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# miR-365-3p mediates BCL11A and SOX6 erythroid-specific coregulation: A new player in HbF activation

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**Hemoglobin switching is a complex biological process not yet fully elucidated. The mechanism regulating the suppression of fetal hemoglobin (HbF) expression is of particular interest because of the positive impact of HbF on the course of diseases such as β-thalassemia and sickle cell disease, hereditary hemoglobin disorders that affect the health of countless individuals worldwide. Several transcription factors have been implicated in the control of HbF, of which BCL11A has emerged as a major player in HbF silencing. SOX6 has also been implicated in silencing HbF and is critical to the silencing of the mouse embryonic hemoglobins. BCL11A and SOX6 are co-expressed and physically interact in the erythroid compartment during differentiation. In this study, we observe that *BCL11A* knockout leads to post-transcriptional downregulation of SOX6 through activation of microRNA (miR)-365-3p. Downregulating SOX6 by transient ectopic expression of miR-365-3p or gene editing activates embryonic and fetal β-like globin gene expression in erythroid cells. The synchronized expression of BCL11A and SOX6 is crucial for hemoglobin switching. In this study, we identified a BCL11A/miR-365-3p/SOX6 evolutionarily conserved pathway, providing insights into the regulation of the embryonic and fetal globin genes suggesting new targets for treating β-hemoglobinopathies.**

## INTRODUCTION

Mutations in the β-globin gene cause β-thalassemia and sickle cell disease (SCD), the most common autosomal recessive disorders worldwide.[1–5] The human β-globin locus on chromosome 11 contains embryonic (ε), fetal (Gγ and Aγ), and adult (δ and β) globin genes. The stage-specific switch from human fetal (γ) to adult (β) expression is of particular interest because of its clinical importance; indeed, the reactivation of fetal hemoglobin (HbF) in adulthood can improve the two major β-hemoglobin disorders. Increased γ-globin expression reduces the imbalance of the α- and β-globin chains in β-thalassemia and interferes with HbS polymerization improving disease symptoms in SCD.[3,6] Numerous studies have shed light on the molecular mechanisms regulating hemoglobin switching and the potential therapeutic reactivation of HbF in adult hematopoietic cells.[4,7] Genome-wide association studies have identified *BCL11A*, a gene encoding a zinc-finger transcription factor, as a major HbF regulator.[8–11] BCL11A represses γ-globin expression in adult erythroid cells by binding to the TG(A/T)CCA motif in γ-globin promoters and other sites along the β locus.[12,13] BCL11A interacts with the NuRD (nucleosome remodeling and deacetylase) complex as well as transcription factors GATA1, FOG1, and SOX6 to mediate γ-globin gene silencing.[14,15]

Sex-determining region Y (SRY)-box 6 (SOX6) is a transcription factor that belongs to Sry-type HMG (high-mobility group) box family. SOX6 is involved in various cell types and developmental processes, such as the nervous system,[16] chondrogenesis,[17] and cardiac and skeletal muscle formation.[18] SOX6 appears to be crucial for establishing definitive erythropoiesis.[19–22] It has also been implicated in hemoglobin switching. SOX6 silences the embryonic murine globin gene ε in definitive erythropoiesis by binding to its proximal promoter.[20] It has been reported to cooperate with BCL11A to silence γ-globin expression in adult erythroid cells.[14,15,23] SOX6 and BCL11A are co-expressed during erythroid development. They physically interact and, together with GATA1, co-occupy sites in the human β-globin cluster.[4,15,23]

The molecular mechanisms coordinating the synchronous expression of SOX6 and BCL11A during erythroid differentiation have not been elucidated. Here, we explored the hypothesis that a post-transcriptional mechanism, such as microRNA (miRNA)-mediated silencing, may contribute to the coexpression of BCL11A and SOX6 in the erythroid compartment.

miRNAs are small, noncoding RNA molecules (20–25 nucleotides [nt]) that guide the RNA-induced silencing complex (RISC) to target

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sequences in mRNAs.[24–26] miRNAs regulate cell- or tissue-specific gene expression by inducing translational repression and/or degradation of their target RNAs.[27] Increasing studies have implicated miRNAs in the control of γ-globin expression.[28,29]

In *BCL11A*-null mice, SOX6 expression is strongly decreased, and the human transgenic γ-globin gene is activated.[15] We identified several differentially expressed miRNAs via genome-wide miRNA expression profiling of embryonic day (E) 14.5 fetal liver from *BCL11A*-null and wild type (WT) mice littermates. In this study we showed that miR-365-3p, overexpressed in *BCL11A*-null fetal livers, directly targets the SOX6 mRNA.

Transient ectopic miR-365-3p expression downregulates SOX6 in erythroid cells and increases the expression of the murine embryonic and human embryonic and fetal β-like globin genes. We have also shown that BCL11A is a direct transcriptional repressor of miR-365-3p, while it does not directly affect SOX6 expression.

The results we report here highlight an evolutionarily conserved regulatory network formed by BCL11A, miR-365-3p, and SOX6 involved in the fine-tuning coregulation of BCL11A and SOX6 expression in erythroid cells.

Given the therapeutic potential of fetal globin in β-hemoglobinopathies,[30] we aimed to study the effects of a stable decrease in SOX6 caused by targeted CRISPR-Cas9 genome editing in HUDEP-2 cells.[31] Stable HUDEP-2 *SOX6*-edited cells produced much more ε and γ-globin than cells with only transient SOX6 inhibition.

These results may lead to new strategies for activating embryonic and fetal β-like globin genes in adult erythroid cells as a therapy for β-hemoglobinopathies.

## RESULTS

### SOX6 expression is downregulated in BCL11A-null mice and is not influenced by BCL11A overexpression

BCL11A and SOX6 are coregulated in mouse fetal liver[15] and cooperate in silencing γ-globin transcription. SOX6 expression is decreased in *BCL11A* knockout (KO) mice,[15] indicating that the mechanism by which these two genes are coregulated is directed from BCL11A toward SOX6.

RT-qPCR experiments conducted on E14.5 fetal liver cells from *BCL11A*+/+ (WT), *BCL11A*+/− (heterozygous), and *BCL11A*−/− (KO) mice confirmed a prior observation[15] that *SOX6* is significantly underexpressed in *BCL11A* KO mice. Moreover, SOX6 expression showed an intermediate level in fetal livers from heterozygous *BCL11A* mice compared to *BCL11A* KO and WT mice (Figure 1A).

One possible mechanism by which BCL11A could affect SOX6 expression is by acting as a transcriptional activator. To identify BCL11A transcription factor binding sites (TFBSs) in the *SOX6* locus, we used optimal IDR (irreproducible discovery rate) peaks of chromatin immunoprecipitation sequencing (ChIP-seq) data for BCL11A[32] from HUDEP-2 cells[31] downloaded from the GEO database[33] (GEO: GSE103445). We visualized the data using the University of California, Santa Cruz (UCSC) genome browser[34] (https://genome.ucsc.edu). The nearest BCL11A TFBS to *SOX6* was approximately 300 kb from the 3′ end of *SOX6* and mapped to an enhancer region in the GeneHancer database (code: GH11J015649). However, *SOX6* was not reported as a target gene of this enhancer. The second nearest BCL11A TFBS was 690 kb from the 5′ end of *SOX6* in the *PIK3C2A* gene. These observations would suggest the absence of BCL11A binding sites in known *SOX6* regulatory regions.

To experimentally evaluate the transcriptional activation potential of BCL11A on *SOX6*, we transfected K562 and HUDEP-2 cells with the pCMV6XL5-BCL11A-XL vector (Figure 1B). *BCL11A* has four alternatively spliced isoforms, of which three are detectable by RT-qPCR in K562 and HUDEP-2 cells (Figure S1). The BCL11A-XL isoform is the most abundant in both cell types (Figure S1) and is known to be the most relevant to globin repression.[12]

A comparison of SOX6 mRNA expression levels in K562 and HUDEP-2 cells with and without BCL11A overexpression (Figures 1C, 1D, 1F, and 1G) failed to detect activation of SOX6 expression (Figures 1E and 1H). This result indicates that BCL11A does not transcriptionally activate *SOX6* and suggests that a post-transcriptional mechanism, such as miRNA-mediated silencing, could be responsible for the coregulation of BCL11A and SOX6.

### Differentially expressed miRNAs in BCL11A-null mice

To identify miRNAs differentially expressed between *BCL11A*+/+ and *BCL11A*−/− E14.5 fetal livers, we performed next-generation sequencing (NGS) of total RNA using Illumina's high-throughput sequencing technology (see section "materials and methods"), generating 10 million reads per sample on average. We determined the expression level for 3,870 miRNAs using the LC Sciences quantification pipeline with the DESeq2 software (Wald test).

Among the differentially expressed miRNAs, we selected those targeting SOX6 using three prediction programs (TargetScan, DIANA-microT, and RNA 22).

Two miRNAs were differently expressed in the NGS data analysis: miR-365-3p was upregulated in *BCL11A*−/− samples (fold change = 1.73 $p = 9.93 \times 10^{-3}$), and miR-144-3p was downregulated (fold change = −0.64, $p = 4.42 \times 10^{-3}$). RT-qPCR analysis of E14.5 fetal livers from *BCL11A*+/+, *BCL11A*+/−, and *BCL11A*−/− mice confirmed the direction and significance of the effect (Figures 2A and 2B).

### Stage-specific expression of miR-365-3p, miR-144-3p, SOX6, and BCL11A during mouse development

We performed an RT-qPCR experiment during embryo development at E10.5 (yolk sac), E12.5, and E14.5 (fetal liver) in *BCL11A* WT mice to assess the possible stage-specific expression of miR-365-3p and miR-144-3p. The expression of miR-365-3p was highest at E10.5

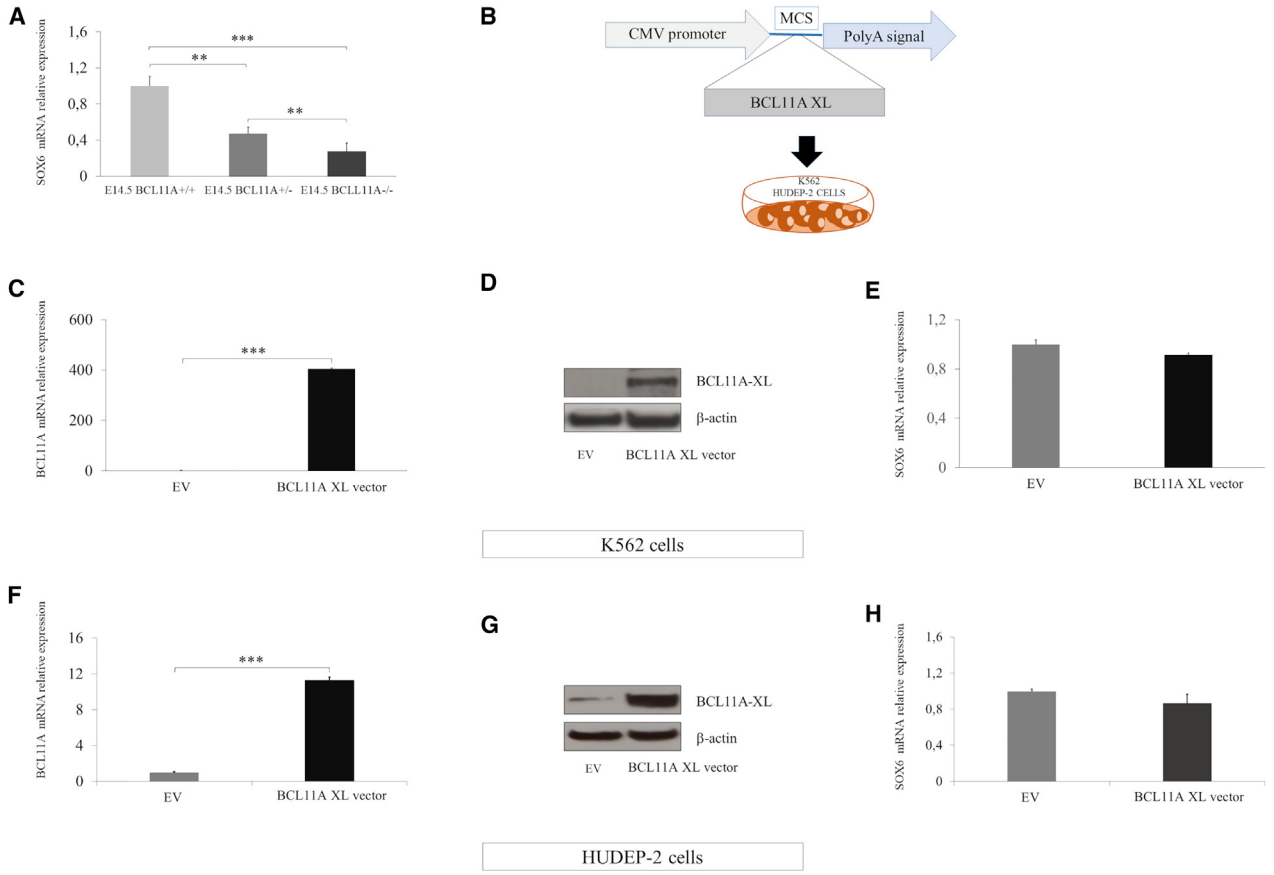


**Figure 1. SOX6 expression in BCL11A KO mice and in BCL11A over-expressing K562 and HUDEP-2 cells**

(A) SOX6 mRNA expression level in *BCL11A*+/+, *BCL11A*+/−, and *BCL11A*−/− E14.5 fetal livers (n = 10 for each genotype). (B) Schematic representation of constructs used to transfect K562 and HUDEP-2 cells. (C and F) BCL11A mRNA expression level after transfection of BCL11A-XL vector or empty vector (EV) in (C) K562 and (F) HUDEP-2 cells. (D and G) Western blot analysis of BCL11A protein levels in (D) K562 and (G) HUDEP-2 cells after transfection of BCL11A-XL or EV. (E and H) SOX6 mRNA expression level after transfection of BCL11A-XL vector or EV in (E) K562 and (H) HUDEP-2 cells. Data are means of three independent experiments. Levels of significance calculated by t test are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

and decreased during development (Figure 3A). The expression of miR-144-3p was lowest at E10.5 and increased at E12.5 and E14.5 (Figure 3B).

These results demonstrate the stage-specific expression of these two miRNAs, which show opposing patterns: miR-144-3p correlated positively and miR-365-3p correlated negatively with *SOX6* and *BCL11A* expression. Indeed, SOX6 and BCL11A expression was decreased at E10.5 and increased at E12.5 and E14.5 (Figures 3C and 3D).

### miR-365-3p directly targets the SOX6 3′ UTR

We identified putative miRNA binding regions for both miRNAs using three target-prediction tools (TargetScan, DIANA-microT, and RNA 22).

We identified two miR-365-3p binding sites: one mapped to positions 990–996 of the SOX6 3′ UTR and was conserved in different species (seed 1). The second site mapped to positions 3,407–3,341 and was less conserved (seed 2). A single miR-144-3p binding site was identified at positions 5,024–5,030 of the SOX6 3′ UTR. We performed a luciferase assay using a reporter vector to functionally validate the interaction of the miRNAs with the SOX6 3′ UTR (Figure 4A). Since miR-365-3p had two seed regions, we first cloned two SOX6 3′ UTR fragments into the reporter vector (Figure 4A). The first contained only seed region 1 (WT1) and the other seed region 2 (WT2). In addition, two mutant constructs containing mutated seed 1 (MUT1) or seed 2 (MUT2) were created by site-direct mutagenesis. Then, we co-transfected each construct (WT and MUT) in HeLa cells with a miR-365-3p mimic. As negative controls, co-transfection of WT constructs with a non-targeting miRNA mimic or transfection of an empty vector (EV) were carried out. Luciferase activity was assayed 48 h after transfection.

Figure 4B shows that transfection of the miR-365-3p mimic into HeLa cells significantly decreased the activity of the WT1 SOX6 3′

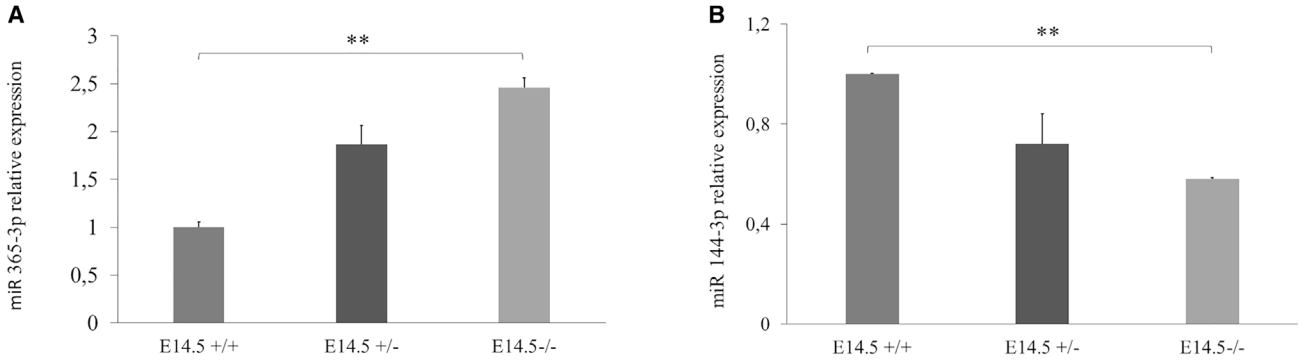


**Figure 2. miR-365-3p and miR-144-3p expression level in BCL11A-KO mice fetal liver**

(A) Expression level of miR-365-3p in *BCL11A*+/+, *BCL11A*+/−, and *BCL11A*−/− E14.5 fetal liver (n = 6 for each genotype). (B) Expression level of miR-144-3p in *BCL11A*+/+, *BCL11A*+/−, and *BCL11A*−/− E14.5 fetal liver (n = 6 for each genotype). Levels of significance calculated by t test are indicated (*p < 0.05, **p < 0.01, ***p < 0.001).

UTR compared to negative controls. However, the MUT1 binding site did not show this inhibitory effect. WT2 did not show significant differences from the controls after treatment with the miR-365 mimic (Figure 4C).

Based on these findings, we cloned the full-length SOX6 3′ UTR with the WT1 or MUT1 sequence downstream of the luciferase reporter gene and analyzed its activity in HeLa cells co-transfected with the miR-365-3p mimic. Figure 4D shows that transfection of the miR-365-3p mimic into HeLa cells significantly decreased the activity of the WT1 full-length SOX6 3′ UTR compared to the negative controls. In contrast, the MUT1 binding site abrogated the miR-365-3p-dependent repression. These results indicate that SOX6 3′ UTR is directly targeted by miR-365-3p at the evolutionarily conserved seed 1 region, consistent with *in silico* prediction. In contrast, the poorly conserved seed 2 region was not targeted in our assay.

To functionally validate the interaction of miR-144-3p with the SOX6 3′ UTR, the full-length 3′ UTR with the WT or mutated miR-144-3p binding site was co-transfected with the miR-144-3p mimic. As negative controls, co-transfection of WT constructs with a non-targeting miRNA mimic or transfection of an EV were carried out. Figure 4E shows no significant difference in luciferase activity after miR-144-3p mimic treatment compared to negative controls. Since these results revealed no functional effect of miR-144-3p targeting the 3′ UTR of *SOX6*, it was not further investigated.

### miR-365-3p inhibits the expression of endogenous SOX6 in mouse fetal liver cells

To investigate whether miR-365-3p regulates the expression of endogenous SOX6, we examined the effect of miR-365-3p overexpression by transfecting a miRNA mimic and a non-targeting mimic in E14.5 immortalized mouse fetal liver cells. These cells had been obtained from a homozygous *p53*-KO mouse model as described by Papadopoulos et al.[35] Figure 5A shows that SOX6 mRNA levels decreased in E14.5 fetal liver cells transfected with the miR-365-3p mimic compared to the negative control. Western blot analysis of the same samples confirmed the inhibitory effect of miR-365-3p at the protein level (Figures 5B and 5C). The expression of other validated modulators of γ-globin expression (BCL11A, KLF1, LRF/ZB7BA, and MYB) was unaffected by the miR-365-3p mimic (Figure S2A).

These results demonstrated that miR-365-3p significantly reduces the mRNA and protein levels of endogenous SOX6 in fetal liver cells, confirming the involvement of miR-365-3p in *SOX6* post-transcriptional regulation.

SOX6 is known to silence the embryonic ε-globin gene in definitive erythroid cells since this gene is ectopically expressed in the fetal liver of *SOX6*-null mice.[20] The *βH1* embryonic globin gene is also derepressed in *SOX6*-null mice, although it appears to be silenced in the late fetal liver stage.[20] Therefore, RT-qPCR analysis was performed after miR-365-3p mimic treatment, revealing increased expression of the embryonic genes $\varepsilon^{y}$ and βH1 compared to the negative controls (Figure S3).

### miR-365-3p modulates the expression of endogenous SOX6 and enhances fetal and embryonic globin expression in HUDEP-2 cells

To investigate whether miR-365-3p affects SOX6 expression in human cells, we transfected the miR-365-3p mimic or a non-targeting miRNA into HUDEP-2 cells.[31] We confirmed that SOX6 mRNA and protein levels were significantly decreased in miR-365-3p mimic-treated human cells compared to controls (Figures 6A, 6D, and 6E). The expression of other validated modulators of γ-globin expression (BCL11A, KLF1, LRF/ZB7BA, and MYB) was unaffected by the miR-365-3p mimic (Figure S2B).

SOX6 is known to be a crucial silencing factor for the mouse ε-globin gene.[20] SOX6 has also been implicated in cooperating with BCL11A in silencing human γ-globin transcription.[15] To investigate whether miR-365-3p-mediated SOX6 downregulation could affect both fetal and embryonic human globin gene expression, γ- and ε-globin

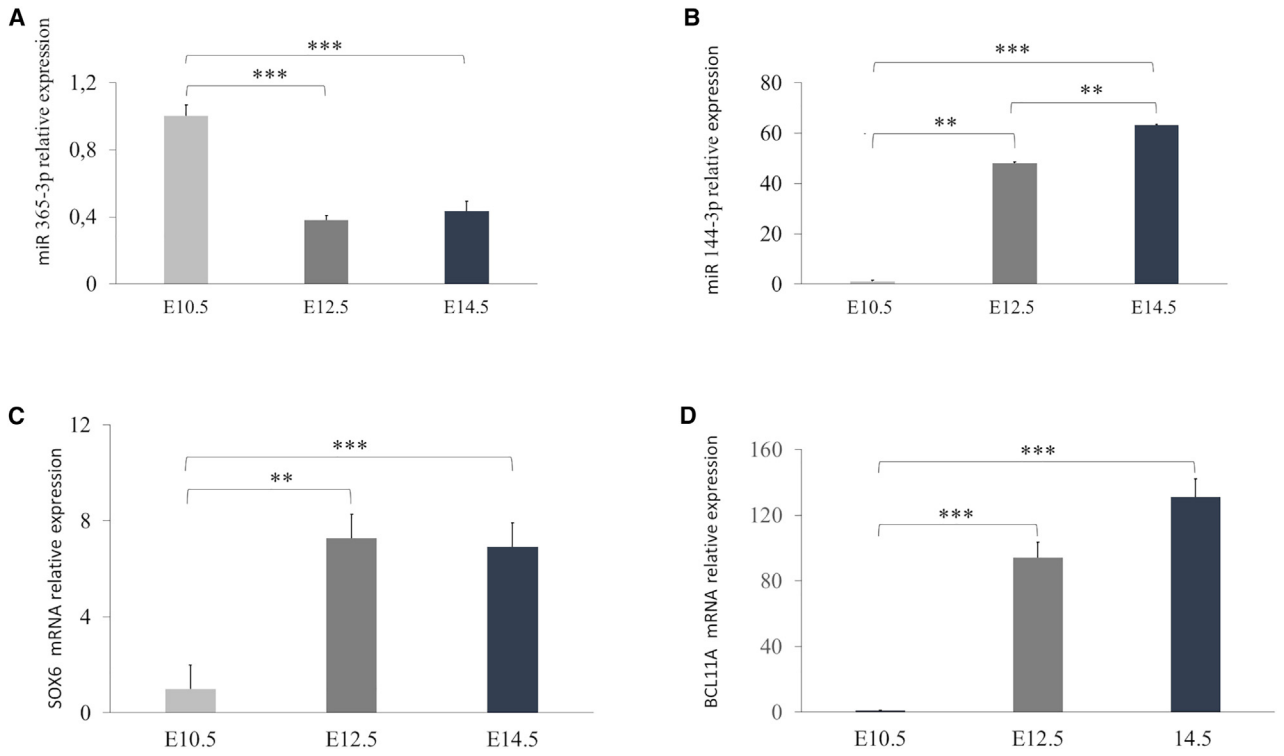


**Figure 3. Expression of mir-365-3p and miR-144-3p during mouse development**

(A and B) Expression levels of miR-144-3p and miR-365-3p during embryo development: E10.5 (yolk sac), E12.5, and E14.5 (fetal liver) (n = 6 for each time point). (C and D) SOX6 and BCL11A mRNA expression level during embryo development: E10.5 (yolk sac), E12.5, E14.5 (fetal liver) (n = 6 for each time point). All genotypes were WT for BCL11A. Levels of significance calculated by t test are indicated (*p < 0.05, **p < 0.01, ***p < 0.001).

mRNA levels were measured by RT-qPCR analysis in induced HUDEP-2 cells after miR-365-3p mimic exposure. Figure 6B shows that the miR-365-3p-mediated post-transcriptional reduction in SOX6 expression significantly increased γ-globin gene expression in HUDEP-2 cells; ε-globin gene expression also increased slightly, although it was not statistically significant (Figure 6C). We then investigated whether the inhibitory effect of miR-365-3p was also detectable at the protein level. Figures 6D and 6E shows that treatment with the miR-365-3p mimic decreased SOX6 but increased γ- and ε-globin levels compared to the negative control.

### BCL11A is a transcriptional repressor of miR-365-3p

We have shown that miR-365-3p is overexpressed in *BCL11A*-null mice, suggesting that BCL11A might directly repress miR-365-3p expression.

To experimentally evaluate whether overexpression of *BCL11A* could affect miR-365-3p expression, we transfected K562 cells with the pCMV6XL5-BCL11A-XL vector as previously described (Figures 1B, 1C, and 1E). A comparison of the miR-365-3p expression levels in K562 cells with and without BCL11A overexpression showed a significant reduction in miR-365-3p expression with BCL11A overexpression (Figure 7A), suggesting that BCL11A is a direct transcriptional repressor of miR-365-3p.

In humans, miR-365-3p is encoded by two distinct genes, located on different chromosomes: *MIR365A* on chromosome 16 and *MIR365B* on chromosome 17. To identify TFBSs for BCL11A in the *MIR365A* and *MIR365B* transcriptionally regulatory regions, we exploited previous ChIP-seq data for BCL11A from HUDEP-2 cells.[32] Data were downloaded from the GEO database[33] (GEO: GSE103445), and the UCSC genome browser[34] (https://genome.ucsc.edu) was used to visualize the data, as described previously.

The nearest BCL11A ChIP-seq signal to the *MIR365A* gene is a 370-bp sequence 23 kb from its 5′ end that maps to an enhancer region in the GeneHancer database[36] (code: GH16J014283; Figure S4). GH16J014283 is *in silico* predicted to be an enhancer of the *MIR193BHG* gene,[36] a long noncoding RNA gene hosting *MIR365A* (Figure S4). This 370-bp sequence does not contain a perfect canonical binding motif for BCL11A (Figure S5A). Therefore, we focused on suggestive putative, non-canonical binding motifs containing the highly conserved motif TGNCC.[32] We identified two putative binding sites (Figure S5A). For subsequent experiments, we also considered an additional suggestive putative binding motif positioned in the center of the ChIP-seq signal and showing good homology (six out of eight bases) to the *in vivo* BCL11A consensus motif (C/G) TG(A/T)CCA(C/G)[32] (Figure S5A). The nearest BCL11A ChIP-seq signal for *MIR365B* was a 389-bp sequence mapping to a candidate

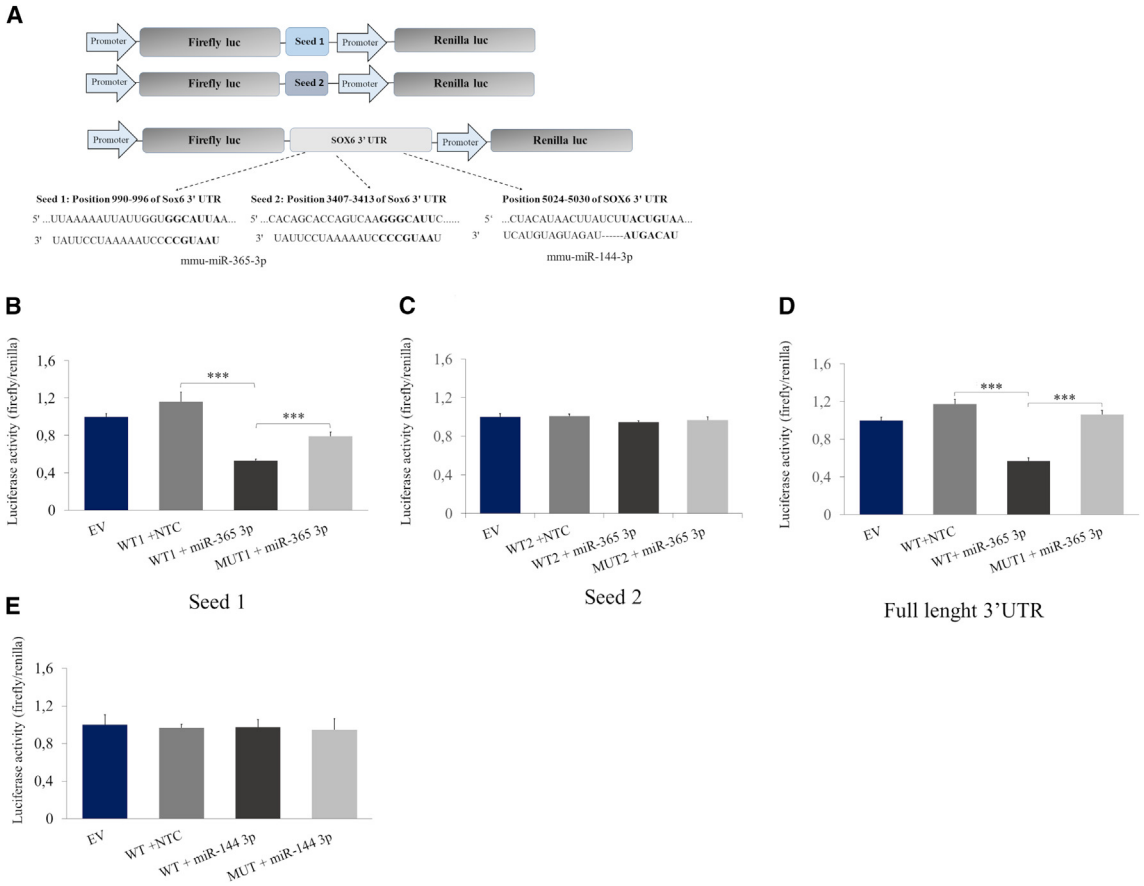


**Figure 4. miRNA target gene validation of SOX6 3′ UTR using a luciferase reporter gene assay**

(A) Schematic representation of luciferase constructs used for reporter assays. Fragment of the SOX6 3′ UTR containing the two putative miRNA-365-3p binding sequences (seed 1 and seed 2), WT and mutated, and a third construct containing the full-length SOX6 3′ UTR, WT or mutated at seed 1, were cloned into the pmirGLO vector. (B) Luciferase activity in HeLa cells co-transfected either with a non-targeting control (NTC) or an miR-365-3p mimic and seed 1 construct WT (WT1) or mutated (MUT1) and EV. (C) Luciferase activity in HeLa cells co-transfected with an NTC or an miR-365-3p mimic and seed 2 construct WT (WT2) or mutated (MUT2) and EV. (D) Full-length SOX6-3′ UTR construct, WT or MUT1 co-transfected with an NTC or an miR-365-3p mimic and EV. (E) Luciferase activity in HeLa cells co-transfected with full-length SOX6 3′ UTR (WT or mutated), miR-144-3p, and an NTC. Firefly luciferase levels were normalized to Renilla luciferase activity. Data are the means of three independent experiments. Levels of significance calculated by t test are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

promoter/enhancer region (ENCODE)[39] of the *MIR365BHG* gene, a long noncoding RNA gene hosting *MIR365B* (Figure S6). One canonical consensus binding motif (TGTCCA)[32] for BCL11A exists near the center of this ChIP-seq peak (Figure S5B).

We confirmed the binding of BCL11A to two (TFBS1 and TFBS2) out of three putative binding sites in the enhancer region GH16J014283 (*MIR365A*) and to the single binding site (TFBS3) in the candidate promoter/enhancer region of *MIR365BHG* (*MIR365B*) in induced HUDEP-2 cells using the cleavage under targets and release using nuclease (CUT&RUN) assay (Figures S5C–S5E).

In order to evaluate the functional role of these BCL11A TFBSs on miR-365-3p expression, we CRISPR-Cas9-mediated genome edited each of them in HUDEP-2 cells. After puromycin-positive selection, total efficiency was 70% for TFBS1, 41% for TFBS2, and 52% for TFBS3, calculated by the tracking of insertions or deletions (indels) by decomposition (TIDE) assay based on the percentage of sequences with indels compared to the same regions in HUDEP-2 cells treated with Cas9 (Figure S7).

Cut&RUN assays were performed on the induced edited HUDEP-2 cells to confirm that BCL11A binding was impaired after editing. Figure 7 shows significantly decreased binding efficiency at TFBS1 (Figure 7B), TFBS2 (Figure 7C), and TFBS3 (Figure 7D) in edited pools compared to Cas9 control pools. Our assay used the distal BCL11A TFBS of the human γ-globin gene's promoter[12] as the positive control (Figure 7E). RT-qPCR analysis of the same pools showed a significant increase in miR-365-3p expression in the TFBS1, TFBS2, and TFBS3 edited pools compared to the Cas9-treated pool (Figure 7F).

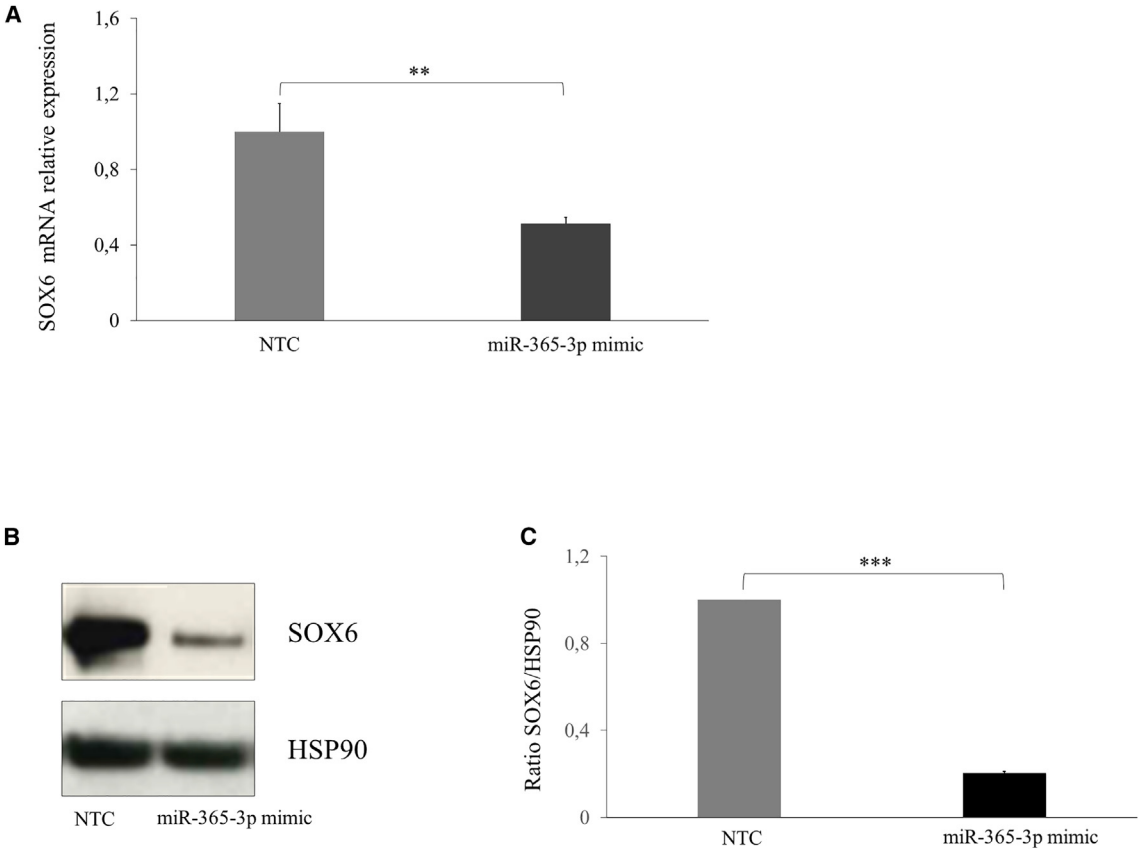


**Figure 5. miR-365-3p downregulates SOX6 expression in mouse fetal liver cells**

(A) SOX6 mRNA levels in mouse *p53−/−* fetal liver cells after mimic negative control or miR-365-3p mimic transfection. (B) Western blot analysis of SOX6 protein levels in mouse fetal liver cells after mimic negative control or miR-365-3p mimic transfection. (C) Quantification of protein levels was performed using ImageJ and the ratio was normalized to the negative control. Data are means of four independent experiments. Levels of significance calculated by t test are indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Altogether, these results demonstrate that BCl11A represses miR-365-3p transcription by binding to regulatory regions in both *MIR365A* and *MIR365B* in human erythroid cells. In addition, the binding of BCL11A in the promoter region of the murine orthologous gene *Mir365-2* in E14.5 fetal liver cells suggests that the molecular mechanism by which BCL11A represses miR-365-3p expression is evolutionarily conserved (supplemental results and Figure S8).

### Targeted deletion of SOX6 by CRISPR-Cas9 strongly enhances ε- and γ-globin gene expression

We have shown that the transient overexpression of miR-365-3p can decrease SOX6 expression, increasing the expression of both the fetal (γ) and embryonic (ε) human globin genes. Given the therapeutic potential of these globins in β-hemoglobinopathies, we aimed to study the effects of a stable decrease in SOX6 obtained by disrupting its gene.

Therefore, we CRISPR-Cas9-mediated genome edited the *SOX6* locus to stably disrupt its expression in HUDEP-2 cells (Figure 8). After puromycin-positive selection, an efficiency of 81% was calculated by the TIDE assay, as previously described (Figure S9). The edited pool showed a 62% decrease in *SOX6* mRNA levels and a 64% decrease in SOX6 protein levels compared to the Cas9-treated pool (Figures 8A–8C). The expression of other validated modulators of γ-globin expression (BCL11A, KLF1, LRF/ZB7BA, and MYB) was unaffected by *SOX6* editing (Figure S2C). No significant differences were evident between the edited and unedited cell pools during *in vitro* erythroid differentiation, indicating that *SOX6* knockdown does not impair HUDEP-2 cell differentiation (supplemental results and Figure S10A).

We then used RT-qPCR to evaluate whether globin gene expression was affected by reduced SOX6 expression in induced HUDEP-2 cells.[37] Measuring the ratios of γ/γ + ε + β mRNAs, we found highly significant γ-globin overexpression in the HUDEP-2 *SOX6*-edited pool compared to the control (Figure 8D). We also explored whether SOX6 downregulation impaired ε-globin gene expression as well. Therefore, we compared the percentage of ε/ε +γ + β mRNAs in the *SOX6*-edited and Cas9-treated HUDEP-2 pools. Consistent with the variation in fetal globin, we detected a highly significant

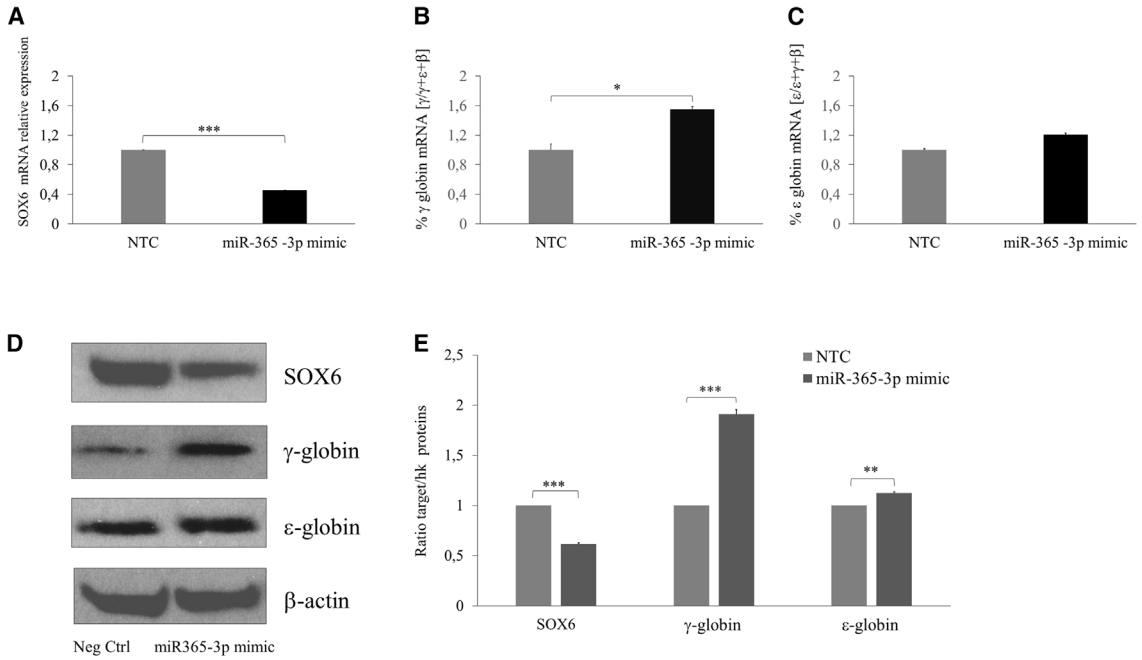


**Figure 6. miR-365-3p downregulates SOX6 and increases ε- and γ-globin expression in human HUDEP-2 cells**

(A) SOX6 mRNA levels after mimic negative control or miR-365-3p mimic transfection. (B) Percentage of γ-globin mRNA (γ/γ + ε + β) after mimic negative control or miR-365-3p mimic transfection. (C) Percentage of ε-globin mRNA (ε/γ + ε + β) after mimic negative control or miR-365-3p mimic transfection. (D) Western blot analysis of SOX6, γ-globin, and ε-globin protein levels in HUDEP-2 cells mimic negative control or miR-365-3p mimic transfection. (E) Quantification of protein levels was performed using ImageJ. Data are means of three independent experiments. Levels of significance calculated by t test are indicated (*p < 0.05, **p < 0.01, ***p < 0.001).

increase in ε-globin level in the *SOX6*-edited HUDEP-2 pool (Figure 8E). Western blot analysis showed a substantial increase in both γ-globin (21 ± 1.25-fold increase) and ε-globin (10 ± 0.26-fold increase) at the protein level (Figures 8F and 8G), confirming the repressing role of SOX6 on the expression of the γ- and ε-globin genes in HUDEP-2 cells. Moreover, the increased γ-globin expression in *SOX6*-edited HUDEP-2 cells was accompanied by more cells expressing HbF (supplemental results and Figure S10B).

These results demonstrate that robust ε- and γ-globin gene expression can be achieved in adult erythroid cells by stable repression of SOX6.

## DISCUSSION

BCL11A has emerged as a key regulator of the hemoglobin switching that represses HbF expression in adult life. Approaches aimed at specifically repressing BCL11A expression in erythroid cells underlie strategies to increase HbF as a therapy for β-hemoglobinopathies.[1–7] It has recently been elucidated that BCL11A acts through direct binding and transcription factor competition at the γ-globin gene promoter.[12,13] Several studies have implicated SOX6 in erythropoiesis[22] and hemoglobin switching.[15] It directly silences the murine embryonic ε-globin gene in definitive erythropoiesis by binding to its promoter[20] and cooperates with BCL11A in silencing the human fetal γ-globin gene.[15] The two transcription factors are most likely part of a bigger protein complex that also includes the NuRD and GATA1 corepressor complexes.[15,20] BCL11A and SOX6 physically and functionally interact, are co-expressed during erythroid development and differentiation, and co-occupy discrete sites in the human β-globin cluster with GATA1.[15] More recently, SOX6 has been shown to interact directly with NonO in human erythroid cells, a potential new player in the transcriptional silencing of fetal globin expression.[23]

The network underlying the erythroid-specific coregulation of BCL11A and SOX6 is unknown. Highlighting the molecular mechanisms that regulate their coordinated expression may help efforts to therapeutically modulate hemoglobin switching. SOX6 was downregulated in the fetal liver of *BCL11A*-deficient mice. Here, we have shown that SOX6 expression in heterozygous *BCL11A*-KO mice is intermediate between WT and *BCL11A*-null mice. These observations suggested that BCL11A might directly or indirectly participate in regulating SOX6 expression in erythroid cells.

BCL11A functions mainly as a transcriptional repressor crucial in the development of several systems and γ- to β-hemoglobin switching. The observation that SOX6 is downregulated in *BCL11A*-null mice argues against the possibility that it might function as a repressor of SOX6. While rare, BCL11A has also been reported to function as a transcriptional activator.[38–40] However, we have shown that the concerted expression of BCL11A and SOX6 in erythroid cells does not involve direct BCL11A transcriptional activation of *SOX6*.

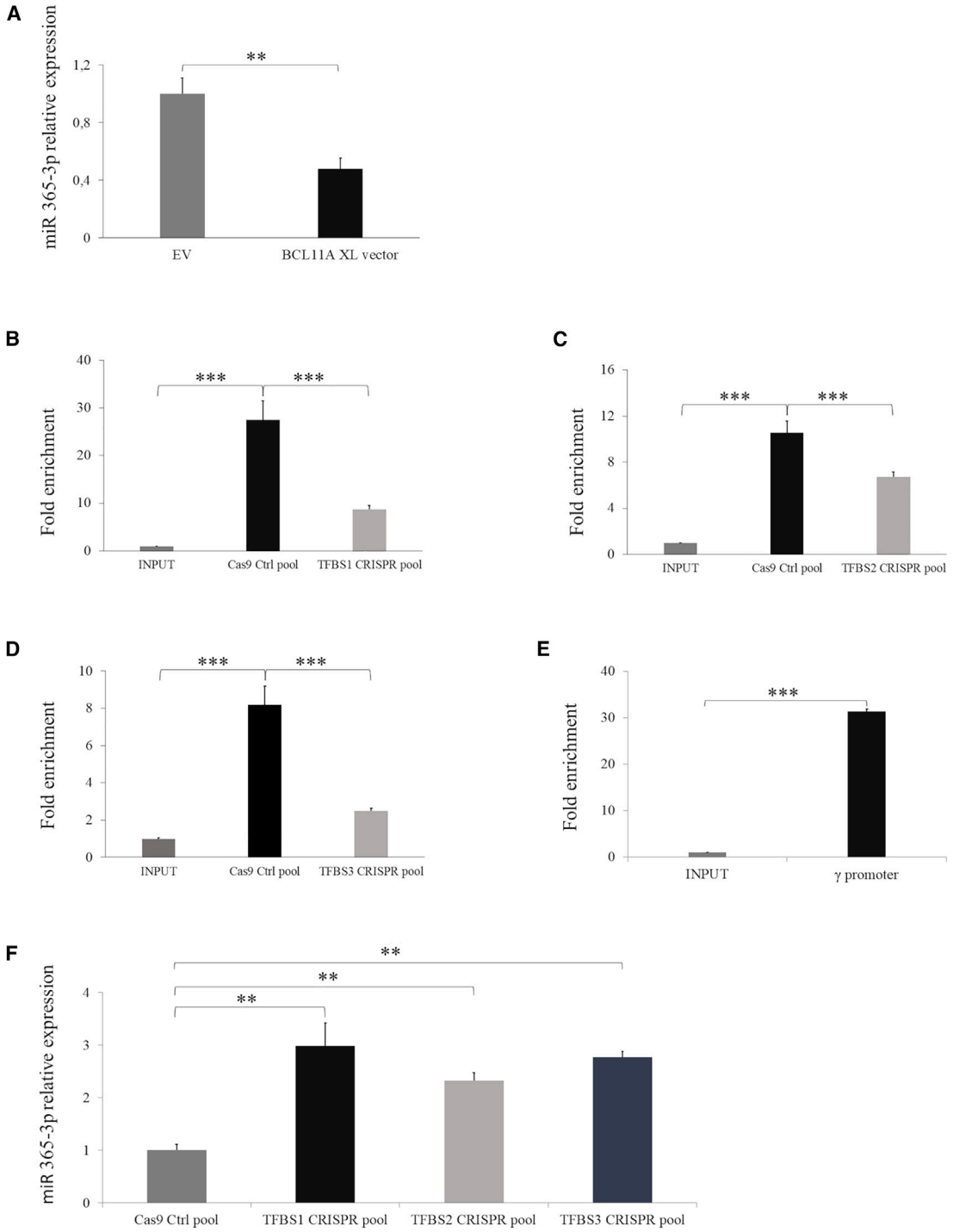


**Figure 7. BCL11A is a transcriptional repressor of miR-365-3p**

(A) RT-qPCR analysis of miR-365-3p after transfection of EV or BCL11A XL vector in K562 cells (n = 6). (B–D) CUT&RUN qPCR assay in HUDEP-2 cells. Analysis of BCL11A binding to TFBS1, TFBS2, and TFBS3 CRISPR genome-edited pools compared to Cas9 control pools. (E) CUT&RUN qPCR of BCL11A binding to the distal TGACCA motif of the human γ-globin promoter[13] compared to input. Data are means of three independent experiments. Data show means of $2^{-\Delta\Delta}$ Ct values normalized to input. (F) RT-qPCR analysis of miR-365-3p after genome editing of TFBS1, TFBS2, and TFBS3 CRISPR genome-edited pools compared to Cas9 control pool. Data show means of $2^{-\Delta\Delta}$ Ct values normalized to Cas9 Ctrl pool. Data are means of three independent experiments. Level of significance calculated by t test is indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

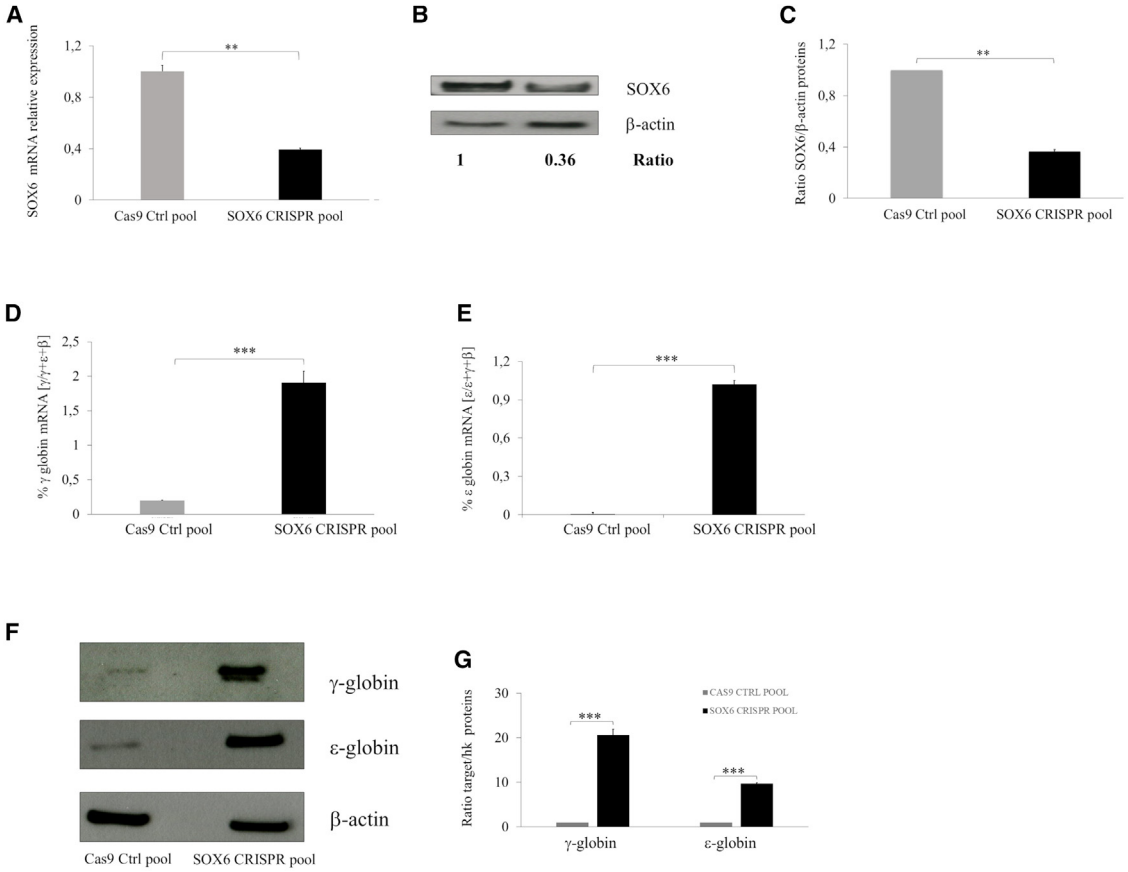


**Figure 8. SOX6 downregulation enhances globin gene expression in HUDEP-2 cell line**

(A) RT-qPCR analysis of SOX6 CRISPR genome-edited pool compared to Cas9 control pool (n = 3 each). (B and C) Western blot assays and relative ratios between SOX6 and β-actin proteins showing the downregulation of SOX6 protein synthesis in SOX6 CRISPR genome-edited pool compared to Cas9 control pool (n = 3 each). (D) RT-qPCR analysis of γ-globin gene expression (percentage γ-globin mRNA [γ/γ + ε + β]) in the SOX6 CRISPR genome-edited pool compared to the Cas9 control pool. (E) RT-qPCR analysis of ε-globin gene expression (percentage ε-globin mRNA [ε/γ + ε + β]) in the SOX6 CRISPR genome-edited pool compared to the Cas9 control pool. (F) Western blot analysis of SOX6, γ-globin, and ε-globin protein levels in the SOX6 CRISPR genome-edited pool compared to the Cas9 control pool. (G) Quantification of protein levels performed using ImageJ. Data are means of three independent experiments. Levels of significance calculated by t test are indicated (*p < 0.05, **p < 0.01, ***p < 0.001).

Given this observation, we explored the possibility that a post-transcriptional gene regulatory mechanism mediated by miRNAs could be involved in their coordinated expression.

Several studies have investigated the role of miRNA expression during murine and human erythropoiesis,[41–45] uncovering mechanisms where the hemoglobin switching molecular network involves post-transcriptional regulation by miRNAs.[46–49]

Increasing experimental data support an association between miRNAs and γ-globin expression. Some miRNAs have been associated with γ-globin activation by inhibiting transcriptional repressors, and others with γ-globin suppression by silencing transcriptional activators.[28,29] Among the miRNAs associated with γ-globin or HbF activation, several have been found to target BCL11A, the major silencer of γ-globin gene expression. Other miRNAs have been shown to target other factors important in γ-globin gene silencing and hemoglobin switching, such as *KLF1* and *MYB*.[28,29] Fewer miRNAs have been associated with γ-globin (*HBG*) suppression, of which one directly targets the γ-globin genes (miR-96).[49]

Analyzing the differential expression in murine fetal liver has allowed us to discover several differentially expressed miRNAs in *BCL11A*-null mice. We focused our attention on miRNAs that were predicted to target SOX6. We have validated miR-365-3p as being differentially expressed and targeting a predicted seed region strongly conserved among vertebrates in the SOX6 3′ UTR. We also found that miR-365-3p has a stage-specific expression pattern, being more highly expressed in the yolk sac than in the fetal liver, contrasting with BCL11A and SOX6.

In addition, miR-144-3p was differentially expressed in *BCL11A*-null mice and showed a stage-specific expression pattern. However, our results do not functionally validate miR-144-3p targeting the 3′ UTR of SOX6. Nevertheless, some indirect effects of miR-144-3p

on SOX6 expression in erythroid cells cannot be ruled out and will require further investigation.

CRISPR-Cas9-mediated genomic editing and CUT&RUN assays demonstrated that BCL11A represses miR-365-3p transcription in HUDEP-2 cells by binding to regulatory regions in both the *MIR365A* and *MIR365B* genes.

In addition, miR-365-3p effectively decreased the expression of the endogenous *SOX6* gene at the mRNA and protein levels in immortalized mouse fetal liver cells, further validating its function as a post-transcriptional repressor of SOX6. The downregulation of SOX6 was associated with the increased expression of the embryonic genes $\varepsilon^y$ and βH1, supporting previous results.[20,50]

Transfection experiments in HUDEP-2 cells indicate that miR-365-3p is also a post-transcriptional repressor of SOX6 expression in human erythroid cells. In these cells, miRNA-365-3p-mediated SOX6 downregulation was associated with a significant slight increase in the expression of ε- and γ-globin genes. These results agree with previous data indicating that SOX6 is a suppressor of γ-globin gene expression[15] and highlight its role in the silencing of the human embryonic ε-globin gene.

Our finding that miR-365-3p contributes to the silencing of ε- and γ-globin genes via post-transcriptional inhibition of SOX6 expression may lead to new strategies for activating embryonic and fetal globin genes in adult erythroid cells by miRNA drugs. RNA modulation based on miRNAs is an emerging field in treating many diseases.[51] The use of miRNA-based therapies has the advantage that miRNAs occur naturally in cells. Moreover, miRNAs often have multiple targets within the same pathway, inducing a broader but specific response.[51,52] Therefore, using or targeting "natural" miRNAs could be a promising novel therapeutic approach for several diseases, including β-hemoglobinopathies.[51–53] However, despite their high therapeutic potential, several obstacles and concerns must be considered as one moves toward clinical use. They include immunological and off-target adverse effects caused by miRNAs influencing the expression of multiple targets, which can produce unwanted outcomes and/or activate oncogenes or inactivate tumor suppressor genes.[54]

The γ-globin gene is a validated target for treating β-thalassemia and SCD.[7,13] The ε-globin gene appears to be an excellent anti-sickling agent in SCD *in vivo*.[55,56] It is unknown whether ε-globin might be a functionally suitable replacement for the adult β-globin gene in β-thalassemia, and preclinical studies are needed.

In our experiments, the increase in the expression of both globin genes, mediated by transient ectopic expression of miR-365-3p, was modest. Further studies would be necessary to develop more effective and stable miRNA-based approaches to increase their levels. However, the Cooperative Study of Sickle Cell Disease[57,58] and more recent studies[59,60] have provided evidence that even small increases in HbF levels should have clinically significant effects on this disease and may improve survival because even a small decrease in the HbS concentration inside erythrocytes may affect the delay time (the period before HbS fibers appear). Therefore, more erythrocytes may escape the capillaries of peripheral tissues before sickling occurs.[59,60]

To explore whether a more pronounced and stable downregulation of SOX6 could produce a more robust effect, we investigated the result of a stable decrease in SOX6 expression in HUDEP-2 cells obtained by disrupting its gene through CRISPR-Cas9-mediated genome editing.

Earlier studies have shown that, in a single case, heterozygous disruption of human *SOX6* was insufficient to overcome the HbF silencing.[61] These previous data suggest that a 50% reduction in the expression of SOX6 does not reach the critical threshold necessary to derepress the fetal globin genes or that a dosage compensation from the intact allele was acting in the patient.[61] Here, we have shown that an erythroid-specific stable downregulation of the SOX6 gene by 64% was sufficient to increase γ-globin expression (about 20-fold) to a level that could potentially result in partial amelioration of clinical severity.[59,60,62,63] This potential therapeutic effect could also be enhanced by the simultaneous increase (about 10-fold) in the embryonic ε-globin level.[55,56]

We conducted our experiments using the immortalized human erythroid line HUDEP-2, which predominantly expresses adult hemoglobin (HbA) with very limited expression of HbF.[31] Erythroid cells derived from CD34+ hematopoietic stem and progenitor cells (HSPCs) could also be investigated. However, these cells tend to have high background levels of HbF.[71]

In summary, miRNA sequencing of fetal liver murine cells showed that miR-365-3p is overexpressed in *BCL11A*-null mice. We established that this miRNA directly targets SOX6 mRNA and that its ectopic expression modulates SOX6 protein levels in immortalized murine fetal liver and HUDEP-2 cells. Consequently, murine and human embryonic and fetal β-like globin genes are upregulated. Stable SOX6 downregulation by >60% strongly derepressed ε- and γ-globin genes.

We also showed that BCL11A is a direct transcriptional repressor of miR-365-3p, highlighting an evolutionarily conserved regulatory network formed by BCL11A, miR-365-3p, and SOX6 that contributes to the fine-tuning and coregulation of BCL11A and SOX6 expression in erythroid cell development and differentiation, which is critical for the suppression of embryonic and fetal globin genes in adults (see the graphical abstract).

Our results may be of interest for developing new therapeutic strategies for β-thalassemia and SCD centered on the modulation of SOX6 expression either by miRNA therapeutics or gene-editing approaches.

## MATERIALS AND METHODS

### Mice

The original BCL11A heterozygous and WT were bought from Riken Bio Resource Center, Japan. All procedures conducted on the animals

were in accordance with the rules and regulations set by the Ethical Committee (OPBA) of University of Cagliari (approval number: n. 299/2021-PR).

### Genotyping

Genotypes were determined by PCR from genomic DNA using the following primers: CCATGACGGCTCTCCCACAAT, ATCCCGAC TCCAGACTGGGAC, and ACGAGTTCTTCTGAGGGGATC to discriminate between the WT and KO BCL11A gene.

### Cell culture

Human erythroleukemic K562 cells were cultured in RPMI 1640 (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Lonza), L-glutamine, and antibiotics (penicillin-streptomycin 100 U/100 μg/mL) in a humidified 5% $CO_2$ atmosphere at 37°C. K562 cells were induced to differentiate by hemin using standard protocols.

HeLa cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented by 10% FBS (Euroclone) with penicillin and streptomycin (100 U/mL) (Euroclone) in an atmosphere of 5% $CO_2$ at 37°.

Fetal livers of E14.5 mouse embryos (p53−/− mice) were resuspended in 1 mL of serum-free stem cell expansion medium (StemPro-34; Life Technologies). Cells were passed through a 70-μm Nylon cell strainer (Euroclone), washed and seeded at $4 \times 10^6$ cells/mL into StemPro-34 medium supplemented with human erythropoietin (EPO; 2 U/mL), murine recombinant stem cell factor (SCF; Sigma-Aldrich, USA, 100 ng/mL), dexamethasone (Dexa; Sigma, $10^{-6}$ M); cell density was maintained between 3 and $7 \times 10^6$ cells/mL by daily dilution with fresh medium containing 2× factors as previously described.[64]

Human umbilical-derived erythroid progenitor -2 (HUDEP-2) cells were maintained in culture as previously described.[37] For expansion, the cells were grown to 0.2–0.8 $\times$ $10^6$ cells/mL in StemSpan serum-free expansion medium (Stem Cell Technologies, 9650) in the presence of 50 ng/mL SCF, 3 IU/mL EPO, 1 μM dexamethasone, 1 μg/mL doxycycline, and 1% penicillin-streptomycin. To induce erythroid maturation, HUDEP-2 cells were grown to 0.7–1.4 $\times$ $10^6$ cells/mL in Iscove modified Dulbecco's medium (Invitrogen) supplemented with 2% FBS, 3% human serum albumin, 3 IU/mL EPO, 10 μg/mL insulin, 1000 μg/mL holotransferrin, 3 U/mL heparin, and 1 μg/mL doxycycline for days 1–3. Cells were grown to 1–2 $\times 10^6$ cells/mL in the same medium for days 4 and 5. HUDEP-2 cells were provided by the RIKEN BRC through the National Bio Resource Project of the, Japan.

### BCL11A overexpression inK562 and HUDEP-2 cell lines

pCMV6XL5 vector containing human BCL11A-XL cDNA (Origene, N. cat TC 314796) was transfected into K562 and HUDEP-2 cells using nucleofector Amaxa 96-well Shuttle protocol.

### RNA isolation and real-time qPCR

Total RNA was isolated using the miRneasy mini kit (QIAGEN) according to the manufacturer's protocol. For mRNA qPCR, RNA was subjected to reverse transcription with Superscript III (Invitrogen) and random primers according to protocols provided by the manufacturer. The gene's expression was detected by Syber Green chemistry, while, for miRNAs, assays were performed using the miRCURY LNA universal RT miRNA PCR method (QIAGEN).

Normalization was performed using miR-223-3p miR-16-5p miR-143-3p.[65–67] Assessing gene expression stability of putative normalizer genes was done using Normfinder. Spike in was used as interplate calibrator.

Real-time PCR was carried out using an ABI PRISM 7900 thermocycler (Applied Biosystems, Foster City, CA). The reactions were performed on at least three different samples in triplicate. The analysis of RT-qPCR data was performed using the ΔΔCT method.

### miRNA library preparation and sequencing

miRNA sequencing was performed by LC Sciences (Houston, TX, USA) using Illumina's high-throughput sequencing technology. A small RNA library was generated from our customer sample using the Illumina TruSeq Small RNA Preparation kit according to Illumina's TruSeq Small RNA Sample Preparation Guide (TruSeq Small RNA instructions). The purified cDNA library was used for cluster generation on Illumina's Cluster Station and then sequenced on an Illumina GAIIx following the vendor's instructions for running the instrument. Raw sequencing reads (40 nt) were obtained using Illumina's Sequencing Control Studio software version 2.8 (SCS v2.8) following real-time sequencing image analysis and base calling by Illumina's Real-Time Analysis version 1.8.70 (RTA v1.8.70). A mean of 10 million reads per sample were produced.

### MiRNA sequencing data analysis

By using a proprietary pipeline script, ACGT101-miR v4.2 (LC Sciences), miRNA sequencing reads were processed and a total of 3,870 miRNA were quantified. The LC Sciences quantification pipeline outputs miRNA expression levels in two formats: read counts and normalized read counts. Read counts were used to determine differentially expressed miRNAs with DESeq[68] (with Wald and LRT methods) and edgeR[69] (with the exact and LRT [likelihood ratio test] method) between genotypes. False discovery rate (FDR) was computed using the Benjamini-Hochberg method.[70] Methods and criteria used for various mappings were documented in the ACGT-101 User's Manual.

### *In silico* prediction of miRNA targeting of SOX6 3′ UTR

To identify candidate miRNAs targeting the SOX6 3′ UTR, the results of three prediction programs were integrated: TargetScan (http://www.targetscan.org/), DIANA-microT (http://diana.cslab.ece.ntua.gr/), and RNA 22 (https://cm.jefferson.edu/rna22/).

### Luciferase constructs

A pmirGLO Dual-Luciferase miRNA target expression vector (Promega) was used to generate the 3′ UTR constructs for the luciferase assays. We generated three constructs: two fragments of the SOX6 3′ UTR (each approximately 500 bp) containing the two putative miRNA-365-3p binding sequences and a third construct containing the full-length SOX6 3′ UTR. All fragments were amplified by PCR and cloned downstream of the firefly luciferase gene using the primers listed in Table S1.

### Site-directed mutagenesis

To generate SOX6 3′ UTRs carrying mutated sequences in the seeding regions of miRNA-365-3p and miRNA-144-3p, site-directed mutagenesis was performed using the QuickChange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions.

### Luciferase miRNA target reporter assay

For luciferase reporter experiments, 100 nM miRNA-365-3p and miRNA-144-3p mimics or an miRNA mimic negative control (Ambion) were co-transfected with 500 ng of the three generated reporter constructs into HeLa cells. miRNA mimics are designed to simulate naturally occurring mature miRNAs, resulting in downregulation of target mRNA translation due to mRNA sequestration or degradation. $1 \times 10^6$ cells were seeded into 12-well plates and, after overnight incubation, cells were treated with Lipofectamine RNAiMAX Transfection Reagent and mimic in Opti-MEM I Reduced Serum Medium (Life Technologies) according to the manufacturer's recommendations. Reporter assays were carried out 48 h after transfection; the relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and a Synergy 2 Plate Reader (BioTek). Firefly luciferase activity was normalized to Renilla luciferase activity. All the experiments were independently repeated six times for statistical validation.

### miRNA targeting of human and mouse SOX6

Here, $2 \times 10^5$ HUDEP-2 cells in erythroid maturation medium, as described before, or $2 \times 10^5$ p53 −/− mouse fetal liver cells were transfected in 12-well plate with 100 nM miRNA-365-3p mimic or miRNA mimic negative control (Qiagen). After 48 h, cells were collected and analyzed by RT-qPCR. All the experiments were independently repeated six times for statistical validation.

### Western blotting

Proteins from HUDEP-2 and p53−/− mouse fetal liver E14.5 cells were collected using RIPA (radio-immunoprecipitation assay) buffer, fractionated by SDS-PAGE, and transferred using a blotting chamber (Bio-Rad) onto a polyvinylidene fluoride (PVDF) membrane (Amersham-Biosciences). Blotted membrane was incubated with BCL11A, SOX6, γ-globin, ε-globin, or β-actin antibodies, then incubated with secondary antibodies and developed with an enhanced chemiluminescence (ECL) detection system (Amersham-Biosciences) according to the manufacturer's instructions. Quantification of protein levels detected by immunoblotting was performed using ImageJ and the ratio was normalized relative to the control sample.

The following antibodies were used: SOX6 (Santa Cruz Biotechnology sc-393314), β-actin (Santa Cruz Biotechnology sc-69879), HPS90 (Santa Cruz Biotechnology sc-101494), HBG (γ-globin) (Thermo Fisher Scientific PA5-77998) HBE (ε-globin) (Thermo Fisher Scientific, PA5-77997), and BCL11A (Thermo Fisher Scientific, MA5-34678).

### CUT&RUN assay

The CUT&RUN assay (Cell Signaling Technology, MA, USA) on BCL11A was performed according to the manufacturer's instructions. Briefly, $1 \times 10^5$ induced HUDEP-2 cells[64] or fetal liver cells of E14.5 mouse embryos were collected and washed with 1× wash buffer (including spermidine and protease inhibitor cocktail) and centrifuged at $600 \times g$ for 5 min at room temperature. Cells were then resuspended with 400 mL of buffer. Then 100 μL of cells were transferred to a new 1.5-mL tube and stored at 4°C, and these were taken as input samples for further verification. The remaining cells were marked as the positive control group, negative control group, and BCL11A group. Ten microliters of activated concanavalin A-coated magnetic bead in suspension were added to these groups to capture cells. These groups were incubated with antibodies targeting tri-methyl-histone H3, immunoglobulin (Ig) G control, and BCL11A at 4°C for 2 h. Tri-methyl-histone H3 and IgG antibody served as the positive and negative control, respectively. Protein pAG-MNase enzyme was added into immunoprecipitated samples and incubated for 60 min at 4°C. Then, 2 mM $CaCl_2$ was added into immunoprecipitated samples to activate protein pAG-MNase on ice for half an hour. Stop buffer was added to these samples to stop the reaction at 15 min at 37°C, and then these samples were centrifugated at 4°C and the supernatant containing the CUT&RUN fragments were collected. DNA of CUT&RUN fragments was purified using a DNA Purification Kit (Cell Signaling Technology, MA, USA). For RT-qPCR assays, positive control group, negative control, input DNA group, and an experimental group were used to determine each gene's binding site's capacity and efficiency of amplification.

### CRISPR-Cas9-mediated genome editing

sgRNAs targeting the *SOX6* coding sequence, BCL11A TFBS1, TFBS2, and TFBS3, were designed using the CRISPR design tool (crispr.mit.edu). Top- and bottom-strand oligonucleotides were annealed and ligated into pSpCas9-(BB)-2A-PURO CRISPR plasmid (#48139, Addgene). HUDEP-2 cells were transfected to generate CRISPR-Cas9 KOs with 5 μg of plasmid using the Amaxa 2B Nucleofector (Lonza) and protocol U-08. Forty-eight hours post transfection, cells were positively selected by adding 1 μg/mL of puromycin to the medium.

Efficiency: DNA was extracted from the puromycin-selected HUDEP-2-edited pool, PCR amplified, and Sanger sequenced for TIDE analysis (Deskgen) and indel detection at the genomic predicted target site.

Cas9 CTRL pool: HUDEP-2 transfected with the pX459 plasmid expressing only Cas9.

*SOX6* CRISPR pool: HUDEP-2 transfected with the pX459 plasmid expressing the SOX6 sgRNA (5′-GACCCAGGATTTAACCTCAA-3′) and Cas9.

TFBS1 CRISPR pool: HUDEP-2 transfected with the pX459 plasmid expressing the TFBS1 sgRNA (5′-GAAGTCTGGGTGGTCCAGCCT -3′) and Cas9.

TFBS2 CRISPR pool: HUDEP-2 transfected with the pX459 plasmid expressing the TFBS2 sgRNA (5′- CACCGTAAGGTCCGCAAA TTTGGC-3′) and Cas9.

TFBS3 CRISPR pool: HUDEP-2 transfected with the pX459 plasmid expressing the TFBS3 sgRNA (5′- GTTTGTGTGGACAACTG CAGT-3′) and Cas9.

The mRNA of human SOX6 and β cluster genes (HBE, HBG, and HBB) was analyzed using RT-qPCR.

### Primers

The list and sequence of primers used in this paper are reported in Table S1.

## DATA AND CODE AVAILABILITY

The data that support the findings of this study are available from the corresponding author (M.S.R.) upon request.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2023.09.002.

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## AUTHOR CONTRIBUTIONS

M. Simbula designed and performed the experiments, analyzed the data, prepared the figures, and contributed to the writing of the manuscript. M.F.M. and M.M. performed the experiments, contributed to the interpretation of the data, and contributed to the writing of the manuscript. I.A., C.A.C., and L.P. performed the experiments. M.P. performed bioinformatics analysis. M.C. and M. Shah provided unpublished material and results. M.C. and P.M. revised the manuscript. M.S.R. designed the study and experiments, supervised the research, analyzed and interpreted the data, and wrote the manuscript. All authors contributed to the discussion and approved the final manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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