Mitigation of age-dependent accumulation of defective mitochondrial genomes

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Abstract

Unknown processes promote the accumulation of mitochondrial DNA (mtDNA) mutations during aging. Accumulation of defective mitochondrial genomes is thought to promote the progression of heteroplasmic mitochondrial diseases and degenerative changes with natural aging. We used a heteroplasmic Drosophila model to test 1) whether purifying selection acts to limit the abundance of deleterious mutations during development and aging, 2) whether quality control pathways contribute to purifying selection, 3) whether activation of quality control can mitigate accumulation of deleterious mutations, and 4) whether improved quality control improves health span. We show that purifying selection operates during development and growth but is ineffective during aging. Genetic manipulations suggest that a quality control process known to enforce purifying selection during oogenesis also suppresses accumulation of a deleterious mutation during growth and development. Flies with nuclear genotypes that enhance purifying selection sustained higher genome quality, retained more vigorous climbing activity, and lost fewer dopaminergic neurons. A pharmacological agent thought to enhance quality control produced similar benefits. Importantly, similar pharmacological treatment of aged mice reversed age-associated accumulation of a deleterious mtDNA mutation. Our findings reveal dynamic maintenance of mitochondrial genome fitness and reduction in the effectiveness of purifying selection during life. Importantly, we describe interventions that mitigate and even reverse age-associated genome degeneration in flies and in mice. Furthermore, mitigation of genome degeneration improved well-being in a Drosophila model of heteroplasmic mitochondrial disease.

mitochondria | mtDNA | heteroplasmy | aging | mutations

Unlike nuclear genotype, which is largely stable during one’s lifetime, when genetically distinct mitochondrial genomes co-reside (heteroplasmy), their relative proportions shift during growth, development, and aging. This shift is not random (1–7). Mutant mitochondrial DNA (mtDNA) variants accumulate during aging and in the progression of some mitochondrial diseases (1–5). Stereotyped changes in abundance of particular alleles in different tissues in human and mouse indicate that selective forces favor different mitochondrial genomes (3, 6, 7). However, despite the importance of mitochondrial function to health and well-being, we have limited understanding of the processes underlying the accumulation of mitochondrial mutations with age.

The nuclear genome encodes mechanisms of quality control that survey the function of mitochondria and eliminate or compromise the proliferation of defective mitochondria (8, 9). Two described mechanisms use PINK1, the product of a gene discovered as one of the causes of early-onset familial Parkinson’s disease, as a sensor of mitochondrial function. PINK1 accumulates on the surface of mitochondria having a reduced membrane potential, and its kinase activity in this location signals several downstream events (10–15). In one pathway defined largely in a cell culture model, PINK1 activates PARKIN, the product of another Parkinson’s disease gene, which then triggers elimination of compromised mitochondria by mitophagy (16). In a second pathway, acting in the Drosophila female germ line (17, 18), PINK1 acts in a PARKIN-independent pathway that targets a protein called Larp to inhibit its role in promoting biogenesis of the mitochondria (18) (SI Appendix, Fig. S2).

Since deleterious mtDNA mutations compromise electron transport of mitochondria, it seems that quality control would put genomes carrying such mutations at a disadvantage, creating a purifying selection that leads to their elimination. However, this outcome is far from certain. Various factors such as the sharing of gene products among mitochondria as a result of dynamic fission and fusion could mask the consequences of heteroplasmic mutations, shielding them from quality control. Indeed, a number of studies suggest a contrast between the germ line, which exhibits purifying selection, and adult somatic tissues, which often show accumulation of mutations. Studies in Drosophila show that purifying selection acting in the female germ line eliminates deleterious mutations in a few generations (18–21). Genetic dissection revealed that this purifying selection...
selection depends on the PINK1/LARP pathway of quality control (18). On the other hand, an elegant study that induced heteroplasmic deletions in the flight muscle of the adult fly detected no substantial indications of purifying selection unless additional stressors were introduced (22). Furthermore, a detailed study in Drosophila carrying a proofreading defective mitochondrial DNA polymerase (mutator line) showed that adult flies accumulate a spectrum of mutations biased toward deleterious mutations (23). Since deleterious mutations would potentially be removed by purifying selection, this finding argues either that it was not operating or that it was opposed by a stronger selection favoring deleterious mutations (23). Similarly, studies in mice suggest a discordance between germ line and soma. When mutations in the mitochondrial genome that were introduced in a mutator line were passed through subsequent generations in a wild-type background, there was selective elimination of deleterious mutations, a strong signal of purifying selection (7). In contrast, zygotically accumulated mutations in the mutant mouse exhibited high levels of deleterious mutations, suggesting a lack of purifying selection in the soma. Furthermore, Parkin mutant mice did not show a significant increase in mutations in adult wild-type or mutator mice, suggesting that Parkin-dependent quality control does not contribute to purifying selection (16). While these studies suggest major changes in the efficiency of purifying selection, other studies have suggested continued purifying selection in some circumstances. For example, adult human T cells of mitochondrial disease patients show exceptionally low heteroplasmy levels, suggesting that selection and to understand the nature of quality control in the soma during growth, development, and aging using an experimental model developed in Drosophila.

A previously described heteroplasmic line of Drosophila melanogaster, which carries a wild-type mitochondrial genome (Yak-mt) from another species, Drosophila yakuba, and a D. melanogaster genome (Mel-mt) crippled by a temperature-sensitive mutation in cytochrome oxidase subunit 1 (mt:ColT300I) (Fig. 1A and SI Appendix, Fig. S1). The Mel-mt+ genome has an intrinsic advantage in replication. At a permissive temperature, it gradually displaces the Yak-mt genome, resulting in the loss of the D. yakuba genome in a few generations (25). At 29°C, a temperature at which the ColT300I mutant cannot support viability (26), purifying selection counters the replicative advantage of the mutant Mel-mt+ genome, preventing it from taking over (25). qPCR gives a measure of the ratio of Yak-mt/total-mt. The difference in this ratio between permissive and restrictive temperatures allows us to assess the impact of a functional disparity on competition between the two genomes and provides a measure of purifying selection. Past work using this heteroplasmic line focused on changes in the relative abundance of the two genomes from one generation to the next and uncovered the action of purifying selection during oogenesis (18–21, 25, 27). It is notable that this selection depends on quality control, occurs by competition between mitochondrial within the oocyte, and does not involve selection for organismal fitness (18, 21). Changes in the ratio of Yak-mt/total-mt during the lifetime of the fly suggested that maintenance of this ratio is dynamic (25). Here, we examined changes in the relative abundance of the two genomes in the soma. Shifts in the ratio of Yak-mt/total-mt provide a measure of the effectiveness of purifying selection during the life of the fly and a means of assessing whether purifying selection can be genetically or pharmacologically modified. We found evidence that purifying selection is active in the soma during growth and development. The influence of mutations in quality control genes suggests that somatic mechanisms of quality control overlap those operating in the germ line. However, the effectiveness of purifying selection declines with age and has no obvious impact in tested tissues beyond 5 d after eclosion of adult flies. Importantly, we found that quality control can be stimulated in the adult by genetic alterations or by feeding kinetin and that such measures forestall mutational accumulation and aging phenotypes in Drosophila and can reverse mutational accumulation in aged mice.

**Results**

**Somatic Selection of Mitochondrial Genomes during Development and Aging.** To assess the action of selection in somatic tissues during development, we collected eggs from heteroplasmic flies at 29°C, allowed development to young adulthood at either 22°C or 29°C, and measured the ratios of mitochondrial genomes in whole flies (Fig. 1B). For analysis of events in the soma, we focused on males because high ovarian mtDNA levels result in a large germ line influence in females (25). At 22°C, the ratio of Yak-mt/total-mt declined dramatically from egg to adult, representing the outcome of competition when both genomes were functional. At 29°C, Yak-mt/total-mt was maintained at a higher level than in flies raised at 22°C. This difference reflects a response to functional disparity between the genomes and shows selection against the detrimental mutation. However, the Yak-mt/total-mt ratio in the 5-d adult males was lower than in freshly laid eggs, suggesting that this selection is weaker during growth to adulthood than it is in the female germ line (Fig. 1B).

To assess the influence of a functional disparity during early development, we examined larval tissues after development at 22 or 29°C. Again, the Yak-mt/total ratio declined at 22°C. The extent of the decline was different in the different tissues. Although the basis for this difference is not known, it might be attributed to tissue-specific influences on the replicative abilities of the two different genomes (3). However, regardless of the cause, the Yak-mt/total ratio at 22°C serves as a control for change induced when mt:Col is inactivated by increasing the temperature to 29°C. Total mtDNA levels were similar at both temperatures (SI Appendix, Fig. S1), suggesting that copy number is not responsive to the difference in mt:Col function. In contrast, the ratio of Yak-mt to total-mt was higher at 29°C in all tissues examined (Fig. 1C). We conclude that some form of purifying selection contributes to mitochondrial genome quality in diverse larval tissues during development to the late larval stage.

Despite early purifying selection in larval stages and maintenance of the ratio at 29°C, the Yak-mt/total-mt ratio in the central nervous system (CNS)/brain declined during continued development (Fig. 1D). Indeed, 5 d after eclosion from the pupal case (5-d adult), the ratio was nearly at the level seen in the brains of flies that were raised the entire time at 22°C (Fig. 1D). Measuring Yak-mt/total-mt every 2 h after eclosion revealed a rapid decline of the ratio (Fig. 1E). Thus, the transition to adulthood is accompanied by a rapid shift in the ratio of mitochondrial genomes that erases the benefits of earlier purifying selection in the brain. A decline in the effectiveness of purifying selection would precede its consequence on the Yak-mt/total-mt ratio. Thus, the rapidity of the posteclosion readjustment of Yak-mt/total-mt reveals a dynamic requirement for purifying selection.

Aging of adult flies from 5 to 20 d did not result in further decline of Yak-mt/total-mt in brain at either temperature (Fig. 1F). In contrast, the Yak-mt/total-mt fell further in the highly proliferative gut. However, it is notable that this decrease in the gut was the same at both temperatures. The temperature independence of Yak-mt/total-mt during aging indicates a lack of effective purifying selection against the detrimental mutation after 5 d of adulthood.
Nuclear Genes Modulate Purifying Selection of Mitochondrial Genomes in the Soma. Since PINK1 acts as a sensor in quality control pathways, we tested its influence on purifying selection in the soma. A loss-of-function mutation in the X chromosomal *Pink1* locus dramatically decreased *Yak-mt/total-mt* in 5-d adult male flies raised at 29°C (Fig. 2A). This decline was significantly rescued by an autosomal copy of wild-type *Pink1*. We conclude that *Pink1* contributes to somatic purifying selection in flies.

**Fig. 1.** Quality control modulates the ratio of heteroplasmic mitochondrial genomes during development. (A) Heteroplasmy for schematized *Yak-mt* and *Mel-mt* genomes was established by transferring cytoplasm of *D. yakuba* embryos into *D. melanogaster* embryos carrying the doubly mutant genome *mt:ND2del + mt:ColT300I* (*Mel-mt*). (B) The proportion of *Yak-mt* (*Yak-mt/total-mt*) following development from egg to adult shows action of quality control. Eggs (2-h collection at 29°C) were assayed (gray bar) or allowed to develop to 5 d after eclosion at 22°C (blue) or 29°C (amber). Adult females have a large contribution from oocyte mtDNA. (C) Quality control operates in multiple tissues with different effectiveness. The blue and amber bars (22°C or 29°C, respectively) show the proportion of *Yak-mt* in different tissues of late third instar larvae. (D) The impact of purifying selection declined with age in the CNS. (E) The decline in relative abundance of wild-type, indicated by the dashed regression lines (orange for 29°C and blue for 22°C), *Yak-mt* following eclosion was fast. (F) The signature of quality control is absent during maturation of adults. *Yak-mt/total-mt* ratios from gut and brain taken from 5-d (gray bars) or 20-d adults aged at 22°C or 29°C (blue and amber bars, respectively). Here and below, *P* < 0.05; **P** < 0.01; and ***P*** < 0.001 by one-way ANOVA/Tukey’s multiple comparison test. Data represent eight independent biological repeats, with each repeat being an average of ratios assessed in three samples of eggs or adults. For tissues, data represent tissues dissected from eight individuals. Error bars represent standard error. In E, slopes differ (*P* < 0.05) by linear regression. Cyto, cytoplasm; AED, after egg deposition; ns, not significant.
To assess quality control events downstream of Pink1, we reduced the gene dose of larp, a mediator of Pink1 quality control in the germline (SI Appendix, Fig. S2) (18). Although changes in gene dose usually produce subtle or no phenotype, we reasoned that the decline in the Yak-mt/total-mt ratio in brains during the transition from pupa to adulthood might provide an especially sensitive point to monitor the impact of quality control in somatic tissues. Heterozygosity for larp compromised purification selection so that the brains of newly eclosed flies had much reduced Yak-mt/total-mt (Fig. 2B). Additionally, a genome-wide screen showed that reduction in the dose of the gene tamas (tam), which encodes the mitochondrial DNA polymerase catalytic subunit (POLγA), enhanced elimination of mitochondrial genomes with deleterious mutations during oogenesis (27).

When we reduced the dose of tam during growth and development, a higher Yak-mt/total-mt persisted in brains, indicating stronger purifying selection (Fig. 2B and SI Appendix, Figs. S3–S5). These findings show parallels between germ line and somatic purifying selection and implicate a common quality control pathway in the processes.

Adult Yak-mt/total-mt heteroplasmic flies are lethargic and short-lived (about 20 d) at 29 °C. Since heteroplasmic Pink1 mutants had reduced mitochondrial genome quality, we examined the influence of Pink1 on the health of heteroplasmic flies. Pink1 mutant flies that are otherwise wild type are viable at 22 and 29 °C. In contrast, while Pink1 flies heteroplasmic for Yak-mt and Mel-mt survive at 22 °C, they die during the first few days of adulthood at 29 °C. This finding shows that PINK1 contributes to survival of heteroplasmic flies when function of Mel-mt is compromised. The death is likely the consequence of the much-reduced abundance of the functional mitochondrial genome (Fig. 2A).

We then examined the influence of reducing the dose of tam during aging. When raised at 22 °C when both genomes are functional, there was no detected difference in Yak-mt/total-mt between control and tam heterozygous as they aged to 20 d (SI Appendix, Fig. S6). However, when raised at 29 °C, the heterozygous (tamKO/+ ) flies sustained a higher level of Yak-mt/total-mt for 14 d into adulthood (Fig. 3A). The relative impact of tam dose at the two temperatures is in accord with previous findings indicating that tam dose acts to enhance purifying selection (27).

To assess possible benefits of increased Yak-mt/total-mt, we examined the consequence of reducing the dose of tam at 29 °C. The tam heterozygotes showed more vigorous climbing at least until 11 d after eclosion (Fig. 3B). Neuronal cell loss has been associated with age-associated degenerative conditions. To test for such an association, we tested the PPL1 cluster of dopaminergic (DA) neurons for cell loss in control and in tamKO heterozygous flies. In the absence of heteroplasmy, normal flies exhibit an invariant number of PPL1 DA neurons throughout adult life (28). In contrast, DA neuron number dropped between 5 and 20 d of adulthood in the heteroplasmic flies. This decline was not detected in the tamKO heteroplasmic line (Fig. 3D). The phenotypic benefits associated with maintenance of a higher proportion of functional genomes suggests that mitochondrial genome quality impacts age-associated degeneration.

Kinetin Treatment Enhances Purifying Selection during Aging in Flies and Mice. Encouraged by findings that genetic manipulations altered the balance of mitochondrial genomes, we wondered whether pharmaceutical activation of quality control might reverse mutational accumulation in aging animals. Kinetin is a modified form of adenine and a cytokine in plants (29). Kinetin and its ribosyl derivative have been shown to activate PINK1 in mammalian cells (30, 31). We tested the influence of kinetin during aging of adult flies. We fed 3-d-old adults raised either at 29 °C or 22 °C vehicle control (dimethyl sulfoxide [DMSO]), the natural purine (adenine, 100 μM), or kinetin (100 μM) and followed the ratio of mitochondrial genomes. While Yak-mt/total-mt was...
relatively stable in controls, in flies raised at 29°C the ratio rose progressively in the kinetin-fed flies (Fig. 4A), while kinetin feeding had no effect at 22°C (SI Appendix, Fig. S7), when we expect no discordance in the abilities of the two genomes to support electron transport. The effect of kinetin was dose dependent and was neutralized by adenine competitor (SI Appendix, Fig. S8). We concluded that kinetin treatment promotes purifying selection in our heteroplasmic line during aging.

Since PINK1-mutant heteroplasmic flies do not survive into later adulthood, to test whether the kinetin action depends on PINK1 function, we examined heteroplasmic larvae. Kinetin (50 μM) feeding increased Yak-mt/total-mt in the CNS of control heteroplasmic larvae at 29°C. This action of kinetin was not detected in PINK1 loss-of-function mutant larvae, consistent with the expectation that kinetin acts by stimulating PINK1 (Fig. 4B). Alternatively, while kinetin increased Yak-mt/total-mt in control heteroplasmic larvae at 29°C, it had no effect on larvae at 22°C (SI Appendix, Fig. S8C), paralleling results in adult flies (SI Appendix, Fig. S7). This temperature dependence suggests that the effect requires the functional distinction between the genomes as expected if kinetin acts by stimulating quality control.

We then tested whether increased Yak-mt/total-mt in older kinetin-treated flies is associated with improved well-being. Kinetin feeding sustained climbing activity (Fig. 4C) and maintained the DA neurons in PPL1 in aged heteroplasmic flies (Fig. 4D). Thus, kinetin treatment promotes purifying selection and mitigates the decline of vigor of heteroplasmic flies.

As in the fly model, competition between mutant and wild-type mitochondrial genomes ought to influence accumulation of mutations in mammals. If failing capabilities of purifying selection in aged mammals contribute to age-associated accumulation of mutations as in flies, perhaps kinetin treatment would suppress or reverse age-associated accumulation of a mitochondrial mutation in mice.

We used a sensitive digital PCR assay to measure age-associated accumulation of a naturally emerging deletion in the minor arc of mtDNA in liver of WT C57BL6/J mice (Fig. 4F). Note that because mutations are rare events, first emergence of the deletion will vary from mouse to mouse, leading to some quantitative variation. Nonetheless, mutational abundance rises dramatically in older mice, with a near-exponential response to age. We fed control or kinetin-containing food to 16.5-mo-old mice for 6 wk and assessed levels of the minor arc deletion in liver (Fig. 4G). Moreover, kinetin feeding reduced the level of the minor arc deletion to well below that of similarly aged mice fed control food (Fig. 4G). This shows that a PINK1 activator reverses the age-associated increase of this mutant allele.

Discussion

Random segregation of mitochondrial genomes can lead to stochastic variation in the relative abundance of co-resident...
genomes within the cells of an individual (6, 32), but selective forces often bias the outcome to drive directional changes (6, 19, 20, 22, 32–35). In the female germ line of Drosophila, quality control promotes biogenesis and proliferation of those mitochondria that maintain a useful membrane potential (18, 19, 36). This puts mitochondria compromised by deleterious mutations at a proliferative disadvantage, resulting in their elimination within a few generations. It has, however, been less clear how selective forces and quality control mechanisms in influence the abundance of a deleterious mutation. The accumulation of mitochondrial mutations is important in the progression of heteroplasmic mitochondrial disease and impacts normal aging.

A genetic model for heteroplasmia in Drosophila provided a means of assessing selective forces that influence the abundance of a deleterious mutation. While the overall abundance of the genome with the deleterious mutation is influenced by a variety of selective forces, by using a temperature-sensitive mutation and assessing the results at two different temperatures, we can isolate the influence of purifying selection. By introducing mutations altering quality control pathways, we can assess the role of the affected pathway in purifying selection. This allowed us to show that purifying selection operates in somatic tissues during development and growth (Fig. 1) and that a PINK1-dependent pathway contributes importantly to this purifying selection (Fig. 2A). Sensitivity to reduction in the dose of larp and enhancement by reduction in the dose of tam suggest that the mechanism operating in the soma is similar to that described for purifying selection during oogenesis (18), but there may be additional mechanisms contributing.

Selection can only influence the relative proportion of competing mitochondrial genomes if there is replication and/or turnover of the genomes. Thus, stability might contribute to the absence
of observed purifying selection in the adult. However, this cannot explain periods of dynamic change in Yak-mt/total-mt. For example, the Yak-mt/total-mt ratio in the gut falls during adulthood, but falls the same amount at both temperatures (Fig. 1F). Additionally, we characterized a very rapid decline in Yak-mt/total-mt in the young adult following eclosion (Fig. 1D and E). Because this decline erased the benefits of earlier purifying selection, we interpret the change as the dynamic resetting of Yak-mt/total-mt ratio after a major reduction in the effectiveness of purifying selection.

We measured the impact of purifying selection rather than the process itself. Consequently, a rise in counteracting selection, rather than a loss in purifying selection, might be responsible for the reduction in the impact of purifying selection. The unexpected prevalence of deleterious mutations in adult mutant flies led to the suggestion that such mutations might be positively selected (23). Although we do not know how such a “destructive” selection would occur, if it exists, it would act in opposition to purifying selection and so reduce its impact. Accordingly, it is possible that a rise in destructive selection accounts for our recorded changes in adulthood.

Perhaps our most impactful finding is that purifying selection can be at least somewhat restored in later adulthood either genetically or pharmacologically (Figs. 3 and 4). In addition to improving mitochondrial genome quality, these treatments enhanced the climbing ability of aged heteroplasmic flies and suppressed loss of DA neurons (Figs. 3 and 4). This ability to reverse negative effects of heteroplasmy on the aging of flies suggests that such treatment might have the potential to reverse detrimental consequences of heteroplasmic mitochondrial diseases and perhaps suppress age-associated accumulation of mitochondrial mutations. Such possibilities are further bolstered by our finding that kinetin, the pharmacologic agent promoting purifying selection in adult flies, also worked in mice to suppress age-associated increase in a detrimental mtDNA mutation (Fig. 4).

Our findings raise questions about how the quality control mechanisms decline with age. A previous study, using a different model that assessed the levels of a deletion of mtDNA that was induced in adult Drosophila flight muscle, also found negligible levels of purifying selection (22). This study went on to demonstrate that a number of genetic alterations enhanced purifying selection. This led to the conclusion that “wild-type levels of key gene products are not set to maximize mtDNA quality control.” Specifically, this work implied that such processes as mitochondrial fusion and fission as well as the ability of mitochondrial adenosine 5′-triphosphate synthase to run in reverse are not optimized for quality control in the adult. Together with our findings, this report suggests a number of different pathways that are candidates for pharmacological modification that will benefit mtDNA quality during aging. While the findings also suggest that many different pathways might limit quality control in the adult, they do not explain why function is not optimized.

While it is puzzling why there is a lapse of purifying selection in the adult, we suggest that it may be fallout of the shifting priorities of evolution at different stages of life. Growth and development of metazoans occur in protected stable environments where organismal performance is not a factor, but maintenance of genome quality is key to the success of the future individual and to the production of future generations. Under these circumstances, evolution succeeds by prioritizing purifying selection. In contrast, the survival and reproductive potential of the adult depends on performance in a challenging and varied environment. At this life stage, evolution would select for elite performance and might optimize the energy-producing capacity of mitochondria by increasing the DNA content of weak mitochondria, even at the expense of triggering amplification of less functional genomes.

Regardless of the underlying reasons, we showed a decline in the impact of quality control in the adult and that a decline in vigor parallels reductions in the average quality of mitochondrial genomes during aging. These observations, together with substantial literature (6, 10–12, 18–22, 25, 32–35), suggest that the mitochondrial “genotype” of a metazoon is plastic and molded by selective forces acting within the organism. Importantly, we showed that mitochondrial genome quality can be genetically and pharmacologically modified during an organism’s lifetime to benefit well-being (Figs. 3 and 4). These findings bode well for development of therapies that modulate selective forces to enhance mitochondrial quality to benefit health span and lessen the impact of mitochondrial disease.

Materials and Methods

Fly Stocks. The heteroplasmic fly (Mel-mt°/Yak-mt) containing both a D. melanogaster mt genome with two mutant alleles (mt.ND22985 + mt.CaP3010) and a D. yakuba mitochondrial genome was previously described (25). We used a derived stock of the original heteroplasmic line (25) that carries a higher proportion of Yak-mt. Mel-mt°/Yak-mt females were backcrossed to males of a laboratory version of Canton S for 10 generations to homogenize the nuclear background (the laboratory stock was itself created by ingress of a Canton S background into w1118). The tam° and tam° alleles are congenic transgenic flies with a restored wild-type allele of tam (control) and a knockout allele, respectively (gifts from Dr. Anna Wredenberg, Karolinska Institutet, Stockholm, Sweden [37]). tam°/tam° originated from Dr. David M. Glover, University of Cambridge, Cambridge, UK and was a gift from Dr. Hong Xu, NIH, Bethesda, MD (17). tam°, tam°, PINK1°, PINK1°, and Dipl(3)Oeco were obtained from the Bloomington Drosophila Stock Center. The stocks were cultured at 22 and 29 °C on standard fly medium.

Mice. Male C57BL/6J mice were obtained from The Jackson Laboratory, housed in a specific pathogen-free facility with a standard 12-h light/dark cycle at the University of California, San Francisco, and given food and water ad libitum. Experiments were conducted in accordance with institutional guidelines approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

DNA Isolation. Total DNA of adult flies was extracted as follows. Three adult flies were pooled and mechanically homogenized with a plastic pestle in 105 μL homogenization buffer (100 mM Tris-HCl [pH 8.8], 0.5 mM ethylenediaminetetraacetic acid (EDTA), and 1% sodium dodecyl sulfate [SDS]). The homogenate was incubated at 65°C for 30 min, followed by addition of 15 μL potassium acetate (8 M) and incubation on ice (30 min) to precipitate protein and SDS. Subsequently, the homogenate was centrifuged at 13,000 rpm for 15 min at 4°C. DNA was recovered from the supernatant by adding 0.5 volume 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 20 μL double distilled water (dH2O). DNA was extracted from tissue samples as follows. A single dissected fly tissue (brain, wing disk, or gut) was placed on a clean cover slide, mixed with 10 μL lysis buffer (10 mM Tris-HCl [pH 8.8], 1 mM EDTA, 25 mM NaCl, 1% SDS, and 8 μL/mL Proteinase K, NEB) for 3 min at room temperature. The tissue/lysis mixture was transferred to a 0.2-mL tube and incubated at 37°C for 30 min and heat inactivated by 95°C for 5 min.

Mice were anesthetized by intraperitoneal injection of 0.3 cc 17.5 mg/mL Ketamine and 10 mg/mL Xylazine mixture in saline and perfused with 15 to 20 mL sterile phosphate-buffered saline (PBS) through the heart left ventricle using the Surflo winged infusion set (Terumo Corporation, SV-218LK attached to a 30-mL syringe. After perfusion, livers were collected, immediately frozen on dry ice, and stored at −80°C until DNA isolation. Two small liver fragments (~1 mm2) per animal were analyzed. Fragments were lysed in 90 μL lysis solution (50 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 0.5% SDS, and 2 mg/mL...
proteinase K (Promega, v3021)) at 37°C overnight followed by addition of 10 μL of 3M potassium acetate and incubation at room temperature for 20 min. Next, lysates were centrifuged at 21,000 × g for 10 min, and supernatants were transferred to silica membrane spin columns (Macherey-Nagel) and centrifuged at 11,000 × g for 1 min. Flow-throughs were heat inactivated at 70°C for 30 min and cleaned up using homemade magnetic beads [1:1 ratio (38)]. After cleanup, DNA was eluted in 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and stored at 4°C.

qPCR Analyses. For all qPCR assays, SYBR Select Master Mix (Applied Biosystems, 4470908) 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 128-bp mt:IrRNA region present in both mtDNA genotypes was performed (primer NAC-CAACCTGATTAACCG and TGCGACCTTGATTGTG) and normalized to nuclear genome copy number of the e tub84D gene (primer ATGCCCGAATGATCCTATCC and AGGTGTAACAGGTGCTACCC). To measure copy number of D. yakuba mtDNA, the qPCR of the 71-bp D. yakuba mt:COXI region was performed (primer AGTGGAAACGAGCCTGTTGCA and CTCACCATGACGCTATCCG). The efficiency and specificity of D. yakuba mt:COXI and total mt:IrRNA primers in qPCR reaction were normalized each time by analyzing the ratio between the two primer sets in DNA samples from a D. melanogaster wild-type stock of Canton S. The background for the Yak-specific reaction was also assessed in every qPCR experiment and was always less than 0.005% of total mtDNA samples from Canton S. qPCR was performed with the following reaction conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 5 s and 62.5°C for 15 s. For each 20-μL qPCR reaction, 0.375% of an adult fly or 0.667% of a tissue’s total genomic DNA was used as a template. The percentage of D. yakuba mtDNA was calculated by dividing D. yakuba mtDNA copy number by the total mtDNA copy number. The threshold cycle (CT) values used ranged from 18 to 30.

ddPCR Analyses. The droplet digital polymerase chain reaction (ddPCR) analysis was performed on a QX100 system (Bio-Rad). Two assays designed to detect minor allele deletion (primer LD_F CCAAGATTTGACCATG, primer LD_R CITCCTGAAATTATGTC and probe LD Common_F CTGCAGAATGCTGTTTCTGAC and probe LD_common_R AGTCCTACATGCTTTC, probe LD_common_AGCCTCAAGGCTTAAA) were used simultaneously. Template DNA concentration was adjusted to be below 3,500 mitochondrial genome copies per microliter of ddPCR reaction mixture. The reaction mixture (total volume 22 μL) contained 11 μL of 2× ddPCR Super Mix for Probes (Bio-Rad, 1863024), 1.1 μL of four primers mixture, 18 μL each, 1.1 μL of 5 μM probe LD common labeled with HEX (IDT), 1.1 μL of 5 μM probe LD labeled with FAM (IDT), 0.5 μL of Alu1 restriction enzyme (NEB, R0137L), 2.2 μL of ddH2O, and 5 μL of template DNA. Then, 20 μL of the reaction mixture and 70 μL of oil (Bio-Rad, 1863005) were loaded on a D8 cartridge (Bio-Rad, 1864007) for droplet generation on QX100 Droplet Generator (Bio-Rad), and 40 μL of droplet emulsion were transferred to 96-well plate (Bio-Rad, 12001925) and sealed with a pierceable foil (Bio-Rad, 1814040) using PX1 PCR plate sealer (Bio-Rad). The optimized PCR thermal cycling was conducted on a conventional PCR machine (Bio-Rad, C100 Touch) using the following conditions: 10 min activation period at 95°C, followed by 40 cycles of denaturation at 94°C for 30 s, ramp rate 3°C s⁻¹, and combined annealing-extension at 54°C for 1 min, ramp rate 2°C s⁻¹, and one cycle of 98°C for 10 min. After thermocycling, samples were analyzed on the QX100 Droplet Reader (Bio-Rad). Results were analyzed with QuantaSoft Analysis Software v 1.0.596 software (Bio-Rad).

Behavior Assay. A number of independent crosses, 15, 22, or 25, were carried out to produce flies of each genotype: wild-type, tam(K0)+, or tam(F1)+, respectively. Ten male flies were collected from each cross to form 62 independent cohorts. Each cohort was challenged in a climbing test done at days 1, 5, 11, and 20. In each test, climbing ability was defined as the ability of the adult fly to climb 5 cm within 10 s. If the fly could accomplish the task (<10 s), it was given a score of 1; otherwise, it was given a score of 0 (>10 s). All surviving flies were scored, and the % success of the surviving flies was tabulated and plotted. In total, 248 independent tests were conducted.

Immunocytochemistry and Confocal Microscopy. Adult brains were dissected in PBT (0.3% Tween 20 in PBS) and incubated with fixation solution (4% formaldehyde in PBT) for 20 min, followed by blocking for 1 h with 1% bovine serum albumin in PBT. Samples were immunostained with rabbit anti-Tyrosine hydroxylase (AB152; EMD Millipore Corporation) at 1:200 and Alexa 488-conjugated anti-rat (ab150156; AbCam). Samples were imaged with a 20X dipping 2-mm working distance objective on a Zeiss Confocal Laser Scanning Microscope 780 with identical imaging parameters among different genotypes in a blind fashion. Images were processed with Photoshop CS4 using only linear adjustment of contrast.

Kinetin Treatment in Fly. For larval treatment, 0- to 6-h eggs were collected and grown on regular fly food (10 mL containing DMSO (0.1%) with or without 50 μM kinetin. Six hours after egg collection, a 200 μL solution of 5% DMSO without or with 5 mM kinetin was applied on the top of the fly food for control and experimental larvae, respectively. Tissues were dissected from late third instar larvae.

For adult treatment, 3- to 8-old male adults were collected and first kept for 48 h in vials with a cotton ball moistened with 2 mL of a 10% sucrose, 2% DMSO solution (control) or the same supplemented with 100 μM kinetin, 100 μM adenine, or both 100 μM kinetin and 100 μM adenine. Then, the flies were transferred to vials prepared as described below, and passaged to similar fresh vials every 2 d until dissection. Recipient vials contained 10 mL of regular fly food with 0.1% DMSO (control) or the same supplemented with 100 μM kinetin, 100 μM adenine, or both 100 μM kinetin and 100 μM adenine. Prior to use, 200 μL of 5% DMSO (control), or the same supplemented with 5 mM kinetin, 5 mM adenine, or both 5 mM kinetin and 5 mM adenine was applied on the surface of the fly food and air-dried.

Kinetin Chow. Kinetin (Sigma-Aldrich) was delivered orally to mice in their chow following published reports (37). Rodent chow (Purina, 5053) was formulated by Research Diets to contain 3.50 g kinetin/kg chow for mice. These amounts of kinetin were well tolerated during trials according to a previous report (39). Chow was stored at ~80°C. Fresh chow was provided at least every 4 d. The delivery of chow was not blinded with respect to drug treatment. The 16.5-mo-old mice were fed with kinetin or control chow for 6 wk before sample collections at 18 mo of age. Body weights and chow intake were monitored at least twice weekly.

Data Availability. All study data are included in the article and/or SI Appendix.

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