Molecular analysis of the erythroid phenotype of a patient with *BCL11A* haploinsufficiency

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Key Points

- A patient with *BCL11A* haploinsufficiency displays >12% fetal hemoglobin; genome-wide analyses indicate otherwise normal erythropoiesis.

- The *HBG1/2* promoters display increased chromatin accessibility but no loss of DNA methylation.

The *BCL11A* gene encodes a transcriptional repressor with essential functions in multiple tissues during human development. Haploinsufficiency for *BCL11A* causes Dias-Logan syndrome (OMIM 617101), an intellectual developmental disorder with hereditary persistence of fetal hemoglobin (HPFH). Due to the severe phenotype, disease-causing variants in *BCL11A* occur de novo. We describe a patient with a de novo heterozygous variant, c.1453G>T, in the *BCL11A* gene, resulting in truncation of the BCL11A-XL protein (p.Glu485X). The truncated protein lacks the 3 C-terminal DNA-binding zinc fingers and the nuclear localization signal, rendering it inactive. The patient displayed high fetal hemoglobin (HbF) levels (12.1-18.7% of total hemoglobin), in contrast to the parents who had HbF levels of 0.3%. We used cultures of patient-derived erythroid progenitors to determine changes in gene expression and chromatin accessibility. In addition, we investigated DNA methylation of the promoters of the γ-globin genes *HBG1* and *HBG2*. HUDEP1 and HUDEP2 cells were used as models for fetal and adult human erythropoiesis, respectively. Similar to HUDEP1 cells, the patient's cells displayed Assay for Transposase-Accessible Chromatin (ATAC) peaks at the *HBG1/2* promoters and significant expression of *HBG1/2* genes. In contrast, *HBG1/2* promoter methylation and genome-wide gene expression profiling were consistent with normal adult erythropoiesis. We conclude that HPFH is the major erythroid phenotype of constitutive *BCL11A* haploinsufficiency. Given the essential functions of BCL11A in other hematopoietic lineages and the neuronal system, erythroid-specific targeting of the *BCL11A* gene has been proposed for reactivation of γ-globin expression in β-hemoglobinopathy patients. Our data strongly support this approach.

Introduction

De novo mutations affecting 1 allele of the *BCL11A* gene are the cause of Dias-Logan syndrome (OMIM 617101), an intellectual developmental disorder with hereditary persistence of fetal hemoglobin (HPFH).[1] A variety of genetic variations, such as deletions, as well as stop codon, frameshift, and missense variants, have been reported in the literature.[1-7] *BCL11A* encodes a transcriptional repressor.

Through alternative splicing, a number of isoforms can be produced; the longest is called BCL11A-XL.[8] Amplifications and translocations involving the *BCL11A* gene indicate that the protein is involved in malignancies of diverse cellular origins.[8-22] In addition, it has been linked to the risk of developing type 2 diabetes.[16,23-29] Remarkably, in the erythroid lineage, BCL11A was identified as a direct repressor of the *HBG1/2* genes encoding γ-globin, the fetal β-like globin.[30-33] Expression of fetal hemoglobin (HbF; $\alpha_2\gamma_2$) in adults is clinically relevant, because it ameliorates the symptoms of patients with β-hemoglobinopathies. In the case of β-thalassemia, γ-globin chains can functionally replace the missing β-globin chains. In sickle cell anemia, which is caused by a pathological variant of β-globin (p.E6V), increased expression of γ-globin leads to reduced expression of aberrant β-globin. The reduction in sickle hemoglobin (HbSS) decreases the propensity of the cells to change shape as a result of the polymerization of HbSS that occurs under low-oxygen conditions. Hemoglobin switching from HbF to adult hemoglobin (HbA; $\alpha_2\beta_2$) starts perinatally and is completed during the first year of life. Recently, significant progress has been made in understanding the molecular mechanism of hemoglobin switching. A regulatory network involving the transcription factors MYB, KLF1, and BCL11A has been proposed, of which BCL11A acts as a direct repressor of the *HBG1/2* genes.[34] Additional players in hemoglobin switching are cofactors, such as members of the NuRD[35,36] and BAP1/CoREST[37,38] complexes; the LRF transcription factor (encoded by *ZBTB7A*), which is also a direct repressor of the *HBG1/2* genes[39]; and factors involved in stress erythropoiesis, such as ATF4[40] and HKI.[41] In addition, LIN28B has been identified as a repressor of BCL11A expression in fetal erythroid cells.[42,43] Functional analysis to investigate the role of these proteins in hemoglobin switching has traditionally relied on the use of genetically modified mice and cell lines, such as human K562 and mouse MEL. The emerging power of genome-wide association studies and linkage analysis, followed by application of next-generation sequencing (NGS), has provided strong support for the role of variants of *MYB*, *KLF1*, *BCL11A*, and the *HBB* locus itself in the regulation of HbF levels in adults.[44-46] For *BCL11A* a number of SNPs associated with an erythroid-specific enhancer are linked to expression levels of the protein in erythroid progenitor cells, with lower-expressing alleles linked to higher HbF levels.[30,31,33] Because of its important role in neurological development, pathologic variants of *BCL11A* affecting the protein occur as de novo events.[1] Although the initial reports described patients with microdeletions that included *BCL11A* and a few genes located nearby,[3,4] nonsense, frameshift, and missense variants have been reported more recently in patients with clinical phenotypes characteristic of what is now termed Dias-Logan syndrome (OMIM 617101).[1] Here, we describe a patient with a de novo heterozygous variant c.1453G>T in the *BCL11A* gene, resulting in truncation of the BCL11A-XL protein (p.Glu485X). At the age of 3 years and 10 months, the patient displayed an HbF level of 18.7%, whereas other blood parameters were within normal range. The parents displayed 0.3% HbF, a normal level for adults. To study the effects of *BCL11A* haploinsufficiency on genome-wide gene expression and chromatin accessibility, we established cultures of primary erythroid progenitors derived from the patient. These data were compared with those obtained from the HUDEP1 and HUDEP2 erythroid progenitor cell lines, which are models for human fetal and adult erythropoiesis, respectively.[47] In addition, because methylation of the *HBG1/2* promoters has been associated with silencing of the genes,[48-50] we studied DNA methylation at cytosine guanine dinucleotide (CpG) residues in the promoters of the *HBG1/2* genes.

Materials and methods

Human samples

Clinical exome sequencing was performed as part of a diagnostic work-up, and written informed consent was given for further hematological and molecular studies, according to the requirements of the Institutional Review Board of Erasmus MC's Medical Ethics Review Committee. The study was conducted in accordance with the Declaration of Helsinki. All other materials and methods are described in supplemental Materials and methods.

Results

Patient description and whole-exome sequencing

The female patient is the second child of nonconsanguineous parents with no family history of intellectual disability or congenital abnormalities. She was born after an uneventful pregnancy with a birth weight of 3660 g. At birth, the head circumference was measured as −1 standard deviation [SD]. She was first seen in the pediatric ward at the age of 3 months because of feeding difficulties. Physical examination at that time showed a head circumference of 37 cm (−2 SD) and facial dysmorphism consisting of strabismus, bilateral epicanthi, thin upper lip, and fine hair. She showed no hypotonia, and eye contact was normal. She was treated with a stomach tube at home because of the feeding difficulties. At the age of 10 months her head circumference had declined further compared with healthy children; it was measured as 41.5 cm (−2.7 SD). She developed a significant motor developmental delay. More specifically, she was able to sit unsupported at 16 months; she walked with support at 2 years and independently at 4 years. At the age of 16 months cognitive testing using the Bayley Scales of Infant and Toddler Development, second version, Dutch language (BSID-II-NL) showed a cognitive delay of 7 months and a developmental index of 55. Head circumference evolved from −2.5 SD up to the age of 3 years to within normal range at 7 years (Figure 1). Her length and weight were always normal. Ophthalmological examination showed hypermetropia (+3.5 oculus dexter and oculus sinister) and amblyopia of the left eye. Brain magnetic resonance imaging at the ages of 1 and 5 years revealed no abnormalities. At the age of 7 years she was evaluated more extensively. She still presented with gait abnormalities and weak motor coordination skills, falling easily. Her build was strikingly muscular, without joint laxity. Behavior showed recurrent hand flapping. Despite speech therapy she was unable to develop meaningful speaking skills, and communication was performed through pictograms. Facial features included strabismus divergens, mild left ptosis, flat midface, flat philtrum, thin upper lip, and protruding ears with thin helixes and attached earlobes (Figure 1).

Whole-exome sequencing (WES), performed in the patients and her parents, revealed a de novo heterozygous variant c.1453G>T (p.Glu485X) in exon 3 of the *BCL11A* gene (NM_022893 [BCL11A_i001]), resulting in truncation of the BCL11A-XL protein. The c.1453G>T variant is predicted to deleteriously affect isoform L (GenBank: NM_018014.3, Ensembl: ENST00000356842) and isoform XL (NM_022893.3, ENST00000335712) of BCL11A.

Figure 1. A patient with *BCL11A* haploinsufficiency due to de novo p.Glu485X variant.
Patient at age 4 months (A) and at age 3 years and 6 months (B). Facial features included strabismus divergens, mild left ptosis, flat midface, flat philtrum, thin upper lip, and protruding ears with thin, flat helixes, and attached earlobes. (C) A muscular build and instable gait present at 3 years and 6 months. (D) Head circumference evolved from -2.5 SD up to the age of 3 years to within normal range at 7 years.
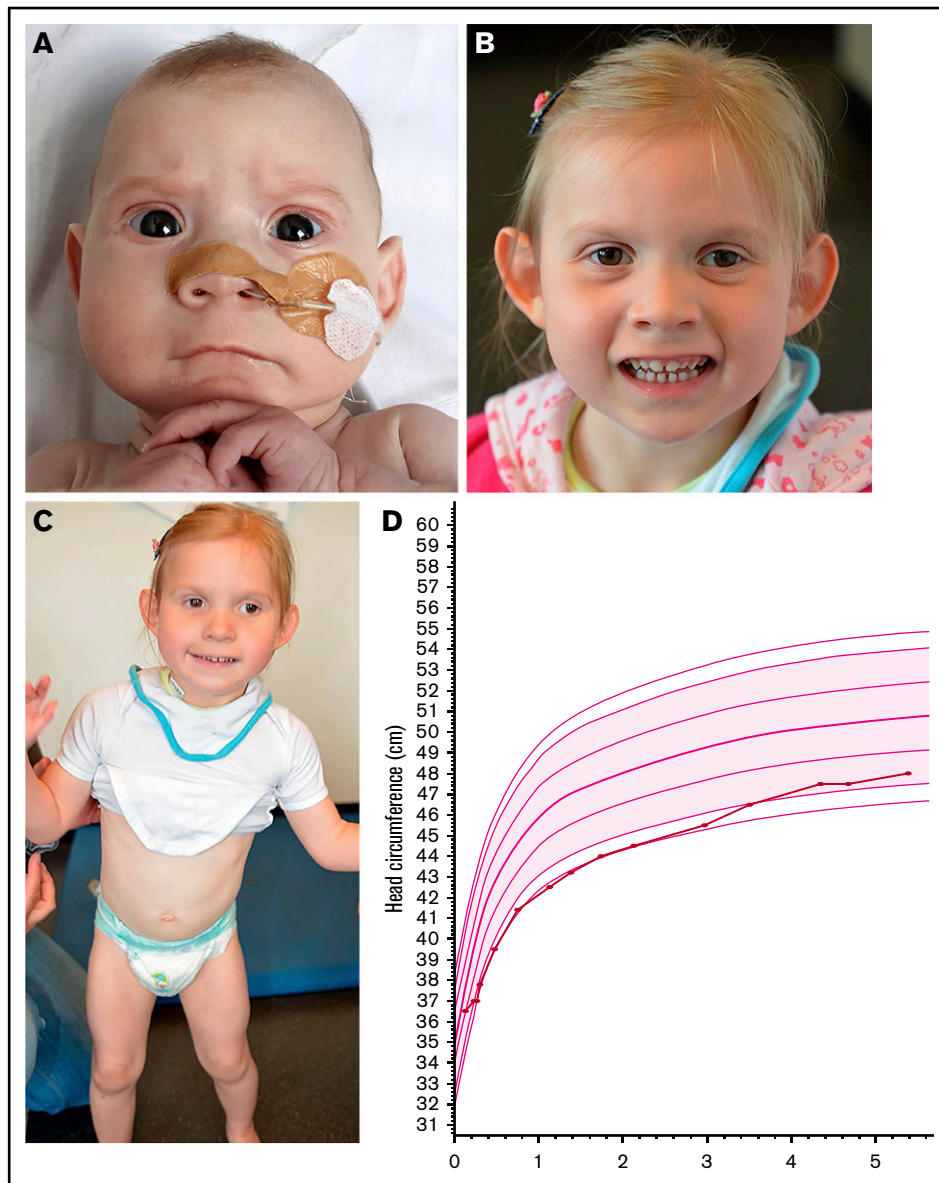
Thus, the patient was diagnosed with Dias-Logan syndrome (OMIM 617101) due to haploinsufficiency for *BCL11A*.

Analysis of hematological parameters

To explore the erythroid phenotype of the patient, we first performed complete blood counts to assess the hematological parameters of the patient and her parents (supplemental Table 1). At the time of blood collection, the patient's age was 3 years and 10 months. At the age of 7 years and 3 months, HbF was measured again. The complete blood count results were unremarkable; all parameters measured were within normal range expected for the age group. Flow cytometry analysis of CD117 (KIT), CD71 (transferrin receptor), CD44 (Indian blood type), and CD235a (glycophorin A), which are markers for erythroid development, showed that these proteins were expressed at levels similar to those observed in control blood (supplemental Figure 1). We then measured HbF levels by high-performance liquid chromatography, which revealed

that both parents had an HbF level of 0.3%, which is within normal range for adults (0-1%). In contrast, the child's HbF level was 18.7% at the age of 3 years and 10 months and 12.1% at the age of 7 years and 3 months, which is much higher than the normal range for these age groups (0-1%) (Figure 2). Persistent expression of HbF is a hallmark of Dias-Logan syndrome[1] and is consistent with the notion that BCL11A is a dose-dependent repressor of the *HBG1/2* genes.[51]

Patient-derived primary erythroid progenitor cultures

We wished to explore the impact of *BCL11A* haploinsufficiency on erythroid cells in more detail. To this end, we isolated the buffy coat from ~7 mL of peripheral blood collected from the patient and used these cells to establish cultures of primary human erythroid progenitors (HEPs).[52] HEPs can be expanded in defined medium in the presence of stem cell factor, erythropoietin, and the glucocorticoid hormone dexamethasone. At days 14 and 17 of
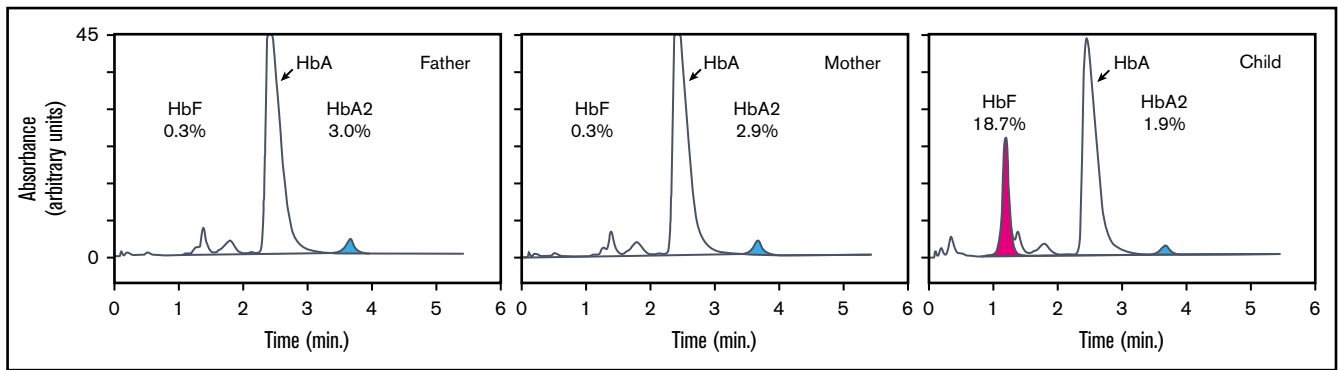
Figure 2. Hemoglobin analysis of peripheral blood. Hemoglobin subtypes of peripheral blood were analyzed by high-performance liquid chromatography. The percentages of total hemoglobin are indicated for HbA ($\alpha_2\beta_2$), HbA2 ($\alpha_2\delta_2$; blue), and HbF ($\alpha_2\gamma_2$; pink).

expansion culture, as well as 48 hours after the induction of differentiation (removal of stem cell factor and dexamethasone, increase in erythropoietin, and addition of iron-saturated transferrin), we harvested patient-derived HEPs to isolate RNA and protein for gene expression analysis by quantitative reverse transcription polymerase chain reaction (RT-qPCR), RNA sequencing (RNA-seq), and western blotting. DNA was isolated to assess methylation of CpG residues in the *HBG1/2* promoters by bisulfite sequencing. In addition, 50 000 cells were used to gauge chromatin accessibility with ATAC-seq (Assay for Transposase-Accessible Chromatin with NGS).[53] We reasoned that *BCL11A* haploinsufficiency might confer fetal-like characteristics to the patient's erythroid cells; therefore, we used HUDEP1 and HUDEP2 cells[47] as benchmarks for comparative analyses. These cells are widely used as models to study human fetal and adult erythropoiesis and hemoglobin switching.[39,54-56]

## High HbF levels are maintained in patient-derived HEPs

To characterize the patient-derived HEPs, we investigated the expression levels of the α-like and β-like globin genes, as well as of MYB, KLF1, LIN28B, BCL11A and LRF, because these factors are important regulators of hemoglobin switching. We first used RNA-seq to measure expression of these genes at the messenger RNA (mRNA) level (supplemental Figure 2A; supplemental Table 2). The patterns of differential expression between HUDEP1 and HUDEP2 cells reflected the expected profile for the fetal and adult stages represented by these cells. HUDEP1 cells almost exclusively expressed γ-globin (>90% of total β-like globins) and up to 10% of embryonic ϵ-globin. HUDEP2 cells predominantly expressed adult β-globin (~99% of total β-like globin), ~1% δ-globin, and virtually undetectable levels of fetal and embryonic globins. This was confirmed by RT-qPCR analysis (supplemental Figure 3A).

Compared with HUDEP1 cells, expression of MYB was approximately eightfold higher and expression of BCL11A was approximately twofold higher in HUDEP2 cells, whereas there was no change for KLF1 and even a small reduction for LRF (~0.87-fold). Most notably, expression of LIN28B was downregulated >150-fold in HUDEP2 cells compared with HUDEP1 cells (supplemental Figure 2; supplemental Table 2). LIN28B represses BCL11A protein expression in human fetal erythroid progenitors, and forced expression of LIN28B causes adult human erythroblasts to differentiate with a more fetal-like phenotype.[42,43] Collectively,

these data show that these distinctive features of human fetal and adult erythropoiesis are faithfully reproduced by the HUDEP1/HUDEP2 model system.

We then compared expression of these genes in patient-derived HEPs. We observed much higher γ-globin mRNA levels (~17% of total β-like globin) compared with HUDEP2 cells, establishing that the HPFH phenotype is sustained throughout the culture period (supplemental Figure 2; supplemental Table 2). This was confirmed by RT-qPCR analysis (~11% γ-globin of total β-like globin in patient RNA samples; supplemental Figure 3A). In contrast, expression levels of MYB, KLF1, BCL11A, and LRF were similar to those observed in HUDEP2 cells. This could be expected for MYB, KLF1 and LRF, because these factors are not thought to be regulated by BCL11A.[31] Of note, we observed that LIN28B was not expressed in patient-derived HEPs. Thus, LIN28B has no role in the HPFH phenotype of the patient, and its absence sets the patient-derived HEPs apart as adult erythroid progenitors. This is further supported by the low level of embryonic ζ-globin expression in patient-derived HEPs, whereas it was expressed at substantial levels in HUDEP1 cells (supplemental Figure 2; supplemental Table 2; ~15% ζ-globin of α-like globin in differentiated HUDEP1 cells). RNA-seq data indicated that the BCL11A p.Glu485X variant may not trigger nonsense-mediated RNA decay, because wild-type and mutant allele mRNA were detected in the RNA-seq data. Therefore, it was important to assess the impact of *BCL11A* haploinsufficiency on protein expression levels. We performed western blots on whole-cell extracts of HUDEP1, HUDEP2, and patient-derived HEP cells (Figure 3). We observed a clear reduction in the expression of the BCL11A-XL isoform in patient-derived HEPs compared with HUDEP2 cells. Furthermore, a protein with the expected molecular weight of p.Glu485X BCL11A was detected in the patient sample only (Figure 3A).[55] Of note, domain mapping of BCL11A-XL indicates that the BCL11A p.Glu485X variant is unable to repress *HBG1/2* genes.[55] MYB was expressed at similar levels in patient-derived HEPs and HUDEP2 cells but at much lower levels in HUDEP1 cells (Figure 3B, top panel). In contrast, expression of LIN28B was only detectable in HUDEP1 cells (Figure 3B, middle panel). The transcriptional regulators KLF1 and LRF were expressed at apparently similar levels in all 3 cell types (Figure 3C-D). These results are all consistent with the RNA-seq data. Finally, western blot analysis of β- and γ-globin expression (Figure 3E-F) was in agreement with the RNA-seq and RT-qPCR data

Figure 3. Protein expression analysis of transcription factors, LIN28B, and globins. Western blots showing expression of BCL11A (XL isoform and p.Glu485X) (A), MYB and LIN28B (B), KLF1 (C), LRF (D), β-globin (E), and γ-globin (F). β-tubulin was used as loading control. Protein extracts were derived from HUDEP1 cells (H1), HUDEP2 cells (H2), and patient HEPs (P).
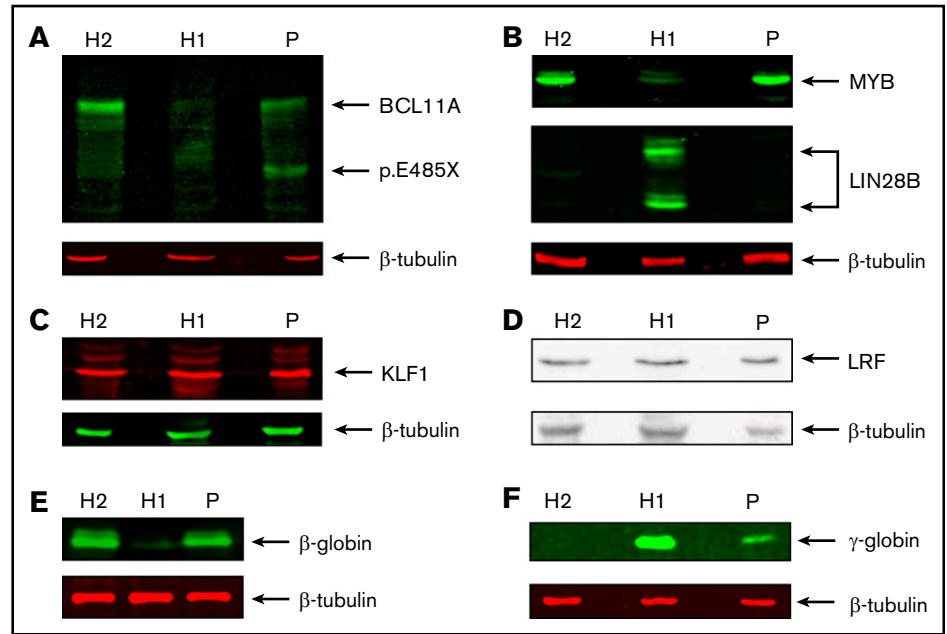
(supplemental Figures 2 and 3A). Collectively, our results strongly support the notion that the HPFH phenotype of the patient is caused by reduced expression of the BCL11A-XL isoform.

Methylation of the *HBG1/HBG2* promoters

The promoters of the *HBG1/2* genes contain a number of CpGs that become methylated during human development.[50] Of note, treatment of a small number of β-hemoglobinopathy patients with the DNA demethylating agent 5-azacytidine led to increased HbF levels.[48,49] This experimental treatment has seen a recent revival, as reflected by a randomized phase 1 study in which the 5-azacytidine derivative decitabine was used.[57] To this day it remains controversial whether demethylation of the *HBG1/2* promoters is the direct cause of the therapeutic effect of 5-azacytidine.[58] Therefore, it is of interest to investigate the methylation status of the *HBG1/2* promoters in the setting of *BCL11A* haploinsufficiency. We performed bisulfite sequencing to assess methylation of 7 CpGs, located between positions −252 and +50 of the *HBG1/2* promoters (Figure 4). We observed that these 7 CpGs are virtually unmethylated in HUDEP1 cells, as expected in cells representing human fetal erythropoiesis.[50] In contrast, they are almost fully methylated in HUDEP2 cells (Figure 4). We then investigated the methylation status of the *HBG1/2* promoters in HEPs derived from the patient with *BCL11A* haploinsufficiency. We observed that, despite the considerable level of γ-globin expression, DNA methylation was present at a high level for most CpGs. Of note, the methylation level of the −162 CpG was between that of HUDEP1 and HUDEP2 cells, indicating that methylation of this residue might serve as a biomarker for activity of the *HBG1/2* promoters. We conclude that the fetal methylation profile of the *HBG1/2* promoters is not maintained in adult erythroid cells with *BCL11A* haploinsufficiency.

Genome-wide gene expression analysis

Having validated the HEP culture system as a tool to study the impact of *BCL11A* haploinsufficiency on human erythropoiesis, we

were interested in further exploring gene expression using RNA-seq data. Principal component analysis of the RNA-seq profiles showed that the adult/fetal erythropoiesis state was the main source of variation between the samples. Principal component 1 (PC1) separated fetal HUDEP1 samples from adult HUDEP2 and patient samples, whereas PC2 separated the samples according to their state of differentiation (Figure 5A). Thus, the global gene expression profile of the patient's cells was much more similar to that of HUDEP2 cells than to HUDEP1 cells. This was also reflected in the number of differentially expressed genes (DEGs): 3812 in patient cells vs HUDEP2 cells and 4930 in patient cells vs HUDEP1 cells (2log fold change > |1| and adjusted P value < .01; Figure 5B-C;
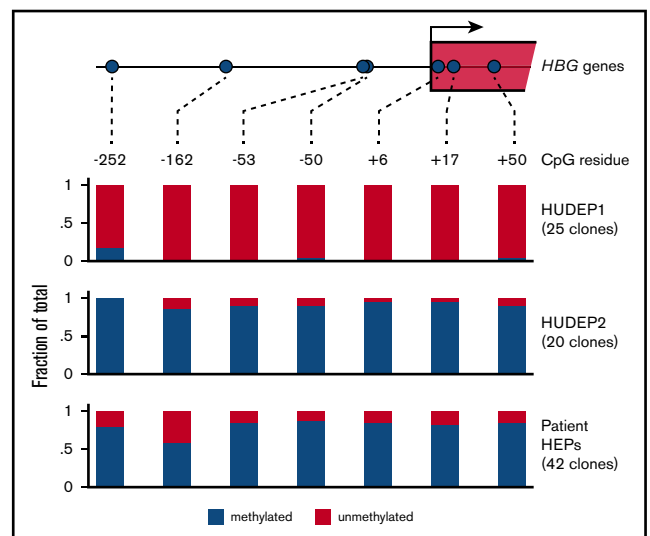

Figure 4. DNA methylation of the *HBG1/2* promoters. Genomic DNA was isolated from HUDEP1 and HUDEP2 cells and patient-derived HEPs. DNA methylation of the *HBG1/2* promoters was analyzed by bisulfite sequencing. The number of clones sequenced for each bisulfite-converted DNA sample is indicated.
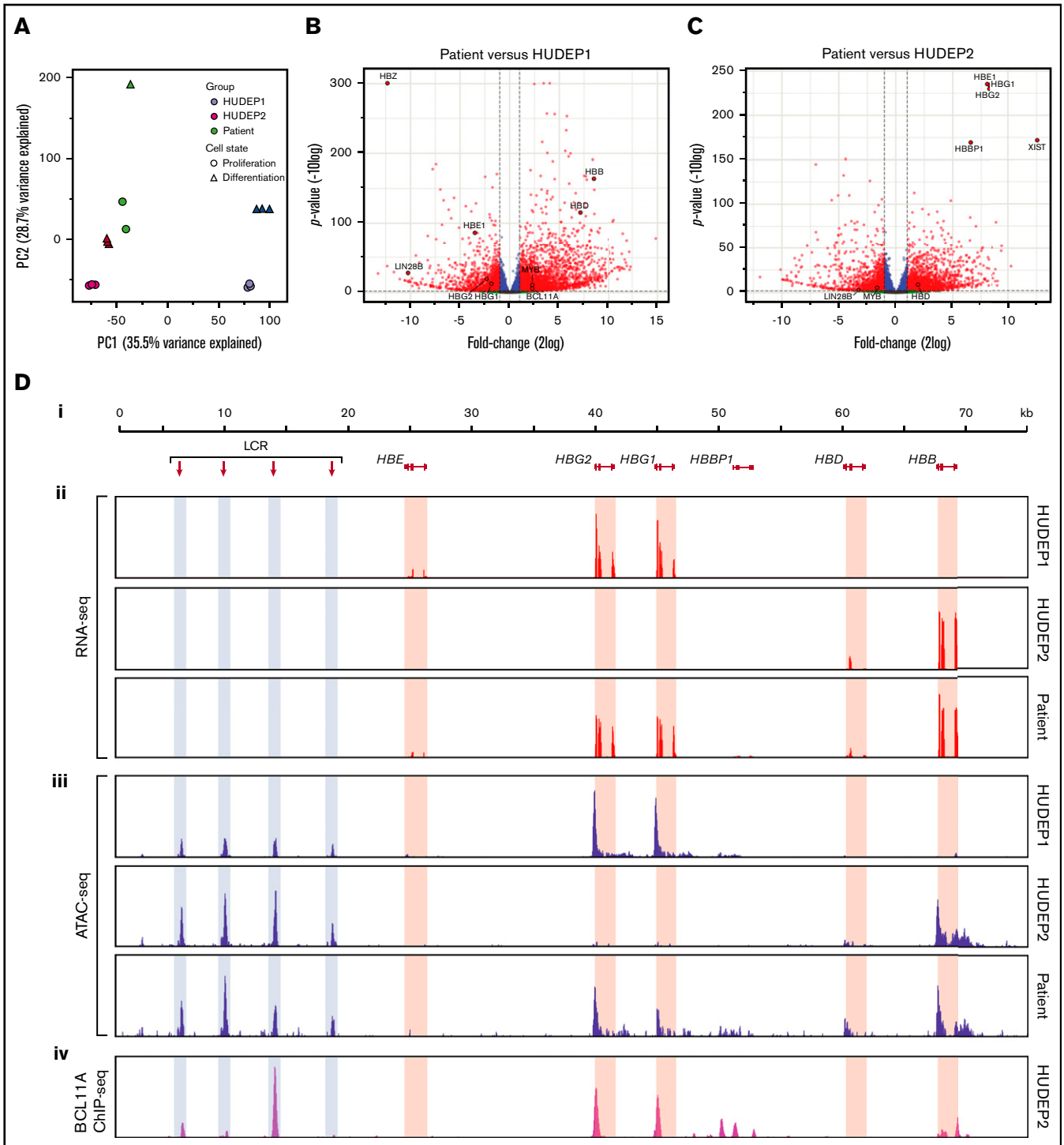
Figure 5. RNA expression and chromatin accessibility at the *HBB* locus. (A) Principal component analysis of RNA-seq data. PC1 separates fetal cells (HUDEP1) from adult cells (HUDEP2 and patient). PC2 separates cells according to differentiation status. (B) Volcano plot of HUDEP1 cells vs patient RNA-seq data. (C) Volcano plot of HUDEP2 cells vs RNA-seq data. (D) An overview of the *HBB* locus (Di) with RNA expression (RNA-seq; red) (Dii) and chromatin accessibility (ATAC-seq; blue) (Diii) assays on HUDEP1 and HUDEP2 cells and patient HEPs. (Div) Binding of BCL11A to the *HBB* locus in HUDEP2 cells, as assessed by chromatin immunoprecipitation followed by NGS (ChIP-seq); data are from Martyn et al.[64] *HBB*, β-globin gene; *HBBP1*, β-globin pseudogene 1; *HBD*, δ-globin gene; *HBE*, ε-globin gene; *HBG1*, Aγ-globin gene; *HBG2*, Gγ-globin gene; LCR, a series of enhancers marked by hypersensitive sites.

supplemental Figure 4; supplemental Table 2). Considering that 1849 DEGs were shared between HUDEP1 and HUDEP2 cells (supplemental Figure 4), we could narrow this down to 1963 DEGs in patient cells vs HUDEP2 cells and 3081 DEGs in patient cells vs HUDEP1 cells. We used volcano plots of all expressed genes (15 488; supplemental Table 2) to highlight the differences in number and magnitude of DEGs in the patient cells vs HUDEP1 cells (Figure 5B) or HUDEP2 cells (Figure 5C). These plots also illustrate the differential expression of several globin genes and key regulators that were discussed earlier (Figures 2 and 3; supplemental Figures 2 and 3; supplemental Table 2). Compared with HUDEP2 cells, we observed high expression of XIST in the cells from our patient (Figure 5C). This is consistent with the fact that HUDEP2 cells were derived from a male donor, whereas HUDEP1 cells were derived from a female donor (R.K. and Y.N., unpublished data, 30 October 2020).

RNA-seq analysis of the HBB locus

To visualize expression of the β-like globin genes in HUDEP1 cells, HUDEP2 cells, and the patient's cells, RNA-seq data were displayed on the HBB locus (Figure 5D). From these tracks, we inferred the expression ratios of the HBG1 (encoding Aγ-globin) and HBG2 (encoding Gγ-globin) genes. In Aγ-globin mRNA, codon 136 reads G**C**A encoding alanine, whereas in Gγ-globin mRNA it reads G**G**A encoding glycine. During hemoglobin switching there is a gradual increase in the Aγ-globin/Gγ-globin mRNA ratio. In HUDEP1 cell cultures this ratio was 0.24/0.76. HUDEP2 cells express too little γ-globin mRNA to determine the Aγ/Gγ ratio reliably. Therefore, we used RNA-seq data obtained from control adult HEP cultures (Steven Heshusius, Laura Grech, N.G., R.W.W.B., Xander T. den Dekker, W.F.J.v.IJ., Benjamin Nota, Alex E. Felice, T.B.v.d., Marieke von Lindern, Joseph Borg, Emile van den Akker, and S.P., manuscript submitted March 2021). We found that these cells displayed an Aγ/Gγ ratio of 0.34:0.66, whereas this ratio was 0.30:0.70 in the patient's cells. Thus, our data indicate that, in addition to increasing γ-globin expression, BCL11A haploinsufficiency yields an Aγ-globin/Gγ-globin mRNA ratio close to the one that is characteristic of the adult stage of human erythropoiesis. Of note, expression of the HBBP1 pseudogene was much higher in patient-derived HEPs than in HUDEP1 and HUDEP2 cells (supplemental Figures 2 and 3B; supplemental Table 2). We also observed an increase in HBBP1 expression in BCL11A-null HUDEP2 cells (supplemental Figure 3D). HBBP1 has been implicated in HbF expression,[59-61] chromatin architecture of the HBB locus,[62] and erythropoiesis.[63]

Chromatin accessibility of the HBB locus

First, we used our ATAC-seq data to assess chromatin accessibility at the HBB locus in HUDEP1 and HUDEP2 cells. In HUDEP1 cells, the distal regulatory elements of the locus control region (LCR) and the HBG1/2 promoters displayed strong ATAC peaks, whereas the promoter of the HBB gene was inaccessible (Figure 5Diii; HUDEP1 cells). In HUDEP2 cells, strong ATAC peaks were observed at the LCR and the HBB gene promoter but not at the HBG1/2 promoters (Figure 5Diii; HUDEP2 cells). This is consistent with the notion that BCL11A binds to the promoters of the HBG1/2 genes in adult erythroid cells[64] (Figure 5Div), thus precluding promoter activation and rendering the local chromatin in a closed and inaccessible structure.[55,64] Remarkably, in patient-derived HEPs we observed, in addition to the LCR peaks, strong ATAC peaks at the promoters of

the HBG1/2 genes and at the promoter of the HBB gene. We conclude that reduced levels of BCL11A (Figure 3A) profoundly affect chromatin accessibility at the HBG1/2 promoters, even in the presence of apparently normal levels of 2 other key regulators of hemoglobin switching,[39,65] LRF and KLF1 (Figure 3B-C), and high DNA methylation levels (Figure 4).

Discussion

We analyzed the erythroid phenotype of a patient with BCL11A haploinsufficiency. This patient carries a de novo variant, c.1453G>T, resulting in an inactivating truncation of the BCL11A-XL protein (p.Glu485X). Previous work identified BCL11A as a direct repressor of the HBG1/2 genes in adult erythropoiesis.[55,64] In mice, the complete absence of BCL11A results in neonatal lethality, accompanied by hematopoietic and neuronal abnormalities.[66,67] In the hematopoietic system, the stem cell and lymphoid compartments are particularly affected.[68] In the neuronal system, BCL11A is required for neuronal differentiation and the formation of sensory circuits.[66] Using conditional knockout alleles in combination with hematopoietic-specific Cre recombinase lines, such as Mx1-Cre and EpoR-Cre, BCL11A was established as a dose-dependent repressor of the embryonic and fetal hemoglobin genes.[39,51,69,70] In the patient, we found that all blood parameters were within normal range, with the exception of high HbF levels (12.1-18.7% of total hemoglobin). RNA-seq and RT-qPCR revealed high expression of the HBG1/2 genes in cultured primary erythroid progenitors derived from peripheral blood of the patient. This was paralleled by the results of chromatin accessibility mapping by ATAC-seq, which established that promoters of the HBG1/2 genes displayed an open chromatin conformation. Because expression of KLF1 and LRF, 2 other key regulators of hemoglobin switching,[39,65] was not affected at the RNA or protein level, our data indicate that activity of the HBG1/2 promoters in BCL11A haploinsufficiency is maintained via a direct mechanism involving reduced levels of BCL11A protein. This is consistent with the western blot analysis of BCL11A expression in the patient-derived HEP cells. The high level of HBBP1 expression in patient-derived HEPs and BCL11A-null HUDEP2 cells is an intriguing observation. HBBP1, a β-like globin pseudogene, is expressed in human erythroid cells as a long noncoding RNA.[63] Single-nucleotide polymorphisms in HBBP1 have been associated with HbF levels[59-61]; deletion of HBBP1 increases HBG1/2 expression and affects the 3-dimensional chromatin architecture of the HBB locus.[62] Because BCL11A binds to the HBBP1 region,[54,64] our data support the notion that BCL11A is directly involved in the function of the HBBP1 region.[62] In addition, the HBBP1 transcript may stabilize TAL1 mRNA, boosting expression of this essential transcription factor in erythroid cells.[63] Whether HBBP1 expression has a role in the erythroid phenotype of BCL11A deficiency is an interesting topic for follow-up research.

We used the HUDEP1 and HUDEP2 erythroid progenitor cell lines as benchmarks for human fetal and adult erythropoiesis, respectively.[47] Our comparative analysis strongly supports the notion that BCL11A haploinsufficiency does not involve maintenance of the fetal phenotype of the erythroid compartment. Notably, the LIN28B gene, which is highly expressed in human fetal erythropoiesis,[42,43] is not expressed in HUDEP2 cells and patient-derived HEPs. Furthermore, the methylation pattern of the HBG1/2 promoters closely resembles the hypermethylated state found in

adult erythroid cells, rather than the hypomethylated state typical of fetal erythroid cells.[50] Principal component analysis of the RNA-seq data also places the patient-derived HEPs more closely to adult erythroid progenitors than to fetal erythroid progenitors. The erythroid phenotype of *BCL11A* haploinsufficiency is different from that observed for *KLF1* haploinsufficiency, which is also associated with HPFH.[46,65] KLF1 is an activator of the *BCL11A* and *ZBTB7A* genes; therefore, its effects on *HBG1/2* expression are indirect.[56,65] Moreover, in contrast to BCL11A, KLF1 orchestrates the entire terminal erythroid differentiation program; consequently, *KLF1* haploinsufficiency has a much broader impact on the erythroid transcriptome.[46] For instance, *KLF1* haploinsufficiency is associated with the In(Lu) blood type[46] that is characterized by reduced expression of BCAM and CD44, which is not the case in our patient.

Because BCL11A deficiency does not impede steady-state or stress erythropoiesis, it is a very attractive molecular target for reactivation of γ-globin expression in β-hemoglobinopathy patients. Given the essential functions of BCL11A in other hematopoietic lineages and the neuronal system, erythroid-specific targeting of the *BCL11A* gene has been proposed as a strategy for reactivation of γ-globin expression.[54,71-75] Deletion of the erythroid-specific enhancer located in intron 2 of the gene in hematopoietic stem cells, using genome editing tools such as zinc finger nucleases (NCT03432364, NCT03653247) and CRISPR/Cas9 (NCT03655678, NCT03745287),[76] is being explored in clinical trials. In addition, erythroid-specific knockdown of BCL11A mRNA is being tested using a lentiviral gene transfer approach (NCT03282656).[77] In this context, it is relevant to study the impact of constitutive *BCL11A* haploinsufficiency, as occurs in patients with Dias-Logan syndrome, on human erythropoiesis. Reassuringly, our data show that constitutive *BCL11A* haploinsufficiency is associated with maintenance of high HbF expression without generally affecting the adult characteristics of erythropoiesis. Collectively, our results extend the observations from previous studies on cellular and mouse models of BCL11A deficiency and fully support the approach to reduce BCL11A activity in erythroid cells as a means to reactivate HbF expression in β-hemoglobinopathy patients.

Authorship

Contribution: M.W.W., D.E.B., M.C.G.N.v.d.H., W.F.J.v.IJ., and S.P. designed research; M.W.W., T.B.v.D., N.G., K.L.J.S., K.v.L., D.S.V., S.C., S.A.d.M., R.P., Z.A., R.W.W.B., and M.C.G.N.v.d.H. performed research; M.W.W., M.H.C., D.S.V., S.C., R.K., Y.N., R.W.W.B., D.E.B., and M.C.G.N.v.d.H. contributed vital new reagents, patient data, or analytical tools; M.W.W., M.H.C., T.B.v.D., K.v.L., D.S.V., R.P., D.E.B., M.C.G.N.v.d.H., W.F.J.v.IJ., and S.P. analyzed data; and M.W.W., M.H.C., D.S.V., D.E.B., M.C.G.N.v.d.H., W.F.J.v.IJ., and S.P. wrote the manuscript.

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References

1. Dias C, Estruch SB, Graham SA, et al; DDD Study. BCL11A haploinsufficiency causes an intellectual disability syndrome and dysregulates transcription. *Am J Hum Genet.* 2016;99(2):253-274.

2. Balci TB, Sawyer SL, Davila J, Humphreys P, Dyment DA. Brain malformations in a patient with deletion 2p16.1: a refinement of the phenotype to BCL11A. *Eur J Med Genet.* 2015;58(6-7):351-354.

3. Basak A, Hancarova M, Ulirsch JC, et al. BCL11A deletions result in fetal hemoglobin persistence and neurodevelopmental alterations. *J Clin Invest.* 2015;125(6):2363-2368.

4. Funnell AP, Prontera P, Ottaviani V, et al. 2p15-p16.1 microdeletions encompassing and proximal to BCL11A are associated with elevated HbF in addition to neurologic impairment. *Blood.* 2015;126(1):89-93.

5. Hancarova M, Simandlova M, Drabova J, Mannik K, Kurg A, Sedlacek Z. A patient with de novo 0.45 Mb deletion of 2p16.1: the role of BCL11A, PAPOLG, REL, and FLJ16341 in the 2p15-p16.1 microdeletion syndrome. *Am J Med Genet A.* 2013;161A(4):865-870.

6. Peter B, Matsushita M, Oda K, Raskind W. De novo microdeletion of BCL11A is associated with severe speech sound disorder. *Am J Med Genet A.* 2014;164A(8):2091-2096.

7. Yoshida M, Nakashima M, Okanishi T, et al. Identification of novel BCL11A variants in patients with epileptic encephalopathy: expanding the phenotypic spectrum. *Clin Genet.* 2018;93(2):368-373.

8. Nakamura T, Yamazaki Y, Saiki Y, et al. Evi9 encodes a novel zinc finger protein that physically interacts with BCL6, a known human B-cell proto-oncogene product. *Mol Cell Biol*. 2000;20(9):3178-3186.

9. Satterwhite E, Sonoki T, Willis TG, et al. The BCL11 gene family: involvement of BCL11A in lymphoid malignancies. *Blood*. 2001;98(12):3413-3420.

10. Fukuhara N, Tagawa H, Kameoka Y, et al. Characterization of target genes at the 2p15-16 amplicon in diffuse large B-cell lymphoma. *Cancer Sci*. 2006;97(6):499-504.

11. Boelens MC, Kok K, van der Vlies P, et al. Genomic aberrations in squamous cell lung carcinoma related to lymph node or distant metastasis. *Lung Cancer*. 2009;66(3):372-378.

12. Yin B, Delwel R, Valk PJ, et al. A retroviral mutagenesis screen reveals strong cooperation between Bcl11a overexpression and loss of the Nf1 tumor suppressor gene. *Blood*. 2009;113(5):1075-1085.

13. Jiang BY, Zhang XC, Su J, et al. BCL11A overexpression predicts survival and relapse in non-small cell lung cancer and is modulated by microRNA-30a and gene amplification. *Mol Cancer*. 2013;12(1):61.

14. Kasaian K, Wiseman SM, Walker BA, et al. The genomic and transcriptomic landscape of anaplastic thyroid cancer: implications for therapy. *BMC Cancer*. 2015;15(1):984.

15. Khaled WT, Choon Lee S, Stingl J, et al. BCL11A is a triple-negative breast cancer gene with critical functions in stem and progenitor cells. *Nat Commun*. 2015;6(1):5987.

16. Kong X, Zhang X, Xing X, Zhang B, Hong J, Yang W. The association of type 2 diabetes loci identified in genome-wide association studies with metabolic syndrome and its components in a Chinese population with type 2 diabetes. *PLoS One*. 2015;10(11):e0143607.

17. Er TK, Su YF, Wu CC, et al. Targeted next-generation sequencing for molecular diagnosis of endometriosis-associated ovarian cancer. *J Mol Med (Berl)*. 2016;94(7):835-847.

18. Tao H, Ma X, Su G, et al. BCL11A expression in acute myeloid leukemia. *Leuk Res*. 2016;41:71-75.

19. Yin J, Zhang F, Tao H, et al. BCL11A expression in acute phase chronic myeloid leukemia [published correction appears in *Leuk Res*. 2017;52:67]. *Leuk Res*. 2016;47:88-92.

20. Zhang X, Wang L, Wang Y, et al. Inhibition of FOXQ1 induces apoptosis and suppresses proliferation in prostate cancer cells by controlling BCL11A/MDM2 expression. *Oncol Rep*. 2016;36(4):2349-2356.

21. Santuario-Facio SK, Cardona-Huerta S, Perez-Paramo YX, et al. A new gene expression signature for triple negative breast cancer using frozen fresh tissue before neoadjuvant chemotherapy. *Mol Med*. 2017;23(1):101-111.

22. Tian Q, Deng WJ, Li ZW. Identification of a novel crizotinib-sensitive BCL11A-ALK gene fusion in a nonsmall cell lung cancer patient. *Eur Respir J*. 2017;49(4):1602149.

23. Staiger H, Machicao F, Kantartzis K, et al. Novel meta-analysis-derived type 2 diabetes risk loci do not determine prediabetic phenotypes. *PLoS One*. 2008;3(8):e3019.

24. Simonis-Bik AM, Nijpels G, van Haeften TW, et al. Gene variants in the novel type 2 diabetes loci CDC123/CAMK1D, THADA, ADAMTS9, BCL11A, and MTNR1B affect different aspects of pancreatic beta-cell function. *Diabetes*. 2010;59(1):293-301.

25. Klimentidis YC, Divers J, Casazza K, Beasley TM, Allison DB, Fernandez JR. Ancestry-informative markers on chromosomes 2, 8 and 15 are associated with insulin-related traits in a racially diverse sample of children. *Hum Genomics*. 2011;5(2):79-89.

26. Cauchi S, Ezzidi I, El Achhab Y, et al. European genetic variants associated with type 2 diabetes in North African Arabs. *Diabetes Metab*. 2012;38(4):316-323.

27. Langberg KA, Ma L, Sharma NK, et al; American Diabetes Association GENNID Study Group. Single nucleotide polymorphisms in JAZF1 and BCL11A gene are nominally associated with type 2 diabetes in African-American families from the GENNID study. *J Hum Genet*. 2012;57(1):57-61.

28. Jonsson A, Ladenvall C, Ahluwalia TS, et al. Effects of common genetic variants associated with type 2 diabetes and glycemic traits on α- and β-cell function and insulin action in humans. *Diabetes*. 2013;62(8):2978-2983.

29. Tang L, Wang L, Ye H, et al. *BCL11A* gene DNA methylation contributes to the risk of type 2 diabetes in males. *Exp Ther Med*. 2014;8(2):459-463.

30. Menzel S, Garner C, Gut I, et al. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. *Nat Genet*. 2007;39(10):1197-1199.

31. Sankaran VG, Menne TF, Xu J, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science*. 2008;322(5909):1839-1842.

32. Sedgewick AE, Timofeev N, Sebastiani P, et al. BCL11A is a major HbF quantitative trait locus in three different populations with beta-hemoglobinopathies. *Blood Cells Mol Dis*. 2008;41(3):255-258.

33. Uda M, Galanello R, Sanna S, et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. *Proc Natl Acad Sci USA*. 2008;105(5):1620-1625.

34. Borg J, Patrinos GP, Felice AE, Philipsen S. Erythroid phenotypes associated with KLF1 mutations. *Haematologica*. 2011;96(5):635-638.

35. Amaya M, Desai M, Gnanapragasam MN, et al. Mi2β-mediated silencing of the fetal γ-globin gene in adult erythroid cells. *Blood*. 2013;121(17):3493-3501.

36. Gnanapragasam MN, Scarsdale JN, Amaya ML, et al. p66Alpha-MBD2 coiled-coil interaction and recruitment of Mi-2 are critical for globin gene silencing by the MBD2-NuRD complex. *Proc Natl Acad Sci USA*. 2011;108(18):7487-7492.

37. Shi L, Cui S, Engel JD, Tanabe O. Lysine-specific demethylase 1 is a therapeutic target for fetal hemoglobin induction. *Nat Med*. 2013;19(3):291-294.

38. Yu L, Jearawiriyapaisarn N, Lee MP, et al. BAP1 regulation of the key adaptor protein NCoR1 is critical for γ-globin gene repression. *Genes Dev*. 2018; 32(23-24):1537-1549.

39. Masuda T, Wang X, Maeda M, et al. Transcription factors LRF and BCL11A independently repress expression of fetal hemoglobin. *Science*. 2016; 351(6270):285-289.

40. Huang P, Peslak SA, Lan X, et al. The HRI-regulated transcription factor ATF4 activates BCL11A transcription to silence fetal hemoglobin expression. *Blood*. 2020;135(24):2121-2132.

41. Grevet JD, Lan X, Hamagami N, et al. Domain-focused CRISPR screen identifies HRI as a fetal hemoglobin regulator in human erythroid cells. *Science*. 2018;361(6399):285-290.

42. Basak A, Munschauer M, Lareau CA, et al. Control of human hemoglobin switching by LIN28B-mediated regulation of BCL11A translation. *Nat Genet*. 2020;52(2):138-145.

43. Lee YT, de Vasconcellos JF, Yuan J, et al. LIN28B-mediated expression of fetal hemoglobin and production of fetal-like erythrocytes from adult human erythroblasts ex vivo. *Blood*. 2013;122(6):1034-1041.

44. Thein SL. Molecular basis of beta thalassemia and potential therapeutic targets. *Blood Cells Mol Dis*. 2018;70:54-65.

45. Liu D, Zhang X, Yu L, et al. KLF1 mutations are relatively more common in a thalassemia endemic region and ameliorate the severity of β-thalassemia. *Blood*. 2014;124(5):803-811.

46. Perkins A, Xu X, Higgs DR, et al; KLF1 Consensus Workgroup. Krüppeling erythropoiesis: an unexpected broad spectrum of human red blood cell disorders due to KLF1 variants. *Blood*. 2016;127(15):1856-1862.

47. Kurita R, Suda N, Sudo K, et al. Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. *PLoS One*. 2013;8(3):e59890.

48. Ley TJ, DeSimone J, Anagnou NP, et al. 5-azacytidine selectively increases gamma-globin synthesis in a patient with beta+ thalassemia. *N Engl J Med*. 1982;307(24):1469-1475.

49. Ley TJ, DeSimone J, Noguchi CT, et al. 5-Azacytidine increases gamma-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. *Blood*. 1983;62(2):370-380.

50. van der Ploeg LH, Flavell RA. DNA methylation in the human gamma delta beta-globin locus in erythroid and nonerythroid tissues. *Cell*. 1980;19(4):947-958.

51. Sankaran VG, Xu J, Ragoczy T, et al. Developmental and species-divergent globin switching are driven by BCL11A. *Nature*. 2009;460(7259):1093-1097.

52. van den Akker E, Satchwell TJ, Pellegrin S, Daniels G, Toye AM. The majority of the in vitro erythroid expansion potential resides in CD34(−) cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. *Haematologica*. 2010; 95(9):1594-1598.

53. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods*. 2013;10(12):1213-1218.

54. Canver MC, Smith EC, Sher F, et al. BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. *Nature*. 2015;527(7577):192-197.

55. Liu N, Hargreaves VV, Zhu Q, et al. Direct promoter repression by BCL11A controls the fetal to adult hemoglobin switch. *Cell*. 2018;173(2):430-442.e17.

56. Norton LJ, Funnell APW, Burdach J, et al. KLF1 directly activates expression of the novel fetal globin repressor *ZBTB7A/LRF* in erythroid cells. *Blood Adv*. 2017;1(11):685-692.

57. Molokie R, Lavelle D, Gowhari M, et al. Oral tetrahydrouridine and decitabine for non-cytotoxic epigenetic gene regulation in sickle cell disease: a randomized phase 1 study. *PLoS Med*. 2017;14(9):e1002382.

58. Mabaera R, Greene MR, Richardson CA, Conine SJ, Kozul CD, Lowrey CH. Neither DNA hypomethylation nor changes in the kinetics of erythroid differentiation explain 5-azacytidine's ability to induce human fetal hemoglobin. *Blood*. 2008;111(1):411-420.

59. Giannopoulou E, Bartsakoulia M, Tafrali C, et al. A single nucleotide polymorphism in the HBBP1 gene in the human β-globin locus is associated with a mild β-thalassemia disease phenotype. *Hemoglobin*. 2012;36(5):433-445.

60. Kerdpoo S, Limweeraprajak E, Tatu T. Effect of Swiss-type heterocellular HPFH from XmnI-Gγ and HBBP1 polymorphisms on HbF, HbE, MCV and MCH levels in Thai HbE carriers. *Int J Hematol*. 2014;99(3):338-344.

61. Roy P, Bhattacharya G, Mandal A, et al. Influence of BCL11A, HBS1L-MYB, HBBP1 single nucleotide polymorphisms and the HBG2 XmnI polymorphism on Hb F levels. *Hemoglobin*. 2012;36(6):592-599.

62. Huang P, Keller CA, Giardine B, et al. Comparative analysis of three-dimensional chromosomal architecture identifies a novel fetal hemoglobin regulatory element. *Genes Dev*. 2017;31(16):1704-1713.

63. Ma Y, Liu S, Gao J, et al. Genome-wide analysis of pseudogenes reveals HBBP1's human-specific essentiality in erythropoiesis and implication in β-thalassemia. *Dev Cell*. 2021;56(4):478-493.e11.

64. Martyn GE, Wienert B, Yang L, et al. Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. *Nat Genet*. 2018;50(4):498-503.

65. Borg J, Papadopoulos P, Georgitsi M, et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. *Nat Genet*. 2010;42(9):801-805.

blood advances

66. John A, Brylka H, Wiegreffe C, et al. Bcl11a is required for neuronal morphogenesis and sensory circuit formation in dorsal spinal cord development. *Development*. 2012;139(10):1831-1841.

67. Liu P, Keller JR, Ortiz M, et al. Bcl11a is essential for normal lymphoid development. *Nat Immunol*. 2003;4(6):525-532.

68. Luc S, Huang J, McEldoon JL, et al. Bcl11a deficiency leads to hematopoietic stem cell defects with an aging-like phenotype. *Cell Rep*. 2016;16(12): 3181-3194.

69. Esteghamat F, Gillemans N, Bilic I, et al. Erythropoiesis and globin switching in compound Klf1:Bcl11a mutant mice. *Blood*. 2013;121(13):2553-2562.

70. Xu J, Sankaran VG, Ni M, et al. Transcriptional silencing of {gamma}-globin by BCL11A involves long-range interactions and cooperation with SOX6. *Genes Dev*. 2010;24(8):783-798.

71. Bjurström CF, Mojadidi M, Phillips J, et al. Reactivating fetal hemoglobin expression in human adult erythroblasts through BCL11A knockdown using targeted endonucleases. *Mol Ther Nucleic Acids*. 2016;5:e351.

72. Brendel C, Guda S, Renella R, et al. Lineage-specific BCL11A knockdown circumvents toxicities and reverses sickle phenotype. *J Clin Invest*. 2016; 126(10):3868-3878.

73. Chang KH, Smith SE, Sullivan T, et al. Long-term engraftment and fetal globin induction upon *BCL11A* gene editing in bone-marrow-derived CD34$^+$ hematopoietic stem and progenitor cells. *Mol Ther Methods Clin Dev*. 2017;4:137-148.

74. Li J, Lai Y, Shi L. BCL11A down-regulation induces γ-globin in human β-thalassemia major erythroid cells. *Hemoglobin*. 2018;42(4):225-230.

75. Psatha N, Reik A, Phelps S, et al. Disruption of the BCL11A erythroid enhancer reactivates fetal hemoglobin in erythroid cells of patients with β-thalassemia major. *Mol Ther Methods Clin Dev*. 2018;10:313-326.

76. Frangoul H, Altshuler D, Cappellini MD, et al. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. *N Engl J Med*. 2021;384(3):252-260.

77. Esrick EB, Lehmann LE, Biffi A, et al. Post-transcriptional genetic silencing of *BCL11A* to treat sickle cell disease. *N Engl J Med*. 2021;384(3):205-215.