ESKO KEMPPAINEN

Genetic and Metabolic Suppression of Mitochondrial Disease Phenotype in *Drosophila*

ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE
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Abstract

Mutations in genes encoding the components of the mitochondrial translational apparatus give rise to various human mitochondrial disorders with a range of clinical phenotypes. We have used a *Drosophila* mutant harbouring a missense mutation in the gene *technical knockout (tko)*, which encodes the highly conserved mitoribosomal protein *S12*, to elucidate the pathophysiology and metabolic adaptation to such defects in multicellular organisms. The *tko<sup>25t</sup>* mutant flies suffer from a severe oxidative phosphorylation and ATP deficiency, and exhibit a phenotype of developmental delay occurring during larval stages, sensitivity to paralytic and epileptic seizures and hearing impairment, features analogous with common clinical manifestations of mitochondrial disease in humans.

Based on my studies, this phenotype is exacerbated when mutant flies are reared on high-sugar diet. Although the exact mechanism of this sugar toxicity remains unknown, rearing the mutant flies on low-sugar diet results in significant phenotypic recovery which is associated with partial rescue of severe ATP and NADPH deficiencies as well as decreased pyruvate and lactate burden. The *tko<sup>25t</sup>* phenotype is also rescued by overexpression of the endogenous allele in suppressor lines carrying duplications of the *tko* gene. Yet, the mutant larvae do not respond to mitochondrial dysfunction by inducing mitochondrial biogenesis or by over-expressing components of the mitochondrial translational apparatus. In turn, the mutant larvae exhibit transcriptionally a large-scale metabolic reprogramming highlighted by down-regulation of sugar metabolism and increased catabolism of lipids - changes indicating shift in fuel-preference from carbohydrates to fats and fatty acids. The results also suggest that the primary cause underlying the pathophysiology of the disease-like phenotype might be severe metabolic crisis rather than isolated ATP or NADPH deficiency.

Altogether, this study highlights the importance of understanding the molecular basis of disease and the therapeutic potential of dietary intervention in the treatment of mitochondrial disorders.
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List of original communications

This thesis is based on the following original communications, which are referred to in the text by their roman numerals I-III.


III) Kemppainen E, George J, Garipler G, Soga T, Dunn C, Tuomela T, Kiviranta E, Jacobs HT. Mitochondrial dysfunction plus high-sugar diet provokes a metabolic crisis that inhibits growth. 2015.

*Equal contribution.

II) Publication will be included in the doctoral dissertation “Complementation of Cytochrome c Oxidase Deficiency by Transgenic Expression of the Alternative Oxidase in Drosophila” by Kia Kemppainen, University of Tampere.

III) Manuscript submitted to Cell Metabolism.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>6GPDH</td>
<td>6-phosphogluconate dehydrogenase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>adPEO</td>
<td>Autosomal dominant Progressive External Ophthalmoplegia</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocator</td>
</tr>
<tr>
<td>AOX</td>
<td>Alternative oxidase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cI-V</td>
<td>Respiratory chain complexes I-V</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CPTx</td>
<td>Carnitine palmitoyltransferase</td>
</tr>
<tr>
<td>cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERMES</td>
<td>Endoplasmic reticulum–mitochondria encounter structure</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide (oxidised)</td>
</tr>
<tr>
<td>FADH2</td>
<td>Flavin adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>G3P</td>
<td>Glycerol-3-phosphate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose 6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>GS</td>
<td>GeneSwitch</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>$H_2O_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSP</td>
<td>Heavy strand promoter</td>
</tr>
<tr>
<td>HTRA2</td>
<td>High Temperature Requirement Protein A2</td>
</tr>
<tr>
<td>IDH</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>IMM</td>
<td>Mitochondrial inner membrane</td>
</tr>
<tr>
<td>IMS</td>
<td>Intermembrane space</td>
</tr>
<tr>
<td>kdn</td>
<td>knockdown</td>
</tr>
<tr>
<td>KSS</td>
<td>Kearns–Sayre syndrome</td>
</tr>
<tr>
<td>L1-3</td>
<td>Drosophila larval instars 1-3</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long-chain fatty acids</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LHON</td>
<td>Leber's hereditary optic neuropathy</td>
</tr>
<tr>
<td>LRPPRC</td>
<td>Leucine-rich pentatricopeptide repeat containing</td>
</tr>
<tr>
<td>LSP</td>
<td>Light strand promoter</td>
</tr>
<tr>
<td>MAMS</td>
<td>Mitochondrial Associated ER Membranes</td>
</tr>
<tr>
<td>MCFA</td>
<td>Medium-chain fatty acids</td>
</tr>
<tr>
<td>MELAS</td>
<td>Mitochondrial encephalopathy with lactic acidosis and stroke-like episodes</td>
</tr>
<tr>
<td>MERRF</td>
<td>Myoclonus epilepsy with ragged red fibers</td>
</tr>
<tr>
<td>MFRTA</td>
<td>Mitochondrial free radical theory of ageing</td>
</tr>
<tr>
<td>MIRAS</td>
<td>Mitochondrial recessive ataxia syndrome</td>
</tr>
<tr>
<td>MNGIE</td>
<td>Mitochondrial Neurogastrointestinal Encephalopathy</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Manganese superoxide dismutase</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>MRG</td>
<td>Mitochondrial RNA granules</td>
</tr>
<tr>
<td>MRP</td>
<td>Mitoribosomal protein</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>mRpS12</td>
<td>Mitoribosomal protein S12</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>MTERF</td>
<td>Mitochondrial transcription termination factor</td>
</tr>
<tr>
<td>MTPAP</td>
<td>Mitochondrial Poly(A) Polymerase</td>
</tr>
<tr>
<td>mtSSB</td>
<td>Mitochondrial single-stranded DNA-binding protein</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide (oxidised)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidised)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NARP</td>
<td>Neuropathy, ataxia, and retinitis pigmentosa</td>
</tr>
<tr>
<td>Ndi, Nde</td>
<td>NADH dehydrogenases (alternative)</td>
</tr>
<tr>
<td>nDNA</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O₂⁻⁻</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>OH⁺</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OMM</td>
<td>Mitochondrial outer membrane</td>
</tr>
<tr>
<td>OXA1</td>
<td>Mitochondrial oxidase assembly protein 1</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>PEO</td>
<td>Progressive external ophthalmoplegia</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator activated receptor γ co-activator-1α</td>
</tr>
<tr>
<td>Pᵢ</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>POLG</td>
<td>Polymerase γ</td>
</tr>
<tr>
<td>POLRMT</td>
<td>Mitochondrial RNA polymerase</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SCFA</td>
<td>Short chain fatty acid</td>
</tr>
<tr>
<td>TEFM</td>
<td>Mitochondrial transcription elongation factor</td>
</tr>
<tr>
<td>TIM</td>
<td>Translocator of innermembrane</td>
</tr>
<tr>
<td>TORC1/2</td>
<td>Target of Rapamycin complex ½</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TOM</td>
<td>Translocator of outer membrane</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer-RNA</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>VLCFA</td>
<td>Very long chain fatty acid</td>
</tr>
</tbody>
</table>
1. Introduction

Pathogenic mutations in mitochondrial DNA (mtDNA) and nuclear encoded mitochondrial proteins give rise to a large and heterogeneous group of mitochondrial disorders, usually characterized by defective oxidative phosphorylation. Mitochondrial diseases are often multi-syndromic but manifest predominantly in the muscle and nervous system due to the high energy expenditure of these tissues (Schon, DiMauro, & Hirano, 2012; Wallace, Fan, & Procaccio, 2010). Mitochondrial disorders have classically had an orphan disease status but recent epidemiological studies have shown that collectively these disorders affect more than 1 in 10000 people in the western countries (Greaves et al., 2012).

The majority of these pathological mutations affect the machinery of mitochondrial translational machinery. Two of the most common pathogenic mitochondrial DNA mutations, 3243A>3G (Goto et al. 1990) and 8344A>G (Shoffner et al. 1990), both affect mitochondrial transfer-RNAs and manifest as progressive multi-syndromic disorders characterized by epileptic seizures and lactic acidosis (Douglas C Wallace et al., 2010). A third highly prevalent mutation 1555A>G (Prezant et al., 1993), affects the 12S mitoribosomal RNA (rRNA), conferring aminoglycoside sensitivity and predisposing to sensorineural deafness. While these mitochondrial tRNA and rRNA mutations have relatively high incidence, mutations in nuclear genes encoding polypeptide components of the translation machinery are extremely rare, but equally deleterious (Boczonadi & Horvath, 2014).

A point mutation in the Drosophila tko (technical knockout) gene, encoding the conserved mitochondrial ribosomal protein S12 (mRpS12), results in a phenotype highly analogous with the clinical manifestations of human mitochondrial protein synthesis disorders. The *tko*<sup>25t</sup> mutant phenotype is characterized by developmental delay, hearing impairment and malfunction of the central nervous system, manifesting as susceptibility to epileptic and paralytic seizures (Toivonen et al., 2001; Toivonen et al., 2003). The underlying molecular defect involves instability of the small subunit of the mitoribosome which impairs the synthesis of mitochondrially encoded subunits of the OXPHOS complexes, and ultimately
results in respiratory chain deficiency. Yet, how this gives rise to the organismal phenotype, remains largely unknown (Toivonen et al., 2001; Toivonen, Boocock, & Jacobs, 1999; Toivonen et al., 2003). Because of the similarity between the tko\textsuperscript{25t} and clinical disease phenotypes, and the numerous benefits \textit{Drosophila} offers as a model organism, the mutant flies provide a convenient tool for studying the pathogenic mechanisms of human mitochondrial disorders, and a platform for evaluating potential therapeutic strategies.

The vast majority of mitochondrial disorders currently have no effective cures. The forms of treatment currently under investigation or already in clinical practice can be roughly divided into four distinct functional categories: antioxidant treatments to protect cells from increased oxidative damage caused by mitochondrial dysfunction, endurance exercise and small molecule compounds aimed at improving cellular energy metabolism or enhancing it by inducing mitochondrial biogenesis, gene therapy designed to compensate for a loss of gene activity or to modulate mtDNA heteroplasmy, and nutritional therapy (Kerr, 2010; Peralta et al., 2015; Pfeffer et al., 2013). In this work, we’ve focused on evaluating the amenability and effects of different nutritional interventions and OXPHOS bypass gene therapy using the tko\textsuperscript{25t} model.

One of the possible gene therapeutic strategies is based on nuclear expression of mitochondrially targeted single subunit respiratory chain complex alternatives, such as the alternative oxidase (AOX), which is found mostly in plants. AOX can provide a functional by-bass for the entire cytochrome segment of the respiratory chain and prevent the formation of superoxide radicals when the ubiquinone pool is over-reduced while relieving the blockage of mitochondrial substrate oxidation due to impaired cytochrome \textit{bc}\textsubscript{1} (Complex III) or cytochrome \textit{c} oxidase (Complex IV) complex activities (El-Khoury et al., 2013; Fernandez-Ayala et al., 2009). In addition to providing a therapeutic tool, bypassing segments of defective respiratory chain can help in identifying disease-relevant metabolic phenomena, such as ATP deficiency, elevated reactive oxygen species (ROS) production and impaired substrate oxidation, and their relative contribution to the disease pathogenesis.

A compelling alternative for gene and drug therapy is nutritional intervention. Dietary supplements, such as carnitine and coenzyme Q\textsubscript{10} have already been used to treat human mitochondrial disorders with some degree of success (Horvath, 2012; Valero, 2014), and dietary intervention has shown significant promise, particularly
in murine disease models (Ahola-Erkkilä et al., 2010; Tischner et al., 2014). The tko\(^{25t}\) model can be easily exploited to address how modulating the balance of principal dietary components, carbohydrates, fats and proteins would affect the mutant phenotype, due to the ease of manipulating Drosophila diet.

Finally, one of the clear advantages Drosophila offers over many other multi-cellular model organisms is its genetic tractability. In earlier studies, the tko\(^{25t}\) phenotype has been shown to be attenuated by maintaining the mutant flies as an inbred stock (Toivonen et al., 2001). This provides a unique opportunity to discover novel genetic factors which can act to suppress phenotypes resulting from mitochondrial protein synthesis defects.
2. Review of literature

2.1 Mitochondria - Structure, functions and organization

Mitochondria are organelles harboured by virtually all eukaryotic cells and organisms. The few exceptions include certain cell types, such as mature erythrocytes, which lose their mitochondria during cell differentiation. The few amitochondriate eukaryote species existing today, such as microsporidia, have likely evolved from mitochondria-bearing ancestors (Palmer, 1997). Mitochondria have originated and evolved through a symbiotic relationship between a once free-living prokaryotic ancestor, engulfed by a host cell, the ancestral eukaryote (McInerney et al., 2014). While considerable disagreement still exists concerning the phylogenetic and evolutionary details of this endosymbiotic process, comparative genomic studies of eukaryotic mitochondrial DNA have shown that mitochondria are of bacterial ancestry, originating from the phylum α-proteobacteria (Gray, 2012).

During eukaryotic evolution, mitochondria have lost a considerable degree of their genetic independence and physiological functions. Yet, their widespread presence implies that mitochondria still participate in several vital physiological processes in eukaryotic cells. Perhaps the best appreciated of these is the ATP synthesis-coupled oxidation of nutrients, a process known as oxidative phosphorylation (OXPHOS), which provides cells with energy. The catabolism of virtually all dietary fats, proteins and carbohydrates ends in, or at least impinges on mitochondria, culminating in the flux of carbon into the tricarboxylic acid cycle (TCA cycle), in the form of acetyl-CoA and TCA cycle intermediates (Lehninger, Nelson & Cox, 2005) (Fig. 2). In addition to their central role in energy metabolism, mitochondria participate in several other key metabolic processes such as heme biosynthesis from succinyl-CoA (TCA cycle intermediate), which is catalyzed primarily by mitochondrial enzymes (Ponka, 1997). Mitochondria also host both, part of the mevalonate pathway and the urea cycle, enzymes catalyzing the initial steps of steroid hormone biosynthesis from cholesterol, as well as two enzymes which participate in the first steps of gluconeogenesis: pyruvate carboxylase which
converts pyruvate into oxaloacetate and the mitochondrial isoform of phosphoenolpyruvate carboxykinase which catalyzes the conversion of oxaloacetate into phosphoenolpyruvate. (Lehninger, Nelson & Cox, 2005). Mitochondria and the TCA cycle also have a crucial role in amino acid catabolism and biosynthesis. The generation of Fe/S clusters is catalyzed by mitochondrial scaffold proteins and enzymes (Rouault & Tong, 2005). Likewise, ketogenesis, i.e. the production of ketone bodies acetoacetate, acetone and β-hydroxybutyrate, occurs exclusively in liver cell mitochondria. Finally, mitochondria together with the endoplasmic reticulum (ER), constitute a Ca\textsuperscript{2+} storage and buffering compartment and thus participate in the regulation of intra-cellular calcium fluxes (Williams et al., 2013).

Mitochondria are enclosed by two membranes. The outer mitochondrial membrane (OMM) is rich in voltage-dependent anion channels (VDACs), formed by rings of porin proteins. These channels facilitate the passage of small hydrophilic molecules through the outer membrane. The inner mitochondrial membrane (IMM) is largely impermeable to solutes and import through the IMM thus relies on dedicated channel and transport proteins. The inner membrane forms highly dynamic structures, called cristae, which protrude from the IMM into the lumen of mitochondria, known as the mitochondrial matrix. The inner membrane and crista house the numerous mitochondrial solute transporters, protein complexes of the electron transport chain (ETC), the ATP synthase and protein translocases of the inner membrane (TIMs), making it one of the most protein rich membranes known (60-70% protein by weight) (Becker et al., 2009). The inner mitochondrial membrane is also characterized by the presence of cardiolipin, a phospholipid exclusive to bacteria and the IMM. The outer and inner membranes encapsulate a compartment called the intermembrane space (IMS).

The mitochondrial matrix houses the remainder of the mitochondrial proteome consisting of e.g. the pyruvate dehydrogenase complex (PDH) and the enzymes of the TCA cycle, urea cycle, β-oxidation and anaplerotic pathways. Most of these processes feed electrons to the electron transport chain (ETC) via the electron carriers, NADH and FADH\textsubscript{2}.

Mitochondria vary greatly in size, morphology and number between different cell types and tissues. Classically, mitochondria were described and perceived as spherical or oval, thread- and granule-like organelles with a static structure and an ascertainable copy number within the cell. The term mitochondrion originates from
the Greek words *mitos* (thread) and *chondron* (grain), and was coined by the microbiologist Carl Benda, who was among the first to discover and make observations on the shape these organelles (Benda, 1898). With the advancement of modern microscopy, it is now appreciated that, in many cell types, mitochondria actually form dynamic reticular networks which are constantly being reshaped by fission and fusion events (Bereiter-Hahn & Vöth, 1994; Lewis & Lewis, 1914; Smith, 1931), and that these transformations are genetically regulated and linked to alterations in the bioenergetic and redox state of the cell, mitochondria or distinct compartments within the mitochondrial network (Nasrallah & Horvath, 2014; Scorrano, 2013).

The mitochondrial network interfaces with the endoplasmic reticulum (ER) in many cell types. In mammalian cells, these regions of interaction between ER and mitochondria are known as Mitochondrial Associated ER Membranes or MAMS (endoplasmic reticulum–mitochondria encounter structure, or ERMES, in yeast), and have recently been shown to have distinct biochemical properties, and to provide a physical domain for many physiological processes (Naon & Scorrano, 2014). These contact sites are important for mitochondrial phospholipid biosynthesis which requires phospholipid trafficking between the two organelles (Osman, Voelker, & Langer, 2011). MAMS also provide microdomains for Ca$^{2+}$ trafficking between the ER and mitochondria. Besides stimulating TCA cycle enzymes and OXPHOS, mitochondrial Ca$^{2+}$ fluxes are also associated with the regulation of apoptosis, creating a link between MAMS and programmed cell death (Gunter et al., 2004; Naon & Scorrano, 2014).

### 2.1.1 Mitochondrial genetics and DNA metabolism

Mitochondria contain their own circular, double-stranded DNA genome, which encodes a small, but functionally very significant fraction of the approximately 1500 mitochondrial polypeptides (Calvo et al., 2006). Based on comparative genomics, mitochondrial genomes have been under heavy reductive pressure during evolution which has resulted in a substantial loss of mitochondrially encoded genes due to redundant overlap in processes carried out by both mitochondria and the ancestral host cells, and through gene transfer from mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) (Bernt et al., 2013). Species within the eukaryotic domain thus now
carry a small and somewhat variable number of mtDNA-encoded genes. Plants have generally retained more mitochondrial genes than animals but, in both kingdoms, the complement of mtDNA encoded genes appears to have become fixed (Bullerwell & Gray, 2004).

Figure 1. The organization of the human mitochondrial DNA. Abbreviations: ATPx – ATP synthase, COXx – cytochrome c oxidase, CYTB – cytochrome b, D-loop – displacement loop, HSPx – heavy strand promoter, LSP – light strand promoter, NDx – NADH dehydrogenase, O_H – origin of replication (heavy strand), OL – origin of replication (light strand). The single letter abbreviations designate transfer-RNA genes of the corresponding amino acid, according to standard IUPAC nomenclature.
The mammalian mtDNA encodes only 13 polypeptides, all components of the five inner membrane OXPHOS complexes (Fig. 1). These include 7 subunits of complex I (ND1-4, -4L, -5 and -6), one subunit of complex III (cytochrome b, encoded by the CYTB gene), three subunits of complex IV (COX I-III) and two subunits of complex V (subunits 6 or a, and A6L, encoded, respectively, by the ATP6 and ATP8 genes). In addition to the polypeptide encoding genes, mtDNA encodes 2 ribosomal RNAs (12S and 16S) and 22 transfer-RNAs, all of which are components of the mitochondrial translation machinery. The human mitochondrial genome is 16,569 bp long and contains only a single significant non-coding region (Anderson et al., 1981). The human mitochondrial coding sequences are intronless, and organized on the two strands in a contiguous and partially overlapping manner. The mtDNAs of other mammals and most metazoans are similar in these respects. The two strands of human mtDNA are denoted the heavy and the light strand, based on their guanine content and differential buoyant density. Mitochondrial genes are spread on both strands with a significant bias; the human light strand only carries a single protein-coding gene (ND6) (Fig. 1).

Mitochondrial DNA is maternally inherited and does not undergo germline recombination (Hällberg & Larsson, 2014). It also exists in multiple copies per cell, implying that several mtDNA genotypes can co-exist in a single cell. This condition is known as heteroplasm, whereas the existence of only a single mtDNA genotype is called homoplasm. Since mitochondrial DNA has a high mutation rate, an order of magnitude higher than that of nuclear DNA (Pakendorf & Stoneking, 2005), and is uniparentally transmitted, this could potentially give rise to a phenomenon known as the Muller’s ratchet, or the irreversible accumulation of deleterious mutations in an asexual population (Muller, 1964), leading to loss of fitness and eventual extinction. Several poorly understood processes are thought to exist to counter this threat. One of these is the so-called mitochondrial bottleneck, a phenomenon resulting from the fact that primordial germ cells are apportioned only a small fraction of the mitochondria of the developing embryo, and thus only a subset of the mother’s mtDNA molecules are transmitted to the offspring (Shoubridge & Wai, 2007). Since this could also result in the rapid segregation of novel and potentially pathogenic mtDNA genotypes, a mechanism of purifying selection which would remove potentially deleterious mtDNA molecules, has been inferred to exist in the germ line (Stewart et al., 2008). Transmission of mtDNA mutations through the germline is also suppressed by the early embryonic lethality of e.g. pathogenic...
tRNA mutations (Freyer et al., 2012) and the general decline in fertility caused by high levels of female germline mtDNA mutations (Ross et al., 2013).

Mitochondrial DNA copy number can vary by orders of magnitude between different cell types and tissues, ranging from the few thousands in human fibroblasts, cardiac and skeletal muscles (Kukat et al., 2011; Miller, 2003) to tens of thousands in oocytes (Chen et al., 1995). mtDNA molecules are arranged and compacted into nucleoids, nucleoprotein complexes with a mean diameter of approximately 100 nm (Brown et al., 2011; Kukat et al., 2011). Mitochondrial nucleoids have been reported to consist of 1-10 copies of mitochondrial DNA molecules (Bogenhagen, 2009; Kukat et al., 2011) and a varying complement of proteins (Bogenhagen et al., 2003; Garrido et al., 2003; Holt et al., 2007). The primary mtDNA packaging factor is the high mobility group (HMG)-box protein TFAM which binds and bends mtDNA in a histone-like manner (Ngo, Kaiser, & Chan, 2011; Rubio-Cosials et al., 2011)

Mitochondrial DNA replicates independently of nDNA and using a somewhat different mechanism. mtDNA is replicated on nucleoid-like DNA/protein complexes called replisomes, at the core of which are the two subunits of the mitochondrial DNA polymerase gamma (pol γ). This trimeric protein complex is composed of the 140 kDa pol γ catalytic subunit, encoded by POLG gene and two 55 kDa accessory subunits, encoded by the POLG2 gene (Chinnery & Hudson, 2013). In addition to synthesizing DNA, the catalytic subunit possesses a 3′-5′ exonuclease activity required for proofreading, and a deoxyribose-5-phosphate (5′dRP) lyase activity necessary for enzymatic DNA repair (Copeland, 2012). Because of the exonucleolytic proofreading activity and its high nucleotide selectivity, pol γ has extremely high fidelity, such that misinsertions during replication occur only at a rate of 1 in every 500,000 nucleotides (Copeland & Longley, 2014). The replisome also includes the 5′-3′ DNA helicase Twinkle which unwinds the double-stranded DNA. These minimal replisome components are sufficient for template directed DNA synthesis in vitro, but the rate of replication is greatly enhanced by the addition of the mitochondrial single stranded binding protein (mtSSB), which stabilizes the transiently single-stranded DNA template at replication forks (Korhonen et al., 2004).
2.1.2 Mitochondrial transcription

Mammalian mitochondrial RNA synthesis yields long polycistronic RNAs (Montoya et al., 1982). The basal machinery required for transcription initiation consists of the mitochondrial RNA polymerase (POLRMT), the mitochondrial transcription factor TF2BM and the dual role protein, TFAM. Transcription of the light strand, which in mammals only encodes the ND6 subunit of CI and eight tRNAs, initiates from a single light-strand promoter (LSP), located just outside of the D-loop region (Fig. 1). According to a long-standing hypothesis, heavy strand transcription is assumed to initiate at two sites adjacent to the D-loop, approximately 100 bp apart (Martin et al., 2005). Transcription from heavy-strand promoter 1 (HSP1) is posited to abruptly terminate immediately after the rRNA genes, where the transcription machinery encounters the mitochondrial transcription termination factor 1 (MTERF1), which is bound to its target site within the tRNA\textsubscript{L(UUR)} gene (Kruse et al. 1989; Martin et al. 2005). Thus, transcription initiated from the second heavy strand promoter (HSP2) would cover the entire strand with 2 rRNAs, 12 mRNAs and 14 tRNAs. This model has been implicitly challenged by data provided by a recent knockout of the mouse Mterf1 gene pair. The Mterf1 knockout in these mice does not affect rRNA gene expression, but rather the corresponding anti-sense transcripts, suggesting that MTERF1 has a role in blocking the light strand transcription unit prior to it reaching the rRNA gene region (Terzioglu et al., 2013).

Like TFAM, the transcription factors TFB2M and its homolog B1 (TFB1M) carry dual functionality. Both of them can activate transcription but also additionally act as methyltransferases for rRNAs (Cotney & Shadel, 2006; Metodiev et al., 2009; Seidel-Rogol, McCulloch, & Shadel, 2003). In vitro, though, TFB2M has been reported to possess a several fold higher capacity to stimulate transcription (Falkenberg et al., 2002) whereas TFB1M has a more potent methyltransferase based on in vivo assays performed in bacteria (Metodiev et al., 2009). In addition, three other transcription regulatory factors have been characterized in mammals: the transcription initiation regulator MTERF2 (Wenz et al., 2009) and the negative regulator MTERF3 (Park et al. 2007), both belonging to the same MTERF-family together with MTERF1. The third factor, c17orf42, is also the first putative mitochondrial transcription elongation factor (TEFM) to be identified (Minczuk et al., 2011). Elongation factors are common elements in other transcription systems (Sims, Belotserkovskaya, & Reinberg, 2004), and since mitochondrial transcription...
produces long, polycistronic RNAs, their existence in mitochondria would seem plausible.

The fact that mitochondrial transcription yields polycistronic RNAs implies, that the transcripts require further processing to yield the fully mature mRNAs, tRNAs and rRNAs. The first step in this process is the cleavage of the individual RNA species. The mitochondrial precursor RNAs contain pre-mRNAs punctuated by mitochondrial pre-tRNAs. The 5’ and 3’ ends of these RNAs serve as cleavage sites for two different enzymes. The mitochondrial RNase P complex, comprising the MRPP1, MRPP2 and MRPP3 subunits (Holzmann et al., 2008), cleaves the polycistronic RNAs at the 5’ end of tRNAs, while the nuclease ELAC2 catalyzes the 3’ cleavage (Brzezniak et al., 2011). Two gene pairs, ATP8/ATP6-COIII and ND5-CYTB, present an exception to this rule since these genes are not flanked by tRNAs on both sides. The post-transcriptional processing of these pre-mRNAs is still somewhat unclear, although the pentatricopeptide repeat domain protein (PTCD2) has been reported to be involved in the cleavage of the ND5 and CYTB transcripts (Xu et al., 2008).

The mt-mRNAs undergo polyadenylation catalyzed by the mitochondrial RNA poly-A polymerase (MTPAP) (Nagaike et al., 2005; Piechota et al., 2006). Polyadenylation is a required to inhibit mitochondrial mRNA decay (Piechota et al., 2006) but also to generate UAA stop codons, which in some cases are not encoded by the mtDNA (Anderson et al., 1981). rRNAs are also heavily modified post-transcriptionally. These modifications include methylation, 2’-O-ribose methylation and pseudouridinylation at specific positions (reviewed by Hällberg & Larsson, 2014). Similarly, tRNAs must undergo nucleoside modifications in order to assume stability and proper folding and function (Suzuki et al. 2011; Hällberg & Larsson 2014). One of the most significant universal mt-tRNA modifications is the addition of a CCA-sequence into the acceptor stem in the 3’ end of each tRNA, in a reaction catalyzed by the mitochondrial CCA-adding enzyme (TRNT1) (Nagaike et al., 2001).

Recent studies suggest that at least some of the early steps of mitochondrial transcript processing occurs co-transcriptionally in distinct foci named mitochondrial RNA granules (MRGs) (Antonicka et al., 2013; Jourdain et al., 2013), and in the close vicinity of mtDNA nucleoids (Lee et al. 2013). Furthermore, the initial steps in mitoribosome assembly and rRNA processing have been shown to
occur at, or in the close proximity of nucleoids (Bogenhagen, Martin, & Koller, 2014; Lee et al., 2013), suggesting that many key aspects of mitochondrial DNA metabolism and gene expression could be intimately linked and coordinated through physical interactions.

2.1.3 Mitochondria and cellular energy metabolism

Mitochondria and the oxidative phosphorylation system release and convert the chemical energy stored in organic compounds into the high-energy bonds linking the phosphate groups of adenosine triphosphate (ATP) molecules. This process is arguably one of the most important responsibilities of mitochondria in most eukaryotes. Numerous metabolic pathways supply mitochondria and the TCA cycle with carbon. Virtually all amino acid, carbohydrate and lipid catabolic pathways end or at least impinge on mitochondria (Fig. 2).

The breakdown of dietary carbohydrates in most cases proceeds through at least some of the steps of the glycolytic pathway, resulting in the production of pyruvate. While glycolysis provides rapid means of producing ATP from carbohydrates, the energy efficiency, i.e. the ATP yield per unit of carbohydrate, of this process is insufficient to satisfy the energy requirements of many cell types and tissues. Thus, a proportion of the pyruvate produced in the cytosol is transported into mitochondrial matrix, where it is decarboxylated to acetyl-CoA by the enormous 9.5 MDa (Gray, Tompkins, & Taylor, 2014) pyruvate dehydrogenase complex. Acetyl-CoA is then combined with the TCA-cycle intermediate oxaloacetate in a condensation reaction catalyzed by the mitochondrial citrate synthase, to supply the cycle with carbon. Acetyl-CoA is also generated by mitochondrial β-oxidation, a stepwise oxidation of fatty acids. The oxidation of short (SCFA, 4-8 carbon atoms) and medium chain (MCFA, 6-12) fatty acids is generally mitochondrial, whereas the oxidation of long chain fatty acids (LCFA, 10-16) occurs both in mitochondria and peroxisomes, and that of very long chain fatty acids (VLCFA, 17-26) is exclusively peroxisomal (Panini, 2013). Short and medium chain fatty acids can diffuse into mitochondria relatively freely but LCFA require a special import process. Prior to import into mitochondria, LCFA are “activated” in the cytoplasm through a ligation with CoA. This reaction is ATP-dependent and catalyzed by acyl-CoA synthetases (or acyl-CoA ligases) (Nelson & Cox, 2005). The fatty acyl-CoAs then enter the intermembrane space where carnitine palmitoyltransferase 1 (CPT-I)
transfers the fatty acyl-groups to carnitine. The resulting fatty acyl-carnitine intermediates are then transported into the mitochondrial matrix by the carnitine-acylcarnitine translocase (CACT). Finally, in the matrix, the fatty acyl carnitines are acted on by a second carnitine palmitoyl transferase (CPT-II), which regenerates fatty acyl-CoA, substrate for β-oxidation. The mitochondrial β-oxidation is a four-reaction process with each full round of oxidation resulting in a sequential removal of two-carbon units (acetyl-CoA) at the β-position of fatty acyl-CoA molecule. This process can fully degrade only even-chain fatty acids, though, as the final step in the breakdown of odd-chain fatty acids results in the production of acetyl-CoA and propionyl-CoA, rather than two molecules of acetyl-CoA. In this case, the propionyl-CoA is converted into the TCA-cycle intermediate succinyl-CoA through a methylmalonyl-CoA intermediate. These intermediate steps are also required for the full degradation of methionine and the branched chain amino acids valine, leucine and isoleucine. The deamination and breakdown of each of these amino acids yields propionyl-CoA and can thus supply carbon to the TCA-cycle in the form of succinyl-CoA. Other amino acids can also be catabolized to provide energy. While the contribution of amino acids to total energy production in humans on a well-balanced diet is quite low, some 10-15% (Pasquale, 2007), this figure can be substantially higher e.g. for those on a low-carbohydrate diet. Amino acids are divided into two sub-classes based on the end-products of their catabolism: glucogenic and ketogenic. The deaminated carbon skeletons of glucogenic amino acids can provide carbon to the TCA cycle, in addition to succinyl-CoA, in the form of pyruvate (alanine, cysteine, glycine, serine, threonine and tryptophan), oxaloacetate (asparagine and aspartate), fumarate (aspartate, phenylalanine and tyrosine) and α-ketoglutarate (arginine, glutamate, glutamine, histidine and proline). Ketogenic amino acids (isoleucine, leucine, lysine, tryptophan and tyrosine) in turn, are degraded to acetyl-CoA. The dehydrogenases of the TCA cycle (isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase) oxidize their respective substrates while reducing the electron acceptors NAD+ and FAD+ to NADH and FADH2. These high-energy electron carriers, in turn, supply electrons to the electron transport chain.
2.1.4 Mitochondrial OXPHOS system

The production of ATP from ADP and organic phosphate is energetically unfavorable. The required energy is provided by the oxidation of the two electron carriers, NADH and FADH$_2$, in a chain of oxidation/reduction reactions catalyzed by four multisubunit protein complexes (complexes I-IV, CI-IV) of the inner mitochondrial membrane, known as the electron transport chain (ETC). Three of these complexes couple the transport of electrons to translocation of protons across the inner mitochondrial membrane. This proton motive force ($\Delta p$) is then used to drive ATP synthesis, catalyzed by another multimeric protein complex (complex V, CV). These two, coupled processes are collectively called the oxidative phosphorylation (OXPHOS) or cellular respiration system.

Structurally, the five OXPHOS or respiratory chain complexes consist of 92 polypeptides, 13 of which are mitochondrially encoded (Chinnery & Hudson, 2013). In addition, numerous proteins are transiently associated with the complexes during their assembly (assembly factors), or participate in their regulation and coordination. The largest of these OXPHOS complexes, complex I (NADH:ubiquinone oxidoreductase), comprises 44 subunits: 7 nDNA- and 7 mtDNA-encoded polypeptides form the functional core of CI while a further 30 nDNA encoded subunits are required for its stability and regulation (Hirst, 2011; Kmita & Zickermann, 2013). The small, four-subunit complex II (succinate: ubiquinone oxidoreductase, succinate dehydrogenase) is the only respiratory chain complex which contains no mitochondrially encoded polypeptides. It is also an enzyme of the TCA cycle. Complex III (ubiquinol:cytochrome c oxidoreductase) contains a single mtDNA- and 10 nDNA-encoded subunits. Complex IV (cytochrome c oxidase, COX) consists of three mitochondrially encoded core subunits, and an additional 11 encoded by the nDNA. Finally, complex V (F$_0$F$_1$-ATP synthase) comprises of 17 nDNA encoded and 2 mtDNA encoded subunits.

The core function of complexes I and II is to receive electrons from the freely diffusible NADH and the protein bound FADH$_2$, respectively, and transfer them to ubiquinone [coenzyme Q10 (CoQ)], reducing it first to ubisemiquinone and then to ubiquinol (CoQH$_2$). CoQ transports electrons to CIII, which relays them further to another diffusible electron carrier, cytochrome c. In the final electron transport step, cytochrome c donates electrons to CIV, which uses them to reduce molecular oxygen, the terminal electron acceptor in mammalian mitochondria, into water.
Pyruvate dehydrogenase (NAD+ to NADH), the TCA cycle (NAD+ to NADH and FAD to FADH2) and β-oxidation (NAD+ to NADH and FAD to FADH2) are generally considered the primary source of reducing equivalents. However, other metabolic pathways also contribute to the mitochondrial redox-balance to a varying degree, depending on the cell and tissue type. For instance, enzymes participating in the glycine cleavage system and other reactions of mitochondrial amino-acid catabolism, such as proline oxidase and the branched-chain α-ketoacid dehydrogenase complex, can also reduce NAD+ and FAD+. In addition, the mitochondrial glycerol-3-phosphate dehydrogenase, which participates in the α-glycerophosphate shuttle, transfers electrons from the cytosol into the ubiquinone pool.

The energy released by the flow of electrons down the electron transport chain is used by complexes I, III and IV to pump protons from the matrix, across the inner membrane, into the inner membrane space. This generates an electrochemical gradient across the IMM. The potential energy of the proton gradient is used for multiple purposes: to produce ATP, to import proteins and other ions, and to generate heat by dissipating the Δp through a process called uncoupling (Ledesma, de Lacoba, & Rial, 2002). The flow of protons from the inner membrane space into the matrix through the membrane-spanning part (Fo) of complex V powers the conversion of ADP + P_i to ATP (Boyer, 1997). The inner membrane adenine nucleotide translocators (ANTs) are used to transport the mitochondrially synthesized ATP into the cytosol in exchange for ADP.

Respiratory chain complexes of intact mitochondria are arranged in larger assemblies, called supercomplexes or respirasomes. The supercomplexes of mammalian cells mainly comprise complexes I, III and IV in different stoichiometric combinations (Acín-Perez et al., 2008; Dudkina et al., 2011). Functionally, supercomplex formation stabilizes the individual complexes and provides a means for substrate channeling (Acín-Pérez et al., 2004; Schägger et al., 2004), which significantly improves the efficiency of electron flow through them (Bianchi et al., 2004). The individual complexes may also produce and release less reactive oxygen species (ROS) when bundled into supercomplexes (Maranzana et al., 2013).
2.1.4.1 Alternative respiratory chain enzymes

The OXPHOS system with its five multisubunit complexes is generally considered to provide the most energy efficient means for the terminal oxidation steps in nutrient catabolism and ATP synthesis. However, lower eukaryotes present exceptions to the generally highly conserved organization of the OXPHOS system. Baker’s yeast, *Saccharomyces cerevisiae*, for example, is completely devoid of complex I, but instead uses two rotenone-insensitive, single-subunit NADH dehydrogenases, Nde1p and Ndi1p, to transfer electrons to ubiquinone, either from the cytosol (Nde1p), or from the mitochondrial matrix (Ndi1p) (Yagi et al., 2001). As opposed to complex I, neither enzyme is capable of translocating protons between mitochondrial or cellular compartments. In addition to *S. cerevisiae*, plants also carry several alternative NAD(P)H dehydrogenases, but unlike the case of yeast, these complement a standard electron transport chain (reviewed by Rasmusson, Soole, & Elthon, 2004).

Plants, in particular, utilize a second alternative enzyme which by-passes the cytochrome segment (CIII and CIV) of the respiratory chain (Siedow & Umbach, 2000). This alternative oxidase (AOX) receives electrons from ubiquinol and passes them directly to molecular oxygen. The AOX of plants is integral to the inner mitochondrial membrane and is believed to operate as a homodimer, with no proton translocation activity (Juszczuk & Rychter, 2003). Although the exact physiological function of AOX in plants is unknown, biochemical evidence suggests that AOX can facilitate the maintenance of mitochondrial substrate oxidation and TCA-cycle activity when high levels of ATP are being produced concomitantly by photosynthesis (Noguchi & Yoshida, 2008; Rustin & Lance, 1986). AOX is enzymatically inactive *in vivo* under normal physiological conditions, since it has a very low affinity for ubiquinol and is only activated by a significant over-reduction of the quinone pool (Yoshida et al., 2010).

In recent years, the alternative respiratory chain enzymes have been extensively studied in the context of respiratory chain dysfunction, and its compensation using the molecular bypass of dysfunctional complexes provided by these enzymes. AOX has been successfully used to rescue cytochrome *c* oxidase deficiency in human cells (Dassa et al., 2009; Hakkaart et al., 2006), flies (Kemppainen et al., 2014) and, most recently, in rodents (El-Khoury et al., 2013). Likewise, the yeast Ndi1p has been shown to ameliorate the pathological outcomes of complex I deficiency in all
three species (Bai et al. 2001; Burman et al. 2014; Cho et al. 2012; Marella et al. 2010; Park et al. 2007; Sanz et al. 2010). Furthermore, both enzymes can be used to dissect the pathological mechanisms underlying complex OXPHOS disorders by providing a bypass for isolated segments of the respiratory chain, decreasing ROS production and alleviating the metabolic block in substrate oxidation imposed by dysfunctional electron transport.

2.1.4.2 Mitochondrial reactive oxygen species

Mitochondria and the electron transport chain are often considered the primary source of ROS in the cell. While the evidence to support this notion is inconclusive or insufficient, mitochondria are certainly one of the major sources of ROS, even under normal physiological conditions (reviewed by Brown & Borutaite, 2012). Mitochondrial ROS production occurs primarily via electron leakage from the redox active prosthetic groups of complexes I and III, although many mitochondrial enzymes, such as α-ketoglutarate dehydrogenase, monoamine oxidase and mitochondrial glycerol-3-phosphate dehydrogenase can contribute to total ROS production (Balaban, Nemoto, & Finkel, 2005; Starkov, 2008). Reactive oxygen species include a number of oxygen-derived, highly reactive molecules. Most of them are derivatives of either the superoxide anion (O$_2^-$) or hydrogen peroxide (H$_2$O$_2$) (Brown & Borutaite, 2012). The superoxide anion is produced when escaping electrons react with, and reduce O$_2$. Superoxide is detoxified by converting it to hydrogen peroxide, in a reaction catalyzed by the mitochondrial manganese superoxide dismutase (MnSOD or SOD2). The freely diffusible H$_2$O$_2$ itself is detoxified by decomposition to water, catalyzed by catalases or glutathione peroxidases. Alternatively, in the presence transition metals, such as Fe$^{2+}$, hydrogen peroxide may react to produce the hydroxyl radical (OH$^-$) in the so-called Fenton reaction (Winterbourn, 2013). H$_2$O$_2$ is the least reactive and most stable of the three reactive oxygen species, while OH$^-$ has the shortest half-life time but also the highest reductive potential (Holmström & Finkel, 2014).

Cells process endogenous ROS by a variety of antioxidant mechanisms, including the aforementioned detoxifying enzymes, and the glutathione system, which decomposes H$_2$O$_2$ to water. This reaction is catalyzed by glutathione peroxidases, and results in the oxidation of glutathione (GSH) to glutathione disulfide (GSSG).
Glutathione is regenerated by glutathione reductase which reduces the oxidized glutathione back to GSH, in an NADPH-dependent manner (Wu et al., 2004). These antioxidant defenses may become insufficient when ROS-production is elevated for prolonged periods of time, such as in the case of high Δp, NADH/NAD⁺ and CoQH₂/CoQ ratios, resulting from respiratory chain dysfunction (reviewed by Murphy, 2009). The close proximity of the mtDNA molecule to the site of production of the highly reactive ROS, O₂⁻ and OH⁻, makes it exceptionally prone to oxidative lesions.

To counter the DNA lesions inflicted by ROS and other environmental stressors, mitochondria have several repair mechanisms. Such mechanisms have been shown to include at least mismatch repair, base excision repair and degradation and regeneration of damaged mtDNA molecules (Alexeyev et al., 2013; Copeland & Longley, 2014; Kazak, Reyes, & Holt, 2012).

### 2.1.5 Mitochondrial protein homeostasis

#### 2.1.5.1 Mitochondrial protein synthesis

The existence of mitochondrial translation machinery was postulated in the early 1960’s. The first mitochondrial ribonucleoprotein complexes were isolated from rat liver a few years later. Due to the endosymbiotic origin of mitochondria, prokaryotic and mitochondrial proteins synthesis systems have some fundamental similarities, yet considerable structural divergence has occurred between mitoribosomes and the ancestral system of bacteria. The mammalian 55S mitoribosome consists of two subunits, the 28S small subunit and the 39S large subunit. The backbones of the small and large subunits are formed by the 12S and 16S rRNAs, respectively. The rest of the mitoribosome comprises approximately 80 nuclear-coded polypeptides, of which 15 SSU and 20 LSU polypeptides display no significant homology to bacterial proteins, suggesting that these are newly acquired ribosomal proteins (Koc et al. 2000, 2001; Suzuki et al. 2001; Cavdar et al. 2001). The prokaryotic (70S) ribosomes likewise consist of small (30S) and large (50S) subunits, and are generally smaller than mitoribosomes despite having higher sedimentation coefficients. This disparity in metrics is explained by the higher protein content, and thus more porous structure of the mitoribosome. Mammalian mitoribosomes have a
protein to RNA ratio of 2 to 1, whereas the opposite is true for prokaryotic ribosomes (Sharma et al., 2003).

The rRNA stem-loops of the small subunit coordinate codon-anticodon pairing, making the SSU largely responsible for the decoding process and fidelity of translation. The large subunit in turn interacts with the acceptor arms of tRNAs and covalently links amino acids to the nascent polypeptide chain, thus carrying out the peptidyl transferase reaction.

Mitochondrial protein synthesis proceeds in four discernible steps, but many aspects of the process remain poorly understood, largely due to the lack of an in vitro mitochondrial translation system (Rorbach et al., 2007). One of the most enigmatic questions in mitochondrial protein synthesis remains how translation is initiated i.e. how ribosomes are directed to the initiation codon. Mitochondrial mRNAs lack 7-methylguanylate cap structures in their 5'-ends and upstream leader sequences which facilitate initiation in the eukaryotic cytoplasm, and also lack the Shine-Dalgarno sequences of the prokaryotic mRNAs (Montoya, Ojala, & Attardi, 1981). Two initiation factors, IF2_{mt} and IF3_{mt}, have been identified in mammalian mitochondria (Koc & Spremulli, 2002; Liao & Spremulli, 1991). The absence of the prokaryotic IF1 in mammalian systems could simply be explained by functional redundancy: the Escherichia coli IF1 fails to stimulate translation initiation of the 55S ribosomes, while IF2_{mt} alone can catalyze translation initiation of the 70S ribosomes, in the absence of IF1 (Spremulli et al., 2004). IF2_{mt}, like its bacterial orthologue, is believed to promote tRNA-SSU interaction (Spencer & Spremulli, 2004).

In bacteria, translation commences with the formation of an initiation complex, consisting of 30S SSU, mRNA, N-formylmethionylated tRNA (tRNA^{fMet}), the three IFs and GTP (Boelens & Gualerzi, 2002). Initiation factors assist the first methionyl-tRNA to access the ribosomal P-site. All the subsequent aminoacylated-tRNAs will enter the ribosome at the A-site. IF3, which interacts with the small subunit at the vicinity of the P-site, is required to block the premature association of the two ribosomal subunits (Brock, Szkaradkiewicz, & Sprinzi, 1998) and to proofread the correct selection of tRNA during initiation (Meinnel et al., 1999). The GTP-bound form of IF2 forms a ternary complex with tRNA^{fMet} resulting in the release of IF3. The dissociation of IF3 primes subunit adhesion, which in turn triggers IF2 to hydrolyze GTP to GDP and the subsequent release of all initiation
Translation elongation requires three accessory proteins, EF-Ts, EF-Tu and EF-G, in bacteria and mitochondria alike, with the exception of yeast, which lacks EF-Ts. Although mitochondrial polypeptide chain elongation has not been extensively studied, the substantial homology between the mitochondrial elongation factors and their bacterial counterparts suggests that the process proceeds in a similar fashion in both systems (Rorbach et al., 2007). Elongation begins with the aminoacylated-tRNA entering the ribosomal A-site in a ternary complex with EF-Tu and GTP. The anticodon site of the tRNA interacts with the mRNA, causing GTP hydrolysis and the release of EF-Tu-GDP, while the ribosomal peptidyl transferase center catalyzes the formation of a new polypeptide bond. The nascent polypeptide chain then translocates together with the peptidyl-carrier tRNA to the P-site exposing the A-site for a new aminoacyl-tRNA. This translocation is promoted by ribosome bound EF-G-GTP and GTP-hydrolysis. Simultaneously, the ribosome advances a single codon length along the mRNA. The inactive EF-Tu-GDP is activated to EF-Tu-GTP by EF-Ts. Once the tRNA has translocated through the A and P-sites, it exits the ribosome at an E-site. The existence of a bacteria-like E-site in mitoribosomes is unclear since the contact sites between the ribosome and tRNA are poorly conserved (Mears et al., 2002; Mears et al., 2006). This elongation cycle continues until the ribosome encounters a stop codon.

Translation termination is the least well-studied phase of mitochondrial protein synthesis. The translation termination and release factor family consists of four members, identified based on sequence homology: mtRF1 (Nozaki et al., 2008), mtRF1a (Soleimanpour-Lichaei et al., 2007), ICT1 (Richter et al., 2010) and C12orf65 (Antonicka et al., 2010). Termination in mitochondria is signaled by one of the four stop codons: the non-conventional AGA and AGG, and the canonical UAA and UAG, found in 11 of the 13 mitochondrial open reading frames. The latter two stop codons are decoded by the mitochondrial release factor RF1a (mtRF1a). Two competing theories exist on how the remaining two, non-canonical stop codons AGA and AGG are decoded. The first possibility is that the mitoribosome undergoes a -1 frameshift upon the entry of either of these triplets on to the A-site, thus effectively placing a standard UAG in to the A-site (Temperley et al., 2010).
The second possibility is that AGA and AGG are truly stop codons but translation termination is catalyzed by ICT1 instead of mtRF1a (Akabane et al., 2014; Lind, Sund, & Aqvist, 2013).

After translation termination, one more crucial step is required before the mitoribosome is ready for a new round of protein synthesis. This process of ribosome recycling involves dissociation of the ribosome-mRNA-tRNA complex and the two mitoribosomal subunits from each other, assisted by special recycling factors (RFs). Two mitochondrial RFs, RRF_{mt} and EF-G2 (also referred to as RRF2_{mt}), have been characterized in mammalian mitochondria so far (Tsuboi et al., 2009; Zhang & Spremulli, 1998) but the exact mechanistic details of the recycling process are still under investigation.

2.1.5.2 Mitochondrial protein import

The mitochondrial genome encodes only 13 polypeptides while proteomics studies have estimated the mitochondrial proteome to comprise approximately 1500 polypeptides in mammals and 1000 in yeast (Pagliarini et al., 2008; Reinders et al., 2006; Sickmann et al., 2003). This implies that the vast majority of mitochondrial proteins are synthesized on cytosolic ribosomes and imported through the mitochondrial double membrane in a controlled fashion (reviewed by Dudek et al., 2013). Synthesis of mitochondrially targeted proteins occurs in the proximity of the mitochondrial outer membrane, but protein import is considered to take place post-translationally, with SOD2 and fumarase amongst the few known exceptions (reviewed by Dudek et al., 2013).

Protein targeting into mitochondria requires a specific targeting sequence, which is usually an N-terminal, 15 to 55 amino acid-long presequence containing amphipathic α-helices and a net positive charge (Vögtle et al., 2009).

Transport across the outer membrane is performed by the Translocase of the Outer Membrane (TOM complex). Transported proteins are initially docked to Tom20 and Tom70 proteins, which proofread the existence of a correct import signal. Hydrophobic proteins are escorted to the docking site with the assistance of chaperone proteins, which prevent premature and misfolding. Upon entry to the intermembrane space, proteins destined to the outer membrane are bound by small
Tim chaperones and transported into the Sorting and Assembly Machinery (SAM), which integrates them into the OMM. The various inner membrane metabolite carriers are inserted into the IMM by the Translocase of the Inner Membrane 22 (TIM22 complex), with the help of membrane potential. Protein translocation to the matrix continues directly from the TOM complex to the presequence translocase, or the Translocase of the Inner Membrane 23 (TIM23 complex). In addition to the matrix, the TIM23 complex can also sort proteins laterally to the inner membrane. IMM sorting and integration is driven by membrane potential alone, while protein translocation into the matrix requires additionally the Presequence translocase-Associated import Motor (PAM) and ATP.

The mitochondrial import presequence is removed in the IMS, IMM or the matrix by either one of the classical presequence peptidases, MPP (the mitochondrial presequence peptidase), IMP (the inner membrane peptidase) and Oct1, or other mitochondrial proteases (Mossmann et al. 2012).

2.1.6 Mitochondrial quality control

The term mitochondrial quality control (QC) comprises of all of the various cellular pathways responsible for the controlled turnover of mitochondrial proteins and lipids, even entire organelles, and the processes involving the regulation of mitochondria dynamics and morphology. The goal of the QC machinery is to maintain and restore the integrity of mitochondrial function and to protect the cell from the deleterious consequences of organelle failure. Defective quality control has been most prominently linked to neurodegenerative disorders, such as mitochondrial forms of Parkinson’s disease.

QC at the macromolecular level inside mitochondria involves highly conserved proteases and chaperone proteins which monitor the folding and assembly of mitochondrial proteins, and remove damaged and misfolded polypeptides (reviewed by Tatsuta & Langer, 2008). On the organellar level, functionality may be restored via fusion between damaged and intact mitochondria, or alternatively, segments of the mitochondrial network may be removed by fission. Both of these processes are regulated by the factors governing mitochondrial dynamics (Detmer & Chan, 2007). Finally, if mitochondria are damaged beyond repair, individual organelles can be
recycled through an autophagy-like process, termed mitophagy (Kim et al. 2007). This protects the cells from the inadvertent release of pro-apoptotic proteins from dysfunctional mitochondria (reviewed by Maiuri et al., 2007).

2.1.6.1 *Mitochondrial protein degradation and unfolded protein response*

The mitochondrial proteolytic QC machinery recognizes and degrades misfolded and damaged proteins in the intermembrane space and in the matrix. This process is catalyzed by ATP-dependent proteases which cleave their substrate proteins into short peptides, which are then further acted on by various oligopeptidases. Since many mitochondrial proteins are components of multi-protein assemblies, their degradation requires the help of mitochondrial chaperone proteins.

Two proteases of the AAA+ superfamily, Lon and ClpXP, chaperone and degrade mitochondrial proteins in the matrix, while a third family member, m-AAA is an inner membrane protease which cleaves membrane proteins on the matrix side (Goard & Schimmer, 2014). The Lon protease, in particular, has been shown to be assisted by the Hsp70 and Hsp100 family chaperone proteins, which prevent or dissolve protein aggregates and thus enable proteolysis (Tatsuta & Langer, 2008). The m-AAA cleaves membrane protein domains protruding into the matrix, whereas the i-AAA protease catalyzes analogous proteolysis in the inter-membrane space (Leonhard et al., 2000). The mitochondrial proteases are also involved in a number of processes in a regulatory capacity. For example, in mice and yeast, the m-AAA protease is required to process the preprotein form of the mitoribosomal protein L32, and failure to do so results in protein synthesis deficiency (Bonn et al., 2011; Nolden et al., 2005). The Lon protease in turn, has been demonstrated to regulate mitochondrial gene expression and mtDNA copy number through its target, TFAM, at least in *Drosophila* (Matsushima, Goto & Kaguni, 2010).

Under some circumstances, the processing capacity of the mitochondrial proteases and chaperones may be exceeded, resulting in a mitochondrial proteotoxic stress. This may trigger the mitochondrial unfolded protein response UPR_{mt}, a response distinct from the much better studied ER-specific UPR. UPR_{mt} may be induced, for instance, when mitochondrial gene expression is inhibited, creating a disequilibrium between cytosolically and mitochondrially synthesized polypeptides inside
mitochondria (Martinus et al., 1996; Zhao et al., 2002). Induction of the mitochondrial UPR results in the up-regulation of mitochondrial chaperones and proteases, namely Hsp60, Hsp\textsuperscript{70\textit{mt}}, Hsp10 and ClpXp (Haynes & Ron, 2010). The retrograde signaling which induces the UPR\textit{mt} is still somewhat poorly characterized. In mammals, the transcription factor CHOP regulates at least some of the unfolded protein responsive genes while in \textit{C. elegans}, a shift in the localization of a transcription factor ATFS-1 from cytosol/mitochondria to nucleus has been shown to activate UPR\textit{mt} (Haynes & Ron, 2010; Nargund et al., 2012; Pellegrino, Nargund, & Haynes, 2013).

### 2.1.6.2 Mitochondrial dynamics and quality control

Mitochondrial QC is also exerted through the regulation of mitochondrial network dynamics. Four dynamin-related GTPases have been identified as the central mediators of mitochondrial fission and fusion: the cytosolic DRP1 is recruited to the outer membrane to catalyze mitochondrial fission, while the mitofusins MFN1 and MFN2 in the outer membrane, and OPA1 in the inner membrane, control membrane fusion (Cerveny et al., 2007; Hoppins & Nunnari, 2009).

Mitochondrial fission creates smaller mitochondria, which can \textit{e.g.} facilitate mitophagy. Upon its activation and translocation to the outer membrane, DRP1 multimerizes and forms a ring-like structure, which constricts and separates a fraction of the larger mitochondrion (Lee et al., 2004; Zhu et al., 2004).

Mitochondrial fusion allows content mixing between mitochondria. This process can potentially dilute damaged mitochondrial content such as, mutated DNA, by replenishing it with healthy macromolecules. Fusion is mediated by the outer membrane mitofusins, which form homodimeric or heterodimeric coiled-coil linkages between adjacent mitochondria. Mitofusins are degraded in mitophagy through PINK1/PARKIN-mediated ubiquitination and proteolysis (Tanaka et al. 2010).
2.1.6.3 Mitophagy

Once mitochondria become damaged beyond rescue by fusion, parts of the mitochondrial network may be sequestered. These mitochondrial fragments are removed by mitophagy. A cascade of events resulting in mitophagy can be triggered by the loss of membrane potential, which causes OPA1 inactivation and inhibits mitochondrial fusion. Two genes, PINK1 and PARKIN, are intimately linked to the regulation of mitophagy of depolarized mitochondria (Clark et al., 2006; Greene et al., 2003). PINK1 is a serine/threonine kinase which accumulates on mitochondria upon loss of membrane potential and recruits PARKIN from the cytosol (Matsuda et al., 2010; Narendra et al., 2008). The E3 ubiquitin ligase PARKIN, polyubiquitinates its substrates, which include mitofusins and VDACs, and thus induces the engulfment and subsequent degradation of mitochondria by the autophagosome-lysosome pathway (Narendra et al. 2008; Geisler et al. 2010; Tanaka et al. 2010). Mutations in both PINK1 and PARKIN have been linked to the pathogenesis of neurodegenerative disorders, most notably Parkinson’s disease.

Recently, an alternative PINK1 and PARKIN-mediated pathway for recycling mitochondrial content via mitochondrially derived vesicles (MDVs) was discovered (McLelland et al., 2014). This pathway operates independently of DRP1 and canonical mitophagy, and is triggered by oxidative stress, rather than membrane depolarization. Furthermore, the MDVs only appear to transport a selected subset of proteins to degradation at lysosomes.

2.1.6.4 Mitochondria and programmed cell death

Severe mitochondrial dysfunction may ultimately result in necrotic or apoptotic cell death (Lemasters et al., 1999). Mitochondria were first linked to apoptosis through the discovery that a core component of the apoptotic machinery, Bcl-2, was anchored to the outer mitochondrial membrane in C. elegans (Hengartner & Horvitz, 1994). For a while, mitochondria were thought to act merely as a signaling scaffold for the apoptotic machinery, but it is now well understood, that mitochondria can actively regulate programmed cell death (Galluzzi et al., 2012). The exact mechanistic details of the early steps after the induction of apoptosis are still not fully resolved, and may vary between taxa. The dissipation of the
mitochondrial membrane potential is known to be one of the inducing signals for the tightly regulated multi-step process resulting eventually in cell death. The loss of membrane potential induces outer membrane permeabilization, which in apoptosis is mediated by two Bcl-2 family members, BAX and BAK (Breckenridge and Xue, 2004; Green and Kroemer, 2004). The permeabilization also releases a number of mitochondrial proteins, including cytochrome c, SMAC/Diablo and HtrA2/Omi, which initiate the apoptotic caspase cascade (Galluzzi et al., 2012).

2.1.7 Mitochondrial disorders

Mitochondrial disorders form a large and heterogeneous group of individually rare syndromes with a highly variable age of onset, severity and tissue specificity, and which generally arise as a result of respiratory chain dysfunction caused by mutations in mtDNA or genes nuclear-coded mitochondrial proteins (Nunnari & Suomalainen, 2012; Schon et al., 2012; Wallace et al., 2010). Respiratory chain dysfunction can occur as an isolated deficiency of a single OXPHOS complex, often caused by a mutation in a gene for a structural subunit or assembly factor, such as in the case of Leigh syndrome with COX deficiency, which results from mutations in the cIV assembly factor SURF1. More often though, most of the respiratory chain is affected, e.g. as a result of point mutations in the mtDNA-encoded mitochondrial tRNAs or rRNAs, or substantial mtDNA depletion. In such cases, the individual’s and tissue-specific heteroplasmy levels play a major role in determining the disease outcome. Due to the involvement of both genomes, mitochondrial disorders resulting from non-sporadic mutations can be transmitted either maternally or in Mendelian fashion. Largely due to reasons of mtDNA heteroplasmy and stochastic segregation in post-mitotic tissues, mitochondrial disorders may not manifest in every generation or affected individual, or the same mutation may result in a different disease outcome in even closely related individuals.

The first case of a patient suffering from a mitochondrial disorder was reported already over half a century ago (Luft et al. 1962), but the era of mitochondrial disease research genuinely started in the late 80’s after the discovery of the first pathogenic mtDNA mutations (Holt, Harding, & Morgan-Hughes, 1988; Wallace et al., 1988). Since then, 150 distinct mitochondrial disorders have been characterized and more than 300 mtDNA lesions have been associated with clinical
manifestations. Altogether, the different respiratory chain disorders are currently predicted to affect at least 1 in 5000 individuals (Skladal, Halliday, & Thorburn, 2003; Vafai & Mootha, 2012).

Mitochondrial disorders can manifest essentially in only a single organ, such as in the eye, in the case of Leber’s hereditary optic neuropathy (LHON), or can affect multiple organs and tissues, with at least the muscle and central nervous system usually being affected. Mitochondrial DNA mutations, in particular, can manifest with extreme phenotypic variability. Syndromes which involve multiple deletions or loss of mitochondrial DNA, such as progressive external ophthalmoplegia (PEO), MIRAS (mitochondrial-associated recessive ataxia syndrome), SANDO (Sensory ataxia, neuropathy, dysarthria, ophthalmopaesis), IOSCA (infantile onset spinocerebellar ataxia), the Alpers-Huttenlocher syndrome or MDS (mitochondrial DNA depletion syndrome) can display either autosomal dominant or recessive inheritance (Rötig & Poulton, 2009). These disorders result from mutations in nuclear encoded genes involved in mtDNA maintenance, such as POLG, Twinkle, or in deoxyguanosine kinase (dGK) and thymidine kinase 2, two enzymes of the mitochondrial nucleoside salvage pathway (El-Hattab & Scaglia, 2013; Rötig & Poulton, 2009). Mitochondrial DNA point mutations in tRNA and rRNA genes are the cause of some of the most prevalent mitochondrial disorders, such as MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes), MERRF (myoclonus epilepsy with ragged red fibers) (Rötig 2011; Vafai & Mootha 2012). In addition to point mutations and deletions, mtDNA duplications are associated with mitochondrial diseases. Although generally rare, duplications are often detected in patients suffering from Kearns-Sayre syndrome (KSS) (Poulton et al., 1995).

Occasionally, mitochondrial disorders involving respiratory chain dysfunction arise from mutations not affecting the respiratory chain or mtDNA maintenance and expression machinery directly. Examples of this include impaired mitochondrial Fe-S cluster assembly which may result in Friedreich’s ataxia, and Coenzyme Q10 deficiency which is associated with familial mitochondrial cytopathies (González-Cabo & Palau, 2013; Ogasahara et al., 1989). Defective mitochondrial quality control, in turn, has been linked to neurodegenerative disorders such as Parkinson’s disease (Nunnari & Suomalainen, 2012).
Finally, diseases which are not necessarily characterized by features of classical mitochondrial disorders, may be of mitochondrial origin. Alterations in mitochondrial metabolism, for example, are being increasingly associated with many pathological conditions such as obesity, type 2 diabetes and cancer (Arruda et al., 2014; Chandel, 2014; Montgomery & Turner, 2014).

2.1.8 Disorders of mitochondrial protein synthesis

The mitochondrial protein synthesis machinery is responsible for translating only 13 mtDNA encoded polypeptides, but altogether approximately 100 nuclear genes are required to encode the translation machinery itself. Pathogenic mutations could theoretically affect any of these genes, which include those encoding mitoribosomal proteins, tRNA-modifying enzymes and aminoacyl-tRNA synthetases, translation initiation and elongation factors, and translational activators. Likewise, mtDNA-encoded tRNAs and rRNAs are common targets of pathogenic, protein synthesis impairing mutations.

The first mtDNA mutations affecting mitochondrial protein synthesis were described over 20 years ago. One of the most prevalent, the A3243G mutation in the tRNA<sup>L(UUR)</sup> gene, results in the childhood onset MELAS syndrome, a mitochondrial cytopathy characterized by lactate buildup and stroke-like episodes (Goto et al. 1990). A second highly prevalent mitochondrial tRNA mutation is the A8344G in the tRNA<sup>Lys</sup> gene. This mutation accounts for approximately 80% of MERRF cases (Shoffner et al., 1990b). A third mitochondrial tRNA gene often found mutated is that encoding tRNA<sup>Ile</sup>. Mutations in tRNA<sup>Ile</sup> are usually heteroplasmic and present as cardiomyopathy and PEO (Rötig, 2011). More than 150 tRNA mutations with different clinical manifestations (e.g. myopathy, encephalomyopathy, Leigh syndrome, chronic progressive external ophthalmoplegia) have been characterized, but mutations in these three tRNA genes comprise over half of them (Rötig, 2011).

tRNA mutations can affect translation in multiple ways: by causing defective tRNA folding, preventing nucleotide modifications, affecting tRNA stability or by causing amino acid misincorporation and thus decreasing the stability of mtDNA-encoded proteins (Börner et al., 2000; Sasarman, Antonicka, & Shoubridge, 2008). The mitochondrial tRNAs are frequently pseudouridylated, which stabilizes their secondary and tertiary structure. Mutations in the mitochondrially targeted isoform of pseudouridine synthase (PUS1), which converts uridine to pseudouridine, have
been found in patients suffering from mitochondrial myopathy, lactic acidosis and sideroblastic anemia (MLASA) (Bykhovskaya, Casas, Mengesha, Inbal, & Fischel-Ghodsian, 2004).

Mitoribosomal rRNA mutations have been associated with aminoglycoside-induced sensorineural hearing loss. The A1555G mutation in the 12S rRNA is one of the best-characterized mtDNA mutations. The mutation is usually homoplasmic and has a very high incidence in clinical populations, particularly in Asia (Rötig, 2011).

Pathogenic mutations in mitoribosomal protein genes themselves are very rare, possibly due to their early lethality. Two proteins of the small subunit, MRPS16 and MRPS22, have been found to be mutated in patients suffering from muscle hypotonia and lactic acidemia (Miller et al., 2004; Saada et al., 2007). In both cases, the patients had decreased levels of 12S rRNA and significantly decreased activities of complexes I, III and IV. Likewise, mutations in two large subunit genes, MRPL3 and MRPL44 have been recently discovered using exome-sequencing (Carroll et al., 2013; Galmiche et al., 2011). In the case of the MRPL3, MRPL44 and MRPS22 mutations, all patients exhibited cardiomyopathy.

Finally, an increasing number of pathological mutations in mitochondrial translation elongation and terminations factors, as well as aminoacyl-tRNA synthetases, are being identified. The manifestations of these mutations generally involve respiratory chain deficiency and clinical features commonly seen in other mitochondrial disorders.
Drosophila - a genetically tractable model organism

Research on the genetic and molecular basis of the pathophysiology of complex human disorders has traditionally relied heavily on vertebrate model organisms, such as the mouse and the rat. Physically small, and genetically and physiologically simple invertebrates such as the nematode Caenorhabditis elegans and the fruit fly Drosophila melanogaster are, however, well suited for studying many aspects of human disorders. Drosophila melanogaster, in particular, has been the model system of choice in genetics and developmental biology over the last century. Drosophila was used extensively e.g. by the evolutionary biologist, geneticist Thomas Hunt Morgan and his students, in their work with the chromosome theory of heredity in the early 20th century. This pioneering work laid foundations for modern-day genetics and contributed greatly to the transformation of biology into a more of an experimental science.

The small fruit flies are easy and cheap to rear, and have a short life cycle (10 days) and lifespan (60-100 days). Thus, establishing genetic crosses or inbred lines is much faster compared to rodents. Adult males reach sexual maturity within hours after eclosing and females are capable of laying hundreds of eggs during their reproductive life cycle. Many Drosophila tissues and organs are functionally analogous to their mammalian counterparts, but often slightly down-scaled in terms of number of cells/cell types and functional complexity. These include the gut, the central nervous system, heart and skeletal muscle, the Malpighian tubules (analogous to the kidney) and the fat body, the fly equivalent of the liver and white adipose tissue.

Fly genetics have many unique features which can be exploited for research purposes. The fly genome is compact, only approximately 5% of the size of the human genome, but still carries over 13,000 predicted protein-coding genes (Celniker & Rubin, 2003; Rubin, 2000), and approximately 75% of known human disease genes have orthologues in Drosophila (Bier, 2005). The genome is distributed on five chromosomes (X, Y, 2L/R, 3L/R and 4) which can be paired experimentally with balancer chromosomes, i.e. artificially generated chromosomes containing multiple inversions that suppress meiotic recombination with the corresponding endogenous chromosome. Balancer chromosomes also carry point mutations resulting in visible phenotypic traits, such as curly wings or stubble bristles. Balancers are especially useful in maintaining recessive, often lethal
mutations, and tracking their segregation with the help of the phenotypic markers they carry. Likewise, transgenes are usually linked with an eye colour marker, allowing their tracking in genetic backgrounds lacking eye colour.

Transgene expression in *Drosophila* is easily achieved using the bipartite GAL4-upstream activating sequence (UAS) derived from yeast. The GAL4/UAS-system (Elliott & Brand, 2008) allows for the transgenic expression of any UAS-controlled construct, *e.g.* short hairpin RNAs (shRNA), to be induced ubiquitously or in a tissue specific manner. The expression may also be temperature or drug induced, as in the case of the progesterone analogue (RU486, mifepristone) activated GeneSwitch-GAL4 system (Roman et al., 2001).

The *Drosophila* genome is highly accessible for manipulation using chemical/irradiation mutagenesis, P element-mediated mutagenesis or methods for producing defined genomic deficiencies and mutations. One of Morgan’s students, Herman J. Muller, was the first to demonstrate the mutagenicity of X-rays, a discovery which has since been vastly exploited alongside the use of the mutagenic agent EMS, in impairing and deducing functions of previously uncharacterized genes in *Drosophila*. P element-mediated mutagenesis strategies take advantage of transposable elements unique to *Drosophila*, which can be mobilized to generate imprecise excisions in the genome (Rio, Laski, & Rubin, 1986). Large libraries of predefined P element insertions (gene disruptions) and excisions generated using other transposable elements, are commercially available. Targeted mutagenesis in *Drosophila* is currently possible using either the CRISPR/Cas9 (Bassett & Liu, 2014) system or homologous recombination (Maggert, Gong, & Golic, 2008).

This palette of tools and characteristics has made *Drosophila* a widely used model organism in a diverse field of research ranging from neurodegeneration, obesity, aggression, and alcoholism, to learning and memory.

### 2.2.1 The development and life cycle of *Drosophila*

*Drosophila* development proceeds through four discrete stages: embryogenesis, the larval (juvenile) stage, pupal development which also includes sexual maturation, and reproductive adulthood (reviewed by Tennessen and Thummel, 2013). These developmental stages are also distinctive morphologically. The transparent embryos
develop for a day when reared at 25 °C and hatch from the eggs as worm-like larvae. This is followed by the first two larval stages (L1 and L2), both of which last for one day. After the third and final larval stage (L3), and the 4th day of larval development, growth is terminated and a puparium forms. Metamorphosis proceeds to produce a pharate adult, which ecloses after 4-5 days of pupal development. Progression through these stages is under neuroendocrine regulation. Pulses of the insect steroid molting hormone, 20-hydroxyecdysone (20E) control transitions from one developmental stage to another (Riddiford, Cherbas, & Truman, 2000; Thummel, 2001). The prehormone ecdysone is synthesized from cholesterol in the insect endocrine organ, the prothoracic gland, and released into the circulation. Ecdysone is further processed in the peripheral tissues to produce the active 20E form of the hormone (Huang, Warren, & Gilbert, 2008; Thummel, 2001), which can then exert its action through the ecdysone receptor. The 20E receptor is a heterodimer of two nuclear receptors, the ecdysone receptor (EcR) and Ultraspiracle (USP), and its activation triggers expression cascades of stage specific genes (Gilbert & Warren, 2005; Huang et al., 2008).

Under normal circumstances, growth in Drosophila is restricted to the larval stages, during which larvae increase in body mass up to 200 fold (Tennessee & Thummel, 2011). The correct timing of the high 20E titer at the end of the L3 stage is thus crucial, since this marks not just the end of the exponential growth phase, but also the end of the feeding stage during which the larva has had to accumulate sufficient nutrient reserves to survive through the pupal stage. This implies that the hormonal regulation must be under tight genetic control, and sensitive to the nutritional status of the animal.

Two somewhat poorly understood major transitions occur in larvae towards the end of the juvenile stage. The first one is surpassing a minimal viable weight around the L2 to L3 molt, which signifies that larvae have reached sufficient body mass to complete development in the absence of nutrients (C. Mirth, Truman, & Riddiford, 2005). Poor nutrient conditions after this point will only delay the final 20E pulse and the onset of metamorphosis, but not prevent it. Next, larvae need to reach a second checkpoint, which is the critical weight. The attainment of this milestone commits the larvae to metamorphosis within a definite period of time (C. K. Mirth & Riddiford, 2007; Tennessee & Thummel, 2011). Further feeding or starvation beyond this time point will only produce large or smaller adults, respectively, but does not affect the timing of the onset of pupariation.
Metamorphosis triggers the maturation of adult tissues. Most larval tissues, e.g. the larval fat body, are degraded during pupal development to provide energy and macromolecules for the biosynthesis of adult tissues. Many adult tissues, especially the appendages such as the wings, legs and antennae, develop from special tissue termed the imaginal discs. Imaginal discs consist of progenitor cells allocated largely during embryogenesis, and which remain quiescent during the larval stages. Upon metamorphosis, these cells are activated and start differentiating according to their predetermined fates.

2.2.1.1 Conserved signalling network regulates development in Drosophila

All of these aforementioned processes, and their timing in particular, are orchestrated by a conserved network of cellular growth regulators and diffusible signaling factors, which coordinate the growth of peripheral tissues based on several cues, such as nutritional status. Damage to key tissues, such as the imaginal discs, can also stall development prior to metamorphosis through systemic signaling. Two signaling pathways form the backbone of the growth and metabolic regulatory network. The first one is the Target of rapamycin complex (TOR), which in various configurations is present all through Eukaryota. TOR is a major regulator of processes required for cell growth, such as translation, transcription and autophagy (Kapahi et al. 2010; Albert & Hall 2014). The second, likewise highly conserved pathway is induced by eight insulin/insulin-like growth factors in Drosophila, known as the insulin-like peptides (ILPs). These circulating messengers are structurally and functionally related to mammalian insulins, and activate the sole fly insulin receptor (InR), and subsequently the downstream phosphatidylinositol 3-kinase (PI3K)/AKT protein kinase signaling pathway. The TOR and PI3K/AKT pathways are interlinked, but may also act independently of each other (Hietakangas & Cohen, 2009).

The TOR kinase is present in two signaling complexes: TOR complexes 1 (TORC1) and 2 (TORC2) (Loewith et al., 2002). TORC1 is the primary mediator of amino acid and energy sensing while the TORC2 modulates insulin signaling through the PI3K/AKT pathway. The major phosphorylation targets of the TORC1 include the ribosomal protein S6 kinase (S6K) and the eukaryotic initiation factor 4E binding protein (4E-BP) (Miron, Lasko, & Sonenberg, 2003). In the phosphorylated state, 4E-BP is blocked from inhibiting cytosolic translation by binding to initiation factor
eIF4E. S6K phosphorylation primes it for activation via 3-phosphoinositide-dependent protein kinase 1 (PDK1)-mediated phosphorylation. The activated S6K further phosphorylates ribosomal protein S6 and eIF4B to promote the formation of the translation preinitiation complex (Holz et al., 2005). *Drosophila* S6K mutants are severely growth retarded and have a small body size due to reduced cell size (Montagne et al., 1999), whereas 4E-BP mutations have a much milder phenotype, characterized by sensitivity to starvation and oxidative stress (Teleman, Chen, & Cohen, 2005; Tettweiler et al., 2005).

The PI3K/AKT pathway is activated in response to circulating insulins and insulin-like growth factors (IGFs). These ligands activate receptor tyrosine kinases, such as the insulin receptors (InR), on the cell surface. Actived insulin receptor recruits both the adaptor protein insulin receptor substrate (IRS, *chico* in *Drosophila*) and the catalytic subunit of the PI3K, resulting in its activation (Engelman, Luo, & Cantley, 2006). Activated PI3K then phosphorylates the plasma membrane phosphatidylinositol-4,5-diphosphate (PIP2) to yield phosphatidylinositol-triphosphate (PIP3). The phosphatidylinositol-triphosphates further recruit the PDK1 and AKT kinases to the plasma membrane, where AKT is activated by PDK1 via phosphorylation of Thr308 (Alessi et al., 1997; Yang et al., 2006). This AKT phosphorylation precedes another catalyzed by TORC2 at Ser473 (Ser505 in *Drosophila*). Two key AKT targets are the TORC1 inhibitors tuberous sclerosis 2 (TSC2) (Inoki et al., 2002; Potter, Pedraza, & Xu, 2002) and the proline-rich substrate of AKT 40 (PRAS40) (Sancak et al., 2007; Vander Haar et al., 2007). Another well-characterized AKT regulated protein in *Drosophila* is the Forkhead box class O (FoxO) transcription factor. The *Drosophila* Akt actively phosphorylates FoxO to retain its cytosolic localization (Brunet et al., 1999). If Akt activity is low under adverse conditions, FoxO becomes nuclearly localized and activates the transcription of its target genes, including the translation inhibitor Thor (encoding *Drosophila* 4E-BP) (Jünger et al., 2003).

The insulin receptor ligands in *Drosophila* comprise of 8 insulin-like peptides (ILP1-8). These peptides are considered to be functionally analogous to the mammalian insulin and insulin-like growth factors. The various ILPs are secreted from different fly organs, and most have well characterized spatiotemporal expression patterns. All ILPs share a similar domain structure and sequence homology with mammalian insulin (Grönke et al., 2010) and e.g. ILP5 can active the human InR (Sajid et al., 2011). In a nutrient-rich environment, ILP2, ILP3 and
ILP5 are synthesized primarily by the median neurosecretory cells of the fly brain (Brogiolo et al., 2001; Ikeya et al., 2002). ILP6 synthesis begins at the end of the 3rd larval stage and is highest in the fat body (Bai, Kang, & Tatar, 2012). The most recently discovered ILP, ILP8, is expressed predominantly in the ovaries but is also secreted from imaginal discs during the larval stages in response to tissue damage, in order to prevent progression to metamorphosis and allow for tissue regenerative processes to proceed (Colombani, Andersen, & Léopold, 2012; Garelli et al., 2012).

### 2.2.1.2 Nutrition and energy sensing regulate growth

As illustrated by the relationship between size, feeding behavior and developmental timing in larvae, nutrition is a key regulator of growth and growth signaling. In this context, the fat body is perhaps the most pivotal organ in sensing nutritional status in *Drosophila*.

The fat body is the primary insect nutrient store and is functionally analogous with mammalian liver and white adipose tissue. It also has the capability to regulate the growth of peripheral tissues: silencing of the exclusively fat body-specific cationic amino acid transporter *slimfast (slif)*, inhibits growth and produces small adults (Colombani et al., 2003). This effect is mediated by the TOR complex as demonstrated by the fact that inactivation of TOR specifically in the fat body phenocopies *slif* loss-of-function, while overexpression of a TOR downstream target, the S6 kinase (S6K), partially rescues it (Colombani et al., 2003; Oldham et al., 2000; Zhang et al., 2000). The exact nature of the fat body-derived, amino acid responsive humoral signal which regulates the growth of the entire organism remains unknown (Géminard, Rulifson, & Léopold, 2009). The fat body is known to respond to dietary sugars and lipids through a cytokine, Unpaired 2 (Upd2), which regulates *ILP* secretion (Rajan & Perrimon, 2012). The TOR-mediated response to amino-acid starvation is somewhat better understood, although it is unclear which amino acids are key to this regulation. Leucine, arginine and glutamine have all been implicated in this process, while the leucyl-tRNA synthetase has been linked functionally to TOR activation in response to leucine levels in human cells (reviewed by Jewell et al. 2013).

In addition to nutrients, cellular energy supply is a crucial determinant of growth rate. A decrease in cellular ATP levels results in an elevated AMP/ATP ratio. AMP
binds the γ subunit of the AMP-activated protein kinase (AMPK) and allows AMPK to be phosphorylated by the LKB1 kinase (Hardie, 2011). Activated AMPK inhibits growth primarily via regulating TORC1 activity through phosphorylation of two target proteins: the TORC1 inhibitor TSC2 (Inoki et al. 2003) and the TORC1 component RAPTOR (Gwinn et al., 2008).

2.2.2 tko^25t

Many proteins of eukaryotic mitochondrial ribosomes have no apparent orthologues in prokaryotes and thus represent a class of novel mitoribosomal genes, whereas some ribosomal proteins and rRNA tertiary structures have been preserved over hundreds of millions of years of evolution. The mitoribosomal small subunit protein S12 is one such highly conserved ribosomal protein and a key component of the ancient ribosome decoding centre. It has been characterized in numerous organisms including bacteria, mammals and Drosophila (Royden, Pirrotta, & Jan, 1987; Toivonen et al., 1999; Toivonen et al., 2001). Mutations in the bacterial S12 have been shown to result in hyperaccurate translation and a reduced rate of elongation (Bilgin et al., 1992).

The Drosophila mitoribosomal protein S12 (mRpS12), is encoded by the gene *technical knock-out* (*tko*). The *tko* gene maps to the X-chromosome and is expressed ubiquitously at a relatively low level throughout development ([www.flybase.org](http://www.flybase.org)). The *tko* gene derives its name from the stress (“bang”) sensitive behavioural phenotype exhibited by one of its few known non-lethal mutant alleles, *tko*^25t^ (Judd et al., 1972; Shannon et al., 1972; Royden et al., 1987; Shah et al., 1997). The bang sensitive mutants form a very heterogeneous group of genetic abnormalities, all of which result in a phenotypic high susceptibility to hyperactivity and paralytic and epileptic seizures, induced by mechanical or electric stimulus. Some of the other mitochondrial bang sensitivity loci include the adenine nucleotide translocase *sesB* (Zhang et al., 1999), the mitochondrial voltage-dependent anion channel *porin* (Graham et al., 2010), mitochondrial fission protein *Drp1* (Verstreken et al., 2005) and the Drosophila mitochondrial citrate synthase gene *knockdown* (Fergestad, Bostwick, & Ganetzky, 2006). The large number of mitochondrial mutants exhibiting stress-sensitivity is not surprising since mitochondrial function is particularly indispensable for signaling over neuromuscular junctions. In
*Drosophila*, mitochondrial dysfunction has been shown to specifically impair the mobilization of reserve pool vesicle during intense neurotransmission (Verstreken et al., 2005).

The hypomorphic *tko*<sup>25t</sup> mutation creates one of the best-studied defects of mitochondrial protein synthesis in *Drosophila*. It results in the substitution of a conserved leucine residue with histidine at position 85 (L85H). The mutant phenotype is associated with a severe OXPHOS deficiency and a decreased steady-state level of the 12S rRNA, indicating impaired assembly or stability of the mitoribosomal small subunit (Toivonen et al. 2001, 2003). Similar observations have been made in the two reported cases of human mitoribosomal SSU mutations (Miller et al., 2004; Smits et al., 2011). The *tko*<sup>25t</sup> phenotype in *Drosophila* further includes developmental delay (slow larval growth), hearing impairment and defective male courtship. However, rather surprisingly, the *tko*<sup>25t</sup> mutants exhibit no gross morphological changes with the exception of small and thin sensory bristles.
3. Aims of research

The primary aim of this study was to identify genetic and nutritional factors modulating the phenotypic outcome of impaired mitochondrial protein synthesis and OXPHOS deficiency, in a multi-cellular disease model (tko\textsuperscript{25i}), which recapitulates some of the most common manifestations of human mitochondrial disorders. We also attempted to discover some of the naturally occurring adaptive metabolic responses deployed by the mutant flies during development. This work was subdivided into three independent studies, presented in the corresponding articles (I and II) and manuscript (III):

I) The goal of the first study was to exploit the naturally occurring phenotypic recovery of the tko\textsuperscript{25i} mutants, achieved by selective inbreeding, to identify phenotype-suppressing alleles.

II) The aim of the second study was to test whether the expression of the alternative respiratory enzymes could alleviate some of the major phenotypic features of the tko\textsuperscript{25i} mutants and to use the Ciona intestinalis alternative oxidase (AOX) to dissect the relationship between particular OXPHOS and phenotypic manifestations of mitochondrial protein synthesis defects.

III) The aim of the final study was to investigate the metabolic adaptation to mitochondrial dysfunction during development, using primarily metabolomics and transcriptomics methods. In addition, we investigated how mutant flies responded to dietary intervention.
4. Materials and methods

4.1 *Drosophila* stocks and maintenance

Unless otherwise indicated, the *tko*\textsuperscript{25s} mutant and wild-type flies and larvae used were of Oregon-R genetic background and had been maintained inbred in a controlled laboratory for hundreds of generations. Additionally, a Canton-S and Oregon-R/Canton-S hybrid background was used for some experiments. The transgenic lines A1 and A3, carrying either a single, or multiple insertions of the *tko*\textsuperscript{25s} allele have been described in (Toivonen et al. 2003) and the UAS-AOX and UAS-Ndi1 transgenic constructs and flies in (Fernandez-Ayala et al. 2009) and (Sanz et al. 2010), respectively.

Unless otherwise stated, flies were maintained on a standard medium (high-sugar, HS) consisting of 1% agar (Oriola), 1.5 % (w/v) sucrose, 3% glucose, 3.5% Instant Dry yeast, 1.5% maize flour, 1% soy flour, 3% Lyle’s black treacle (Tate & Lyle, UK), 0.5% propionic acid (Sigma-Aldrich) and 1.0% methyl 4-hydroxybenzoate (Sigma), and a 12 h light/dark cycle at 25 °C. Variations of this medium were as follows:

ZS (‘zero-sugar’ medium) – 3.5% dried yeast, 1% soya flour, 1.5% maize flour.

Sugar series – ZS medium but with variable sucrose content, from zero to 10%.

Isocaloric media with decreased sugar and increased protein and/or fat content: LS (low sugar) medium – 5.5% dried yeast, 2.5% maize flour, 2.5% soya flour, 3% treacle; HP (high protein) medium – 5.5% dried yeast, 1% maize flour, 1% soya flour, 3% soya protein, 3% treacle; HF (high fat) medium – 5.5% dried yeast, 1.5% maize flour, 1.5% soya flour, 0.75% olive oil, 3% treacle.

Sugar type series – HS medium but with all of the sugars as sucrose, fructose or glucose (denoted S, F and G, respectively)
Various supplements, as indicated in figures and legends, were generally added from stock solutions after medium was cooled to 50 °C, including sodium pyruvate or lactate (25 mg/ml), ornithine (5 or 20 µg/ml), cycloheximide (up to 250 µg/ml)

4.2 Developmental time

Less than 5 day-old virgin females and males were pre-mated for 2 days, tipped into fresh vials or bottles every 24 h 3-4 times in succession, and then discarded. Eclosing progeny were counted once per day. Mean eclosion time ± SD was generated for each genotype from a minimum of three independent replicates of this experimental setup.

4.3 Bang sensitivity

Flies were anesthetized using CO₂, sexed, separated into individual empty culture vials and allowed to recover from anesthesia for a minimum of 3 h. Individual flies were then vortexed at maximum speed for 10 s in order to induce temporary paralysis. The duration of the paralytic/epileptic seizures was timed and means ± SD of the time taken for recovery of normal posture and mobility were generated using a minimum of 25 flies per genotype.

4.4 Larval feeding behaviour

Larval feeding behaviour was determined based on ingestion of a die-supplemented medium. Briefly, individual larvae grown on HS or ZS medium were placed on petri dishes of the same medium containing 0.16% erioglaucine for 20 min, washed with PBS, dried, and then homogenized in 100 µl PBS. Homogenates were centrifuged at 12000 g_{max} and dye uptake was measured spectrophotometrically (absorbance at 630 nm) using the supernatant fractions.
4.5 DNA extraction

25-40 anesthetized flies or larvae were collected in a 1.5 ml microcentrifuge tube on ice. Flies or larvae were homogenized in 400 µl of lysis buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl and .5% w/v SDS). Samples were incubated at 65 °C for 30 min. 800 µl of buffer consisting of 1.43 M potassium acetate and 4.29 M lithium chloride was then added, and samples were incubated on ice for 1 h. Samples were centrifuged at 12,000 g_max for 15 min and the resulting supernatant was transferred to a fresh microcentrifuge tube. DNA was precipitated by adding 600 µl of isopropanol and centrifuging at 12000 g for 15 minutes. After centrifugation, the supernatant was discarded and the pellets washed twice with 70% ethanol. DNA was dissolved in TE buffer and stored at -20 °C.

4.6 Construction of mRpL14 transgenic flies

Arguably this section should appear in Results. Transgenic lines carrying insertions of the Drosophila mRpL14 gene with its putative promoter were generated as described in (I). Briefly: mRpL14 was amplified from genomic DNA together with 750 bp segment upstream and 430 bp segment downstream of the transcription unit. This fragment was cloned and amplified using Zero Blunt TOPO PCR Cloning kit (Invitrogen). The insert was then introduced into a modified pGREEN-H-Pelican vector (Barolo et al. 2000, obtained through Drosophila Genomics Resource Center, USA) which carries gypsy insulator elements in order to prevent read-through transcription. The plasmid was amplified, extracted and phenol/chloroform purified using standard procedures. Transgenic flies were generated by Vanedis Drosophila Injection Service (Oslo, Norway) using germ cell micro-injection and P element mediated transgenesis, followed by several consecutive generations of crosses of the transgene-harboring progeny into the w^1118 background. Insertion site mapping was performed as described below.

4.7 Insertion site mapping using inverse-PCR

3.5 µg of genomic DNA from transgenic flies was digested with 10 U of ScrFI (Fermentas) in manufacturer’s buffer for 3 h at 37°C in a total volume of 25 µl. The
enzyme was heat-inactivated and the digested DNA was self-ligated with 4 Weiss units of T4 DNA ligase (Fermentas) in a total volume of 400 µl. Ligated DNA was ethanol precipitated and resuspended in 100 µl of 10 mM Tris-HCl (pH 8.0). 10 µl of the resuspended DNA was used as a template in a PCR-reaction with pGREEN-H-Pelican vector specific inverse-PCR primers. The resulting PCR-products were sequenced using the same primers in order to identify the genomic insertion site of the P element construct, by BLAST searching against the genomic DNA sequence.

4.8 RNA extraction

For RNA-sequencing, RNA was extracted from flash-frozen groups of 30 larvae using miRNA Easy Mini Kit (Qiagen) according to manufacturer’s instructions. Three biological replicate samples were produced by pooling 4 independent preparations to produce each replicate.

For Quantitative Reverse Transcription-PCR (QRTPCR), RNA was extracted using the TRIzol-method. Briefly, 100 mg of flies or larvae (approx. 30 females, 40 males or 25 larvae) were homogenized in 1 ml of TRIzol-reagent (Life Technologies). The homogenate was incubated at room temperature for 5 min after which 200 µl of chloroform was added to the samples. Samples were then incubated at room temperature for 3 min and centrifuged at 12000 g for 10 minutes at 4 °C. 600 µl of the upper aqueous phase was transferred into a fresh microcentrifuge tube and RNA was precipitated by adding an equal volume of 2-propanol and incubating at room temperature for 10 min. RNA was then pelleted by centrifuging at 12000 gmax for 10 min at 4 °C. The pellets were washed with 75% ethanol in DEPC-dH_{2}O and resuspended in 200 µl of DEPC-dH_{2}O.

Prior to cDNA synthesis, RNA samples were DNase I treated with 0.05 U/ml of DNase I (Thermo Scientific) in DNase I buffer (100 mM Tris-HCl, 25 mM MgCl_{2}, 1 mM CaCl_{2}) at 37 °C for 1 h. RNA was then phenol:chloroform purified, ethanol precipitated and washed, and resuspended in DEPC-treated dH_{2}O.
4.9 Analysis of gene expression using Quantitative Reverse Transcription-PCR (QRTPCR)

One µg of RNA was used for cDNA synthesis using the High-Capacity cDNA synthesis kit (Life Technologies) according to manufacturer’s instructions. Two replicate reactions of each sample were pooled and diluted 1:20 with dH₂O. 2 µl of the pooled and diluted sample was used for quantitative (real-time) PCR performed using Fast SYBR Green Master Mix (Life Technologies) and 0.5 mM final primer concentrations. Triplicate real-time PCR reactions were performed and data was acquired using a StepOne Plus instrument and software (Life Technologies). Gene expression was analyzed relative to the housekeeping gene RpL32.

For microarray data verification, RNA was transcribed using random hexamers (Amersham Biosciences) and M-MuLV reverse transcriptase (Fermentas). Real-time PCR was performed using QuantiTect SYBR Green PCR Master Mix (Qiagen) and Light Cycler (Roche) instrument following manufacturers’ instructions. The data was acquired and analyzed using Light Cycler data collection software version 3.5 (Roche).

Gene-dosage analysis was performed as described above but using approximately 500 ng of genomic DNA and 0.5 mM primer concentration in a final reaction volume of 20 µl. Two biological and three technical replicates per genotype were used for each gene analyzed and gene dosage was measured relative to RpL32.

4.10 Analysis of gene expression using RNA-sequencing

RNA sequencing analysis was performed on Hiseq 2500 sequencers (Illumina) using a paired-end library and 100 bp read-length and otherwise standard protocols. Expression analysis was performed using the Chipster. Sequencing reads were mapped to the Drosophila reference genome (BDGP release 5.72) using TopHat version 2.0.9, and differential expression analysis was performed using CuffDiff.
4.11 Analysis of gene expression by Western blotting

Total protein was extracted from 20 frozen larvae per sample. Three replicate samples per condition per experiment were pooled to produce a single individual sample. Experiments were repeated independently at least three times. Larvae were homogenized in 200 µl of buffer containing 150 mM NaCl, 1 mM EDTA, 2 M urea, 1.3 % (w/v) SDS, 10 mg/ml cOmplete protease inhibitor mix (Roche) and PhosSTOP phosphatase inhibitor mix (Roche). Homogenates were centrifuged for 1 min at 12000 g_{\text{max}} at 4 °C. The supernatant was mixed with 4 x Laemmli loading buffer consisting of 40% (w/v) glycerol, 8% (w/v) SDS, 250 mM Tris-HCl (pH 6.8), 0.015% (w/v) bromophenol blue and 200 mM DTT, heated for 7 min at 95 °C and cooled on ice. Approximately 30 µg of protein was loaded on Criterion TGX AnyKD precast SDS-PAGE gels (Bio-Rad) and run in ProSieve EX Running buffer (Lonza). Protein was transferred to 0.45 µm Hybond ECL nitrocellulose membrane (Amersham, GE Heathcare Life Sciences) in ProSieve EX Transfer Buffer (Lonza) using Criterion blotters (Bio-Rad). Membranes were blocked at room temperature for 1 h in either TBS-Tween with 2.5% (w/v) milk or with PBS-Tween with 2.5% (w/v) BSA. Primary antibody incubations were carried out overnight at 4 °C in in PBS-Tween with 2.5% (w/v) BSA. Membranes were then washed 3 x 5 min in PBS-Tween and incubated with HRP-conjugated secondary antibody for 1 h at room temperature in PBS-Tween with 2.5% (w/v) milk. After this, the unbound secondary antibody was washed off 3 x 5 min in PBS-Tween and finally once in PBS. Protein was detected and visualized using SuperSignal West Femto Chemiluminescent Substrate (Pierce, Thermo Scientific) and ChemiDoc imager (Bio-Rad).

Primary antibodies used were: Anti-Akt #4691 (Cell Signaling), Anti-phospho-Akt #4054 (Cell Signaling), anti-AMPK #80039 (Abcam) and anti-phospho-AMPK #4188 (Cell Signaling). HRP-conjugated secondary antibodies were Peroxidase Horse Anti-Mouse IgG #PI-2000 (Vector Labs) and Peroxidase Goat Anti-Rabbit IgG #PI-1000 (Vector Labs). Primary and secondary antibodies were used as 1:1000 and 1:10000 dilutions, respectively. Alpha-Tubulin #52866 (Abcam) was used as loading control in a 1:10000 dilution.
4.12 Metabolic assays

4.12.1 Metabolomics

Fifteen mid-third instar larvae were harvested, washed with PBS and snap-frozen in liquid nitrogen. Frozen samples were completely homogenized by a cell disrupter (Shake Master NEO; Bio Medical Science, Tokyo, Japan) at 4°C, after adding 500 µl of methanol-containing internal standards [20 µM each of methionine sulfone, 2-(N-morpholino)-ethanesulfonic acid (MES) and D-Camphol-10-sulfonic acid(CSA)]. The homogenate was then mixed with 200 µl of Milli-Q filtered water and 500 µl of chloroform and centrifuged at 9100 g max for 4 hr at 4°C. Subsequently, the aqueous solution was centrifugally filtered through a 5-kDa cut-off filter (Millipore) to remove proteins. The filtrate was centrifugally concentrated and dissolved in 50 µl of dH2O containing reference compounds (200 µM each of 3-aminopyrrolidine and trimesate). Prior to CE-TOFMS analysis, the sample solution for anion was diluted five times and that for cation was diluted 10 times with dH2O. Note that these procedures for metabolite analysis were performed by the laboratory of Dr. Tomoyoshi Soga, Keio University, Japan, using biological materials supplied by myself.

4.12.2 Triglycerides and free glycerol

The assay was slightly modified from (Tennessen et al. 2014). 20 mid 3rd instar larvae were collected, rinsed several times with PBS in a 1.5 ml microcentrifuge tube and snap frozen in liquid nitrogen. Frozen larvae were homogenized in 100 µl of cold PBS + 0.05% Tween 20. Ten µl of the homogenate was diluted 1:10 with PBS and set aside to measure protein content using the Bradford assay. The larval lysate was incubated at 70 °C in order to inactivate endogenous lipases. One 20 µl aliquot of the heat-treated lysate was mixed with 20 µl of triglyceride reagent (Thermo Scientific, Konelab, Indiko) and another with 20 µl of PBS. Both reactions were then incubated at 37 °C for 1 h. After this, 100 µl of free glycerol reagent (Sigma) was added to each sample. Samples were mixed well, incubated at 37 °C for 5 min and finally centrifuged for 3 min at full speed. 100 µl of the supernatant was used to measure absorbance at 540 nm. Glycerol solution (0.26 mg/ml in PBS, equaling 2.5 mg/ml of triolein) was used to generate a standard curve. The
triglyceride content of each sample was determined by subtracting the glycerol content of the untreated sample from the triglyceride reagent-treated aliquot.

4.12.3 ATP, lactate and pyruvate assays

Twenty larvae or adult flies were homogenized in 100 µl of 6 M guanidine hydrochloride on ice. The homogenate was incubated at 95 °C for 5 min and centrifuged at 12000 g_{max} for 5 min at 4 °C. The supernatants were transferred to fresh microcentrifuge tubes and stored at -80 °C.

The samples were diluted 1:10 and with PBS (pH 7.4) for analysis. Steady-state ATP levels were assayed using an ATP determination kit (Molecular Probes, Life Technologies) according to the manufacturer’s protocol. 90 µl of the ATP reaction mix was combined with 10 µl of the diluted sample to initiate the reactions and luminescence was measured immediately at 535 nm using a Hidex Chameleon plate reader. ATP standards were used to generate a standard curve and ATP concentrations were normalized to soluble protein in the samples, measured with the Bradford assay.

Pyruvate and lactate were measured using 1:10 diluted samples with commercially available fluorescence based determination kits (Abcam) according to manufacturer’s instructions. Ten µl of sample was combined with 50 µl of either lactate or pyruvate reaction mix. Reactions were incubated at room temperature for 30 min after which fluorescence was measured (excitation at 535 nm/emission at 590 nm) using a plate reader. Lactate and pyruvate standards were used to generate a standard curves and concentrations were normalized to soluble protein as before.

4.12.4 Enzymatic assays

Enzymatic assays were adapted from (Hinman & Blass 1981), (Merritt et al. 2005) and (Merritt et al. 2009). 20 frozen larvae were homogenized on ice in 200 µl of ice-cold 100 mM Tris-HCl (pH 7.4). The reaction conditions for each enzyme were as follows (all at pH 7.4): malic enzyme activity was assayed in the presence of 100 mM Tris-HCl, 0.34 mM NADP, 50 mM MnCl₂, 50 mM DL-malic acid; glucose-6-phosphate dehydrogenase was assayed in the presence of 100 mM Tris-HCl, 0.34 mM NADP, 3.5 mM D-glucose-6-phosphate; 6-phosphogluconate dehydrogenase
was assayed in the presence of 100 mM Tris-HCl, 0.34 mM NADP, 3.5 mM 6-phosphogluconate; isocitrate dehydrogenase activity in the presence of 100 mM Tris-HCl, 0.34 mM NADP, 0.84 mM MgCl$_2$, 1.37 mM DL-isocitric acid. Enzyme inhibitors sodium gluconate, sodium pyruvate and sodium lactate were used at 100 mM final concentration where indicated. Reactions were initiated by combining 10 µl of the larval homogenate with 90 µl of each reaction mixture. Increase in absorbance at 510 nM was detected in the presence of .6 mM iodonitrotetrazolium chloride and 6.5 µM phenazine methosulfate. Absorbance was measured kinetically at 1-2 min intervals for 15 min and NADPH standards were used to transform absorbance values into changes in NADPH concentration. Enzyme activity was normalized to total soluble protein, measured with the Bradford assay.

Isocitrate dehydrogenase and glutamate dehydrogenase activities were also analyzed separately using a commercially available IDH activity kit (Abcam) in order to evaluate the relative contributions of the NAD$^+$/NADH- and NADP$^+$/NADPH-dependent isoforms to the total enzyme activity. For this, 20 frozen larvae were homogenized as before in 200 µl of cold manufacturer’s IDH assay buffer. Enzyme activities of IDH and GDH were analyzed using manufacturer’s IDH substrate and 100 mM L-glutamate as substrates, respectively, and either NAD$^+$ or NADP$^+$ as enzyme co-factors. Reactions were initiated by combining 50 µl of the reaction mix with 10 µl of sample. Absorbance measurement (at 450 nm), enzyme activity determination and normalization were carried out as before.

### 4.12.5 Hemolymph glucose measurement

30-50 freshly collected larvae were rinsed with PBS, dried and transferred into a 0.2 ml vial with small holes in the bottom. Larvae were then punctured several times with a 27 gauge ½” needle and the small vial inserted inside of a larger vial. Larvae were then centrifuged at 1500 g$_{max}$ for 5 min at 4 °C in order to drain hemolymph. Two µl of hemolymph was transferred into a fresh vial containing 8 µl of ice-cold TBS (pH 6.6). Samples were incubated at 70 °C for 5 min and subsequently centrifuged at 12000 g$_{max}$ for 1 min at RT. The supernatant was transferred into a fresh vial and stored frozen at -20°C until analysis. All possible steps were performed on ice.
Trehalose and free glucose were measured using Glucose (HK) reagent (Thermo Scientific). Three µl of hemolymph were incubated with 0.5 µg of porcine kidney trehalase (Sigma-Aldrich) overnight at 37 °C. This was mixed with 150 µl of the glucose reagent, after which reactions were incubated at 37 °C for 1 h and absorbance at 530 nm was measured with a Hidex Chameleon plate reader. Non-trehalase treated samples were analyzed in parallel and the values subtracted from the trehalase-treated aliquots to give an estimate of free glucose in the sample. Glucose standards were used to determine the absolute glucose levels in the samples. Glucose levels were normalized to the amount of soluble protein in the samples, as determined using the Bradford assay. A minimum of eight replicate samples per genotype and condition were used.

4.13 Mitochondrial isolation

Mitochondria were isolated from 40 freshly harvested larvae or adults. Flies or larvae were homogenized with mortar and pestle in 2 ml of isolation buffer containing 250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA and 0.1% (w/v) BSA (pH 7.4). The homogenate was filtered and transferred into a microcentrifuge and centrifuged at 150 g_{max} for 3 min. The supernatant was then decanted into a fresh microcentrifuge tube and centrifuged at 9000 g_{max} for 10 min. Finally, the supernatant was discarded and the mitochondrial pellet resuspended in 50 µl of isolation buffer without BSA. All steps were carried out at 4 °C.

4.14 ROS measurements

H_{2}O_{2} production was measured using 0.05 mg/ml of isolated mitochondria in 100 µl of assay buffer containing 120 mM KCl, 5 mM KH_{2}PO_{4}, 3 mM HEPES, 1 mM EGTA, 1 mM MgCl_{2} and 0.2% (w/v) (BSA pH 7.2), 0.05 U/ µl superoxidase dismutase (Sigma-Aldrich), 0.1 U/µl of horseradish peroxidase (Life Technologies) and 10 µM Amplex Red (Life Technologies). Substrates and inhibitors were used at the following concentrations: 5 µM pyruvate, 5 µM proline, 20 mM sn-glycerol-3-phosphate, 1 mM ADP, 0.5 µM rotenone and 0.5 µM antimycin A.
5. RESULTS

5.1 Phenotypic rescue of \( tko^{25t} \) is achieved by modulating gene dosage (I)

5.1.1 Selective inbreeding rescues the \( tko^{25t} \) phenotype in suppressor lines

Based on our previous observations, we knew that maintaining the \( tko^{25t} \) mutants inbred over several generations would result in a gradual attenuation of the mutant phenotype (Toivonen et al., 2001; Toivonen et al., 2003). We decided to exploit this phenomenon in order to discover possible genetic suppressors of the \( tko^{25t} \) mitochondrial disease phenotype. Thus, we first backcrossed the mutant allele from a long-maintained laboratory stock into two different wild-type strains, Oregon-R (OR) and Canton-S (CS), over 12 generations. This crossing procedure was considered sufficient to eliminate possible pre-existing phenotypic suppressor alleles. We then mated flies of these two strains to produce an F1 generation with a severe and fully penetrant mutant phenotype (CS/OR \( tko^{25} \)). We picked individual mating pairs and placed these under selection for accelerated development, \( i.e. \) choosing the first eclosing males and females in each generation to establish the next. Over 12 generations, this resulted in all of the inbred lines exhibiting stable but characteristically different degrees of rescue of both developmental delay and bang sensitivity. Remarkably, three of these lines, denoted \textit{Weebles} 1-3 (\textit{Wb}1-3), exhibited an especially pronounced rescue of the disease-like phenotype (Fig. 3). We verified by sequencing that these three lines still carried the \( tko^{25t} \) mutation (Fig. 3A) and had not acquired additional mutations within the \( tko \) coding region. We then characterized these suppressor lines in further detail. All three \textit{Weebles} lines showed nearly complete rescue of developmental delay compared with \( tko^{25t} \) in a hybrid genetic background (Fig. 3B). The degree of rescue was moderate but still substantial also in the three other suppressor lines (A-C).
Figure 3. A) Sequencing of the suppressor and control lines at the region of the \( tko^{25t} \) point mutation region. B) Developmental time (determined as mean eclosion day + SD) at 25 °C. C) Bang sensitivity of <1 day old adults (mean recovery time from paralysis). D) Ratios of mitoribosomal 12S and 16S RNAs, determined using Q-RT-PCR. Sex, strain and phenotype as indicated. CS – Canton S, OR – Oregon R, CS/OR – F1 hybrid of Canton S females and Oregon R males, A-C – moderate suppressor lines, \( Wb^{1-3} \) – Weeble suppressors.

The Weeble lines, likewise, no longer exhibited bang sensitivity while the mild suppressor lines still exhibited a moderate phenotype (Fig. 3C). Finally, we quantified the ratio of mitochondrial ribosomal RNAs (12S:16S), which is considered representative of the ratio of small and large mitoribosomal subunits, and a diagnostic marker for the \( tko^{25t} \) phenotype (Toivonen et al. 2001, Toivonen et al., 2003). We observed the expected decrease in the steady-state ratio (Fig. 3D) in the mutant background, whether outbred (CS/OR \( tko^{25t} \)) or inbred (A-C), attributable to the depletion of the 12S mitoribosomal RNA. The ratio was significantly recovered in the suppressor lines (\( Wb^{1-3} \)) in both sexes, consistent with the improvement in phenotype.
5.1.2 Suppressor lines carry X-chromosomal duplications surrounding the tko locus

In order to identify the Weeble suppressor, multiple genetic experiments were performed to discover its genomic location. Based on a series of crosses between \( tko^{25t} \) and \( Wb^1 \), the suppressor allele appeared to be dominant and X-linked, irrespective of the parent of origin (Fig. 4A). This also implied that mitochondrial genotype did not contribute to the phenotypic suppression. We then performed a transcriptomic analysis of the Weeble suppressor lines and outbred wild type and \( tko^{25t} \) flies. This revealed a contiguous set of X-chromosomal loci, including the \( tko \) gene, to be consistently approximately two-fold elevated in expression in both suppressor lines (Fig. 4B). Since this was suggestive of a corresponding
chromosomal duplication, we performed a gene dosage analysis using southern blotting, targeting *tko* and two other X-chromosomal genes *CG8636* and *CG12498* (I). The first gene chosen was located within the region exhibiting the 2-fold elevated expression in *Wb*² but not in *Wb*¹, and the latter was outside of this region in both. Southern blotting confirmed that the copy number of *tko* was indeed elevated in both suppressor lines, while that of *CG8636* only in *Wb*². *CG12498* and *RpL32*, which was used as a single copy control, both had uniform copy number throughout the sample set. We verified these findings using qPCR, and further extended the analysis to cover all three suppressor lines, and genes within, and just outside of the potentially amplified region. Using genomic DNA of males, we confirmed that the amplification spanned a wide region of the X-chromosome surrounding the *tko* locus in all *Weebles* (Fig. 4B). We mapped the left end of the duplication to a region between the transposable element, *Tirant 24*, and the gene *CG14050* in all suppressor lines. The right breakpoint in *Wb*¹ was discovered to be located within the region between *trol* and *CG13758*, whereas in *Wb*² and *Wb*³ the duplication extended some 60-65 kB further downstream, with the right end mapping to a region between the genes *CG8636* and *Tsp3A*.

### 5.1.3 Ubiquitous ectopic expression of *tko*²⁵ᵗ or *mRpL14* does not suppress the *tko*²⁵ᵗ phenotype

Based on previous data, we knew that ectopic copies of a transgenic *tko*²⁵ᵗ allele in the mutant background did not confer significant phenotypic rescue, not even when the extra alleles were expressed at a level several fold higher than that of the endogenous gene (Toivonen et al. 2003). Since the duplicated region in all suppressor lines also carried a second mitoribosomal gene, *mRpL14*, we hypothesized that the amplification of both of the genes is required for a more substantial rescue of the *tko*²⁵ᵗ phenotype. We therefore cloned *mRpL14* together with its putative promoter region of approximately 750 bp, and established several transgenic fly lines carrying ectopic copies of the gene. We then chose two suitable transgenic lines, both of which, based on an insertion site analysis, had only a single genomic integration site on either the chromosome arm 2R (14-2R) or 3R (14-3R), did not interrupt the coding sequence of any known gene and did not reside within large heterochromatic regions. Finally, we performed a series of crosses to introduce the *mRpL14* transgenes into the *tko*²⁵ᵗ background, and produced *mRpL14* transgenic
mutant flies with and without additional ectopic copies of the tko\textsuperscript{25t} allele. We used the previously characterized (Toivonen et al. 2001) transgenic lines, A1 and A3, to provide the extra copies of the tko\textsuperscript{25t} allele, under the regulation of the endogenous promoter sequence. While the \textit{Wb\textsuperscript{1}} suppressor allele was sufficient to rescue male and female tko\textsuperscript{25t} mutants, both transgenes, whether alone or in combination, failed to complement the mutant phenotype (Fig. 5A, 5B and 5C), despite causing an increase in transcript levels comparable with that of the suppressors (Fig. 5D).

**Figure 5.** Ectopic expression of tko\textsuperscript{25t} and \textit{mRpL14} does not alleviate tko\textsuperscript{25t} phenotype. A) and B) Developmental time of male and female flies expressing transgenic copies of tko\textsuperscript{25t} (A1 and A3) and \textit{mRpL14} (14-2R and 14-3R). C) The effect of transgene expression on bang sensitivity. D) Relative (to wild-type of the same sex) expression levels of tko\textsuperscript{25t} and \textit{mRpL14} expressing flies.
5.1.4 The phenotypic rescue in suppressor lines is dosage and chromosomal context dependent

After we failed to rescue the \textit{tko}^{25t} mutants by introducing the extra copies of \textit{mRpL14} and \textit{tko}^{25t}, both of which produced approximately 2-fold elevated expression, we decided to further investigate the gene dosage effect and its dependence on chromosomal context. Hence, we combined \textit{Weeble} suppressors \textit{X}-chromosomes with a \textit{tko} null allele, \textit{tko}^{3}. We also introduced the extra autosomal copies of the \textit{tko}^{25t} allele carried by the transgenic construct A3 to the fully \textit{tko} deficient \textit{tko}^{3} background and characterized the resulting progeny. Females with the combination of a \textit{Wb} allele and \textit{tko}^{3} behaved essentially as females homozygous for \textit{tko}^{25t}, eclosing with a considerable developmental delay and exhibiting bang sensitivity (Fig. 6A and 6B). Increased gene copy number of the \textit{Wb}/\textit{tko}^{25t} and \textit{Wb}/\textit{Wb} females was sufficient to rescue both phenotypes. Consistent with our previous findings, similar or even higher \textit{tko}^{25t} copy number, achieved by introducing the A3 transgenic alleles into the \textit{Wb}/\textit{tko}^{3} background, was insufficient

\textbf{Figure 6.} \textit{Weeble} suppression is dependent on elevated \textit{tko}^{25t} copy number. \textbf{A}) and \textbf{B}) Developmental time and bang sensitivity. \textbf{C}) Unlike \textit{Weeble}, extra ectopic copies of \textit{tko}^{25t} (A3) are insufficient to rescue the mutant phenotype. Genetic nomenclature as before. FM7 indicates a balancer chromosome wild-type for \textit{tko}. 

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to rescue the mutant phenotype (Fig. 6C). This and the previous results thus strongly implied that while increase in gene dosage and transcript levels is necessary for the phenotypic rescue of the tko$^{25t}$ mutants, coordinate expression provided only by the Weebles amplifications is critical for the full suppressor effect.

5.2 Targeting the OXPHOS deficiency in tko$^{25t}$ with alternative respiratory chain enzymes (II)

5.2.1 AOX expression does not rescue tko$^{25t}$

The sea squirt Ciona intestinalis alternative oxidase (AOX) has been successfully used to compensate multiple respiratory chain complex defects in flies (Fernandez-Ayala et al. 2009), particularly those affecting the cytochrome c oxidase (Kemppainen et al. 2014). We therefore decided to test whether bypassing the cytochrome segment of the respiratory chain would alleviate the tko$^{25t}$ phenotype. AOX was expressed using the drug-inducible GeneSwitch-system (Barolo et al. 2000), a modified version of the UAS-GAL4 system (Brand & Perrimon, 1993), combined with a ubiquitous $\alpha$-tubulin promoter, and a concentration of the inducing drug RU486 that was found to produce maximal expression during development and adulthood. For further details see (II).

When expressed during development in a tko$^{25t}$ background, AOX had no obvious effect on either eclosion time or bang sensitivity (data shown on II), compared to either non-induced (no drug) or driver or transgene-only controls. Likewise, when the induction was initiated upon eclosion, AOX did not mitigate bang sensitivity after reaching its maximum expression level in two-day-old adults (II). We also tested prolonged AOX expression during adulthood, but even after 30 days, we failed to detect any rescue of the phenotype. However, it is noteworthy that we did not detect any further functional deterioration in the aged mutant adults either, as determined by the recovery times in the bang sensitivity assay (II).
5.2.2 Ndi1 and its co-expression with AOX throughout development is lethal to \( tko^{25t} \)

After failing to rescue the \( tko^{25t} \) phenotype using AOX, we tested whether an equivalent bypass of complex I could do so. Complex I is quantitatively the most severely affected of the respiratory chain complexes in \( tko^{25t} \), at least during development (Toivonen et al., 2003). For the CI bypass, we used the yeast NADH dehydrogenase Ndi1 (Sanz et al. 2010). Ndi1 expression was induced in the \( tko^{25t} \) background throughout development using the ubiquitous daughterless-GAL4 (\( da\-\)GAL4) driver (detailed crossing scheme in II). When expression was induced during development at the standard experimental temperature of 25 °C, Ndi1 was almost fully lethal to both mutant male (\( tko^{25t}/Y \)) and female (\( tko^{25t}/tko^{25t} \)) flies (Table 1). Ndi1 expression also conferred partial lethality upon phenotypically wild-type heterozygous \( tko^{25t} \) (\( tko^{25t}/FM7 \)) females. The lethality was equally penetrant when Ndi1 was co-expressed with AOX at 18 °C during development (Table 2). We thus concluded that, unlike AOX-expression, which simply failed to rescue the \( tko^{25t} \) phenotype, the transgenic expression of Ndi1 is selectively lethal to mutant flies.

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**Table 1.** The effect of Ndi1 expression on \( tko^{25t} \). Total number of eclosed progeny of each genotype class, reflecting survival through development. FM7, CyO and Ser are essentially wild-type balancers for the X, 2\(^{nd}\) and 3\(^{rd}\) chromosomes, respectively.
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**Table 2.** The effect of Ndi1 and AOX co-expression on tko^{2St}. Total number of eclosed progeny of each genotype class, reflecting survival through development. FM7, CyO and Ser are essentially wild-type balancers for the X, 2^{nd} and 3^{rd} chromosomes, respectively.
5.2.3 ATP and ROS do not correlate with phenotype in Ndi1 expressing tko^{25t} flies

We have previously show that the tko^{25t} mutants exhibit elevated ROS production and decreased ATP levels (Chen et al., 2012). In order to understand how these physiological changes contribute to the phenotype in tko^{25t}, we repeated the measurements in the presence of the bypass enzymes, hoping that this might also elucidate why the two bypass enzymes failed to rescue tko^{25t}. This time the expression of both transgenes was induced independently in the mutant background using da-GAL4, and at a more permissive temperature of 18 °C, in order to obtain enough flies to quantify H$_2$O$_2$ production and the steady-state level of ATP in induced and non-induced tko^{25t} mutant flies, and phenotypically non-mutant controls.

![A] H$_2$O$_2$ production

![B] [ATP]

**Figure 7.** A) H$_2$O$_2$-production and B) steady state ATP-levels in AOX and Ndi1-expressing (daGAL4) and non-expressing, phenotypically wild-type (25t/FM7) and tko^{25t} mutant (25t/25t) females.
Consistent with previous findings, we again observed elevated mitochondrial ROS production and lower steady-state ATP levels in homozygous mutants compared to heterozygous controls (Fig. 7A). Ubiquitous expression of either AOX or Ndi1 had no significant effect on ATP, and only Ndi1 was able to slightly alleviate ROS production (Fig. 7B). Despite this modest positive effect, the overall effect of Ndi1 expression is deleterious to ttko25t, implying that ROS production is not a crucial determinant of the disease-like phenotype.

5.3 Phenotypic rescue of ttko25t can be achieved by modulating diet

5.3.1 ttko25t phenotype is sensitive to diet composition but not calorie content

Since rescuing the OXPHOS deficiency in ttko25t using the alternative respiratory chain enzymes had failed, we rationalized that the mutant phenotype could be due to a complex metabolic defect that was not simply the inability to produce sufficient ATP by OXPHOS, or a simple redox disturbance that AOX or Ndi1 should be able to correct. This was supported by the fact that neither ROS production nor ATP deficiency in adults appeared to correlate with the severity of the phenotype. We therefore decided to investigate how the mutant phenotype would respond to disturbances of metabolic homeostasis through dietary manipulation.

We first performed the bang sensitivity and developmental time assays on five different isocaloric diets (see Materials and Methods). These diets, denoted high sugar (HS), intermediate sugar (IS), high fat (HF), high protein (HP) and low sugar (LS), were generated by replacing some of the sugar components of the high sugar medium with equicaloric amounts of fats (olive oil), proteins (soy protein extract) or both. Wild-type flies were largely unaffected by the dietary modulation while mutant flies exhibited high correlation between the dietary sugar content and severity of the phenotype (Fig. 8A and 8B): both developmental delay and bang sensitivity were substantially rescued on the three diets with least sugar, HP, HF and LS. In order to corroborate this initial result, we performed the same experiment with five diets with identical composition, with the exception of dietary sugar...
(sucrose). Again, the developmental delay in tko\textsuperscript{25t}-mutants was aggravated by elevated sugar content in a dose-dependent manner while wild-type flies remained relatively unaffected by the diet composition (Fig. 8C). Finally, we verified that the apparent developmentally toxic effects of the high sugar diets were not specific to a certain type of source of sugar by alternating between glucose, sucrose and fructose as the primary sugar component in the diet. We observed little to no difference on developmental time between the alternative sugars (Fig. 8D).

Encouraged by these observations, we decided to explore the relationship between the tko\textsuperscript{25t} phenotype and dietary sugar content, and chose two diets upon which to carry out further experiments: a high-sugar diet (HS) and a zero-sugar diet (ZS), referred to as the 0% diet in the sugar concentration series. Both of these diets were

![Figure 8](image_url)

**Figure 8.** Dietary sugar retards growth in tko\textsuperscript{25t} irrespective of other dietary components. A) Mean eclosion day on isocaloric diets. B) Bang sensitivity of tko\textsuperscript{25t} on isocaloric diets. C) and D) Mean eclosion day on medium with different sugar concentrations and different sugar types. Genotypes as indicated. HS – High sugar, IS – intermediate sugar, HF – High fat, HP – High protein, LS – Low sugar, S – Sucrose, F – Fructose and G – Glucose.
based on the same dietary formulation, the only difference being the omission of the pure sugar components of the HS medium, with consequently decreased calorie content (Fig. 9A). We verified that neither wild-type nor mutant larvae exhibited compensatory feeding on the lower calorie diet by quantifying feeding behavior and food intake on both diets during a short period of time. Based on the feeding assay, all larvae fed less on the ZS diet while the tko25t larvae generally exhibited lower food consumption than wild-type (Fig. 9B). We also tested how the dietary sugar load, together with feeding behaviour and genetic background were reflected in the combined circulating trehalose and glucose levels in the larvae. Trehalose is the primary circulating form of sugar in Drosophila, but it is rapidly interconverted with glucose inside cells. Zero-sugar diet markedly decreased sugar levels, while tko25t larvae exhibited lower circulating sugar levels than wild-type larvae (Fig. 9C).

**Figure 9.** A) Dietary content of the (standard) high-sugar and zero-sugar diets. B) Feeding behaviour of larvae on HS and ZS diets. C) Circulating trehalose and glucose (combined) levels of larvae reared on HS and ZS diets.
5.3.2 Metabolic homeostasis is disturbed by mitochondrial dysfunction in *tko*\textsuperscript{25t}

In order to characterize how the mitochondrial dysfunction is reflected in global metabolism in the *tko*\textsuperscript{25t} mutants, we performed a small molecule CE/TOFMS analysis and compared the metabolic profiles of mutant and wild-type mid-third instar larvae reared on high- and zero-sugar diets. The metabolomics data was filtered for metabolites, which were not reliably detected in replicates of at least one sample type and analyzed for statistical significance between sample groups using two-tailed Student's t-test (Appendix I). Changes with a p-value < 0.01 were considered significant, although in the case of up- or down-regulated metabolic pathways, changes with a p-value of < 0.05 were considered due to the small sample size.

Of particular interest were metabolites, which exhibited either increased or decreased levels in *tko*\textsuperscript{25t} larvae reared on high-sugar in comparison with wild-type, but were present at significantly more wild-type like levels in mutants on zero-sugar diet. We considered this to be a possible indication of accumulation or depletion of an individual metabolite or products of a metabolic pathway being detrimental for growth. Quantitatively, the abundance of a much higher number of metabolites was affected by genotype than by diet. 24 metabolites exhibited decreased and 11 increased levels, in *tko*\textsuperscript{25t} larvae on both diets, while only 4 metabolites were up and 5 down in both genotypes on high-sugar versus zero-sugar diet.

The metabolites elevated on HS in wild-type and mutant larvae included alanine, ornithine, GABA, gluconate and 3-hydroxykynurenine while isocitrate, glucuronate, carnitine, citrulline and N-epsilon-Acetyllysine were partially depleted on the same diet.

Metabolites which were detected at significantly lower levels in *tko*\textsuperscript{25t} on both diets included *e.g.* multiple amino acids and their derivatives (see below), glycolytic and pentose phosphate pathway intermediates fructose-1,6-bisphosphate (Fig. 10E) and sedoheptulose-7-phosphate, putrescine and β-alanine. As expected, the metabolomics analysis also confirmed the steady-state ATP deficiency in *tko*\textsuperscript{25t} (Fig. 10B). Perhaps most intriguingly, the level of NADPH, which is required by the glutathione system and many biosynthetic processes, such as fatty acid biosynthesis, was down in *tko*\textsuperscript{25t} on both diets (Fig. 10G).
The concentrations of only three compounds: alanine, methionine sulfoxide and saccharopine, were significantly elevated in HS \( tko^{25t} \) larvae and concomitantly markedly reduced on ZS diet. Vice versa, the steady-state levels of seven compounds: glucuronate, adenylosuccinate, UDP-N-acetylglucosamine, glutamate, arginine, dopamine and perhaps most notably ATP and NADPH, were significantly down in \( tko^{25t} \) on HS diet but at least partially recovered on the ZS diet.

### 5.3.2.1 Amino acid homeostasis

Most amino acids were relatively unaffected by either diet or the \( tko^{25t} \) mutation reflecting metabolic plasticity of Drosophila (see Appendix I, Fig. 10D). Alanine and histidine levels were elevated on the high-sugar diet whereas those of glutamate and threonine were higher on the zero-sugar diet. Again, the \( tko^{25t} \) mutation had a substantially greater impact on amino acid homeostasis than diet. Glycine, alanine, serine and threonine were elevated while aspartate, asparagine, lysine, glutamate, histidine and tryptophan were partially depleted in mutant larvae on both diets. This amino acid depletion in \( tko^{25t} \) was also reflected in metabolites which are direct downstream products of amino acid metabolism. 3-hydroxykynurenine and histamine, for example, are products of tryptophan and histidine degradation, respectively, and both were partially depleted in \( tko^{25t} \). Furthermore, aspartate is a biosynthetic precursor for multiple compounds and other amino acids, such as \( \beta \)-alanine, adenylosuccinate, argininosuccinate and asparagine (through asparagine synthase), all of which were decreased in \( tko^{25t} \) larvae.

Due to the presumed absence (see Discussion) of a canonical urea cycle in insects, the pathways resulting in nitrogen removal are likely to be very different between flies and mammals. Nevertheless, metabolites putatively involved in the removal of nitrogenous waste products exhibit distinct imbalance between the two diets and genotypes. The mammalian urea cycle intermediates ornithine and citrulline (Fig. 10D), in particular exhibit very different patterns of abundance. Ornithine levels were generally higher on the high-sugar diet whereas citrulline exhibited the opposite trend. Citrulline levels were in turn systematically higher in mutant larvae while the lowest ornithine level was detected in \( tko^{25t} \) on ZS and highest on HS. The accumulation of ornithine in the HS \( tko^{25t} \) larvae may be indicative of increased arginase activity, since the concentration of arginine is concomitantly reduced on
HS, and rescued to a wild-type like level on ZS in \( tko^{25t} \). The two common nitrogenous waste products in insects, urate and urea, were substantially and systematically increased and decreased, respectively, in \( tko^{25t} \) larvae on both diets.

### 5.3.2.2 Lipid metabolism

The methodology chosen for the global metabolomics analysis unfortunately does not detect the vast majority of the different lipid species. However, we did observe a two-fold increase in the concentration of phosphorylcholine in \( tko^{25t} \) on both diets. Moreover, \( \alpha \)-acetylcarnitine, a derivative of L-carnitine and CoA, were approximately two-fold increased in \( tko^{25t} \) larvae. Finally, three saturated medium chain fatty acids, caprylic acid (octanoate), capric acid (decanoate) and lauric acid (dodecanoate), were detected at approximately equal levels in wild-type larvae, and in \( tko^{25t} \) on the ZS diet, but were either below detection level, or nearly depleted in mutant larvae on the HS diet. This suggests that the catabolism of MCFAs is particularly high in \( tko^{25t} \) larvae on the high-sugar diet. Yet, despite the apparent increase in fatty acid catabolism, we measured very high levels of stored triglycerides in \( tko^{25t} \) larvae on both diets (Fig. 10C).

### 5.3.2.3 Redox balance indicators

We have previously shown that lactate dehydrogenase (LDH) is upregulated in \( tko^{25t} \) adults, implying that the mutant flies are converting pyruvate to lactate in order to regenerate NAD\(^+\) via pathway alternative to the respiratory chain (Fernández-Ayala et al., 2010). The NADH/NAD\(^+\) ratio is crucial for ATP production and the equilibrium is typically shifted towards the overreduced state when the OXPHOS is inhibited. Surprisingly, this did not appear to be the case in \( tko^{25t} \) on either diet, despite the severe multi-complex OXPHOS deficiency (Appendix I, Fig 10G). We did observe highly elevated levels of pyruvate and lactate, though, consistent with the idea of inhibited mitochondrial substrate oxidation. This elevation was also confirmed by enzymatic assays (Fig. 10A). We also found the concentrations of both metabolites to be slightly but significantly lower on low-sugar diet, in both wild-type and mutant larvae. Serum lactate was likewise several fold higher in \( tko^{25t} \) (Fig. 10A), but no difference was observed
between HS and ZS, indicating that lactate clearance is likely to be operating at maximum capacity in mutant larvae under both conditions. Although the accumulation of pyruvate and lactate could merely signal increased glycolytic flux and thus reflect adaptation to both OXPHOS deficiency, we found both compounds to phenocopy the developmental retardation of \( tko^{25t} \) in wild-type larvae, when administered through diet (III). The pyruvate and lactate supplementation of zero sugar diet also aggravated the phenotype in \( tko^{25t} \) larvae and thus partially phenocopied the high sugar-effect (III).

Nicotinamide adenine dinucleotides (NADH/NAD\(^+\)) and nicotinamide adenine dinucleotide phosphates (NADPH/NADP\(^+\)) both participate in similar redox-dependent processes but in different cellular functions. The NADPH/NADP\(^+\) ratio is maintained primarily through regeneration of NADPH by three subsets of enzymes: two enzymes of the pentose phosphate pathways, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), and mitochondrial and cytosolic isoforms of the isocitrate dehydrogenase (IDH) and malic enzyme (ME). NADPH availability is vital for many biosynthetic processes, but also a crucial determinant of cellular antioxidant capacity. NADPH is required by glutathione reductase, which regenerates reduced glutathione (GSH) from the oxidized form (GSSG). In the parallel, analogous ROS detoxifying system, NADPH is used by the thioredoxin reductase (TrxR) to reduce oxidized thioredoxin molecules (Lu & Holmgren, 2014). Finally, NADPH can maintain catalase activity in the presence of high \( \text{H}_2\text{O}_2 \) by preventing its substrate-induced inactivation (Kirkman et al., 1999). On the high-sugar diet, \( tko^{25t} \) larvae exhibited a dramatic NADPH depletion in comparison to wild-type. On zero-sugar compared with high-sugar diet there was a notable increase in NADPH levels in both genotypes (Fig. 10G), sufficient to restore the NADPH/NADP ratio above 1. In order to investigate how the NADPH/NADP ratio is modulated in \( tko^{25t} \), and on the two diets, we assayed the maximal activity of the primary NADPH-generating enzymes in larval homogenates (Fig. 11A). The activities of both pentose phosphate pathway enzymes glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) were down on ZS diet, but also generally slightly lower in \( tko^{25t} \) than in wild-type, implying that the maximal pentose phosphate pathway throughput is modulated by diet. Likewise, the total activity of the malic enzyme was slightly higher on the HS than on ZS diet, but significantly higher in mutant than wild-type larvae on both diets. Last, we tested the combined maximal capacity
of the various IDH isoforms. In this case, we observed only a modestly increased activity in \textit{tko}^{25t} larvae, irrespective of the diet.

Since these measurements only reflect the \textit{in vitro} activity of these enzymes in larval homogenates in the presence of a large excess of substrate, we also measured the enzyme activities in the presence of potential enzyme inhibitors, namely pyruvate, lactate and gluconate, all of which were elevated in \textit{tko}^{25t}, particularly on high sugar. Gluconate, which is the product of glucose dehydrogenase-catalyzed glucose oxidation, was in fact only detected in significant quantities in wild-type and mutant larvae reared on the HS diet (Fig. 10F). We used the quantitative metabolomics data to determine approximately physiological substrate/inhibitor ratios. In the repeat experiments, we found all three compounds to be potent inhibitors of IDH, either each alone, or all three in combination (Fig. 11B). The inhibitory effect appeared not to be additive, \textit{i.e.}, the inhibitory effect was no greater when all three compounds were applied simultaneously (in equimolar concentrations) than individually. All three inhibitors had only marginal effects on the activities of G6PDH and 6PGDH, while malic enzyme was substantially inhibited by lactate and its enzymatic product, pyruvate, but not by gluconate (Fig. 11B).
Figure 10. Selected metabolic changes exhibited by tko^{25}. A) Pyruvate, lactate and serum lactate concentrations relative to wild-type on HS. B) Steady-state ATP levels. C) Total body triglycerides relative to wild-type on HS. D) Amino acids exhibiting statistically significant changes between to diets or genotypes (relative to wild-type on HS). E) Fructose-1,6-bisphosphate levels. F) Gluconate levels. G) NAD^{+}/NADH and NADP^{+}/NADPH levels.
Figure 11. Maximal activities of the primary NADPH-generating enzymes in whole body larval homogenates. A) Maximal enzyme activities of malic enzyme, glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and isocitrate dehydrogenase (IDH). B) The effect of enzyme inhibitors in scrambled extracts. Enzyme activities were determined as the rate of NADP⁺-reduction.
5.3.3 Transcriptional analysis of tko\textsuperscript{25t} mutant larvae

Transcriptome-wide analysis of tko\textsuperscript{25t} adults identified systematic changes in the expression of genes involved with core nutrient metabolism, namely up-regulation of gut-specific proteases and lipases, with the concomitant down-regulation sugar transporters and glucosidases (Fernández-Ayala et al., 2010). We conducted a similar, global transcriptomic analysis, using RNA sequencing, on feeding mid-to-late wild-type and tko\textsuperscript{25t} 3\textsuperscript{rd} instar larvae, reared on either HS or ZS diet. We mapped the sequencing reads to the well-annotated Drosophila transcriptome, and performed differential gene expression analysis comparing wild-type and tko\textsuperscript{25t} larvae on each diet, and each genotype on the two different diets. In addition, we selected all genes exhibiting at least two-fold up- or down-regulation in a given pairwise comparison and performed a functional annotation enrichment analysis using the DAVID (Database for Annotation, Visualization and Integrated Discovery, http://david.abcc.ncifcrf.gov) gene ontology database tools. These results are summarized and presented in Appendix II as KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) enlisted pathways and biological process gene ontologies exhibiting enrichment of pathway components among the up- and down-regulated gene sets. Hundreds of genes showed substantial differential regulation to either direction in each pairwise comparison. Through this analysis, we were able to identify systematic changes with regards to adaptation to the different dietary conditions or to mitochondrial dysfunction during larval growth. Only substantial and coordinate changes in expression patterns are discussed below, while a more detailed analysis of differentially expressed genes can be found in the Discussion, and a summary, using a slightly different approach, in (III).

5.3.3.1 Metabolic reprogramming

One of the most noticeable patterns of differential transcriptional regulation involved core nutrient and energy metabolic processes: glycolysis/pentose phosphate pathway, pyruvate metabolism, the TCA cycle and oxidative phosphorylation. These processes/pathways were systematically down-regulated on the zero-sugar diet in wild-type flies, presumably as a part of a natural adaptation to the nutritional environment. Markedly, the same processes were also down-regulated in tko\textsuperscript{25t} mutant larvae on both diets, in comparison with wild-type larvae.
reared on the high-sugar diet. This suggests that the metabolic changes in mutant larvae mimic those observed in adaptation to low-sugar diet. It is also noteworthy, that the transcriptional regulation of the TCA cycle and oxidative phosphorylation appears to be tightly coupled with that of glycolysis and the pentose phosphate pathway in larvae. Curiously, testis-specific isoforms of enzymes falling into the aforementioned pathways were up-regulated in *tko*\(^{25}\) mutants. Carbohydrate metabolism was clearly inhibited on zero-sugar diet in wild-type larvae, and on both diets in *tko*\(^{25}\) while gut- and adipose tissue-specific triacylglyceride lipases and phospholipases were consistently and significantly induced under the same conditions. This indicates that the metabolic re-programming is not limited to down-regulation of carbohydrate catabolism, but involves a shift in fuel preference from sugars to lipids.

### 5.3.3.2 Protein homeostasis and proteotoxic stress responses

Protein synthesis is one of the most ATP-dependent process in cells, accounting for an estimated 30% of total cellular energy consumption (Buttgereit & Brand, 1995). Hence, it is unsurprising that we found numerous genes encoding components of the cytosolic translation machinery to be down-regulated in *tko*\(^{25}\), particularly on the HS diet, while in wild-type larvae, the high dietary sugar appears to systematically induce the expression of cytoribosome components (III). A similar trend was observed with a large number of genes encoding structural components of muscle, indicating that muscle biosynthesis is closely linked to cytosolic protein synthetic capacity.

We did not observe any global, differential regulation of genes involved in either ER or mitochondrial unfolded protein responses although we had previously identified a mitochondrial stress responsive small heat-shock protein Hsp22, to be significantly up-regulated in *tko*\(^{25}\) (Fernández-Ayala et al., 2010). The transcriptome-wide analysis of larvae did not identify *Hsp22* as being differentially regulated which might have resulted from an *in silico* mapping error in the RNA-sequencing data analysis, since the *Hsp22* coding sequences overlap with the open reading frame of another heat shock protein gene (*Hsp67*). Analysis of *Hsp22* transcript levels using quantitative PCR confirmed it to be significantly up-regulated
in tko\textsuperscript{25t} during the larval stages as well (Fig. 12C). The induction of a stereotypical mitochondrial chaperone protein (Morrow et al., 2000) was an indication of mitochondrial proteotoxic stress resulting potentially from oxidative damage or aberrant translation of mitochondrial proteins, or an imbalance between cytosolically and mitochondrially translated polypeptides caused by inhibition of mitochondrial protein synthesis. In order to test the latter hypothesis, we performed a developmental time experiment using media containing low levels of cycloheximide (CHX), a well-known inhibitor of eukaryotic cytosolic translation elongation (Schneider-Poetsch et al., 2010). We hypothesized that sublethal concentrations of CHX might alleviate possible proteotoxic stress. Unsurprisingly, both concentrations of CHX tested significantly retarded development in wild-type flies, as well as in phenotypically wild-type tko\textsuperscript{25t} heterozygotes (Fig. 12B). In a striking contrast to the response in wild-type flies, the homozygous and hemizygous mutant flies were unresponsive to inhibition of cytosolic protein synthesis (Fig. 12B).

5.3.4 Growth signaling network responds to mitochondrial dysfunction during development

The core growth-regulatory signaling network is highly conserved from \textit{Drosophila} to mammals. Since the developmental abnormalities in tko\textsuperscript{25t} do not appear to impact the gross morphology of the fly, we hypothesized that the growth inhibition is a result of a coordinated signaling process. We thus chose a few key signaling modules as indicators of activation or inactivation of the three core growth regulatory signaling complexes, the AMP-activated kinase (AMPK), TORC1 and TORC2. We used Western blotting and pan- and phospho-specific antibodies to probe for the selected targets in whole 3\textsuperscript{rd} instar larval homogenates. Representative blots are presented in (Fig. 12A).

We observed, a modest increase in the phosphorylation of Akt at ser\textsuperscript{505}, an indicator of TORC2 activity and insulin receptor stimulus (Hietakangas & Cohen, 2009), in larvae reared on high-sugar medium. Yet, this putative increase in Akt/TORC2 signaling was not associated with increased growth rate in either genotype. The phosphorylation of thr\textsuperscript{398} of the translational regulator S6K is a widely accepted readout for TORC1 complex activity (Magnuson, Ekim, & Fingar, 2012). This
TORC1-dependent S6K-phosphorylation was more intense in wild-type larvae on both diets, indicating lower TORC1 activity in tko^{25t}. Strikingly, also the total S6K protein level was significantly depleted in tko^{25t} larvae on HS diet. One of the known inhibitors of the TOR-complex, the AMP-kinase is activated in response to elevated AMP(ADP)/ATP ratio. AMPK activation was analyzed by detecting phosphorylation of thr^{172}, a site which is phosphorylated to activate the kinase in an AMP(ADP) responsive manner (Pan & Hardie, 2002). The AMPK-phosphorylation was substantially increased in tko^{25t} larvae, especially when grown on high-sugar medium, consistent with the severe ATP deficiency.

**Figure 12.** A) Representive Western blot illustrating changes in the abundance and phosphorylation status of key signalling molecules. B) Mean eclosion day on cycloheximide (CHX) supplemented diets. C) Relative transcript levels of Hsp22 in wild-type (OR, oregon-R) and tko^{25t} (25t) larvae.
6. Discussion

The primary aims of this study were to increase our understanding of the molecular and metabolic basis of mitochondrial disease pathogenesis resulting from mild mitochondrial dysfunction. We approached this by first trying to identify genetic factors modifying the disease phenotype. Next, we attempted to understand how the primary molecular defect manifests at the organelle level. Finally, we investigated how multi-cellular organisms can adapt metabolically to often highly deleterious pathological states, and how nutrition modulates these adaptive responses. The *Drosophila tko*\(^{25t}\) mutant offered an appropriate tool for these purposes. The molecular defect in this disease model had already been well-characterized (Toivonen et al., 2001; Toivonen et al., 2003). Furthermore, the primary phenotypic features of the mutant flies, developmental delay and bang sensitivity, are quantifiable and correspond to common and relevant clinical manifestations of human mitochondrial disorders. The *tko*\(^{25t}\) phenotype is also mild enough to permit genetic and environmental manipulations, which are detrimental to the mutant flies, yet allow for viable flies to develop for analyzing the outcome of such treatments. Although morphologically very different from humans, fruit flies exhibit a high degree of conservation in the composition, structure and function of mitochondria and mitochondrial processes as well as major tissues, organ systems and many developmental processes. At the organismal level, core metabolic pathways and regulatory processes are likewise highly conserved between *Drosophila* and mammals, allowing for clinically relevant research to be conducted using fly mitochondrial disease models, so long as all results and findings are critically assessed.

6.1 Controlled over-expression of the mutant allele completely rescues the *tko*\(^{25t}\) phenotype (I)

We had previously observed that inbreeding of homozygous *tko*\(^{25}\) females and hemizygous mutant males results in a gradual improvement of the disease
phenotype (Jacobs et al., 2004). This raised two possible explanations: either the expression of the hypomorphic allele was increased, which would compensate for the defective gene product, or the inbreeding and the concomitant selection for fast developing and fertile individuals had resulted in the emergence of phenotype suppressing alleles of one or more other genes. Since the ectopically expressed copies of the mutant allele had previously failed to rescue the phenotype, it appeared likely that the phenotypic recovery is achieved through the action of genetic suppressors. Thus, my aim was to identify such genetic factors, and for this purpose, we imposed inbreeding and a selection regime, starting with a highly heterogenous genetic background, in order to obtain phenotypically rescued tko\textsuperscript{25t} strains. We managed to obtain several such strains, three of which exhibited full recovery of bang sensitivity and developmental delay (Fig. 3). In each of the three cases, the rescue resulted from a segmental duplication of an X-chromosomal region containing the tko\textsuperscript{25t} allele (Fig. 4). The Weeble duplications also carried a second mitoribosomal gene, mRpL14. As in the case of the transgenic tko\textsuperscript{25t} alleles, autosomal ectopic expression of extra copies of mRpL14 under the regulation of its own promoter, did not confer notable phenotypic rescue. Finally, by producing different combinations of the Weeble alleles, the tko null allele (tko\textsuperscript{3}) and the endogenous tko\textsuperscript{25t}, we were able to demonstrate that over-expressing the tko\textsuperscript{25t} allele alone in its natural chromosomal milieu is sufficient for the rescue (Fig. 5). We further verified this by mapping the suppressor allele to the tko locus by combining chromosomes carrying deletions flanking the tko allele with the Weeble suppressor chromosomes (see I for details and results).

Although the genetic evidence strongly implies that the elevated expression of the tko\textsuperscript{25t} allele alone is sufficient for the Weeble suppressor effect, we cannot completely exclude the possibility that another gene within the minimal common duplicated interval could at least contribute to the rescue. Yet, we found no reasons to believe this is the case, based on diligent review of the literature with regards to the other duplicated genes (I).
6.1.1 Molecular mechanisms of \( tko^{25t} \) rescue in the \textit{Weeble} suppressors

Transgenic expression of the wild-type \( tko \) allele can complement \( tko^{25t} \) whereas expressing the mutant allele, even at relatively high levels, does not result in significant phenotypic rescue. In this respect, the fact that the \( Wb \) alleles can fully compensate the \( tko^{25t} \) mutation despite lower total transcript levels, implies that distal transcriptional enhancers are required to coordinate the increase in expression to a specific developmental stage and/or tissue. Moreover, the substantial recovery of the 12S:16S rRNA ratio in the \textit{Weeble} lines does not bring the ratio close to that observed in wild-type, despite the nearly complete rescue of the phenotype. This further enforces the idea that stringent spatiotemporal regulation is important for \( tko \) expression, and potentially also for other mitochondrial/mitoribosomal genes. The incomplete rescue of the 12S:16S ratio might, alternatively, indicate that very sharp thresholds operate for tolerance of decreased mitoribosomal RNA levels, or that the ratio is not as reflective of the mitoribosome subunit levels as we’ve hypothesized. The \( tko^{25t} \) larvae for instance, exhibit a peculiar pattern of dysregulation of the mitochondrial transcripts: multiple mitochondrial mRNAs, including ATP6, ATP8, ND1 and ND5 are up-regulated while the transcript levels of most mtDNA-encoded genes, including the 12S rRNA, are decreased in \( tko^{25t} \) compared with wild-type larvae on both diets (III). This suggests that the steady state 12S rRNA level might reflect a more global transcriptional defect or response than just the abundance of the mitoribosomal SSU.

6.2 Alternative respiratory chain enzymes do not rescue \( tko^{25t} \) (II)

We hypothesized that the mutant phenotype is primarily a consequence of impaired OXPHOS resulting from a limited supply of mitochondrially encoded complex subunits. The obvious potential downstream consequences of the OXPHOS deficiency are shortage of ATP and disturbed redox homeostasis due to a build-up of reduced electron carriers. Under such conditions, the availability of ATP itself could become rate-limiting for the numerous growth-promoting anabolic processes, while the accumulation of NADH, in turn, could signal back to the TCA cycle by inhibiting many of the involved enzymes (Nelson & Cox, 2005). This attenuation of
mitochondrial catabolism is a necessary adaptation to a decreased flux of electrons through the ETC but also disruptive for cellular macromolecule biosynthesis, which the TCA cycle supplies with precursors.

We thus hypothesized that providing a by-pass for a segment of the respiratory chain using the alternative respiratory chain enzymes *Ciona intestinalis* AOX or the *Saccharomyces cerevisiae* Ndi1 would allow for mitochondrial metabolism to proceed, although less efficiently in terms of biological energy conversion, but nonetheless alleviate the disease-like phenotype in tko25t. Furthermore, correlations between changes in phenotype resulting from OXPHOS complex by-pass and those in key physiological parameters, such as ATP levels or ROS-production, could help to dissect the pathogenic processes underlying the mutant phenotype.

Despite the previously demonstrated potency of AOX to compensate for respiratory chain deficiency in flies (Fernandez-Ayala et al., 2009), its ubiquitous expression throughout development, or during adulthood had no obvious phenotypic effects (II) in tko25t flies. Although this did not exclude the possibility that OXPHOS deficiency underlies the organismal phenotype in tko25t, it strongly suggested that the growth limitation might occur at the level of complex I. We therefore performed analogous experiments using Ndi1. Ndi1 had been previously shown to be able to compensate even severe CI deficiency in *Drosophila* (Sanz et al. 2010; Cho et al. 2012), suggesting that the genetic complementation could robustly demonstrate whether CI was the sole underlying cause of the tko25t phenotype. Unlike AOX expression, which had no clear impact on tko25t, Ndi1 expression during development was semi-lethal and highly deleterious selectively to homozygous tko25t mutant flies (Table 1). We then finally tested the co-expression of AOX and Ndi1 at 18 °C (as opposed to 25 °C), but again, expressing Ndi1 in the tko25t background proved equally deleterious, with or without the co-expression of AOX (Table 2).

To investigate why AOX or Ndi1 expression failed to alleviate the tko25t mutant phenotype, we analyzed two key physiological parameters, ROS-production and steady-state ATP levels, in adult progeny. Our measurements confirmed previous findings of decreased ATP and elevated ROS in the mutant flies (Chen et al., 2012).

In accordance with the fact that AOX expression had no effect on the tko25t phenotype, it also had no significant impact on either physiological parameter. Several interpretations of this finding, taken in isolation, are plausible. First, the expression of the mutant phenotype in tko25t might involve only processes not
affected by AOX. Second, the finding is consistent with the possibility that the developmental defect is fully the result of ATP deficiency, resulting from lack of proton-pumping activity at complex III and/or IV since although AOX restores electron flow to oxygen, it does not facilitate proton pumping at the ETC complexes that it bypasses. The expression or co-expression of Ndi1 together with AOX, aggravated the phenotype, despite having only a negligible effect on both ROS production and ATP levels. This suggests that the severity of the mutant phenotype is determined rather by complex metabolic factors, than simply limited ATP supply, although a significant recovery in steady-state ATP-levels was associated with phenotypic rescue in the dietary experiments (III, Fig. 10A).

6.3 OXPHOS dysfunction and its impacts on physiology, development and the CNS in Drosophila (II,III)

The tko25t flies exhibit mild to severe developmental delay depending on the diet and genetic background. The controlled inhibition of growth rate appears to be the only obvious gross developmental abnormality resulting from the rather severe OXPHOS dysfunction in these mutants, with the exception of thin mutant bristles. Interestingly, a tko25t-like phenotype is exhibited by several Drosophila ATP-deficient mitochondrial mutants, including the mitochondrial adenine nucleotide translocase sesB1 and citrate synthase (knock-down, kdn) mutants (Fergestad et al., 2006), implying similar underlying pathological mechanisms. Both of the aforementioned mutants also exhibit the bang-sensitive behavior characteristic of tko25t. Despite a wealth of studies on Drosophila bang-sensitive mutants, the precise molecular mechanisms behind this phenotype remain unknown and are probably heterogeneous. Insufficient supply of ATP to support normal neuromuscular function has been proposed as one of the possible underlying pathogenic mechanisms in all three mutants since all of them suffer from a variable degree of ATP deficiency (Fergestad et al., 2006). In neurons, ATP is particularly crucial for the maintenance of ionic gradients over cell membranes (Beal et al. 1993; Attwell & Laughlin 2001). This is in agreement with the phenotype of several Drosophila ion-channel mutants, such as the Na+/K+ ATPase alpha-subunit (Atpα) and the Na(+) channel gain-of-function para mutants, which exhibit bang sensitivity as well
(Fergestad et al., 2006; Howlett et al., 2013). Nevertheless, the fact that we found no signs of age-dependent deterioration associated with the bang sensitivity (II), strongly implied that, in \textit{tko}^{25t}, this phenotype is developmentally determined, rather than resulting from an acute ATP deficiency during adulthood.

As we have demonstrated in \textit{Drosophila} and as has been documented in other organisms and context, OXPHOS deficiency can also result in metabolite build-up, most notably of that of pyruvate, lactate and TCA cycle intermediates, resulting from a block in the electron transport chain, which inhibits TCA cycle and the flux of pyruvate into mitochondria. The accumulating cytosolic pyruvate needs to be shunted into lactate in order to regenerate NAD\(^+\) and maintain high glycolytic flux. Fluctuations of pyruvate and lactate homeostasis are thus directly linked to the cellular NAD\(^+\)/NADH equilibrium. Surprisingly, despite the elevated pyruvate and lactate in \textit{tko}^{25t} larvae, we did not observe significant disturbance of this balance when comparing with wild-type larvae (Fig. 10). Yet, pyruvate and lactate themselves appear to be contribute to the metabolic crisis as both can partially phenocopy both \textit{tko}^{25t} and the effect of high-sugar diet on the mutant larvae (III).

Whilst the NAD\(^+\)/NADH homeostasis in \textit{tko}^{25t} remained superficially unaffected, another key redox metabolite, NADPH, was severely depleted in \textit{tko}^{25t} larvae on the high-sugar medium (Fig. 10). NADPH is required primarily by biosynthetic reactions, such as fatty acid synthesis and, importantly, for ROS detoxification, providing hints of the processes possibly handicapping development of \textit{tko}^{25t} mutant larvae. While the shortage of ATP, NADPH, other metabolites or macromolecules might underlie the developmental delay in \textit{tko}^{25t}, the growth inhibition must nevertheless be carefully regulated to provide a means of completing development in the presence of elevated levels of toxic compounds, such as pyruvate and lactate, allowing time for excretion or detoxification processes (e.g. lactate clearance). The metabolomics analysis also identified other metabolites, the levels of which were elevated in \textit{tko}^{25t} larvae on HS and normalized on the ZS diet. These metabolites included \textit{e.g.} ornithine, which has been shown to inhibit larval growth when administered through diet (Hinton, 1956), although we failed to reproduce these findings (III). Similar approach could naturally be employed to testing most of the metabolites, which showed depletion or accumulation in \textit{tko}^{25t} larvae, particularly on the HS diet, but this was unfortunately not in the scope of this study.
One of the peculiar features of mitochondrial dysfunction is the often highly tissue-specific manner in which symptoms manifest. In the case of mtDNA mutations, this phenomenon is often explained with a drift in heteroplasmy levels and tissue-specific threshold for mitochondrial mutations (DiMauro, 2013). The tendency of muscle and nervous system to be the predominantly affected tissues in mitochondrial disorders, likely stems from the fact that these tissues are highly dependent on mitochondrial ATP synthesis, as well as coordinated fluxes of Ca\(^{2+}\) ions, a process in which mitochondria are considered to be involved in (Ylikallio & Suomalainen, 2012). Muscle and nervous system dysfunction is also heavily implicated in \(tko^{25t}\) as exemplified by the bang sensitivity and the down-regulation of multiple structural genes of the muscle in \(tko^{25t}\) larvae on HS, suggestive of a decrease in muscle biosynthesis (III). Intriguingly, the temperature sensitive expression of a wild-type copy of the \(tko\)-allele specifically in the gut, suggested that mitochondrial function in this tissue could also be a crucial determinant of the viability of the entire organism (III).

### 6.3.1 Protein homeostasis

The role of proteotoxic stress is becoming increasingly well appreciated in the pathogenesis of many metabolic disorders (Hutt, Powers, & Balch, 2009). In the context of mitochondrial dysfunction, particularly when involving defective mitochondrial protein synthesis, toxic accumulation of unfolded or damaged proteins may occur as a result of aberrant translation. Decreased mitochondrial protein synthesis capacity could also create an imbalance between mitochondrially and cytosolically translated proteins.

We did not have direct evidence of the impaired mitochondrial translation resulting in truncated, mutated or misfolded proteins. Neither did the transcriptomics data suggest induction of large-scale mitochondrial or ER unfolded protein response. Nevertheless, the \(tko^{25t}\) larvae exhibited prominent induction of a mitochondrial chaperone protein Hsp22 (Fig. 12). This small heat-shock protein has been shown to be induced \(e.g\). by oxidative stress and during aging (Gruenewald et al., 2009; King & Tower, 1999) and we thus decided to test whether the mutant larvae would show differential sensitivity to the cytosolic protein synthesis inhibitor cycloheximide compared to wild-type. We hypothesized that if the defective mitochondrial translation resulted in a cytosolic-mitochondrial protein imbalance, inhibiting
cytosolic protein synthesis might relieve such stress conditions. To our surprise, the mutant larvae appeared to be unresponsive to inhibition of cytosolic protein synthesis, unlike wild-type controls, in which the CHX administration resulted in significant developmental delay (Fig. 12). One interpretation of this is that the tko\textsuperscript{25t} larvae already exhibit significant developmental delay, irrespective of the cause, which simply masks the effects of the CHX treatment. On the other hand, inhibition of translation initiation (see below) might be epistatic to that of elongation, and thus the effect of CHX to tko\textsuperscript{25t} could be minimal. Nevertheless, the phenomenon implies that cytosolic protein synthetic capacity is not growth limiting in larvae.

6.3.2 Growth signaling

OXPHOS dysfunction occurring in specific tissues and cell-types is very likely to have, not only systemic metabolic consequences, but also to affect growth regulation through systemic (endocrine) signaling. Since the developmental delay of the mutant larvae appears to reflect a coordinated inhibition of larval growth rather than a catastrophic failure in development processes in general, we attempted to identify markers of changes in the growth-regulatory signaling network in whole larval homogenates, accounting for the growth inhibition. Unsurprisingly, AMP-activated kinase activity was induced in tko\textsuperscript{25t} larvae on HS, as indicated by the increased phosphorylation at Thr172 (Fig. 12). AMPK activation accords with the significantly decreased ATP to AMP/ADP ratio resulting from ATP depletion. Besides its direct targets, such as the acetyl-CoA carboxylase, AMPK also interacts with other pathways, such as the TOR-signaling pathway (Hardie, 2014). We therefore tested specific targets of both of the TOR-signaling complexes. The TORC1 target, S6K exhibited decreased activation through phosphorylation in tko\textsuperscript{25t} larvae on both diets, consistent with the observed activation of AMPK and the known roles of S6K and TORC1 in promoting growth (Fig. 12). Quite remarkably, we also consistently observed a decrease in the total S6-kinase protein levels in HS-grown tko\textsuperscript{25t} larvae, suggestive of an additional, previously uncharacterized mechanism regulating its expression in mutant larvae, under the most stressful conditions. Finally, we analyzed the activation of TORC2-dependent regulation of the Drosophila Akt kinase. Based on TORC2-sensitive phosphorylation at Ser505, Akt activity was slightly higher in wild-type larvae, again consistent with the higher larval growth rate (Fig. 12). Akt phosphorylation at this particular serine is also sensitive to insulin-Pi3K-signaling, at least in Drosophila (Parisi et al., 2011),
implying that this growth/metabolic signaling pathway is modestly affected in tko^{25t} as well. Consistent with this finding, we found Thor (4E-BP), a transcriptional target of FoxO, to be up-regulated in tko^{25t} larvae (III). FoxO itself is inactivated by Akt signaling, by restricting its subcellular localization to the cytosol (Teleman et al., 2008).

Since the analysis was performed in whole body lysates, we do not know whether growth signaling is differentially affected in the various tissues of the developing larvae. However, it is tempting to hypothesize that mitochondrial dysfunction, specifically in cells in the brain responsible for the secretion of insulin-like peptides, or the neuroendocrine organ, prothoracic gland, could significantly inhibit growth systemically. For example, AMPK activation in the silkworm Bombyx mori prothoracic gland has been shown to inhibit the secretion of the molting hormone ecdysone (Gu et al., 2013). Furthermore, developmental delay resulting from nutrient deprivation can be fully compensated by activating TOR solely in the Drosophila prothoracic gland (Layalle, Arquier, & Léopold, 2008).

6.4 Excess dietary sugar is selectively developmentally toxic to tko^{25t} mutants (III)

Irrespective of the underlying molecular and physiological mechanisms, our findings indicate that, in the tko^{25t} model, OXPHOS dysfunction impairs the metabolic plasticity that wild-type flies exhibit with respect to being able to adapt to diets of variable composition. In the dietary experiments, diets with highly variable sugar, fat, protein and calorie content supported normal development of wild-type flies. tko^{25t} flies, in contrast, showed dose-dependent sensitivity to increasing concentrations of dietary sugar, while being insensitive to alterations in relative dietary fat and protein content (Fig. 8).

Very high concentrations of dietary sugar have been shown to inhibit larval growth in wild-type Drosophila larvae as well (Havula et al., 2013; Na et al., 2013). However, it should be noted that the concentrations of dietary sugar used in this study were well tolerated by wild-type flies, resulting in no apparent developmental abnormalities. In this respect, tko^{25t} and its aggravated sugar sensitivity provides a paradigm for understanding the underlying genetic and metabolic reasons for sugar
tolerance. OXPHOS deficiency may be just one of many physiological conditions which could subject organisms to such carbohydrate-induced growth retardation. However, the role of mitochondria in nutrient metabolism and growth is undeniably central.

In addition to providing insight into the pathogenic mechanisms of sugar toxicity, the observation that tko\textsuperscript{25t} mutants develop faster and exhibit less severe bang sensitivity on low-sugar diet implies that dietary modulation of the disease phenotype provides a means of investigating how the respiratory chain deficiency impacts growth rate. We thus focused on investigating the metabolic and physiological changes occurring with respect to genotype, \textit{i.e.} wild-type versus mutant larvae, but also their different responses to dietary sugar.

Through a mechanism still unclear to us, the ZS diet resulted in a dramatic recovery of steady-state ATP-levels in tko\textsuperscript{25t} larvae. We did not identify a single clear explanation for the dramatic rescue of the ATP deficiency, such as induction of mitochondrial biogenesis, from the pattern of transcriptional changes. On the contrary: mitochondrial transcripts were similarly up- and down-regulated in mutant larvae on both diets, while transcripts of respiratory chain complex subunits, TCA cycle and glycolytic enzymes were systematically down-regulated in tko\textsuperscript{25t} larvae (Appendix II). Thus, a complex network of metabolic changes is more likely to underlie the adaptive advantage of the zero-sugar diet, as discussed below.

Correlating with the rescue of the ATP deficiency of tko\textsuperscript{25t} larvae by low dietary sugar, the depleted NADPH-levels were significantly elevated on the ZS diet (Fig. 10). Despite the hypothetically reduced flux through the NADPH-regenerating pentose phosphate pathway, indicated by the decreased maximal activity of G6PDH and 6PGDH (Fig. 11), the lower concentrations of lactate and pyruvate on the ZS diet might eventually result in a higher total rate of NADPH-production because of decreased inhibition of malic enzyme (Fig. 11). Furthermore, the ability to protect cells from the toxic effects of excess sugar, such as spontaneous reactions with the lysine residues of proteins via the Maillard reaction (Grandhee & Monnier, 1991), is likely to be partially dependent on the availability of NADPH. We observed high levels of gluconate, the inert product of glucose dehydrogenase catalyzed glucose oxidation (Gld) (Cavener & MacIntyre, 1983), in larvae reared on the high-sugar

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diet (Fig. 10). The exact identity of the primary electron acceptor of glucose dehydrogenase is unknown, but its regeneration in the cytosol is likely to depend on the NADPH-consuming cytochrome P450 system (Ferri, Kojima, & Sode, 2011).

6.5 Metabolic reprogramming provides means of adapting to mitochondrial dysfunction (III)

Understanding the metabolic requirements of larval development is pivotal in interpreting the physiological rationale of the metabolic changes exhibited by the tko-25t larvae. During mid-embryogenesis, developing Drosophila initiate the larval metabolic program, which relies heavily on aerobic glycolysis to support the exponential increase in biomass throughout larval development (Tennessen et al., 2014). This shift in metabolism resembles the classic Warburg effect (Vander Heiden, Cantley, & Thompson, 2009) and is accordingly accompanied by the induction of expression of glycolytic enzymes, mediated in Drosophila by the estrogen-related receptor (ERR) (Diaz-Ruiz, Rigoulet, & Devin, 2011; Tennessen et al., 2011). Compared to early stage embryos, towards the end of embryogenesis, several metabolites, such as glycerol-3-phosphate (G3P), β-alanine, serine, urate and the TCA cycle intermediates citrate, isocitrate, α-ketoglutarate and succinate exhibit significant accumulation, while others, such as glutamate and aspartate, become depleted (III, Fig. 10). Many of these metabolic changes, e.g. the increase in G3P, urate and citrate, and the decrease in glutamate and aspartate levels, are identical to the metabolic changes observed in tko-25t larvae compared with wild-type, towards the end of larval development. This implies that the metabolic changes necessary to adapt to mitochondrial dysfunction are at least partially overlapping with those required to meet the metabolic requirements of the developing larva, or growth dependent on aerobic glycolysis, in general. Quite fittingly, Otto Warburg initially interpreted his observations of the increased glycolytic flux in cancer cells as a sign of mitochondrial respiratory impairment (Vander Heiden et al., 2009; Warburg, 1956).

The metabolic switch in mid-embryogenic larvae includes the coordinate up-regulation of glycolytic enzymes and respiratory chain components, suggesting that despite the canonical view of glycolytic metabolism being crucial for ATP production while TCA cycle is largely responsible for producing precursors for fatty
acid and amino acid biosynthesis, the respiratory chain is nevertheless pivotal for this process in highly proliferative cells. This also provides another possible explanation for why the respiratory chain deficiency in tko\textsuperscript{25t} might impair development: the mutant larvae may be starving for macromolecules rather than the energy to synthesize them. Under such conditions, low-sugar diet might be beneficial since this condition appears to naturally increase the ingestion of fatty acids (as discussed below), perhaps compensating for the inability to sufficiently produce them \textit{de novo} in tko\textsuperscript{25t}. In accordance with this hypothesis, we observed higher steady-state levels of ATP and NADPH (both of which are consumed by biosynthetic processes), in both genotypes on the ZS diet.

In addition to inhibition of carbohydrate metabolism, the transcriptional and metabolic profiling of mutant and wild-type larvae also provides evidence for a switch from glucose metabolism to the use of fatty acids in mutant larvae, particularly on the high-sugar diet. First, lipolytic enzymes were systematically induced in tko\textsuperscript{25t} larvae while gut-specific sugar transporters were concomitantly down-regulated (\textit{Appendix II}, III). Secondly, medium chain fatty acids (octanoate, decanoate and dodecanoate) were either detected at very low level, or completely depleted in HS reared tko\textsuperscript{25t} larvae, suggesting that either their catabolism is considerably elevated, or that their availability is limited (\textit{Appendix I}, III). Since the ZS diet also appears to induce the expression of lipolytic enzymes, the availability of FAs is likely to be higher on the low-sugar diet, supporting the hypothesis of increased fatty acid catabolism.

In apparent contradiction of this hypothesis, the tko\textsuperscript{25t} mutants also exhibit elevated triglyceride levels on both diets (\textit{Fig. 10}). This puzzling finding could be explained by multiple physiological reasons. First and foremost, a block in electron transport chain will inevitably result in increased acetyl-CoA and citrate concentrations, as many TCA cycle enzymes become inhibited by the elevated NADH. Both, the excess citrate and acetyl-CoA are transported into the cytosol, where they are converted to malonyl-CoA, the substrate for \textit{de novo} fatty acid synthesis, but also an inhibitor of CPTI, and subsequently mitochondrial FA catabolism (Foster, 2012). The CPTI inhibition promotes fatty acid esterification and thus prevents the futile oxidation of newly synthesized fatty acids. In agreement with the TCA cycle and ETC inhibition, tko\textsuperscript{25t} larvae from both diets, exhibit increased levels of citrate and the acetylated form of carnitine (o-acetylcarnitine), which can act as a acetyl-group sink under such pathological conditions (Lindeboom et al., 2014). All of the
metabolic transformations described above can promote lipogenesis by elevating the cytosolic acetyl-CoA. Furthermore, the significant rise in the glycerol-3-phosphate levels in the mutant larvae can also contribute to the increased lipogenesis, a process, which has been shown to protect *Drosophila* from the deleterious effects of excess dietary sugar (Musselman et al., 2013). G3P is generated through multiple pathways: via phosphorylation of glycerol (catalyzed by glycerol kinase) originating from TAG lipolysis, or via glyceroneogenesis from e.g. pyruvate and lactate. G3P also lies in a crucial intersection between different metabolic processes: in addition to the aforementioned reactions, G3P is an intermediate of the α-glycerophosphate cycle, an interconversion between the glycolytic intermediate dihydroxyacetone phosphate (DHAP) and G3P, catalyzed by the mitochondrial and cytosolic glycerol-3-phosphate dehydrogenases. These two dehydrogenases also transport electrons from the cytosolic NADH pool to the mitochondrial ubiquinone. In the case of OXPHOS deficiency, the cytosolic NADH/NAD$^+$ ratio is often high and the ubiquinone pool overreduced. Under these circumstances, G3P may accumulate since the conversion of G3P to DHAP, is coupled with the reduction of NAD$^+$ to NADH and would thus be unfavorable.

Finally, despite the fact that changes in levels of amino acids and the expression of proteolytic enzymes appear to be superficially inconsistent, the potential impact of alterations in protein and amino acid metabolism to the *tko*$_{25t}$ metabolic phenotype cannot be overlooked. We observed a very high increase in urate levels in *tko*$_{25t}$ larvae reared on both diets. Terrestrial insects such as *Drosophila*, are generally ureotelic organisms, i.e. excrete uric acid as the primary nitrogenous waste product (Singer, 2003). The increase in urate would thus imply increased amino acid deamination, and possibly energy production reliant on anaplerotic flux of carbon to the TCA cycle. In addition, urate is the end product of purine metabolism. Purine metabolism, like amino acid catabolism, can provide the TCA cycle with carbon skeletons and, furthermore, the conversion of purines to uric acid can maintain a presumably ancient and quite inefficient form of ATP production, which nonetheless, has been proposed to be of significance in e.g. skeletal muscle during exercise (Lowenstein & Goodman, 1978).

What then, is finally the outcome of all of the metabolic transformations for *tko*$_{25t}$ during development? Stochiometrically, a switch from sugars to fatty acid oxidation results theoretically in proportionally more electrons being transferred to ubiquinone through complex II and the electron transferring flavoprotein dehydrogenase, than
through complex I. This could hypothetically relieve the electron transfer activity of
CI, which has been proposed to be the major site of electron leak ROS production in
mitochondria (see Review of literature). Complex I also carries the most
mitochondrially encoded subunits of all the respiratory chain complexes, and is
perhaps the most severely affected respiratory chain complex in $tko^{25t}$ larvae
(Toivonen et al., 2001). Although we did not assay ROS-production directly in vitro
in larvae, but elevated methionine sulfoxide (Stadtman, Moskovitz, & Levine, 2003)
in $tko^{25t}$ larvae indicated increased ROS derived damage. Notably, this oxidative
protein damage marker is detected at relatively high levels only in $tko^{25t}$ on HS diet,
and rescued to wild-type levels by the ZS diet (Appendix I). It is also compelling
to draw parallels between the growth impairment caused by the OXPHOS
deficiency in $tko^{25t}$ larvae and the anti-tumorigenic effect of biguanides (such as
metformin and phenformin), which is presumably achieved by inhibiting complex I
(Evans et al., 2005; Shackelford et al., 2013). Unfortunately, as in the case of $tko^{25t}$,
the exact molecular mechanisms of the biguanide-induced growth inhibition are
unknown, but are likely not limited to disruption of bioenergetics (Andrzejewski et
al., 2014; Wheaton et al., 2014). Nevertheless, the fact that understanding how the
delicate balance between the biosynthetic and bioenergetic processes is achieved in
proliferating cells, is currently of utmost interest in the field of cancer research, will
also inevitably benefit mitochondrial research as it will provide clues on how we
could address the numerous metabolic perturbations caused by mitochondrial
dysfunction. Our study highlights the potentially critical role of sufficient NADPH
regeneration during growth and development, and the fact, that this process could be
compromised by OXPHOS deficiency. Maintaining a steady supply of ATP and a
stable NADH/NAD$^+$ ratio, while ensuring the production of biosynthetic precursor
molecules and limiting ROS-production are widely accepted as perhaps the most
central requirements for cell viability, growth and proliferation, but what tends to
get overlooked is the fact that NADPH is the currency at the expense of which many
of the relevant processes are operating, and that the shortage of NADPH could
constrain growth equally well as e.g. that of ATP.
7. CONCLUSIONS

We have previously shown that ectopically expressed copies of the hypomorphic tko\(^{25t}\) allele fail to rescue the mutant phenotype, but a partial phenotypic suppression can be achieved by successive inbreeding of the mutant flies (Toivonen et al., 2001; Toivonen et al., 2003). In the present work, we successfully reproduced the phenotypic rescue by inbreeding tko\(^{25t}\) flies, and generated three Weebles suppressor lines, all of which exhibited near full reversal of the mutant phenotype. All three Weebles lines carried a duplication of a segment of the X-chromosome, which in all cases contained the tko\(^{25t}\) allele itself. We further validated that the duplication of another mitoribosomal gene (mRpL14) within the amplified interval, did not contribute to the phenotypic rescue. It thus seems very likely that the increased copy number of the tko\(^{25t}\) allele alone is sufficient for the near full rescue of the phenotype, but that the natural chromosomal milieu is required to coordinate the elevated expression to a correct tissue or developmental stage.

We also analyzed the ability of two alternative, single subunit respiratory chain enzymes (AOX and Ndi1), to compensate the OXPHOS deficiency in tko\(^{25t}\). AOX expression did not improve the gross mutant phenotype while ubiquitous expression of Ndi1 in the mutant background proved highly deleterious. The fact that AOX failed to compensate the tko\(^{25t}\) phenotype, superficially implied that the residual activity of complexes III and IV was sufficient to support adequate respiratory chain activity in the mutant flies. This further suggests that complex I deficiency might largely underlie the phenotype in tko\(^{25t}\). Yet, rather than ameliorating the phenotype, Ndi1 expression, alone or in combination with AOX, caused lethality in tko\(^{25t}\) flies. This strongly implies that the residual proton-pumping activity of the endogenous complex I remained vital for tko\(^{25t}\), and Ndi1 as a non-proton translocating enzyme attenuated the NADH-driven generation of the proton gradient. On the other hand, we did not observe a drop in steady-state ATP levels or elevation in ROS production when Ndi1 was expressed in the mutant background, suggesting that rather than a mere OXPHOS deficiency, a more complex metabolic disturbance might underlie the tko\(^{25t}\) phenotype or that crucial
developmental stage or tissue specific phenomena are being ignored by performing all of the experiments in whole tissue homogenates.

In support of the metabolic crisis hypothesis, the metabolic and transcriptional characterization of tko\textsuperscript{25t} larvae revealed significant changes in core cellular metabolism. More precisely, mitochondrial dysfunction triggers a large-scale metabolic reprogramming in tko\textsuperscript{25t} larvae, suggestive of intolerance to dietary sugar. This was indicated by the systematic induction of genes involved in dietary lipid metabolism, namely gut-specific triglyceride lipases and phospholipases, and the concomitant down-regulation of sugar metabolism, implying a shift in fuel preference from sugars to fats and fatty acids. But despite the apparent induction of fat catabolism, the mutant larvae still accumulated more triglycerides than wild-type controls. This was potentially due to the impaired mitochondrial substrate oxidation resulting from diminished OXPHOS capacity. Indicative of this, the mutant larvae also exhibited elevated levels of citrate, acetyl-L-carnitine and glycerol-3-phosphate associated with transcriptional down-regulation of the components of the glycerophosphate shuttle, all of which can promote \textit{de novo} lipogenesis by providing it with precursors.

Consistent with the metabolic re-programming, the mutant larvae responded favourably to low dietary sugar content. Low-sugar diet nearly fully suppressed the developmental delay exhibited by tko\textsuperscript{25t} larvae, irrespective of the dietary fat and protein content, implying that dietary glucose and sucrose or their metabolism, or the products of their metabolism are developmentally toxic to mutant larvae. Although this study did not reveal the exact cause of this sugar toxicity, the careful analysis of mutant larvae reared on both “favourable and unfavourable” diet (ZS and HS, respectively) provided hints of possible mechanisms. For example, excess production of pyruvate (and concomitantly of that of lactate) by glycolytic breakdown of glucose and fructose might overburden both the maintenance of cellular redox balance (NAD+/NADH ratio) and lactate clearance, under conditions in which both are already operating at, or near maximum capacity due to the OXPHOS dysfunction and the high dependence on aerobic glycolysis during larval growth (Tennessen et al., 2014). Pyruvate or lactate, both of which accumulated in tko\textsuperscript{25t} larvae, showed toxicity when administered via diet to either wild-type or mutant larvae. Irrespective of the mechanism, low-sugar diet corrected several crucial physiological parameters in tko\textsuperscript{25t} larvae, such as pyruvate overload and severe ATP and NADPH deficiencies.
Altogether, our findings raised the intriguing question why is it necessary for the tko^{25t} flies to deploy a large scale metabolic response in order to adapt to the disturbances of mitochondrial homeostasis while a mere increase in mitochondrial translational capacity would appear sufficient to compensate for the underlying molecular defect. One possible explanation is that many of the processes required for the maintenance of mitochondrial function are coordinately regulated, and correcting for distinct imbalances between different aspects of mitochondrial metabolic homeostasis might thus be difficult or impossible. Understanding the reasons underlying this phenomenon is of particular interest with regards to mitochondrial disease treatment since some of the potential novel therapeutic methods are based on inducing large-scale mitochondrial biogenesis (e.g. via PGC-1a activation) rather than targeting specific imbalances potentially contributing to the disease pathogenesis. Based on our findings with tko^{25t}, such approaches might thus not be universally applicable in mitochondrial disorders. Furthermore, different forms of dietary intervention carry significant potential as exemplified by tko^{25t} and other mitochondrial disease models, yet again, understanding the disease etiology remains crucial in order to avoid unexpected complications from drastic changes in diet. Dietary sugar for example, provides an “easily accessible” source of energy, which can be indispensable for some patients suffering from deficient supply of ATP to cells and tissues, but when certain mitochondrial functions are impaired, its metabolism may have toxic effects. This also underlines the need for more research on the interactions between the different dietary components and the various mitochondrial defects.
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always been equally welcoming for a young undergraduate and often times tired and grumpy graduate student (both referring to yours truly). I’m particularly thankful for Janne and Rimmy, the early team tko, for taking me under their wings as a first year undergraduate (and introducing me to the O’C’s pub quiz), and Jack, my associate in crime the last few years, for never being too busy for my endless rants in the lab.

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## Appendix I

### Metabolites decreased in tk025t on both diets

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<th>Average wt ZS</th>
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### Metabolites increased in tk025t on both diets

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### Metabolites increased on HS in both genotypes

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### Metabolites decreased on HS in both genotypes

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### Metabolites increased in tko25t on HS but “rescued” by ZS

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### Metabolites decreased in tko25t on HS but “rescued” by ZS

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Up-regulated on ZS vs. HS in Wd-Tk

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Down regulated on ZS vs. HS in Tko

KEGG term, Count, Biological process term, Count

00010: Glycolysis / Gluconeogenesis
00190: Oxidative phosphorylation
00020: Citrate cycle (TCA cycle)
00030: Pentose phosphate pathway
00250: Protein digestion and absorption
00260: Glucagon mediated secretion
00300: Neuronal inhibitory ligand-receptor interaction

KEGG term, Count

00040: Potassium ion transport
00050: Nucleotide excision repair
00060: Cell cycle control
00070: Chaperone-mediated protein transport
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**KEGG Term**

| 00:041020.7:Cellular amino metabolic process |
| 00:041021.7:Cellular aspartate metabolism process |
| 00:041022.7:Cellular fumarate metabolism process |
| 00:041023.7:Cellular malate metabolism process |
| 00:041024.7:Cellular succinate metabolism process |
| 00:041025.7:Cellular pyruvate metabolism process |
| 00:041026.7:Cellular acetate metabolism process |
| 00:041027.7:Cellular lactate metabolism process |
| 00:041028.7:Cellular sugar metabolism process |
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| 00:041031.7:Cellular mannose metabolism process |
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| 00:041033.7:Cellular xylose metabolism process |
| 00:041034.7:Cellular galacturonic acid metabolism process |
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| 00:041039.7:Cellular amino sugar metabolism process |
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| 00:041045.7:Cellular deoxyribonucleotides metabolism process |
| 00:041046.7:Cellular amino sugar and nucleotide metabolism process |
| 00:041047.7:Cellular nucleobase and nucleoside metabolism process |
| 00:041048.7:Cellular nucleotide and NA metabolism process |
| 00:041049.7:Cellular nucleotide and NA biosynthesis process |
| 00:041050.7:Cellular nucleotide and NA catabolic process |
| 00:041051.7:Cellular nucleotide and NA metabolic process |

**Biological Process Term**

| 00:044100.7:Cellular amino metabolic process |
| 00:044101.7:Cellular aspartate metabolism process |
| 00:044102.7:Cellular fumarate metabolism process |
| 00:044103.7:Cellular malate metabolism process |
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| 00:044128.7:Cellular nucleotide and NA metabolism process |
| 00:044129.7:Cellular nucleotide and NA biosynthesis process |
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**Up-regulated in KEGG**

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| 00:041021.7:Cellular aspartate metabolism process |
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| 00:041023.7:Cellular malate metabolism process |
| 00:041024.7:Cellular succinate metabolism process |
| 00:041025.7:Cellular pyruvate metabolism process |
| 00:041026.7:Cellular acetate metabolism process |
| 00:041027.7:Cellular lactate metabolism process |
| 00:041028.7:Cellular sugar metabolism process |
| 00:041029.7:Cellular glucose metabolism process |
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| 00:041031.7:Cellular mannose metabolism process |
| 00:041032.7:Cellular galactose metabolism process |
| 00:041033.7:Cellular xylose metabolism process |
| 00:041034.7:Cellular galacturonic acid metabolism process |
| 00:041035.7:Cellular rhamnose metabolism process |
| 00:041036.7:Cellular ribose metabolism process |
| 00:041037.7:Cellular deoxyribose metabolism process |
| 00:041038.7:Cellular sialic acid metabolism process |
| 00:041039.7:Cellular amino sugar metabolism process |
| 00:041040.7:Cellular nucleoside metabolism process |
| 00:041041.7:Cellular nucleotide metabolism process |
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Down regulated in kdo vs. or n25

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Downregulated in kdo vs. or n25
Original communications
Phenotypic suppression of the Drosophila mitochondrial disease-like mutant tko^{25t} by duplication of the mutant gene in its natural chromosomal context

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Abstract

A mutation in the Drosophila gene technical knockout (tko^{25t}), encoding mitoribosomal protein S12, phenocopies human mitochondrial disease. We isolated three spontaneous X-dominant suppressors of tko^{25t} (designated Weebles), exhibiting almost wild-type phenotype and containing overlapping segmental duplications including the mutant allele, plus a second mitoribosomal protein gene, mRpL14. Ectopic, expressed copies of tko^{25t} and mRpL14 conferred no phenotypic suppression. When placed over a null allele of tko, Weebles retained the mutant phenotype, even in the presence of additional transgenic copies of tko^{25t}. Increased mutant gene dosage can thus compensate the mutant phenotype, but only when located in its normal chromosomal context.

1. Introduction

Human mitochondrial diseases affecting the oxidative phosphorylation (OXPHOS) system can result from a large number of different mutations, both in the nuclear genome or in the maternally inherited mitochondrial DNA (mtDNA) (Schon, 2000; Smeitink, 2003). Many such pathological mutations impact the apparatus of intramitochondrial protein synthesis (Jacobs and Turnbull, 2005; Schepet et al., 2007), including mtDNA-encoded rRNAs and tRNAs, as well as nuclear DNA-encoded mitoribosomal proteins, translation elongation factors, RNA modification enzymes and aminoacyl-tRNA ligases. Although the tissues most obviously involved, are not well understood. In particular, the severity of disease can vary greatly between individuals bearing the identical pathological mutation. Although in part explicable by variable patterns of heteroplasmy in the case of mtDNA mutations, the causes of such phenotypic heterogeneity remain largely unknown.

We have been studying a mutant of Drosophila melanogaster (tko^{25t}) that constitutes a model for mitochondrial diseases, especially those affecting intramitochondrial protein synthesis. The mutant carries a mis-sense mutation (L85H) in the X-linked nuclear gene for mitoribosomal protein S12 (technical knockout, tko), resulting in a phenotype of developmental delay, bang-sensitivity (temporarily paralysis induced by mechanical shock), impaired responsiveness to sound and defective male courtship. Compared with wild-type strains, tko^{25t} exhibits decreased activities of the OXPHOS enzymes dependent on intramitochondrial translation products and a relative deficiency of mitoribosomal small subunit rRNA relative to large subunit rRNA (Toivonen et al., 2001). The gross organismal phenotype is shared with other mutants impaired in mitochondrial bioenergy metabolism, such as sesB, encoding the adenine nucleotide translocase (Zhang et al., 1999), or knockdown, encoding citrate synthase (Fergestad et al., 2006). Null alleles of tko, such as tko^{25t}, are homozygous lethal at the larval stage, confirming that tko is an essential gene; in fact, tko^{25t} is the only viable tko mutant allele yet isolated.

Flies transgenic for a tko^{25t} allele which has been reverted to leucine at amino-acid 85, still in the tko^{25t} background, display a fully wild-type phenotype (Toivonen et al., 2001), whereas flies with an unreverted transgenic copy of tko^{25t} retain a fully mutant phenotype (Toivonen et al., 2001, 2003), even when the ectopic tko^{25t} transgene is expressed in adult flies at levels comparable with, or even in excess of, that of the endogenous gene (Toivonen et al., 2003). In earlier studies we also noticed that, when maintained as an inbred stock over many generations, the phenotype of tko^{25t} flies became attenuated (Toivonen et al., 2001). To investigate this further, we backcrossed tko^{25t} into two different wild-type backgrounds (Canton S and Oregon R), then mated tko^{25t}...
Canton S females to tko25t Oregon R males, creating an F1 hybrid that exhibited a much more pronounced mutant phenotype (Jacobs et al., 2004), characterized by so-called semi-lethality (i.e. the failure of most progeny to complete development and eclose as adult flies). Inbred lines were then established from individual mating pairs from this cross and maintained under selection for accelerated development over at least 12 generations (see Section 2). Six such inbred lines were analysed phenotypically, and found to eclose at a time intermediate between that of the original F1 hybrid and wild-type flies (Jacobs et al., 2004). Two of the lines showed a particularly pronounced suppression of the mutant phenotype and were selected for more detailed study.

In the present work we present the phenotypic analysis of these lines, plus one additional suppressor line obtained from the reciprocal cross, and establish the molecular nature of the suppressor. Surprisingly, we found that all three suppressors contain local duplications of overlapping regions of the X-chromosome, including the (still mutant) tko25t allele, as well as one other mitoribosomal protein gene, mRpl14. Genetic analysis of the suppressors, in combination with deletion and point mutants of the tko allele, but that it must be in its natural chromosomal context on the X-chromosome. Increased gene dosage of mRpl14 appears not to be required for suppression. The findings provide insight into genetic factors which may underlie phenotypic differences in mitochondrial disease, and suggest possible ways to intervene therapeutically.

2. Materials and methods

2.1. Drosophila stocks and maintenance

Wild-type (Canton S and Oregon R), w1118, FM7a, CyO and TM3Sb balancer, tko1 mutant and deficiency stocks were obtained from stock centres. Bloomington deficiency stocks 733, 6892 and 935 have, respectively, the following genotypes: Df(1)w258-42/y.FM1;[w];Df(1)JC19/FM7c and Df(1)TEM7, tko1 mutant flies were originally supplied by Dr. C.-F. Wu. tko1 was maintained over the FM7a balancer. Transgenic lines A1 and A3, containing P(tko25t) transgenes, respectively on chromosomes 3 and 2, were described previously (Toivonen et al., 2003). Flies were maintained at 25 °C on a 12 h light/dark cycle in standard medium containing 1.5% (w/v) sucrose (Merck), 3% glucose (Sigma), 3.5% Instant Dry Baker’s Yeast (European), 1.5% maize flour (Oriola), 1% wheat germ (Oriola), 1% soya flour (Oriola), 1% agar (Oriola) and 3% Lyle’s black treacle (Tate & Lyle, UK), to which was added 0.1% methyl 4-hydroxybenzoate (Sigma) and 0.5% (v/v) propionic acid (JT Baker).

2.2. Selection of tko25t suppressor lines

tko25t was backcrossed over >12 generations into wild-type strains Canton S and Oregon R, which were then mated to create an F1 generation with a fully penetrant mutant phenotype. Inbred suppressor lines were initiated from single mating pairs, whose progeny were then maintained under selection for >12 generations by selecting the first eclosing six females and three males from the progeny in each generation to create the next.

2.3. Eclosion timing

Mean developmental time to eclosion at 25 °C was measured by setting up 10 parallel crosses of 10 females and 5 males for each experiment, which was itself repeated at least three times. In each cross, flies were allowed to copulate for one day, then transferred to fresh food vials at a fixed time, after which the first set of vials was discarded. The flies were transferred to a fresh set of vials twice more, allowing the flies to lay eggs for exactly 24 h each time. After having laid eggs for 24 h in the third and last set of vials, the mated flies were discarded. The number of eclosing offspring was counted every day at noon.

2.4. Bang-sensitivity

Bang-sensitivity was measured essentially as previously (Toivonen et al., 2001). Freshly eclosed flies were anaesthetized using CO2 and separated into empty food vials (one fly per vial). Sexed and separated flies were transferred to 25 °C for 2–3 h to recover from anesthesia. Individual flies were vortexed for 15 s, and time to recovery recorded (defined as the time taken for the flies to be standing upright with apparently normal locomotor activity).

2.5. RNA extraction

Total RNA was extracted from aliquots of 100 mg of flies (30 females or 40 males) anaesthetized on ice and immediately frozen at 80 °C. Frozen flies were ground using a plastic homogenizer in 200 µl of Trizol reagent (Invitrogen). An additional 800 µl of Trizol was added, and the extract was thoroughly mixed. The homogenate was incubated at room temperature for 5 min, after which 200 µl of chloroform was added and the mix incubated for an additional 3 min before centrifugation for 15 min at 12,000 gmax at 4 °C. The upper, aqueous phase was transferred to a fresh vial and RNA precipitated by adding 500 µl of isopropanol and incubating at room temperature for 10 min. The precipitate was pelleted by centrifugation for 10 min at 12,000 gmax at 4 °C, washed with 75% ethanol, air-dried, resuspended in 89 µl DEPC-treated water, to which was added 1 µl (10 units) DNase (Amersham Biosciences) and 10 µl of 10× DNase I buffer (60 mM MgCl2, 400 mM Tris–HCl, pH 7.5), and incubated for 1 h at 37 °C. The RNA was recovered by phenol/chloroform extraction and ethanol precipitation and finally resuspended in 50 µl of DEPC-treated water.

2.6. Quantitative RT-PCR

Complementary DNAs were synthesized from 2 µg aliquots of RNA denatured at 90 °C for 3 min in reaction volumes of 11 µl containing 1 µl of 10 mM dNTP mix (Finnzymes) and 1 µl of 0.0125 U/µl random hexamers (Amersham Biosciences). The reactions were transferred to ice and 4 µl of 5× reaction buffer (250 mM Tris–HCl, 250 mM KCl, 20 mM MgCl2, 50 mM DTT, pH 8.3) plus 0.5 µl of 40 U/µl RNase inhibitor (Fermentas) and 2.5 µl of DEPC-treated water were added. After incubation at 25 °C for 10 min, 2 µl of 20 U/µl MMuLV reverse transcriptase (Fermentas) were added and the reactions incubated further at 25 °C for 10 min then at 37 °C for 1 h. Reactions were stopped by heating at 70 °C for 10 min. The levels of mitochondrial 125 and 16S rRNA, as well as tko and mRpl14 mRNA, were measured relative to an internal standard (RpL32) using custom-designed (Tib MolBiol) hybridization probes on a Roche LightCycler 1.5 instrument (see Supplementary data for full details). All RNA extractions were performed in triplicate, and each of these extractions was treated as an individual experiment. Each total RNA extract was used as a template for three separate cDNA synthesis reactions, which were then pooled and analysed in triplicate, producing a total of 9 data points per fly line or developmental stage.
2.7. Genomic DNA extraction

Genomic DNA was extracted from aliquots of 100 mg of flies (30 females or 40 males) anesthetized on ice and ground for 1 min using a plastic homogenizer in 200 μl of lysis buffer (100 mM Tris–HCl, 100 mM EDTA, 100 mM NaCl, 0.5% SDS, pH 7.5). An additional 300 μl of lysis buffer was added and the mixture was homogenized for a further 1 min, or until only cuticles visibly remained. For DNA to be used in Southern blots, the homogenates were incubated at 37 °C for 1 h. Nucleic acids were isolated by gentle phenol–chloroform extraction, followed by centrifugation at 5000 gmax for 5 min at room temperature, and precipitation of 400 μl of the upper, aqueous phase by the addition of 200 μl 5 M potassium acetate (pH 5.6) and 400 μl of isopropanol. The vial was inverted with care to mix all solutions and DNA was recovered by centrifugation at 12,000 gmax for 15 min at room temperature, washed with 70% ethanol, air-dried and resuspended without pipetting in 150 μl of Tris–HCl (pH 7.5) for one day at 4 °C. For DNA to be used in PCR-based methods, homogenates were incubated at 65 °C for 30 min, after which 800 μl of collection buffer (1.4 M potassium acetate, 4.3 M LiCl) was added to each sample prior to incubation on ice for 30 min. Homogenates were centrifuged for 15 min at 12,000 gmax, and 1 ml of each resulting supernatant was transferred to a fresh microcentrifuge tube and mixed with 600 μl of isopropanol to precipitate nucleic acids. The centrifugation was repeated and the supernatants discarded. DNA pellets were washed with 70% ethanol, air-dried and resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.8. Gene dosage analysis by Southern blots

Twenty micrograms of DNA was digested with Ndel (manufacturer's buffer and recommended conditions; NEB) and fractionated on a 0.75% agarose gel. Depurination, capillary blotting to nylon membrane and UV-crosslinking were performed as described previously (Toivonen et al., 2001). Blots were prehybridized for 30 min at 65 °C in hybridization solution (250 mM sodium phosphate, 1 mM EDTA, 7% SDS, pH 7.2) and hybridized overnight in the same solution with PCR-derived probes as indicated in Supplementary data. After hybridization, the blot was washed at 65 °C twice for 15 min with 5× SSC plus 0.1% SDS, once for 15 min with 1× SSC plus 0.1% SDS, and finally for 15 min in 0.1× SSC plus 0.1% SDS. Finally, the blot was exposed to autoradiography films (Kodak BioMax MS). Images were digitized and analysed using Scion Densitometry Software (NIH, USA).

2.9. Gene dosage analysis by quantitative PCR

Two DNA extracts from both males and virgin females of a given genotype were pooled and used as a template, diluted to a final concentration of approximately 100 ng/μl. Amplification reactions were performed in 20 μl final volume consisting of 5 μl of template DNA, 10 μl of Quantitect SYBR Green PCR Master Mix (Qiagen) and 1 μl of 10 μM forward and reverse primers (see Supplementary data for primer sequences and full details).

2.10. Construction of transgenic lines

The mRpl14 gene region was amplified from genomic DNA using customized primers (see Supplementary data for primer information and full cloning details), producing a 1.8 kb product containing the gene itself, plus 730 bp upstream and 430 bp downstream of the transcription unit (see Supplementary Fig. 1). The product was cloned using Zero Blunt TOPO PCR Cloning Kit (Invitrogen) under manufacturer’s recommended conditions. After sequence verification, the mRpl14 insert was liberated by digestion with XbaI (Fermentas) and SpeI (Fermentas) and introduced into a modified version of the vector pGREEN-H-Pelican (Barolo et al., 2000), obtained from Drosophila Genomics Resource Center, Bloomington, IN, USA (see Supplementary Fig. 1 for details). The construct leaves transgenic mRpl14 under the control of its own promoter, but protected from readthrough transcription by flanking gypsy insulator elements. The final plasmid, following sequence and restriction-map verification, was purified by standard phenol/ chloroform extraction and ethanol precipitation and sent to Vandes Drosophila Injection Service (Oslo, Norway) for microinjection. Transgenic progeny were identified by the w+ eye colour marker in the w1118 background, then sib-crossed to establish stable homozygous transgenic lines, from which males were crossed to white-eyed double-balancer females (w1118/w1118; +/CyO; +/Tm3Sb), to identify insertions on chromosomes 2 or 3.

2.11. Insertion site mapping by inverse PCR

Genomic DNA (3.5 μg) from homozygous transgenic flies of each line was digested with 10 units of ScrFI (Fermentas) in manufacturer’s buffer for 3 h at 37 °C, in a total volume of 25 μl. The enzyme cuts in the foot segment of the Pelican vector, between the P-element 3’ end and the upstream insulator, and at a high frequency in genomic DNA. After heat inactivation of the restriction enzyme, the digested DNA was circularized by self-ligation overnight at 4 °C in a total volume of 400 μl, using four Weiss units of T4 DNA ligase (Fermentas) in the manufacturer’s buffer. Ligated DNA was ethanol precipitated and resuspended in 100 μl of 10 mM Tris–HCl (pH 8.0). Ten microliters of this was used for PCR amplification using the foot-segment specific primer set (see Supplementary data), and products were sequenced using the same primers to identify transgene insertion sites by comparison with the genomic DNA reference sequence.

2.12. Analysis of gene expression by Affymetrix microarrays

RNA extraction and purification, and cRNA probe synthesis was performed using Affymetrix® protocols and kits provided by Qiagen, from three independent groups of 40 males of wild-type, tko25t, Weeb1 and Weeb2 strains. Each of the three replicates of cRNA was hybridized to independent GeneChip® Drosophila Genome 2.0 Arrays (Affymetrix). Data extraction, cell intensity calculation and computational analysis were performed using GeneChip® Operating Software and Expression Console Release Software from Affymetrix. For each pair-wise comparative analysis, the signal value (MASS and RNA algorithms, at linear scale) of each probe was compared as follows: wild-type to tko25t, Weeb1 and Weeb2, and tko25t to Weeb1 and Weeb2. Statistics were performed with Microsoft® Office Excel 2003. Firstly, the data were prefiltered according to their detection p-value, selecting those with p-value <0.05. Secondly, SAM (Significance Analysis of Microarrays, Stanford Tools) was performed as described (http://www-stat.stanford.edu/~tibs/SAM/sam.pdf) using s0 = 20 and minimum fold change (R) = 1.5 as fixed parameters, and Δ-value so that False Discovery Rate (FDR) <2.5%. Finally, probes showing significantly altered expression in both Weeb1–wild-type and Weeb2–tko25t comparisons were selected for further analysis. Probes showing significantly altered expression in both Weeb2–wild type and Weeb2–tko25t comparisons were similarly selected.

3. Results

3.1. Isolation of tko25t suppressors under selection

As described in Section 2, inbred lines of Drosophila carrying the tko25t mutation were established by crossing tko25t homozygous fe-
males and \(tko^{25t}\) hemizygous males outbred into different wild-type backgrounds (Canton S and Oregon R, respectively), then establishing lines from individual mating pairs. These were then placed under selection for accelerated development over >12 generations, developmental delay being one of the cardinal phenotypes of \(tko^{25t}\). This procedure generated sets of inbred lines which showed stable and characteristically different degrees of phenotypic rescue (Jacobs et al., 2004). Two lines, denoted \(\text{Weeb}^1\) (\(\text{Weeb}^1\)) and \(\text{Weeb}^2\) (\(\text{Weeb}^2\)) showed an especially pronounced phenotypic rescue (Fig. 1) and were selected for further study. We initially confirmed by sequencing that both retained the original \(tko^{25t}\) mutation, with no wild-type copy of the \(tko\) gene (Supplementary Fig. 2A), and no other mutations within the \(tko\) transcription unit or its immediate flanking sequences. Both lines exhibited an almost wild-type developmental timing (Fig. 1A) and were no longer bang-sensitive (Fig. 1B). Note that flies of both sexes were studied in all experiments, since \(tko\) is located on the X-chromosome, and differential expression in males and females would be one potential complication of any suppressor effect. In crosses to \(tko^{25t}\), \(\text{Weeb}^1\) behaved as a (semi-) dominant X-linked suppressor (Supplementary Fig. 2B), regardless of the parent of origin (Supplementary Fig. 2C). In both of the \(\text{Weeb}\) lines the ratio of 12S:16S mitochondrial rRNAs, which represents a marker for the relative amount of small and large mitoribosomal subunits, and is diagnostic for the mutant phenotype, was found to be intermediate (Fig. 1C) between that seen in outbred \(tko^{25t}\) flies (between 0.3 and 0.4) and in wild-type flies (approximately 1.0). The improvement was mainly due to a recovery in 12S rRNA levels (Supplementary Fig. 2D). A third line, designated \(\text{Weeb}^3\), showing a similar degree of phenotypic recovery (Fig. 1), was obtained from a similar selection procedure using the reciprocal cross (Oregon R \(tko^{25t}\) females, Canton S \(tko^{25t}\) males).

3.2. \(\text{Weeb}\) suppressors contain overlapping X-chromosomal duplications

To map \(\text{Weeb}^1\) more precisely, having established that it was X-linked, we crossed \(\text{Weeb}^1\) males to \(tko^{25t}\) females homozygous also for the \(w^-\) (\(\text{white}^-\)) mutation, located on the X-chromosome close to \(tko\), and conferring white eye colour. This approach allowed us to determine the location of \(\text{Weeb}^1\) with respect to \(\text{white}\), using a standard recombination mapping approach. Female progeny from the cross were crossed to wild-type males. The resulting male progeny carry either parental or recombinant X-chromosomes, which can be distinguished on the basis of the combined eye colour and eclosion day phenotypes. Our reasoning was that, if \(w^-\)
and Weeb are closely linked, then most red-eyed males should eclose before their white-eyed male sibs. From the results (Fig. 2A), it is clear that Weeb1 does indeed map close to w. A conservative estimate, considering all red-eyed progeny eclosing after day 13 as recombinants, would place Weeb1 within six map-units of w, an interval including tko itself.

A transcriptomic analysis revealed independently that Weeb1 and Weeb2 adult males each showed approximately 2-fold elevated expression of most members of a contiguous set of genes in overlapping segments of the X-chromosome, including tko (Fig. 2B), suggesting gene duplication as the mechanism of suppression. An increased copy number of the mutant tko gene in both Weeb1 and Weeb2 males was confirmed by Southern blotting (Fig. 2C). The gene CG8636, some 167 kb to the right of tko, and located amongst a group of genes showing 2-fold elevated expression in Weeb2 but not in Weeb1, was found by Southern blotting to have elevated copy number in Weeb2 but not Weeb1. A gene outside of the region which had shown elevated expression, CG12498, was at normal copy number in both Weeb1 and Weeb2. To investigate the issue further, Q-PCR was carried out on genomic DNA of males from the Weeble lines, using primer pairs specific for tko and for each of a number of genes or non-coding DNA elements from the surrounding region of the X-chromosome. This confirmed the duplication of tko in both Weeb1 and Weeb2, as well as in Weeb1 (Fig. 2D). In all cases, the duplication was inferred to span a region of the X-chromosome in which tko was positioned approximately centrally. The left break-point of the duplication in all three Weeble lines was mapped to a non-coding region between the right end of the transposable element Tirant 24, and the gene CG14050. The right-hand duplication break-point of Weeb1 was mapped to the region between the genes trol and CG13758, implying a duplication of approximately 228 kb, whereas the right-hand break-point of both Weeb2 and Weeb1 was located a further 60–65 kb along the chromosome, between the genes CG8636 and Tsp3A.

3.3. Autosomal tko25t and mRpL14 transgenes do not suppress the tko25t mutant phenotype

Previous data indicated clearly that additional, ectopic copies of the mutant tko gene in the tko25t background did not confer any significant phenotypic rescue, even where these transgenes were expressed in adult flies at up to several times the expression level of the endogenous tko gene (Toivonen et al., 2003). We noted that the duplicated interval in the three Weeble suppressors of tko25t also included the gene for a second mitoribosomal protein mRpL14. We reasoned that phenotypic suppression of tko25t might require additional copies of both mRpL14 and tko25t. To test this we established transgenic lines carrying additional, ectopic copies of mRpL14 and crossed them into the tko25t background with or without additional transgenic copies of tko25t, then tested for any rescue of the tko25t mutant phenotype. To minimize any transgene-specific, chromosome-specific or other background effects, we conducted all experiments in the same Canton S/Oregen R tko25t Fl hybrid background as used to profile the effects of tko25t and the suppression by Weeble. The presence of an mRpL14 transgene failed to rescue development (Fig. 3A and B). Ectopic copies of the mutant allele tko25t produced, as previously, no rescue of bang-sensitivity (Fig. 3C) and only a minimal rescue of developmental delay (Fig. 3A and B), even in the case of flies carrying the tko25t transgene P(tko25t(A3)) on chromosome 2, which were found to express tko25t in males at approximately 5-fold higher levels than the original mutant (Fig. 3D). The combination of an mRpL14 and a tko25t transgene produced no further improvement in phenotype (Fig. 3A–C), even though both transgenes were expressed in such adults at levels comparable with their expression in Weeb1 adults (Fig. 3D). The ratio of 12S to 16S RNA in adult females carrying the P(tko25t(A3)) transgene was the same as in adult tko25t females (Fig. 3E), although there was a partial rescue towards a more wild-type ratio in males. However, the ratio remained lower than in Weeb1 adults. The additional presence of the mRpL14 transgene did not affect the 12S:16S RNA ratio.

3.4. Increased dosage of tko25t in its normal chromosomal context is necessary and sufficient for action of the Weeble suppression

Extra copies of both mRpL14 and tko25t, producing 2-fold elevated expression of each of these genes in adults, thus gave only a minimal phenotypic rescue, whereas local duplication of the region of the X-chromosome containing these genes gave substantial rescue even in females containing the duplication on only one X-chromosome (Supplementary Figs. 2B and C; Fig. 3). Since the transgenic constructs used for both mRpL14 and tko25t contained all intronic and flanking DNA, including regions overlapping the upstream transcription unit and extending at least a further 0.5 kb downstream, this finding implies that some other, more distant sequence element within the duplication must be involved in the phenotypic rescue, and that doubled expression of both genes in adult flies is insufficient to bring about suppression of the tko25t mutant phenotype. To confirm and investigate this issue further, we set up crosses to combine Weeble X-chromosomes with test chromosomes containing deletions or point mutations of the tko region. All three Weeble chromosomes in combination with the null allele tko25t gave a female phenotype that was still completely mutant, both for developmental delay (Fig. 4A) and bang-sensitivity (Fig. 4B). Consistent with this, the tko25t mutant phenotype was regenerated by combining any of the Weeble chromosomes with an X-chromosomal deletion including the tko gene, but not with X-chromosomal deletions affecting flanking regions that do not include tko (Fig. 4C and D). Finally, the combination of Weeb1 and tko25t on the X-chromosome retained the mutant phenotype even when an expressed, ectopic copy of tko25t was also present (Fig. 4E). Thus, although increased gene dosage of tko25t is required for suppression, it must be in its natural chromosomal context to be effective.

4. Discussion

Previously we found that ectopic, expressed copies of the mutant tko25t gene failed to complement the tko25t mutation (Toivonen et al., 2001, 2003). In the present study, we isolated three suppressors of tko25t, carrying overlapping duplications of a portion of the X-chromosome including the (mutant) tko25t allele (Fig. 2). These Weeble suppressors showed almost complete rescue of the tko25t mutant phenotype, with restoration of the ratio of 12S to 16S RNA to an intermediate level (Fig. 1). The Weeble duplications include a second mitoribosomal protein gene, mRpL14 (Fig. 2), but ectopic, expressed copies of it had no effect on phenotype, even when combined with ectopic tko25t (Fig. 3). Finally, combining Weeble and other chromosomes in various combinations (Fig. 4) led to the conclusion that suppression requires additional copies of tko25t in their correct chromosomal milieu.

4.1. Molecular mechanism of suppression

Ectopic expression of wild-type tko results in complementation of the tko25t mutant phenotype (Toivonen et al., 2001, 2003; Jacobs et al., 2004), whereas similar expression of the mutant allele does not (Toivonen et al., 2001, 2003; Fig. 3). The fact that the 12:16S rRNA ratio was only restored to an intermediate level, even when the organosomal phenotype was virtually wild-type, implies the operation of rather sharp threshold effects. If mitoribosome biogenesis falls below such a threshold, we posit that mitochondrial
protein synthesis is no longer sufficient for maintaining physiological homeostasis, resulting in a pathological phenotype, as inferred also in humans (Miller et al., 2004; Saada et al., 2007).

Increased expression of \( tko^{25t} \) directed by ectopic \( tko^{25t} \) transgenes is associated with a modestly improved 12S:16S rRNA ratio (Fig. 3D and E). However, the expression level of the \( tko^{25t} \) mutant allele in adults of these lines is comparable with, or even exceeds that seen in \( Weeb^1 \), where the 12S:16S rRNA ratio is improved further, and the organismal mutant phenotype is suppressed. The expression level of \( tko^{25t} \) in adult flies thus does not correlate...
strictly with phenotypic suppression, even though increased gene dosage appears to be necessary for it. One simple way to reconcile these findings is to postulate that the phenotype is determined by the tko expression level at an earlier, perhaps specific time in development, or in a restricted set of cells, for which the RNA level in whole adult flies is not a good marker. Alternatively, the tko mRNA level may not correlate with synthesis of the tko protein, which could depend on expression of another function supplied in cis. Some other genes in the duplicated region, with undiscovered roles in mitoribosome biogenesis, may contribute to the phenotypic rescue. However, there are no good candidates, and the experiments shown in Fig. 4 imply that their role, if any, must be minor.
4.2. Cis-regulation of tko expression

An additional copy of the mutant gene on the X-chromosome complements the tko25t mutant phenotype, whereas an ectopic copy does not. The transgenic construct used to create the ectopic lines contains a 3.2 kb insert of genomic DNA from the tko25t mutant strain (Fig. 5). This includes the entire tko coding sequence, the single intron, the full 5' and 3' untranslated (utr) sequences of tko mRNA, plus 1027 bp of 5' flanking and 539 bp of 3' flanking DNA. The 5' flanking DNA includes 677 bp of overlap with the 3' utr of the mRNA of the upstream gene, boi (brother of iHog). We infer that this transgenic construct must lack more distant regulatory information directing expression at a critical developmental stage or cell-type, or else responding directly to mitochondrial stress.

Enhancers and other regulatory elements of Drosophila genes are generally located in the kilobase of 5' flanking DNA, plus the first intron and occasionally the 3' flanking region, although many exceptions are known, including some genes finely regulated during development. The Hox genes, for example, are governed by compartmen-specific enhancers located sometimes tens of kilobases from the transcription start site, often inside very long introns (Zhou and Levine, 1999; Akbari et al., 2008). A recent study showed that even where cis-regulatory information such as an enhancer is located close to the gene, distant secondary enhancers, often embedded within other genes, can play an important accessory role in regulation (Hong et al., 2008). Moreover, specific enhancers are able to skip over intervening regulatory elements, including insulators, to activate target genes (Zhou and Levine,
Another factor influencing the behaviour of cis-regulatory sequences is the local chromosomal context, which can determine whether a specific element functions as an activator or insulator (Soshnev et al., 2008). Major chromosomal rearrangements, such as the Weeble duplications, may disturb or modify the action of such context-dependent elements. Regulation by very distant elements is also seen in mammalian genomes (Kleinjan and van Heyningen, 2005). Based on these precedents, we propose that tko expression is regulated in a developmentally specific manner by distant X-chromosomal elements, whose action might depend on signalling of OXPHOS insufficiency, as we have shown recently for the promoter of the mammalian tko homologue Mrps12 (Zanotto et al., 2008).

The location of such response elements need not be within the region duplicated in the Weeble strains. However, an obvious candidate location would be within the 13 kb non-coding region immediately downstream of tko (Fig. 5), although such expanses are relatively common in the Drosophila genome (nine more are found within 500 kb of tko, for example). The segment downstream of tko contains no transposable elements or any other type of repetitive DNA. However, it does contain two non protein-coding transcribed regions, one of which has a short region conserved in Drosophila erecta.

The region upstream of the tko transcription unit comprises only 200 bp before the 3' end of boi, but includes also a 50 bp insertion found in tko25t but not in wild-type genomic DNA. Regulatory elements influencing tko may also lie within the boi transcription unit, which extends a further 26 kb upstream and overlaps that of zeste (z) on the opposite strand. To identify such element(s) by conventional transgenesis would be a major undertaking. Defining the critical cell-types and developmental stages for rescue, using the GAL4 system, may facilitate an approach using suitable reporter constructs.

Whatever the nature of regulation by distant elements, it is clearly unnecessary for phenotypic complementation by the wild-type allele, which brought about complete rescue and even full dosage compensation in males, using the equivalent construct (Toivonen et al., 2003). The hypothesized distant element is thus required only to boost expression of the hypomorphic tko25t allele.

4.3. Mitochondrial disease relevance

As far as we are aware, segmental duplication of a chromosome region is unprecedented as a mechanism of suppression of a missense mutation. However, there are many cases where such a lesion is associated with pathology, attributable to abnormal gene dosage (Lupski, 1998). For example, Charcot–Marie Tooth Disease Type 1A is most commonly associated with a 1.5 Mb duplication on human chromosome 17 (Lupski et al., 1991; Valentijn et al., 1992), which includes the gene for peripheral myelin protein 22 (PMP-22). Many cases of Pelizaeus–Merzbacher syndrome result from a contiguous gene duplication on the X-chromosome (Ellis and Malcolm, 1994; Woodward et al., 2005) which can vary in size from 0.1–4.6 Mb. Germline duplications have been reported to cause autism (Ullmann et al., 2007) and early-onset Alzheimer dementia (Rovelet-Lecrux et al., 2006), and somatic duplications are commonly associated with the development and progression of many types of cancer (Redon et al., 2006). Examples are also known where segmental duplications provide protection against pathological insults. The copy number of the CCL3L1 chemokine receptor gene influences both HIV susceptibility (Gonzalez et al., 2005) and the risk of developing the auto-immune disease sys-

![Fig. 5. Schematic map of tko25t transgenic construct and tko region of X-chromosome. Bar-scale (top) indicated physical map position. Introns denoted in black, exons in white, untranslated segments of tko mRNA cross-hatched.](image-url)
temic lupus erythematosus (Mamanti et al., 2008), which is also affected by FCGR3 gene copy number (Aitman et al., 2006).

Segmental duplications with no obvious phenotype are common in humans and other species (see http://www-projects.tcgac.ca/variation). The human genome contains a surprisingly large number, covering approximately 6% of the genome in ‘wild-type’ individuals (Bailey and Eichler, 2006), and these have been postulated to provide an important reservoir of genetic variation (Redon et al., 2006; Bailey and Eichler, 2006; Jakobsson et al., 2008; Shen et al., 2008; Symmons et al., 2008). Our findings may thus have a more general relevance in mitochondrial disease. Copy number variation (CNV) and single-nucleotide polymorphisms (SNPs) both contribute to inter-individual differences in gene expression, and both may play roles in the expression of mitochondrial disease phenotypes. In mitochondrial diseases, especially those caused by mutations in genes for the protein synthetic apparatus such as tRNAs, there is a wide range of phenotypic variation, both in severity and tissue-specificity (Zeviani and Di Donato, 2004; Wong, 2007; Haas et al., 2007). Whilst some of this is explicable by heteroplasm, variable expression of many genes involved in mitochondrial protein synthesis is likely to play a key role. Finding ways to enhance their expression might therefore be a useful approach therapeutically.

The tko26 mutation results in a deficiency of mitoribosomal small subunits, causes by their defective assembly or instability (Toivonen et al., 1999, 2001). In bacterial ribosomes S12 is one of the second group of polypeptides to be assembled (Culver, 2003), but is also an essential component of the small subunit. Increased expression of the mutant tko protein may thus compensate partially for the inefficiency of its incorporation into assembled subunits. However, pathological mutations in MRPS16 and MRPS22 have a similar mitoribosome assembly phenotype (Miller et al., 2004; Saada et al., 2007; Emdadul Haque et al., 2008), therefore increased expression of MRPS12 alone may be insufficient to alleviate defects in mitochondrial translation. Further studies of mitoribosome assembly may reveal a limiting step that could provide a useful pharmacological target. Another type of target might be trans-acting factors governing the machinery of mitochondrial biosynthesis more globally, such as nuclear respiratory factors NRF-1 and NRF-2 (Scarpulla, 2008), NF-Y (Fontanesi et al., 2008; Zanotto et al., 2008) or co-activators of the PGC-1 family (Lin et al., 2005; Scarpulla, 2008).

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Appendix A. Supplementary material


References


The Alternative Oxidase AOX Does Not Rescue the Phenotype of tko^{25t} Mutant Flies

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ABSTRACT A point mutation [technical knockout^{25t} (tko^{25t})] in the Drosophila gene coding for mitoribosomal protein S12 generates a phenotype of developmental delay and bang sensitivity. tko^{25t} has been intensively studied as an animal model for human mitochondrial diseases associated with deficiency of mitochondrial protein synthesis and consequent multiple respiratory chain defects. Transgenic expression in Drosophila of the alternative oxidase (AOX) derived from Ciona intestinalis has previously been shown to mitigate the toxicity of respiratory chain inhibitors and to rescue mutant and knockdown phenotypes associated with cytochrome oxidase deficiency. We therefore tested whether AOX expression could compensate the mutant phenotype of tko^{25t} using the GeneSwitch system to activate expression at different times in development. The developmental delay of tko^{25t} was not mitigated by expression of AOX throughout development. AOX expression for 1 d after eclosion, or continuously throughout development, had no effect on the bang sensitivity of tko^{25t} adults, and continued expression in adults older than 30 d also produced no amelioration of the phenotype. In contrast, transgenic expression of the yeast alternative NADH dehydrogenase Ndi1 was synthetically semi-lethal with tko^{25t} and was lethal when combined with both AOX and tko^{25t}. We conclude that AOX does not rescue tko^{25t} and that the mutant phenotype is not solely due to limitations on electron flow in the respiratory chain, but rather to a more complex metabolic defect. The future therapeutic use of AOX in disorders of mitochondrial translation may thus be of limited value.

Drosophila provides a useful animal model for human genetic diseases (Lloyd and Taylor 2010; Lu and Vogel 2009), including those associated with mitochondrial dysfunction (Sánchez-Martínez et al. 2006, Palladino 2010). Prominent among the latter are the many diseases caused by deficiency or malfunction of components of the machinery of mitochondrial protein synthesis (Pearce et al. 2013). These can be caused by point mutations of mitochondrial DNA (mtDNA), by large mtDNA deletions, or by nuclear gene lesions, and can involve interactions with environmental factors, including some antibiotics. Although their clinical phenotypes vary, a common thread is deficiency of multiple respiratory chain complexes, including ATP synthase, which include mtDNA-encoded subunits. The resulting metabolic crisis then produces a developmental and physiological disease condition, which can be widespread, severe, and often fatal.

We have previously investigated a Drosophila model of such diseases; tko^{25t} carries a (recessive) point mutation in the gene for mitoribosomal protein S12 (Royden et al. 1987; Shah et al. 1997). tko^{25t} flies exhibit developmental delay, sensitivity to seizures induced by mechanical stress (“bang sensitivity”), and a set of linked phenotypes that share features with human mitochondrial disease, including hearing impairment and sensitivity to antibiotics that impair mitochondrial protein synthesis (Toivonen et al. 2001). At the molecular level, tko^{25t} shows decreased abundance of mitoribosomal small subunits, multiple respiratory chain and ATP synthase deficiency (Toivonen et al. 2001), and altered gene expression indicative of a metabolic shift toward glycolytic lactate production and anaplerotic pathways (Fernández-Ayala et al. 2010).

The phenotype of tko^{25t} flies can be partially suppressed by segmental duplication of the mutant gene in its natural chromosomal milieu (Kemppainen et al. 2009), by hybridization to specific suppressor
cytoplasmic (mtDNA) backgrounds (Chen et al. 2012), or by over-expression of spargel (Chen et al. 2012), the Drosophila homolog of PGC1-α, proposed to function as a master regulator of mitochondrial biogenesis (Scarpulla 2011). In other studies, we found that toxic inhibition of complex III (cIII) by antimycin or cIV by cyanide, or phenotypes resulting from mutations or knockdown of cIV subunits or the cIV assembly factor Surfy in Drosophila, could be mitigated by concomitant expression of the mitochondrial alternative oxidase (AOX) from Ciona intestinalis (Fernández-Ayala et al. 2009; Kemppainen et al. 2014).

AOX is widespread in eukaryotes, being found in plants, fungi, and many animal phyla, although not in arthropods or vertebrates (McDonald et al. 2009). It provides a nonproton-translocating bypass of the cytochrome segment of the mitochondrial respiratory chain, maintaining electron flow under conditions in which it would be inhibited by high membrane potential, toxic inhibition, or insufficient capacity of cIII and/or cIV. tko25t flies exhibit multiple respiratory chain deficiency, including profoundly decreased activity of both cIII and cIV (Toivonen et al. 2001). However, whereas lactate dehydrogenase can theoretically compensate, at least in part, for the lack of cI (Fernández-Ayala et al. 2010), ubiquinone-linked dehydrogenases, such as succinate dehydrogenase (complex II, cII), require the cytochrome chain for onward electron transfer to oxygen to reoxidize ubiquinol. Thus, even though it cannot directly support ATP production, AOX expression in tko25t should facilitate intermediary metabolism, leading to an amelioration of the mutant phenotype if that phenotype is due to limitations on electron flow through cIII and cIV.

We therefore set out to test whether expression of Ciona AOX in Drosophila at different times in the life-cycle could correct the major organismal phenotypes of tko25t, namely bang sensitivity and developmental delay.

**RESULTS**

**Transgenic expression of AOX in Drosophila using an inducible driver**

We previously documented the amount of expression of AOX at the RNA level in transgenic flies containing single and double copies of the UAS-AOX transgene activated by different ubiquitously acting drivers (Fernandez-Ayala et al. 2009). In the same study, using the drug-inducible tubulin-GeneSwitch driver (tub-GS), we determined the minimal level of the inducing drug RU486 (10 μM) that would sustain maximal AOX expression throughout development when flies were cultured in drug-containing food. To be able to induce and sustain AOX expression at different times during adult life, we first conducted further tests using the tub-GS driver (Figure 1). Expression of AOX was induced in 1-d-old adults using different concentrations of RU486 and was measured 24 hr later using UAS-AOX–bearing flies with no driver or with the highly active da-GAL4 driver as controls (Figure 1A). Even without drug, the tub-GS driver supported AOX expression at a three-fold to 10-fold higher level than in the absence of any driver. As observed previously using various drivers (Fernandez-Ayala et al. 2009), expression in males was always approximately three-fold higher than in females, which is probably a feature of the standard UAS transgenic construct and/or dosage compensation elements associated with the linked mini-white marker gene. RU486 even at low doses increased expression at least 10-fold further, and expression reached a plateau at a drug concentration of 100 μM. To be sure of fully activating expression, we thereafter routinely used 200 μM RU486 as the activating condition.

Next, we determined the kinetics of induced expression and the effects of sustained drug exposure or its withdrawal (Figure 1, B and C). AOX expression already reached a plateau level after 1 d of drug exposure in females (Figure 1B) and males (Figure 1C); thereafter, it remained constant if flies were maintained on drug-containing food. If drug was withdrawn by switching to drug-free food at day five, then expression decreased to a new plateau level by 1 d later. However, this level was two-fold to three-fold higher than that of flies never exposed to drug. Flies endowed with UAS-AOX and tub-GS were cultured continuously on RU486-containing food for many weeks and remained phenotypically indistinguishable from flies grown on drug-free food.

**Adult-specific induction of AOX does not rescue bang sensitivity of tko25t**

Bang sensitivity is generally considered to arise from a functional defect of nerve conduction during high-frequency stimulation in the giant fiber pathway (Pavlidis and Tanouye 1995; Lee and Wu 2002; Fergestad et al. 2006; Ueda et al. 2008). Bang-sensitive mutants with an underlying mitochondrial defect, including kdb (citrate synthase) and sesB1 (adenine nucleotide translocase) as well as tko25t display a characteristic seizure pattern (Fergestad et al. 2006). We therefore decided to test whether expression of AOX in tko25t mutant flies could compensate for the mitochondrial defect and thus alleviate bang sensitivity. We crossed tub-GS into the tko25t background using a balancer chromosome strategy to analyze progeny from a single experimental cross that generated flies carrying tko25t, tub-GS, and/or UAS-AOX in all eight possible combinations. Bang sensitivity was tested in 2-d-old males and females of each class, either with or without transfer 24 hr earlier to food containing 200 μM RU486 (Figure 2). Unambiguously, the results indicate that AOX is unable to modify the bang-sensitive phenotype of tko25t adults, and it does not induce any detectable bang sensitivity in control flies. In fact, applying Student’s t test with Bonferroni correction confirmed that there were no significant
differences between any of the classes that were mutant for tkot, irrespective of sex, transgene, driver, or RU486 induction. Similarly, there were no significant differences between any of the classes that were wild-type for the tko gene, irrespective of these other parameters. As expected, the difference between tkot mutant flies of each class and the corresponding class without tkot was significant ($P < 0.01$) in every case.

Continuous induction of AOX throughout development does not rescue tkot

Considering an alternative hypothesis, that the bang-sensitive phenotype of tkot is established during development, we conducted similar crosses but used fly food containing RU486. In our previous study (Fernandez-Ayala et al. 2009), we established that 10 μM RU486 was sufficient to induce maximal transgene expression during the larval stages, so we

Figure 1  AOX expression in adult flies driven by tubulin-GeneSwitch. (A) Relative AOX expression, determined by QRT-PCR normalized to RpL32 control RNA, in 2-d-old UAS-AOX flies bearing the drivers indicated exposed to different concentrations of RU486 for 24 hr. Means ± SD of three biological replicates. Note the logarithmic scale. (B and C) Relative AOX expression in adult UAS-AOX flies bearing the indicated drivers and exposed to 200 μM RU486 as shown. Means ± SD of three biological replicates.

Figure 2  Bang sensitivity is unaffected by AOX induction in adult flies. Bang sensitivity (square-root of recovery time from vortexing) of 2-d-old flies of the sex and genotype indicated, with or without 24 hr of prior treatment with 200 μM RU486. Means ± SD for groups of 30 individually analyzed flies.
used this concentration of the drug along with drug-free control vials. This procedure allowed us also to analyze effects on the second canonical phenotype of \(tko^{25t}\), developmental delay, which was previously found to occur uniquely during the larval (growth) stages (Toivonen et al. 2001).

Once again, we observed no rescue of the mutant phenotype that was attributable to AOX expression (Figure 3). The developmental delay of \(tko^{25t}\) mutant flies (Figure 3A) was slightly greater in males than in females, as observed previously (Kemppainen et al. 2009), and an additional delay of approximately 1 d was produced in flies of all genotypes and both sexes by the presence of RU486 in the food. The UAS-AOX transgene, the tub-GS driver, and the two in combination did not produce any significant change in developmental timing of \(tko^{25t}\) mutant flies, although there was a slight delay produced by AOX expression in wild-type flies, as reported previously using the da-GAL4 driver. The bang sensitivity of the progeny flies showed no significant change according to any of the parameters tested, except for the presence of the \(tko^{25t}\) mutation itself (Figure 3B).

**Prolonged adult induction of AOX does not rescue bang sensitivity of \(tko^{25t}\)**

To test whether correction of the \(tko^{25t}\) phenotype in adult flies requires long-term expression of AOX, we cultured \(tko^{25t}\) flies generated in the previous crosses continuously for a period of 30 d on food either with or without RU486 at the inducing concentration of 200 \(\mu M\), noting the previous result that sustained expression requires continuous exposure to the drug. This also enabled us to check the stability of the phenotype during adult life, which, to our knowledge, has not previously been studied systematically.

Bang sensitivity was unaffected by any of the parameters tested in this experiment (Figure 4). There was no rescue (or worsening) of the phenotype either by basal or by induced AOX expression, no effect of age, no difference between the sexes, and no effect of tub-GS.

**Ndi1 expression during development is lethal to \(tko^{25t}\)**

Because AOX expression at any stage of the fly life-cycle had no effect on the major phenotypic features of \(tko^{25t}\) mutants, we considered the hypothesis that the steps in mitochondrial electron flow that AOX bypasses may not be crucial determinants of the phenotype. The \(tko^{25t}\) mutation impacts all four of the enzymatic complexes of the oxidative phosphorylation (OXPHOS) system that contain mitochondrial translation components (Toivonen et al. 2001), but it is unclear which is limiting for respiration or ATP synthesis. Because complex I (cI) activity is severely affected by the mutation, we considered the alternative hypothesis that a decreased capacity for electron flow through cI alone underlies the \(tko^{25t}\) mutant phenotype, and that decreased capacity of complexes III and/or IV is immaterial, thus accounting for a failure of AOX expression to modify the phenotype.

To test this idea, we set up a genetic cross (Figure 5A) to investigate whether an analogous bypass of cI using the nonproton-pumping NADH dehydrogenase from yeast (Ndi1) could rescue the phenotype. Ndi1 expression was shown previously to be benign in *Drosophila* and to rescue the lethality of severe knockdown of cI subunits (Sanz et al. 2010b). We introduced the ubiquitously acting da-GAL4 driver and a UAS-Ndi1 transgene separately into the \(tko^{25t}\) mutant strain and then crossed females heterozygous both for \(tko^{25t}\) and UAS-Ndi1 with \(tko^{25t}\) males carrying da-GAL4 (Figure 5A). The cross repeatedly gave...
a low number of \(tko^{25t}\) progeny (Table 1). However, almost all of them carried the balancer marker in place of UAS-Ndi1, indicating that the combination of da-GAL4, \(tko^{25t}\) and UAS-Ndi1 is semi-lethal. Expression of Ndi1 in \(tko^{25t}\) heterozygotes had a far less dramatic effect. We conclude that, far from rescuing \(tko^{25t}\), expression of Ndi1 is selectively deleterious to \(tko^{25t}\) mutant flies.

This result raises the possibility that although neither Ndi1 nor AOX can individually rescue \(tko^{25t}\), the co-expression of both transgenes might do so. This would be the case, for example, if the \(tko^{25t}\) phenotype were due to a combined limitation on electron flow at both cI and at cIII+cIV of similar magnitude. Although co-expression of Ndi1 and AOX at 25° was previously shown to be synthetically lethal even in wild-type flies (Sanz et al. 2010b), in trial experiments we were able to obtain co-expressing flies when cultured at 18°. We therefore implemented the experimental cross illustrated in Figure 5B to determine whether Ndi1 and AOX co-expression can rescue \(tko^{25t}\). As shown in Table 2, although control flies were now obtained, and again there were only a few Ndi1-expressing flies in the \(tko^{25t}\) mutant background, no doubly expressing \(tko^{25t}\) flies eclosed. We conclude that, far from rescuing \(tko^{25t}\), combined expression of the two transgenes is more deleterious than of either alone.

**Effects on ATP or ROS do not correlate with modulation of \(tko^{25t}\) phenotype**

In previous studies we found decreased steady-state ATP levels in extracts from \(tko^{25t}\) mutant flies, as well as elevated production of ROS in isolated \(tko^{25t}\) mitochondria (Chen et al. 2012). However, the relevance of these observations to the organismal phenotype remains to be conclusively demonstrated. The effects of AOX and Ndi1 expression on the \(tko^{25t}\) phenotype provided an opportunity to test this relationship further. To obtain a sufficient number of \(tko^{25t}\) flies expressing Ndi1 to conduct this experiment, flies were reared at 18° instead of 25° (see previous section).

We confirmed the previous observation of decreased ATP levels in \(tko^{25t}\) homozygotes compared with heterozygous controls (Figure 6A) but found no significant alteration thereof when either AOX or Ndi1 was expressed. Mitochondrial ROS production in \(tko^{25t}\) homozygotes was also elevated in every case compared with heterozygous controls (Figure 6B). This was unaffected by expression of AOX but modestly alleviated by Ndi1 expression, despite the fact that the effect of Ndi1 on the overall organismal phenotype was deleterious. This, plus the wide variation in ROS production according to genetic background (reflecting different balancer chromosomes), implies that the \(tko^{25t}\) organismal phenotype is also not directly determined by ROS.

**DISCUSSION**

In this work we set out to determine whether AOX from Ciona intestinalis can ameliorate the mutant phenotype of \(tko^{25t}\), which carries a mutation in mitochondrial protein S12, resulting in globally decreased OXPHOS capacity. We found that induced AOX expression, whether during development, in freshly eclosed adults, or maintained in adults over a period of 30 d, has no effect on \(tko^{25t}\), nor does it produce a phenocopy of \(tko^{25t}\) in wild-type flies. In contrast, ubiquitous expression of Ndi1, the alternative NADH dehydrogenase from yeast, was highly deleterious to \(tko^{25t}\) during development and was lethal when combined with both \(tko^{25t}\) and AOX.

**Failure of AOX rescue suggests that a complex metabolic defect underlies the \(tko^{25t}\) phenotype**

\(tko^{25t}\) exhibits a functional deficiency of all four OXPHOS complexes containing mitochondrial translation products (Toivonen et al. 2001), but it is unclear which of these is limiting for electron transfer. Because AOX provides a functional bypass of complexes III and IV, its failure to rescue the organismal phenotype can be interpreted in one of several ways. The first would be that the residual activity of cIII/cIV is not limiting for mitochondrial electron transport in \(tko^{25t}\), and that the phenotype is entirely due to cI dysfunction. The second postulates that AOX is unable to rescue \(tko^{25t}\) because, as a nonproton-motive enzyme, it does not support the synthesis of ATP, and ATP deficiency is what underlies the mutant phenotype. A third possibility is that the phenotype is a consequence of one or more processes on which AOX does not impinge, such as elevated ROS production, or proteotoxicity due to the protein synthesis defect. Although none of these can be entirely eliminated, the fact that Ndi1 expression worsens the phenotype, either alone or in combination with AOX, and that changes in ATP level or mitochondrial ROS production do not correlate with it, suggest that the mutant phenotype is determined either by a complex interplay of factors or by other metabolic effects that are as yet unknown. Disrupted redox homeostasis resulting from a cI defect should be rescuable by Ndi1. A combined limitation on electron flow at cI and cIII and/or cIV should be alleviated by combined expression of Ndi1 and AOX. Manifestly, these predictions are inconsistent with our findings.
Ndi1 is constitutively active (Sanz et al. 2010b), consistent with the fact that in its natural setting (in budding yeast) cl is absent. By diverting electrons away from cl, it may act to decrease net ATP production still further, but this seems unlikely to be the explanation for its effect on tk025t because the apparent additional decrease in ATP level (Figure 6A) was modest and not statistically significant. However, the low number of successfully eclosing flies may represent the tail of a distribution, with those individuals suffering further ATP depletion simply unable to complete development. Effects on mitochondrial ROS production also did not correlate with the organismal phenotype. Although we confirmed elevated ROS production in tk025t flies (Figure 6B), it was more affected by genetic background than by the expression of the alternative respiratory chain enzymes, and the effect of Ndi1 was again paradoxical. Note, however, that all metabolic assays were conducted on materials from flies reared at 18°C, whereas for most of the phenotypic experiments reported here flies were cultured at 25°C. This may have some bearing on the findings.

Proteotoxicity due to imbalance between cytosolic and mitochondrial protein synthesis has been implicated as a longevity mechanism, acting hormetically via the induction of the mitochondrial unfolded protein response (Figure 5). Genetic crosses used to test rescue of tk025t. Crosses used to test rescue by (A) Ndi1 or (B) Ndi1 plus AOX combined. Progeny classes are color-coded as indicated to denote their meaning in the experiment. The results of the cross are shown in Table 1. Note that FM7 / Y males do not contain an unmanipulated X-chromosome, so they are not strictly a wild-type control.
these reasons, as well as the arguments stated above, we feel the hypothesis Bang sensitivity of mitochondrial ATP production, sesB1. Bang sensitivity is a commonly observed mutant phenotype in et al. synthesis, adenine nucleotide transport and the TCA cycle (Fergestad et al. et al. logical pathways, including, in addition to mitochondrial protein and is due to lesions affecting a variety of cellular or physio-
delay in adenine nucleotide translocase (Zhang et al. 2014). However, decreased levels of NAD+ are associated with being the primary mechanism underlying the tko25t phenotype. The failure of AOX to rescue bang sensitivity and developmental defects (Engel and Wu 1994), they fall into two classes depending on whether motor neurons are directly affected (Fergestad et al. 2006). Some of them show a clear degenerative phenotype with drastically shortened lifespan, whereas others, including tko25t, show only a modestly decreased lifespan and associated neuropathology (Fergestad et al. 2008). In the current study, we found no significant change in the bang sensitivity of tko25t over 30 d of adult life, in contrast to the synergistic and progressive effects on bang sensitivity seen when tko25t is combined with other bang-sensitive mutants (Fergestad et al. 2008). We conclude that the bang sensitivity of tko25t is a developmentally determined phenotype, at least in an otherwise wild-type genetic background.

**Therapeutic implications for AOX in mitochondrial disease**

AOX has been proposed as a therapeutic tool relevant to a wide variety of mitochondrial disorders (El-Khoury et al. 2014). The present work indicates important limitations of this concept, whatever the precise link between mitochondrial translational dysfunction and the organismal phenotype in tko25t. Despite profound effects on flies exposed to toxins or mutations directly or indirectly affecting cytochrome oxidase (Fernandez-Ayala et al. 2009; Kempainen et al. 2014), or even the pleiotropic phenotypes caused by partial knockdown of DNA polymerase γ (Humphrey et al. 2012), AOX expression produced no detectable modification to the tko25t phenotype.

### Table 1 Test of ability of Ndi1 expression to rescue tko25t

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Number of Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>tko25t / FM7 ; CyO / 2 ; daGAL4 / Ser</td>
<td>Female</td>
<td>152</td>
</tr>
<tr>
<td>tko25t / FM7 ; UAS-Ndi1b20 / 2 ; daGAL4 / Ser</td>
<td>Female</td>
<td>72</td>
</tr>
<tr>
<td>tko25t / Y ; CyO / 2 ; daGAL4 / Ser</td>
<td>Female</td>
<td>57</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>FM7 / Y ; UAS-Ndi1b20 / 2 ; daGAL4 / Ser</td>
<td>Male</td>
<td>65</td>
</tr>
<tr>
<td>tko25t / Y ; CyO / 2 ; daGAL4 / Ser</td>
<td>Male</td>
<td>25</td>
</tr>
<tr>
<td>tko25t / Y ; UAS-Ndi1b20 / 2 ; daGAL4 / Ser</td>
<td>Male</td>
<td>20</td>
</tr>
<tr>
<td>tko25t / Y ; daGAL4 / Ser</td>
<td>Male</td>
<td>1</td>
</tr>
</tbody>
</table>

Output from cross shown in Fig. 5A.

### Table 2 Test of ability of Ndi1 and AOX co-expression to rescue tko25t

<table>
<thead>
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<th>Sex</th>
<th>Number of Progeny</th>
</tr>
</thead>
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<tr>
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</tr>
<tr>
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<td>34</td>
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<td>Female</td>
<td>35</td>
</tr>
<tr>
<td>tko25t / Y ; CyO / 2 ; daGAL4 / Ser</td>
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<td>23</td>
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<tr>
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<td>tko25t / Y ; UAS-Ndi1b20 / 2 ; daGAL4 / Ser</td>
<td>Female</td>
<td>5</td>
</tr>
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<tr>
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<tr>
<td>FM7 / Y ; UAS-Ndi1b20 / 2 ; daGAL4 / Ser</td>
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</tr>
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<tr>
<td>tko25t / Y ; daGAL4 / UAS-AOX</td>
<td>Male</td>
<td>0</td>
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</tbody>
</table>

Output from cross shown in Fig. 5B.

a A repeat experiment gave similar results.
tko25t has been considered as a model for mitochondrial diseases, exhibiting not only seizures and developmental delay but also hearing impairment (Toivonen et al. 2001). It is of particular relevance to those disorders where the primary defect is in the mitochondrial translation system, which applies to many of the commonest pathological mtDNA mutations such as the 3243G > A MELAS mutation, as well as an increasingly recognized subset of nuclear gene mitochondrial disorders exhibiting multiple OXPHOS deficiencies (Pearce et al. 2013). The implementation of respiratory chain bypasses such as AOX or Ndi1 should, in theory, alleviate pathological phenotypes associated with restrictions on electron transport, depending on which segments of the respiratory chain are affected. In cases where multiple OXPHOS complexes are affected, both bypasses in combination might be needed to restore electron flow. tko25t constitutes a model for such diseases, yet neither AOX nor Ndi1 ameliorated the phenotype, and Ndi1 was even deleterious. As already indicated, Ndi1 and AOX do not restore proton pumping at the respiratory chain segments that they bypass, nor can they alleviate, a priori, all other aspects of mitochondrial dysfunction. Their uses in eventual therapy for disorders of mitochondrial translation therefore may be limited and clearly requires a fuller understanding of the pathophysiological mechanism case by case.

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LITERATURE CITED


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