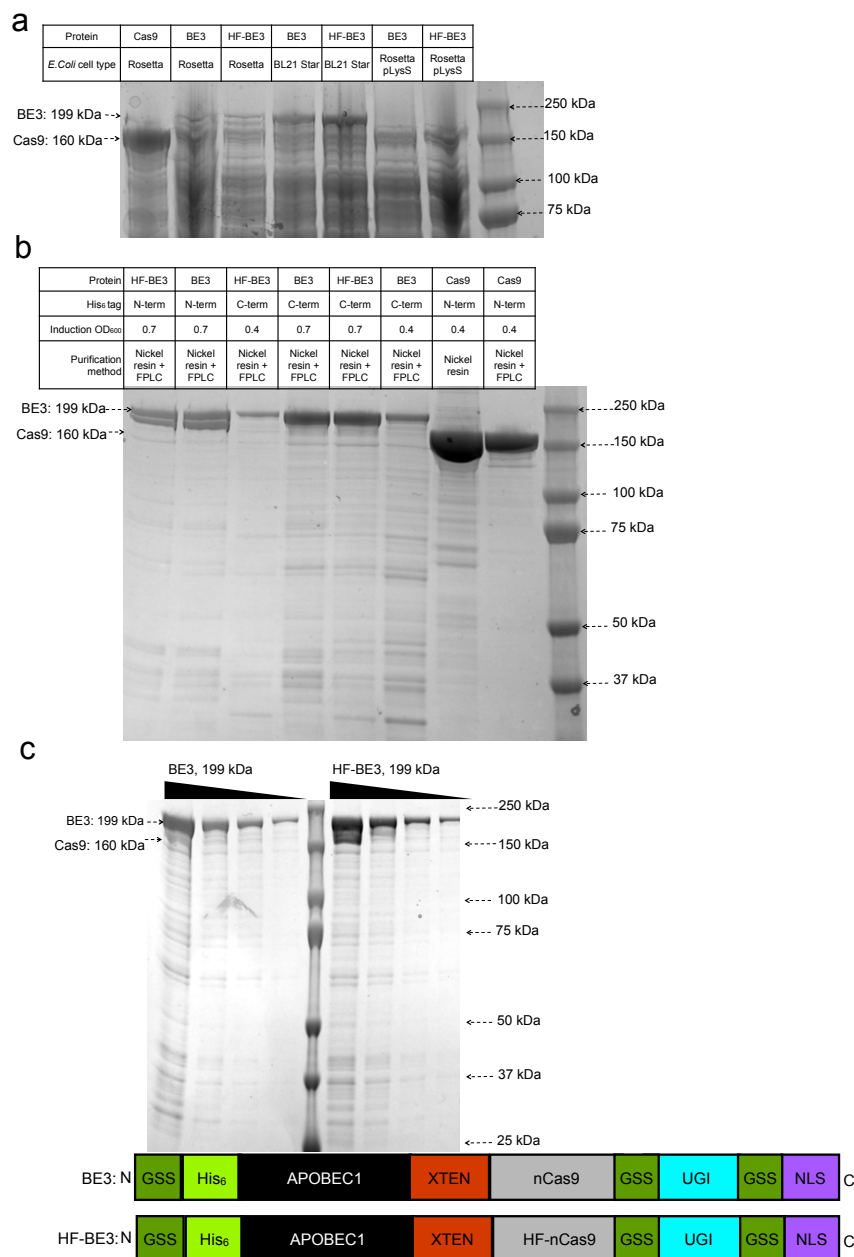
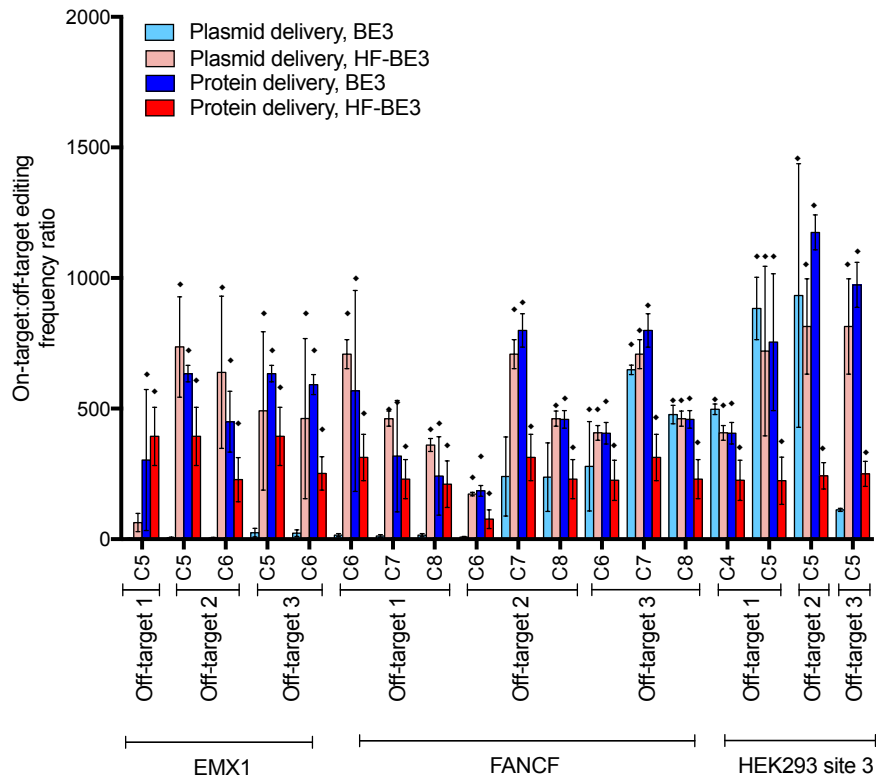


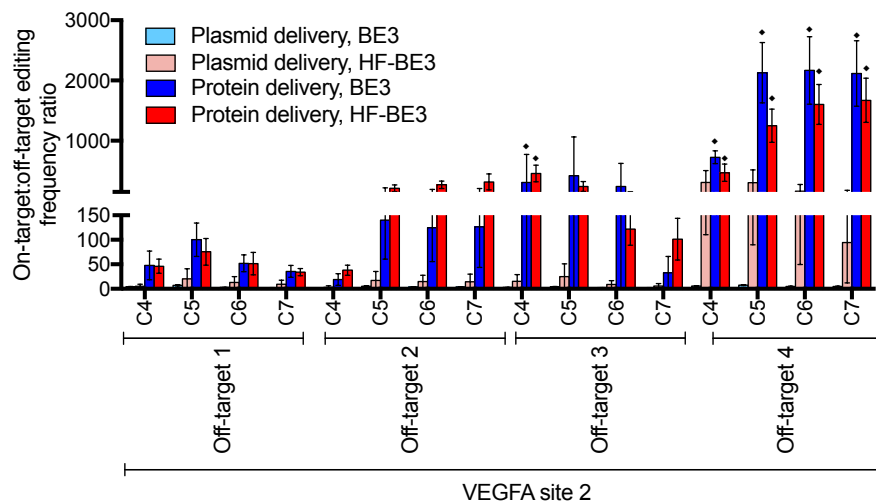


Supplementary Figure 1: Purification of base editor proteins. (a): Selection of optimal *E. coli* strain for base editor expression. After IPTG-induced protein expression for 16 h at 18 °C, crude cell lysate was analyzed for protein content. BL21 Star (DE3) (Thermo Fisher) cells showed the most promising post-expression levels of both BE3 and HF-BE3 and were used for expression of base editors. (b): Purification of expressed base editor proteins. Placing the His₆ tag on the C-terminus of the base editors lead to production of a truncation product for both BE3 and HF-BE3 (lanes 1 and 2). Unexpectedly, this truncation product was removed by placing the His₆ tag on the N-terminus of the protein (lanes 3-6). Inducing expression of base editors at a cell density of OD₆₀₀ = 0.7 (lanes 4-5), later than is optimal for Cas9 expression (OD₆₀₀ = 0.4)¹, improves yield of base editor proteins. Purification was performed using a manual HisPur resin column followed by cation exchange FPLC (Akta). (c): Purified BE3 and HF-BE3. Different concentrations of purified BE3 and HF-BE3 were denatured using heat and LDS and loaded onto a polyacrylamide gel. Protein samples are representative of proteins used in this study. Gels in (a), (b), and (c) are BOLT Bis-Tris Plus 4-12% polyacrylamide (Thermo Fisher). Electrophoresis and staining were performed as described in Methods.

a

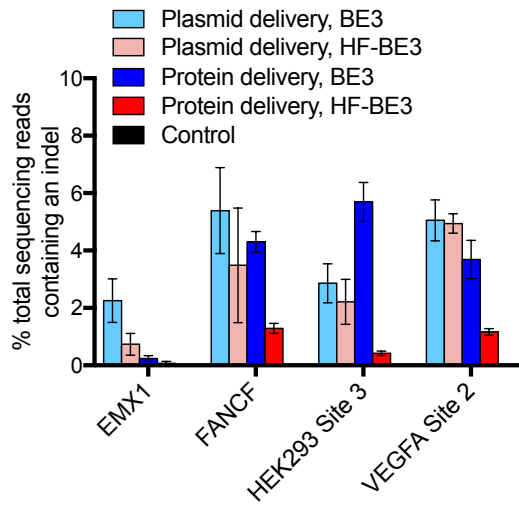


b

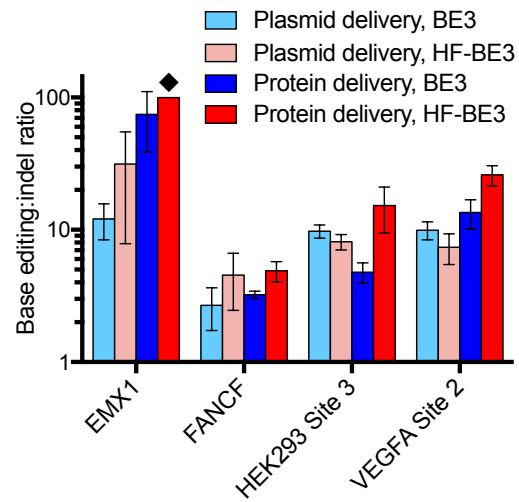


Supplementary Figure 2: **On-target:off-target base editing frequency ratios for plasmid and protein delivery of BE3 and HF-BE3.** Base editing on-target:off-target specificity ratios were calculated by dividing the on-target editing percentage at a particular cytosine in the activity window by the off-target editing percentage at the corresponding cytosine for the indicated off-target locus (see Methods). When off-target editing was below the threshold of detection (0.025% of sequencing reads), we set the off-target editing to the limit of detection (0.025%) and divided the on-target editing percentage by this upper limit. In these cases, denoted by ♦, the specificity ratios shown represent lower limits. Specificity ratios are shown for non-repetitive sgRNAs FANCF, HEK 293 site 3, and FANCF (a) and for the highly repetitive sgRNA VEGFA site 2 (b). Values and error bars reflect mean $\pm$ S.D. of three independent biological replicates performed on different days.

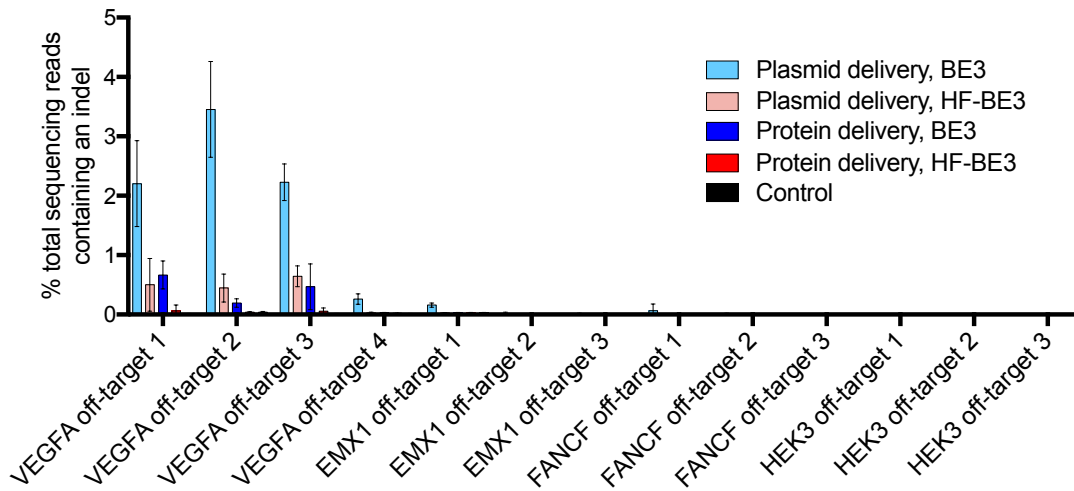
a



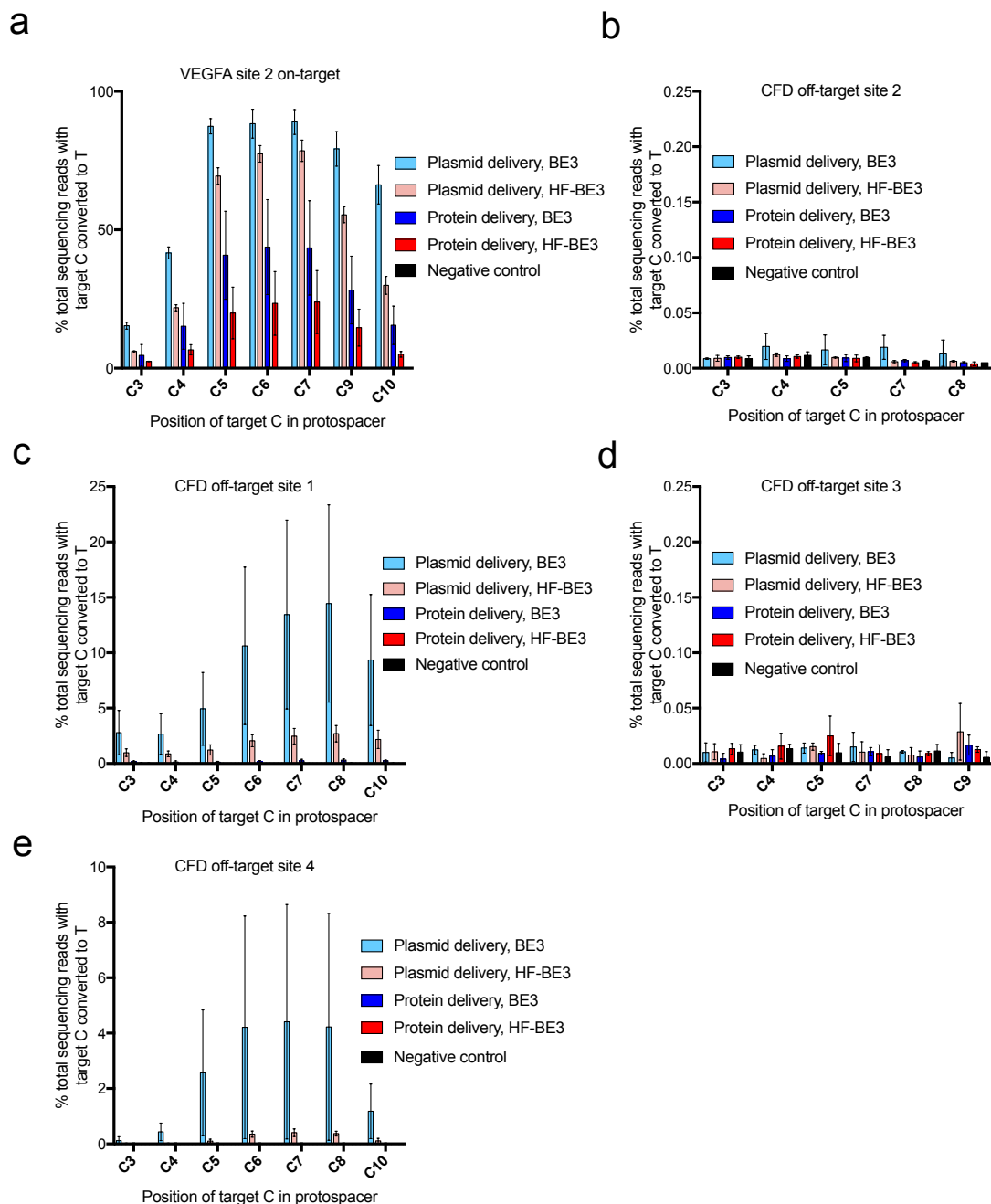
b



c

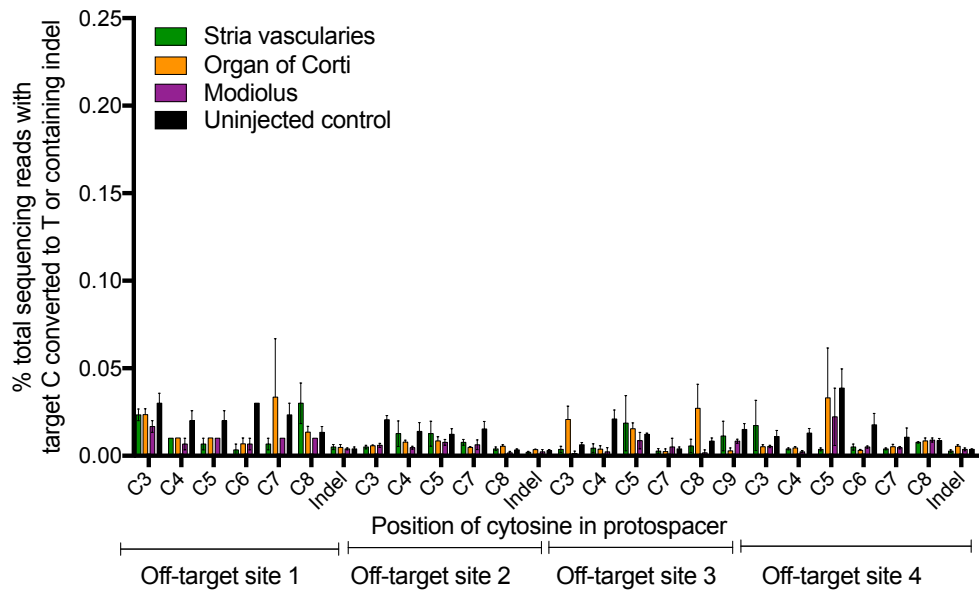


Supplementary Figure 3: **Indel formation associated with base editing at genomic loci.** (a): Indel frequency at on-target loci for VEGFA site 2, EMX1, FANCF, and HEK293 site 3 sgRNAs. (b): Ratio of base editing:indel formation. The diamond (♦) indicates no indels were detected (no significant difference in indel frequency in the treated sample and in the untreated control). (c): Indels observed at the off-target loci associated with the on-target sites interrogated in (a). Values and error bars reflect mean $\pm$ S.D. of three independent biological replicates performed on different days.

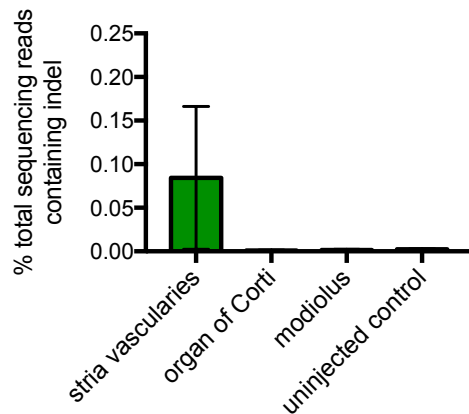


Supplementary Figure 4: **On- and off-target base editing in murine NIH/3T3 cells.** (a): On-target base editing associated with the 'VEGFA site 2' sgRNA (See Figure 5E for sequences). The negative control corresponds to cells treated with plasmid encoding BE3 but no sgRNA. Values and error bars reflect mean $\pm$ S.D. of three independent biological replicates performed on different days. (B-E): Off-target editing associated with this site was measured using high-throughput DNA sequencing at the top four predicted off-target loci for this sgRNA (sequences shown in Figure 5E). (b): off-target 2, (c): off-target 1, (d): off-target 3, (e): off-target 4. Values and error bars reflect mean $\pm$ S.D. of three independent biological replicates performed on different days.

a



b



Supplementary Figure 5: **Off-target base editing and on-target indel analysis from *in vivo*-edited murine tissue.** (a): Editing is plotted for each cytosine in the base editing window of off-target loci associated with VEGFA site 2. (b): Indel rates at the on-target base editor locus. Values and error bars reflect mean $\pm$ S.E.M of three injected and three control mice.

Supplementary Note 1: Python script used to analyze quality-filtered *in vitro*-edited DNA.

```
1. from __future__ import print_function
2. from __future__ import division
3.
4. import Bio #This will import the BioPython suite
5. from Bio import SeqIO #Necessary to read/write sequence handles
6. from Bio.Seq import Seq
7. import os
8. import collections
9. import csv
10.
11. inputfile = "please_specify_your_input_file_here_containing_filtered_reads" #specify the file
    names that contain sequences
12. filenames = []
13.
14. for file in os.listdir(inputfile):
15.     if file.endswith(".fastqsanger"):
16.         filenames.append(file)
17.
18. spacer = []
19. list_of_filenames = []
20.
21. for file in filenames:
22.     site = {}
23.     output = open(file + ".txt", "w")
24.     list_of_filenames.append(file + ".txt") #allows calling of the txt files that come from f
    astq files later
25.     for rec in SeqIO.parse(file, "fastq"):
26.         split1=rec.seq.tostring().split("GTTTCGCGGCGATCG") #14-
    base pair constant_region_before_protospacer
27.         if len(split1)>=2:
28.             split2=split1[1].split("TGGATCGCCTGGCA") #14-
    base pairc constant_region_after_protospacer
29.             site=split2[0]
30.             if len(site)==20:
31.                 output.write(site + "\n")
32.
33. BASES = 'ATGCN'
34. UNRECOGNIZED = 'X'
35. BASE_SEPERATOR = dict(zip(BASES, '.,.,.\n'))
36. a_index = 0
37. t_index = 1
38. g_index = 2
39. c_index = 3
40. n_index = 4
41.
42. def get_counts_by_column(base, count, library):
43.     current_count = library[count]
44.     if base == 'A':
45.         current_count[a_index] += 1
46.     elif base == 'T':
47.         current_count[t_index] += 1
48.     elif base == 'G':
49.         current_count[g_index] += 1
50.     elif base == 'C':
51.         current_count[c_index] += 1
52.     elif base == 'N':
53.         current_count[n_index] += 1
54.
55. def dna_counts(list_of_sequences, sample):
56.     first_oligo = list_of_sequences[0]
57.     for i in range (len(first_oligo)):
```

```
58.         sample.append([0,0,0,0,0])
59.     for j in range(len(first_oligo)):
60.         for i in range(len(list_of_sequences)):
61.             get_counts_by_column(list_of_sequences[i][j], j, libname)
62.
63.
64. for file in list_of_filenames:
65.     spacer_list = open(file).read().splitlines()
66.     output2=[]
67.     dna_counts(spacer_list, output2)
68.     with open(file + ".csv", "wb") as f:
69.         writer = csv.writer(f)
70.         writer.writerows(output2)
```

Supplementary Note 2: Sequences of proteins used in this study

Protein sequence of expressed BE3

MGSSHHHHHHSSETGPVAVDPTLRRRIEPHEFEVFFDPREL RKETCLLYEINWGGRH SIWRHTSQNTNKHVEV
NFIEKF TTERYFCPNTRCSITWFLSWSPCGECSRAITEFLSRYPHVTLFIYIARLYHHADPRNRQGLRDLISSGVTI
QIMTEQESGYCWRNFVNYSNPSNEAHWPYPHLLWVRLYVLELYCIILGLPPCLNLRKQPKLTFFTIALQSCHYQ
RLPPHILWATGLKSGSETPGTSESATPESDKKYSIGLAIGTNSVGWAVITDEYKVP SKKFKVLGNTDRHSIKKNLI
GALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRL EESFLVEEDKKHERHPIFGNV
DEVAYHEKYPTIYHLRKKLVDSTDKADLRLLIYLA LAHMIKFRGHFLIEGDLNPDNSDVKLFIQLVQTYNQ LFEENP
INASGVDAKAILSARLSKSRLENLIAQLPGEKKNGLFGNLIASLGLTPNFKSNFDLAEDAKLQLSKD TYDDDL
NLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQ LPEKYKEIFFDQ
SKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKNLREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFY
PFLKDNREKIEKILTRIPYYVGPLARGNSRFAWMTRKSEETITPWNFE EVVDKGASAQSFIERMTNFDKNLPNE
KVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEIS
GVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTG
WGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMLIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPA IK
KGILQTVKVVDELVKVMGRHKPENIVIAMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNE
KLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYW
RQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKS
KLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVG TALIKKYPKLESEFVYGDYKVYDVRKMIKSEQEIGKATAK
YFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVL SMPQVNIVKKTEVQTGGFSKESILP
KRNSDKLIARKKDWDPKKYGGFDSP TVAYSVLVVAKEKVKSKKLKSVKELLGITIMERS SFEKNPIDFLEAKGY
KEVKKDLIIKLPKYSLFELENG RKRLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQH
KHYLDEIIEQISEFSKRVLADANLDKVL SAYNKHDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKE
VLDATLIHQ SITGLYETRIDLSQLGGDSGGSTNLSDIIEKETGKQLVIQESILMLPEEVEEVIGNKPSDILVHTAYD
ESTDENVMLLTSDAPEYKPWALVIQDSNGENKIKMLSGGSPKKKRKV

Protein sequence of expressed HF-BE3

MGSSHHHHHHSSETGPVAVDPTLRRRIEPHEFEVFFDPREL RKETCLLYEINWGGRH SIWRHTSQNTNKHVEV
NFIEKF TTERYFCPNTRCSITWFLSWSPCGECSRAITEFLSRYPHVTLFIYIARLYHHADPRNRQGLRDLISSGVTI
QIMTEQESGYCWRNFVNYSNPSNEAHWPYPHLLWVRLYVLELYCIILGLPPCLNLRKQPKLTFFTIALQSCHYQ
RLPPHILWATGLKSGSETPGTSESATPESDKKYSIGLAIGTNSVGWAVITDEYKVP SKKFKVLGNTDRHSIKKNLI
GALLFDSGETAEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFHRL EESFLVEEDKKHERHPIFGNV
DEVAYHEKYPTIYHLRKKLVDSTDKADLRLLIYLA LAHMIKFRGHFLIEGDLNPDNSDVKLFIQLVQTYNQ LFEENP
INASGVDAKAILSARLSKSRLENLIAQLPGEKKNGLFGNLIASLGLTPNFKSNFDLAEDAKLQLSKD TYDDDL
NLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLKALVRQQ LPEKYKEIFFDQ
SKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKNLREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFY
PFLKDNREKIEKILTRIPYYVGPLARGNSRFAWMTRKSEETITPWNFE EVVDKGASAQSFIERMTAFDKNLPNE
KVLPKHSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEIS
GVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVMKQLKRRRYTG
WGALSRKLINGIRDKQSGKTILDFLKSDGFANRNFMA LIHDDSLTFKEDIQKAQVSGQGDSLHEHIANLAGSPA IK
KGILQTVKVVDELVKVMGRHKPENIVIAMARENQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNE
KLYLYYLQNGRDMYVDQELDINRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYW
RQLLNAKLITQRKFDNLTKAERGGLSELDKAGFIKRQLVETRAITKHVAQILDSRMNTKYDENDKLIREVKVITLKS
KLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVG TALIKKYPKLESEFVYGDYKVYDVRKMIKSEQEIGKATAK
YFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVL SMPQVNIVKKTEVQTGGFSKESILP
KRNSDKLIARKKDWDPKKYGGFDSP TVAYSVLVVAKEKVKSKKLKSVKELLGITIMERS SFEKNPIDFLEAKGY
KEVKKDLIIKLPKYSLFELENG RKRLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQH
KHYLDEIIEQISEFSKRVLADANLDKVL SAYNKHDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKE
VLDATLIHQ SITGLYETRIDLSQLGGDSGGSTNLSDIIEKETGKQLVIQESILMLPEEVEEVIGNKPSDILVHTAYD
ESTDENVMLLTSDAPEYKPWALVIQDSNGENKIKMLSGGSPKKKRKV

Supplementary Note 3: Sequences of oligonucleotides used in this study

Unpublished Primers used to amplify off target genomic DNA for HTS in human cells

fwd_VEGFA_site2_off_target_1_human	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNTCCTACAAGTAACAGTCCAAGAA
rev_VEGFA_site2_off_target_1_human	TGGAGTTCAGACGTGTGCTCTTCCGATCTTTCTGCAACTTAACCTACGTGAAA
fwd_VEGFA_site2_off_target_2_human	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNACCAAGCCCATTGTCCAGG
rev_VEGFA_site2_off_target_2_human	TGGAGTTCAGACGTGTGCTCTTCCGATCTTCTTCTTTTGAGCTTTGGGC
fwd_VEGFA_site2_off_target_3_human	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNTCCTACCAGCAGCAGTTCC
rev_VEGFA_site2_off_target_3_human	TGGAGTTCAGACGTGTGCTCTTCCGATCTCTCACCTCAGCTCCTGCAC
fwd_VEGFA_site2_off_target_4_human	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCCCACTGATTCTACACCATGGT
rev_VEGFA_site2_off_target_4_human	TGGAGTTCAGACGTGTGCTCTTCCGATCTGGAGTTCCCAACCTTTTGACA

Other primers (for off target sites associated with HEK_3, EMX1, FANCF) were previously published

Primers used to amplify off target genomic DNA for HTS in murine cells

fwd_VEGFA_site2_off_target_1_murine	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCTGGCTGGAGATTCAGAGACAC
rev_VEGFA_site2_off_target_1_murine	TGGAGTTCAGACGTGTGCTCTTCCGATCTTGCCCTTCTGACACACATAC
fwd_VEGFA_site2_off_target_2_murine	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNACCCCTCAAGGCTTGACATTTC
rev_VEGFA_site2_off_target_2_murine	TGGAGTTCAGACGTGTGCTCTTCCGATCTTGAAAAGTTGGGAGAGGGGATG
fwd_VEGFA_site2_off_target_3_murine	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNTTGTACCCAGTCCCCTCATC
rev_VEGFA_site2_off_target_3_murine	TGGAGTTCAGACGTGTGCTCTTCCGATCTTGAAGTTACGGGGATGTCACTTG
fwd_VEGFA_site2_off_target_4_murine	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNTTAACATCCAGTCTCCCAAACACA
rev_VEGFA_site2_off_target_4_murine	TGGAGTTCAGACGTGTGCTCTTCCGATCTACACACACACACTACTAGGACA

Primers used to amplify on target genomic DNA for HTS in murine cells

fwd_VEGFA_site2_on_target_murine	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCGCTACTACGGAGCGAGAAG
rev_VEGFA_site2_on_target_murine	TGGAGTTCAGACGTGTGCTCTTCCGATCTACAGGGGCAAAGTGAGTGAC

Primers used for generating PCR products to serve as substrates for T7 transcription of sgRNAs

rev_sgRNA_T7: used in all cases	AAAAAAGCACCGACTCGGTGCCAC
fwd_sgRNA_T7_EMX1	TAATACGACTCACTATAGGGAGTCCGAGCAGAAGAAGAAGTTTTAGAGCTAGAAATAGCA
fwd_sgRNA_T7_FANCF	TAATACGACTCACTATAGGGGAATCCCTTCTGCAGCACCGTTTTAGAGCTAGAAATAGCA
fwd_sgRNA_T7_HEK_site_3	TAATACGACTCACTATAGGGGCCCAGACTGAGCACGTGAGTTTTAGAGCTAGAAATAGCA
fwd_sgRNA_T7_VEGFA_site_2	TAATACGACTCACTATAG GACCCCTCCACCCCGCCTCGTTTTAGAGCTAGAAATAGCA

fwd_sgRNA_T7_TC_repeat_in_vitro	TAATACGACTCACTATAGGTCTCTCTCTCTCTCTCTCTCGTTTTAGAGCTAGAAATAGCA
---------------------------------	--

Primers used for generating sgRNA transfection plasmids

The pFYF1320 plasmid was used as template as previously described (Komor et al). The sequence of other sgRNA plasmids was previously reported

rev_sgRNA_plasmid	GGTGTTCGTCCTTTCCACAAG
fwd_VEGFA_site_2	GACCCCTCCACCCCGCCTCGTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGC

Sequences of ssDNA substrates used in in vitro deaminase assays

fwd_TC_repeat_substrate	ACGTAAACGGCCACAAGTTCGCGGCGATCGTCTCTCTCTCTCTCTCTGGATCGCCTGGCATCTTCT TCAAGGACG
rev_TC_repeat_substrate	CGTCCTTGAAGAAGATGCCAGGCGATCCAGAGAGAGAGAGAGAGAGACGATCGCCGCGAACTTGTG GCCGTTTACGT

Previously published primers used to amplify off target genomic DNA for HTS in human cells

fwd_EMX1_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNCAGCTCAGCCTGAGTGTTGA
rev_EMX1_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTCTCGTGGGTTTGTGGTTGC
fwd_FANCF_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNCATTGCAGAGAGGCGTATCA
rev_FANCF_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGTCCCAGGTGCTGAC
fwd_HEK293_site3_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNATGTGGGCTGCCTAGAAAGG
rev_HEK293_site3_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTCCCAGCCAACTTGTC AACC
fwd_EMX1_off1_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNAGTAGCCTCTTCTCAATGTGC
rev_EMX1_off1_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTGCTTTACAAGGATGCAGTCT
fwd_EMX1_off2_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNAGAGCTAGACTCCGAGGGGA
rev_EMX1_off2_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTTCTCGTCTGCTCTCACTT
fwd_EMX1_off3_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNAGAGGCTGAAGAGGAAGACCA
rev_EMX1_off3_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTGGCCCAGCTGTGCATTCTAT
fwd_FANCF_off1_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNAACCCACTGAAGAAGCAGGG
rev_FANCF_off1_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTGGTGCTTAATCCGGCTCCAT
fwd_FANCF_off2_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNTCCAGTGTTTCCATCCCGAA
rev_FANCF_off2_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTCCTCTGACCTCCACAACCTCT
fwd_FANCF_off3_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNCTGGGTACAGTTCTGCGTGT
rev_FANCF_off3_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTTCACTCTGAGCATCGCCAAG
fwd_HEK293_site3_off1_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNNTCCCCTGTTGACCTGGAGAA

rev_HEK293_site3_off1_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTCACTGTACTTGCCCTGACCA
fwd_HEK293_site3_off2_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNTTGGTGTGACAGGGAGCAA
rev_HEK293_site3_off2_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTCTGAGATGTGGGCAGAAGGG
fwd_HEK293_site3_off3_HTS	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNTGAGAGGGAACAGAAGGGCT
rev_HEK293_site3_off3_HTS	TGGAGTTCAGACGTGTGCTCTTCCGATCTGTCCAAAGGCCCAAGAACCT

Primers used to amplify on target genomic DNA for HTS in zebrafish

fwd_TYR1_zebrafish	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNGTCCCCGAGTCTGCACCT
rev_TYR1_zebrafish	TGGAGTTCAGACGTGTGCTCTTCCGATCTCGAACTTGCAATCGCCGCAA
fwd_TYR2_zebrafish	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNTTCTGCCTTGGCATCGGGTG
rev_TYR2_zebrafish	TGGAGTTCAGACGTGTGCTCTTCCGATCTCACCATACCGCCCCTAGAACTAACATTC
fwd_TYR3_zebrafish	ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNACAAGTCTTCCATGGTGTGT
rev_TYR3_zebrafish	TGGAGTTCAGACGTGTGCTCTTCCGATCTTCCAGGGCTTTCGTGGAGA

Locus and cytosine position	P values (Student's two-tailed t-test) for comparisons between listed treatments									
	plasmid BE3 vs plasmid HF-BE3	plasmid BE3 vs protein BE3	plasmid BE3 vs protein HF-BE3	plasmid BE3 vs control	plasmid HF-BE3 vs protein BE3	plasmid HF-BE3 vs protein HF-BE3	plasmid HF-BE3 vs control	protein BE3 vs protein HF-BE3	protein BE3 vs control	protein HF-BE3 vs control
EMX1, C5	0.053	0.000	0.001	0.000	0.416	0.056	0.003	0.023	0.000	0.004
EMX1, C6	0.065	0.000	0.000	0.000	0.445	0.023	0.004	0.001	0.000	0.003
FANCF, C6	0.152	0.017	0.003	0.000	0.137	0.003	0.000	0.002	0.000	0.004
FANCF, C7	0.591	0.554	0.007	0.000	0.914	0.007	0.000	0.008	0.000	0.006
FANCF, C8	0.011	0.026	0.004	0.000	0.958	0.018	0.000	0.023	0.000	0.007
FANCF, C11	0.524	0.948	0.019	0.001	0.363	0.010	0.000	0.010	0.000	0.021
HEK site 3, C3	0.061	0.001	0.071	0.000	0.002	0.002	0.005	0.00199	0.001	0.003
HEK site 3, C4	0.048	0.924	0.010	0.004	0.001	0.001	0.001	0.00004	0.000	0.001
HEK site 3, C5	0.291	0.592	0.016	0.006	0.243	0.243	0.002	0.00022	0.000	0.001
VEGFA site 2, C3	0.060	0.416	0.239	0.010	0.042	0.280	0.002	0.475	0.002	0.018
VEGFA site 2, C4	0.036	0.191	0.047	0.004	0.032	0.803	0.002	0.066	0.000	0.005
VEGFA site 2, C5	0.098	0.650	0.028	0.003	0.044	0.169	0.002	0.004	0.000	0.001
VEGFA site 2, C6	0.452	0.781	0.118	0.004	0.165	0.239	0.002	0.013	0.000	0.001
VEGFA site 2, C7	0.401	0.683	0.172	0.003	0.120	0.454	0.002	0.026	0.000	0.001
VEGFA site 2, C9	0.225	0.308	0.254	0.004	0.504	0.828	0.004	0.624	0.000	0.002
VEGFA site 2, C10	0.064	0.061	0.023	0.005	0.732	0.257	0.009	0.057	0.000	0.003
EMX1, C5 off-target 1	0.003	0.003	0.002	0.002	0.119	0.036	0.035	0.269	0.255	0.643
EMX1, C5 off-target 2	0.013	0.013	0.013	0.013	0.158	0.294	0.521	0.390	0.058	0.054
EMX1, C6 off-target 2	0.024	0.024	0.024	0.024	0.285	0.560	0.954	0.420	0.103	0.306
EMX1, C5 off-target 3	0.022	0.019	0.019	0.019	0.297	0.297	0.300	>0.99999	0.882	0.815
EMX1, C6 off-target 3	0.017	0.015	0.015	0.015	0.296	0.296	0.328	>0.99999	0.051	0.025
FANCF, C5 off-target 1	0.031	0.031	0.031	0.031	0.314	0.530	0.333	0.337	0.349	0.618
FANCF, C6 off-target 1	0.016	0.016	0.016	0.016	0.347	0.786	0.930	0.338	0.344	0.678
FANCF, C7 off-target 1	0.028	0.028	0.028	0.027	0.374	0.039	0.106	0.353	0.346	0.639
FANCF, C8 off-target 1	0.014	0.014	0.014	0.014	0.341	0.932	0.685	0.343	0.318	0.605
FANCF, C11 off-target 1	0.007	0.007	0.007	0.007	0.374	0.001	0.000	>0.99999	0.475	0.016
FANCF, C6 off-target 2	0.099	0.099	0.032	0.036	0.599	0.475	0.912	0.914	0.393	0.060
FANCF, C7 off-target 2	0.080	0.080	0.027	0.030	0.898	0.638	0.819	0.539	0.859	0.530
FANCF, C8 off-target 2	0.123	0.123	0.045	0.050	0.789	0.538	0.960	0.047	0.539	0.252
FANCF, C10 off-target 2	0.093	0.093	0.029	0.033	0.630	0.509	0.847	0.768	0.670	0.482
FANCF, C11 off-target 2	0.264	0.264	0.127	0.107	0.599	0.658	0.326	>0.99999	0.047	0.345
FANCF, C6 off-target 3	0.872	0.872	0.492	0.108	0.239	0.493	0.129	0.469	0.584	0.252
FANCF, C7 off-target 3	>0.99999	>0.99999	0.859	0.016	0.537	0.866	0.116	0.572	0.272	0.595
FANCF, C8 off-target 3	0.886	0.886	0.246	0.757	>0.99999	0.001	0.648	0.495	0.780	0.650
FANCF, C10 off-target 3	0.566	0.566	0.284	0.202	0.053	0.387	0.260	0.453	0.913	0.541
FANCF, C11 off-target 3	0.422	0.422	0.145	0.145	0.495	0.230	0.230	0.658	0.658	>0.99999
HEK293 site 3, C3 off-target 1	>0.99999	0.910	0.412	0.326	0.910	0.412	0.326	0.293	0.223	0.480
HEK293 site 3, C4 off-target 1	>0.99999	0.994	0.437	0.391	0.994	0.437	0.391	0.495	0.451	0.614
HEK293 site 3, C5 off-target 1	>0.99999	0.616	0.814	0.337	0.616	0.814	0.337	0.459	0.116	0.481
HEK293 site 3, C3 off-target 2	0.285	0.473	0.100	0.473	0.141	0.735	0.141	0.038	>0.99999	0.038
HEK293 site 3, C5 off-target 2	0.375	0.294	0.177	0.294	0.687	0.428	0.687	0.064	>0.99999	0.064
HEK293 site 3, C9 off-target 2	0.053	0.624	0.374	0.624	0.554	0.154	0.554	0.872	>0.99999	0.872
HEK293 site 3, C3 off-target 3	0.067	0.116	0.768	0.435	0.519	0.230	0.561	0.349	0.768	0.643
HEK293 site 3, C5 off-target 3	0.011	0.011	0.011	0.011	0.016	0.643	0.435	0.184	0.025	0.346
HEK293 site 3, C9 off-target 3	>0.99999	0.374	0.652	0.811	0.132	0.539	0.776	0.609	0.643	0.893
VEGFA site 2, C4 off-target 1	0.101	0.015	0.014	0.012	0.041	0.032	0.025	0.117	0.012	0.001
VEGFA site 2, C5 off-target 1	0.060	0.013	0.012	0.010	0.078	0.062	0.044	0.201	0.009	0.012
VEGFA site 2, C6 off-target 1	0.019	0.005	0.005	0.004	0.080	0.062	0.045	0.087	0.002	0.012
VEGFA site 2, C7 off-target 1	0.017	0.004	0.004	0.003	0.080	0.060	0.037	0.076	0.001	0.002
VEGFA site 2, C9 off-target 1	0.230	0.088	0.037	0.011	0.667	0.256	0.051	0.134	0.004	0.007
VEGFA site 2, C10 off-target 1	0.535	0.136	0.106	0.035	0.283	0.211	0.050	0.717	0.028	0.010
VEGFA site 2, C4 off-target 2	0.038	0.004	0.003	0.003	0.087	0.051	0.048	0.063	0.048	0.134
VEGFA site 2, C5 off-target 2	0.033	0.004	0.004	0.004	0.078	0.061	0.059	0.028	0.020	0.248
VEGFA site 2, C6 off-target 2	0.026	0.005	0.005	0.004	0.051	0.038	0.038	0.043	0.038	0.783
VEGFA site 2, C7 off-target 2	0.053	0.006	0.005	0.005	0.072	0.056	0.055	0.078	0.064	0.704
VEGFA site 2, C8 off-target 2	0.071	0.006	0.006	0.006	0.079	0.065	0.065	0.118	0.107	0.703
VEGFA site 2, C9 off-target 2	0.193	0.008	0.007	0.006	0.103	0.090	0.084	0.068	0.007	0.217
VEGFA site 2, C10 off-target 2	0.063	0.003	0.003	0.002	0.116	0.107	0.090	0.545	0.016	0.346
VEGFA site 2, C4 off-target 3	0.005	0.003	0.003	0.003	0.091	0.031	0.030	0.116	0.107	0.158
VEGFA site 2, C5 off-target 3	0.011	0.007	0.005	0.005	0.211	0.048	0.042	0.220	0.177	0.001
VEGFA site 2, C6 off-target 3	0.020	0.005	0.003	0.003	0.142	0.038	0.033	0.193	0.149	0.015
VEGFA site 2, C7 off-target 3	0.045	0.006	0.003	0.003	0.101	0.035	0.030	0.093	0.060	0.083
VEGFA site 2, C8 off-target 3	0.069	0.007	0.005	0.005	0.087	0.045	0.039	0.120	0.067	0.041
VEGFA site 2, C9 off-target 3	0.093	0.006	0.005	0.005	0.041	0.032	0.028	0.396	0.195	0.005
VEGFA site 2, C10 off-target 3	0.342	0.011	0.008	0.007	0.109	0.081	0.069	0.273	0.098	0.036
VEGFA site 2, C3 off-target 4	0.001	0.001	0.001	0.001	0.374	0.374	0.230	0.271	0.358	0.633
VEGFA site 2, C4 off-target 4	0.007	0.006	0.006	0.006	0.137	0.137	0.137	0.592	0.862	0.690
VEGFA site 2, C5 off-target 4	0.007	0.007	0.007	0.007	0.026	0.017	0.018	0.461	0.655	0.279
VEGFA site 2, C6 off-target 4	0.005	0.004	0.004	0.004	0.021	0.018	0.018	0.398	0.546	0.149
VEGFA site 2, C7 off-target 4	0.007	0.006	0.006	0.006	0.051	0.048	0.050	0.373	0.720	0.029
VEGFA site 2, C8 off-target 4	0.007	0.006	0.006	0.006	0.092	0.092	0.092	0.325	0.014	0.275
VEGFA site 2, C9 off-target 4	0.016	0.007	0.007	0.007	0.150	0.150	0.150	0.502	1.000	0.615
VEGFA site 2, C10 off-target 4	0.213	0.009	0.009	0.009	0.261	0.261	0.261	0.653	0.575	0.660

Supplementary Table 1: **P-values for differences in base editing under different treatment conditions at all loci evaluated in this study.** *p*-values were calculated using the Student's two tailed t-test as described in the Materials and Methods. When the *p*-value indicated a significant difference (*p* < 0.05), the corresponding entry has been highlighted in red.

Site	Sequence	GUIDE-Seq count
EMX1 on-target	GAGT <u>C₅C₆</u> GAGCAGAAGAAGAAGGG	4,521
EMX1 off-target 1	GAGT <u>C₅</u> <u>TA</u> AGCAGAAGAAGAAAGAG	1,445
EMX1 off-target 2	GAGG <u>C₅C₆</u> GAGCAGAAGAAAGACGG	700
EMX1 off-target 3	GAGT <u>C₅C₆</u> <u>TAG</u> CAGGAGAAGAAGAG	390
HEK293 site 3 on-target	GGC <u>C₄C₅</u> AGACTGAGCACGTGATGG	2,074
HEK293 site 3 off-target 1	<u>CAC</u> <u>C₄C₅</u> AGACTGAGCACGTGCTGG	327
HEK293 site 3 off-target 2	<u>GACA</u> <u>C₅</u> AGACTGGGCACGTGAGGG	306
HEK293 site 3 off-target 3	<u>AGCT</u> <u>C₅</u> AGACTGAGCAAGTGAAGG	136
VEGFA site 2 on-target	GA <u>C₃C₄C₅C₆C₇T</u> <u>C₉C₁₀</u> ACCCCGCCTCCGG	540
VEGFA site 2 off-target 1	<u>CTA</u> <u>C₄C₅C₆C₇T</u> <u>C₉C₁₀</u> ACCCCGCCTCCGG	1,925
VEGFA site 2 off-target 2	<u>ATT</u> <u>C₄C₅C₆C₇C₈C₉C₁₀</u> ACCCCGCCTCAGG	1,549
VEGFA site 2 off-target 3	<u>ACA</u> <u>C₄C₅C₆C₇C₈C₉C₁₀</u> ACCCCGCCTCAGG	1,178
VEGFA site 2 off-target 4	<u>TG</u> <u>C₃C₄C₅C₆C₇C₈C₉C₁₀</u> ACCCCACTCTGG	1,107
FANCF on-target	GGAAT <u>C₆C₇C₈TT</u> <u>C₁₁</u> TGCAGCACCTGG	4,816
FANCF off-target 1	GGAA <u>C₅C₆C₇C₈GT</u> <u>C₁₁</u> TGCAGCACCAAGG	2,099
FANCF off-target 2	GGAGT <u>C₆C₇C₈T</u> <u>C₁₀C₁₁</u> <u>TAC</u> AGCACCAAGG	524
FANCF off-target 3	<u>AGAGG</u> <u>C₆C₇C₈C₉</u> <u>T</u> <u>C₁₁</u> TGCAGCACCAAGG	150

Supplementary Table 2: **Protospacer and PAM sequences for the on- and off-target human genomic loci studied in this work.** The off-target sites were chosen based on their GUIDE-Seq read count². Cytosines within the editing window for a particular sgRNA are shown in red and numbered. The PAM sequence is shown in blue. Protospacer bases in off-target loci that differ from their respective on-target loci have been underlined. For genomic sequences interrogated in murine samples, see Figure 5E.

Site	Sequence	CFD score	Description of locus
On-target	G <u>AC</u> CCCCCTCCACCCCGCCTC <u>CGG</u>		VEGFA site 2
Off-target 1	T <u>CC</u> CCCCCTCCACCCACCTC <u>CGG</u>	0.7857	intergenic:mmu-mir-21c-Nrp1/Mir1903
Off-target 2	TG <u>CCC</u> ACCTCACCCCGCCTC <u>TGG</u>	0.65	intron:Vipr1
Off-target 3	GC <u>CCCT</u> CCC <u>A</u> ACCCACCTC <u>TGG</u>	0.6323	intron:Nos1ap
Off-target 4	CAC <u>CCCCCT</u> CACCCCGCCTC <u>AGG</u>	0.625	intergenic:Unc5b-mmu-mir-6408

Supplementary Table 3: **Protospacer and PAM sequences for the predicted off-target loci in the mouse genome associated with the VEGFA site 2 sgRNA.** CFD scores ³ were calculated using CRISPOR ⁴. Positions in the off-target protospacers that differ from the on-target sequence are underlined.

Site	Sequence
TYR1	GT ^{C₃} AGGT ^{C₈} GAGGGTTCTGTC ^{AGG}
TYR2	CTT ^{C₄C₅} AGGATGAGAACACAG ^{AGG}
TYR3	CAAC ^{C₄C₅} A ^{C₇} TGCTCAAAGATGCT ^{TGG}

Supplementary Table 4: **Protospacer and PAM sequences for the zebrafish genomic loci studied in this work.**

HEK cell samples			
Sample Description	Replicate		
	1	2	3
Protein, BE3	237256	295609	159391
Protein, HF-BE3	383480	389874	383467
Plasmid, BE3	315213	280891	335668
Plasmid, HF-BE3	196323	251965	369201
Control	390748	395523	353614
Protein, BE3	19280	26472	24799
Protein, HF-BE3	36383	30007	39193
Plasmid, BE3	35580	29557	22243
Plasmid, HF-BE3	40371	27187	28248
Control	35106	37274	34939
Protein, BE3	82978	124689	83840
Protein, HF-BE3	142404	140482	142220
Plasmid, BE3	112027	117071	100894
Plasmid, HF-BE3	114187	98876	122553
Control	76854	90547	89271
Protein, BE3	14514	25515	19325
Protein, HF-BE3	24678	24361	25312
Plasmid, BE3	16945	19918	10225
Plasmid, HF-BE3	12200	14769	17797
Control	8739	13648	7818
Protein, BE3	17924	111693	173909
Protein, HF-BE3	243899	300503	276139
Plasmid, BE3	208476	291370	155430
Plasmid, HF-BE3	117174	154033	199152
Control	119263	170436	121686
Protein, BE3	237799	262947	185371
Protein, HF-BE3	313253	233699	244922
Plasmid, BE3	243094	230316	234421
Plasmid, HF-BE3	170958	160091	140693
Control	158691	148720	137270
Protein, BE3	28684	28237	43315
Protein, HF-BE3	49300	42576	57690
Plasmid, BE3	55008	55813	54310
Plasmid, HF-BE3	55199	11384	7659
Control	63741	42878	48524
Protein, BE3	104822	181792	161090
Protein, HF-BE3	204580	175561	177303
Plasmid, BE3	178584	152264	206863
Plasmid, HF-BE3	191297	138425	160789
Control	190303	190061	190516
Protein, BE3	146089	95113	135015
Protein, HF-BE3	155947	136541	157991
Plasmid, BE3	150036	128438	158905
Plasmid, HF-BE3	371077	123642	142562
Control	130322	134545	141833
Protein, BE3	145058	175338	161837
Protein, HF-BE3	212337	178993	179887
Plasmid, BE3	186452	166500	80441
Plasmid, HF-BE3	163732	118453	134719
Control	131461	134470	155608
Protein, BE3	41986	61678	67890
Protein, HF-BE3	41057	55850	86411
Plasmid, BE3	39114	48575	70074
Plasmid, HF-BE3	41617	55638	75718
Control	68852	59422	81265
Protein, BE3	113462	80529	191344
Protein, HF-BE3	202662	233981	203024
Plasmid, BE3	208912	202044	107234
Plasmid, HF-BE3	86494	113989	86807
Control	92255	72386	56661
Protein, BE3	96271	117442	84374
Protein, HF-BE3	105624	102312	105343
Plasmid, BE3	101002	98747	70052
Plasmid, HF-BE3	308966	69787	83184
Control	99986	100344	100639
Protein, BE3	25524	182451	65388
Protein, HF-BE3	71858	75553	71785
Plasmid, BE3	60980	57360	78169
Plasmid, HF-BE3	68316	34659	85718
Control	49685	57388	60418
Protein, BE3	46981	90793	79439
Protein, HF-BE3	58629	71186	61575
Plasmid, BE3	70817	82736	75706
Plasmid, HF-BE3	77038	71123	78511
Control	62183	48574	68439
Protein, BE3	165905	257565	142888
Protein, HF-BE3	148339	151300	130712
Plasmid, BE3	101950	103226	203004
Plasmid, HF-BE3	167969	175193	97010
Control	101476	150435	102327
Protein, BE3	136738	213438	118711
Protein, HF-BE3	123413	126114	109375
Plasmid, BE3	85576	86600	169592
Plasmid, HF-BE3	140317	145738	137050
Control	84818	125139	85454
Protein, BE3	11940	36593	24946
Protein, HF-BE3	26762	31566	36377
Plasmid, BE3	32420	21547	14659
Plasmid, HF-BE3	31427	16592	17385
Control	17385	28128	32717

Murine Samples from NIH 3T3 cell treatment			
Sample Description	Replicate		
	1	2	3
Plasmid, BE3	16641	102216	46361
Plasmid, HF-BE3	89330	126545	100993
Protein, BE3	88998	81697	51124
Protein, HF-BE3	128218	29193	131515
Control	18767	38866	58985
Plasmid, BE3	174782	167504	182565
Plasmid, HF-BE3	167120	182520	192389
Protein, BE3	230569	212805	138144
Protein, HF-BE3	228668	211457	183370
Control	171738	191117	20879
Plasmid, BE3	206475	227332	206089
Plasmid, HF-BE3	213809	203028	199078
Protein, BE3	215995	275754	249969
Protein, HF-BE3	250918	272063	241059
Control	193760	175959	249693
Plasmid, BE3	60388	126278	7328
Plasmid, HF-BE3	89045	128508	5178
Protein, BE3	167195	330046	11163
Protein, HF-BE3	82120	309352	10393
Control	83204	176939	5661
Plasmid, BE3	192846	113709	171078
Plasmid, HF-BE3	205601	151434	188943
Protein, BE3	218194	181993	208398
Protein, HF-BE3	211966	148976	186838
Control	183933	130318	197476

Mouse Cochlea Samples			
Sample Description	Replicate		
	1	2	3
Stria vasculares	37889	205706	62091
Organ of Corti	148447	175004	29075
Modiolus	182806	181382	61269
Uninjected control	228222	241979	272759
Stria vasculares	44457	244487	244466
Organ of Corti	136335	118318	34747
Modiolus	67176	209543	68699
Uninjected control	343100	342717	379015
Stria vasculares	72962	319883	265793
Organ of Corti	198456	131430	60530
Modiolus	92014	251509	81413
Uninjected control	399138	345965	483920
Stria vasculares	8325	80322	142556
Organ of Corti	81014	45976	1810
Modiolus	9928	75555	11341
Uninjected control	399138	345965	483920
Stria vasculares	232194	397770	554054
Organ of Corti	313472	285309	176872
Modiolus	230105	371399	258142
Uninjected control	524503	637946	624709

Zebrafish samples			
Sample Description	1	2	3
	1	2	3
Treated zebrafish	72355	49498	81061
Scrambled sgRNA	107919	98502	92429
Treated zebrafish	51434	48014	41547
Scrambled sgRNA	61466	62374	66765
Treated zebrafish	6487	57247	75883
Scrambled sgRNA	64596	71234	75624

Mouse Cochlea Samples - treated with unrealated sgRNA			
Sample Description	Sample		
	Stria	Corti	Modiolus
537459	249767	389274	On Target (VEGFA)

Supplementary Table 5: Number of HTS reads that align to the reference sequence and pass the quality filters described in Materials and Methods.

Supplementary References

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