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Transcriptional regulation of *Elf-1*: locus-wide analysis reveals four distinct promoters, a tissue-specific enhancer, control by PU.1 and the importance of *Elf-1* downregulation for erythroid maturation

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ABSTRACT

Ets transcription factors play important roles during the development and maintenance of the haematopoietic system. One such factor, *Elf-1* (*E*74-*l*ike *f*actor *1*) controls the expression of multiple essential haematopoietic regulators including Scl/Tal1, Lmo2 and PU.1. However, to integrate *Elf-1* into the wider regulatory hierarchies controlling haematopoietic development and differentiation, regulatory elements as well as upstream regulators of *Elf-1* need to be identified. Here, we have used locus-wide comparative genomic analysis coupled with chromatin immunoprecipitation (ChIP-chip) assays which resulted in the identification of five distinct regulatory regions directing expression of *Elf-1*. Further, ChIP-chip assays followed by functional validation demonstrated that the key haematopoietic transcription factor PU.1 is a major upstream regulator of *Elf-1*. Finally, overexpression studies in a well-characterized erythroid differentiation assay from primary murine fetal liver cells demonstrated that *Elf-1* downregulation is necessary for terminal erythroid differentiation. Given the known activation of PU.1 by *Elf-1* and our newly identified reciprocal activation of *Elf-1* by PU.1, identification of an inhibitory role for *Elf-1* has significant implications for our understanding of how PU.1 controls myeloid–erythroid differentiation. Our findings therefore not only represent the first report of *Elf-1* regulation but also enhance our understanding of the wider regulatory networks that control haematopoiesis.

INTRODUCTION

The formation and subsequent differentiation of haematopoietic stem cells (HSCs) is tightly controlled requiring the sequential and coordinated action of multiple transcription factors. The Ets family is one of the largest families of transcriptional regulators involved in the regulation of haematopoiesis. All Ets proteins contain a region of conserved sequence, corresponding to the ETS domain, which constitutes the DNA binding domain and recognizes a core GGAW sequence in the major groove of DNA. Binding of ETS proteins to this sequence is often facilitated by synergistic interaction with transcriptional partners on composite DNA elements (1).

The Ets-transcription factor *Elf-1* was originally cloned from a human T-cell cDNA library by hybridization with a probe encoding the DNA binding domain (ETS domain) of the human Ets-1 cDNA (2). Based on its preferential expression in embryonic lymphoid organs (thymus and spleen), a wide variety of epithelial cells and fetal liver (3) as well as in adult haematopoietic tissues, including thymus, spleen and bone marrow (4,5), *Elf-1* emerged as a potential key regulator of haematopoietic gene

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expression. Consistent with this notion, *Elf-1* has been shown to be a direct upstream regulator of genes important for haematopoiesis such as Scl, Fli-1, Lyl-1, Runx1 and Lmo2 (6–11). Like other members of the Ets family such as Ets-1 and Ets-2, no major haematopoietic phenotypes have been observed in *Elf-1*$^{-/-}$ mice (12). However, given that *Elf-1*, like Ets-1, is a member of a small subfamily, a likely explanation for the lack of a strong phenotype in loss of function assays is compensation by other Ets proteins which are also known to be expressed in the haematopoietic system.

Elf-1 has also been shown to be important for blood vessel development, a process that is closely linked to early haematopoiesis during embryonic development. *Elf-1* has been reported to take part in the transcriptional control of major regulators of blood vessel development such as Tie1, Tie2, angiopoietin-2, the vascular endothelial growth factor receptor 1 (VEGFR1), the endothelial nitric-oxide synthase (eNOS) and endoglin (3,13–16). Moreover, a recent landmark study demonstrated that *Elf-1* constitutes a potentially very attractive target for the development of new cancer therapies as systemic application of an ELF-1 blocking peptide was able to inhibit tumour angiogenesis in a mouse model of melanoma (17).

Functional activity of Ets proteins is modulated at multiple levels. For example, it is known that ELF-1 appears in the cytoplasm as a 80 KDa protein that is *O*-glycosylated and phosphorylated in order to be translocated into the nucleus where it can be detected as a 98 KDa protein. After dephosphorylation, the protein is degraded through the proteasome pathway (18–20). In resting T cells, ELF-1 binds to the underphosphorylated form of the retinoblastoma protein pRb and after T-cell activation, the phosphorylation of pRb results in the release of ELF-1 (21).

Despite its function as an important transcriptional regulator in both blood and endothelium, nothing is known about the transcriptional regulation of the *Elf-1* gene itself. Consequently, the position of *Elf-1* within the wider regulatory networks controlling blood cells had remained obscure. In this study, we have identified and characterized five regulatory elements within the *Elf-1* locus. Four regulatory regions functioned as promoters and one region as a lineage-specific enhancer. Furthermore, we showed that the transcription factor PU.1 plays a major role in the regulation of *Elf-1* and that downregulation of *Elf-1* is important for terminal erythroid differentiation.

MATERIALS AND METHODS

Sequence analysis and custom arrays

Genomic *Elf-1* sequences were downloaded from Ensembl, aligned using multi-Lagan (22) and displayed using mVista (23). Primers to generate the *Elf-1* oligo tiling array were designed using Primer3 (24) on repeat masked sequence spanning *Elf-1* and flanking genes (chr14:74 160 804–74 269 970, coordinates in build mm7). Oligos were spotted in triplicate using a BioRobotics MicroGrid II Total Array System. Array design files have been submitted to ArrayExpress.

ChIP-chip assays

ChIP assays were performed as previously described (25). Briefly, cells were treated with formaldehyde and cross-linked chromatin was sonicated to 300 bp averaged size. Immunoprecipitations were performed using anti-acetyl histone H3 antibody (Millipore, 06-599), anti-H3 trimethyl lysine 4 (Millipore, 04-745 and gift from Dr L. O'Neill, Birmingham, UK) and anti-PU.1 (Santa Cruz, sc-352X). ChIP material was labelled with Cy3 and Cy5 fluorochromes and hybridized as described (26). Microarrays were scanned using an Agilent scanner and median spot intensities quantified using GenePix Pro 6.0 with background subtraction. A Perl script was developed to normalize the resulting data and calculate mean ratios of normalized ChIP signals over input (27) using the triplicate values on the array. Resulting data were plotted using the Variable WidthBar Graph Drawer (http://hscl.cimr.cam.ac.uk/genomic_tools.html). All experiments have been deposited in ArrayExpress with accession numbers E-MEXP-2664, E-MEXP-2665 and E-MEXP-2666.

Gene expression analysis

Total RNA was isolated using Tri-Reagent (Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions. Contaminating genomic DNA was removed by DNAseI treatment with Turbo DNA free (Applied Biosystems/Ambion, Warrington, UK). Total RNA (125 ng) was used for cDNA synthesis, using oligo(dT) 17-mer primer and MMLV reverse transcriptase (Sigma-Aldrich). Mouse *Elf-1,* mouse *PU.1* and mouse *18s* mRNA levels were measured by real-time PCR using the primer sets listed in Supplementary Table S1. Relative expression was calculated as a ratio to the 18 s signal.

Cell culture, reporter constructs and transfection assays

The myeloid progenitor cell line 416B, murine erythroleukaemia cell line F4N, endothelial cell line MS1 and the T-cell line BW5147 were maintained as previously described (28,29). Fetal liver and adult thymus cell suspensions were obtained by direct pipetting of freshly dissected tissues from mice. *Elf-1 luciferase* reporter constructs were amplified from mouse genomic DNA using primers listed in Supplementary Table S1, confirmed by sequencing and cloned into pGL2basic (Promega, Southampton, UK). These regions were selected based on the combined results of comparative genomics and ChIP-chip experiments (Supplementary Figures S1–S5). Cell lines were stably transfected by electroporation as described (29). G418 or puromycin was added 24 h post-transfection and cells were assayed 7–27 days later, depending on the cell line. For transactivation assays, 293T cells were transfected with 1 μg 'luciferase' construct in combination with 3μg pcDNA3mPU.1 (generous gift from Dr J. Frampton, Birmingham, UK) or the empty vector pcDNA3 as control using ProFection Mammalian Transfection System-Calcium Phosphate (Promega). Each transfection

and transactivation was performed on at least two different days in triplicate.

In vitro erythroid differentiation assay

In vitro erythroid differentiation assays were performed as previously described (30,31). Briefly, livers from E14.5 (C57Bl/6 × CBA) F1 murine embryos were extracted by blunt dissection and homogenizsed. Lineage-negative haematopoietic progenitor cells were isolated using the MACS Murine Lineage Cell Depletion Kit (Miltenyi Biotec GmbH, Gladbach, Germany) according to the manufacturer's instructions. 2×10^5 lineage-negative cells were plated onto fibronectin coated 12-well plates (BD Discovery Labware, Bedford, MA, USA) and grown in Erythroid Differentiation Medium [IMDM supplemented with 15% fetal bovine serum, 1% detoxified bovine serum albumin (BSA) (both from Stem Cell Technologies, Vancouver, Canada), 600 μg/ml holo-transferrin (Sigma-Aldrich), 10 μg/ml recombinant human insulin (Sigma-Aldrich), 2 mM L-glutamine, 10^{-4} Mβ-mercaptoethanol, and 2 U/ml human recombinant erythropoietin].

Retroviral transfection

Plasmid containing murine whole-length *Elf-1* cDNA (6) was digested with PstI, blunted with T4 polymerase, digested again with XhoI and subcloned into a retroviral overexpression vector containing MSCV pGKprom-Puro-IRES-EGFP (PIG). Retrovirus production was carried out using the pCL-Eco Retrovirus Packaging Vector (Imgenex, San Diego, CA, USA) and the 293T cell line. Murine fetal liver cells were infected with retrovirus by centrifugation at 2000 r.p.m. at 30°C for 1.5 h with 4 μg/ml polybrene (Sigma-Aldrich) after which the retroviral supernatant was replaced with Erythroid Differentiation Medium (30).

Flow cytometry analysis and cell sorting

Samples for FACS analysis were stained using antibodies against the surface markers Ter119 [APC conjugated, 1 in 100 dilution] (BD, Oxford, UK) and CD71 [PE conjugated, 1 in 100 dilution] (BD). Dead cells were excluded using the 7-aminoactinomycin (7AAD) stain. All analysis was performed using a FacsCalibur analyser (BD) and sorting was performed using a Dakocytomation MoFlo cell sorter.

RESULTS

Locus-wide comparative genomic analysis identifies five non-coding conserved regions representing candidate Elf-1 regulatory elements

Cross-species sequence conservation of non-coding regions has been successfully used in the past to identify regulatory elements (27,32). To locate candidate regulatory elements within the m*Elf-1* gene locus, we compared the human, dog and opossum *Elf-1* loci to 109 kb of the mouse locus containing the entire *Elf-1* gene and extending into upstream and downstream flanking genes. All coding regions were highly conserved between species analysed (Figure 1A). Non-coding sequence conservation was more limited but comparison with the opossum sequence revealed five highly conserved non-coding regions that represented candidate regulatory elements of m*Elf-1* gene. Four of those regions were located 55, 21, 14 and 8.5 kb upstream of the ATG start codon of the open reading frame and the fifth region was located just upstream of the start codon which from here on will be referred to as the proximal region.

Although *Elf-1* is expressed in most haematopoietic cells (33), levels vary between cell types. In order to identify cellular systems for functional validation of candidate regulatory elements, expression levels of *Elf-1* were assessed in different cell types (Figure 1B). We analysed two sources of primary cells (E13.5 fetal liver and whole adult thymus) and four different cell lines: F4N (erythroleukaemia), 416B (early myeloid progenitor), BW5147 (T-lymphoid) and MS1 (endothelial). Finally, we also included in the study the embryonic stem (ES) cell line HM-1 since ES cells express low levels of *Elf-1* mRNA (Supplementary Figure S6). As shown in Figure 1B, all cell types expressed significant levels of *Elf-1* mRNA when compared to the ES cell line HM-1. Particularly, high expression was detected in the whole adult thymus and in the myeloid progenitor cell line 416B.

Locus-wide ChIP-chip analysis identifies five Elf-1 candidate regulatory elements

We had previously shown that the combination of locus-wide comparative genomics and ChIP-chip analysis provides an effective strategy for the identification of bona fide regulatory elements (16,27). We therefore performed ChIP-chip analysis using a 109 kb tiling array that spanned the *Elf-1* locus and flanking genes with chromatin prepared from the four *Elf-1* expressing cell lines (erythroleukaemia F4N, myeloid progenitor 416B, T-lymphoid BW5147 and endothelial MS1) as well as two sources of primary cells (E11.5 fetal liver and whole-adult thymus). Acetylation of histone H3 lysine 9 (H3AcK9) is a histone mark associated with an open chromatin conformation and is generally observed at promoters and enhancers of active genes. The acetylation pattern across the *Elf-1* locus in the highly expressing myeloid progenitor cell line 416B included an enriched region close to the ATG start codon of the gene. This region was also enriched in the endothelial and T-lymphoid cell lines but it was practically absent in the erythroid cell line. Myeloid and T-lymphoid cell lines presented a broad enriched region that co-localized with the −21 and −14 regions identified by comparative genomic analysis, whereas the endothelial and erythroid cell lines only showed H3K9 acetylation around the −21 region. In the vicinity of the 5′-flanking gene *Wbp4*, around 55 kb upstream of the ATG start codon, another enriched region was detected in all cell types analysed. Therefore, with the exception of the −8.5 region all homology peaks identified by comparative genomics displayed histone H3K9 acetylation. Consistent with its predominant erythroid nature, the pattern of acetylation found in fetal liver

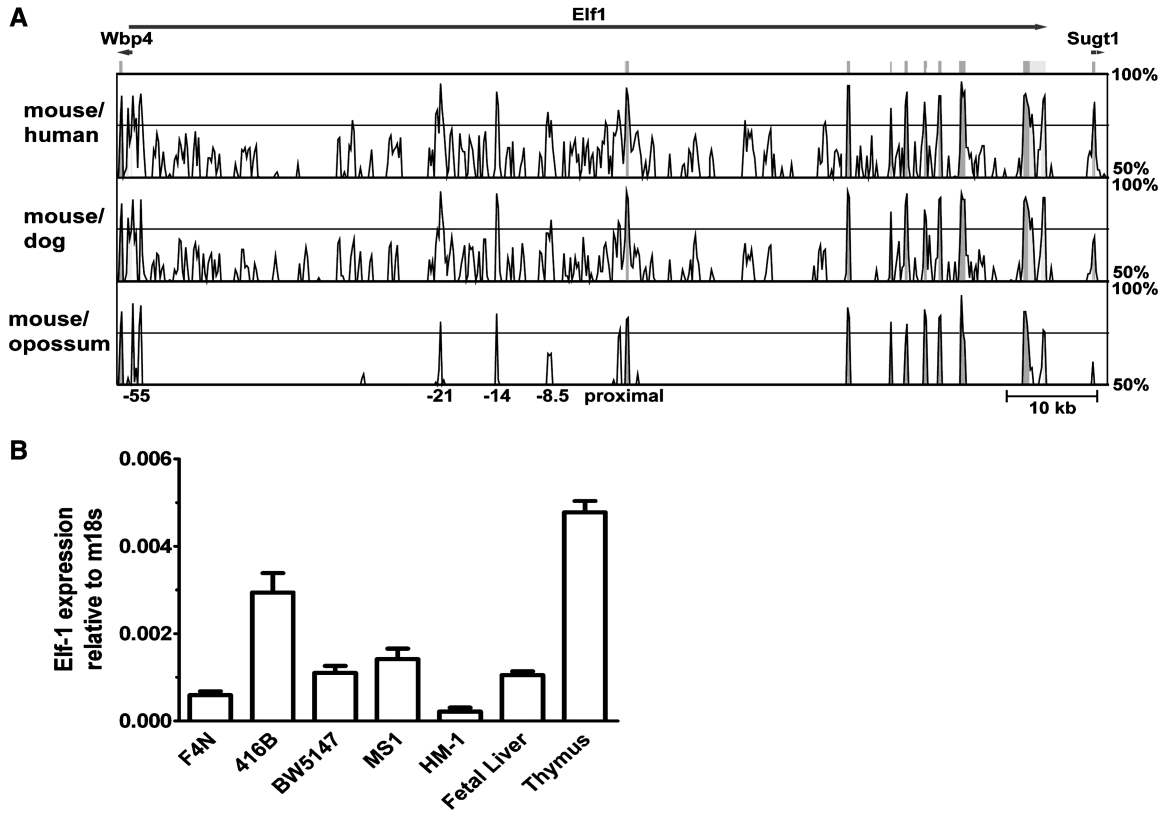

Figure 1. Identification of five non-coding conserved regions by comparative analysis. (**A**) Sequence conservation of the *Elf-1* locus. MVista representation of sequence conservation across the mouse *Elf-1* locus showing mouse/human, mouse/dog and mouse/opossum alignments. The conservation plots show regions with at least 50% of conservation (*y*-axis) across the 109 kb tiling path spanning the mouse *Elf-1* locus. Peaks of sequence conservation in coding regions of the genome are shown in dark grey, those in transcribed but not translated regions (3′UTR and 5′UTR) are shown in pale grey. Neighbouring genes are also depicted. Arrows indicate the direction of transcription. Positions of the conserved regions are named relative to the ATG of *Elf-1*. (**B**) *Elf-1* expression in haematopoietic cell lines. Real-time PCR analysis of mouse *Elf-1* expression in the murine erythroleukaemia cell line F4N, the myeloid progenitor cell line 416B, the T-lymphoid cell line BW5147, the endothelial cell line MS1, the ES cell line HM-1 as well as in two sources of primary cells (fetal liver and whole-adult thymus). Values are expressed relative to m*18s* expression. Error bars indicate SD.

was most similar to erythroid F4N cells and, similarly, the pattern for the thymus resembled that of the T-lymphoid BW5147 cells. Interestingly, a region 49 kb upstream of the *Elf-1* ATG (−49 region) showed substantial levels of acetylation in low-expressing *Elf-1* cells, while it was largely absent in highly expressing cells. This region is conserved in human and dog but it is not conserved in opossum. Taken together therefore, comparative genomics combined with histone acetylation ChIP-chip analysis identified five candidate regulatory regions, namely the −55, −49, −21 −14 and proximal regions (Figure 2).

Four of the five Elf-1 histone acetylation peaks represent candidate alternative promoters

Interrogation of EST databases as well as the database of transcriptional start sites (DBTSS) suggested that the acetylation peaks identified above might represent alternative promoters of the *Elf-1* gene (Supplementary Figure S7). We therefore also assessed trimethylation of histone H3 lysine 4 (H3Me3K4) by ChIP-chip analysis (Figure 3). This chromatin modification is specifically associated with active promoter regions and the results correlated very closely to those previously obtained using antibodies against acetylation. When the high *Elf-1* expressing myeloid cell line 416B was analysed, a high enrichment could be detected for the regions corresponding to the −55, −21 and proximal elements but not for the −14 element, suggesting that this element could act as an enhancer within this locus. As in the acetylation analysis, the trimethylation patterns of fetal liver and erythroid F4N cells were similar, showing robust enrichments at the −55 and −49 regions. T-lymphoid cells and adult thymus showed similar patterns, with peaks at the −55 and −21 regions and the endothelial MS1 cell line only showed major enrichment at the −55 region.

In summary, the ChIP-chip survey allowed us to identify five candidate regulatory elements: −55, −49, −21, −14 and proximal regions. Four of these regions (−55, −49, −21 and proximal) displayed both acetylation and trimethylation marks suggesting that they represent alternative *Elf-1* promoters. Transcripts that could be promoted by three of these regions (−55, −21 and proximal) have been previously reported in mouse or rat (Supplementary Figure S7). Although the region −49 also displayed both acetylation and trimethylation marks, no

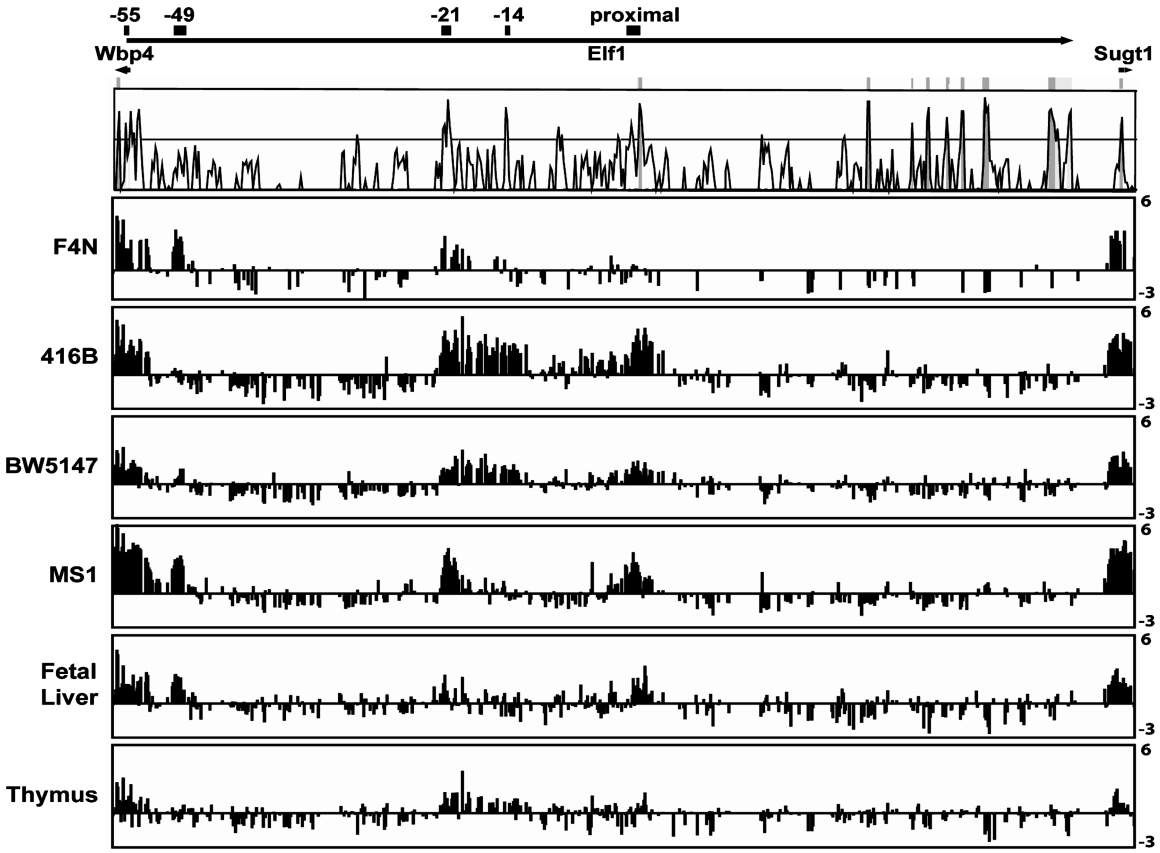

Figure 2. Locus-wide ChIP-chip of acetylation of histone H3 lysine 9 identifies five potential regulatory elements. MVista representation of mouse/human sequence conservation is shown on the top together with all candidate regulatory elements. Annotations as in Figure 1A. ChIP-chip for the murine erythroleukaemia cell line F4N, the myeloid progenitor cell line 416B, the T-lymphoid cell line BW5147, the endothelial cell line MS1, E11.5 fetal murine liver and adult murine thymus. Enrichment values are calculated as fold enrichment over the mean intensity across the whole locus and expressed as log base 2.

transcripts originating from this element have been reported so far. DNAse I hypersensitivity analysis by ChIP-chip performed in the primary tissues fetal liver and thymus showed that this region was highly hypersensitive (Supplementary Figure S8). Taken together, these results are consistent with the hypothesis that this region could represent an as yet unidentified promoter involved in *Elf-1* regulation. Finally, one region (−14) only carried acetylation marks, suggesting that it may function as an enhancer. Four of these regions overlapped with regions identified in the comparative genomic analysis, further validating these results.

The Elf-1 locus contains four distinct regions with promoter activity

To validate the results obtained by integration of the comparative genomic sequence analysis and ChIP-chip approaches, the four candidate promoter regions were cloned into the pGL2 basic luciferase vector and stably transfected into the erythroleukaemia cell line F4N, myeloid progenitor cell line 416B, the T-lymphoid cell line BW5147 and endothelial cell line MS1. In three of the four cell lines, the proximal element showed the highest promoter activity. However, different profiles were observed according to the proportional activity of the different regions (Figure 4A). In the high-expressing myeloid cell line 416B and in T-cell line BW5147, proximal and −21 elements presented higher activity than the other two elements. Proximal and −55 elements showed strong activity in the F4N cell line defining a different profile. Finally, in the endothelial cell line MS1, proximal, −21 and −55 regions had similar activity.

The activity of the −49 element was low in all four cell lines analysed, although this region was acetylated and methylated in our ChIP-chip assays. Genome-wide ChIP-Seq binding profiles for GATA-1 have been reported in erythroid cells (34). We extracted the data corresponding to the *Elf-1* locus that demonstrated specific binding of GATA-1 to the −49 region (Supplementary Figure S9). We therefore tested whether this element might in fact be an enhancer and generated reporter constructs with the −49 element cloned up- and downstream of the luciferase gene under the control of a minimal region of the SV40 promoter (pGL2 promoter). No significant enhancer activity could be detected in any of the cell lines when this element was present (data not shown). However, a reproducible decrease in the activity can be detected in F4N when this element was located downstream of the reporter gene. Then, antisense promoter activity of this element was also tested to explore the

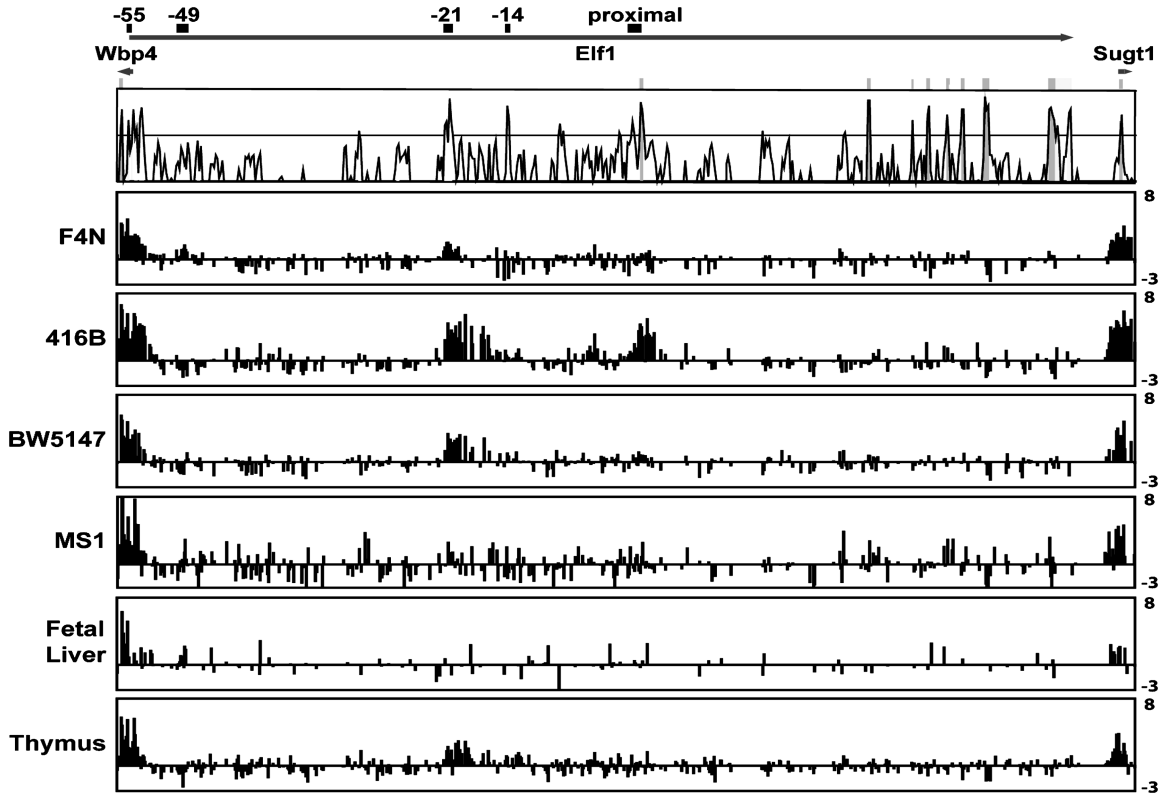

Figure 3. Locus-wide ChIP-chip of trimethylation of histone H3 lysine 4 identifies four potential promoter elements. MVista representation of mouse/human sequence conservation is shown on the top together with all candidate regulatory elements. Annotations as in Figure 1A. ChIP-chip for the murine erythroleukaemia cell line F4N, the myeloid progenitor cell line 416B, the T-lymphoid cell line BW5147, the endothelial cell line MS1, whole E11.5 fetal murine liver and whole-adult murine thymus. Enrichment values are calculated as fold enrichment over the mean intensity across the whole locus and expressed as log base 2.

possibility of transcriptional interference. However, no activity was detected in any of the four cell lines when stably transfected with this construct in an antisense orientation (data not shown).

The Elf-1 −14 region functions as a lineage-specific enhancer

The −14 region is highly conserved between species and it is strongly acetylated in several cell types, which suggests an important function for this region in the regulation of the locus. As it only carried acetylation marks, we hypothesized it may function as an enhancer. Thus, we tested the enhancer activity of this element when cloned downstream of the luciferase gene under the control of the SV40 minimal promoter (pGL2 promoter). Stable transfection assays using 416B myeloid progenitor and BW5147 T-lymphoid cell lines demonstrated that the −14 element functioned as a very strong enhancer in the myeloid progenitor cell line 416B and showed moderate activity in the T-lymphoid cell line BW5147 (Figure 4B). In contrast, the −14 element showed no activity in the F4N erythroleukaemia and MS1 endothelial cell lines. These results were entirely consistent with the ChIP-chip analysis which had shown cell-type-specific acetylation and therefore demonstrate that the −14 region contains a lineage-specific transcriptional enhancer element.

PU.1 binds to −21 and −14 elements *in vivo* and controls the activity of regulatory elements of Elf-1

We also explored which transcription factors could be responsible for the activation of the newly identified regulatory elements of *Elf-1* and performed ChIP-chip using antibodies directed against known key haematopoietic transcription factors such as PU.1, SCL and GATA2. PU.1 is a transcription factor known to be important for haematopoiesis that has frequently been reported to co-operate with *Elf-1* in gene regulation (6,35–37). In addition, interrogation of the Supplementary Data for a recent large-scale system biology analysis of myeloid differentiation (38) demonstrated that knock-down of PU.1 expression in the myeloid cell line THP-1 leads to a reduction of *Elf-1* gene expression (Supplementary Figure S10A). In order to analyse whether PU.1 is directly involved in the transcriptional regulation of *Elf-1*, we performed ChIP-chip assays in the *Elf-1* high-expressing cell line 416B using an antibody against PU.1 (Figure 5A). Binding of PU.1 can be detected at both the −21 and −14 elements. ChIP-chip analysis with antibodies against GATA-2 and SCL were also performed but no binding was detected across the *Elf-1* locus even though clear binding peaks were observed in regions from the *Scl* locus included on the same array as positive controls (data not shown).

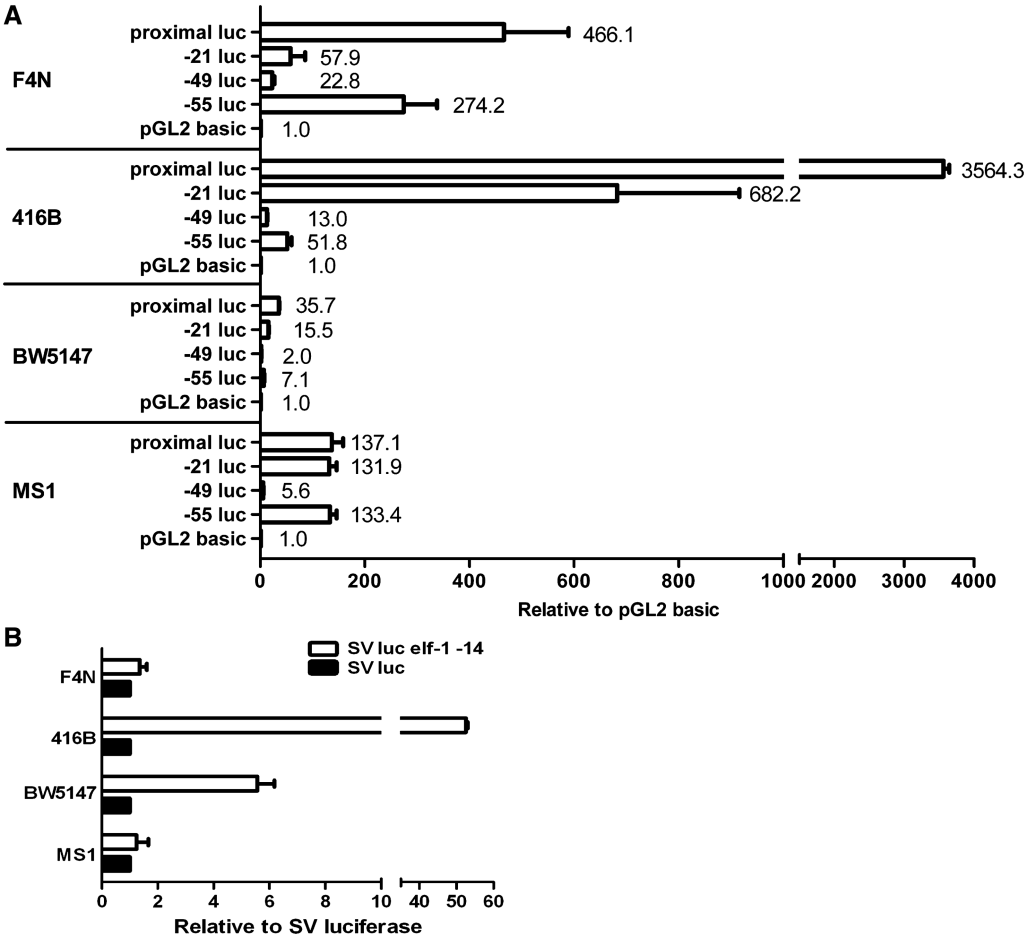

Figure 4. Functional validation of candidate regulatory elements. Promoter (**A**) and enhancer (**B**) activity of candidate regulatory regions was assayed. Stable transfection of the murine erythroleukaemia cell line F4N, the myeloid progenitor cell line 416B, the T-lymphoid cell line BW5147, the endothelial cell line MS1 with luciferase reporter constructs containing *Elf-1* candidate regulatory elements. Mean and SEM for two (A) or three (B) independent transfections (each one performed in triplicate) are shown. (A) −55, −49, −21 and proximal elements of *Elf-1* were inserted upstream of a luciferase reporter gene. Values are expressed relative to empty vector, pGL2 basic. (B) A 550-bp length of DNA corresponding to the *Elf-1* −14 element was inserted downstream of a luciferase reporter gene under the transcriptional control of the minimal SV promoter. Values are expressed relative to empty vector, pGL2 promoter.

To validate whether binding of PU.1 signified direct activation of *Elf-1* by PU.1, transactivation assays were performed in human embryonic kidney 293T cells where a PU.1 expressing plasmid was co-transfected with plasmids carrying the four promoter elements and the enhancer region. No transactivation could be detected on the proximal, −49 or −55 elements but a significant increase in luciferase activity was detected when the −21 element and *PU.1* were co-transfected (Figure 5B). We failed to detect transactivation on the −14 element (data not shown), likely because additional factors and/or integration into chromatin may be required. The transactivation assays were therefore not only consistent with the ChIP-chip data by showing specific activation by PU.1 at the only promoter identified by ChIP-chip to be bound by PU.1, but also importantly validated PU.1 as an upstream activator of *Elf-1* expression. As an additional validation of the regulation of *Elf-1* by PU.1, we performed transient co-transfections of the −21 element with a PU.1 knock-down or control construct in the myeloid progenitor cell line 416B. These experiments revealed a specific reduction of the activity of the *Elf-1* −21 promoter when PU.1 levels are reduced thus corroborating further the role of PU.1 as an upstream regulator of *Elf-1* expression (Supplementary Figure S10B).

Elf-1 downregulation is important for erythroid differentiation

The enrichment levels obtained when using H3Me3K4 antibodies for the erythroleukaemia cell line F4N and fetal liver were modest, especially when compared to myeloid progenitor cells. Since downregulation of other Ets factors is important for erythroid development (39,40), we therefore investigated whether this may also be true for *Elf-1*. Erythroid differentiation can be monitored by labelling fetal liver cells for erythroid-specific TER119 and non-erythroid-specific transferrin receptor (CD71; 30). To investigate *Elf-1* expression during murine fetal liver erythropoiesis, E14.5 fetal liver cells

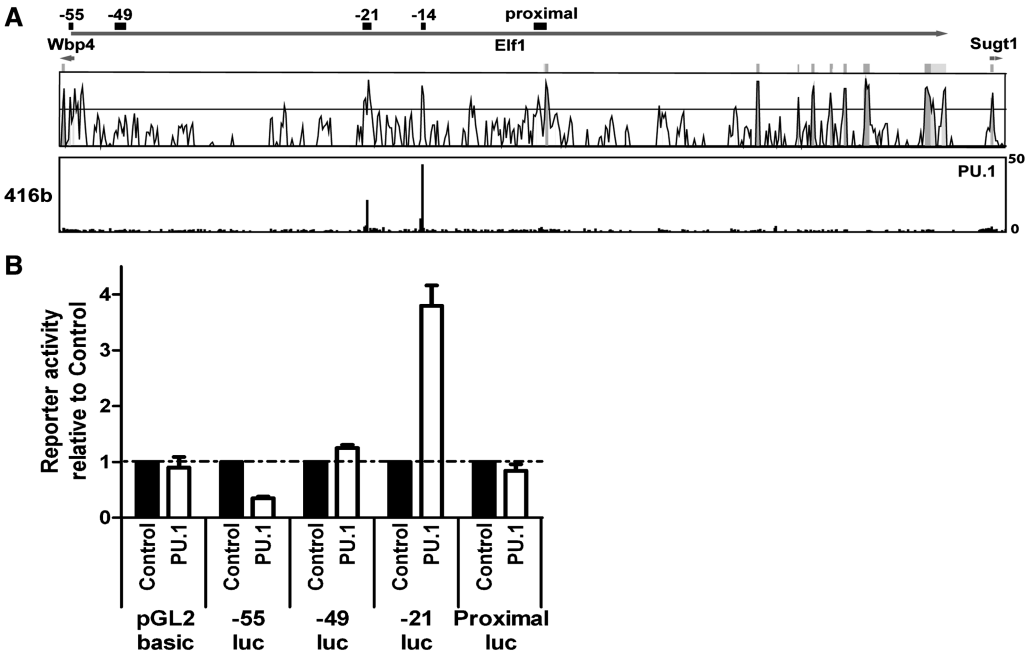

Figure 5. PU.1 regulates expression of *Elf-1*. (**A**) PU.1 ChIP-chip assays. MVista representation of mouse/human sequence conservation is shown on the top together with all candidate regulatory elements. Annotations are the same as in Figure 1A. ChIP-chip for the myeloid progenitor cell line 416B across the 109 kb tiling path spanning the mouse *Elf-1* locus. Enrichment values are expressed as fold above mean intensity across the locus. (**B**) Transactivation of candidate regulatory elements by PU.1. Transient co-transfections of 293T cells with luciferase reporter constructs containing *Elf-1* candidate regulatory elements in combination with either pcDNA3mPU.1 (PU.1) or the empty vector pcDNA3 (control). Values are expressed relative to the control, pcDNA3. Mean and SEM for two independent transfections (each one performed in triplicate) are shown.

were flow sorted into four populations representing sequential phases of erythroid differentiation (31) and the levels of *PU.1* and *Elf-1* transcripts in each population determined. Expression levels of m*PU.1* and m*Elf-1* were determined by real-time qPCR in $CD71^{med}TER119^{low}$, $CD71^{high}TER119^{med/low}$, $CD71^{high}TER119^{high}$ and $CD71^{med/low}$ $TER119^{high}$ cells, corresponding to regions RI–RIV, respectively (Figure 6A and Supplementary Figure S11A). The $CD71^{low}TER119^{high}$ population are largely enucleated cells, and were therefore excluded from this analysis. Interestingly, a very similar pattern of downregulation was seen for PU.1 and *Elf-1*, which was consistent with our previous results identifying PU.1 as an upstream regulator of *Elf-1* and also suggesting that downregulation of *Elf-1* may be necessary for erythroid differentiation.

To further explore the biological significance of our results, we took advantage of an extensively validated *in vitro* culture system that supports terminal erythroblast proliferation and differentiation in a manner that closely mimics the *in vivo* terminal proliferation and maturation of erythroid cells (30). In this system, differentiation of CFU-E progenitors can be followed step by step and in a quantitative manner using flow cytometry. Essentially, lineage negative E14.5 fetal liver cells are purified and cultured *in vitro*. Differentiation is then analysed at various time points by double labelling for TER119 and CD71, which makes it possible to distinguish at least five distinct populations of cells: $CD71^{med}TER119^{low}$ that contains primitive progenitor cells and proerythroblasts; $CD71^{high}TER119^{low}$, proerythroblasts and early basophilic erythroblasts; $CD71^{high}TER119^{high}$, early and late basophilic erythroblasts; $CD71^{med}TER119^{high}$ chromatophilic and orthocromatophilic erythroblasts; $CD71^{low}TER119^{high}$ late orthocromatophilic erythroblasts and reticulocytes (Figure 6B and Supplementary Figure S11B, regions R1–R5, respectively). $TER119^{high}$ surface expressing cells constitute committed erythroid cells. During the course of erythroid differentiation, a decrease of cell size is also observed.

We therefore overexpressed m*Elf-1* cDNA in purified $TER119^{low}$ cells with the use of a retroviral vector (MSCV-*Elf-1*-PGKprom-puro-IRES-GFP) that also encodes GFP. Thus, transduced cells were labelled with GFP and overexpressed m*Elf-1* (Supplementary Figure S11C); uninfected GFP^- cells served as internal controls. We then analysed erythroid-differentiation profiles in the *in vitro* culture system. Figure 6B–D show the results of a representative experiment. Overexpression of *Elf-1* did not cause any significant changes in FACS profiles after 24 h of differentiation. After 48 h of differentiation, however, overexpression of *Elf-1* impaired terminal erythroid differentiation, as evidenced by the statistically significant (Student's *t*-test, $P \leq 0.05$) accumulation of more immature cells in R3 at the expense of more mature cells in R4 when compared to the empty vector control (Figure 6B and D). Moreover, a significantly larger proportion of *Elf-1* transduced cells displayed a larger cell size consistent with the presence of more immature cells (Figure 6C). These results indicate that downregulation of *Elf-1* is important for terminal differentiation of erythrocytes.

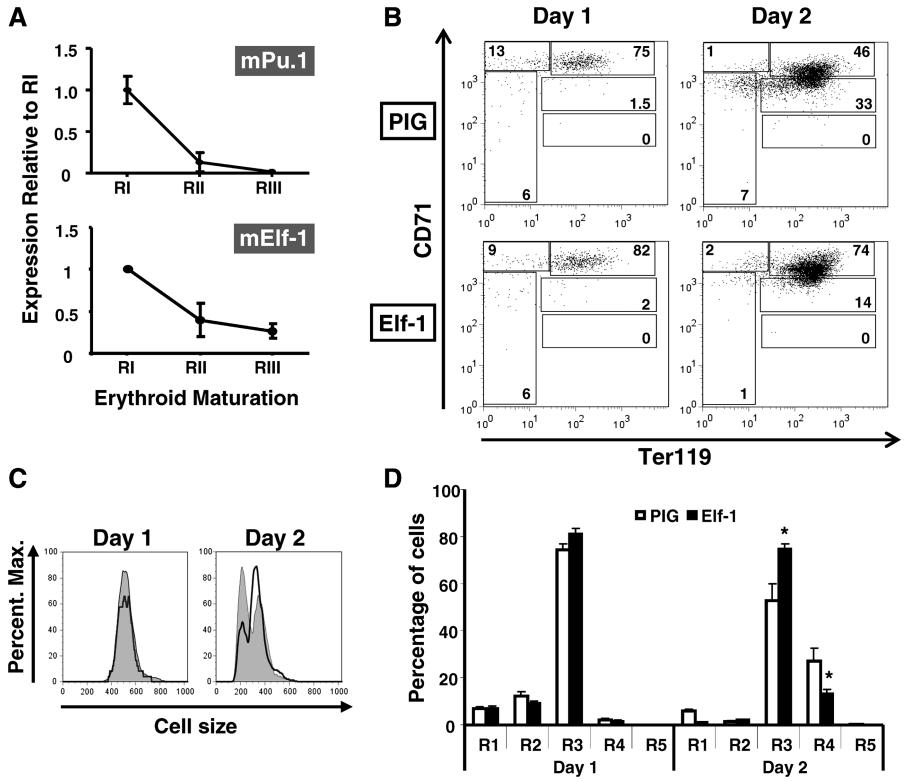

Figure 6. Overexpression of *Elf-1* impairs terminal erythroid differentiation. (**A**) Expression of PU.1 and *Elf-1* during *in vivo* fetal liver terminal erythroid differentiation. Real-time PCR analysis of mouse PU.1 and *Elf-1* expression in three populations of murine E14.5 fetal liver cells: (RI) $CD71^{med}TER119^{low}$, (RII) $CD71^{high}TER119^{med/low}$ and (RIII) $CD71^{high}TER119^{high}$; sorted by flow cytometry and representing sequential stages of erythroid differentiation (Supplementary Figure S11A). m*18s* ribosomal RNA gene expression was used to normalize expression of each population. Values are expressed relative to the expression at RI stage. Error bars indicate SD. (**B**) Representative FACS plots for Ter119 and CD71 expression during *in vitro* differentiation in lineage negative E14.5 fetal liver cells overexpressing *Elf-1* or a control (PIG) vector after 24 h (Day 1) or 48 h (Day 2). Five populations corresponding to stages of erythroid differentiation from progenitors to enucleated cells are gated: (R1) $CD71^{med}TER1119^{low}$, (R2) $CD71^{high}TER119^{low}$, (R3) $CD71^{high}TER1119^{high}$, (R4) $CD71^{med}TER1119^{high}$ and (R5) $CD71^{low}TER1119^{high}$ (Supplementary Figure S11B). Percentage of GFP^{+} cells in each of these stages are indicated. (**C**) Representative FACS plots for cellular size in lineage negative E14.5 fetal liver cells overexpressing *Elf-1* (black line) or a control (PIG) (grey shaded area) vector after 24 h (Day 1) or 48 h (Day 2) of *in vitro* culture. (**D**) Bar charts indicating the proportion of cells at each of the five stages of erythroid differentiation (R1–R5) after 24 h (Day 1) and 48 h (Day 2) of *in vitro* culture of lineage negative E14.5 fetal liver cells retrovirally transfected with an overexpressing *Elf-1* or control (PIG) vector. Representative plot (mean and SD) for one of five independent experiments, each one performed in triplicate. $P \leq 0.05$ (Student's *t*-test) are indicated with an asterisk.

DISCUSSION

Reconstruction of regulatory networks is essential to understand the complex and dynamic interactions between the multiple regulators that control the development and subsequent differentiation of blood stem cells. Definition of transcriptional regulatory networks requires both knowledge of the transcription factors involved and also the DNA sequences to which they bind. Therefore, identification and subsequent functional analysis of gene regulatory elements remains an essential area of research, in particular when it serves to clarify upstream hierarchies of key regulators thus providing firm anchor points for major regulatory network nodes. In this article, we have analysed the transcriptional regulation of *Elf-1*, which itself encodes a major transcriptional regulator of blood and endothelial cells.

In this study, an integrated analysis of the *Elf-1* locus using comparative genomic analysis, ChIP-chip and functional studies allowed us to define five distinct regulatory regions spread over 50 kb, including the identification of a lineage-specific enhancer element. The comparative genomic analysis showed the presence of five regions conserved between mouse, human, dog and opossum. Of these conserved regions, four contained high levels of acetylation at H3K9, a chromatin mark associated with areas of open chromatin. One additional region, conserved between mouse, human and dog but not opossum, was also enriched for this chromatin mark. Further ChIP-chip assays were performed to analyse trimethylation of lysine 4 on the tail of histone H3, a modification that is associated with recently transcribed regions. These experiments led to the discovery that four of the five regions identified by elevated histone acetylation display promoter activity in functional assays. The fifth region, which carried active chromatin marks but no transcription marks, was validated as an enhancer in functional analysis. We also demonstrate that PU.1 is a major upstream regulator of *Elf-1* since PU.1 binding could be detected by ChIP-chip in one of the four promoter regions. We validated this result with functional analysis

where we showed that only the promoter bound by PU.1 could be transactivated in its presence.

The expression profile of *Elf-1* has been widely analysed during embryonic development. In chicken embryos, *Elf-1* is expressed along the lining of the larger caliber and smaller caliber branching blood vessels, in the developing heart at Day 10 (3), in the developing dorsal aorta of the mouse embryos (17), in embryonic lymphoid organs (thymus and spleen) and a wide variety of epithelial cells and fetal liver (3). *Elf-1* is specifically expressed in multiple haematopoietic cell types (Supplementary Figure S6) and we now show for the first time that *Elf-1* downregulation is important for erythroid differentiation. Scl and Lmo2 are known targets of *Elf-1*, and together with GATA-1, LDB1 and E2A constitute the major transcriptional activator complex operating in erythroid cells (41). In light of our findings that *Elf-1* downregulation is critical for erythroid differentiation, *Elf-1* activation of Lmo2 and Scl therefore seems at first glance contradictory. However, *Elf-1* specifically activates only those regulatory elements within Scl and Lmo2 that are responsible for directing the expression of these genes in endothelial and blood stem/progenitor cells. In contrast, the erythroid specific regulatory elements of Scl and Lmo2 require autoregulation through Scl/Lmo2 containing complexes (6,7,28). Our results therefore highlight the need to understand the dynamical nature of regulatory hierarchies and the need to perform functional assays in multiple lineages. Identification of lineage-specific upstream regulators therefore will be essential to reconstruct differentiation state-specific regulatory networks as well as state transitions during differentiation.

The control of myeloid versus erythroid differentiation of multipotent progenitors by PU.1 and GATA-1 has been the subject of extensive investigations (40,42–44). Current models suggest that PU.1 and GATA-1 are positive drivers of myeloid and erythroid differentiation, respectively, and direct cross-antagonistic interactions between the two determine myeloid versus erythroid cell fate choice. Our observations that (i) PU.1 is a direct upstream activator of *Elf-1* in progenitor cells and (ii) *Elf-1* itself inhibits terminal erythroid differentiation suggest that the model of PU.1/GATA-1 cross-antagonism as a simple molecular switch may need to be revised. Other known myeloid targets of PU.1, such as *c-fms*, promote myeloid differentiation but we now show that *Elf-1* itself actually inhibits erythroid differentiation. *Elf-1* has been shown to activate the PU.1 −14 enhancer element (45) and is also known to be able to repress GATA-1 target genes (46,47). It is therefore possible that *Elf-1* exerts its effect largely through enhancing the antagonistic interaction of PU.1 with GATA-1. To this end, PU.1 and *Elf-1* could also collaborate to directly repress GATA-1. *Elf-1* is known to co-operate and interact with other members of the GATA family such as GATA-2 (48) and GATA-3 (49) to promote transcription but this does not exclude the possibility that *Elf-1* interacts with GATA-1 to inhibit its action or repress its expression in a similar fashion to PU.1. Moreover, pRb, a binding partner of *Elf-1* (21), is itself intrinsically required for terminal erythroid differentiation (50,51) suggesting that *Elf-1* may have a wider role and thus link previously unsuspected regulators with the phenotype of PU.1 mediated suppression of erythroid differentiation.

This study reports the first description of the transcriptional regulation of the Ets factor *Elf-1* and therefore represents a critical advance in integrating this important regulator into the wider haematopoietic regulatory networks. Moreover, identification of reciprocal activation between *Elf-1* and PU.1 together with identification of a functional role for *Elf-1* as a negative regulator of terminal erythroid differentiation have provided important new insights into the transcriptional control of myelo-erythroid differentiation. Therefore, taken together, these results have expanded our understanding of haematopoietic regulatory networks and provided new insights into the molecular mechanisms that control erythropoiesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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