GENE EDITING

To BE or not to BE, that is the question

In vivo studies indicate that cytosine but not adenine base editors induce elevated levels of genome-wide off-target substitutions.

Jia Chen, Bei Yang and Li Yang

A variety of cytosine or adenine base editors (CBEs or ABEs) that combine native cytidine deaminases or in vitro evolved adenosine deaminases with CRISPR–Cas9 have been developed to induce targeted C-to-T or A-to-G base conversions with high efficiency and precision[1]. Recently, Zuo et al.[2] and Jin et al.[3] reported in *Science* that an early-developed and commonly used CBE, BE3, induces a large number of unintended base substitutions in mouse embryos and rice plants; in contrast, ABEs demonstrated high editing specificity in both studies. The off-target sites detected for BE3 in both in vivo studies had low levels of sequence similarity to on-target sites, showing that these off-target sites are generated in a sgRNA-independent manner. By contrast, an in vitro study comparing the editing specificity of an ABE (7.10), a CBE (BE3) and unmodified CRISPR–Cas9 published independently last month in *Nature Biotechnology*[4] found that the two base editors have similar numbers of sgRNA-dependent off-target sites and fewer than wild-type Cas9, and that they often recognize different off-target sites. Together, these studies highlight the necessity and urgency of developing more precise CBEs and show that our understanding of the mechanisms causing different types of off-target effects for CBEs, ABEs and Cas9 is still limited.

Base editors (BEs) have several advantages over CRISPR–Cas9 for making single point mutations in a genome. In CRISPR–Cas9 editing, the Cas9 endonuclease generates a double-strand break (DSB) at a target site complementary to its single-guide RNA (sgRNA). The DSB is repaired in most cases by non-homologous end joining, which can result in random insertions or deletions (indels) of nucleotides (Fig. 1a, bottom left). Less frequently, the DSB is resolved by homology-directed repair using a donor DNA template (Fig. 1a, bottom right), a pathway that can be exploited to introduce specific point mutations. Because BEs catalyze the deamination of cytidine or adenosine at target sites without generating

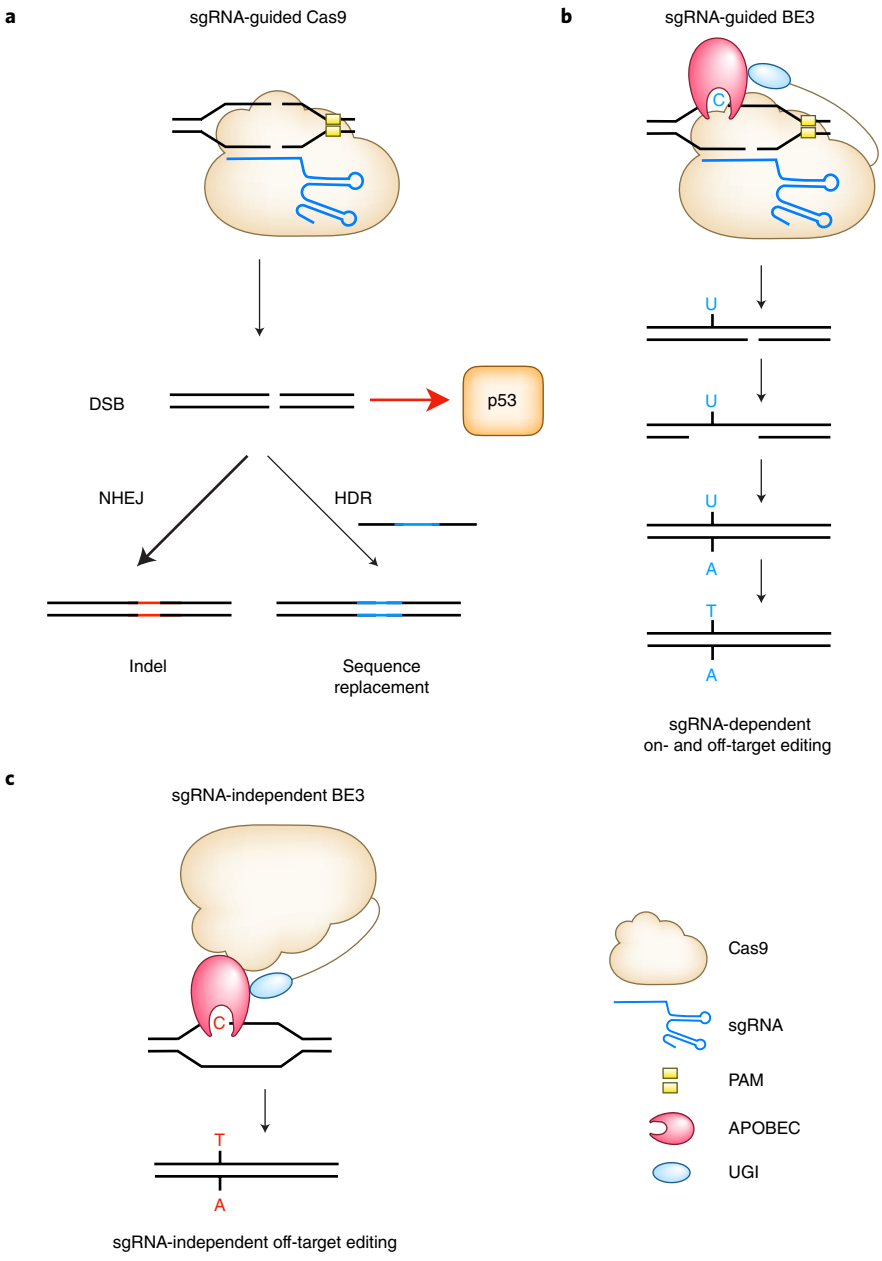

Fig. 1 | Cas9- and BE-mediated editing. a, Gene editing and p53-mediated DNA damage response induced by Cas9. **b**, sgRNA-dependent on-target and off-target base editing induced by BE3. **c**, sgRNA-independent off-target editing induced by BE3. NHEJ, non-homologous end joining; HDR, homology-directed repair; PAM, protospacer-adjacent motif; UGI, uracil DNA glycosylase inhibitor.

DSBs[1], they generally do not lead to indels, and because they do not rely on cell-cycle- or cell-type-dependent homology-directed repair, they are much more efficient than Cas9-mediated single-base editing. The subsequent DNA replication or repair processes incorporate a C-to-T or A-to-G substitution in the sgRNA-targeted genomic region (Fig. 1b) and have been shown to work in various cells and in living organisms. These features make base editing a promising system for correcting disease-associated genetic mutations.

BEs theoretically should also avoid the unexpected on-target side effects of Cas9 identified in recent studies, such as triggering the p53-mediated DNA damage response[5] (Fig. 1a, middle right) or inducing large genomic deletions at target sites[6]. These effects are elicited by DSBs and may lead to cell growth arrest or even neoplasia[5,6].

Cas9 has long been known to cleave DNA at off-target sites that are partially complementary to the sgRNA. Because BEs use catalytically dead Cas9 proteins or Cas9 nickases as the 'locus locator', base editing has a similar potential for off-target activity[4,7]. However, this problem has been greatly reduced for both types of editing by developing or conjugating engineered versions of Cas9 with improved targeting specificity (e.g., HF1-BE3)[1].

Recently, Zuo et al.[2] and Jin et al.[3] applied genome-wide methods to detect and compare global C-to-T conversions in living organisms treated with Cas9 or BE3, a conjugate of a Cas9 nickase with the rat APOBEC1 cytidine deaminase (rA1). Surprisingly, they found that BE3 caused more off-target base conversions than the ABE in both mouse embryos[2] and rice[3] and more single nucleotide variants than wild-type Cas9 in mouse embryos (with no statistically significant difference in the number of indels)[2]. Although the underlying mechanism was not fully addressed, several lines of evidence suggest that the observed off-target C-to-T conversions could be sgRNA–Cas9 independent. First, these off-target sites edited by BE3 exhibited low levels of, or even no, sequence similarity to on-target sites. In addition, BE3 triggered high levels of off-target base conversions in the absence of sgRNA. Working in rice, Jin et al.[3] also found that HF1-BE3 induced even more off-target base conversions than BE3.

Second, the off-target editing sites observed in two independent mouse embryos microinjected with the same sgRNA and BE3 did not overlap[2]. This indicates that the generation of these off-target editing events is non-sgRNA–Cas9-specific. Given that APOBEC family members are well known as DNA 'mutators' to induce C-to-T substitutions in single-stranded genomic regions[8] that can be generated during various cellular processes, all these observations suggest that the detected off-target editing[2,3] may be caused by the rA1 moiety of BE3 (Fig. 1c). Also, the fact that these substantial differences are notobserved in vitro[4] suggests that the mechanism underlying sgRNA-independent and sgRNA-dependent off-target effects needs to be explored in the context of the intracellular environment and/or active cellular processes.

How can the rA1-related off-target events that arise during BE3-mediated base editing be reduced? The discovery that ABEs do not induce substantial off-target editing[2–4] sheds some light on this question. As the adenine deaminases used in ABEs are evolved from a tRNA-specific adenosine deaminase, these enzymes may have lower DNA binding or catalytic activity than rA1. Thus, it is possible that the use of native or engineered APOBEC proteins with relatively low DNA binding or catalytic activity[9,10] may help to reduce off-target base editing. Some previous studies have already shown that the use of engineered APOBECs with lower DNA binding activity in CBEs narrows the editing windows[9,10], thus reducing proximal off-target editing[1]. Whether these engineered CBEs[9,10] also bear lower sgRNA-independent off-target editing than BE3 awaits further investigation.

However, it is worth noting that reducing DNA binding or catalytic activity may lead to other concerns. Although the delivery of BEs or their expression vectors into zygotes by microinjection often gives high on-target editing efficiencies, the delivery efficiencies in tissues or somatic cells (for example, primary blood cells) and in plants are generally low in practice. Thus, the use of activity-reduced BEs may lead to inefficient on-target editing. Because high on-target activity is essential for broad applications of base editing, especially for therapeutic applications in somatic cells, it may be prudent to adopt strategies other than using activity-reduced deaminases when developing high-precision BEs.

In summary, the finding that the commonly used BE3 induces unexpected off-target base substitutions[2,3] not only highlights important considerations for the safety of base editing, but also, more importantly, suggests directions for the development of new BEs with greater fidelity. Given the rapid evolution of the gene editing field, there is every reason to think that these revelations about off-target activity will spur the development of ever-more-precise BEs going forward. ❐

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Published online: 18 April 2019

https://doi.org/10.1038/s41587-019-0119-x

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Competing interests

The authors declare no competing financial interests.