An APOBEC3A-Cas9 base editor with minimized bystander and off-target activities

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Base editor technology, which uses CRISPR-Cas9 to direct cytidine deaminase enzymatic activity to specific genomic loci, enables the highly efficient introduction of precise cytidine-to-thymidine DNA alterations1–6. However, existing base editors create unwanted C-to-T alterations when more than one C is present in the enzyme’s five-base-pair editing window. Here we describe a strategy for reducing bystander mutations using an engineered human APOBEC3A (eA3A) domain, which preferentially deaminates cytidines in specific motifs according to a TCR>TCA>VCN hierarchy. In direct comparisons with the widely used base editor 3 (BE3) fusion in human cells, our eA3A-BE3 fusion exhibits similar activities on cytidines in TC motifs but greatly reduced editing on cytidines in other sequence contexts. eA3A-BE3 corrects a human β-thalassemia promoter mutation with much higher (>40-fold) precision than BE3. We also demonstrate that eA3A-BE3 shows reduced mutation frequencies on known off-target sites of BE3, even when targeting promiscuous homopolymeric sites.

In contrast to gene-editing nucleases7–9, base editors do not require double-strand breaks or exogenous donor DNA templates, and they induce lower levels of unwanted variable-length insertion/deletion mutations (indels)1,2,10, but their ability to edit all Cs within their editing window can potentially have deleterious effects. Mutations in the cytidine deaminase enzyme can shorten the length of the editing window and thereby partially address this limitation but these base editor variants still do not discriminate among multiple cytidines within the narrowed window and also possess a more limited targeting range11.

To engineer base editors with greater precision within the editing window, we leveraged the natural diversity of cytidine deaminases to identify one with greater sequence specificity than the rat APOBEC1 (rAPO1) deaminase present in the widely used BE3 architecture. BE3 consists of a Streptococcus pyogenes Cas9 nuclease, bearing a mutation that converts it into a nickase (nCas9), fused to rAPO1 and a uracil glycosylase inhibitor (UGI) (Fig. 1a). We replaced rAPO1 in BE3 with the human APOBEC3A (A3A) cytidine deaminase to create A3A-BE3 (Fig. 1a). We used A3A because previous in vitro studies showed preferential deamination of cytidines in a TCR motif (where R = A/G)12–14. To test the precision of A3A-BE3, we used a guide RNA (gRNA) targeted to a single integrated EGFP reporter gene in human U2OS cells, which bears both a cognate motif (TCG) and a non-cognate bystander (GCT) motif within its expected editing window. Surprisingly, A3A-BE3 did not preferentially edit the cytidine in the TCG motif over the GCT motif (Fig. 1b).

We hypothesized that the lack of expected sequence preference by A3A-BE3 on the EGFP site might have been due to the increased proximity of A3A secondary to its recruitment to that site. We envisioned that sequence selectivity might be restored by reducing the non-specific binding of A3A for its substrate DNA. Based on co-crystal structures of A3A and a single-stranded DNA substrate, and of A3A alone, we identified 11 residues that appear to mediate base-specific or non-specific contacts to the DNA or that are directly involved with dimerization or reside proximal to the dimer interface (Fig. 1c).13–15. Guided by this, we created 14 mutant A3A-BE3 proteins bearing one or more amino acid substitutions at each of these positions (Fig. 1b). Testing of these A3A-BE3 variants showed that most retained high activity on both the bystander and cognate motifs but that those bearing mutations in position N57 had drastically reduced bystander motif alteration while they retained near-wild-type activity on the cognate motif (Fig. 1b). We also found the preference of A3A for its cognate TCR motif could be further strengthened by combining point mutations at residues N57, K60, or Y130 (Supplementary Fig. 1). This strategy yielded the N57Q/Y130F (QF) variant, which has similar sequence preferences to the N57A/G single-mutation variants. We conclude that mutation of A3A can restore its cytidine deaminase sequence preference in the context of a base editor fusion.

We next sought to more broadly assess the precision of our A3A-BE3 fusions on a larger number of endogenous human gene sites. We tested 12 different gRNAs targeted to three different human genes and directly compared the editing activities of seven base editor fusions: three A3A-BE3 variants (bearing N57G, N57A, and N57Q/Y130F mutations in A3A), the original BE3, and three previously described BE3 variants, YE1, YE2, and YEE BE3 (YE BE3s), that have mutations...
in rAPO1 designed to slow its kinetic rate and thereby restrict the editing window\(^1\) (Fig. 1d; s.d. values shown in Supplementary Table 1). Among the seven base editors tested, A3A (N57G)-BE3 displayed the highest activity at cognate motifs while minimizing bystander cytidine editing at all of the sites tested. At eight of 12 tested sites, A3A (N57G)-BE3 induced 5- to 264-fold (median of 11.2-fold) higher editing of cognate motifs than bystander motifs in the editing window. At the remaining four tested sites, A3A (N57G)-BE3 induced less than fivefold higher editing of cognate-to-bystander motifs, but still edited bystander motifs at much lower frequencies than observed with BE3 while retaining high activity at the cognate motif. As expected, all three A3A-BE3 variants maintained a five-nucleotide editing window (approximately 5 to 9 nucleotides downstream from the 5′ end of the targeted sequence) similar to that of wild-type BE3, YE BE3s, and various A3A-BE3 variants at 12 endogenous human gene target sites, each bearing a cognate cytidine preceded by a 5′ T (indicated with a black arrow) and one or more bystander cytidines within the editing window. All editing efficiencies shown represent the mean of \(n = 3\) biologically independent samples.
A3A-BE3. Introduction of the 32-amino-acid linker between eA3A and nSpCas9 (from the recently described BE4 fusion\textsuperscript{10}) did not substantially increase editing activity or alter editing window length (data not shown). YE1 BE3 narrowed the editing window to approximately three nucleotides in most cases while still retaining catalytic activity at the cognate motif. YE2 BE3 failed to produce fewer bystander mutations compared to YE1 BE3, and YEE BE3 lost significant activity at nine sites. Based on these results, we chose the A3A (N57G)-BE3 variant for additional characterization and refer to it hereafter as eA3A-BE3 (for engineered A3A-BE3).

To examine the purity of alleles produced by eA3A-BE3, we analyzed high-throughput sequencing results obtained at the 12 endogenous human gene target sites (Fig. 1d) and found that eA3A-BE3 showed substantial differences in the frequencies of unwanted alterations compared with the original BE3. At 11 of the 12 sites, eA3A-BE3 showed altered frequencies of unwanted base substitutions (i.e., C to A or G) from those observed with BE3, with increases at 7 of the 12 sites (Supplementary Fig. 2a). This finding supports the previously proposed hypothesis that processing of genomic lesions with multiple uracils by endogenous DNA repair machinery differs from those with single uracils\textsuperscript{16}. Interestingly, we observed that eA3A-BE3 induced fewer indels than BE3 at 8 of the 12 sites (Supplementary Fig. 2b), suggesting that single-nucleotide editing does not generally produce indels at substantially different frequencies than multi-base editing.

To attempt to improve the precision of eA3A-BE3 at sites that had cognate-to-bystander editing ratios of <5, we sought to further reduce the catalytic efficiency of the eA3A-BE3 deaminase. Our rationale stemmed from the observation that the majority of bystander deamination events at these sites occur on the same DNA strand as (i.e., in cis with) a cognate event; bystander deamination without cognate deamination is found at fewer than 2% of all alleles at these sites while deamination of the cognate cytidine alone was found in at least 20% of alleles (Supplementary Fig. 3). To obtain a protein with lower catalytic rate, we added mutations to eA3A at positions homologous to three residues (E38, A71, or 196) previously shown to modulate the catalytic activity of the human AID enzyme\textsuperscript{16} and then tested these on three sites that had retained bystander deamination when edited with eA3A-BE3 (Supplementary Fig. 4). Mutations made to residues 196 and A71 greatly decreased mutation of bystander motifs at each of the three target sites while retaining 50–75% of eA3A-BE3 activity at the cognate motif. These results suggest that it may be possible to further modify eA3A-BE3 using a set of defined mutations to tune precision at sites with suboptimal cognate-to-bystander editing ratios.

We next sought to characterize and optimize the potential off-target activity of eA3A-BE3. We did this using three different gRNAs (targeted to the EMX1, FANCF, and VEGFA genes) (Supplementary Table 2), for which a number of off-target sites had been previously identified with BE3 by either Digenome-seq (performed with Rapo1-nSpCas9 also known as “BE3 ΔUGI”\textsuperscript{17}) or GUIDE-seq (performed with SpCas9 nuclease)\textsuperscript{1,18}. We also identified two potential off-target sites for a fourth gRNA (targeted to the CTNNBI1 gene) using GUIDE-seq performed with SpCas9 nuclease (Supplementary Fig. 5) and some additional closely matched sequences in the human reference genome using the in silico Cas-OffFinder program\textsuperscript{19}. We performed targeted amplicon sequencing of these 60 sites to assess base editing events induced by the BE3 and eA3A-BE3 with these four gRNAs in human HEK293T cells. For two of the four gRNAs, on-target base editing efficiency of the cognate motif with eA3A-BE3 either matched or outperformed the original BE3, although we observed small to moderate decreases in editing efficiency with the CTNNBI1 site 1 or VEGFA site 2 gRNAs (Fig. 2a–d and Supplementary Fig. 6). For 36 of the 60 potential off-target sites we examined, BE3 induced significant base editing events (compared to control amplicons from untransformed cells) (Fig. 2a–d and Supplementary Table 3). Notably, at 34 of these 36 off-target sites, eA3A-BE3 induced significantly lower frequencies of base editing events and with no significant detectable editing at 21 of these 36 sites (Fig. 2a–d). The A3A N57G mutation is critical for the higher specificity observed because an A3A-BE3 fusion lacking this alteration showed higher off-target mutations with the EMX1 site 1 and FANCF site 1 gRNAs (Supplementary Fig. 7). Addition of mutations that improve the genome-wide specificity of SpCas9 (the “HF1” and “Hypa” mutations\textsuperscript{20,21}), together with a second UGI domain further reduced off-target base editing events (reducing them to undetectable levels for all but 5 of the 15 sites that still showed detectable edits with eA3A-BE3) (Fig. 2a–d). These higher-specificity variants also improved base editing product purity and reduced frequencies of indels at on-target sites (Supplementary Fig. 8), consistent with earlier studies that used similar strategies to improve outcomes for the original BE3 (refs. 10,22).

To test eA3A-BE3 on a disease-relevant mutation, we examined its activity on a common β-thalassemia allele, found in China and in some Southeast Asian populations\textsuperscript{23,24}, for which single-nucleotide editing is critical. Mutation of position –28 of the human HBB promoter from A to G (and, therefore, T to C on the complementary strand) results in β-thalassemia (Fig. 3a). The HBB –28 C mutation can be corrected using a gRNA that has this C within its predicted editing window. However, another C (at position –25 of the HBB promoter) is also present within the editing window of this gRNA, and previous work has shown that mutation of this base can cause a β-thalassemia phenotype in humans, independent of the nucleotide present at the –28 position (Fig. 3a)\textsuperscript{25,26}. We directly compared the abilities of the original BE3, the YE BE3s, and eA3A-BE3 to edit an integrated copy of 200 bp of mutant HBB promoter sequence encompassing the –28 C and –25 C in HEK293T cells. (For technical reasons, all experiments targeting the HBB –28 (A>G) allele used a gRNA expressed with a self-cleaving hammerhead ribozyme on its 5′ end.) As expected, eA3A-BE3 showed higher precision than BE3 and the YE BE3s for selectively editing the –28 C relative to the –25 C (Fig. 3b). This resulted in substantially higher levels of perfectly corrected alleles bearing only a –28 C-to-T edit: 22.48% for eA3A-BE3 compared with 0.57%, 1.04%, 0.92%, and 0.76% for BE3, YE1 BE3, YE2 BE3, and YEE BE3, respectively (Fig. 3c). Analysis of eight potential off-target sites for the HBB-targeted gRNA (three identified by GUIDE-seq with SpCas9 nuclease (Supplementary Fig. 5) and five by in silico methods; Online Methods) showed that eA3A-BE3 induced significant off-target editing at two sites whereas BE3 induced significant editing at these same two sites and an additional third site, all at higher frequencies (Fig. 3d). As expected, the eA3A-HF1-BE3-2xUGI and eA3A-Hypa-BE3-2xUGI fusions had undetectable frequencies of off-target edits at all eight sites examined (Fig. 3d). Both high-fidelity base editor fusions also exhibited improved product purity, resulting in a reduction of unwanted –28 C-to-G edits (also known to cause β-thalassemia) from 16.3% with eA3A-BE3 to 8.8% and 7.5% with the HF1 and Hypa variants, respectively (Fig. 3c).

We next sought to determine whether eA3A-BE3 could edit the –28 (A>G) mutation at the endogenous HBB locus in erythroid precursor cells derived from human CD34\textsuperscript{+} hematopoietic stem and progenitor cells. We purified eA3A-BE3 and A3A (N57Q)-BE3 proteins to near homogeneity (Supplementary Fig. 9a) and electroporated them as ribonucleoprotein (RNP) complexes (with the HBB gRNA) into human erythroid precursors obtained from a compound heterozygous β-thalassemia patient bearing a 4-bp deletion in exon 1 of one HBB.
Our study illustrates how changing and engineering the cytidine deaminase in base editors can optimize on-target precision and reduce off-target effects. We envision that a large suite of base editor fusions can be engineered by exploiting both the rich diversity of naturally occurring cytidine deaminase domains and by modifying these enzymes using protein evolution. In our study, mutation of the N57 residue in the human A3A deaminase was critical to restoring its native target sequence precision in the context of a base editor and also to lowering its off-target editing activity. Introduction of additional mutations at I96 and A71 further refined this precision, albeit at the expense of desired cognate activity. Furthermore, the eA3A deaminase we engineered might be incorporated into and used to reduce the off-target effects of other base editor architectures that use different Cas9 orthologs for which high-fidelity variants have not yet been described (e.g., SaCas9 from *Staphylococcus aureus*).

Relative to previously published studies, our strategy of using alternative and engineered cytidine deaminases provides an orthogonal approach to improve the precision of on-target editing. An earlier study introduced mutations into the rAPOBEC1 part of BE3 that narrows the editing window but this reduces targeting range and does not permit predictable discrimination of base deamination when multiple cytidines are present in the window (as is the case with the β-thalassemia *HBB*–28 promoter mutation we successfully modified.
with eA3A-BE3). We also note that the YE BE3 variants that show the highest discrimination among multiple cytidines typically show the greatest reductions in their overall editing activity.

One limitation of eA3A-BE3 is a decreased targeting range due to the increased sequence requirements flanking the target cytidine, a restriction that might be addressed by using engineered SpCas9 protospacer adjacent motif (PAM) recognition variants and naturally occurring Cas9 orthologs with different PAM specificities. In this regard, we constructed eA3A-BE3 derivatives using the engineered VRQR or xCas9 variants of SpCas9 that have been reported to recognize sites with an NGA or NGN PAM sequence, respectively.20,28. We found that eA3A-BE3(VRQR) robustly edited nine sites bearing NGA PAMs with high efficiencies and precision (Supplementary Fig. 10). eA3A-BE3(xCas9) efficiently edited a subset of two target sites with NGT PAMs we tested, while showing lower activities on five other sites with NGT, NGC, or NGA PAMs (Supplementary Fig. 10); for all seven sites, eA3A-BE3(xCas9) generally showed higher efficiencies and higher precision than BE3(xCas9) (Supplementary Fig. 10). eA3A-BE3(VRQR) also retained its improved off-target specificity relative to the original BE3(VRQR) with two gRNAs targeted to sites containing NGA PAMs (Supplementary Fig. 11). Taken together, these results show that Cas9 variants with altered PAM recognition can be used with our eA3A-BE3 platform, suggesting that (like BE3) it behaves in a modular fashion with retention of higher on-target precision and off-target specificity even when constructed with engineered SpCas9 variants. To further expand the targeting range of the eA3A platform it may also be possible to engineer or evolve different sequence specificities into APOBEC enzymes in the context of a base editor architecture, as has been done with APOBEC enzymes in isolation.13,29. Thus, in the longer term, we envision that targeting range restriction might eventually be overcome by creating a larger series of different base editors that collectively recognize cytidines embedded in any sequence context.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.
Highly efficient RNA-guided base editing in mouse embryos. The ssDNA mutator APOBEC3A is regulated by cooperative
Crystal structure of APOBEC3A bound to single-stranded DNA
High-fidelity CRISPR-Cas9 nucleases with no detectable
5. Shimatani, Z.
4. Rees, H.A.
3. Kim, K.
2. Nishida, K.
1. Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A. & Liu, D.R. Programmable editing
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specific deaminase domains in base editor architectures.
are inventors on a patent application that has been filed for engineered sequence-
reviewed and are managed by Massachusetts General Hospital and Partners
Biopharmaceuticals. J.K.J. holds equity in Endcadia, Inc. J.K.J. ’s interests were
Biotechnologies, Pairwise Plants, Poseida Therapeutics, and Transposagen
performed experiments in
analysis. O.C. performed all experiments with assistance from J.M.G. Y .W . and J.Z.
J.M.G. conceived of the project, designed experiments, and performed data
and comments.
AUTHOR CONTRIBUTIONS
J.M.G. conceived of the project, designed experiments, and performed data
analysis. O.C. performed all experiments with assistance from J.M.G. Y.W. and J.Z.
performed experiments in β-thalassemia cells, and Y.W., J.Z., and D.E.B. designed
and analyzed these experiments. Data analysis was performed by M.K.C. with
assistance from L.P. J.K.J. conceived of experiments and directed the research. J.K.J.
and J.M.G. wrote the manuscript with input from all the authors.
COMPETING INTERESTS
J.M.G. is currently a full-time employee of and holds equity in Beam Therapeutics.
J.K.J. has financial interests in Beam Therapeutics, Editas Medicine, Monitor
Biotecknologies, Pairwise Plants, Poseida Therapeutics, and Transposagen
Biopharmaceuticals. J.K.J. holds equity in Endcadia, Inc. J.K.J.’s interests were
reviewed and are managed by Massachusetts General Hospital and Partners
HealthCare in accordance with their conflict of interest policies. J.M.G. and J.K.J.
are inventors on a patent application that has been filed for engineered sequence-
specific deaminase domains in base editor architectures.

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Sequences of proteins and their expression

Electroporation was performed using Lonza 4D+ because they were discovered and these sites represent the most comprehensive off-target characterization with Digenome-seq, an unbiased approach to discover BE3-specific off-target sites.

ml
5% human solvent detergent pooled plasma AB, 3 IU ml−1 medium (EDM) consisting of IMDM supplemented with 330 µg ml−1 glutamine, and 1% penicillin/streptomycin. During days 0–7 of culture, cultures were incubated overnight at 16 °C in an ice-water bath.

Targeted amplicon sequencing. On- and off-target sites were amplified from ~100 ng genomic DNA from three biological replicates for each condition. PCR amplification was performed with Phusion High Fidelity DNA Polymerase (NEB) using the primers listed in Supplementary Tables 2 and 4. 50 µl PCR reactions were purified with 1× volume magnetic beads. Amplification fidelity was verified by capillary electrophoresis on a Qiaxcel instrument. Amplicons with orthogonal sequences were pooled for each triplate transfection, and Illumina flow-cell-compatible adapters were added using the NEBNext Ultra II DNA Library Prep kit according to manufacturer instructions. Illumina i5 and i7 indices were added by an additional ten cycles of PCR with Q5 High Fidelity DNA Polymerase using primers from NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1) and purified using 0.7× volume magnetic beads. Final ampiclon libraries containing Illumina-compatible adapters and indices were quantified by droplet digital PCR and sequenced with 150-bp paired-end reads on an Illumina MiSeq instrument. Sequencing reads were demultiplexed by a MiSeq Reporter, then analyzed for base frequency at each position by a modified version of CRISPResseq60. Indels were quantified in a 10-bp window surrounding the expected cut site for each sgRNA.

Expression of HBB –28 (A>G) gRNAs. In order to use eA3A base editors with the HF1 or Hpya mutations that decrease genome-wide off-target editing, it was necessary to use 20 nucleotides of spacer sequence in the gRNA with no mismatches between the spacer and target site60,31,32. We expressed the HBB –28 (A>G) gRNA from a plasmid using the U6 promoter, which preferentially initiates transcription at a guanine nucleotide at the +1 position. To preserve perfect matching between the spacer and target site, we appended a self-cleaving 5’ hammerhead ribozyme that is able to remove the mismatched guanine at the 5’ of the spacer32. This strategy rescued activity of HF1 eA3A BE3.9 or Hpya eA3A BE3.9 by ~1.4-fold compared to the gRNA with a 5’ mismatched guanine (Supplementary Fig. 12).

Protein purification. Proteins were expressed and purified as previously described4. Briefly, 8 liters of BL21 STAR (DE3) Escherichia coli containing the plasmids pET-6xHis-eA3A-BE3 or pET-6xHis-A3A (N57Q)-BE3 were grown to OD600 = 0.7 in LB broth then cooled to 16 °C in an ice-water bath. Protein expression was then induced by the addition of 0.5 mM IPTG, and cultures were incubated overnight at 16 °C. Cells were harvested by centrifugation then lysed by sonication. Proteins were purified by Ni-NTA immobilized metal affinity chromatography then cation exchange using SP Sepharose resin. Following cation exchange, the elution buffer was diluted to a final salt concentration of 150 mM, and the proteins were concentrated to 20 mg/ml and snap-frozen in liquid nitrogen.

RNP electroporation. Electroporation was performed using Lonza 4D Nucleofector (V4XP-3032 for 20 µl Nucleocuvette Strips) per the manufacturer’s instructions. For 20 µl Nucleocuvette Strips, the RNP complex was prepared by mixing SpCas9 (200 pmol) and chemically modified synthetic sgRNA (200 pmol) purchased from Synthego and incubated for 15 min at room temperature immediately before electroporation. 50K erythroid precursors

ONLINE METHODS

Plasmids and oligonucleotides. Sequences of proteins and their expression plasmids used in this study are listed in the Supplementary Information. gRNA target sites and sequences of oligonucleotides used for on-target PCR amplicons for high-throughput sequencing in this study can be found in Supplementary Table 4. Sequences of oligonucleotides used to investigate off-target editing sites can be found in Supplementary Table 2. Base editor expression plasmids containing amino acid substitutions were generated by PCR and standard molecular cloning methods. gRNA expression plasmids were constructed by ligating annealed oligonucleotide duplexes into MLM3636 cut with BamBI. All gRNAs except those targeting the HBB –28 (A>G) and CTNNB1 sites were designed to target sites containing a 5’ guanine nucleotide.

Human cell culture and transfection. U2OS.EGFP cells containing a single stably integrated copy of the EGFP-PEST reporter gene and HEK293T cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM GlutaMax, penicillin and streptomycin at 37 °C with 5% CO2. The media for U2OS.EGFP cells was supplemented with 400 µg ml−1 Geneticin. Cell line identity was validated by STR profiling (ATCC), and cells were tested regularly for mycoplasma contamination. U2OS.EGFP cells were transfected with 750 ng of plasmid expressing BE and 250 ng of plasmid expressing sgRNA according to the manufacturer’s recommendations using the DN-100 program and s.e.m. cell line kit on a Lonza 4-D Nucleofector. For HEK293T transfections, 75,000 cells were seeded in 24-well plates and 18 h later were transfected with 500 ng of plasmid expressing the base editor and 200 ng of plasmid expressing sgRNA using TransIT-293 (Mirus) according to the manufacturer’s recommendations. For all targeted amplicon sequencing and GUIDE-seq experiments, genomic DNA was extracted 72 h post-transfection. Cells were lysed in lysis buffer containing 100 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, and 0.05% SDS and incubated overnight at 55 °C in an incubator shaking at 250 r.p.m. Genomic DNA was extracted from lysed cells using carboxyl-modified Sera-Mag Magnetic Speedbeads resuspended in 2.5 mM NaCl and 18% PEG-6000 (magnetic beads).

The HEK293T:HBB cell line was constructed by cloning a 200-bp fragment of the HBB promoter upstream of an EF1a promoter driving expression of the puromycin resistance gene in a lentiviral vector. The HBB –28 (A>G) mutation was inserted by PCR and standard molecular cloning methods. The lentiviral vector was transfected into HEK293T cells and media containing viral particles was harvested after 72 h. Media containing viral particles was serially diluted and added to 10-cm plates with ~10 million HEK293T cells. After 48 h, media was supplemented with 2.5 µg ml−1 puromycin, and cells were harvested from the 10-cm plate with the fewest surviving colonies to ensure single-copy integration.

Peripheral blood was obtained from β-thalassemia patients following Boston Children’s Hospital institutional review board approval and patient informed consent. CD34+ hematopoietic stem and progenitor cells (HSPCs) were isolated using the Miltenyi CD34 Microbead kit (Miltenyi Biotec). HSPCs were cultured with X-VIVO 15 (Lonza, 04-4180) supplemented with 100 ng ml−1 human SCF, 100 ng ml−1 human thrombopoietin (TPO) and 100 ng ml−1 recombinant human Flt3-ligand (Flt3-l) 3 days for expansion. For in vitro erythroid differentiation, HSPCs were cultured with erythroid differentiation medium (EDM) consisting of IMDM supplemented with 330 µg ml−1 holo-human transferrin, 10 µg ml−1 recombinant human insulin, 2 IU ml−1 heparin, 5% human solvent detergent pooled plasma AB, 3 IU ml−1 erythropoietin, 1% l-glutamine, and 1% penicillin/streptomycin. During days 0–7 of culture, EDM was further supplemented with 1 µM hydrocortisone (Sigma), 100 ng ml−1 human SCF, and 5 ng ml−1 human IL-3 (R&D). After day 7 erythroid precursors were cryopreserved. 1 day after thawing, 50,000 erythroid precursors were electroporated using the Lonza 4-D electroporator. During days 7–11 of culture, EDM was supplemented with 100 ng ml−1 human SCF only. During days 11–18 of culture, EDM had no additional supplements.

Off-target site selection and amplicon design. Two of the sites characterized here, EMX1 site 1 and FANCF, were previously characterized by modified Digene-seq, an unbiased approach to discover BE3-specific off-target sites. All off-target sites discovered by modified Digene-seq were investigated, and these sites represent the most comprehensive off-target characterization because they were discovered de novo using BE3. The VEGFA site 2 target is a promiscuous, homopolymeric gRNA that was previously characterized by GUIDE-seq. Because the VEGFA site 2 gRNA has over 100 nuclease off-target sites, we selected the 20 off-target sites with the highest number of GUIDE-seq reads that also reside in loci for which we were able to design unique PCR amplification primers for characterization here. The CTNNB1 and HBB –28 (A>G) gRNAs had not been previously characterized with respect to BE or nuclease off-target sites. We performed GUIDE-seq as previously described38 using these gRNAs to determine the SpCas9 nuclease off-target sites, and used Cas-OffFinder to predict all of the potential off-target sites with one RNA bulge and one mismatch. (GUIDE-seq and Cas-OffFinder analyses were performed using the hg38 reference genome.) This class of off-targets is more prevalent in BE3 relative to nucleases37, and thus sites that we were unlikely to discover by GUIDE-seq. Primers were designed to amplify all off-target sites such that potential edited cytidines were within the first 100 bp of Illumina high-throughput sequencing reads. A total of six primer pairs encompassing EMX1 site 1, VEGFA site 2, and CTNNB1 site 1 off-target sites did not amplify their intended amplicon and were thus excluded from further analysis.

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resuspended in 20 µl P3 solution were mixed with RNP and transferred to a cuvette for electroporation with program EO-100. The electroporated cells were resuspended with EDM for in vitro differentiation.

RT-qPCR quantification of globin induction. RNA isolation was performed with RNeasy columns (Qiagen, 74106) according to the manufacturer’s instructions. Reverse transcription was performed with the iScript cDNA synthesis kit (Bio-Rad, 170-8890). RT-qPCR was performed with iQ SYBR Green Supermix (Bio-Rad, 170-8880). The induction of globin gene expression was measured using primers amplifying HBG1/2 (5′-GTTATCAATAAGCTCTAGTCC and ACAACCAGGACCTTTCCCA-3′), HBB (5′-TGAGGAGAGTCTGCGTTACA-3′ and 5′-ACCACCAGCAGCACCTGCCA-3′) and HBA1/2 (5′-GCCCTGGAGAGGATGTTC-3′ and 5′-TTCTGTCCGTCGGCGCTA-3′)33.

Statistical testing. All statistical testing was performed using two-tailed Student’s t-test according to the method of Benjamini, Krieger, and Yekutieli, without assuming equal variances between samples.

Ethical regulation compliance. All work was performed in compliance with relevant ethical regulations.

Life Sciences Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. High-throughput sequencing reads have been deposited in the NCBI Sequence Read Archive under SUB4137121.

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When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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- [ ] Clearly defined error bars
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Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection
High-throughput sequencing data was collected and demultiplexed by an Illumina MiSeq instrument.

Data analysis
High-throughput sequencing data was analyzed by CRISPResso v2 for base editing efficiencies. CRISPResso v1 was used to analyze sequencing reads for the presence of indels.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
Sample size of \( n = 3 \) was chosen for experiments where statistical analyses were performed

Data exclusions
No data was excluded

Replication
All replications were successful.

Randomization
Samples were not randomized.

Blinding
Authors were not blinded to samples.

Reporting for specific materials, systems and methods

Materials & experimental systems  Methods

| n/a | Involved in the study | n/a | Involved in the study |
|-----|-----------------------|-----|-----------------------|
| ☐ | Unique biological materials | ☐ | ChIP-seq |
| ☐ | Antibodies | ☐ | Flow cytometry |
| ☒ | Eukaryotic cell lines | ☒ | MRI-based neuroimaging |
| ☒ | Palaeontology | ☒ | |
| ☒ | Animals and other organisms | ☒ | |
| ☐ | Human research participants | ☐ | |

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  ATCC

Authentication  STR profiling

Mycoplasma contamination  Cells were tested for mycoplasma contamination bi-weekly and all results were negative for contamination.

Commonly misidentified lines (See ICLAC register)  No cell lines were used that are in the ICLAC register.

Human research participants

Policy information about studies involving human research participants

Population characteristics  Genotype: beta-zero/beta-plus: compound heterozygous for cd41/42 (-CCCT) and -28 (A-G) (promoter TATA box)  Gender: Male  Age: 25  Diagnosis is beta-thalassemia major

Recruitment  Patient cells that were previously collected by another group were obtained according to genotype.

Nature Biotechnology: doi:10.1038/nbt.4199