REVIEW ARTICLE

CRISPR/Cas9-mediated base editors and their prospects for mitochondrial genome engineering

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Base editors are a type of double-stranded break (DSB)-free gene editing technology that has opened up new possibilities for precise manipulation of mitochondrial DNA (mtDNA). This includes cytosine and adenosine base editors and more recently guanosine base editors. Because of having low off-target and indel rates, there is a growing interest in developing and evolving this research field. Here, we provide a detailed update on DNA base editors. While base editing has widely been used for nuclear genome engineering, the growing interest in applying this technology to mitochondrial DNA has been faced with several challenges. While Cas9 protein has been shown to enter mitochondria, use of smaller Cas proteins, such as Cas12a, has higher import efficiency. However, sgRNA transfer into mitochondria is the most challenging step. sgRNA structure and ratio of Cas protein to sgRNA are both important factors for efficient sgRNA entry into mitochondria. In conclusion, while there are still several challenges to be addressed, ongoing research in this field holds the potential for new treatments and therapies for mitochondrial disorders.

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) has provided the opportunity to create double-stranded breaks (DSBs) at specific points of the DNA using a programmable guide RNA molecule (gRNA) [1]. The genomic DNA with the identical sequence to the gRNA (notwithstanding uracil substituting for thymine in RNA) is called the protospacer. Each protospacer is directly followed by a specific motif for targeting, known as the protospacer adjacent motif (PAM). Cas9 from different bacterial species uses different PAM sequences, such as the NGG PAM sequence for SpCas9. As a result, only DNA immediately flanking a PAM is interrogated by the Cas9-gRNA complex (also called ribonucleoprotein or RNP complex). Several human diseases are caused by a single-base mutation (SBM) [2]. The capacity to correct SBMs has been dramatically improved by recent progress in genetic engineering tools. Combining DSBs with an oligonucleotide donor can mediate precise editing, including correction of SBMs via the homology-directed repair (HDR) mechanism [3]. Although advancements have been made in HDR efficiency, CRISPR/HDR-editing is still associated with high levels of undesired mutations, namely insertions/deletions (indels) both on-target and off-target [4, 5]. Aside from the off-target issue, CRISPR/Cas9-induced DSBs can lead to plasmid [6], lentiviral [7], and retrotransposon [7] insertions into the host genome and also chromosomal rearrangements, such as chromosomal deletion and translocation [6, 8]. To overcome these problems, Cas9-fused base and prime editors

have been developed that make specific substitutions of single or multiple bases in genomic DNA without inducing any DSB [9]. In this review, we provide an overview of the basics, mechanisms, state of the art, and prospects of base editing technologies and their potential applications in mitochondrial genome editing.

BASE EDITORS

Base editing enables replacement of a target base pair in a programmable manner without inducing a DSB [10]. Following the first report of base editing by Komor et al. [10], three other independent groups also demonstrated the first types of base editors by fusion of endonuclease-deficient Cas9 (dCas9) and different sources of cytidine deaminases in the same year [11–13]. Afterwards, base editors were upgraded by breaking a single strand of DNA using the Cas9 nickase (nCas9).

Cytidine base editors
Fusion of dCas9 and cytidine deaminase. The first type of cytidine base editor to be developed—base editor 1 (BE1)—was a fusion protein comprising a catalytically inactivated (dCas9) form of *Streptococcus pyogenes* Cas9 (SpCas9) and a cytidine deaminase, such as APOBEC1, that was capable of converting cytidines into uridines [10]. The authors found that BE1 activity in NC dinucleotides followed the order TC ≥ CC ≥ AC > GC, with the highest editing efficiency occurring when the target C was located at or near position 7 of the protospacer (Table 1). BE1 can convert

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Table 1. Attributes of Cytidine base editors (CBEs), Adenosine base editors (ABEs), and Glycosylase base editors (GBEs) used for mammalian genetic engineering.

Base editor	Name	Cas9 type	Deaminase structure	Base conversion	Preferred base	Editing window (PAM:21–23)	On-target editing efficiency (%)[a]	Off-target rate (%)[a]	Addgene plasmids	References
CBE	BE1	dCas9	APOBEC*	C-to-T	C5-6	C4-8	0.8–7.7		#73019, #73018	[10]
	BE2	dCas9	APOBEC-UGI	C-to-T	C5-6	C4-8	4–20		#73020	[10]
	TAM-dCas9	dCas9	AIDx-UGI	C-to-T			4–30	0.5	#83260, #85414	[11, 12]
	Target-AID-dCas9	dCas9	AID-UGI	C-to-T	C5-6	C2-6	12.5	0.2	#91692, #83260	[13]
	BE3	nCas9	APOBEC-UGI	C-to-T	C5-6	C4-8	35–50	<0.1	#100803, #100804, #73021, #87437, #163531	[10]
	YE1-BE3	(Sa)nCas9	Mutated APOBEC1-UGI	C-to-T		C5-7	50–70		#132943, #85174	[44]
	Target-AID -nCas9	nCas9	AID-UGI	C-to-T	C5-6	C2-6	74.7	7.6	#188647, #188648, #188649	[13]
	rBE3	nCas9	hAID	C-to-T	C5-6	C4-8	8–73	2–13	#145086, #145087, #143736	[15]
	rBE5	nCas9	hAID*D	C-to-T	C5-6	C2-6	57	72	Not detected	[19]
	rBE9	nCas9	hAID*D-UGI	C-to-T	C5-6	C4-8	11–69	0–28	Not detected	[19]
	AID-BE4max	nCas9	AIDmax	C-to-T	C5-6	C3-13	18–45	3.6	#174696, #157948, #158582	[51]
	A3G-BE4	nCas9	APOBEC3G-2x UGIs	C-to-T	C5-6	C4-15	50–80	<0.1	#163636, #163637, #163638	[20, 36]
	SECURE-BEmax	nCas9	rAPOBEC1-UGI	C-to-T	C5-6	C3-12	20–60	0	#140251	[119]
	EvoAPOBEC1-BE4max	nCas9	PACE APOBEC1-UGI	C-to-T	C4-8	C3-9	60–80	6–20	#122611, #125616, #137175, #137183, #137183	[21]
	CE-CBE	nCas9	APOBEC1-1048-1063	C-to-T	C6	C3-10	75	<0.1		[39]
	Sa-CBE-693	(Sa)nCas9		C-to-T	C6-14	C6-17	40–58	4–7		[40]
	SpNRRH and SpNRCH	nCas9 (SpNRRH/SpNRCH)	PACE APOBEC1-UGI	C-to-T	C6-8	C6-18	14–21			[43]
	SpRY-CBE4max	nCas9 (SpRY)	PACE APOBEC1-UGI	C-to-T	C5-8	C3-12	50–70	8–94		[42]

Table 1. continued

Base editor	Name	Cas9 type	Deaminase structure	Base conversion	Preferred base	Editing window (PAM:21–23)	On-target editing efficiency (%)[a]	Off-target rate (%)[a]	Addgene plasmids	References
ABE	ABE7.10	nCas9	TadA-TadA*	A-to-G	A5-6	A4-9	30-95, 10-15	0.2	#181753, #124447	[29, 120]
	ABEmax	nCas9	Codon optimized TadA-TadA*	A-to-G	A5	A3-8	30-50	1.6-2	#112095, #112098, #112101, #119814, #119813, #125647	[120, 121]
	miniABEmax	nCas9	TadA*	A-to-G	A7-12	A4-16	6-40	0.1	#187062, #135362, #131311	[121]
	SECURE-ABE	nCas9	TadA*	A-to-G	A5-6	A3-9	15-70	0	#131313, #131312	[119]
	GhABE7.10n	nCas9	TadA7.10	A-to-G	A5-6	A4-7	2-64	0		[122]
	GhABE7.10dCpf1	dCpf1	TadA7.10	A-to-G	A5-6	A4-7	0.45			[122]
	ABE8e	nCas9	PA(N)CE* TadA-TadA8	A-to-G	A5-7	A4-8	10-80, 90	0.1-1.6	#138489, #138491, #138500	[23, 36, 38, 123]
	CE-ABE	nCas9	TadA*-TadA 1048-1063	A-to-G	A5	A3-9	45-50	<0.1		[39]
	Sa-ABE-693	(Sa)nCas9	TadA8e-693	A-to-G	A6-13	A6-17	15-40			[40]
	CE-8e-SpRY	nCas9 (SpRY)	nCas9 N-terminus-Tad8e-nCas9 C-terminus	A-to-G	A6-9	A2-12	45			[41]
GBE	GBE	nCas9	APOBEC1-nCas9-UNG	C-to-G	C6	C5-7	15	0.4	#140252	[25]
	GBE2.0	nCas9	APOBEC1(R33A)-nCas9-Rad51-Ung1	C-to-G	C6	C5-7	31	0.4	#140256, #140257, #140254	[26]
	SpRY-GBE	nSpRyCas9	NLS-VP64-APOBEC1-nSpRy-NLS-UDG	C-to-G	C6	C5-8		0.5	#170105	[27]
	gGBE	nCas9	NLS-nCas9 (D10A)-MPG-NLS	G-to-Y	G7	G1-12	55	0-4	#202629, #202630	[28]
CBE & ABE	SPACE	nCas9	TadA*-nCas9-AID-UGI	C-to-T / A-to-G	C3-4 / A5	C2-7 / A4-7	22 / 13	1.6 / 1.6		[50]
	ACBE	nCas9	NLS-ecTadA-32aa-nCas9-NLS-10aa-SH3-CDA1-9aa-UGI-NLS	C-to-T / A-to-G	C5 / A6	C1-7 / A4-8		0 / 0		[49]
	ACBEmax	nCas9	NLS-TadA-nCas9-rAPOBEC1-UGI	C-to-T / A-to-G	C8 / A5-6	C2-8 / A4-8	30-40 / 45	<0.1 / <0.1		[48]
	A&C-BEmax	nCas9	ABEmax and AID-BE4max	C-to-T / A-to-G	C7-10 / A6-7	C2-17 / A5-8	2-30 / 40-78		#157949, #157948	[51]

Table 1. continued

Base editor	Name	Cas9 type	Deaminase structure	Base conversion	Preferred base	Editing window (PAM:21–23)	On-target editing efficiency (%)[a]	Off-target rate (%)[a]	Addgene plasmids	References
	miniAGBE	nCas9	NLS-hAPOBEC1-48aa-ecTadA8e-nCas9-NLS	C-to-T C to G A-to-G	C7 to T C4 to G A5-6	C2-11 C2-11 A4-7	15–60 12–17 50–78	0 0 0		[52]
	TadDE	nCas9	TadDE (engineered TadA8e)	C-to-T A-to-G	C4-5 A6	C2-9 A2-9	73 80	0.1–0.6 <0.1	#193837, #193846	[36]
	Sa-CABE	Sa(nCas9)	APOBEC1-Xten-TadA8e	C-to-T A-to-G	C6-14 A6-13	C6-17 A6-15	15–25 15–40	4–7		[40]
GBE & ABE	GGBE1.3	nCas9	TadA8e-APOBEC1-HMG1-nCas9	C-to-G A-to-G	C6 A5	C4-12 A2-14	25–30 65–70	3 23		[53]

[a]On- and off-target editing efficiencies are depicted as a range or a mean value. This largely depends on the cell line, transfection method, and target sequence. The mean values are an approximate average from different cells and target genes from the same study.

*Apolipoprotein B mRNA editing enzyme catalytic polypeptide 1 (APOBEC1), also known as C-to-U-editing enzyme, is a cytidine deaminase enzyme; uracil DNA glycosylase (UNG); uracil glycosylase inhibitor (UGI); activation-induced cytidine deaminase (AID); tRNA adenine deaminase (TadA); high mobility group nucleosome binding domain 1 (HMGN1), phage-assisted continuous evolution (PACE); phage-assisted non-continuous evolution (PA(N)CE). All Cas9 are derived from Streptococcus pyogenes (Sp), unless otherwise stated (e.g., Staphylococcus aureus or Sa); SElective Curbing of Unwanted RNA Editing (SECURE).

all C nucleotides within a five-nucleotide window of the spacer sequence into T nucleotides [10]. Despite this, the editing efficiency of BE1 in mammalian cell lines was low, ranging from 0.8 to 7.7%. Due to the promiscuity of the cytidine deaminase, application of base editor technology may be limited to protospacers that only include C nucleotides that are intended for substitution, since the base editor may convert any C to a T within the editing window. To improve the base editing efficiency, BE1 was fused with an inhibitor of base excision repair; uracil glycosylase inhibitor (UGI). The same study demonstrated a second type of base editor, BE2, that is a fusion of APOBEC–XTEN–dCas9–UGI [10]. The base editing efficiency was considerably increased using BE2 compared to BE1, ranging from 4 to 20% in different human cell lines.

In the same year, Ma et al. (2016) reported a base editor that was equivalent to the BE1 and BE2 systems developed by Komor et al. (2016). It consisted of dCas9 fused to activation-induced cytidine deaminase (AID), encoded by the *AICDA* gene. AID is a B-cell-specific cytidine deaminase that induces somatic hypermutations at a rate of 1 per 1000 bases by converting cytidines to uridines, resulting in C-to-T mutations [11]. The dCas9-AID system was able to correct an inserted premature stop codon (TAG) in a stably integrated GFP reporter by converting the final base of the codon to a T or a C with a 2% efficiency in the human embryonic kidney cell line 293 T. Ma et al. (2016) further improved the editing efficiency to 4% by engineering the catalytic domains and deleting the C-terminal nuclear export domain of the AID protein to form the dCas9-AIDx system. dCas9-AIDx induced a 4% gain-of-function in the reporter gene using a pool of sgRNAs (single guide RNAs). Further analysis of the GFP sequence showed the presence of 20% C-to-T mutations in a cluster around the sgRNA-targeted sequences, with a less than 0.5% indel rate and no off-target effects [11]. Unexpectedly, dCAs9-AIDx converted the C or G to all three other bases with a more even propensity; the rate of C-to-T, C-to-G, and C-to-A was 40%, 30%, and 30%, respectively, while the rate of G-to-A, G-to-C, and G-to-T was 50%, 30%, and 20%, respectively. This all-base substitution using the hyperactive variant of AID in the dCas9-AIDx system uses an abasic repair for the U:G mispairs and provides substitution of all three bases instead. To overcome the non-specific C/G-editing, dCas9-AIDx was co-expressed with a uracil DNA glycosylase (UNG) inhibitor (UGI, a protein isolated from bacteriophage PBS1). This dCas9-AIDx-UGI resulted in a five-fold increase in the C-to-T base editing with no abasic conversion [11]. When combined with a single sgRNA, the target site of dCAs9-AIDx-UGI was confined to the protospacer sequence, with the highest activity at −12 bp and −16 bp upstream of the PAM (protospacer adjacent motif) sequence (C4-C8, counting from the first base at the 5′ end of the protospacer). The fused dCas9-AIDx-UGI protein induced gain-of-function mutations in chronic myelogenous leukemia by C-to-T conversion at an unknown efficiency since the editing was followed by selection under drug treatment. However, combining the dCas9-AIDx-UGI with pooled sgRNAs resulted in C-to-T conversion in sequences other than the protospacer, specifically the regions between sgRNAs.

In the same issue of *Nature Methods* in which the dCas9-AIDx system was published, Hess et al. (2016) reported another type of dCas9-fused AID base editor, similar to the BE1 system developed by Komor et al. (2016). Named CRISPR-X, it also used a hyper-activated variant of AID via deletion of the nuclear export signal (NES). AID normally functions by migrating with the RNA polymerase II complex during transcription of immunoglobulin genes and mutating specific hotspot sequence motifs. Analysis of the target site for C-to-T substitutions induced by CRISPR-X showed that the direction of transcription is a more important factor than the protospacer sequence itself – the mutational hotspot region was located from +12 to +32 bp downstream of the PAM relative to the direction of transcription rather than the

polarity of the guide RNA target strand [12]. However, the C-to-T conversion rate using the CRISPR-X system was 1%—lower than that of the dCas9-AIDx system [11]. Similar to the dCas9-AID system [11], the CRISPR-X system also induced C/G substitution with all three nucleotides with comparable efficiency [12].

Considering AID orthologues as a possible route to increase the C-to-T conversion rate, dCas9 was fused with the *Petromyzon marinus* cytidine deaminase 1 AID (PmCDA1) to form a synthetic complex (Target-AID) by an N-terminal fusion of AID to dCas9 [13]. Although Target-AID could convert C4-C5 in the editing window with no off-target and minimal indel effects, the editing efficiency was not increased compared to the previous variants of dCas9 CBEs [13].

Overall, the N-terminally fused protein of dCas9 with either APOBEC1 or AID induces C-to-T conversion in the C4-C8 editing window [10–12]. Substitution of C with other bases has been seen with different cytidine base editors [10–12]. To minimize the non-specific substitution and maximize the C-to-T conversion, fusion of 1–2 UGI molecules to the C-terminus of dCas9 protein was an efficient approach [10]. However, low base editing efficiency remains the main drawback of dCas9 CBEs.

Fusion of nCas9 and cytidine deaminase. The third generation of CBEs (BE3) increased editing efficiency by replacing dCas9 with nCas9 – capable of nicking the non-edited strand containing the G nucleotide. This provided a 2–4-fold increase (up to 37 %) in base editing efficiency in various cell lines (7). BE3 was used to correct two single-nucleotide polymorphisms (SNPs) in the APOE4 gene with 35–50% efficiency using the nucleofection approach. However, the substitution of dCas9 with nCas9 was associated with a higher rate of off-target base editing [10]. Nishida et al. (2016) further improved editing efficiency by combining the nCas9 BE with AID, demonstrating higher efficiency than AID-dCas9 in both yeast and CHO cells, although it also generated more indels in mammalian cells [13]. The higher indel rate was reduced to 0.2% following the inclusion of the UGI, although this did not reduce the gRNA-mediated off-target rate of 1.6% seen with the nCas9-AID without UGI [13]. Expression of the BE3 cassette under either the maize ubiquitin promoter or the CaMV35S promoter was associated with a similar rate of on-target C-to-T conversion (ranging from 5 to 20%) and the C-to-G substitution issue [14, 15]. Codon-optimization of BE3 by removing six potential polyadenylation sites in the nCas9 sequence and also addition of an N-terminal NLS considerably increased the base editing efficiency to >70% within the editing window of positions 4–7 in human cell lines [16]. In addition, it has been shown that engineering BE3 protein (BE3$^{W90Y/R126E}$) could considerably reduce the high rate of RNA off-target mutations, from 8000–10,000 RNA SNVs (single nucleotide variants) using BE3 to 1000 RNA SNVs using BE3$^{W90Y/R126E}$ [17]. CBEs are also reported to have out-of-protospacer and target-strand editing, which refers to off-target editing that occurs at the Cas9 protein's targeted strand [18]. Out-of-protospacer editing occurs in nucleotides located from close vicinity to hundreds of bases away from the protospacer so that >50% of Cas9-dependent off-target sites identified by the whole genome Detect-Seq sequencing were considered out-of-proto-spacer, mainly taking place on the PAM distal side rather than PAM proximal side [18].

Further engineering of human AID was made by excluding its NES signal from the hAID sequence (termed hAID*D) (14). The rice codon-optimized hAID*D was attached to the N- terminal end of nCas9-NLS using the XTEN linker and the engineered protein was designated rBE5, carrying hAID*D-XTEN-nCas9-NLS chimeric protein [19]. Using this rBE5 system improved the C-to-T conversion rate to 30-60% in an editing window of C2-C6. However, the indels rate was also increased in the engineered version of CBE (from 2–13% in rBE3 to 72% in rBE5) [19]. To improve the fidelity of rBE5 and reduce the off-target and indel rate, the C-terminus of the rBE5 protein was fused to UGI to form hAID*D-XTEN-NCas9-NLS-UGI protein (designated as rBE9) (14). rBE9 maintained the high rate of C-to-T conversion, with a lower rate of indel formation compared to the rBE5 system [19]. However, the indel rate was positively correlated with the base editing efficiency using the nCas9-based CBEs [10, 14, 19]. Further studies showed that fusing two UGIs to the engineered APOBEC3G (A3G) could improve the editing efficiency in the CC dinucleotide (Fig. 1a). The developed A3G-BE mediated C-to-T conversion in the editing window of C4-15 of the protospacer with high efficiency and fidelity and minimum off-target effects [20].

In all studies using different versions of APOBEC1 and AID-based CBEs, it was evident that C-to-T base editing efficiency depends on the context of the sequence, with the preference of TC > CC ≥ AC > GC [10, 14, 15]. To increase CBE efficiency in GC dinucleotides, a phage-assisted continuous evolution (PACE) method was applied to find a GC-efficient CBE based on random mutations in the fused protein [21]. Thuronyi et al. (2019) developed a collection of BE-PACEs through the creation of additional mutations in APOBEC1. This not only enhanced the deaminase efficiency but also resolved the issue of context sequence-dependency of CBEs [21]. Briefly, PACE benefits from a continuous selection circuit that involves *E. coli* host cells containing a plasmid encoding a selection circuit, called gene III, and a mutagenesis plasmid [22]. The expression of gene III is linked to the expression level of a biomolecule mediator encoded in M13 bacteriophage. A mutagenesis plasmid in the host cells directs continuous mutagenesis of the phage genome, and only those variants of genome-mutated phage that obtained a desired activity of the biomolecule marker mediate the expression of gene III in the host cells [21, 23, 24]. The phage is cultured in a fixed-volume vessel that is continuously diluted with host-cell culture. As a result, only phages with a faster propagation rate than the dilution rate can remain and evolve [24]. This resulted in the identification of a CBE variant with a 29% reduction in size and higher editing activity on GC targets as well non-GC targets. The C-to-T conversion rate in GC dinucleotides was increased from <5% in BE4max to 70% in EvoAPOBEC1-BE4max [21].

Glycosylase base editors. Glycosylase base editors (GBEs) were originally developed as a type of cytosine base editor that can introduce C-to-A and C-to-G base changes in DNA [25]. The GBEs consist of a nCas9, a cytidine deaminase, and a uracil-DNA glycosylase (UNG) [25]. The UNG enzyme excises the U base created by the deaminase, creating an apurinic/apyrimidinic (AP) site that triggers DNA repair. GBEs have shown differing base editing activity in prokaryotes and eukaryotes: in *Escherichia coli*, GBEs were able to convert specifically C-to-A with an efficiency of 87%, while the GBEs converted C-to-G at specifically targeted sites in mammalian cells with 5-53% efficiency [25]. Further improvement in GBE base editing was achieved by replacing the human UNG in GBE with UNG1 from Saccharomyces cerevisiae, R33A engineering in APOBEC1, and inclusion of Rad51 at C-terminus of nCas9 (APOBEC(R33A)-nCas9-Rad51-UNG1) [26]. The resulting GBE2.0 editor had increased the average C-to-G editing efficiency from 15% using GBE to 31% with 36-93% specificity. The editing window for GBE is currently C5-7, with the highest activity at C6 [26]. Fusion of VP64 and SpRy to the GBE system (NLS-VP64-APOBEC1-SpRynCas9-NLS-UDG) could also enlarge the editing window to C5-8, increasing the C-to-G base editing efficiency while increasing the indel rate compared to the original GBE system [27]. Recently, in an innovative study based on glycosylase enzyme only and without using APOBEC1, a deaminase-free guanine base editor was also developed called gGBE: NLS-nCas9 (D10A)-MPG (engineered N-methylpurine DNA glycosylase protein)-NLS [28]. This glycosylase enzyme directs G-to-T/C conversion in the spacer sequence and subsequently C-to-A/G conversion in the sgRNA complementary sequence. gGBE v6.1
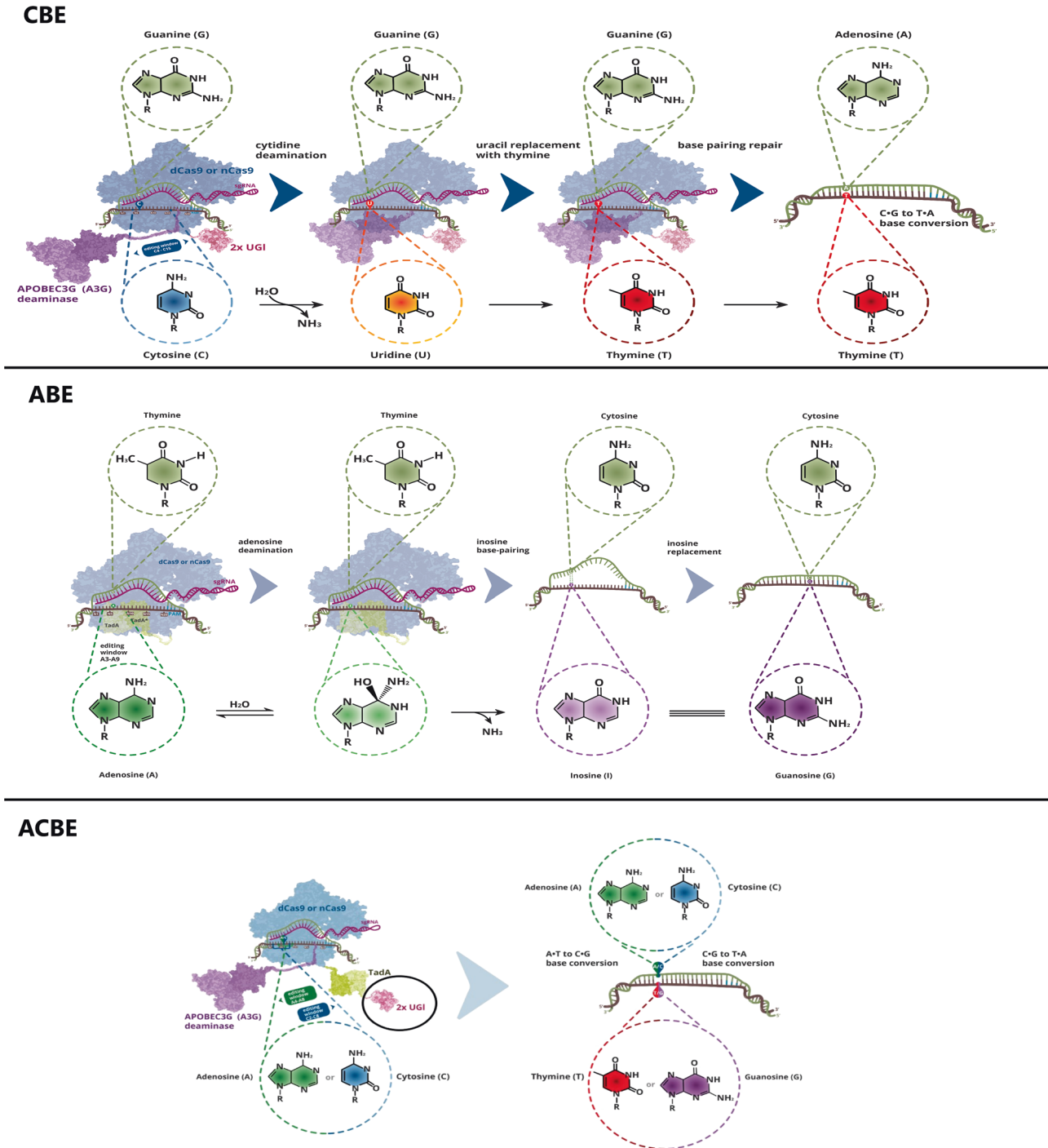
Fig. 1 Schematic presentation of base editors. Cytidine base editor (CBEs): APOBEC3G-nCas9-2x UGIs, in brief A3G-BE4. Adenosine base editors (ABEs): Codon optimized TadA-TadA*-nCas9 or ABEmax. Combined adenosine and cytidine base editors (ACBE): NLS-TadA-nCas9-rAPOBEC1-UGI or ACBEmax.

demonstrated on average 50% G-to-T/C (i.e., G-to-Y) conversion ratio for the G7 base in the spacer sequence or C-to-A/G conversion in the sgRNA complementary sequence in both cultured human cells and mouse embryos [28].

In summary, combining cytosine base editors with nCas9 instead of dCas9 and adding an N-terminal NLS increased cytidine to thymidine conversion efficiency [10, 13, 16]. However, the off-target and indel rates increased relative to the increase in on-target efficiency. Combining a PACE-derived CBE variant with a fused 2×UGI may allow for high on-target C-to-T conversion

efficiency in a sequence-independent manner [21]. Otherwise, C-to-G/A conversion is unavoidable. In contrast, including a UNG into CBEs could enhance uracil glycosylation and increase the rate of C-to-G/A to C-to-A.

Adenosine base editors (ABEs)

ABE history. Adenosine base editors (ABE) were developed by David Liu's group (Table 1) [29]. Since fusion of nCas9 had a higher efficiency of cytidine base editing than those fused with dCas9, ABEs were structured based on nCas9 fused with an adenosine

deaminase to mediate A-to-G conversion [29]. However, the development of an A-to-G convertor was initially challenging as no natural adenosine deaminase is available for DNA [29]. Adenosine deaminases that act on RNA (ADARs), originally called dsRNA adenosine deaminase (DRADA) [30], have been characterized in various organisms including mice and humans [31]. As well as duplex RNA, ADARs can be effective on single-stranded DNA when a DNA-RNA hybrid is formed by using guide RNAs [32]. Adenosine deamination activity was maximized in YAG motifs, where Y denotes T or C, with 81-94% efficiency [32]. It is worth noting that the first attempt to deliberately convert A to G was performed by adapting the BE2 system for gain-of-function in the ablated chloramphenicol antibiotic resistance gene [11, 29]. This was achieved through the use of *E. coli*-derived tRNA adenosine deaminase (TadA) in a stepwise adenosine-to-inosine-to-guanine process [33]. nCas9 fused with the N-terminus of TadA carrying A106V + D108N mutations (ABE1.2) with 3% A-to-G conversion efficiency in human genomic DNA [29]. Engineering TadA protein (ABE1.2 + D147Y + E155V) and fusing it with nCas9 resulted in 11% efficiency (20). In both BE and ABE systems, only N-terminus fusion of deaminases to the (n/d)Cas9 showed base editing activity. Evolution of ABEs from ABE1 to ABE5 was carried out by using larger linkers between TadA and Cas9 protein, engineering TadA peptide, and combining two TadA monomers, called TadA*. Analysis of the edited sequences showed that ABE3.1 strongly prefers the A substrate in the target sequence of YAC (Y = T or C) with up to 65% efficiency. However, ABE utility was still strongly limited to a specific target site [29]. To increase ABE efficiency in non-YAC targets, further generations of ABE were developed. ABE5.3 (containing heterodimeric wtTadA–TadA*–Cas9 nickase with two 32-residue linkers) allowed 39% A-to-G editing efficiency, as well as 22–33% A-to-G conversion rate in non-YAC targets with A located at positions 3-6 in the protospacer (A3-6) [29]. Although the editing window was slightly enlarged (positions 3-7 of the protospacer) using the ABE6.4 variant, the base editing efficiency was unfavorably reduced to 20–40% [29].

Development of ABE7.10. The seventh generation of ABEs was developed by implementing P48S + A142N + W23R + S48A + R152P mutations in the TadA* polypeptide of the wtTadA-TadA*-dCas9 fused protein having two 32-residue linkers [29]. ABE7.10 had a 50% efficiency for A-to-G conversion in YAC codons and 54% for non-YAC templates [29]. In a plant study, the codon-optimized TadA*7.10 version could effectively generate A-to-G conversion with up to 64% efficiency in an A5-A7 editing window using nCas9, whereas the dCas9-fused version was ineffective [34]. More importantly, zygotic injection of ABE7.10 mRNA and the sgRNAs could induce A-to-G base editing in a A3-7 editing window with 83-100% and 85% efficiency in mouse and rat, respectively highlighting the high base editing efficiency of ABE7.10 [35]. The influence of transfection method (direct microinjection vs lipofection or electroporation) on editing efficiency is also a critical factor for consideration.

ABE8 versions. All deoxyadenosine deaminases have been developed based on ABE7.10 [36]. The efficiency of ABE7.10 was improved by Gaudelli et al. (2020) who generated the eighth generation of ABEs, ABE8, with further engineered TadA protein (ABE7.10 + I76 + V82 + Y147R + Q154R) [37]. Gaudelli et al. (2020) also developed ABE8x with a heterodimeric fusion of wtTadA and TadA promoter, and ABE8m with an engineered single TadA promoter, making a 500-bp shorter sequence [37]. The core target site on the protospacer was A4-7 for ABE7.10 and A3-9 for the ABE8 system. In a parallel experiment, ABE8 doubled the editing efficiency compared to ABE7.10 and caused on average 60% A-to-G gain-of-function rate in human gamma globin genes and 98–99% target modification in primary human T cells [37]. They also confirmed that ABE8s induce no significant off-target adenine

deamination in genomic DNA, but very low levels of A-to-G conversion in mRNA [37]. Engineering of ABE8 was carried out to shorten the fused protein and also increase the editing window.

Liu's group developed ABE8e (Fig. 1b), with the addition of eight extra mutations to TadA7.10 that considerably enhanced the deaminase activity compared to the ABE7.10 along with a higher turnover rate (590 fold) [23, 38]. ABE8e was developed using a series of phage-assisted non-continuous evolution (PANCE) and PACE methods. PANCE is a manual version of PACE with the same selection circuit, but instead of continuously diluting the medium in the vessel as in the PACE method, the fresh culture medium is manually mixed with an aliquot from the proceeding passage. The TadA8e-dimer containing the TadA8e variant (wildtype TadA + TadA8e) induced a higher A-to-G base editing activity and a lower indel rate compared to other TadA8 variants. Moreover, shortening the ABE8e by removing the wildtype TadA could induce a similar base editing activity compared to that of ABE8e-dimer (wildtype TadA + TadA8e). However, the shorter ABE8e is unfavorably associated with a higher off-target rate (2–7%) compared to both full-length ABE8e-dimer (0.1–1.6%) and ABE7.10 (0.1–0.3 %) [23]. More importantly, combining other variants of nCas9 with the ABE8e could expand the editing window to protospacer positions A3–14. Moreover, the editing efficiency at the boundaries of the editing window was increased by an average of 2.9-fold for CP1028-ABE8e and 2.5-fold for CP1041-ABE8e compared to the corresponding ABE7.10 variants, without substantial changes in indel frequencies [23]. Also, the base editing efficiency was higher when plasmids were used to encode the ABE8e compared to the ribonucleoprotein (RNP) reagents [23]. ABEs have also been shown to have a high rate of RNA off-target [17]. However, engineering of ABE7.10 (ABE7.10^{F148A}) could reduce the number of RNA SNVs from 4,000 RNA using ABE7.10 to <1,000 RNA using ABE7.10^{F148A} while it maintained its on-target A-to-G conversion rate for A5 [17].

In summary, the evolved TadA8e based on the phage-assisted selection system is the most efficient version of ABE. To avoid off-target base editing using non-viral transfection systems, ABE8e-dimer with a wildtype TadA+TadA8e is preferable.

Fusion of Cas9 variants with base editors
Cas9-embedded ABEs and CBEs. It has been evident that while N-terminus fusion of deaminases to Cas9 (both nickase and dead versions) achieve efficient editing, they also result in off-target effects [10, 29]. It was hypothesized that off-target effects could be reduced by embedding a deaminase within nCas9 instead of fusing at its N-terminus [39]. An all-in-one plasmid was developed expressing the nCas9 gene, the ampicillin-resistance gene with a C > T mutation that caused a premature stop codon in beta-lactamase, and a sgRNA that could direct base conversion in the premature stop codon of the ampicillin-resistance gene to restore its function. Mu transposase was then used to randomly insert a TadA*-TadA-encoding transposon randomly into the plasmid, with a proportion inserting within the nCas9 coding sequence [39]. Only resulting ABE editors capable of converting A-to-G (T-to-C in the opposite strand) would be capable of restoring function to the ampicillin-resistance gene. Resistant clones were sequenced and a tolerant region wtihin nCas9 (amino acid positions 1048–1063) was identified for embedded TadA*-TadA. Thus, CE1048-1063-ABE was engineered with TadA embedded between positions 1048–1063 within nCas9 rather than at its N-terminus. The A-to-G base editing of CE1048-1063-ABE was similar to the ABEmax, with high activity at the editing window of A3–A9, maximizing at A5 with 45–50 % efficiency. A similar approach was used for CE-CBEs by embedding APOBEC1 between positions 1048–1063. The embedded version had a similar efficiency as the N-terminus-fused version. The editing window (C3–C10) was the same as that of BE4max, with the maximum C-to-T efficiency of 75% for C6. The main improved feature of the embedded versions of CE-ABE and

CE-BCE was a reduction in off-target effects [39]. A similar strategy was implemented to embed the APOBEC1 and TadA8e into the SaCas9 nickase [40]. The editing window was enlarged to C6–C17 in the resultant Sa-CBE-693 with 40% efficiency on different target loci [40]. The rate of on-target to off-target editing was 30% using Sa-CBE-N, while it increased to 900% using the embedded Sa-CBE. The same method was also applied for embedding the deaminase into the SpRY Cas9 protein, an engineered SpCas9, and could reduce the off-target rate while having a high rate of base conversion [41]. The mechanism by which Cas9-embedding enhances editing fidelity is not yet understood. However, we speculate that the embedded protein might increase the Cas9 specificity or decrease the Cas9-DNA binding affinity. In summary, embedding deaminases into different variants of nCas9 proteins is a very promising approach to have a more flexible editing scope and lower off-target editing than those with N-terminus-fused deaminases [40].

Nearly PAMless SpCas9 (SpRY, SpNRRH, and SpNRCH). Like other CRISPR/Cas9 systems, the CRISPR-Cas9 DNA base editing typically requires a PAM sequence to effectively interact with the target DNA. This requirement limits the number of possible target sequences within a genome. A near-PAMless variant of SpCas9 named SpRY was developed that does not require the canonical NGG PAM but can also utilize NRN (R stands for purine bases of Guanine and Adenine) and to a lesser extent NYN PAMs (Y stands for pyrimidine bases of Cytosine and Thymine) [42]. SpRY nuclease and base editor variants can target almost all PAMs in mammalian cell lines, however the off-target rate was higher than its CBEmax counterpart [42]. Another modified version of SpCas9 with non-G PAM was developed based on PACE/PANCE methods [43]. Using this method, variants of SpCas9 were detected targeting the non-G PAMs NRRH and NRCH (H = adenine, cytosine, and thymine). Fusing SpNRRH and SpNRCH to BE4max induced C-to-T conversion at rates ranging from 2–21% depending on the PAM sequence, comparable to the standard nCas9 fused proteins [43]. The off-target was reduced to half of that observed for the nCas9 protein [43]. The development of nearly PAMless Cas9 systems is progressing rapidly. Embedding the TadA-8e monomer into SpRY-nCas9 resulted in an efficient Cas9-embedded adenosine base editor, designated CE-8e-SpRY [41]. This CE-8e-SpRY could target almost all genomic sites with a high efficiency (on average 45%, with the maximum efficiency of 80% for A6–A9) and reduced RNA and DNA off-targeting activities (less than 10% of that for 8e-N-SpRy) [41].

Staphylococcus aureus Cas9 (SaCas9). *Staphylococcus aureus* Cas9 (SaCas9), which requires an NNGRRT PAM, has been considered as an alternate to SpCas9. Replacement of the Cas9 nickase form of SpCas9 with that of SaCas9 in BE3 to generate APOBEC1–SanCas9–UGI (SaBE3) showed a 50–70% C-to-T conversion [44]. Moreover, the use of an engineered SaCas9 variant containing three mutations (SaKKH-Cas9) could relax the variant's PAM requirement to NNNRRT [45] and achieve a 50% conversion efficiency [44]. Interestingly, they found that shortening the gRNA sequence from 20 nt to 16 nt did not reduce the editing window length [44]. Yang et al. (2018) replaced SpnCas9 with SanCas9-KKH (PAM: NNNRRT) and nCas9-VQR (PAM: NGA) to generate SaKKH-ABE and VQR-ABE, respectively. Microinjection of ABE mRNA and sgRNA in mouse zygotes induced A-to-G conversion with 16% efficiency for SaKKH-ABE and 20% efficiency for VQR-ABE. Yang et al. (2018) also found a < 0.2% off-target mutation rate using the ABE system which is similar to wild-type controls, demonstrating that ABE might have very few or no off-target effects at these tested sites [35]. In another study, substitution of SpCas9 with SaCas9 resulted in an extended editing window of A5-14 and A4-15 for Sa-ABE7 and Sa-ABE8, respectively, although with lower editing efficiency (ranging from 20–40%) [37]. In summary, SaCas9

base editors are associated with a lower base editing efficiency than SpCas9 versions.

Compact Cas9 variants. A compact type of Cas9 has also been developed from *Neisseria meningitidis* (Nme) [46]. This Nme2Cas9 has a dinucleotide (N4CC) PAM requirement that provides for high on-target and low off-target editing efficiency. An all-in-one AAV (adeno-associated virus) vector has been developed comprising NmeCas9 and sgRNA modules that collectively span 4.7 kb between the ITRs, which was efficiently used for in vivo editing of the mouse genome [46]. Similarly, a short type of Cas9 has also been derived from *Campylobacter jejuni* (CjCas9), with a 2.9-kb coding sequence compared with 3.2 kb for *S. aureus* and 4.1 kb for *S. pyogenes* [47]. CjCas9 does, however, requires a 10-bp PAM with ACAC at positions 5-8. The main advantage of this CjCas9 was that no off-target activity was detected using in vitro and in vivo transfection of the CjCas9-encoding plasmid. Moreover, the sgRNA module (with U6 promoter) and the Cas9-EGFP module (with EF1a promoter) were able to be packed into just 4.7-kb [47]. Therefore, it is likely that Nme2Cas9 and CjCas9 will be fused with base editor systems for in vivo applications in the future.

Combination of cytidine and adenosine base editors

In 2020, five reports were published on the development of dual-base editors by combining ABE and CBE [48–51] (Fig. 1c). All fused proteins could simultaneously convert four base substitutions (C-to-A, G-to-T, A-to-G, and T-to-C) using a single gRNA. However, compared to the previously developed single-base editors, the on-target efficiencies were lower. Sakata et al. (2020) developed the ACBE system with NLS-TadA-nCas9-rAPOBEC1-UGI, designated ACBEmax. ACBEmax had an average of 20% efficiency for simultaneous editing of C-to-T and A-to-G conversions, with a relatively low off-target to on-target ratio of 0.1 % (Table 1). The ACBEmax editing window was C2-C8 and A4-A8 positions, whereas the maximum efficiency was observed for C8 and A5-A6 positions [48]. Gruenwald et al. (2020) created a CRISPR-Cas9-based synchronous programmable adenine and cytosine editor (SPACE) by combining nCas9 with both miniABEmax (Table 1) and Target-AID to produce a dual base editor [50]. Using various gRNAs in human HEK293T cells, the overall efficiency of SPACE for A-to-G editing was 13%, slightly lower than that of miniABEmax-V82G (18%), and averaged 22% for C-to-T editing efficiency comparable to that of Target-AID (25%) [50]. Moreover, a higher editing efficiency for both C-to-T and G-to-A was observed using SPACE, as a single fused protein carrying both miniABEmax and Target-AID, related to co-transfection of individual miniABEmax and Target AID, whereas the indel frequency was lower for SPACE compared to the co-transfected groups with ABE and CBE. The off-target efficiency of SPACE was 1.6% for both cytidine and adenosine bases [50]. Also, Xie et al. (2020) reported ACBE, with the same name also applied to an independently developed dual base editor [48], which is a more complex fusion of NLS-ecTadA-32aa-nCas9-NLS-10aa-SH3-CDA1-9aa-UGI-NLS. The editing efficiency was 28% for C-to-T and 19% for A-to-G, whereas the highest dual editing efficiency (20%) was observed for the C5A6 dinucleotide. No off-target editing was observed for the ACBE dual base editor [49]. In another study, to create the A&C-BEmax, a cytidine base editor followed by two TadA molecules and nCas9 was fused with two copies of UGIs and then added to the N-terminus of the nCas9 [51]. The simultaneous C-to-T and A-to-G conversion rate ranged from 2 to 30%(?), while the off-target rates were reduced compared to single AID-BE4max and ABEmax [51]. Liang et al. (2022) fused a CGBE (glycosylase base editor) with ABE to develop a new type of dual deaminase-mediated base editing system, AGBE, that can simultaneously introduce four types of base conversions (C-to-G, C-to-T, C-to-A, and A-to-G) as well as indels with a single sgRNA in mammalian cells [52].

However, the AGBEs were mainly triple base editors, with A/C-to-G and C-to-T abilities. In the same study, Liang et al. (2022) developed miniAGBEs by fusing human APOBEC1 (hAPOBEC1) to the N-terminus of ecTAdA8e followed by nCas9 and another NLS at the C-terminus of the protein (Table 1). The editing windows were C2-C11 and A4-A7, with the highest efficiency for C4 to G (17%), C7 to T (60%), and A5-A6 to G (80%) [52]. No significant difference was found between different types of miniAGBEs for base editing [52]. Indeed, miniAGBE does not involve UGI. It has also previously been shown that deaminated cytidine using both AID and APOBEC1 can be substituted with any of the three bases, with the preference $T > G > A$ [10, 12]. Therefore, the current C-to-G transition is due to a lack of UGI in the fused protein.

More recently, Neugebauer et al. (2022) developed an ABE-based CBE following mutating residues that interact with the DNA backbone close to the deamination active site and developed a TadA-CBE with comparable C-to-T base editing efficiency to BE4max and lower off-target effects [36]. Further engineering of the TadA8e could create a TadDE or TAD dual editor that could simultaneously convert adenosine and cytidine base editing with 50-70% efficiency [36]. TadDE performed very similar levels of adenine and cytosine base editing (ABE:CBE ratio = 1.1), on average 73-80% for a plasmid target and 35% for a genomic target (Table 1). Since ABEs offer lower levels of Cas-independent off-target editing compared to CBEs, the ABE-based TadDE has a smaller size and substantially lower Cas-independent DNA and RNA off-target editing activity compared to other CBEs [36]. Its off-target DNA editing ranged from 0.1 to 0.6%, much lower than that of CBEs [36]. Compared to other dual editors, such as those produced by fusing both cytidine and adenosine deaminases to a Cas domain [51, 52], TadDE is smaller and more efficient [36]. TadDE has the editing window of C2-C9 and A2-A9, with the highest efficiency for C6 and A6, respectively. This suggests that if C and A nucleotides are located in positions 4 to 6, the application of TadDE is highly recommended as it had >60% efficiency for dual conversions [36]. Using the Cas9-embedded approach, both TadA-8e and APOBEC3A were tandemly inserted between amino acids 693 and 694 of SaCas9 using 16aa XTEN linkers to form Sa-CABE-693 [40]. While maintaining a similar base editing efficiency for a C3–C9 editing window, Sa-CABE-963 converted C10-C17 bases with a higher efficiency than that of the standard N-terminus-fused protein [40]. The editing window was enlarged and the off-target effects were reduced using the embedded nCas9 compared to that of N-terminus-fused deaminases.

GBEs have also been combined with ABEs [53]. Replacing glycosylase factor with a chromatin-associated factor (HMG1) could induce 25–30% C-to-G conversion rate along with 65–70% A-to-G conversion efficiency using engineered TadA8e. This result is in agreement with C-terminus fusing of UGI to CBEs to exclude C-to-G conversion and support C-to-T base editing [11].

In summary, the combination of CBEs and ABEs can be achieved via the co-transfection of both enzymes. Furthermore, fusion of both deaminases with nCas9 has been carried out either by fusion of TadA to the N-terminal of nCas9 and the AID/APOBEC1 to the C-terminal of the nCas9 protein, both TadA and AID/APOBEC1 together at the N-terminus of nCas9, or embedding TadA8e and APOBEC1 within the nCas9 protein. For the N-terminus-fused deaminases, the addition of one or two UGIs to the C-terminus of the fused protein is highly recommended for decreasing indel rates and specifically inducing C-to-T conversion. Using glycosylases could increase the base editing options from C-to-T and G-to-A to C-to-G and G-to-C/T conversion [25–28]. However, there is a large room for improvement of the editing window and base editing efficiency using glycosylases and other chromatin-associated factors.

PROSPECTS: APPLICATION OF CRISPR/CAS9 TO EDIT MITOCHONDRIAL GENOMES
Mitochondrial genetic disorders
Mitochondrial DNA (mtDNA) is a double-stranded circular molecule of approximately 16.5 kbp in mammals that encodes 13 protein subunits in the oxidative phosphorylation system and a full set of 22 tRNAs and 2 rRNAs. Some of the unique characteristics of mtDNA include circularity, maternal inheritance, high G + T content in one strand of the chromosome [54], high copy number, and heteroplasmy [55]. Although more than 99% of the proteins in mitochondria are encoded by the nuclear genome (nDNA-encoded), the integrity of mtDNA is critical for mitochondrial functions. Mitochondrial dysfunction is known to cause over 200 disorders, including neurologic, muscular, cardiac, gastro-intestinal, and ophthalmologic diseases [56, 57]. Interestingly, the majority of these mutations are heteroplasmic—mtDNA coexists in both wild type and mutated forms in the same cell – while the occurrence and strength of the disease depends on the heteroplasmy level [58].

In regard to the source of these heteroplasmic mutations, replication of mtDNA is carried out by the heterotrimeric DNA polymerase γ (Pol γ). This DNA polymerase has a $3' \rightarrow 5'$ exonuclease activity that accurately proofreads the newly synthesized DNA strand with a lower than 1×10^{-6} error rate [59, 60]. However, despite mtDNA being located in a high-ROS environment, the majority of mtDNA mutations are produced by DNA replication but not via ROS-inducement [60]. It has been shown that the exonuclease domain of Pol γ is a hotspot for oxidation and therefore becomes severely oxidized, generating a net negative charge around the active site for mismatch repair, rendering it enzymatically deficient [60]. Indeed, under oxidative conditions, the Pol γ exonuclease domain is far more sensitive to oxidation than its polymerase domain [60, 61]. Therefore, mtDNA is always prone to mutations under stress conditions. Thus there is a need to develop efficient strategies for correcting mitochondrial mutations that cause disease.

Transfer of protein into mitochondria
Specific signals are necessary for the transfer of therapeutic nucleases into mitochondria reviewed by [62]. Transport of a protein into mitochondria requires a mitochondrial targeting sequence (MTS; also called mitochondrial localization signal (MLS)). It has also been shown that addition of MTS sequence from ATP2P gene to the 5′ end of mRNA increased the mRNA localization onto the surface of yeast mitochondria [63]. Moreover, the translated MTS has been defined as a short (15–70 amino acids) motif located at the N-terminus of a protein mediates the mitochondrial transport of the fused protein in mammalian cell lines and yeast [62, 63]. Bearing positively charged basic residues, these canonical sequences consist of an alternating pattern of hydrophobic and positively charged residues that form an amphipathic helix [62]. Following entry into the mitochondrial matrix, the MTS is cleaved by mitochondrial processing peptidase [64]. In addition, the use of NES sequences, a 9-11-residue motif located at the 3′N-terminus of the engineered fused protein, has also been necessary to ensure the exclusive localization of these proteins to mammalian mitochondria [65]. The presence of four hydrophobic leucine residues are required for a fully functional NES [66, 67].

Transfer of RNA into mitochondria
Import of RNA molecules by mitochondria has been a challenging topic and therefore, should be considered as an important factor for CRISPRing mitochondrial genome [68]. Here, we discussed the most reproducible discoveries and methods in this field. In yeast, tRNA$^{Lys}_{CUU}$ (referred to as tRK1), is partially addressed into mitochondria, while tRNA$^{Lys}_{UUU}$ (tRK2) is localized only in the

cytoplasm [69, 70]. Selective import of tRNAs into cellular organelles requires cytoplasmic factors. The mitochondrial entry of tRNAs is an RNP-mediated process that involves interaction of RNA molecule with specific enzymes in yeast, [71]. The cytoplasmic lysyl-tRNA synthetases (referred to KRS) involves in aminoacelyation of lysyl-tRNAs, while mitochondrial lysyl-tRNA synthetase (referred to MSK) is able to form stable RNP complexes with the aminoacylated tRNA and translocate the tRNA across the mitochondrial membranes [71]. Although KRS depletion and N-terminal truncation of MTS signal from MSK enzymes showed the blockade of the RNA entry into the mitochondria, having cytosolic factors are also required for the mitochondrial entry [71].

Because mammalian mitochondria encodes all required tRNAs, for a long time, it was believed that they do not import any cytoplasmic tRNA. Further studies in the last two decades showed that human mitochondria are also able to internalize yeast tRNA derivatives as 5S rRNA with a similar transportation mechanism that found in yeasts [72, 73]. Indeed, similar to the yeast cytosolic extract, human cytosolic extract also possess all requirement factors to import tRK1 or 5S rRNA following incubation with MSK [72, 73]. These results suggest the possibility of developing a tRNA mitochondrial import system in human cells [72]. It was suggested that expression of yeast pre-MSK in human cells is sufficient to provide RK1 mitochondrial import in vivo [72].

Then, tRNA importation in rat and human mitochondria was also docuemnted [74]. Rigorous analysis of subcellular RNA fractions from mitochondria evidenced that some small non-coding RNAs, such as nucleus-encoded tRNA^{Gln} and also the synthetic transcripts of yeast tRNA^{Lys} are internalized by yeast and human mitochondria [74]. They also showed that two stem-loop-structured RNAs with 56 and 110 nt could be imported into the mitochondria in an mitochondrial ATPase-dependent manner [74]. Apart from tRNAs, there are small RNAs that are expressed in the nucleus, but present in mitochondria. For example, the RNA component of RNase MRP, an endoribonuclease involve in primer RNA cleavage during replication of mitochondrial DNA, the RNA component of RNase P, an endoribonuclease involved in processing of 5′ ends of tRNAs, and 5 S ribosomal RNA which acts as a scaffold interconnecting several functionally important sites on the ribosome [70, 75].

Apart from the importance of RNP-dependent internalization of some tRNAs into mitochondria, RNA import is also a sequence specific process [76]. The RNA sequence of the aminoacceptor helix of tRNA is an important factor for the mitochondrial entry so that replacement of bases 1:72 in tRK2 by those of tRK1 makes the engineered tRK2 importable into mitochondria with a similar transportation efficiency to the natural tRK1 [76].

Mitochondria from all organisms possess translocator proteins expressed in the outer mitochondrial membrane (TOMs) that contains a recognition motif with high affinity to cholesterol involve in the import of lipophilic molecules into mitochondria [77]. However, RNA trafficking into mitochondria is taking place but with a low efficiency [75, 78, 79]. Therefore, there is a huge room for improvement in this area. In an elaborative study, based on RNA secondary structure rearrangement, a carrier-free targeting approach for mitochondrially importable RNAs was developed for human cells [80]. RNA molecule conjugated with a cleavable linker of hydrazone bond to cholesterol entered into mitochondrial matrix (on average, 15% of mitochondria uptake the RNA) [81]. A more recent study showed that the addition of a hairpin structure at 3′ end of sgRNA (downstream of the scaffold hairpins, called hairpin F) increased the RNA transportation efficiency compared to the standard sgRNAs which devoid the additional hairpin structure [78]. In this study, using rigorous checkpoints, Northern hybridization confirmed that isolated mitochondria contained intact transcripts which were devoid of nuclear or cytosolic contamination (5.8S rRNA) [78].

In overall, the mitochondrial import of hairpin-structured RNAs, such as tRNAs, gRNAs, and synthetic RNAs, has been reproducibly evidenced [74, 78]. The mitochondrial import of these structured RNAs is an RNP-mediated process directed by specific mitochondrial and cytosolic proteins. Engineering RNA structures for a more efficient RNP formation can act as a key factor to enhance sgRNA import into mitochondria.

Current state of mitochondrial genome editing

In principle, genetic engineering of mitochondria can be carried out by two strategies: the anti-replicative strategy that benefits from the induction of DSBs in the mitochondrial genome, and the genome correction strategy that uses nucleases, such as endonucleases, ZFNs, TALENs, and CRISPR/Cas9-based base editors (Fig. 2).

The anti-replicative strategy. One potential strategy for treating mitochondrial diseases is to selectively inhibit the replication of mutation-carrying mtDNA [82]. As depicted in Fig. 2, the anti-replicative strategy aims to shift the heteroplasmy level specifically by targeting the replication of mutant mtDNA, inducing degradation of mutation-bearing mitochondria, and subsequently giving a propagative advantage to wild-type genomes [82]. The classical NHEJ does not exist in mitochondria and therefore, mitochondrial genomes bearing DSBs are rapidly degraded [83, 84], resulting in a shift in the mitochondrial heteroplasmy [85, 86]. Therefore, only genomes with single-stranded breaks or no breaks can escape from degradation and remain after the genome editing process. In agreement with this, <0.1% indel rate was detected in the mitochondrial genome of human, mouse, and rat embryos using the DdCBE technology—low enough to be attributable to sequencing artifacts [87–90]. The mechanism for mtDNA elimination has been discovered to be mediated via exonucleolytic activities that are major components of the mtDNA replication machinery [91]. CRISPR-mediated inactivation of the mitochondrial 5′ → 3′ exonuclease MGME1 and the 3′ → 5′ exonuclease activity of the mitochondrial DNA polymerase γ and siRNA-mediated knockdown of the mitochondrial DNA helicase TWNK led to the accumulation of abnormal linear and rearranged mtDNA. The inactivation of these three genes, located in the nuclear genome but acting in the mitochondria, showed that the removal of linearized mtDNA is important for the maintenance of the proper mitochondrial genome [91].

In an in-vitro study, it was demonstrated that synthetic peptide nucleic acid (PNA) oligomers that complement the mutant mtDNA to generate stable PNA/DNA complexes can specifically inhibit the replication of the mutant genome, without interacting with the wildtype mtDNA templates [82]. However, because of the natural impediment of mitochondrial membranes, introducing PNA molecules into mitochondria is yet to be achieved [92].

Early studies on endonuclease-mediated mitochondrial depletion showed that fusion of the signal peptide of cytochrome c oxidase subunit IV (pCoxIV) to the N-terminus of an endonuclease could mediate transfer of the nuclease into mitochondria without affecting the nuclear DNA integrity [93]. Therefore, the inclusion of the MTS coding sequence into the mRNA through the mitochondrial-specific gene cassette in the N-terminus of the protein sequence is a safe and effective pathway to transfer the desired protein into the mitochondria [63, 94]. This strategy has been demonstrated by fusing a MLS to the N-terminus of bacterial restriction endonucleases, which resulted in their mitochondrial importation (for review, see [95]).

Genetic engineering has been widely used for the selective degradation of mutated mtDNA [96]. Zinc finger nucleases (ZFNs) —nucleases that are conjugated to a zinc-finger peptide (ZFP)— were used for the selective degradation of particular mtDNA sequences. In an innovative method, instead of relying on the necessary dimerization of two ZFN molecules, Minczuk et al.
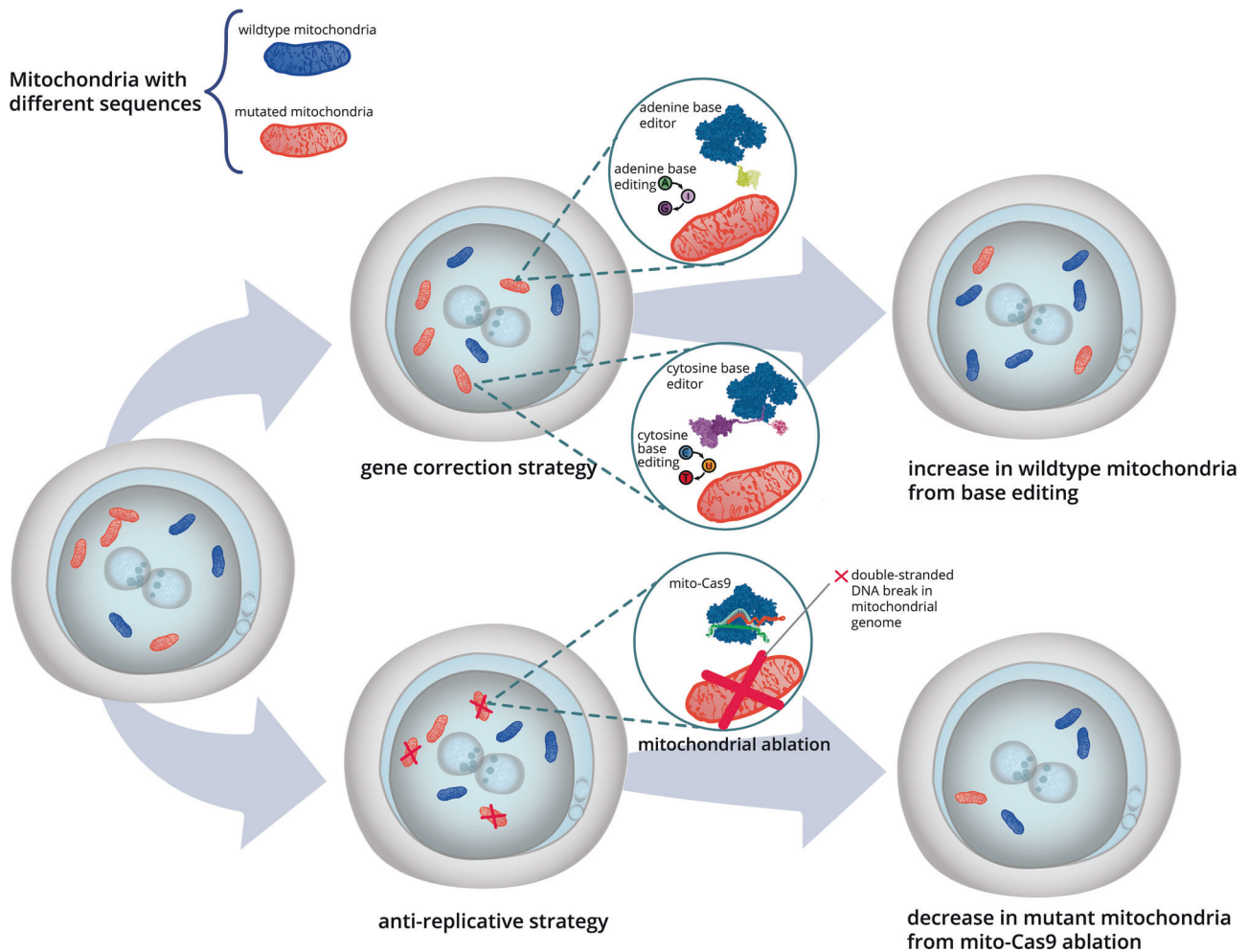
Fig. 2 Mitochondrial genome engineering in zygotes. Two gene editing approaches are depicted: anti-replicative vs gene correction approaches. The wild-type mitochondria is in blue color and the mitochondria with mutated genome is depicted with red color. In the anti-replicative strategy, only the mito-Cas9 protein and sgRNA was injected that caused to the degradation of the mutated mitochondria. In the gene correction strategy, mt-nCas9-base editor (CBE/ABE) was injected with sgRNA. This caused base conversion and gene correction.

(2018) created a single-chain ZFN by conjugating two FokI nuclease domains connected by a flexible linker to a single ZFP and a 51-aa N-terminal MTS from human ATP synthase F1β subunit. They showed that the single-chain ZFN was efficiently transported into mitochondria, bound to mtDNA in a sequence-specific manner (detecting a single base pair mutation in a 12-bp sequence) and cleaved dsDNA at the predicted site adjacent to the mutation, which resulted in selective degradation of the mutated mtDNA and increased the proportion of wild-type mtDNA molecules in the heteroplasmic cells [96]. Selective elimination of mutated mtDNA and driving mtDNA heteroplasmy has been demonstrated to prevent germline transmission of an associated disease by using a mouse strain containing both BALB and NZB mtDNA haplotypes and selectively degrading BALB mtDNA in oocytes, embryos, and live animals using mitochondrion-targeted restrictionendonuclease and TALEN [97]. Both restriction endonuclease and TALEN were fused with the MLS derived from ATP5B. While maintaining normal nuclear genomic integrity, they also reduced levels of mutated mtDNA responsible for a series of human mitochondrial diseases in mammalian oocytes using mitochondrion-targeted TALENs (mit-TALENs) [97].The CRISPR/Cas9 approach has also been used to degrade mitochondria containing a mutated genome. Transfection of HEK-293T cells with engineered sgRNAs with an extra hairpin

downstream of the scaffold RNA (considered as mito-sgRNA) and MTS^COX8A-hCas9 (considered as mito-Cas9) showed 2-3-fold depletion of mtDNA when two mito-sgRNAs were co-transfected, though mito-Cas9 combination with one mito-sgRNA did not induce mtDNA depletion [78]. They showed the mitochondrial entry of mito-sgRNA and mito-Cas9 as well as mtDNA depletion in the transfected cells [78]. However, no point mutation or indels were detected at the target sites [78]. Bi et al. (2022) could also detect sgRNAs and mito-Cas9 (MTS ^COX8A -Cas9 [78]) in the mitochondria of transfected HEK cells [98]. Similar to previous reports on indirect evidence of the effectivity of the CRISPR/Cas9 system in mitochondria [78, 99], the copy number of mtDNA was reduced significantly compared to cells transfected with NLS-wildtype Cas9-NLS and the sgRNA individual groups [98]. A hybrid sgRNA construct has recently been reported in which the 20-nucleotide RNA stem-loop structure of RNASe P was added at the 5′ end of the sgRNA sequence [100]. Although this hybrid sgRNA and mito-Cas9 combination showed high levels of hybrid sgRNA in mitochondria [100], their approach was not reproducible by other groups [79]. Moreover, addition of a hairpin structure similar to the RNase P structure at the 5′ end of sgRNA blocked the Cas9 cleavage effect in vitro and therefore was excluded from further experiments, while only hairpin structures at the 3′ end of sgRNA were successfully implemented [78].

The genome correction strategy. The majority of DNA deaminases were developed based on ssDNA/RNA editors. This includes the APOBEC and TadA families, which naturally deaminate ssDNAs and RNAs, respectively [101–103]. ZFN and TALEN editors do not generate ssDNA and, therefore are not compatible with ssDNA deaminases. Therefore, dsDNA editors have been widely used with ZFN and TALEN to successfully edit mitochondrial genomes [87]. A cytosine deaminase system has recently been developed for base editing the mitochondrial genome by exploiting an interbacterial toxin, DddA, that catalyses the deamination of cytidines in dsDNA [87]. The DddA toxic domain (DddA$_{tox}$)—sufficient for deaminase activity—was split in half so that each half-DddA$_{tox}$ was non-toxic and inactive until bound together on target DNA by adjacently bound programmable DNA-binding proteins. Fusions of each DddA$_{tox}$ half with a transcription activator-like effector array (TALE) protein and a uracil glycosylase inhibitor (UGI) produced two synthetic TALE-DddA$_{tox}$-UGI proteins that together formed a sgRNA- and Cas9-free DddA-derived cytosine base editor (DdCBE). DdCBE catalyzed C-to-T conversion in human mtDNA with high target specificity and product purity (>95% efficiency) as well as negligible off-target editing (<0.4%). Microinjection of plasmids expressing the DdCBE components into mouse zygotes generated C-to-T substitutions that were detected in the resultant F$_0$ newborns and transmitted to their F$_1$ progeny [88]. Also, the injection of plasmid and mRNA encoding DdCBE components into rat, mouse, zebrafish, and human zygotes generated high efficient base editing in mitochondrial target genes with 0.1–0.3% off-target editing [89, 90, 104, 105]. The DdCBE system was also implemented for highly efficient mitochondrial genome editing in human and mouse cells [90, 104]. MitoKO DdCBE is a TALE-based library developed for knocking out all 13 protein-coding genes in the mouse mitochondrial genome [106]. In this innovative strategy, the tryptophan TGA codon was converted to the TAA stop codon via a C-to-T conversion in the opposite strand, with 40-70% efficiency. Successive transfections of the MitoKO DdCBE constructs could be used to improve the knock-out of hetero-plasmic alleles in both cellular and animal models. Moreover, engineering the MitoKO DdCBE construct by reducing the expression level of the DddA$_{tox}$ half-split reduced the off-target rate [107].

In a more recent study, DddA$_{tox}$ halves were fused to mitochondrion-targeting ZFDs (mtZFD) that were produced by N-terminus linking MTS and NES sequences and C-terminus fusion of the DddA$_{tox}$ halves to ZFDs, targeting eight mitochondrial genes [108], the C-to-T base editing efficiency averaged 11% in HEK cells within an editing window of C2-C11, with no preference for the CT motif. The on- and off-target editing efficiency was dose-dependent by which increasing the plasmid or mRNA concentration increased both on-target (on average 25%) and off-target rate (ranging from 5–20%) [108].

Recently, ABEs have been developed for mitochondrial genome editing. It called TALED that is a TALE-linked deaminase which composed of an MTS, a catalytically impaired, full-length DddA variant or split DddA, and an engineered TadA protein, which induce targeted A-to-G editing in human mitochondria [109]. TALED induces A-to-G conversion with up to 10–40% efficiency for A(/T)7–12 bases located between two TALE interaction sites. Importantly, they found that the presence of DddA is a prerequisite for effective TadA functionality [109]. This might be due to this fact that TadA is a ssDNA deaminase. However, TALE-DddA may facilitate production of ssDNA that is a substrate for TadA function.

Although single-stranded DNA deaminases have been combined to Cas9 protein to direct deamination of single-stranded DNA in the nucleus (already discussed in previous sections) [101–103], ssDNA deaminases have rarely been used with the CRISPR/Cas9 system for mitochondrial genome editing. Apart from the difficulties and natural obstacles for mitochondrial genome editing, there are limited groups in the world studying in this field. Nonetheless, although targeted base editing has not yet been demonstrated, the import of CRISPR-based CBEs into mitochondria and non-targeted base editing of mitochondrial genome have recently been reported in HEK cells, producing up to 1.1% C-to-T conversion in a guide-independent manner [110]. This highlights guide-independent off-target editing in mitochondria as an important issue to resolve. This study also concluded that inefficiency in the sgRNA trafficking into mitochondria was the main reason for this low efficiency [110]. Furthermore, this low efficiency might be partly attributable to the cell transfection method—in this case the calcium phosphate method. the use of more robust cell transfection methods, such as electroporation and nucleofection, can increase Cas9 and sgRNA delivery into the mitochondria and subsequently be an important factor for improving gene editing efficiency [111].

Prospective approaches for CRISPR-mediated mitochondrial genetic engineering

Eukaryotic cells already possess mechanisms for importing macromolecules into mitochondria [112]. Similar to mitochondrial ZFNs [108] and TALENs [87], mito-Cas9 has been engineered by fusion of MTS to the N-terminus of the Cas9 protein [98, 110]. The gene editing efficiency of mtZFD (on average 25%) [108] and mit-TALEN (up to 95%) [87] was higher than that of mito-Cas9 approach (1–6%) [98, 110]. There are considerable differences in size among programmable nucleases: zinc finger arrays are the smallest, encoded by 2×0.3-0.6 kbp DNA, TALENs are medium-sized nucleases encoded by 2×1.7-2 kbp, and Cas9 from *S. pyogenes* is the largest encoded by a 4.1 kbp sequence [113]. Protein size can affect both the cargo delivery approach and transfer rate into the mitochondria. Although Cas9 protein import into mitochondria has been reproducibly demonstrated [68, 78, 110], use of smaller-sized Cas9 protein can enhance its delivery to mitochondria. Supporting this idea, use of mito-Cas12a, with less hydrophobicity and about 70% of the size of mito-Cas9, could improve Cas protein entry into mitochondria [68]. Also, ZFD- and TALEN-encoding genes can be readily packaged in an AAV vector with limited cargo space, whereas CRISPR/Cas9-encoding genes require to be split into two AAV vectors for in-vivo studies and gene therapy applications [108]. Therefore, the larger size of the Cas9 protein can be a limiting factor for the entrance and efficiency of the CRISPR/Cas9 system compared to the ZFN and TALEN systems.

Unlike the ZFN and TALEN editors, which incorporate both target specificity and nuclease functions into a single molecule, CRISPR/Cas9 is a two-component system, comprising an RNA and a nuclease protein. The import of sgRNA into mitochondria is an additional factor for achieving highly efficient CRISPR editing of the mitochondrial genome. It has been well verified that the RNA mitochondrial import pathway is the only known natural mechanism of nucleic acid delivery into mitochondria [92, 114, 115]. Several promising approaches are reviewed and suggested by Sieber and colleagues to facilitate RNA transport into mitochondria [112]. In summary, inner membrane for tRNA import complex (RIC) derived from *Leishmania tropica* [116], tRNALys derivatives [72], 5S rRNA derivatives [117], and structured hairpin at the 3' of sgRNA [78] have each been shown to facilitate mitochondrial entry of recombinant RNAs. The above-mentioned mitochondrial RNA import receptors could potentially be used to target sgRNA and enhance its import rate into mitochondria. In a proof-of-concept study, combining mito-Cas9 with two sgRNAs carrying an extra hairpin clearly showed depletion of mtDNA. It was interesting that individual sgRNAs with this extra hairpin structure could not reduce mtDNA copy number [78]. Since mitochondrial entry of RNAs is an RNP-dependent process that require interaction of endogenous proteins (such as TOMs and cytosolic proteins), sgRNA interaction with mito-Cas9 might

compete with the RNP transport system and subsequently reduce CRISPR efficiency. However, simultaneous transfection of two sgRNAs having additional hairpin structure could provide a higher chance to have free sgRNAs (sgRNAs that are not bound to mito-Cas9 protein) in the cytosol and increase the chance of RNP-mediated import of sgRNAs. Formation of CRISPR RNPs inside the mitochondria was also reported recently [68]. Therefore, transfection of a higher amount of sgRNAs or lower amount of Cas9 protein may increase the escaping chance of sgRNAs from RNP complex formation with Cas9 and increase the sgRNA availability inside mitochondria. Considering the recent achievements in CRISPR-editing mammalian mitochondria, it is expected that CRISPR-mediated genome editing for therapeutic applications in mammalian mitochondria will continue to grow in feasibility, due partly to RNA-assisted importers for sgRNAs overcoming its current limitations [78, 95]. Apart from the sgRNA mitochondrial trafficking, it is highly likely that Cas9 binds to sgRNA and forms the RNP complex prior to entering mitochondria. Although the presence of Cas9 protein into the mitochondria has been improved using the MTS-mediated mito-Cas9 translocation [78, 98, 99], the low efficiency of gRNA transport into mitochondria indicates that mitochondrial translocation of Cas9 protein is not in the Cas9-gRNA or RNP complex form. It still remains to be elucidated if the folded Cas9-gRNA complex is not the preferred substrate for the mitochondrial translocation system, or if the Cas9-gRNA RNP undergoes unfolding throughout its translocation process which destabilizes Cas9-gRNA binding and prevents co-transport of bound gRNA, or both scenarios are the case. Understanding this translocation process might help to develop pathways to have a proper number of gRNA molecules inside of the mitochondria along with the imported Cas9 molecules. Moreover, although cytosine base editors have only been used for mitochondrial genome editing, Tad-based editors can also be used for adenosine base editing in mitochondrial genomes (Fig. 2). With the development of the dual base editor TadDE (detailed description is provided in section "Combination of cytidine and adenosine base editors"), it is possible to use this dual-purpose Tad protein to simultaneously target cytosine and adenine with a small recombinant protein. TadDE can be combined with either CRISPR/Cas9, ZFD, or TALEN systems.

Apart from mitochondrial RNA-assisted approaches, electroporation of mRNAs has been helpful for mRNA transport into plant mitochondria [118]. One of the reasons for the low efficiency of CRISPR-based mitochondrial gene editing can be attributed to the use of lipofections, such as Lipofectamine 2000/3000 [98] and calcium phosphate methods [110]. By contrast, electroporation of rat and human cells with DdCBE-encoding plasmids efficiently mediated base editing in mitochondrial target genes [89, 90]. Therefore, electroporating sgRNAs with Cas9 protein/plasmid/mRNA can be a promising approach for mitochondrial genome engineering.

CONCLUSION
This review discussed the advantages and challenges of on-target editing of the mitochondrial genome. In summary, the correction of mutations in mtDNA is a plausible approach using base editor technologies. The current status of mtDNA editing approaches has mainly involved combining base editors with ZFD and TALE techniques, while CRISPR/Cas9-fused base editors have been less frequently used. However, the development of fused proteins with mitochondrial targeting sequences as well as engineering the scaffold structure of sgRNA have opened a new window to exploiting the advantages of RNPs. Implementing smaller Cas proteins with base editors as well as optimization of the Cas protein: sgRNA ratio can be important factors to increase their mitochondrial entry and editing efficiency. We envisage that, in the near future, clinical gene correction of mitochondrial DNA will be performed by base editors.

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