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Publication Date

2021-06-01

DOI

10.1016/j.ggedit.2021.100005

Peer reviewed

HHS Public Access

Author manuscript

Gene Genome Ed. Author manuscript; available in PMC 2021 August 05.

Published in final edited form as:

Gene Genome Ed. 2021 June ; 1: . doi:10.1016/j.ggedit.2021.100005.

Base Editors: Expanding the Types of DNA Damage Products Harnessed for Genome Editing

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Abstract

Base editors are an innovative addition to the genome editing toolbox that introduced a new genome editing strategy to the field. Instead of using double-stranded DNA breaks, base editors use nucleobase modification chemistry to efficiently and precisely incorporate single nucleotide variants (SNVs) into the genome of living cells. Two classes of DNA base editors currently exist: deoxycytidine deamination-derived editors (CBEs, which facilitate C•G to T•A mutations) and deoxyadenosine deamination-derived base editors (ABEs, which facilitate A•T to G•C mutations). More recently, the development of mitochondrial base editors allowed the introduction of C•G to T•A mutations into mitochondrial DNA as well. Base editors show great potential as therapeutic agents and research tools, and extensive studies have been carried out to improve upon the original base editor constructs to aid researchers in a variety of disciplines. Despite their widespread use, there are few publications that focus on elucidating the biological pathways involved during the processing of base editor intermediates. Because base editors introduce unique types of DNA damage products (a U•G mismatch with a DNA backbone nick for CBEs, and an I•T mismatch with a DNA backbone nick for ABEs) to facilitate genome editing, a deep understanding of the DNA damage repair pathways that facilitate or impede base editing represents an important aspect for the further expansion and improvement of the technologies. Here, we first review canonical deoxyuridine, deoxyinosine, and single-stranded break repair. Then, we discuss how interactions among these different repair processes can lead to different base editing outcomes. Through this review, we hope to promote thoughtful discussions on the DNA repair mechanisms of base editing, as well as help researchers in the improvement of the current base editors and the development of new base editors.

1. Introduction

Single nucleotide variants (SNVs) account for over 96% of currently identified human genetic variation.[1] While the majority of observed genetic variants (over 99%) currently lack a clinical interpretation, of those known to cause genetic diseases, roughly 55% are SNVs. [2] Therefore, the ability to precisely and efficiently introduce SNVs into the genome

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11.Competing Interests

A.C.K. is a member of the SAB of Pairwise Plants, and is an equity holder for Pairwise Plants and Beam Therapeutics. A.C.K.'s interests have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies

of living cells holds great potential for the treatment of genetic diseases, as well as for modelling the impact that currently unclassified genetic variants have on cellular function and human health. The development of CRISPR-associated (Cas) nucleases as genome editing agents has led to a revolution and paved the way for the development of next generation genome editing agents, such as base editors, the subject of this review. [3,4]

Originally functioning as adaptive immune systems in bacteria and archaea, Cas nucleases (such as Cas9 and Cas12) have been repurposed to perform genome editing in a wide variety of cells and organisms. Their ability to create double-stranded DNA breaks (DSBs) with high efficiencies in a programmable, RNA-guided manner has enable their rapid adoption by the genome editing community at large. To direct Cas nucleases to a genomic locus of interest, researchers simply change the spacer sequence of the guide RNA (gRNA). This then facilitates Cas:gRNA-DNA binding via Watson-Crick-Franklin base pairing rules between the spacer sequence of the gRNA and the protospacer sequence of the target locus. The protospacer must also be directly next to a protospacer adjacent motif (PAM). Once bound to its target DNA sequence, the Cas nuclease will introduce a DSB.

However, the introduction of a DSB is only the first step of the genome editing process. The next step involves the cellular response to this DNA damage product. In fact, all current genome editing technologies (including “nontraditional” genome editing agents, such as base editors and prime editors, which are discussed later) employ a two-step process to perform their function: 1) Deliberately introduce a DNA lesion at a user-programmed location in the genome, followed by 2) Manipulate or rely on the endogenous DNA damage repair pathways to process the lesion into a desired editing outcome. In the case of DSB repair (which has been extensively studied and reviewed elsewhere [5,6]), multiple pathways compete with each other to process the lesion, resulting in a mixture of outcomes. In brief, processing by the non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) pathways results in insertions, deletions, translocations, and other rearrangements at the site of the break, which are particularly useful for gene disruption studies.[7,8] Alternatively, precise DNA edits can be installed via the homology-directed repair (HDR) pathway through the use of an exogenously supplied DNA donor template. This donor template is designed to contain the desired sequence modification flanked by homology arms; the HDR machinery will use this as a template to repair the DSB and in the process incorporate the modified sequence into the genome.[9] HDR-mediated genome editing can be used to install any type of sequence modification of interest, including SNVs, but it is highly cell cycle dependent (and only active during G2 and S phases), and usually outcompeted by NHEJ for the repair of DSBs. [10,11]

DNA base editing is a recent technology specifically developed to combat these challenges inherent to DSB-reliant genome editing methods. [12,13] Base editors facilitate the efficient and precise introduction of SNVs using non-DSB DNA damage intermediates. DNA base editors are generally protein fusion constructs consisting of a catalytically impaired Cas nuclease (dCas9, dCas12, or Cas9n) linked to a single-stranded DNA (ssDNA)-specific nucleobase modifying enzyme (Figure 1A). Upon localization of the Cas protein to its target DNA sequence, hybridization of the gRNA to its complementary DNA sequence displaces and exposes a small stretch of ssDNA at the PAM-distal region of the non-complementary

strand. This exposure allows the ssDNA modifying enzyme to gain access to and chemically modify its target base within this ssDNA window. To date, by harnessing hydro-deamination chemistry performed by deaminase enzymes, two major classes of base editors have been developed. Cytosine base editors (CBEs) catalyze the transition mutation of C•G base pairs to T•A base pairs via the deamination of deoxycytidine to deoxyuridine as the mutagenic intermediate (Figure 1B). Adenine base editors (ABEs) catalyze the transition mutation of A•T base pairs to G•C base pairs via the deamination of deoxyadenosine to form deoxyinosine as the mutagenic intermediate (Figure 1C). Because both deoxyuridine and deoxyinosine are noncanonical DNA nucleotides, they are recognized as DNA lesions by the cell and repaired by cellular DNA repair mechanisms distinct from those utilized in DSB-reliant methods. To improve base editing efficiencies, the catalytically dead dCas9 protein can be changed to the nickase version, Cas9n, to introduce a nick on the non-modified DNA strand. This nick serves to bias the cellular repair mechanisms to preferentially replace this strand and use the mutagenic intermediate as a template for repair.

Base editors therefore use unique types of DNA damage: a mutagenic, noncanonical DNA nucleotide on one strand, and a DNA backbone nick on the other strand, 5’ upstream from the modified nucleotide (Figure 1D). A variety of comprehensive reviews have been reported elsewhere on the development of base editing technologies, including the many modifications of the original systems. [14-18] Therefore, here we focus on the potential DNA repair mechanisms of base editing. We hope to aid researchers in the improvement of current base editors and promote thoughtful discussion on the identification of potential intermediate to use for new base editors.

2. Deoxycytidine Deamination-Derived Base Editors: C•G to T•A Base editors (CBEs), C•G to G•C Base Editors (CGBEs) and mitochondrial C•G to T•A Base editors (DdCBEs)

The first DNA base editor utilized a naturally occurring ssDNA-specific cytidine deaminase, rAPOBEC1, fused to dCas9 to convert target deoxycytidines to deoxyuridines. As deoxyuridine has the base-pairing properties of deoxythymidine, this base editor was engineered to introduce C•G to T•A base pair conversions in a programmable manner. [12] Further optimizations led to the use of Cas9n and the addition of the uracil glycosylase inhibitor (UGI) peptide. The UGI component served to prevent excision of the uridine intermediate by the cellular DNA repair machinery (discussed in more detail below). Extensive optimizations of the original CBE construct have created a plethora of different CBEs, which are reviewed elsewhere. [14,15,19]

While CBEs were expected to cleanly introduce C•G to T•A SNVs, concomitant C•G to non-T•A conversions (such as C•G to G•C or A•T) at the target deoxycytidine were commonly observed. Knock-out studies demonstrated these conversions required the base excision repair enzyme uracil N-glycosylase (UNG).[20] CBE constructs lacking the UGI component have been used for targeted random mutagenesis, as they display elevated rates of C•G to non-T•A conversions compared to constructs containing UGI. [21,22] Additionally, C•G to G•C base editors (CGBEs) have been developed by fusing the UNG

enzyme instead of UGI to the CBE architecture. These CGBEs promote the generation of abasic sites at the target cytidine, facilitating the C•G to G•C mutations in a sequence-dependent manner.[23,24] More recently, XRCC1 (X-ray repair cross-complementing protein 1), a scaffolding protein for base excision repair (BER), was fused to the CBE construct, which also lead to an increase in C•G to G•C editing efficiencies. [25]

Mitochondrial DNA (mtDNA) genome editing has long been elusive due to the challenges associated with using mitochondrial DSB repair pathways for editing, and a lack of methods for delivering nucleic acids (and thus CRISPR gRNAs) into the mitochondrion. [26] However, CRISPR-free mtDNA CBEs (DdCBEs) were recently reported that perform C•G to T•A base editing in mtDNA. [27] In contrast to CRISPR-based CBEs, DdCBEs are constructed by fusing two halves of a dsDNA cytidine deaminase (DddA) to separate TALE (transcription activator-like effector) proteins that have been engineered to bind at neighboring “half sites” (Figure 1E). DdCBEs demonstrated for the first time efficient and precise genome editing in human mtDNA, using deoxyuridine intermediates.

3. Deoxyadenosine Deamination-Derived Base Editors: A•T to G•C Base Editors (ABEs)

One year after the publication of the first CBE, the first ABE was developed.[13] Unlike cytidine deaminases, there are no naturally occurring adenosine deaminases capable of using DNA as a substrate. The TadA* (mutant TadA) enzyme used to produce the final ABE construct (ABE7.10) was developed from seven rounds of directed evolution on the *E. coli* TadA enzyme that naturally deaminates adenosines in tRNA. Later studies reported ABE8 variants (engineered through additional rounds of directed evolution), which demonstrate enhanced editing activity and compatibility with Cas9 and Cas12a homologs. [28,29] Unlike the C•G to non-T•A conversions observed with CBEs, ABEs produce more precise editing outcomes, with no A•T to non-G•C conversions and minimal indel formation.

4. Processing of Base Editing Intermediates by DNA Repair Pathways

The most innovative aspect of CBEs, DdCBEs, and ABEs were their utilization of new types of DNA damage as genome editing intermediates; previous tools had almost exclusively relied on DSBs and single-stranded breaks (SSBs) as intermediates. CBEs and DdCBEs on the other hand generate a U•G mismatch intermediate at the site of interest, and ABEs utilize an I•T mismatch intermediate. In addition, the most commonly used CBE and ABE variants employ a Cas9n to introduce an SSB on the opposite strand from the deoxyuridine or deoxyinosine. Depending on the location of the target deoxycytidine or deoxyadenosine within the protospacer, this nick is usually located 3 to 14 nucleotides away in the 5’ direction from the mismatched site (Figure 1D). Here, we review canonical deoxyuridine, deoxyinosine, and SSB repair pathways individually, and analyze how the combination of these types of damage can result in different base editing outcomes.

4.1. Deoxyuridine Repair

Deoxyuridine is a common DNA damage product caused by the spontaneous deamination of deoxycytidine, and its repair has been studied extensively.[6,30,31] Deoxyuridine in DNA is efficiently recognized by the glycosylase enzyme UNG (Uracil N-Glycosylase) as the first step of the base excision repair (BER) pathway (Figure 2). [32] There are two splice variants produced from the *UNG* gene, UNG1, which is mitochondrially-localized, and UNG2, which is localized to the nucleus.[33] In addition to UNG, mammalian cells have at least three additional glycosylases, SMUG1 (Single-strand selective monofunctional uracil DNA glycosylase), TDG (G/T mismatch-specific thymine DNA glycosylase) and MBD4 (Methyl-CpG-binding domain protein 4) that also have the ability to recognize and remove deoxyuridine from DNA. [34-36] Upon deoxyuridine recognition, all four of these glycosylases hydrolyze the N-glycosidic bond between the uracil nucleobase and the 2-deoxyribose sugar backbone to generate an abasic site (Figure 2, step 1). The abasic site is then recognized by an apurinic/apyrimidinic endonuclease, most likely APE1 (Apurinic/Apyrimidinic Endonuclease 1) but potentially APE2 as well, which cleaves the sugar phosphate backbone immediately 5’ to the abasic site to produce a 3’-hydroxyl (3’-OH) terminus and a 5’-terminal 2-deoxyribose-5-phosphate (5’-dRP, Figure 2, step 2).[37,38] Following this incision, there are two sub-pathways of BER available to further process the nicked, abasic site-containing strand: short-patch (Figure 2, step 3) and long-patch BER (Figure 2, step 4).[39-41] Short-patch BER (SP-BER) involves the replacement of only the abasic site-containing nucleotide, which is first mediated by the DNA polymerase Pol β. Pol β removes the 5’ dRP moiety through its dRP lyase activity and then incorporates the correct nucleotide onto the 3’-hydroxyl end via its polymerase activity, using the intact strand as a template. The final ligation step is performed by the XRCC1/LIG3 (DNA Ligase 3) complex. Alternatively, long-patch BER (LP-BER) can process the nicked, abasic site-containing intermediate by replacing 2-15 nucleotides on this strand. This process relies on enzymes that normally participate in DNA replication, such as the DNA polymerases Pol δ or Pol ε, RFC (Replication factor C), and PCNA (Proliferating cell nuclear antigen). The newly synthesized strand displaces the old strand to form a 5’ flap, which is then digested by FEN1 (Flap endonuclease 1) or DNA2 (DNA replication helicase/nuclease 2). Instead of XRCC1/LIG3, the final ligation step in LP-BER is carried out by the PCNA/LIG1 (DNA ligase 1) complex. How the cell chooses between SP- and LP-BER is not currently well-understood, but several factors are thought to influence this decision including the type of damaged base, the stage of the cell cycle, and the cell differentiation state.[42] Repair of uracil through both SP- and LP-BER have been observed.[39,43]

Deoxyuridine in mitochondrial DNA is processed through both LP- and SP-BER as well. [44-47] Some nuclear BER factors, including APE1, FEN1, DNA2, LIG3, are imported into the mitochondrion, while others have distinct mitochondrial counterparts. Instead of UNG2, uracil in mitochondria is excised by UNG1. Following incision of the abasic site by APE1, DNA synthesis in both SP- and LP-BER is mediated by the mitochondrial-specific replicative polymerase Pol γ, and the ligation is then performed by LIG3 alone without XRCC1.[48]

4.2. Deoxyinosine Repair

In mammalian cells, deoxyinosine is also mainly repaired through BER (Figure 2). Hypoxanthine (the nucleobase of deoxyinosine) is recognized and excised by MPG (N-methylpurine-DNA glycosylase, also known as AAG), resulting in the generation of an abasic site.[49,50] Similar to the repair of deoxyuridine, this abasic site is then processed by APE1, followed by either short-patch or long-patch DNA synthesis and ligation. Recently, an alternative excision repair (AER) pathway, initiated by EndoV (endonuclease V), was found to play a major role in deoxyinosine repair in *E. coli* instead of BER. [51] EndoV, unlike DNA glycosylases, recognizes deoxyinosine and cleaves the DNA sugar phosphate backbone at the second phosphodiester bond 3' to the lesion. In *in vitro* experiments, the deoxyinosine can then be excised by the *E. coli* DNA polymerase Pol I through its 3'-to-5' proofreading exonuclease activity.[52] The resulting gap is filled through DNA synthesis and ligation. The human homologue of EndoV, ENDOV, was recently identified and found to exhibit deoxyinosine 3'-endonuclease activity on ssDNA. [53,54] However, this AER pathway has yet to be confirmed *in vivo* in humans.

4.3. Cas-induced Nick Processing and Strand Replacement

4.3.1. SSB repair (SSBR)—Naturally, SSBs are produced by certain DNA-damaging agents, including most commonly by endogenous reactive oxygen species (ROS). Because SSBs are intermediates in the BER pathway, SSBR and BER have certain machinery in common, but additional factors are required for SSB detection and processing (Figure 3A). [42,55] SSBR is initiated when the SSB is recognized by PARP1 (Poly [ADP-Ribose] Polymerase 1), which then recruits the remainder of the SSBR machinery to the site of the break via its interactions with the scaffolding protein XRCC1. [56,57] Damaged 3' ends (such as 3'-phosphate and 3'-phosphoglycolate) and 5' ends (such as 5'-hydroxyl and 5'-adenosine monophosphate) are then further processed by enzymes such as PNKP (Bifunctional polynucleotide phosphatase/kinase), TDP1 (Tyrosyl-DNA phosphodiesterase 1) and APTX (Aprataxin).[58-60] The next DNA strand replacement and ligation processes are identical to the BER pathway, which can be carried by either a short-patch (SP-SSBR) or a long-patch mechanism (LP-SSBR). However, SSBs introduced by Cas nucleases have "clean" 3'-hydroxyl and 5'-phosphate ends, and thus do not need to be end-processed. [61,62] Cas-induced SSBs therefore can potentially be re-ligated directly by either the XRCC1/LIG3 or PCNA/LIG1 complexes.[63]

4.3.2. Mismatch repair (MMR)—As mentioned previously, CBEs and ABEs generate a unique type of DNA damage, which can be thought as of a lesion-containing "mismatch" accompanied by a nick on the opposite strand, 5' downstream from the mismatched site (Figure 1D). This damage resembles the intermediates processed by the replication-uncoupled, noncanonical mismatch repair (ncMMR) pathway.[31,64] Canonical MMR (cMMR) is associated with DNA replication, and therefore its substrates are mismatched base pairs that consist of two canonical, or undamaged, nucleotides produced by replication errors.[65] In contrast, ncMMR operates independently of replication (predominantly during the G1-phase of the cell cycle), and recognizes mismatches containing damaged nucleotides that have been formed as a result of deamination, oxidation, and alkylation of DNA bases. [64,66] Although ncMMR was initially identified as a player in somatic hypermutation in B

cells, later studies demonstrated its presence in other cell types, including nondividing cells.[67] Base editors have been shown to be compatible with many cell types, indicating that ncMMR may play an important role in processing base editing intermediates.[68] During ncMMR, the “mismatch” is recognized by the mismatch recognition complex MutSα (which is comprised of MSH2 and MSH6, Figure 3B). Conventionally, MutSα recruits the MutLα complex (comprised of MLH1 and PMS2) to mismatched sites to introduce a nick on either the 5’ or 3’ side of the mismatch.[69] Unlike cMMR, which utilizes pre-existing strand discontinuities (such as Okzaki fragment termini) to direct the repair to the nascent strand during DNA replication, ncMMR lacks strand discrimination signals. In fact, MutLα can nick either strand during ncMMR, and not necessarily the lesion-containing strand.[64,70,71] In the case of base editing, the strand discrimination signal is potentially provided by the nick introduced by Cas9n on the non-lesion-containing strand, and therefore downstream repair factors could be directly recruited to the nick

Nick-containing mismatches can be processed through an EXO1 (exonuclease 1)-dependent (Figure 3B, route 1) or an EXO1-independent pathway (Figure 3B, route 2).[72] In the EXO1-dependent pathway, MutSα directly recruits EXO1 to the site of the nick and activates it.[73] EXO1 is a 5’-to-3’ exonuclease and will degrade the nicked strand, creating a ssDNA gap of up to 150nt. It is important to note that in the case of base editing intermediates, the directionality of this exonuclease activity will generate a gap across from the deoxyuridine or deoxyinosine. DNA resynthesis then fills in the gap via normal DNA replication factors, including RPA (Replication protein A, which binds to the ssDNA of the gap to protect it from further damage), RFC, PCNA and Pol δ.[74] When DNA resynthesis encounters a replication-blocking lesion, such as an abasic site, DNA translesion synthesis (TLS) factors may be recruited to bypass the lesion (discussed in more details later).[31,64,66,75] It is also worth noting that the ssDNA gap generated by EXO1 may expose additional deoxycytidines within this region to the ssDNA cytidine deaminase component of CBEs.[31] Indeed, recent work that analyzed base editing outcomes in high-throughput revealed statistically significant cytosine editing spanning from the −15nt position 5’ upstream of the protospacer to the +17nt position where the nick is introduced.[76] This observation could be explained by EXO1-dependent strand replacement during MMR being involved in base editing.

The EXO1-independent pathway resembles LP-BER.[77] The MutSα and MutLα complexes can recruit PCNA and Pol δ to the nick to carry out strand displacement synthesis spanning the mismatched site. The displaced 5’ flap is then digested by FEN1 or DNA2, and the resulting nick is sealed by a DNA ligase.

5. CBE Outcomes in Genomic DNA (gDNA): An Interplay Between Deoxyuridine Repair and Cas-induced Nick Processing

Cytosine base editing in genomic DNA (gDNA) can result in four different outcomes: Repair back to the original C•G base pair, C•G to T•A editing, C•G to non-T•A editing, and indel outcomes. We discuss here potential DNA repair mechanisms that can cause these different outcomes (Figure 4).

5.1. Repair back to C•G

A U•G, nick-containing intermediate can be repaired back to an intact C•G base pair via sequential processing by SSBR and BER. The nick has to be sealed by SSBR first, followed by BER reverting the U•G base pair back to a C•G base pair (Figure 4A). If the deoxyuridine is processed by BER before the re-ligation of the nick, incision of the abasic site by APE1 in concert with the pre-existing nick would generate a DSB, which could lead to indel formation.[70,78]

5.2. C•G to T•A outcomes

To produce a C•G to T•A outcome, DNA synthesis needs to occur across the deoxyuridine (Figure 4B). This can be done during normal DNA replication (during S-phase of the cell cycle) if the U•G intermediate is still intact, or if Cas-induced nick processing and strand replacement by either LP-SSBR or ncMMR occurs prior to uracil excision by UNG2. However, the strand replacement needs to extend long enough that the polymerase reads across the uracil – 3 to 14 nucleotides long for typical base editing intermediates. Uracil can be readily bypassed by the replicative polymerases Pol δ and Pol ε, thus TLS activation is not required.[79-81] These scenarios would both produce a U•A intermediate, and the deoxyuridine would eventually be replaced by a deoxythymidine by BER.

5.3. C•G to non-T•A outcomes

C•G to non-T•A outcomes can only occur in cells with UNG2.[20] Therefore, these outcomes most likely originate from an abasic site-containing intermediate. We believe this occurs via a very nuanced scenario, when nick processing and strand replacement by LP-SSBR or ncMMR occur after uracil excision by UNG2, but before the abasic site has been incised by APE1 (Figure 4C).[31,82,83] While not a C•G to non-T•A outcome, it is worth mentioning that replicative DNA polymerases have the ability to bypass abasic sites, and most likely incorporate a dAMP (deoxyadenosine monophosphate) opposite them. This is known as the “A-rule”, and would result in the desired C•G to T•A outcome.[84] However, this bypass efficiency is low, and replicative DNA polymerases are severely stalled at abasic sites, which leads to the activation of TLS.[81,85]

There are two TLS pathways known to rescue stalled DNA replication machinery and bypass abasic sites: the REV1-dependent and REV1-independent pathways. In the REV1-dependent pathway, REV1 is recruited to a stalled PCNA via its N-terminal domain.[86,87] REV1 is a dCMP (deoxycytidine monophosphate) transferase capable of inserting dCMPs opposite abasic sites, which would lead to a C•G to G•C outcome (Figure 4C, route 1).[88-90] Alternatively, instead of inserting dCMP, the C-terminal domain of REV1 can recruit other error-prone Y-family TLS polymerases, such as Pol $_{\eta}$, Pol $_{\iota}$ and Pol $_{\kappa}$, to the damaged site (Figure 4C, route 2).[86,91] All these TLS polymerases can bypass abasic sites.[84] Notably, Pol $_{\eta}$ and Pol $_{\iota}$ can incorporate a dTMP (deoxythymidine monophosphate) opposite abasic sites *in vitro* fairly efficiently, which would lead to a C•G to A•T outcome. However, their roles *in vivo* have not yet been confirmed. On the other hand, the REV1-independent pathway relies on monoubiquitylation of the stalled PCNA (Figure 4C, route 3).[92,93] Monoubiquitylated PCNA (PCNA-Ub) can recruit TLS polymerases directly to the stalled site. A recent study showed that the C•G to G•C outcome

of cytosine base editing does not clearly correlate with REV1 expression, suggesting that the REV1-independent pathway may be involved in this process.[76]

Several studies have observed that the deaminase components of CBEs can affect C•G to non-T•A outcomes.[23,76] It has been suggested that these deaminases may possess a previously unrecognized role in directing repair pathways, while others suggest that the dynamics of the deaminase may affect editing outcomes. It is interesting to note that in both studies, rAPOBEC1 and eA3A, the two cytidine deaminase enzymes with the fastest kinetics, facilitated the highest levels of C•G to non-T•A outcomes.[94,95] Additionally, editing of multiple cytidines concurrently within the base editing window has shown to dramatically decrease C•G to non-T•A outcomes.[20] Overall, we believe the kinetics of cytidine deamination impact C•G to non-T•A outcomes due to the unique timing of events as described above required for these outcomes to occur.

5.4. Indel outcomes

We attribute indel outcomes to the generation of DSBs *in situ* (Figure 4D). A DSB can be generated if incision of the abasic site happens before re-ligation of the nick. Furthermore, if the nick escapes global SSBR and persist until DNA replication, it will be turned into a DSB upon helicase unwinding. [96] Template switching or HDR pathways can repair the DSB back to the original sequence, whereas NHEJ or MMEJ pathways would both lead to the formation of indels.[7,9]

6. Cytosine Base Editing Outcomes in Mitochondrial DNA (MtDNA): Interplays Among Deoxyuridine Repair, MtDNA Replication, and Degradation

MtDNA cytosine base editing is distinct from genomic cytosine base editing in three major aspects. First, each cell contains thousands copies of mtDNA, meaning there are thousands of target sites for the mitochondrial base editor in contrast to typically two target alleles for genomic CBEs.[97,98] Despite this, DdCBEs demonstrated up to 50% editing efficiencies, implying that many uracils may simultaneously be present in mtDNA during editing. This in turn may pose challenges to the mitochondrial uracil repair machinery. Second, the two TALE proteins used in DdCBEs do not introduce DNA breaks of any kind (therefore, no nicks will be present in the intermediate). Third, DddA is a dsDNA cytidine deaminase, resulting in a wider editing window (14-18bp) than CBEs. This can result in deamination of multiple cytidines at a time, and on both strands. SSBs or DSBs can be generated as a result of BER processing of these multi-U intermediates. Despite this, the only observed editing outcomes of DdCBEs are repair back to the original C•G base pair and the C•G to T•A outcome. We will discuss how interactions among deoxyuridine repair, mtDNA replication, and mtDNA degradation can lead to these different outcomes (Figure 5).

6.1. Repair back to C•G

The U•G intermediates of DdCBEs can be repaired back to C•G base pairs by mitochondrial BER in an analogous manner to nuclear BER (Figure 5A). However, we believe additional

repair mechanisms may be at play here as well for two reasons. First, there is a lack of C•G to non-T•A and indel outcomes observed with DdCBEs despite the sheer number of uracils they are introducing into mtDNA.[27] Furthermore, TLS and NHEJ/MMEJ, which we hypothesize to be responsible for C•G to non-T•A and indel outcomes, respectively, with genomic CBEs, exist in human mitochondria.[44,99-101]

The DNA degradation pathway is a prevalent DNA repair pathway in mitochondria, which we believe contributes to the high precision of DdCBEs.[101-103] While DNA degradation is not an option for genomic DNA, a significant number of mtDNA molecules can be degraded without compromising mitochondrial functions, as there are typically thousands copies of mtDNA within each cell. MtDNA degradation is triggered by DSBs, and there are three possible scenarios during the processing of mitochondrial base editing intermediates that can produce DSBs (Figure 5B). First, Pol γ could stall at a UNG1-generated abasic site during mtDNA replication. Although Pol γ can bypass abasic sites *in vitro*, this activity *in vivo* is low (Figure 5B, route 1).[101] Second, DSBs can be formed when mtDNA replication encounters a SSB (which would be produced by APE1 incision of abasic sites) (Figure 5B, route 2). And third, if two uracils (or more) are on the opposing strands and are both processed by UNG1 followed by APE1, this would result in a DSB (Figure 5B, route 3). Once DSBs form, rapid degradation of mtDNA is mediated by the 5'-to-3' exonuclease MGME1 (Mitochondrial Genome Maintenance Exonuclease 1), the 3'-to-5' exonuclease activity of Pol γ, and the mitochondrial replicative DNA helicase TWNK (Twinkle protein).[104] A decrease in mtDNA copy number was not observed during base editing with DdCBE, but mtDNA replication can quickly rescue the loss of mtDNA.

6.2. C•G to T•A outcomes

The target C•G to T•A outcome is most likely achieved by Pol γ incorporating an A opposite the U during mtDNA replication (Figure 5C). In rare cases in which Pol γ bypasses abasic sites, it obeys the A-rule, which would lead to C•G to T•A outcomes as well.[101]

7. Adenine Base Editing Outcomes: An Interplay between Deoxyinosine Repair and Cas-induced Nick Processing

ABEs feature highly precise editing profiles as well, with mostly A•T to G•C outcomes and repair back to A•T observed (Figure 6). Indel outcomes are minimal but do sometime occur. Notably, despite ABE8 variants producing much higher editing efficiencies than ABE7.10, their indel formation is not significantly increased.[28,29] Because ABEs and CBEs generate very similar base editing intermediates, the high product purity of ABEs indicates a lack of TLS or NHEJ/MMEJ activation, which we attribute to inefficient deoxyinosine excision. Indeed, MPG, the DNA glycosylase responsible for excising of a wide range of damaged bases including hypoxanthine, has a k_{chem} (the rate constant for hydrolysis of the N-glycosidic bond) of 0.033 s^{-1}.[105] This is orders of magnitude slower than the k_{chem} Of UNG (115 s^{-1}).[106,107] Deoxyinosine formation in DNA occurs much less often than deoxyuridine, as spontaneous deoxyadenosine deamination occurs at only 2-3% of the rate of deoxycytidine deamination.[108] Therefore, a much lower selection pressure occurred during the evolution of an enzyme for deoxyinosine repair. This observation in turn suggests

that the development of future base editors would benefit from using more rarely occurring DNA damaged intermediates.

7.1. Repair back to A•T

Similar to CBEs, an I•T, nick-containing intermediate can be repaired back to an intact A•T base pair by SSBR-mediated nick re-ligation followed by MPG-initiated BER (Figure 6A). However, ABE experiments performed in MPG knock-out cells did not exhibit a significant increase in editing efficiency compared to wild-type controls. We therefore think this process is quite inefficient.[13]

7.2. A•T to G•C outcomes

The A•T to G•C outcome requires DNA synthesis extended across the deoxyinosine. This could be achieved through strand replacement of the nicked, unedited strand by LP-SSBR, ncMMR, or normal DNA replication analogous to the corresponding CBE outcome (Figure 6B).

7.3. Indel outcomes

As excision of hypoxanthine by MPG is inefficient, and indel rates do not scale with increasing base editing efficiencies, we believe ABE-mediated indels are more likely a result of DSBs formation due to helicase unwinding of the Cas9n-induced nick during DNA replication (Figure 6C).

8. Conclusion

Base editors demonstrated a new genome editing strategy of harnessing non-DSB DNA damage intermediates. Extensive engineering efforts from many researchers have been devoted to tackle the limitations of base editors, including off-target DNA editing, off-target RNA editing, and bystander editing.[14,15] An additional key limitation of base editors was their inability to introduce transversion mutations. Researchers have begun to chip away at this limitation through the manipulation of the DNA repair pathways involved in cytosine base editing. This highlights the importance of understanding the DNA repair mechanisms that produce different base editing outcomes. We anticipate that the development of additional new base editors will require exploiting new nucleic acid modification chemistries and/or DNA repair manipulation strategies.

In addition to base editors, prime editors are another class of editing agents that use non-DSB intermediates.[109] Prime editors can mediate the incorporation of insertions, deletions, and all possible SNVs in mammalian cells by using a 5’-flap-containing mismatch or bulge intermediate. These intermediates are introduced by a reverse transcriptase-Cas9n fusion construct using a 3’-extension on the gRNA as a template for the reverse transcriptase. Although the mechanisms involved in the processing of prime editor intermediates are outside the scope of this review, prime editors highlight the value of using new DNA damage products as genome editing intermediates for the development of new genome editing tools.

Base editors showcase great potential as therapeutics and research tools due to their high efficiency, precision, and compatibility with many cell types. In fact, base editing technologies have already been applied in the ongoing development of treatments for various genetic diseases and cancer immunotherapy.[14,68] However, they are not without room for improvement. As first proposed by Thuronyi and co-workers, while increasing base editor expression levels and deaminase activity led to improvements in overall editing efficiencies, maximum editing levels reach saturation at a certain limit imposed by editor-independent factors, which could include cell cycle states, local chromatin structures, and DNA repair processes.[28,29,110-112] Specifically, some sites seem to be intrinsically difficult to edit (even when highly active deaminases are used), potentially due to low saturation limits at these sites. Raising the saturation limits at these sites will require modulating editor-independent factors, which can be achieved through an expanded understanding of the mechanisms by which base editors work (Figure 7). For example, a recent study demonstrated that using small molecule inhibitors of histone deacetylases can increase base editing efficiencies by artificially inducing an open chromatin state and facilitating better base editor access to target loci.[113] Similarly, while nucleosomes impede DNA binding and cutting by Cas enzymes *in vitro* and *in vivo*, the addition of nucleosome remodeling (sliding) factors restore activity at previously occluded target sites *in vitro*.[114,115] Due to the fact that Cas enzymes were not evolved to explore the complex, chromatin-bound eukaryotic genomes, fusion of chromatin remodelers to base editors represents an unexplored avenue to raise the saturation limit at poorly edited sites. In addition, previous studies have already demonstrated that DNA repair processes can be modulated to raise the saturation limits. Base editing outcomes are the result of an equilibrium among competing DNA repair pathways, and tilting the balance towards certain pathways can favor certain desired outcomes. As for CBEs, the inhibition of UNG-initiated BER by fusing UGI peptides to the editor suppresses the major repair pathways that would lead to repair back to C•G and to undesired C•G to non-T•A or indel outcomes, thus greatly promoting the C•G to T•A outcome (Figure 4). For both CBEs and ABEs, we hypothesize that inhibiting the direct reversal of the Cas-induced nick (direct ligation and SP-SSBR) while promoting the processing of the Cas-induced nick by LP-SSBR or ncMMR could further boost the C•G to T•A and A•T to G•C efficiency, respectively. For CGBEs, just as fusing UNG boosted transversion efficiencies, suppressing the incision of abasic sites (APE1) while facilitating the recruitment of error-prone polymerases may increase the efficiency of C•G to G•C outcomes. Future research that illuminates the detailed biological pathways involved in base editing will facilitate the continued optimization of base editors to maximize their efficiencies, targeting abilities, and safety. Research that directly studies the mechanisms of base editing remains to be an indispensable building block for the further expansion and improvement of these technologies.

Acknowledgments

A.C.K. and S.G. are partially funded by NIH grant R35 GM138317. Q.T.C. is supported by the Molecular Biophysics Training Grant, NIH Grant T32 GM008326.

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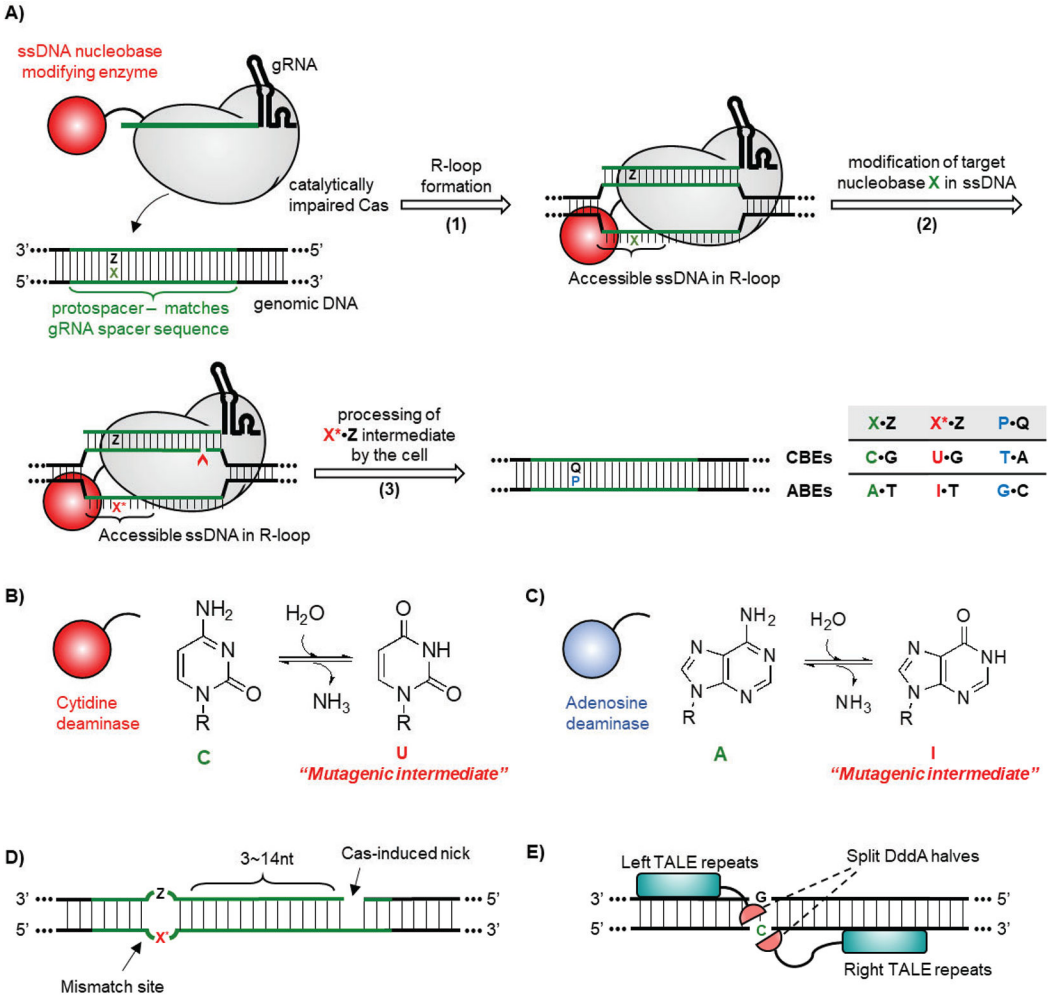

Figure 1: Overview of DNA base editing technologies.

A) DNA base editors are generally protein fusion constructs consisting of a catalytically impaired Cas nuclease (dCas9, dCas12, or Cas9n) linked to a ssDNA-specific nucleobase modifying enzyme. (1) Binding of a base editor to its target locus forms a DNA/RNA hybrid R-loop, which exposes the target nucleotide X in a stretch of ssDNA outside of the Cas protein. (2) The ssDNA nucleobase modifying enzyme now chemically modifies its target base X into X*, a noncanonical nucleotide that serves as a mutagenic intermediate. (3) X* is further processed by cellular repair or replication machinery, resulting in its conversion to P. For CBEs, X•Z, X*•Z and P•Q are C•G, U•G, and T•A, respectively. For ABEs, they are A•T, I•T, and G•C, respectively. **B)** The ssDNA nucleobase modifying enzymes used in CBEs are cytidine deaminases, which chemically modify deoxycytidine to deoxyuridine via hydro-deamination chemistry. **C)** Adenosine deaminases are used in ABEs, which chemically modify deoxyadenosine to deoxyinosine via hydro-deamination chemistry. **D)** The unique DNA damage intermediate harnessed for base editing, featuring a modified nucleotide(X*)-containing mismatch and a nick located 3 to 14 nucleotides away in the 5' direction from the mismatched site on the unmodified strand. **E)** DdCBEs are constructed by

fusing two halves of a dsDNA cytidine deaminase DddA to separate TALE proteins that bind at neighboring “half sites”.

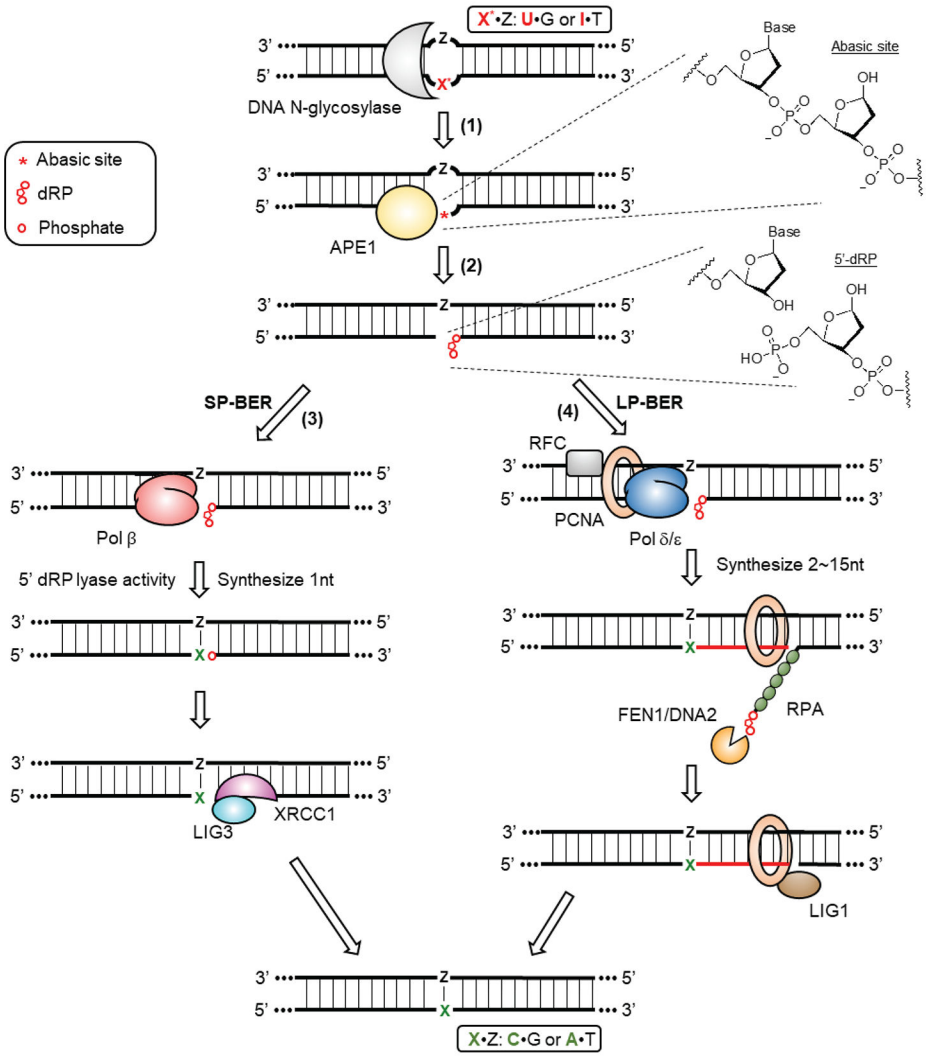

Figure 2: Base excision repair.

(1) Base excision repair is initiated by the recognition of modified nucleotides (X*) by DNA N-glycosylases. Deoxyuridine is recognized by four DNA N-glycosylases, including UNG, SMUG1, TDG and MBD4. Deoxyinosine can be recognized by MPG. DNA N-glycosylases hydrolyze the N-glycosidic bond between the nucleobase and the 2'-deoxyribose, leading to the formation of abasic sites. (2) APE1 recognizes abasic sites and incises the DNA sugar backbone to result in a SSB containing a 3'-OH and a 5'-dRP terminus. Further processing of the SSB diverts into two potential pathways. (3) SP-BER involves Pol β removing the 5' dRP moiety and incorporating the correct nucleotide X back using Z as a template. The final ligation is performed by the XRCC1/LIG3 complex. (4) LP-BER involves the recruitment of DNA replication factors to the site of the SSB, including RFC, PCNA and Pol δ/ε. Synthesis of a new DNA strand (colored in red) displaces the old strand to form a 5'-flap, which is later digested by FEN1 or DNA2. The final ligation is performed by the PCNA/LIG1 complex.

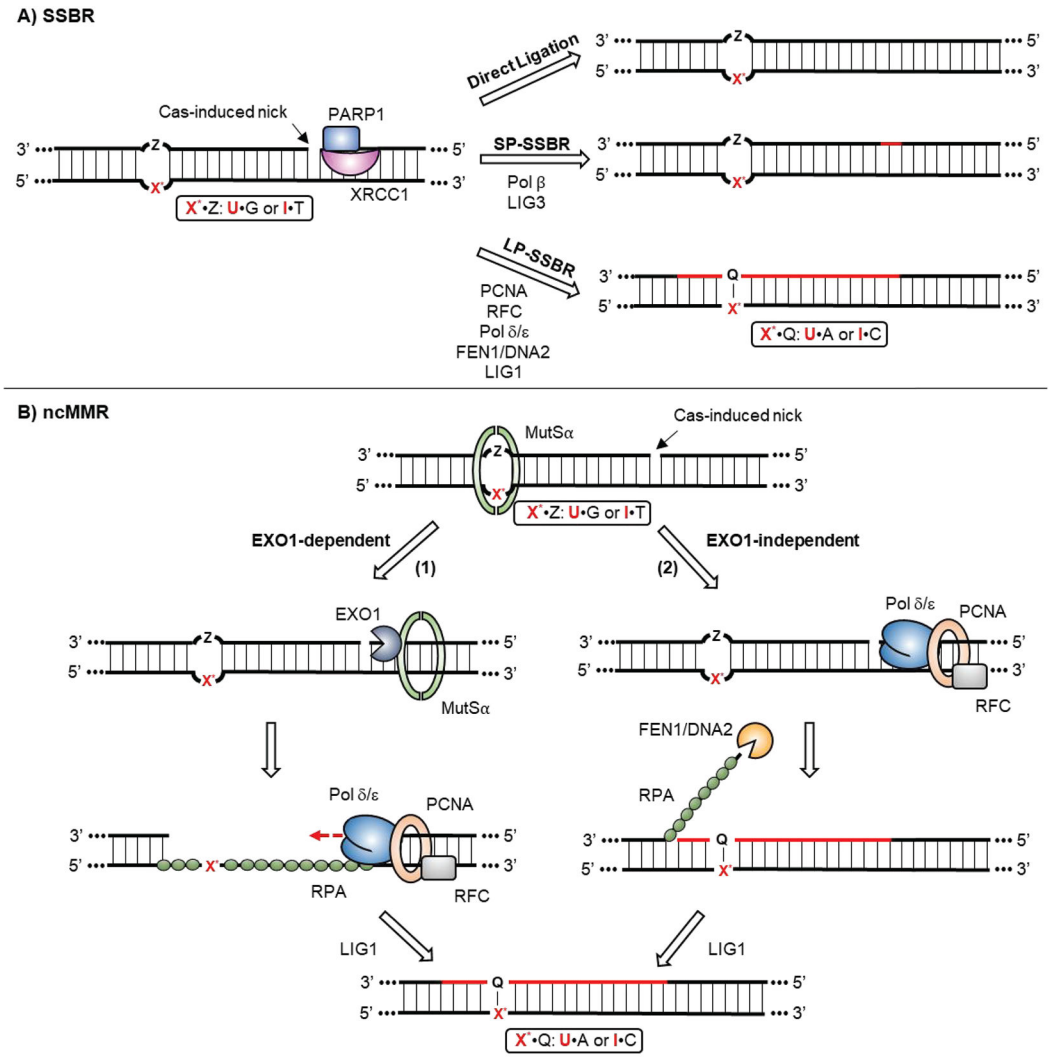

Figure 3: Cas-induced nick processing and strand replacement.

A) SSBR is initiated by detection of the Cas-induced nick by the XRCC1/PARP1 complex. Then, either direct ligation, SP-SSBR or LP-SSBR can be involved to re-seal the nick. Newly synthesized strands are colored in red. **B)** ncMMR is initiated by detection of the X*•Z mismatch by MutSα. Subsequently, there are two potential pathways that can process the Cas-induced nick: the EXO1-dependent and -independent pathways. (1) EXO1 is directly recruited and activated at the site of the nick by MutSα. It digests the nicked strand in a 5'-to-3'direction across from the mismatch, leading to formation of a gap across from the X*-containing strand, which becomes coated by RPA. DNA replication factors, including RFC, PCNA and Pol δ/ε, are then recruited to fill in the gap, using the X*-containing strand as a template. (2) In the EXO1-independent pathway, the DNA replication complex is directed recruited to the nick to carry out strand replacement synthesis. The displaced 5'-flap is then digested by FEN1 or DNA2. In both pathways, the final ligation is performed by LIG1, with the X*•Z mismatch being turned into a X*•Q base-pair intermediate.

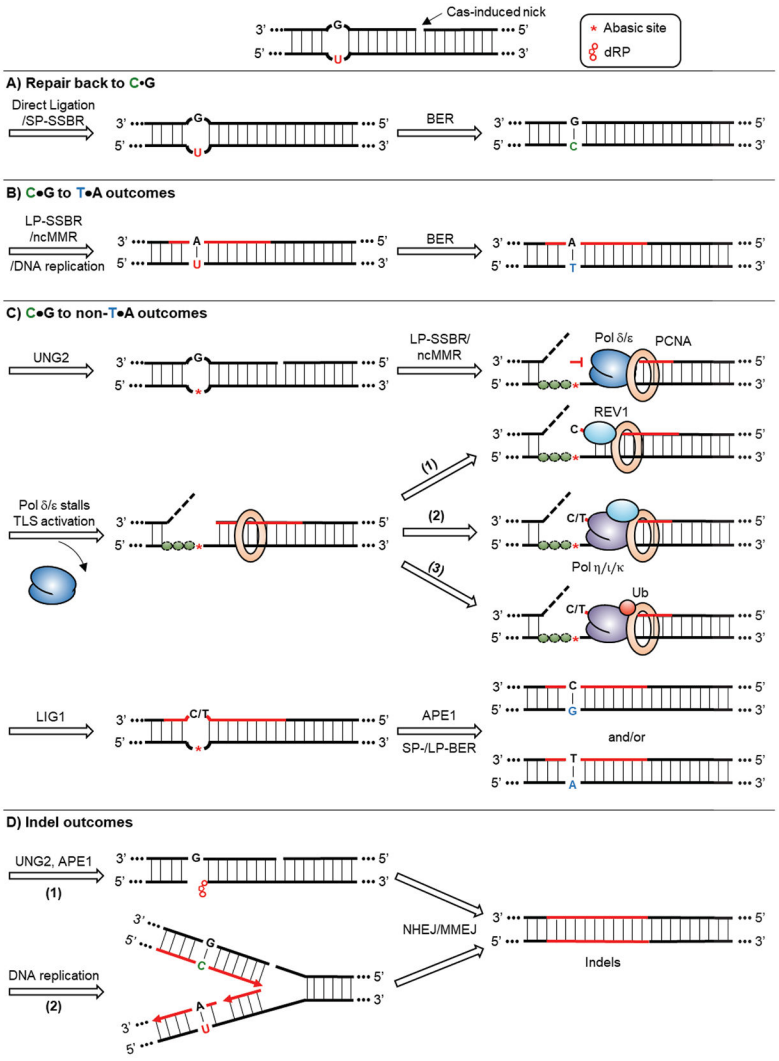

Figure 4: CBE outcomes in gDNA.

A typical CBE intermediate features a U•G mismatch accompanied by a 5’ Cas-induced nick. **A)** The CBE intermediate can be repaired back to C•G by first re-ligation of the nick through SSBR, followed by the deoxyuridine being processed by BER. **B)** The CBE intermediate can lead to C•G to T•A outcomes when DNA synthesis occurs across the deoxyuridine, which can be achieved by either the LP-SSBR, ncMMR, or normal DNA replication. The resulting U•A base pair can be resolved into a T•A base pair by BER. **C)** The CBE intermediate can also result in C•G to non-T•A outcomes, which is mediated by DNA synthesis across abasic sites. Abasic sites are generated by excision of uracil by UNG2. DNA synthesis to replace the nicked strand is then carried out by LP-SSBR or ncMMR. Depending on the pathway involved, either an RPA-coated ssDNA gap or a 5’-flap would form, which are depicted by dashed lines. Replicative polymerases Pol δ and Pol ε stall at abasic sites, leading to a subsequent TLS activation to rescue the stalled DNA synthesis and bypass the lesion. There are three potential downstream routes. (1) REV1 is recruited to PCNA. It can insert a dCMP opposite abasic sites. (2) Alternatively, REV1 can recruit other error-prone polymerases, such as Pol $_{\eta}$, Pol $_{\iota}$ and Pol $_{\kappa}$, to the stalled site. They

are capable of incorporating both dCMP and dTMP opposite abasic sites. (3) The REV1-independent pathway depends on the recruitment of error-prone polymerases by monoubiquitylation of PCNA. Ligation of the newly synthesized strand by LIG1 forms an intact abasic site-containing intermediate, which is further processed by APE1 and the rest of the BER machinery. This eventually leads to the formation of C•G to G•C and C•G to A•T outcomes. **D)** The CBE intermediate can be processed into indels, which we attribute to *in situ* DSB formation. (1) DSBs can arise from processing of deoxyuridine by UNG2 and APE1 before the re-ligation of the Cas-induced nick. (2) DSBs can also be originated from helicase unwinding of the nicked strand during DNA replication. DSB processing by both NHEJ and MMEJ can lead to indels.

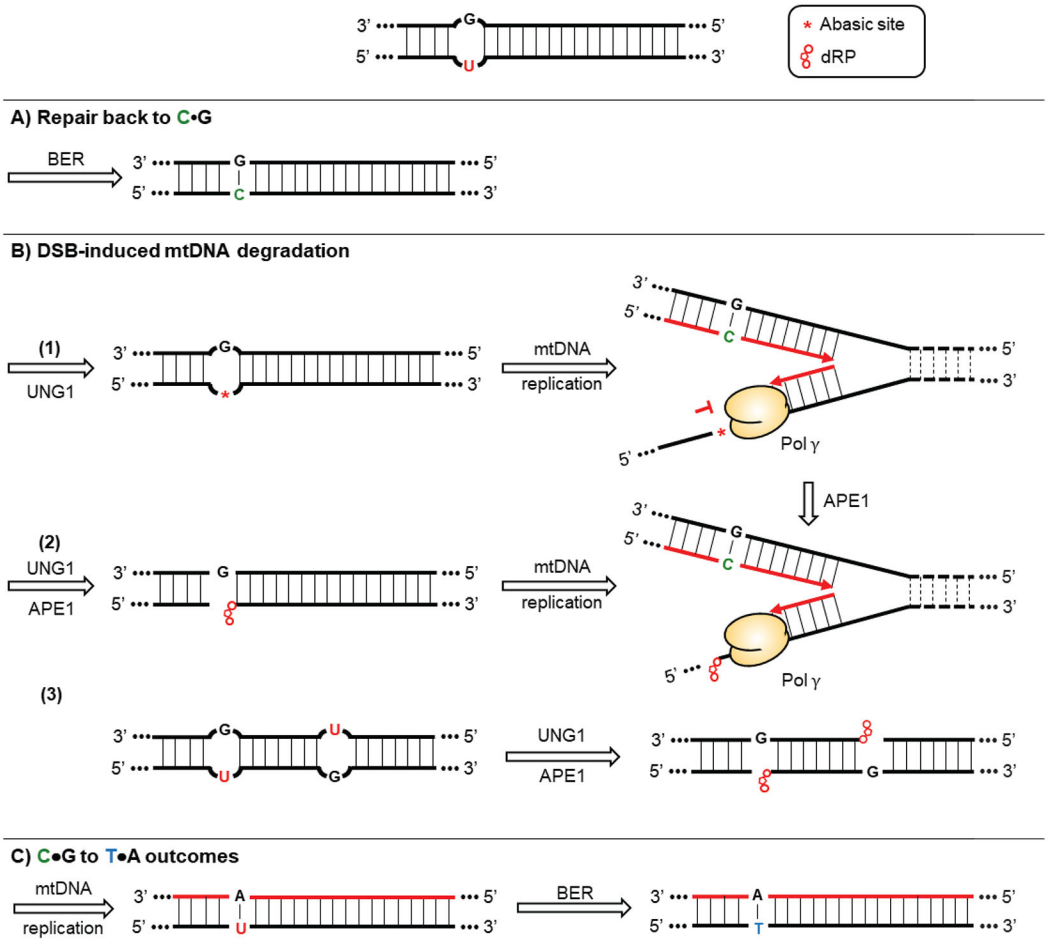

Figure 5: Cytosine base editing outcomes in mtDNA.

Unlike CBE in gDNA, a DdCBE intermediate in mtDNA contains a U•G mismatch (or multiple) without any nicks. **A)** This intermediate can be repaired back to C•G by mitochondrial BER. **B)** There are several scenarios in which this intermediate can result in DSB formation, and eventually mtDNA degradation. (1) Pol γ stalls at abasic sites generated by UNG1 during mtDNA replication. (2) mtDNA replication encounter SSBs generated by APE1. Note that the leading-strand and the lagging-strand synthesis during mtDNA replication are uncoupled.[116] Hence, the replication forks are depicted with dashed lines. DNA synthesis based on the unmodified strand leads to repair back to C•G. (3) If two (or more) deoxycytidines are deaminated on opposite strands, processing of these deoxyuridines by UNG1 and APE1 can lead to DSBs. **C)** The U•G intermediate can be resolved into a T•A outcome by DNA synthesis across the deoxyuridine during mtDNA replication, followed by the deoxyuridine being replaced by a deoxythymidine through mitochondrial BER.

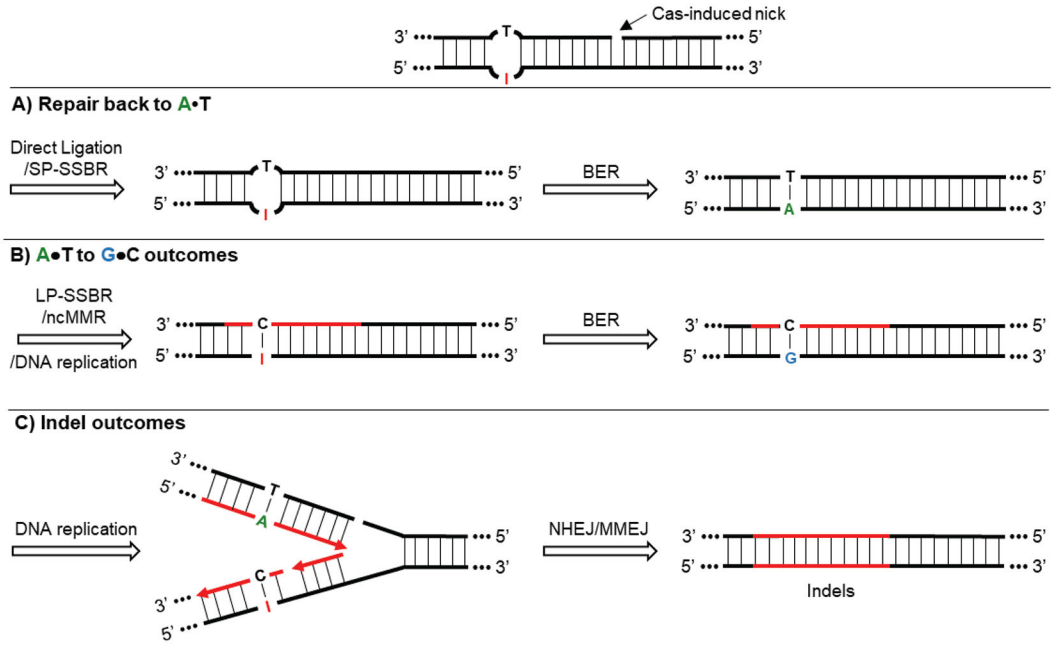

Figure 6: ABE outcomes.

A typical ABE intermediate contains an I•T mismatch with a 5' Cas-induced nick. **A)** The ABE intermediate can be repaired back to an intact A•T base pair by sequential processing of nick re-ligation and BER. **B)** The ABE intermediate can be resolved into a T•A outcome when DNA synthesis occurs across the deoxyinosine during LP-SSBR, ncMMR, or normal DNA replication. The resulting I•C base pair can be converted to a G•C base pair by BER. **C)** Indel outcomes are attributed to helicase unwinding of the nicked strand during DNA replication, followed by processing of the DSB through NHEJ/MMEJ.

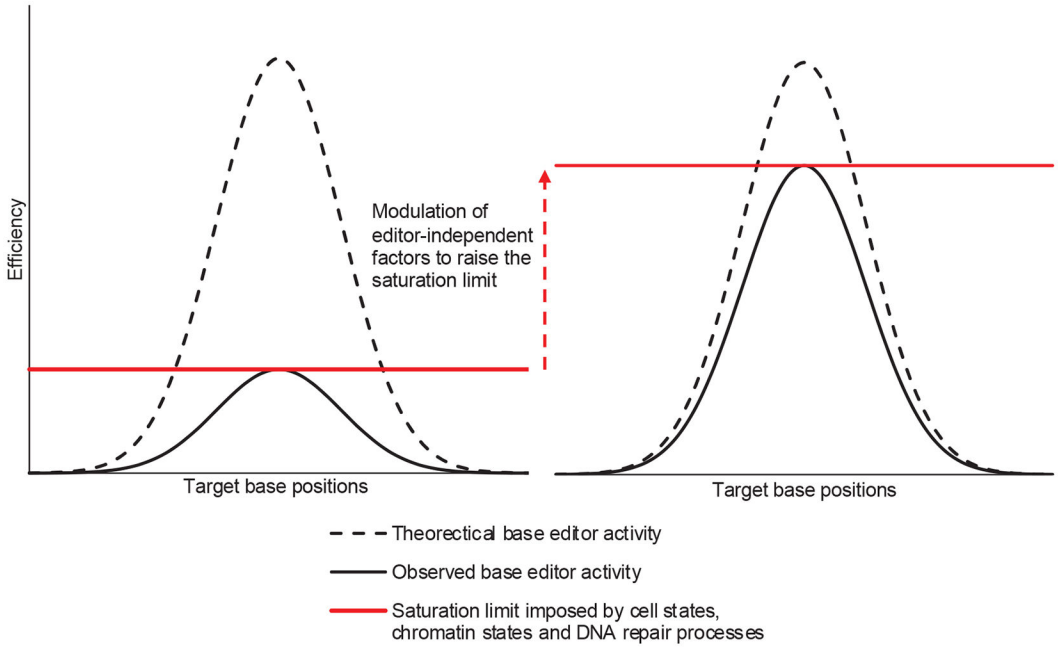

Figure 7: A conceptual model of base editing.

Editing efficiency reaches a maximum at the optimal target base position. At certain poorly edited sites, the observed base editing efficiencies are low even when a highly active base editor is used. We reason that the low saturation limits of those sites are imposed by editor-independent factors, such as cell states, chromatin structures, and DNA repair processes. Raising the limits by modulation of these editor-independent factors can potentially increase editing efficiencies at these sites.