

# Lineage-specific activators affect $\beta$ -globin locus chromatin in multipotent hematopoietic progenitors

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**During development, the regulated expression of tissue-specific genes can be preceded by their potentiation, that is, by chromatin activation in progenitor cells. For example, the human  $\beta$ -like globin genes are potentiated in a gene- and developmental-specific manner in hematopoietic progenitors. Developmental regulation of human  $\beta$ -gene expression in erythroid cells is mostly determined by transcriptional activators; however, it is not clear how gene-specific potentiation is set in hematopoietic progenitors. Using human and transgenic multipotent hematopoietic progenitors, we demonstrate that human  $\beta$ -globin locus activation is characterized by TBP, NF-E2, CBP and BRG1 recruitment at both the Locus Control Region and human  $\beta$ -gene promoter. Our results further indicate that in hematopoietic progenitors, EKLF influences chromatin organization at the human  $\beta$ -globin locus and is instrumental for human  $\beta$ -gene potentiation. Thus, we show that lineage-specific transcriptional activators expressed at basal levels in progenitor cells can participate in gene potentiation.**

*The EMBO Journal* (2006) 25, 3586–3595. doi:10.1038/sj.emboj.7601232; Published online 13 July 2006

**Subject Categories:** chromatin & transcription; development

**Keywords:** chromatin; epigenetics; globins; hematopoietic progenitors; transcriptional activators

## Introduction

The expression of lineage-specific genes can be potentiated before transcriptional activation (Bonifer, 1999). Gene potentiation is characterized by an active chromatin organization in multipotent progenitor cells (Jimenez *et al*, 1992; Bottardi *et al*, 2003) and can be accompanied by partial occupancy of gene regulatory regions by transcriptional activators. Gene

expression in differentiated cells requires synergy among activators, co-activators, general transcription factors, chromatin remodeling and histone modifying complexes (Struhl, 2005). In hematopoietic stem cells (Ye *et al*, 2003) and in multipotent hematopoietic progenitor cells (HPC) (Hu *et al*, 1997), the ‘promiscuous’ expression of lineage-specific factors such as PU.1, C/EBP $\alpha$ , PAX5, SCL, GATA-1, GATA-2 and GATA-3 does not alter the biological potential of these cells, but ostensibly provides a proper environment for stochastic lineage commitment mainly by repressing or activating groups of genes (Cantor and Orkin, 2002; Graf, 2002). However, it is not known if promiscuously expressed transcriptional activators influence gene potentiation in multipotent progenitor cells.

In mammals, the human  $\beta$ -(hu $\beta$ -) globin locus is a well-characterized multigenic locus, and therefore a good model to identify mechanisms that control epigenetic regulation of gene expression during hematopoiesis. The hu $\beta$ -globin locus consists of five developmentally regulated genes ( $\epsilon$ - $\gamma$ - $\gamma$ - $\delta$ - $\beta$ ), whose high-level expression in erythroid cells (EryC) depends upon the Locus Control Region ( $\beta$ LCR) comprised of four erythroid-specific DNaseI hypersensitive sites (HS). The  $\beta$ LCR enhances gene transcription through direct interaction with globin promoters (Carter *et al*, 2002; Tolhuis *et al*, 2002; Palstra *et al*, 2003). Even though DNaseI sensitivity at the endogenous murine locus is not lost when the  $\beta$ LCR is deleted (Bender *et al*, 2000), in EryC the human  $\beta$ LCR is a major determinant of chromatin organization at the hu $\beta$ -globin locus (Grosveld *et al*, 1987). In progenitor cells, the  $\beta$ -globin locus is in an active chromatin organization (Jimenez *et al*, 1992) and potentiation of hu $\beta$ -like genes, most likely mediated by as yet unidentified transcriptional activators, is developmental-specific (Bottardi *et al*, 2003).

EKLF, GATA-1 and the p45 subunit of NF-E2 (p45) are among the best-characterized transcriptional activators involved in hu $\beta$ -gene regulation in EryC. EKLF is essential for adult  $\beta$ -globin gene transcription and EKLF knockout (KO) mice suffer from severe anemia and die at approximately 14.5 d.p.c. (days *postcoitus*) (Nuez *et al*, 1995; Perkins *et al*, 1995). EKLF binds to  $\beta$ LCR and globin promoters (Bieker, 2001) and it is required for  $\beta$ LCR- $\beta$  major ( $\beta$ maj) gene direct interaction (Drissen *et al*, 2004). EKLF recruits the erythroid-specific SWI/SNF chromatin remodeling complex 1 (E-RC1) to the  $\beta$ -globin locus (Armstrong *et al*, 1998) and the absence of EKLF leads to reduced DNaseI HS formation at the  $\beta$ maj and hu $\beta$ -promoters (Wijgerde *et al*, 1996). GATA-1 binds to  $\beta$ LCR HSs and globin promoters and is critical for primitive and definitive erythroid cell differentiation (Pevny *et al*, 1991; Fujiwara *et al*, 1996). It interacts with CBP, SWI/SNF, ACF/WCRF and NuRD complexes (Blobel *et al*, 1998; Kadam *et al*, 2000; Hong *et al*, 2005; Rodriguez *et al*, 2005). GATA-1 is also required for  $\beta$ LCR- $\beta$ maj direct interaction (Vakoc *et al*, 2005). NF-E2 is comprised of a ubiquitously expressed subunit,

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Received: 19 January 2006; accepted: 20 June 2006; published online: 13 July 2006

MafK, and the hematopoietic-specific activator p45 (Andrews *et al*, 1993), and it is recruited to mouse and human  $\beta$ LCR and promoter regions (Daftari *et al*, 1999; Forsberg *et al*, 2000; Sawado *et al*, 2001; Leach *et al*, 2003). NF-E2 is necessary for globin gene expression in differentiated MEL (mouse erythroleukemia) cells but p45 KO mice exhibit only mild effects on erythropoiesis and no significant influence on globin gene expression (Shivdasani and Orkin, 1995).

Interestingly EKLf, GATA-1 and p45 are also promiscuously expressed in hematopoietic progenitors (Hu *et al*, 1997). p45 could be involved in priming/potentialization of the mouse  $\alpha$ -globin locus (Anguita *et al*, 2004), whereas PU.1-GATA-1 interaction influences lineage commitment (Cantor and Orkin, 2002; Graf, 2002). However, it has never been investigated whether these factors play roles in developmental-specific potentialization of the hu $\beta$ -gene and globin locus activation in multipotent HPC.

To understand the molecular events leading to hu $\beta$ -like globin gene potentialization, we studied the influence of promiscuously expressed transcriptional activators on recruitment of general transcription factors as well as chromatin remodeling and histone modifying activities at  $\beta$ LCR HS2, hu $\gamma$ - and hu $\beta$ -promoter in primary multipotent HPC. Our results, obtained with human cells and transgenic mice expressing the hu $\beta$ -globin locus, suggest that EKLf is instrumental for globin gene potentialization in HPC, facilitating p45, CBP, BRG1 and TBP recruitment at the hu $\beta$ -promoter. Finally, we provide insight into the cooperative role of EKLf and p45 for promoting appropriate chromatin activation in HPC, as well as for Pol II recruitment and subsequent transcriptional elongation in EryC. Based on these results, we suggest a model whereby EKLf is a key regulator of hu $\beta$ -gene potentialization in HPC.

## Results

### HPC purification and characterization

Mouse HPC were isolated from 13.5 d.p.c. fetal livers of line 2 mice (ln2 HPC), which are transgenic for the entire hu $\beta$ -globin locus and express the hu $\beta$ -globin genes normally (Strouboulis *et al*, 1992). Mouse HPC corresponds to a Ter119<sup>-</sup>, Gr-1<sup>-</sup>, B220<sup>-</sup>, CD31<sup>high</sup> population, which represents 1–2% of the total fetal liver and does not contain mature or late committed cells (see below). The sorted population was always  $\geq 98\%$  pure. The hematopoietic potential of these cells was ascertained by *in vitro* clonal assays in methylcellulose (Table I, ln2 HPC). On average, out of 100 colonies, 85 originated from progenitors with multilineage potential (CFU-GEMM: colony forming unit-granulocyte, erythrocyte, megakaryocyte, macrophage; CFU-GM: colony forming unit-granulocyte, megakaryocyte), whereas 15

showed unilineage commitment (BFU-E: Burst forming unit-erythrocyte). No CFU-E (colony forming unit-erythrocyte) was detected.

To further characterize this population, ln2 HPC cDNA was used to study the expression of marker genes. SCL, GATA-1, p45, GATA-2, EKLf, C/EBP $\alpha$  and globin transcripts are all expressed, whereas transcripts corresponding to GATA-3 are not detected (Figure 1A; ln2 HPC). This expression pattern suggests that the Ter119<sup>-</sup>, Gr-1<sup>-</sup>, B220<sup>-</sup>, CD31<sup>high</sup> cell population is mainly composed of multipotent HPC (Akashi *et al*, 2000), namely common myeloid and megakaryocyte/erythrocyte lineage-restricted progenitors, a finding supported by our *in vitro* clonal assays (see above). As evaluated by quantitative real-time reverse transcriptase (RT)-PCR, the levels of p45, EKLf and hu $\beta$ -globin mRNA are, respectively,  $\sim 7$ -,  $\sim 6$ - and  $\sim 100$ -fold higher in EryC than in HPC (Supplementary Figure 1). Accordingly, Western blot analyses revealed that GATA-1, p45 and EKLf protein levels are approximately, 12-, 6- and 11-fold higher in EryC than in HPC (Figure 1B).

Since an EKLf KO background is extensively used in this study, we investigated whether the absence of EKLf causes a general failure of cellular differentiation during hematopoiesis because EKLf regulates other genes crucial for final EryC differentiation (Drissen *et al*, 2005). HPC purified from 13.5 d.p.c. ln2 EKLf KO fetal livers (ln2 EKLf<sup>-/-</sup> HPC) represent 1–2% of total fetal liver (as observed in ln2 HPC) and, as expected (Nuez *et al*, 1995; Perkins *et al*, 1995), give rise to erythroid colonies in methylcellulose (Table I). Furthermore, the gated populations and marker gene expression analyses (with the exception of globin genes) do not reveal any apparent difference between ln2 HPC and ln2 EKLf<sup>-/-</sup> HPC (Figure 1A). In particular, GATA-1 and p45 expression levels, as evaluated by quantitative real-time RT-PCR and Western blot analysis, do not change significantly among the two populations (Figure 1B–E). Thus, the absence of EKLf does not preclude normal hematopoietic differentiation of HPC.

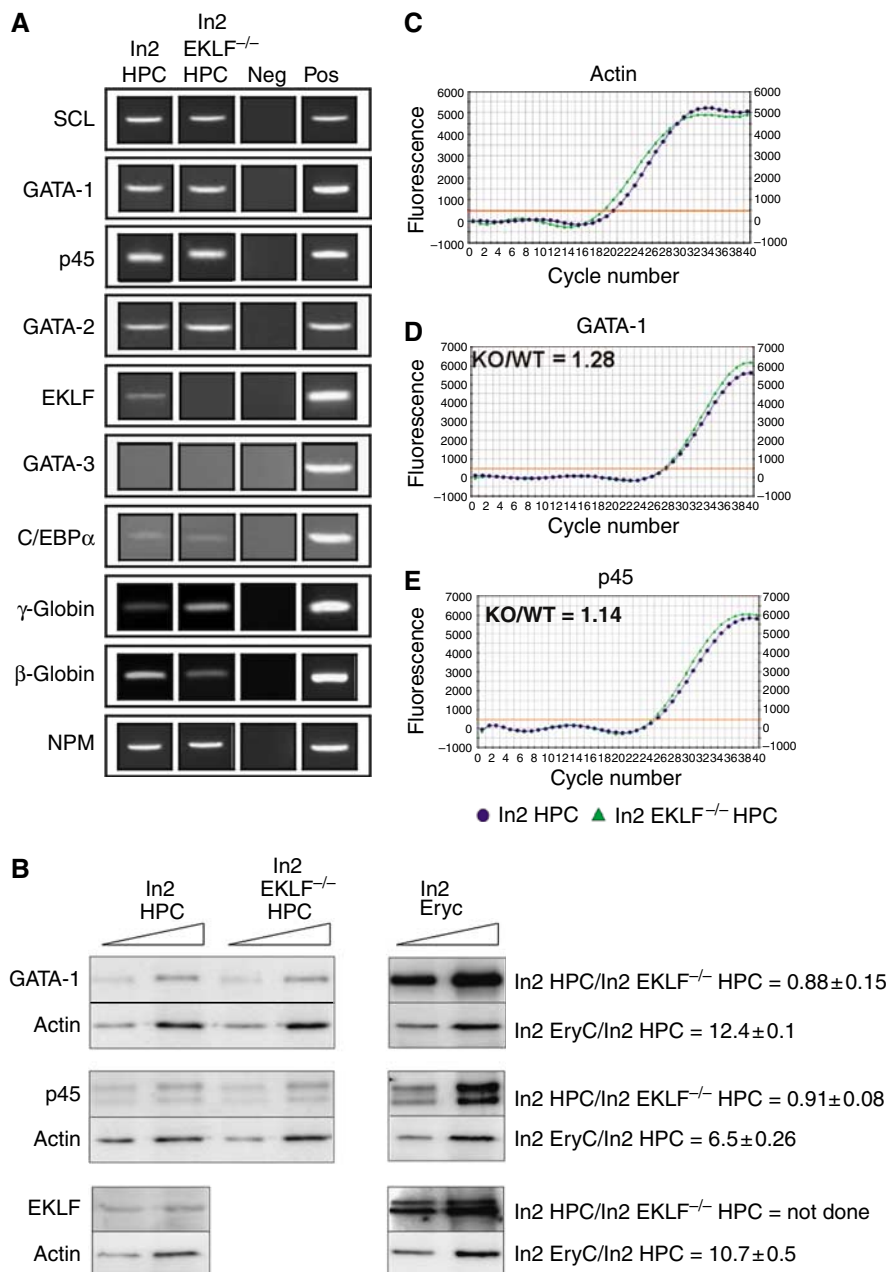
### Transcriptional activators recruitment at the hu $\beta$ -globin locus in HPC

Our previous results suggested that in bone marrow-derived ln2 HPC and human CD34<sup>+</sup> cells, chromatin at the hu $\beta$ -globin LCR is in an active state, and the hu $\beta$ -promoter is potentialized for transcriptional activation (Bottardi *et al*, 2003). We now investigate how histone covalent modification and chromatin remodeling activities are recruited at HS2, hu $\beta$ - and hu $\gamma$ -promoters during hematopoiesis. Chromatin immunoprecipitation (ChIP) assays were performed on 13.5 d.p.c. fetal liver-derived hematopoietic cells. In 13.5 d.p.c. fetal livers, the hu $\beta$ -gene is expressed and hu $\gamma$ -genes are mostly silent as judged by the fact that hu $\gamma$ -transcripts represent  $\sim 10\%$  of total hu $\beta$ -globin level (Strouboulis *et al*, 1992). HS2 was chosen because it is important for high-level hu $\beta$ -gene expression in EryC (Morley *et al*, 1992), and moreover HS2 deletion can abrogate epigenetic regulation of globin genes in EryC (Milot *et al*, 1996) as well as locus activation in HPC (Bottardi *et al*, 2005).

Histone acetylation of the hu $\beta$ -globin locus in fetal liver ln2 HPC (Figure 2B; Supplementary Figure 2A) is similar to the pattern observed in bone marrow HPC (Bottardi *et al*, 2003), suggesting that  $\beta$ LCR and hu $\beta$ -promoter are in an active/potent chromatin organization also in 13.5 d.p.c.

**Table I** Clonal assays in methylcellulose: 150 HPC purified from 13.5 d.p.c. ln2 or ln2 EKLf<sup>-/-</sup> fetal livers were plated onto methylcellulose plates

Clonogenic ability of Ter119 <sup>-</sup> , Gr-1 <sup>-</sup> , B220 <sup>-</sup> , CD31 <sup>high</sup> cells				
	CFU-GEMM (%)	CFU-GM (%)	BFU-E (%)	Colonies/10 <sup>3</sup> cells
ln2 HPC	38.9	44.8	16.3	210 $\pm$ 40
ln2 EKLf <sup>-/-</sup> HPC	34	48.2	17.8	240 $\pm$ 35



**Figure 1** Expression of marker genes in In2 HPC and In2 EKLf<sup>-/-</sup> HPC. (A) Semiquantitative RT-PCR performed on equal amounts of RNA purified from In2 HPC or In2 EKLf<sup>-/-</sup> HPC. PCR samples were resolved onto agarose gel.  $\gamma$ -Globin: fetal human ( $\gamma$ ) and mouse embryonic ( $\beta$ H1) transcripts;  $\beta$ -globin: adult human ( $\beta$  and  $\delta$ ) and mouse ( $\beta$ min and  $\beta$ maj) globin transcripts; NPM: ubiquitously expressed nucleophosmin transcript, used as internal control; Neg: negative control; Pos: positive control; (B) Western blot analysis of In2 HPC, In2 EKLf<sup>-/-</sup> HPC and In2 EryC; 4 and 8  $\mu$ g of whole-cell protein extract were loaded in each lane of a 10% SDS-PAGE. Anti-GATA-1 and -p45 antibodies were purchased from SantaCruz; anti-actin antibodies were purchased from LabVision; anti-EKLf serum is a generous gift of S Philipsen. Protein levels in In2 HPC versus In2 EKLf<sup>-/-</sup> HPC or In2 EryC versus In2 HPC were calculated using actin as internal control and they are shown on the right side of each panel together with their standard error of means; (C-E) representative examples of quantitative real-time RT-PCR; the relative level of GATA-1 or p45 gene expression in In2 EKLf<sup>-/-</sup> HPC versus In2 EKLf HPC were calculated according to Pfaffl (2001), using mouse actin as internal control and expressed as KO/WT ratio; x axis: cycle number; y axis: derivative of SYBR Green fluorescence. Blue dots: In2 HPC; green triangles: In2 EKLf<sup>-/-</sup> HPC.

fetal liver HPC. As shown in Figure 2C, GATA-1 is not significantly crosslinked at HS2, hu $\beta$ - or hu $\gamma$ -promoters while, as reported previously (Anguita *et al*, 2004), HS-12 of the mouse  $\alpha$ -globin locus is slightly enriched (Supplementary Figure 3A). p45 is detected at HS2 and hu $\beta$ -promoter in In2 HPC and in CD34<sup>+</sup> cells (Figure 2D; Supplementary Figure 3B). p45 is also detected at hu $\gamma$ -

promoters in In2 HPC (Figure 2D). Thus, p45 is recruited to the hu $\beta$ -globin locus at early stages of hematopoiesis.

Since EKLf is expressed at basal levels in HPC, we investigated whether it can influence hu $\beta$ -gene potentiation in HPC. Commercially available antibodies against EKLf are not suitable for ChIP analysis (Feng and Kan, 2005). Thus, we used a genetic approach in comparing In2 HPC with In2

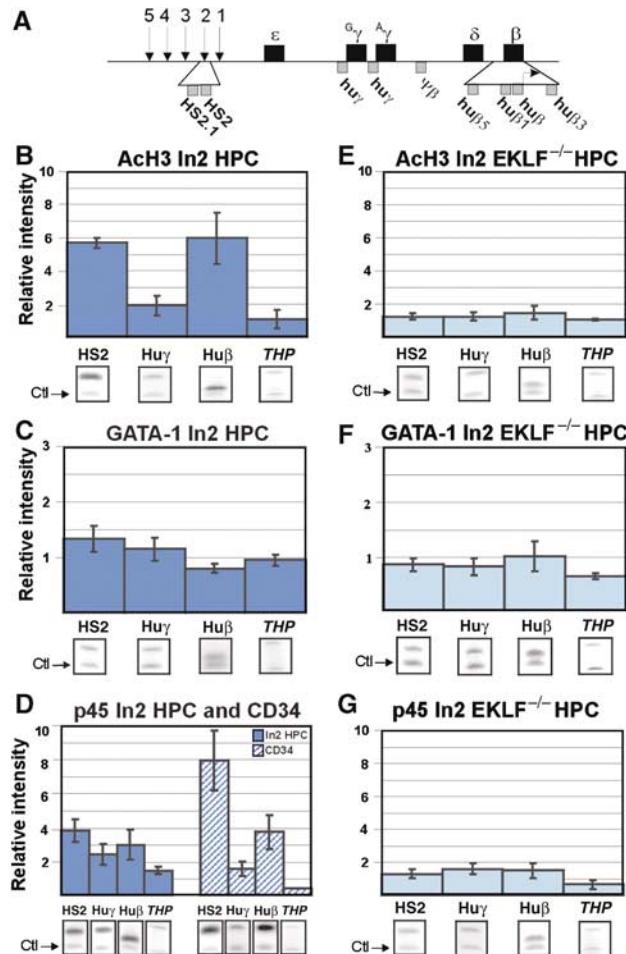
EKLF<sup>-/-</sup> HPC. As shown in Figure 2E, histone H3 acetylation at the hu $\beta$ -globin locus decreases significantly in Ln2 EKLF<sup>-/-</sup> HPC relative to Ln2 HPC, whereas no major differences are detected at HS-29 of the mouse  $\alpha$ -globin locus (Supplementary Figure 3C). (Unless specified, 'significant' refers to a statistically significant difference between wild type and KO cells, based on unpaired student's *t*-test *P*-values <0.05). As in Ln2 HPC, GATA-1 is not detected at HS2 or

globin promoters in Ln2 EKLF<sup>-/-</sup> HPC (Figure 2F). p45 binding to HS2 and hu $\beta$ -promoter is significantly affected in Ln2 EKLF<sup>-/-</sup> HPC (Figure 2G), suggesting that EKLF can influence p45 recruitment to these regions and that p45 as well as EKLF participate in locus organization in HPC.

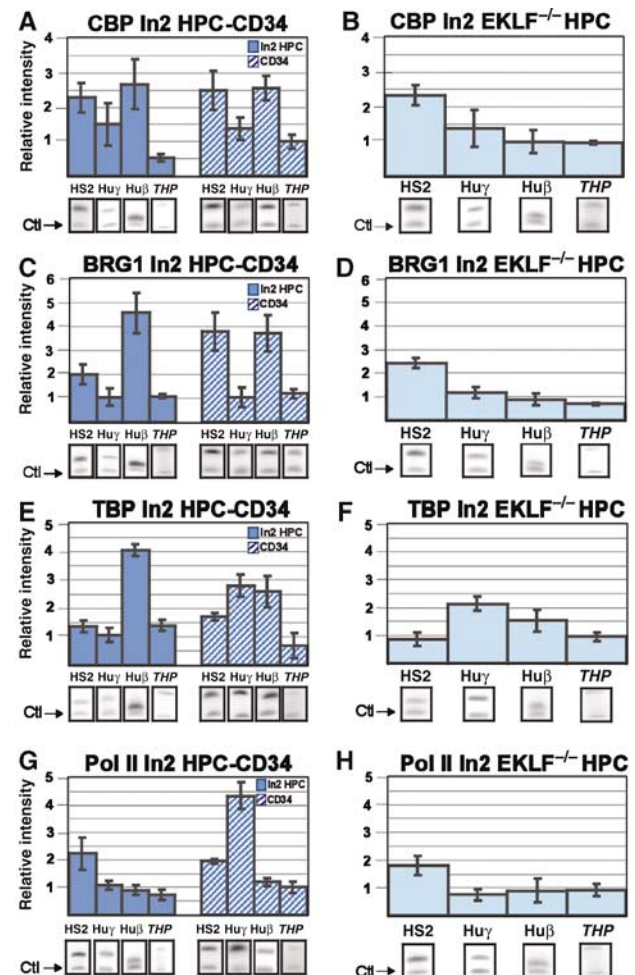
### Recruitment of histone modifying and chromatin remodeling activities at the hu $\beta$ -globin locus in HPC

Based on the above results, we investigated whether the role of p45 and EKLF in globin potentiation includes their capacity to recruit histone modifying and chromatin remodeling activities to the globin locus.

CBP is detected at HS2 and hu $\beta$ -promoter in Ln2 HPC and CD34<sup>+</sup> cells (Figure 3A; Supplementary Figure 3D). However, CBP binding is significantly reduced at hu $\beta$ -promoter in Ln2 EKLF<sup>-/-</sup> HPC (Figure 3B). p45 (Hung *et al*, 2001) and EKLF (Zhang and Bieker, 1998) interact with and are acetylated by CBP, but unlike p45 (Johnson *et al*, 2001), the ability of EKLF to recruit CBP to any region of the globin locus has never been demonstrated. The fact that in Ln2 EKLF<sup>-/-</sup>



**Figure 2** Histone H3 acetylation, GATA-1 and p45 recruitment at the hu $\beta$ -globin locus in HPC. (A) A map of the hu $\beta$ -globin locus; genes are shown as black boxes and the location of  $\beta$ LCR HSs is indicated by arrows. Amplified regions used for ChIP analyses are indicated by grey boxes. (B–G) ChIP assays: immunoprecipitated and unbound (input) chromatin samples were subjected to duplex radioactive PCR with one primer set specific for hu $\beta$ -globin regions and another primer set specific for mouse *zfp37* (ZFP, zinc-finger protein 37) or human *pax6* (pax6, paired box protein 6) regulatory regions ('Ctl', indicated by arrows), two genes that are not expressed in hematopoietic cells. All PCR reactions were performed in parallel under conditions of linear amplification. Products were quantified by PhosphorImager. The level of enrichment of globin regions relative to the control and input samples is represented by bars, with their corresponding standard deviations. A value of 1 indicates no enrichment. Mouse THP/ZFP or human THP/pax6 controls are included (THP, kidney-specific Tamm-Horsfall gene) to confirm that no enrichment is detected at regulatory regions of non-hematopoietic genes. To obviate for weak signals, THP/ZFP PCR reactions were run longer but always in conditions of linear amplification. Representative gel images are shown below each panel; AcH3: anti-acetylated histone H3 antibodies; dark blue bars: Ln2 HPC; dark blue hatched bars: human CD34<sup>+</sup> cells; light blue bars: Ln2 EKLF<sup>-/-</sup> HPC.



**Figure 3** CBP, BRG1, TBP and Pol II recruitment at the hu $\beta$ -globin locus in HPC. Ln2 HPC, human CD34<sup>+</sup> cells, or Ln2 EKLF<sup>-/-</sup> HPC were subjected to ChIP analyses with anti-CBP (A, B), -BRG1 (C, D), -TBP (E, F), or -Pol II (G, H) antibodies. Analysis and quantification of immunoprecipitated samples were performed as described in Figure 2. Dark blue bars: Ln2 HPC; dark blue hatched bars: human CD34<sup>+</sup> cells; light blue bars: Ln2 EKLF<sup>-/-</sup> HPC.

HPC H3 acetylation and p45 recruitment are affected at both HS2 and hu $\beta$ -promoter (Figure 2E and G), whereas CBP binding is reduced only at hu $\beta$ -promoter, suggests that the absence of EKLF could either: (i) directly impede CBP recruitment at the hu $\beta$ -promoter; or (ii) preclude p45 and CBP recruitment at the hu $\beta$ -promoter with consequent hypoacetylation. Therefore, the role of p45 in locus acetylation was verified in HPC purified from 13.5 d.p.c. In2 p45 KO fetal livers (In2 p45<sup>-/-</sup> HPC). The absence of p45 does not significantly change histone H3 acetylation level at HS2 (data not shown), whereas H3 acetylation and CBP recruitment at the hu $\beta$ -promoter significantly decrease in In2 p45<sup>-/-</sup> HPC (Supplementary Figure 4). Thus, it appears that p45-mediated recruitment of CBP, which is facilitated by EKLF, contributes to histone acetylation of the hu $\beta$ -promoter in HPC. The detection of histone H3 acetylation at HS2 in In2 p45<sup>-/-</sup> HPC suggests that other activator/s capable of interacting with histone acetyltransferases can bind HS2 in HPC.

The remodeling complex E-RC1 contains, among other proteins, the yeast homologue BRG1 and EKLF (Armstrong *et al*, 1998). We therefore wondered whether EKLF might be part of a remodeling complex in HPC. Since the EKLF-BRG1 interaction is sufficient to remodel a chromatin-assembled  $\beta$ -globin promoter (Kadam *et al*, 2000), and BRG1 is the first among the SWI/SNF components to be recruited to promoter regions, we verified whether EKLF could influence hu $\beta$ -gene potentiation through BRG1 recruitment and hence chromatin remodeling. The comparison between In2 HPC or CD34<sup>+</sup> cells and In2 EKLF<sup>-/-</sup> HPC (Figure 3C and D) reveals a significant reduction of BRG1 binding at hu $\beta$ -promoter in In2 EKLF<sup>-/-</sup> HPC. As expected, BRG1 binding at the c-myc promoter (Nagl *et al*, 2006) is similar in In2 HPC versus In2 EKLF<sup>-/-</sup> HPC (Supplementary Figure 3E). Based on these results, we propose that in HPC EKLF facilitates chromatin remodeling of the hu $\beta$ -promoter through BRG1 recruitment.

### Transcription preinitiation complex assembly and globin gene potentiation in HPC

The preinitiation complex (PIC) includes Pol II, general transcription factors, for example TBP, and additional cofactors. Basal levels of hu $\beta$ -gene expression in HPC (Figure 1A) prompted us to ask whether gene potentiation in these cells requires PIC assembly. TBP was detected at hu $\beta$ -promoter in In2 HPC and CD34<sup>+</sup> cells (Figure 3E). Even though bound to the hu $\gamma$ -promoter in CD34<sup>+</sup> cells (see Discussion), TBP is not found at hu $\gamma$ - (Figure 3E) or hu $\epsilon$ - (data not shown) promoters in In2 HPC. Thus, globin potentiation in In2 HPC is developmental-specific and is associated with TBP recruitment at the hu $\beta$ -promoter before the onset of high-level transcriptional activity. This is supported by our results obtained in EKLF KO cells. In EKLF KO mice, the switch from  $\gamma$ -to- $\beta$ -globin expression is delayed; indeed, around 13.5 d.p.c.  $\gamma$ -globins are expressed in a greater number of fetal liver EryC derived from these mice relative to the situation for wild-type counterparts (Perkins *et al*, 1996; Wijgerde *et al*, 1996). Consequently, in In2 EKLF<sup>-/-</sup> HPC the TBP binding significantly decreases at hu $\beta$ -promoter, whereas it increases at hu $\gamma$ -promoters (Figure 3F).

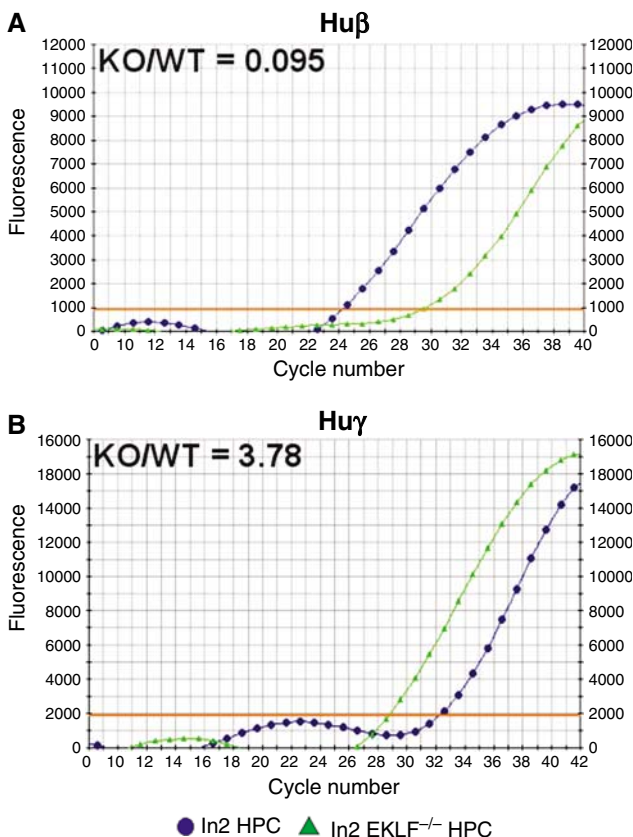
It has been proposed that NF-E2-TAF<sub>II</sub>130 interaction facilitates PIC assembly at globin promoters (Amrolia *et al*, 1997). Accordingly, in In2 p45<sup>-/-</sup> HPC, TBP recruitment at the hu $\beta$ -promoter is significantly lower than in wild-type cells

(Supplementary Figure 4). Altogether, these results suggest that EKLF, either directly or through p45, contributes to hu $\beta$ -gene potentiation also by modulating the developmental-specific recruitment of TBP at the hu $\beta$ -promoter in HPC.

In2 HPC were then subjected to ChIP analyses with an antibody that binds Pol II in a phosphorylation-independent manner. Pol II is detected at HS2, but not at the hu $\beta$ -, hu $\gamma$ - (Figure 3G and H) or  $\beta$ maj- (Supplementary Figure 5) promoters, suggesting that HS2-bound Pol II is not efficiently transferred to globin promoters in In2 HPC or In2 EKLF<sup>-/-</sup> HPC. However, Pol II is crosslinked at hu $\gamma$ -promoters in CD34<sup>+</sup> cells (see Discussion). Thus, the presence of TBP but not Pol II suggests that the PIC is partially assembled at the hu $\beta$ -promoter and contributes to globin gene potentiation in HPC.

### Basal levels of globin gene expression in HPC

Globins are expressed at basal levels in multipotent progenitors (Figure 1A) (Hu *et al*, 1997; Bottardi *et al*, 2003). EKLF is essential for hu $\beta$ -gene expression in EryC, but it is not known whether EKLF is also important for basal levels of globin gene expression in HPC. By quantitative real-time RT-PCR (Figure 4), we show that hu $\beta$ -gene expression is 10-fold lower and hu $\gamma$ -gene expression is 4-fold higher in In2 EKLF<sup>-/-</sup> HPC than in In2 HPC. Thus, fetal liver-derived



**Figure 4** Quantitative real-time RT-PCR of In2 HPC and In2 EKLF<sup>-/-</sup> HPC. Representative examples of hu $\beta$ - (A) and hu $\gamma$ -gene (B) expression were evaluated by real-time RT-PCR. The relative levels of globin gene expression in In2 EKLF<sup>-/-</sup> HPC versus In2 HPC were calculated according to Pfaffl (2001), using mouse actin as internal control (as described in Figure 1) and expressed as KO/WT ratios. Blue dots: In2 HPC; green triangles: In2 EKLF<sup>-/-</sup> HPC.

HPC express  $hu\beta$ - and  $hu\gamma$ -globin genes and the absence of EKLK favors  $hu\gamma$ - to the detriment of  $hu\beta$ -gene basal levels of expression.

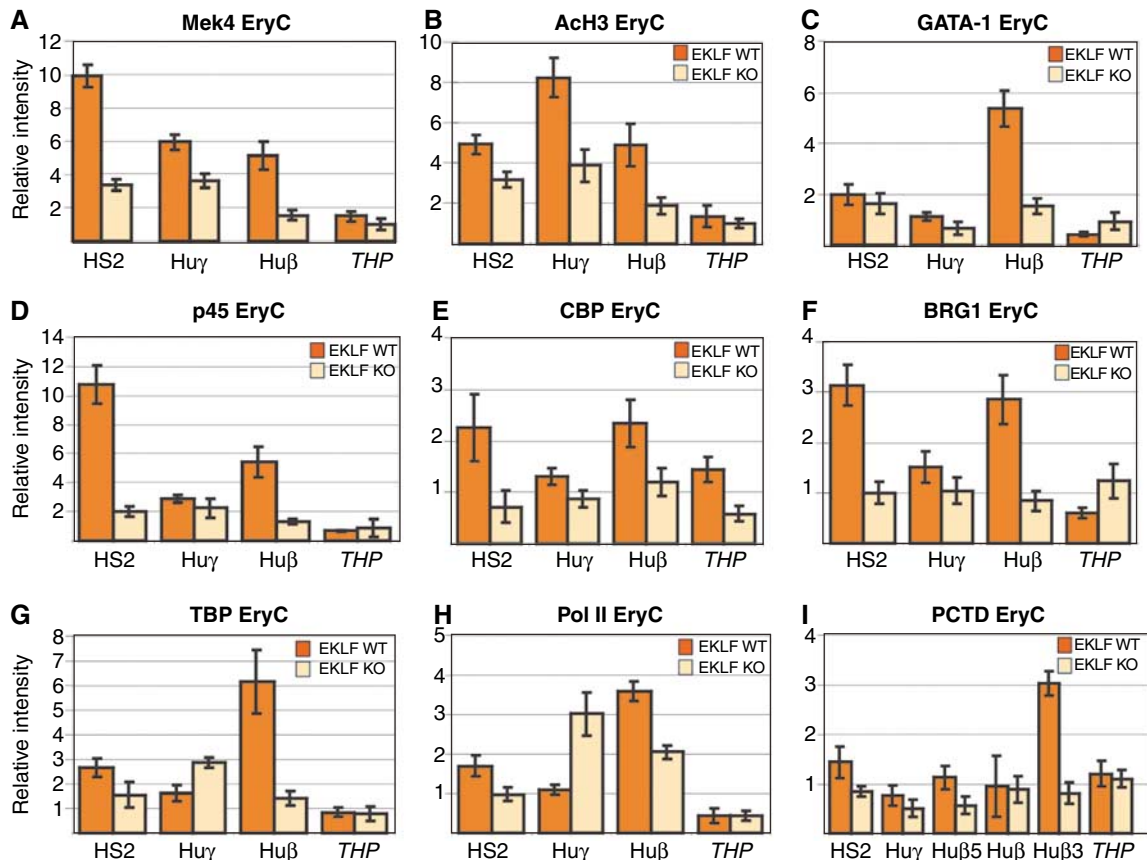
**Chromatin organization at the  $hu\beta$ -globin locus in EryC**

EKLK is required for enucleation and final erythrocyte formation during *in vitro* culture of definitive erythrocytes, whereas EKLK is dispensable for the first steps of erythroid cell differentiation (Table I; Drissen *et al*, 2005). Wright-Giemsa staining of ln2 EKLK wild type or ln2 EKLK KO 13.5 d.p.c. fetal livers revealed that, as expected (Perkins *et al*, 1995), late erythroblasts and enucleated red cells are less represented in 13.5 d.p.c. EKLK KO than wild-type fetal liver. However, only minor variations in the ratio of erythroid versus nonerythroid cells and in the percentage of primitive erythroid cells are observed among the two backgrounds (Supplementary Figure 6). In view of the above, ln2 EKLK wild type (ln2 EryC) as well as ln2 EKLK KO (ln2 EKLK<sup>-/-</sup> EryC) 13.5 d.p.c. fetal livers were used for ChIP analyses. First, compared with ln2 EryC, H3 lysine 4 (K4) dimethylation and H3 acetylation levels in ln2 EKLK<sup>-/-</sup> EryC (Figure 5A and B), though still considerable (except for K4 dimethylation at  $hu\beta$ -promoter), are lower at HS2,  $hu\gamma$ - and  $hu\beta$ -promoters. GATA-1 and p45 are crosslinked to HS2 and  $hu\beta$ -promoter in ln2 EryC (Figure 5C and D). However, in ln2 EKLK<sup>-/-</sup> EryC, GATA-1 recruitment at the  $hu\beta$ -promoter (Figure 5C) and p45 recruitment at HS2 and  $hu\beta$ -promoter (Figure 5D) are reduced. Likewise, in

ln2 EKLK<sup>-/-</sup> EryC CBP, BRG1, TBP and Pol II recruitment are affected at HS2 and  $hu\beta$ -promoter (Figure 5E-H). Pol II binding is also reduced at the  $\beta$ major promoter in ln2 EKLK<sup>-/-</sup> EryC (Supplementary Figure 5). Interestingly, in ln2 EKLK<sup>-/-</sup> EryC, TBP (Figure 5G) and Pol II (Figure 5H) binding to  $hu\gamma$ -promoters is greater than in ln2 EryC. These results are in agreement with previous observations indicating that  $\gamma$ -genes are expressed at higher levels in EKLK KO than EKLK wild-type 13.5 d.p.c. fetal livers (Perkins *et al*, 1996; Wijgerde *et al*, 1996). To ascertain the significance ( $P < 0.05$ ) of the variations in histone acetylation as well as p45, CBP, and BRG1 recruitment in ln2 EryC versus ln2 EKLK<sup>-/-</sup> EryC, other regions were analyzed (Supplementary Figure 7A-D). Since no significant differences are observed at any of the additional regions analyzed, it is likely that the EKLK effect at the  $\beta$ -globin locus is not related to a global defect in transcriptional activation. Thus, the defect in  $hu\beta$ -gene expression documented in ln2 EKLK<sup>-/-</sup> EryC is most likely due to abnormal recruitment of chromatin proteins and histone modifying/chromatin remodeling activities, transcriptional activators, TBP, and Pol II at the  $\beta$ LCR, and, in particular, at the  $hu\beta$ -globin promoter.

**Pol II phosphorylation and transcriptional elongation**

In the absence of EKLK, transgenic mice carrying the  $hu\beta$ -globin locus do not express mouse or human adult globin genes in EryC (Perkins *et al*, 1996; Wijgerde *et al*, 1996).



**Figure 5** Chromatin organization at the  $hu\beta$ -globin locus in EryC. (A-I) ChIP analyses of ln2 EryC (dark orange bars) or ln2 EKLK<sup>-/-</sup> EryC (light orange bars). Analysis and quantification of immunoprecipitated samples, as well as antibodies used for ChIP assays are as described in Figures 2 and 3; MeK4: anti-lysine 4 dimethylated histone H3 antibodies; PCTD: anti-phospho-Ser2 Pol II CTD antibodies.

Nonetheless, we detect Pol II at the hu $\beta$ -promoter in ln2 EKL $F^{-/-}$  EryC (Figure 5H). To elucidate this conflicting result, ln2 EryC or ln2 EKL $F^{-/-}$  EryC were subjected to ChIP analyses with an antibody that recognizes phospho-Ser2 at the C-terminal domain (CTD) of Pol II (PCTD). It is known that the overall phosphorylation level of Pol II increases during transcriptional elongation. In fact, during promoter clearance and elongation, Pol II CTD is phosphorylated and this post-translational modification renders Pol II a transcription- and elongation-competent enzyme. As shown in Figure 5I, PCTD detection at the 3' end of the hu $\beta$ -gene (Hu $\beta$ 3) is significantly lower in ln2 EKL $F^{-/-}$  EryC than in ln2 EryC. This result suggests that in EKL $F^{-/-}$  EryC, Pol II is not efficiently phosphorylated on Ser2 and is stalled at the hu $\beta$ -promoter.

## Discussion

### **Transcriptional activators involved in globin gene potentiation in HPC**

Previous studies have shed light on the importance of EKL $F$  during ontogeny (Xue *et al*, 2004), and for adult  $\beta$ -genes expression in EryC (Nuez *et al*, 1995; Perkins *et al*, 1995; Wijgerde *et al*, 1996). EKL $F$  is not essential for early hematopoietic differentiation. However, observations here and elsewhere that EKL $F$  is promiscuously expressed in multipotent HPC and progenitor cell lines (Hu *et al*, 1997; Figure 1) prompted us to explore whether EKL $F$  is required for hu $\beta$ -gene potentiation in HPC. In EryC, EKL $F$  and BRG1 participate in the remodeling complex E-RC1 (Armstrong *et al*, 1998). Interestingly, EKL $F$ –BRG1 interaction appears to be crucial since the *Brg1* hypomorphic mutant mice exhibit abnormal definitive erythroid cell differentiation, which very much resembles to the phenotype observed in EKL $F$  KO mice (Bultman *et al*, 2005). Since BRG1 recruitment to the hu $\beta$ -promoter in HPC is EKL $F$ -dependent, we postulate that the influence of EKL $F$  on hu $\beta$ -gene potentiation is linked to chromatin remodeling. Chromatin remodeling activity increases hu $\beta$ -promoter accessibility for transcriptional activators as exemplified by the situation for p45, whose binding to the hu $\beta$ -promoter depends upon EKL $F$ .

We previously identified histone H3 hyperacetylation as a hallmark of developmental-specific potentiation of the hu $\beta$ -globin gene in HPC (Bottardi *et al*, 2003, 2005). We now provide strong evidence that in transgenic and human HPC, histone acetylation at HS2 and hu $\beta$ -promoter correlates with CBP recruitment, which is likely to be facilitated by transcriptional activators. CBP detection at the hu $\beta$ -promoter is impaired in ln2 EKL $F^{-/-}$  HPC and in ln2 p45 $^{-/-}$  HPC. Since EKL $F$  affects p45 recruitment in HPC, we propose that the influence of EKL $F$  on CBP recruitment at hu $\beta$ -promoter is mediated by p45. Indeed, even if EKL $F$  can interact with p300 and CBP, it would not be able to directly recruit CBP to the locus (Zhang and Bieker, 1998). However, in EryC p45–CBP interaction (Hung *et al*, 2001) favors histone acetylation at the  $\beta$ major promoter (Johnson *et al*, 2001), suggesting that p45 can interact with CBP while bound to the locus.

p45 interactions with CBP (Hung *et al*, 2001) and TF $_{II}$ 130 (Amrolia *et al*, 1997) favor PIC assembly and  $\beta$ -globin gene expression in MEL cells (Johnson *et al*, 2001; Sawado *et al*, 2001). We demonstrate that TBP recruitment at hu $\beta$ -promoter is markedly affected in p45 $^{-/-}$  HPC. Additionally, disruption

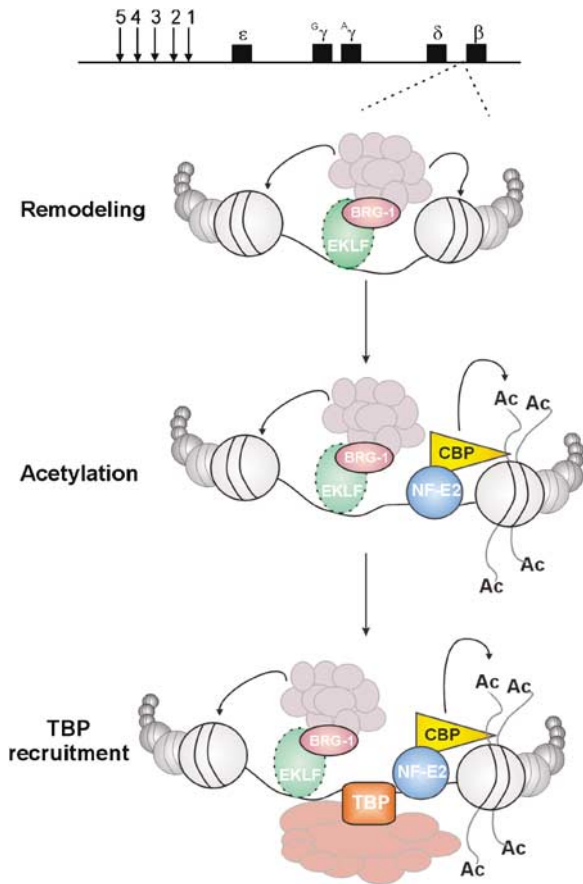
of globin gene potentiation in ln2 p45 $^{-/-}$  HPC is associated with a significant decrease in hu $\beta$ -gene expression in ln2 p45 $^{-/-}$  EryC, which is, on average, three-fold lower than in ln2 EryC (Supplementary Figure 8). The influence of p45 on hu $\beta$ -gene expression is particularly intriguing because adult mouse  $\alpha$ - and  $\beta$ -globin gene expression are not significantly modified in p45 KO mice (Shivdasani and Orkin, 1995; Supplementary Figure 8). The different consequences of p45 KO on the regulation of adult mouse and human  $\beta$ -globin gene may be explained by: (i) variations in the epigenetic regulation of the two  $\beta$ -globin loci during hematopoiesis (Bottardi *et al*, 2003); and (ii) the fact that in human and transgenic ln2 EryC the hu $\beta$ -globin locus appears to reside in a chromosomal environment more restrictive for transcription compared with its murine counterpart (Forrester *et al*, 1990; Milot *et al*, 1996; Epner *et al*, 1998). Thus, we suggest that p45 is important for hu $\beta$ -gene potentiation and, since p45 is also detected at HS2 and hu $\gamma$ -promoters, possibly also for locus activation in HPC.

MafK can dimerize with different partners (Blank and Andrews, 1997). For instance, p45 is replaced by Bach1 to form a repressive Bach1/MafK complex in erythroid precursors (Brand *et al*, 2004), where p45 expression is mostly abolished (Anguita *et al*, 2004). In ln2 p45 $^{-/-}$  HPC CBP recruitment and histone acetylation at hu $\beta$ -promoter are impaired. Thus, MafK partners (if any) that replace p45 in these cells do not promote CBP recruitment and subsequent histone acetylation at the hu $\beta$ -promoter.

GATA-1 and GATA-2 are expressed in HPC, but neither could be detected by ChIP at either HS2 or hu $\beta$ -promoter in ln2 HPC (Figure 2C and data not shown). Thus, it appears that the aforementioned factors do not bind to these regions in HPC. However, in HPC, GATA-1 and/or GATA-2 could bind to other regions along the locus or be components of multiprotein complexes (Anguita *et al*, 2004; Rodriguez *et al*, 2005), which might impede their immunoprecipitation by ChIP.

Although the absence of EKL $F$  severely impairs p45, CBP and BRG1 recruitment to the hu $\beta$ -promoter in HPC, chromatin organization and transcriptional activator recruitment are also affected at the  $\beta$ LCR HS2, which was chosen because of its role in chromatin activation and hu $\beta$ -gene potentiation in HPC (Bottardi *et al*, 2005). In EryC, HS2 requires the presence of EKL $F$  to participate in the active chromatin hub (Drissen *et al*, 2004). However, in EKL $F$  KO EryC, DNase I sensitivity is specifically affected at HS3 (Wijgerde *et al*, 1996). Therefore, we also evaluated the influence of EKL $F$  at HS3 and observed that in ln2 EKL $F^{-/-}$  HPC and ln2 EKL $F^{-/-}$  EryC BRG1 recruitment is affected at HS3 as well (data not shown). Thus, EKL $F$  action at HS2 might be either direct or indirect and requires the contribution of HS3, particularly in EryC, where HS2 and HS3 have been found to functionally interact.

It has been observed that general transcription factors can bind the mouse  $\lambda$ 5 locus in embryonic stem cells and that the recruitment of specific transcription factors occurs in B cell-restricted progenitors (Szutorisz *et al*, 2005). Here, we show that lineage-specific transcriptional activators are recruited at the hu $\beta$ -globin locus in multipotent HPC and they participate in the locus potentiation. Altogether, our results indicate that: (i) EKL $F$  is critical for the recruitment of p45, CBP and BRG1 at the hu $\beta$ -promoter in HPC; and (ii) these transcriptional activators contribute to locus chromatin activation and hu $\beta$ -



**Figure 6** Model of hu $\beta$ -globin gene potentiation in In2 HPC. Schematic representation of hu $\beta$ -gene potentiation in multipotent In2 HPC (see Discussion for more details). Nucleosomes are represented by beads and transcriptional activators are indicated as colored objects.

gene potentiation in HPC. Based on this we propose a model (Figure 6) where basal-level expression of erythroid-specific transcriptional activators, for example, EKLf and p45, either sequentially or simultaneously, allow appropriate hu $\beta$ -gene potentiation in freshly isolated human and transgenic HPC. Reminiscent of what has been found in other systems with distinct transcriptional activators (Agalioti *et al*, 2002; de la Serna *et al*, 2005), EKLf, p45 (NF-E2) and TBP could cooperate to induce or stabilize a potent chromatin necessary for the recruitment of additional transcriptional activators during differentiation and to support hu $\beta$ -gene expression in EryC.

#### **TBP and Pol II association to the hu $\beta$ -globin locus in HPC**

We demonstrate for the first time that the absence of EKLf in HPC affects basal levels of hu $\beta$ -gene expression and, to some extent, benefits hu $\gamma$ - over hu $\beta$ -gene expression. This is also supported by the preferential recruitment of TBP at hu $\beta$ -promoter in In2 HPC, and at hu $\gamma$ -promoters in In2 EKLf<sup>-/-</sup> HPC. Hu $\beta$ - and hu $\gamma$ -promoters should not compete for  $\beta$ LCR interaction in hematopoietic progenitors (Palstra *et al*, 2003). However, EKLf might contribute to hu $\gamma$ -gene silencing in adult HPC. Indeed, EKLf can interact with Sin3A and histone deacetylase 1 (HDAC1) corepressors, and it can induce repression of a reporter gene in hu $\gamma$ -expressing cell lines (Chen

and Bieker, 2004). A similar repression mechanism involving EKLf could take place in HPC.

Since Pol II is not detected at hu $\beta$ - or hu $\gamma$ -promoters in HPC, it is likely that basal levels of hu $\beta$ -gene expression result from a limiting number of Pol II molecules (difficult to detect by ChIP) engaged at the promoter at any given time, and also from stochastic Pol II-hu $\beta$ -promoter interactions. Indeed, only a subset of In2 HPC express hu $\beta$ - or hu $\gamma$ -genes (Bottardi *et al*, 2003), a circumstance that might further affect ChIP sensitivity. We also demonstrate that Pol II is recruited at  $\beta$ LCR HS2 in multipotent HPC. This observation is reminiscent of what has been observed at the mouse globin  $\beta$ LCR in EryC and cell lines (Johnson *et al*, 2001). The fact that Pol II binding to  $\beta$ LCR HS2 in In2 EKLf<sup>-/-</sup> HPC is not significantly affected suggests that, as in the case of MEL cells (Johnson *et al*, 2003), Pol II-HS2 interaction occurs independent of histone acetylation/methylation patterns or transcriptional status.

Throughout this study, very similar observations were made in In2 HPC versus human CD34<sup>+</sup> cells. However, TBP and Pol II binding to hu $\gamma$ -promoters are, respectively, three- and four-fold higher in CD34<sup>+</sup> cells than in In2 HPC. This discrepancy is likely due to the fact that CD34<sup>+</sup> cells were purified from apheresis samples of normal patients who underwent SCF and G-CSF treatment to increase CD34<sup>+</sup> cell mobilization. SCF can induce  $\gamma$ -globin expression in adult human erythroblasts (Bhanu *et al*, 2005). Even though we did not detect a significant difference in hu $\gamma$ /hu $\beta$ -gene basal levels of expression between CD34<sup>+</sup> cells and In2 HPC (data not shown), increased TBP and Pol II binding at hu $\gamma$ -promoters in CD34<sup>+</sup> cells suggests that SCF might modify globin gene potentiation at early stages of hematopoiesis.

#### **Chromatin organization of the hu $\beta$ -globin locus in EryC**

In 13.5 d.p.c. fetal liver EryC, globin expression is linked to GATA-1, p45, CBP, BRG1 and TBP recruitment at the locus, and to efficient Pol II CTD phosphorylation at the 3' end of hu $\beta$ -gene. Analyses of EKLf<sup>-/-</sup> EryC allowed us to highlight some of the molecular defects associated with EKLf ablation. Specifically, H3 acetylation/methylation, GATA-1, p45, CBP, BRG1 and TBP recruitment at HS2 and hu $\beta$ -promoter are significantly affected. These results support and extend previous studies (Wijgerde *et al*, 1996; Armstrong *et al*, 1998) by suggesting that in EryC EKLf influences chromatin organization at the hu $\beta$ -promoter and, to a lesser extent, at the  $\beta$ LCR. In addition, the delayed switching from hu $\gamma$ - to hu $\beta$ -gene expression in the absence of EKLf (Perkins *et al*, 1996; Wijgerde *et al*, 1996) correlates well with increased TBP and Pol II binding to the hu $\gamma$ -promoters in In2 EKLf<sup>-/-</sup> EryC. However, the fact that in In2 EKLf<sup>-/-</sup> EryC hyperphosphorylated Pol II is absent at the 3' end of the hu $\beta$ -gene strongly suggests that transcriptional elongation is also impaired in these cells. Accordingly, it has been reported that EKLf is important for  $\beta$ LCR- $\beta$ major promoter interactions (Drissen *et al*, 2004), which favor transcriptional elongation (Sawado *et al*, 2001).

In summary, we provide the first evidence that the lineage-associated transcriptional activators EKLf and p45, which are expressed at basal levels in multipotent progenitor cells, can be functional and involved in gene potentiation in multipotent HPC, that is, before transcriptional activation. Finally, based on the results presented here, we propose a model that

recapitulates the significant 'steps' in the chain of events required to activate chromatin and to render the hu $\beta$ -globin gene and promoter permissive for transcriptional machinery assembly during hematopoiesis.

## Materials and methods

### Mouse transgenic lines

Homozygous  $\ln2$  mice (Strouboulis *et al*, 1992) were bred with CD1 females and  $\ln2^{+/-}$  13.5 d.p.c. fetal livers were isolated. Otherwise,  $\ln2^{+/-}$ :EKL $F^{+/-}$  (Nuez *et al*, 1995) or  $\ln2^{+/-}$ :p45 $^{+/-}$  (Shivdasani and Orkin, 1995) mice were crossed with EKL $F^{+/-}$  or p45 $^{+/-}$  mice respectively and 13.5 d.p.c.  $\ln2^{+/-}$ :EKL $F^{-/-}$  or  $\ln2^{+/-}$ :p45 $^{-/-}$  fetal livers were isolated.

### HPC purification and cell culture

Human CD34 $^{+}$  cells were purified as previously described (Bottardi *et al*, 2003). Five up to 10 13.5 d.p.c. fetal livers were first incubated with rat anti-Ter119, -B220 and -Gr-1 antibodies (Ab) followed by anti-rat FITC-conjugated Ab. After a brief wash in PBS 5% heat inactivated rat serum, cells were stained with biotinylated anti-CD31 Ab followed by SAV-TC Ab. For *in vitro* clonal assays, HPC were plated onto MethoCult M3434 medium (StemCell Technology) and colony types were scored at days 3 and 14.

### ChIP and duplex PCR analyses

ChIP assays were carried out using at least  $3 \times 10^5$  HPC or  $10^6$  CD34 $^{+}$  cells or EryC, as per the manufacturer's instruction (Upstate Biotechnology) and chromatin was reduced in size by sonication in order to obtain fragments of 400-bp average size. Antibodies were raised against acetylated histone H4, H3 or dimethylated (K4) histone H3 (Upstate Biotechnology); TBP (SI-1), NF-E2 (C-19), GATA-1 (N6), Pol II (N-20), CBP (A-22) and BRG1 (H-88) (Santa Cruz); or Ser2 phosphorylated Pol II (H5) (Covance). For the latter, chromatin pre-clearing was carried out with anti-mouse IgM

antibodies coupled to agarose beads (Sigma). On average, 1/30 of each ChIP sample was used in quantitative duplex PCR, as previously described (Bottardi *et al*, 2003). Primer sequences are available on request.

### RT-PCR and quantitative real-time PCR

Total RNA was extracted by Trizol (Invitrogen), treated with DNaseI-RNase free (Invitrogen) and used for cDNA synthesis with oligo(dT)<sub>15</sub> primers and SuperScript Reverse Transcriptase (Invitrogen). Semiquantitative PCR was carried out with primers specific for murine SCL, GATA-1, p45, GATA-2, EKLF, GATA-3, C/EBP $\alpha$ , NPM and fetal human ( $\gamma$ ) and mouse embryonic ( $\beta$ H1) globin transcripts (Epner *et al*, 1998) or adult human ( $\beta$  and  $\delta$ ) and mouse ( $\beta$ min and  $\beta$ maj) globin transcripts (Reik *et al*, 1998). Primer sequences are available on request.

Quantitative real-time RT-PCR analysis was carried out as in (Bottardi *et al*, 2005) with minor changes. Total RNA was treated with DNaseI-RNase free (Invitrogen) and used for cDNA synthesis with oligo(dT)<sub>15</sub> primers and SuperScript Reverse Transcriptase (Invitrogen). Real-time PCR was performed with Qiagen QuantiTect probes specific for hu $\beta$ - or hu $\gamma$ -globin cDNA. Mouse actin, GATA-1, p45, EKLF or  $\beta$ min/ $\beta$ maj globin cDNA were detected by Sybr Green (Bio-Rad). Primer sequences are available on request.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

## Acknowledgements

We thank E Drobetsky, M Trudel and W de Laat for useful discussions and critical reading of the manuscript, G D'Angelo and J Hebert for Wright-Giemsa staining analysis, DC Roy for human apheresis samples, RA Shivdasani for kindly providing p45 knock-out mice and V Bourgoïn and A Orimoto for technical assistance. VB is supported by the CIHR. EM is a scholar of the CIHR and this work was supported by a grant from the CIHR to EM.

## References

- Agalioti T, Chen G, Thanos D (2002) Deciphering the transcriptional histone acetylation code for a human gene. *Cell* **111**: 381–392
- Akashi K, Traver D, Miyamoto T, Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**: 193–197
- Amrolia PJ, Ramamurthy L, Saluja D, Tanese N, Jane SM, Cunningham JM (1997) The activation domain of the enhancer binding protein p45NF-E2 interacts with TAF<sub>II</sub>130 and mediates long-range activation of the alpha- and beta-globin gene loci in an erythroid cell line. *Proc Natl Acad Sci USA* **94**: 10051–10056
- Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, Orkin SH (1993) Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. *Nature* **362**: 722–728
- Anguita E, Hughes J, Heyworth C, Blobel GA, Wood WG, Higgs DR (2004) Globin gene activation during haemopoiesis is driven by protein complexes nucleated by GATA-1 and GATA-2. *EMBO J* **23**: 2841–2852
- Armstrong JA, Bieker JJ, Emerson BM (1998) A SWI/SNF-related chromatin remodeling complex, E-RC1, is required for tissue-specific transcriptional regulation by EKLF *in vitro*. *Cell* **95**: 93–104
- Bender MA, Bulger M, Close J, Groudine M (2000) Beta-globin gene switching and DNase I sensitivity of the endogenous beta-globin locus in mice do not require the locus control region. *Mol Cell* **5**: 387–393
- Bhanu NV, Trice TA, Lee YT, Gantt NM, Oneal P, Schwartz JD, Noel P, Miller JL (2005) A sustained and pancellular reversal of gamma-globin gene silencing in adult human erythroid precursor cells. *Blood* **105**: 387–393
- Bieker JJ (2001) Kruppel-like factors: three fingers in many pies. *J Biol Chem* **276**: 34355–34358
- Blank V, Andrews NC (1997) The Maf transcription factors: regulators of differentiation. *Trends Biochem Sci* **22**: 437–441
- Blobel GA, Nakajima T, Eckner R, Montminy M, Orkin SH (1998) CREB-binding protein cooperates with transcription factor GATA-1 and is required for erythroid differentiation. *Proc Natl Acad Sci USA* **95**: 2061–2066
- Bonifer C (1999) Long-distance chromatin mechanisms controlling tissue-specific gene locus activation. *Gene* **238**: 277–289
- Bottardi S, Aumont A, Grosveld F, Milot E (2003) Developmental stage-specific epigenetic control of human beta-globin gene expression is potentiated in hematopoietic progenitor cells prior to their transcriptional activation. *Blood* **102**: 3989–3997
- Bottardi S, Bourgoïn V, Pierre-Charles N, Milot E (2005) Onset and inheritance of abnormal epigenetic regulation in hematopoietic cells. *Hum Mol Genet* **14**: 493–502
- Brand M, Ranish JA, Kummer NT, Hamilton J, Igarashi K, Francastel C, Chi TH, Crabtree GR, Aebersold R, Groudine M (2004) Dynamic changes in transcription factor complexes during erythroid differentiation revealed by quantitative proteomics. *Nat Struct Mol Biol* **11**: 73–80
- Bultman SJ, Gebuhr TC, Magnuson T (2005) A Brg1 mutation that uncouples ATPase activity from chromatin remodeling reveals an essential role for the SWI/SNF-related complexes in beta-globin expression and erythroid development. *Genes Dev* **19**: 2849–2861
- Cantor AB, Orkin SH (2002) Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene* **21**: 3368–3376
- Carter D, Chakalova L, Osborne CS, Dai YF, Fraser P (2002) Long-range chromatin regulatory interactions *in vivo*. *Nat Genet* **32**: 623–626
- Chen X, Bieker JJ (2004) Stage-specific repression by the EKLF transcriptional activator. *Mol Cell Biol* **24**: 10416–10424
- Daftari P, Gavva NR, Shen CK (1999) Distinction between AP1 and NF-E2 factor-binding at specific chromatin regions in mammalian cells. *Oncogene* **18**: 5482–5486
- de la Serna IL, Ohkawa Y, Berkes CA, Bergstrom DA, Dacwag CS, Tapscott SJ, Imbalzano AN (2005) MyoD targets chromatin remodeling complexes to the myogenin locus prior to forming a stable DNA-bound complex. *Mol Cell Biol* **25**: 3997–4009

- Drissen R, Palstra RJ, Gillemans N, Splinter E, Grosveld F, Philipsen S, de Laat W (2004) The active spatial organization of the beta-globin locus requires the transcription factor EKLf. *Genes Dev* **18**: 2485–2490
- Drissen R, von Lindern M, Kolbus A, Driegen S, Steinlein P, Beug H, Grosveld F, Philipsen S (2005) The erythroid phenotype of EKLf-null mice: defects in hemoglobin metabolism and membrane stability. *Mol Cell Biol* **25**: 5205–5214
- Epner E, Reik A, Cimbora D, Telling A, Bender MA, Fiering S, Enver T, Martin DI, Kennedy M, Keller G, Groudine M (1998) The beta-globin LCR is not necessary for an open chromatin structure or developmentally regulated transcription of the native mouse beta-globin locus. *Mol Cell* **2**: 447–455
- Feng D, Kan YW (2005) The binding of the ubiquitous transcription factor Sp1 at the locus control region represses the expression of beta-like globin genes. *Proc Natl Acad Sci USA* **102**: 9896–9900
- Forrester WC, Epner E, Driscoll MC, Enver T, Brice M, Papayannopoulou T, Groudine M (1990) A deletion of the human beta-globin locus activation region causes a major alteration in chromatin structure and replication across the entire beta-globin locus. *Genes Dev* **4**: 1637–1649
- Forsberg EC, Downs KM, Bresnick EH (2000) Direct interaction of NF-E2 with hypersensitive site 2 of the beta-globin locus control region in living cells. *Blood* **96**: 334–339
- Fujiwara Y, Browne CP, Cunniff K, Goff SC, Orkin SH (1996) Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci USA* **93**: 12355–12358
- Graf T (2002) Differentiation plasticity of hematopoietic cells. *Blood* **99**: 3089–3101
- Grosveld F, van Assendelft GB, Greaves DR, Kollias G (1987) Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell* **51**: 975–985
- Hong W, Nakazawa M, Chen YY, Kori R, Vakoc CR, Rakowski C, Blobel GA (2005) FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1. *EMBO J* **24**: 2367–2378
- Hu M, Krause D, Greaves M, Sharkis S, Dexter M, Heyworth C, Enver T (1997) Multilineage gene expression precedes commitment in the hemopoietic system. *Genes Dev* **11**: 774–785
- Hung HL, Kim AY, Hong W, Rakowski C, Blobel GA (2001) Stimulation of NF-E2 DNA binding by CREB-binding protein (CBP)-mediated acetylation. *J Biol Chem* **276**: 10715–10721
- Jimenez G, Griffiths SD, Ford AM, Greaves MF, Enver T (1992) Activation of the beta-globin locus control region precedes commitment to the erythroid lineage. *Proc Natl Acad Sci USA* **89**: 10618–10622
- Johnson KD, Christensen HM, Zhao B, Bresnick EH (2001) Distinct mechanisms control RNA polymerase II recruitment to a tissue-specific locus control region and a downstream promoter. *Mol Cell* **8**: 465–471
- Johnson KD, Grass JA, Park C, Im H, Choi K, Bresnick EH (2003) Highly restricted localization of RNA polymerase II within a locus control region of a tissue-specific chromatin domain. *Mol Cell Biol* **23**: 6484–6493
- Kadam S, McAlpine GS, Phelan ML, Kingston RE, Jones KA, Emerson BM (2000) Functional selectivity of recombinant mammalian SWI/SNF subunits. *Genes Dev* **14**: 2441–2451
- Leach KM, Vieira KF, Kang SH, Aslanian A, Teichmann M, Roeder RG, Bungert J (2003) Characterization of the human beta-globin downstream promoter region. *Nucleic Acids Res* **31**: 1292–1301
- Milot E, Strouboulis J, Trimborn T, Wijgerde M, de Boer E, Langeveld A, Tan-Un K, Vergeer W, Yannoutsos N, Grosveld F, Fraser P (1996) Heterochromatin effects on the frequency and duration of LCR-mediated gene transcription. *Cell* **87**: 105–114
- Morley BJ, Abbott CA, Sharpe JA, Lida J, Chan-Thomas PS, Wood WG (1992) A single beta-globin locus control region element (5' hypersensitive site 2) is sufficient for developmental regulation of human globin genes in transgenic mice. *Mol Cell Biol* **12**: 2057–2066
- Nagl Jr NG, Zweitzig DR, Thimmapaya B, Beck Jr GR, Moran E (2006) The c-myc gene is a direct target of mammalian SWI/SNF-related complexes during differentiation-associated cell cycle arrest. *Cancer Res* **66**: 1289–1293
- Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F (1995) Defective haematopoiesis in fetal liver resulting from inactivation of the EKLf gene. *Nature* **375**: 316–318
- Palstra RJ, Tolhuis B, Splinter E, Nijmeijer R, Grosveld F, de Laat W (2003) The beta-globin nuclear compartment in development and erythroid differentiation. *Nat Genet* **35**: 190–194
- Perkins AC, Gaensler KM, Orkin SH (1996) Silencing of human fetal globin expression is impaired in the absence of the adult beta-globin gene activator protein EKLf. *Proc Natl Acad Sci USA* **93**: 12267–12271
- Perkins AC, Sharpe AH, Orkin SH (1995) Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLf. *Nature* **375**: 318–322
- Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'Agati V, Orkin SH, Costantini F (1991) Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349**: 257–260
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45
- Reik A, Telling A, Zitnik G, Cimbora D, Epner E, Groudine M (1998) The locus control region is necessary for gene expression in the human beta-globin locus but not the maintenance of an open chromatin structure in erythroid cells. *Mol Cell Biol* **18**: 5992–6000
- Rodriguez P, Bonte E, Krijgsveld J, Kolodziej KE, Guyot B, Heck AJ, Vyas P, de Boer E, Grosveld F, Strouboulis J (2005) GATA-1 forms distinct activating and repressive complexes in erythroid cells. *EMBO J* **24**: 2354–2366
- Sawado T, Igarashi K, Groudine M (2001) Activation of beta-major globin gene transcription is associated with recruitment of NF-E2 to the beta-globin LCR and gene promoter. *Proc Natl Acad Sci USA* **98**: 10226–10231
- Shivdasani RA, Orkin SH (1995) Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2. *Proc Natl Acad Sci USA* **92**: 8690–8694
- Strouboulis J, Dillon N, Grosveld F (1992) Developmental regulation of a complete 70-kb human beta-globin locus in transgenic mice. *Genes Dev* **6**: 1857–1864
- Struhl K (2005) Transcriptional activation: mediator can act after preinitiation complex formation. *Mol Cell* **17**: 752–754
- Szutorisz H, Canzonetta C, Georgiou A, Chow CM, Tora L, Dillon N (2005) Formation of an active tissue-specific chromatin domain initiated by epigenetic marking at the embryonic stem cell stage. *Mol Cell Biol* **25**: 1804–1820
- Tolhuis B, Palstra RJ, Splinter E, Grosveld F, de Laat W (2002) Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol Cell* **10**: 1453–1465
- Vakoc CR, Letting DL, Gheldof N, Sawado T, Bender MA, Groudine M, Weiss MJ, Dekker J, Blobel GA (2005) Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol Cell* **17**: 453–462
- Wijgerde M, Gribnau J, Trimborn T, Nuez B, Philipsen S, Grosveld F, Fraser P (1996) The role of EKLf in human beta-globin gene competition. *Genes Dev* **10**: 2894–2902
- Xue L, Chen X, Chang Y, Bieker JJ (2004) Regulatory elements of the EKLf gene that direct erythroid cell-specific expression during mammalian development. *Blood* **103**: 4078–4083
- Ye M, Iwasaki H, Laiosa CV, Stadtfeld M, Xie H, Heck S, Clausen B, Akashi K, Graf T (2003) Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. *Immunity* **19**: 689–699
- Zhang W, Bieker JJ (1998) Acetylation and modulation of erythroid Kruppel-like factor (EKLf) activity by interaction with histone acetyltransferases. *Proc Natl Acad Sci USA* **95**: 9855–9860