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

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Abstract

Though mitochondrial DNA is prone to mutation and few mtDNA repair mechanisms exist[1], crippling mitochondrial mutations are exceedingly rare[2]. Recent studies demonstrated strong purifying selection in the mouse female germline[3,4]. However, the mechanisms underlying the positive selection of healthy mitochondria remain to be elucidated. We visualized mtDNA replication during *Drosophila* oogenesis. We found that mtDNA replication commenced prior to oocyte determination during the late germarium stage, and was dependent on mitochondrial fitness. We isolated a temperature-sensitive lethal mtDNA mutation, *mt:CoI*^{T300I}, which displayed reduced mtDNA replication in the germarium at the restrictive temperature. Additionally, the frequency of *mt:CoI*^{T300I} in heteroplasmic flies was decreased both through oogenesis and over multiple generations at the restrictive temperature. Furthermore, we determined that selection against *mt:CoI*^{T300I} overlaps with the timing of selective replication of mtDNA in the germarium. These findings establish a previously uncharacterized developmental mechanism for selective amplification of healthy mtDNA, which may be evolutionarily conserved to limit transmission of deleterious mutations.

Drosophila oogenesis commences with a germline stem cell (GSC) at the anterior tip of the germarium (Fig. 1a). Asymmetric division of GSCs produces a cyst that continues to divide four times with incomplete cytokinesis to form 16 interconnected germ cells: one eventual oocyte, and 15 nurse cells. These 16 developing germ cells are interconnected by a large cytoplasmic structure called the fusome (Fig. 1a), which protrudes through the ring canals connecting cells of the cysts and mediates intercellular transport from nurse cells to the oocyte[5]. To explore the inheritance mechanism of mtDNA, we first asked when and where mtDNA proliferation occurs during oogenesis. We used incorporation of a thymidine analog, 5-ethynyl-2′-deoxyuridine (EdU), to determine the pattern of DNA replication in the

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Note: Supplementary Information and Source Data are available in the online version of the paper.

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.

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ovary. Pre-incubation with aphidicolin, an inhibitor of nuclear DNA polymerase-α, significantly reduced EdU incorporation into nuclear DNA, and allowed visualization of many punctate structures in the cytoplasm (Supplementary Fig. 1). Importantly, these puncta localized with mitochondria marked by ATP synthase (Supplementary Fig. 1b), and were absent in ovaries treated with ethidium bromide, a potent mtDNA replication inhibitor[6] (Supplementary Fig. 2), validating that they indeed label replicating mtDNA. mtDNA replication displayed 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 (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 greater than 10-fold 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, 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 fusomes[7]. However, EdU incorporation appeared normal in stem cells and post-germarium egg chambers in *hts* mutants (Fig. 2b), suggesting that the fusome, not Hts *per se*, was essential for this developmentally regulated mtDNA replication at region 2B.

Next we tested whether mitochondrial fitness impacted mtDNA replication in the germline. We disrupted mitochondrial function by RNAi-mediated knockdown of *cytochrome c oxidase* (COX)*Va (CoVa),* which is required for the assembly of COX[9]. 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, FCCP, which dissipates mitochondrial membrane potential and impairs ATP production with a wide dynamic range[10]. Similar to *CoVa* knockdown, high concentration of FCCP (10 μM) completely abolished mtDNA replication throughout the germarium (Fig. 2f), while incubation with DMSO had no impact on mtDNA replication (Fig. 2c). Lower doses of FCCP (5 μM or 2 μM) had no obvious effect on mtDNA replication in region 1, 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 posterior cysts of region 2B (Supplementary Fig. 4d, e). 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 impact mtDNA inheritance, we examined the replication and transmission of a deleterious mtDNA mutation. Applying a method based on a mitochondrially targeted restriction enzyme[11], we isolated a temperature sensitive lethal mtDNA mutation, *mt:CoI*^{T300I}, a threonine to isoleucine substitution in *cytochrome c oxidase subunit I* (CoI) (Fig. 3a). Homoplasmic *mt:CoI*^{T300I} 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:CoI*^{T300I} flies died within 5 days. *mt:CoI*^{T300I} mitochondria displayed 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 of *mt:CoI*^{T300I} extracts rapidly diminished over the course of 40 minutes at 29°C (Supplementary Fig. 5b), whereas wild type COX had little change over the same span. Importantly, *mt:CoI*^{T300I} flies displayed a gradual decline in COX activity (Fig. 3c) and a significant reduction in respiratory control ratio (Supplementary Fig. 5a) after shifting to 29°C, an indication of electron transport chain impairment[12].

Since *mt:CoI*^{T300I} mitochondria are de-energized, we reasoned that mtDNA replication would be impaired in *mt:CoI*^{T300I} ovaries. Ovaries from *mt:CoI*^{T300I} flies 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 stem cells and post-germarium egg chambers of *mt:CoI*^{T300I} 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:CoI*^{T300I} germaria was significantly reduced to less than 50% of that of wild type under the same condition (Fig. 3d, e). These results further support that mtDNA replication is dependent on the organelles' fitness, and we reasoned that impaired replication of the mutant genome would limit its transmission.

To test whether transmission of *mt:CoI*^{T300I} is restricted, we generated heteroplasmic flies containing both wild type and *mt:CoI*^{T300I} mtDNA by germ plasm transplantation[13]. 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 the 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:CoI*^{T300I} (Fig. 3a). XhoI digestion of a PCR fragment flanking the XhoI site from heteroplasmic flies produced one XhoI resistant band of *mt:CoI*^{T300I} mtDNA and two smaller wild type fragments (Fig. 4b), thereby allowing us to quantify their relative levels. We monitored the levels of *mt:CoI*^{T300I} in two populations of heteroplasmic flies that were maintained at 18°C or 29°C over multiple generations. Heteroplasmic animals maintained a relatively stable level of mutant mtDNA throughout 6 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:CoI*^{T300I} was restricted from transmission and gradually purged from the population at the non-permissive condition. We also compared the level of heteroplasmy of each individual larval progeny produced 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 lack of selection during somatic development, demonstrate that selection against *mt:CoI*^{T300I} occurs during oogenesis.

It takes approximately 7 days for a stem cell to develop into the first egg chamber budding from the germarium, and another 3 days to develop into a mature egg at 25°C[14] (Fig. 1a).

This defined schedule of oogenesis allowed us to estimate the timing of selection. In another temperature shift experiment, we compared the levels of heteroplasmy in progeny produced at 18°C to their siblings produced on successive days after the mother was shifted to 29°C. We did not observe significant reduction of the *mt:CoI*^{T300I} genome in progeny produced on the first 2 days at 29°C (Fig. 4d). In contrast, the level of mutant genomes was significantly reduced in the progeny produced after 3 days 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 three days after shifting suggests that selection occurs at a late germarium stage.

Multiple mechanisms have been proposed to restrain the transmission of harmful mtDNA mutations. Genetic bottleneck promotes random sampling of mtDNA variants into different primordial germ cells[15]. The germ cells with high mutational loads would have compromised mitochondrial activities and be eliminated[15, 16], or have impaired proliferation. We did not observe a significant increase in germarium cell death in heteroplasmic *mt:CoI*^{T300I} flies compared to wild type at 29°C (Supplementary Fig. 7), nor did heteroplasmic flies display reduced fecundity[17], 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 based on the notion that mutations on structural genes are limited from transmission even at levels that are too low to impair the overall cellular function[3,4,18]. We demonstrated that the selection occurs at a late germarium stage that is temporally associated with mtDNA replication. Importantly, mtDNA replication is coupled to mitochondrial fitness and is particularly sensitive to mitochondrial disruption in regions 2B and 3. We propose a selection on the organelle level based on the collective functional readout of mtDNAs within. Each mitochondrion usually contains multiple copies of mtDNAs. Mitochondria containing a high level 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 the mtDNA copy number per mitochondrion would impact the effectiveness of selection due to intra-organelle complementation. The lower the copy number of mtDNA, the more effective selection would be (Supplementary Fig. 8b). Interestingly, mtDNAs are not actively replicated in cysts until the 16-cell stage in region 2B. Meanwhile, mitochondria divide continuously[8]. Thus, mtDNA copy number per mitochondrion would be reduced to prepare for effective selection (Supplementary Fig. 8a). Selective replication of mtDNA variants has been demonstrated in murine ovarian follicles[19], suggesting that replication competition could also be involved in the purifying selection in mammals[3,18].

Mitochondria undergo a series of changes to their morphology and position in developing germaria[8] (Fig. 1a, Supplementary Fig. 9). In region 2A, mitochondria are dispersed throughout the cysts, and then recruited to the fusome in region 2B[8]. We found that mitochondrial localization around the fusome was dependent on microtubules and mitochondrial activity (Supplementary Fig. 10), and *mt:CoI*^{T300I} mitochondria were dispersed in region 2B germaria at 29°C (Supplementary Fig. 11). These results further

substantiate the model that 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 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 the selection in the germline. It would be interesting to test whether the extent and the speed of selection against deleterious mutations would be reduced in the mutants with impaired mitochondria transport[20]. Of note, the Balbiani body exists in the developing oocytes of various metazoans[21, 22] and it has been noted that mitochondria in the Balbiani body have a higher fitness[23], implying a conserved role of the Balbiani body in mitochondrial inheritance.

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 wild type. Heteroplasmic flies were maintained at 18°C, unless otherwise stated. Homoplasmic *mt:CoI*^{T300I} flies were isolated by mitochondrially targeted restriction enzyme, mitoXhoI as described previously[11]. Hts^{01103}, *Df (2R)BSC26* and *P{UAS-Dcr-2.D}1; P{GAL4-nos.NGT}40* (Bloomington # 25751) were from Bloomington Stock Center. *CoVa* RNAi line (VDRC# 44490) was from Vienna Drosophila RNAi Center. Homoplasmic *mt:CoI*^{T300I} female flies were backcrossed to w^{1118} males at least 5 generations, to isogenize the nuclear genetic background, prior to conducting experiments. Survival rate was calculated by counting the number of adults that eclosed from pupae at the respective temperatures.

Generation of heteroplasmic flies

Germ plasm from w^{1118} embryos was injected into homoplasmic *mt:CoI*^{T300I} embryos by Genetic Services, Inc., as described previously[13]. Each individual surviving female from injected embryos was crossed to w^{1118} males at room temperature, and female progeny of that cross were mated en masse to w^{1118} males at room temperature for 3–4 days, and the 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 heteroplasmy

A 4 kb mtDNA fragment spanning the XhoI site in *mt:CoI* was PCR amplified from total animal DNA as described previously[11], and purified using the Thermo Scientific gel purification kit. 500 ng of the resultant product was digested with 20 units of XhoI at 37°C overnight. Following agarose gel electrophoresis, DNA was visualized with an ImageQuant LAS4000 and band intensity was quantified with Image Quant TL 7.0 software (GE Healthcare). Proportion of wild type DNA was calculated by adding the intensity of the 2.4 kb and 1.6 kb XhoI fragments and divided by the sum of the intensities of the undigested 4 kb fragment plus 2.4 kb and 1.6 kb XhoI fragments. To avoid the complication that resistance to XhoI digestion may be due to heteroduplex formation during PCR[24], wild type and *mt:CoI*^{T300I} DNA were mixed in a range of proportions in 10% increments from 100%

wild type to 100% *mt:CoI*^{T300I} and utilized as template in a PCR with *mt:CoI* primers. PCR products (500 ng) were subjected to XhoI digestion and the proportion of wild type DNA was quantified. The mixed product standard curve was generated by mixing wild type and *mt:CoI*^{T300I} PCR products in a series of proportions and digesting with XhoI (Supplementary Figure 12a).

mtDNA selection in female germ line, over generations and through development

To test mtDNA selection in the female germ line, a single heteroplasmic female was mated with several w^{1118} male flies at 18°C for 3 days, then transferred to a new vial and cultured at 29°C. It takes about 72 hours from first post-germarium stage, stage-2 egg chamber to mature oocytes[14]. We discarded the embryos produced during the first 3 days at 29°C considering that they were derived from the germarium developed at 18°C, and collected progeny produced from day 4–6 at 29°C. Because of the technical difficulty of obtaining consistent PCR products from a single embryo, and the fact that there is no change in heteroplasmy during the development from embryo to adult (Supplementary Figure 12b), we collected larvae from each temperature setting, and assayed their heteroplasmy. To assay the heteroplasmy over generations, two groups of heteroplasmic flies were constantly maintained at 18°C or 29°C. At each generation, a group of more than 50 flies (mixed male and female) were collected after laying eggs and stored at −80°C until assaying for heteroplasmy en mass. To quantify heteroplasmy at different developmental stages, a cage of heteroplasmic flies was set up and the eggs were collected for 8 hours on grape juice plates at 18°C. An approximately equal number of eggs were transferred to vials and cultured at 29°C, and the animals were collected at different developmental stages and stored at −80°C until being assayed en mass. The experiment was carried out in triplicate, and the average heteroplasmy of each stage was calculated.

EdU incorporation and immunostaining

Ovaries (5–10 pairs) from 4–5 day old female flies were dissected in Schneider's media/10% fetal bovine serum (FBS) and pretreated for 3 hours at room temperature in 7 μM aphidicolin (Sigma) diluted in Schneider's media/10% FBS. This was followed by a 2-hour incubation in 10 μM EdU/7 μM aphidicolin in Schneider's media/10% FBS. Following two 3-minute washes in media, ovaries were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for 20 minutes at room temperature. After fixation, ovaries were washed twice for 5 minutes in 3% BSA/PBS. Permeabilization was carried out in 0.5% Triton X-100/PBS for 20 minutes. EdU detection was carried out using the Click-iT EdU labeling kit (Invitrogen) according to manufacturer's instructions. Following the Click-iT reaction, tissues were rinsed twice with 3% BSA/PBS, and then blocked in 0.2% BSA/0.1% Triton-X 100/PBS (PBSBT) for 30 minutes. Incubation with primary antibody was carried out overnight at 4°C. Ovaries were then washed 3X 10 minutes and 2X 30 minutes in PBSBT. Secondary antibody incubation was done at room temperature for 2 hours in PBSBT. Previous washes were repeated, and ovaries were mounted in Vectashield mounting medium with DAPI (Vector laboratories). In experiments where drug treatments were performed during EdU incorporation, 2.5 μg/ml Ethidium Bromide, specified concentrations of FCCP or 1 mM 2,4-Dinitrophenol (DNP) were included. For immunostaining in the absence of EdU incorporation, ovaries were fixed as described above, and washed 3X 10

minutes in 0.1% Triton X-100/PBS (PBST) and blocked for 2 hours in PBSBT. Primary and secondary antibody incubations were carried out as mentioned above. The following antibodies were used: mouse α-Hts RC (DSHB) 1:1000; mouse α-Hts 1B1 (DSHB) 1:100; mouse α-ATP synthase subunit alpha (MitoSciences, 15H4C4) 1:1000; Alexa Fluor 568 goat α-mouse IgG (Invitrogen) 1:200. For drug treatments, CCCP was diluted in Schneider's medium/10% FBS to 10 μM, and incubated with ovaries for 1 hour following dissection. For colchicine treatment, flies were reared on food containing colchicine (50 μg/ml) for 2–3 days before ovary dissection[25]. All images were collected with a Perkin Elmer Ultraview Vox and processed with Volocity software. The efficacy of aphidicolin on inhibiting nuDNA replication was variable. Images of germaria with strong nuDNA labeling, which may lead to overestimation of mtDNA puncta, were excluded from data analyses. We observed that later stage egg chambers had strong EdU signal, and 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, due to the strong out of focus signal.

Confocal image quantification

EdU quantification was conducted by eye, counting the number of green (Alexa Fluor 488) puncta within a region of interest (ROI) spanning a particular developmental region of the germarium. The volume of the ROI was calculated using Volocity 6.1.1 software (Perkin Elmer), and the ratio of the number of EdU puncta per ROI was calculated for 5–10 germaria. Statistical analysis was completed with Microsoft Excel and a two-tailed student's t-test was used to calculate statistical significance.

Cytochrome c oxidase activity

The COX activity was measured according to a previously described protocol[11]. 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 at 550 nm of 29.5 $mM^{-1}cm^{-1}$. For each experiment, the thoraces were separated from heads and abdomens from 6 male flies and homogenized in 100 μl sodium phosphate buffer containing 0.05% Tween-80. The supernatants were collected by centrifugation at 4000 g for 1 minute. 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 sec for 2 min. The temperature of reaction was controlled by connected circulating water bath. The data was analyzed using SWIFT II Wavescan software (version 2.06, Biochrom Ltd) and normalized with protein concentrations determined by Coomassie Blue G-250 based reagent (Thermo scientific). Each data is the average of at least 3 independent experiments.

Mitochondrial Isolation and Respiration

Approximately 400 live flies were immobilized by chilling briefly on ice and placed in a chilled tube. 500 μl of ice-cold isolation medium (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, 1%(w/v) bovine serum albumin (fatty acid free), pH 7.4 at 4°C) was added and the flies were grounded gently with a pestle to avoid shearing. The liquid was passed through

two layers of absorbent muslin (Johnson and Johnson) and centrifuged at 500 g for 3 min at 4°C. The supernatant was passed through two layers of muslin and centrifuged at 9000 g for 10 min. The pellet was rinsed twice in BSA-free mitochondrial isolation buffer and resuspended in 100 μl BSA-free buffer. Mitochondrial respiration rate was determined using a fiber optic oxygen probe connected to the computer-operated oxygraph control unit (Instech Laboratories, Inc.) 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 $MgCl_2$ and 0.2 % BSA, pH 7.2) and allowed to equilibrate for 2 min. The NAD^+-linked substrates (5 mM pyruvate plus 5 mM proline) were then added to the chamber and equilibrated for 3 min, followed by the addition of 1 mM ADP. The resulting slope was used to calculate the rate of state 3 respiration. The state 4 respiration was calculated by the slope without ADP treatment. 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 after the isolation of mitochondria. Respiratory control ratios (RCR) were calculated by dividing state 3 respiration rate with 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. Based on the result of F test, two-sided *t* test was used for statistical analysis. Difference was considered statistically significant when $P<0.05$.

Modeling the impact 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 unhealthy 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 as "*n*", the frequency of mutant mtDNA as "*f* "and the mtDNA copy number per mitochondrion as "*c*", then the probability, "*P*" that a given mitochondrion contains 100% mutant mtDNA can be deduced as $P=\prod_1^c \frac{nf-(c-1)}{n-(c-1)}$. We estimated that there are 100 mtDNAs per cell (n=100) based on the relative quantification of nuclear DNA and mtDNA level in *Drosophila*[26]. Graphs showing *P* as a function of *f* and *c* were computed in MS excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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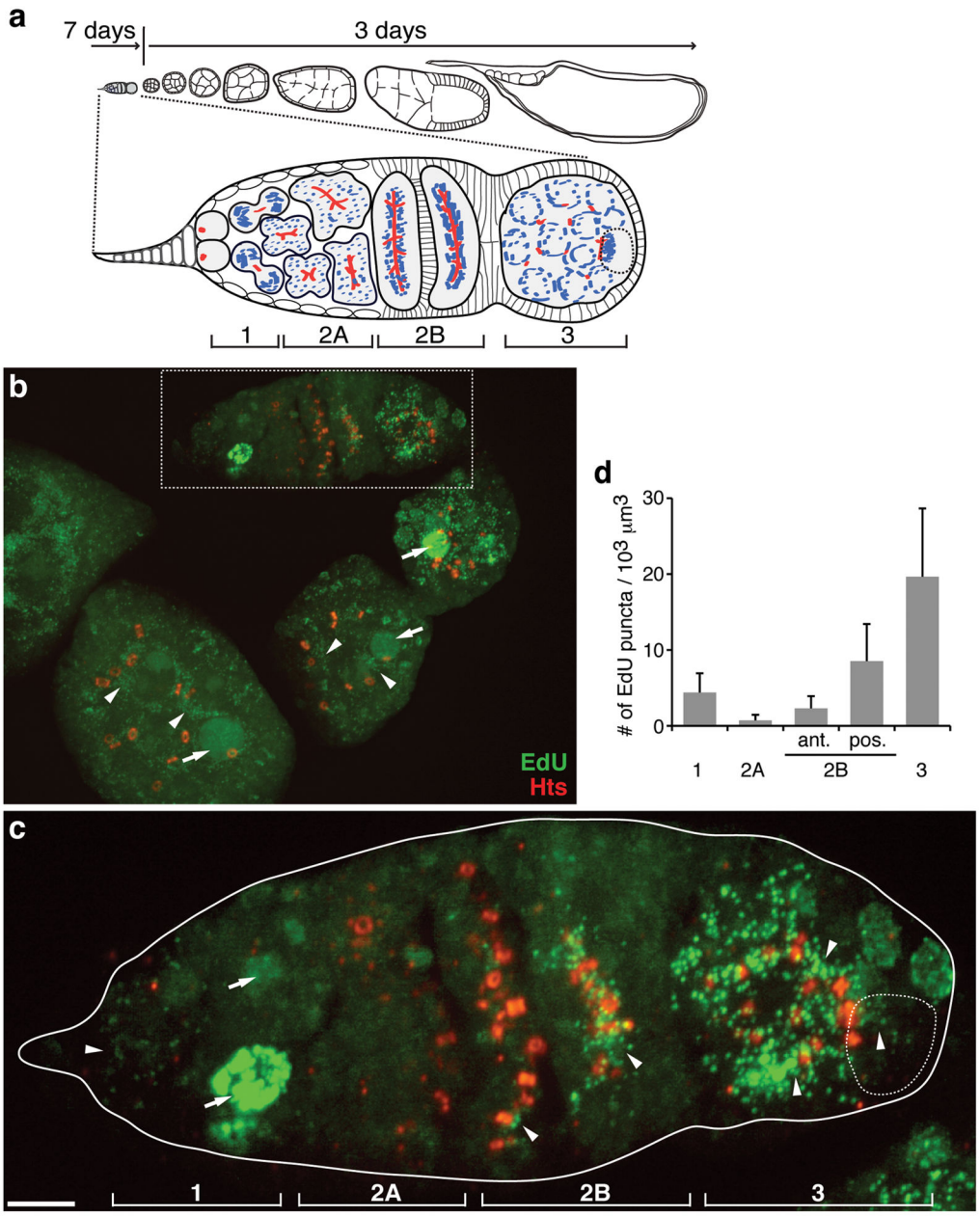

Figure 1.

mtDNA replication occurs around the fusome in the germarium. (**a**) Diagram of a *Drosophila* ovariole, illustrating anterior to posterior, successive stages of development and a magnified view of the germarium. The durations of germarium and post-germarium stages at 25°C are labeled[14]. Mitochondrial morphology and subcellular distribution changes in the germarium are illustrated[8] and described in detail in the main text. Fusome (red), mitochondria (blue) and future oocyte (broken line) are shown. (**b**) Representative Z-stack projection of an wt ovariole (>50 repetitions), showing 5-ethynyl-2′-deoxyuridine (EdU) (green) incorporation into mtDNA (arrowheads), and nuclei (arrows) in the presence of aphidicolin, and α-Hts RC (red) staining at 25°C. Scale bar, 10 μm. (**c**) Magnified view of

boxed region in (**b**) showing 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. 2B ant., region 2B anterior; 2B pos., region 2B posterior. $P< 0.05$ for each data point compared to region 2A.

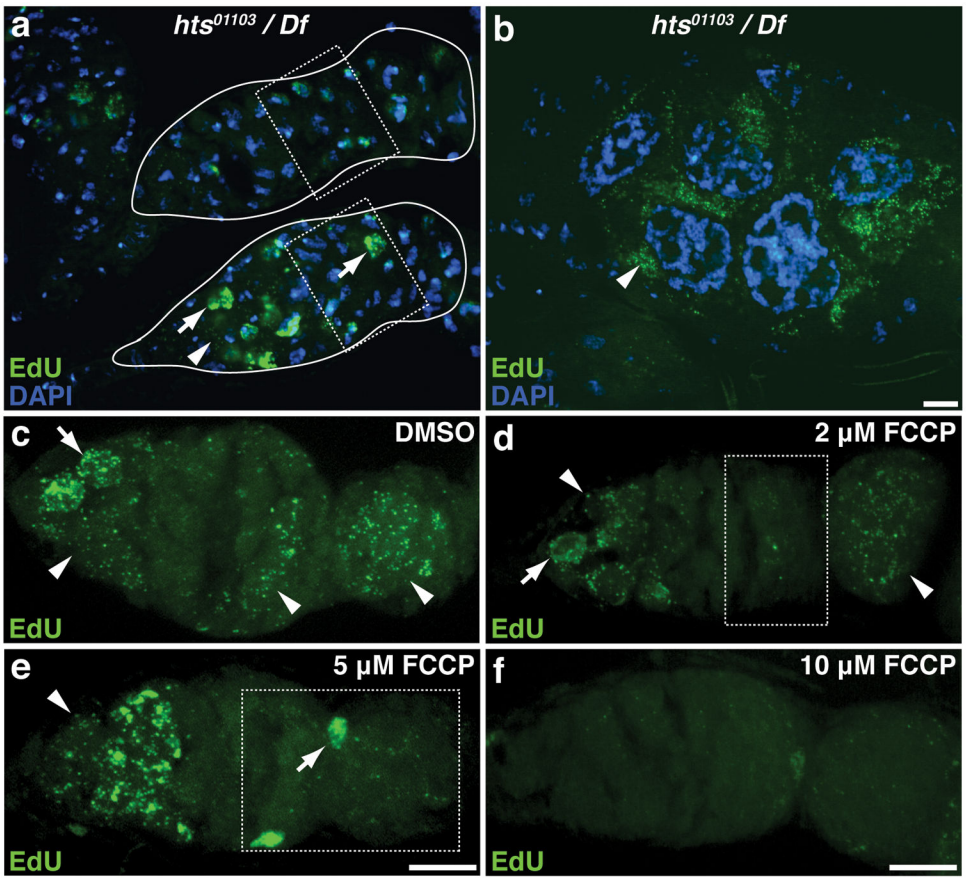

Figure 2.

The fusome and mitochondria activity are essential for mtDNA replication in region 2B cysts. (**a**) A representative image from > 10 repetitions showing EdU incorporation into nuclear DNA, labeled by DAPI, but not mtDNA, in *hts* (*hts*^{01103}*/Df (2R)BSC26*) mutant germaria. Boxes, region 2B. (**b**) Loss of Hts protein does not perturb mtDNA replication in post-germarium egg chambers. (**c-f**) Z-stack projections of wt germaria subjected to DMSO (**c**), or the mitochondria uncoupler FCCP at concentrations of 2 μM (**d**), 5 μM (**e**) and 10 μM (**f**) during incorporation of EdU (green). Note the lack of EdU puncta in region 2B with 2 μM FCCP and in both region 2B and 3 with 5 μM FCCP (boxes). Arrow, nucleus; Arrowheads, mtDNA; Scale bars, 10 μm.

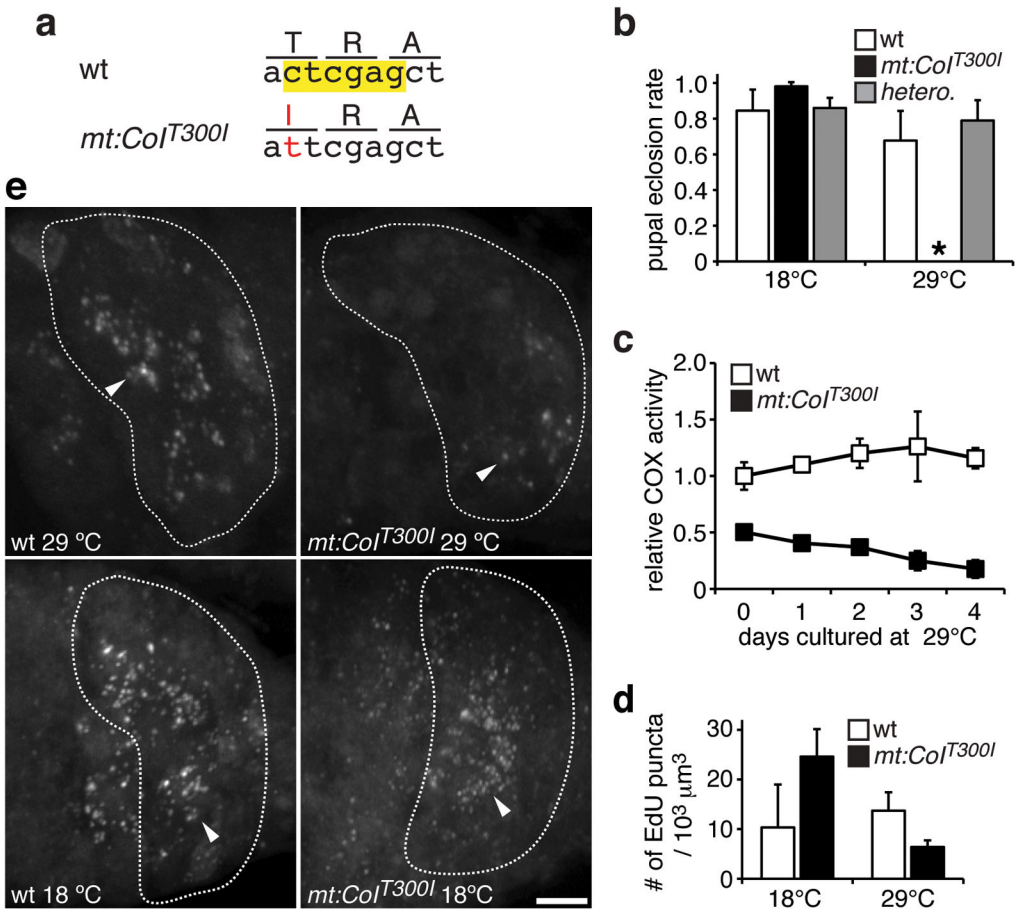

Figure 3.

mtDNA replication is reduced in 16-cell cysts of homoplasmic *mt:CoI*^{T300I} germaria at 29°C. (**a**) Mutated nucleotide and amino acid residue (red) in *mt:CoI*^{T300I}, including XhoI site (yellow). (**b**) Eclosion rate (mean±s.d., from total >50 animals in 5 groups) of wt, homoplasmic and heteroplasmic *mt:CoI*^{T300I} animals at 18°C and 29°C. * indicates zero *mt:CoI*^{T300I} eclosed at 29°C. (**c**) COX activities of wt and *mt:CoI*^{T300I} flies were examined on consecutive days shifting to 29°C. Data represent 3 biological replicates. Values were normalized with average COX activities of wt at 29°C 0 day and shown as mean±s.d. (**d**) Quantification of EdU puncta (mean±s.d.) in posterior cyst of region 2B at 18°C (wt n= 7, *mt:CoI*^{T300I} n= 7) or 29°C (wt n= 5, *mt:CoI*^{T300I} n= 8). The number of EdU puncta in *mt:CoI*^{T300I} at 29°C is significantly less than that at 18°C or wild type at 29°C ($P< 0.0005$). (**e**) Z-stack projections showing EdU incorporation in region 2B of germaria from wt and *mt:CoI*^{T300I} at 18°C and 29°C. Arrowheads, mtDNA. Scale bar, 5 µm.

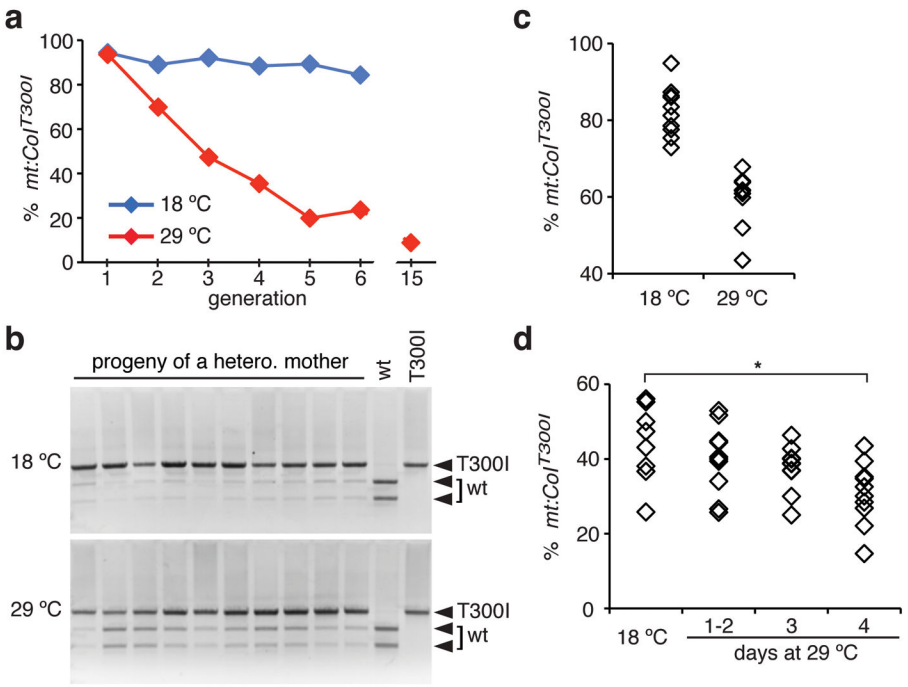

Figure 4.

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

Table 1

Inhibition of mtDNA replication by FCCP. Relative levels of mtDNA replication in the presence of different concentrations of FCCP at indicated regions of the germarium. # of EdU puncta was normalized to control (DMSO) in the same region. Value, mean±s.d. (n=6).

FCCP (μM)	1	2B	3
2	86±23%	25±7%**	61±23%*
5	107±35%	36±22%**	39±14%**
10	15±5%	17±13%**	19±14%**

* $P < 0.05$,

** $P < 0.001$ for each data point compared to respective control.