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Perfecting Targeting in CRISPR

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Abstract

CRISPR-based genome editing holds promise for genome engineering and other applications in diverse organisms. Defining and improving the genome-wide and transcriptome-wide specificities of these editing tools are essential for realizing their full potential in basic research and biomedical therapeutics. This review provides an overview of CRISPR-based DNA- and RNA-editing technologies, methods to quantify their specificities, and key solutions to reduce off-target effects for research and improve therapeutic applications.

1. INTRODUCTION

guide RNA (gRNA): provides the specificity of an RNA-guided endonuclease, comprising a targeting sequence (CRISPR RNA) and a recruiting sequence (*trans*-activating CRISPR RNA) for Cas9 or only CRISPR RNA for Cas13

CRISPR (clustered regularly interspaced short palindromic repeats) is a natural bacterial defense system against bacteriophage infection that has recently been harnessed for genome and transcriptome editing in a wide range of organisms based on the generation of double-strand DNA breaks (DSBs) and RNA cleavage (3, 24, 32, 47, 52, 58, 73, 76, 79, 91, 127). On the basis of engineered type II (Cas9) and type VI (Cas13) programmable nucleases, DNA and RNA base editing, prime editing, and CRISPR interference/activation (CRISPRi/a) editing have been developed and expanded, enabling the correction and installation of mutations related to genetic diseases and the investigation of basic biological processes (4, 10, 37, 38, 40, 69–71, 87, 105, 115, 135), such as transcriptional perturbation (138) and epigenetic modulation (94). These DNA-based editors were generated by fusing dead Cas9 (dCas9) without DSB activity or Cas9 nickase (Cas9n) with only nickase activity to cytosine deaminases (e.g., APOBEC and AID for C-to-T editing) or transfer RNA (tRNA) adenosine deaminases (e.g., TadA for A-to-I editing) (37, 71, 89). RNA editing systems were formed by fusing dCas13b/dCas13d/dCas13X without RNA cleavage activity to an adenosine deaminase domain (e.g., $ADAR2_{DD}$ for A-to-I editing) or an engineered cytosine deaminase domain (e.g., $ADAR2_{dd}$ for C-to-U editing) (4, 25, 87, 135). To enable sequence-specific genomic regulation, dCas proteins also were fused to multiple gene-regulatory effectors, such as reverse transcriptase (10), transcriptional repressors and activators (40, 101), and epigenetic regulators (17, 99).

For therapeutic and genome-modification applications, one of the most prominent concerns is increasing the specificity of these wild-type and modified nucleases and base editors (BEs) as genome editing tools to prevent undesired mutagenesis or activity on nontargeted sequences. Unwanted local DNA cleavage from Cas9 can result in genomic instability, and unwanted RNA cleavage from Cas13 can lead to host dormancy, both of which are high risk factors for applications in future gene therapies (5, 92, 123). Thus, it is essential to quantitatively evaluate DNA and RNA off-target effects and even single-nucleotide variations (SNVs) generated by these tools. Many researchers have designed a number of unbiased methods for detecting off-target DNA and RNA edits from BEs, adenine BEs (ABEs), RNA editing for programmable adenosine (A) to inosine (I) replacement (REPAIR), and RNA editing for specific cytosine (C) to uracil (U) exchange (RESCUE) (4, 25, 28, 43, 55, 57, 63, 64, 82, 147, 150), as well as further engineered or evolved novel high-fidelity DNA/RNA editing tools using different Cas variants, modified guide RNA (gRNA), and/or fused deaminase variants (4, 25, 28, 44, 77, 87, 104, 135, 136).

In this review, we provide a comprehensive overview of the current methods for quantifying and eliminating off-target effects of CRISPR-based DNA/RNA targeting and editing. First, we briefly introduce the methods and optimized approaches for detecting and/or minimizing off-target effects of the CRISPR-Cas9 system. Next, we highlight unbiased methods available for detecting genome- and transcriptome-wide off-target effects from both Cas9-based DNA base editing and Cas13-based RNA targeting/editing systems, as well as various strategies to improve the specificity of these editors. Finally, we discuss other emerging CRISPR-based editing tools for therapeutic application and basic biological research, including prime editing, CRISPRi/a, and epigenome editing.

2. THE CRISPR-CAS9 SYSTEM

The type II CRISPR-Cas9 system has been widely used for genome editing in a variety of organisms (24, 32, 52, 58, 79, 91, 127). The most commonly used CRISPR-Cas9 system involves two critical components: a Cas9 nuclease derived from *Streptococcus pyogenes* (Sp), and approximately 20 nucleotides of single-guide RNA (sgRNA) that are complementary to the

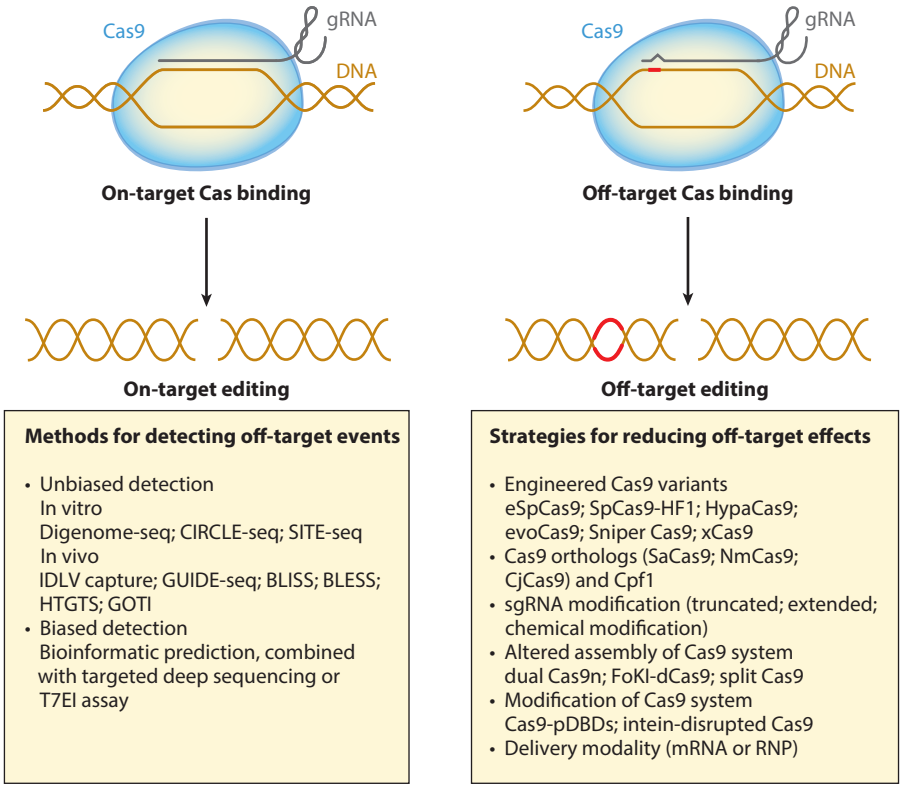


Figure 1

Schematic of CRISPR-Cas9-mediated on-target and off-target genome editing. Directed by a gRNA, the Cas9 nuclease generates double-strand DNA breaks at targeted sites (on-target editing). Cleavage at unintended sites (off-target editing) mainly results from sequence similarity between the gRNA and the off-target sites. Several biased and unbiased methods have been developed to detect off-target effects (26, 35, 39, 61, 122, 131, 137), while various strategies have assisted in reducing undesired off-target effects to improve the specificity (19, 21, 51, 67, 74, 77, 99, 110). Abbreviations: BLESS, direct in situ breaks labeling, enrichment on streptavidin, and next-generation sequencing; BLISS, breaks labeling in situ and sequencing; CIRCLE-seq, circularization for in vitro reporting of cleavage effects by sequencing; Digenome-seq, digested genome sequencing; GOTI, genome-wide off-target analysis by two-cell embryo injection; gRNA, guide RNA; GUIDE-seq, genome-wide unbiased identification of double-strand breaks evaluated by sequencing; HTGTS, high-throughput genome-wide translocation sequencing; IDLVs, integrase-deficient lentiviral vectors; mRNA, messenger RNA; pDBD, programmable DNA-binding domain; RNP, ribonucleoprotein; sgRNA, single-guide RNA; SITE-seq, selective enrichment and identification of adapter-tagged DNA ends by sequencing. **Figure 1** adapted with permission from Reference 60.

target DNA site, adjacent to a 5′-NGG protospacer adjacent motif (PAM) (24, 58, 91). Cas9 adopts a bilobed architecture composed of a nuclease (NUC) lobe—which contains the HNH and RuvC-like nuclease domains, a PAM-interacting (PI) domain, and a wedge domain (WED)—and an α-helical recognition (REC) lobe—which contributes to the recognition of gRNA-target DNA (59, 96, 97).

However, despite gRNAs guiding the target specificity of Cas9, unwanted mutations induced by Cas9 still occur at off-target loci, which can confound gene function research and impede the therapeutic applications (24, 43, 50, 100, 123) (**Figure 1**).

HNH nuclease domain: the domain containing a catalytic motif composed of histidine (H), asparagine (N), and aspartic acid (D) used to cleave the DNA strand complementary to the guide RNA

2.1. Methods for Detecting Off-Target Effects of Cas9

Various approaches for detecting the genome-wide off-target cleavage events have been established and can be divided into three main categories: in silico, in vitro, and in vivo techniques (60, 62) (**Figure 1**). In general, in silico detection methods based on computational algorithms are easily performed (1, 48, 113), such as with E-CRISP (**http://www.e-crisp.org/E-CRISP/**), Cas-OFFinder (**http://www.rgenome.net/cas-offinder/**), and CCTop (**https://crispr.cos.uni-heidelberg.de/**) software, but there are recognized limitations, such as putative off-target sites that are only partially predicted and not effectively ranked. Current in vivo methods are based on the identification, through deep sequencing, of cells containing off-target genomic DNA loci with DSBs for nucleases (such as Cas9); these genomic DNA loci are detected in the context of chromatin architecture and epigenetic status, which could influence off-target effects. These methods, which selectively label genomic regions with DSBs followed by amplification and whole-genome sequencing (WGS), include integrase-deficient lentiviral vector (IDLV) capture (35); genome-wide unbiased identification of DSBs evaluated by sequencing (GUIDE-seq) (122); high-throughput genome-wide translocation sequencing (HTGTS) (39); direct in situ breaks labeling, enrichment on streptavidin, and next-generation sequencing (BLESS) (26); breaks labeling in situ and sequencing (BLISS) (137); and discovery of in situ Cas off-target effects and verification by sequencing (DISCOVER-seq) (131). In vitro methods are based on the isolation of purified genomic DNA for in vitro cleavage reactions to determine the positions of DSBs as a function of Cas9-gRNA ribonucleoprotein (RNP) concentration, which could identify potential off-target sites, although these may not occur in the cellular context. In vitro methods include digested genome sequencing (Digenome-seq) (61), circularization for in vitro reporting of cleavage effects by sequencing (CIRCLE-seq) (120), and selective enrichment and identification of adapter-tagged genomic DNA ends by sequencing (SITE-seq) (16).

2.2. Methods for Reducing Off-Target Effects of Cas9

Strategies for reducing genome-wide off-target effects of CRISPR-Cas9 have been well developed, and can be grouped into four categories: engineered Cas9, engineered gRNA, modified Cas9 system, and changed delivery system (**Figure 1**). First, off-target effects might be minimized by eliminating the nonspecific interactions between SpCas9 and DNA sites. Based on the structural analysis of SpCas9 complexed with gRNA and target DNA, researchers have carried out rational design by amino substitutions in the Cas9 protein (8, 54, 97), generating various high-fidelity nucleases including eSpCas9 (110), SpCas9-HFI (67), Hypa-Cas9 (21), HeFspCas9 (74), SpCas9-NG (98), and HiFiCas9 (125). As an alternative to the structure-based rational design approach, directed evolution methods employ randomized libraries to screen for variants with improved specificity. Via various directed evolution platforms, xCas9 (51), Sniper-Cas9 (77), and EvoCas9 (19) were successfully selected with increased specificity and/or activity.

Second, engineered gRNAs provide an alternative, feasible, optimized strategy for reducing off-target effects of SpCas9. The use of more active gRNA architectures can increase DNA cleavage activity both in vitro and in cells but also at off-target sites (100). There is a trade-off between activity and specificity both in vitro and in cells as a shorter, less active gRNA is more specific than a longer, more active gRNA (100). Studies have demonstrated that off-target effects can be somewhat reduced by using alternative methods, including truncated gRNAs (tru-gRNAs) with destabilized cleavage complex formation (34), extended gRNAs with two extra guanine nucleotides at the 5′ end (23), and chemically modified gRNAs with deoxyribose nucleotides substituted for ribose nucleotides at the 5′ end (139). In addition, many strategies have been shown to reduce off-target genome editing, such as limiting the expression level of SpCas9 or gRNA (33, 75), delivery system modification with preassembled Cas9 RNPs or messenger RNA (mRNA) (65, 102), fusion

of Cas9 with a DNA-binding domain (Cas9-pDBD) (12, 13), and an intein-disrupted SpCas9 variant (27). Different modifications of Cas9-based systems, such as RNA-guided FokI-dCas9 nucleases, paired Cas9 nickases, and split Cas9 proteins, were also demonstrated to improve specificity (45, 90, 103, 121, 133, 141).

3. GENOME EDITING WITH BASE EDITORS

Most human genetic diseases are due to point mutations. The ability to precisely correct these point mutations has been of great interest for the treatment of genetic disorders. Tools have been developed to utilize dCas9 or Cas9n for site-specific editing without generating DSBs. Deaminases, which induce single-nucleotide conversion, are fused with dCas9 or Cas9n, forming BEs (**Figure 2*a*,*b***). The Liu group (71) fused a rat cytidine deaminase (APOBEC1) to dCas9 and found that it can convert C to U at a fixed point, leading to the conversion of a C:G base pair to T:A during the subsequent DNA replication process. However, cellular DNA repair responses can antagonize this process and repair edited bases (71, 89, 95). To prevent base excision repair and increase the editing efficiency, a uracil glycosylase inhibitor (UGI) was added to the 3′ terminus of a cytosine BE (CBE) (71). A third-generation editor (BE3) containing APOBEC1 fused to a 16-residue XTEN linker, Cas9n, and UGI [APOBEC1-XTEN-dCas9 (A840H)-UGI] can achieve permanent conversion of ∼15–75% of a target nucleotide in mammalian cells; this is currently the most widely used CBE (71) (**Figure 2*a***). BEs have been developed with human APOBEC3A for use in human and plant cells (38, 129, 149). To achieve the conversion from A:T to G:C, the Liu group (37) added the tRNA adenosine deaminase (TadA) from *Escherichia coli* to Cas9n and obtained adenine BEs (ABEs) (**Figure 2*b***). Optimized and enhanced CBEs and ABEs include BE4max, AncBE4max, and ABEmax (69).

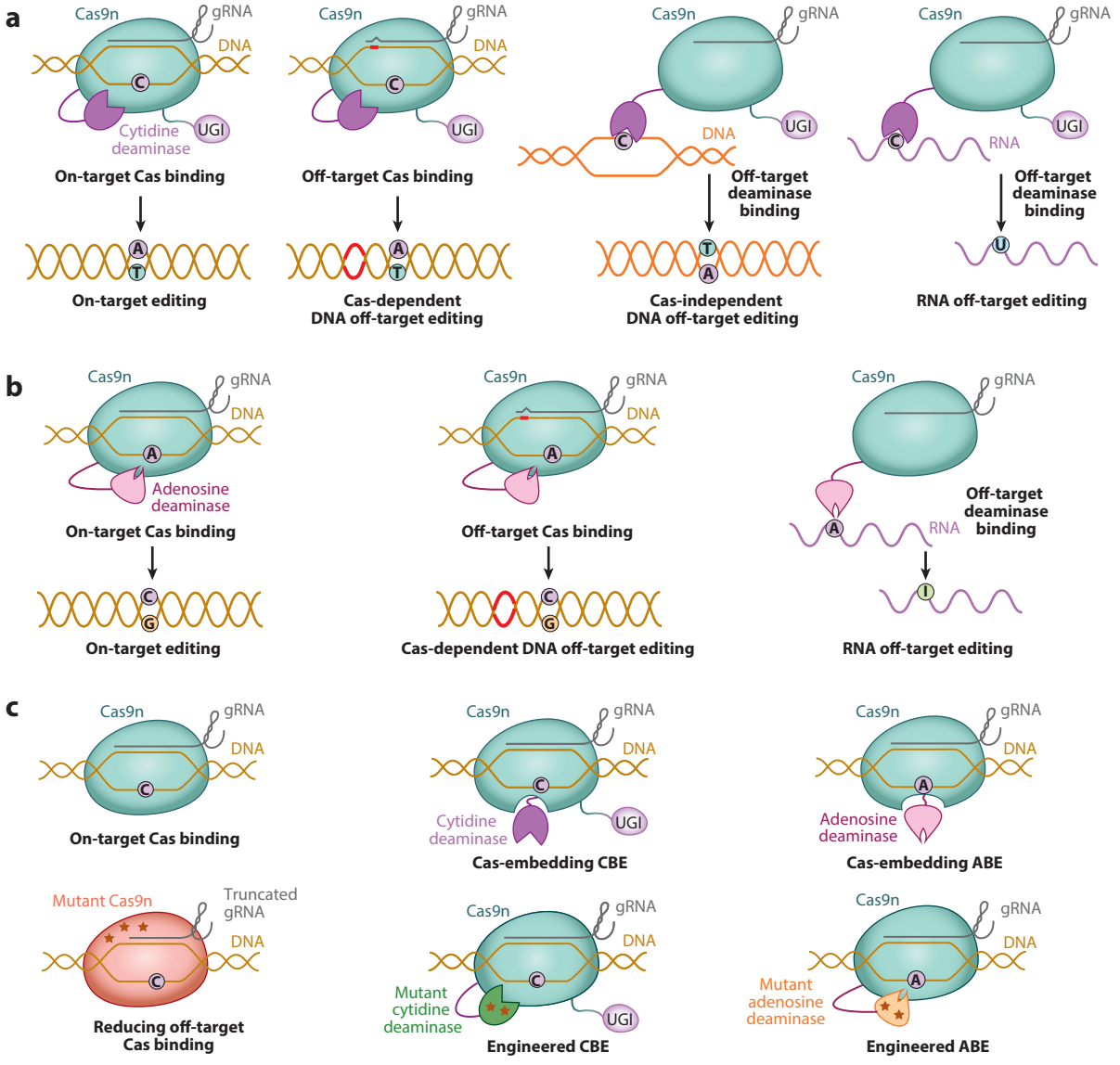
3.1. Off-Target Effects of Base Editors

There are two types of off-target activities of CBEs and ABEs: Cas-dependent and Cas-independent off-target events (**Figure 2*a*,*b***). Like CRISPR-Cas9 systems, the Cas-dependent off-target effects can be caused by the similarity of the sequences between gRNA-targeted sites and off-target sites within a given mismatch tolerance (119). One study using chromatin immunoprecipitation sequencing (ChIP-seq) on mouse embryonic stem cells (mESCs) transfected with programmable gRNAs found genome-wide binding of dCas9-sgRNA, with a majority of the dCas9-sgRNA complexes bound outside of the target site, suggesting that applications based on dCas9 or dCas9-effector fusions will be complicated by substantial off-target edits (134). The Cas-independent off-target effects of Cas9 effectors mainly result from random binding of deaminase from BEs (150). In addition, the lack of discrimination between the Cs (or As) within the base-editing window may cause editing of nontarget Cs (or As), referred to as bystander editing (14, 38, 105).

3.2. Detection of Off-Target Effects of Base Editors

To quantify the two types of off-target effects from BEs, various methods have been established and can be used to specifically measure Cas-dependent off-target or Cas-independent off-target edits (**Table 1**).

3.2.1. Cas-dependent off-target effects detection. Approaches based on deep sequencing that detect Cas-dependent off-target cleavage in the whole genome have been reported by several groups. Both endonuclease V sequencing (EndoV-seq) (82) and Digenome-seq (63, 64) are in vitro assays to investigate the Cas-dependent off-target effects of genome editing tools. These



- High-fidelity Cas domain ★
  HF-Cas9n
  (N497A, R661A, Q695A, Q926A)
  eCas9n (1.1)-pBE
  (K848A, K1003A, R1060A)
  Cas9n-HF2-pBE
  (N497A, R661A, Q695A,
  Q926A, D1135E)
  HypaCas9n-pBE
  (N692A, M694A, Q695A, H698A)
- gRNA truncations
- Delivery modality
  mRNA, RNP or LNP

- Cas-embedding CBE
  Insertion of cytidine deaminase of CBEs in the middle of Cas9n
- Engineered cytidine deaminase variants ★
  - For Cas-independent DNA off-target effects
    AALN rAPOBEC1 (R33A, K34A, H122L, D124N);
    YE1 rAPOBEC1 (W90Y, R126E); PpAPOBEC1 (WT, or H122A, or R33A); RrA3F (WT, or F130L); AmAPOBEC1; SsAPOBEC3B (WT, or R54Q); A3Bctd-VHM-BE3; A3Bctd-KKR-BE3
  - For RNA off-target effects
    SECURE rAPOBEC1 (R33A, or R33A and K34A);
    YE1 rAPOBEC1(W90Y, R126E); Human APOBEC3A (R128A, or Y130F)
    PpAPOBEC1 (WT, H122A, or R33A); RrA3F (WT, F130L)
    AmAPOBEC1; SsAPOBEC3B (WT, R54Q)

- Cas-embedding ABE
  Insertion of adenosine deaminase of ABEs in the middle site of Cas9n
- Engineered adenosine deaminase variants ★
  - For RNA off-target effects
    ABE7.10 TadA (F148A) -TadA* (F148A)
    ABEmax TadA (E59A) -TadA* (V106W)
    miniABEmax TadA* (V82G, or K20A /R21A)

(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

General overview of CRISPR/Cas9-based on-target and off-target base editing. (*a*) CBEs consisting of cytidine deaminase fused to the Cas9n-gRNA complex to generate a targeted C-to-T transition in DNA within the base-editing window. However, off-target base changes can occur in DNA because the deaminase can be led to off-target sites by Cas9n (owing to gRNA mismatch and/or randomly binding at nontarget ssDNA). Off-target base editing occurs in RNA, mainly resulting from the natural affinity of deaminases to RNA (panel *a* adapted with permission from Reference 60). (*b*) Adenosine deaminase in the ABEs catalyzes a targeted A-to-G conversion. The gRNA mismatch at undesired off-target sites results in off-target editing in DNA, while the deaminase's accessibility to RNA results in off-target base editing in RNA (panel *b* adapted with permission from Reference 60). (*c*) Several strategies have been developed to reduce Cas-dependent and Cas-independent DNA off-target effects of CBEs, Cas-dependent DNA off-target effects of ABEs, and RNA off-target effects of both CBEs and ABEs. Abbreviations: A, adenosine; ABE, adenine base editor; Cas9n, Cas9 nickase; CBE, cytosine base editor; C, cytosine; G, guanine; gRNA, guide RNA; I, inosine; LNP, lipid nanoparticle; mRNA, messenger RNA; RNP, ribonucleoprotein; ssDNA, single-stranded DNA; T, thymine; tRNA, transfer RNA; U, uracil; UGI, uracil glycosylase inhibitor; WT, wild type.

Table 1 Assays for detecting off-target mutations caused by base editors

Detection Assays	Description	Application	Reference(s)
Modified Digenome-seq 1	Purified genomic DNA is treated with the base editor and a mixture of DNA-modifying enzymes in vitro to produce DNA DSBs at uracil-containing sites. Off-target edits are computationally identified from WGS data.	Cas-dependent DNA off-target edits for CBE	64
EndoV-seq	Purified genomic DNA deaminated by ABE in vitro is treated with endonuclease V to nick the inosine-containing DNA strand, followed by WGS. Off-target edits are computationally identified.	Cas-dependent DNA off-target edits for ABE	82
Modified Digenome-seq 2	Purified genomic DNA is treated with ABE7.10 and either endonuclease V or a combination of human alkyladenine DNA glycosylase and endonuclease VIII in vitro. Off-target edits are computationally identified from WGS data.	Cas-dependent DNA off-target edits for ABE	63
GOTI	One blastomere of a two-cell mouse embryo is edited, followed by applying WGS of FACS-purified progeny cells of edited and nonedited blastomeres at embryonic day 14.5 to identify genome-wide off-target single-nucleotide variants in edited cells.	Cas-dependent and Cas-independent DNA off-target edits for CBE, Cas-dependent DNA off-target edits for ABE	150
Agrobacterium-mediated transformation of rice callus cells	Rice callus cells are edited by base editors with *Agrobacterium*-mediated transformation. After 11–12 weeks, regenerated T_0 plants derived from the transformed calli are analyzed by WGS to identify genome-wide single-nucleotide variants and indels.	Cas-dependent and Cas-independent DNA off-target edits for CBE, Cas-dependent DNA off-target edits for ABE	57
Bacterial rifampin resistant assay	In addition to editing the on-target C-to-T mutation site in the inactivated chloramphenicol acetyltransferase gene, which rescued the impaired chloramphenicol resistance, the deaminase-catalyzed off-target C-to-T mutations in the *rpoB* gene enable *Escherichia coli* resistance to the antibiotic rifampin. Thus, off-target C-to-T mutations are evaluated by counting the number of rifampin-resistant colonies.	Cas-independent DNA off-target edits for CBE	28
Bacterial thymidine kinase toxicity assay	As deaminase-catalyzed C-to-T mutations in the promoter of the *HSV-TK* gene inactivate this kinase and promote *E. coli* survival in the presence of the nucleoside analog dP, off-target C-to-T mutations are evaluated by counting the number of dP-resistant colonies.	Cas-independent DNA off-target edits for CBE	28

(*Continued*)

Table 1 (*Continued*)

Detection Assays	Description	Application	Reference(s)
Human cell orthogonal R-loop assay	Plasmids encoding an SpCas9-based CBE and an SpCas9 on-target sgRNA are cotransfected into human HERK293T cells with a catalytically dead SaCas9 (dSaCas9) and an SaCas9 sgRNA targeting a genomic locus unrelated to the on-target site; this can generate long-lived R-loop (DNA:RNA hybrids and the associated ssDNA), which magnifies SpCas9 sgRNA-independent deamination at this specific locus. Then targeted amplicon sequencing of the orthogonal R-loop site is performed to monitor the off-target deamination.	Cas-independent DNA off-target edits for CBE	28
Orthogonal R-loop assay in plants	Plasmids encoding CBEs based on SpCas9 are cotransformed into rice protoplasts with an ssDNA-generating SaCas9 nickase and an SaCas9 sgRNA targeting a given genetic site. The nSaCas9-sgRNA can generate an orthogonal R-loop that is more susceptible to cytidine deaminase fused to SpCas9. The off-target deamination is then evaluated by targeted amplicon sequencing of the orthogonal R-loop site.	Cas-independent DNA off-target edits for CBE	55
RNA-seq	Bulk RNA sequencing or single-cell RNA sequencing	Cas-independent RNA off-target edits for CBE and ABE	43, 106, 147

Abbreviations: ABE, adenine base editor; CBE, cytosine base editor; Digenome-seq, digested genome sequencing; dP, 6-(β-d-2-deoxyribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c][1,2]oxazin-7-one; DSB, double-strand break; EndoV-seq, endonuclease V sequencing; FACS, fluorescence-activated cell sorting; GOTI, genome-wide off-target analysis by two-cell embryo injection; gRNA, guide RNA; *HSV-TK*, herpes simplex virus thymidine kinase; RNA-seq, RNA sequencing; sgRNA, single-guide RNA; ssDNA, single-stranded DNA; WGS, whole-genome sequencing.

methods mostly rely on the generation of nicks on the genome and thus are not applicable to detect Cas-independent off-target effects. Additionally, Arbab et al. (11) developed and integrated a library of 38,538 target sequences into the genome in mammalian cells and characterized the sequence-activity relationships among several CBEs in the BE4max architecture (BE4max, BE4-CP, ecoA-BE4, AID, CADA1-BE4, and eA3A-BE4) and ABEs in the ABEmax architecture (ABEmax and cp1041-ABEmax) on those targets. Based on the above analysis, they further trained and described the machine learning model (BE-HIVE), which could accurately predict base-editing genotypic outcomes and efficiency, and discovered unpredictable C-to-G or C-to-A bystander off-target edits (11).

3.2.2. Cas-independent off-target DNA/RNA effects detection. Sensitive detection of off-target effects is important for CRISPR BE application to human therapeutics. Recently developed methods to detect Cas-independent off-target DNA activities caused by BEs include WGS, genome-wide off-target analysis by two-cell embryo injection (GOTI), the bacterial rifampin-resistant assay, the bacterial thymidine kinase toxicity assay, and the orthogonal R-loop assay. For detecting off-target RNA edits, the conventional, classic approaches are transcriptome-wide bulk RNA sequencing (RNA-seq) and single-cell RNA-seq.

3.2.2.1. Whole-genome sequencing. Several previous studies applied WGS, an unbiased and precise method, to detect potential off-target effects of CRISPR/Cas9 (9, 53, 117, 132). However, while WGS at heterogeneous cells can provide direct genome-wide information on specificity, it is difficult to distinguish true off-target SNVs induced by BEs and intrinsic differences reflecting

biological heterogeneity in analyte cells (56). WGS analysis of rice callus cells based on clonally derived systems treated with BEs overcomes the complicated analysis of large genomes from heterogeneous cells (57). Jin et al. (57) performed a comprehensive analysis of genome-wide off-target effects from BEs, including BE3, high-fidelity BE3 (HF1-BE3), and ABE, which revealed that BE3 and HF1-BE3 induce substantial off-target edits.

3.2.2.2. Genome-wide off-target analysis by two-cell embryo injection. Zuo et al. (150) established an off-target detection technology called GOTI to overcome high background mutation rates and the heterogeneity present in large pools of examined populations. They selectively edited one blastomere from a two-cell mouse embryo, and then applied WGS to identify potential off-target edits. This method avoids the noise caused by in vitro amplification of single cells. In addition, since the experimental and control groups are from the same embryo, they have the same genetic background. Therefore, the differences between the genomes of two groups can be considered to be caused by the editing tools. GOTI can be applied to evaluate the off-target effects induced by CRISPR-Cas9, CBEs, and ABEs.

3.2.2.3. Bacterial rifampin-resistant assay. Doman et al. (28) described a rifampin resistant assay used to measure Cas9-independent deamination by CBEs in *E. coli*. This assay is based on survival rates on chloramphenicol plates compared to survival rates on rifampin plates, which reflect high on-target editing activity and Cas-independent deamination activity, respectively. Chloramphenicol resistance is rescued by the CBE-catalyzed C-to-T mutation of an inactivated variant of chloramphenicol acetyltransferase (i.e., carrying an inactivated T-to-C edit). Rifampin resistance is reliably generated based on the Cas-independent deamination–catalyzed C-to-T mutation of the *rpoB* gene in *E. coli*. This assay allows rapid and cost-effective measurement of Cas-independent off-target effects.

3.2.2.4. Bacterial thymidine kinase toxicity assay. Similar to the bacterial assay described above, the thymidine kinase toxicity assay was designed to detect deaminase activity via catalyzing off-target C-to-T editing on the promoter of the herpes simplex virus thymidine kinase (*HSV-TK*) gene. The *HSV-TK* gene is inserted into the *E. coli* chromosome; the additive nucleoside analog 6-(β-d-2-deoxyribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-c] [1,2] oxazin-7-one (dP) is phosphorylated by HSV-TK and incorporated into the genome of *E. coli* cells, resulting in cell death. The off-target editing on the promoter inactivates the *HSV-TK* gene expression and thus leads to the survival of *E. coli* in the presence of dP (28).

3.2.2.5. Orthogonal R-loop assay. Doman et al. (28) developed an assay to measure Cas9-independent deamination by CBEs in human cells, which is not dependent on WGS. The catalytically inactive *Staphylococcus aureus* Cas9 (dSaCas9) and the SaCas9 sgRNA targeting the genomic DNA unrelated to an on-target site are coexpressed in HEK293T with a dSpCas9-based CBE and an on-target sgRNA. SpCas9-independent deamination by CBEs at the unrelated target site is magnified by the generation of a long-lived R-loop [DNA:RNA hybrids and the associated single-stranded DNA (ssDNA)]. Therefore, this off-target deamination can be measured easily by high-throughput sequencing. This R-loop assay allows the detection of Cas-independent off-target DNA editing in a high-throughput, time-saving, and cost-effective manner. Jin et al. (55) combined the nSaCas9-mediated R-loop assay and WGS to analyze the specificity of CBEs in plants.

3.2.2.6. Transcriptome-wide RNA sequencing. Transcriptome-wide RNA-seq was performed to detect off-target editing of bases at nontarget sites in RNA. Three initial reports show that,

through transcriptome-wide RNA-seq, both CBEs and ABEs cause substantial (such as tens of thousands of) off-target RNA SNVs in human cells (43, 106, 147). These RNA-seq studies were performed by Zhou et al. (147) with the RNA-seq data (125× coverage depth) for HEK293T cells with BE3 and ABE7.10 and the RNA-seq (∼70–100 million reads per library) of BE3-expressing HepG2 cells by Grünewald et al. (43). To avoid the loss of random off-target signals in bulk cells, single-cell RNA-seq was performed to confirm off-target effects from BE3 and ABE7.10 (147).

3.3. Strategies to Reduce Off-Target Effects of Base Editors

The specificity of CRISPR-based DNA BEs could be enhanced in various ways. Methods include reducing Cas-dependent off-target effects by modified gRNA/Cas9 and Cas-independent off-target effects through engineered high-fidelity cytidine/adenosine deaminase (**Figure 2*c***; **Table 2**). Additionally, alteration of the delivery systems could reduce both off-target effects described above.

3.3.1. Modified guide RNAs and engineered Cas9. Many off-target effects result from nonspecific binding of gRNA with possible mismatches. Efforts focused on modifying the gRNA scaffold to increase the targeting specificity, such as truncating the 3′ end of gRNA, can significantly improve specificity. In addition to modified gRNA, Cas9 engineering has been applied to increase the targeting specificity to combat Cas-dependent off-target effects. High-fidelity Cas9 variants, as described in Section 2.2, can be incorporated into classic CBE architecture to increase specific binding and/or reduce tolerance for mismatched DNA binding (104, 136). Cas9-HF, eCas9, Cas9n-HF, HypaCas9n, and circularly permuted CP1028 in the form of nickase were incorporated into CBE architecture, significantly decreasing Cas-dependent off-target effects (28, 104, 136). These variants may be applied in therapeutic base editing.

A Cas-embedding BE approach introduced deaminase (APOBEC1 or TadA-TadA*) into the middle of Cas9n, which is different from the conventional approach of fusion to the N terminus. Using MuA-transposon-based random insertion and genetic screening, Liu et al. (88) identified the Cas9n site that can be tolerant of deaminase insertion, which reduces DNA off-target effects, perhaps by increasing steric hindrance.

3.3.2 Delivery modality. Delivery methods that allow continuous, uncontrolled BE and sgRNA expression even after the on-target locus has been edited increase the opportunity for genome editing at off-target loci (104). Off-target levels can be increased along with sustained exposure to the BE; thus, high concentrations of gRNA:BE complexes can generate mutations at off-target loci within the PAM if the concentration of the enzyme is not limited. In contrast to plasmid-based delivery, direct delivery of the BE and/or sgRNA as the RNP complex or mRNA/sgRNA form (31, 63, 80, 104) or delivery of BE mRNA and sgRNA using lipid nanoparticles (LNPs) (126) results in more transient BE activity and hence improved specificity with fewer off-target effects.

3.3.3. Engineered cytidine deaminase. Except modified sgRNA and engineered Cas9 to reduce Cas-dependent off-target effects, the major strategy to reduce Cas-independent off-target effects of CBEs is to engineer cytidine deaminase, producing various high-specificity CBE variants (**Table 2**).

3.3.3.1. SECURE-BE3. Based on previous reports that various rAPOBEC1 mutations reduce RNA C-to-U editing, the Joung group (43) engineered selective curbing of unwanted RNA editing (SECURE) variants and identified two of these variants (BE3-R33A and BE3-R33A-K34A).

Table 2 Strategies for reducing Cas-independent off-target edits caused by base editors

Class	Base editors	Engineered deaminases	Initial base editor	Major properties	Reference(s)
CBE	SECURE-BE3	rAPOBEC1 (R33A or R33A/K34A)	BE3	Reduce RNA off-target edits, narrow the editing window	43
	eA3A-BE3	hAPOBEC3A (N57G, Y130F, or R128A)	BE3	Reduce bystander editing and DNA off-target edits (for N57G), reduce RNA off-target edits (for Y130F or R128A)	38, 147
	hA3G-CBE	hAPOBEC3G (NTD truncation) in A3G-BE4.4; hAPOBEC3G (NTD truncation /P200A/N236A/P247K/Q318K/Q322K/H248N/K249L/H250L/G251C/F252G/L253F/E254Y/Y315F) in A3G-BE5.13; hAPOBEC3G (NTD truncation/P200A/N236A/P247K/Q318K/Q322K/L234K/C243A/F310K/C321A/C356A/Y315F) in A3G-BE5.14	BE4max	Reduce DNA off-target edits, reduce RNA off-target edits for A3G-BE5.13, broaden the editing window	78
	AALN-BE4	rAPOBEC1 (R33A/K34A/H122L/D124N)	BE4max	Reduce DNA off-target edits, broaden the editing window	28
	YE-rAPOBEC1-BE	rAPOBEC1 (W90Y/R126E, W90Y/R132E, R126E/R132E, or W90Y/R126E/R132E)	BE3	Reduce DNA off-target edits, narrow the editing window	66
		rAPOBEC1 (W90Y/R126E, W90Y/R132E, R126E/R132E, or W90Y/R126E/R132E)	BE4max	Reduce DNA/RNA off-target edits, narrow the editing window	28
		rAPOBEC1 (W90Y/R126E)	BE3	Reduce DNA/RNA off-target edits	147, 151
		rAPOBEC1 (W90Y/R126E/FNLS)	BE3	Reduce DNA/RNA off-target edits, narrow the editing window, enhance on-target editing efficiency	151
	Other BEs	RrA3F (WT or F130L); SsAPOBEC3 (WT or R54Q); PpAPOBEC3B (WT, H122A, or R33A); AmAPOBEC1	BE4	Reduce DNA/RNA off-target edits	140
		hA3Bctd (T214V/D314H/Y315M or R211K/R311K/D314R)	BE3	Reduce DNA off-target edits	55
ABE	ABE7.10-F148A	TadA (F148A)-TadA* (F148A)	ABE7.10	Reduce RNA off-target edits, narrow the editing window	147
	ABEmaxAW	TadA (E59A)-TadA* (V106W)	ABEmax	Reduce RNA off-target edits, improve DNA editing specificity	106
	ABEmaxQW	TadA (E59Q)-TadA* (V106W)	ABEmax	Reduce RNA off-target edits, improve DNA editing specificity	106
	SECURE-ABE	TadA (deletion)-TadA* (K20A/R21A or V82G)	ABEmax	Reduce RNA off-target edits	44
	ABE8e-V106W	TadA (deletion)-TadA* (A109S/T111R/D119N/H122N/Y147D/F149Y/T166I/D167N/V106W)	ABEmax	Increase editing efficiency, reduce DNA/RNA off-target edits compared with ABE8e	107

Abbreviations: ABE, adenine base editor; BE, base editor; CBE, cytosine base editor; NTD, N-terminal domain; SECURE, selective curbing of unwanted RNA editing; TadA, transfer RNA adenosine deaminase; WT, wild type.

Compared to BE3, the variants showed reduced RNA off-target edits, narrowed the on-target editing window at C5–C7 (BE3-R33A) and the even-more-restricted editing window C5–C6 (BE3-R33A-K34A) with a relatively stringent 5′-TC requirement. Based on the predicted three-dimensional-structure model of variants, these mutation sites do not locate adjacent to the deaminase catalytic residue and thus do not affect on-target DNA editing. By fusion with engineered Cas9s containing altered PAM recognition, the variants have high specificity and a broadened target site, which will be advantageous in therapeutic applications.

3.3.3.2. eA3A-BE3. Similar to the rat APOBEC1, human APOBEC3A deaminase was fused into BE3 to obtain A3A-BE3, which showed high on-target C-to-T DNA-editing activity but also high off-target activity. Gehrke et al. (38) introduced a N57G mutation in the hA3A domain, forming eA3A-BE3, and Zhou et al. (147) introduced Y130F or R128A in the hA3A domain; all of these mutations reduced RNA off-target effects, probably due to reduced binding between RNA and the ssDNA-binding domain of hA3A. Here, we collectively refer to A3A(N57G)-BE3, A3A(R128A)-BE3, and A3A(Y130F)-BE3 as eA3A-BE3.

3.3.3.3. hA3G-CBE. Current CBEs edit all Cs within their activity windows, generating undesired bystander mutations. Lee et al. (78) identified and engineered the human APOBEC3G (A3G) deaminase; when fused to the Cas9n, the resulting A3G-BEs exhibit selective editing of the second C in the 5′-CC-3′ motif in human cells. On the basis of the two-cell embryo injection method and RNA-seq analysis, A3G-BEs showed minimum genome- and transcriptome-wide off-target effects, achieving high targeting fidelity.

3.3.3.4. AALN-BE4. To improve the efficiency of CBE, the fourth-generation BEs, BE4 (rAPOBEC1-Cas9n-UGI-UGI), were generated by appending a second copy of UGI to BE3 (72). BE4max, still referred to as BE4, has further optimized codon usage and nuclear localization signals (NLSs) (69). On the basis of SECURE-BE3, an R33A-K34A mutation was introduced into BE4 to reduce off-target effects, with the additional amino acid changes H122L and D124N made to enable the efficient deamination of 5′-GC rather than being limited to a 5′-TC substrate (28). Compared with BE4, AALN-BE4 showed much lower levels of DNA off-target deamination via the orthogonal R-loop assay and additionally reduced Cas-dependent DNA off-target editing based on GUIDE-seq.

3.3.3.5. YE-rAPOBEC1-BE. Previous reports indicate that W90Y/W90F mutations decrease the hydrophobicity of catalytic sites of rat APOBEC3G/APOBEC1, and R126E or R132E mutations influence ssDNA accessibility (22, 49, 66). Combined with these mutations, various rAPOBEC1 variants that also contain YE1 (W90Y-R126E), YE2 (W90Y-R132E), EE (R126E-R132E), and YEE (W90Y-R126E-R132E) were obtained and fused to Cas9n; these editors showed narrowed editing windows and reduced base-editing activity (66). Via WGS, GOTI, and RNA-seq, these variants fused with BE4max (BE4) and exhibited reduced Cas-independent DNA/RNA off-target editing while retaining on-target DNA edits, especially YE1-BE3 and YE1-BE4 (28, 147, 151).

3.3.3.6. Other APOBEC CBEs. Other than rAPOBEC1, cytosine deaminases such as APOBECs from additional species, e.g., RrA3F, AmAPOBEC1, SsAPOBEC3B, or SsAPOBEC3B, display a high in *cis*/in *trans* ratio at selected sites (140). The high-fidelity (HiFi) mutations R33A, W90F, K34A, R52A, H122A, H122, and/or Y120F provide additional improvements to rAPOBEC1 (140). Based on the variants described above and a high-throughput screening for comparisons against BE4, BE4 with RrA3F (WT, F130L), AmAPOBEC1, SsAPOBEC3 (WT, R54Q), and PpAPOBEC3B (WT, H122A, R33A) (140) were found to display high on-target DNA-editing activity (28) and minimized unguided DNA and RNA off-target activity. Additionally, A3Bctd-BE3, constructed with human A3Bctd (a truncated APOBEC3B deaminase) and fused to BE3, exhibited improved cytosine base editing in plant cells. Through further rational engineering, two variants, A3Bctd-VHM-BE3 and A3Bctd-KKR-BE3, were obtained, and both showed efficient on-target editing and reduced Cas-independent DNA off-target editing as demonstrated by WGS and an R-loop assay (55).

3.3.4. Engineered adenosine deaminase. Other than modified sgRNA and engineered Cas9 to reduce Cas-dependent off-target effects, the major strategy to reduce Cas-independent off-target effects of ABEs is to engineer adenosine deaminase, forming high-specificity ABE variants (**Table 2**).

3.3.4.1. ABE7.10-F148A. Based on transcriptome-wide RNA-seq at an average depth of 125×, the Yang group (147) identified substantial off-target RNA SNVs induced by ABE7.10, and they subsequently rational-designed the substitution of F148A in both the TadA and TadA* domains, which nearly abolished the RNA off-target effects and narrowed the editing window, further increasing the precision of DNA base editing.

3.3.4.2. ABEmaxAW/ABEmaxQW. Based on ABE7.10, codon and nuclear localization sequence optimization were performed to generate ABEmax, improving the efficiency of ABE (69) but also leading to increased off-target RNA mutations (106). Guided by the TadA and TadA* structures, Rees et al. (106) introduced E59A/E59Q to TadA and V106W to TadA* to generate ABEmaxAW (ABEmax, TadA E59A/E59Q, TadA* V106W). These mutations inactivated the deaminase activity of wild-type TadAs and altered the binding space between TadA* and ssRNA, which greatly minimized RNA off-target editing (106).

3.3.4.3. SECURE-ABE. SECURE-ABE was designed based on a principle similar to the development of SECURE-CBE. Considering that the wild-type TadA domain from ABEmax might introduce nontarget RNA binding and editing, the Joung group (44) removed the wild-type TadA domain form ABEmax to generate miniABEmax. Guided by the structural information, they further modified the TadA* of miniABEmax by the substitution of amino acids (K20A-R21A or V82G). Both variants exhibited reduced off-target RNA-editing activity while retaining comparable high on-target DNA-editing activity.

3.3.4.4. ABE8e-V106W. Furthermore, the Liu group (107) evolved ABE7.10 (the optimized version of initial ABE7.10, also named miniABEmax) to ABE8e via phage-assisted noncontinuous and continuous evolution (PANCE and PACE) with eight additional mutations. While significantly improving (~590-fold) on-target DNA-editing activity compared to that of the earlier ABE version, ABE8e also shows increased off-target RNA- and DNA-editing activity. By introducing a V106W substitution into the TadA domain, as ABE8e-V106W, DNA/RNA off-target edits were substantially reduced, without affecting the increased on-target activity.

4. CRISPR-CAS13-BASED RNA TARGETING AND EDITING

Type VI CRISPR-Cas systems with the single effector protein Cas13, which includes six subtypes—VI-A (Cas13a, known as C2c2), VI-B (Cas13b/C2c6), VI-C (Cas13c/C2c7), VI-D (Cas13d), VI-X (Cas13X), and VI-Y (Cas13Y)—were identified through metagenomic discovery (5, 29, 30, 73, 112, 135). CRISPR-Cas13 exclusively targets RNA via an intrinsic RNase activity that can be activated by the binding of a CRISPR RNA (crRNA) gRNA and has the potential to be developed as a promising technology for RNA biology (3).

Akin to a DNA BE, Cas13 could be utilized for RNA editing in cells. Catalytically inactivated Cas13 proteins (dCas13) have been fused to natural RNA deaminases to develop CRISPR-derived RNA base-editing systems that directly mediate nucleotide conversions, such as REPAIR (RNA editing for programmable adenosine (A) to inosine (I) replacement) from adenosine deaminase (ADAR) from *Xenopus* oocytes (25). Using an evolved ADAR domain, cytidine deaminase

(C-to-U) edits of dCas13-ADAR2$_{dd}$ (RESCUE) were achieved (4). In contrast to DNA base cleavage/editing with irreversible, permanent changes to the genome, RNA base cleavage/editing enables reversible modifications in genetic material and allows more flexible control of gene function. However, off-target effects from Cas13 may impede the development of its in vivo applications.

4.1. On-Target RNA Cleavage and Applications

Previous structural studies of Cas13a, Cas13b, and Cas13d show that Cas13 proteins implement RNA cleavage activity with two distant catalytic sites in a two-step process (30, 73, 84, 85, 111, 142, 143) (**Figure 3*a***). The first step is termed crRNA maturation, wherein pre-crRNA binding, structural rearrangement, and processing occur at the Cas13 crRNA recognition (REC) lobe. The second step involves crRNA-guided target RNA binding and cleavage, through the formation of a guide-target RNA duplex via base pair complementarity, further introducing significant conformational changes in the nuclease (NUC) lobe, such that the two higher eukaryote and prokaryote nucleotide-binding (HEPN) domains are exposed to an outside surface, thus forming one catalytic site to cut target or collateral RNAs.

With the advantage of on-target RNA interference ability, CRISPR-Cas13 has been widely applied for transcriptome editing by inducing RNA cleavage at defined loci in eukaryotic cells. LwaCas13a exhibits broad and high efficacy for targeted knockdown of endogenous transcripts in mammalian and plant cells (3). In addition, Cas13a has been used for virus transcript knockdown against RNA viruses in tobacco (7, 145). Due to their compact size, RfxCas13d derived from *Ruminococcus flavefaciens* XPD3002 (termed CasRx) and Cas13X/Cas13Y derived from microorganisms in hypersaline habitats could be packaged into a single adeno-associated virus (AAV) vector particle to generate RNA knockdown across diverse endogenous transcripts with high efficiency and specificity, compared to that of RNA interference in human cells (73, 135). Recent reports show that CasRx induces efficient *Vegfa* knockdown in a mouse model of age-related macular degeneration (146), *Pcsk9* knockdown in the mouse liver for metabolism modulation (47), and maternal and zygotic mRNA knockdown in zebrafish embryos (76). Cas13-mediated *Ptbp1* knockdown was shown to induce a glia-to-neuron conversion in Parkinson or a retinal injury mouse model (148). Additionally, as an antiviral strategy, Cas13X can effectively degrade RNA from coronaviruses SARS-CoV-2, and CasRx degrades RNA from both SARS-CoV-2 and live influenza A virus in human lung epithelial cells through prophylactic antiviral CRISPR in human cells (PAC-MAN) (2, 135). CasRx has also been adopted as a useful tool for the discovery of functional circular RNAs (81).

4.2. Off-Target RNA Cleavage and Detection Methods

As the applications of CRISPR-Cas13 are progressing toward the clinic and in vivo therapeutic applications, one of the most frequent concerns is the specificity or off-target effects of the nucleases. Like those of Cas9, off-target effects of Cas13 likely are mainly due to base mismatch within gRNA and nuclease/Cas collateral-dependent off-target effects. To improve RNA knockdown safety, it is critical to be able to precisely measure and quantify off-target effects. Methods for transcriptome-wide identification of potential off-target effects include bioinformatics prediction, fluorescence probes in vitro, fluorescence reporters and unbiased transcriptome-wide RNA-seq in vivo (**Figure 3*a***).

4.2.1. Guide RNA mismatch-dependent off-target effects. Through the systematic analysis of CasRx targeting of endogenous transcripts, studies of massively parallel screens of

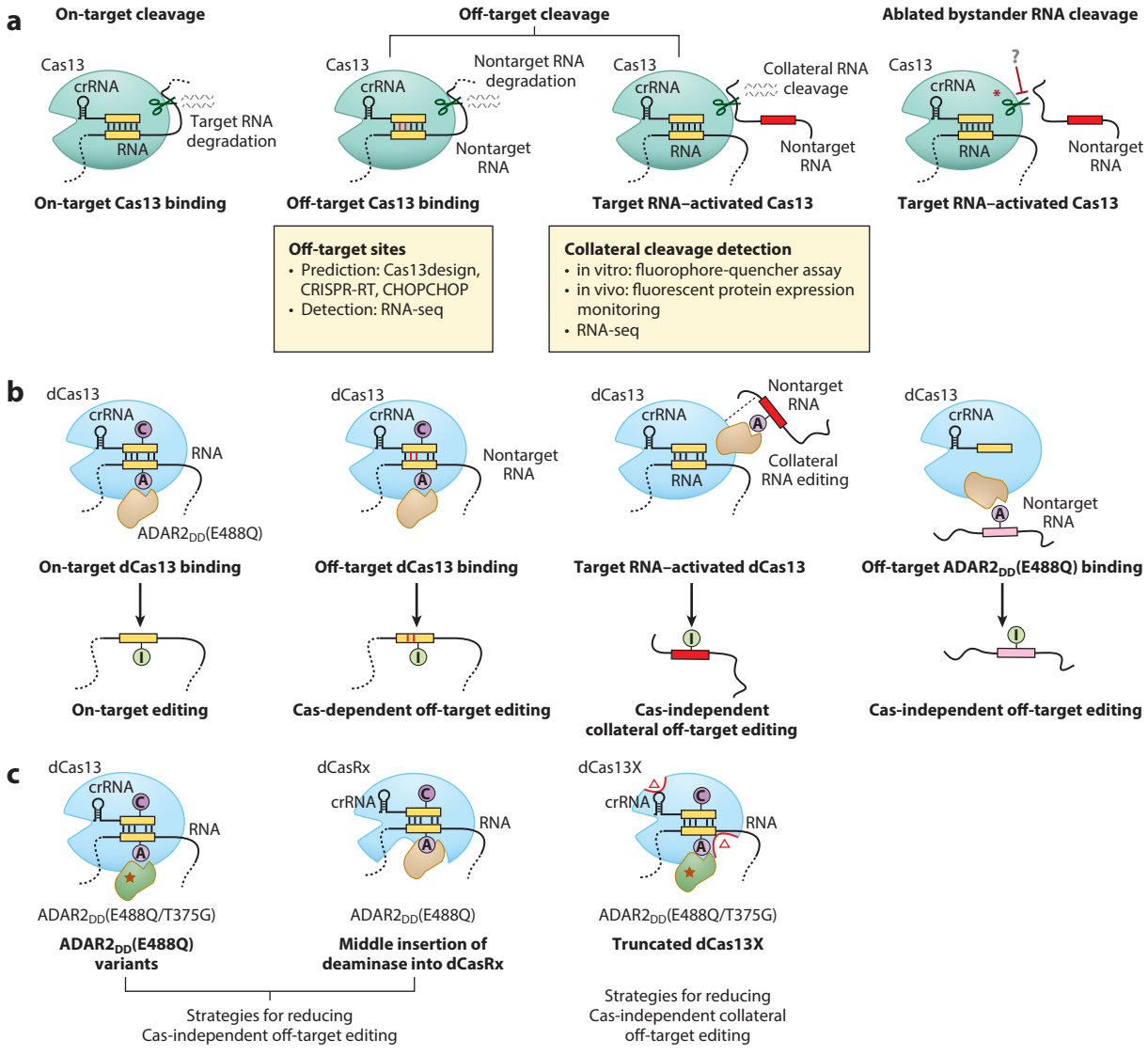


Figure 3

General overview of Cas13-based RNA targeting and editing. (*a*) Cas13 cleaves target RNA guided by the crRNA. There are two types of off-target RNA cleavages: Cas-dependent off-target effects caused by mismatches between the crRNA and the off-target sites and collateral nontarget RNA cleavage caused by the target RNA–activated RNase activity of Cas13. Cas13 variants that are unable to bind to bystander RNAs are likely to reduce the collateral cleavage. Methods for detecting off-target RNA cleavage are listed. The asterisk represents one of our hypotheses that mutations at this asterisk site near the HEPN domain likely reduce the collateral cleavage. The on-target cleavage and collateral RNA cleavage portions of panel *a* are adapted with permission from Reference 30. (*b*) REPAIR RNA base editors consisting of dCas13 and ADAR2$_{DD}$(E488Q) DD introduce RNA A-to-I alterations. Off-target RNA editing can occur due to Cas-dependent mismatches between the crRNA and undesired off-target sites. Cas-independent collateral off-target editing can be caused by target RNA–activated dCas13 binding to bystander RNAs or by binding of ADAR2$_{DD}$(E488Q) itself to nontarget RNAs. (*c*) Current strategies for reducing off-target base editing mainly reduce the nontarget binding between ADAR2$_{DD}$(E488Q) and RNA sites, including applying ADAR2$_{DD}$ (E488Q/T375G) variants, introducing middle insertion of deaminase into dCasRx, and employing the truncated form of dCas13X (miniCas13X). The red delta indicates truncated dCas13X. The red star indicates engineered ADAR2$_{DD}$ (E488Q/T375G). Abbreviations: A, adenosine; ADAR2$_{DD}$, adenosine deaminase acting on RNA type 2; crRNA, CRISPR RNA; DD, deaminase domain; I, inosine; REPAIR, RNA editing for programmable adenosine (A) to inosine (I) replacement; RNA-seq, RNA sequencing.

gRNA have shown the impact of different numbers or sites of mismatches between Cas13 and target sequences on Cas13 efficacy (130). While single, double, and triple mismatches in the seed region (at 15–21 spacer nucleotides distal from the direct region) significantly reduced or even abrogated the target knockdown activity of Cas13d, disruption of the base proximal stem increased the activity. Those mismatches also change the spectrum on off-target sites. Off-target effects from gRNA mismatch could be predicted in silico using gRNA design software, such as CRISPR-RT (**http://bioinfolab.miamioh.edu/CRISPR-RT**), Cas13design (**https://cas13design.nygenome.org/**), or CHOPCHOP (**http://chopchop.cbu.uib.no/**).

4.2.2. Cas collateral-dependent off-target effects. In addition to specific nucleic acid cleavage, Cas13 has another nonspecific degradation activity toward RNA in general, termed collateral activity or *trans*-cleavage, which further enhances immunity within bacterial cells (5). Making full biotechnological use of the collateral activity of Cas13 in vitro, specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) has been developed for nucleic acid detection (41, 42). Previous studies have shown that collateral RNA degradation generated by Cas13a or Cas13d occurs in prokaryotic cells, such as *E. coli* or *Listeria seeligeri* cells (25, 73), by transcriptome-wide RNA-seq and growth phenotype analysis. Although such activity has not been observed in these eukaryotic cells, increasing evidence indicates the presence of collateral activity in cancer cells (128) or *Drosophila* (15), when Cas13 is used for RNA interference in vivo.

4.3. Off-Target RNA Edits and Detection Methods

The discovery of the Cas13 system led to the development of an RNA-guided, CRISPR-RNA BE, with an inactivated Cas13b from *Prevotella* sp. fused to an ADAR2 containing only the catalytic domain carrying the hyperactivating mutation E488Q (dCas13b-ADAR2$_{DD}$-E488Q) (25) (**Figure 3*b***). Although the specifically guided CRISPR-RNA BE shows high precision, there are still high levels of unintended off-target effects from the A-to-I alterations of REPAIRv1 or the C-to-U alterations of RESCUEv1 upon fusion with the deaminase or endogenous ADARs (4, 25). In addition, off-target editing is also caused by gRNA mismatch or dCas13-dependent collateral binding to the nonspecific transcript (87, 135). These edits on nonspecific RNA can be detected by RNA-seq.

4.4. Strategies Against Off-Target Effects

Significant optimization of gRNA, Cas13/dCas13, or deaminase systems has been achieved for potential therapeutic applications (**Figure 3*c***).

4.4.1. Modified guide RNA. Generally, optimizing strategies for reducing gRNA mismatch-dependent off-target effects include truncation or chemical modification of gRNA and large-scale screening of gRNAs based on in silico (130) or improved experimental design (46). Screening of gRNA by introducing one mismatch enables the Cas13 system to specifically cleave the *KRAS-G12D* transcript in cancer cells, not wild-type *KRAS* in normal cells, and this may be used for the development of a potential pancreatic cancer therapy (144).

4.4.2. Engineered Cas13. Different Cas13 proteins exhibit distinct specificities. Fusions of dCas13d or truncated dCas13X (miniCas13X), which have smaller sizes and increased specificity compared to dCas13b, to ADAR2$_{DD}$-E488Q function well in the nucleus with on-target activity and reduced off-target effects (135). Liu et al. (87) characterized various fusion strategies, and found that an ADAR2$_{DD}$-E488Q insertion into dCasRx, termed REPAIRvx, showed precision,

high efficiency for on-target editing, and reduced off-target editing, as examined by fluorescence-activated cell sorting (FACS) screening and RNA-seq.

Substantial off-target effects and severe host nucleic acid degradation leading to host dormancy mainly result from the collateral activity of Cas13 (5, 92). However, to our knowledge, target and collateral cleavage activity probably are activated by target RNA binding, and the precise mechanism of dual cleavage is still unclear. Currently, aside from employing Cas13 with minimal collateral activity, no method has been developed for eliminating collateral activity for in vivo therapeutic applications.

4.4.3. Engineered ADAR. Since the RNA-binding domain of full-length ADARs can generate off-target edits, ADARs have been truncated to only retain the catalytic domain ($ADAR2_{DD}$) (25). Yet $ADAR2_{DD}$ still can recognize and edit RNA duplexes, causing massive off-target editing. The Zhang group (25) generated $ADAR2_{DD}$-E488Q, termed REPAIRv2, impairing the $ADAR2_{DD}$–RNA interaction with a site-specific mutation of T375G, which indeed dramatically reduced off-target effects. REPAIRv2 not only minimizes undesirable off-target effects, but also reduces on-target RNA editing activity. Using a rational mutagenesis and protein evolution approach, researchers engineered RESCUE(v1) from REPAIR(v1) (A-to-I editing) to produce C-to-U editing at target sites. Via RNA-seq, RESCUEv1 displays C-to-U and substantial A-to-I off-target editing (4). Zhang and colleagues (4) introduced a S375A mutation into RESCUEv1, forming a high-fidelity version, RESCUEv2, that also had decreased on-target activity.

5. OTHER CRISPR-CAS-BASED EDITING APPLICATIONS

Based on CRISPR-Cas systems, in addition to conventional BEs, other editors produced by the fusion of dCas or nCas to various types of proteins have been developed as practical tools for understanding and controlling biological functions, such as prime editing, CRISPRi, CRISPRa, and epigenome editing. One of the main considerations for these editors, as useful tools for understanding biological functions or in vivo applications, is still the off-target effects.

5.1. Prime Editing

Prime editing is a recent genome-editing technology that enables all 12 possible point mutations, multiple base pair insertion, or deletion in a targeted way (10). The prime editor consists of a fusion protein between a Cas9n and an engineered reverse transcriptase (Cas9n-RT) as well as a prime-editing guide RNA (pegRNA). The pegRNA contains the sgRNA, a primer binding site (PBS) and a reverse RNA template for a reverse transcriptase to generate the desired edit in the DNA. Three prime-editing versions, PE1, PE2, and PE3/PE3b, with increasing editing efficiency, have been designed. Prime editing has been applied in various human cell lines and mouse embryos, with varying efficiencies (10, 86, 116). Likely owing to three hybridization events (conventional protospacer-spacer hybridization, nicked target strand–PBS hybridization, and 3′ flap–target strand hybridization), Cas9-dependent off-target effects of prime editing were found to be much lower than what is observed for other BEs. Many problems remain to be addressed, such as whether prime editing causes other undetectable off-target editing, or whether a suitable delivery system can improve the efficiency of prime editing for in vivo applications.

5.2. CRISPRi/a Editing

The fusion of dCas9 to transcriptional repressors or activators has been developed as a toolbox for transcriptional downregulation or upregulation, called CRISPRi/a (40, 101). Various gene-regulatory domains from repressors KRAB, KRAB-MeCP2, or MS2-HP1a-KRAB have been

fused with dCas9 for CRISPRi (118, 138), and those from activators p65, VP64, SAM, VPR, or VP64-SunTag have been fused with dCas9 for CRISPRa (18, 20, 109). Compared to Cas cleavage, CRIPSRi/a is inducible and reversible. Theoretically, there are also a few off-target effects due to two hybridization events. In a CRISPRi/a screen, Tycko et al. (124) found a distinct set of off-target edits causing strong confounding fitness effects. Thus, specific sgRNAs and a high-fidelity dCas screening are required.

5.3. Epigenome Editing

Various epigenetic regulators have been fused to dCas for modulating the epigenome, such as histone (de)acetylation or DNA (de)methylation (17, 94, 99). Those regulators include repressors or activators, which can be considered a special type of CRISPRi/a, and chromatin editors; both types are used in epigenome editing. These editors could be used to manipulate changes in the specific gene expression, chromatin state, and cellular behavior by editing chromatin marks and also to depict high-density maps that show how alterations in chromatin marks within a specific DNA locus affect gene expression (6, 68, 93, 114). In widespread application of chromatin editors, except in editing efficiency at targeted chromatin sites, specificity is considered, and off-target edits at other genomic loci are a risk for cellular states, probably influencing undesirable biological functions. Recent studies reported that the overexpression of dCas-fused DNMT3A showed an increase in CpGme at multiple untargeted sites across the genome, probably owing to Cas-dependent off-target effects (36, 83, 108). Further studies are needed to detect off-target effects of other chromatin modulators and to engineer high-fidelity Cas epigenome editing systems.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

CRISPR-based DNA- and RNA-editing technologies have already shown great, revolutionary therapeutic potential, but more studies are needed to assess off-target concerns, delivery options, and their immunogenicity. Regarding off-target concerns, it will be helpful to establish more sensitive and reliable methods to identify the specificity of various genome tools. Each off-target detection method has its own limitations and scope of application, and each genome-editing tool often induces more than one type of off-target mutation. For CRISPR-based tools, including DNA/RNA BE, prime editor, CRISPRi/a, CRISPR-epigenome editor, it is necessary to detect off-target editing at not only the DNA level but also the RNA level and sometimes at the epigenetic level. Therefore, the elimination of both Cas-dependent gRNA-guided and fusion-protein-induced off-target editing is critical for the targeting specificity of CRISPR-based tools.

Future directions in the quest to perfect CRISPR targeting could be divided into three goals. The first goal is to obtain the high-fidelity Cas nuclease with minimized Cas-dependent gRNA-guided off-target editing. The second goal is to reduce fusion-protein-induced off-target editing. The final goal is to make editing tools that are expressed transiently or controllably for in vivo applications (e.g., their expressed time, inducible systems, and kinetics, etc.). Nonetheless, future work on CRISPR-based therapeutics will need to continue to examine the impact of genome modification to fully understand and ensure the safety of these approaches in biomedicine.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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