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Supplemental Information

TALEN-Mediated Gene Editing of *HBG* in Human Hematopoietic Stem Cells Leads to Therapeutic Fetal Hemoglobin Induction

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SUPPLEMENTAL MATERIALS

Supplemental Figures:

Supplemental Figure 1). TALEN indel Screen. T7 gel analysis of a screening transfection using 3 alternative TALEN designs in hPBSCs to assess cleavage activity at target sites within *HBG1* and *HBG2*. * = band at ~210bp indicates cleavage of PCR product due to presence of indels.

Supplemental Figure 2). ddPCR to assess for intergenic deletions. Combined data from ddPCR assays designed to detect small deletions in each gene vs. larger deletions that occur following cleavage events in both the *HBG1* and *HBG2* promoters. These large deletional events likely account for the decreased detection of editing at the *HBG1* locus compared to *HBG2*.

Supplemental Figure 3). Representative FACS analysis of human hematopoietic cell lineages within the bone marrow of a recipient W41 mouse at time of sacrifice. Data shown are from a recipient of transplanted with TALEN-edited hPBSCs. Multi-lineage human engraftment is demonstrated including erythroid maturation. Decreased reticulocytes are seen due to a brief RBC lysis step required to clear mouse erythrocytes. Parental gates are color matched for reference.

Supplemental Figure 4). Relative frequency of indels by size before and after transplant. a) Heat map demonstrating the relative shift in indel size post-transplant. Negative values (red) indicate a relative decrease in frequency and positive values (green) a relative increase (Upper axis color bar). Deletion size is indicted on the lower axis (bp). Data suggest a trend for retention of smaller deletions post-transplant. b) Plot of the indel size post-transplant in both *HBG1* and *HBG2*. Negative values indicate a decrease and positive value an increase in frequency, respectively, relative to input. There is a general trend towards smaller deletions being retained post-transplant compared to larger deletions. n=5 animals.

Supplemental Figure 5). Indel frequency by size at harvest of primary and secondary transplant. *HBG1* and *HBG2* indel frequency as measured by Next Gen Sequencing of bone marrow cells harvested from primary and secondary transplant recipients. Data suggest that a similar profile of deletions is maintained from primary to secondary transplant. Values are the percentage of all sequence reads corresponding to a given insertion or deletion size. A deletion of zero is excluded and represents wild type sequence. n=5 animals.

Supplemental Figure 6). Off target screening for TAL1 pair. Table represents summary of Prognos off-target analysis. Gel represents T7 analysis of mock and TALEN-edited samples for each of the seven off-target genes.

Supplemental Figure 7). Post-transplant induction of HbF detected by methylcellulose colony forming assay. Red circles represent detection of editing in the *HBG* promoter (*HBG1* and/or *HBG2*). Black circles represent no editing detected. Grey circles represent HPLC data with insufficient gDNA for genomic analysis. Data from one post-transplant mouse. t test p-values *<0.05

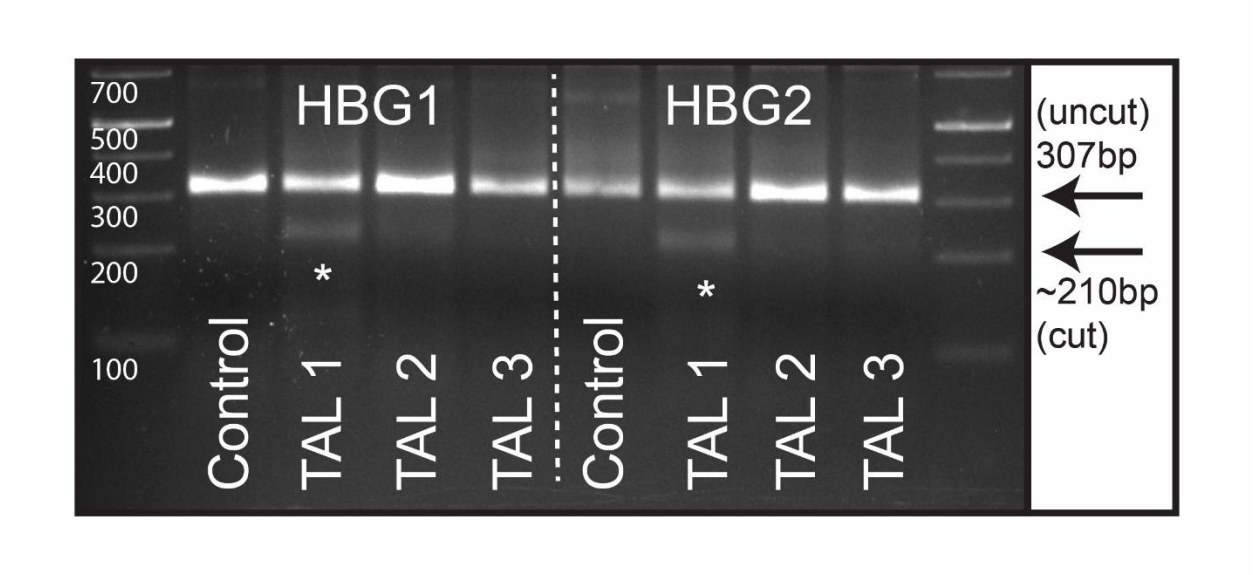

700
500
400
300
200
100
HBG1
HBG2
Control
TAL 1
TAL 2
TAL 3
Control
TAL 1
TAL 2
TAL 3
*
*
(uncut)
307bp
~210bp
(cut)

Figure S1

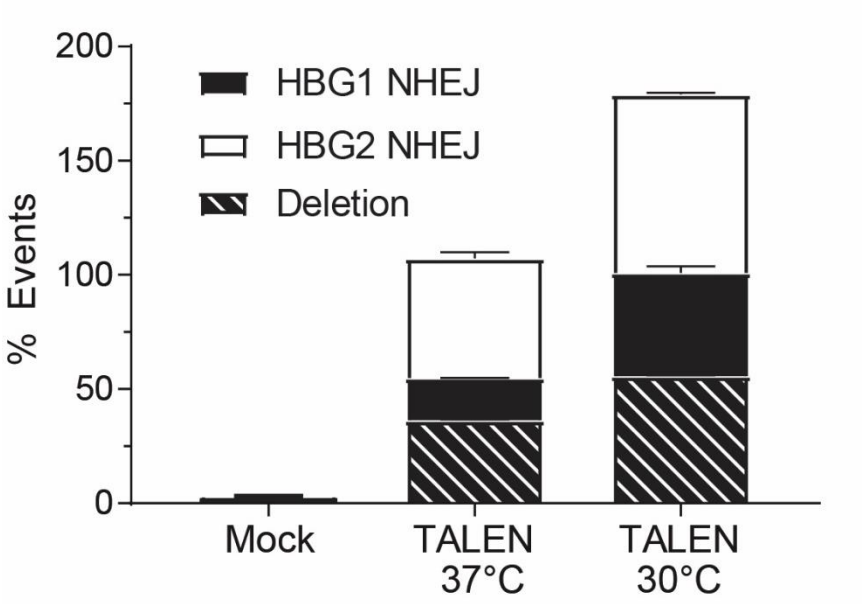

200
150
100
50
0
% Events
HBG1 NHEJ
HBG2 NHEJ
Deletion
Mock
TALEN 37°C
TALEN 30°C

Figure S2

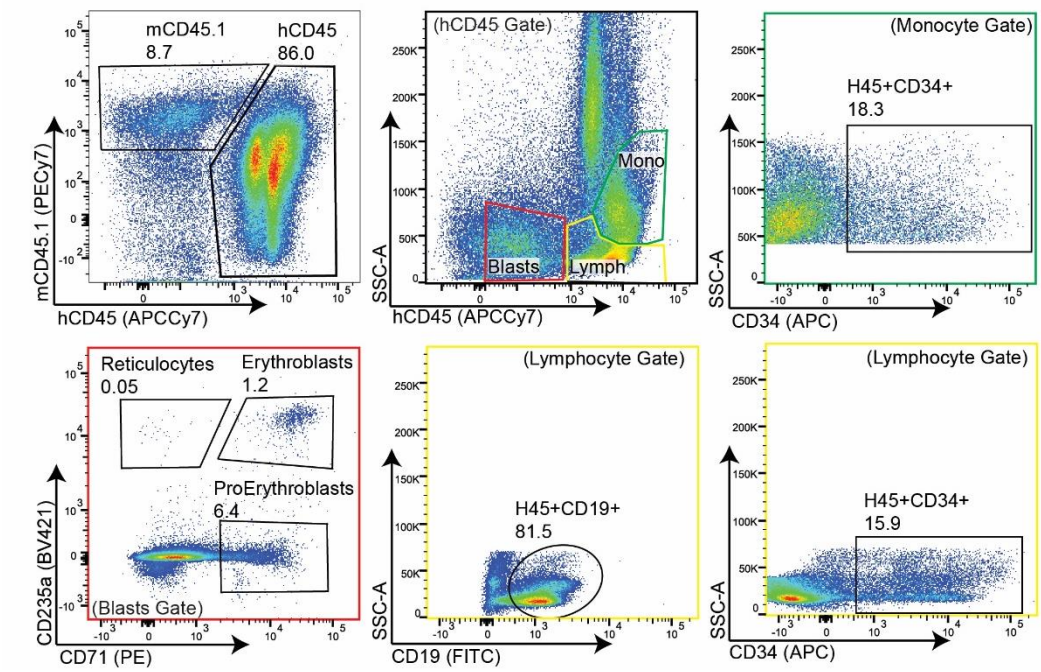

mCD45.1
8.7
hCD45
86.0
mCD45.1 (PECy7)
hCD45 (APCCy7)
(hCD45 Gate)
Mono
Blasts
Lymph
SSC-A
hCD45 (APCCy7)
(Monocyte Gate)
H45+CD34+
18.3
SSC-A
CD34 (APC)
Reticulocytes
0.05
Erythroblasts
1.2
ProErythroblasts
6.4
(Blasts Gate)
CD235a (BV421)
CD71 (PE)
(Lymphocyte Gate)
H45+CD19+
81.5
SSC-A
CD19 (FITC)
(Lymphocyte Gate)
H45+CD34+
15.9
SSC-A
CD34 (APC)

Figure S3

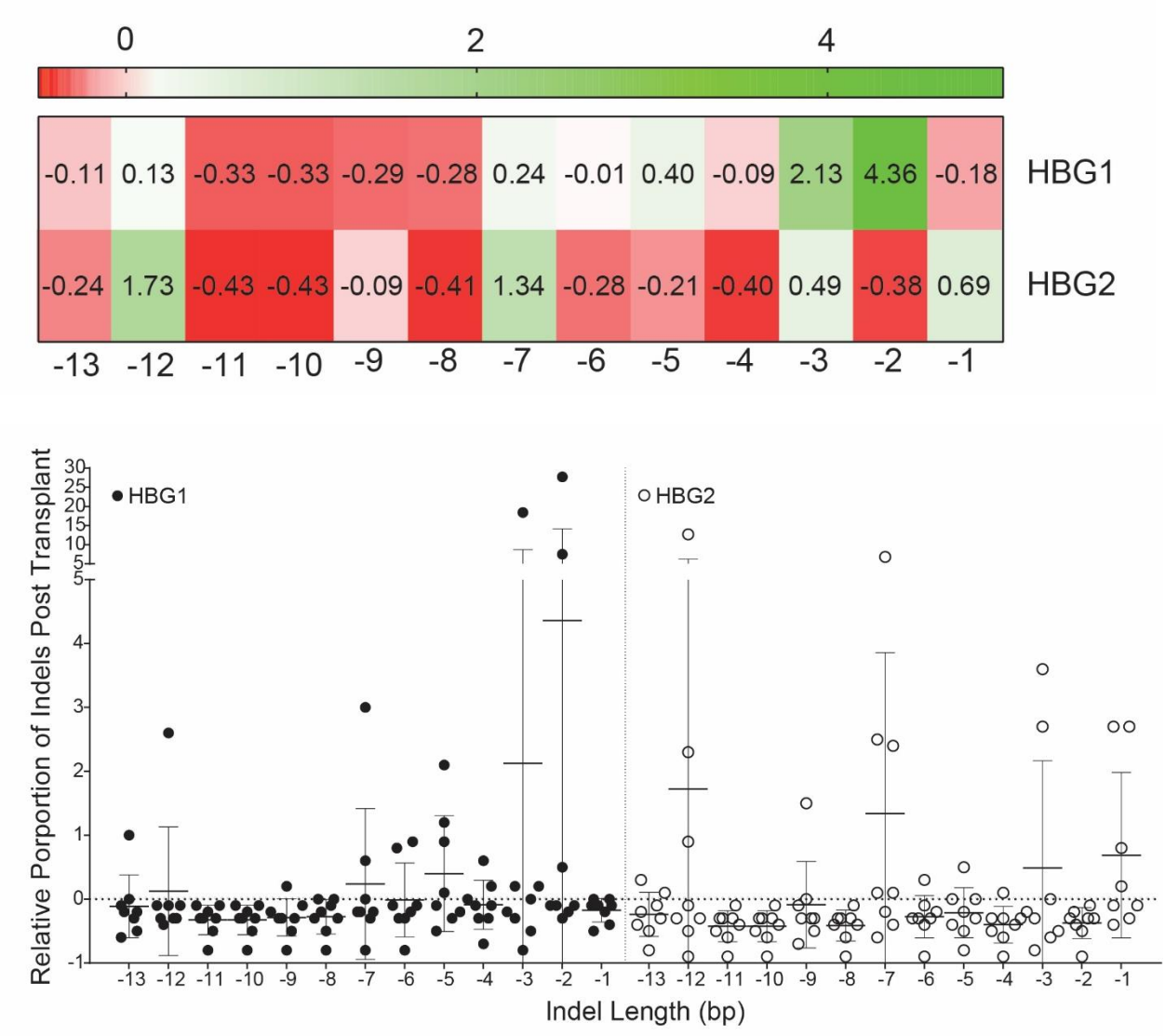

0
2
4
-0.11 0.13 -0.33 -0.33 -0.29 -0.28 0.24 -0.01 0.40 -0.09 2.13 4.36 -0.18
HBG1
-0.24 1.73 -0.43 -0.43 -0.09 -0.41 1.34 -0.28 -0.21 -0.40 0.49 -0.38 0.69
HBG2
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1
HBG1
HBG2
Relative Porportion of Indels Post Transplant
Indel Length (bp)

Figure S4

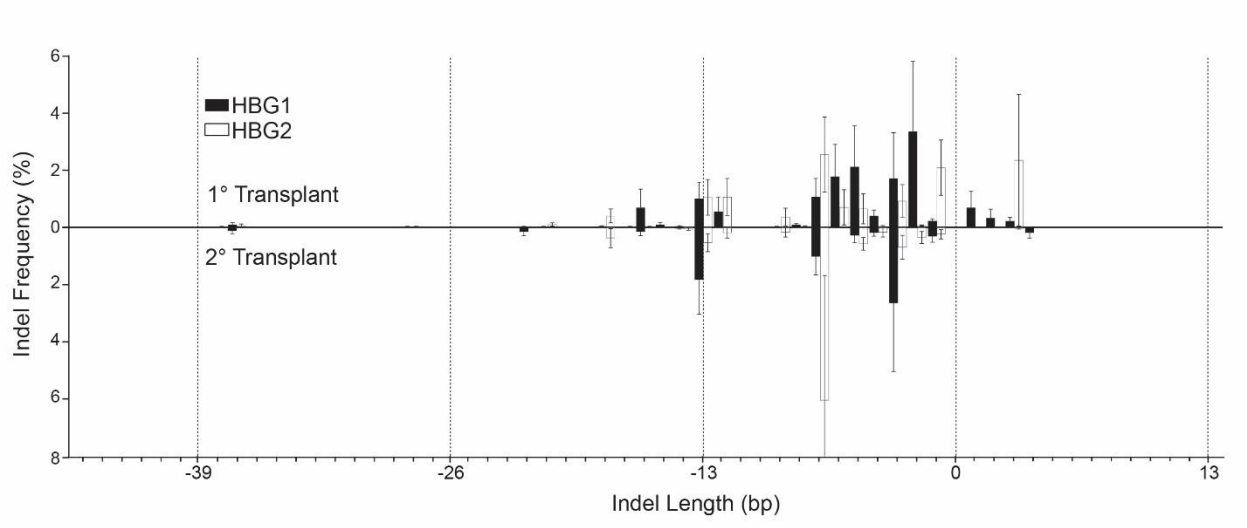
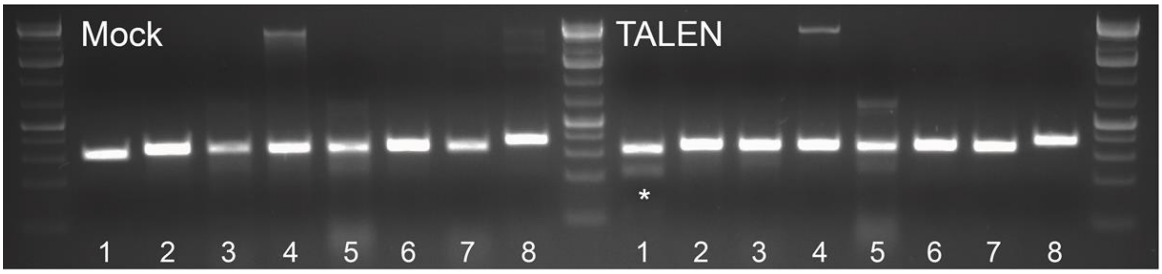

HBG1
HBG2
1° Transplant
2° Transplant
Indel Frequency (%)
6
4
2
0
2
4
6
8
-39
-26
-13
0
13
Indel Length (bp)

Figure S5



Lane	Primer Target	Prognos Homology Score	Genomic Region	Closest Gene	Distance to Gene (bp)	Chromosomal Location	Cutting Detected	Binding Type (Spacer)
1	HBG1	98	Promoter	HBG1	114	chr11:5271173-5271225	YES	R(17)L
2	Off Target 1	34	Intergenic	ZDHHC7	9,725	chr16:84998290-84998342	NO	L(19)L
3	Off Target 2	29	Intergenic	ADRA2C	48,165	chr4:3818418-3818471	NO	L(20)L
4	Off Target 3	29	Intergenic	NPBWR1	54,569	chr8:53908023-53908076	NO	L(20)L
5	Off Target 4	29	Intergenic	AUTS2	735,964	chr7:68327888-68327941	NO	L(20)L
6	Off Target 5	29	Intergenic	O3FAR1	263	chr10:95350092-95350145	NO	L(20)L
7	Off Target 6	26	Exon	FAM123B	0	chrX:63407207-63407259	NO	L(19)L
8	Off Target 7	26	Promoter	ZMYND8	642	chr20:45986116-45986171	NO	R(20)L

Figure S6

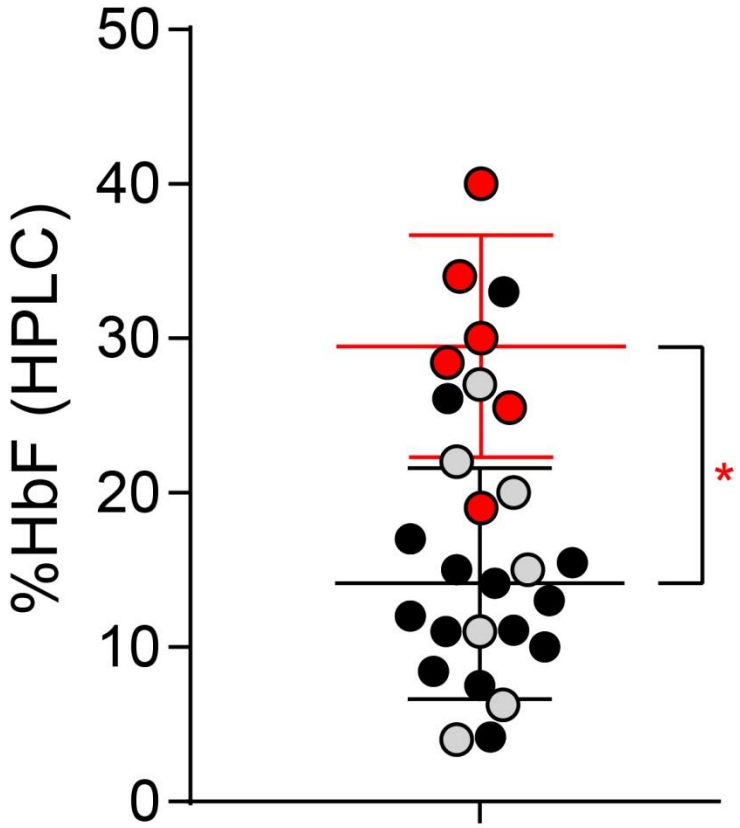


Figure S7

TABLES

Supplemental Table 1 – TALEN Targeter 2.0 Results.

	TAL1 RVDs	TAL2 RVDs	Spacer Length	Plus Strand	RE Sites	HD/ NN/ NH	Prognos Off Target Sites (Mismatches)
TAL1	NH NH NH NG NG NH NH HD HD NI NH HD HD NG NG NH	NH NH NH NG NG NH NH HD HD NI NH HD HD NG NG NH	17	GGGTTGGCCAG CCTTG ccttgaccaatagcctt GACAAGGCAAA CTTGACC	None	59	0 and 0: 2 100% Homology between *HBG1*&2
TAL2	NG NH NH HD HD NI NH HD HD NG NG NH HD HD NG NG	NG NH NH NG HD NI NI NH NG NG NG NH HD HD NG NG NH	15	TGGCCAGCCTT GCCTT gaccaatagccttga CAAGGCAAACT TGACCA	None	55	0 and 0: 2 100% Homology between *HBG1*&2
TAL3	NG NH NH HD HD NI NH HD HD NG NG NH HD HD NG NG NH NI	NI NG NG NH NH NG HD NI NI NH NG NG NG NH HD HD NG	15	TGGCCAGCCTT GCCTTGA ccaatagccttgaca AGGCAAACTTG ACCAAT	None	51	0 and 0: 2 100% Homology between *HBG1*&2

Supplemental Table 2 – Primer and Probe Sequences

Type	Name	Sequence
T7/MiSeqPrimers	*HBG1* FP	GCGCTGAAACTGTGGCTTTA
	HBG1 RP	AGCATGCACACACCACAAAC
	HBG2 FP	TCCTGCACTGAAACTGTTGCTTTA
	HBG2 RP	AAAGTGTGGAGTGTGCACATGA
NestedPCR Primers	FP	GCCCCTTCCCCACACTATCT
	RP	CCCCACAGGCTTGTGATAGT
ddPCR Probes and Primers	HBG FP	AAACTACAGGCCTCACTGGA
	HBG RP	GCTGAAGGGTGCTTCCTTTT
	HBG1 FAM Probe	ATCCTCTATGATGGGAGAAGGAA
	HBG2 FAM Probe	TTCTATGGTGGGAGAAGAAAACT
	NHEJ HEX Probe	CCTTGACCAATAGCCTTGACA
ddPCR Thermocycler Program	95C 10min, 95C 30s, 58C 1 min, 72C 1 min, x45 cycles, 98C 10min	

Supplemental Methods

hPBSC culture

Human peripheral blood stem cells (hPBSCs) were apheresed from healthy donors by the Fred Hutchinson PBMC Donor Program. Cells were maintained in culture in SCGM media (CellGenix 20802-0500) containing 100ng/mL SCF (PeproTech 300-07), 100ng/mL TPO (PeproTech 200-18), 100ng/mL Flt3-L (PeproTech 300-19) and 100ng/mL IL-3 (PeproTech 200-03). Cells were culture at 37°C, 5% CO_2 and maintained at a concentration of $1x10^6$/mL.

TALEN Transfection

hPBSCs were cultured for 48 hours post thaw. Cells were counted, centrifuged and suspended in Neon buffer T with TALEN mRNA (0 to 4ug / 2e5 cells / 10uL Neon Tip). Cells were electroporated using the Neon Transfection System (Invitrogen) (1300V, 20ms, 1 pulse) and placed in SCGM media with cytokines. Transient cold shock was carried out on some samples with overnight (~16h) culture at 30°C, 5% CO_2.

Flow Cytometry

Antibodies and catalog numbers (CD19 BD Pharmingen 555412, CD71 BD555537, CD3 BD641397 (APC-H7), CD33 BD333946, CD235a Miltenyi Biotec 35842, mCD45.1 BD565278, CD34 BD563778, hCD45 BD564914, HBF Thermo Fisher MHFH05. Fixation and permeabilization for HBF staining carried out using Paraformaldehyde and Triton-X or the BD Cytofix/Cytoperm solution kit per manufacturer's protocol. Stained cells were visualized and analyzed using the Becton Dickinson Fortessa cell analyzer.

Erythroid Differentiation

hPBSCs underwent erythroid differentiation by culture in IMDM media with 20% Certified Fetal Bovine Serum (Gibco 160000-044) with 20ng/mL hSCF (PeproTech 300-07), 1ng/mL IL-3 (PeproTech 200-03), and 2IU/mL Erythropoietin (R&D 287-TC). Cells were maintained at a low cell density of $3\text{-}5x10^6$/mL to decrease background HbF induction for 12-15 days. Erythroid differentiation was monitored by CD235a staining and visual pellet color assessment.

CFU Assay

Bone marrow samples from transplanted mice were plated in MethoCult Colony-Forming Unit (CFU) (Stemcell Technologies) assays per manufacturer's protocol. Individual erythroid colonies were isolated, washed and split with one portion analyzed by HPLC and the other for genomic DNA isolation and genotyping.

T7 Assay

Rapid screening of allele specific (*HBG1* or *HBG2*) cleavage at the TALEN target site was carried out by T7 assay following nested PCR. Primer lists in Supplemental Table 2. Off target cleavage screening was also performed using site specific primers and T7 Endonuclease I. PCR products were cleaned using the

Zymo clean and concentrate kit and cut and analyzed using T7 Endonuclease I (NEB E3321) following manufacturer's protocol. Products were run on a 2% agarose gel containing ethidium bromide and imaged on the BioRad GelDoc XR+.

Deep Sequencing

Amplicon sequencing was performed by first amplifying the unique *HBG1* and *HBG2* sites followed by Illumina MiSeq on pooled barcoded samples with a mean number of sequences of 8,230 (range 5,896-12,454) per allele per condition (minimum 3 replicates). Sequences were analyzed using the CRISPResso software as designed but modified to accommodate TALEN edited sequences[47].

HPLC

Following erythroid differentiation hemoglobin protein levels were assessed by Reverse Phase HPLC (globin chain analysis) on a Shimadzu Prominence UFLC chromatograph using an Aeris 3.6um Widepore C4 250 x 4.6mm column (Phenomenex). Mobile phases: A: Water 0.1% TFA (trifluoroacetic acid), B: Acetonitrile 0.08% TFA. Detection 220nm.

Data Analysis

All data analysis was carried out in GraphPad Prism unless otherwise specified. Significance indicated with * =$p<0.05$, **=$p<0.005$, ***=$p<0.0005$.