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Embryonic erythropoiesis and hemoglobin switching require transcriptional repressor ETO2 to modulate chromatin organization

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ABSTRACT

The underlying mechanism of transcriptional co-repressor ETO2 during early erythropoiesis and hemoglobin switching is unclear. We find that absence of ETO2 in mice interferes with down-regulation of PU.1 and GATA2 in the fetal liver, impeding a key step required for commitment to erythroid maturation. In human β-globin transgenic *Eto2* null mice and in human CD34$^+$ erythroid progenitor cells with reduced ETO2, loss of ETO2 results in ineffective silencing of embryonic/fetal globin gene expression, impeding hemoglobin switching during erythroid differentiation. ETO2 occupancy genome-wide occurs virtually exclusively at LDB1-complex binding sites in enhancers and ETO2 loss leads to increased enhancer activity and expression of target genes. ETO2 recruits the NuRD nucleosome remodeling and deacetylation complex to regulate histone acetylation and nucleosome occupancy in the β-globin locus control region and γ-globin gene. Loss of ETO2 elevates LDB1, MED1 and Pol II in the locus and facilitates fetal γ-globin/LCR looping and γ-globin transcription. Absence of the ETO2 hydrophobic heptad repeat region impairs ETO2-NuRD interaction and function in antagonizing γ-globin/LCR looping. Our results reveal a pivotal role for ETO2 in erythropoiesis and globin gene switching through its repressive role in the LDB1 complex, affecting the transcription factor and epigenetic environment and ultimately restructuring chromatin organization.

INTRODUCTION

Erythropoiesis involves a series of coordinated cellular events and precise regulation of a gene expression program leading to progressive hemoglobinization and the production of mature red blood cells, or erythrocytes from hematopoietic stem cells (HSC). Mammalian α- and β-globin loci encode developmentally regulated embryonic and adult globin genes (1). In humans, the β-globin locus also contains two fetal γ-globin genes, *HBG1* and *HBG2*. The repression of *HBG1/2* and increased production of *HBB* (adult β-globin) occurs perinatally and is referred to as hemoglobin switching. A critical component of the globin switch is the exchange of locus control region (LCR) enhancer contacts with the γ-globin genes for contacts with the β-globin gene (2,3). How this change is orchestrated is not fully understood but is important because continued production of fetal hemoglobin in adult life, as occurs in Hereditary Persistence of Fetal Hemoglobin (HPFH), improves the clinical course of Sickle Cell Disease and β-thalassemia (1).

BCL11A and LRF/ZBTB7A are direct negative regulators of γ-globin transcription (4). These factors recruit co-repressors LSD1/CoREST, DNMT1 and NuRD to specific recognition motifs in the γ-globin promoters (5–8). Naturally occurring γ-globin promoter mutations associated with HPFH occur within BCL11A and LRF binding motifs and reduce factor occupancy, providing a satisfying explanation for relief of γ-globin gene repression (9). Moreover, recent studies have demonstrated the potential for small molecule inhibition of NuRD function as a therapeutic approach to treat β-globin hemoglobinopathies (10). However, it is not clear from these studies how long-range association of the LCR with active γ-globin genes is antagonized by BCL11A or LRF. While BCL11A and LRF provide a large component of γ-globin silencing, it remains possible that other repressors contribute significantly, possibly through down-regulation of their expression or other mechanisms. For example, HRI and POGZ are recently uncovered novel γ-globin repressors working in large part through repression of BCL11A (11,12).

Chromosomal looping protein LDB1 is required for global activation of erythroid genes, including globin genes, and in myeloid cells LDB1 regulates transcription of PU.1,

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the master regulator of myelopoiesis (13–16). The LDB1 complex, including DNA-binding transcription factors GATA1 and TAL1 and scaffolding protein LMO2, binds to erythroid enhancers and mediates contact with target promoters (17–19). Co-repressor ETO2 confers negative function upon the LDB1 complex to repress target genes and the stoichiometry of ETO2 to TAL1 or LDB1 is important for LDB1 complex negative versus positive function (20–25). The LDB1 complex binding motif in the γ-globin promoter lies between the BCL11A and LRF binding sites. An HPFH mutation within this motif (−175 T to C) creates a stronger binding site for TAL1 and increases looping of the promoter to the LCR (26). Whether co-repressor ETO2 participates in the regulation of long-range looping to antagonize with LDB1 complex function is still unclear.

We focused on the potential involvement of ETO2 in embryonic erythropoiesis and γ-globin silencing. Studies of *Eto2*$^{-/-}$ mice revealed that ETO2 is required for normal maturation of mouse erythroid progenitors. Loss of ETO2 in mice and in human erythroid progenitors results in delayed hemoglobin switching and persistence of embryonic/fetal globins in adults. ETO2 functions as a repressor virtually exclusively through the LDB1 complex at enhancers and inhibits LCR-γ globin looping by interacting with NuRD complex, making ETO2 of potential interest as a drug target for treatment of β-globin hemoglobinopathies.

MATERIALS AND METHODS

Mouse lines

Eto2$^{-/-}$ mice (27) were crossed with animals carrying a human β-globin transgene and with a deletion of the mouse β-globin LCR (28) to obtained *Eto2*$^{-/-}$ /hTg$^{+/+}$ animals. Mouse protocols were approved by the NIDDK Animal Care and Use Committee in accordance with AALAC specifications.

Cell culture

K562 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. Primary CD34+ umbilical cord cells were obtained from Lonza (cat. 2C-101) and were cultured as described (29). HUDEP cells were a kind gift of Drs. R. Kurita and T. Maeda and were cultured as described (30).

ETO2 CRISPR/Cas9 gene editing, RNAi and over-expression

The website (http://crispr.mit.edu/) was used to design sgRNAs for ETO2 knockout, which were cloned into pSpCas9(BB)-2A-GFP (Addgene #48138). A vector containing an shRNA targeting *ETO2* was purchased from Open Biosystems (clone IDs: TRCN0000020164). CRISPR/Cas9 sgRNAs were electroporated into K562 cells with Nucleofector kit V (VCA1003). ETO2 KO single cell clones were isolated after FACS sorting for GFP expression. The shRNA plasmid was electroporated into CD34^{+} cells at day 6 of expansion using human CD34^{+} cell Nucleofector kit (VAPA-1003). Cells were collected at D8-D18 of differentiation at 2 day intervals. For sgRNAs and shRNA sequences see Supplemental Table S1. The pMY-IRESNeo vector (Cell Biolabs) was used to express full length ETO2 in WT and ETO2 KO K562 cells. ETO2 domain mutants were generated from this plasmid by Q5 Site-Directed Mutagenesis Kit (E0554S).

RNA isolation and RT-qPCR

RNA was isolated using the RNeasy mini kit (QIAGEN, 74104) for K562 cells, PicoPure RNA isolation kit (Thermo Fisher Scientific, KIT0202) for fetal liver or yolk sac cells, and Mouse RiboPure-Blood RNA Isolation Kit (Thermo Fisher Scientific, AM1951) for blood samples. After RNA isolation, DNaseI was used to remove genomic DNA following manufacturers' protocols (Roche, 03539121103). cDNA was generated using the SuperscriptIII First-Strand Synthesis SuperMix (ThermoFisher Scientific, 18080-400) with random hexamers. Quantitative PCR was conducted using iTag Universal SYBR Green Super Mix (BioRad, 172-5120) and the 7900HT Real-Time PCR System (Applied Biosystems). For PCR primer sequences, see Supplemental Table S1.

Western blotting and co-immunoprecipitation

Nuclear protein extraction was performed with hypotonic buffer solution (20 mM Tris−HCl, pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$) and Cell Extraction Buffer (ThermoFisher Scientific, FNN0011) following the manufacturer's instructions. 400 ug protein was used for immunoprecipitation with Dynabeads Protein A Immunoprecipitation Kit (ThermoFisher, 10006D). Western blotting was performed as described (18). For antibodies see Supplemental Table S2.

Fluorescence-activated cell sorting (FACS)

Freshly prepared fetal livers were dissected from embryos at E14.5 and manually dissociated into a single cell suspension. Samples were stained with APC rat anti-mouse Ter119 (BD Pharmigen, 557909), PE rat anti-mouse CD71 (BD Pharmigen, 553267), and 4′,6-diamidino-2-phenylindole (DAPI, Thermo Scientific, 62247). Cell populations were purified using the BD FACSAria III Cell Sorter. Gating thresholds were set to eliminate unviable cells and separate multiple events (populations S0-S4/5). Bone marrow cells were isolated from 3-mouth-old mice and stained with APC rat anti-mouse Ter119 (BD Pharmigen, 557909), PE rat anti-mouse CD71 (BD Pharmigen, 553267).

ChIP-qPCR and ChIPmentation

ChIP and ChIPmentation were performed essentially as described (31,32). Briefly, 30×10^6 K562 cells or mouse fetal liver cells were fixed with 1% formaldehyde, sonicated and immunoprecipitated with antibodies. For ETO2, MED1, CHD4, HDAC1 and LSD1 ChIP, formaldehyde and EGS crosslinking was performed. The comparative CT method

was applied to calculate the relative enrichment of the protein of interest over input DNA abundance. H3K27ac and H3K9ac signals were normalized to H3 signals. For ChIP primers, see Supplemental Table S1. Antibodies used for ChIP are listed in Supplemental Table S2.

After final washing in the ChIP procedure, precipitated DNA was tagmentated on magnetic beads with 0.2ul of tagmentation enzyme (Nextera XT DNA Library Preparation Kit, Illumina # FC-131–1024) for 10 min at 37°C while shaking (1000 rpm). The beads were washed and tagementated DNA was eluted and de-crosslinked in ChIP elution buffer for 1 h at 55°C and incubated at 65°C overnight. DNA was purified using SRI beads (1.8×) and PCR amplified with Nextera primers. Libraries were purified (1.8×) and size selected (0.65×/0.15×) with SRI beads. Library quality was validated using a High Sensitivity DNA Chip in the Agilent Bioanalyzer 2100. Samples were sequenced on a HiSeq instrument (Illumina) by the NIDDK Genomics Core.

RNA sequencing

RNA sequencing libraries were generating from 1 ug (K562 cells, yolk sac cells or total fetal liver cells) or 10 ng (FACS sorted fetal liver cells) of RNA using the Illumina TruSeq Stranded mRNA Library Preparation kit (20020594) following manufacturer's protocol. Library quality was validated using a High Sensitivity DNA Chip in the Agilent Bioanalyzer 2100. Samples were sequenced on a HiSeq instrument (Illumina) by the NIDDK Genomics Core.

Chromosome conformation capture (3C)

10^7 K562 cells were crosslinked with 1% formaldehyde and 3C was carried out as described using EcoRI cleavage (33). Relative crosslinking between the anchor fragment and fragments of interest was analyzed with SYBR Green Supermix on the 7900HT Real-Time PCR System. Interaction between two fragments within the α-tubulin gene was used for the internal normalization control. 3C primers were previously published (25).

MNase digestion

Digestion was performed as described (34) using Micrococcal nuclease (ThermoFisher Scientific, 88216). For primers see Supplemental Table S1.

Data analysis

For complete data analysis, see Supplementary Materials and Methods.

RESULTS

ETO2 promotes a critical commitment step in murine erythropoiesis *in vivo* and represses embryonic globin genes during maturation of mouse erythroid cells

Homozygous disruption of *cbfa2t3* gene, encoding ETO2, revealed its importance for murine hematopoietic progenitor proliferation and stress-dependent erythropoiesis in adult, although *Eto2*$^{-/-}$ animals are viable and fertile and exhibit only a mild anemia at 8 weeks of age: embryonic erythropoiesis was not studied (Supplementary Figure S1) (27). At E14.5 we observed that WT and *Eto2*$^{-/-}$ livers and embryos were phenotypically similar with no difference in cellularity (Figure 1A). To examine maturational progression, E14.5 fetal liver cells of WT and *Eto2*$^{-/-}$ animals were sorted by FACS after staining with CD71 and Ter119 antibodies (Figure 1B) (35). There was a more than 2-fold increase in immature progenitors (S0 low and S0 medium) for *Eto2*$^{-/-}$ cells, compared to controls, and a substantial decrease in more mature cell populations (S1, S2 and S3), indicating a significant impediment to erythroid lineage commitment (Figure 1C).

The log 2-fold change in density between WT and *Eto2*$^{-/-}$ sorted cells affirmed accumulation of immature CD71$^-$/Ter119$^-$cells and a unique mutant-enriched population of CD71$^+$/Ter119$^+$ in the fetal livers of mutant compared to WT embryos (Figure 1D, upper right panels). Sub-gating of S3 cells separated populations enriched in WT (S3-c) and mutant (S3-m) embryos (Figure 1C, shaded area). Data-driven spatial clustering identified four populations enriched in either WT or *Eto2*$^{-/-}$ cells (Figure 1D, lower panels), which were separately analyzed for forward scatter, a proxy for cell size. Immature CD71-/Ter119- cells that accumulate in *Eto2*$^{-/-}$ livers have an increased fraction of larger cells compared to WT (Figure 1E, cluster 3) but other clusters showed no difference. In summary, *Eto2*$^{-/-}$ embryos have a block in passage from S0 to S1 early in erythropoiesis that is manifest by (1) delayed acquisition of Ter119/CD71 cell surface markers and (2) delayed reduction in cell size, as well as delayed loss of CD71 at later stages of maturation.

We performed RNA-seq of FACS sorted cells to examine transcriptomic changes across erythroid maturation due to loss of *Eto2*. Two broad responses to ETO2 loss emerged: a similar effect in all populations or no effect in most populations except for population S4/5 (Supplementary Figure S2A, B). This suggests that while ETO2 depletion leads, in part, to delayed differentiation, those cells that do mature have strongly disrupted transcription (Supplemental Table S3, Supplementary Figure S2C). Notably, S1 cells have strongly increased levels of PU.1 and GATA2 ($\log_2$-fold change 3.59, padj 2.44E−07 and 4.86, padj 7.94E−15, respectively), regulators whose reduction across the S0-S1 boundary is key to erythroid lineage choice (35). In contrast to most genes, embryonic α- and β-genes were strongly up-regulated across sorted populations upon *Eto2* loss, particularly in population S4/5 (Figure 1F and Supplementary Figure S3). *Hbb-h1* was increased less strongly in population S4/5, whereas, the adult globin genes showed no change. PU.1 and GATA2 remained elevated in S4/5 cells from *Eto2*$^{-/-}$ embryos and GATA1 remained low compared to WT cells.

Overall, these results show disturbed erythroid differentiation kinetics and persistence of embryonic globin transcripts in the most mature E14.5 cells upon loss of *Eto2*. Consistent with the lack of anemia of *Eto2*$^{-/-}$ embryos, total production of β-like globin transcripts was

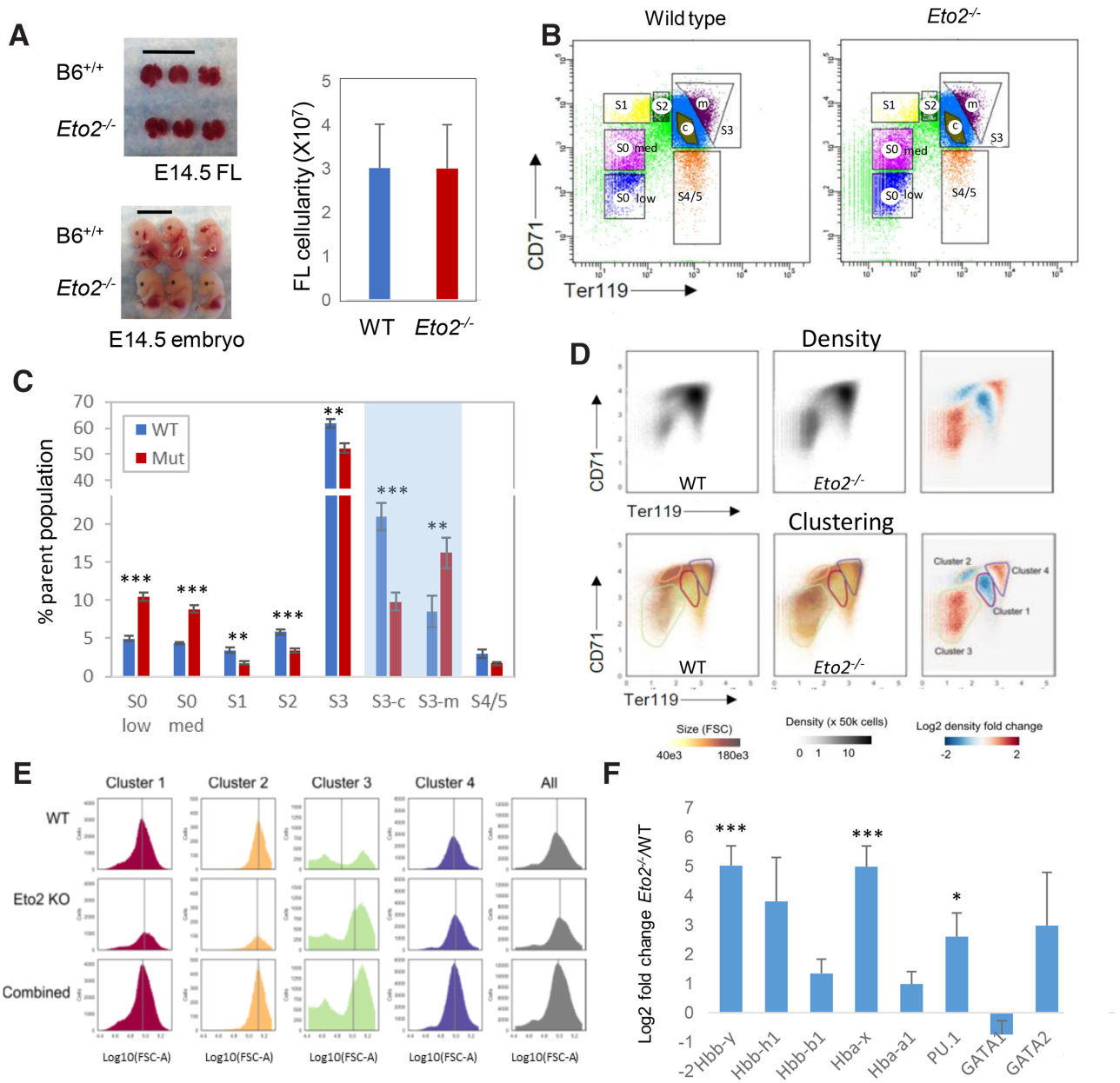

Figure 1. Maturational defect in erythropoiesis and persistence of embryonic globin gene transcription in *Eto2*$^{-/-}$ mice. (**A**) E14.5 embryos and livers of *Eto2*$^{-/-}$ and littermate control C57BL/6 mice are similar in size and appearance (left) and cellularity (right). (**B**) Flow cytometry with gates set to separate maturational stages S0–S4/5 for E14.5 fetal liver cells of *Eto2*$^{-/-}$ (right) and wildtype (WT, left) littermates. S0 low and S0 medium are defined as low GATA1 and medium GATA1, respectively (35). The x-axis depicts Ter119 (Glycophorin A, GYPA) staining intensity while the y-axis depicts CD71 (Transferrin receptor) intensity. (**C**) Quantification of each population S0 to S4/5 (panel B) from control (blue) and *Eto2*$^{-/-}$ (Mut, red) E14.5 fetal livers. Shaded area represents sub-gating of population S3. Error bars indicate SD. $N = 11$ biological replicates. $^{**}P < 0.01$, $^{***}P < 0.001$ by two-tailed Student's *t*-test. (**D**) Flow cytometry densities of WT control (top left panel), *Eto2*$^{-/-}$ (top center panel) fetal liver cells from embryos at E14.5 and $\log_2$ fold ratio of *Eto2*$^{-/-}$ compared to WT control (top right panel). Bottom left and bottom center show the same points but colored by magnitude of forward scatter. Bottom right shows difference plot again but with convex hulls describing clusters overlaid. (**E**) Top. histograms of forward scatter in each of the clusters indicated in D. Vertical line in each panel indicates the mean. (**F**) $\log_2$ fold change of expression for selected genes in population S4/5 RNA-seq for *Eto2*$^{-/-}$ compared to WT cells. Error bars represent standard error of the $\log_2$ fold change. P_{adj} values shown *<0.05, ***<0.001.

not reduced, and was even slightly elevated, in the most mature S3 and S4/5 populations (TPM 117% and 128%, respectively) compared to WT (data not shown). This suggests direct induction of embryonic globin transcripts by loss of ETO2, rather than an effect on β-like globin gene transcription more generally, via a different mechanism. We conclude that *Eto2* is essential for effective erythropoiesis and full silencing of embryonic β-like globin genes during mouse development.

Loss of ETO2 delays the globin switch from embryonic/fetal to adult β-globin transcription

ETO2 function in silencing of embryonic β-like globin genes prompted us to investigate the influence of ETO2 specifically on hemoglobin switching *in vivo*. We carried out timed mating of $Eto2^{-/-}$ animals and matched controls and collected E8.5 yolk sacs, and E12.5 and E14.5 fetal livers, spanning the time course of globin switching during development, for analysis of RNA by RT-qPCR. In C57BL/6 animals at E12.5, mouse globin switching was complete and the adult β-maj globin gene was predominately expressed (Figure 2A, top). By contrast, $Eto2^{-/-}$ embryos predominately expressed embryonic Ey-globin at E12.5, at a level more than 3.5-fold higher than in WT embryos (Figure 2A, bottom).

To extend this investigation to human globin gene switching, $Eto2^{-/-}$ animals were bred to mice homozygous for a single copy of a human β-globin locus transgene and deletion of the endogenous mouse LCR (28) (Supplementary Figure S4A, B). $Eto2^{-/-}$/mLCR$^{+/-}$/huTg$^{+/+}$ mice heterozygous for the mouse LCR (mLCR) were viable and fertile but no live pups with the $Eto2^{-/-}$/mLCR$^{-/-}$ /huTg$^{+/+}$ genotype were born. However, embryos with this genotype were viable up to E16.5, allowing us to study fetal liver erythropoiesis during development. Consistent with the contemporaneous switch of transgenic fetal γ-globin to adult β-globin with embryonic mouse globin gene silencing (65), human globin switching in $Eto2^{+/+}$/mLCR$^{-/-}$ /huTg$^{+/+}$ was substantially complete by E12.5 (Figure 2B). However, a dramatic delay in switching from human embryonic/fetal to adult globin gene expression was observed at E12.5 in $Eto2^{-/-}$/mLCR$^{-/-}$ /huTg$^{+/+}$ mice with persistence of γ-globin at 10% of total globin transcripts at E14.5, suggesting continued transcription of fetal globin genes upon ETO2 loss. Although transcribed at a very low level because of deletion of the mouse LCR, hemoglobin switching in the endogenous mouse locus of these transgenic mice is also delayed (Supplementary Figure S4C).

Hemoglobin switching from γ-globin to β-globin during differentiation of human umbilical cord CD34$^+$ primary erythroid cells upon knock down of ETO2 mirrored the delay observed in $Eto2^{-/-}$ embryos and embryonic/fetal globins persisted at 53% of total globins compared to 28% for control cells at day 18 of differentiation (Figure 2C, D, Supplementary Figure S5). We also examined the effect of ETO2 loss in human umbilical cord derived erythroid progenitor cells (HUDEP2), an immortalized CD34$^+$ hematopoietic stem cell-derived erythroid precursor cell line (36). These cells reflect a time point after the switch when γ-globin transcripts are exceptionally low. ETO2 shRNA elevated γ-globin transcription both before and after differentiation, while ε-globin transcripts were more modestly affected (Figure 2E, F). Therefore, in both mouse and human erythroid progenitors, the loss of ETO2 results in ineffective silencing of embryonic and fetal globin genes during hemoglobin switching and can elevate γ-globin transcription in the HUDEP2 model. Altogether, these *in vivo* and *in vitro* results support dual roles for ETO2 during murine embryonic erythropoiesis: ETO2 is required for effective erythrocyte maturation as well as to regulate sequential expression of β-globin genes.

ETO2 is an LDB1-associated enhancer-binding transcriptional repressor of erythroid genes in human K562 cells

To investigate the key role of ETO2 in repression of erythroid genes, we knocked out ETO2 in human K562 cells by two different CRISPR/Cas9 strategies to dissect its mechanistic function (Supplementary Figure S6A). Clones with undetectable ETO2 protein and strongly elevated γ- and ε-globin were obtained (25) (Supplementary Figure S6). RNA-seq was performed for control and ETO2 KO-1 cells (Figure 3A). Erythroid fingerprint genes (19) were enriched in both up- and down-regulated categories, but the magnitude of enrichment was greater for up-regulated genes (odds ratio 2.08, FDR $<$ 2e−16) than for downregulated genes (odds ratio 1.62, FDR $<$ 3.96e−8). Human embryonic and fetal globin genes *HBZ, HBE1, HBG1/2* were up regulated upon ETO2 loss, as occurred *in vivo* in $Eto2^{-/-}$ human transgenic mice.

Overlap of differentially expressed genes upon ETO2 KO in K562 cells revealed 103 up-regulated genes shared with previous knock-down studies that had been performed in murine G1E and MEL cells (23,37,38) (Supplemental Table S4). This core repressive erythroid ETO2 regulatory signature includes heme synthetic enzymes, erythrocyte membrane proteins and erythroid master transcription factors (Figure 3B). In addition, our results reveal that down-regulated genes upon ETO2 loss include transcription factors *ETS1* and *KLF8* that play negative roles in globin gene expression and hemoglobin synthesis (39,40) and downregulated genes are highly enriched for histone deacetylase complex function (Supplementary Figure S7A).

To follow transcriptomic changes in ETO2 targets during differentiation of erythroid progenitors, we performed RNA-seq for CD34$^+$ cells progenitor cells at differentiation days 8 and 12. We found that genes that are de-repressed upon ETO2 KO in K562 cells are up regulated during differentiation of CD34$^+$ cells, including all but one (TSPAN33) of the core repressive erythroid ETO2 regulatory signature genes in Figure 3B, as well as embryonic/fetal globin genes HBE1 and HBG1/HBG2 (Supplementary Figure S7B, C). In addition, RNA-seq of WT and $Eto2^{-/-}$ mouse E8.5 yolk sac and E12.5 and E14.5 fetal liver cells showed that all the genes representing the core repressive ETO2 regulatory signature (Figure 3B) are upregulated at E8.5 in $Eto2^{-/-}$ cells compared to control

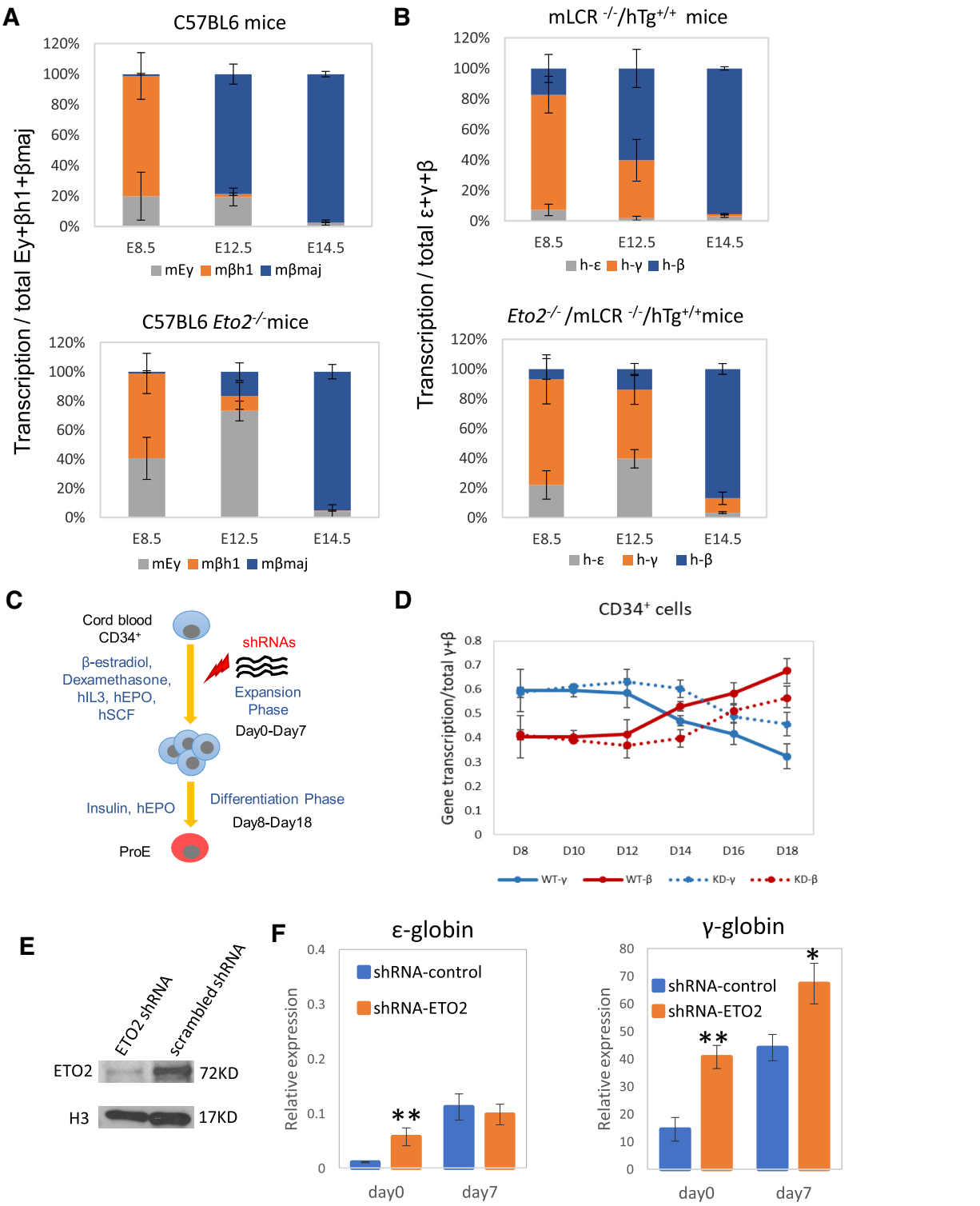

Figure 2. Absence of *Eto2* delays the hemoglobin switch and results in ineffective embryonic/fetal globin gene silencing during development. (**A**) Mouse globin gene expression as a % of total β-globin gene expression detected in C57BL/6 and $Eto2^{-/-}$ mice at E8.5, E12.5 and E14.5. Error bars indicate SD. $N = 3$ biological replicates. (**B**) Expression of human globin genes is shown as a % of total β-globin gene expression in *Eto2* WT mice ($Eto2^{+/+}$mLCR$^{-/-}$ hTg$^{+/+}$) and $Eto2^{-/-}$ mice ($Eto2^{-/-}$ mLCR$^{-/-}$ hTg$^{+/+}$) at E8.5, E12.5 and E14.5. (**C**) Schema for ETO2 knock down in human primary erythroid cells (CD34^{+} cells). (**D**) RT-qPCR shows expression of globin genes as a % of total β-globin gene expression during differentiation of CD34^{+} cells over 18 days. Error bars indicate SD. N = three biological replicates. (**E**) Western blot of extracts from HUDEP2 cells differentiated with an ETO2 shRNA or a scrambled shRNA as control. (**F**) RT-qPCR was performed for ε-globin γ-globin at day 0 and day 7 of differentiation. ε-globin levels were >100-fold lower than γ-globin. Error bars indicate SD. $N = 3$ independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ by two-tailed Student's *t*-test. For Primers, see Supplemental Table S1. For antibodies, see Supplemental Table S2.

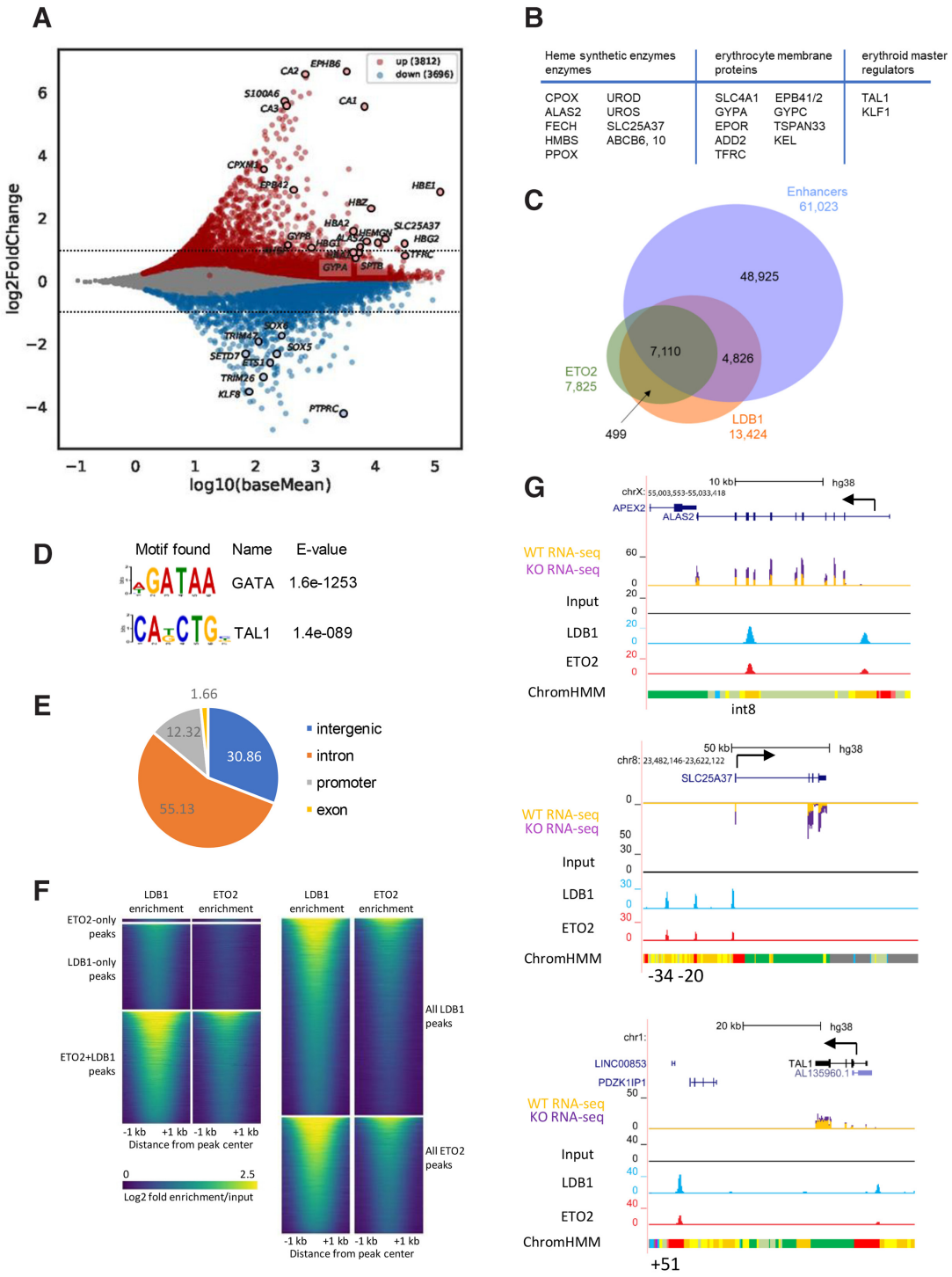

Figure 3. ETO2 and LDB1 co-occupy enhancers and primarily represses erythroid-related and β-globin gene expression. (**A**) M−A plot showing differential gene expression between WT (no sgRNA) and ETO2 knock out in K562 cells. Of 57 992 annotated genes, loss of ETO2 resulted in differentially expression of 7508 genes of which 3812 were up-regulated (red) and 3696 were down-regulated (blue) ($\log_2$-fold change > 0, $P_{adj} < 0.05$). RNA-seq was performed in triplicate. (**B**) Core repressive erythroid ETO2 regulatory signature genes shared across platforms in which ETO2 KO or KD studies have been performed include heme synthetic enzymes, erythrocyte membrane proteins and erythroid master transcription factors (23,37,38). (**C**) Venn diagram showing colocalization of enhancers (from the Ensembl Regulatory Build segmentation, total enhancers, blue) and ChIPmentation peaks for LDB1 (gold) and ETO2 (green). Number of peaks or calls is indicated. (**D**) Top motifs from *de novo* motif searching of ChIPmentation data under the ETO2+LDB1 co-binding sites. (**E**) The distribution and percentage of ETO2 binding sites in different genomic elements. (**F**) Heatmaps of normalized ChIPmentation signal enrichment within subsets of peaks. Left subpanel shows subsets of peaks; right subpanel shows all peaks for each signal. (**G**) Representative screenshots of RNA-seq signal in WT (yellow) or ETO2 KO (purple) K562 cells and ChIPmentation signals at select loci. Human equivalents of known mouse enhancers of the selected genes are labeled. Y-axes represent CPM (number of reads per base / million mapped reads). Bottom-most track is the K562 segmentation track from ChromHMM.

cells (Supplementary Figure S7D). Importantly, these genes are not significantly affected at E12.5 or E14.5. These results are consistent with a model in which ETO2 represses the erythroid transcriptional program early in differentiation (38). They are also consistent with partial compensation for loss of ETO2 repressive activity during embryonic development in the mouse.

To identify direct targets of ETO2, we performed ChIPmentation for K562 cells (31). Using antibodies to unmodified proteins, we uncovered 13,424 and 7,825 high confidence LDB1 and ETO2 peaks, respectively (Figure 3C). Interestingly, ETO2 occupancy was almost completely co-incident with LDB1 (97%, Figure 3C). A *de novo* MEME search revealed strong enrichment for the LDB1 complex GATA1 and TAL1 binding motifs at LDB1/ETO2 co-occupied sites (Figure 3D). Nearly 86% of these sites were intergenic or intronic, with limited binding to proximal promoters, indicating that ETO2 and LDB1 function together primarily in genomic regions likely to encompass enhancers (Figure 3E, Supplementary Figure S8A, B). In support, the venn diagram in Figure 3C illustrates that a large percentage of LDB1-bound sites co-occupied by ETO2 overlapped with enhancers called by the Ensemble regulatory build (41). Although a significant number (42%) of LDB1 called peaks lacked ETO2 in this analysis, the ETO2 signal was, overall, weaker than the LDB1 signal suggesting that LDB1-unique sites may have low levels of ETO2 too weak to be called as a peak (Figure 3F). Genome browser views in Figure 3G illustrate localization of LDB1 and ETO2 at enhancers of prominent erythroid genes upregulated upon loss of ETO2.

Integration of transcriptomic data and genome localization results together with long range interaction data for K562 cells (42) revealed that LDB1 and ETO2 peaks align with Ensemble enhancers that interact with 3,201 genes that are either up-regulated (1399 genes) or down-regulated (1802 genes) upon ETO2 loss (Supplemental Table S5). Genes looped to LDB1 enhancers included 64% of the genes upregulated upon ETO2 loss across mouse and human erythroid cells (Supplemental Table S4). The looped genes also included 18 of the 22-core repressive ETO2 regulatory signature genes (Figure 3B): genes for UROS, ABCB10, KEL and EPB42 proteins were missing but manual inspection of these gene loci suggests they may also be regulated by LDB1/ETO2 regulated enhancers. As we have seen (Supplementary Figure S7D), these core ETO2-repressed looped genes are also mis-regulated at E8.5 in *Eto2*$^{-/-}$ mice, indicating the central involvement ETO2 in enhancer long range regulation of erythroid genes in cell lines and *in vivo*. Overall, the results are consistent with ETO2 playing a broad and primarily negative regulatory role for erythroid genes, including embryonic and fetal globin genes, by occupying LDB1-complex regulated enhancers.

ETO2 negatively affects enhancer activity and chromatin looping in the β-globin locus

To explore how ETO2 influences enhancer activity, we focused on the γ-globin gene, whose expression *in vivo*, in CD34^{+} progenitor cells and in K562 cells is regulated by the LCR through LDB1 complex occupancy and long-range interactions (2,25) (Figure 4A). ETO2 occupancy, as determined by ChIP-qPCR, is low at the active γ-globin promoters in K562 cells (Figure 4A). This result is in agreement with earlier studies showing low ETO2 occupancy at active γ-globin promoters in primary erythroid progenitors differentiated to produce high levels of γ-globin, whereas, in the same cells differentiated to produce low levels of γ-globin transcripts there is notable occupancy of ETO2 at silent γ-globin promoters (43). ChIP-qPCR further revealed loss of ETO2 from the β-globin locus upon ETO2 KO and increased enrichment of LDB1 and complex members GATA1 and TAL1 (Figure 4B, C; Supplementary Figure S9A, B). Moreover, increased Mediator (MED1) and RNA Polymerase II was detected in these sites upon of ETO2 loss, consistent with increased transcription (Figure 4D, E).

Increased enrichment of LDB1 looping complex members upon ETO2 KO prompted us to investigate β-globin locus chromatin looping changes in ETO2 KO cells. We performed 3C and observed interaction between the LCR and γ-globin genes was significantly increased in ETO2 KO cells compared to WT cells (Figure 4F). Inversely, overexpression of ETO2 (Supplementary Figure S9C) impaired LCR-γ globin looping. ETO2 loss also increased ε-globin looping to the LCR. Moreover, ETO2 loss increased non-coding eRNA transcription from the intergenic BGLT3 enhancer, which contributes to activation of γ-globin genes, and increased BGLT3/γ-globin gene interaction (44) (Supplementary Figure S9D−F). Overall, these loss and gain of function studies show that antagonizing LDB1 enhancer looping is a critical component of ETO2 control of LDB1 complex positive versus negative regulation of target genes and suggests that ETO2 is a toggle switch for such enhancer function.

Involvement of NuRD complex in ETO2 co-repressor activity

LDB1 complex member GATA1 pulls down NuRD components in megakaryocyte and erythroid backgrounds, suggesting NuRD may have a role in hematopoietic gene regulation (45,46). NuRD components associate with both GATA1-repressed and activated genes in G1E cells, through a NuRD/GATA/FOG axis (47,48). However, the γ-globin gene does not seem to be silenced primarily through NuRD/GATA/FOG (49,50). To ask if ETO2/LDB1 repression of γ-globin involves the NuRD complex, we performed co-IP experiments with an ETO2 antibody and blotted the precipitated protein with NuRD component antibodies.

We observed that ETO2 pulls down CHD4 as well as HDAC1, which is a component of several co-repressor complexes (51,52) (Figure 5A). Reciprocally, CHD4 pulls down ETO2. In addition, IP with antibodies to NuRD components MTA2 and RBBP4 show that they too pull down ETO2. Occupancy of CHD4, HDAC1, MTA2 and RBBP4 at the LCR and γ-globin promoter was disrupted by ETO2 loss (Figure 5B−E). At the same time, decreases in nucleosome occupancy and increases in histone H3K9 and H3K27 acetylation in the LCR and γ-globin gene provided evidence for CHD4 and HDAC1 activity in the

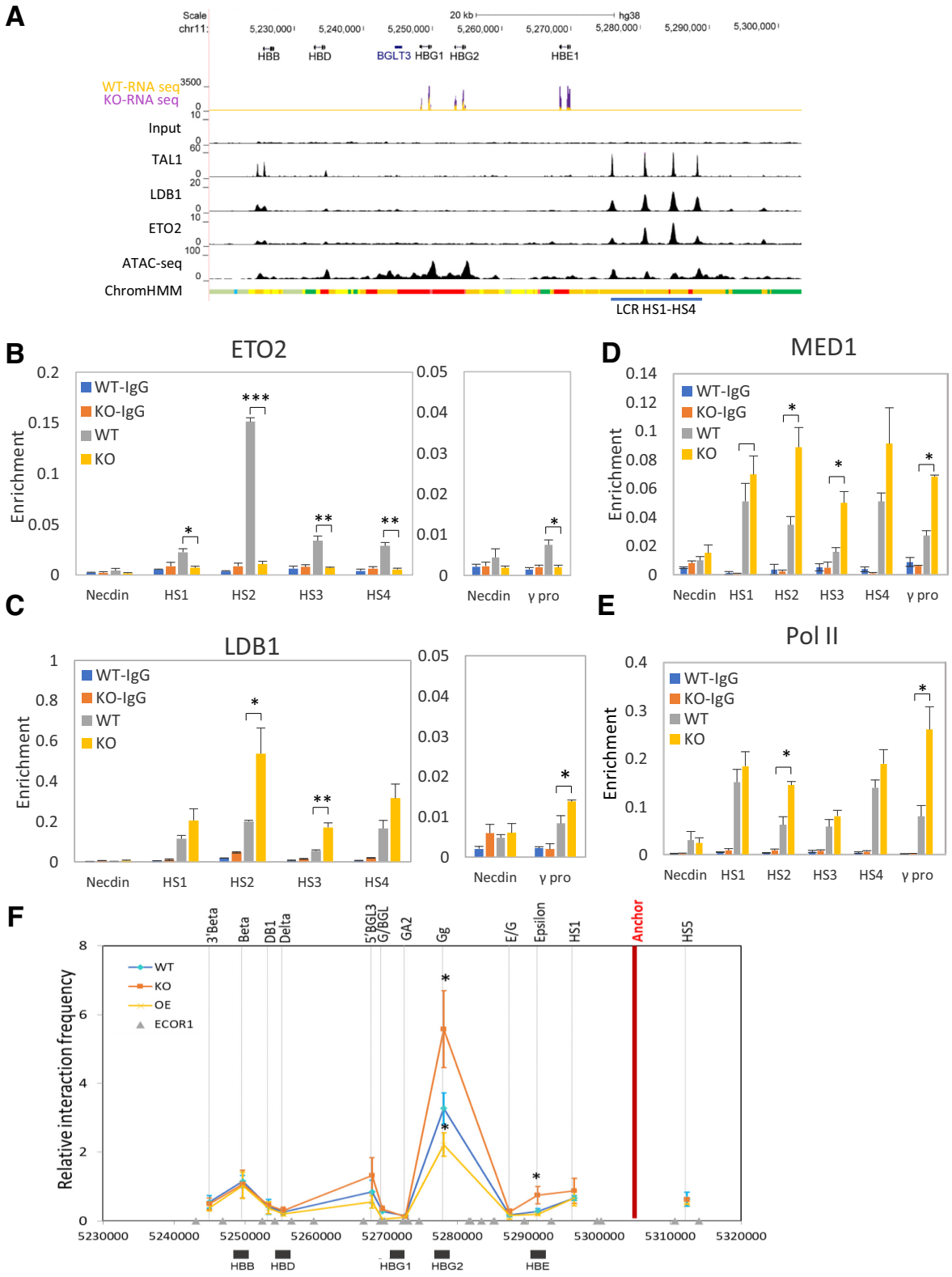

Figure 4. ETO2 antagonizes LDB1-complex enhancer activity and chromatin looping between the β-globin locus LCR enhancer and active genes. (**A**) Representative screenshots of RNA-seq, TAL1, LDB1 and ETO2 ChIPmentation signals in the β-globin gene locus. (**B–E**) ChIP-qPCR of ETO2, LDB1, MED1 and Pol II occupancy in the LCR HS1-HS4 and γ-globin promoter for WT and ETO2 KO K562 cells. (**F**) Chromosome conformation capture (3C) indicates LCR/globin gene interaction frequency in WT, ETO2-KO and ETO2-overexpressing cells. LCR serves as anchor. Ggamma primer does not distinguish between A/Ggamma genes and signal represenrs the interaction frequency for both genes. Error bars indicate SD. $N = 3$ biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-tailed Student's *t*-test.

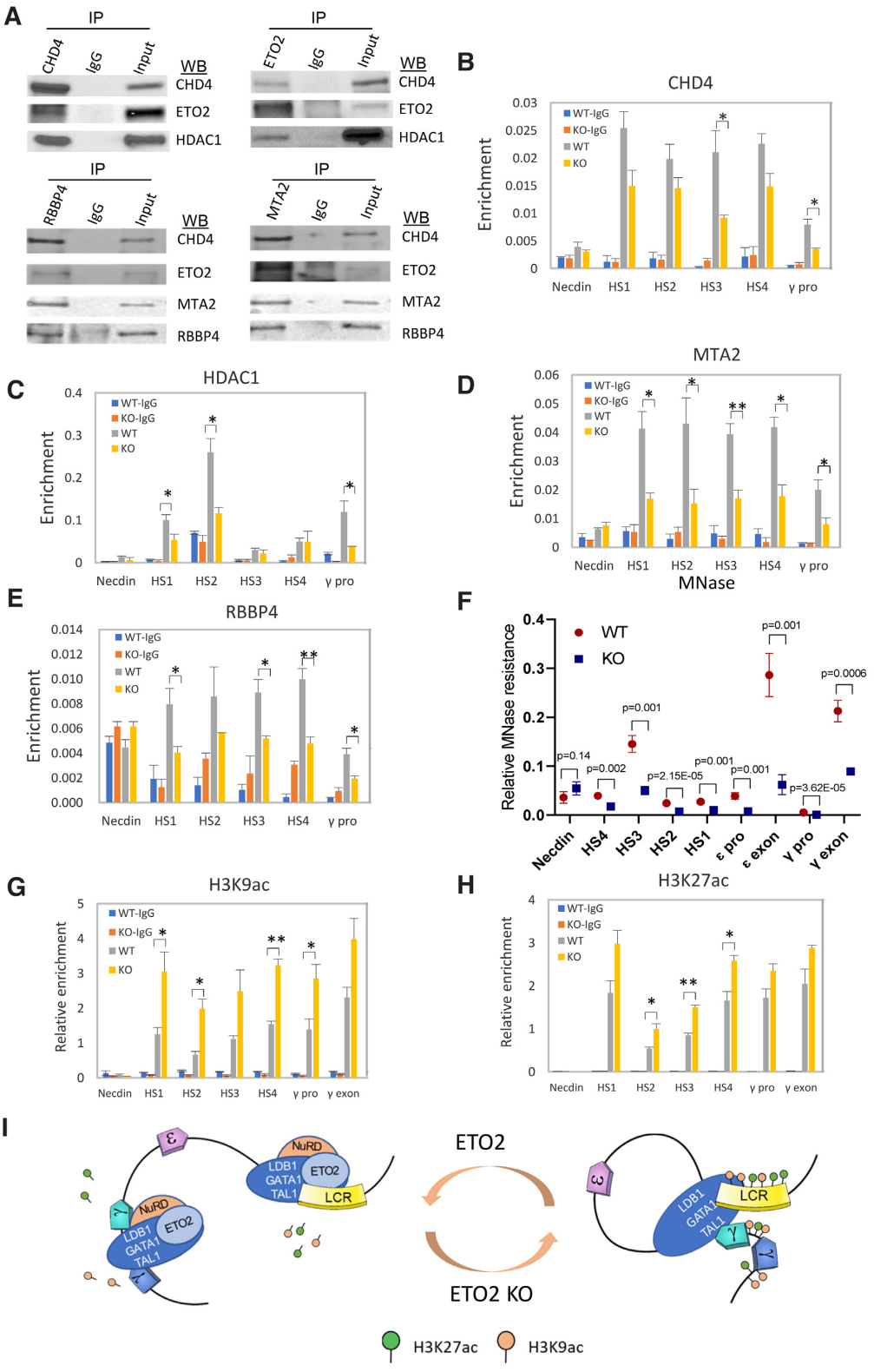

Figure 5. ETO2 occupancy affects the enrichment of nucleosome remodeling and deacetylating NuRD complex in the β-globin locus. (**A**) Co-IP analysis of reciprocal interactions between ETO2 and CHD4. (**B–E**) ChIP-qPCR for CHD4, HDAC1, MTA2 and RBBP4 occupancy at the LCR HSs and γ-globin gene in WT and ETO2-KO cells. (**F**) MNase-qPCR showing sensitivity to MNase digestion of the globin gene locus in WT and ETO2-KO cells. (**G, H**) ChIP-qPCR for H3K9ac and H3K27ac in LCR HSs and γ-globin gene in WT and ETO2-KO cells. (**I**) Model depicting reduced ETO2/LDB1 ratio, elevated H3K27ac and H3K9ac modification and γ-globin looping to the LCR after loss of ETO2. Error bars indicate SD. $N = 3$ biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$ by two-tailed Student's *t*-test.

locus and their disruption by ETO2 deletion (Figure 5F-H). Consistent with these results, persistent transcription of Ey-globin in E12.5 ETO2 null fetal liver cells (Figure 2A) correlated with increased H3K27ac and MNase sensitivity, compared to WT fetal liver, *in vivo* (Supplementary Figure S10). This difference was not noted at E14.5 when Ey-globin transcription had significantly decreased.

To validate these results in a different system we used HUDEP2 cells. When HUDEP2 cells are induced to undergo erythroid differentiation, γ-globin (Figure 2F) and erythroid fingerprint genes that are negatively regulated by ETO2, including EPOR, SLC4A1, EPB41, ALAS2 and TAL1 are de-repressed (36,53). To directly compare de-repression of ETO2 targets to up-regulation by ETO2 KO, we first used co-immunoprecipitation to show that ETO2 interacts with CHD4 NuRD component in HUDEP2 cells (Supplementary Figure S11A). We then performed ChIP for ETO2, LDB1 and CHD4 in these cells at the LCR and γ-globin promoter and at ALAS2 and TAL1 enhancers before (D0) and after (D7) induction. In each case, we observed an increase in LDB1 occupancy accompanied by decreased ETO2 and CHD4 following induction (Supplementary Figure S11B-F). These results parallel those observed in the LCR and γ-globin gene after ETO2 KO in K562 cells.

Taken together, these results indicate that ETO2 interacts with multiple NuRD components and recruits NuRD complex to regulate the chromatin status at target genes including in the β-globin locus. NuRD reduction upon loss of ETO2 is associated with stabilization of H3K27 and H3K9 acetylation in the β-globin locus, modulating the γ-globin epigenetic environment to favor LCR/γ-globin looping and transcription activation (Figure 5I).

Functional domains of ETO2 in repression of erythroid genes

To provide support for the idea that ETO2 is important for NuRD occupancy in the β-globin locus, we defined domains of ETO2 involved in NuRD interaction. ETO2 shares four domains (NHR1–4) with conserved homology (50–70%) with *D. melanogaster* Nervy [for review, see (54)] (Figure 6A). NHR1 is required for interaction of ETO2 with TAL1 in erythroid cells (55). Several HDACs and the SIN3A co-repressor interact with ETO2 through NHR2 and this region also supports oligomerization of the ETO2/GLIS2 fusion protein characteristic of a large fraction of acute megakaryoblastic leukemias (56). The zinc finger C-terminal NHR4 domain contributes to interaction of ETO2 with co-repressor complexes N-Cor/SMRT and SIN3A. The function(s) of NHR3 is less clear.

We stably expressed WT-ETO2 (FL) and deleted versions (NHR1, FL-ΔD1; NHR2, FL-ΔD2; NHR4 FL-ΔD4) in the background of ETO2-KO K562 cells (Figure 6A, Supplementary Figure S12A). Loss of NHR1 reduced the interaction between ETO2 and TAL1, but NHR2 also influenced this interaction (Figure 6B). Loss of NHR2 disrupted ETO2 interaction with HDAC1 and was also the primary region of interaction with CHD4. Therefore, NuRD components depend on similar interactions with ETO2 NHR2 as do other co-repressor complex components that are functionally important for repression/activation of ETO2 target genes (54). To strengthen these results, we performed ChIP for ETO2 KO cells expressing WT-ETO2 (FL) or deleted versions using antibodies to CHD4 and MTA2, which are found only within the NuRD complex. Absence of NHR2 rather than other functional domains of ETO2 impeded the binding of CHD4 and MTA2 to the LCR and γ-globin promoter. The results clearly affirm that CHD4 and MTA2 interact with ETO2 through NHR2 (Figure 6C, D).

RNA-seq was performed to evaluate the ability of mutant ETO2 proteins to rescue expression of genes dysregulated by loss of ETO2 in KO cells (Supplementary Figure S12B). Rescue was defined as restoration of expression to a level not significantly different from WT. Expression of 2,318 genes was rescued by FL ETO2 in ETO2 KO cells (both up and down-regulated upon ETO2 KO. Deletion of any single NHR significantly reduced the ability of ETO2 to function in this fashion and almost 60% of the genes that could be rescued by FL ETO2 were dependent on NHR2 (Supplementary Figure S12C). A large fraction of rescued genes (30%), including γ-globin genes, required all three regions for positive or negative gene regulation by ETO2 (Figure 6E, Supplementary Figure S12C). This result implicates ETO2 interaction with both co-repressor complexes and with TAL1 as critical for its function in gene regulation.

We further interrogated the role of ETO2 domains in LCR/γ-globin chromatin looping using 3C. Expression of full length ETO2 in ETO2 KO cells repressed LCR/γ-globin loop formation to a level similar to that seen in control cells but absence of any ETO2 functional domain resulted in failure to confer this repression (Figure 6F). These results support the relevance of each NHR of ETO2 for the regulatory function of ETO2 on erythroid gene expression. They also bolster the conclusion that ETO2 recruits NuRD to the β-globin locus through NHR2 to antagonize LCR/γ-globin looping.

DISCUSSION

Transition of E14.5 mouse fetal liver erythroid cells through the S0-S1 boundary represents a crucial commitment step in erythropoiesis marking the onset of erythropoietin dependence, chromatin opening at the β-globin locus and activation of GATA1 function (35). S0 to S1 erythroid progression requires decreased PU.1, which favors myeloid lineage choice, and decreased GATA2, initiating the GATA switch (57). However, S1 cells of *Eto2*$^{-/-}$ E14.5 fetal liver cells retain elevated PU.1 and GATA2 compared to WT cells. PU.1 is regulated by LDB1 complex long-range enhancer looping (16). Therefore, loss of ETO2 would be expected to favor retention of long-range enhancer contacts by this gene and continued transcription. GATA2 also has LDB1-occupied enhancers but dependence of long-range regulation of GATA2 on LDB1 is less clear (58). Thus, one feature of erythroid lineage choice may be the direct role of ETO2 in antagonizing enhancer looping. The full influence of ETO2 loss on direct and indirect target genes during erythroid maturation deserves further investigation.

Genome-wide studies indicate that ETO2 carries out its co-repressor role in partnership with the LDB1 complex

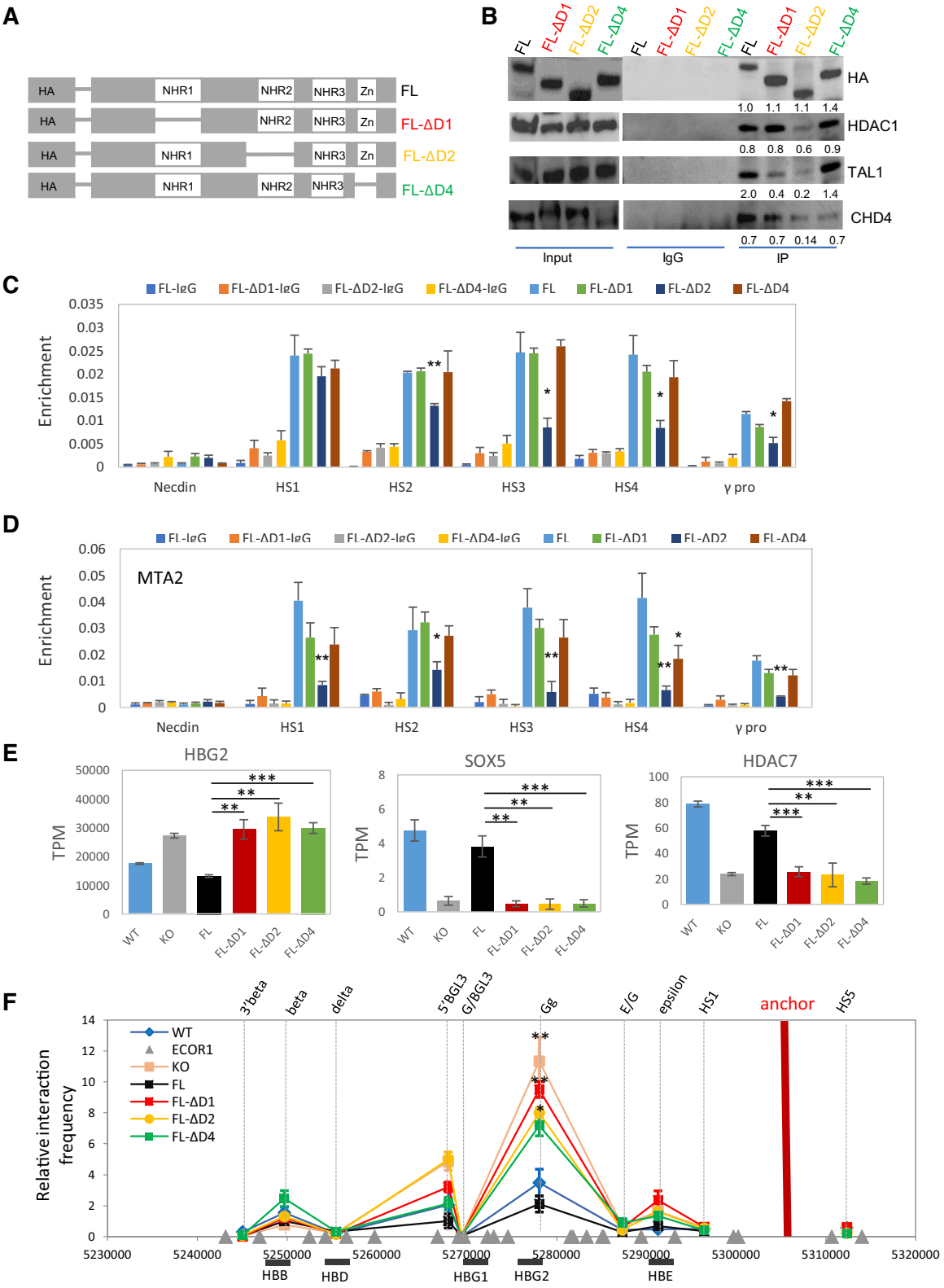

Figure 6. ETO2 depends on three functional domains to play a regressive role in epigenetic regulation of erythroid genes and LCR/γ-globin looping. (**A**) Diagram of HA-tagged ETO2 FL and domain deletion variants expressed in ETO2 KO cells. Full length, ETO2; ETO2 with NHR1 deletion (domain 1 deletion, FL-ΔD1); ETO2 with NHR2 deletion (domain 2 deletion, FL-ΔD2); ETO2 with NHR4 (Zn) deletion (domain 4 deletion, FL-ΔD4). (**B**) Co-IP for interactions between ETO2 variant proteins and HDAC1, TAL1 and CHD4. (**C, D**) ChIP-qPCR for CHD4 and MTA2 in ETO2 KO cells expressing ETO2 or different ETO2 variant proteins. E. Transcripts Per Million (TPM) of HBG2 (negatively regulated by ETO2) and SOX5 and HDAC7 genes (positively regulated by ETO2) in the ETO2 WT, KO, FL, FL-ΔD1, FL-ΔD2, FL-ΔD4 RNA-seq. F. 3C shows interaction frequency between the LCR and globin genes in ETO2 KO cells expressing ETO2 or different ETO2 variant proteins. LCR serves as anchor. Error bars indicate SD. $N = 3$ biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$ by two-tailed Student's t-test.

across erythroid enhancers. Loss and gain of function studies using the γ-globin genes as a model, showed that the abundance of ETO2 at an LDB1-regulated enhancer serves as a toggle switch between looping/activation and loss of contacts/repression. The positive effect of ETO2 loss on enhancer chromatin looping may directly reflect elevated LDB1 occupancy dynamically mediating increased gene contacts, while ETO2 recruitment of NuRD complex components may antagonize looping indirectly through nucleosome compaction and maintenance of low levels of H3K9ac and H3K27ac at enhancers and target genes (59). While the temporal relationship between looping and epigenetic modifications cannot be determined in our studies, other data invoke the determinative relationship of enhancer looping to gene activation (17,60,61). The suggestion is that ETO2 tunes enhancer looping and gene expression through influencing LDB1 abundance and stabilizes the outcome by altering the epigenetic landscape via NuRD partnership.

ETO2-mediated repression remains incompletely understood. GFI1B, HDACs, NCOR/SMRT MBD2 and SIN3A co-repressors have been implicated as ETO2 partners in diverse cell types, as well as LSD1/CoREST in MEL cells (20,23,38,50,54,62). Our data invoke the recruitment of the NuRD complex by ETO2 through the NHR2 domain and demonstrate the importance of this domain for occupancy of NuRD in the β-globin locus. The ETO2 NHR2 interface is essential for maintenance of ETO2-GLIS2-driven leukemia though mediating homo- and heterodimerization (51). Interestingly, we found that loss of the NHR2 abrogated the repressive role of ETO2 in LCR/γ-globin looping, raising the possibility that ETO2 dimerization antagonizes LDB1 dimerization to confer a negative influence on enhancer long-range interactions.

The NuRD component CHD4 was known to occupy the γ-globin gene and CHD4 KD upregulated γ-globin transcription in human progenitor cells (6,50,61,63). NuRD and other co-repressor activities are also recruited to γ-globin by BCL11A and LRF whose sites of occupancy flank the LDB1 complex binding site in the promoter (6,7). Moreover, BCL11A loss affects γ-globin/LCR looping, although BCL11A is not known to be involved in long-range enhancer contacts (8,64). It seems reasonable to propose that BCL11A and LRF antagonize LCR/γ-globin LDB1 occupancy/looping through epigenetic mechanisms occurring at the gene promoter, but other/additional mechanisms are possible. In contrast, because LDB1 mediates LCR/globin gene looping through protein dimerization, ETO2 repression through the LDB1 complex appears to be directly related to looping (17,18).

Despite the importance of ETO2 for the kinetics of normal erythropoiesis and suppression of embryonic/fetal globins, embryonic viability is unaffected in *Eto2* null animals. Moreover, ETO2 appears to be dispensable for maintenance of steady-state adult erythropoiesis and $Eto2^{-/-}$ adult mice have only a mild anemic phenotype (27). Potential compensatory co-repressors include ETO2 partner IRF2BP2, ZBTB7A, BCL11A and SOX6 (7,38,64,65). However, RNA-seq of E14.5 fetal liver erythroid cells revealed that ETO2 loss has only modest effects on transcription of these repressors (Supplementary Figure S13). Further experiments will be necessary to investigate this issue. Interestingly, embryonic mouse globins can be detected, albeit at low levels (<0.001% of total β-type globins), in adult blood cells of *Eto2* null animals after they are completely repressed in normal animals and a delay in erythroid maturation can also be observed (Supplementary Figure S14). These results illustrate the importance of ETO2 for stable embryonic globin gene silencing. ETO2 interaction with the LDB1 complex and/or ETO2 recruitment of NuRD complex may be suitable targets for small molecule interference that might be beneficial in β-globin hemoglobinopathies.

DATA AVAILABILITY

The raw and processed RNA-seq and ChIPmentation data from this work has been submitted to the NCBI Gene Expression Omnibus (GEO: http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSE142228 and GSE156306.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Author contributions: X.G., J.P.B. and A.D. designed the experiments. X.G., J.P. and I.K performed experiments. R.D. performed bioinformatic analyses. X.G., J.P. and A.D wrote the paper, which was edited by all authors.

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