Mutations of mitochondrial DNA are not major contributors to aging of fruit flies

Timo E. S. Kauppila[a,1], Ana Bratic[a,1,2], Martin Borch Jensen[b], Francesca Baggio[a], Linda Partridge[c], Heinrich Jasper[b], Sebastian Grönke[c], and Nils-Göran Larsson[a,d,2]

[a]Department of Mitochondrial Biology, Max Planck Institute for Biology of Ageing, D-50931 Cologne, Germany; [b]The Buck Institute for Research on Aging, Novato, CA 94945; [c]Department of Biological Mechanisms of Ageing, Max Planck Institute for Biology of Ageing, D-50931 Cologne, Germany; and [d]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-17177 Stockholm, Sweden

Edited by Ruth Lehmann, New York University Medical Center, New York, NY, and approved August 27, 2018 (received for review December 13, 2017)

Mammals develop age-associated clonal expansion of somatic mtDNA mutations resulting in severe respiratory chain deficiency in a subset of cells in a variety of tissues. Both mathematical modeling based on descriptive data from humans and experimental data from mtDNA mutator mice suggest that the somatic mutations are formed early in life and then undergo mitotic segregation during adult life to reach very high levels in certain cells. To address whether mtDNA mutations have a universal effect on aging metazoans, we investigated their role in physiology and aging of fruit flies. To this end, we utilized genetically engineered flies expressing mutant versions of the catalytic subunit of mitochondrial DNA polymerase (DmPOLγA) as a means to introduce mtDNA mutations. We report here that lifespan and health in fruit flies are remarkably tolerant to mtDNA mutations. Our results show that the short lifespan and wide genetic bottleneck of fruit flies are limiting the extent of clonal expansion of mtDNA mutations both in individuals and between generations. However, an increase of mtDNA mutations to very high levels caused sensitivity to mechanical and starvation stress, intestinal stem cell dysfunction, and reduced lifespan under standard conditions. In addition, the effects of dietary restriction, widely considered beneficial for organismal health, were attenuated in flies with very high levels of mtDNA mutations.

mtDNA | aging | lifespan | dietary restriction | intestinal stem cells

Mitochondria have been estimated to contain ∼1,200 proteins (1), most of which are encoded by the nuclear genome and imported into mitochondria. Consistent with the high number of proteins, mitochondria are involved in many important cellular processes, such as oxidative phosphorylation (OXPHOS), iron–sulfur cluster biogenesis, fatty acid oxidation, reactive oxygen species (ROS) production, and apoptosis. In contrast to other organelles, mitochondria possess their own DNA, which in most metazoans encodes 13 proteins of key importance for OXPHOS, as well as the tRNAs and rRNAs needed to translate the corresponding mRNAs on mitochondrial ribosomes (2). Mutations affecting mtDNA or nuclear genes encoding mitochondrial proteins can impair OXPHOS function and cause different types of mitochondrial diseases (3). Furthermore, accumulation of somatic mtDNA mutations has been reported to play a role in certain age-related diseases and has gained significant attention as a contributor to the naturally occurring aging process (4). Several studies in mammals have shown that there is a progressive decline in mitochondrial function with age, accompanied by a concomitant increase of point mutations and deletions of mtDNA (5–9). The origins of these mutations have been heavily investigated and most studies suggest they originate from replication errors (4). Mutations can be present only in a fraction of all (heteroplasmy) or in all (homoplasmy) mtDNA copies of a cell or tissue. Furthermore, the levels of mtDNA mutations can fluctuate dramatically in mitotic tissues owing to random segregation of mtDNA during cell division. There is segregation of mtDNA mutations also in postmitotic tissues because of the continuous cell cycle-independent replication of mtDNA. The levels of pathogenic mtDNA mutations can thus drift over a cell's lifetime and once a certain threshold is reached they cause respiratory deficiency in a subset of cells in different tissues (10).

Several studies have investigated whether the levels of mtDNA mutations are changing during the life of an organism, and indeed this seems to be the case in flies, mice, rats, rhesus monkeys, and humans (11–15). In addition to observational studies, experimental studies in the mouse have shown that high levels of somatic mtDNA mutations cause a premature aging syndrome (16, 17). Therefore, both observational and experimental studies support the hypothesis that mitochondrial dysfunction contributes to the aging process in mammals.

There are currently few strategies to counteract these age-associated changes in mitochondrial function. We have recently shown that increasing the absolute amount of mtDNA is a powerful approach to rescue defects caused by heteroplasmic mtDNA mutations (18). Additionally, some pharmacological approaches have yielded promising results (19–21). Also, nutritional interventions, such as dietary restriction (DR), are known to have beneficial health effects (22). To date, studies investigating the possible connection between DR, mitochondrial function, and lifespan extension have produced contradicting results. DR has been reported to decrease, have no effect on, or even increase oxygen consumption rates (23–26). Additionally, some genetic and pharmacological approaches to decrease mitochondrial function have been reported to extend lifespan (27–30), but it has also been

Significance

Mutations of mtDNA accumulate in aging humans and other mammals to cause mitochondrial dysfunction in a subset of cells in various tissues. Furthermore, experimental induction of mtDNA mutations causes a premature aging syndrome in the mouse. To study if mitochondrial dysfunction is universally involved in shortening life span in metazoans, we generated a series of fruit fly lines with varying levels of mtDNA mutations. Unexpectedly, we report that fruit flies are remarkably tolerant to mtDNA mutations, as exemplified by their lack of effect on physiology and lifespan. Only an artificially induced, very drastic increase of the mtDNA mutation load will lead to reduced lifespan, showing that mtDNA mutations are unlikely to limit lifespan in natural fruit fly populations.

Author contributions: T.E.S.K., A.B., L.P., H.J., S.G., and N.-G.L. designed research; T.E.S.K., A.B., M.B.J., and F.B. performed research; T.E.S.K., A.B., and M.B.J. analyzed data; and T.E.S.K., A.B., and N.-G.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]T.E.S.K. and A.B. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: Ana.Groenke@age.mpg.de or nils-goran.larsson@ki.se.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1721683115/-/DCSupplemental.

Published online September 24, 2018.

reported that mitochondrial function is necessary for DR-mediated lifespan extension (31, 32).

As summarized above, mammalian studies support the connection between mtDNA mutations and aging. However, mathematical models suggest that mtDNA mutations should not limit the lifespan of short-lived organisms, such as fruit flies, due to the slow drift of somatic mtDNA mutations (33). To experimentally test whether mtDNA mutations can limit the lifespan of fruit flies, we utilized genetically engineered flies harboring mutated alleles that lead to the expression of DmPOLγA variants with decreased polymerase or exonuclease activities (34). Impairing proofreading of DmPOLγA increases mtDNA mutation load, whereas low polymerase activity leads to mtDNA depletion. Using these genetic models we studied the accumulation of mtDNA mutations in mitotic and postmitotic tissues of flies as well as the impact of these mutations on adult physiology and longevity. Strikingly, we did not observe an increase in the levels of somatic mtDNA mutations with age of adult flies. Moreover, moderately increasing the somatic mtDNA mutation load did not have any impact on fly lifespan or physiology. Even after accumulation of mutations for multiple generations, the lifespan of fruit flies was remarkably tolerant toward mtDNA mutations, likely due to the slow clonal expansion of mtDNA mutations across generations, which, in turn, is explained by the relatively large genetic bottleneck in flies. In fact, inheritance of mtDNA mutations for multiple generations in the presence of an experimentally decreased genetic bottleneck was required to cause mitochondrial dysfunction, which was manifested as defects in locomotor activity, stem cell dysfunction, low tolerance to mechanical stress, sensitivity to starvation, and, eventually, shortened lifespan. Interestingly, the positive effect of DR on fly longevity was attenuated only in the presence of a strong mitochondrial dysfunction.

Results

Clonal Expansion of mtDNA Mutations in the Female Germline of mtDNA Mutator Flies. Mitochondrial function is essential for fly viability because high levels of mtDNA mutations or strong mtDNA depletion lead to arrested fly development at late larval stages (34). To further study how the decline in mitochondrial function affects viability and physiology of adult flies, we utilized genetically engineered fruit flies expressing a catalytic subunit of DmPOLγA with impaired polymerase (H1038A and Q1009A) or exonuclease (D263A) activities (34).

Ongoing mtDNA replication in adult flies was investigated by using pulse-chase BrdU labeling as a proxy for mtDNA turnover. Four days of BrdU feeding resulted in robust labeling of mtDNA, and this label was rapidly lost in adult flies, with females showing a somewhat faster decay of the BrdU signal than males (Fig. 1*A* and *SI Appendix*, Fig. S1 *A* and *B*). The more rapid loss of mtDNA labeling in female flies is likely explained by the high levels of mtDNA replication during oogenesis in the ovaries (35) as the BrdU turnover was similar between female and male flies without the abdomen (*SI Appendix*, Fig. S1 *C* and *D*).

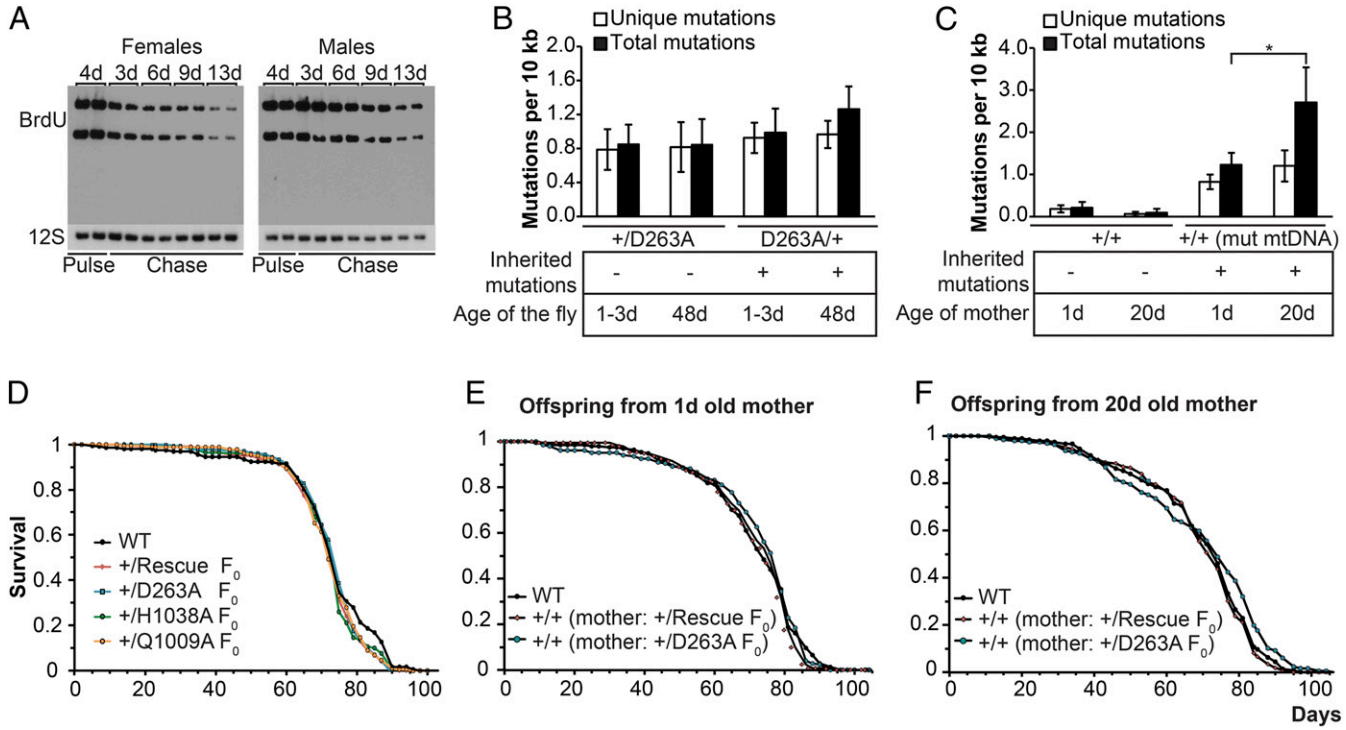

Fig. 1. Accumulation of mtDNA mutations with age, mtDNA turnover, and lifespan effects of *DmPOLγA* alleles. (*A*) Pulse-chase experiments with BrdU to detect mtDNA turnover in adult fruit flies. MtDNA was extracted from flies after pulse labeling and after 3, 6, 9, and 13 d of chase. MtDNA was digested using SacI, producing three fragments: 10.3, 5.2, and 4 kb (*SI Appendix*, Fig. S1*A*). The two shorter fragments were used for Southwestern blotting to detect mtDNA incorporated BrdU and the 10.3-kb fragment was detected using P^{32} -12S probe and used as a loading control. (*B*) Quantification of unique (white bar) and total (black bar) mtDNA mutation loads in thorax from young (1–3 d) and old (48 d) heterozygous mtDNA mutator flies with only somatic (+/D263A) or both inherited and somatic mutations (D263A/+). Error bars represent SD. Student's two-tailed *t* test. (*C*) Quantification of unique (white bar) and total (black bar) mtDNA mutation loads from the WT progeny of young (1 d) and old (20 d) WT and heterozygous mtDNA mutator female flies. Error bars represent SD. Student's two-tailed *t* test. *$P < 0.05$. (*D*) Lifespan analyses of flies inheriting *DmPOLγA* alleles paternally and therefore carrying only somatic mtDNA mutations. +/Rescue F_0 (red), +/D263A F_0 (blue), +/H1038A F_0 (green), and +/Q1009A F_0 (orange). $P < 0.0001$, log-rank test. (*E* and *F*) Lifespan analyses of flies originating from either (*E*) young (1 d) or (*F*) old (20 d) female flies. Only the progeny with WT nuclear background was used for experiments. Progeny from WT mother (black), +/Rescue F_0 mother (red), and +/D263A F_0 mother (blue). $P < 0.0001$, log-rank test. Flies labeled as +/D263A inherited the D263A allele paternally (no inherited mtDNA mutations), whereas D263A/+ flies inherited the D263A allele maternally (inherited and somatic mtDNA mutations). In this way, fly genotypes are always presented as follows: *DmPOLγA* allele from mother/*DmPOLγA* allele from father.

To investigate whether the ongoing mtDNA replication contributes to a lifetime accumulation of mtDNA mutations in adult flies, we quantified the mtDNA mutation load in thorax of young (1–3 d) and old (48 d) heterozygous mtDNA mutator flies harboring either exclusively somatic (+/D263A) or both maternally transmitted and somatic mtDNA mutations (D263A/+). Surprisingly, the levels of somatic mtDNA mutations did not increase with age of +/D263A flies (Fig. 1*B*). The levels of total mtDNA mutations in D263A/+ flies showed a small increase with age, likely because of clonal expansion of maternally transmitted mtDNA mutations, but the difference was not statistically significant (Fig. 1*B*). These results suggest that the mtDNA replication in fly thorax is not high enough to result in a robust increase of the mtDNA mutation load during the short lifespan of adult fruit flies.

Although there were no changes in the mtDNA mutation load with age in a postmitotic fly tissue, such as the thorax, mtDNA mutations may accumulate in highly proliferative tissues, such as the female gonads. During 20 d, a female fly lays hundreds of eggs (*SI Appendix*, Fig. S1*E*), and this ongoing proliferation of germline stem cells could allow mtDNA mutations to accumulate in the female germline. To avoid sampling errors caused by alterations of the ovarian structure as flies age, we did not directly measure the mtDNA mutation load in ovaries, but instead assessed the load in the progeny of young and old female flies. Almost no mtDNA mutations could be detected in the progeny of either young or old WT flies (Fig. 1*C*). Although the load of unique mtDNA mutations was increased to a similar extent in the progeny of young and old mtDNA mutator flies (+/D263A) (Fig. 1*C*), the total mtDNA mutation load was more increased in the progeny of old mtDNA mutator flies (Fig. 1*C*). These findings are consistent with ongoing clonal expansion of mtDNA mutations in the ovaries of aging mtDNA mutator flies.

Somatic mtDNA Mutations Do Not Limit the Lifespan of Fruit Flies. Inherited (36) or a combination of inherited and somatic mtDNA mutations (16, 17) have been reported to cause age-related phenotypes in the mouse. However, experimental evidence for a similar role for somatic mtDNA mutations in aging of short-lived species, such as fruit flies, is lacking. As expected, neither of the polymerase-deficient *DmPOLγA* alleles (Q1009A, H1038A) had any effect on fly lifespan (Fig. 1*D*, *SI Appendix*, Fig. S2 *A* and *B*, and Dataset S1), which is in line with our recent findings showing that two dominant negative *DmPOLγA* alleles (Y873C and Y873H) do not limit the lifespan of fruit flies (37). Surprisingly, an increase in the somatic mtDNA mutation load did not have any effect on the lifespan in exonuclease-deficient (+/D263A F_0) flies (Fig. 1*D*, *SI Appendix*, Fig. S2 *A* and *B*, and Dataset S1).

To investigate how inherited mtDNA mutations affect the lifespan of flies with a WT nuclear background, we took advantage of the fact that old females transmit more mtDNA mutations to their offspring than young females (Fig. 1*C*). We generated two fly strains with a WT nuclear background by selecting progeny from young (1 d) and old (20 d) heterozygous mtDNA mutator flies. Interestingly, we did not observe any changes in the lifespan of the offspring of mtDNA mutator flies (+/D263A F_0) relative to controls with engineered WT locus (Rescue) or WT flies (Fig. 1 *E* and *F*). The progeny of 20-d-old mothers showed poorer survival in comparison with the progeny of 1-d-old mothers (Fig. 1 *E* and *F*, *SI Appendix*, Fig. S2 *C* and *D*, and Dataset S1). However, this difference was independent of the fly genotype, and therefore also of mtDNA mutations, and was reminiscent of the Lansing effect (38, 39). Taken together, our results show that mtDNA mutations can expand clonally with age in the proliferating female germline, but these mutations do not limit the lifespan of the offspring.

Lifespan of Fruit Flies Is Highly Tolerant Toward High Levels of mtDNA Mutations. Flies heterozygous for the mtDNA mutator allele (+/D263A F_0) did not have a shorter lifespan despite an increase in mtDNA mutation load (Fig. 1*D*), which is in stark contrast to the well-documented premature aging phenotype of the mtDNA mutator mouse (16, 17) and shortened lifespan of WT mice with inherited mtDNA mutations (36). However, the heterozygous mtDNA mutator flies have only half of the mutation load of the WT mice with inherited mtDNA mutations, that is, 0.9×10^{-4} mutations per bp versus 2.1×10^{-4} mutations per bp, respectively (40). It is therefore possible that the mtDNA mutation levels do not reach the critical threshold level required to limit fly lifespan. To define this level, we established fly lines with variable levels of mtDNA mutations. Maternal lineages of heterozygous mtDNA mutator flies show slow accumulation of mtDNA mutations in successive generations (34). We analyzed offspring of these fly lines after 1, 6, and 15 generations of breeding to test whether the obtained increase in mtDNA mutations would limit fly lifespan. Surprisingly, these flies did not show any consistent changes in lifespan compared with WT controls (Fig. 2 *A–C*, *SI Appendix*, Fig. S3 *A–F*, and Dataset S1). We further tested 2-y-intercrossed mtDNA mutator flies that were outcrossed to remove the mtDNA mutator allele [$^{+/+}$ (mut mtDNA) $>F_{30}$]. These flies have a WT nuclear background and high levels of mutated mtDNA (Fig. 2*J*), which cause a strong developmental delay that can be rescued by introducing WT mtDNA (34). However, the lifespan of these flies was indistinguishable from that of controls (Fig. 2*D*, *SI Appendix*, Fig. S3*G*, and Dataset S1). To further increase the mtDNA mutation load, we used the recently published compound heterozygous flies (D263A/H1038A) that carry one allele encoding an exonuclease-deficient and one allele encoding a proofreading-deficient version of DmPOLγA (*SI Appendix*, Fig. S4*A*) (34). Similar to the heterozygous mtDNA mutator flies, compound heterozygous flies accumulate mtDNA mutations, but additionally they also show a rapid clonal expansion of mtDNA mutations across generations because of a decreased genetic bottleneck size (34). The compound heterozygous flies had a lifespan similar to that of controls (Fig. 2*E*, *SI Appendix*, Fig. S4*B*, and Dataset S1) despite harboring high levels of somatic mtDNA mutations. To further increase mtDNA mutation load, compound heterozygous flies containing both clonally expanded and somatic mtDNA mutations were generated by breeding the corresponding heterozygous stocks for four generations before crossing them (*SI Appendix*, Fig. S4*A*) (34). These flies have more mtDNA mutations than the short-lived WT nuclear mice with inherited mtDNA mutations, 9.7×10^{-4} and 2.1×10^{-4} mutations per bp, respectively (34, 36, 40). However, also these compound heterozygous flies had lifespans similar to those of WT flies (Fig. 2*F*, *SI Appendix*, Fig. S4*C*, and Dataset S1). These findings show that neither the complementation as such nor the resulting high levels of mtDNA mutations limit the lifespan of fruit flies.

These results raise the question of why the intercrossed mtDNA mutator flies have normal lifespan whereas flies carrying near-homoplasmic mtDNA mutations have been shown to have shortened lifespans in several reports (41–43). One explanation for these differences could be that the mtDNA mutations in the intercrossed mtDNA mutator flies have not clonally expanded to reach a threshold level sufficient to cause mitochondrial dysfunction and compromise lifespan. Indeed, we and others have shown that clonal expansion of mtDNA mutations is a slow process in flies in comparison with mammals (34, 44). The post-PCR cloning and sequencing method is sensitive and detects low-level mtDNA mutations, but the covered region is only 1.2 kb of the mtDNA. In contrast, sequencing long-range PCR-amplified mtDNA allows one to detect mutations in the whole coding region, but only mutations present above the Sanger sequencing detection threshold level (~25%) can be detected. Therefore, to better assess how mutations expand clonally in the full coding region of mtDNA, we PCR-amplified and Sanger-sequenced the full coding region of mtDNA of different fly lines and categorized the identified heteroplasmic mutations depending on the mutation level [i.e., low (<33%), medium (33–66%) and high (>66%) mutation levels]. Using this method, heterozygous mtDNA mutator flies with only somatic mutations (+/D263A) did not show any clonally expanded mutations. Flies carrying both somatic and inherited mtDNA mutations (D263A/+) were intercrossed for multiple generations, but even after 15 generations of consecutive intercrossing of heterozygous mtDNA

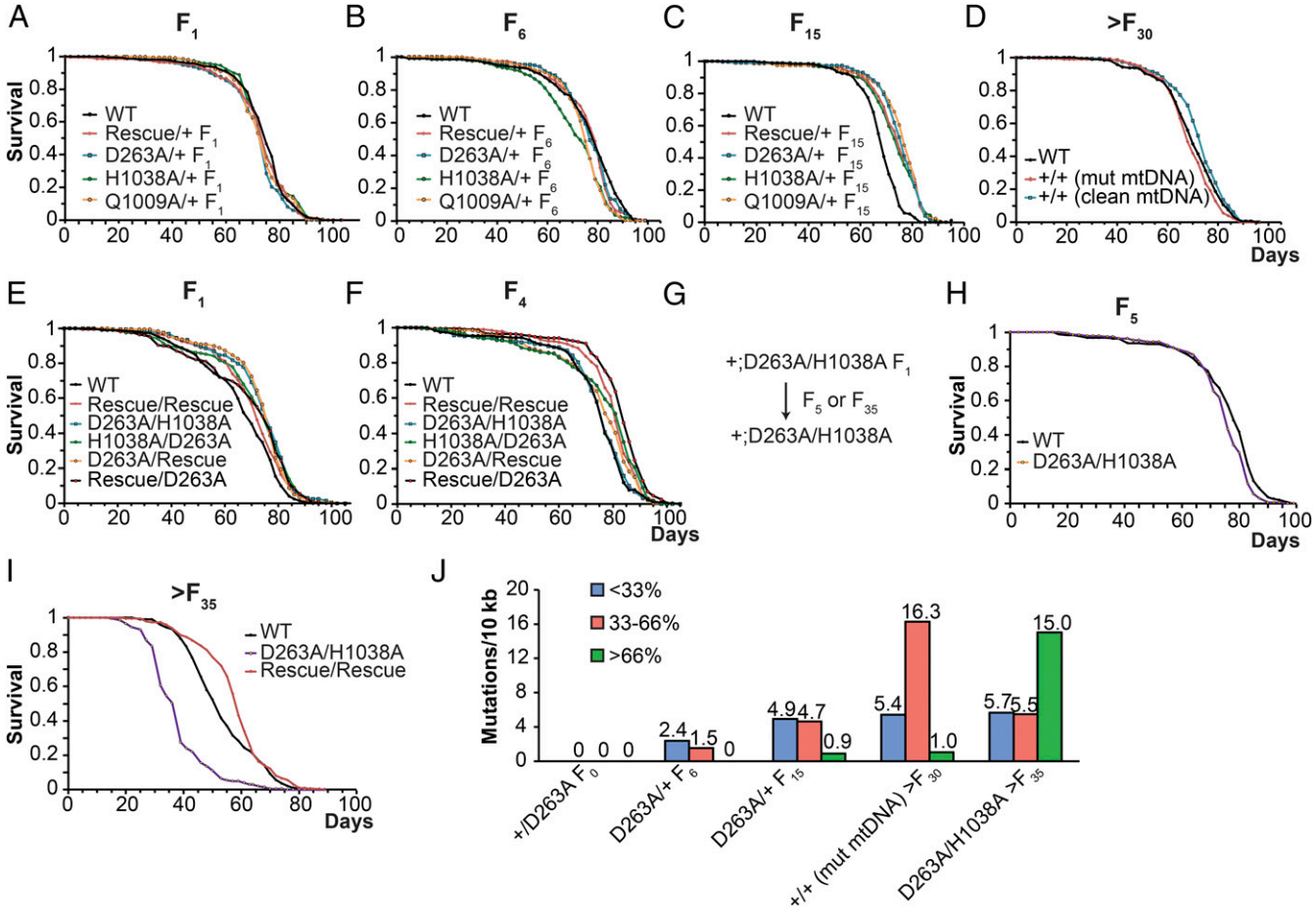

Fig. 2. Fruit fly lifespan is tolerant against mtDNA mutations. (*A*–*C*) Lifespan analyses of flies inheriting different *DmPOLγA* alleles maternally for (*A*) 1, (*B*) 6, and (*C*) 15 generations and therefore carrying both somatic and inherited mtDNA mutations. Lifespan curves of WT (black), Rescue/+ (red), D263A/+ (blue), H1038A+/(green), and Q1009A+/(orange) flies. $P < 0.0001$, log-rank test. (*D*) Lifespan analysis of outcrossed heterozygous mtDNA mutator flies. Heterozygous mtDNA mutator flies were intercrossed for more than 30 generations to accumulate mtDNA mutations. These flies were further outcrossed to WT nuclear background to remove the mtDNA mutator (D263A) allele. WT flies (black), flies with WT nuclear background and inherited mtDNA mutations [$^{+/+}$ (mut mtDNA), green], and flies with WT nuclear background and reintroduced WT mtDNA [$^{+/+}$ (clean mtDNA), red]. $P < 0.0001$, log-rank test. (*E*) Lifespan analyses of compound heterozygous flies with no mtDNA mutations [WT (black) and Rescue/Rescue (red)], with only somatic mtDNA mutations [H1038A/D263A (green), Rescue/D263A (black line, red circle)], or with somatic and inherited mtDNA mutations [D263A/H1038A (blue), D263A/Rescue (orange)]. $P < 0.0001$, log-rank test. (*F*) Lifespan analyses of flies that have been intercrossed for four generations before made compound heterozygous (crossing scheme shown in *SI Appendix*, Fig. S4*A*). WT (black), Rescue/Rescue (red), D263A/H1038A (blue), H1038A/D263A (green), D263A/Rescue (orange), and Rescue/D263A (black line with red circle). $P < 0.0001$, log-rank test. (*G*) Crossing scheme to generate 5 and >35 generations intercrossed D263A/H1038A compound heterozygous flies used in *H* and *I*. (*H* and *I*) Lifespan analyses of compound heterozygous flies intercrossed for (*H*) five generations or (*I*) more than 35 generations as shown in *G*. $P < 0.0001$, log-rank test. (*J*) Quantitative and qualitative analysis of high-heteroplasmy-level mtDNA mutations detectable by Sanger sequencing in heterozygous D263A/+ flies intercrossed for 1, 6, or 15 generations, $^{+/+}$ (mut mtDNA) $>F_{30}$ flies and in compound heterozygous flies intercrossed for more than 35 generations (D263A/H1038A $>F_{35}$). Mutations were categorized to low (<33%, blue), middle (33–66%, red), and high (>66%, green) heteroplasmic levels.

mutator flies (D263A/+ F_{15}) there were hardly any mutations present at a sufficiently high level required to induce mitochondrial dysfunction (Fig. 2*J*). To allow sufficient time for mtDNA mutations to expand clonally, we intercrossed a compound heterozygous fly population for 5 or >35 generations (Fig. 2*G*). We did not observe any changes in the lifespan of the compound heterozygous fly population intercrossed for five generations (Fig. 2*H* and Dataset S1). However, compound heterozygous flies D263A/H1038A intercrossed for >35 generations had a severely reduced lifespan (Fig. 2*I*, *SI Appendix*, Fig. S4*D*, and Dataset S1), whereas control flies (Rescue/Rescue) did not show any consistent changes in lifespan after the same amount of intercrossing (Fig. 2*I* and *SI Appendix*, Fig. S4*D*). The intercrossed compound heterozygous flies carried both synonymous and nonsynonymous mutations at high levels (Fig. 2*J* and Dataset S2). Additionally, these flies had mtDNA depletion (*SI Appendix*, Fig. S4 *E* and *F*), whereas mtDNA deletions could not be detected (*SI Appendix*, Fig. S4*E*). Altogether these results further support the notion that the rate of clonal expansion of mtDNA mutations across generations is a relatively slow process in flies and that mtDNA mutations will only limit lifespan when present at high levels.

Clonally Expanded mtDNA Mutations Impair Mitochondrial Function. To verify that the phenotypes of the intercrossed compound heterozygous flies (D263A/H1038A $>F_{35}$) were caused by mitochondrial dysfunction, we assessed the levels of assembled respiratory chain complexes by blue native (BN) PAGE. We found that flies carrying high levels of mtDNA mutations (D263A/H1038A $>F_{35}$) had reduced levels of complex I, complex IV, complex V, and supercomplexes (Fig. 3*A* and *SI Appendix*, Fig. S5 *A* and *B*). This reduction in steady-state levels of assembled complexes was accompanied by reduced in-gel activity of complex I and complex IV (Fig. 3*A*) without any clear relation to the age of the flies (*SI Appendix*, Fig. S5*C*). Compound heterozygous flies that

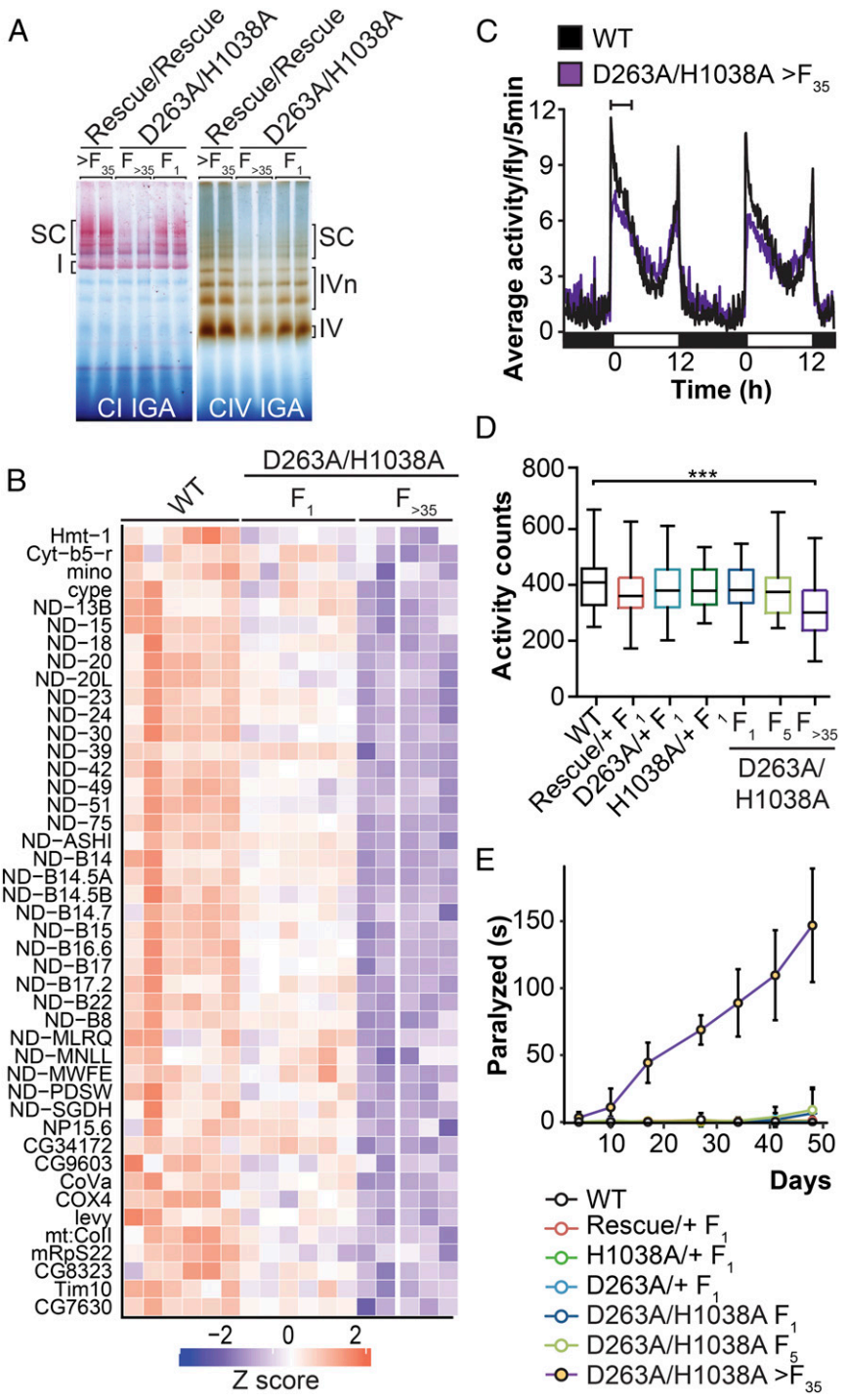

Fig. 3. MtDNA mutations lead to loss of OXPHOS complexes, decrease locomotor activity, and cause mechanical stress sensitivity. (*A*) BN-PAGE analyses were performed to determine the steady-state levels of OXPHOS complexes. In gel activity assays for complex I (*Left*) and complex IV (*Right*) were performed on mitochondrial extracts isolated from control flies (Rescue/Rescue $>F_{35}$) and compound heterozygous flies with low levels of inherited and somatic mutations (D263A/H1038A F_1) and high levels of clonally expanded mtDNA mutations (D263A/H1038A $>F_{35}$). (*B*) Mass spectrometry analysis of Percoll gradient-purified mitochondrial proteins from WT flies, compound heterozygous flies with low-level inherited and somatic mutations (D263A/H1038A F_1), and compound heterozygous flies with clonally expanded mutations (D263A/H1038A $>F_{35}$). False discovery rate = 0.05. (*C*) A representative example of average locomotor activity of WT flies (black) and D263A/H1038A $>F_{35}$ flies (red) during 48 h. Scale bar represents morning bout (8:00–12:00) used for statistical analysis shown in *D*. (*D*) Locomotor activity of heterozygous flies with different *DmPOLγA* alleles (Rescue/+, D263A/+, H1038A/+) and compound heterozygous flies with low (D263A/H1038A F_1), medium (D263A/H1038A F_5) or high (D263A/H1038A $>F_{35}$) levels of mtDNA mutations during the morning bout (8:00 AM–12:00 PM). Whiskers extend to minimum and maximum. One-way ANOVA with Dunnett's post hoc test. ***$P < 0.001$. (*E*) Mechanical stress sensitivity assay was performed on aging heterozygous flies carrying different *DmPOLγA* alleles (Rescue/+, D263A/+, and H1038A/+) and on compound heterozygous flies with low (D263A/H1038A F_1), medium (D263A/H1038A F_5), and high (D263A/H1038A $>F_{35}$) levels of clonally expanded mtDNA mutations. Error bars represent SD.

had accumulated somatic mtDNA mutations for only one generation (D263A/H1038A F_1) did not show any changes in the complex I or IV activities (Fig. 3*A*), which is in line with previous respiration measurements from compound heterozygous larvae (34).

As an independent way of assessing levels of OXPHOS enzymes, we performed mass spectrometry analyses from Percoll gradient-enriched mitochondria. Supporting the results from the BN-PAGE assays, several complex I and IV subunits were decreased in abundance in the D263A/H1038A $>F_{35}$ intercrossed compound heterozygous flies (Fig. 3*B*). Many proteins related to metabolism, OXPHOS, and mtDNA gene expression were increased in abundance in flies with a high mtDNA mutation load (*SI Appendix*, Fig. S5*D*). Intriguingly, it has been reported that the complex V subunit alpha (ATP5A1) is highly up-regulated in the mtDNA mutator mouse heart (45). In flies with mtDNA mutations, several complex V subunits were up-regulated, including *bellwether* (*blw*), the fly ortholog of ATP5A1 (*SI Appendix*, Fig. S5*D*). The increase of ATP5A1 is likely explained by a conserved mitochondrial dysfunction response present in both flies and mammals.

Impaired Neuromuscular Function in Flies with mtDNA Mutations. We proceeded to study the physiological consequences of different levels of mtDNA mutations in flies. In humans, mtDNA mutations affect mostly high-energy-demanding tissues, for example the neuromuscular system (46). This is true also in model organisms such as flies, where mutations in nuclear-encoded mitochondrial proteins or mutations in mtDNA are known to impair the function of neurons and muscles (47). We quantified the locomotor activity of flies with various levels of mtDNA mutations (Fig. 3 *C* and *D*). Fly lines with low or medium levels of mtDNA mutations (D263A/+, D263A/H1038A F_1, and D263A/H1038A F_5) or various *DmPOLγA* alleles (Rescue/+, H1038A/+, and D263A/+) did not have any measurable impairment in their locomotor activity (Fig. 3*D*). However, flies with high levels of clonally expanded mtDNA mutations (D263A/H1038A $>F_{35}$) showed decreased physical activity during the morning bout (Fig. 3 *C* and *D*), consistent with neuronal and/or muscular defects.

To address whether clonally expanded mtDNA mutations can cause neuronal defects, we tested the mechanical stress sensitivity of mutant flies. Mutations in several mitochondrial proteins, including mRpS12, ANT, citrate synthase, and dYme1L, are known to make flies sensitive to mechanical stress (a.k.a. bang-sensitive) (48, 49). The impairment of mitochondrial function is believed to lead to neuronal hyperexcitability as these phenotypes can be suppressed by anticonvulsants (49). Therefore, to study whether the bang-sensitivity phenotype can be caused by mutations in mtDNA, we exposed flies with different *DmPOLγA* alleles and different mtDNA mutation levels to mechanical stress. Similar to the locomotor assay, flies carrying different *DmPOLγA* alleles or low levels of mtDNA mutations did not show any increase in bang sensitivity, whereas flies with clonally expanded mtDNA mutations (D263A/H1038A $>F_{35}$) were increasingly susceptible to mechanical stress-induced paralysis as they aged (Fig. 3*E*).

In summary, our results show that the different *DmPOLγA* alleles, some of which cause somatic mutagenesis of mtDNA, do not have any effect on fly physiology as determined by lifespan measurement, locomotor activity, or sensitivity to mechanical stress. In contrast, the health span of flies is markedly affected by mutations of mtDNA that have had a substantial amount of time to expand clonally over several generations.

Fruit Flies with High Levels of Clonally Expanded mtDNA Mutations Are Sensitive to Starvation. Mass spectrometry analysis of protein expression showed that several mitochondrial enzymes related to metabolism were up-regulated in flies with clonally expanded mtDNA mutations (*SI Appendix*, Fig. S5*D*). We therefore assessed the effects of mtDNA mutations on fly physiology and metabolism by performing a starvation assay. The presence of various *DmPOLγA* alleles (+/Rescue F_0, +/H1038A F_0, +/D263A F_0, H1038A/D263A F_1, and H1038A/D263A F_5) did not affect the starvation sensitivity of flies and only the presence of high levels of clonally expanded mtDNA mutations (D263A/H1038A $>F_{35}$) made flies starvation-sensitive (Fig. 4 *A* and *B* and Dataset S1). In this respect, it is interesting to note that mtDNA haplotypes have been reported to affect the starvation resistance of *Drosophila simulans* (50) and that *Drosophila melanogaster* strains carrying a homoplasmic mutation in ND2 have impaired fat storage (51).

To understand whether the starvation sensitivity was caused by changes in the amount of stored lipids and/or failure to mobilize lipids, we quantified fly lipid content (Fig. 4 *C* and *D*). The presence of *DmPOLγA* alleles (Rescue/+, H1038A/+, and D263A/+) or low levels of mtDNA mutations (D263A/+ and D263A/H1038A F_1) did not affect total body lipid content (Fig. 4*C*), consistent with the normal response to starvation. However, D263A/H1038A $>F_{35}$ flies with clonally expanded mtDNA mutations showed a significant decrease in total body fat content (Fig. 4 *C* and *D*) and were sensitive to starvation. Quantification of lipid content after starvation showed that flies, including those with high levels of mtDNA mutations, were able to mobilize their lipid stores upon starvation (Fig. 4*D*). Additionally, the total lipid content was correlated with the total body weight as flies with high levels of mtDNA mutations (D263A/H1038A $>F_{35}$) had a small but significant decrease in total body mass (Fig. 4*E*). These results suggest that increased mtDNA mutagenesis does not impair the function of β-oxidation.

The observation that flies with mtDNA mutations were passive in locomotor assays and had slightly decreased total body weight and fat content prompted us to further study their behavior. To this end, we quantified the feeding activity of flies with different *DmPOLγA* alleles and variable levels of mtDNA mutations by counting proboscis extensions onto the food surface in regular time intervals (52). In line with the previous results, only D263A/H1038A $>F_{35}$ flies with high levels of mtDNA mutations showed decreased feeding activity (Fig. 4*F*), probably explaining the observed decline in total body mass and lipid content. The decreased feeding could originate from impaired neuronal function as indicated by the locomotor and bang-sensitivity assays.

Mitochondrial Dysfunction Abrogates DR-Mediated Lifespan Extension. Given the observed metabolic effects caused by high levels of mtDNA mutations, we investigated the response to DR in flies with low (D263A/+ F_1, H1038A/D263A F_1, and H1038A/D263A F_5), medium (D263A/H1038A F_5), or high (D263A/H1038A $>F_{35}$) levels of mtDNA mutations. We investigated how DR will affect lifespan by using food sources with four different yeast concentrations [0.1×, 0.5×, 1×, and 2× sucrose–yeast–agar (SYA) food]. The WT flies showed the longest lifespan under 0.5× and 1× SYA conditions, whereas a shorter lifespan was observed under low or high (0.1× and 2× SYA) yeast concentrations (Fig. 5, *SI Appendix*, Fig. S6 *A* and *B*, and Dataset S1). Flies with low and medium levels of mtDNA mutations responded to DR in a similar way as controls (Fig. 5, *SI Appendix*, Fig. S6 *A* and *B*, and Dataset S1). However, flies with high levels of mtDNA mutations showed only an initial response to 0.1× SYA food but failed to respond to all other DR conditions (Fig. 5*B*, *SI Appendix*, Fig. S6*B*, and Dataset S1). It has been previously shown that knockdown of certain nuclear-encoded mitochondrial proteins inhibits a proper response to DR (31, 32). Interestingly, our results show that also mitochondrial dysfunction originating from an increased mtDNA mutation load can attenuate the response to DR in flies.

High Levels of mtDNA Mutations Deteriorate Intestinal Stem Cell Function. It has previously been reported that decreased lipid levels in flies often correlate with intestinal barrier dysfunction (53) and that intestinal function is important for the proper DR response (54). MtDNA mutations have been shown to accumulate in somatic stem cells in stomach, liver, and colonic crypts of

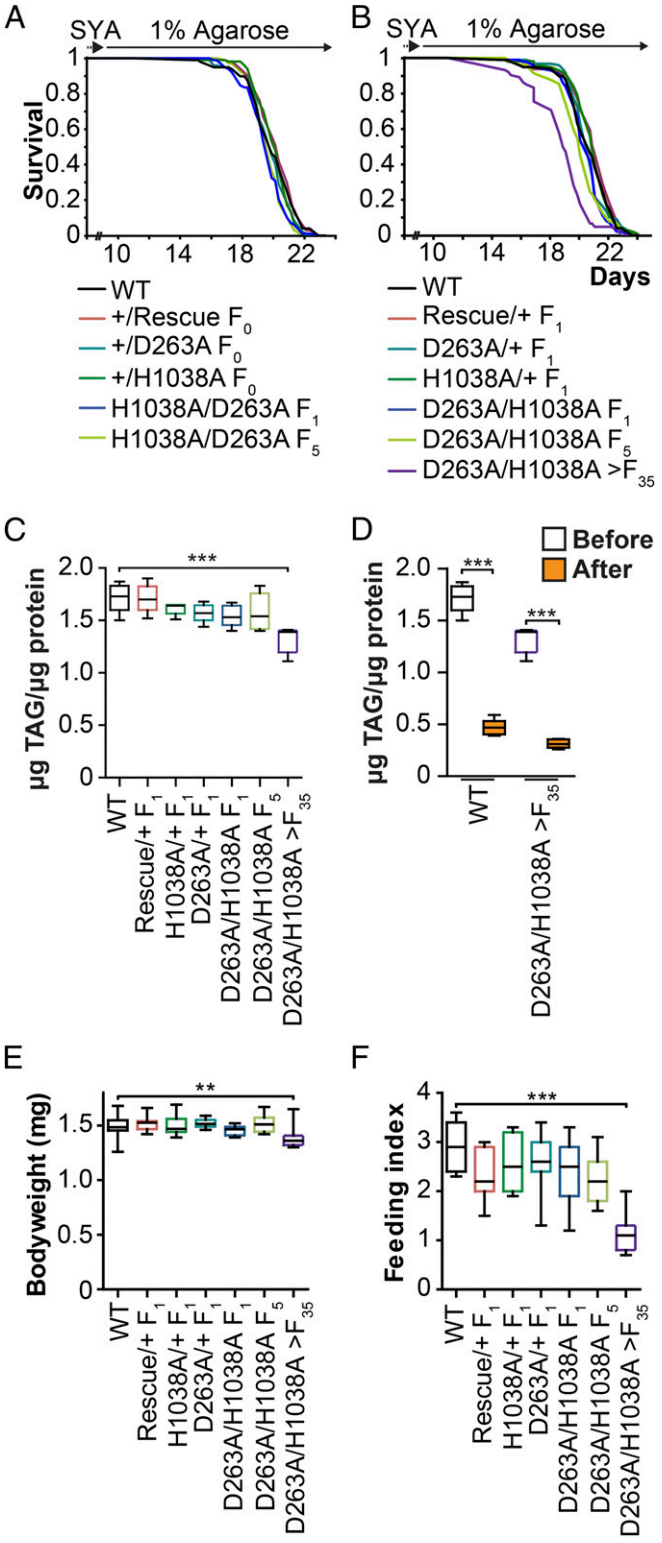

Fig. 4. Fruit flies with high levels of clonally expanded mtDNA mutations are sensitive to starvation. (*A* and *B*) Starvation assay of heterozygous flies with different *DmPOLγA* alleles inherited (*A*) paternally or (*B*) maternally and compound heterozygous flies with (*A*) only somatic (H1038A/D263A F_1 and H1038A/D263A F_5) mutations or (*B*) low (D263A/H1038A F_1), medium (D263A/H1038A F_5), and high (D263A/H1038A $>F_{35}$) levels of mtDNA mutations. Flies were kept 10 d on normal 1× SYA food before being transferred to 1% agarose. $P < 0.0001$, log-rank test. (*C* and *D*) Quantification of lipids in adult flies (*C*) before and (*D*) before and after starvation carrying different *DmPOLγA* alleles as heterozygous and compound heterozygous flies with low (D263A/H1038A F_1), medium (D263A/H1038A F_5), and high (D263A/H1038A $>F_{35}$) levels of mtDNA mutations. ***$P < 0.001$. (*C*) One-way ANOVA with Dunnett's post hoc test. (*D*) Student's two-tailed *t* test. (*E* and *F*) (*E*) Body weight and (*F*) feeding activity of adult heterozygous flies with different *DmPOLγA* alleles (Rescue/+, H1038A/+, and D263A/+) and of compound heterozygous flies with low (D263A/H1038A F_1), medium (D263A/H1038A F_5), and high (D263A/H1038A $>F_{35}$) levels of mtDNA mutations. ***$P < 0.001$. One-way ANOVA with Dunnett's post hoc test.

humans and mice (55–58). In addition, mtDNA mutations have been reported to impair fat absorption in the intestine of the mtDNA mutator mouse (59). The absence of DR-mediated lifespan extension in flies with high levels of mtDNA mutations could therefore stem from intestinal stem cell (ISC) dysfunction. The proliferative capacity of the ISCs is important for preserving intestinal integrity under different stress or dietary conditions (60, 61), and mitochondrial dysfunction is known to contribute to tissue degeneration and aging by affecting homeostasis of somatic stem cells (20, 53, 58).

To test whether mtDNA mutations can compromise ISC function, we exposed 7-d-old and 14-d-old adult flies to a mitogenic condition by feeding them *Erwinia carotovora carotovora 15* (Ecc15), a known inducer of stem cell proliferation in the gut of *Drosophila* (62). Among young flies (7 d old), only short-lived flies (D263A/H1038A $>F_{35}$) showed failed proliferation of ISC and loss of ISCs as indicated by the lack of the Delta marker (Fig. 6 *A* and *E*) and an M-phase-specific cell-cycle marker (phosphorylation at Ser10 of histone-3, PH3) (Fig. 6*B*). This phenotype was not driven by complementation per se as ISCs of compound heterozygous flies with low levels of mtDNA mutations (H1038A/D263A) were able to proliferate (Fig. 6*C*). Therefore, to assess the contribution of inherited mtDNA mutations alone to the observed ISC phenotype, D263A/H1038A $>F_{35}$ flies were outcrossed to WT nuclear background while maintaining mtDNA mutations [$^{+/+}$ (mut mtDNA) $>F_{35}$; see *SI Appendix*, Fig. S7]. These flies showed loss of surviving stem cells after mitogenic stress (Fig. 6 *A* and *D*) and decreased stress-induced ISC proliferation at older age (14 d old) (Fig. 6*C*), although to lesser extent in comparison with the parental D263A/H1038A $>F_{35}$ line. These results suggest that the nuclear background of compound heterozygote flies is contributing to the observed phenotypes. As expected, D263A/H1038A $>F_{35}$ flies outcrossed to a WT nuclear background and WT mtDNA [$^{+/+}$ (clean mtDNA) $>F_{35}$] were able to respond to mitogenic stress similar to WT controls (Fig. 6 *A*–*D*).

These results are in line with findings from other organisms where mitochondrial dysfunction has been shown to impair stem cell function (20, 63) and suggest that clonally expanded mtDNA mutations may be the driving force behind the observed decline in fly lifespan.

Discussion

The role of mitochondria in aging has attracted a lot of interest ever since it was shown that increasing the amount of somatic mtDNA mutations in mice causes premature aging phenotypes (16, 17), and even mimics some phenotypes of aging humans (58). The amount of mtDNA mutations is known to increase in various species with advancing age (11–15), but the role of mtDNA mutations in aging of short-lived organisms has not been fully elucidated. To address this question, we examined whether mtDNA mutations can accelerate aging phenotypes and affect physiology of fruit flies. Surprisingly, we report that adult fruit flies are highly tolerant toward high levels of mtDNA mutations, which suggests that mtDNA mutations are not a major contributor to aging in fruit flies.

We demonstrate here that mtDNA mutations accumulate in the female germline of mtDNA mutator flies with age. Consequently, older females transmit more mtDNA mutations to their progeny than young females. Interestingly, transmission of mtDNA mutations from either young or old females had no effect on the lifespan of the progeny. This somewhat surprising

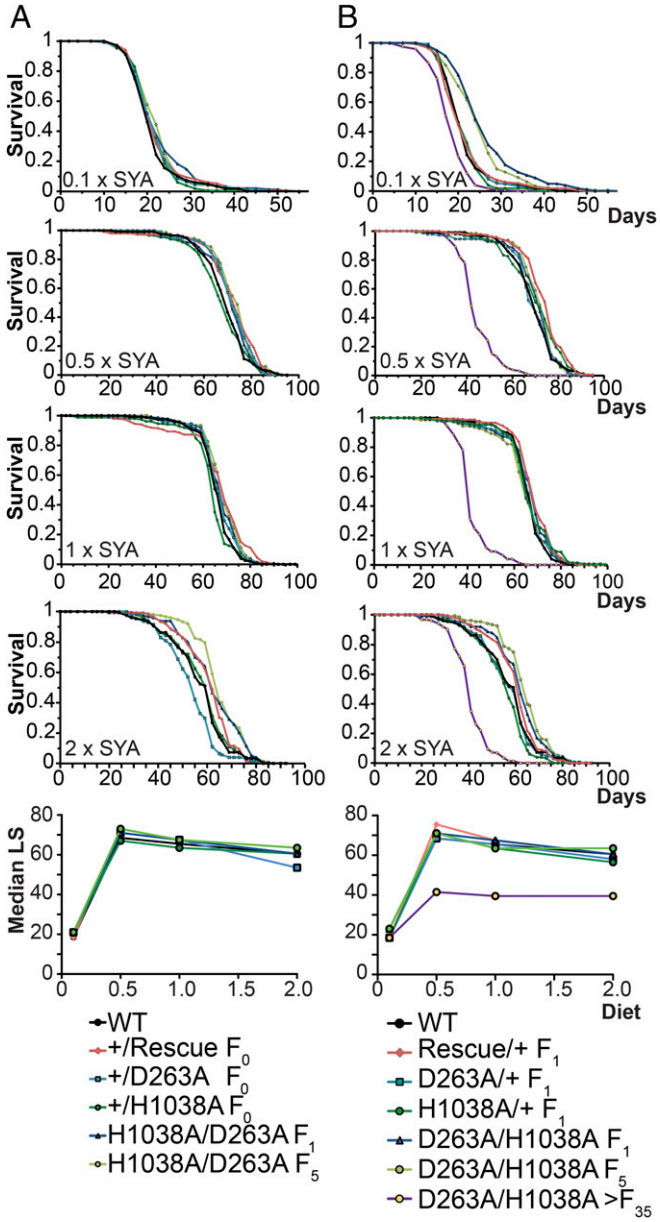

Fig. 5. Flies with high levels of mtDNA mutations do not respond to DR. (*A*) Lifespan analysis of flies with only somatic mtDNA mutations. The effect of different yeast concentrations (0.1×, 0.5×, 1×, and 2× SYA) on fly lifespan was determined. Heterozygous flies (+/Rescue F_0, +/D263A F_0, and +/H1038A F_0) and compound heterozygous (H1038A/D263A F_1 and H1038A/D263A F_5) flies, all of which inherited D263A allele paternally, were used to test if somatic mtDNA mutations affect DR-mediated lifespan extension. Parametric survival analysis with logistic distribution (lowest Akaike information criterion) was used to analyze responses to DR. $P < 0.0001$, log-rank test. (*B*) Lifespan analysis of flies with inherited mtDNA mutations. Heterozygous flies (Rescue/+ F_1, D263A/+ F_1, and H1038A/+ F_1) and compound heterozygous flies with low (D263A/H1038A F_1), medium (D263A/H1038A F_5), and high levels (D263A/H1038A $>F_{35}$) of clonally expanded mtDNA mutations, all of which inherited D263A allele maternally, were fed on different DR conditions (0.1×, 0.5×, 1×, and 2× SYA) to test if clonally expanded mtDNA mutations attenuate beneficial effects of DR on fly lifespan. Parametric survival analysis with logistic distribution (lowest Akaike information criterion) was used to analyze responses to DR. $P < 0.0001$, log-rank test.

finding may be explained by the fact that most of the replication errors of mtDNA are made in early development of fruit flies (34), zebrafish (64), mice (65), and humans (66). In somatic tissues, the levels of mtDNA mutations fluctuate because of random genetic drift (58, 66), and in mammals this leads to focal OXPHOS dysfunction in a subset of cells in aging tissues (10). Based on mathematical modeling, it has been predicted that humans require decades before a de novo mtDNA mutation can reach a threshold level sufficient to cause a biochemical defect (67). Indeed, WT mice, in contrast to humans, show limited age-associated clonal expansion of mtDNA mutations, likely due to the shorter lifespan (33, 68). In flies, the female germline is one of the tissues with highest proliferation. Despite this, the maximal shift in heteroplasmy levels across generations is only up to 12% (44), suggesting that postmitotic tissues of the fly have minimal shifts in mtDNA heteroplasmy with age. Therefore, it is highly unlikely that somatic mtDNA mutations can reach the threshold needed to limit the lifespan of short-lived organisms such as fruit flies. To test this hypothesis, we investigated how somatic mtDNA mutations accumulate in postmitotic tissues with age and how these mtDNA mutations affect the fly lifespan. Our results show that somatic mtDNA mutations do not accumulate in postmitotic tissues of the aging fly and are therefore unlikely to limit the lifespan.

The absence of effects of mtDNA mutations on lifespan and/or health span in adult flies is in strong contrast with our previous finding that even low levels of mtDNA mutations cause developmental delay in flies (34). This could be an indication that the energetic demands in developing larvae are substantially higher than in adult flies and therefore even low levels of mtDNA mutations may be sufficient to affect development. Some additional support for this hypothesis is provided by studies showing that knockdown of certain OXPHOS subunits causes developmental lethality in flies, whereas knockdown in adulthood even can have lifespan-extending effects (27). The low impact of mtDNA mutations on fly lifespan can also be explained by the fact that mtDNA mutations cause focal respiratory chain dysfunction in human tissues only when present above a certain critical threshold. In humans and other mammals rapid shifts in mtDNA genotypes can occur in just a few generations, whereas we show here that similar genotype shifts do not occur in fruit flies, consistent with our previous report that mtDNA point mutations only accumulate slowly between generations (34). Even after 15 generations of intercrossing of mtDNA mutator flies (D263A/+), we could not detect any impact of mtDNA mutations on fly lifespan. To address whether mtDNA mutations can eventually reach critical levels to limit the fly lifespan, we extensively intercrossed D263A/H1038A compound heterozygous flies to generate flies with high levels of clonally expanded mtDNA mutations. These flies displayed strong mitochondrial dysfunction causing severe, often age-dependent, physiological alterations, such as decreased locomotor activity, decreased feeding, and sensitivity to mechanical stress. Interestingly, many of these same phenotypes have been seen in flies with mutations in nuclear-encoded mitochondrial proteins (69). We further showed that short-lived D263A/H1038A $>F_{35}$ flies have a marked reduction in proliferative capacity of ISCs, which is an important determinant of *Drosophila* aging (53, 60). This is in line with previous findings showing that mitochondrial dysfunction can impair cell-cycle progression and stem cell maintenance in fruit flies (70, 71). Depending on the type of dysfunction, activation of AMPK signaling or ROS-mediated activation of JNK signaling can occur. It has also been proposed that the progeroid phenotype of mtDNA mutator mice can be at least partly attributed to somatic stem cell dysfunction due to altered ROS signaling (20). Intestinal fly stem cells are considerably better defined than mammalian stem cells and the fly lines we presented here will provide powerful models to further study the connection between mitochondrial function and stem cell maintenance under different stress or dietary conditions.

In conclusion, we report here that fruit flies are less sensitive to mtDNA mutations in adulthood than during development. Moreover, our findings give experimental support to mathematical models suggesting that only limited clonal expansion of somatic mtDNA mutations can occur in short-lived organisms

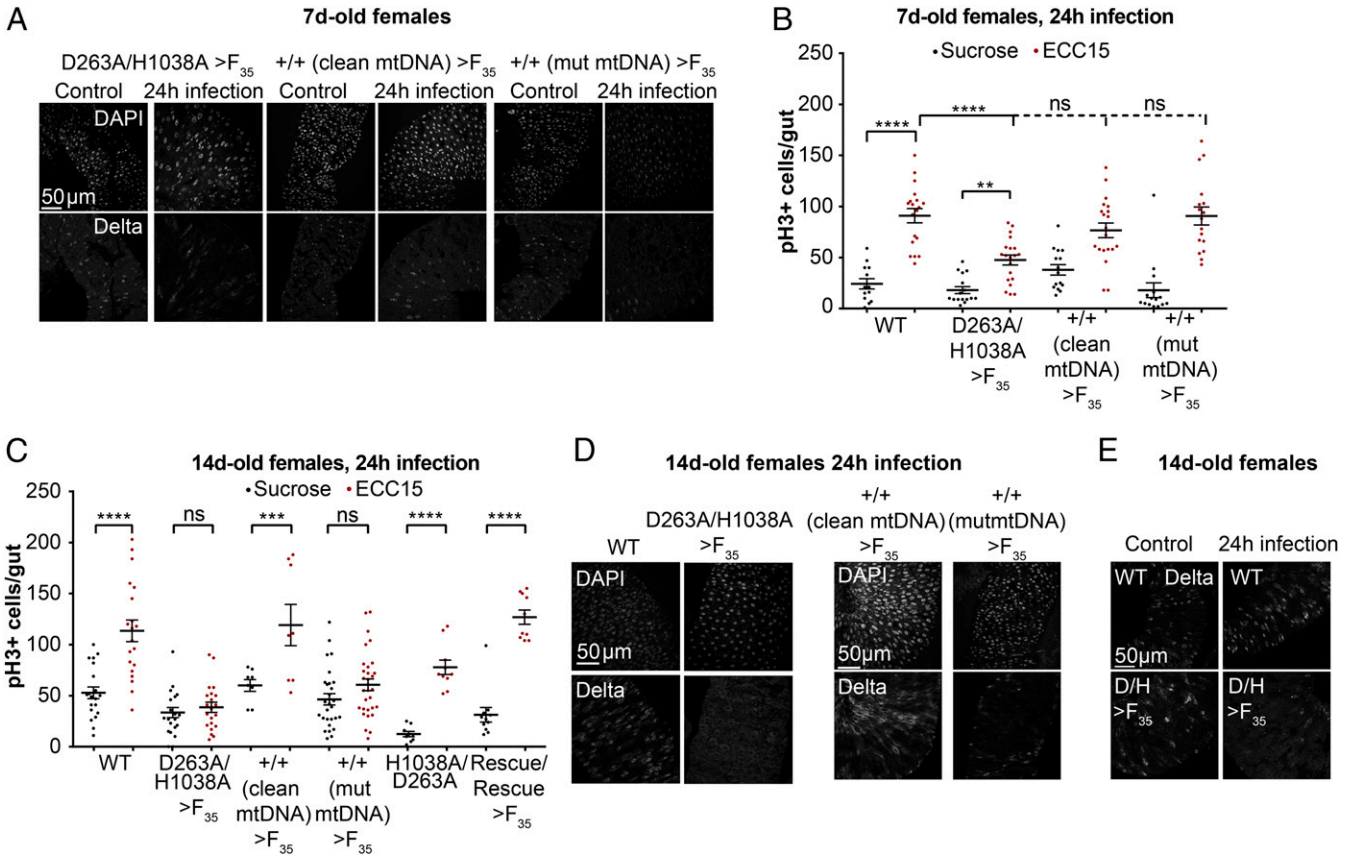

Fig. 6. mtDNA mutations affect proliferation of ISCs. (*A*) The number of ISCs is reduced upon infection with Ecc15 in 7-d-old D263A/H1038A >F_{35} flies but not in 7-d-old D263A/H1038A >F_{35} outbred flies without [$^{+/+}$ (clean mtDNA) >F_{35}] and with [$^{+/+}$ (mut mtDNA) >F_{35}] mtDNA mutations compared with corresponding controls. In all panels, nuclear DNA is stained with DAPI, whereas ISCs are detected by immunohistochemistry using a Delta antibody. (*B* and *C*) Proliferation of ISCs is reduced upon infection with Ecc15 in young (7-d-old) and older (14-d-old) flies with high levels of mtDNA mutations (D263A/H1038A >F_{35}), respectively. D263A/H1038A >F_{35} outbred flies with mutated mtDNA [$^{+/+}$ (mut mtDNA) >F_{35}] showed reduced proliferation of ISCs only after 14 d of age. Proliferation of ISCs was determined by staining the cells with pH3 antibody. (*D* and *E*) The number of ISCs is reduced upon infection with Ecc15 in 14-d-old D263A/H1038A >F_{35} flies and 14-d-old D263A/H1038A >F_{35} outbred flies with mutated mtDNA [$^{+/+}$ (mut mtDNA) >F_{35}] but not in 14-d-old D263A/H1038A >F_{35} outbred flies without mtDNA mutations [$^{+/+}$ (clean mtDNA) >F_{35}] compared with corresponding controls. In all panels, nuclear DNA is stained with DAPI, whereas ISCs are detected by immunohistochemistry using a Delta antibody. ***$P < 0.01$, ****$P < 0.001$, ns (nonsignificant) > 0.05, one-way ANOVA.

like fruit flies. Furthermore, extensive intercrossing of flies with decreased proofreading capacity and a decreased genetic bottleneck can be used to establish fly lines with clonally expanded mtDNA mutations. These mtDNA mutations have various physiological consequences, such as intestinal barrier dysfunction and neuronal and muscle dysfunction, resulting eventually in shortened adult lifespan of fruit flies. Our results show that mtDNA mutations do not have a major role in limiting the health- or lifespan of natural fly populations, because the experimentally obtained very high mtDNA mutation levels that we report here are unlikely to be found in nature.

Materials and Methods

See *SI Appendix* for additional methods.

Lifespan, DR, and Starvation Assays. For lifespan 200 female flies were distributed equally with 10 flies per vial on 1× SYA medium and transferred into new vials with fresh food every 2–3 d. Dead flies were counted during every transfer. All lifespans were done at least twice. For DR experiments, 150–200 female flies were distributed equally with 10 flies per vial and transferred into new vials with fresh food every 2–3 d. Flies were kept on 0.1×, 0.5×, 1×, or 2× SYA food. DR experiments were done using the optimized DR protocol described in ref. 72 and repeated twice.

For starvation assays, 100 female flies per genotype were distributed at a density of 20 flies per vial onto 1× SYA food and aged for 10 d. Flies were then moved to vials with starvation medium (1% agarose) and mortality was determined by counting dead flies three times per day. Results from all assays are expressed as the proportion of survivors ±95% confidence interval.

Gut Staining. One day before indicated ages, adult female *Drosophila* guts were starved for 4 h to synchronize feeding then transferred to 5% sucrose ± Ecc15 from 15 mL of overnight culture. After 24 h guts were dissected in 1× PBS, fixed for 45 min at room temperature (100 mM glutamic acid, 25 mM KCl, 20 mM $MgSO_4$, 4 mM sodium phosphate, 1 mM $MgCl_2$, and 4% formaldehyde), washed for 1 h at 4 °C (1× PBS, 0.5% BSA, and 0.1% Triton X-100), and then incubated with primary antibodies (4 °C overnight) and secondary antibodies (4 °C for 2 h) in washing buffer, washing three times for 10 min after each antibody. Further details are provided in *SI Appendix*.

Statistical Analysis. GraphPad Prism (GraphPad Prism Software, Inc.) was used for statistical analyses. Comparison of two samples was done using two-tailed Student's *t* test. For multiple comparisons one-way ANOVA with Dunnett's post hoc test was used. Parametric survival analysis with logistic distribution (lowest Akaike information criterion) was used to analyze responses to DR.

Log-rank test was used for lifespan, DR, and starvation analyses and an alpha of 0.001 was used as the cutoff for statistical significance. Comparisons between ISC proliferation counts were done by one-way ANOVA, using Sidak's post hoc test. Each dataset comprises two or more independent experiments.

ACKNOWLEDGMENTS. We thank Xinping Li (Proteomic Core Facility of the Max Planck Institute for Biology of Ageing) for liquid chromatography mass spectrometry analyses, Luke Tain for assistance in statistical analyses, and Stefanie Kipschull and Avan Taha for technical assistance. This work was supported by the Max Planck Society, Swedish Research Council Grant 2015-00418, and the Knut and Alice Wallenberg Foundation (N.-G.L.). F.B. was supported by AXA Research Fund.

1. Calvo SE, Clauser KR, Mootha VK (2016) MitoCarta2.0: An updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Res* 44:D1251–D1257.
2. Hällberg BM, Larsson N-G (2014) Making proteins in the powerhouse. *Cell Metab* 20: 226–240.
3. Gorman GS, et al. (2016) Mitochondrial diseases. *Nat Rev Dis Primers* 2:16080.
4. Kauppila TES, Kauppila JHK, Larsson N-G (2017) Mammalian mitochondria and aging: An update. *Cell Metab* 25:57–71.
5. Müller-Höcker J (1989) Cytochrome-c-oxidase deficient cardiomyocytes in the human heart–An age-related phenomenon. A histochemical ultracytochemical study. *Am J Pathol* 134:1167–1173.
6. Fayet G, et al. (2002) Ageing muscle: Clonal expansions of mitochondrial DNA point mutations and deletions cause focal impairment of mitochondrial function. *Neuromuscul Disord* 12:484–493.
7. Ferguson M, Mockett RJ, Shen Y, Orr WC, Sohal RS (2005) Age-associated decline in mitochondrial respiration and electron transport in Drosophila melanogaster. *Biochem J* 390:501–511.
8. Bua E, et al. (2006) Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. *Am J Hum Genet* 79:469–480.
9. Herbst A, et al. (2016) Latent mitochondrial DNA deletion mutations drive muscle fiber loss at old age. *Aging Cell* 15:1132–1139.
10. Larsson N-G (2010) Somatic mitochondrial DNA mutations in mammalian aging. *Annu Rev Biochem* 79:683–706.
11. Itsara LS, et al. (2014) Oxidative stress is not a major contributor to somatic mitochondrial DNA mutations. *PLoS Genet* 10:e1003974.
12. Yui R, Ohno Y, Matsuura ET (2003) Accumulation of deleted mitochondrial DNA in aging Drosophila melanogaster. *Genes Genet Syst* 78:245–251.
13. Williams SL, Mash DC, Züchner S, Moraes CT (2013) Somatic mtDNA mutation spectra in the aging human putamen. *PLoS Genet* 9:e1003990.
14. Schwarze SR, et al. (1995) High levels of mitochondrial DNA deletions in skeletal muscle of old rhesus monkeys. *Mech Ageing Dev* 83:91–101.
15. Khaidakov M, Heflich RH, Manjanatha MG, Myers MB, Aidoo A (2003) Accumulation of point mutations in mitochondrial DNA of aging mice. *Mutat Res* 526:1–7.
16. Trifunovic A, et al. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 429:417–423.
17. Kujoth GC, et al. (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* 309:481–484.
18. Jiang M, et al. (2017) Increased total mtDNA copy number cures male infertility despite unaltered mtDNA mutation load. *Cell Metab* 26:429–436.e4.
19. Ahlqvist KJ, et al. (2015) MtDNA mutagenesis impairs elimination of mitochondria during erythroid maturation leading to enhanced erythrocyte destruction. *Nat Commun* 6:6494.
20. Ahlqvist KJ, et al. (2012) Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in Polg mutator mice. *Cell Metab* 15: 100–109.
21. Cerutti R, et al. (2014) NAD(+)-dependent activation of Sirt1 corrects the phenotype in a mouse model of mitochondrial disease. *Cell Metab* 19:1042–1049.
22. Fontana L, Partridge L (2015) Promoting health and longevity through diet: From model organisms to humans. *Cell* 161:106–118.
23. Masoro EJ (2002) Caloric restriction-induced life extension of rats and mice: A critique of proposed mechanisms. *Biochim Biophys Acta* 1790:1040–1048.
24. Ramsey JJ, Hagopian K (2006) Energy expenditure and restriction of energy intake: Could energy restriction alter energy expenditure in companion animals? *J Nutr* 136(Suppl 7):1958S–1966S.
25. Selman C, et al. (2005) Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition. *Mech Ageing Dev* 126:783–793.
26. Hunt ND, et al. (2006) Bioenergetics of aging and calorie restriction. *Ageing Res Rev* 5: 125–143.
27. Copeland JM, et al. (2009) Extension of Drosophila life span by RNAi of the mitochondrial respiratory chain. *Curr Biol* 19:1591–1598.
28. Zuryn S, Kuang J, Tuck A, Ebert PR (2010) Mitochondrial dysfunction in Caenorhabditis elegans causes metabolic restructuring, but this is not linked to longevity. *Mech Ageing Dev* 131:554–561.
29. Dell'agnello C, et al. (2007) Increased longevity and refractoriness to Ca(2+)-dependent neurodegeneration in Surf1 knockout mice. *Hum Mol Genet* 16:431–444.
30. Mookerjee SA, Divakaruni AS, Jastroch M, Brand MD (2010) Mitochondrial uncoupling and lifespan. *Mech Ageing Dev* 131:463–472.
31. Zid BM, et al. (2009) 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. *Cell* 139:149–160.
32. Bahadorani S, Hur JH, Lo T, Jr, Vu K, Walker DW (2010) Perturbation of mitochondrial complex V alters the response to dietary restriction in Drosophila. *Aging Cell* 9:100–103.
33. Kowald A, Kirkwood TBL (2013) Mitochondrial mutations and aging: Random drift is insufficient to explain the accumulation of mitochondrial deletion mutants in short-lived animals. *Aging Cell* 12:728–731.
34. Bratic A, et al. (2015) Complementation between polymerase- and exonuclease-deficient mitochondrial DNA polymerase mutants in genomically engineered flies. *Nat Commun* 6:8808.
35. Hurd TR, et al. (2016) Long Oskar controls mitochondrial inheritance in Drosophila melanogaster. *Dev Cell* 39:560–571.
36. Ross JM, Coppotelli G, Hoffer BJ, Olson L (2014) Maternally transmitted mitochondrial DNA mutations can reduce lifespan. *Sci Rep* 4:6569.
37. Siibak T, et al. (2017) A multi-systemic mitochondrial disorder due to a dominant p. Y955H disease variant in DNA polymerase gamma. *Hum Mol Genet* 26:2515–2525.
38. Priest NK, Mackowiak B, Promislow DE (2002) The role of parental age effects on the evolution of aging. *Evolution* 56:927–935.
39. Lansing AI (1947) A transmissible, cumulative, and reversible factor in aging. *J Gerontol* 2:228–239.
40. Ross JM, et al. (2013) Germline mitochondrial DNA mutations aggravate ageing and can impair brain development. *Nature* 501:412–415.
41. Burman JL, et al. (2014) A Drosophila model of mitochondrial disease caused by a complex I mutation that uncouples proton pumping from electron transfer. *Dis Model Mech* 7:1165–1174.
42. Celotto AM, Chiu WK, Van Voorhies W, Palladino MJ (2011) Modes of metabolic compensation during mitochondrial disease using the Drosophila model of ATP6 dysfunction. *PLoS One* 6:e25823.
43. Xu H, DeLuca SZ, O'Farrell PH (2008) Manipulating the metazoan mitochondrial genome with targeted restriction enzymes. *Science* 321:575–577.
44. Solignac M, Génermont J, Monnerot M, Mounolou JC (1987) Drosophila mitochondrial genetics: Evolution of heteroplasmy through germ line cell divisions. *Genetics* 117:687–696.
45. Edgar D, et al. (2009) Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice. *Cell Metab* 10:131–138.
46. Chinnery PF (2015) Mitochondrial disease in adults: What's old and what's new? *EMBO Mol Med* 7:1503–1512.
47. Sen A, Cox RT (2017) Fly models of human diseases: Drosophila as a model for understanding human mitochondrial mutations and disease. *Curr Top Dev Biol* 121:1–27.
48. Qi Y, Liu H, Daniels MP, Zhang G, Xu H (2016) Loss of Drosophila i-AAA protease, dYME1L, causes abnormal mitochondria and apoptotic degeneration. *Cell Death Differ* 23:291–302.
49. Pandey UB, Nichols CD (2011) Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacol Rev* 63:411–436.
50. Ballard JW, Melvin RG, Katewa SD, Maas K (2007) Mitochondrial DNA variation is associated with measurable differences in life-history traits and mitochondrial metabolism in Drosophila simulans. *Evolution* 61:1735–1747.
51. Wang A, Mouser J, Pitt J, Promislow D, Kaeberlein M (2016) Rapamycin enhances survival in a Drosophila model of mitochondrial disease. *Oncotarget* 7:80131–80139.
52. Wong R, Piper MDW, Wertheim B, Partridge L (2009) Quantification of food intake in Drosophila. *PLoS One* 4:e6063.
53. Rera M, Clark RI, Walker DW (2012) Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila. *Proc Natl Acad Sci USA* 109: 21528–21533.
54. Regan JC, et al. (2016) Sex difference in pathology of the ageing gut mediates the greater response of female lifespan to dietary restriction. *eLife* 5:e10956.
55. McDonald SA, et al. (2008) Mechanisms of field cancerization in the human stomach: The expansion and spread of mutated gastric stem cells. *Gastroenterology* 134:500–510.
56. Fellous TG, et al. (2009) Locating the stem cell niche and tracing hepatocyte lineages in human liver. *Hepatology* 49:1655–1663.
57. Taylor RW, et al. (2003) Mitochondrial DNA mutations in human colonic crypt stem cells. *J Clin Invest* 112:1351–1360.
58. Baines HL, et al. (2014) Similar patterns of clonally expanded somatic mtDNA mutations in the colon of heterozygous mtDNA mutator mice and ageing humans. *Mech Ageing Dev* 139:22–30.
59. Fox RG, Magness S, Kujoth GC, Prolla TA, Maeda N (2012) Mitochondrial DNA polymerase editing mutation, PolgD257A, disturbs stem-progenitor cell cycling in the small intestine and restricts excess fat absorption. *Am J Physiol Gastrointest Liver Physiol* 302:G914–G924.
60. Jasper H (2015) Exploring the physiology and pathology of aging in the intestine of Drosophila melanogaster. *Invertebr Reprod Dev* 59(Suppl 1):51–58.
61. Biteau B, et al. (2010) Lifespan extension by preserving proliferative homeostasis in Drosophila. *PLoS Genet* 6:e1001159.
62. Buchon N, Broderick NA, Kuraishi T, Lemaitre B (2010) Drosophila EGFR pathway coordinates stem cell proliferation and gut remodeling following infection. *BMC Biol* 8:152.
63. Norddahl GL, et al. (2011) Accumulating mitochondrial DNA mutations drive premature hematopoietic aging phenotypes distinct from physiological stem cell aging. *Cell Stem Cell* 8:499–510.
64. Otten ABC, et al. (2016) Replication errors made during oogenesis lead to detectable de novo mtDNA mutations in zebrafish oocytes with a low mtDNA copy number. *Genetics* 204:1423–1431.
65. Ameur A, et al. (2011) Ultra-deep sequencing of mouse mitochondrial DNA: Mutational patterns and their origins. *PLoS Genet* 7:e1002028.
66. Greaves LC, et al. (2014) Clonal expansion of early to mid-life mitochondrial DNA point mutations drives mitochondrial dysfunction during human ageing. *PLoS Genet* 10:e1004620.
67. Elson JL, Samuels DC, Turnbull DM, Chinnery PF (2001) Random intracellular drift explains the clonal expansion of mitochondrial DNA mutations with age. *Am J Hum Genet* 68:802–806.
68. Greaves LC, Barron MJ, Campbell-Shiel G, Kirkwood TBL, Turnbull DM (2011) Differences in the accumulation of mitochondrial defects with age in mice and humans. *Mech Ageing Dev* 132:588–591.
69. Fergestad T, Bostwick B, Ganetzky B (2006) Metabolic disruption in Drosophila bang-sensitive seizure mutants. *Genetics* 173:1357–1364.
70. Owusu-Ansah E, Yavari A, Mandal S, Banerjee U (2008) Distinct mitochondrial retrograde signals control the G1-S cell cycle checkpoint. *Nat Genet* 40:356–361.
71. Owusu-Ansah E, Banerjee U (2009) Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. *Nature* 461:537–541.
72. Bass TM, et al. (2007) Optimization of dietary restriction protocols in Drosophila. *J Gerontol A Biol Sci Med Sci* 62:1071–1081.