Off-target Editing by CRISPR-guided DNA base editors

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Abstract

Base editing is a genome editing strategy that induces specific single nucleotide changes within genomic DNA. Two major DNA base editors, Cytosine base editors (CBEs) and Adenine base editors (ABEs), have been developed that consist of a Cas9 protein linked to a deaminase enzyme that catalyzes targeted base conversion directed by a sgRNA. This strategy has been used widely for precise genome editing because, unlike CRISPR-Cas nuclease-based genome editing systems, this strategy does not create double strand DNA breaks (DSBs) that often result in high levels of undesirable indels. However, recent papers have reported that DNA base editors can cause substantial off-target editing in both genomic DNA and RNA. The off-target editing described in these studies is primarily guide RNA-independent arising from promiscuous reactivity of the deaminase enzymes used in DNA base editors. In this perspective, we discuss the development of DNA base editors, the guide RNA independent off-target activity reported in recent studies, and strategies that improve the selectivity of DNA base editors.

Graphical Abstract
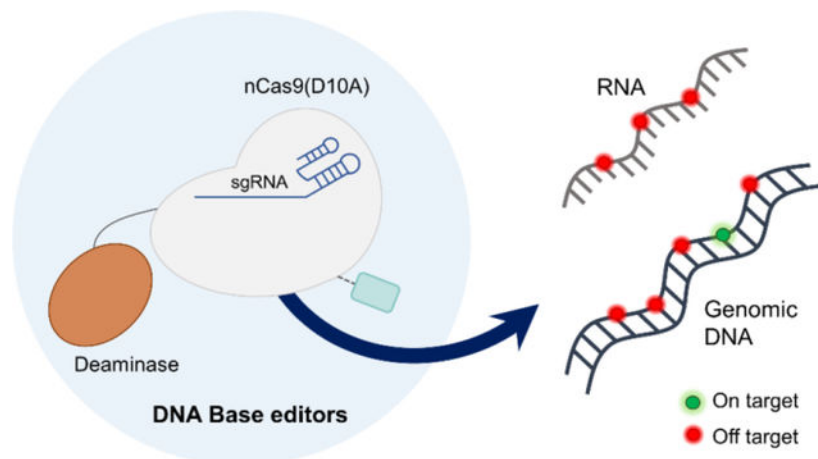
Keywords

genome editing; CRISPR-Cas; APOBEC; TadA; cytidine deaminase; adenosine deaminase

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Introduction

The majority of human genetic diseases arise from single nucleotide changes[1–2]. Thus, therapeutic strategies that could allow one to correct single point mutations hold promise as powerful tools to treat genetic disorders. CRISPR (clustered regularly interspaced short palindromic repeat)-Cas(CRISPR-associated protein nuclease)-based systems have revolutionized the field of genome engineering[3–5]. Several CRISPR-Cas systems have been developed to induce double strand DNA breaks (DSBs) at specific sequences directed by single guide RNAs (sgRNAs)[5–11]. Such induced DSBs are then repaired by cellular DNA repair pathways including the non-homologous end-joining (NHEJ) pathway and the homology-directed repair (HDR) pathway[12]. Indeed, CRISPR-Cas-mediated selective cleavage of duplex DNA coupled with HDR using appropriately designed donor DNA fragments has become a popular approach for introducing specific sequence changes in genomes[4, 13–14]. However, HDR competes with NHEJ which often results in unwanted indels (insertions or deletions)[15–16].

Unwanted off-target changes in the genome resulting from DSBs and subsequent NHEJ is a significant concern for the therapeutic application of typical CRISPR-Cas genome editing strategies[17–18]. Therefore, manipulating the genome sequence without DSBs could be more powerful. Base editing is a genome editing strategy originally developed in the laboratories of David Liu at Harvard University that changes a specific single nucleotide within genomic DNA directly without introducing DSBs[19–20]. The base editors are composed of a catalytically defective Cas protein fused to a deaminase enzyme capable of direct modification of the target nucleotide guided by the sequence of a sgRNA. Since base editing does not introduce DSBs, creation of undesired indels is minimized.[19–21] In addition, DNA base editing can be applied more broadly since the application of CRISPR-Cas genome editing is largely limited to actively dividing cells where the HDR pathway is most efficient[12, 22]. However, off-target editing by DNA base editors has been observed[19–21, 23–27]. Earlier work described approaches to measure the extent of guide RNA-dependent off-target editing and to reduce this type of off target activity[23–25, 28–29]. However, more recent reports suggest that promiscuous reactivity of deaminase domains present in the DNA base editors can lead to guide RNA-independent off target editing in both DNA and RNA[30–34]. In this Perspective, we review the development of the base editor genome editing enzymes, recent reports of a guide RNA-independent off target activity and discuss strategies for improving target selectivity.

DNA Base editors

Two main classes of base editors have been developed; Cytosine Base Editors (CBEs) that convert C-to-T[19, 21] and Adenine Base Editors (ABEs) that convert A-to-G[20].

Cytosine Base Editors (CBEs)

Cytosine Base Editors (CBEs) are composed of catalytically inactive Cas nuclease and an APOBEC/AID cytidine deaminase[19, 21]. The Cas protein is directed by a sgRNA to bring the deaminase to a specific base editing target site[4–5]. APOBECs (Apolipoprotein B mRNA Editing Catalytic Polypeptide-like) and AID (activation-induced cytidine deaminase) are

members of a family of enzymes that catalyze hydrolytic deamination of cytosine (C) to generate uracil (U) in DNA and RNA[35]. Since uracil is replicated like thymine, the deamination of cytidine leads to a C-to-T change in DNA. Fusion of rat APOBEC1 and catalytically dead Cas9 nuclease (dCas9) from *Streptococcus pyogenes* created the first generation of cytosine base editor (BE1)[19]. Since the development of BE1, additional studies led to incorporation of uracil DNA glycosylase inhibitor (UGI) to prevent removal of U by Uracil DNA glycosylase (UDG) activity (BE2)[19] and replacing dCas9 with Cas9 D10A nickase (nCas9) (BE3)[19] which creates a nick on the non-edited DNA strand leading to preferential replication of the edited strand (Figure 1). When the BE3-sgRNA complex binds to target genomic DNA, this results in formation of a DNA-RNA hybrid structure near the target site and a ssDNA loop that is not bound to sgRNA[19]. This ssDNA is then available for rAPOBEC1-catalyzed cytosine deamination within a 5-nucleotide window[19]. Although the BE3-sgRNA system still induces a small number of indels, this level is substantially lower than CRISPR-Cas systems that induce DSBs[19, 24]. Therefore, it has been used widely for various applications[27, 36].

Adenine Base Editors (ABEs)

To increase the scope of genomic DNA base editing, Adenine Base Editors (ABEs) were developed for Adenosine (A) to Guanosine (G) conversion[20]. Adenosine deaminases carryout hydrolytic deamination of Adenosine(A) to generate Inosine (I)[37]. Although A is not directly converted to G by adenosine deaminase activity, inosine is recognized as G during replication[37]. Thus, A-to-I editing is interpreted as an A-to-G conversion. The major challenge in the development of ABEs was the fact that there are no known adenosine deaminases that act on ssDNA. *E. coli* TadA (ecTadA) is a tRNA adenosine deaminase that catalyzes the conversion of A43 in tRNA^{Arg2} to inosine[38]. In an impressive feat of in vitro evolution, the Liu lab converted ecTadA into an enzyme that can readily deaminate 2'-deoxyadenosine in ssDNA[20]. This created the first generation of ABE, TadA*-dCas9 (TadA* = evolved TadA)[20]. ABEs were further optimized to increase the efficiency of editing by incorporating wild-type TadA[20], accounting the fact that wild-type ecTadA catalytic activity requires homodimer formation[39]. This resulted in ABE 7.10 (ecTadA-ecTadA*-nCas9)[20] (Figure 1). Like BE3, when treated with ABE, the base editor complex is directed to target a specific site in genomic DNA. After formation of the sgRNA-DNA complex, ssDNA is available for base modification by evolved ecTadA* within a 4–7 nucleotide window[20].

Genome and transcriptome wide off-target editing by DNA base editors

There have been several studies looking at the off-target effects of CRISPR-Cas-based genome engineering methods[40–43]. However, less was known about the genome wide off-target activity of DNA base editors. Recently, two papers in *Science* reported analyses of genome-wide off-target mutation induced by DNA base editors[31–32]. Each study took a different approach to accurately analyze the off-target mutations. Zuo et al.[32] developed a method called Genome-wide Off-target analysis by Two-cell embryo Injection (GOTI) to determine genome wide off-target mutations in mice (Figure 2A). Their GOTI method uses two-cell embryos derived from Ai9 (CAG-LoxP-Stop-LoxP-tdTomato) mice[44] which

display a strong tdTomato fluorescence when Cre recombinase is expressed. They injected a total of 11 different combinations of CRISPR-Cas9, BE3, or ABE with or without sgRNAs along with Cre mRNA into one blastomere of two-cell embryos, which they referred to as the edited blastomere[32]. Then, the edited blastomere and non-edited blastomere were sorted by FACS based on the tdTomato expression level at embryonic day 14.5 (E14.5)[32]. This process enabled them to distinguish spontaneous mutations from off-target mutations generated by CRISPR-Cas9 or DNA base editors[32]. Cells with CRISPR-Cas9 or base editors (tdTomato[+]) and without CRISPR-Cas9 or base editors (tdTomato[-]) were sequenced separately by Whole genome sequencing (WGS) followed by variant calling processes for the tdTomato[+] samples to detect single nucleotide changes and indels using tdTomato[-] samples as reference[32].

The method of Jin et al.[31] used a clonally derived rice plant system to rule out spontaneous mutations caused by cellular processes from off-target mutations by DNA base editors (Figure 2B). In addition, they included control plants in their analysis to remove background mutations possibly caused by tissue culture and transformation processes[31]. A total of 14 combinations of different base editors with or without sgRNAs were transformed into rice via *Agrobacterium* transformation[31]. Off-target mutations from each regenerated rice plant with or without sgRNAs along with control plants were investigated by WGS followed by variant calling to identify single nucleotide changes and indels[31].

Although the two studies used different strategies to investigate genome wide off-target mutations caused by DNA base editors, both studies found that there were no significant changes in the number of single nucleotide changes upon treatment with ABE compared to that of controls[31-32]. However, the amount of unintended off-target single nucleotide changes was significantly increased by BE3, predominately having C>T mutations, compared to that of ABE or controls[31-32]. Interestingly, both studies indicated that the observed off-target mutations were sgRNA independent[31-32]. This suggests that off-target mutations caused by BE3 originate from promiscuous reaction of the cytidine deaminase fused to BE3. In fact, both groups reported that off-target mutations caused by BE3 were high in transcribed regions where ssDNA is available due to R-loop formation during transcription[31-32]. This ssDNA could be accessible to the cytidine deaminase domain of the base editor, resulting in off-target mutations by BE3 throughout the genome. Therefore, both studies suggest that the rAPOBEC1 present in cytosine base editors should be optimized to increase the specificity of target base editing and reduce unintended off-target mutations[31-32].

The deaminase enzymes rAPOBEC1 and mutant ecTadA* were fused to nCas9 to yield site-specific Cytosine base editors and Adenine base editors, respectively[19-20]. However, the extent to which the APOBEC1 or TadA domains in these proteins might edit different sites in the transcriptome was not fully defined when the DNA base editors were originally developed[19-20]. Indeed, rAPOBEC1 is capable of converting C-to-U in both ssDNA and RNA[35, 45]. Also, ecTadA was selected for efficient A-to-I modification on ssDNA resulting in an evolved enzyme bearing several mutations[20]. Although these mutations convert wild type ecTadA to mutant ecTadA* suitable for DNA base editing, these mutations do not

guarantee the loss of RNA editing activity of ecTadA*. Furthermore, RNA editing activity could originate from wild type ecTadA that is also a part of the ABE design[33–34].

Three recent papers investigated the transcriptome wide RNA off-target mutations caused by DNA base editors[30, 33–34]. For instance, in a paper recently published in *Nature* by Grünewald[30], the authors first transfected cultured mammalian cells with BE3 and ABE plasmids fused to eGFP along with sgRNA for specific genome editing[30]. Controls for each DNA base editor lacking deaminase domains (BE3 without rAPOBEC1 and ABE without ecTadA dimer) were also tested[30]. Transfected cells were sorted based on eGFP level and DNA and total RNA samples from fluorescent cells were analyzed for on-target DNA editing as well as off-target RNA editing[30] (Figure 3A). They identified BE3-induced C-to-U modifications transcriptome wide, having a preference for the sequence motif ACW (W=A or U) which is identical to the preferred editing context for wild-type rAPOBEC1[30]. They further analyzed RNA off-target sites by Whole-exome sequencing (WES) to rule out the possibility of mutations caused by DNA editing[30]. These experiments confirmed that the RNA off-target modifications were not caused by corresponding genomic DNA editing[30]. They also showed that these RNA off-target modifications are sgRNA-independent by comparing off-target modifications caused by BE3 with sgRNAs targeting a specific human genome sequence and sgRNA having a sequence that cannot be found in human genome[30]. To reduce RNA off-target modifications, this group introduced specific mutations in the rAPOBEC1 domain previously reported to reduce C-to-U editing on RNA (R33A or R33A/K34A)[30, 46–47]. They refer to these as SECURE (SElective Curbing of Unwanted RNA Editing) variants of the Cytidine base editor[30]. These new variants showed a substantial reduction in RNA off-targets, while maintaining similar DNA on-target editing with a narrowed editing window[30]. In addition to BE3, ABE RNA off-target editing was also addressed in this paper[30]. These authors showed that ABE induces a significant amount of RNA off-target modifications throughout the transcriptome, showing a UA motif editing preference matching that of wild-type ecTadA[30]. These results were corroborated by two additional recent papers on this topic, Rees et al.[33] and Zhou et al.[34], both reporting off-target modifications in RNA induced by ABEs. Furthermore, both of these studies indicated that RNA off-target activity of ABEs is transient and can be controlled by mutagenesis in the TadA domains. Rees et al.[33] showed that ABE RNA off-target editing originates from both the wild-type ecTadA domain and the evolved ecTadA* domain. Thus, wild-type ecTadA, evolved ecTadA*, or both were inactivated by point mutation (E59A). Only when wild-type ecTadA was inactivated with this mutation could the RNA off-target activity be reduced and the DNA on-target editing be maintained[33]. The DNA editing specificity of the evolved ecTadA* was further optimized by mutating three residues identified from the crystal structure of *S. aureus* TadA[39] bound to RNA. These authors hypothesized that introducing a steric clash between one of these three residues and RNA by mutating each to bulky or hydrophobic amino acids could reduce unintended RNA editing activity (Figure 3B). As a result, a new ABE variant, ABEmaxAW with ecTadA E59A and ecTadA* V106W mutations, showed a substantial reduction in RNA off-target editing while maintaining efficient DNA on-target base editing[33]. Similarly, Zhou et al.[34] incorporated a single point mutation (D53E or F148A) identified from previous studies[38, 48–50] within both wild-type ecTadA and evolved ecTadA* to prevent high level RNA off-target activity while retaining a

similar level of DNA on-target activity. Taken together, these studies indicate that one must consider not only genome-wide but also transcriptome-wide off-target effects from the DNA base editors. In addition, this work clearly indicates that one effective strategy to reduce transcriptome off-targets is by strategic mutation of the deaminase domains present in the base editors[30, 33–34].

Each of the studies discussed above indicated that substantial guide RNA-independent off-target base editing arises from promiscuous reactivity of the deaminase enzymes present in each base editor (rAPOBEC1 in BE3 and ecTadA in ABE). Thus, additional control of the enzymatic activity (i.e. catalysis and substrate binding) of these domains is required to achieve precise targeted base editing without unwanted genome or transcriptome modification (Figure 4). Fortunately, there are several strategies available to modulate the activity of the base editor deaminase domains. Since the development of the first DNA base editors, variants have been reported with different deaminases, linker lengths, and Cas nucleases[27]. Each system has a slightly different editing window in the ssDNA in the Cas-sgRNA-target complex. For instance, BE3 has a 5-nucleotide editing window and ABE has a 4–7 nucleotide window[19–20, 27]. Within each editing window, any C or A base can be modified by the corresponding deaminase domain, resulting in bystander off-target modifications. Therefore, a narrow editing window is preferred, ideally targeting a single, specific nucleotide for modification. Recent reports suggest that this type of off-target editing can be controlled by tuning the activity of the deaminase in the editor enzyme. For example, different cytosine base editors (YE1-BE3, EE-BE3, YE2-BE3, and YEE-BE3) were developed by incorporating two or three mutations in rAPOBEC1 that reduce catalytic activity and substrate binding of rAPOBEC1 enzyme resulting in a narrow editing window. [23] Mutations used in these base editors were identified from the APOBEC3G enzyme that has 42% sequence similarity with rAPOBEC1[45]. With those mutations, off-target editing was reduced while still maintaining similar base modification efficiency for on-target DNA base editing. In addition, the SECURE variants of cytosine base editors reported by Grünewald et al.[30] described above reduced transcriptome off-target editing with mutations that were previously shown to decrease RNA editing activity of wild-type APOBEC1. They used the R33A and K34A mutations for this purpose. Interestingly, R33 and K34 are part of the N-terminal basic amino acid cluster of APOBEC1[47, 51]. It has been reported that this part of protein binds the ACF cofactor, which is an RNA binding protein[52–54]. Mutating these residues likely inhibits interaction with ACF, reducing off target RNA binding affinity and hyper editing activity. Also, it is likely to continue to be useful to test other deaminase enzymes in the context of new base editors, particularly deaminases for which structural information is available. For example, hA3A-BE3 was developed using engineered human APOBEC3A (hA3A) guided by available high resolution structures for hA3A[55]. hA3A-BE3 shows higher editing specificity while reducing bystander off-target editing compared to cytosine base editor variants with rAPOBEC1 mentioned above. Furthermore, it has been reported that BE3 fused with an hA3A mutant (R128A or Y130F) further reduces unwanted RNA off-target editing, while retaining similar DNA on-target editing[34].

Other factors should also be considered to further increase on-target specificity for DNA base editors (Figure 4). As mentioned before, UGI was incorporated into cytosine base editors to prevent the removal of U in DNA, thus increasing the overall efficiency of

cytosine base editor activity. However, the presence of UGI can also increase the level of unwanted C-to-T mutations by blocking Uracil N-glycosylase (UNG) activity[56–57]. Therefore, the development of highly efficient cytosine base editors without UGI should be considered. Also, the level of overexpression and the cellular half life of DNA base editors provide additional means of optimization since high cellular concentrations of long-lived deaminase enzymes are likely to lead to undesirable off-target editing[58–59]. Indeed, RNP delivery of DNA base editors improves on-target specificity compared to plasmid delivery[24]. Thus, different delivery strategies should be considered carefully to control exposure time as a means of further minimizing off-target editing[24, 33]. Finally, increasing the targetable sequences by using different Cas nuclease variants with different PAM requirements will continue to expand the application of the base editors[23, 60].

Additional insight from site-directed RNA editing approaches

Since substantial off-target editing by DNA base editors originates from their deaminase activity on RNA, experience with existing site-directed RNA editing strategies (RNA base editors) could provide some insight for reducing RNA off-target editing by DNA base editors. There have been several site-directed RNA editors developed using the Adenosine Deaminases Acting on RNA (ADARs) that convert A-to-I within duplex RNA. Site-directed RNA editing strategies can be classified into two approaches. One approach uses an antisense oligonucleotide to redirect endogenous ADARs for site-specific editing. For example, both RESTORE[61] and LEAPER[62] systems use antisense oligonucleotides to recruit endogenous ADARs to the target editing site of interest. Because these methods harness endogenous ADARs rather than overexpressing ADARs, RNA off-target editing from high expression levels of the deaminase is reduced[61–63]. The other approach uses an antisense guide RNA (gRNA) to direct engineered ADAR fusion proteins for site-specific editing. Several systems have been developed that link ADAR fusions to a gRNA including SNAP tagging (SNAP-ADAR)[64–68], λN peptide (λN-ADAR)[69–71], or a Cas protein (dCas13b-ADAR or REPAIR)[72]. These ADAR fusions are then directed by the gRNA that forms a duplex at the target site for site-specific RNA editing. It is noteworthy that both approaches take advantage of ADARs' preference for duplex RNA and editing A in the context of an A•C mismatch[61–62, 66, 70, 72]. Site directed RNA editing strategies that use fusion proteins containing ADAR catalytic domains must also contend with guide RNA-independent off targeting. For instance, Rosenthal addressed this issue for λN-ADAR by targeting the fusion proteins to the nucleus where off-target editing is less efficient[63]. To reduce off-target RNA editing in the REPAIR system, Zhang and colleagues made use of high-resolution structures of human ADAR2 bound to RNA[73] to introduce mutations at sites known to contact RNA, thus reducing substrate binding affinity of the deaminase domain itself[72]. Finally, our lab described a bump-hole approach for increasing directed RNA editing specificity[74]. This approach uses a mutant deaminase domain of human ADAR2 with a bulky residue that causes a steric clash with the nucleobase opposite the targeted A[74]. Only when this clash is relieved with a gRNA containing an abasic site opposite the A can efficient reaction occur, thus increasing the specificity of directed RNA editing[74]. While high resolution structures that include human APOBECs and *S. aureus* TadA have been helpful in guiding mutations in DNA base editors, no structures are currently available for

the DNA base editors themselves bound to DNA. Such structures would be highly valuable for guiding further optimization of editor efficiency and specificity.

Conclusion and future outlook

DNA editing systems can be powerful tools with various applications from basic research to therapeutics. The DNA base editors described here are promising systems that introduce specific C-to-T or A-to-G changes in genomes. However, optimization of DNA base editors has been necessary to reduce guide RNA-independent base modifications in cellular DNA and RNA[30–34]. The promiscuous editing activity of the deaminase enzymes used in base editor systems appears to be a primary cause of off-target base editing[30–34]. rAPOBEC1 in BE3 retains ssDNA and RNA editing activity which results in off-target modifications in both the genome and transcriptome, whereas ecTadA in ABE retains RNA editing activity, leading to transcriptome wide off-target base modifications[30–34]. Initial efforts have shown that strategic use of mutation within the deaminase domains can decrease guide RNA-independent off-target editing for DNA base editors[30, 33–34]. However, it is often true that mutations that reduce off-target base editing can also lead to a decrease in on-target base editing. Therefore, it is important to fully understand features of these enzymes that control catalysis and substrate binding such that maximum editing activity can be delivered precisely to the desired site of reaction. Additional biochemical and structural studies with nucleic acid modifying deaminases, alone or in the context of base editors, will inform these efforts.

Acknowledgments

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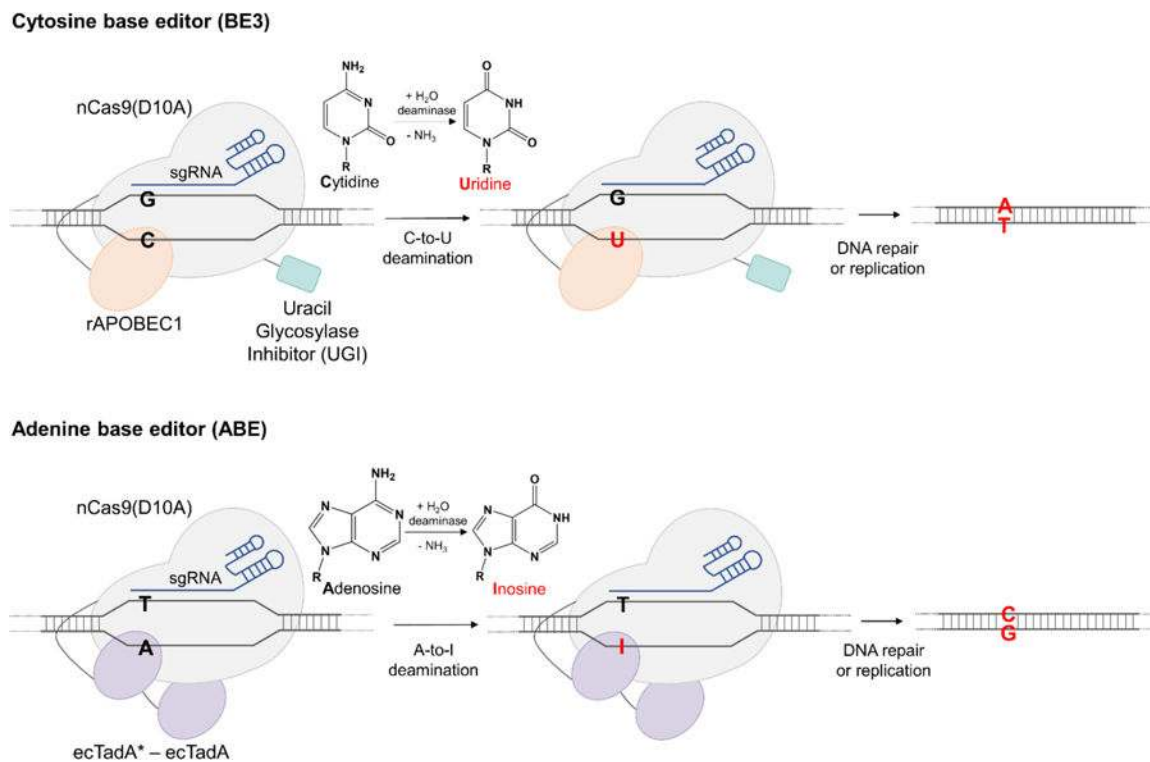
Cytosine base editor (BE3)

Adenine base editor (ABE)

Figure 1.
Cytosine base editor and Adenine base editor. (Top) Cytosine base editor can be directed to target DNA by nCas9 complex[19]. A cytidine deaminase (rAPOBEC1) deaminates a target cytidine (C) to uridine (U). This modification results in conversion of the original C•G base pair to T•A base pair by DNA repair or replication process[19]. (Bottom) When Adenine base editor is directed to target DNA by the nCas9 complex, an adenosine deaminase (ecTadA) deaminates a target adenosine (A) to inosine (I)[20]. Inosine is recognized as guanosine (G) by cellular machinery[37]. Therefore, base modification by adenine base editor leads to conversion of the original A•T base pair to G•C base pair by DNA repair or replication process[20].
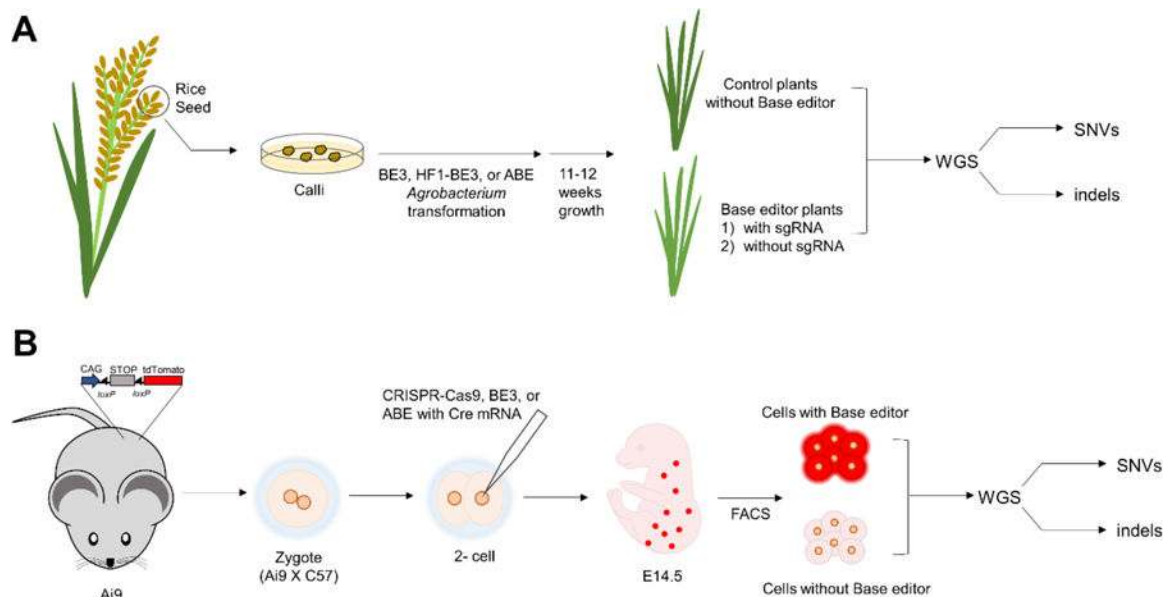
Figure 2.
Experimental designs to study off-target editing of DNA base editors. (**A**) Zuo et al. developed a method called Genome-wide Off-target analysis by Two-cell embryo Injection (GOTI) to determine genome wide off-target mutations in mouse[32]. (B) Workflow used in Jin et al. to identify identified the unintended genome wide off-target conversions in rice plants[31].
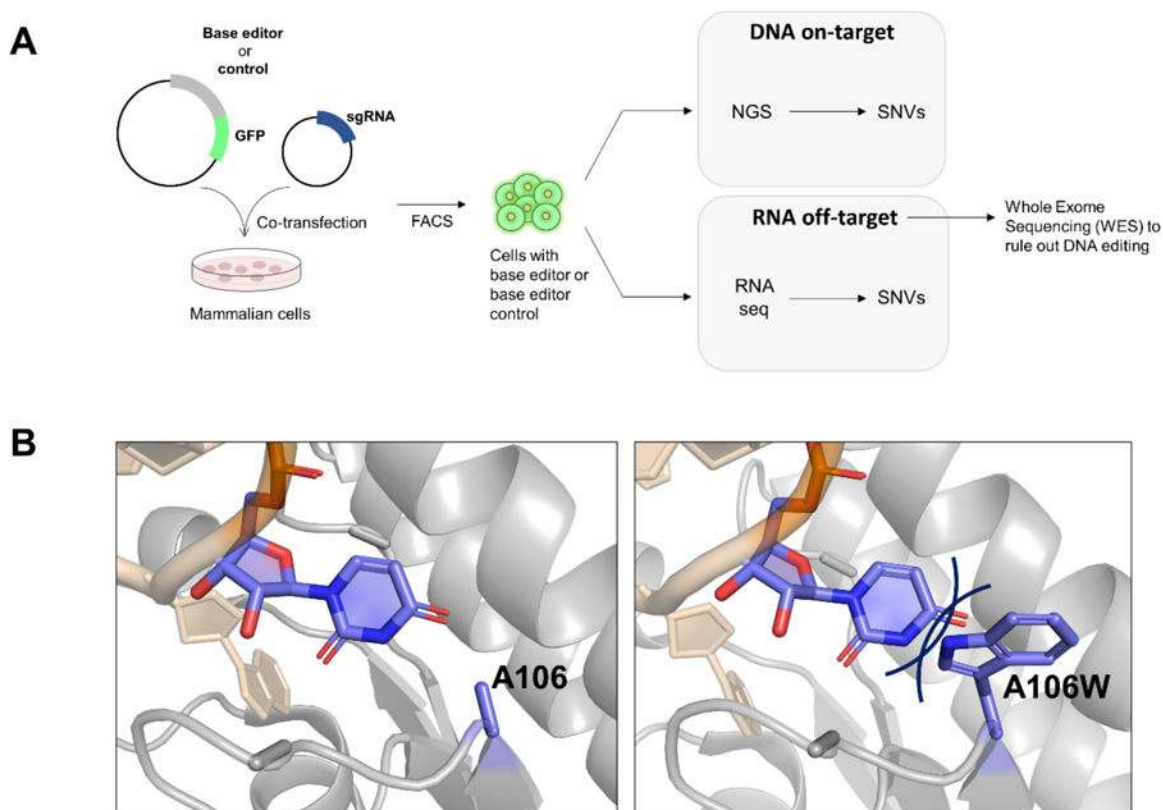
Figure 3.

(A) General experimental workflow from Grünewald et al.[30] to investigate the transcriptome wide RNA off-target mutations caused by DNA base editors. (B) Crystal structure of *S. aureus* TadA bound to a minimized version of its native substrate (tRNA[Arg2]) (PDB id: 2B3J)[39] showing Ala 106, a residue that corresponds to V106 in ecTadA*. The V106W mutation in ecTadA* (shown here as an A106W mutation in *S. aureus* TadA) is expected to result in a steric clash with RNA reducing undesired RNA off-target editing[33].
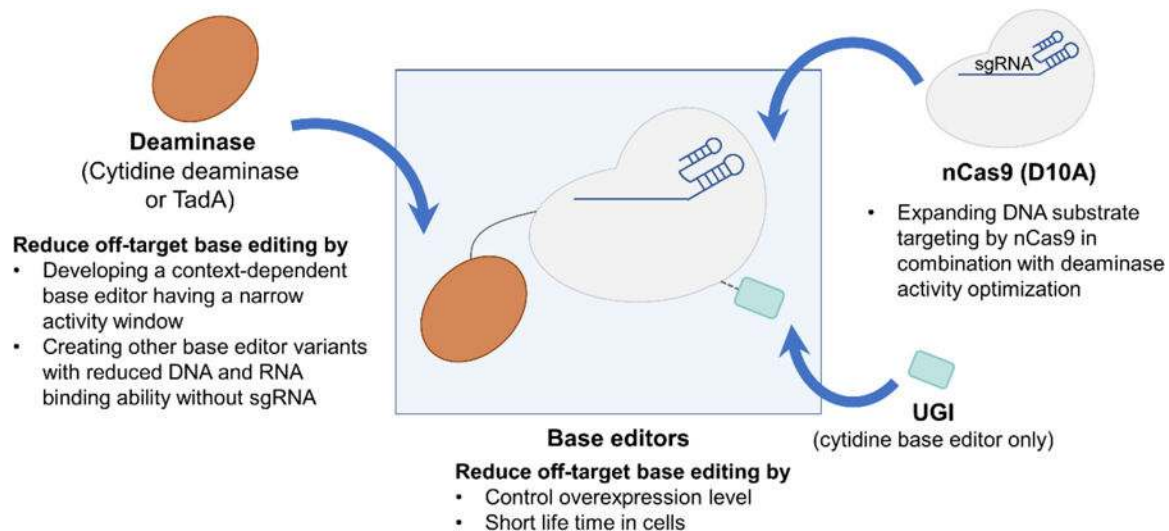
Figure 4.
Components of base editors and what can be optimized to minimize off-target editing, while maximizing the efficiency of base editing by DNA base editors.