# Reactivation of Developmentally Silenced Globin Genes by Forced Chromatin Looping

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## SUMMARY

**Distal enhancers commonly contact target promoters via chromatin looping. In erythroid cells, the locus control region (LCR) contacts β-type globin genes in a developmental stage-specific manner to stimulate transcription. Previously, we induced LCR-promoter looping by tethering the self-association domain (SA) of Ldb1 to the β-globin promoter via artificial zinc fingers. Here, we show that targeting the SA to a developmentally silenced embryonic globin gene in adult murine erythroblasts triggers its transcriptional reactivation. This activity depends on the LCR, consistent with an LCR-promoter looping mechanism. Strikingly, targeting the SA to the fetal γ-globin promoter in primary adult human erythroblasts increases γ-globin promoter-LCR contacts, stimulating transcription to approximately 85% of total β-globin synthesis, with a reciprocal reduction in adult β-globin expression. Our findings demonstrate that forced chromatin looping can override a stringent developmental gene expression program and suggest a novel approach to control the balance of globin gene transcription for therapeutic applications.**

## INTRODUCTION

Long-range enhancers physically contact their target promoters to form chromatin loops. The β-globin locus has been at the forefront of studies on the dynamics and mechanisms of chromatin looping and gene regulation. In erythroid cells, a powerful distal enhancer called the locus control region (LCR), composed of multiple DNase I hypersensitive sites (HSs), is in physical proximity to the β-globin genes in a developmentally dynamic manner (Carter et al., 2002; Tolhuis et al., 2002). In primitive murine erythroid cells, the LCR loops to the embryonic-type β-globin gene promoters (εy and βh1), whereas in definitive erythroid cells, the LCR exclusively contacts the adult-type β-globin genes (β-major and β-minor), with the intervening embryonic globin genes looped out (Palstra et al., 2003). Humans additionally bear fetal stage-specific β-like globin (γ-globin) genes that contact the LCR and are transcribed from the beginning of fetal liver erythropoiesis until birth when blood formation gradually shifts to the bone marrow. Several transcription factors have been implicated in loop formation at the β-globin locus, including Klf1, GATA1, and its coregulators Ldb1 and FOG1 (Drissen et al., 2004; Song et al., 2007; Vakoc et al., 2005). In previous work we addressed whether any of these factors might be sufficient for chromatin looping and whether looping is a causal event during transcription initiation or merely a reflection of it (Deng et al., 2012). Specifically, in immature murine erythroblasts in which the β-globin promoter is not in contact with the LCR, tethering of Ldb1 to the promoter via artificial zinc-finger (ZF) proteins established an LCR-promoter loop similar to that observed in mature erythroid cells and stimulated transcription (Deng et al., 2012). The self-association (SA) domain of Ldb1 was sufficient for this activity. These results demonstrated that forced juxtaposition of an enhancer and promoter via chromatin looping can trigger gene activation and identified a relatively small functional domain of Ldb1 as a powerful mediator of such long-range interactions. Therefore, it should be possible to activate any other similarly regulated nearby gene by producing an interaction between it and the active LCR.

The molecular mechanisms mediating the developmental switch in globin gene expression have been widely investigated. A variety of nuclear complexes contribute to the silencing of the

embryonic and fetal globin genes in adult erythroid cells (Sankaran et al., 2010). Among these, arguably the most powerful one is nucleated by Bcl11a (Sankaran et al., 2008, 2009), which does not bind directly to promoters of the silenced human fetal globin genes but seems to repress them via a mechanism involving higher-order chromatin looping (Kiefer et al., 2011; Xu et al., 2010). Hence, manipulating chromatin loops might be a viable strategy to reverse the globin switch. The overarching interest in these questions stems in part from the clinical importance of understanding hemoglobin switching. Patients affected by sickle cell anemia and β-thalassemia experience a milder course of the disease if they express elevated levels of fetal hemoglobin in adulthood (Platt et al., 1994; Weatherall, 2001). This observation in particular provided a major impetus for studies over the last decades aimed at unraveling the mechanisms by which the fetal globin genes are silenced with the ultimate goal of reactivating their expression.

Here, we tested whether developmentally silenced embryonic or fetal globin genes can be reactivated in adult erythroid cells by juxtaposing them with the LCR and whether reactivation would lead to a concomitant reduction in the adult-type globin genes. We found that ZF mediated tethering of the SA domain of Ldb1 to the murine embryonic βh1-globin promoter activated transcription in an adult-type erythroblast cell line and primary definitive erythroid cells. This activity was abrogated in erythroid cells from mice in which the LCR had been deleted, demonstrating that embryonic globin gene activation was dependent on a long-range LCR interaction. Primary human erythroid cells expressing the SA domain fused to a ZF moiety that targets the γ-globin promoters produced high quantities of γ-globin mRNA and protein in the vast majority of cells with an accompanying reduction in adult-type globin expression. The reciprocal alteration in fetal versus adult globin gene transcription was reflected in corresponding changes in contact frequencies with the LCR.

Together, these studies demonstrate that stringent developmental regulation of a gene can be overcome through the manipulation of higher-order chromatin structure and that such manipulations have the potential for therapeutic applications.

## RESULTS

### βh1-SA-Mediated Activation of an Embryonic Globin Gene in Adult Erythroblasts

To examine the potential of forced gene looping in reprogramming the β-globin locus, we began by using G1E erythroblasts. G1E is a murine adult-type erythroid cell line that lacks the erythroid transcription factor GATA1 (Weiss et al., 1997). As a result, a productive interaction between the LCR and the adult β-globin (β-major) promoter fails to form, and β-major mRNA production is low (Vakoc et al., 2005). GATA1 deficiency is associated with little to no Ldb1 recruitment to the promoter (Tripic et al., 2009). Tethering Ldb1 or its SA domain to the β-major promoter restored looping in a manner highly similar to that in normal erythroid cells or G1E cells upon GATA1 restoration (Deng et al., 2012). Here, we asked whether a similar approach could be employed to achieve a distinct goal, which is to activate the embryonic βh1-globin gene by tethering the Ldb1 SA domain to its promoter to induce an interaction with the LCR (Figure 1A). We designed custom ZF proteins and screened them for binding to βh1-globin promoter sequences in vitro (see the Experimental Procedures). Functional proteins were fused to the SA domain of Ldb1 (βh1-SA) and introduced into G1E cells by retroviral infection. Infected cells were isolated by fluorescence-activated cell sorting (FACS). Chromatin immunoprecipitation (ChIP) revealed specific binding of βh1-SA to the βh1-globin promoter, but not to the promoters of the other β-like globin genes (Figure 1B). We observed a low but reproducible ChIP signal at the DNase 1 hypersensitive (HS) sites of the LCR, likely due to βh1-SA binding to endogenous Ldb1-containing complexes (Deng et al., 2012). We next examined the effects of βh1-SA on βh1-globin transcription by qRT-PCR. βh1-globin mRNA levels were increased 368-fold over nonexpressing G1E cells or cells that express a ZF protein targeting HS2 of the LCR (L-SA) (Figure 1C).

Restoration of GATA1 activity in G1E cells via expression of an estradiol-inducible form of GATA1 (GATA1-ER) leads to erythroid maturation and high-level induction of β-major transcription, as well as low-level induction of βh1-globin (Figures 1C and 1D) (Weiss et al., 1997; Welch et al., 2004). Hence, we investigated whether βh1-SA expression was capable of raising βh1-globin transcription in differentiating β-major producing erythroid cells. Notably, βh1-SA induced βh1-globin levels 26-fold over control cells, amounting to ~15% of total β-globin expression compared to ~0.5% in vector only containing cells (Figures 1C and 1D). Tethering the SA domain to the LCR (L-SA in Figure 1) or β-major promoter (Deng et al., 2012; data not shown) did not activate βh1-globin expression, confirming that the effects of βh1-SA are specific. Basal-level βh1-globin expression and βh1-SA-induced βh1-globin transcription were higher in GATA1 replete cells when compared to parental G1E cells, likely due to increased LCR and promoter activities in the presence of GATA1 (Figure S1A available online). The substantial increase in βh1-globin expression was associated with a trend toward lower β-major expression (see also below) but without significant changes of other erythroid genes, including those encoding α-globin and Kit, indicating that the effects of βh1-SA are not simply a consequence of differentiation induction (Figures 1D and S1B).

### βh1-SA-Mediated Activation of Embryonic Globin Gene Expression in Primary Adult Erythroblasts

To address whether βh1-SA activates expression of βh1-globin in murine primary erythroid cells, we isolated immature definitive (adult-type) erythroid progenitor cells from E13.5 wild-type fetal livers by sorting for CD71 medium/low and Ter119 low populations (S0 cells) as described previously (Figure 2A) (Pop et al., 2010). Cells were infected with a retroviral vector expressing βh1-SA (infection rate >75%; data not shown) and expanded for 48 hr, followed by measurements of transcript levels by RT-qPCR (reverse transcription quantitative polymerase chain reaction). βh1-SA activated βh1-globin transcription by almost 800-fold compared to empty vector control (Figure 2B), exceeding 30% of total β-globin synthesis (Figure 2C). We noted that the zinc finger alone also raised βh1-globin expression but only to ~1/6 of the levels achieved by βh1-SA (Figures 2B and 2C). Although the cause for this activity is unclear, we surmise that zinc-finger binding might increase chromatin occupancy of

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