Corepressor-dependent silencing of fetal hemoglobin expression by BCL11A

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Reactivation of fetal hemoglobin (HbF) in adults ameliorates the severity of the common β-globin disorders. The transcription factor BCL11A is a critical modulator of hemoglobin switching and HbF silencing, yet the molecular mechanism through which BCL11A coordinates the developmental switch is incompletely understood. Particularly, the identities of BCL11A cooperating protein complexes and their roles in HbF expression and erythroid development remain largely unknown. Here we determine the interacting partner proteins of BCL11A in erythroid cells by a proteomic screen. BCL11A is found within multiprotein complexes consisting of erythroid transcription factors, transcriptional corepressors, and chromatin-modifying enzymes. We show that the lysine-specific demethylase 1 and repressor element-1 silencing transcription factor corepressor 1 (LSD1/CoREST) histone demethylase complex interacts with BCL11A and is required for full developmental silencing of mouse embryonic β-like globin genes and human γ-globin genes in adult erythroid cells in vivo. In addition, LSD1 is essential for normal erythroid development. Furthermore, the DNA methyltransferase 1 (DNMT1) is identified as a BCL11A-associated protein in the proteomic screen. DNMT1 is required to maintain HbF silencing in primary human adult erythroid cells. DNMT1 haploinsufficiency combined with BCL11A deficiency further enhances γ-globin expression in adult animals. Our findings provide important insights into the mechanistic roles of BCL11A in HbF silencing and clues for therapeutic targeting of BCL11A in β-hemoglobinopathies.

gene regulation | globin switching | hematopoiesis

Fetal hemoglobin (HbF, $\alpha_2\gamma_2$) is a major genetic modifier of the phenotypic heterogeneity in patients with the major β-globin disorders sickle cell disease (SCD) and β-thalassemia (1). The synthesis of hemoglobin undergoes switching during ontogeny such that HbF is the predominant hemoglobin produced during fetal life and is gradually silenced and replaced by adult hemoglobin (HbA, $\alpha_2\beta_2$) around birth. Because increased γ-globin expression in adults can substitute for the mutant or absent β-globin in SCD and β-thalassemia, respectively, the fetal-to-adult globin switch is critical to the pathogenesis of these conditions. As a result, intense efforts have been aimed to elucidate the molecular mechanisms of HbF silencing and to develop target-based therapeutics to enhance HbF production (2).

Recent genomewide association studies (GWAS) led to the identification of a new HbF-associated locus on chromosome 2, located within the gene *BCL11A* (3). Subsequent studies demonstrated that BCL11A, a zinc-finger transcription factor, is a bona fide repressor of HbF expression (4–7). BCL11A protein is developmentally regulated and is required to maintain HbF silencing in human adult erythroid cells (4, 5). KO of BCL11A in mice carrying a human β-globin cluster transgene leads to profound delay in globin switching and impaired HbF silencing in adult erythroid cells (5, 8). Previously silenced γ-globin genes can also be reactivated by loss of BCL11A in adult animals (8). Most importantly, inactivation of BCL11A alone is sufficient to ameliorate the hematologic and pathologic defects associated with SCD through high-level HbF induction in humanized mouse models (8). These genetic studies provide persuasive evidence that BCL11A functions as a central modulator of HbF expression and globin switching in vivo. Further molecular studies revealed that BCL11A interacts with several erythroid regulators including GATA1, FOG1, and SOX6, and with the nucleosome remodeling and deacetylase complex (NuRD) (4, 6). BCL11A acts within the β-globin cluster by associating with several discrete regions, including sequences specifically deleted in patients with hereditary persistence of fetal hemoglobin (HPFH). However, BCL11A does not bind detectably to the γ-globin promoter in erythroid chromatin, suggesting that its mode of action is more complex than simply blocking transcription at the proximal promoter. This observation is consistent with a role of BCL11A in promoting long-range chromosomal interactions within the β-globin locus (6, 7, 9). Therefore, in an effort to elucidate further the precise molecular mechanisms by which BCL11A coordinates the switch, it is relevant to identify systematically BCL11A-interacting partner proteins, and use functional and genetic approaches to assess their individual roles in HbF regulation and erythroid cell maturation. In addition to providing important insights into the molecular mechanisms through which BCL11A controls hemoglobin switching, our data suggest opportunities, as well as challenges, for therapeutic induction of HbF in patients with the major hematologic disorders.

Results

BCL11A-Interacting Partner Proteins in Erythroid Cells. We characterized BCL11A-containing multiprotein complexes by a proteomic affinity screen using a metabolic biotin tagging approach (10). Murine erythroleukemia (MEL) cell lines that stably express the bacterial biotin ligase *BirA* and the recombinant BCL11A (XL isoform) containing a FLAG epitope tag together with a *BirA* recognition motif fused to its amino terminus were generated (FB-BCL11A; Fig. 1*A*). Clones were selected that express the recombinant BCL11A at levels similar to endogenous BCL11A (Fig. S1).

Single streptavidin affinity purification or tandem anti-FLAG immunoaffinity and streptavidin affinity purification was performed on nuclear extracts from MEL cells stably expressing BirA alone or BirA together with FB-BCL11A. Copurified proteins were fractionated in SDS/PAGE, followed by liquid chromatography and tandem MS peptide sequencing (Fig. 1*A*). We identified numerous peptides of BCL11A and nearly all previously established interacting proteins, including FOG1, the entire NuRD complex, and the nuclear matrix protein MATRIN-3 (4). Besides previously recognized partners, we also identified a panel of previously undiscovered interacting complexes, including the hematopoietic regulators RUNX1 and IKZF1 (or IKAROS), several transcriptional corepressor complexes, and the SWI/SNF chromatin remodeling complex (Fig. 1*B*). The BCL11A-interacting corepressors include

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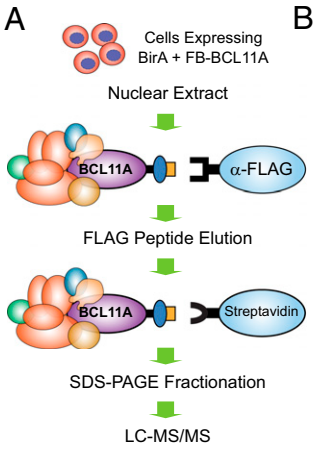

B

Protein Identity	# of Peptides MEL	# of Peptides K562
BCL11A	13[a], 11[a], 12[b]	17[a], 14[a], 11[a], 12[b]
Transcription factors		
FOG1 (ZFPM1)	1, 2, 0	0, 0, 0, 0
RUNX1	1, 4, 0	0, 0, 0, 1
IKAROS (IKZF1)	1, 3, 0	0, 0, 0, 0
NuRD complex		
Mi-2β (CHD4)	8, 8, 1	21, 10, 21, 10
Mi-2α (CHD3)	0, 0, 0	4, 4, 9, 2
MTA1	0, 0, 0	6, 5, 2, 1
MTA2	0, 0, 1	23, 16, 13, 2
MTA3	0, 0, 0	2, 2, 0, 0
HDAC1	7, 7, 5	19, 16, 8, 4
HDAC2	5, 4, 2	12, 15, 6, 2
RBBP4	3, 3, 1	4, 5, 5, 1
RBBP7	2, 1, 1	7, 6, 6, 1
MBD3	0, 0, 0	7, 6, 0, 0
P66α (GATAD2A)	0, 0, 0	29, 10, 4, 1
P66β (GATAD2B)	2, 1, 0	12, 12, 5, 2
LSD1/CoREST complex		
LSD1 (KDM1A)	15, 27, 8	1, 5, 12, 5
CoREST (RCOR1)	8, 9, 3	8, 8, 3, 1
NCoR/SMRT complex		
NCoR (NCOR1)	6, 14, 3	0, 0, 2, 0
SMRT (NCOR2)	13, 16, 2	0, 0, 8, 0
TBLR1	0, 2, 0	4, 0, 2, 0
TBL1	0, 0, 0	3, 0, 0, 0
CORO2A	2, 1, 0	0, 0, 0, 0
KAISO (ZBTB33)	7, 7, 6	26, 21, 18, 8
SIN3 complex		
SIN3A	5, 1, 1	6, 4, 4, 2
SIN3B	0, 0, 0	1, 2, 1, 0
Other corepressors		
BCOR	17, 31, 6	1, 0, 6, 2
TRIM28	11, 12, 5	2, 11, 13, 4
SWI/SNF complex		
SNF5 (SMARCB1)	0, 1, 0	2, 3, 3, 1
BRG1 (SMARCA4)	7, 9, 2	8, 1, 12, 2
BRM (SMARCA2)	0, 0, 0	7, 1, 11, 0
BAF57 (SMARCE1)	2, 1, 0	5, 2, 3, 0
BAF60A (SMARCD1)	0, 0, 0	10, 3, 3, 0
BAF60B (SMARCD2)	0, 0, 0	7, 3, 5, 0
BAF155 (SMARCC1)	10, 6, 0	16, 5, 10, 2
BAF170 (SMARCC2)	3, 4, 0	15, 3, 10, 0
SNF2H (SMARCA5)	2, 5, 0	12, 2, 12, 0
BAF180 (PB1)	0, 0, 0	5, 1, 15, 0
ASH2L	0, 0, 0	2, 1, 5, 0
Other nuclear factors		
DNMT1	14, 22, 3	9, 1, 3, 1
JMJD1A (KDM3A)	1, 1, 0	0, 0, 0, 0
JMJD1B (KDM3B)	0, 1, 0	0, 0, 0, 0
JARID1A (KDM5A)	0, 1, 0	0, 0, 0, 0
CARM1	1, 1, 0	0, 0, 0, 0
YLPM1	16, 16, 0	0, 2, 9, 0
MSH2	3, 6, 0	0, 2, 3, 0
Nuclear matrix		
MATRIN-3 (MATR3)	15, 8, 5	3, 6, 13, 5

[a]Single streptavidin affinity purification
[b]Tandem α-FLAG and streptavidin affinity purification

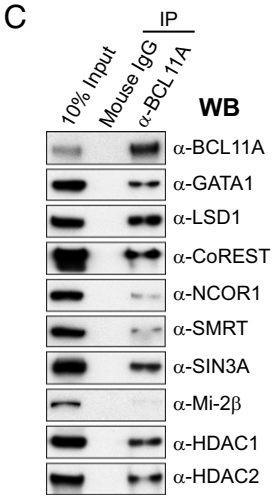

Fig. 1. Identification of BCL11A-interacting proteins in erythroid cells by a proteomic affinity screen. (*A*) Schematic diagram of experimental design. (*B*) List of identified proteins is shown with the number of peptides obtained in each trial. Official gene symbols of the identified proteins are shown in parentheses. (*C*) Confirmation of interactions between BCL11A and identified proteins by coimmunoprecipitation in primary human erythroid cells.

the histone demethylase complex lysine-specific demethylase 1 (LSD1)/CoREST, the nuclear receptor corepressors NCoR/SMRT, the SIN3 deacetylase complex, the BCL6 corepressor BCOR, the DNA methyltransferase 1 (DNMT1), and histone demethylases (Fig. 1*B*).

To identify BCL11A-interacting proteins in human erythroid cells, we performed a similar proteomic screen in human erythroleukemia (K562) cells. Remarkably, the identified partner proteins are largely similar to those found in MEL cells (Fig. 1*B*), suggesting that BCL11A acts within functionally conserved protein complexes. By coimmunoprecipitation (co-IP) analysis, we confirmed that both the epitope-tagged and the endogenous BCL11A associate with the identified partner proteins in erythroid cell lines and primary human adult erythroid cells, respectively (Fig. S1; Fig. 1*C*).

Functional RNAi Screen in Primary Human Erythroid Cells. To assess the functional roles of BCL11A-interacting proteins in HbF regulation, we performed a lentiviral RNAi screen in primary adult human CD34$^+$ hematopoietic stem/progenitor cells (HSPCs) undergoing ex vivo erythroid maturation (Fig. 2*A*). Primary CD34$^+$ HSPCs were transduced with lentiviruses containing shRNAs targeting BCL11A or its interacting proteins during an expansion phase, followed by erythroid differentiation for 5–7 d. Knockdown of each gene was confirmed by qRT-PCR, and shRNAs that resulted in >75% decrease of target mRNA expression were selected for subsequent analyses (Table S1; Figs. S2 and S3). KLF1 (or EKLF) is a critical erythroid regulator required for adult β-globin transcription (11). Recently it was shown that KLF1 occupies the BCL11A promoter and activates its expression (12, 13). Consistent with previous analysis, knockdown of BCL11A or KLF1 results in marked increase in γ-globin expression and serves as positive controls for the RNAi screen. We first ranked the genes according to the level of human γ-globin expression (Fig. 2*B*). Notably, the top of the list consists of subunits of several transcriptional corepressor complexes, including the NuRD complex (CHD4, HDAC1, HDAC2, and MBD2), DNMT1, SIN3A, NCOR1, and the polycomb repressive complex 2 (PRC2; EZH2, EED, and SUZ12). shRNA-mediated depletion of BCL11A-interacting proteins also results in derepression of the human embryonic ε-gene. We next ranked the genes according to the level of ε-globin expression (Fig. 2*C*). The highest ranked genes include subunits of the PRC2 complex (EED, EZH1, EZH2, and SUZ12), several histone demethylases (KDM7A, KDM3A, and KDM5D) and SIN3A. Importantly, depletion of KLF1, but not BCL11A, significantly increases ε-globin expression (Fig. 2*C*), indicating a distinct role of KLF1 in regulating human embryonic globin expression. These results demonstrate that BCL11A-interacting proteins are differentially required for silencing of human embryonic or fetal globin expression, suggesting that they may form distinct subcomplexes depending on the chromatin context.

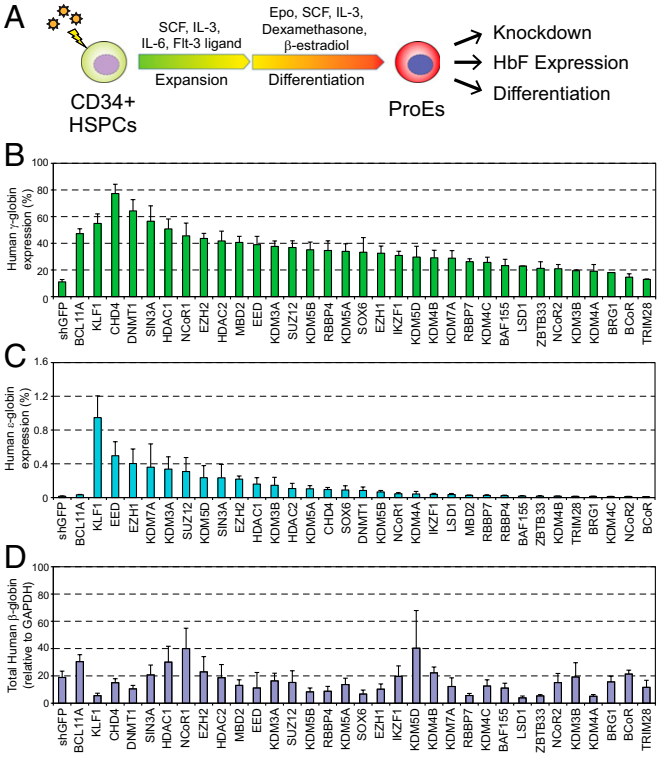

Fig. 2. Functional RNAi screen of BCL11A-interacting proteins in primary human erythroid cells. (*A*) Schematic diagram of experimental design. (*B*) Expression of human γ-globin mRNA was measured by qRT-PCR. Data are plotted as percentage of γ-globin over total β-like human globin gene levels (two to five shRNAs per gene). Genes are ranked based on the level of γ-globin expression (highest to lowest). (*C*) Genes are ranked based on the level of ε-globin expression (highest to lowest). (*D*) Expression of human total β-like globin mRNA normalized to GAPDH mRNA level. Results are the means ± SD of at least three independent experiments.

Besides the reactivation of HbF expression, depletion of several BCL11A-interacting proteins impairs erythroid cell maturation ex vivo, as revealed by reduced expression of erythroid cell surface markers CD71 and CD235a (Fig. S3). Perturbation of total globin synthesis is a secondary consequence of defective red cell differentiation. Notably, depletion of BCL11A-interacting proteins results in variable effects on the synthesis of total human β-like globin mRNAs (Fig. 2*D*). Although knockdown of many genes has little effect, knockdown of several genes leads to a greater than twofold increase (NCOR1 and KDM5D) or decrease (KLF1, KDM5B, SOX6, RBBP7, LSD1/KDM1A, ZBTB33, and KDM4A) in expression of total β-like globin mRNAs (Fig. 2*D*; Fig. S3), suggesting that their gene products influence processes required for normal erythroid development.

Chromatin Occupancy of BCL11A-Interacting Proteins. The identification of BCL11A-interacting proteins and subsequent functional RNAi screen led to prioritization of candidate HbF regulators for detailed mechanistic studies. If BCL11A and its interacting partners regulate HbF expression in a functional protein complex, ablation of one critical component of the complex may lead to destabilization of the larger complex and/or its chromatin occupancy. We next determined whether the chromatin occupancy of BCL11A-interacting proteins is dependent on the presence of BCL11A. ChIP was performed in primary human adult erythroid cells transduced with control or BCL11A lentiviral shRNA. In control cells, ChIP analysis revealed that BCL11A occupies several discrete regions within the human β-globin cluster, including the DNase I hypersensitive site 3 (HS3) within the locus control region (LCR), the ε-globin promoter, and the γδ-intergenic region, consistent with previous analyses (4, 6). Knockdown of BCL11A abolishes its occupancy at the above regions, consistent with the near absence of BCL11A protein in the knockdown cells (Fig. 3*B*). Interestingly, loss of BCL11A leads to a marked increase of H3K4me2, a histone mark associated with active transcription, at the Aγ-globin promoter and the γδ-intergenic region, with a concomitant decrease of the H3K9me1 mark (Fig. 3 *C* and *D*). Because both H3K4me2 and H3K9me1 are substrates for the histone demethylase LSD1, which is identified as a BCL11A-interacting protein (Fig. 1), we next examined the chromatin occupancy of LSD1 and its cofactor CoREST. Loss of BCL11A also impairs chromatin occupancy of the LSD1/CoREST complex within the β-globin locus, particularly at the ε-promoter and the γδ-intergenic region (Fig. 3 *E* and *F*), suggesting that BCL11A is required for efficient recruitment or stable association of the LSD1/CoREST complex within the β-globin cluster. Similarly, the binding of the NuRD complex (Mi-2β and HDAC1) and EZH2 is also decreased on BCL11A knockdown (Fig. 3 *G–I*). Notably, RNA Pol II occupancy at the Aγ promoter is enhanced on knockdown, consistent with transcriptional reactivation of the γ-globin genes in the BCL11A-depleted adult erythroid cells (Figs. 2*B* and 3*J*). These results indicate that loss of BCL11A leads to impaired chromatin occupancy of a subset of its interacting corepressors in human adult erythroid cells.

Loss of LSD1/CoREST Reactivates Mouse Embryonic β-Like Globin Genes. We next determined whether the LSD1/CoREST complex is required for the expression of β-like globin genes in vivo. Mouse strains carrying floxed alleles of *Lsd1* or *Rocr1* (encoding CoREST) were generated by gene targeting. We first examined the expression of mouse embryonic β-like globin genes (εy- and βh1-globin) in fetal livers of *Lsd1*- or *Rcor1*-deficient embryos. Erythroid-specific deletion of LSD1 by EpoR-Cre results in a 6.2- and 26.7-fold increase in expression of the εy- and βh1-globin mRNAs in embryonic day 12.5 (E12.5) fetal liver definitive erythroid cells, respectively (Fig. 4*A*; $P < 0.05$). Similarly, loss of CoREST results in a 13.0- and 15.4-fold increase in εy- and βh1-globin mRNAs in E13.5 fetal livers (Fig. 4*B*; $P < 0.05$). Because germ-line or erythroid-specific deletion of LSD1 is embryonic lethal, we studied LSD1 conditional KO (cKO) adult mice using the hematopoietic-selective and IFN inducible Mx1-Cre allele (14). Acute deletion of LSD1 in adult bone marrow also results in a modest increase (5.8- and 10.6-fold) in expression of εy- and βh1-globin genes (Fig. 4*D*; $P < 0.05$). These results provide genetic evidence that the LSD1/CoREST complex is required to maintain full silencing of mouse embryonic β-like globin genes in definitive erythroid cells

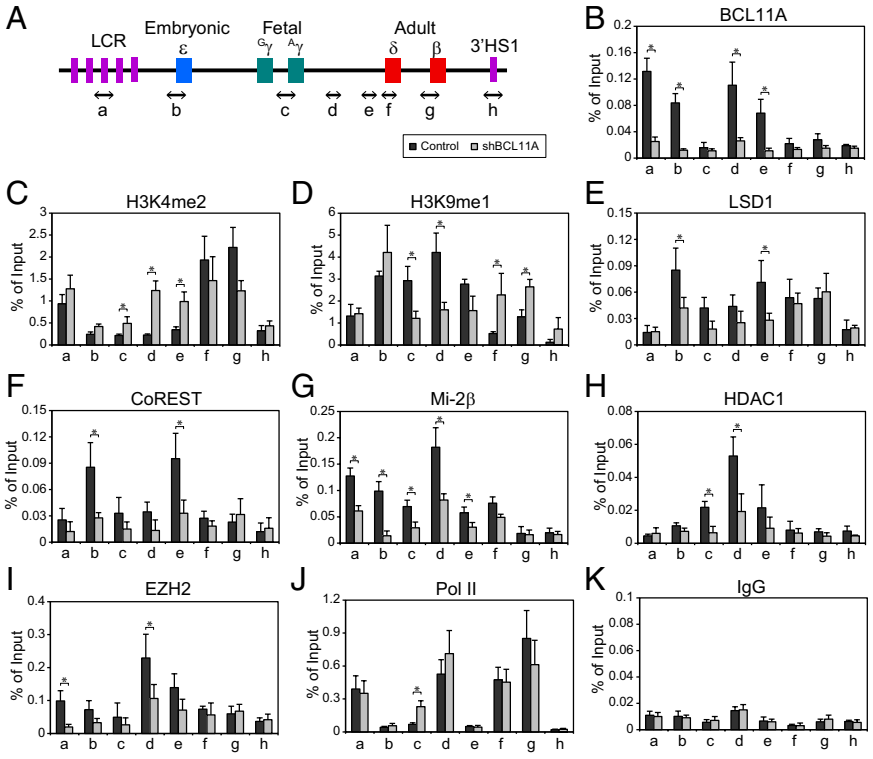

Fig. 3. Chromatin occupancy of BCL11A-interacting proteins in primary human erythroid cells. (*A*) In vivo chromatin occupancy in primary human erythroid cells transduced with lentiviral shRNAs against GFP (Control) or BCL11A (shBCL11A). Primers were designed to amplify discrete regions across the β-globin locus, including (*a*) HS3 within the LCR, (*b*) ε-globin promoter, (*c*) Aγ-globin promoter, (*d*) +3-kb region downstream of Aγ-globin gene, (*e*) −1-kb region upstream of δ-globin gene, (*f*) δ-globin promoter, (*g*) β-globin promoter, and (*h*) 3′HS1 site. (*B–K*) ChIP-qPCR analysis of BCL11A, H3K4me2, H3K9me1, LSD1, CoREST, Mi-2β, HDAC1, EZH2, RNA Pol II, and rabbit IgG in primary human erythroid cells in the presence or absence of BCL11A knockdown, respectively. Results are means ± SD; *$P < 0.05$.

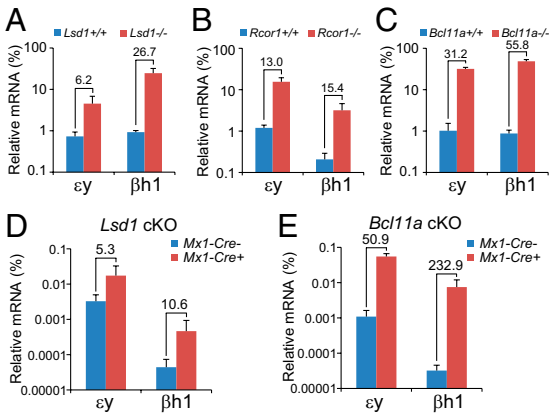

Fig. 4. The LSD1/CoREST complex is required for silencing of mouse embryonic β-like globin genes. (*A*) Expression of mouse embryonic (εy and βh1) globin genes was monitored by qRT-PCR in E12.5 fetal livers of control (*Lsd1*$^{+/+}$) and LSD1 KO (*Lsd1*$^{-/-}$; by EpoR-Cre) embryos. All data are shown as percentage (%) of total mouse β-like globin expression. The fold changes of globin mRNAs are indicated. Results are means ± SD ($n \geq 5$ per genotype; $P < 0.05$). (*B*) Expression of εy- and βh1-globin genes in E13.5 fetal livers of control and CoREST KO (*Rcor1*$^{-/-}$) embryos ($n \geq 3$ per genotype; $P < 0.05$). (*C*) Expression of εy- and βh1-globin genes in E12.5 fetal livers of control and BCL11A KO (*Bcl11a*$^{-/-}$; by EpoR-Cre) embryos ($n \geq 3$ per genotype; $P < 0.01$). (*D*) Expression of εy- and βh1-globin genes in PB of control and LSD1;Mx1-Cre cKO mice (3 wk post-pIpC, 9–15 wk old; $n \geq 5$ per genotype; $P < 0.05$). (*E*) Expression of εy- and βh1-globin genes in PB of control and BCL11A;Mx1-Cre cKO mice (3 wk post-pIpC, 9–15 wk old; $n \geq 3$ per genotype; $P < 0.01$).

in vivo. However, deletion of BCL11A in mouse results in much greater increases (31.2- to 232.9-fold) of embryonic globin genes in definitive fetal liver and bone marrow erythroid cells (Fig. 4 *C* and *E*) (8), indicating that the extent of induction by LSD1 deletion is quite modest compared with that of BCL11A deletion (Fig. 4; compare *A* and *B* with *C* or *D* with *E*).

LSD1 Cooperates with BCL11A in Silencing γ-Globin Expression. Because the repression of the endogenous εy- and βh1-globin genes is modestly and severely impaired in definitive fetal liver and bone marrow erythrocytes of *Lsd1*-deficient and *Bcl11a*-deficient mice, respectively (Fig. 4) (5), and LSD1 physically interacts with BCL11A (Fig. 1), we next examined whether LSD1 and BCL11A function collaboratively in silencing human γ-globin genes in vivo. We generated BCL11A and LSD1 compound KO mice carrying the human β-locus transgene [β-yeast artificial chromosome (β-YAC)]. Because erythroid-specific loss of LSD1 by EpoR-Cre results in embryonic lethality, we obtained compound BCL11A homozygous LSD1 heterozygous (*Bcl11a*$^{-/-}$::*Lsd1*$^{+/-}$) cKO adult mice. Erythroid-specific loss of BCL11A alone leads to 383-fold increase in γ-globin mRNA in the peripheral blood (from 0.022% in *Bcl11a*$^{+/+}$::*Lsd1*$^{+/+}$ control mice to 8.40% in *Bcl11a*$^{-/-}$::*Lsd1*$^{+/+}$ mice). The expression of γ-globin is further induced to 13.53% in *Bcl11a*$^{-/-}$::*Lsd1*$^{+/-}$ compound KO adult mice (Fig. 5*A*; $P = 0.0006$). To determine the effect of complete loss of BCL11A and LSD1 on γ-globin silencing, we generated compound homozygous knockout adult mice using the inducible Mx1-Cre allele. Loss of LSD1 alone leads to a slight increase of γ-globin mRNA in the peripheral blood (from 0.025% in *Bcl11a*$^{+/+}$::*Lsd1*$^{+/+}$ mice to 0.04% in *Bcl11a*$^{+/+}$::*Lsd1*$^{-/-}$ mice; Fig. 5*B*). Loss of BCL11A alone reactivates γ-globin mRNA to 12.88%. Combined loss of BCL11A and LSD1 further induces γ-globin mRNA to 18.19% in *Bcl11a*$^{-/-}$::*Lsd1*$^{-/-}$ compound KO adult mice (Fig. 5*B*; $P = 0.03$). These data demonstrate that depletion of LSD1 by itself has a modest effect on transgenic γ-globin expression in vivo. However, loss of LSD1 further enhances the effect of BCL11A deficiency in HbF derepression, suggesting that BCL11A serves as a major barrier to induction by LSD1 deficiency.

Depletion of LSD1 Induces HbF Expression and Impairs Erythropoiesis. We initially observed that erythroid-specific loss of LSD1 resulted in embryonic lethality at E13.5 with severe anemia. We next examined hematologic parameters in LSD1 and BCL11A compound KO adult mice (by EpoR-Cre). Consistent with previous analysis (8), erythroid- or hematopoietic-specific loss of BCL11A has little effect on total red blood cell (RBC) number and hemoglobin (Hgb) level (Fig. S4). In contrast, the *Bcl11a*$^{-/-}$::*Lsd1*$^{+/-}$ compound KO mice are mildly anemic (Fig. S4*A*), indicating that LSD1 is required for normal erythroid development. Hematopoietic-selective loss of LSD1 also leads to profound decrease in total number of white blood cells (WBCs; Fig. S4*B*), consistent with a role for LSD1 in terminal differentiation of other hematopoietic lineages (15).

To examine more directly the role of LSD1 in HbF silencing and erythropoiesis, we depleted its expression in primary human adult erythroid progenitors. Transduction of two independent lentiviral shRNAs against LSD1 leads to efficient knockdown of LSD1 protein in CD34^{+} HSPC-derived erythroid progenitors (Fig. 5*C*). Although knockdown of BCL11A expression leads to a substantial increase of human γ-globin mRNA (from 9.5% to 50.3% at day 7 of erythroid differentiation), depletion of LSD1 expression results in modest increases in γ-globin expression (sh1: 21.3%; sh5: 32.3%; Fig. 5*D*). Of note, depletion of LSD1 also results in ∼65% decrease in total β-globin mRNAs in day 7 erythroid progenitors; therefore, much of the observed increase in relative γ-globin expression is due to reduced β-globin expression rather than γ-globin induction per se (Fig. 5*D*, *Lower*). This finding is in contrast to the changes in relative globin expression on BCL11A knockdown, which are predominantly characterized by induction of γ-globin itself. Consistent with decreased total globin production, LSD1-depleted cells retain expression of CD34 antigen and fail to express maturing erythroid cell-specific markers CD71 and CD235a (Fig. S5*A*). In fact, the majority of LSD1-depleted cells exhibit proerythroblast and basophilic erythroblast morphology, whereas the more mature polychromatophilic and orthochromatic erythroblasts are nearly absent at day 9 of differentiation (Fig. S5*B*). Collectively, these data indicate that LSD1 is required for both full HbF silencing and erythroid cell maturation.

Several small molecule inhibitors have been used to target LSD1 by inhibiting its histone demethylase activity (16). We next tested two LSD1 inhibitors, pargyline and trans-2-phenylcyclopropylamine [tranylcypromine (TCP)], in reactivation of HbF expression in primary human adult erythroid cells. Pargyline treatment by itself has little effect on HbF expression. Combining pargyline treatment and BCL11A knockdown results in modest increases in HbF expression compared with BCL11A knockdown alone (Fig. S6*A*). In contrast, TCP treatment induces HbF expression in both control and BCL11A knockdown cells (Fig. S6*A*). However, TCP treatment results in a profound decrease in production of total β-like globin mRNAs (Fig. S6*B*). Additionally, TCP-treated cells fail to express CD71 and CD235a (Fig. S6*D*), indicating that the induction of HbF expression mediated by chemical inhibitors of LSD1 is also associated with impaired erythroid maturation.

DNMT1 Is Required for Maintenance of HbF Silencing. The proteomic screen identified the DNA methyltransferase DNMT1 as another protein with intrinsic enzymatic activity in association with BCL11A (Fig. 1). DNA methylation plays an important role in globin gene expression, and DNA demethylating agents have been shown to induce HbF expression in various model systems and patients (8, 17, 18). However, the role of the methyltransferase DNMT1 in γ-globin silencing in an intact animal has not been previously assessed by formal genetic experiments. Thus, we determined whether DNMT1 is directly involved in HbF silencing in vitro and in vivo. Upon shRNA-mediated knockdown of DNMT1 expression in primary human adult erythroid cells, HbF expression is significantly induced, whereas the amount of total β-like globin mRNAs is modestly reduced (Figs. 2*B* and 5*E*). Similarly, inhibition of DNMT1 activity by chemical inhibitors leads to enhanced HbF expression in primary erythroid cells (Fig. S7).

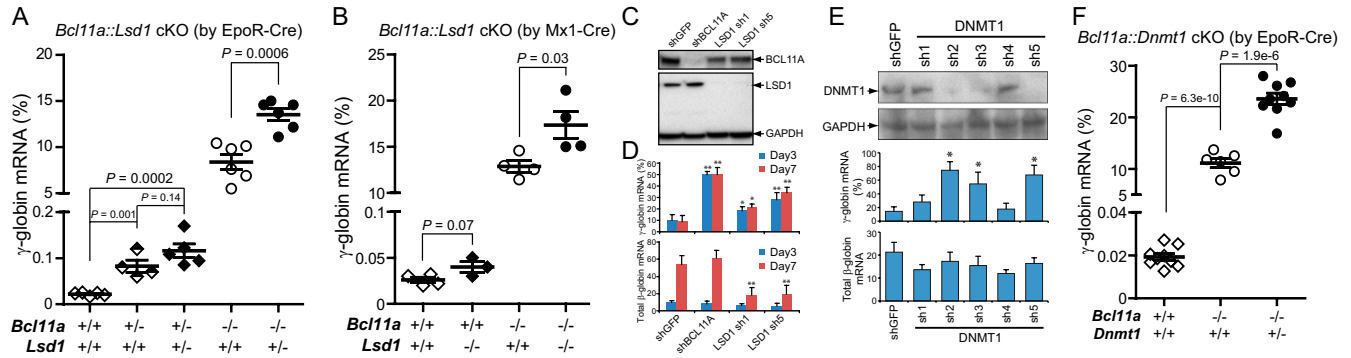

Fig. 5. Compound KO of *Bcl11a*, *Lsd1*, or *Dnmt1* enhances γ-globin expression. (*A*) Expression of γ-globin mRNA was measured by qRT-PCR in peripheral blood (PB) of *Bcl11a::Lsd1* cKO animals (by EpoR-Cre; 8–12 wk old). Results are the means ± SD. *P* values were determined by a two-tailed *t* test. (*B*) Expression of γ-globin in PB of *Bcl11a::Lsd1* cKO animals (by Mx1-Cre; 8–12 wk old). (*C*) Lentiviral shRNA-mediated knockdown of *BCL11A* and *LSD1* proteins in primary human erythroid cells. (*D*) Knockdown of BCL11A or LSD1 results in increased γ-globin mRNAs (*Upper*). Knockdown of LSD1 reduces total β-like globin mRNAs (*Lower*). Data are shown as means ± SD; $*P < 0.05$, $**P < 0.01$. (*E*) shRNA-mediated knockdown of DNMT1 in primary human erythroid cells (*Upper*) results in increase of γ-globin mRNAs and modest decrease in total β-like globin mRNAs (*Lower*). Data are shown as means ± SD; $*P < 0.05$. (*F*) Expression of γ-globin in PB of *Dnmt1::Bcl11a* cKO animals (by EpoR-Cre; 8–12 wk old).

To assess the role of DNMT1 in HbF silencing in vivo, we generated DNMT1 and BCL11A compound erythroid-specific cKO β-YAC mice (by EpoR-Cre). Although homozygous loss of DNMT1 results in embryonic lethality, compound BCL11A homozygous DNMT1 heterozygous ($Bcl11a^{-/-}::Dnmt1^{+/-}$) KO mice are viable despite a mild anemic phenotype (Fig. S8). Of note, DNMT1 haploinsufficiency leads to a 2.1-fold increase of γ-globin mRNA in the absence of BCL11A (Fig. 5*F*; from 11.16% in $Bcl11a^{-/-}::Dnmt1^{+/+}$ mice to 23.58% in $Bcl11a^{-/-}::Dnmt1^{+/-}$ mice, $P = 1.9 \times 10^{-6}$). Collectively, these data provide important genetic evidence that DNMT1 haploinsufficiency in combination with BCL11A deficiency can further enhance HbF expression.

Discussion

We characterized interacting partner proteins of BCL11A in primary human erythroid cells that may participate in HbF regulation. We infer that BCL11A acts within multiprotein complexes consisting of transcriptional corepressors and chromatin-modifying subunits. Knockdown of several BCL11A-interacting corepressor proteins induces HbF expression in primary human erythroid cells, whereby the effect on total hemoglobin production and erythroid maturation is variable. The chromatin occupancy of several BCL11A-interacting complexes, including the LSD1/CoREST and NuRD complexes, within the β-globin cluster is dependent on the presence of BCL11A in human erythroid cells. These results suggest that BCL11A coordinates the hemoglobin switch and HbF silencing by assembling transcriptional corepressor complexes within the β-globin cluster.

The identification of BCL11A-interacting proteins provides clues to target BCL11A and/or its cofactors for therapeutic HbF induction in β-hemoglobinopathies. To define favorable molecular targets for HbF induction, it is imperative to evaluate several criteria, including quantitative effects on HbF expression, limited effects on expression of nonglobin genes and on erythroid maturation, minimal impact outside the erythroid lineage, and feasibility of therapeutic intervention (2). Several favorable features, such as potency in HbF silencing, dose dependence, and minimal influence on erythropoiesis, recommend BCL11A as a target. However, its roles in other cell lineages, including B lymphocytes and the central nervous system, may present challenges (19, 20). As transcription factors have traditionally been viewed as undruggable due to the lack of catalytic domains, alternative strategies involving interference with protein–protein interactions or targeting partner proteins with enzymatic activities may be considered. Our results show that BCL11A interacts with several chromatin-modifying enzymes, such as the LSD1/CoREST demethylase complex, DNMT1, and the NuRD complex. Small molecule inhibitors for these enzymes are in various phases of clinical development, including those in current medical practice. In principle, targeting enzymatic partner proteins of BCL11A constitutes a strategy for indirectly targeting BCL11A function.

Our in-depth characterization of the roles of two BCL11A-interacting enzymatic partners, LSD1 and DNMT1, in HbF regulation provides additional insights. KO of LSD1 in mice leads to derepression of mouse embryonic β-like globin genes and transgenic human γ-globin genes in definitive erythroid cells. Interference with LSD1 by shRNA or small molecule inhibitors reactivates HbF expression in primary human adult erythroid cells. Consistent with a role of LSD1 in HbF silencing, while this paper was under review, it was reported that RNAi or chemical inhibition of LSD1 enhances γ-globin expression in human erythroid cells and quite modestly β-locus transgenic mice (21). Although these findings illustrate favorable features of LSD1 as a potential target, we also demonstrate that LSD1 serves critical roles more broadly in erythroid cell maturation and overall globin expression. LSD1 homozygous KO mice are embryonic lethal with severe anemia. Deletion of LSD1 in adult bone marrow results in impaired function of hematopoietic stem cells, neutropenia, and markedly reduced number of leukocytes (15), indicating that LSD1 is necessary for specification and terminal differentiation of several hematopoietic lineages. Similarly, interference with LSD1 by shRNAs or inhibitors impairs erythroid maturation from human $CD34^+$ HSPCs ex vivo (Figs. S5 and S6). Accordingly, its potential as a therapeutic target for HbF induction is compromised by its multifaceted roles in hematopoiesis. Our study also shows that DNMT1 haploinsufficiency augments HbF expression elicited by BCL11A deficiency. However, complete loss of DNMT1 is incompatible with normal hematopoietic development (22). Therefore, the therapeutic window appears to be relatively narrow whereby DNMT1 activity may be inhibited without perturbing normal erythroid functions.

The nucleosome remodeling and histone deacetylase (NuRD) complex is also identified as a BCL11A-interacting corepressor complex. The NuRD complex consists of several enzymatic subunits, including the ATPase subunit Mi-2β and the histone deacetylases (HDAC1 and HDAC2). Depletion of Mi-2β results in a profound increase in γ-globin expression in primary human erythroid cells (the top-ranked gene CHD4; Fig. 2*B*; Fig. S3). Consistent with a role in HbF silencing, knockdown or KO of Mi-2β reactivates γ-globin genes in the β-YAC transgenic mice (23, 24). However, the degree of γ-globin induction is substantially less than that seen with BCL11A deficiency (8). Additionally, depletion of HDAC1 and HDAC2 reactivates γ-globin expression in primary human erythroid cells (Fig. 2*B*). HDAC1 and HDAC2 have also been identified as HbF inducers by a chemical genetic screen (25). These results collectively provide strong support for important

roles of several enzymatic subunits within the NuRD complex in globin expression, yet the extent to which various effects can be ascribed directly to action in concert with BCL11A or independently are difficult to discern.

Our studies used two model systems, including the primary human erythroid culture and the β-YAC transgenic mice, to evaluate HbF regulation and erythroid maturation. To interpret results meaningfully, it is important to acknowledge the potential limitations unique to each system. Although cultured primary human erythroid cells model several important aspects of human erythroid development such as an adult-stage pattern of globin profile, the background level of γ-globin mRNA is much higher than that present in adult erythroid cells of normal individuals. Moreover, $CD34^+$ HSPCs appear relatively permissive for HbF induction by various agents, thus rendering them sensitive but not necessarily specific indicators to assay for the effects of manipulations inducing HbF (2). Changes in growth kinetics and differentiation status of $CD34^+$ HSPCs affect HbF expression. In contrast to primary human cells, the baseline expression of transgenic γ-globin gene in adult β-YAC mice is far lower than anticipated from normal individuals, possibly due to differences in the transacting environment between mouse and human (5). Thus, interventions seeking to derepress γ-globin expression face a much greater quantitative hurdle. Although some manipulations, such as inhibition of LSD1 or DNMT1, may induce transgenic γ-globin expression several fold above this low level, the effects are nonetheless small compared with those elicited by BCL11A KO. Care needs to be exercised in extrapolating findings in the available model systems to nominate targets for therapeutic manipulation.

Materials and Methods

Experimental Animals. The β-globin locus transgenic (β-YAC) mouse strain was created as described (26). Mice containing a *Bcl11a* floxed allele were created as described (8). Mice containing an *Lsd1* floxed allele (M.A.K. and S.H.O.) or a *Rocr1* (encoding CoREST) floxed allele (H.Y. and G.M.) were generated through gene targeting approaches. $Dnmt1^{fl/fl}$ mice were created as previously described (22, 27). To obtain the *Bcl11a* cKO mice, the $Bcl11a^{fl/fl}$ mice were crossed with the EpoR-Cre knock-in mice (28) or the Mx1-Cre transgenic mice (14). These mice were crossed with $Lsd1^{fl/fl}$ or $Dnmt1^{fl/fl}$ mice to create compound cKO mice. Polyl:ployC (plpC) was prepared in PBS and administrated via i.p. injection daily at a dose of 25 μg/kg for 7 d. Peripheral blood was isolated via the retroorbital plexus and analyzed on a HEMAVET HV950 hematology system (Drew Scientific). All experiments were performed with the approval of the Children's Hospital Boston Animal Ethics Committee.

Cell Culture. Primary adult human $CD34^+$ HSPC-derived erythroid progenitors were generated ex vivo as described (6). The MEL-BirA (MBirA), MEL-FLAG-Bio-BCL11A (MBB1.4), K562-BirA (KBirA), and K562-FLAG-Bio-BCL11A (KBB2.4) stable cell lines were generated as described (10).

Protein Affinity Purification and Proteomic Analysis. BCL11A-interacting multiprotein complexes were purified and characterized as described (29). Copurified proteins were separated by SDS/PAGE, followed by whole lane LC-MS/MS using an LTQ linear ion-trap mass spectrometer. A subtractive approach including parallel pull-down in parental MEL-BirA (MBirA) or K562-BirA (KBirA) cells was used.

Lentiviral RNAi. Lentiviral shRNA clones in the pLKO.1-puro vector were obtained from Sigma-Aldrich (two to five shRNAs per gene; Table S1). Primary human $CD34^+$ HSPCs were transduced by spin infection at day 3 of expansion. Cells were washed three times with PBS and seeded in fresh media 24 h after infection. Selection (1 μg/mL puromycin) was initiated at 48 h after infection, followed by erythroid differentiation for 5–7 d.

ChIP. ChIP was performed as described previously (29) using the following antibodies: H3K4me2 (07-030; Millipore), H3K9me1 (ab9045; Abcam), BCL11A (ab19487 and ab18688; Abcam), LSD1 (ab17721; Abcam), CoREST (07-455; Millipore), Mi-2β (provided by Stephen Smale, University of California, Los Angeles, CA), HDAC1 (06-720; Millipore), EZH2 (07-689; Millipore), RNA Pol II (sc-899; Santa Cruz Biotechnology), and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology).

RNA Isolation and qRT-PCR. RNA was extracted using the QIAamp RNA Blood Mini Kit or RNeasy Plus Mini Kit (Qiagen). RNA was reverse-transcribed and analyzed with the iQ SYBR Green Supermix using the iCycler Real-Time PCR Detection System (Bio-Rad). Primer sequences are listed in Table S2.

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