sites. The layered H3K27Ac track shows levels of enrichment of the H3K27Ac histone mark, which is often found near active regulatory elements, and this peak overlaps the potential NBEAL2 enhancer. Chromatin interaction analysis with paired-end tag sequencing (or ChIA-PET) data for K562 cells further shows a physical interaction between this enhancer region and the *NBEAL2* promoter (Figure 4A). The final lane displays the 100 vertebrates basewise conservation by PhyloP.

In addition, data were subtracted from a recent study that determined the genome-wide binding sites for the 5 key hematopoietic transcription factors, GATA1, GATA2, RUNX1, FLI1, and TAL1/SCL, in primary human MKs.<sup>35</sup> Interestingly, the NBEAL2 enhancer region is covered with ChIP sequencing peaks that are specific for GATA1 and GATA2 (Figure 4B). The exact location of these 2 peaks was also marked in Figure 4A at the top panel. The first binding peak contains 3 potential GATA binding sites of which 2 have the reverted sequence (referred to as BS-1, BS-2, BS-3) while the 2<sup>nd</sup> peak contains 2 potential GATA binding sites (referred to as BS-4 and BS-5) (Figure 5A).



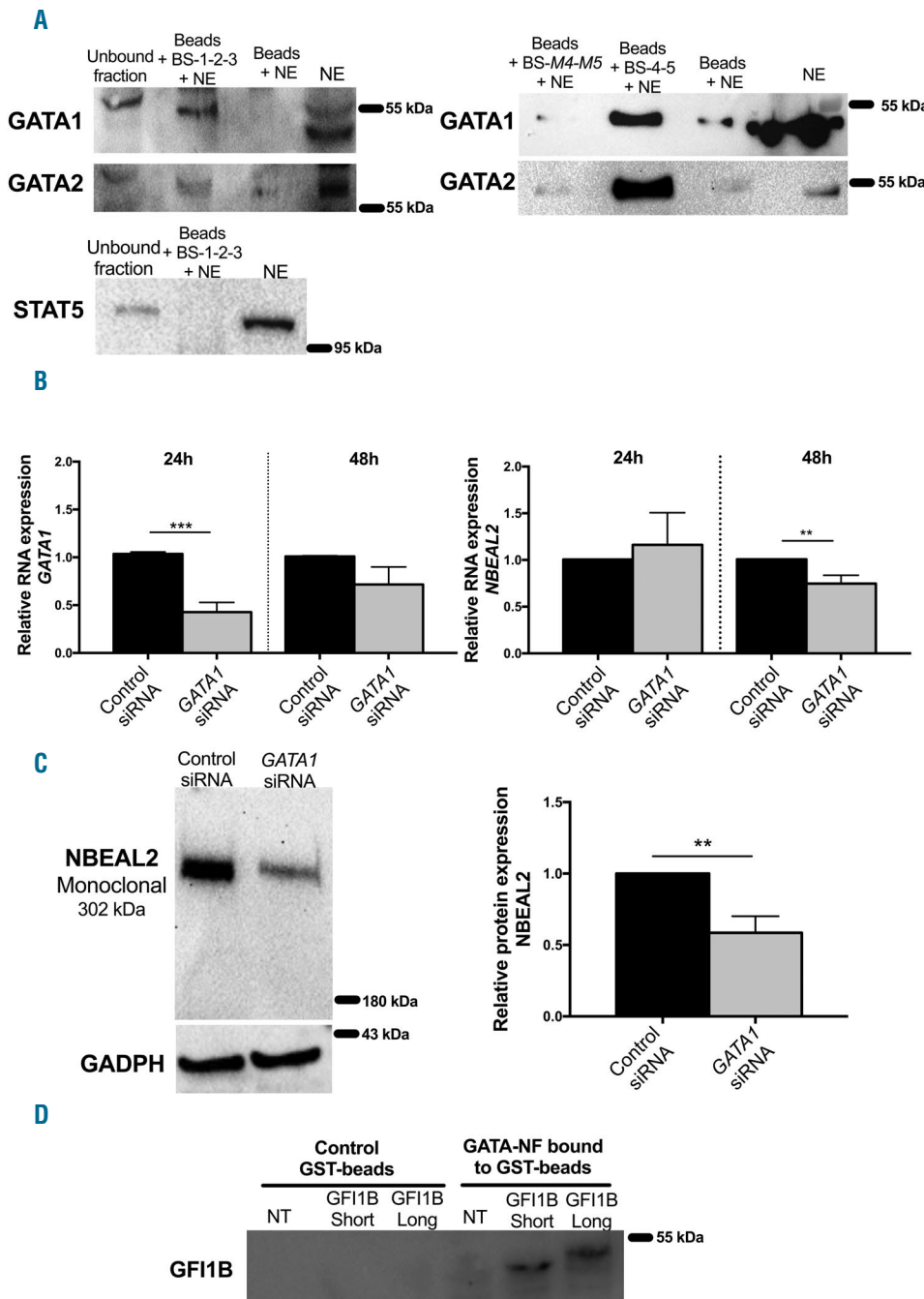
**NBEAL2 enhancer activity using a luciferase reporter assay**

The two enhancer peaks were cloned as overlapping fragments (BS-1-2-3 and BS-4-5) in the pGL3 promotor vector to obtain luciferase reporter constructs (Figure 5A). Polymerase chain reaction (PCR) mutagenesis was used to obtain the following mutant luciferase reporter constructs: BS-M1, BS-M2, BS-M3 and BS-M4-M5 that lack the GATA binding site and will not bind to GATA1/2 (Figure 5A). We compared the enhancer activity of the different luciferase constructs in K562 that express high GATA1 and lower GATA2 levels (Figure 5B). Both enhancer fragments BS-1-2-3 and BS-4-5 were able to significantly increase luciferase expression and mutagenesis of only GATA BS-2, and BS-3 blocked this enhancing activity. The mutants BS-

1, BS-4 and BS-5 did not significantly change the enhancing activity. The combination of BS-1-2-3 and BS-4-5 showed a cumulative effect on the enhancer activity and the combination of BS-1-M2-3 and BS-M4-M5 blocked this enhancer activity (Figure 5C).

**DNA binding assay confirms binding of GATA to NBEAL2 enhancer**

We next performed a DNA binding assay using biotin-labelled DNA fragments that cover the two NBEAL2 enhancer peaks (again referred to as BS-1-2-3 and BS-4-5). These biotinylated DNA fragments, bound to superparamagnetic streptavidin beads, were incubated with NE from GATA1 or GATA2 overexpressing HEK293 cells. The enhancer fragment BS-1-2-3 interacts with GATA1 and



**Figure 6. DNA binding assays for NBEAL2 enhancer region with GATA1 and GATA2 and the effect of GATA1 depletion on NBEAL2 expression.** (A) DNA fragments BS-1-2-3 and BS-4-5 were labeled with biotin and bound to superparamagnetic streptavidin beads. NE were used from GATA1 or GATA2 overexpression HEK293 cells. (left) Immunoblots for GATA1, GATA2 and STAT5 were performed on proteins bound to BS-1-2-3. STAT5 served as negative control. The unbound fraction is also shown. (right) Immunoblots for GATA1 and GATA2 were performed on proteins bound to BS-4-5 and BS-M4-M5. GATA1 and GATA2 bind the wild-type, but not the mutant DNA fragments. (B) qRT-PCR was performed on K562 cells with a GATA1 siRNA depletion to determine the expression of GATA1 (left) and NBEAL2 (right) relative to GADPH after 24 and 48 hours. (left)  $**P=0.0073$ , t-test; (right)  $***P=0.0004$ , t-test. (C) (left) Immunoblots analysis for NBEAL2 and integrin  $\beta 3$  on protein lysates collected after 48h. (right) Quantification of NBEAL2 expression.  $**P=0.0050$ , t-test. (D) Immunoprecipitation using GST control beads and GST-GATA1 NF beads and protein lysates from HEK293 cells transfected with the long and short GFI1B isoforms. These results are representative for three independent experiments. Control GST-beads (only GST sequence) and non-transfected (NT) HEK293 cells were used as negative controls. siRNA: small interfering RNA; NE: nuclear extracts; RNA: ribonucleic acid; GADPH: glyceraldehyde 3-phosphate dehydrogenase; GST: Glutathione S-Transferase; BS: binding sites; NF: N-terminal finger.

GATA2, but not with STAT5 for which there is no recognition site in the enhancer region (Figure 6A). Also the 2nd peak BS-4-5 interacts strongly with GATA1 and GATA2 while mutagenesis of both GATA BS-4 and BS-5 in this fragment completely inhibits these interactions (Figure 6B). However, as mutagenesis of BS-4 and BS-5 did not change the enhancer activity (Figure 5C), these GATA sites might not be important for this enhancer region.

#### siRNA mediated GATA1 depletion in K562 cells results in decreased NBEAL2

We performed a GATA1 knockdown in K562 cells using SMARTpool GATA1 siRNA and compared it to cells transfected with SMARTpool control siRNA (Figure 6B). After 24h, qRT-PCR showed a significant reduction in *GATA1* expression while *NBEAL2* expression remained normal. However, after 48h a significantly decreased *NBEAL2* expression was observed. This was confirmed by using immunoblot analysis, with strongly decreased *NBEAL2* levels 48h after transfection (Figure 6C).

#### GATA1 binding to GFI1B

FOG1 is known as a transcriptional co-regulator of GATA1 *via* interaction with its zinc fingers and the N-terminal GATA1 zinc finger (NF).<sup>13,14</sup> We hypothesized that the zinc fingers of GFI1B might also interact with this NF GATA1 domain, as a strong phenotype homology was noticed between GATA1 and GFI1B macrothrombocytopenia. Therefore, a pull-down assay was performed that showed a physical interaction between the GATA1 NF and the large and short GFI1B isoform (Figure 6D).

## Discussion

Important similarities, but also differences exist between clinical phenotypes caused by *NBEAL2*, *GATA1* and *GFI1B* germline defects in humans.<sup>36</sup> They share the presence of large platelets with  $\alpha$ -granule defects, bone marrow fibrosis with the presence of emperipoiesis, platelet dysfunction and different degrees of bleeding. However, these diseases have a different mode of inheritance (recessive, X-linked and dominant for *NBEAL2*, *GATA1* and *GFI1B* defects, respectively). More importantly, GATA1 and GFI1B, but not *NBEAL2* defects are characterized by red blood cell abnormalities, and the severity of their  $\alpha$ -granule defect is also different. Quantification of  $\alpha$ -granule numbers for two *NBEAL2* and two *GATA1*-deficient patients using EM indeed showed decreased  $\alpha$ -granule numbers for all, but also a significant difference between both groups with almost no  $\alpha$ -granules for *NBEAL2* defects. Nevertheless, because of the important resemblance in the  $\alpha$ -granule defect, we hypothesized that the transcription factor GATA1 could regulate *NBEAL2* expression during megakaryopoiesis.

Analogous to the recent findings from Di Buduo *et al.*<sup>29</sup> who studied megakaryopoiesis using blood stem cells from GPS patients, stem cells from *GATA1*-deficient patients showed normal MK differentiation, but a severe reduction in proplatelet formation with decreased  $\alpha$ -granules. Abnormal MK maturation with severely impaired cytoplasmic maturation, including fewer platelet-specific granules, has been described for *Gata1*-deficient mice that lack an upstream regulatory region that controls *Gata1* expression in MKs.<sup>22,37</sup> Moreover,

reduced proplatelet and  $\alpha$ -granule formation have also been described for V205G *Gata1* transgenic mice that phenocopy patients with X-linked macrothrombocytopenia, as this mutation blocks binding of the GATA1 NF to FOG1.<sup>38</sup> The exact pathogenic mechanism of missense variants in GATA1 NF is not really understood, but our previous *in vitro* binding assays with D218G and D218Y showed normal direct interaction with DNA and decreased interactions with FOG1.<sup>13,14</sup> However, more recent murine erythroid cell-based studies could only confirm the defective interaction between FOG1 and GATA1 D218Y, while D218G was shown to interfere with the GATA1-TAL1 cofactor complex.<sup>39</sup> If GATA1 D218G and D218Y are predicted to have different effects on DNA binding, they both resulted in abnormal *NBEAL2* expression. Moreover, GATA1 depletion *via* deletion of a MK-specific enhancer in mice or using siRNA also resulted in decreased *NBEAL2* expression. Therefore, our studies indicate that *NBEAL2* expression can be regulated by changes in GATA1 levels or *via* functional changes in GATA1 NF. However, more studies are needed to evaluate how these GATA1 NF variants could change direct or indirect DNA binding in gene promoters and enhancer regions. In addition, for GFI1B patients, bone marrow MKs showed extensive peripheral cytoplasm with irregular proplatelets and were largely devoid of cell organelles,<sup>12</sup> while more recent *in vitro* differentiation assays with mutant GFI1B constructs in stem cells showed abnormal proplatelet formation.<sup>40</sup> Further studies are needed to discover if GATA1-GFI1B co-transcriptional complexes could regulate *NBEAL2* expression during megakaryopoiesis.

To our knowledge, it was not yet known that *NBEAL2* is a GATA1 target gene. GATA1 drives many important target genes for red blood cell and MK formation such as the globin genes, *EPO*, *EPOR*, *GATA2*, *NFE2*, *GP1BA*, *GP1BB*, *PF4*, *MPL* and others.<sup>37,41</sup> Interestingly, a Bernard-Soulier syndrome patient with macrothrombocytopenia was described with a variant in the promoter of the *GP1BB* gene altering GATA1 binding together with a 22q11 deletion (involving the *GP1BB* gene) on the other allele.<sup>42</sup> This could mean that large platelets in *GATA1* patients are due to defective *GP1BB* expression. This also implicates that the macrothrombocytopenia phenotype caused by *GATA1* deficiency is complex and can be the result of many genes that show aberrant *GATA1*-drive expression, but part of the overall phenotype and especially the  $\alpha$ -granule defect could be due to *NBEAL2* loss. Moreover, a long-distance enhancer region 31 kb upstream of the *NBEAL2* gene was discovered in data from ENCODE for K562 cells that express high levels of GATA1 and whole genome ChIP sequencing data for GATA1 and GATA2 binding sites using primary MKs (Figure 4).<sup>35</sup> Typical WGATAR recognition sequences (GATA1/2 BS) are present in this region, and a physical interaction between this enhancer region and the *NBEAL2* promoter was predicted based on ChIA-PET experiments using K562 cells.<sup>43</sup> It is known that long-distance enhancers come into close proximity of target promoters through looping.<sup>44</sup> Transcription factors and their complexes mediate such enhancer-promoter loop formation. The enhancer-gene loop is necessary for transcriptional upregulation, but how the looping changes the transcriptional output is still unclear. Chromatin looping has been shown to be relevant for the reactivation of globin genes,<sup>45</sup>

but examples of such complex transcriptional regulation *via* interactions between enhancers and promoters important for gene expression during megakaryopoiesis remains largely unstudied. Insights into such mechanisms would be highly relevant to understand genome-wide association study (GWAS) findings that showed associations of mostly noncoding variants with changes in platelet count and volume.<sup>46</sup> It might therefore be that the noncoding variant influences expression of a long-distance gene rather than the closest gene. In the study herein, the NBEAL2 enhancer is actually located within an intron of another gene, *CCDC12* (Figure 4). We confirmed the binding of GATA1 and GATA2 to this NBEAL2 enhancer region with a DNA binding assay, and showed that in luciferase reporter assays it functions as an enhancer element that depends mainly on GATA BS-2 and BS-3. Such GATA binding sites can of course be occupied by GATA1 or GATA2. GATA2 is essential for the maintenance of HSCs and progenitor cells, whereas GATA1 drives the differentiation of HSCs into megakaryopoiesis. GATA1 represses GATA2 transcription, and this involves GATA1-mediated displacement of GATA2 from chromatin, a process that is

called a GATA switch.<sup>47</sup> It is known that GATA switches play an important role in the differentiation of blood stem cells.<sup>48</sup> Such a GATA switch can occur for the GATA binding sites in the enhancer of the *NBEAL2* gene; further studies are needed to unravel this exact mechanism as this might identify the precise moment that NBEAL2 is expressed during megakaryopoiesis.

In conclusion, the study herein provides an explanation for the phenotypic resemblance between NBEAL2 and GATA1 defective macrothrombocytopenia with paucity of  $\alpha$ -granules, as GATA1 is important for NBEAL2 expression during megakaryopoiesis *via* a long-distance enhancer.

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