

Original Article

MYB represses ζ -globin expression through upregulating ETO2

Zejun Dong^{1,2}, Yuhua Ye^{1,2}, Wei Zhang^{1,2}, Hualei Luo^{1,2}, Jialong Li^{1,2}, Qianqian Zhang^{1,2}, Xinhua Zhang³, Xiang Guo^{4,*}, and Xiangmin Xu^{1,2,*}

¹Innovation Center for Diagnostics and Treatment of Thalassemia, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China, ²Department of Medical Genetics, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China, ³Department of Hematology, 923rd Hospital of the People's Liberation Army, Nanning 530021, China, and ⁴Institute of Blood Diseases, Department of Hematology, Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu 610072, China

*Correspondence address. Tel: +86-20-61648293; E-mail: xixm@smu.edu.cn (X.X.) / Tel: +86-18884895329; E-mail: xianguo@uestc.edu.cn (X.G.)

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Abstract

Reactivating the embryonic ζ -globin gene represents a potential therapeutic approach to ameliorate the severe clinical phenotype of α -thalassemia and sickle cell disease. The transcription factor MYB has been extensively proven to be a master regulator of the γ -globin gene, but its role in the regulation of ζ -globin remains incompletely understood. Here, we report a mechanistic study on the derepression of ζ -globin both *in vivo* and *in vitro*. We show that MYB depletion in mouse models and human hematopoietic stem cells leads to consistent and remarkable reactivation of ζ -globin. Furthermore, multiomics analysis and functional validation of MYB-knockout and wild-type cell lines reveal that ETO2 functions as a novel repressor of ζ -globin through coordination with NuRD nucleosome remodeling and the deacetylation complex to modulate histone deacetylation of ζ -globin. Additionally, we evaluate the clinical significance of these findings by knocking out ETO2 in primary CD34⁺ cells from nondeletional hemoglobin H patients, which results in a significant increase in ζ -globin expression. The RNA-seq data reveal that key erythroid genes are more co-regulated by Myb and Eto2 than by Myb and Klf1, highlighting a distinctly enhanced erythroid-specific transcriptional impact within the MYB-ETO2 regulatory axis. Compared with ETO2 knockout alone, codepletion of ETO2 and BCL11A did not significantly activate ζ -globin, suggesting that the MYB-ETO2 pathway primarily silences ζ -globin. Our study reveals a linear MYB-ETO2 signaling pathway crucial for ζ -globin repression and offers new targets for treating α -thalassemia and sickle cell disease.

Key words gene switch, thalassemia, hematopoiesis, zeta-globin

Introduction

Thalassemia is among the most prevalent monogenic disorders worldwide and poses a significant health and economic burden. Approximately 1%–5% of the world's population is reported to carry α -thalassemia mutations, and 3% is reported to carry β -thalassemia mutations [1–3]. Over the last three decades, considerable progress has been achieved in β -thalassemia treatment, highlighted by research on γ - β hemoglobin switching and pharmacological reactivation of fetal hemoglobin [4–10]. However, gaps are pronounced in the case of α -thalassemia. Mammalian α - and β -like globin genes, which are arranged on the basis of their

expression during development, transition from embryonic/fetal to adult forms, reflecting their role across different gestational stages and erythroblast maturation sites. The α -like globin locus in both humans and mice harbors three functional genes, including an embryonic ζ -globin gene and two duplicated adult α -globin genes [11]. The embryonic ζ -globin gene is specifically expressed in primitive erythrocytes derived from the yolk sac, followed by transcriptional repression as a transition to definitive erythropoiesis occurs [12]. Previous experimental evidence has confirmed that the induction of embryonic ζ -globin, by acting as a substitute for deficient adult α -globin, rescues the viability of transgenic mouse

models of α -thalassemia and sickle cell disease (SCD), suggesting a potential therapeutic approach for both conditions [13,14]. However, relatively limited attention has been given to the regulatory mechanisms of ζ -globin reactivation.

Erythropoiesis is a differentiation process in which hematopoietic stem cells progressively transform into mature red blood cells through a series of cellular events and the precise regulation of globin gene expression, which is highly regulated by specific transcription factors and growth factors. Substantial efforts have been made over the past few decades to investigate the genetic mechanisms underlying globin gene expression and fetal-to-adult hemoglobin switching, leading to the identification of BCL11A, MYB, KLF1 and LRF as the major modifiers in the regulation of γ -globin gene expression [15–17]. The transcription factor MYB plays an indispensable role not only in definitive erythropoiesis but also in its capacity to repress embryonic (ε -globin) and fetal gene (γ -globin) expression within the β -globin locus. The traditional model posits that MYB action occurs through KLF1, affecting the downstream factors BCL11A and LRF to regulate γ -globin gene expression [16,18–20]. Biallelic deleterious mutations in KLF1 reportedly cause hemolytic anemia with a dramatic increase in ζ -globin expression [21]. Recent studies provided evidence that BCL11A and LRF, two master γ -globin regulators, might have a direct effect on ζ -globin repression, both by associating with NuRD complex and possibly recruiting HDAC proteins as well [22–24]. Sankaran VG *et al.* [25] reported that patients with trisomy 13 (Patau syndrome) have elevated ζ -globin level as a result of decreased level of the transcription factor MYB. However, the mechanism by which MYB regulates ζ -globin level remains unclear.

By generating *Myb* homozygous knockout (KO) mice and MYB gene KO HUDEP-2 and CD34⁺ cells, along with multiomics analysis of MYB-KO data, we revealed a novel MYB-ETO2 pathway responsible for the genetic silencing of ζ -globin, which may serve as a potential therapeutic target for α -thalassemia and SCD. Taken together, our study identified the trans-acting factors responsible for the transcriptional silencing of ζ -globin genes and revealed a major regulatory signaling pathway from MYB to ETO2 to ζ -globin.

Materials and Methods

Mice

Female C57BL/6 mice weighing 22 ± 2 g and 8 weeks old were procured from Southern Medical University Laboratory Animal Co. (Guangzhou, China), and housed in a strictly pathogen-free (SPF) setting, adhering to uniform conditions, which included a consistent 12-h light/12-h dark cycle and a temperature range of 22°C with a tolerance of $\pm 2^\circ\text{C}$. Male *Myb*-KO mice were generated via a CRISPR/Cas9-mediated genome editing system (Cyagen Biosciences Inc., Suzhou, China). All the animals were maintained on the C57BL/6 background by backcrossing with C57BL/6 mice. The animals had unrestricted access to both food and water. The execution of all experimental procedures involving animals (approval No. L2020051) was in strict accordance with the protocols for the care and use of laboratory animals as stipulated by Southern Medical University. Genotyping of the mice was performed using polymerase chain reaction (PCR) analysis. Comprehensive details about the primer sequences can be found in the [Supplementary Table S1](#).

Study participants

Written informed consent was obtained from each donor. The study

was approved by the Medical Ethics Committee of the 923rd Hospital of the People's Liberation Army, Nanning, China, and was conducted in accordance with the Declaration of Helsinki (923LL-2022KY-01-001).

RNA sequencing

RNA was extracted from E12.5 fetal liver cells via TRIzol reagent (Invitrogen, Carlsbad, USA). Total RNA integrity and quantity were assessed via the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, USA). The amplified libraries were purified with AMPure XP magnetic beads (Beckman Coulter, Brea, USA) and quantified with a Qubit 2.0 fluorometer (Invitrogen). The RNA-seq library was sequenced via the Illumina NovaSeq 6000 (Illumina, San Diego, USA) following the manufacturer's instructions.

Lentiviral vector construction and virus transduction

Lentiviral overexpression vectors or sgRNA constructs were cotransfected with psPAX2 and pMD2G (3: 3: 2) into 293T cells in 10-cm dishes via polyethyleneimine (PEI, no. 40815ES0; YEASEN, Shanghai, China). The cDNA cloning and sgRNA primers used are listed in [Supplementary Table S1](#). After infection, the cells were incubated at $37^\circ\text{C}/5\% \text{CO}_2$, and the medium was changed after 12 h. Lentiviral supernatants were harvested at 72 h post-transfection, filtered through a $0.45 \mu\text{m}$ filter and frozen at -80°C . HUDEP-2 cells were plated in 12-well plates, transfected with different viruses and polybrene, and then incubated for 24 h. The transduced HUDEP2 cells were selected by puromycin treatment ($1 \mu\text{g}/\text{mL}$) for 3 days or sorted for GFP-positive cells via BD FACS Aria III (BD Biosciences, San Jose, USA).

Ribonucleoprotein transfection

For CRISPR editing in HUDEP-2 and CD34⁺ HSPCs, sgRNAs were synthesized by GenScript as previously described [10,23]. Peripheral blood CD34⁺ cells were isolated and cultured for 2 days before ribonucleoprotein (RNP) transfection was performed via the Neon Transfection System (MPK5000; Invitrogen). A total of 1×10^5 cells were prepared and resuspended in an RNP mixture containing 9 μL of Buffer T (supplied with a Neon Transfection System 10 μL Kit; MPK1025; Invitrogen), 200 pmol of sgRNA and 20 μg of Alt-R S.p. Cas9 Nuclease V3 (IDT, no. 1081058) prior to electroporation.

RNA isolation, RT-PCR, and real-time PCR

Total RNA was extracted via TransZol Up (R40403; TransGen Biotech, Beijing, China) following the manufacturer's protocol. The RNA was reverse-transcribed via an Evo M-MLV RT Mix Kit (AG11728; Accurate Biology, Changsha, China). The cDNA was amplified with ChamQ Universal SYBR qPCR Master Mix (Q311-02; Vazyme, Nanjing, China) on a LightCycler 96 system (Roche, Basel, Switzerland). All experiments were conducted in triplicate, and gene expression was normalized to that of *GAPDH* for each experiment. All primers used in this study were synthesized by Sangon (Shanghai, China). Detailed information regarding the primers is available in [Supplementary Table S1](#).

Western blot analysis

Total protein was extracted from different cell types via RIPA lysis buffer (P001B; Beyotime, Shanghai, China). The cells were

incubated in RIPA lysis buffer for 30 min. Protein concentrations were determined with a BCA protein assay kit (P0012; Beyotime). A total of 15–20 μ g of protein extract was separated by 12.5% SDS-PAGE (PG113; EpiZyme, Shanghai, China) and transferred onto PVDF membranes. Membranes were blocked and incubated overnight at 4 °C with primary antibodies. The primary antibodies used were anti-MYB (ab45150, 1:2000; Abcam, Cambridge, UK), anti-ETO2 (ab129444, 1:3000; Abcam), anti-HBZ (GT1544, 1:1000; Invitrogen), and anti-GAPDH (184095, 1:2500; Abcam). The PVDF membranes were subsequently incubated with the corresponding secondary antibodies: anti-rabbit (7074S, 1:3000; CST, Danvers, USA), anti-mouse (7076S, 1:3000; CST) at room temperature for 2 h, and detected using a Tanon imaging system (5200; Tanon, Shanghai, China).

Fluorescence-activated cell sorting (FACS)

Fetal livers harvested from E12.5 and E13.5 embryos were mechanically processed into single-cell suspensions. The cells were then stained with APC-conjugated anti-Ter119 (17-5921-81, 1:200; Invitrogen), PE-conjugated anti-CD71 (12-0711-81, 1:100; eBioscience, San Diego, USA), and a panel of five FITC-conjugated mouse lineage antibodies (anti-CD3e, anti-CD41, anti-CD11b, anti-CD45R, and anti-Ly-6G/6C; BD Biosciences) at 4 °C for 30 min. After staining with DAPI (236276; Roche), cell sorting was performed on a FACSria III cell sorter (BD Biosciences). Gating strategies were applied to exclude cellular debris and separate multiple events (populations S0-S4/5), and the data were analyzed with FlowJo software (version 10.8.1). HUDEP-2 cells were isolated on day 0 and day 3 of differentiation and stained with CD71 (CD71-APC; BD Pharmingen, San Diego, USA) and CD235a (CD235a-PE, BD Pharmingen) surface markers at 4 °C for 30 min. Data were acquired on a BD Fortessa X-20 machine (BD Biosciences) and analyzed with FlowJo software (version 10.8.1).

Quantitative polymerase chain reaction followed by chromatin immunoprecipitation (ChIP-qPCR)

In total, 5×10^6 HUDEP-2 cells were harvested for ChIP via the SimpleChIP Plus Sonication Chromatin IP Kit (56383; CST). All procedures were performed according to the manufacturer's instructions. Immunoprecipitations were carried out on wild-type, MYB-KO, ETO2-KO HUDEP-2 cells (5×10^7). Cross-linking was done with 1% formaldehyde for 10 min, followed by glycine quenching. DNA was fragmented to 100–500 bp by sonication using a Covaris M220 (Covaris Inc., Woburn, USA). Specific antibody or rabbit IgG was used to precipitate the crosslinked DNA overnight at 4 °C, then magnetic beads (56383; CST) were added and incubated for 1 h. After wash with washing buffer, elution was done at room temperature for 15 min. DNA was extracted following reversal of crosslinks using NaCl at 65 °C for 5 h. The following antibodies were used at the indicated staining dilutions: ETO2 (A303-616A-T, 1:50; Proteintech, Wuhan, China), LMO2 (ab91652, 1:100; Abcam), MED1 (A300-793A, 1:100; Abcam), CHD4 (ab240640, 1:50; Abcam), MTA2 (ab8106, 1:100; Abcam), H3K9ac (39137, 1:100; Active Motif, Carlsbad, USA), and H3K27ac (39133, 1:100; Active Motif). The percent input method was applied to calculate the enrichment efficiency on the basis of the enrichment over the input. The signals from H3K9ac and H3K27ac were normalized against the total histone H3 signal. The ChIP primers used are listed in [Supplementary Table S1](#).

ATAC sequencing

Nuclei were extracted from WT, MYB-KO, and ETO2-KO HUDEP-2 cell lines and resuspended in a Tn5 transposase reaction mixture. The transposition reaction was carried out at 37 °C for 30 min. Following transposition, adapters 1 and 2 were added, and the library was amplified via PCR using the ATAC-seq Kit (Novogene, Madison, USA). Libraries were purified via AMPure beads, and their quality was assessed with a Qubit fluorometer (Invitrogen). The sequencing libraries were clustered on a cBot Cluster Generation System with the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) and sequenced on the Illumina NovaSeq platform (Illumina), producing 150 bp paired-end reads at Novogene Bioinformatics Technology Co., Ltd (Tianjin, China).

Genotyping

Mouse genomic DNA was extracted from toe biopsies and fetal heads via the alkaline lysis method. The mice were genotyped via PCR analysis. (i) For the wild-type allele, the forward primer (5'-TGGCTTAACCTTTTAGGGGGCT-3') and the reverse primer (5'-GGACCTATCGAACCAGCAAGATGA-3') yielded products of 141 bp. (ii) For the *Myb* null allele, the forward primer (5'-TTGGCTTAACCTTTTAGGGGGC-3') and the reverse primer (5'-GGCTCCTTTCATGTTTCAGAGGTTAGC-3') yielded an amplification product of 246 bp. (iii) For the heterozygous allele, products of 141 bp and 281 bp were generated from the wild-type and mutant loci, respectively.

CD34⁺ HSC selection

Peripheral blood stimulated with granulocyte colony-stimulating factor was collected from healthy individuals and HbH disease patients with the ^{SEA}/ α^{cs} mutation with the TT and GG genotypes of rs7776054. Mononuclear cells were isolated from mobilized peripheral blood via Ficoll-Paque PLUS medium (17144003; GE, Guangzhou, China) and subsequently washed three times with cold PBS to remove platelets. Positive CD34⁺ cells were collected via the EasySep Human CD34 Positive Selection Kit II (17856; STEMCELL, Vancouver, Canada) according to the manufacturer's instructions.

Detection of genome editing events

Genomic DNA from control or CRISPR-edited cells was extracted via the standard proteinase K and phenol-chloroform methods. A 600-bp-long fragment covering the sgRNA-targeted sites was amplified via the high-efficiency and high-fidelity PCR enzyme KOD FX (No. KFX-101; Toyobo, Osaka, Japan). To assess the KO efficiency of the sgRNAs, we performed PCR, followed by Sanger sequencing and Syntheso analysis (decomposing and tracking insertions/deletions). The effects of gene KO and overexpression were also confirmed by qRT-PCR and western blot analysis.

Public RNA sequencing data analysis

The ChIP-seq data for MYB were derived from E13.5 fetal liver cells (GSE112717). ATAC-seq data of E13.5 fetal livers were also obtained from the GEO database (GSE112717). RNA-seq data comparing WT and knockdown MYB strains from GEO GSE85187 were derived from K562 cells. Data from GSE71396 and GSE142226, which include *Klf1* and *Eto2* WT vs KO files, were derived from E14.5 fetal liver cells. To integrate the *Eto2* KD data, we downloaded the *Eto2* WT vs KD files from GEO: GSE59859, derived from MEL cells, and GSE24333, derived from G1E cells, mapping the provided

gene symbols to Ensembl IDs. Erythroid fingerprint genes were obtained from <http://doi.org/10.5281/zenodo.189503>.

Statistical analysis

Student's *t* test and one-way ANOVA were performed to calculate statistical significance via GraphPad Prism (version 9; GraphPad Software, La Jolla, USA). *P* < 0.05 indicated significant difference.

Results

MYB is a key factor for the repression of the embryonic ζ -globin gene during murine erythropoiesis

MYB is highly conserved across vertebrates, exhibiting approximately 80% coding sequence homology and 91.04% amino acid sequence homology between humans and mice (Supplementary Figure S1A). Analysis of the BioGPS microarray database indicated that *Myb* is abundantly expressed in murine hematopoietic stem and progenitor cells (HSPCs), bone marrow, and T cells (Supplementary Figure S1B). To elucidate the physiological role of *Myb* in hematopoietic development, we employed CRISPR-Cas9 genome editing to generate an *in vivo* study model in which exons 2–6 of the *Myb* gene were removed, abrogating *Myb* expression (Supplementary Figure S1C–G). We detected few *Myb*^{−/−} progeny from the mating of *Myb*^{+/-} mice at E15.5 and no *Myb*^{−/−} progeny postnatally (Supplementary Table S2). Compared with wild-type (WT) embryos, the *Myb*^{−/−} embryos, which were smaller in size, appeared anemic at E12.5 (Figure 1A). As expected, fewer fetal liver cells were detected in *Myb*^{−/−} embryos than in their WT littermates (Supplementary Figure S2A). Additionally, we observed a corresponding decrease in the number of erythroid cells (S0–S4) in the fetal livers of *Myb*-KO mice (Supplementary Figure S2D). Cells from the livers of WT and *Myb*^{−/−} littermates isolated from E12.5 fetuses were separated into six populations by staining with the cell surface markers CD71 and Ter119 (S0–S5) (Supplementary Figure S2B). We detected increased frequencies of immature cell populations (S0) and decreased percentages of more mature cell populations (S1, S3) in *Myb*^{−/−} fetal livers, suggesting an inhibitory effect on erythroid differentiation (Figure 1B and Supplementary Figure S2C). To elucidate the mechanism by which *Myb* governs the transcription of the globin gene during erythropoiesis, we performed real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) and found that ζ -globin (*Hba-x*) expression was significantly upregulated upon *Myb* loss at E12.5 and E13.5 (Figure 1C). ζ -globin expression remained elevated in E13.5 fetal liver cells, whereas the expression of other embryonic globin genes showed only mild induction (Figure 1C). Notably, the ζ - to α -globin switch was delayed at E12.5 in *Myb*^{−/−} embryos compared with controls, subverting the predominant expression of α -globin in wild-type fetal liver-sorted cells at E12.5 (Figure 1D). We then conducted RNA-seq experiments on E12.5 fetal liver cells. Principal component analysis (PCA) was performed with two components, PC1 and PC2, accounting for 92.52% of the variance (PC1 = 82.62%, PC2 = 9.9%) (Supplementary Figure S3A). As expected, ζ -globin transcripts were significantly induced, and the expressions of known targets of *Myb* (*Klf1*, *Bcl11a*, and *Zbtb7a*) were downregulated in *Myb*^{−/−} mice (Figure 1E). Gene set enrichment analysis (GSEA) comparing *Myb*-KO with WT mice revealed that the downregulated differential gene sets were enriched in processes associated with erythrocyte differentiation and the regulation of chromatin binding (Figure 1F). Gene Ontology (GO) and Kyoto Encyclopedia of Genes

and Genomes (KEGG) analyses revealed enrichment of condensed chromosomes, centromeric regions and hematopoietic cell lineages in the *Myb*-KO and WT RNA-seq datasets (Supplementary Figure S3B). Taken together, these findings suggest that MYB functions as a key factor for the repression of the embryonic ζ -globin gene in murine erythropoiesis.

Identification of ETO2 as a direct target gene of MYB in the regulation of ζ -globin

To investigate whether MYB regulates ζ -globin by binding to its promoter, we performed data mining via the ChIP-seq database (GSE112717) of the murine fetal liver. No MYB binding peaks were observed on the ζ -globin promoter. To elucidate the indirect function of MYB on ζ -globin, we analyzed MYB ChIP-seq data (GSE112717) and *Myb*^{−/−} RNA-seq data and identified 44 MYB-binding genes (Figure 2A and Supplementary Figure S3C). Given that MYB is a conserved regulator in mammals, we merged the genes regulated by the knockdown of MYB in human K562 cell lines (GSE85187) with the 44 overlapping genes mentioned above (Figure 2A and Supplementary Figure S3C). Five MYB target genes, *SPTB*, *LMO2*, *CCRL2*, *ETO2*, and *VAMP8*, were identified. Among those genes, only *ETO2* and *LMO2* have been implicated as erythroid transcription factors and cofactors (Supplementary Figure S3D) [26,27]. We selected ETO2 for further intensive study. Data mining of the ENCODE database revealed that the transcription start site (TSS) from −1551 to −1558 in the *Eto2* promoter coincided precisely with the MYB-binding motif predicted by JASPAR (Figure 2B and Supplementary Figure S4). We further performed qRT-PCR on FACS-sorted cells (S3) taken from E12.5 fetal livers. The expression of ETO2 was highly decreased in *Myb*^{−/−} sorted cells, which was in agreement with the results of the positive control (*Klf1*, *Bcl11a*, and *Lrf*), which demonstrated similar trends (Figure 2C). ChIP-qPCR confirmed that MYB binds to the active *ETO2* promoter and that MYB binding is markedly reduced in MYB-KO cells (Figure 2D). We explored the regulatory influence of MYB on ETO2 by merging RNA-seq datasets from E12.5 MYB-KO and ETO2-KO fetal liver cells. Analysis revealed 2411 coregulated genes (adjusted *P* < 0.05), and 54 of 2411 genes were associated with erythroid function (Figure 2E). Correspondingly, 1050 genes were found to be coregulated, including 25 erythroid genes out of a total of 104 erythroid genes in human cell lines (Supplementary Figure S3E). GO analysis revealed that these genes were significantly associated with erythroid differentiation, chromosome, centromeric region, condensed chromosome, and transcription coregulator activity (Figure 2F and Supplementary Figure S3F). Given that ETO2 is a corepressor of globin gene expression, we propose that *Myb* likely regulates ζ -globin to α -globin switching by affecting ETO2 expression during human and mouse development.

MYB expression regulates ETO2 expression to repress ζ -globin

To validate our hypothesis that MYB regulates ζ -globin expression via ETO2, exon 5 of the MYB gene was targeted via CRISPR-Cas9 genome editing to abrogate MYB expression in HUDEP2 cells (Supplementary Figure S5A–D). Compared with those in wild-type cells, ETO2 level was markedly reduced, whereas ζ -globin level was significantly increased in MYB-KO HUDEP-2 cells (Figure 3A,B and Supplementary Figure S5E). Notably, the ζ -globin/(ζ -globin + α -globin) gene expression ratio was markedly increased in MYB-KO

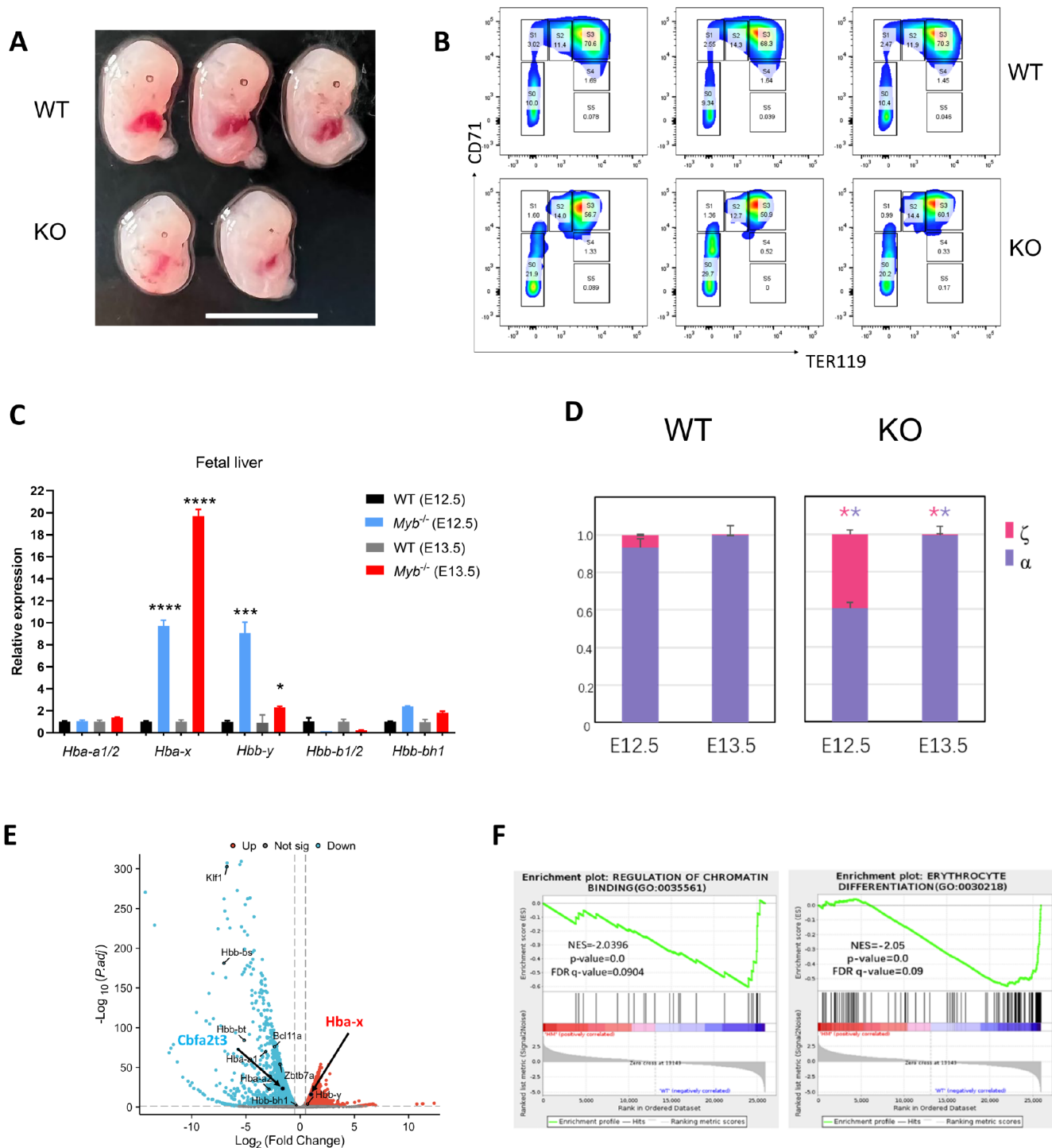


Figure 1. Erythroid maturation defects and delayed hemoglobin switch (ζ - α) in *Myb*^{-/-} mice (A) Gross morphology of E12.5 *Myb*^{+/+} and *Myb*^{-/-} embryos. (B) Erythroid differentiation in E12.5 fetal liver, stained with CD71 and Ter119. Fetal liver cells at E12.5 from *Myb*^{-/-} (KO, lower panel) and *Myb*^{+/+} (WT, upper panel) littermates were gated and separated into six subsets (S0–S5) according to the erythroblast maturation state via flow cytometry analysis. (C) RT-qPCR analysis was performed to assess the expression levels of embryonic globin genes (*Hba-x*, *Hbb-y*, *Hbb-bh1*) and adult globin genes in *Myb*^{-/-} fetal liver cells, which were normalized to their respective WT expression levels at E12.5 and E13.5. The experiments were performed in triplicate, and data are presented as the mean $\pm$ SD. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001. (D) Expression of globin genes was determined by RT-qPCR. The data are presented as a fraction of total α -like globin (ζ + α) expression at E12.5 and E13.5. The experiments were performed in triplicate, and data are presented as the mean $\pm$ SD. Pink asterisks indicate the ζ -globin expression fraction of total α -like globin (ζ + α) expression in *Myb*-KO embryos relative to WT, while purple asterisks represent the α -globin expression fraction of total α -like globin (ζ + α) expression in *Myb*-KO embryos relative to WT embryos. **P* < 0.05; error bars indicate standard deviations. (E) RNA-seq analysis revealing differential gene expression in *Myb*^{-/-} E12.5 fetal liver cells compared with that in their WT littermates. The data are plotted to represent the average log₂-fold change in the mRNA levels of two independent biological replicates. The genes encoding globin genes and *Myb*-direct target genes are highlighted. (F) GSEA of the WT and *Myb*-KO RNA-seq datasets.

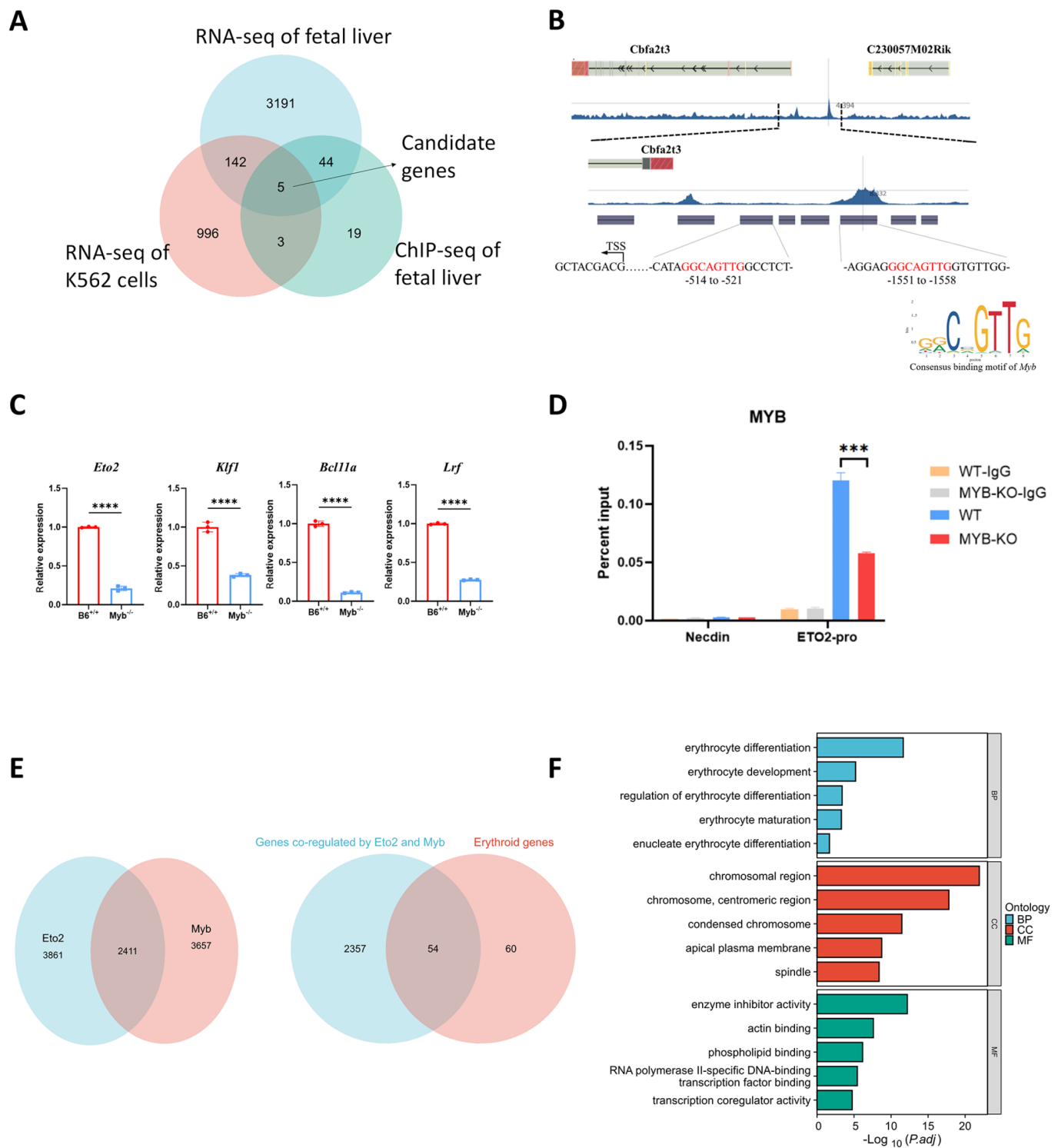


Figure 2. Identification of ETO2 as a regulator of ζ-globin repression mediated by MYB (A) Venn diagram illustrating *Myb* target selection. (B) MYB enrichment at the gene promoter of *Eto2* from MEL cells obtained from ENCODE. The de novo motif logo of MYB in the mouse genome is provided below. (C) RT-qPCR analysis was performed to assess the expression levels of selected genes in population S3 of *Myb*^{-/-} fetal liver cells at E12.5. (D) ChIP-qPCR analysis of MYB occupancy at the promoter region of the *ETO2* gene in WT and MYB-KO HUDEP-2 cells. The abscissa represents the ζ-globin promoter and *Necdin* (negative control), whereas the ordinate represents the relative enrichment level of MYB. (E) Left panel: Intersection analysis of genes whose expression level changed in *Myb*^{-/-} and *Eto2*^{-/-} fetal liver cells at E12.5. Fold change > 2 and *P* < 0.05 were used as cutoffs for identifying DEGs. Right panel: Intersection analysis of genes co-regulated by *Eto2* and *Myb*, as well as erythroid-specific genes. The *Eto2* knockout (KO) data originated from the GEO database (GSE156306). (F) GO analysis of genes co-regulated by *Eto2* and *Myb*. *P* < 0.05 were used as cutoffs for identifying DEGs. ****P* < 0.001, *****P* < 0.0001.

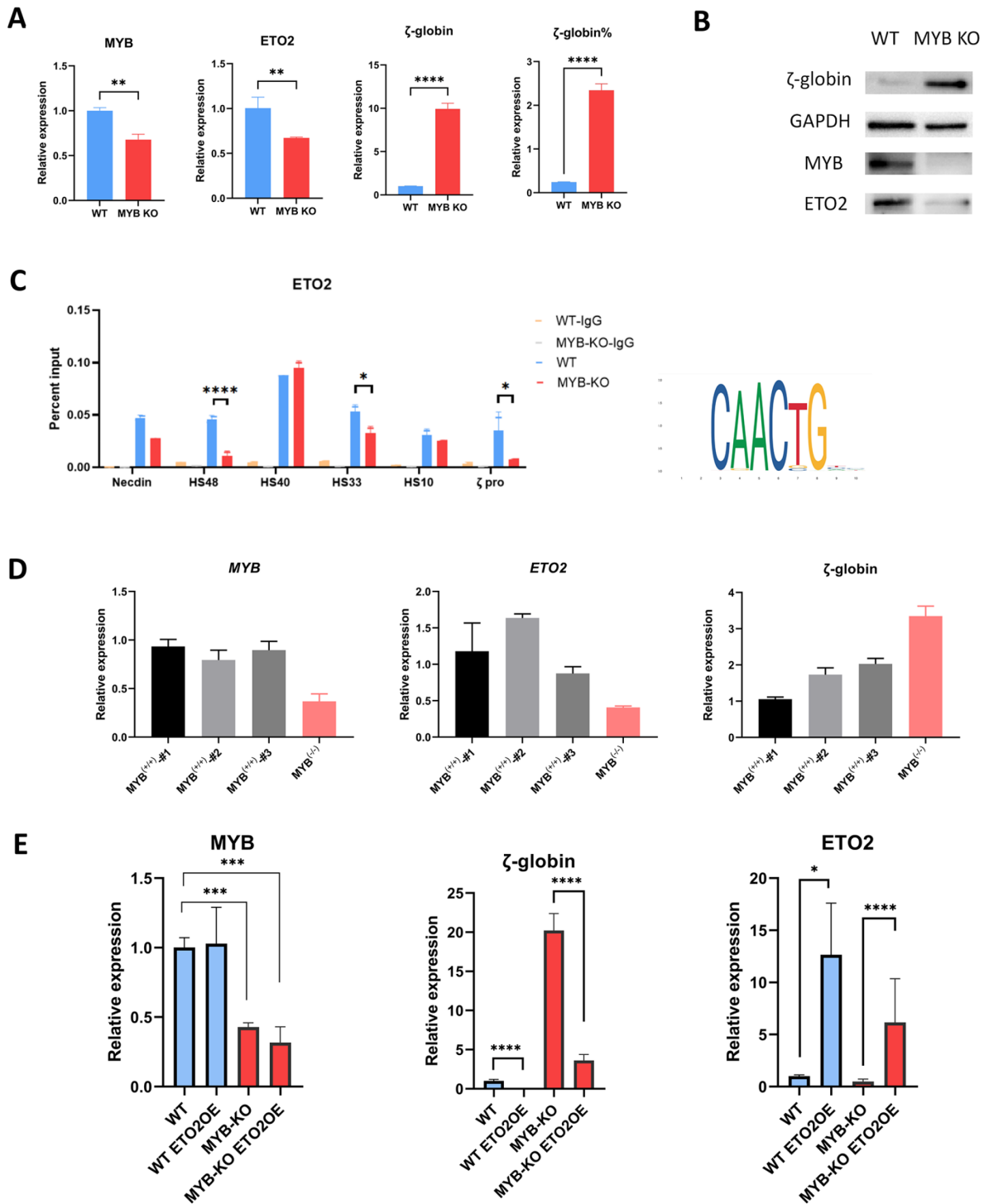


Figure 3. The downregulation of MYB expression regulates a reduction in ζ -globin expression by modulating ETO2 (A) qPCR detection of MYB, ETO2, and ζ -globin expression, along with the percentage of ζ -globin mRNA relative to the total α -like globin expression of MYB-KO HUDEP-2 cell lines. (B) Western blot analysis of ζ -globin, MYB and ETO2 in MYB-KO HUDEP-2 cells. GAPDH served as a negative control. (C) ChIP-qPCR for ETO2 occupancy at locus control region hypersensitive sites (LCR HSs) and the ζ -globin promoter in WT and MYB-KO HUDEP-2 cells. The abscissa represents the LCR HSs, ζ -globin promoter and *Necdin* (negative control), whereas the ordinate represents the relative enrichment level of ETO2. The de novo motif logo of MYB in the human genome is provided on the right. (D) RT-qPCR results showing the expressions of MYB, ETO2 and ζ -globin in CD34⁺ cells mobilized from HbH disease patients (α -SEA/ α -CS α) on D12 versus D6 of differentiation. Individuals with the GG genotype of rs7776054 were classified as MYB^(-/-) individuals, whereas those with the T/T genotype were labeled as MYB^(+/+) individuals, with both groups carrying the α -CS α / α -SEA deletion. The "G" allele of rs7776054 is correlated with elevated ζ -globin expression and reduced MYB and ETO2 expression levels. (E) qPCR detection of MYB, ETO2, and ζ -globin expression in MYB-KO ETO2-overexpressing HUDEP-2 cell lines. Each experiment was conducted in triplicate. Data are presented as the mean $\pm$ SD. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; ns, not significant.

cells (Figure 3A and Supplementary Figure S5E). Alexandre Fagnan *et al.* [28] also reported significant enrichment of the histone marker H3K27ac at the α -globin locus, particularly in the R2 and R4 regions, in the shMYB HEL cell line compared with the shREN HEL cell line, suggesting an association with active chromatin (Supplementary Figure S6). Western blot analysis revealed an efficient reduction in ETO2 protein expression and a substantial increase in ζ -globin protein level in MYB-KO HUDEP-2 cells (Figure 3B). To test whether MYB can silence *HBZ* through direct regulation of ETO2, we performed ChIP-qPCR and found that loss of MYB decreased the enrichment of ETO2 in the α -globin cluster (Figure 3C). To further determine the regulatory role of MYB in ETO2 and ζ -globin, CD34⁺ HSPCs were selected from the peripheral blood of HbH disease patients and differentiated along the erythroid lineage. Individuals with the mutant GG genotype of rs7776054 were classified as MYB^(-/-), whereas those with the TT genotype were labeled as MYB^(+/+), with both groups carrying the $\alpha^{cs}\alpha^{-SEA}$ deletion. A previous study revealed that, compared with WT/WT control cells, cells cultured *ex vivo* from SNP/SNP individuals (including rs7776054) presented consistently lower MYB level [29]. MYB and ETO2 expression levels were markedly reduced, whereas ζ -globin level was markedly increased, compared with those in wild-type cells (MYB^(+/+)) carrying -^{cs} and -^{SEA} deletions ($\alpha^{cs}\alpha^{-SEA}$) (Figure 3D). To determine whether ETO2 is a key factor involved in the regulatory pathway of MYB- ζ -globin, we transduced lentiviral overexpression vectors of ETO2 into MYB-KO HUDEP-2 cells. We found that overexpression of ETO2 decreased ζ -globin level in MYB-KO HUDEP-2 cells (Figure 3E). Our results suggest that ETO2 is directly regulated by MYB, which drives the downregulation of ζ -globin expression.

ETO2 silences ζ -globin and affects the chromatin environment at the α -globin locus through the recruitment of NuRD complex

A previous study reported that ETO2 loss leads to increased enhancer activity and expression of γ -globin [10]. To investigate whether ETO2 also serves as a repressor of ζ -globin regulation, we transduced HUDEP-2 and CD34⁺ HSPCs with lentiviral sgRNA vectors targeting the human *ETO2* gene (Supplementary Figure S7A–F). RT-qPCR and western blot analyses revealed that ζ -globin levels were increased upon ETO2 loss on day 0 and day 7 of differentiation in HUDEP-2 cells (Figure 4A–D). The ζ -globin/(ζ -globin + α -globin) gene expression ratios were increased in *ETO2*-KO HUDEP-2 cells on day 0 and day 7 of differentiation (Figure 4A, B). We then purified CD34⁺ cells from mobilized, peripheral human blood and transduced these cells with ETO2 sgRNA lentiviral vectors (Supplementary Figure S7F). Consistently, ζ -globin gene expression was markedly upregulated in *ETO2*-KO healthy and HbH disease patients during erythroid differentiation from day 6 to day 12, which was consistent with the changes in ζ -globin expression levels in *ETO2*-knockdown MEL and G1E cells (Figure 4E and Supplementary Figure S8A,B). Previous studies have shown that ETO2 silences embryonic and fetal globin genes by antagonizing LDB1 looping and recruiting NuRD complex to the β -globin locus [10]. To determine whether ETO2 regulates embryonic ζ -globin through a similar pathway as in the β -globin locus, we conducted ChIP-qPCR to examine the occupancy of LDB1 and the NuRD complexes. Studies have shown that LMO2 is critical in the assembly of LDB1 complex, enabling the interactions between

GATA1 and other factors to form a loop in the β -globin locus for gene regulation [30–32]. Given this crucial role, we focused on LMO2 as a representative of the LDB1 complex in our ChIP-qPCR analysis. As expected, disruption of NuRD complex component (CHD4, MTA2) occupancy and a marked increase in LMO2 and MED1 were detected upon ETO2 loss. Additionally, H3K9ac and H3K27ac were significantly enriched in the enhancers and the ζ -globin promoter (Figure 4F,G and Supplementary Figure S9A–C). These results indicate that ETO2 recruits NuRD complex to repress ζ -globin expression.

MYB and ETO2 downregulate ζ -globin expression through similar effects on erythroid differentiation

We then performed ATAC-seq on the WT, MYB-KO, and *ETO2*-KO HUDEP-2 cell lines to assess chromatin accessibility across the genome, including at the ζ -globin locus. We detected a significant increase in chromatin accessibility at the ζ -globin gene region in both MYB-KO and *ETO2*-KO cells compared with WT cells, indicating that the ζ -globin gene is indeed reactivated upon knockout of MYB or *ETO2* (Figure 4H). We then examined the effects of MYB and ETO2 on erythroid differentiation. MYB depletion and ETO2 loss had similar effects on erythroid maturation and differentiation, as assessed by CD235a and CD71 expressions (Figure 5A). Progressive erythropoiesis was assessed by flow cytometry analysis of the CD71⁺CD235a⁻ (Q1), CD71⁺CD235a⁺ (Q2), and CD71⁻CD235a⁺ (Q3) populations, which corresponded to increased erythroid differentiation from CFU-E to orthochromatic erythroid progenitors [33,34]. Fluorescence-activated cell sorting (FACS) revealed increased frequencies of more mature cell populations (Q2) in both MYB-depleted and ETO2-depleted HUDEP-2 cells on day 0 and day 3 of differentiation (Figure 5B). Erythroid genes were more enriched in the *Myb* and *Eto2* co-regulated categories than in the *Myb* and *Klf1* co-regulated categories in murine fetal liver cells (Figure 5C,D). To address whether the repressor BCL11A is involved in the MYB-ETO2- ζ -globin regulatory pathway, we assessed ζ -globin expression in *ETO2/BCL11A* double-KO HUDEP-2 cells (Supplementary Figure S10A–C). Compared with that in *BCL11A*-KO cells, ζ -globin expression was significantly increased in *ETO2*-KO cells. However, codepletion of ETO2 and BCL11A did not trigger significant ζ -globin activation compared with that in *ETO2*-KO cells, indicating that MYB-ETO2, rather than MYB-BCL11A, regulates ζ -globin gene expression (Figure 5E). Together, these results delineate a linear MYB-ETO2- ζ -globin pathway and highlight its critical role in histone acetylation at the α -globin locus.

Discussion

The cellular and molecular mechanisms underlying the switch from γ -globin to β -globin gene regulation have been largely elucidated, leading to the development of targeted therapeutic approaches aimed at reactivating γ -globin production. Studies have revealed that reactivating the expression of normal embryonic ζ -globin could also fully compensate for the activity of the missing or faulty adult genes underlying α -thalassemia and SCD [13,14]. Therefore, investigating the underlying mechanisms of ζ -globin repression is beneficial.

MYB is essential for definitive erythropoiesis and has been implicated in the silencing of embryonic and fetal genes at the β -globin locus [16]. Interestingly, ζ -globin is elevated in patients with

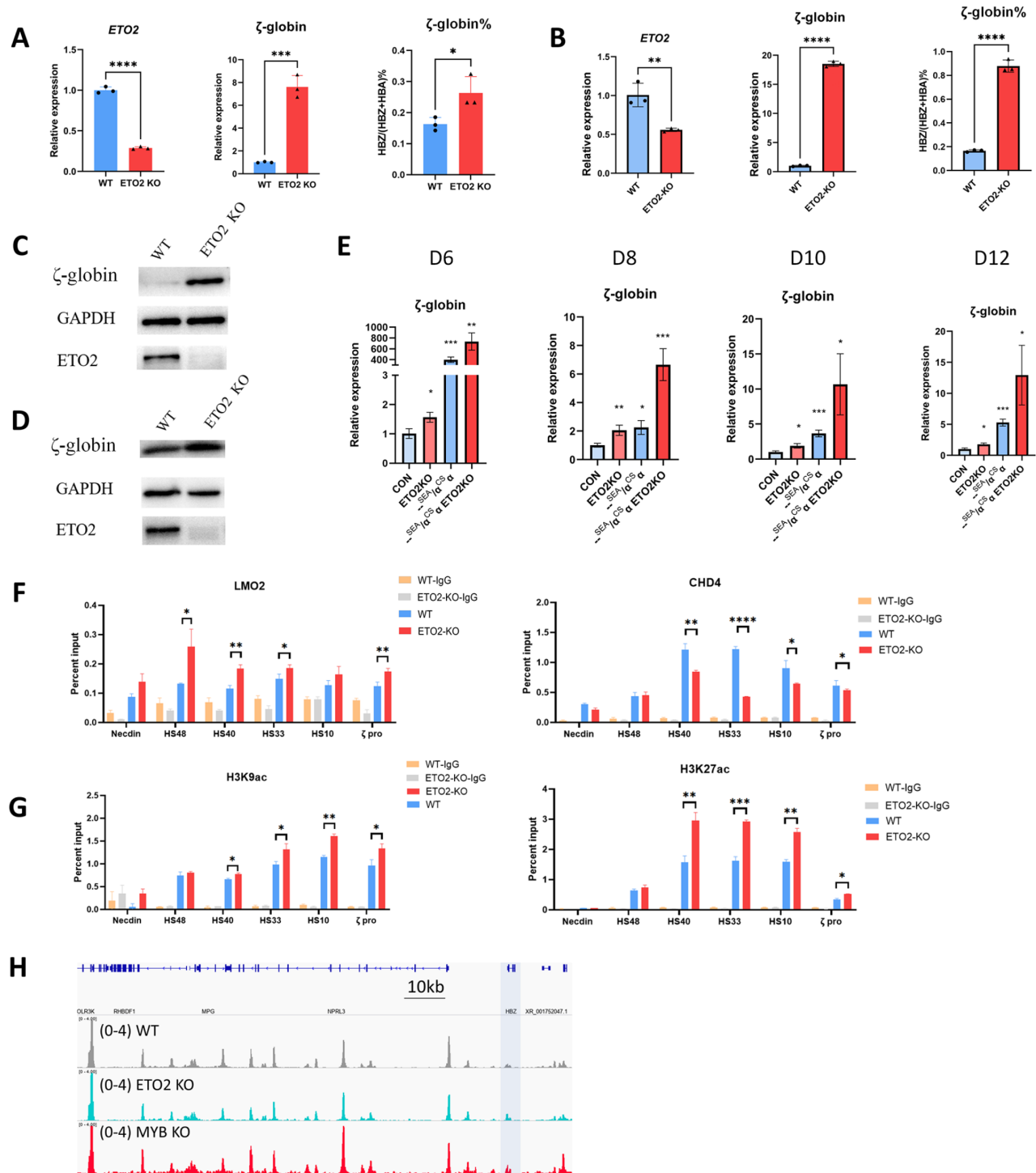


Figure 4. The absence of ETO2 leads to ineffective silencing of the embryonic globin gene (A,B) qPCR analysis was conducted to evaluate the expression levels of ETO2 and ζ -globin and the percentage of embryonic ζ -globin mRNA relative to total α -like globin (α -globin + ζ -globin) expression in *ETO2*-KO HUDEP-2 cell lines on days 0 and 7 of differentiation. (C,D) Western blot analysis of ETO2 and ζ -globin in *ETO2*-KO HUDEP-2 cells on days 0 and 7 of differentiation; GAPDH served as a negative control. (E) qPCR analysis was performed to detect ζ -globin expression in *ETO2*-KO CD34⁺ cells mobilized from both healthy donors and individuals diagnosed with HbH disease ($-\text{SEA}/\alpha^{\text{CS}}\alpha$) during 12 days of differentiation. (F) ChIP-qPCR for LMO2 and CHD4 occupancy at the LCR HSs and ζ -globin gene promoter in WT and *ETO2*-KO HUDEP-2 cells. (G) ChIP-qPCR for H3K9ac and H3K27ac occupancy at the LCR HSs and ζ -globin gene promoter in WT and *ETO2*-KO HUDEP-2 cells. The abscissa represents the LCR HSs, ζ -globin promoter and *Necdin* (negative control), whereas the ordinate represents the relative enrichment level of the occupied protein. (H) ATAC-seq screenshots of MYB-KO (red, bottom-most) and *ETO2*-KO (blue, middle) HUDEP-2 cells indicating the chromatin accessibility of MYB and ETO2 loss. WT (grey, upper) was used as a negative control. The ζ -globin (HBZ) gene is indicated by the light blue bar. Each experiment was conducted in triplicate. Data are presented as the mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns, not significant.

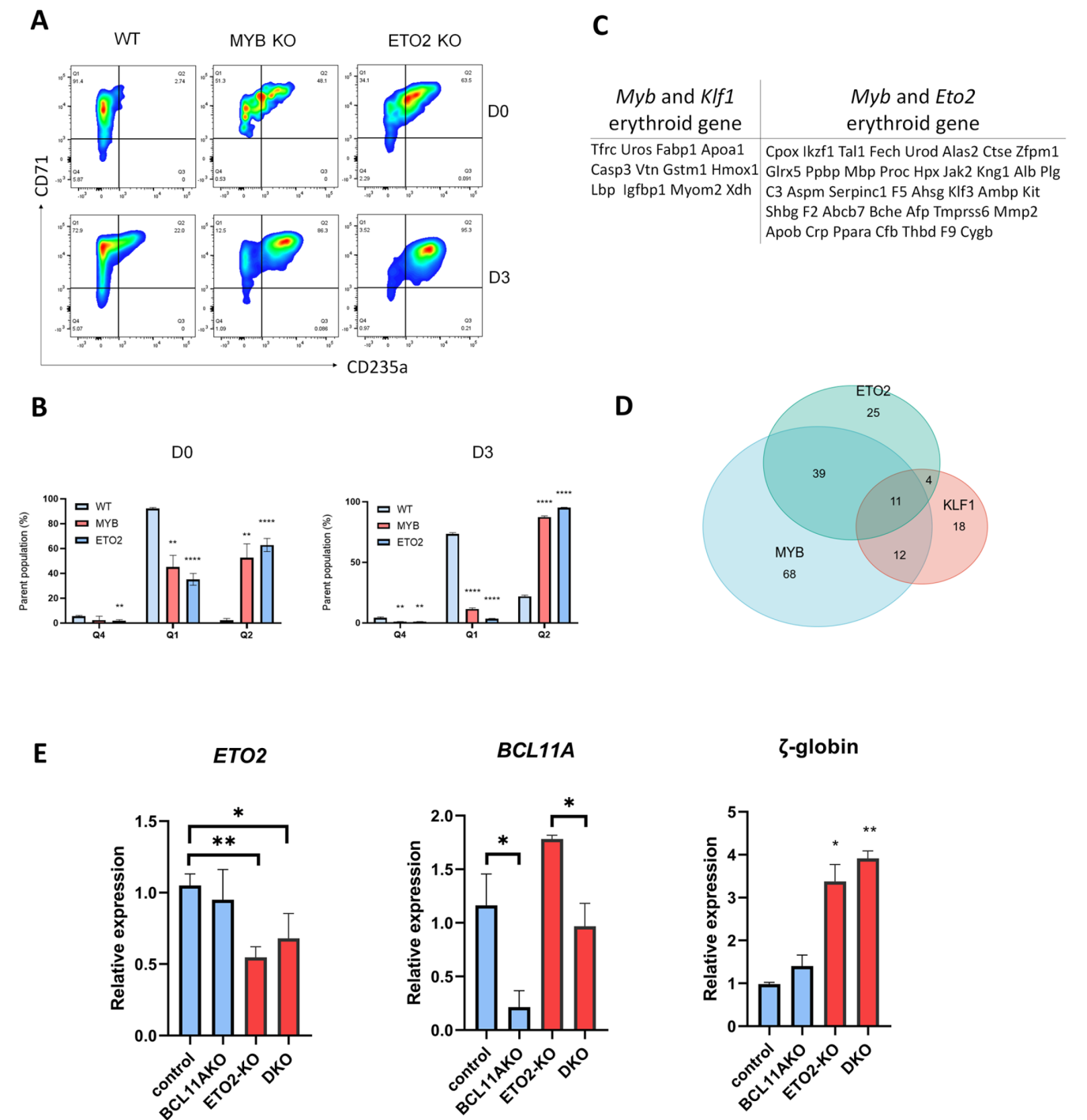


Figure 5. MYB and ETO2 downregulate ζ-globin expression through similar effects on erythroid differentiation (A) Progressive erythropoiesis was assessed by flow cytometry analysis of CD71 and CD235a antibodies. The cells can be separated into four subsets (Q4-Q1-Q2-Q3), corresponding to increased erythroid differentiation. Erythroid differentiation in WT, MYB-KO and ETO2-KO HUDEP-2 cells on day 0 and day 3 and staining with CD71 and CD235a antibodies. Flow cytometry analysis was conducted on MYB-KO HUDEP-2 cells (middle panel), ETO2-KO HUDEP-2 cells (right panel) and WT HUDEP-2 cells (left panel). (B) Quantification of each population (Q4-Q1-Q2-Q3) of WT, MYB-KO and ETO2-KO HUDEP-2 cells. The calculation of Q3 is not performed because of the low number of cells. (C,D) Left panel: Intersection analysis of genes co-regulated by Klf1 and Myb, as well as erythroid-specific genes. Right panel: Intersection analysis of genes co-regulated by Eto2 and Myb, as well as erythroid-specific genes. Lower panel: Venn diagram showing the intersection analysis of genes co-regulated by MYB, ETO2 and KLF1 as well as erythroid-specific genes. The Klf1 KO and Eto2 KO data for E14.5 fetal liver cells were obtained from the GEO database (GSE71396 and GSE142226). Fold change > 2 and adjusted $P < 0.05$ were used as cutoffs for identifying differentially expressed genes in Myb^{-/-} and Eto2^{-/-} fetal liver cells at E12.5. $P < 0.05$ were used as cutoffs for identifying differentially expressed genes in Klf1^{-/-} and fetal liver cells at E14.5. (E) qPCR detection of ETO2, BCL11A and ζ-globin in ETO2-KO, BCL11A-KO, and double-KO HUDEP-2 cells. Each experiment was conducted in triplicate. Data are presented as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, ns, not significant.

trisomy 13 (Patau syndrome), driven by two microRNAs (miR-15a and miR-16-1) that regulate the decreased level of the transcription factor MYB [25]. To elucidate the transcriptional network affected by MYB in the reactivation of embryonic ζ -globin, we conducted a multi-omics analysis of MYB knockout RNA-seq and ChIP-seq data. We determined that MYB stimulates the expression of ETO2 by binding to its promoter. Previous studies have reported that ETO2, which participates in erythropoiesis and globin gene switching, functions as a novel regulator of embryonic and fetal globin gene transcription [10]. The consensus binding site GGCAGTTG, identified from JASPAR and ChIP sequencing studies [35], is present in two locations within the *ETO2* promoter for MYB binding. Interestingly, we did not observe binding peaks in the ζ -globin promoter region, which is in agreement with the finding that additional transcription factors cooperate with MYB in the regulation of ζ -globin repression. MYB depletion and ETO2 loss had similar effects on erythroid maturation and differentiation, suggesting that ETO2 participates in the MYB regulatory mechanism in erythropoiesis and globin gene expression. Notably, in the comparative analysis of the RNA-seq datasets, the intersection of downstream erythroid-specific genes identified between MYB/ETO2 and MYB/KLF1 revealed a significantly enriched gene set related to MYB/ETO2 pairing. This observation highlights a more substantial overlap and a distinctly enhanced erythroid-specific transcriptional impact within the MYB-ETO2 regulatory axis, underscoring the critical role of ETO2 in mediating MYB-driven erythropoiesis. Taken together, these findings indicate that the MYB-ETO2 axis may serve as a therapeutic target in the treatment of α -thalassemia and SCD.

ζ -globin is normally expressed in primitive erythroid cells and is nearly undetectable during definitive erythropoiesis. Andrew J. King *et al.* [22] demonstrated that an inhibitor (HC toxin) of specific histone deacetylases (HDAC1 and HDAC2) targeting definitive cells partially reactivates ζ -globin expression in adult cells, suggesting that histone deacetylation contributes to the silencing of the ζ -globin gene. Additionally, the removal of *BCL11A* or *LRF* *in vivo* in definitive murine erythroid cells leads to some degree of ζ -globin derepression and increased chromatin accessibility [22]. More specifically, our study identified a novel repressor, ETO2, which participates in ζ -globin regulation by enhancing repressive NuRD complex recruitment and histone deacetylation of the ζ -globin region of chromatin. *ETO2* knockout in mouse erythroid cells has only modest effects on the transcription of repressors such as *BCL11A* [10]. Notably, ζ -globin expression was significantly increased in *ETO2*-KO cells compared with that in *BCL11A*-KO cells, indicating that ETO2 may have a greater influence on ζ -globin activation. Additionally, as ETO2 and *BCL11A* exert their ζ -globin repression effects by coassociating with the NuRD complex, we knocked out both *ETO2* and *BCL11A*. The codepletion of *ETO2* and *BCL11A* did not trigger significant ζ -globin activation, suggesting that the functions of these two genes seem to be partially dependent. Unlike *Klf1*-null embryos, which succumb to severe anemia, *Eto2*^{-/-} adult mice exhibit only a mild anemic phenotype [10,36]. Embryonic ζ -globin is still expressed in adult mouse blood cells, albeit at low levels [10]. Based on these findings, ETO2 may be a potential drug target for the treatment of α -thalassemia and SCD.

The current findings suggest that the regulation of α - and β -like globin genes might be a developmentally specific yet highly ordered process in hemoglobin switching involving a shared set of upstream

regulators for the balanced and coordinated synthesis of stage-specific hemoglobin tetramers. Therefore, exploring the roles of these transcription factors in ζ -globin repression and the ζ - to α -globin switch, which is a fundamental issue prior to developing reliable genetic therapies for α -thalassemia patients, is highly important. In our investigation, we evaluated the ζ -globin/ ζ -globin + α -globin gene expression ratios and observed an increase in the percentage of embryonic genes in MYB- or *ETO2*-KO HUDEP-2 cells. This finding further confirms the indispensable role of MYB and ETO2 in regulating ζ -to- α -hemoglobin switching.

In summary, we identified therapeutic targets for ζ -globin derepression and delineated the regulatory pathway MYB-ETO2- ζ -globin, suggesting that current strategies developed for treating β -hemoglobinopathies could also benefit severe α -thalassemia patients. Overall, targeting the MYB-ETO2- ζ -globin pathway represents a promising approach for the development of novel therapies for α -thalassemia and SCD.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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