SUPPLEMENTARY APPENDIX

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

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Supplementary figures and methods for

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

Supplementary figure S1

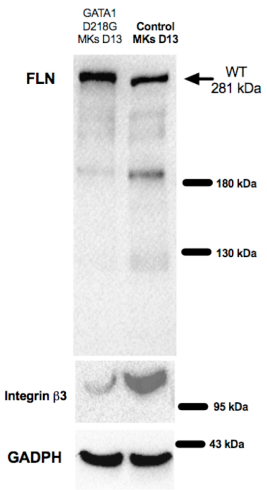

Figure S1. Immunoblot analysis to study FLN expression in day 13 differentiated MKs from peripheral blood-derived CD34+ HSC of the GATA1 D218G patient and a healthy control. No difference in expression level was observed and no evidence for increased FLN degradation in the D218G sample could be noticed.

Supplementary figure S2.

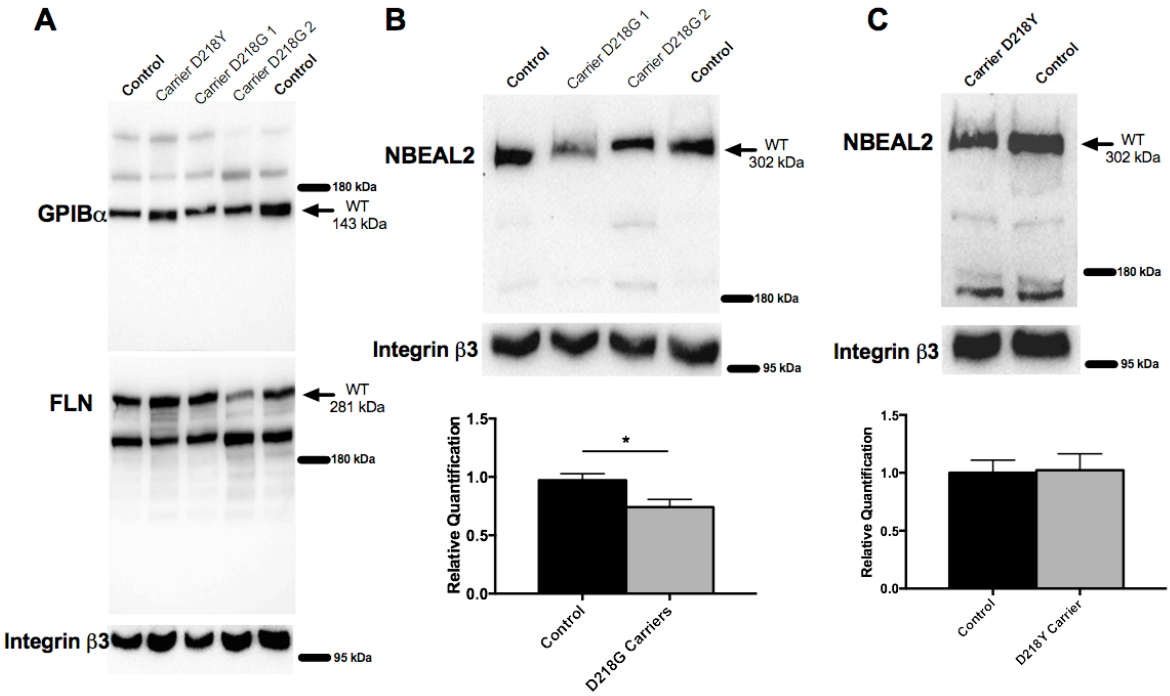

Figure S2. (A) Immunoblot analysis showed no significant difference in FLN and GPIBα expression levels between platelets of the GATA1 D218G or D218Y female carriers and unrelated controls. No degradation bands related to these proteins were observed for the carrier platelets. (B) Immunoblot analysis was performed to compare NBEAL2 protein expression levels in platelets of GATA1 D218G or D218Y female carriers. No difference in NBEAL2 expression between the GATA1 D218Y female carrier and control platelets was observed while female carriers with GATA1 D218G had mildly reduced NBEAL2 levels in their platelets. We previously showed skewed X-inactivation and no mutant GATA1 mRNA in platelets from the D218Y carrier while the D218G carrier had only weak skewing of X-inactivation with the presence of the D218G mutation in platelet RNA (4). This could mean that the D218G GATA1 mutation in her platelets resulted in slightly reduced NBEAL2 expression. However, this mild reduction in NBEAL2 seems not to have an effect on the phenotype as all GATA1 female carriers have normal platelet counts and no clinical bleeding symptoms (2,4).

Supplementary figure S3

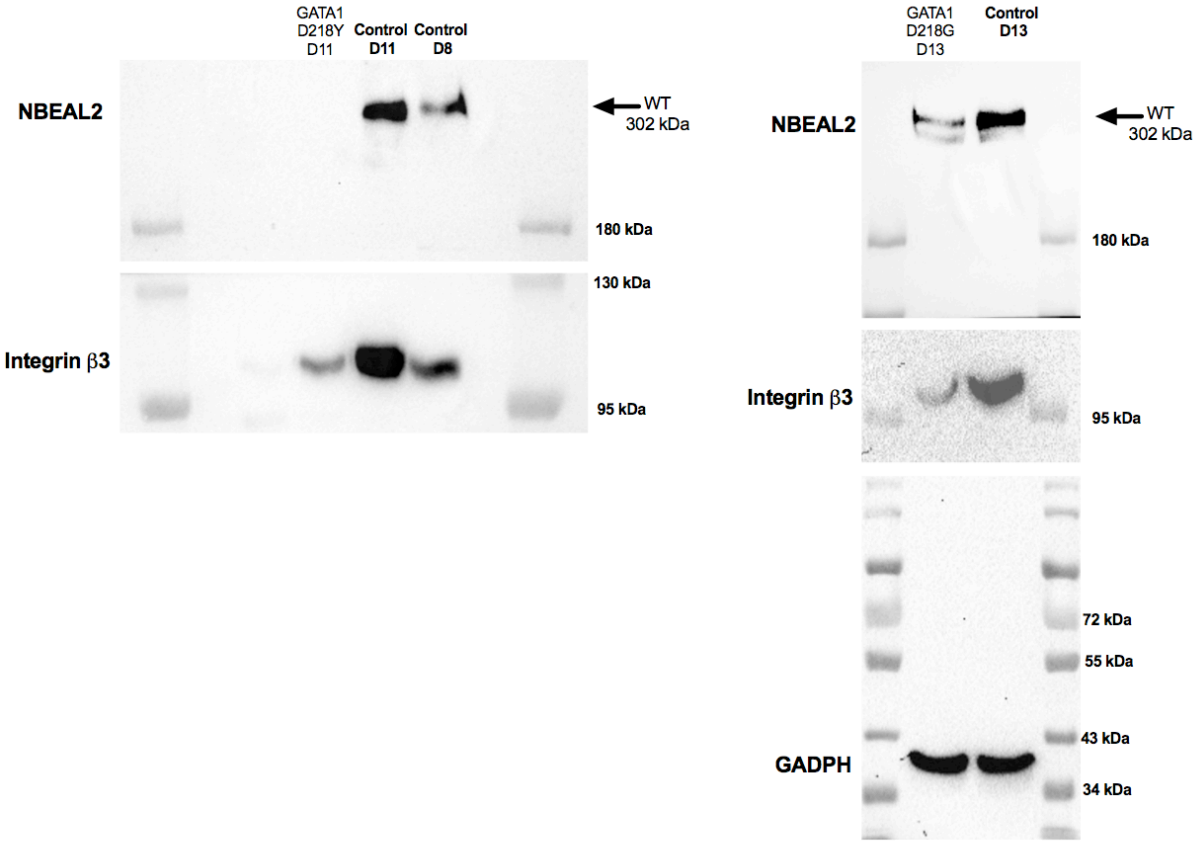

Figure S3. Uncropped blots for Figure 2C to show absence of NBEAL2 degradation products in the D218G and D218Y samples.

Supplementary figure S4

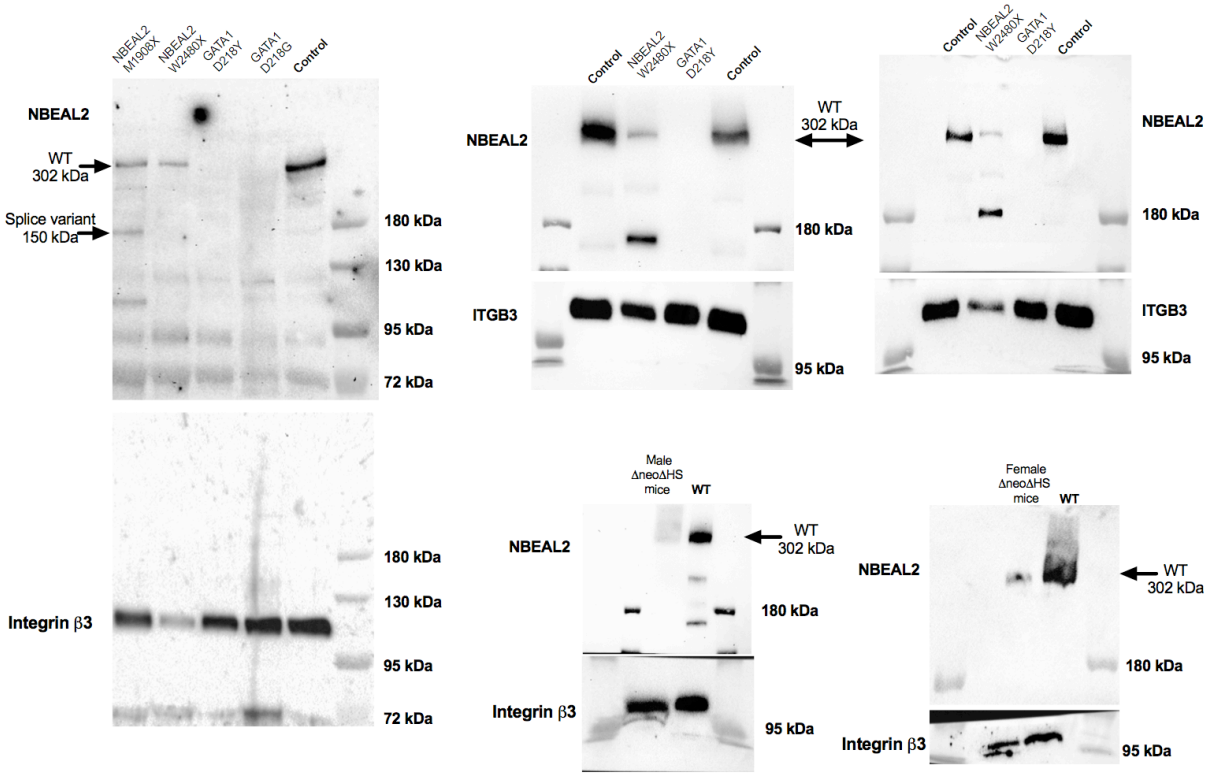

Figure S4. Uncropped blots for the different panels in Figure 3.

2. SUPPLEMENTARY METHODS

Patient studies

The NBEAL2 variants will be deposited in the LOVD database (under submission). The GATA1 variants have already been deposited (ClinVar: http://www.ncbi.nlm.nih.gov/clinvar/variation/10427/ for D218Y and http://www.ncbi.nlm.nih.gov/clinvar/variation/10424/ for D218G).

Megakaryocyte cultures, proplatelet formation and immunohistochemistry

The MK differentiation assays was performed twice with different controls. HSC were cultured in StemSpan SFEM medium (Stem Cell Technologies), supplemented with 20 ng/ml TPO, 10 ng/ml SCF, 10 ng/ml IL-6 and 10 ng/ml FLT-3 (Peprotech). Immunostaining experiments were performed at days 8 and 11 after incubation of MK for 4 hours on fibrinogen-coated coverslips. They were performed with anti-von Willebrand factor (VWF)

(A0082; Dako) and an Alexa fluor 488-labelled anti-rabbit secondary antibody (Invitrogen) in combination with Alexa Fluor 594 phalloidin (A12381; Life technologies). Microscopy pictures were taken at 63x magnifications with the Zeiss AxioVert 200M microscope and Zeiss Axiovision software. Quantification of the fluorescence signal was performed with ImageJ software.

Immunoblot analysis

The concentration of total protein was determined by Bradford analysis and equal protein fractions (40μg) were resolved by SDS-PAGE on 5% acrylamide gels and transferred to ECL-nitro-cellulose membrane. The blots were blocked for 1h at room temperature in Tris-buffered saline with tween (TBS-T; 0,1% Tween-20) supplemented with 5% non-fat dry milk. Blots were incubated overnight at 4°C (the dilution for all antibodies was 1:1000 in 1X TBS-T 5% non-fat dry milk) with the following antibodies: rabbit polyclonal anti-NBEAL2 (ab151150; Abcam), rabbit monoclonal anti-NBEAL2 (ab187162; Abcam), rabbit monoclonal anti-GADPH (14C10; Cell Signaling), mouse polyclonal anti-Filamin (CP72; Calbiochem), mouse monoclonal anti-GPIBα (G27CG) (previously described[4]), and anti-integrin β3 (sc-14009; Santa Cruz Biotechnology). Blots were washed 3 times for 10 min with 1X TBS-T. Membranes were incubated with HRP-conjugated secondary antibodies (Dako) (dilution 1:1000 in 1X TBST 5% nonfat dry milk) for 2-3h at RT and staining was performed with ECL detection reagent (Life Technologies). Chemiluminescent blots were imaged with the ChemiDoc MP imager (Bio-Rad) and ImageJ software was used for densitometry analysis.

Luciferase reporter assays to measure NBEAL2 enhancer activity

PCR was used to modify the 5 potential GATA sites and are referred to as BS-*M1*-2-3, BS-1-*M2*-3, BS-1-2-*M3* (all single mutants) and a double mutant BS-*M4*-*M5*. The primer sets to clone the two different enhancer regions were: 1) BS-1-2-3 forward 5'-AGGAAGCTTAGTGTCACCTTGTGCCCATC-3' (*Hin*dIII site) and BS-1-2-3 reverse 5'-CAGAAGCTTTGAGAACATCTGATAAG-3' (*Hin*dIII site) and 2) BS-4-5 forward 5'-GACTAGCTAGCAAGAGGCTGGGA-3' (*Nhe*I site) and BS-4-5 reverse 5'-GGATCCCCGGGGACTTGGGCTGG-3' (*Sma*I site*)*. These constructs were used as templates for PCR mutagenesis. For the BS-*M1*-2-3 construct BS-1-2-3 was used as a template. The primer sets to clone the pGL3-BS-*M1*-2-3 were the BS-1-2-3 forward and the BS-*M1*-2-3 reverse 5'-

GTTCAGCTCACTTCTCAACACTCCATCTTC-3' together with the BS-*M1*-2-3 forward 5'-CTGAAGATGGAGTGTTGAGAAGTGAGCTGAAC-3' and BS-1-2-3 reverse. Those PCR products were combined to make the pGL3-BS-*M1*-2-3 construct. The BS-1-2-3 construct was also used as a template to make the BS-1-*M2*-3 construct. The primer sets to make the pGL3-BS-1-*M2*-3 were the BS-1-2-3 forward and the BS-1-*M2*-3 reverse 5'-TGGTCTGGTCAGCAGCTCGAGTGGATGCTCG-3' together with the BS-1-*M2*-3 forward 5'-CGAGTATCCACTCTATCTGCTGACCA-3' and BS-1-2-3 reverse. Those two PCR products were also combined to create the pGL3-BS-1-*M2*-3 construct. To make the BS-1-2-*M3* construct the BS-1-2-3 construct was used as template. The BS-1-2-3 forward together with the BS-1-2-*M3* reverse primer 5'-CAGAAGCTTTGAGAAC ATCTGAATAAG-3'was used to create the BS-1-2-*M3* construct. The pGL3 promotor vector without enhancer sequence was used as control construct. The transfection of K562 cells was performed with the Cell line nucleofector Kit V (Lonza) using the Amaxa electroporation system according to the manufacturer's instruction (method T016). pEGFP (Clontech) was added to the transfections to ensure equal transfection efficiencies as done before[1]. Cell extracts were prepared after 48 hours using 1x reporter lysis buffer and 40 µl of cell extract was mixed with 50 µl of luciferase assay reagent (Promega) to determine luciferase activity in a luminescence counter (EG&G Berthold MicroLumat Plus LB96V) as described[1,2]. Each plasmid was assayed in triplicate in six separate transfection experiments.

qRT- PCR to quantify *GATA1*, *GATA2*, *NBEAL2* and *ITGB3* expression

The *GATA1* forward 5'-GATGAATGGGCAGAACAGGC-3', *GATA1* reverse 5'-AGCTTGTAGTAGAGGCCGC-3', GATA2 forward 5'-CCAAGTCCTCCGGTTCTTCC-3', *GATA2* reverse primer 5'-AGAGAGGGAAGCCAGAGGAG-3', *NBEAL2* forward 5'-TCCTAGCCAGACTCCCAGAG-3', *NBEAL2* reverse 5'-CGCAATGGTGTTGAGTTGCA—3', *ITGB3* forward 5'-CGGTACGTGATATTGGTGAAGG-3' and *ITGB3* reverse 5'-AAACTCCTCATCACCATCCAC-3' were used.

GATA1 knockdown in K562 cells with SiRNA

GATA1 depletion in K562 cells was obtained after transfection with SMARTpool GATA1 siRNA (Dharmacon) or negative control SMARTpool siRNA (Dharmacon). K562 cells (10^6) were resuspended in 100µl of Nucleofector solution V (Lonza) and transfected with

160 pmol of SMARTpool GATA1 siRNA (Dharmacon) or SMARTpool control siRNA (Dharmacon). This GATA1 siRNA was previously shown to be highly effective.[5] Cells were transfected with the Nucleofector II (Lonza) using the T-016 program and were collected at 24h and 48h post transfection for real time PCR and immunoblot analysis. qRT-PCR was performed to study expression of *GATA1* and *NBEAL2* and immunoblot analysis to detect protein expression of NBEAL2 and GADPH.

DNA binding assay

Biotynylated DNA fragments for the DNA binding assay were generated by PCR. Overlapping DNA fragments of 980 bp (for BS-1-2-3) and 194 bp (for BS-4-5) were PCR amplified using the following primer sets: biotin-labelled forward 5'-CATAGTGCCTGAAGGTGACAG-3' and biotin-labelled reverse primer 5'-ACTCTTGGTGAAGCACTGAA-3' for BS-1-2-3 and forward primer 5'-GAACAGCGAGGCTAGAGATAAGGACTGGAGATAAGGACGG-3' and biotin labelled reverse primer 3'-CTCTTTGTCCCCTAAGTGCCA-5' for BS-4-5. A mutant DNA fragment with BS-*M4-M5* was also amplified by PCR using forward primer 5'-GAACAGCGAGGCTACTTGAGGACTGGACTTGAGGACGG-3' (mutant BS-4 and BS-5 are underlined) and a biotin labelled reverse primer 3'-CTCTTTGT CCCCTAAGTGCCA-5'.

The biotinylated PCR fragments were bound to Superparamagnetic streptavidin beads (Hyglos) and next added to NE isolated from HEK293 cells transfected with pcDNA3.1 containing GATA1 or GATA2. This binding reaction was performed with 50 μg NE and 1 μg of Poly(dI-dc) (Thermo Scientific) that were added to 1.5 μg biotinylated DNA for 3 hours at room temperature. Next beads were washed 7 times with 10xSSC (1.5 M Sodium Chloride, 0.15 M sodium citrate, pH 7) to remove unbound DNA. After addition of loading buffer, samples were loaded on a 7% SDS-PAGE. The blots were stained with anti-STAT5 (9363; Cell Signaling), anti-GATA1 (Sc-265; Santa Cruz Biotechnology) or anti-GATA2 antibody (polyclonal rabbit antibody previously described[3]).

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

Immunoprecipitation was performed in triplicate with GATA1-NF GST-beads incubated with total protein lysates from HEK293 cells after transfection with pWPT (Trono lab, Addgene) containing the GFI1B long (NM_004188) or short (NM_001135031) isoform[26]

using jetPRIME (Polyplus). About 1 mg of HEK293 lysate was added to 500 µl RIPA buffer, with GATA1-NF GST or control GST beads for overnight incubation. After incubation and washing, the bound fraction was loaded on gel and immunoblots were performed with GFI1B antibody (sc-22795; Santa Cruz Biotechnology).

Statistical analysis

For quantitative assays, treatment groups were reported as mean ±SEM and compared using the unpaired Student t-test and one-way analysis of variance (ANOVA) test with Bonferroni's correction using Prism6 (GraphPad Software Inc.).

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