Modeling Pathogenic Mutations of Human Twinkle in Drosophila Suggests an Apoptosis Role in Response to Mitochondrial Defects

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Abstract

The human gene C10orf2 encodes the mitochondrial replicative DNA helicase Twinkle, mutations of which are responsible for a significant fraction of cases of autosomal dominant progressive external ophthalmoplegia (adPEO), a human mitochondrial disease caused by defects in intergenic communication. We report the analysis of orthologous mutations in the Drosophila melanogaster mitochondrial DNA (mtDNA) helicase gene, d-mtDNA helicase. Increased expression of wild type d-mtDNA helicase using the UAS-GAL4 system leads to an increase in mtDNA copy number throughout adult life without any noteworthy phenotype, whereas overexpression of d-mtDNA helicase containing the K388A mutation in the helicase active site results in a severe depletion of mtDNA and a lethal phenotype. Overexpression of two d-mtDNA helicase variants equivalent to two human adPEO mutations shows differential effects. The A442P mutation exhibits a dominant negative effect similar to that of the active site mutant. In contrast, overexpression of d-mtDNA helicase containing the W441C mutation results in a slight decrease in mtDNA copy number during the third instar larval stage, and a moderate decrease in life span in the adult population. Overexpression of d-mtDNA helicase containing either the K388A or A442P mutations causes a mitochondrial oxidative phosphorylation (OXPHOS) defect that significantly reduces cell proliferation. The mitochondrial impairment caused by these mutations promotes apoptosis, arguing that mitochondria regulate programmed cell death in Drosophila. Our study of d-mtDNA helicase overexpression provides a tractable Drosophila model for understanding the cellular and molecular effects of human adPEO mutations.

Introduction

The majority of cellular ATP is generated by oxidative phosphorylation carried out within the mitochondrial inner membrane. Animal mitochondrial DNA encodes 13 polypeptides involved in OXPHOS, whereas all the factors essential for mtDNA replication are encoded in the nuclear genome [1]. As a consequence, the biogenesis of the OXPHOS system is subject to a highly-coordinated, dual genetic control [2]. Mutations in genes encoding the factors essential for mtDNA replication results in both loss of mtDNA integrity via base substitution mutations, duplications and deletions, and in mtDNA depletion [3].

Autosomal dominant progressive external ophthalmoplegia is a human mitochondrial disease associated with the presence of multiple deletions in the mtDNA [3,4]. The disease has an adult-onset at 20–40 years of age. Its symptoms include muscle weakness, wasting, exercise intolerance, ataxia, hearing loss, cardiomyopathy and peripheral neuropathy [3]. Most adPEO families carry heterozygous mutations in one of three genes: ANT1 (adenine nucleotide translocator 1), POLG (mitochondrial DNA polymerase), or C10orf2/Twinkle (mtDNA helicase) [3,5,6,7].

Whereas the nuclear DNA replication machinery is very complex, the number of factors needed to replicate the mitochondrial DNA is comparatively small [8,9,10]. The human
gene C10orf2 encodes the essential mitochondrial replicative DNA helicase, Twinkle. Twinkle shares high homology with the bacteriophage T7 gene 4 protein (T7 gp4), which contains both helicase and primase catalytic activities located in its carboxyl- and amino-terminal halves, respectively [6]. The amino acid sequence of the T7 gp4 helicase domain is well conserved in Twinkle, but varies substantially in the primase domain. Twinkle co-localizes with mtDNA structures designated as mitochondrial nucleoids [6], and it has been shown to modulate mtDNA copy number in vivo [11]. Studies in human cell culture and in a transgenic mouse expressing a Twinkle variant with an in-frame duplication of amino acids 353–365 that is analogous to a human adPEO mutation showed the accumulation of multiple mtDNA deletions and mitochondrial dysfunction [12,13].

Twinkle mutations are mainly associated with adPEO, but have recently been linked to SANDO [14] and Infantile-Onset Spinocerebellar Ataxia (JOSCA) [15]. Surprisingly, neither mtDNA deletions nor point mutations have been found, suggesting that JOSCA mutations in Twinkle affect mtDNA stability in a physiological and tissue specific manner.

Recently, a novel protein with nuclease/helicase activity localized within the mitochondria has been described and designated as DNA2. In humans, hDNA2 forms a complex with the mitochondrial DNA polymerase to stimulate its DNA polymerase activity. Although little is known about this protein, it appears to play a role in processing mtDNA intermediates during replication and repair [16]. However, only a single mtDNA helicase (d-mtDNA helicase) has been identified in Drosophila.

Several studies have demonstrated that mitochondria play a crucial role in the apoptotic pathway in mammals [17], and thus mutations that affect mitochondrial function such as adPEO, could critically impact this pathway. Despite the fact that almost all of the proteins implicated in cell death are highly conserved in metazoans, the role of mitochondria in apoptosis in Drosophila remains controversial [18,19,20]. However, one study indicates a significant role for mitochondria in programmed cell death in the fly, where upon apoptosis, the Reaper and HID proteins cause mitochondrial fragmentation and release of cytochrome c in both cultured S2 cells and in the developing fly embryo [21]. It is known that factors involved in mitochondrial dynamics play a key role in the segregation of dysfunctional mitochondria [22], and deregulation of these pathways could trigger mitochondrially-mediated apoptosis.

In this study, we overexpressed various adPEO mutations of d-mtDNA helicase in Drosophila melanogaster to characterize their effects in a tractable animal model, and to analyze the role of dysfunctional mitochondria in apoptosis. Our data show that overexpression of adPEO mutations in the fly causes a severe depletion of mtDNA and as a consequence, an increase in programmed cell death. Taken together, our previous [23,24] and present results give evidence in vivo of the crucial role of mitochondria in apoptosis in Drosophila melanogaster, providing some clues for the understanding of the pathophysiology of human mitochondrial diseases.

**Results**

A Drosophila Model to Study adPEO Mutations

Genomic analysis has identified a number of mutations associated with adPEO in the human mtDNA helicase gene, C10orf2 [6]. The Drosophila genome encodes a highly conserved orthologue in the CG3924 gene, with the two proteins sharing 54.6% identity and 73% similarity (as evaluated by Mobyle Pasteur MATCHER). Most of the amino acid residues mutated in adPEO patients are conserved in the Drosophila protein [24], raising the possibility to establish Drosophila as a tractable animal model to study them. With this aim, we used the inducible UAS-GAL4 system to express d-mtDNA helicase variants containing human adPEO mutations. UAS lines with four versions of the gene were generated: the wild type (wt) version was used as a control, and three versions containing the “Walker A” [27] K388A active site mutation, analogous to K318 in the helicase domain of T7 gp4, and the W441C and A442P mutations, which are analogous to the W474C and A475P mutations found in adPEO patients [6]. These mutations map within the linker region and helicase domain, respectively (Figure 1). Previous results in Drosophila S2 cells showed severe mtDNA depletion when d-mtDNA helicase containing the K388A or A442P mutations were overexpressed, whereas cells that overexpressed d-mtDNA helicase containing the W441C mutation did not show a significant phenotype. In contrast to patients harbouring these mutations, multiple deletions in mtDNA were not observed in cultured fly cells [24].

To characterize the effects of overexpression of the variants of d-mtDNA helicase in Drosophila, the daughterless-GAL4 driver line was used to induce constitutive and ubiquitous expression. Overexpression of the K388A and A442P d-mtDNA helicase mutations caused complete lethality in third larval instar and pupal stages, respectively (Figure 2C–E). However, the overexpression of W441C variant and the wild type version did not show any observable phenotype (Figure 2, D). These results were quantified (Figure 2F) and corroborated with multiple independent lines for each mutation. Immunoblot analysis of third instar larvae expressing the d-mtDNA helicase variant and control lines showed relatively similar levels of d-mtDNA helicase protein among the different lines; in contrast, d-mtDNA helicase was undetectable in non-transgenic control animals under the same conditions indicating normally low levels of endogenous expression and the relatively high levels of transgene expression (Figure 2G).

**Constitutive Overexpression of Mutant d-mtDNA Helicase Induces mtDNA Depletion and OXPHOS Impairment in Drosophila**

The effect of overexpression of d-mtDNA helicase variants on mtDNA levels was evaluated by quantitative real time PCR analysis. We found a significant decrease in mtDNA copy number in third instar larvae of the lines overexpressing K388A, W441C and A442P mutations as compared with the control lines (Figure 3A). Overexpression of wild type d-mtDNA helicase did not cause decrease in mtDNA. However, despite the presence of multiple deletions in mtDNA being a common feature in skeletal muscle in adPEO patients, we found no evidence by Southern Blot (Figure 3B) and long range-PCR analysis (data not shown) of multiple deletions in the mutant or control lines. These results are comparable with data analysing overexpression of d-mtDNA helicase active site mutants in Drosophila Schneider cells [24]. However, in contrast to the in vivo study [24], we also observed significantly decreased levels of mitochondrial transcripts in vivo (Figure 3C). To analyze the biochemical consequence of the mtDNA depletion and the decrease in mtDNA-encoded transcripts caused by mutated d-mtDNA helicase, we measured the enzymatic activity of cytochrome c oxidase (complex IV) in larvae of the control and mutant lines. As expected, we observed a significant decrease in complex IV activity in the K388A and A442P expressing lines (Figure 4A), which showed a clear impairment of the OXPHOS function. Complex IV activity in the W441C line was similar to the control line, consistent with the
lack of an apparent phenotype produced by the moderate decrease in mtDNA levels.

To evaluate if the OXPHOS impairment produces any change in the levels of reactive oxygen species (ROS), we employed two different approaches: we used the MitoSox probe that binds specifically to the mitochondrial superoxide anion, and reactive dihydroethidium (DHE), which permeates cell membranes freely, as indicators of cytosolic superoxide production. Neither approach revealed any significant different between the control lines and the overexpression of the \( d^- \)mtDNA helicase variants, even in the presence of OXPHOS impairment (Figure 4B, C). This finding is consistent with that observed in adPEO patients, where no increase of ROS was found.

Constitutive Overexpression of W441C d-mtDNA Helicase does not Impair OXPHOS Function, but Slightly Reduces Life Span

Patients carrying adPEO mutations in the \textit{Twinkle} gene have adult onset symptoms between the ages of 20 and 40 years, suggesting that these mutations are only moderately deleterious for enzyme function. Expression of the W441C mutation at levels comparable to those of the other pathogenic mutations did not produce development defects, despite causing mild mtDNA depletion in the third instar larval stage. Hence, this line allowed us to study the effects of mutant \( d^- \)mtDNA helicase variants, even in the presence of OXPHOS impairment (Figure 4B, C). This finding is consistent with that observed in adPEO patients, where no increase of ROS was found.


d-mtDNA Helicase Mutations Decrease Cell Proliferation and Increase Apoptosis in vivo

Various mutations in the \( d^- \)mtDNA helicase gene cause a significant depletion of mtDNA. We have shown previously that impairment of mtDNA replication and/or maintenance in \textit{Drosophila} causes mtDNA depletion and an increase in apoptosis [23,28]. To address this in our \( d^- \)mtDNA helicase mutant lines, we analysed the levels of apoptosis and cell proliferation in the wing imaginal discs of third instar larvae, in lines overexpressing the variants of \( d^- \)mtDNA helicase under the control of different GAL4-drivers. We used anti-caspase3 immunocytochemistry to highlight cells undergoing apoptosis, and anti-phosphohistone antibody to label proliferating cells. Compared with controls, wing imaginal discs from the K338A and A442P mutant lines showed an increase in apoptosis and a decrease in cell proliferation (Figure 6A–L). In contrast, the phenotypes of the lines overexpressing the wild type version and W441C mutant were similar to that of controls (Figure 6K, L). To corroborate these results, we overexpressed the variants of the \( d^- \)mtDNA helicase in the posterior compartment of wing discs using the driver \textit{en-GAL4} and evaluated apoptosis such that the anterior compartment could be used as an internal control with the same cellular background. As shown in Figure 6, we observed an increase in apoptotic clusters in the posterior compartment (Figure 6N,P) as compared to the anterior compartment (Figure 6G–L), suggesting that the elevated levels of apoptosis are due specifically to the documented mitochondrial dysfunction, arguing that mitochondria are key players in this pathway.

Figure 1. Sequence alignment and location of amino acid substitutions in mtDNA helicase. Schematic diagram of the sequence organization of mtDNA helicase; eight amino acid sequence motifs common to ring primases and helicases are indicated in gray. The bacteriophage T7 gp4 linker region is indicated in the middle of the diagram. Mutations in the human mtDNA helicase gene (\textit{Twinkle}/C10orf2) found in adPEO are shown above the scheme (reviewed in [46]), the orthologous adPEO mutations W441C and A442P in the \textit{Drosophila} mtDNA helicase gene (\( d^- \)mtDNA helicase), and the “Walker A” mutation K388A are shown below the scheme. The underlined mutations indicate autosomal recessive PEO mutations. The positions of mutations in \( d^- \)mtDNA helicase used in this study are shown in bold. Sequence alignment of the regions containing altered amino acids is shown in the lower panel. \( Dm \), fly; \( Ag \), mosquito; \( Mm \), mouse; \( Hs \), human; \( T7 \), bacteriophage T7.

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A previous study of d-mtDNA helicase knockdown in Drosophila Schneider cells using RNAi [24] showed cellular phenotypes of slow growth, reduced viability and a 5-fold reduction in mtDNA copy number. Whereas overexpression of the wild type helicase modestly increased mtDNA copy number, overexpression of an active site mutant K338A resulted in a dose-dependent mtDNA depletion, more severe than that observed upon d-mtDNA helicase knockdown. Furthermore, whereas overexpression of the A442P mutant significantly reduced mtDNA copy number, overexpression of the W441C mutant did not.

To extend these findings and in consideration of the need to develop tractable animal models to study mitochondrial diseases,
we employed the UAS-GAL4 system to examine the effect of the
K338A, W441C, and A442P mutations in d-mtDNA helicase, and
have established a Drosophila model of nucleo-mitochondrial
communication defect, in a system in which the physiological
conditions of the whole organism can be evaluated to understand
pathophysiological disorders. We observed a severe decrease in
mtDNA copy number and mtDNA-encoded transcripts in the
K388A and A442P mutant lines, which caused lethality at the
third larval instar and pupal stages, respectively. However, the
overexpression of the mutant W441C caused only slight mtDNA
depletion without apparent developmental effects.

Interestingly, unlike the findings reported for adPEO patients
[29], and in line with the observations in human cells [13], in the
Drosophila system, mtDNA deletions were not found either in S2
cell lines or in animals. A likely explanation might be that the
generation of deleted molecules in Drosophila cells is slower than in
mammals. Another explanation for the findings observed in this
and previous studies [13] could be that replication stalling caused
by overexpression of mutant versions of Twinkle might induce an
increased rate of mtDNA turnover, via mitophagy [12,30]. The
molecular mechanism by which a dominant mutation in mtDNA
helicase generates multiple mtDNA deletions is unknown [31],
and the accumulation of deleted mtDNA molecules varies widely
in different postmitotic tissues in adPEO patients [29,32].
Similarly, in a transgenic mouse model expressing a pathogenic
adPEO duplication in the Twinkle/C10orf2 gene, mtDNA deletions
were detected only after 18 months of life [12] suggesting that
mtDNA defects may be a direct consequence of age-associated
deterioration of the repair mechanisms (discussed in [13]).
Despite this discrepancy, and in contrast to that observed in human
patients, the absence of mtDNA deletions is a common observa-
tion in other genetics defects affecting the nDNA-mtDNA
intergenomic communication [33,34,35].

In adPEO patients, many of the mutations in the Twinkle gene
map within a small region, which corresponds to the linker region
of bacteriophage T7 gp4. The linker region separates the primase
and helicase domains and is important for hexamer formation
[36]. We found that the A442P mutant displays a dominant
negative effect like the helicase active site mutant K338A. Lys338 is
analogous to Lys318 in T7 gp4, and is not essential for T7 gp4

Figure 3. Differential changes in mtDNA copy number and mitochondrial transcript levels. (A) Relative mtDNA copy number was
measured by qRT-PCR in third instar larvae of Drosophila lines expressing mutant d-mtDNA helicase. (B) Total DNA (10 µg) was fractionated in an 0.8%
agarose gel, blotted to nylon membrane, and hybridized with radiolabeled probes for the mtDNA to observe multiple deletions. (C) Relative
mitochondrial mRNA levels of the ND5 and COX I genes was measured by qRT-PCR. Data represent the mean ± s.d., *P<0.05, **P<0.005 as compared
with wild-type (w1118).
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hexamer formation [37,38]. Thus, the mutant K338A likely forms hexamers with the same efficiency as the wild type polypeptides. However, in the T7 gp4 enzyme, analogous residue A475 in the helicase domain is in close contact with analogous residues R374 and F370 in the linker region, suggesting that it may be required to form stable multimers. The finding that the A475P adPEO variant fractionates mainly as a monomer in gel filtration supports this interpretation for the human enzyme [39]. Likewise, the loss of helicase activity due to hexamer instability could thus explain the phenotype observed in this work in the overexpression of the A442P mutation in flies; whereas hexameric proteins were detected in mitochondrial extracts from Schneider cells, the conformation and/or stability of the oligomers may be compromised.

We found that the overexpression of the wild type form of d-mtDNA helicase slightly increases mtDNA copy number in adults, as it does both in Schneider cells and in mice [11,24], indicating that d-mtDNA helicase is modestly limiting for mtDNA replication under physiological conditions. Overexpression of the W441C mutant reduces the mtDNA content in the larval stages, but is recovered in adult cells. This suggests that mtDNA depletion occurs during embryonic and/or larval development but that replacement of mtDNA in postmitotic cells is relatively slow and may not be affected significantly by the W441C mutation, at least during the relatively short life of Drosophila. Interestingly, the life

Figure 4. OXPHOS enzymatic activities and ROS levels in Drosophila lines expressing mutant d-mtDNA helicase. (A) Activities of complex IV were measured in mitochondrial protein extracts obtained from third instar larvae of each genotype expressing the different variants with da-GAL4. Data represent the mean ± s.d., *P≤0.05, as compared with wild-type (w1118), of at least three independent determinations. (B) Imaginal discs from third instar larvae were dissected and digested with trypsin prior to incubation with MitoSox (5 mM). The cell suspension was analysed using a BD Biosciences FACS Vantage SE instrument, and the data were processed using Cell Quest Pro Software. Data represent the mean ± s.d. as compared with wild-type (w1118). (C–F) Overexpression of d-mtDNA helicase with the engrailed driver (en-GAL4) (posterior compartment, P) in wing imaginal discs, stained with dihydroethidium (DHE 30 μM) that binds superoxide anions. C, w;en-GAL4;UAS helicaseWT; D, w;en-GAL4;UAS helicaseK388A; E, w;en-GAL4;UAS helicaseW441C; F, w;en-GAL4;UAS helicaseA442P.

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span of these animals was slightly reduced as compared to animals overexpressing the wild type form. In the T7 gp4 enzyme, the amino acid orthologous to W441C in flies is not predicted to interact directly with the linker region and in theory, this mutant is able to form hexamers. However, in vitro helicase activity of the equivalent W474C mutant in human cells was reduced by 70%, leading to a decrease in mtDNA levels in vivo [6,13,39]. 2D-PAGE analysis showed that overexpression of this mutant produces an altered pattern of replication intermediates, suggestive of a specific effect in initiation. Furthermore, nucleoid morphology was perturbed in the W474C expressing cells, suggesting a mtDNA segregation defect [13].

The most obvious phenotype in the Drosophila lines overexpressing the K388A and A442P mutations is premature death in the larval and pupal stages, respectively, with a decrease in mtDNA copy number as compared with control lines. We explored the effect on mitochondrial function of this mtDNA depletion, and found reduced activity of complex IV, indicating an impairment of OXPHOS function. Increased levels of ROS have been documented to be a direct consequence of OXPHOS impairment [40]. Nonetheless, we found no evidence of an increase of ROS or of oxidative damage in any of the Drosophila lines overexpressing the d-mtDNA helicase mutants, consistent with observations in adPEO patients [41]. In fact, there are notable differences in comparing the effects produced by other mitochondrial-associated mutations in Drosophila. For instance, overexpression of the catalytic core of pol γ, the product of the tamos gene, interferes with mtDNA replication leading to mtDNA depletion, OXPHOS defects and an increase in oxidative stress [28]. It is known that the mitochondrial quality control pathway is regulated strictly by various factors [22], such that defective mitochondria may be removed by mitophagy before the accumulation of ROS occurs, suggesting that different alterations in respiratory chain function may affect ROS production in diverse ways.

A direct consequence of the overexpression of the K388A and A442P helicase mutants and likely due to the mtDNA depletion is an increase in apoptosis and a decrease in cell proliferation. We reported previously that silencing or overexpression of genes involved in replication and expression of mtDNA in Drosophila increases significantly the number of apoptotic cells [23,42]. It has also been shown that the mitochondria play a central role in the

Figure 5. Characterization during adult life of the wild type and W441C mutant d-mtDNA helicase. (A) Longevity curve of lines overexpressing the wild type and W441C mutants using a constitutive and ubiquitous driver (da-GAL4). 100 adult males (0–2 days old) were collected in an empty tube with standard medium and the number of surviving flies was counted every 2 days. Kaplan-Meier survival curve was performed (p = 0.045, n = 3 biological experiments). (B) Quantitation of mtDNA copy number by qRT-PCR at 5, 25 and 50 days of life in both lines.
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apoptosis pathway in mammals [17]. Even though nearly all of the proteins involved in cell death pathways are conserved throughout metazoan evolution, the importance of mitochondria in the Drosophila apoptotic pathway is both a controversial and an active field of research [18,19,20]. Drosophila lacking one of the d-cyt-c genes was reported to exhibit severely delayed apoptosis in the developing retina [43], and mutations in d-cyt-c were found to have reduced caspase activation during spermatid individualization [44]. These and other findings have led to the hypothesis that alterations in mitochondrial morphology might lead to formation of a cytochrome c-dependent apoptosome at the mitochondrial surface as part of a feed-forward signal in caspase activation [44,45]. Although the mechanism remains unclear, the results obtained here and in our previous studies [23,42] allow us to conclude that mitochondrial dysfunction associated with defects in replication and expression of mtDNA induces apoptosis, arguing that an intrinsic mitochondrially-mediated apoptotic pathway is active in Drosophila, and offering the possibility to explore its role in pathophysiological processes.

Figure 6. Apoptosis and proliferation levels in wing imaginal discs from third instar larvae. (A–J) Overexpression of the different versions of d-mtDNA helicase with the daughterless-Gal4 driver in wing imaginal discs. (A–E) Monoclonal anti-caspase 3 antibody was used for immunocytochemistry to assess apoptotic levels in lines expressing d-mtDNA helicase. (F–J) Monoclonal anti-phosphohistone 3 antibody was used for immunocytochemistry to evaluate cell proliferation levels in lines expressing d-mtDNA helicase. (K–L) Quantification of apoptosis and cell proliferation levels using a ubiquitous and constitutive driver (da-GAL4). Data represent the mean ± s.d., *P<0.05, **P<0.005 as compared with controls. (M–P) Overexpression of d-mtDNA helicase with the engrailed (en-GAL4) driver (posterior compartment, P) probed by staining with anti-caspase 3 antibody in third instar larvae’s wing imaginal discs. Overexpression of the K388A mutant shows an increase in apoptosis clusters when compared with the anterior, the control compartment. (M) w;en-GAL4;UAS helicaseWT; (N) w;en-GAL4;UAS helicaseK388A; (O) w;en-GAL4;UAS helicaseW441C; (P) w;en-GAL4;UAS helicaseA442P.

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Materials and Methods

Fly Stocks and Culture
Flies were raised on standard yeast-glucose-agar medium at 25°C and 65% relative humidity in 12 h light/dark cycles, unless otherwise indicated. To determine the chromosomal location of the transgenes and for the manipulation of transgenic lines, the stock w; CyO/If(TM3)BR/Me4 was used. Other GAL4 stock lines used were: w; da-GAL4, w; y-GAL4 and w; en-GAL4-UAS GFP, obtained from the Bloomington Drosophila Stock Center at Indiana University. For more details of Drosophila lines, see Information S1.

Generation of Transgenic UAS-d-mtDNA Helicase Lines
The constructs used to overexpress the different versions of the d-mtDNA helicase gene comprised cDNA fragments from d-mtDNA helicase cloned into the pUAST Drosophila transformation vector. The d-mtDNA helicase insert was obtained as previously described [24]. Recombinant pUAST-d-mtDNA helicase constructs were transformed into DH5α competent bacterial cells (Stratagene). Transgenic lines were generated by microinjection of the pUAST-d-mtDNA helicase plasmids in y w1118 embryos following standard procedures [24]. Ten independent transgenic lines for each construct were obtained; all of them overexpressed d-mtDNA helicase. Each of them carried a single independent insertion in chromosome II or III, which was brought to homozygosis. For more information see Information S1.

UAS-GAL4 Experiments

d-mtDNA helicase fragments were overexpressed using the UAS-GAL4 system described by Brand and Perrimon [23]. The daughterless-GAL4 driver (da-GAL4) yields a high and ubiquitous transgenic expression throughout development and was used to induce the overexpression of d-mtDNA helicase. The homozygous da-GAL4 driver was crossed to homozygous UAS lines and the progeny were subjected to study. The same procedure was followed with the w; en-GAL4 (posterior compartment) driver. Crosses were set up in standard food vials and environmental conditions, and the flies were passed every three days. Additional details on Drosophila lines are presented in the Information S1 tables.

Viability and Longevity Curves of D. Melanogaster
For viability counts, 100 embryos from lines overexpressing the various mutations of d-mtDNA helicase were collected. Embryos were incubated at 25°C with wet yeast, and the number of animals reaching the developmental stages of third instar larvae, pupae and adults were scored. For longevity curves, 200 adult males were collected from the same lines and placed in a vial containing fresh culture medium. Flies were transferred to fresh medium three times a week, and dead flies were counted every day and a Kaplan-Meier survival curve was performed.

Real-time RT-PCR
Total RNA was extracted using TRIzol reagent (Invitrogen), and 1 μg was converted into cDNA using the QIAGEN quantitect reverse transcription kit following the manufacturer’s instructions. Genomic DNA was isolated from larvae by standard methods. Real-time PCR was performed using the 7900 HT Fast Real-Time PCR System (Applied Biosystems), and PCR products were quantified fluorometrically using the taqman probe (Applied Biosystems); d-mtDNA helicase: Dm01809992_g1; d-COX I (Fw): TACAGTCCCCTCCGCTTTTTT, d-COX I (rev): ACGGGATATAGGTTGATATAACAGTT, FAM probe: TATCCAGCTCGATTCCC, d-ND5 (Fw): AGGATTTT-TAAAATTAGCTATATTTATTTAATACCTGATCTTTT, d-ND5 (rev): ACCCCCTATTTAAACGATATTTCTTGAGA, FAM probe: CCCAGACATATAAC. At least three independent amplifications were performed in triplicates for each transcript, and mean values were normalized to the mean value of the reference rRNA L18S (Fw): AGGCTTCCGCTTTATTT-GACT, (rev): CCTACACCTTAGTGTGCACGTG and FAM probe: AAGACGGGAAAGCTT. The PCR amplification program was 95°C for 15 s, 60°C for 1 min, 40 cycles with an initial step of 2 min 50°C and 95°C for 10 min. For mtDNA/nuclear-DNA relative quantification genomic DNA from Drosophila third instar larvae was purified and quantified by standard methods. 10 ng of DNA were added to Master Mix-PCR Applied Biosystem. The PCR amplification program was 95°C for 15 s, 60°C for 1 min, 40 cycles with an initial step of 2 min 50°C and 95°C for 10 min. Software used was SDS 2.3 (Applied Biosystems). Assays were performed with two different taqman probes: ND5 and 18S.

Immunocytochemistry in Imaginal Wing Discs
Imaginal discs from third instar larvae were dissected in PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. They were blocked in PBS containing 1% bovine serum albumin and 0.3% Triton X-100 for 1 h, incubated with the primary antibody overnight at 4°C (1:50 dilution), washed four times in blocking buffer, and then incubated with the appropriate secondary antibody (1:200 dilution), for 2 h at room temperature in the dark. Finally, they were washed and mounted in Vectashield (Vector Laboratories). Primary antibodies used were: rabbit anti-phosphohistone 3 (Sigma-Aldrich) and rabbit anti-caspase 3 (Cell Signaling Technology). Secondary antibodies were coupled to the fluorochromes Alexa Fluor 647 (Invitrogen) or Alexa 555 (Invitrogen). In addition, the nucleus was stained with TO-PRO-3 iodide (Invitrogen) and actin with Phalloidin-TRITC (Sigma-Aldrich). Preparations were imaged with a Leica TCS SP2 laser-scanning microscope (Heidelberg, Germany). The number of apoptotic cells was measured using analySIS FIVE software (Olympus Digital Imaging Solutions).

Oligonucleotides
All oligonucleotides were purchased from Isogen (the Netherlands) and Sigma-Aldrich. All sequences are given in the 5’ to 3’ direction. Probes used for Southern and Northern hybridization were PCR products obtained from the amplification of Drosophila nuclear and mitochondrial DNA using the following primers: d-mtDNA helicase (dir): ATAGAGCAGCGCGGTATTTAA; d-mtDNA helicase (rev): TCAGTTTCTCGGATGGCTTCT; d-COX I (dir): ACACACGGAAACAGAATGCTCG; d-COX I (rev): AGGGAAGCCAAAGCCCTGTAAGG. Oligonucleotides used for quantitative PCR (qPCR) are as follows: d-mtDNA helicase. TaqMan Gene Expression Assays Dm01809992_g1; d-COX I (Fw): TGACTACATCCTCCTGCTTTT, d-COX I (rev): ACGGGATAAGGTTGATATAACAGTT, FAM probe: TTTCCAGCTCGATTCCC, d-ND5 (Fw): AGGATTTTTAAAATTAGCTATATTTATTTAATACCTGATCTTTT, d-ND5 (rev): ACGCCCTATTTAAACGATATTTCTTGAGA, FAM probe: CCCAGACATATAAC. For more details see Information S1.

Quantitative RT-PCR Assays
~10 frozen larvae from each genotype were used for RNA extraction with TRIzol reagent (Invitrogen). Total RNA from each sample (2 μg) was reverse transcribed into cDNA using oligo(dT) as a primer and the SuperScript III First-strand
Synthesis System for RT-PCR (Invitrogen), according to the manufacturer’s instructions. The gene encoding the 18S rRNA was used as a normalizing internal control [25]. Exon-specific oligonucleotide primers for the d-mtDNA helicase were designed with Primer3 software. Real time PCRs were performed in a Rotor Gene thermocycler (Corbett Research) using Taqman probes (Applied Biosystems) as double-stranded DNA binding dye and under the following conditions: 50°C for 2 min, 95°C for 10 min; 40 cycles (95°C for 15 sec and 60°C for 1 min annealing/extension. Each sample contained 1X Taqman probe, 0.2 mM dNTPs, 1X enzyme buffer, 4 mM MgCl₂, 0.5 units of AmpliTaq Gold DNA polymerase enzyme, 0.3 μM of each oligonucleotide, and 4 μl of a 1/20 dilution of the reverse transcribed products in a final volume of 20 μl. Three separate samples were collected from each genotype and triplicate measurements were performed.

**Immunoblotting**

Protein extracts (30 μg) obtained from mitochondria purified by differential centrifugation were fractionated by 10.5% SDS-PAGE and transferred to nitrocellulose filters. Filters were pre-incubated for 1 h with 5% skim milk in TBS, followed by incubation for 1 h with the corresponding primary antibody. Polyclonal antibody against d-mtDNA helicase was used as previously described [24]. As a loading control, an antibody against Drosophila VDAC-porin was used at a 1:2000 dilution.

**DNA Extraction and Southern Blotting**

Aliquots of 20 frozen third instar larvae were ground in 800 μl of homogenization solution that contained 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 60 mM NaCl, 0.5% SDS. Precipitated material was removed by centrifugation and the supernatant fractions were extracted once with phenol/ chloroform (1:1), treated with 10 μg/ml RNase A at 37°C for 15 min, re-extracted with phenol/ chloroform (1:1), and precipitated with 2 volumes of ethanol. DNA precipitates were washed with 80% ethanol and resuspended in 50 μl of H₂O. Southern analysis was performed as described previously [25].

**Mitochondrial Preparations**

For enzymatic measurements, aliquots of fresh third instar larvae were ground in buffer containing 250 mM sucrose, 2 mM EDTA, 100 IU/liter heparin, 10 mM Tris-HCl, pH 7.4 and mitochondria were isolated by differential centrifugation; purified mitochondria were sonicated for 6 s at 4°C, frozen and thawed. Protein determination was performed using the DC Protein Assay kit (Bio-Rad).

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