Transcriptional regulators Myb and BCL11A interplay with DNA methyltransferase 1 in developmental silencing of embryonic and fetal β-like globin genes

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ABSTRACT **The clinical symptoms of hemoglobin disorders such as β-thalassemia and sickle cell anemia are significantly ameliorated by the persistent expression of γ-globin after birth. This knowledge has driven the discovery of important regulators that silence γ-globin postnatally. Improved understanding of the γ- to β-globin switching mechanism holds the key to devising targeted therapies for β-hemoglobinopathies. To further investigate this mechanism, we used the murine erythroleukemic (MEL) cell line containing an intact 183-kb human β-globin locus, in which the ^{G}γ- and β-globin genes are replaced by DsRed and eGFP fluorescent reporters, respectively. Following RNA interference (RNAi)-mediated knockdown of two key transcriptional regulators, Myb and BCL11A, we observed a derepression of γ-globin, measured by DsRed fluorescence and qRT-PCR ($P<0.001$). Interestingly, double knockdown of Myb and DNA methyltransferase 1 (DNMT1) resulted in a robust induction of ε-globin, (up to 20% of total β-like globin species) compared to single knockdowns ($P<0.001$). Conversely, double knockdowns of BCL11A and DNMT1 enhanced γ-globin expression (up to 90% of total β-like globin species) compared to single knockdowns ($P<0.001$). Moreover, following RNAi treatment, expression of human β-like globin genes mirrored the expression levels of their endogenous murine counterparts. These results demonstrate that Myb and BCL11A cooperate with DNMT1 to achieve developmental repression of embryonic and fetal β-like globin genes in the adult erythroid environment.—Roosjen, M., McColl, B., Kao, B., Gearing, L. J., Blewitt, M. E., Vadolas, J. Transcriptional regulators Myb and BCL11A interplay with DNA methyltransferase 1 in developmental silencing of embryonic and fetal β-like globin genes. *FASEB J.* 28, 1610–1620 (2014). www.fasebj.org**

Key Words: RNA interference · β-hemoglobinopathies · epigenetic repressor · molecular mechanism

THE β-HEMOGLOBINOPATHIES, SUCH as sickle cell anemia (SCA) and β-thalassemia, are the most common inherited red blood cell disorders in the world, with at least 300,000 severely affected individuals born annually (1). The pathological effects of the β-hemoglobinopathies result from mutations within the β-globin gene. In the case of β-thalassemia, a large number of mutations exist that cause reduced or absent expression of β-globin, whereas SCA results from the presence of Glu-6-Val acid substitution in the β-globin protein. Standard treatments include lifelong blood transfusions, which are complicated by the accumulation of iron within multiple tissues. Ongoing chelation therapy is therefore required to minimize iron-related pathologies, which may affect vital organs such as the heart, liver, and kidney.

One promising approach for the treatment of these conditions is through the increased expression of fetal hemoglobin (HbF; $\alpha_2\gamma_2$). HbF is the main hemoglobin expressed in red blood cells during fetal development but diminishes to <1% of total hemoglobin soon after birth,

Abbreviations: BCL11A, B-cell lymphoma/leukemia 11A; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DNMT1, DNA methyltransferase 1; DRED, direct-repeat erythroid-definitive protein; DsRed, red fluorescent protein; eGFP, enhanced green fluorescent protein; FCS, fetal calf serum; HbA, adult hemoglobin; HbF, fetal hemoglobin; HPFH, hereditary persistence of fetal hemoglobin; KLF1, Krüpple-like factor 1; LCR, locus control region; LSD1, lysine-specific demethylase 1; MEL, murine erythroleukemia; MOI, multiplicity of infection; NuRD, nucleosome-remodeling deacetylase; RNAi, RNA interference; SCA, sickle cell anemia; SCFA, short-chain fatty acid; shRNA, short hairpin RNA

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being replaced by the major adult hemoglobin (HbA; $\alpha_2\beta_2$). This postnatal transition from HbF to HbA is known as "hemoglobin switching" and coincides with the clinical manifestation of symptoms of β-thalassemia and SCA around 1 yr after birth. However, some individuals maintain expression of HbF into adulthood, a nonpathological phenomenon known as hereditary persistence of fetal hemoglobin (HPFH). Coinheritance of an HPFH genotype with SCA or β-thalassemia results in a less severe form of the disorder due to the compensatory effects of HbF expression. Therefore, inducing HbF expression in non-HPFH individuals has great potential for the treatment of β-hemoglobinopathies.

At present, hydroxyurea remains the only U.S. Food and Drug Administration (FDA)-approved agent for treatment of SCA. Hydroxyurea treatment ameliorates the clinical symptoms of SCA and substantially reduces the frequency of pain crises. Recent clinical trials have identified hydroxyurea to also be beneficial in patients with β-thalassemia, as it increases HbF expression and thereby reduces the necessity for transfusion (2). Other agents, such as 5-azacytidine and decitabine, increase HbF by inhibiting DNA methyltransferase (3). Moreover, short-chain fatty acid (SCFA) compounds, such as butyrate and its derivatives, have received particular attention due to their activity as inducers of HbF. SCFAs function by inhibiting histone deacetylases, leading to increased chromatin accessibility and thereby relieving epigenetic silencing of gene expression (4). From this body of work and subsequent investigations has come the recognition of globin switching as an epigenetic process. Numerous studies have now examined the potential for pharmacological induction of HbF using agents that affect epigenetic processes such as DNA methylation and histone acetylation (5–7). However, several HbF inducers under clinical investigation demonstrate unpredictable responses and poor specificity and are associated with toxicity and potential carcinogenicity (8). Therefore, a comprehensive understanding of the regulatory mechanisms governing globin gene expression, especially the γ- to β-globin switching mechanism immediately after birth, would undoubtedly help to identify key targets for the development of specific therapeutic strategies.

Many insights into γ-globin switching have come from the study of individuals with HPFH (9–11). Several γ-globin promoter mutations and deletions in the β-globin locus have been reported to cause HPFH. In addition, recent genome-wide association studies examining the genetic basis of HPFH identified single-nucleotide polymorphisms located within the *HBS1L-MYB* intergenic region, as well as within the gene encoding the transcription factor B-cell lymphoma/leukemia 11A (*BCL11A*; ref. 12). In addition, family studies have revealed that a number of individuals with HPFH have mutations in the gene encoding the critical erythroid transcription factor Krüppel-like factor 1 (KLF1; ref. 10). Recent molecular studies into the switching mechanism have identified numerous other determinants of hemoglobin switching, such as the erythroid transcription factors GATA1 (13), friend of PRMT1 (FOP1; ref. 14) and SOX6 (12). Emphasizing the epigenetic nature of globin switching, modifiers of chromatin, such as lysine-specific demethylase 1 (LSD1/KDM1; ref. 15) and DNA methyltransferase 1 (DNMT1), which catalyzes the methylation of cytosine bases of CpG dinucleotides within the γ-globin promoter, are crucial mediators of gene expression. Multimeric transcriptional regulators, such as the direct-repeat erythroid definitive (DRED) complex (16), CoREST, and nucleosome remodeling deacetylase (NuRD), are also involved in γ-globin gene regulation (17–19). This complexity demonstrates the challenges involved in fully understanding the molecular mechanism responsible for γ-globin switching.

To further investigate this clinically important developmental switch, a number of studies have employed cultured human primary erythroid cells to identify and evaluate γ-globin gene regulators. However, the limited availability of primary human erythroid cells imposes restraints on high-throughput analyses (20). To gain a comprehensive understanding of the regulatory mechanisms involved in γ-globin gene expression, a novel fluorescent-based cellular reporter assay system was developed (21). Using homologous recombination, two fluorescent reporter genes, red fluorescent protein (DsRed) and enhanced green fluorescent protein (eGFP), were inserted into an intact 183-kb human β-globin locus, replacing the coding regions of the ^{G}γ- and β-globin genes, respectively. Modified constructs were stably transfected into adult murine erythroleukemic (MEL) cells and human embryonic/fetal erythroleukemic K562 cells, wherein high-level expression of human fetal or adult globin gene expression was observed, depending on the specific developmental phenotype of the parent cell line.

In this study, we exploited the multifunctional aspect of the dual reporter MEL cell model system to assess the effect of Myb, BCL11A, and DNMT1 on γ-globin gene regulation. Following RNA interference (RNAi) knockdown of Myb and BCL11A, we noted elevated levels of both ^{A}γ-globin and DsRed gene expression. However, when expression of Myb and DNMT1 was inhibited simultaneously, we noted a robust induction of ε-globin. Corepression of BCL11A and DNMT1 resulted in increased γ-globin expression. Interestingly, this activity was mirrored by their endogenous murine counterparts, εy- and βh1-globin genes. Taken together, our studies support the conclusion that Myb and BCL11A serve to define the developmental-stage specific repression of the embryonic and fetal β-like globin genes mediated by DNMT1.

MATERIALS AND METHODS

Cell culture

The MEL cell line, MEL^{G}γDsRed-βeGFP, was created by generating stable clones containing a 183-kb human genomic frag-

ment containing the entire β-globin locus, modified to replace the reading frame of ^{G}γ-globin with that of DsRed, and the β-globin codons replaced by those of eGFP (ref. 21 and Supplemental Fig. S1). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere supplemented with 5% CO_2. Erythroid differentiation and hemoglobinization of the MEL cell line was performed by incubating cells for 7 d in the presence of 2% dimethyl sulfoxide (DMSO) prior to analysis. DsRed or eGFP expression was determined by measuring the median fluorescent intensity (MFI) by flow cytometry, while relative messenger RNA (mRNA) levels were measured by real-time quantitative RT-PCR (qRT-PCR).

Lentiviral and retroviral RNAi

All short hairpin RNA (shRNA) sequences are listed in Supplemental Table S1. Lentiviral shRNA constructs targeting Myb (TRCN0000042500 and TRCN0000042501) and BCL11A (TRCN0000096553) in the pLKO.1 vector were obtained from the MISSION shRNA Library (Sigma-Aldrich, St. Louis, MO, USA; refs. 21, 22). The scrambled control pLKO.shScr vector (plasmid 1864) was obtained from Addgene (Cambridge, MA, USA). The shRNA hairpin targeting DNMT1 was contained within the miR30 cassette of an LMP retroviral vector (23) modified to express blue fluorescent protein (LMP-EBFP2), and was provided by M.E.B. The DNMT1 shRNA was designed using the Designer of Small Interfering RNA (DSIR) website (http://biodev.extra.cea.fr/DSIR/), and cloned as described previously (24). Lentivirus (22) and VSV-G pseudotyped retrovirus (25) were prepared and cells transduced as per previously described techniques (22). Briefly, 2×10^5 cells were resuspended in DMEM + 10% FCS medium supplemented with polybrene (8 μg/ml). Viruses were added at a multiplicity of infection (MOI) of 5. At 24 h post-transduction, cells were washed 3 times with PBS and seeded in fresh medium containing puromycin (1 μg/ml). Following 5 to 7 d of puromycin selection, cells were seeded into differentiation medium containing 2% DMSO and differentiated for 7 d.

Cell proliferation and hemoglobin production

The rate of MEL cell proliferation following shRNA knockdown was determined in normal growth medium after 7 d of puromycin (1 μg/ml) selection. MEL cells were seeded at 0.2×10^5 cells/well in normal growth medium and counted daily for 8 d using a hematocytometer and trypan blue exclusion. For measurement of hemoglobin, MEL cells (5×10^6) were harvested from expansion medium and concentrations determined using Drabkin's reagent (Sigma-Aldrich) with reference to a standard curve prepared using lyophilized mouse hemoglobin (Sigma-Aldrich).

Flow cytometry

Approximately 5×10^5 MEL cells expressing the fluorescent reporters DsRed and/or eGFP were washed and resuspended in PBS + 2% FBS, then analyzed using an LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Data acquisition and analysis were performed using BD FACsDiva software (Becton Dickson) and FlowJo 10 (Tree Star, Inc., Ashland, OR, USA).

RNA isolation and qRT-PCR analysis

RNA was extracted from approximately 5×10^5 MEL cells at 8 d post-transduction (d 0 of differentiation) and at d 7 of 2% DMSO differentiation. RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen; Life Technologies, Grand Island, NY, USA). Real-time qRT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems; Life Technologies) and run on an Applied Biosystems 7300 Real-Time PCR system. Relative expression was quantified using the Pfaffl method as described previously (26). The ribosomal protein gene (*Rpl32*) was used as a reference gene to normalize gene expression. Primers used in this study are listed in Supplemental Table S2.

Western blot method, antibodies, and reagents

Approximately 1×10^6 cells were suspended in lysis buffer (20 mM Tris, pH 8.0; 150 mM NaCl; 1% Triton X-100; and 10% protease inhibitor cocktail). Expression of BCL11A, Myb, and DNMT1 were assessed using antibodies staining Ab18B12DE6 (AbCam, Cambridge, MA, USA), 05-175 (Millipore, Billerica, MA, USA) and H-300 (SC20701; Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively, as described previously (12). Antibody staining for β-actin (A5441; Sigma-Aldrich) was used as a loading control. Western blots were visualized using the Image Quant LAS 400 (GE Healthcare, Rydalmere, NSW, Australia). Relative expression was calculated using Image Quant TL 7.0 software (GE Healthcare).

Statistical analysis

All data are presented as means ± SD. For RNAi studies, unpaired Student's *t* test was used to calculate the statistical significance ($P \leq 0.05$) between the untreated and RNAi treated samples. All statistical analysis and graph visualization was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

This study was performed using the previously described dual reporter cell line, MEL^{G}γREDβeGFP, in which the human ^{G}γ-globin promoter drives a DsRed reporter, and the β-globin promoter controls an eGFP reporter. Manipulation of the regulatory networks governing globin gene expression can therefore be rapidly detected by fluorescence-activated cell sorting (FACS). RNAi knockdown of Myb, BCL11A, and DNMT1 was undertaken to examine the functional interplay between key regulators of globin gene expression.

Myb modulates multiple γ-globin regulators

Recent studies of γ-globin regulation have shown that Myb is an indirect upstream regulator of BCL11A through KLF1 transactivation in human cells, and that reduced expression of Myb was associated with an increase in γ-globin gene expression (27). Therefore, the role of Myb was examined in MEL^{G}γREDβeGFP

cells by lentiviral shRNA-mediated knockdown. Transduced MEL cells underwent 7 d of puromycin selection prior to measurement of Myb expression at both the mRNA (**Fig. 1**) and protein level (**Fig. 2**). As determined by qRT-PCR, shMyb500 reduced Myb mRNA by >25%, while shMyb501 produced a much greater reduction of Myb mRNA (>75%), relative to scramble shRNA negative control (Fig. 1). Similarly, Western blot analysis revealed that both shMyb vectors were able to reduce Myb expression at the protein level, with shMyb501 producing the greatest reduction in Myb expression (>70%; Fig. 2 and Supplemental Fig. S2; refs. 28, 29).

Since Myb has been identified as a critical upstream regulator of BCL11A and KLF1, we next examined BCL11A and KLF1 mRNA expression levels following Myb knockdown. As expected, both shMyb constructs reduced BCL11A and KLF1 mRNA levels. shMyb500 reduced BCL11A and KLF1 by >40% while shMyb501 reduced BCL11A and KLF1 by > 70% (Fig. 1). In a recent study, Myb was also identified as an upstream regulator of the TR2 and TR4 orphan nuclear receptors (30). TR2 and TR4 comprise the DNA-binding core of the DRED binding complex, which binds to the direct-repeat elements in the embryonic and fetal globin gene promoter (31, 32). Therefore, following Myb knockdown, we also measured TR2 and TR4 expression levels by qRT-PCR. As predicted, TR2 and TR4 mRNA levels were significantly reduced following Myb knockdown, with shMyb501 producing the greatest reduction of TR2 and TR4 expression (>40 and >50%, respectively; Fig. 1).

These results demonstrate that Myb is a crucial upstream modulator of multiple γ-globin regulators. Notably, Myb knockdown exhibited a dose-dependent effect on the level of BCL11A, KLF1, TR2, and TR4 expression, with shMyb501 producing the greatest reduction. Moreover, while BCL11A and DNMT1 are also important γ-globin regulators, their reduction by RNAi knockdown did not modify Myb, KLF1, TR2, or TR4 expression (Fig. 1), suggesting that BCL11A and DNMT1 do not directly regulate these genes (12).

Myb modulates cell proliferation and differentiation

Myb is highly expressed in immature proliferating hematopoietic cells and is down regulated in terminally differentiating cells, suggesting that Myb is linked to

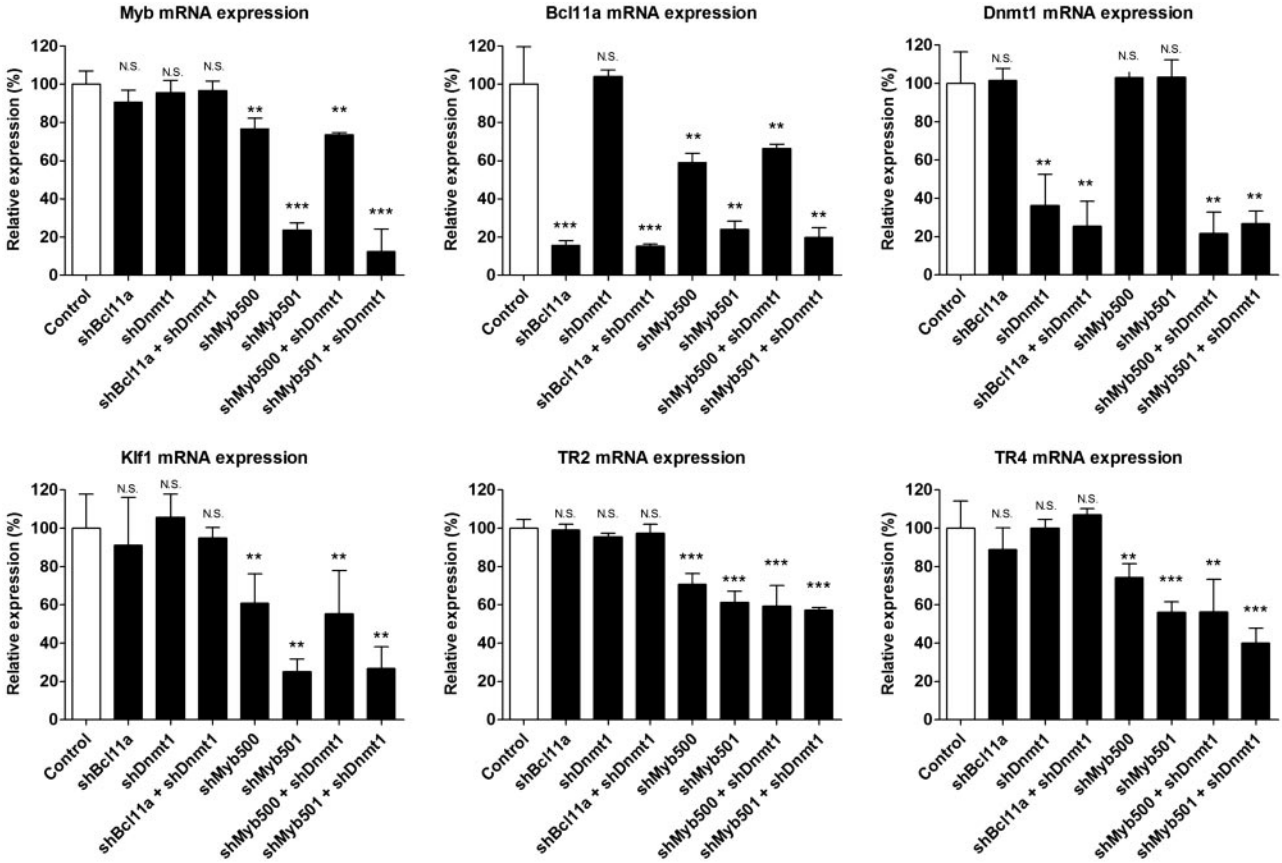

Figure 1. Analysis of globin gene regulators following RNAi knockdown of Myb, BCL11A, and DNMT1. Expression of globin gene regulators was measured by qRT-PCR following transduction of MEL^{G}γDsRed-βeGFP cells with virally encoded shRNA at an MOI of 5 and puromycin selection. Total RNA was extracted and used to measure Myb, BCL11A, DNMT1, KLF1, and TR2/TR4 expression. Knockdown of Myb reduced BCL11A, KLF1, and TR2/TR4 mRNA expression levels, whereas RNAi knockdown of BCL11A and DNMT1 had no effect on Myb, KLF1 and TR2/TR4 mRNA levels. The ribosomal protein gene Rpl32 was used as a reference gene to normalize gene expression. Data represent means ± SD *vs.* control of ≥3 independent experiments. N.S., not significant. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

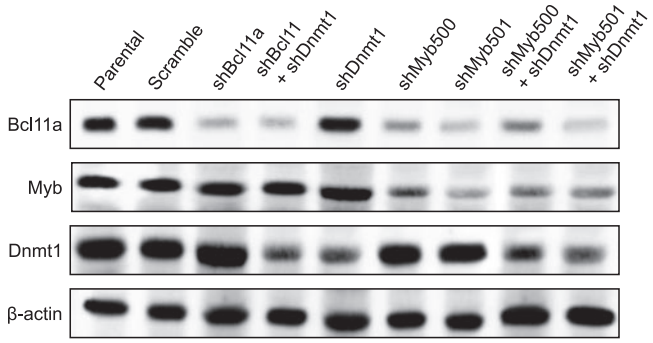

Figure 2. Western blot analysis of γ-globin-silencing factors following RNAi knockdown of Myb, BCL11A, and DNMT1. Cells were harvested following viral transduction and 7 d of puromycin selection. Whole-cell lysates were prepared and analyzed by Western blot using mAb against Myb, BCL11A, and DNMT1. RNAi knockdown of Myb generated a robust reduction in BCL11A expression, confirming that Myb transactivates BCL11A. RNAi knockdown of BCL11A and DNMT1 had no effect on Myb, expression. β-Actin was used as a loading control. Relative expression was calculated using Image Quant (Supplemental Fig. S2).

the transition between proliferation and differentiation (33–35). Interestingly, during our investigation of the MEL reporter cell line transduced with shMyb501, which reduced Myb by >80%, we noted a significant increase in rate of cell proliferation (Supplemental Fig. S3*A*), while the less effective shMyb500 had no measurable effect on cell proliferation. Since Myb is known to be reduced in expression as differentiation proceeds, we next investigated erythroid maturation by measuring hemoglobin levels using Drabkin's reagent. Knockdown of Myb caused the MEL cells to increase hemoglobin production in the absence of other prodifferentiation stimuli. Interestingly, the amount of hemoglobin produced was correlated with the level of Myb; cells treated with shMyb501, which caused the greatest reduction of Myb expression produced more hemoglobin than those treated with the less effective shMyb500. No statistically significant production of hemoglobin was detected in cells treated with the scramble control shRNA (Supplemental Fig. S3*B*).

Reporter gene expression following knockdown of Myb, BCL11A, and DNMT1

Recent *in vitro* and *in vivo* functional studies have revealed that Myb, BCL11A, and DNMT1 are critical regulators of γ-globin (35–37). We next asked whether depletion of these regulators could be used to modify expression of γ-globin. To do so, we carried out shRNA-mediated knockdown of Myb, BCL11A, and DNMT1 in MEL^{G}γREDβeGFP cells under standard culture conditions, and examined the subsequent expression of the two fluorescent reporter genes.

MEL cells can be induced to undergo terminal erythroid differentiation by treatment with DMSO, leading to reorganization of protein complexes at the β-globin locus, and increased β-globin expression (38). We subsequently examined expression of the reporter genes during DMSO-induced differentiation to determine the effect of Myb, BCL11A, and DNMT1 knockdown, both singly and in combination.

Following 3 d of DMSO treatment, depletion of either Myb or DNMT1 increased the DsRed:eGFP fluorescence ratio, indicating preferential activation of the γ-globin promoter (**Figs. 3** and **4**). No significant alteration was observed in response to BCL11A knockdown at that time point; however, following 7 d of DMSO treatment, the DsRed:eGFP fluorescence ratio in BCL11A knockdown cells had approximately doubled relative to the scramble shRNA control. From these results, it can be seen that knockdown of the chosen

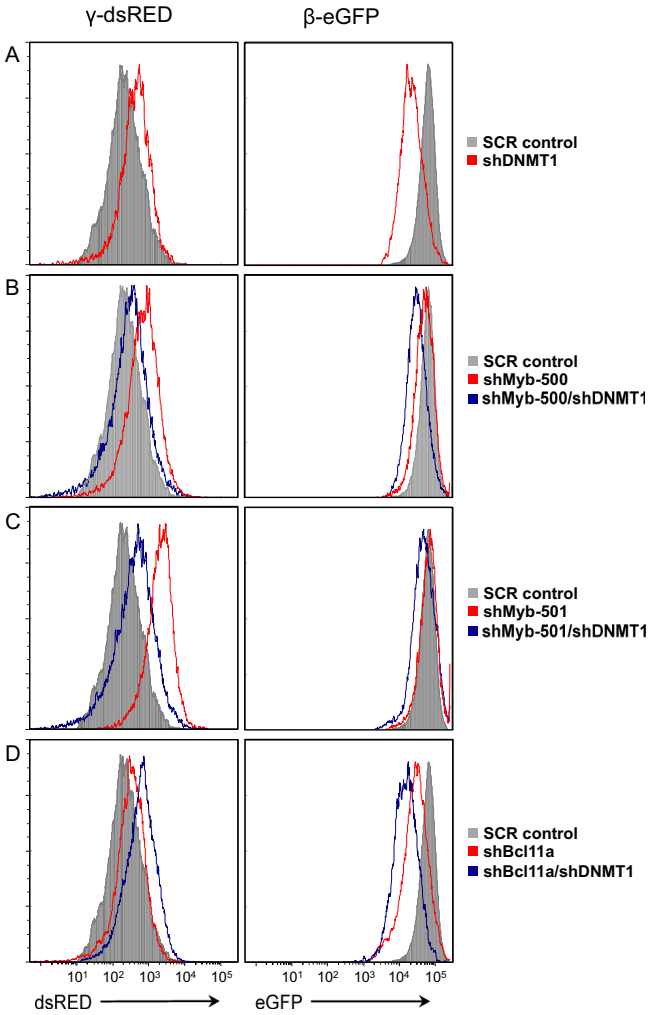

Figure 3. Analysis of DsRed and eGFP expression. Relative DsRed and eGFP fluorescence was measured in MEL ^{G}γRedβeGFP cells by flow cytometry following shRNA knockdown of DNMT1 (*A*), Myb-500 (*B*), Myb-501 (*C*), and BCL11A (*D*). Left panels: DsRed fluorescence profiles. Right panels: eGFP fluorescence profiles. Increased expression of DsRed was observed following RNAi knockdown of Myb, BCL11A, and DNMT1, and was associated with a modest reduction in eGFP expression. A robust induction of DsRed expression was observed following double knockdown of BCL11A and DNMT1, while double knockdown of Myb and DNMT1 had minimal effect on DsRed expression relative to scrambled control (shaded).

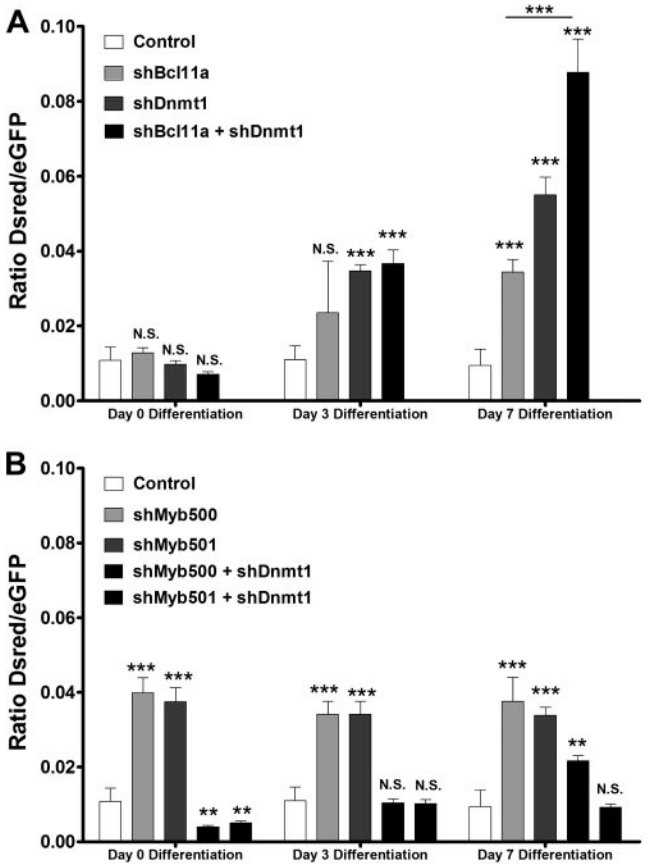

Figure 4. DsRed:eGFP ratio at different time intervals following shRNA treatment. Comparisons of DsRed fluorescence relative to eGFP (DsRed:eGFP) in the MEL $^{G}\gamma$RedβeGFP cell line, as measured by flow cytometry, following shRNA knockdown of BCL11A and DNMT1 (*A*) and Myb and DNMT1 (*B*). As seen on d 7 of differentiation, a significant increase in the DsRed:eGFP ratio was observed following double knockdown of BCL11A and DNMT1 relative to BCL11A and DNMT1 alone or scrambled control. A significant increase in the DsRed:eGFP ratio was observed following Myb knockdown, while double knockdown of Myb and DNMT1 had minimal effect on DsRed:eGFP ratio relative to scrambled control. Data represent means ± SD *vs.* control of ≥3 independent experiments. N.S., not significant. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

γ-globin regulators resulted in an increase in activity of the γ-globin promoter relative to that of β-globin.

Knockdown of the 3 regulators was undertaken in combination to gain insight into their functional relationships. Knockdown of BCL11A in combination with DNMT1 resulted in an approximately additive increase in the DsRed:eGFP fluorescence ratio, reflecting the results observed when either of these regulators was inhibited individually (Fig. 4). However, simultaneous knockdown of Myb and DNMT1 decreased the DsRed:eGFP ratio to levels less than those observed in cells in which only one of the regulators was depleted.

BCL11A and DNMT1 cooperate in silencing γ-globin and βh1-globin gene expression

Since the modified β-globin locus retains the native $^{A}\gamma$-globin, expression of fetal globin was assessed by qRT-PCR of the unmodified $^{A}\gamma$-globin gene. Following BCL11A knockdown, we noted a substantial increase in $^{A}\gamma$-globin expression (**Fig. 5**), and a concomitant reduction in expression of eGFP mRNA from the β-globin promoter. When BCL11A knockdown was combined with DNMT1, we noted a further increase in the DsRed:eGFP fluorescence ratio and $^{A}\gamma$-globin gene expression compared to BCL11A or DNMT1 knockdown alone, suggesting BCL11A and DNMT1 work collectively to silence γ-globin. Furthermore, we also measured ε-globin gene expression following BCL11A and DNMT1 depletion. While a small but significant increase in ε-globin gene expression was measured following BCL11A and DNMT1 knockdown, it was considerably lower than that observed for the $^{A}\gamma$-globin gene (Fig. 5).

We next extended our investigation to examine the expression pattern of the endogenous murine embryonic β-like globin (εy- and βh1-globin) genes. Following BCL11A depletion, we noted a small but significant increase in both εy-globin and βh1-globin gene expression (4.9 and 9.3%, respectively; Fig. 5). Interestingly, DNMT1 depletion resulted in a substantial reactivation of βh1-globin expression (52.0%), whereas there was only a modest increase in εy-globin gene expression (10%) (Fig. 5). Moreover, when both BCL11A and DNMT1 were depleted, we noted a synergistic reactivation of βh1-globin gene expression (64.0%), with only minimal effect on εy-globin gene expression (12.0%) (Fig. 5). Furthermore, depletion of both BCL11A and DNMT1 led to a significant reduction of the endogenous murine β-globin genes (76.0% reduction), which is consistent with the observed transcriptional decline in human β-globin promoter activity. This study provides important additional evidence to support the hypothesis that BCL11A and DNMT1 cooperate to silence γ-globin gene expression and its murine counterpart βh1-globin, whereas BCL11A and DNMT1 only weakly cooperate to reactivate ε-globin and its endogenous murine counterpart εy-globin in the adult erythroid environment.

Myb and DNMT1 cooperate in silencing ε-globin and εy-globin gene expression

Since Myb also plays a pivotal role in γ-globin gene expression, we next wanted to determine whether Myb and DNMT1 function collaboratively in silencing γ-like globin genes. We first examined DsRed and eGFP expression following Myb or DNMT1 knockdown. As previously noted, knockdown of Myb led to a substantial increase in $^{G}\gamma$-globin promoter-linked DsRed expression. However, when we examined the double Myb and DNMT1 knockdown, using two independent lentiviral shMyb constructs, we unexpectedly observed only a modest increase in DsRed reactivation relative to cells treated with scrambled control shRNA (Fig. 3). To further examine this reduced response and the role of Myb and DNMT1 in globin gene regulation, we used qRT-PCR to investigate the expression pattern of en-

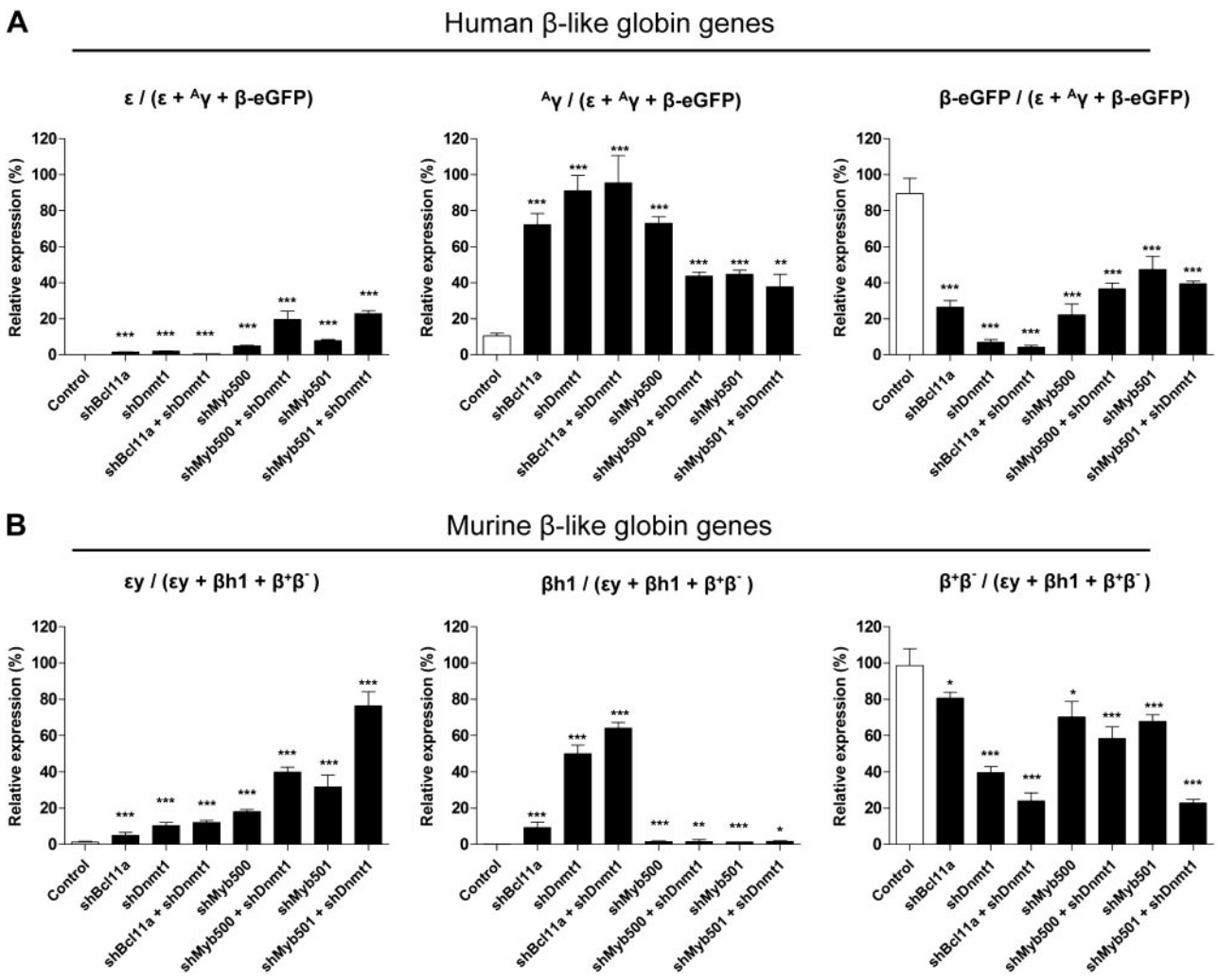

Figure 5. Human and murine globin mRNA expression levels following shRNA treatment. *A*) Measurement of human globin transgene expression in MEL ^{G}γRedβeGFP cells following shRNA-knockdown of BCl11A, DNMT1, and Myb. A significant increase in human ε- and γ-globin gene expression was observed following knockdown of BCL11A, DNMT1 and Myb. A greater increase in γ-globin gene expression was observed following double knockdown of BCL11A and DNMT1 relative to BCL11A and DNMT1 alone. Increase in γ-globin and ε-globin gene expression was associated with a decrease in β-eGFP expression. *B*) Measurement of murine globin gene expression following RNAi knockdown of BCL11A, DNMT1, and Myb. A significant increase in murine εy- and βh1-globin expression was observed following shRNA-knockdown of BCL11A, DNMT1, and Myb. A greater increase in βh1-globin gene expression was observed when BCL11A was knocked down in conjunction with DNMT1, whereas a robust increase in εy-globin gene expression was observed following double knockdown of Myb and DNMT1. Increase in εy- and βh1- globin gene expression was associated with a decrease in murine β-globin gene expression. Data represent means ± SD *vs.* control of ≥3 independent experiments. Human or murine β-like globin gene expression is presented as a percentage of total β-like globin transcripts. Analysis of human β and γ corresponds to eGFP and ^{A}γ-globin mRNA, respectively. Relative expression of individual globin genes is presented in Supplemental Figs. S4 and S5. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

dogenous murine and human β-like globin genes in the murine adult erythroid environment. We first examined the expression of the embryonic β-like globin genes. Surprisingly, knockdown of Myb together with knockdown of DNMT1 produced a significant induction in εy- and ε-globin gene expression, with the shMyb501 and shDNMT1 combination producing the strongest reactivation (76.3 and 22.3%, respectively; Fig. 5). Interestingly, βh1-globin gene expression remained largely resistant to reactivation, while induction of ^{A}γ-globin was significantly lower when compared to BCL11A and DNMT1 knockdown. Collectively, our results illustrate that under normal erythroid conditions Myb and DNMT1 repress εy- and ε-globin gene expression while BCL11A and DNMT1 cooperate to substantially repress βh1-globin and ^{A}γ-globin gene expression.

DISCUSSION

Elevated expression of HbF has the capacity to provide a clinically significant reduction in the symptoms of β-thalassemia and SCA. Consequently, the investigation of the postnatal silencing of γ-globin has uncovered a remarkably complex network of regulators responsible

for globin switching. However, further improvements in our understanding of these regulatory networks hold the key to devising targeted and more effective therapies for β-hemoglobinopathies. In this study, we take advantage of a previously described reporter system consisting of the intact genomic 183-kb human β-globin locus in an adult murine erythroid environment (21). Notably, the concurrent expression of DsRed and eGFP fluorescent reporter genes, under the control of the $^{G}\gamma$ and β-globin promoters, respectively, permit corresponding globin gene expression to be measured in a developmental-stage-specific manner. Our aim was to use the fluorescent assay system to examine the mechanisms governing γ-globin gene expression. In addition, the multifunctional aspect of this cell line permitted parallel investigation of human and murine β-like globin gene regulation following RNAi knockdown of key regulatory elements.

In this study, we focused on Myb, BCL11A and DNMT1, as these factors have previously been identified to be associated with fetal globin gene silencing. Myb is an essential hematopoietic transcription factor that acts as a master regulator, highly expressed in immature, proliferating cells of all hematopoietic lineages and down-regulated during terminal differentiation (39). In human erythroid progenitor cells, reduced levels of Myb expression results in increased HbF synthesis, while increased expression of Myb in the human embryonic/fetal K562 cell line inhibits γ-globin expression (27, 40). Myb promotes the expression of a critical erythroid transcription factor, KLF1. KLF1, in turn, influences hemoglobin switching directly by activating β-globin expression through binding to sequences in the β-globin promoter (35). More recently, KLF1 has been demonstrated to directly regulate Myb *via* an intergenic enhancer at −80 kb (29) and also directly regulates KLF3 and KLF8, known repressors of γ-globin gene expression in definitive erythroid cells (35, 41). Furthermore, KLF1 plays an indirect role in silencing γ-globin by driving the expression of BCL11A, which plays a crucial role in hemoglobin switching (42). BCL11A coordinates hemoglobin switching by interacting with erythroid transcription factors GATA1, FOG1, and SOX6, and with the NuRD complex (12, 19). In addition, BCL11A occupies critical sites within the β-globin locus, including the upstream locus control region (LCR) and γδ-intergenic regions of the β-globin cluster in adult human erythroid progenitors. This interaction is believed to support long-range interactions between the LCR and the β-globin gene, promoting an epigenetic state favoring β-globin gene expression at the expense of γ-globin (12, 43).

A recent study of a mouse model expressing the Hbs1l-Myb HPFH phenotype implicated the TR2 and TR4 orphan nuclear receptor genes as components of the Myb-mediated regulation of ε- and γ-globin gene expression (30). TR2 and TR4 have previously been identified as the core DNA-binding subunits of a large macromolecular repressor complex, DRED, that suppresses ε- and γ-globin gene expression in definitive erythroid cells (31, 32). TR2 and TR4 form heterodimers or homodimers and recognize direct-repeat (DR) promoter elements comprised of AGGTCA sequences separated by 0–6 nt. The human ε-globin promoter contains 2 DR elements, while the γ-globin promoter only contains 1 DR element. The promoters of the mouse εy and βh1 genes contain equivalent regions (32).

In this study, following Myb knockdown in the MEL reporter system, a significant reduction of TR2 and TR4 expression was detected (Fig. 1), which was associated with increased expression of ε- and γ-globin genes (Fig. 5). Interestingly, we also noted a significant reduction of KLF1 and BCL11A gene expression, which is consistent with observed changes in human erythroid progenitor cells following Myb knockdown (30). However, this is at odds with the work of Suzuki *et al.* (30), which indicates that the Myb-KLF1 pathway is not effective in the murine environment. Notably, we observed that the Myb-KLF1-BCL11A and Myb-TR2/TR4 pathways are both active in the murine background but very much dependent on Myb expression levels (Fig. 1). We therefore suggest that the discrepancy between our study and that of Suzuki *et al.* (30) may be due to the different level Myb expression achieved in their experimental murine systems. The Hbs1l-Myb$^{Tg/+}$ transgenic mouse investigated by Suzuki *et al.* (30) displayed a small reduction of Myb expression but only in a small subset of analyzed erythroid cells, while KLF1 expression remained largely unaltered, leading to the conclusion that Myb in mouse is not an upstream regulator of KLF1. However, when Suzuki *et al.* (30) investigated RNAi-mediated knockdown of Myb in human erythroid cells, a substantial reduction in Myb (>80%), and KLF1 (>40%), expression was achieved, consistent with our observation in MEL cells (Fig. 1).

Recent epigenetic profiling of the β-globin locus has provided further insights into globin gene regulation. Perhaps one of the best-characterized epigenetic factors associated with globin gene expression is DNA methylation. DNMT1 was shown to methylate the β-globin promoter in embryonic and fetal development, and subsequently modifies the γ-globin promoter in adulthood. The role of DNMT1 in HbF repression is further demonstrated by the observation that siRNA depletion of DNMT1 in baboon erythroid progenitor cells results in a reduction in methylation of the ε- and γ-globin gene promoters, leading to elevated expression of both genes (16, 36). In addition, DNMT1 inhibitors such as decitabine have been shown to hypomethylate the γ-globin promoter and induce HbF in model systems and patients (44, 45).

Recently, a proteomic screen for corepressors of γ-globin showed DNMT1 to associate with BCL11A (19). Our in-depth analysis of DNMT1 and BCL11A revealed that knockdown of either increased γ-globin gene expression and to a lesser extent ε-globin. However, when both DNMT1 and BCL11A were knocked down we noted a greater induction of γ-globin expression, mirrored by its murine counterpart, βh1-globin

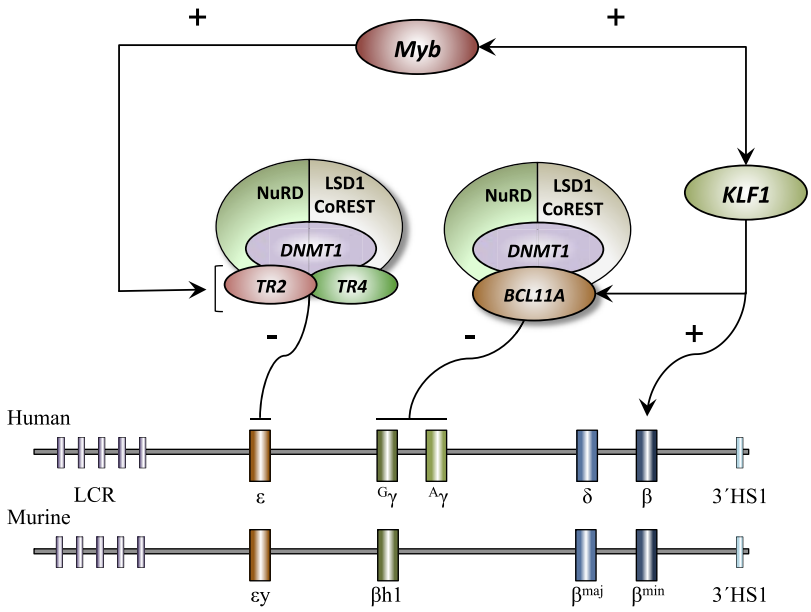

Figure 6. Schematic diagram of the human and mouse β-globin loci, summarizing the multiple regulatory networks of Myb in globin gene expression. Both human (top) and murine (bottom) β-globin loci contain multiple β-like globin genes, arranged in the order of their developmental expression. Upstream of the β-like globin genes is a cluster of erythroid-specific hypersensitive sites, known as the LCR. Myb promotes the expression of KLF1, which, in turn, influences globin switching by activating β-globin expression and indirectly repressing γ-globin gene expression *via* BCL11A gene regulation. Moreover, the regulatory network of KLF1 within erythroid progenitor cells also promotes Myb transcription (29). Myb promotes the expression of TR2/TR4, which interacts physically with DNMT1 to directly repress human ε- and murine εy-globin genes during erythroid differentiation (16). BCL11A also interacts physically with DNMT1 to collaboratively silence γ- and βh1-globin transcription in adult erythroid cells. The TR2/TR4 and BCL11A are also encompassed into multimeric transcriptional regulators comprised of LSD1/CoREST and NuRD complexes to achieve stage-specific repression of embryonic and fetal globin-like genes (Adapted from Tanabe *et al.*, ref. 32; Cui *et al.*, ref. 16; and Xu *et al.*, ref. 12).

gene (Fig. 5). Moreover, our results provide further support that BCL11A and DNMT1 cooperate in silencing γ-globin gene expression. Furthermore, our observations are in good agreement with the recent study by Xu *et al.* (19), who showed that compound-knockout mice for both BCL11A and DNMT1 genes could further augment γ-globin gene reactivation in mice carrying the human β-globin locus.

Since Myb is located upstream in the KLF1-BCL11A pathway, we hypothesized that the combination of Myb and DNMT1 knockdown would similarly synergize to reactivate γ-globin gene expression. Surprisingly, we noted lower levels of DsRed and ^{A}γ-globin gene reactivation following double knockdown of Myb and DNMT1, relative to Myb or DNMT1 knockdown alone. On further investigation, it was noted that knockdown of Myb and DNMT1 in combination had a far greater effect on ε- and εy-globin reactivation. This intriguing preferential reactivation of ε- and εy-globin reactivation can be explained by the differential binding affinities of the Myb-regulated TR2 and TR4 factors to ε- and εy-globin promoters *vs.* those of the γ- and βh1-globin genes. As described above, the human ε- and murine εy-globin promoters contain 2 DR elements, while human γ- and murine βh1-globin promoters have only 1 DR element (32). Furthermore, the DNA-binding affinity of TR2 and TR4 to the εy-promoters is higher than the βh1-promoter region, suggesting that binding affinity is a function of DR copy number (16, 36). Interestingly, Cui *et al.* (16) recently reported that the binding of TR2 and TR4 to the εy-globin promoter was enhanced on MEL cell differentiation, whereas their binding to the βh1-globin promoter was diminished after differentiation. This differential TR2/TR4 binding to the εy-globin *vs.* βh1-globin DR elements further supports our data, which suggest that Myb/DNMT1 preferentially elicits adult stage-specific silencing of ε- and εy-globin *via* the TR2/TR4 transcriptional corepressors. Furthermore, TR2 and TR4 associate specifically with an array of well-defined transcriptional corepressors to silence ε-like globin genes through epigenetic modifications (16). Notably, TR2 and TR4 have been shown to physically interact with DNMT1, as does BCL11A, strongly supporting the functional significance of this interaction in maintaining specific silencing of ε- and εy-globin gene in adult erythroid cells (16).

In this study, we demonstrate the utility of the multifunctional MEL cell-based fluorescence reporter assay system to assess murine and human globin gene regulation following knockdown of Myb, BCL11A, and DNMT1. We conclude that Myb and BCL11A display differential roles in maintaining stage-specific silencing of embryonic and fetal β-like globin gene expression in an adult erythroid environment. Since Myb is not only located within the KLF1-BCL11A pathway but also regulates TR2/TR4, components of the DRED complex, our studies further reveal the complex nature of globin switching (**Fig. 6**). Notably, further characterization of the function of epigenetic cofactors such as NuRD and LSD1/CoREST will serve to further delineate the chromatin modifications responsible for maintaining stage-specific silencing of embryonic and fetal β-like globin gene expression. In addition, a comprehensive analysis of this system will significantly enhance our understanding of the regulatory networks that govern globin gene expression. Moreover, this cellular assay can potentially facilitate the discovery and evaluation of new therapeutic targets for the treatment of β-hemoglobinopathies. FJ

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Transcriptional regulators Myb and BCL11A interplay with DNA methyltransferase 1 in developmental silencing of embryonic and fetal β-like globin genes

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