Direct evidence of CRISPR-Cas9-mediated mitochondrial genome editing

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GRAPHICAL ABSTRACT

PUBLIC SUMMARY
- We designed a mitochondria-targeted Cas9 system for successful mtDNA editing
- Cas9-edited mtDNA was confirmed by the PCR-free third-generation sequencing
- RAD51 agonist RS-1 significantly enhanced mtDNA knockin efficiency
Direct evidence of CRISPR-Cas9-mediated mitochondrial genome editing

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Pathogenic mitochondrial DNA (mtDNA) mutations can cause a variety of human diseases. The recent development of genome-editing technologies to manipulate mtDNA, such as mitochondria-targeted DNA nucleases and base editors, offer a promising way for curing mitochondrial diseases caused by mtDNA mutations. The CRISPR-Cas9 system is a widely used tool for genome editing; however, its application in mtDNA editing is still under debate. In this study, we developed a mito-Cas9 system by adding the mitochondria-targeted sequences and 3’ untranslated region of nuclear-encoded mitochondrial genes upstream and downstream of the mtDNA target site. Successful knockin of exogenous ssODNs into mtDNA was further validated using polymerase chain reaction (PCR) and/or PCR-based assays. Successful knockin of exogenous ssODNs into mtDNA was further validated using polymerase chain reaction-free third-generation sequencing technology. We also demonstrated that RS-1, an agonist of RAD51, significantly increased knockin efficiency of the mito-Cas9 system. Collectively, we provide direct evidence that mtDNA can be edited using the CRISPR-Cas9 system. The mito-Cas9 system could be optimized as a promising approach for the treatment of mitochondrial diseases caused by pathogenic mtDNA mutations, especially those with homoplasmic mtDNA mutations.

INTRODUCTION

Mitochondria play essential roles in cell metabolism, energy production, apoptosis, calcium homeostasis, and immunity.1,2 Mammalian mitochondria are double-membrane organelles with their own genome ( mitochondrial DNA [mtDNA]). Human mtDNA is a double-stranded circular molecule composed of 16,569 base pairs (bp), containing 37 genes encoding 13 respiratory chain subunits, 22 transfer RNAs, and 2 ribosomal RNAs (tRNAs).3 Mitochondrial dysfunction resulting from mtDNA mutations can cause a variety of human diseases.1,2 There are 100–100,000 copies of the mtDNA genome in a cell, depending on the cell type.3,4 Mutant and wild-type mtDNA can co-exist in one cell, known as heteroplasmasy.5 The level of heteroplasmasy in pathogenic mtDNA mutations can affect disease onset and clinical phenotype, with a general threshold of 60%–95% of mutant mtDNA causing biochemical and clinical defects.6,8

Currently, curing mitochondrial diseases remains a daunting task. Gene therapy through allotropic expression of mitochondrial genes shows promise for the treatment of Leber hereditary optic neuropathy7 caused by mtDNA mutations.8 Another approach is mitochondrial replacement therapy, which transfers the patient’s spindle, pronuclear, or polarg body genome into healthy enucleated donor oocytes or embryos to circumvent mother-to-child mtDNA disease transmission.9,10,11 With the rapid development of genome-editing technology, several approaches for manipulating mtDNA in vitro and in vivo have been established in recent years,12–19 which exploit the rapid degradation of damaged mtDNA with double-strand breaks (DSBs) via mtDNA replisome components.20,21 For instance, mitochondria-targeted DNA nucleases specific to mutant mtDNA, including mitochondrial endonucleases,15 mitochondrial transcription activator-like effector nuclease (mito-TALENs),16,18,19 mitochondrial zinc-finger nucleases (mito-ZFNs),19,21,22 and mitochondrial meganucleases,23,24 can specifically induce DSBs and promote the degradation of mutant mtDNA. These approaches can effectively shift the heteroplasmic level of mutant mtDNA but are limited by the availability of homoplasmic pathogenic mtDNA mutations. A bacterial cytidine deaminase fused with mito-TALEN (DdCBE) was recently established to induce base editing for C > T transition in mtDNA,25 with successful application in mice, rats, zebrafish, plants, and human embryos.26–31 More recently, small-sized zinc-finger deaminases (ZFDs) were engineered for precise C > T base editing of nuclear and mitochondrial genes,32–35 offering several advantages in therapeutic application. The same research team also developed an A > G base editor for mtDNA (TALED, transcription activator-like effector-linked deaminases), providing a broader scope for mtDNA editing.36 However, although base editors are promising tools for mtDNA editing, their substantial off-target effects on nuclear genes remain poorly resolved.37,38 Moreover, no current tools are able to induce or edit other types of mutations such as insertions, deletions, and transversions in mtDNA molecules.

The CRISPR-Cas9 system is a widely used tool for genome editing.36 The core principle is to introduce DSBs in the targeted genomic region, then complete genome editing via DSB repair pathways, including the non-homologous end joining (NHEJ), microhomology-mediated end joining (MMJE), and homologous recombination (HR) pathways.37 The CRISPR-Cas9 system is user friendly and flexible, and can circumvent the limitations of TALENs and ZFNs.38 However, the suitability and efficiency of mtDNA editing by CRISPR-Cas9 remains controversial.39 First, DSB repair is inefficient in mammalian mitochondria4,21 but is essential for successful editing by CRISPR-Cas9. Second, delivery of small-guide RNA (sgRNA) and Cas9 protein complexes into mitochondria is challenging. Several recent studies have attempted to manipulate mtDNA using the CRISPR-Cas9 system,40–45 which confirmed that the Cas9 protein can be transported into mitochondria, and that sgRNAs show mitochondrial localization.46 Most of these studies assessed successful manipulation of mtDNA mediated by CRISPR-Cas9 based on a decrease in mtDNA copy number40–43 and positive signals of allele-specific polymerase chain reaction (PCR) and/or PCR-based sequencing.44,45 Although these studies suggest the possibility of using CRISPR-Cas9 to manipulate mtDNA, PCR-based methods may be limited in their detection of edited mtDNA due to “template switching” or other technical artifacts.46–48 Furthermore, evidence showing successful mtDNA
We established the mito-Cas9 system by replacing the nuclear localization sequence (NLS) at the N terminus of Cas9 with the mitochondria-targeting signal (MTS) of MT-ND4, and the NLS at the C terminus of Cas9 with the mitochondria-targeting signal (MTS) of COX8A. 

In this study, we constructed a mito-Cas9 system, and confirmed that the system enabled successful knockin of exogenous single-stranded DNA oligonucleotides (ssODNs) into mtDNA using PCR-free third-generation sequencing technology. Furthermore, overexpression or activation of RAD51 significantly increased the knockin efficiency of the mito-Cas9 system. These results provide direct evidence of successful CRISPR-Cas9-mediated mitochondrial genome editing.

RESULTS
Mito-Cas9 system targeted mitochondria and decreased mtDNA content

We established the mito-Cas9 system by replacing the nuclear localization sequence (NLS) at the N terminus of Cas9 in the px330-mCherry vector with the MTS of COX8A, and the NLS at the C terminus of Cas9 with the 3′ untranslated region (UTR) of SOD2 (Figure S1). The MTSs and 3′ UTR of the respective mitochondrial genes efficiently mediated the mitochondrial localization of mRNA-49,50 Small-guide RNA, sgRNA1\(^{ND4}\) (Table S1), was designed to target m.11 697–11 716 in the M7-ND4 gene, with a G added to the 5′-position of the 20-bp guide sequence to obtain efficient U6 transcription of sgRNA and overall efficiency of the CRISPR-Cas9 system,\(^{37,51,52}\) and was cloned to create the mito-Cas9 construct sgRNA1\(^{ND4}\)-MTS\(^{COX8A}\)-Cas9-UTRSOD2 (Figure S1). Previous studies have shown that unmodified sgRNAs can co-fractionate with mitochondria,\(^{43,49,50}\) therefore we did not further modify the sgRNAs. We constructed other mito-Cas9 constructs with sgRNA2\(^{ND4}\) and MTSSs of mitochondrial genes COX10 and SOD2 using a similar strategy (Figure S1).

As expected, HEK293T cells transfected with the Cas9 construct containing the MTS of COX8A (sgRNA1\(^{ND4}\)-MTS\(^{COX8A}\)-Cas9-UTRSOD2) contained more Cas9 protein in the cytoplasmic components than cells transfected with the same amount of px330-mCherry vector or sgRNA APP-NLS-Cas9 vector (Figures 1A and 1B). We performed protease protection assays and confirmed the successful translocation of the Cas9 protein into the mitochondria of cells transfected with the mito-Cas9 system, albeit with low efficiency (Figure 1C). As shown in Figure 1C, the Cas9 proteins showed multiple bands (around 80–160 kDa) in samples with or without proteinase K treatment. A small fraction of the Cas9 protein with MTSSs, together with COXIV (positive control for mitochondrial inner membrane proteins), was protected from proteinase K digestion by the mitochondrial proteinase K for 30 min on ice, followed by western blotting, with 10 μg of crude mitochondrial fraction as a control. (D) Immunofluorescence assay showing mitochondrial localization of the Cas9 protein in the cytoplasmic components than cells transfected with the same amount of px330-mCherry vector or sgRNA APP-NLS-Cas9 vector (Figures 1A and 1B). We performed protease protection assays and confirmed the successful translocation of the Cas9 protein into the mitochondria of cells transfected with the mito-Cas9 system, albeit with low efficiency (Figure 1C). As shown in Figure 1C, the Cas9 proteins showed multiple bands (around 80–160 kDa) in samples with or without proteinase K treatment. A small fraction of the Cas9 protein with MTSSs, together with COXIV (positive control for mitochondrial inner membrane proteins), was protected from proteinase K digestion by the mitochondrial proteinase K for 30 min on ice, followed by western blotting, with 10 μg of crude mitochondrial fraction as a control. (D) Immunofluorescence assay showing mitochondrial localization of the Cas9 protein in
membrane. In contrast, the cytoplasmic protein β-actin and mitochondrial outer membrane protein MFN2 (used as negative controls) were completely digested by proteinase K (Figure 1C). Unlike COXIV, which was completely localized in the mitochondria, Cas9 (with or without MTSSs) was partially localized in the mitochondria and its protein level decreased significantly upon protease K treatment.

In the immunofluorescence assays, the Cas9 protein with MTSSs showed preferred co-localization with mitochondrial green fluorescent protein (Figure 1D).

Notably, despite the lack of NLS or MTSS, overexpression of the Cas9 protein was still observed in the nucleus and crude mitochondria (Figures 1A–1D). However, the reason for this Cas9 distribution pattern remains unknown.

We extracted mitochondria from HEK293T cells co-transfected with 6-carboxyfluorescein (FAM)-labeled sgRNA1N4D (100 bp) and pDsRed2-mito vector (expressing mitochondria-targeted red fluorescent protein [mito-RFP]). We observed significant co-localization of FAM-labeled sgRNA1N4D and mito-RFP (Figure 1E). We further used flow cytometry to quantify the proportion of labeled mitochondria in the total mitochondrial extract. FAM-labeled sgRNA1N4D signals were detected in the mitochondria labeled with mito-RFP, albeit at a relatively low frequency (Figure S2A). We observed similar results using MitoTracker to label mitochondria (Figure S2B). These results suggest that Cas9 with MTSSs and sgRNA can be (partially) transported into mitochondria.

Within cells, mtDNA with DSBS is rapidly degraded, therefore, a reduction in mtDNA copy number can be used as a marker for mtDNA manipulation.42-43 Consistent with previous studies,40-43 we observed a significant reduction in mtDNA copy number in HEK293T cells overexpressing the mito-Cas9 construct sgRNA1N4D-MTS-COX8A-Cas9-UTRSDO2 compared with cells overexpressing nuclear-targeted Cas9 (sgRNA1PP-NLS-Cas9) or non-targeted controls (MTS-COX8A, Cas9-UTRSDO2, mito-Cas9 without sgRNA) (Figure 1F).

Our further assessed the effect of the mito-Cas9 system on mitochondrial function. Cells transfected with mitochondria-targeted Cas9 showed significantly increased levels of reactive oxygen species (ROS) and significantly decreased levels of adenosine triphosphate compared with cells transfected with nuclear-targeted Cas9 or Cas9 without any targeting sequence (Figure S3A), indicating decreased mitochondrial function upon mito-Cas9 expression. To investigate whether the reduction in mtDNA copy number in mito-Cas9 transfected cells was caused by increased ROS, we determined the mtDNA copy number in cells treated with vitamin K3 (vitK3, reported to increase cellular ROS level53) and melatonin (reduced ROS, we determined the mtDNA copy number in cells treated with vitamin K3 (vitK3, reported to increase cellular ROS level53) and melatonin (reduced ROS, we determined the mtDNA copy number in cells treated with vitamin K3 (vitK3, reported to increase cellular ROS level53) and melatonin (reduced ROS). We found that the mito-Cas9 system significantly diminished levels of ROS compared with cells transfected with ssODN1 significantly diminished with time and was barely detected at 144 h (Figure S7). In contrast, cells transfected with ssODN1 and the mito-Cas9 system contained a significantly higher level of the EcoRI site-specific PCR product relative to cells transfected with ssODN1 alone at each time point (Figure S7). Of note, a reduction in the EcoRI site-specific PCR product was observed with time (Figure S7), which might be attributed to the higher proliferation rate of cells with wild-type mtDNA compared with cells with edited mtDNA.

To further exclude potential contamination from other sources of mtDNA (e.g., leakage of mtDNA fragments in cytosol from damaged mitochondria) or mtDNA-like fragments (e.g., nuclear mitochondrial pseudogenes in the nuclear genome) outside the mitochondria, which could potentially be recognized as a target by the mito-Cas9 system, we performed a DNase protection assay to validate the successful knockin of exogenous ssODNs into mtDNAs located within the mitochondria (Figure 2F). Upon DNase I treatment, no PCR product could be amplified for the nuclear APP gene (Figure 2F), whereas the mtDNA PCR product (amplified by primer pair L11338/H11944) and EcoRI site-specific PCR product were visible, indicating that exogenous ssODNs were inserted into mtDNA and were protected from DNase I digestion by the mitochondrial membrane (Figure 2F).

Quantification of the EcoRI site-specific PCR products showed that the mito-Cas9 construct sgRNA1N4D-MTS-COX8A-Cas9-UTRSDO2 significantly increased the knockin efficiency of ssODN1 in both crude mtDNA (DNase−) and purified mtDNA (DNase+) (Figure 2B). The EcoRI site-specific PCR product was also detected when the target and knockin sites were changed (Figures S4B–S4D). Compared with cells transfected with ssODN2 alone or with a combination of ssODN2 and MTS-COX8A-Cas9-UTRSDO2 (no sgRNA), cells transfected with a combination of sgRNA2N4D-MTS-COX8A-Cas9-UTRSDO2 and ssODN2 showed a significant increase in EcoRI site-specific PCR product (Figure S4D), indicating that mito-Cas9 system-mediated knockin can be applied to any site suitable for ORISPR-Cas9 editing. These results demonstrate that the mito-Cas9 system can mediate knockin of exogenous ssODNs into mtDNA through the HR repair pathway.

### Third-generation sequencing verified knockin of exogenous ssODNs into mtDNA through mito-Cas9 system

To exclude the possibility that the EcoRI site-specific PCR products were caused by PCR artifacts, such as template-switching artifacts46-48 between exogenous ssODNs and mtDNA, we performed PCR-free third-generation sequencing to verify the knockin of exogenous ssODNs into mtDNA (Figure 3A). The ssODN1 donor template and mito-Cas9 construct sgRNA1N4D-MTS-COX8A-Cas9-UTRSDO2 were co-transfected into HEK293T cells for 48 h. Cells transfected with ssODN1 only or with a combination of ssODN1 and MTS-COX8A-Cas9-UTRSDO2 (no sgRNA) were considered as controls. BamHI digestion was used to linearize purified ssODN1.
mtDNA isolated from DNase I-treated mitochondria (Figure 3A). Total genomic DNA was completely digested by BamH I and showed no clear bands on the gel, whereas successfully linearized mtDNA was evidenced by a single band of mtDNA after BamH I digestion (Figure S8A). We also measured the efficiency of linearization by quantifying the mtDNA fragment flanking the BamH I site using qRT-PCR with the primer pair L14054/H14573 (Table S1; linearized mtDNA could not be amplified by L14054/H14573) and observed a significant decrease in the PCR product for purified mtDNA subjected to BamH I compared with undigested samples (Figure S8B), indicating that mtDNA was thoroughly linearized. Direct sequencing of linearized mtDNA using PCR-free PacBio sequencing technology yielded a distinct sequence peak at /C24 kb (Figure 3B, left), indicating full-length capture and sequencing of the mtDNA genome. Reads Mapping using the

Figure 2. Mito-Cas9 system mediated efficient knockin of exogenous ssODNs into mtDNA (A) Design of mito-Cas9-mediated knockin system. (B) Quantification of knockin efficiency of mito-Cas9. (Upper) Results of qRT-PCR. HEK293T cells were transfected with or without a combination of Cas9 constructs and ssODN1. Content of mtDNA with successful knockin of EcoRI site (edited mtDNA, amplified by primer pair L11338/EcoRI-R) was normalized to whole mtDNA (total mtDNA, amplified by primer pair L394/H475) using total genomic DNA as the template. (Below) Agarose gel image showing representative PCR product for each group of cells with different transfections. (C) Knockin efficiency of different mito-Cas9 constructs with catalytically dead Cas9 (dCas9): sgRNA1ND4-MTSCOX8A-dCas9-UTRSOD2, expressing SpCas9 protein with mutations p.D10A and p.H840A. Knockin efficiency was quantified using the same qRT-PCR procedure as in (B), with total genomic DNA from respective transfected cells as the template. (D) Sequencing chromatogram of EcoRI site-specific PCR product. Targeting region of sgRNA1ND4 is marked with a box on the mtDNA revised Cambridge reference sequence (rCRS). (E) Knockin efficiency of different mito-Cas9 constructs with different MTSs. HEK293T cells were transfected with ssODN1 and mito-Cas9 construct fused with indicated MTSs of mitochondrial genes COX8A, COX10, or SOD2. Knockin efficiency was quantified using the same qRT-PCR procedure as in (B), with total genomic DNA from respective transfected cells as the template. NC, cells without transfection. (F) DNase protection assay confirmed the mitochondrial source of EcoRI site-specific PCR product amplified from edited mtDNA in mitochondria. Crude mitochondria were extracted from HEK293T cells transfected with sgRNA1ND4-MTScox8A-Cas9-UTRSOD2 and ssODN1 for 48 h, then treated with DNase I at 37°C for 1 h. PCR amplifications for nuclear APP (amplified by primer pair APP-F/APP-R), total mtDNA (amplified by primer pair L11338/H11944), and edited mtDNA (amplified by primer pair L11338/EcoRI-R) were performed using mtDNA template extracted from mitochondria with or without DNase I treatment, respectively. (G) Quantification of knockin efficiency in mtDNAs from crude mitochondria treated with DNase I (DNase+) or without DNase I (DNase−). Bars are mean ± SD. ns, not significant; **p < 0.01, ***p < 0.001, ****p < 0.0001; one-way ANOVA test adjusted by Tukey’s multiple comparisons test for (B, C, and E); two-tailed Student’s t test for (G).
mtDNA reference sequence and nuclear mitochondrial (NUMTs) reference sequences showed that most reads mapped to NUMTs were <16 kb (Figure 3C, left). Therefore, we only used reads >16 kb in length, and with higher mapping percentage and concordance to mtDNA than to NUMTs, for the following analyses, thus reliably excluding the potential effects of NUMTs and other artifacts. We identified precise insertion of GAATTC at the sgRNA1ND4 target site in mtDNA reads from the entire mitochondrial genome (Figure 3D). Of note, a higher frequency of GAATTC insertion at the sgRNA1ND4 targeting site was observed for cells transfected with sgRNA1ND4-MTSCOX8A-Cas9-UTRSOD2 + ssODN1 (2/411,780, 0.0047%) than for control cells transfected with MTSCOX8A-Cas9-UTRSOD2 (no sgRNA) + ssODN1 (3/22,995, 0.0087%) (Figure 3E, top). In both samples, we observed randomly distributed GAATTC insertions across the mtDNA genome at extremely low frequencies (Figure 3E, top), which may be caused by potential errors in the third-generation sequencing technology. To exclude the possibility that these targeted insertions were introduced by sequencing errors in PacBio Sequel II, we used another PCR-free sequencing method, Nanopore sequencing, to sequence mtDNAs extracted from cells transfected with sgRNA1ND4-MTSCOX8A-Cas9-UTRSOD2 + ssODN1. In the captured reads across the entire mtDNA genome (peak near 16,569 bp; Figures 3B and 3C, right), we confirmed a higher rate of targeted GAATT insertion in cells transfected with sgRNA1ND4-MTSCOX8A-Cas9-UTRSOD2 + ssODN1 (2/411,780) compared with cells transfected with ssODN1 alone (1/314,840) (Figure 3E, bottom). We observed several untargeted GAATT insertions across the mtDNA genome in the PacBio data (Figure 3E, top), but these insertions were not replicated in the Nanopore sequencing data (Figure 3E, bottom), suggesting that most untargeted insertions were introduced by PacBio sequencing errors. Further analysis of the number of targeted insertions in the mtDNA sequencing data of different lengths. We identified a higher frequency of precise GAATT insertions in mtDNA sequences larger than 10 kb at the sgRNA1ND4 target region in cells transfected with sgRNA1ND4-MTSCOX8A-Cas9-UTRSOD2 + ssODN1 (n = 6) than in cells transfected with ssODN1 alone (n = 2) (Figure 3F). Overall, the total numbers of targeted insertions in the mtDNA sequences with different length cutoffs (from 10 to 16 kb) were consistently higher in cells transfected with the mito-Cas9 system than cells transfected with ssODN1 alone (Figure 3F). Therefore, the higher rate of targeted insertions in cells transfected with the mito-Cas9 system is unlikely to be due to random sequencing errors, but may be indicative of the editing capability of the mito-Cas9 system. The occurrence of targeted insertions in cells transfected with ssODN1 alone, even at a very low frequency, deserves further attention and focused study in the future, which may suggest an
unprecedented role of ssODN1 in the initiation of mtDNA replication in the absence of the mito-Cas9 system (Bi et al., unpublished data).

**RAD51 activation enhanced mito-Cas9 system knockin efficiency**

We further tested whether modulating those factors involved in genome stability maintenance and repair pathways could improve the knockin efficiency of the mito-Cas9 system. HR is essential for maintaining mitochondrial genome integrity.58,66,67 Key factors in the HR pathway, such as RAD51 and XRCC3, can be recruited to mitochondria and participate in mtDNA maintenance under DNA damage stress.58,66,67 We confirmed the presence of RAD51 in mitochondria using a proteinase protection assay (Figure 4A). As our mito-Cas9 system was based on HR-mediated events, we hypothesized that enhancing RAD51 function would increase the knockin efficiency of ssODNs into mtDNA, as shown by the Cas9-mediated knockin efficiency for nuclear DNA.68-70 We treated HEK293T cells transfected with the mito-Cas9 construct sgRNA1<sup>ND4-L</sup>-MTSCOX8A-Cas9-UTRSOD2 and/or ssODN1 with RAD51 agonist RS-1 (10 μM) for 42 h, then quantified the level of edited mtDNA (Figure 4B). Stimulation of RAD51 with RS-1 significantly increased the knockin efficiency of the mito-Cas9 system (Figure 4B), whereas RS-1 had no significant effect on cells transfected with ssODN1 alone (Figures 4B and S9A) or on the other three controls (mito-Cas9 without ssODN1, mito-Cas9 without sgRNA, and mito-Cas9 with sgRNA2<sup>ND4</sup>-targeting another region) (Figures 4B and S9A). Overexpression of the RAD51 protein resulted in a significant increase in the knockin efficiency of the mito-Cas9 system, similar to the treatment of the RAD51 agonist (Figures 4C and S9B). However, knockdown of RAD51 or inhibition of RAD51 by RI-1<sup>71</sup> had no or a weak effect on the knockin efficiency of the mito-Cas9 system (Figures 4C, 4D, S9C, and S9D), suggesting a potential compensatory effect in cells with RAD51 knockdown or inhibition during the maintenance of genome stability. Interestingly, an increase in the EcoRI site-specific PCR product signal was observed in cells transfected with unexpected role of ssODN1 in the initiation of mtDNA replication in the absence of the mito-Cas9 system (Bi et al., unpublished data).

We further quantified the knockin efficiency of the mito-Cas9 system using second-generation sequencing technology. Using a primer pair outside of ssODN1, we amplified a fragment flanking the knockin site (m.11 600–11 820) in crude and purified mtDNAs (Table 1; Figure S9E), respectively, then subjected the PCR products to second-generation sequencing. Knockin of ssODNs was identified in the sequencing reads (Table 1; Figure S9E). Consistent with the qRT-PCR results, HEK293T cells transfected with the mito-Cas9 construct sgRNA1<sup>ND4-L</sup>-MTSCOX8A-Cas9-UTRSOD2 and ssODN1, RS-1 treatment resulted in a nearly 5-fold increase in knockin efficiency in the crude mtDNA (0.14%) relative to that in cells without RS-1 treatment (0.03%; p < 2.2 × 10<sup>-16</sup>; Fisher’s exact test) (Table 1). Knockin frequency further increased in the purified mtDNA (0.23%; p < 2.2 × 10<sup>-16</sup>; Fisher’s exact test) (Table 1). Of note, the relative number of sequence reads with knockin was significantly lower (p < 2.2 × 10<sup>-16</sup>; Fisher’s exact test) in cells transfected with ssODN1 alone than in cells transfected with mito-Cas9 constructs and ssODN1 (Table 1). Furthermore, no significant changes in knockin efficiency were observed in the ssODN1-transfected cells with or without RS-1 treatment, suggesting that sequence reads with GAATTC knockin in cells transfected with ssODNs alone were not HR mediated (Table 1).

Collectively, these results suggest that RAD51 is a key factor for the knockin of exogenous ssODNs into mtDNA. Small-molecule RS-1 increased knockin efficiency of the mito-Cas9 system through RAD51 activation, further supporting HR-mediated knockin of exogenous ssODNs into mtDNA. Although template switching artifacts or other potential factors may introduce some noise in regard to the interpretation of knockin frequency, this noise is unlikely to have contributed to the significant differences observed between groups.

**Lack of evident off-target sites in nuclear genome by the mito-Cas9 system**

We performed whole-genome sequencing (WGS) for genomic DNA isolated from HEK293T cells transfected with sgRNA1<sup>ND4-L</sup>-MTSCOX8A-Cas9-UTRSOD2 and ssODN1 and sgRNA2<sup>ND4-L</sup>-MTSCOX8A-Cas9-UTRSOD2 + ssODN2, respectively. Mean sequence depth for the two samples was about 34×. The nuclear genome was screened for potential off-target sites with ‘NGG’ or ‘NAG’ protospacer adjacent motif and with up to nine mismatches relative to sgRNA. No insertions or
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Table 1. Frequency of reads with GAATTC knockin based on second-generation sequencing

| Sample                          | Total no. of reads | No. of reads with GAATTC insertion | Knockin ratio |
|--------------------------------|--------------------|-----------------------------------|--------------|
| Crude mtDNA                    |                    |                                   |              |
| ssODN1                         | 5,028,392          | 343                               | 0.00006821   |
| ssODN1 + RS-1                  | 6,838,719          | 324                               | 0.00004738   |
| mito-Cas9 + ssODN1             | 5,367,579          | 1608                              | 0.00029958   |
| mito-Cas9 + ssODN1 + RS-1      | 6,208,098          | 8839                              | 0.00142379   |
| Purified mtDNA                 |                    |                                   |              |
| ssODN1 + RS-1                  | 5,991,432          | 210                               | 0.00003505   |
| mito-Cas9 + ssODN1 + RS-1      | 4,992,779          | 11,714                            | 0.000234619  |

Counts for sequencing reads with the GAATTC insertion in a short PCR fragment (region m.11 600–11 820 in mtDNA) flanking the knockin site. Crude mtDNA and purified mtDNA were used as respective templates for PCR, and PCR products were sequenced by second-generation sequencing technology. Crude mtDNA, mtDNAs isolated from crude mitochondria in HEK293T cells transfected with ssODN1 alone, mito-Cas9 system (sgRNA^N-D^MTSCoxA-Cas9-UTP^D^D2 and ssODN1), and with or without RS-1 treatment; purified mtDNA, mtDNAs isolated from crude mitochondria after DNase I digestion.

deletions were identified in the potential off-target sites of sgRNA^N-D^ and sgRNA^D^ (Table S2). We further screened the whole genome for GAATTC insertions using the WGS data. No nuclear DNA reads with the GAATTC insertion were detected, indicating that GAATTC knockin in the mtDNA reads of third-generation sequencing data was not caused by potential off-target and/or unspliced knockin in nuclear DNA or from NUMTs.²²

mtDNA pathogenic mutation generation using the mito-Cas9 system

Intragenic inversion mutation m.3902_3908INV (m.3902_3908 ACCTTGC>GCAAAGT) in the MT-ND1 gene is reported to cause fatal infantile lactic acidosis and mitochondrial myopathy.⁷³,⁷⁴ At present, however, the underlying mechanism remains unclear. Here, using the same strategy, we designed the mito-Cas9 system to introduce the m.3902_3908inv mutation into HEK293T cells (Figure 5A). Successful knockin of ssODN^D2^ in mtDNA within the mitochondria was demonstrated using a DNase protection assay (Figure 5B). Consistent with previous observations from the ssODN^N-D^ and ssODN^N-N^ knockin experiments, cells co-transfected with the mito-Cas9 constructs and ssODN^D2^ showed a significant increase in m.3902_3908INV-specific PCR products compared with cells transfected with ssODN^N-D^ alone or with a combination of ssODN^D2^ and MTSCoxA-Cas9-UTP^D^D2 (no sgRNA) (Figure 5C). In addition, mutation m.3902_3908inv was identified in the second-generation sequencing reads (Figure 5D). The knockin efficiency of mutation m.3902_3908inv was about 0.05% (2,432 reads successfully edited among 5,284,619 raw reads). These results suggest that the mito-Cas9 system may serve as a promising approach for targeted knockin in mtDNA and could be optimized as a workable model to establish cellular models for studying mtDNA pathogenic mutations.

DISCUSSION

Currently, whether mtDNA can be edited by CRISPR-Cas9 remains controversial.⁶⁹,⁷⁰,⁷¹ Due to the lack of NEHEJ repair, mtDNA with DBSs degrades rapidly,⁷² resulting in a reduction in mtDNA copy number, and thus mtDNA-editing products have not been directly detected in previous research.⁴⁰,⁴⁷ In this study, in addition to adding MTSs to Cas9,⁴⁰,⁴¹ we optimized the mitochondria-targeted Cas9 system by adding the 3’ UTR of the SO2 gene to the downstream region of the Cas9 gene to facilitate efficient mitochondrial localization of Cas9 mRNA. We confirmed that the developed mito-Cas9 system transported Cas9 into the mitochondria (Figure 1C) and enabled mtDNA manipulation. Importantly, we developed a PCR-free third-generation sequencing technology that effectively avoids artifacts caused by PCR amplification (e.g., template switching artifacts) and verified the accurate knockin of exogenous ssODNs into mtDNA using the mito-Cas9 system (Figure 3). Another straightforward approach to confirm the effectiveness and efficiency of mitochondria-targeted CRISPR-Cas9 system can be engineered by introducing mtDNA-based drug resistance. Currently, we are attempting to establish a drug-resistant cell line with the mtDNA mutation m.2991T>C in the 16S rRNA of mtDNA, which could facilitate selection by chloramphenicol.⁷⁶

Previous studies have shown that homologous arms longer than 40 bp exhibit higher HR efficiency than shorter arms.⁴⁷,⁵⁸ Based on this observation, we designed the homologous arms of three ssODNs (ssODN1, ssODN2, and ssODN^D2^), which were all 45 bp in length. We also investigated the knockin efficiency of ssODN^D2^ with shorter arms (22 bp). Results showed that the knockin efficiency of ssODN^D2^ was significantly lower than that of ssODN1 (Figure S10). Thus, the knockin efficiency of ssODN may be affected by its length, and optimizing homologous arm length to balance the stability and mitochondrial transport efficiency of ssODN could be helpful for increasing the knockin efficiency of the mito-Cas9 system.

The mechanism that maintain mitochondrial genome stability remain elusive. Notably, for the two main DNA repair pathways, NEHEJ cannot be detected in mitochondria and the existence of HR in mitochondria is controversial.⁵⁹,⁶⁰,⁶¹,⁶²,⁶³ Consistent with previous study,⁶³ we found that RAD51, a key nuclear factor of the HR repair pathway, was translocated into the mitochondria (Figure 4A) and its activation with agonist RS-1 enabled a 2- to 5-fold increase in knockin efficiency of ssODNs into mtDNA (Figure 4B). In addition, the effects of RAD51 activation appeared to be specific to cells transfected with the mito-Cas9 system (Figure 4, Table 1). These findings and third-generation sequencing results suggest that mtDNA can be affected by the HR pathway, and activation of the HR pathway by RAD51 stimulation may enhance CRISPR-Cas9-mediated knockin in mtDNA. One unresolved question is how ssODN is imported into mitochondria, which requires further research. Several studies have shown that DNA and RNA can be transported into mitochondria in animal cells and in plants,⁷⁴,⁷⁵,⁷⁶ and our study provides further evidence that FAM-labeled sgRNA and ssODN1 can be transported into mitochondria (Figures 1E, S2, and S6A).

Enthusiasm for mtDNA editing stems from the clinical need for the treatment of mitochondrial diseases caused by pathogenic mtDNA mutations, most of which are in a heteroplasmic state. Both mito-ZFN⁷⁷,⁷⁸,⁷⁹ and mito-TALEN⁸⁰,⁸¹ are effective in altering the heteroplasmic level of mutant mtDNA. For homoplasmic mtDNA mutations, base editing⁷¹,⁸² and CRISPR-Cas9-mediated HR knockin can be used to edit mutant mtDNA. However, one of the key problems with mtDNA editing is that each cell may have hundreds to thousands of mtDNA copies and the editing of each copy is unlikely. Thus, employing mtDNA-editing technology to cure mitochondrial diseases caused by mtDNA mutations remains a considerable challenge. In our study, knockin efficiency of the mito-Cas9 system was rather low (0.03%–0.23%) compared with the recently developed DdCBE method (5%–50%).⁵⁷ We speculate that the low efficiency may be due to inefficient mitochondrial transport of the editing system and limited editing efficiency of the Cas9 protein to mtDNA. Therefore, mito-Cas9 system optimization, either by improving the mitochondrial transport efficiency such as mitochondrial RNA transport (although we did not achieve better mtDNA editing efficiency in cells overexpressing PNPASE, which can regulate RNA-import to mitochondria,⁸³ compared with cells overexpressing RAD51 [data not shown]) or by using engineered Cas proteins with higher editing efficiency,⁵² is essential for the application of this mtDNA editing system. Furthermore, the knockin efficiency varied for different types of mutations, which may be due to different sequence features of the targeted region (i.e., GC% content) or different targeting efficiencies of the sgRNAs. Despite its limited editing efficiency, the mito-Cas9 system has the potential for wider scope of variant replacement and can introduce accurate knockin of target variants without changing other loci in the same editing window, thereby serving as an alternative strategy for manipulating mtDNA, especially considering recent findings that mitochondrial base editors may induce extensive off-target editing in the nuclear genome.⁴³,⁴⁵ In addition, inducing a small fraction of wild-type mtDNA using the mito-Cas9 system, then altering the heteroplasmic level of the wild-type mtDNA using mito-ZFN or mito-TALEN technology, offers a promising strategy for editing homoplasmic pathogenic mtDNA mutations.
In conclusion, we established an mtDNA editing system based on CRISPR-Cas9-mediated knockin via the HR pathway and found that RAD51 agonist RS-1 significantly enhanced mtDNA knockin efficiency. Using PCR-free third-generation sequencing, we provide direct evidence for mtDNA editing mediated by the CRISPR-Cas9 system. Future studies devoted to increasing editing efficiency are essential for expanding the application and safety of the mito-Cas9 system in the treatment of mitochondrial diseases caused by pathogenic mtDNA mutations.

**MATERIALS AND METHODS**

Detailed materials and methods are included in the supplemental information.

**DATA ACCESS**

The data that support the findings of this work are available from the corresponding author upon reasonable request. The sequencing data were deposited at GSA (https://rgdc.cncb.ac.cn/gsa/) under accession number HRA001435.

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**Figure 5. Introduction of pathogenic mutation m.3902_3908inv to mtDNA of HEK293T cells**

(A) Design of sgRNA and mito-Cas9-mediated knockin of mtDNA mutation m.3902_3908inv. (B) Site-specific PCR of mutation m.3902_3908inv. Crude mitochondria were extracted from HEK293T cells transfected with sgRNA3902-targeted region (amplified by primer pair 3902F/H4227) were performed using the DNA template extracted from mitochondria with or without DNase I treatment. (C) Quantification of knockin efficiency of mutation m.3902_3908inv. HEK293T cells were transfected with or without a combination of Cas9 constructs and ssODN3902. Proportion of mtDNA with successful m.3902_3908inv knockin (amplified by 3902F/H4227 (3902F-H4227)) was normalized to whole mtDNA (amplified by L394-H475). (D) Detection strategy and presence of m.3902_3908inv knockin in mtDNA reads generated by second-generation sequencing.
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AUTHOR CONTRIBUTIONS
Y.-G.Y., R.B., X.H., H.Z., and P.Z. designed the study. R.B. and Y.L. constructed the mito-Cas9 system. M.X. performed third-generation sequencing and analyzed the data. Q.Z., B.X., and X.Z. extracted DNA and performed PCR analysis. D.-F.Z. and X.L. carried out secondary generation sequencing and analyzed the results. R.B. and G.M. performed flow cytometry. Y.-G.Y., R.B., M.X., and Y.L. wrote the first draft of the paper. All authors contributed to and approved the final version of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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LEAD CONTACT WEBSITE
http://sourcedb.kiz.cas.cn/yw/zjrc/sc/200908/t20090825_2446617.html.
Supplemental Information

Direct evidence of CRISPR-Cas9-mediated mitochondrial genome editing

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Online Supplemental Information

Materials and Methods

Construction of mitochondrial-targeting CRISPR/Cas9 system
In this study, mito-Cas9 was constructed using the px330-mCherry vector (Addgene plasmid #98750) 1. Briefly, the nuclear localization sequence (NLS) and 3’×flag sequences at the N-terminus of SpCas9 in the px330-mCherry vector were replaced with the mitochondrial-targeting sequence (MTS) of mitochondrial genes (COX8A, COX10, or SOD2), and the NLS at the C-terminus of SpCas9 was replaced with the 3’-UTR of the SOD2 gene using a ClonExpress® MultiS One Step Cloning Kit (Vazyme, C113) (Figure S1). Two sgRNAs targeting the MT-ND4 gene, including sgRNA1ND4 targeting the mtDNA region 11,697-11,716 (mt.11,697-11,716) and sgRNA2ND4 targeting m.11,851-11,868 (Table S1), were designed using the Breaking-Cas tool (https://bioinfogp.cnb.csic.es/tools/breakingcas) 2 and cloned into the mito-Cas9 constructs. Two ssODNs (ssODN1, relative to sgRNA1ND4; ssODN2, relative to sgRNA2ND4) were designed, with each having a 45 bp homologous arm flanking a 6 bp insertion of the EcoRI restriction site “GAATTC” (Table S1). The sgRNA targeting the nuclear APP gene (coding amyloid-beta precursor protein) (sgRNAAPP) was cloned to the px330-mCherry vector to obtain the nuclear targeting Cas9 construct sgRNAAPP-NLS-Cas9. All constructs were validated by sequencing. Overall, the mito-Cas9 system designed in this study contained three main elements: 1) MTS of a mitochondrial gene (COX8A, COX10, or SOD2) inserted at the N-terminus of Cas9; 2) 3’-UTR of SOD2 inserted downstream of Cas9; and 3) sgRNA (sgRNA1ND4 or sgRNA2ND4) targeting mtDNA. Constructs of MTS−COX8A−Cas9-UTR−SOD2 (no sgRNA) and sgRNA1ND4−MTS−COX8A−dCas9-UTR−SOD2 (catalytically dead Cas9 (dCas9), SpCas9 with mutations p.D10A and p.H840A) 3 were used as controls (Figure S1). The dCas9 was sub-cloned from the pST1374-N-NLS-flag-linker-Cas9-D10A vector 4 with mutation p.H840A that was provided by Dr. Jiankui Zhou.

In order to further demonstrate the feasibility of introducing the m.3902_3908inv mutation using the mito-Cas9 system, we designed sgRNA targeting to the m.3892_3918 region of the MT-ND1 gene and a ssODN containing this pathogenic mutation (ssODN3902) using the same strategy (Table S1).

Cell culture, transfection, and sorting
The HEK293T cells were obtained from the Kunming Cell Bank, Kunming Institute of Zoology, and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher) at 37 °C in 5% CO2. Cells were seeded in a 6-well plate at a density of 5×105 cells/well for 12 h before transfection. The mito-Cas9 constructs (2.5 µg each) were transfected with or without ssODN (50 pmol each) into cells using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer’s protocols. Co-transfection of 6-carboxyfluorescein (FAM)-labeled sgRNA1ND4/ssODN1 and pDsRed2-mito vector (Clontech, which expresses mitochondrial targeting red fluorescent protein, mito-RFP) were performed using the same strategy. At 48 h after transfection, cells were harvested and analyzed using flow cytometry (BD, Influx, USA) at 610 nm to detect cells with successful transfection of the mito-Cas9 constructs or the pDsRed2-mito vector, and at 535 nm to detect cells with successful transfection of FAM-labeled sgRNA1ND4/ssODN1.

Proteinase and DNase protection assays
Crude mitochondrial preparations were isolated using a Mitochondria Crude Isolation Kit (GMS10006, GENMED, China). For the proteinase protection assay, crude mitochondrial fraction (20 µg) was treated with 50 µg/mL proteinase K (Axygen) for 30 min on ice to remove proteins outside the mitochondria, followed by treatment with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, P7626) to stop the proteinase K reaction and collect purified mitochondria.
For the DNase protection assay, crude mitochondrial fraction (20 μg) was treated in 50 μL of reaction buffer containing 0.5 U/μL DNase I (Takara) at 37 °C for 1 h to remove DNA molecules outside the mitochondria, with purified mtDNA then extracted using an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen). PCR amplifications of nuclear APP gene (amplified by primer pair APP-F/APP-R), total mtDNA (amplified by primer pair L11338/H11944), and edited mtDNA (amplified by primer pair L11338/EcoRI-R, or 3902F/H4227) were performed using the DNA template extracted from mitochondria before and after DNase I treatment, respectively. The PCR reactions were conducted in a total volume of 20 μL containing 1× PCR buffer, 1 unit of LaTaq (TaKaRa), 175 μmol/L of each dNTP, 0.2 μmol/L of each primer (Table S1), and about 50 ng DNA template. The following PCR procedures were used: a pre-denaturation cycle at 94 °C for 5 min; 35 amplification cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 35 s; and a final extension cycle at 72 °C for 7 min.

Western blotting
Nuclear and cytoplasmic components from the HEK293T cells transfected with the mito-Cas9 constructs (2×10⁶ cells for each construct) were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, P0027) following the manufacturer’s instructions. For collection of total cell protein, the HEK293T cells were lysed in cell lysis buffer (Beyotime, China, P0013) and protein concentration was determined using a BCA Protein Assay Kit (Beyotime, P0012). Protein (20 μg) was separated using 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, 162-0177). After blocking with 5% (w/v) non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h at room temperature, the membrane was incubated with respective primary monoclonal antibodies overnight at 4 °C. After three washes with TBST, the membrane was incubated with anti-mouse/rabbit IgG peroxidase-conjugated secondary antibodies (KPL), then the epitope was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500). Primary antibodies included antibodies against Cas9 (Cell Signaling Technology, 14977T), Flag tag (Abmart, M20008L), ATP5A (Proteintech, 14675-1-AP), H3 (Cell Signaling Technology, 4499S), GAPDH (Proteintech, 60004-1-lg), MFN2 (Proteintech, 12186-1-AP), COXIV (Cell Signaling Technology, 4850P), β-actin (Abmart, P30002F), RAD51 (Proteintech, 14961-1-AP) and α-tubulin (Enogene, E1C601). ImageJ (National Institutes of Health, Bethesda, MD) was used to quantify the protein expression level.

Quantification of mtDNA copy number and knock-in efficiency
Quantitative real-time PCR (qRT-PCR) was performed to measure the mtDNA copy number and knock-in efficiency of the mito-Cas9 system using the 2^-ΔΔCT method, as described in our previous study 5. In brief, mtDNA content was measured using primer pairs L394/H475 and L11718/H11944 (Table S1) and was normalized to a single-copy nuclear β-globin gene measured with primer pair HBB502f/HBB614r to determine the relative mtDNA copy number 5. The proportion of mtDNA with successful knock-in of the EcoRI site “GAATTC” was measured using the EcoRI site-specific primer pair L11338/EcoRI-R (Table S1) and normalized to total mtDNA content measured using primer pair L394/H475. The ratio of mtDNA with the EcoRI site “GAATTC” relative to total mtDNA content was used to determine the knock-in efficiency of the mito-Cas9 system. A total of 20 ng of DNA was subjected to qRT-PCR using iTaq Universal SYBR Green Supermix (172-5125; Bio-Rad Laboratories) with the above indicated primer pairs on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories). The knock-in efficiency of ssODN2 and ssODN3 were determined with the same strategy (Table S1).

Examine the effect of RAD51 modulation on the knock-in efficiency mediated by mito-Cas9 system
We used RAD51 agonist 6 RS-1 (Sigma, R9782) and inhibitor 7 RI-1 (Merck Millipore, 553514) to activate and inhibit the RAD51 activity, respectively, and tested the potential
effect on the knock-in efficiency of the mito-Cas9 system. Briefly, RS-1 and RI-1 were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and 20 mM, respectively. After the HEK293T cells were transfected with the mito-Cas9 constructs for 6 h, the medium was changed with fresh growth medium supplemented with 10 μM RS-1, 20 μM RI-1, or an equal volume of DMSO (negative control). Cells were harvested for subsequent assays at 48 h post-transfection. We also evaluated the effect of RAD51 overexpression or knockdown on the knock-in efficiency of the mito-Cas9 system. The HEK293 cells were grown in 6-well plate for transfection of RAD51 overexpression vector (pcDNA3.1-RAD51, 1.25 μg/well) (Public Protein/Plasmid Library) or control vector (pcDNA3.1, 1.25 μg/well), together with mito-Cas9 construct sgRNA1ND4-MTS_COX8A-Cas9-UTR_SOD2 (1.25 μg/well) and ssODN1 (50 pmol/well). For knockdown assay, control siRNA (siRNA NC, 25 pmol/well) or siRNA targeting RAD51 mRNA (siRNA RAD51, 25 pmol/well) (Table S1) was co-transfected with mito-Cas9 construct sgRNA1ND4-MTS_COX8A-Cas9-UTR_SOD2 (2.5 μg/well) and ssODN1 (50 pmol/well). Transfections were performed using Lipofectamine 3000 (Thermo Fisher) according to the manufacturer’s protocols. Cells were harvested at 48 h after transfection for evaluating the knock-in efficiency.

Second-generation sequencing
We used second-generation sequencing technology (for mtDNA region m.11 600-11 820 amplified using mtDNA from HEK293T cells transfected with mito-Cas9) and third-generation sequencing technology (for mtDNA isolated from mitochondria and without PCR amplification) to identify edited mtDNA. For second-generation sequencing, purified mtDNA (mtDNA extracted from DNase I-treated mitochondria) and crude mtDNA (mtDNA extracted from crude mitochondria) from HEK293T cells transfected with or without a combination of the mito-Cas9 construct sgRNA1ND4-MTS_COX8A-Cas9-UTR_SOD2 and ssODN1 were used as templates for amplifying the mtDNA region m.11 600-11 820, which contained the potential knock-in of the EcoRI site (Table S1). The library was constructed using PCR products for paired-end sequencing on the Illumina NovaSeq platform. Raw reads were trimmed to remove sequencing adapters and low-quality reads using fastp v0.20.0. The clean reads were aligned to the revised Cambridge reference sequence (rCRS, GenBank Accession No. NC_012920) using Burrows-Wheeler Aligner (BWA) v0.7.17-r1188. Mapped reads <101 bp long were discarded to avoid potential noise from ssODNs (with a length of 96 bp), which might exist in the mtDNA extracts. Reads with the “GAATTC” insertion at the target region of sgRNA1ND4 were extracted using an in-house Perl script, which was available at the MitoTool (mitotool.kiz.ac.cn) web server (http://mitotool.kiz.ac.cn/lab/Extract_readswith_GAATTC_insertion.pl). Sequence depth for each library was estimated using SAMtools v1.7. A fragment covering region m.3809-4058 was amplified and was subjected to second-generation sequencing using the same strategy to estimate the knock-in efficiency of ssODN_SOD2.

Third-generation sequencing
A total of 5×10⁷ HEK293T cells were transfected with a combination of the mito-Cas9 construct sgRNA1ND4-MTS_COX8A-Cas9-UTR_SOD2 and ssODN1 for 48 h before harvesting to isolate crude mitochondria. We treated crude mitochondria with DNase I at 37 °C for 1 h to remove any DNA molecules outside the mitochondria, with the digested fraction then subjected to DNA extraction using an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen) to obtain purified mtDNA. Around 10 μg of purified mtDNA was linearized by BamHI (100 U at 37 °C for 1 h, R0136S, New England Biolabs) at position m.14 258, followed by library construction based on the standard protocols for the Single-Molecule Real-Time (SMRT) long-read sequencing developed by Pacific Biosciences (PacBio). Purified mtDNA extracted form HEK293T cells transfected with a combination of the MTS_COX8A-Cas9-UTR_SOD2 (no sgRNA) and ssODN1 was considered as control and was subjected to the same procedure for library construction. The library was sequenced on the PacBio Sequel II platform. In order to distinguish reads of nuclear DNA of mitochondrial origin (NUMT) from reads of mtDNA, we first generated circular consensus sequences (ces)
from subreads using ccs v5.0.0 (https://github.com/PacificBiosciences/ccs). The ccs were then mapped to ±30 kb region of all NUMTs reference sequences and the extended version of rCRS with pbmm2 v1.3.0 (https://github.com/PacificBiosciences/pbmm2), respectively. The extended version of the reference sequence was composed of two complete rCRS sequences starting at the BamHI linearized site m.14,258. Ccs with MAPQ less than 30, mapped percentage (length mapped to reference sequence/total sequence length) less than 95%, and mapped concordance less than 95% were discarded. Subreads from ccs with higher mapped percentage and mapped concordance to NUMTs than to rCRS were considered as potential NUMTs. Subreads with a “GAATTC” insertion at the sgRNA1 target region were extracted using an in-house Perl script (http://mitotool.kiz.ac.cn/lab/Extract_reads_with_GAATTC_insertion.pl) and were displayed using Integrative Genomics Viewer (IGV) v2.8.9.

We also performed the third-generation sequencing using the Nanopore sequencing. Briefly, purified mtDNA extracted from HEK293T cells transfected with sgRNA1-MTS-Cas9-UTR+ssODN1 was sequenced using PromethION sequencing platform of Oxford Nanopore Technologies. Purified mtDNA extracted from HEK293T cells transfected with ssODN1 was used as a control. Libraries were prepared following the standard procedures. DNA fragments with length between 10000-20000 bp were selected by agarose gel to obtain relative intact mtDNA molecules. Sequencing reads with average quality score less than 15 were discarded. The remaining reads were mapped to the extended version of rCRS and ±30 kb region of all NUMTs reference sequences using minimap2 (https://github.com/lh3/minimap2) respectively. The mapped reads were analyzed using the same pipeline as described above. Because Nanopore has a relative higher sequencing error rate than PacBio, we used a relative loose threshold for mapped concordance (80%) when analyzing the Nanopore data. For a read with “GAATTC” insertion, the quality of flanking sequence ±10 bp of the insertion was estimated, and an average quality score less than 15 was discarded.

**Off-target estimation**

Genomic DNA was extracted from HEK293T cells grown in a 6-well plate that were transfected with sgRNA1-MTS-Cas9-UTR+ssODN1 and sgRNA2-MTS-Cas9-UTR+ssODN2, respectively. About 2 μg genomic DNA per sample was used to prepare the whole-genome sequencing (WGS) library (150 bp paired-end), and sequenced on the DNBSEQ-T7 platform (Beijing Genomics institution, BGI). The quality of WGS data were checked by FastQC v0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Sequencing adapters and low-quality reads were removed by using the Trimmomatic v0.33. The clean reads were then mapped to human reference genome GRCh38.p7 (hg38) using the Burrows-Wheeler Aligner. Cas-Offinder was used to predict potential off-target sites of sgRNAs. Genomic sites with “NGG” or “NAG” PAM motifs and with up to nine mismatches with sgRNA1 or sgRNA2 were defined as potential off-target sites (Table S2). The predicted sites were subjected to CRISPRessoWGS of the CRISPResso2 software to explore the off-target events using the WGS data. Reads with low sequencing quality (quality <20) or mapping quality (MAPQ<60) were filtered from analysis. Frequencies of insertions, deletions, or substitutions within each potential off-target site in cells transfected with different sgRNAs were compared by Fisher’s exact test to identify the potential off-target events.

**Measurement of cellular reactive oxygen species (ROS) level and ATP level**

The cellular ROS level and ATP level were determined using our previously described methods. In brief, HEK293T cells transfected with different Cas9 constructs, including: 1) Cas9 (PST1374-Cas9 vector), expresses Cas9 protein without any targeting sequence; 2) NLS-Cas9: construct sgRNAAPP-NLS-Cas9 with removal of mcherry; 3) MTS-Cas9: construct sgRNA1-MTS-Cas9-UTR+ssODN2 with removal of mcherry. After transfection for 24 h, cells were treated with vitamin K3 (vitK3; 7.5 μM) or with melatonin (100 μM) for another 24 h. Then, cells with and without treatment were harvested and incubated with...
phosphate buffer saline (PBS) containing 0.5 μM DCFH-DA probe (Sigma-Aldrich, D6883) at 37 °C for 20 min. Cells were washed with PBS and analyzed by using flow cytometry (BD, Vantage SE, USA) at 535 nm. For ATP measurement, cells seeded in 24-well plate were lysed in 100 μL lysis buffer (GENMED, China, GMS10050). 10 μL of cell lysate was subjected to ATP measurement according to the manufacture’s manual for ATP Determination Kit (Invitrogen) on GloMax 96 Luminometer (Promega). The final ATP level was normalized by protein concentration of each sample.

**Immunofluorescence assay**

HEK293T cells were cultured on slides and were transfected with combinations of mito-GFP vector (expresses mitochondrial targeting green fluorescent protein (GFP)) and different Cas9 constructs (NLS-Cas9: construct sgRNA<sup>APP</sup>-NLS-Cas9 with removal of mcherry; MTS-Cas9: construct sgRNA<sup>ND4</sup>-MTS<sup>Cox8A</sup>-Cas9-UTR<sup>SOD2</sup> with removal of mcherry). Cells were fixed in 4 % paraformaldehyde for 30 min and were incubated with the Cas9 primary antibody (1:500, Cell Signaling Technology, 14697T) overnight at 4˚C. After three washes with PBS (5 min each), cells were incubated with Alexa Fluor 594-conjugated secondary antibody (1:500, ab150116, abcam) for 1 h at room temperature. Nuclear were stained by DAPI (1:1000; Invitrogen, D1306) for 15 min. The slides were visualized under an Olympus FluoView 1000 confocal microscope (Olympus).

**Statistical analysis**

Differences in mtDNA copy number, ROS level, ATP level and knock-in efficiency among cells transfected with different constructs were quantified by two-tailed Student’s t-test using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Multiple comparisons were analyzed by one-way ANOVA test with adjustment of Tukey’s multiple comparisons using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). P<0.05 was considered statistically significant.
Supplementary Figure S1. Structure of the Cas9 constructs used in this study. The vector name was listed on the left of the schematic profile of each vector. UTR, 3'-untranslated region; MTS, mitochondrial-targeting sequence; NLS, nuclear localization sequence; CBh, chicken β-actin promoter; dCas9, catalytically dead Cas9, SpCas9 with mutations p.D10A and p.H840A\(^{3,4}\). The two sgRNAs targeting to the \(MT-ND4\) gene are labeled as sgRNA\(^{1\text{ND4}}\) and sgRNA\(^{2\text{ND4}}\), respectively. The sgRNA targeting to the nuclear \(APP\) gene and the sgRNA targeting m.3892-3918 region for introducing m.3902_3908\(^{\text{ACCTTGCGAAGGT}}\) are labeled as sgRNA\(^{\text{APP}}\) and sgRNA\(^{3\text{902}}\), respectively.
Supplementary Figure S2. Quantification of FAM-labeled sgRNA^{ND4} in mitochondria using flow cytometry. (A) HEK293T cells were co-transfected with FAM-labeled sgRNA^{ND4} and pDsRed2-mito vector (Clontech, expresses mitochondrial targeting red fluorescent protein, mito-RFP). Cells without any transfection were used as the negative control (NC). Crude mitochondria were isolated from cells at 48 h after transfection, and were subjected to flow cytometry. (B) HEK293T cells were transfected with FAM-labeled sgRNA^{ND4} for 48h, then cells were incubated with 100 nM mitotracker (Molecular Probe, USA, M22425) for 30 min. Crude mitochondria were isolated from cells and were subjected to flow cytometry. Cells without any transfection and staining were used as the negative control (NC).
Supplementary Figure S3. Alterations of cellular reactive oxygen species (ROS) and ATP levels in cells transfected with the mito-Cas9 system. HEK293T cells were transfected with expression vector for Cas9 without any targeting sequence (Cas9), construct of Cas9 with mitochondrial targeting sequence (MTS-Cas9: construct sgRNA1ND4-MTSCOX8A-Cas9-UTRSD2 with removal of mcherry), and construct of Cas9 with nuclear targeting sequence (NLS-Cas9: construct sgRNAAPP-NLS-Cas9 with removal of mcherry), respectively. (A) Cells were measured for the ROS levels at 48 h after transfection by using flow cytometry. (B) Measurement of the ATP levels in HEK293T cells after transfection for 48 h. (C) Measurement of cellular ROS levels and mtDNA copy number in transfected HEK293T cells with or without treatment of vitamin K3 (vitK3, 7.5 μM), melatonin (100 μM). Bars are mean ± SD. ns, not significant; *, P < 0.05; **, P < 0.01; one-way ANOVA test adjusted by Tukey’s multiple comparisons tests.
Supplementary Figure S4. Editing of mtDNAs using the mito-Cas9 system with sgRNA2<sup>ND4</sup>. (A) Quantification of mtDNA copy number for HEK293T cells transfected with nuclear-targeting Cas9 vector (sgRNA<sup>APP-NLS</sup>-Cas9), mitochondrial-targeting Cas9 without sgRNA (MTS<sup>COX8A</sup>-Cas9-UTR<sup>SOD2</sup>) and mito-Cas9 construct (sgRNA2<sup>ND4-MTS</sup>COX8A-Cas9-UTR<sup>SOD2</sup>). The mtDNA content was quantified by qRT-PCR with primer pair L394/H475, and was normalized to a single copy nuclear β-globin gene. (B) Design of the mito-Cas9 mediated knock-in system with sgRNA2<sup>ND4</sup> and ssODN2. (C) EcoRI site-specific PCR with primer EcoRI-F/H11944. (D) Quantification of knock-in efficiency of ssODN2 by mito-Cas9 system using qRT-PCR. HEK293T cells were transfected with or without a combination of Cas9 constructs and ssODN2. Content of mtDNA with successful knock-in of EcoRI site (amplified by primer pair EcoRI-F/H11944) was normalized to whole mtDNA (total mtDNA, amplified by primer pair L394/H475). Bars are mean ± SD. ns, not significant; ***, P < 0.001; ****, P < 0.0001; one-way ANOVA test adjusted by Tukey’s multiple comparisons tests.
Supplementary Figure S5. Amplification curve (A) and melting curve (B) of the EcoRI-specific quantitative real-time PCR (qRT-PCR) products. The PCR products were amplified by using EcoRI-specific primer pair L11338/EcoRI-R with genomic DNA as the template. The genomic DNA samples were extracted from HEK293T cells with transfection of (1) MTS\textsuperscript{COX8A-Cas9-UTR\textsuperscript{SOD2}}, (2) ssODN1, (3) sgRNA1\textsuperscript{ND4}-MTS\textsuperscript{COX8A-Cas9-UTR\textsuperscript{SOD2}}+ssODN1, respectively, for 48 h.
Supplementary Figure S6. Localization of the FAM-labeled ssODN1 in mitochondria and flow cytometry analyses of HEK293T cells with transfection of the FAM-labeled ssODN1 and/or Cas9 constructs. (A) Fluorescence microscopy assay of isolated mitochondria from HEK293T cells with transfection of the FAM-labeled ssODN1 and pDsRed2-mito vector (Clontech, expresses mitochondrial targeting red fluorescent protein, mito-RFP) for 48 h. (B) Flow cytometry analyses of the HEK293T cells transfected with or without the combination of FAM-labeled ssODN1 and different Cas9 constructs for 48 h. Cells were analyzed using flow cytometry (BD, Influx, USA) at 610 nm to detect mcherry that representing successful transfection of different Cas9 constructs, and at 535 nm to detect successful transfection of FAM-labeled ssODN1.
Supplementary Figure S7. Knock-in efficiency of the mito-Cas9 system in HEK293T cells. The HEK293T cells were transfected with ssODN1, with or without the mito-Cas9 system. The potential knock-in efficiency was quantified by qRT-PCR for transfected cells harvested at 48 h, 96 h and 144 h after transfection. Bars are mean ± SD. ****, P < 0.0001; one-way ANOVA test adjusted by Tukey’s multiple comparisons tests.
Supplementary Figure S8. Preparation of purified mtDNA for the third-generation sequencing. (A) Linearization of the mtDNA with BamHI at site m.14258. Total DNA, total genomic DNA, including nuclear and mitochondrial DNA; Purified mtDNA, mtDNA extracted from DNase I treated crude mitochondria. The digestion was performed in a total volume of 100 µL containing 10 µg DNA and 100 unit of BamHI at 37 °C for 3 h. (B) Quantification of non-linearized mtDNA molecules in purified mtDNAs with or without BamHI digestion. qRT-PCR was performed using primer pair L14054/H14573 flanking the BamHI site and primer pair L394/H475, to estimate the proportion of non-linearized mtDNA molecules. Linearized mtDNA could not be amplified by L14054/H14573. Bars are mean ± SD. ****, P < 0.0001.
Supplementary Figure S9. Effects of RAD51 on the knock-in efficiency mediated by mito-Cas9 system. (A) Quantification of knock-in efficiency of different Cas9 groups with or without RAD51 activation. Four control groups were designed: 1) MTSCOX8A-Cas9-UTR SOD2; 2) ssODN1 only; 3) sgRNA2 ND4-MTS COX8A-Cas9-UTR SOD2+ssODN1, sgRNA2 ND4 targeting another region; 4) MTS COX8A-Cas9-UTR SOD2+ssODN1, mito-Cas9 without sgRNA. HEK293T cells were transfected with Cas9 construct or ssODN1, or with a combination of Cas9 construct and ssODN1 for 48 h. Purified mtDNA was extracted from Dnase I treated crude mitochondria from transfected HEK293T cells treated with DMSO or RS-1 (10 μM). Content of mtDNA with successful knock-in of EcoRI site (amplified by primer pair L11338/EcoRI-R) was normalized to whole mtDNA (amplified by primer pair L394/H475). (B) Overexpression of RAD51 in HEK293T cells. The HEK293T cells were co-transfected with pcDNA3.1-RAD51 or empty vector, together with mito-Cas9 construct and ssODN1. The RAD51 protein level was quantified and normalized to α-tublin. (C-D) Knockdown of RAD51. HEK293T cells were transfected with control siRNA (siRNA NC) or siRNA targeting RAD51 mRNA (siRNA RAD51), together with mito-Cas9 construct and ssODN1. The mRNA level of RAD51 was quantified by qRT-PCR (C). The protein level of RAD51 was analyzed by Western blotting, and was normalized to α-tublin (D). Bars are mean ± SD. ns, not significant; *, P<0.05; **, P<0.01, one-way ANOVA test adjusted by Tukey’s multiple comparisons tests in (A); two-tailed Student’s t test in (B-D). (E) “GAATTC” insertions were identified in second-generation sequencing reads.
Supplementary Figure S10. The ssODN with long homologous arms (45 bp) had better knock-in efficiency than that with short homologous arms (22 bp). The HEK293T cells were transfected with ssODN1 (with a 45 bp homologous arm in each side) or ssODN50 (with a 22 bp homologous arm in each side), together with or without the mito-Cas9 system. The knock-in efficiency was quantified by qRT-PCR for transfected cells after transfection for 48 h. Bars are mean ± SD. ****, *P* < 0.0001; one-way ANOVA test adjusted by Tukey’s multiple comparisons tests.
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