

Editing the Genome Without Double-Stranded DNA Breaks

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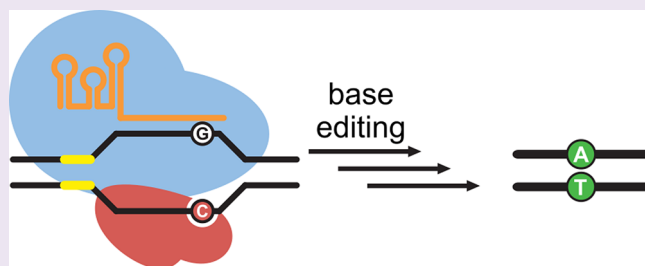
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ABSTRACT: Genome editing methods have commonly relied on the initial introduction of double-stranded DNA breaks (DSBs), resulting in stochastic insertions, deletions, and translocations at the target genomic locus. To achieve gene correction, these methods typically require the introduction of exogenous DNA repair templates and low-efficiency homologous recombination processes. In this review, we describe alternative, mechanistically motivated strategies to perform chemistry on the genome of unmodified cells without introducing DSBs. One such strategy, base editing, uses chemical and biological insights to directly and permanently convert one target base pair to another. Despite its recent introduction, base editing has already enabled a number of new capabilities and applications in the genome editing community. We summarize these advances here and discuss the new possibilities that this method has unveiled, concluding with a brief analysis of future prospects for genome and transcriptome editing without double-stranded DNA cleavage.



Traditional approaches to edit genomes in living cells introduce a double-stranded DNA break (DSB) at a desired genomic locus. Genomic DNA near the DSB can be replaced with an exogenous donor DNA template by using the endogenous homology-directed repair (HDR) pathway. HDR requires that the donor DNA be homologous to the targeted locus to precisely and predictably result in gene modification.¹ Nonhomologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and single-strand annealing (SSA) are complementary endogenous DSB repair pathways that ultimately result in the accumulation of stochastic insertions and deletions (indels) or translocations at the site of the DSB.^{2–5} Notably, DSBs are typically repaired more efficiently through these pathways than through HDR, especially in nonmitotic cells.

CRISPR systems are RNA-guided protein endonucleases that have stimulated a renaissance in the field of genome editing, allowing researchers to introduce a DSB at nearly any location in the genome with unprecedented ease. In CRISPR systems, Cas endonuclease proteins form effector complexes with “guide RNA” molecules, which program the resultant complex to localize to and cleave a target nucleic acid sequence (the protospacer) through canonical Watson–Crick base-pairing. Protospacer sequences are constrained by the requirement for a nearby protospacer-adjacent motif (PAM), a Cas protein-dependent short nucleic acid sequence.⁶ Reprogramming the complex to recognize and cut an alternative DNA or RNA sequence simply requires replacing the spacer of the guide RNA with the new sequence of interest.^{7,8} The advent and implementation of the CRISPR–Cas systems for use in

eukaryotic genome editing^{9–11} has led to an explosion of advances in the life sciences over a remarkably short time.¹²

Despite significant progress to improve HDR-mediated genome editing outcomes in cultured cells, largely through the inhibition of NHEJ, synchronization of cells, and donor template designs,^{13–17} current strategies to precisely modify eukaryotic genomes using HDR under therapeutically relevant conditions remain inefficient, especially in unmodified, non-replicating cells, and frequently result in stochastic mixtures of genome modifications. Motivated by these limitations, we and others have developed alternative approaches to genome editing that do not rely on HDR. Base editing, a novel genome editing strategy, integrates concepts drawn from chemical biology and genome editing to enable the direct chemical conversion of one target genomic DNA base into another at an intended locus without inducing DSB formation.^{18–21} In this review article, we discuss base editing and other strategies for genome editing that do not require DSBs, with an emphasis on recent applications and prospects for the future of this emerging field.

■ OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS

Site-directed mutagenesis is a powerful and enabling molecular cloning technology. This method integrates a short DNA oligonucleotide homologous to a region of interest but

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containing a desired mutation,²² enabling DNA modification in a locus-specific manner. This strategy was successfully adapted for genome editing in yeast by transforming *S. cerevisiae* with oligonucleotides harboring the desired modifications, introducing multiple mutations with efficiencies reaching 0.1%.²³ Mechanistic analysis later revealed that *in vivo* oligonucleotide-directed mutagenesis (ODM) did not require DSB formation but rather involved annealing of the oligonucleotide with the genomic DNA during replication to form an Okazaki fragment-like intermediate, followed by incorporation of the mutation *via* the mismatch repair (MMR) pathway.²⁴

Unsurprisingly, ODM efficiencies are governed by the initial annealing between the oligonucleotide and the targeted genomic locus.²⁵ To improve *in vivo* hybridization efficiencies, researchers used “chimeraplasts,” chimeric DNA–RNA oligonucleotides containing hairpin-capped ends (Figure 1).²⁶ The

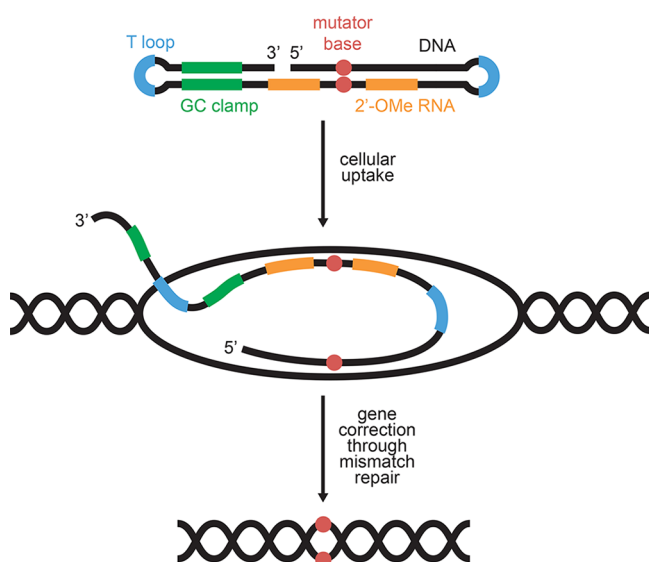


Figure 1. Genome editing using oligonucleotide-directed mutagenesis (ODM). The chimeraplast (top) is a DNA–RNA hybrid double hairpin containing a mutated sequence (red) homologous to the target locus of interest. 2'-OMe RNA (orange) is incorporated in the homology region to increase the binding affinity between the chimeraplast and genomic DNA. A GC clamp (green) enhances nuclease resistance, while hairpin loops (blue) at the very ends of the chimeraplast prevent concatamerization. Following cellular uptake, the chimeraplast anneals to the genomic DNA, forming mismatches at the mutator base. The endogenous cellular mismatch repair pathway will then repair the mismatches at low frequencies using the chimeraplast as a template instead of the genomic DNA.

DNA segment is complementary to the gene of interest and bears the desired mutation(s), while the RNA segments improve hybridization with genomic DNA.²⁷ The resulting genomic DNA:chimeric oligonucleotide heteroduplex is recognized by the endogenous cellular mismatch repair machinery and repaired, albeit infrequently, using the chimeraplast as a template instead of the genomic DNA.²⁸

Further optimization has resulted in the successful use of chimeraplasts in eukaryotic cells *in vitro*^{26,29–31} and *in vivo*.^{32,33} Unfortunately, the generality of this approach is limited by its dependence on many factors, including the target sequence, transcription level of the target genetic locus, target cell type, cellular replication state, and abundance of the Rad51 and MSH2 proteins.^{34–36} Collectively, these dependencies result in

large variations in efficiency.^{37,38} Such unpredictable outcomes have made ODM (in its current form) unsuitable for some research applications and most therapeutic uses. However, ODM has been extensively optimized for use in plant genome editing despite requiring single-nucleotide polymorphism screening to discover the desired genetic change(s). Such editing pipelines have facilitated genome modification in tobacco,^{39,40} corn,^{41,42} rice,⁴³ wheat,⁴⁴ and rapeseed.⁴⁵ Notably, ODM has been used to develop a nontransgenic plant breeding technology that has led to a commercial, nontransgenic herbicide-resistant canola.⁴⁶

To combat the inconsistencies with the ODM method, Storici and co-workers developed the two-step double selection method “*delitto perfetto*.”⁴⁷ The first step uses homologous recombination (HR) to incorporate a cassette containing a counterselectable marker and a reporter gene into a genomic locus of interest without explicitly creating DSBs. Cells that have successfully incorporated the cassette are isolated using the reporter gene. Next, an oligonucleotide that includes the mutation of interest is delivered into the cells and incorporated into the genome while excising the cassette, again *via* HR. Counterselection yields those cells with the desired mutation. This method has been successfully used in both yeast⁴⁸ and mammalian cells,^{49,50} but due to its reliance on multiple HR events and double selection, its use is restricted to those cell types that express proteins such as Rad52 that are required for HR, and its intrinsic efficiency is low.

BASE EDITING

We recently reported the development of base editing, a genome editing methodology that allows the precise, irreversible conversion of one base pair to another in a programmable manner, obviating the need for DSB formation or HDR.¹⁸ Other laboratories have since developed and reported related systems, demonstrating the robustness and scope of the base editing strategy.¹⁹ Our initial base editors used a single-stranded DNA-specific cytosine deaminase enzyme tethered to a catalytically impaired Cas9 protein (dCas9) to convert a C·G base pair to a T·A base pair at a target locus of interest (Figure 2a). The deaminase-dCas9 fusion catalyzes the hydrolytic deamination of cytosine to uridine within a small (~3- to 5-nucleotide) window of the target protospacer by exploiting a short segment of accessible single-stranded DNA in the “R-loop” of the Cas9:guide RNA:DNA ternary complex.⁵¹ To enhance conversion of the U·G intermediate to the desired T·A product in eukaryotic cells, we fused an 83 amino acid uracil glycosylase inhibitor (UGI) to the deaminase-dCas9 construct to inhibit uracil excision following deamination (Figure 2b). In addition, we reverted an inactivating catalytic mutation in the HNH domain of dCas9 to enable DNA nicking of the G-containing strand opposite the newly formed uracil, thereby inducing host MMR to repair the U·G mismatch into a U·A pair (Figure 2b).⁵² The combination of these innovations resulted in BE3, a single protein consisting of a tripartite fusion between the *Rattus norvegicus* APOBEC1 cytosine deaminase, *Streptococcus pyogenes* Cas9n(D10A), and *Bacillus subtilis* bacteriophage PBS2 UGI (Figure 2a). BE3 results in permanent C·G-to-T·A correction and does not rely on DSB formation, yielding higher base editing efficiencies than HDR (typically, 15–75% for BE3) and much lower indel frequencies than nuclease-mediated approaches (typically <5% for BE3). In addition, multiple studies have shown that BE3 has fewer off-target editing events than

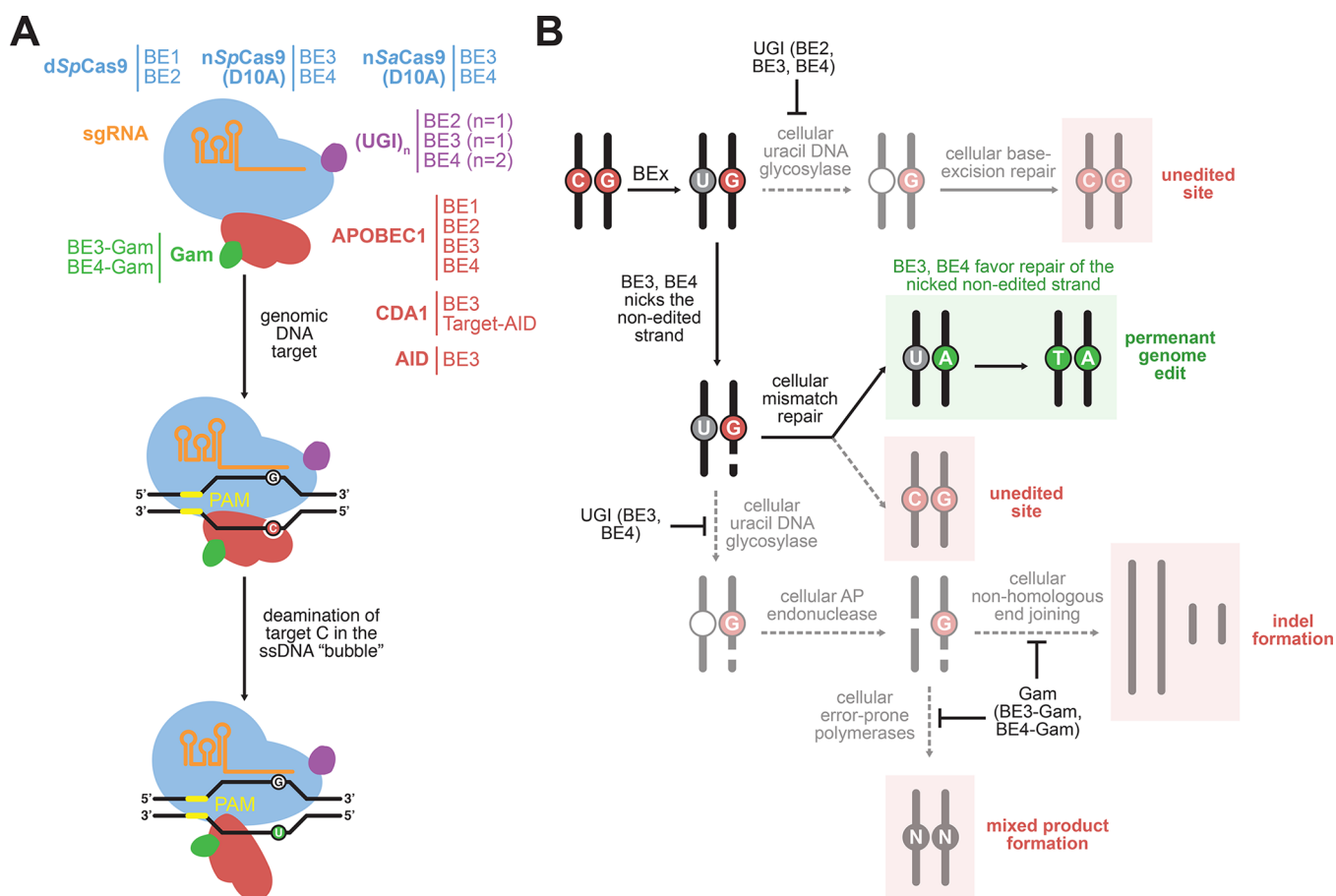


Figure 2. Overview of C-G-to-T-A base editing. (A) The components of C-G-to-T-A base editors. The sgRNA (orange) complexes with a Cas9 homologue (blue) to direct the base editor to a genomic locus of interest. A ssDNA-specific cytidine deaminase enzyme (red) catalytically deaminates cytosine nucleobases within the single-stranded portion of the R-loop to uracil nucleobases. The addition of one or more copies of uracil glycosylase inhibitor (UGI, purple) and the bacteriophage Mu DSB-binding protein Gam (green) help to maximize resolution of the U-G intermediate to a T·A base pair. (B) Possible outcomes following formation of the U-G base editing intermediate. Uracil DNA glycosylase (UNG) can excise uracil from the U-G mismatch, resulting in an abasic site that is ultimately converted back to a C-G base pair. The UGI component of base editors (in BE2, BE3, and BE4) suppresses uracil excision. The BE3 and BE4 editors incorporate a Cas9 nickase to selectively nick the G-containing strand opposite the converted uracil. This nick guides the mismatch repair machinery to preferentially replace the G-containing strand, resulting in the desired T·A base pair. Excision of uracil from the nick-containing intermediate by UNG, followed by abasic site processing by AP endonuclease can yield a DSB. The DSB can be processed by NHEJ to produce indels, or by error-prone polymerases to yield mixed products such as G·C or A·T base pairs.

Cas9.^{18,53,54} This chemoselective genomic modification can be efficiently achieved in living cells and organisms^{54–56} without resorting to the introduction of synonymous mutations *via* HDR to limit Cas9 re-engagement,¹³ or subjecting cells to perturbative conditions that enhance HDR efficiency.^{14–16}

In addition to BE3, convergent strategies for genome editing have been developed by the community to address the inherent limitations of using DSBs for gene correction. Target-AID is a BE3-like base editor that uses an alternative architecture and deaminase domain, resulting in a C-G-to-T·A genome editing agent with a shifted deamination window compared to BE3.¹⁹ CRISPR-X²¹ and targeted AID-induced mutagenesis (TAM)²⁰ are techniques that implement dCas9-cytidine deaminase fusions for targeted saturation mutagenesis of gRNA-dictated genomic regions. In the absence of any uracil glycosylase inhibitor such as UGI, the U-G intermediate is converted to an abasic site by the base excision repair enzyme uracil DNA glycosylase and can be further resolved by error-prone DNA repair processes to form G·C and A·T base pairs, rather than T·A products (Figure 2b).⁵⁷ CRISPR-X and TAM exploit this

alternate repair outcome to stochastically mutate the targeted C·G base pairs, enabling the generation of genetically encoded mutant libraries.

Despite its relatively recent addition as a genome editing tool, base editing has already enabled new applications. BE3 was used to develop CRISPR-STOP and iSTOP, general methods to generate gene knockouts through the introduction of stop codons.^{58,59} As base editing largely avoids the introduction of indels, the resulting gene knockout edits are predictable, and the formation of potentially cytotoxic or confounding DSB intermediates and their byproducts is avoided. A ligand-responsive base editor has also been engineered by incorporating a small-molecule activated self-cleaving element into the guide RNA used by BE3, enabling robust spatiotemporal control over base editing activity.⁶⁰ In addition, BE3 variants that collectively expand the targeting scope of base editing have been developed, including variants with altered PAM requirements (increasing the number of potential genomic sites amenable to base editing by 2.5-fold),⁶¹ narrowed editing windows (enabling high-resolution discrim-

ination of neighboring cytidines in the protospacer),⁶¹ and reduced off-target editing (producing higher confidence genome editing selectivity).⁵⁴ We recently engineered a fourth generation base editor, BE4, with improved uracil glycosylase inhibition that halves the frequency with which the target C-G is converted to base pairs other than the desired T·A product (Figure 2).⁵⁷ Fusion of this optimized construct to the DSB-binding protein Gam from bacteriophage Mu yielded BE4-Gam, a base editor that retains the optimized properties of BE4 while further reducing indel formation (Figure 2).⁵⁷

Base editing has been used in a wide range of organisms. As the first example of successful base editing in plants, BE3 was used to edit the *OsNRT1.1B*, *OsSLR1*, *OsPDS*, and *OsSBE1b* genes in rice, with C·G-to-T·A editing efficiencies between 12 and 20%.^{62,63} These studies were complemented by additional work using BE3 to edit multiple genes in rice, wheat, and corn with C·G-to-T·A efficiencies as high as 44%.⁶⁴ Furthermore, Target-AID was used to introduce herbicide-resistant C·G-to-T·A point mutations in rice at efficiencies up to 32% and modify genes involved in plant hormone signaling in tomatoes with up to 21% of progeny containing homozygous, heterozygous, or biallelic base substitutions (including various transversions and transitions).⁶⁵ Recently, BE3 mRNA micro-injection was shown to catalyze the introduction of C·G-to-T·A mutations in the *Dmd* and *Tyr* genes of mouse embryos with frequencies approaching 100%.⁵⁵ Additionally, purified BE3:sgRNA complexes have been used for DNA-free genome editing in zebrafish embryos and live mice inner ears.⁵⁴ These exciting studies illustrate the robustness of a C·G-to-T·A base in a variety of organisms ranging from microbes to plants to mammals.

FUTURE DIRECTIONS

The major limitation of base editing thus far has been an inability to achieve other types of base-to-base conversions beyond C·G-to-T·A mutations. Numerous DNA-modifying enzymes, most notably methyltransferases, have been harnessed in combination with Cas9 as epigenome editing tools,^{66–68} yet the repertoire of naturally occurring enzymes capable of chemically modifying DNA bases in ways that alter their base pairing properties is quite limited. In contrast to the dearth of DNA-modifying catalysts, RNA is extensively post-transcriptionally modified by a variety of naturally occurring enzymes.^{69–71} The development of programmable RNA base editors has the potential to modulate the biological activity of RNAs with exquisite temporal resolution and thus enable novel therapeutics without the risk of permanently modifying the native genetic information.

Indeed, the double-stranded RNA adenosine deaminase editing enzyme ADAR2 (which deaminates adenosine to inosine, which is read as guanine by both translational and splicing enzymes)⁷² has already been repurposed for sequence-specific A-to-I RNA editing in mammalian cells.^{73–78} Researchers attached the catalytic domain of ADAR2 (which acts only on dsRNA) to an antisense RNA oligonucleotide using either a SNAP-tag^{75,77,78} or the λ -phage N protein-boxB RNA hairpin interaction.^{74,76} Coexpression of these fusion proteins with their corresponding antisense RNA results in localization of ADAR2 to mRNA that is complementary to the antisense oligonucleotide. A-to-I editing then occurs at adenosines within the resulting double-stranded RNA region. Using a similar strategy, the wild-type ADAR2 enzyme was redirected to mRNA regions of interest using an antisense RNA

oligonucleotide fused to the natural ADAR2 RNA substrate.⁷³ The recent discovery of the RNA-guided endoribonuclease C2c2 (Cas13a)⁷⁹ will likely enable additional opportunities for RNA-guided sequence-specific RNA editing. A combination of chemistry, protein engineering, directed evolution, and/or molecular biology could be used to transform these (and other) enzymes into new classes of genome editing tools for the transient, sequence-specific modification of RNAs.

CONCLUSION

Methods to convert one target base pair to a different base pair without requiring DSBs are promising technologies for the study and potential treatment of genetic diseases. By circumventing the introduction of cytotoxic and mutagenic DSBs, the cell cycle-dependent HDR repair pathway, and the stochastic NHEJ and related repair pathways, DSB-free genome editing methods such as base editing facilitate precise and controllable genome editing in a robust and general manner. Recently, studies have illustrated the potential of these approaches to address the limitations that arise from DSBs and demonstrate the promise of applying chemical principles to the field of genome editing.

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Notes

The authors declare the following competing financial interest(s): A.C.K. and D.R.L. have filed provisional patent applications on base editing through Harvard University. D.R.L. is a consultant and co-founder of Editas Medicine and Beam Therapeutics, companies that are developing genome-editing therapeutics.

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