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Dynamic long-range chromatin interactions control *Myb* proto-oncogene transcription during erythroid development

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The key haematopoietic regulator *Myb* is essential for coordinating proliferation and differentiation. ChIP-Sequencing and Chromosome Conformation Capture (3C)-Sequencing were used to characterize the structural and protein-binding dynamics of the *Myb* locus during erythroid differentiation. In proliferating cells expressing *Myb*, enhancers within the *Myb-Hbs1l* intergenic region were shown to form an active chromatin hub (ACH) containing the *Myb* promoter and first intron. This first intron was found to harbour the transition site from transcription initiation to elongation, which takes place around a conserved CTCF site. Upon erythroid differentiation, *Myb* expression is downregulated and the ACH destabilized. We propose a model for *Myb* activation by distal enhancers dynamically bound by KLF1 and the GATA1/TAL1/LDB1 complex, which primarily function as a transcription elongation element through chromatin looping.

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Introduction

The differentiation of stem and progenitor cells into mature differentiated cells requires a tight control of progenitor cell expansion, proliferation arrest and terminal differentiation. The *Myb* proto-oncogene encoding the c-Myb transcription factor (TF) is expressed in stem and progenitor cells of all haematopoietic lineages and plays a central role in the control of their proliferation (Mucenski *et al*, 1991; Sandberg *et al*, 2005; Vegiopoulos *et al*, 2006; Ramsay and Gonda, 2008; Lieu and Reddy, 2009). Lack of *Myb* is lethal (E15) due to the complete absence of definitive erythroid cells (Mucenski *et al*, 1991). Conditional knockout models revealed additional essential non-erythroid roles of *Myb*, mainly in the lymphoid system (Bender *et al*, 2004; Thomas *et al*, 2005), and the self-renewal and multi-lineage differentiation potential of adult haematopoietic stem cells (Lieu and Reddy, 2009). *Myb* is highly expressed in immature proliferating haematopoietic cells and is strongly downregulated in terminally differentiating cells (Gonda and Metcalf, 1984; Emambokus *et al*, 2003), suggesting that *Myb* is linked to the transition between proliferation and differentiation. Aberrant *Myb* expression in leukaemic cells is consistent with this idea (Ramsay and Gonda, 2008), correlating with increased proliferation and a loss of differentiation. Despite its importance, the control of *Myb* expression during haematopoiesis is poorly understood. Early work suggested a regulatory role for sequences in the first intron, primarily in blocking transcription elongation (Bender *et al*, 1987; Reddy and Reddy, 1989; Hugo *et al*, 2006). Recently, microRNAs were shown to be involved in regulating c-Myb protein levels (Xiao *et al*, 2007; Lu *et al*, 2008). However, the transcriptional regulatory elements and associated *trans*-acting factors controlling *Myb* expression during development remain mostly uncharacterized.

The mouse *Myb* gene on chromosome 10 is flanked by the *Ahi1* and *Hbs1l* genes, which have no known function during haematopoiesis. Several studies pointed out a potential role for the 135 kb *Myb-Hbs1l* intergenic region in the regulation of *Myb*: (i) transgene integration within the intergenic region led to severe downregulation of *Myb* expression (Mukai *et al*, 2006); (ii) ChIP-on-chip data showed an open chromatin structure (i.e., H3Ac and H4Ac) of the region in human erythroid cells expressing *MYB* (Wahlberg *et al*, 2009); and (iii) several studies showed that SNPs in the human *MYB-HBS1L* intergenic region (possibly affecting *MYB* expression) were strongly associated with variation in several clinically relevant erythrocyte traits (Thein *et al*, 2007; Lettre *et al*, 2008; Ganesh *et al*, 2009). For example, specific SNPs associate with elevated fetal haemoglobin (HbF), which ameliorates β-haemoglobinopathy severity and has therapeutic potential. Thus, important regulatory elements appear to reside in the *Myb-Hbs1l* intergenic region, but they have not been localized precisely or characterized in any way.

Erythroid development is controlled by an array of TFs, including GATA1, its associated partners LDB1, TAL1, KLF1 and c-Myb (Cantor and Orkin, 2001). A complex of the haematopoietic TFs GATA1/TAL1/LDB1 together with the ETO2/MTGR1 cofactors (the 'LDB1 complex') binds regulatory regions of developmentally regulated genes (Fujiwara *et al*, 2009; Yu *et al*, 2009; Kassouf *et al*, 2010; Soler *et al*, 2010; Tallack *et al*, 2010) and controls their activation upon terminal erythroid differentiation (Soler *et al*, 2010). The LDB1 complex preferentially binds at large distances from promoters (up to 300 kb) in intergenic regions, providing long-range candidate regulatory elements. An example is the long-range control of the β-globin genes by *cis*-regulatory elements spread over 100 kb, forming the locus control region (LCR). When β-globin is expressed, the LCR folds into a three-dimensional (3D) active chromatin hub (ACH) (Tolhuis *et al*, 2002; Palstra *et al*, 2003), where distal enhancers reside in close proximity to the expressed genes. Structural proteins such as CTCF and Cohesin are known to participate in such 3D interactions (Ong and Corces, 2011). TFs also have a role in long-range gene regulation, for example, LDB1, GATA1, FOG1 and KLF1 are required to maintain such interactions within the β-globin locus and other loci (Drissen *et al*, 2004; Vakoc *et al*, 2005; Song *et al*, 2007; Jing *et al*, 2008).

We report here that the activating LDB1 complex, KLF1 and CTCF occupy multiple regulatory elements within the *Myb-Hbs1l* intergenic region, which have the chromatin hallmarks of active enhancers. Chromosome Conformation Capture (3C) and high-throughput sequencing (3C-Seq) show that these elements and the actively transcribed *Myb* gene cluster together in the nuclear space to form an ACH *in vivo*, bringing the enhancers in close proximity to the *Myb* gene promoter and first intron. The latter contains a highly conserved CTCF binding site around which productive transcription elongation starts. The ACH is lost when cells terminally differentiate, concomitant with the downregulation of *Myb* and a decreased binding of TF complexes at the distal enhancers.

Results

The LDB1 complex binds distal enhancers in the Myb-Hbs1l intergenic region

ChIP-Sequencing (ChIP-Seq) was used to identify the genome-wide binding sites of key erythroid TFs in mouse erythroleukaemia (MEL) cells and in primary mouse fetal liver (FL) cells (Soler *et al*, 2010). This showed preferential intragenic and intergenic binding of the LDB1 complex away from promoter sequences, suggesting it is involved in long-range gene regulation, a hypothesis supported by other studies (Song *et al*, 2007). Five LDB1 complex binding sites were detected in the *Myb-Hbs1l* intergenic region, −36, −61, −68, −81 and −109 kb upstream of the *Myb* transcription start site, in MEL cells and primary mouse erythroid progenitors from E13.5 FL (Figure 1A; Supplementary Figure S1A and B). These intergenic binding sites harboured all components of the activating LDB1 complex (GATA1/LDB1/TAL1/ETO2) in erythroid progenitors, consistent with active transcription of both *Myb* and *Hbs1l* genes (Supplementary Figure S1C). Additionally, in MEL and primary FL cells, the −81 kb binding site was found co-occupied by KLF1 (Figure 1B), a key erythroid TF primarily associated with gene activation, in agreement with a recent KLF1 ChIP-Seq experiment performed using primary mouse erythroid progenitors (Tallack *et al*, 2010; Supplementary Figure S2B and C). None of these TFs were found to bind the *Myb* or *Hbs1l* promoters. Next, all intergenic sites were shown to possess characteristic features supporting enhancer activity, that is, the presence of the histone acetyl transferase p300 (Visel *et al*, 2009), RNA polymerase II (polII), monomethylated histone 3 Lysine 4 (H3K4me1), and acetylated H3K27 (Heintzman *et al*, 2009; Figure 1C). PolII occupancy was especially abundant on the LDB1/KLF1 bound −81 kb sequence, showing similar enrichments to the highly active *Myb* promoter. In order to show that these LDB1 binding sites can indeed act as enhancers, they were cloned upstream of a minimal *Myb* promoter controlling a firefly luciferase reporter gene. Transfection into MEL cells

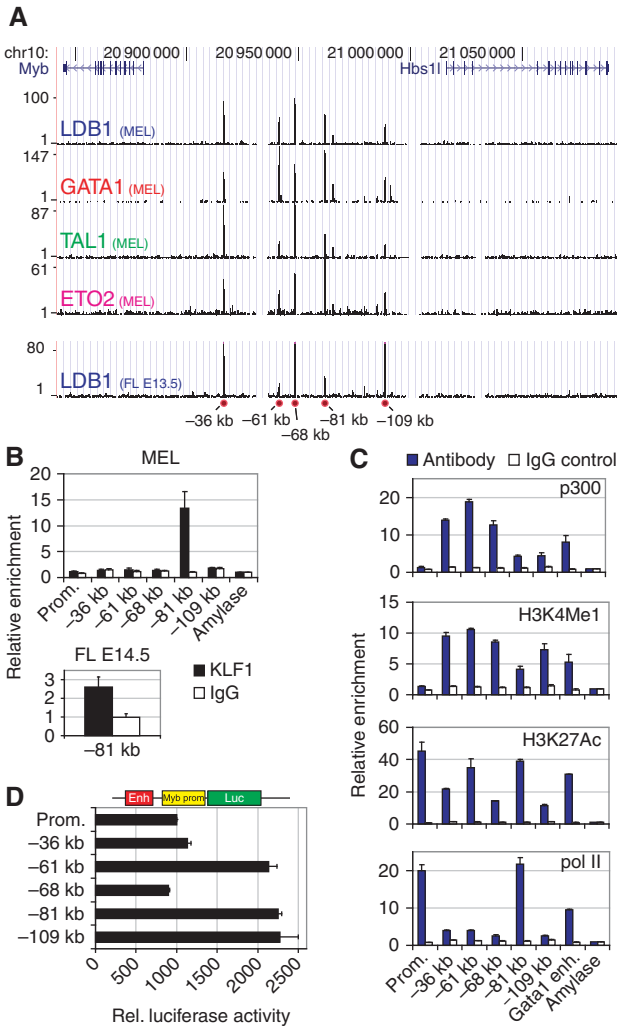

Figure 1 The *Myb-Hbs1l* intergenic region contains transcriptional enhancers. (**A**) ChIP-Seq of the LDB1 complex components LDB1, GATA1, TAL1 and ETO2 in the *Myb-Hbs1l* locus in MEL cells (MEL). LDB1 binding in primary E13.5 fetal liver erythroid progenitors is also shown (FL E13.5). The position of the intergenic binding sites relative to the *Myb* TSS is indicated at the bottom (red circles). (**B**) ChIP analysis showing intergenic KLF1 occupancy in MEL cells and in E14.5 fetal liver cells. (**C**) ChIP analysis showing the binding of p300, polII and the presence of the enhancer-associated histone modifications H3K4me1 and H3K27ac at the intergenic region in MEL cells. (**D**) Luciferase reporter assays in MEL cells showing the enhancer activity of the different intergenic elements. ChIP enrichments were calculated versus a negative control region (amylase). Results are presented as the mean ± s.e.m. of at least two independent experiments.

showed that the −61, −81 and −109 kb elements are able to enhance luciferase activity (Figure 1D). In summary, these results suggest that the intergenic LDB1 complex binding sites represent active regulatory elements in erythroid progenitors, some possessing enhancer activity *in vitro*.

In-vivo conformation of the Myb-Hbs1l locus

We next performed 3C-Seq (Soler *et al*, 2010) experiments (Supplementary Figure S3) to investigate whether the *Myb* promoter was interacting with the intergenic regulatory elements. 3C-Seq was first performed on fresh mouse E12.5 FL tissue (primarily containing erythroid progenitors) using the *Myb* promoter as the viewpoint. Fetal brain (FB) samples were processed in parallel as a control, since *Myb* expression is much lower in brain tissue and it lacks the erythroid-specific LDB1 complex. Furthermore, a previous p300 ChIP-Seq performed in FB tissue showed no enrichments within the *Myb-Hbs1l* intergenic region (Supplementary Figure S2A). Multiple promoter-interacting elements located in the intergenic region were detected in FL, of which most were either absent or showed a low signal in FB (Figure 2A and B), thus revealing erythroid-specific long-range communication between the *Myb* promoter and intergenic elements. In addition, 3C-Seq signals were shown to correlate with binding of the LDB1 complex, KLF1 and CTCF, which have all been implicated in mediating long-range chromatin interactions (Drissen *et al*, 2004; Vakoc *et al*, 2005; Splinter *et al*, 2006; Song *et al*, 2007; Figure 2B). Statistical analysis (Poisson distribution/running-mean comparison, $P \leqslant 0.001$) of the FL and FB 3C-Seq data sets confirmed the erythroid specificity of the majority of intergenic interactions (Supplementary Figure S4). Quantitative 3C-qPCR experiments were carried out to confirm these results. This shows a very similar long-range interaction pattern (Figure 2C), with the exception of the −68-kb LDB1 complex binding site that was not detected by 3C-Seq but was found interacting by 3C-qPCR. These data show *in-vivo* nuclear proximity between LDB1 complex, KLF1 and CTCF-bound intergenic sequences and the *Myb* promoter, further implying they represent regulatory elements involved in *Myb* transcriptional regulation.

To further confirm the *Myb* promoter 3C(-Seq) data, the 3C-Seq was repeated using the −36 and −81 kb LDB1 complex binding sites as viewpoints. This showed that both sites interact with the *Myb* promoter and the adjacent CTCF-bound intron 1 fragment (Figure 2B). Additionally, there were multiple interactions detected between the −36 kb/−81 kb LDB1 complex binding sites and other TF and CTCF binding sites (Figure 2B). Collectively, the 3C data show that the active erythroid *Myb* promoter and intron 1 cluster with intergenic TF-bound elements to form a complex higher order chromatin structure. Of note, these data indicate that whereas the *Myb* gene promoter is found in close proximity to the distal enhancers, both the −36 and −81 kb regions also show a strong interaction with the intron 1 CTCF site as well.

The intron 1 CTCF element marks the start of productive transcription elongation and interacts with elongation factor-bound distal enhancers

Several studies have shown that *Myb* expression is regulated at the level of transcription elongation through an attenuation site in the first intron (Bender *et al*, 1987; Watson, 1988; Reddy and Reddy, 1989; Hugo *et al*, 2006) ∼2 kb downstream of *Myb* exon 1, in the vicinity of the CTCF binding site identified in our study. Since this region interacts with the distal −36 and −81 kb elements, the intronic CTCF-bound element may actually mark the site of productive transcription elongation. Hence, ChIP experiments were carried out in erythroid progenitors to map the appearance of Serine 2 (Ser2)-phosphorylated polII (polII Ser2-P) and the H3K36 trimethylation (H3K36me3) mark, which are specifically associated with transcription elongation and peak within the transcribed region of genes (Brookes and Pombo, 2009; Buratowski, 2009; Figure 3A and C). As expected, no polII Ser2-P or H3K36me3 enrichments were detected at the promoter and upstream regions, whereas a sharp increase was seen starting around the CTCF binding site and increasing into the gene body (Figure 3A and C). Ser5-P polII on the other hand, representing the initiating polII state, specifically accumulated upstream of the CTCF site. In order to more precisely localize the transition from transcription initiation to productive elongation, ChIP-Seq for polII Ser5-P and H3K36me3 was performed in MEL cells. As shown in Figure 3B and D, the initiating polII signal covers the 5′ end of the gene and extends up to the intronic CTCF site. In contrast, H3K36me3 starts to appear after the CTCF binding site in MEL cells. In addition, a recently published H3K36me3 data set from mouse primary erythroid progenitors (Wong *et al*, 2011) and data obtained from the human erythroid cell line K562 show a similar pattern (Supplementary Figure S5). These data suggest that the transition to productive transcription elongation occurs around the intronic CTCF site. ChIP experiments were used next to analyse the presence of the elongation factors CDK9 and TIF1γ at the *Myb* locus. CDK9 is a kinase that phosphorylates the Ser2 residue of the polII C-terminal domain (CTD), and is known to bind the LDB1 complex (Meier *et al*, 2006). TIF1γ was recently identified as a component of the LDB1 complex, regulating transcription elongation in haematopoietic cells, at least in part by allowing CDK9 recruitment to its target sites (Bai *et al*, 2010). CDK9 and TIF1γ showed only minor enrichments at the promoter and first intron (where polII Ser2-P appears), but surprisingly showed a much stronger occupancy at the upstream regulatory elements (Figure 3E and F). These experiments suggest that productive elongation is stimulated around the intronic CTCF site by positive elongation factors bound at the distal enhancer elements. These factors are likely to be brought in physical proximity to the elongation site by dynamic chromatin looping, where they can transiently carry out their enzymatic function. In support of this notion, depletion of TIF1γ in primary human erythroid cells resulted in a severe reduction of *Myb* mRNA levels (Bai *et al*, 2010). In addition, in order to prove that the Ser5-P polII enrichments observed in the *Myb* first intron were specific and independent from productively elongating polII (Ser2-P), MEL cells were treated with the Cdk9 inhibitors DRB or flavopiridol. Under these conditions, a global loss of phosphorylated Ser2 RNA polII was observed (Figure 4A) and *Myb* transcription was almost completely abolished (primary transcripts are decreased by >95%, Figure 4B). Importantly, ChIP experiments showed that the Ser5-P polII pattern on the *Myb* promoter and first intron was similar in vehicle-treated cells and cells treated with CDK9 inhibitors, while Ser2-P polII enrichments were lost (Figure 4C). Thus, the Ser5-P polII occupancy of the *Myb* promoter and first intron up to the CTCF site is independent of ongoing transcriptional elongation.

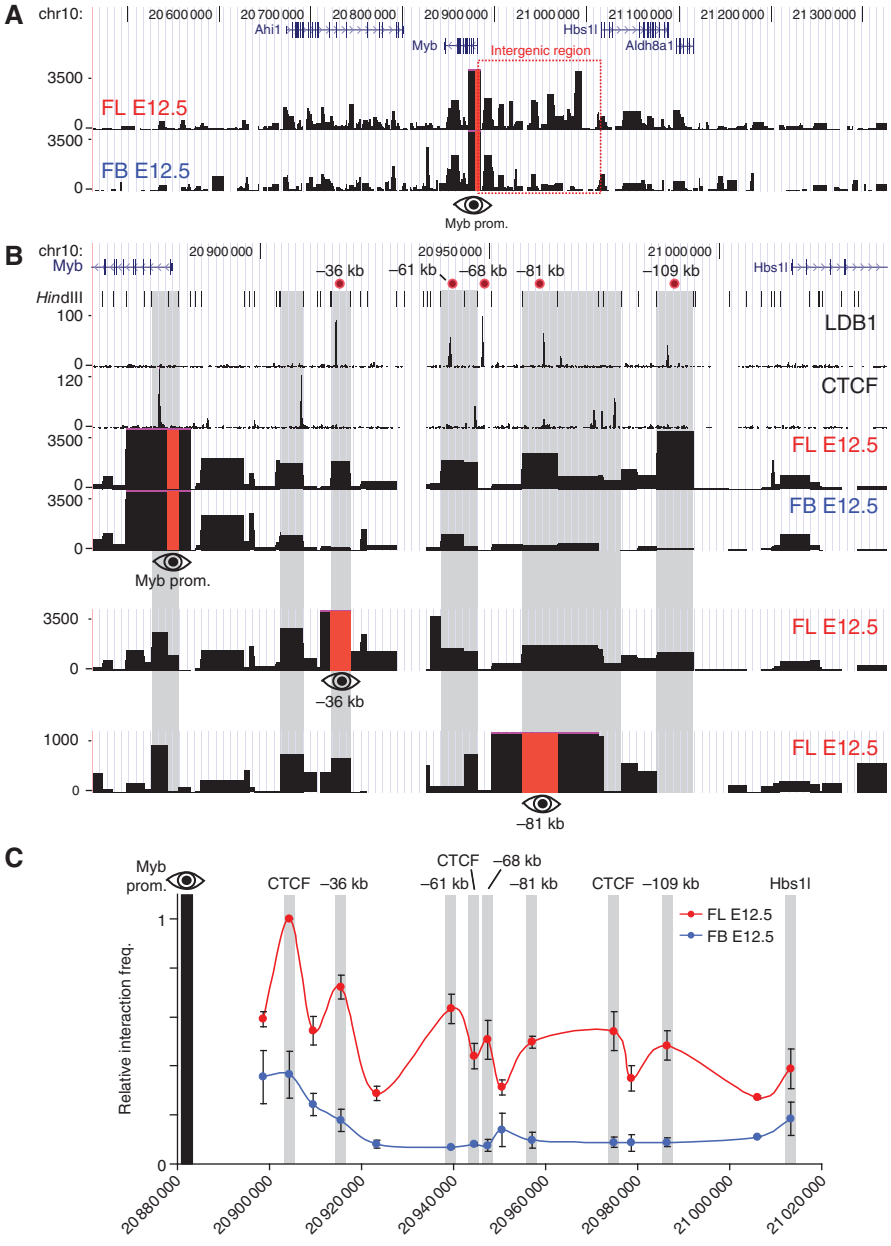

Figure 2 Long-range genomic interactions within the *Myb-Hbs1l* locus. (**A**) 3C-Seq analysis of the *Myb* promoter-associated regions *in vivo*, using E12.5 mouse fetal liver (FL E12.5) and fetal brain (FB E12.5). Signals are presented as reads per millions per *Hin*dIII restriction fragment (vertical axis). The viewpoint (*Myb* promoter) is indicated by a red bar with an eye symbol. (**B**) Zoom-in view of the *Myb-Hbs1l* intergenic region. The ChIP-Seq profiles for LDB1 and CTCF (MEL) are shown together with the 3C-Seq signals obtained using the *Myb* promoter (top), the −36 kb (middle) and the −81 kb elements (bottom) as viewpoints (indicated by a red bar and eye symbol). Grey shading of *Hin*dIII fragments indicates sites where long-range interactions and transcription factor binding colocalize. The position of the *Hin*dIII restriction sites and the intergenic enhancers (relative to the *Myb* TSS) is indicated at the top. (**C**) Locus-wide crosslinking frequencies analysed by 3C-qPCR using the *Myb* promoter as viewpoint. Relative crosslinking frequencies observed in E12.5 FL (red) and FB (blue) are shown. Highest crosslinking frequencies per FL/FB pair tested were set to 1. The *x* axis shows the genomic coordinates of the interacting fragments in the locus. Data are plotted as mean ± s.e.m. of at least three independent experiments.

In support of this, while Cdk9 inhibition results in a loss of full-length transcripts, transcription of the 5′ end of the gene is maintained and much less sensitive to Cdk9 inhibition (Figure 4D). The 40–50% decrease in 5′ transcripts compared with vehicle-treated cells can be accounted for by the general ∼50% decrease of Ser5-P polII at the promoter under these conditions (Figure 4C). Importantly, these data show that in the absence of Ser2 phosphorylation, RNA polII is still able to engage at the *Myb* gene and is still transcribing the first ∼2 kb (i.e., up to the CTCF site) but is unable to bypass this site and progress throughout the gene efficiently. Interestingly, the long-range interactions are maintained upon DRB treatment (Figure 4E).

Erythroid differentiation is accompanied by decreased Myb expression and a loss of chromatin looping

In order to correlate the long-range interactions observed in the *Myb-Hbs1l* locus with *Myb* transcriptional activity, *Myb* expression and locus structure were analysed during erythroid differentiation. Differentiation of MEL cells or mouse

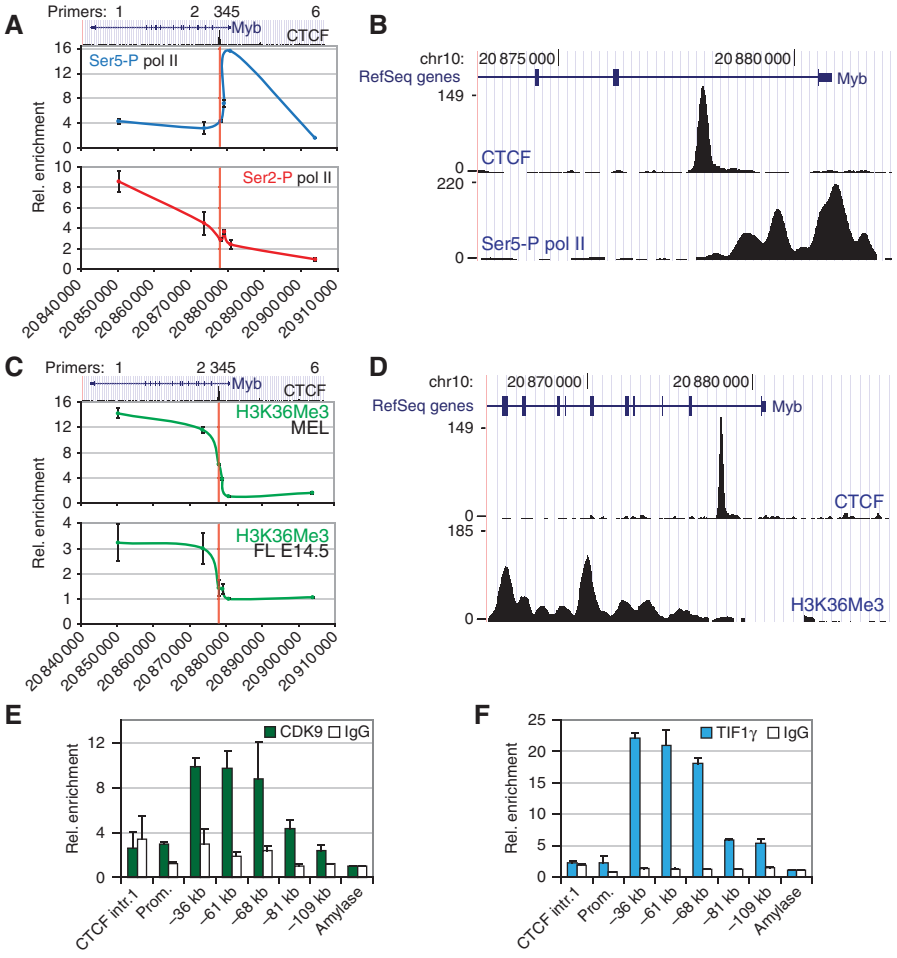

Figure 3 Transcription elongation starts in the vicinity of the *Myb* first intron CTCF element. (**A**, **C**) ChIP analysis showing the distribution of (**A**) polII phosphorylated at Ser5 (Ser5-P) and Ser2 (Ser2-P) in MEL cells, and (**C**) H3K36me3 in MEL and E14.5 fetal liver cells. UCSC Genome browser pictures depicting the *Myb* gene and CTCF binding in the first intron (ChIP-Seq) are shown above the graph. Primer pairs (1–6) used for PCR are indicated above the gene. The *x* axis shows the genomic coordinates and the position of the CTCF binding site is indicated at the top (red vertical line). (**B**, **D**) ChIP-Seq profiles of CTCF, (**B**) Ser5-P pol II and (**D**) H3K36me3 obtained from MEL cells. (**E**, **F**) Occupancy of the *Myb-Hbs1l* intergenic region by the elongation factors (**E**) CDK9 and (**F**) TIF1γ in MEL cells as shown by ChIP. Enrichments were calculated versus a negative control region (amylase) and presented as the mean ± s.e.m. of at least two independent experiments.

E13.5 FL primary erythroid progenitors resulted in a strong decrease in *Myb*, but not *Hbs1l* or *Ahi1* primary transcription (Figure 5A). Erythroid maturation of MEL and FL cells was monitored by analysing the activation of the two terminal differentiation markers Glycophorin A (*Gypa*) and Beta-Major (*Hbb-b1*) (Supplementary Figure S6A), as well as the characteristic decrease in cell size of the primary progenitors (Supplementary Figure 6B). Thus, significant downregulation of *Myb* transcription occurs upon terminal erythroid differentiation, while the flanking genes show stable or modestly increasing expression levels. 3C-Seq was subsequently carried out using the *Myb* promoter as viewpoint in MEL cells before and after differentiation, representing stages of high and low *Myb* expression, respectively (Figure 5B). In non-differentiated MEL cells, the *Myb* promoter showed a long-range interaction pattern very similar to that seen in primary erythroid progenitors (Figure 2B). However, opposite to what was observed for the β-globin locus (Palstra *et al*, 2003), the frequency of most intergenic contacts was strikingly diminished upon differentiation. This loss of interaction was observed essentially for all LDB1 complex, KLF1- and CTCF-bound fragments of the locus (Figure 5B). 3C-Seq experiments using mouse dermal fibroblasts (MDF, which do not express *Myb*) confirmed the erythroid-specific nature of the interactions (Figure 5B). The loss of chromatin looping in both MEL cells and primary erythroid progenitors upon erythroid differentiation was confirmed by the more quantitative 3C-qPCR method (Figure 5C). These data show that *Myb* downregulation upon erythroid differentiation is accompanied by a loss of communication between the *Myb* promoter and the intergenic TF-bound enhancers.

Decreased transcription and elongation factor occupancy at the intergenic enhancers upon erythroid differentiation

The long-range interactions between *Myb* and the intergenic enhancers are lost upon differentiation, clearly paralleling *Myb* downregulation. However, it is unclear what underlies the loss of looping and expression. To address this question, quantitative ChIP experiments were performed in MEL cells before and after differentiation to analyse intergenic TF occupancy during erythroid maturation. An overall decrease in LDB1 complex (Figure 6A), KLF1 (Figure 6B) and elongation factor (Figure 6C) occupancy was seen at the intergenic

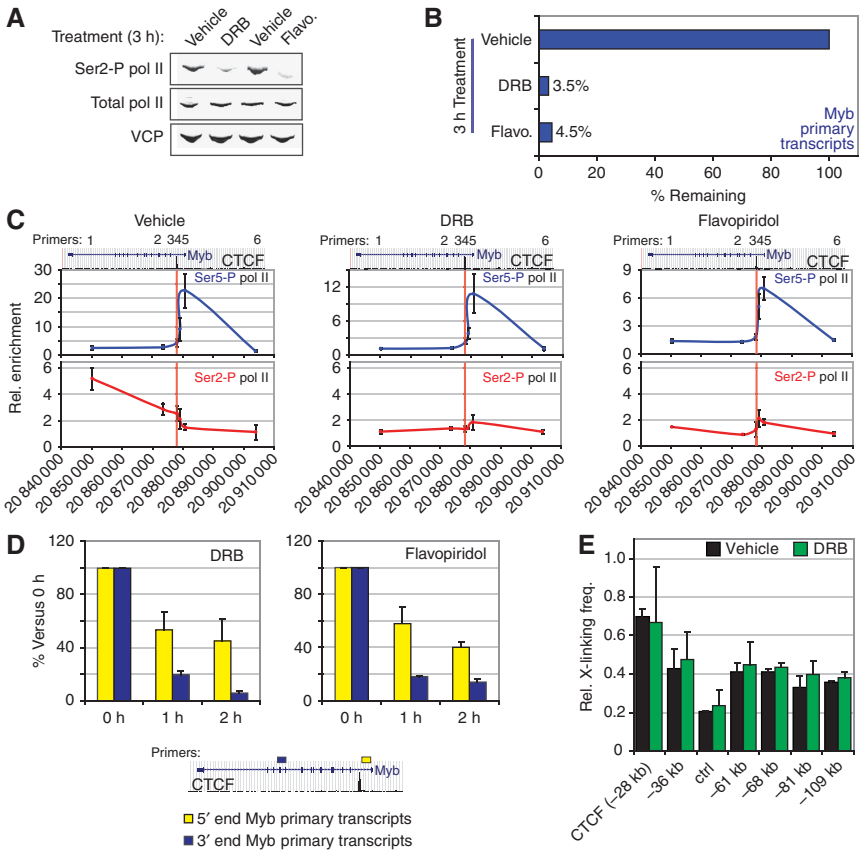

Figure 4 Effect of CDK9 inhibition on phosphorylated pol II occupancy, transcription and chromatin looping. (**A**) Western blot analysis of Ser2-P pol II and total pol II levels in vehicle-, DRB- or Flavopiridol (Flavo.)-treated MEL cells. Valosin Containing Protein (VCP) served as a loading control. (**B**) *Myb* primary transcripts measured by RT–qPCR after treatment with the indicated compounds. Signals were normalized to 18S rRNA expression and transcript levels in vehicle-treated cells were set to 100%. (**C**) ChIP analysis of Ser5-P and Ser2-P pol II binding at the *Myb* transcriptional unit in vehicle-, DRB- and Flavopiridol-treated MEL cells. Genomic coordinates, gene location, CTCF occupancy and PCR primers are indicated above each graph (as in Figure 3). (**D**) Time-course CDK9 inhibition in MEL cells using DRB and Flavopiridol. *Myb* 5′ end and 3′ end transcripts were measured by RT–qPCR. Primer locations within the gene are depicted by coloured rectangles in a schematic below the graphs. (**E**) 3C-qPCR analysis on vehicle- or DRB-treated MEL cells. The *Myb* promoter *Hin*dIII fragment was used as a viewpoint. Data are plotted as mean ± s.e.m. of at least two independent experiments.

binding sites upon differentiation. In agreement with this, the levels of enhancer-associated histone modifications and proteins often decrease as well (Supplementary Figure S7A). Furthermore, ChIP-Seq showed changing polII occupancy of the *Myb* transcription unit during differentiation (Supplementary Figure S7B). In both undifferentiated and differentiated cells, polII accumulates at the promoter and first intron (high signals), up to the CTCF site. In undifferentiated cells, polII actively bypasses this site and progresses into the gene, whereas in differentiated cells a strong reduction of polII beyond the CTCF site is seen (Supplementary Figure S7B). Thus, a loss of activating proteins at the intergenic regulatory elements upon differentiation coincides with losses of long-range interactions, polII progression into the gene body, and expression. Interestingly, initiation still appears to take place, as previously suggested (Bender *et al*, 1987).

LDB1 and KLF1 are essential for high Myb expression in erythroid progenitors

LDB1 and KLF1 have recently been implicated in long-range gene regulation (Drissen *et al*, 2004; Song *et al*, 2007; Tallack *et al*, 2010). KLF1 selectively occupies the −81-kb site in the *Myb-Hbs1l* intergenic region, while LDB1 binds all five regulatory elements (Figure 1). Since intergenic binding of both proteins decreased as *Myb* transcription is downregulated, we hypothesized that loss of KLF1 or LDB1 in erythroid progenitors would result in decreased *Myb* expression. To verify this, short hairpin RNAs (shRNAs) against *Klf1* or *Ldb1* mRNA were used to reduce their respective protein levels in MEL cells. A 50–80% decrease in mRNA and protein was observed when compared with cells transduced with a control lentivirus (Figure 7A and B). Both knockdowns resulted in a 50% decrease of *Myb* transcription, while the flanking *Hbs1l* and *Ahi1* genes were not significantly affected (Figure 7C and D). Similarly, knocking down the expression of CTCF also results in a significant reduction of *Myb* transcription, without affecting *Hbs1l* or *Ahi1* (Supplementary Figure S8C). The decrease in *Myb* primary transcripts was not caused by cellular differentiation or a change in TF levels due to LDB1 or KLF1 depletion, as we observed no changes compatible with erythroid maturation in the expression of late erythroid markers (*Gypa* and *Hbb-b1*) or key erythroid TFs (Supplementary Figure S8A and B). These results are in agreement with reports showing reduced *Myb* expression *in vivo* in *Klf1*$^{-/-}$ FL (Pilon *et al*, 2008), and a 50% decrease of *Myb* expression in bone marrow haematopoietic progenitors conditionally depleted for LDB1 (Li *et al*, 2011). Since LDB1 is a scaffold-like protein important for TF complex assembly

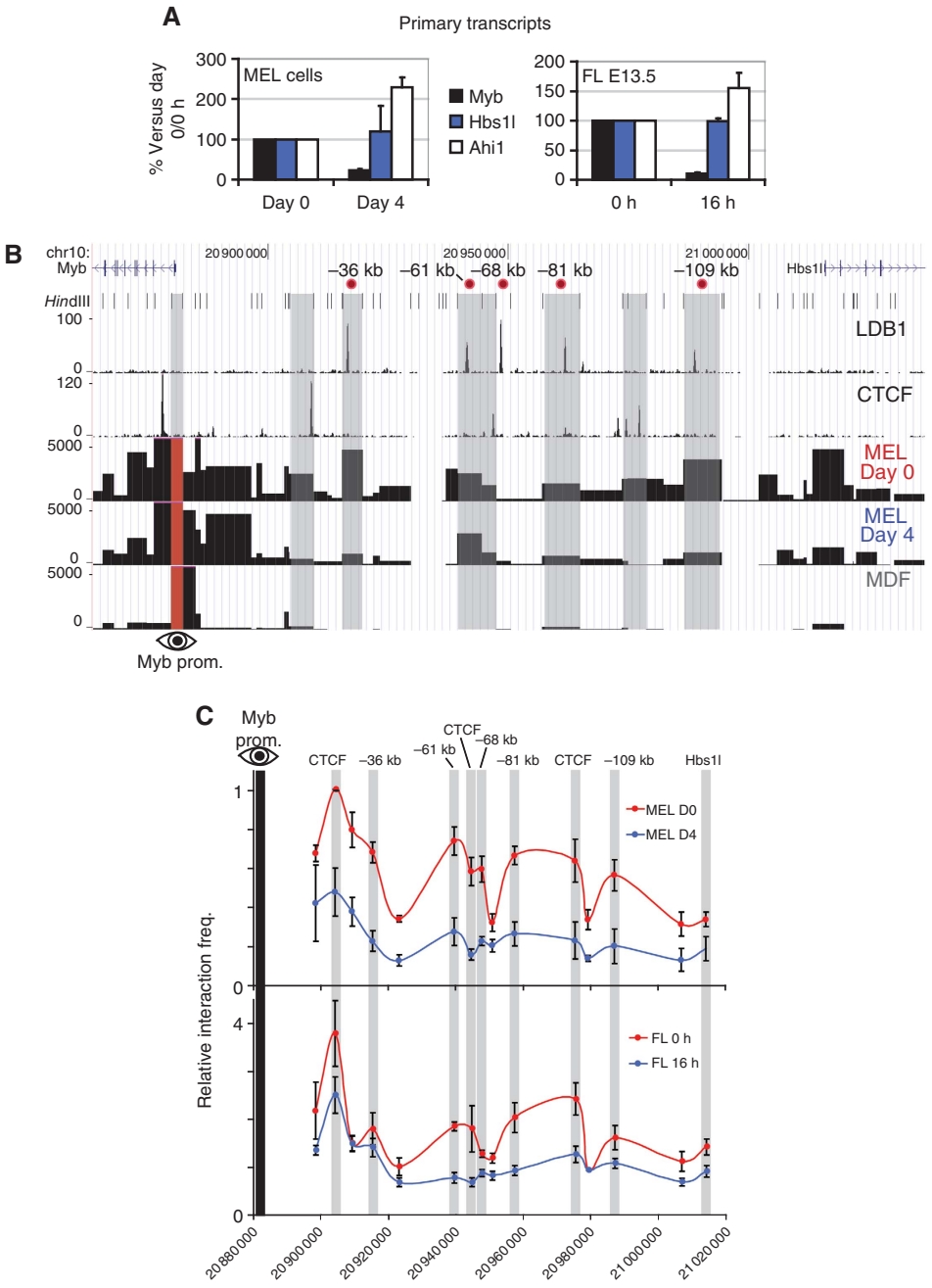

Figure 5 Erythroid differentiation is accompanied by a loss of *Myb* transcription and long-range genomic interactions. (**A**) Primary transcript levels of *Myb*, *Hbs1l* and *Ahi1* during terminal differentiation of MEL (left panel) and E13.5 fetal liver (FL) erythroid progenitors (right panel). MEL cells were induced for 4 days in the presence of 2% DMSO. Fetal liver cells were cultured *ex vivo* for 16 h in differentiation medium. Data are expressed as percentages of expression versus day 0 (MEL) or 0 h (FL) of differentiation. Signals were normalized to *Rnh1* or *Calr* expression, and day 0 (MEL) or 0 h (primary cells) values were set to 100 (i.e., undifferentiated cells). Results are plotted as mean ± s.e.m. of three independent experiments. (**B**) 3C-Seq analysis of the *Myb* promoter-associated regions in undifferentiated MEL cells (MEL day 0) and differentiated MEL cells (MEL day 4). Mouse dermal fibroblasts (MDFs) were used as a negative control (no *Myb* expression). Results are represented as in Figure 2. (**C**) Analysis of the *Myb-Hbs1l* locus conformation by 3C-qPCR in differentiating MEL and FL cells. See Figure 2C for details.

and chromatin looping, locus conformation was analysed by 3C-qPCR after LDB1 knockdown (Figure 7E). This showed that LDB1 depletion indeed results in reduced long-range promoter–enhancer contacts (Figure 7E), further emphasizing its key role in chromatin loop formation.

In summary, these data suggest that LDB1 and KLF1 form a regulatory module to maintain high levels of *Myb* transcription, consistent with the gene-activating role described for these factors in erythropoiesis (Soler *et al*, 2010; Tallack *et al*, 2010).

Discussion

The expression of the *Myb* proto-oncogene in haematopoietic cells is subjected to very tight control to properly coordinate cellular proliferation and differentiation. Given that enforced

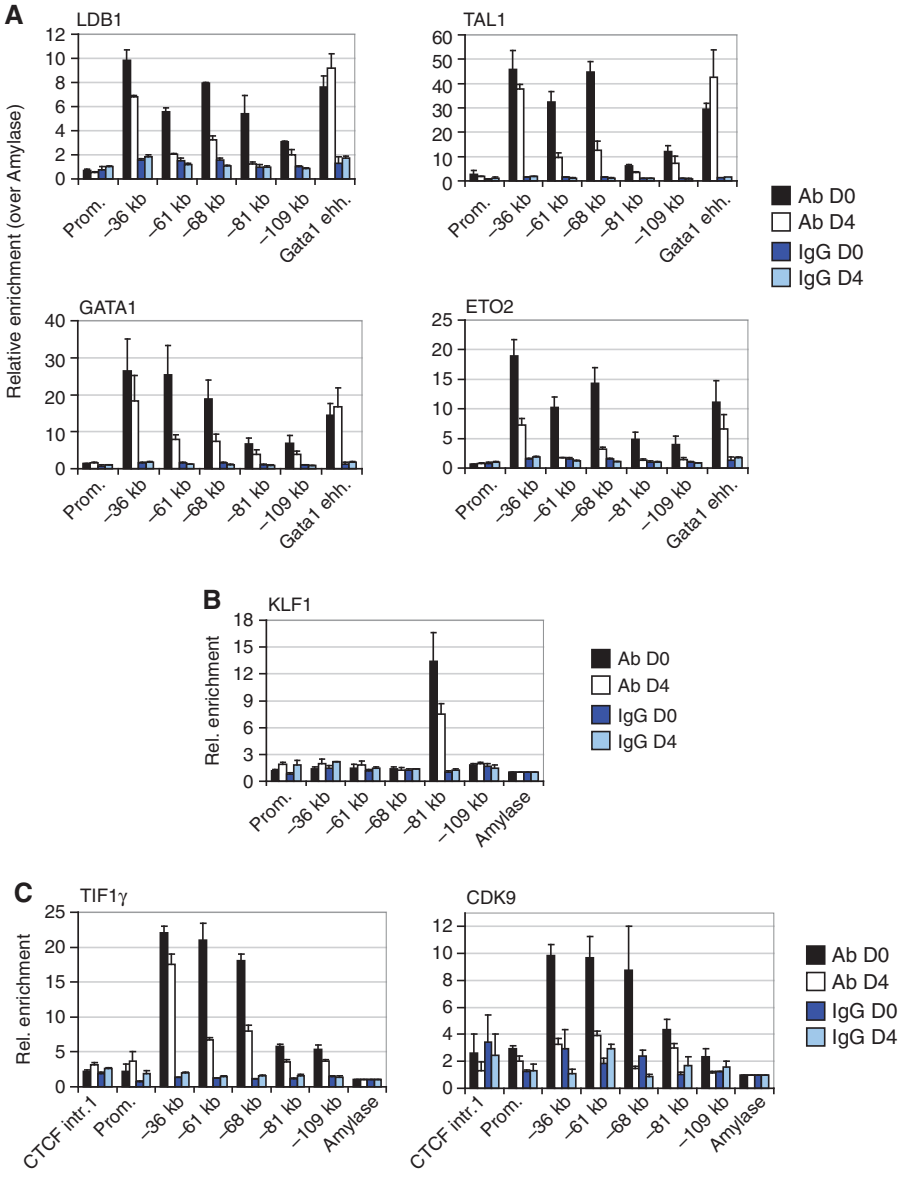

Figure 6 Erythroid differentiation induces a dramatic decrease of transcription factor occupancy within the *Myb-Hbs1l* intergenic region. (**A–C**) MEL cells were treated with 2% DMSO for 4 days to induce erythroid differentiation. Chromatin occupancy of (**A**) LDB1, TAL1, GATA1, ETO2, (**B**) KLF1, (**C**) TIF1γ and CDK9 was examined by ChIP in undifferentiated (D0) and differentiated (D4) MEL cells. Amylase served as a negative control region. Data are presented as mean ± s.e.m. of 2–4 independent experiments.

Myb expression impairs haematopoietic differentiation and that aberrant *Myb* expression associates with haematopoietic malignancies, deciphering *Myb* transcriptional control is crucial for a better understanding of both normal haematopoietic development and associated disorders.

TF binding and long-range interactions at the Myb-Hbs1l locus

A combination of ChIP-Seq and 3C-Seq was used to map the genome-wide binding sites of critical transcription and structural factors, and to characterize the spatial interactions within the *Myb* locus. 3C-Seq offers an advantage over array-based 4C technology to map long-range genomic interactions at the level of a single locus (in addition to a genome-wide level), since it does not suffer from saturating signals surrounding the viewpoints (Simonis *et al*, 2006; Soler *et al*, 2010). It is therefore well suited to analyse locus-wide chromatin looping within tens of kilobases up to megabases without prior knowledge of the interaction sites. Combining ChIP-Seq and 3C-Seq shows that the *Myb-Hbs1l* intergenic region harbours important regulatory elements controlling *Myb* expression, that bind either the structural protein CTCF or the essential erythroid TFs GATA1, LDB1, TAL1 and KLF1. The sites that bind KLF1 and the GATA1/TAL1/LDB1 complex are transcriptional enhancers, confirming the positive role of these factors on erythroid gene expression (Figure 1; Soler *et al*, 2010; Tallack *et al*, 2010).The 3C-Seq genomic interaction profiles show an erythroid-specific pattern of interactions between the *Myb* promoter, first intron and intergenic enhancers (Figure 2), which is highly similar for primary erythroid progenitors and MEL cells. CTCF, KLF1 and GATA1/TAL1/LDB1 binding sites were shown to mark the sites of long-range genomic interactions. The reproducibility be-

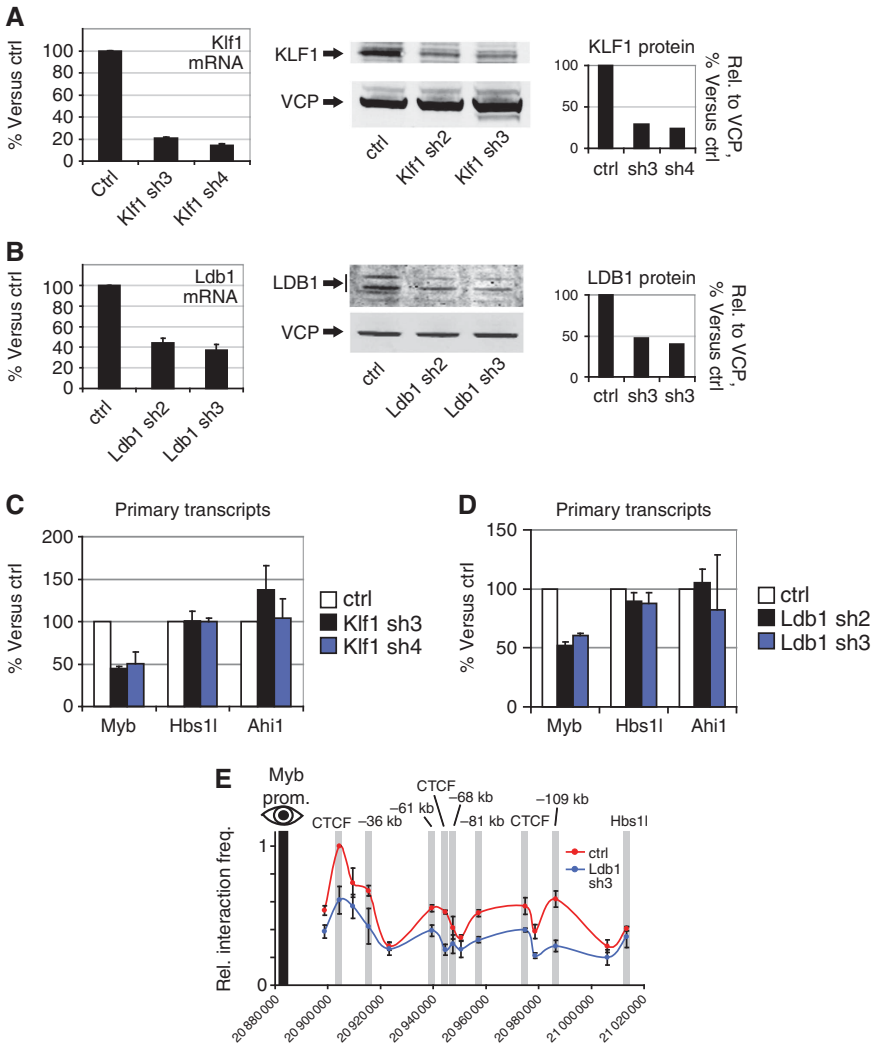

Figure 7 LDB1 and KLF1 positively regulate *Myb* expression. (**A**, **B**) Two independent shRNAs were used to decrease (**A**) *Klf1* and (**B**) *Ldb1* expression in MEL cells. Knockdown efficiency was measured at the mRNA and protein levels. Results are compared with a non-targeting scrambled shRNA. Valosin Containing Protein (VCP) served as a loading control for protein analysis. (**C**, **D**) Effect of (**C**) *Klf1* and (**D**) *Ldb1* knockdowns on *Myb*, *Hbs1l* and *Ahi1* primary transcript levels. (**E**) The effect of LDB1 depletion on chromatin looping was measured by 3C-qPCR using the *Myb* promoter as viewpoint. The interaction frequencies in control and LDB1-depleted samples are shown in red and blue, respectively. Data are plotted as mean ± s.e.m. of at least three independent experiments.

tween different biological materials, 3C-qPCR validations and the clear overlap between long-range interactions and TF binding further validate the specificity of the 3C-Seq profiles.

Both KLF1 and LDB1 activate Myb expression, and the LDB1 complex is required to establish spatial proximity between Myb and the distal intergenic enhancers

We show here a requirement for KLF1 and LDB1 in maintaining high levels of *Myb* expression in erythroid progenitors (Figure 7). Reducing the level of either of these factors results in a 50% decrease of *Myb* transcription without inducing erythroid differentiation (Supplementary Figure S8). This suggests that *Myb* downregulation coincides with, but is not a driver of differentiation. The DNA-binding erythroid Kruppel-like factor KLF1 is the founding member of the mammalian Kruppel-like family of zinc-finger TFs. It recognizes CACCC-box motifs often found in erythroid-specific gene promoters and is required for their activation. KLF1 binds a single location in the *Myb-Hbs1l* locus at the −81-kb enhancer, which contains a conserved CACCC-box motif. The positive role of KLF1 on erythroid gene expression is confirmed by our finding that KLF1 activates *Myb* transcription. Interestingly, $Klf1^{-/-}$ mouse embryos die around E15 from a lack of definitive erythropoiesis, resulting in severe anaemia (Nuez *et al*, 1995; Perkins *et al*, 1995). This phenotype shares similarities with the lethal anaemia of $Myb^{-/-}$ embryos which die around E15 (Mucenski *et al*, 1991). It has been shown that E13.5 $Klf1^{-/-}$ FL-derived erythroid cells fail to progress through the last cell cycles of terminal erythroid differentiation, in part due to misregulation of the G1-to-S phase transition TFs E2F2 and E2F4 (Pilon *et al*, 2008; Tallack *et al*, 2009). The phenotypic similarities between $Klf1^{-/-}$ and $Myb^{-/-}$ mouse models, the strong downregulation of *Myb* in $Klf1^{-/-}$ FL cells (Pilon *et al*, 2008) and the implication of Myb in the G1-to-S transition (Oh and Reddy, 1999) suggest that *Myb* misregulation in $Klf1^{-/-}$ cells also contributes significantly to the observed proliferative defect.

The widely expressed nuclear adaptor LDB1 functions as a core component of multiprotein complexes, regulating the development of many tissues. The LDB1 protein itself has no known DNA-binding or enzymatic activities. In erythroid cells, LDB1 forms a complex with the DNA-binding TFs GATA1, TAL1 (SCL), E2A and the cofactors LMO2/LMO4 and ETO2/MTGR1. In addition, the LDB1 complex interacts with transcription elongation factors, like TIF1γ and CDK9, a kinase known to regulate transcription elongation through phosphorylation of the polII CTD at Ser2. Consistent with its essential functions, $Ldb1^{-/-}$ mouse embryos do not develop beyond the E10 stage and show dramatic developmental defects including a lack of haematopoiesis (Mukhopadhyay *et al*, 2003; Li *et al*, 2010). Due to the early lethal phenotype, the role played by LDB1 during haematopoiesis *in vivo* remained largely unexplored. Recent data, however, showed a continuous requirement for LDB1 in the maintenance and differentiation of haematopoietic stem cells, and in the development of the lymphoid, erythroid and megakaryocytic lineages (Li *et al*, 2010, 2011). LDB1 is required to activate the late erythroid gene expression program (Li *et al*, 2010; Soler *et al*, 2010) and it exerts this function at least in part by facilitating long-range interactions between remote enhancers and their target genes (Song *et al*, 2007). Our analysis of the *Myb-Hbs1l* locus conformation shows that in erythroid progenitors expressing *Myb* at high levels, the enhancers are clustered in the nuclear space to form an ACH structure resembling the one observed within the active β-globin locus. We show here that LDB1 is required for the maintenance of the long-range interactions between the *Myb* gene and the upstream enhancers. Reducing the level of LDB1 in erythroid progenitors results in a decrease of *Myb* promoter–enhancer interactions and transcription (Figure 7). Interestingly, transcription of the neighbouring genes *Hbs1l* and *Ahi1* remains unaffected under these conditions, even though *Ahi1* harbours a binding site for the LDB1 complex in its first intron (Soler *et al*, 2010). During the course of erythroid differentiation, when *Myb* transcription is downregulated dramatically, the long-range interactions are reduced, resulting in a loss of the ACH (Figure 5). This loss of long-range communication is explained by the decreased occupancy of the LDB1 complex at the intergenic enhancers (Figure 6). Interestingly, decreasing the level of LDB1 results in a loss of all interactions, not just those bound by LDB1. This suggests that in order to be maintained and stabilized, the chromatin hub requires several if not all the interactions (i.e., the enhancer sites and the CTCF sites). Accordingly, affecting the binding of LDB1 on some sites would induces a destabilization of the whole structure, and thus have an impact on sites not bound by the protein but normally present in the hub.

Strikingly, this observation contrasts with the general increase of binding of the LDB1 complex on induced erythroid genes during terminal differentiation (Soler *et al*, 2010). A mechanistic explanation for this selective loss of the LDB1 complex from the *Myb-Hbs1l* locus could be that, in the late stages of differentiation, additional TFs start competing for binding or induce a local destabilization or degradation of the complex.

Heterogeneity between the distal enhancer elements

It is not clear whether *Myb* requires the entire intergenic region for full activation, because the individual contributions of the different intergenic enhancers are unknown. They could play an additive role to ensure high local concentrations of positive transcriptional regulators and therefore high levels of transcription. Alternatively, they might be required to stabilize the chromatin hub at the *Myb* gene and first intron. Such a multi-component complex structure has already been observed for developmentally regulated genes like globins. In that case the activity of the elements appears additive, although they are individually clearly different in structure and activity. For the *Myb* locus, the elements also appear to be different in function. They show different enhancer activity *in vitro*, and differ in protein occupancy. Indeed, whereas all elements are enriched for the core components of the LDB1 complex and enhancer-associated histone modifications/proteins, the −81-kb enhancer shows a 5- to 7.5-fold higher enrichment for polII and is the only one bound by KLF1 (Figure 1), a factor essential for *Myb* transcription (Figure 7). The −81-kb element also shows a high degree of sequence conservation between mouse and human. This regulatory element is therefore likely to play a key role in the transcriptional activation of the locus. Conditional deletion of the individual enhancers will provide crucial information about their role(s) *in vivo*, in particular whether the −81-kb element represents an enhancer with a specialized function.

Transcription and elongation factors at distal regulatory elements: a model for Myb transcriptional activation during development

Our data are in agreement with previous reports highlighting the regulatory potential and the importance of the *Myb-Hbs1l* intergenic region for *Myb* transcriptional regulation (Mukai *et al*, 2006; Wahlberg *et al*, 2009). In addition, the presence of regulatory elements within the *Myb* first intron affecting transcription elongation has been reported >20 years ago, although their role is still not fully understood (Bender *et al*, 1987; Hugo *et al*, 2006). An attenuation element was mapped in the first intron, where a poly-T tract was predicted to yield a stem-loop structured nascent RNA. Based on this finding, it was speculated that the stable intronic stem-loop transcript might provide a docking site for RNA-binding proteins to overcome the transcription elongation block in a way similar to the HIV TAR stem-loop RNA (Ramsay and Gonda, 2008). Although we cannot exclude this hypothesis, our data indicate that the intronic transcription elongation region corresponds to a domain containing a highly conserved CTCF binding site (coinciding with the start of the Ser2-P polII and H3K36me3 elongation signature, Figure 3), which appears to function in combination with the upstream elements. For example, the −36 and −81 kb enhancers loaded with erythroid TFs, polII and the elongation factors CDK9 and TIF1γ loop towards the *Myb* intron 1 CTCF site (Figure 2B). As the intergenic elements actively cluster together (Figure 2B) and are also bound by transcription and elongations factors (Figures 1 and 3), they are likely to contribute to the stimulation of transcription elongation. To further support this idea, we carried out CDK9 inhibition experiments (Figure 4). As stated above, CDK9 is primarily bound to the upstream regulatory elements. Its inhibition resulted in a loss of elongating (Ser2-P) polymerase and 3′ *Myb* transcription, while the initiating (Ser5-P) polymerase and 5′ transcription were retained, without affecting looping. A plausible explanation would therefore be that CDK9 is

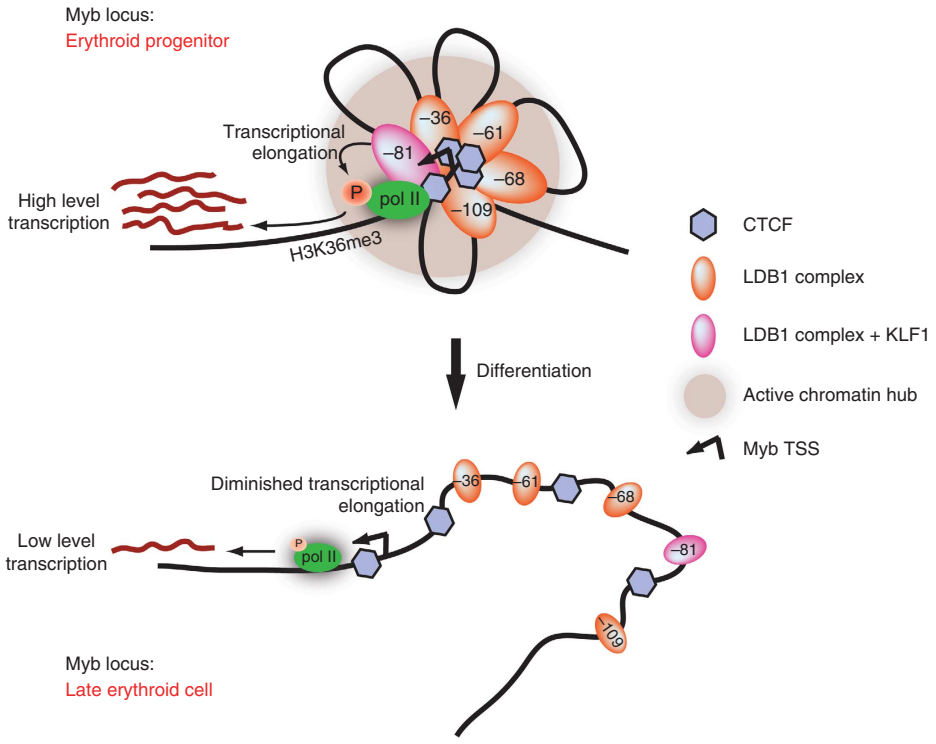

Figure 8 Model of the dynamic transcriptional regulation of *Myb* in differentiating erythroid cells. The *Myb* Active Chromatin Hub (ACH, grey sphere) is a structured nuclear compartment containing clustered *cis*-regulatory elements enriched for activating transcription factor complexes containing transcription elongation factors (orange and pink ovals) and CTCF (blue diamonds). The ACH provides a local high concentration of polII, transcription and elongation factors around the *Myb* gene, allowing for high-level expression in erythroid progenitors. During differentiation, intergenic transcription factor occupancy decreases (small ovals) at the *cis*-regulatory elements, leading to a destabilization of the ACH and a dramatic decrease of *Myb* transcription, allowing cells to terminally differentiate.

brought to the intronic transition site by looping, as represented in our model (Figure 8). As the chromatin loops were still able to form under these conditions (Figure 4E), they may have become 'non-functional' due to an inability to provide kinase activity.

Interestingly, a role for the β-globin LCR in the transition from transcriptional initiation to elongation has been proposed (Sawado *et al*, 2003). Indeed, both CDK9 and TIF1γ bind the LCR (unpublished observation). It remains to be tested whether the *Myb* and globin ACHs fulfil similar tasks in the transition to productive elongation. In the *Myb* locus, the presence of CTCF is likely to play a key role in orchestrating the long-range interactions (Splinter *et al*, 2006) and its presence is required for high level *Myb* expression (Supplementary Figure S8C). The intronic CTCF site may mark a transcriptional barrier element preventing polII from progressing further into the gene body (Supplementary Figure S7B). However, when the distal enhancers are loaded with TFs, polII and elongation factors, it would serve as an anchoring site for the enhancers to form an ACH. Clustering all the factors around the *Myb* promoter and intronic productive elongation site would then override the transcriptional block in erythroid progenitors to allow *Myb* transcription at a high rate (Figure 8, upper half). The presence of a previously suggested structured nascent RNA (Thompson *et al*, 1997; Hugo *et al*, 2006; Ramsay and Gonda, 2008) could locally cause polII to slow down, thereby increasing the chance of phosphorylation by the elongation factors bound at the distal elements. Both mechanisms could thus participate in the elongation checkpoint operating at the *Myb* intronic attenuation region. During terminal differentiation, the ACH is destabilized due to a loss of intergenic TF occupancy, resulting in decreased *Myb* transcription to allow the cells to fully mature (Figure 8, lower half).

Implications for development and disease

Since fluctuations in *Myb* expression are a common feature of differentiating haematopoietic cells, it is expected that similar mechanisms will take place in different lineages, probably using (part of) the intergenic regulatory elements described here, but bound by other lineage-specific TF complexes. Recent genome-wide studies in early haematopoietic stem/progenitor cells revealed the binding of several haematopoietic TFs on some of the *Myb* intergenic enhancers (Wilson *et al*, 2010; Li *et al*, 2011). It will be interesting to track enhancer usage and ACH formation during the course of haematopoietic stem/progenitor cell differentiation to the different lineages (e.g., myeloid versus lymphoid), and to investigate how the locus structure is affected in haematopoietic diseases like leukaemia. Importantly, our data provide a framework for further comparative analysis in human erythroid cells, where *MYB-HBS1L* allelic variants strongly associate with clinically relevant red blood cell traits and high fetal globin gene expression (Thein *et al*, 2007; Lettre *et al*, 2008; Ganesh *et al*, 2009; Galarneau *et al*, 2010), a crucial feature decreasing the severity of β-thalassaemia and sickle-cell anaemia. Several intergenic enhancers have high sequence conservation between mouse and human. Considering that the intronic CTCF and transcription elongation transition sites also seem to be conserved in human

erythroid cells (Supplementary Figure S5A), a careful examination of the impact of intergenic SNPs on TF binding, chromatin looping and *MYB* expression in individuals bearing these SNPs will be of primary interest. A preliminary analysis of highly associated SNPs showed that some fall close to or within the conserved intergenic sequences, suggesting that they may affect regulation of *MYB* expression. However, to date we did not find clear examples where the variants either create or destroy a GATA1/LDB1 binding sequence motif. A more systematic analysis needs to be performed in order to better understand the functional impact of SNPs in the *MYB-HBS1L* intergenic region. It is likely that the impact of the variants may only have a mild effect on *MYB* expression, which may complicate the analyses. However, with recent reports implicating c-MYB in the regulation of human fetal haemoglobin expression (Jiang et al, 2006; Sankaran *et al*, 2011) and the maintenance of leukaemia in mice (Zuber *et al*, 2011), modulation of c-MYB levels could become an attractive therapeutic approach in the treatment of β-haemoglobinopathies and leukaemia.

Materials and methods

ChIP and ChIP-Seq procedures

ChIP and ChIP-Seq procedures were performed as described (Soler *et al*, 2010, 2011). ChIP-Seq samples were sequenced (36 bp reads) on the Illumina GAII platform and analysed by NARWHAL (Brouwer *et al*, 2011). Data were visualized using a local mirror of the UCSC genome browser.

3C and 3C-Seq procedures

The 3C and 3C-Seq libraries were prepared as described previously (Simonis *et al*, 2006; Soler *et al*, 2010; Supplementary Figure S3). *Hin*dIII was used as the primary restriction endonuclease. The 3C PCR signals were normalized as described (Palstra *et al*, 2003), with the highest crosslinking frequency set to 1. For 3C-Seq, either NlaIII (*Myb* prom and −36 kb viewpoints) or DpnII (−81 kb viewpoint) were used as secondary restriction enzymes. The 3C-Seq library was sequenced (76 bp reads) on the Illumina GAII platform.

For more detailed Materials and methods, see the Supplementary data.

Accession codes

The ChIP-Seq and 3C-Seq data sets were deposited to the Sequence Read Archive (the accession numbers for the ChIP-Seq were previously published (Soler *et al*, 2010). 3C-Seq data can be obtained using accession number SRA048225).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: ES and FG conceived the study; RS, RJP, FG and ES designed the experiments; RS, CAS, EdB, AvdH, MS and ES performed the experiments; DE provided critical reagents and helpful comments; BL supervised informatics analyses; BL and ST designed the 3C-Seq analysis pipeline; ST, JCB and BL performed ChIP-Seq analysis; CK, AvdS and WvIJ performed ChIP-Seq and 3C-Seq DNA library preparation and Illumina sequencing; MvdH performed Illumina sequences alignments and data export; ES, FG and RS wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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