Selective propagation of functional mitochondrial DNA during oogenesis restricts the transmission of a deleterious mitochondrial variant

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Although mitochondrial DNA (mtDNA) is prone to mutation and few mtDNA repair mechanisms exist1, crippling mitochondrial mutations are exceedingly rare2. Recent studies have demonstrated strong purifying selection in the mouse female germline3,4. However, the mechanisms underlying positive selection of healthy mitochondria remain to be elucidated. We visualized mtDNA replication during Drosophila melanogaster oogenesis, finding that mtDNA replication commenced before oocyte determination during the late germarium stage and was dependent on mitochondrial fitness. We isolated a temperature-sensitive lethal mtDNA allele, mt:CoIT300I, which resulted in reduced mtDNA replication in the germarium at the restrictive temperature. Additionally, the frequency of the mt:CoIT300I allele in heteroplasmic flies was decreased, both during oogenesis and over multiple generations, at the restrictive temperature.

Next, we tested whether mitochondrial fitness affected mtDNA replication in the germ line. We disrupted mitochondrial function by RNA interference (RNAi)-mediated knockdown of cytochrome c oxidase (COX) subunit Va (CoVa), which is required for the assembly of COX5. This severe mitochondrial disruption nearly abolished mtDNA replication throughout the entire germarium (Supplementary Fig. 4a). Next, we subjected wild-type ovaries to a classic mitochondrial protonophore, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), which dissipates mitochondrial membrane potential and impairs ATP production with a wide dynamic range6. Similar to CoVa knockdown, high FCCP concentration (10 µM) completely abolished mtDNA replication throughout the germarium (Fig. 2f), whereas incubation with DMSO had no impact on mtDNA replication (Fig. 2c). Lower doses of FCCP (2 or 5 µM) had no obvious effects on mtDNA replication in region 1 punctate structures in the cytoplasm (Supplementary Fig. 1). Notably, these puncta localized with mitochondria marked by staining for ATP synthase (Supplementary Fig. 1b) and were absent in oocytes treated with ethidium bromide, a potent mtDNA replication inhibitor6 (Supplementary Fig. 2), validating the notion that these puncta indeed label replicating mtDNA. mtDNA replication showed a striking spatial pattern in the germarium. We observed a moderate level of mtDNA replication, indicated by the number of EdU puncta, at the anterior tip of the germarium (region 1) where the stem cells reside. In contrast, there was almost no EdU incorporation in region 2A (Fig. 1c,d). mtDNA replication resumed in region 2B of the germarium, where the number of EdU puncta in the posterior cyst was more than tenfold higher than in region 2A (Fig. 1c,d). Specifically, intense EdU staining was concentrated in the middle of 16-cell cysts, surrounding hu li tai shao (Hts)7 protein, a marker for ring canals and the fusome (Fig. 1c and Supplementary Fig. 3). mtDNA continued to replicate at a high level in region 3 of the germarium (Fig. 1c,d) and in post-germarium egg chambers (Fig. 1b). We found that EdU puncta were completely absent in region 2B of germaria from hts-mutant flies (Fig. 2a), which lack fusomes7. However, EdU incorporation appeared normal in stem cells and post-germarium egg chambers in hts mutants (Fig. 2b), suggesting that the fusome and not Hts per se was essential for developmentally regulated mtDNA replication in region 2B.

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Figure 1 mtDNA replication occurs around the fusome in the germarium. (a) Diagram of a Drosophila ovary, illustrating, anterior (A) to posterior (P), successive stages of development and a magnified view of the germarium. The durations of the germarium and post-germarium stages at 25 °C are labeled13. Changes in mitochondrial morphology and subcellular distribution in the germarium are illustrated18 and described in detail in the main text. The fusome (red), mitochondria (blue) and future oocyte (broken line) are shown. (b) Representative z-stack projection of a wild-type ovary (>50 repetitions), showing EdU incorporation into mtDNA (arrowheads) and nuclei (arrows) in the presence of aphidicolin and staining for Hts (clone RC) at 25 °C. Scale bar, 10 µm. (c) Magnified view of the boxed region in b showing the germarium with developmental regions indicated. Scale bar, 5 µm. (d) Quantification of mtDNA replication indicated by the number (mean ± s.d., n = 5) of EdU puncta in different regions of the germarium. A, anterior region 2B; P, posterior region 2B. P < 0.05 for each data point compared to region 2A.

but suppressed replication in regions 2 and 3, with strong suppression in region 2B (Fig. 2d–f and Table 1). Furthermore, moderate mitochondrial disruption by a mild uncoupler, 2,4-dinitrophenol (DNP), also significantly reduced mtDNA replication in the posterior cysts of region 2B (Supplementary Fig. 4d). Taken together, these data demonstrate that mtDNA replication is coupled to mitochondrial activity and that germarium region 2B and, to a lesser extent, region 3 are particularly sensitive to mitochondrial disruption.

To test how this developmentally regulated and fitness-dependent mtDNA replication might affect mtDNA inheritance, we examined the replication and transmission of a deleterious mtDNA genome. Applying a method based on a mitochondrially targeted restriction enzyme10, we isolated a temperature-sensitive lethal mtDNA allele, mt:CoIT300I, which encodes a threonine-to-isoleucine substitution in cytochrome c oxidase subunit 1 (Col) (Fig. 3a). Homoplasmic mt:CoIT300I flies developed normally at 18 °C (Fig. 3b) but failed to eclose from pupae at 29 °C. When shifted to 29 °C after eclosion at the permissive temperature, mt:CoIT300I flies died within 5 d. mt:CoIT300I mitochondria showed normal respiration (Supplementary Fig. 5a) despite having reduced COX activity at 18 °C, suggesting that COX might not be the rate-limiting step in the Drosophila respiratory chain. Mutant COX was extremely temperature sensitive. COX activity in mt:CoIT300I extracts rapidly decreased over the course of 40 min at 29 °C (Supplementary Fig. 5b), whereas there was little change in wild-type COX activity over the same time period. Notably, mt:CoIT300I flies showed a gradual decrease in COX activity (Fig. 3c) and a significant reduction in the respiratory control ratio (RCR; Supplementary Fig. 5a) after shifting to 29 °C, an indication of impairment of the electron transport chain11.

Because mt:CoIT300I mitochondria are de-energized, we reasoned that mtDNA replication would be impaired in mt:CoIT300I ovaries. Ovaries from mt:CoIT300I flies cultured at 18 °C displayed EdU incorporation patterns similar to those of wild-type flies reared at either 18 °C or 29 °C (Fig. 3e). mtDNA replication in the stem cells and post-germarium egg chambers of mt:CoIT300I flies appeared normal at 29 °C (Supplementary Fig. 6a,b), indicating that the mitochondrial replication machinery remained functional. However, EdU incorporation in region 2B of mt:CoIT300I germaria was significantly reduced to less than 50% of the incorporation seen in this region in wild-type germaria under the same conditions (Fig. 3d,e). These results further support the idea that mtDNA replication is dependent on the organelle’s fitness, and we reasoned that impaired replication of the mutant genome would limit its transmission.

To test whether transmission of the mt:CoIT300I genome was restricted, we generated heteroplasmic flies containing both
wild-type and mt:CoIT300I mtDNA by germplasm transplantation. The initial founder heteroplasmic flies contained less than 10% wild-type mtDNA, which was enough to restore viability at 29 °C (Fig. 3b), thereby avoiding complications caused by pleiotropic deficiencies in the homoplasmic mutant at the non-permissive temperature. Wild-type mtDNA has a single XhoI site that is disrupted in mt:CoIT300I mtDNA (Fig. 3a). XhoI digestion of a PCR fragment spanning the XhoI site from heteroplasmic flies produced one XhoI-resistant band of mt:CoIT300I mtDNA and two smaller wild-type fragments (Fig. 4b), thereby allowing us to quantify their relative levels. We monitored the levels of mt:CoIT300I mtDNA in two populations of heteroplasmic flies that were maintained at 18 °C or 29 °C over multiple generations. Heteroplasmic flies maintained a relatively stable level of mutant mtDNA throughout six generations at 18 °C (Fig. 4a). In contrast, the proportion of mutant mtDNA in heteroplasmic flies was dramatically reduced from 90% to less than 20% by the fifth generation at 29 °C (Fig. 4a) and was below 10% by generation 15 (Fig. 4a), demonstrating that the mt:CoIT300I genome was restricted from transmission and was gradually purged from the population at the non-permissive temperature. We also compared the extent of heteroplasy in each individual larval progeny generated by a single mother at either 18 °C or 29 °C. Larvae produced at 18 °C contained 73–95% mutant mtDNA (Fig. 4c), whereas the level of mutant mtDNA decreased to 43–68% in progeny produced at 29 °C. This result, together with the notion of a lack of selection during somatic development, demonstrates that selection against mt:CoIT300I mtDNA occurs during oogenesis.

It takes approximately 7 d for a stem cell to develop into the first egg chamber budding from the germarium and another 3 d for it to develop into a mature egg at 25 °C (Fig. 1a and ref. 13). This defined schedule of oogenesis allowed us to estimate the timing of selection. In another temperature-shift experiment, we compared the extent of heteroplasy in progeny produced at 18 °C to that found in their siblings produced on successive days after the mother was shifted to 29 °C. We did not observe a significant reduction in the abundance of the mt:CoIT300I genome in the progeny produced on the first 2 d after the shift to 29 °C (Fig. 4d). In contrast, the levels of the mutant genome were significantly reduced in the progeny produced after 3 d at 29 °C (Fig. 4d). The temporal resolution of this temperature-shifting scheme is imprecise, and the asynchrony of ovariole development complicates this analysis. Nonetheless, the onset of selection 3 d after the temperature shift suggests that selection occurs at a late germarium stage.

Multiple mechanisms have been proposed to restrain the transmission of harmful mtDNA mutations. A genetic bottleneck promotes random sampling of mtDNA variants into different primordial germ cells, and germ cells with high mutational loads would have compromised mitochondrial activities and would be eliminated or have impaired proliferation. We did not observe a significant increase in germarium cell death in heteroplasmic mt:CoIT300I flies compared to wild-type flies at 29 °C (Supplementary Fig. 7), nor did heteroplasmic flies show reduced fecundity, a plausible consequence of impaired germ cell proliferation. Thus, the selection that we observed is unlikely to have occurred at the cell level.

Strong purifying selection at the organelle level has been suggested on the basis of the notion that mutations in structural genes are limited from transmission, even at frequencies that are too low to impair overall cellular function. We demonstrated that selection occurs at a late germarium stage that is temporarily associated with mtDNA replication. Notably, mtDNA replication is coupled to mitochondrial fitness and is particularly sensitive to mitochondrial disruption in regions 2B and 3. We propose that selection occurs on the organelle level according to the collective functional readout of the mtDNAs within the mitochondrion. Each mitochondrion usually contains multiple copies of mtDNA. Mitochondria containing a high proportion of wild-type mtDNA would replicate faster than those containing more mutant genomes, leading to a decrease in the proportion of mutant mtDNA through oogenesis (Supplementary Fig. 8a). Our model predicts that mtDNA copy number in each mitochondrion would have an impact on the effectiveness of selection owing to intraorganellar complementation. The lower the copy number of mtDNA, the more effective selection would be (Supplementary Fig. 8b).

Interestingly, mtDNA is not actively replicated in cysts until the 16-cell stage and 29 °C. We did not observe a significant increase in mtDNA replication by FCCP (Fig. 4d). Values were normalized to the average COX activities for wild-type flies at 29 °C at 0 d and are shown as mean ± s.d. Data represent three biological replicates. Values were normalized to the average COX activities for wild-type flies at 29 °C at 0 d and are shown as mean ± s.d. Data represent three biological replicates. Values were normalized to the average COX activities for wild-type flies at 29 °C at 0 d and are shown as mean ± s.d. Data represent three biological replicates. Values were normalized to the average COX activities for wild-type flies at 29 °C at 0 d and are shown as mean ± s.d. Data represent three biological replicates.
mouse ovarian follicles, suggesting that replicative competition could also be involved in purifying selection in mammals.

Mitochondria undergo a series of changes to their morphology and position in developing germaria (Fig. 1a and Supplementary Fig. 9). In region 2A, mitochondria are dispersed throughout the cysts and are then recruited to the fusome in region 2B. We found that mitochondrial localization around the fusome was dependent on microtubules and mitochondrial activity (Supplementary Fig. 10), and mt:ColT300I mitochondria were dispersed in region 2B germaria at 29 °C (Supplementary Fig. 11). These results further substantiate the model in which healthy mitochondria are selectively recruited to the fusome and the genomes within are preferentially replicated. Some fusome-associated mitochondria will be transported to the oocyte and will form an evolutionarily conserved structure, the Balbiani body, which supplies mitochondria to the future germ cells of the next generation. Thus, selective transport of healthy mitochondria to the Balbiani body could act synergistically with selective mtDNA replication to further strengthen selection in the germ line. It would be interesting to test whether the extent and the speed of selection against deleterious mutations would be reduced in mutants with impaired mitochondrial transport. Of note, the Balbiani body exists in the developing oocytes of various animals, and it has been noted that mitochondria in the Balbiani body have higher fitness, implying a conserved role of the Balbiani body in mitochondrial inheritance.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

H.X. conceived and designed the experiments. J.H.H., Z.C. and H.X. performed the experiments, analyzed the data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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**REFERENCES**

1. Wallace, D.C. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* **39**, 359–407 (2005).
2. Rand, D.M. Mitigating mutational meltdown in mammalian mitochondria. *PLoS Biol.* **6**, e35 (2008).
3. Fan, W. et al. A mouse model of mitochondrial disease reveals germline selection against severe mtDNA mutations. *Science* **319**, 958–962 (2008).
4. Freyer, C. et al. Strong purifying selection in transmission of mammalian mitochondrial DNA. *PLoS Biol.* **6**, e10 (2008).
5. de Cuevas, M., Lilly, M.A. & Spradling, A.C. Germline cytoskeleton in *Drosophila*. *Annu. Rev. Genet.* **31**, 405–428 (1997).
6. O’Farrell, P.H. & Holt, C.E. Specific inhibition by ethidium bromide of mitochondrial DNA synthesis in physicsum polycephalum. *J. Cell Biol.* **49**, 546–553 (1971).
7. Lin, H., Yue, L. & Spradling, A.C. The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cytoskeletal rearrangements. *Dev. Cell* **120**, 947–956 (1994).
8. Formusoka, D. et al. Novel insights into the assembly and function of human nuclear-encoded cytochrome c oxidase subunits 4, 5a, 6a, 7a and 7b. *Biochem. J.* **428**, 363–374 (2010).
9. Lou, P.-H. et al. Mitochondrial uncouplers with an extraordinary dynamic range. *Biochem. J.* **407**, 129–140 (2007).
10. Xu, H., DeLuca, S.Z. & O’Farrell, P.H. Manipulating the animal mitochondrial genome with targeted restriction enzymes. *Science* **321**, 575–577 (2008).
11. Chance, B., Williams, G.R. Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J. Biol. Chem.* **217**, 409–427 (1955).
12. Niki, Y., Chigusa, S.I. & Matsuura, E.T. Complete replacement of mitochondrial DNA in Drosophila. *Nature* **341**, 551–552 (1989).
13. Spradling, A.C. *In The Development of Drosophila melanogaster* eds. Bate, M. & Martinez, A. 1–70 (CSHL Press, Cold Spring Harbor, NY, 1993).
14. Chinnery, P.F. et al. The inheritance of mitochondrial DNA heteroplasmy: random drift, selection or both? *Trends Genet.* **16**, 500–505 (2000).
15. Freyer, C. et al. Variation in germline mtDNA heteroplasmy is determined prenatally but modified during subsequent transmission. *Nat. Genet.* **44**, 1282–1285 (2012).
16. Ma, H., Xu, H. & O’Farrell, P.H. Transmission of mitochondrial mutations and action of purifying selection in *Drosophila melanogaster*. *Nat. Genet.* doi:10.1038/ng.2919 (9 March 2014).
17. Shoubridge, E.A. & Wai, T. Medicine. Sistedenting mutational meltdown. *Science* **319**, 914–915 (2008).
18. Cox, R.T. & Spradling, A.C. A Balbiani body and the fusome mediate mitochondrial inheritance during *Drosophila* oogenesis. *Development* **130**, 1579–1590 (2003).
19. Wai, T., Teoli, D. & Shoubridge, E.A. The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. *Nat. Genet.* **40**, 1484–1488 (2008).
20. Cox, R.T. & Spradling, A.C. Miton control the early acquisition of mitochondria by *Drosophila* oocytes. *Development* **133**, 3371–3377 (2006).
21. Kloc, M., Bilinski, S. & Enkin, L.D. The Balbiani body and germ cell determinants: 150 years later. *Curr. Top. Dev. Biol.* **59**, 1–36 (2004).
22. Pepling, M.E., Wilhelm, J.E., O’Hara, A.L., Gephardt, G.W. & Spradling, A.C. Mouse oocytes within germ cell cysts and primordial follicles contain a Balbiani body. *Proc. Natl. Acad. Sci. USA* **104**, 187–192 (2007).
23. Zhou, R.R., Wang, B., Wang, J., Schatten, H. & Zhang, Y.Z. Is the mitochondrial cloud the selection machinery for preferentially transmitting wild-type mtDNA between generations? Rewinding Müller’s ratchet efficiently. *Curr. Genet.* **56**, 101–107 (2010).

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**Figure 4** Germline selection against the mt:ColT300I genome at the restrictive temperature. (a) Frequency of the mt:ColT300I mutation in heteroplasmic flies maintained at 29 °C or 18 °C over generations. (b) Xhol digestion of the PCR fragment spanning mt:Col, amplified from single larvae produced by the same heteroplasmic mother at 18 °C or 29 °C. T300I, mt:ColT300I allele. (c) Proportion of mutant mtDNA in ten single larvae at 18 °C or 29 °C, calculated by quantifying band intensity in b. Average proportion of mutant mtDNA: 18 °C, 83 ± 5%; 29 °C, 60 ± 9%; n = 10; *P* < 0.0005. (d) Proportion of mutant mtDNA in ten single larvae from the same mother at 18 °C (45 ± 9%) or produced on the first 2 d (40 ± 9%), day 3 (37 ± 6%) or day 4 (37 ± 8%) after being shifted to 29 °C. n = 10, *P* < 0.005.
**ONLINE METHODS**

*Drosophila* genetics. Flies were maintained on cornmeal medium at 25 °C unless otherwise stated. w*1118* flies containing wild-type mtDNA were used as the wild-type strain. Heteroplasmic flies were maintained at 18 °C unless otherwise stated. Homoplasmic mt:CoIT300I flies were isolated by mitochondrially targeted restriction enzyme, mt*10Xhol*, as described previously10. Hto10133[DF(2)R]BSC26 and P[UAS-Der-2.2]1; P[GAL4-nos.NGT]40 flies were from the Bloomington Stock Center (25751). The CoVa RNAi line was from the Vienna Drosophila RNAi Center (44490). Homoplasmic mt:CoIT300I female flies were backcrossed to w*1118* males for at least five generations to isogenize the nuclear genetic background before conducting experiments. Survival rate was calculated by counting the number of adults that eclosed from pupae at the respective temperatures.

**Generation of heteroplasmic flies.** Germlasm from w*1118* embryos was injected into homoplasmic mt:CoIT300I embryos by Genetic Services, Inc., as described previously12. Each individual surviving female from injected embryos was crossed to w*1118* males at room temperature, and female progeny were mated *en masse* to w*1118* males at room temperature for 3–4 d. Embryos were incubated at 29 °C to select for adult escaper progeny. Female escapers were crossed to w*1118* males to maintain the stock as the founder line.

**Molecular confirmation and quantification of heteroplasmia.** A 4-kb mtDNA fragment spanning the XhoI site in mt:CoI was PCR amplified from total-fly DNA as described previously10 and purified using the Thermo Scientific gel purification kit. The resultant product (500 ng) was digested with 20 units of XhoI at 37 °C overnight. After agarose gel electrophoresis, DNA was visualized with an ImageQuant LAS4000, and band intensity was quantified with SWIFT II Wavescan software (version 2.06, Biochrom) and were normalized with the specified concentrations of FCCP or 1 mM DNP was included. For immunostaining in the absence of EdU incorporation, ovaries were fixed with primary and secondary antibodies were carried out as described above. The following antibodies were used: mouse antibody to Hts RC (DSHB), 1:1,000 dilution; mouse antibody to ATP synthase subunit α (MitoSciences, 15H4C4), 1:1,000 dilution; and Alexa Fluor 568 goat antibody to mouse IgG (Invitrogen), 1:200 dilution. For drug treatment, carbonyl cyanide m-chlorophenyl hydradrazone (CCCP) was diluted in Schneider’s medium supplemented with 10% FBS and was then incubated at 29 °C for 2 h in PBS. Incubations with primary and secondary antibodies were carried out as described above. The following antibodies were used: mouse antibody to Hts RC (DSHB), 1:1,000 dilution; mouse antibody to ATP synthase subunit α (MitoSciences, 15H4C4), 1:1,000 dilution; and Alexa Fluor 568 goat antibody to mouse IgG (Invitrogen), 1:200 dilution. For drug treatment, carbonyl cyanide m-chlorophenyl hydradrazone (CCCP) was diluted in Schneider’s medium supplemented with 10% FBS and was then incubated with ovaries for 1 h. For colchicine treatment, flies were reared on food containing colchicine (50 µg/ml) for 2–3 d before ovary dissection25. All images were acquired with a PerkinElmer UltraVIEW VoX and processed with Volocity software. The efficacy of aphidicolin in inhibiting nuclear DNA replication was variable. Images of germaria with strong nuclear DNA labeling, which might lead to overestimation of the number of mtDNA puncta, were excluded from data analyses. We observed that later-stage egg chambers had strong EdU signal and that the epithelial sheath had strong background fluorescence. Thus, images of a particular germarium that was overlapped by or close to other tissues or later-stage egg chambers were also excluded from data analyses owing to the strong, out-of-focus signal.

**Confocal image quantification.** EdU quantification was conducted by eye, counting the number of green (Alexa Fluor 488–labeled) puncta in a region of interest (ROI) spanning a particular developmental region of the germarium. The volume of the ROI was calculated using Volocity 6.1 software (PerkinElmer), and the number of EdU puncta per ROI was calculated for 5–10 germaria. Statistical analysis was performed with Microsoft Excel, and a two-tailed Student’s *t* test was used to calculate statistical significance.

**Cytochrome c oxidase activity.** COX activity was measured according to a previously described protocol10. Cytochrome c (10 mg/ml; equine heart, EMD Millipore) was reduced with 10 mg/ml sodium ascorbate (Sigma) in 0.1 M sodium phosphate buffer (pH 7.2), which was further dialyzed out overnight at 4 °C in sodium phosphate buffer. The concentration of reduced cytochrome c was measured using the extinction coefficient of 550 nm of 29.5 M⁻¹ cm⁻¹. For each experiment, thoraces were separated from the heads and abdomens of six male flies and homogenized in 100 µl of sodium phosphate buffer containing 0.05% Tween-80. Supernatants were collected by centrifugation at 4,000g for 1 min. The reaction was initiated by adding 25 µl of supernatant to 750 µl of 25 µM reduced cytochrome c, and the optical density at 550 nm was recorded every 2 s for 2 min. The temperature of the reaction was controlled by a connected circulating water bath. Data were analyzed using SWIFT II Wavescan software (version 2.06, Biochrom) and were normalized by protein concentrations determined by Coomassie Blue G-250–based reagent (Thermo Scientific). Each data point is the average of at least three independent experiments.

**Mitochondrial isolation and respiration.** Approximately 400 live flies were immobilized by chilling briefly on ice and were placed in a chilled tube.

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**Mitochondrial isolation and respiration.** Approximately 400 live flies were immobilized by chilling briefly on ice and were placed in a chilled tube.
Ice-cold isolation medium (500 µl; 250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, 1% BSA (fatty acid free), pH 7.4 at 4 °C) was added, and the flies were gently ground with a pestle to avoid shearing. The liquid was passed through two layers of absorbent muslin (Johnson & Johnson) and centrifuged at 500g for 3 min at 4 °C. The supernatant was passed through two layers of muslin and centrifuged at 9,000g for 10 min. The pellet was rinsed twice in BSA-free mitochondrial isolation buffer and resuspended in 100 µl of BSA-free buffer. The mitochondrial respiration rate was determined using a fiber-optic oxygen probe connected to the computer-operated oxygraph control unit (Instech Laboratories). The temperature was maintained at 22 °C or 29 °C, and the total volume was 1.1 ml. Freshly isolated mitochondria (150 µg) were added to the respiration buffer (120 mM KCl, 5 mM potassium phosphate, 3 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, and 0.2% BSA, pH 7.2) and allowed to equilibrate for 2 min. NAD⁺-linked substrates (5 mM pyruvate plus 5 mM proline) were added to the chamber and equilibrated for 3 min, and 1 mM ADP was then added. The resulting slope was used to calculate the rate of state 3 respiration. State 4 respiration was calculated from the slope without ADP addition. State 4 respiration is too low to be measured reliably at 18 °C; thus, we conducted the experiment at 22 °C for the permissive temperature control. All measurements were performed within 1–2 h of the isolation of mitochondria. RCRs were calculated by dividing the state 3 respiration rate by the state 4 respiration rate, and the RCR remained stable during the time interval.

**Statistical analysis.** The F test was used to compare the equality of variances between two groups. On the basis of the result of the F test, a two-sided t test was used for statistical analysis. Differences were considered to be statistically significant with P < 0.05.

**Modeling the effect of mtDNA copy number and mutant frequency on selection.** The proposed model of selection is based on the fitness of an individual mitochondrion. The presence of wild-type mtDNA will complement the mutant genome in a mitochondrion. If only the deleterious mitochondria containing 100% mutant genomes were selected against, the efficacy of selection would be determined by the probability of a given mitochondrion containing 100% mutant genomes. Given the total mtDNA copy number in a cell (n), the frequency of the mutant mtDNA (f) and mtDNA copy number per mitochondrion (c), the probability P that a given mitochondrion contains 100% mutant mtDNA can be deduced as follows:

\[
P = \prod_{i=1}^{n} \frac{n f - (c - 1)}{n - (c - 1)}
\]

We estimated that there are 100 mtDNA copies per cell (n = 100) on the basis of the relative quantification of nuclear DNA and mtDNA levels in *Drosophila* (2014). Graphs showing P as a function of f and c were computed in Microsoft Excel.

24. Yoneda, M., Tanno, Y., Tsuji, S. & Attardi, G. Detection and quantification of point mutations in mitochondrial DNA by PCR. *Methods Enzymol.* 264, 432–441 (1996).
25. Koch, E.A. & Spitzer, R.H. Multiple effects of colchicine on oogenesis in *Drosophila*: induced sterility and switch of potential oocyte to nurse-cell developmental pathway. *Cell Tissue Res.* 228, 21–32 (1983).
26. Calleja, M. et al: Mitochondrial DNA remains intact during *Drosophila* aging, but the levels of mitochondrial transcripts are significantly reduced. *J. Biol. Chem.* 268, 18891–18897 (1993).