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Correction of Sickle Cell Disease in Adult Mice by Interference with Fetal Hemoglobin Silencing

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Persistence of human fetal hemoglobin (HbF, $\alpha_2\gamma_2$) in adults lessens the severity of sickle cell disease (SCD) and the β -thalassemias. Here, we show that the repressor BCL11A is required in vivo for silencing of γ -globin expression in adult animals, yet dispensable for red cell production. BCL11A serves as a barrier to HbF reactivation by known HbF inducing agents. In a proof-of-principle test of BCL11A as a potential therapeutic target, we demonstrate that inactivation of BCL11A in SCD transgenic mice corrects the hematologic and pathologic defects associated with SCD through high-level pancellular HbF induction. Thus, interference with HbF silencing by manipulation of a single target protein is sufficient to reverse SCD.

The switch from fetal (HbF, $\alpha_2\gamma_2$) to adult hemoglobin (HbA, $\alpha_2\beta_2$), a paradigm for transcriptional control in development, is

critical to the pathogenesis of sickle cell disease (SCD) and the β -thalassemias. As increased HbF lessens the severity of these conditions (1, 2), elu-

citation of mechanisms to relieve HbF silencing in adult erythroid cells has been a long-sought goal. Here, we demonstrate that inactivation of one component involved in HbF regulation, the transcription factor BCL11A, provides phenotypic correction of mice that model SCD. Our findings provide a crucial proof of principle for targeted reactivation of HbF.

SCD, the first “molecular disease,” is caused by substitution of valine for β -6 glutamic acid in the β -globin chain of adult hemoglobin (3). The

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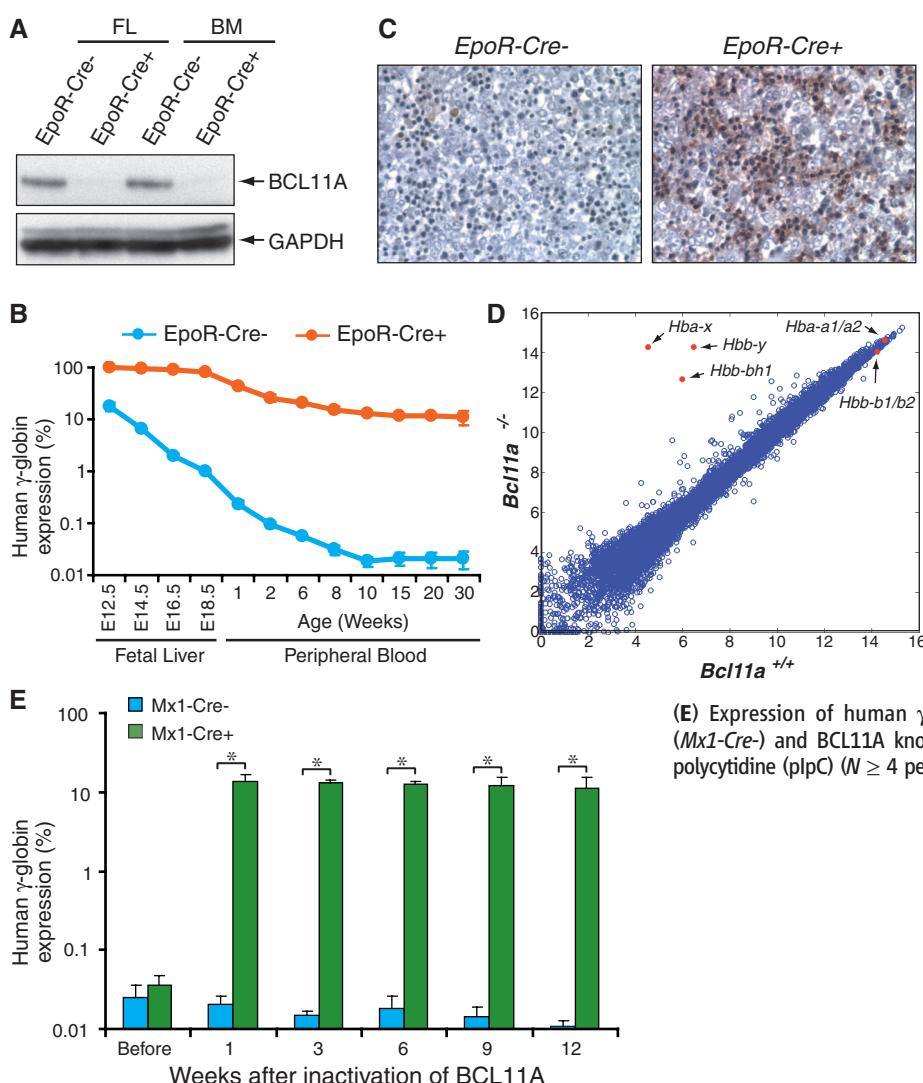


Fig. 1. BCL11A loss in adult mice reverses γ -silencing. (A) Expression of BCL11A protein in CD71⁺Ter119⁺ fetal liver (FL) and bone marrow (BM) cells of control (*EpoR-Cre⁻*) and BCL11A knockout (*EpoR-Cre⁺*) β -YAC mice. Glyceraldehyde phosphate dehydrogenase (GAPDH) was analyzed as a loading control. (B) Expression of human fetal (γ) globin genes was monitored by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in FL cells (E12.5 to E18.5) or peripheral blood of postnatal animals (1 to 30 weeks old). Data are plotted as percentage of γ -globin over total β -like human globin gene levels in control (*EpoR-Cre⁻*) and BCL11A knockout (*EpoR-Cre⁺*) β -YAC mice ($N \geq 4$ per genotype at each time point). Results are means \pm SD. All γ -globin levels for the different genotypes are significantly different ($P < 1 \times 10^{-5}$, two-tailed *t* test). (C) Immunohistochemistry for HbF was performed on E16.5 FLs from *EpoR-Cre⁻* and *EpoR-Cre⁺* animals. (D) Transcriptional profiling of control (*Bcl11a*^{+/+}) and *Bcl11a*^{-/-} (*EpoR-Cre⁺*) CD71⁺Ter119⁺ erythroid cells ($N = 3$ per genotype). Probes corresponding to mouse α - and β -globin genes are indicated by arrows. *Hba-x*, ζ -globin; *Hba-a1/a2*, α -globin; *Hbb-y*, ϵ -globin; *Hbb-bh1*, β h1-globin; *Hbb-b1/b2*, β -globin. (E) Expression of human γ -globin genes was monitored by qRT-PCR in control (*Mx1-Cre⁻*) and BCL11A knockout (*Mx1-Cre⁺*) mice before and after polyinosinic-polycytidine (pIpc) ($N \geq 4$ per genotype at each time point; $*P < 1 \times 10^{-5}$).

mutated, assembled hemoglobin, HbS ($\alpha_2\beta^S_2$), undergoes polymerization upon deoxygenation, resulting in erythrocyte deformation, hemolysis, and morbid complications secondary to microvascular occlusion. HbS polymerization is highly sensitive to inhibition by HbF (4).

The level of HbF varies among adult individuals and is inherited as a quantitative trait. Genome-wide association studies (GWAS) recently provided critical insight into loci controlling HbF. Three loci harboring genetic variants

with large effects were identified, including the β -globin cluster itself, a *HBSIL-MYB* gene interval, and the gene encoding BCL11A (5–7). Functional studies demonstrate that BCL11A serves as transcriptional repressor of HbF expression (8–10). BCL11A controls the developmental switch from embryonic to adult β -globin in the mouse and the silencing of HbF expressed from human β -globin locus transgenes in mouse fetal liver. Moreover, BCL11A contributes to HbF silencing in cultured primary human erythroid cells

(8, 11). While providing compelling support for BCL11A as a regulator of globin switching and HbF silencing in development, these findings do not address its *in vivo* role at the adult stage and potential as a therapeutic target for reactivation of HbF. Besides the GWAS-identified regions, numerous nuclear factors have been implicated in globin switching and/or HbF silencing (12). Apart from their proposed roles in globin regulation, many of these are essential for proper maturation of erythroid cells, thereby complicat-

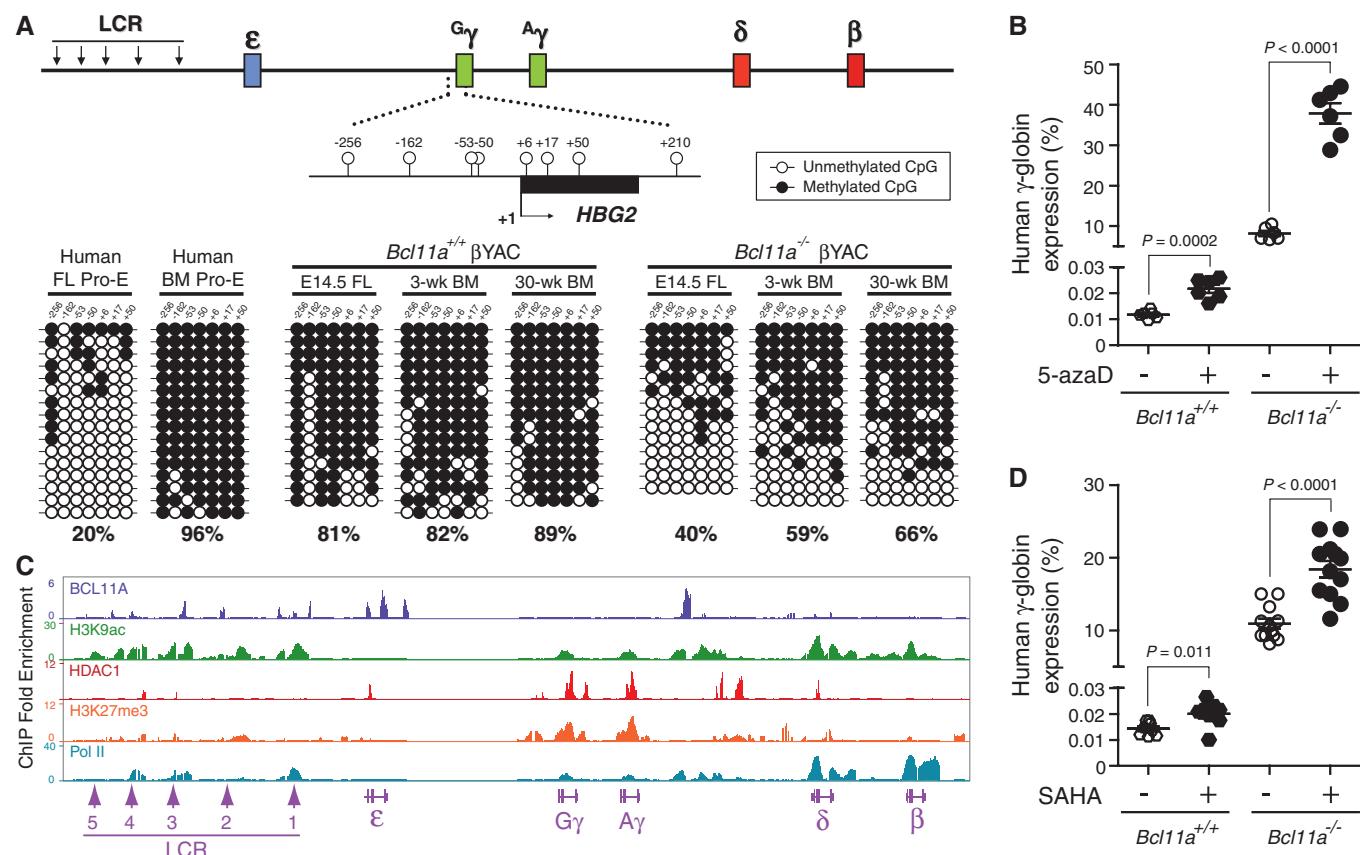


Fig. 2. DNA demethylation and HDAC inhibition enhance residual γ -globin expression. (A) CpG methylation within the $G\gamma$ promoter was measured by bisulfite sequencing analysis in $CD71^+$ $Ter119^+$ erythroid progenitors in control ($Bcl11a^{+/+}$) and $Bcl11a^{-/-}$ (*EpoR-Cre*) β -YAC mice at various development stages. Human primary FL and BM proerythroblasts (Pro-E) were analyzed as controls. The percentage of methylated CpG dinucleotides is shown for each sample. A diagram of the human β -globin cluster is shown on the top. (B) Expression of human γ -globin genes was monitored by qRT-PCR in control ($Bcl11a^{+/+}$, $N = 8$) and $Bcl11a^{-/-}$ (*EpoR-Cre*, $N = 12$) β -YAC mice before and after 5-azaD treatment ($N = 6$ per genotype per treatment). (C) In vivo chromatin occupancy of BCL11A, acetyl-H3 lysine 9 (H3K9ac), HDAC1, trimethyl-H3 lysine 27 (H3K27me3), and RNA Pol II was determined by ChIP-chip in primary human erythroid progenitors. A genome browser representation of binding patterns at the human β -globin cluster is shown. (D) Expression of human γ -globin genes was monitored by qRT-PCR in control ($Bcl11a^{+/+}$, $N = 8$) and $Bcl11a^{-/-}$ (*EpoR-Cre*, $N = 12$) β -YAC mice before and after SAHA treatment.

Table 1. Correction of hematologic parameters and urine concentration defect in SCD mice by inactivation of BCL11A. All animals were analyzed at 8 to 10 weeks after birth. Data are means \pm SEM. P values were calculated by two-tailed t test between the SCD and SCD/ $Bcl11a^{-/-}$ animals; * $P < 0.002$, ** $P < 1 \times 10^{-5}$.

Mice	RBC ($\times 10^6/\mu\text{l}$)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	Retic (%)	RDW (%)	Urine concentration (mOsm)
Control	10.1 ± 0.2	13.1 ± 0.3	44.2 ± 1.0	44.1 ± 1.4	13.0 ± 0.4	29.7 ± 0.6	3.1 ± 0.6	19.0 ± 0.7	2440 ± 213
SCD	6.4 ± 0.5	7.8 ± 0.6	28.3 ± 1.9	44.8 ± 1.6	12.3 ± 0.6	27.5 ± 0.6	38.2 ± 3.9	26.9 ± 0.5	1037 ± 82
SCD/ $Bcl11a^{-/-}$	$9.8 \pm 0.4^*$	$13.6 \pm 0.7^{**}$	$46.2 \pm 1.4^{**}$	47.2 ± 0.9	13.8 ± 0.2	29.3 ± 0.8	$7.0 \pm 0.3^*$	$23.4 \pm 0.4^*$	$2133 \pm 333^*$

$(Bcl11a^{+/+})$ and $Bcl11a^{-/-}$ (*EpoR-Cre*) β -YAC mice before and after 5-azaD treatment ($N = 6$ per genotype per treatment). (C) In vivo chromatin occupancy of BCL11A, acetyl-H3 lysine 9 (H3K9ac), HDAC1, trimethyl-H3 lysine 27 (H3K27me3), and RNA Pol II was determined by ChIP-chip in primary human erythroid progenitors. A genome browser representation of binding patterns at the human β -globin cluster is shown. (D) Expression of human γ -globin genes was monitored by qRT-PCR in control ($Bcl11a^{+/+}$, $N = 8$) and $Bcl11a^{-/-}$ (*EpoR-Cre*, $N = 12$) β -YAC mice before and after SAHA treatment.

ing their consideration as targets for therapeutic manipulation.

To address in vivo roles of BCL11A, we employed stringent genetic tests in mice carrying the human β -globin gene cluster as a yeast artificial chromosome transgene (β -YAC mice). Knockout of BCL11A interrupts silencing of endogenous β -like embryonic genes and human γ -globin genes in mouse fetal liver (9). Because BCL11A-null mice are postnatally lethal, we examined the contribution of BCL11A to γ -silencing in adults through conditional inactivation of BCL11A with erythroid-selective EpoR-GFP Cre alleles (13) (Fig. 1A). Mice harboring erythroid-specific inactivation of BCL11A developed normally. Erythropoiesis in fetal liver and adult bone marrow appeared normal in the absence of BCL11A (fig. S1). As in the conventional knockout, hemoglobin switching failed to occur in fetal liver, such that γ constituted >80% of the β -like human globins (Fig. 1B and fig. S2). HbF was robustly expressed in definitive erythroid cells of E16.5 (embryonic day 16.5) fetal liver (Fig. 1C and fig. S3). After birth, the level of γ -globin declined progressively to a residual level of ~11% in 30-week-old adults (Fig. 1B and fig. S2). Mouse embryonic β -like globin genes (ey and bh1) were also up-regulated in BCL11A-null erythroid cells throughout development (fig. S4).

We further examined several experimental features (YAC copy number and time of Cre-mediated gene inactivation) that might contribute to the observed silencing and showed that they were noncontributory (see SOM text and fig. S5).

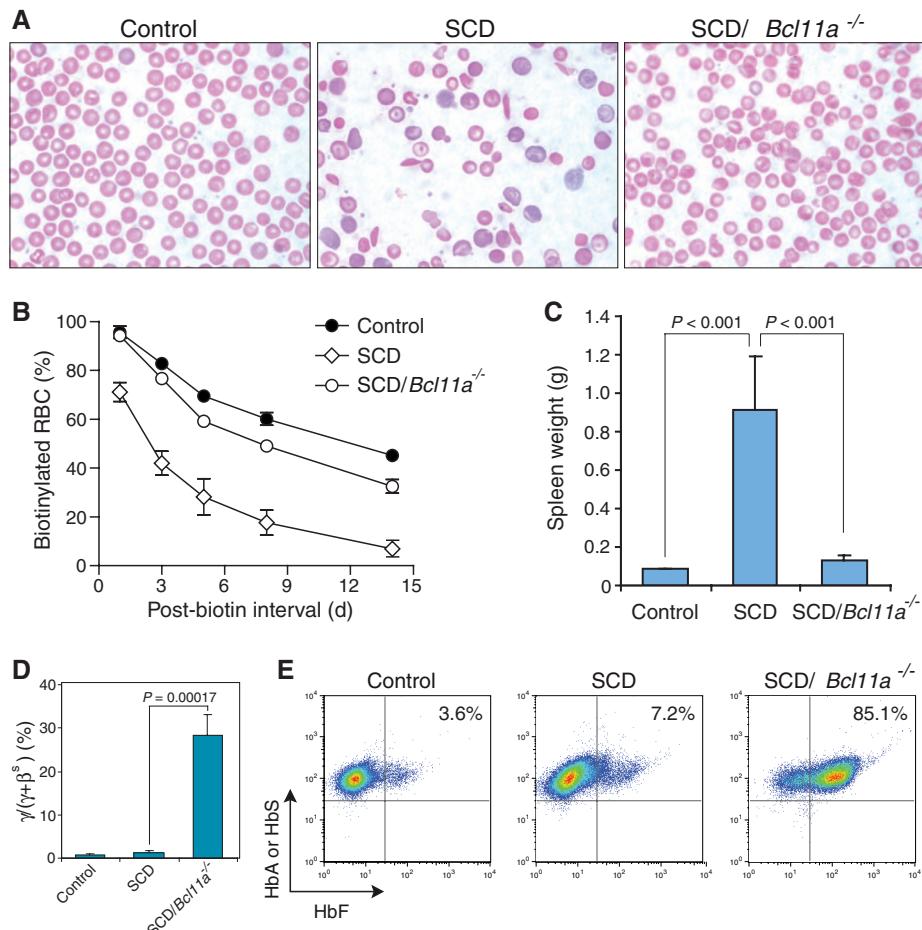
Transcriptional profiling of BCL11A-null erythroid cells purified from adult bone marrow was used to assess the quality of erythroid maturation. BCL11A-null and wild-type erythroid cells exhibited highly similar patterns of gene expression, characterized by a Pearson correlation coefficient (r^2) of 0.9736 for the log₂ normalized intensities (Fig. 1D). The expression of known erythroid transcriptional regulators, including GATA1, FOG1, NF-E2, KLF1, SOX6, and MYB, was comparable between the groups. The most differentially expressed genes were mouse embryonic β -like and α -like globin genes (Fig. 1D, fig. S6, and tables S2 and S3). Thus, BCL11A is highly selective in controlling targets in erythroid cells, and only expression of the globin genes is substantially affected in its absence.

These findings establish roles for BCL11A in HbF silencing but fail to demonstrate whether γ -globin genes that are fully silenced during normal development can be reactivated upon loss of BCL11A. Thus, we introduced the interferon-inducible Mx1-Cre allele into BCL11A floxed β -YAC mice (fig. S7). Efficient excision of floxed

BCL11A alleles in adult mice was not associated with significant changes in blood counts (fig. S8) except for a decline in total B cells, consistent with a role in lymphopoiesis (14). Developmentally silenced γ -globins were reexpressed to 13.8% of total β -like human globins 1 week after inactivation of BCL11A and sustained thereafter (Fig. 1E). As this level of γ -derepression closely approximates that in EpoR-Cre BCL11A conditional mice, the substantial component of HbF silencing dependent on BCL11A is reversible. Similarly, the mouse embryonic ey - and bh1 -globin genes were derepressed on BCL11A loss (fig. S9).

Partial silencing of γ -globin expression in BCL11A-null erythroid cells points to additional silencing pathways that act independently of BCL11A. Two epigenetic pathways, DNA methylation and histone deacetylation, have been implicated in HbF control (15–17). DNA methylation of the γ -globin promoters progressively increased in BCL11A-null erythroid cells in correlation with the gradual, but partial, silencing of γ -globin expression (Fig. 2A). Administration of the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-azaD) to normal β -YAC mice led to a very small increment in γ -globin mRNA (Fig. 2B). In contrast, 5-azaD treatment was synergistic to BCL11A loss, leading to 37.9% γ -globin mRNA.

Fig. 3. Inactivation of BCL11A rescues sickle cell defects in humanized SCD mice. **(A)** Representative blood smears of control, SCD, and $\text{SCD}/\text{Bcl11a}^{-/-}$ mice are shown at 1000x magnification. **(B)** RBC life span is significantly extended in $\text{SCD}/\text{Bcl11a}^{-/-}$ mice at every time point compared with SCD mice ($N \geq 4$; $P < 0.01$). Results are means \pm SEM. **(C)** Correction of splenomegaly in $\text{SCD}/\text{Bcl11a}^{-/-}$ mice ($N \geq 3$ per genotype). Results are means \pm SEM. **(D)** Expression of fetal (γ) and sickle adult (β^s) globin genes was monitored by qRT-PCR in the peripheral blood of control, SCD, and $\text{SCD}/\text{Bcl11a}^{-/-}$ animals (8 to 10 weeks old; $N = 5$, 6, and 4, respectively). Results are means \pm SEM. **(E)** Distribution of HbF in red cells. Representative graphs for control, SCD, and $\text{SCD}/\text{Bcl11a}^{-/-}$ animals are shown. The same scale is used in all three graphs, and the mean percentage of F cells (HbF/HbA double-positive) is shown ($N = 5$, 6, and 4, respectively).



By chromatin immunoprecipitation coupled to microarrays (ChIP-chip) analysis, we observe that histone deacetylase 1 (HDAC1) occupies the γ -globin promoters in primary adult human erythroid precursors (Fig. 2C). Notably, the binding peaks of HDAC1 overlap peaks of trimethyl-H3 Lys27, consistent with the very-low-level expression of γ -globin genes in adult erythroid cells. Administration of an HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), also synergized with BCL11A in derepression of γ -globin mRNA (Fig. 2D). The level of histone H3 acetylation was elevated in bone marrow cells and within γ -promoter regions after treatment (fig. S10), suggesting that the enhanced transcription of γ -globin genes is related to the increase in histone acetylation. These results show that loss of BCL11A markedly enhances the effects of DNA demethylation and HDAC inhibitors in reactivating HbF expression and suggest that BCL11A down-regulation might be combined with known HbF inducers for efficient HbF augmentation.

The above findings illustrate favorable features of BCL11A as a target for reactivation of HbF in the β -hemoglobinopathies. HbF silencing in the adult is strongly, although not exclusively, dependent on BCL11A. Red cell production is largely unaffected by its absence. Moreover, loss of BCL11A enhances effects of known HbF inducers. We next explored whether impairment of BCL11A is sufficient to ameliorate disease symptoms in a mouse model of SCD. Transgenic knockout mice expressing exclusively human sickle hemoglobin faithfully recapitulate the principal hematologic and histopathologic features of the human disease and are widely used to evaluate pharmacologic and genetic therapies (18–22). “Berkeley” SCD mice (19), which contain targeted deletions of murine α and β globins plus human fetal ($^G\gamma$, $^A\gamma$) and adult (δ , β^s) globin transgenes in a normal genomic configuration and express low levels of HbF in adults, have been employed in our primary analysis.

We introduced BCL11A-null alleles into SCD mice (SCD $Bcl11a^{fl/fl}$ EpoR-Cre $^+$, hereafter called SCD/ $Bcl11a^{-/-}$ mice). As in typical human patients, SCD mice exhibit severe hemolytic anemia, reticulocytosis, and shortened RBC survival (Fig. 3, A and B, and Table 1). Hematologic parameters, including RBC counts and hemoglobin (Hb) content, were corrected in SCD/ $Bcl11a^{-/-}$ mice (Table 1). Reticulocyte counts, red cell distribution width, and numbers of white blood cells (WBCs) and platelets were near control values (Table 1 and fig. S11). In stark contrast to SCD mice, sickled cells were absent in SCD/ $Bcl11a^{-/-}$ mice (Fig. 3A), and red cell survival was markedly improved (Fig. 3B). Accordingly, spleen size, a measure of compensatory erythropoiesis, was dramatically reduced (Fig. 3C). In humans and SCD mice, RBC sickling leads to reduced medullary blood flow and impairs urine-concentrating ability. Urine osmolality of SCD/ $Bcl11a^{-/-}$ mice was normal, heralding improved renal function (Table 1). Organ histopathology present in SCD

mice was also reversed in SCD/ $Bcl11a^{-/-}$ mice (fig. S12).

To relate these phenotypic findings to HbF reactivation, we determined the steady-state level of γ -globin expression and cellular distribution of HbF in SCD/ $Bcl11a^{-/-}$ mice. Expression of γ -globin genes was greatly elevated ($P=0.00017$) in adult SCD/ $Bcl11a^{-/-}$ mice compared with SCD mice (28.3% versus 1.3% of total β -like human globins) (Fig. 3D). The cellular distribution of HbF in red cells was assessed by staining for F cells using antibodies against HbF and HbA (which also recognizes HbS). Peripheral blood of control and SCD mice contained few F cells (3.6% and 7.2%, respectively) (Fig. 3E). In contrast, peripheral blood of SCD/ $Bcl11a^{-/-}$ mice exhibited strong panacellular staining of HbF, and F cells accounted for 85.1% of total RBCs ($P=3.9 \times 10^{-5}$) (Fig. 3E). The level of HbF expression achieved in the absence of BCL11A exceeds the estimated ~15 to 20% HbF thought to be necessary to virtually eliminate SCD phenotypes in patients (4). In addition, we introduced BCL11A-null alleles into an independent SCD mouse strain (22). Similar to the Berkeley SCD mice, inactivation of BCL11A corrected the sickle cell phenotypes due to HbF reactivation (fig. S13). Thus, our findings are not limited to a single SCD mouse model.

Described more than 100 years ago (23), SCD is the first genetic disorder for which a causative mutation was identified (3). Despite extensive study of sickle hemoglobin containing erythrocytes and globin expression, effective therapy has been elusive and empirical. The correction of the phenotypes of SCD mice with removal of BCL11A provides proof of principle and critical in vivo evidence on behalf of therapeutic targeting of BCL11A in patients with SCD. We anticipate that our findings should apply similarly to the β -thalassemias. Several formidable barriers need to be overcome before these findings are translated into new therapies. As a transcription factor, BCL11A is a challenging therapeutic target. Interfering with BCL11A expression and/or function can be pursued by several strategies. Depletion of BCL11A mRNA by systemic administration of RNA inhibitory molecules (24, 25) or by somatic gene transfer of short hairpin RNA (26) merits exploration. Substantial advances in the transplantation of gene-modified autologous hematopoietic stem cells (27) make the latter approach attractive, particularly in that reactivation of γ -globin expression is accompanied by reciprocal down-regulation of mutant β -globin on the same chromosome. Screening for small molecules (or peptides) that inhibit BCL11A function, either directly or through disruption of protein-protein interactions, represents another approach (28). Recent advances in chemical biology enhance the prospects for discovery of drugs to accomplish this goal (29). The correction of SCD in mice by genetic manipulation of a single component involved in globin gene regulation constitutes a requisite step in translating new insights

in HbF silencing into mechanism-based, improved therapy for the major hemoglobin disorders.

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Supporting Online Material

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Materials and Methods

SOM Text

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Tables S1 to S3

References (30–43)

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