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Review

# Human mitochondrial DNA diseases and *Drosophila* models

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ABSTRACT

Mutations that disrupt the mitochondrial genome cause a number of human diseases whose phenotypic presentation varies widely among tissues and individuals. This variability owes in part to the unconventional genetics of mitochondrial DNA (mtDNA), which includes polyploidy, maternal inheritance and dependence on nuclear-encoded factors. The recent development of genetic tools for manipulating mitochondrial genome in *Drosophila melanogaster* renders this powerful model organism an attractive alternative to mammalian systems for understanding mtDNA-related diseases. In this review, we summarize mtDNA genetics and human mtDNA-related diseases. We highlight existing *Drosophila* models of mtDNA mutations and discuss their potential use in advancing our knowledge of mitochondrial biology and in modeling human mitochondrial disorders. We also discuss the potential and present challenges of gene therapy for the future treatment of mtDNA diseases.



## 1. Introduction

Mitochondria are unique organelles in that they contain their own genome, but also rely on many nuclear-encoded factors for their structure, function and propagation. Consequently, mutations in either the nuclear or mitochondrial genome may impair mitochondrial function and ultimately lead to human diseases. The first study identifying a molecular lesion in mitochondrial DNA (mtDNA) as the cause for a human disease dates back to 1988 (Holt et al., 1988; Wallace et al., 1988). Since then, more than 700 mtDNA mutations have been linked to a range of human diseases (Lott et al., 2013). An even larger number of nuclear mutations leading to mtDNA defects have also been associated with mitochondrial diseases.

Our understanding of mtDNA-associated diseases is complicated by their variable clinical presentations. This variability stems in great part from the genetics of mtDNA, which differs from classic Mendelian genetics in many aspects. Mitochondria are transmitted exclusively through the maternal lineage in most metazoans, and each cell contains hundreds to thousands of mtDNA molecules, which randomly segregate during cell division. As a result, the severity of a phenotype can vary according to the proportion of mutant mtDNA that an individual inherited. The phenotype may also progress with age, and it often displays disparities among different tissues in the same individual. Such complex relationship between genotype and phenotype makes the clinical diagnosis of mtDNA-related diseases and the study of their etiology challenging, which in turn greatly hampers the development of therapies. Mitochondrial DNA dysfunctions are currently estimated to affect one in 5000 individuals worldwide (Schaefer et al., 2004, 2008; Gorman et al., 2015). Yet, at this point, there is no effective treatment for mtDNA-related disorders.

To better understand the physiopathology of mitochondrial diseases, and to develop effective therapies, animal models that simulate a variety of symptoms associated with these complex human disorders are required. There have been a few mammalian models carrying mtDNA mutations (Marchington et al., 1999; Lin et al., 2012). However, the number is quite limited due to technical hurdles in manipulating mitochondrial genomes of most metazoans. *Drosophila melanogaster* (*D. melanogaster*) has recently emerged as a promising exception, thanks to the development of various tools to modify its mitochondrial genome. These tools now afford this powerful model organism as an attractive alternative to mammalian systems for studying diseases arising from mtDNA deficiencies.

In this review, we provide an overview of the mitochondrial genetic system and of human mitochondrial diseases caused by mtDNA mutations. We further discuss existing *D. melanogaster* models of mtDNA mutations and discuss their potential use in advancing our knowledge of mitochondrial biology and in

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modeling human mitochondrial disorders. Last, we discuss potential new therapeutic interventions in mtDNA diseases, particularly the use of gene therapy, and highlight the current challenges in manipulating the mitochondrial genome and the potential use of *Drosophila* to expand our toolkit.

## 2. The mitochondrial genetic system

The mitochondrial genome follows genetic rules that differ from the Mendelian rules governing nuclear genome inheritance. The most prominent differences are maternal inheritance and polyploidy, the presence of multiple copies of mtDNA within a single cell. Before we can understand how mtDNA mutations arise and lead to complex disorders, it is important to understand mitochondrial genetics and the basic mechanism of mtDNA replication and transcription.

### 2.1. Overview of mitochondrial functions

Mitochondria play a number of pivotal roles in eukaryotic cells, the most essential of which is to produce cellular ATP through the oxidative phosphorylation (OXPHOS) system. Located on the heavily folded cristae of the mitochondrial inner membrane, OXPHOS includes four electron-transport chain complexes (Complexes I–IV) and the ATP synthase (Complex V). The concerted action of these transmembrane complexes, composed of more than 80 different polypeptides, produces most of ATP in cell (Fig. 1). Mitochondria are also involved in other cellular pathways, including $Ca^{2+}$ homeostasis, anabolic and catabolic processes, ROS signaling and apoptosis (Nunnari and Suomalainen, 2012; Tait and Green, 2012).

### 2.2. Organization of the mitochondrial genome

The human mitochondrial genome consists of multicopy, 16.6 kb circular double-stranded DNA molecules (Fig. 2A). Aside from encoding 13 proteins of the OXPHOS system, it also encodes the RNAs necessary for their translation within the organelle (22 tRNAs and 2 rRNAs). The remaining components of OXPHOS, as well as proteins required for mtDNA replication and transcription, and additional components of the translation machinery, are all encoded in the nucleus, synthesized in the cytoplasm and imported into mitochondria post-translationally (Taylor and Turnbull, 2005).

The human mitochondrial genome is extremely compact (Fig. 2A): intergenic sequences are absent or limited to a few bases, and introns are altogether absent (Taanman, 1999). The only long stretch of non-coding region (NCR) serves as a regulatory element for mtDNA replication and transcription. The individual strands of the mtDNA molecules are designated as the heavy (H) and light (L) strand due to their difference in the buoyant densities in a cesium chloride gradient. The 1 kb NCR, also known as the D-loop, harbors the replication origin for H-strand DNA replication ($O_H$). Another origin for L-strand DNA replication ($O_L$) is located outside of D-loop, which is approximately 11 kb downstream of $O_H$ (Clayton, 1982). The NCR also contains three promoters that drive expression of the mtDNA transcripts, two heavy strand promoters (HSP1 and HSP2) and one light strand promoter (LSP) (Montoya et al., 1982).

The *D. melanogaster* mitochondrial genome is ~3 kb bigger than human mtDNA, largely because of an elongated NCR that contains 90%–96% deoxyadenylate and thymidylate residues (the A + T rich region). Other than that, mitochondrial genomes in human and fruit flies are highly conserved and their gene contents are identical, although the organization of their genomes differ slightly (Fig. 2B) (Garesse and Kaguni, 2005). Given the relative lack of non-coding sequence, the chances that a random mutation affects mitochondrial function are high.

### 2.3. Mitochondrial DNA replication and transcription

While nuclear DNA is replicated during the synthesis phase (S phase) of the cell cycle and synthesized precisely once per cell division, mtDNA replication is only loosely coupled with the cell cycle (Bogenhagen and Clayton, 1977; Zhang et al., 2015), and may change in response to the bioenergetic demands of the cell. The exact mechanism of mtDNA replication is still debatable. Two distinct models have been proposed. In the strand-displacement model (Clayton, 1982; Phillips et al., 2017), the replication of the leading strand (also known as the heavy strand) initiates at the origin $O_H$ located in the NCR. When leading-strand replication has reached two-thirds of the genome, the replication origin on the light strand, $O_L$, is exposed, and L-strand synthesis initiates and proceeds in the opposite direction. The second model involves a conventional coupling of leading- and lagging-strand synthesis initiating at $O_H$ (Holt et al., 2000). The two models of mtDNA replication may operate under different conditions, depending on whether copy number is being modulated or maintained in a

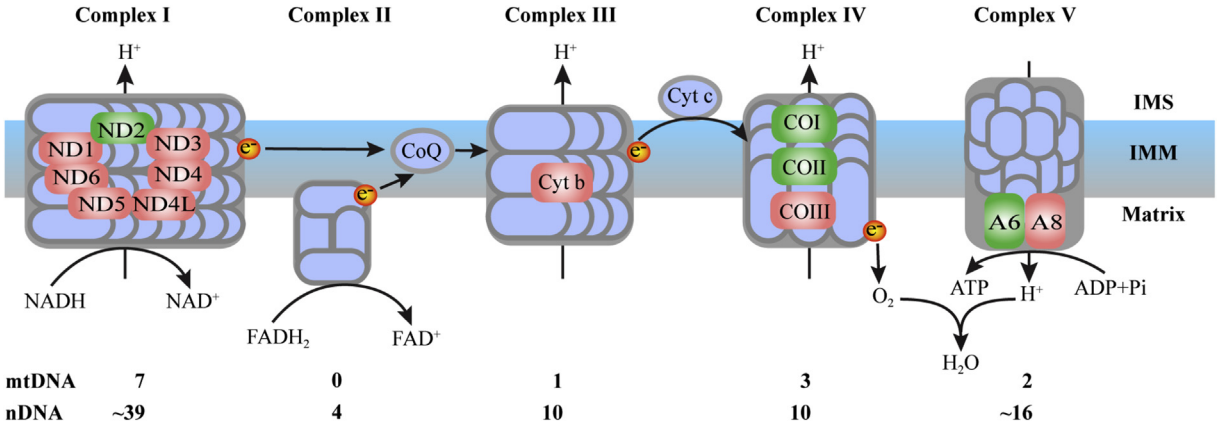


**Fig. 1.** The mitochondrial oxidative phosphorylation system. The four electron-transport chain complexes (Complexes I–IV) and the ATP synthase (Complex V) are represented. The mtDNA-encoded subunits are labelled in red and green (in green are the subunits for which *Drosophila* mtDNA mutants are available), and nuclear DNA (nDNA)-encoded subunits are labelled in blue. Electrons from reducing equivalents (NADH and $FADH_2$) pass along the electron transport chain complexes embedded in the inner mitochondrial membrane (IMM), via two electron carriers, coenzyme Q (CoQ) and cytochrome c (Cyt c). Meanwhile, the protons are pumped from the mitochondrial matrix into the intermembrane space (IMS). The resulting electrochemical proton gradient is used by ATP synthase to convert ADP and inorganic phosphate (Pi) to ATP as protons flow back into the matrix.

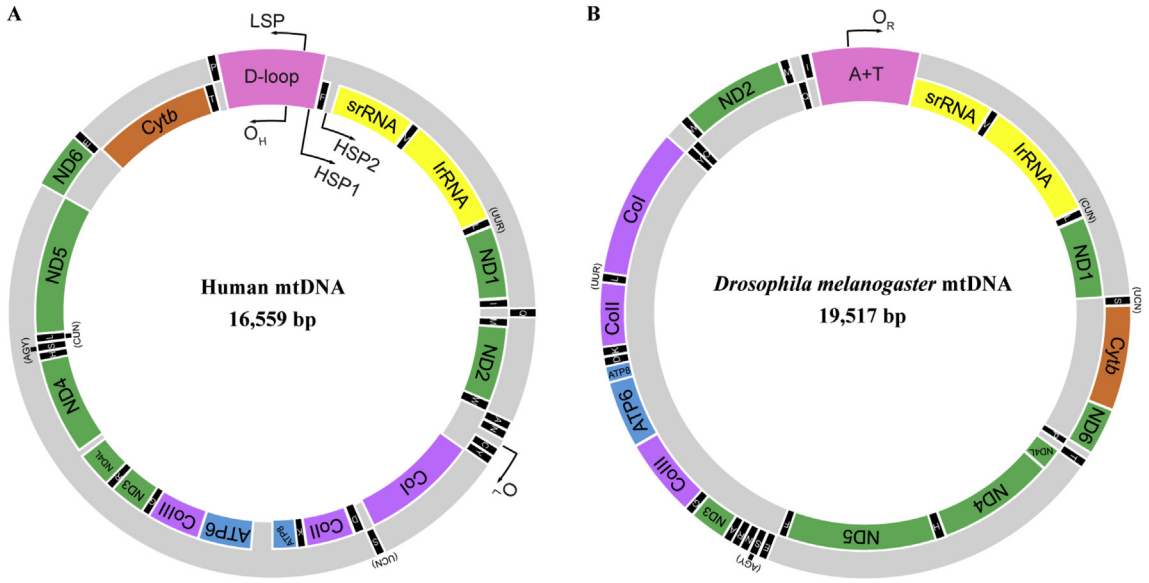


**Fig. 2.** Organization of the human and *Drosophila melanogaster* mitochondrial DNA genomes. **A**: The human mitochondrial genome (NC_012920.1) encodes 13 polypeptides, 22 tRNAs and 2 rRNAs. ND, NADH dehydrogenase; Cyt *b*, cytochrome *b*; Co, cytochrome c oxidase; ATP, ATP synthase; srRNA, 12S ribosomal RNA; lrRNA, 16S ribosomal RNA. The 22 tRNAs are represented by the amino acid they carry, using the single-letter nomenclature. The origins of heavy- and light-strand replication ($O_H$ and $O_L$), as well as the promoters of heavy- and light-strand transcription (HSP and LSP) are labelled. A non-coding region of approximately 1 kb, the D-loop, harbors transcription promoters and one of the replication origins. **B**: The *Drosophila melanogaster* mitochondrial genome (U37514) encodes the same set of transcripts as the human genome (represented in the same colors), but in a slightly different genomic order. The *Drosophila* mtDNA is approximately 3-kb bigger than its human counterpart, predominantly due to an expanded "A + T rich" region in its regulatory region. This is also where the putative origin of replication ($O_R$) of *Drosophila* mtDNA is located.

steady state.

Several proteins have been found necessary to reconstitute a minimal replisome *in vitro* (Korhonen et al., 2004). Mitochondrial DNA polymerase γ (POLγ) is the only known DNA polymerase devoted to mtDNA synthesis (Ropp and Copeland, 1996). This heterotrimeric DNA polymerase is composed of a catalytic subunit with exonuclease activity (POLγA) and a dimeric accessory subunit (POLγB) that increases processivity (Carrodeguas et al., 1999, 2001). MtSSB, the single-stranded DNA binding protein, and Twinkle, a mitochondrial helicase with 5′−3′ directionality, act together with POLγ to form the core components of the mtDNA minimal replisome. Mitochondrial transcriptional factor A (TFAM), the major mtDNA packaging factor (Alam et al., 2003), associates with these proteins and with mtDNA to form structures called nucleoids. The nucleoids, which may contain one or more copies of mtDNA, are recognized as the segregation units of mtDNA transmission and inheritance (Legros et al., 2004; Kukat et al., 2011).

Transcription from three promoters in mtDNA gives rise to a series of polycistrons in both human and *Drosophila melanogaster*. HSP1, one of the promoters on the heavy strand, generates a transcript containing two tRNAs and two rRNAs (12S and 16S), while HSP2, the other heavy-strand promoter, produces a transcript spanning almost the entire genome. LSP, located on the light strand, controls the transcription of eight of the tRNAs and of the MT-ND6 gene (Chang and Clayton, 1984). Mature mRNAs and rRNAs are produced by excision of the tRNA molecules from the polycistronic transcripts. Since most protein-coding and two rRNAs genes are separated by at least one tRNA gene, like words separated by punctuation marks, this mode of RNA processing is called the "tRNA punctuation model" (Ojala et al., 1981). The machinery of mtDNA transcription include TFAM, mitochondrial RNA polymerase (POLRMT) and one of the mitochondrial transcription factor B paralogues, TFB1M or TFB2M (Falkenberg et al., 2002). All proteins essential for mtDNA replication and transcription are highly conserved between human and *Drosophila*, and the orthologs have been extensively studied by using versatile genetic toolkits of *D. melanogaster* (Garesse and Kaguni, 2005).

### 2.4. Peculiarities of mitochondrial genetics

The genetics of mtDNA is different from classic Mendelian genetics in almost all aspects. The most prominent characteristics that influence these differences are polyploidy and maternal inheritance.

Polyploidy results from the fact that each cell contains many mitochondria, and each mitochondrion usually contains multiple copies of mtDNA. Depending on the cell type, mtDNA copy number can vary from hundreds to thousands. Polyploidy has important consequences on the transmission of mtDNA mutations. During cell division, mtDNA molecules sort randomly between the two daughter cells. If the mother cell harbors a mix of wild-type and mutant mtDNAs, a situation known as heteroplasmy, each daughter cell may receive different proportions of mutated molecules. Neutral mtDNA variants may maintain a constant level of heteroplasmy in a cell population, although their proportions vary among dividing cells (Stewart and Chinnery, 2015). But the random segregation of deleterious mutations may cause certain cells to inherit an amount of unhealthy mutant mtDNA, with potential adverse effects on the function of these cells or the tissues they give rise to.

The problem of the random segregation of mtDNA mutations during cell division is compounded by maternal inheritance. Although there have been a few cases demonstrating the paternal origin of mtDNA in humans (Schwartz and Vissing, 2002; Luo et al., 2018), in most animals, mtDNA mutations are inherited exclusively through the maternal lineage, because paternal mtDNA is eliminated from the gametes or the zygotes (Sato and Sato, 2013). This situation prevents the correction of mutations by recombination between the parental genomes, as normally happens on nuclear genome during sexual reproduction. As a result, mtDNAs have a high mutation rate, estimated to be 100- to 1000-fold higher than that of nuclear DNA (Wallace and Chalkia, 2013).

Without the benefits of recombination, mtDNA mutations would be expected to continually accumulate over generations, which would lead to the eventual genetic meltdown of mitochondria, known as Muller's ratchet (Muller, 1964). However, the successful evolution of mitochondria and their host organisms argues that mechanisms must exist to prevent the transmission of deleterious mtDNA mutations through the female germline. A prevailing hypothesis in mammals, called the genetic bottleneck hypothesis, is based on the observation that the offspring of a heteroplasmic mother can have mtDNA heteroplasmy ratios that differ significantly from each other and/or from the mother (Koehler et al., 1991). According to this hypothesis, mitochondria go through a genetic bottleneck during oocyte maturation, such that only a fraction of the mtDNA molecules present in the mother are represented in the mature eggs. The resulting offspring may then be subjected to selection based on individual fitness, which in turn prevents the spread of deleterious mtDNA mutations.

Recent studies in mammals (Stewart et al., 2008) and *Drosophila* (Hill et al., 2014; Ma et al., 2014) also suggest a strong purifying selection against harmful mtDNA mutations at the organelle level. Our studies in *D. melanogaster* led us to propose that wild-type mtDNA replicates much more vigorously than mutant mtDNA, so that the load of mutant mtDNA declines through oogenesis (Hill et al., 2014; Zhang et al., 2019). These mechanisms, acting at different levels, together restrict the transmission of mtDNA mutations from one generation to the next and prevent mitochondrial genetic meltdown. However, the selective transmission process is not perfect, and sometimes defective mitochondria carrying deleterious mtDNA mutations leak through, which can eventually cause severe mtDNA disorders.

## 3. Human mtDNA diseases

Genetic epidemiological studies suggest that the incidence of mitochondrial diseases caused by the most common mtDNA mutations is about one in 5000 people (Schaefer et al., 2004, 2008; Gorman et al., 2015). Considering that mtDNA mutations share some clinical features and can be found in association with diseases such as hypertension (Wilson et al., 2004) and diabetes (Mootha et al., 2003; Petersen et al., 2003), the incidence could be much higher. Hence, pathogenic mtDNA mutations are very common and the number is continually rising. In this part, we review some common mtDNA diseases.

### 3.1. Phenotypic characteristics of mtDNA diseases

There is dramatic variability in the phenotypic presentation of mtDNA disorders. The same mutations in mitochondrial genome can cause various clinical presentations in different patients or different tissues within an individual. Conversely, the same clinical features can be caused by different mtDNA mutations. For example, the variant known as m.3243A > G can cause Chronic Progressive External Ophthalmoplegia (CPEO) (Moraes et al., 1993), diabetes mellitus and deafness (van den Ouweland et al., 1994), or a severe encephalopathy with recurrent strokes and epilepsy (Pavlakis et al., 1984). On the other hand, a group of clinically indistinguishable individuals with external ophthalmoplegia may include individuals with a large deletion of mtDNA, others with a m.3243A > G single nucleotide variant, and others yet with a pathogenic variant in a nuclear gene causing secondary mtDNA defects. This phenotypic variability can be attributed to the unconventional genetics of mtDNA, including threshold effect, tissue mosaicism and progressivity with age.

#### 3.1.1. Threshold effect

Most pathogenic mtDNA mutations identified in human diseases were found in a heteroplasmic state, mixed with normal mtDNA within the cells. Heteroplasmic mtDNA mutations typically do not lead to biochemical defects and pathological phenotypes until they reach a threshold, typically 60%–90% of mutant mtDNA. Although higher proportions of mutant mtDNA are generally associated with more severe clinical symptoms, the threshold depends on the particular mtDNA mutation and varies among different tissues and individuals (DiMauro and Schon, 2003; Taylor and Turnbull, 2005).

#### 3.1.2. Tissue mosaicism

Some mtDNA diseases may affect a single organ, but the majority impair multiple organ systems. Mitochondrial DNA diseases predominantly affect tissues with higher energy demand, such as nervous system and muscle, and often present with dramatic neurologic and myopathic features. However, they can also affect other systems such as the endocrine system and the gastrointestinal system (Wallace, 2005). The most unpredictable aspect of mitochondrial genetics is the segregation of mtDNA molecules in heteroplasmic situations, both in the female germline and in somatic tissues (Wallace and Chalkia, 2013). Indeed, the proportion of heteroplasmic alleles can shift during both mitotic and meiotic cell division, a process known as replicative segregation (Wallace and Chalkia, 2013). As a result, tissues and organs differentiated from the same heteroplasmic embryo could have distinct proportions of normal and mutant mtDNA (Wallace, 2005). In addition, different tissues or organs have different sensitivity to mitochondrial disruption. Thus, genetic mosaicism causes bioenergetic mosaicism and phenotypic complexity in an organ-specific manner.

#### 3.1.3. Progressivity

Clinically relevant mtDNA diseases are mostly progressive and develop with age. Patients who inherit a heteroplasmic mtDNA disorder from their mother often do not present symptoms until late childhood or early adult life. Individuals who did not inherit an mtDNA mutation may still develop mtDNA disorders with time, because somatic mtDNA mutations accumulate with age (Wallace, 1999). Elderly people typically carry a wide range of different mtDNA deletions in post-mitotic tissues, such as brain and skeletal muscles (Cortopassi and Arnheim, 1990; Corral-Debrinski et al., 1992). But specific mtDNA mutations can accumulate to very high levels over time both in mitotic tissues, including individual cells or stem cell lineages, and in post-mitotic tissue. This phenomenon, referred to as clonal expansion, results in part from the fact that mtDNA turns over independently of the cell cycle, and can therefore replicate and expand even in post-mitotic cells. Although the dynamics by which mtDNA mutations occur and clonally expand are debated (Elson et al., 2001; Carelli et al., 2015), the delayed onset and progressive course of mtDNA defects suggest their importance in the ageing process. Overall, the age of mtDNA diseases' onset reflects the level of mutation and the severity of the biochemical defect, but other factors, such as nuclear background or environmental factors, may also contribute to the expression of the disease (Tuppen et al., 2010).

Although threshold effect, mosaicism and progressivity could partially explain the extremely heterogenous clinical phenotypes of mtDNA diseases, some questions are still unanswered. For instance, how do we explain clinical variability in the case of diseases due to homoplasmic mtDNA mutations? One such example is a point mutation located in the *ND4* gene, which causes Leber's Hereditary Optic Neuropathy (LHON), a common mitochondrial disease related to degeneration of the retinal ganglion cells (Yu-Wai-Man et al., 2002). The mtDNA mutations are usually homoplasmic, yet the

age of visual loss initiation varies within a large range and the disease affects adult men predominantly. How can a homoplasmic mtDNA mutation, which should affect all tissues at any given time, display both sex dimorphism and disparity of age onset of the disease?

In addition, there are many examples of mtDNA diseases whose clinical severity shows no correlation with the proportion of mutant mtDNA (Chinnery et al., 2001). How, and to what extent, can a particular mutated mtDNA molecule affect mitochondrial function? Although most mutations affecting mtDNA are expected to disrupt the respiratory chain, it is very unlikely that compromised mitochondrial respiration is the only reason for the variability of mtDNA clinical presentations. Indirect effects of respiratory dysfunction on $Ca^{2+}$ homeostasis, ROS signaling, apoptosis and other mitochondrial pathways might also contribute to the etiology of the diseases. So far, our knowledge of the biochemical and molecular mechanisms linking the mtDNA genotypes and phenotypes is very limited. Model systems that recapitulate the complexity of common mtDNA diseases may provide new perspectives on their etiology (see below).

### 3.2. mtDNA mutations and common mtDNA diseases

Mitochondria are under dual genetic control of both nuclear and mitochondrial genomes. Consequently, mitochondrial diseases can arise from mtDNA defects *per se* or mutations in nuclear genes that are involved in mitochondrial genome maintenance and expression. According to their origin, mtDNA diseases can be classified into defects caused by point mutations, deletions and depletion of mitochondrial genome. To better demonstrate the features and genotype-phenotype relationships of mtDNA disorders, we describe some examples of common mtDNA diseases categorized by their origins. A current compendium of all known pathogenic mtDNA mutations can be found on the MitoMAP database (Lott et al., 2013).

#### 3.2.1. mtDNA point mutations

These mutations may affect any of the products encoded by mtDNA: tRNAs, rRNAs and proteins. Point mutations in mitochondrial protein-coding genes disrupt the specific protein and its corresponding respiratory chain complex. Point mutations in tRNAs may impair overall mitochondrial translation by shortening the supply of specific tRNAs. Mutations in tRNA genes account for around 60% of all human diseases related to mtDNA mutations (Sen and Cox, 2017). This is an unusually large number considering that tRNA genes only occupy 9% of the mtDNA sequence (Stewart et al., 2008; Sen and Cox, 2017). This phenomenon may be explained by the less deleterious nature of mutations in tRNA genes, thus a weaker selection against them during mtDNA maternal transmission.

Leber's Hereditary Optic Neuropathy (LHON) is the most common mitochondrial disease caused by mtDNA point mutation in a protein-coding region. Approximately 95% of the LHON patients have one of three mtDNA mutations (m.11778G > A, m.3460G > A, and m.14484T > C) (Yu-Wai-Man et al., 2002), which are located in the ND4, ND1 and ND6 subunits of Complex I of the OXPHOS system, respectively. The mtDNA mutations are usually homoplasmic, so all offspring and all tissues will be affected by the mutation. However, LHON typically only causes degeneration of retinal ganglion cells (RGCs), which leads to an acute or subacute loss of central vision. This degeneration occurs in about 50% of men, but only 10%–15% of women mutation carriers (Kirches, 2011; Sadun et al., 2011). So far, the underlying mechanisms of LHON's incomplete penetrance and gender bias are not clear. Mitochondrial haplogroups and susceptibility alleles in the nuclear genome have been proposed to affect the probability of disease development in mutation carriers (Kirches, 2011; Carelli, 2016).

As mentioned earlier, mtDNA mutations in tRNA regions are much more frequent than in protein-coding regions. Two major syndromes caused by tRNA point mutations are Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS) (Goto et al., 1990), and Myoclonic Epilepsy and Ragged Red Fibers (MERRF) (Shoffner et al., 1990). MELAS usually affects multiple organs, particularly the brain and muscles. It has an early age of onset with mild symptoms, including muscle weakness, recurrent headaches, vomiting and seizures. As the disease progresses, stroke-like episodes accompanied by seizures appear, causing damage to the brain, muscles and visual system. Most people with MELAS have a high concentration of lactic acid in their body, leading to vomiting, fatigue and muscle weakness. More than 80% of patients with MELAS have the m.3243A > G mutation within mitochondrial $tRNA^{Leu}$ gene (Goto et al., 1990; DiMauro and Hirano, 1991). Other mutations in tRNA (Taylor et al., 1996) and protein-coding region (Santorelli et al., 1997) may also cause MELAS. MERRF is a progressive neurodegenerative disease, which often presents in childhood or early adulthood. The primary features of MERRF include myoclonus, seizures, cerebellar ataxia, myopathy and ragged red fibers (RRFs) on muscle biopsy. Around 80% of patients with MERRF exhibit the point mutation m.8344A > G, which maps to mitochondrial $tRNA^{Lys}$ gene (Shoffner et al., 1990; Silvestri et al., 1993).

#### 3.2.2. mtDNA deletions

mtDNA deletions vary in size from 1.3 kb to 7.6 kb (Schon et al., 1989). The size and location of the deletions do not correlate with the presentation or severity of the ensuing diseases. Instead, the proportion of mtDNAs bearing the deletion and their tissue distribution are the more influential determinants of clinical symptoms (Moraes et al., 1995). mtDNA deletions are usually caused by mutations in the nuclear genes encoding mtDNA maintenance proteins or proteins involved in mitochondrial nucleotide metabolism. Examples include the DNA polymerase subunit POLγA (encoded by *POLG*) (Hudson and Chinnery, 2006), the mtDNA replication helicase Twinkle (encoded by *PEO1*) (Spelbrink et al., 2001), and the ADP/ATP translocase SLC25A (encoded by adenine nucleotide translocator 1, *ANT1*) (Kaukonen et al., 2000). Most mtDNA deletions share similar characteristics: they are located between the two mtDNA replication origins ($O_H$ and $O_L$) and are flanked by short direct repeats (Samuels et al., 2004). Current models suggest that mtDNA deletions are generated during mtDNA replication (Shoffner et al., 1989) or repair (Krishnan et al., 2008; Phillips et al., 2017).

The two classic syndromes associated with mtDNA deletions are Chronic Progressive External Ophthalmoplegia (CPEO) and Kearns-Sayre Syndrome (KSS). CPEO is characterized by a progressive paralysis of the eye muscles causing compromised eye movement and ptosis. The symptoms usually appear in late childhood or early adulthood. KSS is a more severe syndromic variant of CPEO, which usually occurs before the age of twenty and involves more organs. Its major clinical presentation involves progressive external ophthalmoplegia and the development of retinitis pigmentosa. Additional symptoms may include mild skeletal muscle weakness, hearing loss, impaired cognitive function, and diabetes mellitus. Both CPEO and KSS are mainly caused by sporadic large-scale single deletion or multiple deletions (Moraes et al., 1989). These deletions range in size from 1.3 kb to 7.6 kb and map to different sites on the mtDNA. Among them, a 4977 bp deletion between a 13-bp repeat was found in one-third of the patients, which encodes 5 of the 22 tRNA genes and 7 polypeptides of the OXPHOS, including 4 subunits of Complex I, 1 subunit of Complex IV, and 2 peptides of

Complex V. It is generally thought that the percentage of mtDNA molecules carrying the deletion influences the severity of disease. However, the precise mechanism by which the same deletion causes different biochemical phenotypes and clinical presentation is not clear.

#### 3.2.3. Depletion mutations

Mutations in nuclear genes that function in mitochondrial nucleotide synthesis or mtDNA replication can also cause mitochondrial DNA depletion syndrome, which is characterized by a severe reduction in mtDNA copy number leading to the impaired production of energy in affected tissues. For instance, mutations in the proteins that maintain the mitochondrial deoxyribonucleoside triphosphate (dNTP) pool will decrease the amount of DNA building blocks within mitochondria and, subsequently, lead to mtDNA depletion. Such proteins include thymidine kinase 2 (TK2) (Oskoui et al., 2006), adenosine diphosphate (ADP)-forming succinyl CoA ligase beta subunit (SUCLA2) (Elpeleg et al., 2005), and deoxyguanosine kinase (DGUOK) (Kasapkara et al., 2013). Mutations in POLγ and Twinkle decrease the efficiency of mtDNA synthesis, leading to insufficient mtDNA copy number and eventually to the depletion of mtDNA during mtDNA turnover and cell divisions (El-Hattab and Scaglia, 2013). Over the last two decades, a group of genes specifically linked to mtDNA instability, and found to cause mtDNA depletion and deletion in human diseases, has emerged (Table 1). A detailed description of these genes can be found in other excellent reviews (Young and Copeland, 2016).

## 4. *Drosophila* models of mitochondrial DNA diseases

While much has already been learned about a number of mtDNA-related diseases from clinical studies, animal models are necessary to further understand the physiopathology and to develop therapeutic approaches. Ideally, an animal model would simulate a variety of symptoms seen in mtDNA-related diseases in humans. In addition, it should be amenable to genetic manipulations. However, currently, there is no effective way to introduce specific sequence alterations on mitochondrial genome of metazoans. Therefore, animal models carrying mtDNA mutations or deletions found in human diseases are rare.

In *D. melanogaster,* however, several genetic tools have been developed over the past decade to manipulate mtDNA. Notably, by expressing restriction enzymes in mitochondria, we were able to isolate inheritable mutations in *Drosophila* mtDNA (Xu et al., 2008). Generation of heteroplasmic flies containing different mtDNA alleles (Hill et al., 2014; Ma et al., 2014) and the ability to engineer mosaicism in *Drosophila* to study mtDNA mutations in a tissue-specific manner (Chen et al., 2015) have made this powerful model organism uniquely attractive to study mtDNA-related diseases. *Drosophila* also presents several additional advantages as a model for human diseases. First, nuclear and mitochondrial genes are highly conserved between *Drosophila* and humans: 75% of human disease genes are conserved in *Drosophila* (Bier, 2005; Pandey and Nichols, 2011); the gene composition of *Drosophila* mtDNA is the same as in vertebrates; the proteins responsible for mtDNA

**Table 1**
Nuclear loci leading to mtDNA deletions or mtDNA depletion and their associated clinical disorders.

| Gene | Protein | Function | mtDNA genotype | Clinical phenotype | Reference |
|---|---|---|---|---|---|
| **mtDNA maintenance** | | | | | |
| *POLG* | POLγA | Mitochondrial DNA polymerase catalytic subunit | Depletion and deletions | Progressive external ophthalmoplegia, ataxia, encephalopathy and hepatopathy | Hudson and Chinnery, 2006 |
| *POLG2* | POLγB | Mitochondrial DNA polymerase processivity subunit | Depletion and deletions | Myopathy and ophthalmoplegia | Longley and Clark, 2006 |
| *PEO1* | Twinkle | mtDNA helicase | Depletion and deletions | Progressive external ophthalmoplegia | Spelbrink et al., 2001 |
| *RNASEH1* | RNaseH1 | Nuclear and mitochondrial ribonuclease H1 | Deletions | Encephalomyopathy and myopathy | Reyes et al., 2015 |
| *DNA2* | DNA2 | Mitochondrial and nuclear helicase/nuclease | Deletions | Myopathy | Ronchi et al., 2013 |
| *MGME1* | MGME1 | RecB-type exonuclease | Depletion and deletions | Myopathy | Kornblum et al., 2013 |
| **Mitochondrial nucleotide metabolism** | | | | | |
| *TK2* | TK2 | Mitochondrial thymidine kinase | Depletion | Ophthalmoplegia and myopathy | Oskoui et al., 2006 |
| *DGUOK* | DGUOK | Deoxyguanosine kinase | Depletion | Hepatopathy and encephalopathy | Kasapkara et al., 2013 |
| *SUCLA2* | SUCLA2 | ATP-dependent succinate-CoA ligase | Depletion | Encephalopathy and myopathy | Elpeleg et al., 2005 |
| *SUCLG1* | SUCLG1 | GTP-dependent succinate-CoA ligase | Depletion | Encephalopathy and myopathy | Ostergaard et al., 2007 |
| *ABAT* | ABAT | 4-aminobutyrate aminotransferase | Depletion | Encephalopathy and myopathy | Besse et al., 2015 |
| *TYMP* | TP | Thymidine phosphorylase | Depletion and deletions | Mitochondrial neurogastrointestinal encephalopathy | Hirano, 1993 |
| *RRM2B* | RRM2B | p53-ribonucleotide reductase, small subunit | Depletion and deletions | Mitochondrial neurogastrointestinal encephalopathy | Fratter et al., 2011 |
| *ANT1* | SLC25A | Adenine nucleotide translocator | Deletions | Cardiac and skeletal myopathy, progressive external ophthalmoplegia | Kaukonen et al., 2000 |
| *AGK* | AGK | Acylglycerol kinase, translocase of the inner membrane 22 (TIM22) complex | Depletion | Sengers syndrome, cardiac and skeletal myopathy and cataract | Mayr et al., 2012 |
| *MPV17* | MPV17 | Mitochondrial inner membrane protein with unknown function | Depletion and deletions | Neuropathy and hepatopathy | Spinazzola et al., 2006 |
| **Mitochondrial dynamics** | | | | | |
| *OPA1* | OPA1 | Mitochondrial dynamin like GTPase | Depletion and deletions | Optic atrophy and neuropathy | Hudson et al., 2008 |
| *MFN2* | MFN2 | Mitofusin 2 | Deletions | Optic atrophy and neuropathy | Rouzier et al., 2012 |
| *FBXL4* | FBXL4 | Mitochondrial LLR F-Box protein | Depletion | Encephalopathy and myopathy | Bonnen et al., 2013 |
| **Others** | | | | | |
| *GFER* | GFER | Mitochondrial disulfide relay system protein | Deletions | Myopathy with cataract and combined respiratory-chain deficiency | Di Fonzo et al., 2009 |
| *AIFM1* | AIF1 | Unknown | Depletion | Encephalomyopathy | Ghezzi et al., 2010 |

maintenance and mitochondrial biogenesis are also largely conserved (Garesse and Kaguni, 2005). Second, *Drosophila* has many organs frequently affected by mtDNA defects, including a complex brain and nervous system, highly developed skeletal muscles and a cardiac muscle. Several nuclear *Drosophila* mutants have been shown to display brain degeneration (Celotto et al., 2006), cardiomyopathy (Antonicka et al., 2003), and deafness (Toivonen et al., 2001). These properties make the *Drosophila* models of mtDNA mutations currently available valuable tools to gain insights into mitochondrial biology and to test potential therapeutic interventions for mtDNA diseases.

### 4.1. ATP6 mutant

The *Drosophila ATP6*$^{1}$, which carries a G-to-A point mutation on the conserved amino acid residue in *ATP6* locus, was the first report of a pathogenic mtDNA mutation in an intact animal model (Celotto et al., 2006). It was discovered by chance in a *Drosophila* stock carrying a mutation in *ANT1*, a nuclear gene whose protein facilitates the transport of ATP and ADP across the inner mitochondrial membrane. As *ANT1* mutations can cause mtDNA instability in humans, the *ATP6*$^{1}$ mutation may reflect the existence of a similar mutator effect for ANT1 in *Drosophila*.

ATP6 is an essential component of the mitochondrial $F_1F_0$-ATP synthase (Complex V) in OXPHOS, which is responsible for the final step of ATP generation through the coupling of hydrogen ion transport with rotary ATP catalysis. Point mutations in the human *ATP6* gene can cause a variety of mitochondrial syndromes, including Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP) (Holt et al., 1990; Fryer et al., 1994), Maternally Inherited Leigh's Syndrome (MILS) (Campos et al., 1997) and Familial Bilateral Striatal Necrosis (FBSN) (Thyagarajan et al., 1995). The *ATP6*$^{1}$ phenotype in *Drosophila* includes conditional paralysis, shortened lifespan, neural dysfunction, and muscular degeneration, thus recapitulating the key features of the maternally inherited Leigh's syndrome in humans. The ATP synthase activity was almost completely lost in *ATP6*$^{1}$ mutants; however, their respiration rate was unaltered. The *ATP6*$^{1}$ mutants were found to compensate for their loss of oxidative phosphorylation by enhancing glycolysis, ketogenesis and Kreb's cycle activity during the early stage of pathogenesis. This *Drosophila* mutant therefore demonstrated the dynamic nature of metabolic compensatory mechanisms when mitochondria function was disrupted (Celotto et al., 2011). It was also useful for the development of dietary therapies against Mitochondrial Encephalomyopathies (MEs) (Fogle et al., 2016), as a high fat/ketogenic diet was found to be effective against the ME seizures.

### 4.2. CoI mutants

Ten years ago, a method was developed to select for heritable mtDNA mutations in *Drosophila* by targeting restriction enzymes to mitochondria (Xu et al., 2008). The restriction enzymes linearize wild-type mtDNA molecules, which impair mtDNA replication and cause their eventual loss during cell proliferation. However, mtDNA molecules carrying a random mutation abolishing the recognition site are preserved and are able to repopulate the germline. The resulting escaper progeny carry mtDNA mutations in a homoplasmic state (one type of mtDNA). In *Drosophila* mtDNA, there are 31 single restriction sites spread over 8 protein-coding genes, 2 rRNA genes and 3 tRNA genes. Thus, this method could target the majority of the *Drosophila* mtDNA-encoded genes and generate a variety of mtDNA alleles.

Expression of a mitochondria-targeted *Xho*I enzyme (mitoXhoI) created multiple homoplasmic lines containing different mutations on the *Xho*I site, which maps to the *Cytochrome C Oxidase subunit I* gene (*CoI*). *mt:CoI*$^{R301L}$ mutants are healthy, but male-sterile. *mt:CoI*$^{R301S}$ mutants exhibit a wide range of defects, including growth retardation, age-dependent neurological and muscular dysfunctions and reduced longevity. *mt:CoI*$^{T300I}$ is a temperature-sensitive lethal mutation: when raised at 29 °C, flies do not eclose from the pupal cases. The viability can be fully restored by expressing an alternative oxidase (AOX), which specifically bypasses the cytochrome chains and restores the electron flow (Chen et al., 2015). This result proves that expression of AOX may serve as a potential therapeutic approach for cytochrome chain disorders.

By transplanting the germ plasm during early embryogenesis (Matsuura et al., 1989), heteroplasmic lines containing both wild-type and various types of mtDNA mutations were generated (Hill et al., 2014; Ma et al., 2014). A genetic scheme similar to the one used for studying essential nuclear genes was developed to induce tissue-specific homoplasmy in otherwise heteroplasmic background by expressing mitoXhoI in particular tissues (Chen et al., 2015). This system allowed us to probe the phenotypic consequences of an lethal mtDNA mutation, *mt:CoI*$^{T300I}$ in an age-related and tissue-specific manner. We found that *mt:CoI*$^{T300I}$ homoplasmy in the eye caused severe neurodegeneration, which could be suppressed by improving mitochondrial $Ca^{2+}$ uptake (Chen et al., 2015). This result suggests that $Ca^{2+}$ mishandling might be the underlying mechanism of the *mt:CoI*$^{T300I}$ pathogenesis. Whether this result can be translated to human diseases remains unclear, as none of the CoI mutations associated with human diseases map to the same residue as the *mt:CoI*$^{T300I}$ mutation. Nevertheless, our results demonstrate the potential application of this system to modeling mtDNA diseases. Furthermore, the *mt:CoI*$^{T300I}$ mutation has allowed us to discover the mechanism by which deleterious mtDNA mutations were selectively eliminated during *Drosophila* oogenesis (Hill et al., 2014; Ma et al., 2014; Zhang et al., 2019), thus showing that *Drosophila* models can be used to unravel many mysteries of mitochondrial genetics.

### 4.3. ND2 mutants

Using the same strategy as above with the *Bgl*II restriction enzyme, two *Drosophila* lines carrying mutations in *NADH Dehydrogenase subunit 2* (*ND2*) gene were recovered (Xu et al., 2008). Unlike the single base changes in *mt:CoI*, one *ND2* mutation is an in-frame deletion and the other is a single residue insertion. Dysfunction of Complex I, to which ND2 belongs, is a common cause of early-onset mitochondrial diseases such as Leigh syndrome and MELAS (Triepels et al., 2001). The *ND2* mutants were therefore recognized as a suitable animal model to explore the mechanism by which Complex I deficiency leads to mitochondrial diseases (Burman et al., 2014). Indeed, the 3-residue deletion mutant (mt:*ND2*$^{del1}$) phenocopies the symptoms of Leigh syndrome, such as reduced life span, age-dependent neurodegeneration, seizures, heat intolerance, decreased Complex I activity and contents, and reduced mitochondrial membrane potential. Biochemical analysis suggested that mt:*ND2*$^{del1}$ leads to energy deficits and neurodegeneration by decoupling electron transfer from proton pumping. This study provided an explanation for the pathogenesis of Complex I-associated neurodegeneration.

### 4.4. “Male harming” mtDNA mutant

Natural selection for mtDNA fitness does occur in *Drosophila*, but only in the female germline, because of the strict maternal inheritance of mtDNA. Mutations that affect male fertility would exclusively escape such selection, a conundrum called “mother's curse” (Frank and Hurst, 1996). Based on this assumption, Malik's lab has successfully isolated a “male-harming” mtDNA mutation,

*mt:COII*$^{G177S}$ that affects *Cytochrome C Oxidase subunit II* on mtDNA by repetitive outbreeding (Patel et al., 2016). This hypomorphic mutation specifically impairs male fertility by compromising sperm production and activity, whereas all other male or female physiological traits appear normal. The age- and temperature-dependent decrease in male fertility was correlated with the reduction of Complex IV activity. These findings suggest the importance of mtDNA function in male fertility.

The authors further found that the fertility defect of the *mt:COII*$^{G177S}$ *Drosophila* could be completely or partially suppressed in different nuclear backgrounds, indicating that the phenotypic presentation of mtDNA mutations is largely dependent on the nuclear background. In humans, some mtDNA point mutations (Folgero et al., 1993; Holyoake et al., 2001) and large deletions (Cummins et al., 1994) were reported in infertile individuals. However, one intriguing phenomenon is the low frequency of male infertility among the reported carriers of the mutations. A lot of these mutations or deletions have been only reported in single individuals or at the most two members of a family (Rajender et al., 2010). The observations made with the *mt:COII*$^{G177S}$ *Drosophila* mutants may provide an explanation for this phenomenon, and make this model an excellent tool for exploring the interactions between nuclear and mitochondrial genomes.

## 5. Future therapeutic approaches for mtDNA diseases

Despite advances in understanding of mitochondrial genetics and disease pathogenesis, effective treatments are still lacking. Current treatments largely aim to mitigate symptoms and prevent complications. The administration of vitamin supplements, pharmacological agents and exercise therapies has been used in isolated cases and has met with limited success (Pfeffer et al., 2012). Currently, several strategies are being actively pursued to address mtDNA-associated pathologies by gene therapy. We would like to stress that delivering nucleotides to mitochondria remains a challenge (Lightowlers, 2011; Gammage et al., 2018), which limits the application of gene therapy to directly edit mitochondrial genome. However, targeting proteins into mitochondria is a routine practice. A protein with low complexity can be effectively targeted to the mitochondria matrix as a fusion protein containing a mitochondrial targeting sequence at its N terminus. Based on this premise, two strategies have been developed to manage mtDNA diseases. One approach is targeting sequence-specific endonucleases into mitochondria to selectively attack disease-causing mtDNA mutations. The other one is to rescue mtDNA defects by “allotopic” or “xenotropic” expression of nuclear transgenes encoding affected molecules or their enzymatic alternatives. Here, we give a brief overview of each strategy and highlight their limitations. We also present the potential of *Drosophila* as a model to develop mitochondrial transformation, which would lay the foundation for future mitochondrial genome editing.

### 5.1. Shifting heteroplasmy

In principle, expression of a mitochondrially-targeted deoxyribonuclease that specifically recognizes the mutant sequence could either remove mutant mtDNA, or at least reduce its abundance in a heteroplasmic background. Restriction endonucleases (Bayona-Bafaluy et al., 2005; Bacman et al., 2012), zinc finger nucleases (ZFNs) (Gammage et al., 2014) and transcription activator-like effector nucleases (TALENs) (Bacman et al., 2013) have been tested and demonstrated various levels of success. While the use of restriction enzymes to attack mtDNA is effective and technically straightforward, the happenstance that a pathogenic mtDNA mutation lies in a unique restriction enzyme site is rare. Engineered TALENs or ZFNs are much more versatile in term of their recognition sequences, although some engineered endonucleases demonstrate limited activity (Patananan et al., 2016). Nonetheless, mitochondrial targeted TALENs have successfully eliminated mtDNAs with specific point mutations or large deletions in patient-derived cells (Bacman et al., 2013), as well as shifted the mtDNA heteroplasmy ratio in heteroplasmic mouse germ cells (Reddy et al., 2015). Despite their great promise, these gene cleaving enzymes have limitations. One major concern is that eliminating a high percentage of mutant mtDNA may inadvertently decrease the total mtDNA copy number to below a functional threshold (Patananan et al., 2016).

### 5.2. Allotopic expression

Allotopic expression refers to the nuclear expression of proteins that are encoded by the mtDNA. Instead of being produced inside of mitochondria, the correct version of mutated proteins is expressed and translated in the nuclear-cytosol compartment and imported into mitochondria. Allotopic expression of MT-ATP6 and MT-ND1 has been shown to correct respiratory defects in cybrid cells (cytoplasmic hybrid cells that contain nuclear genes from one individual, and mitochondrial genes from another) harboring the corresponding mutations (Guy et al., 2002; Manfredi et al., 2002). However, the highly hydrophobic nature of mtDNA-encoded proteins concerns the efficacy of this approach and cautions unintended side effects during their transit from cytosol to mitochondria (Perales-Clemente et al., 2011). A variation of allotopic expression, in which mRNAs or tRNAs are expressed on nuclear genome and imported into mitochondria, has also been demonstrated (Karicheva et al., 2011; Wang et al., 2012). A few nuclear-derived RNAs have been found inside of mitochondria (Kamenski et al., 2007; Schneider, 2011; Towheed et al., 2014). Mitochondrial RNA targeting sequences have been identified (Wang et al., 2010), and applied to mediate mitochondrial import of chimera RNAs when inserted at the 5′ end of mtDNA-encoded mRNAs, tRNAs or exogenous RNAs. Additionally, a tRNA importing complex of *Leishmania* has been reported to be able to facilitate the transport of tRNAs from cytosol to mitochondria in mammalian cultured cells (Goswami et al., 2006). Despite all the claimed successes, targeting RNAs into mitochondria remains a controversy, as pointed out by others (Schekman, 2010; Lightowlers, 2011; Gammage et al., 2018). We do not intend to question the validity of these methiodides. Nonetheless, it is necessary to study literature on this important topic with perusal.

An alternative to the allotopic expression is the xenotropic expression of proteins functioning in similar pathways from other species. Two examples are the *Saccharomyces cerevisiae* (*S. cerevisiae*) single subunit NADH oxidase (Ndi1) and the sea squirt *Ciona intestinalis* alternative oxidase (AOX). Ndi1 functions like NADH dehydrogenases, but without translocating protons across the inner mitochondrial membrane. Targeting the yeast Ndi1 into mitochondria has been shown to rescue Complex I deficiency in human cells (Yagi et al., 2001) and in flies that have Complex I assembly defects (Cho et al., 2012). AOX catalyzes electron transfer from ubiquinone to molecular oxygen, providing an alternative electron flow pathway and bypassing Complex III and IV. Xenotropic expression of AOX confers resistance to inhibitors of respiratory chain Complexes III and IV in cultured cells (Hakkaart et al., 2006). Notably, AOX can completely rescue the viability of the *mt:CoI*$^{T300I}$ *Drosophila* mutants (Chen et al., 2015), and has no obvious effect on wild-type flies. These results provide encouraging evidence for xenotropic expression of Ndi1 or AOX as a potential therapeutic approach for disorders caused by mitochondrial mutations.

### 5.3. Genetically editing the mitochondrial genome

Over the last decades, there have been many attempts to directly edit the mitochondrial genome. However, methods for mitochondrial transformation are only available in yeast *S. cerevisiae* (Fox et al., 1988; Johnston et al., 1988) and the green alga *Chlamydomonas reinhardtii* (Remacle et al., 2006), no reliable method has been developed in other species (Lightowlers, 2011). The lack of effective ways to directly edit mtDNA sequence has become a significant hurdle to further understand the basic mitochondrial biology and pathophysiology of mtDNA diseases. Developing efficient and reliable methods for mitochondrial transformation posts great challenges. However, recent advances in *Drosophila* mitochondrial genetics may provide insights and serve as a potential model for manipulating mitochondrial genome.

Editing mtDNA is complicated for several reasons. First of all, there is no effective way to import nucleic acids through the double-layered mitochondrial membranes. Although mitochondrially-targeted endonucleases can cleave mtDNA at specific sequences (Reddy et al., 2015), no targeted correction of mtDNA can be obtained, due to the inability of delivering a DNA template. Liposome-based nanoparticles (Yamada et al., 2008) and bacterial conjugation (Yoon and Koob, 2005) have been found to facilitate the import of DNA or RNA into mitochondria *in vitro*. Additionally, the introduction of foreign mitochondria into the germ plasm of *Drosophila* embryos through transplantation has proven feasible, and led to the creation of heteroplasmic flies (Matsuura et al., 1989). Thus, it is tempting to test the possibility of delivering mitochondria transformed by liposome nanoparticles or bacterial conjugation into fly embryos and follow their propagation in whole animals. In addition, the nanoparticles and donor bacteria carrying the modified mtDNA may be directly injected into *Drosophila* embryos, where they might fuse with mitochondria in the same fashion as *in vitro*.

The second obstacle to mtDNA editing is that even if nucleic acids can be delivered to the mitochondrial matrix, DNA templates have to be stably recombined into the endogenous mtDNA. In the nucleus, substituting or inserting nucleotides with genome editing tools such as TALEN and CRISPR/Cas9 systems requires a homology-directed repair mechanism. However, machineries for either homologous recombination or non-homologous end joining do not seem to be particularly active, even if they do present in animal mitochondria (Alexeyev et al., 2013). In fact, the frequency of mitochondrial genome recombination is exceedingly rare in most species under normal physiological condition. O'Farrell's lab recently demonstrated efficient recombination between two mtDNA haplotypes in *D. melanogaster*, by artificially creating double-strand breaks using mitochondrially-targeted restriction enzymes. This work proved in principle that recombination of animal mitochondrial DNA could be achieved (Ma and O'Farrell, 2015).

Finally, there are hundreds of copies of mtDNA in each cell. To ensure efficient selection of the edited mtDNA, the ideal recipient should offer the least competition from endogenous mtDNA. Currently, mammalian ρ0 cells, which devoid of endogenous mitochondrial genome, are most frequently used. However, there are only a limited collection of ρ0 cell lines, and some seem incompatible with specific mutant mtDNAs (Hashiguchi and Zhang-Akiyama, 2009). Additionally, the generation of new ρ0 cells involves DNA intercalating agents, which are potent nuclear DNA mutagens, and hence are not desirable (Patananan et al., 2016). Mitochondrially-targeted restriction enzymes or DNA endonucleases that specifically attack wild-type mtDNA may again come in handy. These enzymes can not only generate double-strand breaks on endogenous mtDNA to facilitate recombination, but also remove non-recombined molecules and thereby select for recombined molecules.

Given the many challenges that remain, manipulating the mitochondrial genome will require long-term and collective efforts. Such efforts are however warranted, as the ability to introduce specific mtDNA alternations will not only provide fundamental insights into mitochondrial biology, but also potentially offer strategies for treating mtDNA-related diseases.

## 6. Perspectives

Mitochondrial DNA diseases are estimated to affect 1 in 5000 people in the world. Given the increasing evidence that mtDNA mutations are associated with common clinical disorders, such as diabetes and Parkinson's disease, the incidence could be much higher. Although our current knowledge demonstrates the importance of mtDNA mutations in causing diseases, how these mutations affect human health and lead to such a broad spectrum of clinical phenotypes remains to be determined. More importantly, so far there have been no curative therapies and only very limited treatments of these diseases. Due to the diverse roles that mitochondria play in cellular processes, establishing animal models that simulate the pathogenic mitochondrial DNA mutations would improve our understanding of the specific mitochondrial deficiencies and cellular processes involved, as well as facilitate developing effective treatment. Recent progress in manipulating mtDNA mutants in *Drosophila* has opened new avenues for studying mtDNA genetics. Success at developing new techniques for genetically altering mtDNA may create more possibilities for treatment of mtDNA disorders and improve our understanding of fundamental mitochondrial biology.

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