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Original Article

CABE-RY: A PAM-flexible dual-mutation base editor for reliable modeling of multi-nucleotide variants

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Multi-nucleotide variants (MNVs) represent an important type of genetic variation and have biological and clinical significance. To simulate MNVs, we designed four dual-mutation base editors combining hA3A(Y130F), TadA8e(V106W), and protospacer adjacent motif (PAM)-flexible SpRY and selected cytosine and adenine base editor-SpRY (CABE-RY), which had the best editing performance, for further study. Characterization and comparison showed that CABE-RY had a smaller DNA editing window and lower RNA off-target edits than the corresponding single base editors. Thus, we have established a versatile tool to efficiently simulate MNVs over the genome, which could be very useful for functional studies on MNVs in humans.

INTRODUCTION

Sequencing technologies have rapidly advanced our understanding of human genetic variants. Many disease-associated variants have been identified in patients. Single-nucleotide variants (SNVs), which represent one type of variant, are usually included in genetic evaluations with phenotypic information.[1] Other kinds of genetic variants may also have influences on diseases. Therefore, precisely elucidating how SNVs contribute to causal relationships between genotypes and phenotypes remains a major challenge.

Multi-nucleotide variants (MNVs) represent another variant type that is considered to be related to disease. For example, Kaplanis et al.[2] found significant enrichment of MNVs in genes associated with diagnosing developmental disorders (DDD), showing that MNVs are on average more harmful than SNVs. Usually, there are two or more nearby variants on the same haplotype in an individual.[2,3] When nearby variants are within the same codon, the amino acid changes are different than if the separate SNVs are annotated independently. Due to neglect of MNVs, most existing variant callers mistake MNVs for SNVs with incorrect amino acid change predictions,[4,5] which probably hampers scientific research and clinical practice.

Recently, the widespread application of CRISPR-based gene-editing technologies has revolutionized genetics and disease research. CRISPR-based base editors, which are derived by fusing deoxynucleoside deaminase to the nickase Cas9 and are recruited by a guide RNA to their target DNA region, efficiently make C-to-T or A-to-G nucleotide changes, improving simulation of SNVs to enable exploration of the relationship between genotype and phenotype. To further expand the editing capabilities of base editors, dual-mutation base editors, which combine cytosine base editors (CBEs) and adenine base editors (ABEs), have been successfully developed.[6–10] Theoretically, dual-mutation base editors make simultaneous A/C conversions, the most frequent adjacent dinucleotide MNVs,[2] more conveniently than single-mutation base editors. However, existing dual-mutation base editors have narrow application ranges due to protospacer adjacent motif (PAM) restriction. Although prime editors (PEs) can theoretically simulate most variants, including MNVs,[11] the efficiency of PEs is low, especially *in vivo*.[12] Therefore, better dual-mutation base editors that can simulate MNVs need to be developed.

SpRY is a mutant of SpCas9 that is highly PAM-compatible.[13] The near-PAMless SpCas9 shows powerful advantages in PAM recognition. However, although it exhibits robust activity on sites with NRN PAMs (where R is A or G), it exhibits low activity on those with NYN PAMs (where Y is C or T). Considering that one of the limitations of using existing dual-mutation base editors is that they require target recognition of the NGG motif in the genome, in

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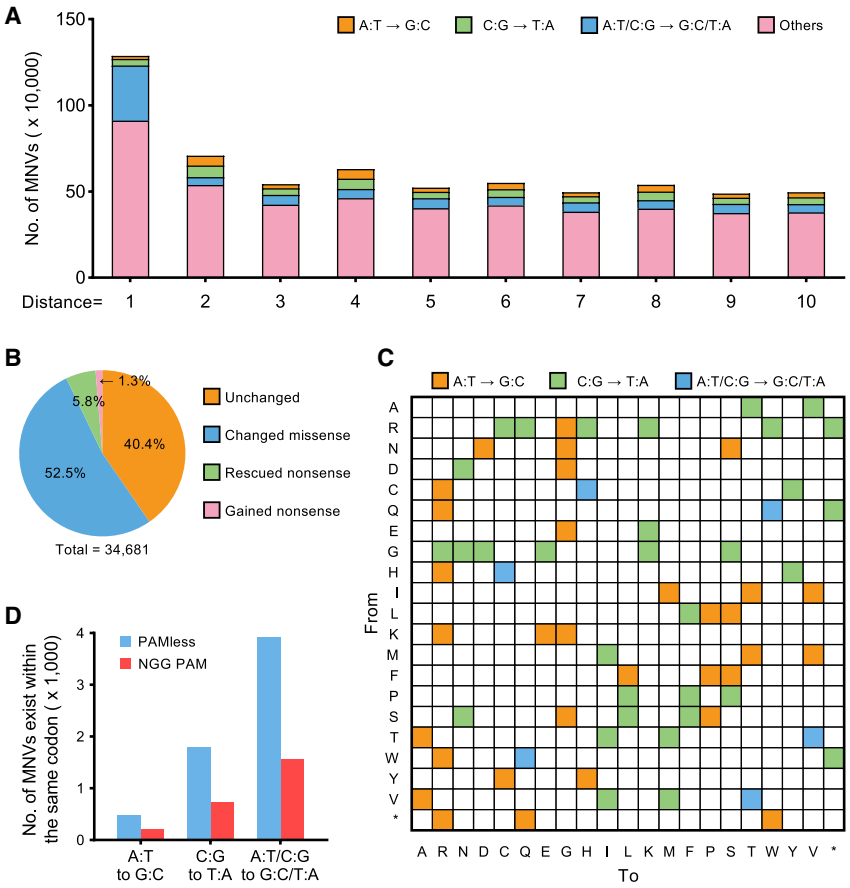


Figure 1. Necessity of a dual-mutation base editor with minimal PAM restriction for simulating MNVs

(A) Substitution pattern of MNVs from gnomAD. The x coordinate is the distance between two SNVs. (B) Differences in functional impacts on the protein between MNVs and individual SNPs. (C) Heatmap showing the amino acid changes that can be simulated by the dual-mutation base editor. (D) The PAM-flexible dual-mutation base editor has a broadened scope to target MNVs within the same codon. A broadened editing window (positions 3–10) was used to calculate the targeting scope.

this study, we used SpRY to construct a dual-mutation base editor with minimal PAM restriction. To this end, we fused hA3A(Y130F), which has efficient C-to-T editing, including in G/C rich regions,[14] and TadA8e(V106W), which has the highest efficiency of A-to-G editing among editors developed to date,[15] to the N terminus of SpRY to produce a dual-mutation base editor named cytosine and adenine base editor-SpRY (CABE-RY). With this tool, we effectively performed simultaneous A/C conversion and successfully simulated MNVs.

RESULTS

Necessity of a dual-mutation base editor with minimal PAM restriction for simulating MNVs

An increasing number of MNVs have been discovered and deposited in the Genome Aggregation Database (gnomAD).[16] To better understand the biology of MNVs, we analyzed the MNV mutation type in gnomAD. The results showed that there was more simultaneous A/C conversion than AA or CC conversion, particularly in two adjacent mutations (Figure 1A). When two nearby mutations are located in the same codon, they may have a different functional impact on the protein than the individual mutations (Figure S1A). Indeed, among a total of 31,575 MNVs, 52.5% of them resulted in different missense mutations from those caused by the individual mutations, while 5.8% of MNVs rescued nonsense mutations caused by the individual mutations, and 1.3% of MNVs produced nonsense mutations that were not caused by the individual mutations (Figure 1B). This difference may affect understanding of functional research or clinical diagnosis of genetic diseases. The dual-mutation base editor allows us to better understand the functional impacts of MNVs by simulating these MNVs.

Ideally, a dual-mutation base editor should be able to effectively simulate 70 amino acid substitutions (Figure 1C), including six that cannot be achieved by existing CBEs and ABEs (Figure S1B). However, the previously reported dual-mutation base editors can only target sites with NGG PAMs, which greatly limits their application. Therefore, we speculated that the near-PAMless engineered CRISPR-Cas9 variant SpRY could be used to construct a PAM-flexible dual-mutation base editor without strict PAM restriction. We analyzed the editing scopes between a conventional NGG PAM editor and the PAM-flexible editor. Considering that dual-mutation editors usually have a broader editing window than conventional editors,[9] we calculated the editable MNVs with an editing window spanning positions 3–10 (versus the common window spanning positions 4–8), which presumably increased the number of targetable MNVs. As expected, the PAM-flexible dual-mutation base editor was found to target ~2.7 times the number of MNVs and specific codons than the conventional NGG PAM editor (Figure 1D; Figure S1C), suggesting that the PAM-flexible dual-mutation base editor is a potential tool with an expansive editing scope for modeling MNVs.

Establishment of a PAM-flexible dual-mutation base editor, CABE-RY

To create a PAM-flexible dual-mutation base editor, we combined SpRY with cytosine deaminase and adenine deaminase from the previously described hA3A(Y130F), which has efficient C-to-T editing, including in G/C rich regions,[14] and TadA8e(V106W), which has the highest efficiency of A-to-G editing among editors developed thus far.[15] Four constructs were generated based on

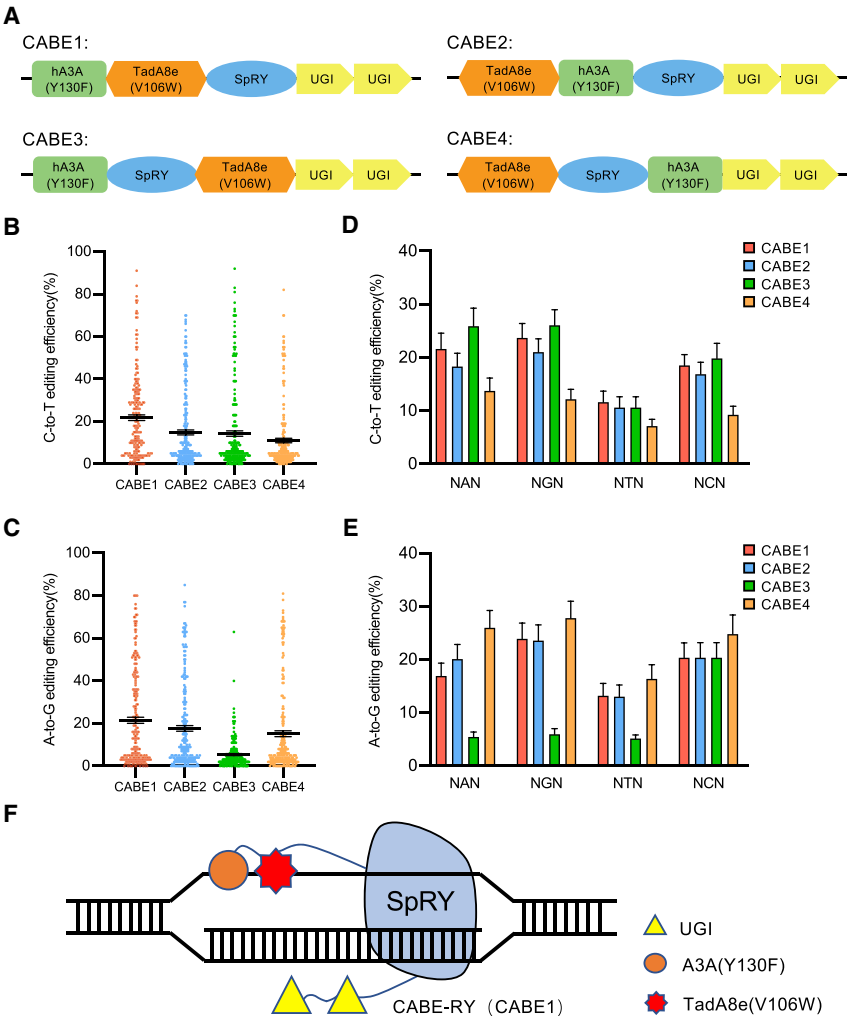


Figure 2. Establishment of a PAM-flexible dual-mutation base editor, CABE-RY, in HEK293T cells

(A) Schematic diagram for construction of four dual-mutation base editors, CABE-1, CABE-2, CABE-3, and CABE-4. (B and C) Comparison of the C-to-T (B) and A-to-G (C) editing efficiencies of the four CABE editors. The horizontal black lines represent the mean editing efficiencies at 48 endogenous sites for each CABE editor. Each dot represents the editing efficiency of each edited base (n = 3 independent replicates). The error bars represent the standard error of the mean (SEM) values. (D and E) Mean C-to-T (D) and A-to-G (E) editing efficiency plots for the four CABE editors with different PAMs. The data are presented for each edited base at 48 endogenous sites (n = 3 independent replicates). The error bars represent the SEM values. (F) Schematic overview of CABE-RY. hA3A(Y130F)-TadA8e(V106W) is linked to the N terminus of SpRY.

the locations of SpRY and the two deaminases (Figure 2A; Sequence S1).

Then, we compared the on-target editing efficiency of the four constructs across 48 endogenous sites. Each single-guide RNA (sgRNA), together with four individual constructs, was cotransfected into HEK293T cells. The editing efficiency was analyzed by Sanger sequencing, and the results showed that all 4 editors induced simultaneous A/C conversions. The mean efficiency of CABE-1 was higher than that of CABE-2 (21.5% versus 17.71% for A-to-G; 21.8% versus 14.88% for C-to-T). The mean A-to-G editing efficiency of CABE-3 was far lower than that of CABE-1 (5.4% versus 21.5%), while the mean C-to-T editing efficiency of CABE-4 was far lower than that of CABE-1 (11.1% versus 21.8%) (Figures 2B and 2C). We further analyzed the C-to-T and A-to-G editing activities of CABE-1 at sites of different PAMs (Figures 2D and 2E). Similarly, the CABE-1 had the best performance. Therefore, we chose CABE-1, in which hA3A(Y130F)-TadA8e(V106W) was fused to the N terminus of SpRY, for further study and named it CABE-RY (Figure 2F).

Determination of the on-target DNA editing efficiency of CABE-RY

Next, amplicon deep sequencing was used for the target sites to thoroughly characterize CABE-RY performance. Related simultaneous A/C conversions of some sites are shown in Figure 3A. The C-to-T editing window (positions 4–14) of CABE-RY was narrower than that of hA3A(Y130F)-RY (positions 1–14) (Figure S2A). Similarly, the A-to-G editing window (positions 3–9) was slightly smaller than that of ABE8e(V106W)-RY (positions 3–11) (Figure 3B; Figure S2B). Analysis of the 48 targets containing both As and Cs in the editing window revealed that the C-to-T editing efficiency was 46.9%, while the A-to-G editing efficiency was 48.3%. Then, we examined the C-to-T and A-to-G editing activities of CABE-RY in different PAMs. For NYN PAMs, we observed that the mean activities of both C-to-T and A-to-G editing were lower than those for NRN PAMs (36.7% versus 58.0% for C-to-T; 38.8% versus 59.8% for A-to-G) (Figure 3C; Figures S3A–S3C). This is consistent with the characteristics of SpRY.[13] In addition, CABE-RY had a low insertion or deletion (indel) frequency of 1.60% (Figure S3D).

We further compared the editing efficiency between CABE-RY and coexpressed ABE8e(V106W)-RY and hA3A(Y130F)-RY in HEK293T cells (Figures S4A–S4C) and found that CABE-RY showed a slightly higher editing efficiency than the two coexpressed base editors (54.7% versus 47.8%). Additionally, robust base editing of CABE-RY was also observed in murine N2a cells (29.4%) (Figure S4D), suggesting that CABE-RY works universally for different species.

Determination of the off-target RNA editing efficiency of CABE-RY

One of the concerns regarding base editors is RNA off-target mutagenesis.[17] To detect transcriptome-wide RNA off-target effects,

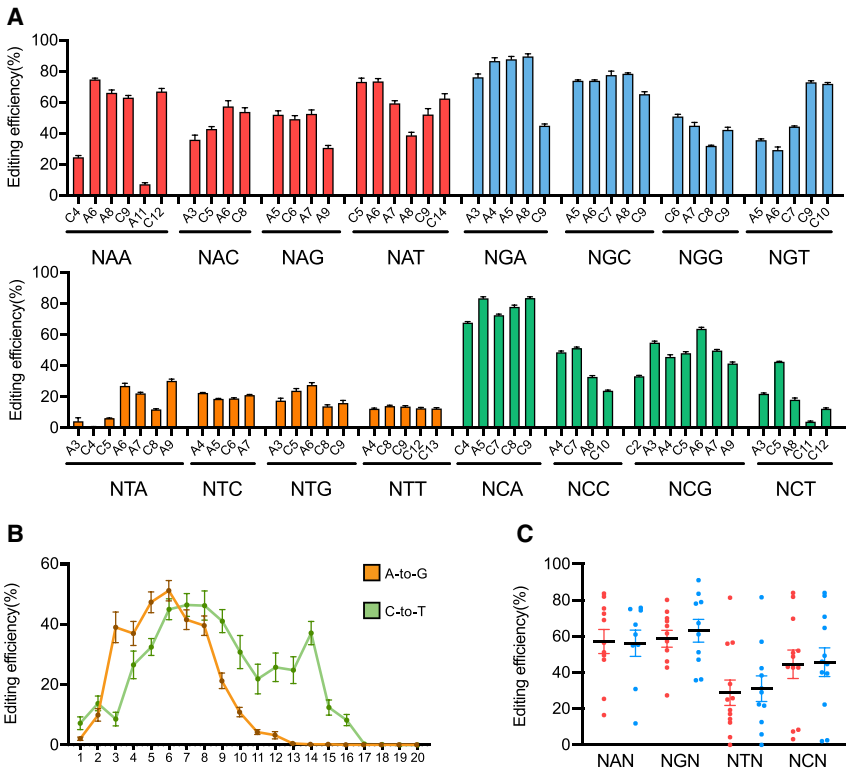


Figure 3. Determination of the on-target DNA editing efficiency of CABE-RY in HEK293T cells

(A) Relative C-to-G and A-to-T base editing efficiencies of CABE-RY with different PAMs at 16 human genomic target DNA sites (n = 3 independent replicates). The error bars represent the SEM values. (B) Sequencing analysis of the base editing window of CABE-RY across NNN PAMs in HEK293T cells. The error bars represent the SEM values. The data are presented for each edited base separately at 48 endogenous sites (n = 3 independent replicates). (C) Aggregate distribution of C-to-T (red) and A-to-G (blue) edits made across the editing window with CABE-RY. The error bars represent the SEM values. The data are presented for each edited base in the editing window at 48 endogenous sites.

we performed RNA sequencing (RNA-seq) on HEK293T cells coexpressing CABE-RY, ABE8e(V106W)-RY, hA3A(Y130F)-RY, and GFP (negative control) with a sgRNA targeting NAT SITE3 (Table S1), which had high DNA on-target editing efficiency (Figure 4A). As expected, ABE8e(V106W)-RY introduced thousands of RNA edits in the transcriptome, while hA3A(Y130F)-RY had a slight effect (Figure 4B; Figure S5A). Interestingly, the number of RNA off-target edits of CABE-RY was one-third that of ABE8e(V106W)-RY (Figure 4B; Figure S5A). In addition, these editors did not cause significant differences in gene expression at the whole-transcriptome level (Figures S5B–S5D).

Then, we further analyzed the specific types of RNA off-target edits and found that the main off-target edit type for CABE-RY and ABE8e(V106W)-RY was A-to-I RNA editing, while that of hA3A(Y130F)-RY was C-to-U RNA editing (Figures 4C and 4D). Interestingly, CABE-RY showed lower A-to-I RNA editing than the other editors (nearly 3,000) and undetectable C-to-U RNA editing (comparable to the editing of the GFP control) (Figures 4C and 4D). A possible explanation of the performance may be the embedding of the deaminase domains in nCas9 for minimization of off-target effects of base editors;[18] in addition, the combination of hA3A(Y130F) and TadA8e(V106W) may create possible steric hindrance to reduce off-target edits.

Successful simulation of MNVs by CABE-RY in HEK293T cells

Given these findings, we tested ten sites in gnomAD and directly compared the editing efficiency of CABE-RY with that of ABE8e(V106W)-RY or hA3A(Y130F)-RY in HEK293T cells. Compared with those of the single base editors, the editing efficiencies for C-to-T and A-to-G editing of CABE-RY were slightly lower (25.8% versus 31.4% for C-to-T, 38.1% versus 43.9% for A-to-G) (Figure 5A). However, CABE-RY created more mutation types and amino acid types than the single base editors (Figures S6 and S7) and showed high simultaneous A/C conversion efficiency (31.8%) in terms of simulating MNVs, which is unfeasible for a single base editor. It is worth noting that CABE-RY induced bystander edits like those induced by ABE and CBE. To better understand how bystander edits confound MNV simulation using CABE-RY, we first analyzed the potential bystanders flanking two adjacent MNVs with gnomAD (Figure S8) and found that 18.9% of MNVs may not be affected by bystanders, while 14.4% of MNVs may be affected by bystanders. Thus, CABE-RY is still a potentially useful tool for modeling MNVs.

PEs can theoretically simulate most variants, including MNVs, with no bystander edits. Therefore, we compared the simulation efficiency between PE3 and CABE-RY. A pegRNA with a 13 nt prime binding site (PBS) length and a 12 nt reverse transcriptase (RT) template length was designed as suggested.[19] As expected, both CABE-RY and PE3 successfully induced MNVs, but CABE-RY exhibited significantly higher simulation efficiency than PE3 (31.8% versus 2.8%; Figure 5B). Notably, perfect simulation of MNVs without bystander edits was successfully achieved in 8.7% of cases on average (Figure 5C). These results demonstrate that CABE-RY is a good tool for simulating MNVs.

To further explore the accuracy of MNV simulation by CABE-RY, we analyzed the proportion of perfectly simulated MNVs. The results showed that the influence of bystander edits was site dependent. Some sites retained a relatively high percentage of perfect MNV simulations (35.47% for the WDR90 site, 13.47% for the SYNM), while some sites had significantly reduced proportions of perfect MNV

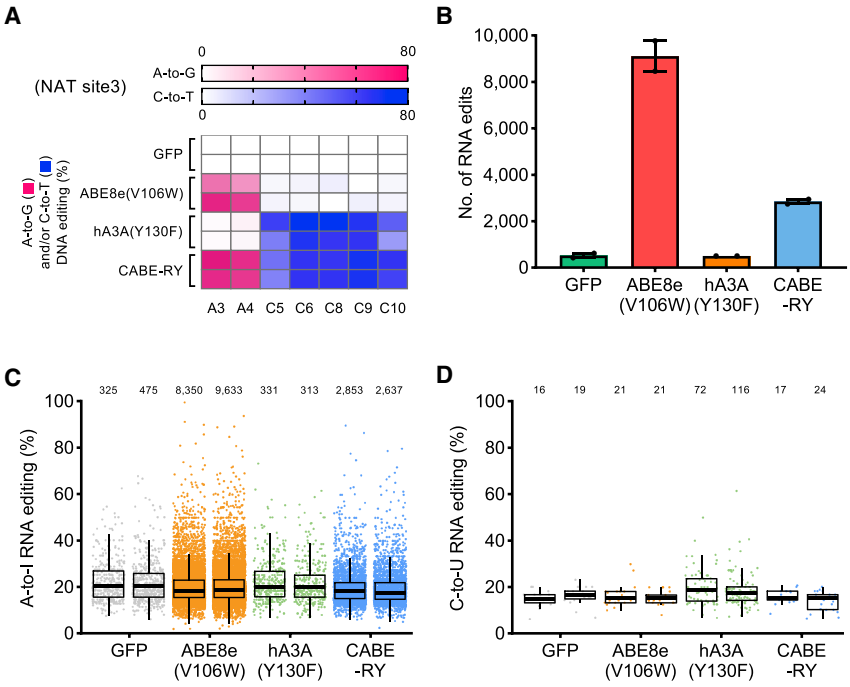


Figure 4. Determination of the off-target RNA editing efficiency of CABE-RY

(A) Heatmaps showing the on-target editing frequencies of GFP (control), ABE8e(V106W), hA3A(Y130F), and CABE-RY in NAT SITE3. A-to-G edits are indicated in pink, and C-to-T edits are indicated in blue. (B) Total number of RNA off-target edits detected in RNA-seq experiments for GFP (control), ABE8e(V106W), hA3A(Y130F), or CABE-RY. The error bars represent the SEM values. (C and D) Jitter plots showing the efficiencies of A-to-I (C) or C-to-U (D) RNA off-target editing. The data shown are from two independent replicates. The number of edits is represented on the top.

simulations (Figure S6). Taken together, the results indicate that CABE-RY can efficiently simulate MNVs and is a versatile tool for dissection of the functions of MNVs.

DISCUSSION

The data in gnomAD reveal that simultaneous A/C conversion has a higher mutation frequency than AA or CC conversion, particularly in two adjacent mutations for MNVs, and this mutation type can be simulated by reported dual-mutation base editors.[6–10] However, the published dual-mutation base editors are restricted to sites with NGG PAMs, which greatly limits their application. Here, we constructed a dual-mutation base editor, CABE-RY, by combining hA3A(Y130F), TadA8e(V106W), and SpRY. This editor was found to effectively induce simultaneous A/C conversion over the whole genome with a sgRNA after successful verification. Characterized by the presence of SpRY, CABE-RY edits many more sites than existing dual-mutation editors with minimal PAM restriction.

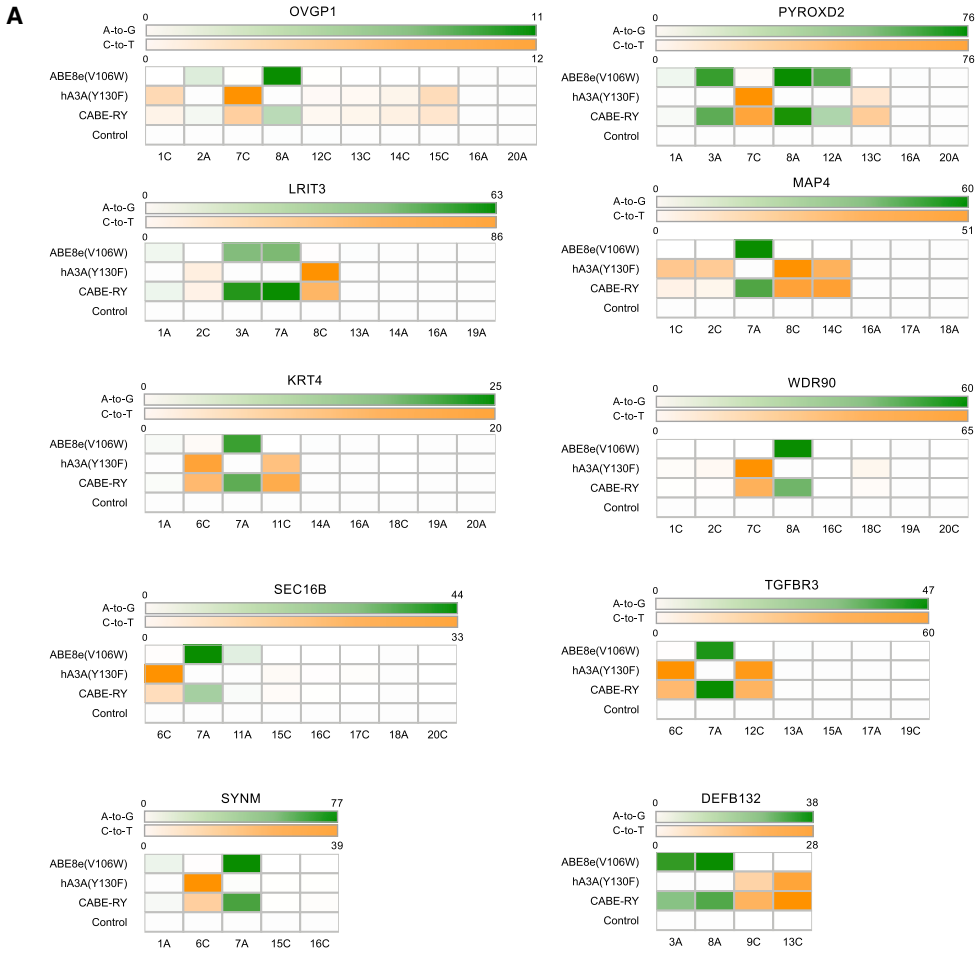
We further characterized the editing of CABE-RY and revealed that CABE-RY efficiently edited DNA (46.9% efficiency for C-to-T edits and 48.3% efficiency for A-to-G edits). Interestingly, CABE-RY induced less RNA off-target mutagenesis, including less A-to-I RNA editing and undetectable C-to-U RNA editing, than the other editors tested. To illustrate that CABE-RY is an appropriate tool to simulate MNVs, we compared the simulation efficiency between PE3 and CABE-RY. CABE-RY exhibited higher simulation efficiency than PE3 (31.8% versus 2.8%), even when we focused only on perfect MNV (8.7% versus 2.0%). Here, we have confirmed that CABE-RY can efficiently generate simultaneous A/C conversions. Nevertheless, several concerns remain. For example, self-targeting of sgRNA is unavoidable, and it may lead to wrong sgRNA generation, resulting in reduction of editing efficiency and even unpredictable targeting. Meanwhile, CABE-RY exhibits robust activities with NRN PAMs but lower activities with NYN PAMs, which limits the use of CABE-RY over the genome. Considering other SpCas9 variants may have stronger affinity with NYN PAMs, such as FnCas9RHA on YG PAMs,[20] M44 on TTTN PAMs,[21] AsCas12a-K949A on TTTV PAMs, and [22] Nme2Cas9 on NNNNCC PAMs,[23] we can choose these SpCas9 variants instead of SpRY, which may improve editing efficiency at specific sites. Nevertheless, to create a fully PAMless Cas9 in the future is desired. In fact, ortholog mining and protein engineering have produced many PAM variants. The combination of these strategies may further relieve PAM restrictions and create a truly PAMless Cas9.[24]

In addition, we also compared CABE-RY editing frequencies with those of single base editors. Our results demonstrated that compared with a single base editor, CABE-RY has several advantages. First, CABE-RY can efficiently generate simultaneous A/C conversions to simulate MNVs. Second, CABE-RY can generate more mutation types than single base editors; thus, it can effectively simulate 70 amino acid substitutions, including six that cannot be achieved by existing single base editors, which is helpful for understanding disease-related mutations. For example, the unique p.Cys91His mutation has been discovered in Niemann-Pick disease and p.Gln326Trp in intervertebral disc disease.[25,26] In addition, it's reported that simultaneous A/C conversion induced higher hemoglobin subunit gamma (HBG) reactivation.[9] In conclusion, CABE-RY is a versatile tool for the field of gene editing and is especially useful for simulating MNVs.

MATERIALS AND METHODS

Cell culture

HEK293T cells were purchased from the American Type Culture Collection (ATCC). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. We maintained the cell lines at 37°C in a 5% CO_2 cell culture incubator.



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Plasmid construction

To construct base editor expression plasmids, human codon-optimized DNA sequences of hA3A(Y130F), TadA8e(V106W), and UGI were synthesized by GenScript and cloned into the pCMV-SpRY(D10A) backbone containing C-terminal-fused EGFP and blasticidin (BSD). To construct sgRNA expression vectors, synthesized oligos were annealed and ligated into a BsaI-digested sgRNA expression vector (pGL3-U6-sgRNA-mCherry plasmid). To construct pegRNA expression vectors, synthesized oligos were annealed and ligated into pegRNA expression vector (pGL3-U6-pegRNA-mCherry plasmid). Sequences of sgRNA and pegRNA constructs used in this work are listed in Tables S1 and S2.

Analysis of on-target editing

To evaluate editing efficiency, HEK293T cells were seeded into 24-well plates 1 day before the analysis. The base editor expression plasmids (1,000 ng) and corresponding sgRNA plasmids (500 ng) were cotransfected using Lipofectamine 2000 (Life Technologies) as the manufacturer's protocol recommended. Seventy-two hours after transfection, ~10,000 cells with dual fluorescence signals (GFP and mCherry) were collected by fluorescence-activated cell sorting (FACS) to improve efficiency (Figure S9), and these cells were harvested for genomic DNA extraction using QuickExtract DNA Extraction Solution (Lucigen) according to the manufacturer's protocols. The genomic regions encompassing the target sites were amplified from the genomic DNA with Phanta Max Super-Fidelity DNA polymerase (Vazyme, P505-03). The primers used are listed in Table S3. The PCR products were analyzed by Sanger sequencing or high-throughput sequencing as indicated. For Sanger sequencing, the chromatograms were quantified using EditR. For high-throughput sequencing, the PCR products were sequenced on an Illumina HiSeq X Ten (2 × 150 paired-end) at the Novogene Bioinformatics Institute (Beijing, China). The sequencing data were analyzed using CRISPResso2.

Analysis of RNA off-target editing

HEK293T cells were seeded into 6-cm dishes and transfected with 4 μg of CABE-RY, hA3A(Y130F)-RY, ABE8e(V106W)-RY, and GFP (control) plasmids and 2 μg of sgRNA expression vector using Lipofectamine 2000 at ~70% confluency. Two days after transfection, the top 15% of the GFP signal-positive cells were harvested by FACS. RNA was immediately extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA samples were subjected to deep sequencing (~20 million reads per sample) on an Illumina HiSeq X Ten platform (2 × 150 paired-end) at the Novogene Bioinformatics Institute (Beijing, China). The clean data were first mapped to the human reference genome (version: hg38) with annotations from GENCODE version 30 by STAR software (version 2.5.1). After removing duplicates, GATK HaplotypeCaller (version 4.1.2) was used to identify and filter the edits. All edits were verified, and the efficiency was calculated using the bam-readcount program with the parameters -q 20 -b 30. Importantly, for the reference allele in the wild-type sample, the depth of a given edit had to be least 10×, and all the edits had to be present in at least 99% of reads.

Statistics and reproducibility

All statistical analyses mentioned above were performed on data from at least 3 biologically independent experiments (n = 3). The data shown in this research were statistically analyzed by unpaired two-tailed Student's t test using GraphPad Software (GraphPad Prism 8). A p value smaller than 0.05 was considered to indicate statistical significance.

Data availability

The high-throughput sequencing data have deposited. The accession code is NCBI Sequence Read Archive database: PRJNA688630. All other data are available upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.07.016.

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AUTHOR CONTRIBUTIONS

X.H. and X.X. conceived, designed, and supervised the project. W.T. and Q.L. performed most experiments with the help of other authors. S.H. analyzed and interpreted the data. W.T., Q.L., and X.H. wrote the paper with inputs from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Figure 5. Evaluation of MNV simulation by CABE-RY in HEK293T cells

(A) Heatmaps showing the A-to-G (green) and C-to-T (orange) editing frequencies of ABE8e(V106W), hA3A(Y130F), and CABE-RY at ten sites (n = 3 independent replicates). The number and the letter at the bottom represent the related position and the respective base in the protospacer sequence. (B) Proportion of mutation types edited by CABE-RY (left) and PE3 (right) in different sites. Data are analyzed by deep sequencing and summarized from three independent experiments. Perfect MNV (orange) represents the mutation type that just alters the target MNV site without bystanders. Others (green) represent the mutation types that just alters the target MNV site within bystanders. WT (gray) represents unedited type. (C) Comparison of simulated perfect MNV efficiency between CABE-RY and PE3 on 10 endogenous sites. The error bars represent the SEM values.

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Supplemental information

CABE-RY: A PAM-flexible dual-mutation base editor for reliable modeling of multi-nucleotide variants

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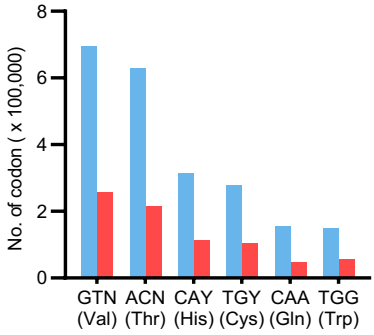
A

ACT (Thr) → ATT (Ile) ACT (Thr) → TCT (Ser) ACT (Thr) → TTT (Phe) AGA (Arg) → ACA (Thr) AGA (Arg) → AGT (Ser) AGA (Arg) → ACT (Thr) CTT (Leu) → CTA (Leu) CTT (Leu) → TTT (Phe) CTT (Leu) → TTA (Leu) CGG (Arg) → AGG (Arg) CGG (Arg) → CGT (Arg) CGG (Arg) → AGT (Ser)	Changed missense
GAG (Glu) → TAG (STOP) GAG (Glu) → GTG (Val) GAG (Glu) → TTG (Leu)	Rescued nonsense
TCC (Ser) → TCA (Ser) TCC (Ser) → TGC (Cys) TCC (Ser) → TGA (STOP)	Gained nonsense

B

A/C simultaneous conversion		
GTN (Val)	→	ACN (Thr)
TGG (Trp)	→	CAA (Gln)
ACN (Thr)	→	GTN (Val)
TGY (Cys)	→	CAY (His)
CAA (Gln)	→	TGG (Trp)
CAY (His)	→	TGY (Cys)

C

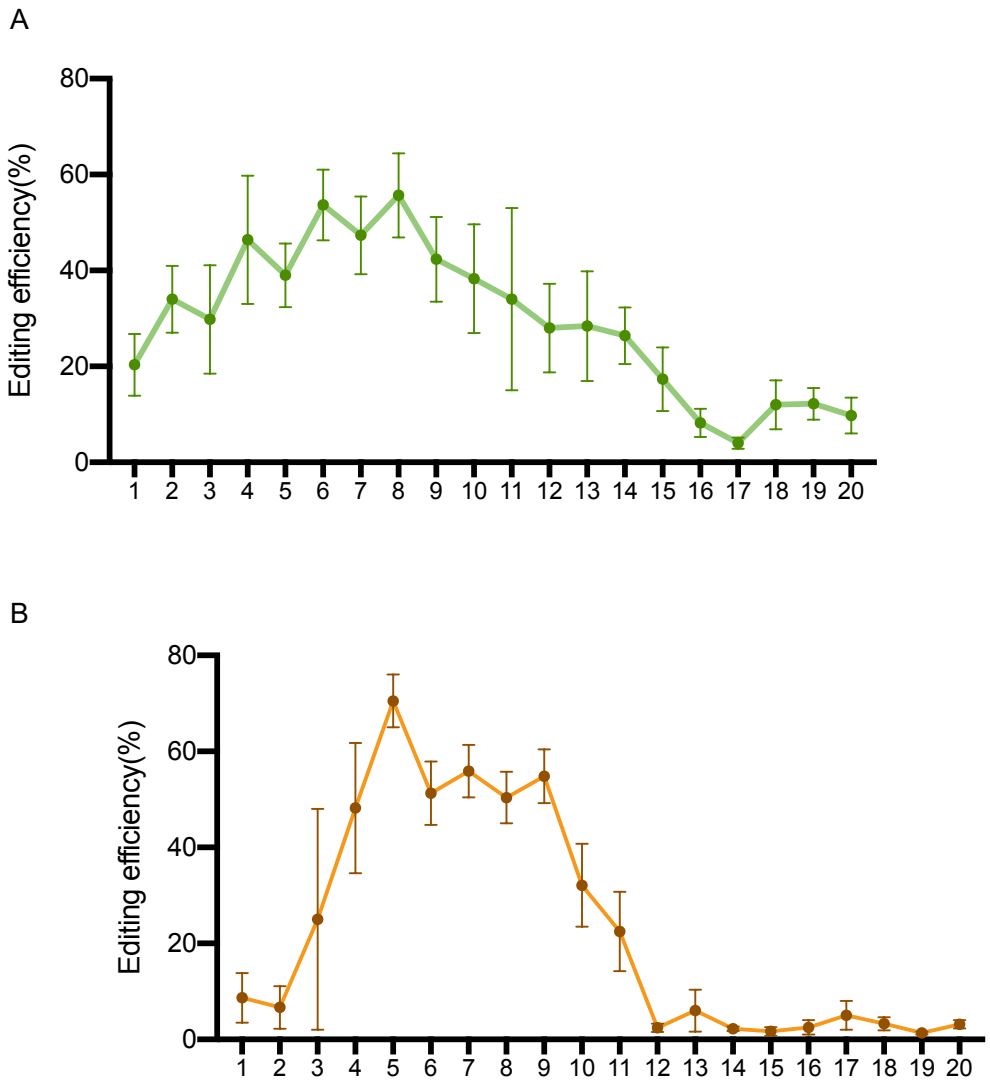


Supplemental Figure 1. Further analysis of MNVs.

(A) Examples of the differences in functional impacts on the protein between MNVs and individual SNVs.

(B) Six amino acid changes can be achieved uniquely by simultaneous A/C conversion.

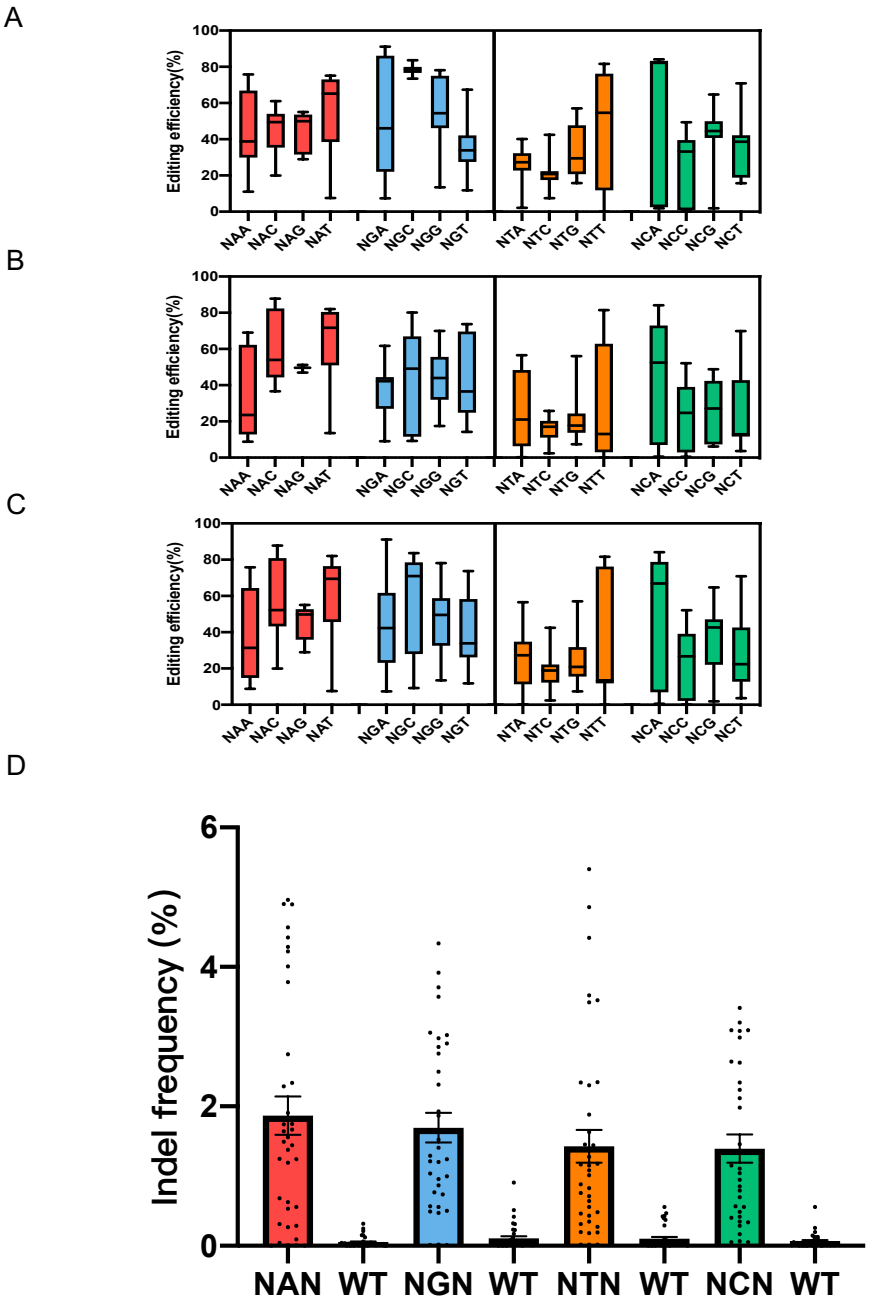
(C) The PAM-flexible dual-mutation base editor can target more specific codons in the human genome than existing editors.



Supplemental Figure 2. Mean nuclease activity plots for a single base editor across NNN PAMs in human cells.

(A) Mean nuclease activity plots for ABE8e(V106W)-RY across NNN PAMs in human cells. The error bars represent the standard error of the mean (SEM) values.

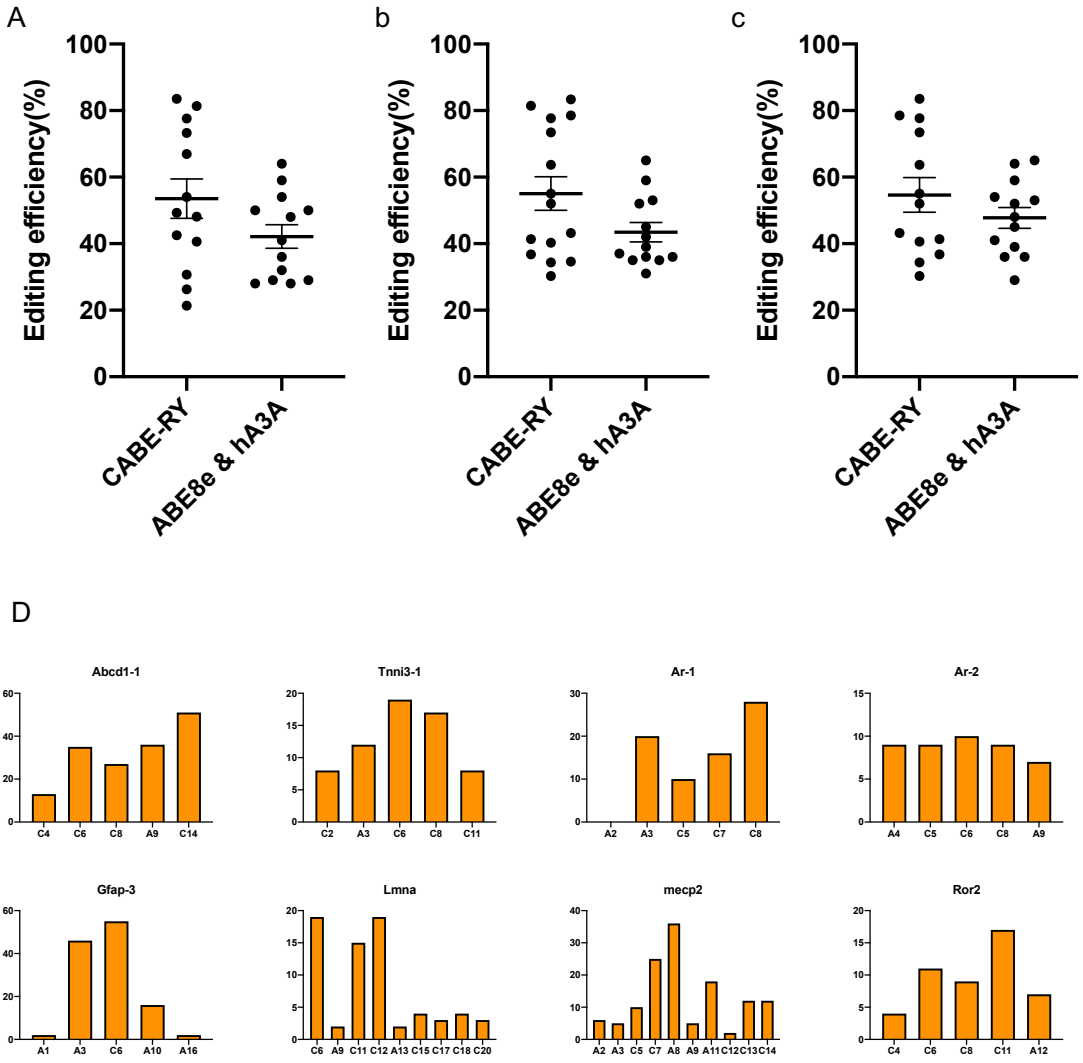
(B) Mean nuclease activity plots for A3A(Y130F)-RY across NNN PAMs in human cells. The error bars represent the standard error of the mean (SEM) values.



Supplemental Figure 3. Other characteristics of CABE-RY at 48 endogenous target sites.

(A, B, C) Aggregate distribution of C-to-T edits (A), A-to-G edits (B) and overall edits (C) across the editing window with CABE-RY.

(D) Box plots indicating the average indel frequencies. The error bars represent the standard error of the mean (SEM) values.



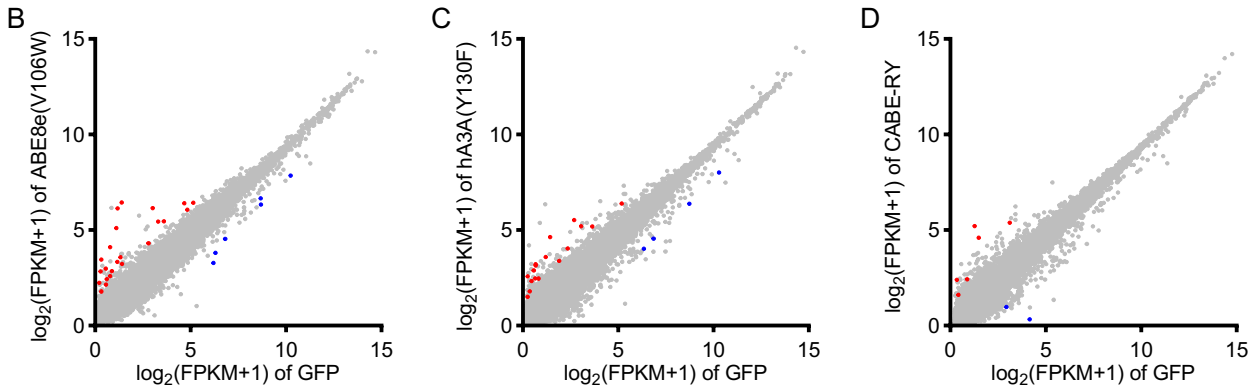
Supplemental Figure 4. Further analysis of CABE-RY.

(A, B, C) Comparison of simultaneous A/C conversion between CABE-RY and coexpressed ABE8e(V106W)-RY and hA3A(Y130F)-RY in HEK 293T cells. The scatter plot shows the efficiency of C-to-T (A), A-to-G (B) and overall edits (C) across the editing window with CABE-RY.

(D) Editing efficiency of CABE-RY in the N2a cell line.

A

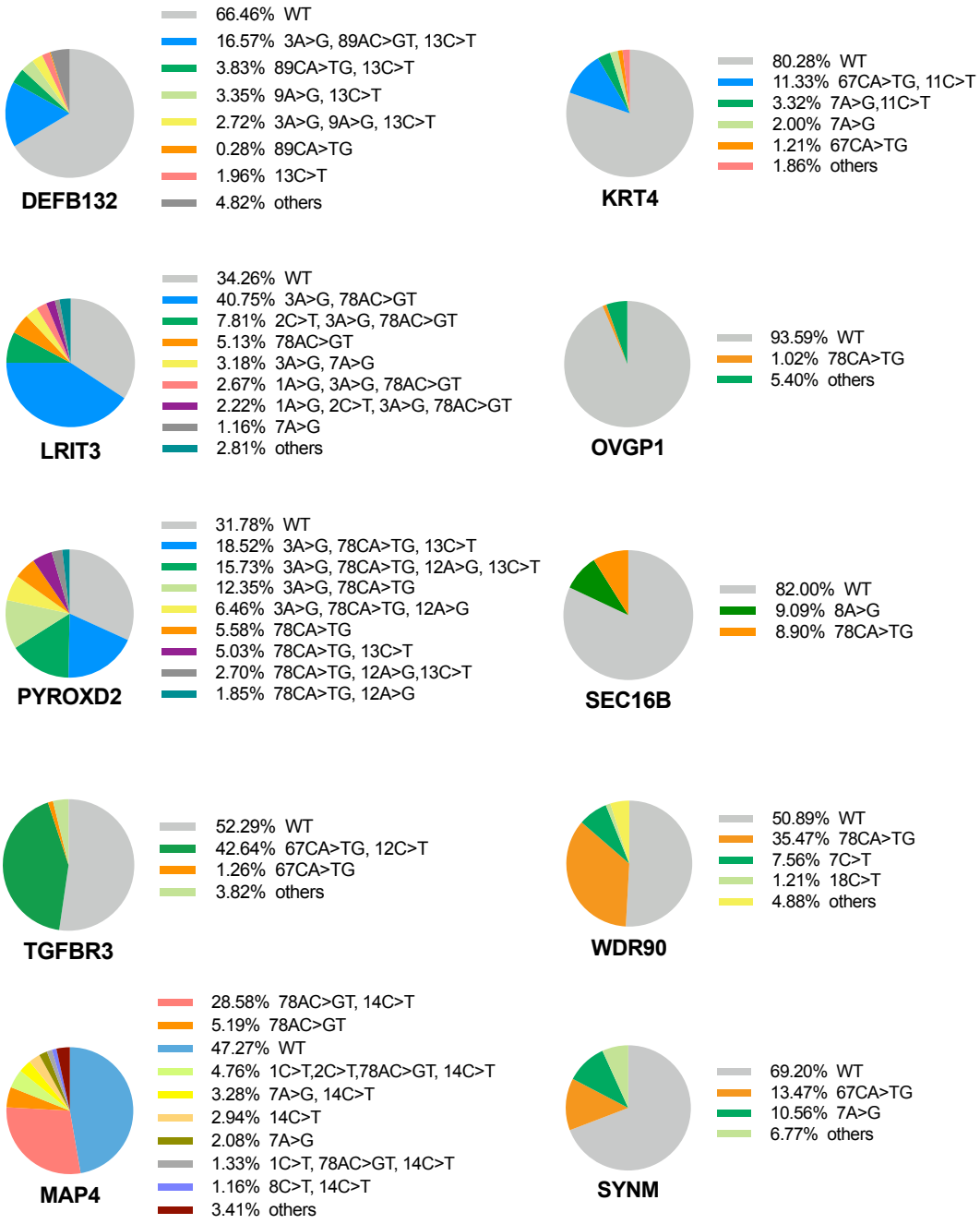
Sample	Raw reads	Mapped reads	Off-target edits (totals)	Off-target edits (A-to-I)	Off-target edits (C-to-U)
GFP-1	24,043,676	21,021,190	428	325	16
GFP-2	22,677,528	20,233,880	618	475	19
ABE8e(V106W)-1	21,999,661	17,071,710	8,462	8,350	21
ABE8e(V106W)-2	19,862,432	15,371,901	9,765	9,633	21
hA3A(Y130F)-1	20,294,961	15,491,241	508	331	72
hA3A(Y130F)-2	20,592,803	15,613,140	506	313	116
CABE-RY-1	21,168,535	17,376,848	2,944	2,853	17
CABE-RY-2	20,020,981	16,466,648	2,748	2,637	24



Supplemental Figure 5. Further description of the RNA-seq data for transcriptome-wide off-target analysis.

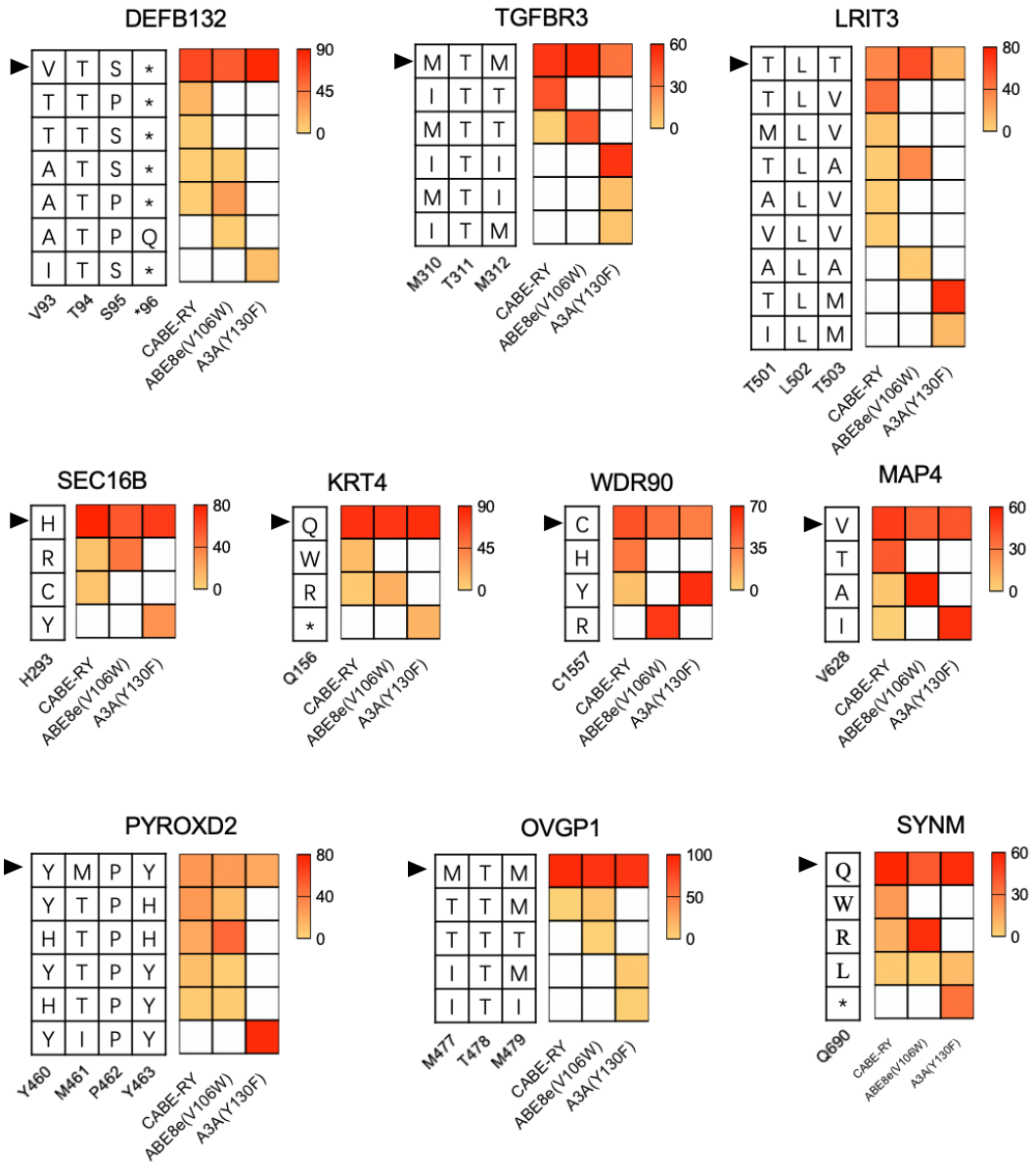
(A) Sequencing information and the number of off-target edits for each sample.

(B, C, D) Transcriptome-wide expression levels of ABE8e(V106W) (B), hA3A(Y130F) (C) and CABE-RY (D) compared to GFP (control). Shown is the mean of two biological replicates. The upregulated genes are labeled in red, and the downregulated genes are labeled in blue.



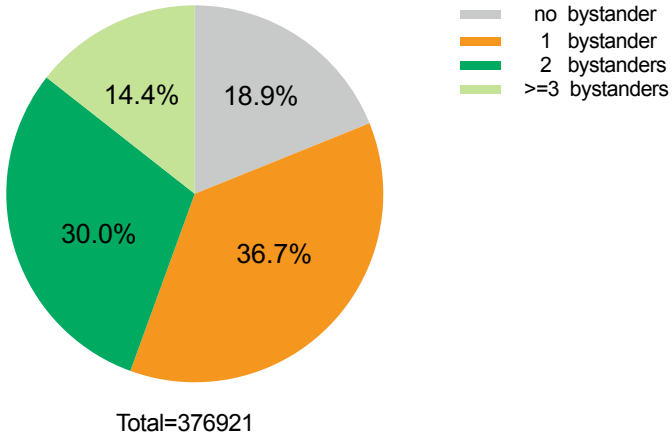
Supplemental Figure 6. Mutation types of simulated MNVs.

The pie chart shows the proportions of mutation types at the ten sites. Only alleles with at least 1% abundance are included. The proportion of perfect MNV is indicated with orange.



Supplemental Figure 7. Amino acid changes in MNV models.

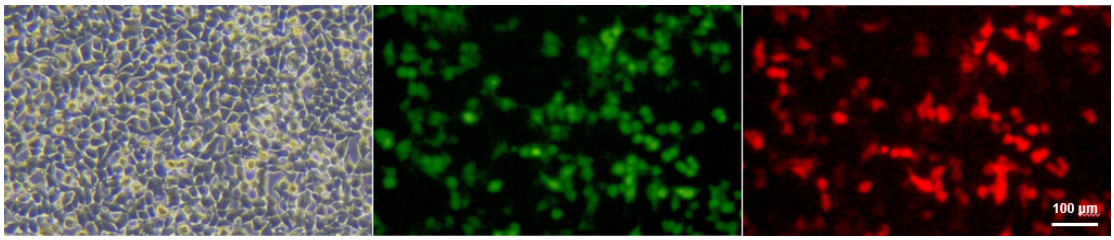
The amino acid edits are summarized in the map on the left; the mean frequencies of amino acid edits are shown in the heatmap. The wild-type (unedited) allele is indicated with a black triangle. Only alleles with at least 1% abundance are included in the heatmap.



Supplemental Figure 8. Bystander effect in gnomAD.

The pie chart shows the proportions of bystanders obtained for modeling of two adjacent MNVs in gnomAD. The main editing window (5-10 for C-to-T, 3-8 for A-to-G) was used for analysis.

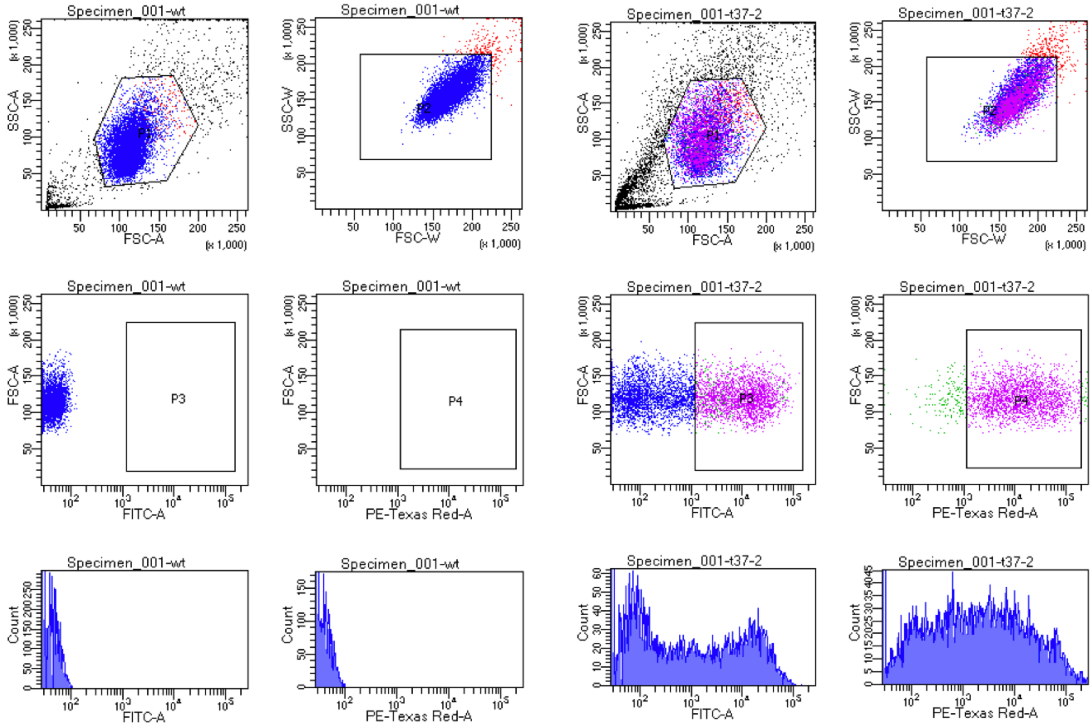
A



Bright field GFP mcherry

B

Negative Control (no transfection) CABE-RY (GFP) + sgRNA (mcherry)



Tube: wt

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	8,433	84.3	84.3
P2	8,205	97.3	82.0
P3	0	0.0	0.0
P4	0	####	0.0

Tube: t37-2

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	5,758	57.6	57.6
P2	5,457	94.8	54.6
P3	2,328	42.7	23.3
P4	2,136	91.8	21.4

Supplemental Figure 9. Profiles for transfection and FACS analysis.

(A) Images of transfected HEK293T cells with fluorescence markers.

(B) FACS gating profiles for GFP- and mCherry-positive HEK293T cells.

Supplementary Sequences 1. DNA sequences used this study for cell transfection. Within base editor sequences, BPNLS sequences are highlighted in cyan, A3A(Y130F) sequences are in pink, TadA8e sequences are in yellow, linkers are in blue, SpRY are in red, UGIs are in tan, P2A sequences are in grey, EGFP sequences are in green and BSD sequences are in purple.

CABE-RY

atgaaacggacagccgacggaagcgagttcgagtcaccaaagaagaagcggaaagtcGAGGCATCTCCAGCAAGCGG
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gggctaa

Supplemental Table 1. sgRNA sequences.

Number	Site	Protospacer sequence
1	NGG SITE 1	GAACACAAAGCATAGACTGC
2	NTT SITE 1	ACATCATCAGATATTCTGCA
3	NTC SITE 1	GCTGCAAACAAGTGCAGAAT
4	NTG SITE 1	TGGCAGGACGTCTGCCCAAT
5	NAC SITE 1	ACCAACAATAGAGGCCCATT
6	NAG SITE 1	GTTTACATAAAAGATCTTCA
7	NGT SITE 1	AATACAAATAGTTAAGAACA
8	NGC SITE 1	CTGGAACACAAAGCATAGAC
9	NCT SITE 1	TCCTAAACCAGTGTCAGGGA
10	NCC SITE 1	AAAGATCTTCACAGGCTACC
11	NCG SITE 1	CCAACAATAGAGGCCCATTA
12	NAT SITE 1	CTGTCAAACTGTGCGTATGA
13	NAA SITE 1	TACCAACAATAGAGGCCCAT
14	NCA SITE 1	CTGCAGCCCAAGCCTCAGTG
15	NGA SITE 1	AGGTCCTAAACCAGTGTCAG
16	NTA SITE 1	CTACCAACAATAGAGGCCCA
17	NTT SITE 2	GACAGTTCCTTCCAATTCCA
18	NTC SITE 2	AACAACAGTACTTGCGACAG
19	NTG SITE 2	GCAGCAGCCTGGAAAAGTAC
20	NGT SITE 2	TTGGAACTCCTGCTTGCAAG
21	NGC SITE 2	AAGGCCAAGCTTGCCTGCCC
22	NCC SITE 2	GAGATGCAGCGAATGTGAAA
23	NCT SITE 2	TCATCTTAGGCCTTCAAGGA
24	NGG SITE 2	CCTGCCCTGCATTTTATCAA
25	NCG SITE 2	GAGCAGGGAAGAAGGAATCA
26	NAT SITE 2	TACTTGCGACAGTTCCTTCC
27	NAC SITE 2	CATTTCCCTCTACGCTCGCT
28	NAG SITE 2	TTGAACAACAGTACTTGCGA
29	NAA SITE 2	CCTGCTTGCAAGTGTCAACC
30	NCA SITE 2	GATGCAGCGAATGTGAAATC
31	NGA SITE 2	GAAAAGTACTTGGGGACCAA
32	NTA SITE 2	CCTCTTCTGGAAAGGGGTAC
33	NTT SITE 3	CTTAGGGGGCACTTCGACCA
34	NTC SITE 3	TCCACCCGCTGTGCGTCCCA
35	NTG SITE 3	TGGCCGAATGCAAAGGTTCT
36	NGT SITE 3	TCGGCCAATGGGGCACAAGG
37	NGC SITE 3	CCCAAAAGTGGGGCGTACA
38	NCC SITE 3	GAGTTCCACCCGCTGTGCGT
39	NCT SITE 3	TGGACTCAGATGCTCCAACG
40	NGG SITE 3	GGTTACACCAAAGGGCTAGA

41	NCG SITE 3	ATGCAAAGGTTCTCTGCTAG
42	NAT SITE 3	GTAACCTCCCTTGAAAGGGG
43	NAC SITE 3	GGAGCATCTGAGTCCAGGGG
44	NAG SITE 3	ACAGCGGGTGGAACTCCCAA
45	NAA SITE 3	CTGCTAGACGACAGCGCAGG
46	NCA SITE 3	TGCGTCCCACTCCTTGTGCC
47	NGA SITE 3	GAATGCAAAGGTTCTCTGCT
48	NTA SITE 3	CCGAATGCAAAGGTTCTCTG
49	DEFB132	ATTATGATGTTACGGTCGTT
50	KRT4	AGGTGCAGTTCTTAGAGCAA
51	LRIT3	ACATTGACGTGGAATATGAT
52	OVGP1	CATGGTCATTGCCCCAGTGA
53	PYROXD2	ATAGGGCATGTACTGAGTGA
54	SEC16B	TGGTGCATGTAGGTCCCAGC
55	TGFBR3	TTGGTCATTGTCATAGATCT
56	WDR90	CCTGTGCAGGGGTGGCTCAC
57	MAP4	CCTGTGACGGTTTCTAAAGG
58	SYNM	AGGGGCAGGGTGGGCCGGGG

Supplemental Table 2. pegRNA sequences.

pegRNA	spacer sequence	3 ‘extension sequence	PBS length(nt)	RT template length(nt)
DEFB132	CAGTGGTTATTATGATGTTA	ACAAACGACCACAACATCATAATAA	13	12
KRT4	GCTGGTGCCTTTATCAACAC	CTCTAAGAACCACACCTGTGTTGAT	13	12
LRIT3	AAGAGAGTGTGACATTGACG	CATATTCCACACCAATGTCACACTC	13	12
OVGP1	AGAGAAGACTGAGATCACTG	AAGTCATGGTTGTTGCCCCAGTGAT	13	12
PYROXD2	TTGCCTCCAGCCAGCGTATA	ACTCAGTACACACCCTATACGCTGG	13	12
SEC16B	TTTCGGGCCAGGAGGTCAGC	GGGACCTACACACACCAGCTGACCT	13	12
TGFBR3	AATGTCATCTCTTATTGATT	TCTATGACAACAACCAAATCAATAA	13	12
WDR90	ACGGAAGGTTGTCCCTGTGC	GAGCCACCCCCACACAGGGACAACC	13	12
MAP4	CTTTTTCCCCGTTCCTGTGA	TTTAGAAACCACCACAGGAACGGGG	13	12
SYNM	TGTCCGCCCTCACCAGAGAG	CGGCCCACCCCACCCCTCTCTGGTG	13	12
nicking sgRNA	**spacer sequence**			
DEFB132	AACCACTGGCAATCAAGGAG			
KRT4	TGGTCGTCTGCTGCTGGAGC			
LRIT3	CAGGTCCTTCCCACCATACT			
OVGP1	AGGGATACAGTTTCCTTTGT			
PYROXD2	TTCCTCGCTGGACCCCACCC			
SEC16B	CATGCTGTGCAGTTCAACAA			
TGFBR3	GAATCTGGTGAAGTGGGCTT			
WDR90	CCAGGTCAGACTGTCCTCTC			
MAP4	GATTCTGTGTTAGAAAAACT			
SYNM	CGGAACTGCCACCAGCACCC			

Supplemental Table 3. Primer sequences.

Site	Forward sequence (5'-3')	Reverse sequence (5'-3')
NGG SITE 1	ATAACAAGACCTGGCTGAGC	TCAAGCAGGTGATTACAGGA
NTT SITE 1	CAGCATGTGGTAATTTTCCA	CACATGACAGTTAAGGTTTG
NTC SITE 1	CAAGACCTGGCTGAGCTAAC	TTTAGTCTTTCAAGCAGGTG
NTG SITE 1	AGGCTACCCCCTAAGTCTAG	GTTTCCTTTACAGGGCCAGC
NAC SITE 1	GATCTTCACAGGCTACCCCC	CCACATGCTGTCACAGTTAG
NAG SITE 1	GCAGTGTTTAGAAGGAGACT	GTTGGTAGAATGGCAGTGCA
NGT SITE 1	GGCCCTGTAAAGGAAACTGG	TGTTTAGTCTTTCAAGCAGG
NGC SITE 1	ATGATAACAAGACCTGGCTG	TCTTTCAAGCAGGTGATTAC
NCT SITE 1	AGAAGGAGACTTGTGCACAT	ATTGTTGGTAGAATGGCAGT
NCC SITE 1	GTTTAGAAGGAGACTTGTGC	GGTAGAATGGCAGTGCAATA
NCG SITE 1	AAAGATCTTCACAGGCTACC	CCAGCGGGCTGGAAAATTAC
NAT SITE 1	ATGTGGTAATTTTCCAGCCC	CAAGCACATGACAGTTAAGG
NAA SITE 1	CTTCACAGGCTACCCCCTAAG	GAAAATTACCACATGCTGTC
NCA SITE 1	CCTAAACCAGTGTCAGGGAG	TGCTTTGTGTTCCAGTTTCC
NGA SITE 1	GGAGACTTGTGCACATTCTA	TCTATTGTTGGTAGAATGGC
NTA SITE 1	CACAGGCTACCCCCTAAGTC	GGCTGGAAAATTACCACATG
NTT SITE 2	GTGGAACATGGTGAGTGCTT	TAGCAGAGGAATCAGGCAAG
NTC SITE 2	ACACTGCAATGTTTTTGTGG	AGGCAAGAAATAAGCAACTC
NTG SITE 2	GTGGAGATGCAGCGAATGTG	GAAAAGCACTCACCATGTTC
NGT SITE 2	GAGGAGTAGCCAAAGACCAT	TCCATAACAAAAGGAAGCAC
NGC SITE 2	TGAACCCTCTTCTGGAAAGG	CTGTTGTTCAAAGTTCCTTG
NCC SITE 2	GTGCTTCCTTTTGTTATGGA	GATTCCTTCTTCCCTGCTCC
NCT SITE 2	ATGGGGGTCCACACTGCAAT	CCGAAGTCACGAAGTAACTT
NGG SITE 2	CTCTTCTGGAAAGGGGTACC	GTCGCAAGTACTGTTGTTCA
NCG SITE 2	CTGGAAAGGGGTACCTATTA	TTGGAAGGAACTGTCGCAAG
NAT SITE 2	TGTTTTTGTGGAACATGGTG	GAATCAGGCAAGAAATAAGC
NAC SITE 2	GACTTGTGGAGATGCAGCGA	CACTCACCATGTTCCACAAA
NAG SITE 2	GGTCCACACTGCAATGTTTT	ATAAAGCCGAAGTCACGAAG
NAA SITE 2	GAGTAGCCAAAGACCATCAG	CTTCCATAACAAAAGGAAGC
NCA SITE 2	GCTTCCTTTTGTTATGGAAG	GATGATTCCTTCTTCCCTGC
NTA SITE 2	CTTGTGGAGATGCAGCGAAT	AAGCACTCACCATGTTCCAC
NGA SITE 2	AGATCTTGAACCCTCTTCTG	GTTCAAAGTTCCTTGAAGGC
NTT SITE 3	AGGAAGCAGTATCCGAAGGC	CCAATGGGGCACAAGGAGTG
NAC SITE 3	CAGTATCCGAAGGCAGCAGC	CATTCGGCCAATGGGGCACA
NGC SITE 3	ACAATTACTTAGGGGGCACT	ACTCAGCAGTATCTTCAGTG
NAG SITE 3	TACTTAGGGGGCACTTCGAC	AAGGGGAATACTCAGCAGTA

NGT SITE 3	GGCACTTCGACCATTTCTGA	TATCTTCAGTGCTCTTGCCT
NCT SITE 3	GACAACGCCAAGGAGTTGTG	AGCCCTTTGGTGTAACCTCC
NCC SITE 3	CGCCAAGGAGTTGTGTAAGG	TCTCGCCTTCTAGCCCTTTG
NTC SITE 3	GGAGTTGTGTAAGGCAGTGT	CCTAGGCTCTCGCCTTCTAG
NCA SITE 3	ACCATTTCTGACAACGCCAA	TTTGGTGTAACCTCCCTTGA
NTA SITE 3	CGTTGGAGCATCTGAGTCCA	GACGGCAGTTCAAGTGTCCC
NTG SITE 3	TGGAGCATCTGAGTCCAGGG	GGTAGACGGCAGTTCAAGTG
NAA SITE 3	GAGCATCTGAGTCCAGGGGA	GACAGGGTAGACGGCAGTTC
NCG SITE 3	GAACAGCTTCGGGGGGATTG	CTTGTAGAGAGACAGGGTAG
NGA SITE 3	GGGATTGCATGTACGCCCCA	CCAGTGCTCCGGACTTGTAG
NGG SITE 3	GTACGCCCCACTTTTGGGAG	GTCCAGTGCTCCGGACTTGT
NAT SITE 3	CACTCCTTGTGCCCCATTGG	GTTGTAGTAGTCGCGACTCT
DEFB132	GGGAGACAGCATTGTTCATG	TAAAGAGGAGCCTTGGCTAG
KRT4	GTTCTTGGGCCGTGTAGTTC	CCAAGGTATCTAGCTGCTTC
LRIT3	AGTAAGCTTCCTCCAGCCAG	GGGTTCCAAGCCATCTATGG
OVGP1	TGAGATCCACGGAAAGTGTG	AGGGGTCACAGACTGATAAC
PYROXD2	GTCCCAAGTCATCTCGGATG	CGTGCAGCTGTCTTTACCTC
SEC16B	GGGCAGAAATACATCTGTGG	TCCAGGGACACTGGGTCTTC
TGFBR3	TCATCGTTCTTAGCCCAAGG	AAGCCCACTTCACCAGATTC
WDR90	TGTTGCCTTCTCCACCGATG	GGGCTGCTTTACCTCTTTGC
MAP4	GCCCCTTGTAGGAGAACTCC	CCTAACTTACCTGAGGTCTC
SYNM	TGAAGGGCATCTCCTCCAAG	TCTTTGCTCAGCTCCTCCAG