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Transcription factors LRF and BCL11A independently repress expression of fetal hemoglobin

Takeshi Masuda¹, Xin Wang², Manami Maeda¹, Matthew C. Canver³, Falak Sher³, Alister P. W. Funnell⁴, Chris Fisher⁵, Maria Suci⁵, Gabriella E. Martyn⁴, Laura J. Norton⁴, Catherine Zhu¹, Ryo Kurita⁶, Yukio Nakamura^{6,7}, Jian Xu^{3,8}, Douglas R. Higgs⁵, Merlin Crossley⁴, Daniel E. Bauer³, Stuart H. Orkin^{3,9}, Peter V. Kharchenko^{2,*}, and Takahiro Maeda^{1,*}

¹Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.

²Department of Biomedical Informatics, Harvard Medical School, Boston, MA 02115, USA.

³Division of Hematology/Oncology, Boston Children's Hospital, Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Stem Cell Institute, Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA.

⁴School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia.

⁵Medical Research Council, Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, Oxford University, Oxford, UK.

⁶Cell Engineering Division, RIKEN BioResource Center, Tsukuba, Ibaraki, Japan.

⁷Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan.

⁸Children's Research Institute, Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX 75390, USA.

⁹Howard Hughes Medical Institute, Boston, MA 02115, USA.

Abstract

Genes encoding human β -type globin undergo a developmental switch from embryonic to fetal to adult-type expression. Mutations in the adult form cause inherited hemoglobinopathies or globin disorders, including sickle cell disease and thalassemia. Some experimental results have suggested that these diseases could be treated by induction of fetal-type hemoglobin (HbF). However, the mechanisms that repress HbF in adults remain unclear. We found that the LRF/ZBTB7A transcription factor occupies fetal γ -globin genes and maintains the nucleosome density necessary for γ -globin gene silencing in adults, and that LRF confers its repressive activity through a NuRD

*Corresponding author. peter.kharchenko@post.harvard.edu (P.V.K.); tmaeda@partners.org (T.M.).

SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text

Figs. S1 to S14

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repressor complex independent of the fetal globin repressor BCL11A. Our study may provide additional opportunities for therapeutic targeting in the treatment of hemoglobinopathies.

During human development, the site of erythropoiesis changes from the embryonic yolk sac to the fetal liver and then, in newborns, to the bone marrow, where it persists through adulthood. Coincidentally, there is a “globin switch” from embryonic to fetal globin genes in utero, and then a second switch from fetal to adult globin expression soon after birth. This process has been studied for more than 60 years (1). The latter transition from fetal to adult hemoglobin is marked by a switch from a fetal tetramer consisting of two α and two γ subunits (HbF: $\alpha_2\gamma_2$) to an adult tetramer containing two α -like and two β -like globin subunits (HbA: $\alpha_2\beta_2$).

Mutations in the adult globin gene cause hemoglobinopathies such as thalassemia and sickle cell disease (SCD). These diseases are among the most common monogenic inherited human disorders and represent emerging public health challenges (2). For example, the number of children born with SCD is expected to exceed 14 million worldwide in the next 40 years (3).

Molecular genetic and clinical evidence indicates that elevated levels of fetal-type hemoglobin (HbF) in adults ameliorate SCD and β -thalassemia pathogenesis (1, 4). Thus, a promising approach is to pharmacologically inactivate a silencer(s) of fetal globin expression in order to reactivate HbF production in adult erythroid cells. Nuclear factors that regulate globin switching have been identified, but how they function cooperatively or independently in fetal globin repression is not fully understood.

Leukemia/lymphoma-related factor (LRF), encoded by the *ZBTB7A* gene, is a ZBTB transcription factor that binds DNA through C-terminal C2H2-type zinc fingers and presumably recruits a transcriptional repressor complex through its N-terminal BTB domain (5). To assess the effects of LRF loss on the erythroid transcriptome, we inactivated the *Zbtb7a* gene in erythroid cells of adult mice (6). We then performed RNA sequencing (RNA-seq) analysis using splenic erythroblasts from control and LRF conditional knockout (*Zbtb7a^{F/F} Mx1-Cre⁺*) mice (LRF KO mice) (Fig. 1A). Efficient *Zbtb7a* deletion was confirmed by Western blot and RNA-Seq reads (fig. S1, A and B) (7). Wild-type mice express two embryonic β -like globin genes: *Hbb-bh1* and *Hbb-y* (8, 9). Although both genes are expressed at early embryonic stages, *Hbb-bh1* is the ortholog of human γ -globin (10, 11). LRF-deficient adult erythroblasts showed significant induction of *Hbb-bh1*, but not *Hbb-y*, with a moderate reduction in adult globin levels (fig. S2A). These results were validated by quantitative polymerase chain reaction (qPCR) (fig. S2B). Isoelectric focusing of peripheral blood hemolysates revealed unique bands corresponding to embryonic globin proteins in peripheral blood from LRF KO mice (Fig. 1B).

We used a humanized mouse model to investigate whether LRF loss would reactivate human fetal globin expression in vivo. To do so, we established LRF KO mice harboring the human β -globin gene cluster as a yeast artificial chromosome transgene (β YAC) (12) (fig. S2C). Human γ -globin transcripts, but not those of embryonic β -globin (HBE1), were significantly induced in LRF-deficient erythroblasts and constituted 6 to 12% of total human

β -like globins in peripheral blood (Fig. 1C and fig. S2D). The magnitude of γ -globin induction in LRF/bYAC mice approximated that seen in BCL11A/ β YAC mice (13).

We next determined whether LRF loss could induce HbF in human erythroid cells. To this end, we used human CD34⁺ hematopoietic stem and progenitor cell (HSPC)-derived primary erythroblasts and determined γ -globin expression levels upon short hairpin RNA (shRNA)-mediated LRF knockdown (LRF KD) (fig. S3A). LRF expression was markedly induced upon erythroid differentiation over a 2-week period (Fig. 2A). LRF KD significantly increased the percentage of γ -globin mRNA (Fig. 2B and fig. S3, B and C) and protein expression (fig. S3D) relative to adult globin. HbF levels in LRF KD cells were greater than those seen in parental or scrambled-shRNA transduced cells (Fig. 2C and fig. S3E). Because LRF KO mice exhibit a mild macrocytic anemia due to inefficient erythroid terminal differentiation (14), we assessed the effects of LRF deficiency on human erythroid differentiation. We observed a delay in differentiation upon LRF KD relative to controls (fig. S4A and supplementary text).

HSPC-derived erythroid cells tend to display relatively high basal levels of HbF (Fig. 2C). Moreover, it is difficult to exclude the possibility that the effects of LRF KD may be the result of a sub-population of cells expressing aberrantly high HbF levels. To circumvent these problems, we used a human immortalized erythroid line (HUDEP-2), which undergoes terminal differentiation upon doxycycline removal (fig. S5A) (15). This line possesses an advantage over lines currently used for globin switching studies because it expresses predominantly adult hemoglobin (HbA), with very low background HbF expression (15). Using CRISPR/cas9 gene modification, we knocked out *ZBTB7A* in HUDEP-2 cells (fig. S5B). We did not observe a significant difference in erythroid differentiation between control and *ZBTB7A* KO HUDEP-2 cells, as evidenced by morphologic and fluorescence-activated cell sorting (FACS) analyses (fig. S5, C and D). To evaluate genome-wide gene expression changes promoted by *ZBTB7A* deletion, we performed RNA-seq analysis. Wild-type HUDEP-2 cells exhibited gene expression patterns similar to those of HSPC-derived basophilic erythroblasts (fig. S6). As expected, γ -globin transcripts, but not those of embryonic ϵ -globin, were markedly induced in *ZBTB7A* KO HUDEP-2 cells (Fig. 2D and fig. S7A). Levels of adult β -globin transcripts in *ZBTB7A* KO cells were approximately half those seen in control cells (fig. S7A); γ -globin transcripts constituted more than 60% of total β -like globins (fig. S7B). Induction of γ -globin was also validated at the protein level (fig. S7, C and D). We then established three independent *ZBTB7A* KO HUDEP-2 clones (fig. S7E) and determined HbF levels in each by high-performance liquid chromatography (HPLC). All three clones exhibited HbF levels greater than 60%, whereas that of parental cells was less than 3% (Fig. 2E). Notably, the HbF reactivation occurred without changes in levels of transcripts encoding known HbF repressors, including BCL11A (fig. S7F). BCL11A protein levels were also unchanged in *ZBTB7A* KO cells (fig. S5B).

To determine LRF occupancy sites genome-wide, we performed chromatin immunoprecipitation and sequencing (ChIP-seq) with an antibody to LRF, using HSPC-derived proerythroblasts and HUDEP-2 cells. These experiments exhibited strong correlations and concordance among the replicates (fig. S8A). We identified 5684 and 10,385 LRF binding sites in HSPC-derived proerythroblasts and HUDEP-2 cells,

respectively (fig. S8B). The most enriched motif identified in either cell type was consistent with that previously identified in vitro using CAST (cyclic amplification and selection of targets) analysis (16), confirming antibody specificity (figs. S9 and S10). Genes differentially expressed in control and *ZBTB7A* KO cells were significantly enriched for LRF binding sites (Fisher's exact test, $P < 1.6 \times 10^{-5}$ and $P < 8.3 \times 10^{-13}$ for undifferentiated and differentiated conditions, respectively). It is also notable that LRF occupancy sites differ from those of the known γ -globin repressors SOX6 and BCL11A (17) (fig. S8C).

In support of a direct role of LRF at the β -globin cluster, we observed several significant LRF-ChIP binding signals at adult (*HBB*) and fetal (*HBG1* and *HBG2*) globin genes and at upstream hypersensitivity (HS) sites within the locus control region (LCR) (Fig. 3 and supplementary text). To assess the local chromatin accessibility at the β -globin cluster in the presence or absence of LRF, we performed ATAC-seq [assay for transposase-accessible chromatin with high-throughput sequencing (18)]. In control HUDEP-2 cells, the *HBB* gene and LCR HS sites, but not the γ -globin genes, exhibited ATAC-seq nucleosome-free signals (Fig. 3). In contrast, strong chromatin accessibility was evident at the γ -globin genes in *ZBTB7A* $^{\Delta\Delta}$ cells before differentiation, and the signal was amplified upon differentiation. Strikingly, differential enrichment of ATAC signals in *ZBTB7A* $^{\Delta\Delta}$ cells was evident only at the γ -globin genes (Fig. 3) but not at the *HBB* gene or HS sites, indicating that chromatin in the latter is accessible regardless of *ZBTB7A* genotype. Thus, although LRF binds to the *HBB* gene and HS sites as well as to the γ -globin genes, LRF depletion specifically opens chromatin at the γ -globin genes.

To identify a repressor complex interacting with LRF in an unbiased fashion, we performed a yeast two-hybrid screen with the human LRF-BTB domain as bait. A total of 360 positive clones were processed out of 52.1 million potential binding events. LRF-interacting proteins with high confidence included four ZBTB proteins, three NuRD/CHD family proteins, and two chromatin remodelers (fig. S11, A and B). Given their abundance in erythroid cells (fig. S12A) and their potential repressor function, we focused on three factors (GATAD2B, CHD3, and CHD8) for further validation. Interactions of LRF with each were validated by immunoprecipitation (fig. S12B). Because NuRD complex components are implicated in globin switching (17, 19, 20), we examined the LRF-GATAD2B interaction in more detail. Immunoprecipitation of human proerythroblast lysates with an antibody to LRF pulled down GATAD2B and other NuRD complex components (Fig. 4A). The interactions were also validated in mouse erythroid cells (fig. S10C). Although BCL11A reportedly interacts with NuRD components (21, 22), we did not detect BCL11A in the NuRD complexes containing LRF in either human or mouse erythroid cells (Fig. 4A and fig. S12C). For reciprocal validation, we performed immunoprecipitation in human erythroid cell lysates with antibodies to GATAD2B or MTA2; as expected, both antibodies pulled down LRF (Fig. 4B). BCL11A was readily detected in MTA2-containing protein complexes, as reported (22). Consistent with our findings, LRF was not identified as a BCL11A-interacting protein in proteomic affinity screens in erythroid cells (21, 22).

Finally, to determine whether LRF and BCL11A suppress γ -globin expression via distinct mechanisms, we established *ZBTB7A/BCL11A* double-knockout (DKO) HUDEP-2 cells

(fig. S12D) and compared HbF in these cells to that in *ZBTB7A* or *BCL11A* single-KO HUDEP-2 cells. DKO cells exhibited a significantly greater fetal/adult β -globin ratio than did either *ZBTB7A* or *BCL11A* single-KO cells (fig. S12, E and F). The HbF levels of DKO cells were at 91 to 94% of total Hb (Fig. 4, C and D). These data suggest that LRF and BCL11A represent a primary fetal globin repressive activity in adult erythroid cells (fig. S13).

Our results show that LRF is a potent repressor of embryonic/fetal β -like globin expression in adult erythroid cells. We postulate that LRF depletion opens local chromatin at the γ -globin genes, thereby enabling erythroid transcriptional activators to induce γ -globin expression. Furthermore, we propose that LRF silences γ -globin expression independently of BCL11A, as implied by our observations that (i) LRF inactivation in mice specifically reactivates *Hbb-bh1*, but not *Hbb-y*, expression, whereas BCL11A depletion induces both of these embryonic globins (13); (ii) LRF directly binds to the *HBG1* gene, whereas BCL11A reportedly targets intergenic region(s), the LCR, and sequences between *HBG1* and *HBD* (17, 23); (iii) the LRF-NuRD complex in adult erythroid cells lacks BCL11A; and (iv) *ZBTB7A/BCL11A* DKO HUDEP-2 cells exhibit significantly greater γ -globin expression than do either *ZBTB7A* or *BCL11A* single-KO cells.

Our work suggests that the two NuRD-associated pathways, in which LRF and BCL11A are respectively involved, are responsible for turning off fetal globin expression in order to switch over to adult globin. These findings may enable the development of therapies to turn on fetal globin expression in individuals with human hemoglobinopathies displaying defective adult globin gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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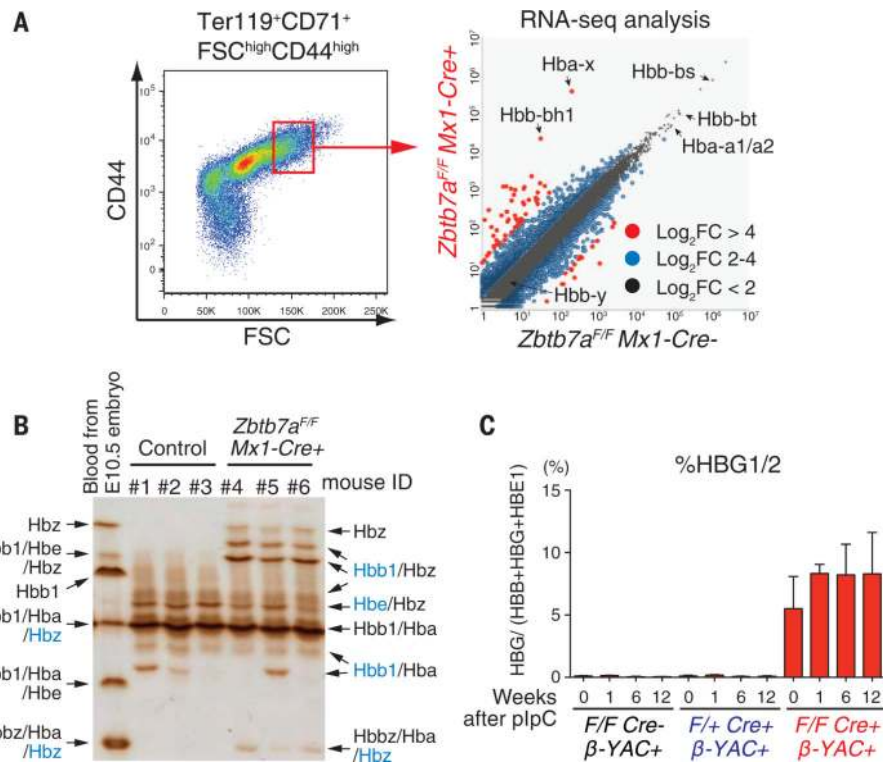


Fig. 1. Induced *Zbtb7a* deletion reactivates embryonic/fetal globin expression in adult mice (A) RNA-seq analysis of splenic erythroblasts from control and LRF KO mice. Mice were injected with polyinosinic-polycytidylic acid, and splenic erythroblasts were harvested 2 months later. Each dot represents an individual gene; differentially expressed genes are depicted according to FPM (fragments per million mapped reads) values. (B) Isoelectric focusing of peripheral blood hemolysates and subsequent peptide mass fingerprinting with matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS). Embryonic globins (Hbz and Hbbz) are evident in samples from LRF KO mice. Globins shown in blue were detected at a much lower level. (C) Levels of human γ -globin (HBG) transcripts were monitored by qPCR before and after LRF depletion. Error bars denote SD. We observed a low level of γ -globin expression before induction of LRF KO, likely due to leaky Cre activity (24).

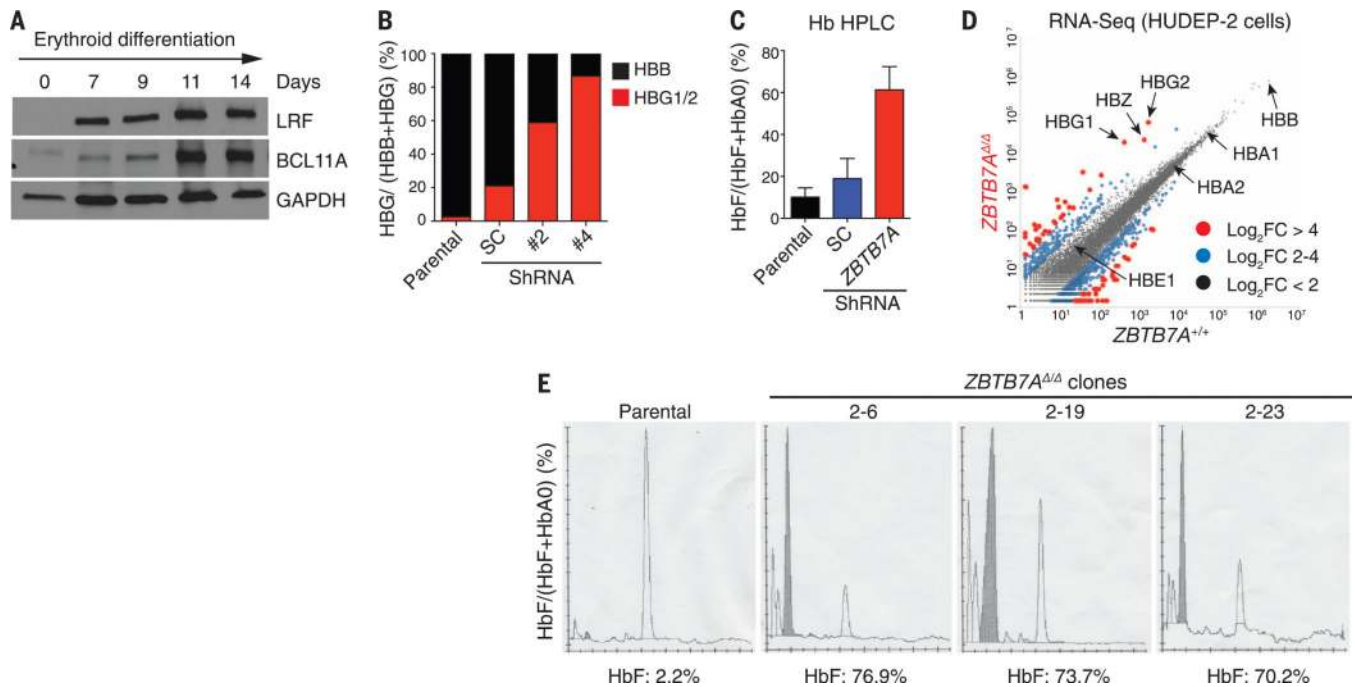


Fig. 2. *ZBTB7A* deletion reactivates γ -globin expression in human erythroblasts

(A) Time course analysis of LRF and BCL11A protein levels by Western blot. GAPDH was used as a loading control. (B) Bar graphs show proportions of γ -globin to total β -globin transcripts measured by qPCR on day 15. (C) Bar graphs show proportions of HbF relative to adult globin (HbA0) on day 15. Means of two independent samples per condition are shown. Error bars denote SD. (D) RNA-seq analysis of control and LRF KO HUDEP-2 cells. Differentially expressed genes are indicated according to FPM values. (E) Representative HPLC profiles of control and *ZBTB7A*^{ΔΔ} HUDEP-2 clones. Control: HUDEP-2_Cas9.

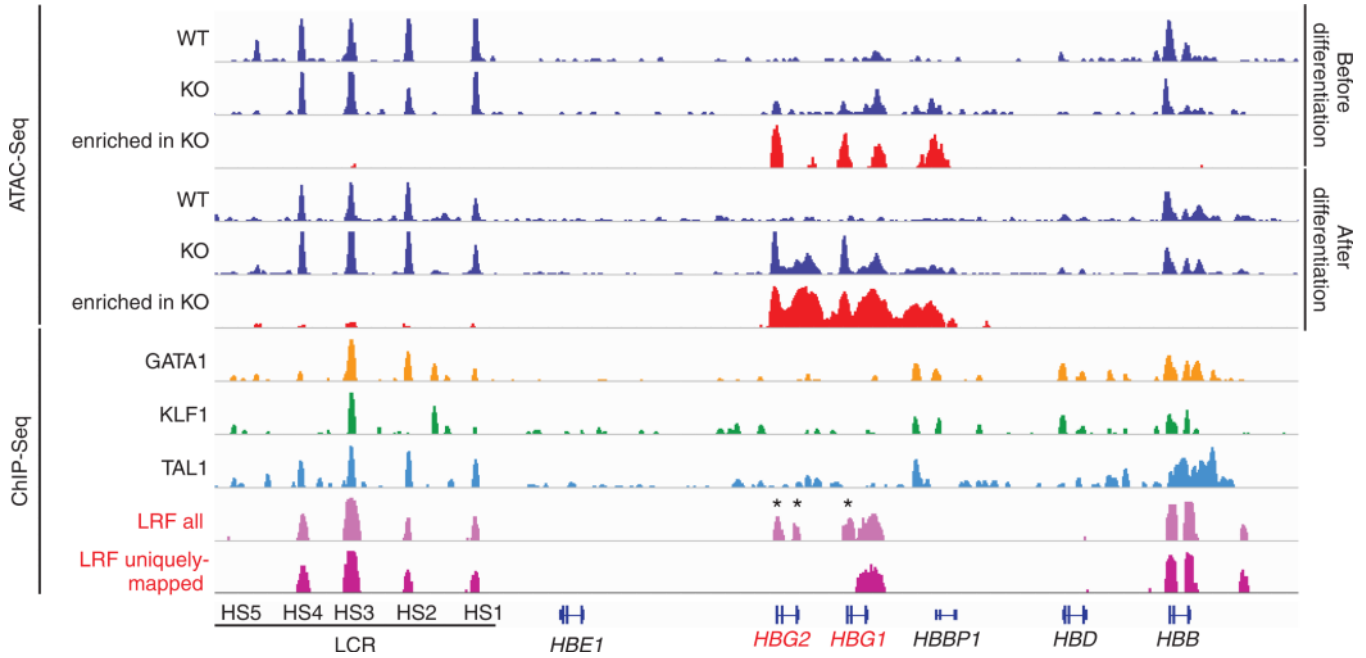


Fig. 3. LRF occupies the γ -globin gene and maintains local chromatin compaction
 LRF ChIP-seq and ATAC-seq signals at the β -globin cluster (HUDEP-2 cells) are shown, along with ChIP-seq enrichment for GATA1, KLF1, and TAL1 (25). Regions showing statistically significant ATAC-seq differences between LRF KO and wild-type HUDEP-2 cells are depicted in red. Because of high sequence similarity of *HBG1* and *HBG2*, we analyzed LRF occupancy sites at the γ -globin locus using two different mapping methods: one mapping all mappable fragments (“LRF all”) and the other mapping only uniquely mappable fragments (“LRF uniquely mapped”). In the “LRF all” track, fragments mappable to either *HBG1* or *HBG2* were randomly distributed between both genes (marked by asterisks).

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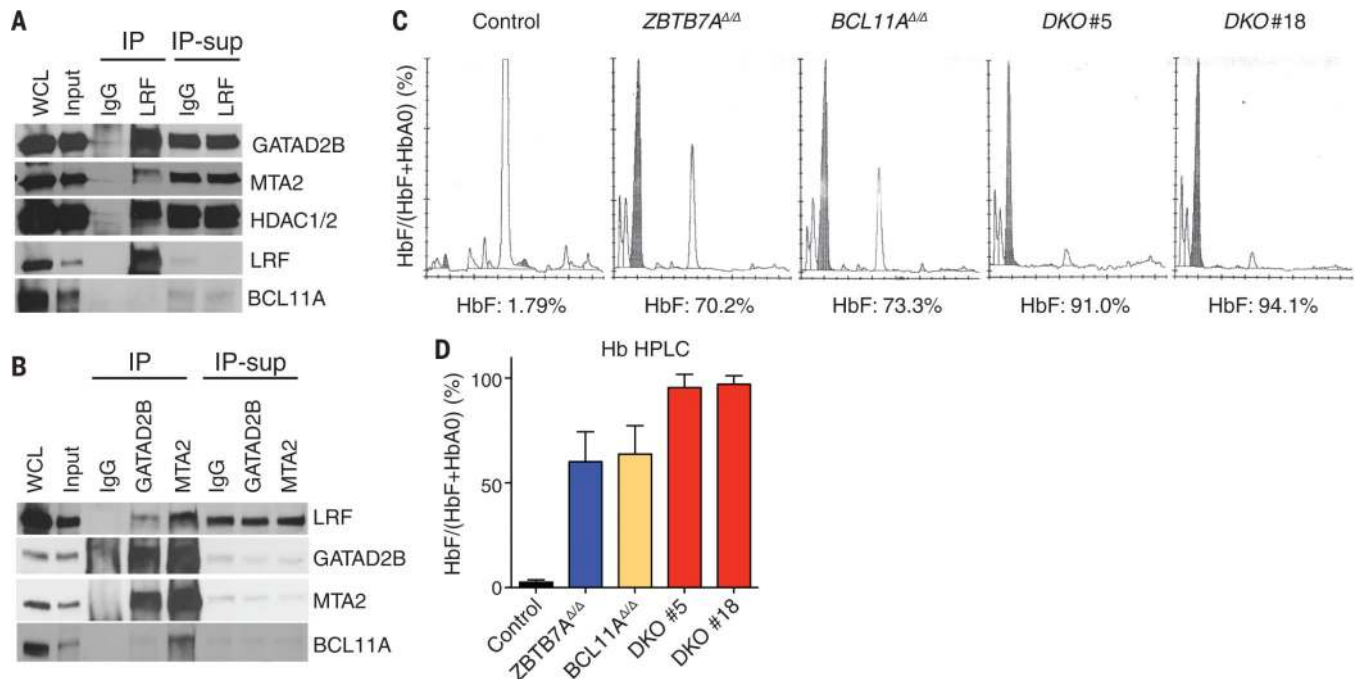


Fig. 4. LRF and BCL11A silence γ -globin expression through distinct mechanisms

(A) Immunoprecipitation with antibody to LRF confirmed LRF-GATAD2B interaction in HSPC-derived erythroblasts. BCL11A was not detected in the LRF-containing NuRD complex. IP-sup denotes supernatant after immunoprecipitation (unbound fraction). (B) Reciprocal validation using antibodies to GATAD2B or MTA2. (C) Representative HPLC profiles of control (HUDEP-2_Cas9), *ZBTB7A* KO, *BCL11A* KO, and *ZBTB7A/BCL11A* DKO (clones 5 and 18) HUDEP-2 cells. (D) Bar graphs show proportions of HbF relative to adult globin (HbA0). Means of two independent samples per clone are shown. Error bars denote SD.