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Review article

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Base editing of organellar DNA with programmable deaminases

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Abstract

Mitochondria and chloroplasts are organelles that include their own genomes, which encode key genes for ATP production and carbon dioxide fixation, respectively. Mutations in mitochondrial DNA can cause diverse genetic disorders and are also linked to ageing and age-related diseases, including cancer. Targeted editing of organellar DNA should be useful for studying organellar genes and developing novel therapeutics, but it has been hindered by lack of efficient tools in living cells. Recently, CRISPR-free, protein-only base editors, such as double-stranded DNA deaminase toxin A-derived cytosine base editors (DdCBEs) and adenine base editors (ABEs), have been developed, which enable targeted organellar DNA editing in human cell lines, animals and plants. In this Review, we present programmable deaminases developed for base editing of organellar DNA in vitro and discuss mitochondrial DNA editing in animals, and plastid genome (plastome) editing in plants. We also discuss precision and efficiency limitations of these tools and propose improvements for therapeutic, agricultural and environmental applications.

Sections

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Introduction

Mitochondria and chloroplasts are two organelles in eukaryotic cells that have key roles in the carbon cycle. Chloroplasts are plant-specific organelles responsible for photosynthesis, by which plants convert light energy into chemical energy and fix carbon dioxide (CO_2) to produce carbohydrates. The resulting carbohydrates and their derivatives are consumed by animals and are fully oxidized through the citric acid cycle and oxidative phosphorylation in mitochondria to yield ATP and carbon dioxide, thereby completing a cycle (Fig. 1). According to the endosymbiotic theory[1], these organelles have originated from once free-living microbes, which were engulfed by another organism, giving rise to a eukaryotic cell, billions of years ago. Although most organellar proteins are encoded in the nuclear genome, presumably because their genes were transferred to the nucleus during the course of evolution, both mitochondria and chloroplasts retain their own genome of double-stranded DNA (dsDNA) in circular form. Mammalian mitochondrial DNA (mtDNA) contains 37 genes encoding 13 proteins, 22 tRNAs and 2 ribosomal RNAs (rRNAs)[2] (Box 1); chloroplast DNA of land plants contains approximately 120 genes encoding approximately 100 proteins and regulatory RNAs[3]. A single eukaryotic cell harbours hundreds of mitochondria and chloroplasts (in plants), each of which contains multiple (approximately 10 to 100) copies of the organelle's genome. The different organellar genomes in a single cell can be homogeneous or identical in their sequence (termed homoplasmy) or heterogenous (termed heteroplasmy).

Mitochondrial genetic diseases are caused by mutations in either mtDNA or in nuclear genes encoding mitochondrial proteins. mtDNA is much more likely to be mutated than nuclear DNA, mainly because of the high levels of reactive oxygen species in mitochondria generated during oxidative phosphorylation[4]. Approximately 1 in 5,000 individuals harbours a clinically confirmed pathogenic mtDNA mutation, which can be either homoplasmic or heteroplasmic[4–6]. Most of these mtDNA mutations are either A>G (= T>C on the other DNA strand; 45%) or C>T (= G>A; 42%) conversions (Box 1), which can be corrected by cytosine or adenine base editors, respectively. Mitochondrial genetic diseases are diverse and involve degeneration of many tissues and organs; such diseases include Leber hereditary optic neuropathy (LHON); Leigh syndrome; mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS); and myoclonic epilepsy with ragged red fibres. Currently, there are no cures for these diseases.

Box 1

Human mtDNA and pathogenic mtDNA mutations at a glance

- Size of the human mitochondrial genome: 16,569 bp
- Number of genes: 37, including 13 protein-coding genes, 2 ribosomal RNA genes and 22 tRNA genes
- Number of clinically confirmed pathogenic mitochondrial DNA (mtDNA) mutations: 96
- Mutation types: 43 A>G (=T>C) single-nucleotide variations (SNVs; 44.8%), 40 C>T (=G>A) SNVs (41.7%), 8 other SNVs (8.3%) and 5 indels or inversions (5.2%)
- Prevalence of pathogenic mtDNA mutations: approximately 1 in 5,000

Sources of information: Mitomap and refs. 4,79.

Mitochondrial genetic disorders in humans share some similarities with cytosolic male sterility in plants, a trait by which a plant cannot produce functional pollen. First, they are caused by mtDNA mutations; second, phenotypes of cytosolic male sterility mutations and certain human mtDNA mutations are sex-linked. For example, males with LHON mutations are about five times more likely to develop optic neuropathy than females with the same mutations[7]. Mutations in mtDNA that are fatal in males but are not deleterious (or are rather beneficial) in females can remain in a gene pool owing to the maternal inheritance of mtDNA, whereas mtDNA mutations that are equally harmful in both males and females fail to be inherited[8].

Targeted mutagenesis is a powerful and often essential method for studying the function of a gene of interest and enhancing the activity of its encoded protein. CRISPR nucleases[9,10] repurposed from prokaryotic adaptive immune systems, and CRISPR RNA-guided base editors[11,12] and prime editors[13,14] are now broadly used for targeted genetic modifications in nuclear DNA[15]. However, they have been found unuseful for manipulating organellar DNA, largely because of the

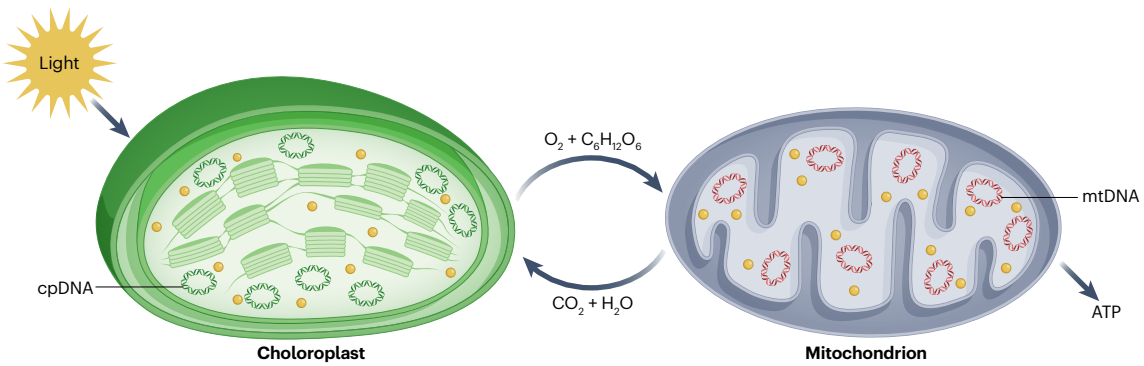


Fig. 1 | The carbon cycle mediated by mitochondria and chloroplasts. In chloroplasts, light energy is transformed into chemical energy through the absorption of carbon dioxide (CO_2) and water (H_2O) and the generation of oxygen (O_2) and sugar ($C_6H_{12}O_6$). In mitochondria, the sugar is consumed and oxidized through the citric acid cycle and oxidative phosphorylation to yield CO_2, H_2O and ATP, the energy currency of the cell. As a result, carbon is cycled between chloroplasts and mitochondria, two crucially important organelles in the ecosystem. cpDNA, chloroplast DNA; mtDNA, mitochondrial DNA.

Table 1 | Summary of organellar base editing

Organelle	Cells or organisms	Target genes	Type of base editing	Vector or delivery method	Nomenclature of base editors	Ref.
Mitochondria	Human cells	*ND1, ND2, ND4, ND5, ATP8*	C>T	Plasmid	DdCBE	24
Mitochondria	Human cells	*ND1, ND2, ND4, ND4L, ND5, CO1, CO2, CYB*	C>T	Plasmid, mRNA, protein	ZFD	30
Mitochondria	Human cells	*ND1, ND3, ND4, ND5, CYB, CO2, CO3, ATP8, MT-RNR2*	A>G	Plasmid	TALED	34
Mitochondria	Human cells	*ND1, ND4, ND6*	C>T	Plasmid, AAV	mt-DdCBE	32
Mitochondria	Human cells	*ND1, ND4, ND5, ND6, ATP8*	C>T	Plasmid	HiFi-DdCBE	46
Mitochondria	Human cells	*ND4*	A>G	Circular RNA	mitoABE$^{Nt.BspD6I(C)}$	35
Mitochondria	Human embryos	*ND1*, tRNA^{Lys}	A>G	mRNA	DdCBE	42
Mitochondria	Mouse	*ND5*	C>T	mRNA	DdCBE	36
Mitochondria	Mouse	*ND5*, tRNA^{Ala}, *MT-RNR2*	C>T	mRNA	DdCBE–NES	27
Mitochondria	Zebrafish	*ND1, ND5*	C>T	mRNA	DdCBE	44
Mitochondria	Rat	tRNA^{Glu}, tRNA^{Lys}	C>T	mRNA	DdCBE	40
Mitochondria	Mouse	*ND1, ND5*, tRNA^{Lys}	C>T	mRNA	DdCBE	38
Mitochondria	Zebrafish	*CO1, CO3*, tRNA^{Leu1}	C>T	mRNA	DdCBE	45
Mitochondria	Mouse	*ND3*	C>T	AAV	DdCBE	43
Mitochondria	Mouse	*ND5*	C>T	mRNA	DdCBE	49
Mitochondria	Mouse	*ND1*, tRNA^{Lys}	C>T	AAV	ZF-DdCBE	31
Mitochondria	Mouse	*ATP6*	C>T	mRNA	DdCBE	39
Mitochondria	Rat	*ATP8, ND1*	C>T	mRNA	DdCBE	41
Mitochondria	Mouse	*ND1*, tRNA^{Val}, tRNA^{Leu2}	C>T	mRNA	mitoCBE2.1	29
Mitochondria	Rapeseed, lettuce	*ATP6, rps14*	C>T	mRNA	mt-DdCBE	61
Mitochondria	*Arabidopsis thaliana*	*ATP1*	C>T	Agrobacterium	mitoTALECD	63
Chloroplasts	Rapeseed, lettuce	*rrn16, psbA, atpB*	C>T	mRNA, plasmid	cp-DdCBE	61
Chloroplasts	*A. thaliana*	*rrn16, rpoC1, psbA*	C>T	Agrobacterium	ptpTALECD	62
Chloroplasts	Rice	*psaA*	C>T	Agrobacterium	cp-DdCBE	64
Chloroplasts	Lettuce, *A. thaliana*	*rrn16, psbA, psaA, rbcL*	A>G	mRNA, agrobacterium	TALED	65
Chloroplasts	*A. thaliana*	*psbA, rbcL, rrn16, ndhB*	C>T	Agrobacterium	ptpTALECE	78

AAV, adeno-associated virus; cp-DdCBE, DdCBE with a chloroplast transit peptide; DdCBE, DNA deaminase toxin A-derived cytosine base editor; DdCBE–NES, DdCBE with a nuclear export signal; HiFi-DdCBE, high-fidelity DdCBE; mitoABE$^{Nt.BspD6I(C)}$, mtDNA adenine base editors with Nt.BspD6I(C); mitoCBE2.1, mitochondrial CBE2.1; mitoTALECD, mitochondrial TALE-CD-UGI; mt-DdCBE, DdCBE with a mitochondrial targeting sequence; ptpTALECD, TALECD with a plastid-targeting signal peptide; rRNA, ribosomal RNA; TALE, transcription activator-like effector; TALED, TALE-linked deaminase; ZFD, zinc-finger deaminase; ZF-DdCBE, zinc-finger DdCBE.

difficulty of delivering their guide RNA (gRNA) into organelles[16,17]. Mitochondria-targeted zinc-finger nucleases (ZFNs)[18,19] and transcription activator-like effector (TALE) nucleases (TALENs)[20] can selectively cleave and degrade mutant mtDNA molecules in a heteroplasmic state but cannot create mtDNA mutations or correct a pathogenic mtDNA mutation in a homoplasmic state. Instead, recently developed CRISPR-free, protein-only base editors enable targeted single-nucleotide conversions in mtDNA and chloroplast DNA without DNA cleavage (Table 1).

In this Review, we first introduce various tools and methods developed for mitochondrial base editing in mammalian cells and describe their applications in model animals. We then discuss challenges of therapeutic mtDNA editing, with a focus on off-target editing and bystander editing. Lastly, we describe organellar DNA editing in plants and discuss agricultural and environmental applications. We focus on programmable deaminases for organellar DNA editing rather than on engineered nucleases that cleave DNA, such as ZFNs and TALENs, which have been extensively covered in recent reviews[5,6].

Tools and methods for mitochondrial base editing

So far, mitochondrial base editing has been demonstrated in mammalian cells using double-stranded DNA deaminase toxin A (DddA)-derived cytosine base editors (DdCBEs), TALE-linked deaminases (TALEDs), zinc-finger deaminases (ZFDs) and mtDNA adenine and cytosine base editors (mitoABEs and mitoCBEs, respectively). In this section, we discuss the development and improvement of these mitochondrial base editors.

DddA-derived cytosine base editors

DdCBEs, TALEDs and ZFDs are all based on the dsDNA-specific deaminase DddA. DdCBEs and TALEDs both contain TALE arrays, whereas ZFDs contain zinc-finger proteins (ZFPs).

The development of DdCBEs. Eukaryotic cytidine and adenosine deaminases used in nuclear base editors strongly prefer single-stranded DNA (ssDNA)[21,22], which can be formed in the R-loop region at the target site by the CRISPR–Cas ribonucleoprotein complex[23], but not by TALE arrays or ZFPs. Thus, a deaminase that can catalyse nucleobase deamination in dsDNA is required for efficient base editing in organellar DNA. DddA is an interbacterial toxin identified in *Burkholderia cenocepacia*; it is a cytidine deaminase that can operate on dsDNA[24]. As intact dsDNA-specific cytidine deaminases can cause numerous C>T mutations or even DNA double-strand breaks (DSBs) throughout the genome, $DddA_{tox}$, the deaminase domain in DddA, can be highly toxic and trigger cell death. To reduce its toxicity, $DddA_{tox}$ is split into two inactive halves, each of which is fused to a mitochondrial targeting sequence (MTS), a TALE array and a uracil DNA glycosylase inhibitor (UGI), together creating a DdCBE pair (Fig. 2a). When the two TALE–DddA–UGI fusion proteins — one including the $DddA_{tox}$ N-terminal and the other including the C-terminal — bind to the target-flanking regions, the functional DddA enzyme is assembled at the target site (Fig. 2a) and triggers C>U deamination on both DNA strands; the fused UGIs inhibit the activity of mitochondrial uracil DNA glycosylases to maintain the resulting uracils, which are converted to thymine following mtDNA replication. With this splitting strategy, DdCBEs were successfully used to induce C>T base editing in both mitochondrial and nuclear genomes. Unlike CRISPR RNA-guided CBEs that induce C>T conversions predominantly on the non-target strand (not complementary to the gRNA), DdCBEs induce C>T (= A>G) editing on both DNA strands.

Expanding and enhancing DdCBEs. Like most cytidine deaminases in the apolipoprotein B mRNA editing catalytic polypeptide-like (APOBEC) family[22,25], DddA strongly prefers to deaminate cytosines in a 5′-TC context. To enhance the editing efficiency of DdCBEs, phage-assisted evolution was used to obtain the DddA6 variant with improved editing efficiency (3.3-fold on average)[26]. Alternatively, the fusion of a nuclear export signal (NES) to DdCBE led to the development of DdCBE–NES (Fig. 2b), which also had improved editing efficiency[27]. By cleaving, and thereby degrading, unedited mtDNA with a TALEN, editing efficiencies of DdCBE and DdCBE–NES could be further improved. Furthermore, DddA was evolved against non-TC contexts to expand the editing scope of DdCBEs, resulting in the DddA11 variant that could edit cytosines in the 5′-HC contexts (that is, AC, CC and TC)[26] (Fig. 2b). Later on, by searching for DddA homologues in protein databases, two groups separately identified *Simiaoa sunii* DddA (*Ss*DddA)[28] and *Roseburia intestinalis* DddA (*Ri*DddA)[29], both of which can deaminate cytosines in the GC context. By fusing TALEs with the inactive halves of DddA11, *Ss*DddA or *Ri*DddA, the corresponding DdCBE11, DdCBE_Ss and mitoCBE2.0 (with *Ri*DddA) tools expanded the base editing range in the mitochondrial genome (Fig. 2b).

Compact cytosine base editors. Two compact CBEs have been developed, which can be delivered using a single adeno-associated virus (AAV) vector with a limited cargo space of <4.7 kb. Two groups independently replaced TALE arrays with ZFPs, which are much smaller than TALE arrays, in DdCBEs to develop ZFDs[30] (termed ZF-DdCBEs in ref. 31) (Fig. 2c). As ZFPs can spontaneously penetrate into mammalian cells, purified ZFD proteins were also directly delivered into mammalian cells[30]. Unfortunately, the ZFDs were not sufficiently specific compared with TALE-containing DdCBEs, inducing hundreds of off-target mutations in the mitochondrial genome. In addition, a ZFD monomer and a DdCBE monomer can be combined to induce targeted base editing[30] (Fig. 2c). Interestingly, some hybrid ZFD–TALE-DdCBE pairs achieved single-base editing without bystander editing.

Alternatively, through screening of $DddA_{tox}$ variants containing random mutations, a variant with four amino acid substitutions, termed GSVG, was characterized as a non-toxic, full-length DddA deaminase, which could be expressed in mammalian cells without splitting[32]. Fusion of the full-length GSVG variant with a single TALE array and UGI produced monomeric DdCBEs (mDdCBEs) that can be packaged in a single AAV (Fig. 2c). AAV-mediated long-term expression of mDdCBEs achieved nearly homoplasmic (>99%) C>T editing in human cells[32].

Adenine base editing in the mitochondrial genome

A>G (= T>C) editing in the nuclear genome is catalysed by adenine base editors (ABEs), which are composed of a Cas9 nickase and an engineered bacterial tRNA-specific adenosine deaminase (TadA)[21]. Following eight rounds of directed evolution, the *Escherichia coli* TadA, which catalyses the deamination of adenosine to generate inosine (I) in tRNA, was evolved to catalyse A>I deamination in DNA with high efficiency[33]. Nevertheless, the evolved TadA variants (for example, TadA8e) still operate on ssDNA and, therefore, cannot be fused to TALE arrays or ZFPs for efficient mtDNA editing. Interestingly, however, the UGI in one subunit of a DdCBE pair was replaced with TadA8e to develop a split TALED (sTALED) pair (with the other subunit containing UGI), which generated concurrent A>G and C>T editing in mtDNA in cultured human cells[34] (Fig. 2d). Next, the UGI in the other subunit was removed to construct a UGI-free sTALED pair, which induced only A>G editing (Fig. 2d).

Glossary

Bystander editing
Refers to the formation of unintended mutations in the editing window of a base editor.

Chloroplast transit peptide
(CTP). An N-terminal portion in a nuclear-encoded protein that directs the transport of the protein into chloroplasts. The CTP is cleaved off during the transport by a peptidase.

Interbacterial toxin
A toxin secreted from a bacterium that inhibits growth of competing bacteria.

Mitochondrial targeting sequence
(MTS). A peptide of 15–70 amino acids at the N terminus of a nuclear-encoded protein, which facilitates transport of the protein into mitochondria. The MTS is cleaved off during the transport by a peptidase.

Nickase
An enzyme that cleaves one strand of double-stranded DNA to produce a single-strand break (nick).

Phage-assisted evolution
A directed evolution methodology for protein engineering that exploits the phage life cycle in bacteria.

R-loop
A DNA–RNA hybrid structure consisting of a DNA strand hybridized with an RNA and a displaced DNA strand.

TALE arrays
Transcription activator-like effector (TALE) arrays bind to a target DNA sequence. The arrays are composed of highly homologous repeats of 34 amino acids, derived from the bacterial genus *Xanthomonas*.

Transfer DNA
(T-DNA). A DNA segment in a tumour-inducing plasmid, derived from agrobacterium, that is transferred to the genome of a host plant.

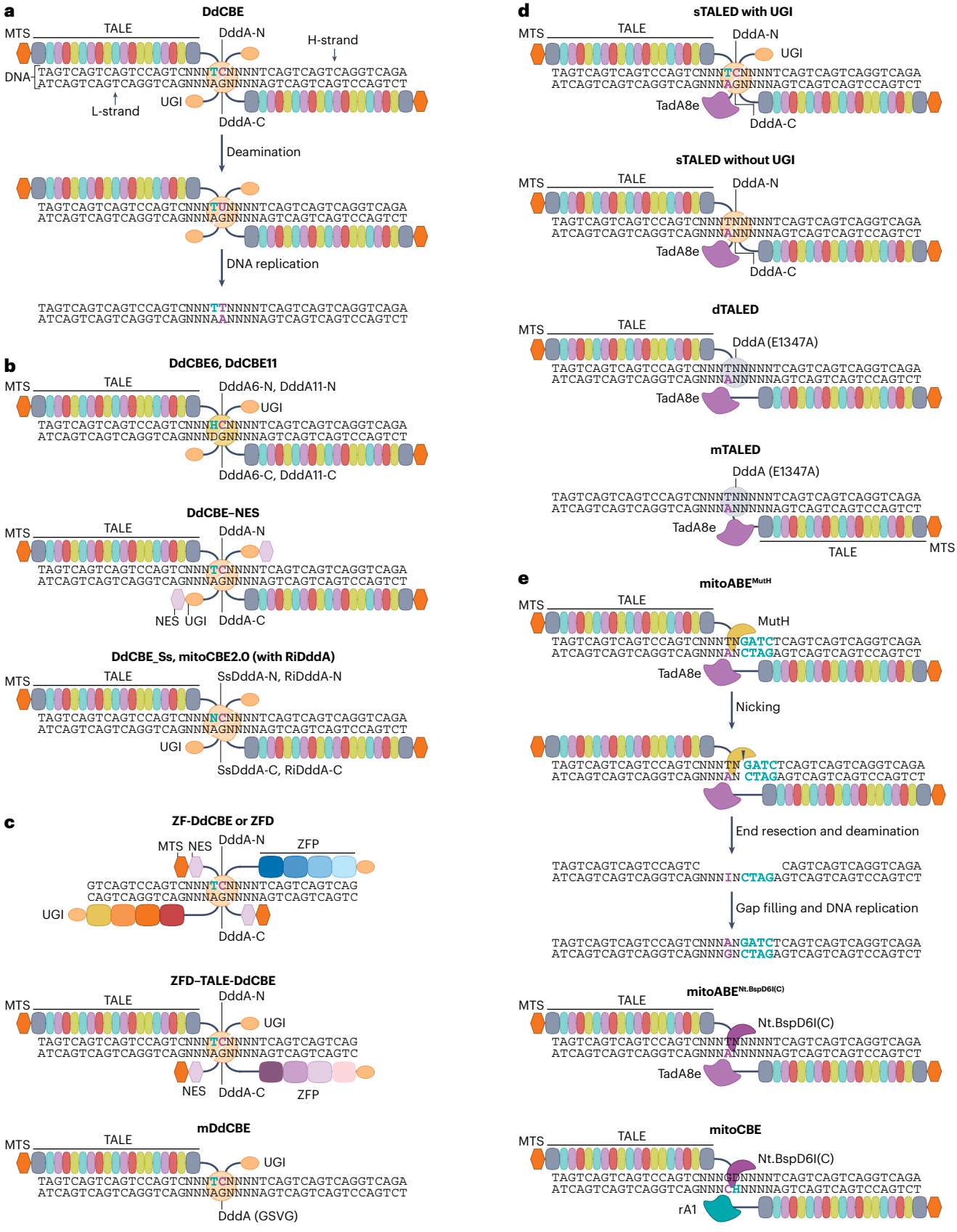

a
DdCBE
MTS
TALE
DddA-N
H-strand
DNA
TAGTCAGTCAGTCCAGTCNNNTCNNNNNTCAGTCAGTCAGGTCAGA
ATCAGTCAGTCAGGTCAGNNNAGNNNNNAGTCAGTCAGTCCAGTCT
L-strand
UGI
DddA-C
Deamination
TAGTCAGTCAGTCCAGTCNNNTTNNNNNTCAGTCAGTCAGGTCAGA
ATCAGTCAGTCAGGTCAGNNNAANNNNNAGTCAGTCAGTCCAGTCT
DNA replication
TAGTCAGTCAGTCCAGTCNNNTTNNNNNTCAGTCAGTCAGGTCAGA
ATCAGTCAGTCAGGTCAGNNNAANNNNNAGTCAGTCAGTCCAGTCT
b
DdCBE6, DdCBE11
DddA6-N, DddA11-N
UGI
TAGTCAGTCAGTCCAGTCNNNHCNNNNNTCAGTCAGTCAGGTCAGA
ATCAGTCAGTCAGGTCAGNNNDGNNNNNAGTCAGTCAGTCCAGTCT
DddA6-C, DddA11-C
DdCBE–NES
NES
DdCBE_Ss, mitoCBE2.0 (with RiDddA)
SsDddA-N, RiDddA-N
SsDddA-C, RiDddA-C
c
ZF-DdCBE or ZFD
ZFP
GTCAGTCCAGTCNNNTCNNNNNTCAGTCAGTCAG
CAGTCAGGTCAGNNNAGNNNNNAGTCAGTCAGTC
ZFD–TALE-DdCBE
TAGTCAGTCAGTCCAGTCNNNTCNNNNNTCAGTCAGTCAG
ATCAGTCAGTCAGGTCAGNNNAGNNNNNAGTCAGTCAGTC
mDdCBE
DddA (GSVG)
d
sTALED with UGI
TadA8e
sTALED without UGI
dTALED
DddA (E1347A)
mTALED
e
mitoABE^MutH
MutH
Nicking
End resection and deamination
Gap filling and DNA replication
mitoABE^Nt.BspD6I(C)
Nt.BspD6I(C)
mitoCBE
rA1

Fig. 2 | The development of mitochondrial DNA base editors. a, The double-stranded DNA deaminase toxin A (DddA)-derived cytosine base editor (DdCBE). Each of the DdCBE monomers contains a mitochondrial targeting sequence (MTS), a transcription activator-like effector (TALE) array, one-half of $DddA_{tox}$ (the DddA deaminase domain, here named 'DddA' for simplicity) — the N-terminal ($DddA_{tox}$-N) or the C-terminal ($DddA_{tox}$-C) — and a uracil DNA glycosylase inhibitor (UGI). Following cytidine deamination by the recombined $DddA_{tox}$ in the mitochondrial DNA heavy (H)-strand and/or light (L)-strand (deamination in the H-strand is shown in the diagram as an example), the replication of mitochondrial DNA results in both C>T and G>A editing. The target nucleotides (C or A) and context-defining nucleotides (for example, T or H(= A/C/T)) are shown in purple and in cyan, respectively. **b**, DdCBEs with enhanced editing efficiency or expanded editing scope. DdCBE6 and DdCBE11 contain DddA6 and DddA11, respectively, which are evolved DddA versions with enhanced deamination activity in a TC context and deamination activity expanded to the HC context, respectively. DdCBE–NES contains a nuclear export signal (NES) at the C terminus of each monomer and provides improved mitochondrial DNA editing efficiency. DdCBE_Ss and mitoCBE2.0 contain *Simiaoa sunii* DddA (*Ss*DddA) and *Roseburia intestinalis* DddA (*Ri*DddA), respectively, which can deaminate cytosines in an N(= A/C/G/T)C context. **c**, Compact cytosine base editors. Zinc-finger DdCBEs (ZF-DdCBEs, also known as zinc-finger deaminases (ZFDs)) contain zinc-finger proteins (ZFPs) instead of TALE arrays for DNA targeting. ZFD–TALE-DdCBEs are hybrids of a ZFD monomer and a TALE-DdCBE monomer. Monomeric DdCBE (mDdCBE) contains an intact $DddA_{tox}$ variant (with the GSVG mutations). **d**, Adenine base editors. Slit TALE-linked deaminase (sTALED) with UGI comprises a monomer that includes $DddA_{tox}$-N and a UGI and a monomer that includes $DddA_{tox}$-C and a single-strand-specific adenosine deaminase, TadA8e; therefore, it can concurrently generate A>G and C>T edits. A sTALED version lacking a UGI can generate only A>G edits. Dimeric TALED (dTALED) comprises a monomer with inactive $DddA_{tox}$ (with the E1347A mutation) and a monomer with TadA8e. Monomeric TALED (mTALED) includes both TadA8e and $DddA_{tox}$ (E1347A) and, thus, functions as a monomer. **e**, Strand-selective base editors. Mitochondrial DNA adenosine base editor (mitoABE)^{MutH} comprises a TALE monomer with the nickase MutH and a monomer with TadA8e. MutH generates a nick in the DNA, which enables resection of the nicked strand and deamination of the adenosines in the resulting single-stranded DNA region by TadA8e. During gap filling, the resected DNA is resynthesized using the edited sequence as template, leading to conversion of the target nucleotide. In contrast to mitoABE^{MutH}, one monomer of mitoABE$^{Nt.BspD6I(C)}$ contains the nickase Nt.BspD6I(C), which has no observable sequence constraints. The mitochondrial DNA cytosine base editor (mitoCBE) comprises a monomer with Nt.BspD6I(C) and a monomer with the single-strand-specific cytidine deaminase, rat APOBEC1 (rA1). D, guanine, adenine or thymine; RiDddA-N, N-terminal *Ri*DddA; RiDddA-C, C-terminal *Ri*DddA; SsDddA-N, N-terminal *Ss*DddA; SsDddA-C, C-terminal *Ss*DddA.

Furthermore, to increase editing specificity (on the expense of efficiency), a catalytically deficient, full-length $DddA_{tox}$ ($DddA_{tox}$ E1347A) was fused to one TALE array, whereas TadA8e was fused to another TALE, thereby forming a dimeric TALED (dTALED) (Fig. 2d). Finally, to reduce the size of the TALE-linked ABEs, a single TALE array was fused to both TadA8e and $DddA_{tox}$ E1347A, generating a monomeric TALED (mTALED) (Fig. 2d). As the catalytically deficient $DddA_{tox}$ is used in dTALEDs and mTALEDs, these two editors, in general, are less efficient and more specific than sTALEDs.

Strand-selective base editing with DNA nicking

Recently, strand-selective base editors were developed for mtDNA editing[35]. By fusing a wild-type or engineered *E. coli* MutH nickase to one TALE array and fusing a TadA variant (TadA8e-V106W) to another TALE array, mitoABE^{MutH} was developed. MitoABE^{MutH} deaminates adenosines in both DNA strands at the target site and induces a nick in one of the DNA strands, the identity of which depends on the distance between the binding site of the TALE–MutH fusion protein and the nearest available MutH recognition sequence (5′-GATC-3′) (Fig. 2e). The nicked strand is resected, forming a ssDNA region and, thus, enabling adenosine deamination by the ssDNA-targeting TadA8e. During gap filling, the resected DNA is resynthesized according to the edited sequence of the unnicked strand, leading to conversion of the target site. Thus, A>G editing is induced in only one DNA strand by MitoABE^{MutH}, which confers higher editing precision than the DddA-derived mitochondrial base editors. Although mitoABE^{MutH} achieved high strand specificity in mtDNA base editing, its editing scope is limited by the requirement of the MutH recognition sequence (5′-GATC-3′) at the target site. To develop more versatile mitoABEs with no sequence constraints, screens for nickases with no or reduced sequence preferences were performed. Using such a nickase, Nt.BspD6I(C), mitoABE$^{Nt.BspD6I(C)}$ was developed to induce editing with no observable sequence constraints and to correct the LHON-causing *ND4* mutation in a patient-derived cell line (Fig. 2e). However, strand preference was compromised, possibly because the nickase is less stringent and recognizes sequences in both DNA strands. Using the rat cytidine deaminase APOBEC1 (ref. 35), strand-specific mitoCBEs were similarly developed (Fig. 2e).

Mitochondrial DNA base editing in animals

DddA-derived mitochondrial base editors have been used to induce mtDNA editing in the embryos of mammalian species and zebrafish and also in newborn and adult mice.

mtDNA editing in mammalian embryos

DdCBEs have been successfully used to install mtDNA mutations in the embryos of mice and rats (Fig. 3a). A point mutation (G12918A), which is associated with Leigh disease, MELAS syndrome and LHON–MELAS overlap syndrome, and a premature stop codon, both in the NADH–ubiquinone oxidoreductase chain 5 gene (*ND5*), were introduced separately in mouse embryos using in vitro transcripts encoding DdCBEs[36]. The resulting F_0 mice exhibited a wide range of editing efficiencies — 0.25–23% for G12918A and 0.22–57% for the premature stop codon — suitable for mitochondrial disease models. In another study, DdCBE–NES alone or DdCBE–NES with mitoTALEN was used to edit *Nd5* in mouse embryos and to obtain F_0 mice with the G12918A mutation; the mice displayed a hunchback appearance and damaged mitochondrial structures in kidney and brown adipose tissues[27]. One F_1 mouse showed enlarged lateral ventricles and asymmetrical hippocampal atrophy, indicative of cognitive decline, as observed in the human neurodegenerative disease associated with the G12918A (G13513A in human mtDNA) mutation[37].

Likewise, DdCBE mRNAs were injected into one-cell and two-cell mouse embryos to introduce disease-associated mtDNA mutations in *ND1*, *ND5* and the $tRNA^{Lys}$ gene in F_0 mice[38]. After backcrossing the edited F_0 female mice with wild-type male mice, the mutations were detected in F_1 mice, showing that the mitochondrial mutations caused by DdCBEs can be transmitted to offspring (Fig. 3a). The recently developed mitoCBE2.1 was also used to install a C>T mutation in a 5′-GC context in one-cell mouse embryos with a frequency of 3.6–8.3%[29].

Rather than targeting one gene at a time, a library of DdCBEs called MitoKO was constructed, which was designed to generate a premature

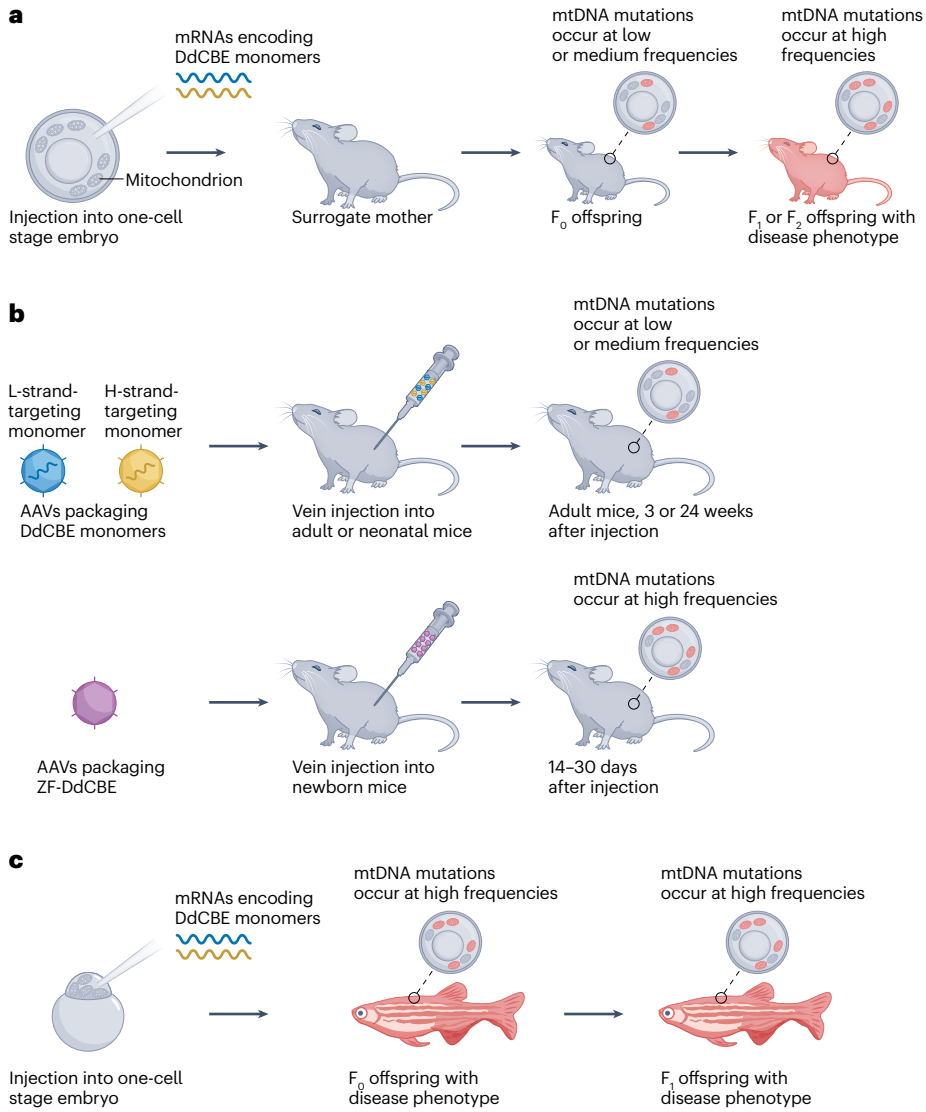
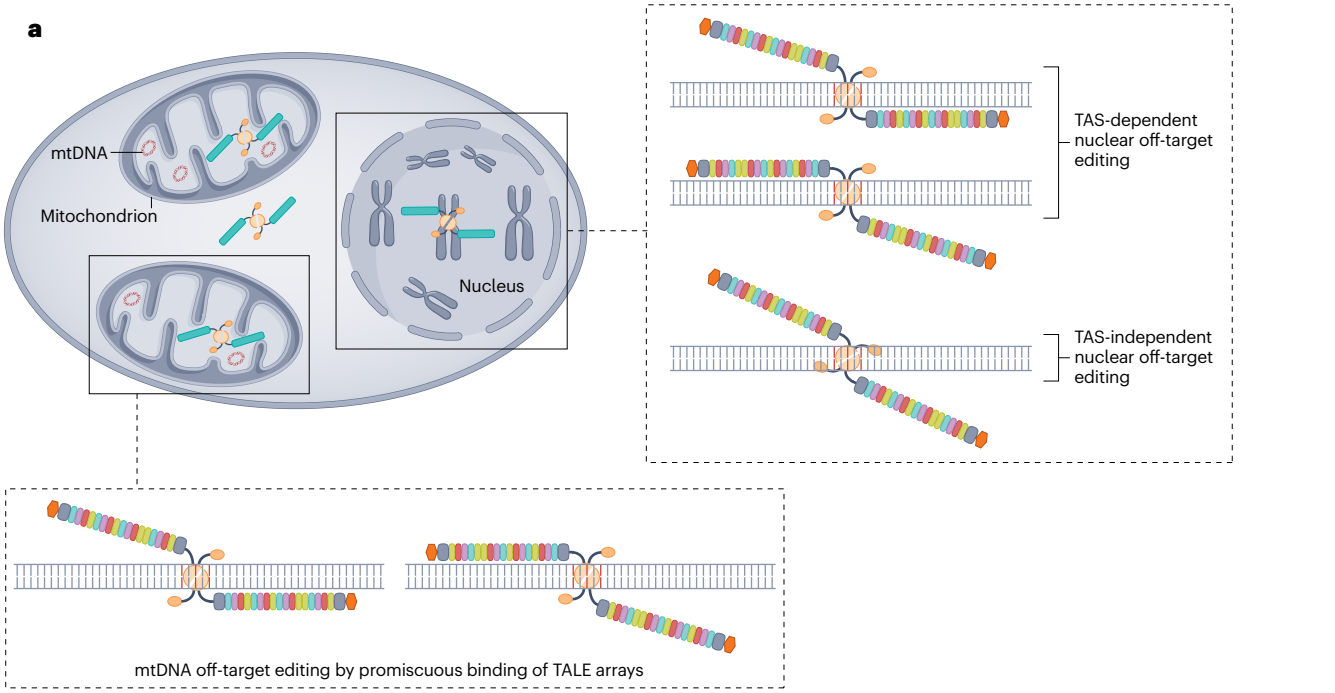


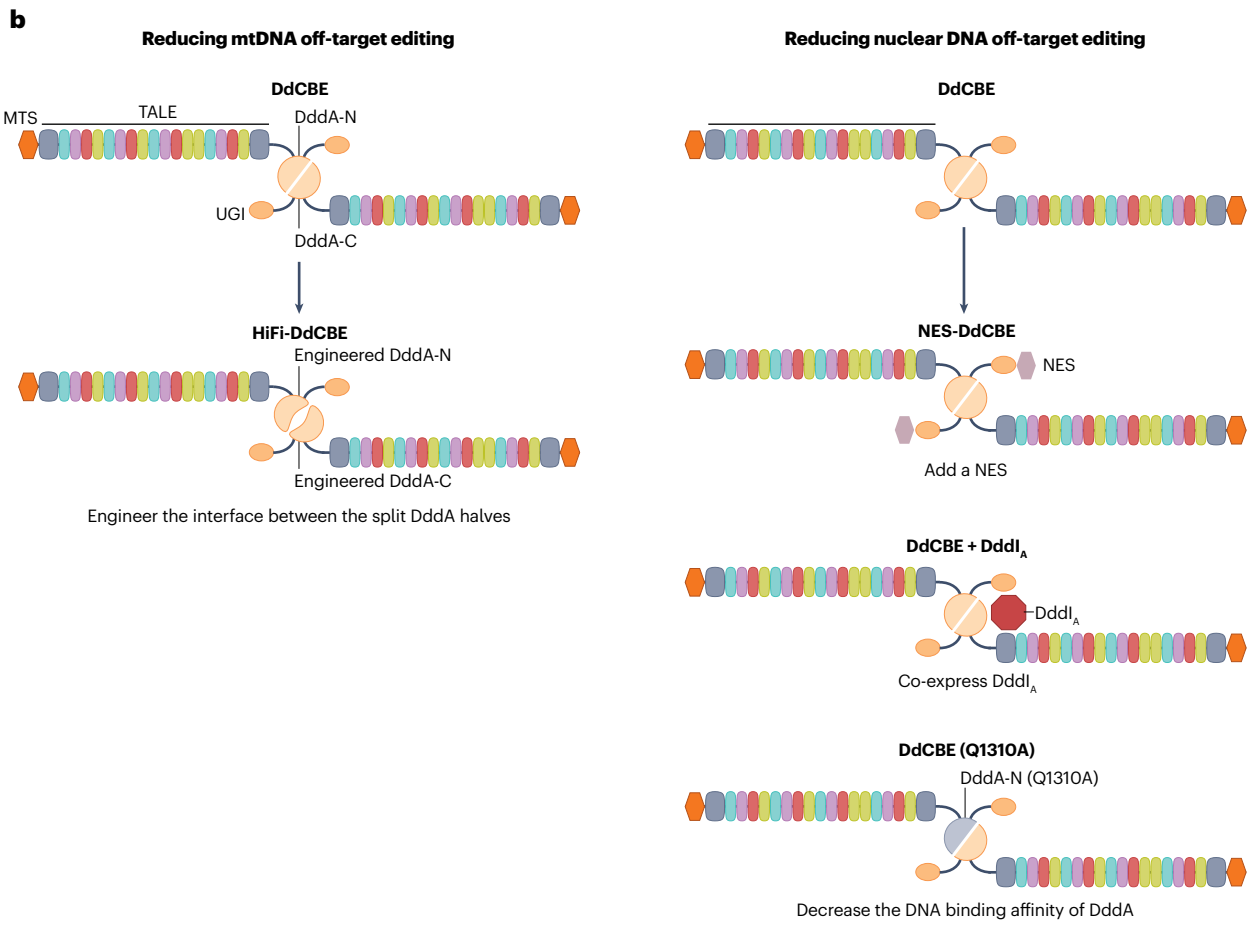
Fig. 3 | Mitochondrial base editing in animals. **a**, Mitochondrial DNA (mtDNA) editing in mouse and rat embryos. RNAs encoding monomers of a DNA deaminase toxin A-derived cytosine base editor (DdCBE) can be injected into one-cell mouse or rat embryos, after which the injected embryos are transplanted into surrogate mothers. The obtained F_0 offspring generally contain mtDNA mutations at low or medium frequencies; following backcrossing, F_1 or F_2 mice can have mtDNA mutations at high frequencies and display disease phenotypes[27,36]. **b**, Adeno-associated virus (AAV)-mediated mtDNA editing in mice. Separate AAV vectors for the different DdCBE monomer sequences (light (L)-strand-targeting monomer and heavy (H)-strand-targeting monomer) were delivered into adult and newborn mice through vein injection. In the adult mice, varying levels of mtDNA mutations were detected 3 weeks (low levels) or 24 weeks (medium levels) after injection whereas, in the newborn mice, the levels of mtDNA mutations were higher[43]. AAV vectors packaging zinc-finger DdCBE (ZF-DdCBE) sequences were delivered into newborn mice through vein injection, and high levels of mtDNA mutations were detected 14–30 days after injection[43]. **c**, mtDNA editing in zebrafish. mRNAs encoding DdCBE monomers were injected into one-cell zygotes. mtDNA mutations were detected in F_0 fish at high frequencies and were passed on to F_1 offspring. Both F_0 and F_1 fish displayed disease phenotypes[44,45].

stop codon in every protein-coding gene in the mouse mitochondrial genome[39]. The ATP synthase 6 (*ATP6*)-targeting MitoKO DdCBE was injected into one-cell mouse embryos and produced F_0 mice with moderate editing efficiencies (3–10%). Following breeding of the F_1 and F_2 female mice with the highest heteroplasmy levels, F3 offsprings with mutation frequencies of over 50% were obtained.

DdCBEs have also been tested in other laboratory animals. DdCBE-encoding sequences were cloned into the PiggyBac vector and then the corresponding mRNAs were injected into rat zygotes[40]. The mutations – in the $tRNA^{Lys}$ and $tRNA^{Glu}$ genes – were detected in F_0 rats and were transmitted to F_1 offspring, which displayed phenotypes typically found in humans with corresponding mitochondrial diseases.

Fig. 4 | Off-target mutations induced by mitochondrial base editors and strategies to reduce their frequency. **a**, Off-target mutations are induced by DNA deaminase toxin A (DddA)-derived cytosine base editor (DdCBEs) in both nuclear DNA and mitochondrial DNA (mtDNA). In mtDNA, DdCBEs induce off-target editing through nonspecific binding of transcription activator-like effector (TALE) arrays. In nuclear DNA, DdCBEs induce TALE array sequence (TAS)-dependent off-target mutations, which can be caused by nonspecific binding of TALE arrays, and TAS-independent off-target mutations, which is associated with other DNA-binding proteins. **b**, Strategies to reduce off-target editing by DdCBEs. In mtDNA, off-target editing can be minimized by using high-fidelity DdCBEs (HiFi-DdCBEs), in which the interface between the split DddA halves is engineered to suppress spontaneous interactions between them. In nuclear DNA, off-target editing can be reduced by including a nuclear export signal (NES) in the DdCBE, co-expressing the DddA inhibitor $DddI_A$ or introducing mutations that decrease the DNA binding affinity of DddA. DddA-C, C-terminal DddA; DddA-N, N-terminal DddA; MTS, mitochondrial targeting sequence; UGI, uracil DNA glycosylase inhibitor.


a
mtDNA
Mitochondrion
Nucleus
TAS-dependent nuclear off-target editing
TAS-independent nuclear off-target editing
mtDNA off-target editing by promiscuous binding of TALE arrays


b
Reducing mtDNA off-target editing
DdCBE
MTS
TALE
DddA-N
UGI
DddA-C
HiFi-DdCBE
Engineered DddA-N
Engineered DddA-C
Engineer the interface between the split DddA halves
Reducing nuclear DNA off-target editing
DdCBE
NES-DdCBE
NES
Add a NES
DdCBE + DddI$_A$
DddI$_A$
Co-express DddI$_A$
DdCBE (Q1310A)
DddA-N (Q1310A)
Decrease the DNA binding affinity of DddA

Moreover, using DdCBEs and the Cre–*loxP* system, conditional knockout rat strains of six protein-encoding mtDNA genes were generated[41]. Specific knockout of *ATP8* in the heart and neurons resulted in heart failure and postnatal death, respectively.

Editing of mtDNA has also been demonstrated in human embryos[42]. DdCBEs injected into tripronuclear zygotes introduced pathogenic C>T mutations in *ND1* and *MT-TK* (encoding $tRNA^{Lys}$) with frequencies that ranged from 3% to 59%.

AAV-mediated mtDNA editing in mice

Base editors can be packaged into AAVs for in vivo editing (Fig. 3b). The sequences encoding a dimeric DdCBE targeting *ND3* were packaged into separate AAV vectors and delivered into the hearts of adult and newborn mice through vein injection[43]. The editing efficiency was low (1–2%) in the heart tissue of adult mice 3 weeks after injection, but 24 weeks after injection, it increased to 10–20%. Interestingly, the editing efficiency by the same DdCBE through AAV delivery reached 20–30% in the heart of newborn mice, suggesting that younger animals are more amenable to AAV-mediated mtDNA editing. Taking advantage of the relatively small size of ZF-DdCBEs compared with that of TALE-linked CBEs, a dimeric ZF-DdCBE sequence was packaged into a single AAV vector and delivered into newborn mice to induce mtDNA editing in vivo with high efficiency (up to 60%)[31].

mtDNA editing in zebrafish

In zebrafish, DdCBE mRNAs were injected into one-cell zygotes to obtain F_0 fish, in which two mitochondrial genes were successfully edited with editing efficiencies of up to 88.32%[44]. These mutations were passed on to F_1 offspring, which showed defective motility. In another study, DdCBEs (called FusXTBEs) introduced mtDNA mutations with high editing efficiencies of up to 90% in zebrafish embryos[45]. The resulting F_0 fish showed impaired mitochondrial functions, demonstrating the potential of using zebrafish as a model for human mitochondrial diseases (Fig. 3c).

Towards therapeutic mitochondrial gene editing

To date, almost 100 pathogenic mutations in human mtDNA have been reported, most (≈85%) of which are transitions (that is, purine-to-purine or pyrimidine-to-pyrimidine conversions) (Box 1). Thus, the majority of pathogenic mtDNA mutations can theoretically be repaired by cytosine and adenine base editors. However, the formation of off-target mutations by the base editors is a major safety concern for therapeutic mtDNA editing. Mitochondrial genome-wide deep sequencing showed that some DdCBEs can cause considerably higher mutation rates than those observed in controls, which could be attributed to the nonspecific binding of TALE arrays[24] or to TALE-independent DddA deaminase activity (Fig. 4a). To reduce mtDNA off-target editing by DdCBEs, high-fidelity DdCBEs (HiFi-DdCBEs) were developed by substituting alanine for key residues at the interface between the split $DddA_{tox}$ halves, which could prevent the spontaneous assembly of an intact $DddA_{tox}$ at nonspecific sites[46] (Fig. 4b). Because the binding of TALE arrays could properly place the two $DddA_{tox}$ halves at the target site, the alanine substitutions that reduced the interactions between the halves could largely maintain the on-target editing efficiency. The HiFi versions of the evolved DdCBE6 and DdCBE11 were also developed by introducing the same amino acid substitutions to minimize off-target mutations.

Although DdCBEs are designed to induce mtDNA editing, unexpectedly, DdCBEs also caused off-target mutations in the nuclear genome. Detect-seq (dU-detection enabled by C>T transition during sequencing), a method previously designed to evaluate off-target mutations induced by CRISPR-derived base editors[47], was used to determine the occurrence of nuclear off-target mutations caused by DdCBEs. Two types of nuclear genome off-target mutations were identified: TALE array sequence (TAS)-dependent and TAS-independent off-target mutations[48] (Fig. 4a). Use of another off-target-detection method, GOTI (genome-wide off-target analysis by two-cell embryo injection), again revealed that DdCBEs caused substantial levels of off-target mutations in the nuclear genome[49]. Fortunately, nuclear off-target mutations could be largely avoided by adding a NES to DdCBEs, co-expressing the natural DddA inhibitor $DddI_A$ or introducing mutations to reduce the DNA binding affinity of DddA[27,48] (Fig. 4b).

In addition to off-target editing, bystander editing could also hinder therapeutic applications of mitochondrial base editors. If a specific pathogenic mutation is to be eliminated for therapy, bystander editing could cause a new pathogenic mutation in the same gene. The frequency of bystander editing can be reduced by narrowing the editing window, an effect that has been well established for CRISPR-derived base editors[50–55]. In the future, new base editors with high editing precision and narrowed editing windows should be developed for the therapy of diseases caused by mtDNA mutations.

Organellar DNA editing in plants

In addition to mitochondria, plant cells have chloroplasts, which contain their own genomic DNA. The number of genes encoded in chloroplast DNA can vary depending on the species. The plastid genomes (plastomes) of land plants, for example, contain approximately 100 protein-coding genes, as well as rRNA and tRNA genes. The proteins encoded in the plastome, some of which are targets of herbicides[56], function in photosynthesis and carbon fixation.

Compared with mtDNA in animal cells, mitochondrial genomes in plant cells are relatively large and complex[57]. The size of plant mtDNA generally ranges from less than 100 kb to more than 10 Mb, at least four and up to several thousand times larger than that of animal mtDNA (≈16.5 kb). Whereas animal mitochondrial genomes are single circular DNA molecules, their plant counterparts exist as a mixture of linear, circular and branched molecules. Despite these size and structural differences, plant mitochondrial genomes do not contain many additional protein-coding genes compared with their animal counterparts.

Homologous recombination of a donor DNA, which typically includes a selection marker gene, into organellar DNA has been used for targeted genetic modifications in several model plant species[58]. To obtain homoplasmic modifications, multiple rounds of regeneration and selection are required, which is time-consuming and cumbersome. Furthermore, this method leaves a marker gene or a scar (following removal of the marker gene with a recombinase) in the organellar genome. TALENs with a chloroplast transit peptide (CTP) or a MTS can be delivered to plant organelles to boost the efficiency of homologous recombination. However, TALEN-mediated organellar genome modifications are hindered by the lack of efficient DSB repair mechanisms in organelles and also by ectopic homologous recombination causing unexpected genomic rearrangements[59,60]. In this section, we describe base editing with programmable deaminases in plant organelles, which does not require donor DNA and does not trigger error-prone DSB repair.

Targeted C>T editing in plant organelles

CRISPR RNA-free base editors enable site-specific single-nucleotide conversions in plant organelles (Fig. 5). Plant codon-optimized DdCBEs with a MTS (mt-DdCBEs) or a CTP (cp-DdCBEs) were used for base editing of organellar DNA in lettuce and rapeseed protoplasts[61] (Fig. 5a).

Transient transfection of plasmids encoding cp-DdCBEs and mt-DdCBEs into protoplasts caused C>T editing at target sites in calli regenerated from the protoplasts with frequencies of up to 38% in chloroplasts and 25% in mitochondria. Nearly homoplasmic editing (≈99%) of the chloroplast 16S rRNA gene (*rrn16*) into a variant resistant to binding by the antibiotic spectinomycin was achieved in lettuce plantlets regenerated in the presence of spectinomycin. Although no off-target mutations were detectably introduced in spectinomycin-resistant clones in the vicinity of the target site, off-target C>T edits were observed at low frequencies (1.2–4.1%) in protoplasts transiently transfected with the cp-DdCBE expression plasmid. The use of in vitro transcripts encoding the cp-DdCBE rather than plasmid DNA largely avoided these off-target edits without reducing the efficiency of target editing. Apparently, DdCBE mRNA is more rapidly degraded in protoplasts than its encoding plasmid DNA, thereby minimizing off-target activity.

Agrobacterium was also used to express plant codon-optimized DdCBEs, termed plastid-targeting peptide-TALE cytidine deaminases (ptpTALECDs), in *A. thaliana* and to obtain T1 plants with site-specific C>T mutations in three chloroplast genes, *rrn16*, the RNA polymerase C1 gene (*rpoC1*) and the photosystem II protein D1 gene (*psbA*)[62] (Table 1). Importantly, in addition to heteroplasmic edits, homoplasmic C>T edits were observed at the target sites and were transmitted to the next generation, independently of the DdCBE-encoding transfer DNA (T-DNA), which was incorporated in the nuclear genome (Fig. 5b). The resulting transgene-free segregant T2 plants with point mutations in their plastome are likely to be exempted from genetically modified organism regulations in many countries, including the USA, Japan and China. Whole-genome sequencing of T1 and T2 plants showed that off-target mutations were rarely introduced in the plastome. mtDNA editing in *A. thaliana* was also achieved, using T-DNA encoding

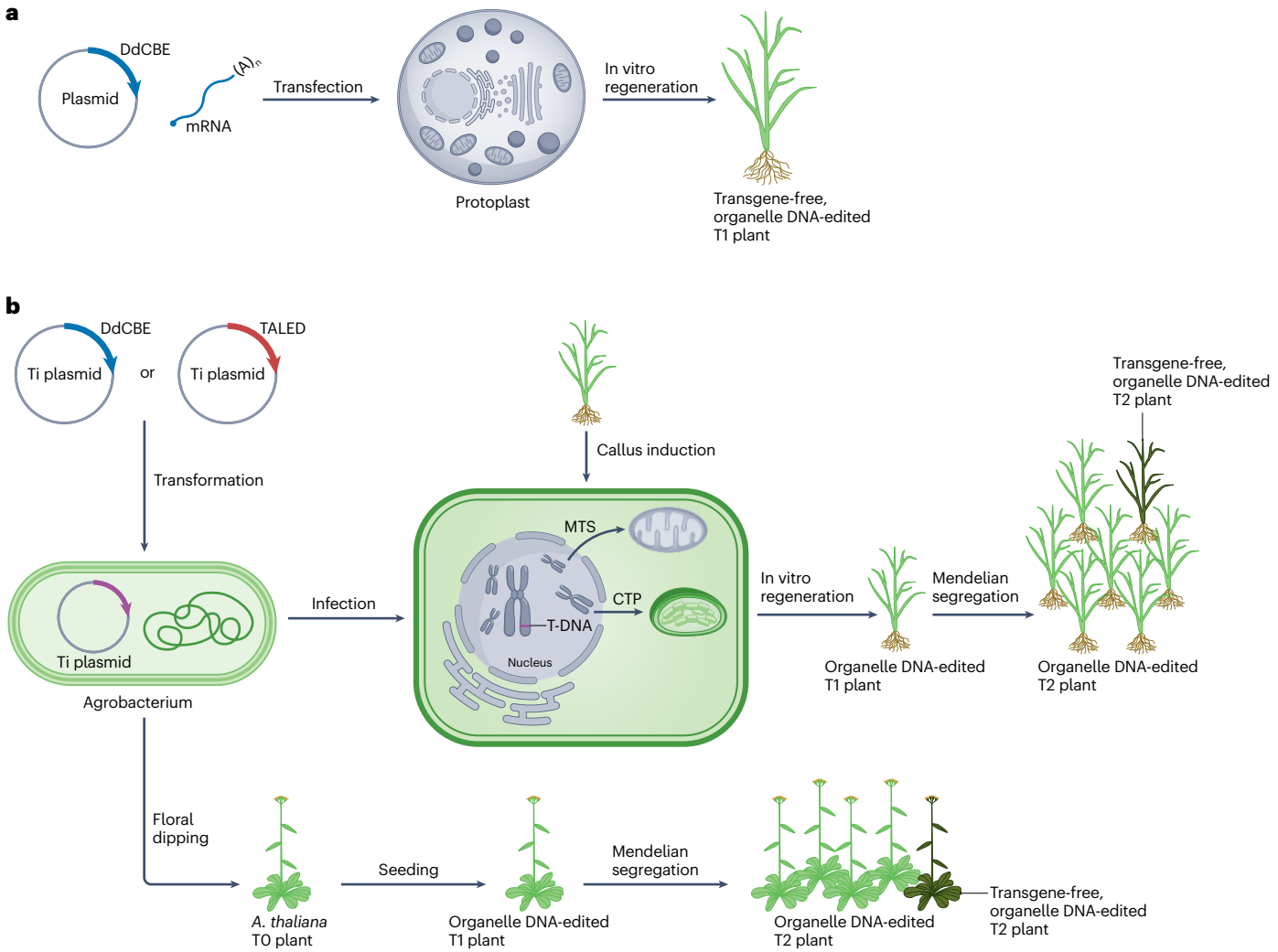


Fig. 5 | Organellar DNA editing in plants. a, Transgene-free organellar base editing in plants. Plasmids or mRNAs encoding base editors such as DNA deaminase toxin A-derived cytosine base editors (DdCBEs) and transcription activator-like effector-linked deaminase (TALEDs; adenine base editors) with a mitochondrial targeting sequence (MTS) or a chloroplast transit peptide (CTP) are transfected into protoplasts, from which organelle DNA-edited plants are regenerated in vitro. **b**, Agrobacterium-mediated transformation. Plant cells are infected with agrobacterium carrying tumour-inducing (Ti) plasmids, and transfer DNA (T-DNA) encoding the base editor is inserted into the genome of T1 plants. Transgene-free, organelle DNA-edited T2 plants are obtained through Mendelian segregation. Floral dipping is a convenient method of transforming *Arabidopsis thaliana* by dipping inflorescences into a solution containing agrobacterium.

mitoTALECDs[63]. Some T1 plants had homoplasmic C>T edits, which were stably inherited by the T2 plants.

Plastid genome editing was also demonstrated in rice using cp-DdCBEs targeting the photosystem I P700 chlorophyll *a* apoprotein A1 (*psaA*) gene[64], which encodes an essential protein component of the photosystem I complex. Agrobacterium-mediated expression of the *psaA*-specific DdCBEs in rice induced the formation of a premature stop codon as a result of C>T editing with high frequencies of up to 98%. The transgenic rice lines with nearly homoplasmic editing had an albino phenotype of white or yellow leaves, indicating reduced chlorophyll production.

Targeted A>G editing in plant organelles

Adenine base editing of plastid DNA in plants has been demonstrated using plant codon-optimized TALEDs[65] (Fig. 5b). As expected, plants with homoplasmic mutations disrupting the *psaA* start codon had an albino or pale-green phenotype, whereas T2 plants with a homoplasmic mutation in *rrn16* were resistant to spectinomycin. Homoplasmic A>G mutations in the ribulose-bisphosphate carboxylase gene (*rbcL*) encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), which catalyses CO_2 fixation, also caused partial albinism in T1 plants, suggesting that the resulting mutant proteins had low activity. Unlike human mitochondria, in which UGI-free TALEDs induce A>G edits without C>T edits, whereas UGI-containing TALEDs induce concurrent A>G and C>T edits[34], UGI-free TALEDs induced C>T edits in addition to A>G edits at some but not all target sites in protoplasts and whole plants. Apparently, uracil bases produced by UGI-free TALEDs in plastid DNA are less efficiently removed by endogenous DNA glycosylase compared with those in mtDNA in human cells.

Agricultural and environmental applications

Base editing in plant organellar DNA is a powerful method for studying the functions of organellar genes and phenotypes associated with naturally occurring single-nucleotide variations (SNVs). DdCBEs, TALEDs and other CRISPR RNA-free base editors can be used for installing a premature stop codon (for example, CAA can be changed to TAA using DdCBEs) or disrupting the start codon (ATG can be changed to GTG or ACG using TALEDs) in a gene of interest. Likewise, they can induce C>T and A>G SNVs in a strain or species of interest. A library of TALE-guided base editors could introduce transition mutations in an organellar gene of interest at almost-saturating levels, thereby enabling phenotype-based screening and directed evolution. For instance, SNVs in chloroplast genes that cause herbicide resistance could be identified through base editor-driven targeted mutagenesis and selection in the presence of the herbicide of interest[61]. Likewise, in plant, mutagenesis and screening may allow the creation of RuBisCo variants with enhanced catalytic efficiency and improved substrate specificity. In addition to CO_2 fixation, RuBisCo can catalyse oxidation to yield toxic by-products, which must be metabolized in an energy-wasting process known as photorespiration. RuBisCo variants with enhanced carboxylation rates and reduced affinity for oxygen could increase crop productivity and contribute to carbon neutralization to mitigate the climate crisis, especially when expressed in trees.

Conclusion and future outlook

For the past decade, since CRISPR–Cas9 has been successfully repurposed for nuclear genome editing in human cells[66–69], we have witnessed remarkable improvements in CRISPR RNA-guided tools, enabling more convenient, efficient and precise gene editing in diverse systems. In the coming years, organellar DNA editors could similarly be further improved and expanded for broad applications. For example, current tools are limited to transition mutagenesis. New systems enabling transversion (purine-to-pyrimidine or pyrimidine-to-purine) mutagenesis in organellar genomes would be of broad interest and utility. In this regard, it is of note that glycosylase–Cas9 nickase fusion proteins have been developed for C>G or A and A>C or T conversions in nuclear DNA[70–74]. DdCBEs and TALEDs fused to glycosylases may allow transversions in organellar genomes. An open question is whether organellar genome editing could be 'CRISPRized'[16]. If gRNA could be delivered into organelles, CRISR RNA-guided base editors[21,75,76] and prime editors[77] could install all possible transitions, transversions, insertions and deletions in organellar DNA.

Organellar base editors are also limited by their unwanted bystander editing at the target site. ZFDs or ZFD–TALE-DdCBE hybrid pairs often avoid bystander edits[30,31], presumably because they have narrowed editing windows compared with DdCBEs or TALEDs. However, ZFDs are much less specific than TALE arrays, often inducing hundreds of off-target mutations in the mitochondrial genome. Next-generation base editors without off-target and bystander editing activities are needed for disease modelling in cell lines and animals and, more importantly, for therapeutic and agricultural applications in the future. Just as CRISPR–Cas9 and other programmable nucleases have paved the way for nuclear genome editing, programmable deaminases are now set to open up a new avenue for organellar genome editing, which will be broadly useful for basic research, medicine and biotechnology.

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Author contributions
Both authors contributed equally to all aspects of the article.

Competing interests
J.C. is a scientific co-founder and adviser of CorrectSequence Therapeutics, a company that uses gene-editing technologies. J.-S.K is a founder and shareholder of ToolGen, Edgene. and GreenGene, which are focused on genome editing.

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