Associated Protocol 2: Off-target editing

ANIMALS. All mice experiments were approved by the Broad Institute IACUC. Wild-type adult C57BL/6J mice (000664) were purchased from the Jackson Laboratory. All mice were housed in a room maintained on a 12 h light and dark cycle with *ad libitum* access to standard rodent diet and water. Animals were randomly assigned to various experimental groups.

RETRO-ORBITAL INJECTIONS. 50 mL of VLPs (containing $4x10^{11}$ or $7x10^{11}$ VLPs) were centrifuged for 10 min at 15,000 *g* to remove debris. The clarified supernatant was diluted to 120 mL in 0.9% NaCl (Fresenius Kabi; 918610) right before injection. $1x10^{11}$ viral genomes (vg) of total AAV was diluted to 120 mL in 0.9% NaCl (Fresenius Kabi; 918610) right before injection. Anesthesia was induced with 4% isoflurane. Following induction, as measured by unresponsiveness to bilateral toe pinch, the right eye was protruded by gentle pressure on the skin, and an insulin syringe was advanced, with the bevel facing away from the eye, into the retrobulbar sinus where VLP or AAV mix was slowly injected. One drop of Proparacaine Hydrochloride Ophthalmic Solution (Patterson Veterinary; 07-885-9765) was then applied to the eye as an analgesic. Genomic DNA was purified from various tissue using Agencourt DNAdvance kits (Beckman Coulter; A48705) following the manufacturer's instructions.

CIRCLE-SEQ. Circularization for In vitro Reporting of Cleavage Effects by sequencing (CIRCLE-seq) was performed and analyzed as described previously (Tsai et al., 2017) save for the following modifications: For the Cas9 cleavage step, guide denaturation, incubation, and pro- teinase K treatment was conducted using the more efficient method described in the CHANGE-seq protocol (Lazzarotto et al., 2020). Specifically, the sgRNA with the guide sequence ''GCCCATACCTTGGAGCAACGG'' was ordered from Synthego with their standard chemical modifications, 2'O-Methyl for the first three and last three bases, and phosphorothioate bonds between the first three and last two bases. A 5' ''G'' nucleotide was included with the 20-nucleotide specific guide sequence to recapitulate the sequence ex- pressed and packaged into VLPs. The sgRNA was diluted to 9 mM in nuclease-free water and re-folded by incubation at 90 C for 5 min followed by a slow annealing down to 25 C at a ramp rate of 0.1 C/second. The sgRNA was complexed with Cas9 nuclease (NEB; M0386T) via a 10 min room temperature incubation after mixing 5 mL of 10x Cas9 Nuclease Reaction Buffer provided with the nuclease, 4.5 mL of1 mM Cas9 nuclease (diluted from the 20mM stock in 1x Cas9 Nuclease Reaction Buffer), and 1.5 mL of9 mM an- nealed sgRNA. Circular DNA from mouse N2A cells was added to a total mass of 125 ng and diluted to a final volume of 50 mL. Following 1 h of incubation at 37 C, Proteinase K (NEB; P8107S) was diluted 4-fold in water and 5 mL of the diluted mixture was added to the cleavage reaction. Following a 15 min Proteinase K treatment at 37 C, DNA was A-tailed, adapter ligated, and USER-treated, and PCR-amplified as described in the CIRCLE-seq protocol (Tsai et al., 2017). Following PCR, samples were loaded on a prepar- ative 1% agarose gel and DNA was extracted between the 300bp and 1kb range to eliminate primer dimers before sequencing on an Illumina MiSeq. Data was processed using the CIRCLE-seq analysis pipeline and aligned to the human genome Hg19 (GRCh37) with parameters: ''read_threshold: 4; window_size: 3; mapq_threshold: 50; start_threshold: 1; gap_threshold: 3; mismatch_threshold: 6; merged_analysis: True''.

AMPLICON SEQUENCING OF OFF-TARGET SITES NOMINATED BY CIRCLE-SEQ. We observed in prior work that exhaustively assessed ABE8e off-target sites nominated by CIRCLE-seq that off-target editing effi- ciency did not track well with the CIRCLE-seq read count (Newby et al., 2021). However, nominated off-target sites where editing was observed shared some striking similarities. Namely, over 90.7% of the 54 off-target sites with validated off-target editing had zero mismatches or one mismatch to the guide in the 9 nucleotides proximal to the PAM. The few sites with more than 1 mismatch in this region were all edited with low efficiency (the bottom half of sites, when ranked by editing efficiency). Based on this knowledge, we chose to assess 12 off-target sites in our CIRCLE-seq list that showed one or fewer mismatches in the 9 nucleotides of the protospacer proximal to the PAM to increase the chance that we sequence a true off-target

site.

Description	Spacer	Gene
On-target	CCCATACCTTGGAGCAACGG *CGG*	Pcsk9
OT1	**GA**CATACCTT**AA**AGCAA**A**GG *AGG*	Intron; *ELP3*
OT2	CCC**C**TACCTTGG**G**GCAAC**A**G *TGG*	Intergenic
OT3	CCCA**CC**C**T**TTGGAG**-**AACGG *TGG*	LncRNA; LINC02006
OT4	CCCA**G-**CCTTGG**G**GCAACGG *AGG*	Intergenic
OT5	C**A**CATA**T**CT**A**GGAGCAA**-**GG *AGG*	Intergenic
OT6	CCCA**C**ACCC**-G**GAGCAACGG *GGA*	Intron; *DDX6*
OT7	**T**CCATACC**C-**GGAGCAACG**A** *GGG*	LncRNA; RP11-314D7.4
OT8	**TT**CAT**-**CCTTGGAGCAACGG *TGA*	LncRNA; FAM66D
OT9	**T**C**TG**TACC**A**TGGAGCAA**A**GG *CGG*	LncRNA; RIKEN cDNA 4933424G05 gene
OT10	**A**CCATA**A**C**CAA**GAGCAAC**A**G *GGG*	Intron; *Klhl3*
OT11	**T**CCATA**A**CT**CA**GAGCAAC**A**G *TGG*	Intergenic
OT12	**G**CCATACC**C**TGG**G**GCA**G**C**A**G *TGG*	Intron; *NCAM1*
OT13	**G**C**A**A**C**ACCTTGGAGCAAC**T**G *AGG*	Intron; *SNRNP40*
OT14	**GA**CAT**-**CCTTGGAGCAAC**T**G *TGG*	Intron; *Fry*

Mismatches are denoted in red

HIGH-THROUGHPUT SEQUENCING OF GENOMIC DNA. Genomic DNA was isolated as described above. Following genomic DNA isolation, 1 mL of the isolated DNA (1–10 ng) was used as input for the first of two PCR reactions. Genomic loci were amplified in PCR1 using PhusionU polymerase (Thermo Fisher Scientific). PCR1 primers for genomic loci are as follows: forward primer 5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNGGCTGCACTTAGAGACCACC-3'; reverse 5'- TGGAGTTCAGACGTGTGCTCTTCCGATCTATGAAGAGCTGATGCTCGCC-3'. PCR1 was performed as follows: 95 C for 3 min; 30–35 cycles of 95 C for 15 s, 61 C for 20 s, and 72 C for 30s; 72 C for 1 min. PCR1 products were confirmed on a 1% agarose gel. 1 mL of PCR1 was used as an input for PCR2 to install Illumina barcodes. PCR2 was conducted for nine cycles of ampli- fication using a Phusion HS II kit (Life Technologies). Following PCR2, samples were pooled and gel purified in a 1% agarose gel using a Qiaquick Gel Extraction Kit (Qiagen). Library concentration was quantified using the Qubit High-Sensitivity Assay Kit (Thermo Fisher Scientific). Samples were sequenced on an Illumina MiSeq instrument (paired-end read, read 1: 200–280 cycles, read 2: 0 cycles) using an Illumina MiSeq 300 v2 Kit (Illumina).

HIGH-THROUGHPUT SEQUENCING DATA ANALYSIS. Sequencing reads were demultiplexed using the MiSeq Reporter software (Illumina) and were analyzed using CRISPResso2 (Clement et al., 2019) as previously described (Doman et al., 2020). Batch analysis mode (one batch for each unique amplicon and sgRNA com- bination analyzed) was used in all cases. Reads were filtered by minimum average quality score (Q > 30) prior to analysis. The following quantification window parameters were used: -w 20 -wc -10. Base editing efficiencies are reported as the percentage of sequencing reads containing a given base conversion at a specific position. Prism 9 (GraphPad) was used to generate dot plots and bar plots.

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