Supplemental data

Supplemental Methods

Isolation of CD34$^+$ hematopoietic stem and progenitor cells (HSPCs)
Circulating G-CSF–mobilized human mononuclear cells were obtained from deidentified healthy adult donors (Key Biologics, Lifeblood). Plerixafor-mobilized mononuclear cells were isolated from SCD patients (Uchida et al. manuscript under review). Enrichment of CD34$^+$ cells was performed by immunomagnetic bead selection using an AutoMACS instrument (Miltenyi Biotec). After enrichment, the CD34$^+$ cell fraction was 95% of the total, with <0.2% CD3$^+$ cells and <0.1% CD19$^+$ cells.

Optimization of *HBG1/HBG2* promoter editing in human CD34$^+$ cells via Cas9:sgRNA-1 RNP, using the Neon Transfection System
Purified recombinant Cas9 protein was obtained from Berkeley Macrolabs. Single guide RNAs were synthesized by TriLink BioTechnologies or Synthego. Chemically modified sgRNA included 2′-*O*-methyl 3′-phosphorothioate (MS) modifications at 3 terminal nucleotides at both the 5′ and 3′ ends.[1] We did not observe clear differences in activity using sgRNAs synthesized by the two manufacturers. Ribonucleoprotein complexes (RNPs) were formed by incubating different amounts of Cas9 (6.4 to 128 pmol) with sgRNA-1 (supplemental Table 3) (12.8 to 256 pmol) in 5 μL of 10 mM HEPES (Thermo Fisher Scientific, catalog # 15630080), 150 mM NaCl (Thermo Fisher Scientific, catalog # 9759).

Cryopreserved CD34$^+$ cells were thawed and prestimulated for 48 h in maintenance medium (see supplemental Table 2). The cells were then washed, resuspended in T buffer (Thermo Fisher Scientific), mixed with RNP (described below), and electroporated with 1600 V, 3 pulses of 10 ms, using a Neon Transfection System (Thermo Fisher Scientific, catalog # MPK5000). Cells were grown in culture in StemSpan™ SFEM with SCF, Flt-3l, and TPO for 4 days before the indel frequency was measured. As controls, cells were either electroporated with Cas9 alone or not electroporated.

For pilot experiments to optimize genome-editing activity, 2×10^5 CD34$^+$ cells were mixed with 6.4 to 128 pmol of RNP (as pmol of Cas9) in a volume of 10 μL before electroporation. For *in vitro* erythroid differentiation studies, 2×10^5 CD34$^+$ cells were mixed with 32 pmol of RNP (as pmol Cas9) in a volume of 10 μL. For xenotransplantation studies, 5×10^6 CD34$^+$ cells were mixed with 800 pmol of RNP (as pmol Cas9) in a volume of 100 μL or scaled at the same ratios.

Measurements of on-target indel frequencies
After editing, *HBG2* and *HBG1* promoter regions surrounding the on-target BCL11A binding site were amplified with oligonucleotide primers If1 and Ir1 to generate 322-nt and 318-nt PCR products for *HBG1* and *HBG2* respectively, then analyzed by next-generation sequencing (NGS) (see main Figure 4A and supplemental Table 3).

Two methods were used to approximate larger deletions consistent with the loss of a 4.9-kb *HBG1-HBG2* intergenic fragment that could arise after simultaneous on-target editing of both gene promoters (see main Figure 4A). **First**, the oligonucleotide primers ΔHBG2.F and ΔHBG2.R were used to amplify exon 3 of both *HBG1* and *HBG2* to generate gene-specific amplicons that differ by 1 nucleotide at cDNA position 410 (G or C at *HBG2* and *HBG1*, respectively) and analyzed by targeted next generation sequence, with reduced G reads in the common amplicon reflecting the deletion of *HBG2* exon 3. **Second**, we used a TaqMan primer (Del.p) and probe sets (Del.f-Del.r) (Thermo Fisher Scientific) to detect the loss of a sequence from 481 to 683 upstream of the Cas9/sg-

RNA1 cleavage site in *HBG1* (see main Figure 4A[2]). Quantitative PCR was performed on genomic DNA from gene-edited cells, and the results were compared to those obtained with control HUDEP-2 clones containing 0, 1, or 2 4.9-kb–deleted alleles. $\Delta\Delta C_T$ values were determined using amplification of the RNaseP gene (*RPPH1*) as a copy-number reference. Inversions of the 4.9-kb *HBG1-HBG2* intervening sequence were detected by PCR amplification of edited genomic DNA, using the primers HBG2.inv.F and HBG2.inv.R (main Figure 4D). The amplicons were fractionated by agarose gel electrophoresis and analyzed by NGS.

Libraries for NGS were made using a 2-step PCR protocol, in which the target genomic site of interest was amplified first with primers containing partial Illumina sequencing adaptors (step 1) and then with primers containing indices and the remaining necessary Illumina sequencing adapters (step 2). Briefly, target regions were amplified with locus-specific primers (If and Ir) containing universal 5′ tails. PCR amplifications were performed with MyTaq 2× Master Mix (Bioline, catalog # BIO-25041), CloneAmp™ HiFi PCR Premix (Takara, catalog # 639298), or Platinum SuperFi PCR master mix (Thermo Fisher Scientific, catalog # 12358250) according to the manufacturer's protocol. The step 1 PCR product was indexed by using 0.1× the volume from step 1 with indexing primers (P5-dual-index.F and P7-dual-index.R). Melting at 94°C for 2 min was followed by 5 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 40 s. The Illumina MiSeq platform was used to generate 2 × 250 paired-end sequencing reads at the Hartwell Sequencing Center (at St. Jude). Sequences were analyzed by joining paired reads and analyzing amplicons for indels or the desired test sequence using *CRIS.py*[3]. Indels were reported as the number of reads without the WT amplicon length.

Karyotyping and fluorescence *in situ* hybridization (FISH)

Human $CD235a^-$ cells from mouse bone marrow at 17 weeks after xenotransplantation were grown in culture in StemSpan SFEM (100 ng/mL hSCF, Flt3-L, and TPO) with colcemid (20 ng/mL) for 4 h then analyzed by G-banding and fluorescence *in situ* hybridization (FISH). For G-banding, cells were air dried on slides with trypsin and Wright's stain, using standard cytogenetic methods. Twenty metaphase cells were characterized per treatment condition. For metaphase FISH, two bacterial artificial chromosomes (BACs) were used to detect the deletion or translocation of chromosome arm 11p (the *HBB* locus). The probe telomeric to *HBB* (11p15) was labeled with red-dUTP (Alexa Fluor 594; Molecular Probes), and a probe centromeric to *HBB* (11q13) was labeled with green-dUTP (Alexa Fluor 488; Molecular Probes) by nick translation. Both labeled probes were combined with sheared human DNA and hybridized to metaphase cells derived from each sample, using routine cytogenetic methods in a solution containing 50% formamide, 10% dextran sulfate, and 2× saline-sodium citrate (SCC; Fisher Scientific). The cells were then stained with 4,6-diamidino-2-phenylindole (DAPI) and analyzed. FISH for deletion of *HBG2* was performed as described previously.[2] In brief, a 5.2-kb probe encompassing the region between the sgRNA-1 cleavage sites in *HBG2* and *HBG1* was generated by PCR amplification and cloned using TA vector (Promega). Nick translation was used to label purified DNA with red–dUTP (Alexa Fluor 594; Molecular Probes) and to label a control HBB probe (RP11-1205H24) independently with green–dUTP (Alexa Fluor 488, Molecular Probes). The probes were hybridized simultaneously with interphase and metaphase cells in 50% formamide, 10% dextran sulfate, and 2× SCC. Metaphase cells were stained with DAPI and scored for signals representing the potentially deleted region (red) and *HBB* (green).

Erythroid cell culture

Erythroid differentiation of $CD34^+$ cells was performed using a 3-phase protocol[4] (and supplementary Table 2). Phase 1 (days 1–7): IMDM with 2% human blood type AB plasma, 3% human AB serum, 1% penicillin/streptomycin, 3 units/mL heparin, 10 µg/mL insulin, 3 units/mL EPO, 200 µg/mL holo-transferrin, 10 ng/mL SCF, and 1 ng/mL interleukin IL-3. Phase 2 (days 8–14): Phase 1 medium without IL-3. Phase 3 (days 15–21): The holo-transferrin concentration was increased to 1 mg/mL, and SCF was withdrawn.

Erythroblast maturation was monitored by immuno-flow cytometry for the cell surface markers CD235a, CD49d, CD71, and Band3 (supplemental Table 1). To quantify erythroblast enucleation, $1.5–4 \times 10^5$ $CD34^+$-derived erythroid cells were incubated with Hoechst 33342 for 20 min at 37°C, fixed with 0.05% glutaraldehyde, and permeabilized with 0.1% Triton X-100. Cells were stained with FITC mouse anti-human CD235a and anti-human HbF-APC then analyzed by flow cytometry.

To assess morphology, 1.5×10^5 cells were deposited on glass slides by centrifugation at 250 rpm for 5 min, using a Cytospin™ 4 cytocentrifuge (Thermo Scientific). The cells were stained for 2 min with May-Grünwald solution (Sigma, catalog # MG1L-1L), rinsed in water, stained for 10 min with Giemsa solution (Sigma, catalog # GS500-500mL, diluted 20×), rinsed in water, and air dried, then Cytoseal™ 60 mountant (Thermo Scientific, catalog # 8310-4) was applied before the slides were examined microscopically.

To analyze erythroid BFU-E progenitors, 500 $CD34^+$ cells in 1.0 mL of MethoCult™ H4230 methylcellulose (Stemcell Technologies, catalog # 04230) with 2 U/mL EPO (EPOGEN®, Amgen), 10 ng/mL SCF (R&D Systems, catalog # 255-SC), and 1 ng/mL IL-3 (R&D Systems, catalog # 203-IL) were incubated in 35-mm tissue culture dishes. After 14 days, single colonies were enumerated, then they were lysed in hemolysate reagent (Helena Laboratories, catalog # 5125) for high-performance liquid chromatography (HPLC) and DNA-sequencing analyses to quantify indels.

HbF quantification

High performance liquid chromatography (HPLC) quantification of hemoglobin tetramers and individual globin chains was performed using ion-exchange and reverse-phase columns on a Prominence HPLC System (Shimadzu Corporation). Proteins eluted from the column were identified at 220 and 415 nm with a diode array detector. The relative amounts of hemoglobins or individual globin chains were calculated from the area under the 415-nm peak and normalized based on the DMSO control. % HbF = [HbF/(HbA + HbF)] × 100; % γ-globin = [(Gγ-chain + Aγ-chain)/β-like chains (β + Gγ + Aγ)] × 100.

***In vitro* sickling assay**

Magnetic-Activated Cell Sorting (MACS®, Miltenyi Biotec)-purified human SCD $CD235a^+$ erythroid cells (1.0×10^5 cells) from mouse bone marrow 16–18 weeks after transplantation were grown in culture in phase 3 ED medium under hypoxic conditions (2% O_2) for 48 h. The IncuCyte® S3 Live-Cell Analysis System (Sartorius) with a 20× objective was used to monitor cell sickling, with images being captured every 2 h. The percentage of sickling was measured by manual counting of sickled cells versus normal cells based on morphology and was performed in a double-blinded manner by 2 observers.

Xenotransplantion of gene-edited $CD34^+$ HSPCs into NOD.Cg-*Kit^{W-41J} Tyr^+ $Prkdc^{scid}$ $Il2rg^{tm1Wjl}$*/ThomJ (NBSGW) mice

NBSGW mice were purchased from The Jackson Laboratory (stock no. 026622). Gene-edited or control $CD34^+$ cells were administered at a dose of 1×10^6 per mouse by tail-vein injection in female mice aged 8–12 weeks. Chimerism post-transplantation was evaluated at 6, 11, and 16 weeks in the periphery (see supplemental Table 1, Panel 1 [Peripheral blood]) and at 17 weeks in the bone marrow at the time of euthanasia (see supplemental Table 1, Panel 1 [Bone marrow]). Cell lineage composition was determined in the bone marrow by using human-specific antibodies (see supplemental Table 1, Panel 1 [Bone marrow]), and lineages were sorted using a FACSAria III cell sorter (BD Biosciences). $CD34^+$ HSPCs or $CD235a^+$ erythroblasts were isolated with magnetic beads, using the human-specific CD34 MicroBead Kit UltraPure, human, (Miltenyi Biotec Inc., catalog # 130-100-453) and CD235a (glycophorin A) MicroBeads, human, (Miltenyi Biotec Inc., catalog # 130-050-501)].

Analysis of off-target mutations

CIRCLE-seq was performed on genomic DNA as previously described.[4,5] The top 26 off-target sequences predicted by CIRCLE-seq were selected for analysis in gene-edited cells. A primer set was developed for each off-target sequence by using Primer3[6] (supplemental Table 3), and amplicons were generated from edited or control DNA for high-throughput sequencing on a MiSeq instrument. On average, the amplicons were sequenced to a depth of approximately 100,000 paired-end reads.

Editing of CD34$^+$ HSPCs with the Lonza 4D-Nucleofector™ system (performed at St. Jude Children's Research Hospital) (Figure 7A)

Cas9-2xNLS was purified in 20 mM HEPES, 5% glycerol, 150 mM KCl, and 1 mM TCEP, pH 7.5. Cas9-3xNLS protein was purified in 50 mM HEPES, 5% glycerol, 150 mM KCl, and 1 mM TCEP, pH 7.5. Both proteins were purified in the St. Jude protein core facility.

CD34$^+$ HSPCs were thawed in Iscove Modified Dulbecco Medium (IMDM) (Gibco, catalog # 12440053) and pre-stimulated for 48 h in StemSpan SFEM II (Stem Cell Technologies, catalog # 9655) with 100 ng/mL human SCF, 100 ng/mL human thrombopoietin (TPO), 100 ng/mL recombinant human Flt3-ligand (Flt3-L), and 1% penicillin/streptomycin (Fisher, catalog # 1507063).

Cas9-NLS variants (50 pmol) were mixed with sgRNA (150 pmol, synthesized by Synthego) at a 1:3 molar ratio for RNP complexation and incubated for 15 min at room temperature (RT). Then, 2×10^5 cells were resuspended in P3 solution (20 µL) mixed with RNPs (5 µL) and 20 µL was transferred to a nucleofection strip (Lonza, catalog # V4SP-3096). Electroporation was performed using a Lonza 4D-Nucleofector™ according to the manufacturer's instructions, with different pulse codes (DN-100, DS-130, DS-150, and EO-100). Electroporated cells were recovered with SFEM II medium including cytokines. Genomic DNA was extracted on culture day 3 by using Agencourt DNAdvance (Beckman Coulter, catalog # A48705) then analyzed by NGS for indels.

NGS libraries were prepared with a 2-step PCR protocol. In the first step, the targeted genomic site was amplified by PCR with Phusion Hot Start Flex 2X Master Mix (New England BioLabs, catalog # M0536L) and primers with partial Illumina sequencing adaptors (If2 and Ir2) (supplemental Table 3). Amplification conditions were as follows: 98°C for 3 min, followed by 35 cycles of 98°C for 10 s, 60°C for 10 s, 72°C for 7 s, and 72°C for 3 min. In the second step, PCR was performed with a KAPA HiFi HotStart ReadyMix PCR Kit (Roche, KK2602) to add Illumina sequencing adapters (P5-dual-index and P7-dual-index) to the purified PCR product of the first step. Amplification conditions were as follows: 98°C for 45 s, followed by 10 cycles of 98°C for 15 s, 65°C for 30 s, 72°C for 30 s, and 72°C for 1 min. The Illumina MiSeq platform was used to generate FASTQ sequences with 150-bp paired-end reads, and these were analyzed by joining paired reads and analyzing amplicons, using *CRISPResso* for indel measurement.[7]

Editing of CD34$^+$ HSPCs with the Lonza 4D-Nucleofector™ and xenotransplantation (performed at Boston Children's Hospital) (Figure 7B-F)

Cas9-3xNLS was purified and reconstituted in 20 mM HEPES, 150 mM NaCl, pH 7.4. The modified synthetic sgRNA (2′-O-methyl 3′ phosphorothioate modifications in the first and last 3 nucleotides) was obtained from Synthego. The sgRNA concentration was calculated based on the full-length product reporting method. For RNP complexation, Cas9 (500 pmol) was mixed with sgRNA at a 1:3 molar ratio and incubated for 15 min at RT. A 30% glycerol solution (7.9 µL) was added to the Cas9 protein before sgRNA addition to obtain a final concentration of 2% glycerol in the RNP mix.

CD34$^+$ HSPCs were thawed and grown in culture for 24 h in X-VIVO 15 medium (Lonza, catalog # 04-418Q) supplemented with the human cytokines stem cell factor (SCF), thrombopoietin (TPO), and Flt3-L. For

xenotransplantation, 4 million cells were resuspended in 100 µL of P3 solution and mixed with RNPs. $CD34^+$ cells were transferred to 100-µL nucleofection cuvettes (Lonza, catalog # V4XP-3024) and electroporated with a Lonza 4D-Nucleofector™ using the EO-100 program in accordance with the manufacturer's recommendation. Edited cells were recovered and grown in culture in X-VIVO medium supplemented with cytokines. Twenty-four hours after electroporation, the cells were transferred to erythroid differentiation medium and were harvested on day 5. Genomic DNA was extracted using a Qiagen Blood and Tissue kit. PCR was performed with KOD Hot Start DNA Polymerase with TIDE.F and TIDE.R primers (supplemental Table 3). Amplification conditions were as follows: 95°C for 3 min; then 35 cycles of 95°C for 20 s, 60°C for 10 s, and 70°C for 10 s; followed by 70°C for 5 min. PCR products were Sanger-sequenced and analyzed for indels by using the TIDE algorithm with a 40-bp decomposition window[8], comparing genomic DNA from edited to unedited cells.

Gene-edited or control human $CD34^+$ donor cells were transplanted by retro-orbital injection into 4–5-week-old female NBSGW recipients at a dose of 8×10^5 cells per mouse. To determine donor chimerism levels and indel frequencies at 16 weeks after xenotransplantation, recipient bone marrow cells were incubated with human lineage-specific antibodies (supplemental Table 1, Panel 2), separated on a FACSAria II cell sorter (BD Biosciences), and analyzed by PCR followed by TIDE-seq.

Supplemental Tables

Supplemental Table 1. Antibodies used in flow cytometry panels

Panel	*Antibody*	*Clone*	*Manufacturer*	*Catalog #*
Erythropoiesis and fetal hemoglobin expression				
	FITC Mouse Anti-Human CD235a	GA-R2 (HIR2) (RUO)	BD Pharmingen™	559943
	Hoechst 33342	Stock 10 mM = 2000×	Millipore Sigma	B2261
	PE Anti-Human CD49d	9F10	BioLegend	304304
	PE/Cy7 Anti-Human CD71	CY1G4	BioLegend	334112
	APC Anti-Human Band3		New York Blood Center	Gift from X. An
	Anti-Fetal Hemoglobin-APC, human	REA533	Miltenyi Biotec	130-108-243
Chimerism after *xenotransplantation Panel 1*				
(Peripheral blood)	FITC Mouse Anti-Human CD45	HI30 (RUO)	BD Pharmingen™	555482
	APC-Cy™7 Rat Anti-Mouse CD45	30-F11 (RUO)	BD Pharmingen™	557659
(Bone marrow)	BV786 Rat Anti-Mouse CD45	30-F11 (RUO)	BD Horizon™	564225
	PerCP-Cy™5.5 Rat Anti-Mouse TER-119/Erythroid Cells	TER-119 (RUO)	BD Pharmingen™	560512
	BV605 Mouse Anti-Human CD45	HI30 (RUO)	BD Horizon™	564047
	PE-Cy™7 Mouse Anti-Human CD33	P67.6 (RUO (GMP))	BD Biosciences	333946
	APC-Cy™7 Mouse Anti-Human CD3	SK7 (Leu-4) (RUO)	BD Pharmingen™	557832
	CD19 (Leu™-12) PE	4G7 (IVD)	BD Biosciences	349209
	CD34 FITC	8G12 (HPCA2) (ASR)	BD Biosciences	340668
	APC Mouse Anti-Human CD235a	GA-R2 (HIR2) (RUO)	BD Pharmingen™	551336
	DAPI			
Chimerism after *xenotransplantation Panel 2*				
	Human TruStain FcX		BioLegend	422302
	TruStain fcX	93	BioLegend	101320
	V450 Mouse Anti-Human CD45	HI30	BD Biosciences	560367
	PE-eFluor 610 mCD45 Monoclonal	30-F11	Thermo Fisher	61-0451-82
	FITC Anti-Human CD235a	HI264	BioLegend	349104
	PE Anti-Human CD33	P67.6	BioLegend	366608
	APC Anti-Human CD19	HIB19	BioLegend	302212
	Fixable Viability Dye eFluor 780		Thermo Fisher	65-0865-14

Supplemental Table 2. Media and cytokines

Medium	*Component*	*Manufacturer*	*Catalog #*	*Final Concentration*
$CD34^+$ maintenance				
	StemSpan™ SFEM (base)	Stemcell Technologies	09650	
	Human Stem Cell Factor (hSCF)	R&D systems	rhSCF, 255-SC/CF	100 ng/mL
	Thrombopoietin (TPO)	R&D systems	rhTPO # 288-TP/CF	100 ng/mL
	FLT3-Ligand (FLT3-L)	R&D systems	rhFlt-3 # 3088-FK/CF)	100 ng/mL
	Penicillin-Streptomycin	Thermo Fisher Scientific	15070063	Penicillin: 50 U/mL Streptomycin: 50 µg/mL
Erythroid differentiation	*Common to all phases:*			
	IMDM (base)	Thermo Fisher Scientific	12440061	
	Human Male AB Plasma	SeraCare	1810-0001	2%
	Human AB Serum	Atlanta Biologicals	S40110	3%
	Heparin	Sagent Pharmaceuticals	NDC # 25021-401-02	3 UI/mL
	Insulin	Lilly	Humulin® R U-100, NDC # 0002-8215-17	10 µg/mL
	EPO	Amgen	EPOGEN®, NDC # 55513-144-01	3 UI/mL
	Penicillin-Streptomycin	Thermo Fisher Scientific	15070063	Penicillin 50 U/mL Streptomycin 50 µg/mL
Erythroid differentiation (phase I)	*Add the following to common components:*			
	Human Holo-Transferrin	Millipore Sigma	T0665	200 µg/mL
	Human Stem Cell Factor (hSCF)	R&D systems	rhSCF, 255-SC/CF	10 ng/mL
	Human IL-3	R&D systems	rhIL-3, 203-IL/CF	1 ng/mL
Erythroid Differentiation (phase II)	*Add the following to common components:*			
	Human Holo-Transferrin	Millipore Sigma	T0665	200 µg/mL
	Human Stem Cell Factor (hSCF)	R&D systems	rhSCF, 255-SC/CF	10 ng/mL
Erythroid differentiation (phase III)	*Add the following to common components:*			
	Human Holo-Transferrin	Millipore Sigma	T0665	1 mg/mL

Supplemental Table 3. Nucleic sequences of guide RNA and primers

Assay	*Name*	*Sequence (5' to 3')*
Single guide RNA		*Underlined sequence corresponds to target*
	sgRNA-1	G*C*U*UGUCAAGGCUAUUGGUCAGUUUUAGAGCUAGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAG UCGGUGCU*U*U*U * 2'-O-methyl modification and phosphorothioate internucleotide linkage. *Chemically modified versions of sgRNA-1 contained 2'-O-methyl modifications and phosphorothioate linkages in the first and last 3 nucleotides. gRNAs synthesized by Trilink contain an additional 5' G, while gRNAs synthesized by Synthego do not.*
Indels by NGS at HBG2/1 CCAAT box		*Underlined sequences correspond to universal 5' tails*
	If1	CACTCTTTCCCTACACGACGCTCTTCCGATCTACTGAATCGGAACAAGG CAAAGGCT
	Ir1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACCCATGGCGTCTGG ACTAGGAGCT
	If2	CACTCTTTCCCTACACGACGCTCTTCCGATCTTGACTGAATCGGAACAA GGCAAAGG
	Ir2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTATTCTTCATCCCTAG CCAGCCGC
		Underlined sequences correspond to bar coding
	P5-dual-index	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNACACTCTTTCC CTACACGACGCTCTTC
	P7-dual-index	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAG ACGTGTGCTC
	P5-dual-index.F	AATGATACGGCGACCACCGAGATCTACACNNNNNNACACTCTTTCCCT ACACGACGCTCTTC
	P7-dual-index.R	CAAGCAGAAGACGGCATACGAGATNNNNNNNNNNGTGACTGGAGTTCAGA CGTGTGCTC
Indels by TIDE at HBG2/1 CCAAT box		
	TIDE.F	GCCTACCTTCCCAGGGTTTC
	TIDE.R	TGATGGGAGAAGGAAACTAGC
Deletion of HBG2 by NGS		*Underlined sequences correspond to universal 5' tails*
	ΔHBG2.F	CACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGGAAGCTTGGTGTG TAG

	ΔHBG2.R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAGCCTATCCTTGAAAGCTCTG
Deletions of HBG2 by qPCR		
	Del.F	ACGGATAAGTAGATATTGAGGTAAGC
	Del.R	GTCTCTTTCAGTTAGCAGTGG
	Del.P	ACTGCGCTGAAACTGTGGTCTTTATGA
	TaqMan™ RNase P Control Reagents Kit	Thermo Fisher Scientific, catalog # 4316844
Inversions of HBG2 by PCR		
	HBG2.inv.F	CTGTTTCTGAGGAGTCCTGTCCTA
	HBG2.inv.R	GGCGTCTGGACTAGGAGCTTATTG
Off-target validation		
	ON-T.F	ACTGAATCGGAACAAGGCAAAGGCT
	ON-T.R	ATAACCTCAGACGTTCCAGAAGCGAGTGTG
	OT-1.F	TGGAGGTTCTTGAATGAGGCCTGA
	OT-1.R	AGAAATCTTGATGCACAGAGGGCT
	OT-2.F	ATTTTTATCCAGCTTGGCAGGGGC
	OT-2.R	CACCCCCAACCCCTTCACATGT
	OT-3.F	GCCTCCAGAAGAAATGACAAAAGGC
	OT-3.R	CCTCCCTGGTTCACGCCA
	OT-4.F	GGATGTAGTCCCACTTGTTTATTTTGT
	OT-4.R	AATTGGCCCTTTATCTGACACCA
	OT-5.F	ACTCACTGTAGGAGCTTGGAGAAGT
	OT-5.R	GGTGCTAAGTGGCAAAGCTGAGG
	OT-6.F	GCCCACCTGTAACGAAATAGAAGAGGG
	OT-6.R	TTTCCCACGGCCACATCACACA
	OT-7.F	GCAGGGGAAGCTGTCTCAAAGC
	OT-7.R	TGTGCTTGCTGGTAAGAAACATCT
	OT-8.F	TTTAGTGCTTGCCCCCTGCTCT
	OT-8.R	ACAGCAAGGCTACTGCGTCCAC
	OT-9.F	AGGGAACTCATGGTCTATCAGACACA
	OT-9.R	TCAGGACTTCAGCCTCCCCATCT
	OT-10.F	AGGATGAGGGACAGAAAACATTGCTG
	OT-10.R	TTGGGGGAGTGACTTGTGCCAG
	OT-11.F	CCTGAAGCAAAGGAATACTGGGCCT
	OT-11.R	GGTCATCAGAGGCACTGGGGGA
	OT-12.F	GCTGCACTTTTTGAAGGCAAAGTCC
	OT-12.R	GGGGAACAGGGTTAGAAAGTCAACA
	OT-13.F	AAATTTGTGTTTGGGTGTGCATGCC
	OT-13.R	AGCTGGCAGTGAGCTGTTCCAG
	OT-14.F	TCTATTTTTGCTTTTGTTGCTTGTGCT
	OT-14.R	TCAACAAGGGTGCCAAGAGTACACA
	OT-15.F	TCCGAAGACACTTTGTATTCCTGGA

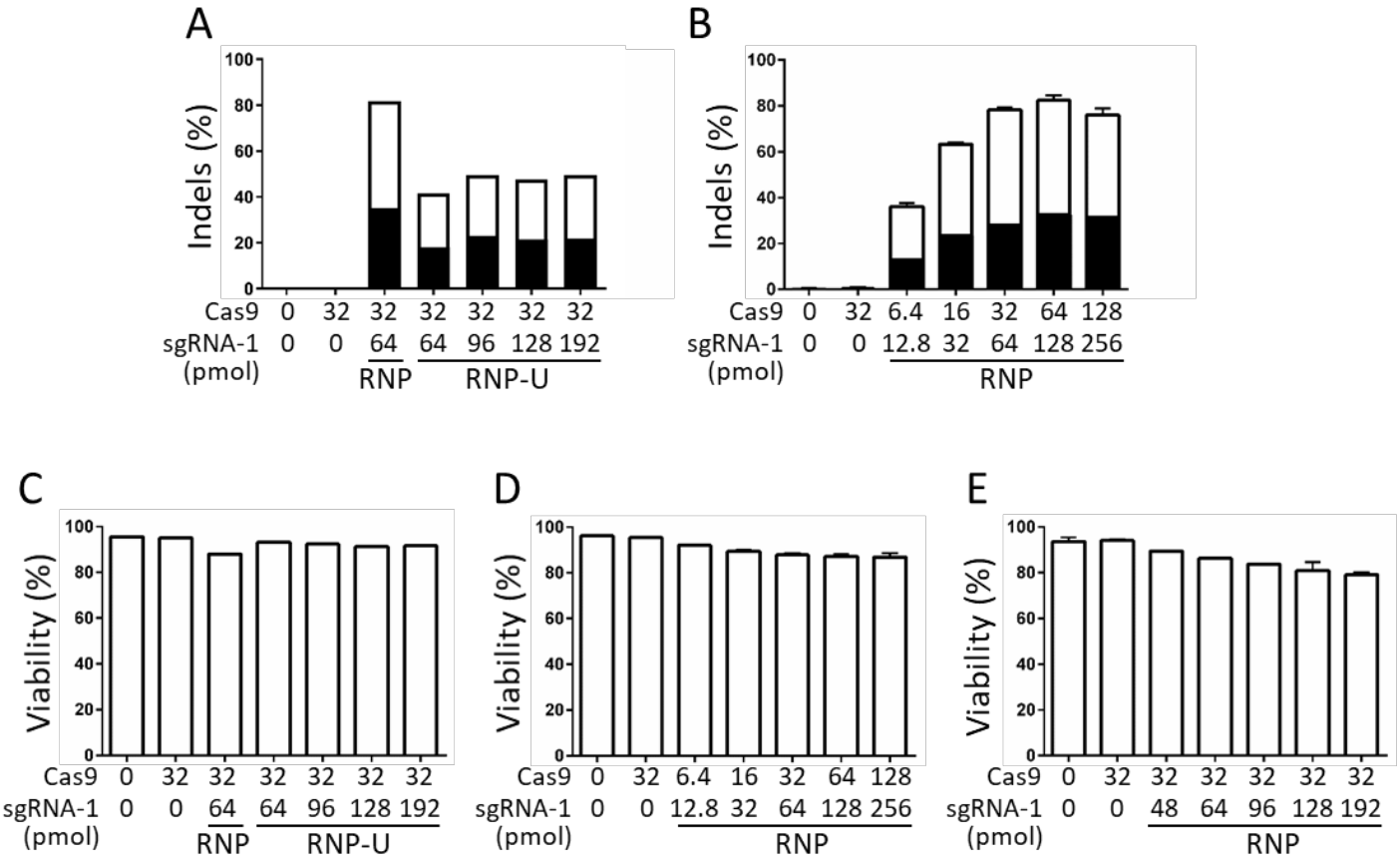
OT-15.R	TCACCACTTGGGGGTCTCCTGT
OT-16.F	ACCATGCAGCAATGTAGCAGAT
OT-16.R	CGCCCGGCCAATACTGGTTCTT
OT-17.F	GTGTCAGCCACTGCATCTGG
OT-17.R	AAGCCCACCGGTTCCTGCAC
OT-18.F	TGCTGTGGAGAAGTTTTGCAGT
OT-18.R	GGTGACAGAGCAAGACCCTC
OT-19.F	TGTTTGCTTGGTTGTTACTTTTTCAATT
OT-19.R	ACTACAGCATGATGCCGATGAAC
OT-20.F	ACTTTTGCTTTTGTTGCCTGAGC
OT-20.R	AGCCCACTAAGAAAACACAGTGGGG
OT-21.F	GGCCAGGCCTGAAATGGAGTCT
OT-21.R	GCAGCTGCTCTCCTTCCTACTCC
OT-22.F	TCTCTAAATGTTATGCCTCTCCATGAAA
OT-22.R	TTCCATGAGGACATTGAGCATGA
OT-23.F	TGTGTGTGCGTGTGTGTACATT
OT-23.R	TGGCCGGTTAAAACTAGTTTTGCCA
OT-24.F	CCACCACGCCTGACTGTGGATA
OT-24.R	ATTCACACAACATAAGCTAGGAAAATTCT
OT-25.F	GGCCAGCTCCCAGTTGTACTGT
OT-25.R	CACCCGTCTCAGCCTCCC
OT-26.F	ATGGCCCACTTGTATTCTGCCC
OT-26.R	GGGCATGGACCTCTGAAAATCTGC

Supplemental Table 4. Quantification of on-target mutations by NGS and TaqMan qPCR before and after bone marrow transplantation

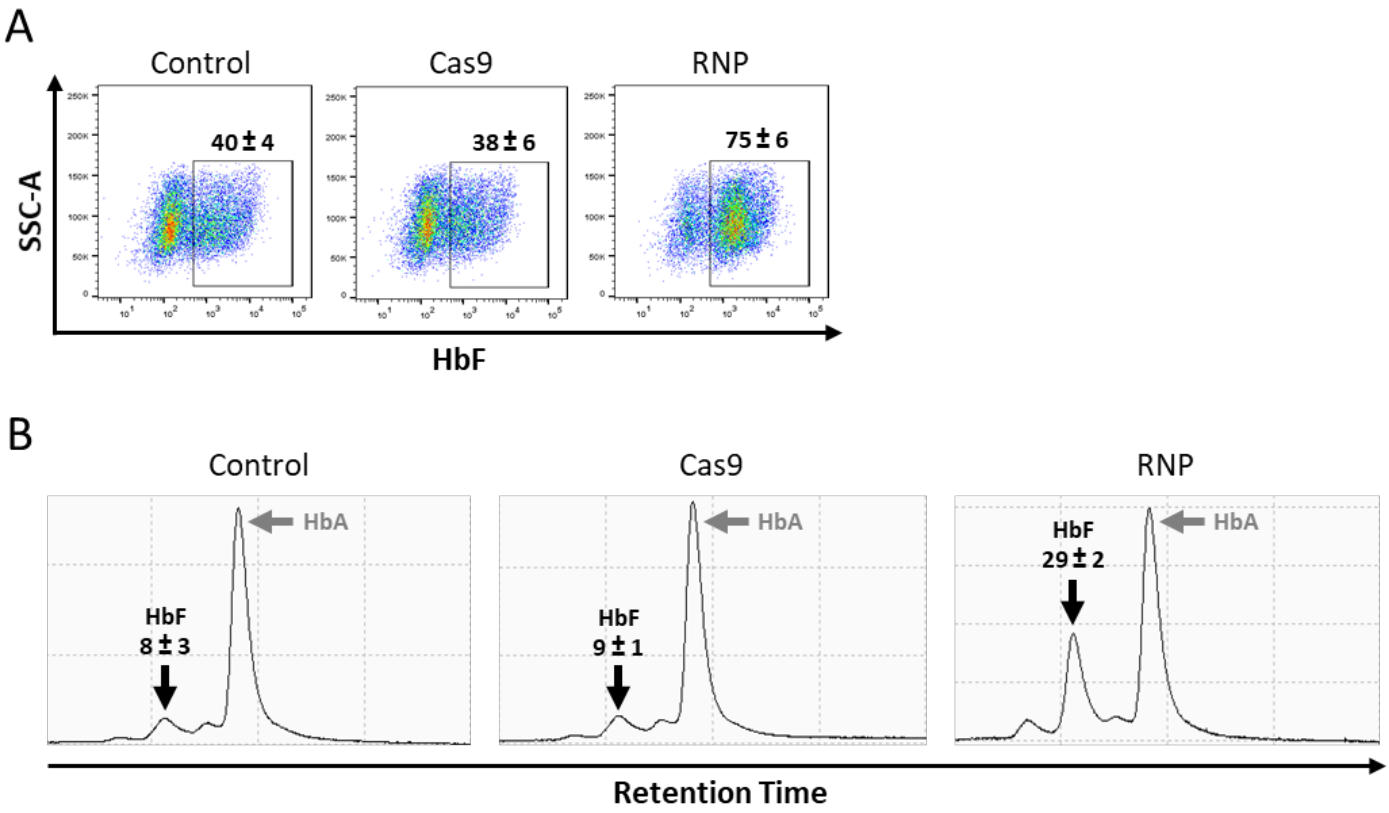
		Pre-BMT			***Post-BMT***			
Donor	**Donor type**	**% Indels (NGS)**	**TaqMan % (qPCR)**	**Δ*HBG2* % (NGS)**	**% Indels (NGS)**	**TaqMan % (qPCR)**	**Δ*HBG2* % (NGS)**	**Δ*HBG2* % (FISH)**
1	ND#1	73	32 (44)	36 (49)	44 ± 9	21 ± 9 (50 ± 26)	11 ± 13 (28 ± 26)	NTD
2	ND#2	76	31 (41)	23 (30)	45 ± 8	8 ± 4 (18 ± 11)	5 ± 2 (12 ± 3)	NTD
3	SCD#1	80	40 (50)	23 (29)	72 ± 3	33 ± 5 (46 ± 5)	23 ± 8 (33 ± 12)	32
4	SCD#2	55	19 (35)	12 (22)	18 ± 2	15 ± 2 (83 ± 13)	14 ± 8 (78 ± 42)	NTD
5	ND#1 3xNLS	96	71 (75)	NTD	90 ± 2	40 ± 6 (44 ± 7)	NTD	NTD
6	ND#2 3xNLS	94	54 (58)	NTD	96 ± 3	22 ± 10 (23 ± 10)	NTD	NTD

All values are percentages. For TaqMan and NGS, the values in parentheses indicate the percentage of *HBG2* loss normalized to small indels (per 100% small indels).
ND indicates a normal donor, whereas SCD indicates a donor with sickle cell disease. NTD: not determined.

Supplemental Figures



Supplemental Figure 1. Effects of gene editing on CD34$^+$ cell viability.
CD34$^+$ HSPCs were electroporated with Cas9 + sgRNA-1 RNP, using the Neon Transfection System, as described in Figure 1B and the Supplemental Methods. Indel frequency was determined by next-generation sequencing (NGS) after culture for 4 days. **A.** Optimization of the Cas9:unmodified sgRNA ratio for indel formation (n = 2 biological replicates). **B.** Optimization of the ribonucleoprotein (RNP) concentration for indel formation (n = 4 biological replicates with 1 donor). Ribonucleoproteins were generated by incubating the indicated amounts of Cas9 with sgRNA in 5 µL of 10 mM HEPES, 150 mM NaCl for 30 min at room temperature. The designation "RNP-U" denotes unmodified sgRNA, whereas "RNP" denotes chemically modified sgRNA, which was used in subsequent studies. For all experiments, RNPs were mixed with 2×10^5 CD34$^+$ cells resuspended in 10 µL of T buffer, electroporated at 1600V, with 3 × 10-ms pulses, incubated in culture for 4 days, then analyzed for indel formation by NGS of PCR products generated using primers located approximately 100 bp to either side of the predicted sgRNA-1 cleavage site (supplemental Table 3). The graphs show the mean ± SD indel frequency on the y-axis. The 13-nt HPFH deletion is shown in black. **C**, **D**, and **E.** Cell viability measured by flow cytometry using DAPI for the cells described in panels A and B of this figure and in main Figure 1B, respectively**.**



Supplemental Figure 2. Induction of HbF in erythroid cells derived from gene-edited normal donor CD34$^+$ cells.
CD34$^+$ cells from were edited by electroporation of Cas9 + sgRNA-1 RNP, using the Neon Transfection System, then grown in culture under erythroid differentiation conditions for 21 days. **A.** Hemoglobin F (HbF)-immunostaining "F-cells" determined by flow cytometry. **B.** HbF levels determined by isoelectric focusing high-performance liquid chromatography (IE-HPLC). Both panels show representative data as the means ± SDs of results from 7 independent experiments using CD34$^+$ cells from 2 independent donors.

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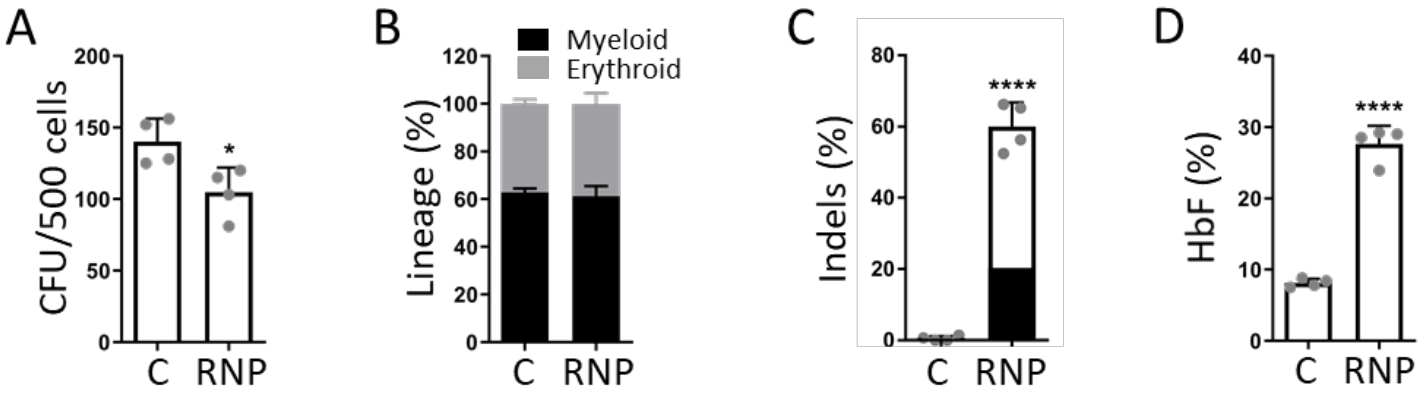
Reference	AAACTCCACCCATGGGTTGGCCAGCCTTG**CCTTGACCAATAGCCTTGACAAG**GCAAACTTG		**% Allele (Read count)**
Input	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTTG	0	30.2 (302)
	AAACTCCACCCATGGGTTGGCCAGCCTT----------GCCTTGACAAGGCAAACTTG	Δ13	28.8 (288)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGA-CAATAGCCTTGACAAGGCAAACTTG	Δ1	6.5 (65)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGA--AATAGCCTTGACAAGGCAAACTTG	Δ2	3.9 (39)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTG---AATAGCCTTGACAAGGCAAACTTG	Δ3	3.9 (39)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTG----ATAGCCTTGACAAGGCAAACTTG	Δ4	2.2 (22)
	AAACTCCACCCATGGGTTGGCC----------AGCCTTGACAAGGCAAACTTG	Δ18	1.5 (15)
	AACTCCACCCATGGGTTGGCCAGCCTTGCCTTGACCCAATAGCCTTGACAAGGCAAACTTG	+1	1.3 (13)
	AACTCCACCCATGGGTTGGCCAGCCTTGCCTTGAACCAATAGCCTTGACAAGGCAAACTTG	+1	1.3 (13)
	AAACTCCACCCATGGGTTGGCCAG----------CCAATAGCCTTGACAAGGCAAACTTG	Δ11	1.2 (12)

Reference	AAACTCCACCCATGGGTTGGCCAGCCTTG**CCTTGACCAATAGCCTTGACAAG**GCAAACTTG		**% Allele (Read count)**
BM CD34⁺ 17w Mouse 1	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTTG	0	35.9 (401)
	AAACTCCACCCATGGGTTGGCCAGCCTT----------GCCTTGACAAGGCAAACTTG	Δ13	34.7 (387)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGA-CAATAGCCTTGACAAGGCAAACTTG	Δ1	7.6 (85)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTG----ATAGCCTTGACAAGGCAAACTTG	Δ4	2.8 (31)
	CCATGGGTTGGCCAGCCTTGCCTTGACAATATTGGCCAATAGCCTTGACAAGGCAAACTTG	+9	2.2 (24)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGA-------CCTTGACAAGGCAAACTTG	Δ7	2.0 (22)
	AACTCCACCCATGGGTTGGCCAGCCTTGCCTTGACCCAATAGCCTTGACAAGGCAAACTTG	+1	1.8 (20)
	AAACTCCACCCATGGGTTGGCCAGCCTTG----------AAACTTG	Δ25	1.6 (18)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTGA--AATAGCCTTGACAAGGCAAACTTG	Δ2	1.5 (17)
	TCCACCCATGGGTTGGCCAGCCTTGCCTTGACAAGGCAATAGCCTTGACAAGGCAAACTTG	+4	1.4 (16)
	AAACTCCACCCATGGGTTGGCCAGCCTTGCCTTG---AATAGCCTTGACAAGGCAAACTTG	Δ3	1.3 (15)

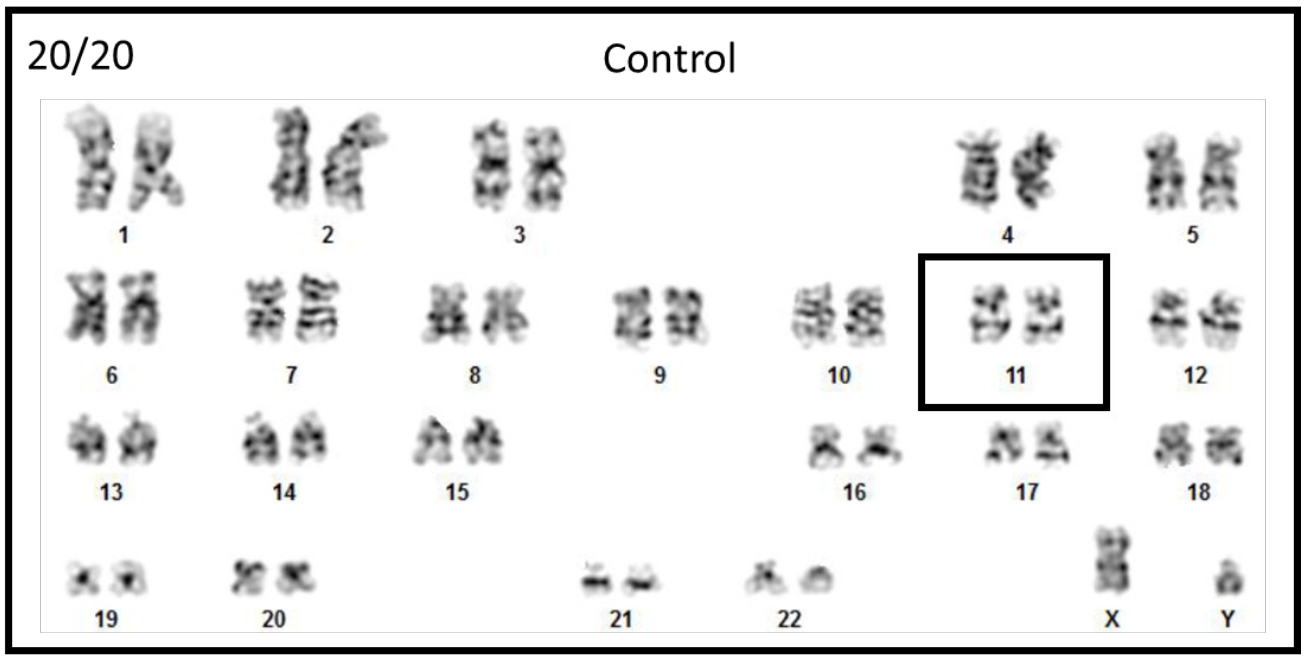
Supplemental Figure 3. Similar indel distributions before and after transplantation
Normal donor #1 CD34$^+$ cells were edited with Cas9 + sgRNA-1 RNP, using the Neon Transfection System. The top panel shows small on-target indels determined by NGS after culture for 4 days. The bottom panel shows indels in donor CD34$^+$ cells purified from the bone marrow of recipient NBSGW mice at 17 weeks after transplantation. Sequence alignments show edited region of *HBG1* and *HBG2*. The reference sgRNA-1 sequence is underlined and in bold, the PAM sequence is shown in red, and the BCL11A binding site is shown and shaded in blue. Deletions are represented by dashes. The percentage of each mutation is shown on the right, with the read counts by NGS in parentheses.



Supplemental Figure 4. Myeloid and erythroid colonies generated from normal donor HSPCs edited with Cas9:sgRNA-1 RNP, using the Neon Transfection System.

A. Five hundred HPSCs were plated in methylcellulose cultures with myeloid and erythroid cytokines. Colonies (CFU) were counted 15 days after plating control (C) (= Cas9 only) or edited (RNP) HSPCs. **B.** Relative frequencies of myeloid and erythroid colonies. **C.** Indels determined by NGS in pooled colonies. **D.** HbF expression in pooled colonies. Gray dots represent 4 technical replicates performed in a single experiment.

A



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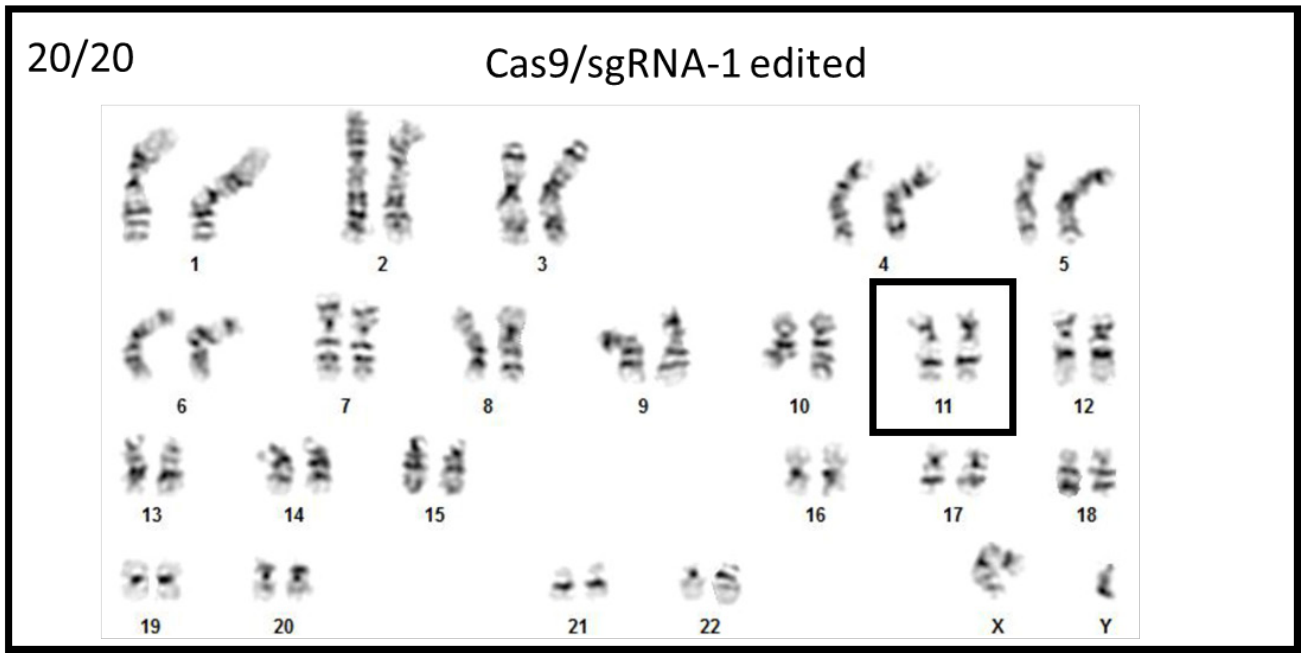
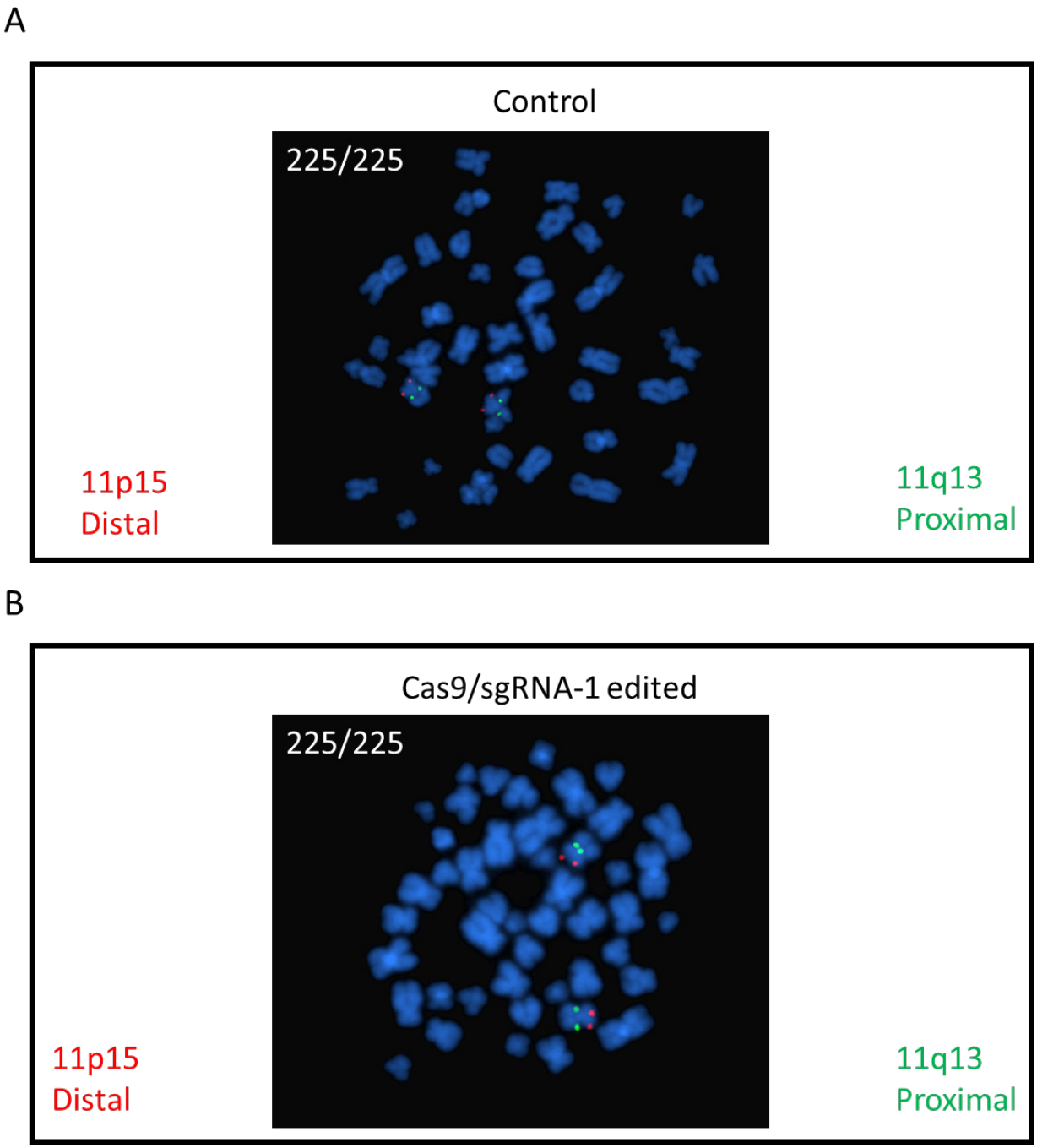
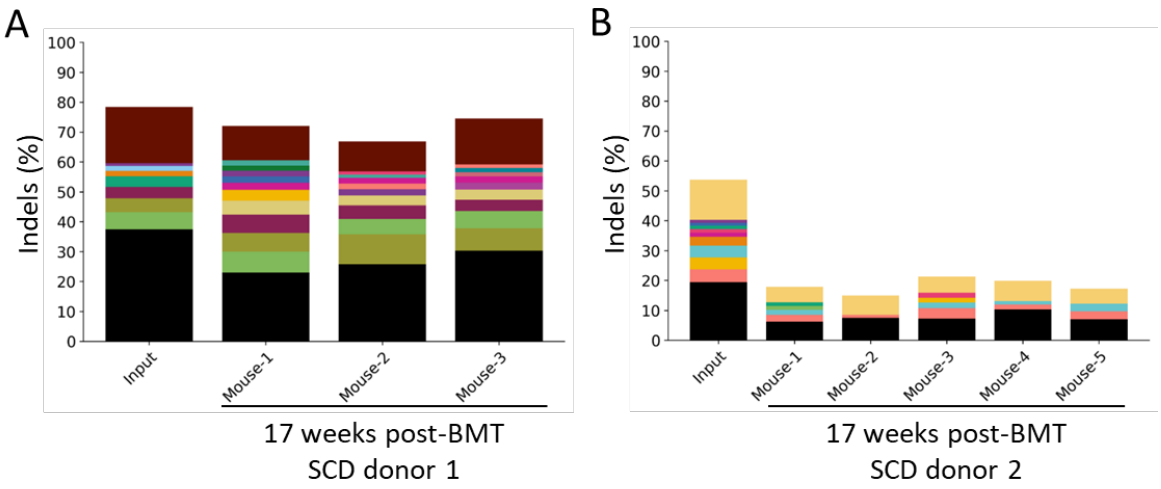


Supplemental Figure 5. G-banded karyotypes of gene-edited human cells after xenotransplantation. Human CD34$^+$ cells edited with Cas9:sgRNA-1 RNP, using the Neon Transfection System, or control unedited CD34$^+$ cells were transplanted into NBSGW mice. At 17 weeks after transplantation, human CD235a$^-$ cells were purified by immunomagnetic bead selection and karyotyped by G-banding. No chromosomal abnormalities were detected in 20 metaphase cells derived from control (**A**) or gene-edited (**B**) erythroblast populations. The frequency of on-target indels in the human donor population was 35%.



Supplemental Figure 6. Metaphase FISH from gene-edited human cells after xenotransplantation.

Human CD34$^+$ cells edited with Cas9:sgRNA-1 RNP, using the Neon Transfection System, or control unedited CD34$^+$ cells were transplanted into NBSGW mice. At 17 weeks after transplantation, human CD235a$^-$ erythroblasts were purified by immunomagnetic bead selection and metaphase fluorescence in situ hybridization (FISH) analysis was performed. The distal portion of chromosome 11 (telomeric to cut-site; 11p15) was labeled with a red-dUTP (Alexa Fluor 594) and a chromosome 11 control (centromeric to cut-site; 11q13) was labeled with a green-dUTP (Alexa Fluor 488). A total of 225 metaphase cells were counted from the control (**A**) or gene-edited (**B**) erythroblast populations. No translocations or truncations of chromosome 11 were detected in either group. The frequency of on-target indels in the human donor population was 35%.



C

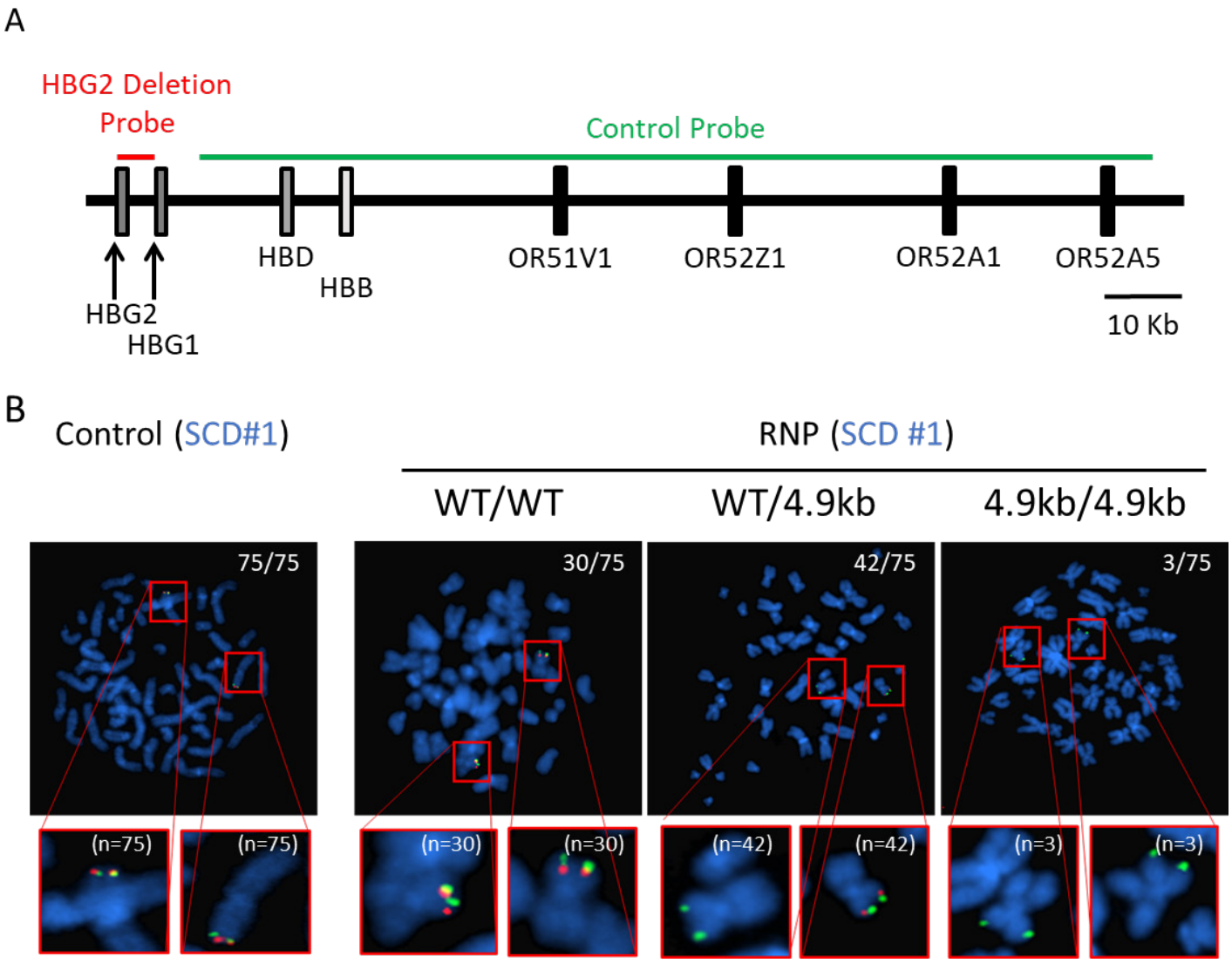
Transplant with SCD donor 1

Reference	TGGCCAGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTTGACCAATAGT		**% Allele (Read count)**
Input	TGGCCAGCCTT----------GCCTTGACAAGGCAAACTTGACCAATAGT	Δ13	37.4 (187)
	TGGCCAGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	0	21.6 (108)
	TGGCCAGCCTTGCCTTG---AATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ3	5.8 (29)
	TGGCCAGCCTTGCCTTGA-CAATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ1	4.6 (23)
	TGGCCAGCCTTGCCTTGA--AATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ2	3.8 (19)
	TGGCCAGCCTTGCCTTG----ATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ4	3.6 (18)
	TGGCCAGCCTTGCCTTG------AGCCTTGACAAGGCAAACTTGACCAATAGT	Δ6	1.8 (9)
	TGGCC----------AGCCTTGACAAGGCAAACTTGACCAATAGT	Δ18	1.6 (8)
	GGCCAGCCTTGCCTTGAACCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	+1	1.0 (5)

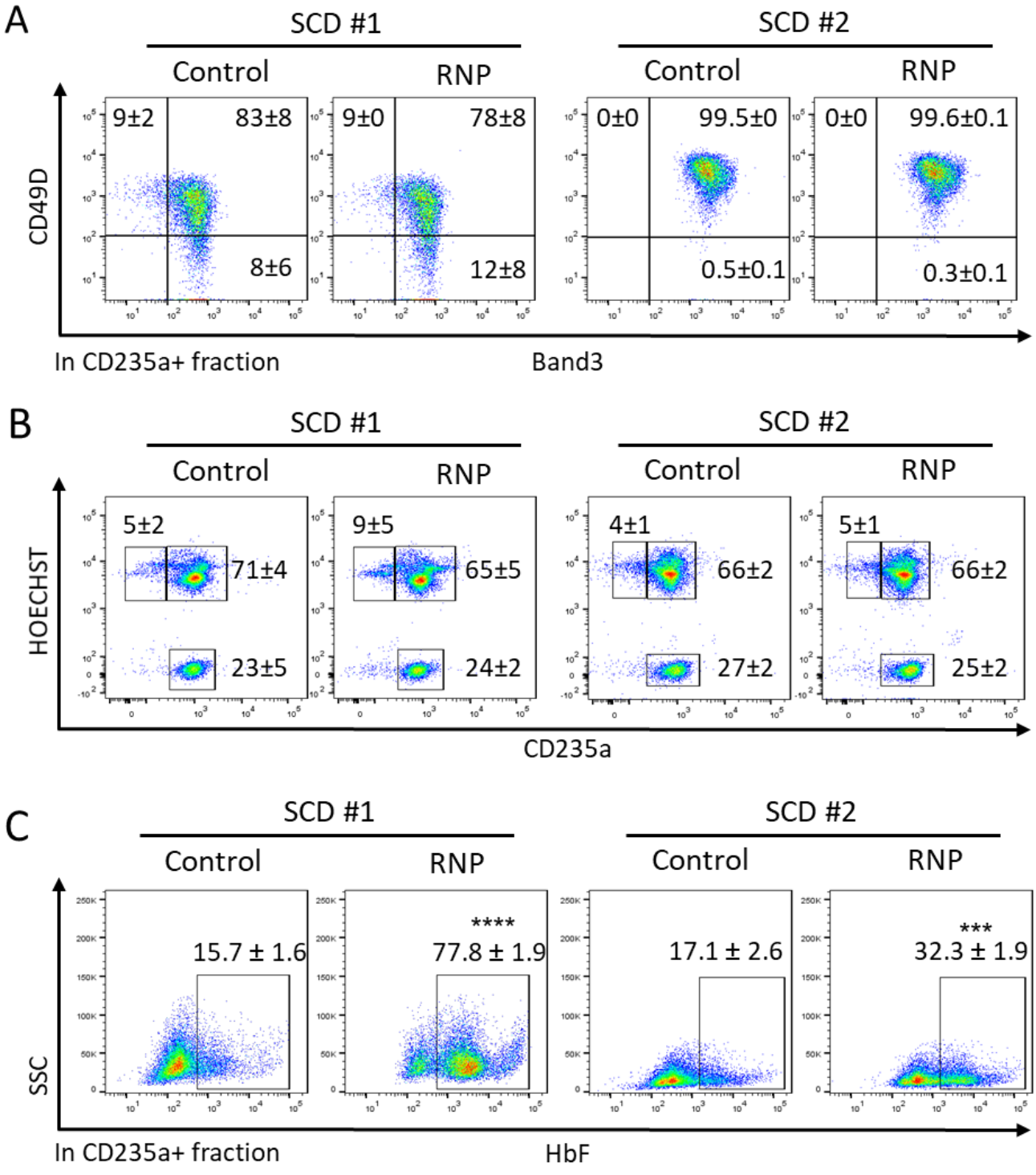
Reference	TGGCCAGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTTGACCAATAGT		**% Allele (Read count)**
BM CD34⁺ 17w Mouse 1	TGGCCAGCCTTGCCTTGACCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	0	27.9 (1115)
	TGGCCAGCCTT----------GCCTTGACAAGGCAAACTTGACCAATAGT	Δ13	23.0 (919)
	TGGCCAGCCTTGCCTTG---AATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ3	6.9 (277)
	TGGCCAGCCTTGCCTTGA-CAATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ1	6.3 (250)
	TGGCCAGCCTTGCCTTGA--AATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ2	6.1 (245)
	TGGCCAGCCTTGCCTTG----ATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ4	4.6 (185)
	TGGCCAGCCTTG------CCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ6	3.7 (147)
	TGGCCAGCCTTGCCTTG-CCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ1	2.3 (91)
	TGGCCAG----------CCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ11	2.1 (85)
	GGCCAGCCTTGCCTTGAACCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	+1	1.9 (77)
	TGGCCAGCC----------TAGCCTTGACAAGGCAAACTTGACCAATAGT	Δ13	1.8 (72)
	GGCCAGCCTTGCCTTGACCCAATAGCCTTGACAAGGCAAACTTGACCAATAGT	+1	1.7 (66)

Supplemental Figure 7. Distributions of on-target γ-globin gene promoter indels before and after xenotransplantation.

Plerixafor-mobilized CD34$^+$ cells from 2 patients with SCD were edited with Cas9:sgRNA-1 RNP, using the Neon Transfection System, and transplanted into NBSGW mice. On-target indels at the γ-globin gene promoter were measured by NGS at 3 days after editing (input) and in donor-derived CD34$^+$ cells isolated from recipient bone marrow at 16 weeks after transplantation. The bar charts show high-frequency indels in cells derived from SCD donor #1 (**A**) and SCD donor #2 (**B**). The height of each bar represents the indel frequency. Each indel is represented by a different color. The 13-nt HPFH deletion is represented in black. The remaining indels with frequencies below 1% are grouped and represented at the top of each bar in burgundy (**A**) or yellow (**B**). **C.** Sequence alignments showing the most common on-target indels determined by NGS of edited CD34$^+$ cells from SCD donor #1. The reference sgRNA-1 sequence is underlined and in bold, the PAM sequence is shown in red, and the BCL11A binding site is shown and shaded in blue. Deletions are represented by dashes. The percentage of each mutation is shown on the right, with the NGS read counts in parentheses. See also main Figure 6.



Supplemental Figure 8. Quantification of the 4.9-kb *HBG2-HBG1* intergenic deletion by fluorescence *in situ* hybridization (FISH).
CD34$^+$ cells from SCD donor #1 were edited with Cas9:sgRNA-1 RNP, using the Neon Transfection System, and transplanted into NBSGW mice. After 16 weeks, human donor CD34$^+$ cells were purified from recipient bone marrow, grown in culture under erythroid differentiation conditions for 14 days, and analyzed by FISH. **A.** Map of the region showing nearby genes and FISH probes used to detect the 4.9 kb-deleted segment and the adjacent region. The arrows indicate Cas9:sgRNA-1 cleavage sites. **B.** Metaphase FISH analysis. The HBG2-HBG1 intergenic region lost in the 4.9-kb deletion is marked by the red probe, and the adjacent control region is marked in green. The deletion was absent in 75 control (non-edited) cells shown in the leftmost panel. Among 75 RNP-edited cells (in the other 3 panels), 30 had no 4.9-kb deletion (wt/wt), 42 were heterozygous (wt/4.9kb), and 3 were homozygous (4.9kb/4.9kb).



Supplemental Figure 9. Xenotransplantation of SCD-CD34$^+$ cells into immunocompromised NBSGW mice. **A.** Erythroid maturation was measure by flow cytometry on the human CD235a$^+$ fraction with antibodies against Band3 (a marker of differentiation) and CD49d (a marker of erythroid blasts). **B.** Enucleation was monitored using Hoechst stain (nuclear dye) and CD235a$^+$. **C.** Representative scatter plot for F-cells determined by flow cytometry for the control and RNP groups. Unpaired t-tests were performed for statistical analysis; **** indicates a P-value of <0.0001.

Supplemental References

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