

Precise, minimally evolved adenine base editors generated through mutation reversion analysis

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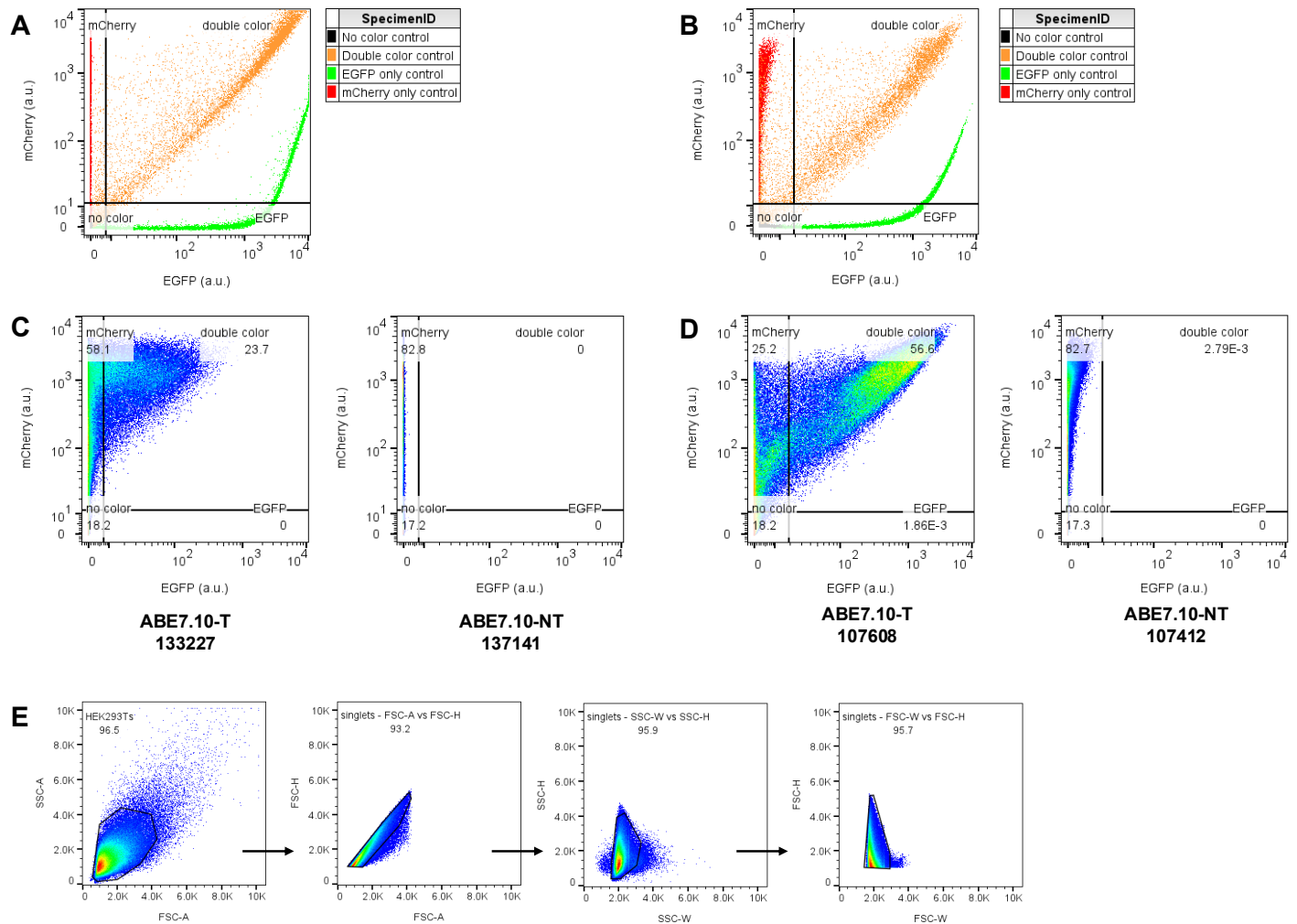
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residue	wtTadA	TadA*7.10	TadA*8e	TadA*8.20
23	W	R	R	R
36	H	L	L	L
48	P	A	A	A
51	R	L	L	L
76	I	I	I	Y
82	V	V	V	S
84	L	F	F	F
106	A	V	V	V
108	D	N	N	N
109	A	A	S	A
111	T	T	R	T
119	D	D	N	D
122	H	H	N	H
123	H	Y	Y	<u>H</u>
146	S	C	C	C
147	D	Y	<u>D</u>	<u>R</u>
149	F	F	Y	F
152	R	P	P	P
155	E	V	V	V
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156	I	F	F	F
157	K	N	N	N
166	T	T	I	T
167	D	D	N	D

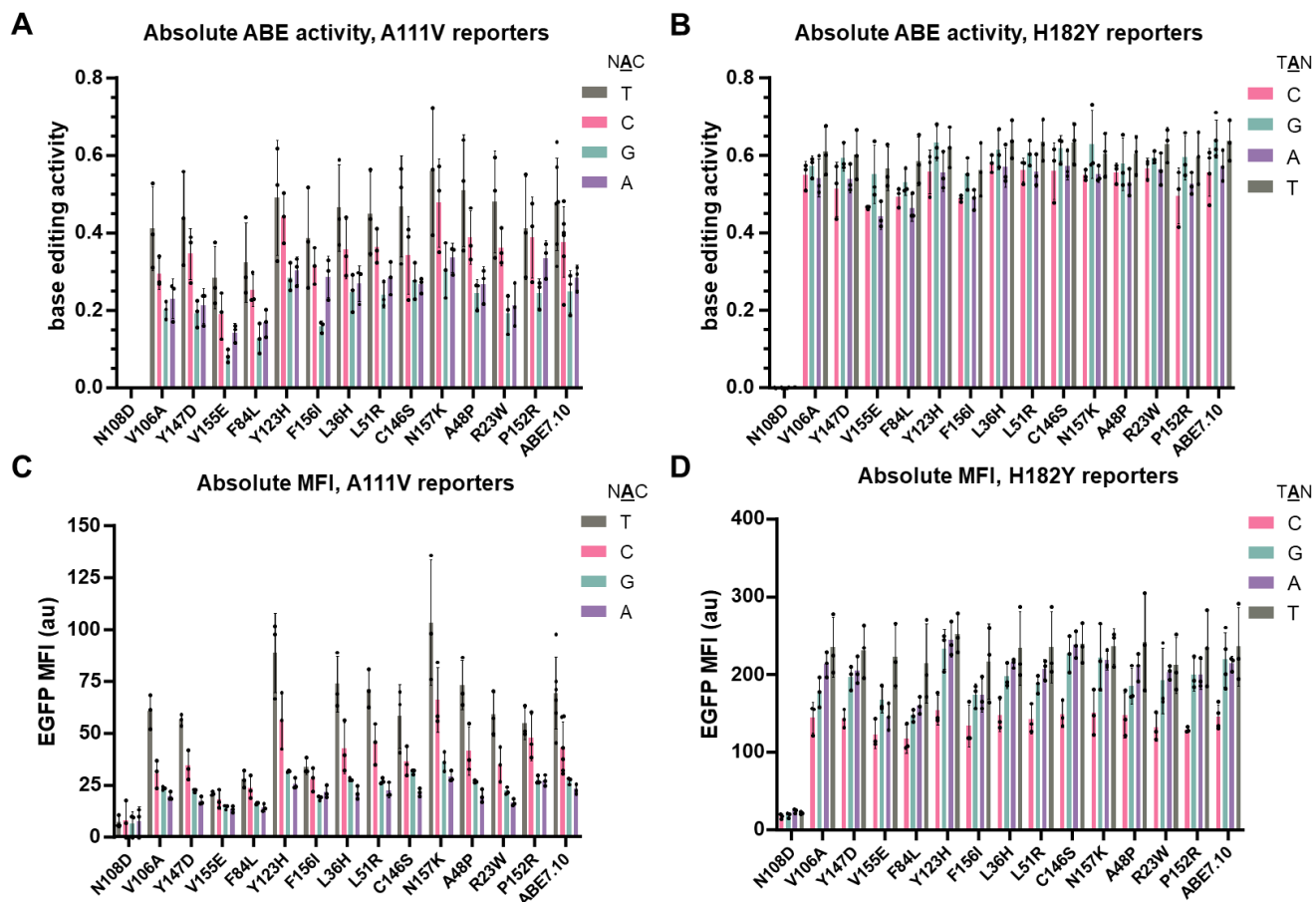
Supplementary Figure 1 Identities of mutated residues in evolved TadA variants

Mutated residues are colored by the variant they were first evolved in – blue for ABE7.10, pink for ABE8e, and peach for ABE8.20. Outlined boxes with underlined amino acids indicate a residue position that was further evolved from an earlier generation modification.



Supplementary Figure 2 Example flow cytometry gating scheme for EGFP turn-on fluorescent reporters

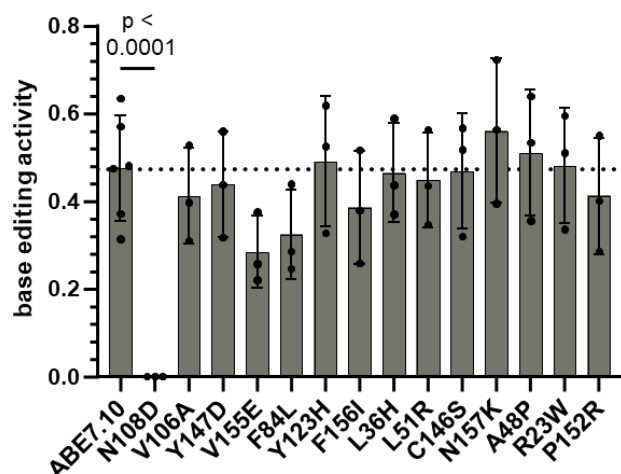
A-B. Representative color control gates for the A111V family of reporters (**A**) and H182Y family of reporters (**B**) (taken from one of six transfections). Color controls were collected using HEK293T cells transfected with a non-targeting gRNA plasmid, ABE0.1 plasmid, and appropriate reporter plasmid: mCherry-P2A-dEGFP(A111V - **A** or H182Y - **B**) for mCherry only control, dmCherry(Y72H)-P2A-EGFP for EGFP only control, mCherry-P2A-EGFP for double color control, and dmCherry(Y72H)-P2A-dEGFP(A111V - **A** or H182Y - **B**) for no color control. **C-D.** Gating was validated for each family of reporters using the ABE7.10 editor with targeting (T) or non-targeting (NT) gRNA. Representative plots here are shown for the A111V **GAC** reporter (**C**), and for the H182Y **TAT** reporter (**D**). **E.** A representative gating strategy used to identify live, singlet cells is shown for ABE7.10 with T gRNA and the A111V **GAC** reporter plasmid.



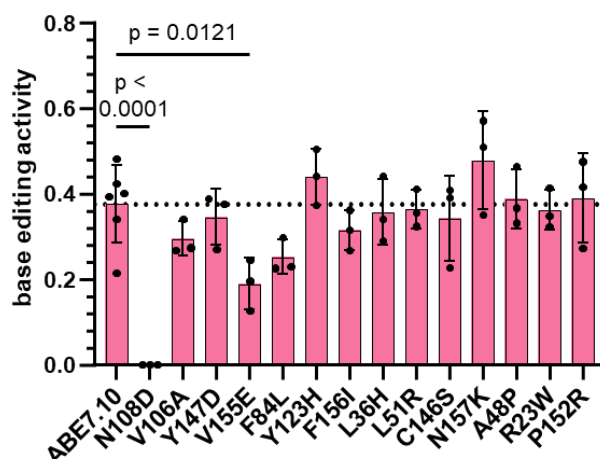
Supplementary Figure 3 EGFP turn-on fluorescent reporter absolute editing activity of single-residue reversion editors

HEK293T cells were transfected with a mixture containing one of the eight reporter plasmids, its respective gRNA plasmid, and a base editor plasmid encoding one of the 14 ABE7.10 single-residue reversion editors or ABE7.10. Cells were incubated for 72 hours before analysis by flow cytometry. Cells were analyzed for both mCherry and EGFP fluorescence, and their “BE activity” score was calculated by dividing the percent of edited cells (those with double color mCherry & EGFP fluorescence) by the total percent of transfected cells (those with mCherry fluorescence). Shown are average absolute editing activity scores (**A-B**) and median EGFP fluorescence intensity (MFI) of double color positive cells (**C-D**) for all fourteen ABE7.10 single-residue reversion editors and ABE7.10 at all A111V -1 base reporters (**A** and **C**) and H182Y +1 reporters (**B** and **D**). Bars and error bars represent the average and standard deviation. All single-residue reversion editors were measured with $n = 3$ biological replicates, ABE7.10 was measured with $n = 6$ biological replicates for A111V TAC and CAC reporters, $n = 4$ biological replicates for H182Y TAC and TAG reporters, and $n = 3$ biological replicates for all others.

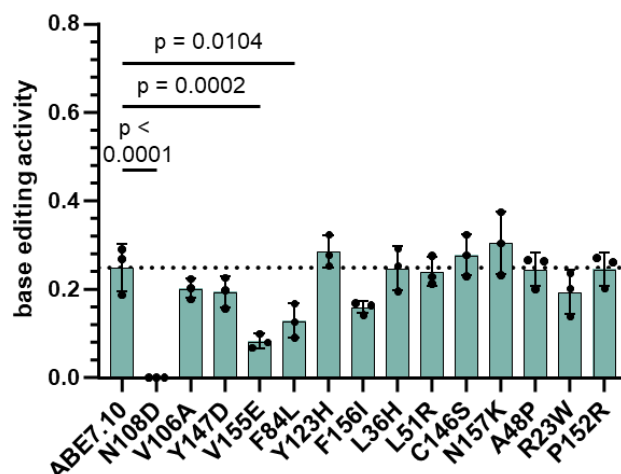
Absolute ABE activity, A111V TAC reporter



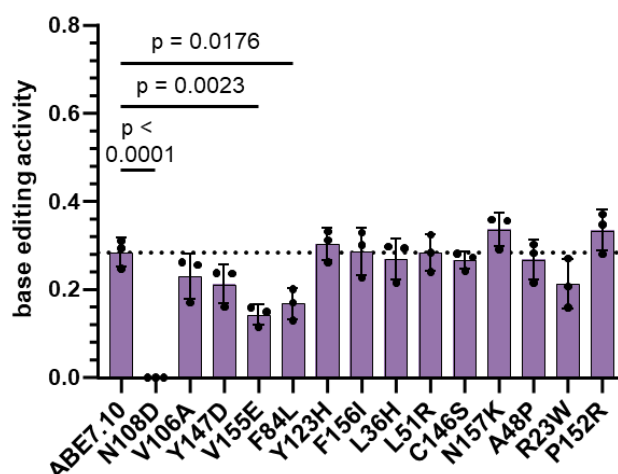
Absolute ABE activity, A111V CAC reporter



Absolute ABE activity, A111V GAC reporter



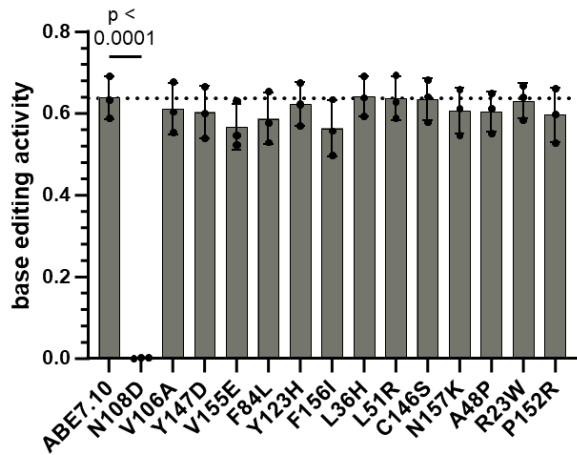
Absolute ABE activity, A111V AAC reporter



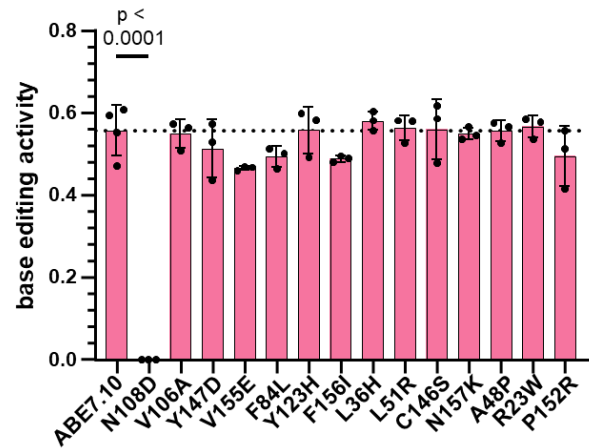
Supplementary Figure 4 Absolute editing activity for all single-residue reversion editors against the A111V family of fluorescent turn-on reporters, broken down by reporter

Cells were treated as described in Supplementary Figure 3. Shown are BE activity scores for the A111V family of reporters, broken down by sequence context. Bars and error bars represent the average and standard deviation for $n = 3$ biological replicates (all single-residue reversion editors) while ABE7.10 was measured with $n = 6$ biological replicates for A111V TAC and CAC reporters and $n = 3$ biological replicates for all others. Dashed horizontal line indicates average BE activity score of ABE7.10, for a given reporter. Data were analyzed by ordinary one-way ANOVA with Holm-Šidák multiple comparison test to ABE7.10 with significant p-values reported ($p \geq 0.05$ not significant, not indicated).

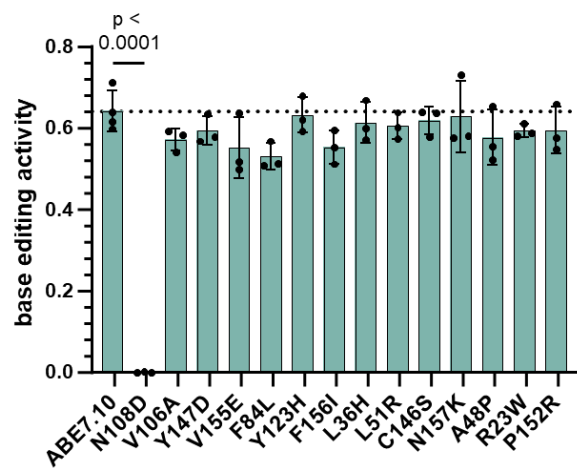
Absolute ABE activity, A111V TAT reporter



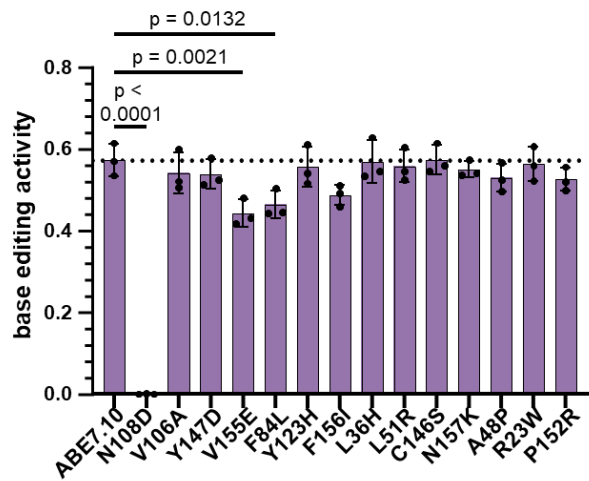
Absolute ABE activity, H182Y TAC reporter



Absolute ABE activity, H182Y TAG reporter

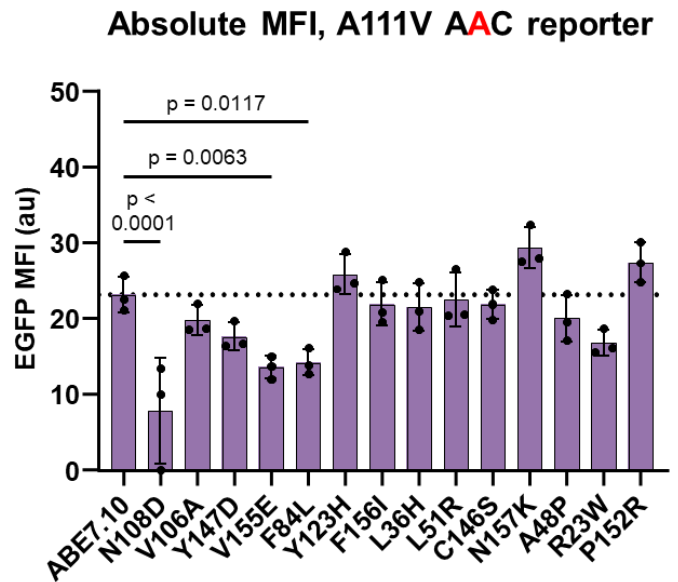
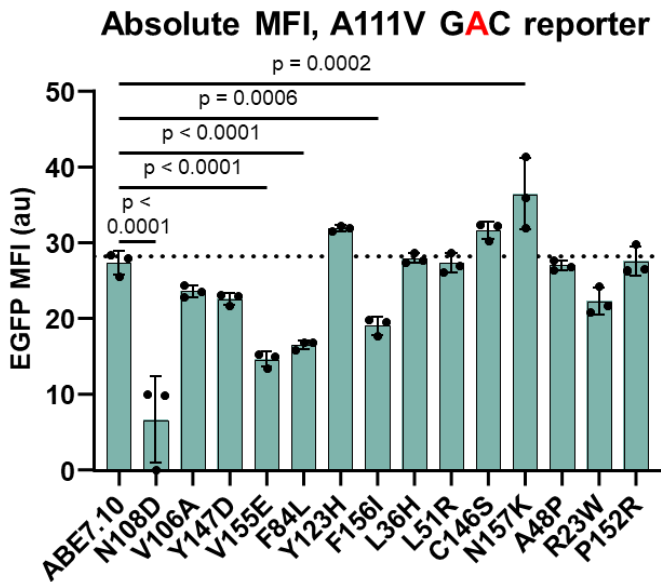
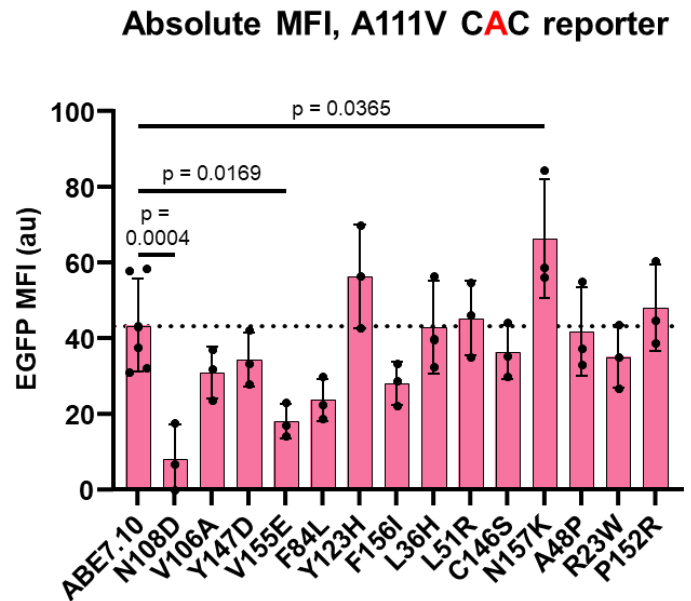
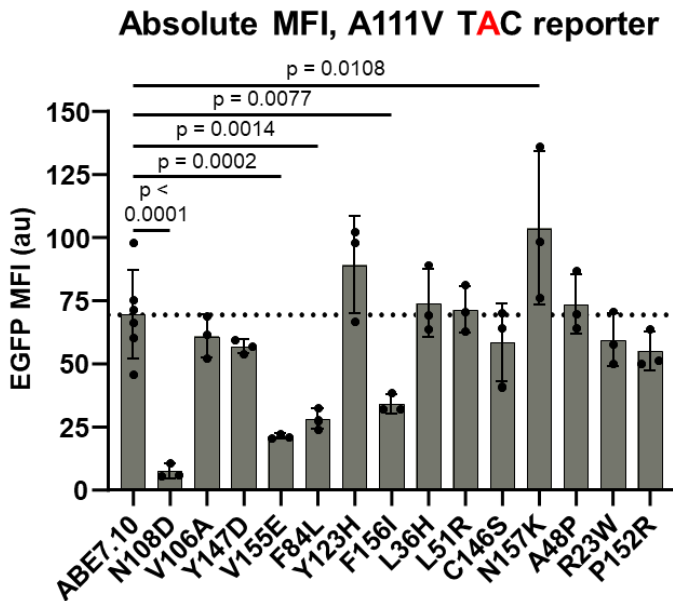


Absolute ABE activity, H182Y TAA reporter



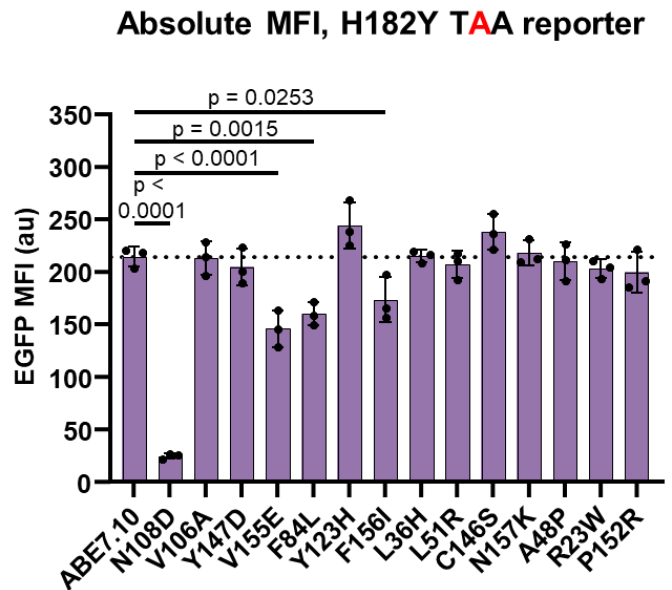
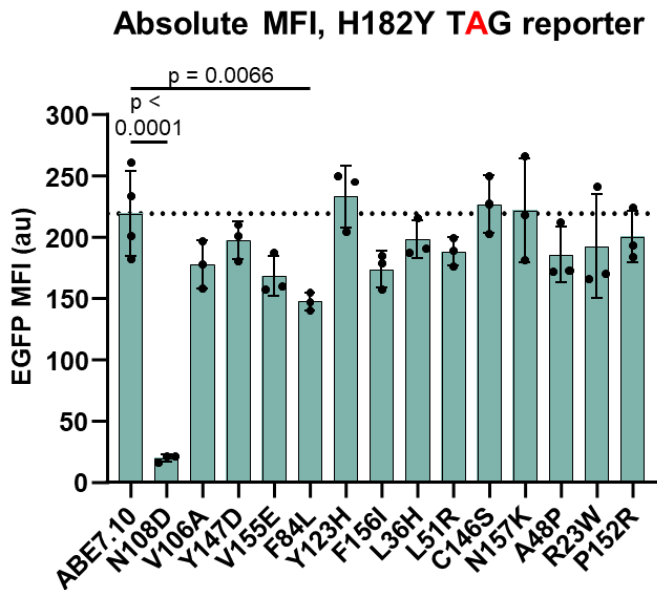
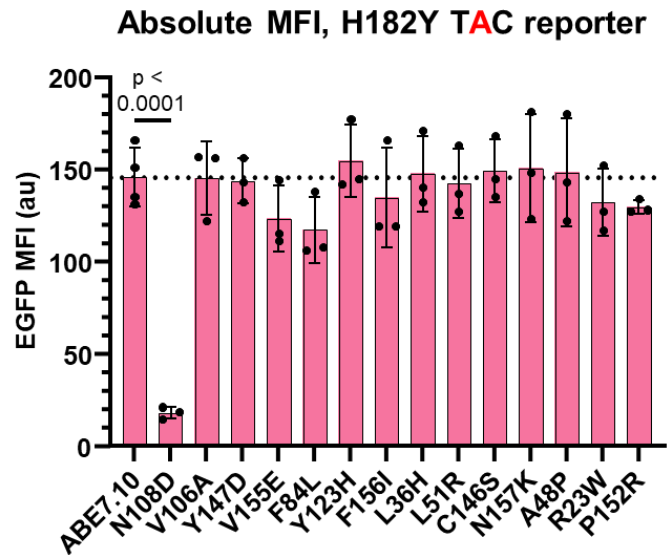
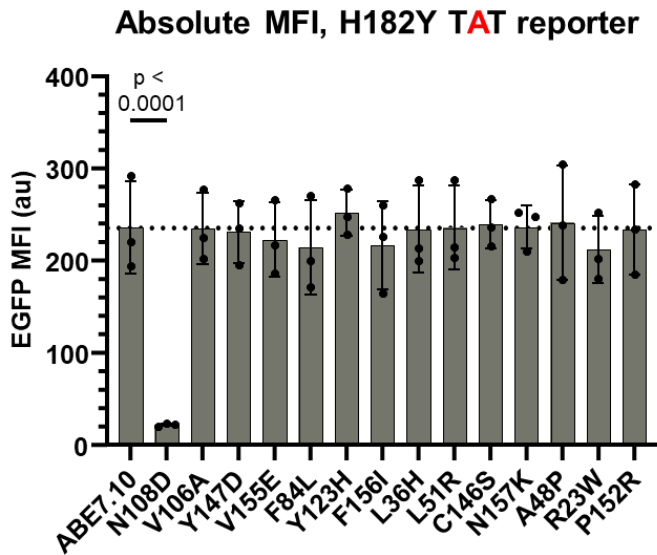
Supplementary Figure 5 Absolute editing activity for all single-residue reversion editors against the H182Y family of fluorescent turn-on reporters, broken down by reporter

Cells were treated as described in Supplementary Figure 3. Shown are BE activity scores for the H182Y family of reporters, broken down by sequence context. Bars and error bars represent the average and standard deviation for $n = 3$ biological replicates (all single-residue reversion editors) while ABE7.10 was measured with $n = 4$ biological replicates for H182Y TAC and TAG reporters, and $n = 3$ biological replicates for all others. Dashed horizontal line indicates average MFI of ABE7.10 for a given reporter. Data were analyzed by ordinary one-way ANOVA with Holm-Šídák multiple comparison test to ABE7.10 with significant p -values reported ($p \geq 0.05$ not significant, not indicated).



Supplementary Figure 6 Absolute MFI values for all single-residue reversion editors against the A111V family of fluorescent turn-on reporters, broken down by reporter

Cells were treated as described in Supplementary Figure 3. Shown are MFI values for the A111V family of reporters, broken down by sequence context. Bars and error bars represent the average and standard deviation for $n = 3$ biological replicates (all single-residue reversion editors) while ABE7.10 was measured with $n = 6$ biological replicates for A111V TAC and CAC reporters and $n = 3$ biological replicates for all others. Dashed horizontal line indicates average BE activity score of ABE7.10, for a given reporter. Data were analyzed by ordinary one-way ANOVA with Holm-Šídák multiple comparison test to ABE7.10 with significant p-values reported ($p \geq 0.05$ not significant, not indicated).



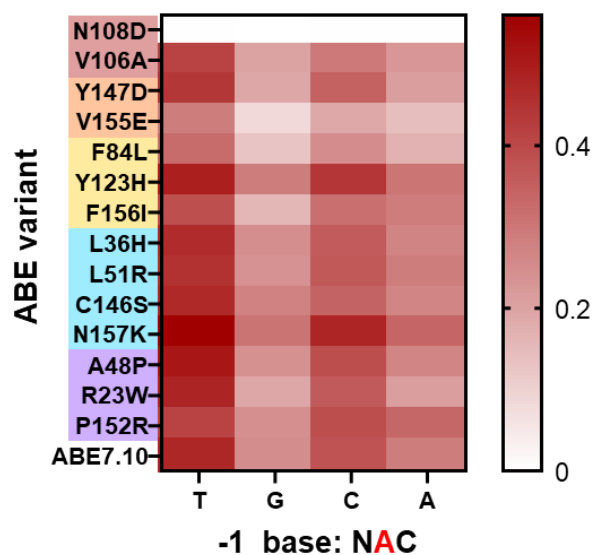
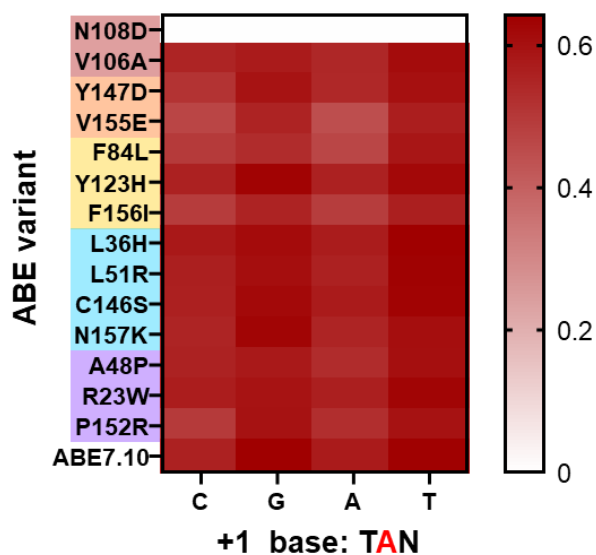
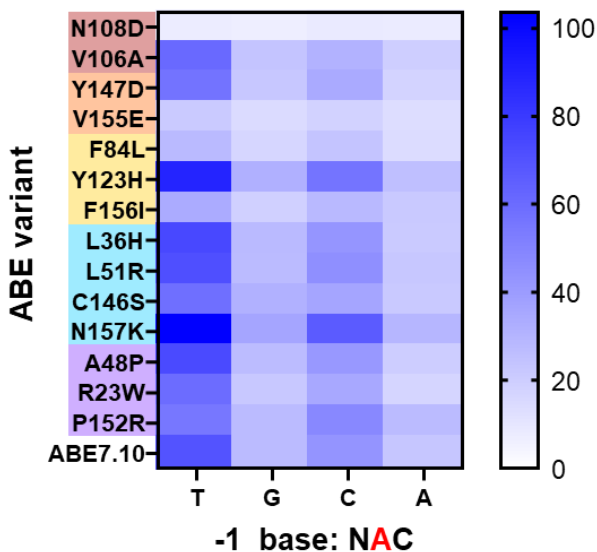
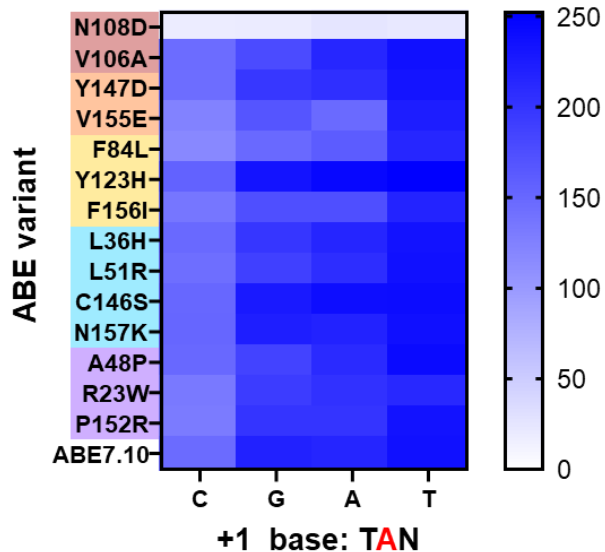
Supplementary Figure 7 Absolute MFI values for all single-residue reversion editors against the H182Y family of fluorescent turn-on reporters, broken down by reporter

Cells were treated as described in Supplementary Figure 3. Shown are MFI values for the H182Y family of reporters, broken down by sequence context. Bars and error bars represent the average and standard deviation for $n = 3$ biological replicates (all single-residue reversion editors) while ABE7.10 was measured with $n = 4$ biological replicates for H182Y T_{AC} and T_{AG} reporters, and $n = 3$ biological replicates for all others. Dashed horizontal line indicates average MFI of ABE7.10, for a given reporter. Data were analyzed by ordinary one-way ANOVA with Holm-Šidák multiple comparison test to ABE7.10 with significant p -values reported ($p \geq 0.05$ not significant, not indicated).

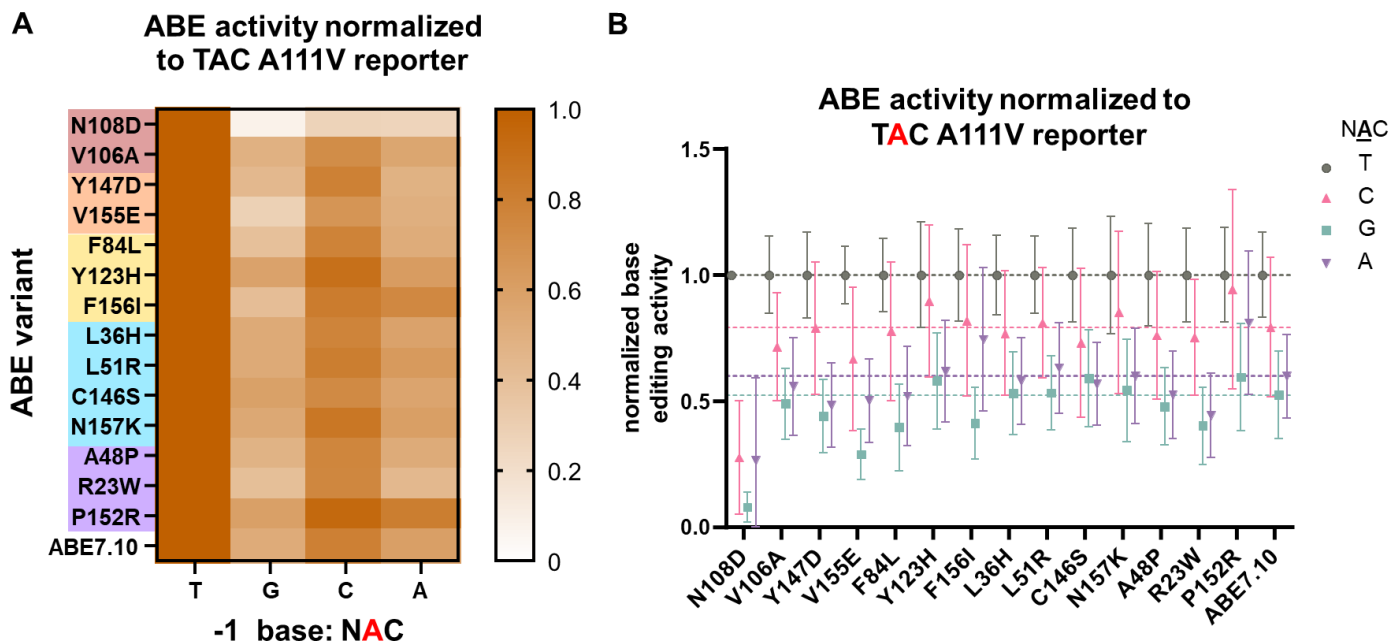
A	sample	average BE activity score across -1 reporters	average BE activity score across +1 reporters	overall average BE score across all reporters	B	sample	average MFI across -1 reporters	average MFI across +1 reporters	overall MFI across all reporters
	ABE7.10-N157K	1.21±0.23	0.97±0.06	1.09±0.12		ABE7.10-N157K	1.40±0.22	1.03±0.16	1.22±0.14
	ABE7.10-Y123H	1.10±0.20	0.98±0.06	1.04±0.10		ABE7.10-Y123H	1.22±0.18	1.10±0.14	1.16±0.11
	ABE7.10	1.00±0.18	1.00±0.06	1.00±0.10		ABE7.10	1.00±0.15	1.00±0.14	1.00±0.10
	ABE7.10-C146S	0.99±0.19	0.99±0.06	0.99±0.10		ABE7.10-L36H	1.00±0.15	0.98±0.13	0.99±0.10
	ABE7.10-L36H	0.97±0.18	1.00±0.06	0.98±0.09		ABE7.10-C146S	0.95±0.13	1.03±0.11	0.99±0.08
	ABE7.10-L51R	0.97±0.16	0.98±0.05	0.97±0.09		ABE7.10-P152R	1.02±0.14	0.95±0.14	0.99±0.10
	ABE7.10-A48P	1.01±0.18	0.94±0.06	0.97±0.10		ABE7.10-L51R	1.01±0.14	0.95±0.11	0.98±0.09
	ABE7.10-P152R	1.02±0.18	0.92±0.06	0.97±0.10		ABE7.10-A48P	0.97±0.14	0.96±0.15	0.97±0.10
	ABE7.10-R23W	0.88±0.16	0.98±0.05	0.93±0.09		ABE7.10-V106A	0.83±0.11	0.95±0.11	0.89±0.08
	ABE7.10-Y147D	0.84±0.15	0.93±0.06	0.89±0.08		ABE7.10-Y147D	0.80±0.10	0.97±0.12	0.88±0.08
	ABE7.10-V106A	0.82±0.15	0.94±0.06	0.88±0.08		ABE7.10-R23W	0.80±0.12	0.87±0.10	0.84±0.08
	ABE7.10-F156I	0.82±0.14	0.87±0.05	0.85±0.07		ABE7.10-F156I	0.69±0.09	0.86±0.13	0.78±0.08
	ABE7.10-F84L	0.61±0.12	0.86±0.05	0.74±0.06		ABE7.10-F84L	0.54±0.07	0.79±0.11	0.67±0.07
	ABE7.10-V155E	0.48±0.09	0.84±0.05	0.66±0.05		ABE7.10-V155E	0.46±0.06	0.83±0.13	0.64±0.07
	ABE7.10-N108D	2.07E-4 ±9.5E-5	1.04E-3 ±3.1E-4	6.2E-4 ±1.6E-4		ABE7.10-N108D	0.22 ±9.3E-2	0.10 ±1.3E-2	0.16 ±4.7E-2

Supplementary Figure 8 Normalized average BE activity and MFI scores for single-residue reversion editors evaluated with EGFP turn-on fluorescent reporters

A. Average normalized BE activity scores for cells treated as described in Supplementary Figure 3. Base editing activity for all single-residue reversion editors was averaged across $n = 3$ biological replicates, while base editing activity for ABE7.10 was averaged across $n = 6$ biological replicates for A111V TAC and CAC reporters, averaged across $n = 4$ biological replicates for H182Y TAC and TAG reporters, and averaged across $n = 3$ biological replicates for all other reporters. For each editor with each reporter, fold-change in editing activity with respect to ABE7.10 (normalized BE activity) was calculated by dividing the reversion editor's average editing activity by that of ABE7.10 at the same motif, with propagated error considering standard deviation of the reversion editor and ABE7.10's base editing activity. Further averaging and propagating error for these normalized BE activity scores across all members of a reporter family (-1 or +1) is reported above, as well an additional average and associated propagated error across all eight fluorescent EGFP turn-on reporters (overall average BE score). Single-residue reversion variants have been ordered by decreasing overall average normalized BE activity score, and cells are colored by the reversion editor's relative activity with respect to ABE7.10 across all eight reporters. Green indicates reversion editors that increased normalized editing activity (score > 1.00), blue indicates variants with mostly neutral activity (score range 0.95-0.99), yellow indicates variants that mildly decreased editing activity (score 0.80-0.94), light orange indicates variants that moderately decreased editing activity (score 0.64-0.79), while dark orange indicates variants that severely decreased editing activity (score < 0.64). **B.** Average normalized MFI values were determined as in part A, and have similarly been ordered by decreasing overall normalized MFI. Cell colors match those for normalized BE activity scores.

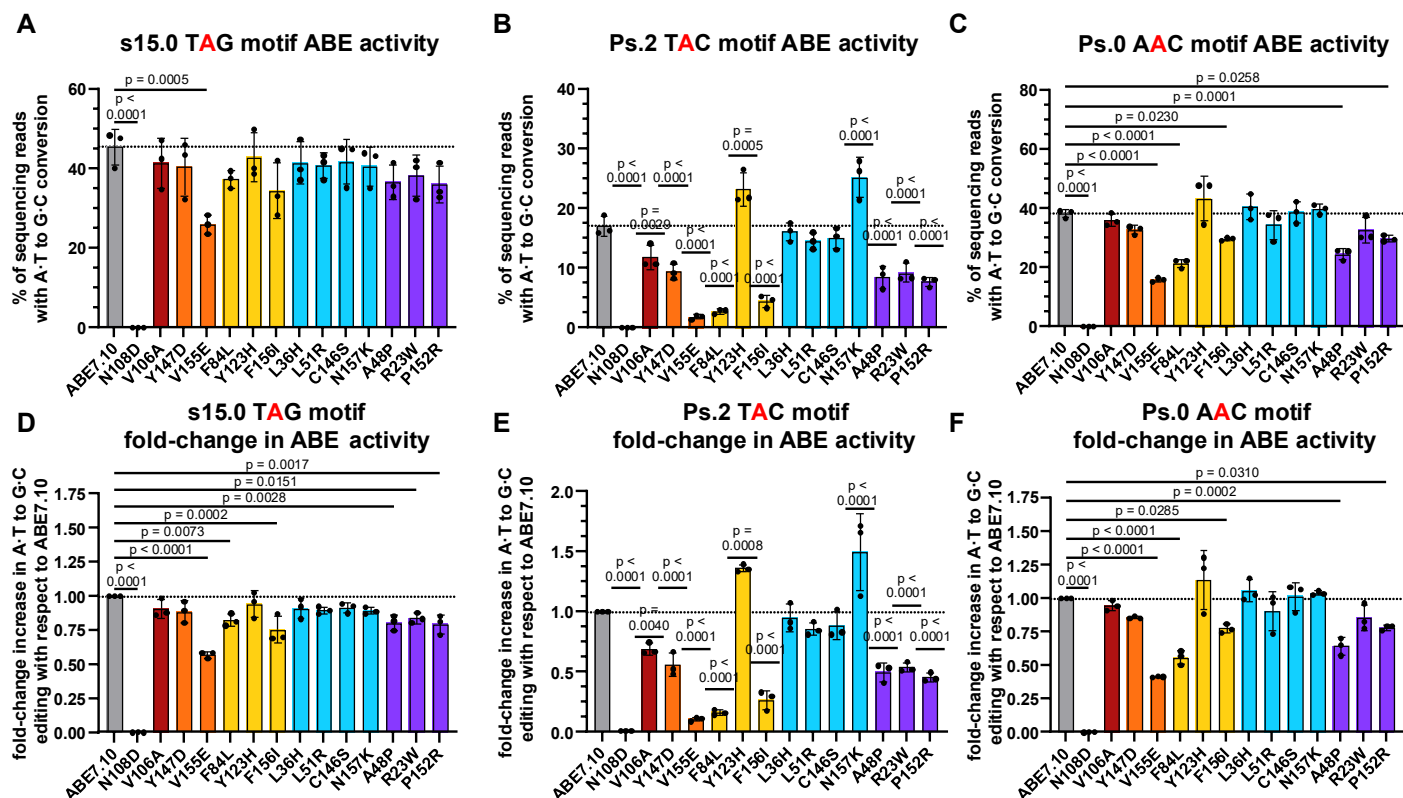
A Absolute ABE activity, A111V reporters**B** Absolute ABE activity, H182Y reporters**C** Median fluorescence intensity, A111V reporters**D** Median fluorescence intensity, H182Y reporters**Supplementary Figure 9 Heat maps of EGFP turn-on fluorescent reporter absolute editing activities and MFIs**

Cells were treated as described in Supplementary Figure 3. Color gradients indicate average absolute editing activity (not fold-change) scores for A111V -1 reporters (**A**) and H182Y +1 reporters (**B**) and average absolute EGFP MFI (not fold change) for A111V -1 reporters (**C**) and H182Y +1 reporters (**D**). Averages were drawn from $n = 3$ biological replicates for all 14 ABE7.10 single-residue reversion editors, while ABE7.10 was measured with $n = 6$ biological replicates for A111V TAC and CAC reporters, $n = 4$ biological replicates for H182Y TAC and TAG reporters, and $n = 3$ biological replicates for all others.



Supplementary Figure 10 Sequence context preference analyses of reversion editors

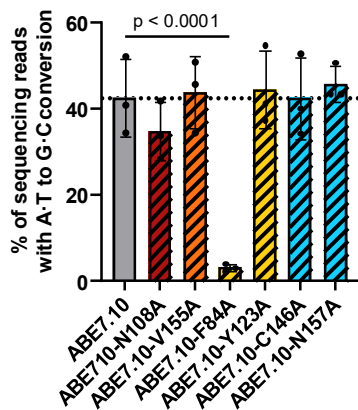
Cells were treated as described in Supplementary Figure 3. Only data points collected with the -1 A111V reporter family were analyzed. The average base editing activity values for each reversion editor were collected from $n = 3$ biological replicates for all single-residue reversion editors while ABE7.10 was measured with $n = 6$ biological replicates for A111V TAC and CAC reporters and $n = 3$ biological replicates for all others. For a given editor at a given motif, values were then normalized to the average activity of the same editor at the favored TAC sequence motif. Data is presented as heat map where color gradient indicates sequence normalized average editing activity (**A**). The same data set has also been illustrated in **B** where points show the sequence normalized average editing activity, and error bars illustrate propagated error measurements taken from the standard deviation of base editing activity at the NAC motif, and standard deviation of the base editing activity at the TAC motif for a given editor. Horizontal dotted lines indicate normalized average editing activity for the ABE7.10 editor at different NAC sequence motifs, with colors corresponding to their respective -1 N base.



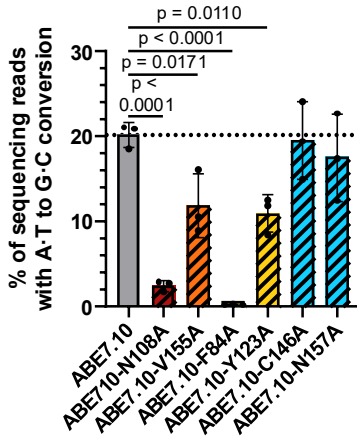
Supplementary Figure 11 Single-residue reversion editor activity at three endogenous mammalian sites

A-C. HEK293T cells were transfected with an ABE plasmid encoding ABE7.10 or one of the 14 single-residue reversion editors indicated along with one of three gRNA plasmids targeting the protospacers indicated in Extended Data Figure 1A. Cells were harvested after 72 hours and genomic loci of interest were analyzed for editing activity with NGS. Shown are average editing activities (percent of DNA sequencing reads with the target A•T converted to G•C) at the s15.0 (**A**), Ps.2 (**B**), and Ps.0 (**C**) sites. **D-E.** Shown are the average fold-change values in endogenous editing activity relative to ABE7.10 for each of the 14 single-residue reversion editors at the s15.0 (**D**), Ps.2 (**E**), and Ps.0 (**F**) sites. Bars and error bars represent the average and standard deviation of $n = 3$ biological replicates, with individual data points shown. Dotted line indicates the average editing efficiency of ABE7.10. Data were analyzed by ordinary one-way ANOVA with Holm-Šidák multiple comparison test to ABE7.10 with significant p-values reported ($p \geq 0.05$ not significant, not indicated).

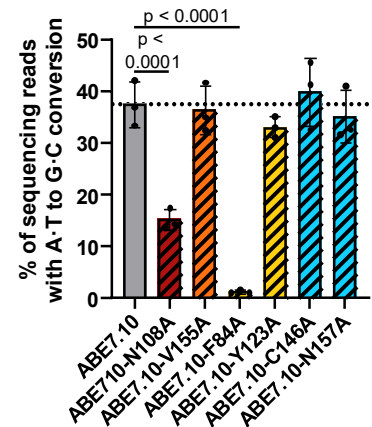
A s15.0 TAC motif ABE activity,
select alanine mutations



B Ps.2 TAC motif ABE activity,
select alanine mutations

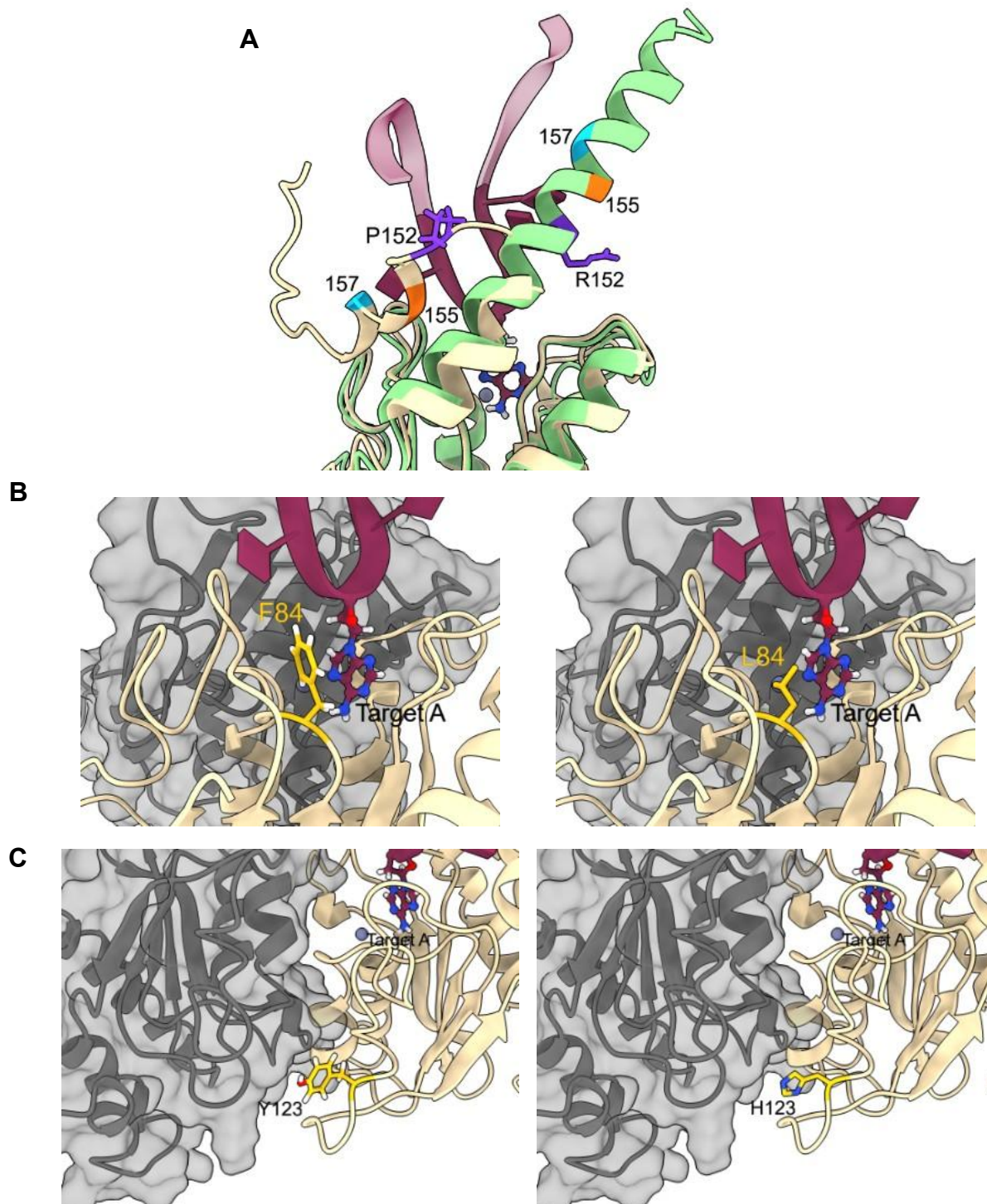


C Ps.0 AAC motif ABE activity,
select alanine mutations



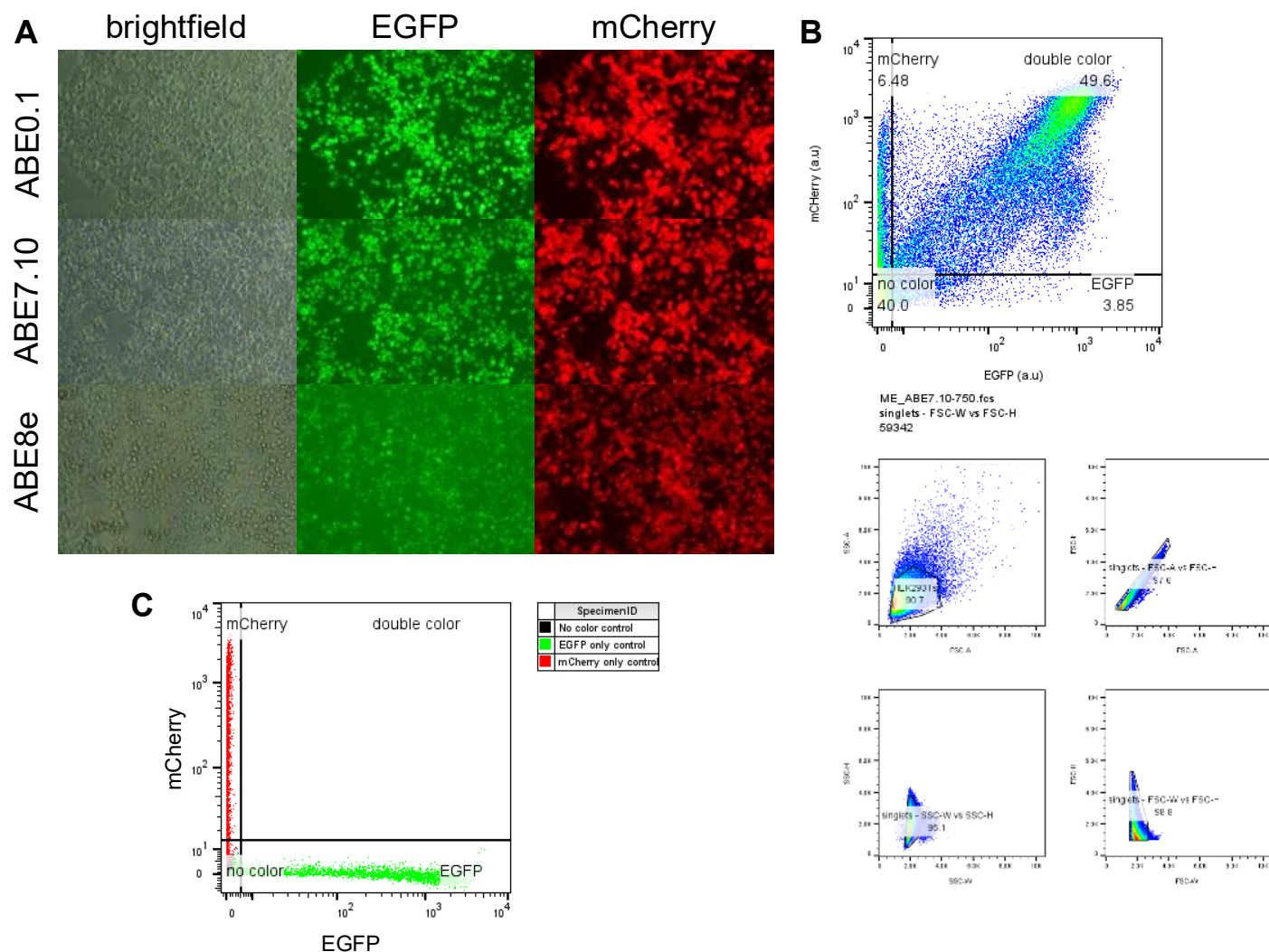
Supplementary Figure 12 Single-residue alanine substitution editing activity at three endogenous mammalian sites

A-C. HEK293T cells were transfected with an ABE plasmid encoding ABE7.10, a single-residue reversion or an alanine substitution editor as indicated, along with one of three gRNA plasmids targeting the protospacers listed in Extended Data Figure 1A. Cells were harvested after 72 hours and genomic loci of interest analyzed for editing activity with NGS. Shown are average editing activities (percent of DNA sequencing reads with the target A•T converted to G•C) at the s15.0 (**A**), Ps.2 (**B**), and Ps.0 (**C**) sites. Dotted line indicates the average editing efficiency of ABE7.10. Bars and error bars represent the average and standard deviation of $n = 3$ biological replicates, with individual data points shown. Data were analyzed by ordinary one-way ANOVA with Holm-Šídák multiple comparison test to ABE7.10 with significant p-values reported ($p \geq 0.05$ not significant, not indicated).



Supplementary Figure 13 Modeling illustrates relative positioning of key residues

ABE variants are modeled using the crystal structure of ABE8e (PDB ID: 6VPC) and substituting the catalytic TadA* domain (tan ribbon) with an AlphaFold2-predicted TadA*7.10^{9,12}. The “docking domain” (in grey-solid) was modeled using the ChimeraX swapaa function to revert the TadA*8e residues to TadA*7.10. Note that while all constructs were developed as monomers containing only the “catalytic” domain, the possibility of in trans dimerization merits consideration of how substitutions/reversions may impact potential dimerization interfaces. **A.** A focus on the C-terminal $\alpha 5$ helix for TadA*7.10, and TadA0.1 (green, modeled from PDB ID: 1Z3A with AlphaFold predictions for unmodeled regions). The P152 (TadA*7.10) and R152 (wtTadA) residues are highlighted to illustrate the differential “kink” observed in the C-terminal helix for evolved TadA variants. **B.** The left model highlights the F84 residue as found in the final ABE7.10 variant, while right illustrates the modeled ABE7.10–F84L, shown in relation to the target A. **C.** The left model illustrates the ABE7.10 Y123 residue positioning at the dimer interface, while the right models the ABE7.10-H123Y variant.

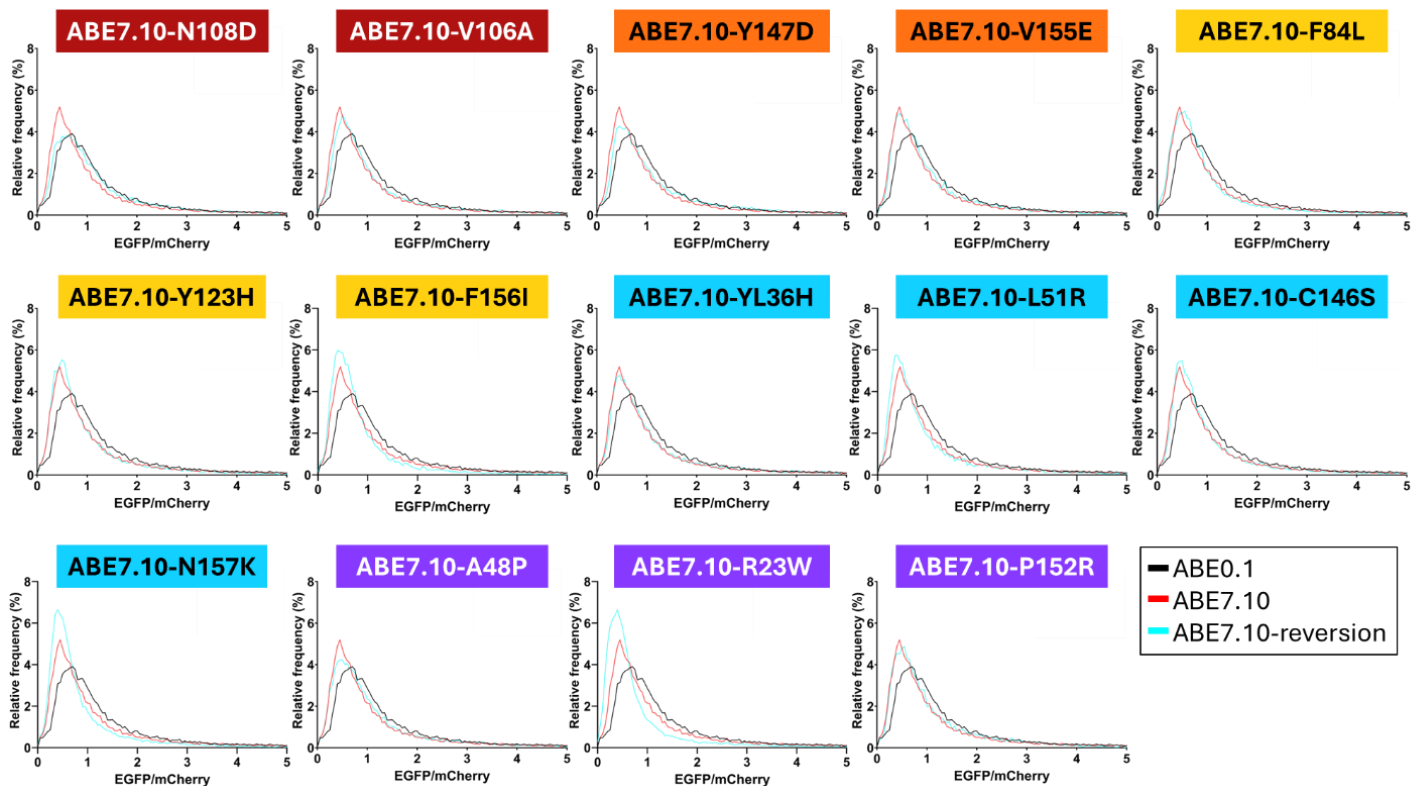


Supplementary Figure 14 Stability/expression reporter benchmarking

A. Fluorescence microscopy images of HEK293T cells transfected with the stability reporters indicated in Extended Data Figure 2A for ABE0.1, ABE7.10, and ABE8e. Images are representative of those taken from $n = 3$ independent transfections.

B. Representative gating strategy used to identify double color fluorescent cells (top) for further analysis of ABE stability of expression, illustrated using the ABE7.10 stability reporter. Gating strategy used to identify live, singlet cells is shown below.

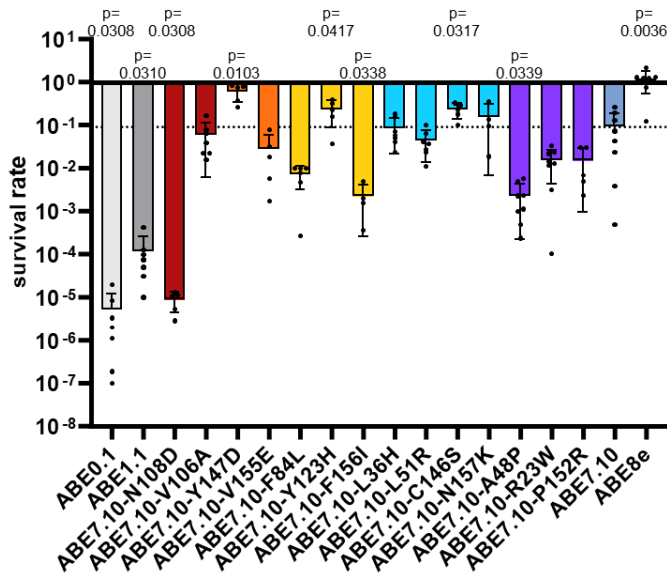
C. Single color plasmid controls, establishing fluorescent protein marker gates. mCherry only utilized an mCherry-P2A-ABE0.1-dEGFP(A111V) construct, EGFP only a dmCherry(Y72H)-P2A-ABE0.1-EGFP construct, and no color a dmCherry(Y72H)-P2A-ABE0.1-dEGFP(A111V) construct.



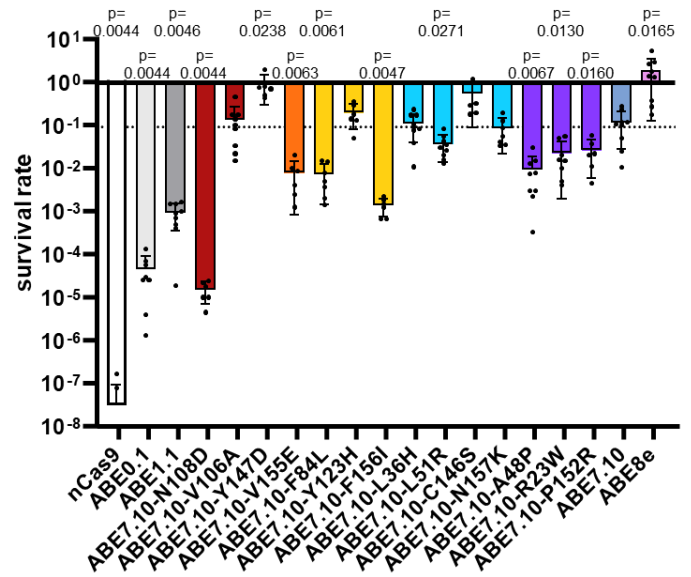
Supplementary Figure 15 EGFP/mCherry fluorescence ratios for ABE7.10 single-residue reversion editors

HEK293T cells were transfected with stability of expression reporter plasmids containing mCherry-P2A-ABE-EGFP expression cassettes (Extended Data Figure 2A). Following 48 hours of incubation, cells were analyzed via flow cytometry using the gating shown in Supplementary Figure 14, and EGFP/mCherry ratios were calculated for individual double color cells. The frequency of distribution of EGFP/mCherry ratios for all double color cells are plotted for each single-residue reversion, along with distributions of ABE7.10 and ABE0.1. EGFP/mCherry ratios are tabulated in bins of 0.05 width. Plots show a representative replicate from $n = 3$ independent transfections.

A TAG (H193Y) bacterial ABE activity reporter

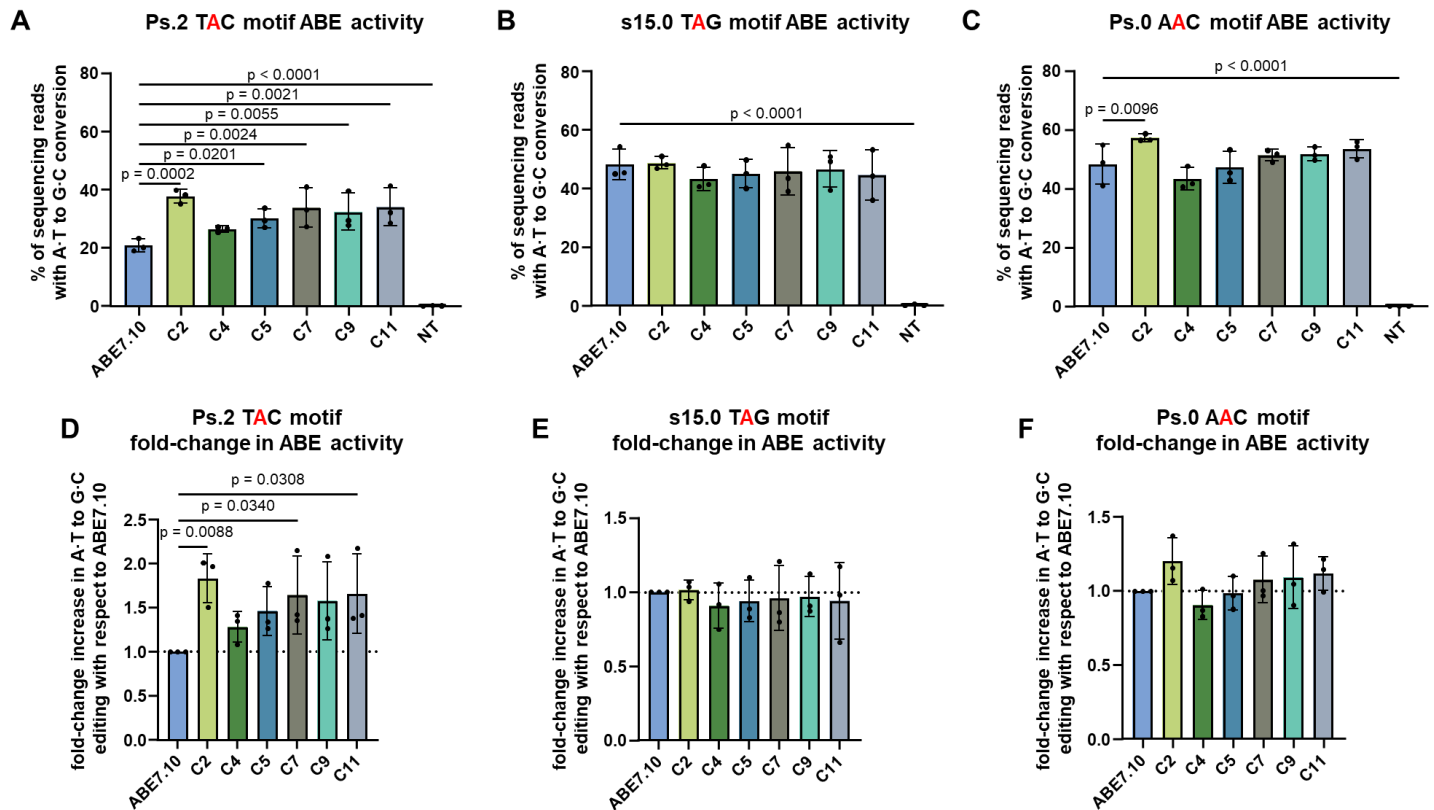


B TAC (Q122*) bacterial ABE activity reporter



Supplementary Figure 16 Absolute survival rate data for bacterial base editing activity assay

Survival rates measure ABE activity in bacterial host systems. Briefly, a plasmid with one of two chloramphenicol resistance inactivating point mutations (pCam^R) requiring an A•T to G•C correction to restore antibiotic resistance functioned as an ABE activity reporter. S1030 *E. coli* with pCam^R were transformed with plasmids harboring bacteria-optimized ABE variants (pbABE) of the single-residue reversion ABEs, or benchmark editors ABE0.1, ABE1.1, ABE7.10 or ABE8e. pbABE plasmids also contained an appropriate gRNA for targeting the mutation in pCam^R to restore chloramphenicol resistance, as well as a theophylline inducible riboswitch to temporally control expression of the base editor. Bacteria were allowed to grow overnight in liquid culture with theophylline, before splitting the culture and dilution plating each sample on +/- chloramphenicol plates. For a given editor, colony count on chloramphenicol containing plates was divided by colony count on chloramphenicol absent plate to determine survival rate of a given single-residue reversion editor in bacteria. These absolute survival rates are reported for the H193Y **TAG** reporter (**A**) and Q122* **TAC** reporter (**B**). Bars and error bars represent the average and standard deviation. Samples were measured for n = 9 +/- chloramphenicol plating paired replicates and survival rate was calculated prior to discarding ROUT 1% outliers. Note nCas9 control never grew colonies on + chloramphenicol plate for the H193Y **TAG** reporter system and is omitted here. Dotted line indicates average survival rate for ABE7.10. Data were analyzed with Brown-Forsythe and Welch's ANOVA test, with comparison tests to ABE7.10 using unpaired t-test with Welch's correction. Significant p-values are reported above samples, p ≥ 0.05 was not significant and is not indicated.



Supplementary Figure 17 Activity of multi-residue reversion editors at 3 endogenous sites in HEK293Ts

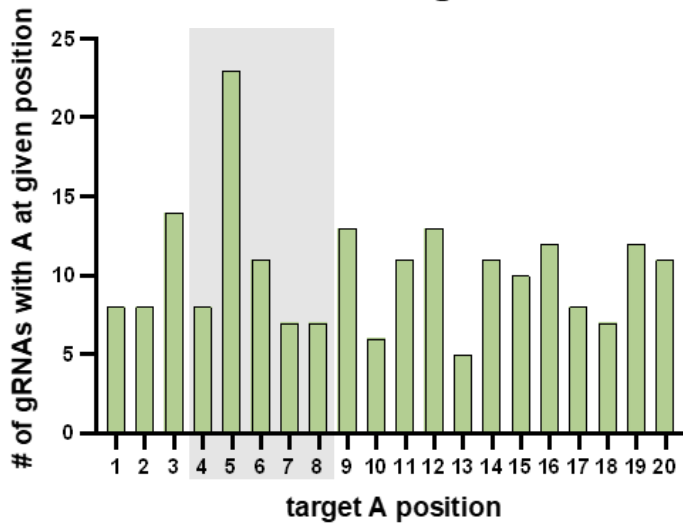
A-C. Percent of sequencing reads with target A•T to G•C conversion for multi-residue reversion editors. ABE plasmids were transfected into HEK293Ts cells alongside one of three gRNA plasmids (protospacers shown in Extended Data Figure 1A) or a non-targeting (NT) gRNA and allowed to incubate for 72 hours. Genomic DNA was then harvested, target amplicons amplified, and BE activity (percent of reads with A•T converted to G•C) analyzed via NGS. Absolute editing efficiency is shown as measured at Ps.2 (**A**), s15.0 (**B**), and Ps.0 (**C**) sites. **D-E.** Further normalization to ABE7.10 displays the fold-change in ABE activity for multi-residue reversion editors compared to ABE7.10 at Ps.2 (**D**), s15.0 (**E**), and Ps.0 (**F**) sites. Bars and error bars represent the average and standard deviation of $n = 3$ biological replicates. Dotted horizontal line indicates average activity of ABE7.10. Data were analyzed with one-way ANOVA, uncorrected Fisher's LSD test comparison to ABE7.10, single pooled variance with significant p-values reported ($p \geq 0.05$ not significant, not indicated).

site	gRNA	most edited A target motif	% indels	gRNA plasmid
s17.0*	GACAAAGAGGAAGAGAGACG	AA <u>A</u> ₅ A	28	pME335
s7.1*	AGTAATATAGAGTTCCACAG	T <u>A</u> ₇ T	44	pME329
s7.2*	ATAGAGTTCCACAGTGGGCA	GA <u>A</u> ₅ G	44	pME330
s7.3	GGATAAGGTAAGTAATATAG	T <u>A</u> ₅ A	47	pME331
Hi.4*	GCAAAGTGAAGACCAAGGAT	AA <u>A</u> ₅ G	36	pME349
Hi.1*	GCAACAACAACAAAACGGAC	CA <u>A</u> ₆ A	46	pME350
Hi.5	TCTCAGCAACAACAACAAAA	CA <u>A</u> ₅ G	30	pME351
Hi.0*	GAAGACCAAGGATAGACTGC	GA <u>A</u> ₅ C	43	pME352
Hi.3	CGCTATGATGCCCAGGCTGT	T <u>A</u> ₅ T	27	pME353
H2.0*	GAACACAAAGCATAGACTGC	CA <u>A</u> ₅ C	44	pME341
H2.1	CAAGACCTGGCTGAGCTAAC	GA <u>A</u> ₅ C	50	pME342
H2.3*	GAATAGCTGCAAACAAGTGC	T <u>A</u> ₅ G	46	pME343
Rn.1*	CAACATACAGAAGTCAGGAA	T <u>A</u> ₇ C	30	pME356
Rn.2	GGCATATGAGACGTGTAAAC	T <u>A</u> ₆ T	44	pME357
H3.1	AGAAAGGCATGGATGAGAGA	AA <u>A</u> ₅ G	33	pME344
H3.0	GGCCCAGACTGAGCACGTGA	CA <u>A</u> ₆ G	63	pME345
H3.2*	CTCTCATCCATGCCTTTCTA	CA <u>A</u> ₆ T	44	pME346
H3.3	GCAGAAATAGACTAATTGCA	GA <u>A</u> ₅ A	39	pME347
H3.4	CGTGATGGCAGAGGAAAGGA	GA <u>A</u> ₅ T	54	pME348
Em.3	ACAAACCCACGAGGGCAGAG	AA <u>A</u> ₅ C	57	pME336
Em.4	GCAGAAGCTGGAGGAGGAAG	GA <u>A</u> ₅ A	26	pME338
Em.1*	AAGTACAAACGGCAGAAGCT	T <u>A</u> ₅ C	43	pME340
s15.1	AGGAAACTGAATCTTAGGGA	AA <u>A</u> ₆ C	38	pME332
s15.2	AGAGATCCTTTCTGATGCAG	GA <u>A</u> ₅ T	25	pME333
s15.3*	GTCTAAGCTTTCTAGACAGT	T <u>A</u> ₅ A	44	pME334
Ps.3	AACCACAGTGTGTTGATGAA	CA <u>A</u> ₅ C	43	pME354
Em.2	GGGCAACCACAAACCCACGA	AA <u>A</u> ₆ C	44	pME337
NT	GTATTACTGATATTGGTGGG	-	-	pME313

Supplementary Figure 18 Sequences of the 27 endogenous sites for profiling ABE activity

gRNAs used to profile ABE activities at endogenous sites. The most edited A (bold/underlined) was determined by identifying the A within the protospacer with the highest average editing (percent of reads with A•T to G•C conversion) across all editors tested. % indels reports the average percentage of sequencing reads with indels as measured by transfecting HEK293T cells with wtCas9 and the given gRNA, incubating for 72 hours, harvesting genomic DNA, and amplifying the target site for sequencing via NGS (n = 2). Sites indicated with * were used to compare editing efficiencies and precision of ABE7.10, ABE7.10-HRHSK, ABE8e, ABE8e+V106W and ABE9 editors.

Distribution of A's amongst selected 27 endogenous sites

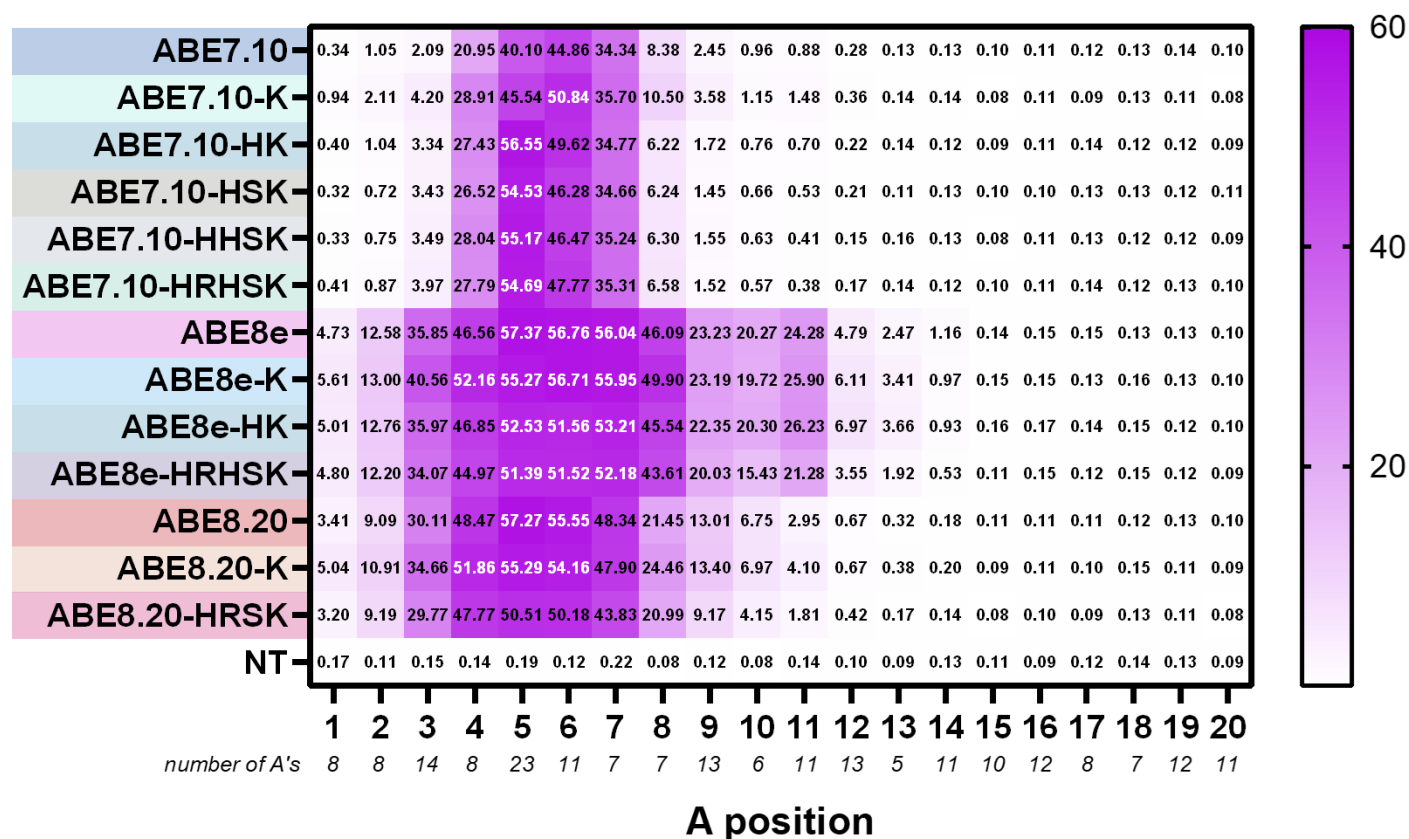


motif	number of A's in 20nt window	number of A's in active window	number of sites with most edited A
AAA	18	8	1
AAC	19	5	3
AAG	21	6	2
AAT	8	2	0
CAA	21	7	1
CAC	9	2	2
CAG	16	3	2
CAT	7	3	1
GAA	19	3	2
GAC	12	3	2
GAG	19	2	1
GAT	10	3	2
TAA	8	3	2
TAC	2	2	2
TAG	11	1	1
TAT	5	3	3

Supplementary Figure 19 Distribution of available A's at 27 endogenous sites

27 endogenous genomic sites were selected for further ABE activity profiling. Protospacer and gRNA information for the 27 sites can be found in Supplementary Figure 18. Within these 27 sites, 205 A's were present, with the positional distribution shown by the above bar graph. The active window, as defined by ABE7.10, consists of positions 4 to 8 from the PAM-distal end and is shown with the highlighted box. A's were also defined by their local tri-motif NAN, and followed the distribution outlined above. The most edited A for a given site was determined by taking the average editing across all editors tested for each A within that sites protospacer, and identifying the position (and tri-motif) with the highest average editing (percent of reads with A•T to G•C conversion).

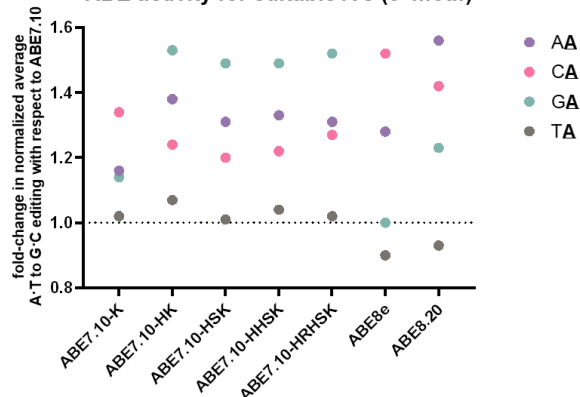
Relative A•T to G•C editing activity



Supplementary Figure 20 Relative editing activity of ABE multi-residue reversion variants as a function of target A position

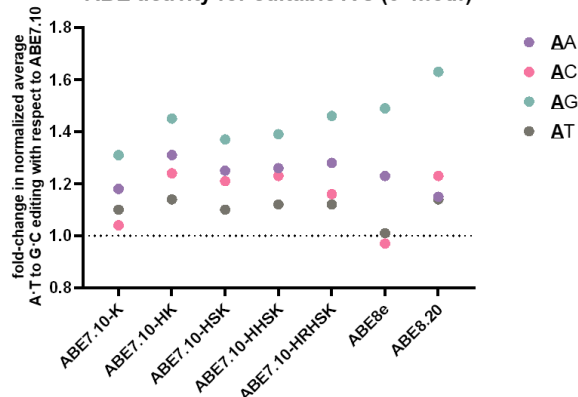
HEK293T cells were transfected with an ABE plasmid encoding ABE7.10 or variant editor indicated along with one of the 27 gRNA plasmids from Supplementary Figure 18, or a non-target (NT) gRNA. Cells were harvested after 72 hours and genomic loci of interest analyzed for editing activity with NGS. Within a given transfection replicate, absolute editing efficiencies for all A's were normalized to the editing activity of ABE7.10 at the H2.0 A₅ position (the maximally edited A, across all sites and in every replicate). Average normalized editing activity values for ABE7.10-derived editors are reported by target A position within the protospacer window. Darker shaded cells indicate higher average normalized editing activity. Averages were determined from n = 3 biological replicates for ABE7.10-derived and ABE8-derived reversion editors, and n = 6 biological replicates for ABE7.10, ABE8e, and ABE8.20.

A Fold-change in ABE7.10 normalized average ABE activity for editable A's (5' motif)



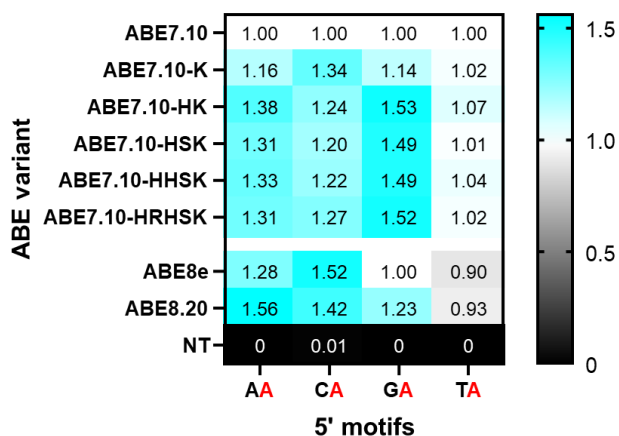
# of editable A's (5' motifs)				
	AA	CA	GA	TA
ABE7.10	19	13	8	9
ABE7.10-K	19	13	8	9
ABE7.10-HK	19	13	8	9
ABE7.10-HSK	19	13	8	9
ABE7.10-HHSK	19	13	8	9
ABE7.10-HRHSK	19	13	8	9
ABE8e	34	31	22	13
ABE8.20	26	20	14	10

B Fold-change in ABE7.10 normalized average ABE activity for editable A's (3' motif)

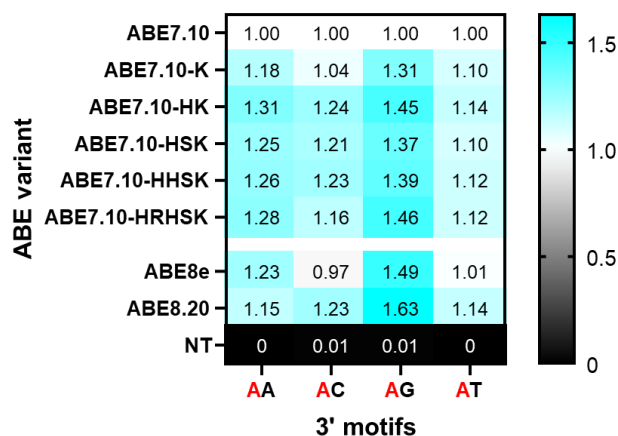


# of editable A's (3' motifs)				
	AA	AC	AG	AT
ABE7.10	17	11	11	10
ABE7.10-K	17	11	11	10
ABE7.10-HK	17	11	11	10
ABE7.10-HSK	17	11	11	10
ABE7.10-HHSK	17	11	11	10
ABE7.10-HRHSK	17	11	11	10
ABE8e	34	22	28	16
ABE8.20	25	14	18	13

Fold-change in ABE7.10 normalized average ABE activity for editable A's (5' motif)

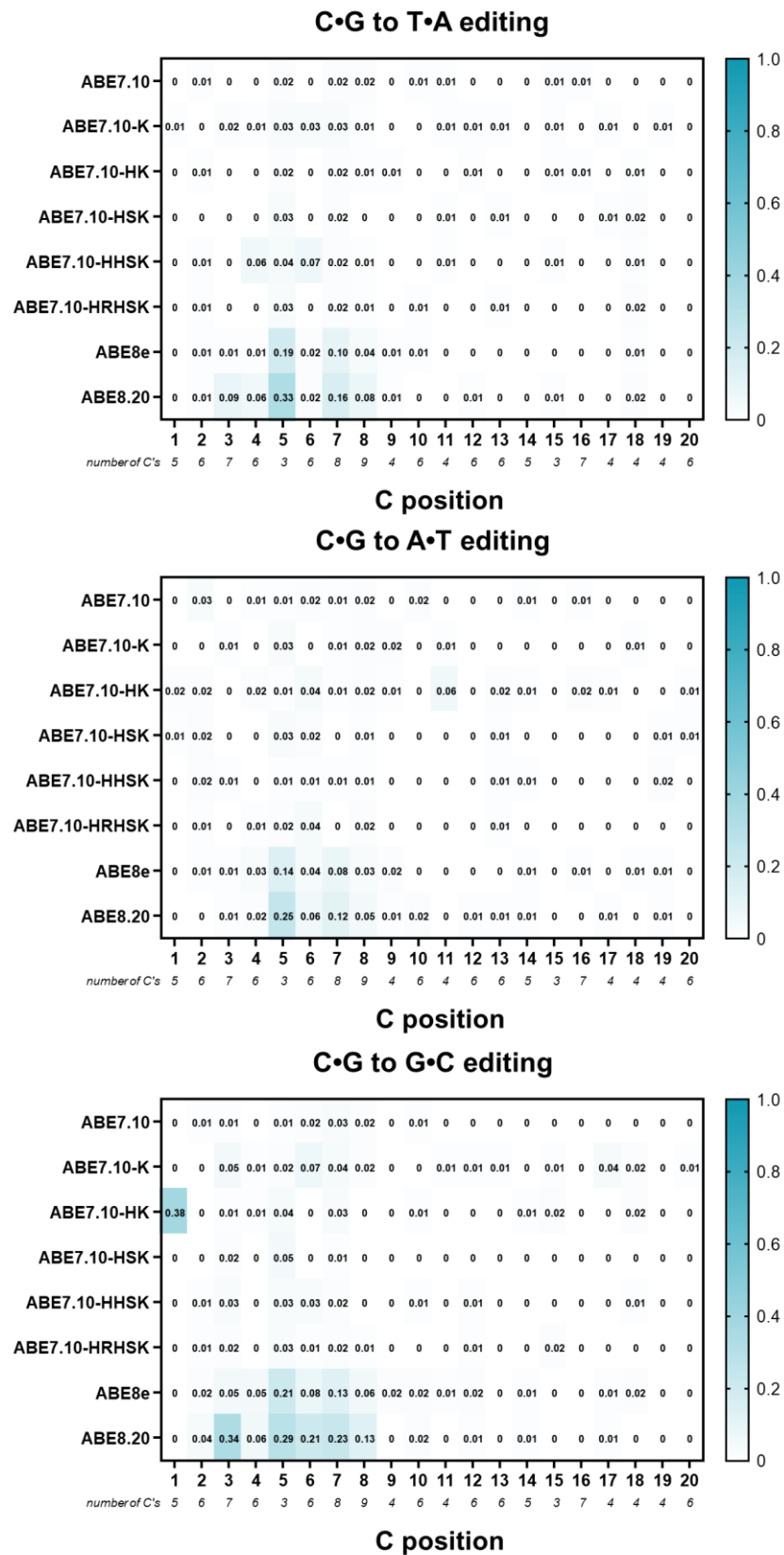


Fold-change in ABE7.10 normalized average ABE activity for editable A's (3' motif)



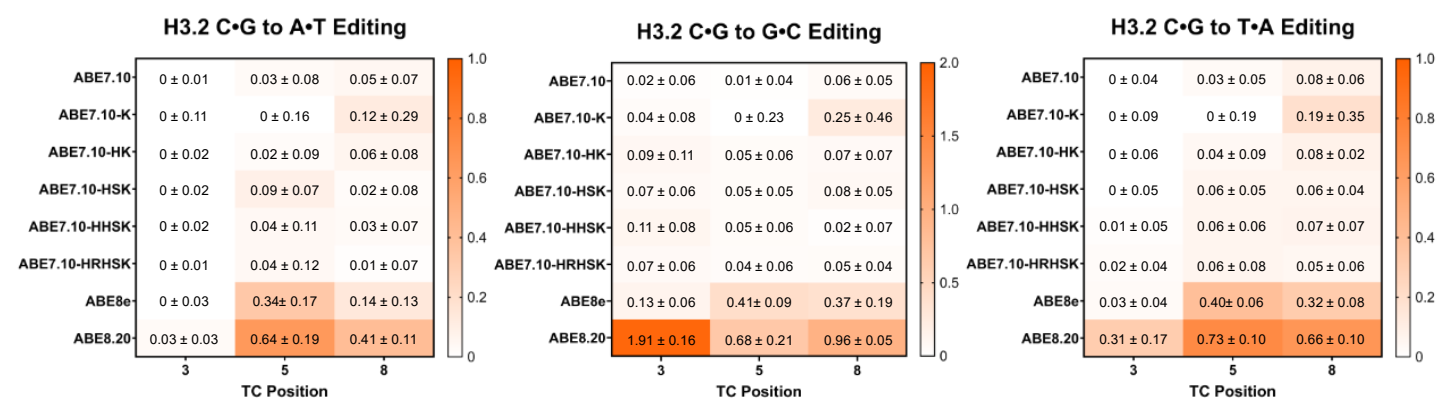
Supplementary Figure 21 Relative ABE activity of editable A's, analyzed by 5' and 3' motif

HEK293T cells were treated and data further normalized as described in Supplementary Figure 20. The subset of "editable A's" (A's that reside in a protospacer position where the average normalized editing score for a given editor across all tested protospacers was 15% or greater) are considered here to study editing patterns with respect to sequence motif. Data are presented as average fold-changes in normalized editing activity relative to ABE7.10 for all editable A's with a given sequence motif. All 5' motif combinations (**A**) and 3' motif combinations (**B**) are represented (top), with number of A's considered editable given below for each editor at each **NA** and **AN** motif (bottom). The dashed horizontal line indicates relative activity of ABE7.10. Heat maps show fold-change in average normalized editing scores for a given motif's editable A's for all indicated editors as compared to ABE7.10. Scale has been set so greyscale indicates activity less than ABE7.10, white shows benchmark ABE7.10 activity, and cyan indicates improved editing activity relative to ABE7.10.



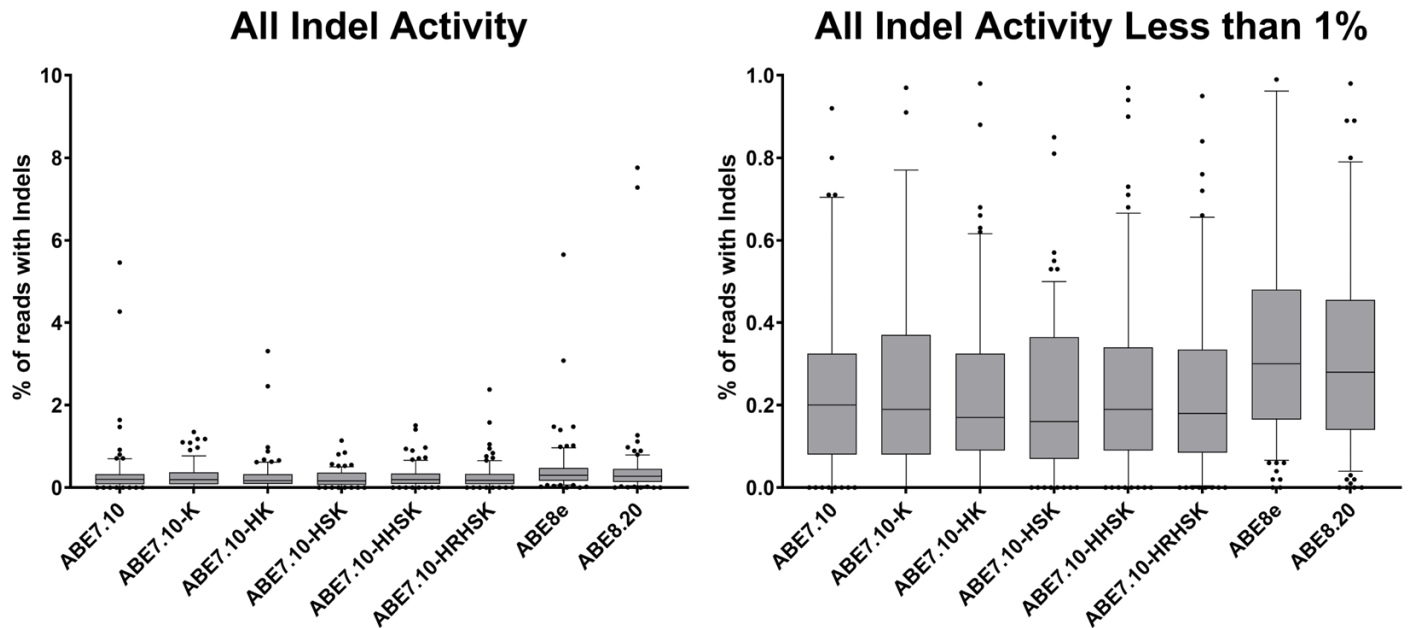
Supplementary Figure 22 Cytosine modification by ABE multi-residue reversion variants as a function of C position

HEK293T cells were treated as described in Supplementary Figure 20. Average editing activity values at cytosine bases for ABE7.10-derived editors are reported by target C position within the protospacer window. Darker shaded cells indicate higher average normalized editing activity. Averages were determined from $n = 3$ biological replicates for all editors, with subtraction of background editing by a same-transfection non-targeting gRNA control.



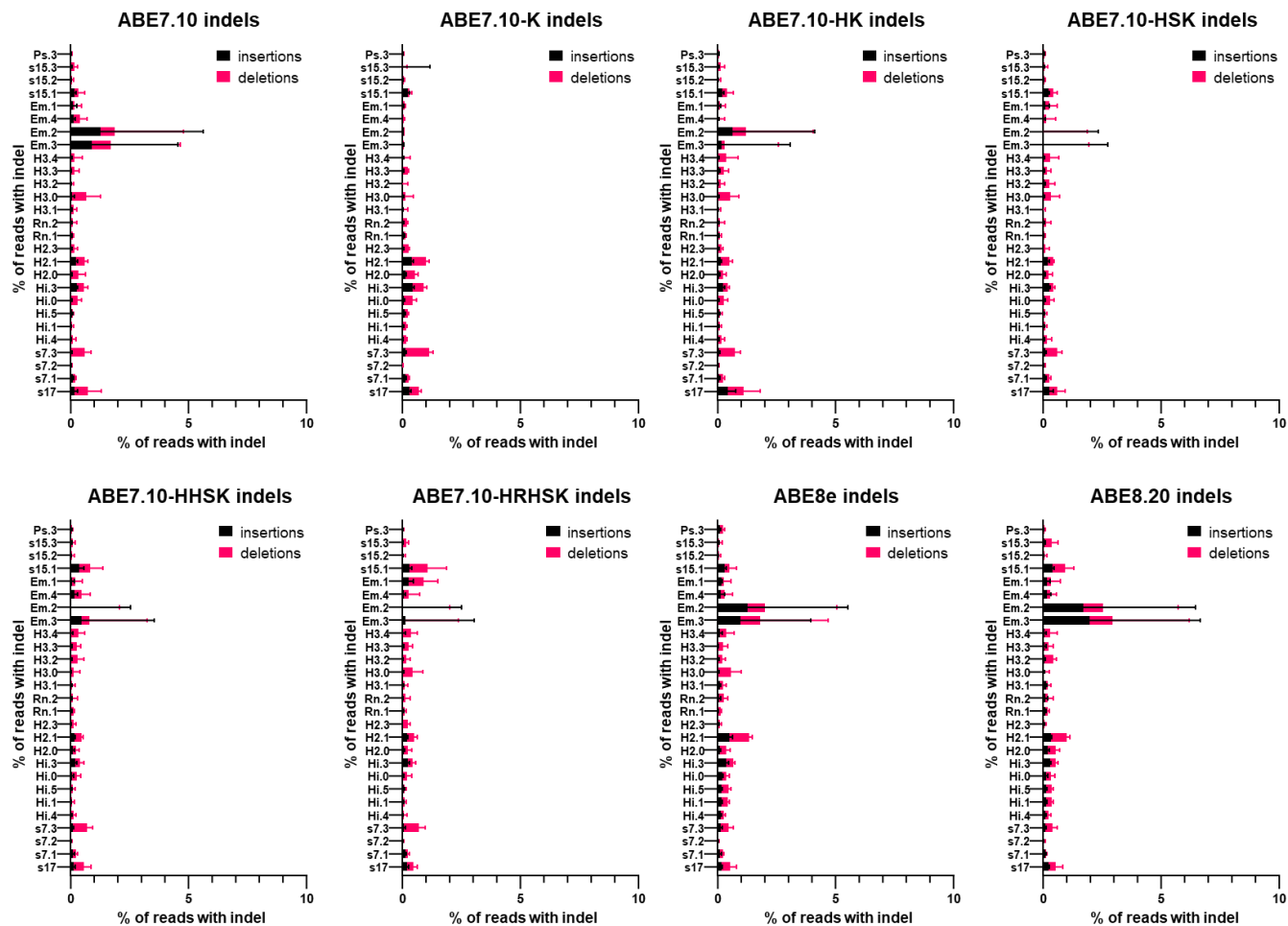
Supplementary Figure 23 Cytosine modification by ABE multi-residue reversion variants at the H3.2 TC motif rich site

HEK293T cells were treated as described in Supplementary Figure 20 with the H3.2 gRNA (CTC₃TC₅ATC₈CATGCCTTTCTA). Average editing activity values are reported by position. Darker shaded cells indicate higher average normalized editing activity. Averages were determined from n = 3 biological replicates for all editors, with subtraction of background editing by a same-transfection non-targeting gRNA control. Values and errors are mean and propagated error of editor and control standard deviations, respectively.



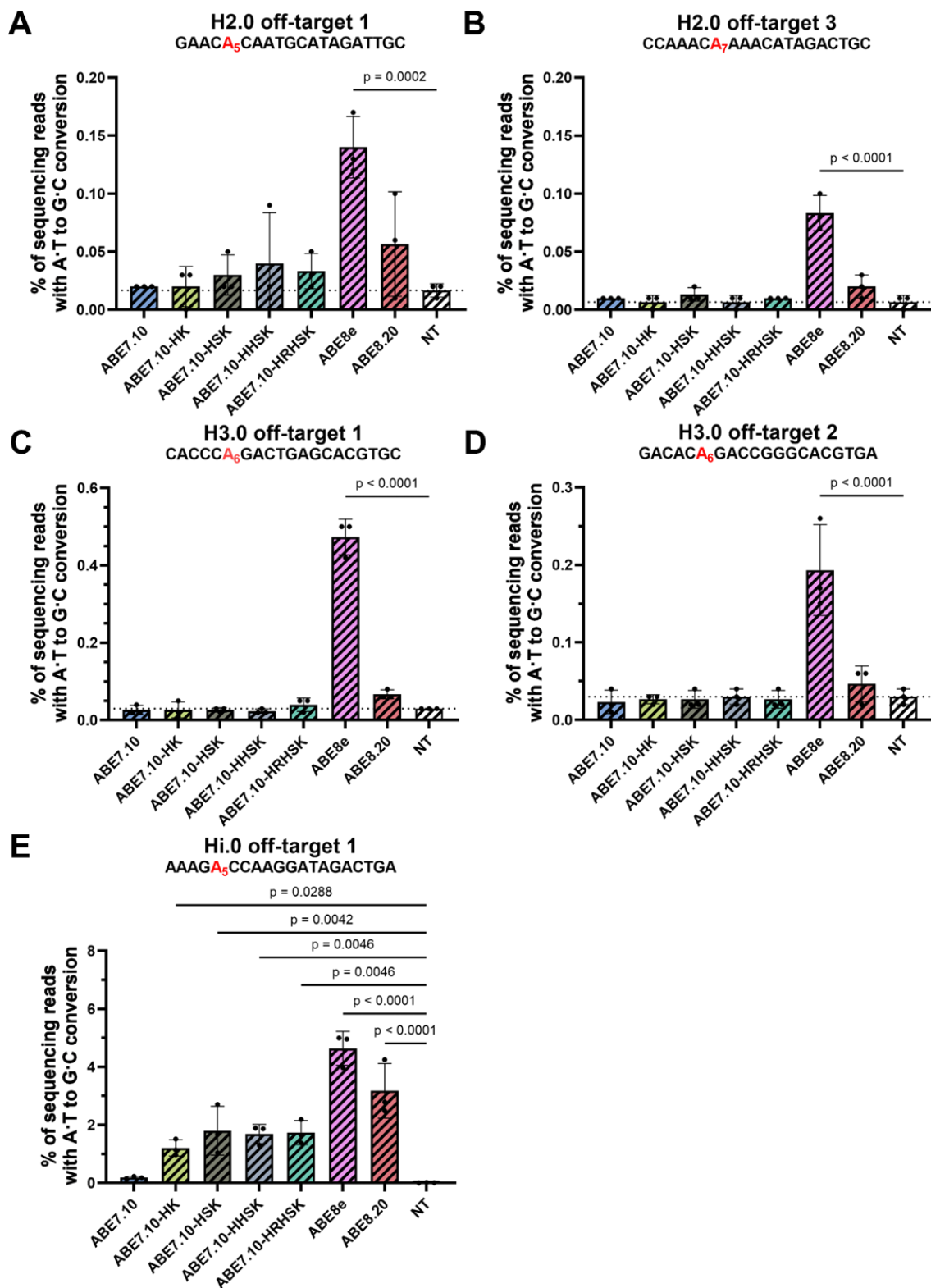
Supplementary Figure 24 Indel activity introduced by multi-residue reversion editors across 27 endogenous sites

HEK293T cells were treated as described in Supplementary Figure 20. Indel activity is shown for $n = 3$ biological replicates for each editor, all values are corrected by subtraction of background indel editing present in a same-transfection non-targeting gRNA control. Box and whiskers indicate 10th and 90th percentile, line shows median, and points illustrate measured indel activity outside these percentiles. The same data are represented in the graphs on the left and right, with the right graph zoomed in on indel activity less than 1%.



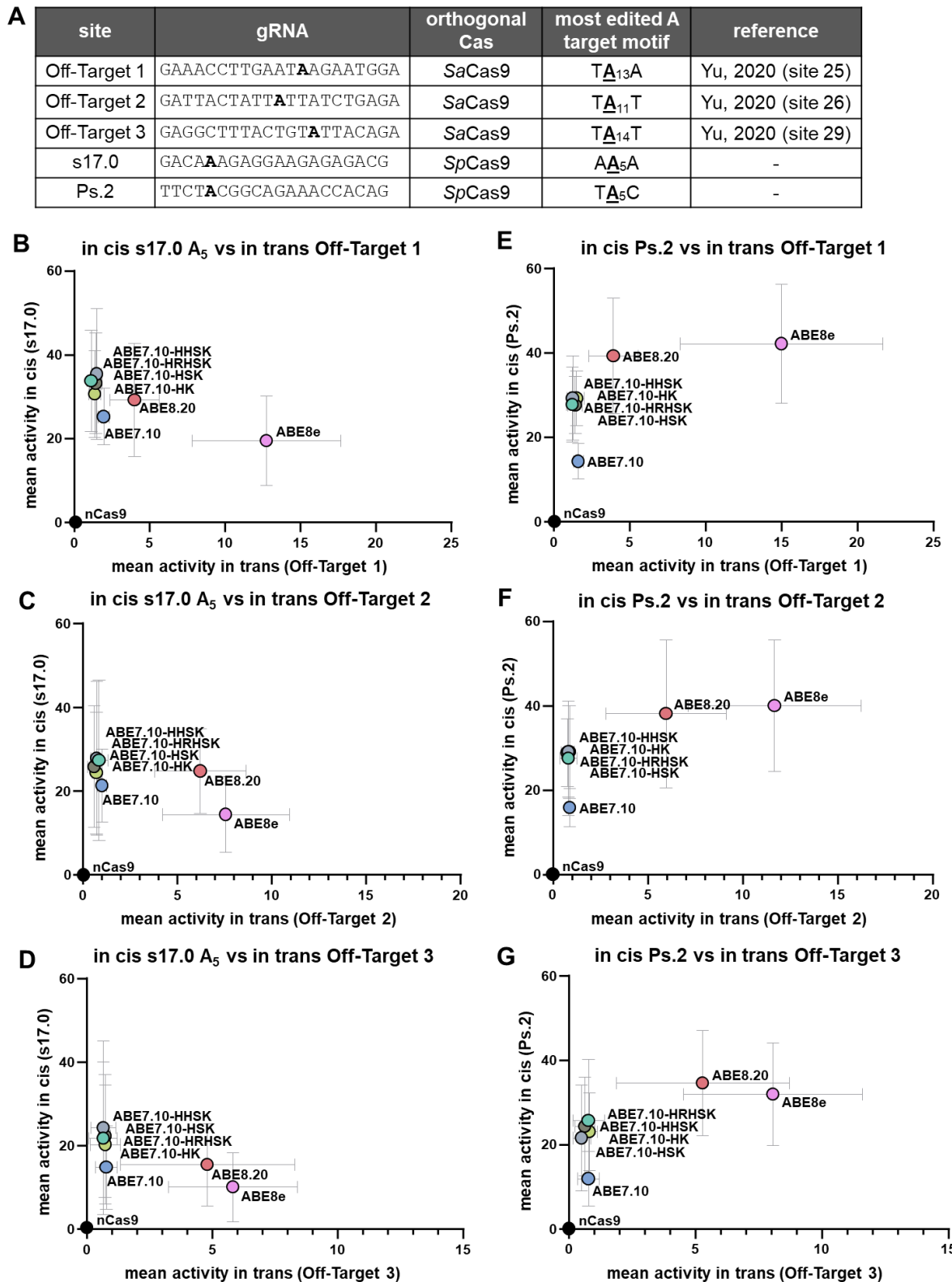
Supplementary Figure 25 Indel activity introduced by multi-residue reversion broken down by endogenous sites

HEK293T cells were treated as described in Supplementary Figure 20. Indel activity is shown for $n = 3$ biological replicates for each editor, broken down by site and indel type (insertion or deletion). All values are corrected by subtraction of background indel editing present in a same-transfection non-targeting gRNA control. Bars represent average of $n = 3$ biological replicates, error bars show propagated error resulting from standard deviation present in samples and non-targeting controls.



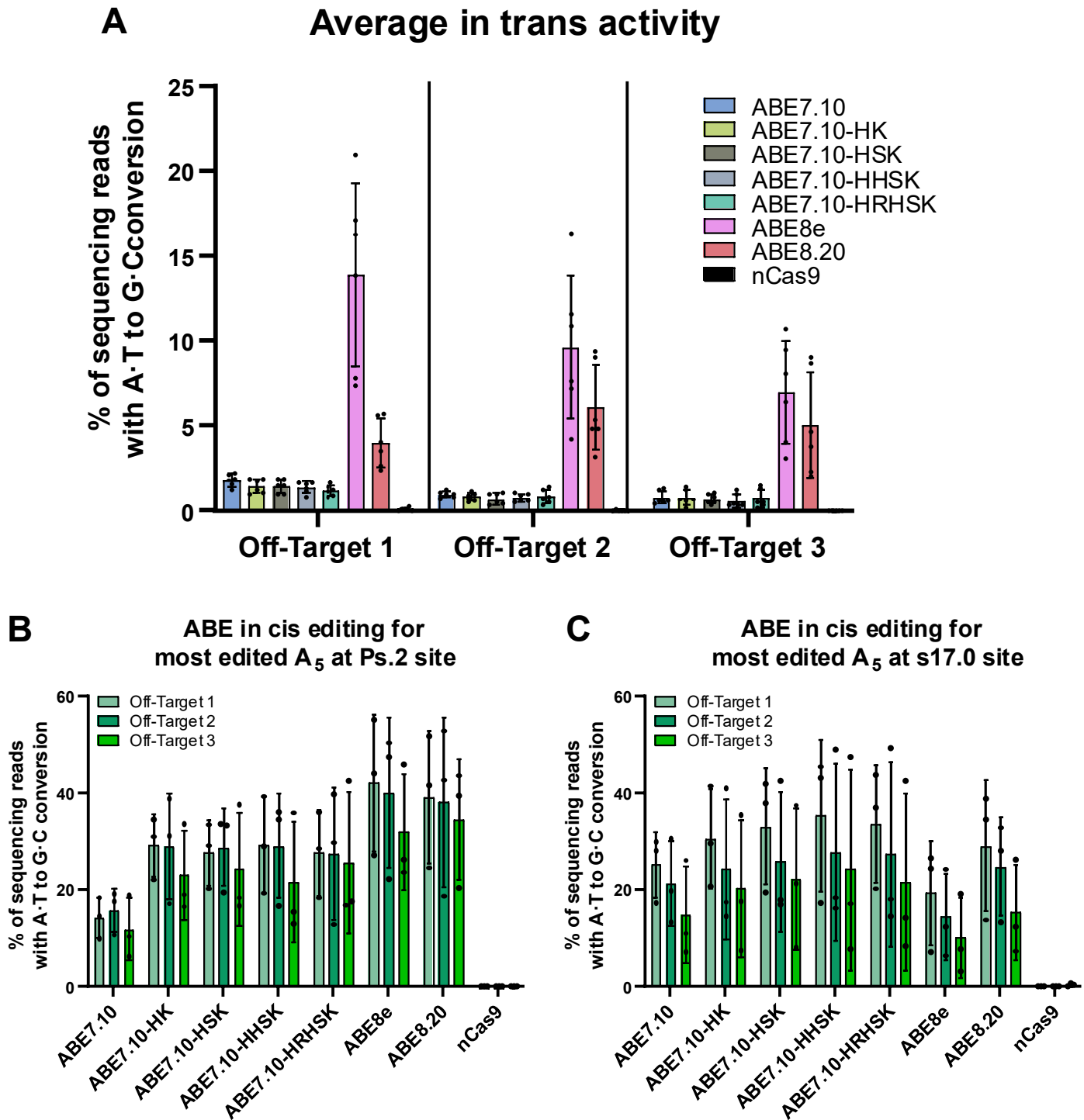
Supplementary Figure 26 gRNA-dependent DNA off-target activity measured at five verified sites

HEK293T cells were transfected with plasmids encoding each of the ABE variants indicated and an on-target gRNA (see Figure 4C). 72 hours after transfection, both the on-target loci and previously validated gRNA-dependent off-target genomic loci were amplified and evaluated for A•T to G•C editing via NGS. Off-target “protospacers” are shown in with the most edited A indicated in red. Bars show average editing measured for $n = 3$ biological replicates, with error bars showing standard deviation. Data were analyzed with one-way ANOVA, Dunnet’s test comparison to non-targeting (NT) sample, single pooled variance with significant p-values reported ($p \geq 0.05$ not significant, not indicated).



Supplementary Figure 27 Orthogonal R-loop assay on- and off-target sites absolute activity

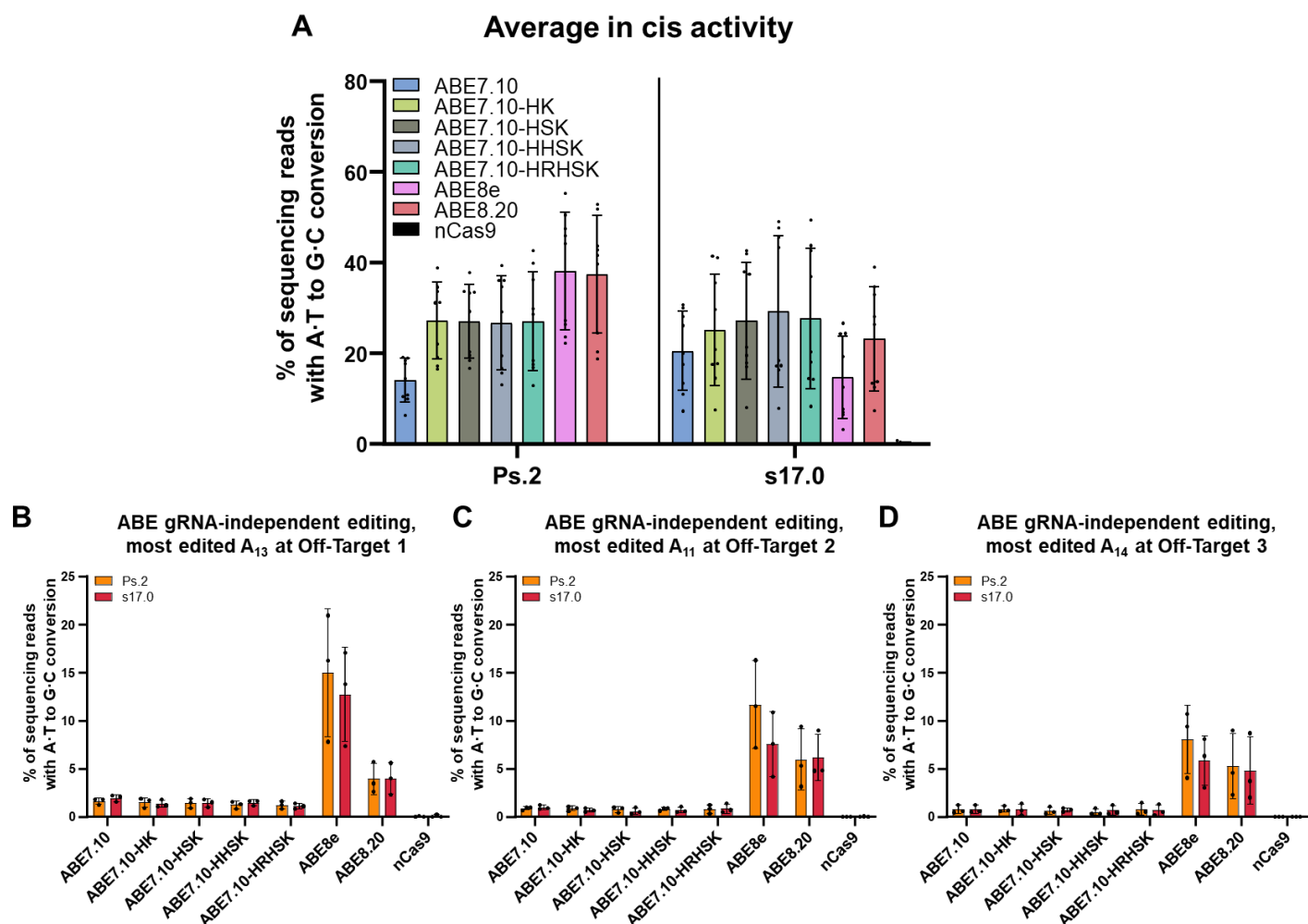
A. Sa-gRNAs used for three independent, previously verified, ABE off-target (in trans) sites, and Sp-gRNAs for comparison of on-target (in cis) activity. **B-G.** HEK293T cells were transfected with plasmids encoding one of the indicated Sp-nCas9 ABEs, one of two Sp-gRNAs, Sa-dCas9 and one of three Sa-gRNAs. Cells were allowed to incubate for 72 hours, following which genomic DNA was harvested, on-target and off-target loci amplified, and sequences analyzed via NGS. x-y plots represent the average editing activity for the most edited A in a given target for $n = 3$ individual biological replicates with error bars showing standard deviation. Each combination of on- and off-target gRNAs has been plotted independently.



A.

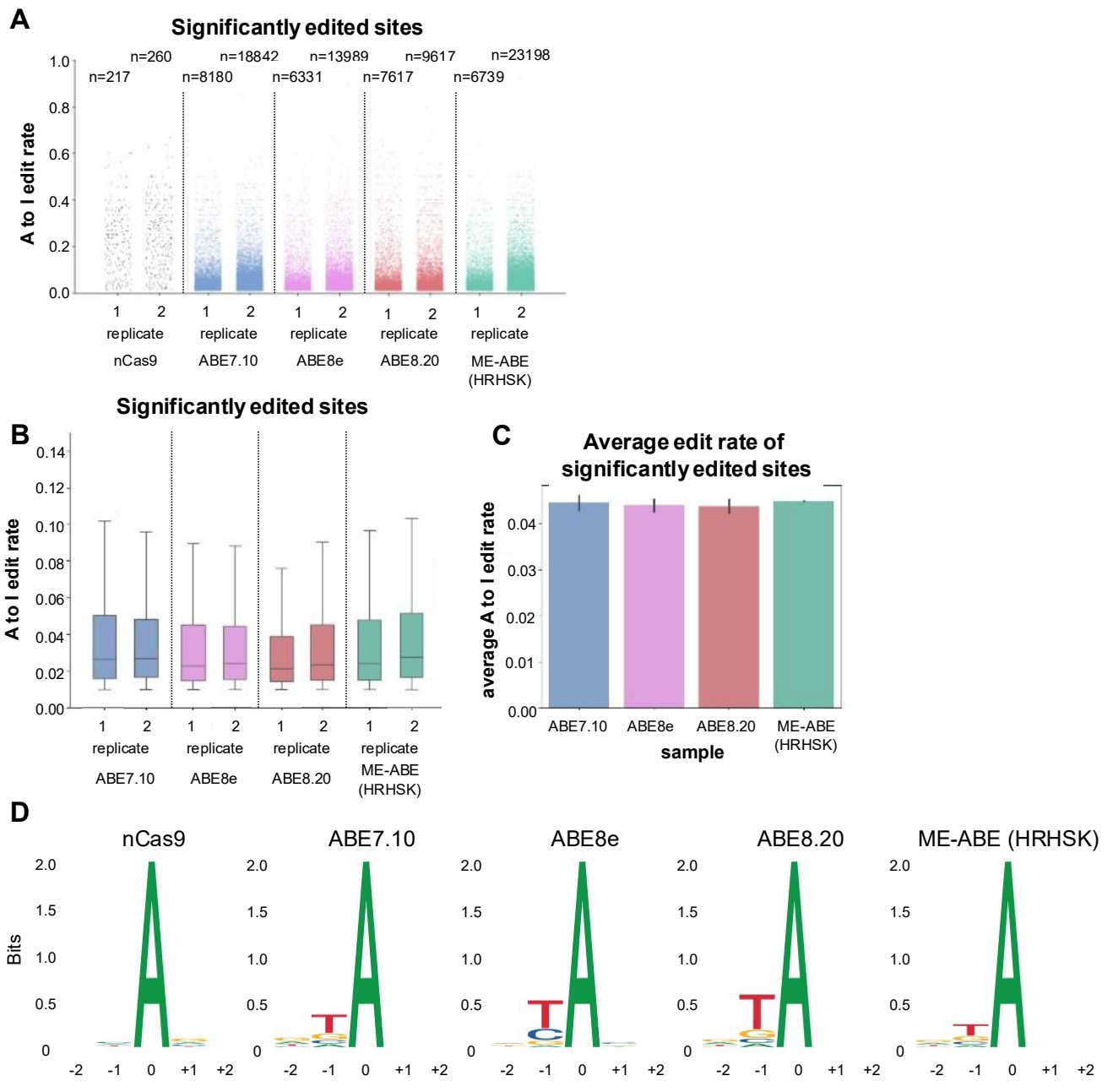
Supplementary Figure 28 Breakdown of in trans and in cis editing measured by orthogonal R-loop assay for ME-ABEs

A. Cells were treated as described in Supplementary Figure 27. Average in trans (off-target) editing activity of ME-ABEs and canonical editors was measured by the orthogonal R-loop off-target assay and is broken down by off-target gRNA. **B-C.** Average in cis (on-target) editing activity at Ps.2 (**B**) and s17.0 (**C**) for ME-ABEs and canonical editors was measured by the orthogonal R-loop off-target assay and is broken down by off-target gRNA. Bars represent average editing ($n = 3$ biological replicates per on/off gRNA combo), error bars display standard deviation.



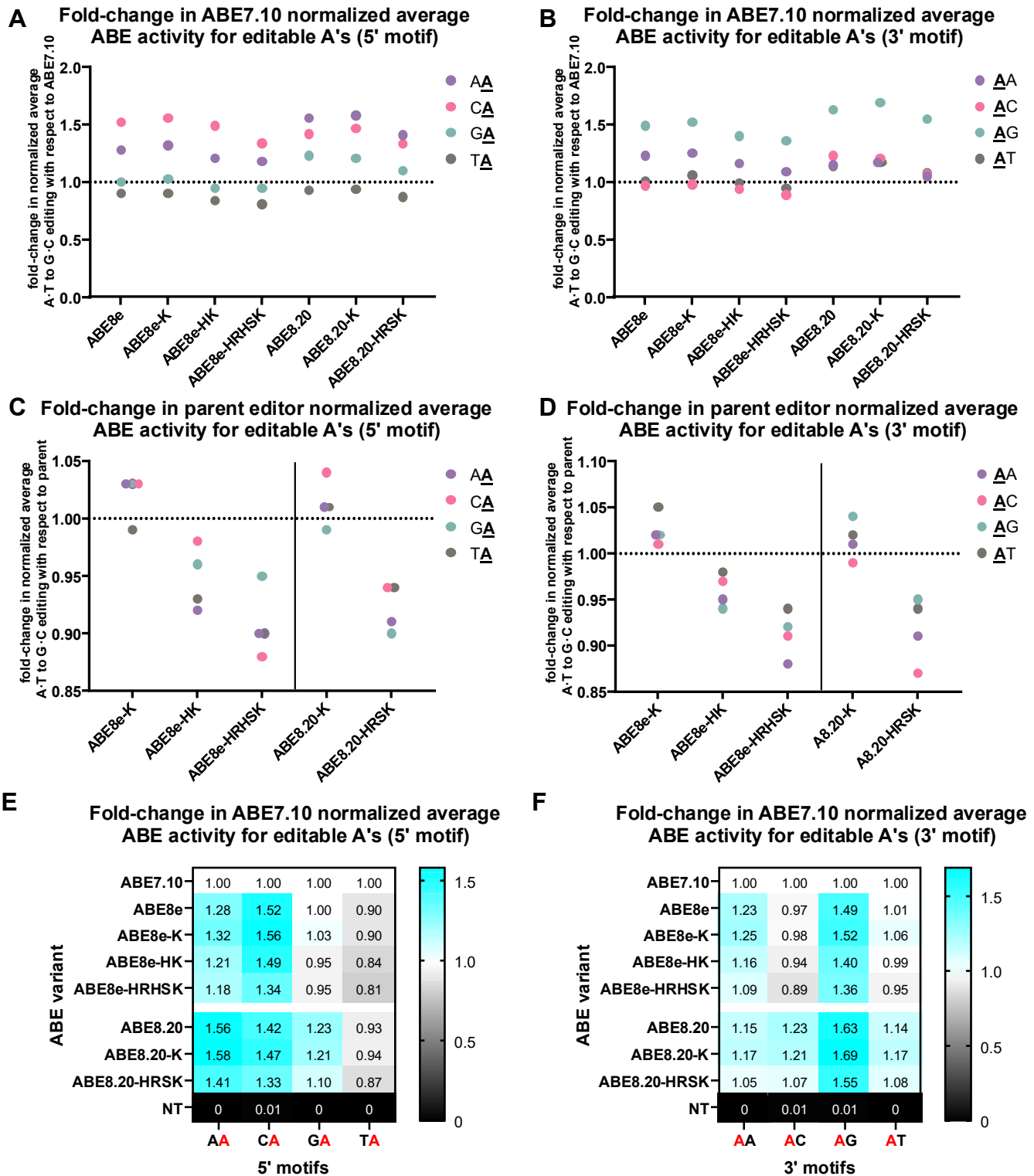
Supplementary Figure 29 Breakdown of in cis and in trans editing measured by orthogonal R-loop assay for ME-ABEs

A. Cells were treated as described in Supplementary Figure 27. Average in cis (on-target) editing activity of ME-ABEs and canonical editors was measured by the orthogonal R-loop off-target assay and is broken down by on-target gRNA. **B-D.** Average in trans (off-target) editing activity at Off-Target site 1 (**B**), Off-Target site 2 (**C**), and Off-Target site 3 (**D**) for ME-ABEs and canonical editors was measured by the orthogonal R-loop off-target assay and is broken down by on-target gRNA. Bars represent average editing ($n = 3$ biological replicates per on/off gRNA combo), error bars display standard deviation.



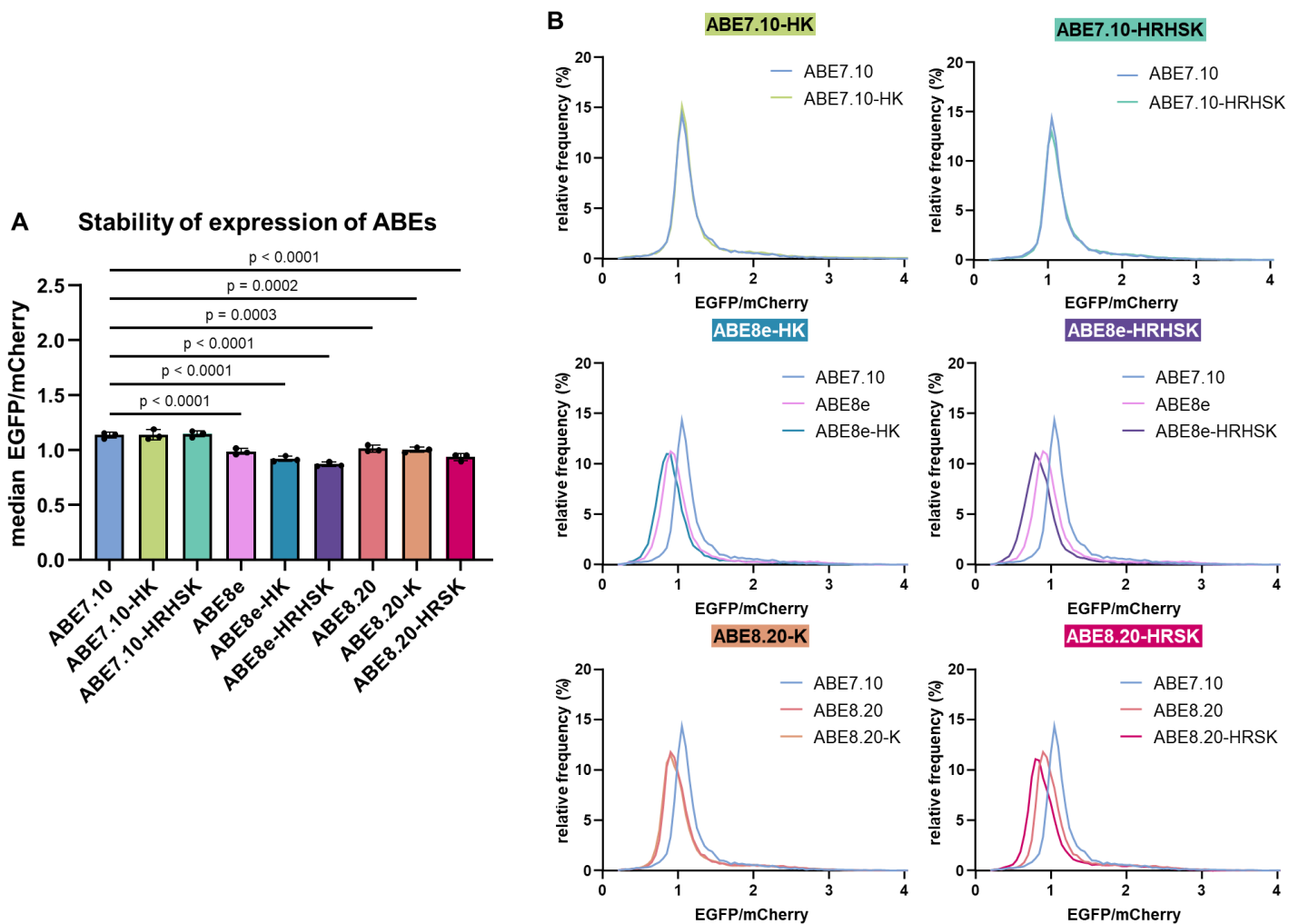
Supplementary Figure 30 ME-ABEs demonstrate RNA off-target activity similar to other established ABEs

A. HEK293T cells were transfected with plasmids encoding indicated ABE (or mCherry control plasmid) and the H2.0 gRNA. 72 hours post-transfection, total RNA was harvested and prepared for RNA-Seq. Variants were identified using REDITools3. Editing at a given position was filtered to exclude SNPs present in dbSNP151, and was only considered significant if read coverage at that position was greater than or equal to 10, editing rate of collected reads was greater than or equal to 1%, and a comparison to the mCherry control passed a Fisher's Exact t-test, $p < 0.05$. Editing rates and number of significant sites for $n = 2$ independent biological replicates are shown. **B.** RNA-Seq data prepared as in **A** is presented as box and whiskers plot, with outliers (sites with editing outside of inter-quartile range) not shown. Boxes indicate quartiles, whiskers indicate edge of distribution, line indicates median. **C.** Average editing rate of significantly edited sites for ABE editors for $n = 2$ biological replicates. **D.** Logo plots for a representative replicate illustrate sequence preferences of significantly edited A's. Note that because RNA-Seq was generated as cDNA, T can be read as U.



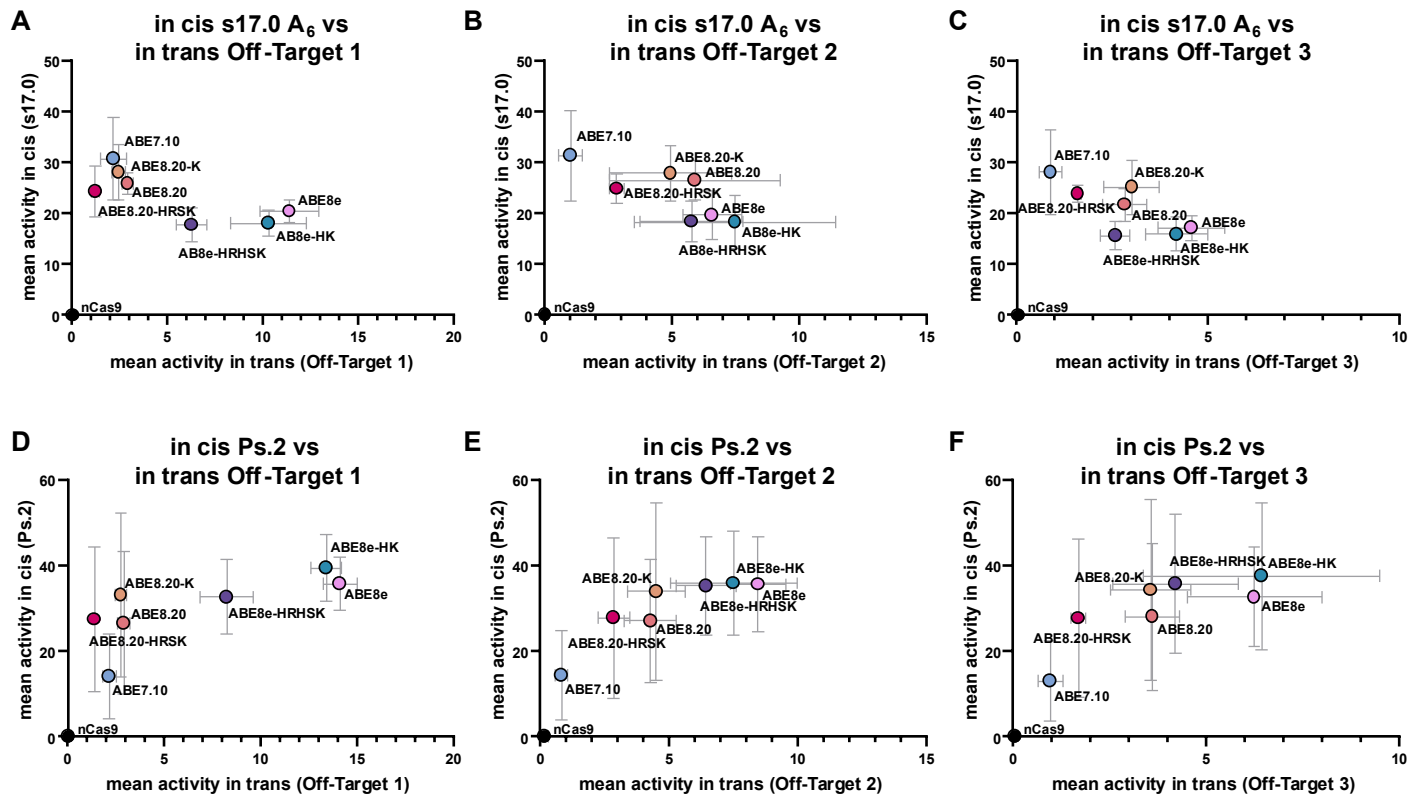
Supplementary Figure 31 Relative ABE activity of reversion editors considering editable A's, analyzed by 5' and 3' motif

HEK293T cells were treated and data further normalized as described in Supplementary Figure 20. "Editable A's" (as described in Supplementary Figure 21) were sorted by 5' and 3' motif. Average fold-change in normalized ABE activity as compared ABE7.10 (**A/B**), and to each reversion editors parent editor (**C/D**) is presented. All 5' motif combinations (**A/C**) and 3' motif combinations (**B/D**) are represented. **E-F**. Heat map shows fold-change in average normalized editing scores for a given motifs editable A's for indicated editors as compared to ABE7.10. Scale has been set so greyscale indicates activity less than ABE7.10, white shows benchmark ABE7.10 activity, and cyan indicates improved editing activity relative to ABE7.10.



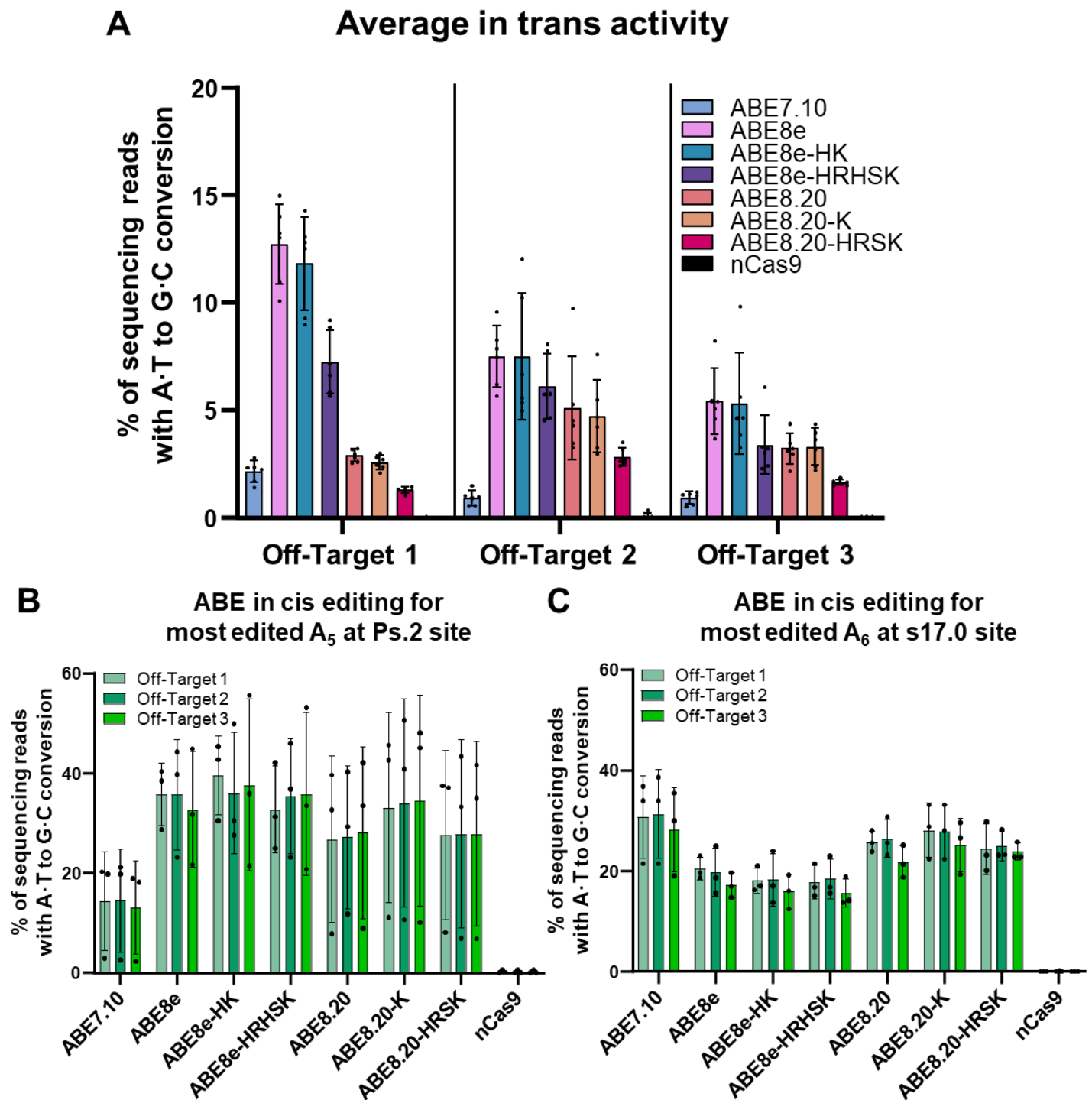
Supplementary Figure 32 Stability of expression for ABE7.10 multi-residue reversion variants and ABE8-derived reversion editors

A. HEK293Ts cells were transfected with stability reporter plasmids containing mCherry-P2A-ABE-EGFP expression cassettes as described in Extended Data Figure 2. Following 48 hours of incubation, cells were analyzed via flow cytometry where EGFP/mCherry ratios were calculated for individual double color events. Median EGFP/mCherry ratio from $n = 3$ biological replicates are shown, error bars how standard deviation. Data were analyzed with an ordinary one-way ANOVA with Holm-Šidák multiple comparison test to ABE7.10 with significant p-values reported ($p \geq 0.05$ not significant, not indicated). **B.** Frequency of distribution of EGFP/mCherry ratios are plotted for each reversion editor along with distributions of ABE7.10 and respective parent editor when relevant. EGFP/mCherry ratios have been tabulated in bins of 0.05 width. Plots represent values calculated from events analyzed from a single representative transfection, ($n = 3$ biological replicates).



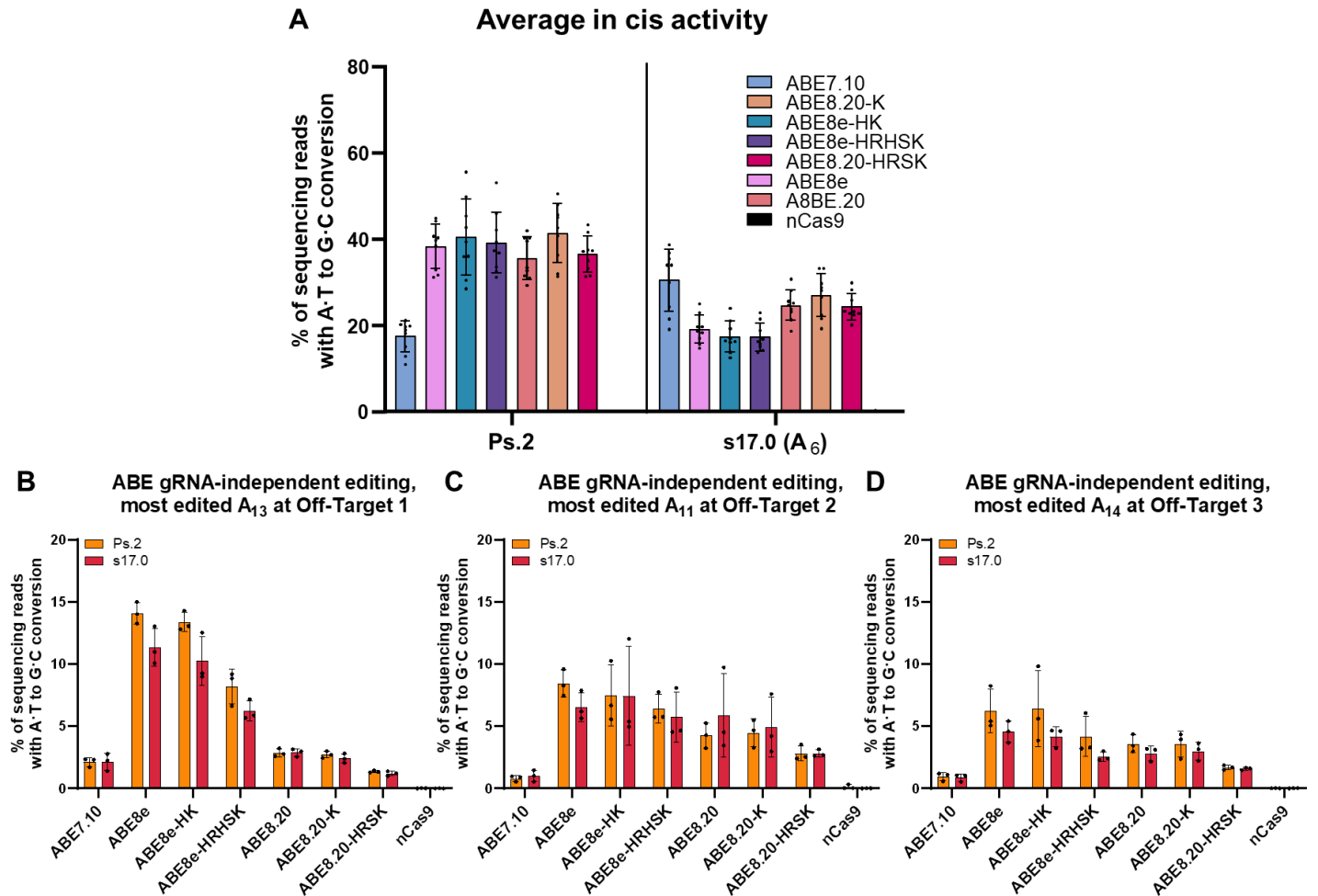
Supplementary Figure 33 Orthogonal R-loop assay for ABE8-derived reversions editors on- and off-target sites absolute activity

Cells were treated as described in Supplementary Figure 27. **A-F.** x-y plots represent the average editing activity for the most edited A in a given on/off combination target for $n = 3$ individual biological replicates with error bars showing standard deviation. Each combination of on- and off-target gRNAs has been plotted independently. Note for ABE8-derived editors, the s17.0 most edited A falls at position 6.



Supplementary Figure 34 Breakdown of in trans and in cis editing measured by orthogonal R-loop assay for ABE8 family of multi-residue reversion editors

A. Cells were treated as described in Supplementary Figure 27. Average in trans (off-target) editing activities of a subset of ABE8-derived combination reversion editors, as measured by the orthogonal R-loop off-target assay are shown, broken down by off-target gRNA. **B-C.** Average in cis (on-target) editing activity at the Ps.2 (**B**) and s17.0 A₆ (**C**) sites for a subset of ABE8-derived combination reversion editors, as measured by the orthogonal R-loop off-target assay, are shown, broken down by off-target gRNA. Bars represent average editing ($n = 3$ biological replicates per on/off gRNA combo), error bars display standard deviation.



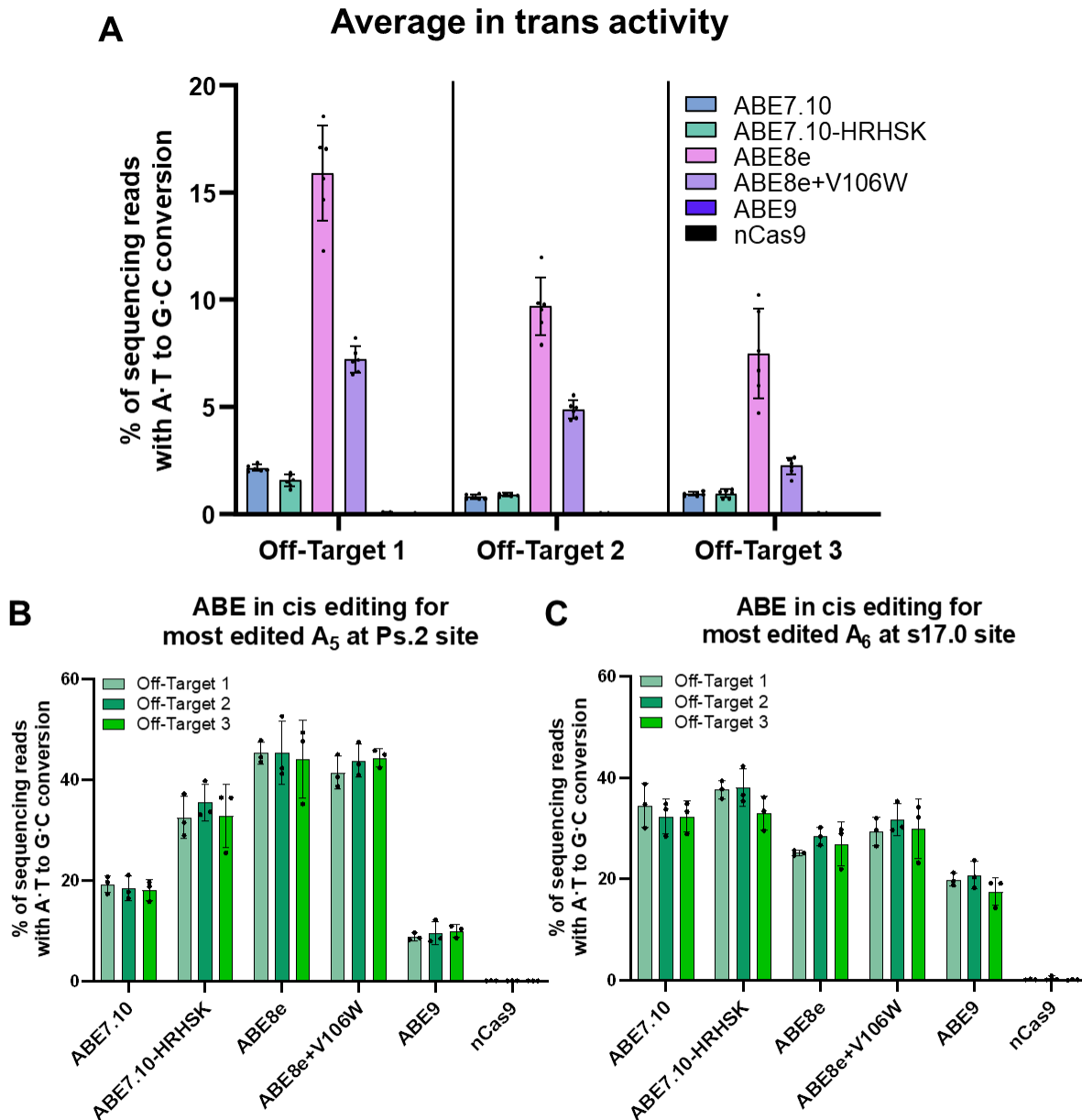
Supplementary Figure 35 Breakdown of in cis and in trans editing measured by orthogonal R-loop assay for ABE8 family of multi-residue reversion editors

A. Cells were treated as described in Supplementary Figure 27. Average in cis (on-target) editing activities of a subset of ABE8-derived combination reversion editors, as measured by the orthogonal R-loop off-target assay, are shown, broken down by on-target gRNA. **B-D.** Average in trans (off-target) editing activity at Off-Target site 1 (**B**), Off-Target site 2 (**C**), and Off-Target site 3 (**D**) for a subset of ABE8-derived combination reversion editors, as measured by the orthogonal R-loop off-target assay, are shown, broken down by on-target gRNA. Bars represent average editing ($n = 3$ biological replicates per on/off gRNA combo), error bars display standard deviation.

A position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Number of A's	3	6	7	4	10	4	5	4	7	3	5	7	3	6	3	8	2	2	4	3
ABE7.10	0.49%	0.15%	1.69%	26.86%	54.66%	49.69%	39.26%	3.11%	3.15%	0.80%	1.35%	0.33%	0.09%	0.16%	0.05%	0.20%	0.62%	0.13%	0.13%	0.06%
ABE7.10+HRHSK	0.43%	0.16%	1.71%	35.61%	66.14%	48.39%	38.29%	1.83%	1.82%	0.51%	0.48%	0.14%	0.12%	0.11%	0.09%	0.10%	0.27%	0.11%	0.10%	0.12%
ABE8e	9.82%	1.84%	32.56%	50.84%	63.82%	61.16%	56.30%	32.45%	21.80%	12.23%	32.57%	7.27%	3.93%	1.32%	0.14%	0.15%	0.20%	0.14%	0.11%	0.10%
ABE8e+V106W	4.08%	2.69%	25.71%	53.20%	72.93%	75.73%	69.44%	43.19%	22.45%	16.33%	22.73%	3.40%	1.92%	0.34%	0.10%	0.13%	0.20%	0.13%	0.17%	0.08%
ABE9	0.16%	0.09%	0.36%	11.94%	42.39%	24.93%	8.04%	0.48%	0.18%	0.09%	0.10%	0.08%	0.06%	0.14%	0.07%	0.17%	0.44%	0.12%	0.15%	0.07%
NT	0.05%	0.08%	0.13%	0.12%	0.21%	0.25%	0.31%	0.11%	0.12%	0.07%	0.09%	0.07%	0.03%	0.12%	0.05%	0.09%	0.26%	0.13%	0.15%	0.06%

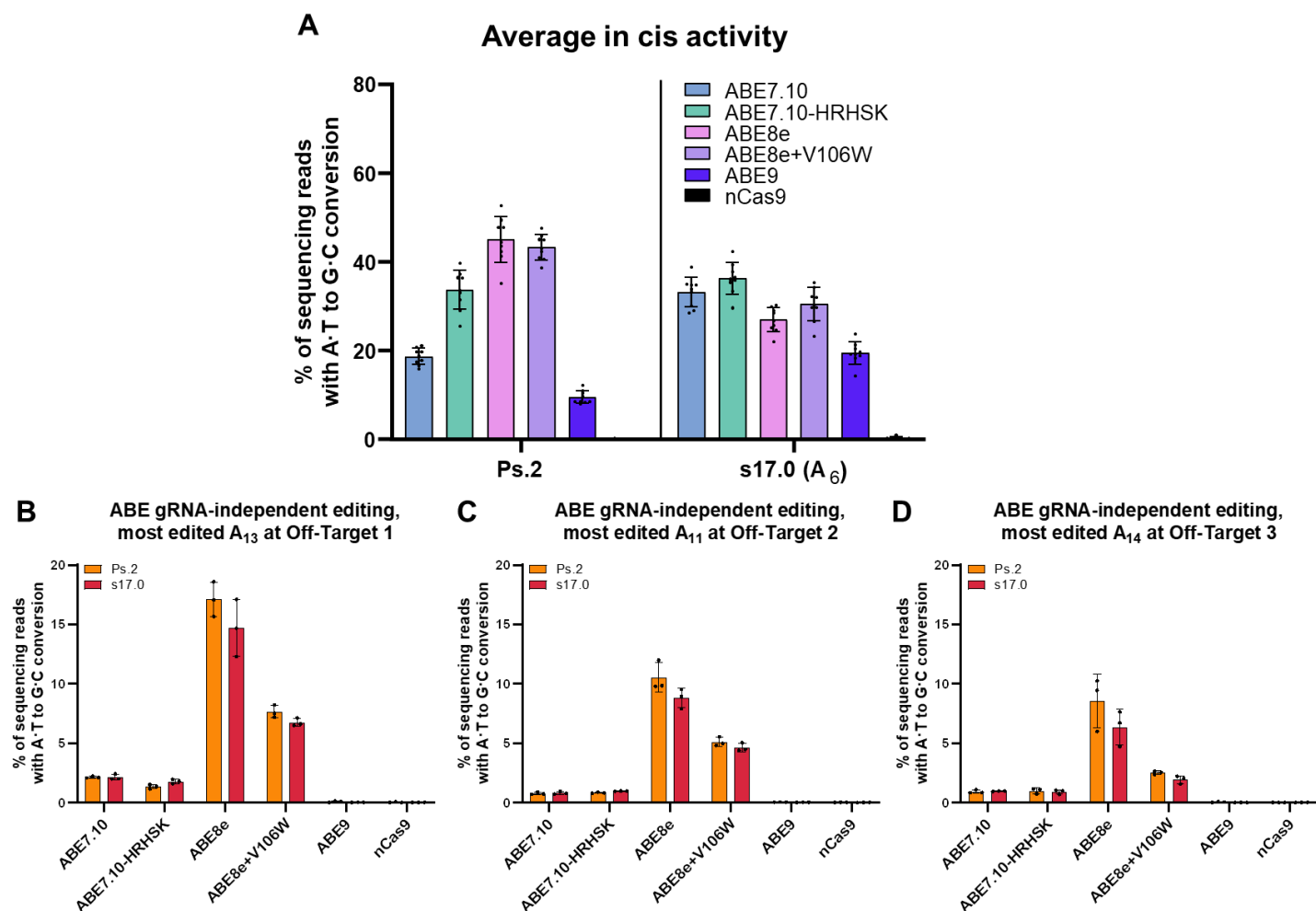
Supplementary Figure 36 Relative editing activity of ABE multi-residue reversion variants compared to ABE8e-V106W and ABE9 as a function of target A position

HEK293T cells were treated and data further normalized as described in Supplementary Figure 20 for a subset of 12 target sites. Average normalized A·T to G·C editing activity values for listed editors are reported by Target A position within the protospacer window. Darker shaded cells indicate higher average normalized editing activity. Averages were determined from n = 3 biological replicates for all editors.



Supplementary Figure 37 Breakdown of in trans and in cis editing measured by orthogonal R-loop assay for precision editors

A. Cells were treated as described in Supplementary Figure 27. Average in trans (off-target) editing activities of a set of precision editors, as measured by the orthogonal R-loop off-target assay are shown, broken down by off-target gRNA. **B-C.** Average in cis (on-target) editing activity at the Ps.2 (**B**) and s17.0 A₆ (**C**) sites for a set of precision editors, as measured by the orthogonal R-loop off-target assay, are shown, broken down by off-target gRNA. Bars represent average editing (n = 3 biological replicates per on/off gRNA combo), error bars display standard deviation.



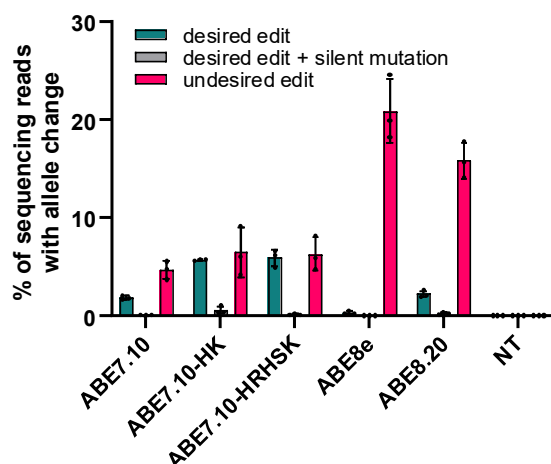
Supplementary Figure 38 Breakdown of in cis and in trans editing measured by orthogonal R-loop assay for precision editors

A. Cells were treated as described in Supplementary Figure 27. Average in cis (on-target) editing activities of a set precision editors, as measured by the orthogonal R-loop off-target assay, are shown, broken down by on-target gRNA. **B-D.** Average in trans (off-target) editing activity at Off-target site 1 (**B**), Off-Target site 2 (**C**), and Off-Target site 3 (**D**) for a set of precision editors, as measured by the orthogonal R-loop off-target assay, are shown, broken down by on-target gRNA. Bars represent average editing (n = 3 biological replicates per on/off gRNA combo), error bars display standard deviation.

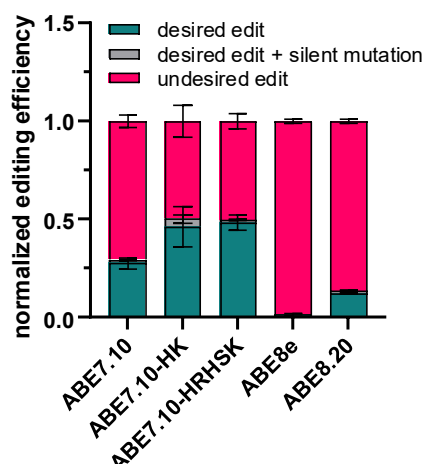
	H H ₅₅₈ T S L L V	% reads with allele change				
		ABE7.10	ABE7.10-HK	ABE7.10-HRHSK	ABE8e	ABE8.20
H558R	CACCGCACATCACTGCTGGT	1.91	6.69	6.71	0.24	3.02
	CACCGCACGTCACCTGCTGGT					
	CACCGCACGTCGCTGCTGGT					
	CACCGCACATCGCTGCTGGT					
T559A	CACCACGATCACTGCTGGT	3.40	2.95	1.49	0.55	0.72
H558R/T559A	CACCGCGCATCACTGCTGGT	1.42	5.85	6.40	21.65	16.27
	CACCGCGGTCACCTGCTGGT					
	CACCGCGGTCGCTGCTGGT					
-	CACCACACGTCACCTGCTGGT	0.75	0.37	0.29	0.00	0.08
H557R/H558R/T559A	CGCCGCGGTCACCTGCTGGT	0.00	0.00	0.00	2.43	0.75

G - Target A to G conversion
 G - Silent A to G conversion
 G - Missense A to G conversion

SCN5A H558R SpRY variants
CACCAC₅CACATCACTGCTGGT



SCN5A H558R SpRY variants
precision scores



Supplementary Figure 39 Modeling SCN5A H558R SNV of clinical interest

ABE variants were targeted to model the *SCN5A* H558R SNVs of clinical interest in HEK293T cells. Editors and appropriate gRNA were delivered via plasmid and allowed to incubate for 72 hours prior to extraction, amplification, and analysis of genomic DNA via NGS. Editing outcome data are presented three ways: (Top) For a single replicate (out of $n = 3$), editing percentages as a function of allele sequences are shown. Sequences producing the desired allele are highlighted in grey, with the corresponding A to G conversions in the protospacer color-coded - green for Target A to G, yellow for silent A to G, red for missense A to G. (Bottom left) Absolute editing efficiencies are presented as measured in $n = 3$ biological replicates, error bars indicate standard deviation. Desired edits (teal) account for sequences with only the target A edited, with desired edits + silent mutations (grey) shown when applicable. Undesired edits (red), include any sequences in which the target A may or may not have been edited alongside additional bystander mutations that resulted in alternative missense mutations, as well as those in which the target A was not edited, but additional bystander mutation that resulted in silent mutations were. (Bottom right) Data are also presented as normalized precision scores, illustrating the average ($n = 3$ biological replicates) fraction of edited sequencing reads to contain the desired, desired + silent, or undesired mutations. Error bars indicate standard deviation.

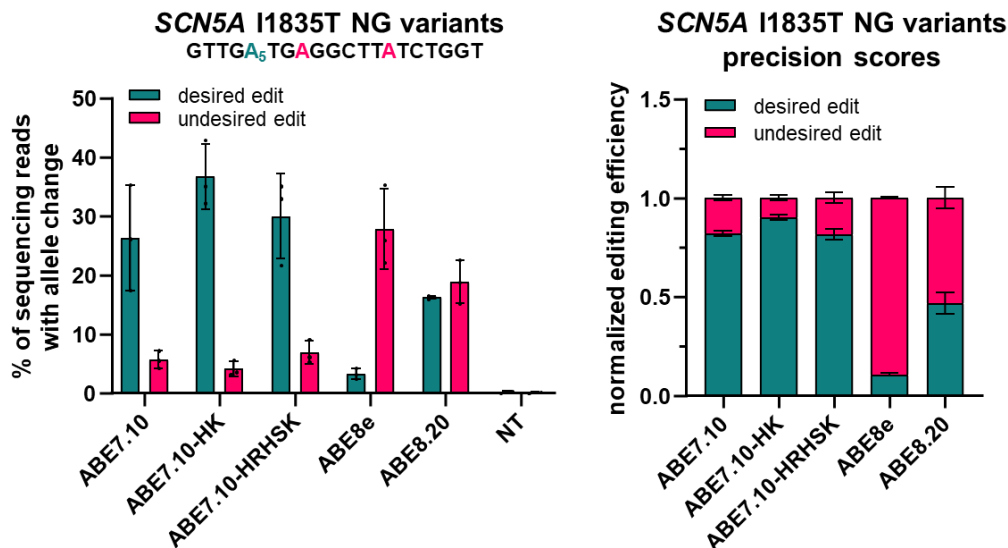
	L I ₁₈₃₅ N M D L P	% reads with allele change				
		ABE7.10	HK	HRHSK	ABE8e	ABE8.20
I1835T	GTTG GT GAGGCTTATCTGGT	35.30	42.83	35.11	4.25	15.92
L1834P	GTTGATG GG GCTTATCTGGT	2.23	0.11	0.27	0.02	0.04
L1834P/I1835T	GTTG GT G GG GCTTATCTGGT	4.97	5.54	8.71	33.65	22.17
I1832T/L1834P/I1835T	GTTG GT G GG GCTT GT CTGGT	0.00	0.00	0.11	1.29	0.12
I1832T/I1835T	GTTG GT GAGGCTT GT CTGGT	0.01	0.01	0.01	0.35	0.21

- G

- Target A to G conversion
- G

- Silent A to G conversion
- G

- Missense A to G conversion



Supplementary Figure 40 Modeling SCN5A I1835T SNV of clinical interest

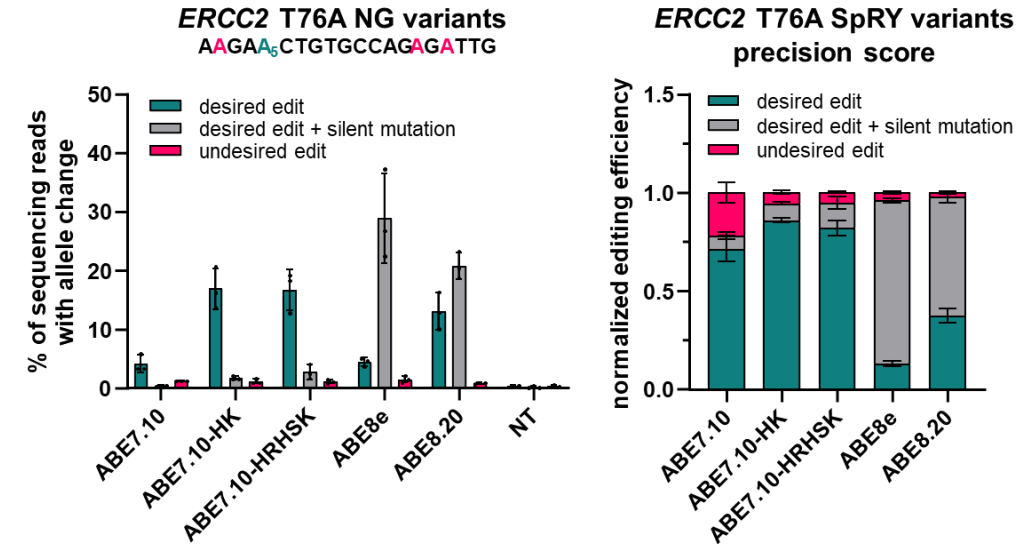
ABE variants were targeted to model the SCN5A I1835T SNVs of clinical interest in HEK293T cells. Data were generated and are presented as described in Supplementary Figure 39.

	S R T ₇₆ V P E I E	% reads with allele change				
		ABE7.10	HK	HRHSK	ABE8e	ABE8.20
T76A	AAGAGCTGTGCCAGAGATTG	6.14	22.65	23.21	42.23	39.50
	AAGGGCTGTGCCAGAGATTG					
	GAGGGCTGTGCCAGAGATTG					
	AAGGGCTGTGCCGAGATTG					
-	AAGAACTGTGCCAGAGATTG	0.66	1.20	0.99	1.33	0.30
	GAGAACTGTGCCAGAGATTG					
I80V	AAGAACTGTGCCAGAGATTG	0.20	0.15	0.15	0.13	0.13
E79G	AAGAACTGTGCCAGAGATTG	0.17	0.11	0.14	0.12	0.10
R73G	AAGAACTGTGCCAGAGATTG	0.14	0.10	0.12	0.05	0.08
R73G/T76A	AAGGGCTGTGCCAGAGATTG	0.00	0.00	0.01	0.43	0.00
	GGGGCTGTGCCAGAGATTG					

G - Target A to G conversion

G - Silent A to G conversion

G - Missense A to G conversion



Supplementary Figure 41 Modeling ERCC2 T76A SNV of clinical interest

ABE variants were targeted to model the ERCC2 T76A SNVs of clinical interest in HEK293T cells. Data were generated and are presented as described in Supplementary Figure 39.

	Y <u>E₉₅</u> K Q E G E	% reads with allele change				
		ABE7.10	HK	HRHSK	ABE8e	ABE8.20
E95G	TATGGAAGCAGGAGGGCGA	10.10	22.26	19.45	3.88	7.46
K96E	TATGAGAGCAGGAGGGCGA	2.28	1.22	1.06	0.36	0.00
E95G/K96E	TATGGAGAGCAGGAGGGCGA	1.57	4.38	9.28	8.04	10.44
K96R	TATGAGAGCAGGAGGGCGA	0.77	0.36	0.26	0.01	0.01
K96E/Q97R	TATGAGAGCGGGAGGGCGA	0.54	0.00	0.00	0.00	0.00
E95G/K96G	TATGGGGCAGGAGGGCGA	0.10	0.59	0.97	9.83	8.89
K96G	TATGAGGGCAGGAGGGCGA	0.15	0.39	0.00	0.23	0.00
E95G/K96R	TATGGAGGCAGGAGGGCGA	0.32	0.34	1.67	0.58	0.35
E95G/Q97R	TATGGGAAGCGGGAGGGCGA	0.18	0.33	0.14	0.05	0.00
Y94C	TGTGAGAAGCAGGAGGGCGA	0.33	0.17	0.01	0.02	0.19
E95G/K96E/Q97R	TATGGAGCGGGAGGGCGA	0.15	0.00	0.36	1.35	0.01
E95G/K96G/Q97R	TATGGGGCGGGAGGGCGA	0.00	0.00	0.00	5.49	0.13
Y94C/E95G/K96G/Q97R	TGTGGGGCGGGAGGGCGA	0.00	0.00	0.00	3.33	0.18
Y94C/E95G/K96G	TGTGGGGCAGGAGGGCGA	0.00	0.00	0.00	1.05	6.32
Y94C/E95G/K96E	TGTGGGAGCAGGAGGGCGA	0.02	0.08	0.00	0.59	3.82
E95G/K96G/E98G	TATGGGGCAGGAGGGCGA	0.00	0.00	0.00	0.21	0.00
E95G/K96E/E98G	TATGGGAGCAGGGGGCGA	0.00	0.00	0.00	0.19	0.00
Q97R	TATGAGAAGCGGGAGGGCGA	0.17	0.03	0.02	0.11	0.01
Y84C/E95C	TGTGGAAGCAGGAGGGCGA	0.00	0.00	0.00	0.00	0.55

G

- Target A to G conversion

G

- Silent A to G conversion

G

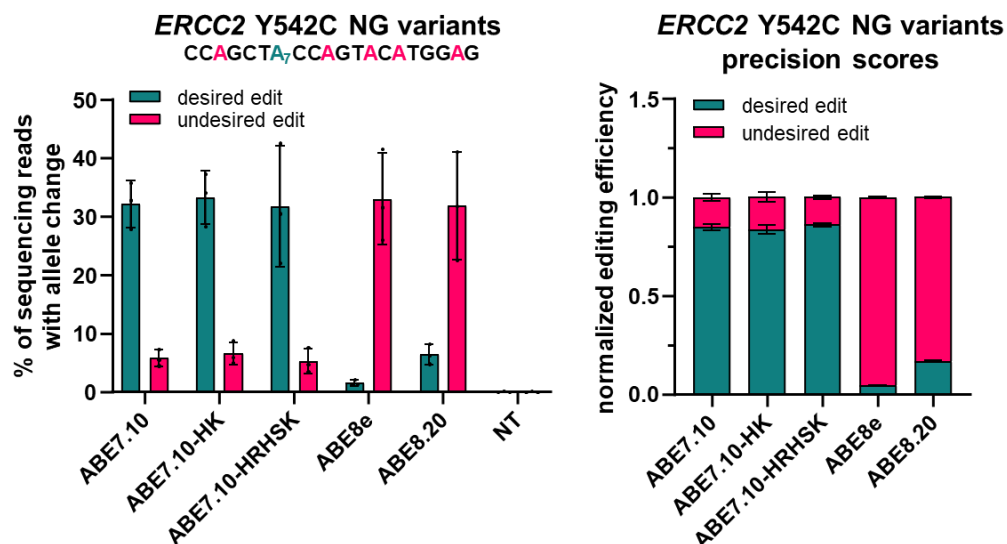
- Missense A to G conversion

Supplementary Figure 42 Modeling ERCC2 E95G SNV of clinical interest

ABE variants were targeted to model the ERCC2 E95G SNVs of clinical interest in HEK293T cells. Data were generated and are presented as described in Supplementary Figure 39. Note that average editing efficiency and average precision of editing data for ERCC2 E95G can be found in the main manuscript, Figure 6F-G.

	T S Y ₅₄₂ Q Y M E	% reads with allele change				
		ABE7.10	HK	HRHSK	ABE8e	ABE8.20
Y542C	CCAGCT G CCAGTACATGGAG	35.77	37.25	42.51	2.06	8.24
S541G/Y542C	CC G GCT G CCAGTACATGGAG	6.11	8.29	6.85	24.40	37.33
Y542C/Q543R	CCAGCT G CC G TACATGGAG	0.71	0.21	0.34	0.19	0.63
S541G	CC G GCTACCAGTACATGGAG	0.41	0.21	0.38	0.03	0.18
S541G/Y542C/Q543R	CC G GCT G CC G TACATGGAG	0.05	0.00	0.00	12.28	1.74
S541G/Y542C/Q543R/Y544C	CC G GCT G CC G T G CATGGAG	0.00	0.00	0.00	2.21	0.31
S541G/Y542C/Y544C	CC G GCT G CCAGT G CATGGAG	0.04	0.00	0.00	1.70	0.81
S541G/Y542C/M545V	CC G GCT G CCAGTAC G TGGAG	0.00	0.00	0.00	0.53	0.00
S541G/Y542C/Q543R/Y544C/M545V	CC G GCT G CC G T G C G TGGAG	0.00	0.00	0.00	0.13	0.00

G - Target A to G conversion
G - Silent A to G conversion
G - Missense A to G conversion

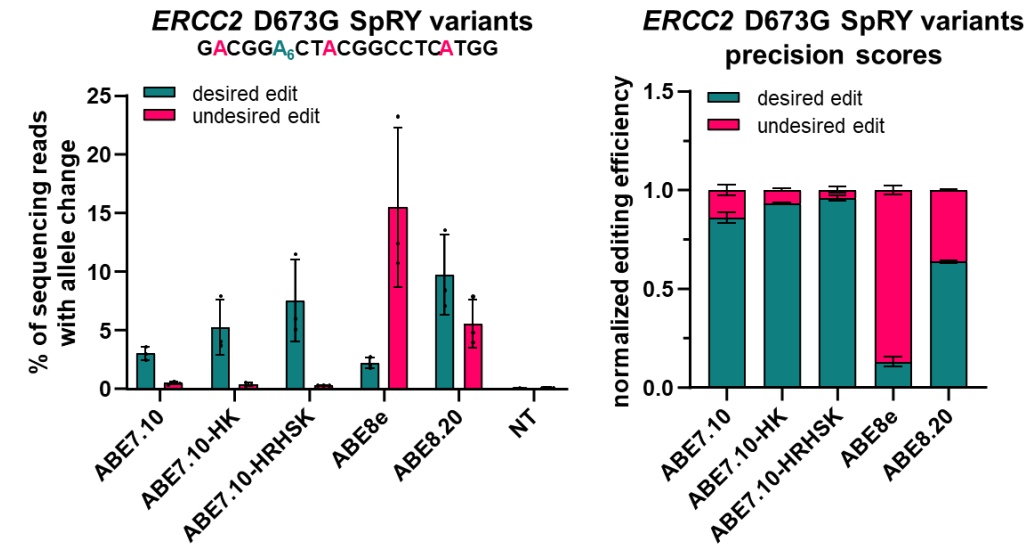


Supplementary Figure 43 Modeling *ERCC2* Y542C SNV of clinical interest

ABE variants were targeted to model the *ERCC2* Y542C SNVs of clinical interest in HEK293T cells. Data were generated and are presented as described in Supplementary Figure 39.

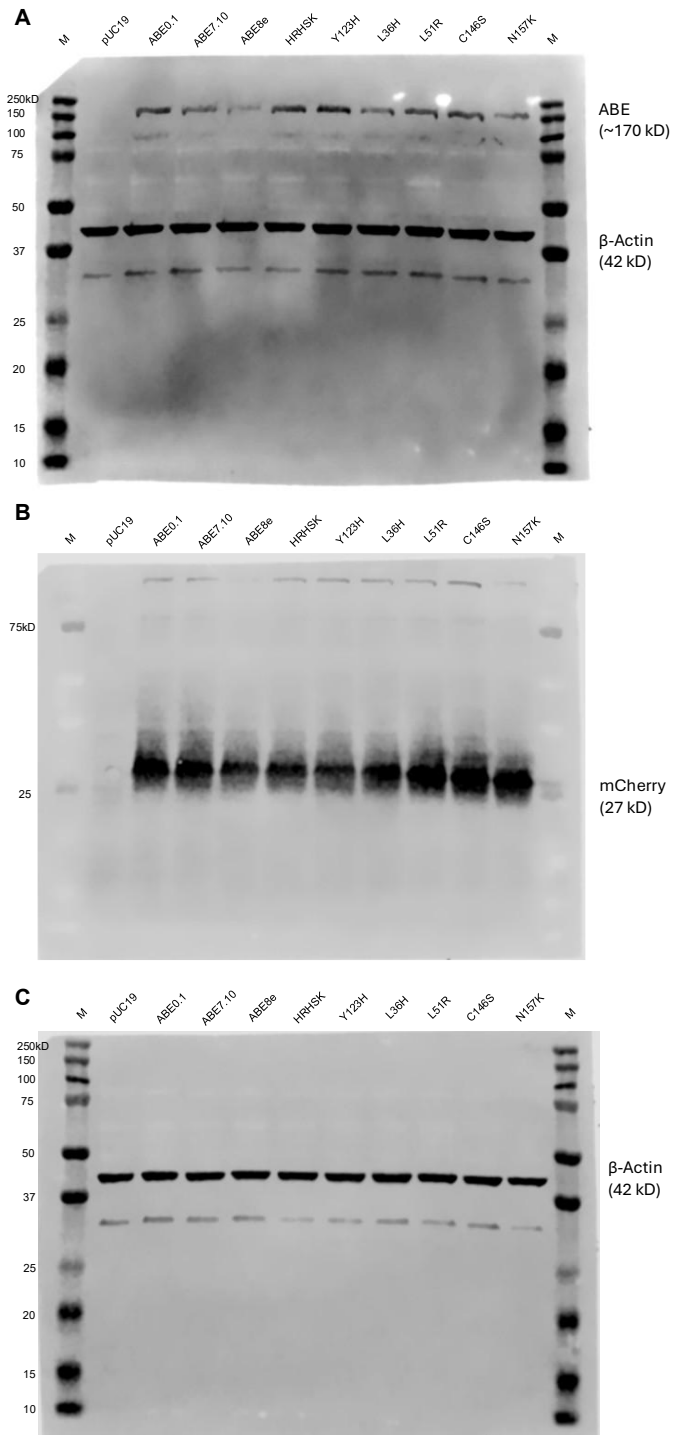
	K T D ₆₇₃ Y G L M V	% reads with allele change				
		ABE7.10	HK	HRHSK	ABE8e	ABE8.20
D673G	GACGG G CTACGGCCTCATGG	3.59	7.93	11.48	2.71	13.54
Y674C	GACGGACT G CGGCCTCATGG	0.24	0.31	0.08	0.51	0.14
D673G/Y674C	GACGG G T CGGCCTCATGG	0.20	0.12	0.09	20.35	7.06
T672A/D673G/Y674C	G G CGGCT G CGGCCTCATGG	0.00	0.00	0.00	2.29	0.29
T672A	G G CGGACTACGGCCTCATGG	0.05	0.05	0.04	0.06	0.37
T672A/D673G	G G CGGCTACGGCCTCATGG	0.00	0.07	0.08	0.00	0.02

G - Target A to G conversion
G - Silent A to G conversion
G - Missense A to G conversion



Supplementary Figure 44 Modeling *ERCC2* D673G SNV of clinical interest

ABE variants were targeted to model the *ERCC2* D673G SNVs of clinical interest in HEK293T cells. Data were generated and are presented as described in Supplementary Figure 39.



Supplementary Figure 45 Uncropped western blot images to analyze expression levels of ABEs

Shown are the uncropped representative western blots monitoring ABE expression levels from Extended Data Figure 1C. Blots are representative from $n = 3$ independent blots performed on separate days. HEK293T cells were transfected with plasmids expressing either negative control pUC19, or the indicated editor in an ABE-P2A-mCherry architecture. Cells were allowed to grow for 48 hours before harvesting and quantifying total protein. Samples were evenly split and ran in parallel with 40 μ g total protein loaded per gel, separated via electrophoresis, transferred to 0.45 μ m nitrocellulose membranes, following which parallel membranes were sequentially stained. Marker (M) is BioRad precision plus Protein in Dual Color.

A. ABE (protein of interest) was examined relative to β -Actin (housekeeping protein). Blot is stained and imaged for ABE and β -Actin. Image displayed represents black and white composite of both 700 nm and 800 nm scans post-sequential staining for β -Actin. **B.** On a separate membrane, mCherry was detected following a 800 nm scan. **C.** Sequential staining of membrane in **B** for β -Actin with a 700 nm scan.

Supplementary Discussion:

Supplementary Discussion 1: Description of episomal EGFP fluorescence turn-on reporters of ABE activity

The episomal fluorescence turn-on reporter used to measure ABE activity has been previously reported¹⁰. Briefly, it relies on a plasmid coding for two independently translated fluorescent proteins: mCherry, which acts as a transfection marker, and a deactivated enhanced green fluorescent protein (dEGFP), which acts as the reporter for base editing activity (Figure 2A). The dEGFP protein is deactivated by either an A111V or H182Y mutation, which results in deficient EGFP fluorescence that can be restored by a single A•T to G•C conversion in the gene⁵⁶. The sensitivity of these reporters is an order of magnitude greater than next generation sequencing (NGS), enabling the detection of editing activity by low efficiency variants (such as ABE0.1)¹⁰. Both reporters embed the target A within a TAC motif as the native substrate of TadA is the UAC motif in the tRNA_{Arg} anticodon loop¹⁸, and we previously found that most evolutionary variants of TadA retain this sequence context preference¹⁰. The target A resides on the non-coding strand (to avoid EGFP turn-on due to editing of the mRNA transcript³²), and is positioned such that the base directly 5' (-1) or 3' (+1) from the target A falls at a wobble codon position in the A111V and H182Y reporters, respectively. This allows for substitution of any base N at the upstream or downstream position, enabling evaluation of editing efficiencies for all ABE variants at all NAC and TAN sequence motifs, using eight unique reporter plasmids

Supplementary Discussion 2: Analysis of 5' base impact on sequence motif preferences for single-residue reversion editors

We sought to assess how individual mutations impacted sequence motif preferences independently of their differences in overall editing efficiencies. Given the larger dynamic range of the -1 family of reporters, we focused on this dataset. Absolute BE activity scores (not normalized to ABE7.10) for a given editor were each normalized to that editor's activity score at the TAC motif (Supplementary Figure 10). The resulting "sequence normalized" values of the reversion editors were then compared to those of ABE7.10 (Supplementary Figure 10B, dotted lines). We observed that the majority of reversion editors had sequence normalized scores within error of ABE7.10, and general sequence motif preferences matched those of ABE7.10 (TAC > CAC > AAC ~ GAC, Supplementary Figure 10). Notably, the N108D reversion editor saw the greatest difference in sequence motif editing efficiencies relative to ABE7.10, with a significant aversion to all VAC motifs (where V = A/C/G). This is in accordance with earlier findings that suggest the initial D108N mutation broadened the sequence motif preference of TadA¹⁰, and suggests that the reversion of this residue to Asp108 reduces the enzyme's sequence motif compatibility. We also found the V155E variant to disfavor the GAC motif. Given the positioning of this residue adjacent to the -1 base in the ABE8e structure, the transition from Glu155 to Val155 during development of TadA7.10 may have relaxed editor sequence preference due to sterics. Finally, neither the Y123H nor N157K variants (which increased overall editing activities) exhibited a notable difference in sequence motif editing preferences relative to ABE7.10, suggesting these variants improved editing efficiency in a sequence motif agnostic manner.

Supplementary Discussion 3: Activity of single-residue reversion variants at endogenous target sites

HEK293T cells were transfected with each ABE plasmid and one of three gRNA plasmids targeting the protospacers indicated in Extended Data Figure 1A. After 72 hours, genomic DNA was harvested, target sites were amplified, and base editing efficiency was analyzed via next generation sequencing (NGS). At each site, A•T to G•C editing efficiencies for each ABE7.10 single-residue reversion variant were normalized to that of ABE7.10 within each experimental replicate and are presented as fold-change values in Extended Data Figure 1B. Of the three sites tested, the largest dynamic range in editing activity was observed at the Ps.2 site (TAC target motif), followed by the Ps.0 site (AAC motif), followed by the s15.0 site (TAG motif) (Supplementary Figure 11A-C). Consistent with the fluorescent reporter data, ABE7.10-N108D once again caused the largest decrease in editing across all target sites (on average 155 ± 32 -fold, 595 ± 300 -fold, and 319 ± 185 -fold lower than ABE7.10 at the TAC, TAG, and AAC sites, respectively, Supplementary Figure 11D-F). ABE7.10-V155E demonstrated the second largest decrease in editing activity (9.2 ± 2.1 -fold at the TAC site), followed by ABE7.10-F84L (6.2 ± 1.1 -fold at the TAC site) and ABE7.10-F156I (3.8 ± 1.0 -fold at the TAC site), with similar trends at TAG and AAC sites.

Most reversion variants derived from mutations accumulated during round five of directed evolution (including ABE7.10-L36H, ABE7.10-L51R, and ABE7.10-C146S) did not have a significant impact on editing ability compared to ABE7.10 across all three endogenous target sites, consistent with the data from the fluorescent reporter assay (Extended Data Figure 1B and Supplementary Figure 11). Furthermore, the ABE7.10-N157K reversion (which increased activity at all NAC motifs in the fluorescent reporter, Figure 2B) increased editing activity 1.5 ± 0.2 -fold compared to ABE7.10 at the most sensitive TAC Ps.2 site (Extended Data Figure 1B and Supplementary Figure 11B and E). Similarly, the ABE7.10-Y123H reversion variant, which also increased editing as observed in the fluorescent reporter assay, increased editing activity 1.4 ± 0.2 -fold compared to ABE7.10 at the TAC Ps.2 site, and 1.1 ± 0.2 -fold at the AAC Ps.0 site (Extended Data Figure 1B and Supplementary Figure 11B, C, E, and F).

Supplementary Discussion 4: Analysis of alanine substitution variants

A subset of ABE7.10 mutations were further selected for analysis by alanine substitution, based on the data from their respective ABE7.10 single-residue reversion variant's endogenous and episomal editing. We selected two residues that increased activity when reverted to the wild-type amino acid (N157K and Y123H), three residues that decreased activity when reverted to the wild-type amino acid (N108D, V155E, and F84L), and one residue that had a neutral impact when reverted back to wild-type (C146S). These residues were selected for alanine substitution as replacement of these charged, aromatic, or polar side chains could alter critical biochemical interactions, providing insight into the native or mutated residues' role in base editing activity. We generated ABE7.10 alanine variants, in which each of these six mutations were substituted for alanine in the background of ABE7.10, and measured their editing activities at endogenous sites (Extended Data Figure 1A). The fold-changes in editing activity with respect to ABE7.10 are shown in Extended Data Figure 1C-F, plotted alongside their respective reversion editor's activity.

We generally observed consistent trends in relative editing activities for a given editor across all three sites, with the largest dynamic ranges observed at the Ps.2 TAC site (Supplementary Figure 12). At this site, the ABE7.10-N108D editor had an ABE7.10 normalized activity score of $(6.4 \pm 1.3) \times 10^{-3}$, while that of its corresponding alanine mutant ABE7.10-N108A was 0.12 ± 0.03 , indicating a partial rescue of activity (a 19 ± 6 -fold increase, Extended Data Figure 1E). The relative activity scores of aspartic acid < alanine < asparagine are consistent with our previous computational studies, which show that asparagine 108 (present in ABE8e and ABE7.10) engages in a favorable hydrogen bond interaction with the substrate DNA backbone directly 5' to the target A^{10,17}. However, the negative charge of aspartic acid results in unfavorable electrostatic repulsion with the DNA backbone, causing the residue to rotate away from the DNA substrate, and a subsequent loss in activity. This unfavorable interaction would be mitigated with the alanine mutant, but would lack the ability to form a favorable hydrogen bond with the target DNA, reducing editing activity for the alanine mutant relative to the asparagine variant.

Residues 155 (E in wtTadA, V in TadA*7.10) and 157 (K in wtTadA, N in TadA*7.10) reside in the C-terminal alpha helix of TadA (residues 138 through 165). During evolution from wtTadA, a "kink" and resulting large structural shift was introduced in the middle of this helix following the introduction of the R152P mutation (Supplementary Figure 13A), bringing both of these residues within 3 Å of the DNA substrate in the ABE8e

structure¹³ and our ABE7.10 simulations^{10,17}. We have previously hypothesized that this structural shift may have relaxed the +1 base sequence specificity, leading to enhanced editing activity. Alanine substitutions of these C-terminal residues in the context of the ABE7.10 background (with the R152P-induced “kink”) would therefore probe the importance of electrostatic and hydrogen-bonding interactions between the proximal C-terminus and DNA substrate. The ABE7.10-V155E editor displayed a normalized activity score of 0.11 ± 0.02 at the TAC site, compared to 0.59 ± 0.19 for ABE7.10-V155A (a 5.4 ± 2.1 -fold increase, Extended Data Figure 1E). At the other two sites, ABE7.10-V155E also performed worse than ABE7.10, while ABE7.10-V155A performed within error of ABE7.10 (Extended Data Figure 1C). An aversion to glutamic acid at this position may be due to unfavorable electrostatic interactions. The alanine substitution mutant ABE7.10-N157A demonstrated a 1.7 ± 0.6 -fold decrease in activity score relative to ABE7.10-N157K at the TAC site and was within error of ABE7.10 (Extended Data Figure 1E). The positively charged lysine residue may interact favorably with the DNA backbone and could be responsible for the observed increase in activity. However, this interaction may not be possible in ABE0.1 due to the fully extended nature of the C-terminal helix, which causes residue 157 to reside farther than 3 Å away from the DNA substrate. This may explain why residue 157 evolved into asparagine in round 5 of evolution (before the presence of the kink), while lysine is more optimal in the ABE7.10 background. These data also illustrate how the impact of a given mutation (or reversion) is dependent on the context of additional mutations.

When analyzing the residue 84 mutants, we observed a further loss in activity by the ABE7.10-F84A mutant over the ABE7.10-F84L reversion (a 12 ± 5 -fold decrease in activity score at the TAC site, Extended Data Figure 1E), suggesting an important role for phenylalanine at this position. Indeed, this residue has been hypothesized to pi-stack with the target A base and orient it optimally for deamination in the ABE8e structure and our ABE7.10 and ABE8e simulations⁵⁷. Such an interaction would not be possible with leucine or alanine at position 84 (Supplementary Figure 13B). Conversely, alanine substitution at position 146 (ABE7.10-C146A) showed no significant impact on activity scores compared to ABE7.10-C146S (which had activity scores within error of ABE7.10, Extended Data Figure 1E), suggesting the identity of this surface-exposed, C-terminal residue is flexible in the context of the kinked $\alpha 5$ -helix.

Finally, we found a preference for histidine at position 123; while the ABE7.10-Y123H reversion was consistently more active than ABE7.10 (Extended Data Figure 1B), the ABE7.10-Y123A mutant was generally

less active or equivalent to ABE7.10 (2.5 ± 0.6 -fold decrease in activity score compared to ABE7.10-Y123H at the TAC site). As this residue lies at the modeled TadA dimer interface (Supplementary Figure 13C), this preference (alanine < tyrosine < histidine) may reflect interactions between monomers, or possible solvent interactions. While all ABE7.10 reversion and mutation constructs from this work were developed as monomers, this does not preclude the possibility that dimerization between monomeric BE constructs may take place in situ^{13,25}. This subset of alanine substitutions demonstrates that for each residue probed, the optimal amino acid for editing activity was either the wild-type residue or the ABE7.10 mutation.

Supplementary Discussion 5: Two independent reporters examine ABE stability of expression

We adapted a fluorescent stability of expression reporter by covalently attaching ABE variants to EGFP via a flexible linker, while co-expressing this product with mCherry fluorescence marker via the ribosomal skipping sequence P2A (Extended Data Figure 2A). Thus, the entire mCherry-P2A-ABE-EGFP reading frame is transcribed in the same mRNA transcript, but mCherry and ABE-EGFP are translated independently. mCherry fluorescence therefore acts as a transfection normalization factor, reporting on the relative amount of transcript within each cell (i.e. cells that receive more plasmid copies will have higher mCherry fluorescence, and mutations or conditions that impact transcript levels can be compensated for by mCherry fluorescence normalization). The ratio of EGFP to mCherry fluorescence intensities in double color positive cells is then calculated to report on the amount of ABE protein within each cell relative to the amount of plasmid/transcript expressed. As such, this assay accounts for ABE variants that impact either protein expression and/or stability in a cellular environment.

To calibrate this assay, HEK293T cells were transfected with stability reporter plasmids containing ABE0.1, ABE7.10, or ABE8e, incubated for 48 hours to allow ABE expression, and then analyzed for ABE expression qualitatively via fluorescence microscopy (Supplementary Figure 14A) and quantitatively via flow cytometry (Extended Data Figure 2B). We found that the ABE8e editor resulted in cells with a much lower median EGFP/mCherry fluorescence compared to ABE7.10, which in turn was slightly lower than that of ABE0.1. To confirm that this pattern of reduced editor expression for ABE8e was not an artifact of its fusion with EGFP, HEK293T cells were transfected with ABE construct plasmids without covalent EGFP fusion (ABE-P2A-mCherry). Total protein was harvested 48 hours post-transfection and samples were analyzed via western blot and stained for Cas9 (indicative of ABE) and mCherry protein levels (Extended Data Figure 2C). Consistent with the flow cytometry results, the more evolved ABE8e variant showed a stark decrease in relative ABE protein level compared to ABE7.10 and ABE0.1. These results suggest lower expression and/or stability for the more evolved ABE variants in a cellular context with plasmid delivery.

Encouraged by these data demonstrating that the flow cytometry-based assay could detect differences in expression/stability of ABE variants and that these expression results were verifiable in an independent western blot analysis, we utilized the fluorescence read-out assay to measure stability of expressing for all 14 single-residue reversion editors. We found that the reversion of mutations introduced in later rounds of

evolution caused decreases in expression/stability compared to ABE7.10 (purple and blue bars, Extended Data Figure 2D and Supplementary Figure 15). In particular, ABE7.10-R23W (in which a mutation introduced in the final round of directed evolution was reverted) saw the greatest decrease in expression/stability (1.34 ± 0.18 -fold decrease relative to ABE7.10). The loss in expression/stability of ABE7.10-R23W and other late-round reversion editors suggests they may have acted in a compensatory manner, contributing to improvements in stability of the ABE following rearrangements brought on by earlier evolutionary changes. In contrast, reversion of mutations that were introduced earlier in the evolutionary process generally caused increases in expression/stability compared to ABE7.10 (red and orange bars, Extended Data Figure 2D), suggesting these mutations may have been destabilizing. For example, ABE7.10-N108D, which reverts the first mutation of the evolutionary process back to wild-type, had an average EGFP/mCherry fluorescence intensity ratio similar to that of ABE0.1, representing a 1.25 ± 0.08 -fold increase compared to ABE7.10 (Extended Data Figure 2D). Notably, while this reversion mutation *increased* the relative amount of intracellular ABE protein, it *decreased* the base editing activity of the editor (Figure 2B). Therefore, its negative impact on activity cannot be ascribed to loss of protein expression. Conversely, ABE7.10-N157K had a lower median EGFP/mCherry fluorescence intensity ratio compared to ABE7.10, representing a 1.26 ± 0.05 -fold decrease (Extended Data Figure 2D). This reversion mutation *decreased* the relative amount of intracellular ABE protein while causing an *increase* in the base editing activity of the editor (Figure 2B). These results suggest that protein expression/stability was not the driving factor behind altered ABE activity observed in our single-residue reversion variants.

Supplementary Discussion 6: Description of assay used to measure ABE activity in bacteria

We adapted from the selection process used to evolve ABE7.10 by converting the selection plasmid into a method for quantitatively measure ABE activity. Previously, a plasmid encoding an inactive chloramphenicol acetyl transferase (Cam^R) was used to apply selection pressure. Cam^R was non-functional, requiring a single A•T to G•C conversion to re-establish chloramphenicol resistance. We created two variations of this reporter to assay editing activity at two different sequence motifs: a Cam^R H193Y reporter (target motif TAG, previously used in selection round 1 and 2 of ABE7.10 development), and a Cam^R Q122* reporter (target motif TAC - Figure 3A). Competent S1030 *E. coli* cells harboring either of these two reporter plasmids were generated and transformed with a bacterial expression optimized base editor variant (pbABE) plasmid. pbABE plasmids contained a theophylline-inducible ABE variant and an appropriate gRNA to direct the ABE to the Cam^R target site (Figure 3A). Following overnight expression of the ABE variant, bacterial cultures were split onto plates containing maintenance antibiotic kanamycin either with or without survival-limiting antibiotic chloramphenicol. Base editing activity was measured via survival rate, where the number of colonies observed on the chloramphenicol-containing plate was compared to that on the chloramphenicol-free plate.

Supplementary Discussion 7: Reversion variants that led to increased editing activity are combined to create ABE7.10 multi-residue reversion editors

Given that several reversion variants had a minimal or sometimes beneficial impact on base editing activity, we postulated that combinations of these reversions may lead to a similar or synergistic effect on editing activity. We focused on the two single-residue reversion variants that most consistently improved editing in mammalian cell-based reporters, Y123H and N157K, as well as reversions of additional mutations from the same rounds of evolution as Y123H and N157K (Figure 1). While both residues that were selected for in the same round of evolution as H123Y (L84F and I156F) showed a deleterious effect when reverted, residues that were co-evolved with K157N (H36L, R51L, and S146C) were generally found to have a neutral effect when reverted. We therefore chose to include these reversion variants in various combinations with Y123H and N157K (Combinations C2-C10, Extended Data Figure 3A). In particular, the L36H and L51R reversions had minimal impact on editing activity across all mammalian reporter assays, and both are located on external surfaces of the protein, making minimal contacts with other residues within the protein (Figure 1). We therefore hypothesized that they may have co-evolved with functional mutations during directed evolution without contributing to changes in activity. We posited that combining them with each other (combination C1) would have negligible impact on editing activity, while combining them with other reversion variants that improved editing (combinations C3-C5, C8, and C10) could either further improve editing activity or have no impact. We included one combination editor with the P152R and N157K reversions (combination C11). Given our hypothesis that the beneficial impact of the N157K reversion may be attributed to favorable electrostatics between substrate DNA and the charged lysine, we reasoned this interaction may be negated with reversion of the P152R C-terminal helix “kinking”¹⁰.

The eleven ABE7.10 multi-residue reversion variants listed in Extended Data Figure 3A were first evaluated for their impact on base editing activity using the episomal A111V TAC motif EGFP fluorescence turn-on reporter. Nearly half of these multi-residue reversion variants showed editing activity within error of ABE7.10, suggesting none of our selected combinations severely disrupted editing activity (Extended Data Figure 3B-C). This included C1, supporting our hypothesis that the two surface-facing H36L and R51L mutations played little role in ABE activity. Additionally, the N157K/P152R combination (C11) saw no significant difference in activity relative to ABE7.10, but a 1.27 ± 0.31 -fold decrease in BE activity relative to the ABE7.10-N157K single-residue reversion. These findings suggest that the subsequent P152R reversion negates

favorable BE activity mediated by the N157K variant, further supporting our hypothesis that favorable interaction between the kinked C-terminal helix and the substrate DNA are responsible for improved editing activity. The remaining combos (C2, C4, C5, C7, C8, and C10) all demonstrated *increased* editing activity relative to ABE7.10, with combos C2, C8, and C10 increasing editing scores by 1.24 ± 0.08 -fold, 1.33 ± 0.19 -fold, and 1.37 ± 0.13 , respectively (Extended Data Figure 3B). This was particularly remarkable for C8 given it reverts over a third of the mutations originally introduced to ABE7.10. The trends in relative activity scores (as measured by percent of transfected cells with EGFP fluorescence) were also consistent with the relative changes measured by EGFP MFI of double color positive cells (Extended Data Figure 3C).

We further evaluated the editing efficiencies of the six multi-residue reversion variants that exhibited significant increases in editing activity over ABE7.10 in the episomal editing assay (those highlighted in Extended Data Figure 3A) at three endogenous genomic sites previously tested (Extended Data Figures 2A and 3D). We again observed the greatest dynamic range of editing activities at the Ps.2 site (TAC motif) where, consistent with the editing activity changes observed with the fluorescence turn-on reporter (which shares a TAC sequence motif), all six of these multi-residue reversion variants demonstrated increased editing activities relative to ABE7.10. This ranged from 1.28 ± 0.17 -fold for combo C4 to 1.83 ± 0.28 -fold for combo C2 (Supplementary Figure 17A and D). Editing efficiencies of the six multi-residue reversion variants at the s15.0 site (TAG motif) were within error of ABE7.10 (Extended Data Figure 3D and Supplementary Figure 17B and E), while at the Ps.0 site (AAC motif), the C2 combo editor improved editing efficiency by 1.20 ± 0.16 -fold over ABE7.10, and all other multi-residue reversion variants demonstrated editing efficiencies within error of ABE7.10 (Extended Data Figure 3D and Supplementary Figure 17C and F).

Combo C8, in which five of the fourteen mutations are reverted to wild-type, had increased or equivalent editing efficiency to ABE7.10 as measured at the episomal fluorescence turn-on reporter and across all three sites (Extended Data Figure 3B-D and Supplementary Figure 17). Given these data, we elected to carry out further characterization on combos C2, C7, C8, and C10, all containing Y123H and N157K, and subsequently introducing additional “neutral” reversions C146S, L36H, and L51R.

Supplementary Discussion 8: Selection of endogenous target sites and generation/normalization of ABE activity data

We measured the activity and window size of canonical editors ABE7.10, ABE8e, and ABE8.20, and ME-ABEs at 27 endogenous sites (Supplementary Figure 18). We also included the single-residue reversion variant ABE7.10-N157K in these studies as this reversion caused the greatest increase in editing activity among all the single-residue reversion editors. These 27 endogenous target sites, which encompassed coding and non-coding regions, contained 205 unique A's. Each protospacer contained at least one A at either favored position 5 or 6 (as counted from the PAM-distal end of the protospacer), and all 16 possible tri-motif sequences **NAN** were represented (distribution information can be found in Supplementary Figure 19). HEK293T cells were transfected with plasmids encoding an ABE variant and one of 27 gRNAs (or a non-targeting NT control). Cells were allowed to incubate for 72 hours before harvesting genomic DNA, amplifying and sequencing target sites. To account for variability introduced by differing transfection efficiencies, editing values for all 205 A's were normalized to the editing percentage of ABE7.10 at the A₅ position for the H2.0 site (the most edited A for all editors, across all sites and in all transfections) within a given biological replicate. We defined the "editing window" for any given ABE variant as the positions within the protospacer where the average normalized A•T to G•C editing efficiency across all tested A's within this position was greater than 15% (which is equivalent to an average absolute editing efficiency greater than 8%).

Supplementary Discussion 9: Analysis of sequence motif preferences for ME-ABEs across 27 endogenous sites

We next analyzed all editors for sequence motif preferences. To compare motif preferences, the 205 A's were filtered down to only include those that were present in the editing window of each ABE variant (positions 4-7 for all ABE7.10 variants, positions 3-8 for ABE8.20, and positions 3-11 for ABE8e). This resulted in 49 A's for the ABE7.10 variants, 70 A's for ABE8.20, and 100 A's for ABE8e, which were then grouped by their 5' or 3' motifs (Extended Data Figure 5 and Supplementary Figure 21). While a different number of A's were analyzed for each editor, in this manner only "editable A's" were considered for each editor. The 5' motif is generally considered to play a greater role in sequence context preferences of ABEs, with TadA-derived editors favoring TA motifs²¹. The results from our A111V EGFP fluorescence turn-on assay supported this as the TAC motif was the most efficiently edited motif by ABE7.10 and all functional reversion variants (Supplementary Figures 9-10). Similarly, at the larger panel of endogenous sites, all ABE variants tested exhibited a preference for the TA motif (Extended Data Figure 5). The ME-ABEs displayed similar average normalized editing efficiencies compared to ABE7.10 across the sites with TA motifs in their active windows. For example, ABE7.10-HK and ABE7.10-HRHSK had a 1.07-fold and 1.02-fold increase over ABE7.10, respectively. Notably, the further evolved ABE8e and ABE8.20 variants had slightly lower normalized editing efficiencies than ABE7.10 when considering all editable TA's, with only 0.90-fold and 0.93-fold activity compared to ABE7.10, respectively.

To our surprise, the ME-ABEs increased average normalized editing efficiencies compared to ABE7.10 at GA and AA motifs. Traditionally, TadA-based ABEs have favored YA (Y = T or C) motifs²¹, a preference attributed to the wtTadA enzyme's native UAC substrate. An analysis of endogenous sites found that at editable GA motifs, the editors ABE7.10-HK and ABE7.10-HRHSK increased average editing efficiencies 1.53-fold and 1.52-fold compared to ABE7.10, respectively (Supplementary Figure 21A). Furthermore, for editable A's in this sequence context, the ME-ABEs displayed average editing activity greater than that of ABE8e and ABE8.20 (1.00-fold and 1.23-fold that of ABE7.10, respectively). For AA motifs, ABE7.10-HK and ABE7.10-HRHSK increased average editing efficiencies 1.38-fold and 1.31-fold compared to ABE7.10, respectively. These values represent greater or equal average editing activities compared to ABE8e (with a 1.28-fold increase compared to ABE7.10 at this motif), but slightly lower activities than ABE8.20 (with a 1.56-fold increase compared to ABE7.10 at this motif). At both 5' purine sequence motifs, the single-residue reversion editor ABE7.10-K demonstrated average activity scores between that of ABE7.10 and the ME-ABEs, with a

1.14-fold increase relative to ABE7.10 at GA sites, and 1.16-fold increase at AA sites, demonstrating the dual-reversion, at a minimum, improves editing in these sequence contexts.

We observed substantial differences between the average normalized editing efficiencies of all ABE7.10 reversion variants compared to the more evolved ABE8 editors at the editable CA motif sites. The ABE8 editors showed a much stronger preference for CA motifs than ABE7.10, with ABE8e and ABE8.20 increasing average normalized editing activity relative to ABE7.10 by 1.52-fold and 1.42-fold, respectively (Supplementary Figure 21A). While the ME-ABEs still offered some advantage over ABE7.10 at these sites, they were lower than that of both ABE8 variants, with ABE7.10-HK and ABE7.10-HRHSK only facilitating a 1.24-fold and 1.27-fold increase in activity relative to ABE7.10. The single-residue reversion editor ABE7.10-K showed a slight advantage over the ME-ABEs at these sites (1.34-fold increase relative to ABE7.10), but still lower than both ABE8 variants.

We additionally analyzed all editors for 3' motif preferences. Average normalized editing efficiencies were highest at AT motifs for all editors (Extended Data Figure 5), which matched our findings from the H182Y EGFP fluorescence turn-on results (Supplementary Figures 9B and D). All ME-ABEs improved editing activity for editable AT's compared to ABE7.10 (Supplementary Figure 21B). ABE7.10-HK and ABE7.10-HRHSK had normalized average editing efficiencies of 1.14-fold and 1.12-fold to ABE7.10, respectively. While ABE8.20 had a similar increase (1.14-fold compared to ABE7.10), ABE8e editing activity was on-par with that of ABE7.10 at editable AT motifs (1.01-fold increase).

At all other 3' sequence motifs, ME-ABEs showed improvement over ABE7.10 with varying effectiveness compared to the further evolved ABE8 variants (Supplementary Figure 21B). At AC motifs, this increase in average normalized editing activity over ABE7.10 ranged from 1.16-fold for ABE7.10-HRHSK to 1.24-fold for ABE7.10-HK, which was on par with ABE8.20 (a 1.23-fold increase relative to ABE7.10). ABE8e demonstrated an average 0.97-fold activity relative to ABE7.10 at AC motifs. At AA motifs, the ME-ABEs increased average normalized editing activity relative to ABE7.10 (ABE7.10-HK – 1.31-fold, ABE7.10-HRHSK – 1.28-fold), more so than either ABE8e (1.23-fold) or ABE8.20 (1.15-fold). Only at AG motifs did the ABE8 editors offer an advantage over the ME-ABEs (ABE8.20 had a 1.63-fold increase, and ABE8e had a 1.49-fold increase over ABE7.10), though all ME-ABEs still improved editing over ABE7.10 (ABE7.10-HK and ABE7.10-HRHSK increased activity 1.45-fold and 1.46-fold, respectively).

Supplementary Discussion 10: Analysis of cytosine editing at endogenous sites finds minimal editing by ME-ABEs

There were 109 unique C's to evaluate across 26 of the 27 sites (s7.3 lacks C's). We calculated average C•G to T•A editing frequencies across all C's within a given protospacer position and found these values to be within error of non-targeting controls for ABE7.10 and all ME-ABEs for all positions (Supplementary Figure 22). Average C•G to T•A editing frequencies for ABE8e and ABE8.20 were greater than that of the ME-ABEs, but still within error of non-targeting controls (averaging $0.19 \pm 0.19\%$ and $0.33 \pm 0.31\%$ at the highest edited position 5, respectively), due to global averaging. C•G to A•T and C•G to G•C editing values followed these trends as well (Supplementary Figure 22). We examined each C individually and found cytosine editing to be highest at TC motifs, consistent with the native motif preference of TadA. Editing at the H3.2 site, which contains three TC motifs at positions 3, 5, and 8, is shown in Supplementary Figure 23 and demonstrates statistically significant levels of C•G to T•A editing for ABE8e and ABE8.20 ($0.40 \pm 0.06\%$ and $0.73 \pm 0.10\%$ at position 5, respectively), while C•G to T•A editing by ABE7.10 and all ME-ABEs was at or below the limit of detection.

Supplementary Discussion 11: Description of orthogonal R-loop assay for detection of gRNA-independent off-target activity

This assay uses an orthogonal catalytically dead Cas9 variant from *S. aureus* (*Sa*-dCas9) combined with an independent gRNA (*Sa*-gRNA) to create an artificial loop of exposed ssDNA at a genomic location determined by the *Sa*-gRNA. This ssDNA serves as an off-target substrate for any TadA enzymes within the cell present as part of an *S. pyogenes*-constructed ABE (*Sp*-ABE). The *Sp*-ABE is delivered with an orthogonal gRNA (*Sp*-gRNA) that targets a different locus than the *Sa*-dCas9-exposed off-target site. Both the *Sa*-gRNA and *Sp*-gRNA loci are then sequenced, and a ratio of on-target “in cis” editing to off-target “in trans” editing can be calculated to evaluate the gRNA-independent off-target propensities of the base editor. We performed this assay using two *Sp*-ABE on-target sites (Ps.2 and s17.0). These sites were chosen based on independent target A sequence motifs (TAC vs AAA), as well as a wide dynamic range of editing efficiencies for ABE7.10, the ME-ABEs, and the ABE8 variants. We selected three *Sa*-dCas9 off-target sites that had been previously evaluated for gRNA-independent off-target editing by ABE7.10 using the orthogonal R-loop assay (Supplementary Figure 27A)⁴⁶. HEK293T cells were transfected with plasmids encoding one of the ABE variants shown in Figure 4D, an on-target *Sp*-gRNA, *Sa*-dCas9, and an off-target *Sa*-gRNA (all six on-target/off-target combinations were tested for each editor). Cells were allowed to incubate for 72 hours before the genomic DNA was harvested and both the in cis and in trans target loci were amplified and analyzed with NGS. A•T to G•C editing efficiencies averaged across the three off-target sites and the two on-target sites for each editor are plotted in Figure 4D.

Supplementary Discussion 12: Analysis of RNA off-target activity by ABE7.10-HRHSK and benchmark editors

Whole transcriptome RNA-seq analysis of ME-ABE ABE7.10-HRHSK and benchmark editors ABE7.10, ABE8e, and ABE8.20 was performed in duplicate (Supplemental Figure 30). The absolute number of significant A to I edits for all four editors were within 1.3-fold (replicate 1) or 2.4-fold (replicate 2) of each other for a given replicate. While trends in numbers of significant A to I edits varied between replicates, both average and median editing rates of sites that were significantly edited were within error for all four ABEs. Differences in transfection efficiencies, editor expression levels, and/or endogenous transcript expression levels potentially contributed to the variation in absolute numbers of significant A to I edits across the various biological replicates and editors. The average editing rate of significantly edited A's was $4.48 \pm 0.01\%$ for ABE7.10-HRHSK, while those of ABE7.10, ABE8e, and ABE8.20 were $4.44 \pm 0.22\%$, $4.39 \pm 0.18\%$, and $4.37 \pm 0.21\%$, respectively (Supplementary Figure 30C). Analysis of the sequence motifs of significantly edited A's showed all editors favored the TadA native sequence motif UA, with ABE7.10-HRHSK showing a slightly diminished preference for this consensus motif relative to ABE8e and ABE8.20 (Supplementary Figure 30D).

Supplementary Discussion 13: Multi-residue reversions that improved editing in the ABE7.10 background do not further improve editing activity when introduced to ABE8e and ABE8.20 backgrounds

Following our findings that introducing multiple residue reversions into the ABE7.10 background improved on-target editing activity without altering the editing window or increasing off-target activity, we elected to introduce these same reversions into the ABE8e and ABE8.20 backgrounds. ABE8e and ABE8.20 are both derived from ABE7.10 and were developed using independent evolution strategies, though both utilized bacterial hosts (Supplementary Figure 1)^{11,12}. ABE8e acquired an additional eight mutations on top of the 14 in ABE7.10, one of which was the reversion Y147D which we found increased activity significantly when introduced into the ABE7.10 background in bacterial assays (Figure 3B-E), but decreased editing activity in mammalian assays (Extended Data Figure 1B). ABE8.20 acquired an additional five mutations on top of the 14 in ABE7.10, including the reversion Y123H (present in all ME-ABEs) and Y147R. Given the similar behavior we observed when comparing the ME-ABEs to one another (Figure 4), we elected to introduce the minimum number of reversions (Y123H and N157K) as well as the maximum number of reversions (L36H, L51R, Y123H, C146S, and N157K) into both ABE8e and ABE8.20 backgrounds. Because ABE8.20 already contains the Y123H mutation, its “minimal reversion editor” is the ABE8.20-K variant. We also elected to try the single-residue reversion variant ABE8e-K (which contains only the N157K reversion mutation) to further test whether this single reversion alone is enough to alter editing patterns. All ABE8 reversion variants tested are listed in Extended Data Figure 6A.

All five ABE8 reversion editors were profiled for A•T to G•C editing activity against the previously described panel of 27 endogenous genomic sites (Supplementary Figure 18) alongside ABE7.10, ABE8e, and ABE8.20. Their absolute editing efficiencies were normalized against ABE7.10 editing at the A₅ position of the H2.0 site within a given experimental replicate. Averaging normalized editing efficiencies for A's at a given position across all sites allowed for determination of editor window using previously described criteria (Extended Data Figures 4, 6B and Supplementary Figure 20), leading to the finding that all reversion editors maintained the same editing window as their respective parent editor. Both single-residue reversion editors ABE8e-K and ABE8.20-K showed slight increases in average normalized editing efficiencies over their respective parent editors at positions 3, 4, and 8 (Supplementary Figure 20). ABE8e-K had on average a 1.13-fold, 1.12-fold, and 1.08-fold increase in editing over ABE8e at positions 3, 4, and 8, respectively. Similarly, ABE8.20-K had on average a 1.15-fold, 1.07-fold, and 1.14-fold increase over ABE8.20 at positions 3, 4, and 8,

respectively. At most other positions within the editing window, the average normalized editing efficiencies of the ABE8 multi-residue reversion editors were slightly lower than that of their parent editors, with the maximally modified editors seeing the greatest loss in activity. This ranged from a 1.04-fold decrease at position 4 to a 1.31-fold decrease at position 10 for ABE8e-HRHSK compared to ABE8e, and ranged from 1.01-fold decreases at positions 3 and 4 to a 1.13-fold decrease at position 5 for ABE8.20-HRSK compared to ABE8.20.

We additionally analyzed all ABE8 reversion variants for sequence motif preferences, focusing only on a given editor's editable A's (Extended Data Figure 6C-D and Supplementary Figure 31). None of the introduced reversions altered the parent editors' relative 5' or 3' sequence motif preferences. In a few cases, we did observe small improvements in editing over their parental counterparts (for example, ABE8e-K and ABE8.20-K improved editing over their respective parent editors at **CA** motifs by 1.03- and 1.04-fold, respectively). However, we noted a general decrease in editing activity by the ABE8 reversion variants compared to their respective parental ABE8 editors across most sequence motifs, with the maximally reverted variants decreasing activity more than the minimal-reversion editors (Supplementary Figure 31C-F).

In general, these data indicate that introducing these five reversions into the ABE8 background had a detrimental effect on editing, in contrast to their impact when introduced into the ABE7.10 background. To assess if these decreases in editing were related to intracellular ABE expression levels, we repeated our stability of expression studies using mCherry-P2A-ABE-EGFP constructs (Extended Data Figure 2A) on the ABE7.10-HK and ABE7.10-HRHSK ME-ABEs, as well as all ABE8 variants (Supplementary Figure 32). We found both ME-ABEs to have median EGFP/mCherry fluorescence intensity ratios within error of their ABE7.10 parental counterpart. Western blot analysis of ABE7.10-HRHSK supported the conclusion that protein expression levels for this ME-ABEs was on par with ABE7.10, and greater than that of ABE8e (Extended Data Figure 2C). Both ABE8e and ABE8.20 had significantly reduced expression/stability levels compared to ABE7.10 as assessed with the fluorescent reporter. The median EGFP/mCherry fluorescence intensity ratio for ABE8e and ABE8.20 were 0.99 ± 0.03 and 1.01 ± 0.03 , compared to 1.13 ± 0.03 for ABE7.10 (Supplementary Figure 32A). Finally, both ABE8e-HRHSK and ABE8.20-HRSK demonstrated 1.13 ± 0.04 -fold and 1.08 ± 0.05 -fold further decreases in expression/stability levels compared to their respective parental editors. These data collectively suggest that these reversions are destabilizing to the protein in either ABE8 background, but not in the context of the ABE7.10 background. These results are not unexpected and reinforce the importance of

epistatic interactions when analyzing extensively mutated systems. The additional mutations accumulated during evolution of the ABE8 editors are likely negating or counteracting the favorable interactions made by the five reversions tested, resulting in destabilizing interactions and reduced activity when new mutations and reversions are combined.

Our five ABE8e and ABE8.20 reversion variants were further evaluated for gRNA-independent DNA off-target activity using the orthogonal R-loop assay as before (Extended Data Figure 6E and Supplementary Figures 33). It has previously been shown that ABE8e editors exhibit higher off-target editing when compared to ABE8.20¹⁴, a finding we replicated (Extended Data Figure 6E, and Supplementary Figures 34A and 35B-D). However, unlike the ME-ABEs, where all four tested editors behaved the same, ABE8-derived reversion editors exhibited notable differences. ABE8.20-K and ABE8e-HK had average in trans editing activities within error of their respective parental editors (3.52 ± 0.32 for ABE8.20-K vs 3.72 ± 0.75 for ABE8.20, and 8.20 ± 1.57 for ABE8e-HK vs 8.54 ± 1.51 for ABE8e), while the maximally modified ABE8.20-HRSK and ABE8e-HRHSK variants had reduced in trans editing activities compared to their respective parental editors (1.91 ± 0.07 for ABE8.20-HRSK and 5.56 ± 1.08 for ABE8e-HRHSK). The reduced off-target activity for the maximally reverted variants may be a function of overall reduced deamination ability. While the levels of in cis editing observed for ABE8-derived variants were similar to their given parent editor's as measured by the two sites chosen for this assay (Supplementary Figure 35A), our more extensive study of on-target editing across the 27 endogenous sites suggested the maximally reverted editors generally decreased on-target editing activity. Overall, our identification of the five reversions with desirable on- and off-target properties was specific to the ABE7.10 background they were initially evaluated against; introducing them into the ABE8e and ABE8.20 backgrounds offered a slight decrease in gRNA-independent DNA off-target editing activity, but did not improve on-target activity and had no impact on editing windows.

Supplementary Discussion 14: ME-ABEs function in HeLa cells

To further characterize ME-ABEs, we profiled their endogenous editing activity in HeLa cells. ABE7.10-HRHSK was encoded on a plasmid containing the fluorescent protein marker mCherry and transfected into HeLa cells alongside one of six gRNA plasmids targeting a variety of endogenous sites previously investigated in HEK293Ts. FACS was used to separate successfully transfected mCherry⁺ HeLa cells, their genomic DNA was harvested, and target sites were amplified for analysis via NGS. Editing patterns in HeLa matched those previously observed in HEK293Ts (Figure 6A). ABE7.10-HRHSK improved editing in HeLa cells over ABE7.10 at five of the six sites, ranging from a 1.17-fold increase (H3.4) to a 3.54-fold increase (s7.2). Furthermore, ABE7.10-HRHSK editing in HeLa cells maintained a narrower editing window than ABE8e and ABE8.20 (Figure 6B and Extended Data Figure 7). For example, at the Hi.4 A₅ site, $89.8 \pm 1.4\%$ of edited reads contained a single A•T to G•C edit when edited with ABE7.10-HRHSK. This was in contrast to ABE8e and ABE8.20 which had single A precision edit rates of $7.6 \pm 0.6\%$ and $9.9 \pm 2.1\%$, respectively, and instead had a majority of reads which contained multiple edited A's. These findings demonstrate that the favorable editing properties of ME-ABEs are not cell-type dependent.

Supplementary Discussion 15: Site selection and data generation/normalization for testing ME-ABEs ability to model SNVs of clinical interest

ME-ABEs were challenged to model six SNVs of clinical interest associated with the genes *SCN5A*, and *ERCC2*. Point mutations in the *SCN5A* gene, which encodes for a sodium channel, are associated with arrhythmias including Brugada syndrome and long QT syndrome^{43–45}. Likewise, mutations in the *ERCC2* gene, which encodes for a component of the transcription/repair complex factor TFIIH, are associated with extreme UV sensitivity, leading to trichothiodystrophy and increased cancer risk associated with xeroderma pigmentosum^{46,47}.

For each SNV of interest, we designed a protospacer that placed the target A in position 5, 6, or 7, taking into account the presence of PAM motifs (Figure 6C). Several targets required us to generate ME-ABEs with the “nearly PAMless” SpRY Cas architecture, allowing us to simultaneously test ME-ABE compatibility with an alternate Cas variant^{48,49}. Plasmids containing an appropriate base editor were transfected alongside the relevant gRNA plasmid into HEK293T cells, cells were incubated for 72 hours, genomic DNA was extracted, and target amplicons sequenced to evaluate editing efficiencies. Data were analyzed for both absolute editing efficiencies (percent of sequencing reads with only the target A•T converted to G•C) as well as precision (fraction of all edited reads with only the target A•T, and no bystanders, converted to G•C).

Supplementary Note

Supplementary Note 1 Processing of RNA-Seq Data

RNA-Seq data was processed as outlined in the Methods section. The following scripts indicate usage of REDITools3 and custom python script to identify significant RNA off-target edits by position and editing rate.

```
#!/usr/bin/env python3

"""
REDITOOLS PROCESSING SCRIPT
A→G ONLY
Processes BOTH control and experimental files.
Matches each replicate with its matching control replicate.
"""

import os
import pandas as pd
import numpy as np
from scipy import stats

# ----- CONFIG -----
INPUT_DIR = "/home/user"      # directory with all *_repX.txt files
OUTPUT_DIR = "/home/user"     # where outputs will be saved

SNP_FILE = "/home/user"      # path to SNP database

MIN_COVERAGE = 10
MIN_EDIT_RATE = 0.01
REF_MATCH_THRESHOLD = 0.99
ALPHA = 0.05
CHUNK_SIZE = 40_000_000
# -----

# =====
# HELPERS
# =====

def read_reditools(fp):
    return pd.read_csv(fp, sep="\t", comment="#", low_memory=False)

def split_basecounts(df):
    bc = (df["BaseCount[A,C,G,T]"]
          .astype(str)
          .str.replace(r'^\[|\]$ ', '', regex=True)
          .str.replace(" ", ""))

    parts = bc.str.split(",", expand=True).astype(int)
    parts.columns = ["A", "C", "G", "T"]
    df[["A", "C", "G", "T"]] = parts
    return df

# =====
# CONTROL PROCESSING
# =====
```

```

def process_control(path):
    """Return a control dataframe containing only A-reference sites with ≥99%
    reference match."""
    df = read_reditools(path)

    # Keep chr*
    df = df[df["Region"].astype(str).str.startswith("chr")]

    # Coverage filter
    df["Coverage-q30"] = pd.to_numeric(df["Coverage-q30"],
    errors="coerce").fillna(0).astype(int)
    df = df[df["Coverage-q30"] >= MIN_COVERAGE]

    # A reference only
    df = df[df["Reference"] == "A"]

    # No strand filtering
    df = split_basecounts(df)

    # Reference match filter (A / Coverage)
    df["ref_match"] = df["A"] / df["Coverage-q30"].replace(0, np.nan)
    df = df[df["ref_match"] >= REF_MATCH_THRESHOLD]

    return df

# =====
# EXPERIMENTAL PROCESSING
# =====

def process_experimental(path):
    df = read_reditools(path)

    # Keep chr*
    df = df[df["Region"].astype(str).str.startswith("chr")]

    # Coverage filter
    df["Coverage-q30"] = pd.to_numeric(df["Coverage-q30"],
    errors="coerce").fillna(0).astype(int)
    df = df[df["Coverage-q30"] >= MIN_COVERAGE]

    # A reference only
    df = df[df["Reference"] == "A"]

    df = split_basecounts(df)

    # Edit rate
    df["edit_rate"] = df["G"] / df["Coverage-q30"].replace(0, np.nan)
    df = df[df["edit_rate"] >= MIN_EDIT_RATE]

    return df

# =====
# MERGE + FISHER + SNP
# =====

```

```

def merge_with_control(exp, ctrl):
    ctrl = ctrl.rename(columns={
        "A": "A_ctrl",
        "G": "G_ctrl",
        "Coverage-q30": "cov_ctrl"
    })
    exp = exp.rename(columns={
        "A": "A_exp",
        "G": "G_exp",
        "Coverage-q30": "cov_exp"
    })

    merged = exp.merge(
        ctrl[["Region", "Position", "A_ctrl", "G_ctrl", "cov_ctrl"]],
        on=["Region", "Position"],
        how="inner"
    )

    merged["rate_exp"] = merged["G_exp"] / merged["cov_exp"].replace(0, np.nan)
    merged["rate_ctrl"] = merged["G_ctrl"] / merged["cov_ctrl"].replace(0,
np.nan)

    return merged[merged["rate_exp"] >= merged["rate_ctrl"]]

def fisher_row(row):
    table = [
        [row["G_exp"], row["A_exp"]],
        [row["G_ctrl"], row["A_ctrl"]]
    ]
    return stats.fisher_exact(table, alternative="greater")[1]

def build_output(df, sample, rep):
    df["pval"] = df.apply(fisher_row, axis=1)
    df = df[df["pval"] < ALPHA]

    out = pd.DataFrame({
        "Region": df["Region"],
        "Position": df["Position"],
        "Sample": sample,
        "Replicate": rep,
        "P-value": df["pval"],
        "Sample_edit_ct": df["G_exp"],
        "Sample_ref_ct": df["A_exp"],
        "Control_edit_ct": df["G_ctrl"],
        "Control_ref_ct": df["A_ctrl"],
        "Experimental_coverage": df["cov_exp"],
        "Control_coverage": df["cov_ctrl"],
        "Experimental_edit_rate": df["rate_exp"],
        "Control_edit_rate": df["rate_ctrl"]
    })

    return out

def remove_snps(df):

```

```

df = df.rename(columns={"Region": "Chrom"})
for chunk in pd.read_csv(SNP_FILE, sep="\t", usecols=["Chrom", "End"],
chunksize=CHUNK_SIZE):
    chunk = chunk.rename(columns={"End": "Position"})
    merged = df.merge(chunk, on=["Chrom", "Position"], how="left",
indicator=True)
    df = merged[merged["_merge"] == "left_only"].drop(columns="_merge")
    if df.empty:
        break
return df

```

```

# =====
# MAIN PIPELINE
# =====

```

```
def main():
```

```

    # detect control files
    controls = [f for f in os.listdir(INPUT_DIR)
                 if f.startswith("mCherry_rep") and f.endswith(".txt")]

    for ctrl_file in controls:
        rep = ctrl_file.split("_rep")[1].split(".")[0]
        ctrl_path = os.path.join(INPUT_DIR, ctrl_file)

        print(f"\n=== Processing control rep {rep} ===")
        ctrl_df = process_control(ctrl_path)

        # find experimental files for same rep
        exp_files = [f for f in os.listdir(INPUT_DIR)
                     if f.endswith(f"_rep{rep}.txt") and not
f.startswith("mCherry")]

        for exp_file in exp_files:
            sample = exp_file.split("_rep")[0]
            exp_path = os.path.join(INPUT_DIR, exp_file)

            print(f"  Sample {sample}, rep{rep}")

            exp_df = process_experimental(exp_path)
            merged = merge_with_control(exp_df, ctrl_df)

            sig = build_output(merged, sample, rep)
            sig = remove_snps(sig)

            out_path = os.path.join(OUTPUT_DIR,
f"{sample}_rep{rep}_AtoG_significant.csv")
            sig.to_csv(out_path, index=False)

            print(f"    → {len(sig)} significant A→G sites saved")

```

```

if __name__ == "__main__":
    main()

```

