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# Advances in Mitochondrial Genome Editing for Disease Modeling and Precision Therapeutics

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## ABSTRACT

Mitochondria play a pivotal role in cellular energetics and metabolism, housing their own distinct genome, the mitochondrial DNA (mtDNA). Dysregulation or mutations in mitochondrial genes can lead to a spectrum of diseases, including inherited mitochondrial disorders, neurodegenerative conditions, and age-related disorders. The emerging field of mitochondrial genome editing has garnered attention as a potential avenue for therapeutic interventions and disease modeling.

This review comprehensively explores the diverse approaches and technologies employed in mitochondrial genome editing. It delves into the mechanisms, applications, and challenges associated with using various molecular tools such as mitochondrial gene editing by CRISPR/Cas9 and CRISPR/Cas12a systems, zinc finger nucleases (ZFN), high-fidelity DdCBEs, transcription activator-like effectors nuclease (TALEN), pAgo-based systems, and mitochondrial DNA base editing for therapeutic and disease modeling purposes.

Furthermore, the review sheds light on the potential applications of mitochondrial genome editing in the context of treating mitochondrial diseases, correcting pathogenic variants, and modeling disease pathogenesis for research purposes. Additionally, the discussions encompass the strategies for optimizing specificities, enhancing on-target editing, and addressing potential off-target effects to ensure the clinical translatability of mitochondrial genome editing.

The insights gleaned from this review provide a comprehensive understanding of the current landscape of mitochondrial genome editing, presenting promising avenues for the development of novel therapeutic interventions and disease modeling for various mitochondrial disorders and related conditions.

Overall, this comprehensive review presents a thorough overview of the complexities, opportunities, and future prospects in the field of mitochondrial genome editing, offering a roadmap for advancing the understanding and clinical applications of these innovative technologies.

***Keywords:*** mitochondria, non-infectious diseases, inflammation, precision medicine, mitochondrial diseses, mtDNA

## MITOCHONDRIA

Mitochondria are semi-autonomous organelles located in eukaryotic cells, primarily responsible for aerobic respiration. They generate energy for cellular functions through the processes of the electron transport chain and oxidative phosphorylation. Additionally, mitochondria are involved in crucial metabolic pathways such as the Krebs cycle, β-oxidation, as well as the synthesis of lipids and cholesterol. They also play significant roles in the generation of reactive oxygen species and the regulation of apoptosis [1,2].

These organelles contain their own distinct genome that resembles bacterial DNA, separate from the nuclear genome. A key component of this genome is mitochondrial DNA (mtDNA). When researchers sequenced mtDNA, they discovered that human mtDNA comprises two strands—heavy and light chains—totaling 16,569 base pairs, which encode 2 rRNAs,

22 tRNAs, and 13 protein subunits [3,4]. The proteins produced from mtDNA can vary between species and across different tissues. The expression of mitochondrial proteins is principally regulated by nuclear DNA, which controls the transcription and translation processes by interacting with a specific noncoding region of the mitochondrial genome known as the 1 kb D-loop. The 13 genes within mtDNA produce peptides that work in conjunction with over 60 nuclear-encoded proteins, forming the mitochondrial respiratory chain essential for aerobic metabolism [5,6]. Therefore, the functionality of mitochondria is heavily dependent on the interaction between nuclear and mitochondrial genetics. Mutations in either the nuclear or mitochondrial genomes can lead to mitochondrial diseases [7].

Inherited mitochondrial diseases commonly manifest as defects in oxidative phosphorylation, stemming from mutations in either mitochondrial or nuclear DNA that affect structural mitochondrial proteins or those involved in mitochondrial functions. These conditions rank among the most prevalent genetic metabolic disorders and are also significant contributors to genetic neurological disorders [8,9]. Despite its compact size, the mitochondrial genome is a critical factor in genetic disease. When heteroplasmy—where mutant and normal mtDNA coexist—reaches a certain threshold, various mitochondrial diseases can manifest. Recent studies have linked mitochondrial genes to specific human disorders, such as Leigh syndrome, Leber Hereditary Optic Neuropathy (LHON), and NARP syndrome (Neuropathy, Ataxia, and Retinitis Pigmentosa). Progress has been made in understanding mitochondrial genetics and the relationship between gene mutations and disease manifestations, along with the identification of acquired mtDNA mutations in relation to aging and cancer [10]. Presently, many mutations in mitochondrial genes and their associated diseases lack effective treatments. However, advancements in gene editing technologies offer hope for correcting mitochondrial mutations and developing gene therapies for these conditions [11,12].

## MITOCHONDRIAL GENE EDITING

One of the state-of-the-art therapeutic interventions used for treating mitochondrial diseases is mitochondrial transplantation. Nevertheless, to keep the therapeutic results, it is necessary to perform mitochondrial transplantation several times. Moreover, the effect of mitochondrial transplantation is affected by such factors as mitochondrial isolation, mitochondrial sources, and the route of administration. According to a number of studies, upon mitochondrial transplantation, there is immune response, and finding a way to store mitochondria long term is a significant problem. Furthermore, as mitochondrial diseases occur because of the mutation of mtDNA up to certain degree, which leads to functional disorders, the technology of gene editing aiming at mitochondrial genes specifically will be a promising method of treating mitochondrial diseases [13,14]. This strategy aims to reduce the prevalence of mutated mitochondrial DNA (mtDNA) by modifying the defective mitochondrial gene, thereby addressing mitochondrial disorders. Unlike mitochondrial transplantation, mitochondrial gene editing technologies may have broader applications. The primary techniques currently used for mitochondrial gene editing consist of restriction endonucleases (RE), zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and the CRISPR/Cas9 system [15].

## MITOCHONDRIAL GENOME EDITING USING THE CRISPR/CAS9 SYSTEM

Efforts to create a mitochondrial-adapted CRISPR/Cas9 system have focused on combining mitoCas9 with small guide RNAs (sgRNAs) that include a stem-loop structure. One study revealed that the stem-loop sequence derived from H1 RNA could enhance the delivery of sgRNA into mitochondria. In experiments using mouse cells, complexes of the modified sgRNA and mitoCas9 successfully diminished the levels of mtDNA containing the 11205G mutation in the ND4 gene (mtND4) [16]. Both modified sgRNAs featuring the H1 RNA stem-loop and unmodified versions were found in the mitochondrial compartment; however, modified sgRNAs were notably more abundant within the mitochondria of transfected cells than the unmodified controls. A limitation of this study was the failure to remove the outer mitochondrial membrane during extraction, which may have led to contamination from outer membrane and cytosolic RNAs, affecting the quantification of sgRNA levels in mitochondrial samples [17]. Additionally, assessments of mtDNA levels in the control and experimental groups at 48 and 72 hours post-transfection were not conducted, indicating a need for thorough long-term evaluations of mtDNA content to substantiate claims regarding the reduction of mitochondrial genomes due to the CRISPR/Cas9 intervention.

In another investigation, a combination of modified sgRNAs incorporating an F-arm motif from yeast cytosolic tRNALys (CUU) led to decreased mtDNA levels in cybrids affected by Kearns-Sayre Syndrome after the introduction of mitoCas9. However, employing a single modified sgRNA did not result in significant changes to mtDNA levels or heteroplasmy. Both the modified sgRNAs with the F-arm motif and the original sgRNAs displayed roughly 35% efficiency in mitochondrial entry, suggesting that this mitochondrial-adapted CRISPR/Cas9 system may not effectively target or reduce

mutant mtDNA levels. Furthermore, the F-arm motif did not significantly enhance sgRNA import into mitochondria [18]. The sgRNA cleavage activity, whether utilizing F-arm or D-hairpin motifs, showed partial to complete inhibition, indicating that the stem-loop structure might impact the interaction between sgRNA and the Cas9 nuclease, as well as the binding efficiency of sgRNA to its target DNA [19].

Additional research indicated that the unmodified sgRNA/mitoCas9 complexes initiated a variety of insertion/deletion (InDel) events in mtDNA, albeit at a low frequency of less than 0.05%. This rate is considerably lower than the levels of mutated mtDNA removal achieved by mitoTALENs or ZFNs. Moreover, modified sgRNAs employing stem-loop motifs from 7-2 RNA or H1 RNA did not show an increase in InDel frequency compared to the original sgRNA [20]. This suggests that the stem-loop motifs from 7-2 RNA and H1 RNA do not significantly facilitate the import of sgRNA into mitochondria. Additionally, the presence of nuclear mitochondrial DNA sequences (NUMTs) can complicate the identification of mtDNA variants through Next Generation Sequencing (NGS), which can make it challenging to determine the true source of the sequences. As a result, InDel events detected via NGS may be overrepresented [21].

## MITOCHONDRIAL GENOME EDITING USING CRISPR/CAS12A SYSTEM

The CRISPR/Cas12a system distinguishes itself from CRISPR/Cas9 by employing a single crRNA, typically ranging from 42 to 44 nucleotides, which serves as the guide RNA (gRNA). Recently, researchers have developed a mitochondria-specific version of this system, referred to as mitoCRISPR/Cas12a, aimed at editing mitochondrial DNA (mtDNA). Studies demonstrate that LbCas12a nucleases effectively localize to mitochondria, resulting in less mitochondrial damage compared to other nucleases like SpCas9 and SaCas9 [22]. This highlights LbCas12a's enhanced ability for mitochondrial import.

Both unmodified and modified sgRNAs, including those featuring the H1 RNA stem-loop or the yeast tRNALys (CUU) motifs, have been identified in both nuclear and mitochondrial fractions, although the latter was found in lower quantities. This suggests a mechanism for sgRNA transport into mitochondria that operates independently of a mitochondrial localization sequence (MLS) [23]. It is important to note that previous studies may overestimate sgRNA levels within mitochondria due to residual outer membrane contaminants remaining after isolation. Although the efficiency of crRNA delivery into mitochondria, whether with or without an MLS, warrants further investigation, the functionality of mitoLbCas12a/crRNA complexes—with and without the H1 RNA stem-loop—has been tested in MELAS cybrids [24,25].

Theoretically, mitoLbCas12a/crRNA complexes are expected to diminish mtDNA content. However, findings indicate that both modified and unmodified crRNAs can, counterintuitively, lead to an increase in mtDNA levels, which appears to be influenced by mitoLbCas12a. Thus, the efficacy of the CRISPR/Cas12a system for editing mtDNA in mammalian models remains to be fully established [26].

## ZINC FINGER NUCLEASE (ZFN)

Zinc Finger Nucleases (ZFNs) are made up of two principal elements: the zinc finger DNA-binding domain, which identifies specific DNA sequences, and the FokI nuclease domain, which catalyzes DNA cleavage. This cleavage facilitates either the insertion of new DNA fragments or the generation of frameshift mutations within target genes via cellular repair processes. The zinc finger domain can be classified into three categories, with each category containing roughly 30 amino acids that coordinate around a zinc ion, allowing binding to three base pairs of DNA. Importantly, the FokI domain operates non-specifically as a dimer, requiring the design of separate domains for both 5' to 3' and 3' to 5' orientations. ZFN technology has proven effective for editing nuclear genomes, enabling processes such as gene addition, modification, and deletion [27].

More recently, this technology has been adapted for mitochondrial gene editing. Mitochondrial Targeting Sequences (MTS) and Nuclear Export Signals (NES) are incorporated to direct ZFN proteins specifically to the mitochondria, thereby minimizing the cytotoxic risks associated with modifications to nuclear DNA. Two distinct monomers are engineered to interact with mtDNA: one targets the wild-type sequence, and the other is designed for the mutated variant. The FokI enzyme's homologous dimers can cleave mtDNA, resulting in linear DNA fragments. Since mitochondria do not possess a non-homologous end joining (NHEJ) repair pathway to handle double-strand breaks, the damaged DNA is generally subject to degradation. Nonetheless, this approach presents challenges related to efficiency and an increased likelihood of off-target effects [29].

To enhance accuracy, researchers have developed a single-strand ZFN protein with dual FokI domains, which simplifies the editing process. However, this single-strand ZFN still faces limitations, such as difficulties in addressing large deletions like common deletions (CD), and its lack of dimerization may lead to unintended cytotoxic effects due to persistent nuclease activity [30]. Efforts to improve traditional dimer ZFN have included the use of heterologous dimers to minimize off-target effects and adjusting the arrangement of NES, fusion proteins, and DNA-binding proteins to enhance stability. This approach has resulted in a fivefold increase in the proportion of wild-type mtDNA, surpassing the twofold increase observed with the single ZFN protein with dual FokI editing. This method also allows for the identification of CD, with two monomers targeting wild-type sequences flanking the deletion, enabling effective nuclease activity in cases of deletion mutations [31].

Given the high demand for functional mitochondria in tissues like muscle and brain, and the specific symptoms that arise when mitochondrial gene mutations reach critical levels—such as movement disorders and retinitis pigmentosa—cell models with relevant mitochondrial mutations are essential for further research. Using mtZFN to knock out mutated genes significantly reduced the proportion of mutant mtDNA, offering promise for gene therapy in mitochondrial diseases [32,33]. However, this method also led to a slight decrease in the proportion of normal mtDNA. Moreover, in cells with a high burden of mutated mitochondrial genes, the overall number of mitochondria may decline significantly, potentially leading to cell death. Consequently, strategies to reduce knockout efficiency and iterative knockout approaches have been explored. Additionally, researchers have investigated using hammerhead ribozymes (HHR) to modulate mtZFN expression levels. Genetic verification has been complemented by confirmation of mutant gene knockout at both organelle and cellular levels; improvements in ATP production and energy charge were observed following mtZFN modifications [34]. Notably, the levels of citric acid and aconitic acid, key metabolites in mitochondrial metabolism, were increased, further demonstrating the efficacy of mtZFN [35].

For further validation of mtZFN's effectiveness, in vivo studies have been conducted. One such study involved a mouse model with a tRNA (Ala) mutation at m.5024C>T in cardiac myocytes. Following intravenous injection of an associated viral vector, mtZFN expression was confirmed in heart cells. Results showed an increase in pyruvic acid and aspartic acid levels, alongside a decrease in lactic acid levels, indicating an enhancement in mitochondrial respiratory energy production at the expense of the glycolytic pathway, thereby confirming the viability of mtZFN in vivo [36].

Despite these advancements, mtZFN is characterized by relatively low editing efficiency, and iterative editing protocols require considerable time, averaging about a week per mtZFN application. Furthermore, each zinc finger protein, consisting of around 30 amino acids coordinated with a zinc atom, binds to three base pairs of DNA, which may contribute to inaccuracies in targeting [28,37].

## DDDA6- AND DDDA11-CONTAINING DDCBES

Enhanced variants of DdCBEs featuring the split wild-type DddAtox have been engineered to achieve improved activity and expanded targeting capabilities. Two primary methodologies were employed for the development of these optimized biomolecules: phage-assisted continuous evolution (PACE) and phage-assisted non-continuous evolution (PANCE). Initially, PANCE was harnessed to enhance DdCBEs' activity at TC target sites, leading to the identification of the DddAtox (T1380I) mutant, now referred to as DddA1 [38]. Following this, DddA1 underwent further refinement through PACE, resulting in additional variants labeled DddA2 through DddA5, with DddA5 demonstrating significantly greater editing efficiencies in mtDNA. It was proposed that the T1314I mutation present in DddA4 played a role in reconstituting the split DddAtox halves, contributing to the development of DddA6. Collectively, DdCBEs incorporating DddA6 enhanced mtDNA editing rates in TC contexts by an average factor of 3.3 compared to traditional DdCBEs [39].

Subsequently, efforts were made to create DdCBEs capable of targeting non-TC sequences, utilizing mutagenic drift alongside context-specific versions of PANCE and PACE. This endeavor led to the emergence of DddA7 through DddA11 variants. The mutants produced through PACE exhibited improved targeting ability in assays involving bacterial plasmids and in vitro editing of mtDNA. Notably, DdCBEs with DddA11 achieved enhanced editing efficiencies at TC, CC, and AC sites [40]. However, in tests involving a single spacer with various editable substrates, these variants displayed the greatest editing activity at TC contexts, followed by CC and AC contexts. Despite multiple attempts to boost editing effectiveness at GC sequences using PACE or PANCE methods, suitable DddAtox variants for GC substrates were not successfully developed in these bacterial assays. Nevertheless, DdCBEs incorporating DddA11 were able to edit GC targets at the MT-ND4 site, though at relatively low frequencies [41].

The previously established guidelines for designing DdCBEs still hold relevance today. Recent research indicates that spacers as short as 12 base pairs can effectively facilitate editing by DdCBEs in bacterial contexts. When utilizing traditional cloning techniques, such as Golden Gate assembly, it is advisable to select parental plasmids that contain the desired DddAtox variant [42]. Both DddA6 and DddA11 derive from a split at position G1397, which implies that DdCBEs employing the G1397 split configuration may have superior activity compared to those utilizing the G1333 split. In practical applications, DdCBEs incorporating DddA6 and DddA11 within the G1333 arrangement showed diminished on-target editing efficiency for mtDNA relative to those adopting the G1397 configuration.

Concerning editing windows, both canonical and evolved DdCBEs exhibited analogous mutation patterns when tested with artificial all-TC spacers, with DddA11 typically demonstrating a broader editing window for spacers longer than 15 base pairs [43]. The evaluation of mitochondrial genome-wide target specificity for these evolved base editors revealed that DdCBEs containing DddA6 or DddA11 at two mtDNA target sites led to an increased incidence of off-target edits compared to conventional DdCBEs [44]. It is essential to recognize that potentially promiscuous TALEs could affect specificity in both canonical and evolved DdCBE pairs. Furthermore, the impact of DdCBEs on inducing nuclear off-target mutations has not been thoroughly assessed.

Conversely, research involving nuclear-targeted DdCBEs with evolved DddAtox variants revealed off-target editing. Using the prediction tool PROGNOS, 19 nuclear off-target sites were identified for two nuclear DdCBEs. The data suggested that off-target editing in the nucleus was similar between canonical and evolved DdCBEs, indicating that DddA6 and DddA11 did not significantly compromise DdCBE specificity in nuclear contexts [45,46].

For the delivery approach, HEK293T cells were co-transfected with plasmids that encode each component of a DdCBE pair, with samples collected three days after transfection. Targeted amplicon sequencing detected C•G-to-T•A conversions in mtDNA, achieving frequencies of around 30% in cells treated with either DddA6- or DddA11-containing DdCBEs [17,38]. To enable mtDNA editing in various human cell lines, the left and right arms of a DdCBE pair targeting MT-ND5 were fused with mCherry and eGFP, respectively, utilizing a self-cleaving P2A sequence that separates each arm from its corresponding marker. The cells underwent electroporation with each expression plasmid and were subsequently sorted by fluorescence three days later using fluorescence-activated cell sorting (FACS) to enrich for double-positive cells. This strategy significantly enhanced average editing rates, increasing from below 1% to between 4% and 31% in HeLa cells, an 11-fold improvement in K562 cells, and a 1.5-fold boost in U2OS cells, as confirmed by targeted amplicon sequencing [47,48].

Moreover, DdCBEs with DddA11 were employed to introduce mtDNA variants associated with disease at non-TC positions within HEK293T cells. Specifically, the missense m.11696G>A variant linked to Leber's hereditary optic neuropathy and the nonsense m.13297G>A variant associated with renal oncocytoma were successfully introduced. These alterations were sufficient to impact mitochondrial function, highlighting their potential for modeling mitochondrial diseases [49].

## MONOMERIC DdCBEs (mDdCBEs)

While DdCBEs and mitoZFDs are highly adaptable, their dimeric structures introduce considerable limitations. The requirement for two distinct components creates challenges in synthesis, complicating delivery methods, especially for the larger DdCBEs compared to mitoZFDs. To overcome these challenges, monomeric DdCBEs (mDdCBEs) have been engineered using safe, full-length variants of DddAtox that exhibit a reduced affinity for double-stranded DNA. This strategy, which utilizes targeted mutagenesis, was preferred over dividing DddAtox into two inactive fragments [50]. The monomeric design not only streamlines the production and delivery processes but also results in distinct editing patterns that differ from those of their dimeric versions, thereby expanding the potential for organellar genome editing. Furthermore, the simplified assembly process enhances the efficiency of base editing screens, although the issue of bystander editing still needs to be addressed.

To create an effective, non-toxic full-length variant of DddAtox for base editing applications, a library of DddAtox variants was developed using error-prone PCR techniques [51]. These variants were assessed for their deamination capacity in vitro by fusing them with Cas9 and directing them to a specific site in the nuclear gene TYRO3 in HEK293T cells. This approach led to the successful identification of a modified DddAtox variant, referred to as the GSVG variant, which contains four point mutations. This modified variant was then attached to the C-terminus of transcription activator-like effector (TALE) arrays targeting various locations within the mitochondrial DNA, resulting in the formation of mDdCBEs [39]. When

transiently expressed in human cell cultures, mDdCBEs demonstrated the ability to perform targeted C•G-to-T•A conversions in the mitochondrial genome with efficiencies reaching up to 50%, comparable to those of their dimeric counterparts [50].

The framework of mDdCBEs closely resembles a single arm of a dimeric DdCBE, with the key difference being that a complete DddAtox variant is incorporated instead of a split fragment. Notably, mDdCBEs utilizing the DddAtox E1347A active-site mutant, although less efficient than those utilizing DddAtox GSVG, still permitted editing in mtDNA [50,51]. The structural arrangement of mDdCBEs consists of a specific sequence: starting from the N-terminus to the C-terminus, they include a mitochondrial targeting signal (MTS) derived from SOD2 or COX8A, followed by an epitope tag (HA or FLAG), a TALE domain, a two-amino-acid linker, either the DddAtox GSVG or E1347A, a four-amino-acid linker, and UGI. In terms of functionality, mDdCBEs are designed to exclusively edit cytosines found in TC contexts, similar to their canonical and dimeric analogs. They prefer to target TCs positioned 4 to 11 nucleotides downstream of the TALE-binding sequence, but they can also cause bystander edits up to 61 base pairs downstream from the beginning of the TALE-binding site [51].

The creation of mDdCBEs utilized the same TALE array designs as those used for dimeric DdCBEs, resulting in four distinct mDdCBE versions designated as L/R-GSVG/E1347A. To assess genome-wide target specificity, all four mDdCBE formats were tested at the MT-ND1 and MT-ND6 loci, alongside dimeric DdCBEs and both split and full-length variants of DddAtox as controls [39]. At the MT-ND1 locus, all mDdCBE formats displayed a lower specificity compared to the dimeric DdCBEs, particularly the L-GSVG variant. However, at the MT-ND6 site, both mDdCBEs and dimeric DdCBEs exhibited comparable levels of off-target editing, with the L-E1347A and R-E1347A variants showing higher specificity than the dimeric versions. This suggests that mDdCBEs do not necessarily have reduced specificity compared to split DdCBE pairs. Interestingly, using mRNA to encode mDdCBEs during transfection led to fewer off-target edits, although this was accompanied by decreased efficiencies in on-target editing [39].

The nuclear off-target mutations resulting from mDdCBEs were analyzed using targeted amplicon sequencing on the nuclear pseudogene MTND4P12, which shares significant sequence homology with the targeted site MT-ND4. Notably, off-target editing was not observed at this nuclear pseudogene; however, a broader analysis of the nuclear genome did reveal several off-target editing occurrences [44].

For the delivery method, HEK293T cells were transfected with plasmids or mRNA encoding mDdCBEs. The cells were harvested three days after transfection for targeted amplicon sequencing to evaluate on-target editing. Additionally, HEK293T cells were transduced with AAV2 vectors carrying mDdCBE constructs at multiplicities of infection between 10,000 and 500,000. Six days post-transduction, the cells were collected to assess AAV-mediated base editing, achieving efficiencies of up to 99.1% at the MT-ND4 locus and 59.8% at the MT-ND1 locus [52]. These results highlight that mDdCBEs can be efficiently packaged and delivered using single recombinant AAV vectors in vitro, suggesting the potential for achieving nearly homoplasmic mutations (>99%) in the mitochondrial DNA of cultured human cells through AAV-mediated base editing.

## HIGH-FIDELITY DdCBEs (HiFi-DdCBEs)

Recent assessments of the target specificity of mtDNA base editing technologies have uncovered substantial off-target effects occurring also within the nuclear genome. Standard DdCBEs, evolved variants, mDdCBEs, and mitoZFDs have all been shown to trigger off-target mutations in the mitochondrial DNA. A comprehensive study evaluating the nuclear target specificity of traditional DdCBEs indicated that these base editors often resulted in a high frequency of off-target mutations during in vitro experiments [53]. In contrast, investigations into the nuclear off-target potential of enhanced DdCBEs, mDdCBEs, and mitoZFDs have generally focused on targeted amplicon sequencing of a limited number of predicted off-target sites. A more extensive investigation into both mtDNA and nuclear DNA (nDNA) off-target modifications is crucial for further progress in this field [54].

The challenges posed by the imprecision of mtDNA base editing methods may limit their utility in disease modeling and therapeutic applications. To tackle this issue, high-fidelity DdCBEs (HiFi-DdCBEs) have been engineered, utilizing interface-modified split DddAtox variants. The standard split DddAtox fragments are capable of spontaneously reassembling without the necessity for TALE-DNA interactions, which can lead to unintended off-target mutations in both nDNA and mtDNA [55]. To mitigate this, specific amino acids at the interface of the split halves were replaced with alanine, a non-bulky and inert side chain, allowing for the requirement of TALE arrays in order to reform a functional

deaminase domain. HiFi-DdCBEs have been shown to maintain on-target editing efficiencies similar to traditional DdCBEs while significantly decreasing off-target editing events in human mtDNA [56].

Two split DddAtox variants are recommended for use in HiFi-DdCBEs: T1391A and K1389A. These variants are effective in both G1397- and G1333-split configurations. For optimal specificity, the T1391A variant is suggested, although it may exhibit lower activity compared to standard DdCBEs [57]. In contrast, the K1389A variant is favored when higher activity is more critical than specificity. Importantly, these variants can be utilized in conjunction with DddA6 and DddA11, enabling the development of HiFi-DdCBEs with improved activity and targeting range. Since DddA6 and DddA11 are derived from G1397-split DddAtox, it is advisable to retain this configuration in the design of HiFi-DdCBEs that utilize these components. Additionally, the previously established design parameters for DdCBEs remain relevant, stipulating TALE-binding sequences that span 10 to 18 base pairs, with spacer regions of 12 to 18 base pairs [43].

Comprehensive evaluations of off-target editing from HiFi-DdCBEs have been performed within the context of the mitochondrial genome. For instance, using a wild-type G1397-split DdCBE targeting the MT-ND1 locus resulted in 238 detectable off-target edits in mtDNA, with at least 1% frequency. In contrast, the HiFi-DdCBE variants featuring the K1389 modification showed only five off-target mutations, while the T1391A variant eliminated off-target editing in mtDNA entirely [40]. The on-target editing efficiency at the MT-ND1 site was comparable to that observed with traditional DdCBEs. Furthermore, similar specificity patterns were noted across four distinct regions of human mitochondrial DNA.

When it comes to nuclear off-target editing, HiFi-DdCBEs demonstrated an advantage over conventional DdCBEs by avoiding TALE-dependent off-target events in three nuclear pseudogenes. They also significantly reduced TALE-independent off-target activity across five potential nuclear sites [58].

In terms of delivery methods, all experiments involved the transfection of HEK293T cells with plasmids encoding the DdCBE constructs, each administered at a concentration of 500 ng. The cells were harvested four days after transfection for the purpose of genomic DNA extraction. Targeted amplicon sequencing was utilized to analyze both on-target editing in mtDNA and off-target editing in nDNA, while complete mitochondrial genome sequencing was employed to evaluate mtDNA off-target effects [59].

## ZINC FINGER DdCBEs (ZF-DdCBEs)

Building upon the advancements made with DdCBEs, researchers developed zinc finger DdCBEs (ZF-DdCBEs). Like mitoZFDs, ZF-DdCBEs utilize zinc finger (ZF) arrays for their DNA-binding functions. However, the optimization process for ZF-DdCBEs was much broader compared to that of mitoZFDs, which primarily involved adjusting the linker lengths connecting the ZF arrays and the split halves of DddAtox as well as experimenting with various spacer lengths and arrangements. In contrast, ZF-DdCBE optimization took into account factors such as mitochondrial import, nuclear export, residual activity of uracil-DNA glycosylase in cells, varied lengths of the ZF arrays, scaffold modifications, and enhancements to DddAtox's activity. Importantly, head-to-head comparisons of mitoZFDs and ZF-DdCBEs at various mtDNA sites demonstrated that ZF-DdCBEs consistently achieved superior on-target editing frequencies [51].

Initially, researchers incrementally shortened the N- and C-terminal fragments of DddAtox in the G1397-split format, testing the variants for both on-target and off-target editing across different experimental setups. This method, however, did not significantly enhance specificity. Subsequently, scanning mutagenesis was employed to identify specific point mutations in the C-terminal fragment that destabilized the interaction between the split halves, thereby improving the specificity of ZF-DdCBEs [51]. Moreover, variants of ZF-DdCBEs that had a greater negative charge at the DddAtox termini showed slight gains in specificity while maintaining high levels of on-target editing. Additionally, placing a catalytically inactive N-terminal fragment of the G1397-split DddAtox downstream of the C-terminal section greatly enhanced the specificity of ZF-DdCBEs compared to canonical variants. These approaches culminated in the creation of five high-specificity (HS) ZF-DdCBE variants [30].

As the design of ZF-DdCBEs was optimized, the new high-specificity variants were tested across mtDNA with configurations ranging from v8HS1 to v8HS5. Generally, the v8HS ZF-DdCBEs exhibited reduced off-target editing relative to earlier versions. However, this increase in specificity was occasionally accompanied by a slight decrease in overall activity levels [30]. Some v8HS ZF-DdCBEs successfully improved both specificity and efficiency of on-target editing. In particular, HS1 featured a single point mutation, N18K, in the DddAtox structure, while HS2 incorporated both N18K and P25A mutations, and HS3 included N18K and P25K. HS4 introduced N18K and P25A along with a truncation

of three amino acids in the C-terminal region of the N-terminal fragment of G1397-split DddAtox. Lastly, HS5 differed from HS4 by replacing the P25A mutation with P25K [51].

A variety of zinc finger (ZF) scaffolds was developed to enhance the efficacy of ZF-DdCBEs, specifically including X1, AGKS, V2, and V20. Each of these scaffolds is characterized by a combination of beta and alpha motifs, along with a flexible linker, featuring unique sequences in the zinc fingers that comprise each ZF array [51]. To optimize performance, ZF-DdCBEs were engineered using ZF arrays that contained uniform scaffolds, with variations limited to the DNA-binding residues present in each finger. The X1 scaffold is based on the well-known ZNF268-type zinc fingers, whereas the AGKS scaffold originates from the human transcription factor Sp1C. The V2 and V20 scaffolds were discovered through an extensive survey of the human proteome. Notably, ZF-DdCBE combinations that utilized these newly designed ZF scaffolds consistently outperformed traditional ZF-DdCBEs in terms of on-target editing effectiveness [30].

Similar to mitoZFDs, the DddAtox fragments in ZF-DdCBEs can be linked to a ZF array at either the N- or C-terminal ends, resulting in four possible configurations: C-terminal + C-terminal, C-terminal + N-terminal, N-terminal + C-terminal, or N-terminal + N-terminal. For instance, a C-terminal v8 ZF-DdCBE construct comprises the following components in sequential order from N- to C-terminus: a mitochondrial targeting signal (MTS) derived from the human ATP5F1B gene, a FLAG tag, a nuclear export signal (NES) from MVM, a two-amino-acid linker, another MAPKK NES, a second two-amino-acid linker, an enhanced ZF array, a 13-amino-acid linker, a variant half of the G1397-split DddAtox, a four-amino-acid linker, and UGI. Any of the designated ZF scaffolds (X1, AGKS, V2, or V20) can be employed within the ZF arrays, while the G1397-split DddAtox variant incorporates specific point mutations T1380I, E1396K, and T1413I. Furthermore, to reduce off-target effects while preserving high levels of on-target editing, variants HS1 through HS5 can be incorporated into a v8 ZF-DdCBE framework, creating a v8HS combination [30, 51].

For effective editing of mitochondrial DNA (mtDNA), using the v8 framework paired with the X1 ZF scaffold is recommended. A configuration that utilizes two sets of three zinc fingers each (3ZF + 3ZF) should be adopted, as each ZF unit interacts with three base pairs. Therefore, a binding sequence in a ZF-DdCBE should consist of at least 9 base pairs. The ideal spacer regions separating the ZFs should fall within the range of 4 to 20 base pairs to achieve successful in vitro mtDNA editing. It is also essential to evaluate both orientations of the split DddAtox. Once a proficient ZF-DdCBE pair is determined, further optimization can be performed by extending the ZF array lengths up to six fingers and testing additional ZF scaffolds such as AGKS, V2, and V20. To enhance specificity, the HS1-HS5 variants can be integrated into the refined v8 ZF-DdCBE pair [30, 51].

The implementation of a 3ZF + 3ZF ZF-DdCE pair utilizing the AGKS scaffold successfully introduced the pathogenic m.8340G>A variant in the MT-TK gene in HEK293T cells, achieving an editing efficiency of 31%. In a similar study, an optimized 5ZF + 5ZF ZF-DdCE pair with the AGKS scaffold attained a 39% efficiency in introducing the missense m.3177G>A mutation within the Nd1 gene in C2C12 mouse cells. Additionally, the optimized v8HS1ZF-DdCBE pairs were packaged into single AAV2/9 vectors for conducting in vivo mtDNA base editing in newborn P1 mice [30]. Each ZF-DdCBE pair was regulated by a single CMV promoter, with both arms separated by a P2A peptide. This setup led to the induction of m.7743G>A or m.3177G>A variants in the heart, liver, and quadriceps muscle of injected mice, with editing efficiencies ranging from 12% to 83%. These findings indicate that ZF-DdCBEs can be effectively delivered through single AAV vectors; however, concerns about off-target editing in mitochondria remain [51].

In conclusion, ZF-DdCBEs have been extensively refined to function as modular mitochondrial base editors, enabling both in vivo and in vitro editing through single recombinant AAV vectors. Although they offer versatility, issues surrounding potential off-target activity persist. It is crucial to highlight that evaluations of off-target effects in ZF-DdCBEs have largely been limited to targeted amplicon analyses, lacking comprehensive studies across the entire mitochondrial genome. Moreover, reports of nuclear off-target mutations from mitochondrial-targeted ZF-DdCBEs are still absent. Overall, while DdCBEs are considerably larger, they demonstrate greater specificity and precision than ZF-DdCBEs. The design and enhancement of effective ZF-DdCBEs may also encounter challenges that could limit this technology's broader implementation within the academic community [60].

## TRANSCRIPTION ACTIVATOR-LIKE EFFECTORS NUCLEASE (TALEN)

TALENs are a gene-editing technology that targets specific DNA sequences. They originate from plant pathogenic bacteria, particularly from the Xanthomonas species. The sequence of amino acids found in the DNA-binding domain of the TAL protein is directly related to the corresponding nucleic acid sequence of its target site. This means that the specific

arrangement of amino acids is aligned with the sequence of nucleotides that the protein interacts with [61,62]. Each module of TAL protein recognizes a single base, making it both simple and highly specific. By combining different modules, researchers can create targeted knockouts or regulate the expression of endogenous genes across various nucleotide sequences. TALENs have been successfully employed in multiple organisms, including humans, mice, rats, sheep, pigs, zebrafish, Arabidopsis, and yeast. Their mechanism is similar to that of zinc finger nucleases (ZFNs), comprising a recognition domain and a cleavage domain. The cleavage domain includes the nonspecific nuclease DokI, which mainly facilitates dimer formation [63]. However, TALENs differ in their DNA recognition domains, which consist of a series of TAL proteins—often around 20. Each TAL protein is responsible for recognizing and binding to a corresponding DNA base, thereby enhancing specificity.

TALEN technology has also been utilized for mitochondrial gene editing. The mitochondrial targeting sequence (MTS) of superoxide dismutase (SOD) is modified at the amino terminal of the TALEN monomer to facilitate the targeting of these proteins into mitochondria. Since the nucleic acid enzyme FokI operates only in dimer formations, attention was directed towards addressing fragment missing mutations in mtDNA [64,65]. TALENs were designed to recognize DNA sequences flanking the missing fragment, allowing FokI to come into proximity to form dimers and execute its nuclease function. As a result, the mutated mitochondrial DNA underwent degradation, and a shift from heteroplasmy to wild type. Additionally, the TALEN monomer was configured to target the m.5024C>T mutation, enabling effective knockout strategies. Recent advancements have also shown that two TALEN proteins can be substituted with a single compact TALEN (cTALEN) [66]. Furthermore, mito-TALENs are employed to halt the intergenerational transmission of mitochondrial diseases and reduce levels of mutated mtDNA linked to conditions like LHON and NARP in mammalian oocytes. Considering the maternal inheritance of mitochondria and the limited ability of early embryos to reproduce, significantly reducing mitochondrial numbers in oocytes impacts implantation. Thus, mito-TALEN offers a promising approach to prevent the passage of mitochondrial diseases without the ethical challenges associated with "three-parent" methodologies [67].

Rooted in TALE technology, double-stranded DNA deaminase toxin A (DddA) serves as a catalyst for converting cytosine (C) to uracil (U) in double-stranded DNA, but it is toxic to mammalian cells. This toxin can be divided into two inactive halves known as split-DddA. The split DddA is fused with the TALE protein and uracil glycosylase inhibitors to create a cytosine base editor (DdCBE) that operates without RNA, facilitating the conversion of C•G pairs to T•A in human mtDNA with high specificity and accuracy [68]. The two halves of split DddA only reassemble when the corresponding TALE proteins bind to mtDNA, restoring the catalytic activity needed for base editing. Notably, the inclusion of UGI prevents uracil from being recognized by the DNA repair mechanisms, minimizing the likelihood of removal and substitution with cytosine, which has been shown to enhance editing efficiency by approximately eight times [69]. This MTS-enabled system is capable of precise mtDNA editing, providing an invaluable tool for investigating mitochondrial genetic disorders. Additionally, models of mitochondrial DNA mutations linked to human diseases can be effectively generated with this approach. However, it’s important to note that the DdCBE system using DddA can only target cytosines next to thymine in the genome, which presents significant limitations.

The DNA-binding domain of the TAL protein maintains a one-to-one match with the nucleic acid sequence of the target site, contributing to high accuracy. However, this specificity also increases the molecular weight of mito-TALEN, which can hinder effective viral packaging and cellular import into mitochondria [70].

## pAgo-BASED SYSTEM

Initially discovered in eukaryotic organisms, Argonaute proteins have prokaryotic counterparts known as pAgos, identified in both archaea and bacteria. This family of nucleic acid-guided enzymes shows significant potential for genome editing. In eukaryotes, Argonaute proteins assist in RNA-guided RNA interference, while pAgos primarily use DNA guides to locate and target complementary DNA, providing defense against DNA invasions [71,72].

For instance, the cyanobacterium Synechococcus elongatus (SeAgo) serves as a DNA-guided nuclease that preferentially acts on single-stranded DNA and displays non-specific activity toward double-stranded DNA. Similarly, Clostridium butyricum (CbAgo) targets multicopy genetic elements, creating interference between homologous sequences that results in DNA degradation. The incorporation of small DNA guides by CbAgo relies on its native endonuclease activity and the cellular mechanisms involved in double-strand break repair. Interestingly, both Methanocaldococcus jannaschii (MjAgo) and Thermus thermophilus (TtAgo) are capable of two modes of operation: traditional guide-dependent endonuclease activity and non-guided DNA endonuclease activity [73]. Moreover, CbAgo and Limnothrix rosea (LrAgo) can function as DNA-guided nucleases at physiological temperatures, further enhancing their suitability for genomic applications.

Specific pAgos, like Rhodobacter sphaeroides (RsAgo), utilize guides that are derived from plasmid-based mRNA to target plasmid DNA, thereby decreasing the transcription of reporter genes and the overall prevalence of the plasmid [74].

Recent studies have shown that certain pAgos can facilitate positive selection during RecA-mediated DNA strand exchanges, leveraging interactions between their PIWI domains and the RecA recombinase. This mechanism aids in the homologous recombination of target sequences. Considering the critical involvement of mitochondrial DNA in renewal and replication, it is essential to investigate the use of pAgos with homologous arms for targeting mutations within mitochondrial DNA [75,76]. These arms need to be designed to align with the conventional mtDNA sequence for effective replacement of mutated sequences during replication. If successful, this approach could potentially restore functionality to impaired mitochondria. Nonetheless, the use of pAgo-based systems for mitochondrial DNA editing warrants further research [77].

## ADVANCEMENTS IN MITOCHONDRIAL DNA BASE EDITING: EXPLORING THERAPEUTIC IMPLICATIONS AND INNOVATIVE DISEASE MODELING TECHNIQUES

Base editing techniques are increasingly being utilized to rectify point mutations in nuclear DNA. Recent progress has enhanced our understanding of mitochondrial genetics and how gene mutations correlate with various diseases. Additionally, mutations in mitochondrial DNA (mtDNA) acquired over time have been associated with aging, cancer, and neurodegenerative conditions [78]. As mtDNA editing technologies advance, their application in clinical trials is expected to follow soon. Tools like DdCBE and TALEDs enable precise conversions of C•G to T•A and A•T to G•C in mitochondrial DNA without causing double-strand breaks. These innovative methods show potential for modeling and correcting harmful variants linked to mitochondrial disorders. Current research indicates that among 91 documented mtDNA point mutations associated with diseases, 86 might soon be addressed or modeled with DdCBEs or TALEDs [79].

## CONCLUSION

The exploration of mitochondrial genome editing technologies presented in this review underscores the significant potential of these innovative tools in addressing a wide array of mitochondrial disorders, neurodegenerative conditions, and age-related diseases. From CRISPR/Cas9 and CRISPR/Cas12a systems to zinc finger nucleases, high-fidelity DdCBEs, TALENs, and pAgo-based systems, the diverse molecular approaches offer a rich toolkit for precise manipulation of mitochondrial DNA.

The review highlights the promising prospects for correcting pathogenic variants, modeling disease pathogenesis, and potentially paving the way for therapeutic interventions in the realm of mitochondrial diseases through the targeted editing of mtDNA. By elucidating the mechanisms, applications, and challenges associated with mitochondrial genome editing, this review provides a valuable roadmap for advancing the field's translational potential.

However, the complexities associated with optimizing specificities, enhancing on-target editing, and addressing potential off-target effects underscore the need for continued research and development in the field of mitochondrial genome editing. Moreover, the review emphasizes the importance of exploring these technologies in the context of in vivo models and their potential clinical applications to ascertain their efficacy, safety, and clinical translatability.

In conclusion, the insights offered in this review underscore the transformative potential of mitochondrial genome editing in clinical and research settings. As research in this field continues to progress, robust exploration of therapeutic applications, disease modeling, and precision editing methodologies will be pivotal in unlocking the full potential of mitochondrial genome editing for the benefit of patients and advancing our understanding of mitochondrial biology and disease pathogenesis.

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