Supporting Information

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SI Methods

Plasmid Construction for Electrophoretic Mobility Shift Assays. Double-stranded bis-PNA binding sites were generated by annealing 25- to 36-mer oligonucleotides (Keck Synthesis Facility, Yale University). The sequences of the binding sites, flanked by EcoRI and BamHI sites for insertion into the pBlueScriptII-SK vector, are as follows: IVS2–35 5′GATCTTTTCTTTCCCCTTCTTTTCTATGGTTA and 5′ AATTTAACCATAGAAAAGAAGGGGAAAGAAAA; IVS2–64 5′ GATCGTCATAGGAAG GGGAGAAGTA and 5′AATTTACTTCTCCCCTTCCTATGAC; IVS2–194 5′GATCTTCTTTTTTTTCTTCTCCGC and 5′AATTGCGGAGAAGAAAAAAAAAGAA; IVS2–512 5′GATCTTTAAAAAATGCTTTCTTCTTTTAATATACTT and 5′AATTAAGTATATTAAAAGAAGAAAGCATTTTTTAAA; IVS2–830 5′GATCATACCTCTTATCTTCCTCCCACAGGACTGC and 5′ AATTGCAGTCCTGTGGGAGGAAGATAAGAGGTAT. Linearized pBluescript (Stratagene) vector, predigested with EcoRI/BamHI and purified by electrophoresis and gel extraction, were ligated with the duplex binding sites using the Rapid DNA Ligation Kit (Roche Applied Science). Ligated products were transformed into DH5alpha competent cells (Invitrogen) according to manufacturer's protocols, then selected, grown, and purified with a Qiagen Plasmid Miniprep Kit (Qiagen). The resulting pBlueScript derivatives were sequenced to verify the presence and orientation of the bis-PNA binding site. Two micrograms of each plasmid was used for overnight incubation with the indicated bis-PNAs for EMSA analysis.

CHO Cell Line Construction. The thalassemic beta-globin intron IVS2–1$^{G\rightarrow A}$ or its wild-type equivalent IVS2^{wt} was inserted into the EGFP cDNA sequence of the pEGFP-N1 plasmid (Clontech), between bp 105 and 106, by PCR-based homologous recombination (1), resulting in pGFP/IVS2–1$^{G\rightarrow A}$ and pGFP/IVS2^{wt}, respectively. The HinDIII-NotI fragments of these plasmids, containing the GFP/IVS2 sequence, were subcloned into the multiple cloning site of pcDNA5/FRT (Invitrogen), and the resulting constructs were stably transfected (Fugene 6; Roche) into CHO-Flp cell lines using the Flp-In System (Invitrogen). Colonies were screened for single-copy integration as previously described (2).

Cell-Cycle Synchronization. CHO cells were synchronized by two methods. In the first, cells were serum starved in Ham's F12 media with 0.1% FBS for 72–96 h to achieve G0/G1 cell-cycle synchronization. Following G0/G1 synchrony, cells were treated with 100 μM mimosine or 1.5 mM hydroxyurea for 15 h to achieve synchrony in G1 or S phase, respectively (3). An alternative method for S-phase synchronization was double thymidine block, performed as described in (4). For synchronization in M phase, CHO cells treated with double thymidine block were subsequently incubated with media containing 100 ng/ml nocodazole for 24 h (5). Similar targeting results were obtained with the two methods for S-phase synchrony in CHO cells. To achieve S-phase synchrony in K562 cells, logarithmically growing K562 cells obtained from ATCC (CCL-243), were synchronized with 10 mM hydroxyurea in RPMI media for 24 h before electroporation (6). Cell-cycle profiles were obtained by FACS analysis of propidium iodide cell staining and ModFit software analysis (data not shown).

Cell Transfection. 1×10^6 CHO cells in 100 μl Ham's F12 media were electroporated in a 0.4 cm gap cuvette with a Bio-Rad Gene Pulser at 280V, 960 μFd, and 200 Ω. Following electroporation, cells were plated in F12 media with 10% FBS. 1×10^6 K562 cells were electroporated in 100 μl RPMI media with HBB donor DNA with or without bis-PNA with a single 9 ms, 300V pulse using a BTX 830 Square Wave Electroporator (Harvard Apparatus). Following electroporation, the K562 cells were plated with RPMI supplemented with 10% FBS and harvested 48 h later for genomic DNA analysis. Beta-YAC BMCs (3.5×10^6) were electroporated using the Bio-Rad Gene Pulser at 250V, 960 μF in 250 μl RPMI serum-free media. Cells were incubated on ice for 10 min after electroporation and then transferred into IMDM supplemented with 10% FBS, antibiotics, and AP20187, a chemical supplement required for cell growth in these cells (gift of Ariad Pharmaceuticals). BMCs were harvested 48 h later for purification of genomic DNA or RNA. Human CD34+ cells were obtained from apheresis of G-CSF mobilized peripheral blood from healthy adult donors, then CD34+ selected using a Baxter 300i Isolex Device and cryopreserved (Yale Center of Excellence in Molecular Hematology, Yale University). These cells are >95% CD34+ following thaw in StemSpan Serum-Free Expansion Media (SFEM) supplemented with StemSpan CC100 cytokine mixture [100 ng/ml Flt-3Ligand, 100 ng/ml stem cell factor (SCF), 20 ng/ml interleukin-3 (IL-3), and 20 ng/ml IL-6; StemCell Technologies] 24 h before transfection. Transfection was by Amaxa nucleofection according to manufacturer's protocols (Human CD34+ Nucleofection Kit, Amaxa). Cells were plated with SFEM with 1× cytokine mixture supplementation following nucleofection. At 48 h following nucleofection, the cells were divided into three populations: (1) for erythroid differentiation, with SCF, 2–3 U/ml erythropoietin (EPO) and 50 ng/ml insulinlike growth factor (IGF-1) in SFEM for up to 3 weeks following nucleofection, (2) for neutrophil differentiation, with 50 ng/ml SCF, 100 ng/ml Flt-3L, 5 ng/ml IL-3, 5 ng/ml granulocyte macrophage colony stimulating factor (GM-CSF), and 30 ng/ml G-CSF in SFEM for days 2–5 following nucleofection, then 5 ng/ml IL-3 and 30 ng/ml G-CSF for days 6–9 following nucleofection, thereafter 30 ng/ml G-CSF in SFEM (all cytokines from StemCell Technologies) for up to 14 d following nucleofection, or (3) for genomic DNA harvest for analysis. CD34+-derived cells were harvested by centrifugation at the indicated times for genomic DNA analysis or cell surface staining (see below).

Flow Cytometry. At 48 h following transfection, CHO cells were visualized by fluorescence microscopy with a Zeiss Axiovert 200 microscope, then trypsinized and fixed in 2% paraformaldehyde in PBS for FACS analysis (Becton Dickinson FACSCalibur). For FACS analysis of cell surface markers, 2 to 5×10^5 treated CD34+-derived cells were harvested by centrifugation at various time points following nucleofection, then resuspended in 100 μl staining buffer (PBS with 2% FBS and 0.9% sodium azide). Human Fc receptors were blocked on ice for 10 min using 1 μl of antibodies directed against human CD16 and CD32. The cells were stained with the following antibodies for 20 min, covered in foil, at 4°C: R-phycoerythrin (PE) mouse antihuman CD36, PE mouse antihuman glycophorin A (GPA)/CD235a, FITC mouse antihuman GPA, FITC mouse antihuman CD34, and FITC mouse antihuman CD15, with appropriate isotype controls (BD Biosciences). The cells were then washed twice with PBS, resuspended in PBS, and filtered for flow cytometry (BD

FACSCalibur). For FACS sorting of erythroid cells, 2×10^6 cells were harvested as above and labeled with PE anti-GPA, then FACS sorted for the top quartile of GPA-positive cells (Yale Cell Sorting Facility, Yale University). These sorted cells were collected in SFEM and harvested for genomic DNA for allele-specific PCR analysis.

SiRNA Transfection. XPA knockdown in K562 cells was achieved by transfection (DharmaFECT 4 reagent; Dharmacon, Lafayette CO) of siRNAs designed against the human XPA sequence or GAPD as control (ON-TARGETplus SMART pool siRNA; Dharmacon). Knockdown was confirmed by immunoblotting (see below) at 48 h, 72 h, and 96 h following siRNA transfection. At 48 h following siRNA transfection, K562 cells were electroporated with bis-PNA and HBB donor DNA as described in the main text, and harvested 48 h later for genomic analysis (96 h total siRNA treatment).

Immunoblotting. K562 cells were harvested by centrifugation 48 h, 72 h, and 96 h following XPA siRNA transfection, washed with PBS, and lysed for 30 min on ice with 1× lysis buffer (50 mM Tris, 250 mM NaCl, 5 mM EDTA, 1% Igepal, 0.1% SDS, 10 mM $Na_2P_2O_7$, 10 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, and 1× protease inhibitor mixture). Cell debris was removed by 4°C centrifugation for 10 min at 10,000 rpm, and 10 μg lysate was loaded into 10% Tris-glycine polyacrylamide gels. The protein was transferred to PVDF membrane and probed with an anti-XPA rabbit antibody (7) specific for human XPA protein. HRP-conjugated goat antirabbit secondary antibody (Thermo Fisher Scientific, Waltham MA) and ECL Western Blotting Detection Reagent was used for detection (GE Healthcare, Piscataway NJ). An antiactin monoclonal antibody was used to confirm equal sample loading (Santa Cruz Biotechnology, Santa Cruz CA).

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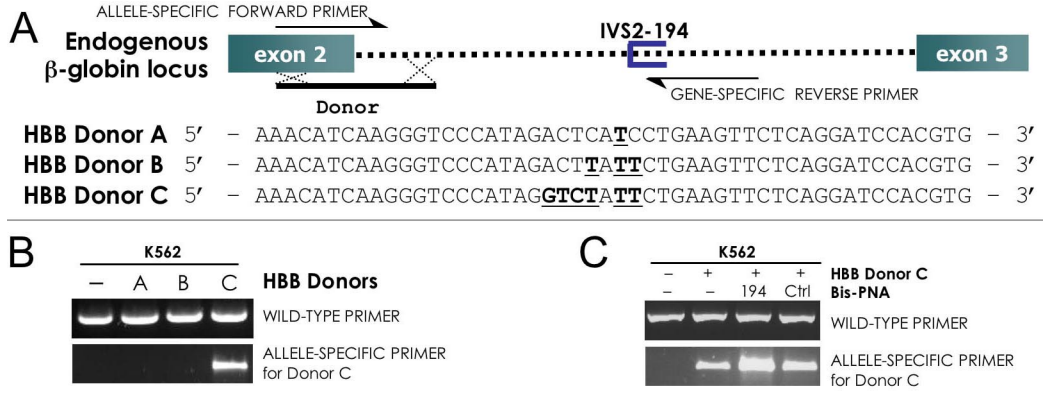

Fig. S1. Allele-specific PCR detects specific mutations introduced by different HBB donor DNAs. HBB donor C was chosen for further work and is the HBB donor used for the experiments in Fig. 4 and Fig. S2. (*A*) Schematic of three single-stranded DNA donor molecules designed for beta-globin gene modification at the exon 2/intron 2 boundary (modifications are underlined). The donor DNA sequences correspond to the template strand of the target site. (*B*) Detection of genomic modification by allele-specific PCR. Only genomic DNA from K562 cells treated with HBB donor C was amplified with the corresponding primer set specific for the sequence change introduced by HBB donor C, indicating that amplification with allele-specific primers was specific for the genomic modification made. (*C*) K562 cells were treated with bis-PNA-194 or a control bis-PNA that binds to an unrelated gene, in conjunction with HBB donor C. The control bis-PNA fails to stimulate gene modification beyond that mediated by HBB donor DNA alone.

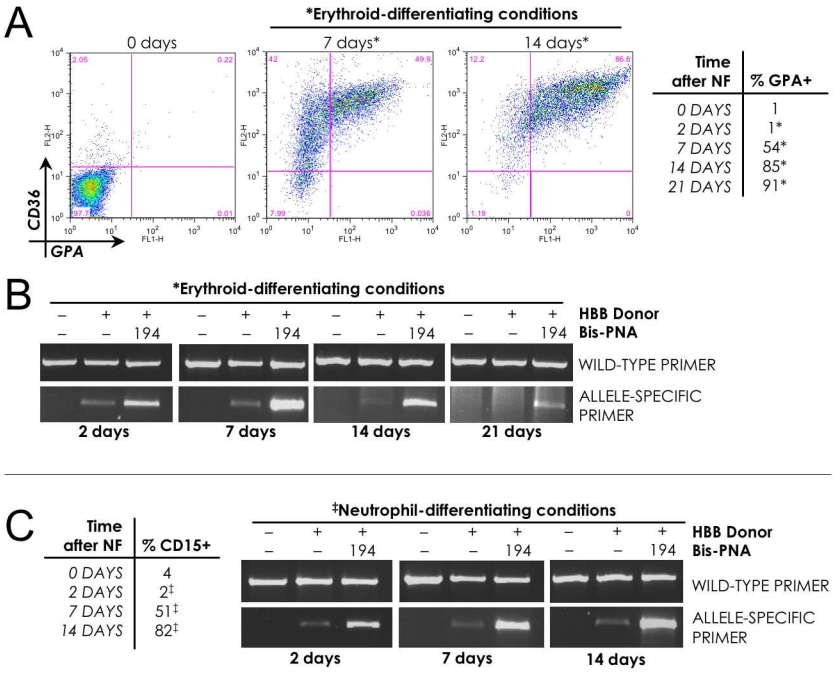

Fig. S2. Bis-PNA and donor DNA-treated human CD34+ cell populations can differentiate into erythroid or neutrophil lineages that retain the targeted gene modification. Human peripheral blood CD34+ cells were nucleofected with bis-PNA-194 and donor DNA, and placed in expansion media. At 48 h postnucleofection, the cells were placed in media containing cytokines appropriate for erythroid differentiation (denoted by * in *A* and *B*) or for neutrophil differentiation (denoted by ‡ in *C*) and maintained for 14–21 days (see SI *Methods* for culture conditions). (*A*) At the time of nucleofection (NF), less than 2% of the CD34+ cell population is positive for the erythroid markers glycophorin A (GPA) or CD36. Seven days postnucleofection, over 90% of the bis-PNA treated cells, growing in erythroid differentiation media, are positive for the erythroid marker CD36, and by 14 days following nucleofection, over 85% are positive for both CD36 and GPA. The table indicates the percentage of cells in the bis-PNA-treated cell population exhibiting surface GPA staining at the indicated day postnucleofection, relative to cell surface staining with an isotype control of the same cell population. (*B*) Genomic DNA from nucleofected hCD34+ cells, growing in erythroid-differentiating conditions, was harvested at the indicated time points following nucleofection. The genomic DNA was then used for allele-specific PCR to detect the mutation introduced by the HBB donor and bis-PNA-194. (*C*) In parallel, nucleofected hCD34+ cells were grown in neutrophil differentiation media. Fourteen days following nucleofection, 82% of the cells are positive for the neutrophil marker CD15. By contrast, only 2% of nucleofected cells grown in erythroid differentiating media are CD15 positive 14 days postnucleofection (data not shown). Allele-specific PCR of genomic DNA harvested from these differentiated cells demonstrate the presence of the mutation introduced by HBB donor and bis-PNA-194.

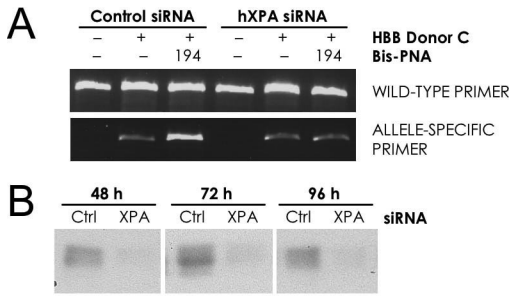

Fig. S3. Nucleotide excision repair is involved in gene modification mediated by bis-PNAs and donor DNA. (*A*) K562 cells were pretreated with siRNA targeting human XPA (hXPA) or GAPD (control) for 48 h before treatment with bis-PNA-194 and donor DNA. After an additional 48 h following bis-PNA treatment, genomic DNA was harvested from the transfected cells and subjected to allele-specific PCR to detect the presence of the specific sequence change introduced by HBB donor DNA. (*B*) Anti-XPA immunoblot of K562 cells treated with the indicated siRNA at 48 h, 72 h, and 96 h post-siRNA transfection.