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) (Driessen 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 reduced 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 artificial 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
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 \( \beta \)-globin locus.

**Tethering Ldb1 to the \( \beta \)-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 \( \beta \)-globin transcription in G1E cells. Because G1E cells lack GATA1, the \( \beta \)-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 \( \beta \)-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 \( \beta \)-globin expression compared to P-Ldb1 by

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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 to the SA domain of Ldb1. Transcript levels were normalized to β-actin.

n ≥ 3. Error bars denote SE. See also Figure S2.
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-D-SA) but left the nuclear localization sequence and LID domain intact. P-D-SA 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). 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 gene, 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.

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**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 ε 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.
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 lends 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+/CD71low 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.
because, in contrast to the latter, primary erythroblasts are replete with transcription factors and produce higher levels of \( \beta \)-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 \( \beta \)-globin expression. The effects were specific to the \( \beta \)-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 \( \beta \)-globin transcription in primary erythroid cells.

**ZF-SA Fusion Protein Induction of \( \beta \)-Globin Transcription Is LCR Dependent**

Targeting of the SA domain to the \( \beta \)-globin locus restores juxtaposition of HS2 with the \( \beta \)-globin gene, Pol II recruitment, and Pol II Ser5ph, strongly suggesting that transcriptional activation is due to LCR-\( \beta \)-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 \( \beta \)-globin transcription simply resulted from SA-induced transcription factor assembly at the \( \beta \)-globin promoter, then ZF-SA should activate transcription independently of the LCR. This distinction is especially important in light of the positive effects on \( \beta \)-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 (\( \Delta \)LCR/+) (Bender et al., 2000). The \( \beta \)-major gene on the \( \Delta \)LCR 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 \( \Delta \)LCR/+ 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 (\( \beta \)-maj-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 \( \beta \)-major gene on \( \Delta \)LCR allele (\( \beta \)-maj-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 \( \beta \)-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.
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 \(D_{\text{LCR}}/D_{\text{LCR}}\) R1 cells were trans-
duced with P-SA, \(\beta\)-globin activation was close to background,
establishing that the low signal obtained with D-specific primers
in \(D_{\text{LCR}}/\text{WT}\) cells was in fact due to cross-hybridization (Fig-
ure 6 B, right). In concert, the results clearly demonstrate that
the activity of ZF-SA proteins is entirely dependent on the pres-
ence 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 chro-
matin fiber. Targeting the SA domain of Ldb1 to the endogenous
\(\beta\)-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 con-
siderable transcriptional activation, indicating that the juxtaposi-
tion 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 \(\beta\)-globin activation. First, 3C
experiments clearly showed that tethering the SA domain to the
\(\beta\)-globin promoter fostered genomic contacts that strongly
resembled those induced by GATA1 with regard to both their
spatial configuration and efficiency. Second, SA domain recruit-
ment completely restored several LCR-dependent functions at
the \(\beta\)-globin promoter, including Pol II recruitment, Ser5ph of
Pol II, and H3K4 methylation. Third, targeted deletion of the
LCR dramatically reduced \(\beta\)-globin transcription without dimin-
ishing 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 \(\beta\)-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-con-
taining complexes that reside at the LCR even in the absence
of GATA1 (Figure S1A) (Tripic et al., 2009). In contradistinction,
Ldb1 association with the \(\beta\)-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

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**Figure 6. LCR Dependence of \(\beta\)-Globin Induction by ZF-SA Proteins**

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

(B) \(\beta\)-major mRNA levels as measured by allele-specific RT-qPCR in R1 cells from WT/\(\Delta\)LCR or \(\Delta\)LCR/\(\Delta\)LCR 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.
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 \( \beta \)-globin expression (W.D., unpublished data).

Thus, 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 bromo-domain 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.

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

Recruitment of Ldb1 to the \( \beta \)-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.
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, 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). 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).

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 separated 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% glucose, 10 ng/ml IL3, 20 ng/ml mIL6, 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% peni-
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REFERENCES


Supplemental Information

EXTENDED EXPERIMENTAL PROCEDURES

ChIP qPCR Primers

HS3: Forward, 5' -CTAGGGACTGAGAGGCTGCTT-3'; reverse, 5'-ATGGGACCTCTTATGACACACCTT-3'. HS2: Forward, 5'-GGTGTGTGGCCAGAGATTG-3'; reverse, 5'-CACTTCTCTCTTGGAATGATCT-3'. HS1: Forward, 5'-CAGATCTCAGAACACTCTCCCATAA-3'; reverse, 5'-TGCCCTTCTCTTCCACATCTTTTCA-3'. ey Promoter: Forward, 5'-GATACCTGGCTCCACCCATT-3'; reverse, 5'-TCTTTGAGCAGCAGCTACCTTG-3'. ey intron 2: Forward, 5'-GCTTCTTCTCTCTCTCTCTTCAACT-3'; reverse, 5'-AATGAACTGAGGAAAGGAAAG-3'. ey intron 3: Forward, 5'-GCCCTGCTCAAGAATGACCAAC-3'; reverse, 5'-TGCAAGCAAGAGCAGAG-3'. 3UTR: Forward, 5'-GCTTCACTGTCAGGATTGACTGGTAC-3'; reverse, 5'-GCCATCGTTAAAGGCAGTTATCACT-3'.

Transcript qPCR Primers

y Promoter: Forward, 5'-GCCCTGCTCAAGAATGACCAAC-3'; reverse, 5'-GCCCTGCTCAAGAATGACCAAC-3'. y intron 2: Forward, 5'-GCTTCTTCTCTCTCTCTCTTCAACT-3'; reverse, 5'-AATGAACTGAGGAAAGGAAAG-3'. y intron 3: Forward, 5'-GCCCTGCTCAAGAATGACCAAC-3'; reverse, 5'-TGCAAGCAAGAGCAGAG-3'.

3C Probes and Primers

β-Globin Locus

LCR-HS2 probe, 56-FAM/TCT GCT CCC TGC CTC GTG A36-TAMSp; Anchor (rHS2), 5'- CAGGGTTTTAGGATATAGGTGAA-3'; reverse, 5'-GAACCTTGCAGGGAATACCTAGTACAG-3'. III (rHS1), 5'-GAATCCTTGAGGAAATACCTAGTACAG-3'; reverse, 5'-GAATCCTTGAGGAAATACCTAGTACAG-3'.

ERCC3 Locus

ERCC3 probe, 56-FAM/TCTAGATTGCAGAAGCCAGCACCACCTTC/36-TAMSp; rERCC3_3, 5'-GCAGTGGAAACAAACACATGT-3'; rERCC3_5, 5'-GGCCCAACCAAGCTTG-3'.

Primers for 3C Digestion Efficiency

HS2 BgIII Cut 1: Forward, 5'-TGAGATCAGGATAGGTGATGAC-3'; reverse, 5'-CAGGGTTTTAGGATATAGGTGAA-3'. HS2 BgIII Uncut 1: Forward, 5'-GGTGTGTGGCCAGAGATTG-3'; reverse, 5'-CACTTCTCTCTTGGAATGATCT-3'. HS2 BgIII Cut 2: Forward, 5'-GCCCTGCTCAAGAATGACCAAC-3'; reverse, 5'-TGCAAGCAAGAGCAGAG-3'. HS2 BgIII Uncut 2: Forward, 5'-AGGAAACAGGCAAGGAGG-3'; reverse, 5'-TCACTGCGTACCTGGTTCTCCTATCT-3'.

SUPPLEMENTAL REFERENCES

Figure S1. ZFs' Targeting of the β-Globin Locus, Related to Figure 1

(A) Anti-GATA1 (top) and anti-TAL1 (middle and bottom) ChIP-seq tracks encompassing the β-globin locus from parental G1E cells (middle) and G1E cells expressing induced GATA1-ER (top, bottom). TAL1 is a reliable indicator for the presence of Ldb1 since their occupancy patterns are virtually identical (Tripic et al., 2009). Note that in the absence of GATA1, TAL1 is completely lost from the β-major globin promoter but not the LCR.

(B–D) L-ZF and P-ZF target HS2 of the LCR (red oval) and the β-major promoter (red triangle), respectively. The DNA sequences used for ELISA experiments are shown, including 18 nucleotides of ZF binding site (uppercase) and the flanking nucleotides (lowercase). Anti-HA ChIP profiles of HA tagged P-ZF (C) and L-ZF (D) in G1E cells were shown. n = 3. Error bars denote SD.
Figure S2. Characterization of ZF-Ldb1 and ZF-SA Activities, Related to Figure 2

(A) β-major mRNA levels in ZF-Ldb1 expressing cells as measured by RT-qPCR with primer pairs for exon 2/3 junction, 5UTR and 3UTR.

(B) Anti-HA ChIP profiles in G1E cells expressing L-SA (top), P-SA (middle), and L-SA+P-SA (bottom). L-SA binds selectively to HS2 of the LCR. Of note, P-SA binds to the promoter and additionally associates with HS1, 2, 3 of the LCR but not to other regions, including the εy, βh1, and βmin genes, an intervening region (IVR16) or an inactive gene (mCD4).

(C and D) mRNA levels of indicated genes as measured by RT-qPCR in G1E cells and derivatives expressing indicated ZF and ZF-SA constructs. Note that while β-major is dramatically activated in P-SA or P-SA/L-SA cells, mRNA levels of GATA1 repressed (Gata2, Kit) and activated (Eraf) genes were largely unchanged by ZF-fusion protein expression.

n = 3. Error bars denote SD. mRNA levels were normalized to β-actin.
Figure S3. 3C Quality Controls, Related to Figure 3
(A) A representative gel electrophoresis of BAC DNA. BAC DNA was purified (lane 2), digested with BgIII (lane 3), and ligated with T4 ligase (lane 4) to generate random ligation products of BgIII fragments that served as standard DNA for the 3C assay.
(B) Linearity of representative 3C primers was tested using serially diluted BAC DNA as template.
(C) Amplification products of representative 3C primers were analyzed by agarose gel electrophoresis to verify primer specificity.
(D) Digestion efficiencies at HS2 from a representative 3C experiment.
Figure S4. CDK9 and H3K4me3 ChIP Studies, Related to Figure 4
(A and B) ChIP profiles with antibodies against CDK9 (A) and H3K4me3 (B) using G1E cells or derivatives expressing activated GATA1-ER or P-SA.
Figure S5. Characterization of Sorted Primary Erythroid Cell Populations, Related to Figure 5

(A and B) mRNA levels of indicated genes in R1 to R4 populations of fetal liver cells as measured by qPCR. N = 3; error bars denote standard deviation.

(C) Differentiation profiles of R1 cells expressing indicated constructs as measured by flow cytometry using Ter119 and CD71 surface markers.
Figure S6. Allele-Specific qPCR, Related to Figure 6

(A–C) β-major mRNA levels of D and S haplotypes as measured by qPCR using allele-specific primers βmaj-D and βmaj-S, respectively. cDNA from D or S haplotypes was prepared from 129 or BL6 mouse strains, respectively. Note that the βmaj-S primer specifically amplified BL6 cDNA but not 129 cDNA. In contrast, the βmaj-D primer cross-amplified BL6 cDNA with an efficiency ~10% of the βmaj-S primer. Allele specificity was further examined by mixing D-cDNA and S-cDNA templates. The observed qPCR signal (C) largely matched the expectation (B) for allele-specific qPCR. The βmaj-S signal remained unchanged with increasing amount of D cDNA, verifying its amplification specificity. However, increasing the proportion of S cDNA led to an augmented signal with the D-specific primers indicative of cross-reactivity up to ~10%.
Figure S7. β-Globin Specific Effects of ZF-SA Fusion Proteins, Related to Figure 6

(A) mRNA levels of indicated genes in R1 cells from WT/ΔLCR fetal livers expressing indicated ZF-SA proteins. R1 cells were treated with erythropoietin (Epo) for 6 hr following transduction with ZF-SA expression constructs. N = 3; error bars denote standard deviation.

(B and C) RT-qPCR measuring mRNA levels of β-major globin (B) or indicated control genes (C) in R1 cells from WT/ΔLCR fetal livers expressing indicated ZF-SA proteins without Epo treatment. mRNA levels were normalized to GAPDH. n = 3. Error bars denote SD.