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Transcriptional silencing of fetal hemoglobin expression by NonO

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ABSTRACT

Human fetal globin (γ-globin) genes are developmentally silenced after birth, and reactivation of γ-globin expression in adulthood ameliorates symptoms of hemoglobin disorders, such as sickle cell disease (SCD) and β-thalassemia. However, the mechanisms by which γ-globin expression is precisely regulated are still incompletely understood. Here, we found that NonO (non-POU domain-containing octamer-binding protein) interacted directly with SOX6, and repressed the expression of γ-globin gene in human erythroid cells. We showed that NonO bound to the octamer binding motif, ATGCAAAT, of the γ-globin proximal promoter, resulting in inhibition of γ-globin transcription. Depletion of NonO resulted in significant activation of γ-globin expression in K562, HUDEP-2, and primary human erythroid progenitor cells. To confirm the role of NonO *in vivo*, we further generated a conditional knockout of NonO by using IFN-inducible Mx1-Cre transgenic mice. We found that induced NonO deletion reactivated murine embryonic globin and human γ-globin gene expression in adult β-YAC mice, suggesting a conserved role for NonO during mammalian evolution. Thus, our data indicate that NonO acts as a novel transcriptional repressor of γ-globin gene expression through direct promoter binding, and is essential for γ-globin gene silencing.

INTRODUCTION

The human β-globin locus consists of an array of 5 homologous globin genes (5′-ε-^{G}γ-^{A}γ-δ-β-3′), which are sequentially activated and silenced throughout development (1). The duplicated fetal γ-globin genes (*HBG1, HBG2*) are silenced shortly after birth, and adults produce predominantly adult hemoglobin (HbA, $\alpha_2\beta_2$), with less than 1% residual fetal hemoglobin (HbF, $\alpha_2\gamma_2$); this shift in expression is commonly referred to as the fetal-to-adult hemoglobin switch (1). Mutations in β-globin genes may generate an abnormal form of hemoglobin, as in sickle cell disease (SCD), or lead to impaired synthesis of β-globin chain, resulting in β-thalassemia (2). Clinical evidence indicates that elevated HbF levels associated with hereditary persistence of fetal hemoglobin (HPFH) attenuate the severity of β-hemoglobinopathies (3). Therefore, a considerable research effort has focused on elucidating the molecular processes of the fetal-to-adult globin switch, in part, with the aim of enabling reactivating γ-globin gene expression in β-hemoglobinopathy patients.

During development, expression of the γ-globin genes is coordinately controlled by *cis*-regulatory elements and *trans*-acting factors, including DNA binding sites within the promoter of γ-globin genes and lineage-specific transcription factors or cofactors, such as GATA1, KLF1, MYB, CP2, NF-E4, TR2/TR4, LYAR, BCL11A and ZBTB7A (4–16). Numerous nuclear regulatory factors controlling HbF levels have been identified, among which, BCL11A is known to account for the majority of γ-globin

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silencing (17–19). The path toward elucidation of the regulatory behaviors of BCL11A in repression of γ-globin genes has been tortuous (7,17,20,21). Initial research showed that BCL11A achieves its regulatory effect on the expression of γ-globin genes through long range interactions and cooperation with SOX6 (7,8,22). By using a CUT&RUN approach to map protein binding sites in erythroid cells, recent studies demonstrated that BCL11A preferentially occupies the distal TGACCA motif on the γ-globin promoter (8). SOX6 was first observed to be involved in the fetal-to-adult globin switch in mice (23). ChIP-Seq data showed that SOX6 strongly binds the ^{G}γ and ^{A}γ proximal promoter (7), although a SOX6 binding motif has not been identified in either the ^{G}γ or ^{A}γ proximal promoter, and the specific regulatory action mediated by SOX6 in γ-globin silencing remains elusive.

In this study, we describe a novel mechanism of γ-globin gene regulation by NonO (also known as p54nrb), a multifunctional DNA/RNA binding protein implicated in transcriptional regulation, mRNA splicing, DNA damage response, circadian rhythm, neuronal development, paraspeckle formation, and innate immune sensing of the HIV capsid in the nucleus (24–28). Through protein-interaction analysis, we found that NonO can interact physically with SOX6. Depletion of NonO significantly reactivated the expression of γ-globin in K562, HUDEP-2 and primary adult human erythroid progenitor cells. We found that NonO bound directly to the core octamer motif, ATGCAAAT, of the γ-globin proximal promoter, accounting for the repressive effect of NonO on transcription of the γ-globin gene. In addition, the action of NonO in regulating globin expression was similar in both humans and mice, indicating its function is evolutionarily conserved.

MATERIALS AND METHODS

Cell cultures

HEK293T cells, K562 cells, MEL cells, and human CD34^{+} primary erythroid progenitors from healthy donors were cultured as described previously (13,29). Briefly, primary human CD34^{+} cells were isolated from healthy human adult peripheral blood (PB) mononuclear cells by magnetic sorting. Cells were cultured initially for expansion in StemSpan SFEM II medium supplied with 1× CC100 cytokine mix (StemCell Technologies, Inc.) and 2% penicillin/streptomycin for 6 days, and cells were then transferred into the same medium with SCF (20 ng/ml), EPO (1 IU/ml), IL-3 (5 ng/ml), dexamethasone (2 μM), β-estradiol (1 μM) and 2% penicillin/streptomycin. Cells were maintained at a density of 1×10^{6} cells per milliliter, and cultures were supplemented every other day with fresh medium during a 12-day differentiation stage. After differentiation, cell surface marker analysis with CD71 and glycophorin A (GPA) indicated that more than 95% of cultured cells were of the erythroid lineage.

HUDEP-2 cells (30) were expanded in StemSpan SFEM II (StemCell Technologies, Inc.) medium supplied with doxycycline (1 μg/ml), dexamethasone (10^{-6} M), SCF (50 ng/ml), EPO (3 IU/ml), 1% L-glutamine and 2% penicillin/streptomycin.

Experimental animals

Transgenic mice carrying a human β-YAC have been described previously (31). β-YAC mice were crossed with mice containing a *NonO* floxed allele (with *lox*P sites flanking exon 4). To obtain the *NonO* conditional knockout (*NonO*$^{fl/fl}$::Mx1-*Cre*$^{+}$::β-YAC) mice, the *NonO*$^{fl/fl}$::β-YAC or *NonO*$^{fl/Y}$::β-YAC mice were crossed with Mx1-*Cre* transgenic mice (32). Poly (I:C) was prepared at 2 mg/ml in PBS and administrated via intraperitoneal (i.p.) injection every other day at the dose of 25 mg/kg for a total 7 days (4 treatment course). The mice were allowed to recover from the induced interferon response for an additional 7 or 14 days before being analyzed. All animal care and handling procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Ethical and Welfare Committee of Nanjing University (Nanjing, China).

Mass spectrometry and protein interaction studies

Anti-FLAG M2 affinity gel immunoprecipitates from FLAG-SOX6 overexpressing K562 cells were separated on SDS-PAGE and stained with Coomassie brilliant blue (CBB). Protein bands of interest were excised and subjected to LC–MS/MS analysis. Immunoprecipitation, immunoblotting, and Glutathione S-transferase (GST) pull-down assays were performed as described previously (12,33). For the SOX6/NonO interaction studies, anti-FLAG M2 agarose (Sigma) was incubated with nuclear extracts in lysis buffer containing 50 mM Na-HEPES, 150 mM NaCl, pH 7.5, 10% glycerol, 1 mM EDTA, 0.5 mM DTT, 0.5% Triton X-100, 1 mM PMSF, and a protease inhibitor cocktail (Sigma) from K562 cells to pull down the FLAG-tagged protein and associated proteins. Specifically bound proteins from stringently washed beads with lysis buffer were visualized by western blot with anti-NonO or anti-FLAG antibody after SDS-PAGE. Antibodies used were: NonO (Millipore, 05–950; or validated homemade polyclonal antibody), SOX6 (Abcam, ab30455), γ-globin (Abcam, ab137096), BCL11A (Abcam, ab19487), HA (Roche, 12CA5), FLAG (Proteintech, 20543-1-AP), GAPDH (ABclonal, AC002).

Lentiviral RNAi and CRISPR

Lentiviral shRNA constructs in the pLL3.7 vector were cloned by inserting the siRNA target sequences of RNA interference with *Xho* I/*Hpa* I sites according to the manufacturer's recommendations (American Type Culture Collection, USA). The targeting oligonucleotides were:

NonO-KD1 (human): GGACCAGTTAGATGATGAA;

NonO-KD2 (human): GCGAAGTCTTCATTCATAA;

SOX6-KD (human): GGATCTCGCTGGAAATCAA;

BCL11A-KD (human): ACGCACAGAACACTCATGGATT;

NonO-KD (mouse): GGACCAGTTAGATGATGAA

For overexpressing SOX6 or NonO, human SOX6 and NonO cDNAs were each cloned into retroviral vector plasmids MSCV-IRES-GFP with unique *EcoR I* and *Xho I* sites.

Retrovirus or lentivirus production in HEK293T cells and infection of K562, HUDEP-2, MEL, or primary human CD34$^+$ cells were performed as described previously (33,34). CD34$^+$ cells were infected with viral supernatants at Day 6 and transduced cells were selected by FACS for GFP expression at Day 12, and were cultured for one more week before collection. Transduced K562, HUDEP-2 and MEL cells were also selected by FACS for GFP expression.

For CRISPR–Cas9 genome editing, a plasmid pSpCas9(BB)-2A-GFP (PX458, Addgene plasmid ID 48138) encoding both the Cas9 protein and the sgRNA was used. CRISPR-Cas9 gene targeting was carried out as previously described (35). The single knockout clones were isolated, and were then confirmed by immunoblotting showing undetectable NonO protein. Frameshifts around the targeted regions in KO cell lines were also verified by sequence analysis. The guiding RNA sequences for NonO knockout were: CTGGACAATATGCCACTCCG.

Cell growth assay, luciferase reporter assay, EMSA, Q-RT-PCR and ChIP Analysis

Cell Counting Kit-8 (CCK-8, Vazyme Biotech Co., Ltd, Nanjing, China, A311-01) was used to assess the *in vitro* viability of the cells following the manufacturer's recommendations. Luciferase reporter assays and EMSA were performed as described previously (13). Q-RT-PCR and ChIP assays were performed as described previously (33). A plasmid DNA encoding γ- or β-globin template was used to generate the standard curve (20–2 000 000 copies) for determination of their copy numbers. Primers for Q-RT-PCR and ChIP are listed in Supplementary Table S1.

Hemoglobin analysis

To assess the expression level of HbF in differentiated CD34$^+$ cells, intracellular staining was performed using an anti-human antibody for HbF (clone HbF-1 with APC conjugation, Life Technologies) according to the protocol of the manufacturer.

RESULTS

Identification of NonO as a SOX6 interacting protein

BCL11A and SOX6 co-occupy the human β-globin cluster and in concert with GATA1 change its conformation, and cooperate in silencing γ-globin transcription in the maturation of human erythroid lineage cells, findings that establish SOX6 as a new master repressor of γ-globin (7,36,37). To identify other potential proteins interacting with SOX6, we performed immunoprecipitation experiments using anti-FLAG M2 affinity Sepharose beads to precipitate SOX6. Associated proteins from nuclear extracts of K562 cells stably overexpressing FLAG-tagged SOX6 were analyzed by mass spectrometric peptide sequencing. NonO (24), a DNA/RNA-binding protein paraspeckle component (25), was identified as a SOX6 interacting protein, and was chosen for further study (Figure 1A and Supplementary Table S2). Of note, we also found that another paraspeckle component SFPQ (25), a NonO heterodimer, was also present. The protein NonO was of particular interest, as it has been found to be co-localized with BCL11A to form paraspeckles in nucleus (38–40) and also identified as a component of the NuRD complex (41) which functions to repress γ-globin gene expression (9,42), albeit not an erythroid-specific protein. The co-immunoprecipitation (Co-IP) assay between NonO and SOX6 performed in HEK293T cells confirmed an interaction between NonO and SOX6 (Figure 1B and C). Further, a GST-pull down assay showed that these two proteins interacted with each other directly (Figure 1D). To further characterize the determinants mediating the association between SOX6 and NonO, we mapped the SOX6 protein domains by Co-IP assays of FLAG-tagged SOX6 expressed transiently in HEK293T cells (Figure 1E and F). These studies revealed a broad region in SOX6 (two terminal domains including a Leucine zipper and an HMG box) which was sufficient for the interaction between SOX6 and NonO, indicating that NonO could interact with SOX6 through multiple domains. Taken together, these results identified NonO as a new interacting protein of SOX6.

NonO represses γ-globin transcription in K562 and HUDEP-2 cells

Since SOX6 has been shown to be able to silence human γ-globin expression and mouse embryonic εy-globin/βh1-globin expression (7,23,43), we next asked whether NonO could regulate γ-globin gene expression as well. We generated two stable NonO knockdown (NonO-KD) K562 cell lines (KD1 and KD2) using specific shRNAs and a scrambled (SCR) control lentiviral constructs. Endogenous NonO and γ-globin protein levels were assessed by western blot, and mRNA levels were quantified by Q-RT-PCR of total RNA from these cells (Figure 2A and B). We found that the expression level of γ-globin was significantly increased in NonO-KD cells compared with SCR cells (Figure 2C), as we expected as a consequence of the reduced protein and mRNA levels of NonO in the NonO-KD cells. Interestingly, Q-RT-PCR also showed that NonO knockdown resulted in a dramatic increase in embryonic ε-globin gene expression (Figure 2C). To further confirm these results, we established two NonO knockout (NonO-KO) K562 cell lines (KO1 and KO2) using one specific guide RNA by CRISPR-mediated knockout. The two NonO knockout cell lines were subjected to Sanger sequencing, and the resulting sequencing data showed that one cell line was homozygous with an identical 1nt insertion on both alleles (KO1), and another line was compound heterozygous with different mutations on two alleles (KO2), which validated the *NonO* gene editing (Supplementary Figure S1). NonO protein was undetectable in KO1 and KO2 by western blot, and the protein level of γ-globin was significantly elevated compared to K562 wild-type (WT) cells (Figure 2D). Similarly, we also observed a significant increase in γ-globin and embryonic ε-globin gene expression in these cells (Figure 2E). Of note, this effect was specific, as the expression of other known erythroid transcriptional regulators, including NF-E2, GATA1, GATA2, SOX6, BCL11A, KLF1 and PRMT5, exhibited no change between the WT and knockout K562 cells (Supplementary Figure S2A). Consistently, NonO depletion did not appear to alter the differentiation of K562 cells, as evidenced by cytospin assays of cell

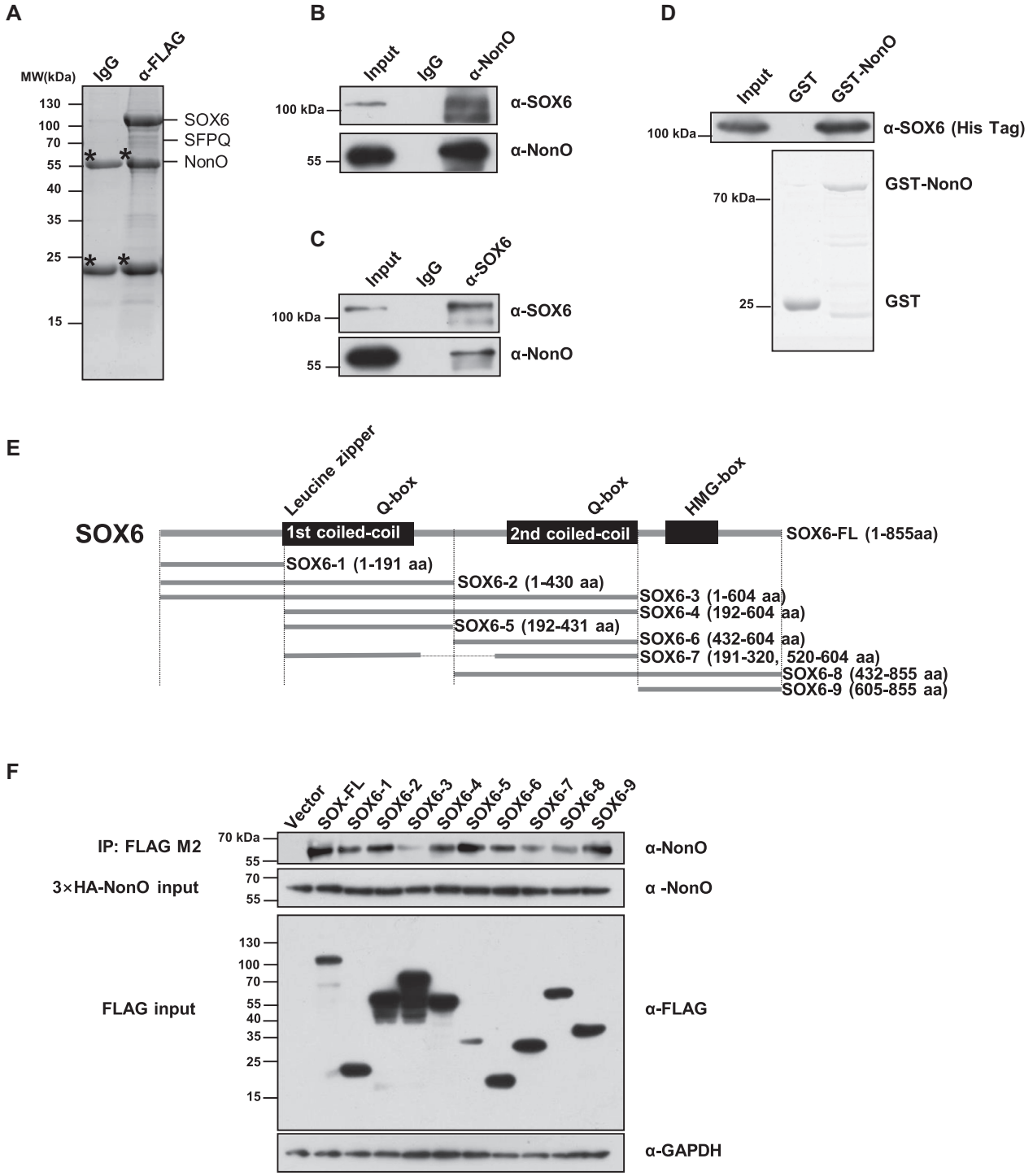


Figure 1. Identification of NonO as a SOX6 interacting protein. (**A**) Coomassie brilliant blue (CBB) stained gel of proteins separated on SDS-PAGE after anti-FLAG M2 affinity gel or IgG (control) immunoprecipitation from K562 cells stably overexpressing FLAG epitope-tagged SOX6 before analysis by mass spectrometry. The bands corresponding to NonO, SOX6, and the known NonO partner protein SFPQ are shown. Asterisks indicate immunoglobulin chains. (**B, C**) Endogenous NonO is co-immunoprecipitated with overexpressed SOX6 in HEK293T cells. Western blot probed with anti-SOX6 or anti-NonO antibodies. IgG serves as a negative control. (**D**) GST-pull down assay (top). Prokaryotic GST and GST-NonO fusion proteins pre-absorbed to Glutathione-Sepharose beads were incubated with purified prokaryotic His-SOX6 fusion protein. Specifically bound protein was eluted from washed beads and visualized by Western blot analysis with anti-SOX6 antibody after SDS-PAGE. Coomassie stained SDS-PAGE gel showing purified prokaryotic GST and GST-NonO fusion proteins (bottom). (**E**) Schematic diagram of SOX6 truncation constructs used in (F). (**F**) Co-immunoprecipitation (Co-IP) of 3 × HA epitope-tagged NonO co-expressed transiently with a FLAG-tagged vector, SOX6-FL (full length, 1–855 amino acids) or SOX6 truncation mutants in HEK293T cells. Whole cell extracts were immunoprecipitated with an anti-FLAG antibody, and co-purified NonO was analyzed by Western blot with an anti-NonO antibody.

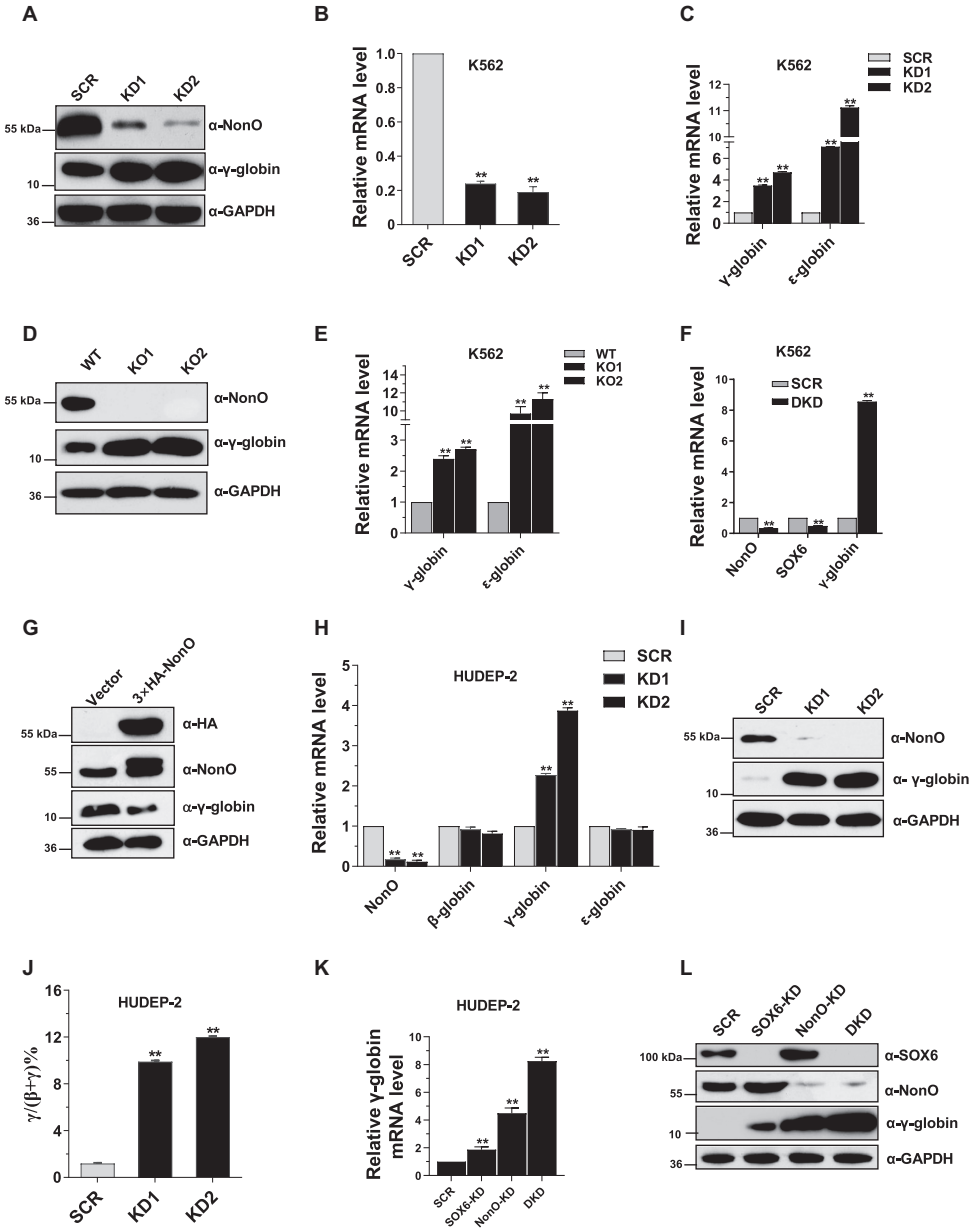


Figure 2. NonO represses γ-globin gene expression in K562 cells and HUDEP-2 cells. (**A**) Western blot analysis with indicated antibodies of cell lysates from NonO-KD1 and NonO-KD2 or SCR control K562 cells; GAPDH served as a loading control. (**B**) Relative NonO mRNA expression levels analyzed by Q-RT-PCR of RNA extracted from NonO-KD1, NonO-KD2 and SCR K562 cells. Results are normalized to GAPDH mRNA, and are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**C**) Human γ- and ε-globin gene expression was analyzed by Q-RT-PCR of RNA extracted from NonO-KD1, NonO-KD2, or SCR K562 cells. Results are normalized to GAPDH mRNA, and are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**D**) Western blot analysis with indicated antibodies of cell lysates from NonO-knockout 1 (KO1) and NonO-knockout 2 (KO2) or WT control K562 cells, GAPDH served as a loading control. (**E**) Human γ- and ε-globin gene expression was analyzed by Q-RT-PCR of RNA extracted from NonO-KO1, NonO-KO2, or WT K562 cells. Results are normalized to GAPDH mRNA, and are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**F**) Human γ-globin gene expression was analyzed by Q-RT-PCR of RNA extracted from NonO-KD and SOX6 KD (Double knockdown, DKD) or SCR K562 cells. Results are normalized to GAPDH mRNA, and are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**G**) Western blot analysis using indicated antibodies of cell lysates from K562 cells stably overexpressing 3 × HA-NonO or vector control. (**H**) Human β-, γ- and ε-globin gene expression was analyzed by Q-RT-PCR of RNA extracted from NonO-KD1, NonO-KD2, and SCR HUDEP-2 cells. Results are normalized to GAPDH mRNA, and are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**I**) Western blot analysis with indicated antibodies of cell lysates from NonO-KD1 and NonO-KD2 or SCR HUDEP-2 cells. GAPDH served as a loading control. (**J**) NonO knockdown results in elevations of γ-globin mRNA levels (as a percentage of total β-like globin gene expression) in HUDEP-2 cells. Results are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$ compared with SCR control. (**K**) Human γ-globin gene expression was analyzed by Q-RT-PCR of RNA extracted from SCR, SOX6-KD, NonO-KD, and NonO/SOX6 Double knockdown (DKD) HUDEP-2 cells. Results are normalized to GAPDH mRNA, and are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**L**) Western blot analysis with indicated antibodies of cell lysates from SCR, SOX6-KD, NonO-KD and NonO/SOX6 Double knockdown (DKD) HUDEP-2 cells. GAPDH served as a loading control.

morphologies (Supplementary Figure S3A). Similar to a previous report in mice (26), the absence of NonO did produce a delay in G2/M phase and an increase in S phase of K562 cells, which was associated with a mild slow-down in cell growth (Supplementary Figure S4A and S4B). Interestingly, we found that there was an additive repressive effect on γ-globin gene expression when NonO and SOX6 were both knocked down simultaneously (Figure 2F, Supplementary Figure S2B and S2C). To further examine the repressive role of NonO in γ-globin gene repression, we stably overexpressed 3× HA-NonO in K562 cells. We found that enforced exogenous NonO expression significantly decreased expression of γ-globin (Figure 2G). However, we observed no changes in binding of SOX6 and BCL11A at the γ-globin promoters when NonO was either knocked out or overexpressed in K562 cells (Supplementary Figure S2D).

Next, to confirm the role of NonO in γ-globin gene repression, we utilized stabilized NonO knockdown HUDEP-2 cells (Human Umbilical Cord Blood-Derived Erythroid Progenitor) (30) to analyze the hemoglobin expression profile in humans. We found similar effects of NonO on γ-globin gene repression, but no increase on ε-globin gene expression, without changes in cell morphologies (Figure 2H–J, and Supplementary Figure S3B). Consistently, there was an additive repressive effect on γ-globin gene expression when NonO and SOX6 were both knocked down simultaneously in HUDEP-2 cells (Figure 2K and L) although BCL11A knockdown showed the highest induction of γ-globin gene expression (Supplementary Figure S2E and S2F). Thus, these results indicate that NonO is a repressor of human γ-globin gene transcription.

NonO binds to the promoter of the γ-globin gene

Next, we sought to identify mechanisms by which NonO regulates γ-globin gene expression. In the proximal promoter of the γ-globin gene, we identified a NonO binding motif (ATGCAAAT) located in both ^{G}γ- and ^{A}γ-globin genes from –182 bp to –175 bp. We constructed a luciferase reporter gene driven by a 1.3 kb DNA sequence upstream of the γ-globin gene. This region has been shown to possess potent promoter activity (44). When this luciferase reporter gene was co-transfected into HEK293T cells with increasing amounts of expression vector containing FLAG-tagged NonO, we observed a significant decrease in relative luciferase activity, suggesting that the γ-globin gene promoter could be suppressed by NonO in a dose-dependent manner (Figure 3A and B). To further confirm whether NonO transcriptionally regulates γ-globin gene promoter activity by binding to ATGCAAAT, a luciferase reporter with mutations of this binding site was constructed (Figure 3B). When the wild-type (WT) reporter was co-transfected with expression vectors containing FLAG-tagged NonO or SOX6 or both into K562 cells, a significant decrease in relative luciferase activity was observed compared to the empty vector. The best inhibitory effect was obtained when both NonO and SOX6 were overexpressed (Figure 3C). However, when the mutant (Mut) luciferase reporter was used, no significant change was found related to NonO overexpression (Figure 3C). Interestingly, a significant increase in relative luciferase activity was observed compared to the empty vector when the –175 T-to-C mutant luciferase reporter was employed (Figure 3C). The reason for this could be that in the context of –175 T-to-C mutant, transcription factors GATA1 and TAL1 complex are recruited to the mutant promoter and thus alter the transcriptional scenario (45,46), or that NonO/SOX6 may sequester repressors away from the mutant promoter. In fact, it has previously been shown that NonO can function as either a transcriptional repressor or an activator, which is possibly context-dependant (25). Further work is required to understand this duality of NonO/SOX6 function in the context of –175 T-to-C mutant on the γ-globin promoter. The expression of the exogenous proteins was verified by western blot analysis (Figure 3D). To verify that NonO can occupy the γ-globin gene promoter, we performed ChIP analysis in K562 and HUDEP-2 cells; the results showed that NonO tended to be enriched in the promoter region between –386 bp and –77 bp (Figure 3E and F). These results are consistent with the NonO ChIP-seq data in K562 cells from the Gene Expression Omnibus (GEO, accession GSE106045, NCBI) (47). Subsequently, we carried out an electrophoretic mobility shift assay (EMSA) using a 31 bp labeled oligonucleotide probe corresponding to the promoter region of the γ-globin gene, containing the NonO binding motif core sequence ATGCAAAT. We found that a WT labeled probe bound to the nuclear extract from K562 cells (Figure 3G, lane 2). This binding could be ablated by competition with unlabeled WT probe, but not by competing mutant probe (Figure 3G, lanes 3, 4). A supershift band was observed when an anti-NonO antibody was added in nuclear extract incubated with WT labeled probe, but not with control IgG (Figure 3G, lanes 5, 6). These results indicate that NonO, or NonO-containing complexes, bound directly to the promoter of γ-globin gene through the motif ATGCAAAT, accounting for its repressive effect on the activity of the γ-globin gene promoter.

NonO contributes to the silencing of γ-globin gene expression in primary adult human erythroid progenitor cells

Human γ-globin genes (*HBG1*/*HBG2*) are developmentally regulated, and are ordinarily silenced after birth. Accordingly, we used human adult primary erythroid progenitor cells from bone marrow as a further model. $CD34^{+}$ hematopoietic progenitors were isolated from healthy adult peripheral blood (PB) mononuclear cells, and were cultured through an expansion stage and differentiation period in different conditional media to generate highly enriched populations (95% purity) of maturing primary erythroblasts *ex vivo* (7) (Figure 4A). We examined NonO protein levels by western blot during the maturation of primary erythroblasts, and found that NonO protein was constitutively highly expressed from early stages to late mature (day 14) erythroblasts (Figure 4B). To examine the effect of altered expression of NonO in adult bone marrow erythroid progenitors, we utilized specific lentiviral shRNA to knock down NonO expression (Figure 4C). To our surprise, the expression of the γ-globin gene increased by a robust ∼90-fold in NonO knockdown cells compared with SCR cells (the level of γ-globin mRNA as a percentage of

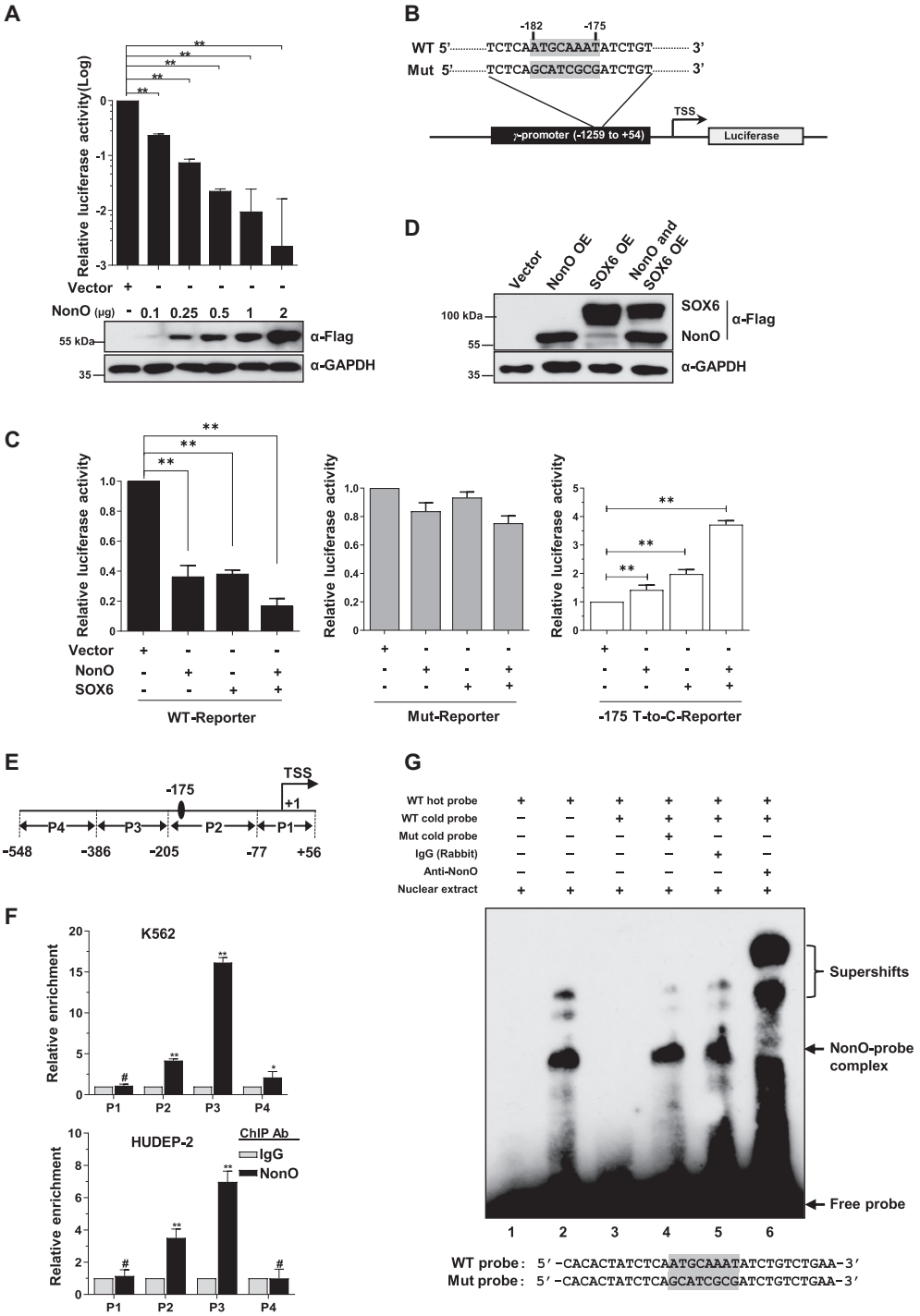


Figure 3. Identification of potential NonO binding site in γ–globin proximal promoter. (**A**) (top) Histogram shows γ–globin proximal promoter activity analyzed by relative luciferase activity in HEK293T cells transfected with an increasing amount of FLAG-NonO protein. (bottom) Western blot analysis of FLAG-NonO protein. Results are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**B**) Schematic representation of the luciferase reporter construct containing γ-globin proximal promoter region (–1259 to +54) with either wild type (WT) or mutant (Mut) potential NonO binding sites. (**C**) Histogram shows γ–globin proximal promoter activity (WT-Reporter, Mut-Reporter or –175 T-to-C Reporter) analyzed by relative luciferase activity in K562 cells transfected with either vector, overexpressed FLAG-tagged NonO, SOX6, or both. Results are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**D**) Western blot analysis of FLAG-tagged NonO and SOX6 from cell lysates of transfected K562 cells analyzed for luciferase activity in (C). GAPDH was used as a loading control. (**E**) Schematic diagram of promoter regions (P1–P4) to be amplified for ChIP analysis spanning the γ-globin proximal promoter. (**F**) ChIP (anti-NonO antibody) analysis of the enrichment of NonO on P1–P4 regions (shown in E) of the γ-globin proximal promoter in chromatin from K562 (top) and HUDEP-2 (bottom) cells. Rabbit IgG served as a negative control. The results are shown as mean ± SD of three independent experiments. Two-tailed Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P > 0.05$ compared with IgG control. Ab, antibody. (**G**) EMSA analysis of the binding of NonO to WT and Mut γ-globin proximal promoter probes (–194 to –164) using K562 nuclear extract. Sequences of WT or Mut probes are listed below.

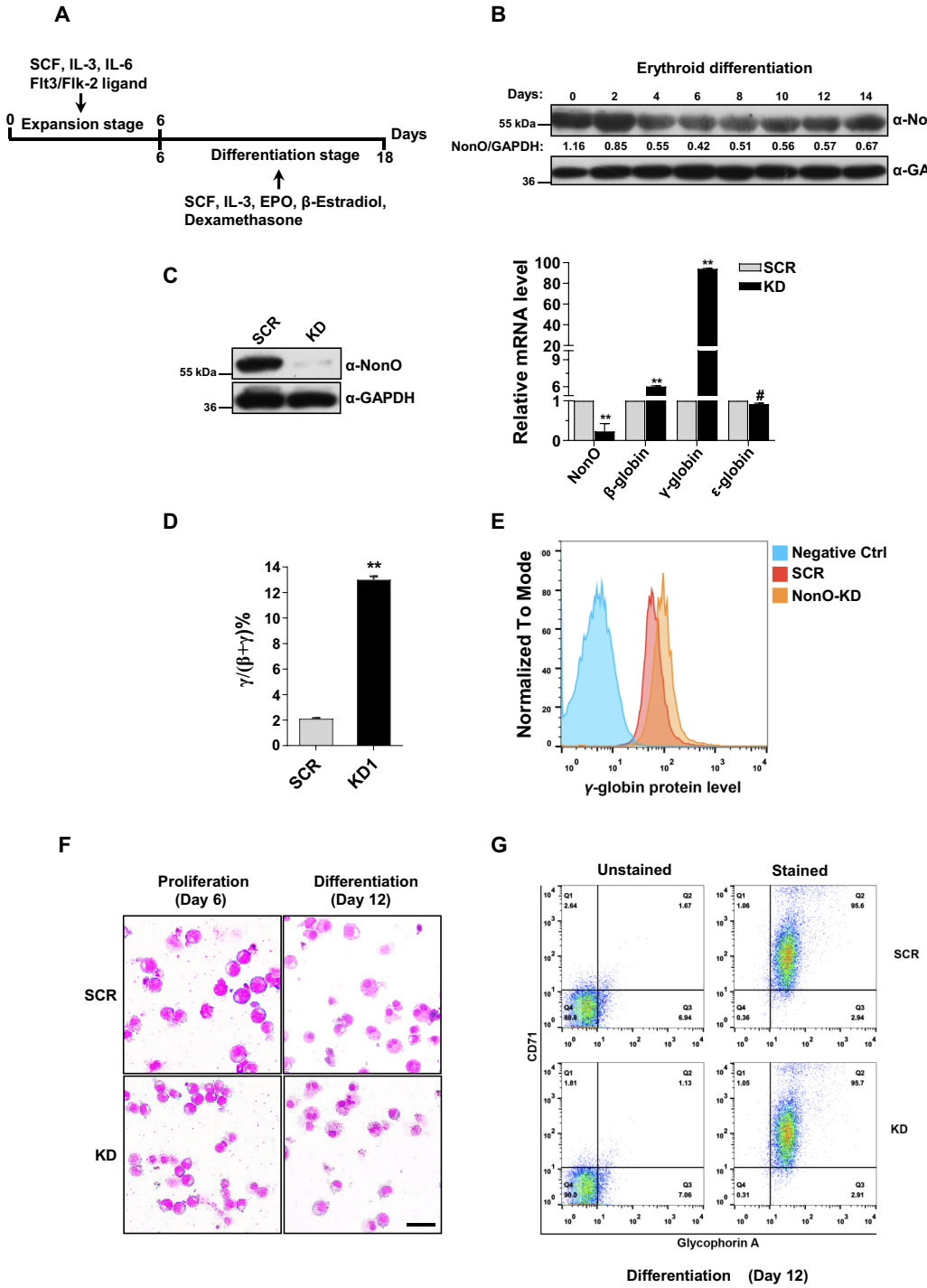


Figure 4. NonO represses γ-globin gene expression in primary adult human erythroid progenitor cells. (**A**) Schematic outline of the procedures for inducing erythroid differentiation of human peripheral blood derived $CD34^+$ cells in a serum-free, two-phase, liquid culture model. (**B**) The expression of human NonO protein measured by western blot from cell lysates of primary adult human erythroid progenitor cells. GAPDH was analyzed as a loading control. The relative band intensities shown between the blots (NonO/GAPDH) were determined using ImageJ software and normalized by the amounts of GAPDH in each sample. (**C**) Western blot analysis of NonO from cell lysates of NonO knockdown (KD) or SCR $CD34^+$ cells; GAPDH was used as a loading control (left panels). Human β-, γ- and ε-globin gene expression analyzed by Q-RT-PCR of RNA extracted from NonO-KD or SCR $CD34^+$ cells. The results are normalized to GAPDH mRNA, and are shown as mean ± SD of three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$, $^{\#}P > 0.05$ (right panels). (**D**) NonO knockdown results in elevations of γ-globin mRNA levels (as a percentage of total β-like globin gene expression) in $CD34^+$ cells. Results are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$ compared with SCR control. (**E**) FACS analysis of fetal hemoglobin (HbF) levels by intracellular staining with APC conjugated anti-human HbF antibody (clone HbF-1) in NonO-KD or SCR $CD34^+$ cells. (**F**) Representative images (Wright-Giemsa-stain) of cytospins prepared on day 6 and day 12 of culture showing the morphology of NonO-KD or SCR $CD34^+$ cells during proliferation and differentiation. Scale bar, 50 μM. (**G**) Representative FACS profiles of NonO-KD or SCR $CD34^+$ cells stained with anti-CD71 and anti-Glycophorin A on day 12 of differentiation.

total β-like globin gene expression was elevated to 12% in NonO knockdown cells compared with 2% in SCR cells), whereas the embryonic ε-globin gene expression remained largely unaffected (Figure 4C and D). We also observed several fold upregulation of β-globin gene expression in NonO knockdown cells compared with SCR cells (Figure 4C). Fetal hemoglobin (HbF) levels were analyzed by intracellular staining with HbF-1 antibodies, and the result showed that NonO knockdown significantly contributed to the induction of HbF protein (Figure 4E). Of note, NonO depletion did not appear to alter the differentiation of erythroblasts, as evidenced by similar cell morphologies, and a similar percentage of CD71 and Glycophorin A double positive mature erythroid cells compared to SCR (Figure 4F and G). These results collectively suggest that NonO may play an important role in silencing human γ-globin expression.

NonO silences mouse embryonic globin genes, and its loss reactivates human γ-globin gene expression in adult β-YAC mice

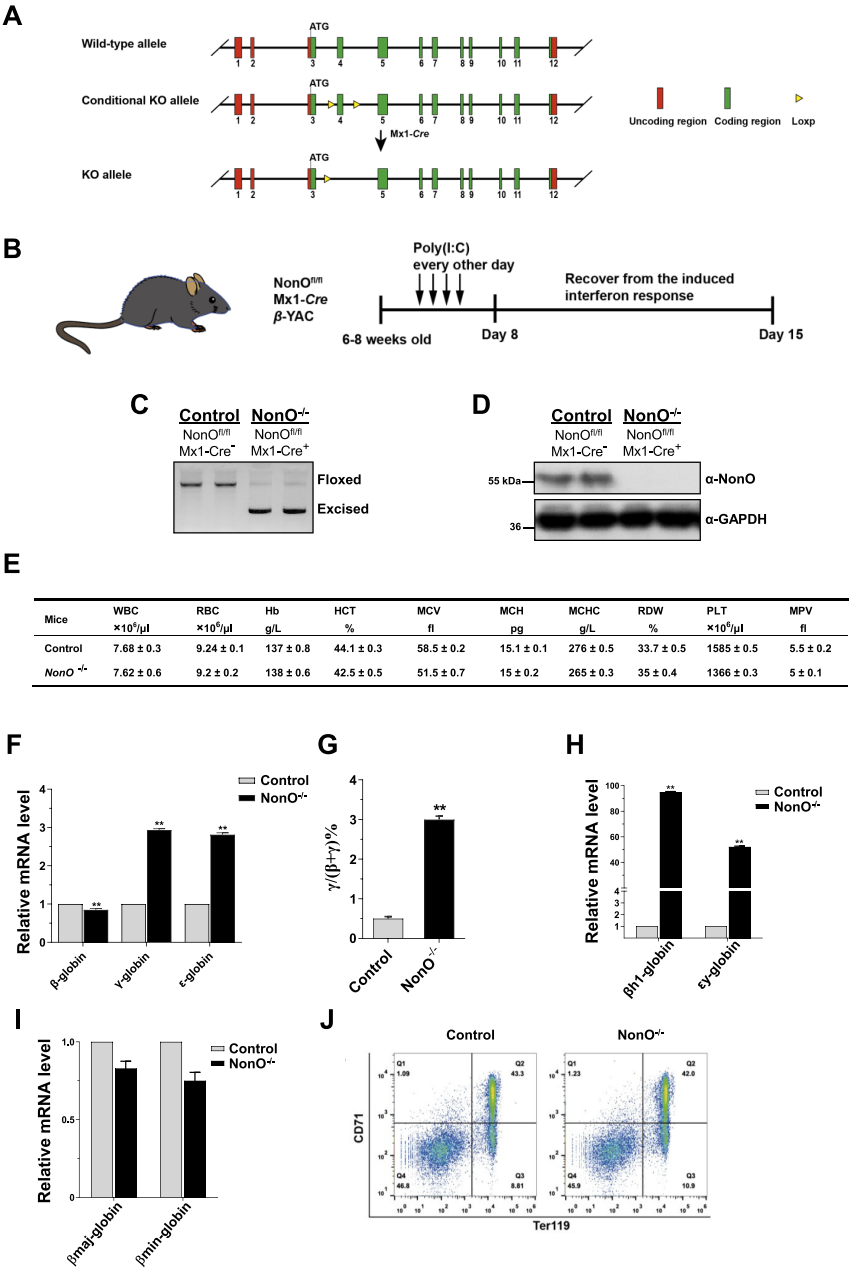
The mouse and human β-globin-like loci are highly conserved, and share many functional elements (7,48). To test whether NonO functions similarly in silencing β-globin-like genes in mouse erythroid cells, we generated lentivirus-mediated RNAi to knock down the expression of NonO in MEL cells, in which mouse embryonic εy- and βh1-globin are expressed at very low levels. Following infection with NonO shRNA lentivirus and subsequent FACS sorting for GFP^+ cells, we examined the consequences of NonO knockdown on murine embryonic globin by Q-RT-PCR. As shown in Supplementary Figure S5A, εy- and βh1-globin expression was significantly elevated upon NonO knockdown in MEL cells, whereas mouse adult β-globins (βmajor and βminor) remained largely unchanged. Knockdown of NonO at the protein level was confirmed by western blot analysis (Supplementary Figure S5B).

To address the role of NonO in regulating globin gene expression during normal development *in vivo*, we constructed a mouse line carrying a *NonO* floxed allele (with *lox*P site flanking exon 4) and harboring the human β-globin gene cluster as a yeast artificial chromosome transgene (β-YAC) and the interferon-inducible Mx1-*Cre* allele. This mouse permits examination of the contribution of NonO to the silencing of the human γ-globin gene in adult mice upon hematopoietic conditional loss of NonO (Figure 5A). Mice (6–8 weeks old) were subjected to 4 courses of poly (I:C)-treatment (Figure 5B). At the end of treatment and recovery, the efficient excision of exon 4 in the bone marrow was verified by PCR (Figure 5C). To confirm that deletion of *NonO* exon 4 resulted in the absence of NonO protein, we analyzed nuclear extracts from bone marrow by western blot. The results showed that NonO was absent from the bone marrow of $NonO^{fl/fl}$::Mx1-Cre^+::β-YAC mice (Figure 5D). These mice exhibited no changes in blood counts compared to control $NonO^{fl/fl}$::Mx1-Cre^-::β-YAC mice (Figure 5E). We then tested relative globin gene expression in bone marrow from mice by Q-RT-PCR. Expression of developmentally silenced γ-globin and ε-globin genes were significantly elevated one week after depletion of NonO, indicating that they had been derepressed (Figure 5F and G). Similarly, mouse embryonic εy-globin and βh1-globin genes were also reactivated upon NonO loss (Figure 5H), whereas mouse adult β-globins (βmajor and βminor) showed no increase (Figure 5I). Of note, NonO depletion did not perturb the overall process of erythroid differentiation, as a similar percentage of double positive (CD71 and Ter119) mature erythroid cells was detected in knockout and control mice (Figure 5J). Collectively, these results suggest that NonO modulates mouse embryonic globin gene silencing, consistent with the role of NonO in regulating γ-globin gene expression in human.

DISCUSSION

ChIP-seq analysis revealed that NonO is associated with transcriptionally active promoters of many genes (49). However, NonO can also negatively regulate transcription by sequestering activators away from target promoters or by binding to silencer motifs in promoters, suggesting that the precise role of NonO is context-dependent (25,50–54). Here, we identify NonO, a critical component of paraspeckle, as a SOX6 interacting partner and a novel repressor of γ-globin gene expression. Among various proteins active in the regulation of γ-globin genes, BCL11A is a major driver, and has been shown to physically interact with, and co-localize with, SOX6 (7) and NonO in nuclear paraspeckles (39), which further helps establish the role of NonO in the developmental γ-globin gene regulatory program.

As a non-POU-domain containing octamer binding protein, NonO was initially isolated from a murine B-cell leukemia line based on its octamer binding motif (ATGCAAAT), which mediates a functional role in regulation of immune-related genes (47). NonO has been shown to function as a chromatin regulator cooperating with Erk to regulate mouse embryonic stem cell (mESC) pluripotency by influencing self-renewing mESCs (55). Genetic studies have shown that NonO conveys circadian gating of the cell cycle important for wound healing, and plays a critical role in energy homeostasis in mice (26,56). However, the role of NonO in erythropoiesis is largely unknown. In our current study, loss of NonO resulted in a significant elevation of γ-globin gene expression in erythroid cells. Similar effects on γ-globin gene expression were observed in knockdown and knockout of NonO in K562 cells, suggesting that reducing the expression of NonO was sufficient for reactivating the expression of γ-globin genes. Intriguingly, knockdown of NonO resulted in much higher transcriptional induction of γ-globin in primary bone marrow erythroid cells than in K562 and HUDEP-2 cells (∼90-fold versus ∼3-fold) suggesting its action on transcription may be context-dependent. Encouragingly, the γ-globin expression level as percentage of total β-like globin gene expression was increased to ∼12% in NonO knockdown of primary bone marrow erythroid cells. Interestingly, knockdown of NonO led to significant transcriptional induction of ε-globin in K562 cells, but no change in transcription of ε-globin in HUDEP-2 cells or primary bone marrow erythroid cells. In line with these results, depletion of NonO in definitive bone marrow erythrocytes of mice resulted in ∼90-fold or ∼50-fold induction of endogenous embryonic βh1-globin or εy-globin, respectively, as well as ∼3-fold induction of



Mice	WBC ×10⁶/μl	RBC ×10⁶/μl	Hb g/L	HCT %	MCV fl	MCH pg	MCHC g/L	RDW %	PLT ×10⁶/μl	MPV fl
Control	7.68 ± 0.3	9.24 ± 0.1	137 ± 0.8	44.1 ± 0.3	58.5 ± 0.2	15.1 ± 0.1	276 ± 0.5	33.7 ± 0.5	1585 ± 0.5	5.5 ± 0.2
NonO -/-	7.62 ± 0.6	9.2 ± 0.2	138 ± 0.6	42.5 ± 0.5	51.5 ± 0.7	15 ± 0.2	265 ± 0.3	35 ± 0.4	1366 ± 0.3	5 ± 0.1

Figure 5. NonO silences mouse embryonic globin genes, and its loss reactivates human γ-globin gene expression in adult β-YAC mice. (**A**) Schematic diagram of Mx1-Cre mediated excision of *exon* 4 (flanked by *lox*P sites) in *NonO* locus to produce *NonO* mutant allele. Coding regions (exons) and non-coding regions are indicated in green and red boxes, respectively, *lox*P sites are represented by yellow triangles. (**B**) Experimental scheme for inducing NonO knockout in adult NonO$^{fl/fl}$::Mx1-Cre+::β^{-}YAC mice by interferon-mediated deletion induced by a 4-day course of poly (I:C) intraperitoneal (i.p.) injections (arrows). (**C**) PCR analysis indicating deletion efficiency in bone marrow cells from Control (NonO$^{fl/fl}$::Mx1-Cre^{-}::β-YAC) and NonO$^{-/-}$ knockout (NonO$^{fl/fl}$::Mx1-Cre^{+}::β-YAC) mice two weeks post-injection of poly (I:C). Ethidium Bromide-stained agarose gel is shown. (**D**) NonO protein monitored by Western blot in extracts from bone marrow from Control (NonO$^{fl/fl}$::Mx1-Cre^{-}::β-YAC) and NonO$^{-/-}$ knockout (NonO$^{fl/fl}$::Mx1-Cre^{+}::β-YAC) mice 2 weeks post-injection of poly (I:C). GAPDH was used as a loading control. (**E**) Comparison of hematologic parameters in Control (*NonO*$^{fl/fl}$::Mx1-*Cre*$^{-}$::*β*-YAC, n = 5) and NonO$^{-/-}$ knockout (*NonO*$^{fl/fl}$::Mx1-*Cre*$^{+}$::*β*-YAC, *n* = 6) mice. All animals were analyzed 8–10 weeks after birth. Data are means ± SEM. WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; PLT, platelets; MPV, mean platelet volume. (**F**) Human β-, γ- and ε-globin gene expression analyzed by Q-RT-PCR in bone marrow cells from Control (NonO$^{fl/fl}$::Mx1-Cre^{-}::β-YAC, *n* = 5) and NonO$^{-/-}$ knockout (NonO$^{fl/fl}$::Mx1-Cre^{+}::β-YAC, *n* = 6) mice two weeks post-injection of poly (I:C). Results are normalized to β-actin mRNA, and are shown as mean ± SD. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**G**) NonO knockout (NonO$^{-/-}$) results in elevations of γ-globin mRNA levels (as a percentage of total β-like globin gene expression) in bone marrow cells from mice. Results are shown as mean ± SD from three independent experiments. Two-tailed Student's *t*-test, $^{**}P < 0.01$ compared with SCR control. (**H, I**) The expression of mouse embryonic (εy, βh1, βmaj, and βmin) globin mRNA was monitored by Q-RT-PCR in bone marrow cells from Control (NonO$^{fl/fl}$::Mx1-Cre^{-}::β-YAC, *n* = 5) and NonO$^{-/-}$ knockout (NonO$^{fl/fl}$::Mx1-Cre^{+}::β-YAC, *n* = 6) mice. Results are normalized to β-actin mRNA, and are shown as mean ± SD. Two-tailed Student's *t*-test, $^{**}P < 0.01$. (**J**) Representative FACS profiles of erythroid markers CD71 and Ter119 assessed in bone marrow cells from Control (NonO$^{fl/fl}$::Mx1-Cre^{-}::β-YAC) and NonO$^{-/-}$ knockout (NonO$^{fl/fl}$::Mx1-Cre^{+}::β-YAC) mice.

transgenic human γ-globin or ε-globin. The difference in embryonic globin or γ-globin expression induction might be cell type- or developmental stage-dependent. Nevertheless, results from both K562, HUDEP-2, primary bone marrow cells, and mice erythroid cells showed that depletion of NonO did not impair the differentiation and maturation of erythroid cells, suggesting the role of NonO in γ-globin gene repression is specific.

NonO protein contains a potential HTH domain followed by a region high in charged amino acids, which facilitates binding of dsDNA (47). There exists an essential octamer motif (–182 bp ATGCAAAT –175 bp) in the proximal promoter region of γ-globin genes, where a T/C substitution at position –175 bp in this octamer sequence changes the ability of some binding proteins (GATA1 or TAL1) to bind the γ-globin promoter accounting for the HPFH mutation (45,46,57). Here, we show that NonO silences γ-globin gene expression by directly binding to this octamer motif in the proximal promoter of γ-globin genes and that –175 T/C HPFH mutation ablates the repressive effects of NonO on γ-globin gene transcription in reporter gene assays. It would be of interest to determine whether the –175 T/C HPFH mutation is associated with alteration of NonO binding.

Several models have been proposed to depict the mechanism of fetal-to-adult hemoglobin switch, including gene competition, autonomous gene silencing, chromosomal looping, and tracking (1,58). With development of innovative research techniques, BCL11A was shown to mediate its repressive function via directly binding to the –115 sites of the γ-globin promoter; these sites largely correspond to the site of the HPFH mutation in the –115 region (8,22). Our study demonstrated that depletion of NonO derepressed γ-globin gene expression without changing activities of key γ-globin regulatory factors including BCL11A, KLF1 and GATA1, suggesting that the reactivation of γ-globin expression is independent of BCL11A. Interestingly, when NonO was knocked down in HUDEP-2 or primary bone marrow erythroid progenitor cells, γ-globin expression was significantly induced, but β-globin expression remained largely unaffected. Similar findings were obtained in the conditional NonO knockout β-YAC mice with either murine globin or human globin expression. This pattern does not quite fit the gene competition model of hemoglobin switch, but the reason for this is not clear. Further analysis of chromatin structures upon alteration of *NonO* gene expression during development may provide more insight into mechanisms of globin gene switching.

NonO is highly conserved in human and mouse at the protein level (24,47). In our current data, conditional depletion of NonO in the adult β-YAC mouse hematopoietic system elevated both human γ-globin and mouse embryonic εy- and βh1-globins, which provides a rationale for possible therapeutic applications in treating SCD and β-thalassemia. It would likely be informative to breed NonO hematopoietic conditional knockout mice into SCD or β-thalassemia transgenic models, and test capacity for correction of the phenotypes of these diseases.

In summary, our data demonstrated a critical repressor role of NonO in the regulation of γ-globin gene transcription. Depletion of NonO can reactivate fetal γ-globin in erythroid cells and adult β-YAC mouse erythroid cells. Moreover, we further demonstrate that the repressive effect of NonO is mediated through direct γ-globin promoter binding at the essential octamer motif (ATGCAAAT), suggesting that NonO may represent a new therapeutic target for the treatment of SCD and β-thalassemia.

DATA AVAILABILITY

Mass spectrometry proteomics raw data are deposited in ProteomeXchange consortium with accession number: PXD024259. Flow cytometry experiments raw data are deposited in FlowRepository with accession IDs: FR-FCM-Z3HF, FR-FCM-Z3HH, FR-FCM-Z3HW, FR-FCM-Z3HJ.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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