OMTN, Volume *17*

Supplemental Information

In Vivo Outcome of Homology-Directed Repair at the *HBB* Gene in HSC Using Alternative Donor Template Delivery Methods

Sowmya Pattabhi, Samantha N. Lotti, Mason P. Berger, Swati Singh, Christopher T. Lux, Kyle Jacoby, Calvin Lee, Olivier Negre, Andrew M. Scharenberg, and David J. Rawlings

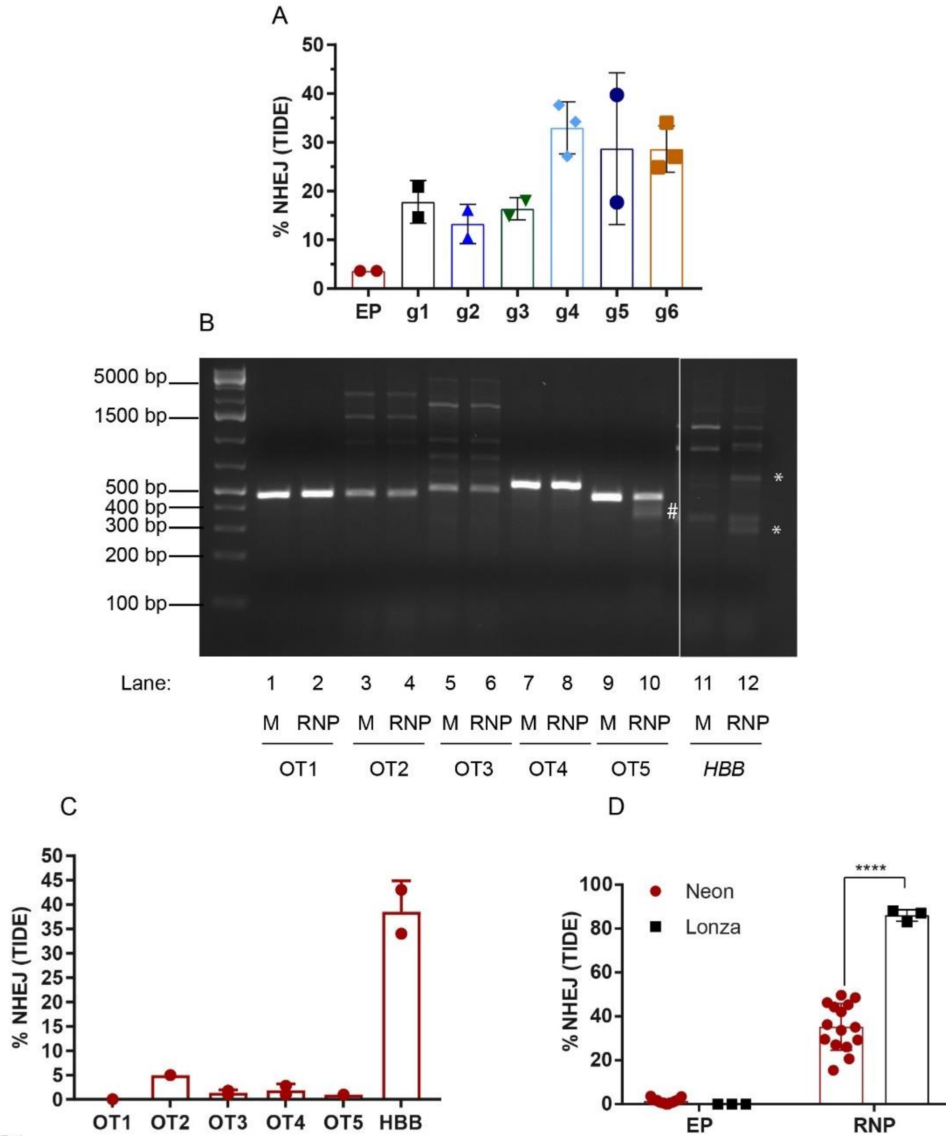


Figure S1. Off-target analysis of sgRNA-g1. (A) Screening of candidate sgRNA (g1-g6, delivered as RNPs) to create DSBs at the *HBB* gene measured by TIDE/ICE sequencing (donor n = 2-3). (B) Off-target analysis of top 5 off-target genes predicted by CCTop algorithm (See Table 1). Gel shows amplicons of top 5 off-target genes amplified from mock-treated (M) and sgRNA-g1 RNP-treated (RNP) samples evaluated by T7 endonuclease assay. (i) OT1: *DENND3* (lane 1-2), (ii) OT2: *MIR7974* (lane 3-4), (iii) OT3: *LINC01206* (lane 5-6) (iv) OT4: *HBD* (lane 7-8) (v) OT5: *TULP4* (lane 9-10) (vi) Target site: *HBB* (lane 11-12). Asterisks (*) represent cleaved bands. # represents a ghost band that does not match any of the potential cleavage fragments (313 bp and 143 bp for OT5: *TULP4*). (C) TIDE/ICE sequencing analysis of top 5 off-target genes (i) OT1: *DENND3* (ii) OT2: MIR7679 (iii) OT3: *LINC01206* (iv) OT4: *HBD* (v) OT5: *TULP4* (vi) Target site: *HBB* (n = 2 experiments). (D) Editing efficiency of sgRNA-g1 delivered as RNP using the NEON electroporation system (donor n = 15) or the Lonza nucleofection system (donor n = 3).

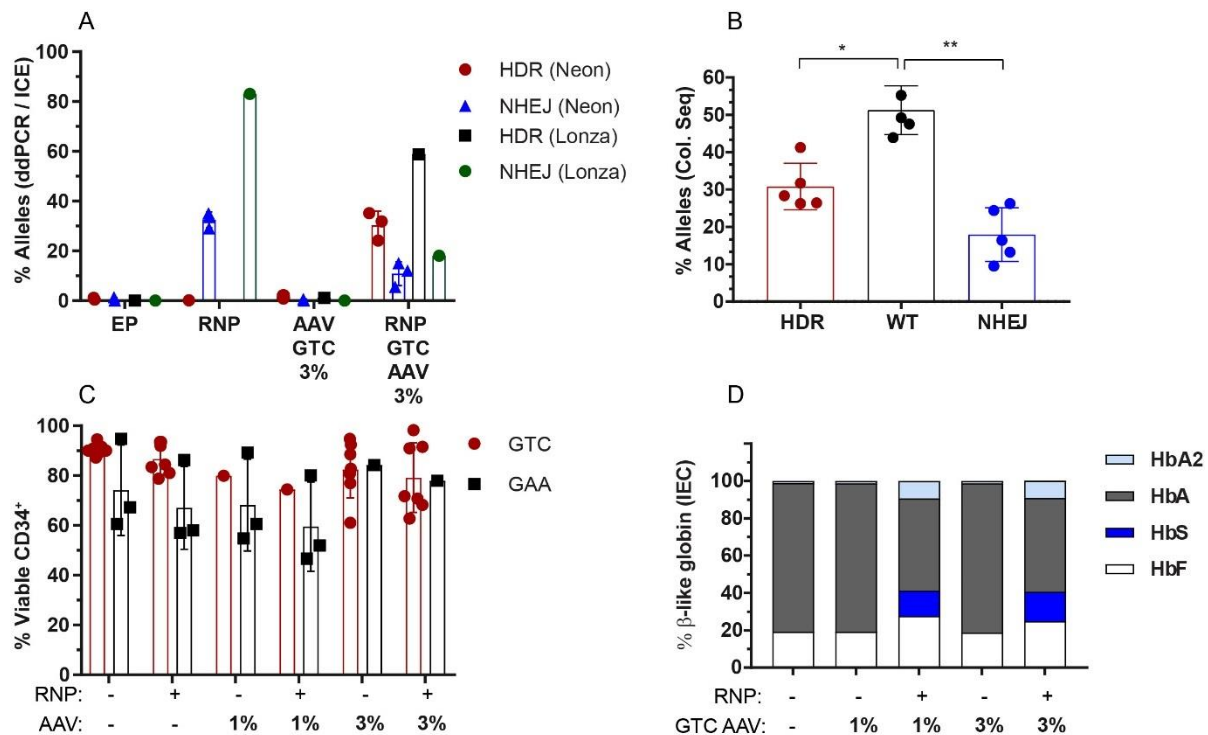


Figure S2

Figure S2. Homology-directed repair using a rAAV6 donor delivery method to drive nucleotide change at the *HBB* locus. (A) HDR and NHEJ outcomes measured by ddPCR and ICE sequencing, respectively, following co-delivery of RNP and GTC (E6V) rAAV6 using either the Neon electroporation system (n = 3) or the Lonza nucleofection system (n = 1). (B) Colony sequencing of samples edited with RNP and transduced with GTC (E6V) rAAV6 (n = 5) using the neon electroporation. (C) Viability of mPBSCs on day 2 post-electroporation and GTC (E6V) or GAA (E6optE) rAAV6 transduction. (D) IEC of erythroid cells to determine globin tetramers *in vitro* in cells treated with rAAV6 alone and RNP plus GTC (E6V) rAAV6 (HbF: Fetal, HbA: Adult, HbA2: Minor adult, HbS: Sickle). All bar graphs show mean ± SD. n represents the number of individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****, $p < 0.0001$. *p*-value was calculated by comparing each sample mean with the respective control sample mean by 2way ANOVA with Dunnett's multiple comparison.

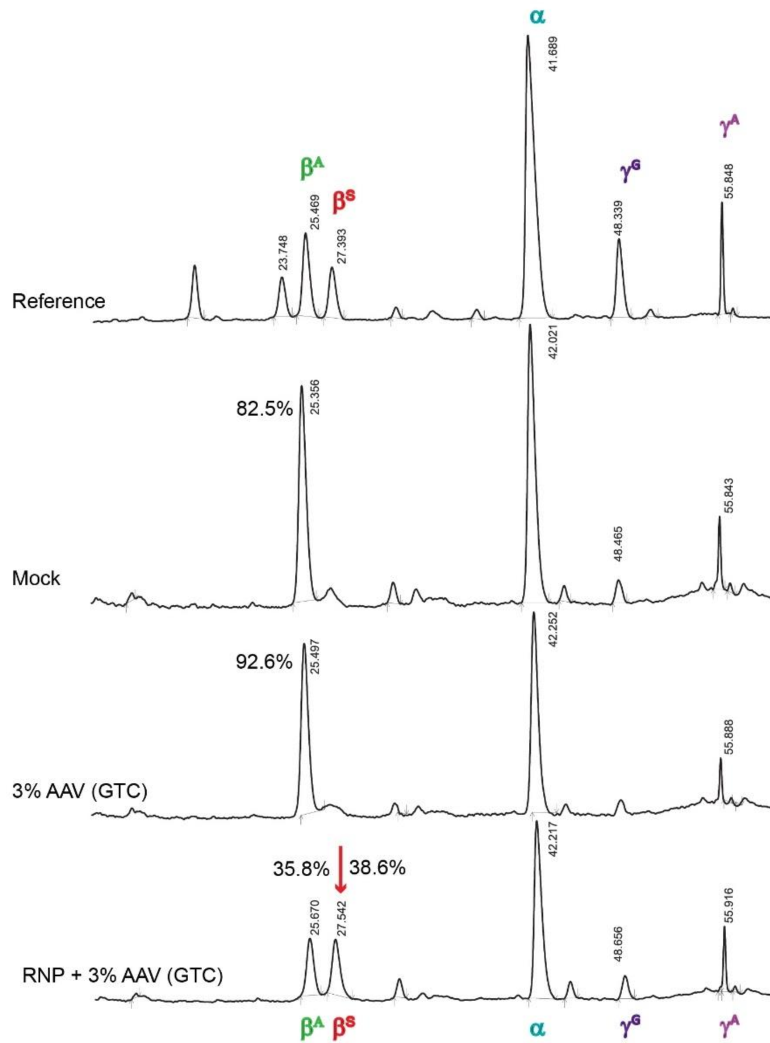


Figure S3. RP-HPLC analysis of edited and differentiated erythroid cells. RP-HPLC chromatogram trace of Reference, Mock, rAAV6 alone and RNP plus GTC (E6V) rAAV6 (3%) transduced cells driving sickle globin expression (α = alpha, β^A = adult, β^S = Sickle, γ^G = Gamma 2, γ^A = Gamma 1). Vertical numbers are HPLC elution times. Lower trace shows sickle globin expression (red arrow).

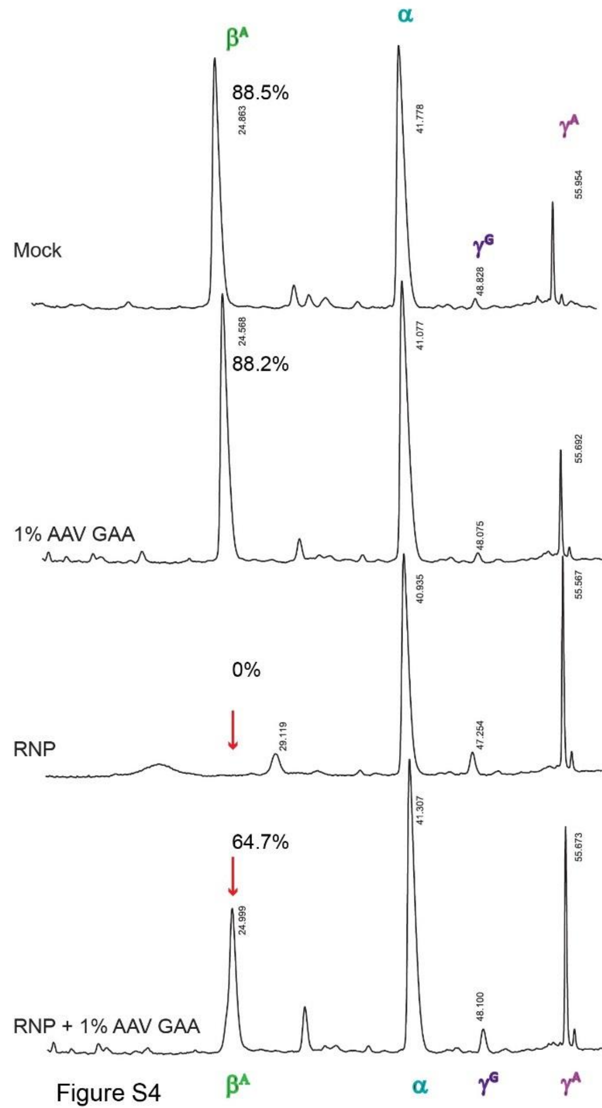


Figure S4. RP-HPLC analysis of edited and differentiated erythroid cells. RP-HPLC chromatogram trace of Mock, rAAV6 alone, RNP alone and RNP plus GAA (E6optE) rAAV6 driving adult globin expression (α = alpha, β^A = adult, β^S = Sickle, γ^G = Gamma 2, γ^A = Gamma 1). Vertical numbers are HPLC elution times. Lower trace shows restoration of adult globin expression.

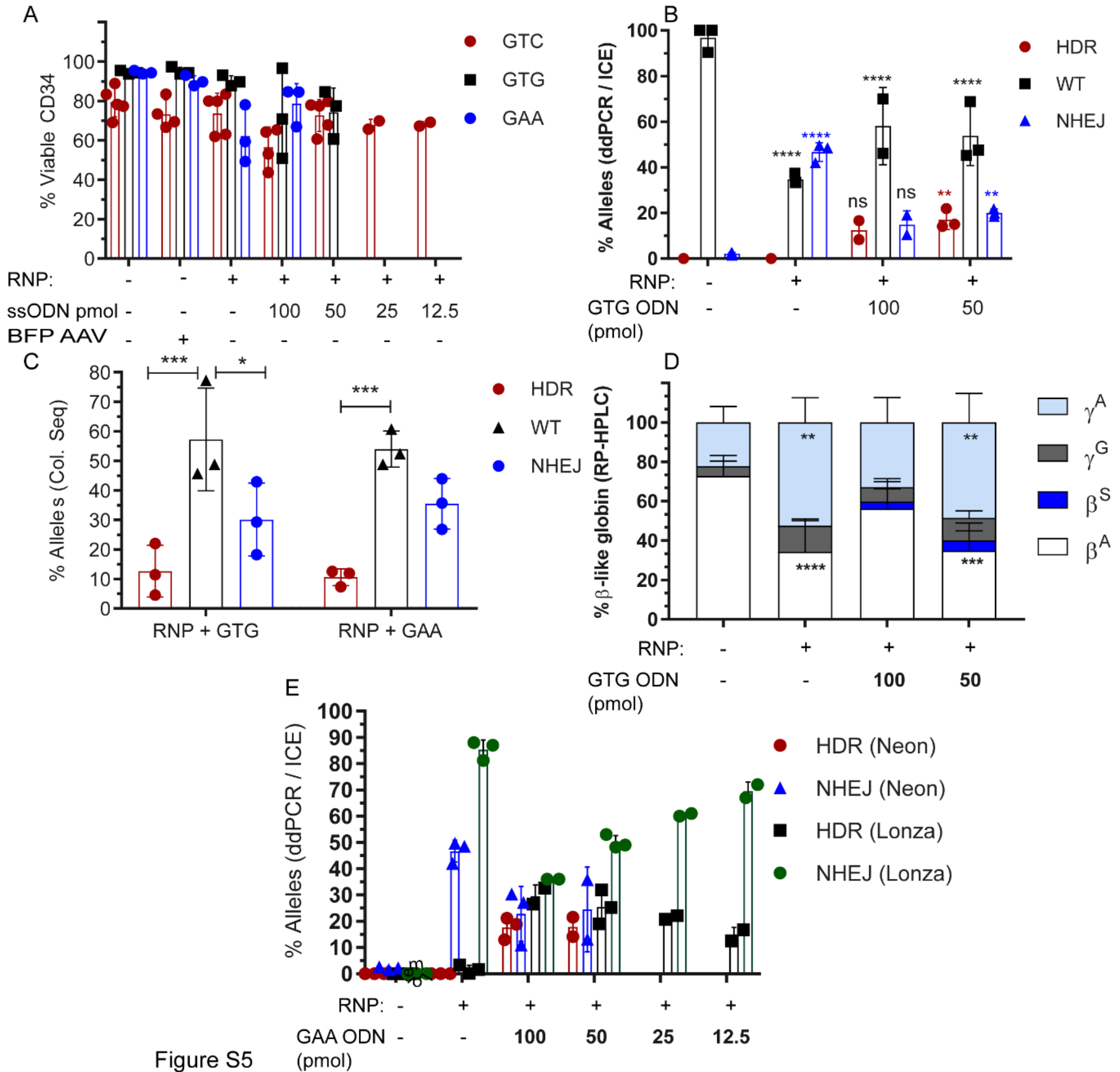


Figure S5: Homology-directed repair using a ssODN donor delivery method to drive nucleotide change at the *HBB* locus. (A) Viability of CD34+ mPBSCs on day 2 post-electroporation with GTC or GTG (E6V) ssODN introducing a sickle mutation or a GAA (E6optE) ssODN introducing a codon optimized SNP change at codon 6 by HDR. (B) WT (%), HDR (%) measured by ddPCR and NHEJ measured by ICE sequencing respectively, following electroporation with RNP alone or co-delivery of RNP and donor GTG (E6V) ssODN at the indicated concentrations (50 pmol ssODN, donor n = 3). (C) Colony sequencing of samples edited with RNP and modified with GTG (E6V) ssODN and GAA (E6optE) ssODN tested with the Neon electroporation system (donor n = 3). (D) RP-HPLC analysis of erythroid cells to determine β-globin expression in edited cells with GTG (E6V) ssODN delivery (donor n = 3). (α = alpha, β^A = adult, β^S = Sickle, γ^G = Gamma 2, γ^A = Gamma 1). (E) WT (%), HDR (%) measured by ddPCR and NHEJ (%) measured by ICE sequencing respectively, following electroporation with RNP alone or co-delivery of RNP and GAA (E6optE) ssODN at the indicated concentrations using either the Neon electroporation system (50 pmol ssODN, n = 2) or the Lonza nucleofection system (50 pmol ssODN, n = 3). All bar graphs show mean ± SD. n represents the number of individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

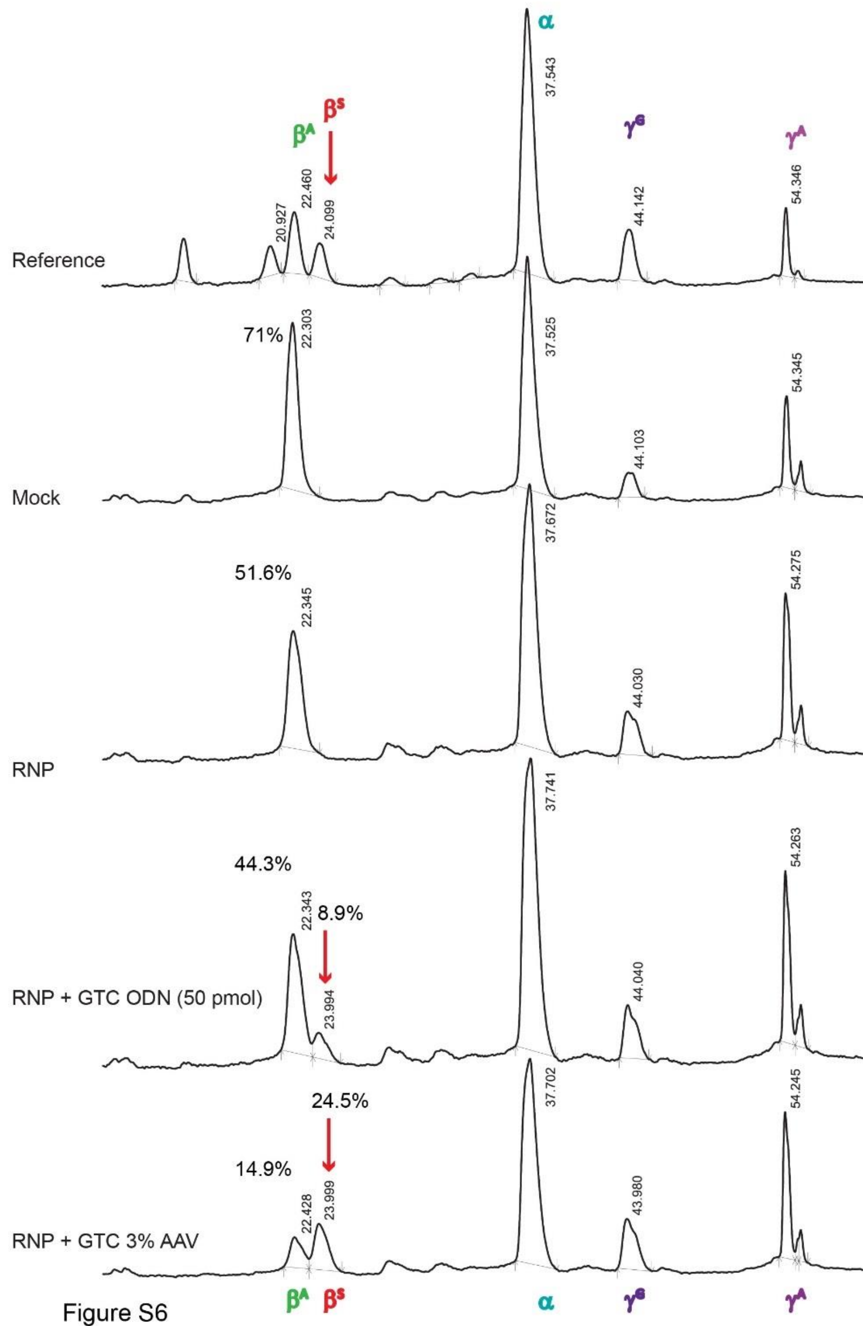


Figure S6. RP-HPLC analysis of edited and differentiated erythroid cells. RP-HPLC chromatogram trace of Mock, RNP alone and RNP plus GTC (E6V) ssODN and rAAV6 donor templates driving sickle globin expression (α = alpha, β^A = adult, β^S = Sickle, γ^G = Gamma 2, γ^A = Gamma 1). Vertical numbers are HPLC elution times. Lower traces show sickle globin expression (red arrow).

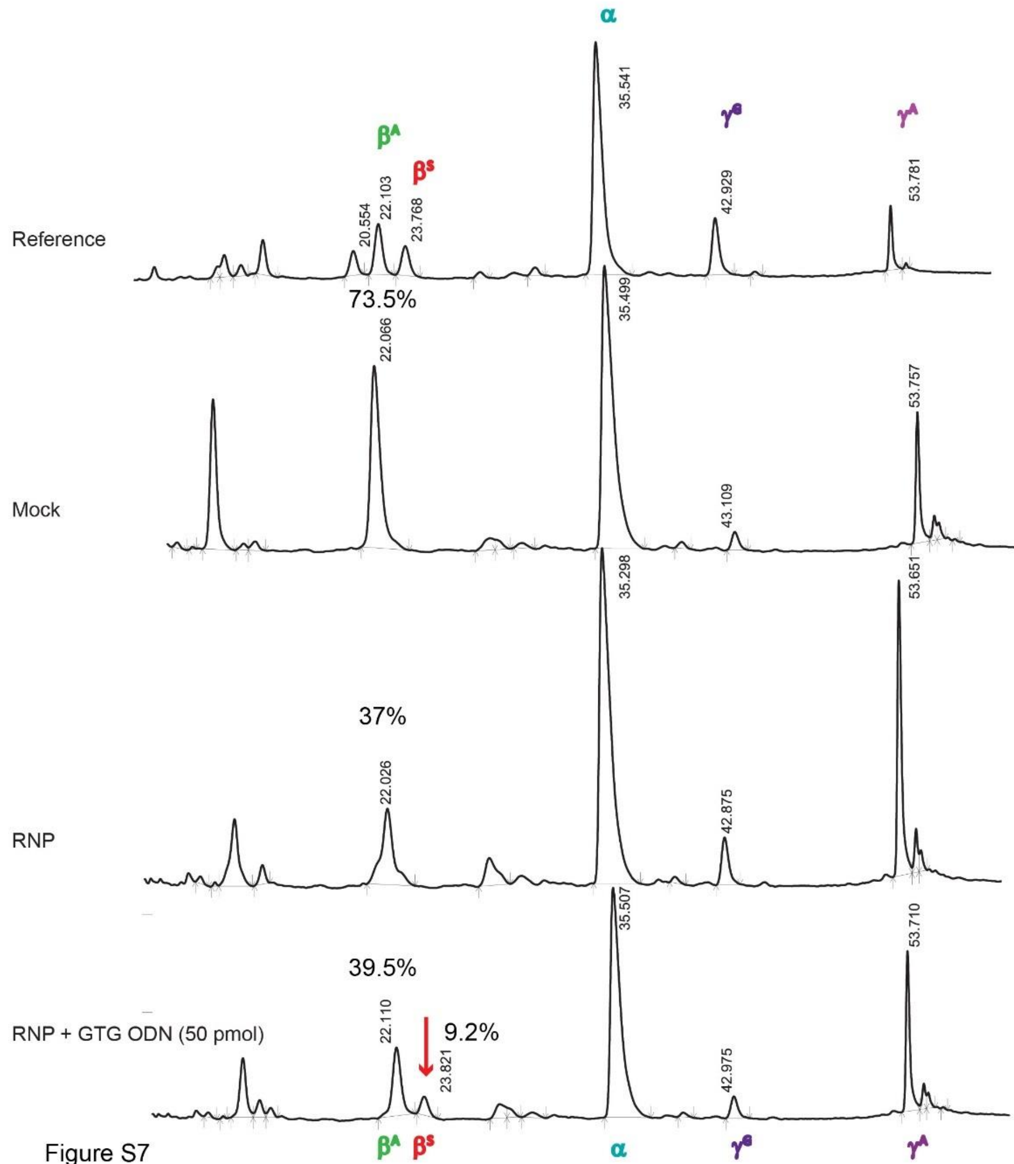


Figure S7. RP-HPLC analysis of edited and differentiated erythroid cells. RP-HPLC chromatogram trace of Reference, Mock, RNP alone and RNP plus GTG (E6V) ssODN driving sickle globin expression (α = alpha, β^A = adult, β^S = Sickle, γ^G = Gamma 2, γ^A = Gamma 1). Vertical numbers are HPLC elution times. Lower trace shows sickle globin expression (red arrow).

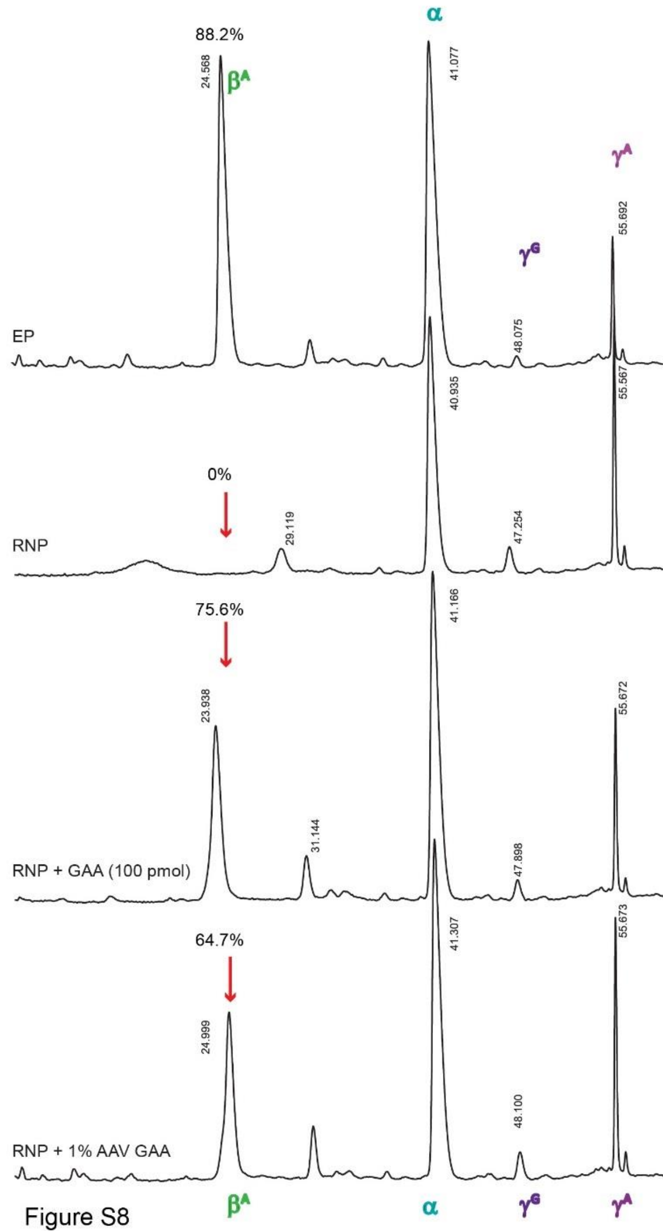
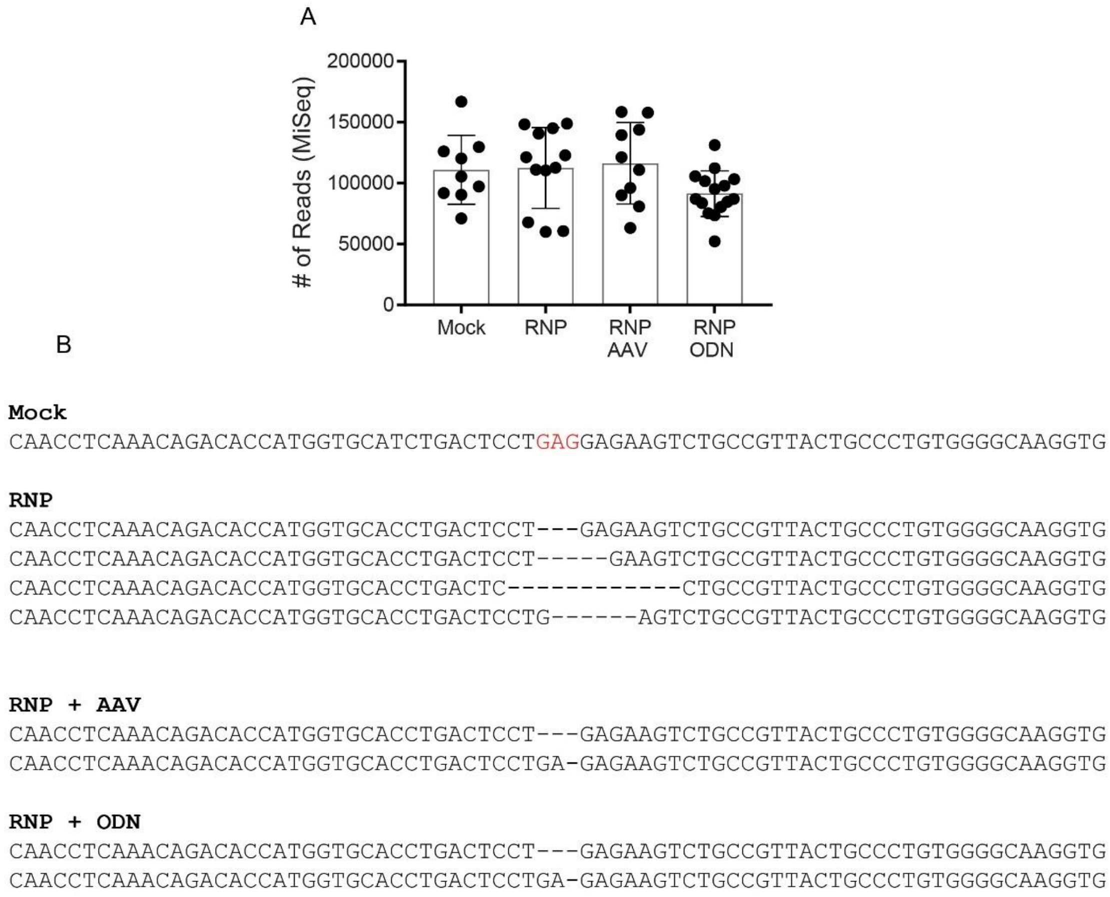


Figure S8. RP-HPLC analysis of edited and differentiated erythroid cells. RP-HPLC chromatogram trace of Mock, RNP alone and RNP plus GAA (Eopt6E) ssODN and rAAV6 donor templates driving adult globin expression (α = alpha, β^A = adult, β^S = Sickle, γ^G = Gamma 2, γ^A = Gamma 1). Vertical numbers are HPLC elution times. Lower traces show restoration of adult globin expression.



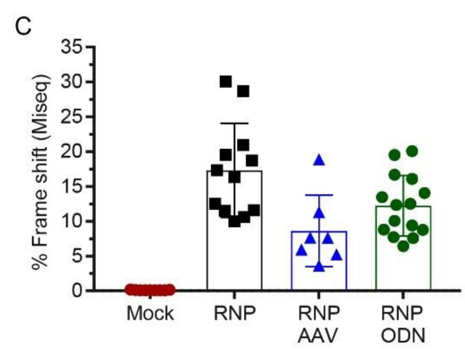


Figure S9

Figure S9. MiSeq data analysis of *in vitro* samples. (A) Number of aligned paired end reads from *in vitro* edited samples. Each dot represents a unique sample. (B) Consensus sequences from predominant NHEJ events observed in Mock, RNP alone, co-delivery of RNP with rAAV6 and RNP with ssODN. (S4C). Quantification of % frame shift mutations in *in vitro* samples by MiSeq analysis in mock, RNP-edited, rAAV6-edited or ssODN-edited samples.

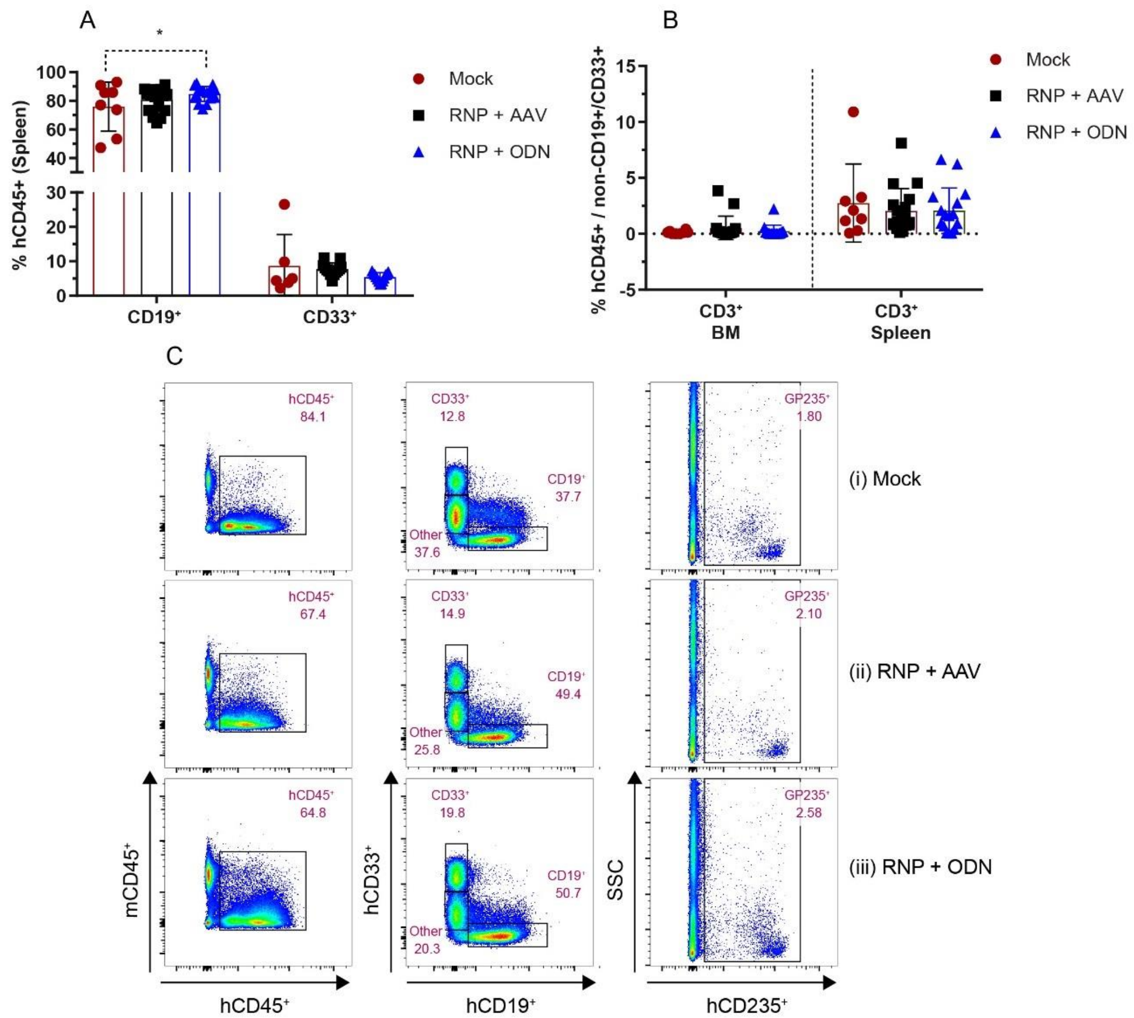


Figure S10. Engraftment potential of rAAV6 vs. ssODN-edited HSCs *in vivo* in NBSGW mice. (A) Human CD19⁺ and CD33⁺ populations in the spleen gated from hCD45⁺ populations. (B) Human CD3⁺ population in the BM and spleen gated from non-CD19⁺ and non-CD33⁺ cells (C) Representative flow plots of human cells (hCD45⁺) within the BM of NBSGW recipient mice transplanted with HSC edited with GTC (E6V) donors. Flow plots demonstrate multi-lineage engraftment including: CD19⁺, CD33⁺ and CD235⁺ cells within the BM of (i) Mock-edited, (ii) rAAV6-edited and (iii) ssODN-edited cells recipients. Gating strategy: Live, Single cells, hCD45⁺ > CD19⁺ CD33⁺. Erythroid cells were gated on mCD45⁻ cells.

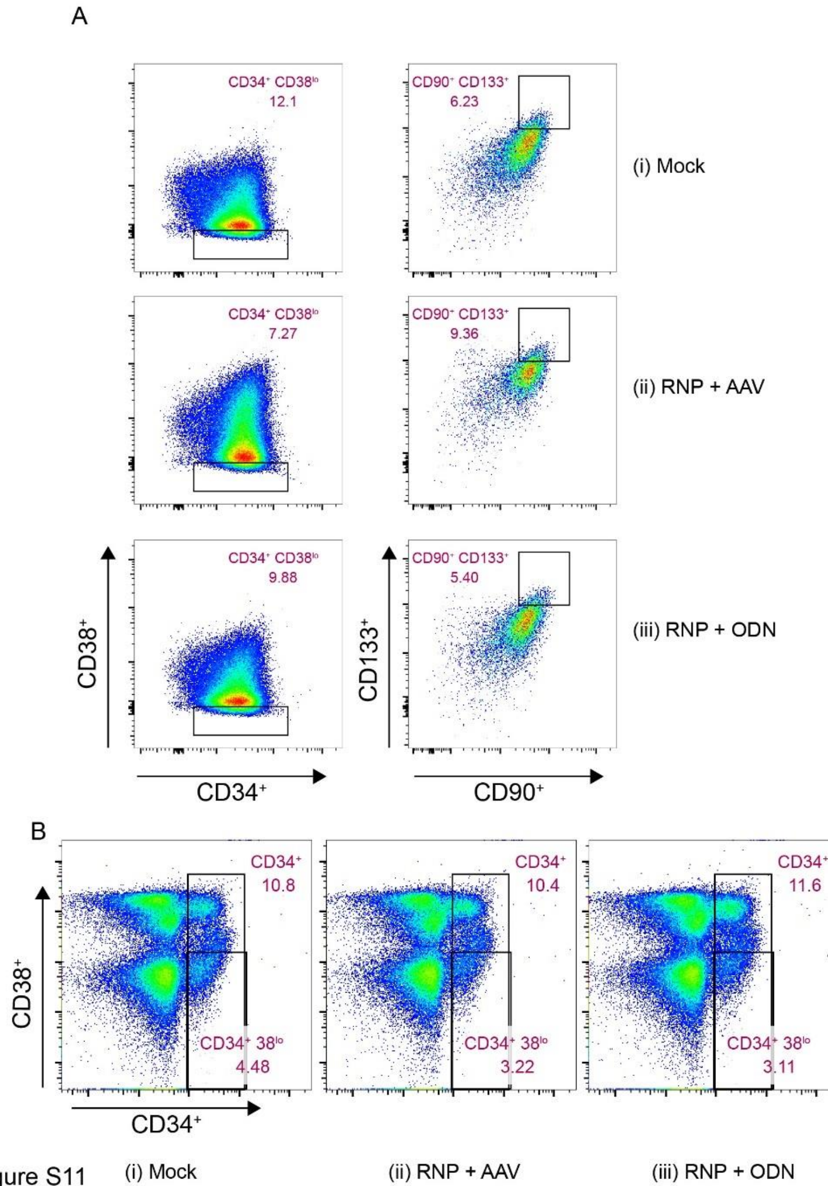


Figure S11. HSC compartment pre- and post-transplant. (A) (*Left panels*) Representative flow plots of $CD34^+$ and $CD34^+$ $CD38^{lo}$ cells pre-transplant showing: (i) Mock-edited, (ii) rAAV6-edited or (iii) ssODN-edited (modified with GTC (E6V) populations. Gating strategy: Live, Single cells, $hCD45^+$ > $CD34^+$ $CD38^+$ > $CD90^+$ $CD133^+$ (*Right panels*) Representative flow plots of $CD34^+CD38^{lo}$ cells using additional markers identify populations enriched for LT-HSC as identified by $CD133^+$ $CD90^+$ double positive cells. (B) Representative flow plots of $CD34^+$ and $CD34^+CD38^{lo}$ compartment from BM of NBSGW mice transplanted with: (i) Mock-edited, (ii) rAAV6-edited or (iii) ssODN-edited cells (GTC (E6V) donor constructs). Gating strategy: Live, Single cells, $hCD45^+$ > $CD34^+$ $CD38^+$.

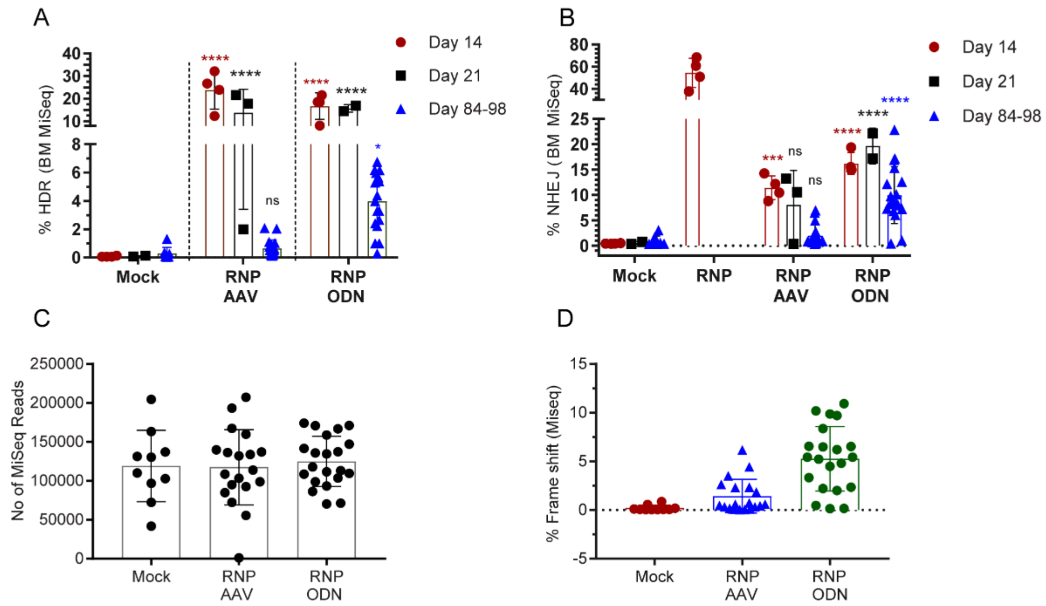


Figure S12

Figure S12. MiSeq analysis of HDR and NHEJ outcomes. (A) HDR rates determined by MiSeq analysis for GTC (E6V) rAAV6 or ssODN treated cells at the indicated time points. (B) NHEJ rates determined by MiSeq analysis for: GTC (E6V) rAAV6 or ssODN treated cells at indicated time points. n represents samples or animals. Input n = 4, (C) Number of aligned paired end reads from *in vivo* BM samples. Each dot represents MiSeq reads from each individual mouse across different groups. (D) Quantification of % frame shift mutations in *in vivo* BM samples by MiSeq analysis in mock, rAAV6-edited or ssODN-edited animals.

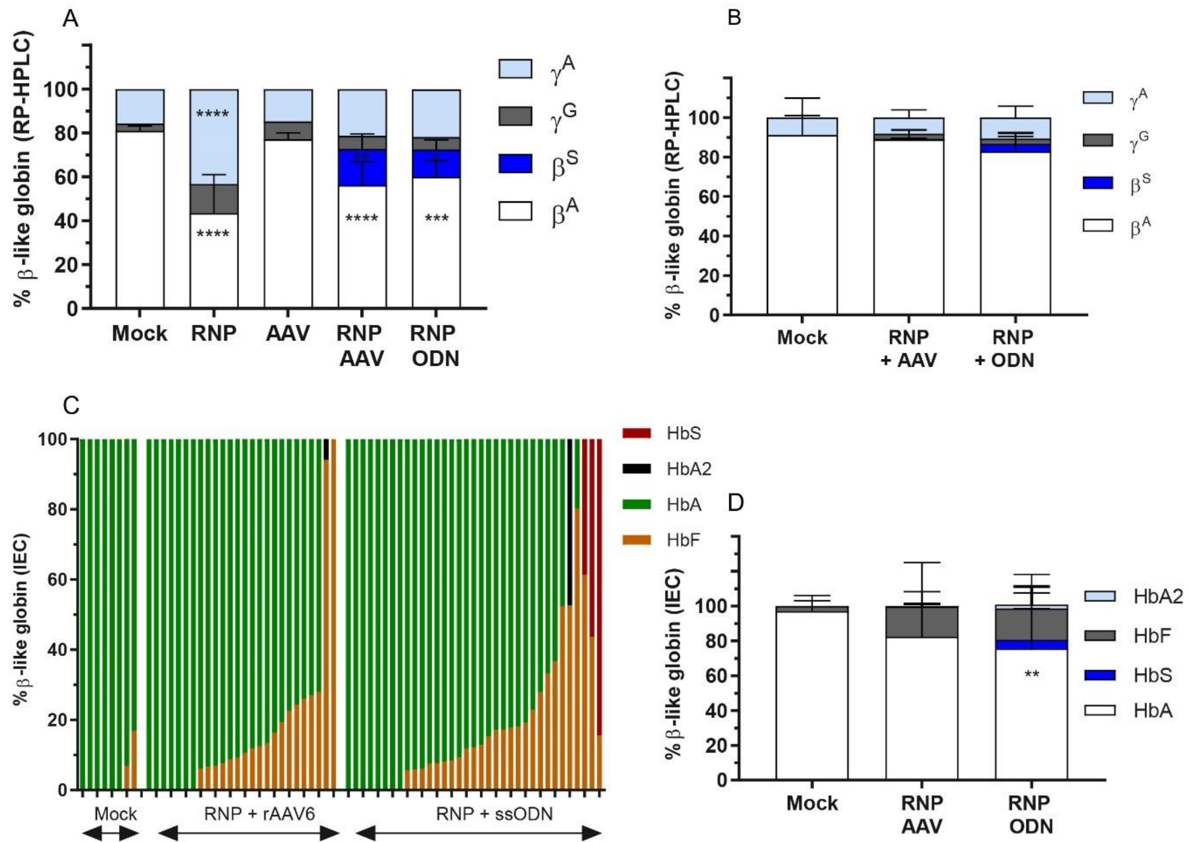


Figure S13. Sickle globin expression pre- and post-transplant. (A) RP-HPLC analysis to measure β-globin subtypes in erythroid cultures following gene editing of CD34$^+$ mPBSCs using GTC (E6V) rAAV6 or ssODN delivery. (B) BM cells isolated at 12-14 weeks from recipient mice transplanted with mock (n = 2), GTC (E6V)-edited rAAV6 (n = 4) or ssODN (n = 3) modified cells, expanded *ex vivo* in erythroid culture conditions for 2 weeks after harvest. RP-HPLC analysis was performed to measure β-globin subtypes expressed (α = alpha, β^A = adult, β^S = Sickle, γ^G = Gamma 2, γ^A = Gamma 1). (C) Ion exchange HPLC of single BFU-E colonies (generated from methocult cultures) to determine globin tetramers expressed following gene editing. (D) Summary of Ion exchange HPLC of single BFU-E colonies to measure globin tetramers expressed in gene edited cells (HbF: Fetal, HbA: Adult, HbA2: Minor adult, HbS: Sickle). All bar graphs show mean ± SD. n represents the number of individual animals. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, *p*-value was calculated by comparing each sample mean with the respective sample mean of the mock or control sample by 2way ANOVA with Dunnett's multiple comparison.

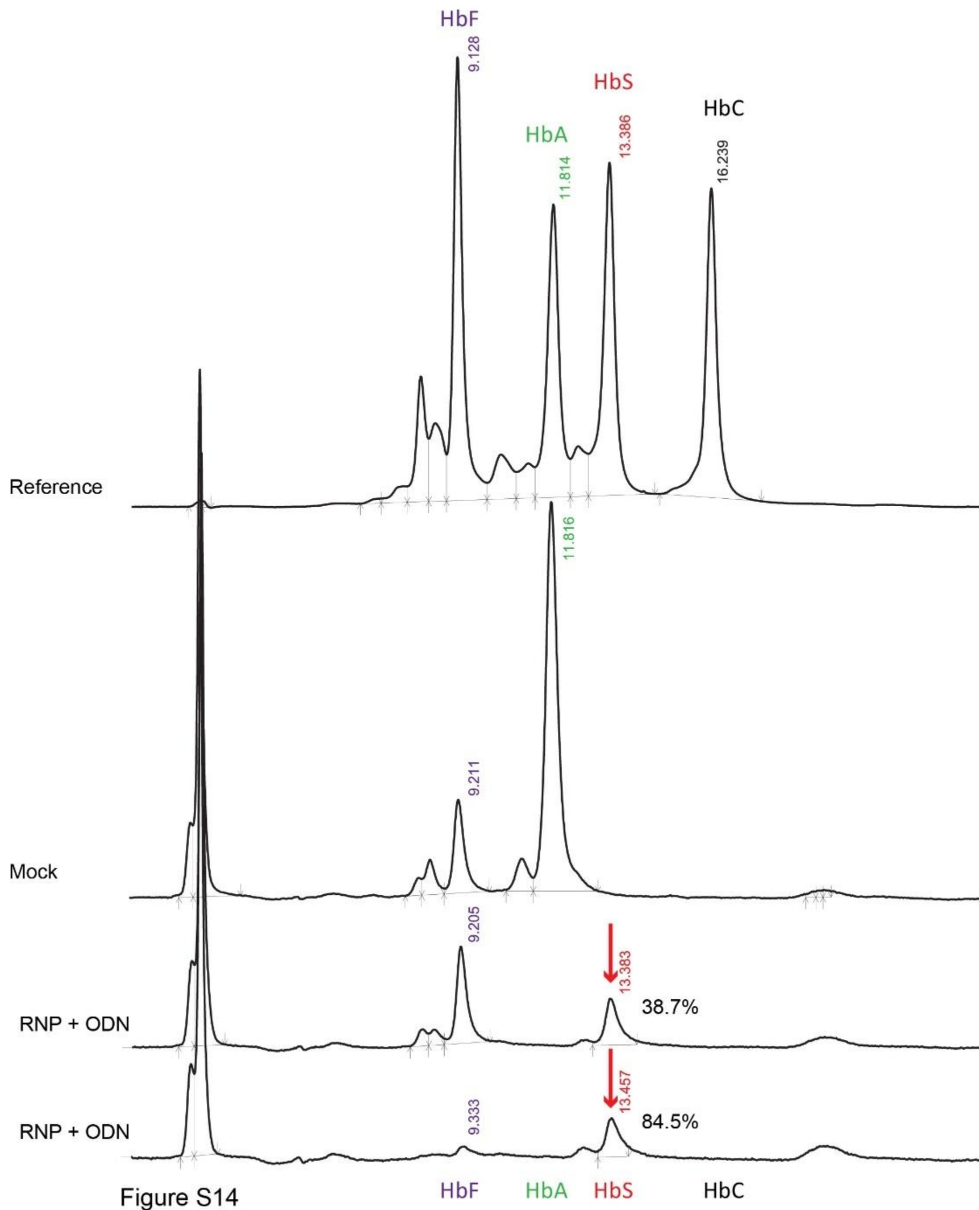


Figure S14. **IEC analysis of edited and differentiated erythroid colonies.** Example of ion exchange HPLC of single BFU-E colonies from transplant 4 to measure globin tetramers (HbF: Fetal, HbA: Adult, HbA2: Minor adult, HbS: Sickle). Lower traces demonstrate sickle globin expression (red arrow) in single colonies derived from engrafted, GTC (E6V) ssODN-edited, HSC. Vertical numbers are HPLC elution times.

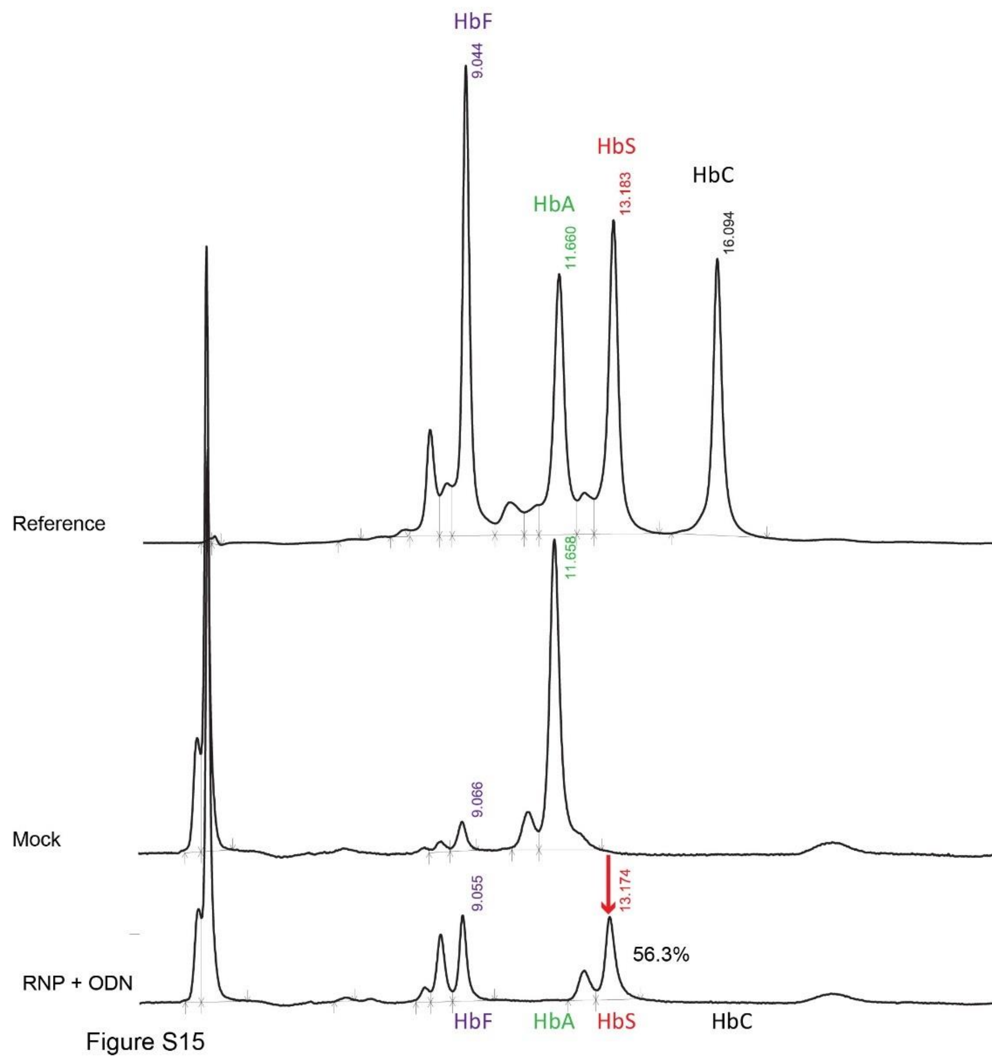


Figure S15. IEC analysis of edited and differentiated erythroid colonies. Ion exchange HPLC of single BFU-E colonies from transplant 3 to determine globin tetramers (HbF: Fetal, HbA: Adult, HbA2: Minor adult, HbS: Sickle). Lower trace demonstrates sickle globin expression (red arrow) in a colony derived from engrafted, GTC (E6V) ssODN-edited, HSC. Vertical numbers are HPLC elution times.

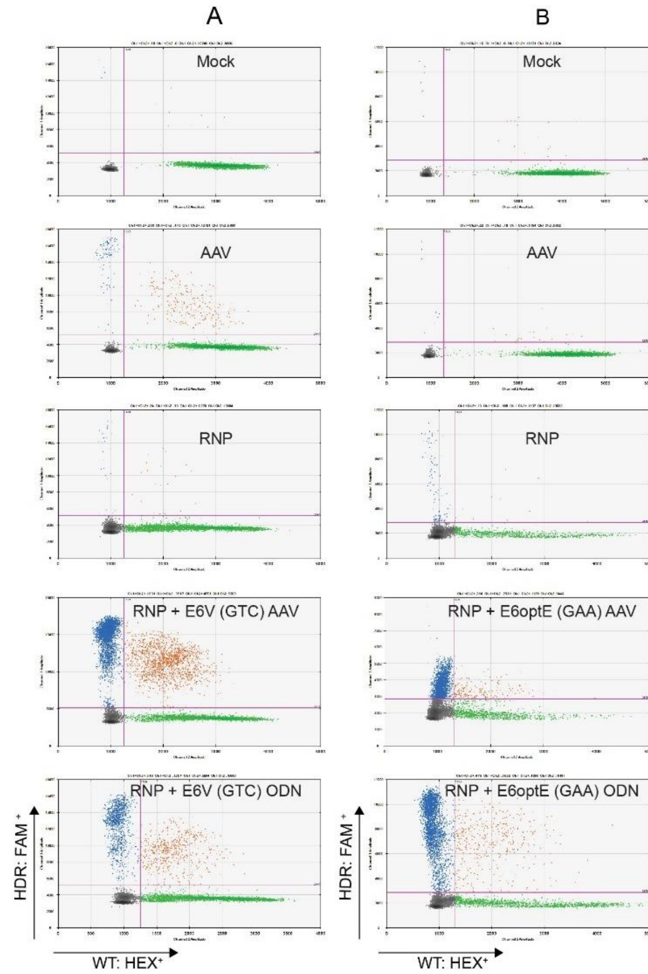


Figure S16

Figure S16. A dual probe assay to detect HDR and WT events using ddPCR. (A) Optimization of ddPCR assay with Mock, GTC (E6V) AAV, RNP-alone, RNP + GTC (E6V) AAV and RNP + GTC (E6V) ODN (B) Optimization of ddPCR assay with Mock, GAA (E6optE) AAV, RNP-alone, RNP + GAA (E6optE) AAV and RNP + GAA (E6optE) ODN.

Supplemental Table 1. Primers and probes used for analysis

Primers	Forward	Reverse
HBB-1250	AGGCTTTTTGTTCCCCCAGA	AGCCTTCACCTTAGGGTTGC
SCL-386	GGGTTGGCCAATCTACTCCC	CCTCTGGGTCCAAGGGTAGA
ddPCR	CATAAAAGTCAGGGCAGAG	GTCTCCTTAAACCTGTCTTG
LINC01206	CAAAAAGCAAAATTTGGGGATA	CTTTTAGCCCAGTGCCAGAC
MIR7974	ATCAGCCCCTCTTTCTGGAT	AGTGCAGTGGTGCCATCATA
HBD	CAGATCCCCAAAGGACTCAA	GCGGTGGGGAGATATGTAGA
TULP4	CACGCCAGGATGTAAGCTCT	TCTGAGGCAAAAGTGCAAGA
DENND3	GGGGGTTTCTATCCCTCACT	CAAGAGGGTCAGGTTGAGGA
Miseq primers with adapters		
HBB-Miseq	[a]**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGGTTGGCCAATCTACTCCC	[a]**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCTCTGGGTCCAAGGGTAGA
HBD-Miseq	[a]**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CACAAACTAATGAAACCCTGCT	[a]**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TCTACACATGCCCAGTTTCCA
Cloning primers		
AAV: 1314: 5' HR: F	GGGGTTCCTGCGGCCGCGATTCAAACTTCCGCAGAACACTTTATTTCACATATACATGCCTCTTA	
AAV: 1314: 5' HR: R	AGTAACGGCAGACTTCTCTTCAGGAGTCAGATGCACCATGG	
AAV: 1314: 3' HR: F	TGGTGCATCTGACTCCTGTCGAGAAGTCTGCCGTTACTGCCCT	
AAV: 1314: 3' HR: R	GGGTTCCTCCTGCAGGGGATCCGATCAGGGAAGAAGGGCTCACAGGACAGTCAAAC	
AAV: 1373: 5' HR: F	GGGGTTCCTGCGGCCGCGATTCAAACTTCCGCAGAACACTTTATTTCACATATACATGCCTCTTA	
AAV: 1373: 5' HR: R	AGTAACGGCAGACTTCTCTTCAGGAGTCAGATGCACCATGG	
AAV: 1373: 3' HR: F	TGGTGCATCTGACTCCTGAAGAGAAGTCTGCCGTTACTGCCCT	
AAV: 1373: 3' HR: R	GGGTTCCTCCTGCAGGGGATCCGATCAGGGAAGAAGGGCTCACAGGACAGTCAAAC	
Probes		
GTC HDR FAM	CTCCTGTCGAGAAGTCTGC	
GAA HDR FAM	CTCCCGAAGAGAAGTCTGC	
GTG HDR FAM	CTCCTGTGGAGAAGTCTGC	
GAG WT HEX	TGACTCCTGTCGAGAAGT	
REF HEX	GTTCACTAGCAACCTCAAACAGACACC	

[a] Sequences in grey are adapter sequences.

Supplemental Table 2. sgRNA and TALEN sequences used for nuclease screen.

Identifier	Strand	Target Sequence	PAM Sequence
sgRNA-g1	Antisense	GUAACGGCAGACUUCUCCUC	AGG
sgRNA-g2	Sense	GUCUGCCGUUACUGCCCUGU	GGG
sgRNA-g3	Sense	UCUGCCGUUACUGCCCUGU	GGG
sgRNA-g4	Sense	AGUCUGCCGUUACUGCCCUG	TGG
sgRNA-g5	Sense	AAGGUGAACGUGGAUGAAGU	TGG
sgRNA-g6	Antisense	CUUGCCCCACAGGGCAGUAA	CGG
TALEN (L)	-	GCATCTGACTCCTGA (15 bp Spacer)	-
TALEN (R)	-	TGCCCCACAGGGCAGTA (15 bp Spacer)	-

Supplemental Table 3. List of antibodies used for analysis.

Antibody	Florochrome	Vendor	Catalog #
CD3	APC-Cy7	BD biosciences	557757
CD71	PE	BD biosciences	555537
h235a	Pacific Blue	BD biosciences	562938
hCD133	PE	Miltenyi Biotec	130-080-801
hCD19	PE/Cy7	Fisher Scientific	50-112-9016
hCD33	Alexa Fluor 700	Fisher Scientific	56-0338-41
hCD33	Alexa Fluor 700	BD biosciences	561160
hCD33	PE	BD biosciences	555450
hCD34	APC/Cy7	BioLegend	343514
hCD38	APC	ThermoFisher Scientific	17-0389-42
hCD38	PerCP-Cy5.5	BD biosciences	551400
hCD45	FITC	Fisher Scientific	50-100-66
hCD45	Pacific Blue	Fisher Scientific	50-163-02
hCD90	PE/Cy7	BD biosciences	561558
mCD45	APC	Fisher Scientific	50-149-71
mCD45	PE	Fisher Scientific	50-103-70
mCD45	PerCP-Cy5.5	Fisher Scientific	50-157-98

Supplemental Table 4. ssODN sequences used for HDR.

ssODN sequences	
E6V GAG > GTC	T*C*AGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAA CCTCAAACAGACACCATGGTGCATCTGACTCCT**GTC**[a]GAGAAGTCTGCCGTTACTGCCCT GTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG*G*T
E6V GAG > GTG	T*C*AGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAA CCTCAAACAGACACCATGGTGCATCTGACTCCT**GTG**[a]GAGAAGTCTGCCGTTACTGCCCT GTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG*G*T
E6optE GAG > CCC GAA	TCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGACACAACTGTGTTCACTAGCAA CCTCAAACAGACACCATGGTGCATCTGACT**CCCGAA**[a]GAGAAGTCTGCCGTTACTGCCCT GTGGGGCAAGGTGAACGTGGATGAAGTTGGTGGTGAGGCCCTGGGCAG*G*T

[a] Sequences in red are changes from WT sequences, * phosphorothioate linkages

Supplemental Methods.

Erythroid cell lysis. Erythroid cells cultured in differentiation media for 14 days were collected and washed in PBS to remove contaminating proteins. A hypotonic lysis of cells in HPLC grade water was performed. The supernatant of hemolysates were centrifuged at 20,000g for 30 minutes at 4°C and 1 - 10 ug of protein were injected into columns.

RP-HPLC analysis of erythroid cells. Following erythroid differentiation, the expression of globin sub-types was assessed by RP-HPLC on a Shimadzu Prominence UFLC chromatograph using an Aeris 3.6 um Widepore C4 250 x 4.6 mm column (Phenomenex). Mobile phases used were: A: Water 0.1% TFA (trifluoroacetic acid), B: Acetonitrile 0.08% TFA at a flow rate of 0.8 ml/min. A gradient from 39% to 50% B was run over a 75-minute timed program. The column oven temperature was 40°C and the sample tray was at kept at 4°C. The peaks were detected at 220 nm. A reference was run to compare the elution times of various globin peaks.

IEC of erythroid cells. The cells after PBS wash were analyzed on PolyCATA 200 x 2.1 mm 5µm 1000Å (PolyC#202CT0510) using the mobile phases: Phase A: Tris 40 mM, KCN 3 mM, in HPLC grade water adjusted to a pH 6.5 with acetic acid, Phase B: Tris 40 mM, KCN 3 mM in HPLC grade water, NaCl 0.2M adjusted to a pH 6.5 with acetic acid. A timed 24-minute program was used to create a 2% to 100% B gradient with a flow rate of 0.3 mL/min. The column oven temperature was 30°C and the sample tray was at kept at 4°C. The peaks were detected at 418 nm. A reference was run to compare the elution times of hemoglobin molecules.

Colony Sequencing. A 1250 bp amplicon around the cut site was amplified with HBB-1250 forward and reverse primers (Supplemental Table 1) from 50 ng of gDNA using GXL DNA polymerase (Takara Bio). The PCR product was purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA) and subcloned into Zero Blunt TOPO PCR Cloning vector (Fisher Scientific, Hampton, NH) and transformed into TOP10 competent cells (Fisher Scientific, Hampton, NH). Kanamycin-resistant colonies were picked and sequenced with SCL-386 primer. Individual sequences were analyzed to determine if sequences were WT, NHEJ or HDR outcome.

T7-Endonuclease Assay. A 1250 bp region around the nuclease cut site was amplified from total gDNA using HBB-1250 primers using GXL DNA polymerase (Takara Bio). The PCR product was purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Bethlehem, PA). 400 ng of PCR product was denatured and re-annealed in 1x Buffer 2 (New England Biolabs, Ipswich, MA) in 19 ul reaction volume. The samples were treated with T7 endonuclease I (New England Biolabs, Ipswich, MA) and incubated at 37°C for 15 minutes and then loaded on a 1% agarose gel and imaged.

Flow cytometry and analysis. Flow cytometric analysis was done on an LSR II flow cytometer (BD Biosciences) and data analysis were done using FlowJo software (TreeStar). The gates were drawn on FSC/SSC populations corresponding to live cells and Singlets drawn using FSC-A/FSC-W.

ssODN Design. Single stranded oligonucleotides (ssODNs) were commercially synthesized by IDT (Ultramer® DNA Oligonucleotides) with phosphorothioate linkages in 2 terminal nucleotides on the 5' and 3' end (Supplemental Table 4) [1]. The ssODN design parameters used the sense strand with asymmetric arms [2,3], 168 bp long and introduced GTC or GAA or GTG change at codon 6 of *HBB*.

rAAV6 design: AAV 1314 and 1373: The 5' homology arm (HA) were amplified from donor genomic DNA (*Homo sapiens* chromosome 11, GRCh38.p12 [5229094,5227003]) with primers AAV: 1314: 5' HR F and R. The 3' HA was amplified from donor genomic DNA (*Homo sapiens* chromosome 11, GRCh38.p12 [5227000,5224938]) with primers AAV: 1314: 3' HR F and R (Supplemental Table 1). The 5' HA, 3' HA, and parent AAV backbone were digested with EcoRV (New England Biolabs, Ipswich, MA), gel purified (Macherey-Nagel, Bethlehem, PA) and assembled with Gibson assembly (New England Biolabs, Ipswich, MA). One Shot Stbl3 cells (ThermoFischer Scientific, Waltham, MA) were transformed with the Gibson assembly product and colonies were sequence verified. The ITR regions were restriction digested using Fast Digest Eam1105 I, SmaI, and BglII (ThermoFischer Scientific, Waltham, MA). **AAV 1321 and 1322:** The gene fragments were synthesized by GeneArt (Thermo Fisher Scientific, Waltham, MA) and cloned into the AAV6 backbone using traditional cloning methods. The plasmid was

transformed into STBL3 cell line (Thermo Fisher Scientific, Waltham, MA), sequence verified and ITR regions verified by restriction digestion.