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REVIEW

Recent advances in CRISPR technologies for genome editing

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Abstract The discovery of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system, and its development into a set of powerful tools for manipulating the genome, has revolutionized genome editing. Precise, targeted CRISPR/Cas-based genome editing has become the most widely used platform in organisms ranging from plants to animals. The CRISPR/Cas system has been extensively modified to increase its efficiency and fidelity. In addition, the fusion of various protein motifs to Cas effector proteins has facilitated diverse set of genetic manipulations, such as base editing, transposition, recombination, and epigenetic regulation. The CRISPR/Cas system is undergoing continuous development to overcome current limitations, including off-target effects, narrow targeting scope, and issues associated with the delivery of CRISPR components for genome engineering and therapeutic approaches. Here, we review recent progress in a diverse array of CRISPR/Cas-based tools. We also describe limitations and concerns related to the use of CRISPR/Cas technologies.

Keywords CRISPR · Cas · Genome editing · Base editors · Prime editors · Epigenetic regulators

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Introduction

The progressive development of genome editing technologies, involving sequence-specific programmable nucleases, has enabled precise genome engineering. Four representative types of endonucleases, zinc-finger nucleases, engineered homing nucleases, transcription activator-like effector nucleases, and Cas nucleases, have been used for genome editing (Kim et al. 1996; Miller et al. 2007; Christian et al. 2010). However, endonuclease-guided target recognition has a major disadvantage, in that it is expensive and difficult to modify the nucleases so that they recognize the desired target sequences (Mushtaq et al. 2018). The discovery of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system, a form of prokaryotic immune system, enabled the far simpler RNA-guided genome editing, overcoming the limitations of the nuclease-guided genome editing tools (Marraffini and Sontheimer 2010). This system has been widely used in various forms of genome editing, such as gene knock-in/knock-out, functional genome screening, and the correction of disease-causing mutations (Liu et al. 2018a, b; Ryu et al. 2018). Further development of the CRISPR/Cas system to improve its editing efficiency through screening and engineering is currently in progress. Structural engineering and random mutagenesis of the Cas nucleases, as well as the creation of Cas fusion proteins, has expanded the utility, versatility, and target range of this system. In particular, Cas fusions with various functional motifs have made possible base editing, prime editing, CRISPR/Cas-mediated transposition, recombination, and epigenetic regulation. Limitations in these systems, such as the occurrence of unwanted genetic byproducts and off-target effects, must be resolved prior to therapeutic applications. We provide a various CRISPR/Cas technologies that have been used for genome editing.

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Mechanism of actions: the CRISPR/Cas system

The CRISPR/Cas system is an evolved RNA-mediated adaptive defense system in bacteria and archaea that functions to protect cells from invading foreign phages and plasmids (Marraffini and Sontheimer 2010). This adaptive immune system is activated by the expression of the Cas gene operon and the CRISPR array, which consists of spacer sequences that exist between a series of direct repeats (Mohanraju et al. 2016). This defense system functions in three progressive phases (Fig. 1): the adaptive phase, expression phase, and interference phase (van der Oost et al. 2009; Jinek et al. 2012; Mohanraju et al. 2016; Rojo et al. 2018). In the adaptive phase, a short fragment of external genetic material (the protospacer) is integrated as a spacer in the CRISPR array (van der Oost et al. 2009; Jinek et al. 2012; Mohanraju et al. 2016; Rojo et al. 2018). When the same foreign material re-invades, the expression phase occurs, during which CRISPR array is transcribed and the precursor transcript is processed into a mature CRISPR RNA (crRNA) that corresponds to the foreign DNA (van der Oost et al. 2009; Jinek et al. 2012; Mohanraju et al. 2016; Rojo et al. 2018). In some systems, trans-activating RNA (tracrRNA) binds to a complementary region in pre-crRNAs to process them to their mature form. The mature crRNA is assembled with the Cas protein to form a CRISPR ribonucleoprotein (crRNP) complex (van der Oost et al. 2009; Jinek et al. 2012; Mohanraju et al. 2016; Rojo et al. 2018). Finally, in the interference phase, crRNPs are guided to specific sequences, complementary to the crRNA and adjacent to a protospacer adjacent motif (PAM) in the targeted DNA strand. The Cas protein then cleaves the foreign nucleic acid, eliminating it from cells (van der Oost et al. 2009; Jinek et al. 2012; Mohanraju et al. 2016; Rojo et al. 2018). As bacteria and phages co-evolved, phages have developed strategies for escaping CRISPR recognition. These strategies include spacer deletion, spacer mutation, and recombination between multiple phase species, making re-infection of previously infected, CRISPR-immune hosts possible (Han and Deem 2017; Westra and Levin 2020; Zhang et al. 2021). When the CRISPR/Cas system is used as a genome editing tool in cells, the CRISPR/Cas-mediated double stranded breaks (DSBs) are repaired using one of two endogenous intracellular DNA repair mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR). The NHEJ pathway results in the generation of small insertions/deletions (indels) at a target site during the repair of a Cas-induced DSB, which can be useful for gene knock-out but undesirable in other cases. Although NHEJ generally more efficient than HDR, it has a disadvantages in that it can cause non-specific gene disruptions such as insertions, deletions, and translocations, resulting in frameshift mutations or nonsense mutations (Zhang 2020).

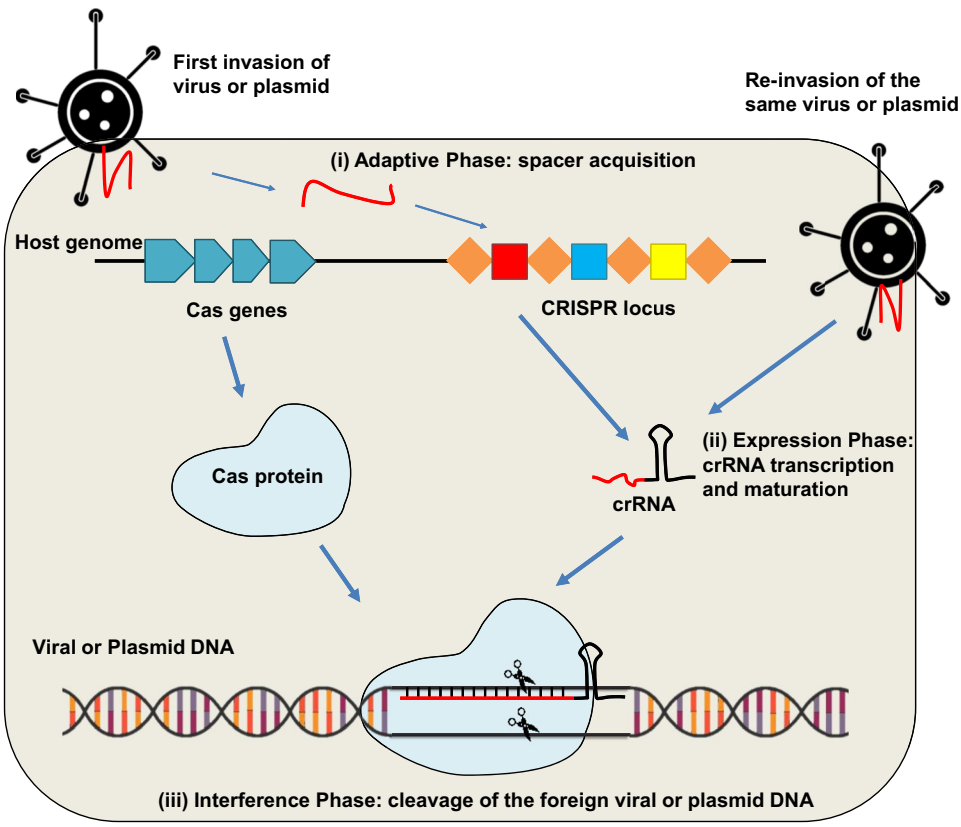

Fig. 1 Schematic summary of the CRISPR/Cas immune process. (i) Adaptive phase. When a virus or plasmid invades a bacterial cell, a short fragment of the foreign genetic material (the protospacer) is integrated as a spacer in the CRISPR array. (ii) Expression phase. When the same type of virus or plasmid subsequently re-invades the cell, the CRISPR array is transcribed and the precursor transcript is processed into a mature CRISPR RNA (crRNA) that corresponds to the foreign DNA. (iii) Interference phase. Finally, a RNP consisting of crRNA and Cas protein cleaves the foreign viral or plasmid DNA

In contrast, the HDR pathway induces precise genome editing in the presence of a donor DNA template containing the desired sequence (Zhang 2020). However, as well as being less efficient than NHEJ, HDR is restricted to specific phases of the cell cycle (G2 and S phases) when sister chromatids are available to accept the template DNA (Chapman et al. 2012; Porto et al. 2020; Yang et al. 2020). Various attempts to increase the efficiency of HDR, such as chemical modulation to inhibit NHEJ (Chu et al. 2015), the use of linear repair templates, optimization of the lengths of the homology regions in the repair template, and the use of modified Cas protein, are underway (Liu et al. 2019).

Classification of CRISPR/Cas systems

CRISPR/Cas systems have been classified into 2 class, 6 types, and 33 subtypes, depending on the number of subunits constituting the Cas protein (the effector module), and the usage of tracrRNA for pre-crRNA processing (Makarova et al. 2015, 2018; Liu and Doudna 2020). The classification of CRISPR/Cas systems is briefly summarized in Table 1. Class I CRISPR/Cas systems, which include types I, III, and IV, are primary systems involving multi-subunit effector complexes (Makarova et al. 2015; Mohanraju et al. 2016; Rojo et al. 2018). In contrast, class II CRISPR/Cas systems, which include types II, V and VI, perform all functions with a single effector protein (Makarova et al. 2015; Rojo et al. 2018; Liu and Doudna 2020). This simplicity has resulted in type II CRISPR/Cas systems being the most widely used for genome editing. The class II Cas9 derived from *Streptococcus pyogenes* (SpCas9) is the most well characterized and frequently used Cas enzyme; it recognizes 5′-NGG-3′ PAMs located at the 3′ end of the target DNA sequence (Pyzocha and Chen 2018). A programmable, single-guide RNA (sgRNA), created by a synthetic fusion of crRNA and tracrRNA, is now the most widely used form for genome editing, given its simplicity and that its use results in similar or higher gene editing efficiencies compared to the two guide RNA system (Shapiro et al. 2020). When the sgRNA, target DNA, and Cas9 effector form a complex, the HNH and RuvC domains of Cas9 respectively cleave the complementary and noncomplementary DNA strands to generate DSBs at a site 3 bp upstream from the PAM (Fig. 2a) (Jinek et al. 2012). Although SpCas9 displays a high gene editing efficiency, it has several features that limit its usefulness for genome editing: (1) large size of Cas9 limits the choice of viral vectors for delivery, (2) the mismatch tolerance between the sgRNA and the target sequence may trigger off-target effects, and (3) pre-existing immunity against Cas9, which has been reported in humans, raises concerns for therapeutic applications. As an alternative to SpCas9, several Cas9 orthologs, including *Staphylococcus aureus* Cas9 (SaCas9) and *Campylobacter jejuni* Cas9 (CjCas9), can be used (Ran et al. 2015; Kim et al. 2017a; Dugar et al. 2018). Because they are smaller than SpCas9, the genes encoding these CRISPR components together with sequences encoding the appropriate sgRNA, can be packaged into small viral vector systems (Jo et al. 2019). Moreover, it has been reported that these nucleases exhibit reduced off-target nuclease activity compared to SpCas9 because they recognize longer PAMs, 5′-NNGRRT-3′ and 5′-NNNVRYAC-3′, respectively (Ran et al. 2015; Yamada et al. 2017; Dugar et al. 2018; Wang et al. 2019). In addition, *Francisella novicida* Cas9(FnCas9) (Hirano et al. 2016), *Neisseria meningitidis* Cas9 (Nme1Cas9, Nme2Cas9) (Zhang et al. 2013), *Brevibacillus laterosporus* Cas9 (BlatCas9) (Gao et al. 2020), *Streptococcus thermophilus* Cas9 (St1Cas9) (Deveau et al. 2008) and *Staphylococcus auricularis* Cas9 (SauriCas9) (Hu et al. 2020) are alternative options for genome editing.

Type V (class II) CRISPR/Cas systems have some similarities with type II systems, but differ in key respects. Cas12a, which recognize T-rich PAMs at the 5′ end of the protospacer, is a representative example of type V CRISPR/Cas systems.

Table 1 Summary of CRISPR/Cas classification

Classification	Type	Effector protein	Target substrate	Signature protein	tracrRNA	PAM motif	References
Class I	Type I	Multi-subunit effector complex	DNA	Cas3	No	Subtype dependent	Makarova and Koonin (2015); Xu et al. (2021)
	Type III			Cas10	No	Subtype dependent	Liu and Doudna (2020)
	Type IV			DinG	No	Subtype dependent	Pinilla-Redondo et al. (2020)
Class II	Type II	Single effector protein		Cas9	Yes	G rich PAM	Koonin et al. (2017)
	Type V			Cas12	subtype dependent	T rich PAM	Zetsche et al. (2015)
	Type VI		RNA	Cas13	No	Subtype dependent	Wang et al. (2020)

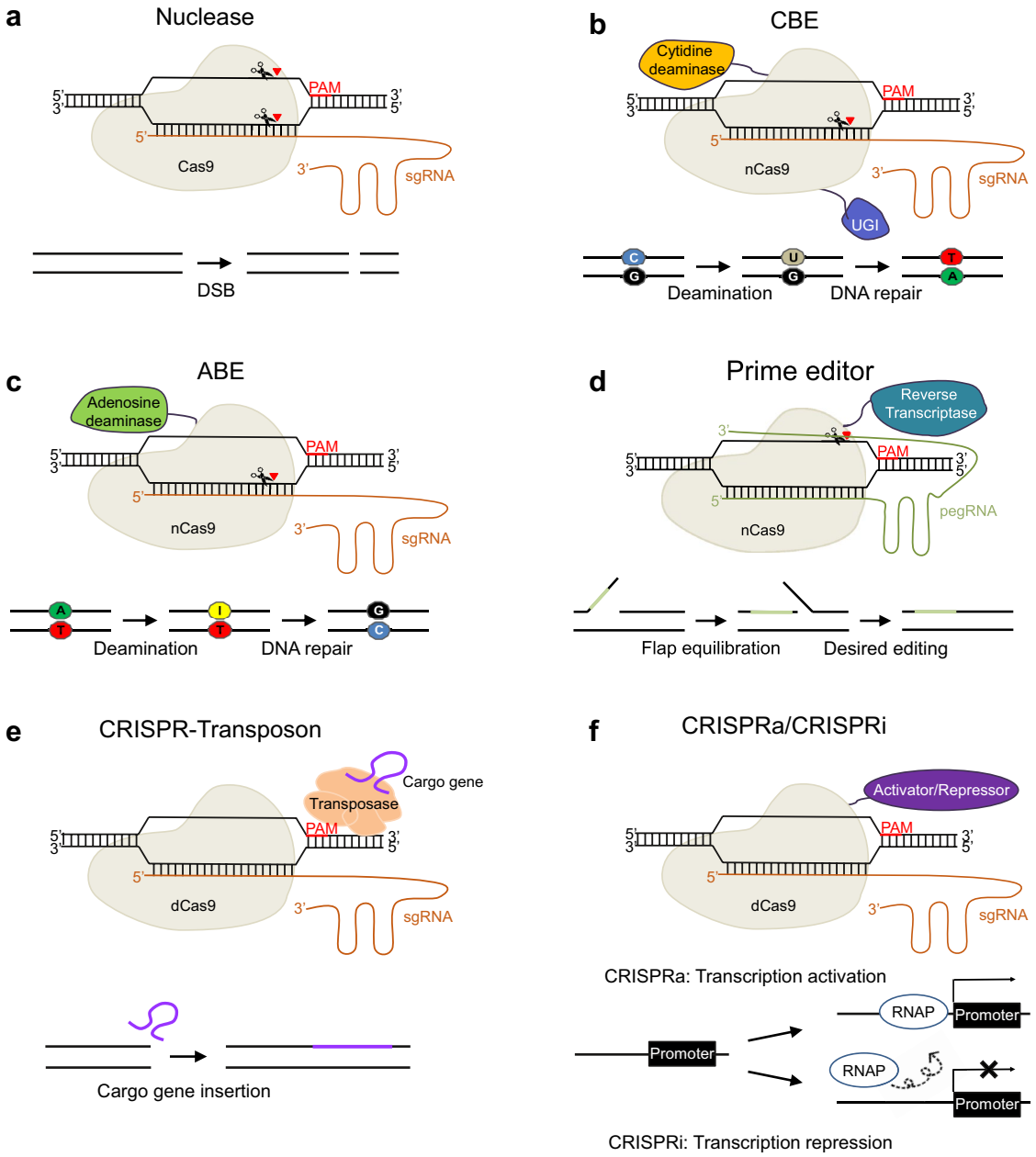

Fig. 2 Schematic summary of CRISPR/Cas tools used for genome editing. **a** The CRISPR/Cas9 system recognizes a PAM and generates DSBs at a site 3 bp upstream from the PAM. **b** CBE consists of an inactive or nickase form of Cas9 (dCas9 or nCas9) fused to a cytidine deaminase and UGI. After the dCas9 or nCas9 domain of a CBE recognizes a specific sequence, the cytosine deaminase deaminates C to generate U. Then, the G in the opposite strand is converted to A by cellular mismatch repair, and C is converted to T. UGI prevents the U from undergoing cellular base excision repair. **c** ABEs are constructed by fusion of nCas9 to *E. coli* tRNA adenosine deaminase, which deaminates A to generate I, which is then converted to G by DNA repair or replication. **d** Prime editors are constructed by fusion of an engineered reverse transcriptase domain to nCas9. A pegRNA binds to the 3′ end of the exposed target DNA strand, which was generated by nCas9. Then, the desired gene edit (which is contained in the pegRNA) is incorporated into the DNA by reverse transcriptase. **e** The Cas-transposon system was developed by fusing a transposase to Cas9. After the targeted sequence is recognized by Cas9 (dCas9, Cas12k or Cascades), the transpose inserts the desired sequence at the site. **f** Tools for CRISPR/Cas-mediated epigenetic regulation are constructed by conjugation of transcriptional repressors or activators to dCas9, generating CRISPRa and CRISPRi systems, respectively. In CRISPRa systems, transcriptional activators recruit RNA polymerase and transcription factors to a promoter and promote transcription. In CRISPRi systems, transcriptional repressors prevent binding of RNA polymerase (RNAP) to the promoter of interest

This protein functions as a nuclease by using only a RuvC domain; it lacks an HNH domain (Zetsche et al. 2015). In addition, Cas12a is guided by a single, relatively compact crRNA and does not require a tracrRNA (Zetsche et al. 2015). The enzyme also exhibits RNase III activity, allowing it to process the precursor crRNA into the mature crRNA. These features have been exploited to allow multiplexed genome editing in which crRNAs for different targets are placed into an array, which is then processed by Cas12a to generate multiple mature crRNAs (Zetsche et al. 2015, 2017). It has been reported that Cas12a exhibits efficiency comparable to that of SpCas9, but higher specificity (Banakar et al. 2020). In contrast to Cas9 (which generates a blunt-ended DSB), Cas12a induces a staggered DSB, generating cuts in the non-targeted and targeted DNA strands at positions 18 and 23, respectively, in the protospacer (Zetsche et al. 2015). Advantageously, the gene encoding Cas12a is relatively short (3.6–3.9 kb), so it has been possible to incorporate it in adeno-associated viral vectors that were used as a gene delivery tool, resulting in efficient in vivo genome editing (Koo et al. 2018).

Some CRISPR/Cas systems protect bacteria from infections by targeting RNA. Cas13a, which belongs to the type VI system, cleaves RNA using two HEPN nuclease domains. Like other systems, the RNA editing process requires crRNA (unlike in the type II systems, tracrRNA is not required). The pre-crRNA undergoes maturation via the RNase function of the Helical-1 domain in the REC lobe (in LshCas13a) or the HEPN2 domain in the NUC lobe (in LbuCas13a). (Shmakov et al. 2015; Kingdom et al. 2017; Liu et al. 2017). The mature crRNA binds to the target RNA after recognition of the protospacer flanking sequence (PFS) located at the 3′ end of the protospacer (Abudayyeh et al. 2016; Burmistrz et al. 2020). After formation of a complex between crRNA-RNA and Cas13a, RNA degradation occurs (Knott et al. 2017). Representative Cas13a enzymes include LseCas13a, LwaCas13a, LshCas13a, LbuCa13a, and LbaCas13a, and the mechanisms through which their pre-crRNAs undergo maturation, the recognized PFSs, and the resulting RNA cleavage patterns are currently under investigation.

Engineered Cas9 and Cas12a variants

The CRISPR/Cas system has been rapidly developed by genetic engineering to expand its targeting scope and improve its specificity. Structure-guided and complimentary evolution-based engineering of Cas9 have led to increased PAM plasticity as summarized in Table 2. SpCas9-EQR, -VQR, -VRER and -VRQR were generated by modifying the Cas9 Arg1333 and Gln1335 residues, which respectively recognize the second and third guanine bases in the NGG PAM (Kleinstiver et al. 2015b, 2016; Anders et al. 2016). These Cas9 variants recognize 5′-NGNG-3′, 5′-NGAN-3′, 5′-NGCG-3′, and 5′-NGAH-3′ PAMs, respectively (Kleinstiver et al. 2015b, 2016; Anders et al. 2016). Through phage-assisted continuous evolution and selection of host cell containing evolved Cas9, xCas9 was developed to recognize 5′-NG-3′ PAMs (Hu et al. 2018). In addition, other SpCas9 variants (SpCas9-NRRH, SpCas9-NRCH and SpCas9-NRTH), which recognize most 5′-NR-3′ PAMs, have been generated by employing phage-assisted continuous and non-continuous evolution (Miller et al. 2020). Moreover, throughout use of a high-throughput PAM determination assay, engineered SpG and SpRY, which respectively recognize 5′-NGN-3′ and 5′-NRN-3′ PAMs were developed (Walton et al. 2020).

Furthermore, high-fidelity Cas variants, with increased targeting specificity and reduced off-target nuclease activity, have been developed. The general strategies for generating high-fidelity Cas9 variants are summarized in Table 2. As one example, structure-guided mutagenesis was used to neutralize the positively charged residues in the non-target strand binding groove in Cas9, leading to a requirement for more stringent base pairing between the sgRNA and the target DNA strand. With this strategy, eSpCas9 1.0 (K810A/K1003A/R1060A) and eSpCas9 1.1 (K848A/K1003A/R1060A) were generated, resulting in reduced off-target effects while maintaining on-target efficiency (Slaymaker et al. 2016). Similarly, SpCas9-HF1 (Kleinstiver et al. 2016), HypaCas9 (Chen et al. 2017), Sniper-Cas9 (Lee et al. 2018), and enAsCas12a-HF1 (Kleinstiver et al. 2019) also showed high-fidelity genome editing.

Cas9 orthologs have also been engineered to widen their targeting scope and increase their editing efficiency. For example, E782K/N968K/R1015H mutations were introduced into SaCas9 to create SaCas9-KKH, which recognizes 5′-NNNRRT-3′ PAMs (Kleinstiver et al. 2015a). RHA FnCas9 (E1369R/E1449H/R1556A), which recognizes 5′-YG-3′ PAMs, was generated by reducing the binding affinity of FnCas9 with the third guanine base in the 5′-NGG-3′ PAM (Hirano et al. 2016).

Cas12a, which recognizes T-rich PAMs, has a wider PAM window than other Cas variants. To take advantage of its wide targeting scope, mutations were introduced into *Acidaminococcus* Cas12a (AsCas12a) to generate the E174R/S542R/K548R variants, which recognize various PAMs, including 5′-TTYN-3′, 5′-VTTV-3′, and 5′-TRTV-3′ (Kleinstiver et al. 2019). To further expand the targeting scope of AsCas12a, additional variants were generated by modifying other residues (variant RR; S542R/K607R and variant RVR; S542R/K548V/N552R) that form hydrogen bonds with the PAM duplex; the RR and RVR variants recognize TYCV and 5′-TATV-3′ PAMs, respectively (Nishimasu et al. 2017; Kleinstiver et al. 2019). To reduce off-target effects of AsCas12a variants while maintaining their high levels of on-target activity, K949A was also introduced into

Table 2 Summary of strategies for engineering of Cas proteins

Nuclease	Origin	Feature	Engineering method	Engineered site	Domain location of mutation	PAM	References
Cas9 nickase(nCas9)	SpCas9	Expanded PAM scope	Structure-guided mutagenesis	D10A or H840A	NUC lobe	NGG	Cong et al. (2013)
SpCas9-EQR	SpCas9		Structure-guided mutagenesis	D1135E/R1335Q / T1337R	NUC lobe	NGNG	Kleinstiver et al. (2015b; Anders et al. (2016)
SpCas9-VQR	SpCas9		Structure-guided mutagenesis	D1135V/R1335Q/ T1337R	NUC lobe	NGAN	Kleinstiver et al. (2015b; Anders et al. (2016)
SpCas9-VRER	SpCas9		Structure-guided mutagenesis	D1135V/G1218R/ R1335E/ T1337R	NUC lobe	NGCG	Kleinstiver et al. (2015b; Anders et al. (2016)
SpCas9-VRQR	SpCas9		Structure-guided mutagenesis	D1135V/G1218R/ R1335Q/T1337R	NUC lobe	NGAH	Kleinstiver et al. (2016)
xCas9	SpCas9		Phage-assisted continuous evolution	A262T/R324L/S409I/ E480K/E543D/M694I/ E1219V	REC lobe and NUC lobe	NG	Hu et al. (2018)
SpCas9-NRRH	SpCas9		Phage-assisted continuous/ non-continuous evolution	R1114G/D1135N/ V1139A/D1180G/ E1219V/Q1221H/ A1320V/R1333K	NUC lobe	NRRH	Miller et al. (2020)
SpCas9-NRCH	SpCas9		Phage-assisted continuous/ non-continuous evolution	R1114G/D1135N/ E1219V/D1332N/ R1335Q/T1337N/ S1338T/H1349R	NUC lobe	NRCH	Miller et al. (2020)
SpCas9-NRTH	SpCas9		Phage-assisted continuous/ non-continuous evolution	R1114G/D1135N/ D1180G/G1218S/ E1219V/Q1221H/ P1249S/E1253K/ P1321S/D1332G/ R1335L	NUC lobe	NRTH	Miller et al. (2020)
SpG*	SpCas9		Structure-guided mutagenesis	D1135L/S1136W/ G1218K/E1219Q/ R1335Q/T1337R	NUC lobe	NGN	Walton et al. (2020)
SpRY*	SpCas9		Structure-guided mutagenesis	A61R/L1111R/D1135L/ S1136W/G1218K/ E1219Q/A1322R/ N1317R/R1333P/ R1335Q/T1337R	REC lobe and NUC lobe	NRN > NYN	Walton et al. (2020)
iSpyMac-Cas9*	SpCas9		Exchanging the PI region	replace SpCas9 PI domain with PAM-Interacting(PI) domain of *Streptococcus macacae* Cas9(SmacCas9)	–	NAAN	Chatterjee et al. (2020)

Table 2 (continued)

Nuclease	Origin	Feature	Engineering method	Engineered site	Domain location of mutation	PAM	References
SaCas9-KKH	SaCas9		Structure-guided mutagenesis	E782K/N968K/R1015H	NUC lobe	NNNRRT	Kleinstiver et al. (2015a)
RHA FnCas9	FnCas9		Structure-guided mutagenesis	E1369R/ E1449H/ R1556A	NUC lobe	YG	Hirano et al. (2016)
AsCas12a-RR*	AsCas12a		Structure-guided mutagenesis	S542R/K607R	NUC lobe	TYCV	Gao et al. (2017)
AsCas12a-RVR*	AsCas12a		Structure-guided mutagenesis	S542R/K548V/N552R	NUC lobe	TATV	Gao et al. (2017)
LbCas12a-RR	LbCas12a		Structure-guided mutagenesis	G532R/K595R	NUC lobe	TYCV	Li et al. (2018)
enAsCas12a	AsCas12a	Improved efficiency	Structure-guided mutagenesis	E174R/S542R/K548R	REC lobe and NUC lobe	VTTV, TTYN, and TRTV	Kleinstiver et al. (2019)
enRR	AsCas12a		Structure-guided mutagenesis	E174R/S542R/K607R	REC lobe and NUC lobe	TYCV	Kleinstiver et al. (2019)
enRVR	AsCas12a		Structure-guided mutagenesis	E174R/S542R/K548V/ N552R	REC lobe and NUC lobe	TATV	Kleinstiver et al. (2019)
eSpCas9 (1.0)	SpCas9	High-fidelity	Structure-guided mutagenesis	K810A/K1003A/ R1060A	NUC lobe	NGG	Slaymaker et al. (2016)
eSpCas9 (1.1)	SpCas9		Structure-guided mutagenesis	K848A/K1003A/ R1060A	NUC lobe	NGG	Slaymaker et al. (2016)
SpCas9-HF1	SpCas9		Structure-guided mutagenesis	N497A/R661A/Q695A/ Q926A	REC lobe and NUC lobe	NGG	Kleinstiver et al. (2016)
HypaCas9	SpCas9		Structure-guided mutagenesis	N692A/M694A/Q695A/ H698A	REC lobe and NUC lobe	NGG	Chen et al. (2017)
Sniper-Cas9	SpCas9		*E. coli*-based selection and DNA shuffling	F539S/M763I/K890N	REC lobe and NUC lobe	NGG	Lee et al. (2018)
SpG-HF1	SpCas9		Structure-guided mutagenesis	N497A/R661A/Q695A/ Q926A/D1135L/ S1136W/G1218K/ E1219Q/R1335Q/T1337R	REC lobe and NUC lobe	NGN	Walton et al. (2020)
SpRY-HF1	SpCas9		Structure-guided mutagenesis	N497A/R661A/Q695A/ Q926A/A61R/ L1111R/D1135L/ S1136W/G1218K/ E1219Q/A1322R/ N1317R/R1333P/ R1335Q/T1337R	REC lobe and NUC lobe	NRN > NYN	Walton et al. (2020)
HiFi-iSpyMac	SpCas9		Structure-guided mutagenesis	R691A	REC lobe	NAAN	Chatterjee et al. (2020)
enAsCas12a-HF1	AsCas12a		Structure-guided mutagenesis	E174R/N282A/S542R/ K548R	REC lobe and NUC lobe	VTTV, TTYN, and TRTV	Kleinstiver et al. (2019)

*Highfidelity Cas variants

the AsCas12a RR and RVR variants (Gao et al. 2017). In addition, G532R/K595R mutations were introduced into *Lachnospiraceae bacterium* Cas12a (LbCas12a) to generate LbCas12a-RR variant so that it recognizes 5′-TYCV-3′ PAMs, broadening its targeting scope (Li et al. 2018).

Base editors

Recently, several DNA base editing systems have been developed that allow single base conversions, also known as base editing, in cells and organisms in a guide RNA-dependent manner. By connecting a deaminase to a Cas9 protein, targeted base editing became possible, broadening the types of editing that could be achieved by CRISPR/Cas systems. Two general types of base editors (cytidine base editor (CBE), and adenine baes editor (ABE) have been developed, both with great potential for targeted base mutagenesis (Fig. 2b and 2c). CBE1, the first version of CBE, is a fusion between an inactive form of Cas9 (dead Cas9 or dCas9) and rat-derived cytosine deaminase apolipoprotein B mRNA editing enzyme catalytic subunit 1 (APOBEC1), a cytidine deaminase. CBE1 enables the direct conversion of a targeted C·G base pair to a T·A base pair in a process involving deamination of a C to create a U, which is then converted to T by the cell's DNA mismatch repair process (Komor et al. 2016). CBE2 was generated by fusing a uracil DNA glycosylase inhibitor (UGI) to CBE1, which prevents the removal of the U by base excision repair involving uracil DNA glycosylase (Komor et al. 2016). Another form, CBE3, was created by exchanging dCas9 for Cas9 nickase (nCas9; D10A mutation). This version exhibits increased base editing efficiency compared to CBE1 and CBE2 (Komor et al. 2016). The development of CBE3 resulted in a six-fold increase in genome editing efficiency compared to CBE2, but it had a limitation in that window in which bases were converted was narrow (positions 4 to 8 in the protospacer, counting from the PAM distal base) (Komor et al. 2016). An improved version of CBE (CBE4) was generated by extending the length of linkers (rAPOBEC-nCas9 linker to 32 amino acids, nCas9-UGI linker to 9 amino acids) and attaching two UGIs to the C-terminus of the constructs. This modification in CBE4 resulted in a 1.5-fold increase in base editing efficiency and a 2.3-fold decrease in non-T product formation compared to CBE3 (Komor et al. 2017). As another base editing system, Target-activation-induced cytidine deaminase (Target-AID) was developed by fusing nCas9 (D10A) to a *Petromyzon marinus* cytidine deaminase 1 (PmCDA1). Target-AID exhibits a different targeting window (positions 1 to 5 in the protospacer) compared to CBEs and ability to edit methylated cytosines. In addition, several attempts have been made to expand the base editing window and increase its specificity as summarized in Table 3.

ABEs were constructed using an evolved version of *Escherichia coli* tRNA adenosine deaminase (TadA). Wild-type TadA can convert adenosine to inosine in tRNA. However, because no known natural adenosine deaminase recognizes DNA, TadA was evolved to exhibit this characteristic through bacterial selection methods, creating TadA* (Gaudelli et al. 2017). The first ABE (ABE1.2) was generated by fusing a mammalian codon-optimized TadA*, containing A106V and D108N mutations, to nCas9 (D10A) (Gaudelli et al. 2017). A later version, ABE7.10, which consists of a heterodimer of the wild-type TadA and TadA* fused with nCas9 (D10A) and exhibits improved base editing efficiency, was constructed through bacterial evolution and protein engineering (Gaudelli et al. 2017). ABE7.10 shows broad sequence compatibility, targeting a window spanning positions 4 to 7 in the protospacer. In addition, ABE7.10 exhibits highly efficient adenine base editing, in human cells (Gaudelli et al. 2017) and mice (Ryu et al. 2018). ABEs have been further engineered to exhibit even higher base editing efficiency as summarized in Table 3. For example, evolved TadA variants with increased deoxyadenosine deamination activity were used to generate several versions of ABE8e based on SpCas9, SaCas9, and LbCas12a, which exhibit higher efficiency and a wider activity window than ABE7.10 (Richter et al. 2020).

Glycosylase base editor (GBE) has also been developed by fusing uracil-DNA glycosylase to a CBE. The uracil-DNA glycosylase removes the U produced by the CBE and creates an apurinic/apyrimidinic site, which activates the DNA repair mechanism. GBE [that is, AID-nCas9-uracil DNA glycosylase] showed efficient C-to-A conversion in *E. coli.* Replacement of AID with rAPOBEC1 generated a GBE (APOBEC-nCas9- uracil DNA glycosylase), that enables C-to-G conversion in mammalian cells (Zhao et al. 2021).

A dual adenine and cytosine base editor (A&C-BE) has been developed that can simultaneously induce C-to-T and A-to-G base editing in the same allele. This dual editor consists of a human AID-TadA-TadA*-nCas9-UGIs fusion (Zhang et al. 2020). For A&C-BE, the A-to-G editing window remained consistent as positions 4–7, whereas the base editing window for C-to-T editing was extended to positions 2–17 in the protospacer (Zhang et al. 2020). Furthermore, synchronous programmable adenine and cytosine editors (SPACEs) were generated by a fusion of miniABEmax (which was generated by removing wild-type TadA domain from ABEmax and introducing V82G mutation) to target-AID (Grünewald et al. 2020). SPACEs can induce targeted A-to-G and C-to-T conversions at positions 4–7 and 2–7 in the protospacer, respectively, and exhibit reduced RNA off-target effects and comparable or lower DNA off-target effects compared to miniABEmax-V82G and Target-AID (Grünewald et al. 2020).

Table 3 Summary of strategies for engineering of base editing systems

Base-Editor	Feature	Architecture	Activity window	References
BE1	C to T conversion	rAPOBEC1-dCas9	4–8	Komor et al. (2016)
BE2		rAPOBEC1-dCas9-UGI	4–8	Komor et al. (2016)
BE3		rAPOBEC1-nCas9-UGI	4–8	Komor et al. (2016)
BE4	Increased editing efficiency	rAPOBEC1-nCas9-UGI-UGI	4–8	Komor et al. (2017)
BE4-Gam		Gam-rAPOBEC1-nCas9-UGI-UGI	4–8	Komor et al. (2017)
BE4max		bpNLS-rAPOBEC1-nCas9-UGI-UGI-bpNLS	4–8	Koblan et al. (2018)
AncBE4max		bpNLS-rAPOBEC1(Anc689)-nCas9-UGI-UGI-bpNLS	4–8	Koblan et al. (2018)
A3A-BE3		hAPOBEC3A-nCas9-UGI	4–8	Gehrke et al. (2018)
BE-PLUS	Widen activity window	SunTag-nCas9, scFv-rAPOBEC1-UGI	4–16	Jiang et al. (2018)
FERNY-BE4max		rAPOBEC1(FERNY)-nCas9-UGI-UGI	3–6	Thuronyi et al. (2019)
CDA1-BE4max		PmCDA1-nCas9-UGI-UGI	1–9	Thuronyi et al. (2019)
evoBE4max		rAPOBEC1(E4K/H109N/H122L/D124N/R154H/A165S/P201S/F205S)-nCas9-UGI-UGI	3–8	Thuronyi et al. (2019)
evoFERNY-BE4max		rAPOBEC1(FERNY_H102P/D104N)-nCas9-UGI-UGI	3–8	Thuronyi et al. (2019)
evoCDA1-BE4max		PmCDA1(F23S/A123V/I195F)-nCas9-UGI-UGI	1–13	Thuronyi et al. (2019)
Target-AID		nCas9-PmCDA1-UGI	2–4	Nishida et al. (2016)
EE-BE3	Reduced off-target effect and bystander effect	rAPOBEC1(R126E/R132E)-nCas9-UGI	5–6	Kim et al. (2017b)
YE1-BE3		rAPOBEC1(W90Y/R126E)-nCas9-UGI	5–6	Kim et al. (2017b)
YE2-BE3		rAPOBEC1(W90Y/R132E)-nCas9-UGI	5–6	Kim et al. (2017b)
YEE-BE3		rAPOBEC1(W90Y/R123E/R132E)-nCas9-UGI	5–6	Kim et al. (2017b)
SECURE(R33A)		rAPOBEC1(R33A)-nCas9-UGI	5–7	Grünewald et al. (2019a)
SECURE(R33A/K34A)		rAPOBEC1(R33A/K34A)-nCas9-UGI	5–6	Grünewald et al. (2019a)
eA3A-BE3		hAPOBEC3A(N57G)-nCas9-UGI	4–8	Gehrke et al. (2018)
A3A(R128A)-BE3		hAPOBEC3A(R128A)-nCas9-UGI	4–8	Zhou et al. (2019)
A3A(Y130F)-BE3		hAPOBEC3A(Y130F)-nCas9-UGI	4–8	Zhou et al. (2019)
ABE1.2	A to G conversion	TadA*-dCas9	5	Gaudelli et al. (2017)
ABE7.10		TadA-TadA*-nCas9	4–7	Gaudelli et al. (2017)

Table 3 (continued)

Base-Editor	Feature	Architecture	Activity window	References
ABEmax	Increased editing efficiency	TadA-TadA*-nCas9	4–7	Koblan et al. (2018)
ABE8e		TadA(8e)-nCas9	4–8	Richter et al. (2020)
LbABE8e	Widen activity window	TadA(8e)-LbCas12a	8–14	Richter et al. (2020)
ABE8s		TadA(8s)-nCas9	3–9	Gaudelli et al. (2020)
ABE7.10(F148A)	Reduced off-target effects and bystander effect	TadA(F148A)-TadA*(F148A)-nCas9	4–6	Zhou et al. (2019)
SECURE-ABE(K20A/R21A)		TadA*(K20A/R21A)-nCas9	4–7	Grünewald et al. (2019b)
SECURE-ABE(V82G)		TadA*(V82G)-nCas9	4–7	Grünewald et al. (2019b)
ABEmaxAW		TadA(E59A)-TadA*(V106W)-nCas9	4–7	Rees et al. (2019)
ABE8e(V106W)		TadA(8e)(V106W)-nCas9	4–8	Richter et al. (2020)
GBE	C to A (E.coli) and C to G (mammalian cell) conversion	AID-nCas9- uracil DNA glycosylase & APOBEC-nCas9- uracil DNA glycosylase	6	Zhao et al. (2021)
A&C-BE	Dual base conversion	hAID-TadA-TadA*-nSpCas9-UGI-UGI	C to T: 2–17 A to G: 4–7	Zhang et al. (2020)
SPACE		MiniABEmax(V82G)-nSpCas9-Target-AID	C to T: 2–7 A to G: 4–7	Grünewald et al. (2020)

Prime editors

Prime editing is a recently developed genome editing technology that is capable of generating targeted insertions, deletions, and substitutions in a precise manner. A key feature of prime editing is that it does not require either a DSB or the HDR pathway for targeted editing. Prime editors are generated by fusing an engineered reverse transcriptase (RT) domain to nCas9 (H840A) (Fig. 2d) (Anzalone et al. 2019). This process requires an engineered prime editing guide RNA (pegRNA), which is similar to a sgRNA but also contains a primer binding site (PBS) and an RT template containing the desired edit. nCas9 first generates a nick in the DNA strand containing the PAM. Then, RT binds to the 3′ end of the exposed target DNA strand and performs reverse transcription, after which the non-edited protruding 5′-flap on the strand containing the PAM is degraded by cellular endonucleases. Finally, DNA ligation and repair occur to generate the desired DNA sequence (Anzalone et al. 2019).

Three versions of prime editors have been developed. Prime editor 1 (PE1) was generated by fusing nCas9 (H840A) to the wild-type Moloney murine leukemia virus-RT. The RT domain was further engineered in PE2, which exhibits increased editing efficiency (Anzalone et al. 2019). PE3 uses an additional sgRNA that leads to the generation of a nick in the non-edited strand by nCas9, resulting in a further two- to four-fold increase in editing efficiency. PE3b uses an sgRNA that targets only the edited strand to decrease the frequency of indels in the non-edited DNA strand (Anzalone et al. 2019). Various attempts to improve prime editing are actively underway; efficient genome editing has been demonstrated in plants and mouse models (Lin et al. 2020; Liu et al. 2020).

Prime editing efficiency is greatly influenced by structure of the pegRNA. Several factors such as SpCas9-induced indel frequencies, GC counts, and the PBS melting temperature must be considered to design the optimal pegRNA (Kim et al. 2020). Several prediction tools, including pegFinder (Chow et al. 2020) and PrimeDesign (Hsu et al. 2020) can be useful for designing pegRNAs and for predicting possible prime editing effects in the whole genome.

Transposase and recombinase fusions to Cas9

The CRISPR/Cas system has also been harnessed to enable target-specific insertions of longer DNA fragments. CRISPR/Cas-associated transposases that function in cyanobacterial cells have recently been reported. Here, a nuclease-deficient Cas effector directs transposition of a cargo gene in an RNA-guided manner (Strecker et al. 2019). Based on this system, a Cas-transposon system was developed by fusing a Tn7 transposase to type V-K Cas12k to facilitate target-specific transposition (Fig. 2e) (Strecker et al. 2019). Another type of reconstituted mariner-family transposase, Himar 1, conjugated to dCas9, resulted in site-specific insertion of DNA into the target TA motifs in the *E.coli* and mammalian cells (Chen and Wang 2019).

Genome editing tool that would enable programmable homologous recombination would also be a valuable addition to the CRISPR toolkit. Recombinase, which functions as a dimer, recognize a strict recombinase site, resulting in recombination; this process can cause the insertions, deletions, or inversions of specific sequences through cleavage, strand exchange, and re-ligation (Chaikind et al. 2016). Because recombinases do not induce cellular DNA repair procedures, recombination does not typically lead to byproducts of error-prone DNA repair such as indels. A fusion of dCas9 with an engineered recombinase, Ginβ, has been generated to overcome the sequence constraints of recombinase (Chaikind et al. 2016). Use of this system resulted in modest genome editing efficiency in mammalian cells, demonstrating its potential as an alternative genome editing tool (Cui et al. 2018).

Epigenetic regulators

CRISPR/Cas-mediated epigenetic regulation became possible by the conjugation of several epigenetic regulator proteins to dCas9, generating CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) systems (Fig. 2f). In CRISPRi systems, the Krüppel-associated box (KRAB) repressor, fused to dCas9, is commonly used as an effector (Gilbert et al. 2013; Thakore et al. 2015). KRAB interacts with heterochromatin-forming complexes, which can induce histone methylation and deacetylation to inhibit binding of RNA polymerases to enhancer or promoter regions, inactivating transcription (Gilbert et al. 2013; Thakore et al. 2015).

In contrast, CRISPRa systems promote transcription by using a transcriptional activator, such as the VP16 activation domain, which can activate transcription by interacting with the TATA-binding protein, TFIIB, and SAGA histone acetylase (Hall and Struhl 2002). Fusion of VP64 (4 copies of VP16) or VP192 (12 copies of VP16) to dCas9 led to transcriptional activation in vivo that was increased compared to that of VP16-conjutated dCas9 (Maeder et al. 2013; Balboa et al. 2015). In addition, fusions of SunTag (an array of repeating peptides that can mobilize multiple copies of an antibody-fusion protein) (Tanenbaum et al. 2014), VPR (composed of VP64, p65, and Rta and act as stronger activators than VP64) (Chavez et al. 2015), p300 (which promotes transcription through increased transactivation capacity) (Hilton et al.

2015), and TET (which performs demethylation by oxidizing methyl groups on proteins) (Liu et al. 2016) to dCas9 also promote transcription.

Challenges

The CRISPR/Cas system is certainly a useful gene editing tool, but there are several limitations to overcome before it can be used in various applications. Off-target effects (unwanted genome editing at unintended sites) are one of the major limitations. These effects can be caused by a tolerance for mismatches between the sgRNA and target DNA sequences (outside of the seed region located 1 to 5 nucleotides proximal to the PAM) (Fu et al. 2013). Furthermore, presence of non-canonical PAMs can decrease Cas specificity (Zhang et al. 2014). To minimize off-target effects, several bioinformatic tools such as Cas-OFFinder (Bae et al. 2014), CCTop (Stemmer et al. 2015), and CT-Finder (Zhu et al. 2016) can be useful for designing appropriate sgRNAs.

To apply the CRISPR/Cas system therapeutically, immunity against CRISPR components must also be resolved. Preexisting adaptive immune responses against Cas9 (anti-SpCas9 and anti-SaCas9 antibodies and Cas9-specific T-cells) have been detected in the majority of human serum samples tested, because the bacterial species that are the sources of these components regularly infect humans (Charlesworth et al. 2019). Another report supports the idea that CRISPR RNAs elicit innate immune responses in human cells (Kim et al. 2018). Recently, a phase 1 clinical trial involving the CRISPR/Cas system has been conducted in lung cancer patients, who were infused with CRISPR-PD 1-edited T cells (Lu et al. 2020). The safety and feasibility of this approach were confirmed without any detectable side effects in this study, but more extensive investigations of safety of CRISPR/Cas-modified cells should be conducted. In addition to the CRISPR/Cas trial in cancer therapy, a range of therapeutic approaches for treating various diseases are being tested in vitro and in vivo, and continuous developments of the CRISPR/Cas system are underway to make these therapeutic applications more feasible.

Conclusions

The development of CRISPR/Cas technology has revolutionized the field of genome engineering. CRISPR/Cas-based tools are the most sophisticated and versatile editing tools to be used in areas ranging from basic research to medical therapy development. In addition, CRISPR/Cas technology allows researchers to perform genome-wide screens to study the impact of changes in gene expression on cell function and link genotypes and phenotypes. As one example, CRISPR/Cas knockout screening was used to search for therapeutic targets in pancreatic cancer cells, leading to the finding that such cells were sensitive to MEK inhibitors (Kanarek et al. 2018; Szlachta et al. 2018; Behan et al. 2019).

Applications of the CRISPR/Cas system include transcriptional regulation made possible by conjugation of functional proteins to Cas9, disease modeling, and gene therapies. Nevertheless, many challenges remain; these include off-target effects, bystander effects, the development of efficient delivery methods, and immunity against CRISPR components, all of which need to be fully addressed. Rapid improvements in CRISPR/Cas-based tools are ongoing to overcome these limitations. These tools should reach their full potential in various applications in the near future.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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