GENETIC APPROACHES TO TREAT BETA-THALASSEMIA

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Beta-thalassemia is one of the most common diseases related to the hemoglobin protein. In this disease, the beta-globin gene is mutated, causing severe anemia and ineffective erythropoiesis. Patients can additionally present with a number of life-threatening co-morbidities, such as anemia and iron overload. Current treatment involves transfusion and iron chelation; allogeneic bone marrow transplant is the only curative option, but is limited by the availability of matching donors and graft-versus-host disease.

As this diseases is a monogenic disease, it makes an attractive setting for genetic therapy at the RNA and DNA level. At the DNA level, gene therapy aims to correct the mutated beta-globin or add back a functional copy of beta-globin. Initial gene therapy work was done with oncoretroviral vectors, but has since shifted to lentiviral vectors. Currently, there are a few clinical trials and preclinical tests underway to test the curative potential of some of these lentiviral vectors, including our new gene therapy vector described here with the ankyrin insulator. IVS2-745 is a splicing mutation that occurs in intron 2 of the beta-globin gene. It creates an aberrantly spliced form that incorporates an extra exon and leads to a premature stop codon. Here we report novel uniform 2'-O-methoxyethyl (2'-MOE) splice switching oligos (SSOs) to reverse this aberrant splicing. Lead 2'-MOE SSOs were generated to redirect splicing in the IVS2-745 pre-mRNA. With these lead SSOs we have demonstrated aberrant to wild type splice switching. This switching led to an increase from 3-6% to up to 80% HbA in erythroid cells from thalassemic patients. Furthermore, we have demonstrated a restoration of the balance between beta-like chains and alpha chains, and up to an 87% reduction in toxic alpha-heme aggregates. While next examining the potential benefit of 2'MOE SSOs to patients with a sickle-thalassemic genotype, we observed reduced sickling as a result of 2'MOE SSO induced HbA. In summary, 2'MOE-SSOs are a promising therapy for splicing forms of beta-thalassemia. Their ability to functionally modulate the thalassemia phenotype by correcting the underlying splicing cause offers a pharmacological treatment that is both direct and specific.

BIOGRAPHICAL SKETCH

Alisa Dong received her B.S. in Bioengineering from Berkeley in 2009. After graduating, she worked at Genentech in Process Development and Early Stage Research; then with John Hogenesch on circadian rhythms at the University of Pennsylvania. In 2012, Alisa joined the Rivella lab when it was still at Cornell Weill for her PhD dissertation. She currently works as an *in abstentia* graduate student with Stefano Rivella at the University of Pennsylvania and The Children's Hospital of Philadelphia on gene therapy and splice switching treatments for thalassemia and sickle cell anemia.

This work is dedicated to my family, colleagues, and friends who have supported and pushed me throughout my life. Most of all, to my loving mom, Adrienne Dong, who I know is smiling down on me with pride for going to, and completing, graduate school, to my cooler than ever dad, Calvin Dong, who always knows I have fun *and* work hard, and to my sister Courtney Dong, who I still remember waking me up (loudly) for school every day, and who has set an example to emulate throughout my life.

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LIST OF ABBREVIATIONS

2'MOE	2’-O-methoxyethyl
AAVS1	adeno-associated virus preferred integration site 1
Ank	ankyrin element
BERK	Berkeley mouse model
BM	bone marrow
BMT	bone marrow transplant
bpA	bovine growth hormone polyadenylation symbol
EMH	extra medullary hematopoiesis
G-CSF	granulocyte colony-stimulating factor
GFP	green flourescent protein
GPA	glycophorin A
Hb	hemoglobin
HbA	adult hemoglobin
HbF	fetal hemoglobin
HbS	sickle hemoglobin
HMGA2	high mobility group AT-hook 2
HPFH	hereditary persistance of fetal hemoglobin
HPLC	High performance liquid chromatography
HSC	hematopoietic stem cell
HSVtk	herepes simplex virus type 1 thymidine kinase
iCASP9	inducible caspase 9
IE	ineffective erythropoiesis
iPSC	induced pluripotent stem cells
IRES	internal ribosomal entry site
IVS	intravenous sequence
LCR	locus control region
LTR	long-terminal repeat
MDR1	multidrug resistance 1
MEL	mouse erythroleukemia cells
NTDT	non-transfusion dependent thalassemia
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
RRE	rev response element
SAD	S-Antilles-D Punjab mouse model
SCA	sickle cell anemia
SCD	sickle cell disease
SSO	splice switching oligos
TALENs	transcription activator-like effector nucleases
TCP	Tranyl-cypromine

TR1	transferrin receptor 1
UTR	untranslated region
VCN	vector copy number
WHO	World Health Organization
WPRE	Woodchuck Post-Regulatory Element
ZF	zinc finger

Chapter One

An Introduction to genetic approaches for the treatment of beta-thalassemia

Sickle cell anemia (SCA) and beta-thalassemia are the most frequently inherited blood disorders worldwide. Altogether, roughly 100,000 Americans are affected by these disorders. Both disorders are characterized by mutations in the beta-globin gene, a subunit of hemoglobin (Hb). SCA is an inherited disorder characterized by only a single mutation in the beta-globin gene, leading to the formation of hemoglobin S (HbS) (1). HbS exhibits a marked decrease in solubility, and an increase in viscosity and polymer formation. Ischemic stroke, caused by large vessel arterial obstruction with superimposed thrombosis is one of SCA's most devastating complications. Blood transfusions are administered to prevent thrombosis. Unfortunately, periodic blood transfusions are associated with significant risks of iron overload and other complications, and must be accompanied by iron chelation (2, 3). Beta-thalassemia on the other hand is characterized by one or more of over 300 various mutations in the beta-globin gene. Based on the combinations of these mutations, patients might be affected by a milder form, indicated as beta-thalassemia intermedia or non-transfusion dependent thalassemia (NTDT), or the most severe form, beta-thalassemia major (4, 5). Beta-thalassemia major requires regular transfusions to sustain life. However, due to the negative progression of this disease, very often NTDT patients become transfusion dependent as well (6, 7). Major problems are

progressive splenomegaly from extra medullary hematopoiesis and iron build-up in the heart and other organs, often resulting in fatal outcomes for some patients in their teens or early 20s (8, 9). Current palliative therapeutic options to treat these two disorders are red blood cell transfusion and iron chelation (2, 10).

In addition to life-threatening anemia, patients may present with inherent and treatment-related complications that exacerbate the pathology. For patients with SCA, common complications include painful episodes, acute chest syndrome, and stroke (1); in patients with thalassemia, hepato-splenomegaly, recurrent infections, and spontaneous fractures (11-13). In both cases, transfusion-associated infections and organ damage are side effects of long-term treatment and unsatisfactory iron chelation. Iron overload is observed also in NTDT patients because of ineffective erythropoiesis (6, 14). Ineffective erythropoiesis triggers a cascade of compensatory mechanisms resulting in erythroid marrow expansion, extramedullary hematopoiesis, splenomegaly, and increased gastrointestinal iron absorption (15). Ineffective erythropoiesis triggers increased iron absorption by reducing the expression of hepcidin, the hormone that controls dietary iron absorption (14, 16-18).

Although both transfusion and iron chelation treatments have remarkably improved over the years and, thus, improving the quality of life, they do not provide a definitive cure, as they do not address the inherent genetic cause. To this end, hematopoietic stem cell (HSC) transplantation is the only presently available cure. Allogeneic bone marrow transplant (BMT) can be curative, but only a small proportion of patients have suitable donors. Furthermore,

myeloablative HSC transplantation carries a 5%–10% mortality rate. Graft-vs-host disease and adverse immune reactions can limit the success of allogenic BMT as well (9). Given these limitations, gene therapy using a patients' own HSCs represents an alternative and potential cure because it aims at the direct recovery of the hemoglobin protein function via the addition of a functional copy of the beta- or gamma-globin gene. The development of gene therapy tools for SCA and beta-thalassemia has been the object of research of the last few decades and has been proved successful in mouse model studies, *in vitro* human cell studies, and thus far in one clinical trial.

At the DNA Level: Gene Therapy

The conditions for a clinical-grade gene therapy vector can be summarized as follows. Figure 1 provides a schematic of gene therapy and key issues found in each stage.

(1) controlled transgene expression: erythroid-specific, stage-restricted, elevated, position-independent, and sustained over time

(2) effective targeting of HSCs

(3) highly efficient and stable transduction

(4) absent or low genomic toxicity

(5) correction of the phenotype in preclinical models.

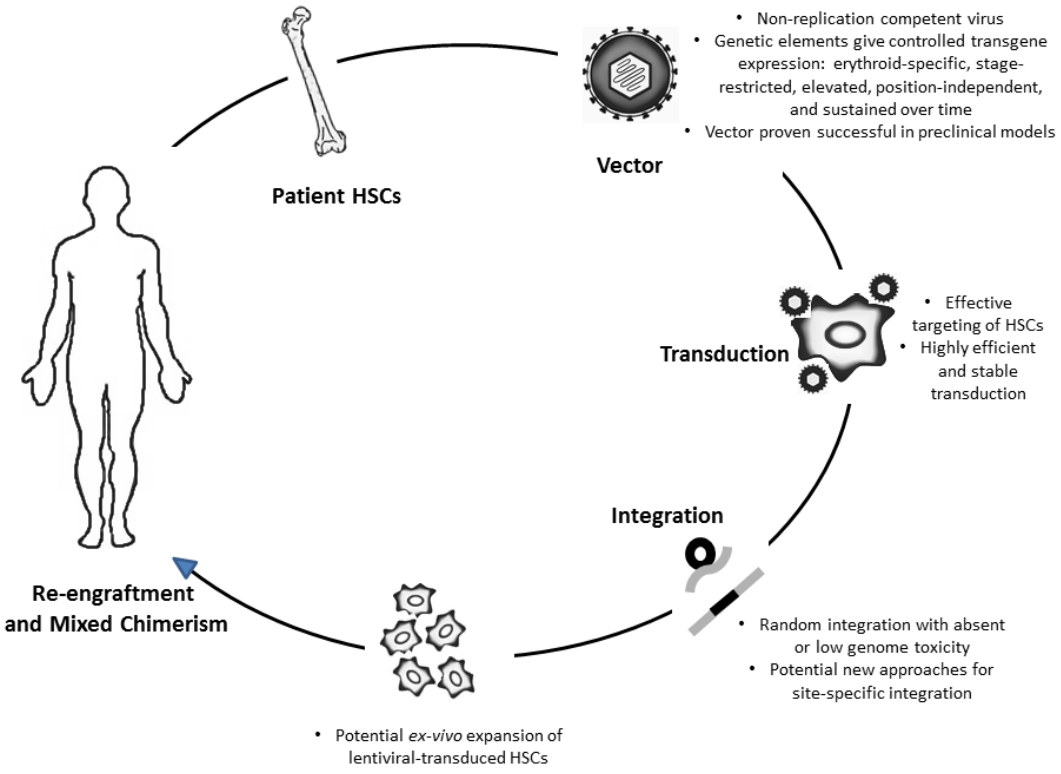


Figure 1: Schematic of gene therapy for beta-thalassmia and sickle cell anemia.

Oncoretroviral Vectors

The first studies of gene addition were done with oncoretroviral vectors and helped paved the way for current lentiviral vectors. Oncoretroviruses, like lentiviruses, belong to the Retroviridae family and are RNA-based viruses(19). Multiple studies showed oncoretroviruses are capable of transferring genetic material without transferring any viral material, and are able to achieve expression of human beta-globin in murine cells. However, the expression of beta-globin was extremely low and nowhere near therapeutic (20-22).

Studies then moved on to determining what other elements needed to be incorporated to achieve higher beta-globin expression. Discovery of the locus control region, or LCR, brought significant advancements to vector design. The

LCR is a regulatory region upstream of the beta-globin locus and is critical for high-level, sustained, erythroid-specific, and position-independent globin expression (23, 24). The LCR is made up of four DNaseI hypersensitive sites (HS) that contain many motifs for transcription factors and chromatin remodeling factors. It is thought to regulate globin gene expression through a looping mechanism, bringing various transcriptional modifiers to the globin promoter (25). Incorporation and modification of these HS sites was done in a number of works. Work by Plavec et al in 1993 (26) showed HS2, HS3, and HS4 elements increased beta-globin expression by 10-fold in mouse erythroleukemia (MEL) cells, but still remained relatively low for mice transplanted with oncoretrovirus-transduced cells: 0.04%-3.2% of endogenous mouse beta-globin RNA. Furthermore, there were problems with stability and high viral titer production. Leboulch and coworkers (27) undertook modification of the LCR in order to overcome these problems. They saw instability in all combinations of LCR sequences, with HS2 alone conferring a single common rearrangement and other combinations showing multiple rearrangements. Beta-globin gene mutagenesis and elimination of a 372 base pair intronic sequence and multiple reverse polyadenytlation and splice sites resulted in higher titer viruses and more stable proviral transmission. Sadelain saw additional success in 1995 (28) with producing a high-titer retroviral beta-globin vector, but unfortunately the vector did not give high position-independent expression and large clonal variation was seen.

Other methods were also tested for their ability to achieve therapeutic levels of expression: addition of a chromatin insulator (29); use of the ankyrin promoter driving the gamma-globin gene (30); use of a mutant gamma-globin enhancer characterized from patients with hereditary persistence of fetal hemoglobin (HPFH) driving gamma globin (31); addition of the HS40 regulatory region from the human alpha-globin gene locus (32); and use of an anti-sickling beta-globin (33). Many important insights were gained by these experiments, however, as with other oncoretroviral studies, their success was limited. Eventually oncoretoviral studies gave way to the lentiviral studies discussed below.

Lentiviral Vectors

In the mid-1990s, lentiviral vectors based on the human immunodeficiency virus (HIV-1) arose as an option for gene transfer. Engineered to be devoid of any pathogenic or replication competency, these viruses are efficiently able to encompass large therapeutic transgene cassettes. Like all retroviruses, lentiviruses exhibit receptor-mediated entry, capsid uncoating, reverse transcription, and integration into the host genome (19). However, lentiviruses have a more intricate genome, notably the Rev response element, or RRE. The RRE helps stabilize the proviral RNA by interacting with the viral protein Rev. This allows for stronger unspliced RNA export from the nucleus (19, 34, 35). Another important element discovered in lentiviruses is the central polypurine tract/central termination sequence element. The cPPT/CTS element is a short

noncoding part of the *pol* gene sequence that increases lentiviral transduction efficiency.

Importantly, for hematological gene therapy purposes, lentiviruses are able to infect dividing and non-diving cells (36). Gene therapy for hematological disorders typically infect HSCs, so that the genetic modification is passed on short term to differentiating cells, and long-term to more stem cells through self-renewal. HSCs, however, are difficult to culture and transduce *ex vivo* due to a delicate balance between dividing/proliferation and engraftment potential. Normally, proliferation and engraftment potential are negatively correlated: increasing the proliferation is detrimental because the cells subsequently do not engraft (37). Thus, ability to infect non-dividing cells that retain engraftment potential is an extremely beneficial property of lentiviruses. Lentiviruses containing regulatory elements, promoters, enhancers, and beta-globin or gamma-globin have been successful with correction of mouse models of thalassemia and SCA, and with *in vitro* correction of human CD34+ peripheral blood (PB) cells. A list of beta-globin vectors can be found in Table 1.

Table 1: Beta-globin vectors

Name	Author and Year	Main Characteristics
TNS9	May 2000 Rivella 2003	- correction of a mouse model of thalassemia intermedia (2000) and prevention of lethality in a mouse model of thalassemia major (2003) - variable expression
$\beta^{A(T87Q)}$	Pawliuk 2001	- correction of two SCA mouse models - Anti-sickling (T87Q) form of beta-globin
BG-1	Puthenveetil 2004	- Full-length cHS4 enhancer used in 3'LTR (1.2kb) - Amount of beta-globin approached normal levels - Low viral titers
T10	Lisowski 2007	- addition of HS1 to HS2-4
Globe	Miccio 2008	- higher titer with removal of HS4
$\beta^{A(T87Q)}$ LentiGlobin	Cavazzana-Calvo 2010	- Used in first European clinical trial to achieve transfusion independence in a β0/βE heterozygous patient - Transgenic $\beta^{(T87Q)}$-globin made up only 1/3 of total Hb, rest combination of HbE/HbF - Integration sites near potential oncogenes - Saw expansion of one clone with a *HMGA2* integration site
G-Globe	Miccio 2011	- no HS4, incorporates HS2 enhancer of the GATA1 gene
AnkT9W T9AnkW	Breda 2012	- Incorporates the Ankyrin insulator - Shows improved β-globin expression over TNS9
CCL-βAS3-FB	Romero 2013	- "FB" insulator containing the minimal 77 bp binding site for CTCF - 3 mutations to beta-globin to confer antisickling properties

In 2000, May (38) and colleagues used the TNS9 vector to correct a mouse model of thalassemia intermedia. Later in 2003, Rivella (39) and colleagues showed that this same vector could be used to rescue lethality in a new model of Cooley's anemia (thalassemia major). TNS9 exhibits position-effect variation though, and in the Cooley's anemia model, was unable to be therapeutic in all mice, with average human beta-globin expression between 3.6 and 9.4 g/dL. Two mice models of SCA were corrected in 2001 by Pawliuk (40) and colleagues: the S-Antilles-D Punjab model (SAD) and the Berkeley (BERK) model. The SAD mouse model expresses human alpha and a human "super S" beta-globin that has two point mutations(41). The BERK model expresses

human alpha and human sickle beta-globin, but additionally does not express any endogenous mouse alpha or mouse beta globin (42). As a result, the BERK model has a more severe phenotype, in part because of suboptimal expression of the human beta-globin gene as compared to the endogenous mouse gene. Pawliuk used a transgenic "βT87Q" form of beta-globin, an antisickling mutant form which has an amino acid substitution at the 87th position. With this vector, Pawliuk saw transgenic Hb could make up to 12% and 52% total Hb for the SAD and BERK models, respectively. In 2013, another antisickling mutant form was tested with three point mutations: T87Q for blocking the lateral contact with HbS, E22A to disrupt axial contacts with HbS, and G16D, which confers a competitive advantage over HbS for interaction with alpha-globin (43). Named "CCL-βAS3-FB", this vector could reduce the relative amount of sickled red blood cells differentiated *in vitro*; and, using vector copy numbers of 0.5-2, could make up 15-25% of total Hb.

In order to increase the safety of lentiviral vectors and improve expression, insulators were tested by Puthenveetil in 2004 (44). An insulator is a genetic element which usually has two properties: (1) enhancer-blocking activity, when placed between an enhancing element and a promoter and (2) preventing the spread of heterochromatin into the integrated transgenic cassette from a nearby heterochromanized region (45-47). By adding an insulator, one can prevent the beta-globin LCR from acting on nearby oncogenes it might have integrated near. It can also help reduce vector silencing, to ensure sustained and high transgene expression. Puthenveetil et alt's vector, named BG-1, added a 1.2 kb cHS4

insulator, taken from the chicken beta-globin hypersensitive site 4. As reviewed by Nienhuis and Persons (48), the cHS4 insulator has a “core” that contains five footprints. The footprints are involved in: recruiting CTCF, an enhancer blocking protein; binding USF proteins in order to recruit histone-modification enzymes that make transcription-activation marks; and binding VEZF1, which prevents DNA methylation in the transcribed region. Human beta-thalassemic cells treated with BG-1 and differentiated *in vitro* showed similar amounts of hemoglobin as non-thalassemic controls. Upon transplantation into immunodeficient mice, treated cells underwent effective erythropoiesis and expressed normal amounts of beta-globin. In 2007, Arumugam (49) and colleagues compared vectors with the cHS4 insulator to those without and consistently saw approximately double the beta-globin expression with the insulator *in vitro* with MEL cells and *in vivo* with transplanted and transduced murine HSCs. While beneficial to expression, the 1.2kb cHS4 insulator causes low viral titers. Thus, in 2009, Arumugam (50) identified a 400 bp extended core region of the cHS4, that still exhibits full insulator activity but does not have a severe an impact on titer. They found the previously identified core only reduced clonal variegation.

Further studies have also been done on the LCR. In 2007, Lisowski et alt (51) showed addition of HS1 from the LCR to HS2-4 significantly increased globin expression. Miccio in 2008 (52) used a “GLOBE” vector containing the HS2 and HS3 regions without the HS4 region. They used it to rescue Cooley’s anemia lethality, but high copy numbers were required for correction (34).

Interestingly, upon transplantation, transduced cells expressing a high level of beta-globin were preferentially selected *in vivo*. Roselli (53) produced preclinical data using the "GLOBE" vector in 2010 on a diverse set of CD34+ patient samples to restore adult Hb (HbA) synthesis. Integration analysis revealed integration preference in transcriptionally active regions but no preference for cancer-related regions. "GLOBE" was later modified into "G-GLOBE" by adding the HS2 enhancer of the GATA-1 gene (54); it too could achieve high expression of beta-globin. The GATA-1 HS2 bound GATA1 and CBP acetyltransferase, leading to the establishment of an open chromatin region.

Table 2: Gamma-globin vectors

Name	Author and Year	Main Characteristics
d432β^{A}γ	Persons 2003	- high variation depending on integration region and vector copy number
mLARβΔγV5 (V5)	Hanawa 2004	- larger LCR reduced position effects and improved expression
G9	Samakoglu 2006	- Includes shRNA against sickle β-globin - Test only *in vitro* on MEL and HeLa cells stably expression sickle beta globin
V5m3	Pestina 2009	- 3'UTR of beta-globin corrected BERK SCA model
V5m3-400	Wilber 2011	- added 400 bp core of cHS4 insulator
GG HI	Papanikolaou 2012	- no LCR elements, but instead contains a gamma-globin promoter with a -117 point mutation associated with HPFH, the HS40 enhancer from the alpha-globin locus, the HPFH-2 enhancer, and the cHS4 insulator -only mild HbF production

Lentiviral transduction of gamma-globin has additionally been shown to be therapeutic. Table 2 lists gamma-globin vectors that have been successfully used. In 2003, Persons (32) et al developed the "d432β^{A}γ" vector, which expressed gamma-globin under beta-globin LCR elements. Although they saw expression of fetal hemoglobin, they still saw high variation due to position and

vector copy number. Hanawa in 2004 (55) used a longer LCR to achieve more consistent expression across animals. In 2006, Samakoglu (56) and colleagues showed that a combination vector could be made that expressed gamma-globin and concurrently knocked-down sickle beta-globin via small hairpin RNA. They tested this vector on HeLa and MEL cells stably expressing sickle beta globin. The discovered that the placement of the shRNA was critical, as it affected interferon response, siRNA production, and the amount of gamma-globin expression. Pestina (57) tested a gamma-globin vector *in vivo* on the BERK SCA model. The gamma-globin was modified to contain a 3'UTR from beta-globin, since proteins are believed to bind the beta-globin 3'UTR and increase mRNA stability. This was further modified by Wilber in 2011 (58) with the addition of the 400bp core of the cHS4 insulator, and tested on human CD34+ PB cells from three beta-thalassemia patients. They saw fetal Hb (HbF) production ranged between 45-60% of total Hb, and up to a 3 fold increase in total Hb content. Papanikolaou in 2012 (59) published a report of a gamma-globin virus without the LCR, but instead containing a gamma-globin promoter with a -117 point mutation associated with HPFH, the HS40 enhancer from the alpha-globin locus, the HPFH-2 enhancer, and the cHS4 insulator. They saw mild improvement of HbF synthesis compared to mock-transduced controls.

Most recently, our laboratory has done work to find new insulators. The aforementioned cHS4 insulator is subject to rearrangements and loss (see Clinical Trials, below). To this end, we generated a new lentiviral vector, which we have named AnkT9W. AnkT9W contains the erythroid-specific ankyrin 5′

hyper-sensitive barrier insulator (60, 61). This insulator does not exhibit enhancer-blocking activity, but does prevent the spread of heterochromatin, and significantly increases expression of beta-globin as compared to vectors without this insulator. This vector was able to maintain high, yet stable levels of Hb synthesis in MEL cells and human CD34+ PBMCs. Analysis indicated that in MEL cells, AnkT9W expressed the transgenic mRNA and hemoglobin at higher levels than the parental T9W (a modified TNS9). Interestingly, AnkT9W was additionally able to correct the phenotype of SCA cells by modifying the *proportion* of sickling vs functional Hb, without changing the overall Hb content. This could be clinically relevant since there is a concern that adding transgenic beta-globin into SCD HSCs increases the total amount of beta-chains, both sick and transgenic. This new total amount might exceed the amount of α-chains, leading to an alpha-thalassemia like phenotype.

Addition of Non-globin Genetic Elements

On top of the numerous studies to add back beta and gamma globin genetic sequences, there are have been additional studies based on adding other genetic elements which can modify beta or gamma-globin gene expression. The gamma-globin repressor *BCL11A* has been identified as target to increase gamma-globin gene expression. Xu and colleagues were able to demonstrate that affecting *BCL11A* alone was able to increase endogenous gamma-globin expression and ameliorate the sickle cell phenotype in mice(62). Since *Bcl11A* knockout is postnatal lethal, they used a floxed *Bcl11A* mice crossed with the *EpoR-GFP Cre* mice, which express Cre recombinase under the erythropoietin

receptor promoter. As in the full *Bcl11A* knockout, the switch from HbF to HbA did not occur. These mice were then bred with SCD mice. In the combination *SCD/Bcl11A*$^{fl/fl}$ mice, sickle cells were absent and blood parameters were markedly improved, thus showing that *Bcl11A* deletion alone was sufficient to ameliorate SCD. Along the same lines, Wilber and colleagues (58) tested a lentiviral construct encoding a *BCL11A* shRNA on CD34+ human PB cells and saw a 3-fold increase in gamma-globin expression. Most recently, Bauer and colleagues (63) have done a genome-wide association study concerning *Bcl11A*. They found a sequence in intron-2 that causes developmentally restricted, erythroid-specific *lacZ* reporter expression in mice. Disruption of this sequence with transcription activator-like effector nucleases (TALENs) in MEL cells lead to reduced expression of *Bcl11A*. As such, this sequence might be a new target to lower *Bcl11A* expression and increase HbF production.

Oct-1 is another gene that negatively regulates gamma-globin gene expression. Oct-1 is a transcription factor that recognizes the octamer ATGCAAAT. The gamma-globin promoter contains three Oct-1 consensus sequences. The -175 consensus sequence has been shown to be associated with HPFH and mutagenesis of the -280 consensus sequence leads to increased gamma-globin expression. Xu and colleagues (64) tested the ability of a “decoy oligonucleotide” to compete for Oct-1 binding, therefore reducing Oct-1 binding at the endogenous gamma-globin locus. In K562 cells, they saw an increase in gamma-globin gene expression after addition of the decoy oligonucleotide.

Genetic elements can also be engineered to affect beta and gamma-globin gene expression. Advances in zinc-finger (ZF) development have allowed the creation of domains able to recognize any 18 base pair DNA sequence. ZF domains can be paired with transcriptional activation domains to create “artificial transcription factors”. In 2010, Wilber and colleagues (65) extensively examined one such engineered ZF transcription factor, termed GG1-VP64. GG1-VP64 recognizes the -117 position of the gamma-globin promoter. The -117 position is the site of a naturally occurring mutation which causes HPFH, and is thus a known region important for modulating gamma-globin gene expression (66). They discovered in wild-type CD34 PB cells that up to 20% HbF could be produced, as compared to 2% in untransduced controls. Later in 2011(58), they tested beta-thalassemic samples and found a therapeutic 20-fold increase in gamma-globin could be achieved. In 2012, Deng et alt published a paper concerning an artificial ZF linked to the protein Ldb1 (25). Ldb1 is a critical part of GATA1-mediated chromatin looping of the LCR to the beta-globin promoter. Deng and colleagues created Ldb1-ZFs that recognized the beta-major promoter (P-ZF) or the HS2 site of the LCR (L-ZF). They showed in murine GATA-1 null cells that beta-major expression could be induced by P-ZF alone or P-ZF and L-ZF, but not by L-ZF alone. Furthermore, they showed that the self-association domain of Ldb1 was sufficient for this activity as well (25).

Clinical Trials

The first successful gene therapy trial for beta-thalassemia was done in Paris and reported by Leboulch in 2010 (67). It was a small trial involving only

two patients. The first patient failed to engraft due to technical issues unrelated to the vector. The second patient, however, has been transfusion independent now for several years. The patient is a compound heterozygote (βE/β0), in which one allele (β0) is nonfunctioning and the other (βE) is an HbE mutant allele whose mRNA may either be spliced correctly (producing a mutated βE-globin) or incorrectly (producing no beta-globin).

The βT87Q LentiGlobin vector was used for this trial (see Table 1). As with the βT87Q vector, this vector expresses a mutated beta-globin distinguishable from transfused beta-globin due to an anti-sickling mutation at the 87th amino acid. It also contains two core copies of the cHS4 insulator. Analysis of the patient's transduced cells revealed an intact coding sequence for the vector, however, with the loss of one copy of the cHS4. Of the twenty-four chromosomal integration sites (IS) found, one of the sites, high mobility group AT-hook 2 (*HMGA2*), caused transcriptional activation of *HMGA2* and became the dominant clone. Cells from the clinical trial patient with a HGMA2 IS showed loss of the 3'UTR of *HGMA2*, preventing the binding of let-7 miRNAs to complementary sequences. Erythroid cells from the *HMGA2* clone exhibited a dominant, myeloid-biased cell clone. *HMGA2* mRNA was undetectable in granulocyte-monocytes, thus the expression was reported to be erythroblast-specific. However, the clonal dominance of *HMGA2* was represented in all populations in similar proportions (erythroblasts, granulocyte-monocyte and LTC-IC cells). The authors hypothesize that this dominance is due to a transient

expression of *HMGA2* in a myeloid-restricted LT-HSC during β-LCR priming, before the β-LCR becomes restricted to the erythroid lineage.

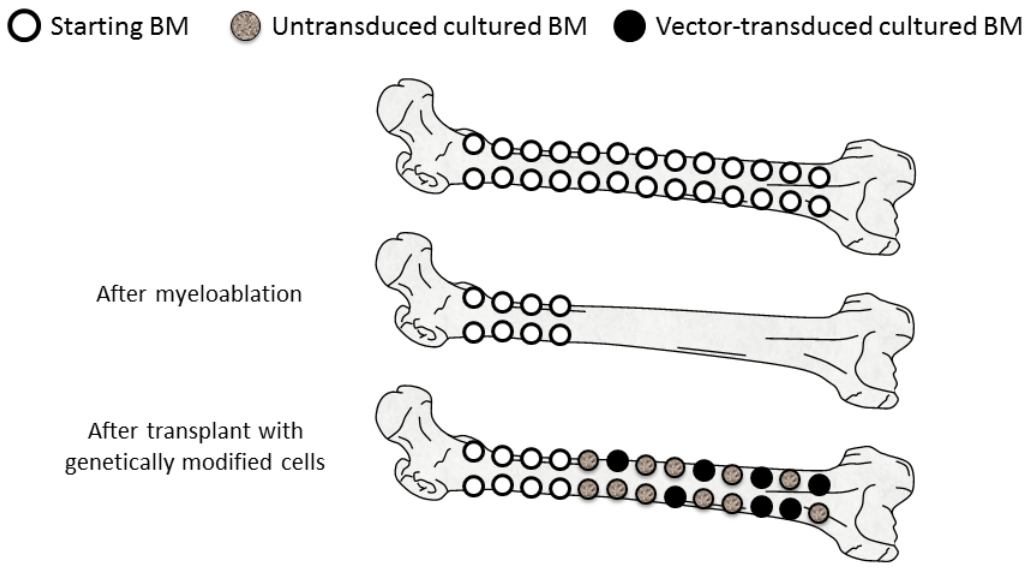
Overexpression of *HMGA2* is found in a number of benign and malignant tumors and can lead to a clonal growth advantage (68). Overexpression is often associated with mutations affecting the 3' untranslated region (UTR), which contains binding sites for the regulatory miRNA let-7 (68). Let-7 miRNA binding to the 3'UTR of *HMGA2* negatively regulates *HMGA2* mRNA and thus the level of protein expression (69).Transgenic mice carrying a *HMGA2* with a shortened 3'UTR expressed increased levels of *HMGA2* protein in multiple tissues including hematopoietic cells. These mice showed splenomegaly, erythropoietin-independent erythroid colony formation, and an increased number of peripheral blood cells in all lineages. Furthermore, BM cells derived from these animals had a growth advantage over wild-type cells. Thus, overexpression of *HMGA2* is associated with clonal expansion at the stem cell and progenitor levels (68).

At the time of reporting, the patient had been transfusion-independent for two years, and showed stable Hb levels from 9-10 g/dL-1. The patient has undergone frequent phlebotomies to increase iron clearance. Therapeutic Hb-βT87Q LentiGlobin however only accounted for 1/3 of the total Hb, with endogenous HbE and HbF making up the rest. Without the additive effect of these endogenous Hb's, this first trial might not have been a success. This suggests that we not only need a predictive *in vitro* model with which to evaluate potential trial patients, but better vectors that can achieve higher therapeutic Hb expression.

Currently the first United States phase I clinical trial has received FDA approval and is enrolling patients. The strategy was briefly described by Sadelain and colleagues in 2010 with the main goals of assessing insertional oncogenesis & replication-competent lentivirus safety, and determining levels of engraftment & vector expression (70). The study plans to use $CD34^+$ cells mobilized by granulocyte colony-stimulating factor (G-CSF). Using G-CSF, Sadelain et al have already achieved successful mobilization of $CD34^+$ cells in three beta-thalassemia patients in amounts sufficient for transduction. In 2002 Li et al (71) studied G-CSF peripheral blood stem cell mobilization in beta-thalassemia patients and found up to a 21.5 fold increase in $CD34^+$ cells could be collected. In 2012, Yannaki and colleagues studied different mobilization methods in 23 patients with beta-thalassemia (72). They studied patients with or without splenectomy, and found that non-splenectomized patients tolerated G-CSF, but splenectomized patients could not tolerate it without a one-month pretreatment with hydroxyurea. They additionally examined Plerixafor, which reversibly inhibits the CXCR4-SDF1 interaction with the BM microenvironment, to mobilize HSCs. Plerixafor proved successful for both splenectomized and non-splenectomized patients.

For the Sadelain trial, the previously described TNS9 vector (38, 39) will be used to induce transgenic expression of beta-globin. Small unpublished modifications have been made to this vector to increase titer, but the gene, promoter, enhancers, and LCR remain intact. Two more trials are also in the works. (1) A trial St. Jude Children's Research Hospital is planned using

gamma-globin coding sequences under control of the beta-globin promoter. (2) The company Bluebird Bio, a company specializing in genetic and orphan diseases, is planning a trial in the San Francisco area using a LentiGlobin BB305 T87Q virus. The identifiers for the TNS9, gamma-globin, and LentiGlobin trials are NCT01639690, NCT00669305, and NCT01745120, respectively; and at the time of writing were all recruiting participants.



Method	○	●	●
Ex-vivo HSC expansion		↑	↑
More intense and more toxic myeloablation	↓		
MGMT/MDR1 selection			↑

Figure 2: Mixed Chimerism. *White:* starting bone marrow of the patient before procedure; *Textured Grey:* Bone marrow that has been cultured but not transduced; *Black:* Bone marrow that has been cultured and successfully transduced with therapeutic vector. *Ex-vivo* expansion would increase the total amount of bone marrow that been cultured. More toxic myeloablation would decrease the amount of residual bone marrow. Selection with *MGMT* or *MDR1* would increase the amount of vector transduced cells.

Mixed Chimerism and In Vivo Selection of Transduced Cells

When undergoing autologous stem cell transplant, patients first need to undergo a myeloablative conditioning regimen. The success of conditioning regimen intensity depends on a balance between toxicity and the amount of mixed transgenic-chimerism, i.e. the amount of BM made up of transplanted cells that carry the vector (Figure 2). Full myeloablation can, theoretically, result in a complete transgenic-chimerism, where the BM is made entirely of cultured cells, with a greater amount of therapeutic vector-transduced cells than in a partial myeloablation setting. However, full myeloablation is more toxic and puts the patient at greater risk, especially in the case of graft failure. A reduced-intensity conditioning regimen can be less toxic, however it can also lead to a lower composition of therapeutic vector-transduced cells due to lower transgenic-chimerism. When a reduced-intensity conditioning regimen is used, beta- globin expression from the vector must be high enough to give transduced cells a survival advantage compared to untransduced cells. In studies done of traditional allogeneic BMT, patients with as low as 20% donor contribution were still able to achieve transfusion independence and normal hemoglobin levels. However, a high initial engraftment (>90% at sixty days post-transplant) is necessary for good chances of stable mixed chimerism (9).

Lucarelli and colleagues identified three Pesaro risk classes for beta-thalassemia patients based on previous iron chelation, hepato- and splenomegaly, and liver fibrosis (73). Patients with irregular iron chelation and more liver damange fall into class 3 while those patients with less iron overload

and liver damage fall into class 1 and 2. A study of 886 beta-thalassemia patients who received transplants from HLA-matched siblings or parents showed a 91% and 84% probability of Thalassemia-free survival with a normal conditioning regimen for class 1 and class 2 patients, respectively(74). There has been a recent trend to lower-intensity, non-myeloablative conditioning regimens though based on the following data: (1) lower morbidity and mortality is associated with these regimens, (2) patients not eligible for the traditional full myeloablative regimen have been safely transplanted with these regimens, and (3) mixed chimerism can be sustained and still lead to amelioration of disease in patients with allografts (75). Multiple groups have had success with reduced-intensity regimens with lower doses of busulfan, or by using alternatives such as thiotepa, treosulfan, fludarabine, busulfex, or antithmocyte globulin (as reviewed in (75, 76)). All of these myeloablation reports, however, relate to beta-thalassemia transplants with HLA-matched donors and not to autologous transplants done with vector-transduced cells. The TNS9 trial with vector-transduced cells will use a reduced-intensity regimen based off of data from successful allogeneic transplants (70) and data from autologous transplants with vector-transduced cells in immunodeficiency disorders(77, 78).

If after transplant a patient were to have a non-therapeutic level mixed chimerism, or suboptimal transgene expression, it would be helpful to have an *in vivo* strategy to increase the chimerism. Conferring a cytoprotective drug resistance to lentiviral-transduced cells is one of these strategies. The human multidrug resistance 1 (*MDR1*) gene, encodes a P-glycoprotein drug efflux pump

that confers resistance to several chemotherapy drugs, including paclitaxel and doxorubicin, both of which under normal conditions are hematopoietically toxic. Researchers discovered a modest positive selection of peripheral blood progenitor cells that had been transduced with *MDR1* could be achieved by giving paclitaxel (79, 80). In a second set of studies, researchers used a mutated *MGMT* gene. *MGMT* encodes the enzyme O^6-methylguanine-DNA methyltransferase, and confers resistance to nitrosoureas and O^6-benzylguanine drugs. A dual vector was created encoding both MGMT under a constitutive promoter and gamma globin under erythroid control elements (V5 from Table 1). Murine wild-type (81) and murine beta-thalassemic bone marrow (82) were transduced with this vector. This dual vector was able to increase the number of fetal hemoglobin expressing cells *in vivo* after treatment with drug. Furthermore, for thalassemic bone marrow, researchers could achieve amelioration of the anemia. Researchers also showed that *ex vivo* selection of lentiviral-transduced and transplantable cells was possible. They pretreated the cells with drug prior to transplantation and saw that a greater number a mice achieved therapeutic fetal hemoglobin levels as compared to untreated controls (82).

MGMT has been studied in both dogs (83) and non-human primates. One study with non-human primates showed mostly mild and transient enrichment of MGMT-transduced cells (84), while another group showed more stable enrichment (85). The dog (83) and one non-human primate study (85) showed no significant enrichment for vector integration sites near proto-oncogenes after drug treatment. In the dog study, two dogs had to be euthanized due to health

complications, but these complications seemed to be unrelated to the MGMT-transduced cells. The other non-human primate study (84) did not extensively study genome toxicity, but also saw no evidence of clonal dominance or leukemic transformation. *MGMT* and *MDR1* have additionally been combined, with bicistronic vectors encoding both genes. The stoichiometry between the two genes has even been examined. With an *MDR1-IRES-MGMT* vector, Maier and colleagues (86) saw a similar cytoprotective effect for monotherapy with paclitaxel or O^6-BG/temozolomide, and a greater cytoprotective effect with the combination therapy. Later studies showed that a F2A provided the best stoichiometry between the two drug resistant genes for the best cytoprotective effect (87).

Concerns and Genome Toxicity

Although lentiviral vectors offer a number of benefits, there are still many unmet concerns. Sustained, high expression is still difficult to achieve, as transgene silencing by chromatin modifications is still a problem. Insulators have helped this situation, but it has not been solved entirely. Additionally, there is a mild concern with replication-competent lentivirus; although as generations of lentiviruses progress, they resemble the original HIV-1 genome less(88). The SIN, or self-inactivating design for a lentivirus removes a 400bp region from the 3' long terminal repeat. This deletion abolishes the enhancer/promoter activity of the virus, therefore reducing transcriptional interference. It is less likely to recombine with cells that have been infected with HIV-1 or make replication-competent lentivirus as it has less similarity (89).

Several studies have been done on non-viral methods to achieve gene transfer (90). However, these methods have not been as efficient and still have difficulty achieving sustained and stable expression. The Sleeping Beauty transposase (SB) system is a non-viral method; it is a synthetic transposon system, reverse engineered from defective copies in fish (91). Sjeklocha and coworkers used SB to transduce human CD34+ cord blood cells. They saw integration and expression of the beta-globin gene, and in studies with K562 cells, saw sustained transgene expression (92).

One of the greatest concerns with lentiviral gene therapy is random integration. Random transgene integration can potentially disrupt a tumor suppressor or cause activation of an oncogene. In trials for X-linked severe combined immunodeficiency, leukemia developed as a result of aberrant gene activation from random integration (93). One method of preventing malignancy with lentiviruses is to analyze the insertion sites before transplantation and select those “safe harbor” sites which are least likely to cause endogenous gene perturbation. A safe harbor is an integration site that is more that 50-100 kilobases away from known coding, miRNA, and ultraconserved regions. In 2011, Papapetrou published a paper concerning genomic “safe harbors” and induced pluripotent stem cells (iPSCs). They found that about 10% of integrations occurred in safe harbors and permitted beta-globin gene expression (94, 95).

Another method is to have a failsafe way of getting rid of vector-transduced cells should they become malignant. In addition to the therapeutic

gene, a suicide gene can also be transduced at the same time. If malignancy occurs, this suicide gene can be induced with drugs to cause apoptosis and ablate vector-transduced cells in the body. Two such suicide genes studied in the context of gene therapy are the herepes simplex virus type 1 thymidine kinase (HSVtk) and inducible caspase 9 (iCasp9) (96). HSVtk-transduced cells can be eliminated with the phosphorylation of acyclovir or ganciclovir by HSVtk. iCasp9 is expressed as a monomer, but upon addition of AP1903, dimerizes and causes apoptosis. In a study with T-cells, iCasp9 effected immediate death, but HSVtk needed 3 days of treatment (97).

Last, site-specific integration—which does not disrupt other genes—is a new area being explored. Site-specific correction of the beta-globin gene has been done with iPSCs. Making iPSCs from thalassemic cells usually requires the addition of four factors: Oct4, Sox2, Klf4, and c-Myc, although a number of other gene combinations have been successfully tried (98, 99). This field alone is a large area of research, and reprogramming can be done in a variety of ways: lentiviruses, episomes, nonintegrating viruses, synthetic RNA, or proteins. In 2009, Ye and colleagues showed iPSCs could be successfully generated from thalassemic patients and upon differentiation, could be stained for HbF (100). Zou (101)and Sebastiano (102)showed in two separate papers that thalassemic iPSCs could undergo site-specific correction of beta-globin using zinc finger nucleases and homologous recombination. This provided the scientific basis for potentially non-integrating *in situ* correction. While the iPSCs generated in the above papers were done with random integration, it is possible to combine a non-

integrating method of iPSC generation and site-specific correction, thus avoiding integration-associated genome toxicity. Most recently, Ma in 2013 used non-integrating episomal technology to create iPSCs and non-integrating TALEN to perform *in situ* correction (103). All of these studies have been met with very limited success though, as iPSCs express extremely low and nowhere near therapeutic levels of beta-globin upon differentiation. Site-specific insertion into the adeno-associated virus preferred integration site (AAVS1) has also been done with limited success. In 2008, Howden and colleagues used bacterial artificial chromosomes and components from adeno-associated virus to preferentially insert beta-globin into the AAVS1 in K562 cells (104). Of the 36 insertion sites analyzed, only 6 of them (17%) occurred in AAVS1, and 5 out of those 6 were intact and functional.

Conclusion

Beta-globin gene addition strategies have come a long way in the past 25 years. Many different vectors with a wide-range of genetic elements have proven successful in preclinical tests and some will be tested in clinical trials. However, work still needs to be done to improve the safety and efficacy. Genomic toxicity and malignancy are some of the largest hurdles to overcome in order to move gene therapy to widespread clinical application. Consistently therapeutic transgene expression for those with thalassemia major is an additional problem. For those vectors that do prove safe and effective, research into increasing the number of engraftable lentiviral transduced cells would help with cases of insufficient mixed chimerism.

At the RNA Level: Splice Switching Oligos

Beta-thalassemia is characterized by over 300 mutations. A subset of these mutations creates new cryptic splice sites and, even though the original splice sites are intact, leads to incorrect splicing. The most common splice mutations involve the creation of a splice site in intron 1 or intron 2 of the beta-globin gene, and are termed IVS1 or IVS2 for intravenous sequence as such. Specific mutations are followed with a number denoting the site of the mutation, such as IVS1-110, IVS2-654, or IVS2-745. Since the correct splice sites are still intact, approaches have been made to create splice switching oligonucleotides, which cover the aberrant splice site and restore splicing to the original sites. A splice switching oligonucleotide has to achieve a number of goals: a) it must bind to the aberrant splice site and prevent it from being recognized by the splicing machinery and b) the duplex it creates must not be recognized by RNaseH, as to prevent degradation of the RNA.

IVS2-745 is a C→G mutation at position 745 within intron 2 that generates an aberrant splice site. Aberrant splicing results in the retention of 165 base pairs of intron 2 in the 745 mRNA, which leads to a missense mutation and premature stop codon. The 745 mutation is relatively common in the Mediterranean area, even reaching as high as 15-20% of thalassemia mutations in regions of Spain, Jordan, Romania, and Serbia (105). A 745 mutation in a homozygous or a compound heterozygous state with other beta-globin mutations results in a wide spectrum of phenotypes from non transfusion dependent thalassemia (NTDT) to transfusion dependent thalassemia (TDT). Any

endogenous increase in hemoglobin production and subsequent reduction in transfusions will not only improve the patient's anemia and co-morbidities, but also improve their quality of life.

Previous studies have shown HbA production *in vitro* with 2′-*O*-methyl (2'OMe) SSOs on HeLa and 3t3 cells (106). The 2'-OMe chemistry adds an O-methyl to the 2' position of the ribose; it is a naturally occurring RNA modification that can be replicated synthetically. When studies were moved to thalassemic patient samples (107), the morpholino chemistry was used, which replaces the sugar moieties with morpholine rings. Morpholino oligos have poor prospects in clinical applications alone though, and need to be further chemically modified and conjugated a cell-penetrating peptide for successful *in vivo* delivery (108). Subsequent work noted difficulty in uptake for other chemistries to erythroid progenitor cells, including the 2'-OMe, 2'-*O*-methoxyethyl, 2'*O*-aminopropyl, locked nucleic acid, phosphoramidate and methylphosphonate derivative chemistries (109).

Our study was done using the uniform 2'-*O*-methoxyethyl, or 2'MOE chemistry. The uniform 2'-MOE chemistry adds a methoxyethyl group to the 2' position of the ribose uniformly throughout the SSO. The substitution contributes to an increased stability and binding affinity for RNA. Uniformly distributed 2'-MOE SSOs do not mediate RNase H degradation when they bind their targets, which may be due to the steric hindrance conferred throughout the oligo by the methoxyethyl group. Safety studies of 2'MOE oligos show they are well tolerated in multiple species from rodents to non-human primates; thus, they make an

attractive setting for clinical applications (109). Over thirty compounds are currently being tested in clinical trials for various indications including cancer and cardiovascular, metabolic, and neurological diseases. Two examples are Mipomersen and Nusinersen: Mipomersen (marketed as Kynamoro) is a 2'MOE antisense oligo that was approved by FDA for the treatment of familial hyper-cholesterolemia (110). Nusinersen, a uniformly modified 2'-MOE SSO, is currently being investigated in multiple phase 3 clinical trials to treat spinal muscular atrophy. Early studies have provided encouraging evidence that Nusinersen treatment significantly improved motor neuron function patients (111).

Chapter Two

Results: Adult Hemoglobin Production, Chain Rebalance, and Splice Correction in IVS2-745 Beta-thalassemia Patient Cells Using 2'-O-methoxyethyl Splice-Switching Oligos

In our study we demonstrate that uniform 2'-MOE SSOs are effective in treating erythroid cells from thalassemia patients without the requirement of additional chemical modification: they induce both splice switching and HbA production. Furthermore, 2'MOE-SSOs alleviate other previously unstudied thalassemic cell parameters, such as a rebalancing of the stoichiometry of alpha to beta chains, a reduction of toxic alpha aggregates, and the correction of erythrocyte deformities.

Cell model system

The 745 allele is a β+ allele: it produces a low level of correctly spliced beta-globin mRNA and HbA. In a study of patients with a 745-sickle genotype (112, 113), the single 745 allele contributes only 3-5% HbA *in vivo*. For our study, we were able to obtain samples from five transfusion-dependent thalassemic patients: four 745/β0 compound heterozygotes (P1-4) and one 745/745 homozygote (P5). The compound heterozyogte samples have a non-functioning secondary allele, termed β0. β0 alleles by definition do not produce HbA, and allow us to fully characterize the potential of the 745 SSO effects. P1-3 have a β039 secondary allele and P4 has a IVS1-1 secondary allele.

$CD34^+$ cells isolated from patients were expanded, differentiated to the red cell lineage, and treated via syringe loading or lipofectamine with 2'-MOE SSOs. As seen in the scramble treated controls (Figure 1 and 2), our *in vitro* model system mimics the HbA levels seen *in vivo* with 745-sickle patients. Hemoglobin HPLC analysis indicated *in* vitro HbA production in the range of 2.60-6.75%, with an average of 4.31 $^+$/- 1.48%. In all samples, similar results were seen with untreated controls (data not shown).

2'MOE-SSOs induce splice switching

Three lead 2'-MOE SSOs were raised against the 745 RNA, referred to as oligos 91, 92, and 93. Upon treatment with these oligos, we observed that 745/β0 heterozygotic patient samples were able to undergo splice reversal at a 1X dose of 4.5nmoles/1e6 cells (Figure 1A and Supplementary Figure 1). As expected, the scramble treated samples exhibit alternative splicing of beta-globin: both the longer 745 alternative mRNA and the shorter WT mRNA are present. Upon treatment, the WT form increases in intensity while the alternatively spliced form decreases in intensity. qPCR results shown in Figure 1B show the amount of correctly spliced WT beta-globin mRNA increases up to 100-fold with a 10X dose. The oligos act with varying levels of effectiveness – 91 is the most effective, follwed by 92 and 93, respectively. The oligos also show a dose-dependent response: in the 10X dosed samples, the 745 form is reduced beyond detection by gel.

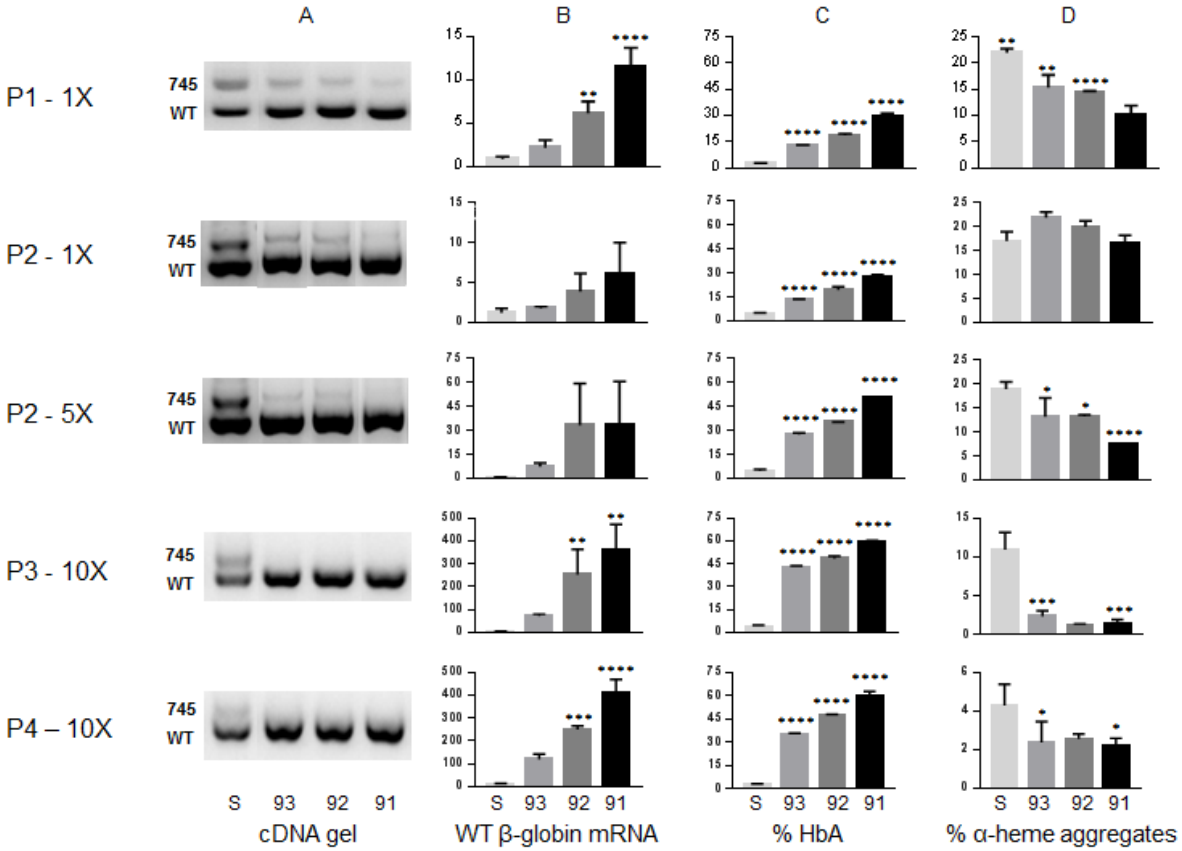
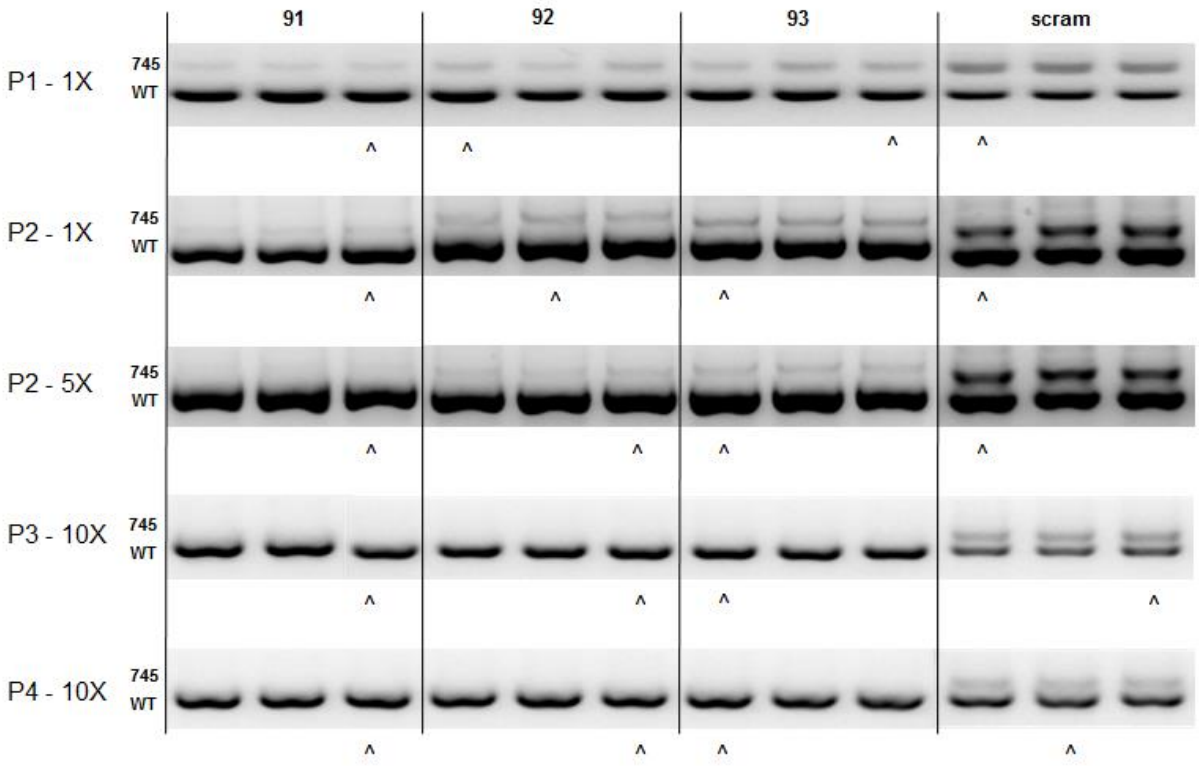


Figure 1: 2'MOE SSOs induce splice switching, HbA production, and alpha-heme tetramer reduction in IVS2-745/β0 heterozygous patient cells. S=scramble control. All statistics (ANOVA/Kruskal-Wallis) are compared to scramble control *p<0.05, **p< 0.01 ***p< 0.001, ****p< 0.0001. [A] PCR for beta-globin cDNA. Each lane=single biological sample (full gels see Supplementary Figure 1). [B] qPCR for only correctly spliced WT beta-globin mRNA. Scale indicates relative expression as normalized to the housekeeping gene GAPDH and red-cell specific gene glycophorin A, n=3. [C] % of HbA and [D] % of alpha-heme aggregates from cell lysates as detected by HPLC, n=3.

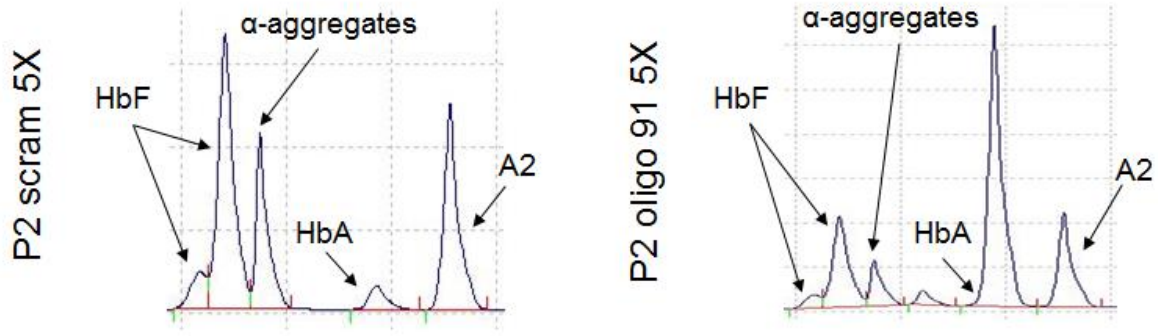


Supplementary Figure 1: Full gels, including all triplicates, of Figure 1A. PCR for beta-globin on cDNA extracted from patient cells. The upper band is the 745 splice mutant form, and the lower band is the WT form. Samples depicted in Figure 1A are marked with a "^".

2'MOE-SSOs restore HbA production in 745 heterozygote patient cells

The uptick in correctly spliced WT beta-globin mRNA corresponded to a robust increase in HbA protein (Figure 1C, Supplementary Figure 2). With only a 1X dose of oligo 91, specimens 1 and 2 increased from their baseline levels of 2.60 $^{+}$/- 0.17% and 4.65 $^{+}$/- 0.46% HbA to 29.21^{+}/- 2.16% and 27.94 $^{+}$/- 1.09%, respectively. A 5X dose of oligo 91 further increased the level of HbA in specimen 2 to 50.83 $^{+}$/- 0.46%. At a 10X dose of oligo 91, the dose-dependent response was seen again, as specimens 3 and 4 went from baseline levels of 4.20 $^{+}$/- 0.75% and 2.97 $^{+}$/- 0.23% HbA to 59.14 $^{+}$/- 1.34% and 60.21 $^{+}$/- 2.61%, respectively. That sums up to a 20-fold increase in HbA in patient 4. As seen at

the RNA level, oligos 92 and 93 were also effective, although the most dramatic results were seem with oligo 91. Oligo 92 induced nearly 50% HbA at a 10X dose in both specimens 3 and 4, up to a 16-fold increase. Oligo 93 induced up to 43% HbA in specimen 4, a 12-fold increase.



Supplementary Figure 2: Representative HPLC profile of the separation of hemoglobins: HbA, alpha-heme aggregates, HbF (fetal hemoglobin, a tetramer of γ-globin and α-globin), and A2 (hemoglobin A2, a tetramer of δ-globin and α-globin)

Reduction of alpha-heme aggregates in 745/β0 heterozygote samples

Due to the imbalance of alpha and beta globin chains in beta-thalassemia, alpha and heme aggregates form in the absence of beta chains (114). These alpha aggregates cause a disruption of terminal erythroid differentiation and apoptosis of erythroid progenitors (115). Any reduction in aggregates could help with the ineffective erythropoiesis of beta-thalassemia. Our results demonstrate that the increase in HbA in the samples from the heterozygous patients led to a concurrent decrease in alpha-heme aggregates (Figure 1D). The baseline aggregate level in heterozygotic cells was variable across samples, with average levels ranging from 22.00 $^{+}$/- 0.76% in P1 to 4.28 $^{+}$/- 1.12% in P4. In P1, a signficant decrease in aggregates was evident across all oligo treatments, and

was most significant in oligo 91 at a 54% reduction. In P2, a significant reduction is seen starting at a 5X dose. Although baseline levels of aggregates in P3 and P4 are lower, oligos 91 and 93 still elicit a significant aggregate reduction at a 10X dose. Consistent with our previous observations, oligo 91 was the most effective, with as high as an 87% reduction in alpha-heme aggregates in P3.

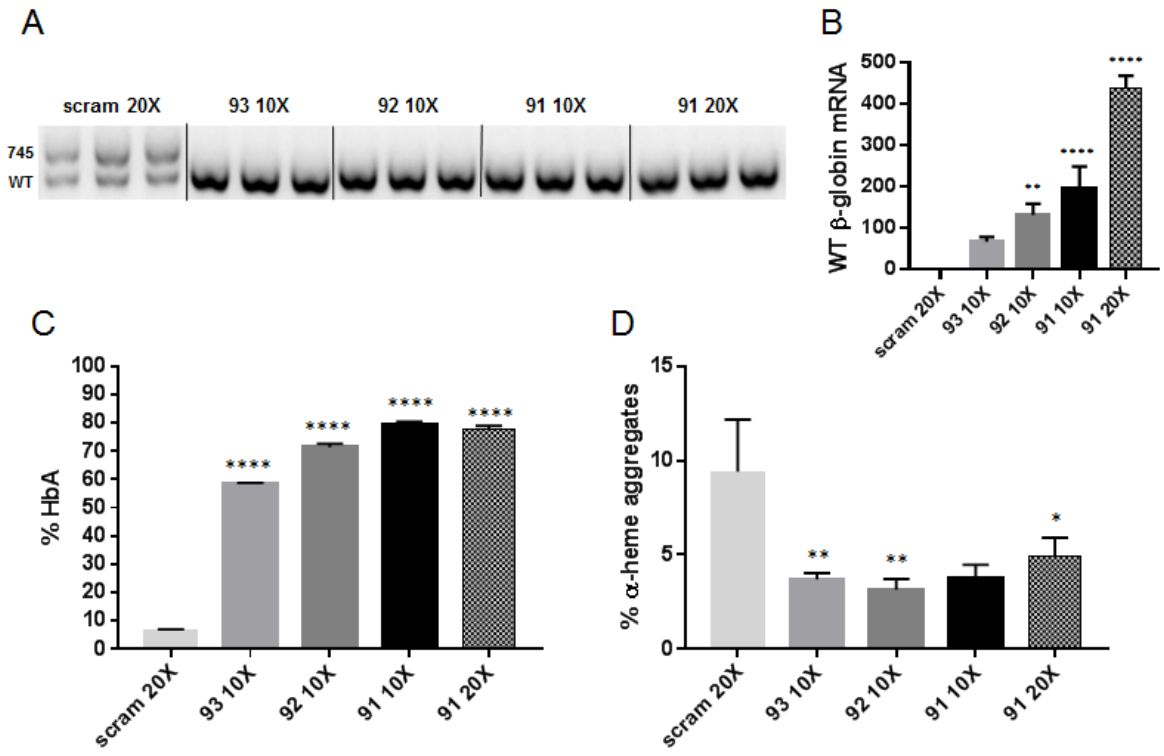


Figure 2: 2'MOE SSOs show the most significant results in homozygous IVS2-745 patient cells. S=scramble control. All statistics (ANOVA/Kruskal-Wallis) are compared to scramble control *p<0.05, **p< 0.01 ***p< 0.001, ****p< 0.0001. [A] PCR for beta-globin cDNA. Each lane represents a single biological sample. [B] qPCR for only correctly spliced WT beta-globin mRNA. Scale indicates relative expression as normalized to the housekeeping gene GAPDH and red-cell specific gene glycophorin A, n=3. [C] % of HbA and [D] % of alpha-heme aggregates from cell lysates as detected by HPLC, n=3.

2'MOE SSOs show the most robust effects on a 745/745 homozygote sample

After demonstrating a dose-dependent response of oligo treatment, we next sought to determine if there was also an "allele" dose-dependency, i.e. whether a homozygote sample would achieve a higher level of HbA than a heterozygote sample. A homozygote sample produces only 745 mutant pre-mRNA, and thus there could be more target 745 pre-mRNA for the oligos to splice switch than in the case of a single 745 allele. Indeed, the homozygous P5 sample baseline level of 6.75 +/- 0.22% HbA is higher than in any heterozygous specimen. This is likely due to the additive contribution of the endogenous WT splicing of the two 745 β+ alleles. As hypothesized, the most striking effects of oligo treatment are seen in the 745 homozygote patient cells (Figure 2). The 745 mutant form is almost undetectable by gel in all treatments; and there is a 300-700 fold increase in correctly spliced WT mRNA for the oligo 91 treated samples (Figure 2A-B). All oligos produced their maximum amount of HbA across all patient samples in the homozygote sample (Figure 2C). Oligo 91 produced 79.46 +/- 0.94% HbA at a 10X dose. The 20X dose produced similar results with 77.92 +/- 1.04% HbA, indicating the effect of this oligo reaches a protein plateau at a 10X dose. Oligo 92 and 93 produced 71.42 +/- 1.22% and 58.48 +/- 0.32% HbA at a 10X dose respectively. As with the heterozygote samples, the increase in HbA led to a decrease in alpha-heme aggregates in the homozygote as well (Figure 2D). The 20X oligo 91 dose in the homozygote led to a 60% reduction in alpha-heme

aggregates.

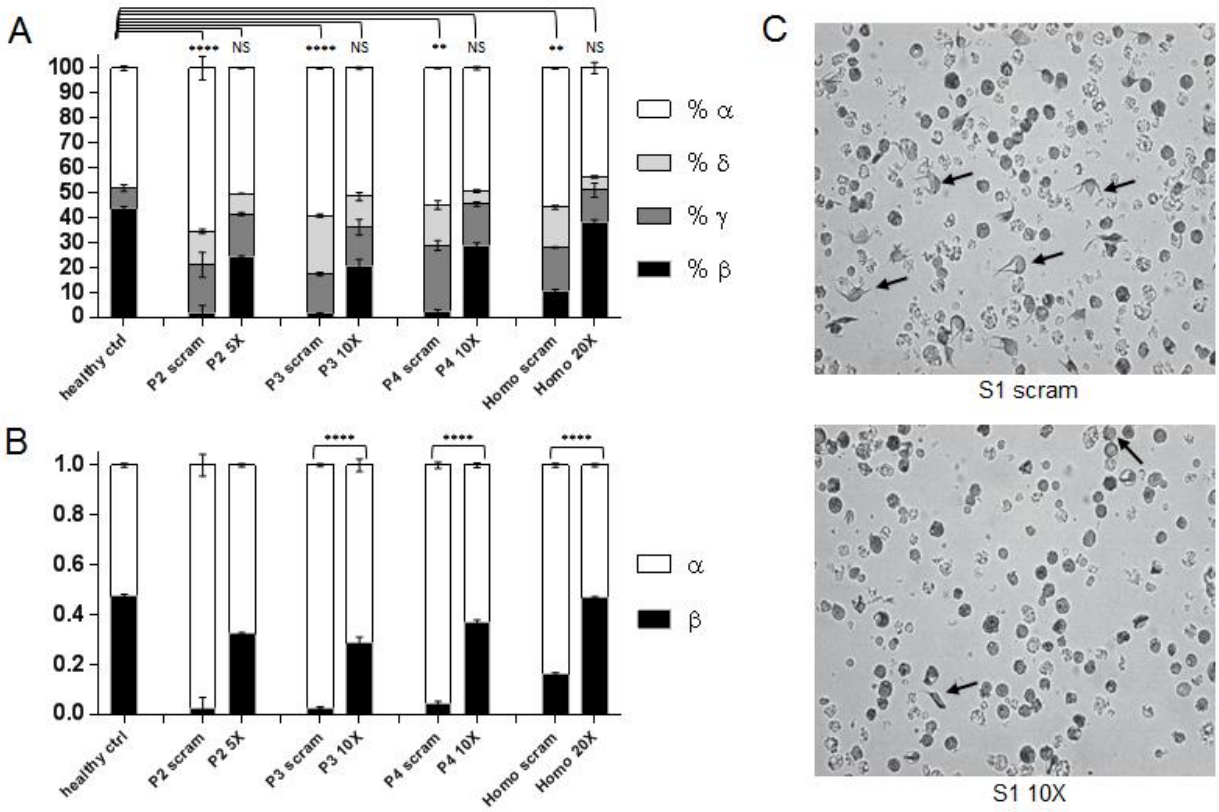


Figure 3: 2'MOE SSO 91 can rebalance the ratio of globins and produce enough functional HbA to combat sickling. [A] Single chain analysis of the balance of alpha chains to beta-like chains (β, γ, δ) or [B] the ratio of alpha chains to beta chains. n=3, scram=scramble treated control at same dose. NS=not significant, **p< 0.01, **** p< 0.0001. [C] *In vitro* sickling assay. 745/Sickle cells were treated with scramble or oligo 91, then exposed to hypoxia for sickling. Barbed cells with long polymers of pointy sickle chains are indicated by black arrows.

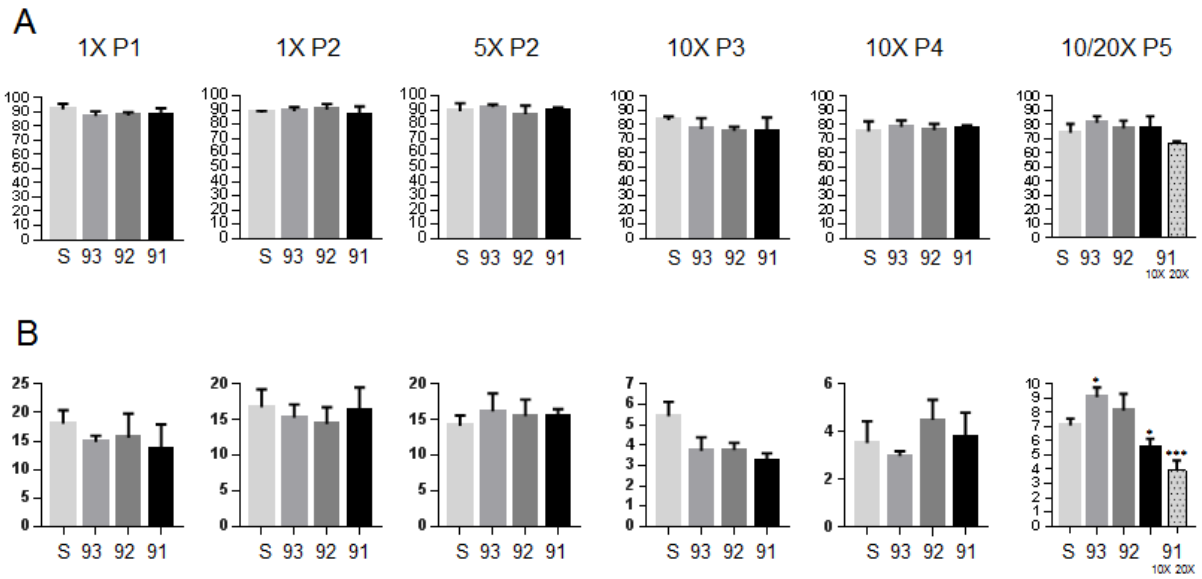
Rebalancing of the alpha-beta globin stoichiometry

In order to examine the effects of 2'MOE SSO treatment on the alpha vs beta globin stoichimetry, cell lystaes were separated using single-chain protein analysis. This allowed separation of the alpha chains from the beta-like chains (β, γ, δ). Without treatment, the ratio of alpha:beta-like chains ranged from 65:35 in P1 to 55:45 in P4/P5, all significantly different from a healthy control at 50:50 (Figure 3A). Single-chain separation showed though, that at as low as a 5X

dose, oligo 91 was able to restore the 50:50 balance of alpha-like chains to beta-like chains. Treated samples have no statistical difference compared to a healthy control. Even when examining only alpha-globin and beta-globin (Figure 3B), the restoration in balance can be seen as the de novo beta-globin is the largest contributor.

2'MOE SSOs combat sickling in samples with a 745/sickle genotype

To address whether the 2'MOE SSOs could induce a functionally relevant amount of HbA, we tested their ability to combat sickling *in vitro*. As we could not obtain a patient sample with the 745/sickle genotype, we created a model system: two homozygous sickle patient specimens (S1 and S2) were transduced with a lentivirus expressing IVS2-745 beta-globin. With vector copy numbers of 2.02 and 1.62 for S1 and S2, respectively, this system replicated a heterozygotic patient sample, as the 2 endogenous sickle alleles were matched. Upon differentiation and exposure to hypoxia, S1 scramble treated cells showed prong-like polymers of sickle chains. Oligo 91 treatment of S1 resulted in an increase in HbA from 8.96 $^{+}$/- 1.88% to 59.82 $^{+}$/- 0.23%, and a decrease in sickle hemoglobin (HbS), from 75.34 $^{+}$/- 2.33% to 30.73 $^{+}$/- 0.32%. This increase in HbA at the expense of HbS led to 50% reduction in the sickling effect (Figure 3C). These results show that 2'MOE SSOs could be indicated for use in compound heterozygous 745/sickle patients to reverse RBC deformities.



Supplementary Figure 3: [A] Viability, as assessed by % not stained by Trypan Blue and [B] Differentiation, as assessed by the number e6 of benzidine positive cells at the end of culture of patient samples. * $p<0.05$, *** $p< 0.001$ (ANOVA/Krunskal-Wallis).

Chapter Three

Methods

Human Ethics

Patients were recruited and samples obtained according to the Declaration of Helsinki, following approvals by the (A) Institutional Ethics Committee of the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Ca' Granda Ospedale Maggiore Policlinico, Milan, #391/2012 for P1, P2, and P4 (B) Cyprus National Bioethics Committee, National Grant ΕΕΒΚ/ΕΠ/2012/05, E.U. Grant ΕΕΒΚ/ΕΠ/2013/23 for P3 and P5 and (C) Children's Hospital of Philadelphia, Institutional Review Board (IRB) #5-012123 for S1. S2 was obtained during automated red cell exchange as part of routine clinical care; as S2 was unlinked and de-identified medical waste, the Montefiore Medical Center IRB deemed it IRB-exempt.

Statistics

For three or more groups, we compared means with a one-way ANOVA test (for samples with normal distributions and equal variances by the Shapiro-Wilks normality test) or medians with a non-parametric Kruskal-Wallis test. For two groups, we compared means with a t-test (for samples with normal distributions by the Shapiro-Wilks normality test) or medians with a Mann-Whitney test. All tests were done using GraphPad Prism software, version 7.

Medias

Phase 1: serum-free StemSpan with 10µL/mL CC-100 cytokine cocktail (both from Stemcell Technologies), 2 U/mL Erytrhopoietin (Amgen), 10^{-6} M dexamethasone (Sigma) and 1% penicillin streptomycin. Freeze Media: 50% characterized FBS (Hyclone), 10% DMSO (Sigma), and 40% Iscove's Modified DMEM(Cellgro). Thaw Media: Iscove's Modified DMEM with 5% characterized FBS. Phase 2: Iscove's Modified DMEM with 3% AB serum (Atlanta Biologicals), 2% Human Plasma (Stemcell Tech), 10ug/mL Insulin (Sigma), 3U/mL Heparin, 200ug/mL Transferrin (Athens Research & Technology), 10ng/mL SCF (Peprotech), 3U/mL EPO (Amgen). Phase III media: same as Phase II, but with 1mg/mL transferrin.

Cell Culture

Whole blood underwent $CD34^+$ selection using immunomagnetic separation (Miltenyi Biotec Inc., Auburn, CA). CD34+ cells were kept undifferentiated in phase 1 media with bi- or tri-weekly media changes, centrifugation to remove both dead and spontaneously differentiating cells and by keeping cell density <0.5e6 cells/mL,. After 8-10 days, cells were frozen in freeze media. Cells were thawed in thaw media, centrifuged 10' at 200g to remove dead cells, and resuspended in phase I. After 12 total days in phase I, cells were transferred into phase II media or a 2:1 mix of phase I and phase III media. SSO treatment occurred on day 14 using 1-2e6 cells/condition; 1X dose was 4.5nmoles/1e6 cells. For syringe loading, cells were resuspended at 1e6/100µL, passed 10 times through a 25 gauge needle, then kept at for 1 hr at 37ºC. Lipofectamine (ThermoFisher) was used according to the manufacturer's protocol, with 150µL

Opti-MEM (ThermoFisher) and 6µL lipofectamine/1e6 cells. Following SSO treatment, cells were plated at 1e6 cells/mL in phase III media. Collection occurred on day 20 for all analyses. Toxicity was assessed by trypan blue staining and level of differentiation was assessed by benzidine staining (116).

RNA, PCR, and real-time qPCR

Total RNA was isolated using Trizol (ThermoFisher). Retrotranscription of total mRNA was done using the SuperScript™ III First Strand Kit (ThermoFisher). PCR reactions were performed with the following primers:Fw: 5′-GGCAAGGTGAACGTGGATGAAGTT -3′; Rev: 5′-TAGGCAGAATCCAGATGCTCAAGG-3′. q-PCR reactions were performed using the ABI 7900HT or Viia7 systems (Applied Biosystems), with either TaqMan (TaqMan PCR 2X Master Mix from ThermoFisher) or SYBR Green (Power SYBR from ThermoFisher or iTaqTM SYBR® Green Supermix from Bio-Rad) chemistry. Quantitative real-time PCR assays of globin, GAPDH, and glycophorin A transcripts were carried out using gene-specific double fluorescently labeled probes. The following primer and probe sequences were used (forward, reverse and probe, when used, of each gene, respectively): WT correctly spliced beta-globin primers=Fw: 5′-CACCTTTGCCACACTGAGTGA-3′; Rev: 5′-GCCCAG GAGCCTGAAGTTCT-3′; 5′-FAM-CACTGTGACAAGCTGCACGTGGATCC-IOWA BLACK-3. The following TaqMan inventoried Gene Expression assays from Thermo Fisher were used: GAPDH: Hs02758991_g1; GYPA: Hs00266777_m1. qPCR results of WT beta-globin were normalized by GAPDH to control for the total amount of cDNA and

GYPA to control for the level of differentiation across samples. For PCR of human beta-globin cDNA, the following primers were used: Fwd 5'-GTGCGAGAGCGTCAGTATTAAG-3', Rev 5'-TCCCTGCTTGCCCATACTA-3'.

Tetrameric and single chain analysis by high performance liquid chromatography (HPLC).

Cell pellets were disrupted with Cytobuster (EMD Millipore) for single chain analysis and water for tetramer analysis. For tetrameric analysis supernatant hemolysates were loaded into a System Gold 126 Solvent Module instrument (Beckman Coulter). Hbs were separated on a weak cation-exchange PolyCAT A column (PolyLC), and detected at a wavelength of 415 nm. The Hbs were bound to the column with mobile phase A (20 mmol/L Bis-Tris, 2 mmol/L KCN, pH 6.96) and eluted with mobile phase B (20 mmol/L Bis-Tris, 2 mmol/L KCN, 200mmol/L NaCl, pH 6.55). Single chain quantification was assessed by reverse-phase HPLC. Hb samples were injected on a Hitachi D-7000 HSM Series apparatus (Hitachi Instruments) using a Zorbax 5 µm 300SB-C8 300 Å, LC 150 x 2.1 mm column (Agilent Technologies) and a gradient from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid in 25 minutes, with UV detection at 215 nm. Standards of HbA, HbF, HbS, and HbC were injected (Analytical Control Systems) and used to determine various Hb peak types (116).

Vector production and Titering

The WT beta-globin coding sequence from the vector AnkCT9W [Breda 2012] was mutagenized to the IVS2-745 beta-globin sequence, creating AnkCT9W-745. Viral stocks were generated by co-transfection of AnkCT9W-745 plasmid

together with the envelope plasmid (VSV-G), the packaging plasmid (pMDLg/p RRE), and the pRSV-REV plasmid into 293T cells (38). An aliquot (5×10^6) of 293T cells was seeded into cell culture dishes (10 cm) 24 hours prior to transfection in Iscove's modification of Eagle's medium (DMEM, Cellgro, Manassas, VA) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C under 5% CO_2. The culture medium was changed 2 hours prior to transfection. The precipitate was formed by adding the plasmids to 450 uL of 0.1× TE (0.1× TE is 10 mM Tris plus 1 mM EDTA) and 50 µL of 2 M $CaCl_2$, then adding 500 µL of 2× HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4) drop wise after which the precipitate was vortexed and immediately added to the cultures. The medium (10 ml) was replaced after 16 hours. Viral supernatants were collected at 24 and 48 hours, cleared by low speed centrifugation, and filtered through cellulose acetate (0.2 µm). Following ultracentrifugation, serial dilutions of concentrated virus (5; 0.5 and 0.05 µL, respectively) were used to infect 1×10^5 NIH 3T3 cells (ATCC, Manassas, VA) in 1 mL of transfection buffer complemented with polybrene (Millipore, Billerica, MA) at a final concentration of 8 µg/mL. Genomic DNA was extracted after 3 days using phenol-chloroform-isoamylic alcohol. The multiplicity of infection (MOI) was calculated using the following formula: number of cells (1×10^5) X dilution factor (1 mL/µL viral preparation) X VCN (measured via real-time PCR, using oligos for WPRE element and Transferrin Receptor gene, see copy number determination) (117).

In vitro RBC sickling and morphological analysis

CD34+ cells were transduced with in expansion (phase I media). 2-3 days later, cells were put in phase II media and genomic DNA was extracted. Copy number was determined as below. SSO treatment with 10X scramble or 10X 91 oligo occurred 2-3 days after the start of phase II; cells were then placed for 6 days in phase III before harvest. We assessed the degree of cell sickling in our experimental samples using previously reported methodology, with slight modifications (117). Briefly, 0.5 - 1 million cells were suspended in isotonic Hemox buffer (TCS Scientific Corp, Southampton, PA), pH 7.4, supplemented with 10 mM glucose and 0.2% bovine serum albumin, in individual wells of a Costar polystyrene 96-well microplate (№ 9017; Corning, Corning, NY). The microplate was then transferred to a Thermomixer R shaker-incubator (Eppendorf), and maintained under hypoxia (Nitrogen gas), with continuous agitation at 500 rpm, at 37° C for 2 hours. At conclusion, aliquots of each sample were collected in 2% glutaraldehyde solution for immediate fixation without exposure to air. Subsequently, fixed cell suspensions were introduced into specialized glass microslides (Dawn Scientific, Inc., Newark, NJ) (118) for acquisition of bright field images (at 40x magnification) of single layer cells on an Olympus BX40 microscope fitted with an Infinity Lite B camera (Olympus) and the coupled Image Capture software.

Copy Number Determination

The number of integrations (VCN) was quantified by Q-PCR using oligos for (Fw: 5'-GTGCGAGAGCGTCAGTATTAAG-3'; Rev: 5'-TCCCTGCTTGCCCATACTA-3') for a specific sequence present in the vector (GAG) and compared it to an

endogenous control present in two copies within the genome (mouse Transferrin=Fw: 5'-TGTTGTAGTAGGAGCCCAGAGAGA-3'; Rev:5'-AGACCTGTTCCCACACTGGACTT-3'; human ID-1=Fw: 5′-AAGGTGAGCAAGGTGGAGATTC-3′; Rev: 5′-TTCCGAGTTCAGCTCCAACTG-3′).

Chapter Four

Discussion and Future Perspectives

2'MOE SSOs have strong therapuetic potential for splice mutants of beta-thalassemia. They effectively correct at the RNA level where the defect occurs, and lead to correction of the thalassemic cell phenotype. The novel production of beta-globin chain leads to HbA production, a coincinding rebalancing of the alpha and beta chains, and a reduction in alpha heme aggregates. As demonstrated by the sickle assay, the oligos even produce enough total HbA to have true functional relevance.

When extrapolating on the clinical potential of 2'MOE SSOs in vivo, we can examine the data of two previously reported patients with a 745/sickle genotype (112). These patients expressed 4.8% HbA and their combined HbA/HbF/HbS levels average 8.6g/dL. If we take these numbers into account, 0.41g/dL of this patient is HbA from the correctly spliced single endogenous 745 allele. 2'MOE SSOs are able to induce up to a 20-fold increase in HbA compared to control – this would translate to over an 8 g/dL increase. As 9g/dL is transufsion-independent, these oligos could very feasibly help pateints achieve transfusion independence. Transfusion independence is a key achievement factor, as transfusion is a contributing factor to iron overload and mutliple organ damage (4).

This is of course contingent on toxicity studies and drug delivery studies. As previously stated, 2'MOE show a favorable tolerability (119). We observed no

significant difference in viability or differentiation for almost all our of treatments (Supplementary Figure 2). If we were to look toward the future though, a side-by-side study would need to be done on multiple patient samples across the doses. For our study, the 1X and 5X dose were used on the same patient and produced no significant differences. However, the 10X and 20X dose were tested on different patient samples. As these samples were harvested from different patients, at separate timepoints, by different collaborators, and shipped separately, we cannot make a true side-by-side comparision. The limited by the availability of donor material and skilled researchers to collect this material prevents us from executing this fully, but better access to local patient databases or training more collaborators could allow such a large-scale study.

Another barrier to clinical translation is the ability to target the oligo to the bone marrow, as 2'MOE oligos accumulate the most in the kidney and liver (120). The spleen though, is the 3rd highest accumluation area, with concentrations between 20-50% of that in the liver. The distribution within the spleen by cell type has not been well characterized, but data shows the most accumulation in the macrophages (119). 2'MOEs need physical or chemical manipluation to enter cultured cells. However *in vivo* and some primary cell cultures studies show natural cellular uptake pathways without this manipulation (119). Of course, *in vitro* systems do not always mimic *in vivo* systems, but as a future indication we could co-culture macrophages with our erythroid progentors to determine if the macrophages are preventing oligo uptake. Per *in vivo,* the lack of an appropriate human 745 beta-globin mouse model prevented us from

fully executing these studies at this time, but other *in vivo* rodent studies show promise. Recent data suggests that the constrained ethyl (cEt) chemistry in antisense studies is successful at targeting the rat and mouse bone marrow (121). The 2'MOE chemistry can potentially be combined with the cEt chemistry for better *in vivo* delivery. Nonetheless, a conjugated strategy that can direct SSO uptake by erythroid cells would be ideal. The oligo can potentially be conjugated to a protein that has an affinity for erythroid progenitors or the bone marrow to achieve specific targeting.

In summary, 2'MOE-SSOs are promising therapeutic tools for certain splicing forms of beta-thalassemia. Their ability to correct the underlying splicing defect offers a pharmacological treatment that is both direct and specific. As such, this therapy could help patients reduce their transfusion dependence or even reach transfusion independence.

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Appendix: ALS10, a new gene therapy vector

*Authorship Note: This work was filed for patent under PCT patent publication number WO 2016118715, with Stefano Rivella, Laura Breda, Gerd Blobel, and Wulan Deng. This work has been moderately reformatted for this thesis, but remains in the general formatting style of the patent application.

VIRAL VECTORS FOR PROPHYLAXIS AND THERAPY OF HEMOGLOBINOPATHIES

CROSS REFERENCE TO RELATED APPLICATIONS
This application claims priority to U.S. Provisional application no. 62/105,829, filed on January 21, 2015, the disclosure of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH
This invention was made with government support under contract no. 1R01HL102449 awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD
The present disclosure related to compositions and methods useful for prophylaxis and/or therapy of hemoglobinopathies.

ABSTRACT
Provided are compositions and methods for inducing expression of human beta-globin in erythrocytes for use in prophylaxis and/or therapy of a hemoglobinopathy in an individual. The method generally entails introducing into CD34+ cells a polynucleotide encoding: i) a 5' long terminal repeat (LTR) and a self-inactivating 3' LTR; ii) at least one polyadenylation signal; iii) at least one promoter; iv) a globin gene locus control region (LCR); v) an ankyrin insulator element (Ank); vi) a Woodchuck Post-Regulatory Element (WPRE) configured such that the WPRE does not integrate into a target genome; and vii) a sequence that is a reverse complement of a sequence encoding human beta-globin, and can include beta-globin that has a βT87Q mutation. Intron 2 of the beta globin gene can be a complete intron. Modified erythrocyte progenitor cells, recombinant vectors and virions comprising recombinant polynucleotides, and methods of making the vectors and virions are included.

BACKGROUND
There are a variety of hemoglobinopathies that affect large portions of the human population. For example, sickle-cell anemia (SCA) is a blood related disorder that affects the structure of the hemoglobin molecules. In SCA the hemoglobin molecule is defective, causing the entire blood cell to change shape (122). After these abnormal hemoglobin molecules release oxygen in circulation, they may cluster together and form long, rod-like structures, which become rigid and

assume sickle shape. Unlike healthy red blood cells, which are usually smooth and donut-shaped, sickled red blood cells cannot squeeze through small blood vessels. Instead, they stack up and cause blockages that deprive organs and tissues of oxygen-carrying blood. This produces periodic episodes of pain and ultimately can damage tissues and vital organs and lead to other serious medical problems. Normal red blood cells live about 120 days in the bloodstream, but sickled red cells die after about 10 to 20 days. Because they cannot be replaced fast enough, the blood is chronically short of red blood cells, leading to anemia. SCA affects millions throughout the world. It is particularly common among people whose ancestors come from Sub-Saharan Africa, South America, Cuba, Central America, Saudi Arabia, India, and Mediterranean countries such as Turkey, Greece, and Italy. In the Unites States, it affects around 72,000 people, most of whose ancestors come from Africa. The disease occurs in about 1 in every 500 African-American births and 1 in every 1000 to 1400 Hispanic-American births. About 2 million Americans, or 1 in 12 African Americans, carry the sickle cell allele.

Beta-thalassemia is one of the two the most common congenital anemias and is due to partial or complete lack of synthesis of beta-globin chains and hemoglobin. Patients affected by beta-thalassemia have mutations in the beta-globin gene. The World Health Organization (WHO) estimates that 50,000 to 100,000 children are born with symptomatic features of beta-thalassemia every year. Cooley's anemia, also known as beta-thalassemia major, the most severe form of this disease, is characterized by ineffective erythropoiesis (IE) and extra medullary hematopoiesis (EMH) requiring regular blood transfusions to sustain life.

In beta-thalassemia intermedia, where a greater number of beta-globin chains are synthesized, the clinical picture is milder and patients do not require frequent transfusions (Musallam, K.M., et al. Non-transfusion-dependent thalassemias. Haematologica 98, 833-844 (2013); Rivella, S. The role of ineffective erythropoiesis in non-transfusion-dependent thalassemia. Blood reviews 26 Suppl 1, S12-15 (2012); Ginzburg, Y. & Rivella, S. beta-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. Blood 118, 4321-4330 (2011)). However, hemoglobin levels often decrease over time, splenomegaly appears, and patients suffer from progressive iron overload due to increased gastrointestinal iron absorption. Current disease management includes prenatal diagnosis, transfusion therapy, iron chelation and allogeneic bone marrow transplantation (BMT), which is limited by finding a compatible bone marrow donor and presents many risks and complications. Beta-thalassemia or Cooley's anemia has a serious impact on the life of those afflicted, as well as on society in general. Therefore, potential gene therapy approaches that provide therapy to these patients would be highly relevant. In this regard, mutations leading to Cooley's anemia can be classified as beta0, such as beta0-39, in which a single point mutation creates a stop codon and no beta-globin protein is produced, or beta+, such as beta+-IVS1-110, where a mutation in the first intron results in alternative splicing and insufficient beta-globin chain synthesis (Musallam, K.M., et al. Non-transfusion-dependent

thalassemias. Haematologica 98, 833-844 (2013); Rivella, S. The role of ineffective erythropoiesis in non-transfusion-dependent thalassemia. Blood reviews 26 Suppl 1, S12-15 (2012); Ginzburg, Y. & Rivella, S. beta-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. Blood 118, 4321-4330 (2011)). Previous research has shown that it is possible to rescue beta-thalassemia in mouse models by lentiviral-mediated beta-globin gene transfer (May, C., et al. Successful treatment of murine beta-thalassemia intermedia by transfer of the human beta-globin gene. *Blood* 99, 1902-1908. (2002); May, C.*, et al.* Therapeutic hemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* 406, 82-86 (2000); Rivella, S., et al. A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human beta-globin gene transfer. *Blood* 101, 2932-2939 (2003)). However, these animals are characterized by a complete deletion of the mouse beta-globin gene. Additional hemoglobinopathies are characterized by mutations in the beta-globin gene that lead to additional aberrant beta-globin chains and patho-physiological sequelae similar to sickle cell anemia and/or beta-thalassemia. Thus, there is an ongoing and unmet need for improved compositions and methods for treating hemoglobinopathies. The present disclosure is pertinent to this need.

SUMMARY

The present disclosure provides compositions and methods for prophylaxis and/or therapy of hemoglobinopathies. In general, hemoglobinopathies comprise disorders that are characterized by reduced synthesis or abnormal structure of the hemoglobin molecule. Thalassemias are considered a type of hemoglobinopathy, with the understanding that certain forms of hemoglobinopathies are typically inherited single-gene disorders that result in abnormal structure of one of the globin chains of the hemoglobin molecule, whereas thalassemias are considered other forms of hemoglobinopathies associated with underproduction of normal globin proteins, such as those cases that arise due to mutations in regulatory genes. Thus, the disclosure is pertinent to individuals in need of treatment for disorders that include but are not necessarily limited to those characterized by altered hemoglobin structure, such as in hemoglobin C disease, hemoglobin S-C disease, sickle cell anemia, as well as in various types of thalassemia that are well known in the art, including but not necessarily limited to beta-thalassemia. This is also pertinent to patients that have mixed hemoglobinopathy features, such as hemoglobin S/thalassemia, for instance.

In one aspect the disclosure provides a method for inducing expression of human beta-globin in erythrocytes for use in prophylaxis and/or therapy of a hemoglobinopathy in an individual. The method generally comprises introducing into erythrocyte progenitor cells, typically CD34+ cells, a polynucleotide encoding: i) a 5' long terminal repeat (LTR) and a self-inactivating 3' LTR (self-inactivating meaning the 3' LTR comprises a deletion relative to its native sequence, and thus results is replication incompetent); ii) at least one polyadenylation signal; iii) at least one promoter; iv) a globin gene locus control

region (LCR); v) an ankyrin insulator element (Ank); vi) a Woodchuck Post-Regulatory Element (WPRE) configured such that the WPRE does not integrate into a target genome; and vii) a sequence that is a reverse complement to a sequence encoding modified human beta-globin comprising a βT87Q mutation (B-globinM). The sequence of human B-globinM is included with this disclosure. The B-globinM is expressed from integrated DNA that is derived from the viral genome. Thus, the B-globinM sequence in the RNA genome of the modified lentiviral vectors of this disclosure is configured so that the B-globinM is expressed in the context of the double stranded DNA that is from integration of the modified lentiviral genome. The expression can in certain embodiments be exclusive to certain cell types, such as erythrocytes. It will be recognized in certain instances that DNA sequences are provided, but those skilled in the art can readily envision the RNA equivalent of the DNA sequence, such as when a DNA sequence is used to illustrate features of a lentiviral genome.

The sequence encoding the B-globinM comprises a first intron (intron 1) between exon 1 and exon 2, and a second intron (intron 2) between exon 2 and exon 3. In certain embodiments intron 2 comprises more than 476 nucleotides of the human B-globinM intron 2 sequence, and may comprise up to the full intron 2 sequence. After introducing the lentiviral construct into erythrocyte progenitor cells the lentiviral construct integrates into one or more chromosomes in the progenitor cells, and the progenitor cells differentiate into erythrocytes. The erythrocytes derived from the erythrocyte progenitor cells produce the beta-globin protein.

The disclosure provides for modified erythrocytes that produce more human beta-globin than a suitable control. In one embodiment the control comprises a human beta-globin value obtained from control cells. In one non-limiting approach the control cells comprise erythrocytes from an individual who has the hemoglobinopathy, wherein the erythrocytes are progeny of progenitor cells into which a control viral vector was introduced. The control viral vector can comprise, for example, the 5' LTR, the 3' LTR, the at least one polyadenylation signal, the at least one promoter, the LCR, the Ank, a WPRE, and the sequence encoding the B-globinM, but the sequence encoding the B-globinM in the control viral vector comprises 476 or fewer nucleotides of the human B-globinM intron 2 sequence, and thus comprises an intron 2 that contains a deletion. In one embodiment, the modified erythrocytes produce increased adult hemoglobin, fetal hemoglobin, B-globinM, or a combination thereof, relative to a control.

In one embodiment the lentiviral vectors can further comprise a sequence encoding a fusion of an Ldb1 transcription factor and a zinc finger (ZF) domain. In certain embodiments the lentiviral vector can comprise a sequence encoding an RNA polynucleotide that is at has reverse complementarity to mRNA encoding transferrin receptor 1. The RNA polynucleotide is capable of decreasing transferrin receptor 1 mRNA by, for example, an RNAi-mediated process.

In certain approaches the disclosure includes modifying erythrocyte progenitor cells that are CD34+ cells. The CD34+ cells can be separated from the individual before the lentiviral vector is introduced into them. The CD34+ cells

can be introduced into the individual subsequent to the introduction of the lentiviral vector. The lentiviral vector can be introduced using any suitable approach. In one embodiment the erythrocyte progenitor cells are infected with viral particles comprising a lentiviral vector, such as a recombinant + strand viral polynucleotide. In certain embodiments, approximately 50 infection particles (IP) per cell are used. In certain embodiments the cells into which the vector is introduced can be enriched for a certain cell type, such as CD34+ cells. In certain approaches the cells are held for a period of from 2-3 days after infection prior to introducing into an individual in need thereof. In embodiments the cells can be frozen and introduced into the patients after they are thawed. The lentiviral vectors can be introduced into a patient using any suitable method. In one approach they are administered by intravenous infusion, which may be performed subsequent to any other technique, such as after depletion of bone marrow cells, i.e., myeloablation. Those skilled in the art will recognize that a cells characterized by a suitable vector copy number (VCN) can be used. In one embodiment the VCN is approximately 1 VCN per cell.

The disclosure includes the lentiviral vectors themselves, the components of which are as described above for use in a method of the disclosure. Lentiviral vectors comprise modified, recombinant polynucleotides, and can comprise RNA or DNA polynucleotides. In certain embodiments a lentiviral vector of this disclosure comprises an isolated polynucleotide, or an isolated preparation of virions that comprise the lentiviral vector. In an embodiment a recombinant lentiviral vector of the disclosure is present in CD34+ cells, wherein the CD34+ cells have been separated from an individual.

Also provided is a method of making a viral particle preparation for use in prophylaxis and/or therapy for one or more hemoglobinopathies. This approach comprises introducing a plasmid encoding a lentiviral vector as described above into packaging cells, wherein the packaging cells comprise a DNA packaging plasmid which encodes at least one virion protein, and wherein the packaging cells comprises a DNA envelope plasmid which encodes an envelope protein. The packaging and envelope plasmids express their respective proteins, which facilitate formation of virions which comprise an RNA lentiviral vector of this disclosure.

DESCRIPTION OF THE FIGURES

Figure 1. The level of HbF/GFP expressing cells increases proportionally to the amount of molecules of GG1-SA vector integrated. (A) Lentiviral construct carrying the bicistronic cassette that expresses the Ldb1 SA domain under the ankyrin promoter (Ank Pr.) and the green fluorescence protein (GFP) through an internal ribosomal entry site (IRES). The Woodchuck hepatitis virus Posttrascriptional Regulatory Element (WPRE) increases RNA stability and protein yield. (B) Percentage of F, S and A_2 hemoglobins measured by HPLC in differentiated untreated erythroid cells (left) or with up to 0.7 copies of ZF-Ldb1-viral molecules integrated on average (middle and right). (C) Increase in HbF and (D) GFP expressing cells measured by flow cytometry in cells untreated or with 0.26 and 0.67copies/cell of ZF-Ldb1-viral molecules integrated.

Figure 2. pCL-ZF-Ldb1 supports high levels of γ-globin/hemoglobin F induction and concurrently reduces sickle globin levels in sickle cell disease (SCD) erythroblasts. (A) β-like globin mRNA content (γ, left, and βs, middle) was measured by Q-PCR and normalized by endogenous GAPDH expression. β-like globin expression changes were further normalized by α-globin expression (which should remain unchanged and is a measure of differentiation across samples). Right: transgenic ZF-Ldb1 mRNA expression in treated samples is plotted against integration of pCL-ZF-Ldb1. (B) HbF (left), HbS (middle) and HbA2 (right) changes in all SCD samples treated with pCL-ZF-Ldb1 LV. (C) Net HbF% increase and HbS%-A2% decrease.

Figure 3. Quantification of tetrameric Hbs and single globin chains in SCD cells untreated and treated with pCL-ZF-Ldb1. (A) Content of HbF increase (left) and all Hbs (right) in erythroid cells without or with pCL-ZF-Ldb1. (B) Single globin quantification by liquid chromatography in denaturing conditions. On left, the quantity of single β- and γ-globin chains (μg) is calculated over the quantity of single α- globin chain. On right, means of γA+G chains/all β-like chains area indicated. μg of Hbs or single globin chains in hemolysates were extrapolated from calibration curves obtained with standard samples with known Hb concentration.

Figure 4. pCL-ZF-Ldb1-transduced SCD cells expressing HbF have a reduced expression of HbS compared to untransduced cells. (A) The number of GFP expressing SCD erythroblasts after pCL-ZF-Ldb1 integration (right) is compared to untransduced erythroblast (left). (B) Percentage of HbF producing cells in permeabilized erythroblasts (left, no anti-HbF Ab), untransduced (center) and pCL-ZF-Ldb1 transduced erythroblasts (right). (C) β-globin expressing cells within the HbF positive populations from B (center and right) are compared to permeabilized cells (left, no anti-HbB Ab). (D) quantification of HbF of SCD samples (from B) analyzed by HPLC.

Figure 5. Expression of γ-globin repressor genes in healthy cells, sickle cells untreated or treated with pCL-ZF-Ldb1 LV. (A) Bcl11A, C-Myb, (B) SOX6 and KLF1 genes' expression is expressed in function of the level of cell differentiation marker glycophorin A (GPA) and normalized by GAPDH expression. The expression of KEL (C, left), the gene that encodes Kell, a blood group antigen, was chosen as internal control. Expression of transgenic Ldb1 in transduced samples (C, right) was confirmed in the same samples analyzed for the other messenger RNAs.

Figure 6. Hemoglobin F increase/ Sickle hemoglobin decrease in CD34+-derived SCD erythroid cells treated with pCL-ZF-Ldb1 LV and with HbF inducers in vitro. (A) (Top) Net increase of HbF% and (bottom) net decrease of HbS% in SCD erythroblasts treated with the HbF inducers decitabine, tranylcypromine, hydroxyurea, pomalidomide, butyrate or with the LV carrying ZF-Ldb1. (B)

Erythroid cell count (hemoglobinized cells, or benzidine+ stained cells) for each treatment is been normalized over the untreated sample. In A-B n=11, except for Hu and pCL-ZF-Ldb1 in which n=10, while for But n=9.

Figure 7. Differential single globin chain synthesis in cells treated with pCL-ZF-Ldb1. (A) Chromatographic profile of representative sickle cells hemolysate untreated (UT) or treated with pCL-ZF-Ldb1. (B) Calibration curve obtained using known hemoglobin concentration of blood from SCD transgenic mouse. (C) Areas under peak of single globin chains extrapolated from the calibration curve (from B) in samples untreated or after treatment with pCL-ZF-Ldb1 (n=5).

Figure 8. Trend of moderately elevated HbF levels in CD34+-derived SCD erythroid cells at steady state. Comparative HPLC assessment of HbF% between healthy and SCD erythroid samples in which γ-globin repressors BCl11a, c-Myb, KLF1 and SOX6 were quantified

Figure 9. Cytotoxicity effect and dose/response calibration. Erythroid cells count measurements (benzidine plus count) of cells (N=2) at different doses of drugs in comparison with integration of ~1 copy/cell of GG1-SA. The arrows in black indicate the dose of each drug chosen for the bulk of the experiments.

Figure 10. Variation of HbF% SCD erythroid cells treated with pCL-ZF-Ldb1 vector and with HbF inducers in vitro at different times. Net increase of HbF% in SCD erythroblasts treated with the HbF inducers 5-aza-cytidine or with the LV carrying ZF-Ldb1, according to tables 1 (L) and S1 (E). Kruskal-Wallis with Dunn's multiple comparison test.

Figure 11. Graphical maps of vectors. Topmost vector map is pCL-ZF-Ldb1, also referred to as pCL20cAnkyrinGG1DDiGFP. Second from vector map is ALS-10. Third from top vector map is CT9Ank. Bottom vector map is ALS-10T. Each vector comprises a 5' and a 3' self-inactivating long terminal repeat (5'LTR and 3'SinLTR, respectively). Also shown is "B-globinM" which is a mutant beta-globin is known as the "βT87Q" form. The B-globinM is configured in the vector such that it is expressed from an integrated DNA that is derived from the RNA genome via a well-known process. "Ank" is an Ankyrin insulator. "IRES" is an internal ribosomal entry site. "P" designates a promoter. "LCR" is a Locus control region. "GFP" is enhanced green fluorescence protein. "pA" is a polyadenylation signal. "WPRE" is the Woodchuck Post-Regulatory Element. "fI1" is a full beta-globin gene intron 1. "fI2" is a full beta-globin gene intron 2. "I1S" is a modified beta-globin gene intron 1 comprising a microRNA targeting the transferrin receptor. "SV40 oriR-pA" is an origin of replication and polyadenylation signal. Beta-globin gene exons 1, 2 and 3 are labeled accordingly.

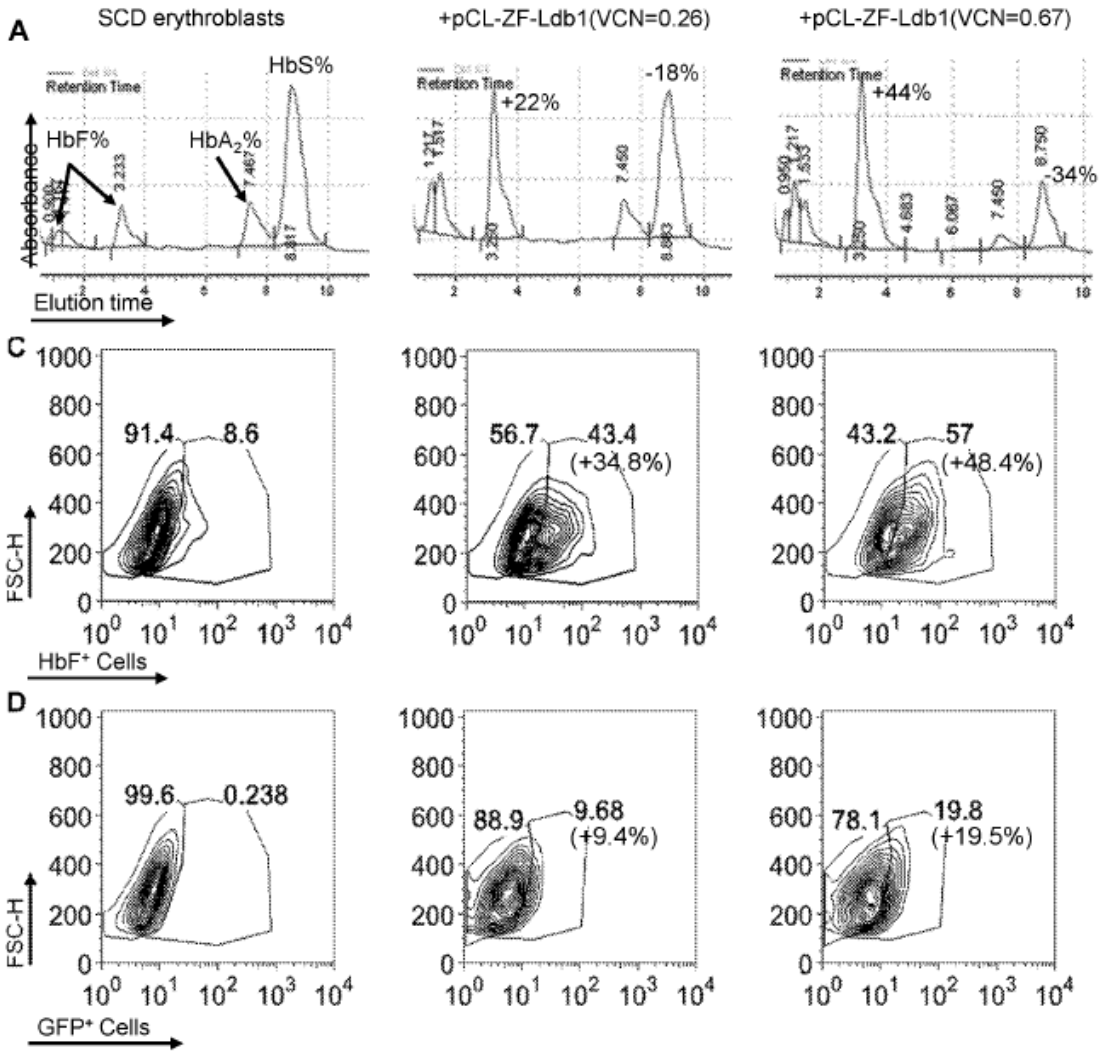
Figure 12 provides a graphical summary of results comparing adult hemoglobin values (HbA) obtained from β0/+ or β0/0 erythroblasts patient CD34+ cells into which the AnkT9W and ALS10 vectors were introduced, as labeled.

Figure 13 provides an annotated polynucleotide and encoded protein sequence of the ZF-Ldb1 vector and the DNA equivalent of the RNA. The nucleotide sequence in Figure 13 is SEQ ID NO:1. The ZF-LDB1 AA sequence (shown as GG1=ZF and DDi=LDB1, with the HA terminal amino acid sequence) is SEQ ID NO:2. Vector features labeled in boxes are shown above the pertinent sequences.

Figure 14 provides an annotated polynucleotide sequence of the ALS-10 vector as the DNA equivalent of the RNA and encoded protein sequence. The nucleotide sequence shown in Figure 14 is provided as SEQ ID NO:3. Vector features in boxes are shown above the pertinent sequences. The location of the initiating βT87Q beta globin methionine codon in its antiparallel configuration is the "CAT" triplet to the immediate left of the beta globin 5' UTR, reading in the 5' to 3' direction.

Figure 15 provides an annotated sequence of the non-mutated beta-globin cDNA. The cDNA sequence is SEQ ID NO:4. Also shown is the beta-globin amino acid sequence which is provided in SEQ ID NO:5. The βT87Q mutation occurs at the Threonine which is shown in the sequence at position 88. The mutation is referred to as βT87Q according to convention wherein the first Methionine at position 1 is not included in the amino acid numbering. In the βT87Q mRNA, the codon for the Threonine at position 88 is replaced by a codon encoding Glutamine.

Figure 1


A
SCD erythroblasts
+pCL-ZF-Ldb1(VCN=0.26)
+pCL-ZF-Ldb1(VCN=0.67)
HbS%
HbF%
HbA2%
Retention Time
Absorbance
Elution time
+22%
-18%
+44%
-34%
C
91.4
8.6
56.7
43.4
(+34.8%)
43.2
57
(+48.4%)
FSC-H
HbF+ Cells
D
99.6
0.238
88.9
9.68
(+9.4%)
78.1
19.8
(+19.5%)
GFP+ Cells

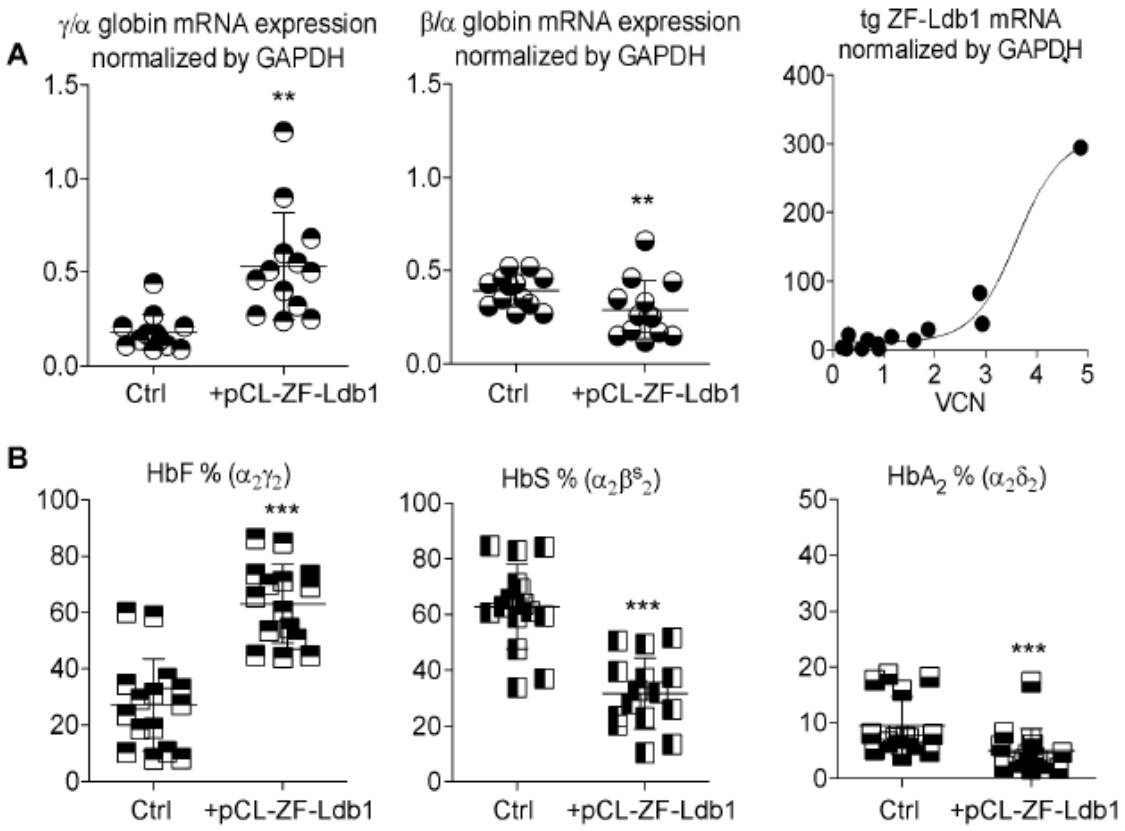

A
γ/α globin mRNA expression normalized by GAPDH
β/α globin mRNA expression normalized by GAPDH
tg ZF-Ldb1 mRNA normalized by GAPDH
Ctrl
+pCL-ZF-Ldb1
VCN
B
HbF % (α2γ2)
HbS % (α2βs2)
HbA2 % (α2δ2)
Ctrl
+pCL-ZF-Ldb1

Figure 2

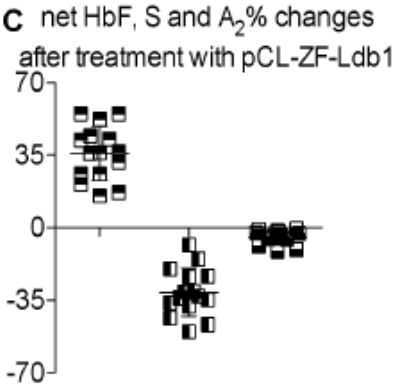
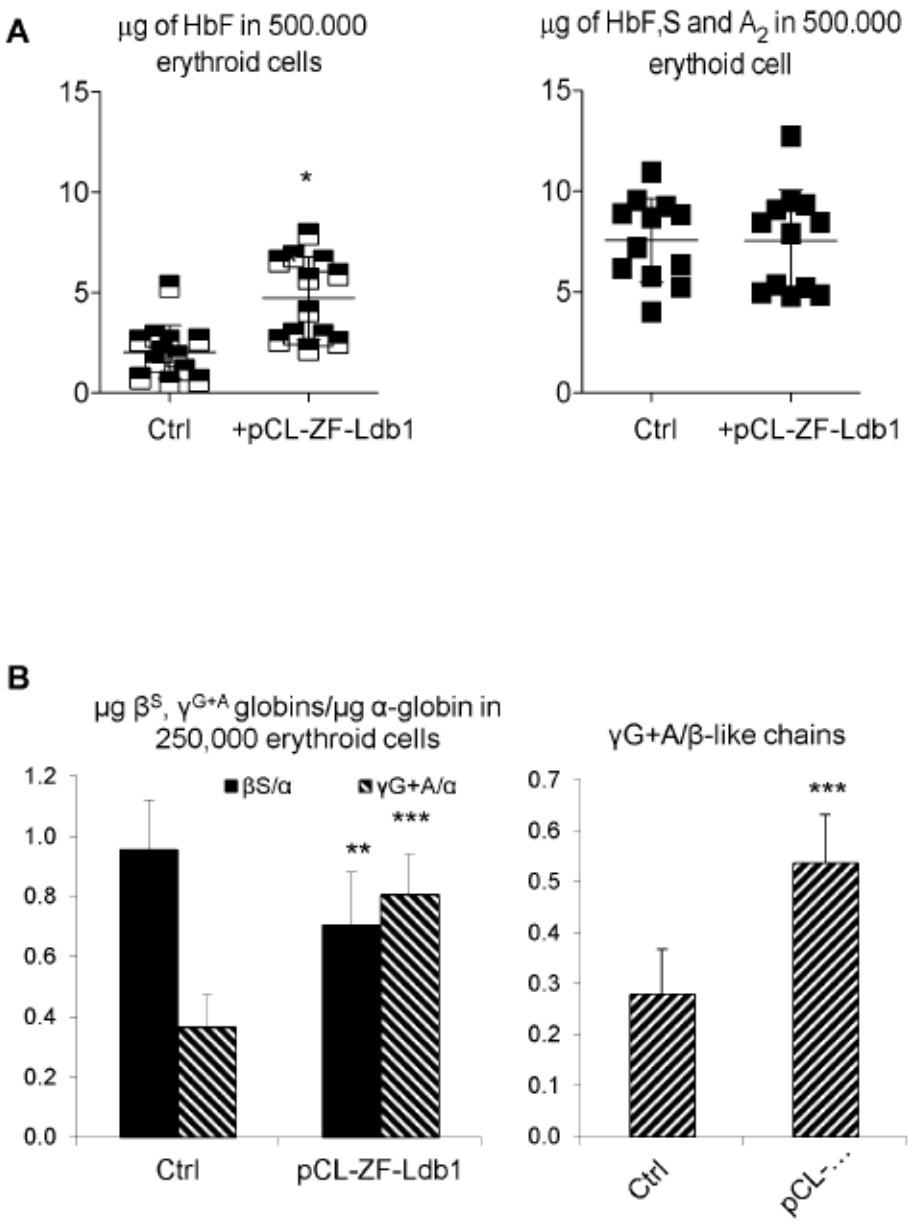

C net HbF, S and A2% changes after treatment with pCL-ZF-Ldb1

Figure 3


A
µg of HbF in 500.000 erythroid cells
15
10
5
0
*
Ctrl
+pCL-ZF-Ldb1
µg of HbF,S and A2 in 500.000 erythoid cell
15
10
5
0
Ctrl
+pCL-ZF-Ldb1
B
µg βS, γG+A globins/µg α-globin in 250,000 erythroid cells
βS/α
γG+A/α
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
Ctrl
pCL-ZF-Ldb1
γG+A/β-like chains
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
Ctrl
pCL-…

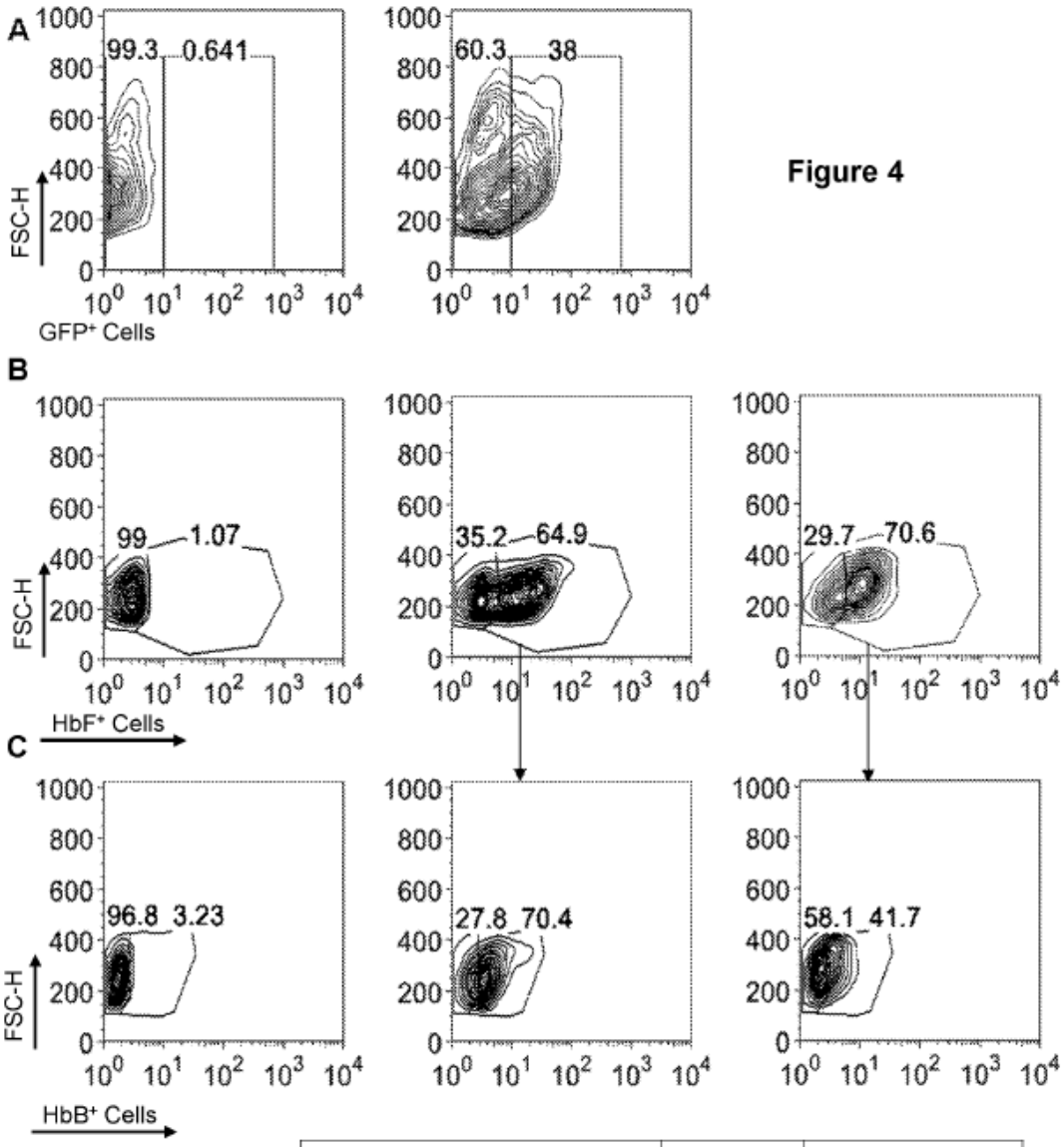


Figure 4

D

	SCD ctrl	SCD + GG1-SA
HbF % (HPLC)	27.3	70
HbF content (pg/erythroblast)	5.2	11.8

Figure 5

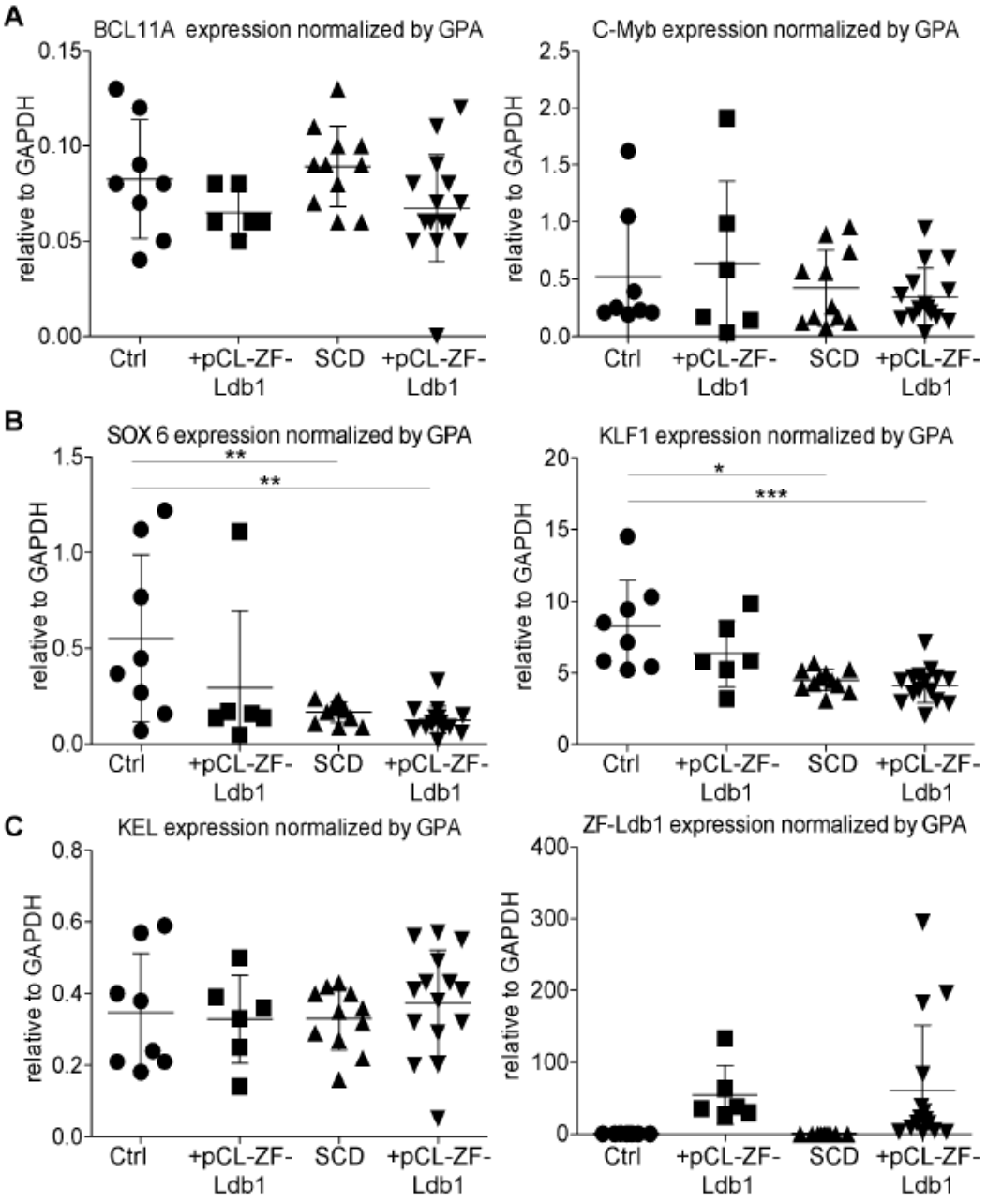

A
BCL11A expression normalized by GPA
relative to GAPDH
0.15
0.10
0.05
0.00
Ctrl
+pCL-ZF-Ldb1
SCD
+pCL-ZF-Ldb1
C-Myb expression normalized by GPA
relative to GAPDH
2.5
2.0
1.5
1.0
0.5
0.0
Ctrl
+pCL-ZF-Ldb1
SCD
+pCL-ZF-Ldb1
B
SOX 6 expression normalized by GPA
relative to GAPDH
1.5
1.0
0.5
0.0
**
**
Ctrl
+pCL-ZF-Ldb1
SCD
+pCL-ZF-Ldb1
KLF1 expression normalized by GPA
relative to GAPDH
20
15
10
5
0
*
***
Ctrl
+pCL-ZF-Ldb1
SCD
+pCL-ZF-Ldb1
C
KEL expression normalized by GPA
relative to GAPDH
0.8
0.6
0.4
0.2
0.0
Ctrl
+pCL-ZF-Ldb1
SCD
+pCL-ZF-Ldb1
ZF-Ldb1 expression normalized by GPA
relative to GAPDH
400
300
200
100
0
Ctrl
+pCL-ZF-Ldb1
SCD
+pCL-ZF-Ldb1

Figure 6

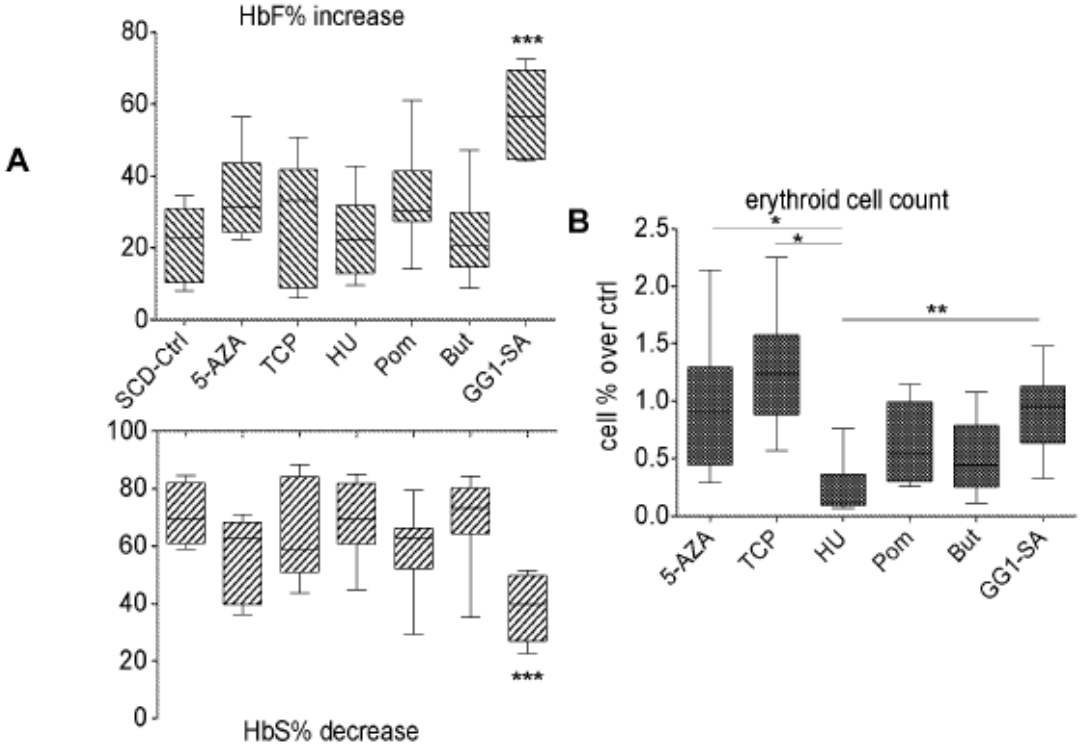

A
HbF% increase
80
60
40
20
0
***
SCD-Ctrl
5-AZA
TCP
HU
Pom
But
GG1-SA
100
80
60
40
20
0
***
HbS% decrease
B
erythroid cell count
cell % over ctrl
2.5
2.0
1.5
1.0
0.5
0.0
*
*
**
5-AZA
TCP
HU
Pom
But
GG1-SA

Figure 7

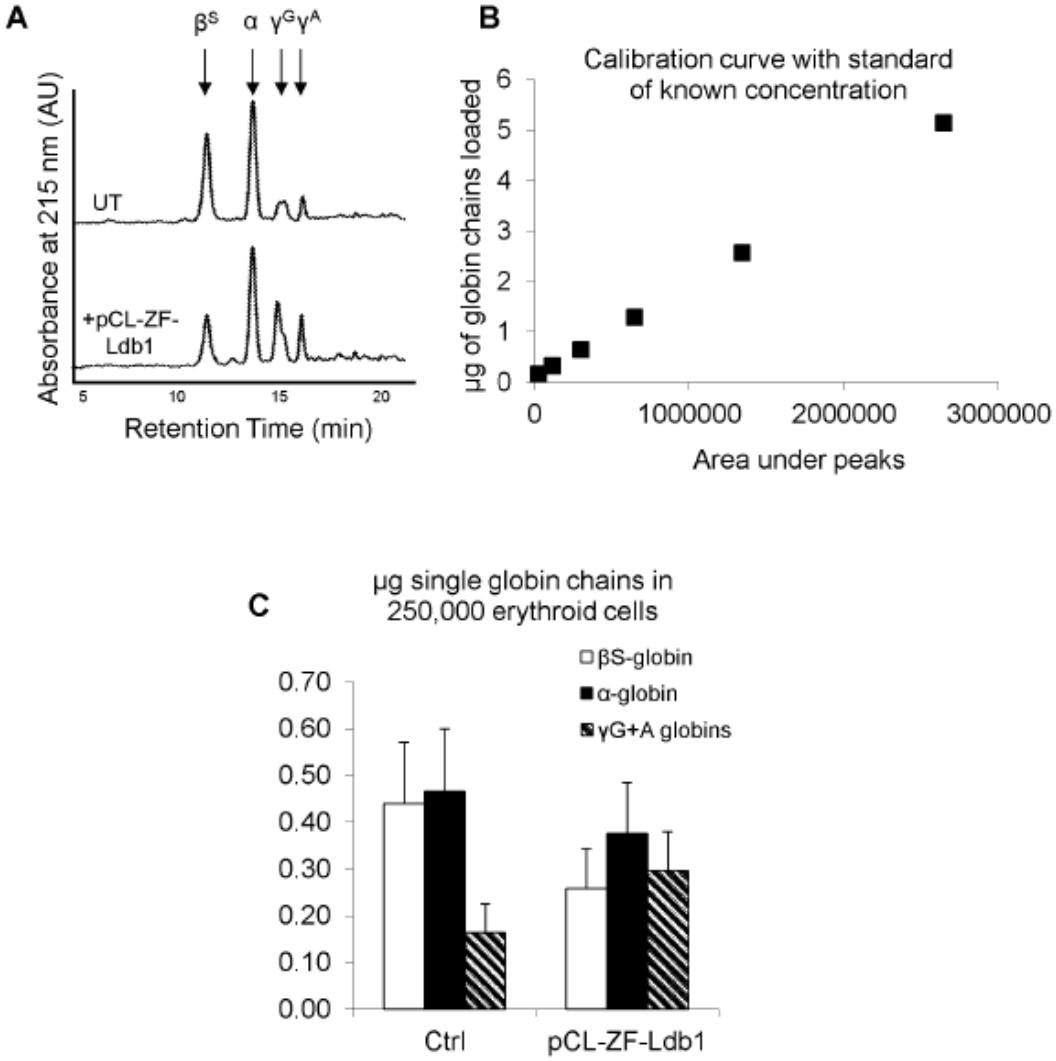

A
βS α γGγA
Absorbance at 215 nm (AU)
UT
+pCL-ZF-Ldb1
5
10
15
20
Retention Time (min)
B
Calibration curve with standard of known concentration
µg of globin chains loaded
0
1
2
3
4
5
6
0
1000000
2000000
3000000
Area under peaks
C
µg single globin chains in 250,000 erythroid cells
βS-globin
α-globin
γG+A globins
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Ctrl
pCL-ZF-Ldb1

Figure 8

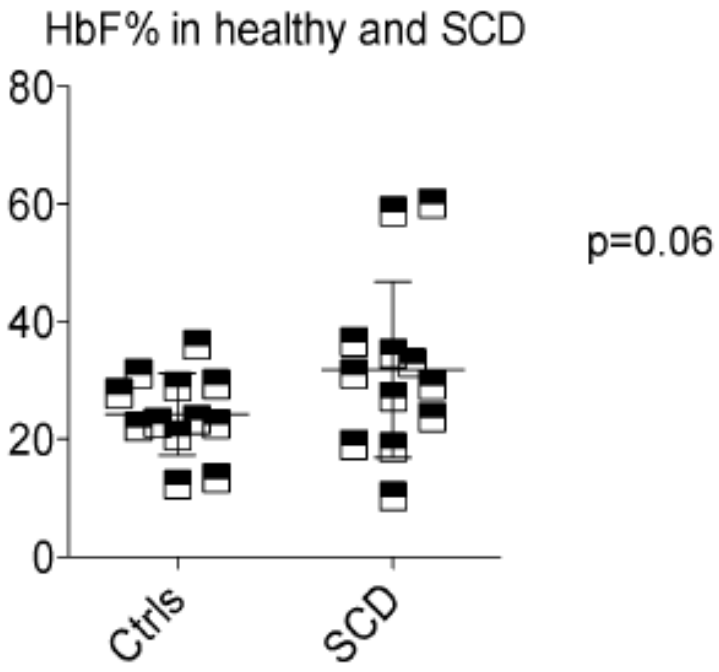


Figure 9

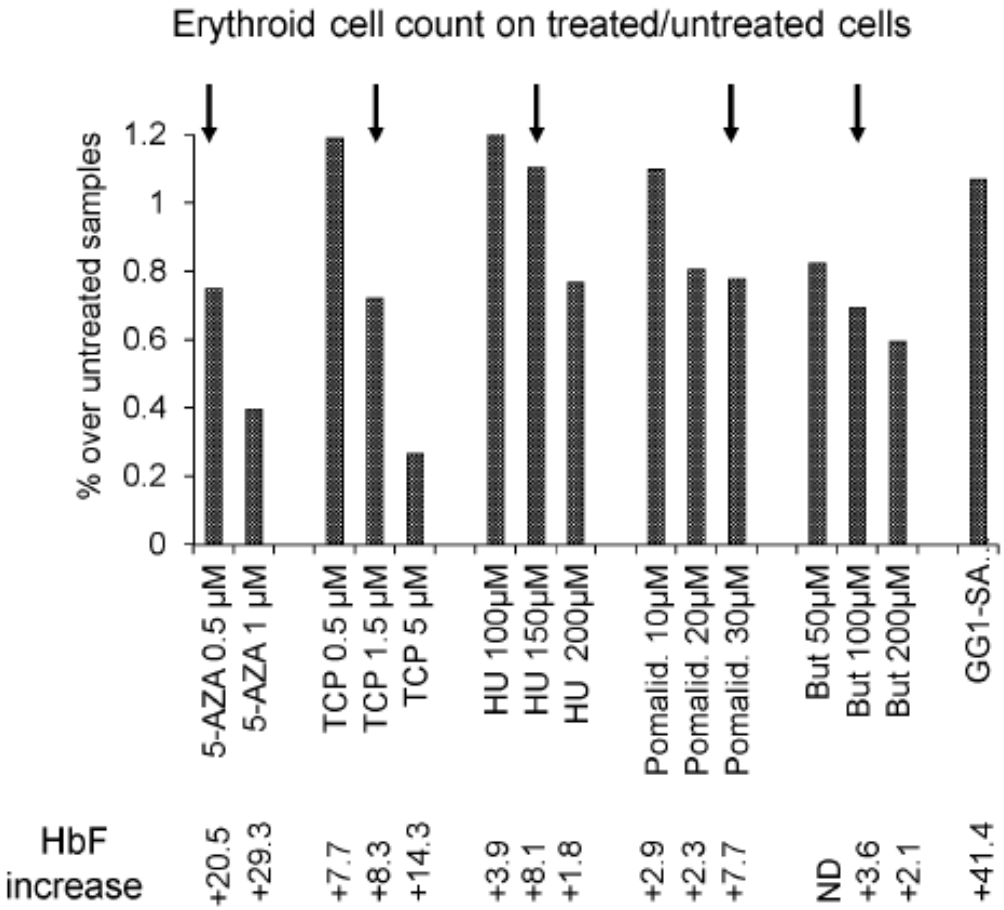
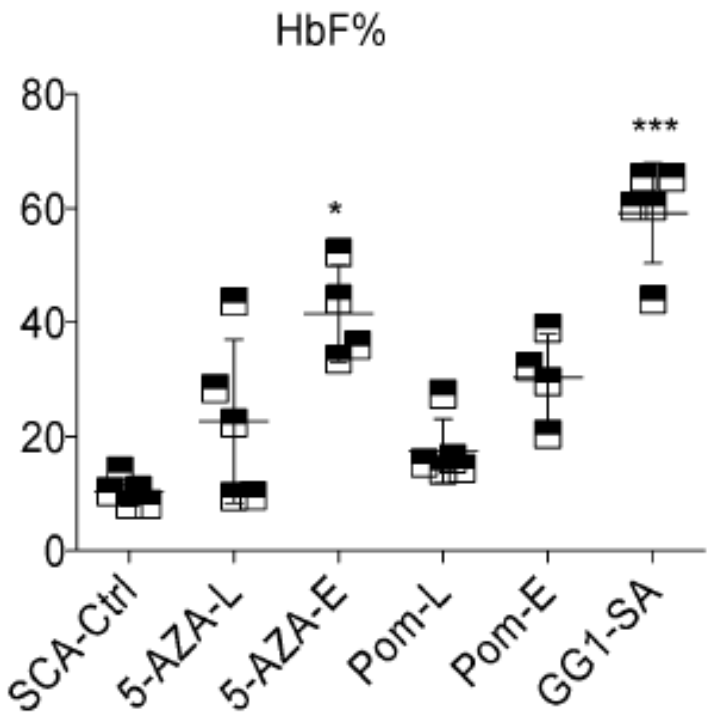

Erythroid cell count on treated/untreated cells
% over untreated samples
1.2
1
0.8
0.6
0.4
0.2
0
5-AZA 0.5 µM
5-AZA 1 µM
TCP 0.5 µM
TCP 1.5 µM
TCP 5 µM
HU 100µM
HU 150µM
HU 200µM
Pomalid. 10µM
Pomalid. 20µM
Pomalid. 30µM
But 50µM
But 100µM
But 200µM
GG1-SA...
HbF increase
+20.5
+29.3
+7.7
+8.3
+14.3
+3.9
+8.1
+1.8
+2.9
+2.3
+7.7
ND
+3.6
+2.1
+41.4

Figure 10



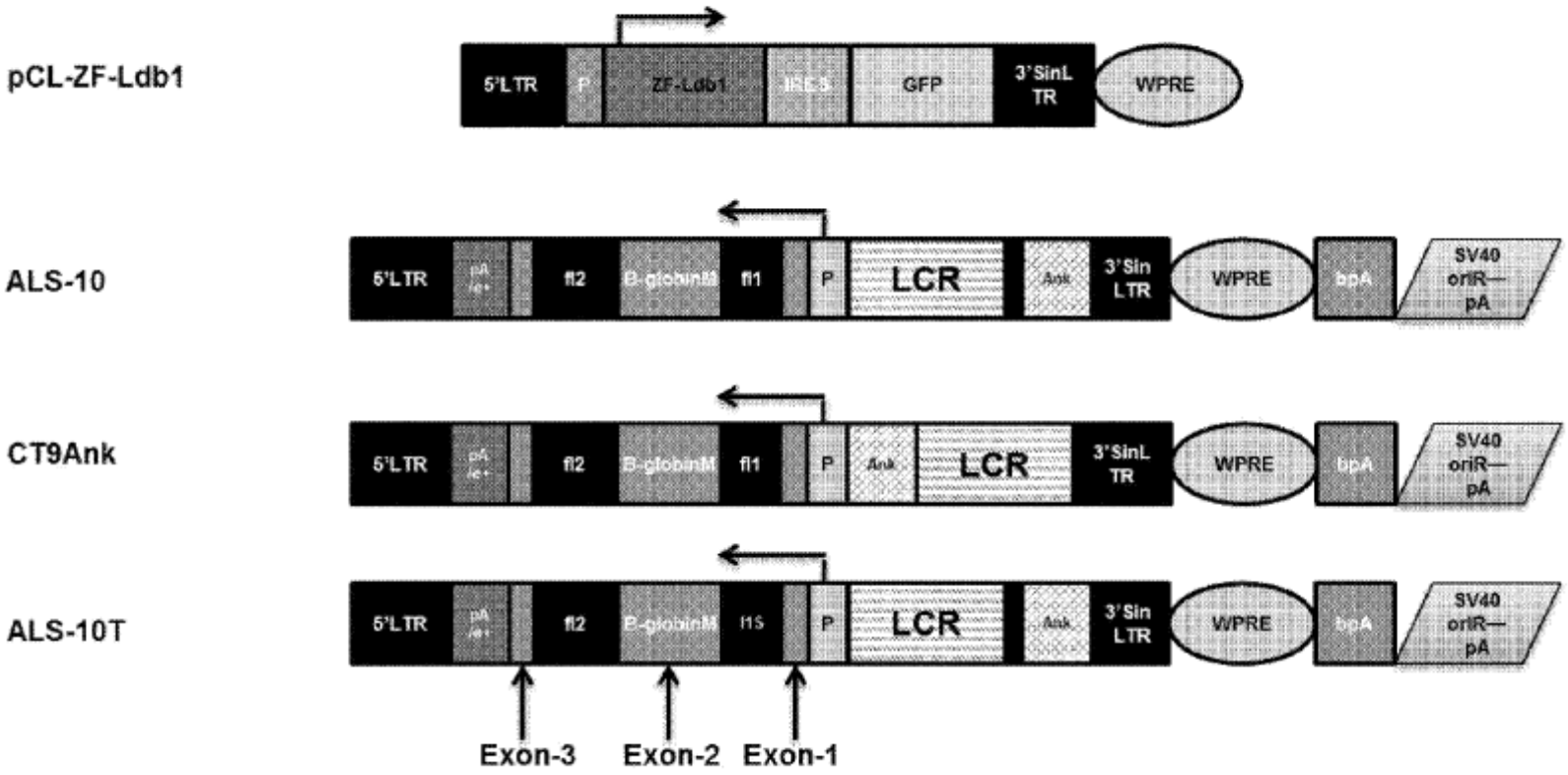

pCL-ZF-Ldb1
5'LTR
P
ZF-Ldb1
IRES
GFP
3'SinL TR
WPRE
ALS-10
5'LTR
fl2
B-globinM
fl1
P
LCR
Ank
3'Sin LTR
WPRE
bpA
SV40 oriR— pA
CT9Ank
5'LTR
fl2
B-globinM
fl1
P
Ank
LCR
3'SinL TR
WPRE
bpA
SV40 oriR— pA
ALS-10T
5'LTR
fl2
B-globinM
fl1S
P
LCR
Ank
3'Sin LTR
WPRE
bpA
SV40 oriR— pA
Exon-3
Exon-2
Exon-1

Figure 11

Figure 12

HbA% in β0/+ or β0/0 erythroblasts treated **AnkT9W** versus **ALS10.**

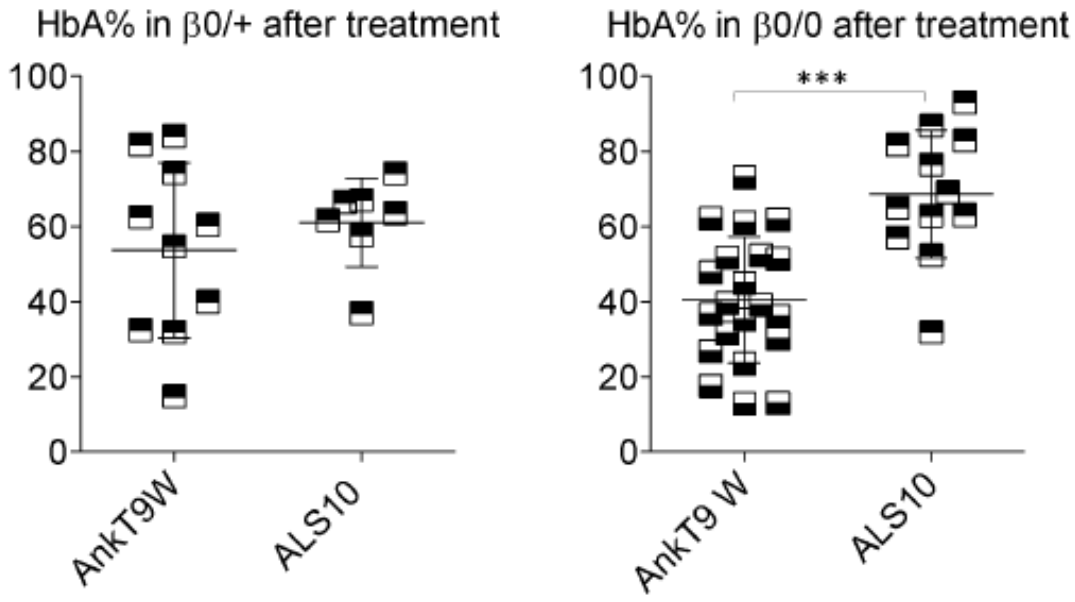


Figure 13

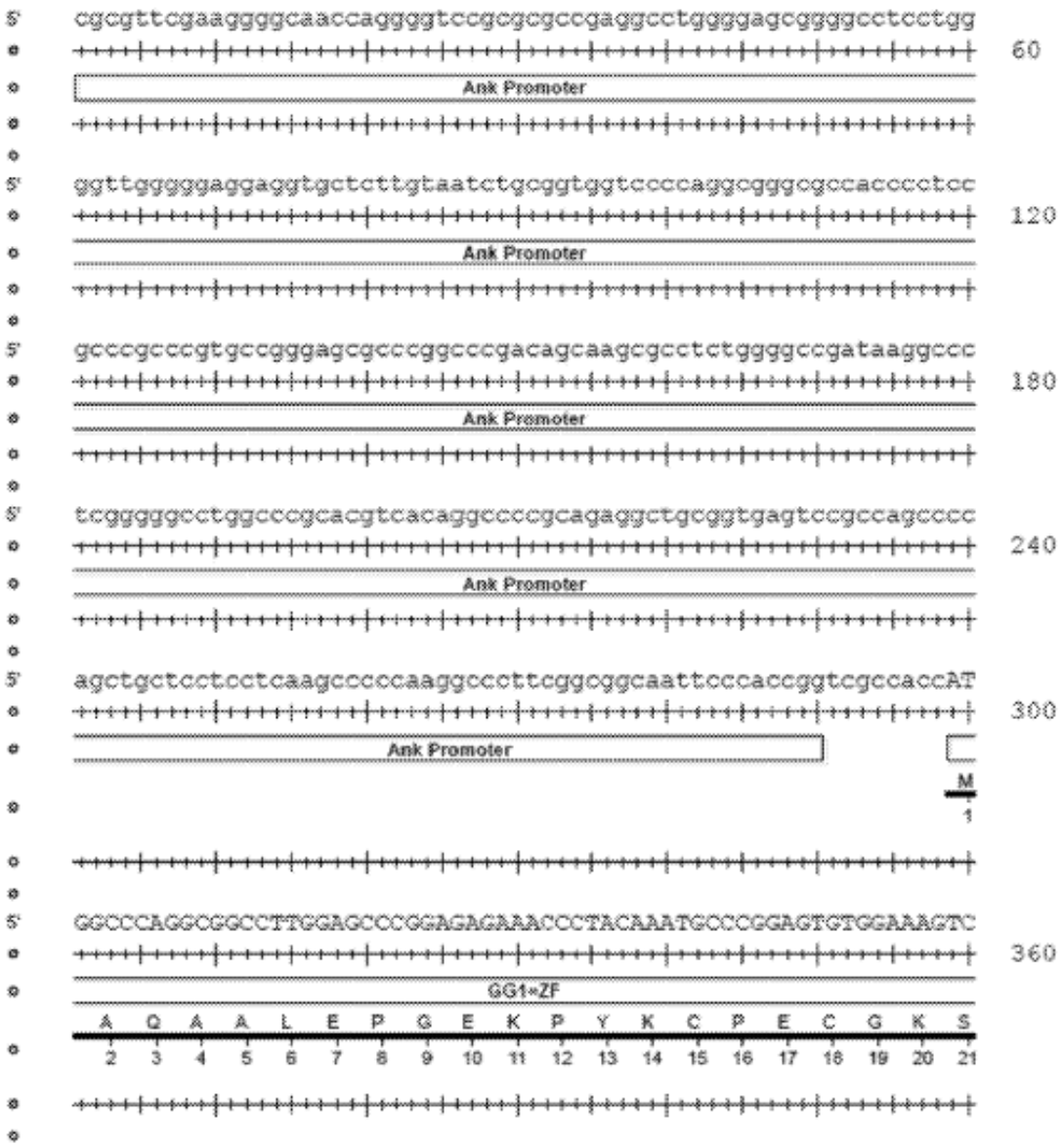

5' cgcgttcgaaggggcaaccaggggtccgcgcgccgaggcctggggagcggggcctcctgg
60
Ank Promoter
5' ggttgggggaggaggtgctcttgtaatctgcggtggtccccaggcgggcgccacccctcc
120
Ank Promoter
5' gcccgcccgtgccgggagcgcccggcccgacagcaagcgcctctggggccgataaggccc
180
Ank Promoter
5' tcgggggcctggcccgcacgtcacaggccccgcagaggctgcggtgagtccgccagcccc
240
Ank Promoter
5' agctgctcctcctcaagcccccaaggcccttcggcggcaattcccaccggtcgccaccAT
300
Ank Promoter
M
1
5' GGCCCAGGCGGCCTTGGAGCCCGGAGAGAAACCCTACAAATGCCCGGAGTGTGGAAAGTC
360
GG1«ZF
A Q A A L E P G E K P Y K C P E C G K S
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Figure 13, continued

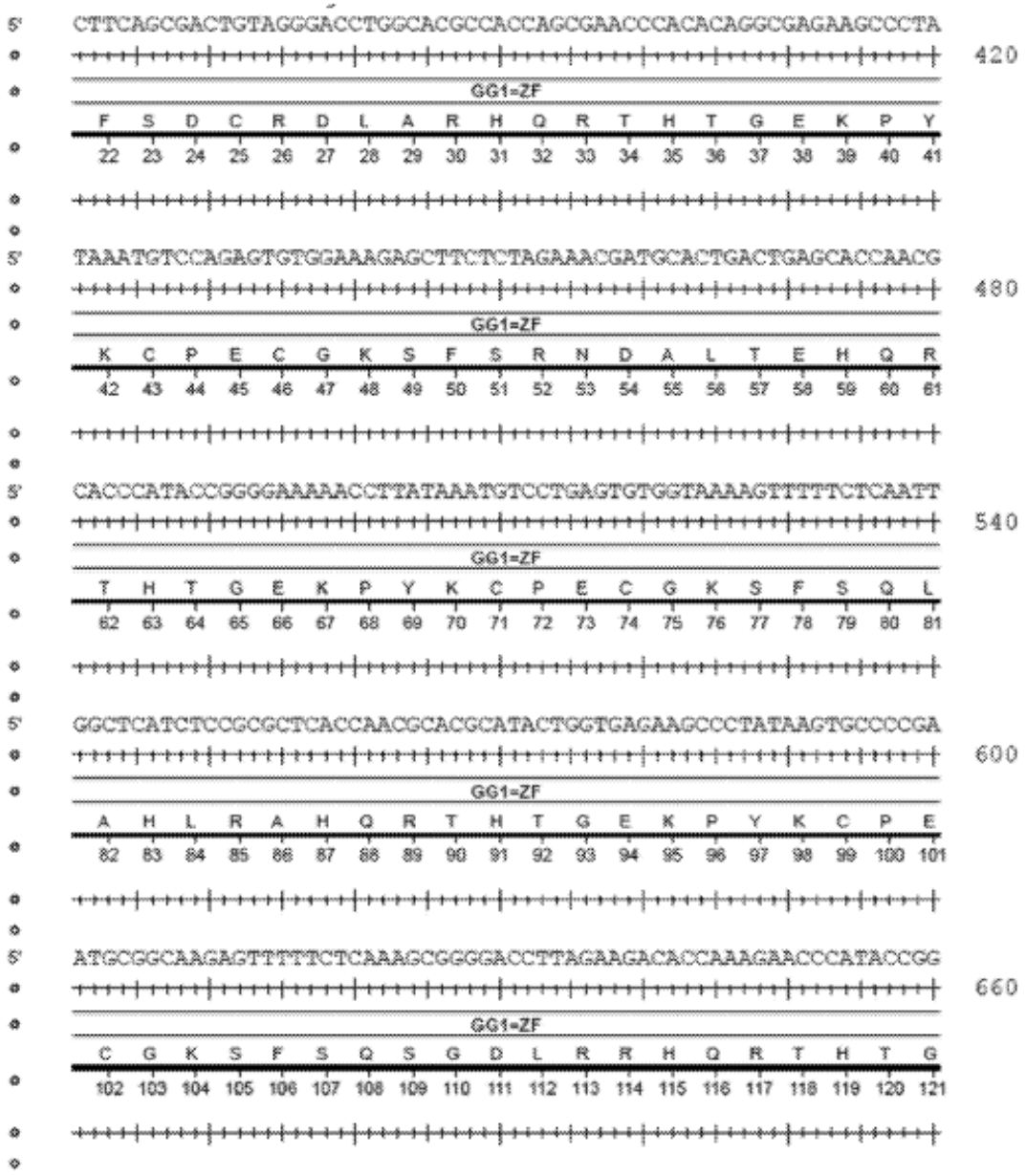


Figure 13, continued

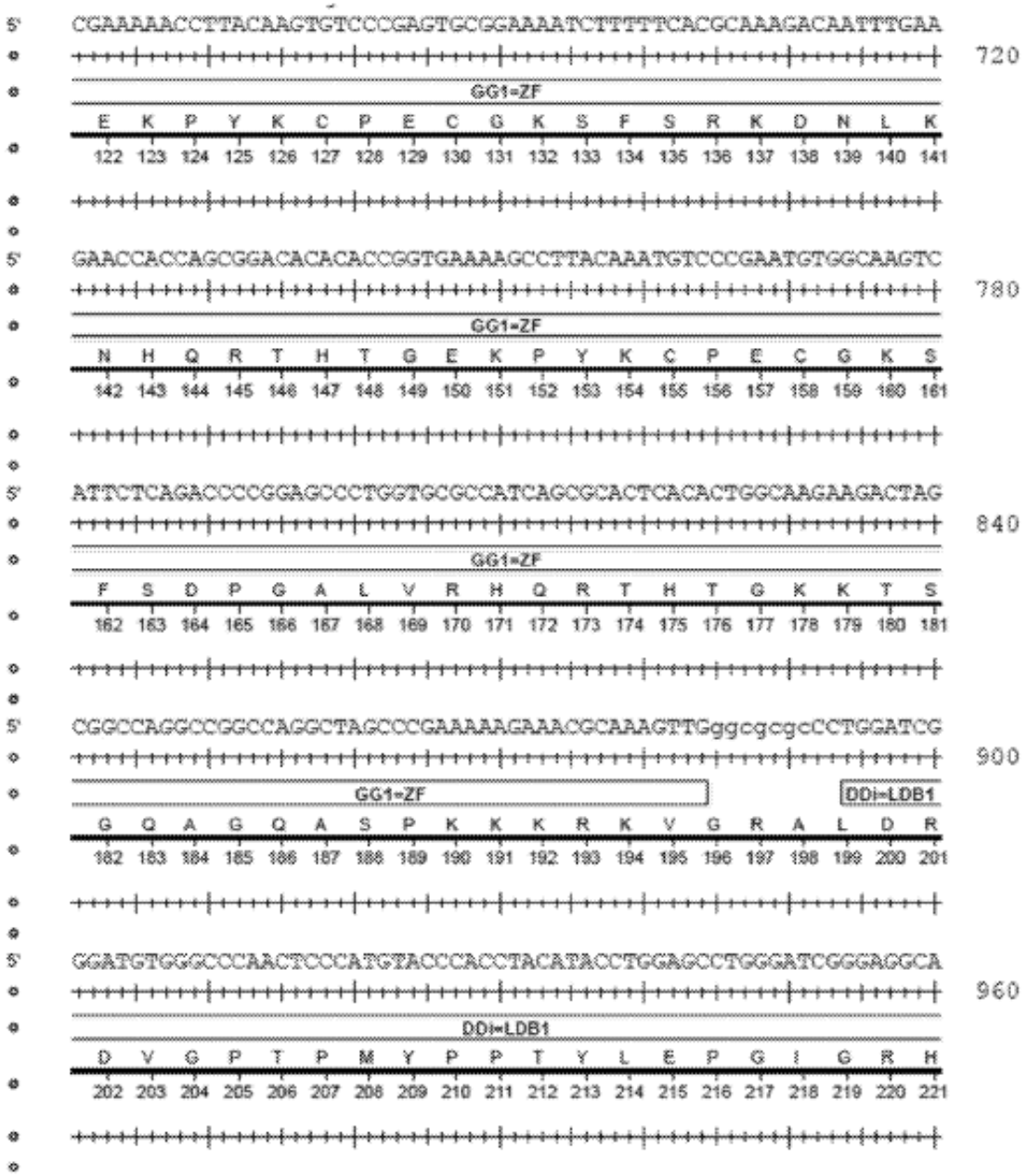


Figure 13, continued

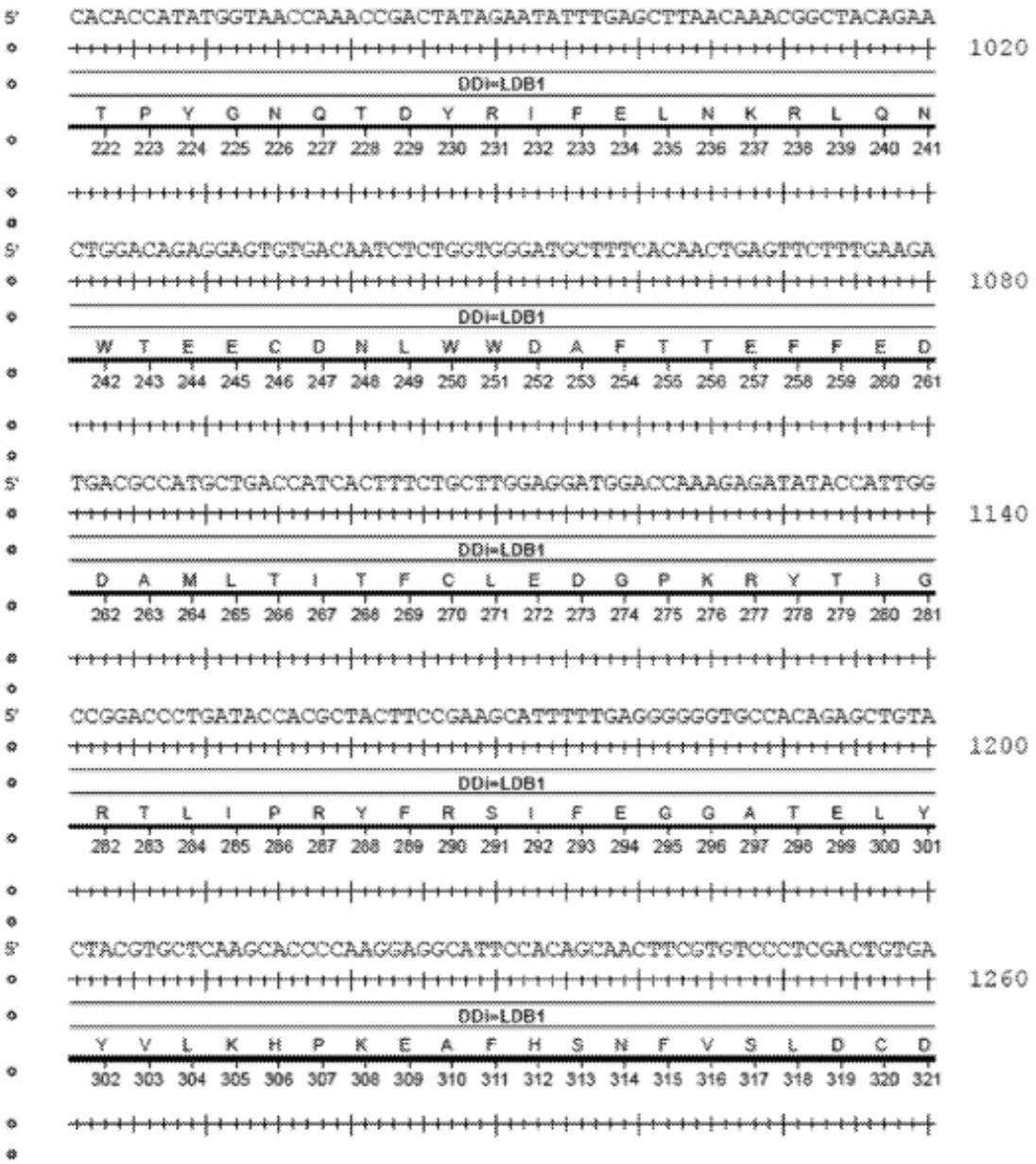


Figure 13, continued

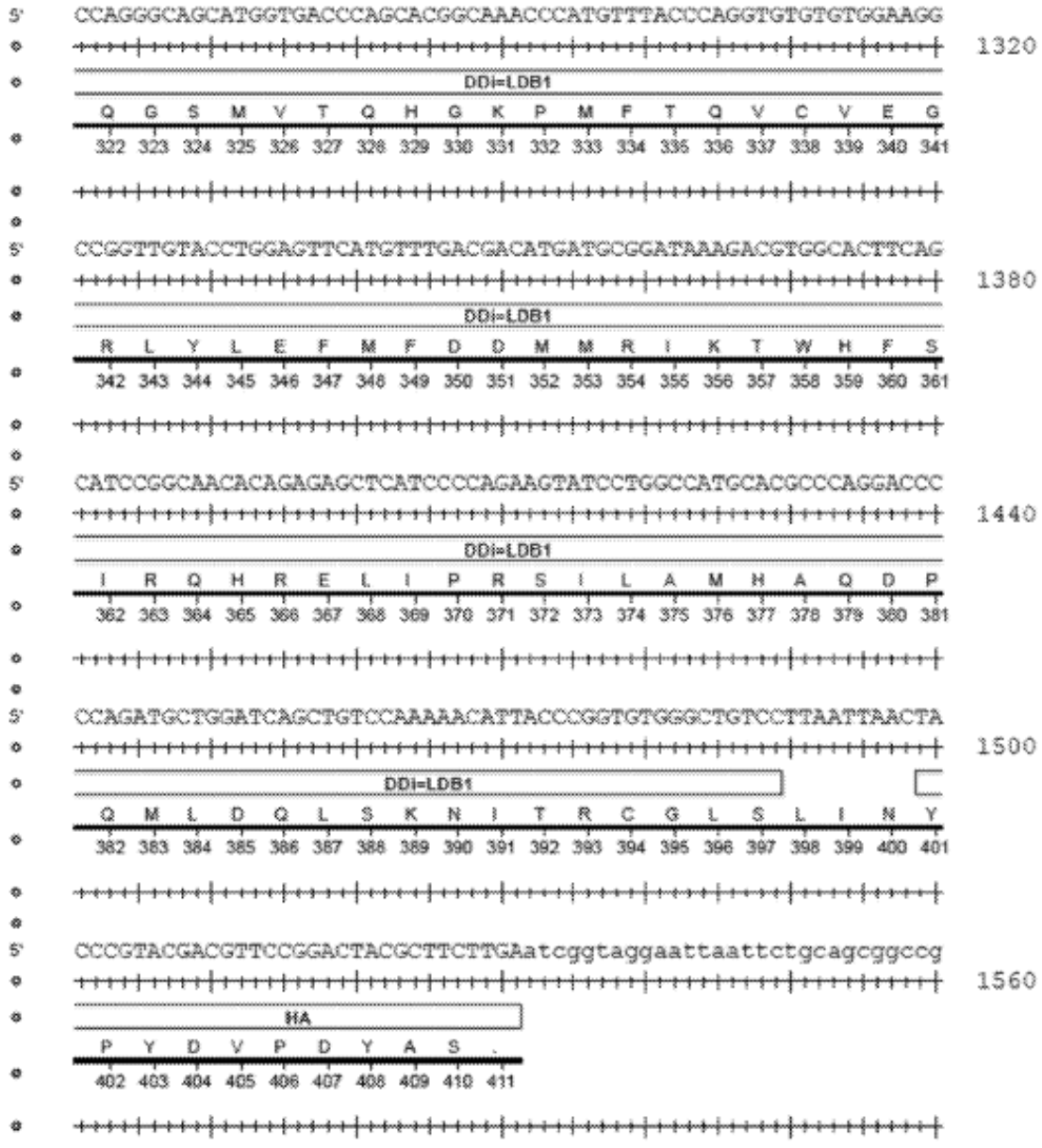


Figure 13, continued

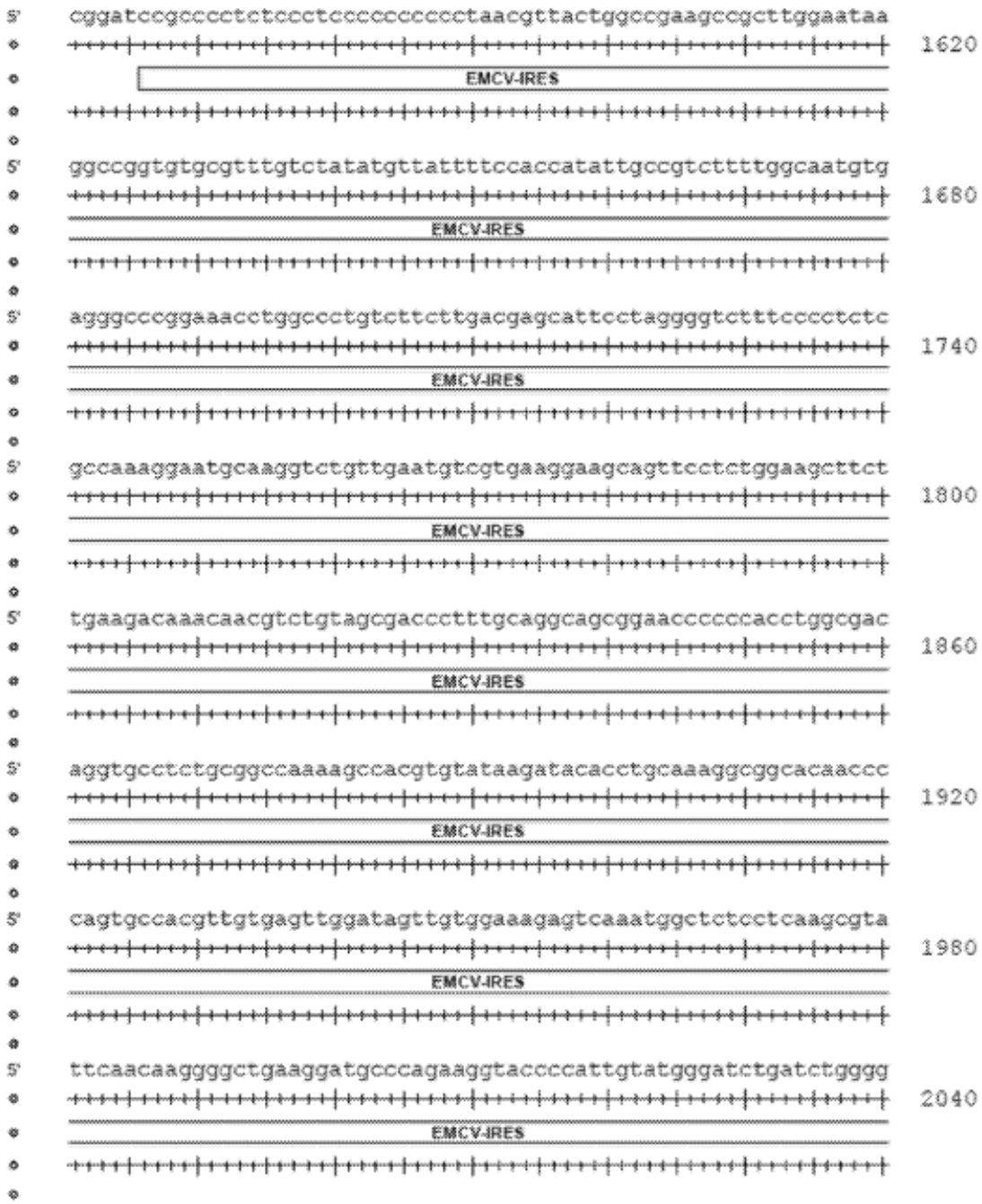


Figure 13, continued

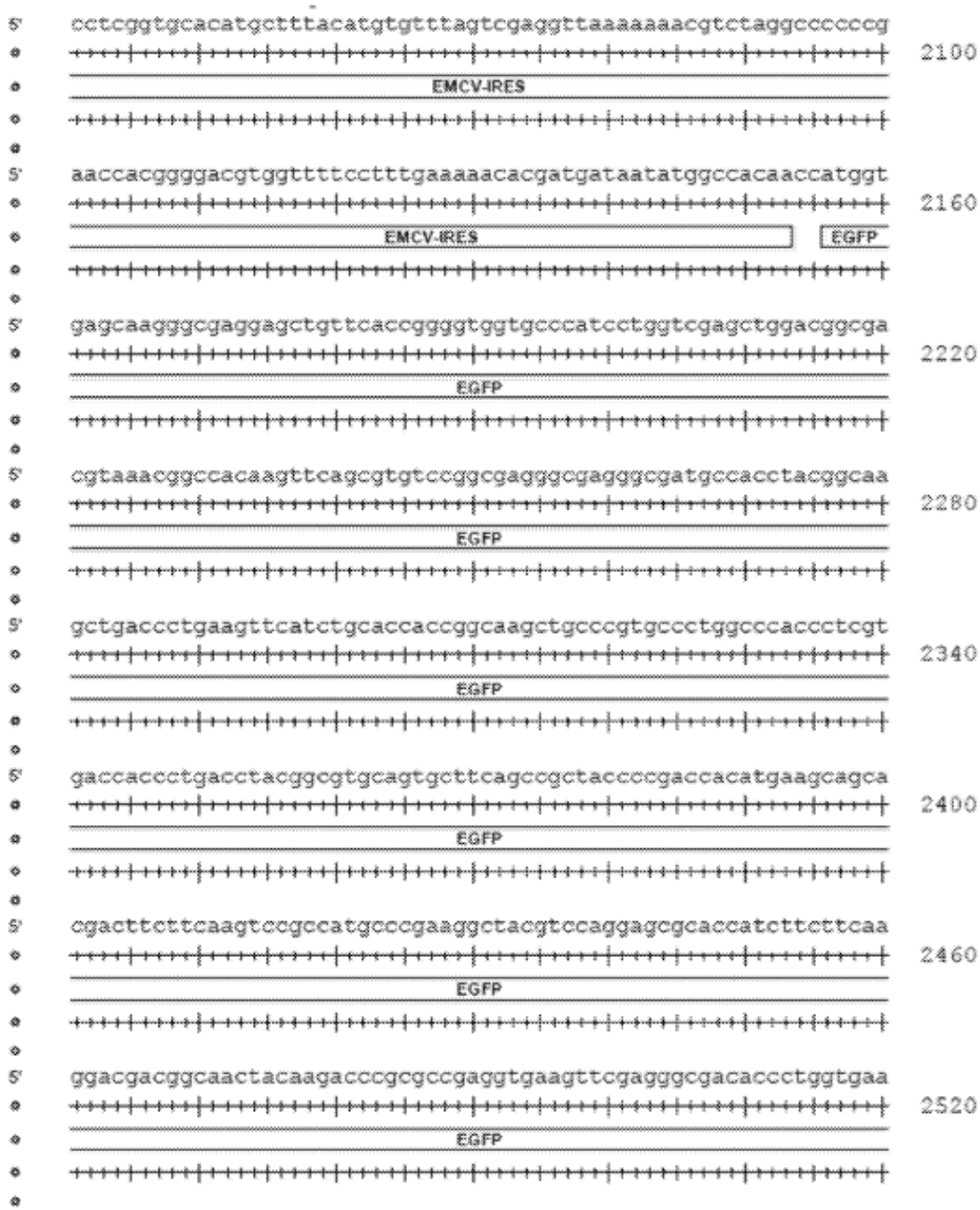


Figure 13, continued

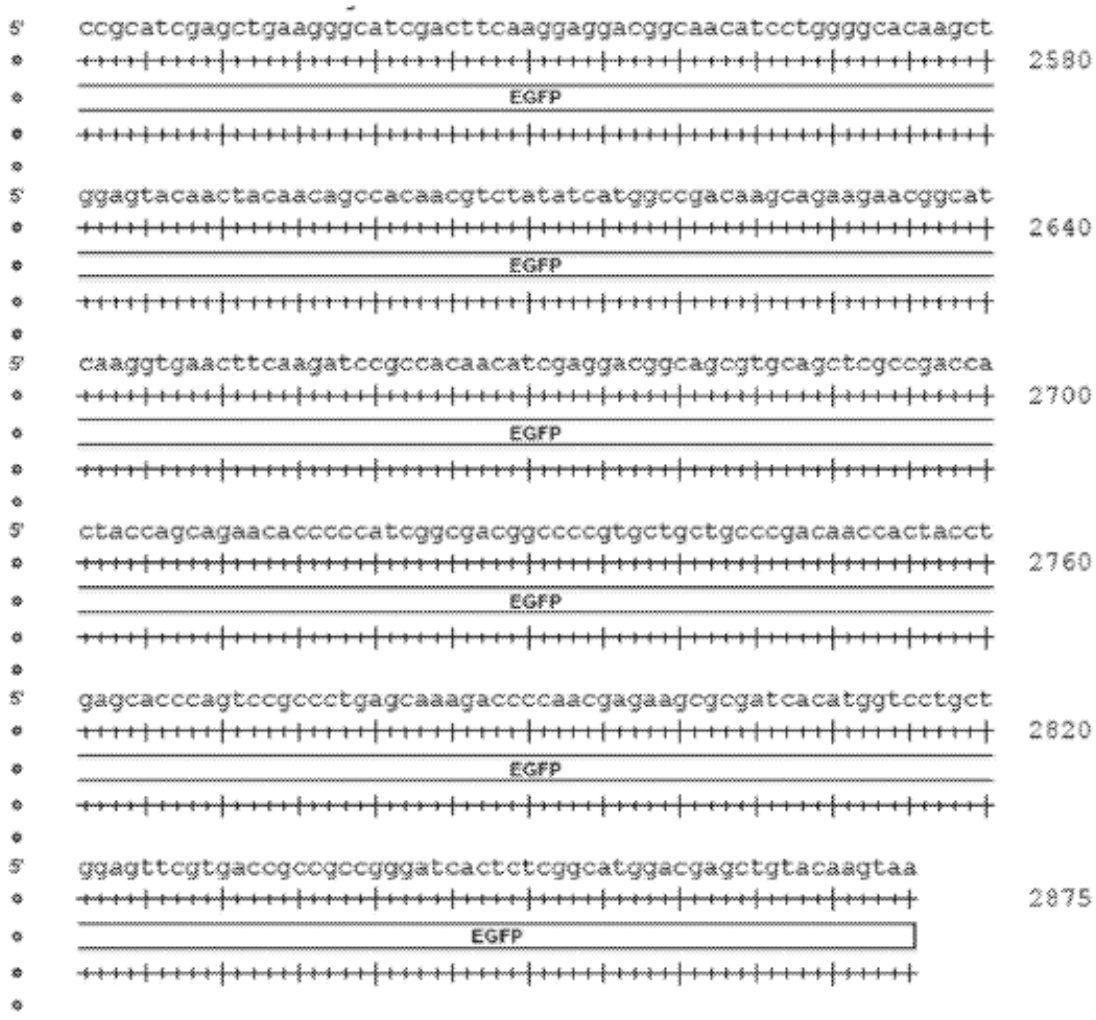
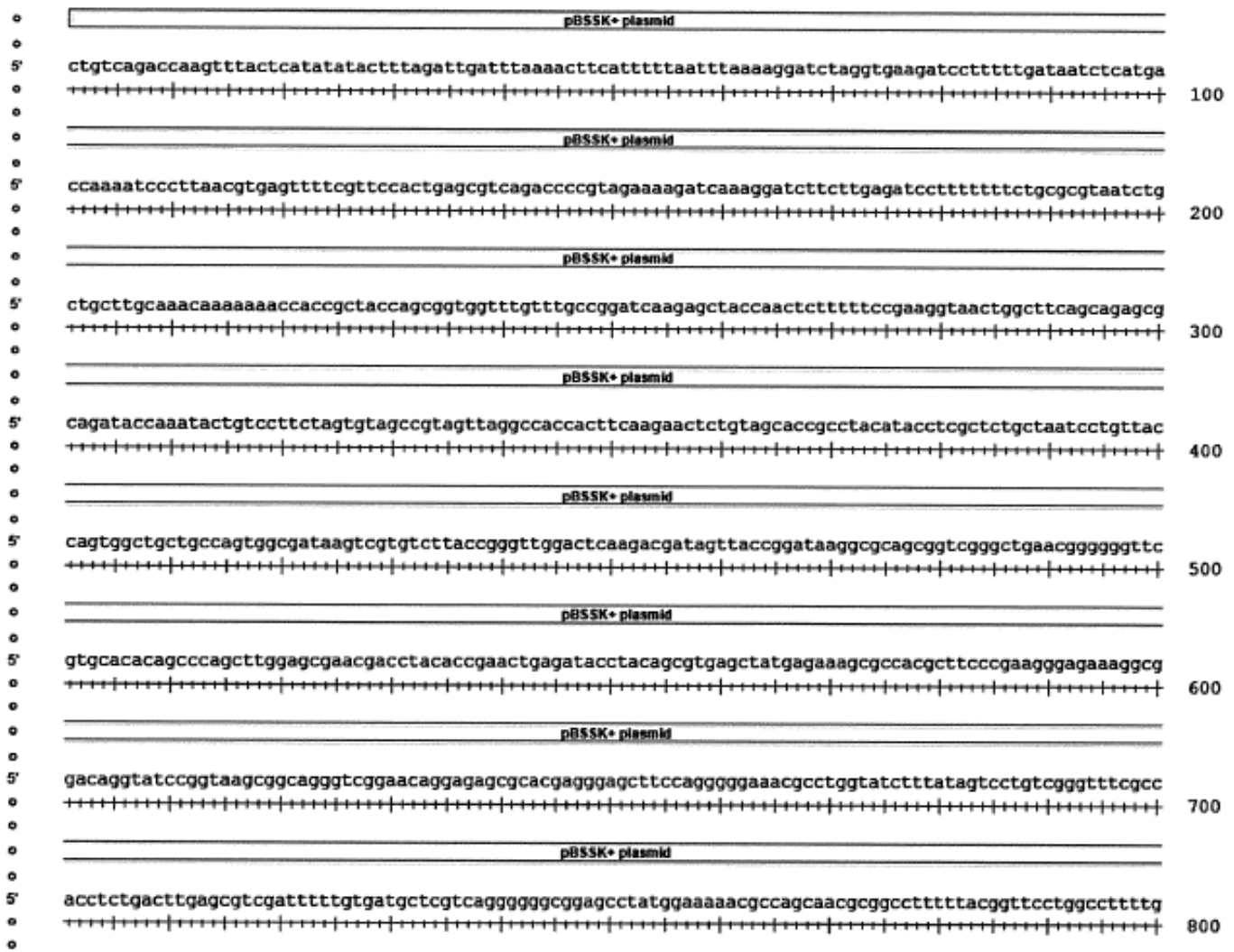


Figure 14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200
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5'
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300
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5'
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500
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5'
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600
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5'
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700
pBSSK+ plasmid
5'
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Figure 14, continued

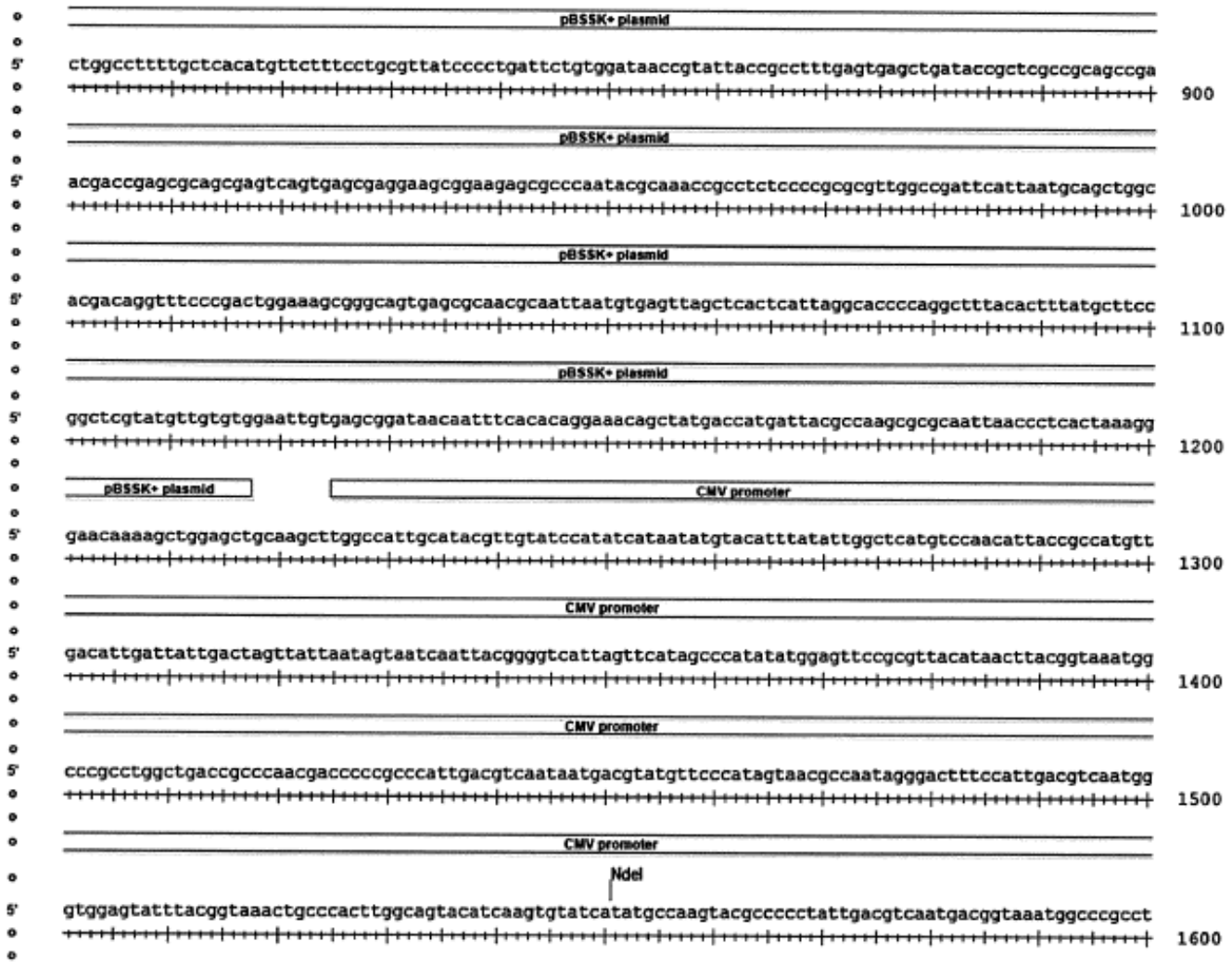
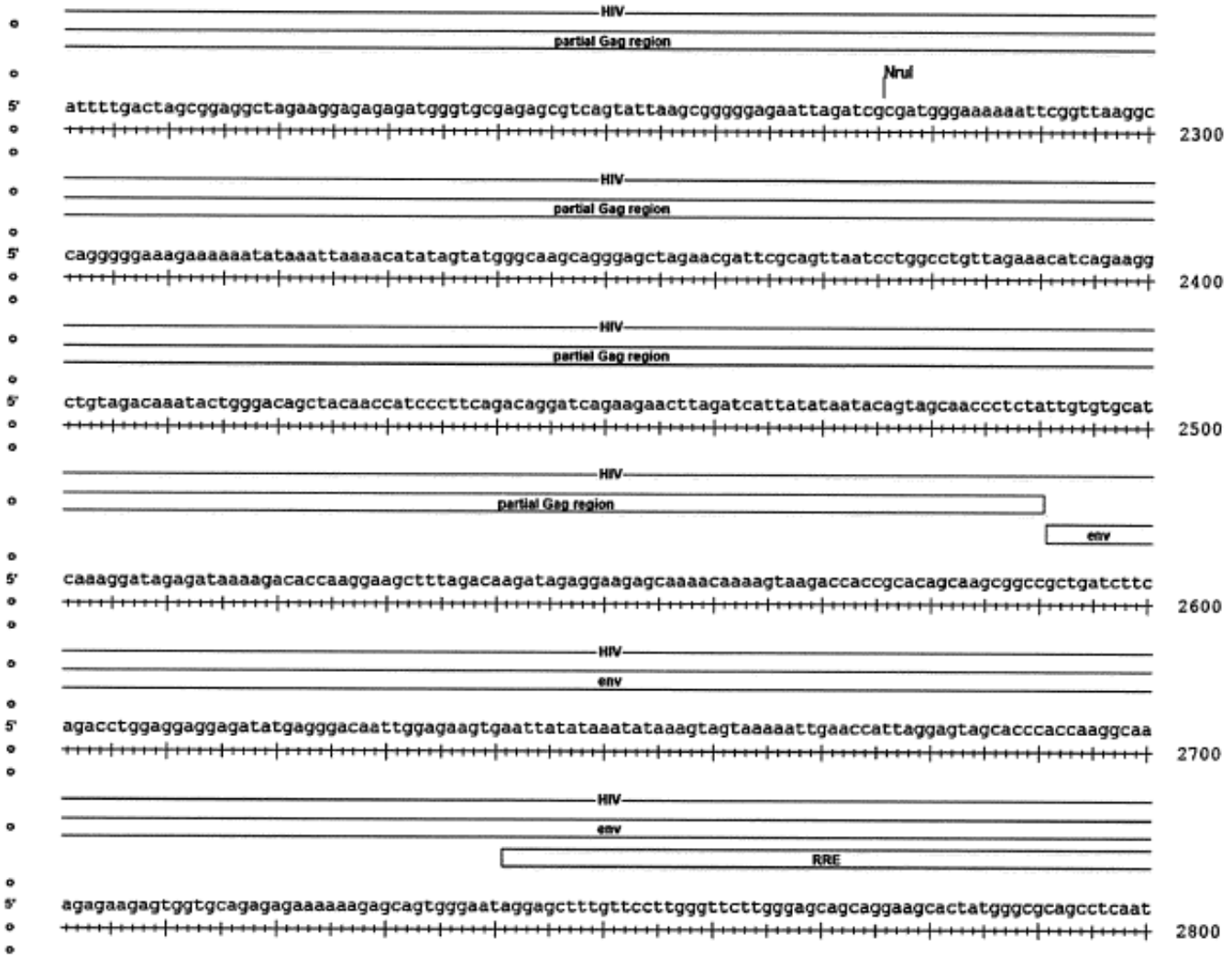


Figure 14, continued

Figure 14, continued



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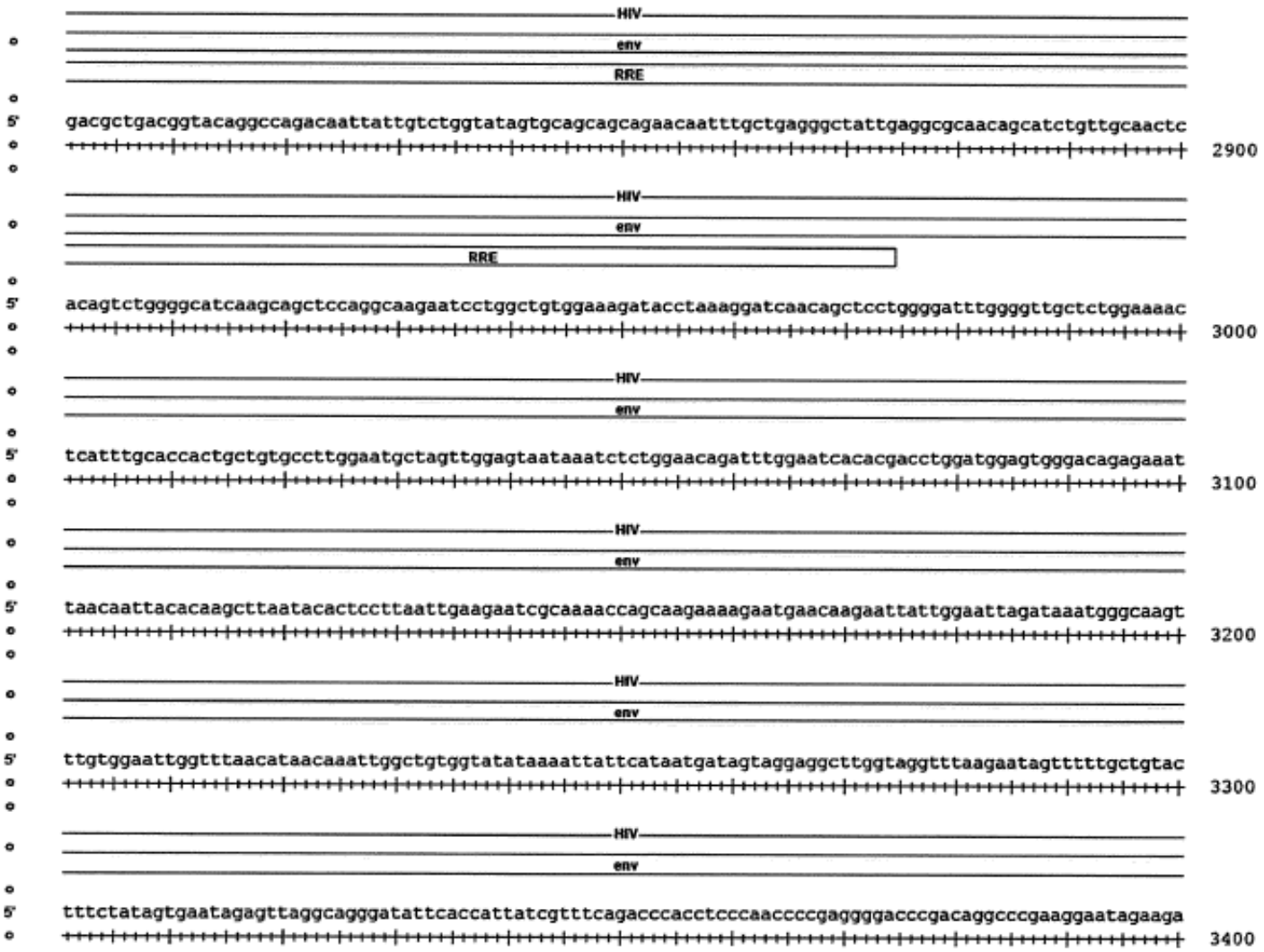

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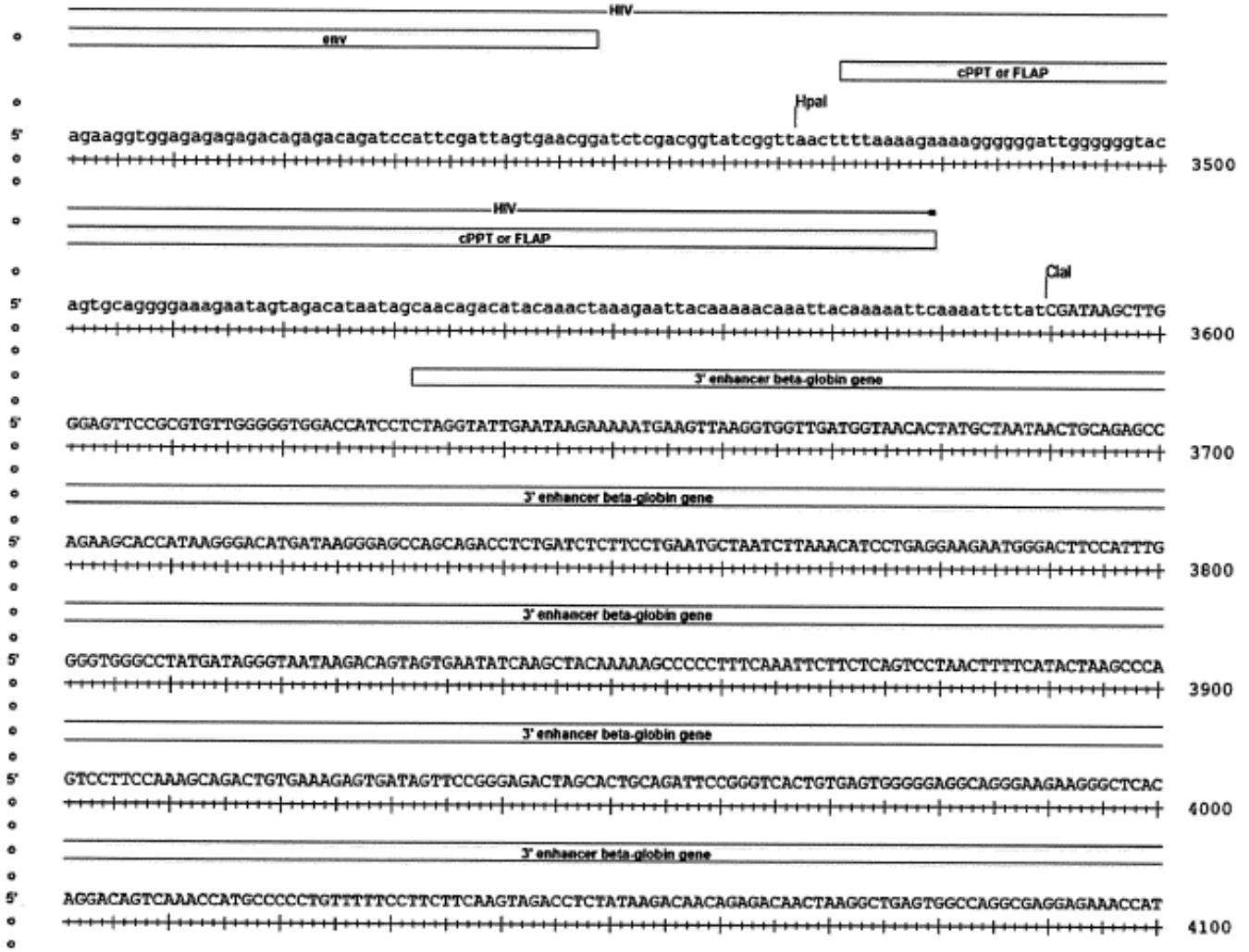

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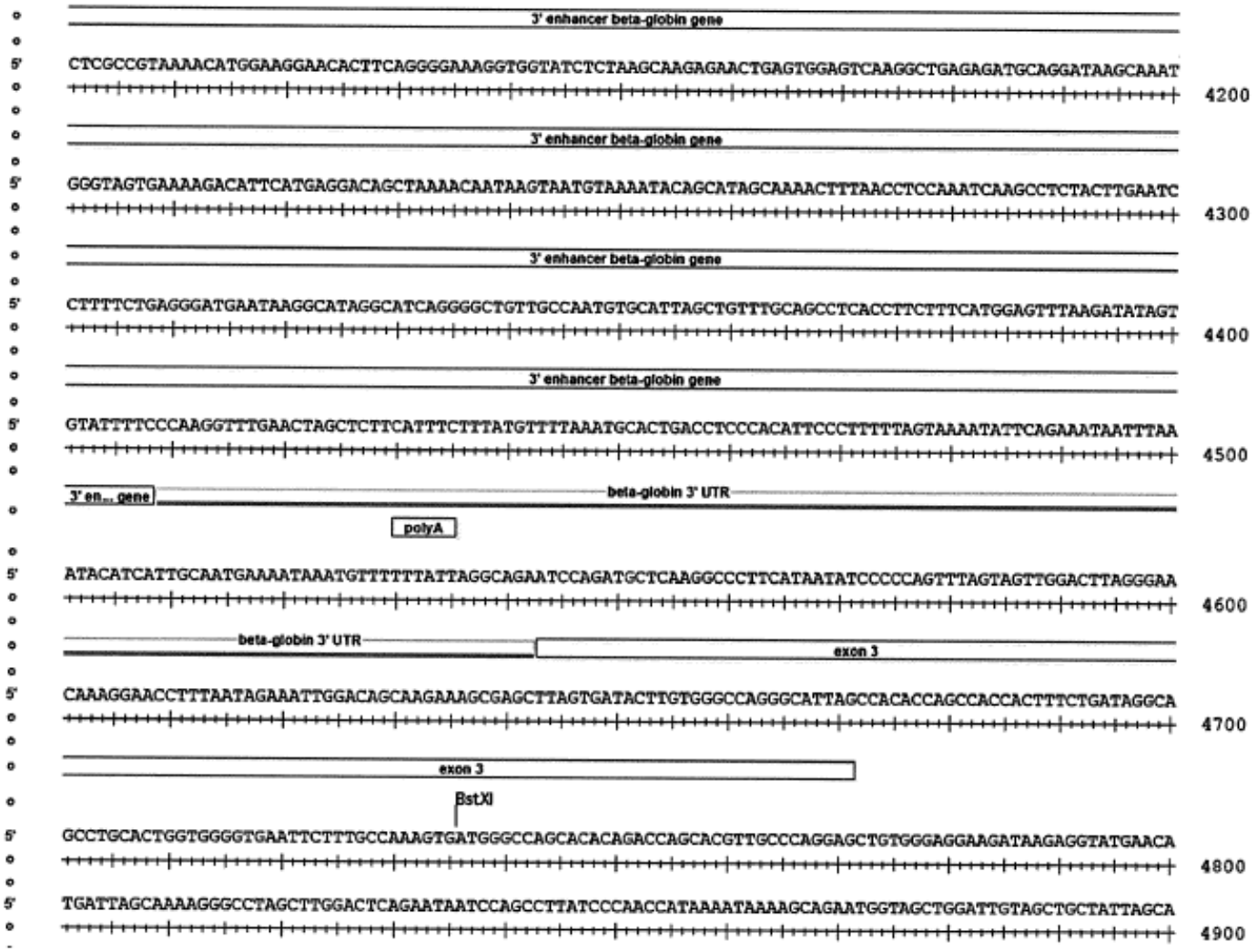
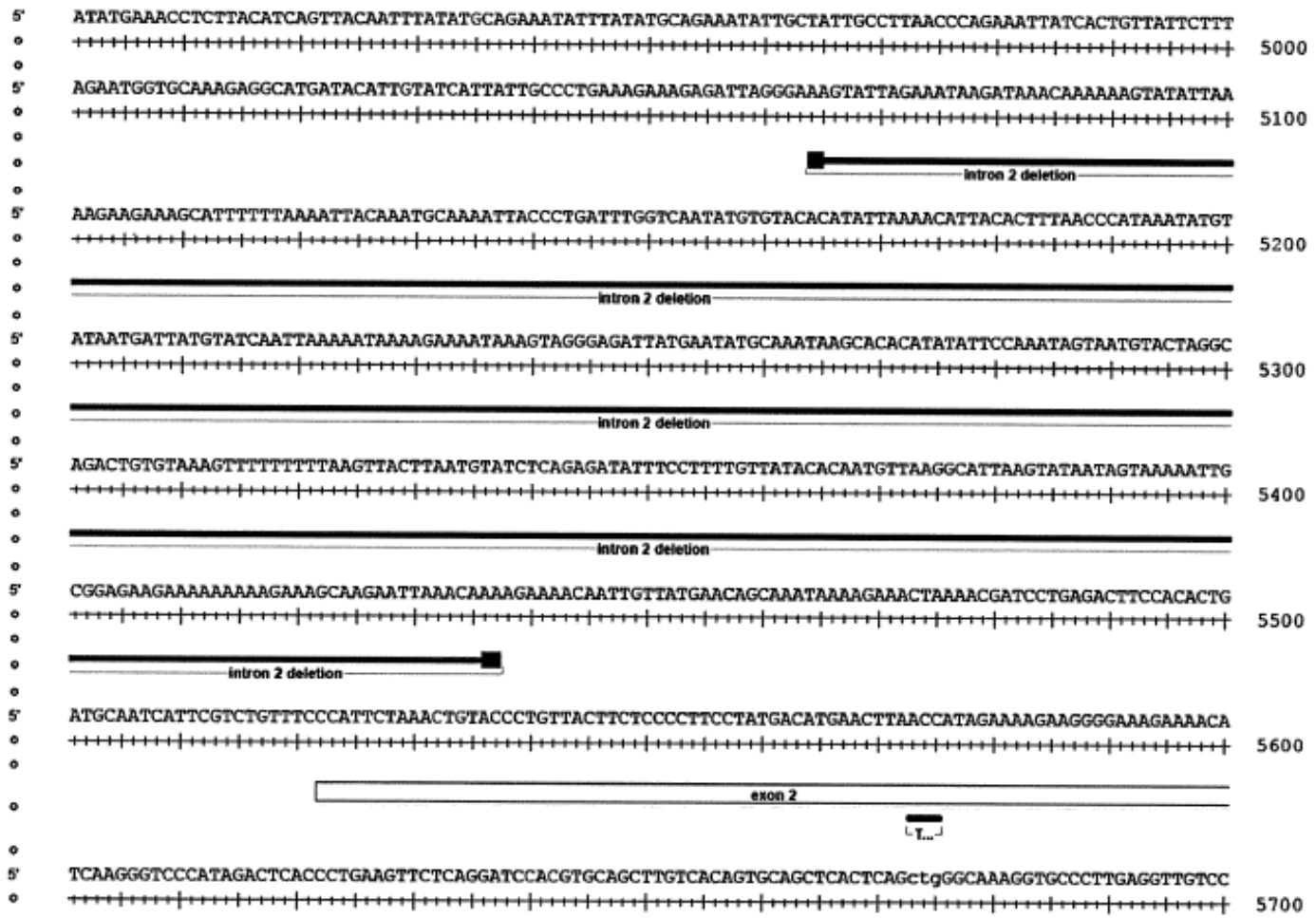


Figure 14, continued



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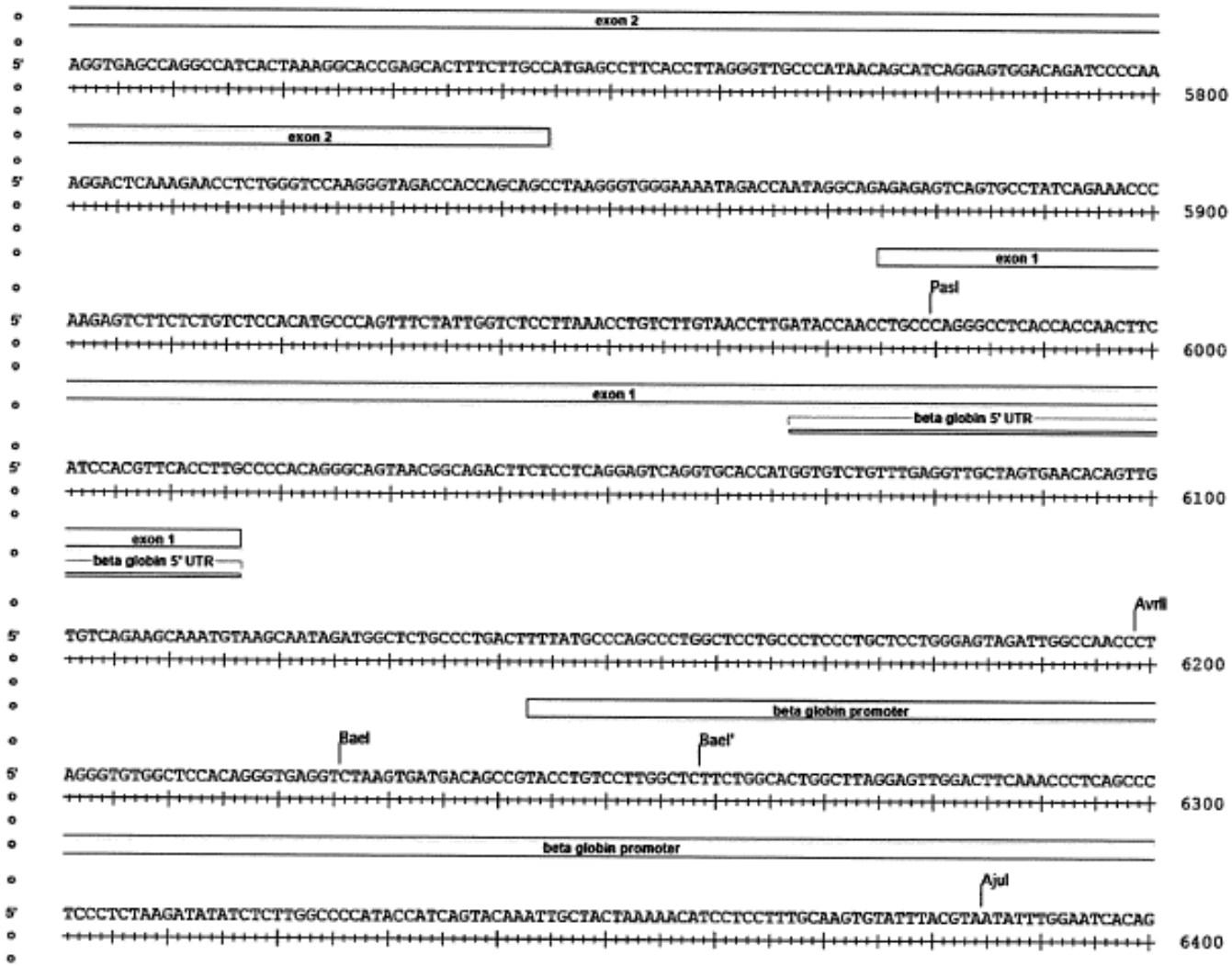

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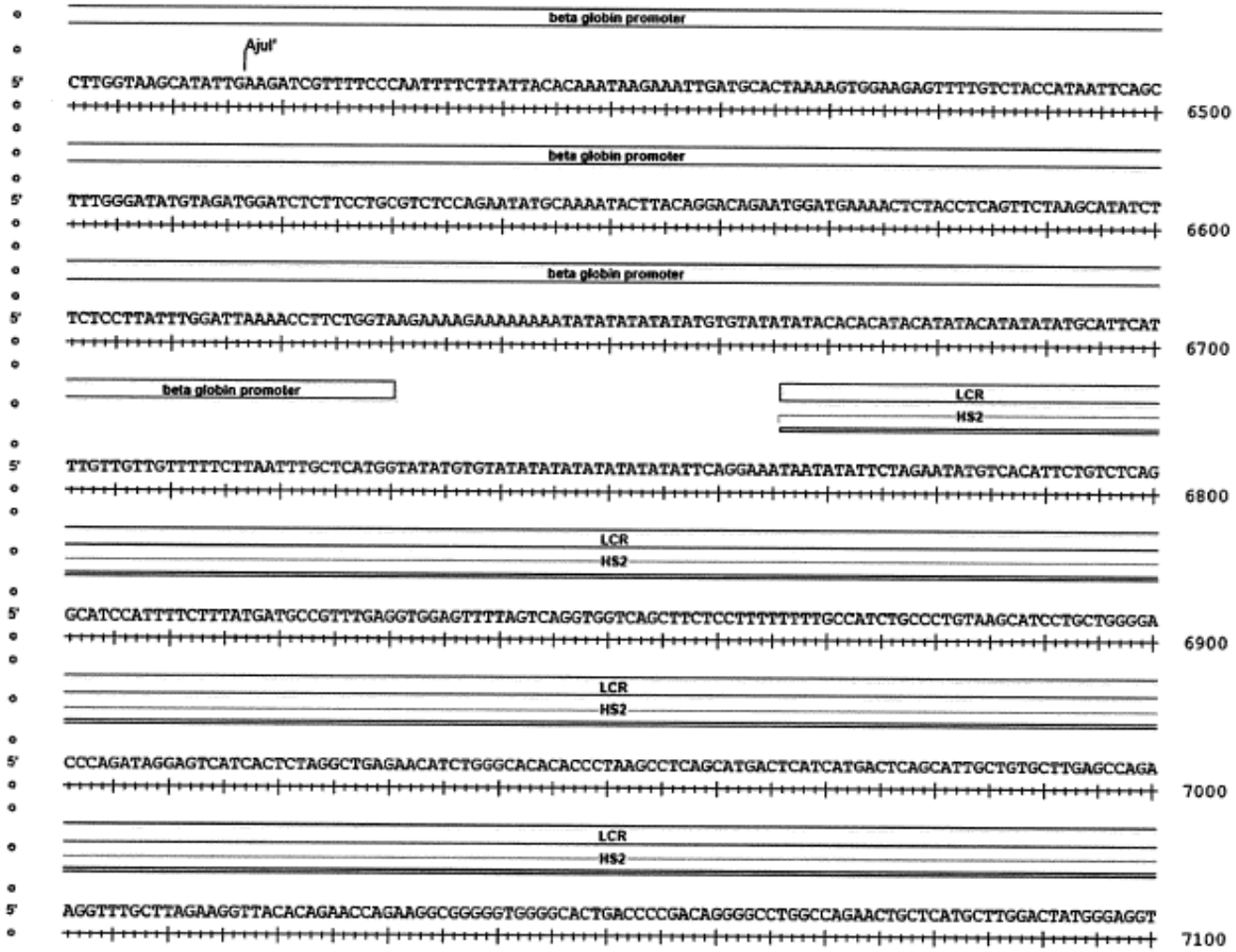

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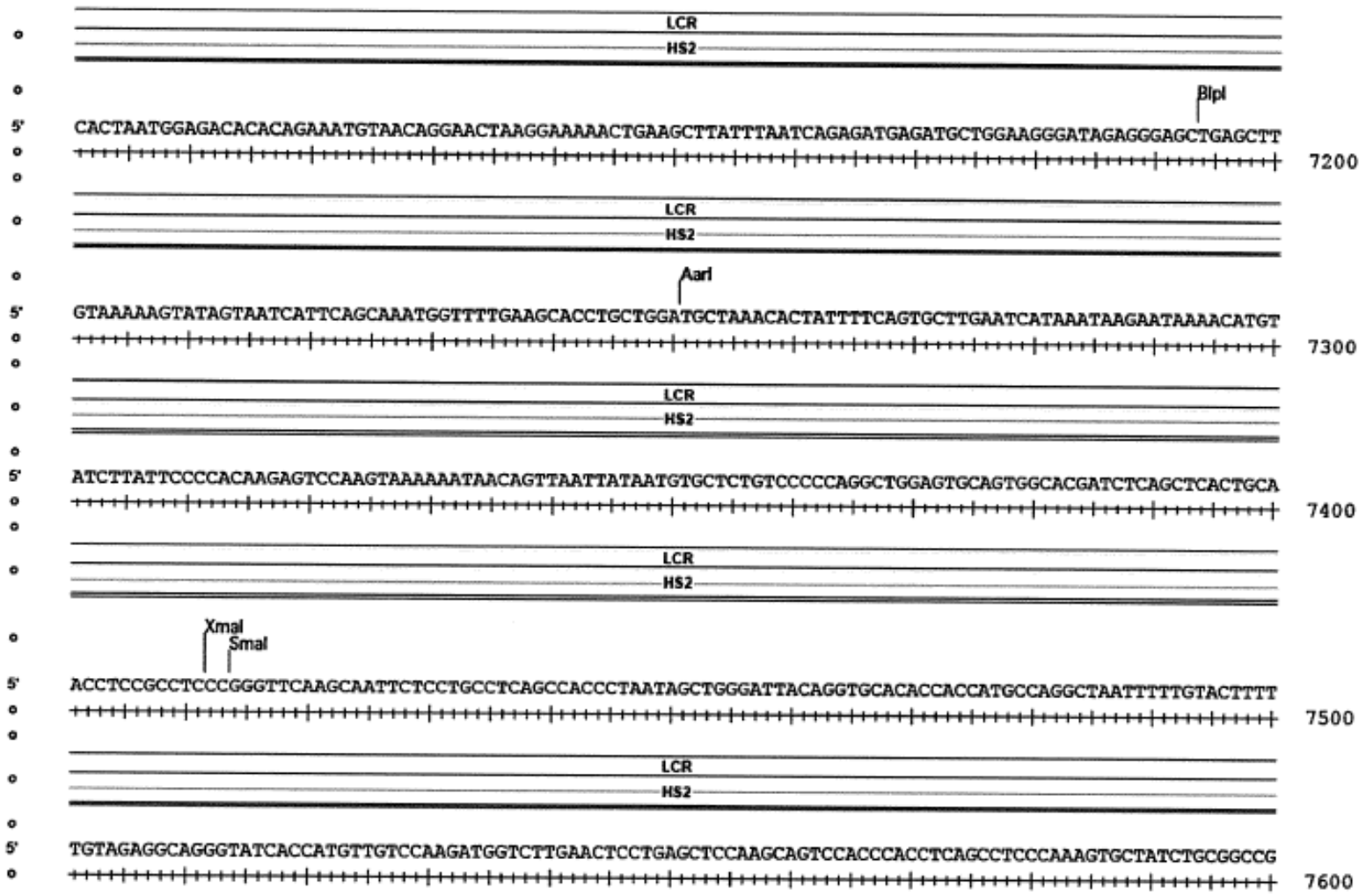


Figure 14, continued

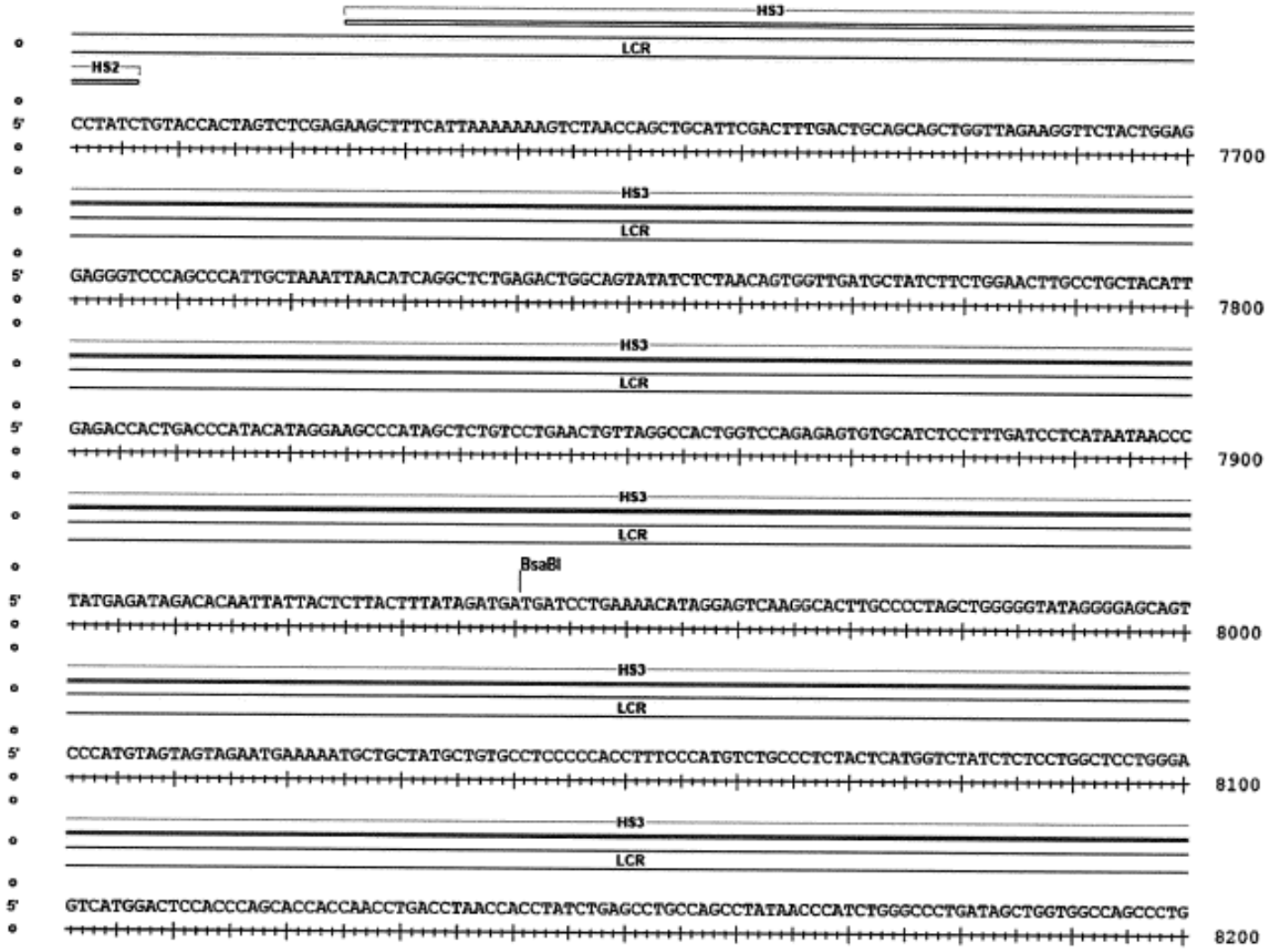


Figure 14, continued

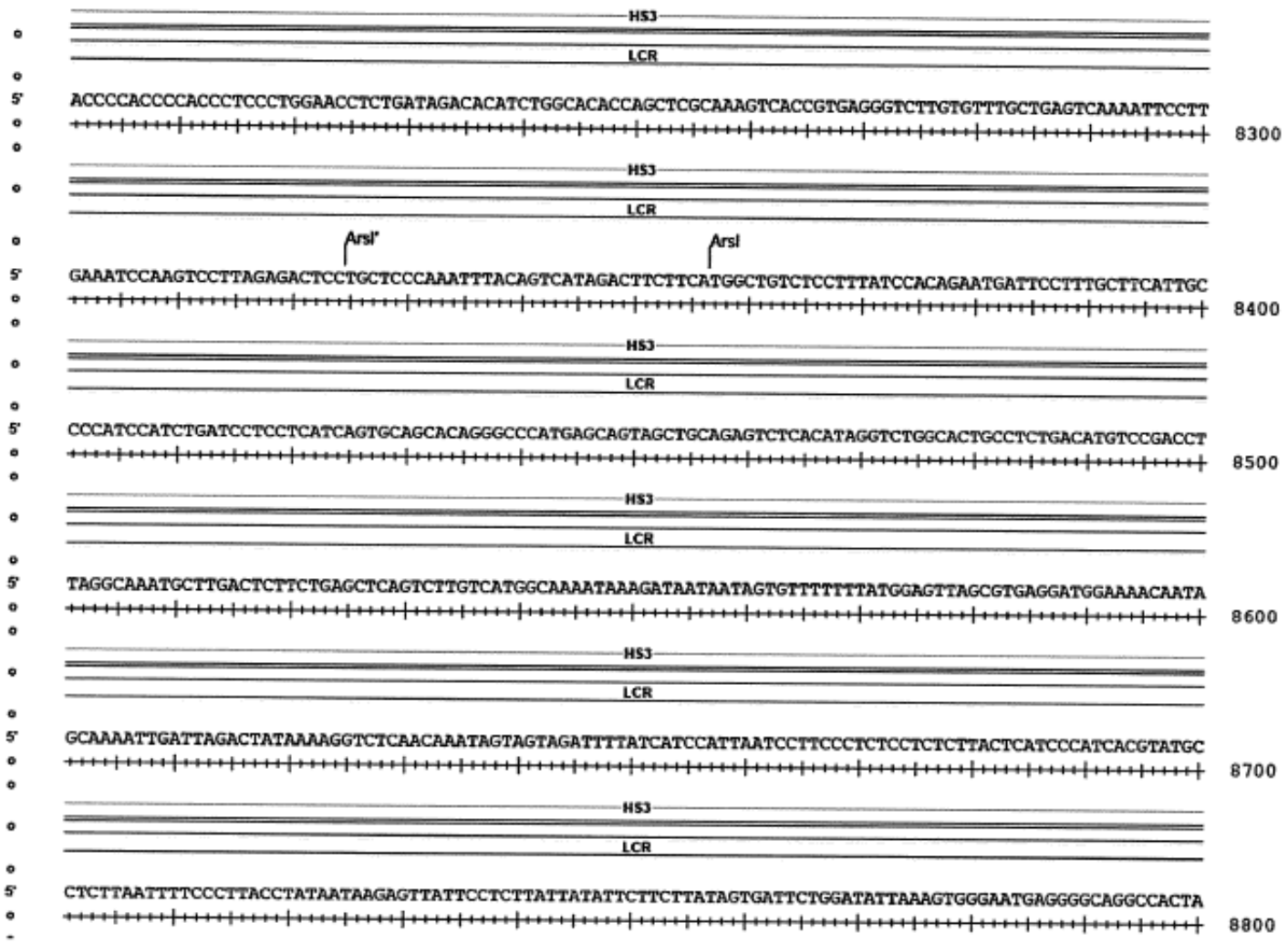
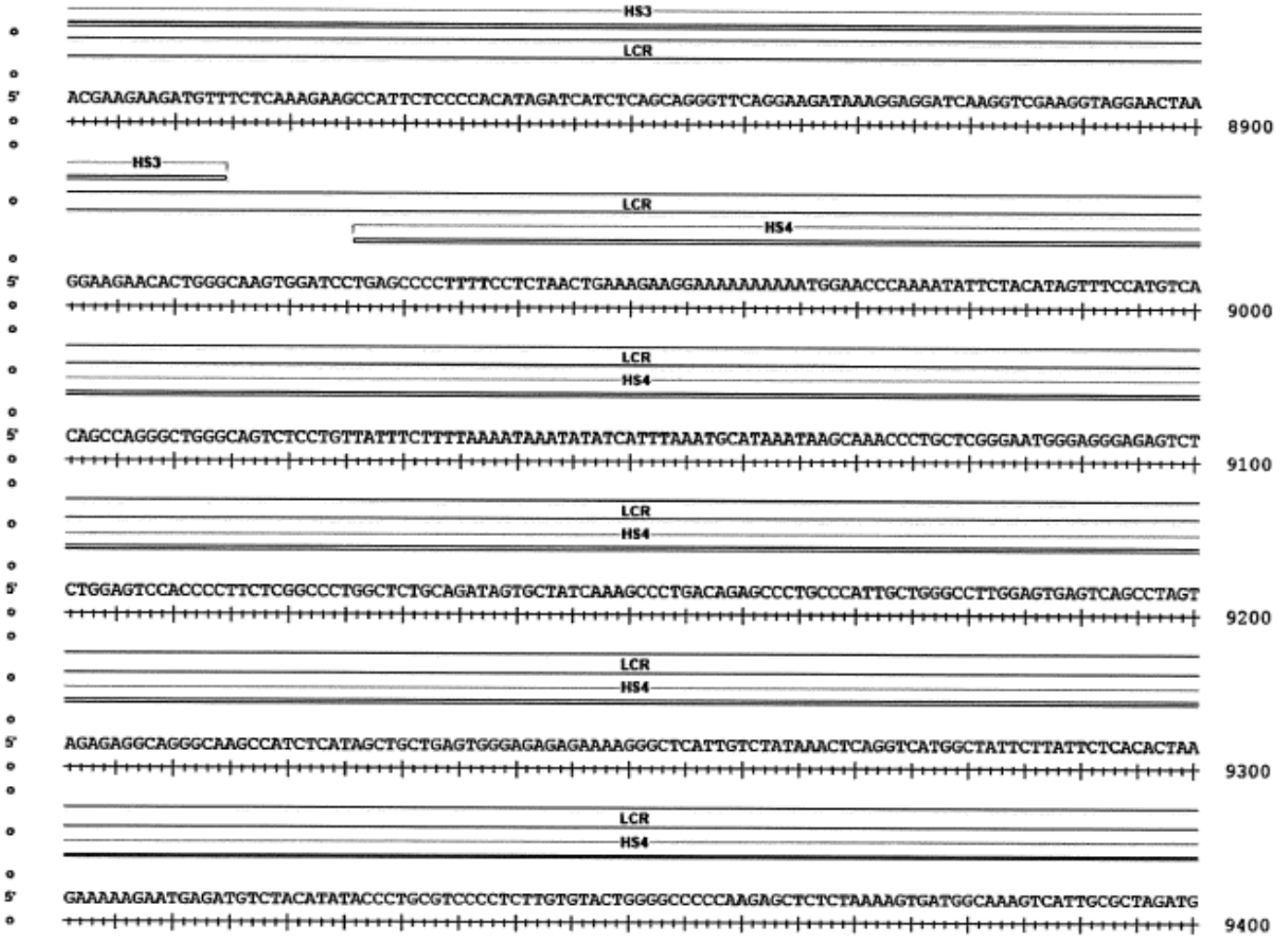


Figure 14, continued



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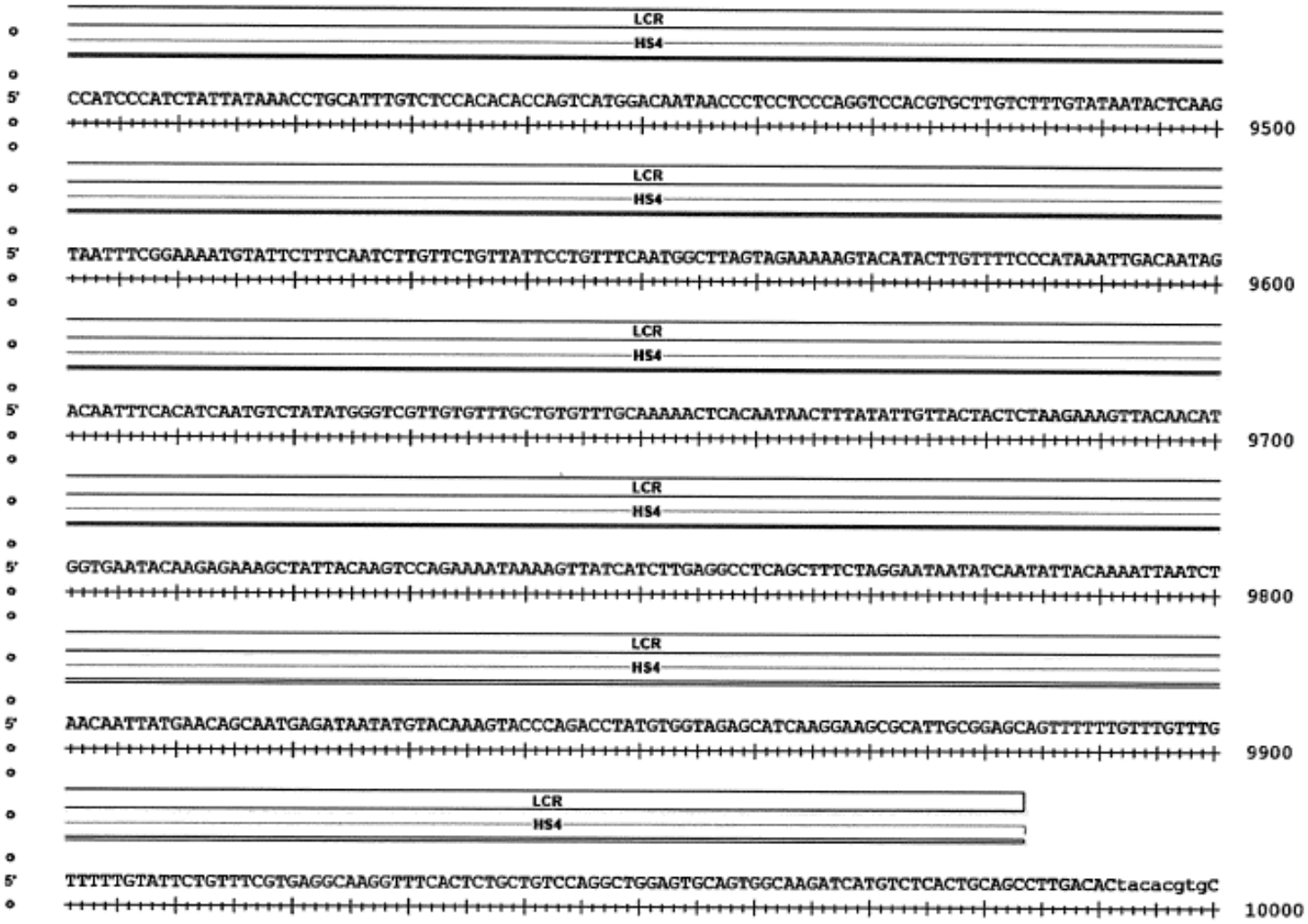

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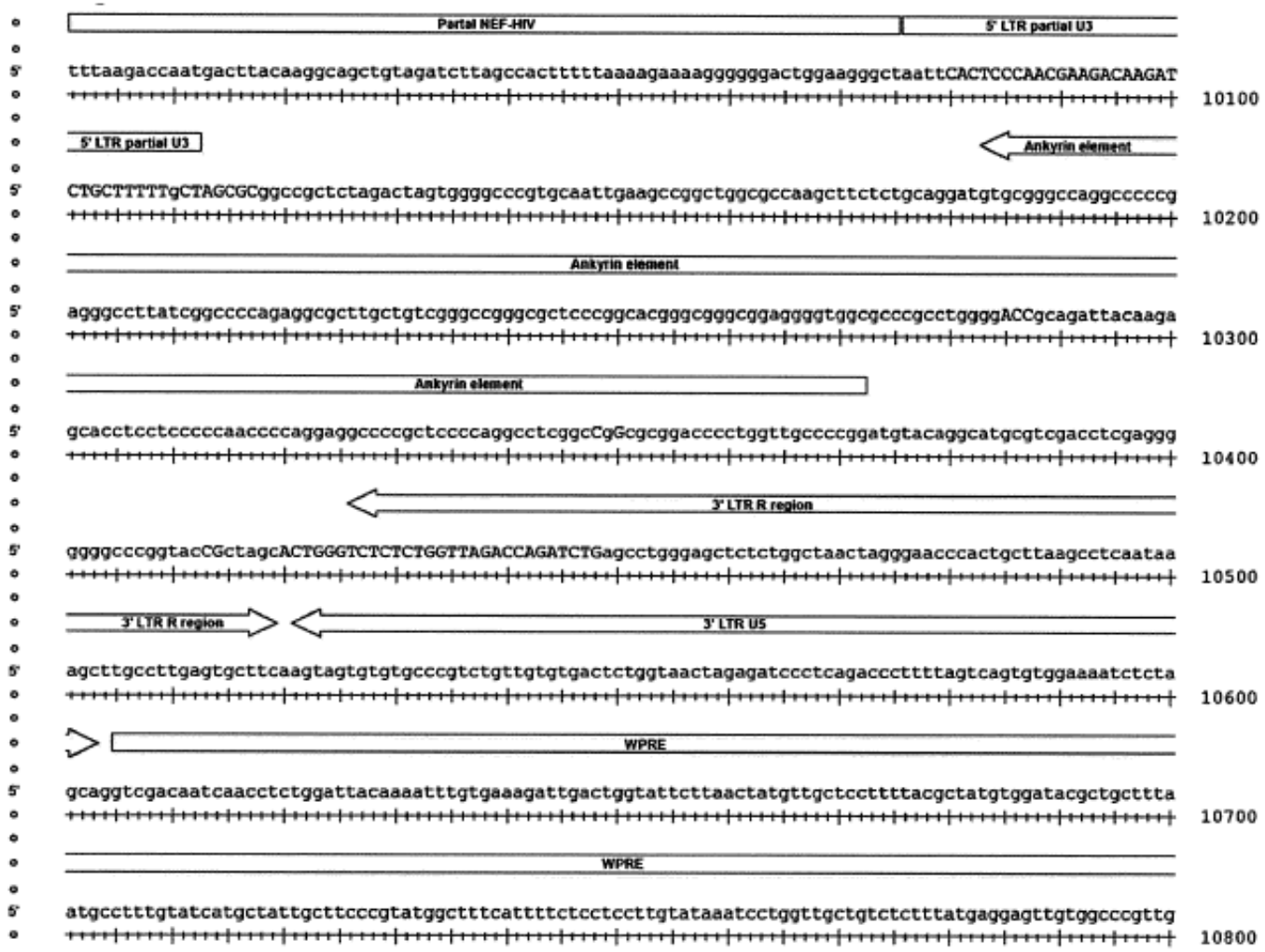

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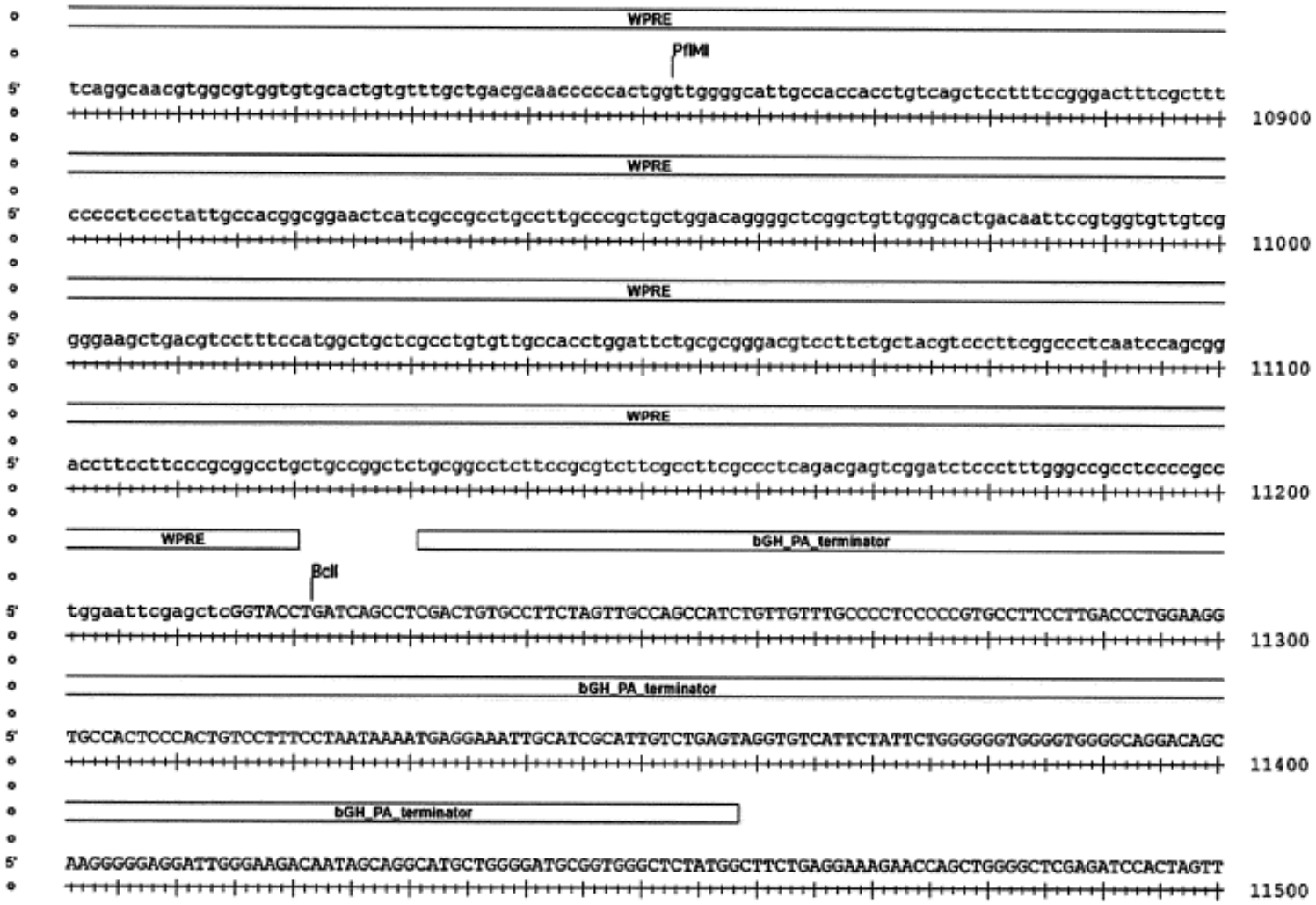

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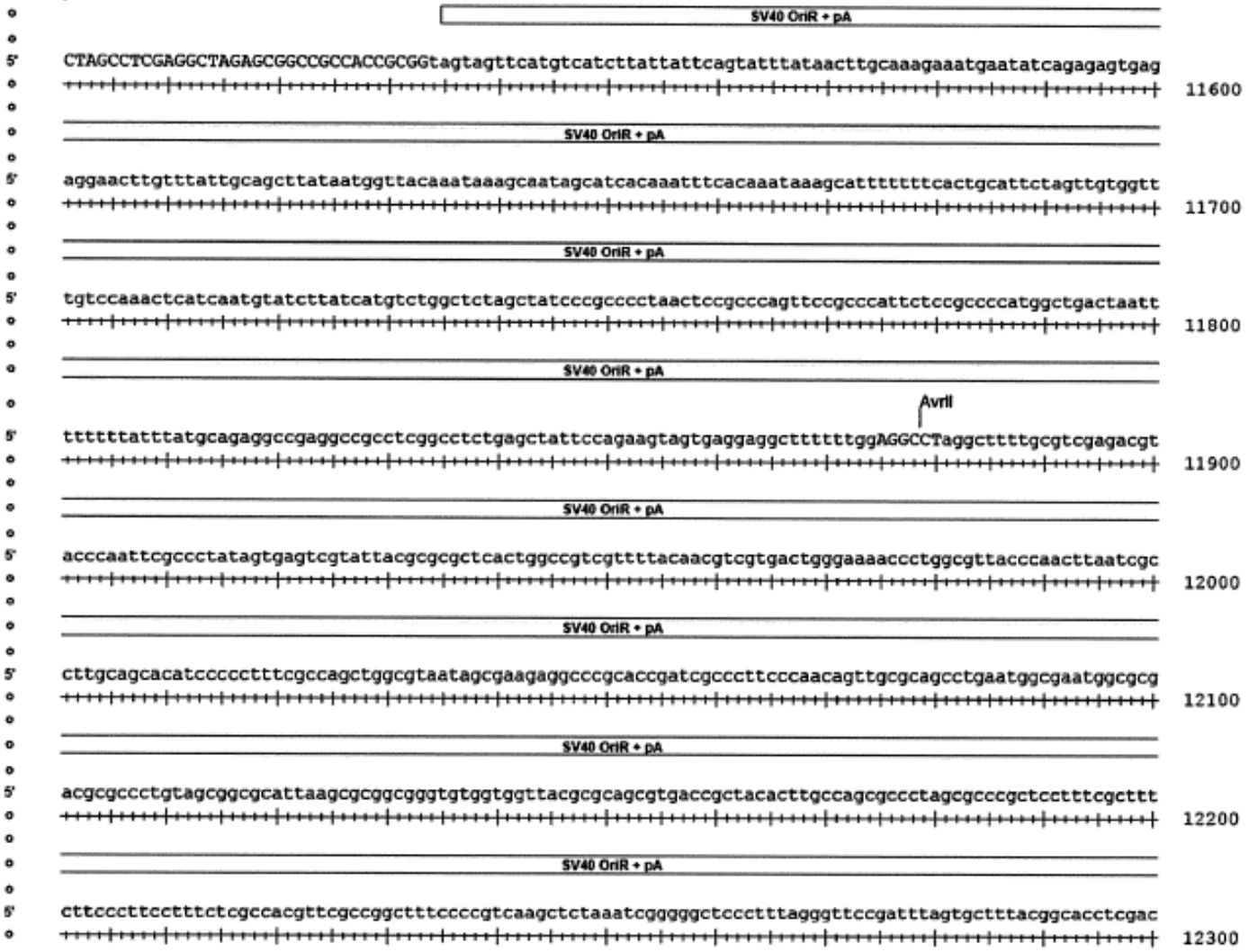


Figure 14, continued

Figure 14, continued

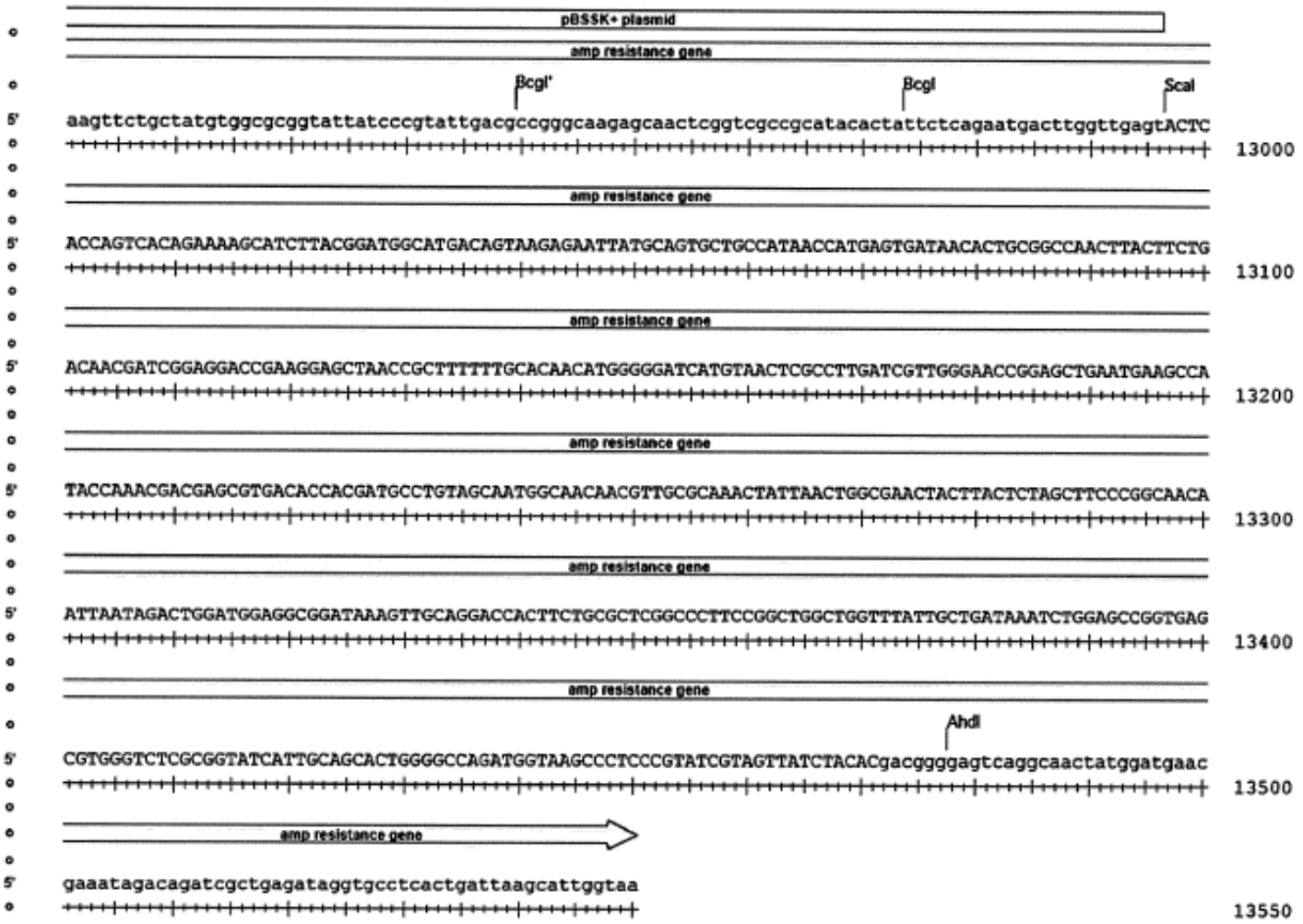
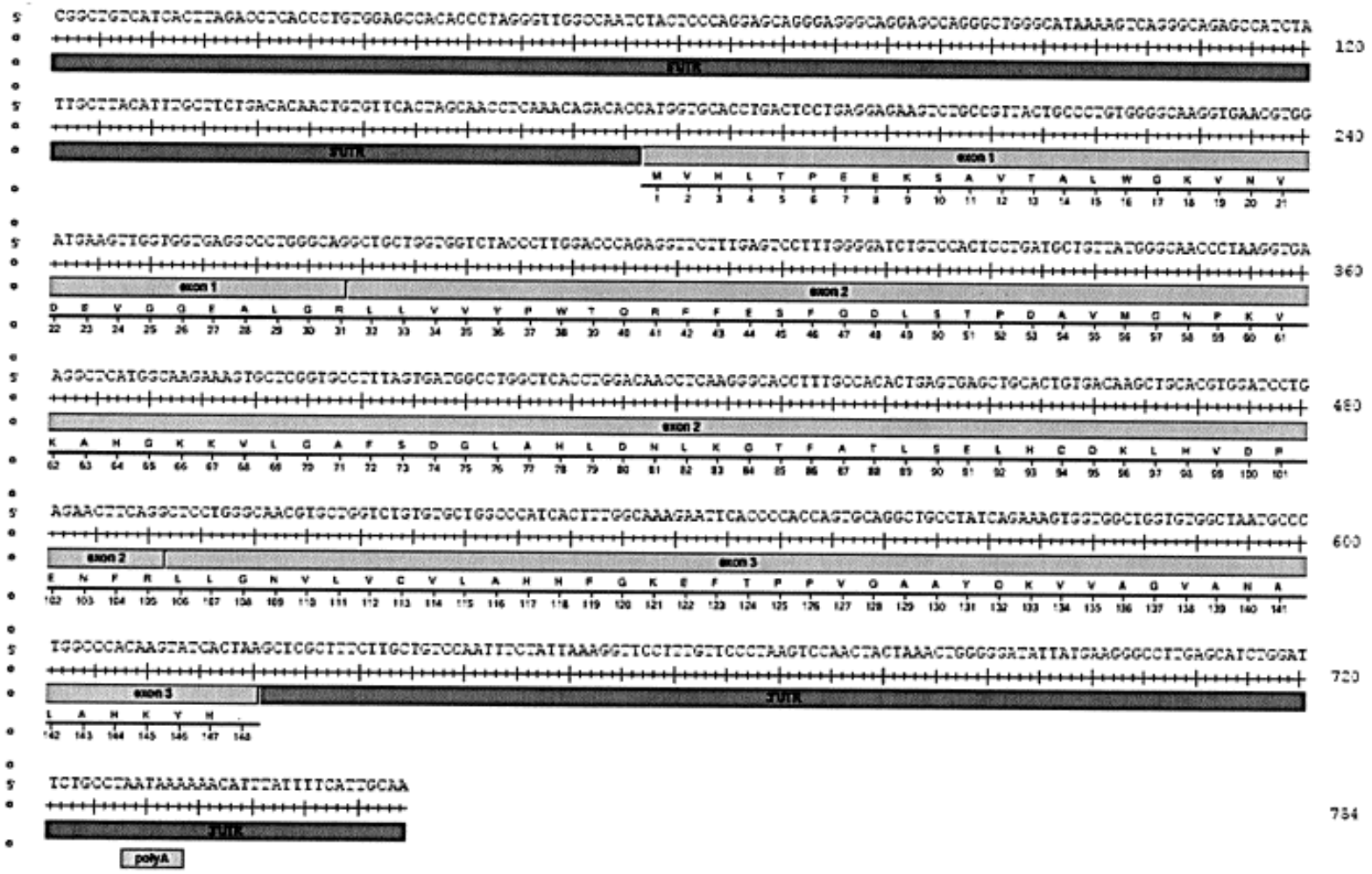


Figure 15


5'
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5'UTR
120
TTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGG
5'UTR
exon 1
M V H L T P E E K S A V T A L W G K V N V
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
240
ATGAAGTTGGTGGTGAGGCCCTGGGCAGGCTGCTGGTGGTCTACCCTTGGACCCAGAGGTTCTTTGAGTCCTTTGGGGATCTGTCCACTCCTGATGCTGTTATGGGCAACCCTAAGGTGA
exon 1
exon 2
D E V D G Q F A L G R L L V V Y P W T Q R F F E S F G D L S T P D A V M G N P K V
22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61
360
AAGGCTCATGGCAAGAAAGTGCTCGGTGCCTTTAGTGATGGCCTGGCTCACCTGGACAACCTCAAGGGCACCTTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTGGATCCTG
exon 2
K A H G K K V L G A F S D G L A H L D N L K G T F A T L S E L H C D K L H V D P
62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101
480
AGAACTTCAGGCTCCTGGGCAACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGTGGCTGGTGTGGCTAATGCCC
exon 2
exon 3
E N F R L L G N V L V C V L A H H F G K E F T P P V Q A A Y Q K V V A G V A N A
102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141
600
TGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTAAAGGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGAT
exon 3
3'UTR
L A H K Y H
142 143 144 145 146 147 148
720
TCTGCCTAATAAAAAACATTTATTTTCATTGCAA
3'UTR
polyA
754

DETAILED DESCRIPTION

The present disclosure provides compositions and methods for prophylaxis and/or therapy for hemoglobinopathies. In this regard, and as is well known in the art, a few months after birth children start producing red cells containing adult hemoglobin, which is the oxygen carrier molecule made of alpha- and beta-globin chains, encoded, respectively, by the alpha- and beta-globin genes. In many hemoglobinopathies, mutations in the gene encoding the beta-globin chain impair the synthesis of adult hemoglobin or lead to the production of abnormal adult hemoglobin. This leads to limited production of red cells or synthesis of abnormal red cells. For these reasons, patients require blood transfusion for survival. Before birth and during the first months of life, children express fetal hemoglobin, which comprises gamma- and alpha-chains. Generally, after birth the gene encoding the gamma-chain is silenced, while the beta-globin gene is activated, switching the production of hemoglobin from fetal to adult. In rare cases, individuals that do not silence the gamma-globin gene and also carry mutations in the beta-globin gene are spared from the disease associated with reduced or abnormal production of adult hemoglobin. Therefore, reactivation of fetal hemoglobin might be therapeutic in ameliorating diseases associated with mutations in the beta-globin gene. Activation and silencing of the gamma-globin gene depends on the proximity of a genomic region called locus control region (LCR), which maps ~40 to 60 kilobases upstream of the gamma- and beta-globin genes and is associated with many factors that activate transcription. This region is required to “loop” and bind the promoter of the gene that needs to be activated. When the gamma-globin gene is expressed, the LCR loops and binds on the gamma-globin promoter. After birth, the LCR moves away from the gamma-globin promoter and loops and binds the beta-globin promoter leading to silencing of the gamma gene and activation of the globin-gene. The transcription co-factor Ldb1 is involved in the LCR looping to the promoters of the gamma- and beta-globin genes. Ldb1 alone does not promote the binding of the LCR to the promoter, but requires additional factors. Artificial zinc-finger (ZF) proteins have the ability of binding specific sequences on the DNA. Ldb1 has been fused to a specific ZF protein that binds the gamma-globin promoter. In transgenic mice, this protein promotes the looping and binding of the LCR to the promoter of the gamma globin gene, activating its expression (Deng, W., et al. Controlling long-range genomic interactions at a native locus by targeted tethering of a looping factor. Cell 149, 1233-1244 (2012)).

An alternative way to approach therapy of hemoglobinopathies associated with mutation in the beta-globin gene is by inserting in the bone marrow cells (and in particular in the hematopoietic stem cells or HSCs) a functional beta-globin gene. Therefore, gene transfer of the non-mutated form of the beta-globin gene by lentiviral vectors could potentially restore production of the human beta-globin protein in patients and be utilized for gene therapy trials. Lentiviral vectors are well characterized for their ability to infect and insert the human beta-globin gene into HSCs. In this regard, the present disclosure provides novel viral vectors that are designed for prophylaxis and/or therapy of hemoglobinopathies. The viral vectors include but are not necessarily limited to lentiviral vectors. In

various embodiments an isolated mammalian hematopoietic progenitor cell or an isolated mammalian stem cell comprising a recombinant lentiviral vector is provided.

Various embodiments of lentiviral vectors that are subjects of this disclosure are shown in Figure 11. Features of these vectors include but are not necessarily limited to the capability to, when introduced to an appropriate cell: 1) reactivate expression of fetal hemoglobin, and/or 2) express a novel transgene adult hemoglobin and/or, concurrently, 3) inactivate the expression of mutant hemoglobin. Certain features of the vectors are known in the art, and Figures 13 and 14 provide representative sequences of them. In particular, any suitable IRES sequence can be used, and those skilled in the art will recognize, given the benefit of this disclosure, which promoter sequences can be adapted for use in embodiments of the invention. Likewise, the LCR sequence is known in the art, as are suitable polyadenylation signals that can function, for example, in erythrocytes. The representative sequences of this disclosure, such as those shown in Figures 13 and 14, can be altered according to well-known parameters, so long as they impart to erythrocytes the ability to produced therapeutically effective amounts of elevated globin. In certain cases polynucleotide sequences can be identical to those presented herein, or they can have least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identity across a contiguous segment of the sequences.

It is expected that lentiviral vectors of this disclosure will result in the expression of therapeutic levels of hemoglobins where previous vectors have failed to do so. In embodiments, these vectors can be specifically adapted to optimize production of beta-globin plus gamma-globin mRNA, and protein levels in presence of different mutations associated with beta-thalassemia, sickle cell anemia and other hemoglobinopathies. Certain aspects of this disclosure involve combinations of genomic elements, specific embodiments of which are depicted schematically in Figure 11. The disclosure includes each and every polynucleotide sequence disclosed herein, the RNA equivalent of every DNA polynucleotide (i.e., where uracil replaces thymine) and every DNA equivalent of every RNA, and the complementary sequence and the reverse complement of every polynucleotide sequence. The disclosure includes every amino acid sequence, and all polynucleotide sequences encoding the amino acid sequences. Contiguous segments of polynucleotides and polypeptides sequences are also included. In non-limiting examples, sequences of this disclosure can comprise or consist of any one or any combination of the contiguous segments of each vector construct disclosed herein, including but not limited to the segments and sequences depicted in Figures 11, 13, 14 and 15, the RNA equivalents thereof, and the DNA and RNA reverse complements thereof. In aspects of the disclosure integration of a lentiviral vector of the disclosure results in an integrated functional globin gene, the expression of which produces a globin molecule that is effective to a participate in providing a therapeutic benefit to an individual with a globinopathy. The gene may exhibit conditional expression, such as being expressed in erythrocytes, but not being expressed in for example, hematopoietic stem cells comprising an integrated

DNA segment derived from the lentiviral construct, wherein the stem cells differentiate into the erythrocytes that express the globin molecule. The disclosure also includes cells, which comprise the recombinant polynucleotides. The present disclosure includes a demonstration of using the ALS10 lentiviral vector depicted in Figure 11 to elevate HbA production in cells from hemoglobinopathy patients. In particular, the disclosure includes a demonstration that the ALS10 vector is superior to previously available approaches for improving HbA production in the most severe thalassemic specimens, i.e., those individuals with β0/0 phenotype. In this regard, the disclosure provides a demonstration of using ALS10 to modify CD34+ cells from β0/0 phenotype patients such that HbA production is improved relative to a suitable control, and those skilled in the art will recognize suitable controls given the benefit of this disclosure. In embodiments, the control can be a single value or a range of values. For example, a control can be a standardized curve or an area on a graph. In one embodiment the control comprises the increase in HbA produced using a construct described in Breda, L., et al., *Therapeutic hemoglobin levels after gene transfer in beta-thalassemia mice and in hematopoietic cells of beta-thalassemia and sickle cells disease patients.* PLoS One, 2012. 7(3): p. e32345, which is known in the art as "AnkT9W."

In more detail, and without intending to be constrained by any particular theory, the approach of the current disclosure was taken in part in an effort to increase adult hemoglobin in thalassemic and SCD cells in a tissue specific manner, and proportionally to the number of viral molecules inserted. To attempt to reduce genome toxicity believed to be caused by random integration of viral vectors, we strived to maintain viral integration within an average of 2 copies/cell.

It has been demonstrated in thalassemic specimens with moderate to medium range of HbA reduction (β+/+ and β+/0, respectively) 0.6 copies of AnkT9W were sufficient to generate HbA levels comparable to those detected in healthy or carrier cells, which is about 80-90% (Breda et al, Plos One, 2012). However, in specimens with the most severe phenotype, β0/0, in which no adult HbA is observed, 0.6 copies of AnkT9W could not meet the level of adult hemoglobin observed in healthy or carrier cells, which was a therapeutic threshold that we strived to achieve in the presently provided approach. In connection with this we modified AnkT9W to generate ALS10. In ALS10, the Woodchuck Post-Regulatory Element (WPRE) was eliminated from the integrating sequence to increase the safety of the vector. The WPRE was part of the integrated portion of AnkT9W since it was placed between the LCR and the 3' LTR. The original purpose of the WPRE was to increase the titer of the lentivirus as it had been previously shown to have that effect, but we have now determined that it is dispensable in the current position. WPRE is a viral sequence and is not required for the expression of the beta-globin gene. It is considered safer to limit as much as possible the amount of viral sequences that are integrated in the genome of patient cells. For this reason, we moved this sequence in the vector to the non-integrating region. This preserves the WPRE's ability to produce high titers of viral particles but excludes the WPRE from the genome of patient cells. In order to preserve the ability of WPRE to increase viral titers without having it in the

integrating sequence, we removed the WPRE from the integrating portion (before the 3'LTR) and added it back after the 3'LTR so that it is positioned outside the sequence that is integrated in the chromosome(s) of target cells. We also added a strong bovine growth hormone polyA tail after the WPRE region (outlined in Figure 11). Our data indicate that the modifications do not decrease viral titer during production of compositions of this disclosure. However, we also modified the segment of the construct that results in expression of HbA in erythrocytes that are derived from CD34+ cells into which the lentiviral vector is introduced. In particular, the portion of the beta-globin gene intron 2 that is deleted in the AnkT9W construct is annotated in Figure 14. As can be seen from that annotation, the beta-globin gene intron 2 encompasses nucleotides 4772 – 5621, inclusive, and is thus 851 nucleotides in length. However, the intron 2 in AnkT9W comprises a deletion of the 375 nucleotides spanning nucleotides 5,164 through 5,537, inclusive (as designated in Figure 14). As a consequence of that deletion, the AnkT9W beta-globin gene intron 2 is 476 nucleotides in length. In contrast, and while not intending to be bound by any particular theory, it is considered that including a longer segment of intron 2 in the context of the ALS10 construct is at least partially responsible for the unexpected and statistically significant increase in HbA in the β0/0 patient cells. In this regard, when compared to AnkT9W, ALS10 showed significant improvement and in particular leads to much higher level of HbA in the most severe thalassemic patient specimens, namely, the β0/0 phenotype. It is therefore reasonable expect that the present approach will benefit patients with hemoglobinopathies that are not necessarily due to a β0/0 phenotype, such as SCD. A summary of the results demonstrating this advantage of ALS10 is presented in Figure 12. The disclosure thus includes lentiviral vectors and cells comprising them, and the integrated construct, wherein the beta-globin gene comprises an intron 2 of more than 476 nucleotides in length, and up to 851 nucleotides in length. The intron is accordingly between 477-875 nucleotides, inclusive, and including all integers and ranges of integers there between. The intron 2 can be thus comprise or consist of 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, 633, 634, 635, 636, 637, 638, 639, 640, 641, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716, 717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728, 729, 730,

731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 827, 828, 829, 830, 831, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 848, 849, 850, 851 nucleotides. The polynucleotide sequence of the human beta globin gene is depicted in Figure 13. It includes the 3' enhancer element, the 3' untranslated region (UTR), the polyA signal, exon 3, intron 2, exon 2, intron 1, exon 1, the 5' UTR, and the beta globin promoter segment. It is shown in the 3'-5' orientation because, as is well known in the art, it is anti-parallel to the depicted remainder of the construct and is expressed only after integration and in the context of a double stranded DNA region wherein one strand of the DNA comprises the beta globin gene sequence presented in Figure 14.

In ALS10 the sequence of the beta-globin gene is also modified to increase its ability to prevent the sickling of the sickle-beta-globin chains (referred to as "B-globinM" in Figure 11). This mutant beta-globin is known as the βT87Q form, due to its amino acid substitution at the 87th position. βT87Q has been used to improve hematological parameters of the SAD and BERK mouse models of SCA (Pawliuk, R.*, et al.* Correction of sickle cell disease in transgenic mouse models by gene therapy. *Science* 294, 2368-2371. (2001)). This form was also used in the first successful clinical trial to correct a patient with β0/βE thalassemia (Cavazzana-Calvo, M.*, et al.* Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature* 467, 318-322 (2010)).

The ankyrin insulator that is well known in the art and was present in AnkT9W is also present in ALS10. ALS10 has the ankyrin insulator in the 3'LTR, while the vector AnkT9W has the ankyrin insulator between the promoter and the LCR.

In one aspect of this disclosure, an intron of the beta-globin gene, or other locations such as the 3'UTR, can be modified to include a polynucleotide that targets and decreases the synthesis of the transferrin receptor 1 (TR1). The rational of this approach is based on our observations that decreased erythroid iron intake can be beneficial in beta-thalassemia and, potentially other hemoglobinopathies, because this decreases formation of heme molecules. Heme is normally included in hemoglobin molecules but in many hemoglobinopathies, due to the reduction in synthesis of beta-globin chains, there is an excess of heme not bound to hemoglobin, and these molecules are toxic to red cells. An excess of heme is responsible for apoptosis of erythroblasts and for altering the intracellular content, production of reactive oxygen species (ROS), and for reducing membrane stability and plasticity of erythrocytes, leading to their reduced lifespan, hemolysis and/or sickling. A combination vector that expressed gamma-globin and concurrently knocked-down sickle beta-globin via small hairpin RNA has been described (Samakoglu, S.*, et al.* A genetic strategy

to treat sickle cell anemia by coregulating globin transgene expression and RNA interference. *Nature biotechnology* 24, 89-94 (2006)) and this approach can be incorporated in embodiments of the present disclosure (i.e., Figure 11, in ALS-10T, signified by "I1S"). With respect to polynucleotides that target TR1 and that can be included in vectors of this disclosure, shRNA is one non-limiting example of an RNAi-mediated approach. But RNAi-based inhibition can be achieved using any suitable RNA polynucleotide that is targeted to TR1 mRNA. With respect to shRNAs, they are known in the art to adopt a typical hairpin secondary structure that contains a paired sense and antisense portion, and a short loop sequence between the paired sense and antisense portions. shRNA is delivered to the cytoplasm where it is processed by DICER into siRNAs. siRNA is recognized by RNA-induced silencing complex (RISC), and once incorporated into RISC, siRNAs facilitate cleavage and degradation of targeted mRNA. In embodiments, an shRNA polynucleotide segment or DNA segment encoding it included for use in suppressing TR1 expression can comprise or consist of between 45-100 nucleotides, inclusive, and including all integers between 45 and 100. The portion of the shRNA that has reverse complementarity to the TR1 mRNA mRNA can be from 21-29 nucleotides, inclusive, and including all integers between 21 and 29. In another approach, a ribozyme that can specifically cleave TR1 mRNA can be included. In another embodiment, microRNA (□RNA) targeted to the TR1 mRNA can be used.

The present disclosure provides an illustration that vector encoding a fusion of an Ldb1 transcription factor and a zinc finger (ZF) domain (ZF-Ldb1) is useful for approaching certain hemoglobinopathies. A ZF-Lbd1 gene can be incorporated into vectors of this disclosure in several configurations. In one non-limiting approach using ALS-10 in Figure 11 as a representative example, the ZF-Ldb1 gene can be positioned such that the vector comprises consecutively (with the same stranded-ness as the beta-globin coding sequence) the 5'LTR, a polyadenylation signal, the ZF-Ldb1 region and a either a promoter that can drive expression of a separate mRNA encoding the ZF-Ldb1 protein, or an IRES so that the mRNA encoding the ZF-Ldb1 protein can be made as a distinct protein, but from the same mRNA that encodes the beta-globin protein. The rational for including ZF-Ldb1 into ALS is twofold: 1) the ZF-Ldb1 will move the LCR from the promoter of the mutant beta-globin gene to that of the gamma-globin gene. In this way the production of mutant RNA will be reduced or shut down, while expression of the functional gamma-globin gene will be activated: 2) The expression of transgenic beta-globin gene (considered to be a potentially curative gene carried by the lentiviral vector) will not be affected in the presence of ZF-Ldb1. Without intending to be constrained by theory this is expected to lead to an additive or synergistic effect by the production of both hemoglobins: fetal hemoglobin (HbF, α2γ2, from the endogenous locus), and adult hemoglobin (HbA or α2β2, from the therapeutic vector). As hemoglobinopathies in humans are characterized by more than 300 mutations, it is reasonable to predict that the many or all hemoglobinopathies could be improved by a vector of this disclosure. In this regard, we demonstrated that a lentiviral vector encoding the ZF-Ldb1 cassette (pCL-ZF-Ldb1) increases synthesis of fetal hemoglobin (HbF, α2γ2) in

CD34-derived erythroid cells from normal individuals and SCA patients. As HbF increased, the synthesis of adult hemoglobin (HbA) or sickle hemoglobin (HbS or α2βs2) diminished, respectively in cells derived from normal individuals or SCA patients. We also investigated the number of vectors integrated per cell, on average. This number is indicated as number of integration per cell or vector copy number (VCN). We observed that the increase in VCN was associated with an increase of HbF both in normal and SCA cells. The number of HbF positive cells measured by flow cytometry also increased proportionally to the VCN. β, γ- and α-globin mRNA levels measured by quantitative PCR also showed that the relative ratio γ/α increased while the β/α or βS/α were reduced in the samples treated with the ZF-Ldb1 vector. Taken together these data suggest that the ZF-Ldb1 can redirect the LCR enhancing effect from the β-globin promoter to the γ-globin promoter. In SCA this effect is particularly beneficial given the toxicity caused by the accumulation of HbS. Examples 1-6 further confirm potential usefulness of including ZF-Ldb1 in ALS10. In particular, these Examples demonstrate infection of hematopoietic stem cells isolated from blood of SCD patients with a lentivirus expressing the ZF-Ldb1 transgene and differentiation of them into mature erythroid cells *in vitro*. HbF synthesis induced by ZF-Ldb1 was compared to that obtained in specimens treated with hydroxyurea and various additional HbF inducers. ZF-Ldb1 increased HbF synthesis and simultaneously reduced sickle Hb (HbS), establishing a balanced synthesis between α- and functional β-like globins. The induction of HbF in cells treated with ZF-Ldb1 was roughly three times higher (+34%), than that observed using decitabine and pomalidomide; tranylcypromine had an intermediate effect, while butyrate and hydroxyurea showed marginal HbF induction. Notably, erythroid cell differentiation and viability remained unaltered in ZF-Ldb1 expressing cells. Thus, lentiviral-mediated ZF-Ldb1 gene transfer appears superior to existing drug regimens for affecting SCD erythroid cells and it is reasonable to expect that combining ZF- to an ALS10 vector will provide an effective approach to prophylaxis and/or therapy of a variety of hemoglobinopathies. The Examples provide also provide a demonstration of the effectiveness of ALS10 in β0/0 patient cells.

Compositions comprising recombinant lentiviral vectors are provided. In certain approaches pharmaceutical compositions are provided and can be prepared by mixing, for example, virions comprising a lentiviral vector of this disclosure and any suitable pharmaceutically acceptable carriers, excipients and/or stabilizers. Some examples of compositions suitable for mixing with IL-8 can be found in: Remington: The Science and Practice of Pharmacy (2005) 21st Edition, Philadelphia, PA. Lippincott Williams & Wilkins. In certain approaches.

The disclosure includes methods of making a virion preparations for use in prophylaxis and/or therapy of hemoglobinopathies. In one embodiment this method comprises introducing a plasmid encoding a lentiviral vector of this disclosure into packaging cells. The packaging cells comprise a DNA packaging plasmid, which encodes at least one virion protein, and a DNA envelope plasmid, which encodes a suitable viral envelope protein. The packaging and envelope plasmids express the respective proteins, which facilitate formation of virions

which comprise a recombinant RNA lentiviral vector of this disclosure. Suitable packaging systems that can be adapted to produce virions of this disclosure are commercially available, such as from Addgene (Australia).

The following Examples are intended to illustrate various aspects of the present disclosure but are not meant to be limiting in any way.

Example 1

This Example demonstrates that lentivirally expressed ZF-Ldb1 raises HbF levels in CD34+-derived sickle erythroblasts. To obtain the data presented here, we used the lentiviral vector pCL-ZF-Ldb1, (Fig. 11, top vector map), which carries a zinc-finger protein that specifically binds the γ-globin promoters, fused to the self-association (SA) domain of Ldb1, as well as a green fluorescent protein under the control of the erythroid specific ankyrin promoter. The effects of ZF-Ldb1 expression on chromatin contacts within the β-globin locus have been shown using 3C experiments in healthy adult erythroblasts. ZF-Ldb1 expression promotes the juxtaposition of the γ-globin promoters with the LCR leading to transcription [Deng, W., et al., Reactivation of developmentally silenced globin genes by forced chromatin looping. Cell, 2014. 158(4): p. 849-60]. This is accompanied by a concomitant reduction in the expression of adult globin genes, compatible with a mechanism in which the fetal and adult promoters compete for LCR enhancer activity. This example describes an attempt to improve the amount of functional hemoglobin in CD34+ cells isolated from sickle cell patients. Sickle CD34+ cells produce predominantly HbS (α2βs2) once differentiated into erythroblasts in vitro. Other hemoglobins, as HbF (α2γ2) and HbA2 (α2δ2) are also produced to a lower degree (Fig. 1A, left). Following infection with pCL-ZF-Ldb1 vector, sickle cells dramatically increased HbF synthesis in a manner proportional to the copy number of viral integrants. In a representative experiment the same SCD derived erythroid cells (shown in Fig. 1A, left) transduced with 0.26 or 0.67 viral molecules on average per cell (VCN or vector copy number) produce 22% or 44% (Fig. 1A, middle and right, respectively) more HbF than the control sample. Concomitantly, with progressively higher viral integration the number of HbF (Fig. 1B) and GFP (Fig. 1C) expressing erythroblasts increased, as determined by flow cytometry measurements. The gain in the fraction of GFP+ cells is somewhat lower, likely the result of reduced translation efficiency that is often observed downstream of the internal ribosomal entry site (IRES) between the ZF-Ldb1 cassette and the GFP gene.

Example 2

This Example demonstrates that transgenic ZF-Ldb1 supports high levels of fetal globin induction and concurrently reduces sickle globin levels in SCD erythroblasts. In particular, we analyzed the mRNA and protein content of erythroblasts derived from 10 SCD subjects. CD34+ cells isolated from peripheral blood mononuclear cells (PBMCs) were frozen and used for independent replicate experiments. Infection with pCL-ZF-Ldb1 was performed on pro-erythroblast within the first 10 days of the expansion phase. At this time cells still

express high levels of CD1117 and CD44 markers and do not express glycophorin A (data not shown) (Breda, L., et al., Therapeutic hemoglobin levels after gene transfer in beta-thalassemia mice and in hematopoietic cells of beta-thalassemia and sickle cells disease patients. PLoS One, 2012. 7(3): p. e32345). To assess the ability of ZF-Ldb1 to reactivate HbF we measured the amount of γ-globin mRNA expressed in cells untreated or after transduction. These values were normalized to GAPDH and to α-globin gene expression whose expression is directly proportional to the erythroid differentiation stage in the cells. Cells with 1.1 copies of pCL-ZF-Ldb1 produced on average a 3-fold increase of the γ/α globin ratio (from 0.2 ± 0.11 to 0.6 ± 0.33), compared to untreated cells and, simultaneously, a reduction of the β/α ratio (from 0.38 ± 0.08 to 0.29 ± 0.16) (Fig. 2A, left and center), confirming the ability of the ZF-Ldb1 construct to partially redirect the LCR from the β- to the γ-globin promoter. These changes were observed only in cells expressing transgenic Ldb1, whose expression was proportional to the level of ZF-Ldb1 integration (Fig. 2A, right).
On average, cells treated with pCL-ZF-Ldb1 produced nearly 40% more HbF (63.10% ± 14.01) compared to untreated cells (27.27% ±16.29) (Fig. 2B, left) and lowered production of HbS (-35.65%) and HbA2 (-5.18%) (Fig. 2B, center and right). Reduction of HbA2 is likely the result of reduced contact frequencies between □-globin and the LCR in the presence of ZF-Ldb1. A summary of net HbA increase and HbS and HbA2 decrease is provided in Fig. 3C.

Example 3

To establish the effect of ZF-Ldb1 treatment on the total amount of cellular Hb we measured absolute Hb content per differentiated cell. Importantly, in spite of significant shifts in the ration of fetal to adult globin, total Hb synthesis changes (HbF+HbS+HbA2) remained essentially unaltered (Fig. 3A, right). To corroborate this evidence is the fact that βS chains are diminished while both γA+γG chains are increased in specimens treated with ZF-Ldb1, analyzed by reversed-phase liquid chromatography (Fig. 3B and S1) which allows for the quantification of single globin chains rather than to tetrameric hemoglobin abundance. The reduced amount of HbS was confirmed by flow cytometry (Fig. 4). ZF-Ldb1-expressing SCD erythroblasts, which can be tracked by GFP expression (Fig. 4A), have a greater fraction of HbF positive cells (Fig. 4B), and within the HbF positive population, a lower fraction of HbS compared to untransduced SCD erythroblasts (Fig 4C). Untreated baseline HbF positive erythroblasts (Fig. 4B, center) were less frequent and contained less than half the HbF/erythroid cell when compared to the ZF-Ldb1 expressing cells (Fig. 4D).

Example 4

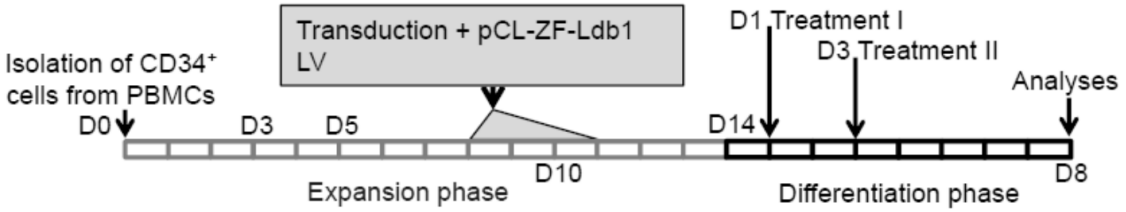
This Example demonstrates that γ-globin gene repressors SOX6 and KLF1 are down regulated in sickle erythroblasts. In particular, BCL11A, SOX6, C-MYb and KLF1 have emerged as salient repressors of γ-globin during erythroid differentiation. We investigated the impact of pCL-ZF-Ldb1 on transcription of these repressors in both wild type and sickle cell disease derived erythroid cells. RT-qPCR analyses indicates that differentiated erythroblasts obtained from SCD

patients present a different pattern of expression for certain negative regulators of γ-globin gene expression, compared to erythroblasts obtained from healthy individuals. BCL11A and C-MYB messenger RNAs present comparable level of expression (Fig. 5A), whereas both KLF1 and SOX6 show significant down regulation in SCD specimens (0.17 ± 0.05 and 4.49 ± 0.76, respectively) compared to healthy ones (0.55 ± 0.43 and 8.29 ± 3.14, respectively) (Fig. 5B). All samples analyzed present similar expression of KEL, an internal control mRNA that increases with level of differentiation. These trends are independent from the level of transgene expression (Fig. 5 and 5C, right). These data could indicate a more permissive chromatin state of the γ-globin gene in SCD cells in culture, as suggested by a trend of higher content of HbF at steady state in these cells compared to healthy ones (Fig. 7).

Example 5

This Example demonstrates that HbF induction in SCD erythroblasts ZF-Ldb1-mediated is greater than induction mediated by pharmacological inducers. A scheme of the experimental procedures is illustrated in Table 1. Briefly, erythroid progenitor SCD cells were infected with pCL-ZF-Ldb1 within days 9-11 in expansion phase, or treated at days 1 and 3 of differentiation phase with pharmacological inducers 5-aza-2'-deoxy-cytidine (0.5μM), tranyl-cypromine (1.5μM), hydroxyurea (150 μM), pomalidomide (30μM), and butyrate (100 μM), These concentrations were determined through evaluation of efficacy (net increase of HbF) versus toxicity (cell death); the original scaling dosage was extrapolated from the recent literature (Fig. 9) [Watanapokasin, Y., et al., *In vivo and in vitro studies of fetal hemoglobin induction by hydroxyurea in beta-thalassemia/hemoglobin E patients.* Exp Hematol, 2005. 33(12): p. 1486-92; Moutouh-de Parseval, L.A., et al., *Pomalidomide and lenalidomide regulate erythropoiesis and fetal hemoglobin production in human CD34+ cells.* J Clin Invest, 2008. 118(1): p. 248-58; Shi, L., et al., *Lysine-specific demethylase 1 is a therapeutic target for fetal hemoglobin induction.* Nat Med, 2013. 19(3): p. 291-4]. Studies were performed on day 8 of differentiation phase, at the orthochromatophilic stage when high amounts of hemoglobin were accumulated. As measured by HPLC, pCL-ZF-Ldb1 had the most robust increase of HbF and decrease in HbS. Specifically, on average the net HbF increase in SCD erythroblast treated with the lentivirus was 34.2% ± 12.53, versus 15.19% ± 12.77 with 5-aza-cytidine ($p<0.01$), 8.08% ± 6.28 with tranyl-cypromine ($p<0.001$), 2.39% ± 2.13 with hydroxyurea ($p<0.001$), 11.84% ± 9.02 with pomalidomide ($p<0.01$), and 4.84% ± 5.03 with butyrate ($p<0.001$). ZF-Ldb1 expressing cells presented HbF significantly higher than untreated cells ($p<0.001$) (Fig. 6A, top). Conversely, on average the net HbS decrease in SCD erythroblast treated with the lentivirus was -31.36% ± 11.61, versus -17.30% ± 15.04 with 5-aza-cytidine, -6.93% ± 6.64 with tranyl-cypromine, -3.48% ± 4.68 with hydroxyurea, 11.61-% ± 9.27 with pomalidomide, and -4.90% ± 7.94 with butyrate (Fig. 6A, bottom).

Table 1. (Top) Experimental flow for expansion, differentiation and treatment of human SCD CD-34+ cells with pCL-ZF-Ldb1 and/or HbF pharmacological inducers. (Bottom) Type of HbF inducer, biological activity and dose used in the study.

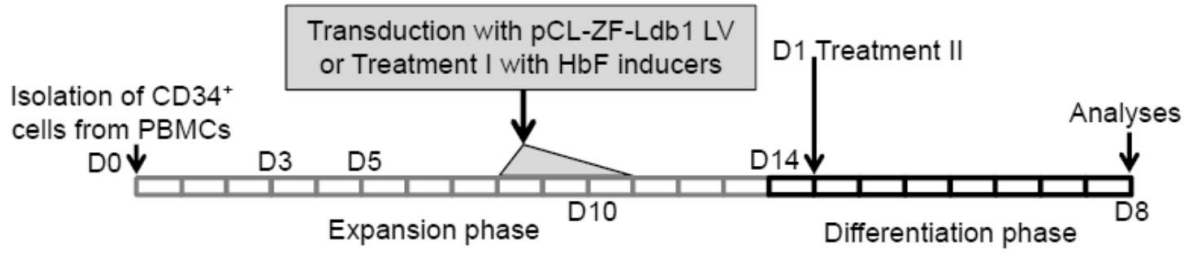


HbF inducers' and doses

Drug	5-Azacytidine (5-Aza)	Tranyl-cypromine (TCP)	Hydroxyurea (HU)	Pomalidomide (Pom)	Butyrate (But)
[C]	0.5 μM	1.5 μM	150 μM	30 μM	100 μM

Along with recovery of greater levels of functional Hb, cells expressing ZF-Ldb1 did not show significant changes in viability compared to untreated samples, whereas cells treated with pomalidomide, butyrate and hydroxyurea showed reduced viability (Fig. 6B). These differences were confirmed in a subset of samples treated with pharmacological inducers at earlier time points (Table 2) to exclude biases due to a delay to drug response (Fig. 10). Taken together, pCL-ZF-Ldb1 was superior to all tested compounds in augmenting HbF and F-Cell levels and importantly was associated with minimal toxicity.

Table 2. (Top) Experimental flow for expansion, differentiation and treatment of human SCD CD-34+ cells with pCL-ZF-Ldb1 or HbF pharmacological inducers. (Bottom) Type of HbF inducer, biological activity and dose used in the study.



It will be apparent from the foregoing that a lentiviral vector carrying the SA Ldb1 domain linked to a ZF protein, which selectively binds the γ-globin promoters, significantly increased HbF synthesis and exceeds previously described pharmacological inducers. It is therefore reasonable that adding an SA Ldb1 domain linked to a ZF protein to an ALS10 vector of this disclosure will likely enhance beneficial properties of the combined vectors.

Example 6

This Example provides a description of the materials and methods used to obtain the results described in Examples 1-5.

Human and Animal Ethics

Peripheral blood samples from SCD patients were obtained during automated red cell exchange as part of their routine clinical care at Montefiore Medical Center. Since the samples were unlinked and de-identified medical waste, the Montefiore Medical Center Institutional Review Board deemed them to be IRB exempt.

Construct

The ZFs targeting HS2 of the human γ-globin promoters are known in the art. The SA domain containing amino acids 1–200 of Ldb1 was inserted C-terminal to the ZF. The SA domain was attached in C-terminal to GG1 tagged with HA.

Vector production and titration

Viral stocks were generated by co-transfection of the gene transfer plasmid (pCL-ZF-Ldb1) together with the envelope plasmid (VSV-G), the packaging plasmid (pMDLg/p RRE), and the pRSV-REV plasmid into 293T cells. An aliquot (5×10^6) of 293T cells was seeded into cell culture dishes (10 cm) 24 hours prior to transfection in Iscove's modification of Eagle's medium (DMEM, Cellgro, Manassas, VA) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C under 5% CO2. The culture medium was changed 2 hours prior to transfection. The precipitate was formed by adding the plasmids to 450 uL of 0.1× TE (0.1× TE is 10 mM Tris plus 1 mM EDTA) and 50 µL of 2 M CaCl2, then adding 500 µL of 2× HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na2HPO4) drop wise after which the precipitate was vortexed and immediately added to the cultures. The medium (10 ml) was replaced after 16 hours. Viral supernatants were collected at 24 and 48 hours, cleared by low speed centrifugation, and filtered through cellulose acetate (0.2 µm). Following ultracentrifugation, serial dilutions of concentrated virus (5; 0.5 and 0.05 µL, respectively) were used to infect 1×10^5 NIH 3T3 cells (ATCC, Manassas, VA) in 1 mL of transfection buffer complemented with polybrene (Millipore, Billerica, MA) at a final concentration of 8 µg/mL. Genomic DNA was extracted after 3 days (Qiagen kit, Valencia, CA). The multiplicity of infection (MOI) was calculated using the following formula: number of cells (1×10^5) X dilution factor (1 mL/µL viral preparation) X VCN (measured via real-time PCR, using oligos for WPRE element and ID gene, see PCR and Real Time PCR).

Real Time (RT)-PCR

Retrotranscription of total mRNA was done using the SuperScript™ II First Strand Kit (Invitrogen, Carlsbad, CA). Q-PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), with either TaqMan (TaqMan PCR 2× Master mix; Applied Biosystems) or SYBR Green (iTaqTM SYBR® Green Supermix, Bio-Rad Laboratories, Hercules, CA) chemistry. Quantitative real-time PCR assays of globin and GAPDH transcripts were carried out using gene-specific double fluorescently labeled probes. The following primer and probe sequences were used (forward, reverse and probe, when used, of each gene, respectively): β: Fw: 5′-CAAGAAAGTGCTCGGTGCCT-3′ (SEQ ID NO:6); Rev: 5′-

GCAAAGGTGCCCTTGAGGT-3′ (SEQ ID NO:7); 5′-FAM-TAGTGATGGCCTGGCTCACCTGGAC-TAMRA-3′ (SEQ ID NO:8); α: Fw: 5′-TCCCCACCACCAAGACCTAC-3′ (SEQ ID NO:9); Rev: 5′-CCTTAACCTGGGCAGAGCC-3′ (SEQ ID NO:10); 5′-FAM-TCCCGCACTTCGACCTGAGCCA-TAMRA-3′ (SEQ ID NO:11); □: Fw: 5′-TGGCAAGAAGGTGCTGACTTC-3′ (SEQ ID NO:12); Rev: 5′-TCACTCAGCTGGGCAAAGG (SEQ ID NO:13); 5′-FAM-TGG GAGATGCCATAAAGCACCTGG-TAMRA-3 (SEQ ID NO:14)′; BCL11A: Fw: 5′-TGATGTGTGTCCATTGGTGTGAGC-3′ (SEQ ID NO:15); Rev: 5′-TGCGAACTTGAACGTCAGGAGTCT, SOX-6 (SEQ ID NO:16): Fw: 5′-AGCTGCTTTCGGCTTTCTCCCTTA-3′ (SEQ ID NO:17); Rev: 5′-CCTTTGCATTTGCAGCAGTTCAGC-3′ (SEQ ID NO:18); C-MYB: Fw: 5′-TCAACCGATCATCCCTCACACTCT-3 ′ (SEQ ID NO:19)′; Rev: 5′-AATCAGCAGCGCTTCCATTCAAGG-3’ ′ (SEQ ID NO:20), KLF-1: Fw: 5′-GCTGCCTCCACCCAAGTG-3′ (SEQ ID NO:21); Rev: 5′-ACCAACTCTGGGCAGTCACAT-3’ (SEQ ID NO:22), Kell: Fw: 5′-AGCAACCACCCATGCCTGCC-3′ (SEQ ID NO:23); Rev: 5′-CTCGGGCCAAAGGCCTCACG-3′ SEQ ID NO:24). For real-time PCR of the reference genes, we used as an endogenous control the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) kit, in which the probe is fluorescently labeled with VIC (Applied Biosystems). The number of integrations (VCN) was quantified by Q-PCR using oligos (Fw: 5′-CGGCTGTTGGGCACTGA-3′ SEQ ID NO:25); Rev: 5′-GGAAGGTCCGCTGGATTGA-3′ SEQ ID NO:26)) and a probe (5′-FAM-ATGGCTGCTCGCCTGTGTTGCC-TAMRA-3′ SEQ ID NO:27) for a specific sequence present in the vector (WPRE) and compared it to an endogenous control present in two copies within the genome (ID-1 Fw: 5′-AAGGTGAGCAAGGTGGAGATTC-3 SEQ ID NO:28)′; Rev: 5′-TTCCGAGTTCAGCTCCAACTG-3′ SEQ ID NO:29).

Two-phase liquid cultures, benzidine staining and transduction

CD34+ cells selection from blood samples was performed by immunomagnetic separation, using the CD34 microbeads kit (Miltenyi Biotec Inc., Auburn, CA). These cells were then expanded following a modified version of the protocol described by Leberbauer and colleagues. Cells were seeded in 5 mL of serum-free StemSpan with 50 µL of StemSpan CC-100 cytokine cocktail (both from Stemcell Technologies, Vancouver, BA, Canada), 2 U/mL Erytrhopoietin (Amgen, Thousand Oaks, CA), 10−6 M dexamethasone (Sigma) and 1% penicillin streptomycin. CD34+ cultures were kept undifferentiated by refreshing the medium twice a week and density gradient centrifugation was used to remove both dead and spontaneously differentiating cells. At this stage cells were either frozen (with 50% characterized Hyclone FBS, 10%DMSO, Sigma, and 40% Iscove's Modified DMEM, Cellgro), or used for experiments. After 10 days in phase I, cells were transferred into phase II media containing α-modified essential medium supplemented with 30% fetal calf serum and 10−5 M β-mercaptoethanol. Erythropoietin was added (5 U/mL) to stimulate erythroid differentiation. Cells were infected with serial dilutions of the virus. Cells were collected on day 7-10 of phase II for all analyses. The level of differentiation was

assessed by benzidine staining. At this stage, cells were analyzed by flow cytometry for GFP, HbF and HbB expression.

Treatment with HbF inducers

We treated peripheral blood derived CD34+ cells (see procedure) with HbF inducer drugs: Hydroxyurea (HU), 5-azacytidine, pomalidomide, sodium butyrate, Tranyl-cypromine (TCP). Tranyl-cypromine (TCP), 5-aza-2'-deoxy-cytidine, pomalidomide and sodium butyrate. We first titrated the drugs to find the most efficient and less toxic concentration according to published data. Hydroxyurea (HU; Sigma-Aldrich), dissolved in water, was added to the culture medium at final concentrations of 150 μM. Tranylcypromine (Sigma-Aldrich) was dissolved in water and added to the culture medium at final concentrations 1.5 μM. 5-azacytidine (DAC; Sigma-Aldrich) was dissolved in water and added to the culture medium at final concentrations of 0.5 μM. Pomalidomide (Sigma-Aldrich) was dissolved in DMSO and was added to the culture medium at final concentrations of 30 μM. Sodium butyrate (Sigma-Aldrich) was dissolved in water and added to the culture medium at final concentration of 100 μM. The treatment timeline is shown in Table I.

Tetrameric and single chain analysis by high performance liquid chromatography (HPLC)

Red cell pellets were lysed with HPLC-grade water, and the resulting membrane-free hemolysates loaded into a System Gold 126 Solvent Module instrument (Beckman Coulter, Fullerton, CA). Hemoglobins were separated on a weak cation-exchange PolyCAT A column (PolyLC, Inc, Columbia, MD), and detected at a wavelength of 415 nm. The Hbs were bound to the column with mobile phase A (20 mmol/L Bis-Tris, 2 mmol/L KCN, pH 6.96) and eluted with mobile phase B (20 mmol/L Bis-Tris, 2 mmol/L KCN, 200mmol/L NaCl, pH 6.55). Single chain quantification was assessed via reversed-phase HPLC. Hb samples in this case were injected on a Hitachi D-7000 HSM Series apparatus (Hitachi Instruments, San Jose, CA) using a Zorbax 5 μm 300SB-C8 300 Å, LC 150 x 2.1 mm column (Agilent Technologies, Santa Clara, CA) and a gradient from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid in 25 minutes, with UV detection at 215 nm. Serial dilutions of a solution with known concentrations of HbA and HbF (Analytical Control System, Inc, Fishers, IN) were used to generate a calibration curve, where the peak areas were plotted against the concentration values. Types and relative quantity of Hbs in samples were assessed by comparison to standard hemoglobin controls.

Example 7

This Example demonstrates that introducing the lentiviral vector ALS10 into CD34+ cells from β0/0 phenotype samples, and thus the most severe thalassemic specimens, results in statistically significantly elevated levels of HbA produced by erythrocytes derived from the modified CD34+ cells. The elevation in HbA is relative to a previously describe construct, which is used in this Example as a comparison control (AnkT9W, from Breda et al, Plos One, 2012), which did not include a complete intron 2. Thus, when compared to the previously described construct, ALS10 showed significant and unexpected

improvement, as demonstrated by the results depicted in Figure 12. To obtain the results presented in Figure 12, the following materials and methods were used.

Vector production and titration

Viral stocks were generated by co-transfection of the gene transfer plasmid (GG1-SA) together with the envelope plasmid (VSV-G), the packaging plasmid (pMDLg/p RRE), and the pRSV-REV vector into 293T cells. An aliquot (5×10^6) of 293T cells was seeded into cell culture dishes (10 cm) 24 hours prior to transfection in Iscove's modification of Eagle's medium (DMEM, Cellgro, Manassas, VA) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C under 5% CO2. The culture medium was changed 2 hours prior to transfection. The precipitate was formed by adding the plasmids to 450 uL of 0.1× TE (0.1× TE is 10 mM Tris plus 1 mM EDTA) and 50 µL of 2 M CaCl2, then adding 500 µL of 2× HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na2HPO4) dropwise after which the precipitate was vortexed and immediately added to the cultures. The medium (10 ml) was replaced after 16 hours. Viral supernatants were collected at 24 and 48 hours, cleared by low speed centrifugation, and filtered through cellulose acetate (0.2 µm). Following concentration by ultracentrifugation, serial dilutions of concentrated virus (5; 0.5 and 0.05 µL, respectively) were used to infect 1×10^5 NIH 3T3 cells (ATCC, Manassas, VA) in 1 mL of transfection buffer complemented with polybrene (Millipore, Billerica, MA) at a final concentration of 8 µg/mL. Genomic DNA was extracted after 3 days (Qiagen kit, Valencia, CA). The multiplicity of infection (MOI) was calculated using the following formula: number of cells (1×10^5) X dilution factor (1 mL/µL viral preparation) X VCN (measured via real-time PCR, using oligos for Psi element and ID gene, by Real Time PCR).

Two-phase liquid cultures, benzidine staining and transduction

Consented patients with β0/0 and healthy individuals donated between 20 and 30 mL of peripheral blood; alternatively 30 to 60 mL of peripheral blood from β0/0 patients were obtained from the discarded blood from the red cell exchange therapeutic procedure. We selected CD34+ cells by immunomagnetic separation, using the CD34 microbeads kit (Miltenyi Biotec Inc., Auburn, CA) and then expanded these cells following a modified version of the protocol described by Leberbauer and colleagues. Cells were seeded in 5 mL of serum-free StemSpan with 50 µL of StemSpan CC-100 cytokine cocktail (both from Stemcell Technologies, Vancouver, BA, Canada), 2 U/mL Erytrhopoietin (Amgen, Thousand Oaks, CA), 10−6 M dexamethasone (Sigma) and 1% penicillin streptomycin. CD34+ cultures were kept undifferentiated by refreshing the medium twice a week and density gradient centrifugation was used to remove both dead and spontaneously differentiating cells. At this stage cells were either frozen (with 50% characterized Hyclone FBS, 10%DMSO, Sigma, and 40% Iscove's Modified DMEM, Cellgro), or used for experiments. After 10 days in phase I, cells were transferred into phase II media containing α-modified essential medium supplemented with 30% fetal calf serum and 10−5 M β-mercaptoethanol. Erythropoietin was added (5 U/mL) to stimulate erythroid differentiation. Cells were infected with serial dilutions of the virus. Cells were

collected on day 7-10 of phase II for all analyses. The level of differentiation was assessed by benzidine staining.

High performance liquid chromatography (HPLC)

Cell pellets were lysed with HPLC-grade water and loaded into a System Gold 126 Solvent Module instrument (Beckman Coulter, Fullerton, CA). Hemoglobins were separated on a PolyCAT A column (PolyLC, Inc, Columbia, MD), which is packed with silica-based material with a bonded coating of polyaspartic acid, and detected at a wavelength of 415 nm. The Hbs were bound to the column with mobile phase A (20 mmol/L Bis-Tris, 2 mmol/L KCN, pH 6.96) and eluted with mobile phase B (20 mmoI/L Bis-Tris, 2 mmol/L KCN, 200mmol/L NaCl, pH 6.55). Serial dilutions of a solution with known concentrations of HbA and HbF (Analytical Control System, Inc, Fishers, IN) were used to generate a calibration curve, where the absorbance detected at 415 nm was plotted against the concentration values. Types and quantity of hemoglobins in samples were assessed by comparison to standard hemoglobin controls.

While the invention has been described through specific embodiments, routine modifications will be apparent to those skilled in the art and such modifications are intended to be within the scope of the present invention.

What is claimed is:

1. A method for inducing expression of human beta-globin in erythrocytes for use in prophylaxis and/or therapy of a hemoglobinopathy in an individual comprising introducing into erythrocyte progenitor cells a polynucleotide encoding:

i) a 5' long terminal repeat (LTR) and a self-inactivating 3' LTR;

ii) at least one polyadenylation signal;

iii) at least one promoter;

iv) a globin gene locus control region (LCR);

v) an ankyrin insulator element (Ank);

vi) a Woodchuck Post-Regulatory Element (WPRE) configured such that the WPRE does not integrate into a target genome; and

vii) a sequence that is a reverse complement of a sequence encoding modified human beta-globin comprising a βT87Q mutation (B-globinM), wherein the sequence encoding the B-globinM comprises a first intron (intron 1) between exon 1 and exon 2, and a second intron (intron 2) between exon 2 and exon 3 of said B-globinM sequence, wherein intron 2 comprises more than 476 nucleotides of human B-globinM intron 2 sequence;

wherein, subsequent to the introducing, the erythrocyte progenitor cells differentiate into erythrocytes, wherein the erythrocytes are present in the individual and the erythrocytes produce more human beta-globin than a control.

2. The method of claim 1, wherein the control comprises a human beta-globin value obtained from control cells, wherein the control cells comprise erythrocytes from an individual who has the hemoglobinopathy, wherein the erythrocytes are progeny of cells into which a control viral vector is introduced, the control viral vector comprising the 5' LTR, the 3' LTR, the at least one

polyadenylation signal, the at least one promoter, the LCR, the Ank, a WPRE, and the sequence encoding the B-globinM, but wherein the sequence encoding the B-globinM in the control viral vector comprises 476 or fewer nucleotides of the human B-globinM intron 2 sequence.

3. The method of claim 1, wherein the lentiviral vector further comprise a sequence encoding a fusion of an Ldb1 transcription factor and a zinc finger (ZF) domain.

4. The method of claim 1, wherein the lentiviral vector further comprises a sequence encoding an RNA polynucleotide that is a reverse complement of mRNA encoding transferrin receptor 1, and wherein the RNA polynucleotide comprises a microRNA or an shRNA sequence and is capable of decreasing transferrin receptor 1 mRNA in an RNAi-mediated process.

5. The method of claim 1, wherein the erythrocyte progenitor cells comprise CD34+ cells, wherein the CD34+ cells are separated from the individual before the introducing the lentiviral vector to the cells, and wherein the CD34+ cells are introduced into the individual subsequent to the introducing of the lentiviral vector.

6. The method of claim 1, wherein the intron 2 comprises 851 nucleotides of the adult human B-globinM intron 2 sequence.

7. The method of any of claims 1, 2, 3, 4, 5 or 6, wherein the increased human beta-globin comprises adult hemoglobin, fetal hemoglobin, B-globinM, or a combination thereof.

8. The method of claim 7, wherein the hemoglobinopathy comprises sickle-cell anemia (SCA) or beta-thalassemia.

9. A lentiviral vector for inducing expression of human beta-globin in erythrocytes and/or erythrocyte progenitor cells for use in prophylaxis and/or therapy of a hemoglobinopathy in an individual, the lentiviral vector comprising:

i) a 5’ long terminal repeat (LTR) and a self-inactivating 3’ LTR;

ii) at least one polyadenylation signal;

iii) at least one promoter;

iv) a globin gene locus control region (LCR);

v) an ankyrin insulator element (Ank);

vi) a Woodchuck Post-Regulatory Element (WPRE) configured such that the WPRE does not integrate into a target genome;

vii) a sequence that is a reverse complement of a sequence encoding modified adult human beta-globin comprising a βT87Q mutation (B-globinM), wherein the sequence encoding the B-globinM comprises a first intron (intron 1) between exon 1 and exon 2, and a second intron (intron 2) between exon 2 and

exon 3 of said B-globinM sequence, wherein intron 2 comprises more than 476 nucleotides of adult human B-globinM intron 2 sequence.

10. The lentiviral vector of claim 9, wherein the lentiviral vector further comprise a sequence encoding a fusion of an Ldb1 transcription factor and a zinc finger (ZF) domain.

11. The lentiviral vector of claim 9, wherein the lentiviral vector further comprises a sequence encoding a RNA polynucleotide that a reverse complement of mRNA encoding transferrin receptor 1, and wherein the RNA polynucleotide comprises a microRNA or a shRNA sequence and is capable of decreasing transferrin receptor 1 mRNA in a RNAi-mediated process.

12. The lentiviral vector claim 9, wherein the lentiviral vector is present in CD34+ cells, wherein the CD34+ cells have been separated from an individual who has a hemoglobinopathy.

13. The lentiviral vector of claim 12, wherein the lentiviral vector is present in a virion.

14. A pharmaceutical composition comprising viral particles, wherein the viral particles each comprise a RNA strand which comprises a lentiviral vector of any one of claims 9, 10, 11.

15. A method of making a viral particle preparation for use in prophylaxis and/or therapy for one or more hemoglobinopathies comprising introducing a plasmid encoding a lentiviral vector of claim 1 into packaging cells which comprise a DNA packaging plasmid which encodes at least one virion protein, and a DNA envelope plasmid which encodes an envelope protein, and allowing expression of the virion protein and the envelope protein such that viral particles form, and separating the viral particles from the packaging cells.