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Supplemental Information

Enzymatic Noncovalent Synthesis for Mitochondrial Genetic Engineering of Cancer Cells

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Peptide synthesis

All amino acid derivatives involved in the synthesis were purchased from GL Biochem (Shanghai) Ltd. The synthesis of all peptide fragments was based on solid-phase peptide synthesis (SPPS). The branched peptides were made via the combination of SPPS and liquid phase synthesis. [1] All crude compounds were purified by HPLC with the yield of 70-80%.

Cell culture

HeLa, HepG2, Saos-2, and HS-5 cells were purchased from American Type Culture Collection (ATCC). HEK293 cells were provided by Prof. Chris Miller from Brandeis University. All the cell lines used in this work were authenticate by authenticated by CellCheck 9 - human (9 Marker STR Profile and Inter-species Contamination Test, IDEXX), confirming 100% match of the cell identity. HeLa, HepG2, and HEK293 cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% (vol/vol) FBS, 0.5% (vol/vol) penicillin (10, 000 unit), and 0.5% (vol/vol) streptomycin (10, 000 unit). Saos-2 cells were cultured in McCoy's 5A with l-glutamine supplemented with 15% (vol/vol) FBS, 10% (vol/vol) FBS, 0.5% (vol/vol) penicillin (10, 000 unit), and 0.5% (vol/vol) streptomycin (10, 000 unit). HS-5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (vol/vol) FBS, 0.5% (vol/vol) penicillin (10, 000 unit), and 0.5% (vol/vol) streptomycin (10, 000 unit). All cells were maintained at 37 °C in a 5% CO2 incubator.

Cell viability assay

All cell viability was determined by MTT cell viability assay via standard protocol.[2]

Plasmid import

For Mito-Flag-assisted delivery of plasmids (pGLO[3] (Bio-Rad), Mito-pGLO (Ordered from Invitrogen, the final construct was verified by sequencing), pMAX-DEST (Addgene, CAT#: 37631), FUNDC1-Myc (OriGene, CAT#: RC208211) and GFP-TP53 (Addgene, CAT# 12091)), the plasmids were dissolved in culture medium (with 10% FBS) followed by the addition of Mito-Flag. The mixtures were incubated in room temperature for 20 min before adding to cells (already adhered). Lipofectamine 3000 (Invitrogen) was used for the general transfection of plasmids following the manufacture's instruction. Arabinose (0.2 wt%) was added in the delivery of pGLO and Mito-pGLO. Cells were incubated at 37 °C in a 5% CO_2 incubator before further analysis. Unless specially mentioned, the final concentration of Mito-Flag is 200 μM. The dose of plasmids is 5 μg per 80, 000 cells. Recommended concentration of plasmid (in medium) is 5 μg/mL. The Incubation time is 48 h.

Virus import

For Mito-Flag-assisted delivery of viral vectors (Baculovirus[4; 5] (Thermo Fisher Scientific, CAT#C10604 and CAT#C10597), AAV (Addgene, CAT#: 37825-AAV5) and lentivirus (abm, CAT# LVP691)), the viruses were mixed in culture medium (with 10% FBS) followed by the addition of Mito-Flag. The mixtures were incubated in room temperature for 20 min before adding to cells (already adhered). Following the manufacture's protocol, cells were directly incubated with

virus in culture medium for the general transfection. Cells were incubated at 37 °C in a 5% CO_2 incubator before further analysis. Unless specially mentioned, the final concentration of Mito-Flag is 200 μM.

Immunofluorescence

Cells were plated on confocal dishes (CellVis) and fixed in 4 wt% paraformaldehyde for 15 min and permeabilized with 1% BSA and 0.1% Tween 20. Fixed cells were incubated in primary antibody at 4 ℃ overnight, washed three times for 5 min each, incubated in secondary antibody for 1 h, washed three times for 5 min each.

Confocal microscopy

80, 000 cells were seeded in 32 mm confocal dishes (CellVis) and incubated in cell incubator at standard condition for 24 h for adherence. After being treated by the condition of interest, cells were analyzed by ZEISS LSM 880 Confocal Microscope and images were taken.

Intracellular fluorescence quantification

Cells were seeded in petri dishes and incubated in standard condition for 12 h before experiment. After being treated by the condition of interest, all cells were washed by PBS (3 time for 5 min). After the washing step, cells were detached from the dishes using trypsin. The trypsin was removed by centrifuge and washing with PBS. The cell suspensions (in PBS) were diluted to 10^5 cell/mL. 100 μL diluted cell suspension was added in a 96-well plate (at least 5 wells per sample). The intracellular fluorescence intensity was immediately determined using a DTX 880 Multimode Detector (Beckman Coulter Inc.). The experiments were repeated, and the intracellular fluorescence intensity was averaged (n>3).

Transmission electron microscopy

Samples of interest were dropped on copper grids and dried. Uranyl acetate was used as negative stain. All TEM images were taken by FEI Morgagni Transmission Electron Microscope.

Mitochondria isolation

Mitochondria were isolated from cells treated by conditions of interest using mitochondria isolation kit (Thermo Fisher Scientific, CAT#89874) following the manufacture's instruction.

Extraction of mitochondrial DNA

The mitochondrial DNA was extracted by Mitochondrial DNA Isolation Kit (Abcam, CAT#ab65321) following the manufacture's protocol.

Western blots of mitochondrial proteins

Mitochondria were isolated from cells treated by conditions of interest using mitochondria isolation kit (Thermo Fisher Scientific). Total mitochondrial protein extracts were prepared in lysis buffer (Cell Signaling Technology, with 1X protease inhibitor cocktail (Abcam)) followed by 5 freeze-thaw circles and 30 min sonication. Protein concentration was determined by Coomassie Blue method. Protein extracts (20 μg per lane) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Western blotting was performed according to

standard protocols. Gel analysis was conducted using ImageJ. All antibodies were purchased from Abcam.

Enterokinase gene knockdown

For each transfection, siRNA duplex was diluted into 100 µL antibiotic-free and FBS-free siRNA transfection medium (Santa Cruz Biotechnology, sc-36868). Recommended final siRNA concentration is 0.1 µM. For each transfection, 6 µL of siRNA transfection reagent (Santa Cruz Biotechnology, sc-29528) was diluted into 100 µL siRNA transfection medium. The siRNA duplex solution was mixed directly into the dilute transfection reagent using a pipette. The mixture was incubated at room temperature for 30 minutes. 0.8 mL siRNA transfection medium was added into the 0.2 mL mixture of siRNA and transfection reagent (total volume 1 mL). Cells (80, 000 per transfection, already adhered) were washed once with 2 mL of siRNA transfection medium and incubated with the 1 mL mixture of siRNA and transfection reagent for 8 h at 37° C in a CO_2 incubator. 1 mL of normal growth medium containing 2 times FBS and antibiotics concentration was added to the cells without removing the transfection mixture. The cells incubated for an additional 24 hours followed by aspirating the medium and replacing with fresh 1x normal growth medium. Cells were incubated for another 48 h before further assay.

mtDNA depletion and the imaging of mitochondrial DNA

Cells were incubated with 1 µM ethidium bromide for 24 h or longer time for mtDNA depletion. PicoGreen was used to check the depletion of mtDNA.[6] After that, cells were washed by PBS 3 times for 5 min. After the washing step, the mtDNA depleted cells were incubated with culture medium in the presence of free pMAX-DEST (Addgene), the mixture of Mito-Flag and pMAX-DEST, or the mixture of lipofectamine and pMAX-DEST for another 24 h. The mtDNA depleted cells incubated with only culture medium were used as mtDNA-negative control. Cells were then washed by PBS 3 times for 5 min, and then stained by PicoGreen (in culture medium) for 2 h before further analysis.

PCR experiment

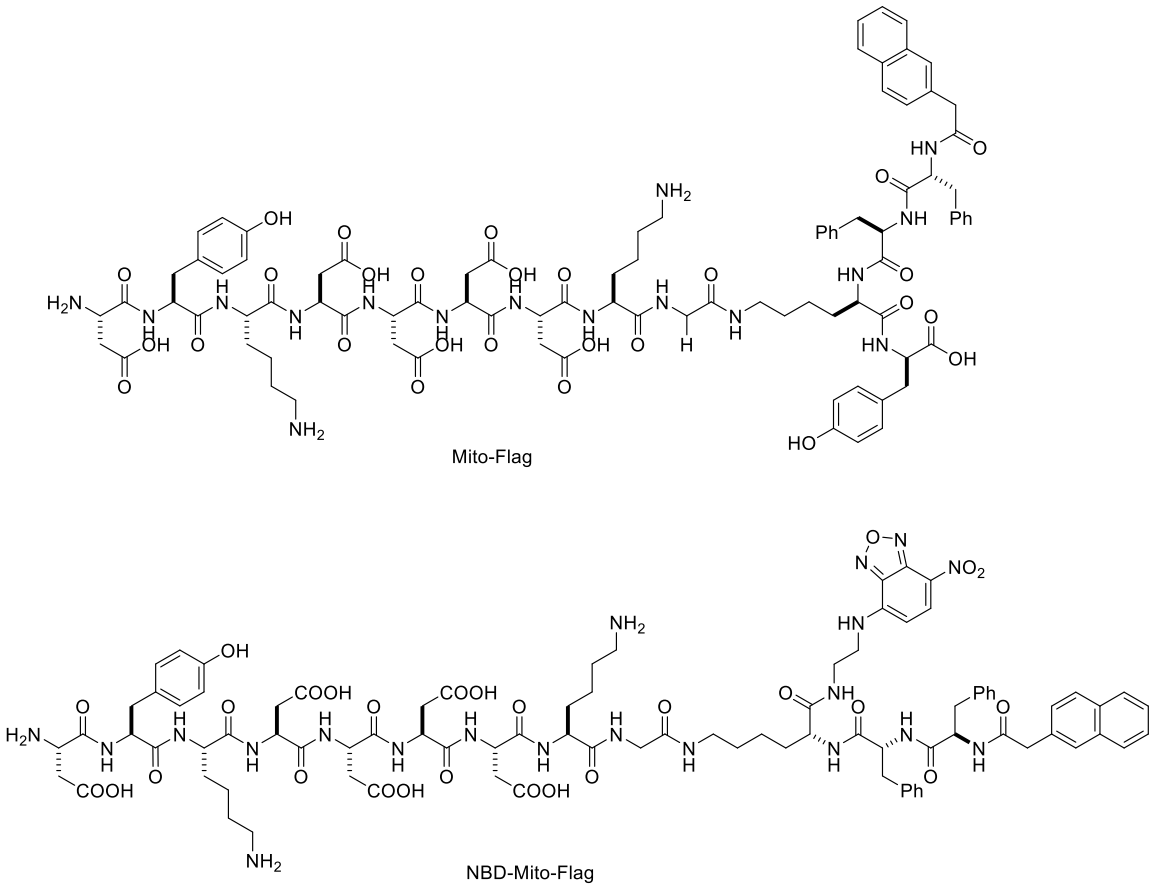
The MT-CO1 gene was amplified by Phusion DNA polymerase (New England Biolabs, CAT#M0530) via corresponding primers (forward: TAAGCACCCTAATCAACTGGC, reverse: GCCTCCACTATAGCAGATGCG) according to the manufacture's protocol. The products were examined by DNA electrophoresis.

DNA encapsulation efficiency

To prove that plasmids are associated with Mito-Flag, free pGLO plasmid (2 µg/mL) and the mixture of Mito-Flag (200 µM) and pGLO plasmid (2 µg/mL) was centrifuged by an Eppendorf 5430 R centrifuge (14, 000 rpm, 1 h). Supernatant was transferred to new centrifuge tubes and the pellets were resuspended in 20 µL TBE buffer. The presence of pGLO plasmid in pellets was determined by DNA electrophoresis. All samples (20 µL per well) for DNA electrophoresis were loaded in 1 wt% agarose. The gel was run at 120 V for 1.5 h. Ethidium Bromide was used for DNA staining in gel. The amount of pGLO plasmid in the supernatant was quantified by PicoGreen assay.[7] Encapsulation efficiency (EE)% was calculated as: %EE = [(DNA added - Free unentrapped DNA)/DNA added]x100%

Statistical analysis and assembly

Data presented are means ± SD (except in the semi-quantification of fluorescent images). All tests were analyzed from n > 9 cells from n > 3 independent experiments per condition. Fluorescent images semi-quantification was conducted using ImageJ. All images were assembled using Photoshop.



Scheme S1. The molecular structures of Mito-Flag and NBD-Mito-Flag.

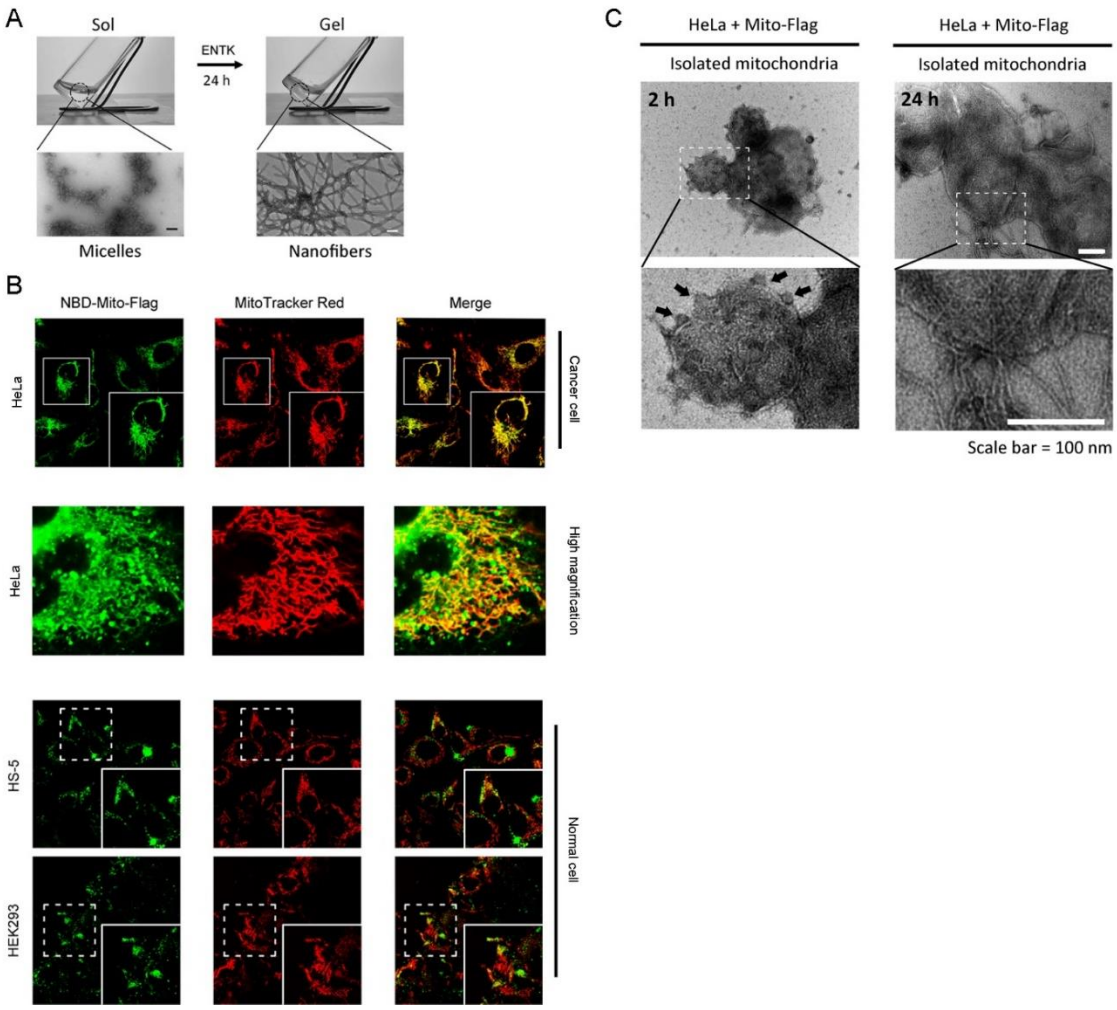


Figure S1. Perimitochondrial ENS of Mito-Flag. (A) Optical and TEM images of the sol to gel transition of Mito-Flag upon ENTK cleavage (2.5 wt%, 10 U/mL ENTK, 24 h, for optical images; 200 μM, 10 U/mL ENTK, 24 h, for TEM images). (B) Confocal fluorescence images of HeLa, HS-5 and HEK293 cells incubated with NBD-Mito-Flag (200 μM, 2 h). (C) TEM images of mitochondria isolated from the HeLa cells that are treated by 200 μM Mito-Flag for 2 h and 24 h, respectively.

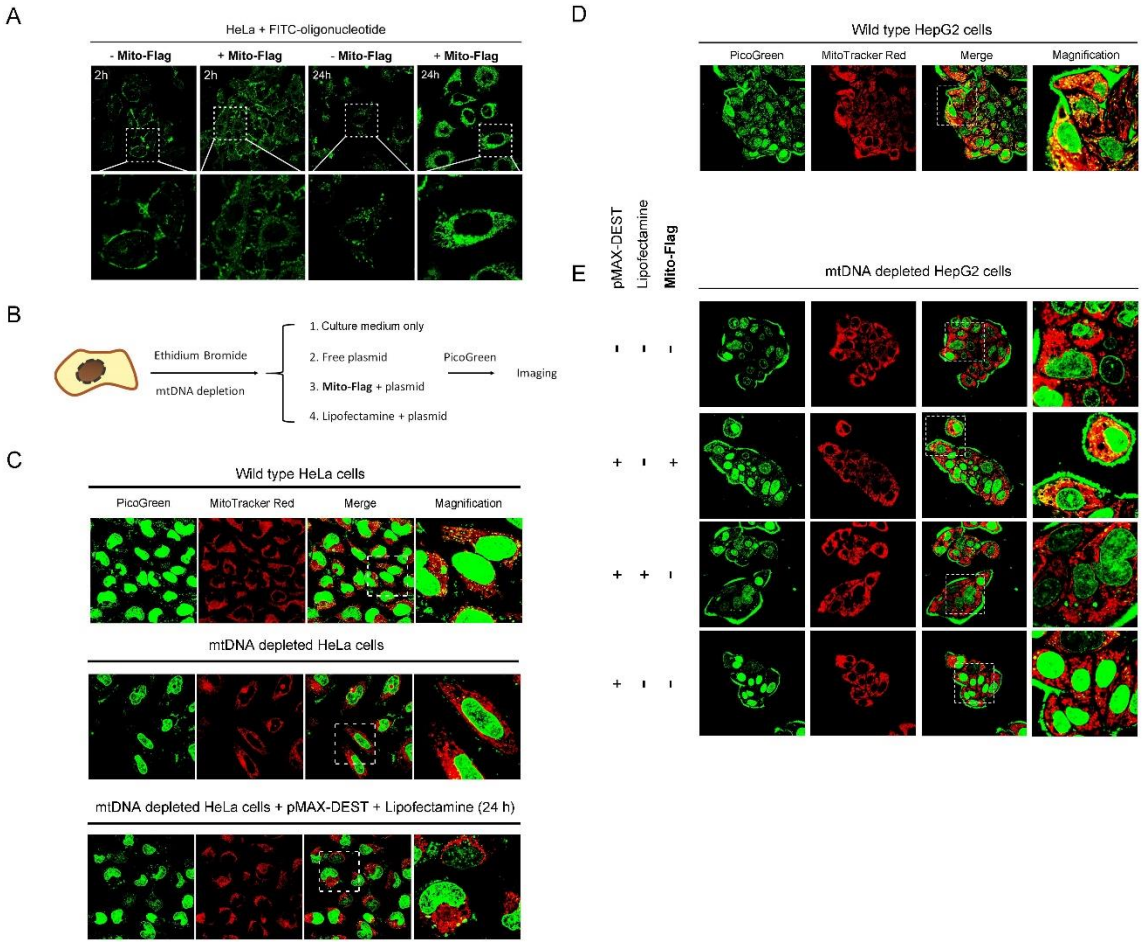
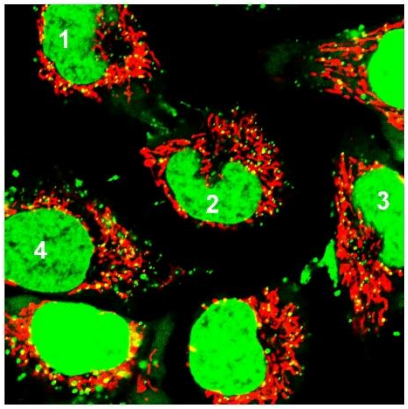


Figure S2. Delivery of FITC-oligonucleotide and pMAX-DEST plasmid (an empty backbone without stuffer) to mitochondria in cells. (A) Fluorescence images of HeLa cells incubated with free FITC-oligonucleotide or the mixture of Mito-Flag and FITC-oligonucleotide for different time. (B) Scheme of the process for the intracellular delivery of pMAX-DEST plasmid into mtDNA depleted cells. (C) Fluorescence images of the mtDNA (stained by PicoGreen) in wide type HeLa cells, mtDNA depleted HeLa cells, and mtDNA depleted HeLa cells incubated with pMAX-DEST mixed with lipofectamine. (D) Fluorescence images of mtDNA (stained by PicoGreen) in wilde type HepG2 cells. (E) Fluorescence images of mtDNA (stained by PicoGreen) in mtDNA depleted HepG2 cells incubated with PBS, the mixture of pMAX-DEST and Mito-Flag, the mixture of pMAX-DEST and lipofectamine, and free pMAX-DEST plasmid.

A

ρ^0 HeLa + plasmid + Mito-Flag



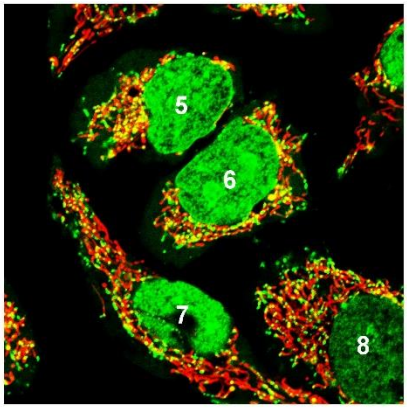
Red = Mitochondria
Green = PicoGreen stained DNA

B

Cell	# of Mitochondria	# of Mitochondria with PicoGreen	Ratio%
1	48	43	90
2	59	50	85
3	64	56	88
4	50	46	92

C

Wild type HeLa control

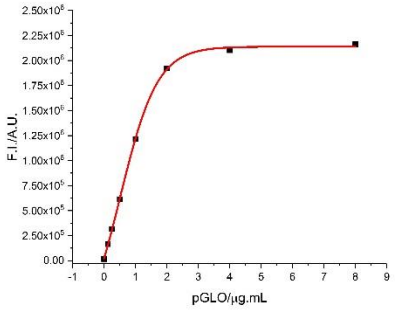


D

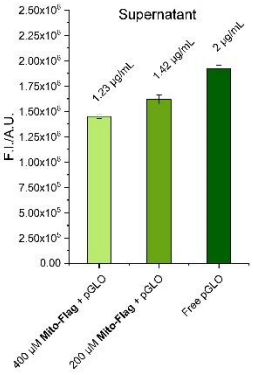
Cell	# of Mitochondria	# of Mitochondria with PicoGreen	Ratio%
5	72	71	99
6	79	78	99
7	109	106	97
8	99	98	99

Figure S3. Ratio of mitochondria overlapped with PicoGreen-stained DNA. (A) Fluorescent images of mtDNA depleted (ρ^0) HeLa cells incubated with Mito-Flag (200 μM) and pMAX plasmid (20 μg/mL, 24 h). (B) Ratio of mitochondria overlapped with PicoGreen-stained DNA in the cells in (A). (C) Fluorescent images of HeLa cells (without EB treatment) stained by PicoGreen and MitoTracker Red. (D) Ratio of mitochondria overlapped with PicoGreen-stained DNA in wild type HeLa cells (without EB treatment).

A



B



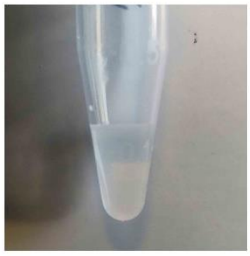
C

Sample ID	Supernatant F.I. / A.U.	DNA concentration in supernatant / μg·mL^{-1}	Encapsulation efficiency (EE)%
Free pGLO (2 μg/mL)	1.92X10^6	2	/
200 μM **Mito-Flag** + pGLO	1.62X10^6	1.42	29.0
400 μM **Mito-Flag** + pGLO	1.45X10^6	1.23	38.5

D

400 μM Mito-Flag + pGLO

E



Free pGLO

F

Time in FBS/h	Encapsulation Efficiency (EE%)
0	30.4%
8	30.8%
24	27.6%

G

PBS Concentration	Ionic Strength/molXL^{-1}	Encapsulation Efficiency (EE%)
1X	0.1751	30.7%
2X	0.3502	33.2%
4X	0.7004	42.3%

Figure S4. DNA encapsulation efficiency. (A) Standard curve of pGLO plasmid determined by PicoGreen assay. (B) Fluorescence intensity and pGLO plasmid concentration in the supernatant of "pGLO plasmid", "200 μM Mito-Flag + pGLO" and "400 μM Mito-Flag + pGLO" after centrifuge. (C) DNA encapsulation efficiency (EE)% of Mito-Flag at different concentrations. (D) and (E) Photos of the pellets (indicated by arrow) after centrifuge. (F) DNA EE% of pGLO plasmid by Mito-Flag (200 μM) dissolved in FBS (with DNase inhibitor) for different time. (G) DNA EE% of pGLO plasmid by Mito-Flag (200 μM) dissolved in solutions of different ionic strength.

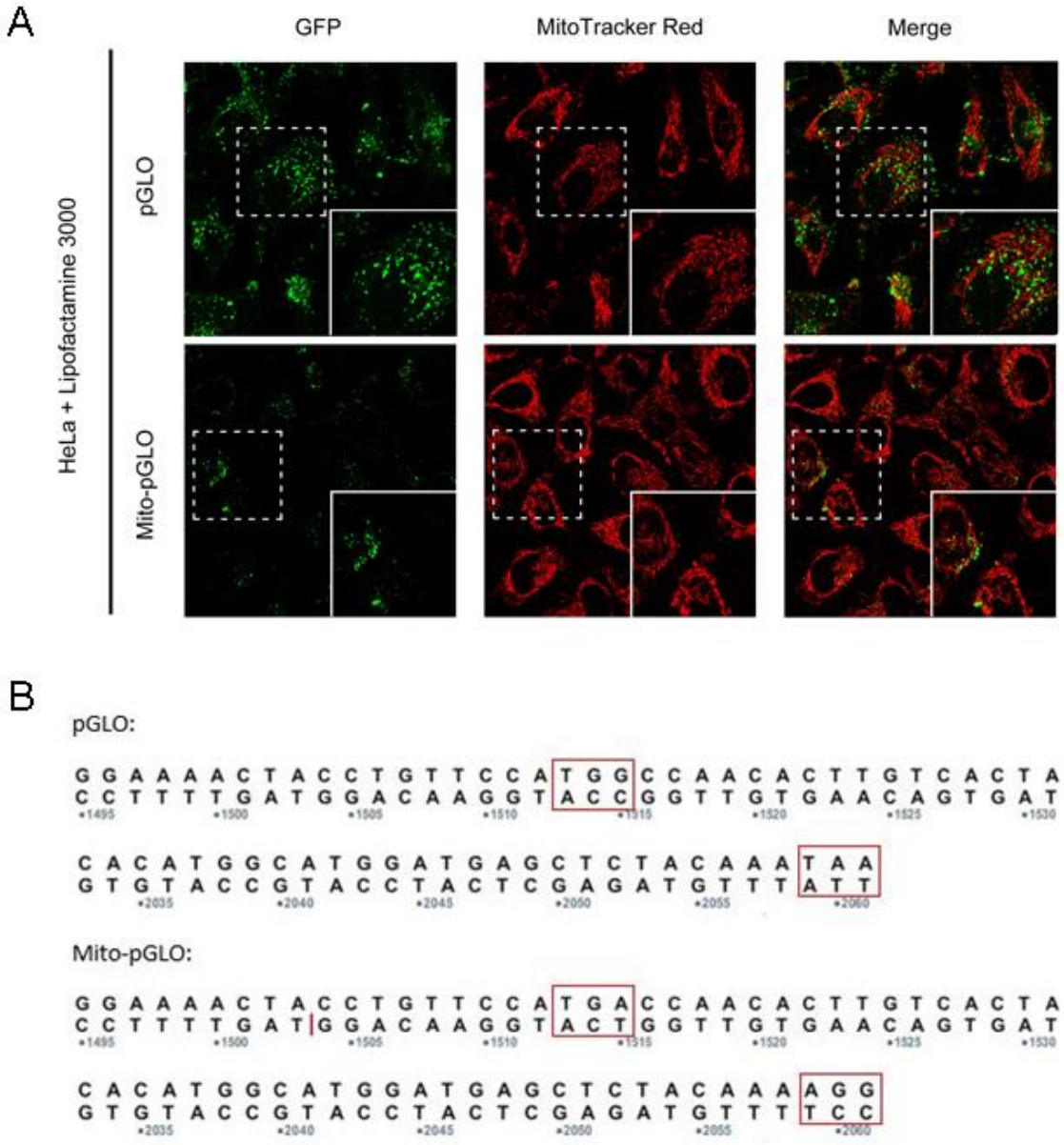


Figure S5. Fluorescent images of the HeLa cells transfected with pGLO or Mito-pGLO plasmid via Lipofectamine 3000. (A) Fluorescent images of HeLa cells transfected with pGLO or Mito-pGLO plasmid via Lipofectamine 3000. HeLa cells incubated with Lipofectamine 3000 and Mito-pGLO plasmid only show sporadic fluorescence around mitochondria, which may due to the DNA import into mitochondria[8] after the cellular uptake of Mito-pGLO plasmid. (B) Partial gene sequence of pGLO and Mito-pGLO (the mutated codons are circled).

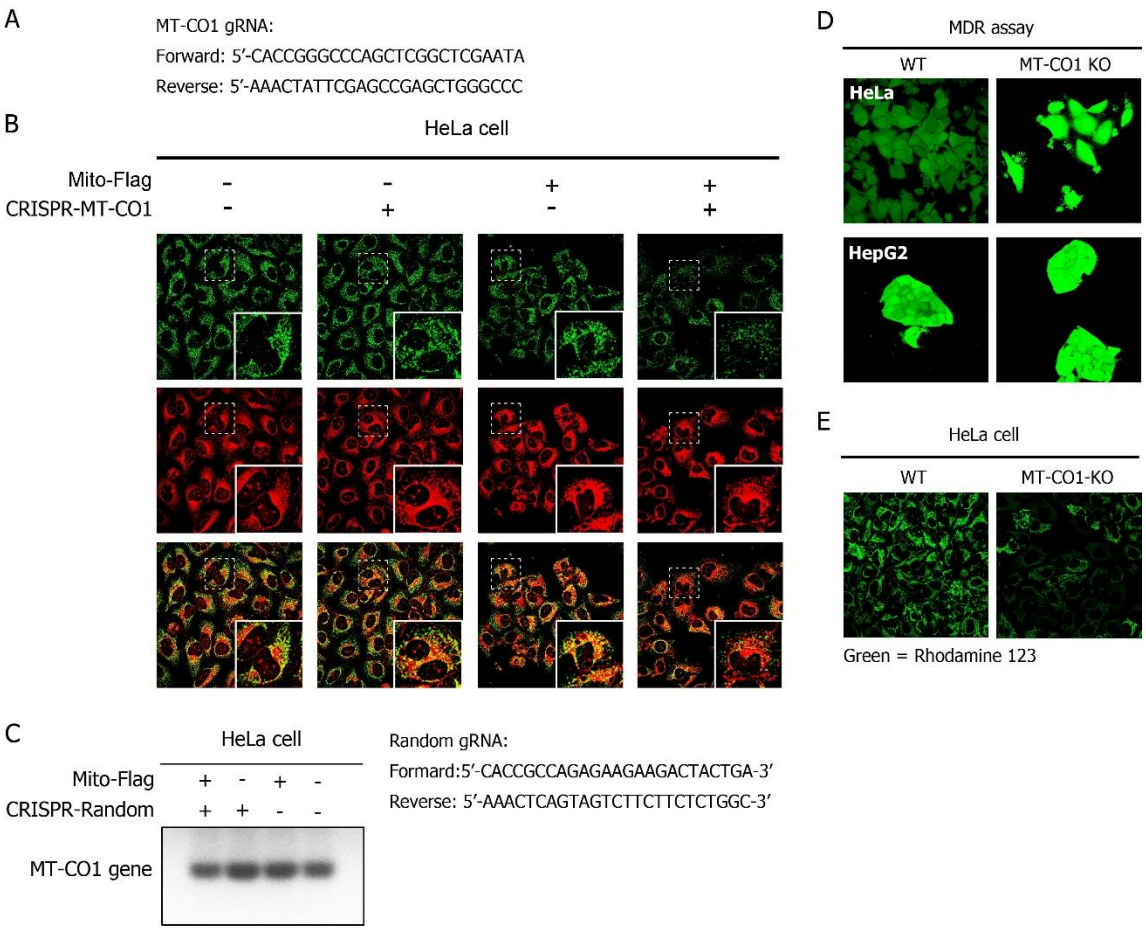


Figure S6. ENS of Mito-Flag delivers CRISPR/Cas9 components into the mitochondria of cancer cells for mitochondrial genome editing. (A) gRNA for MT-CO1. (B) Immunofluorescence staining of MT-CO1 in HeLa cells treated by conditions of interest. (C) DNA electrophoresis of the PCR products from HeLa cells incubated with the mixture of Mito-Flag and the plasmid encoding CRISPR/Cas9 and random gRNA control. (D) MDR assay for wild type (WT) and MT-CO1 KO HeLa and HepG2 cells. (E) Evaluation of mitochondrial membrane potential in WT and MT-CO1 KO HeLa cells via Rhodamine 123.

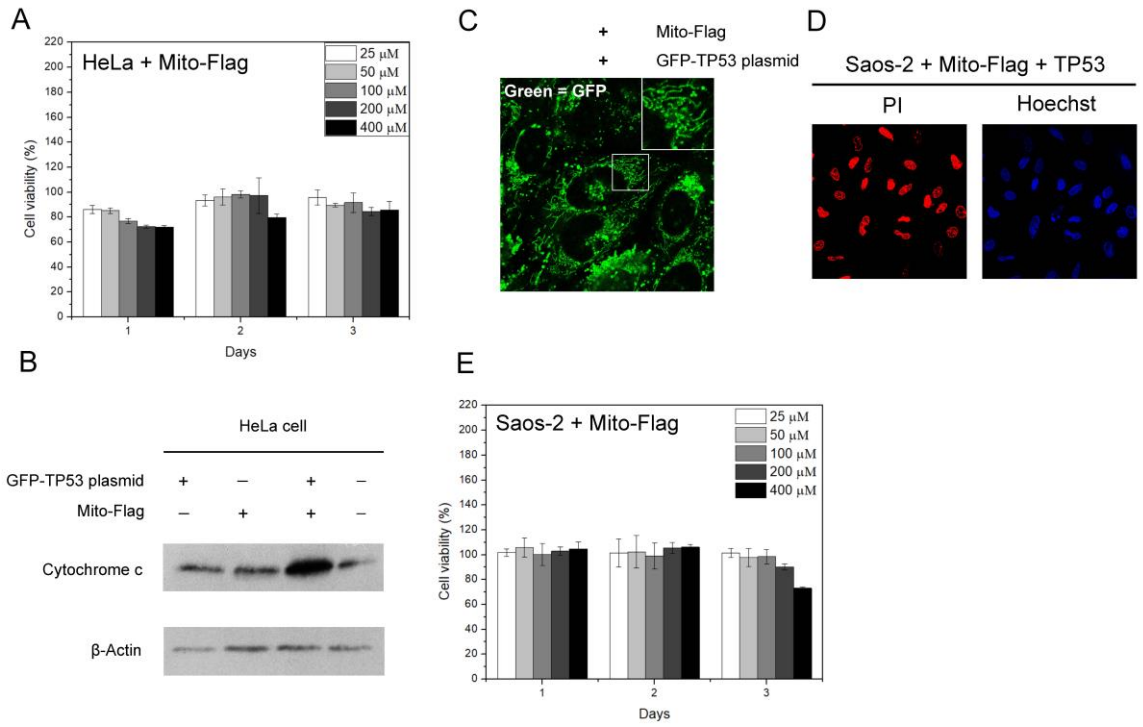


Figure S7. The mixture of Mito-Flag and GFP-TP53 plasmid induce mitochondrial permeabilization and apoptosis. (A) Cell viability assay of HeLa cell incubated with Mito-Flag. (B) Western blot analysis of Cyt C in the cytosolic fraction of HeLa cells treated by conditions of interest. (C) Confocal fluorescence image of Saos-2 cells incubated with the mixture of GFP-TP53 plasmid and Mito-Flag. (D) Apoptosis assay (via PI) of Saos-2 cells incubated with the mixture of GFP-TP53 plasmid and Mito-Flag (3rd day). (E) Cell viability assay of Saos-2 cell incubated with Mito-Flag.

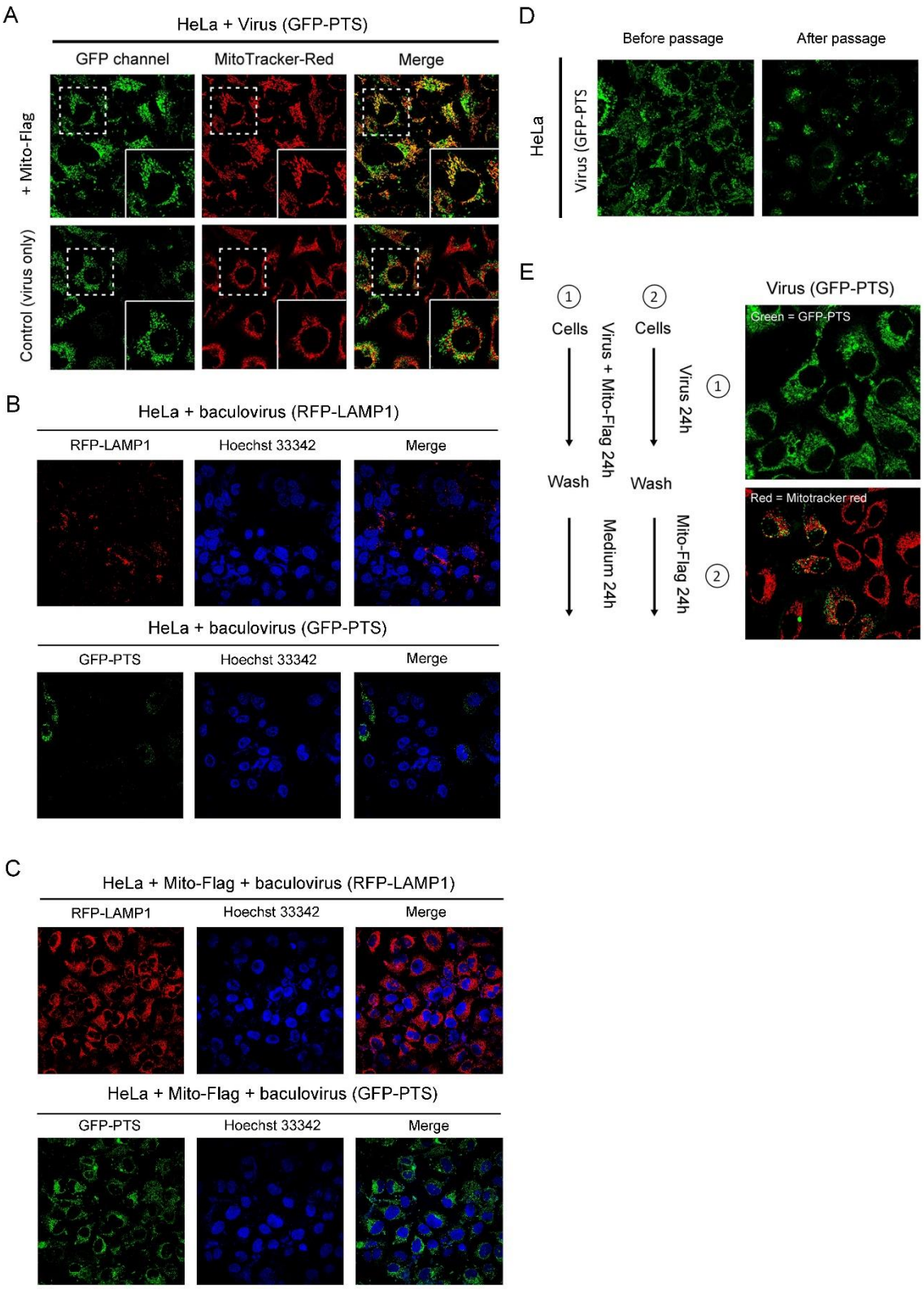


Figure S8. **Mito-Flag improve transfection efficiency for baculoviral vectors.** (A) Fluorescent images of HeLa cells incubated with baculovirus encoding GFP-PTS with/without Mito-Flag. (B)

and (C) Fluorescent images of HeLa cells incubated with baculovirus encoding RFP-LAMP1 or GFP-PTS with (B) and without (C) Mito-Flag. The incubation time is 24 h. Nucleus were stained by Hoechst 33342. The use of Mito-Flag results in more cells expressing RFP-LAMP1 or GFP-PTS compared to the control. (D) Sequential treatment of HeLa cells by baculovirus encoding GFP-PTS and Mito-Flag. (E) The fluorescence intensity from GFP-PTS in mitochondria decreases after cell passage.

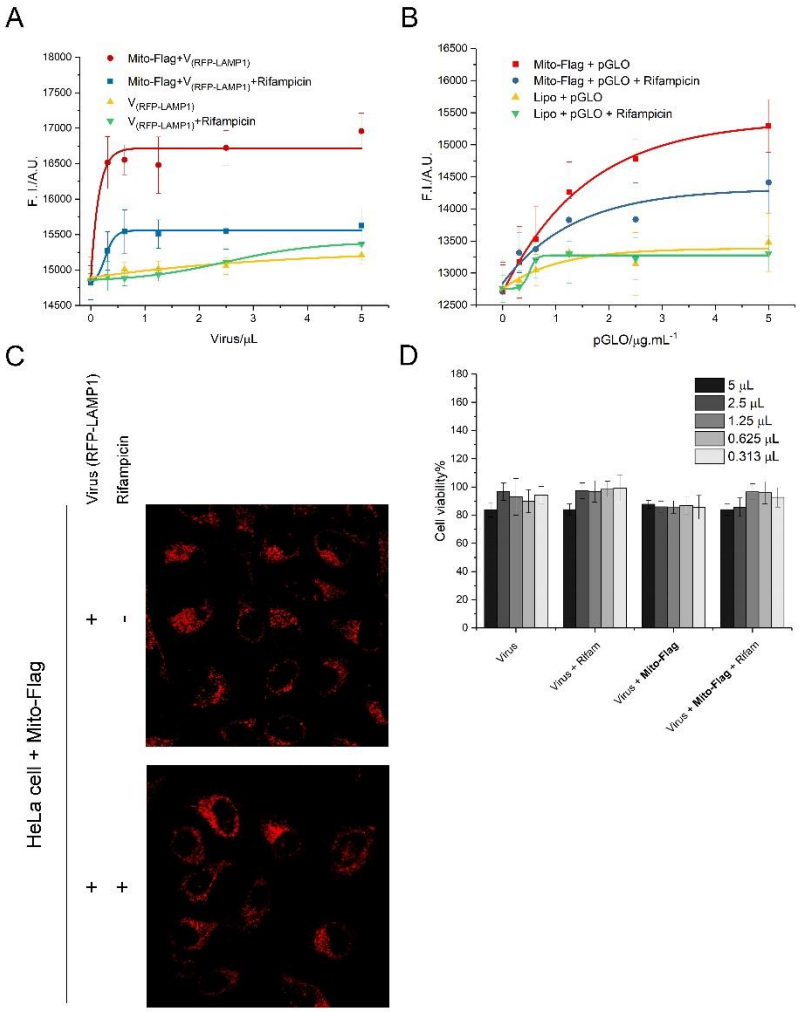


Figure S9. Rifampicin weakens the mitochondrial fluorescence of HeLa cells treated by gene vectors mixed with Mito-Flag. (A) Fluorescent images of the HeLa cells incubated with virus (RFP-LAMP1) with/without rifampicin. No difference in fluorescence intensity was observed, indicating rifampicin hardly effects the gene transcription in nucleus. (B) Quantification of intracellular RFP fluorescence intensity in the HeLa cells treated by "Mito-Flag + virus (RFP-LAMP1)", "Mito-Flag + virus (RFP-LAMP1) + rifampicin", "free virus (RFP-LAMP1)", and "free virus (RFP-LAMP1) + rifampicin". (C) Cytotoxicity of "virus (RFP-LAMP1)", "virus (RFP-LAMP1) + rifampicin", "Mito-Flag + virus (RFP-LAMP1)", and "Mito-Flag + virus (RFP-LAMP1) + rifampicin" against the HeLa cells (24 h). No difference in cell viability suggests that the decreased intracellular fluorescence is not due to cell growth inhibition. (D) Quantification of

intracellular GFP fluorescence intensity in the HeLa cells treated by “Mito-Flag + pGLO”, “Mito-Flag + pGLO + rifampicin”, “Lipofactamin + pGLO”, and “Lipofactamin + pGLO + rifampicin”. The concentration of Mito-Flag is 200 μM, rifampicin is 80 μg/mL.

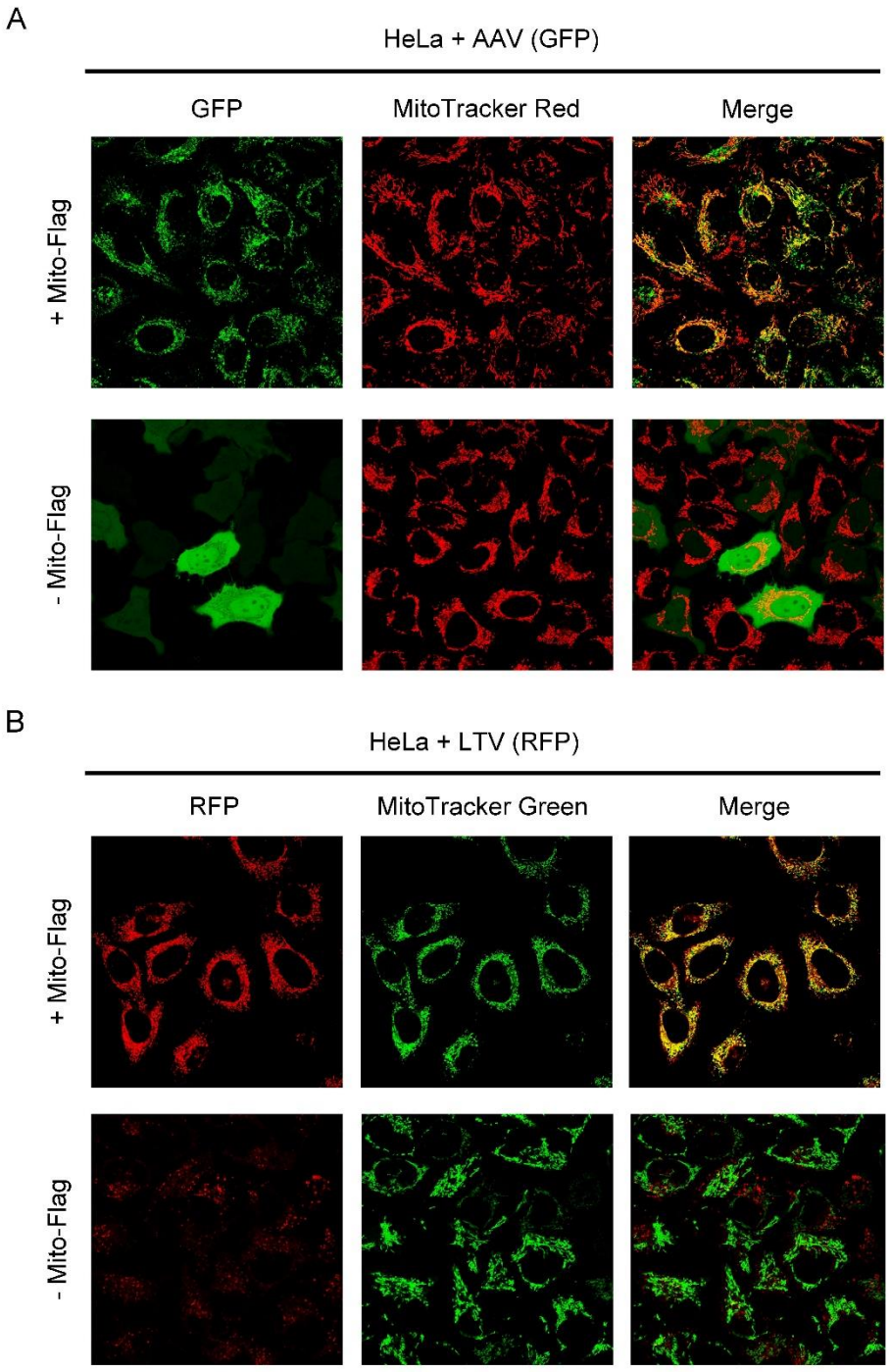


Figure S10. The combination of Mito-Flag and AAV or LTV results in mitochondrial specific gene expression. (A) Fluorescent images of HeLa cells incubated with free AAV or AAV mixed

by Mito-Flag. (B) Fluorescent images of HeLa cells incubated with free LTV or LTV mixed by Mito-Flag. The incubation time is 24 h. Mito-Flag is 200 μM.

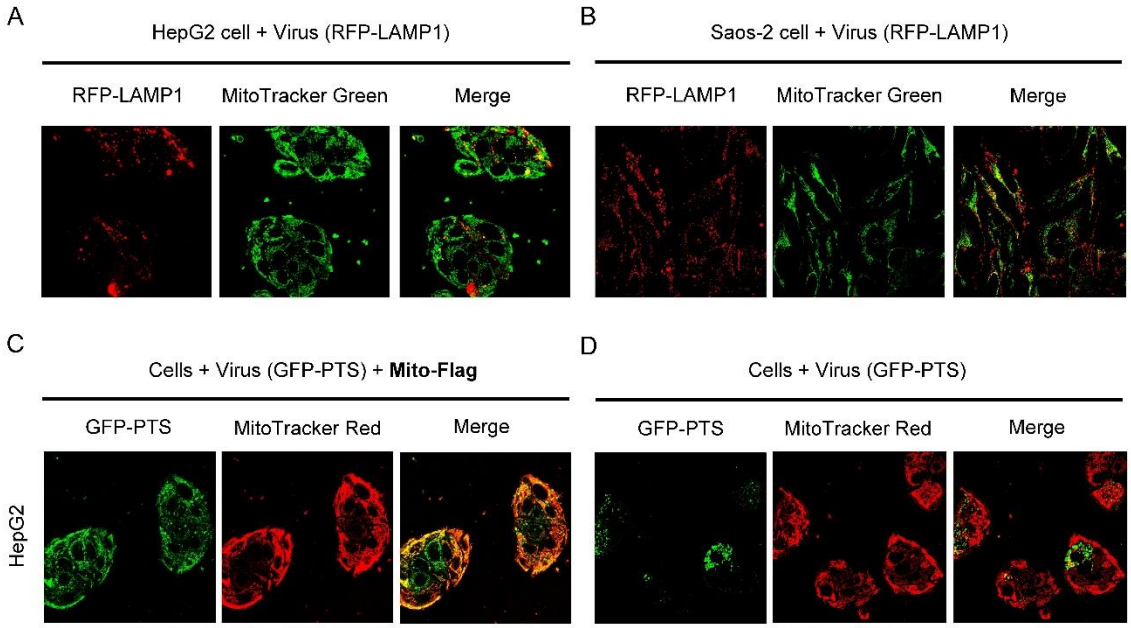


Figure S11. Mito-Flag guides the gene expression of baculoviral vector into the mitochondrial of Saos-2 and HepG2 cells. (A) and (B) Fluorescent images of HepG2 (A) and Saos-2 (B) cells incubated free baculovirus (RFP-LAMP1). (C) and (D) Fluorescent images of HepG2 cells incubated with (C) the mixture of Mito-Flag (200 μM) and virus (GFP-PTS), and (D) free virus (GFP-PTS). The incubation time is 24 h.

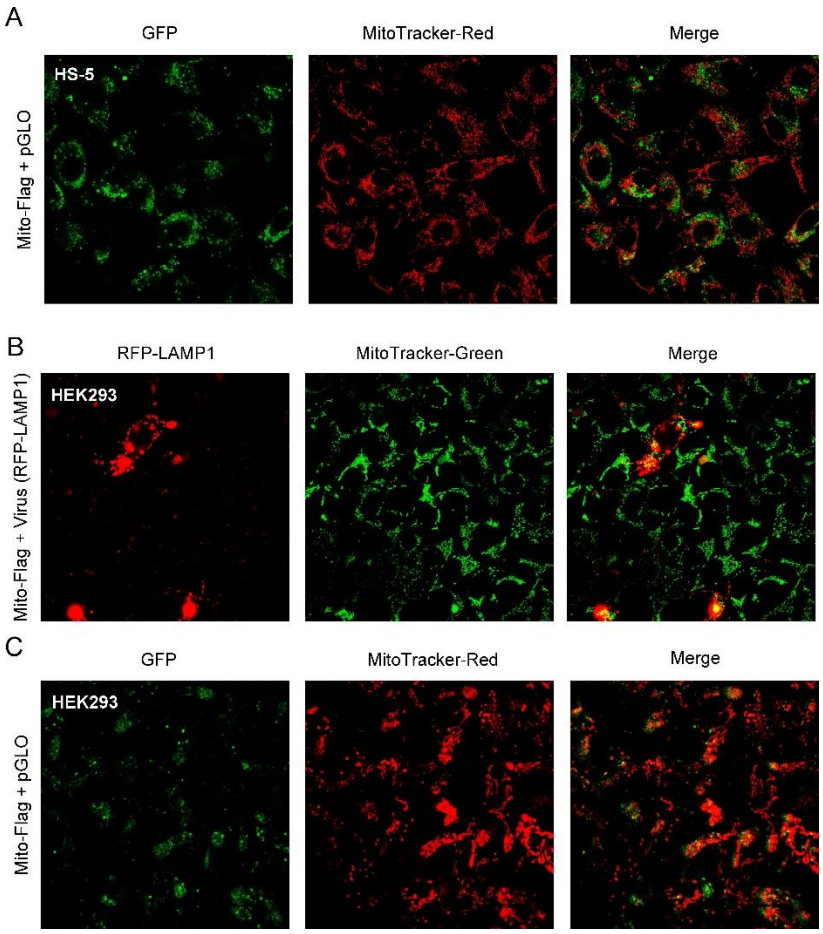


Figure S12. Fluorescent images of HS-5 and HEK293 cells incubated with the mixture of Mito-Flag and gene vectors. (A) HS-5 treated by the mixture of Mito-Flag and pGLO plasmid (5 μg/mL) for 48 h. (B) and (C) HEK293 cells treated by (B) the mixture of Mito-Flag and virus (RFP-LAMP1) or (C) pGLO plasmid (5 μg/mL, 48 h). Mito-Flag hardly translocate the gene expression of gene vectors in the mitochondria of non-cancerous cells. The concentration of Mito-Flag is 200 μM.

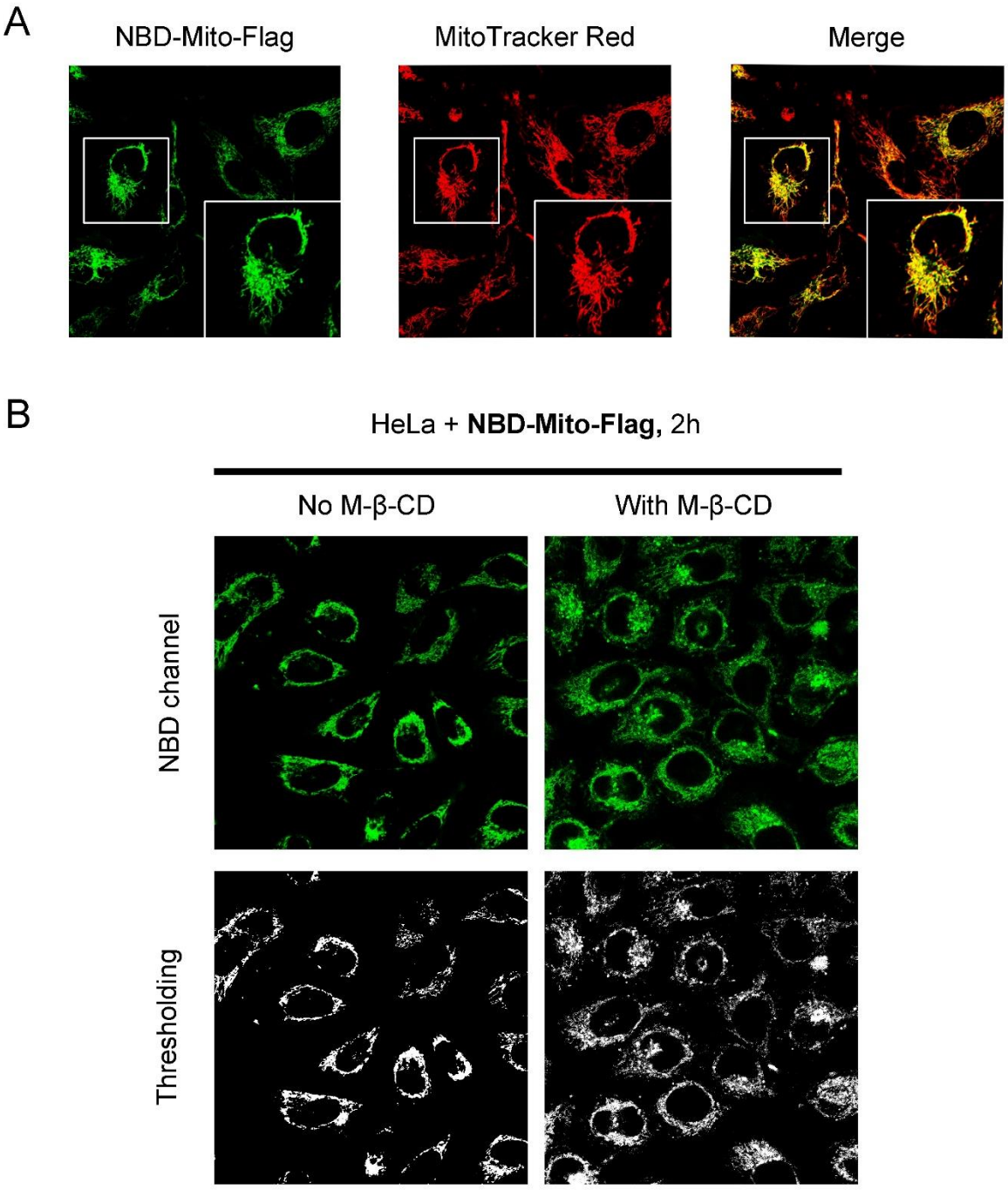


Figure S13. NBD-Mito-Flag accumulates in mitochondria. (A) Fluorescent images of the HeLa cells incubated with NBD-Mito-Flag. (B) M-β-CD (5 mM), a caveolin-dependent endocytosis inhibitor, hardly reduce the cellular uptake of NBD-Mito-Flag (200 μM, 2 h). The concentration of NBD-Mito-Flag is 200 μM, incubation time is 2 h. Mitochondria in HeLa cells were stained by MitoTracker-Red.

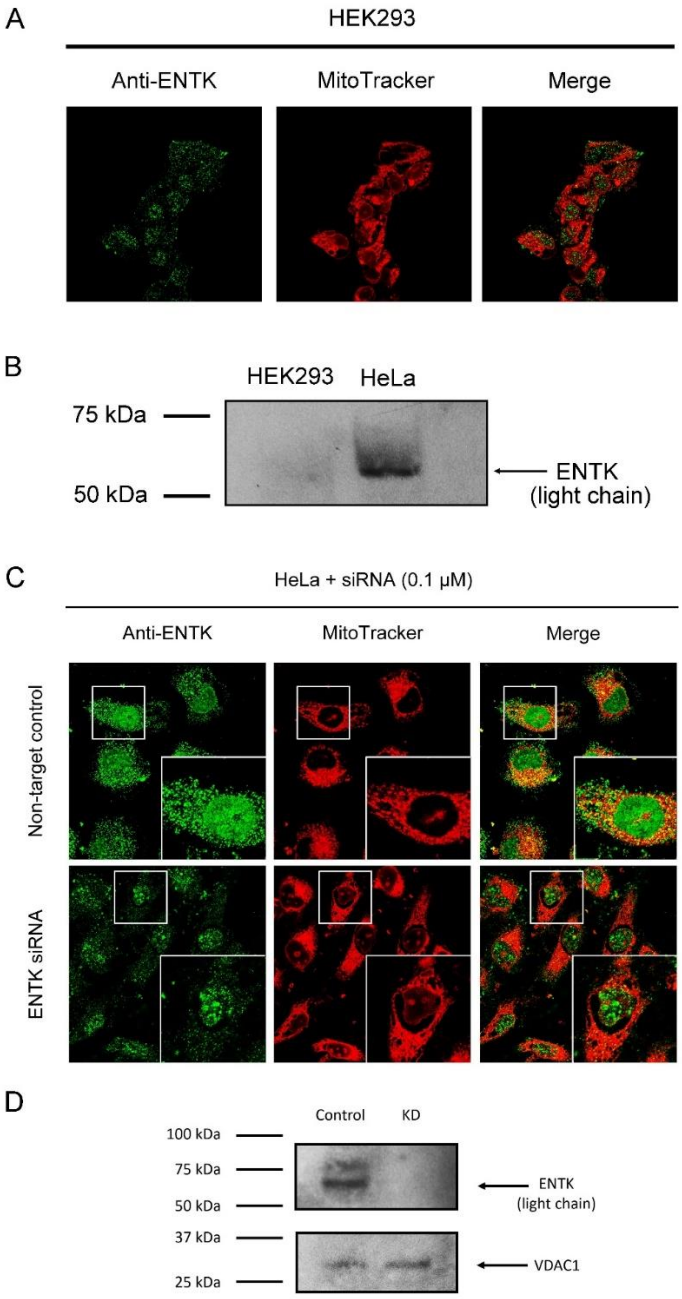


Figure S14. Low mitochondrial ENTK level presents in HEK293 and ENTK-knockdown HeLa cells. (A) immunofluorescent staining images of ENTK in HEK293 cells. (B) western blot analysis of ENTK in the mitochondria isolated from HEK293 and HeLa cells. (C) Antibody staining of ENTK in control and ENTK-knockdown (via siRNA) [9] HeLa cells. (D) western blot analysis of ENTK in mitochondria isolated from control and ENTK-knockdown HeLa cells. VDAC1 serves as mitochondria loading control.

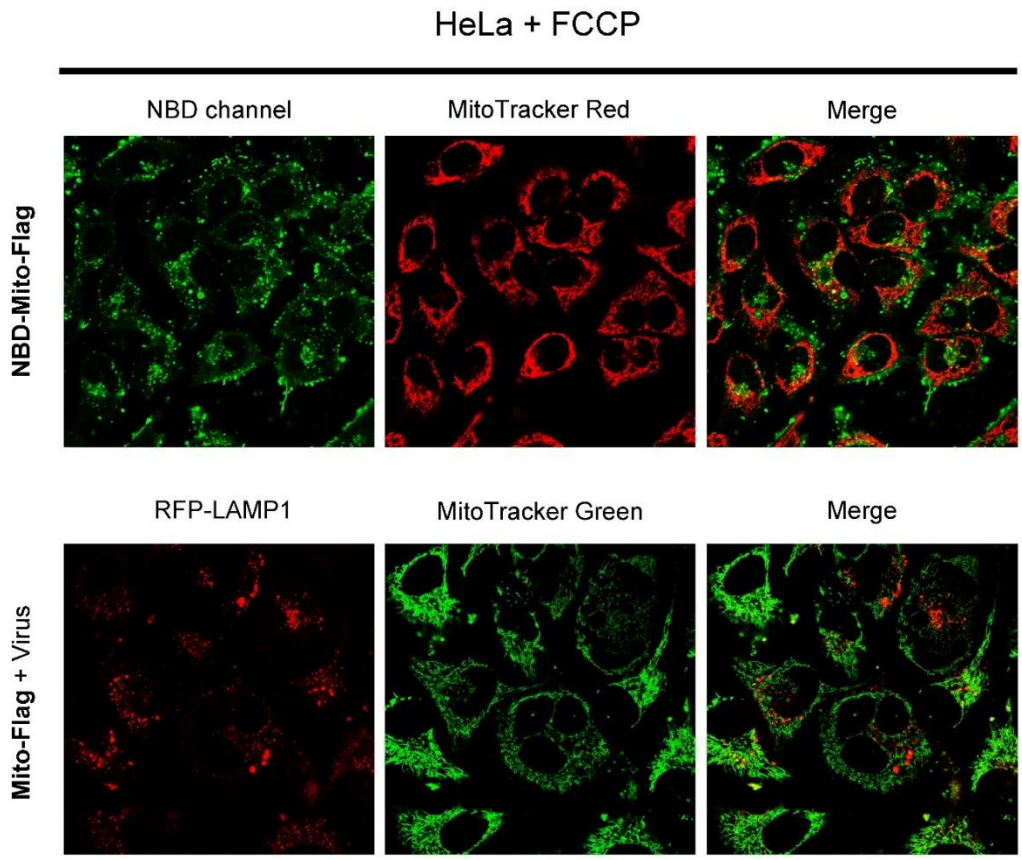


Figure. S15. Dissipating mitochondrial membrane potential inhibits the Flag-tagged peptides from targeting mitochondria. Fluorescent images of the HeLa cells treated by NBD-Mito-Flag (200 μM, 2 h) or the mixture of virus (RFP-LAMP1) and Mito-Flag (200 μM, 24 h) in the presence of FCCP[10] (0.5 μM).

Supplemental References

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