GFI1B controls its own expression binding to multiple sites

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ABSTRACT

Background

Transcription factors play essential roles in both normal and malignant hematopoiesis. This is the case for the growth factor independent 1b (GFI1B) transcription factor, which is required for erythroid and megakaryocytic differentiation and over-expressed in leukemic patients and cell lines.

Design and Methods

To investigate *GFI1B* regulation, we searched for multispecies conserved non-coding elements between *GFI1B* and neighboring genes. We used a formaldehyde-assisted isolation of regulatory elements (FAIRE) assay and DNase1 hypersensitivity to assess the chromatin conformation of these sites. Next, we analyzed transcription factor binding and histone modifications at the *GFI1B* locus including the conserved non-coding elements by a chromatin immunoprecipitation assay. Finally, we studied the interaction of the *GFI1B* promoter and the conserved non-coding elements with the chromatin conformation capture technique and used immunofluorescence to evaluate GFI1B levels in individual cells.

Results

We localized several conserved non-coding elements containing multiple erythroid specific transcription factor binding sites at the *GFI1B* locus. In GFI1B-expressing cells a subset of these conserved non-coding elements and the promoter adopt a close spatial conformation, localize with open chromatin sites, harbor chromatin modifications associated with gene activation and bind multiple transcription factors and co-repressors.

Conclusions

Our findings indicate that *GFI1B* regulatory elements behave as activators and repressors. Different protein levels within a cell population suggest that cells must activate and repress *GFI1B* continuously to control its final level. These data are consistent with a model of *GFI1B* regulation in which GFI1B binds to its own promoter and to the conserved non-coding elements as its levels rise. This would attract repressor complexes that progressively down-regulate the gene. *GFI1B* expression would decrease until a stage at which the activating complexes predominate and expression increases.

Key words: GFI1B, controls, multiple sites.

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The online version of this article has a supplementary appendix.

Introduction

The hematopoietic system achieves the goal of continuous replacement of blood cells through self-renewal of pluripotent hematopoietic stem cells and their subsequent commitment to progenitor and precursor cells of gradually more restricted potential. Lineage-specific transcription factors play essential roles in regulating hematopoietic development, activating gene programs and at the same time exerting inhibitory effects on alternate lineage programs establishing delicate balances essential to maintain normal hematopoiesis and avoiding malignancy.[1]

The growth factor independent 1B (GFI1B) transcription factor is expressed in hematopoietic stem cells, common myeloid progenitors and in megakaryocyte/erythroid progenitors and lineages. There is also evidence of moderate levels of expression in immature B cells, a subset of early T-cell precursors and peripheral blood granulocytes and monocytes.[2,3] During erythroid differentiation *GFI1B* is up-regulated in early erythroblast stages and decreases with terminal differentiation.[2,4,5] Its role in erythropoiesis is crucial for expansion and differentiation of erythroid progenitors.[4,5] Using knockout mice, it has been demonstrated that this gene is required for the development of both erythroid and megakaryocytic lineages.[6] Additionally, *GFI1B* expression has recently been found to be increased in leukemic patients and cell lines.[7,8] The last work also showed a reduction in proliferation of the HEL erythroleukemia cell line after down-regulation of *GFI1B* using small interfering RNA, further supporting its role in this malignancy.[8]

GFI1B has six C-terminal C2H2 zinc-fingers that bind DNA in a sequence-specific manner at sites containing an AATC core sequence (consensus recognition sequence TA**AAT**CACA/TGCA/T) and an N-terminal SNAG transcriptional repression domain.[9-11] GFI1B repression activity is achieved by recruiting lysine-specific demethylase 1 (LSD1 or KDM1), REST corepressor (CoREST) and HDAC 1 and 2 to DNA.[12] At the DNA level GFI1B represses cyclin-dependent kinase inhibitor *p21*^{WAF1},[11] proto-oncogenes *Myc* and *Myb* in association with *GATA1*,[13] tumor suppressor genes *Socs1* and *Socs2*,[14] the anti-apoptotic gene *BCL-xL*,[15] the proto-oncogene *Gfi1*[16,17] and *GFI1B* itself.[18] At the protein level GFI1B interacts with transcription factors SCL,[19,20] E2A,[20] GATA1[13] and co-repressors ETO21 and ETO2.[19,20]

All these facts indicate that correct *GFI1B* expression is important to achieve normal erythroid and megakaryocytic differentiation. It would, therefore, be useful to understand how *GFI1B* expression is regulated, but the only regulatory element identified in erythroid and megakaryocytic cells is the promoter.[12,17,18,22,23]

To gain further insight into *GFI1B* regulation, we used multiple species sequence comparison to identify distant *cis*-acting regulatory elements at the *GFI1B* locus.

Design and Methods

Identification of conserved non-coding elements

Preliminary alignments were assembled with ECR browser (*http://ecrbrowser.dcode.org/*).[24] Genomic sequences spanning the *GFI1B* locus were obtained from NCBI (*http://www.ncbi.nlm.nih.gov/*) and Ensembl (*http://www.ensembl.org/index.htlm*) databases. Subsequent alignments were performed with MacVector software (Accelrys, Cary, NC, USA). Transcription factor binding sites were identified with R-VISTA (*http://rvista.dcode.org/*)[25] and MacVector.

Cell culture

L929 fibroblasts, L8057 megakaryocytic and K562 and MEL 585 erythroid cells were grown in RPMI 1640 medium (Sigma, St Louis, MO, USA) with 50 U/mL penicillin G (Gibco BRL, Rockville, MD, USA), 50 μg/mL streptomycin (Gibco BRL), 2 mM L-glutamine (Gibco BRL) and 10% (v/v) fetal calf serum (Gibco BRL). MEL cells were induced to differentiate with N,N´-hexamethylene-bisacetamide (HMBA) (Sigma) 5 mM and and 10% (v/v) fetal calf serum (Biosera, East Sussex, UK) for 3 days.[26] L8057 cells were induced with 50 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma) for 3 days.[27]

Purification of mouse primary erythroid cells

Primary erythroid cells were isolated as described elsewhere.[28] Briefly, C57BL/6 mice were injected intraperitonally with phenylhydrazine (Sigma), 0.04 mg/g body weight, three times at 12-hour intervals. TER119$^+$ cells were isolated on day 6 by labeling splenocytes with biotin-conjugated rat anti-mouse TER119 antibody (BD, Franklin Lakes, NJ, USA) and incubated with antibiotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The positive fraction was obtained on AUTOMACS (Miltenyi Biotec). The purity of the TER119$^+$ cells, assessed by flow cytometry, was over 80%.

RNA isolation and quantitative real-time polymerase chain reaction expression studies

RNA was extracted with Tri reagent (Sigma). Ribosomal RNA integrity and concentration were checked in a formaldehyde agarose gel. cDNA was obtained with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and primer random (Roche, Basel, Switzerland). *Gfi1b* cDNA was analyzed by real-time polymerase chain reaction (PCR) in a 25 μL reaction containing Taqman Universal PCR Master Mix (Applied Biosystems) and TaqMan(R) Gene Expression Assay Mm01336944_m1 mix with a FAM-NFQ probe between *Gfi1b* exons 1 and 2 (Gene Expression Assays, Applied Biosystems) following the manufacturer's recommendations. *Gfi1b* expression was calculated for each cell type relative to Taqman(R) Gene Expression Assays Mm00839493_m1 DNA-directed RNA polymerase II polypeptide A and normalized with FDCP-Mix results using the -ΔΔCt method.[29]

Western blot

Protein extract was prepared in lysis buffer (60 mM TRIS, 10% glycerol, 2% sodium dodecylsulfate) and sonicated. The concentration was assessed using a DC protein assay (Bio Rad, Hercules, CA, USA). Thirty micrograms of protein were separated by sodium dodecylsulfate (SDS)- polyacrylamide agarose gel electrophoresis (PAGE) on a NuPAGE 3-8% Tris-Acetate gel (Invitrogen, Carlsbad, CA), transferred to an Immobilon-PSQ membrane (Millipore, Billerica, MA), labeled with GFI1B antibody and an anti-goat horse radish peroxidase-conjugated secondary antibody (sc-2020, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected with ECL plus (GE Health care).

Formaldehyde-assisted isolation of regulatory elements

The procedure for formaldehyde-assisted isolation of regulatory elements (FAIRE) has been previously described in detail.[30] Real-time PCR analysis was performed with primers and Taqman

probes shown in the *Online Supplementary Table S1* in an ABI Prism 7000 Sequence Detection System. FAIRE data were normalized with input material in which the cross-link was reverted at 65°C for 4 h. Enrichment of each amplicon was calculated relative to the result at an area previously found insensitive to DNase1 (*c16orf8* or *Dist* gene) (*Online Supplementary Table S1*),[31] using the -ΔΔCt method.[29]

DNase1 hypersensitivity

DNase1 hypersensitive sites were analyzed as described elsewhere.[26] DNA was isolated with phenol/chloroform, digested with BamHI or HindIII restriction enzymes and analyzed by Southern blot using a single-copy probe obtained by PCR (forward primer 5´-GAAAGCTCCTGCTGTGTTTCCC-3´ and reverse primer 5´- GGGAGGCAAGGTTCAGAATTTC-3´).

Chromatin immunoprecipitation assay and real-time polymerase chain reaction analysis

Chromatin immunoprecipitation (ChIP) assays were performed according to the Upstate protocol, as described previously.[31] Cells (1×10^7 per experiment) were fixed with 0.4% formaldehyde for 10 min at room temperature for transcription factors and 1% formaldehyde for 10 min at 37°C in the case of co-factors. Chromatin was sonicated to a size of less than 500 bp. Immunoprecipitation was performed, after an overnight incubation with the appropriate antibody, with protein A agarose (Upstate, Temecula, CA, USA), or with protein G agarose (Roche) according to the antibody. A sample containing no antibody was used as a negative control.

Immunoprecipitated DNA was analyzed by quantitative real-time PCR. Primers and 5'FAM-3'TAMRA Taqman probes were designed by Primer Express software. All primers and probes were validated over a serial dilution of genomic DNA. For a given target sequence, the amount of product precipitated by a specific antibody was determined relative to the amount of non-immunoprecipitated (input) DNA and these results were normalized to a control sequence in the *18S* ribosomal RNA gene (*18S* rRNA control kit, Eurogentec, Liege, Belgium) for human cells and *Gapdh* in mouse studies.[31] Primers and probes from α globin locus control have been published before for both humans[32] and mice.[31] The mouse *Gfi1b* locus and human *GFI1B* and *β-ACTIN* CpG island control are shown in *Online Supplementary Tables S1 and S2*, respectively. The mouse *β-actin* control has been published.[31]

Chromosome conformation capture

The chromosome conformation capture (3C) technique was performed as described elsewhere.[33,34] Briefly, 1×10^7 cells per experiment were fixed with 2% formaldehyde, lysed in 10 mM TRIS pH 8, 10 mM NaCl, 0.2% NP-40 with complete protease inhibitors (Roche), digested with 400 U DpnII (NE biolabs, Ipswich, MA, USA) and ligated with T4 ligase (Fermentas, Burlington, Ontario, Canada). The forward primer and Taqman probe at the restriction fragment including the *Gfi1b* promoter and primers at other restriction fragments are shown in *Online Supplementary Table S3*. Real-time PCR data were normalized with the results of an amplicon without DpnII restriction sites at *α-globin* HS-31 (*Online Supplementary Table S3*)[31] and corrected with the analysis of *Ercc3* locus, these last primers and probe being a generous gift from Dr D Vernimmen.[34]

Immunofluorescence

The immunofluorescence study was performed as described previously.[31] The images were obtained using an Olympus BX 51 microscope with a confocal BioRad Radiance 2000 system. Fluorescence intensities were measured with Metamorph software (Molecular Devices, Sunny Vale, CA, USA).[31] Images were imported into Photoshop and the only manipulation performed was contrast stretch.

Antibodies

We used antibodies against POL II (N-20, sc-899, Santa Cruz), E47 (N-649 X, sc-763 X, Santa Cruz), LMO2 (AF2726, R&D systems, McKinley Place NE, MN, USA), CLIM-2 (LDB1) (N18, sc-11198, Santa Cruz), NF-E2 P45 (C-19, sc-291, Santa Cruz), NF-E2 P18 (C-16, sc-477, Santa Cruz), ETO-2 (G-20, sc-9741, Santa Cruz), c-MYB (H-141, sc-7874, Santa Cruz), PU.1 (T-21, sc-352, Santa Cruz), dimethyl histone H3 lysine 4 (07-030, Upstate), acetyl Histone H4 (06-866, Upstate), acetyl Histone H3 (06-599, Upstate), CoREST (ab24166, Abcam, Cambridge, UK), KDM1/LSD1 (ab17721, Abcam), CoREST (07-455, Upstate), and SCL (a gift from K Porcher), together with specific antibodies for mouse GATA1 (N-6, sc-265, Santa Cruz) and GFI1B (D-19 X, sc-8559 X, Santa Cruz) and human GATA1 (C-20 X, sc-1233 X, Santa Cruz) and human GFI1B (B-7 X, sc-28356 X, Santa Cruz).

Results

Multispecies conserved regions downstream of the GFI1B gene contain erythroid and megakaryocytic transcription factor binding sequences

Cis-acting regulatory elements are frequently identified as multispecies conserved non-coding elements (CNE). In order to study *GFI1B* gene regulation we first analyzed, using the ECR browser and McVector software, sequence conservation over 86 kb of human sequence between the *TSC1* (tuberous sclerosis 1) and *GTF3C5* (general transcription factor IIIC, polypeptide 5) genes that flank *GFI1B* (Figure 1A and *data not shown*). We identified one CNE within the first intron and three downstream of the *GFI1B* gene that contain several highly conserved erythroid/megakaryocytic transcription factor binding sites (Figure 1B, and *Online Supplementary Figures S1-S3*). CNE +1 and +3 have been conserved from human to birds, while CNE+2 is strongly conserved down to marsupials. The intronic area (CNEi) is well conserved in eutherian mammals, but only partly conserved in marsupials.

The downstream CNE +1, +2 and +3, included at least one fully conserved canonical GATA binding site ($^{A}/_{T}$GATA$^{A}/_{G}$),[35] together with other potential GATA recognition sites.[36-39] At least one E-box, SCL/E2A heterodimer binding site, is present in most species at all three regions. In addition, a conserved NF-E2 binding site[40] is located at the most downstream CNE. We did not identify any GFI1/GFI1B consensus binding sequences, although the core AATC sequence has been frequently conserved (Figure 1B, and *Online Supplementary Figures S1 and S2*).

Other recognition sequences for transcription factors involved in hematopoiesis are also present in multiple species at those CNE. One MYB consensus core DNA-binding site has been highly conserved in both CNE+1 and +3, the first of them matching the extended consensus $^{T}/_{C}$AACBG$^{T}/_{C}$C$^{A}/_{G}$[41] in most species (Figure 1B and *Online Supplementary Figure S2*). Finally, multiple conserved ETS

transcription factor binding motifs[42] can be identified in all of these regions (Figure 1B, and *Online Supplementary Figures S1 and S2*).

The CNEi has six potential GATA binding sites, one of which shows a perfect match with the classical GATA binding sequence in most species. There is also a potential MYB binding site and several AATC and ETS binding motifs (*Online Supplementary Figure S3*).

These data suggest that these CNE represent potential *cis*-elements of *GFI1B*.

3' conserved non-coding elements associate with open chromatin regions and recruit RNA polymerase II

We have previously demonstrated that mouse erythropoiesis can be recapitulated with primary cells and cell lines that can be used for ChIP analysis.[31] We, therefore, followed this strategy to study *Gfi1b* regulation through the differentiation process with cells representing different stages of erythroid and megakaryocytic differentiation. The L929 fibroblast cell line was used as a source of differentiated murine non-hematopoietic cells.

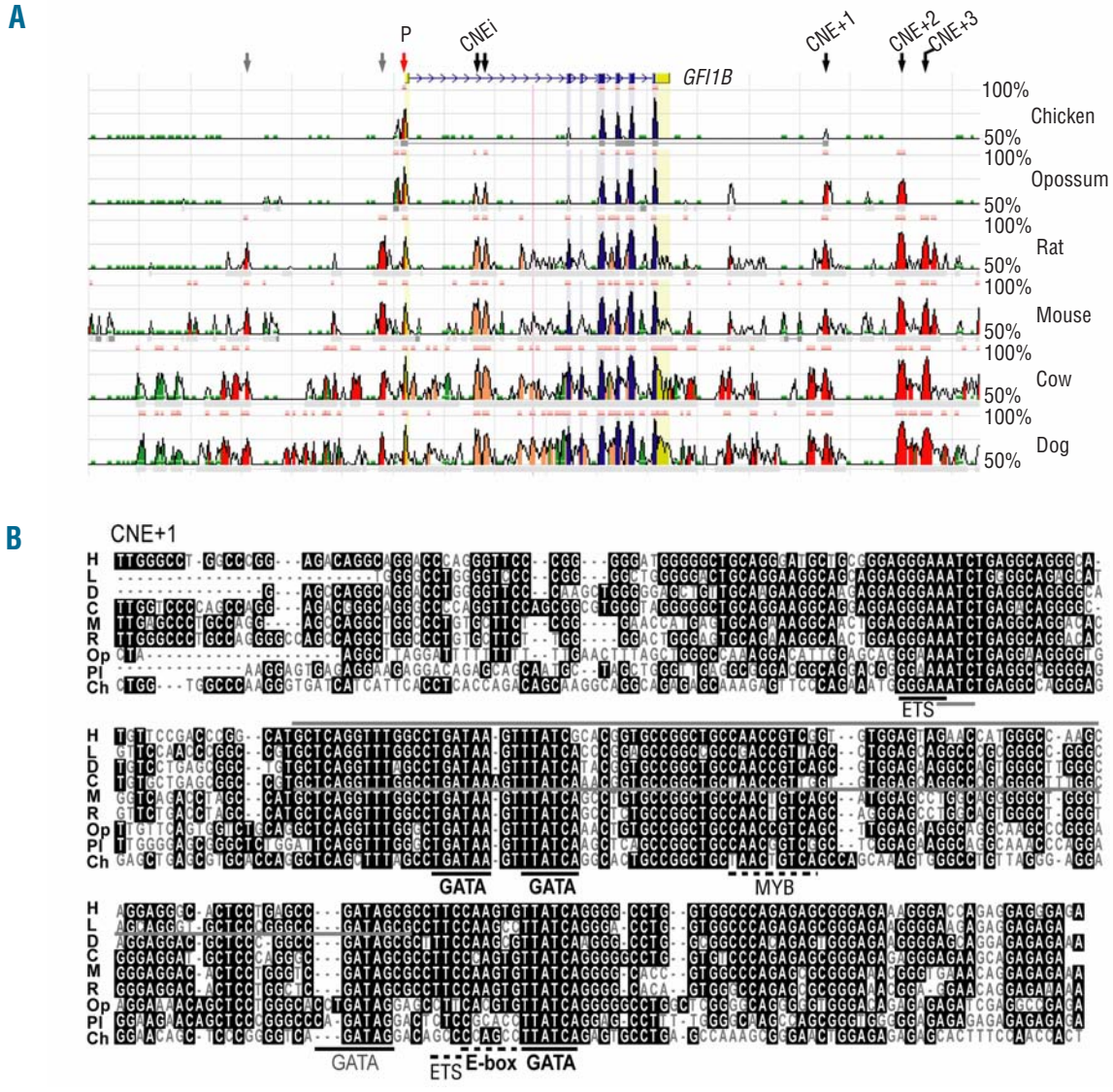

Figure 1. Identification of candidate distant *cis*-acting elements at the *GFI1B* locus. (A) Partial view of ERC browser output showing the main conserved non-coding elements (CNE) analyzed in this study. Intergenic CNE are colored in red, intronic ones are in orange, exons in blue, untraslated regions in yellow and repetitive sequences in green. A red arrow indicates *GFI1B* promoter location (P), black arrows point to the first intron (CNEi) and downstream (+1, +2 and +3) CNE, where conserved erythroid and megakaryocytic recognition sequences are found and gray arrows localize the best conserved through evolution upstream CNE. (B) Example of detailed study of conserved transcription factor binding sites, corresponding to the alignment of nine species at CNE+1. Two exact matches with GATA consensus binding sites (black letter font) and one with a mismatch outside the GAT core in some species (gray letter font) are indicated with a black line below the sequence. An E-box (CANNTG) conserved in most species from human to opossum is shown. One MYB consensus core DNA-binding site (YAACNG) has been conserved in most species, matching in most of them the extended consensus $^{T}/_{c}$AACBG$^{T}/_{c}$C$^{A}/_{G}$. A conserved AATC GFI1B binding site core is shown with a gray line below the sequence. ETS-domain DNA binding motifs (GGA$^{A}/_{T}$) are also indicated. A continuous line symbolizes that a site is present in all species and a dashed line when it is in most cases. Real-time PCR amplicons are shown as a gray line over the corresponding sequence. H indicates human (*Homo sapiens*); L, mouse lemur (*Microcebus murinus*); D, dog (*Canis familiaris*); C, cow (*Bos taurus*); M, mouse (*Mus musculus*); R, rat (*Rattus norvegicus*); Op, opossum (*Monodelphis domestica*); Pl, platypus (*Ornithorhynchus anatinus*); Ch, chicken (*Gallus gallus*).

We first studied *Gfi1b* expression in those cells by real-time PCR. *Gfi1b* RNA was detected in L929 cells at the limit of the sensitivity of the technique, in the order of 10^5 times less than in FDCP-Mix hematopoietic progenitors. Erythroid and megakaryocytic cells expressed higher levels of *Gfi1b*, with the amount decreasing with cell differentiation (Figure 2A). We confirmed this pattern of expression in erythroid cells at the protein level by western blotting (Figure 2B).

Active chromatin at sites associated with transcription regulation adopts an 'open' conformation. To assay this we used the recently developed FAIRE technique,[30] which enriches for nucleosome-free DNA. Real-time PCR analysis of the *Gfi1b* locus showed the promoter, CNE+1 and CNE+3 to be highly enriched in nucleosome-free DNA relative to the genomic DNA in expressing MEL cells, but not in the control L929 cells (Figure 2C). We also confirmed the presence of specific DNase1 hypersensitive sites at CNE+1

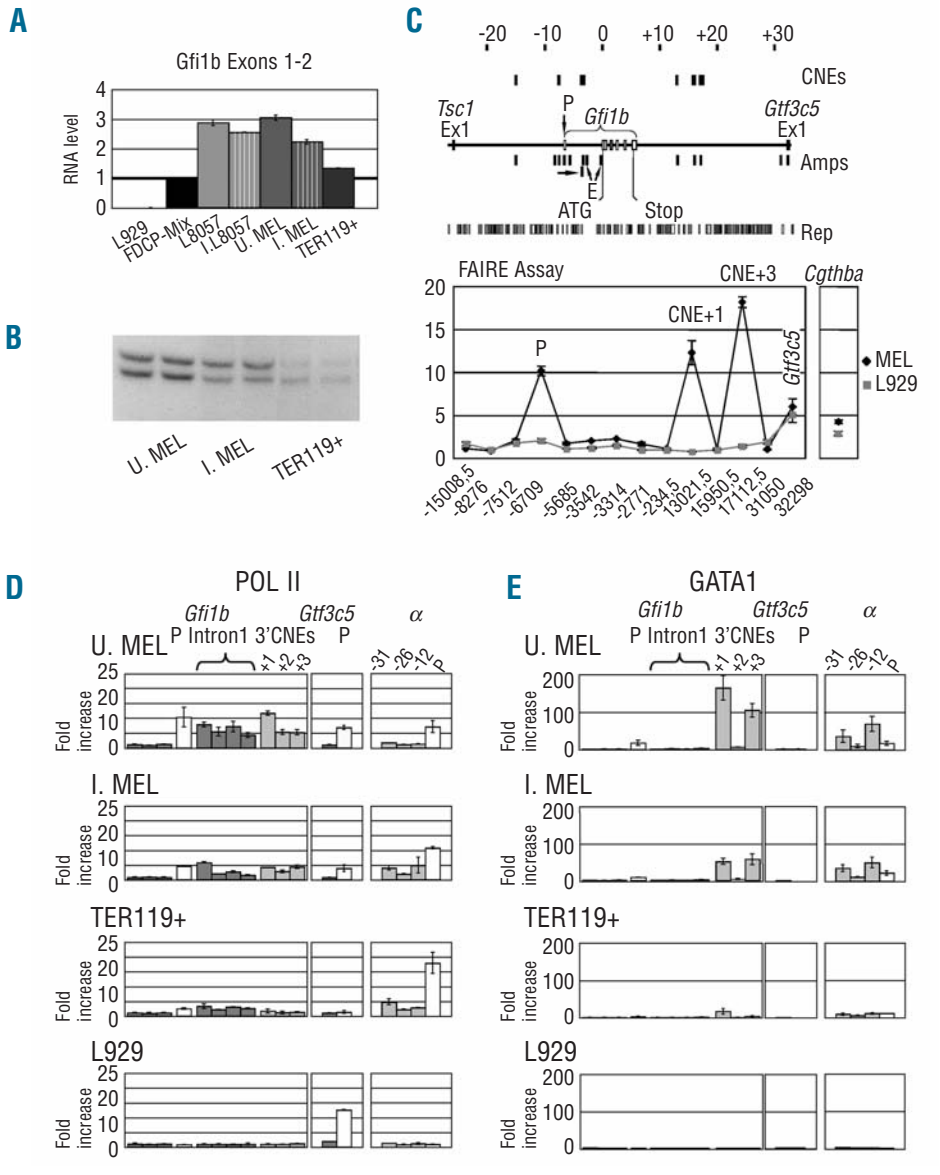

Figure 2. Open chromatin conformation and POL II plus GATA1 recruitment mark *Gfi1b* promoter and downstream elements in expressing cells. (A) *Gfi1b* RNA quantification of cell lines and primary cells by real-time PCR, TaqMan® Gene Expression Assay Mm01336944_m1 with FAM-NFQ probe between *Gfi1b* exons 1 and 2, normalized with the level of RNA polymerase II. The data are relative to factor-dependent cell Paterson subline A4 (FDCP-Mix) cells, which have characteristics of multipotent hematopoietic progenitors; in these cells *Gfi1b* level was set to 1. Error bars correspond to ± 1 standard deviation (SD) from two independent experiments. I. L8057 (induced L8057 cells), U. MEL (uninduced MEL cells), I. MEL (induced MEL cells). (B) Western blot demonstrating the decrease in GFI1B protein level with erythroid differentiation; the same amount of total protein was loaded in duplicate for each cell type.

(C) Top, diagram of mouse *Gfi1b* locus. On the top level, coordinates in relation to ATG start codon. Promoter (P) is pointed with a black arrow; CNE are shown with black boxes over the DNA sequence (black line); white boxes on the sequence are *Gfi1b* exons; black boxes below indicate the position of amplicons used in real time PCR (Amps); white boxes underneath are repetitive sequences (Rep). Location of amplicons at *Gfi1b* first intron E-boxes (E), which have been shown to bind E2A in a T cell lymphoma cell line,[3] are pointed with arrows. A horizontal arrow points to an amplicon only used in FAIRE and *Online Supplementary Figure S5*. Start and stop codons are shown. The first exon (Ex1) of each neighbor gene is indicated. Underneath, FAIRE assay, in gray are L929 data and in black MEL cell results (y-axis). Coordinates at the middle of each amplicon from the start codon are indicated on the x-axis. *Gtf3c5* and *Cgthba* (*c16orf35*) promoter controls are shown. Error bars correspond to ±1 SD from two independent experiments. P, promoter. (D) and (E) ChIP analysis of RNA POL II and GATA1, respectively, in cells representing different stages of erythropoiesis and L929 control. The y-axis indicates the enrichment of ChIP DNA over the input and normalized to *Gapdh* control sequence. POL II binds in all *Gfi1b* expressing cells at the promoter (P), gene (first intron under the brace) and downstream CNE. GATA1 binding corresponds to *Gfi1b* promoter and 3´CNE. Controls at *Gtf3c5* and *α-globin* loci are also shown. Error bars represent ±1 SD from two independent ChIP assays.

and +3 (*Online Supplementary Figure S4*).

Next, we analyzed POL II recruitment to the *Gfi1b* locus by ChIP assays. We confirmed POL II binding at both the *Gfi1b* promoter and the CNEi in all the erythroid cells, but in L929 cells POL II was only enriched at the *Gtf3c5* control gene (Figure 2D). Consistent with the expression data, the level of POL II binding reduced with erythroid differentiation. We also observed POL II recruited to the 3´CNE in erythroid cells, further suggesting that these areas have a regulatory role.

In summary, CNE+1 and +3 become nucleosome-free in *Gfi1b* expressing cells and POL II is recruited to these sites. This is consistent with the idea that these correspond to *cis*-regulatory elements of the *Gfi1b* gene.

GATA/SCL complex binds to Gfi1b *downstream conserved non-coding elements and the promoter, decreasing with erythroid differentiation*

It is known that GATA1 binds to the *GFI1B* promoter in human erythroid K562 cells, where it has a positive regulatory function.[22] Given the conservation and number of potential GATA binding motifs at *Gfi1b* downstream CNE, we first searched for GATA1 binding *in vivo* using ChIP. We confirmed that GATA1 binding at the promoter occurs and found a much higher enrichment of CNE +1 and +3, together with a weak enrichment of CNE+2 (Figure 2E). The CNEi was very slightly enriched over the background level, as we confirmed in a detailed analysis (*Online Supplementary Figure S5*). *Gtf3c5* and *α-globin* loci acted as negative and positive controls, respectively (Figure 2E).

The GATA1/SCL pentameric complex binds to DNA at closely spaced GATA and E-box binding motifs. Besides the canonical E-box/GATA (an E-box situated 8-10 nucleotides upstream of the GATA motif),[43] binding of this complex can also be mediated by variations of this,[44] or by GATA sites alone.[31,45] We, therefore, tested the binding of the remaining components of the pentameric complex, SCL, E2A, LMO2 and LDB1, and observed that they followed GATA1 with an almost identical profile (Figure 3A-B and *Online Supplementary Figure S6 A-B*). These factors were absent from the elements upstream of the *Gfi1b* gene and from the intronic areas. Binding of the pentameric complex decreased with erythroid differentiation at the *Gfi1b* locus, consistently with POL II binding and *Gfi1b* expression.

In conclusion, this coordinate binding of known erythroid transcription factors to the CNE and the promoter, and the strong link between the occupancy, the expression status and POL II recruitment, strongly supports the idea that these elements, particularly CNE+1 and +3, regulate the expression of *Gfi1b* in part via the previously described pentameric complex.

Transcription factor binding at preferential sites of the Gfi1b *locus*

Besides GATA recognition sequences and E-boxes we observed other conserved potential binding sequences for hematopoietic transcription factors. These included NF-E2 at CNE+3, MYB recognition elements at CNE+1, CNE+3 and CNEi and multiple ETS-domain binding sites at all the CNE. The ETS family member PU.1 has been shown to antagonize GATA1 transcriptional activity by binding to it when GATA1 is bound to DNA;[46] this factor is overexpressed in MEL cells, blocking erythroid differentiation.[47]

ChIP analysis showed that both the hematopoietic-restricted 45 kDa (P45) and ubiquitous 18 kDa (P18) subunits of the NF-E2 transcription factor can be identified at CNE+3 (Figure 3C-D). The dynamics of NF-E2 binding followed the pentameric complex, reducing with erythroid differentiation. In MEL cells, c-MYB binding was detected at CNE+1 (*Online Supplementary Figure S6C*) and the *Gfi1b* promoter was highly enriched for PU.1 (*Online Supplementary Figure S6D*).

These data suggest that CNE+1 and +3 have distinct roles in *Gfi1b* regulation and show that PU.1 binding is promoter-specific.

Epigenetic signals for gene activation correlate with pentameric complex binding

We assessed chromatin status by analyzing histone H4 acetylation (AcH4) and dimethylation of lysine 4 of histone H3 (H3diMeK4) which are both marks of gene activation.[48,49] Both histone modifications had similar patterns, with high levels of AcH4 and H3diMeK4 at the *Gfi1b* gene, the 3´CNE and, to a lesser degree, the *Gfi1b* promoter. Unlike the decrease in transcription factor binding, chromatin changes remained similar or even increased with erythroid differentiation. Upstream of the *Gfi1b* promoter, chromatin modifications progressively reduced to background levels (Figure 4). While this paper was under review Laurent B *et al.* published a human *GFI1B* promoter study that also showed that this site is associated with active chromatin marks during erythroid differentiation, in agreement with our data.[50]

Interestingly, the CNE with the highest level of AcH4 and H3diMeK4 in most erythroid cells is CNE+2, which showed the lowest binding of the pentameric complex.

In summary, chromatin modifications suggest that the downstream CNE have a positive regulatory function and that CNE+2 seems to have characteristics distinguishing it from the others.

GFI1B *binds to multiple sites in its own locus. CoREST and LSD1 are found at* GFI1B *binding sites*

GFI1B has been found at its own promoter and this could switch the effect of GATA1 from activation to repression.[18] We, therefore, investigated GFI1B binding to verify whether it has any further binding at its locus. In fact, GFI1B binds not only at its promoter, but also at CNEi, +1 and +3 in MEL cells (Figure 5A and *Online Supplementary Figure S5*).

Co-repressor ETO2 has been shown to interact with the GATA/SCL pentameric complex, mainly during early stages of erythroid and megakaryocytic differentiation, turning its activity into repression.[19,20,23,51] ETO2 recruitment at the *GFI1B* promoter suggests that it may have a repressive activity at this locus.[23] Surprisingly, when we analyzed ETO2 by ChIP in our system, we detected ETO2 at CNE+1 and +3 in MEL cells, but there was no clear enrichment of other GFI1B binding areas in these cells or in terminally differentiated erythroblasts (Figure 5B and *Online Supplementary Figure S5*).

The GFI1B SNAG domain recruits LSD1 and CoREST, and this mediates its transcriptional repression activity.[12]

We detected LSD1 and CoREST *in vivo* at the same sites and cells as where we found GFI1B (Figure 5C-D). In TER119^{+} cells, both co-repressors were mainly present at the promoter, but also remained at a low level at the downstream elements.

We conclude that CNEi, +1 and +3, like the *Gfi1b* promoter, are both gene activators and negative regulators.

Distal regulatory elements bind the same complexes in the megakaryocytic lineage and human erythroid cells

Many specific transcription factors are common to the erythroid and megakaryocytic lineages, including GFI1B. We, therefore, analyzed the binding pattern of POL II, the pentameric complex, GFI1B, LSD1 and CoREST in the megakaryocytic cell line L8057 induced to megakaryocytic differentiation with TPA. We observed an almost identical binding to the one described for erythroid cells (*Online Supplementary Figure S7*).

In the human cell line K562, which has some erythroid features, we confirmed GATA1 binding at the *GFI1B* promoter together with POL II (*Online Supplementary Figure S8*). CNE+1 and +3 were also highly enriched using GATA1 antibody. Again we detected a weak enrichment of CNE+2 compared to the other sites.

We found SCL, E2A (E47) together with LMO2 and LDB1 at the downstream CNE and the promoter. Once more, the enrichment of CNE+1 and +3 was greater than that of the promoter and CNE+2. We also found both subunits of NF-E2 at CNE+3. Human GFI1B binding and histone modifications were similar to those of the mouse (*Online Supplementary Figure S8B*).

In conclusion, the regulatory mechanisms driven by these distal elements have been preserved in erythroid and megakaryocytic lineages and between humans and mice.

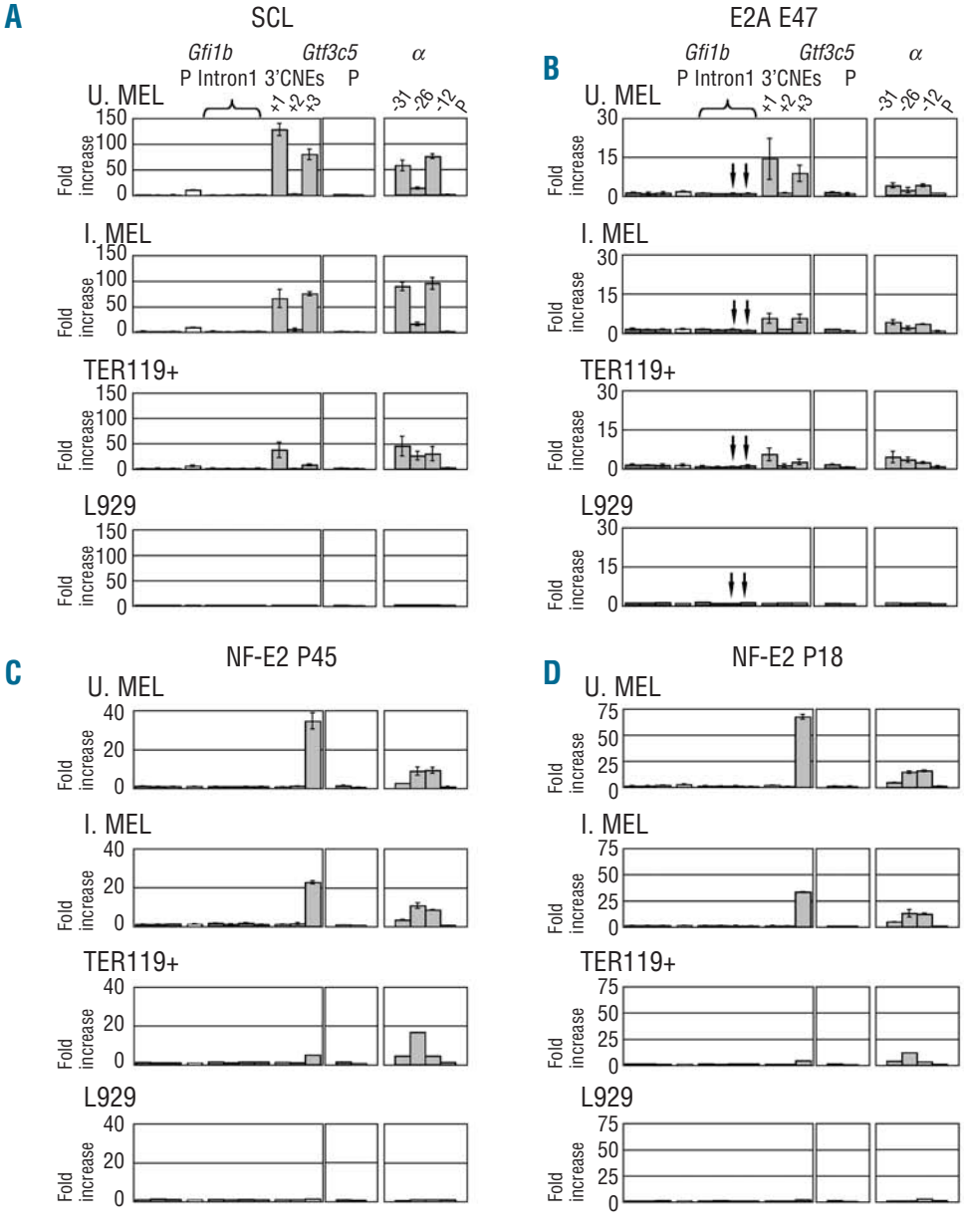

Figure 3. SCL and E2A bind to the mouse *Gfi1b* locus at the promoter and downstream CNE with a similar pattern to GATA1, while NF-E2 specific binding at CNE+3 suggests differential roles for downstream CNE. ChIP analysis of SCL (A), E2A E47 (B), NF-E2 P45 (C) and NF-E2 P18 (D). Plot follows Figure 2. Note that E47 is not present at the intronic E-boxes (arrows), E2A E12 showed a similar pattern (*data not shown*). Both NF-E2 subunits showed binding at CNE+3; reduction in NFE2 binding at this site with erythroid differentiation contrasts with its maintenance or increase at the *α-globin* locus.

Conserved non-coding elements +1 and +3 interact with the Gfi1b *promoter*

If the transcription factor binding elements we found at the *Gfi1b* locus behave as long-distance *cis*-regulators, how do they interact with the gene promoter? Chromosome conformation capture can provide evidence of changes in chromosomal structure due to direct long-range interactions, probably through a looping mechanism.[33,34]

L929 and MEL cells were formaldehyde cross-linked, the cross-linked chromatin digested with restriction enzyme DpnII and then ligated. Interactions between the *Gfi1b* promoter and downstream regions were quantified by real-time PCR with a primer and a Taqman probe at the *Gfi1b* promoter and a primer at each downstream fragment. PCR amplification was ligation-dependent (*data not shown*). L929 and MEL cells showed similar enrichment of the promoter with exon 7 and +8802/+9399 PCR products that decreased in relation to the distance to the promoter in a linear conformation. However, analysis of CNE+1 and CNE+3-promoter interactions showed a greater enrichment in MEL cells over L929 cells, which was not distance-related (Figure 6A), suggesting that these sequences may well interact with the promoter via an erythroid-specific looping mechanism.

GFI1B *is expressed at different level in individual cells*

The co-localization of chromatin modifications and some transcription factors related to gene activation were found in association with the binding of GFI1B protein together with co-repressors. These opposing functions may operate at different stages of maturation or cell cycle, or could be related to varying GFI1B concentrations in different cells within the same population. To examine these possibilities we studied GFI1B protein level by immunofluorescence, which distinguishes the protein concentration in individual cells. When uninduced MEL cells were stained with the GFI1B antibody and studied by confocal microscopy, we observed a high level of cell-to-cell variation in fluorescence intensity, supporting the hypothesis of intercellular heterogeneity (Figure 6B-C). A similar degree of heterogeneity was seen in induced MEL cells but with a decrease in GFI1B intensity with differentiation, in agreement with our previous data (Figure 6C).

Discussion

GFI1B plays a critical role in normal erythropoiesis and megakaryopoiesis[4-6] and is thought to be involved in blood malignancies.[7,8] How the *GFI1B* gene is regulated is largely unknown and the promoter is the only regulatory element identified to date in erythroid and megakaryocytic cells.[17,18,22] Here we have identified additional *GFI1B cis*-regulatory sequences that have been highly conserved through evolution. One element lies in the first intron of the *GFI1B* gene (CNEi) and three lie downstream of the gene (called +1, +2 and +3 according to their proximity to the *GFI1B* coding sequence). All showed multiple conserved binding sites for erythroid/megakaryocytic transcription factors. Two of the sites (+1 and +3) associate with an 'open' chromatin conformation as determined by the FAIRE assay and DNase1 hypersensitivity sites and appear to interact with the *Gfi1b* promoter specifically in expressing cells in the 3C assay. This is the first time that such a study has been performed in primary cells at the *Gfi1b* locus.

The pattern of transcription factor binding to the promoter and the CNE is clearly complex. GATA1 and SCL

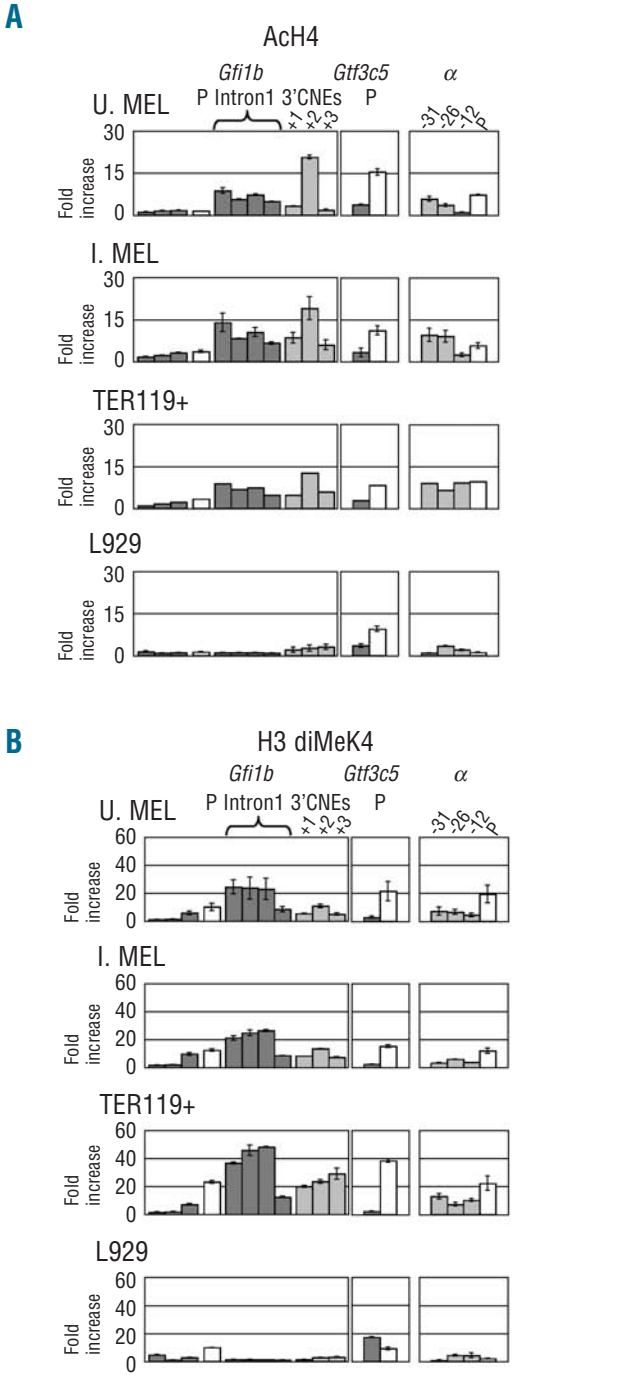

Figure 4. Location of chromatin modifications related to gene activation at the CNE in erythroid cells. Real-time PCR analysis of ChIP with antibodies against acetylated histone H4, AcH4, (A) and dimethylated lysine 4 of histone H3, H3 diMeK4, (B). Chromatin signals of gene activation stay high through erythroid differentiation.

had been previously shown to bind to *GFI1B* promoter and were associated with gene activation.[18,22,23] We now show that the whole pentameric complex, comprising GATA1, SCL, E2A, LMO2 and LDB1, binds to both the promoter and the three downstream elements in human and mouse erythroid and megakaryocytic cells. CNE+1 and +3 show very strong binding of all these factors, while CNE+2 is weakly enriched in ChIP assays. In addition, the most 3´-element binds both subunits of NF-E2 transcription factor (ubiquitous P18 and specific P45). This differential binding may indicate that these elements have unique non-redundant characteristics. A considerable enrichment of histone H4 acetylation and histone 3 diMeK4 at CNE+1, CNE+3, CNEi and promoter is found in the same cells. This combination of histone marks associated with gene activation together with the binding of transcription factors that can have an activator role suggests that the CNE may increase the transcription of the *GFI1B* gene itself.

However, it has been shown that GFI1B down-regulates its own expression by binding to its promoter[18] and recruiting the CoREST/LSD1 repressive complex.[12] We confirmed this binding and also found GFI1B, CoREST and LSD1 at CNE+1, CNE+3 and CNEi. CNE+2, which had no evident GFI1B, CoREST or LSD1 enrichment, had a higher level of AcH4 and H3 diMek4 than the neighboring CNE, showing that there is an inverse correlation between the two events. We also detected co-repressor ETO2 binding at some of the areas (CNE+1 and +3) where GFI1B is present. *In vitro* experiments have indicated that activating complexes of SCL, E2A and GATA1 turn into repressive complexes when associated with ETO2 and GFI1B.[13,19,20] Other elements (CNEi and the *Gfi1b* promoter) do not show ETO2 binding in MEL cells.

How can this apparent paradoxical situation, in which combinations of activating and repressive complexes apparently reside at the same conserved elements, be resolved? A clue may come from the striking heterogeneity in GFI1B protein levels that was observed with immunofluorescence staining of uninduced and induced MEL cells. These populations faithfully recapitulated the decrease in overall GFI1B levels on induction with a downward shift in mean fluorescence but with a similar degree

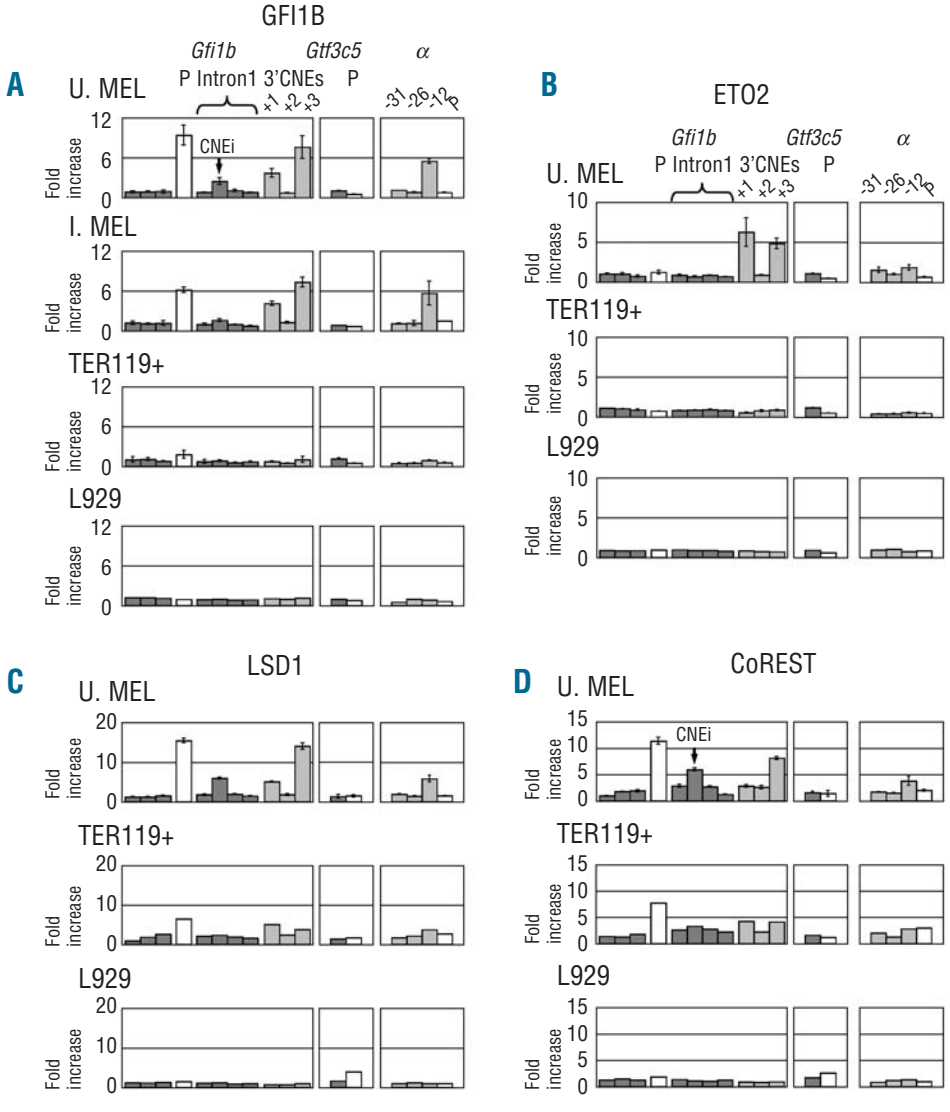

Figure 5. Binding of GFI1B, ETO2 and LSD1 together with REST Co-repressor (CoREST) at CNE+1, CNE+3 and CNEi indicates that these elements can behave as repressors. ChIP assay of GFI1B (A), ETO2 (B), LSD1 (C) and CoREST (D). GFI1B also binds with LDS1 and CoREST at *α-globin* HS-12 in MEL cells, which has been previously suggested to be involved in *α-globin* repression in early erythroid progenitors.[19] GFI1B, LDS1 and CoREST binding with or without ETO2 persists to a low level in terminally differentiated cells at the *Gfi1b* locus, but does not at the *α-globin* locus, consistently with the high level of *α-globin* transcription at this stage.

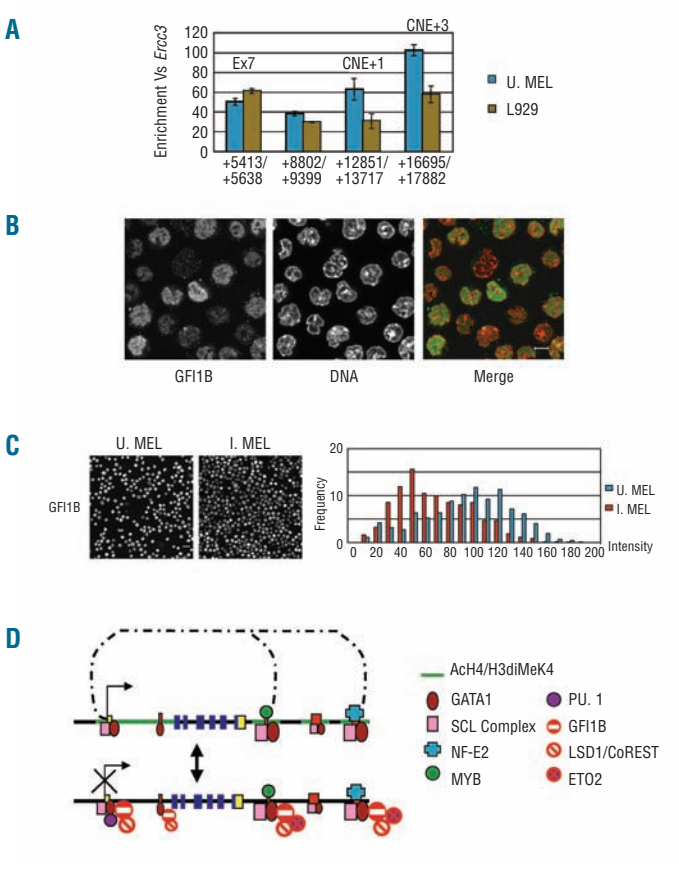

Figure 6. Cellular variation in GFI1B protein levels suggests a continuous repression and activation process in the same cell population. (A) 3C analysis on fixed chromatin digested with DpnII. The bar chart (y-axis) shows the enrichment of PCR products normalized to enrichment on *Ercc3* locus (100%) and a PCR product with no DpnII restriction sites at *α-globin* HS-31. Uninduced MEL cells results are in blue (U MEL) and L929 in brown. Coordinates of DpnII restriction fragments from *Gfi1b* start codon are indicated on the x-axis. Error bars are ±1 SD from two independent experiments. (B) Immunofluorescence in MEL cells illustrating the GFI1B variation in protein level among cells of the same population. Left, GFI1B staining; middle, DNA (TOPRO-3, Molecular Probes); right, merge, in which GFI1B appears in green and the DNA in red. The white bar represents ~5 μm. (C) Left, overview of uninduced and induced MEL cells immunofluorescence with GFI1B antibody. These pictures are representative examples of the images analyzed. The white bar represents ~20 μm. Right, quantification of GFI1B immunofluorescence in uninduced MEL cells (blue) and induced MEL cells (red) the y-axis represents the proportion of cells (%) in each category. The x-axis shows intensity in arbitrary units. After differentiation GFI1B diminishes, but cell variation is strong in both cell populations. (D) Model for *GFI1B* regulation. (Top) When *GFI1B* is expressed the locus is marked with histone acetylation and dimethylation of histone 3 K4 and GATA1/SCL pentameric complex together with other factors such as NF-E2 and c-MYB bind to the downstream CNE transferring POL II to the promoter. (Bottom) After the level of GFI1B in the nucleus reaches a certain threshold level, it starts to bind to its own locus at regulatory elements susch as the promoter, CNEi, +1 and +3, together with ETO2 at some positions, and recruits LSD1 and CoREST. This limits its expression until protein degradation allows the activating forces to overcome the repressive ones and *GFI1B* expression increases. Through erythropoiesis the general reduction in transcription factors produces a slow decline in *GFI1B* expression. SCL complex: SCL, LMO2 and LDB1.

of variability in protein content from cell to cell.

We suggest the following model as consistent with all our data. As GFI1B levels rise in the cell, GFI1B will bind to its own promoter and to the CNE. This will attract repressor complexes that progressively switch off the gene through a feedback loop as protein levels rise (Figure 6D). As GFI1B levels decline, repression of *GFI1B* expression will decrease until a stage is reached at which the activating complexes once more predominate and expression begins again. These fluctuations may be a fine control mechanism to ensure the correct level of the protein either during the cell cycle or during the process of erythroid maturation. As erythroid cells differentiate, a general decrease in transcription factor expression and subsequent loss of binding to these elements correlate with the progressive reduction in *GFI1B* expression. In megakaryocytes other mechanisms could be involved in *GFI1B* repression including the recruitment of octamer-binding transcription factors to the promoter (*unpublished data*).

The validation of this hypothetical model will require further functional experiments. It will also be of great interest to determine whether any of these sites are targets of mutations leading to human disease.

Authorship and Disclosures

EA was the principal investigator and takes primary responsibility for the paper, he designed the study, performed research, analyzed data and wrote the paper; AV analyzed data; FI and AH performed research.

The authors reported no potential conflicts of interest.

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