Reactive oxygen species stimulate mitochondrial allele segregation toward homoplasmyn in human cells

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ABSTRACT Mitochondria that contain a mixture of mutant and wild-type mitochondrial (mt) DNA copies are heteroplasmic. In humans, homoplasmyn is restored during early oogenesis and reprogramming of somatic cells, but the mechanism of mt-allele segregation remains unknown. In budding yeast, homoplasmyn is restored by head-to-tail concatemer formation in mother cells by reactive oxygen species (ROS)–induced rolling-circle replication and selective transmission of concatemers to daughter cells, but this mechanism is not obvious in higher eukaryotes. Here, using heteroplasmic mt.3243A>G primary fibroblast cells derived from MELAS patients treated with hydrogen peroxide (H\(_2\)O\(_2\)), we show that an optimal ROS level promotes mt-allele segregation toward wild-type and mutant mtDNA homoplasmyn. Enhanced ROS level reduced the amount of intact mtDNA replication templates but increased linear tandem multimers linked by head-to-tail unit-sized mtDNA (mtDNA concatemers). ROS-triggered mt-allele segregation correlated with mtDNA-concatemer production and enabled transmission of multiple identical mt-genome copies as a single unit. Our results support a mechanism by which mt-allele segregation toward mt-homoplasmyn is mediated by concatemers.

INTRODUCTION

Eukaryotic cells generally contain hundreds to thousands of copies of mitochondrial DNA (mtDNA). Mutations occur in mtDNA much more frequently than in nuclear genomic DNA; therefore the mitochondrial genome tends toward heteroplasmy, a state associated with mitochondrial disorders, aging, and various human diseases (Holt et al., 1988; Linnane et al., 1989; Wallace, 1994; Ozawa, 1997; Smitink et al., 2006). In humans, one common heteroplasmic mtDNA mutation is the A-to-G transition at nucleotide position (np) 3243 (m.3243A>G), which forms stable heteroplasmyn with wild-type mtDNA (Lehtinen et al., 2000; Raap et al., 2012) and causes mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) disease (Goto et al., 1990) and diabetes (Reardon et al., 1992; Kadowaki et al., 1994). The onset of pathogenic mtDNA-associated diseases depends on an increase in the ratio of mutant to wild-type mtDNA in specific tissues. Mechanisms underlying maintenance of (and alterations in) the proportion of mutant mtDNA in heteroplasmic cells are of considerable clinical importance; however, it remains unclear how mt-allele segregation occurs in higher eukaryotes.

Rapid segregation of mtDNA heteroplasmyn to homoplasmyn (in which all mtDNA copies have an identical sequence) occurs during early oogenesis in metazoan female germlines and is known as the bottleneck phenomenon (Cree et al., 2008; Khrapko, 2008; Wai et al., 2008). A similar phenomenon was observed during reprogramming of somatic cells (Fujikura et al., 2012). The dominant molecular
species of human mtDNA is the circular monomer (one genome unit in size), which replicates by a θ-type mechanism and randomly assorts into sister cells. However, θ-type replication and inheritance cannot explain the rapid segregation of mt-alleles observed in heteroplasmic mammalian cells (Hauswirth and Laipis, 1982; Ashley et al., 1989; Koehler et al., 1991) and single-celled organisms (Birky, 1978a,b). Mathematical modeling predicted that decreasing the number of mtDNA-segregating units to a few copies promotes the segregation of mt-alleles (Birky, 1978a,b). One proposed explanation is a genetic bottleneck, in which mtDNA copy number is dramatically reduced, although the nature (and existence) of such a mechanism remains controversial (Cao et al., 2007; Cree et al., 2008; Khrapko, 2008; Wai et al., 2008). Another mechanism that we found in Saccharomyces cerevisiae is vegetative segregation of mt-alleles caused by formation of head-to-tail tandem multimers (concatamers) of single genome-sized mtDNA in mother cells and their selective transmission into daughter cells.

Concatamers are produced by reactive oxygen species (ROS)-triggered rolling-circle replication, allowing for inheritance of multiple mtDNA genome units as a single segregation unit, thereby allowing most progeny cells to become homoplasmic within eight generations of growth from a heteroplasmic ancestor cell (Ling and Shibata, 2002, 2004; Ling et al., 2011). However, it remains unclear whether ROS production contributes to the segregation of mt-alleles in human cells despite the established role for H$_2$O$_2$ as a signaling molecule in yeast, plants, and animals (Kamata and Hirata, 1999; Neill et al., 2002; Bienert et al., 2006; Ling et al., 2011). In addition, selective mtDNA-concatamer transmission from mother to daughter cells clearly explains homoplasmic restoration in budding yeast and asymmetric cell division, but it is unclear whether this mechanism is applicable to most organisms, including humans, that undergo symmetric cell division.

In this study, using heteroplasmic m.3243A > G primary fibroblast cell lines derived from MELAS patients, we provide evidence that ROS triggers rolling-circle replication, which forms concatamers and stimulates mtDNA-allele segregation toward homoplasmy during vegetative growth.

**RESULTS**

**Induced mt-allele segregation by ROS**

To test the effects of ROS on mt-allele segregation, we analyzed the m.3243A > G mutant fraction in clones from H$_2$O$_2$-treated heteroplasmic fibroblast cell lines derived from MELAS patients (Supplemental Figure S1). Treatment-induced changes to the distribution of mutant mtDNA-allele fractions from a single-peaked to a bimodal distribution (Figure 1A, parental to i) are achieved only by mt-allele segregation from heteroplasmic cells to homoplasmic descendants. A shift of the distribution toward the wild-type or mutant mtDNA side results from selection for cells with either wild-type or mutant mtDNA (Figure 1A, parental to ii and iii, respectively).

MELAS cells treated with H$_2$O$_2$ followed by 6 d of cultivation ("posttreatment cultivation") were subjected to single-cell cloning, and the m.3243A > G mutant fraction in each clone was analyzed (Supplemental Figure S1B). Treatment of cells with H$_2$O$_2$ caused detectable increases in intracellular ROS levels within 15 min (Figure 1B). The m.3243A > G mutant fraction among all mtDNAs within each cell clone was measured using an established PCR-restriction fragment-length polymorphism (RFLP) assay (diagram in Supplemental Figure S1A, left; Goto et al., 1990).

The original MELAS cell culture consisted of cells with 32 ± 3% (n = 5) m.3243A > G mutant mtDNA (Supplemental Figure S1A, right), and all clones isolated from untreated MELAS culture contained m.3243A > G mutant fractions within a range of 25–55% (Figure 1, Ci and D, and Supplemental Figure S2A). Treatment with 100 μM H$_2$O$_2$ for 30 min caused ~40% of the clone population to yield colonies with <25% or >55% m.3243A > G mutant allele content (Figure 1, Civ and D). The shift to a bimodal distribution is indicative of mt-allele segregation (Figure 1Ai). Remarkably, sequencing revealed that some clones (such as clone 1) derived from MELAS cells treated with 100 μM H$_2$O$_2$ for 30 min and cultivated for 6 d contained a very low (<3%) m.3243A > G mtDNA level (Supplemental Figure S2F).

![FIGURE 1: Elevated ROS levels induced segregation of mtDNA alleles in heteroplasmic MELAS cells. (A) Possible distribution profile shifts from parental heteroplasmic cells after growth condition changes. (i) Vegetative segregation toward homoplasy. (ii) Selection for cells with normal mtDNA. (iii) Selection for cells with mutant mtDNA. (B) Intracellular ROS level after H$_2$O$_2$ treatment. MELAS cells were treated with the indicated concentrations of H$_2$O$_2$ and measured at 5-min intervals using the probe CM-H$_2$DCFDA. (C) Distribution of m.3243A > G mutant fraction in clones derived from cells treated for 30 min with (i) 0, (ii) 25 μM, (iii) 50 μM, (iv) 100 μM, or (v) 300 μM H$_2$O$_2$ and then cultivated to allow robust growth of cells. (D) Statistical analysis for distribution of the m.3243A > G mutant fraction in clones derived from cells treated with the indicated concentrations of H$_2$O$_2$. ***p < 0.001, ****p < 0.0001; N.S., not significant. For each concentration of H$_2$O$_2$, the treatment time was 30 min.](image-url)
On the other hand, a higher concentration of H₂O₂ (300 μM) was much less effective at inducing segregation (Figures 1, Cv and D, and Supplemental Figure S2E), presumably due to excess cellular damage. Treatment with 25 or 50 μM H₂O₂ for 30 min did not yield significant changes to m.3243A > G mutant allele content among the measured populations of clones (Figure 1C, ii and iii vs. iv). Therefore only an optimal amount of ROS induced mt-allele segregation toward mutant and wild-type mtDNA homoplasy during vegetative growth.

**Closed circular monomeric mtDNA decreased in H₂O₂-treated MELAS cells**

To determine the effects of ROS on mtDNA species, we treated MELAS cells with 100 μM H₂O₂ and performed analysis using one-dimensional gel electrophoresis followed by Southern hybridization. In untreated cells, the closed circular monomer of one genome-unit size is the major species of mtDNA and is converted to a 16.5-kbp linear form upon treatment with BamHI, which has a single cutting site in human mtDNA (Figure 2A). In H₂O₂-treated cells, we observed that closed circular (cc) mtDNA disappeared, and careful examination revealed a faint signal from linear unit-sized mtDNA and other forms in H₂O₂-treated MELAS cells (Figure 2A).

**Newly synthesized mtDNA was concatemeric in H₂O₂-treated MELAS cells**

Next we sought to elucidate the molecular basis of mt-allelic segregation induced by ROS in human cells. First, we analyzed newly synthesized bromodeoxyuridine (BrdU)-labeled mtDNA by pulsed-field gel-electrophoresis (PFGE), which effectively separates mtDNA molecules from nuclear genomes. We detected BrdU-labeled mtDNA molecules with sizes larger than the unit length from 100 μM H₂O₂-treated MELAS cells but detected only unit-sized mtDNA from untreated cells and no BrdU-signals in ρ0 cells (Figure 2B).

To analyze the forms of larger mtDNA, we prepared plugs from cells treated with or without 100 μM H₂O₂ for 30 min followed by various periods of posttreatment cultivation (Supplemental Figure S3). Finally, we subjected the samples to two-dimensional (2D) gel electrophoresis (Figure 3). In untreated cells, primarily open-circular (oc) and cc mtDNA monomers with unit-sized genomes (16.5 kb) were detected as the major mtDNA species (Figure 3i). After treatment with 100 μM H₂O₂ and 1 h of posttreatment cultivation, cc monomers, linear monomers, and fragmented mtDNAs were detectable (Figure 3, ii and iii). After 5 h of posttreatment incubation, fragmented mtDNAs disappeared, oc monomer signals increased, and a faint signal from cc monomers was detected (Figure 3iv). Of note, after 24 h of posttreatment cultivation, linear mtDNA multimers larger than the unit length (>20 kb; concatemers) were detected, and larger amounts of cc monomers were formed (Figure 3v; see also the positions of the size markers in Supplemental Figure S4). Southern blot analysis revealed no remarkable mtDNA signals from ρ0 cells, indicating that the signals detected in each panel correspond to mtDNA species (Figure 3vi).
FIGURE 3: Profiles of mtDNA species detected by Southern blot after PFGE-coupled 2D gel electrophoresis. (i–vi) Schematic diagrams indicating mtDNA species corresponding to images on the right showing mtDNA species from MELAS cells treated under the indicated conditions.

These results suggest ROS induced de novo synthesis of multimeric mtDNA species in the treated MELAS cells.

Correlation between concatemer formation and mt-allele segregation
To explore the relationship between concatemer formation and mt-allele segregation from heteroplasmic cells, we designed an assay system to monitor signal ratios of mtDNA concatemers to nuclear DNA, using PFGE-coupled 2D gel electrophoresis profiles (Figure 4A). Signals from mtDNA and nuclear DNA in cells treated with various concentrations of H$_2$O$_2$ followed by 24-h posttreatment incubation were detected by Southern blot analysis using $^{32}$P-labeled DNA fragments from the mtDNA OH region and the nuclear $\beta$-actin gene (ACTB), respectively, as probes.

To verify concatemer formation, we excised a piece of agarose gel corresponding to the region predicted to contain linear DNA species with sizes $>$16.5 kb (Figure 4Aiv, right, red-framed box) after PFGE-coupled 2D gel electrophoresis and isolated the DNA species by digesting the agarose gel with $\beta$-agarase I. Large DNA species with branched structures such as the Holliday junction or replication fork–type three-way junctions are unable to enter gels during PFGE and thus should not be included in this linear DNA fraction. The isolated linear DNA was digested with the restriction enzyme BamHI, giving rise to 16.5-kb unit-length fragments (Figure 4B). This result indicates that multimeric mtDNA in H$_2$O$_2$-treated MELAS cells are in a head-to-tail configuration and thus are concatemers.

The major mtDNA species in untreated MELAS cells were oc and cc monomers, with nearly undetectable amounts of concatemers, linear monomers, and linear mtDNA fragments <16.5 kbp (Figure 4Ci). Treatment of MELAS cells with 100 μM H$_2$O$_2$ followed by 24 h of posttreatment incubation significantly increased concatemer signals (Figure 4Civ) relative to cells treated with lower (75 μM: Figure 4Ciii) or higher concentrations of H$_2$O$_2$ (150 μM; Figure 4Cv). Concatemer signals were not significant at 50 μM H$_2$O$_2$ (Figure 4Cii) and became very faint at >200 μM H$_2$O$_2$ (Figure 4C, vi and vii). These results correlate with mt-allele segregation in heteroplasmic cells at the single-cell level (Figure 1C, iv vs. ii, iii, and v). Concatemers are exclusively formed by rolling circle replication or crossing-over–type recombination. Those formed by recombination have discrete lengths (monomers, dimers, trimers, etc.). As shown in Figure 4C, ii–vii, the mtDNA concatemers we detected had continuous lengths and are therefore likely to have been formed by rolling-circle replication.

Detection of rolling-circle replication intermediates from well-bound DNA after PFGE
Concatemers generated by rolling-circle replication are expected to be accompanied by intermediates possessing conjoined circular mtDNA structures at one end. However, some circular structure likely separates from concatemers during experimental processing (Bendich, 1996; Ling and Shibata, 2002, 2004; Ide et al., 2013). In addition, four-way branched structures exist as rolling-circle replication intermediates and may have a role in concatemer-to-monomer resolution (Lewis et al., 2015). DNA species with circular structures, branched DNA with four-way junctions, or replication fork–type three-way junctions (i.e., rolling circle replication intermediates, homologous recombination products, and replication intermediates) are unable to exit PFGE wells but do enter agarose gels during conventional electrophoresis.

We tried to detect rolling-circle replication intermediates (concatemers conjoined with circular mtDNA structures at one end) in samples derived from PFGE wells by two consecutive steps of conventional gel electrophoresis (first through normal agarose and then through low–melting point agarose; schematically illustrated in Figure 5A, i and ii). We isolated DNA species with sizes >16.5 kb from the third electrophoresis round by $\beta$-agarase I digestion (Figure 5A, ii, framed in a red box). After BamHI digestion, the isolated DNA yielded a signal corresponding to the unit size (16.5 kb) of mtDNA (Figure 5B), indicating that the mtDNA species that were well bound during PFGE contained concatemers. We also observed that the...
BglII-digested sample contained well-bound DNA species, using conventional gel electrophoresis (Figure 5B), which had previously been excised from a low-melting point agarose gel after PFGE-coupled 2D electrophoresis, leading us to infer that the large isolated mtDNA species contain impurities from the gel and thus tend to form aggregates spontaneously during DNA handling. On the other hand, because exonuclease III (ExoIII) digests double-stranded DNA in the 3' to 5' direction starting from the 3'-OH end, we predicted that ExoIII treatment would allow for migration of species with attached linear DNA. Digestion of well-bound DNA obtained after the first PFGE run with ExoIII (Kusano et al., 1989) generated signals from circular monomers, which were able to enter the gel during the second PFGE run (Figure 5, C and D). These results indicate that circular structures with attached linear DNAs were among the well-bound DNA species.

**Induced endogenous ROS generation promoted mt-allele segregation**

Next we set out to examine whether inducing endogenous ROS production can also lead to mt-allele segregation. MtDNA polymerase γ (POLG) is essential for replication and repair of mtDNA. Inhibition of POLG by ethidium bromide (EtBr) treatment depletes mtDNA...
Okamaoto et al. (2003) and induces endogenous ROS production (Palmeira et al., 2007; Sasaki et al., 2008). We hypothesized that ROS contribute to mt-allele segregation from heteroplasmy toward homoplasmy upon copy number reduction in heteroplasmic MELAS cell cultures. To test this possibility, we treated MELAS cells with EtBr for 6 d, thereby decreasing mtDNA copy number to <10% of control levels (Figure 6A). Using a flow cytometer and the fluorogenic dye MitoSOX red, an indicator for mitochondrial superoxide in live cells (Robinson et al., 2006), we detected increased ROS levels at 3 and 6 d after EtBr treatment (Figure 6, A and B). Subsequent washing and incubation for 6 d allowed for recovery of mtDNA copy number to a mean value of 79.7% of control levels (Figure 6A) and a decrease in ROS levels to those of untreated MELAS cells at 3 and 6 d after washing out EtBr (Figure 6B). After the recovery process, the m.3243A>G mutant fraction exhibited a much wider distribution (Figure 6Cii and Supplemental Figure S5B), indicating mt-allele segregation.

To further examine the role of ROS in EtBr induced mt-allele segregation, we also treated MELAS cells with EtBr in the presence of ROS scavenger vitamin C or N-acetylcysteine (NAC), respectively. Vitamin C partially but significantly inhibited mt-allele segregation toward lower mutant-allele frequency in EtBr-treated MELAS cells (Figure 6, C, ii vs. iv, and D, and Supplemental Figure S5, B vs. D). In the presence of NAC, we also observed broadening of the distribution of the m.3243A>G mutant fraction (Figure 6, C, ii vs. iii, and D, and Supplemental Figure S5, B vs. C). NAC treatment increases the intracellular level of glutathione, an antioxidant and substrate of glutathione peroxidase. Because EtBr inhibits glutathione peroxidase activity in EtBr-treated rat brains (Abdel-Salam et al., 2012), we suspected that the poor efficacy of NAC was due to inhibition of glutathione peroxidase activity by EtBr. No broadening in the distribution of the m.3243A>G mutant fraction occurred in clones treated with either NAC or vitamin C alone (Figure 6, C, i vs. v and i vs. vi, and Supplemental Figure S5, A vs. E or F). It is very likely that a decrease in mtDNA-encoded electron transport chain (ETC) subunits affected the balance in the relative abundance between the nuclear- and mitochondrial-encoded subunits, thereby disrupting formation of ETC complexes and causing endogenous ROS production.

On the basis of the foregoing observations, we propose a model in which ROS...
production decreases the amount of intact closed-circular mtDNA monomers (potential replication templates) while triggering rolling circle replication on a few residual templates to generate head-to-tail multimers (concatemers) with the same sequence as the template circular DNA. The resulting concatemer formation allows for inheritance of a number of mtDNA genome units into sister cells as a single segregation unit and thus stimulates mtDNA-allele segregation toward wild-type and mutant mtDNA homoplasmy through clonal expansion (Figure 7).

DISCUSSION

In this study, we presented evidence that an optimal level of ROS promoted segregation of heteroplasmic alleles of mtDNA in human cells, and this segregation correlated with the ROS-promoted synthesis of linear tandem multimers (concatemers), which were products of rolling-circle replication. Thus the mechanism for homoplasmy restoration is likely evolutionarily conserved in human mitochondria.

Treatment of human cells with H$_2$O$_2$ causes oxidative damage to DNA bases and mainly results in single-stranded (ss) breaks (Rueff et al., 1993; Villani et al., 2010). Of note, we observed the disappearance of cc mtDNA (Figure 2A) and fragmentation (Figure 3, ii and iii) in H$_2$O$_2$-treated MELAS cells, which contributes to the generation of double-strand breaks (DSBs) in mtDNA molecules. Subsequent processing of newly formed DSBs by a 5’-3’ exonuclease would generate 3’-ssDNA tails, which are known substrates for heteroduplex joint formation directed by a homologous DNA pairing protein. These 3’ ssDNA tails of mtDNA fragments would then be used to initiate rolling-circle replication as in budding yeast (for reviews, see Solieri, 2010; Chen, 2013).
The resulting concatemers allow for inheritance of a number of mtDNA genome units into sister cells as a single segregation unit and thus stimulate mt-allele segregation toward wild-type or mutant mtDNA homoplasmy.

FIGURE 7: Model for ROS-induced segregation of mtDNA alleles. ROS cause a decrease in amounts of intact closed-circular mtDNA monomers, which serve as templates for rolling-circle replication. Rolling-circle replication is initiated by an invading 3′-single-stranded terminus derived from a fragmented mtDNA, as in the case of yeast mitochondria (Ling and Shibata, 2004; Ling et al., 2007, 2011), and generates concatemers consisting of a number of identical sequences. The resulting concatemers allow for inheritance of a number of mtDNA genome units into sister cells as a single segregation unit and thus stimulate mt-allele segregation toward wild-type or mutant mtDNA homoplasmy.

On the other hand, H$_2$O$_2$ treatment causes mtDNA damage, which results in a greatly reduced number of cc monomeric mtDNAs remaining intact (Figures 2A and Figure 3ii). These results suggest that the reduced mtDNA copy number is responsible, at least in part, for inducing mt-allele segregation. Of importance, mathematical modeling predicted that decreasing the number of mtDNA-segregating units to a few copies would promote the segregation of mt-alleles (Birky, 1978a,b). However, human fibroblast cells each contain ≥1000 copies of mtDNA (Lehtinen et al., 2000). Although EtBr treatment reduces mtDNA copy number to 1/10 of the initial level (Figure 6A), ≥100 copies of mtDNA would still remain.

Indeed, we found that treatment with EtBr induced ROS production and promoted mt-allele segregation (Figure 6, A and C). In addition, ROS scavengers such as vitamin C partially but significantly impaired mt-allele segregation in EtBr-treated MELAS cells (Figure 6, C, Civ and D, and Supplemental Figure SSD). These results suggest that ROS play a role in the observed mt-allele segregation. The results taken together indicate that decreasing copy number alone is not sufficient to explain the observed segregation; in other words, reduction in mtDNA replication templates coupled with concatemer formation contributes to mt-allele segregation.

Rapid segregation of heteroplasmic mtDNA occurs at the genetic bottleneck, which is accompanied by remarkable decreases in mtDNA level during oogenesis and increases in mtDNA level during folliculogenesis (Cree et al., 2008; Khrapko, 2008; Wai et al., 2008). Folliculogenesis is regulated by various hormones, including estrogens such as estradiol (Roche, 1996), which induces ROS production at a high level (Okoh et al., 2011). We infer that ROS-mediated concatemer formation might contribute to increases in mtDNA content during folliculogenesis.

In summary, this study, in conjunction with our previous studies in budding yeast (for reviews, see Soleri, 2010; Ling et al., 2011; Chen, 2013), provides new insights into the mechanism underpinning homoplasmy restoration in mammalian cells.

MATERIALS AND METHODS

Cells, cell culture, and reagents

Primary heteroplasmic fibroblast cell lines containing m.3243A > G mutant mtDNA derived from different MELAS patients were obtained from the fibroblast repository at the National Center of Neurology and Psychiatry (NCNP; Tokyo, Japan) under written informed consent and with the approval of the NCNP Institutional Review Board.

Cells were maintained in DMEM (Wako Pure Chemical Industry, Osaka, Japan) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) at 37°C in a humidified incubator in an atmosphere containing 5% CO$_2$. Pyruvate (100 μg/ml) and uridine (50 μg/ml) were added to the culture medium to allow growth of rho- or rho0 cells generated after chemical treatment.

DNA plugs from S. cerevisiae, lambda ladder, and 5-kb DNA ladder were purchased from Bio-Rad Laboratories (Tokyo, Japan). Supercoiled DNA ladder and supercoiled DNA marker sets were purchased from Invitrogen and Epicentre, respectively. These DNA size markers were used as references for the sizes and positions of DNA species.

Southern blot analysis for detection of nuclear DNA and mtDNA molecules

Total cellular DNA (15 μg) was separated by electrophoresis on a 0.5% agarose gel, run at 4°C for 30 h at 1 V/cm, and transferred to a nylon membrane (Amersham Hybond N Plus; GE Healthcare). Signals for mtDNA were detected using the 32P-labeled mtDNA OH region as a probe, as described (Anderson et al., 1981; Lehtinen et al., 2000). Signals for nuclear DNA were detected using a 32P-labeled DNA fragment of the β-actin gene, ACTB (for nuclear DNA), as a probe. Primers designed for the mtDNA OH region were forward, 5′-TAACTCTCACCGGGAGCTCT-3′, and reverse, 5′-AAGGC-TAGGACCAAACTAT-3′. Primers designed for ACTB were forward, 5′-GTCGAGCATTAAAGGAAGGCTGTC-3′, and reverse, 5′-CTC-GTACACTCCCTGCTGGATCC-3′. Signals corresponding to mtDNA and nuclear DNA fragments were quantitated using a Fujifilm BAS-2500 image analyzer (Fujifilm, Tokyo, Japan).

Treatment with chemical compounds and single-cell cloning

Cells were plated at 30–50% confluence 1 d before treatment with chemical compounds. After treatment, cells were allowed to recover in fresh medium for 6 d and then plated at high dilution to create
isolated colonies. Colonies derived from single cells were then collected using cloning rings.

**Measurement of intracellular ROS**
The intracellular ROS level after H$_2$O$_2$ treatment was measured by use of 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H$_2$DCFDA; Molecular Probes, Nacalai Tesque, Kyoto, Japan). Cells (1×10$^3$/ml) were first incubated with 10 μM CM-H$_2$DCFDA for 15 min at 37°C and then treated with H$_2$O$_2$ and analyzed kinetically in a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of ROS after EtBr treatment was performed by flow cytometer. After EtBr treatment, ~1×10$^6$ cells were trypsinized and each cell pellet was resuspended in 1× phosphate-buffered saline (PBS) containing 5 μM MitoSOX (Molecular Probes, M36008). ROS levels were then analyzed by flow cytometry at the Research Resources Center, RIKEN Brain Science Institute (Wako, Japan).

**DNA preparation and a PCR-RFLP assay for m.3243A > G-heteroplasmy**
The heteroplasmy level was estimated by the m.3243A > G mutant fraction, measured using the PCR-RFLP assay described by Goto et al. (1990). Briefly, a 294–base pair mtDNA region containing np 3243 was amplified by PCR using a forward primer (5′-AG-GACAAGAAATAAGGCC; nps 3130–3149), a reverse primer (5′-ACGTGGGCCCCTTGCGTAG; nps 3423–3404), and whole cellular DNA as a template. The Apal digests (182– and 112–base pair mtDNA fragments) of the purified PCR products were separated on a 2% agarose gel by electrophoresis and detected by Southern hybridization using a [32P]dCTP-labeled 294–base pair DNA fragment as a probe. The m.3243A > G mutant fraction was calculated as the proportion of the signals from the 182– and 112–base pair mtDNA fragments to the total signals from the uncut 294–, 182–, and 112–base pair mtDNA fragments.

**Statistical analysis**
The F test was used to determine the statistical significance of differences in the distributions of m.3243A > G allele frequency between two independent groups.

**DNA extraction and real-time PCR for mtDNA copy-number analysis**
Total cellular DNA was extracted using the DNAzol Reagent (Invitrogen). Relative mtDNA copy number was defined as the ratio of mtDNA (represented by the ND1 gene) to nuclear DNA (represented by the β-actin gene). Primers for the ND1 gene were forward, 5′-CCCCTAAAACCCGCCCATCT-3′, and reverse, 5′-GAGC-GATGGTGAGAGCTAAGGT-3′. Primers for the ACTB gene were as follows: forward, 5′-GACAAGAGAAATAAGGCC; nps 3130–3149), a reverse primer (5′-ACGTGGGCCCCTTGCGTAG; nps 3423–3404), and whole cellular DNA as a template. The Apal digests (182– and 112–base pair mtDNA fragments) of the purified PCR products were separated on a 2% agarose gel by electrophoresis and detected by Southern hybridization using a [32P]dCTP-labeled 294–base pair DNA fragment as a probe. The m.3243A > G mutant fraction was calculated as the proportion of the signals from the 182– and 112–base pair mtDNA fragments to the total signals from the uncut 294–, 182–, and 112–base pair mtDNA fragments.

**Exonuclease digestion**
DNA plugs with dimensions of 1 × 2 × 0.15 cm were prepared from 1 × 10$^7$ MELAS cells treated with 100 μM H$_2$O$_2$ for 0.5 h, cultured for 24 h, and then subjected to PFGE. Plugs containing well-bound DNA were digested by ExoIII with attached buffer to a final concentration of 100 units/ml, and then mixed well by pipetting with 5 μl of ExoIII buffer. The digestion was performed for 15 min at 37°C. After digestion, the desired DNA-containing gel segment was excised, rotated 90° counterclockwise, and embedded in a newly poured 1% low–melting point agarose at 50°C. The DNA plugs were then analyzed by adding the suspension to a Bio-Rad DRRI sample mold.

**PFGE-coupled 2D gel electrophoretic analyses**
After PFGE, the desired DNA-containing gel segment was excised, rotated 90° counterclockwise, and embedded in a newly poured 1% agarose gel as a probe. The remaining plug was soaked in 1 M NaOH buffer to remove BrdU, resuspended in 10 μl Tris-EDTA buffer (0.5 M EDTA, 10 mM Tris, pH 7.5), and then mixed well by pipetting with 5 μl of 10% SDS/sodium dodecyl sulfate (SDS) in 10 mM Tris-HCl (pH 8.0) at 65°C. The DNA was then subjected to 2D gel electrophoretic analyses as previously described (Brewer and Fangman, 1987; Ling and Shibata, 2004; Ling et al., 2007). A 5-kbp DNA ladder (Bio-Rad) was used as a size marker. The 2D run was performed at 5 V/cm on a 1% agarose gel or 1% low–melting point agarose at 5°C. Signals for mtDNA and nuclear DNA were detected by Southern hybridization using the 32P-labeled mtDNA OH region or the 32P-labeled DNA fragment of the β-actin gene as a probe.

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**References**
Abdel-Salam OM, Khadrawy YA, Mohammed NA, Youness ER (2012). The effect of gabapentin on oxidative stress in a model of toxic demyelination in rat brain. J Basic Clin Physiol Pharmacol 23, 61–68.
Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, et al. (1981). Sequence and organization of the human mitochondrial genome. Nature 290, 475–465.

Ashley MV, Laipis PJ, Hauswirth WW (1989). Rapid segregation of heteroplasmic bovine mitochondria. Nucleic Acids Res 17, 7325–7331.

Bendich AJ (1996). Structural analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed-field gel electrophoresis. J Mol Biol 255, 564–588.

Bienert GP, Schjoerring JK, Jahn TP (2006). Membrane transport of hydrogen peroxide. Biochim Biophys Acta 1758, 959–1003.

Birky CW Jr (1978a). Transmission genetics of mitochondria and chloroplasts. Annu Rev Genet 12, 471–512.

Birky CW Jr, Strausberg RL, Forster JL, Perlman PS (1978b). Vegetative segregation of mitochondria in yeast: estimating parameters using a random model. Mol Gen Genet 158, 251–261.

Breuer BJ, Fangman WL (1987). The localization of replication origins on ARS plasmids in S. cerevisiae. Cell 51, 463–471.

Cao L, Shitara H, Horii T, Nagao Y, Imai H, Abe K, Hara T, Hayashi J, Yonekawa H (2007). The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. Nat Genet 39, 386–390.

Chen XJ (2013). Mechanism of homologous recombination and implications for aging-related deletions in mitochondrial DNA. Microbiol Mol Biol Rev 77, 476–496.

Cree LM, Samuels DC, de Sousa Lopes SC, Rajasimha HK, Wonnapinij P, Mann JR, Dahl HH, Chinnery PF (2008). A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. Nat Genet 40, 249–254.

Fujikura J, Nakao K, Sone M, Noguchi M, Mori E, Naito M, Taura D, Harada-Mitsuda Y, Kishimoto I, Watanabe A, et al. (2012). Induced pluripotent stem cells generated from diabetic patients with mitochondrial DNA A3243G mutation. Diabetologia 55, 1689–1698.

Goto Y, Nonaka I, Horai S (1990). A mutation in the tRNA(Leu)(UUR) gene associated with a maternal lineage of Holstein cows. Proc Natl Acad Sci USA 79, 4686–4690.

Holst J, Harding AE, Morgan-Hughes JA (1988). Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 334, 717–719.

Ide S, Saka K, Kobayashi T (2013). Rtt109 prevents hyper-amplification of ribosomal RNA genes through histone modification in budding yeast. PLoS Genet 9, e1003410.

Kadowaki T, Kadowaki H, Mori Y, Tobe K, Sakuta R, Suzuki Y, Tanabe Y, Sakura H, Awata T, Goto Y, et al. (1994). A subtype of diabetes mellitus associated with a mutation of mitochondrial DNA. N Engl J Med 330, 962–968.

Kamata H, Horii S, Kato H, Takahashi Y, Matsumoto H, et al. (2004). Mitochondrial DNA formation for generating yeast mitochondrial homoplasmic cells. Mol Biol Cell 15, 2101–2113.

Koehler CM, Lindberg GL, Brown DR, Beitz DC, Freeman AE, Mayfield JE, Myers AM (1991). Replacement of bovine mitochondrial DNA by a replication-defective plasmid through histone modification in budding yeast. Rev Reprod 1, 19–27.

Kovalenko AV, Ohsato T, Nakada K, Isoke K, Spe尔brink JN, Hayashi J, Hamaasaki N, Kang D (2003). Ditercalinium chloride, a pro-antiviral drug, intimately associates with mammalian mitochondrial DNA and inhibits its replication. Curr Genet 43, 364–370.

Kooh Y, Deoraj A, Roy D (2011). Estrogen-induced reactive oxygen species-mediated signalings contribute to breast cancer. Biochim Biophys Acta 1815, 115–133.

Ozawa T (1997). Genetic and functional changes in mitochondria associated with aging. Physiol Rev 77, 429–464.

Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB (2007). Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. Toxicol Appl Pharmacol 225, 214–220.

Raap AK, Jahangir Tafrechi RS, van de Rijke FM, Pyle A, Wahlby C, Suhai K, Raveli RB, de Coo RF, Rajasimha HK, Nilsson M, et al. (2012). Non-random mtDNA segregation patterns indicate a metastable heteroplasmy segmentation unit in m.3243A>G hybrid cells. PLoS One 7, e52080.

Reardon W, Ross RJ, Sweeney MG, Luxon LM, Pembrey ME, Harding AE, Trevathan RC (1992). Diabetes mellitus associated with a pathogenic point mutation in mitochondrial DNA. Lancet 340, 1376–1379.

Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP, Beckman JS (2006). Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proc Natl Acad Sci USA 103, 15038–15043.

Roche JF (1996). Control and regulation of folliculogenesis—a symposium in perspective. Rev Reprod 1, 19–27.

Rueff J, Bras J, Cristovao L, Mexia J, Sa da Costa M, Pires V (1993). DNA strand breaks and chromosomal aberrations induced by H2O2 and 60Co gamma-radiation. Mutat Res 289, 197–204.

Sasaki R, Suzuki Y, Yonezawa Y, Ota Y, Okamoto Y, Demizu Y, Huang P, Yoshida H, Sugimura K, Mizushina Y (2008). DNA polymerase gamma inhibition by vitamin K3 induces mitochondria-mediated cytotoxicity in human cancer cells. Cancer Sci 99, 1040–1048.

Smetnick JA, Zeviani M, Turnbull DM, Jacobs HT (2006). Mitochondrial medicine: a metabolic perspective on the pathology of oxidative phosphorylation disorders. Cell Metab 3, 9–13.

Solari L (2010). Mitochondrial inheritance in budding yeasts: towards an integrated understanding. Trends Microbiol 18, 521–530.

Villani P, Elefent P, Grollino MG, Rescia M, Altavista P, S Pan, M, Pacchierotti F, Cordelli E (2010). Sperm DNA fragmentation induced by DNAase I and hydrogen peroxide: an in vitro comparative study among different mammalian species. Reproduction 140, 445–452.

Wai T, Teoli D, Shoubridge EA (2008). The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. Nat Genet 40, 1484–1488.

Wallace DC (1994). Mitochondrial DNA sequence variation in human evolution and disease. Proc Natl Acad Sci USA 91, 8739–8746.