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Transmission of mitochondrial mutations and action of purifying selection in *Drosophila*

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Summary

It is not known how selection affects mutations on the multicopied mitochondrial genome [1–11]. We transferred cytoplasm between *D. melanogaster* embryos carrying mitochondrial mutations to create heteroplasmic lines transmitting two mitochondrial genotypes. Increased temperature imposed selection against a temperature sensitive mutation in cytochrome oxidase, driving declines in the abundance of the mutant genome over successive generations. Selection did not influence the health or fertility of the flies, but acted during mid oogenesis to influence competition between the genomes. While mitochondria might incur an advantage by selective localization, survival or proliferation, timing and insensitivity to *parkin* mutation suggest that preferential proliferation underlies selection. Selection drove complete replacement of the temperature sensitive mitochondrial genome by a wild type genome, but selection also stabilized multigenerational transmission of two genomes carrying complementing detrimental mutations. While so balanced, stably transmitted detrimental mutations have no phenotype but their segregation could contribute to disease phenotypes and somatic aging.

The proteins encoded by the mitochondrial (mt) genome, while key to oxidative metabolism, do not have obvious roles in replication or transmission of mtDNA. Conventional selection, by eliminating organisms with deficits in oxidative metabolism, could maintain function of these genes. Alternatively, as suggested by biased transmission of mutant mt genomes[2–4], a competition for transmission to the next generation could select among the mt genomes within the organism[2–4]. However, competition could only provide a purifying selection, if the function of mtDNA-encoded genes promotes replication or transmission.

Here, using a rigorous genetic system, we show that selection for function acting within the organism influences the transmission of genomes, and we demonstrate unexpected features of this selection.

To examine selection for mtDNA genotypes, we created heteroplasmic lines of *Drosophila melanogaster* carrying differently marked mt genomes. We combined genotypes by

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injecting poleplasm from one embryo into another[12]. Each input genotype (Figure 1A and Supplementary Table 1) was a homoplasmic mutant derived from a common parental mt genome by selecting for resistance to cutting by mitochondrially-targeted restriction enzymes[13]. Two mutant genotypes are particularly important here: *mt:CoI*^{T300I} and *mt:ND2*^{del1}. Adult flies homoplasmic for *mt:CoI*^{T300I} are viable and healthy at 25°C but die after 4 days at 29°C. This temperature-sensitive (ts) allele allowed us to manipulate selection. A nine base pair deletion *mt:ND2*^{del1} gave us a "negative marker genome" for sensitive, quantitative and selective PCR assays used to track the wild-type (*wt*) *ND2* allele in heteroplasmic lines[14]. Young flies homoplasmic for *mt:ND2*^{del1} are healthy at 25°C, but begin to display mild phenotypes that progressively worsen with age (J.L. Burman, L.S. Itsara; E.B. Kayser; W. Suthammarak; P.G. Morgan; M.M. Sedensky; L.J. Pallanck, in preparation), and they exhibit stronger phenotypes at higher temperatures (Supplementary Table 1).

Seven heteroplasmic combinations were generated in this study (Supplementary Table 2). Some lines, such as those heteroplasmic for the *mt:ND2*^{del1} and the ts mutation (*mt:CoI*^{T300I}) at permissive temperature, transmitted both their genomes without obvious bias for several generations (Figure 1B). Stable heteroplasmy might lead to complementation that would prevent organismal selection of mutant alleles. Indeed, flies heteroplasmic for *mt:CoI*^{T300I} and *mt:ND2*^{del1} were healthy at 29°C, even when the ts genome was at high abundance (~95%). Thus, low abundance *mt:ND2*^{del1} genomes rescued the ts phenotype of *mt:CoI*^{T300I}. Reciprocally, a climbing deficit of *mt:ND2*^{de1l} homoplasmic flies grown at 29°C was absent in the heteroplasmic flies (Supplementary Table 1B). Thus, complementation masks both mutant phenotypes. This complementation could be mediated by co-residence of different mt genomes in mitochondria, or by dispersal of gene products from one mitochondrion to another by fission and fusion. This efficient complementation could suppress elimination of mutant alleles by natural selection.

As progeny randomly sample the mother's pool of mt genomes, abundance will fluctuate, and occasionally, progeny lacking one of the two genomes will be produced, thereby exposing the remaining genome to organismal selection as proposed by the bottleneck model. To quantify homoplasmic offspring from *mt:CoI*^{T300I}/*mt:ND2*^{del1} heteroplasmic mothers, we screened individual progeny for loss of *mt:CoI*^{T300I} by PCR. We only detected such loss when mothers had a low level of the *mt:CoI*^{T300I} genome (e.g. 1.29% in Table 1), and still the rare genome was transmitted to most progeny (Figure 2). Other heteroplasmic lines behaved similarly (Table 1 and Figure 2). When eggs from single heteroplasmic mothers were collected over successive days, the progeny showed progressively more segregation (Supplementary Figure 1): this is expected because additional stem cell divisions are required for later eggs and each division provides another opportunity for chance segregation of a homoplasmic cell. A quantitative analysis of our findings (Supplementary Figure 1 and Supplementary Table 3), agrees closely with a study of segregation of homoplasmic progeny from a naturally heteroplasmic strain of *Drosophila mauritiana*[15,16]. Of relevance here, the stability of heteroplasmy and efficiency of complementation will protect mutations from organismal selection for multiple generations.

We next examined the influence of high temperature on lines carrying a high abundance of the ts genome paired with a *wt* mt genome or with the *mt:ND2*^{del1} genome. Despite complete complementation of ts phenotypes in both heteroplasmic lines, the abundance of the ts genome declined from one generation to the next at 29°C (Figure 3A). The amount of the decline was ~20% when the ts genome was abundant (~90%) and this increment was reduced as abundance declined (Figure 3B). This progressive disappearance appears first order, as if the probability of passing on the ts genome is consistently lower than the probability of passing on the *mt:ND2*^{del1} or *wt* genome.

In three independent lines heteroplasmic for ts and *wt* genomes, the abundance of the ts genome declined to a few percent after 10 generations. By generation 18, the ts genome was no longer detectable in four cultures that we followed (Figure 3A and Supplementary Figure 2A). Thus, when the *mt:CoI*^{T300I} genome was paired with *wt* genomes, high temperature drove a gradual but relentless decline in abundance of the ts genome until it was eliminated.

Lines in which the ts genome was paired with *mt:ND2*^{del1} also showed an early decline in the abundance of the ts genome. However, the decline did not continue to zero but asymptotically approached ~8% (Figure 3A). The ts genome persisted even after 50 generations of counter selection. If persistence represented the absence of selection, random excursions should lead to large variation in the ratios of the two genomes in individual flies. However, analysis of individual flies in two independent lines after 32 generations of selection showed limited variation (Figure 3C), suggesting that the observed balance of the two mutant mt genomes (means of 6.0% and 8.7%) is favored.

In contrast, when we followed lines heteroplasmic for *mt:ND2*^{del1} and a double-mutant genome, *mt:ND2*^{del1}+*mt:CoI*^{T300I}, we found that two of four lines examined lost the ts double-mutant genome after 18 generations of selection (Supplementary Figure 2B), suggesting that in *mt:CoI*^{T300I}/*mt:ND2*^{del1} lines, persistence of the ts genome relied on its ability to provide a *wt ND2* gene. We conclude that neither genome is eliminated when they make complementary contributions to function. Thus, balancing selection for two different functions can stabilize transmission of detrimental genomes.

The balancing selection implies that *mt:ND2*^{del1} does not have full *wt* function. Indeed, a line heteroplasmic for *wt* and *mt:ND2*^{del1} showed progressive decline of the *mt:ND2*^{del1} genome over generations (Supplementary Figure 3A). In accord with its mild phenotype, selection against this genome was weaker than for the ts genome. Selection against the *mt:ND2*^{del1} genome was similar when paired with *mt:CoI*^{R301Q} (Supplementary Figure 3B). However, in another pairing of mild and severe alleles, *mt:ND2*^{del1} and *mt:CoI*^{R301S}, we detected no segregation bias (Figure 1B). We conclude that the rate of selection did not invariably parallel affects on fitness, and note that a severe discordance might allow a detrimental mutation to escape purifying selection.

The selection that we have observed differs from classical organismal selection as neither the health nor the fertility of flies heteroplasmic for the ts genome was compromised at 29°C (Supplementary Figure 4A). Furthermore, a decreased abundance of the ts genome was seen in eggs prior to any opportunity for selection to act on progeny (e.g. Figure 4C). We

conclude that the observed selection involves competition between the mt genomes within the organism, and that, unlike classical selection, this selection places no burden on organismal survival or propagation.

Competition between mt genomes could occur throughout the life of fly, or be limited to a particular stage. When heteroplasmic flies were shifted to restrictive temperature at different stages of development, selection was evident in the eggs that they laid even when the shift was delayed until the completion of the larval stages (Figure 4B). Since the germline initiates oogenesis at the transition from larval to pupal stages[17], this finding suggested that the selection occurs during oogenesis (Figure 4A). Note that selection in the soma was not examined and it may be influenced by independent factors.

To further define the timing of germline selection, we shifted young adult females from 22°C to 29°C, collected their eggs on successive days, and assessed them for the relative abundance of ts genome. The abundance of the ts genome fell over time with the major decline occurring at about 6 days after the shift (Figure 4C), after which the decline was indistinguishable to that seen over a full generation (Figure 3B). As oogenesis at 29°C takes about six days, the temperature-sensitive period appears to start at the beginning of oogenesis and perhaps in the stem cell divisions that support continued oogenesis. If selection occurred during stem cell divisions, selection should increase with the number of divisions. However, no increase in selection was detected over time (Supplementary Figure 4B). We conclude that the selection does not occur in dividing germline stem cells.

An ambiguity compromises interpretation of the temperature upshift experiments. If activity of cytochrome oxidase, the product of *CoI*, persists for some time after the temperature shift, we would not observe immediate selection. This caveat is germane because homoplasmic *mt:CoI*^{T300I} flies shifted to 29°C live for 4 days and only then die, and COX activity in the ts mutant declines gradually at 29°C (cross reference). A double shift experimental paradigm solves this problem. When we shifted late larvae to 29°C and allowed them to mature to adults, their early progeny exhibited selection (Figure 4B). We downshifted these developing flies at different times and assessed selection in their eggs. Because the downshift cannot reverse selection that has already occurred, we can be sure that selection has not yet occurred at the time of downshifts that rescue selection. A transition from no-selection to selection occurred as the time of downshift was changed from day 2 to 4 with about 50% selection on day 3 (Figure 4D). The transition occurred within two days, and at least 50% of the ultimate level of selection had yet to occur at day 3. Thus, a large proportion of the selection occurs in the late germarium or later (Figure 4A).

What could be the basis of competition among mt genomes within the organism? “Winner cells” with a preponderance of good mitochondria might have a proliferative advantage. However, there is no cell division in oogenesis, when selection occurs. Alternatively, oocytes with poor mitochondria might be eliminated, but we see no atrophy of egg chambers (data not shown). Apparently, the mt genomes in the egg chamber compete to contribute to the oocyte. Three categories of mechanism could give mitochondria a transmission advantage: selective localization to germplasm, resistance to an elimination mechanism, or preferential proliferation. Our results are not easily consistent with the first model. Selective

localization to the germ cells predicts selection in the subsequent generation, while we find immediate enrichment of functional genomes in the entire egg. Another model suggests that selective transport of mitochondria from future nurse cells can enrich functional mitochondria in the oocyte as the egg chamber forms in the early germarium[18]. However, our data show that selection occurs after completion of this transport. To examine the second type of model, we tested a popular model in which Parkin promotes culling of mutant mitochondria. Selection was still observed after Parkin knockdown by RNAi or mutation (Figure 4E). Thus, Parkin is not required for selection.

Oogenesis is a period of tremendous growth in the population of mitochondria. A *Drosophila* egg is 100,000 times the size of a normal cell and it has 10 million mt genomes (data not shown). An accompanying paper shows that mtDNA replication is coupled to mitochondrial fitness during the germarium stage (cross reference). We therefore propose that, in the environment of the egg chamber, the genomes in a heteroplasmic line that support more robust oxidative phosphorylation have a small replicative advantage that is amplified during exponential proliferation so as to increase their final contribution to the genomes populating the oocyte.

In conclusion, in addition to showing purifying selection by competition among mt genomes, we have shown an unexpected property of the competition that can perpetuate mutant mt genomes. Selection can stabilize propagation of two defective genomes that complement. In wild populations, such a balancing selection could maintain a mix of mt genomes that provides a reservoir of diversity, thereby promoting an evolutionary plasticity credited to the mt genome[19]. Also, if metabolic demands in different tissues are best supported by distinct mt genotypes, a sustained diversity of genotypes might improve fitness.

Online methods

Fly stocks

The homoplasmic stocks used in this study include the following mutant alleles: *mt:ND2*^{del1}, *mt:ND2*^{ins}, *mt:CoI*^{R301Q}, *mt:CoI*^{sil}, *mt:CoI*^{R301S}, *mt:CoI*^{T300I}, and the two double mutants: *mt:ND2*^{ins}*+mt:CoI*^{sil} and *mt:ND*^{del1}*+mt:CoI*^{T300I}. *w1118* was used a *wt* control for all the experiments. Other strains used included parkin RNAi (Bloomington stock center #38333), *park*^{1} (Bloomington stock center #34747) and *dpk*Δ*21* (obtained from Graeme Mardon) mutants. The stocks were cultured at 18–25°C on standard fly medium.

Generation of heteroplasmic flies

The transfer of mitochondria was carried out by embryo-to-embryo microinjection of poleplasm and the method was modified from[12]. Donor and recipient embryos were collected for 20 min and the chorion was removed by incubating 2 min with 1:1 mixture of household bleach and water followed by thorough washes with water. The embryos were then aligned and affixed to a coverslip with glue made from Scotch 3M double-stick tape dissolved in heptane. Subsequently, both donor and recipient embryos were dehydrated for 8–9 min and covered in halocarbon oil. A portion of the donor poleplasm was sucked out (by inserting the needle from the anterior and pushing it to the posterior end) and transferred

into the posterior end of the recipient embryos. The injected recipient embryos were kept in a humidified chamber at 18°C and hatched larvae were transferred to vials with yeast paste in the next two days and incubated at 22°C until eclosion. Lines were established from the females obtained from the injected embryos, which were systematically mated to males with the recipient mtDNA genotype to eliminate the possibility of paternal leakage. For each of these females, 10–30 F1 females were isolated to establish sublines. Sometimes, females were taken from further generations to found late generation sublines (Supplementary Figure 6). For experiments requiring particularly high abundance or low abundance heteroplasmic lines, we established numerous sublines and among these identified those with the desired distribution of donor and recipient genomes. When not specified, each generation was derived from at least 50 individuals belonging to the previous generation. Individual heteroplasmic flies were named by the mtDNA genotype of the donor followed by that of the recipient: in *mt:CoI*^{R301Q}/*mt:ND2*^{del1} flies, the poleplasm of *mt:CoI*^{R301Q} was introduced into homoplasmic *mt:ND2*^{del1} embryos. A list of heteroplasmic lines generated for this study can be found in Supplementary Table 2.

DNA isolation

Genomic DNA was extracted from adults and eggs as follows. Flies, individuals or groups, were mechanically homogenized with a plastic pestle in 100 µl (or 500 µl) homogenization buffer (100 mM Tris-HCl [pH 8.8], 0.5 mM EDTA, 1% SDS). The homogenate was incubated at 65°C for 30 min, followed by addition of potassium acetate (to 1 M) and incubation on ice (30 min) to precipitate protein and SDS. Subsequently, the homogenate was centrifuged at 20,000 g for 15 min at 4°C. DNA was recovered from the supernatant by adding 0.5 volumes of isopropanol and centrifuging at 20,000 g for 5 min at room temperature. The resultant pellet was washed with 70% ethanol and suspended in 100 µl of ddH_2O per fly. Mitochondrial genotype frequencies were measured in individual founding females, their F1 and further generations via qPCR. When populations were analyzed, we extracted DNA from groups of 30 to 50 individuals.

qPCR Parameters

For all qPCR assays, SensiFast SYBR Green PCR Master Mix (Bioline 98020) was used in 20 µl reactions with 400 nM of each primer. To measure the total mtDNA copy number of heteroplasmic flies, qPCR of a 52 bp region (mt702–753) present in all mtDNA genotypes were performed (primer mt702F and mt753R, Supplementary Table 4). To measure copy number of genomes with *wt* (or *mt:ND2*^{ins}) allele of *mt:ND2* in the presence of deleted genomes, qPCR of the 51 bp *wt mt:ND2* region (or *mt:ND2*^{ins}) was performed (mt774F, mt824R (*wt mt:ND2*) and mt821R (*mt:ND2*^{ins}), Supplementary Table 4). Standard curves were constructed using a series of 10-fold dilutions (5×10^6–5×10^2) of the linearized plasmid containing both the common region and *wt mt:ND2* (or *mt:ND2*^{ins}) region. The efficiency of the 2 primer sets was normalized each time by comparing total mtDNA copy number estimated for the same *wt* DNA sample. qPCR was performed with the following reaction conditions: 95°C for10 min, 40 cycles of 95°C 30 s and 48°C 30 s. For each 20 µl qPCR reaction, 1% of a fly's total genomic DNA was used as template. The percentage of *wt mt:ND2* mtDNA was calculated by dividing *wt mt:ND2* mtDNA copy number by the total mtDNA copy number. The Ct values used ranged from 14 to 34.

Assessing fluctuations in transmission to progeny

A number of heteroplasmic virgins were picked and individually crossed (independent vials) to *mt:ND2*^{del1} males on day 1. The flies (males and single female) of each vial were transferred to new vials every 2–3 days for 11 days at 25°C (when donor genotype is *wt* or *mt:ND2*^{ins}+*mt:CoI*^{sil}) or 14.5 days at 22°C (when donor genotype is *mt:CoI*^{T300I}). Afterwards, individual mothers were sacrificed to estimate the percentage of donor mtDNA by qPCR. For mothers likely to give numerous homoplasmic progeny (i.e. progeny completely lacked the less abundant genome) —those with 1.0–3.0% of donor mtDNA—we analyzed individual progeny by qPCR to establish proportion of donor to total mtDNA. All progeny with less than 0.25% of donor mtDNA (i.e. Ct valued more than 34) based on qPCR measurement were then analyzed by PCR reaction (using primer set 2) and gel electrophoresis. The specificity of detection of properly sized product, rather than dye binding, gives the standard PCR a lower background than the qPCR. Using 30 cycles – this standard PCR gave a positive signal for all samples containing more than 0.012% *wt mt:ND2* (or *mt:ND2*^{ins}) genomes. Since the estimates of the number of segregating units were well below 1,000 (160 here and 239–512 predicted by[13,16]), any progeny that gets a *wt* genome should carry this genome at a level higher than 0.1%. Our PCR could detect the presence of *wt* genomes at levels almost 10-fold lower than 0.1% and for this reason we were confident that the absence of signal defines homoplasmic progeny. The PCR confirmed the presence of the donor mtDNA in some of the progeny and identified flies that lacked donor mtDNA among those having a background signal in qPCR. The calculation of the mean, variance and N (the empirical number of maternal genomes sampled, see below), was based on measurements taken from individual offspring developing from eggs laid in the same vial.

In total, three single mothers and their progeny were used for the study of transmission shown in Table 1. The particular *mt:CoI*^{T300I}/*mt:ND2*^{del1} mother (mother 1 in Table 1) gave rise to 329 adult progeny during her 14.5 day life at 22°C; mtDNA of 316 individuals were extracted and their heteroplasmic levels were measured individually (Supplementary Figure 1). For the other two lines (Table 1, donor mtDNA as *mt:ND2*^{del1} + *mt:CoI*^{sil} or *wt*), the mothers were transferred to a new vial every 3–4 days at 25°C and the study was restricted to the earliest descendants in the first vial (Supplementary Table 5).

To characterize the statistical fluctuations in mt genome transmission we assumed that the sampling of the mothers mt genomes by progeny can be approximated by a model in which progeny samples the maternal pool in a single step. Further, we assumed that if the heteroplasmic mother has two mt genomes, A and B, the probability of selecting the A genome will be directly determined by its abundance so that p_A = [A]/([A] +[B]) and that p_B = 1−p_A. Accordingly, if each progeny selects N heritable units (these might be genomes, homogenous nucleoids or mitochondria carrying genomes of a single type), the probability, P_0, that progeny lack genome A entirely is given by

$$P_0=(1-p_A)^N \text{ and by rearrangement } N=-\ln(P_0)/p_A \quad \text{Formula (1)}$$

Similarly, N can be calculated from other features of the distribution of abundances of genomes among the progeny. Notably the variance (V) is given by

$$V = p_A(1-p_A)/N \text{ and by rearrangement } N = -p_A(1-p_A)/V. \quad \text{Formula (2)}$$

These two methods of estimating N give somewhat different results because the real data does not form a perfect Poison distribution. N was calculated from both P_0 and V according to (1) and (2) and examples of the values obtained by the two methods are compared in Supplementary Table 3.

Previous analyses of the statistical distribution of mt genomes to progeny have usually made an effort to develop a more realistic model in which it is assumed that every cell divison in the lineage producing an oocyte is a selection event and that the final outcome is due to a sequence of such steps. In this case, the measured result (transmission to progeny) is dependent on the number of divisions (k*n*), and the number of segregating units at each of the cell divisions. It is assumed that the number of segregating units per cell division (N_{cd}) is unchanging. This analysis is usually based on variance as follows.

$$Vn = p_A(1-p_A)[1-(1-1/N_{cd})^{kn}]^{22} \quad \text{Formula (3)}$$

V*n* is the variance at the nth generation. If the number of divisions (k*n*) can be independently determined then N_{cd} can be calculated. Since the number of division can only be estimated and constancy of segregation in each division is a dubious simplification, we have avoided this modeling in the presentation given in the text. However, since we know a great deal about the germ line lineage in *Drosophila*, we can make fairly accurate predictions of the number of divisions (k*n*) and thereby generate what should be a pretty reasonable estimate of N_{cd} that can provide a basis for comparison to the literature. For these reasons, we provide this assessment in the supplement. Seven to nine cell divisions separate the egg cytoplasm from the first cohort of progeny[13,15]. The early embryonic divisions of this lineage are expected to make little contribution to variance because they involve large cells that ought have an unusually large number of mt genomes. Thus, we use the lower estimate of 7 for the number of divisions and calculate N_{cd} for progeny from eggs laid during the first day following eclosion. As described in the text the sampling of maternal mt genomes by progeny declines as the mother ages (Supplementary Figure 1), and this effect is thought to be due to extension of the germline lineage as the germline stem cells divide to maintain the egg production in the adult. Assuming that N_{cd} remains constant, we can calculate the number of stem cell divisions needed to account for the observed change (Supplementary Table 3). The calculated number of additional division is roughly what is expected.

Monitoring the proportions of marked mt genomes over generations

To establish a heteroplasmic line, a single heteroplasmic virgin mother (founding mother, G0) was crossed to *mt:ND2*^{del1} males for 2 days at 25°C (when *mt:ND2*^{ins} + *mt:CoI*^{sil}, *wt*, *mt:CoI*^{R301S} or *mt:CoI*^{R301Q} was introduced to *mt:ND2*^{del1} flies as donor mt genotypes).

The mother was then sacrificed for total genomic DNA extraction and the proportion of donor mt genome was estimated by qPCR as described above. The lines were maintained by selfing at 25°C for multiple generations and a group of 30–50 flies from each generation was subjected to DNA extraction followed by qPCR in order to estimate the proportions of donor mt genomes for that generation (Supplementary Figure 6).

To establish *mt:CoI*^{T300I}/*mt:ND2*^{del1}, *wt*/*mt:CoI*^{T300I} + *mt:ND2*^{del1} and *mt:ND2*^{del1}/*mt:CoI*^{T300I} + *mt:ND2*^{del1} heteroplasmic lines, individual virgin females (G0) were crossed to *mt:ND2*^{del1} males for 2 days at 22°C. The progeny of the mothers (G1) were then either maintained at 22°C or shifted to 29°C and maintained at 29°C for multiple generations. The proportions of donor mt genomes were measured in a similar manner as other types of heteroplasmic lines.

To assay the degree of selection over a single generation, we measured the reduction in the proportion of defective genomes in progeny (adult flies) in comparison to their mother. We examined the progeny of single virgin females (born at 29°C) of *mt:CoI*^{T300I}/*mt:ND2*^{del1} and *wt*/*mt:CoI*^{T300I} + *mt:ND2*^{del1} heteroplasmic lines that were crossed to *mt:ND2*^{del1} males for 2 days at 29°C before they were sacrificed to estimate proportions of defective genomes. Their progeny were maintained at 29°C until they reached the young adulthood and sacrificed to measure the proportions of ts genomes.

Temperature shift experiments

Individual females (*mt:CoI*^{T300I}/*mt:ND2*^{del1}, raised at 22°C) were taken from sublines with a high load of *mt:CoI*^{T300I}. They were mated with *mt:ND2*^{del1} males at 22°C in individual vials and then sacrificed to estimate the abundance of *mt:CoI*^{T300I}. We focused on female flies with relatively high abundance of *mt:CoI*^{T300I}. Eggs laid by each mother within a 24 h interval were collected and shifted to 29°C before the beginning of 1st instar larval stage, or as 3rd wandering larva, or as young adults. For all the conditions, egg collection was begun one day after eclosion and eggs laid in the next 1–2 days were collected to measure the proportions of *mt:CoI*^{T300I} genome. The 3rd wandering larva shift experiment was also performed in a *parkin* null line (park^{1}/dpkΔ21) and in females with knocked down Parkin activity in the germline (*nanos*-Gal4>*parkin* RNAi).

For the adult upshift experiment, newly eclosed females (*mt:CoI*^{T300I}/*mt:ND2*^{del1}, raised at 22°C) were mated with *mt:ND2*^{del1} males at 22°C in individual vials and shifted to 29°C. Eggs laid in 12 successive days (i.e. about 2 cycles of oogenesis) were collected to measure the proportions of *mt:CoI*^{T300I} genome.

For the downshift experiment, female flies (*mt:CoI*^{T300I}/*mt:ND2*^{del1}, raised at 22°C) were selected and mated with *mt:ND2*^{del1} males at 22°C in individual vials. Eggs laid within a 12–16 h interval were collected and shifted to 29°C as 3rd wandering larva. These developing flies were then downshifted to 22°C after different durations at 29°C (1–7 days). Eggs laid by these flies were collected one day after eclosion to measure the proportions of *mt:CoI*^{T300I} genome.

Germline stem cell competition assay

Individual young females (*mt:CoI*^{T300I}/*mt:ND2*^{del1}, raised at 29°C) were crossed to *mt:ND2*^{del1} males and their eggs were collected to measure the abundance of the *mt:CoI*^{T300I} genome for the successive 10 days (i.e. more than 1 cycle of oogenesis). The whole experiment was carried out at 29°C.

Quantitative analyses of mt genotypes based on restriction cleavage

In heteroplasmic lines (e.g. *wt*/*mt:CoI*^{T300I}+*mt:ND2*^{del1}) when the abundance of *mt:ND2*^{del1} genome was low, qPCR could no longer be used to confirm the presence of *mt:ND2*^{del1}. We therefore quantified the relative amount of genomes distinguished by mutations affecting restriction enzyme cleavage sites by comparing the ratios of diagnostic restriction fragments. Total mtDNA was collected from 30 adult flies of various heteroplasmic lines at generation 10 or generation 18 as described earlier. A mtDNA region (mt1579–2799) was amplified by PCR (30 cycles of 95°C 30 s, 51°C 30 s and 72°C 75 s). The PCR products were then digested completely with XhoI under the conditions recommended by the suppliers. The digested DNA was separated by gel electrophoresis, and the ratio of cut and uncut DNA was assessed by staining or, for higher sensitivity, by Southern blot.

For Southern blot analysis, digested DNA (which did not cut within the probe region) was separated on a 1.2% agarose gel by electrophoresis and transferred to Hybond N+ membrane by the capillary method. DNA transferred to the membrane was fixed by UV cross-linking (Stratalinker 120 mJ). The blot was hybridized with PCR-generated probes that did not span the cut site being assayed (mt1577–2365) labeled with DIG-11-dUTP using 0.35 mM DIG-11-dUTP, 1.65 mM dTTP and Bioline Velocity Taq DNA polymerase, following the manufacturer's instructions (Roche, Germany). Prehybridization and hybridization were carried out at 41°C overnight in DIG Easy Hyb buffer solution (Roche, Germany). The membrane was washed two or three times with 2×SSC+0.1% SDS at room temperature for 5 min and twice for 15 min with 0.1×SSC+0.1% SDS at 65°C. Hybridized membrane was visualized with NBT/BCIP following the manufacturer's instructions (Roche, Germany). mtDNA primers used to generate the DIG-labeled probe and amplify a region contains the restriction XhoI site are listed in Supplementary Table 4.

Climbing test

Climbing tests were carried out as described in[13]. For each test, thirty 2–3 day old flies were transferred to a plastic cylinder (22 cm long, 1.5 cm diameter) with a mark 10 cm line from bottom. After 1 h for acclimation, the flies were knocked down to the bottom by gently tapping the tubes. The time required for 50% of the flies to climb to the marked 10 cm line was recorded. Three trials were conducted for each group, and three groups were used for each genotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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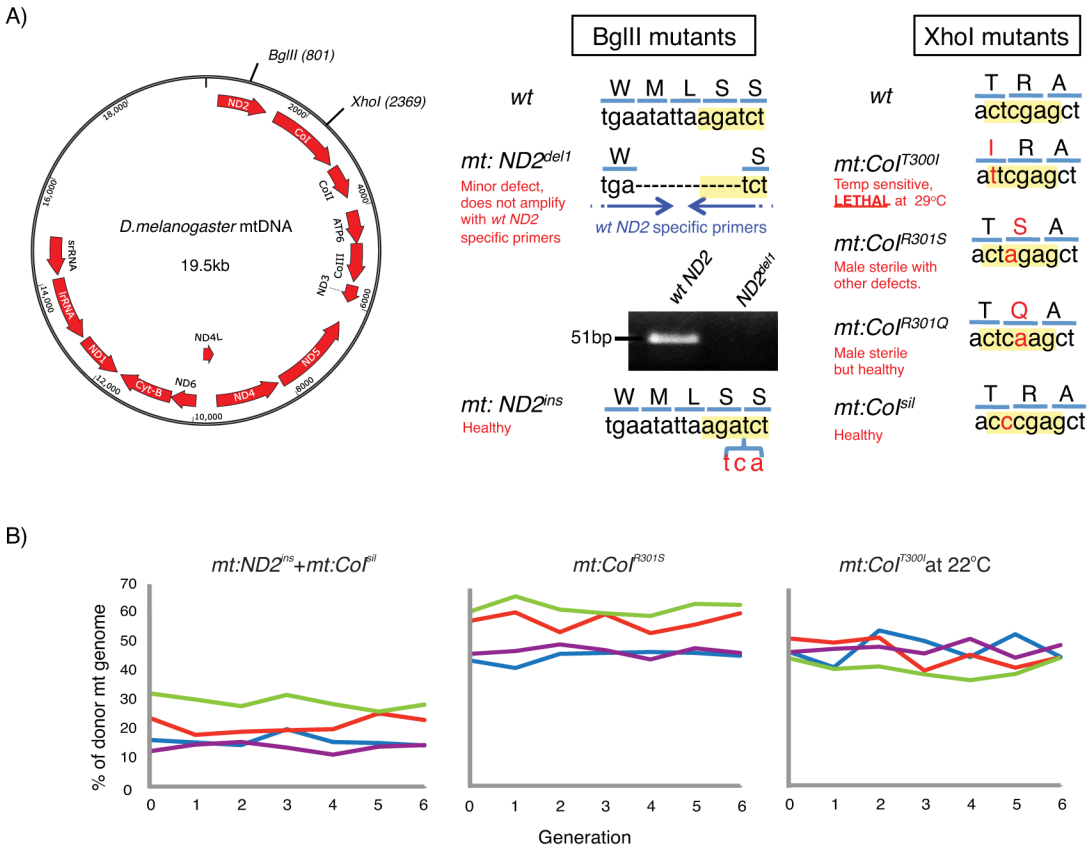

Figure 1.

Stable transmission of genetically marked mt genomes in the background of *mt:ND2*^{del1}. A) Mutants at the BglII and XhoI sites (yellow highlight) of mtDNA used in this study (see Supplementary Table 1 for more detailed phenotypes of these mutants)[13]. *D. melanogaster* mtDNA contains one BglII and one XhoI site in the coding regions of *ND2* and *CoI*, respectively; no PCR product was detected with the *wt mt:ND2* specific primers when *mt:ND2*^{del1} sequence was used as template. B) Stable transmission of heteroplasmy over multiple generations for the indicated three mtDNA genotypes in the background of *mt:ND2*^{del1}. Various heteroplasmic lines were established using *mt:ND2*^{del1} flies as recipient. qPCR defined the proportion of donor mt genotypes to total mtDNA in four independent lineages (colored lines). Growth was at 25°C, except for the lines with *mt:CoI*^{T300I} (which were tested at 22°C, the permissive temperature).

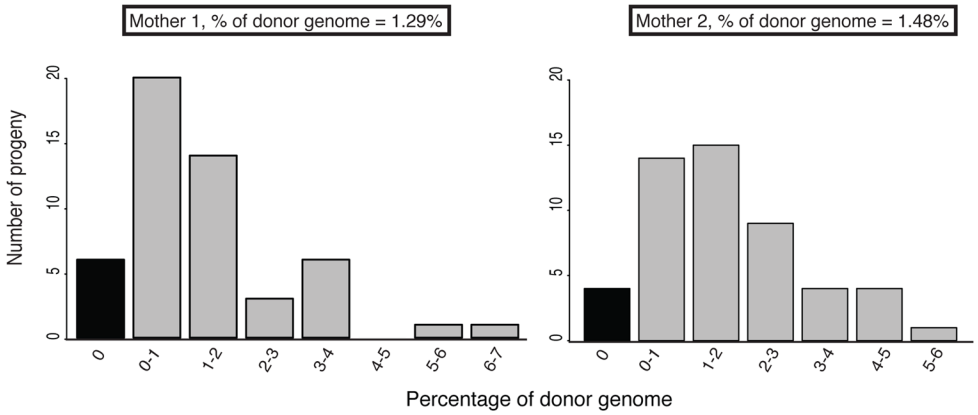

Figure 2.

Segregation of mitochondrial genotypes in progeny. Individual progeny from mothers with a preponderance of the *mt:ND2*^{del1} genotype (see mother 1 and 2 in Table 1) were assayed by qPCR for the abundance of a second genome (i.e. the donor genome). The histograms show the number of early progeny within different abundance intervals for the donor genome. The black bar indicates the number of progeny lacking PCR detectable donor genomes (homoplasmic). See Supplementary Figure 1 for more progeny collected from mother 1 on successive days.

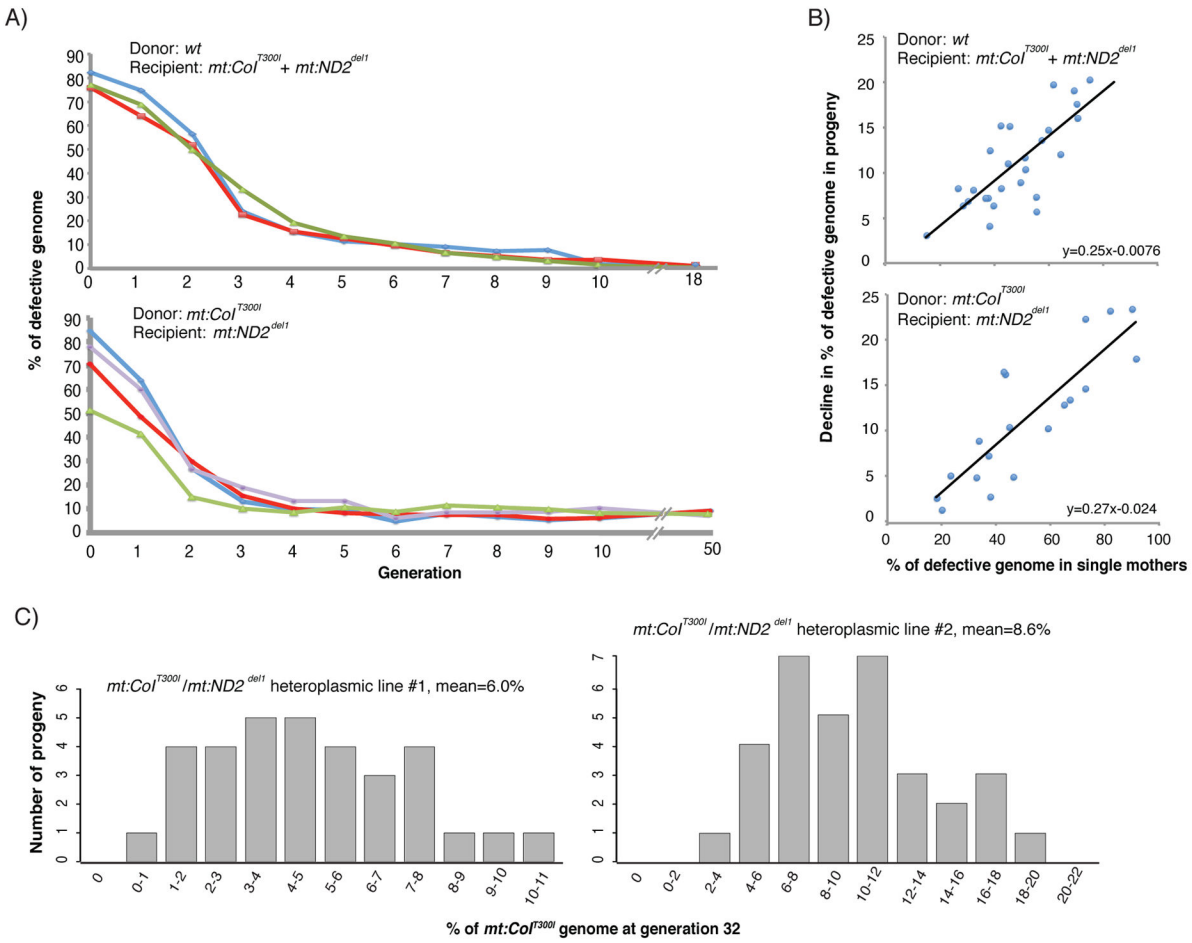

Figure 3.

Purifying selection against the ts genome. A) At the restrictive temperature, abundance of the ts genome declined over multiple generations when co-resident with either *wt* or *mt:ND2*^{del1} at 29°C. Several independent linages are shown (colors: see Supplementary Figure 5 for additional lines). B) Reduction in the abundance of the ts genome over one generation (adult to adult) at 29°C. Abundance in mothers is shown on the x axis and the decline in abundance in a group (30 individuals) of their progeny is shown on the y axis. C) The abundance distribution of the ts genome in individual flies (n=33) of two *mt:CoI*^{T300I}/ *mt:ND2*^{del} lines after 32 generations at 29°C.

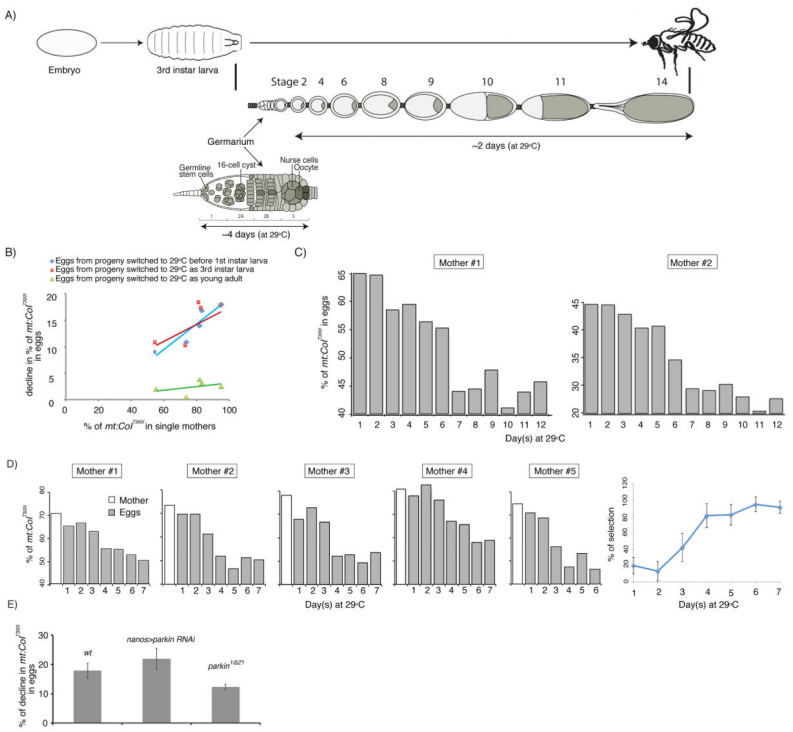

Figure 4.

Temperature shift of mothers followed by analysis of mt genome abundance in eggs reveals the timing of selection in the germline. A) Schematic drawing of an ovariole with the germarium at the anterior tip and egg chambers of increasing age (modified from[20,21]). A group of primordial germ cells differentiates directly into cystoblasts to initiate the first wave of oogenesis at the larval/pupal junction. Subsequently, cystoblasts generated by asymmetric division of germline stem cells follow the depicted progression to continue the production of oocytes. The duration of oogenesis (from the cystoblast to a mature egg) is ~6 days at 29°C. B) Measuring selection during oogenesis. Heteroplasmic mothers (*mt:CoI*^{T301I}/*mt:ND2*^{del1}) were shifted from 22°C to 29°C at the beginning of larval stages (blue diamonds), as 3rd instar wandering larva (red squares), or as newly eclosed adults (green triangles), and abundance of the ts genome in their eggs was measured by qPCR. C) Young adult mothers (*mt:CoI*^{T301I}/*mt:ND2*^{del1}) were shifted from 22°C to 29°C and the abundance of ts genomes was measured in their eggs collected on successive days. Data from two of the ten mothers used in the experiment are shown. D) The progeny from individual heteroplasmic mothers (*mt:CoI*^{T301I}/*mt:ND2*^{del1}) were shifted from 22°C to 29°C at the larval/pupal transition stages, held at 29°C for 1–7 days and then downshifted to 22°C. The abundance of the ts genome in the original mother and the eggs of her double shifted progeny was measured by qPCR. Results from the progeny of five mothers are shown here. The right panel shows the average level of selection after a series of days at 29°C. E) Selection against the ts genome still occurred in a line with reduced Parkin activity (*nanos*-Gal4 driven germline RNAi knockdown) and a *parkin* null mutation. For both lines, declines in the abundance of the ts genome were measured in five mothers and their eggs (the mothers were shifted from 22°C to 29°C at the larval/pupal transition stages). The

percentage of decline was calculated by dividing the absolute decline by the initial abundance the ts genome in the mothers. The error bars represent standard deviation.

Table 1

The frequency of homoplasmic progeny generated by three heteroplasmic mothers with a low abundance of donor mt genomes. The detection of homoplasmic progeny lacking the donor genome was assessed by PCR of individual progeny. These data are for early progeny resulting from egg laying during the first 3 days following eclosion. Growth was at 25°C, except for the lines with *mt:CoI*^{T300I} (which were tested at 22°C, the permissive temperature).

Mother	Donor mt genotye	% of donor mtDNA	# of early progeny	% of homoplasmy
1	*mt:CoI*^{T300I}	1.29	51	11.76
2	*mt:ND2i*^{ns}+*mt:CoI*^{sil}	1.48	51	7.84
3	*wt*	2.65	40	5.00