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(71) Applicants: **THE CHILDREN'S HOSPITAL OF PHILADELPHIA** [US/US]; 3401 Civic Center Boulevard, Philadelphia, PA 19104 (US). **THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA** [US/US]; 3160 Chestnut Street, Suite 200, Philadelphia, PA 19104 (US).

(72) Inventors: **BLOBEL, Gerd**; 107 Colwyn Lane, Bala Cynwyd, PA 19004 (US). **GREVET, Jeremy**; 500 Admiral's Way, Strickland 328, Philadelphia, PA 19146 (US). **SHI, Junwei**; 3737 Chestnut Street, Apt. 1703, Philadelphia, PA 19104 (US). **LAN, Xianjiang**; 772 E. Providence Road, Apt. B109, Aldan, PA 19018 (US).

(74) Agent: **NETTER, Robert, C. Jr.** et al.; Dann, Dorfman, Herrell and Skillman, 1601 Market Street, Suite 2400, Philadelphia, PA 19103-2307 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR HEMOGLOBIN PRODUCTION

(57) Abstract: Methods and compositions for producing fetal hemoglobin and treating a hemoglobinopathy or thalassemia are disclosed.

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COMPOSITIONS AND METHODS FOR HEMOGLOBIN PRODUCTION

By Gerd Blobel

Jeremy Grevet

Junwei Shi

Xianjiang Lan

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This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 62/642,174, filed March 13, 2018. The foregoing application is incorporated by reference herein.

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FIELD OF THE INVENTION

The present invention relates to the field of hematology. More specifically, the invention provides compositions and methods for the production of various forms of hemoglobin, including adult and fetal type hemoglobin.

20

BACKGROUND OF THE INVENTION

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

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Sickle cell disease and thalassemia cause significant worldwide morbidity and mortality (Modell et al. (2008) Bull. World Health Org., 86:480-487; Modell et al. (2008) J. Cardiovasc. Magn. Reson., 10:42). However, effective drugs do not exist for these illnesses. One goal in the treatment of these diseases is to reactivate fetal hemoglobin (HbF). HbF reduces the propensity of sickle cell disease red blood cells to undergo sickling. Indeed, high fetal globin levels are associated with improved outcomes for sickle cell anemia patients (Platt et al. (1994) N. Engl. J. Med., 330:783-784). Elevating HbF also reduces the globin chain imbalance in certain thalassemias, thereby improving symptoms. There is an enormous unmet need to identify compounds that ameliorate the course of these diseases.

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SUMMARY OF THE INVENTION

In accordance with the present invention, compositions and methods are provided for increasing hemoglobin levels (e.g., fetal hemoglobin) in a cell or subject. In a particular embodiment, the method comprises administering at least one speckle-type BTB/POZ protein (SPOP; speckle-type POZ protein) inhibitor to the cell or
5 subject. In a particular embodiment, the subject has a hemoglobinopathy such as sickle cell disease or thalassemia. In a particular embodiment, the cell is an erythroid cell. In a particular embodiment, the SPOP inhibitor is a small molecule. The SPOP inhibitor may be, for example, a MATH domain inhibitor or a BTB domain inhibitor.
10 The SPOP inhibitor may be a CRISPR based or siRNA/shRNA inhibitor of the SPOP gene. The method may further comprise delivering at least one fetal hemoglobin inducer to the cell or subject. The method may exploit additive or synergistic effects with other fetal hemoglobin inducing methods based on pharmacologic compounds and/or various forms of gene therapy.
15 In accordance with another aspect of the instant invention, methods of inhibiting, treating, and/or preventing a hemoglobinopathy (e.g., sickle cell disease or thalassemia) in a subject are provided. In a particular embodiment, the method comprises administering at least one SPOP inhibitor to a subject in need thereof. The SPOP inhibitor may be in a composition with a pharmaceutically acceptable carrier.
20 In a particular embodiment, the subject has a β -chain hemoglobinopathy. In a particular embodiment, the subject has sickle cell anemia. In a particular embodiment, the SPOP inhibitor is a small molecule. The SPOP inhibitor may be, for example, a MATH domain inhibitor or a BTB domain inhibitor. The SPOP inhibitor may be a CRISPR based or siRNA/shRNA based inhibitor of the SPOP gene. The
25 method may further comprise delivering at least one other fetal hemoglobin inducer to the subject.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Figures 1A-1C: Protein-domain based CRISPR-Cas9 screen identifies SPOP
30 as a novel fetal globin repressor. Fig. 1A: Screening strategy. Cas9 expressing HUDEP-2 cells were transduced with a BTB domain and histone modification reader domain-targeting sgRNA library (6 sgRNA/domain). Edited HUDEP-2 cells were then induced to differentiate for 7 days. Differentiated cells were stained by APC conjugated anti-HbF and sorted with HbF high and HbF low by flow cytometry.

Enriched sgRNAs were identified by deep sequencing. Fig. 1B: HbF FACS gating strategy for HbF high and HbF low cell populations. Fig. 1C: Scatter plot of HbF high (y-axis) and HbF low (x-axis) populations as log₂ transformed normalized read counts, each dot represents a sgRNA.

5 Figures 2A-2D: SPOP depletion elevates γ -globin protein and mRNA levels in HDUEP-2 cells. Fig. 2A: SPOP protein domain structure and position of three SPOP sgRNAs within the BTB domain. Fig. 2B: HbF flow cytometry of differentiated cells transduced with indicated sgRNAs. Mean is shown $\pm$ standard deviation (SD) (N=2). Fig. 2C: Immunoblots analysis with indicated antibodies using whole cell lysates from
10 cell pools transduced with sgRNAs. Fig. 2D: Expression of γ -globin mRNA was measured by RT-qPCR, data are plotted as percentage of γ -globin over γ -globin+ β -globin gene levels. Mean is shown $\pm$ SD of at least two biological replicates. *** denotes p -value < 0.001 .

 Figures 3A-3B: SPOP depletion specifically and strongly induces γ -globin in
15 HUDEP-2 cells. Fig. 3A: RNA-seq analysis of cell pools with SPOP sgRNA#1, #2 and #3. Each dot represents an individual gene. Each gene is depicted according to FPKM (Fragments Per Kilobase Million) value. Fig. 3B: Scatter plots showing the results of mass spectrometry analysis of cell pools with SPOP sgRNA#1, #2 and #3 using whole cell lysates. Data represent log₂ protein abundances. Protein abundances
20 were quantified by TMT (Tandem mass tag)-labeled MS. Each dot represents a gene.

 Figures 4A-4F: SPOP depletion elevates γ -globin in primary erythroid CD34+ cells. Fig. 4A: Schematic diagram of experimental design. Fig. 4B: Representative HbF flow cytometry of CD34+ erythroid cells at Day 14 of differentiation. Fig. 4C: Immunoblots analysis with indicated antibodies for differentiated CD34+ cells
25 infected with scrambled or SPOP shRNAs virus. Fig. 4D: HPLC analysis for cells expressing indicated shRNAs at Day 15-16 of differentiation, HbF indicates fetal hemoglobin; HbA indicates adult hemoglobin. Arrow indicates HbF. Fig. 4E: Expression of γ -globin mRNA was measured by RT-qPCR, data are plotted as percentage of γ -globin over γ -globin+ β -globin gene levels. Mean is shown $\pm$ SD of
30 three independent donors. * denotes p -value < 0.01 . Fig. 4F: RNA-seq analysis for CD34+ cells at Day 14 of differentiation. R value denotes Pearson correlation coefficient. Log₂FPKM values were averaged from two independent donors.

 Figures 5A-5D: SPOP-CUL3 complex represses γ -globin independent of BCL11A. Fig. 5A: Immunoblots analysis with indicated antibodies for differentiated

HUDEP-2 cells expressing CUL3 sgRNAs. Fig. 5B: Expression of γ -globin mRNA was measured by RT-qPCR, data are plotted as percentage of γ -globin over γ -globin+ β -globin gene levels. Mean is shown $\pm$ SD of two biological replicates. Fig. 5C: Alignment of human (SEQ ID NO: 11) and mouse (SEQ ID NO: 12) SPOP DNA sequence. Shaded marks SPOP sgRNA#1 sequence. Two mismatched nucleotides upstream of "PAM" in mouse Spop cDNA. Fig. 5D: Expression of γ -globin mRNA was measured by RT-qPCR, data are plotted as percentage of γ -globin over γ -globin+ β -globin gene levels. Mean is shown $\pm$ SD of two biological replicates. Overexpression levels of BCL11A and mSpop cDNAs were controlled by sorting GFP low cell population.

Figures 6A-6C: Overexpression of SPOP mutant elevates γ -globin. Fig. 6A: HbF flow cytometry of differentiated HUDEP-2 cells expressing SPOP Y87N mutant. Fig. 6B: Immunoblots analysis with indicated antibodies for differentiated HUDEP-2 cells expressing empty vector or SPOP mutant. Fig. 6C: Expression of γ -globin mRNA was measured by RT-qPCR, data are plotted as percentage of γ -globin over γ -globin+ β -globin gene levels. Mean is shown $\pm$ SD of two biological replicates.

Figures 7A-7C: SPOP depletion strongly enhances the effect on γ -globin induction by Pomalidomide treatment. Fig. 7A: HbF flow cytometry of indicated HUDEP-2 sgRNAs cell pools treated with or without Pomalidomide. Fig. 7B: Expression of γ -globin mRNA was measured by RT-qPCR, data are plotted as percentage of γ -globin over γ -globin+ β -globin gene levels. Mean is shown $\pm$ SD of two independent experiments. Fig. 7C: BCL11A mRNA levels measured by RT-qPCR. GAPDH was used for normalization. Mean is shown $\pm$ SD of two biological replicates.

Figure 8A provides an example of an amino acid sequence (SEQ ID NO: 8) of human SPOP. Figure 8B provides an example of a nucleotide sequence (SEQ ID NO: 9) encoding human SPOP. Protein coding region is from position 472 to 1593 (excluding stop codon). Underlined sequences and bolded, italicized sequences are examples of target sequences for inhibitory nucleic acid molecules - shRNA and sgRNA, respectively.

DETAILED DESCRIPTION OF THE INVENTION

A major goal in the treatment of sickle cell disease and thalassemia is the reactivation of fetal type globin expression in cells of the adult red blood lineage. In

an unbiased genetic screen, speckle-type BTB/POZ protein (SPOP; speckle-type POZ protein) was identified as a strong regulator of fetal globin production. SPOP (see, e.g., PubMed GeneID: 8405; see, e.g., GenBank Accession Nos. NM_001007226.1 and NP_001007227.1) is a substrate adaptor of the CUL3 ubiquitin ligase complex that mediates the ubiquitination of target proteins, in most cases, causing protein degradation (Pintard et al. (2004) EMBO J., 23(8):1681-1687; Chen et al. (2016). Front. Oncol., 6:113). SPOP binds to its substrates by its N-terminal meprin and traf homology (MATH) domain (e.g., amino acids 28-166) and interacts with culin3 via its C-terminal BTB domain (e.g., amino acids 190-297) (Zhuang et al. (2009) Mol. Cell., 36(1):39-50). The BTB (for BR-C, ttk and bab) or POZ (for Pox virus and Zinc finger) domain is a common structural domain contained within some proteins.

SPOP has been identified as the most frequently mutated gene by exome sequencing in prostate cancer (Barbieri et al. (2012) Nat. Genet., 44(6):685-689). These SPOP mutations observed in human cancers are mainly enriched in its MATH domain which is responsible for substrate binding (Zhuang et al. (2009) Mol. Cell., 36(1):39-50; Barbieri et al. (2012) Nat. Genet., 44(6):685-689). In the context of prostate cancer, a number of substrates have been identified including androgen receptor (AR), DEK, TRIM24, SENP7 and BETs (An et al. (2014) Cell. Rep., 6(4):657-669; Theurillat et al. (2014) Science 346(6205):85-89; Zhu et al. (2015) Cell. Rep., 13(6): 1183-1193; Groner et al. (2016) Cancer Cell., 29(6):846-858; Dai et al. (2017) Nat. Med., 23(9):1063-1071; Janouskova et al. (2017) Nat. Med., 23(9):1046-1054; Zhuang et al. (2009) Mol. Cell., 36(1):39-50). However, it is shown herein that the depletion of SPOP raises fetal hemoglobin levels. Indeed, the genetic screen described herein for HbF inducers in human cells indicates that the loss of SPOP function increases HbF levels. Additional experiments show that the loss of SPOP increases fetal hemoglobin production in human erythroid cells, including primary cells. Without being bound by theory, the mechanism by which this occurs likely involves in the regulation of the protein stability of specific substrates which modulate the transcriptional and/or posttranscriptional upregulation of fetal hemoglobin production. Thus, the role of SPOP in the suppression of fetal and, to a lesser extent, embryonic globin production has been shown herein. This role is exploited herein to treat hemoglobinopathies such as sickle cell anemia and thalassemia.

SPOP binds its substrates for ubiquitination through its MATH domain. A small molecule designed to inhibit the SPOP-substrate protein interaction has been described (Li et al. (2014) *Cancer Cell* 25(4):455-468; Guo et al. (2016) *Cancer Cell* 30(3):474-484). This small molecule appears to mainly inhibit cytoplasmic SPOP-substrate interaction in clear-cell renal cell carcinoma (ccRCC) (Li et al. (2014) *Cancer Cell* 25(4):455-468; Guo et al. (2016) *Cancer Cell* 30(3):474-484). This finding indicates that the SPOP-substrate protein interaction is a target for small molecule design. Although SPOP KO mice is embryonic lethal (Claiborn et al. (2010) *J. Clin. Invest.*, 120(10):3713-3721), proper control of the dosage and tissue-specific delivery of the SPOP inhibitor can ameliorate or avoid potential detrimental side effects.

In accordance with the instant invention, compositions and methods are provided for increasing hemoglobin production in a cell or subject. In a particular embodiment, the method increases fetal hemoglobin and/or embryonic globin expression, particularly fetal hemoglobin. The method comprises administering at least one SPOP inhibitor to the cell, particularly an erythroid precursor cell or erythroid cell, or subject. In a particular embodiment, the subject has a hemoglobinopathy such as sickle cell disease or thalassemia. In a particular embodiment, the subject has sickle cell anemia. In a particular embodiment, the subject has thalassemia, particularly β -thalassemia, and more particularly major β -thalassemia.

The SPOP inhibitor may be administered in a composition further comprising at least one pharmaceutically acceptable carrier. In a particular embodiment, the method further comprises any means by which to induce fetal hemoglobin, such as administering at least one other fetal hemoglobin inducer. Fetal hemoglobin inducers include, without limitation, a lysine-specific demethylase 1 (LSD1) inhibitor (e.g., RN-1 and tranilcypromine (TCP) (Cui et al. (2015) *Blood* 126(3):386-96; Shi et al. (2013) *Nat. Med.*, 19(3): 291-294; Sun et al. (2016) *Reprod. Biol. Endocrinol.*, 14:17)), pomalidomide (Moutouh-de Parseval et al. (2008) *J. Clin. Invest.*, 118(1):248-258), hydroxyurea, 5-azacytidine, sodium butyrate, activators of the Foxo3 pathway (e.g., metformin, phenformin, or resveratrol), histone methyltransferase (HMT) inhibitors (e.g., a histone lysine methyltransferase inhibitor, euchromatic histone-lysine N-methyltransferase 2 (EHMT2; G9a) inhibitor, euchromatic histone-lysine N-methyltransferase 1 (EHMT1; G9a-like protein (GLP))

inhibitor, UNC0638 (2-cyclohexyl-N-(1-isopropylpiperidin-4-yl)-6-methoxy-7-(3-(pyrrolidin-1-yl)propoxy) quinazolin-4-amine), chaetocin, BIX-01294, UNC 0224, UNC 0642, UNC 0631, UNC 0646, A-366 (Sweis et al. (2014) ACS Med. Chem. Lett., 5(2):205-209), etc.), histone deacetylase (HDAC) inhibitors, and eIF2 α K1

5 inhibitors (see, e.g., PCT/US18/15918). In a particular embodiment, the fetal hemoglobin inducer is pomalidomide or hydroxyurea, particularly pomalidomide. The SPOP inhibitor and the fetal hemoglobin inducer can be delivered to the cell or subject sequentially or consecutively (e.g., in different compositions) and/or at the same time (e.g., in the same composition).

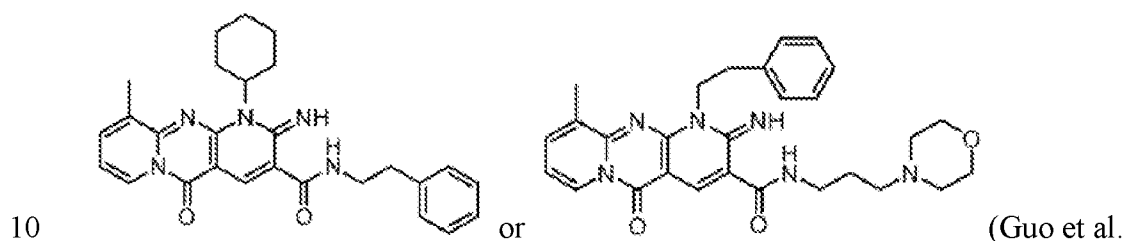
10 In accordance with another aspect of the instant invention, compositions and methods for inhibiting (e.g., reducing or slowing), treating, and/or preventing a hemoglobinopathy or thalassemia in a subject are provided. In a particular embodiment, the methods comprise administering to a subject in need thereof a therapeutically effective amount of at least one SPOP inhibitor. The SPOP inhibitor
15 may be administered in a composition further comprising at least one pharmaceutically acceptable carrier. In a particular embodiment, the hemoglobinopathy is β -thalassemia or sickle cell anemia. In a particular embodiment, the subject has sickle cell anemia. In a particular embodiment, the method further comprises administering at least one other fetal hemoglobin inducer to the subject as
20 described hereinabove. In a particular embodiment, the fetal hemoglobin inducer is pomalidomide or hydroxyurea, particularly pomalidomide. The SPOP inhibitor and the fetal hemoglobin inducer can be administered to the subject sequentially or consecutively (e.g., in different compositions) and/or at the same time (e.g., in the same composition).

25 SPOP inhibitors are compounds which reduce SPOP activity, inhibit or reduce SPOP-substrate interaction, inhibit or reduce SPOP dimerization, and/or the expression of SPOP. Examples of the human amino acid and nucleotide sequence for SPOP are provided in Figure 8. In a particular embodiment, the SPOP inhibitor is specific to SPOP. Examples of SPOP inhibitors include, without limitation, proteins
30 (e.g., dominant-negative SPOP), polypeptides, peptides, antibodies, small molecules, and nucleic acid molecules. In a particular embodiment, the SPOP inhibitor is a MATH domain inhibitor or BTB domain inhibitor. In a particular embodiment, the SPOP inhibitor is a BTB domain inhibitor which inhibits interaction with CUL3. In a particular embodiment, the SPOP inhibitor is an antibody immunologically specific

for the MATH domain or BTB domain. In another embodiment, the SPOP inhibitor is an inhibitory nucleic acid molecule, such as an antisense, siRNA, or shRNA molecule (or a nucleic acid molecule encoding the inhibitory nucleic acid molecule).

In a particular embodiment, the inhibitory nucleic acid molecule targets a sequence within the BTB domain. In a particular embodiment, the SPOP inhibitor is a CRISPR based targeting of the SPOP gene (e.g., with a guide RNA targeting the SPOP gene). The SPOP inhibitor may be a synthetic or non-natural compound.

In a particular embodiment, the SPOP inhibitor is a small molecule. Examples of SPOP small molecule inhibitors include, without limitation,



(2016) Cancer Cell 30(3):474-484) (see, also, Zheng et al. (2017) Sci. China Life Sci., 60:91-93). These compounds have been shown to inhibit SPOP-PTEN and SPOP-DUSP7 protein interaction in kidney cancer (Guo et al. (2016) Cancer Cell 30(3):474-484).

15 Clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9 (e.g., from *Streptococcus pyogenes*) technology and gene editing are well known in the art (see, e.g., Sander et al. (2014) Nature Biotech., 32:347-355; Jinek et al. (2012) Science, 337:816-821; Cong et al. (2013) Science 339:819-823; Ran et al. (2013) Nature Protocols 8:2281-2308; Mali et al. (2013) Science 339:823-826; addgene.org/crispr/guide/). The RNA-guided CRISPR/Cas9 system involves

20 expressing Cas9 along with a guide RNA molecule (gRNA). When coexpressed, gRNAs bind and recruit Cas9 to a specific genomic target sequence where it mediates a double strand DNA (dsDNA) break. The binding specificity of the CRISPR/Cas9 complex depends on two different elements. First, the binding complementarity

25 between the targeted genomic DNA (genDNA) sequence and the complementary recognition sequence of the gRNA (e.g., ~18-22 nucleotides, particularly about 20 nucleotides). Second, the presence of a protospacer-adjacent motif (PAM) juxtaposed to the genDNA/gRNA complementary region (Jinek et al. (2012) Science 337:816-821; Hsu et al. (2013) Nat. Biotech., 31:827-832; Sternberg et al. (2014) Nature

30 507:62-67). The PAM motif for *S. Pyogenes* Cas9 has been fully characterized, and

is NGG or NAG (Jinek et al. (2012) Science 337:816-821; Hsu et al. (2013) Nat. Biotech., 31:827-832). Other PAMs of other Cas9 are also known (see, e.g., addgene.org/crispr/guide/#pam-table). Guidelines and computer-assisted methods for generating gRNAs are available (see, e.g, CRISPR Design Tool (crispr.mit.edu/); Hsu et al. (2013) Nat. Biotechnol. 31:827-832; addgene.org/CRISPR; and CRISPR gRNA Design tool - DNA2.0 (dna20.com/eCommerce/startCas9)). Typically, the PAM sequence is 3' of the DNA target sequence in the genomic sequence.

In a particular embodiment, the method comprises administering at least one Cas9 (e.g., the protein and/or a nucleic acid molecule encoding Cas9) and at least one gRNA (e.g., a nucleic acid molecule encoding the gRNA) to the cell or subject. In a particular embodiment, the Cas9 is *S. pyogenes* Cas9. In a particular embodiment, the targeted PAM is in the 5'UTR, promoter, or first intron. When present, a second gRNA is provided which targets anywhere from the 5'UTR to the 3'UTR of the gene, particularly within the first intron. The nucleic acids of the instant invention may be administered consecutively (before or after) and/or at the same time (concurrently). The nucleic acid molecules may be administered in the same composition or in separate compositions. In a particular embodiment, the nucleic acid molecules are delivered in a single vector (e.g., a viral vector).

In a particular embodiment, the nucleic acid molecules of the instant invention are delivered (e.g., via infection, transfection, electroporation, etc.) and expressed in cells via a vector (e.g., a plasmid), particularly a viral vector. The expression vectors of the instant invention may employ a strong promoter, a constitutive promoter, and/or a regulated promoter. In a particular embodiment, the nucleic acid molecules are expressed transiently. Examples of promoters are well known in the art and include, but are not limited to, RNA polymerase II promoters, the T7 RNA polymerase promoter, and RNA polymerase III promoters (e.g., U6 and H1; see, e.g., Myslinski et al. (2001) Nucl. Acids Res., 29:2502-09). Examples of expression vectors for expressing the molecules of the invention include, without limitation, plasmids and viral vectors (e.g., adeno-associated viruses (AAVs), adenoviruses, retroviruses, and lentiviruses).

In a particular embodiment, the guide RNA of the instant invention may comprise separate nucleic acid molecules. For example, one RNA may specifically hybridize to a target sequence (crRNA) and another RNA (trans-activating crRNA (tracrRNA)) specifically hybridizes with the crRNA. In a particular embodiment, the

guide RNA is a single molecule (sgRNA) which comprises a sequence which specifically hybridizes with a target sequence (crRNA; complementary sequence) and a sequence recognized by Cas9 (e.g., a tracrRNA sequence; scaffold sequence).

Examples of gRNA scaffold sequences are well known in the art (e.g., 5'-

5 GUUUUAGAGC UAGAAAUAGC AAGUUA AAAU AAGGCUAGUC
CGUUAUCAAC UUGAAAAAGU GGCACCGAGU CGGUGCUUUU; SEQ ID
NO: 10). As used herein, the term “specifically hybridizes” does not mean that the nucleic acid molecule needs to be 100% complementary to the target sequence. Rather, the sequence may be at least 80%, 85%, 90%, 95%, 97%, 99%, or 100% complementary to the target sequences (e.g., the complementary between the gRNA and the genomic DNA). The greater the complementarity reduces the likelihood of undesired cleavage events at other sites of the genome. In a particular embodiment, the region of complementarity (e.g., between a guide RNA and a target sequence) is at least about 10, at least about 12, at least about 15, at least about 17, at least about 20, at least about 25, at least about 30, at least about 35, or more nucleotides. In a particular embodiment, the region of complementarity (e.g., between a guide RNA and a target sequence) is about 15 to about 25 nucleotides, about 15 to about 23 nucleotides, about 16 to about 23 nucleotides, about 17 to about 21 nucleotides, about 18 to about 22 nucleotides, or about 20 nucleotides. In a particular embodiment, the guide RNA targets a sequence or comprises a sequence (inclusive of RNA version of DNA molecules) as set forth in the Example provided herein (see, e.g., the sequences provided in Table 1 (e.g., sgRNA1, sgRNA2, or sgRNA3)). In a particular embodiment, the guide RNA targets a sequence or comprises a sequence (e.g., RNA version) which has at least 80%, 85%, 90%, 95%, 97%, 99%, or 100% homology or identity to a sequence set forth in the Example (e.g., Table 1 provided herein; (e.g., sgRNA1, sgRNA2, or sgRNA3 (SEQ ID NO: 1, 2, or 3))). The sequences may be extended or shortened by 1, 2, 3, 4, or 5 nucleotides at the end of the sequence opposite from the PAM (e.g., at the 5' end). When the sequence is extended the added nucleotides should correspond to the genomic sequence.

30 The above methods also encompass *ex vivo* methods. For example, the methods of the instant invention can comprise isolating hematopoietic cells (e.g., erythroid precursor cells) or erythroid cells from a subject, delivering at least one SPOP inhibitor to the cells, and administering the treated cells to the subject. The isolated cells may also be treated with other reagents *in vitro*, such as at least one fetal

hemoglobin inducer, prior to administration to the subject. In a particular embodiment, the cells are not fully mature, enucleated erythrocytes.

The methods of the instant invention may further comprise monitoring the disease or disorder in the subject after administration of the composition(s) of the instant invention to monitor the efficacy of the method. For example, the subject may be monitored for characteristics of low hemoglobin or a hemoglobinopathy.

When an inhibitory nucleic acid molecule (e.g., an shRNA such as shRNA3 or shRNA5) is delivered to a cell or subject, the inhibitory nucleic acid molecule may be administered directly or an expression vector may be used. In a particular embodiment, the inhibitory nucleic acid molecules are delivered (e.g., via infection, transfection, electroporation, etc.) and expressed in cells via a vector (e.g., a plasmid), particularly a viral vector. The expression vectors of the instant invention may employ a strong promoter, a constitutive promoter, and/or a regulated promoter. In a particular embodiment, the inhibitory nucleic acid molecules are expressed transiently. In a particular embodiment, the promoter is cell-type specific (e.g., erythroid cells). Examples of promoters are well known in the art and include, but are not limited to, RNA polymerase II promoters, the T7 RNA polymerase promoter, and RNA polymerase III promoters (e.g., U6 and H1; see, e.g., Myslinski et al. (2001) Nucl. Acids Res., 29:2502-09). Examples of expression vectors for expressing the molecules of the invention include, without limitation, plasmids and viral vectors (e.g., adeno-associated viruses (AAVs), adenoviruses, retroviruses, and lentiviruses). In a particular embodiment, the SPOP inhibitory nucleic acid molecules (e.g., siRNA or shRNA) target and/or encompass the target sequence of SEQ ID NO: 5, 6, or 7, particularly SEQ ID NO: 5 or 6. In a particular embodiment, the SPOP inhibitory nucleic acid molecules (e.g., siRNA or shRNA) target and/or comprise the target sequence which has at least 80%, 85%, 90%, 95%, 97%, 99%, or 100% homology or identity to SEQ ID NO: 5 or 6 (e.g., the RNA version).

As explained hereinabove, the compositions of the instant invention are useful for increasing hemoglobin production and for treating hemoglobinopathies and thalassemias. A therapeutically effective amount of the composition may be administered to a subject in need thereof. The dosages, methods, and times of administration are readily determinable by persons skilled in the art, given the teachings provided herein.

The components as described herein will generally be administered to a patient as a pharmaceutical preparation. The term “patient” or “subject” as used herein refers to human or animal subjects. The components of the instant invention may be employed therapeutically, under the guidance of a physician for the treatment
5 of the indicated disease or disorder.

The pharmaceutical preparation comprising the components of the invention may be conveniently formulated for administration with an acceptable medium (e.g., pharmaceutically acceptable carrier) such as water, buffered saline, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like),
10 dimethyl sulfoxide (DMSO), oils, detergents, suspending agents or suitable mixtures thereof. The concentration of the agents in the chosen medium may be varied and the medium may be chosen based on the desired route of administration of the pharmaceutical preparation. Except insofar as any conventional media or agent is incompatible with the agents to be administered, its use in the pharmaceutical
15 preparation is contemplated.

The compositions of the present invention can be administered by any suitable route, for example, by injection (e.g., for local (direct) or systemic administration), oral, pulmonary, topical, nasal or other modes of administration. The composition may be administered by any suitable means, including parenteral, intramuscular,
20 intravenous, intraarterial, intraperitoneal, subcutaneous, topical, inhalatory, transdermal, intrapulmonary, intraareterial, intrarectal, intramuscular, and intranasal administration. In a particular embodiment, the composition is administered directly to the blood stream (e.g., intravenously). In general, the pharmaceutically acceptable carrier of the composition is selected from the group of diluents, preservatives,
25 solubilizers, emulsifiers, adjuvants and/or carriers. The compositions can include diluents of various buffer content (e.g., Tris HCl, acetate, phosphate), pH and ionic strength; and additives such as detergents and solubilizing agents (e.g., polysorbate 80), anti oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimersol, benzyl alcohol) and bulking substances (e.g., lactose, mannitol). The
30 compositions can also be incorporated into particulate preparations of polymeric compounds such as polyesters, polyamino acids, hydrogels, polylactide/glycolide copolymers, ethylenevinylacetate copolymers, polylactic acid, polyglycolic acid, etc., or into liposomes. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of components of a pharmaceutical

composition of the present invention. See, e.g., Remington: The Science and Practice of Pharmacy, 21st edition, Philadelphia, PA. Lippincott Williams & Wilkins. The pharmaceutical composition of the present invention can be prepared, for example, in liquid form, or can be in dried powder form (e.g., lyophilized for later reconstitution).

5 As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation, as exemplified in the preceding paragraph. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the
10 molecules to be administered, its use in the pharmaceutical preparation is contemplated.

 Pharmaceutical compositions containing a compound of the present invention as the active ingredient in intimate admixture with a pharmaceutical carrier can be prepared according to conventional pharmaceutical compounding techniques. The
15 carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous. Injectable suspensions may be prepared, in which case appropriate liquid carriers, suspending agents and the like may be employed. Pharmaceutical preparations for injection are known in the art. If injection is selected as a method for administering the therapy, steps should be taken
20 to ensure that sufficient amounts of the molecules reach their target cells to exert a biological effect.

 A pharmaceutical preparation of the invention may be formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to a physically discrete unit of the pharmaceutical preparation
25 appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to produce the desired effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate dosage unit are well known to those skilled in the art. Dosage units may be proportionately increased or decreased based on the weight of the patient.
30 Appropriate concentrations for alleviation of a particular pathological condition may be determined by dosage concentration curve calculations, as known in the art. The appropriate dosage unit for the administration of the molecules of the instant invention may be determined by evaluating the toxicity of the molecules in animal models. Various concentrations of pharmaceutical preparations may be administered

to mice with transplanted human tumors, and the minimal and maximal dosages may be determined based on the results of significant reduction of tumor size and side effects as a result of the treatment. Appropriate dosage unit may also be determined by assessing the efficacy of the treatment in combination with other standard

5 therapies.

The pharmaceutical preparation comprising the molecules of the instant invention may be administered at appropriate intervals, for example, at least twice a day or more until the pathological symptoms are reduced or alleviated, after which the dosage may be reduced to a maintenance level. The appropriate interval in a particular case would normally depend on the condition of the patient.

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Definitions

The singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise.

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The terms “isolated” is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity, and that may be present, for example, due to incomplete purification, or the addition of stabilizers.

“Pharmaceutically acceptable” indicates approval by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

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A “carrier” refers to, for example, a diluent, adjuvant, preservative (e.g., Thimersol, benzyl alcohol), anti-oxidant (e.g., ascorbic acid, sodium metabisulfite), solubilizer (e.g., polysorbate 80), emulsifier, buffer (e.g., Tris HCl, acetate, phosphate), antimicrobial, bulking substance (e.g., lactose, mannitol), excipient, auxilliary agent or vehicle with which an active agent of the present invention is administered. Pharmaceutically acceptable carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin.

25 Water or aqueous saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Remington: The Science and Practice of Pharmacy, (Lippincott, Williams and Wilkins); Liberman, et al., Eds., Pharmaceutical

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Dosage Forms, Marcel Decker, New York, N.Y.; and Rowe, et al., Eds., Handbook of Pharmaceutical Excipients, Pharmaceutical Pr.

The term “treat” as used herein refers to any type of treatment that imparts a benefit to a patient suffering from an injury, including improvement in the condition of the patient (e.g., in one or more symptoms), delay in the progression of the condition, etc.

As used herein, the term “prevent” refers to the prophylactic treatment of a subject who is at risk of developing a condition and/or sustaining an injury, resulting in a decrease in the probability that the subject will develop conditions associated with the hemoglobinopathy or thalassemia.

A “therapeutically effective amount” of a compound or a pharmaceutical composition refers to an amount effective to prevent, inhibit, or treat a particular injury and/or the symptoms thereof. For example, “therapeutically effective amount” may refer to an amount sufficient to modulate the pathology associated with a hemoglobinopathy or thalassemia.

As used herein, the term “subject” refers to an animal, particularly a mammal, particularly a human.

A “vector” is a genetic element, such as a plasmid, cosmid, bacmid, phage, transposon, or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication and/ or expression of the attached sequence or element. A vector may be either RNA or DNA and may be single or double stranded. A vector may comprise expression operons or elements such as, without limitation, transcriptional and translational control sequences, such as promoters, enhancers, translational start signals, polyadenylation signals, terminators, and the like, and which facilitate the expression of a polynucleotide or a polypeptide coding sequence in a host cell or organism.

As used herein, the term “small molecule” refers to a substance or compound that has a relatively low molecular weight (e.g., less than 4,000, less than 2,000, particularly less than 1 kDa or 800 Da). Typically, small molecules are organic, but are not proteins, polypeptides, amino acids, or nucleic acids.

An “antibody” or “antibody molecule” is any immunoglobulin, including antibodies and fragments thereof, that binds to a specific antigen. As used herein, antibody or antibody molecule contemplates intact immunoglobulin molecules, immunologically active portions/fragment (e.g., antigen binding portion/fragment) of

an immunoglobulin molecule, and fusions of immunologically active portions of an immunoglobulin molecule. Antibody fragments include, without limitation, immunoglobulin fragments including, without limitation: single domain (Dab; e.g., single variable light or heavy chain domain), Fab, Fab', F(ab')₂, and F(v); and fusions (e.g., via a linker) of these immunoglobulin fragments including, without limitation: scFv, scFv₂, scFv-Fc, minibody, diabody, triabody, and tetrabody.

As used herein, the term “immunologically specific” refers to proteins/polypeptides, particularly antibodies, that bind to one or more epitopes of a protein or compound of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

The phrase “small, interfering RNA (siRNA)” refers to a short (typically less than 30 nucleotides long, particularly 12-30 or 20-25 nucleotides in length) double stranded RNA molecule. Typically, the siRNA modulates the expression of a gene to which the siRNA is targeted. Methods of identifying and synthesizing siRNA molecules are known in the art (see, e.g., Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Inc). Short hairpin RNA molecules (shRNA) typically consist of short complementary sequences (e.g., an siRNA) separated by a small loop sequence (e.g., 6-15 nucleotides, particularly 7-10 nucleotides) wherein one of the sequences is complimentary to the gene target. shRNA molecules are typically processed into an siRNA within the cell by endonucleases. Exemplary modifications to siRNA molecules are provided in U.S. Application Publication No. 20050032733. For example, siRNA and shRNA molecules may be modified with nuclease resistant modifications (e.g., phosphorothioates, locked nucleic acids (LNA), 2'-O-methyl modifications, or morpholino linkages). Expression vectors for the expression of siRNA or shRNA molecules may employ a strong promoter which may be constitutive or regulated. Such promoters are well known in the art and include, but are not limited to, RNA polymerase II promoters, the T7 RNA polymerase promoter, and the RNA polymerase III promoters U6 and H1 (see, e.g., Myslinski et al. (2001) Nucl. Acids Res., 29:2502-09).

“Antisense nucleic acid molecules” or “antisense oligonucleotides” include nucleic acid molecules (e.g., single stranded molecules) which are targeted (complementary) to a chosen sequence (e.g., to translation initiation sites and/or splice

sites) to inhibit the expression of a protein of interest. Such antisense molecules are typically between about 15 and about 50 nucleotides in length, more particularly between about 15 and about 30 nucleotides, and often span the translational start site of mRNA molecules. Antisense constructs may also be generated which contain the entire sequence of the target nucleic acid molecule in reverse orientation. Antisense oligonucleotides targeted to any known nucleotide sequence can be prepared by oligonucleotide synthesis according to standard methods. Antisense oligonucleotides may be modified as described above to comprise nuclease resistant modifications.

The following example is provided to illustrate various embodiments of the present invention. It is not intended to limit the invention in any way.

EXAMPLE

Reactivation of fetal hemoglobin (HbF) production benefits patients with sickle cell disease (SCD) and β -thalassemia. In order to identify new regulators of HbF that might be amenable to pharmacologic control, a protein domain-focused CRISPR-Cas9 library targeting chromatin regulators, including BTB domain containing proteins, was screened. SPOP (Speckle-type POZ protein), a substrate adaptor of the CUL3 ubiquitin ligase complex, emerged as a novel fetal hemoglobin repressor. Depletion of SPOP or overexpression of a dominant negative version significantly raised fetal globin mRNA and protein levels with minimal detrimental effects on normal erythroid maturation, as determined by transcriptome and proteome analyses. SPOP controls HbF expression independently of the major transcriptional fetal hemoglobin repressors BCL11A and LRF. Finally, pharmacologic HbF inducers cooperate with SPOP depletion during fetal hemoglobin upregulation. The study shows SPOP and the CUL3 ubiquitin ligase system in the control of fetal hemoglobin in human erythroid cells and offers new therapeutic strategies for the treatment of β -hemoglobinopathies.

Hemoglobin is comprised of a tetramer containing two α -type and two β -type subunits. The β -type globin gene cluster consists of an embryonic (ϵ -globin), two fetal (γ -globin) and two adult type (δ -globin and β -globin) genes. Mutations in the β -globin gene that underlie sickle cell disease (SCD) and some types of β -thalassemia become clinically relevant after birth when the fetal genes are silenced. Increased fetal hemoglobin (HbF) production due to natural genetic variation or therapeutic

intervention can lower morbidity and mortality in β -hemoglobinopathies (Platt, et al., N. Engl. J. Med. (1994) 330:1639-1644; Miller, et al., N. Engl. J. Med. (2000) 342:83-89). While promising strategies involving gene addition or genome editing are being pursued (Traxler, et al., Nat. Med. (2016) 22:987-990; Canver, et al., Blood (2016) 127:2536-2545; Cai, et al., Stem Cells Transl. Med. (2018) 7:87-97; Wienert, et al., Trends Genet. (2018) 34(12):P927-940), their implementation will be largely restricted to patients with access to sophisticated medical providers. Effective HbF induction by pharmacologic means is therefore needed, but remains a challenge.

BCL11A and LRF (ZBTB7A) are critical direct transcriptional repressors of the γ -globin genes (Menzel, et al., Nat. Genet. (2007) 39:1197-1199; Uda, et al., Proc. Natl. Acad. Sci. (2008) 105:1620-1625; Sankaran, et al., Science (2008) 322:1839-1842; Masuda, et al., Science (2016) 351:285-289). However, as DNA binding proteins with functions in multiple tissues, they remain difficult to target pharmacologically and in an erythroid selective manner. Moreover, LRF depletion delays erythroid differentiation (Masuda, et al., Science (2016) 351:285-289; Maeda, et al., Dev. Cell. (2009) 17:527-540). In spite of a deep understanding of the transcriptional control of the globin genes, the regulatory circuitry outside of DNA binding nuclear factors remains under-explored. Yet, it is clear from targeted depletion experiments that non-DNA binding co-regulatory complexes can play pivotal roles in HbF silencing (Liu, et al., Cell (2017) 170:1028-1043; Grevet, et al., Science (2018) 361:285-290).

To identify new and potentially druggable nuclear HbF regulators, a library that is composed of sgRNAs directed against chromatin associated nuclear proteins containing BTB domain, Chromo domain, PWWP domain, PHD domain and more, HUDEP-2 cells were screened using an improved protein-domain based CRISPR-Cas9 platform (Grevet, et al., Science (2018) 361:285-290; Kurita, et al., PLoS One (2013) 8:e59890; Shi, et al., Nat. Biotechnol. (2015) 33:661-667). These domains have conserved structure and are required to mediate protein-protein interactions. The conserved protein structure of these domains provides a potential surface for docking small molecule inhibitors. Among approximately 600 BTB proteins and E3 ligase components in the library, the screen selectively identified speckle-type POZ protein (SPOP), a substrate adaptor of the CUL3 ubiquitin ligase complex (Chen, et al., Front. Oncol. (2016) 6:113), as a novel repressor of γ -globin transcription.

The repressive role of SPOP on γ -globin levels is at least in part mediated by the CUL3 ubiquitin ligase complex, and disruption of SPOP-substrate interactions by overexpressing a SPOP dominant-negative mutant recapitulated the effect of SPOP depletion on γ -globin expression. The activity of SPOP appears to be carried out independently of BCL11A and LRF. SPOP depletion markedly amplified the effects of pomalidomide, a pharmacologic HbF inducer, indicating that SPOP perturbation is useful in combination with other treatment modalities.

As stated above, a CRISPR screening strategy (Shi et al. (2015) Nat. Biotechnol., 33(6):661-7) was employed to identify regulators of fetal globin expression. Clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9 technology is well known in the art (see, e.g., Sander et al. (2014) Nature Biotech., 32:347-355; Jinek et al. (2012) Science, 337:816-821; Cong et al. (2013) Science 339:819-823; Ran et al. (2013) Nature Protocols 8:2281-2308; Mali et al. (2013) Science 339:823-826). Cas9 possesses two nuclease domains, a RuvC-like nuclease domain and a HNH-like nuclease domain, and is responsible for the destruction of the target DNA (Jinek et al. (2012) Science, 337:816-821; Sapranaukas et al. (2011) Nucleic Acids Res. 39:9275-9282). The two nucleases generate double-stranded breaks. The double-stranded endonuclease activity of Cas9 requires a target sequence (e.g., ~20 nucleotides, see above) and a short conserved sequence (~2-5 nucleotides; e.g., 3 nucleotides) known as protospacer-associated motif (PAM), which follows immediately 3' - of the CRISPR RNA (crRNA) complementary sequence (Jinek et al. (2012) Science, 337:816-821; Nishimasu et al. (2014) Cell 156(5):935-49; Swarts et al. (2012) PLoS One, 7:e35888; Sternberg et al. (2014) Nature 507(7490):62-7). The double strand break can be repaired by non-homologous end joining (NHEJ) pathway yielding an insertion and/or deletion or, in the presence of a donor template, by homology-directed repair (HDR) pathway for replacement mutations (Overballe-Petersen et al. (2013) Proc. Natl. Acad. Sci. U.S.A. 110:19860-19865; Gong et al. (2005) Nat. Struct. Mol. Biol. 12:304-312). The RNA-guided CRISPR/Cas9 system involves expressing Cas9 along with a guide RNA molecule (gRNA). When coexpressed, gRNAs bind and recruit Cas9 to a specific genomic target sequence where it mediates a double strand DNA (dsDNA) break and activates the dsDNA break repair machinery. Specific DNA fragments can be deleted when two gRNA/Cas9 complexes generate dsDNA breaks at relative proximity, and the genomic DNA is repaired by nonhomologous end joining.

Protein domain-focused CRISPR-Cas9 screen identifies SPOP as a potential γ -globin repressor

sgRNAs targeting functional protein domains generates phenotype-altering mutations at a higher rate compared to sgRNAs designed to generate null alleles (Shi, et al., Nat. Biotechnol. (2015) 33:661-667). In order to identify additional potential γ -globin repressors, a library of sgRNAs targeting approximately 600 proteins was designed covering most of BTB domain and histone modification reader domain containing proteins (6 sgRNAs per domain). This library also included 6 sgRNAs for LRF as positive control, and 50 non-targeting sgRNAs as negative controls. This sgRNA library was cloned into the LRG 2.1T lentiviral vector and then introduced into HUDEP-2 cells (Kurita, et al., PLoS One (2013) 8:e59890) stably expressing Cas9 (Figure 1A). Cells were stained using an anti-HbF antibody and sorted the top 10% and bottom 10% HbF expressing cells by FACS, and deep-sequenced the sgRNAs from each population (Figure 1B). As expected, non-targeting sgRNAs were evenly distributed across the HbF high and low populations, and all six sgRNAs of LRF were enriched in the HbF high population (Figure 1C). Moreover, the sgRNAs targeting NuRD complex subunits (CHD4, MBD2 and MTA2) and DNMT1, which are reported to be required for γ -globin silencing (Xu, et al., Proc. Natl. Acad. Sci. (2013) 110:6518-6523), were all enriched in HbF high portion, validating the screen. Interestingly, 5 out of 6 sgRNAs targeting SPOP were significantly enriched in the HbF high population, indicating that it functions as a repressor of γ -globin (Figure 1C).

SPOP is one of the substrate adaptors of the CUL3 ubiquitin ligase complex that mediates ubiquitination of target proteins and, in most cases, causes protein degradation (Chen, et al., Front. Oncol. (2016) 6:113; Pintard, et al., EMBO J. (2004) 23:1681-1687). SPOP binds to its substrates by its N-terminal meprin and traf homology (MATH) domain and interacts with CUL3 via its C-terminal BTB domain (Zhuang, et al., Mol. Cell (2009) 36:39-50) (Figure 2A). SPOP is widely expressed across human tissues (GTEx). In blood, SPOP is highly enriched in erythroid cells (Bloodspot) and its mRNA expression levels are comparable between fetal and adult erythroblasts (Lessard, et al., Hum. Mol. Genet. (2018) 27:1411-1420; Huang, et al., Genes Dev. (2017) 31:1704-1713).

Depletion of SPOP elevates γ -globin levels in HUDEP-2 cells

To validate the results from the screen, 3 of the 6 sgRNAs against the SPOP BTB domain were stably introduced into HUDEP-2-Cas9 cells and allowed the cells to undergo differentiation (Figure 2A; Table 1). All 3 SPOP sgRNAs significantly increased the fraction of HbF expressing cells (Figure 2B). Western blots showed that 1) SPOP protein levels declined somewhat during differentiation; 2) GATA1 protein levels were unchanged upon SPOP depletion, indicating SPOP is not required for normal cell maturation; 3) γ -globin protein levels were significantly increased upon SPOP depletion, consistent with the HbF flow cytometry results; 4) LRF was unchanged, but BCL11A protein levels were modestly decreased (Figure 2C).

<u>CRISPR sgRNA name</u>	<u>Target Sequence</u>
SPOP sgRNA1	CCTCTGCAGTAACCTGTCCG (SEQ ID NO: 1)
SPOP sgRNA2	GAGGACTGTGGGAGAATTCC (SEQ ID NO: 2)
SPOP sgRNA3	TGATGTGCTTCATTTACACG (SEQ ID NO: 3)
Neg2 sgRNA	GACCGGAACGATCTCGCGTA (SEQ ID NO: 4)

Table 1: sgRNA sequences

When measuring the transcriptional effects of SPOP perturbation, robust increases in γ -globin mRNA and pre-mRNA levels were observed, indicating that SPOP impinges on transcriptional regulation of γ -globin. SPOP loss increased ϵ -globin mRNA levels ~2-fold, but β -globin mRNA and per-mRNA levels were similar to controls (Figure 2D). Importantly, there were no notable changes in α -globin, GATA1 and BAND3 mRNA levels (erythroid differentiation makers) and cell morphology, indicating that SPOP loss did not impair erythroid maturation. Varying levels of HbF induction in SPOP depleted clonal HUDEP-2 lines was also observed. In sum, SPOP suppresses γ -globin gene expression.

Effects of SPOP inhibition on the erythroid transcriptome and proteome

To study the impact of SPOP depletion globally, RNA-seq was performed using HUDEP-2 pools independently derived from 3 SPOP sgRNAs and it was found that global gene expression patterns were highly similar between control and SPOP depleted cells (Pearson correlation coefficient above 0.95). γ -globin genes (HBG1/2) appeared to be the most strongly and significantly increased in differentiated cells (Figure 3A). Consistent with the qPCR results, α -globin, β -globin, GATA-1, ALAS2

and BAND3 genes were comparable (Figure 3A). Of note, BCL11A mRNA levels were unchanged (Figure 3A).

Given that SPOP functions as a substrate adaptor of CUL3 E3 ligase complex, SPOP loss might lead to increased protein levels of its substrates. Mass spectrometry experiments were performed to measure protein abundances both in whole cells lysates and nuclear extracts from SPOP depleted cell pools. Among approximately 3000 quantified proteins from whole cell lysates and nuclear extracts, γ -globin was one of the most elevated proteins. However, α -globin and β -globin abundance changed little or not at all upon SPOP depletion, consistent with the RNA-Seq results (Figure 3). Globally, SPOP loss induced γ -globin expression accompanied by relatively few changes in transcriptome and proteome.

Depletion of SPOP elevates γ -globin levels in primary erythroid CD34⁺ cells

The repressive role of SPOP on HbF in a primary human CD34⁺ cell derived culture system was tested (Figure 4A). SPOP depletion by two independent shRNAs significantly increased HbF⁺ cell populations (Figure 4B; Table 2), γ -globin protein levels as shown by Western blot and HPLC (Figure 4C-D), and γ -globin transcript levels by RT-qPCR (Figure 4E). Similar to HUDEP-2 cells, GATA1 and LRF levels were unchanged but BCL11A was reduced approximately two-fold. RNA-seq analysis of SPOP depleted cells showed that 1) global gene expression patterns were highly correlated between control and SPOP depleted cells, 2) γ -globin transcripts were the most elevated with no significant changes of α -globin and β -globin mRNAs, 3) GATA-1, ALAS2 and BAND3 genes were mostly unchanged, and 4) BCL11A and LRF transcript levels were comparable (Figure 4F). These results were further validated by RT-qPCR. Importantly, SPOP depletion did not impair erythroid maturation, as indicated by the similar transcript levels of the differentiation makers, cell surface makers CD71 and CD235a, and cell morphology.

<u>shRNA name</u>	<u>Target Sequence</u>
SPOP shRNA3	CAAGGTAGTGAAATTCTCCTA (SEQ ID NO: 5)
SPOP shRNA5	CAGATGAGTTAGGAGGACTGT (SEQ ID NO: 6)
SC shRNA	AAATTATTAGCGCTATCGCGC (SEQ ID NO: 7)

Table 2: shRNA sequences

SPOP-CUL3 complex represses γ -globin transcription independent of BCL11A

Since CUL3 is the core component of the SPOP-CUL3 E3 ligase complex (Chen, et al., Front. Oncol. (2016) 6:113), it was determined whether CUL3 is involved in the repression of HbF. Indeed, depletion of CUL3 with sgRNAs targeting its Cullin domain elevated γ -globin levels without affecting α - and β -globin levels, and had no significant effects on GATA1, BCL11A and LRF expression (Figure 5A). SPOP protein but not transcript levels were increased upon CUL3 depletion (Figure 5A), consistent with studies showing that SPOP-CUL3 itself can be ubiquitylated (Theurillat, et al., Science (2014) 346:85-89; Gschweidl, et al., Elife (2016) 5:e13841). This upregulation of SPOP in partially CUL3 depleted cells might explain the reduced effect size on γ -globin induction when compared to SPOP depletion. Although CUL3 is known to regulate cell cycle progression by targeting Cyclin E (Singer, et al., Genes Dev. (1999) 13:2375-2387), CUL3 depletion did not ostensibly impair the differentiation of erythroid cells, as evidenced by normal levels of GATA1, α -Globin and BAND3 (Figure 5A). These data also indicate that SPOP regulates γ -globin through substrates of the CUL3 ubiquitin proteasome pathway.

Under SPOP deficient conditions, BCL11A is decreased at the protein but not the mRNA level both in HUDEP-2 cells and primary erythroid CD34+ cells (Figure 2C and Figure 4C), possibly explaining some of the effects on γ -globin regulation. To test this, rescue experiments were performed by re-introducing either BCL11A cDNA or mouse Spop cDNA, which contains 2 mismatched nucleotides upstream of the “PAM” sequence of SPOP gRNA#1 into SPOP depleted cells (Figure 5C). Surprisingly, BCL11A failed to restore repression of γ -globin repression, while mouse Spop cDNA lowered γ -globin to basal protein and mRNA levels (Figure 5D). Moreover, if SPOP represses γ -globin mainly through BCL11A, SPOP depletion should not further raise γ -globin levels in BCL11A deficient cells. However, loss of SPOP in BCL11A depleted cells additively elevated γ -globin levels, indicating that SPOP and BCL11A repress γ -globin transcription through distinct pathways. Finally, given that SPOP functions as a CUL3 E3 ligase adaptor (Dai, et al., Nat. Med. (2017) 23:1063-1071), if BCL11A were one of its direct substrates, depletion of SPOP would be expected to increase, but not decrease, BCL11A protein levels, and overexpression of WT SPOP, but not derivatives unable to bind substrates (Theurillat, et al., Science (2014) 346:85-89); Dai, et al., Nat. Med. (2017) 23:1063-1071), should increase the ubiquitination levels of BCL11A. However, *in vivo* ubiquitination assays showed that

overexpressing WT SPOP or mutant versions had no effect on the ubiquitylation of BCL11A. Taken together, although SPOP depletion moderately alters BCL11A protein level, these results indicate that BCL11A is not the direct downstream effector of the SPOP-CUL3 complex on the repression of HbF.

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Overexpression of a dominant-negative acting SPOP mutant strongly increases γ -globin levels

Given its role as E3 ligase that targets proteins to the proteasome, SPOP-CUL3 likely represses the transcription of the γ -globin genes by promoting the ubiquitylation and degradation of proteins that might function as transcriptional activators of γ -globin. To test this, a SPOP mutant in which Tyr⁸⁷ is replaced by Asn (Y87N) in the MATH domain was expressed. The Y87N mutation disrupts SPOP-substrate interactions in a dominant-negative manner (Zhuang, et al., Mol. Cell (2009) 36:39-50). Indeed, overexpression of the SPOP(Y87N) strongly elevated γ -globin levels and also to some extent the embryonic ϵ -globin gene without significantly affecting α -globin and β -globin levels, and had no measurable effect on BCL11A or LRF levels (Figure 6). Importantly, overexpression of SPOP(Y87N) had no apparent detrimental effect on cell maturation (Figure 6). In concert, the data indicate that the SPOP-CUL3 complex functions by promoting the turnover of protein(s) involved in γ -globin transcriptional regulation.

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Depletion of SPOP cooperates with Pomalidomide during HbF induction

It was determined whether activation of γ -globin expression by SPOP depletion could be amplified by pharmacologic agents that function via distinct pathways. Although the mechanism by which the drug Pomalidomide induces HbF expression remains unclear, it does trigger downregulation of BCL11A mRNA levels (Dulmovits, et al., Blood (2016) 127:1481-1492; Moutouh-de Parseval, et al., J. Clin. Invest. (2008) 118:248-258). Therefore, it was tested whether Pomalidomide amplifies the effects of SPOP depletion in HUDEP-2 cells. This was indeed the case, as the combined treatment elevated the fraction of HbF⁺ cells and increased γ -globin transcription more strongly than either treatment alone (Figure 7A-B). Moreover, BCL11A transcript levels were not changed upon SPOP deletion but significantly decreased upon pomalidomide treatment (Figure 7C). Of note, these conditions did not impair cell differentiation. Together these data indicate that SPOP inhibition in

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combination with other treatments is a therapeutic strategy for HbF induction in patients with hemoglobinopathies.

Here, SPOP, a substrate adaptor of the CUL3 ubiquitin E3 ligase complex, has been identified as a novel regulator of HbF expression. Reduction in SPOP levels increases HbF production in HUDEP-2 and primary human cell cultures without substantial disruption of the cellular transcriptome and proteome. Mechanistically, it is demonstrated that the repressive role of SPOP on γ -globin is dependent on the CUL3 ubiquitin ligase complex. Moreover, ectopic expression of a dominant-negative form of SPOP (Y87N) unable to bind to its substrates substantially induce HbF production. Finally, it found that combining SPOP depletion with pomalidomide exposure induced higher HbF production than either treatment alone.

CUL3 interacts with BTB/POZ domain proteins such as SPOP. Although other BTB-domain containing substrate adaptors of the CUL3 E3 ligase were also included in the sgRNA library, SPOP is the only one involved in HbF repression, reflecting a selective function of SPOP, and consistent with general target selectivity of CUL3 E3 ligases via different BTB-domain containing substrate adaptors. Induction of HbF upon CUL3 depletion was less pronounced compared to SPOP loss, which may be due in part to the compensating effects of increased SPOP protein levels. Moreover, in HUDEP-2 cells, CUL3 depletion impaired cell proliferation, possibly due to deregulation of Cyclin E, but SPOP depletion did not have this effect, indicating that CUL3 targets Cyclin E through another substrate-specific adaptor.

Given the central role of BCL11A in HbF silencing, the moderate decrease of BCL11A in SPOP depleted cells might have contributed to HbF induction. However, restoration of BCL11A levels failed to restore γ -globin silencing. Hence, SPOP likely exerts its function via a different mechanism. Since ubiquitination can in some cases increase protein stability or alter protein activity, it was examined whether SPOP ubiquitinates BCL11A and no changes were observed upon SPOP over-expression, again arguing against a functional link between SPOP and BCL11A. Regardless, the role of SPOP in HbF silencing very likely involves its role as E3 ligase because forced expression of SPOP(Y87N) leads to strong HbF induction. SPOP(Y87N) expression induces γ -globin to levels similar to those observed upon SPOP depletion, but, in contrast to the latter condition, BCL11A levels remained unchanged. These

results are consistent with SPOP functioning via the CUL3 E3 ligase complex and support a mechanism not involving BCL11A.

A means to increase the therapeutic index is to combine therapies that impact different cellular pathways. SPOP depletion had no effect on BCL11A mRNA levels, but pomalidomide is known to lower production of BCL11 mRNA. Thus, the co-operativity of SPOP and pomalidomide during HbF induction was tested. The combination of SPOP depletion and pomalidomide treatment induced HbF production to levels much higher compared to either treatment alone. This indicates that SPOP inhibition can be combined with other treatments to augment effect size and lower adverse effects through dose reduction.

The BTB domain is a highly druggable structure (Kerres, et al., Cell. Rep. (2017) 20:2860-2875). Small molecules designed to inhibit the SPOP-substrate protein interactions have been reported (Guo, et al., Cancer Cell 30:474-484). This indicates that SPOP-substrate protein interactions is a therapeutic target for small molecule inhibitors. Importantly, strong HbF induction achieved through disrupting SPOP-substrate interactions by overexpressing SPOP(Y87N) mutant further supports this position. In summary, SPOP functions as a novel regulator of HbF and offers a therapeutic target for monotherapy or combination therapies for γ -hemoglobinopathies.

20

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

25

What is claimed is:

1. A method of increasing the level of human fetal hemoglobin in a cell or subject, the method comprising administering at least one speckle-type POZ protein (SPOP)
5 inhibitor to the cell or subject.
2. The method of claim 1, wherein the subject has a β -chain hemoglobinopathy.
3. The method of claim 1, wherein the subject has thalassemia.
10
4. The method of claim 1, wherein the subject has sickle cell disease.
5. The method of any of claims 1-4, wherein the SPOP inhibitor is a small molecule.
- 15 6. The method of claim 5, wherein the SPOP inhibitor is a MATH domain inhibitor or a BTB domain inhibitor.
7. The method of any one of claims 1-4, wherein the SPOP inhibitor is an inhibitory nucleic acid molecule.
20
8. The method of claim 7, wherein said inhibitory nucleic acid molecule specifically binds SEQ ID NO: 5 or 6.
9. The method of claim 1, wherein the cell is an erythroid cell, erythroid progenitor
25 cell, or stem cell.
10. The method of claim 1, further comprising administering at least one fetal hemoglobin inducer to the cell or subject.
- 30 11. The method of claim 10, wherein said fetal hemoglobin inducer is pomalidomide.
12. A method of treating a hemoglobinopathy in a subject in need thereof, the method comprising administering a composition comprising at least one speckle-type POZ protein (SPOP) inhibitor and a pharmaceutically acceptable carrier to the subject.

13. The method of claim 12, wherein the subject has a β -chain hemoglobinopathy.
14. The method of claim 12, wherein the subject has thalassemia.
- 5 15. The method of claim 12, wherein the subject has sickle cell anemia.
16. The method of any one of claims 12-15, wherein the SPOP inhibitor is a small molecule.
- 10 17. The method of claim 16, wherein the SPOP inhibitor is a MATH domain inhibitor or a BTB domain inhibitor.
18. The method of any one of claims 12-15, wherein the SPOP inhibitor is an
- 15 inhibitory nucleic acid molecule.
19. The method of claim 18, wherein said inhibitory nucleic acid molecule specifically binds SEQ ID NO: 5 or 6.
- 20 20. The method of claim 12, further comprising administering at least one fetal hemoglobin inducer to the subject.
21. The method of claim 20, wherein said fetal hemoglobin inducer is pomalidomide.
- 25 22. The method of claim 12, wherein the SPOP inhibitor is contained within a cell administered to the subject.
23. A composition comprising at least one speckle-type POZ protein (SPOP) inhibitor and at least one fetal hemoglobin inducer.
- 30 24. The composition of claim 22, wherein said fetal hemoglobin inducer is pomalidomide.

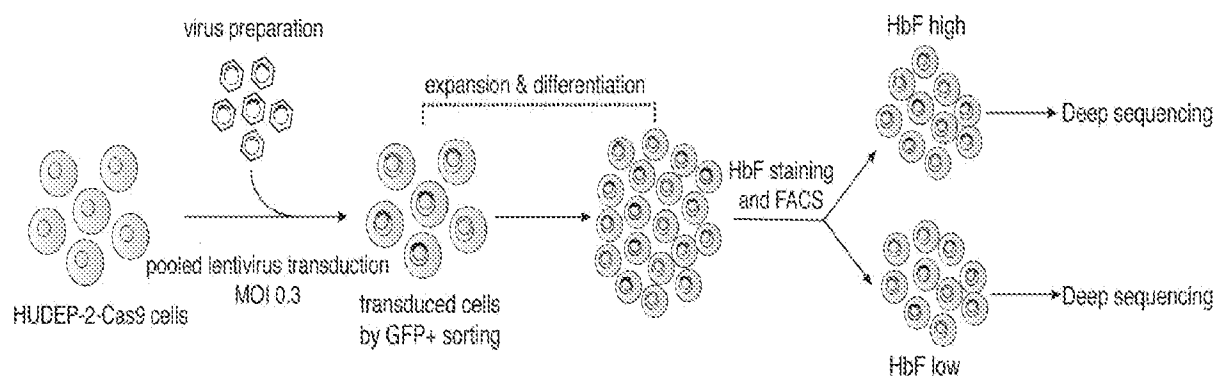


FIG. 1A

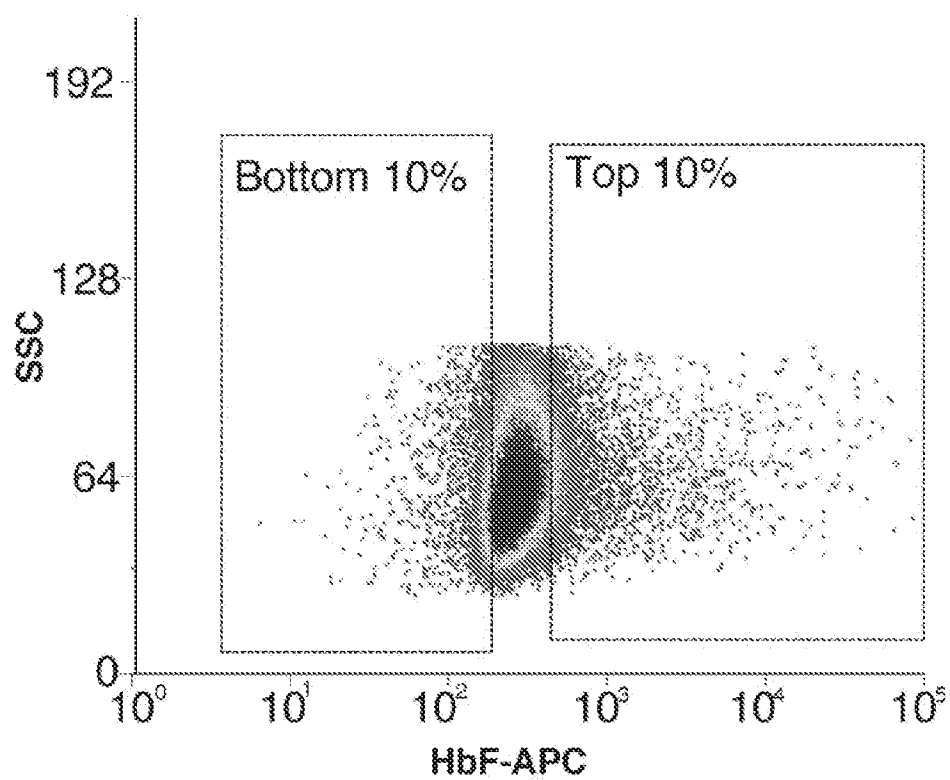


FIG. 1B

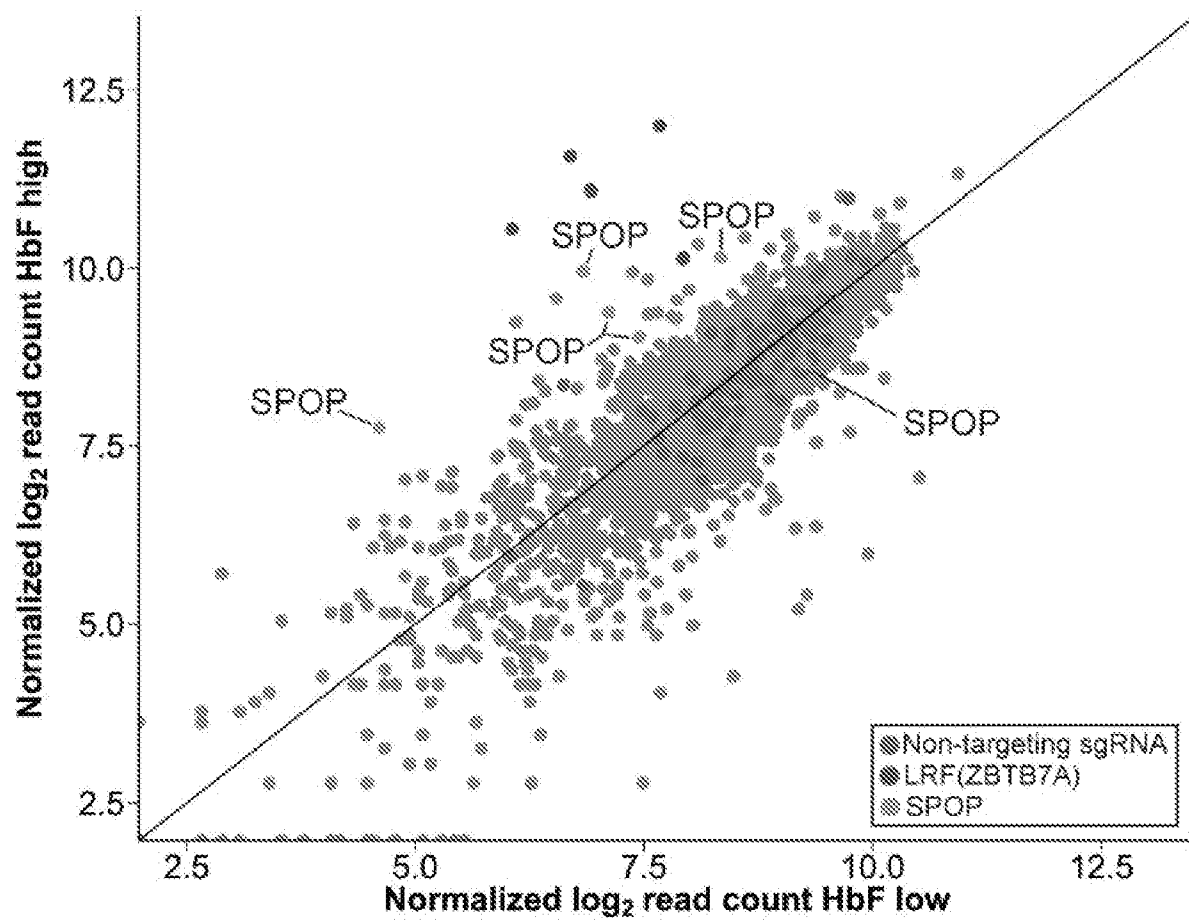


FIG. 1C

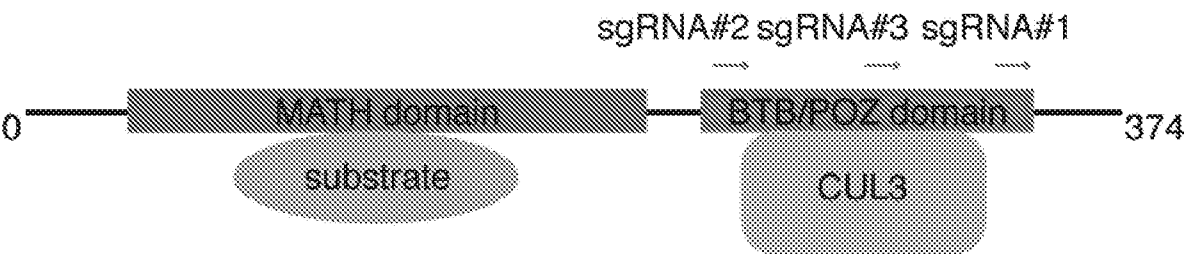


FIG. 2A

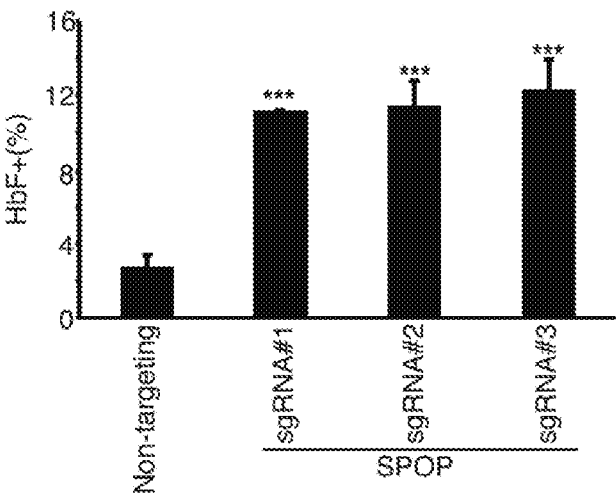


FIG. 2B

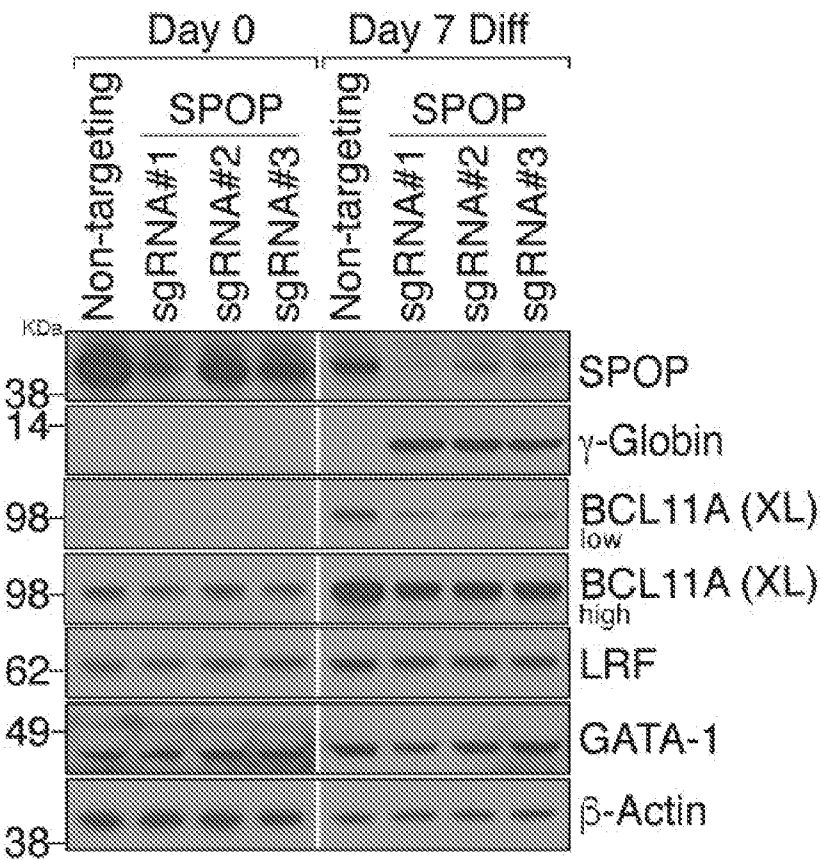


FIG. 2C

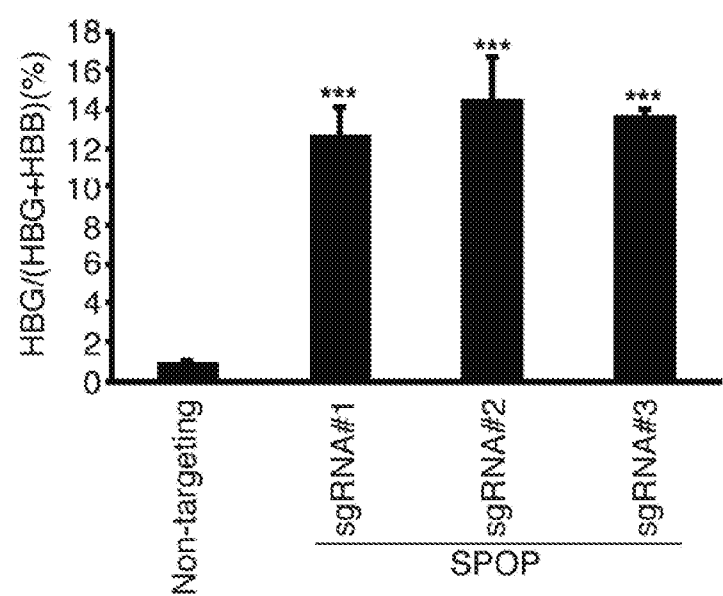


FIG. 2D

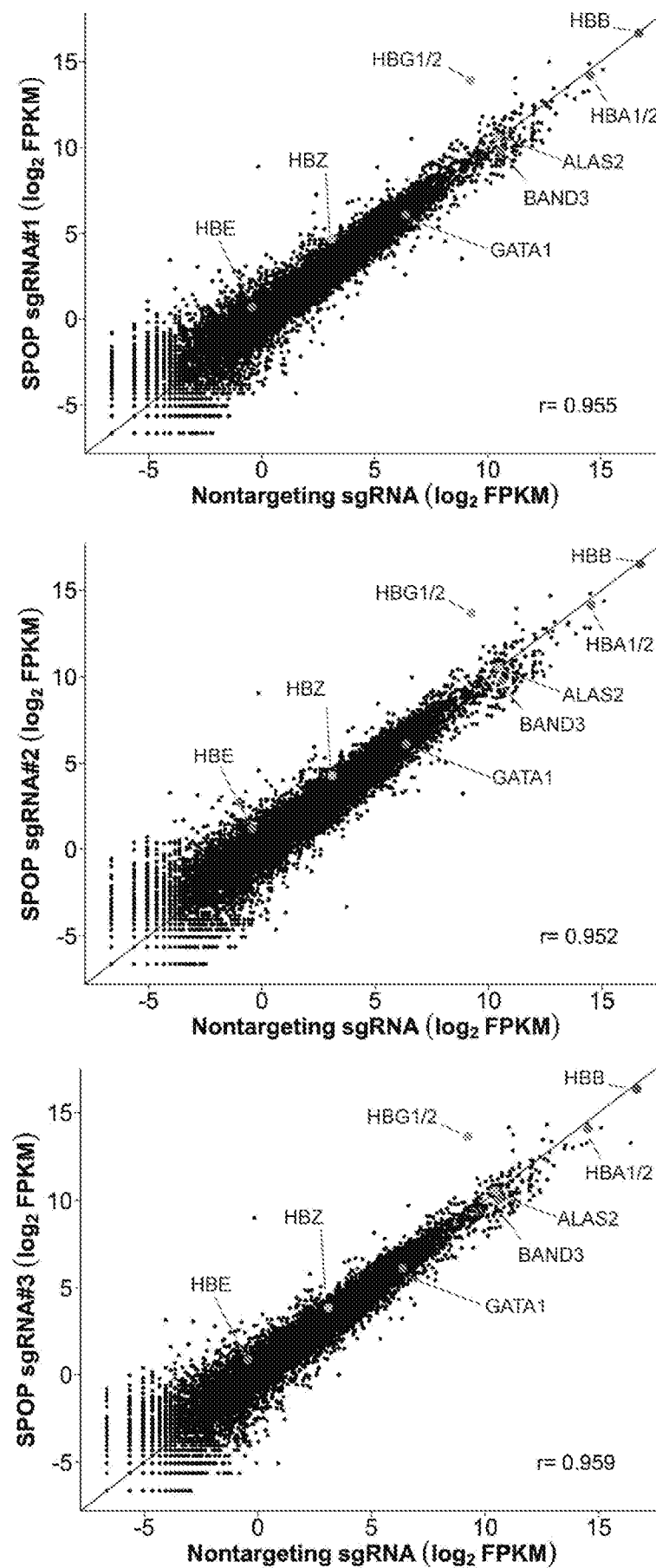


FIG. 3A

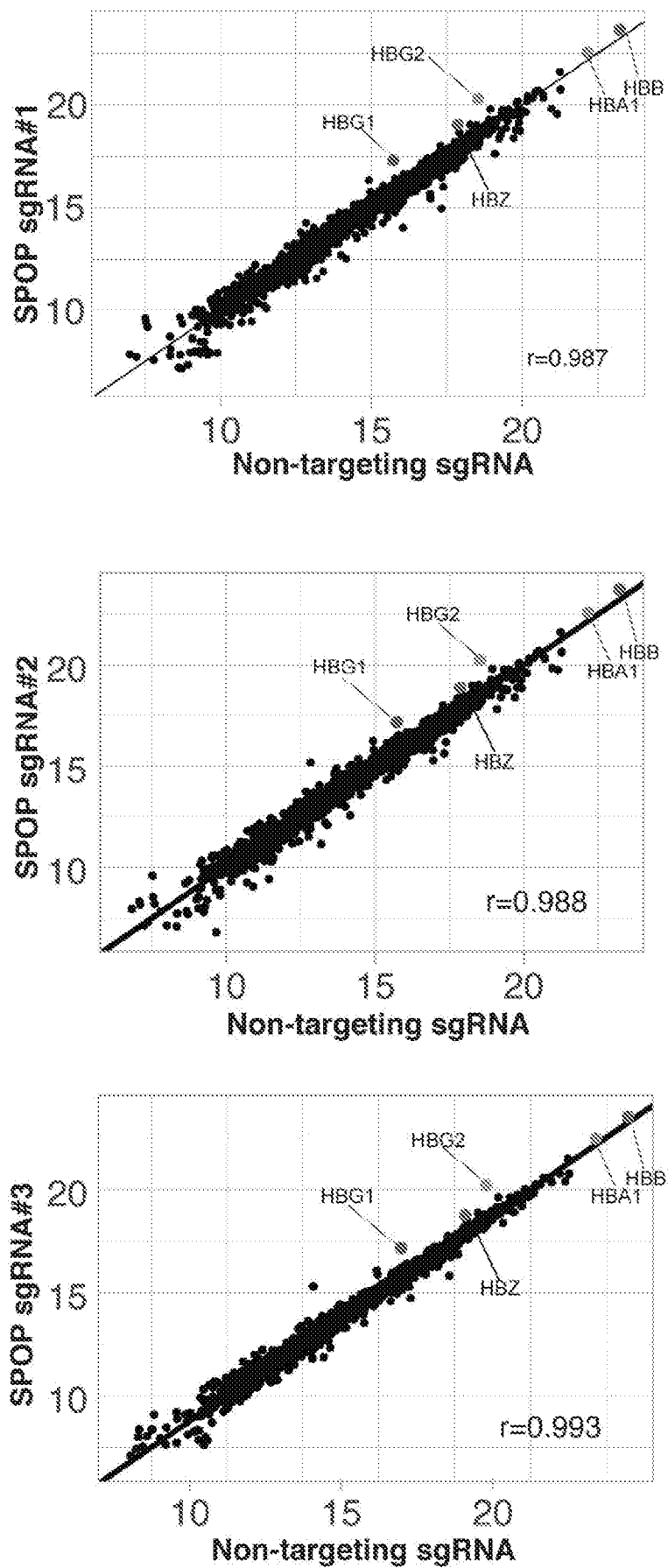


FIG. 3B

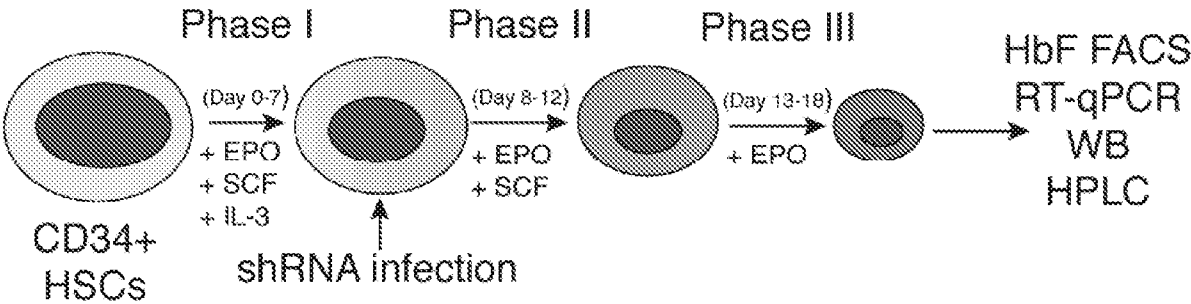


FIG. 4A

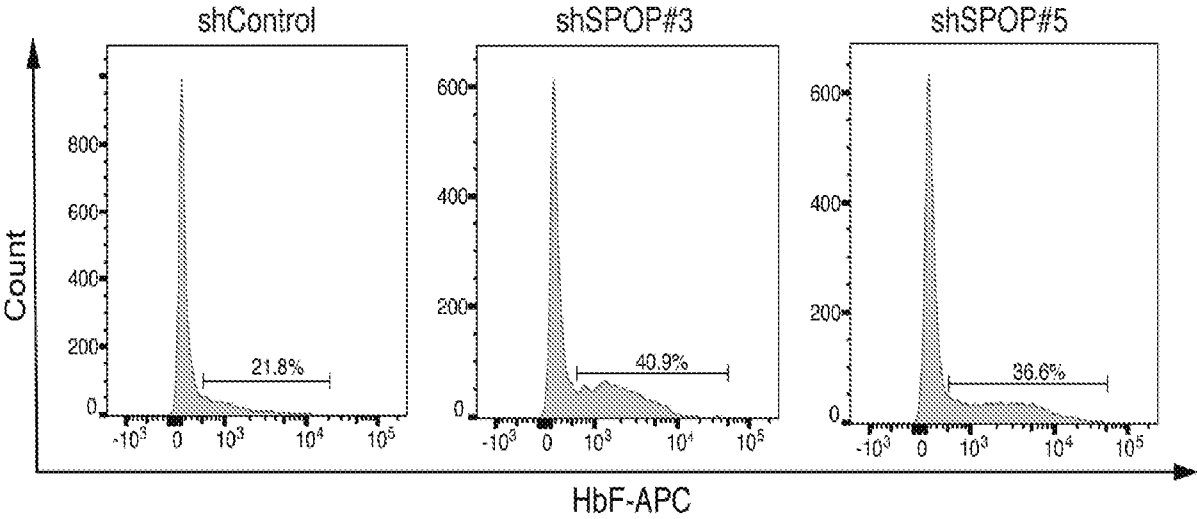


FIG. 4B

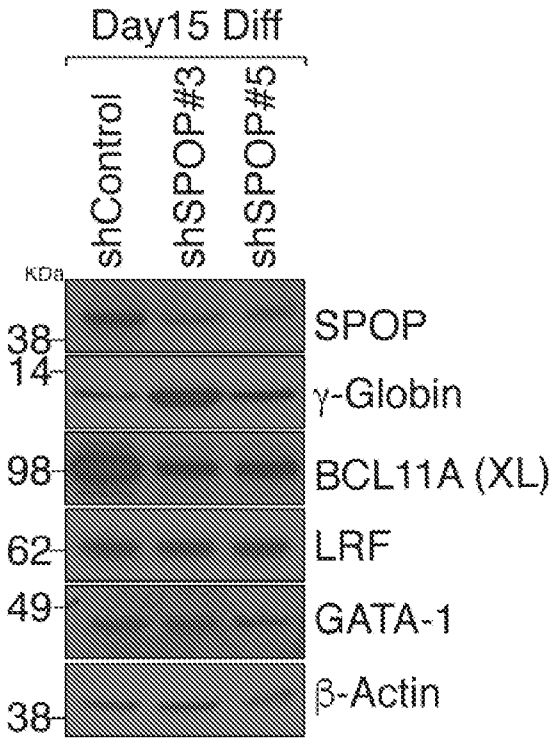


FIG. 4C

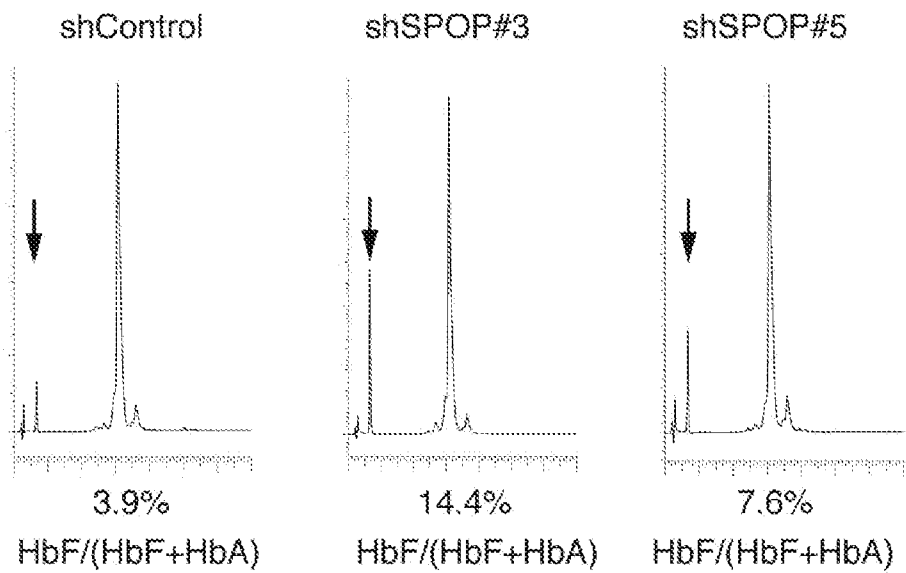


FIG. 4D

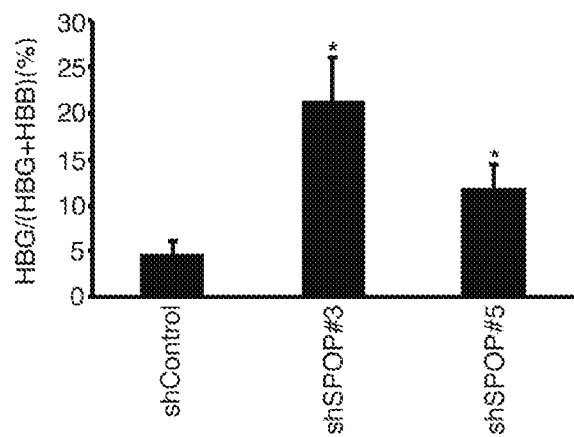


FIG. 4E

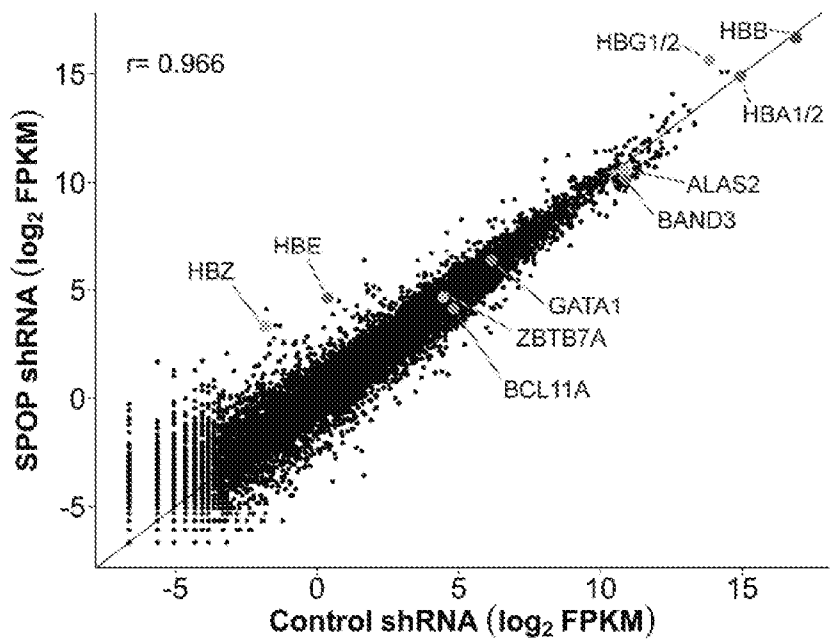


FIG. 4F

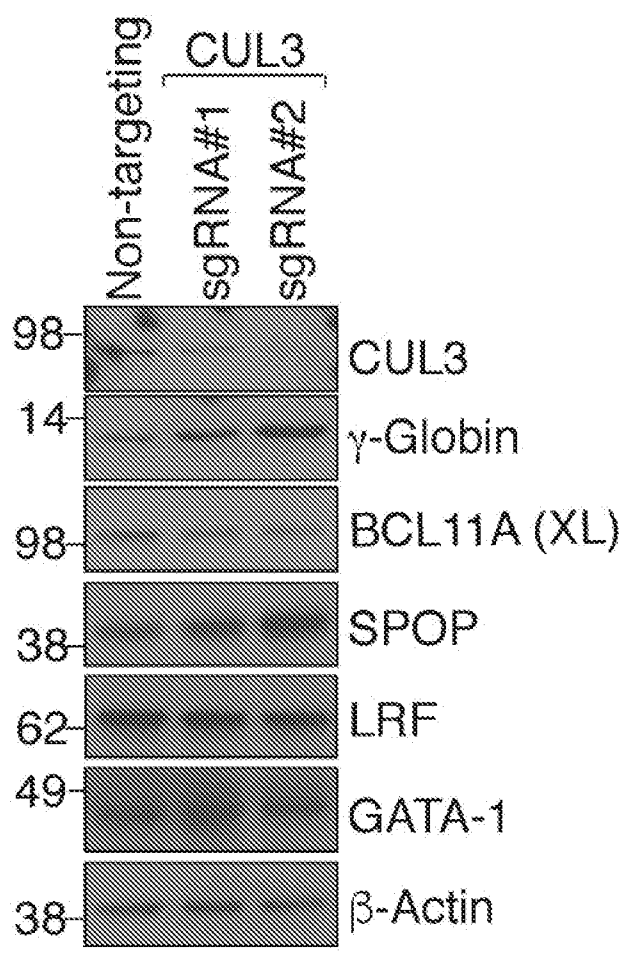


FIG. 5A

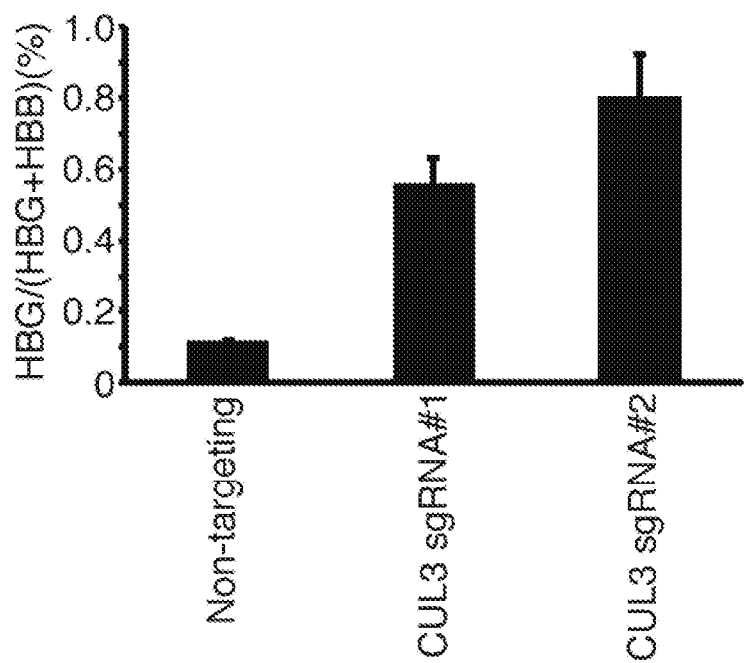
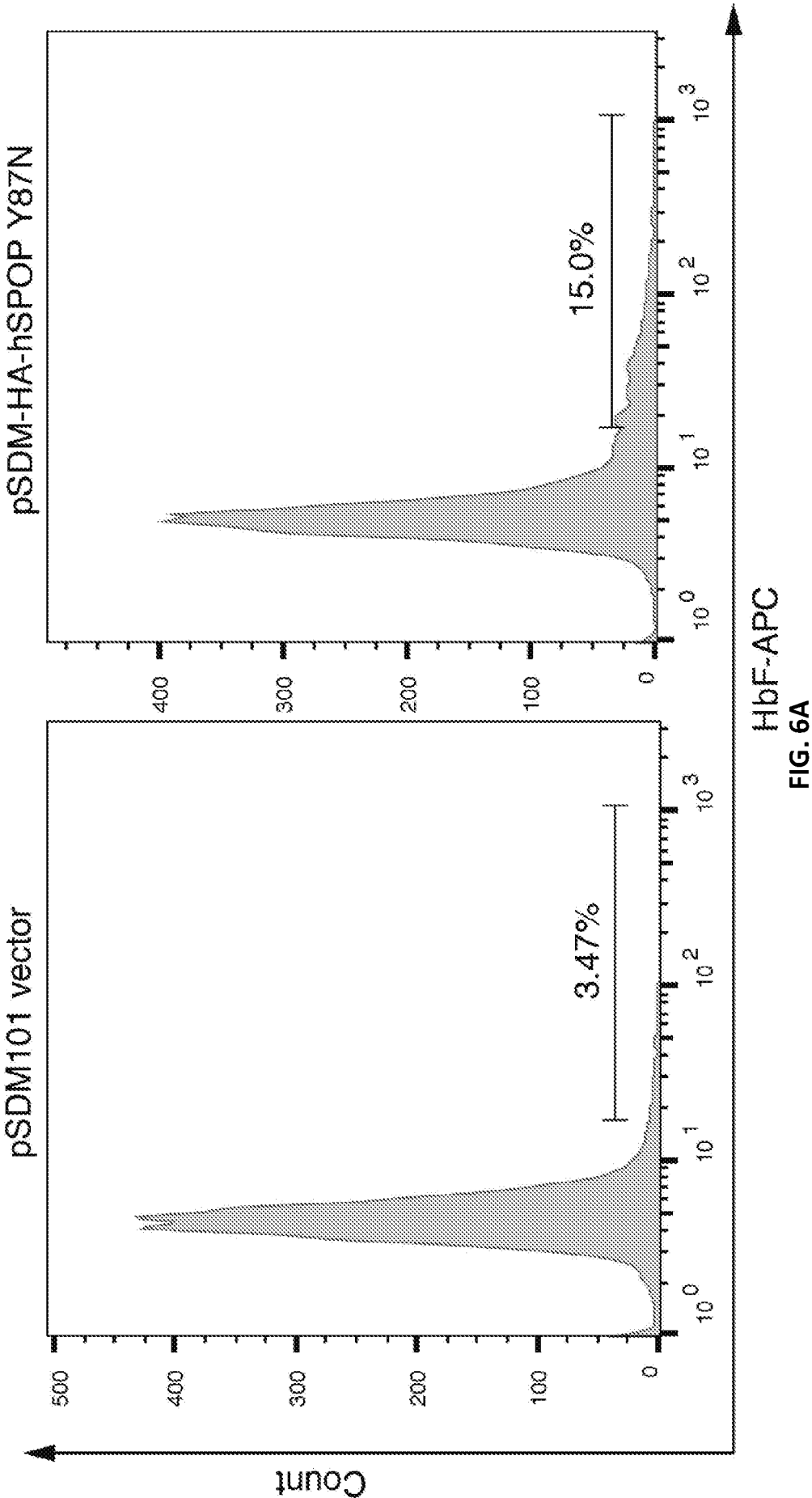


FIG. 5B

Condition	HBG/(HBG+HBB)(%)
Non-targeting + vector	~0.7
SPOP sgRNA#1 + vector	~3.3
SPOP sgRNA#1 + BCL11A	~3.5
SPOP sgRNA#1 + mSpop WT	~0.8

FIG. 5D



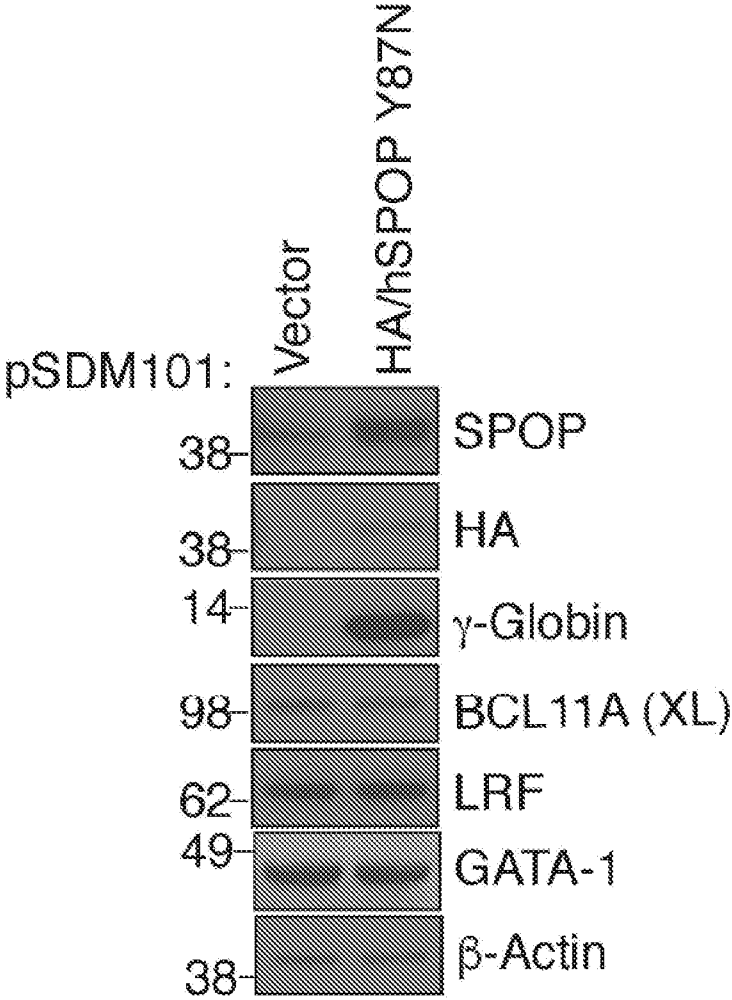


FIG. 6B

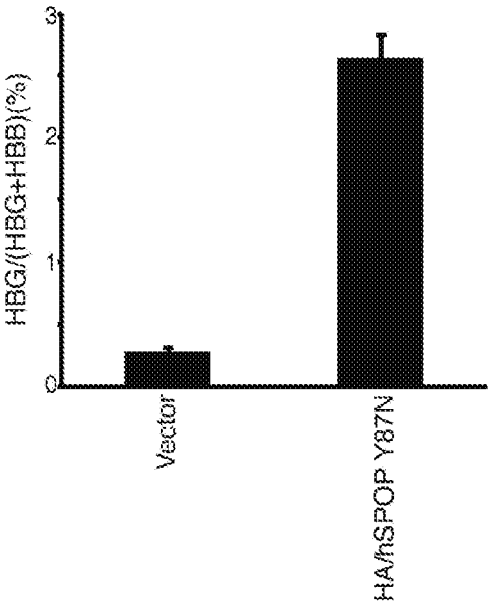
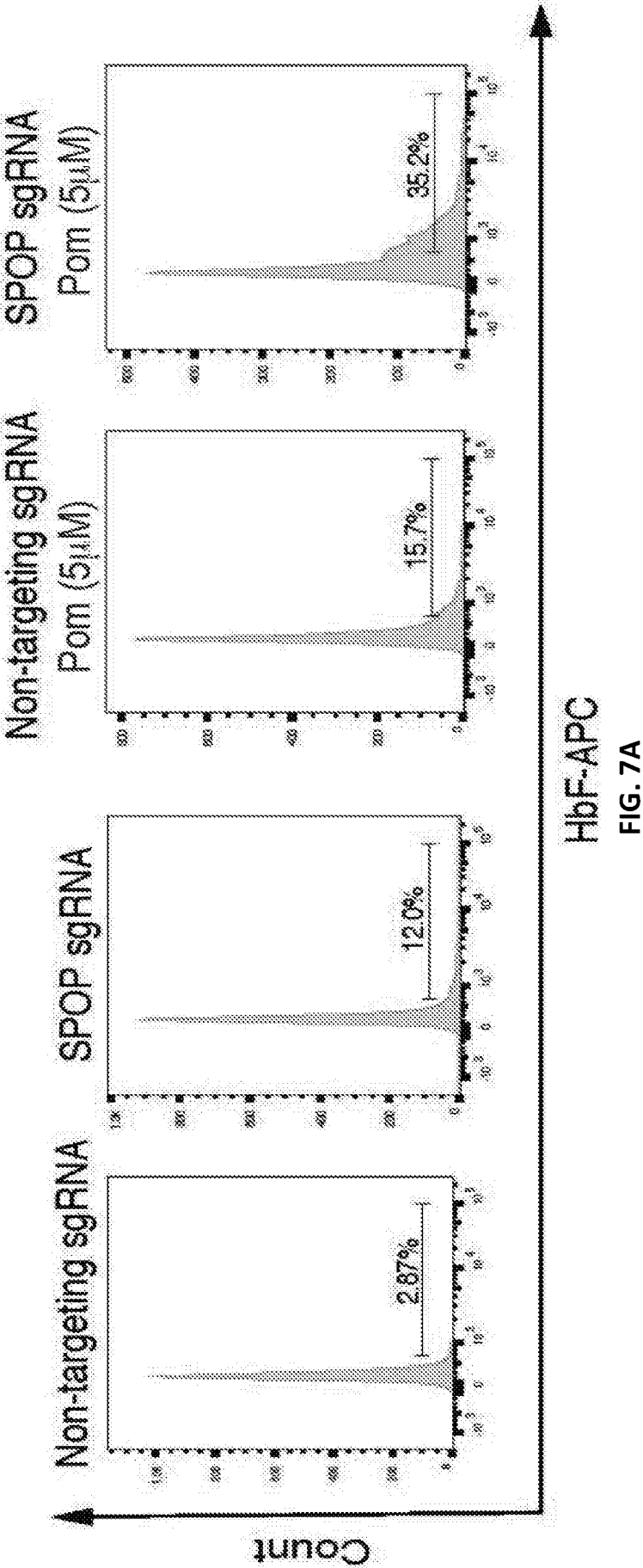


FIG. 6C



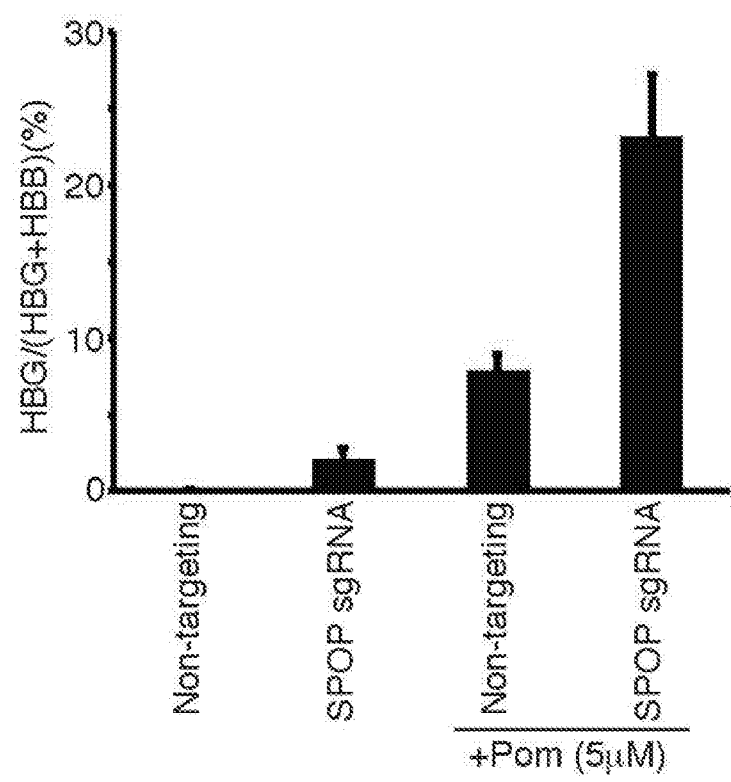


FIG. 7B

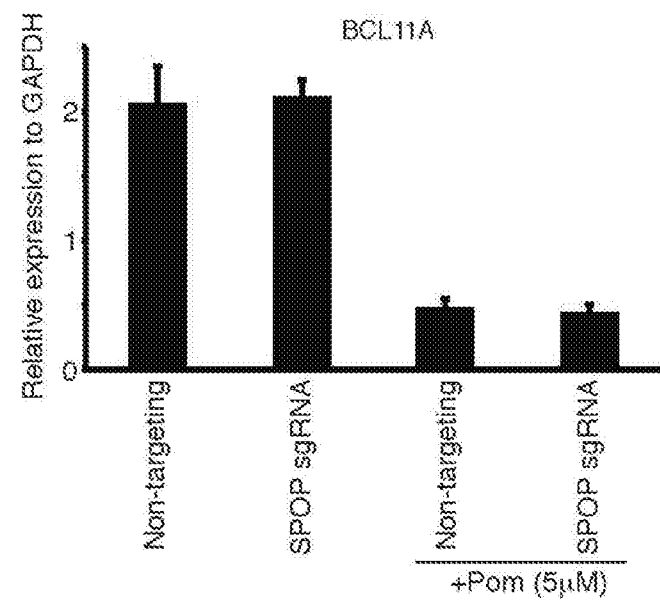


FIG. 7C

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241 VEINDVEPEV FKEMMCFIYT GKAPNLDKMA DDLLAAADKY ALERLKMCE DALCSNLSVE
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361 CPFLGPPRRK LKQS
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Figure 8A

```

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Figure 8B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/21986

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/454, A61K 31/517, A61K 31/7105, G01N 33/50, C12Q 1/68 (2019.01)

CPC - A61K 31/454, A61K 31/517, C12N 15/113, C12N 2310/141, C12N 2320/12, C12N 2320/31, C12N 2330/31, G01N 33/574, G01N 2800/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENG, et al. Prostate cancer-associated mutations in speckle-type POZ protein (SPOP) regulate steroid receptor coactivator 3 protein turnover. PNAS 2013, Vol 110, No 17, pp 6997-7002; Abstract; pg 6999, col 2; Supporting materials, pg 2, Fig S3 and its legend	1-24
A	US 2018/0008725 A1 (CORNELL UNIVERSITY, et al.) 11 January 2018 (11.01.2018) claim 1; para [0021], [0051]	23, 24
X,P	LAN, et al. Domain-Focused CRISPR-Cas9 Screen Identifies the E3 Ubiquitin Ligase Substrate Adaptor Protein SPOP as a Novel Repressor of Fetal Hemoglobin. Blood ePub 21 November 2018, vol. 132 no. Suppl 1, pg 414; in entirety	1-22

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

09 May 2019

Date of mailing of the international search report

06 JUN 2019

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Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
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