

Supporting Information

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SI Materials and Methods

Materials. The following commercial antibodies were used: mouse monoclonal anti-DNA (AC-3010; Progen); anti-Drp1 (D80320) and anti-cytochrome *c* (6H2.B4 or 7F8.2C12; BD Transduction); anti- β -actin (AC-74; Sigma-Aldrich); anti-Cox2 (12C4F12) and MitoProfile Total OXPHOS Rodent WB Antibody Mixture (MitoSciences); anti-mitofusin (Mfn)1 (3C9; Abnova); anti-FLAG (M2; Sigma-Aldrich); mouse polyclonal anti-transcriptional factor A (TFAM) (Abnova); rabbit monoclonal anti-Cox4 (3E11) and anti-GAPDH (14C10; Cell Signaling); rabbit polyclonal anti-Tom20 and anti-Bax (Santa Cruz Biotechnology); anti-mitochondrial fission factor (Mff) (Proteintech); anti-poly(ADP ribose) polymerase (PARP) (Roche); anti-cleaved caspase-3 (Cell Signaling); anti-Smac/DIABLO (Enzo Life Sciences); anti-Sec61 β (Upstate Biotechnology); anti-FLAG (Sigma-Aldrich); and Alexa Fluor 488-, 568-, 647-, or 660-labeled goat anti-mouse IgG, IgG₁, or IgM or anti-rabbit IgG and HRP-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes). Rabbit polyclonal anti-mitofilin antibody was a generous gift from Dr. Toshihiko Oka (Rikkyo University). Mouse monoclonal antibody against Tom22 (IC9-2) (1) and rabbit polyclonal antibodies against Tom40 and Tom70 (2) have been described previously. SYBR Green I was purchased from Molecular Probes; 2'-3'-dideoxycytidine (ddC), actinomycin D (Act D), staurosporine (STS), and Mdivi-1 were purchased from Sigma-Aldrich; and zVAD-fmk was purchased from Peptide Institute. Mammalian expression plasmids of 3xFLAG-rat Drp1, Drp1 K38A, and 3xFLAG-rat Mfn1 were described previously (3, 4).

The target sequences of RNAi oligonucleotides (stealth RNA; Invitrogen) were as follows: 5'-CCUUGUCCAGGUCAGCG-UUUGGCG-3' for *Mff*, 5'-AUUCCUGUAAUCUUGCAUGA-AAUCC-3' for *Mfn1*, and 5'-AAUCCAGAGGGCAGAAC-UUUGUCC-3' for *Mfn2*. siRNAs for *Drp1* (3) and *OPA1* (5) have been described previously.

Immunofluorescence Staining of Fixed Cells. HeLa cells, HeLa cells expressing mitochondria-targeted DsRed (mitRFP) (3), and mouse embryonic fibroblasts (MEFs) (2) were grown in DMEM (Wako) supplemented with 10% FBS (Invitrogen). Lipofectamine 2000 (Invitrogen) was used for plasmid transfection according to the manufacturer's instructions. Cells grown on coverslips were fixed for 15 min with 4% paraformaldehyde, washed twice with PBS, and permeabilized for 5 min with 0.2% Triton X-100 in PBS. After three washes with PBS, cells were blocked for 30 min with 5% skim milk in PBS, then incubated with primary antibodies for 1 h at room temperature. After four washes with PBS, cells were incubated with secondary antibodies for 1 h at room temperature. After four washes with PBS, the coverslips were mounted (SlowFade Gold antifade reagent; Molecular Probes). Samples were observed under a Zeiss LSM700 confocal microscope with a Plano Aplanachromat 63 \times /1.4 numerical aperture oil immersion objective. Confocal images were acquired using Zeiss ZEN 2010LSM software.

Quantitative PCR and RT-PCR. DNA from HeLa cells was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The KAPA SYBR FAST qPCR Kit was used for quantitative PCR with an ABI PRISM 7000 sequence detection system (Applied Biosystems). To produce a

standard curve, 0.5, 1, 2, 4, and 8 ng for mitochondrial DNA (mtDNA) amplification of untreated HeLa cell DNA or 1, 2, 4, 8, and 16 ng for nuclear gene amplification of untreated HeLa cell DNA was used. The primer sets for amplification of mtDNA (65 bp) and β 2M coding nuclear DNA (95 bp) fragments were used as described previously (6).

For RT-PCR, DNA-free RNA samples were prepared using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using the SuperScript First-Strand synthesis system (Invitrogen). The primer sets for mitochondrial transcripts (*ND2*, *ND5*, *COX I*, *COX II*, and *cyt b*) were used as described previously (7). The primer sets for *GAPD* and *HPRT I* (8) served as controls.

EM Studies. Cells were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 37 °C and kept for 30 min at 4 °C. They were then fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4 °C overnight, after which samples were analyzed by Tokai Em, Inc. Samples were rinsed three times with 0.1 M phosphate buffer (pH 7.4) for 30 min each, followed by postfixation with 2% osmium tetroxide (OsO₄) in 0.1 M phosphate buffer (pH 7.4) at 4 °C for 1 h. Fixed samples were dehydrated, embedded in Quetol-812 (Nissin EM), and polymerized at 60 °C for 48 h. The blocks were ultrathin-sectioned at 70 nm with a diamond knife using an Leica ultramicrotome. Sections were placed on copper grids and stained with 2% uranyl acetate at room temperature for 15 min and then rinsed with distilled water, followed by secondary-staining with lead stain solution (Sigma-Aldrich) at room temperature for 3 min. The grids were observed under a JEOL JEM-1200EX transmission electron microscope at an acceleration voltage of 80 kV.

Analysis of Apoptosis. Cells were exposed to 5 μ M Act D or 1 μ M STS with or without 50 μ M zVAD-fmk. For cell fractionation, cells were collected and treated with 0.02% digitonin for 3 min at 37 °C. The cytosolic and organellar fractions were separated by centrifugation and lysed with SDS sample buffer, then evaluated by immunoblot analysis of indicated antibodies as described above.

Oxygen Consumption Measurements. For measurements of glutamate/malate-driven respiration rates, cells (5×10^6) were suspended in respiration buffer [30 mM Hepes, 75 mM sucrose, 20 mM glucose, 5 mM potassium phosphate buffer (pH 7.1), 40 mM KCl, 0.5 mM EDTA, and 3 mM MgCl₂]. Next, an equal volume of respiration buffer I containing 0.01% digitonin was added to the cell suspension, and the mixture was incubated for 5 min at room temperature. Permeabilization was stopped by the addition of 10 volumes of respiration buffer containing 0.3% BSA. These permeabilized cells were collected by centrifugation and resuspended in respiration buffer. Oxygen consumption was measured on whole cells at 30 °C, using Clark's electrode (Oxytherm; Hansatech Instruments), in the presence of 1 mM ADP. Glutamate and malate were added at 5 mM each. Maximum respiration was the oxygen consumption rate after addition of 25 μ M uncoupler dinitrophenol. After a few minutes, respiration was blocked with 0.75 mM KCN.

1. Saeki K, et al. (2000) Identification of mammalian TOM22 as a subunit of the preprotein translocase of the mitochondrial outer membrane. *J Biol Chem* 275(41):31996-32002.

2. Ishihara N, et al. (2009) Mitochondrial fission factor Drp1 is essential for embryonic development and synapse formation in mice. *Nat Cell Biol* 11(8):958-966.

3. Taguchi N, Ishihara N, Jofuku A, Oka T, Mihara K (2007) Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. *J Biol Chem* 282(15):11521–11529.

4. Eura Y, Ishihara N, Yokota S, Mihara K (2003) Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. *J Biochem* 134(3):333–344.

5. Ishihara N, Fujita Y, Oka T, Mihara K (2006) Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. *EMBO J* 25(13):2966–2977.

6. Malik AN, Shahni R, Rodriguez-de-Ledesma A, Laftah A, Cunningham P (2011) Mitochondrial DNA as a non-invasive biomarker: Accurate quantification using real-time

quantitative PCR without co-amplification of pseudogenes and dilution bias. *Biochem Biophys Res Commun* 412(1):1–7.

7. Piechota J, et al. (2006) Differential stability of mitochondrial mRNA in HeLa cells. *Acta Biochim Pol* 53(1):157–168.

8. Vandesompele J, et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3(7):RESEARCH0034.

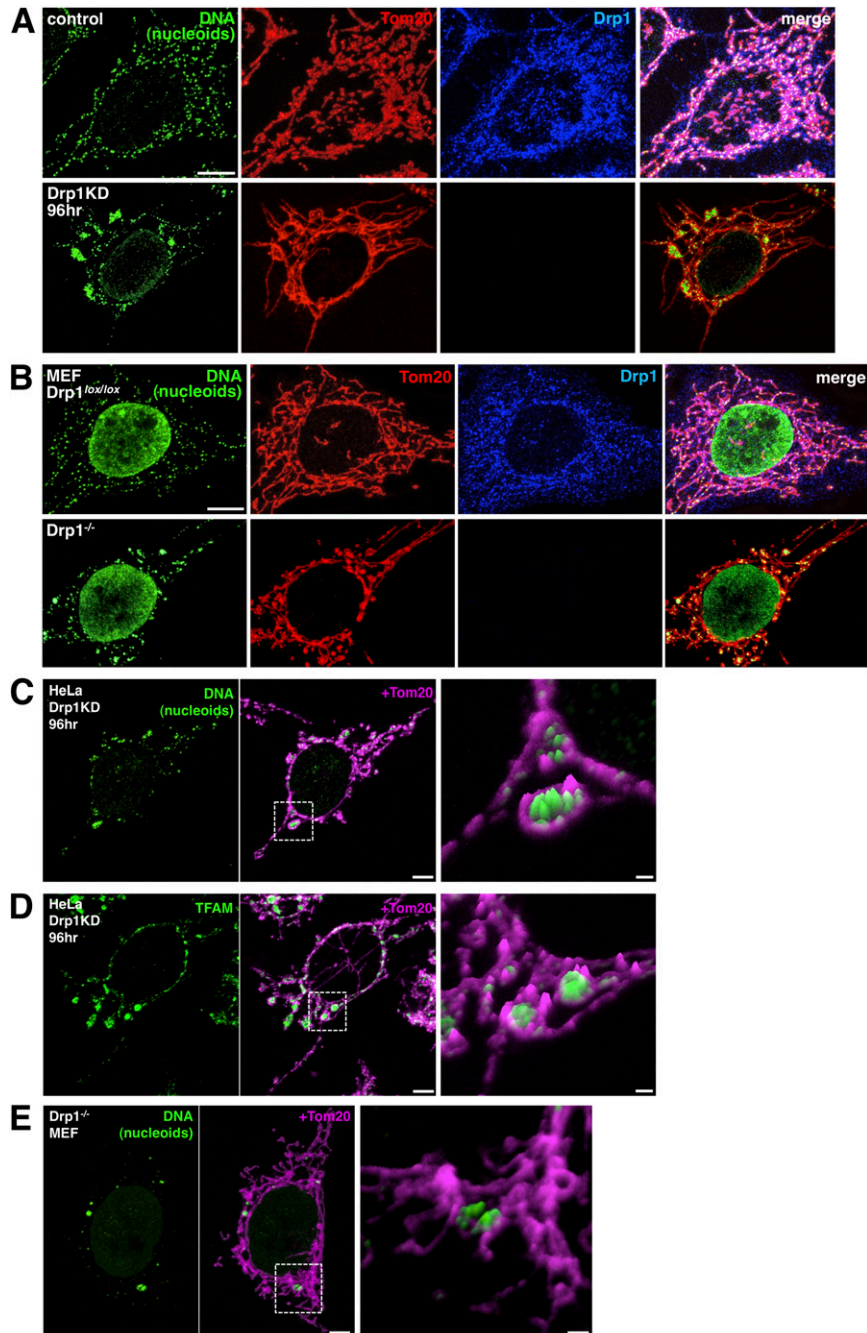


Fig. S1. Enlarged nucleoids in Drp1-deficient cells were formed from clusters of small mtDNA puncta. (A and B) Single-color images of Fig. 1A (A) and Fig. 1B (B) (Scale bar: 10 μ m.) (C–E) Representative images of immunofluorescence staining of Drp1 KD HeLa cells (C and D) and Drp1 KO MEFs (E). (Right) Intensity profile landscapes (2.5D images) of the boxed area in the merged panel. Magenta, Tom20; green, nucleoids (mtDNA) or TFAM. (Scale bars: 5 μ m in Middle; 1 μ m in Right.)

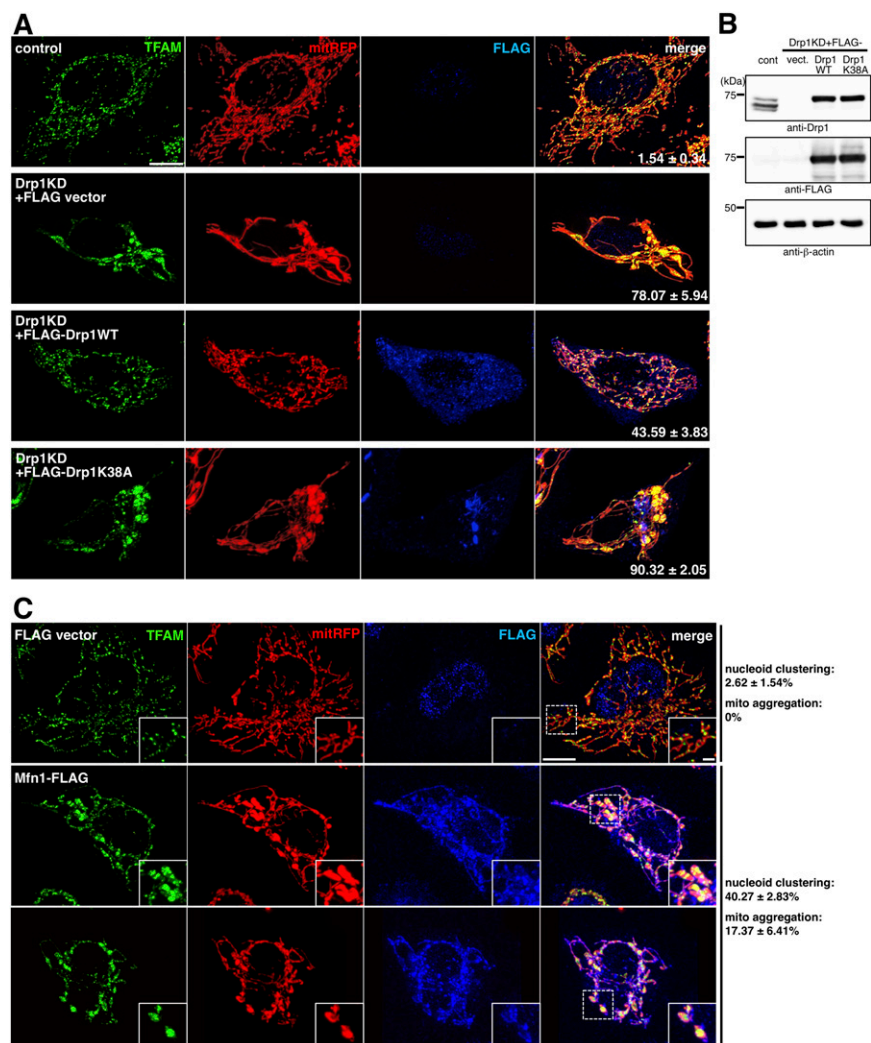


Fig. S2. Mitochondrial fusion/fission factors and nucleoid structures. (A and B) Enlarged nucleoids in Drp1-deficient HeLa cells were reversible. Single color images of Fig. 1C are shown in A. (Scale bar: 10 μ m.) Expression levels of endogenously and exogenously expressed proteins determined by immunoblot analysis are shown in B. (C) Enlarged nucleoids in Mfn1-overexpressing HeLa cells. HeLa cells stably expressing mitRFP were transfected with FLAG-tagged rat Mfn1 or empty vector for 24 h. (Scale bars: 10 μ m; 2 μ m in *Insets*.)

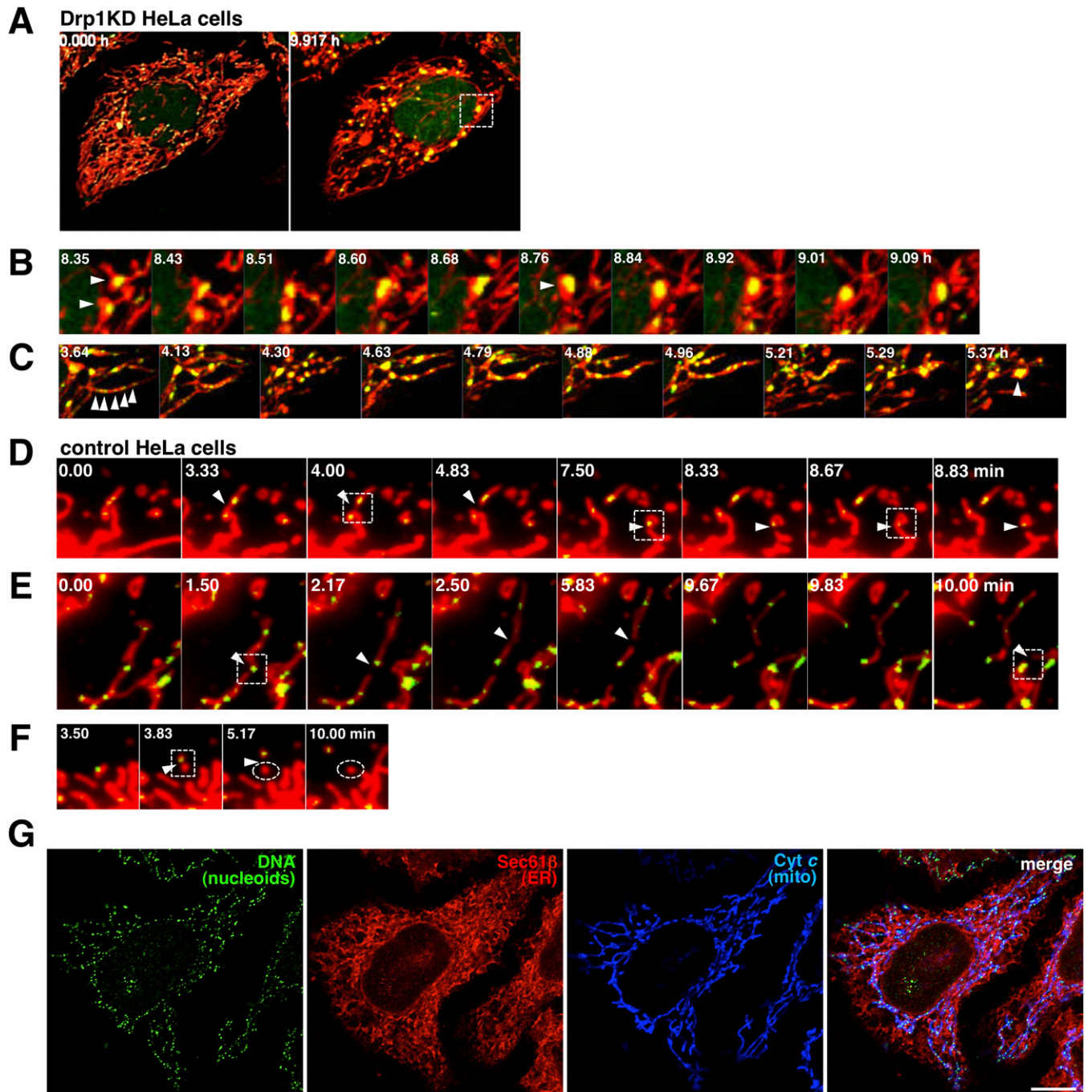


Fig. S3. Live imaging of nucleoids in Drp1-deficient and control cells. (A–F) HeLa cells stably expressing mitRFP were treated with siRNA for Drp1 for 24 h (A–C) or were left untreated (D–F). Cells were stained with SYBR Green 1 for 5 min, and live-cell images were obtained by confocal microscopy (A–C) or fluorescence microscopy (D–F) as in Fig. 2. Overall landscapes and magnified images of the boxed area in [Movie S2](#) are shown in [A](#) and [B](#), respectively. Magnified images of the boxed area in [Movie S3](#) are shown in [C](#). Time-lapse images of mitochondria and nucleoids in [Movies S8](#), [S9](#), and [S10](#) are shown in [D](#), [E](#), and [F](#), respectively. (G) Distribution of ER and nucleoids; overall landscapes of the HeLa cells shown in Fig. 2G. (Scale bar: 10 μ m.)

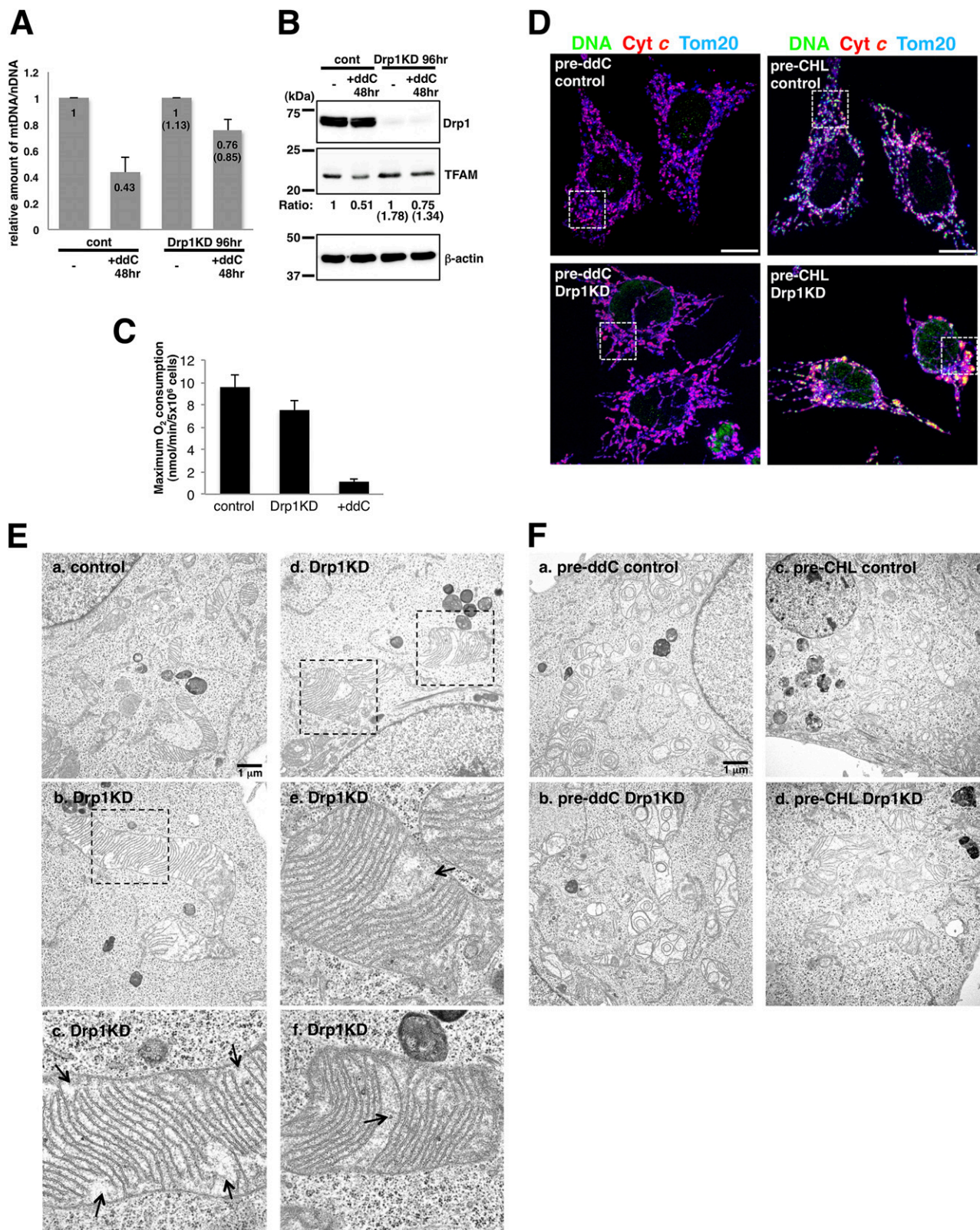


Fig. 54. Mitochondrial cristae are highly stacked in mito-bulbs. (A and B) Effect of mtDNA replication inhibitor on enlarged nucleoids. HeLa cells were treated with siRNA for Drp1 or with control for 96 h, and then treated with 10 μ M ddC to inhibit mtDNA replication or with control vehicle for 48 h. (A) mtDNA content was quantified by quantitative PCR. Results are the average of three independent experiments. (B) Endogenous TFAM protein levels in cells shown in A were determined by immunoblot analysis. Rates of reduction of mtDNA mass and TFAM protein levels by ddC exposure in Drp1 KD cells were moderate compared with control cells. (C) Respiration rates of Drp1KD cells. Maximum oxygen consumption rate in the presence of uncoupler dinitrophenol were measured. Data are expressed as mean \pm SD from independent experiments. Cells treated with 10 μ M ddC for 5 d served as controls. (D) Overall landscapes of each HeLa cell

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shown in Fig. 3 *D* and *E*. (Scale bar: 10 μm .) (*E* and *F*) Overall landscapes of each HeLa cell shown in Fig. 3*F* as obtained by EM. (*E*) Control (*a*) and Drp1 KD cells (*b–f*). Magnified images of the boxed areas in *b* and *d* are shown in *c*, *e*, and *f*. Electron-poor areas in the magnified images are indicated by arrows. (*F*) Overall landscapes of each HeLa cell treated with ddC or chloramphenicol (CHL) shown in Fig. 3*F*.

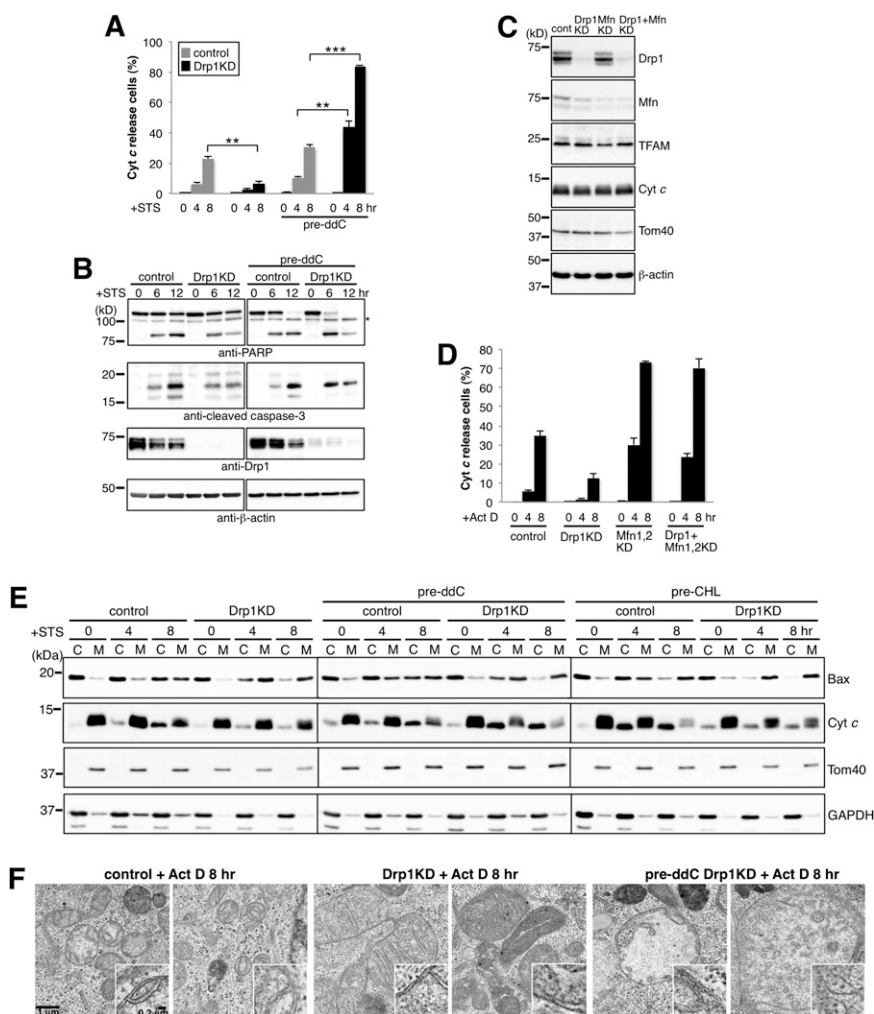
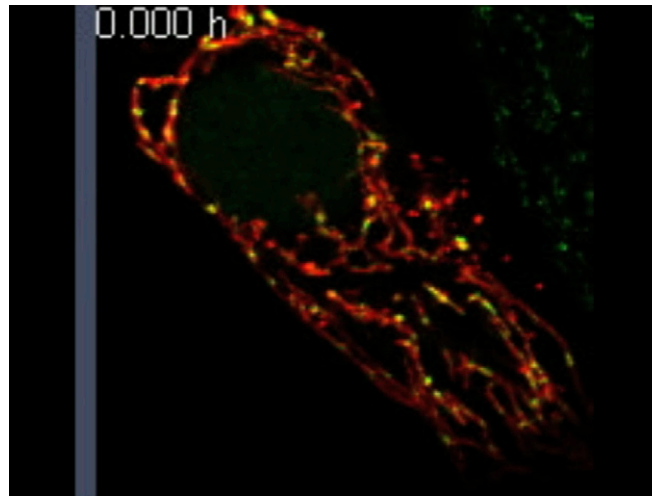
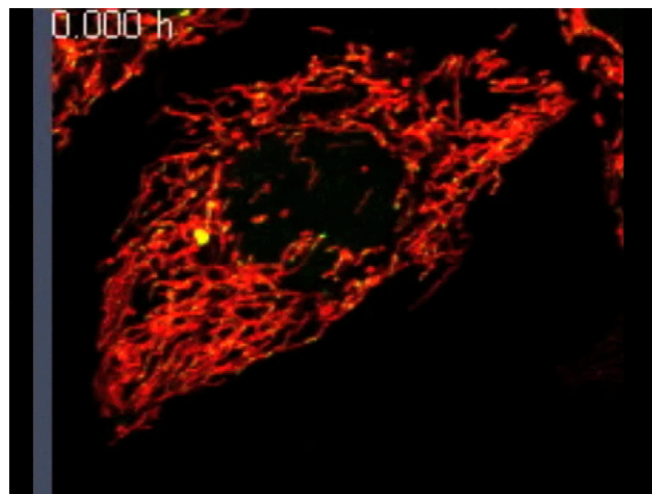


Fig. S5. Nucleoid clustering is required for the apoptosis delay in Drp1-deficient cells. (*A* and *B*) HeLa cells shown in Fig. 3 (pre-ddC and/or Drp1 KD) were treated with 1 μM STS with (*A*) or without (*B*) 50 μM zVAD-fmk for the indicated periods. (*A*) Cyt *c* and Tom20 were stained, and cells with released cyt *c* were counted, as in Fig. 4*A*. Data are expressed as mean \pm SD. $**P < 0.01$; $***P < 0.001$. (*B*) PARP and cleaved caspase-3 were analyzed by immunoblot analysis. The asterisk indicates a nonspecific band. (*C*) Protein levels measured by immunoblot analysis in HeLa cells treated with siRNA for Drp1 and Mfn1/2 for 96 h. (*D*) Cells in *C* were treated with 5 μM Act D as Fig. 4, and cells with released cyt *c* were counted. (*E*) HeLa cells shown in Fig. 3 (pre-ddC, pre-CHL, and/or Drp1 KD) were treated with 1 μM STS in the presence of 50 μM zVAD-fmk for 4 h and 8 h. Cells were subsequently fractionated into membrane (M) and cytosolic (C) fractions, then evaluated by immunoblot analysis as indicated. (*F*) Cells were treated as in Fig. 4, then analyzed by EM.



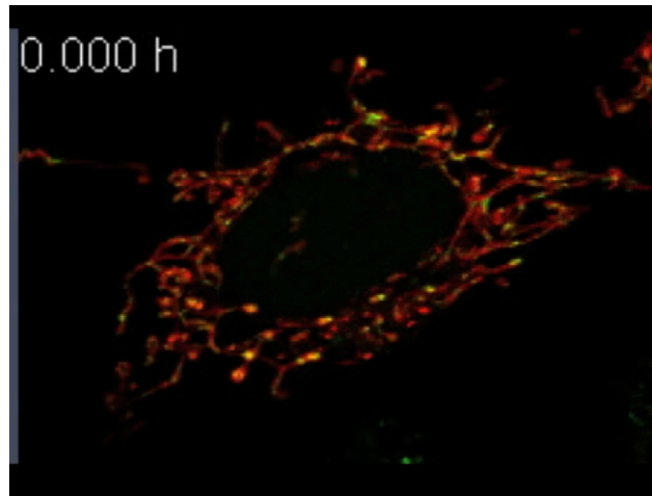
Movie S1. Clustering of nucleoids in Drp1 KD HeLa cells. Original movies of Drp1 KD cells shown in Fig. 2 *A* and *B*. HeLa cells stably expressing mitRFP were treated with Drp1 siRNA for 24 h. Cells were stained with SYBR Green I for 5 min, and live-cell images were obtained by confocal microscopy. The images were obtained every 5 min for 6 h. Overall landscapes are shown first, followed by magnified images of the boxed area. Magnified images are as follows: nucleoids alone [green (SYBR Green I); *Upper Left*], mitochondria alone [red (mitRFP); *Upper Right*], and merged (*Lower*).

[Movie S1](#)



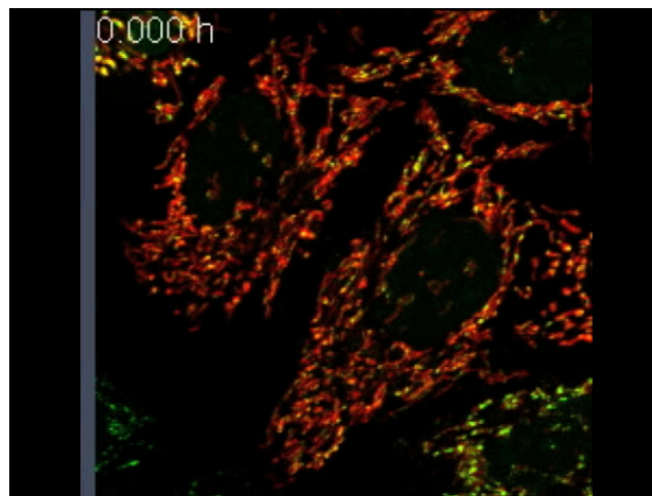
Movie S2. Clustering of nucleoids in Drp1 KD HeLa cells. Original movies of Drp1 KD cells shown in Fig. 3 *A* and *B*. HeLa cells stably expressing mitRFP were treated with Drp1 siRNA for 24 h. Cells were stained with SYBR Green I for 5 min, and live-cell images were obtained by confocal microscopy. The images were obtained every 5 min for 10 h. Overall landscapes are shown first, followed by magnified images of the boxed area. Magnified images are as follows: nucleoids alone [green (SYBR Green I); *Upper Left*], mitochondria alone [red (mitRFP); *Upper Right*], and merged (*Lower*).

[Movie S2](#)



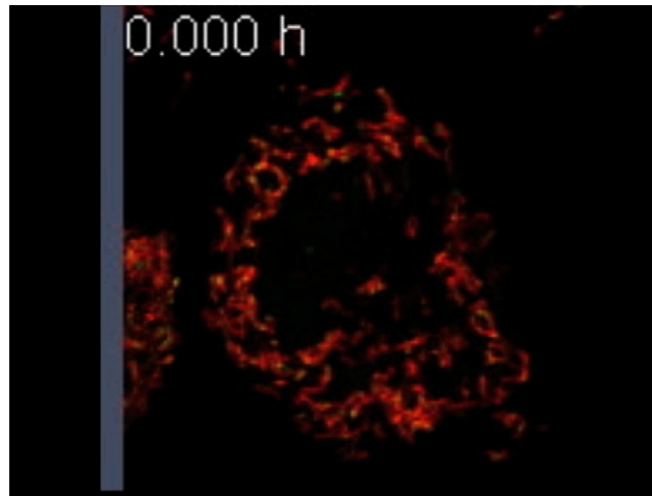
Movie S3. Clustering of nucleoids in Drp1 KD HeLa cells. Original movies of Drp1 KD cells shown in Fig. 3C. HeLa cells stably expressing mitRFP were treated with Drp1 siRNA for 24 h. Cells were stained with SYBR Green I for 5 min, and live-cell images were obtained by confocal microscopy. The images were obtained every 5 min for 10 h. Overall landscapes are shown first, followed by magnified images of the boxed area. Magnified images are as follows: nucleoids alone [green (SYBR Green I); *Upper Left*], mitochondria alone [red (mitRFP); *Upper Right*], and merged (*Lower*).

[Movie S3](#)



Movie S4. No clustering of nucleoids in control HeLa cells and in Drp1-overexpressing HeLa cells. HeLa cells stably expressing mitRFP were treated with control siRNA for 24 h. Cells were stained with SYBR Green I for 5 min, and live-cell images were obtained by confocal microscopy. The images were obtained every 5 min for 6 h. Red, mitRFP; green, nucleoids (SYBR Green I).

[Movie S4](#)



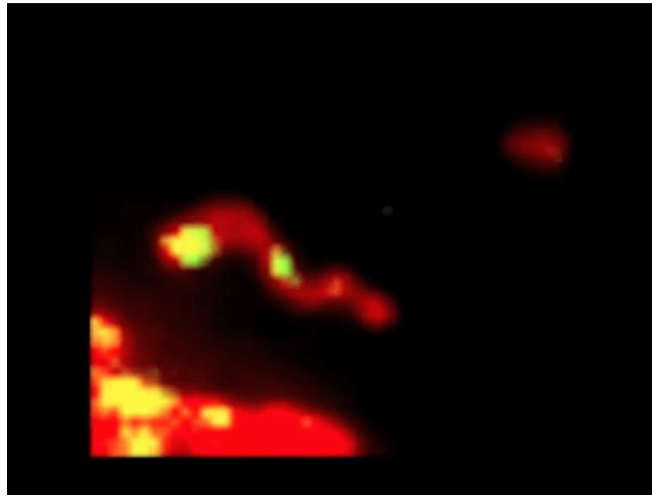
Movie S5. No clustering of nucleoids in control HeLa cells and in Drp1-overexpressing HeLa cells. HeLa cells stably expressing mitRFP were treated with WT FLAG-tagged rat Drp1 expression plasmids for 24 h. Cells were stained with SYBR Green I for 5 min, and live-cell images were obtained by confocal microscopy. The images were obtained every 5 min for 10 h. Red, mitRFP; green, nucleoids (SYBR Green I).

[Movie S5](#)



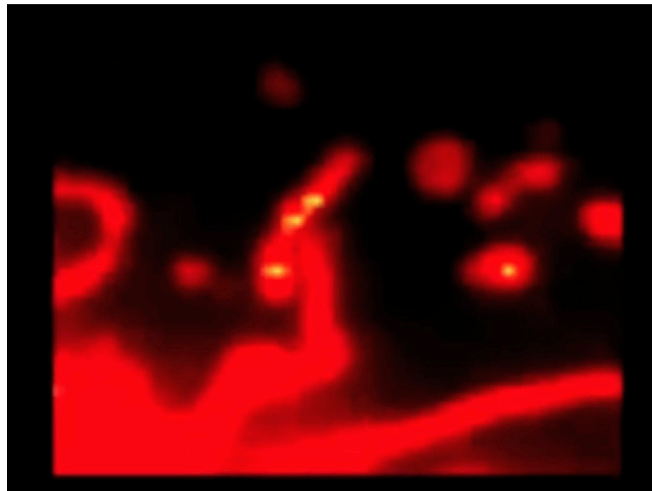
Movie S6. Relationship between mitochondrial fission and nucleoid dynamics in normal HeLa cells. Original movies of control HeLa cells shown in Fig. 2C. HeLa cells stably expressing mitRFP were stained with SYBR Green I for 5 min, and live-cell images were obtained by fluorescence microscopy. The images were obtained every 10 s for 10 min. Red, mitRFP; green, nucleoids (SYBR Green I).

[Movie S6](#)



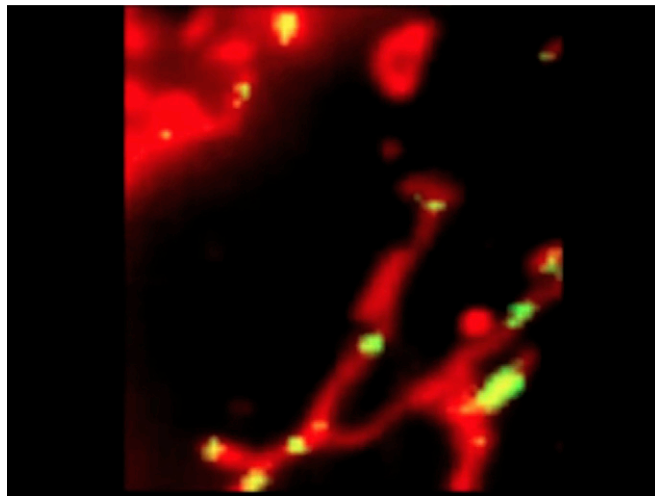
Movie S7. Relationship between mitochondrial fission and nucleoid dynamics in normal HeLa cells. Original movies of control HeLa cells shown in Fig. 2D. HeLa cells stably expressing mitRFP were stained with SYBR Green I for 5 min, and live-cell images were obtained by fluorescence microscopy. The images were obtained every 10 s for 30 min. Red, mitRFP; green, nucleoids (SYBR Green I).

[Movie S7](#)



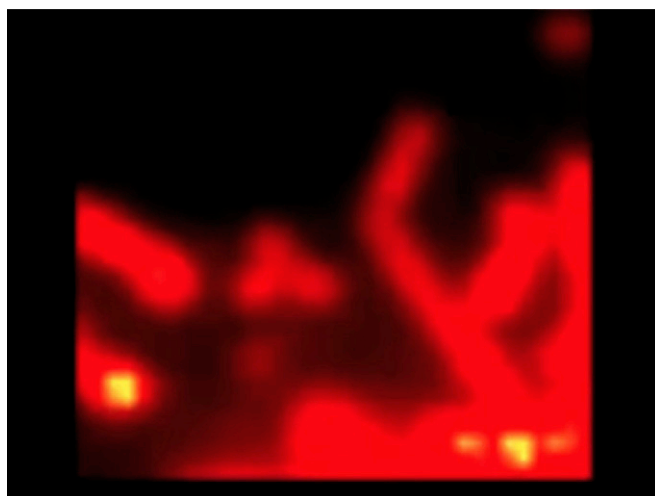
Movie S8. Relationship between mitochondrial fission and nucleoid dynamics in normal HeLa cells. Original movies of control HeLa cells shown in Fig. S3D. HeLa cells stably expressing mitRFP were stained with SYBR Green I for 5 min, and live-cell images were obtained by fluorescence microscopy. The images were obtained every 10 s for 10 min. Red, mitRFP; green, nucleoids (SYBR Green I).

[Movie S8](#)



Movie S9. Relationship between mitochondrial fission and nucleoid dynamics in normal HeLa cells. Original movies of control HeLa cells shown in Fig. S3E. HeLa cells stably expressing mitRFP were stained with SYBR Green I for 5 min, and live-cell images were obtained by fluorescence microscopy. The images were obtained every 10 s for 10 min. Red, mitRFP; green, nucleoids (SYBR Green I).

[Movie S9](#)



Movie S10. Relationship between mitochondrial fission and nucleoid dynamics in normal HeLa cells. Original movies of control HeLa cells shown in Fig. S3F. HeLa cells stably expressing mitRFP were stained with SYBR Green I for 5 min, and live-cell images were obtained by fluorescence microscopy. The images were obtained every 10 s for 10 min. Red, mitRFP; green, nucleoids (SYBR Green I).

[Movie S10](#)