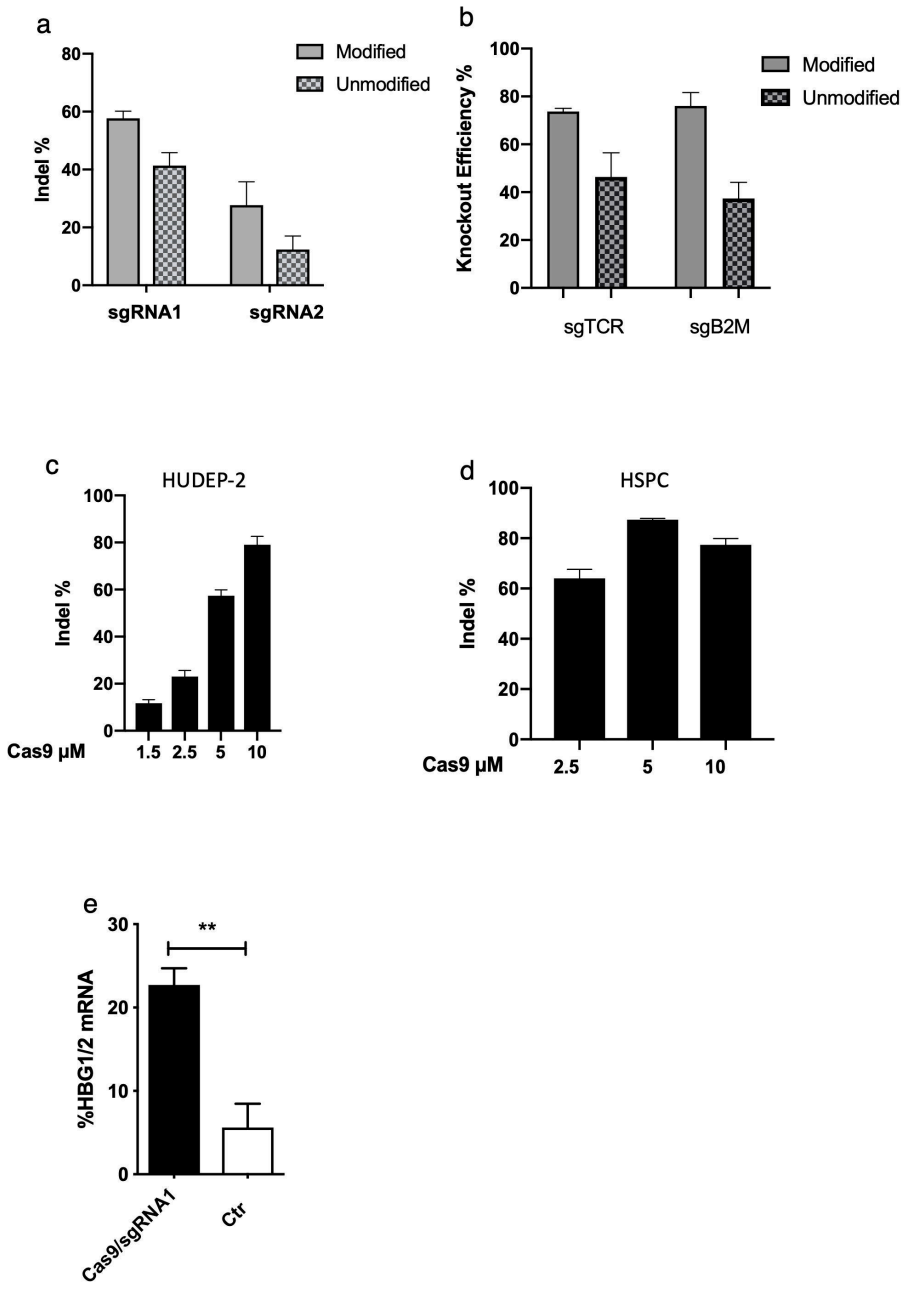

Figure S1. **a-b** Genomic editing efficiencies with modified or unmodified sgRNA on HBG promoter (a) in HUDPE-2 cells or TCR and B2M locus (b) in human primary T cells. **c-d** Indel frequency in HUDEP-2 (c) or HSPCs (d) electroporated with increasing concentrations of RNP complex. **e** γ-globin mRNA level of HUDEP-2 cell line electroporated with Cas9 RNP targeting the HBG promoter. (all data presented in the graph as mean ± s.d., **, $p<0.01$)

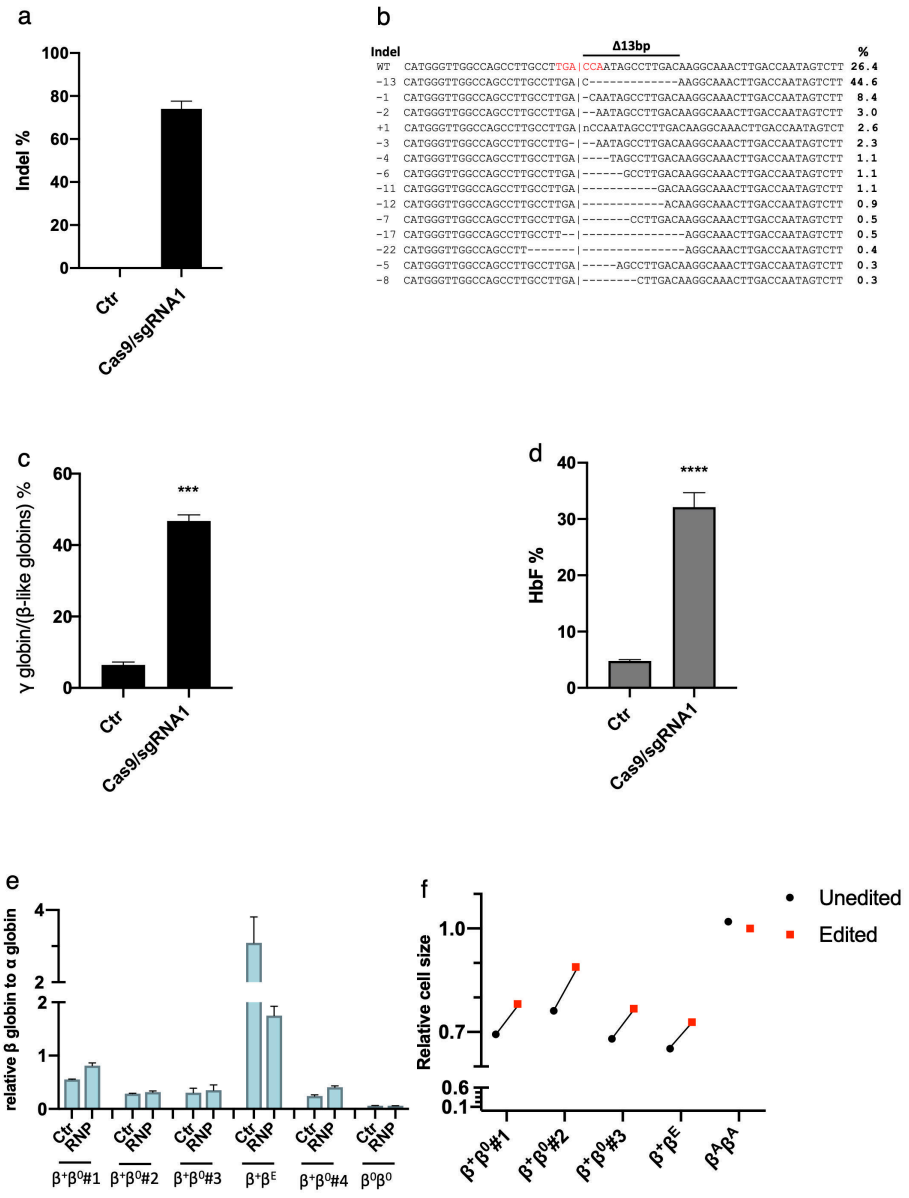

Figure S2. **a** Indel frequency of healthy HSPCs electroporated with Cas9/sgRNA1 complex or Cas9 only. **b** The most frequent indel patterns in HSPCs. Red letters indicate the BCL11A binding site. Pattern frequencies are listed to the right. Δ13bp, 13-bp deletion naturally occurred in HPFH patients. **c-d** Elevation of γ-globin mRNA expression (c) and HbF level (d) in HSPCs by RNP electroporation (data presented as mean ± s.d.). **e** β-globin mRNA expression in different patient cells electroporated with the RNP complex or not (data presented as mean ± s.d.). **f** Cell sizes of edited or control samples measured by relative forward scatter intensity. $\beta^0\beta^0$, β-thalassemia major. $\beta^0\beta^+$, β-thalassemia intermedia. $\beta^+\beta^E$, β-thalassemia intermedia containing heterozygous E26K point on *HBB* gene. $\beta^A\beta^A$, normal alleles.

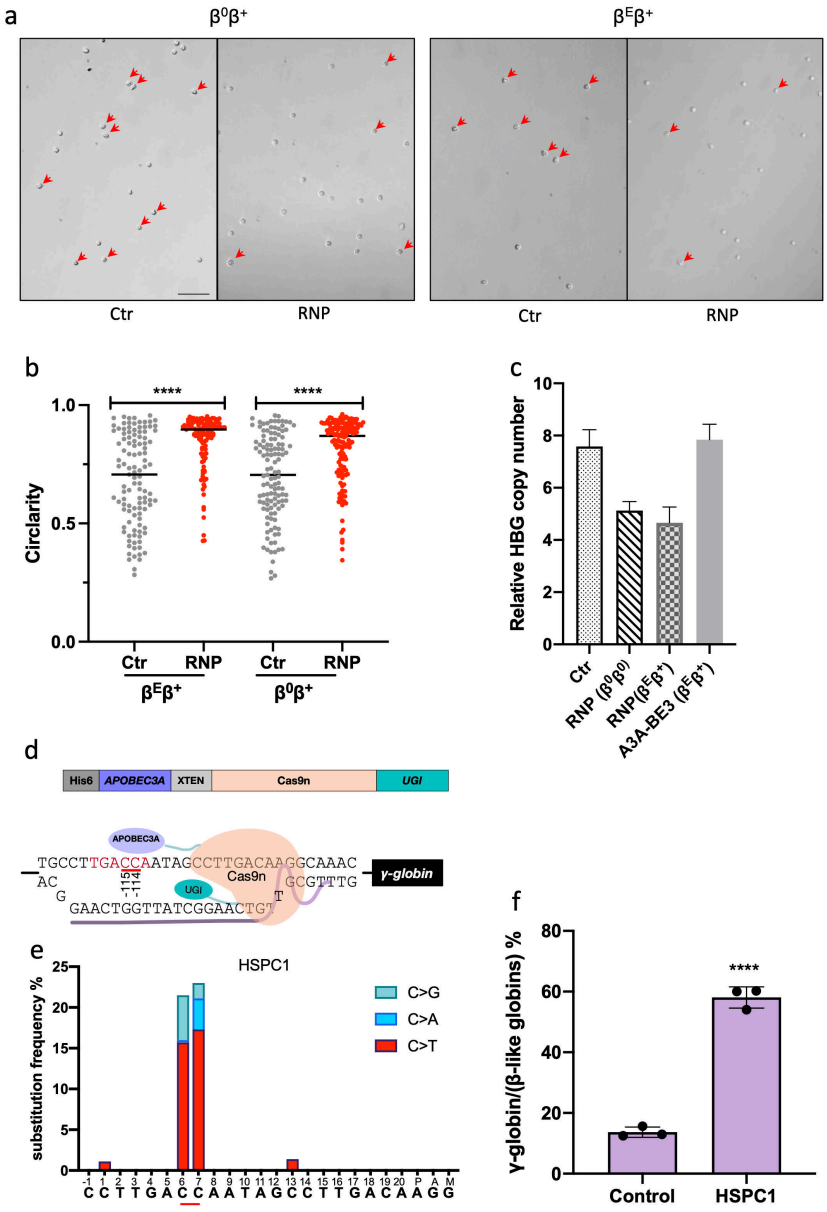

Figure S3 a Representative images of erythroid progenies from unedited/edited patient HSPCs, demonstrating a rounder and uniform cell appearance followed by HBG promoter editing. Red arrows indicate poikilocyte. Scale bar, 50 μm. **b** Cell circularities calculated by imageJ software. **c** Relative HBG copy number calculated by quantitative real time PCR. **d** Schematic view of the hA3A-BE vector and its targeting of the BCL11A binding site. BCL11A binding site is labeled in red, -114C and -115C are underlined. **e** C to D (A, T, or G) substitution frequencies induced by hA3A-BE in the targeted region in healthy HSPCs. The -115C and -114C of the HBG promoter is underlined. **f** γ-globin mRNA level (γ/(γ+β)) in HSPCs edited with hA3A-BE RNP complex or not. (All data presented in the graph as mean ± s.d., ****, $p<0.0001$, n = 3).

Supplementary Table S1

Deep sequencing analysis of the top predicted off-target sites

	target sequence	match	mismatch	type	off target rate
WT	CTTGTCAAGGCTATTGGTCA				
1	CTTG**G**C**TT**GGCTATTGGTCA	17/20	3	intronic	<0.1%
2	CT**A**GTCAAGGCTGTTTGTCA	17/20	3	intronic	<0.1%
3	CTTGTCA**G**GGCT**G**TTGGTC**G**	17/20	3	intronic	<0.1%
4	CTTGTC**C**AGGCT**GC**TGG**G**CA	16/20	4	intergenic	<0.1%
5	CTTGTC**C**AGGCT**CC**TGG**G**CA	16/20	4	intergenic	<0.1%
6	CTTGTC**C**AGGCT**CC**TGG**G**CA	16/20	4	intergenic	<0.1%
7	CTTGTC**C**AGGCT**CC**TGG**G**CA	16/20	4	intergenic	<0.1%
8	CTTGTCAAGGCT**T**T**AT**GT**A**A	16/20	4	intergenic	<0.1%
9	CT**A**GTCAAGGC**A**ATT**A**G**G**CA	16/20	4	intronic	<0.1%
10	**T**TTGTC**T**AGG**A**TATTG**C**TCA	16/20	4	intergenic	<0.1%
11	CTTGT**G**A**T**GGC**G**AT**G**GGTCA	16/20	4	intronic	<0.1%
12	CT**G**G**GA**AAGGCTATTGGT**A**A	16/20	4	intronic	<0.1%

Supplementary Table S2

Deep-seq analysis of indels in Gγ and Aγ target region

Supplementary Table S3

sgRNA-dependent off-target sites analysis of hA3A-BE

Supplementary Table S4

Primers used in this study

Quantification PCR	
ID	**sequence（5’-3’）**
HBA-f	GCCCTGGAGAGGATGTTC
HBA-r	TTCTTGCCGTGGCCCTTA
HBB-f	TGAGGAGAAGTCTGCCGTTAC
HBB-R	ACCACCAGCAGCCTGCCCA
HBG-F	GGTTATCAATAAGCTCCTAGTCC
HBG-R	ACAACCAGGAGCCTTCCCA
HBG_copy_number-f	GCTCCTAGTCCAGACGCC
HBG_copy_number-r	GCCTACCTTCCCAGGGTTTC
Sp1-promoter-f	ACCTCTCCGCCCACTAGGA
Sp1-promoter-r	CAACGGCCAACCAGAATCC
Sanger Sequencing	
ID	**sequence（5’-3’）**
Check F	GCCTACCTTCCCAGGGTTTC
Check R	ATCGGAACAAGGCAAAGGCT
Deep sequencing	
ID	**sequence（5’-3’）**
HBG1/2_F	CTTCCCCACACTATCTCAATG
HBG1/2_R	GTGTGGAACTGCTGAAGGGTG
A_gamma_F	GGCTATAAAAAAAATTAGCAGTATCC
G_gamma_F	GGCTATAAAAAAAATTAAGCAGC
Common_R	ATAACCTCAGACGTTCCAGAAGCGAGTGTG

Materials and Methods

Cell culture. Healthy donors and β-thalassemia patients were from the first affiliated hospital of Guangxi Medical University. Bone marrow (BM) blood of donors or patients was collected following hospital ethics review board approval and informed patient consent. BM blood was then diluted using DPBS (1:1 v/v) and layered on Ficoll-Paque (GE), followed by centrifuging at 500g for 45 min with the acceleration and brake off. Monocytes were harvested after centrifuging and $CD34^+$ cells were enriched using the Diamond $CD34^+$ Isolation Kit according to the manufacturer's protocol. Purified $CD34^+$ cells were cryopreserved before experiments. One day before RNP electroporation, $CD34^+$ cells were thawed and cultured in X-VIVO 15 (Lonza) supplemented with human stem cell factor (hSCF, 100ng/ml, peprotech), human thrombopoietin (hTPO, 100 ng/ml, peprotech), and human Flt3-ligand (hFlt3-L, 100 ng/ml, peprotech). For erythroid differentiation, 24 hours after electroporation (day 0 of differentiation), cells were transferred into IMDM medium supplemented with 1% L-glutamine, 330 μg/ml holo-human transferrin, 10 μg/ml recombinant human insulin, 2 IU/ml heparin, 5% pooled human solvent/detergent-treated plasma AB, 3 IU/ml erythropoietin, 100 ng/ml hSCF, 5 ng/ml human IL-3 (sigma) , 10^{-6}M hydrocortisone, as phase-1. On day 8 of differentiation, IL-3 and hydrocortisone were removed from the medium above, as phase-2. On day 12, hSCF was removed from the the phase-2 medium, as phase-3. Cells were harvested on day 18 for subsequent analysis. HUDEP-2 cells were kept in SFEM medium (stem cell) supplemented with 50ng/ml hSCF, 3 IU/ml Erythropoietin (EPO), 10 μM Dexamethasone (DEX), 1 μg/ml Doxycycline DOX. For erythroid differentiation of HUDEP-2 cells, cells were cultured in phase-2 medium supplemented with 1μg/ml Dox for 4 days and transferred into phase 3 medium for another 3 days before harvesting.

RNP electroporation. Cas9 was purchased from Aldevron. Guide RNAs were synthesized from Synthego (modified) or Genscript (unmodified). $CD34^+$ and HUDEP-2 cells were electroporated using the Lonza 4D Nucleofector. Briefly, cells were spun down at 300g for 10 min and resuspended in 20 µl electroporation buffer prepared according to the V4XP-3032 kit (Lonza). RNPs were prepared by incubating 16 µg Cas9 with 300 pmol guide RNA at room temperature for 10 min. Cells and RNP were mixed together and transferred into 16-well cuvettes for electroporation with program EO-100. After electroporation, cells were incubated at 37 °C for 5 min and washed once using DPBS before they were cultured.

Editing efficiencies and Sequencing. To check editing efficiencies of sgRNA1 and sgRNA2, genomic DNA was extracted from the $CD34^+$ or HUDEP-2 cells using the Genome Extraction kit (Tiangen DP304). Amplicons containing the target sites were amplified using KOD plus DNA polymerase (TOYOBO) and sent for sanger or deep sequencing. Deep sequencing results of on-target editing efficiencies in β-thalassemia patient-derived HSPCs were available at BioProject database: PRJNA558438. Primers used for PCR were provided in supplementary table S4. For off-target events, potential off-target sites were predicted by the web-based algorithm (http://www.rgenome.net/cas-offinder/) followed by PCR amplification, deep sequencing and analyzed by Cas-Analyzer (http://www.rgenome.net/cas-analyzer) (for Cas9) or SAMtools for (hA3A-Cas9). Off-target deep sequencing data is available at BioProject database: PRJNA558438. For knockout efficiencies on TCR and B2M sites, 48 hours after electroporation, cells were stained by anti-human TCR or anti-human B2M antibody at 4 °C for 30 min, washed with PBS for three times and loaded onto the flowcytometry for efficiency determination.

Quantification PCR. For γ-globin expression determination, total mRNA of HUDEP-2 or $CD34^+$ cells were first purified using the PicoPure RNA Isolation Kit (Thermo) and reverse transcribed by Primescript RT Master Mix (TAKARA) according to the manufacturers' protocol. qPCR was performed with Hieff UNICON SYBR Green Master Mix (Yeasen). For HBG copy number determination, genomic DNA from RNP treated or control HSPCs was used as qPCR template. HBG copy number values were normalized to Sp1 promoter numbers. Primers for qPCR are listed in the supplementary table S4.

Hemoglobin HPLC. The fetal hemoglobin level from heathy donors was measured using the Bio-Rad Variant Hemoglobin testing system. For single chain globin variants, 2×10^6 erythroid cells were lysed using 20 μl ddH_2O. Reverse HPLC was performed on an Agilent 1260 infinity II using the 4.6-nm Aeris 3.6mM Widepore C4 LC column.

Measurement of cell viability, size, circularity and enucleation. For the cell viability assay, HSPCs from patient donors were electroporated with the RNP complex. 48 hours later, cells were stained with trypan blue. Cell viability was calculated as total unstained cells/total cells. To calculated the enucleated cells, erythroid progeny cells were stained with the DNA dye Hoechst 33342 (2 μg/ml) for 15 min at room temperature, washed three times and then loaded onto the flowcytometry. Hoechst positive and negative cells were gated and quantitated. Cell size of hoechst negative cells were calculated by median value of forward scatter intensity normalized by the intensity from unedited healthy donors. For cell circularity, hoechst negative cells were sorted. Cell images were taken by Olympus ZX71 inverted microscope. Cell circularity was calculated

by Image J software according to the functions, Circularity = $(4\pi \times Area)/(perimeter)^2$. For each group, about 120 cells were calculated.

hA3A-BE purification. The DNA sequence for hA3A-BE was optimized for E. coli expression, synthesized and cloned into pET28a vector. The resulting fusion construct contained an N-terminal hexahistidine (His6) tag. The plasmid was transformed into E. coli strain BL21 (DE3) (Transgen) and single clone was cultured in 2xTY medium at 25°C until OD 0.8. Then the culture was cooled to 18°C and induced with 0.2 mM IPTG for expression for 17 hours. Cells were lysed using a homogenizer (800 bar) in buffer A (20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM TCEP). Lysate was centrifuged at 12000 rpm for 1 hour and the supernatant was loaded onto a Ni-NTA column (GE Life Sciences). hA3A-BE was eluted via a linear gradient of 0-100% buffer B (20 mM Tris pH 8.0, 500 mM NaCl, 10% glycerol, 1 mM TCEP, 250mM imidazole) followed by ion exchange purification on a 5 ml SP HiTrap column (GE Life Sciences), eluting with a linear gradient of 100 mM – 1 M KCl (GE Life Sciences). The eluted protein was further purified by gel filtration (GE Life Sciences). The final product was concentrated to about 10 mg/ml, flash-frozen in liquid nitrogen and stored at -80°C.