Cas12a Base Editors Induce Efficient and Specific Editing with Low DNA Damage Response

**Graphical Abstract**

**Highlights**
- BEACON induces basal levels of DNA breaks and DNA damage response
- BEACON induces a basal level of RNA off-target mutations
- BEACON induces *in vivo* base editing with high product purity

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**In Brief**
Wang et al. develop a BEACON base-editing system by combining dCas12a with human APOBEC3A and its engineered versions. BEACON induces efficient editing in cells and mouse embryos with basal levels of DNA damage response, RNA off-target mutations, and unintended side products.

Wang et al., 2020, *Cell Reports* 31, 107723
June 2, 2020 © 2020 The Author(s).
https://doi.org/10.1016/j.celrep.2020.107723
Cas12a Base Editors Induce Efficient and Specific Editing with Low DNA Damage Response

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https://doi.org/10.1016/j.celrep.2020.107723

SUMMARY

The advent of base editors (BEs) holds great potential for correcting pathogenic-related point mutations to treat relevant diseases. However, Cas9 nickase (nCas9)-derived BEs lead to DNA double-strand breaks, which can trigger unwanted DNA damage response (DDR). Here, we show that the original version of catalytically dead Cas12a (dCas12a)-conjugated BEs induce a basal level of DNA breaks and minimally activate DDR proteins, including H2AX, ATM, ATR, and p53. By fusing dCas12a with engineered human apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A (APOBEC3A), we further develop the BEACON (base editing induced by human APOBEC3A and Cas12a without DNA break) system to achieve enhanced deamination efficiency and editing specificity. Efficient C-to-T editing is achieved by BEACON in mammalian cells at levels comparable to AncBE4max, with only low levels of DDR and minimal RNA off-target mutations. Importantly, BEACON induces in vivo base editing in mouse embryos, and targeted C-to-T conversions are detected in F0 mice.

INTRODUCTION

The CRISPR-Cas9 system has been successfully applied in various living organisms for genome editing (Hsu et al., 2014; Knott and Doudna, 2018; Komor et al., 2017). The Cas9 nuclease generates DNA double-strand breaks (DSBs) at specific genomic loci under the direction of guide RNAs (gRNAs) (Cong et al., 2013; Jinek et al., 2013; Mali et al., 2013), which are further processed by downstream DNA repair pathways to induce gene editing outcomes. Therefore, the formation of DSBs is required for CRISPR-Cas9-mediated genome editing. Although Cas9 nickase (nCas9) only generates DNA single-strand breaks (SSBs), apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) activation-induced cytidine deaminase (AID) family members (Harris and Liddament, 2004) together with base excision repair proteins (Chen et al., 2014) can lead to DSBs during the repair of these SSBs (Lei et al., 2018). In addition, base editors (BEs) that link nCas9 with APOBEC1 are also associated with DSBs, demonstrated by the induced insertions or deletions (indels) of nucleotides around target sites (Komor et al., 2016; Lei et al., 2018; Nishida et al., 2016).

In most cases, DSBs are repaired through the non-homologous end joining (NHEJ) pathway (Ceccaldi et al., 2016) to yield indels (Chakrabarti et al., 2019; van Overbeek et al., 2016), which lead to knockout of protein-coding genes. Alternatively, DSBs can be resolved through homology-directed repair (HDR) in the presence of a donor DNA (Ceccaldi et al., 2016), which can be used to induce precise sequence replacement. However, DSBs are highly toxic lesions (Chapman et al., 2012) and can trigger downstream DNA damage response (DDR) signaling pathways to disturb cellular homeostasis (Roos et al., 2016; Zhou and Elledge, 2000), e.g., cell proliferation (Haapaniemi et al., 2018; Ihry et al., 2018). Specifically, the generation of DSBs can trigger the autophosphorylation of the protein kinase ataxia-telangiectasia mutated (ATM) (Shiloh and Ziv, 2013). Meanwhile, during the repair of DSB, an end resection process...
generates single-stranded DNA (ssDNA) regions (Ceccaldi et al., 2016) and further activates another protein kinase, ATM and RAD3-related (ATR) (Cimprich and Cortez, 2008). ATM, and to some extent ATR, can phosphorylate the histone variant H2AX and tumor suppressor protein p53, which signal DSB repair (Mah et al., 2010) and then regulate the cell cycle (Bieging et al., 2014).

Cas12a (as known as Cpf1) is another CRISPR-Cas protein that is distinct from Cas9 in many aspects (Zetsche et al., 2015). Cas12a recognizes a T-rich PAM sequence, requires a short gRNA (CRISPR RNA, crRNA) and has been reported to have a generally higher targeting specificity than Cas9 (Kim et al., 2016; Kleinstiver et al., 2016, 2019; Yan et al., 2017). These characteristics render Cas12a a promising gene editing platform. We have recently developed catalytically dead Cas12a (dCas12a)-derived BEs that were conjugated with rat APOBEC1 (rA1) (Li et al., 2018). In theory, dCas12a-derived BEs are unlikely to cause DSBs; therefore, unwanted DDR could be largely avoided. Nevertheless, dCas12a-BEs also induced much lower editing efficiencies than nCas9-BEs did (Gehrke et al., 2018; Huang et al., 2019; Koblan et al., 2018; Li et al., 2018; Thuronyi et al., 2019; Wang et al., 2019), and in vivo base editing by dCas12a-BEs has not been achieved in animals. In this study, we confirmed that the early version of dCas12a-derived BEs induced very low levels of DDR. Furthermore, by screening and engineering different APOBEC/AID family members, we

Figure 1. dCas12a-Derived BEs Triggered DDR Minimally
(A) Schematic diagrams illustrate the gene editing mediated by Cas9 (left), the base editing mediated by dCas12a-derived BEs (right), and the relationship with DNA damage response. DNA double-strand breaks generated by Cas9 nuclease can trigger the phosphorylation and activation of a series of proteins involved in DNA damage response signaling pathways, such as ATM, ATR, H2AX, and p53.
(B) Immunoblots of DDR proteins and their phosphorylated forms triggered by Cas9-mediated gene editing or BE2-, BE3-, and rA1-dCas12a-BE-mediated base editing. The numbers represent individual protein contents relative to those triggered by Cas9 (setting as 100).
(C) Quantification of the relative protein contents normalized with actin in (B) and Figure S1A. Means ± SD are from two independent experiments. See also Figure S1.
identified that highly efficient and specific C-to-T editing could be induced by human APOBEC3A (hA3A)-dCas12a-BEs, referred to as BEACON (base editing induced by human A3A and Cas12a without DNA break). With no toxic DSB and basal levels of DDR and RNA off-target (OT) effects, BEACON also induced in vivo base editing successfully in mouse embryos, and targeted C-to-T conversions were detected in F0 mice.

RESULTS

Low Levels of DDR Induced by dCas12a-BE
Different from Cas9-mediated genome editing, dCas12a-derived BEs only deaminate one or a few cytosines in the ssDNA of the R-loop formed by gRNA at target sites, which will be converted to thymines after DNA replication. Given the fact that dCas12a is used as the Cas moiety, dCas12a-derived BEs theoretically are unlikely to generate DNA breaks or activate DDR cascades (Figure 1A). In contrast, nCas9-derived BEs triggered indel formation as the nCas9-generated nick would be converted to a DSB (Lei et al., 2018), which activates DDR proteins. Indeed, much higher levels of phosphorylated H2AX (γH2AX) were observed in cells treated with Cas9 or a nCas9-derived BE3 than those in cells treated with rA1-dCas12a-BE (Figures 1B, 1C, and S1A). Correspondingly, the levels of phosphorylated p53 (p-p53), ATM (p-ATM), and ATR (p-ATR) in rA1-dCas12a-BE-treated cells were also lower than those in the cells treated with Cas9 or BE3 (Figures 1B, 1C, and S1A). As shown in Figure S1B, rA1-dCas12a-BE induced almost no indel at the target site, whereas high levels of indels were observed with the Cas9 or BE3 treatment. In addition, the catalytically dead Cas9 (dCas9)-derived BE2 also triggered low levels of DDR (Figures 1B, 1C, and S1A) and indels (Figure S1B), but it only induced base editing with limited efficiencies (Figure S1C). These results demonstrated that rA1-dCas12a-BE induced base editing with the least damage on genomic DNA.

Screening of dCas12a-Derived BE
We next compared the editing efficiencies of rA1-dCas12a-BE and the commonly used BE3 at disease-associated target sites (Wang et al., 2019; Figure S2). Although rA1-dCas12a-BE induced purer C-to-T editing products and fewer indels than BE3, it induced ~4-fold lower C-to-T editing frequencies (median, p = 2 × 10^-11; Figure S2E) than BE3 (~13% to 48%; Figure S2B) at seven selected disease-associated genomic loci. The fact that currently available rA1-dCas12a-BEs induce significantly low editing efficiency impedes its broad applications. We sought to develop new dCas12a-derived BEs with high C-to-T editing efficiencies by linking dCas12a with different APOBEC/AID family members. Among three newly constructed BEs (hA3A-dCas12a-BE, hA3B-dCas12a-BE, and hAID-dCas12a-BE) and one previously reported rA1-dCas12a-BE (Figure 2A), hA3A-dCas12a-BE induced the highest editing efficiency, which is ~4-fold higher than the original rA1-dCas12a-BE (median, p = 2 × 10^{-7}; Figure 2C), across 15 genomic loci (Figure 2B). Further analysis showed that hA3A-dCas12a-BE induced the purest C-to-T editing as well (Figure 2D).

Improvement of hA3A-dCas12a-BE
We then aimed to further improve the editing efficiency of hA3A-dCas12a-BE by engineering its hA3A moiety. According to the structure of hA3A in complex with a 5’-TC-containing ssDNA (Shi et al., 2017), the targeted C was usually accommodated in a groove that is formed by hA3A residues from active center loops (AC-loops) 1, 3, 5, and 7 (Figure 3A). Of the residues on these loops, His29, His70, Tyr130, Ser99, Asp131, and Tyr132 are directly involved in substrate coordination (Figure 3A, close up view in right panel). Thus, we opted to avoid mutating these residues and instead to choose aromatic AC-loop residues that are located on the peripheral of the ssDNA binding groove, i.e., Trp98 and Trp104, for subsequent engineering. Meanwhile, Pro134 was also selected for engineering, as proline is generally considered a secondary structure disruptor and the change of Pro134 may have effects on the flexibility of AC-loop 7. Molecular simulation of the corresponding hA3A mutants in complex with ssDNA ligands (Figure 3B) indicated that mutations of W104A and P134Y significantly strengthened the interaction between hA3A and ssDNA, whereas W98A largely destabilized the interaction (Figure 3B). Although the W98Y mutation alone seemed to not affect the binding of the ssDNA ligand (Figure 3B), it might loosen the nucleotide preference at –1 positions (Shi et al., 2017). Thus, we introduced the amino acid changes of W98Y, W104A, and P134Y into the hA3A moiety of hA3A-dCas12a-BE, with the mutation W98A serving as the negative control (Figure 3C). In total, four engineered BEs (hA3AW98A-dCas12a-BE, hA3AW98Y-dCas12a-BE, hA3AV104A-dCas12a-BE, and hA3A–P134Y-dCas12a-BE) were obtained for subsequent comparison.

Among them, three engineered BEs (hA3AW98Y-dCas12a-BE, hA3AV104A-dCas12a-BE, and hA3A–P134Y-dCas12a-BE) induced higher editing frequencies than the wild-type hA3A-dCas12a-BE (median, 1.32-, 1.54-, and 1.40-fold; and p = 6 × 10^{-10}, 8 × 10^{-12}, and 3 × 10^{-16}, respectively), whereas hA3A–W98A-dCas12a-BE showed decreased editing efficiency (Figure 3D). As a control, two mutations at amino acids (C101S and C106S) close to the enzymatic active site eliminated editing efficacy (Figure S3A). Next, we further combined the amino acid changes of W98Y, W104A, and P134Y to test whether these alternations can increase the editing efficiency in a synergistic manner (Figure 3F). The dual-change combinations of W98Y/W104A and
W104A/P134Y induced higher editing frequencies than W98Y/ P134Y (Figure 3G). Interestingly, the triple-change combination of W98Y/W104A/P134Y induced lower editing frequencies than the original hA3A-dCas12a-BE (Figure 3G), suggesting that the triple amino acid changes may compromise the cytidine-deaminase activity of hA3A. Among all engineered BEs, three of them (hA3A_W104A-dCas12a-BE, hA3A_W98Y/W104A-dCas12a-BE, and hA3A_W104A/P134Y-dCas12a-BE) induced high levels of editing efficiencies, i.e., 1.54-, 1.57-, and 1.64-fold of editing efficiencies relative to hA3A-dCas12a-BE (median, \( p = 8 \times 10^{-12}, 2 \times 10^{-13}, \) and \( 5 \times 10^{-21} \), respectively; Figures 3D and 3G), and were used for further engineering.

Next, we also optimized the codons of these four BEs (Figure 4A) for mammalian expression to enhance their editing efficiencies (Koblan et al., 2018). As expected, editing efficiencies of these codon-optimized BEs (hA3A-dCas12a-BE-op, hA3A_W104A-dCas12a-BE-op, hA3A_W98Y/W104A-dCas12a-BE-op, and hA3A_W104A/P134Y-dCas12a-BE-op) were all significantly improved (Figure 4B). Among them, the engineered and codon-optimized ones (hA3A_W104A-dCas12a-BE-op, hA3A_W98Y/W104A-dCas12a-BE-op, and hA3A_W104A/P134Y-dCas12a-BE-op) induced higher levels of editing efficiencies (i.e., 1.55-, 1.71-, and 1.76-fold) than hA3A-dCas12a-BE-op with optimized codons only (Figure 4B, the far right panel). Note, the engineering of hA3A or codon optimization did not substantially affect the product purity induced by corresponding BEs (Figures 3E, 3H, and 4C).

Editing windows of hA3A_W104A-dCas12a-BE-op, hA3A_W98Y/ W104A-dCas12a-BE-op, and hA3A_W104A/P134Y-dCas12a-BE-op were shown to be ~15 bp long (Figures 4D and 3B, positions 6–20, setting the PAM-proximal nucleotide as position 1). Although large editing windows are useful for intended mutagenesis, e.g., creating stop codons to knock out genes (Billon et al., 2017; Kuscu et al., 2017), they were otherwise too wide for precise editing, as multiple cytosines might exist in the editing window (Rees and Liu, 2018). In order to narrow editing windows, we further introduced Y130F or Y132D, which were previously shown to narrow the editing windows of hA3A-conjugated BEs (Wang et al., 2018) into the engineered hA3As. Either Y130F or Y132D narrowed the editing windows while maintaining (or slightly affecting) editing efficiencies (Figures 4D, 4E, 4F, and 3B). Interestingly, the introduction of Y130F or Y132D further reduced the formation of indels (Figures 3C–3F) with an unknown mechanism. However, despite an even further narrowed editing window, simultaneous introduction of Y130F and Y132D led to a reduced editing efficiency (Figures 4F and 3B). Thus, we chose hA3A-dCas12a-BEs containing either Y130F or Y132D for subsequent analysis and designated them as BEACON (base editing induced by human A3A and Cas12a without DNA break). Hereafter, hA3A_W104A/Y132D-dCas12a-BE-op and hA3A_W98Y/W104A/Y130F-dCas12a-BE-op are referred to as BEACON1 and BEACON2, respectively. Both BEACONs exhibited narrowed editing windows and induce low levels of indels (Figures 4D, 3D, and 3E).

**Comparison of BEACON and Other BEs**

With efficiency-maximized and editing-window-narrowed dCas12a-BEs in hand, we next compared base-editing outcomes induced by BEACON1 and BEACON2 with those by BE2, BE3 (Komor et al., 2016), YE1-BE3 (Kim et al., 2017), and AncBE4max (Koblan et al., 2018) at seven disease-related genomic loci (Figure 5A). Across these sites, both BEACON1 and BEACON2 induced significantly higher editing than BE3 (median: 1.88-fold, \( p = 2 \times 10^{-5} \); and median: 2.14-fold, \( p = 1 \times 10^{-5} \), respectively; Figure 5E) and reached levels similar to those of AncBE4max (Figure 5E). At some sites, BEACON2 induced even more efficient C-to-T editing than AncBE4max, e.g., sites BMPR2, PDE6C, and PMS2 (Figure 5B). In addition, the product purity yielded by BEACON2 was higher than that by BE3 (median C-to-T fraction, 99.26% versus 87.28%, \( p = 1 \times 10^{-6} \); Figures 5C and 5F) and similar to that by AncBE4max (median C-to-T fraction, 99.26% versus 97.80%, \( p = 0.0002 \); Figures 5C and 5F). As BEACONs use dCas12a as the Cas moiety, they generated no DSB and only background levels of indels (Figure 5D), which were much lower than those by BE3 and AncBE4max (Figure 5G). Importantly, the levels of DDR proteins in BEACON-treated cells were also lower than those in BE3- and Cas9-treated cells (Figures 5H, S4A, and S4B), consistent with the results that BEACON caused almost no toxic DSB (Figures 5D and 5G). Correspondingly, BEACON2 led to less DSBs (Figure 5C) and fewer negative effects on cell proliferation than AncBE4Max and Cas9 (Figure 5D), as DSBs can activate p53 and then lead to negative effects on cell proliferation (Haapaniemi et al., 2018; Ihry et al., 2018). Of note, although they did not trigger DDR robustly (Figures 5H, S4A, and S4B), BE2 and YE1-BE3 (an engineered BE3 with narrowed editing windows) induced lower levels of editing efficiencies than BEACON2 (Figures 5B and 5E).

**RNA OT Effects**

It has been recently reported that BE3 can generate random transcriptome-wide RNA OT editing (Grünewald et al., 2019;
Zhou et al., 2019). Thus, we performed RNA sequencing (RNA-seq) to determine whether BEACONs also trigger RNA OT mutations with RADAR (RNA-editing analysis pipeline to decode all twelve types of RNA-editing events) (Figures 6 and S4E).

In agreement with previous findings that A-to-I is the most common type of RNA editing event (Eisenberg and Levanon, 2018; Ramaswami et al., 2013; Zhu et al., 2013), only A-to-I RNA editing was found to be predominantly distributed in RNA-seq datasets from untransfected control cells or Cas9-treated cells (Figure 6A). However, C-to-U RNA editing was dramatically increased in RNA-seq datasets from BE3-treated cells, demonstrating widespread and unintended RNA OT effects induced by the rA1 moiety of BE3 (Figures 6B and 6C; Zhou et al., 2019). In contrast, much less RNA OT effects were observed in cells treated with AncBE4max, BEACON1, or BEACON2 (Figure 6B). Of note, the hA3A-Y130F-containing BEACON2 only induced RNA OT mutations similar to the background level (Figures 6B and 6D), which is consistent with a basal level of RNA OT effects induced by previously reported hA3A-Y130F-conjugated BEs (Wang et al., 2018; Zhou et al., 2019).

In Vivo Base Editing Induced by BEACON

To test whether the BEACON system can induce base editing in vivo, one target cytosine in the mouse hydroxysteroid 17-beta dehydrogenase 3 (Hsd17b3) gene was chosen for editing (Figures 7A–7C). We co-injected the mRNA of BEACON1, BEACON2, or rA1-dCas12a-BE with the Hsd17b3-targeted crRNA into one-cell mouse embryos and then analyzed base-editing efficiencies at the blastocyst stage (Figure 7A). Among 8 and 14 embryos injected with BEACON1/crRNA and BEACON2/crRNA, respectively, 1 and 9 were found to bear edited cytosine at the Hsd17b3 locus (Figures 7B, S5C, and S5E). Deep-sequencing analysis confirmed the editing efficiencies at targeted C11 in these embryos (21.04% for BEACON1 and 17.04%–56.84% for BEACON2; Figure 7C). Meanwhile, only a basal level of editing was observed at four other nearby cytosines outside the editing window (C6, C14, C15, and C17; Figure 7C). In contrast, no edited embryo was detected in 11 embryos injected with rA1-dCas12a-BE/crRNA (Figures 7B and S5A). These results further demonstrated the improved in vivo editing efficiency by fusing engineered hA3A with dCas12a.

In addition to comparing BEACONs and rA1-dCas12a-BE in mouse embryos, we also transplanted the embryos into surrogate mothers (Figure 7A). A total of 24 pups were obtained from BEACON1-injected embryos (Figure 7D), and among them, 2 pups were edited (#7 and #11; Figure S5D) with the C11 editing frequencies at 57.73% or 23.10% in tails (Figure 7E). Consistent with the results from mouse embryos, no edited mouse pup was found from the rA1-dCas12a-BE injected embryos (Figure S5B). To further confirm the base editing in mouse offspring, we dissected one BEACON1-edited offspring (#7) and determined the editing frequencies in different tissues. The editing frequencies in heart, liver, spleen, lung, kidney, brain, and testis ranged from 51.34% to 71.24% (Figure 7E), similar to the editing frequency in the tail (Figure 7E). Finally, minimal indels were detected in the embryos or offspring treated with BEACONs (Figure S5F) and only a background level of distal OT editing (the base editing at OT sites where the crRNA potentially binds to) was detected in the tissues of the C11-edited mouse offspring (Figure S6), demonstrating the editing specificity induced by BEACON.

To further compare in vivo editing induced by BEACON2 with BE3 and AncBE4max, we also examined their efficiencies in mouse embryos at a target site within their overlapped editing windows (Wang et al., 2019; Figure S7). Both BE3 and AncBE4-max achieved very efficient C-to-T editing in injected mouse embryos (16 of 16 embryos edited and 14 of 15 embryos edited, respectively), and BEACON2 induced a relatively lower efficiency (8 of 15 embryos edited) (Figures S7A–S7D). These results suggested that the nickase activity is beneficial for in vivo editing efficiency. However, both BE3 and AncBE4max induced significant levels of indels in mouse embryos but BEACON2 did not (Figures S7E–S7G), consistent with the in vitro results (Figures 5D and 5G).

DISCUSSION

Great efforts have been made to develop more efficient and specific BEs for correcting pathogenic-related point mutations to treat relevant diseases (Ranzau and Komor, 2019; Rees and Liu, 2018; Yang et al., 2019), e.g., using nCas9 to boost editing efficiency and introducing alternations in the APOBEC moiety of BEs to suppress unwanted RNA editing (Grunewald et al., 2019; Komor et al., 2016; Nishida et al., 2016; Rees et al., 2019; Zhou et al., 2019). Besides the unwanted OT effects, nCas9-derived BEs also caused the formation of DSBs (Komor...
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A

RLM (Bloom syndrome) target site

Cas9 target site

Cas12a target site

SMPG2 (Primary pulmonary hypertension)

Cas9 target site

Cas12a target site

CHD2 (Epileptic encephalopathy)

Cas9 target site

Cas12a target site

PAFAH1B1 (Lissencephaly 1)

Cas9 target site

Cas12a target site

PDG6C (Cone dystrophy 4)

Cas9 target site

Cas12a target site

PMS2 (Lynch syndrome)

Cas9 target site

Cas12a target site

SPAST (Stoacic atrophy 4)

Cas9 target site

Cas12a target site

B

C-to-T editing frequency (%)

0

20

40

60

80

C

Fraction of cytosine substitutions

D

Indel frequency (%)

0

1

2

BE3

BE2

YE1-BE3

AncBE4max

BEACON1

BEACON2

NT

C-to-A

C-to-G

C-to-T

E

Normalized editing frequency (%)

100

200

300

P = 3 x 10^-10

F

Normalized C-to-T fraction (%)

100

70

60

P = 3 x 10^-10

P = 2 x 10^-9

P = 6 x 10^-10

P = 2 x 10^-9

H

U2OS/Replicate 1

15 kDa

50 kDa

270 kDa

100 12 14 17 28 28 0

100 26 67 25 27 0

100 66 99 99 99 99 99

p-p53

p53

p-ATM

ATM

p-ATR

ATR

Actin

(legend on next page)

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et al., 2016; Lei et al., 2018; Nishida et al., 2016). DSBs can activate downstream DDR proteins, e.g., the phosphorylation of p53 and H2AX, to disturb cellular homeostasis and eventually cause cell death (Haapaniemi et al., 2018; Ily et al., 2018), especially when simultaneous editing at multiple sites is pursued (Niu et al., 2017). In addition, it has been reported that DSBs also trigger large deletions or chromosomal rearrangements (Kosicki et al., 2018). As therapeutic applications of gene editing generally involve stem cells or primary cells, in which DDR pathways are active, the unavoidable DNA breaks induced by nCas9-derived BEs may impede their applications in clinics. Different from nCas9-based BEs, DSBs that are induced by dCas12a-conjugated BEs were barely detectable (Figures 5D and 5G); also, dCas12a-conjugated BEs activate lower levels of DDR proteins, including γH2AX and p53 (Figure 5H), than nCas9-BEs, shedding new light on the potential of dCas12a-conjugated BEs in therapeutics.

The original dCas12a-based BE exhibited relatively low editing efficiency (Li et al., 2018), which hampers its application in vivo. In this study, we screened different APOBEC/AID members and found that the fusion of hA3A-dCas12a could induce efficient base editing (Figure 2). With a series of structure-guided protein engineering, codon optimization, and editing window narrowing (Figures 3 and 4), we further developed the BEACON system, which induced high editing efficiency in mammalian cells (Figure 5) and in mouse embryos (Figure 7). The construction of BEACON also has several advantages in terms of editing precision. First, given that Cas12a has a generally high targeting specificity (Kim et al., 2016; Kleinstiver et al., 2016; Yan et al., 2017), we examined the editing induced by BEACON in vivo at predicted genomic OT sites and found BEACON induced undetectable OT editing at these sites (Figure S6). Moreover, BEACONs with the Y130F or Y132D alternation in the hA3A moiety have narrowed editing windows (Figures 4D, 4E, 4F, and S3B), which enable precise base editing when multiple cytosines locate in a same target site (Figures 7C and 7E). Finally, we also compared the RNA OT mutations triggered by BEACON2 induced only background levels of OT editing transcriptome wide (Figure 6). Of note, the incorporation of hA3A in BEACON may also induce efficient base editing in methylated DNA regions and/or GpC sites, as previously developed hA3A-nCas9-BE did (Wang et al., 2018).

In summary, we developed a dCas12a-based BEACON system and successfully applied it for both in vitro and in vivo editing (Table S1). The BEACON system achieves efficient and specific base editing without generating DNA breaks or triggering DDR cascades, which is essential for its broad applications in mammalian cells and in clinics.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107723.

ACKNOWLEDGMENTS

This work was supported by grants 2018YFC1004602 (J.C.), 2018YFA0801401 (J.C.), and 2019YFA0802804 (L.Y.) from National Key R&D Program of China and 31925011 (L.Y.), 91940306 (L.Y.), 31822016 (L.Y.), 81872305 (J.C.), 31600654 (J.C.), and 31600619 (B.Y.) from National Natural Science Foundation of China. We thank Molecular and Cell Biology Core Facility and Molecular Imaging Core Facility, School of Life Science and Technology, ShanghaiTech University for providing experimental service.
AUTHOR CONTRIBUTIONS

J.C., L.Y., X.H., and Z. Liu conceived, designed, and supervised the project. X.W., C.D., and W.Y. performed most experiments with the help of J. Li, Z. Lu, W.Z., J. Liang, and J. Wu on cell culture, immunofluorescence, and plasmid construction. S.H. performed animal experiments, supervised by Z. Liu. B.Y. analyzed hA3A structure and performed molecular simulation. J. Wei prepared libraries for deep sequencing, supervised by L.Y., Y.W., and Y.-C.X. performed

Figure 6. RNA Off-Target Editing Analysis by RADAR

(A) Histogram to show numbers of all 12 types of RNA editing in different defined regions from cells treated with Cas9, Cas9-BEs, and BEACONs. RNA editing was analyzed and visualized by RADAR (Figure S4E). Means ± SD are from two (NT) or three (Cas9, BE3, AncBE4max, BEACON1, and BEACON2) independent experiments.

(B) Manhattan plot of RNA off-target editing (C-to-U) frequency shown in (A).

(C and D) The RNA off-target editing frequencies and sites induced by BE3 replicate 1 (C) or BEACON2 replicate 1 (D).

See also Figure S4.
A

Supervulate & Mate

CrRNA & BE mRNA

Culture

Blastocysts

for analysis

Transfer

Surrogate mother

Pregnancy & delivery

Offspring for analysis

B

| Target gene | Base editor | No. of examined blastocysts | No. of base-edited blastocysts | Total blastocysts (%) | No. of indel-containing blastocysts | Total blastocysts (%) | No. of proximal OT editing-containing blastocysts | Total blastocysts (%) |
|-------------|-------------|-----------------------------|--------------------------------|-----------------------|-------------------------------------|-----------------------|-------------------------------------------------|-----------------------|
| Hsd17b3     | rA1-dCas12a-BE | 11                          | 0/11 (0%)                      | 0/11 (0%)             | 0/11 (0%)                           | 0/11 (0%)             | 0/11 (0%)                                       | 0/11 (0%)             |
| Hsd17b3     | BEACON1     | 8                           | 1/8 (12.5%)                    | 0/8 (0%)              | 0/8 (0%)                            | 0/8 (0%)              | 0/8 (0%)                                        | 0/8 (0%)              |
| Hsd17b3     | BEACON2     | 14                          | 9/14 (64.3%)                   | 0/14 (0%)             | 0/14 (0%)                           | 0/14 (0%)             | 0/14 (0%)                                       | 0/14 (0%)             |

C

HSD17B3

TTTCA

CAGCC

AGGAC

C14

C15

C17

Editing window

BEACON1

Embryo #5

C6 C11 C14 C15 C17

C6 C11 C14 C15 C17

C6 C11 C14 C15 C17

C6 C11 C14 C15 C17

BEACON2

Embryo #1

Embryo #4

Embryo #7

Embryo #11

Embryo #13

C6 C11 C14 C15 C17

C6 C11 C14 C15 C17

C6 C11 C14 C15 C17

C6 C11 C14 C15 C17

C6 C11 C14 C15 C17

D

| Target gene | Base editor | No. of transferred embryos | No. of offspring | No. of base-edited offspring | Total offspring (%) | No. of indel-containing offspring | Total offspring (%) | No. of proximal OT editing-containing offspring | Total offspring (%) |
|-------------|-------------|----------------------------|------------------|-----------------------------|--------------------|----------------------------------|--------------------|-------------------------------------------------|--------------------|
| Hsd17b3     | BEACON1     | 61                         | 24               | 2/24 (8.3%)                | 0/24 (0%)         | 0/24 (0%)                           | 0/24 (0%)         |
| Hsd17b3     | rA1-dCas12a-BE | 54                         | 8                | 0/8 (0%)                    | 0/8 (0%)          | 0/8 (0%)                            | 0/8 (0%)          |

E

Offspring #7 - tail

C6 C11 C14 C15 C17

Offspring #7 - heart

C6 C11 C14 C15 C17

Offspring #7 - liver

C6 C11 C14 C15 C17

Offspring #7 - spleen

C6 C11 C14 C15 C17

(legend on next page)
bioinformatics analyses, supervised by L.Y. Z.Z. provided technical support. J.C., L.Y., and B.Y. wrote the paper with input from the other authors.

DECLARATION OF INTERESTS

J.C., L.Y., X.H., B.Y., X.W., C.D., and W.Y. have filed patent applications on aspects on this work. The authors declare no competing non-financial interests.

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Figure 7. In Vivo Editing Mediated by BEACONs in Mouse Embryos and Offspring
(A) Schematic diagram illustrating the procedures of in vivo base editing in mouse embryos.
(B) Genotyping of mouse embryos treated with indicated BEs.
(C) HSD17B3 target sequence and C-to-T editing frequency induced by BEACON1 and BEACON2 in mouse embryos at blastocyst stage.
(D) Genotyping of mouse offspring treated with indicated BEs.
(E) C-to-T editing frequencies induced by BEACON1 in the tails of mouse offspring #7 and #11 and C-to-T editing frequencies induced by BEACON1 in different tissues of offspring #7.
See also Figures S5, S6, and S7.

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## Key Resources Table

### Antibodies

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| Rabbit monoclonal anti-phospho-ATM (Ser1981) (D6H9) | Cell Signaling Technology | Cat#5883; RRID: AB_10835213 |
| Rabbit monoclonal anti-phospho-p53 (Ser15) | Cell Signaling Technology | Cat#9284; RRID: AB_331464 |
| Rabbit monoclonal anti-phospho-ATR (Thr1989) | Cell Signaling Technology | Cat#58014; RRID: AB_2722679 |
| Mouse monoclonal anti-ATM [2C1(1A1)] | Abcam | Cat#ab78; RRID: AB_306089 |
| Mouse monoclonal anti-p53 (DO-1) | Santa Cruz | Cat#Sc-126; RRID: AB_628082 |
| Rabbit monoclonal anti-ATR | Abcam | Cat#ab10312; RRID: AB_297050 |
| Rabbit monoclonal anti-gamma H2AX (phosphor S139) [EP854(2)Y] | Abcam | Cat#ab81299; RRID: AB_1640564 |
| Mouse monoclonal anti-beta actin | Absci | Cat#AB21800 |
| Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594) | Abcam | Cat#ab150084; RRID: AB_2734147 |

### Chemicals, Peptides, and Recombinant Proteins

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| Protease inhibitor cocktail (EDTA-free, 100X in DMSO) | Medchemexpress (MCE) | Cat#HY-K0010 |
| Phosphatase inhibitor cocktail (2X Tubes, 100X) | Bimake | Cat#B15001 |
| LIPOFECTAMINE LTX | Life, Invitrogen | Cat#15338100 |
| QuickExtract™ DNA Extraction Solution | Epicenter | Cat#QE09050 |
| Pierce ECL Western Blotting substrate | Thermo | Cat#32106 |
| BisBenzimide H 33342 trihydrochloride | Sigma | Cat#B2261 |
| CC/Mount (TM) tissue mounting medium | Sigma | Cat#C9368 |

### Critical Commercial Assays

| Reagent or Resource | Source | Identifier |
|---------------------|--------|------------|
| MMESSAGE MMACHINE T7 ULTRA 1 KIT | Ambion | Cat#AM1345 |
| RNeasy Mini Kit (50) | QIAGEN | Cat#74104 |
| MEGASHORTSCRIPT T7 KIT 25 RXNS EACH | Ambion | Cat#AM1354 |
| MEGACLEAR KIT 20 RXNS EACH | Ambion | Cat#AM1908 |
| Clone Express®-II One step cloning Kit | Vazyme | Cat#C112-02 |
| Plasmid DNA extraction Kit | TIANGEN | Cat#DP107-T |
| Next Ultra II FS DNA Library Prep Kit | NEB | Cat#E7805L |
| TruSeq Stranded Total RNA Library Prep Kit | Illumina | Cat#RS-122-2201 |

### Deposited Data

- Deep-sequencing data: this paper, GSE145552
- Oligos used for plasmid construction, see Table S2: this paper, N/A
- crRNA and sgRNA target sequences and PCR primers for amplifying genomic DNA, see Table S3: this paper, N/A

*Continued on next page*
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Recombinant DNA     |        |            |
| pCMV-hA3B-dCas12a-BE| this paper | N/A        |
| pCMV-hAID-dCas12a-BE| this paper | N/A        |
| pCMV-hA3A-dCas12a-BE| this paper | N/A        |
| pCMV-hA3A-dCas12a-BE-op| this paper | N/A        |
| hA3A_W98A-dCas12a-BE| this paper | N/A        |
| hA3A_W98Y-dCas12a-BE| this paper | N/A        |
| hA3A_W104A-dCas12a-BE| this paper | N/A        |
| hA3A_W104Y-dCas12a-BE| this paper | N/A        |
| hA3A_P134A-dCas12a-BE| this paper | N/A        |
| hA3A_P134Y-dCas12a-BE| this paper | N/A        |
| hA3A_C101S-dCas12a-BE| this paper | N/A        |
| hA3A_C106S-dCas12a-BE| this paper | N/A        |
| hA3A_W98Y/W104A-dCas12a-BE| this paper | N/A        |
| hA3A_W98Y/P134Y-dCas12a-BE| this paper | N/A        |
| hA3A_W98Y/W104A/P134Y-dCas12a-BE| this paper | N/A        |
| hA3A-dCas12a-BE-op| this paper | N/A        |
| hA3A_W104A-dCas12a-BE-op| this paper | N/A        |
| hA3A_W104A/Y130F-dCas12a-BE-op| this paper | N/A        |
| hA3A_W104A/Y132D-dCas12a-BE-op| this paper | N/A        |
| hA3A_W98Y/W104A-dCas12a-BE-op| this paper | N/A        |
| hA3A_W98Y/W104A/Y130F-dCas12a-BE-op| this paper | N/A        |
| hA3A_W98Y/W104A/Y132D-dCas12a-BE-op| this paper | N/A        |
| hA3A_W104A/P134Y-dCas12a-BE-op| this paper | N/A        |
| hA3A_W104A/P134Y/Y130F-dCas12a-BE-op| this paper | N/A        |
| hA3A_W104A/P134Y/Y132D-dCas12a-BE-op| this paper | N/A        |
| hA3A_W98Y/W104A/Y130F/Y132D-dCas12a-BE-op| this paper | N/A        |
| pCMV-BE3            | Komor et al., 2016 | Addgene Plasmid #73021 |
| pCMV-AncBE4max      | Koblan et al., 2018 | Addgene Plasmid #112094 |
| pBK-YE1-BE3         | Kim et al., 2017 | Addgene Plasmid #85174 |

### Software and Algorithms

| Software and Algorithms | Website | Description |
|-------------------------|---------|-------------|
| FastQC v0.11.4          | http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ |            |
| Trimmomatic v0.36       | Bolger et al., 2014 | http://www.usadellab.org/cms/?page=trimmomatic |
| BWA v0.7.9              | Li and Durbin, 2009 | http://bio-bwa.sourceforge.net/ |
| HISAT2 v2.1.0           | Kim et al., 2019 | https://ccb.jhu.edu/software/hisat2/index.shtml |
| Picard v2.7.1           | https://broadinstitute.github.io/picard/ | https://broadinstitute.github.io/picard/ |
| GATK v4.1.2.0           | McKenna et al., 2010 | https://gatk.broadinstitute.org |
| BLAT v364               | Kent, 2002 | https://genome.ucsc.edu/cgi-bin/hgBlat |
| R v3.5.1                | https://www.r-project.org | https://www.r-project.org |
| RADAR                   | This paper | https://github.com/YangLab/RADAR |
| HPB                     | Zhu et al., 2013 | N/A |
| Zeiss LSM800            | http://www.zeiss.com/microscopy/us/products/confocal-microscopes.html | http://www.zeiss.com/microscopy/us/products/confocal-microscopes.html |
**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jia Chen (chenjia@shanghaitech.edu.cn).

**Materials Availability**
This study did not generate new unique reagents.

**Data and Code Availability**
The original DNA deep-sequencing and RNA sequencing data from this study have been deposited in the NCBI Gene Expression Omnibus (GEO: GSE145552) and the National Omics Data Encyclopedia (accession number OEP000822).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Animals**
All mice experiment procedures were approved by the Animal Care and Use Committee of the Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China. Mice were maintained in standard cages in an Assessment and Accreditation of Laboratory Animal Care credited specific pathogen free facility under a 12 hr dark-light cycle. Sample sizes were estimated based on experiments in similar studies, and the experiments were not randomized or blinded. Female B6D2F1 (C57BL/6 x DBA2J) mice (4-week-old) were superovulated and mated with male B6D2F1 mice (4-week-old). The injected zygotes were cultured in KSOM medium at 37°C (in 5% CO2) and transferred to oviducts of pseudopregnant females (ICR mice, 10-week-old).

**Human cell lines and cell culture**
HEK293T and U2OS cells from ATCC were maintained in DMEM (10566, GIBCO/Thermo Fisher Scientific) + 10% FBS (16000-044, GIBCO/Thermo Fisher Scientific) and regularly tested to exclude mycoplasma contamination.

**METHOD DETAILS**

**Plasmid construction**
Primer sets (hA3A_dCpf1_PCR_F/hA3A_dCpf1_PCR_R) were used to amplify the fragment Human_APOBEC3A with template pUC57-Human_APOBEC3A (synthesized by Genscript). Then the fragment Human_APOBEC3A was cloned into the SacI and Smal linearized dCpf1-BE with plasmid recombination kit Clone Express® (Vazyme, C112-02) to generate the hA3A-dCas12a-BE expression vector pCMV-SV40NLS-hAPOBEC3A-XTEN-dLbCpf1(D832A/E1006A/D1125A)-SV40NLS-SGGS-UGI-SV40NLS. hA3B-dCas12a-BE and hAID-dCas12a-BE expression vectors were constructed with the same strategy.

Two primer sets (hA3A_dCpf1_PCR_F/hA3A_dCpf1_W98A_PCR_R) (hA3A_dCpf1_W98A_PCR_F/hA3A_dCpf1_PCR_R) were used to amplify the W98A-containing fragment hA3A-W98A. Then the fragments were cloned into the Apal and Smal linearized hA3A-dCas12a-BE expression vector to generate the hA3A_W98A-dCas12a-BE expression vector pCMV-SV40NLS-hAPOBEC3A_W98A-XTEN-dLbCpf1(D832A/E1006A/D1125A)-SV40NLS-SGGS-UGI-SV40NLS. hA3A_W98A-dCas12a-BE, hA3A_W104A-dCas12a-BE, hA3A_W104Y-dCas12a-BE, hA3A_W104A/P134A-dCas12a-BE, hA3A_W104A/P134Y-dCas12a-BE, hA3A_W104A/P134D-dCas12a-BE, hA3A_W104A/P134A/P134Y/dCas12a-BE expression vectors were constructed with the same strategy.

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The sequences of the oligos used for plasmid construction are listed in Table S2 and the amino acid sequences of BEs are listed in Table S3.

Transfection
For base editing in genomic DNA, HEK293T and U2OS cells were seeded in a 24-well plate at a density of 1.6 × 10^5 per well and transfected with 200 μL serum-free Opti-MEM that contained 5.04 μL LIPOFECTAMINE LTX (Life, Invitrogen), 1.68 μL LIPOFECTAMINE PLUS (Life, Invitrogen), 1 μg rA1-dCas12a-BE expression vector (or hA3A-dCas12a-BE, hA3B-dCas12a-BE, hAID-dCas12a-BE, hA3AW98Y-dCas12a-BE, hA3AW98Y-dCas12a-BE, hA3A_W104A-dCas12a-BE, hA3A_W104A-dCas12a-BE, hA3A_W104A/P134Y-dCas12a-BE, hA3A_W104A/P134Y-dCas12a-BE, hA3A_W104A/P134Y/Y132D-dCas12a-BE-op, hA3AW98Y/W104A/Y130F-dCas12a-BE-op, hA3AW98Y/W104A/Y132D-dCas12a-BE-op, hA3AW98Y/W104A/Y132D-dCas12a-BE-op, hA3AW98Y/W104A/Y130F-dCas12a-BE-op, hA3A_W104A/P134Y/Y132D-dCas12a-BE-op, BE3 (addgene, #73021), BE2 (addgene, #73020), YE1-BE3 (addgene, #85174) and AncBE4max (addgene, #112094) expression vector) and 0.68 μg crRNA or sgRNA expression vector. After 72hr, the genomic DNA was extracted from the cells with QuickExtract DNA Extraction Solution (QE09050, Epicenter) or the cells were lysed in 2 × SDS loading buffer for western blot.

Cell sorting and RNA extraction
After 40hr after transfection, Cells in the first 15% of the fluorescence intensity were sorted by FACS Ariall. Total RNAs of sorted cells were extracted by using the RNEasy Mini Kit (Qiagen #74104).

In vitro transcription
rA1-dCas12a-BE, hA3A_W104A/Y132D-dCpf1-BE-op, hA3A_W98Y/W104A/Y130F-dCpf1-BE-op, BE3 (addgene, #73021) and AncBE4max (addgene, #73020), YE1-BE3 (addgene, #85174) and AncBE4max (addgene, #112094) expression vector) and 0.68 μg crRNA or sgRNA expression vector. After 72hr, the genomic DNA was extracted from the cells with QuickExtract DNA Extraction Solution (QE09050, Epicenter) or the cells were lysed in 2 × SDS loading buffer for western blot.

Microinjection and embryos transfer
Female B6D2F1 (C57BL/6 × DBA2J) mice (4-week-old) were superovulated and mated with male B6D2F1 mice. One-cell-stage embryos were collected to inject with crRNA/sgRNA (50 ng/ml) and rA1-dCas12a-BE, BEACON1, BEACON2, BE3 and AncBE4max (addgene, #11094) vector. After transfection, the embryos were microinjected into the cytoplasm of zygotes in a droplet of M2 medium containing 5 μg/ml cytochalasin B (CB) using a piezo (Primetech) microinjector. After microinjection, the injected zygotes were cultured in KSOM mediums at 37°C for 8 days. The injected zygotes were cultured under 5% of CO2 in air and transferred to oviducts of pseudopregnant females at 0.5 day post copulation.

Western blot
Transfected cells were lysed in NP40 lysis buffer (50 mM Tris pH 8.0, 150mM NaCl, 1% NP-40, 0.1% SDS, 1mM PMSF, protease inhibitors, and phosphatase inhibitor) for 30 min on ice, then incubated at 97°C for 1 hour after washing in PBST. Before mounting, cells were counterstained with DAPI. Pictures were collected on a confocal microscope (Zeiss LSM800).

Immunofluorescence
Transfected U2OS cells were fixed in 4% PFA for 1 hour at 4°C. After washing in PBS, cells were blocked with PBS containing 0.3% Triton X-100 and 2.5% BSA for 1 hour. Then, cells were incubated overnight at 4°C with primary antibodies (anti-gamma H2AX, 1:300) and secondary antibodies for 1h. Reactive bands were developed in ECL (Thermo Fisher Scientific) and detected with Amersham Imager 680.

DNA library preparation and sequencing
Target genomic sites were PCR amplified by high-fidelity DNA polymerase PrimeSTAR HS (Clonetech) with primers flanking each examined crRNA or sgRNA target site. The PCR primers used to amplify target genomic sequences were listed in Table S4. Indexed DNA libraries were prepared by using the TruSeq ChiP Sample Preparation Kit (Illumina) with some minor modifications. Briefly, the PCR products were fragmented by Covaris S220 and then amplified by using the TruSeq ChiP Sample Preparation Kit (Illumina). After being quantitatively with Qubit High-Sensitivity DNA kit (Life, Invitrogen), PCR products with different tags were pooled together for Illumina sequencing.
We developed a computational pipeline RADAR (RNA-editing Analysis-pipeline to Decode All twelve-types of RNA-editing events, https://github.com/YangLab/RADAR) to detect and visualize all possible twelve-types of RNA editing events from RNA-seq datasets. RADAR consists of three steps (Figure S4).

STEP 1: RNA-seq read mapping. After quality control by FastQC (version 0.11.4, parameters: default), RNA-seq reads are trimmed by Trimmomatic (version 0.36, parameters: TruSeq3-PE-2.fa:2:30:10 TRAILING:25 MINLEN:30) (Bolger et al., 2014) to remove read sequences with low quality, and then mapped to ribosomal DNA (rDNA) sequences by BWA-MEM algorithm (version 0.7.9a, parameters: default) to remove reads mapped to redundant rRNAs. To capture more mismatches for RNA editing candidates, a two-round unique mapping strategy is then applied to align high-quality RNA-seq reads to human hg38 reference genome, sequentially by HISAT2 (version 2.1.0, parameters:--rna-strandness RF--no-mixed-secondary--no-temp-splicesite-known-splicesite-infile--no-soft-clip-score-min L,--16.0-mp 7.7-rfg 0.7-rdg 0.7-max-seeds 20 -k 10-dta) (Kim et al., 2019) with up to two mismatches and by BWA-MEM (version, parameters: default). Unique mapped reads by HISAT2 and BWA-MEM with up to six mismatches are selected and combined for subsequent analysis. After marking duplicate reads identified by Picard (version 2.7.1, parameters: CREATE_INDEX = true VALIDATION_STRINGENCY = SILENT) in the BAM file, uniquely-mapped reads that span exon-exon junctions are split into segments by the GATK (version 4.1.2.0) (McKenna et al., 2010) command, SplitNCigarReads (parameters: default). Base quality scores of all uniquely-mapped reads are recalibrated by two GATK (version 4.1.2.0) commands, BaseRecalibrator (parameters: default) and ApplyBQSR (parameters: default).

STEP 2: RNA editing calling. RNA variants are determined from the BAM file with uniquely-mapped reads by the GATK (version 4.1.2.0) command HaplotypeCaller (parameters:--minimum-mapping-quality 0--stand_call_conf 0--dont-use-soft-clipped-bases true). After filtering out RNA variants overlapped with single nucleotide polymorphisms (SNPs) from dbSNP version 151 (https://www.ncbi.nlm.nih.gov/SNP/), the 1000 Genomes Project (https://www.internationalgenome.org/) and the University of Washington Exome Sequencing Project (https://evs.gs.washington.edu/EVS/), low-quality RNA variants with mapped read numbers < 2, hits per billion-mapped-bases (HPB; Zhu et al., 2013) < 3 or editing ratio < 0.05 were further removed.

RNA variants for potential RNA editing events are classified into three groups according to their genomic locations as previously described (Ramaswami et al., 2013), including in Alu, non-Alu repetitive or non-repetitive regions. Different to those in Alu regions, RNA variants in non-Alu repetitive and non-repetitive regions are further filtered with a series of stringent cutoffs to remove false positive. In brief, RNA variants in simple repeats, in mononucleotide microsatellites ≥ 5 bp or within 4 bp of splice junctions are removed. In addition, BLAST-like alignment tool (BLAT, version 364, parameters: -repMatch = 2253 -stepSize = 5) (Kent, 2002) is used to remove RNA variants within highly similar regions. Finally, RNA variants within bidirectional transcription regions are also removed. All twelve types of RNA editing events in Alu, non-Alu repetitive and non-repetitive regions are eventually determined according to the strands of overlapped genes (Human: hg38 knownGene.txt updated at 2015/6/28).

STEP 3: RNA editing visualizing. All possible RNA-editing events from each given RNA-seq dataset are listed in an Excel file. Numbers of all twelve-types of RNA editing events are plotted by histograms according to their genomic locations in Alu, repetitive non-Alu and non-repetitive regions. Manhattan plots are further used to illustrate RNA editing ratios of selected types of RNA-editing events, such as C-to-U or A-to-G.

**Image analysis/Immunostaining quantification**
The intensities of western blot bands were determined with ImageJ.

**Indel frequency calculation**
For dCas12a-BEs, indels were estimated in the aligned regions spanning from upstream 3 nucleotides to the downstream 48 nucleotides both according to PAM sites (55bp). For dCas9- and nCas9- BEs, indels were estimated in the aligned regions spanning from upstream 8 nucleotides to the target sites to downstream 19 nucleotides to PAM sites (50 bp). Indel frequencies were subsequently calculated through dividing the counts of reads containing at least one inserted and/or deleted nucleotides by the counts of all the mapped reads in the same region. Counts of indel-containing reads and total mapped reads are listed in Table S5.

**Base substitution calculation**
Base substitutions were selected at each position of the examined crRNA or sgRNA target sites that mapped with at least 1,000 independent reads, and obvious base substitutions were only observed at the targeted base editing sites. Counts of reads for each base and total reads are listed in Table S6. Base substitution frequencies were calculated by dividing base substitution reads by total reads.
**C-to-T fraction calculation**

C-to-T fractions were calculated by dividing T reads with the sum of non-C reads (A, G, T) at indicated editing sites.

**Molecular dynamics simulation**

Figures depicting the interaction between hA3A and ssDNA were prepared using the Pymol program (The PyMOL Molecular Graphics System, Version 2.1 Schrödinger, LLC.) All mutant hA3A-DNA complex structures were modified from the structure of hA3A in complex with ssDNA (PDBid:5KEG) and then energy optimized using Protein Preparation Wizard from Schrodinger Suite 2019-1 (https://www.schrodinger.com) following default settings. The simulation systems were solvated with SPC water and neutralized, containing Cl− and Na+ ions at a concentration of 0.15 M to mimic physiological ionic strength. 200 ns molecular dynamics simulations were then performed with Desmond from Schrodinger Suite 2019-1 (https://www.schrodinger.com), using OPLS3 force field. During the simulation, a Desmond implemented muti-stage MD simulation protocol was employed with Temperature T and pressure P kept constant at 310 K and 1 atm, respectively. The RMSD and RMSF of protein and DNA molecule as well as the protein-ligand contacts diagram were calculated with Simulation Interactions Diagram from Schrodinger Suite 2019-1 (https://www.schrodinger.com). The binding energy between hA3A variants and ssDNA along the simulation trajectory was calculated with third-party script thermal_mmgbsa.py from Schrodinger, with a step_size of 2.

**Statistical analysis**

For all figures, error bars show mean ± SD. Statistics and graphs were prepared using Prism software version 8. Statistical significance of differences in C-to-T editing frequency, indel frequency and C-to-T fraction were determined using Student’s t test (one-tailed). For all tests, p values of < 0.05 were considered statistically significant.