The Innovation, Volume *3*

Supplemental Information

Direct evidence of CRISPR-Cas9-mediated mitochondrial genome editing

Rui Bi, Yu Li, Min Xu, Quanzhen Zheng, Deng-Feng Zhang, Xiao Li, Guolan Ma, Bolin Xiang, Xiaojia Zhu, Hui Zhao, Xingxu Huang, Ping Zheng, and Yong-Gang Yao

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 sgRNA1^{ND4} targeting the mtDNA region 11 697-11 716 (m.11 697-11 716) and sgRNA2^{ND4} 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 sgRNA1^{ND4}; ssODN2, relative to sgRNA2^{ND4}) were designed, with each having a 45 bp homologous arm flanking a 6 bp insertion of the *EcoR*I restriction site "GAATTC" (Table S1). The sgRNA targeting the nuclear *APP* gene (coding amyloid-beta precursor protein) (sgRNA^{APP}) was cloned to the px330-mCherry vector to obtain the nuclear targeting Cas9 construct sgRNA^{APP}-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 (sgRNA1^{ND4} or sgRNA2^{ND4}) targeting mtDNA. Constructs of MTS^{COX8A}-Cas9-UTR^{SOD2} (no sgRNA) and sgRNA1^{ND4}-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 (ssODN^{3902}) 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% CO_2. Cells were seeded in a 6-well plate at a density of 5×10^5 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 sgRNA1^{ND4}/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 sgRNA1^{ND4}/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/*EcoR*I-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 (2×10^6 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, 14697T), Flag tag (Abmart, M20008L), ATP5A (Proteintech, 14676-1-AP), H3 (Cell Signaling Technology, 4499S), GAPDH (Proteintech, 60004-1-Ig), MFN2 (Proteintech, 12186-1-AP), COXIV (Cell Signaling Technology, 4850P), β-actin (Abmart, P30002F), RAD51 (Proteintech, 14961-1-AP) and α-tublin (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 $2^{-\Delta\Delta CT}$ 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 *EcoR*I site "GAATTC" was measured using the *EcoR*I site-specific primer pair L11338/*EcoR*I-R (Table S1) and normalized to total mtDNA content measured using primer pair L394/H475. The ratio of mtDNA with the *EcoR*I 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 ssODN^{3902} 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 sgRNA1^{ND4}-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 sgRNA1^{ND4}-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 sgRNA1^{ND4}-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 *EcoR*I 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 [8]. The clean reads were aligned to the revised Cambridge reference sequence (rCRS, GenBank Accession No. NC_012920) [9] using Burrows-Wheeler Aligner (BWA) v0.7.17-r1188 [10]. 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 sgRNA1^{ND4} 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_reads_with_GAATTC_insertion.pl). Sequence depth for each library was estimated using SAMtools v1.7 [11]. 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^{3902}.

Third-generation sequencing

A total of 5×10^7 HEK293T cells were transfected with a combination of the mito-Cas9 construct sgRNA1^{ND4}-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 *BamH*I (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 (ccs)

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 [12] and the extended version of rCRS [9] 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^{ND4} 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. [13].

We also performed the third-generation sequencing using the Nanopore sequencing. Briefly, purified mtDNA extracted from HEK293T cells transfected with sgRNA1^{ND4}-MTS^{COX8A}-Cas9-UTR^{SOD2}+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 [9] and ±30 kb region of all NUMTs reference sequences [12] using minimap2 (https://github.com/lh3/minimap2) [14], respectively. The mapped reads were analyzed using the same pipeline as described above. Because Nanopore has a relative higher sequencing error rate than PacBio [15], 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^{ND4}-MTS^{COX8A}-Cas9-UTR^{SOD2}+ssODN1 and sgRNA2^{ND4}-MTS^{COX8A}-Cas9-UTR^{SOD2}+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 [16]. The clean reads were then mapped to human reference genome GRCh38.p7 (hg38) using the Burrows-Wheeler Aligner [10]. Cas-Offinder [17] 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 [18] 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 [19]. In brief, HEK293T cells transfected with different Cas9 constructs, including: 1) Cas9 (PST1374-Cas9 vector), expresses Cas9 protein without any targeting sequence [20]; 2) NLS-Cas9: construct sgRNA^{APP}-NLS-Cas9 with removal of mcherry; 3) MTS-Cas9: construct sgRNA1^{ND4}-MTS^{COX8A}-Cas9-UTR^{SOD2} 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

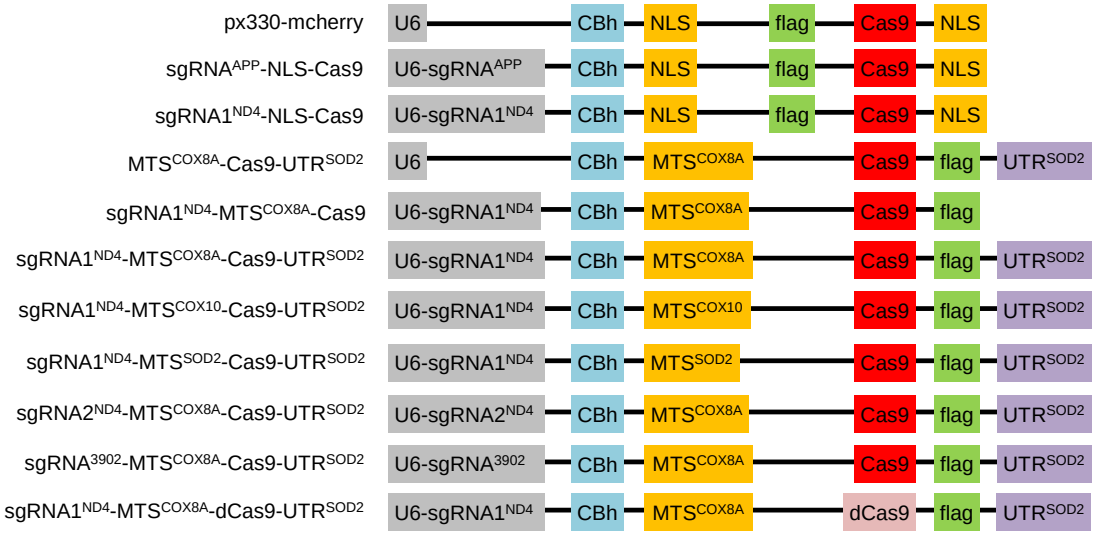
phosphate buffer saline (PBS) containing 0.5 μM DCFH-DA probe (Sigma-Aldrich, D6883) at 37 ºC for 20 min. Cells were washed with PBS and analyzed by using flow cytometry (BD, Vantage SE, USA) at 535 nm. For ATP measurement, cells seeded in 24-well plate were lysed in 100 μL lysis buffer (GENMED, China, 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

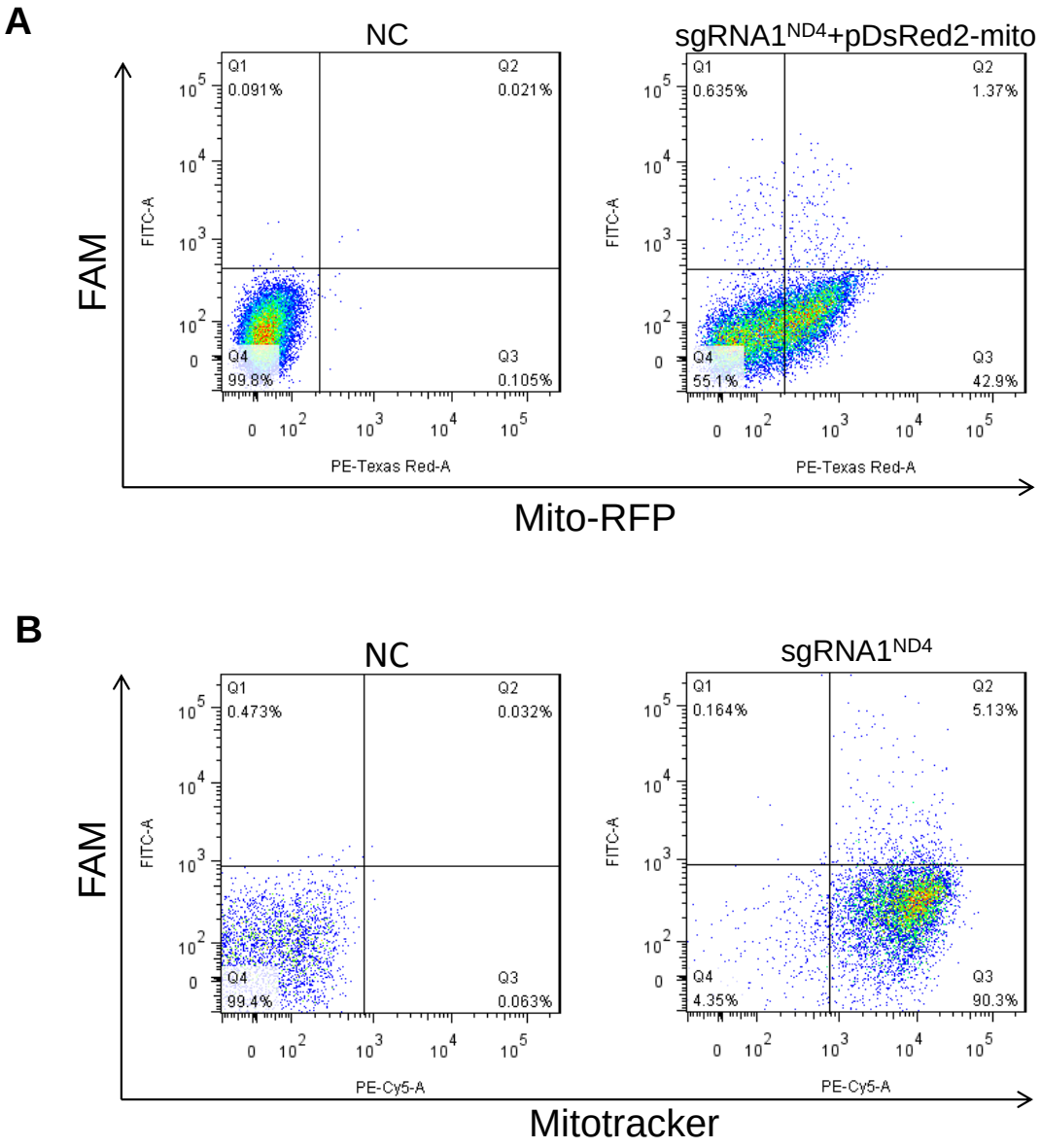
HEK293T cells were cultured on slides and were transfected with combinations of mito-GFP vector (expresses mitochondrial targeting green fluorescent protein (GFP)) and different Cas9 constructs (NLS-Cas9: construct $sgRNA^{APP}$-NLS-Cas9 with removal of mcherry; MTS-Cas9: construct $sgRNA1^{ND4}$-MTS^{COX8A}-Cas9-UTR^{SOD2} 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

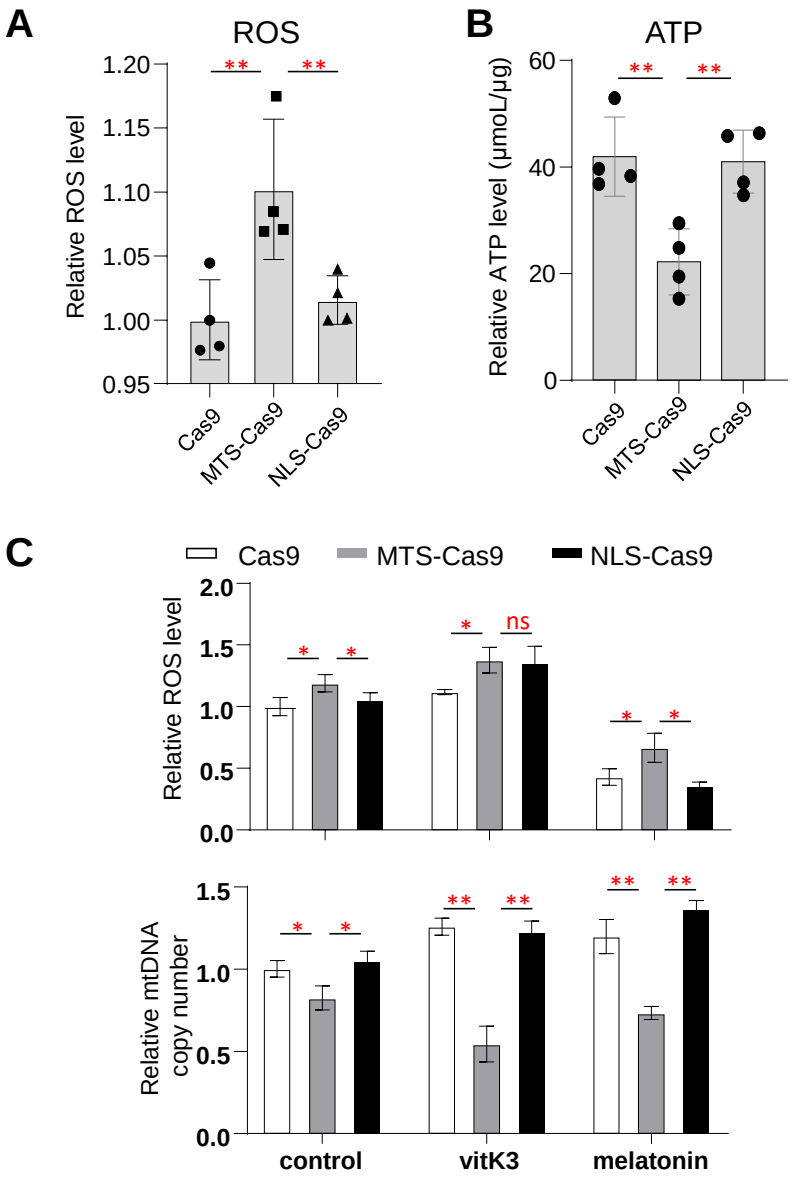
Differences in mtDNA copy number, ROS level, ATP level and knock-in efficiency among cells transfected with different constructs were quantified by two-tailed Student's *t*-test using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Multiple comparisons were analyzed by one-way ANOVA test with adjustment of Tukey's multiple comparisons using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered statistically significant.



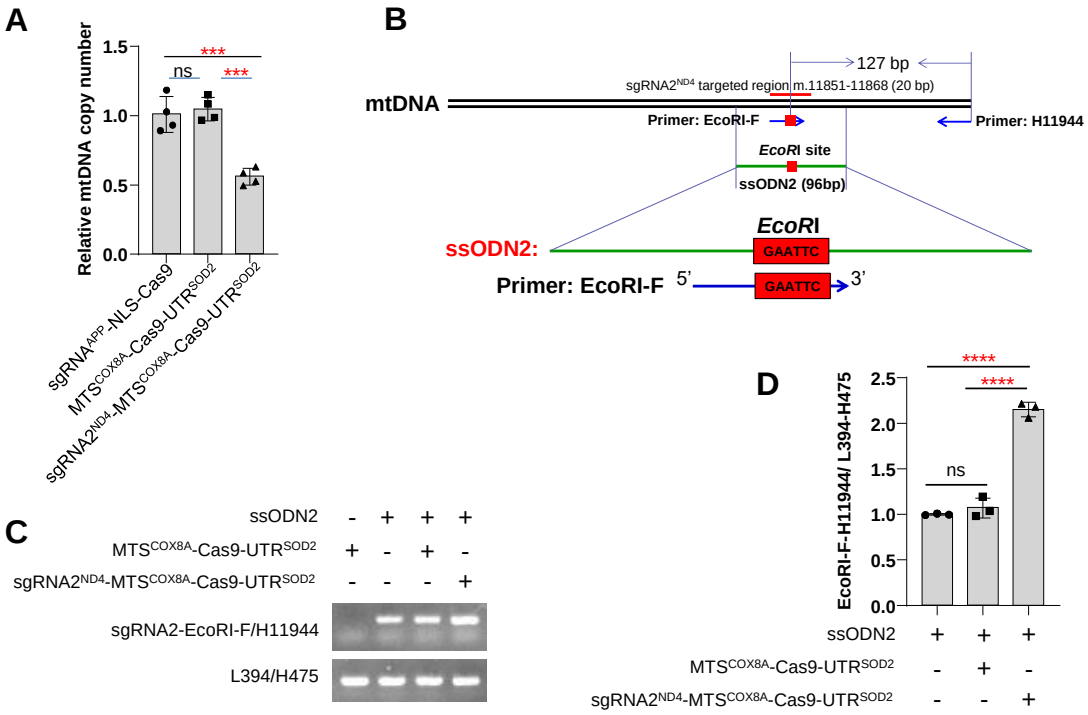
Supplementary Figure S1. Structure of the Cas9 constructs used in this study. The vector name was listed on the left of the schematic profile of each vector. UTR, 3'-untranslated region; MTS, mitochondrial-targeting sequence; NLS, nuclear localization sequence; CBh, chicken β-actin promoter; dCas9, catalytically dead Cas9, SpCas9 with mutations p.D10A and p.H840A [3,4]. The two sgRNAs targeting to the *MT-ND4* gene are labeled as sgRNA1^{ND4} and sgRNA2^{ND4}, respectively. The sgRNA targeting to the nuclear *APP* gene and the sgRNA targeting m.3892-3918 region for introducing m.3902_3908inv (m.3902_3908 ACCTTGC>GCAAGGT) are labeled as sgRNA^{APP} and sgRNA^{3902}, respectively.



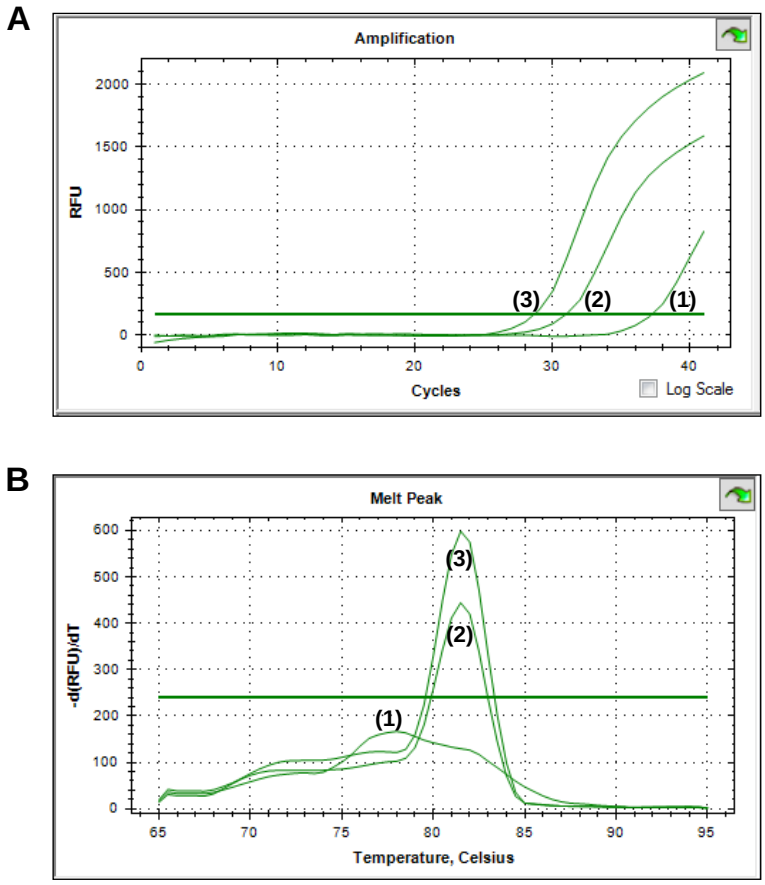
Supplementary Figure S2. Quantification of FAM-labeled sgRNA1^{ND4} in mitochondria using flow cytometry. (A) HEK293T cells were co-transfected with FAM-labeled sgRNA1^{ND4} and pDsRed2-mito vector (Clontech, expresses mitochondrial targeting red fluorescent protein, mito-RFP). Cells without any transfection 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 sgRNA1^{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).



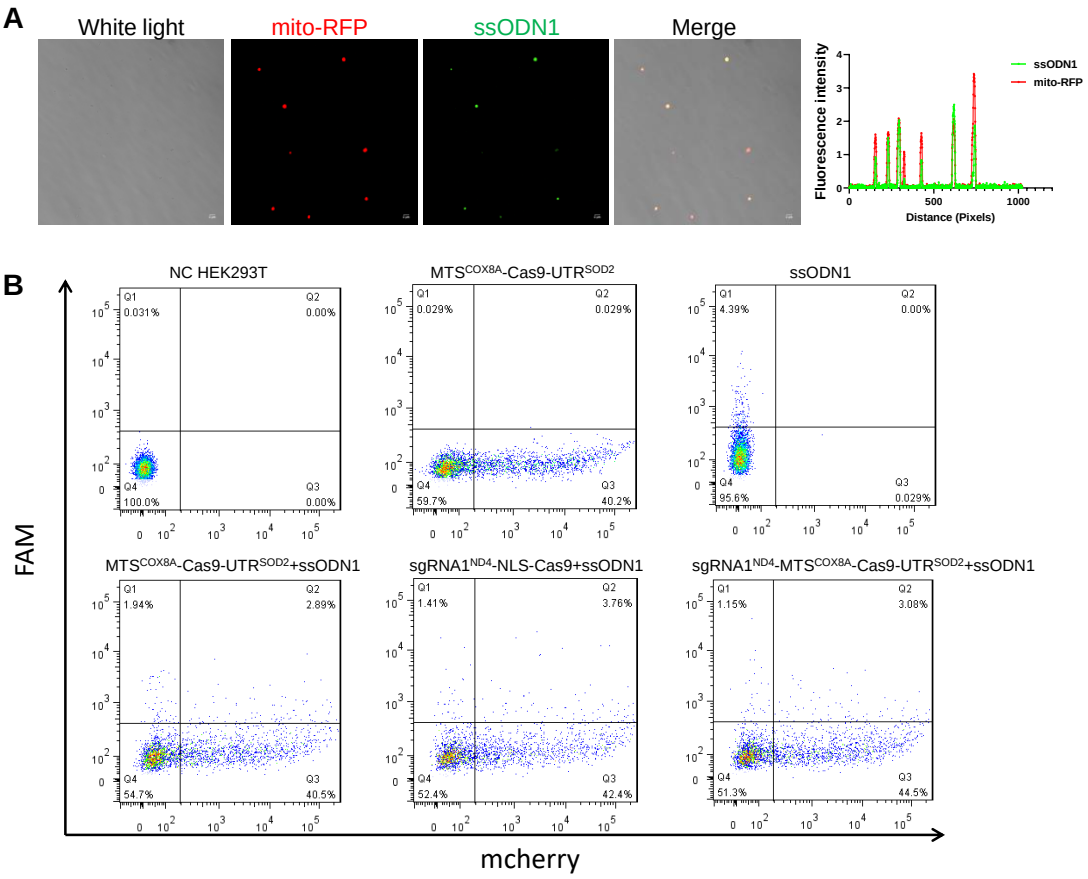
Supplementary Figure S3. Alterations of cellular reactive oxygen species (ROS) and ATP levels in cells transfected with the mito-Cas9 system. HEK293T cells were transfected with expression vector for Cas9 without any targeting sequence (Cas9), construct of Cas9 with mitochondrial targeting sequence (MTS-Cas9: construct sgRNA1^{ND4}-MTS^{COX8A}-Cas9-UTR^{SOD2} with removal of mcherry), and construct of Cas9 with nuclear targeting sequence (NLS-Cas9: construct sgRNA^{APP}-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.



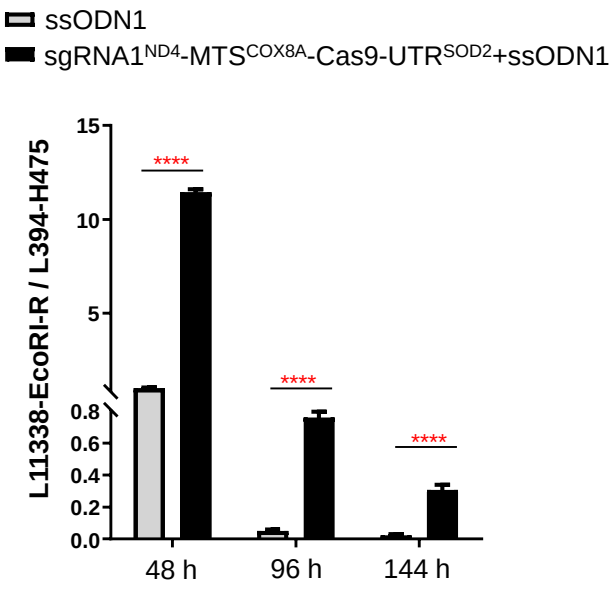
Supplementary Figure S4. Editing of mtDNAs using the mito-Cas9 system with sgRNA2^{ND4}. (A) Quantification of mtDNA copy number for HEK293T cells transfected with nuclear-targeting Cas9 vector (sgRNA^{APP}-NLS-Cas9), mitochondrial-targeting Cas9 without sgRNA (MTS^{COX8A}-Cas9-UTR^{SOD2}) and mito-Cas9 construct (sgRNA2^{ND4}-MTS^{COX8A}-Cas9-UTR^{SOD2}). 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^{ND4} and ssODN2. **(C)** *EcoR*I site-specific PCR with primer *EcoR*I-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 *EcoR*I site (amplified by primer pair *EcoR*I-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.



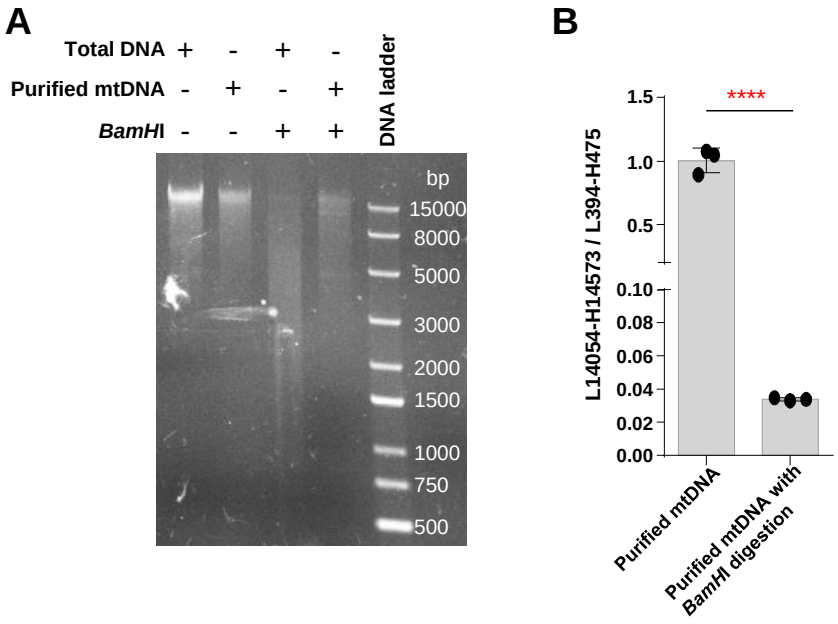
Supplementary Figure S5. Amplification curve (A) and melting curve (B) of the *EcoR*I-specific quantitative real-time PCR (qRT-PCR) products. The PCR products were amplified by using *EcoR*I-specific primer pair L11338/*EcoR*I-R with genomic DNA as the template. The genomic DNA samples were extracted from HEK293T cells with transfection of (1) MTS^{COX8A}-Cas9-UTR^{SOD2}, (2) ssODN1, (3) $sgRNA1^{ND4}$-MTS^{COX8A}-Cas9-UTR^{SOD2}+ssODN1, respectively, for 48 h.



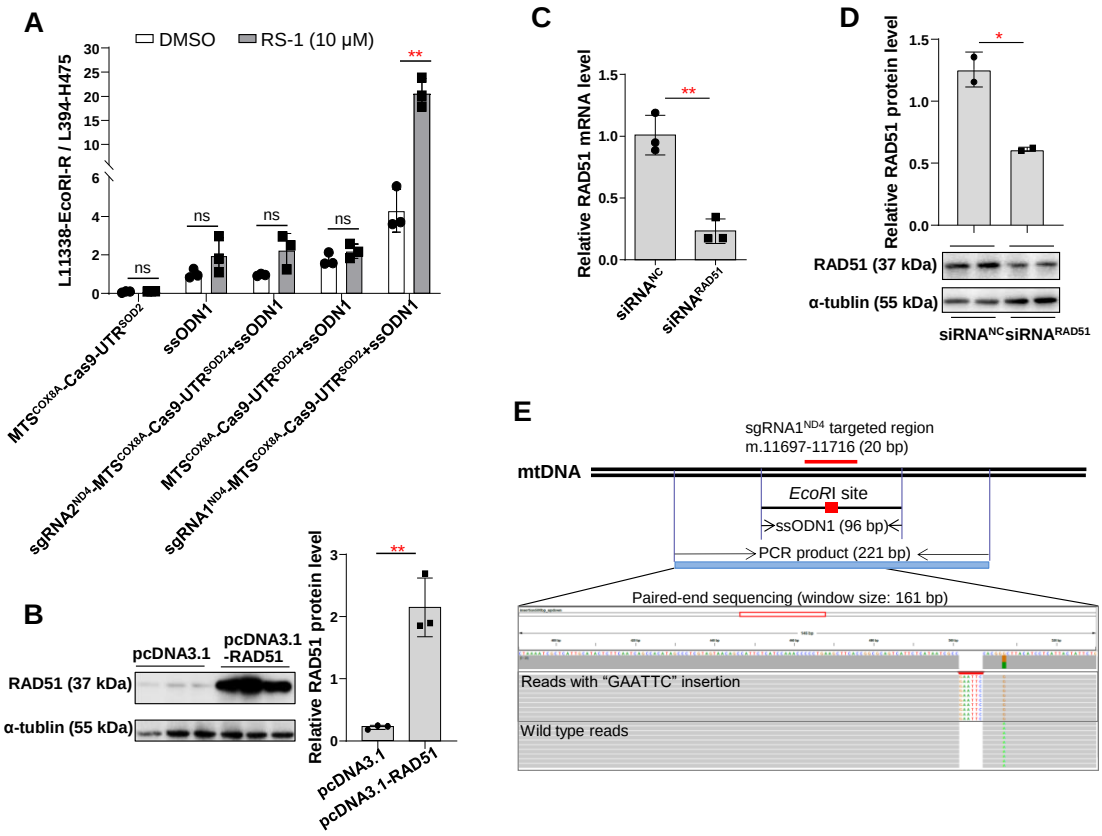
Supplementary Figure S6. Localization of the FAM-labeled ssODN1 in mitochondria and flow cytometry analyses of HEK293T cells with transfection of the FAM-labeled ssODN1 and/or Cas9 constructs. (A) Fluorescence microscopy assay of isolated mitochondria from HEK293T cells with transfection of the FAM-labeled 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.



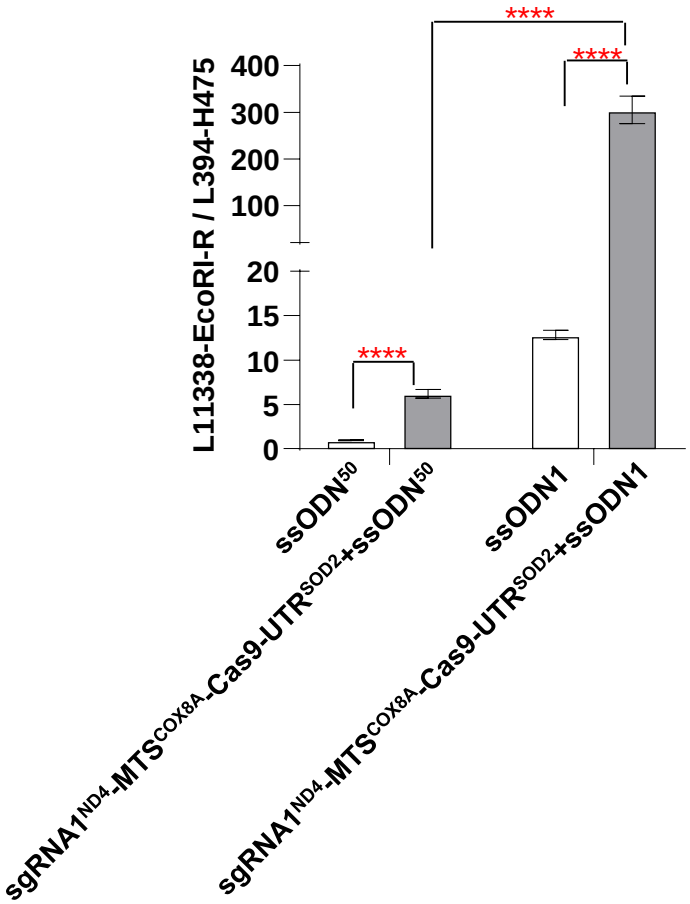
Supplementary Figure S7. Knock-in efficiency of the mito-Cas9 system in HEK293T cells. The HEK293T cells were transfected with ssODN1, with or without the mito-Cas9 system. The potential knock-in efficiency was quantified by qRT-PCR for transfected cells harvested at 48 h, 96 h and 144 h after transfection. 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 *BamH*I 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 *BamH*I at 37 ºC for 3 h. **(B)** Quantification of non-linearized mtDNA molecules in purified mtDNAs with or without *BamH*I digestion. qRT-PCR was performed using primer pair L14054/H14573 flanking the *BamH*I 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) MTS^{COX8A}-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 *EcoR*I 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 ssODN^{50} (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.

References

1. Wu, Y., Liang, D., Wang, Y., et al. (2013). Correction of a genetic disease in mouse via use of CRISPR-Cas9. Cell Stem Cell ***13***, 659-662
2. Oliveros, J.C., Franch, M., Tabas-Madrid, D., et al. (2016). Breaking-Cas-interactive design of guide RNAs for CRISPR-Cas experiments for ENSEMBL genomes. Nucleic Acids Res ***44***, W267-271
3. Qi, L.S., Larson, M.H., Gilbert, L.A., et al. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell ***152***, 1173-1183
4. Shen, B., Zhang, W., Zhang, J., et al. (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. Nat Methods ***11***, 399-402
5. Bi, R., Zhang, A.M., Zhang, W., et al. (2010). The acquisition of an inheritable 50-bp deletion in the human mtDNA control region does not affect the mtDNA copy number in peripheral blood cells. Hum Mutat ***31***, 538-543
6. Jayathilaka, K., Sheridan, S.D., Bold, T.D., et al. (2008). A chemical compound that stimulates the human homologous recombination protein RAD51. Proc Natl Acad Sci U S A ***105***, 15848-15853
7. Budke, B., Logan, H.L., Kalin, J.H., et al. (2012). RI-1: a chemical inhibitor of RAD51 that disrupts homologous recombination in human cells. Nucleic Acids Res ***40***, 7347-7357
8. Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics ***34***, i884-i890
9. Andrews, R.M., Kubacka, I., Chinnery, P.F., et al. (1999). Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nat Genet ***23***, 147
10. Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics ***25***, 1754-1760
11. Li, H., Handsaker, B., Wysoker, A., et al. (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics ***25***, 2078-2079
12. Tsuji, J., Frith, M.C., Tomii, K., and Horton, P. (2012). Mammalian NUMT insertion is non-random. Nucleic Acids Res ***40***, 9073-9088
13. Robinson, J.T., Thorvaldsdottir, H., Winckler, W., et al. (2011). Integrative genomics viewer. Nat Biotechnol ***29***, 24-26
14. Li, H. (2018). Minimap2: pairwise alignment for nucleotide sequences. Bioinformatics ***34***, 3094-3100
15. Delahaye, C., and Nicolas, J. (2021). Sequencing DNA with nanopores: Troubles and biases. PLoS One ***16***, e0257521
16. Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics ***30***, 2114-2120
17. Bae, S., Park, J., and Kim, J.S. (2014). Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics ***30***, 1473-1475
18. Clement, K., Rees, H., Canver, M.C., et al. (2019). CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nat Biotechnol ***37***, 224-226
19. Bi, R., Zhang, W., Yu, D., et al. (2015). Mitochondrial DNA haplogroup B5 confers

genetic susceptibility to Alzheimer's disease in Han Chinese. Neurobiol Aging ***36***, 1604 e1607-1616

20. Shen, B., Zhang, J., Wu, H., et al. (2013). Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. Cell Res ***23***, 720-723