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# Fly Models of Human Diseases: *Drosophila* as a Model for Understanding Human Mitochondrial Mutations and Disease

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# Fly Models of Human Diseases: *Drosophila* as a Model for Understanding Human Mitochondrial Mutations and Disease

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## Abstract

Mitochondrial diseases are a prevalent, heterogeneous class of diseases caused by defects in oxidative phosphorylation, whose severity depends upon particular genetic mutations. These diseases can be difficult to diagnose, and current therapeutics have limited efficacy, primarily treating only symptoms. Because mitochondria play a pivotal role in numerous cellular functions, especially ATP production, their diminished activity has dramatic physiological consequences. While this in and of itself makes treating mitochondrial disease complex, these organelles contain their own DNA, mtDNA, whose products are required for ATP production, in addition to the hundreds of nucleus-encoded proteins. *Drosophila* offers a tractable whole-animal model to understand the mechanisms underlying loss of mitochondrial function, the subsequent cellular and tissue damage that results, and how these organelles are inherited. Human and

*Drosophila* mtDNAs encode the same set of products, and the homologous nucleus-encoded genes required for mitochondrial function are conserved. In addition, *Drosophila* contain sufficiently complex organ systems to effectively recapitulate many basic symptoms of mitochondrial diseases, yet are relatively easy and fast to genetically manipulate. There are several *Drosophila* models for specific mitochondrial diseases, which have been recently reviewed (Foriel, Willems, Smeitink, Schenck, & Beyrath, 2015). In this review, we highlight the conservation between human and *Drosophila* mtDNA, the present and future techniques for creating mtDNA mutations for further study, and how *Drosophila* has contributed to our current understanding of mitochondrial inheritance.

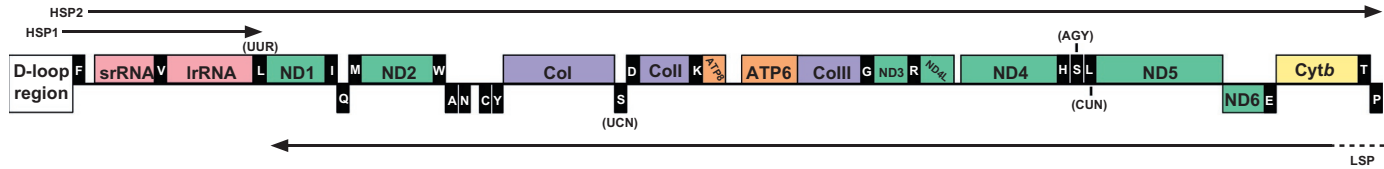


## 1. MITOCHONDRIA PLAY DIVERSE ROLES

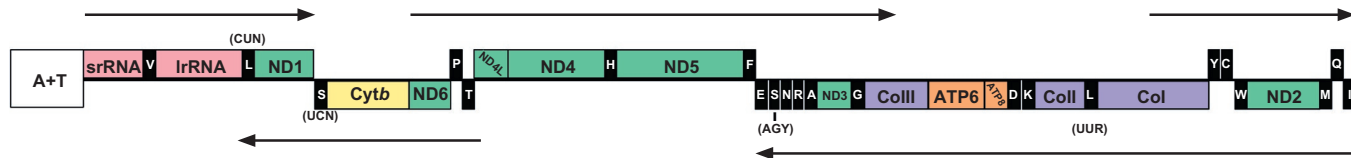
Mitochondria are thought to have arisen through endosymbiosis (Margulis, 1970). As such, in metazoans mitochondria are the only organelle, other than the nucleus, which contains its own DNA, mtDNA. The mtDNA in different species encodes a variable number of products; however, animal mtDNA represents the most stripped-down version of the genome (Gray, 2012). The 16 kb human mtDNA codes for 13 proteins, 22 tRNAs, and 2 rRNAs. All 13 proteins are components of the electron chain complexes (ETC) I, III, IV, and V (the ATP synthase). Almost all the DNA is coding—for example, there are no introns in the resulting mRNAs and very few gaps in coding sequence. *Drosophila* mtDNA encodes the same transcripts as human mtDNA, albeit in a slightly different genomic order (Fig. 1). This fundamental similarity makes the fly an excellent model for studying mitochondrial function.

The evolution between the nucleus and mitochondria has culminated in mitochondria taking on highly specialized functions, offering an environment to support a variety of biochemical reactions required for the cell. Because mitochondria have such a small genome, they rely heavily on imported proteins encoded in the nucleus. The best known mitochondrial product is ATP, produced via the ETC and oxidative phosphorylation (OXPHOS). However, mitochondria are also required for fatty acid beta oxidation, heme, steroid, and nucleotide biosynthesis and are integral to apoptosis. In fact, under specialized conditions it is possible for yeast and cultured cells to survive without functional OXPHOS (i.e., loss of mtDNA); however, they cannot survive in the complete absence of the organelle (Chandel & Schumacker, 1999; Goldring, Grossman, Krupnick, Cryer, & Marmur, 1970; Morais, Gregoire, Jeannotte, & Gravel, 1980; Nagley & Linnane, 1970). All of these

Human 16,559 bp



*Drosophila melanogaster* 19,517 kb



**Fig. 1** Human and *Drosophila* mitochondrial DNA encode the same products. Human mtDNA (*top*) is approximately 3 kb shorter than *Drosophila* mtDNA (*bottom*). The size difference is predominantly due to the expanded “A+T-rich” region in *Drosophila*, which varies among different *Drosophila* species. Human mtDNA is transcribed as three polycistrons (*arrows*): two for the heavy strand (HS), which encodes most of the products, and one for the light strand (LS). The LS promoter (LSP) starts in the D-loop region (indicated by *dashed line*), where the origin of replication is found. HSP1, which includes the rRNAs, expresses at higher levels compared to HSP2. *Drosophila* mtDNA is thought to be transcribed as five polycistrons. Note that the products are relatively evenly encoded on both strands, in contrast to human mtDNA. Some segments maintain the same sequence (e.g., ATP8 → mt:tRNA<sup>Gly</sup>). ATP, ATP synthase (*orange*, Complex V); CO, cytochrome c oxidase (*purple*, Complex IV); Cytb, cytochrome b (*yellow*, Complex III); ND, NADH dehydrogenase (*green*, Complex I).

basic biochemical reactions are required for both *Drosophila* and human cells, and because *Drosophila* mtDNA encodes the same products as human mtDNA, *Drosophila* mitochondria function requires essentially the same nuclear genes as human mitochondria.



## 2. MITOCHONDRIAL DISEASES—CAUSES AND EFFECTS

Human mitochondrial diseases mostly result from a loss of OXPHOS. The term “mitochondrial function” is used broadly so it is important to be as specific as possible when characterizing any particular loss in biochemical function. There are estimated to be between 1000 and 1500 proteins encoded in the nucleus that are imported or associated with mitochondria (Area-Gomez & Schon, 2014). Thus, mitochondrial disease arises through mutations in either nuclear DNA or mtDNA. As with any nuclear gene, mitochondrial disease due to mutations in nuclear genes can be inherited in a Mendelian fashion as either a dominant or recessive trait. In comparison to mutations in disease-causing nucleus-encoded mitochondrial genes, there are over 250 verified disease-causing point mutations in mtDNA. Because mitochondria cannot be made de novo, they are inherited through the mother, and thus mtDNA mutations are exclusively maternally inherited.

The 13 proteins encoded by mtDNA are translated in the mitochondrial matrix using mtDNA-encoded tRNAs (mt:tRNAs) and the mitochondrial ribosome, which consists of the mt:rRNAs and nucleus-encoded proteins. This suite of mt:tRNAs is all that is required for translation in human and *Drosophila* mitochondria. Human and *Drosophila* mtDNA is transcribed as a series of polycistrons (Fig. 1). The mt:tRNAs are thought to function as “punctuation,” with most of the mRNAs separated by at least one mt:tRNA (Ojala, Montoya, & Attardi, 1981). Thus, it is critical that each mt:tRNA and every product of the genome be properly excised allowing each mRNA to be further processed and translated. Of the known disease-causing point mutations in mtDNA, over half are found in mt:tRNAs. This is somewhat surprising, given that the 22 mt:tRNAs encode only 9% of the genome. One explanation could be that mutations in the protein-coding regions are too deleterious and incompatible with life.

The known mutations that cause human mitochondrial disease have been extensively reviewed (Area-Gomez & Schon, 2014; Chinnery, 1993; Dimauro & Davidzon, 2005; Lightowers, Taylor, & Turnbull, 2015). In general, they give rise to defects in the musculature and nervous system. However, while mutations in nuclear DNA and mtDNA would be

expected to cause a decrease in OXPHOS, the different mutations have variable onset and features, from symptoms as mild as eye muscle weakness (external ophthalmoplegia) to infant mortality. There is even evidence that defects in OXPHOS may be a cause of miscarriage (Tay, Shanske, Kaplan, & DiMauro, 2004). Why deficits in the proteins involved in ATP production give rise to such different outcomes largely remains a mystery. In the case of disease caused by mutations in nuclear genes, all cells should have the same genotype. However, for mutations in mtDNA, one reason for differences in tissue deficits could be the threshold effect (Picard et al., 2014; Rossignol et al., 2003; Stewart & Chinnery, 2015). Each cell contains many mitochondria, and each mitochondrion usually contains multiple copies of mtDNAs. Mutations in mtDNAs are usually heteroplasmic (a mixture of wild type and mutant), thus tissues with a higher mutation load would be expected to be more severely affected. But a recent finding suggests that many associated symptoms, secondary to a prominent trademark phenotype that arose beyond the threshold point, can appear even when a particular mutation load is well below the threshold mark. Picard and colleagues found that a predominant pathogenic mutation in a mitochondrial tRNA gene (mtDNA 3243A > G; mutation in mt:tRNA<sup>Leu(UUR)</sup>) has an effect on nuclear gene expression when present at well below the threshold point (Picard et al., 2014). Heteroplasmic cells harboring different mutational loads of this particular mutation have striking variations in their gene expression profile. This could explain why patients with the same mutation manifest different clinical symptoms. For example, patients with mtDNA 3243A > G that have high levels of mutated mtDNA exhibit mitochondrial encephalomyopathy, whereas patients with lower levels can suffer from type II diabetes, deafness, or even be on the autistic spectrum (Goto, Nonaka, & Horai, 1990; Pons et al., 2004; van den Ouweland et al., 1992).



### **3. HOW CAN STUDYING *DROSOPHILA* CONTRIBUTE TO OUR UNDERSTANDING OF HUMAN MITOCHONDRIAL DISEASES?**

As a model organism, *Drosophila* can help our understanding of mitochondrial diseases in several specific ways. The first takes advantage of the rich history of cell biology in *Drosophila*. From dissected tissues taken from wild-type and mutant flies, mitochondria can be clearly visualized at the organelle level in fixed or live tissues (Cox & Spradling, 2006; Sen, Damm, & Cox, 2013). This allows for a detailed and accurate view of

location, numbers, and morphology. In addition, with molecular and biochemical assays, researchers can assay which mitochondrial functions are compromised *in vivo* in *Drosophila* carrying various gene mutations.

The second advantage to using *Drosophila* to study deficits in mitochondrial function is that they have complex central and peripheral nervous systems, and contain the various organ systems that are frequently affected in humans suffering from mitochondrial disease, such as skeletal and heart muscles. These organ systems are far simpler than those in humans, allowing for detailed analysis of the progression of tissue degeneration either during development or aging. Seizures are one feature of mitochondrial diseases such as myoclonic epilepsy with ragged-red fibers (MERRF) and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). This clinical feature is easily mimicked in *Drosophila* by assaying for bang-sensitivity, the paralysis and seizures that can occur after mechanical stimulation (Engel & Wu, 1994; Fergestad, Bostwick, & Ganetzky, 2006; Ganetzky & Wu, 1982). Bang-sensitivity occurs in mutants for several genes involved in metabolism in general, and mitochondrial function in particular (Burman et al., 2014; Celotto et al., 2006; Fergestad et al., 2006; Royden, Pirrotta, & Jan, 1987; Zhang et al., 1999). Another symptom of certain mitochondrial diseases is brain degeneration which can cause cerebellar ataxia, for example, found in MELAS, Leigh syndrome and myoclonic epilepsy, myopathy, and sensory ataxia. *Drosophila* with mtDNA mutations with bang-sensitivity also exhibit progressive brain degeneration (Burman et al., 2014; Celotto et al., 2006). Finally, cardiomyopathy occurs with mutations of multiple nucleus-encoded and mtDNA-encoded genes (Antonicka et al., 2003; Jaksch et al., 2000; Loeffen et al., 2001; Majamaa-Voltti, Peuhkurinen, Kortelainen, Hassinen, & Majamaa, 2002; Papadopoulou et al., 1999; Wahbi et al., 2010). In *Drosophila*, knockdown of several proteins found in the ETC Complex I (NDUFS2, NDUFS7, and NDUFC2) specifically in the heart caused significant, abnormal heart dilation (Tricoire, Palandri, Bourdais, Camadro, & Monnier, 2014).

An additional advantage is the ease of genetic manipulation in *Drosophila*. RNAi knockdown of single proteins in each ETC complex can be temporally and spatially controlled using the UAS/GAL4 system, which has been done for a subset of ETC and OXPHOS proteins (listed in table 1 of Foriel et al., 2015). In addition, mutant analysis has been very helpful in determining the molecular mechanisms underlying mitochondrial dysfunction, sometimes offering unexpected results. For instance, *tko*<sup>25t</sup> (*technical knockout*) is a recessive mutation in the nucleus-encoded mitochondrial

ribosomal protein S12. This bang-sensitive mutation confers a developmental delay and has long been considered a model for mitochondrial disease because the mutation targets the respiratory chain and causes seizures and deafness (Jacobs et al., 2004; Toivonen et al., 2001). The main pathological symptom identified in *tko*<sup>25t</sup> was oxidative stress. Thus, it was assumed that decreasing oxidative stress would ameliorate the effect of the *tko* mutation. However, a recent finding shows that expressing neither the alternative oxidase (AOX, from *Ciona instestinalis*) nor NADH dehydrogenase (*ndi1*, from yeast) could rescue the phenotype of *tko*<sup>25t</sup> mutant flies (Kemppainen, Kemppainen, & Jacobs, 2014). These results imply that the *tko*<sup>25t</sup> mutation affects greater mitochondrial function such that merely targeting oxidative stress cannot afford a remedy.

A major challenge in mitochondrial disease therapy is establishing the root cause against which potential therapeutics can be generated. While the pathological manifestation of a mitochondrial disease looks simple, namely, loss of OXPHOS, it is difficult to dissect what component(s) of that complex structure is compromised. The basic functional moiety of a mitochondrion is its OXPHOS network. For example, if one of the 44 components of Complex I does not function properly, it may be reflected in abnormal Complex I activity. However, a traditional clinical approach may not be adequate to identify the primary cause of the nonfunctional Complex I, whereas a model system allows various forms of experimentation not possible in patients. Even if a particular subunit is mutated, it can be difficult to predict what specific aspect of the molecular complex is deficient. This creates difficulties for rational therapeutic design. Thus, using *Drosophila* offers tremendous potential for identifying the exact molecular mechanism behind any pathological symptoms and could aid in more targeted drug delivery and discovery.



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#### **4. MITOCHONDRIAL DISEASES CAUSED BY mtDNA MUTATIONS**

Since mitochondria cannot be made de novo and contain their own DNA, they are maternally inherited through the egg's cytoplasm. Thus, mutations in mtDNA, and therefore mitochondrial diseases, are inherited through the mother. Common mitochondrial diseases occur from point mutations in all products encoded by mtDNA: protein-coding regions, tRNAs, and rRNAs. For example, maternally inherited forms of Leber hereditary optic neuropathy are due to point mutations in the subunits of



the NADH dehydrogenase complex (Complex I) ND1, ND2, ND4, and ND4L. Point mutations in different mt:tRNAs cause different symptoms and disease (MELAS, MERRF, CPEO as a few of the examples). All of these mutations have effects on the stoichiometry of the protein complexes used for OXPHOS which is the underlying cause of a decrease in mitochondrial output.

What have we learned from flies harboring deleterious mutation in their mtDNA? Currently, there are three fly models to examine specific mutations; however, this is an area with great potential (discussed later). The first model is a serendipitously identified point mutation in mt:ATP6 (Celotto et al., 2006). The bang-sensitivity of *Drosophila* mutant for adenine nucleotide translocase type 1 (ANT1, called stress sensitive (SesB) in *Drosophila*) was separable from a second, cytoplasmically inherited bang-sensitivity found to be caused by a single point mutation in *mt:ATP6*<sup>1</sup>. The *mt:ATP6*<sup>1</sup> mutation exists at near homoplasmy and gives rise to neurodegenerative phenotypes reminiscent of those found in mitochondrial diseases caused by mutations in *ATP6*. This model has been useful for studying the bioenergetic changes that occur before and after neurological symptoms occur (Celotto, Chiu, Van Voorhies, & Palladino, 2011).

The second model used a mitochondrially targeted restriction endonuclease to generate a single-site cleavage in the mtDNA. This idea was first tested in tissue culture cells by the Moraes laboratory, then subsequently successfully used in *Drosophila* to create an intact organism containing a mutation in cytochrome *c* oxidase subunit I (mt:CoI, Complex IV) as well as a small insertion and small deletion in mt:ND2 (*mt:ND2*<sup>ins1</sup> and *mt:ND2*<sup>del1</sup>, respectively) (Bacman, Williams, Pinto, & Moraes, 2014; Srivastava & Moraes, 2001; Xu, DeLuca, & O'Farrell, 2008). In *Drosophila*, a single *XhoI* site is located in *CoI*. Xu et al. conditionally expressed a transgene encoding mitochondrially targeted *XhoI* exclusively in the germline. Upon germline expression, most mtDNA was irreparably cleaved, giving rise to sterile females; however, at a low level, mtDNA with mutations which rendered the DNA impervious to *XhoI* cleavage were selected for. These mutations were able to replicate and repopulate the germline. As would be expected, certain mutations were silent; however, others caused amino acid changes that affected CoI function (Xu et al., 2008). Adults homoplasmic for *mt:CoI*<sup>R301S</sup> mtDNA had 50% of the normal CoI levels, age-related reduction in ATP levels, and neurological and muscular defects, thus largely recapitulating many of the general symptoms exhibited in human mitochondrial diseases.

The *mt:ND2<sup>del1</sup>* mutation, which removes three highly conserved residues at positions 186–188, was recently further characterized (Burman et al., 2014). This work is a good example of how *Drosophila* exhibit tissue phenotypes associated with human mitochondrial disease, while also helping define the molecular function of a particular protein in one of the ETC Complexes. Complex I is the largest complex, with ~44 proteins. Burman et al. found that *mt:ND2<sup>del1</sup>* mutants were bang-sensitive and had reduced life spans, similar to *mt:ATP6<sup>1</sup>* mutants. The mutant adults had neurodegenerative vacuoles in aged fly brains, suggesting a progressive neurodegenerative phenotype, while the musculature remained intact. To pinpoint any defects in respiration, the authors determined that under maximally demanding conditions, the mutants showed a Complex-I-dependent respiratory defect, but no defect in Complex-II- and Complex-IV-dependent respiration. The total amount of assembled CoI was reduced, as was the mitochondrial membrane potential and amount of ATP. Together, these data support a role for *mt:ND2* in the proton pumping mechanism of Complex I, the first time this has been shown in a eukaryote.

There are also disadvantages to using *Drosophila* to study mtDNA mutations. One disadvantage is that any organism with a high mtDNA mutational load will potentially eventually die. Therefore, while it is trivial to maintain a lethal mutation in a nuclear gene in *Drosophila*, maintaining mtDNA mutations can be challenging over many generations. In the case of the *mt:ATP6<sup>1</sup>* mutation, for reasons that are unknown, this mutation is sustained nearly homoplasmically in a background of the *SesB<sup>1</sup>* allele of ANT1. Thus, to examine and characterize the effect of a 100% *mt:ATP6<sup>1</sup>* mutation load, the *SesB* mutation need simply be crossed out of the strain. In the case of the CoI mutation located at the *XhoI* restriction site, while this stock will eventually die, it can be generated reproducibly over and over again by expressing the mitochondrially targeted *XhoI* in the germline.



## 5. DISEASE-CAUSING POINT MUTATIONS ARE MOST PREVALENT IN *mt:tRNAs*—CONSERVATION BETWEEN HUMAN AND *DROSOPHILA*

A thorough and continuously updated compilation of human mtDNA mutations and polymorphisms indicates that there are a total of 305 disease-causing modifications in *mt:tRNAs* and *mt:rRNAs*, with the numbers likely growing (Lehmann et al., 2015; Lott et al., 2013). This is an unusually large number given that only about 9% of mtDNA codes for tRNAs. There are

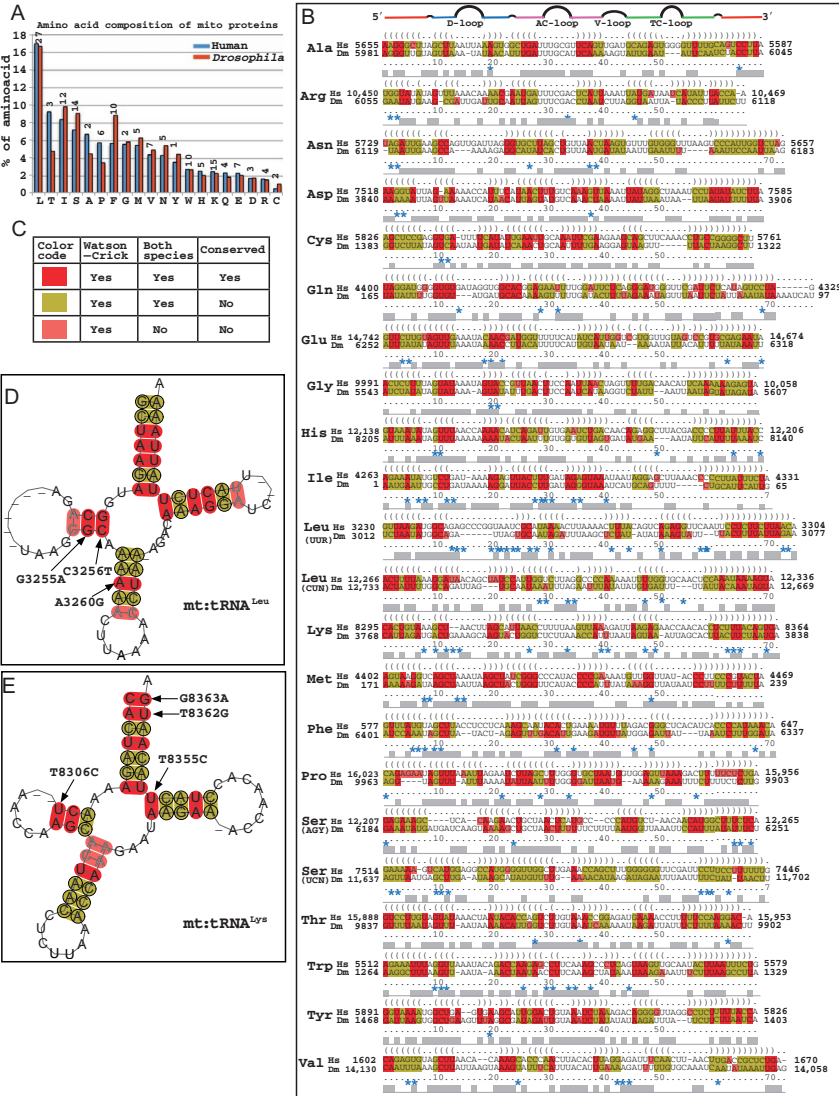
various nucleus-encoded factors that are involved in processing mt:tRNA from their immature transcripts, as well as those that are responsible for post-translational modifications. Mutations in mt:tRNA residues that are sites for processing and modification can cause mitochondrial disease (Powell, Nicholls, & Minczuk, 2015). What are the possible effects of these mutations? mt:tRNA mutations may affect tRNA processing. Mature mt:tRNAs are embedded within the newly synthesized polycistronic transcript (Fig. 1). Due to this “punctuation” model, precursor mt:tRNA processing is critical not only to generate mature mt:tRNAs but also to cleave out the mt:mRNAs (Ojala et al., 1981). Recently, the mitochondrial cognate of RNase Z, the endoribonuclease responsible for 3′-end tRNA cleavage, was identified in *Drosophila*. The authors showed that specific loss of the mitochondrial form affected mt:RNA processing, causing larval lethality, cell-cycle defects, and an increase in reactive oxygen species (Xie & Dubrovsky, 2015). A mitochondrial protein-only RNase P, containing no enzymatic RNA, performs the 5′-end cleavage of mt:tRNAs (Holzmann et al., 2008). Recently, the three *Drosophila* orthologs comprising this complex have been identified and abolishing any of them causes lethality, loss of ATP, and aberrant mt:RNA processing (Sen et al., 2016). Potentially, mutations in the nucleotide residues that participate in the interaction between the mt:tRNA and either of these enzyme complexes responsible for cleavage could hinder processing, leading to not only a reduction in mt:tRNAs but also normal polycistron processing.

A second possible effect of mt:tRNA point mutations is on their unique stem-loop hairpin structure since the primary sequence is responsible for forming this secondary conformation. Mutations in residues contributing to this stable structure may lead to unstable mt:tRNA molecules that could be susceptible to enzymatic degradation. For example, a recent study by Duff et al. described how a single mutation in mt:tRNA<sup>Trp</sup> caused a wide range of defects (Duff et al., 2015). A homoplasmic 5559A > G mutation in cells from a family affected with Leigh syndrome not only altered the processing and stability of mt:tRNA<sup>Trp</sup> but also affected the stability of many other mitochondrial tRNAs, mRNAs, and rRNA.

Another tRNA-specific problem could be due to improper charging. The main function of tRNAs is to read the genetic code and transfer the respective amino acid residues onto the nascent polypeptide. But to do so, tRNAs must get charged with the appropriate amino acid by aminoacyl tRNA synthetase (ARS). In mitochondria, this charging process requires cross talk between nucleus-encoded mitochondrial tRNA synthetase

(mtARS) and mt:tRNAs (Tyynismaa & Schon, 2014). As these two components rely on each other for proper function, mutations in either may lead to a cascade of functional abnormalities. For example, mutations in 9 of 19 total mtARs, encoded by the nuclear genome, are associated with mitochondrial disease in a tissue-specific manner (Konovalova & Tyynismaa, 2013). Despite having wide-spread consequences in causing mitochondrial disease, mutations in mt:tRNAs are not yet treatable. Repairing these mutations is not possible, due to the maternal inheritance of mtDNA. Recent studies have shown that their functional incompatibility can lead to developmental defects in *Drosophila*, and cell growth defects in yeast and mammalian cell culture (Meiklejohn et al., 2013; Perli et al., 2016; Wang et al., 2016). However, researchers have shown that engineered nucleus-encoded mtARs can be used to suppress the effect of deleterious mt:tRNA mutations, which is easier to accomplish than altering mtDNA.

A comprehensive list of diseases related to mutations in mt:tRNAs is available at Mitomap (Brandon et al., 2005; Ruiz-Pesini et al., 2007). Due to their prevalence, there is a great deal of interest in mt:tRNAs point mutations and how they contribute to disease (Yarham, Elson, Blakely, McFarland, & Taylor, 2010). Using *Drosophila* to model human mitochondrial diseases caused by mt:tRNA mutations would be useful to determine the specific effect each mutation has on mt:tRNA processing, stability, and/or modification. To highlight the conservation between *Drosophila* and human mt:tRNAs, Fig. 2 shows a pair-wise structural alignment of all 22 tRNAs using the LocARNA alignment tool (Smith et al., 2010; Will, Joshi, Hofacker, Stadler, & Backofen, 2012). This program compares primary sequences as well as the structural compatibility of input RNA sequences, which is especially important as this indicates any potential disruptions caused by different mutations on secondary structure. Using LocARNA, all mt:tRNA pairs produce highly compatible canonical cloverleaf tRNA structures (examples shown in Fig. 2C and D), except for mt:tRNA<sup>Ser(AGY)</sup> and mt:tRNA<sup>Pro</sup>. The confirmed disease-causing point mutations are marked with asterisks below each pair. We have also marked the residues where unique mutations have been reported (Genebank frequency 0, thus not due to polymorphism). Of a total of 145 mutant residues, 77 are conserved in *Drosophila*, and some of these conserved residues, which are structurally more compatible than others, would be excellent targets for mutagenesis. Using these alignments, along with weighted-based pathogenicity scoring models, allows prioritization of which residues would be best targeted for mutation studies (Blakely et al., 2013; Yarham et al., 2011).



**Fig. 2** mt:tRNA comparison between human and *Drosophila*. (A) A graph showing the amino acid composition (in percent) of mitochondria-encoded proteins in human and *Drosophila*. Amino acids are represented in single letter code. The number of disease-causing point mutations in each mt:tRNA are indicated at the top of each column. (B) Pair-wise alignment between human and *Drosophila* mt:tRNAs using the web-based LocARNA tool. The tRNA sequences were obtained from human (accession # [NC\\_019290](#)) and *Drosophila* (accession # [U37541](#)) mitochondrial genome sequences. The nucleotide start of each sequence is on the left. The gray boxes underneath each alignment indicate conserved nucleotide identity. To show the general location of the stems

Arrows point to conserved disease-causing residues on the consensus structures of mt:tRNA<sup>Lys</sup> and mt:tRNA<sup>Leu</sup> (Fig. 2D and E). These two tRNAs, along with mt:tRNA<sup>Ser</sup>, are the most frequently mutated mt:tRNAs in mitochondrial disease (Lott et al., 2013; Ruiz-Pesini et al., 2007).



## 6. *DROSOPHILA* MODELS OF mtDNA-INDUCED DISEASE: UNTAPPED FUTURE POTENTIAL

There are multiple, devastating maternally inherited mitochondrial diseases. Developing additional fly models containing mtDNA mutations would be very useful for understanding the effect of each specific mutation on assembly and level of ETC complexes, on different tissues, and for determining how and what level of mutation load gives rise to deficits in organ and cell-type function. In addition, being able to generate specific mtDNA mutations at will would allow researchers to determine at the cellular and developmental level the molecular mechanisms governing inheritance. Given that there are only three models for mtDNA mutations in flies (*ATP6*<sup>1</sup>, *mt:CoI*, and *mt:ND2*), what are the future prospects for generating more?

There are several potential ways to generate models of mtDNA-dependent mitochondrial disease in *Drosophila*. The first way is to use the restriction endonuclease method described earlier to generate mutations in mtDNA (Xu et al., 2008). This method has the advantage that it appears robust in manufacturing escaper flies harboring mtDNA mutations through germline selection. Furthermore, this method can be used repeatedly to regenerate the fly stock, since the genetics underlying the technique is relatively simple. However, the disadvantage is that these single-cut endonucleases recognize specific locations in the fly mtDNA genome, which limits the number of positions that would be affected (Table 1). In addition, any given location is not guaranteed to have a deleterious effect.

and loops, a schematic of a canonical tRNA cloverleaf structure in stretched-form is shown at the top, with the complementary stems the same color. (C) The color coding indicates whether the nucleotides are conserved and if they form a Watson–Crick base pair. The color-coding matrix for sequence compatibility was obtained from the LocARNA site (Smith, Heyne, Richter, Will, & Backofen, 2010). (D and E) mt:tRNA<sup>Leu(UUR)</sup> (D) and mt:tRNA<sup>Lys</sup> (E) show generalized compatible secondary structures for each tRNA based on the sequence alignment. The arrows indicate some common point mutations found in human mitochondrial diseases that are conserved in *Drosophila*.

**Table 1** Restriction Endonucleases with Only One Site in *Drosophila melanogaster* Mitochondrial DNA

Endonuclease	Site	Gene
<i>Bsm</i> FI	287	ND2
<i>Bsr</i> BI	558	ND2
<i>Bgl</i> II	800	ND2
<i>Eco</i> RV	1359	tRNA <sup>Cys</sup>
<i>Nru</i> I	1473	COI?
<i>Bsg</i> I	1642	COI
<i>Bst</i> 1107I	2005	COI
<i>Tsp</i> 45I	2182	COI
<i>Ava</i> I	2368	COI
<i>Xho</i> I	2368	COI
<i>Nsi</i> I''	3158	tRNA <sup>Leu</sup>
<i>Sap</i> I	3310	COII
<i>Nci</i> I	3646	COII
<i>Drd</i> I	4245	ATPase 6
<i>Bss</i> SI	4922	COIII
<i>Sty</i> I	4938	COIII
<i>Ple</i> I	5305	COIII
<i>Ahd</i> I	5462	COIII
<i>Hpa</i> I	6751	ND5
<i>Afl</i> II	7417	ND5
<i>Pst</i> I	7514	ND5
<i>Bsu</i> 36I	9613	ND4L
<i>Bsa</i> BI	10,671	CytB
<i>Bsm</i> BI	10,711	CytB
<i>Nde</i> I	11,656	tRNA <sup>Ser</sup>
<i>Bbs</i> I	13,160	lrRNA
<i>Bsr</i> GI	14,207	srRNA
<i>Ban</i> I	14,741	srRNA

8 out of the 13 protein-coding regions have sites and only 3 out of 22 mt:tRNAs have sites.

To get around this problem, a method that is beginning to be explored involves the evolving technology of genome editing combined with mitochondrially targeted nucleases. Transcription activator-like effector nucleases (TALEN) technology has recently been shown to abolish neuro-genic weakness with ataxia and retinitis pigmentosa-associated mtDNA mutations in patient heteroplasmic cells by targeting and cleaving mutated mtDNAs (Reddy et al., 2015). TALEN technology targeting nuclear genes has been shown to be robust in *Drosophila*, thus adapting the modification of mitochondrially targeted TALEN developed for mammals could be used to cleave the *Drosophila* mtDNA genome at any site (Beumer et al., 2013; Katsuyama et al., 2013; Liu et al., 2012; Zhang, Ferreira, & Schnorrer, 2014). This method could potentially generate escaper flies repopulated with nuclease-resistant mtDNA, as is the case with mitochondrially targeted *XhoI* (Xu et al., 2008).

A method with the potential to create any mtDNA mutation on demand in *Drosophila* involves clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology (Sander & Joung, 2014). Recent work indicated this method can cleave mtDNA at the *CoI* locus in HEK cell mitochondria using a mitochondria-targeted Cas9 protein and introducing guide RNAs specific for mtDNA (Jo et al., 2015). While the authors show *CoI* is cut, it is not clear how the guide RNAs get into the mitochondria to mark the *CoI* locus, though various RNAs are known to be imported into eukaryotic mitochondria (Wang, Shimada, Koehler, & Teitell, 2012). CRISPR/Cas9 works very effectively in *Drosophila* on nuclear genes, and this technology appears to be more effective than gene targeting by homologous recombination in flies (Rong & Golic, 2000; Rong et al., 2002). In the nucleus, this genome editing involves homology-directed repair that uses an exogenously supplied oligo DNA encoding the desired change as a template for repair. Thus, for this technology to work on mtDNA for directed mutagenesis, there must be the appropriate repair mechanisms. Homologous recombination between mtDNA molecules has been clearly demonstrated for the first time in *Drosophila*, and the proteins required for double-strand break repair are present in mitochondria (Duxin et al., 2009; Ma & O'Farrell, 2015; Sage, Gildemeister, & Knight, 2010; Tann et al., 2011; Thyagarajan, Padua, & Campbell, 1996). Thus, it may be possible to use CRISPR/Cas9 to induce specific nucleotide changes in mtDNA to mimic human disease-causing mutations.

Point mutations in mtDNA lead to decreases in the proteins comprising the ETC complexes. While this likely occurs through a variety of



mechanisms (e.g., too few tRNAs, unstable mRNAs, nonfunctional proteins), targeted knockdown of individual mt:mRNAs would be useful in order to understand and characterize the resulting developmental and tissue-specific effects. Nucleus-encoded noncoding RNAs are imported into mitochondria in all species (Sieber, Duchene, & Marechal-Drouard, 2011). Wang et al. successfully targeted wild-type mt:tRNAs to mitochondria using a 20-ribonucleotide stem-loop sequence from H1 RNA, the RNA component of RNase P (Wang, Shimada, Zhang, et al., 2012). They demonstrated this method could correct deficits in mt:tRNAs in cultured cells containing mtDNA mutations. Using a variation on this theme in *Drosophila*, Towheed et al. combined a similar approach with the idea of RNA silencing (Towheed, Markantone, Crain, Celotto, & Palladino, 2014). The 5S rRNA was originally thought to be a component of only cytoplasmic ribosomes; however, it is also imported into mitochondria where its function is not entirely clear (Magalhaes, Andreu, & Schon, 1998; Yoshionari et al., 1994). Towheed et al. identified the *Drosophila* ortholog of mitochondrial 5S rRNA and used the stem-loop leader sequence to target antisense RNA to mitochondria (Towheed et al., 2014). This technique resulted in translational inhibition of *mt:ATP6* and a 40–50% reduction in protein levels, which phenocopied *ATP6<sup>1</sup>* mutant flies. Called mitochondrial-targeted RNA expression system (mtTRES), the authors used the GAL4/UAS system to conditionally express the mt:ATP6 antisense mRNA, thus giving them spatial and temporal control.

The final method to create mutated mtDNA that has not been exploited in *Drosophila*, but has much potential, is creating a so-called mutator fly using a proof-reading-deficient mitochondrial polymerase gamma (PolG). PolG, the catalytic subunit of mtDNA polymerase, is a highly processive enzyme that contains three exonuclease domains responsible for excising and repairing mismatched nucleotides during replication (Kaguni & Olson, 1989; Wernette, Conway, & Kaguni, 1988). First described in yeast, PolG mutations were created by mutating conserved residues in the exonuclease domains, which led to an increase in mtDNA mutations as assayed by increased erythromycin resistance (Foury & Vanderstraeten, 1992). Erythromycin, an antibiotic that targets bacterial ribosomes, affects mitochondrial ribosomes due to the conserved mode of action between mitochondrial and bacterial ribosomes. mtDNA mutator mice have been successfully generated that lead to an increase in mtDNA mutations (Trifunovic et al., 2004). The *Drosophila* ortholog of PolG is called Tamas (Iyengar, Roote, & Campos, 1999). Mutations in *tamas* are lethal, and mutations in human PolG are

known to cause mitochondrial disease (Wong et al., 2008). One problem with creating a mutator fly is that ectopically overexpressing *tamas* from a transgene leads to a decrease in mtDNA and lethality (Lefai et al., 2000). Using CRISPR/Cas9 would get around this problem by altering *tamas* at the endogenous locus, and thus the same exonuclease mutations used in yeast and mouse could be introduced under control of the endogenous promoter. A mutator fly would be useful for generating random mtDNA mutations and studying their effect on specific tissues, as well as inheritance. In addition, this would potentially be a good model for examining the effect of increased mtDNA mutation load on aging.



## 7. MITOCHONDRIAL INHERITANCE AND QUALITY CONTROL CHECKPOINTS

MtDNA has a higher mutation rate than nuclear DNA. In *Drosophila*, for example, it is  $10 \times$  higher than the nucleus (Haag-Liautard et al., 2008; Vermulst et al., 2007). Coupled with seemingly more rudimentary DNA repair mechanisms, an outstanding question is how oocytes generally maintain high levels of highly functional mitochondria. During inheritance, mitochondria can undergo a rapid change in genotype, giving rise to the hypothesis that there is a genetic bottleneck. Evidence supports that this bottleneck may take place during oogenesis; however, where and how this happens is not fully understood (Wallace & Chalkia, 2013). Studies in bovine indicated that a change in mtDNA genotype can be rapid, and data from mouse have tried to pinpoint the developmental timing of the bottleneck by estimating changes in mtDNA copy number at different times during fetal oogenesis (Cao et al., 2007; Hauswirth & Laipis, 1982; Jenuth, Peterson, Fu, & Shoubridge, 1996). This bottleneck was thought to be due to random genetic drift; however, there is increasing evidence that it may serve as a purifying mechanism to ensure only the most fit mitochondria populate the oocyte (Fan et al., 2008; Freyer et al., 2012; Stewart et al., 2008).

Women with disease-causing point mutations in mtDNA have a high probability of having children affected by the disease (Taylor & Turnbull, 2005). They may not manifest any disease symptoms until later in life, or at all, and thus may already have children. Prognoses in these cases are hard to make. A woman whose germline is heteroplasmic can have viable oocytes with different levels of mutated mtDNA, thus siblings can inherit different disease severity. There are only limited tools to determine which oocytes

have a lower mutation load. One possible remedy recently advanced is nuclear transfer, using the affected oocyte's nucleus and an enucleated donor egg containing normal mitochondria (Mitalipov & Wolf, 2014). While this method is now legal in the United Kingdom, a better understanding of the molecular mechanisms underlying mitochondrial inheritance is required to provide their patients with an accurate prognosis.

*Drosophila* are an ideal model in which to study the mechanisms responsible for mitochondrial inheritance. *Drosophila* oogenesis is well characterized (Spradling, 1993). The stem cells can be unambiguously identified, each developmental stage is present for examination, and mitochondria can be visualized at the single-organelle level (Cox & Spradling, 2003). *Drosophila* oocyte formation shares a surprisingly large number of similarities with vertebrate oogenesis (Matova & Cooley, 2001). For example, both *Drosophila* and vertebrate germ cells spend part of their life as a cluster of interconnected cells called cysts (Pepling, de Cuevas, & Spradling, 1999). The presence of cysts allows the germ cells to share cytoplasmic components, such as microtubules, Golgi, centrosomes, and mitochondria (Cox & Spradling, 2003; Lei & Spradling, 2016; Pepling & Spradling, 1998). A prominent structure in oocytes is a mitochondrial cloud or Balbiani body (Kloc, Bilinski, & Etkin, 2004). In *Drosophila*, this highly conserved structure forms when a subset of mitochondria from connected sister germ cells moves into the oocyte using the microtubule cytoskeleton and molecular motors (Cox & Spradling, 2003, 2006). Since only a subset of mitochondria is transported into the oocyte to populate the oocyte for the first half of oogenesis, this raises the possibility that these mitochondria may be the most highly functional. Microtubule motor complexes appear to be important, suggesting that the ability of a particular organelle to bind to the motor and be transported may be part of the mechanism; however, this has not been directly tested.

Models of mitochondrial inheritance in *Drosophila* have given insight to the potential mechanisms underlying mitochondrial inheritance during oogenesis. The original studies examining the mitochondrial bottleneck in mouse and *Drosophila* took advantage of natural size differences and neutral polymorphisms between mtDNAs and did not look at competition between deleterious mutations and wild-type mtDNA (Jenuth et al., 1996; Kann, Rosenblum, & Rand, 1998; Solignac, G nermont, Monnerot, & Mounolou, 1984; Solignac, G nermont, Monnerot, & Mounolou, 1987). Recent work has used cytoplasmic injection to create heteroplasmic flies containing wild-type and mutated mtDNAs, followed

by monitoring of mitochondrial purification over time (Hill, Chen, & Xu, 2014; Ma, Xu, & O'Farrell, 2014). The authors of these studies have exploited *Drosophila* to pinpoint the stages when mtDNA purification is taking place during oogenesis.

By carefully analyzing the percent heteroplasmy in flies over multiple generations, Ma et al. were able to demonstrate that there is a mtDNA purifying mechanism that takes place during oogenesis (Ma et al., 2014). They created heteroplasmic flies containing either two mutant mtDNAs (*mt:CoI* and *mt:ND2*) or wild type and mutant. This recreated a more physiological situation, where most mtDNA is wild type, and a small proportion contains a deleterious lesion in the mtDNA. In agreement with previous work, deleterious mtDNA mutations paired heteroplasmically with wild-type mtDNA were lost, giving clear-cut evidence that there is a purifying mechanism for mutated mtDNA. When two mtDNAs for lesions in two different genes were combined, they complemented each other and were maintained, resulting in viable flies. This observation is satisfying, since each cell contains many mtDNA molecules that should be able to complement function; however, this had not been demonstrated. As with mouse, the change in heteroplasmy took place quickly between mothers and their eggs, supporting that mtDNA genotype shifts happen during oogenesis. As one of the mutations was temperature sensitive, the authors were able to perform temperature shift experiments to test when during oogenesis any selection may be occurring. By doing this, they found that the selection occurs after germ cell mitotic expansion, and thus a large proportion of the selection occurs in the later germarium stages or later during oogenesis. This coincides with when the motor-driven Balbiani body formation occurs.

What mechanism could cause this mutant mtDNA selection during oogenesis? One possibility is that wild-type mtDNAs have a replicative advantage over mutated mtDNAs. Hill et al. developed a method using 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analog, to examine mtDNA replication in dissected ovaries (Hill et al., 2014). This was the first time that mtDNA replication had been visualized during oogenesis. In wild-type flies, they found mtDNA replication was particularly high very early in germ cell development. This occurs right after the germ cells have completed their mitoses and have started their meiotic program at stage 2b. In addition, the mtDNA replication was dependent on mitochondrial function and membrane potential as germ cells containing the temperature sensitive, deleterious point mutation *mt:CoI*<sup>T3001</sup> had greatly reduced replication. The timing of increased mtDNA replication occurred around the same

developmental time as [Ma et al. \(2014\)](#) postulated selection occurs. These two elegant studies together demonstrate the advantages of using *Drosophila* to study mtDNA inheritance: the combination of genetic manipulation (through injection to create heteroplasmic flies), the short generation time which allows mtDNA genotypes to be followed over many generations, immunofluorescence, and the ability to generate large sample sizes.



## **8. CONCLUDING REMARKS: LOSS OF MITOCHONDRIAL FUNCTION BROADLY IMPACTS HUMAN DISEASE**

Mitochondrial disease is usually defined by loss of OXPHOS. Human mitochondrial disease is thought to affect as many as 1 in 5000 people, and there are no cures and few effective treatments ([Schaefer, Taylor, Turnbull, & Chinnery, 2004](#)). Because *Drosophila* mtDNA is so similar to human mtDNA, there is much potential to study the cell and developmental consequences of loss of nucleus-encoded mitochondrial proteins, and also mutations in mtDNA. Recent manipulation of deleterious mutant mtDNA has allowed *Drosophila* researchers to start to uncover the molecular mechanisms governing mtDNA inheritance and selection. Of course, mitochondria are responsible for generating many other important metabolites and are also pivotal in cell biological processes such as apoptosis and signaling. Due to a high demand for energy, muscle and neurons are cell types particularly sensitive to alterations in mitochondrial output. Decreases in mitochondrial function can lead to cardiomyopathy and heart problems, Parkinson's disease (reviewed in this issue), as well as other neurodegenerative diseases. Studying mitochondria in *Drosophila* will continue to inform and enlighten researchers about human mitochondrial diseases.

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