Rapamycin increases mitochondrial efficiency by mtDNA-dependent reprogramming of mitochondrial metabolism in *Drosophila*.

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Running title: TOR and mitochondrial metabolism
Summary

Down-regulation of the mTOR pathway by its inhibitor rapamycin is emerging as a potential pharmacological intervention that mimics the beneficial effects of dietary restriction. Modulation of mTOR has diverse effects on mitochondrial metabolism and biogenesis, but the role of mitochondrial genotype in mediating these effects remains unknown. Here we use novel mitochondrial genome replacement strains in Drosophila to test the hypothesis that genes encoded in mtDNA influence the mTOR pathway. We show that rapamycin increases mitochondrial respiration and succinate dehydrogenase activity, decreases H₂O₂ production and generates distinct shifts in the metabolite profiles of isolated mitochondria versus whole Drosophila. These effects are disabled when divergent mitochondrial genomes from D. simulans are placed into a common nuclear background, demonstrating that the benefits of rapamycin to mitochondrial metabolism depend on genes encoded in the mtDNA. Rapamycin is able to enhance mitochondrial respiration when succinate dehydrogenase activity is blocked, suggesting that the beneficial effects of rapamycin on these two processes are independent. Overall, this study provides the first evidence for a link between mitochondrial genotype and the effects of rapamycin on mitochondrial metabolic pathways.

Keywords: Rapamycin, metabolism, mitochondrial genotype
Mitochondria are specialized organelles that convert metabolic substrates into adenosine triphosphate (ATP), the energy currency of cells, via the process of oxidative phosphorylation (OXPHOS). During OXPHOS, NADH and FADH$_2$, which are derived from the mitochondrial tricarboxylic acid (TCA) cycle and fatty-acid oxidation, pass electrons to complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) of the electron transport chain (ETC) respectively. Electrons are then transferred through complex III (cytochrome bc1) to complex IV (cytochrome c oxidase), where they are passed to oxygen and produce water (Scheffler, 2007). This process generates a proton gradient and establishes a membrane potential ($\Delta \psi_m$) across the inner-mitochondrial membrane. Ultimately, the proton gradient is dissipated either at complex V, producing ATP, or through uncoupling proteins producing heat (Scheffler, 2007).

Reactive oxygen species (ROS) are generated during electron transport as a by-product of OXPHOS. Levels of ROS are tightly regulated as ROS serve as a secondary messenger to mediate signal transduction and metabolism (Cheng and Ristow, 2013), while excess ROS can damage DNA, lipids and proteins (Balaban et al., 2005; Murphy et al., 2011).

In addition to ATP production, mitochondria play critical roles in amino acid metabolism, carbohydrates metabolism and fatty acid oxidation.

Although mitochondria contain their own genome and function as distinct organelles separated by lipid membranes, mitochondrial processes are critically dependent on nuclear-encoded gene products and environmental signals in order to accommodate metabolic cellular requirements (Finley and Haigis, 2009; Liu and Butow,
These interactions require continuous communication between mitochondria and the cytosol (Woodson and Chory, 2008). A critical link between cytosolic and mitochondrial metabolism is the enzyme succinate dehydrogenase (complex II of the ETC). Succinate dehydrogenase is the only enzyme shared by the TCA cycle and ETC and lies at the intersection of pathways that connect cell metabolism with mitochondrial respiration (Scheffler, 2007). In the ETC, succinate dehydrogenase acts as an entry point for electrons from FADH₂ via fatty-acid oxidation; in the TCA cycle it oxidizes succinate to fumarate. Notably it is the only complex of the ETC with no mtDNA-encoded subunits.

A pathway that has been identified as a potential mediator of cross-talk between mitochondria and the cytosol is the mammalian target of rapamycin (mTOR) (Finley and Haigis, 2009; Schieke and Finkel, 2006). The mTOR pathway is highly conserved and regulates diverse functions related to nutritional cues and cellular stress (Baltzer et al., 2010; Finley and Haigis, 2009). In yeast, reduced TOR signaling enhances mitochondrial respiration and modulates ROS production to extend chronological life span (Bonawitz et al., 2007; Pan et al., 2012). In mammals, the role of the mTOR pathway in mitochondrial function has tissue-specific effects (Bentzinger et al., 2008; Ramanathan and Schreiber, 2009; Cunningham et al., 2007; Paglin et al., 2005; Polak et al., 2008; Schieke et al., 2006; Düvel et al., 2010). The best characterized function of mTOR is the regulation of translation initiation by mTOR Complex 1 (mTORC1). This involves mTORC1-mediated phosphorylation of the eukaryotic initiation factor 4E-binding protein 1 (eIF4E-BP1) and ribosomal protein S6 kinase 1 (S6K1) in order to up-regulate 5’-cap-dependent protein
translation (Richter and Sonenberg, 2005). In addition to modulation of protein
translation, the mTOR pathway has also been implicated in cell growth, autophagy,
longevity and metabolism (Finley and Haigis, 2009; Laplante and Sabatini, 2012;
Soliman, 2011; Mathew and White, 2012), all key biological processes in health and
homeostasis. Indeed, dysregulation of mTOR signaling is a common condition in human
diseases (Dazert and Hall, 2011).

There is increasing interest in identifying the genetic and cellular mechanisms underlying
the connection between mTOR signaling and mitochondrial function. The majority of
genes that function in mitochondria are encoded by the nuclear genome, but a small and
critical fraction of genes have been retained in the mitochondrial genome since the
endosymbiotic origin of the eukaryotic cell roughly two billion years ago (Lane, 2005).
The majority of studies seeking to understand the role of the mTOR pathway in
mitochondrial function have focused on these nuclear-encoded proteins that are translated
on cytosolic ribosomes and imported into the mitochondrion. The genes encoded in the
mitochondrial genome have received little attention in experimental work related to the
mechanisms of action in the mTOR pathway.

Here we test the hypothesis that the relationship between the mTOR pathway and
mitochondrial physiology is modified by genes encoded in alternative mitochondrial
genomes. To accomplish this we used strains of Drosophila in which different mtDNAs
from both D. melanogaster and D. simulans were placed on a single D. melanogaster
nuclear background (Montooth et al., 2010). We posit that this genetic manipulation will
compromise the co-evolved nature of mito-nuclear cross talk and provide a novel hypomorphic context for analyses of how rapamycin influences mTOR functions. We show that treatment with rapamycin, a specific mTORC1 inhibitor, increases mitochondrial oxidative capacity and respiration rate, as well as decreases the production of mitochondrial H$_2$O$_2$, one of the ROS produced in the mitochondria. This effect was pronounced in *D. melanogaster* genotypes carrying the native *D. melanogaster* mtDNAs, but these beneficial effects of rapamycin were significantly reduced in the strains carrying the ‘foreign’ *D. simulans* mitochondrial genotypes on the *D. melanogaster* nuclear background. Using comparative metabolomics analyses we show that whole fly homogenates have a strong signature of response to rapamycin with only subtle effects of mtDNA genotypes. In contrast, metabolite profiles of isolated mitochondria show strong mtDNA-genotype-dependent responses to rapamycin. The coevolved genotypes carrying the *D. melanogaster* mtDNAs show clear reprogramming of their carbohydrate and amino acid profiles under rapamycin treatment, but the foreign *D. simulans* mtDNAs shift these metabolite levels such that rapamycin has little additional effect. We further show that rapamycin treatment specifically affects the ETC at the level of complex II activity, and does so only for the *D. melanogaster* mtDNA genotypes. Overall, our experiments confirm that rapamycin has multiple beneficial effects on mitochondrial function, and show for the first time that the full effects of these benefits are dependent on the metabolic plasticity mediated by genes encoded in the mtDNA.

**Results**

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6
Rapamycin increases mitochondrial respiration and decreases H$_2$O$_2$ production

To determine the effect of rapamycin on mitochondrial function, we measured OXPHOS capacity of mitochondria isolated from two standard laboratory *D. melanogaster* strains: (i) *white Dahomey* (*w*$_{Dah}$), and (ii) Oregon R (OreR). Flies were fed food containing 200uM rapamycin, a concentration shown to inhibit the phosphorylation of TOR target S6K at T-398, and to affect protein translation (Figure S1, (Bjedov et al., 2010)). We observed that after ADP stimulation (state 3 respiration), mitochondria from flies treated with rapamycin have a higher rate of oxygen consumption than from vehicle-treated flies (Figure 1A), suggesting that rapamycin increases mitochondrial oxidative capacity. Uncoupled mitochondrial respiration rates were measured after the addition of the chemical uncoupler, FCCP. Flies treated with rapamycin display increased uncoupling, indicative of a higher maximum oxidative capacity (Figure 1A). We observed that the absolute values for oxygen consumption could vary significantly from one day to the next, but the relative values of rapa-treated vs. control showed consistent differences in oxygen consumption rates, hence we report ratios to show the rapamycin effect.

As mitochondrial respiration is the main source of ROS in cells, we measured H$_2$O$_2$ in rapamycin treated flies. In both strains, rapamycin decreases the production of H$_2$O$_2$ (Figure 1B).

Effects of rapamycin on ETC complexes and mitochondrial membrane potential

The protein complexes I, III, IV and V of the ETC are jointly encoded by nuclear and mitochondrial genes and work together in the process of OXPHOS. In order to test if the
effect of rapamycin on mitochondrial oxygen consumption is due to an effect in one or
more of the ETC complexes, we studied the \textit{in vitro} activity of the respiratory chain
enzyme complexes (complex I, complex II, complex III, and complex IV) (Figure 1C and
Figure S2A-C) and the enzymatic activity of citrate synthase as a marker of
mitochondrial content and integrity (Figure S2D). Rapamycin-fed flies show increased
complex II enzymatic activity (Figure 1C). No effect was observed in any of the other
enzyme complexes (Figure S2A-C). Interestingly, complex II is the only complex of the
ETC that is solely encoded by nuclear genes, and is an integrator of nutrient catabolism
between glycolytic and respiratory pathways that contribute different reducing
equivalents (NADH vs. FADH)(Scheffler, 2007). As an indicator of hydrogen ion
pumping across the inner membrane during the process of electron transport and
oxidative phosphorylation, we measured changes in membrane potential, $\Delta\psi_m$.
Rapamycin has no significant effect on mitochondrial $\Delta\psi_m$ (Figure S2E).

\textit{Rapamycin effects on respiration are independent of increased Complex II activity}

We then tested the hypothesis that the effect of rapamycin on mitochondrial respiration is
mediated by the increased activity of complex II. We measured mitochondrial complex I
- and complex II-dependent oxygen consumption in flies treated with rapamycin. Oxygen
consumption was quantified with substrates and inhibitors that permit complex I
respiration but block complex II: with pyruvate and malate alone vs. pyruvate and malate
plus malonate (a competitive inhibitor of complex II activity). The reciprocal experiment
was also performed in which oxygen consumption was studied with the complex II
substrate succinate, plus rotenone as an inhibitor of complex I. Rapamycin increases
Divergent mitochondrial genotypes on a common nuclear background respond differently to rapamycin treatment.

To test the hypothesis that the effects of rapamycin are dependent on genes encoded in the mtDNA, four distinct mtDNAs from different strains of *D. melanogaster* (*OreR* and *Zim53*) and *D. simulans* (*sm21* and *si1*) were introgressed onto a single *D. melanogaster* nuclear background, *Oregon R* (*OreR*) (Figure 2A). Offspring from crosses between *D. simulans* and *D. melanogaster* are sterile. However, a rescue of this sterility can be obtained when females from the *D. simulans C167.4* strain are crossed to *D. melanogaster* males (Davis et al. 1996). To transfer mtDNA from *D. simulans* to *D. melanogaster*, *sm21* and *si1* females were backcross for several generations to *D. simulans C167.4* males. Subsequently, females from these backcrosses were crossed to *D. melanogaster* males to obtain the mtDNA replacement strains (Montooth et al., 2010). A series of backcrosses using balancer chromosome stocks and the OreR strain of *D. melanogaster* replaced all *D. simulans* nuclear alleles with OreR nuclear alleles in each of the desired cytoplasmic (mtDNA) backgrounds (Montooth et al., 2010).

Since all mtDNAs were placed on the same OreR nuclear background, differences among the introgressed genotypes could only result from main effects of the distinct mtDNAs or specific interactions of the OreR nuclear background with the different mtDNAs. We observed that rapamycin significantly affects mitochondrial respiration (Figure 2B), $H_2O_2$.
production (Figure 2C), and complex II activity (Figure 2D) in flies harboring mtDNA from *D. melanogaster* (*OreR and Zim53*) but this rapamycin effect was significantly reduced in flies carrying *D. simulans* mtDNA (*sm21 and si1*).

**Rapamycin and mitochondrial genotype alter metabolite profiles of whole flies and isolated mitochondria.**

We performed comparative metabolomics analyses of rapamycin on a subset of our strains, the “responding” (*OreR*) and “non-responding” (*sm21*) mitochondrial genotypes both on the common *OreR* nuclear background after treatment with rapamycin.

Metabolites from whole fly extracts and from mitochondrial extracts were obtained by using gas chromatography mass spectrometry (GC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). We detected 210 metabolites in the whole fly extracts and 230 metabolites in the mitochondrial extracts. These metabolites were separated into 4 categories, those involved in amino-acid metabolism, carbohydrate metabolism, lipid metabolism and those involved in energy homeostasis (cofactors, vitamins and Krebs cycle intermediates) (Figure 3-5, and Table S1). The data were subjected to a principle components analysis (PCA) to visualize the clustering of treatments in metabolite space. For the whole fly extracts, mtDNA genotype has subtle effects on the metabolite profile as revealed by the proximity of the OreR control treated and the sm21 control treated samples in PCA space (Figures 3A and 5A, open symbols). However rapamycin treatment has a significant impact of the metabolite profiles as is evident from the displacement of the rapamycin treated samples to lower values of PC2 (Figures 3A and 5A, solid symbols). Bivariate normal ellipses defining a 95%
The metabolite profiles from mitochondrial isolates analyses are distinct from those of whole fly extracts and identify a genotype-by-rapamycin interaction effect (Figures 4 and 5B). Rapamycin has a strong effect on the metabolite profiles of the responsive mtDNA genotypes (*OreR*). The *OreR* rapamycin treated samples are displaced along the PC1 axis (Figures 4A and 5B). Bivariate normal ellipses defining a 95% confidence area surrounding the *OreR* control treated samples do not overlap with other treatment classes (Figure S3B). For the “non-responding” *sm21* mtDNA genotype, rapamycin has noticeably less of an effect on the shift of the metabolite landscapes (Figures 4A and 5B).

The 95% confidence ellipses for control and rapamycin treated samples are broadly overlapping for this genotype (Figure S3B). This pattern is most evident in the PCA plots based on amino acids (Figure 4B), carbohydrates (Figure 4C, Figure S3D) and metabolites involved in energy homeostasis (cofactors, vitamins and krebs cycle intermediates) (Figure 5B) where rapamycin induces a substantial shift in *OreR*, but has
virtually no impact on the \textit{sm21} genotypes. This pattern is similar but not as pronounced in the lipid analysis (Figure 4D).

Analysis of individual metabolites that are differentially affected in \textit{OreR} and \textit{sm21} flies after rapamycin treatment reflects an imbalance of several mitochondrial pathways. The TCA metabolite fumarate is decreased while malate is increased in \textit{OreR} flies after rapamycin treatment (Table 1). Rapamycin-treated \textit{OreR} mitochondria showed significantly lower levels of fatty acids, reduced hydroxybutyrate (BHBA) and other ketone bodies, and increased carnitine levels. Because the oxidation of exogenous fatty acids is enhanced by rapamycin treatment (Brown et al., 2007), we hypothesized that this imbalance in lipid metabolism may partially result from a decline in \(\beta\)-oxidation as a consequence of the depletion of available endogenous lipids by this time point under rapamycin treatment. Notably, basal levels of BHBA and other ketone bodies significantly differed between \textit{OreR} and \textit{sm21} flies, suggesting differences in basal lipid oxidation between haplotypes (not shown).

Within the amino acids, beta-hydroxybutyrate, beta-alanine and glutamate levels increased after rapamycin treatment in \textit{OreR} but not \textit{sm21}. The rest of amino acids that changed, including glutamine which has been recently shown to be regulated by mTOR (Csibi et al., 2013), were down regulated in \textit{OreR} mitochondria. In \textit{sm21} mitochondria, rapamycin increased the levels of glutamine, histamine and cystine. (Table 1).

Discussion
Mito-nuclear interactions are the result of a two billion year old symbiosis between two genomes with a history of gene transfer from the ancestral mitochondrial genome into the emerging nuclear genome, and a system to import proteins back into the mitochondrial organelle (Rand et al., 2004; Ballard and Rand, 2005; Pesole et al., 2012). As an outcome of this coadaptation the mitochondria and cytosol have established circuits of signaling that ensure homeostasis via cellular plasticity in response to altered environmental conditions. Cytosolic pathways signal to modulate mitochondrial activity (anterograde signaling) and mitochondria signal to the cytosol to alert of changes in mitochondrial metabolism (retrograde signaling) (Liu and Butow, 2006; Woodson and Chory, 2008). Therefore, it is not surprising, that mitochondrial dysfunction is associated with the onset of many diseases (Ristow, 2006; Wiederkehr and Wollheim, 2006; Fukui and Moraes, 2008; Tatsuta and Langer, 2008) and may have an important role in the aging process (Masoro et al., 1982; Tatsuta and Langer, 2008; Finley and Haigis, 2009; Raffaello and Rizzuto, 2011; Vendelbo and Nair, 2011). However, there is controversy over the mechanisms by which mitochondrial activity may modulate longevity. Traditionally ROS production in the mitochondria has been postulated as a cause of aging (Harman, 1956) and manipulations that either increase ROS scavenging or decrease ROS production have extended longevity. Yet, recent studies have challenged the role of ROS as only detrimental in aging, given evidence that ROS is also an important factor promoting longevity via signaling and homeostasis (Ristow and Schmeisser, 2011).
In this study we identify complex II as the only member of the ETC that displays altered activity upon rapamycin treatment. The significant effect on complex II and lack of effects on other complexes points to the interaction of mitochondrial and metabolic energy transduction as an important aspect of mTOR signaling. Participating in both the TCA and ETC, complex II is uniquely situated to coordinate both pathways. Furthermore, complex II is stimulated by FADH₂, which is mainly derived from fatty-acid oxidation. mTOR metabolism has been previously described to be connected to lipid metabolism regulation (Laplante and Sabatini, 2012; Soliman, 2011). Moreover, levels of ketone bodies, carnitine metabolism and free fatty acids significantly differed between OreR and sm21 rapamycin treated flies suggesting lipid oxidation as an important metabolic pathway mediating rapamycin beneficial effects on mitochondrial physiology. Dysfunction of complex II, as well as up-regulation of the mTOR pathway, is associated with precocious aging and cancer (Brière et al., 2005; King et al., 2006; Ishii et al., 2007; Zoncu et al., 2012). Thus, our data suggest that some of the anti-carcinogenic and anti-aging effects of rapamycin treatment (Sharp and Richardson, 2011) might be explained by its effect on succinate dehydrogenase activity.

The relationship between complex II stimulation and mitochondrial respiration warrants further study. Our results show that rapamycin treatment increases the oxygen consumption of complex I and complex II mediated respiration, even though in vitro complex I activity is not affected under treatment with rapamycin (Figure 1D). This implies that the effects of rapamycin are mediated through the actions of other mitochondrial encoded proteins, which agrees with the data obtained by the metabolomic
profiling. Considering this, we hypothesize that metabolic shifts that lead to changes in the pool of reducing equivalents NADH and FADH$_2$ may underlie the effect of rapamycin on the \textit{in vitro} activity of complex II. To test this hypothesis, we measured the NAD$^+$/NADH ratio on mitochondrial isolates from the \textit{D.melanogaster} (OreR) and \textit{D. simulans} (sm21) mtDNA haplotypes. Rapamycin decreases NAD$^+$/NADH ratio only in OreR mitochondria (Figure S2F). Since NADH is a free cofactor to complex I and is added at non-limiting concentrations to the \textit{in vitro} reaction, rapamycin would not affect the \textit{in vitro} activity of complex I. Unlike NADH, FADH$_2$ is attached to complex II through a covalent flavin linkage (Cecchini, 2003), and changes in FADH$_2$ abundance will affect both \textit{in vivo} and \textit{in vitro} complex II activity.

The beneficial effect of rapamycin is diminished in flies carrying \textit{D. simulans} mitochondrial genomes with ~100 amino acid substitutions among the 13 protein coding subunits of OXPHOS complexes. The PCA analysis of metabolites extracted from mitochondrial isolates of \textit{sm21} (\textit{D. simulans}) mitochondrial genotypes showed that “non-responsive” mitochondria shifted the metabolite profile in a manner that did not permit additional shifts from rapamycin treatment. However, the analysis of metabolites extracted from whole flies showed a main effect of rapamycin treatment and parallel metabolic shift of both the “responsive” and “non-responsive” genotypes. Overall our data show that mitochondrial encoded genes are important for rapamycin benefits. These genes are mediating the ability of the drug to reprogram mitochondrial metabolism and increase mitochondrial efficiency. In this study, the enzyme activity and respiration analyses were performed on mitochondria isolated from whole flies. It is possible that...
different tissues have distinct mitochondrial responses to rapamycin, but it is unlikely that
the effects we report here are due to artifacts of tissue-specific activities. For example, if
different tissues have opposing effects of rapamycin, this would reduce our ability to
uncover the effects we see using whole-fly mitochondrial isolation. If some tissues are
responsive and others are unresponsive, again, the signal from whole-fly extractions
would be reduced compared to mitochondrial assays from a single tissue. At a minimum,
the mtDNA-dependent abrogation of the rapamycin effects we have described must be
operating in the majority of cells in the fly since our assays capture the activity of
mitochondria in proportion to the biomass of cell types in a whole fly. Future studies
addressing the specific effects of rapamycin on different tissues will allow us to better
understand the benefits of this drug and identify key tissues that mediate haplotype
differential responses.

The results presented here have uncovered an effect of rapamycin treatment on the
homeostatic nature of metabolic networks with a systems-level response. We described
novel roles for mtDNA-encoded genes in the effects of rapamycin, and offer a new set of
genetic reagents to examine the complex interactions governing TOR and mitochondrial
signaling. We have uncovered a novel association between complex II stimulation,
mitochondrial respiration, and rapamycin treatment that is modified by genes encoded in
the mitochondrial genome. Our experiments established that the stimulation of
respiration by rapamycin is not functionally dependent on the enhanced complex II
activity, implying that rapamycin may act on these two processes independently or
indirectly through shifts in metabolic state. These alternatives remain important
questions for future studies in this system. It would be fruitful to explore the variety of
epistatic interactions between pairs of nuclear and mtDNA genotypes that encode the
proteins mediating the mTOR and OXPHOS pathways.

Experimental Procedures

Fly stocks, husbandry The white Dahomey (w^{Dah}) strain is described in (Bjedov et al.,
2010). Mitochondria-nuclear substitutions strains are described in (Montooth et al.,
2010). All stocks were maintained and conducted under standard conditions (25 °C, 12 h
light:12 h dark) on normal media (11% sugar, 2% autolyzed yeast, 5.2% cornmeal, agar
0.79% w/v in water and 0.2% tegosept -methyl 4-hydroxybenzoate, from Sigma- St.
Louis, MO, USA). All stocks were density controlled in replicate vials using 48-hour
egg lays by 5 pairs of parents for two generations prior to collection of flies for
experimental assays.

Rapamycin treatment

Rapamycin was purchased from LC Laboratories (Woburn, MA, USA.). Rapamycin was
dissolved in ethanol and added to food at the final concentration of 200 µM, as described
in Bjedov et al., 2010. Newly eclosed adult flies were collected within 48 hours and
maintained in rapamycin treated or ethanol vehicle control food for 10 days.

Western immunoblotting

20 female flies per treatment were homogenized in homogenization buffer (1% Triton X-
100, 10mM Tris Base, pH 7.6, 5mM EDTA, 50mM NaCl, 30mM Na pyrophosphate,
50mM Na Fluoride, 100uM orthovanadate, complete protease inhibitor cocktail -Roche,
Following homogenization, samples were incubated on ice for 30 minutes and centrifuged at 16,000 g for 20 minutes. The supernatant was quantified for protein abundance using the BCA Protein Assay Kit from Thermo Scientific (Rockford, IL, USA.). 30 µg of total protein was loaded per lane. Proteins were separated on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and subjected to immunoblotting. Antibodies to dS6K1 were a gift from Thomas Neufeld, phospho-S6K1 was purchased from Cell Signaling Technology (Danvers, MA, USA.).

Mitochondrial/cytosolic isolation and DNA quantification

30 female flies were gently homogenized in 1mL chilled isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.5% fatty acid free BSA, pH 7.2) using a glass-teflon dounce homogenizer. The extracts were centrifuged at 300g for 5 minutes at 4°C. The obtained supernatant was then centrifuged at 6,000g for 10 minutes at 4°C to enrich for mitochondria. The pellet was resuspended in 100 µL of respiration buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5mM KH₂PO₄, pH 7.2). The supernatant was the cytosolic fraction. Freshly prepared mitochondrial isolates were used for respiration and ROS assays, or aliquoted and frozen at -80°C for later enzymatic activity assays. Quantification of mitochondrial protein obtained from the isolation was determined by Bradford Reagent (Sigma-Aldrich, St. Louis, MO, USA) following the manufacture’s protocol. For DNA quantification, RNA from the mitochondrial and cytosolic fractions was removed by RNase A treatment from Quiagen (Valencia CA, USA), and the amount of DNA was determined spectrophotometrically (NanoDrop ND-1000).
Protein synthesis was measured as the incorporation of $^{35}$S-Methionine into protein. 20 female flies were treated with rapamycin for 10 days and maintained in 100uCi/ml $^{35}$S-Methionine plus rapamycin or vehicle control food. After 48 hours of incubation, flies were sorted to separate mitochondrial and cytosolic fraction as described above. To quantify proteins in mitochondria and cytosol isolates using the same method (BCA in this case) a different isolation buffer that lacks BSA (210mM mannitol, 70mM sucrose, 5mM Hepes, 1mM EDTA, pH 7.35) was used to homogenized flies. Proteins were precipitated in 10% trichloracetic acid (TCA) and the quantity of radiolabeled methionine incorporated into protein was measured in both fractions using a Beckam LS 6500 multi porpoise scintillation counter.

Mitochondrial respiration and H$_{2}$O$_{2}$measurement

Respiration rates were determined by oxygen consumption using a Clark-type electrode and metabolic chamber (Hansatech Instruments, Norfolk, UK). 5µM of pyruvate plus 5µM malate, or 10µM succinate plus 0.5µM rotenone was added to an isolated mitochondrial suspension in 1mL or respiration buffer held in the respiration chamber at 30°C. 125 nmol of ADP was added to generate state 3 respiration rates. Uncoupled respiration rates, indicative of maximal rate of O$_{2}$ consumption, were generated by the addition of 0.5 nM of FCCP (Carbonyl cyanide 4-(trifluoro-methoxy) phenylhydrazone), a chemical uncoupler.
H₂O₂ production was measured using an Amplex Red/horseradish peroxidase assay from Invitrogen, (Carlsbad, CA, USA.) following the manufacturer’s protocol. 20 µM of glycerol 3-phosphate or 5µM of pyruvate plus 5µM malate were used as substrate. Qualitatively identical results were obtained with both substrates.

Enzymatic assay and membrane potential measurement and NAD+/NADH measurement

Enzymatic assays were modified from ((Barrientos, 2002) and are described in (Meiklejohn et al., 2013). The specific activity of complex I was determined as the rotenone sensitive rate following the oxidation of NADH at 340 nm with the coenxyme Q analog Decylubiquinone as the electron acceptor (Reaction mixture containing 35 mM NaH₂PO₄, 5 mM MgCl₂, 2.5 mg/mL BSA, 2 mM KCN, 2 µg/mL Antimycin A, 100 µM NADH, 100 µM Decylubiquione, [2 mM Rotenone to inhibit reaction]). The catalytic activity of complex II was monitored by the reduction of DCPIP at 600 nm (Reaction mixture containing 30 mM NaH₂PO₄, 100 µM EDTA, 2 mM KCN, 2 µg/mL Antimycin A, 2 µg/mL Rotenone, 750 µM BSA, 10 mM Succinate, 100 µM DCPIP, 100 µM Decylubiquinone [400mM Malonate to inhibit reaction]). Complex III activity was measured by monitoring the reduction of Cytochrome c at 550 nm (Reaction mixture containing 35 mM NaH₂PO₄, 2.5 mg/mL BSA, 5 mM MgCl₂, 2 mM KCN, 2 µg/mL Rotenone, 50 µM Cytochrome c, 25 µM Decylubiquinol, [5 µg/mL Antimycin A to inhibit reaction]). Potassium borohydride was used to reduce decylubiquione. Complex IV activity was measured by determining the rate of oxidation of reduced cytochrome c at 550 nm (Reaction mixture containing 5 mM MgCl₂, 2 µg/mL Rotenone, 2 µg/mL Antimycin A, 1 mM DDM, 45 µM Cytochrome c [4 mM KCN to inhibit reaction]).
Sodium dithionite was used to reduce cytochrome c. To measure citrate synthase activity, the rate limiting reaction of citrate synthase was coupled to a chemical reaction in which DTNB reacts with CoA-SH and the absorbance of the product is measured at 412 nm (Reaction mixture containing 100 µM DTNB, 300 µM AcetylCoA, 100 mM TrisHCl, 300 µM Oxaloacetic Acid).

JC-1 indicator dye from AnaSpec (Fremont, CA, USA.) was used to measure membrane potential in isolated mitochondria. 30 female flies were gently homogenized and the mitochondria were extracted as described above. The mitochondrial pellet obtained was resuspended in 300µL of respiration buffer. 3uL of a 1µg/uL solution of JC-1 dissolved in DMSO was added to the suspension. Mitochondrial samples were incubated for 15 minutes at 37 degrees protected from light. Samples were centrifuged for an additional 2 minutes at 6000g and resuspended in 600uL of fresh respiration buffer. Fluorescence was measured for red (excitation 550 nm, emission 600 nm) and green (excitation 485 nm, 535 nm) corresponding to the monomeric and aggregate forms of JC-1.

NAD+/NADH and NADH were measured Amplite™ Flourimetric NAD/NADH Ratio assay kit (#15263) from AAT Bioquest (Sunnyvale, CA, USA). Mitochondria from 20 females were extracted as described above. Reaction was performed following the manufacture’s protocol.

Metabolomic profiling

Metabolomic profiles of rapamycin treated flies were performed by a Metabolon (Durham, NC) using Metabolon’s standard solvent extraction methods (proprietary information). The extracted samples were split into equal parts for analysis on the
GC/MS and LC/MS/MS platforms. A total of 6 replicates per sample were performed.

Technical replicate samples were created from a homogeneous pool containing a small amount of all study samples. Values for each sample were corrected by Bradford protein quantification. Each compound was then normalized by median value for each run-day block (block normalization). Missing values were imputed with the observed minimum for that particular compound.

Statistical Analysis

Comparisons between two treatments were performed using the unpaired t-test and linear regression as noted in the figure legends. Analysis of variance was used in cases of multiple comparisons. Differences were considered significant at $p<0.05$, $p<0.025$, $p<0.0125$ as noted in the figure legends. Statistical analyses were performed using the R statistical package unless otherwise specified.

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References


Figure legends

Figure 1. Effects of rapamycin on mitochondrial functions. (A) Oxygen consumption during state 3 (ADP added) and after addition of FCCP uncoupler (uncoupling) in two D. melanogaster laboratory strains (w^{Dah} and OreR) fed with rapamycin for 10 days. Results are shown as the ratio of rapamycin to vehicle control treatment. Ratios are considered
significantly different by Wilcoxon signed rank test. State-3 \([V = 0, p\text{-value} = 0.00]\), uncoupling \([V = 1, p\text{-value} = 0.00]\). \(V\) = sum of ranks in which the ratio 
rapamycin/control is below 1. (B). \(\text{H}_2\text{O}_2\) production of mitochondria isolated from \(w^{\text{Dah}}\) and OreR flies treated with rapamycin or vehicle control for 10 days. * \(p< 0.05\) versus control as determined by ANCOVA: \(w^{\text{Dah}}\) [treatment: \(F\) value: 835.9, \(p\text{-value} < 0.00\)], OreR [treatment: \(F\) value: 161.3, \(p\text{-value} < 0.00\)]. (C) Enzymatic activity of complex II in isolated mitochondria from \(w^{\text{Dah}}\) and OreR flies treated with rapamycin or vehicle control for 10 days. Enzymatic activity was normalized to sample protein content. * \(p< 0.05\) versus control as determined by t-test. \(w^{\text{Dah}}\) t-test \([p\text{-value} = 0.004]\), OreR t-test \([p\text{-value} = 0.003]\). (D) Effects of rapamycin on Complex I and Complex II-mediated respiration. Oxygen consumption during state 3 (ADP added) of complex I (pyruvate and malate, or pyruvate, malate and malonate), complex II (succinate and rotenone) of mitochondria from flies fed with rapamycin for 10 days. Results are shown as the ratio of rapamycin to vehicle control. Ratios are considered significantly different by Wilcoxon signed rank test \([V = 0, p\text{-value} = 0.004]\).

**Figure 2.** Effects of rapamycin on *Drosophila* from divergent mitochondrial-nuclear lineages (A) mtDNA phylogeny between the four mitochondrial haplotype groups used (in boxes). The tree is based on amino acid sequences. Species names are followed by the mitochondrial haplotype. In parentheses are the lines from which the mtDNA was isolated (modified from (Montooth et al, 2010). Oxygen consumption during state 3 and after adding FCCP uncoupler (B), \(\text{H}_2\text{O}_2\) production (C), and enzymatic activity of complex II (D) of mitochondria isolated from the divergent mitochondrial-nuclear lineages after 10 days treatment with rapamycin. Enzymatic activity was normalized to
sample protein content. \( p < 0.0125 \) versus control as determined by t-test. T-test in panel C: [p-value <0.00], panel D: OreR t-test[p-value = 0.004], Zim53 t-test[p-value = 0.007]. Small boxes in (B), (C), and (D) represent the effect of rapamycin in mitochondrial respiration, ROS production and Complex II activity from *D. melanogaster* and *D. simulans* mitochondria. ANOVA: State 3 [F value: 10.258, p-value: 0.004 **], uncoupling [, F value: 9.9567, p-value: 0.005 **], H2O2production [Df:1, F value: 9.9567, Pr(F): 0.005 **], Complex II [ F value: 6.918, p-value: 0.01 *].

**Figure 3. Metabolic reprogramming of whole fly extracts by mtDNA genotype and rapamycin treatment.** Principle components analysis was performed on a sample of 210 metabolites detected in the whole fly extracts from flies carrying OreR and sm21 mtDNA genotypes treated with vehicle control or 200 µm rapamycin. (A) PCA of 210 metabolites identified. (B). PCA of 50 amino acids or amino acid derivatives. (C). PCA of 26 carbohydrates. (D). PCA of 80 lipids. Complete lists of these metabolites are provides in supplementary Table S1. Black open squares = OreR mtDNA on control diet; blue solid squares = OreR mtDNA on rapamycin; red open triangles = sm21 mtDNA on control diet; green solid triangles = sm21mtDNA on rapamycin. Polygons surrounding points are intended to aid the visualization of the six replicate samples for each treatment. See text and Figure S2 for statistical analyses.

**Figure 4. Metabolic reprogramming of mitochondrial extracts by mtDNA genotype and rapamycin treatment.** Principle components analysis was performed on a sample of 230 metabolites detected on the mitochondrial extracts from flies carrying OreR and
sm21 mtDNA genotypes treated with vehicle control or 200 µm rapamycin. (A) PCA of all 230 metabolites. (B) PCA of 39 amino acids or amino acid derivatives. (C) PCA of 20 carbohydrates. (D) PCA of 83 lipids. Complete lists of these metabolites are provided in supplementary Table S1. Black open squares = OreR mtDNA on control diet; blue solid squares = OreR mtDNA on rapamycin; red open triangles = sm21 mtDNA on control diet; green solid triangles = sm21 mtDNA on rapamycin. Polygons surrounding points are intended to aid the visualization of the six replicate samples for each treatment. See text and Figure S2 for statistical analyses.

Figure 5. PCA analysis of metabolites involved in energy homeostasis (cofactors, vitamins and Krebs cycle intermediates) from whole fly extracts (A) and mitochondrial extracts (B). Complete lists of these metabolites are provided as supplementary Table S1. Black open squares = OreR mtDNA on control diet; blue solid squares = OreR mtDNA on rapamycin; red open triangles = sm21 mtDNA on control diet; green solid triangles = sm21 mtDNA on rapamycin. Polygons surrounding points are intended to aid the visualization of the six replicate samples for each treatment.

Table 1. Mitochondrial metabolite change between haplotypes. A list of metabolites within the mitochondrial extract that differs between OreR and sm21 haplotypes after rapamycin treatment. Results are shown as the % of change after rapamycin treatment. Positive and negative signs indicate increased and decreased abundance respectively. nc represents no change. *p* < 0.05 for all metabolites represented.

Figure S1. Effects of rapamycin on protein synthesis. (A) Western blot analysis of mTOR target S6K1. Flies were fed with rapamycin and extracts were made from whole
flies. Antibodies used were against p-S6k1 and dS6K1. (B) Level of $^{35}$S-methionine incorporation in proteins located in the cytosol and in the mitochondria of flies treated with rapamycin and vehicle control (ethanol). *p< 0.05 versus control as determined by t-test. t-test [$t = 3.4572, df = 6, p$-value $= 0.01351$]. (C) Amount of nuclear DNA and mitochondrial DNA in flies treated with rapamycin and vehicle control (ethanol).

**Figure S2. Effects of rapamycin on ETC complexes, citrate synthase, mitochondrial membrane potential and NAD+/NADH ratio. Related to Figure 1.** Enzymatic activity of complex I (A), complex III (B), and complex IV(C), citrate synthase (D) in isolated mitochondria from $w^{Dah}$ and OreR flies treated with rapamycin or vehicle control for 10 days. Enzymatic activity was normalized to sample protein content. (E) Mitochondrial membrane potential (measured as a ratio of fluorescence at 525/590 emission wavelength (monomer/dimer of JC-1 protocol) higher membrane potential have higher 525/590 ratios. (F) (Ratio between NAD$^+$ and NADH in OreR and sm21 mitochondria isolates from flies treated with rapamycin or vehicle control for 10 days. *p< 0.025 versus control as determined by t-test.

**Figure S 3.** PCA analysis using default options of Multivariate Analyses of the statistical software JMP. Principle components were extracted from the correlation matrices for each plot, using the same input data set as used Figure 3, 4 and 5. Some factor axes are reverse relative to Figure 3A and 5A, but all points are in same relative positions. Ellipses are fit as bivariate normal distributions defining 95% confidence limits of the six
replicate samples for each genotype-rapamycin treatment. Non-overlapping ellipses can
be taken as significantly different samples in metabolite space.

Table S1. List of metabolites described on Figure 3, 4 and 5

Table S2. List of PCA summaries from figure 3, 4 and 5
Table 1

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A. All Metabolites

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C. Lipids

D. Carbohydrates

Mitochondrial extracts:
- **OreR Control**
- **OreR Rapa**
- **sm21 Control**
- **sm21 Rapa**
metabolites involved in energy homeostasis

A  Whole fly

B  Mitochondrial pellet

- **OreR Control**
- **OreR Rapa**
- **sm21 Control**
- **sm21 Rapa**