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Controlling Long-Range Genomic Interactions at a Native Locus by Targeted Tethering of a Looping Factor

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SUMMARY

Chromatin loops juxtapose distal enhancers with active promoters, but their molecular architecture and relationship with transcription remain unclear. In erythroid cells, the locus control region (LCR) and β-globin promoter form a chromatin loop that requires transcription factor GATA1 and the associated molecule Ldb1. We employed artificial zinc fingers (ZF) to tether Ldb1 to the β-globin promoter in GATA1 null erythroblasts, in which the β-globin locus is relaxed and inactive. Remarkably, targeting Ldb1 or only its self-association domain to the β-globin promoter substantially activated β-globin transcription in the absence of GATA1. Promoter-tethered Ldb1 interacted with endogenous Ldb1 complexes at the LCR to form a chromatin loop, causing recruitment and phosphorylation of RNA polymerase II. ZF-Ldb1 proteins were inactive at alleles lacking the LCR, demonstrating that their activities depend on long-range interactions. Our findings establish Ldb1 as a critical effector of GATA1-mediated loop formation and indicate that chromatin looping causally underlies gene regulation.

INTRODUCTION

Gene activity is controlled by a combination of proximal and distal regulatory elements that can be separated by up to hundreds of kilobases. Long-standing questions include how these elements interact functionally to regulate gene expression, how gene specificity is achieved, and how unwanted effects on nearby irrelevant genes are avoided. The use of chromosome conformation capture (3C) and its derivatives has revealed that distant chromosomal elements can be juxtaposed to form chromatin loops, thus providing one mechanism of long-range enhancer function (Cullen et al., 1993; Dekker et al., 2002). Chromatin looping has been discovered at numerous gene loci and reflects a widespread organizing principle of the chromatin fiber (for review, see Dean, 2011; Kadauke and Blobel, 2009; Miele and Dekker, 2008; Schoenfelder et al., 2010; Sexton et al., 2009). Although looping can occur at genes prior to their full activation, the onset of transcription is tightly associated with additional looped interactions (Palstra et al., 2003; Spilianakis and Flavell, 2004; Vernimmen et al., 2007). However, based on studies using pharmacological inhibitors of transcription elongation, it has become clear that ongoing transcription is dispensable for sustaining preformed chromatin loops (Mitchell and Fraser, 2008; Palstra et al., 2008). Moreover, chromatin looping is not limited to active genes. For example, upon repression of the Kit gene, loss of an enhancer-promoter loop is accompanied by de novo loop formation within the gene body (Jing et al., 2008). These studies indicate that chromatin loops are highly dynamic and occur at active and repressed genes but leave open the question as to whether these long-range interactions are a cause or consequence of dynamic changes in transcription initiation.

The molecular mechanisms that establish and maintain chromatin loops remain incompletely understood. Fundamental insights into these issues arose from studies of the mammalian β-globin locus, which is among the first gene clusters at which long-range chromosomal interactions between a powerful distal enhancer, the locus control region (LCR), and the target β-globin promoters were described (Carter et al., 2002; Tolhuis et al., 2002). Mechanistic studies defined gene-specific transcription factors that establish LCR-β-globin interactions, including the hematopoietic-restricted factors GATA1 and its cofactor FOG1 (Vakoc et al., 2005), KLF1 (also known as EKLF) (Drissen et al., 2004), and the more broadly expressed protein Ldb1 (Song et al., 2007). Functional disruption of any of these factors was associated with reduced LCR-β-globin interactions and diminished β-globin transcription. However, physical interactions among all of these proteins have been reported (Cantor and Orkin, 2002), making it difficult to distinguish whether they function in linear or parallel pathways. Moreover, whether the loss of

looping underlies the loss of transcription or vice versa remains an open question in these studies.

GATA1 is a DNA-binding protein essential for normal erythroid differentiation and β-globin gene expression (Evans and Felsenfeld, 1989; Pevny et al., 1991; Tsai et al., 1989). GATA elements are present at the β-globin promoter and LCR, suggesting that GATA1 and its cofactors are involved in the juxtaposition of these sites. Our understanding of the mechanisms of GATA1 function has been greatly aided by the use of the GATA1 null proerythroblast cell line G1E. Introduction of an estradiol-inducible version of GATA1 (GATA1-ER) into these cells (G1E-ER4) leads to an estradiol-dependent activation of β-globin gene transcription with concomitant LCR-β-globin looping (Vakoc et al., 2005). The transcription cofactor Ldb1 (also called NLI) does not bind DNA directly but is recruited to E box elements or GATA elements via a multicomponent complex that includes TAL1, LMO2, E2A, and GATA1. GATA1 and Ldb1 display a highly overlapping genomic occupancy pattern, but notably, Ldb1 association strongly favors sites at which GATA1 functions as a transcriptional activator, such as the β-globin locus (Cheng et al., 2009; Kassouf et al., 2010; Soler et al., 2010; Tripic et al., 2009; Wu et al., 2011). Several observations suggest that Ldb1 might be a critical effector of GATA1's looping function. First, knockdown of Ldb1 impairs LCR-β-globin looping (Song et al., 2007). Second, the *Drosophila* homolog of Ldb1, Chip, is required for long-range enhancer action (Morcillo et al., 1997). Third, like GATA1, Ldb1 co-occupies the β-globin promoter and LCR and might therefore function by physically linking the two (Song et al., 2007; Tripic et al., 2009). Fourth, Ldb1 can form homodimers and even higher-order oligomers (Cross et al., 2010; Jurata and Gill, 1997), which might underlie its role in loop formation.

Prior studies in prokaryotes (for review, see Marenduzzo et al., 2007), as well as studies in eukaryotic cells using plasmid constructs, have succeeded in influencing gene expression through forced looping among regulatory elements (Ameres et al., 2005; Mahmoudi et al., 2002; Nolis et al., 2009; Petrascheck et al., 2005). However, the use of plasmids with altered chromatin configuration and the relatively short genomic distances might limit inferences with regard to long-range chromatin interactions at native gene loci.

Here, we devised a strategy to modulate chromatin looping at an endogenous locus in its native environment. This enabled us to address whether forced chromatin looping can activate transcription, to examine the hierarchy of transcriptional regulators in chromatin looping, and to define the ensuing molecular and functional consequences. For our studies, we used G1E erythroid cells because they lack transcription factor GATA1 and thus fail to establish an LCR-β-globin loop and transcribe β-globin. Ldb1 recruitment to the β-globin promoter is entirely GATA1 dependent, whereas substantial amounts of the TAL1/Ldb1 complex remain associated with LCR in the absence of GATA1 (Figure 1A and Figure S1 available online) (Tripic et al., 2009). Therefore, Ldb1 recruitment by GATA1 to the promoter might represent a critical rate-limiting step in juxtaposing the LCR with the promoter to form a loop required for transcription initiation (Figure 1A). We tested this hypothesis by using a ZF targeting approach to tether Ldb1 to the β-globin promoter in G1E cells (Figure 1A). Notably, promoter-bound ZF-Ldb1 was capable of inducing a chromatin loop in G1E cells to an extent similar to that achieved by GATA1 restoration. ZF-Ldb1 constructs completely restored RNA polymerase II (Pol II) recruitment and Pol II serine 5 phosphorylation (Ser5ph) and partially rescued β-globin transcription. Genetic experiments in erythroid cells lacking the LCR confirmed that the ZF-Ldb1 proteins functioned via a long-range looping mechanism. These results reveal that forced juxtaposition of regulatory regions can activate transcription and establish Ldb1 as a critical rate-limiting effector of GATA1 during chromatin looping.

RESULTS

ZF-Mediated Targeting of Ldb1 to the Endogenous β-Globin Locus

As a strategy to tether potential looping factors to the endogenous β-globin locus, we chose artificial ZF proteins because they have been used successfully to target preselected genomic sites in vivo (for review, see Klug, 2010). ZFs were synthesized to target the β-major globin promoter (P-ZF) and DNase1 hypersensitive site 2 (HS2) of the LCR (L-ZF) (Figure 1B), as these sites were previously found to be in close physical proximity (Carter et al., 2002; Tolhuis et al., 2002). Each aritificial ZF protein contained six ZF domains that were linked in tandem to target 18 base pairs of genomic sequence (for review, see Klug, 2010). Target sequences were chosen within the DNase I hypersensitive regions to facilitate access to the ZFs but avoid interference with known transcription factor binding sites (Figure S1B). Binding of the ZFs to their designated DNA sequences was characterized by using a previously described ELISA-based assay (data not shown) (Bartsevich et al., 2003). ZFs were fused to a hemagglutinin (HA) tag and a nuclear localization sequence (NLS), and their chromatin binding profiles were examined by chromatin immunoprecipitation (ChIP) following introduction into G1E cells (Figures S1C and S1D; data not shown). ZFs with suitable binding properties were fused to Ldb1 and introduced into a retroviral vector containing an internal ribosomal entry site (IRES)-green fluorescent protein (GFP) or IRES-yellow fluorescent protein (YFP) cassette. Upon infection of G1E cells, populations of GFP/YFP-positive cells were purified by fluorescence-activated cell sorting (FACS) and subjected to anti-HA ChIP. We identified a P-ZF that strongly bound the β-globin promoter in G1E cells (Figure S1C). Fusion of Ldb1 with P-ZF (P-Ldb1) retained strong binding to the β-major globin promoter but was also detectable at low levels at multiple HSs of the LCR (Figure 1). In the absence of the Ldb1 moiety, this ZF bound to these LCR sites with lower efficiency (Figure S1C), indicating that the association of P-Ldb1 with the LCR is in large part due to its interaction with endogenous Ldb1 complexes at the LCR (Tripic et al., 2009). In addition, L-Ldb1 (L-ZF fused to Ldb1) was found to bind to HS2, but not to the β-globin promoter (Figure 1C), which is consistent with the lack of endogenous Ldb1 complexes in the absence of GATA1 (Figure S1A). Finally, cells coexpressing L-Ldb1 and P-Ldb1 produced comparable ChIP signals at the LCR and β-major promoter (Figure 1D).

It is noteworthy that ChIP results assessing several ZF proteins in erythroblasts or fibroblasts (data not shown) revealed that the

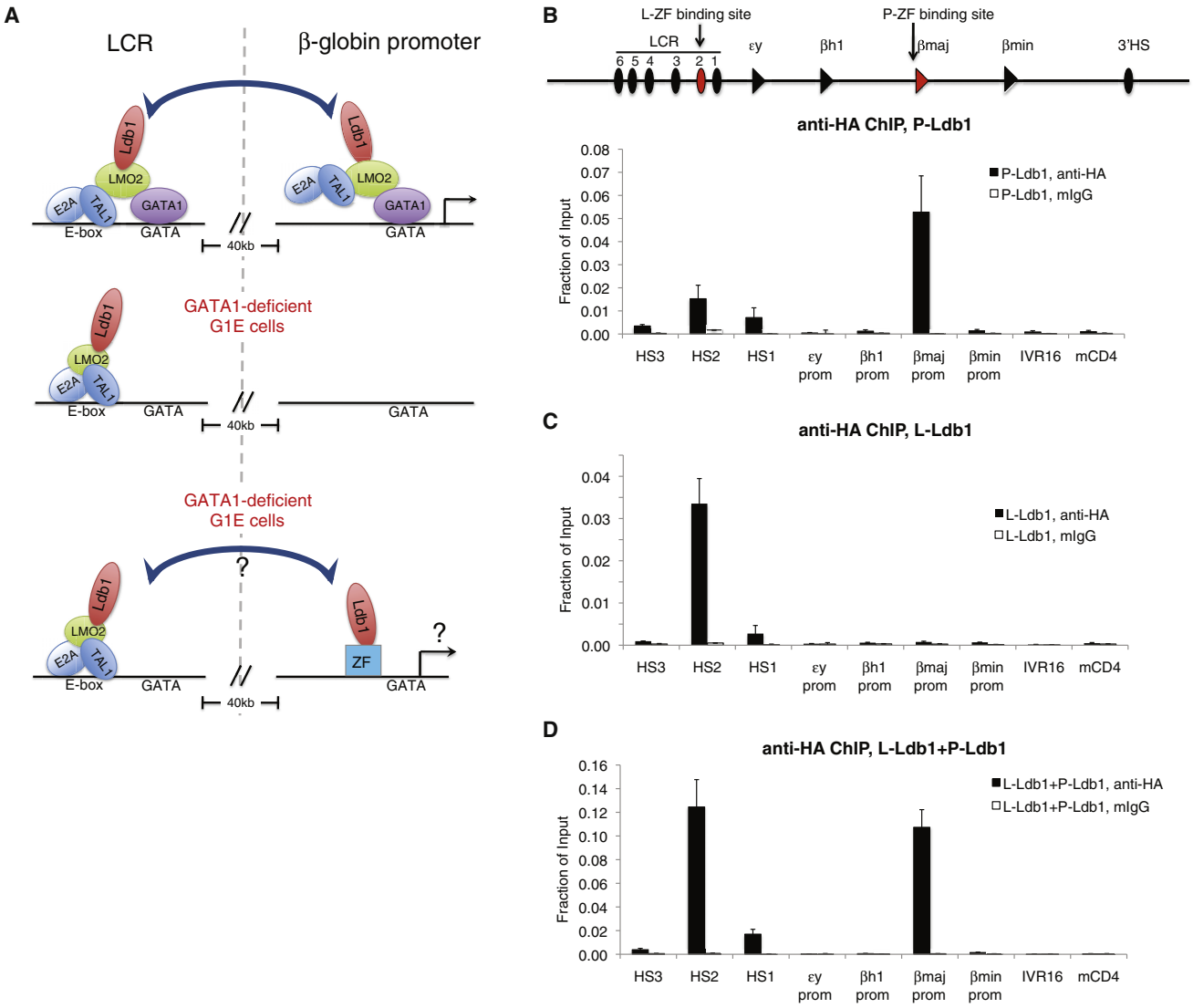

Figure 1. ZF-Mediated Targeting of Ldb1 to the β-Globin Locus

(A) Experimental model. (Top) Wild-type scenario in which GATA1 and the TAL1 complex recruit Ldb1 to promote chromatin looping. (Middle) Lack of GATA1 leads to loss of Ldb1 at the promoter, impaired looping, and reduced transcriptional activation. (Bottom) ZF-mediated Ldb1 tethering to the β-globin promoter is examined for its ability to restore looping and transcription activation.

(B) (Top) P-ZF and L-ZF target the β-major promoter (red triangle) and HS2 of the LCR (red oval), respectively.

(B–D) Anti-HA ChIP in cells expressing P-Ldb1 (B), L-Ldb1 (C), and L-Ldb1+P-Ldb1 (D). L-Ldb1 binds selectively to HS2 of the LCR. Of note, P-Ldb1 binds to the β-major (βmaj) promoter but additionally associates with HS 1, 2, and 3 of the LCR, but not to other regions, including the εy, βh1, and βmin genes, an intervening region (IVR16), or an inactive gene (CD4).

$n \geq 3$. Error bars denote SD. See also Figure S1.

binding properties of ZFs to naked DNA sequences in vitro do not fully predict their binding efficiency in vivo. Nevertheless, we were able to identify a ZF pair capable of targeting Ldb1 to the β-globin locus.

Tethering Ldb1 to the β-Globin Locus Activates Transcription in the Absence of GATA1

LCR promoter looping is required for high-level globin gene expression throughout erythroid development. Therefore, we examined whether promoter- and/or LCR-tethered Ldb1 induces β-globin transcription in G1E cells. Because G1E cells lack GATA1, the β-globin promoter is devoid of Ldb1, whereas the LCR retains significant amounts of Ldb1 mediated by the TAL1 complex bound to E box elements (Figures 1A and S1A). Remarkably, expression of P-Ldb1 activated β-globin transcription over 1,000-fold (Figure 2A), amounting to ~20% of that achieved upon restoration of GATA1 (G1E-ER4 cells) (Figure 2B). L-Ldb1 alone or ZFs without the Ldb1 moiety displayed little activity (Figure 2A). Coexpression of P-Ldb1 and L-Ldb1 failed to further activate β-globin expression compared to P-Ldb1 by

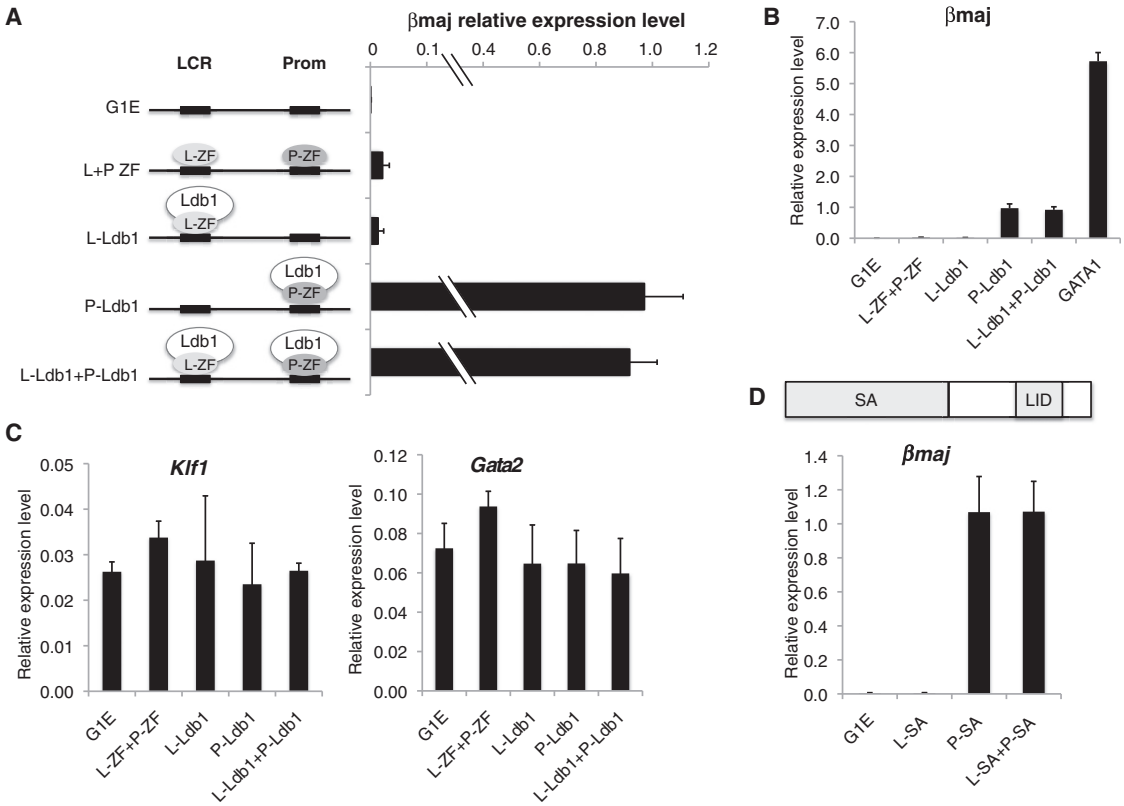

Figure 2. Activation of β-Globin Transcription in GATA1 Null Cells by Tethered Ldb1 or Its SA Domain

(A) β-major globin mRNA levels as measured by RT-qPCR with primer pairs for exon 2 in G1E cells and derivatives expressing indicated ZF and ZF-Ldb1 constructs.

(B) Data in (A) were replotted next to those obtained from induced G1E-ER4 cells (G1E+GATA1). Note that β-major expression achieved by P-Ldb1 or L-Ldb1+P-Ldb1 amounts to ~20% of that induced by GATA1.

(C) Relative expression of indicated erythroid genes as determined by RT-qPCR.

(D) (Top) Schematic of Ldb1. SA, self-association domain; LID, LIM interaction domain. (Bottom) β-major mRNA levels in G1E cells expressing indicated ZF fused to the SA domain of Ldb1. Transcript levels were normalized to β-actin.

n ≥ 3. Error bars denote SE. See also Figure S2.

itself (Figure 2A). Because high-level β-globin expression requires the LCR (Bender et al., 2000), these results suggest that promoter-bound Ldb1 is sufficient to promote long-range contacts with the LCR, presumably via endogenous Ldb1, to activate transcription (see below). Measurements of β-globin expression were confirmed with multiple primer pairs directed against the β-globin transcript (Figure S2A). Moreover, the effects of ZF-Ldb1 expression were gene specific and not simply a consequence of a general differentiation induction because the expression of several additional GATA1-activated (*Klf1*, *Eraf*, and *βh1*) and repressed (*Gata2* and *Kit*) genes was unchanged (Figure 2C; data not shown). The potent activation by ZF-Ldb1 fusion proteins of β-globin transcription is particularly remarkable because it occurred in the absence of GATA1, which is essential for β-globin transcription.

The substantial β-globin transcriptional activation by ZF-Ldb1 strongly implicates an LCR looping mechanism because β-globin transcription is reduced to ~1% of normal when the LCR is deleted (Bender et al., 2000). Moreover, Ldb1 occupancy at the β-globin promoter is normal in the absence of the LCR (Song et al., 2010), indicating that promoter-bound Ldb1 alone is insufficient for β-globin transcription without the LCR. Although β-globin activation by ZF-Ldb1 fusion proteins was substantial, their effects did not match those of GATA1, which is consistent with GATA1 exerting functions in addition to chromatin looping.

Tethering of the Ldb1 Self-Association Domain Is Sufficient for β-Globin Activation

Ldb1 contains an N-terminal self-association (SA) domain that mediates the assembly of higher-order molecular complexes and might account for its looping function (Cross et al., 2010; Xu et al., 2003). Ldb1 also contains a C-terminal LIM interaction domain (LID) that confers binding to LMO2 and its associated GATA1/TAL1/E2A multiprotein complex. To examine whether the SA domain is sufficient for transcription activation, it was fused with L-ZF and P-ZF and introduced into G1E cells. P-SA and L-SA showed very similar genomic binding profiles as the full-length Ldb1 fusion constructs, such that L-SA occupied HS2, whereas P-SA bound the β-major globin promoter and, additionally, the LCR (Figure S2B). Remarkably, expression of

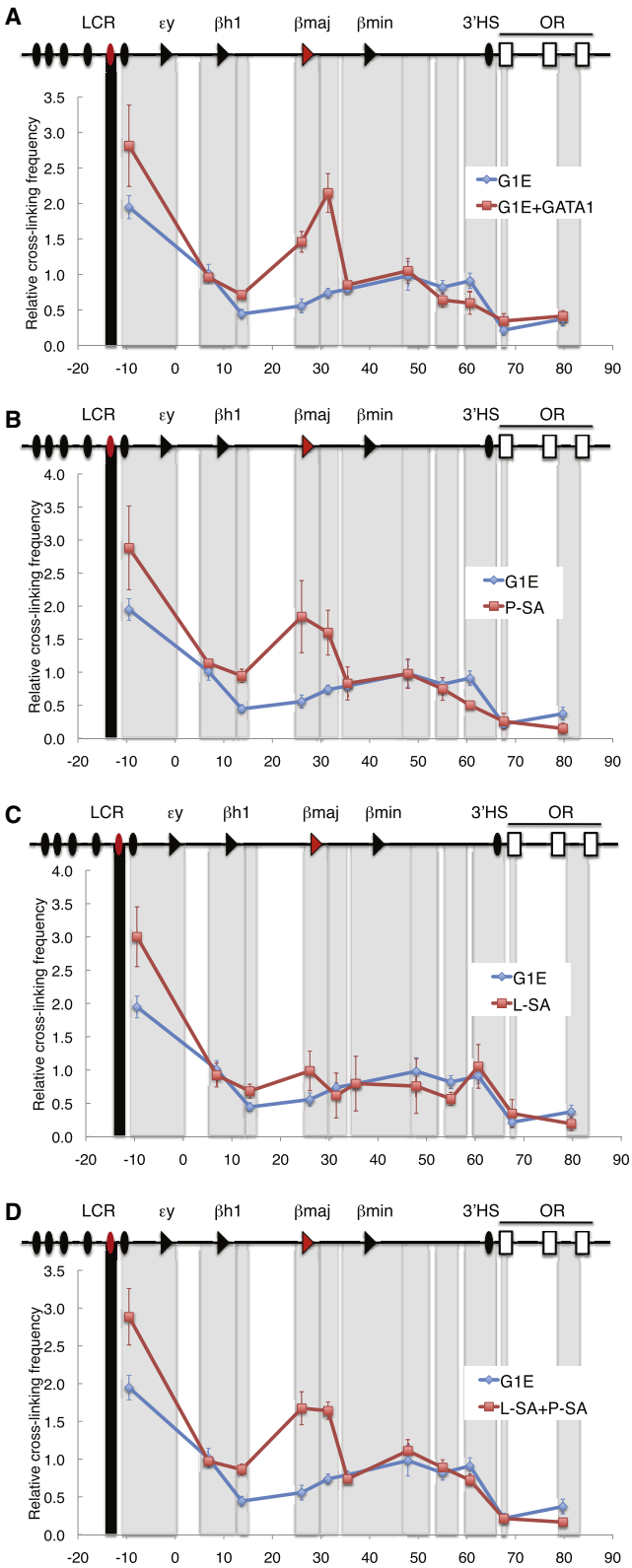

Figure 3. Chromatin Looping by the Tethered Ldb1 SA Domain

(A–D) 3C assay measuring locus-wide crosslinking frequencies in G1E cells (blue), induced G1E-ER4 (G1E+GATA1) (A, red), or G1E cells containing P-SA (B, red), L-SA (C, red), or L-SA+P-SA (D, red). The murine β-globin locus is depicted on top of each graph. The x axis indicates distances (kb) from the εy gene, which represents zero. Black bar denotes the HS2-containing BglII fragment serving as anchor. Gray bars denote analyzed BglII fragments. OR, olfactory receptor genes.

(A, B, and D) n = 3. (C) n = 2. Error bars indicate SEM. See also Figure S3.

P-SA alone or coexpression of L-SA and P-SA activated β-globin gene transcription to virtually the same level as did the full-length Ldb1 fusion proteins (Figures 2D and S2C). Again, the effects of ZF-SA were gene specific and did not globally alter erythroid gene expression (Figure S2C). These results suggest that the Ldb1 self-association domain is sufficient to induce β-globin transcription, further supporting the idea that forced juxtaposition between the LCR and β-globin promoter underlies transcriptional activation.

We also considered the possibility that the remaining portions of Ldb1 might participate in chromatin looping by nucleating higher-order protein complexes. To this end, we generated a ZF-Ldb1 fusion protein lacking the SA domain (P-ΔSA) but left the nuclear localization sequence and LID domain intact. P-ΔSA was capable of inducing β-globin transcription, albeit to a significantly lower degree than P-SA (Figure S2D). Activation never exceeded 50% of that observed with P-SA, even under the most optimal conditions and expression levels (Figure S2D; data not shown). This supports the idea that the SA domain is most efficient in nucleating higher-order complexes required for looping. Nevertheless, these results are also consistent with the possibility that Ldb1 can engage its partner proteins via distinct domains to produce chromatin loops.

Tethering of the Ldb1 Self-Association Domain Induces LCR-Promoter Looping

The strong induction of β-globin transcription by ZF-Ldb1 or ZF-SA implicates an involvement of the LCR and, hence, chromatin looping, because in the absence of the LCR, β-globin transcription is very low (Bender et al., 2000). Therefore, we examined by 3C assay whether expression of ZF-SA constructs juxtaposed the LCR with the β-globin gene to form a chromatin loop (Figure S3). Using HS2 as the anchor region, we found that, in parental G1E cells, the 3C signals generally declined with increasing distance (Figure 3A), which is consistent with our previous observations (Vakoc et al., 2005). In particular, there is no interaction between HS2 and the β-globin genes. Upon GATA1 restoration, the relative proximity of HS2, with two adjacent fragments comprising the β-major globin gene, significantly increased (Figure 3A). HS2 interactions with intervening or downstream segments remained low, indicative of a GATA1-dependent HS2-β-globin chromatin loop (Vakoc et al., 2005). We next determined the chromatin conformation of the β-globin locus in G1E cells expressing ZF-SA proteins. Strikingly, expression of P-SA alone, but not L-SA, produced a strong HS2-β-globin chromatin loop, recapitulating the chromatin conformation induced by GATA1 (Figures 3B and 3C). Thus, recruitment of the SA domain to the β-globin promoter is sufficient for juxtaposition with the LCR, likely via interaction with endogenous LCR-bound Ldb1 (Figures S1A and S2B, model in Figure 1A). Coexpression of P-SA and L-SA triggered juxtaposition of HS2 with the β-globin

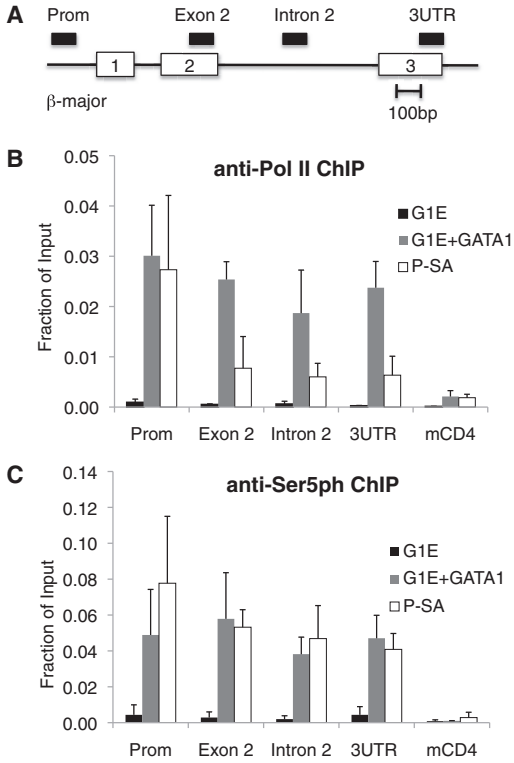

Figure 4. Restoration of Pol II Recruitment and Ser5ph by ZF-SA

(A) Location of amplicons (black bars). Prom, promoter; numbers indicate exons.

(B and C) ChIP with antibodies against total Pol II (B) or Ser5ph (C) using G1E cells, or G1E cells expressing GATA1 or P-SA. Note that, whereas total Pol II binding at the promoter matched that induced by GATA1, Pol II levels in the body of the gene were only partially restored in P-SA cells, which is consistent with incomplete rescue of transcriptional elongation (compare with Figure 2B). n = 3. Error bars denote SD. See also Figure S4.

gene with a similar efficiency as the P-SA alone (Figure 3D). Given the lower levels of occupancy of P-SA at HS2 in comparison to L-SA, it was surprising to find that P-SA was as active as the combination of L-SA plus P-SA or GATA1. It is possible that the ChIP signal for P-SA at the LCR underrepresents the amounts of P-SA because proteins indirectly associated with DNA are not crosslinked as efficiently. Moreover, P-SA association with multiple regions in the LCR via endogenous Ldb1 likely adds to its ability to promote loop formation. Nevertheless, forced LCR-β-globin chromatin looping correlated well with activation of β-globin transcription.

In concert, these results show that tethering the SA domain of Ldb1 to the β-globin promoter is sufficient to produce an LCR-β-globin chromatin loop that is similar, if not identical, to that generated by GATA1. This strongly suggests that Ldb1 is an essential rate-limiting effector of GATA1 during chromatin looping. More generally, juxtaposition of an LCR with a promoter causes strong gene activation.

ZF-SA Expression Produces LCR-Dependent Functions

Two of the key functions of the β-globin LCR are the recruitment of Pol II to the β-globin promoter and the stimulation of Pol II phosphorylation at serine 5 of its C-terminal domain, a modification associated with early transcription elongation (Sawado et al., 2003). Hence, if ZF-SA proteins activate β-globin transcription by promoting LCR-β-globin contacts, they are expected to stimulate Pol II recruitment and Ser5ph. To examine the extent to which ZF-SA fusion proteins restored LCR-dependent functions, we performed ChIP with antibodies against Pol II Ser5ph or with antibodies that react with Pol II regardless of its phosphorylation state. Notably, expression of P-SA triggered Pol II recruitment to the β-globin promoter with an efficiency similar to that achieved by GATA1 expression (Figures 4A and 4B). In contrast, Pol II levels in the body of the gene amounted to ~25%–30% of those found in GATA1-expressing cells, corresponding well with the levels of β-globin mRNA production (Figure 2B). This is consistent with reduced recruitment of the elongation complex P-TEFb to the β-globin promoter and the body of the gene when compared to GATA1-expressing cells (as measured by anti-CDK9 ChIP; Figure S4A). The amounts of Pol II Ser5ph found at the β-globin gene in P-SA-expressing cells were indistinguishable from those observed in GATA1-expressing cells (Figure 4C). As an additional measure of transcription, we determined the level of histone H3 lysine 4 trimethylation (H3K4me3) and found that P-SA restored this mark to levels equal to that produced by GATA1 (Figure S4B). Similar results were obtained in cells coexpressing P-SA and L-SA (data not shown). These results demonstrate that two functions of the LCR, i.e., Pol II recruitment to the β-globin promoter and Pol II Ser5ph, were completely restored by expression of P-SA, lending additional support to the idea that juxtaposition of the LCR with the β-globin promoter underlies the activity of P-SA. The failure to fully restore transcription elongation can be explained by the lack of GATA1 and its cofactors that exert additional looping-independent functions, possibly including the recruitment and activation of P-TEFb complex (Bottardi et al., 2011; Elagib et al., 2008; see Discussion).

Precocious Induction of β-Globin Transcription by ZF-SA Fusion Proteins in Primary Erythroblasts

We examined whether ZF fusion proteins function in primary erythroid progenitor cells to activate β-globin expression. The maturation stage of primary erythroid progenitor cells from E13.5 wild-type (WT) fetal livers was monitored by flow cytometry measuring the expression of the cell surface markers Ter119 and CD71 (Zhang et al., 2003). Cells progress through the R1, R2, R3, and R4 stages of maturation (Figure 5A) and ultimately produce abundant amounts of β-globin (Figure S5A). For the expression of ZF-SA proteins, we purified Ter119^{-} and CD71$^{-/low}$ cells (R1 population in Figure 5A) representing early precursor cells. At this stage, the β-globin genes are not yet highly active, but cells express low levels of essential regulatory factors, including GATA1 and KLF1 (Figure S5B). Following infection with retrovirus-expressing ZF-SA fusion proteins, cells were cultured in defined medium containing cytokines IL-3, IL-6, and SCF to preserve the cells in the precursor state. Remarkably, expression of P-SA only or P-SA/L-SA, but not L-SA alone, precociously activated β-globin transcription (Figure 5B). Note that the fold activation over control was not as pronounced as that observed in the G1E system

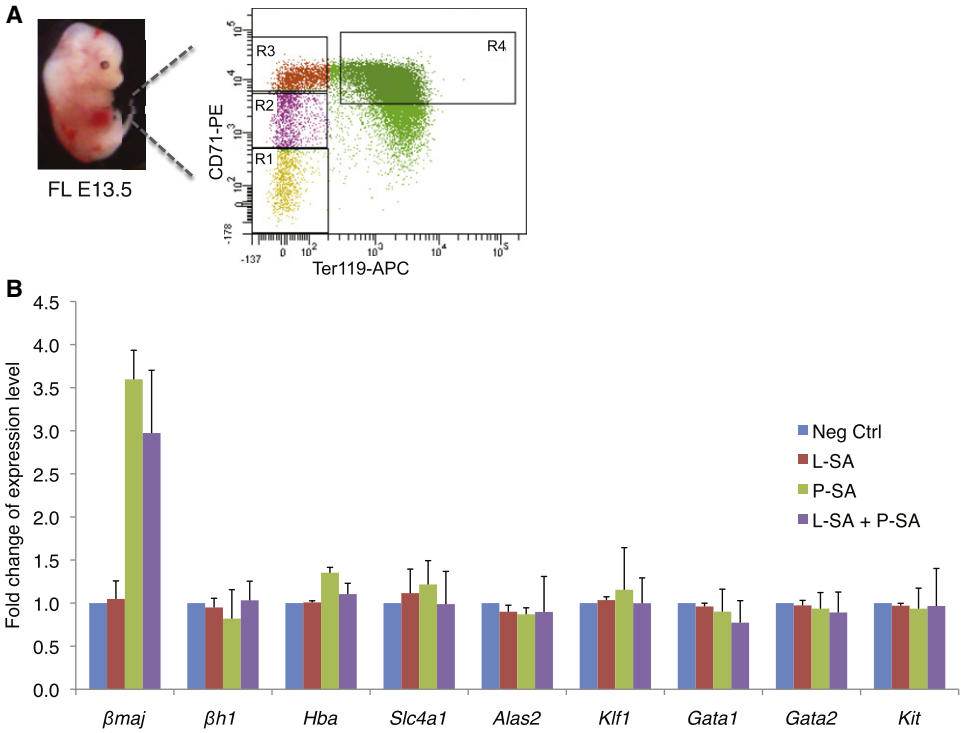

Figure 5. ZF-SA Enhances β-Globin Expression in Primary Erythroid Progenitor Cells

(A) Staging of E13.5 fetal liver erythroid cells by Ter119 and CD71 profiling.

(B) mRNAs from FACS-purified R1 cells transduced with ZF constructs were examined by RT-qPCR with primers for the indicated genes. Negative controls (arbitrarily set to one) (Neg Ctrl) represent cells expressing empty vector. Results were normalized to GAPDH.

n = 3. Error bars denote SD. See also Figure S5.

because, in contrast to the latter, primary erythroblasts are replete with transcription factors and produce higher levels of β-globin, even prior to full maturation. Nonetheless, these results in essence mirrored those from G1E cells in that the same combinations of ZF fusion proteins were capable of activating β-globin expression. The effects were specific to the β-globin locus, as no other erythroid genes examined were altered in their activities (Figure 5B). Moreover, ZF-SA expression did not nonspecifically promote erythroid maturation, as determined by flow cytometry using CD71 and Ter119 surface markers (Figure S5C). Together, these results show that ZF-SA fusion constructs can activate β-globin transcription in primary erythroid cells.

ZF-SA Fusion Protein Induction of β-Globin Transcription Is LCR Dependent

Targeting of the SA domain to the β-globin locus restores juxtaposition of HS2 with the β-globin gene, Pol II recruitment, and Pol II Ser5ph, strongly suggesting that transcriptional activation is due to LCR-β-globin looping. The prediction from these observations is that alleles lacking the LCR would not respond to ZF-SA fusion proteins (Figure 6A). Alternatively, if β-globin transcription simply resulted from SA-induced transcription factor assembly at the β-globin promoter, then ZF-SA should activate transcription independently of the LCR. This distinction is especially important in light of the positive effects on β-globin transcription exerted by the expression of P-SA alone. To definitively distinguish between these possibilities, we examined ZF-SA's functions in E13.5 fetal liver erythroblasts derived from mice that are heterozygous for a deletion of the LCR (ΔLCR/+) (Bender et al., 2000). The β-major gene on the ΔLCR allele is of the D haplotype, whereas that on the WT allele is of the S haplotype. We developed an allele-specific qPCR assay that distinguishes single nucleotide polymorphisms between the transcripts of these alleles (Figure S6), providing an ideal internally controlled experimental setup.

Next, we transduced ΔLCR/+ R1 cells with viral vectors expressing ZF-SA proteins and exposed them to erythropoietin for 6 hr to promote erythroid maturation. Allele-specific RT-qPCR demonstrated that the WT allele (βmaj-S) was activated in cells expressing L-SA together with P-SA or P-SA alone (Figure 6B, left). L-SA had little or no activity similar to ZFs lacking SA that served as negative controls. In striking contrast, the β-major gene on ΔLCR allele (βmaj-D) was expressed at low levels and showed very little response to the P-SA/L-SA or P-SA proteins (Figure 6B, middle). The effects of ZF fusion protein expression were essentially the same in the presence or absence of erythropoietin and were specific to the β-globin locus, as none of the other examined erythroid genes were altered in their activities (Figure S7). The residual signal produced by the D-allele-specific primers was not due to transcription from the D allele but was due to the result of cross-hybridization with S allele cDNA. This

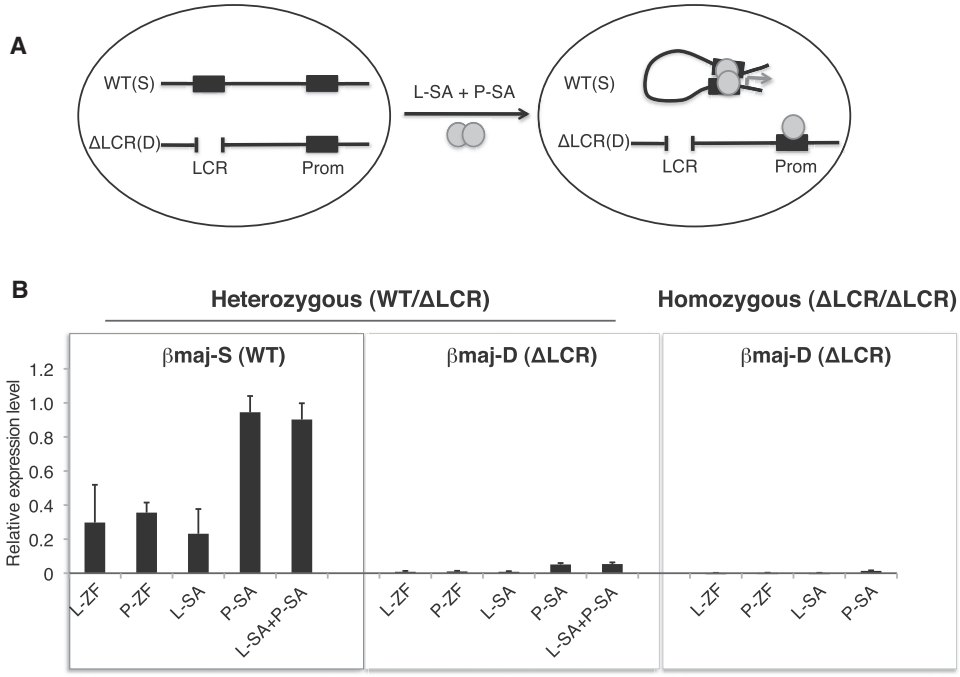

Figure 6. LCR Dependence of β-Globin Induction by ZF-SA Proteins

(A) Experimental concept. The LCR-deleted allele is on the background of the β-major D haplotype, whereas the WT allele is on the background of the β-major S haplotype.

(B) β-major mRNA levels as measured by allele-specific RT-qPCR in R1 cells from WT/ΔLCR or ΔLCR/ΔLCR fetal livers expressing indicated ZF-SA proteins. Transcript levels were normalized to GAPDH.

n = 3. Error bars denote SD. See also Figures S6 and S7.

was demonstrated by template mixing experiments showing that ~10% of the signal produced by the D-allele-specific primers derived from cross-reactivity with the S allele cDNA (Figure S6). Indeed, when homozygous (ΔLCR/ΔLCR) R1 cells were transduced with P-SA, β-globin activation was close to background, establishing that the low signal obtained with D-specific primers in ΔLCR/WT cells was in fact due to cross-hybridization (Figure 6B, right). In concert, the results clearly demonstrate that the activity of ZF-SA proteins is entirely dependent on the presence of LCR and hence on long-range chromatin looping.

DISCUSSION

Here, we employed a ZF targeting strategy to address critical questions concerning the higher-order organization of the chromatin fiber. Targeting the SA domain of Ldb1 to the endogenous β-globin locus compensated to a significant extent for the loss of GATA1, strongly suggesting that Ldb1 serves as an effector of GATA1 during chromatin loop formation. Forced chromatin looping by ZF-SA proteins at a native gene locus caused considerable transcriptional activation, indicating that the juxtaposition of an enhancer with a promoter causally underlies gene induction.

Expression of P-SA by itself produced effects very similar to those of P-SA and L-SA coexpression. Several independent lines of investigation demonstrate that, in P-SA-expressing cells, forced loop formation accounts for β-globin activation. First, 3C experiments clearly showed that tethering the SA domain to the β-globin promoter fostered genomic contacts that strongly resembled those induced by GATA1 with regard to both their spatial configuration and efficiency. Second, SA domain recruitment completely restored several LCR-dependent functions at the β-globin promoter, including Pol II recruitment, Ser5ph of Pol II, and H3K4 methylation. Third, targeted deletion of the LCR dramatically reduced β-globin transcription without diminishing the amounts of promoter-bound Ldb1 (Song et al., 2010). Therefore, tethering Ldb1 or its SA domain to the promoter is not expected to produce such pronounced effects without an involvement of the LCR. Fourth, P-SA and P-SA/L-SA induction of β-globin expression was entirely dependent on the LCR, confirming an underlying looping mechanism. The ability of P-SA to potently induce loop formation is most likely explained by its ability to interact with endogenous Ldb1-containing complexes that reside at the LCR even in the absence of GATA1 (Figure S1A) (Tripic et al., 2009). In contradistinction, Ldb1 association with the β-globin promoter is entirely GATA1 dependent and hence might represent a critical and rate-limiting step during chromatin looping and high-level transcription.

The observation that the SA domain is sufficient to induce long-range chromatin interaction implies that SA of Ldb1 is a major molecular force tying together anchored chromatin regions. Importantly, the SA domain can form multimers (Cross

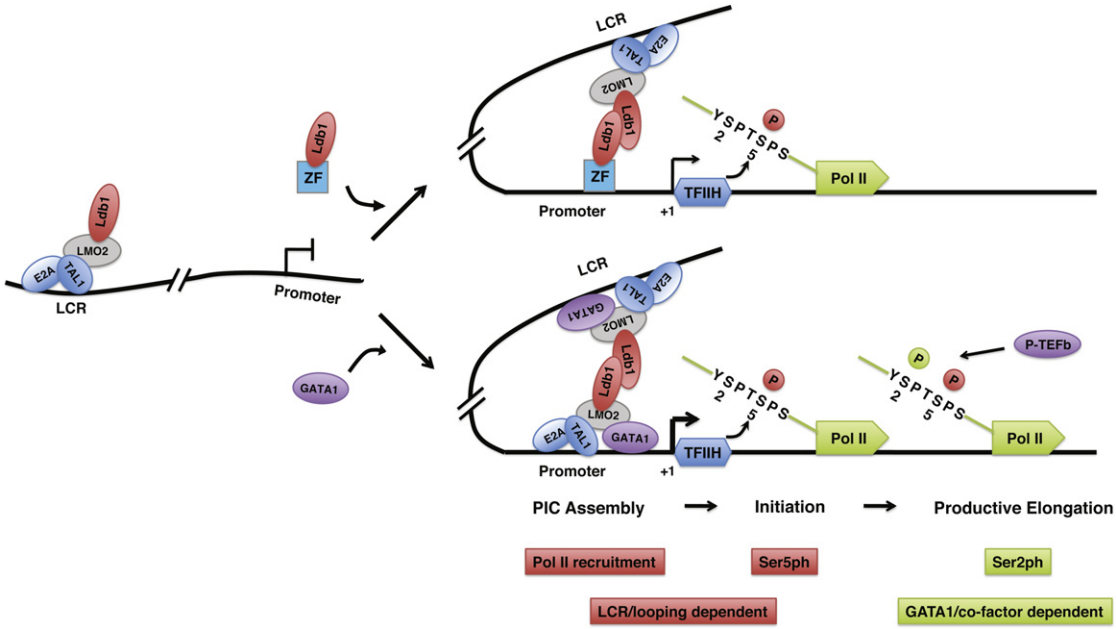

Figure 7. Hypothetical Model Functionally Integrating Chromatin Looping and Transcription Activation

Recruitment of Ldb1 to the β-globin promoter either by ZF proteins or GATA1 promotes LCR promoter looping. Forced chromatin looping by ZF-Ldb1 efficiently restores preinitiation complex (PIC) assembly, Pol II recruitment, Pol II Ser5ph, and transcription initiation. In the absence of GATA1, diminished recruitment of P-TEFb and likely additional GATA1 cofactors account for inefficient transcription elongation. Therefore, chromatin looping can trigger transcription initiation and can occur independently of full transcription elongation.

et al., 2010), allowing for the formation of higher-order complexes that might serve to stabilize interactions between distant chromatin fragments. However, the SA-deleted form of Ldb1 was also active, suggesting that the LID domain is also capable, albeit with lower efficiency, of recruiting the endogenous Ldb1 complex to promote long-range interactions.

Although it is conceivable that, in the simplest terms, the mere dimerization of DNA-bound factors should be capable of inducing chromatin loops, we speculate that multiple contacts are required to provide the requisite specificities and affinities. Moreover, the folding of the chromatin fiber can occur in complex patterns involving simultaneous interactions between multiple segments to form what are called chromatin hubs. Simple protein dimers might be insufficient to accommodate such complex interaction patterns. In agreement, fusion of ZFs with diverse dimerizing domains (lexA, p65NFkB, and the Argent dimerization system) or protein modules that can form multimers, such as the POZ domain of GAGA factor, failed to efficiently activate β-globin expression (W.D., unpublished data). Thus, Ldb1 might have evolved to promote such interactions by forming homomultimers and by engaging numerous gene-specific transcription factors, including the LMO2/TAL1/E2A complex and GATA1. Indeed, a widely used and evolutionarily conserved looping function for Ldb1 is suggested by studies in diverse organisms and cell lineages (Matthews and Visvader, 2003; Morcillo et al., 1997; Thaler et al., 2002).

The cause-effect relationship between chromatin looping and gene regulation has been unclear. By manipulating the chromatin conformation at a native gene locus, we found that juxtaposition of an enhancer with its target gene leads to transcription activation, indicating that looping is a prerequisite for transcription activation. In particular, forced association between the LCR and the β-globin gene sufficed to exert two functions ascribed to the LCR, the formation of a preinitiation complex at the promoter and the generation of early elongating Pol II, as reflected in Ser5ph (Sawado et al., 2003; Song et al., 2010). On the other hand, our observation that ZF-Ldb1 proteins completely rescued chromatin looping but only partially restored transcription elongation agrees with the notion that full transcription is not required for loop formation (Jing et al., 2008; Mitchell and Fraser, 2008; Palstra et al., 2008). We speculate that juxtaposition of the LCR with β-globin promoter increases the concentration of nuclear regulators at the promoter above a threshold critical for preinitiation complex formation and early transcription elongation (see model in Figure 7).

Ldb1 recruitment in GATA1 null cells completely rescued chromatin looping and transcription initiation but only partially restored transcription elongation, indicating that GATA1 contributes additional functions independently of Ldb1 and chromatin looping. Indeed, both the recruitment of the P-TEFb complex and its distribution along the gene were impaired in the absence of GATA1, suggesting GATA1 impacts on P-TEFb regulation at multiple levels, perhaps via direct interaction (Bottardi et al., 2011; Elagib et al., 2008) or indirectly via proteins of the bromodomain and extra terminal domain (BET) family (Lamonica et al., 2011). In addition, GATA1 interacts with many other transcription factors and histone modifiers, the lack of which might account for inefficient transcription elongation.

In concert, these results suggest that Ldb1 functions downstream of GATA1 rather than in a parallel pathway and highlight the usefulness of this system to interrogate protein functions during distinct steps in the transcription cycle. In more general terms, this work illustrates a novel strategy to establish hierarchical orders of transcription factor function. On the background of a transcription factor deficiency, forced tethering of a potential cofactor to a chosen gene can be employed to measure its contribution to defined steps in the transcription cycle, such as loop formation, Pol II recruitment, Pol II phosphorylation, and productive transcription elongation. We believe that this approach is widely applicable for any nuclear factors that can be knocked down or knocked out.

One key general finding of our study is that a single ZF-Ldb1 protein targeted to the β-globin promoter can induce a chromatin loop by interacting with endogenous LCR-bound factors. ZFs have previously been linked to activation domains to successfully activate gene expression (Klug, 2010). However, the use of ZFs to promote interactions with a potent enhancer or LCR is expected to produce more pronounced transcriptional effects. Indeed, we are not aware of any single ZF proteins capable of activating gene transcription by a factor of more than 1,000-fold. Another advantage of a forced looping approach by a single ZF construct, especially in the context of therapeutic applications, is that efficient expression of a single molecule is easier than coexpression of two factors at matching levels.

Finally, specific chromatin loops can occur at repressed genes (Jing et al., 2008), and placing an enhancer and promoter on separate loops can isolate the enhancer to render it inactive (Ameres et al., 2005; Hou et al., 2008). Thus, in addition to activating transcription, we envision that forced chromatin looping could be used to silence gene expression for scientific or therapeutic purposes.

EXPERIMENTAL PROCEDURES

Artificial ZF Design

ZFs, each containing six Cys2-His2 ZF domains and targeting 18–19 bp sites within either the β-major promoter or DNase1 hypersensitive site 2 of the mouse LCR, were designed and assembled from two-finger units as previously described (Bartsevich et al., 2003).

Cell Culture

G1E and G1E-ER4 cells were cultured as described (Weiss et al., 1997). Where indicated, G1E-ER4 cells were treated with 100 nM estradiol (E2) for 21 (3C assays) or 24 hr (RT-qPCR and ChIP assays) to activate GATA1-ER (indicated as G1E+GATA1 in figures).

Isolation of Primary Erythroblasts

WT fetal liver erythroid cells were obtained from CD1 mice (Charles River Laboratories). ΔLCR/ΔLCR mice (129 strain) were described (Bender et al., 2000). To generate ΔLCR/WT mice, ΔLCR/ΔLCR male animals were bred with WT female mice (BL6 strain). E13.5 fetal liver cells were harvested, stained with PE-conjugated anti-CD71 and APC-conjugated anti-Ter119 antibodies, and sorted by FACS. The R1 (Ter119- and CD71-/low) populations were isolated, infected with desired retrovirus, and cultured for 24 hr in proliferation medium containing Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine, 10 ng/ml mIL3, 20 ng/ml m/h IL6, 50 ng/ml mSCF, and 10 ng/ml m/h FLT3L from Peprotech. Where indicated, cells were induced to differentiate by culture in IMDM supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine, 50 ug/ml ascorbic acid, 200 ug/ml holotransferrin (Sigma), and 2 U/ml Erythropoietin ALFA (Epogen).

3C Assay

The 3C assay was performed as described (Jing et al., 2008; Vakoc et al., 2005) with the following modifications. 1×10^7 cells were crosslinked with 1.5% formaldehyde at room temperature for 10 min, followed by glycine quenching, cell lysis, BglII digestion, and T4 ligation. 3C ligation products were quantified in triplicates by quantitative TaqMan real-time PCR. Probes and primers were designed using Primer Express 2.0 software (Applied Biosystems) and tested by serial dilution and gel electrophoresis to ensure specific and linear amplification (Figures S3B and S3C). Digestion efficiencies were monitored by SybrGreen qPCR with primer pairs that amplify genomic regions containing or devoid of BglII digestion sites (Figure S3D). A bacterial artificial chromosome (BAC) containing the entire murine β-globin locus of 129 origin (SourceBioscience, Clone BMQ433I10) was digested with BglII and relegated to generate random ligation products of BglII fragments (Figure S3A). The DNA was serially diluted and used to generate a standard curve to which all 3C products were normalized. The 3C signals at the β-globin locus were further normalized to those from four intervening regions or, alternatively, that of a control locus ERCC3, both producing similar results. Probe and primer sequences are listed in the Extended Experimental Procedures.

ChIP

ChIP was performed as described (Tripic et al., 2009). The following antibodies were used: pan-Pol II (sc-899, Santa Cruz), CDK9 (sc-484, Santa Cruz), Ser5ph (MS-134R, Covance), and H3K4me3 (07-473, Millipore), and anti-HA monoclonal antibody was clone 12CA5. ChIP qPCR primer sequences are listed in the Extended Experimental Procedures.

RT-qPCR

RNA was extracted with Trizol (Invitrogen) from 10^5–10^6 cells. RNase-free glycogen (Invitrogen) was added to aid RNA precipitation. Reverse transcription reactions were performed with random hexamers using Superscript II (Invitrogen). cDNA samples were quantified by SybrGreen qPCR. Allele-specific qPCR was carried out at annealing temperature 62°C (60°C for conventional qPCR as default setting). Data were normalized to β-actin or GAPDH, both producing similar results. Primer sequences are listed in the Extended Experimental Procedures.

Retroviral Infections

Retroviral infections of G1E cells were carried out as described (Tripic et al., 2009). For isolated primary fetal liver cells, spin-infection condition was modified to 2,000 rpm at room temperature for 1 hr, and cells were switched to fresh medium immediately after infection.

Plasmids

Individual ZF protein coding sequences were cloned into MigR1 retroviral vector with three HA tags and an NLS at their N termini. Full-length Ldb1 or the SA domain containing amino acids 1–200 of Ldb1 was cloned in frame C terminal to the ZF. P-ΔSA was generated by deleting the first 256 amino acids of Ldb1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.cell.2012.03.051.

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