METABOLIC DETERMINANTS OF LONGEVITY:
DIETARY SUPPLEMENTATION AND MITOCHONDRIA

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METABOLIC DETERMINANTS OF LONGEVITY:

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Aging is a natural and inevitable process that leads to the irreversible impairment of biological functions and increased vulnerability to death. Great efforts have been spent to study the mechanisms underlying the aging process, with the ultimate goal of developing effective interventions to delay the onset of aging and to extend lifespan. Mitochondria, more than just being the powerhouses of the cell, are involved in a wide range of cellular and metabolic processes. Multiple lines of evidence have suggested that mitochondria play a vital role in the process of aging. Therefore, my research aims to investigate the effect of both dietary supplements and mitochondrial DNA content on lifespan.

Chapter one reviews the contemporary knowledge of mitochondrial DNA genetics and the implication of mitochondrial dysfunction in human diseases. It also summarizes the interaction between mitochondria and different dietary components or dietary patterns. I want to emphasize the importance of investigating the role of mitochondria in the development of complex diseases, and to suggest future directions in identifying nutritional interventions to restore mitochondrial functions.

Chapter two and Chapter three describe the discoveries of two compounds that extend the lifespan of *Drosophila melanogaster*. The first one is konjac glucomannan

hydrolysate, which can promote longevity in both genders across different genetic backgrounds, and regardless of mating status. By investigating the potential underlying mechanisms, we found it extends the lifespan through promoting the growth of gut microbiome and preserving the intestinal proliferative homeostasis. The second compound is oligofructose, the hydrolyzed form of inulin. It can also prolong the lifespan but is restricted to specific genetic backgrounds and mating status. Revealed by the transcriptome analysis, it is very likely that it extends lifespan through the inhibition of mitochondrial respiration and suppression of stress signaling pathways.

Chapter four describes an exploratory project on the relationship between mitochondrial DNA content and lifespan in both normal and stress conditions. By implementing both survival test and transcriptome analysis, we found a possible connection between mtDNA copy number and the lifespan in male flies through regulation of fertility.

BIOGRAPHICAL SKETCH

Yuan Si was born in Zhengzhou, a city in east-central China, and moved to Shanghai with her family later. She grew up in a family with a strong biological background and was exposed to her parents' knowledge of general biology in daily life. With this special family environment, she became interested in biology since her childhood. In high school, she participated in a research project studying the co-evolution of plants and insects, which gave her the first chance to experience the charm and fun of scientific research. With this project, she had the opportunity to expand her horizon in multiple domestic and international scientific competitions. In 2008, she started her undergraduate study at Fudan University in biology major and was determined to pursue an advanced degree in a biology-related field afterward.

Before attending graduate school at Cornell University, Yuan worked for half a year as an intern in Dr. Xu Lin's lab at the Institute for Nutritional Sciences, Chinese Academy of Sciences. This experience brought her to the forefront of nutritional research and convinced her to continue her higher education in this direction. In the past five years, Yuan has been working under the mentorship of Dr. Zhenglong Gu to investigate the effect of dietary supplements and mitochondrial DNA content on longevity using *Drosophila melanogaster* as the model organism.

To my dear parents,

Shengli Si, Xuexia Miao

and my husband,

Kaixiong Ye

for your endless love and support.

这篇论文谨献给我最亲爱的

父亲司胜利，母亲苗雪霞

感谢你们的培养教育和无限的爱

先生叶凯雄

感谢你对我的支持理解和爱

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Chapter 1 – Current knowledge of mitochondrial genetics and the interaction between dietary components and mitochondrial function

1.1 Introduction

Mitochondria are double membrane organelles, presenting in almost all eukaryotic cells. Different from other organelles, mitochondria host their own genome: mitochondrial DNA (mtDNA). The primary function of mitochondria is the production of ATP, supplying over 90% of cellular energy (1). Mitochondria are also responsible for a series of cellular processes, including calcium signaling, iron homeostasis, and cell apoptosis (2, 3). In addition to the well-established mitochondrial diseases, such as Leber hereditary optic neuropathy (LHON), mitochondrial encephalomyopathy, and lactic acidosis and stroke-like episodes (MELAS), a growing spectrum of human diseases have been found to be associated with mitochondrial dysfunction, including cancer, neurodegenerative diseases, and metabolic disorders (3-7). Multiple molecular mechanisms have been proposed to explain the precise roles of mitochondria in these pathological processes, such as the excessive generation of reactive oxygen species (ROS), the accumulation of mtDNA mutations, and the mitochondria-mediated apoptosis. However, there is still no consensus on these issues. On the other hand, understanding the contributions of mitochondrial dysfunction to these common diseases may suggest a new way of intervention just by preserving mitochondrial functions. Besides drugs, many nutrients actively interact with mitochondria and have the potential to protect and improve mitochondrial functions. Disentangling the effects of various nutrients on mitochondria and elucidating the underlying molecular mechanisms will assist the development of new strategies for treating complex diseases.

In this review, we will start with a brief overview of the mtDNA genetics and the implications of mitochondrial dysfunction in human diseases with a focus on mtDNA mutations. We will then summarize the interactions between mitochondria and different dietary components or dietary patterns.

1.2 Current knowledge of mitochondrial DNA

mtDNA genetics

Compelling evidence suggests that mitochondria were once primitive bacterial cells, and were acquired by the host through an endosymbiotic event. Along the way of endosymbiosis, the bacteria became double membrane organelles and gradually transferred genes to their symbiotic cell nucleus, with only a few genetic materials retained as mtDNA (3, 8, 9). Human mtDNA is a circular double-stranded molecule comprising 16,569 base pairs. The two strands are distinguished by their molecular weight: a guanine-rich heavy strand and a cytosine-rich light strand. mtDNA encodes 13 peptides, serving as core subunits of the five enzyme complexes in the oxidative phosphorylation (OXPHOS) system. mtDNA also encodes 2 rRNA and 22 tRNA, which are essential for intra-mitochondrial protein synthesis (Figure 1.1).

Unlike human nuclear DNA (nDNA), mtDNA is condensed with genes. About 93% of its entirety encodes genes, which also lack intronic regions. The 13 protein-coding genes are separated by tRNA or 1~2 non-coding bases. The non-coding region is mainly located in the displacement loop (D-loop), which hosts the mtDNA replication initiation

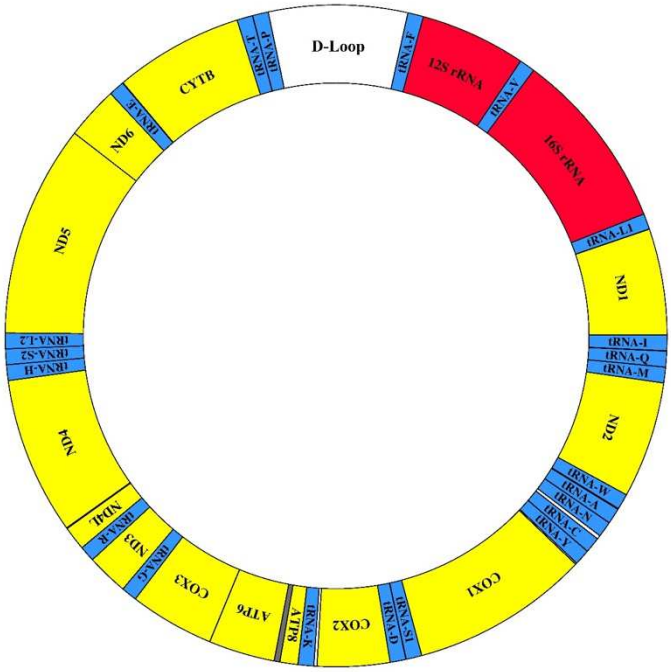

Figure 1. 1 Schematic diagram of the human mitochondrial genome.
Human mtDNA is a 16,569 bp double-stranded circular DNA molecule. 13 protein-encoding genes are shown in yellow blocks, 22 transfer RNA genes are shown in blue blocks and 2 ribosomal RNA genes are shown in red blocks. Figure was generated with mtviz (http://pacosy.informatik.uni-leipzig.de/mtviz)

site and two H-strand transcription promoters. Because of this functionally dense organization, nucleotide substitutions in mtDNA are more likely to cause functional outcomes than nDNA mutations.

mtDNA heteroplasmy

In comparison to only two copies of nDNA in a single cell, there are hundreds to thousands of copies of mtDNA. As a result, the mutation could be present in all copies of mtDNA (homoplasmy) or only a proportion of them (heteroplasmy), as illustrated in Figure 1.2. The proportion of mutant copies is referred as heteroplasmy frequency. This frequency critically determines the phenotypic effect of a specific mutation. It has been suggested that there is a "phenotypic threshold effect". At low heteroplasmy frequencies, the deleterious effect of mutant mtDNA is masked by coexisting wild-type copies, and once exceeding a threshold value (typically 60%-80%), mutant mtDNA will result in an altered phenotype (Figure 1.2) (10-12). This frequency threshold varies across mutations and tissues (3). The heteroplasmy frequency of a specific mtDNA mutation can vary across individuals. Take mutation 3243A>G as an example, which is the most common pathogenic heteroplasmic mutation and can cause multiple mitochondrial diseases, including MELAS, chronic progressive external ophthalmoplegia (CPEO), and Kearns–Sayre syndrome (KSS). Rajasimha *et al.* examined the frequencies of this mutation in 275 individuals and found they range from a few percent to higher than 80% (13). It has also been reported that heteroplasmy frequency is highly tissue-specific within the same individual (14). Heteroplasmy 72T>C showed high frequencies in the liver and kidney, a moderate frequency in skeletal muscle and low frequencies in other

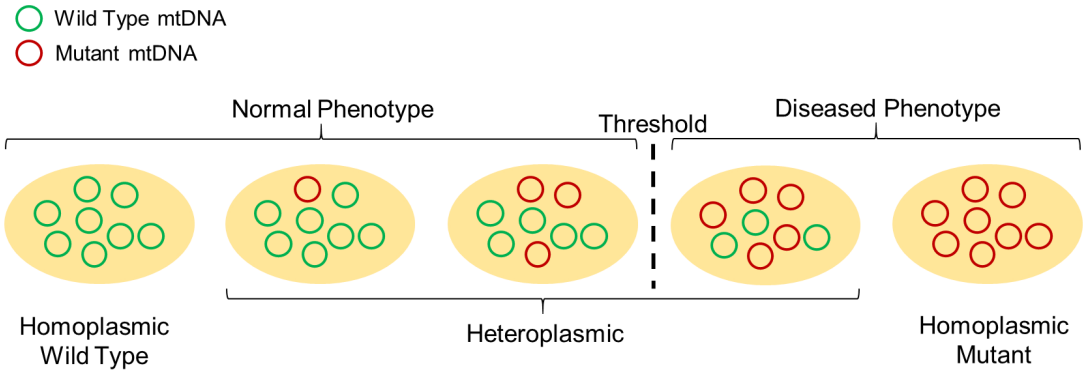

Figure 1. 2 mtDNA heteroplasmy and the threshold effects

The mtDNA within the same cell can be all identical (homoplasmic) or a mixture of wild-type and mutant (heteroplasmic). The cells can contain different proportions of mutated and wild-type mtDNA (referred as heteroplasmy frequency). The heteroplasmy frequency is critical for the mutations' pathogenicity. If the heteroplasmy frequency is below a certain threshold, the cell can maintain normal phenotypes. Once the frequency exceeds the threshold, the cell will show signs of mitochondrial dysfunctions.

tissues (14). While variations across individuals and tissues are well-established, the heteroplasmy frequency variation at the single cell level is still controversial. Jayaprakash *et al.* examined mtDNA heteroplasmy in colonies derived from single cells and found that heteroplasmy frequency was stably maintained in individual daughter cells (15). On the other hand, Neupane *et al.* reported that in mouse embryonic stem cells, heteroplasmy frequency could vary up to 61% between individual cells. This wide variability in stem cells may explain the existence of tissues with different heteroplasmy loads in an individual (16). Our lab is developing an efficient and accurate method to sequence single-cell mtDNA, which will help elucidate the heteroplasmy dynamics at the single-cell level in future studies.

Heteroplasmy sources and changes during lifetime

Taking advantage of the recent advance in next-generation sequencing (NGS) technology, several studies have demonstrated that most of the individuals, if not all, have heteroplasmy in their mitochondrial genomes (17, 18). Heteroplasmic mutations may be inherited mutations from maternal mtDNA, or *de novo* mutations arising during embryonic development. Unlike nuclear genome which is transmitted by sexual reproduction, the human mitochondrial genome is strictly maternally transmitted. Although inherited from a single parent, extensive differences of mtDNA heteroplasmy frequency between mothers and offspring, and among siblings have been observed (19, 20). For example, Li *et al.* found that the average difference in heteroplasmy frequency between mothers and offspring was 10.8%, with a maximal of 78.7% in a Netherlands cohort (21). These variations could be a result of the mitochondrial bottleneck effect:

During oocytes development, only a small number of mtDNA are sampled from primordial germ cells and transmitted to primary oocytes, leading to the drastically varying heteroplasmy frequencies among mature oocytes (Figure 1.3). Because of the bottleneck, low-frequency mutant mtDNA has a relatively lower probability to be transmitted to the next generation. But if they were selected during the bottleneck, the frequency of the mutant mtDNA could dramatically increase. In extreme cases, this process may also allow the frequency of a disease mutation to reach the phenotypic threshold within a single generation, resulting in childhood-onset mitochondrial diseases or even complex diseases, such as autism spectrum disorder (ASD). Additionally, recent evidence has shown that there is a strong purifying selection of mtDNA during the maternal germline development and the embryogenic development (22, 23), which help prevent or reduce the transmission of deleterious mtDNA mutations.

mtDNA heteroplasmy can also be a result of somatic mutations. The triggers of mtDNA somatic mutations are still highly debated. According to a classic theory, it is the immensely oxidative microenvironment of mitochondria that causes mtDNA mutations. However, an emerging hypothesis argues that most mtDNA mutations originate from replication errors because the mtDNA mutation signatures are dominated by transition changes (7, 24-26). mtDNA is replicated constantly through the lifetime and is independent of the cell cycle. Moreover, the mtDNA replication and repairing systems are less accurate than nDNA (27, 28). Therefore, both dividing cells and post-mitotic cells can accumulate mtDNA mutations, especially heteroplasmic mutations over time. A newly introduced mutation in a single mtDNA molecule is possible to clonally expand

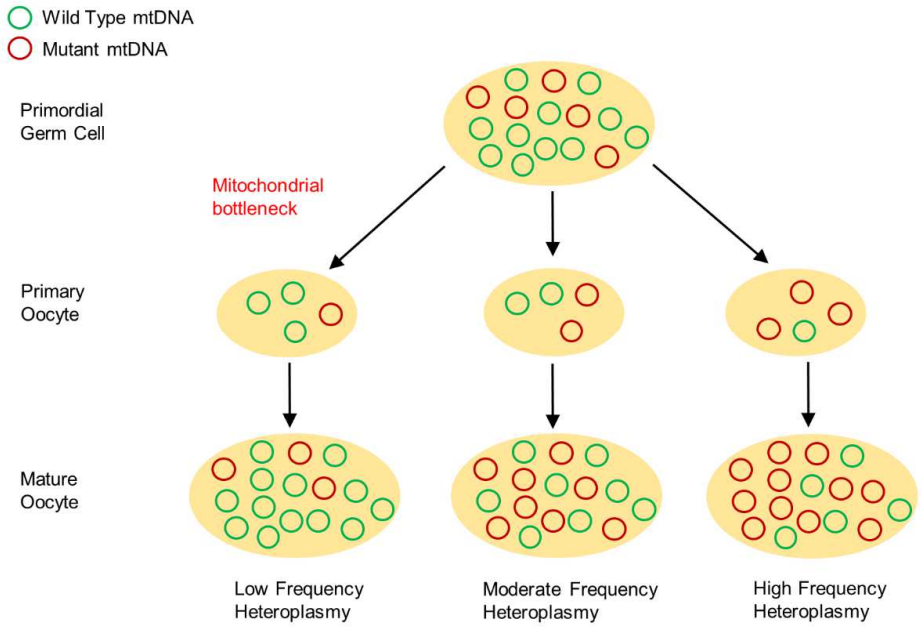

Figure 1. 3 mtDNA heteroplasmy variations caused by the mitochondrial bottleneck. The transmission of mtDNA heteroplasmy from mother to offspring is affected by the mitochondrial bottleneck effect during the egg development. There is a significant reduction of mtDNA copy number from primordial germ cells to primary oocytes, meaning that only a small proportion of mtDNA will be sampled and transmitted to primary oocytes, leading to extensive variation of heteroplasmy frequency in the mature oocytes.

to a higher frequency and even to reach the phenotypic threshold. Computational models are suggesting that mtDNA mutations arose early in life have sufficient time to reach the phenotypic threshold and to cause mitochondrial dysfunctions, simply by random genetic drift (29).

1.3 mtDNA heteroplasmy implication in diseases

Overview

Mitochondrial dysfunction is implicated in a broad spectrum of human diseases. Apart from the classic mitochondrial diseases, such as MELAS and LHON (30-34), emerging evidence associates mtDNA mutations with common human diseases. In early studies, technology limitations drastically affect the sensitivity and resolution to identify mtDNA heteroplasmy, thus the implication of heteroplasmy in common and complex diseases might be overlooked. The advent of NGS technologies offers a new opportunity to revisit the connection between heteroplasmy and different diseases. In the following section, we will provide only a brief recapitulation of the roles of mtDNA mutations in classic mitochondrial diseases, and focus most of our discussion on the recent insights into their implications in common and complex human diseases.

Mitochondrial diseases

"Classic" or "primary" mitochondrial diseases refer to a group of diseases caused by defects in OXPHOS, which are results of mutations in nDNA-or mtDNA-encoded mitochondrial genes. Some mtDNA mutations can contribute to several different mitochondrial DNA diseases. The most common disease-causing mutation 3243A>G is

associated with CPEO, MELAS, maternally inherited diabetes and deafness (MIDD) (35). On the other side, a specific mitochondrial disease can be caused by a set of mutations. To date, mutations located in > 75 genes (in both mitochondrial and nuclear) have been identified to be involved in Leigh syndrome (36). mtDNA mutations 3460G>A, 11778G>A and 14484T>C were found in both homoplasmic and heteroplasmic status in LHON families (37). Advances in NGS technology helped the elucidation of the genetic basis of mitochondrial diseases and their diagnosis, but the treatment of these diseases remains a challenge, the fast development of DNA editing techniques could be a promising direction which has the potential to fix the ETC defects at the DNA level.

Neurodegenerative diseases

The central nervous system is highly energy-demanding and thus heavily depends on the mitochondrial energy production. mtDNA mutations and the associated OXPHOS activity reduction have been observed in several complex neurodegenerative diseases, such as Parkinson's Disease (PD), Alzheimer's Disease (AD) and ASD. Early work revealed that certain mitochondrial haplogroup is associated with different disease risks. Basically, researchers use the differences in mitochondrial DNA to define the mitochondrial haplogroup, with different letters representing different subpopulations. For example, haplogroup J represents a subpopulation originated from Eurasia and has been confirmed to have reduced risk of PD in a meta-analysis study (38), while haplogroup H, U, K, T, I, W and X are associated with increased risks of AD (39-43). However, it is still unclear about the role of heteroplasmic mutations in these diseases,

and we will summarize recent findings of mtDNA heteroplasmy in complex neurodegenerative diseases.

Lin *et al.* first found that mtDNA heteroplasmic mutations are significantly elevated in the substantia nigra of early PD patients (44). As revealed by Coxhead *et al.*, PD patient brains are more vulnerable to mtDNA mutations by analyzing different regions in the brain. Heteroplasmic nonsynonymous variants in several mitochondrial genes are also overrepresented in PD patients (45). Considering AD, it has been reported that mtDNA heteroplasmic mutations are overrepresented in the hippocampus of early-stage patients, and the mutation signature is consistent with replication errors rather than oxidative damages (46). These reports support that the accumulation of mtDNA mutations may contribute to the neurodegeneration in both PD and AD. Unlike PD and AD, Huntington's disease (HD) has elucidated genetic causes, the autosomal dominant repeat expansions in the Huntington gene. However, mitochondria still play an important role in HD progression. While mtDNA depletion and deletion are reported in several studies (47-50), there are very few studies about heteroplasmy. Filosto *et al.* reported the 5613T>C heteroplasmy could cause chronic progressive external ophthalmoplegia in HD patients (51). Our lab is conducting a large-scale study of mtDNA sequencing in HD patients, which will systemically investigate the involvement of mtDNA heteroplasmy in this disease and assist the development of novel strategies to postpone the onset of the disease or even to counteract its progression. In addition to these age-related neurodegenerative diseases, ASD, which usually affects pre-pubertal children, is also associated with mitochondrial dysfunction. Our lab has analyzed 903 ASD children along with their unaffected siblings and mothers. We found that

nonsynonymous and predicted pathogenic heteroplasmic mutations are enriched in autistic probands. Moreover, a large fraction of these mutations is inherited (52), indicating that evaluating mtDNA heteroplasmic mutations in high-risk families may help early diagnosis and treatment of ASD.

Cancer

Reprogrammed energy metabolism is a hallmark of cancer (53). Otto Warburg first observed the rewiring of cancer cell energy metabolism: mitochondrial respiration and OXPHOS are suppressed, substituted by a strong enhancement of glycolysis even in the presence of oxygen. This phenomenon was termed as "aerobic glycolysis" (54-56). The depression of mitochondrial activity in cancer cells may have various reasons. Recently, disruption of mitochondrial respiration complexes due to detrimental mtDNA mutations has been suggested to be one neglected reason.

Several studies have reported that mtDNA somatic mutations are frequently found in tumor tissues (57-59). With precise quantification achieved by NGS, in 10 matched tumor-normal colorectal tissues, He *et al.* found 90% of the cancer tissues had at least one cancer-specific mtDNA point mutation (present in cancer tissue, but not present in the matched normal tissue), and most of these cancer-specific mutations are heteroplasmic rather than homoplasmic (60). Davis *et al.* also found loss-of-function heteroplasmic mutations in NADH dehydrogenase subunit by sequencing mtDNA of 66 chromophobe renal cell carcinomas (61). A more comprehensive study was conducted later by comparing mtDNA sequences from 1,657 cancer and matched normal tissues in 31 cancer types. This study identified 1,907 cancer-specific somatic substitutions,

most of which are heteroplasmic (24). Notably, this study also suggested that the majority of mutations are generated from mtDNA replication errors rather than external mutagens such as ROS, cigarette smoking, and UV light. However, the understanding of how mtDNA mutations affect mitochondrial function and cellular metabolism is still limited. Hardie *et al.* investigated the genotype-phenotype relationships between mtDNA mutations and pancreatic cancer, discovering that heterogeneous genomic landscapes of cancer can converge towards common metabolic phenotypes, including reduced oxygen consumption and increased glycolysis (62).

It is apparent that mtDNA mutations, including heteroplasmic mutations, are widespread in cancer cells. However, the precise role of mtDNA mutations in oncogenesis is currently unresolved. To answer these questions, future investigation should put effort into elucidating the causal mechanisms of mtDNA in cancer.

Aging

Aging is a degenerative process with gradually impaired physiological functions that eventually lead to deterioration of cellular function, disease, and death (7). During last several decades, multiple lines of evidence have shown that impaired mitochondrial function is implicated in aging and age-associated diseases (7, 63-65).

Accumulation of mutations in mtDNA during time can lead to severe impairment of cellular energy production and mitochondrial dysfunction (64). In humans, the accumulation of mtDNA mutations over time has been observed in both dividing cells and non-dividing (post-mitotic) cells, such as brain, muscle, and colon (66-69). The first

experimental evidence for the causative link between accumulation of mtDNA mutations and aging was from the mutator mice model. These mutator mice had proofreading-deficient mtDNA polymerase (Polγ), leading to accumulation of extensive mtDNA mutations. These mice had a reduced lifespan and premature onset of aging-related phenotypes such as weight loss, hair loss, reduced fertility, *etc.* (65, 70).

It has been proposed for decades that ROS generated during cellular metabolism can damage mtDNA, while the resulted mtDNA mutations would further lead to disruption of the electron transport chain (ETC), which produces more ROS, creating a vicious cycle. Recent studies have suggested that the age-associated mtDNA mutation accumulation is not from ROS damage, but rather from the spontaneous errors during the mtDNA replication. These replication errors will arise as low-frequency heteroplasmy and the potential subsequent clonal expansion of these heteroplasmic mutations would disturb the mitochondrial function (3). Therefore, managing the expansion of the mtDNA mutations could be critical for longevity. Eliminating the deleterious mtDNA mutations by targeted editing may become an effective approach to achieve this goal. We will include more details into the discussion about this topic in the last part of the review.

Mitochondria and epigenetics

Epigenetic modifications, such as histone acetylation and DNA methylation, can regulate the gene expressions without changing the DNA sequences. Recently, there is emerging evidence indicating that mitochondria and mtDNA could play a role in regulating nuclear DNA epigenetics. The first proof of this topic comes from Rho^0 cells,

which showed that the depletion of mtDNA would result in significant changes in methylation patterns of many genes, and potentially contribute to tumorigenesis. Bellizzi *et al*. then found individuals and cybrid cells (cells having the same nuclear background, but different mtDNA content) carrying mtDNA of J haplogroup have higher global DNA methylation levels than non-J carriers (71). Similarly, Atilano *et al*. demonstrated varying global methylation patterns between J and H haplogroup in cybrid cells. That study also found the expression of acetylation and methylation-related genes are different between J and H haplogroups, such as HAT1, DNMT1, *etc*., which may explain the methylation profile variations (72). On the other hand, such variations in global methylation profiles may also due to their varied capability to provide intermediate metabolites that are essential for the epigenetic modifications. For instance, S-Adenosyl methionine (SAM), a precursor in the methyl group transfer, is required for histone methylation. The generation of SAM relies on the folate cycle and ATP production, both of which depend on mitochondrial metabolism (73). Therefore, the impaired mitochondrial function may result in a decreased abundance of SAM and may have a further impact on histone methylation. Although we are still in an early stage of understanding the crosstalk between nucleus and mitochondria via epigenetic mechanisms, we believe that future work in this direction may open a new path of modifying epigenetic marks in the nuclear genome by targeting mitochondria.

1.4 Nutrients and mitochondrial function

Nutrients are key determinants of mitochondrial function. Not only that most mitochondrial components are derived from essential nutrients, but also that nutrients

and their metabolites directly modulate mitochondrial structure and function. The key roles of nutrients can be exemplified by observations that certain nutrient deficiency or overdose leads to mitochondrial dysfunction and that some dietary patterns (*e.g.* caloric restriction) may also restore normal mitochondrial activities. In the following section, we will focus on the literature investigating the relationships between mitochondrial functions and key nutrients from different categories such as lipids, vitamins, amino acids, *etc*. In addition, we will discuss mitochondrial interactions with special dietary patterns, such as caloric restriction and protein restriction.

PUFAs

Polyunsaturated fatty acids (PUFAs) are fatty acids containing more than one double bond in their backbones. They are commonly known as the "good" fat with the ability to prevent coronary artery diseases, type 2 diabetes, and atherosclerosis. The beneficial effects of PUFAs may come from their unique properties of being antiarrhythmic, lipid-lowering and anti-inflammatory, which have been intensively studied during the past decades. With rising evidence for the potentially key roles of mitochondria in the development of complex diseases like neurodegenerative diseases, a growing number of studies have been conducted to investigate the effects of PUFAs on mitochondrial functions. Considering that omega-3 PUFAs are among the most studied nutrients, we will summarize the impact of omega-3 PUFAs on mitochondrial functions. Omega-3 PUFAs are a group of PUFAs that have the first double bond at the third carbon from the omega (methyl) end. They are commonly found in flaxseed oil, fish oil, walnut, seafood, *etc.* Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two

well-known long-chain omega-3 PUFAs. Studies on different model organisms have demonstrated that supplementation of omega-3 PUFAs has a broad impact on mitochondrial activities, including increased mitochondrial content (74, 75), elevated expression of genes in regulating mitochondrial functions (76, 77), improved protein quality (78), *etc*. These effects of omega-3 PUFAs on mitochondria may be attributed to its influence on mitochondrial membrane integrity (79).

Mitochondrial membrane is the primary site for several important biochemical pathways including OXPHOS. High levels of omega-3 PUFAs have been observed in the mitochondrial phospholipid bilayers in eukaryotic cells, suggesting that they are essential for normal mitochondrial functions (80). In general, diet-induced changes in the lipid composition of cell membranes affect cell functions by altering membrane fluidity, permeability, structure, *etc*. (81). Maintaining mitochondrial membrane in high quality is essential in achieving critical functions, such as sustaining mitochondrial membrane potential, regulating the Ca2+ signaling, and supporting mitochondrial membrane-associated proteins (79, 82, 83). It is well-known that OXPHOS, one of the most important functions of mitochondria, is also embedded in the mitochondrial inner membrane. It has been shown in multiple studies that omega-3 PUFAs are incorporated into both cellular and mitochondrial membranes following dietary supplementation of fish oil (84). Accumulating evidence suggests that the beneficial effects of omega-3 PUFAs supplementation are mediated through the restoration of mitochondrial functions (85, 86). However, little attention has been paid to the effects of omega-3 PUFAs on mitochondrial membrane conditions. A recent study investigating the effect of fish oil supplementation on brain aging found that fish oil can restore the age-related

decrease in mitochondrial respiration and ATP production. Additionally, they found that fish oil ingestions can partially restore the membrane fluidity, the reduction of which is mainly due to the DHA loss in aged mice (87). Clearly, more studies should be undertaken to elucidate how omega-3 PUFAs exert their beneficial effects, although recovering mitochondrial functions while maintaining mitochondrial membrane integrity could be one of the potential mechanisms.

Short-chain fatty acids

The gut microbiota is a complex of microorganisms residing in the gastrointestinal tract. Research on gut microbiota has gained broad attention due to its association with a wide range of diseases, including cancer (88, 89), metabolic disorders (90-92), autoimmune diseases (93, 94) and even neurological disorders (95-97). Short-chain fatty acids (SCFAs) are the primary products of gut microbiota fermenting dietary fibers in the colon, including acetate, propionate, butyrate, *etc.* SCFAs have been shown to have multiple beneficial effects on host metabolisms and may play a role in the interactions between diet, gut microbiota, and host energy metabolisms (98). Butyrate and propionate are able to regulate gut hormones and protect against obesity induced by high-fat diet in mice (99). Butyrate can also improve insulin sensitivity and increase the energy expenditure in diet-induced obese mice (100). These observations may be explained by the elevated expression of peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) and mitochondrial uncoupling protein-1 (UCP-1) (101). PGC-1α is the master regulator of mitochondrial biogenesis which may enhance fatty acids oxidation by promoting mitochondrial function. Increased expression of

UCP-1 indicates a higher thermogenic activity which may direct more energy to heat generation rather than fat accumulation (100).

In addition to regulating host energy metabolism, SCFAs have been proposed to have the potential in cancer preventative interventions. Both propionate and butyrate can induce apoptosis in colon carcinoma cells (102, 103), while butyrate receives further attention because of its specialized role in colonocytes. Unlike other cells using glucose as the main energy source, colonocytes rely on butyrate to supply ~60–70% of their energy for the rapid cell proliferation of the colonic epithelium (104). Adding butyrate to germ-free colonocytes can rescue their deficit in mitochondrial respiration and prevent them from undergoing autophagy (104). But in most tumor cells including colorectal tumor cells, they have switched to glucose utilization and aerobic glycolysis to supply energy (105). Instead of being metabolized by mitochondria, butyrate accumulates in the nucleus to function as a histone deacetylase (HDAC) inhibitor to regulate genes which promote apoptosis and inhibit cell-cycle progression (106, 107). Apart from being an HDAC inhibitor, butyrate can also induce apoptosis through altering mitochondrial membrane potential (108), enhancing mitochondrial Ca^{2+} accumulation (109), and elevating cytochrome c level (110). Therefore, butyrate is thought to be the crucial mediator for the protective effect of gut microbiota against cancer. Supporting this hypothesis, it has been shown that inoculating mouse cancer models with butyrate-producing bacteria can reduce the tumor burden if a high-fiber diet is provided (107). It is noteworthy that as we discussed above, DHA can also regulate mitochondrial Ca^{2+} accumulation and mitochondrial membrane potential. As expected, the combination of DHA together with butyrate further enhances apoptosis in

carcinoma cells (111, 112). Although most studies on the anti-carcinogenic properties of SCFAs have focused on colon cancer, some researchers found that propionate also reduces cancer cell proliferation in the liver (113). More studies are needed to investigate the effects of other SCFAs on different tissues, and how SCFAs interact with other nutrients.

BCAAs/leucine

Branched-chain amino acids (BCAAs) are the amino acids having aliphatic side-chains with a branch, including three proteinogenic amino acids, leucine, isoleucine, and valine. In addition to being essential amino acids, which means that we have to acquire them from the diet, recent evidence suggests that supplementation of BCAAs also plays a role in controlling weight (114), preventing muscle atrophy (115, 116), and extending lifespan (117). It has been demonstrated that long-term consumption of a BCAA-enriched diet increased average lifespan of middle-aged male mice. This beneficial effect was accompanied by the observation of increased mitochondrial biogenesis and sirtuin 1 expression (118). Multiple independent studies supported this observation that BCAAs, especially leucine, have the ability to enhance mitochondrial functions by promoting mitochondrial biogenesis through the SIRT1 and AMPK pathways (119, 120), and to reduce oxidative burdens by upregulating the ROS scavenging system (121, 122). It is believed that most of the beneficial effects of BCAAs supplementation may be due to its key role in modulating mitochondrial functions (118).

Protein restriction

In addition to caloric restriction, which will be discussed in detail later, researchers have proposed the idea of dietary restriction (DR) to represent a broader scope of dietary interventions that have similar beneficial effects on aging and healthspan, such as restrictions on certain macronutrients and feeding patterns (123). Reduced intake of proteins and amino acids is the most efficient pro-longevity intervention among different types of macronutrient restrictions without limiting calorie intakes (124, 125). Studies on rodents demonstrated that low protein diets were associated with improved healthspan and increased lifespan (126, 127). By analyzing data from the National Health and Nutrition Examination Survey (NHANES), low protein intake was found to be associated with reduced overall mortality in people under 65 years old (128). Furthermore, limiting the intake of a single amino acid is also sufficient for lifespan extension. Animal studies suggested that both methionine and tryptophan restricted diets are able to extend the lifespan and reduce age-dependent deteriorations (129-131). Reduced production of mitochondrial ROS (mtROS) has been observed in both protein and amino acid restrictions, explaining at least part of the lifespan extension phenotype. Neither lipid nor carbohydrate restriction has similar effects on mtROS generation, in agreement with their lack of effect on longevity (132-135). Moreover, excessive administration of methionine leads to increased mtROS generation in rats (136).

Vitamins

Vitamins are a group of organic compounds that are essential for normal growth and metabolism, but most of them cannot be synthesized in human bodies. Although only needed in small quantities, vitamin deficiencies may cause severe outcomes including

the well-known diseases like scurvy (vitamin C), rickets (vitamin D), beriberi (thiamine), *etc*. Most vitamins are involved in metabolic reactions as catalysts or as coenzymes. In mitochondria, vitamins are required not only for the synthesis of some mitochondrial respiratory chain components but also for the essential processes like aerobic respiration and energy production. For example, vitamin B7 is the coenzyme of decarboxylases, which is required for gluconeogenesis and fatty acid oxidation (137). At the same time, vitamins with antioxidant properties such as vitamin C and vitamin E are crucial in protecting proteins, lipids, nuclear acids from oxidative injuries. This is extremely important for mitochondria since most free radicals are produced here. It was reported that vitamin E supplementation was used in treating oxidative phosphorylation disorders (138). Apart from the circumstances mentioned above, emerging evidence also suggests that vitamin D has the potential to improve mitochondrial functions (139-141), but the underlying mechanism is still unknown. In the following section, we will mainly discuss one fat-soluble vitamin and one water-soluble vitamin: vitamin A and vitamin B_3.

Vitamin A

Vitamin A is a group of organic molecules including retinol, retinal, retinoic acids, and carotenoids. They are essential for growth, development, vision, and maintenance of the immune system. Vitamin A can be obtained either directly from the animal source diet or by the intake and conversion of carotenoids from plant source diet. In a study that rats were fed a vitamin A-deficient diet, a decrease in the activity of complex I and complex II was observed in both liver and heart mitochondria and returned to normal

level when refeeding the control diet (142). Another study showed that vitamin A depletion causes oxidative stress and mitochondrial dysfunctions, indicating the role of vitamin A in regulating mitochondrial homeostasis (143). On the other hand, vitamin A supplementation has been demonstrated to impair mitochondrial functions by disrupting membrane organization and increasing ROS production, which may lead to further consequences like apoptosis (144, 145). Therefore, the dosage of vitamin A ingestion is crucial in preserving mitochondrial functions in healthy populations. Furthermore, vitamin A and its derivatives have been widely used as therapeutic agents in treating cancers (mainly leukemia) by triggering cell death or slowing cell cycle progression, thus to inhibit the growth of tumors (146, 147). In a T-cell leukemia cell line, researchers found that the apoptotic signals induced by vitamin A are initiated through reducing mitochondrial membrane potential and down-regulation of Bcl-xL (148). Bcl-xL locates in mitochondria and serves as an inhibitor of programmed cell death. Given the close interactions between vitamin A and mitochondria, if we can elucidate the molecular mechanism of the communication between vitamin A and mitochondria, we will be able to make better use of vitamin A in disease prevention and treatment.

Vitamin B

Vitamin B family is a class of water-soluble vitamins usually serve as coenzymes for the enzymes that are essential for cell metabolism. Whole or unprocessed foods like whole grains are good sources of B vitamins. They are also enriched in meats such as turkey, tuna, and liver. All the B vitamins play vital roles in maintaining mitochondrial functions and have been reviewed elsewhere in details (137). We will focus on niacin

(vitamin B_3) in this review, considering that it is the precursor for nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). Both are involved in the process of nutrient metabolism to generate energy and also in the maintenance of mitochondrial DNA (mtDNA) integrity. First, NAD^+ serves as electron carriers in beta-oxidation, glycolysis, the TCA cycle, and oxidative phosphorylation to eventually generate ATP. In energy-demanding tissues like skeletal muscle, it has been suggested that mitochondrial NAD^+ levels are 2-fold greater than other cell compartments in mice (149). Even upon massive depletion of cytoplasmic NAD^+, mitochondrial NAD^+ levels can be preserved for at least 24 hours to maintain normal cellular energy supply (150, 151). Moreover, it has been shown that reduction in NAD^+ levels may compromise mitochondrial functions and is reversible under NAD^+ precursor supplementation (152). Therefore, vitamin B3 consumption is required to keep NAD^+ at an optimal level that is critical in maintaining proper energy metabolism and cell viability.

Second, NAD^+ also serves as the substrate for other enzymes like poly(ADP-ribose) polymerases (PARPs), which may further impact mtDNA integrity. PARPs can detect single-strand DNA breaks induced by carcinogens and initiate the DNA repair process by synthesizing poly(ADP-ribose) using NAD^+ as the substrate. It has been demonstrated that niacin supplementation exerts protective effects against the development of skin cancer by restoring NAD^+ levels and thus increasing the activity of PARPs (153, 154). Unlike the well-established roles of PARPs in regulating nuclear DNA integrity, its effects on mtDNA maintenance are still unclear. Although several studies have suggested mitochondrial localization of PARP1(155, 156), the most studied

protein in PARP family, its role in mtDNA repair is still a matter of debate (157, 158). There is accumulating evidence indicating that PARP1 is involved in ensuring mtDNA integrity by directly binding mtDNA (159) or regulating mtDNA repair factors in nuclear genome (160). Therefore, as the only substrate for PARP to participate in DNA repair, the abundance of NAD^+ may be critical for the maintenance of mtDNA integrity, which will further impact mitochondrial functions and the development of complex diseases as discussed above. However, PARP1 has also been described as an intensive NAD^+ consumer (161), which cuts down the NAD^+ flow into the above-mentioned energy metabolism and eventually leads to mitochondrial dysfunction. In this sense, inhibiting PARP activity may have beneficial effects by releasing NAD^+, as has been proved by multiple studies (149, 162). Considering its positive roles in DNA repair, restoring NAD^+ pool by NAD^+ precursors supplementation may be a better solution. It is noteworthy that NAD^+ can be synthesized from different forms of vitamin B_3 with different efficiencies. Nicotinamide and nicotinamide riboside, which are derivative forms of vitamin B_3, have been suggested to have higher efficiencies in recovering NAD^+ levels (162, 163).

Coenzyme Q10

Coenzyme Q, also known as ubiquinone, is a lipid-soluble compound that has essential roles in both mitochondrial bioenergetics and free radical scavenging. It is ubiquitous in most eukaryotic organisms, and coenzyme Q10 (CoQ10) is the most predominant form present in human. Biosynthesis is the major source of CoQ10 in human, while it is also enriched in organ meats such as livers and hearts. The importance of CoQ10 lies

in its role in transferring electrons from mitochondrial complexes I and II to mitochondrial complex III (164), which is indispensable for ATP generation. In addition to its vital role in aerobic respiration, CoQ10 is also well known for its antioxidant properties, as it is the only known lipid-soluble antioxidant that can be synthesized *de novo* by animal cells (165). Decades ago, the administration of CoQ10 had already been used in clinical treatment to alleviate symptoms in patients with mitochondrial disorders (166, 167). Now with a better understanding of the possible roles of mitochondria in the development of complex diseases like neurodegenerative diseases, supplementation of CoQ10 has been brought back to be the focus of research as a potential treatment for various complex diseases. CoQ10 has been shown to protect neurons from oxidative stress and severe mitochondrial dysfunction by increasing mitochondrial content in the brain (168), stabilizing the mitochondrial membrane potential (168, 169), and inhibiting mitochondrial permeability transition pore (170). In animal models of PD (Parkinson Disease), dietary CoQ10 prevents the degeneration of dopaminergic neurons, the loss of which is associated with PD (171, 172). Similarly, in animal models of AD (Alzheimer's disease), a decrease in the β-amyloid deposition and improvement in the brain mitochondrial SOD activity were observed after CoQ10 supplementation (173, 174). However, from the results of clinical trials, whether the CoQ10 administration is effective in treating neurological diseases is still controversial. Some studies reported that symptomatic improvements were observed in PD patients following CoQ10 supplementation at different dosages (172, 175, 176), while others found no benefits in PD patients even after a high dosage consumption (177, 178). One possible explanation for the controversy was proposed by Seet *et al.* that the therapeutic response to CoQ10

depends on the dosage and the baseline levels of ubiquinol, the reduced form of CoQ10 (179). In a limited amount of clinical trials of CoQ10 on AD and Huntington diseases, no evidence showed that the use of CoQ10 can slow the functional decline in these diseases (180, 181). More studies with larger sample size are needed to examine whether CoQ10 supplementation is an effective way to treat neurological diseases.

Caloric Restriction

Besides specific nutrient supplementations, reducing calorie intakes has also been suggested to improve mitochondrial functions. Caloric restriction (CR), which usually refers to a 20-40% reduction in calorie intake without malnutrition, is the most promising approach to promote health and extend lifespan across organisms (182-184). Multiple theories have been proposed to explain the underlying molecular mechanisms. As reduced mitochondrial respiratory capacity and increased oxidative stress were reported to be associated with aging (185-187), alteration in mitochondrial metabolism has been considered as a hallmark of aging. It has been proposed that CR may attenuate detrimental effects of aging through increasing mitochondrial biogenesis and reducing oxidative stress. Regarding mitochondrial biogenesis, studies using different organisms have reported that CR increases mtDNA content (188), protein abundance (189) and expressions of genes involved in mitochondrial biogenesis (188, 190). Furthermore, several pathways that are believed to be responsible for the beneficial effects of CR can lead to the enhancement of mitochondrial biogenesis (191, 192). Even in healthy young adults, CR is able to increase the number of mitochondria in skeletal muscle, together with an observation of improvement in mitochondrial function (193). These findings

lead to a widely-accepted hypothesis that CR increases mitochondrial biogenesis, thereby improves mitochondrial functions. There is a growing consensus on the vital roles of mitochondria in CR intervention, but it remains quite contentious regarding whether CR leads to the enhancement of mitochondrial biogenesis. A considerable number of reports demonstrated that no changes in mitochondrial content were observed after CR interventions (194-197). Instead, Lanza *et al.* indicated that CR preserves mitochondrial function mainly through enhanced mitochondrial oxidative efficiency, decreased mitochondrial oxidant generation, and upregulated antioxidant defense (196). Taken together, above evidence suggests that multiple mechanisms may exist in CR-mediated mitochondrial function preservation, and it is likely that elevated mitochondrial biogenesis is a tissue-specific response (198).

The free radical theory of aging was first proposed by Harman in 1956, proposing that mitochondrial energy production decline and mtROS-induced damage are the major causes of aging (199). After that, considerable experimental efforts have been made to elucidate the role of ROS in the process of aging. At the same time, CR has been repeatedly demonstrated to be capable of extending lifespan and delaying the onset of aging-related diseases. Thus, extensive studies have focused on the impact of CR on ROS generation in the past several decades. According to the free radical theory of aging, continuous and progressive production of ROS leads to cellular and tissue damages and eventually to aging. Accumulated data from yeast to human studies suggested that CR improves OXPHOS efficiency and reduces ROS production in mitochondria (200, 201), accompanied by reduced oxidative damage on mitochondrial DNA (202, 203) and proteins (204). The same pattern has also been observed in protein

and amino acids restrictions as discussed above. Accordingly, enormous effort has been made to reduce ROS formation, thus to simulate the effects of CR on the process of aging in various model organisms. However, antioxidants treatment could not mimic the lifespan-extending phenotype of CR (205), and may even abolish the benefits of CR (206). Consistently, altering ROS generation or antioxidant responses failed to modulate lifespan in various organisms (207, 208). With rising evidence in recent years, a new perspective was proposed that ROS may work as signaling molecules to transduce signals from mitochondria to other compartments of the cell. The new theory was named as mitochondrial hormesis or mitohormesis (209). According to this theory, ROS is believed to be essential for adaptive induction of ROS defense and hence to achieve a long-term reduction of oxidative stress (210). Therefore, the concept of mitohormesis is a complement to the free radical theory of aging, explaining the observations of elevated ROS level after CR interventions but with benefits on longevity. Le couteur *et al.* proposed that there may be a threshold of ROS level whereby slightly elevated ROS production is sufficient to induce systematic defense mechanisms, but not to an excessive level to damage mitochondria (211). Therefore, CR may improve mitochondrial functions by reducing the harm from excessive oxidative stress generated by free radicals, back to a physiological level to transduce signals within the cell.

1.5 Conclusions

In the past decades, mitochondrial research has received growing attention, as evidenced by the increasing number of mitochondria-related medical publications (212). Accumulated evidence suggested that mitochondrial dysfunction is involved in the

pathogenesis of many common human diseases. Both mitochondrial genetic perturbations and environmental changes such as dietary challenges may cause mitochondrial dysfunctions. With improved sequencing technology, more pathogenic mtDNA mutations have been revealed. However, no effective interventions targeting mitochondria have been developed to relieve the symptoms or to treat these complex disorders that are known to be associated with mitochondrial dysfunctions. By summarizing the current knowledge, we want to emphasize the importance of investigating the role of mitochondria in the development of complex diseases, and to point out the future directions in identifying better approaches to restore mitochondrial functions. The convenience of implementation and the positive patient compliance make nutritional intervention one of the best candidate strategies to offer long-term prevention and treatment for these complex diseases. Although by now nutritional intervention has only been shown to relieve symptoms of only a few defined disorders, increasing data from animal studies still indicate its potential in a broader spectrum of diseases. In the future, more well-designed clinical trials are needed to confirm these observations in animal studies. Apart from the nutritional approaches, we also propose that an innovative way of mtDNA editing or replacement may become the future treatment for diseases related to mtDNA mutations. We anticipate that increasing research into mitochondria will lead to fruitful discoveries and technologies to help our battle against complex diseases.

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1.7 References

1. Wallace DC. Mitochondrial DNA in aging and disease. Scientific American. 1997;277(2):40-59.
2. Schon EA, DiMauro S, Hirano M. Human mitochondrial DNA: roles of inherited and somatic mutations. Nat Rev Genet. 2012;13(12):878-90.
3. Stewart JB, Chinnery PF. The dynamics of mitochondrial DNA heteroplasmy: implications for human health and disease. Nat Rev Genet. 2015;16(9):530-42.
4. Picard M, Wallace DC, Burelle Y. The rise of mitochondria in medicine. Mitochondrion. 2016;30:105-16.
5. Garcia-Heredia JM, Carnero A. Decoding Warburg's hypothesis: tumor-related mutations in the mitochondrial respiratory chain. Oncotarget. 2015;6(39):41582-99.
6. Chrysostomou A, Turnbull DM. Mitochondria, the Synapse, and Neurodegeneration. In: Reeve AK, Simcox EM, Duchen MR, Turnbull DM, editors. Mitochondrial Dysfunction in Neurodegenerative Disorders. Cham: Springer International Publishing; 2016. p. 219-39.
7. Kauppila TES, Kauppila JHK, Larsson N-G. Mammalian Mitochondria and Aging: An Update. Cell Metabolism. 2017;25(1):57-71.
8. Wallace DC, Chalkia D. Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. Cold Spring Harbor perspectives in biology. 2013;5(11):a021220.
9. Marlow FL. Mitochondrial matters: Mitochondrial bottlenecks, self-assembling structures, and entrapment in the female germline. Stem Cell Research. 2017;21:178-86.
10. Rossignol R, Faustin B, Rocher C, Malgat M, Mazat JP, Letellier T. Mitochondrial threshold effects. Biochem J. 2003;370(Pt 3):751-62.
11. Boulet L, Karpati G, Shoubridge E. Distribution and threshold expression of the tRNA (Lys) mutation in skeletal muscle of patients with myoclonic epilepsy and ragged-red fibers (MERRF). American Journal of Human Genetics. 1992;51(6):1187.
12. Larsson N, Tulinius M, Holme E, Oldfors A, Andersen O, Wahlström J, et al. Segregation and manifestations of the mtDNA tRNA (Lys) A--> G (8344) mutation of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome. American Journal of Human Genetics. 1992;51(6):1201.
13. Rajasimha HK, Chinnery PF, Samuels DC. Selection against Pathogenic mtDNA Mutations in a Stem Cell Population Leads to the Loss of the 3243A→G Mutation in Blood. American Journal of Human Genetics. 2008;82(2):333-43.
14. Li M, Schroder R, Ni S, Madea B, Stoneking M. Extensive tissue-related and allele-related mtDNA heteroplasmy suggests positive selection for somatic mutations. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(8):2491-6.
15. Jayaprakash AD, Benson EK, Gone S, Liang R, Shim J, Lambertini L, et al. Stable heteroplasmy at the single-cell level is facilitated by intercellular exchange of mtDNA. Nucleic Acids Res. 2015;43(4):2177-87.
16. Neupane J, Ghimire S, Vandewoestyne M, Lu Y, Gerris J, Van Coster R, et al. Cellular Heterogeneity in the Level of mtDNA Heteroplasmy in Mouse Embryonic

Stem Cells. Cell Reports. 2015;13(7):1304-9.
17. Ye K, Lu J, Ma F, Keinan A, Gu Z. Extensive pathogenicity of mitochondrial heteroplasmy in healthy human individuals. Proceedings of the National Academy of Sciences. 2014;111(29):10654-9.
18. Ding J, Sidore C, Butler TJ, Wing MK, Qian Y, Meirelles O, et al. Assessing Mitochondrial DNA Variation and Copy Number in Lymphocytes of ~2,000 Sardinians Using Tailored Sequencing Analysis Tools. PLoS Genet. 2015;11(7):e1005306.
19. Ghosh SS, Fahy E, Bodis-Wollner I, Sherman J, Howell N. Longitudinal study of a heteroplasmic 3460 Leber hereditary optic neuropathy family by multiplexed primer-extension analysis and nucleotide sequencing. Am J Hum Genet. 1996;58(2):325-34.
20. Larsson NG, Tulinius MH, Holme E, Oldfors A, Andersen O, Wahlstrom J, et al. Segregation and manifestations of the mtDNA tRNA(Lys) A-->G(8344) mutation of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome. Am J Hum Genet. 1992;51(6):1201-12.
21. Li M, Rothwell R, Vermaat M, Wachsmuth M, Schroder R, Laros JF, et al. Transmission of human mtDNA heteroplasmy in the Genome of the Netherlands families: support for a variable-size bottleneck. Genome Research. 2016;26(4):417-26.
22. Samuels DC, Li C, Li B, Song Z, Torstenson E, Boyd Clay H, et al. Recurrent Tissue-Specific mtDNA Mutations Are Common in Humans. PLOS Genetics. 2013;9(11):e1003929.
23. Liau WS, Gonzalez-Serricchio AS, Deshommes C, Chin K, LaMunyon CW. A persistent mitochondrial deletion reduces fitness and sperm performance in heteroplasmic populations of C. elegans. BMC Genetics. 2007;8:8.
24. Ju YS, Alexandrov LB, Gerstung M, Martincorena I, Nik-Zainal S, Ramakrishna M, et al. Origins and functional consequences of somatic mitochondrial DNA mutations in human cancer. eLife. 2014;3:e02935.
25. Payne BAI, Chinnery PF. Mitochondrial dysfunction in aging: Much progress but many unresolved questions. Biochimica et Biophysica Acta. 2015;1847(11):1347-53.
26. Sevini F, Giuliani C, Vianello D, Giampieri E, Santoro A, Biondi F, et al. mtDNA mutations in human aging and longevity: Controversies and new perspectives opened by high-throughput technologies. Experimental Gerontology. 2014;56:234-44.
27. Holt IJ, Reyes A. Human mitochondrial DNA replication. Cold Spring Harbor Prspectives in Biology. 2012;4(12).
28. Alexeyev M, Shokolenko I, Wilson G, LeDoux S. The Maintenance of Mitochondrial DNA Integrity—Critical Analysis and Update. Cold Spring Harbor Perspectives in Biology. 2013;5(5).
29. Elson JL, Samuels DC, Turnbull DM, Chinnery PF. Random Intracellular Drift Explains the Clonal Expansion of Mitochondrial DNA Mutations with Age. The American Journal of Human Genetics. 2001;68(3):802-6.
30. Wallace DC. Mitochondrial DNA mutations in disease and aging. Environmental and Molecular Mutagenesis. 2010;51(5):440-50.
31. Wallace DC, Chalkia D. Mitochondrial DNA genetics and the heteroplasmy conundrum in evolution and disease. Cold Spring Harbor Perspectives in Biology. 2013;5(11):a021220.
32. Wallace DC. A mitochondrial bioenergetic etiology of disease. The Journal of

Clinical Investigation. 2013;123(4):1405-12.
33. Gorman GS, Chinnery PF, DiMauro S, Hirano M, Koga Y, McFarland R, et al. Mitochondrial diseases. Nature Reviews Disease Primers. 2016;2:16080.
34. Alston CL, Rocha MC, Lax NZ, Turnbull DM, Taylor RW. The genetics and pathology of mitochondrial disease. The Journal of Pathology. 2017;241(2):236-50.
35. Nesbitt V, Pitceathly RD, Turnbull DM, Taylor RW, Sweeney MG, Mudanohwo EE, et al. The UK MRC Mitochondrial Disease Patient Cohort Study: clinical phenotypes associated with the m.3243A>G mutation--implications for diagnosis and management. Journal of Neurology, Neurosurgery, and Psychiatry. 2013;84(8):936-8.
36. Lake NJ, Compton AG, Rahman S, Thorburn DR. Leigh syndrome: One disorder, more than 75 monogenic causes. Ann Neurol. 2016;79(2):190-203.
37. Carelli V, Ghelli A, Ratta M, Bacchilega E, Sangiorgi S, Mancini R, et al. Leber's hereditary optic neuropathy: biochemical effect of 11778/ND4 and 3460/ND1 mutations and correlation with the mitochondrial genotype. Neurology. 1997;48(6):1623-32.
38. Hudson G, Nalls M, Evans JR, Breen DP, Winder-Rhodes S, Morrison KE, et al. Two-stage association study and meta-analysis of mitochondrial DNA variants in Parkinson disease. Neurology. 2013;80(22):2042-8.
39. Krüger J, Hinttala R, Majamaa K, Remes AM. Mitochondrial DNA haplogroups in early-onset Alzheimer's disease and frontotemporal lobar degeneration. Molecular Neurodegeneration. 2010;5(1):8.
40. Lakatos A, Derbeneva O, Younes D, Keator D, Bakken T, Lvova M, et al. Association between mitochondrial DNA variations and Alzheimer's disease in the ADNI cohort. Neurobiology of Aging. 2010;31(8):1355-63.
41. Maruszak A, Canter JA, Styczyńska M, Żekanowski C, Barcikowska M. Mitochondrial haplogroup H and Alzheimer's disease—is there a connection? Neurobiology of Aging. 2009;30(11):1749-55.
42. Santoro A, Balbi V, Balducci E, Pirazzini C, Rosini F, Tavano F, et al. Evidence for sub-haplogroup h5 of mitochondrial DNA as a risk factor for late onset Alzheimer's disease. PLoS One. 2010;5(8):e12037.
43. Van Der Walt JM, Dementieva YA, Martin ER, Scott WK, Nicodemus KK, Kroner CC, et al. Analysis of European mitochondrial haplogroups with Alzheimer disease risk. Neuroscience Letters. 2004;365(1):28-32.
44. Lin MT, Cantuti-Castelvetri I, Zheng K, Jackson KE, Tan YB, Arzberger T, et al. Somatic mitochondrial DNA mutations in early Parkinson's and incidental Lewy body disease. Ann Neurol. 2012;71(6):850-4.
45. Coxhead J, Kurzawa-Akanbi M, Hussain R, Pyle A, Chinnery P, Hudson G. Somatic mtDNA variation is an important component of Parkinson's disease. Neurobiology of Aging. 2016;38:217.e1-.e6.
46. Hoekstra JG, Hipp MJ, Montine TJ, Kennedy SR. Mitochondrial DNA mutations increase in early stage Alzheimer disease and are inconsistent with oxidative damage. Annals of Neurology. 2016;80(2):301-6.
47. Hering T, Birth N, Taanman J-W, Orth M. Selective striatal mtDNA depletion in end-stage Huntington's disease R6/2 mice. Experimental Neurology. 2015;266:22-9.
48. Siddiqui A, Rivera-Sánchez S, Castro MdR, Acevedo-Torres K, Rane A, Torres-

Ramos CA, et al. Mitochondrial DNA damage is associated with reduced mitochondrial bioenergetics in Huntington's disease. Free Radical Biology and Medicine. 2012;53(7):1478-88.
49. Banoei MM, Houshmand M, Panahi MSS, Shariati P, Rostami M, Manshadi MD, et al. Huntington's disease and mitochondrial DNA deletions: event or regular mechanism for mutant huntingtin protein and CAG repeats expansion?! Cellular and Molecular Neurobiology. 2007;27(7):867.
50. Chen C-M, Wu Y-R, Cheng M-L, Liu J-L, Lee Y-M, Lee P-W, et al. Increased oxidative damage and mitochondrial abnormalities in the peripheral blood of Huntington's disease patients. Biochemical and Biophysical Research Communications. 2007;359(2):335-40.
51. Filosto M, Lanzi G, Nesti C, Vielmi V, Marchina E, Galvagni A, et al. A novel mitochondrial tRNAAla gene variant causes chronic progressive external ophthalmoplegia in a patient with Huntington disease. Molecular Genetics and Metabolism Reports. 2016;6:70-3.
52. Wang Y, Picard M, Gu Z. Genetic Evidence for Elevated Pathogenicity of Mitochondrial DNA Heteroplasmy in Autism Spectrum Disorder. PLOS Genetics. 2016;12(10):e1006391.
53. Hanahan D, Weinberg Robert A. Hallmarks of Cancer: The Next Generation. Cell.144(5):646-74.
54. Warburg O. On the origin of cancer cells. Science. 1956;123(3191):309-14.
55. Warburg O. On respiratory impairment in cancer cells. Science. 1956;124(3215):269-70.
56. Warburg O. The Metabolism of Tumours: Investigations from the Kaiser Wilhelm Institute for Biology, translated by Frank Dickens. Constable & Co Ltd. 1930.
57. Wong LJ, Lueth M, Li XN, Lau CC, Vogel H. Detection of mitochondrial DNA mutations in the tumor and cerebrospinal fluid of medulloblastoma patients. Cancer Res. 2003;63(14):3866-71.
58. Jones JB, Song JJ, Hempen PM, Parmigiani G, Hruban RH, Kern SE. Detection of mitochondrial DNA mutations in pancreatic cancer offers a "mass"-ive advantage over detection of nuclear DNA mutations. Cancer Res. 2001;61(4):1299-304.
59. Chatterjee A, Mambo E, Sidransky D. Mitochondrial DNA mutations in human cancer. Oncogene. 2006;25(34):4663-74.
60. He Y, Wu J, Dressman DC, Iacobuzio-Donahue C, Markowitz SD, Velculescu VE, et al. Heteroplasmic mitochondrial DNA mutations in normal and tumour cells. Nature. 2010;464(7288):610-4.
61. Davis Caleb F, Ricketts CJ, Wang M, Yang L, Cherniack Andrew D, Shen H, et al. The Somatic Genomic Landscape of Chromophobe Renal Cell Carcinoma. Cancer Cell. 2014;26(3):319-30.
62. Hardie R-A, van Dam E, Cowley M, Han T-L, Balaban S, Pajic M, et al. Mitochondrial mutations and metabolic adaptation in pancreatic cancer. Cancer & Metabolism. 2017;5(1):2.
63. Bratic A, Larsson N-G, xF, ran. The role of mitochondria in aging. The Journal of Clinical Investigation. 2013;123(3):951-7.
64. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The Hallmarks of

Aging. Cell. 2013;153(6):1194-217.
65. Kujoth G, Hiona A, Pugh T, Someya S, Panzer K, Wohlgemuth S, et al. Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science. 2005;309(5733):481-4.
66. Ross JM, Stewart JB, Hagström E, Brené S, Mourier A, Coppotelli G, et al. Germline mitochondrial DNA mutations aggravate ageing and can impair brain development. Nature. 2013;501(7467):412-5.
67. Bua E, Johnson J, Herbst A, Delong B, McKenzie D, Salamat S, et al. Mitochondrial DNA–Deletion Mutations Accumulate Intracellularly to Detrimental Levels in Aged Human Skeletal Muscle Fibers. The American Journal of Human Genetics. 2006;79(3):469-80.
68. Greaves LC, Elson JL, Nooteboom M, Grady JP, Taylor GA, Taylor RW, et al. Comparison of Mitochondrial Mutation Spectra in Ageing Human Colonic Epithelium and Disease: Absence of Evidence for Purifying Selection in Somatic Mitochondrial DNA Point Mutations. PLOS Genetics. 2012;8(11):e1003082.
69. Greaves LC, Nooteboom M, Elson JL, Tuppen HAL, Taylor GA, Commane DM, et al. Clonal Expansion of Early to Mid-Life Mitochondrial DNA Point Mutations Drives Mitochondrial Dysfunction during Human Ageing. PLOS Genetics. 2014;10(9):e1004620.
70. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature. 2004;429(6990):417-23.
71. Bellizzi D, D'Aquila P, Giordano M, Montesanto A, Passarino G. Global DNA methylation levels are modulated by mitochondrial DNA variants. Epigenomics. 2012;4(1):17-27.
72. Smiraglia DJ, Kulawiec M, Bistulfi GL, Gupta SG, Singh KK. A novel role for mitochondria in regulating epigenetic modification in the nucleus. Cancer Biology & Therapy. 2008;7(8):1182-90.
73. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. Cell Metab. 2017;25(1):27-42.
74. Vaughan RA, Garcia-Smith R, Barberena MA, Bisoffi M, Trujillo K, Conn CA. Treatment of human muscle cells with popular dietary supplements increase mitochondrial function and metabolic rate. Nutr Metab (Lond). 2012;9(1):101.
75. Neschen S, Moore I, Regittnig W, Yu CL, Wang Y, Pypaert M, et al. Contrasting effects of fish oil and safflower oil on hepatic peroxisomal and tissue lipid content. Am J Physiol Endocrinol Metab. 2002;282(2):E395-401.
76. Yoshino J, Smith GI, Kelly SC, Julliand S, Reeds DN, Mittendorfer B. Effect of dietary n-3 PUFA supplementation on the muscle transcriptome in older adults. Physiol Rep. 2016;4(11).
77. Vaughan RA, Garcia-Smith R, Bisoffi M, Conn CA, Trujillo KA. Conjugated linoleic acid or omega 3 fatty acids increase mitochondrial biosynthesis and metabolism in skeletal muscle cells. Lipids Health Dis. 2012;11:142.
78. Johnson ML, Lalia AZ, Dasari S, Pallauf M, Fitch M, Hellerstein MK, et al. Eicosapentaenoic acid but not docosahexaenoic acid restores skeletal muscle mitochondrial oxidative capacity in old mice. Aging Cell. 2015;14(5):734-43.

79. Raza Shaikh S, Brown DA. Models of plasma membrane organization can be applied to mitochondrial membranes to target human health and disease with polyunsaturated fatty acids. Prostaglandins Leukot Essent Fatty Acids. 2013;88(1):21-5.
80. Brenna JT, Diau GY. The influence of dietary docosahexaenoic acid and arachidonic acid on central nervous system polyunsaturated fatty acid composition. Prostaglandins Leukot Essent Fatty Acids. 2007;77(5-6):247-50.
81. Dyall SC. Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA. Front Aging Neurosci. 2015;7:52.
82. Pepe S, Tsuchiya N, Lakatta EG, Hansford RG. PUFA and aging modulate cardiac mitochondrial membrane lipid composition and Ca2+ activation of PDH. Am J Physiol. 1999;276(1 Pt 2):H149-58.
83. Surette ME. The science behind dietary omega-3 fatty acids. CMAJ. 2008;178(2):177-80.
84. Herbst EA, Paglialunga S, Gerling C, Whitfield J, Mukai K, Chabowski A, et al. Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle. J Physiol. 2014;592(6):1341-52.
85. Eckert GP, Lipka U, Muller WE. Omega-3 fatty acids in neurodegenerative diseases: focus on mitochondria. Prostaglandins Leukot Essent Fatty Acids. 2013;88(1):105-14.
86. Putti R, Migliaccio V, Sica R, Lionetti L. Skeletal Muscle Mitochondrial Bioenergetics and Morphology in High Fat Diet Induced Obesity and Insulin Resistance: Focus on Dietary Fat Source. Front Physiol. 2015;6:426.
87. Afshordel S, Hagl S, Werner D, Rohner N, Kogel D, Bazan NG, et al. Omega-3 polyunsaturated fatty acids improve mitochondrial dysfunction in brain aging--impact of Bcl-2 and NPD-1 like metabolites. Prostaglandins Leukot Essent Fatty Acids. 2015;92:23-31.
88. Dapito DH, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. Cancer Cell. 2012;21(4):504-16.
89. Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature. 2013;499(7456):97-101.
90. Wang JL, Chang CH, Lin JW, Wu LC, Chuang LM, Lai MS. Infection, antibiotic therapy and risk of colorectal cancer: a nationwide nested case-control study in patients with Type 2 diabetes mellitus. Int J Cancer. 2014;135(4):956-67.
91. Baothman OA, Zamzami MA, Taher I, Abubaker J, Abu-Farha M. The role of Gut Microbiota in the development of obesity and Diabetes. Lipids Health Dis. 2016;15:108.
92. Cox AJ, West NP, Cripps AW. Obesity, inflammation, and the gut microbiota. Lancet Diabetes Endocrinol. 2015;3(3):207-15.
93. Vieira SM, Pagovich OE, Kriegel MA. Diet, microbiota and autoimmune diseases. Lupus. 2014;23(6):518-26.
94. Wu HJ, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes. 2012;3(1):4-14.

95. Moos WH, Faller DV, Harpp DN, Kanara I, Pernokas J, Powers WR, et al. Microbiota and Neurological Disorders: A Gut Feeling. Biores Open Access. 2016;5(1):137-45.
96. Umbrello G, Esposito S. Microbiota and neurologic diseases: potential effects of probiotics. J Transl Med. 2016;14(1):298.
97. Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, et al. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell. 2016;167(6):1469-80 e12.
98. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res. 2013;54(9):2325-40.
99. Lin HV, Frassetto A, Kowalik EJ, Jr., Nawrocki AR, Lu MM, Kosinski JR, et al. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. PLoS One. 2012;7(4):e35240.
100. Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, et al. Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes. 2009;58(7):1509-17.
101. Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A, Kimura I. Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. Nutrients. 2015;7(4):2839-49.
102. Jan G, Belzacq AS, Haouzi D, Rouault A, Metivier D, Kroemer G, et al. Propionibacteria induce apoptosis of colorectal carcinoma cells via short-chain fatty acids acting on mitochondria. Cell Death Differ. 2002;9(2):179-88.
103. Heerdt BG, Houston MA, Anthony GM, Augenlicht LH. Initiation of growth arrest and apoptosis of MCF-7 mammary carcinoma cells by tributyrin, a triglyceride analogue of the short-chain fatty acid butyrate, is associated with mitochondrial activity. Cancer Res. 1999;59(7):1584-91.
104. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 2011;13(5):517-26.
105. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009;324(5930):1029-33.
106. Donohoe DR, Collins LB, Wali A, Bigler R, Sun W, Bultman SJ. The Warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. Mol Cell. 2012;48(4):612-26.
107. Donohoe DR, Holley D, Collins LB, Montgomery SA, Whitmore AC, Hillhouse A, et al. A gnotobiotic mouse model demonstrates that dietary fiber protects against colorectal tumorigenesis in a microbiota- and butyrate-dependent manner. Cancer Discov. 2014;4(12):1387-97.
108. Heerdt BG, Houston MA, Wilson AJ, Augenlicht LH. The intrinsic mitochondrial membrane potential (Deltapsim) is associated with steady-state mitochondrial activity and the extent to which colonic epithelial cells undergo butyrate-mediated growth arrest and apoptosis. Cancer Res. 2003;63(19):6311-9.
109. Kolar SS, Barhoumi R, Lupton JR, Chapkin RS. Docosahexaenoic acid and

butyrate synergistically induce colonocyte apoptosis by enhancing mitochondrial Ca2+ accumulation. Cancer Res. 2007;67(11):5561-8.
110. Xi L, Chen G, Zhou J, Xu G, Wang S, Wu P, et al. Inhibition of telomerase enhances apoptosis induced by sodium butyrate via mitochondrial pathway. Apoptosis. 2006;11(5):789-98.
111. Ng Y, Barhoumi R, Tjalkens RB, Fan YY, Kolar S, Wang N, et al. The role of docosahexaenoic acid in mediating mitochondrial membrane lipid oxidation and apoptosis in colonocytes. Carcinogenesis. 2005;26(11):1914-21.
112. Kolar SS, Barhoumi R, Callaway ES, Fan YY, Wang N, Lupton JR, et al. Synergy between docosahexaenoic acid and butyrate elicits p53-independent apoptosis via mitochondrial Ca(2+) accumulation in colonocytes. Am J Physiol Gastrointest Liver Physiol. 2007;293(5):G935-43.
113. Bindels LB, Porporato P, Dewulf EM, Verrax J, Neyrinck AM, Martin JC, et al. Gut microbiota-derived propionate reduces cancer cell proliferation in the liver. Br J Cancer. 2012;107(8):1337-44.
114. Qin LQ, Xun P, Bujnowski D, Daviglus ML, Van Horn L, Stamler J, et al. Higher branched-chain amino acid intake is associated with a lower prevalence of being overweight or obese in middle-aged East Asian and Western adults. J Nutr. 2011;141(2):249-54.
115. Yamamoto D, Maki T, Herningtyas EH, Ikeshita N, Shibahara H, Sugiyama Y, et al. Branched-chain amino acids protect against dexamethasone-induced soleus muscle atrophy in rats. Muscle Nerve. 2010;41(6):819-27.
116. Eley HL, Russell ST, Tisdale MJ. Effect of branched-chain amino acids on muscle atrophy in cancer cachexia. Biochem J. 2007;407(1):113-20.
117. D'Antona G, Ragni M, Cardile A, Tedesco L, Dossena M, Bruttini F, et al. Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. Cell Metab. 2010;12(4):362-72.
118. Valerio A, D'Antona G, Nisoli E. Branched-chain amino acids, mitochondrial biogenesis, and healthspan: an evolutionary perspective. Aging (Albany NY). 2011;3(5):464-78.
119. Liang C, Curry BJ, Brown PL, Zemel MB. Leucine Modulates Mitochondrial Biogenesis and SIRT1-AMPK Signaling in C2C12 Myotubes. J Nutr Metab. 2014;2014:239750.
120. Bruckbauer A, Zemel MB. Effects of dairy consumption on SIRT1 and mitochondrial biogenesis in adipocytes and muscle cells. Nutr Metab (Lond). 2011;8:91.
121. Iwasa M, Kobayashi Y, Mifuji-Moroka R, Hara N, Miyachi H, Sugimoto R, et al. Branched-chain amino acid supplementation reduces oxidative stress and prolongs survival in rats with advanced liver cirrhosis. PLoS One. 2013;8(7):e70309.
122. Sun X, Zemel MB. Leucine modulation of mitochondrial mass and oxygen consumption in skeletal muscle cells and adipocytes. Nutr Metab (Lond). 2009;6:26.
123. Lee C, Longo V. Dietary restriction with and without caloric restriction for healthy aging. F1000Res. 2016;5.
124. Mirzaei H, Suarez JA, Longo VD. Protein and amino acid restriction, aging and

disease: from yeast to humans. Trends Endocrinol Metab. 2014;25(11):558-66.
125. Grandison RC, Piper MD, Partridge L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature. 2009;462(7276):1061-4.
126. Pamplona R, Barja G. Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection. Biochim Biophys Acta. 2006;1757(5-6):496-508.
127. Sanz A, Caro P, Barja G. Protein restriction without strong caloric restriction decreases mitochondrial oxygen radical production and oxidative DNA damage in rat liver. J Bioenerg Biomembr. 2004;36(6):545-52.
128. Levine ME, Suarez JA, Brandhorst S, Balasubramanian P, Cheng CW, Madia F, et al. Low protein intake is associated with a major reduction in IGF-1, cancer, and overall mortality in the 65 and younger but not older population. Cell Metab. 2014;19(3):407-17.
129. Sanz A, Caro P, Ayala V, Portero-Otin M, Pamplona R, Barja G. Methionine restriction decreases mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins. FASEB J. 2006;20(8):1064-73.
130. Segall P. Long-term tryptophan restriction and aging in the rat. Aktuelle Gerontol. 1977;7(10):535-8.
131. De Marte ML, Enesco HE. Influence of low tryptophan diet on survival and organ growth in mice. Mech Ageing Dev. 1986;36(2):161-71.
132. Shimokawa I, Higami Y, Yu BP, Masoro EJ, Ikeda T. Influence of dietary components on occurrence of and mortality due to neoplasms in male F344 rats. Aging (Milano). 1996;8(4):254-62.
133. Iwasaki K, Gleiser CA, Masoro EJ, McMahan CA, Seo EJ, Yu BP. Influence of the restriction of individual dietary components on longevity and age-related disease of Fischer rats: the fat component and the mineral component. J Gerontol. 1988;43(1):B13-21.
134. Sanz A, Caro P, Sanchez JG, Barja G. Effect of lipid restriction on mitochondrial free radical production and oxidative DNA damage. Ann N Y Acad Sci. 2006;1067:200-9.
135. Sanz A, Gomez J, Caro P, Barja G. Carbohydrate restriction does not change mitochondrial free radical generation and oxidative DNA damage. J Bioenerg Biomembr. 2006;38(5-6):327-33.
136. Gomez J, Caro P, Sanchez I, Naudi A, Jove M, Portero-Otin M, et al. Effect of methionine dietary supplementation on mitochondrial oxygen radical generation and oxidative DNA damage in rat liver and heart. J Bioenerg Biomembr. 2009;41(3):309-21.
137. Depeint F, Bruce WR, Shangari N, Mehta R, O'Brien PJ. Mitochondrial function and toxicity: role of the B vitamin family on mitochondrial energy metabolism. Chem Biol Interact. 2006;163(1-2):94-112.
138. Gold DR, Cohen BH. Treatment of mitochondrial cytopathies. Semin Neurol. 2001;21(3):309-25.
139. Sinha A, Hollingsworth KG, Ball S, Cheetham T. Improving the vitamin D status of vitamin D deficient adults is associated with improved mitochondrial oxidative function in skeletal muscle. J Clin Endocrinol Metab. 2013;98(3):E509-13.

140. Bouillon R, Verstuyf A. Vitamin D, mitochondria, and muscle. J Clin Endocrinol Metab. 2013;98(3):961-3.
141. Uberti F, Lattuada D, Morsanuto V, Nava U, Bolis G, Vacca G, et al. Vitamin D protects human endothelial cells from oxidative stress through the autophagic and survival pathways. J Clin Endocrinol Metab. 2014;99(4):1367-74.
142. Estornell E, Tormo JR, Marin P, Renau-Piqueras J, Timoneda J, Barber T. Effects of vitamin A deficiency on mitochondrial function in rat liver and heart. Br J Nutr. 2000;84(6):927-34.
143. Chiu HJ, Fischman DA, Hammerling U. Vitamin A depletion causes oxidative stress, mitochondrial dysfunction, and PARP-1-dependent energy deprivation. FASEB J. 2008;22(11):3878-87.
144. Oliveira MR. Vitamin A and Retinoids as Mitochondrial Toxicants. Oxid Med Cell Longev. 2015;2015:140267.
145. Oliveira MR. The neurotoxic effects of vitamin A and retinoids. An Acad Bras Cienc. 2015;87(2 Suppl):1361-73.
146. Ablain J, de The H. Retinoic acid signaling in cancer: The parable of acute promyelocytic leukemia. Int J Cancer. 2014;135(10):2262-72.
147. Wang ZY, Chen Z. Acute promyelocytic leukemia: from highly fatal to highly curable. Blood. 2008;111(5):2505-15.
148. Fujimura S, Suzumiya J, Yamada Y, Kuroki M, Ono J. Downregulation of Bcl-xL and activation of caspases during retinoic acid-induced apoptosis in an adult T-cell leukemia cell line. Hematol J. 2003;4(5):328-35.
149. Pirinen E, Canto C, Jo YS, Morato L, Zhang H, Menzies KJ, et al. Pharmacological Inhibition of poly(ADP-ribose) polymerases improves fitness and mitochondrial function in skeletal muscle. Cell Metab. 2014;19(6):1034-41.
150. Yang H, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, et al. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. Cell. 2007;130(6):1095-107.
151. Houtkooper RH, Canto C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. Endocr Rev. 2010;31(2):194-223.
152. Gomes AP, Price NL, Ling AJ, Moslehi JJ, Montgomery MK, Rajman L, et al. Declining NAD(+) induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging. Cell. 2013;155(7):1624-38.
153. Benavente CA, Schnell SA, Jacobson EL. Effects of niacin restriction on sirtuin and PARP responses to photodamage in human skin. PLoS One. 2012;7(7):e42276.
154. Benavente CA, Jacobson MK, Jacobson EL. NAD in skin: therapeutic approaches for niacin. Curr Pharm Des. 2009;15(1):29-38.
155. Lai Y, Chen Y, Watkins SC, Nathaniel PD, Guo F, Kochanek PM, et al. Identification of poly-ADP-ribosylated mitochondrial proteins after traumatic brain injury. J Neurochem. 2008;104(6):1700-11.
156. Pankotai E, Lacza Z, Muranyi M, Szabo C. Intra-mitochondrial poly(ADP-ribosyl)ation: potential role for alpha-ketoglutarate dehydrogenase. Mitochondrion. 2009;9(2):159-64.
157. Sykora P, Wilson DM, 3rd, Bohr VA. Repair of persistent strand breaks in the mitochondrial genome. Mech Ageing Dev. 2012;133(4):169-75.

158. Szczesny B, Brunyanszki A, Olah G, Mitra S, Szabo C. Opposing roles of mitochondrial and nuclear PARP1 in the regulation of mitochondrial and nuclear DNA integrity: implications for the regulation of mitochondrial function. Nucleic Acids Res. 2014;42(21):13161-73.
159. Rossi MN, Carbone M, Mostocotto C, Mancone C, Tripodi M, Maione R, et al. Mitochondrial localization of PARP-1 requires interaction with mitofilin and is involved in the maintenance of mitochondrial DNA integrity. J Biol Chem. 2009;284(46):31616-24.
160. Lapucci A, Pittelli M, Rapizzi E, Felici R, Moroni F, Chiarugi A. Poly(ADP-ribose) polymerase-1 is a nuclear epigenetic regulator of mitochondrial DNA repair and transcription. Mol Pharmacol. 2011;79(6):932-40.
161. Zhang T, Berrocal JG, Yao J, DuMond ME, Krishnakumar R, Ruhl DD, et al. Regulation of poly(ADP-ribose) polymerase-1-dependent gene expression through promoter-directed recruitment of a nuclear NAD+ synthase. J Biol Chem. 2012;287(15):12405-16.
162. Canto C, Menzies KJ, Auwerx J. NAD(+) Metabolism and the Control of Energy Homeostasis: A Balancing Act between Mitochondria and the Nucleus. Cell Metab. 2015;22(1):31-53.
163. Nikiforov A, Dolle C, Niere M, Ziegler M. Pathways and subcellular compartmentation of NAD biosynthesis in human cells: from entry of extracellular precursors to mitochondrial NAD generation. J Biol Chem. 2011;286(24):21767-78.
164. Littarru GP, Tiano L. Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. Mol Biotechnol. 2007;37(1):31-7.
165. Potgieter M, Pretorius E, Pepper MS. Primary and secondary coenzyme Q10 deficiency: the role of therapeutic supplementation. Nutr Rev. 2013;71(3):180-8.
166. Goda S, Hamada T, Ishimoto S, Kobayashi T, Goto I, Kuroiwa Y. Clinical improvement after administration of coenzyme Q10 in a patient with mitochondrial encephalomyopathy. J Neurol. 1987;234(1):62-3.
167. Yamamoto M, Sato T, Anno M, Ujike H, Takemoto M. Mitochondrial myopathy, encephalopathy, lactic acidosis, and strokelike episodes with recurrent abdominal symptoms and coenzyme Q10 administration. J Neurol Neurosurg Psychiatry. 1987;50(11):1475-81.
168. Matthews RT, Yang L, Browne S, Baik M, Beal MF. Coenzyme Q10 administration increases brain mitochondrial concentrations and exerts neuroprotective effects. Proc Natl Acad Sci U S A. 1998;95(15):8892-7.
169. Duberley KE, Heales SJ, Abramov AY, Chalasani A, Land JM, Rahman S, et al. Effect of Coenzyme Q10 supplementation on mitochondrial electron transport chain activity and mitochondrial oxidative stress in Coenzyme Q10 deficient human neuronal cells. Int J Biochem Cell Biol. 2014;50:60-3.
170. Naderi J, Somayajulu-Nitu M, Mukerji A, Sharda P, Sikorska M, Borowy-Borowski H, et al. Water-soluble formulation of Coenzyme Q10 inhibits Bax-induced destabilization of mitochondria in mammalian cells. Apoptosis. 2006;11(8):1359-69.
171. Horvath TL, Diano S, Leranth C, Garcia-Segura LM, Cowley MA, Shanabrough M, et al. Coenzyme Q induces nigral mitochondrial uncoupling and prevents dopamine cell loss in a primate model of Parkinson's disease. Endocrinology. 2003;144(7):2757-

60.
172. Muller T, Buttner T, Gholipour AF, Kuhn W. Coenzyme Q10 supplementation provides mild symptomatic benefit in patients with Parkinson's disease. Neurosci Lett. 2003;341(3):201-4.
173. Yang X, Yang Y, Li G, Wang J, Yang ES. Coenzyme Q10 attenuates beta-amyloid pathology in the aged transgenic mice with Alzheimer presenilin 1 mutation. J Mol Neurosci. 2008;34(2):165-71.
174. Yang X, Dai G, Li G, Yang ES. Coenzyme Q10 reduces beta-amyloid plaque in an APP/PS1 transgenic mouse model of Alzheimer's disease. J Mol Neurosci. 2010;41(1):110-3.
175. Shults CW, Oakes D, Kieburtz K, Beal MF, Haas R, Plumb S, et al. Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline. Arch Neurol. 2002;59(10):1541-50.
176. Horstink MW, van Engelen BG. The effect of coenzyme Q10 therapy in Parkinson disease could be symptomatic. Arch Neurol. 2003;60(8):1170-2; author reply 2-3.
177. Storch A, Jost WH, Vieregge P, Spiegel J, Greulich W, Durner J, et al. Randomized, double-blind, placebo-controlled trial on symptomatic effects of coenzyme Q(10) in Parkinson disease. Arch Neurol. 2007;64(7):938-44.
178. Parkinson Study Group QEI, Beal MF, Oakes D, Shoulson I, Henchcliffe C, Galpern WR, et al. A randomized clinical trial of high-dosage coenzyme Q10 in early Parkinson disease: no evidence of benefit. JAMA Neurol. 2014;71(5):543-52.
179. Seet RC, Lim EC, Tan JJ, Quek AM, Chow AW, Chong WL, et al. Does high-dose coenzyme Q10 improve oxidative damage and clinical outcomes in Parkinson's disease? Antioxid Redox Signal. 2014;21(2):211-7.
180. Galasko DR, Peskind E, Clark CM, Quinn JF, Ringman JM, Jicha GA, et al. Antioxidants for Alzheimer disease: a randomized clinical trial with cerebrospinal fluid biomarker measures. Arch Neurol. 2012;69(7):836-41.
181. McGarry A, McDermott M, Kieburtz K, de Blieck EA, Beal F, Marder K, et al. A randomized, double-blind, placebo-controlled trial of coenzyme Q10 in Huntington disease. Neurology. 2017;88(2):152-9.
182. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, Beasley TM, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science. 2009;325(5937):201-4.
183. Mair W, Dillin A. Aging and survival: the genetics of life span extension by dietary restriction. Annu Rev Biochem. 2008;77:727-54.
184. Fontana L, Partridge L, Longo VD. Extending healthy life span--from yeast to humans. Science. 2010;328(5976):321-6.
185. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 2006;443(7113):787-95.
186. Trifunovic A, Larsson NG. Mitochondrial dysfunction as a cause of ageing. J Intern Med. 2008;263(2):167-78.
187. Bonawitz ND, Shadel GS. Rethinking the mitochondrial theory of aging: the role of mitochondrial gene expression in lifespan determination. Cell Cycle. 2007;6(13):1574-8.
188. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, et al. Calorie

restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science. 2005;310(5746):314-7.
189. Zid BM, Rogers AN, Katewa SD, Vargas MA, Kolipinski MC, Lu TA, et al. 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. Cell. 2009;139(1):149-60.
190. Sreekumar R, Unnikrishnan J, Fu A, Nygren J, Short KR, Schimke J, et al. Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. Am J Physiol Endocrinol Metab. 2002;283(1):E38-43.
191. Bonawitz ND, Chatenay-Lapointe M, Pan Y, Shadel GS. Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. Cell Metab. 2007;5(4):265-77.
192. Anderson R, Prolla T. PGC-1alpha in aging and anti-aging interventions. Biochim Biophys Acta. 2009;1790(10):1059-66.
193. Civitarese AE, Carling S, Heilbronn LK, Hulver MH, Ukropcova B, Deutsch WA, et al. Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. PLoS Med. 2007;4(3):e76.
194. Hancock CR, Han DH, Higashida K, Kim SH, Holloszy JO. Does calorie restriction induce mitochondrial biogenesis? A reevaluation. FASEB J. 2011;25(2):785-91.
195. Miller BF, Robinson MM, Bruss MD, Hellerstein M, Hamilton KL. A comprehensive assessment of mitochondrial protein synthesis and cellular proliferation with age and caloric restriction. Aging Cell. 2012;11(1):150-61.
196. Lanza IR, Zabielski P, Klaus KA, Morse DM, Heppelmann CJ, Bergen HR, 3rd, et al. Chronic caloric restriction preserves mitochondrial function in senescence without increasing mitochondrial biogenesis. Cell Metab. 2012;16(6):777-88.
197. Miller BF, Robinson MM, Reuland DJ, Drake JC, Peelor FF, 3rd, Bruss MD, et al. Calorie restriction does not increase short-term or long-term protein synthesis. J Gerontol A Biol Sci Med Sci. 2013;68(5):530-8.
198. Ruetenik A, Barrientos A. Dietary restriction, mitochondrial function and aging: from yeast to humans. Biochim Biophys Acta. 2015;1847(11):1434-47.
199. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. 1956;11(3):298-300.
200. Ungvari Z, Parrado-Fernandez C, Csiszar A, de Cabo R. Mechanisms underlying caloric restriction and lifespan regulation: implications for vascular aging. Circ Res. 2008;102(5):519-28.
201. Lopez-Lluch G, Hunt N, Jones B, Zhu M, Jamieson H, Hilmer S, et al. Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. Proc Natl Acad Sci U S A. 2006;103(6):1768-73.
202. Gredilla R, Sanz A, Lopez-Torres M, Barja G. Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart. FASEB J. 2001;15(9):1589-91.
203. Lopez-Torres M, Gredilla R, Sanz A, Barja G. Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. Free Radic Biol Med. 2002;32(9):882-9.
204. Pamplona R, Portero-Otin M, Requena J, Gredilla R, Barja G. Oxidative,

glycoxidative and lipoxidative damage to rat heart mitochondrial proteins is lower after 4 months of caloric restriction than in age-matched controls. Mech Ageing Dev. 2002;123(11):1437-46.
205. Pun PB, Gruber J, Tang SY, Schaffer S, Ong RL, Fong S, et al. Ageing in nematodes: do antioxidants extend lifespan in Caenorhabditis elegans? Biogerontology. 2010;11(1):17-30.
206. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. JAMA. 2007;297(8):842-57.
207. Zhang Y, Ikeno Y, Qi W, Chaudhuri A, Li Y, Bokov A, et al. Mice deficient in both Mn superoxide dismutase and glutathione peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity. J Gerontol A Biol Sci Med Sci. 2009;64(12):1212-20.
208. Yen K, Patel HB, Lublin AL, Mobbs CV. SOD isoforms play no role in lifespan in ad lib or dietary restricted conditions, but mutational inactivation of SOD-1 reduces life extension by cold. Mech Ageing Dev. 2009;130(3):173-8.
209. Tapia PC. Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: "Mitohormesis" for health and vitality. Med Hypotheses. 2006;66(4):832-43.
210. Ristow M, Zarse K. How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). Exp Gerontol. 2010;45(6):410-8.
211. Le Couteur DG, Solon-Biet S, Cogger VC, Mitchell SJ, Senior A, de Cabo R, et al. The impact of low-protein high-carbohydrate diets on aging and lifespan. Cell Mol Life Sci. 2016;73(6):1237-52.
212. Picard M, Wallace DC, Burelle Y. The rise of mitochondria in medicine. Mitochondrion. 2016;30:105-16.

Chapter 2 - Glucomannan hydrolysate promotes gut homeostasis and extends lifespan in *Drosophila melanogaster*

2.1 Introduction

Aging is a natural process that leads to irreversible impairment of physiological functions and increased vulnerability to death. Great efforts have been spent on investigating cellular and molecular mechanisms underlying the aging process, with the ultimate goal of developing effective interventions to delay the onset of aging. It is well known that some drug treatments and nutrient manipulations extend lifespan (1-4). However, most drugs, such as rapamycin (5) and metformin (6), have high risks of side-effects, while nutrient manipulations like calorie restriction have a poor dietary adherence (7). Therefore, it would be desirable to discover new dietary intervention strategies that have high efficiency and low risks.

Konjac glucomannan (KGM) is a natural, odorless fiber extracted from the root of the *Amorphophallus konjac* plant, which is a common food ingredient in Asia (8). KGM is composed of D-mannose and D-glucose monomers with a ratio of 1.6:1 (9). Acids or enzymes can hydrolyze KGM into konjac glucomannan hydrolysates (GMH), a lower-molecular-weight form (10, 11). Both forms of glucomannan from konjac have frequently been consumed and widely studied. KGM has a special feature that it swells and jellifies in the gut, thus helping with weight loss by increasing gastric satiety (12). When hydrolyzed into GMH, the solubility of depolymerized glucomannan is significantly improved, which enhances its efficacy both locally and systemically (13).

GMH has been demonstrated to have prebiotic activity, promoting the growth of beneficial bacteria (11, 14, 15). It stimulates the immune system both in the gut and on the skin (16, 17), and even reduce the levels of serum cholesterol and glucose in diabetic mice (18). Despite these beneficial effects of dietary supplementation of GMH, no studies have investigated its impact on lifespan. To examine whether GMH supplementation extends lifespan, we used *Drosophila melanogaster* as a model organism in which aging is well studied. *Drosophila* is ideal for aging studies because of its relatively short lifespan and ease of environmental and genetic manipulations (19).

The digestive tract is at the frontline of responses to dietary supplementations and a key organ involved in aging (19). It is not only responsible for physiological functions like nutrient absorption but also acts as the defense barrier to control both commensal and pathogenic microbes (20). In the aging intestine, there are dramatic changes in both the quantity and the composition of gut-associated microbes, termed as dysbiosis (21, 22). In a variety of organisms, including humans, changes in the gut microbiota have been associated with disorders like obesity, cancer, and chronic inflammation, potentially due to the dysregulation of the frequent interaction between epithelial cells and commensal bacteria (23-25). Therefore, it has been proposed that manipulating this interaction may be a viable intervention for healthy aging (26, 27). Dietary supplementation of prebiotics that promotes the growth of beneficial bacteria may be a promising approach. Besides the control of commensal bacteria, it is also critical for organisms to maintain gut homeostasis through regenerative processes. As a high-turnover tissue, the intestine undergoes constant regeneration sustained by intestinal stem cells (ISCs), which are the only dividing cells in the *Drosophila* intestine (28, 29). In the aging intestine,

dysregulated ISC proliferation and abnormal differentiation both lead to the accumulation of mis-differentiated cells in the epithelium (30, 31), ultimately leading to epithelial dysplasia and premature death (32). It has been shown that limiting the rate of ISC proliferation in aged flies is sufficient to extend lifespan (30, 33). In *Drosophila*, the proliferation rate of ISCs is regulated by several stress and growth signaling pathways such as JAK/STAT, MAPK, and EGFR pathways (34-36). It has been suggested that the fundamental cause of the aging-associated loss of gut proliferative homeostasis might be the lost control of the interaction between intestinal epithelium and commensal bacteria, which leads to chronically and excessively elevated proliferation of ISCs (21).

In this article, we will present evidence for the effect of GMH on extending lifespan in *Drosophila*. Combining longitudinal transcriptomic and metagenomic analysis with biochemical assays, we show that the lifespan-extending effect of GMH is likely through promoting gut microbiome and proliferative homeostasis.

2.2 Materials and methods

Fly Stocks and Husbandry

All flies were reared under standard laboratory conditions with a 12-h light/dark cycle at 25°C in vials containing agar–dextrose–yeast medium. Nine different strains of wild-type flies were used in this study. Besides Canton-S and Oregon-R, we included one strain (B18) from the Global Diversity Panel and six strains (DGRP-21, DGRP-38, DGRP-40, DGRP-85, DGRP-105, DGRP-136) from Drosophila Genetic Reference

Panel (DGRP). To examine whether *Wolbachia* infection status modulates the lifespan-extending effect, we chose three *Wolbachia* positive strains and three negative strains from the DGRP.

GMH Supplementation and Survival Assay

The GMH supplemented medium was prepared by adding the GMH powder into the control agar-dextrose-yeast medium at a concentration of 0.25% w/v. Flies were collected right after eclosion and housed at a density of 20-30 flies per vial. At least 6 replicates with a minimum of 120 flies were tested for each condition. Flies were transferred to fresh medium every two days for mated females, and every three to five days for virgin females and males. During each transfer, dead flies in each vial were counted. Flies that escaped or died accidentally were recorded as missing. All data were analyzed with Log-Rank test using the Online Application for the Survival Analysis (OASIS) of lifespan assays (37).

Long-term Feeding Assay

Both control medium and GMH supplemented medium were prepared with the addition of a blue indigestible dye, FD&C blue No.1, at a concentration of 1% w/v (38). Dyed growth medium was poured into the cap of an aerated 50 ml Falcon centrifuge tube. Mated females were transferred into Falcon tubes with the medium-containing cap on. Falcon tubes were placed upside down (cap side down) in incubators for two days. Flies were then removed from the tube, and two ml of PBS was added into the falcon tube to wash colored-feces off the sides of tubes. The total amount of food that flies consumed

across two days was measured as a function of the amount of blue in the feces. This was calculated from the optical density of a serial dilution of FD&C blue No.1 solution.

Gut Microbiota Sequencing and Analysis

Guts of flies were dissected in PBS after 10, 20, 30 and 40 days of treatments. DNA was extracted from a pool of 20 guts for each sample using phenol-chloroform extraction method (39). According to Illumina 16s metagenome library preparation guide, 16s ribosomal RNA gene amplicons were prepared for the Illumina MiSeq System. Briefly, two rounds of PCR reactions were carried out to first amplify the hypervariable regions (V3 and V4) of the bacterial 16s rRNA genes, and then to add adaptors and barcodes for Illumina sequencing.

Microbiota derived reads were analyzed following a Bioconductor workflow based on dada2, phyloseq, and EdgeR (40-43). Low-quality reads were filtered: those containing N, with the base quality score less than or equal to two, or with more than two expected errors. Additionally, the first ten bases and all bases after position 230 were trimmed. De-replication was performed to combine identical reads while keeping track of their abundance. The core dada2 ribosomal sequence variants (RSVs) inference algorithm was applied to the de-replicated data to infer sample sequences exactly and resolve differences of as little as one nucleotide by modeling and correcting sample-specific sequencing errors (41). We then merged the inferred forward and reverse sequences, removing paired sequences that do not overlap perfectly. Chimera sequences were further removed. The taxonomy of RSVs was assigned based on the SILVA database (version 128) (44). Sequences assigned to the genus of Wolbachia were removed.

Principal coordinates analysis was performed on log-transformed abundance data using Bray-Curtis dissimilarity. Constrained correspondence analysis was performed to evaluate the contribution of diet and treatment length to the variation in microbiota composition. Alpha diversity was measured with the Shannon index and the Simpson index. Differential abundance across control and supplementation groups was tested with edgeR (43, 45).

Quantification of bacterial load

Flies were individually homogenized in 500 µl of sterile PBS using bead beating with a tissue homogenizer (OPS Diagnostics). The original or diluted homogenates were plated on MRS agar plates by a WASP II autoplate spiral plater (Microbiology International). Plates were incubated at 29 °C for 1-2 days to achieve the optimal colony size, and the CFUs were counted by the software Protocol3. Results were analyzed using a mixed effects model in R.

Gut RNA-Seq and Data Analysis

After 10, 30 and 50 days of GMH treatment, 50 guts were dissected and pooled for each replicate. Total RNA was extracted using Trizol (Invitrogen) and RNeasy mini plus kit (Qiagen). mRNA was isolated using magnetic mRNA isolation kit (NEB). KAPA stranded RNA-Seq library preparation kit was used to construct libraries for Illumina sequencing.

Raw sequencing reads were first processed with Trimmomatic (Version 0.33) (46) to trim adaptor sequences and low-quality bases and then mapped to the reference genome

of *Drosophila melanogaster* (FlyBase Dmel Release 6.09) with STAR (Version 2.5.1b) (47). Differentially expressed genes were identified with edgeR (43). Only genes expressed (defined as count per million > 1) in at least two out of four samples were included in the analysis. Significant differentially expressed genes were defined with FDR < 0.05 and log2(fold change) >= or <= 0.5. Pathway enrichment of differentially expressed genes was evaluated with DAVID 6.8 (48).

ISC proliferation and Gut Length Measurement

Guts were dissected in PBS and fixed for 30 min in PBS with 0.1% Tween 20 (PBT) and 4% paraformaldehyde. They were subsequently rinsed with PBT, and incubated with primary antibodies (1/1000 anti-PH3 (Upstate/Millipore) or 1/1000 anti-GFP, Roche) in PBT 1% BSA. Staining was revealed after a second incubation with Alexa488- or Alexa594-coupled anti-mouse antibodies (Invitrogen), and nuclei were stained with DAPI (Sigma).

2.3 Results

2.3.1 GMH supplementation extends *Drosophila melanogaster* lifespan

We first tested whether GMH supplementation affects the lifespan of *Drosophila melanogaster* while taking into account gender, genetic background, and mating status. Survival tests in mated male and female flies of strain B18 (a wild-type stock) both showed significant lifespan extension (Figure 2.1 A, B). In the control groups, the mean lifespans were 57.39 days for male flies and 58.05 days for female flies. In the GMH supplementation groups, the mean lifespan was increased by 20.2% in males

(p=0.0e+00), and by 14.88% in females (p=6.2e-07). As mating status is known to markedly affect lifespan (49), we also examined the effect of GMH supplementation on virgin B18 females and observed an increase in the mean lifespan by 27.48% (p=0.0e+00) (Figure 2.2). To evaluate the lifespan-extending effect of GMH in different genetic backgrounds, we repeated the survival test in two additional wild-type strains (Oregon-R and Canton-S) and six DGRP strains using mated female flies. GMH supplementation increased the mean lifespan by 11.7% in Oregon-R (p=0.01) (Figure 2.1 C), and 17.66% in Canton-S (p=9.0e-09) (Figure 2.1 D). Five out of six DGRP strains also showed significant results, with a 13.4% increase of the mean lifespan in strain 21 (Figure 2.3 A, p=0.0032), 10.1% in strain 38 (Figure 2.3 B, p=2.2e-05), 4.6% in strain 40 (Figure 2.3 C, p=0.050), 10.0% in strain 85 (Figure 2.3 D, p=0.0083) and 7.3% for strain 136 (Figure 2.3 E, p=5.1e-06). Only strain 105 did not respond to GMH supplementation (Figure 2.3 F). These findings suggest that GMH supplementation can promote longevity in both genders across different genetic backgrounds, regardless of mating status.

2.3.2 GMH supplementation promotes the growth of *Acetobacter* in aged flies

To explore mechanisms underlying the lifespan-extending effect of GMH supplementation, we first evaluated its impact on feeding behavior, as dietary supplementation of GMH might change the food consumption, and calorie restriction would be an effective way to extend lifespan (50). With long-term feeding assays (Figure 2.4 A), we found no significant differences in the amount of food intake between control and GMH treatment groups, ruling out the involvement of calorie restriction in

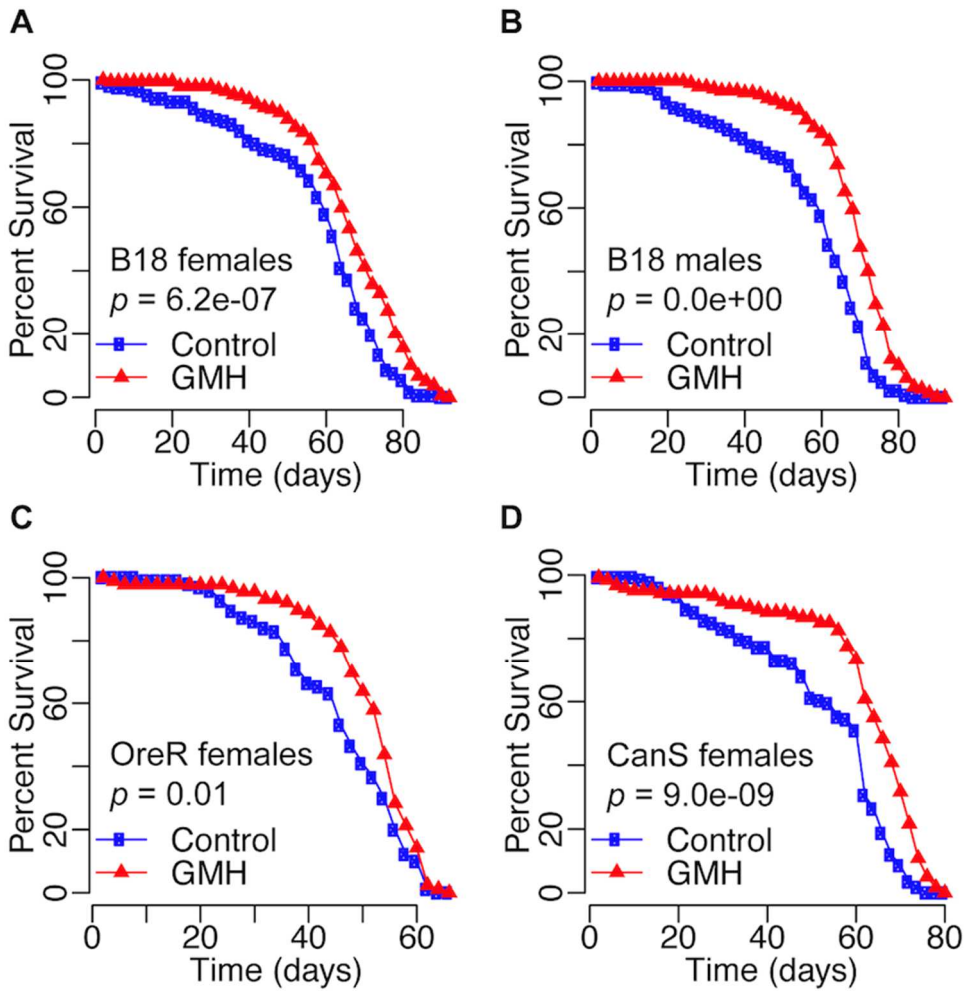

Figure 2. 1 Lifespan extension effect of GMH is observed in strains of B18, Oregon-R and Canton-S

GMH supplementation increase the mean lifespan of (A) mated female flies of strain B18; (B) mated male flies of strain B18; (C) mated female flies of strain Oregon-R; (D) mated female flies of strain Canton-S. For all the survival test, N>120 with replicates.

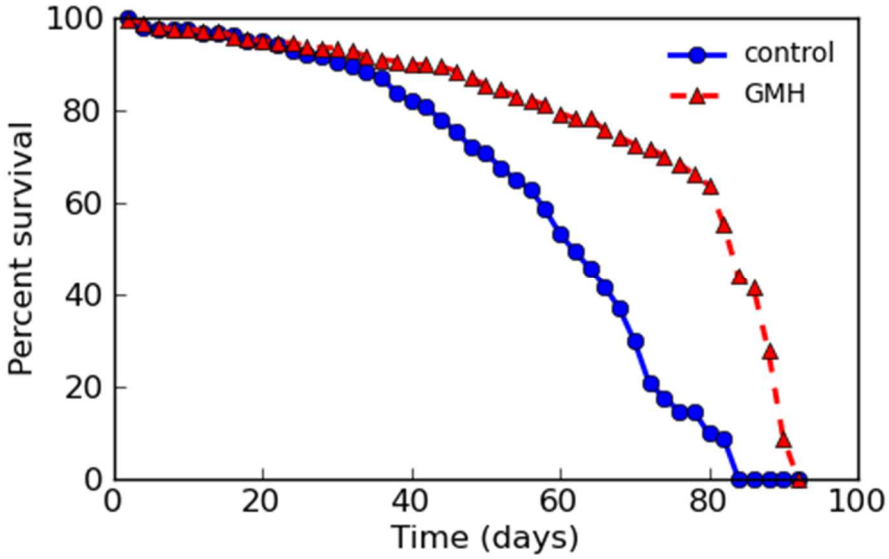

Figure 2. 2 GMH extends lifespan in virgin female flies in strain of B18

The mean lifespan in control group is 58.76 days, and 74.91 days in GMH group. There is a significant increase by 27.5% (*p*= 0.0e+00, N=240)

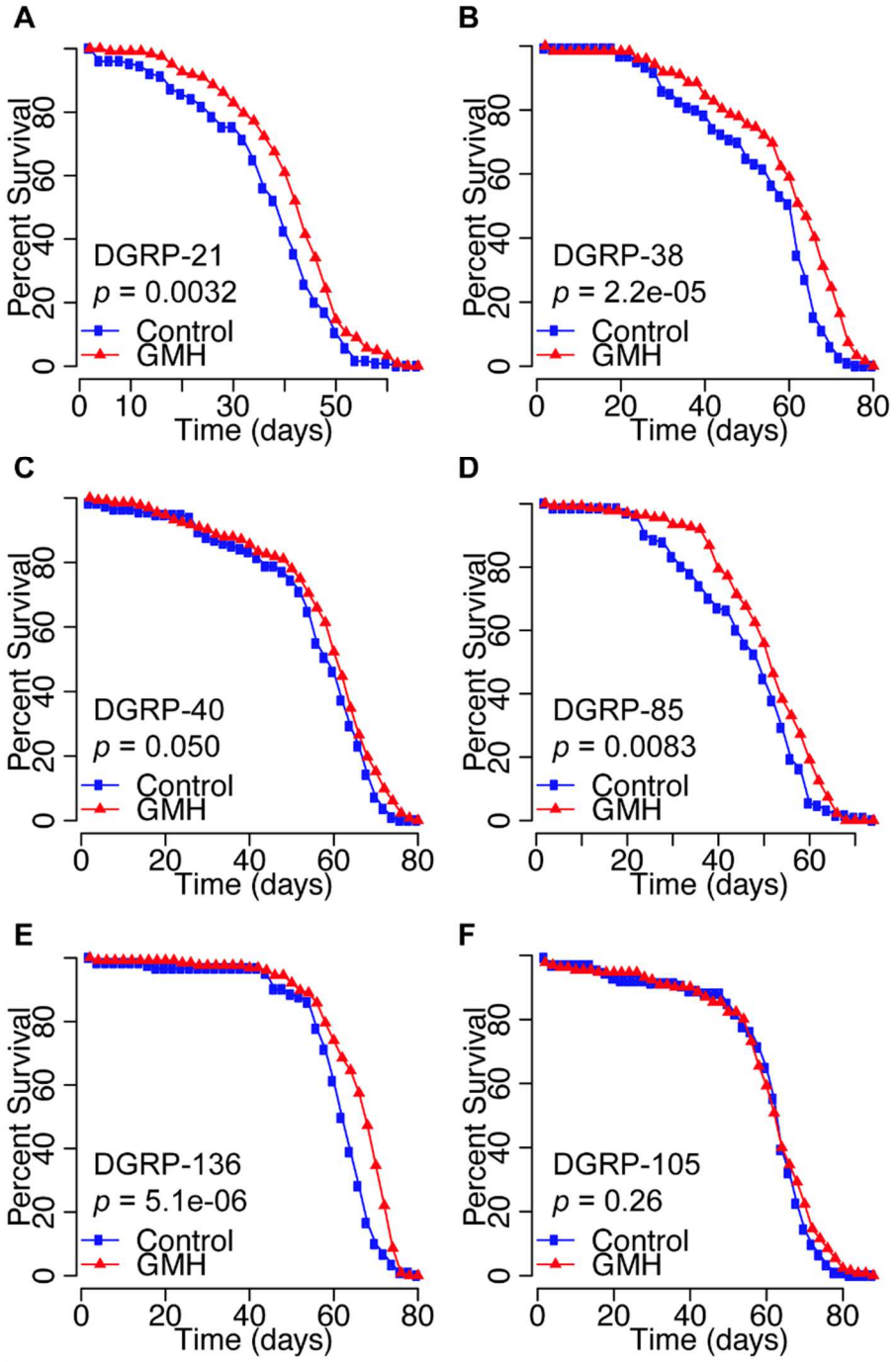

Figure 2. 3 The response to GMH treatment is different in six DGRP strains

GMH significantly extend the mean lifespan of mated female flies of (A) strain DGRP-21; (B) strain DGRP-38; (C) strain DGRP-40; (D) strain DGRP-85; (E) strain DGRP-136; but GMH does not extend the lifespan of flies of (F) strain DGRP-105. For all the survival test, N>120.

the increased lifespan we observed. From existing studies, we know that GMH can function as an effective prebiotic in mice (51, 52). To test if GMH extends lifespan through its potential impact on the gut microbiota, we quantified bacterial load in flies by plating the microbiota (gut microbes are all culturable in *Drosophila*). We estimated bacterial diversity and microbiota composition by sequencing to explore GMH's effect on both overall microbial load and relative abundance of different members of the microbiota. Results from the measurement of representative colony-forming units (CFUs) showed that the number of CFUs in the gut increased exponentially in the process of aging in both control and treatment groups (Figure 2.4 B), as previously shown (21, 31). However, GMH supplemented diet has significantly more CFU count across the three sampling time points ($p = 0.031$), specifically on day 30 ($p = 0.044$) and day 50 ($p = 0.053$), but not on day 10 ($p = 0.74$). The study of colony morphology suggested that Acetobacter species were dominant on both GMH supplemented and control diets. In order to establish what bacterial species could contribute to this increase in bacterial load, we performed 16s rRNA sequencing at four time-points (Day 10, 20, 30 and 40). Consistent with the measurement of CFUs, we observed a shift towards higher relative abundance of *Acetobacter* in old flies (Figure 2.4 C), as previously reported (53), but it was not significantly different between control and GMH groups. The principal coordinates analysis (PCoA) clearly separated samples by sampling times (Figure 2.4 D). However, no apparent clustering was observed for either control or GMH groups. Constrained correspondence analysis further confirmed that the sampling time explains 64.57% ($p < 0.001$) of the variance in microbiome composition while the type of diet explains only 6.17% ($p = 0.255$). Differential abundance analysis at the

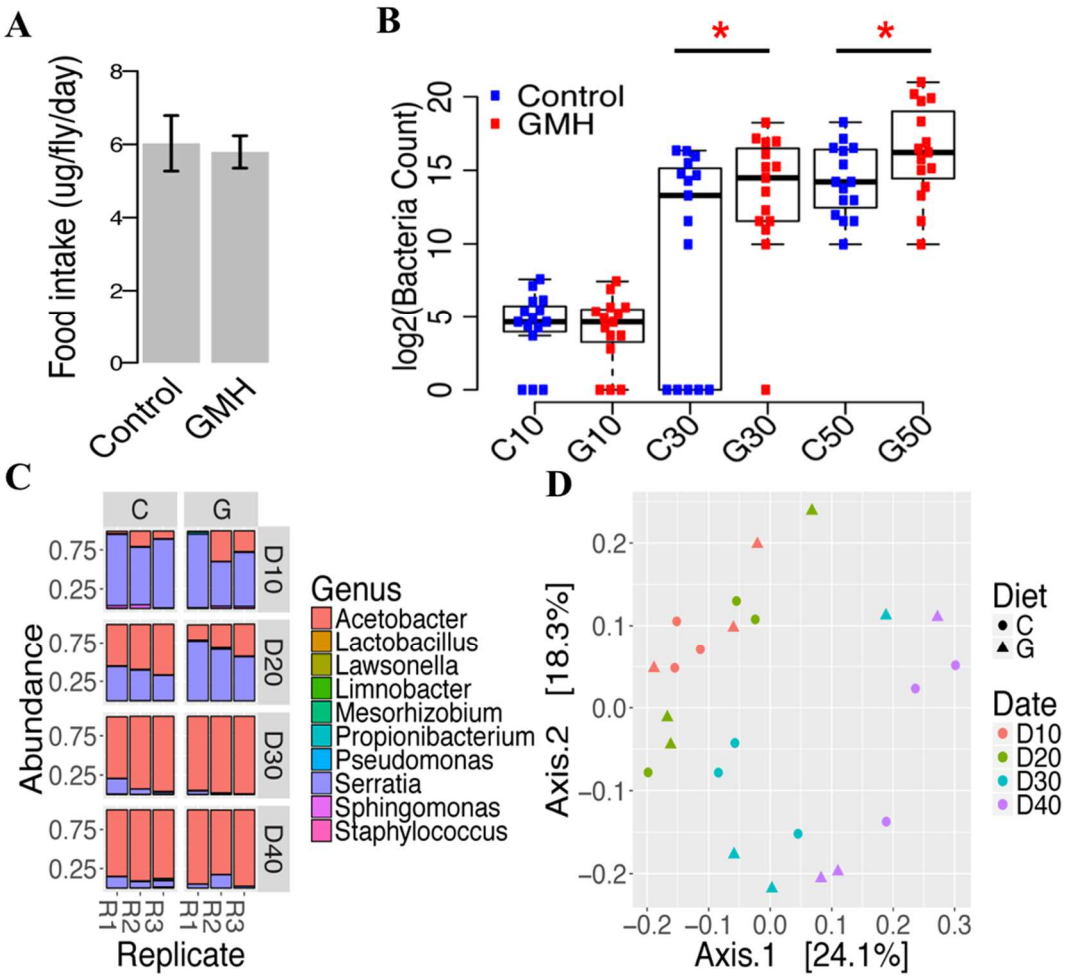

Figure 2. 4 The effect of GMH supplementation on gut microbiome

(A)The amount of food intake when flies are fed on control or GMH food, n = 3 biological replicates with 20 individual flies per replicate; (B)The total bacterial load given as the log number of CFUs of *Acetobacter,* n = 3 biological replicates with 5 individuals per replicate; (C)Each bar represents average relative abundance of each bacterial taxon (top 10 taxa) within a group at genus level; (D) Principal coordinate analysis of gut microbiome compositions in control and GMH groups at different time points.

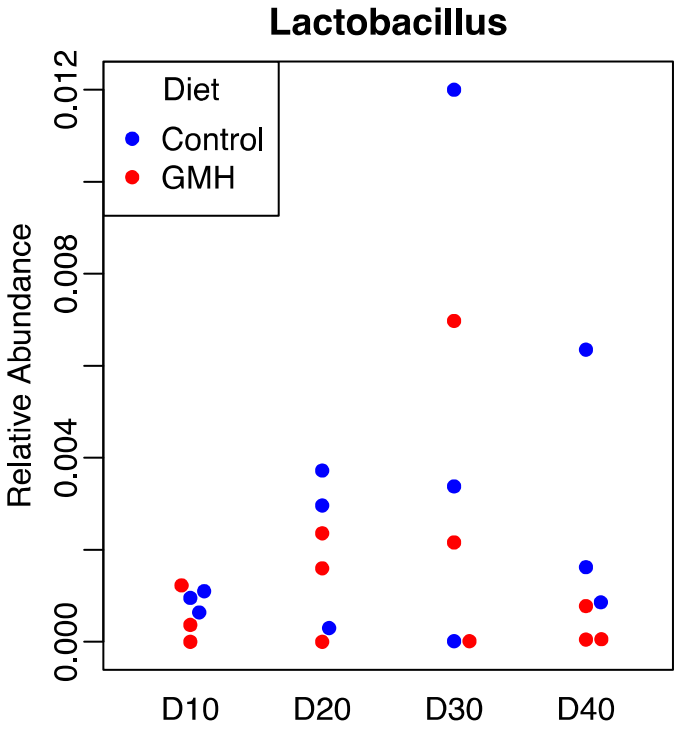

Figure 2. 5 GMH supplementation decreases the relative abundance of *Lactobacillus* There is no difference in the relative abundance of *Lactobacillus* between control and GMH groups from day 10 to day 30. On day 40, the relative abundance of *Lactobacillus* is significantly lower in GMH groups (FDR = 0.0049).

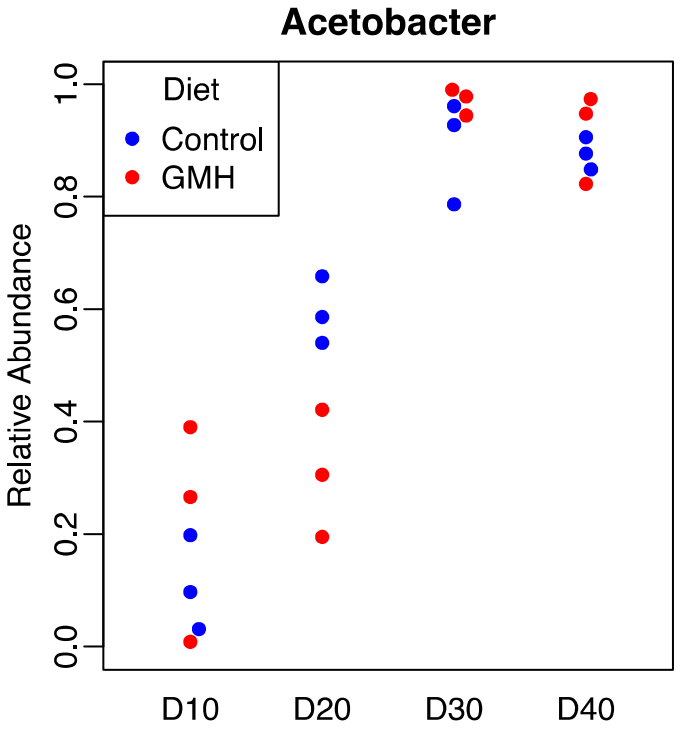

Figure 2. 6 GMH supplementation tends to increase the relative abundance of *Acetobacter*

The relative abundance of *Acetobacter* increases along the process of aging in both control and GMH diets. At day 40, there is a trend that the relative abundance of *Acetobacter* in GMH group is higher than control group, but not significant.

genus level revealed only one genus, *Lactobacillus*, has a significantly lower abundance in GMH group at day 40 (Figure 2.5). For *Acetobacter*, the trend was consistent with the result of CFU measurement suggesting that the GMH group has a higher abundance (Figure 2.6), but this was not significant. Altogether, our results suggest that GMH may work as a prebiotic in the gut to promote the growth of *Acetobacter* and sustain gut health while delaying the onset of aging.

2.3.3 GMH supplementation delays the deterioration of gut integrity

Recent studies have shown that the gut microbiota has a profound influence on host physiology, especially on digestive and immune functions (54). After observing the impact of GMH on the gut microbiota, we evaluated GMH's effect on gut epithelial homeostasis. As the gut is a tissue with high turnover rate, the proliferative activity is critical in maintaining gut integrity (30). Therefore, we measured the rate of stem cell proliferation in the gut by performing immunostaining with an anti-phosphohistone H3 (anti-PH3) antibody, which labels dividing cells. Consistent with previous studies (28, 29), low levels of homeostatic proliferation were observed in young and healthy guts on day 10 and day 30. On day 50, we detected a dramatic increase in the number of PH3 positive cells in both control and GMH groups, but GMH treatment group had significantly fewer dividing cells than the control group ($p = 0.00035$) (Figure 2.7 A, B, C). We also measured gut length as another parameter of the homeostatic status of the gut. Both groups showed significant shortening in gut length along the process of aging, a known phenomenon (55). However, on day 50, guts from GMH treated flies were significantly longer compared to the ones from the control group ($p = 0.0024$) (Figure

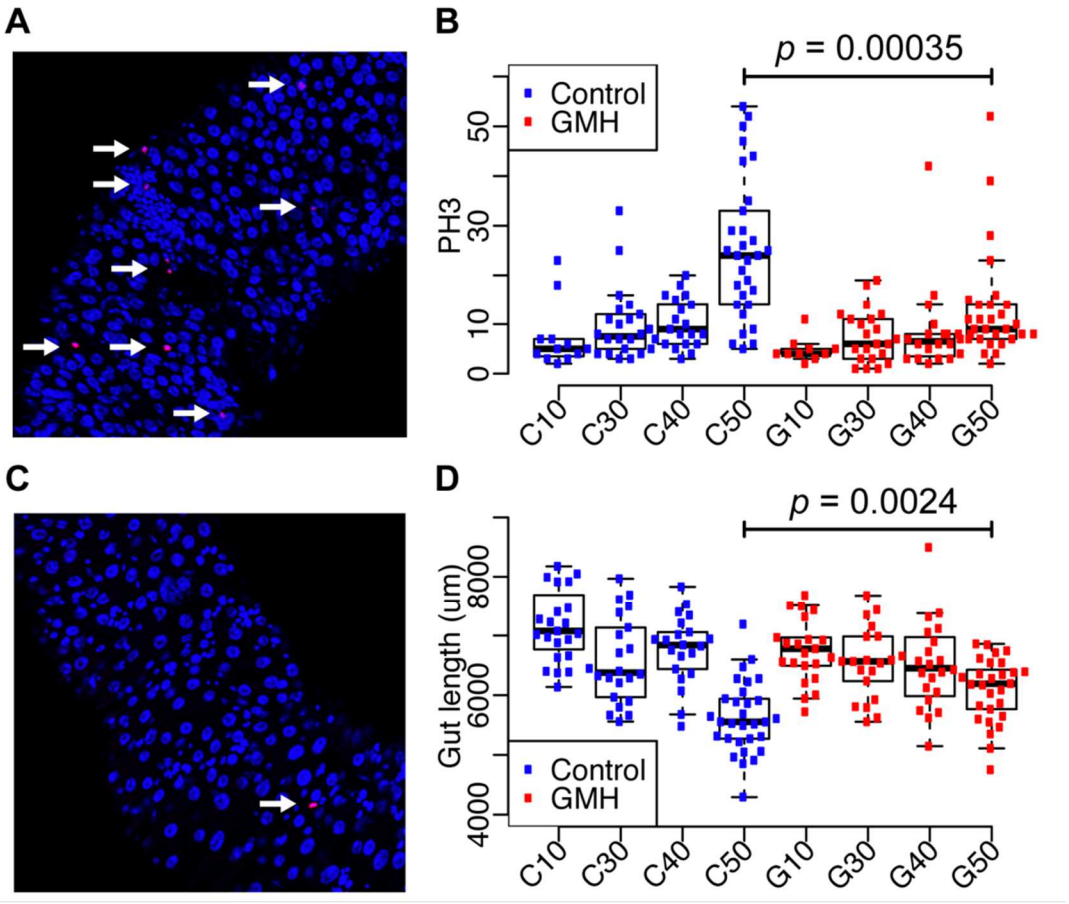

Figure 2. 7 GMH supplementation delays the deterioration of gut integrity

(A) Immunostaining of PH3, a mitosis biomarker, are shown in red (indicated by arrows, DAPI in blue). More PH3 positive cells are observed in flies fed on control diet than (C) the flies fed on GMH supplemented diet at day 50; (B) Quantification of PH3 positive cells in mid-guts from flies fed on either control or GMH supplemented food at different time points. (D) Measurement of the mid-gut length from flies fed on either control or GMH supplemented food at different time points. For panel (B) and (D), n = 3 biological replicates with 10 individual flies per replicate.

2.7 D). In summary, GMH supplementation delays two processes associated with aging – gut stem cell hyper-proliferation and gut-shortening.

2.3.4 GMH supplementation decreases EGFR/MAPK and JAK/STAT pathways

To search for hints of mechanisms underlying the lifespan-extending effect of GMH supplementation, we set out to identify differentially expressed genes between control and supplementation groups. We carried out a mRNA-sequencing experiment using RNA isolated from the guts of flies fed on control or GMH food for 10, 30 and 50 days. Overall, we found 83 genes differentially expressed in guts on day 10 (Figure 2.8 A), 109 genes on day 30 (Figure 2.9 A) and 50 genes on day 50 (Figure 2.8 B). Only a small proportion of the differentially expressed genes overlapped across different time points (Figure 2.9 C). It is known that several growth and stress signaling pathways are involved in regulating ISCs proliferation rate, including MAPK, EGFR, JNK, and JAK/STAT pathways. Therefore, we mainly focused on genes related to these pathways. We found that multiple genes in both JAK/STAT and EGFR/MAPK pathways were down-regulated in GMH groups (Table 2.1, Figure 2.9 B). Genes that encode two key ligands in the JAK/STAT pathway, unpaired2 (*Upd2*) and unpaired 3 (*Upd3*), were both significantly down-regulated on day 50. *Upd3* was also significantly down-regulated on day 30. The negative feedback regulator of JAK/STAT pathway, *Socs36E*, was down-regulated at all three time-points. Moreover, *Ets21c,* a downstream effector of the EGFR/MAPK pathway, was down-regulated in GMH groups at both day 10 and day 30. The phosphatase MAPK phosphatase 3 (*Mkp3*), a negative regulator of the EGFR pathway, was down-regulated at day 10. Another positive regulator of EGFR pathway,

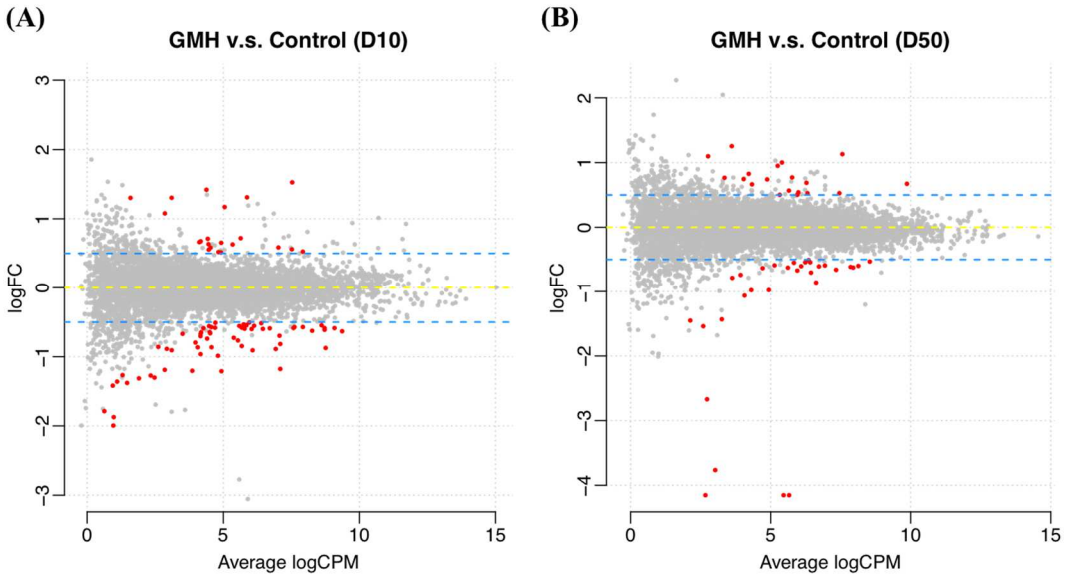

Figure 2. 8 Differentially expressed genes at day 10 and day 50
MA plot show significantly differentially expressed genes at (A) day 10 and (B) day 50

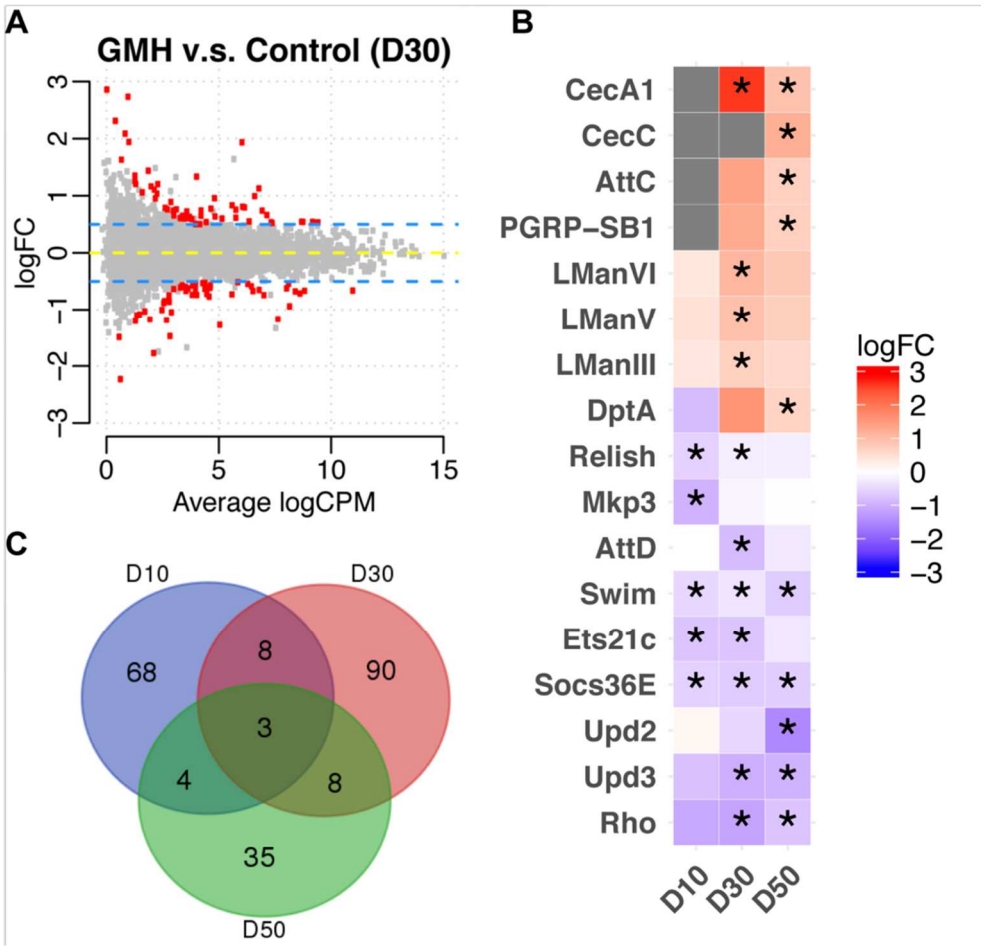

Figure 2. 9 Mid-gut transcriptome analysis shows differences in gene expression levels between control and GMH treatment groups.
(A)MA plot shows the significantly differentially expressed genes between control and GMH groups on Day 30; (B) Heat map shows expression levels of selected genes of interest across three time points, * indicates FDR < 0.05. Genes with low expression levels in half of the samples or more were not included in the differential expression analysis, and indicated as grey here; (C) Venn diagram of differentially expressed genes at three time points.

Table 2. 1 Selected differentially expressed genes between control and GMH treatment at three time points

1. logFC, log fold change; 2. FDR, false discovery rate; 3. AMP, antimicrobial peptide; 4. MMP, mannose metabolic process

Day 10				Day 30				Day50			
name	function	logFC [1]	FDR[2]	name	function	logFC	FDR	name	function	logFC	FDR
Swim	WG signaling	-0.5066	0.01496	CecA1	AMP[3]	2.732	1.81E-05	CecC	AMP	1.2539	1.02E-06
Socs36E	JAK/STAT & EGFR	-0.59	0.03549	LManVI	MMP[4]	1.1272	8.68E-38	CecA1	AMP	0.9497	0.00013
Ets21c	EGFR/ MAPK	-0.7393	0.0015	LManV	MMP	0.992	7.84E-19	AttC	AMP	0.7401	0.02805
Mkp3	EGFR/ MAPK	-0.9869	4.24E-10	LManIII	MMP	0.7395	7.90E-17	DptA	AMP	0.6725	0.02259
				Socs36E	JAK/STAT & EGFR	-0.6497	4.60E-09	PGRP-SB1	IMD signalling	0.7451	0.03088
				Ets21c	EGFR/ MAPK	-0.7395	3.41E-05	Socs36E	JAK/STAT & EGFR	-0.6141	0.01164
				AttD	AMP	-0.8592	0.00672	Swim	WG signaling	-0.6583	0.00048
				Upd3	JAK/STAT	-1.0442	0.00019	Upd3	JAK/STAT	-0.9782	3.65E-05
								Upd2	JAK/STAT	-1.5398	0.00108

Rhomboid (*Rho*), was down-regulated at both day 30 and day 50. Taken together, the suppression of both JAK/STAT and EGFR/MAPK pathways might explain our observation of reduced ISC proliferation rate in GMH-treated flies. Furthermore, consistent with our observations of elevated gut microbial load, several anti-microbial peptide (AMP)-encoded genes were up-regulated in GMH groups at day 30 or day 50, including Cecropin A1 (*CecA1*), Cecropin C (*CecC*), Attacin C (*AttC*), Diptericin A (*DptA*), with only one exception, Attacin D (*AttD*), which was down-regulated at day 30 (Figure 2.9 B). Additionally, as GMH is composed of glucose and mannose monomers, three genes that encode lysosomal alpha-mannosidase were all up-regulated on day 30, further suggesting that GMH or GMH metabolites were absorbed by the gut. For pathway enrichment analysis, although only one pathway survived Bonferroni correction, it showed a consistent pattern that differentially expressed genes are enriched in the above-mentioned pathways (Table 2.2). Overall, the gut RNA-Seq results revealed that GMH supplementation decreases both JAK/STAT and EGFR pathways, and promotes pathways in AMP-involved immune responses, suggesting an impact on gut homeostasis.

2.4 Discussion

In this study, we investigated the effect of life-long GMH supplementation on the lifespan of *Drosophila melanogaster*. We found that in the genetic background of B18, GMH can significantly extend the lifespan of virgin female flies, mated female flies and mated male flies, suggesting that the effect of GMH in B18 is not affected by gender or mating status. To examine whether genetic backgrounds have influences on GMH's

Table 2. 2 GO enrichment pathways at Day 50

Sublist	Category	Term	RT	Genes	Count	%	P-Value	Benjamini
	GOTERM_BP_DIRECT	antibacterial humoral response	RT		4	8.0	1.4E-4	2.2E-2
	GOTERM_BP_DIRECT	innate immune response	RT		5	10.0	7.7E-4	5.9E-2
	GOTERM_BP_DIRECT	humoral immune response	RT		3	6.0	3.5E-3	1.7E-1
	GOTERM_BP_DIRECT	response to bacterium	RT		3	6.0	1.1E-2	3.5E-1
	GOTERM_BP_DIRECT	intestinal epithelial structure maintenance	RT		2	4.0	1.5E-2	3.7E-1
	GOTERM_BP_DIRECT	defense response to bacterium	RT		3	6.0	1.6E-2	3.4E-1
	GOTERM_BP_DIRECT	defense response to Gram-positive bacterium	RT		3	6.0	1.7E-2	3.1E-1
	GOTERM_BP_DIRECT	immune response	RT		3	6.0	2.2E-2	3.6E-1
	GOTERM_BP_DIRECT	paracrine signaling	RT		2	4.0	2.9E-2	4.1E-1
	GOTERM_BP_DIRECT	JAK-STAT cascade	RT		2	4.0	4.8E-2	5.4E-1
	GOTERM_BP_DIRECT	defense response to Gram-negative bacterium	RT		3	6.0	7.4E-2	6.7E-1
	GOTERM_BP_DIRECT	regulation of JAK-STAT cascade	RT		2	4.0	7.6E-2	6.5E-1
	GOTERM_BP_DIRECT	positive regulation of JAK-STAT cascade	RT		2	4.0	9.0E-2	6.8E-1
	KEGG_PATHWAY	Hippo signaling pathway - fly	RT		2	4.0	9.7E-2	4.0E-1

effect, we repeated the experiment using mated females from different wild-type strains, including Oregon-R, Canton-S, and six strains from the DGRP. GMH had a life-extending effect on all strains with only one exception. As the rate of aging is determined by both environmental and genetic factors in *Drosophila* (56, 57), we will discuss this observation from both perspectives. First, environmental factors such as diet, mating status, and fly density are well controlled in our experiment. Since *Wolbachia* infection status is also considered as an environmental factor (58), of six strains chosen from DGRP, three of them were *Wolbachia*-positive, and the other three were not. Among the four strains that were significantly responsive to GMH treatment, two strains were *Wolbachia*-positive, and the other two were not. Therefore, *Wolbachia* infection status does not explain the differences we observed. Second, although the six DGRP strains are from the same species, they differ genetically from each other. Each homozygous strain in DGRP captures a different genome sampled in the natural population (59). The varying response to GMH supplementation indicates the presence of genetic variations moderating its lifespan-extending effect.

We found that GMH can promote the growth of *Acetobacter* in comparison with the control group, suggesting GMH may have prebiotic potential. In young and healthy intestines of *D. melanogaster*, the composition of the gut microbiota is relatively simple with only 5-20 species (60). Major members of the microbiota are benign microbes like *Acetobacter*. Multiple lines of evidence have suggested that *D. melanogaster* has evolved the ability to distinguish beneficial microbes from pathogenic ones so that they can prevent the deleterious induction of immune responses under basal conditions (31, 61). In the aging intestine, there is an increase of both the number and the diversity of

microorganisms (62), termed dysbiosis. This dysbiosis may further exacerbate the age-associated loss of gut homeostasis by chronic activation of stress-signaling pathways and lead to lifespan-shortening (19). However, unlike pathogenic microbes which trigger the stress-signaling pathways and overly stimulate ISCs proliferation, *Acetobacter* only activates the immune responses at a basal level (60). Increased abundance of *Acetobacter* may also outcompete the growth of pathogenic species. Therefore, GMH-mediated increase in the abundance of *Acetobacter* may exert beneficial effects on lifespan by reducing the pressure from age-associated dysbiosis. At the same time, we also implemented 16s rRNA sequencing to investigate the relative composition pattern of the overall gut microbiome after GMH treatment. Apparently, time has a profound effect on gut microbiota composition, with a shift towards a higher relative abundance of *Acetobacter* in older flies of both control and GMH treatment groups. This result is consistent with what we observed in the bacterial load experiment and previous studies (53). It was hypothesized that the gut becomes more toxic in old flies, promoting the growth of aerobic bacteria such as *Acetobacter*, but not anaerobic bacteria like *Lactobacillus.* However, GMH only has a limited effect on the general gut microbiota composition, suggesting that the impact of GMH is mainly on the absolute quantity of gut microbes.

Our results suggest that GMH supplementation has a significant impact on ISC proliferation. As the gut undergoes constant self-renewal, ISC proliferation is required for both normal tissue turnover and epithelial recoveries after damage or infection to maintain intestinal homeostasis in *Drosophila* (63, 64). Generally, low levels of ISC proliferation are observed in young and healthy guts, while strong regenerative activity

is more common when epithelial cells are faced with increased stress and damage at later life stages. As a result of ISCs hyper-proliferation in the aging intestine, disruption and perturbation of normal intestinal organization and function may ultimately lead to the death of the animals. Therefore, the lifespan-extending effect of GMH supplementation is at least partially via the preservation of gut proliferative homeostasis in *Drosophila*. However, the underlying mechanism is still unknown. It is well established that ISC proliferation rates are regulated by several growth and stress signaling pathways, including MAPK, EGFR, JAK/STAT and JNK pathways. From the result of the global gene expression analysis of *Drosophila* midgut, we found that both growth and stress signaling pathways are altered after GMH supplementation. EGFR and MAPK pathways are essential in controlling the rate of ISC proliferation in homeostatic conditions (36). Our results showed that genes in EGFR/MAPK pathways are down-regulated in GMH groups, suggesting that ISC proliferation is inhibited in homeostatic conditions. Under stress conditions, JAK/STAT pathway is activated to promote the differentiation and proliferation of ISCs (31, 34). Our results revealed that genes in JAK/STAT pathway are also down-regulated in GMH groups, suggesting that there are less stress and damage in the intestines of flies with GMH supplementations, or that the activity of these pathways is directly targeted by GMH. Accordingly, lower stress levels in GMH supplemented flies may explain the reduced shrinking of guts in these flies. It is hypothesized that the deregulation of the interaction between intestinal epithelium and the gut microbiome causes the age-related decline of proliferative homeostasis. Therefore, the suppression of JAK/STAT pathway could be a secondary consequence of the increased abundance of *Acetobacter* in the aging intestine, that

would prevent overgrowth of more damaging bacteria. Considered together, GMH improves gut proliferative homeostasis through both growth and stress signaling pathways.

Beyond the tightly-controlled ISC proliferation, other factors like host immune homeostasis also play a vital role in maintaining intestine homeostasis (19, 61). Evidence from RNA-Seq suggested that genes that encode AMPs are significantly up-regulated in GMH groups when flies grow old. Both our study and previous studies showed that the number of bacteria found in the gut increases significantly in old flies (62), and the impairment of the ability to manage the overgrowth of bacteria in aged flies appears to be another potential cause of death(21). One of the strategies to control the growth of the gut microbiota and pathogens is to activate the Imd (Immune deficiency) pathway to induce the expression of AMPs (31, 65). It is noteworthy that other studies also demonstrated that GMH has the ability to enhance immune systems directly by stimulating the gut-associated lymphoid tissue (GALT) system (66) or indirectly by inhibiting the adhesion of pathogens to epithelial cells (67). Given the fact that we observed increased bacterial load in the GMH treatment group, it is possible that elevated expression level of AMPs is related to the changes in the gut microbiome. However, further studies are still needed to elucidate the interaction between the gut microbiome and immune responses after GMH treatment.

As a natural compound, GMH has been shown to exert beneficial effects both locally, by promoting the growth of beneficial gut bacteria, and systematically, by lowering serum cholesterol and glucose levels. For the first time, we demonstrate that GMH

supplementation can extend the lifespan of *Drosophila melanogaster*. Furthermore, our study indicates that GMH intervention can prolong lifespan by altering bacterial load and preserving gut proliferative homeostasis at later life stages. This study should provide insights for future studies to investigate the lifespan extension effect of GMH in other organisms.

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2.6 References

1. Kang HL, Benzer S, Min KT. Life extension in Drosophila by feeding a drug. Proc Natl Acad Sci U S A. 2002;99(2):838-43.
2. Skorupa DA, Dervisefendic A, Zwiener J, Pletcher SD. Dietary composition specifies consumption, obesity, and lifespan in Drosophila melanogaster. Aging Cell. 2008;7(4):478-90.
3. Wu Q, Lian T, Fan X, Song C, Gaur U, Mao X, et al. 2,5-Dimethyl-Celecoxib Extends Drosophila Life Span via a Mechanism That Requires Insulin and Target of Rapamycin Signaling. J Gerontol A Biol Sci Med Sci. 2016.
4. Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, et al. Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. J Gerontol A Biol Sci Med Sci. 2011;66(2):191-201.
5. de Cabo R, Carmona-Gutierrez D, Bernier M, Hall MN, Madeo F. The search for antiaging interventions: from elixirs to fasting regimens. Cell. 2014;157(7):1515-26.
6. Cabreiro F, Au C, Leung KY, Vergara-Irigaray N, Cocheme HM, Noori T, et al. Metformin retards aging in C. elegans by altering microbial folate and methionine metabolism. Cell. 2013;153(1):228-39.
7. Del Corral P, Chandler-Laney PC, Casazza K, Gower BA, Hunter GR. Effect of dietary adherence with or without exercise on weight loss: a mechanistic approach to a global problem. J Clin Endocrinol Metab. 2009;94(5):1602-7.
8. Cescutti P, Campa C, Delben F, Rizzo R. Structure of the oligomers obtained by enzymatic hydrolysis of the glucomannan produced by the plant Amorphophallus konjac. Carbohydr Res. 2002;337(24):2505-11.
9. Yui T, Ogawa K, Sarko A. Molecular and crystal structure of konjac glucomannan in the mannan II polymorphic form. Carbohydr Res. 1992;229(1):41-55.
10. Matsuura Y. Degradation of konjac glucomannan by enzymes in human feces and formation of short-chain fatty acids by intestinal anaerobic bacteria. J Nutr Sci Vitaminol (Tokyo). 1998;44(3):423-36.
11. Blades M, Elamir AA, Tester RF, Al-Ghazzewi FH, Kaal HY, Ghalbon AA, et al. Effects of konjac glucomannan hydrolysates on the gut microflora of mice. Nutrition & Food Science. 2008;38(5):422-9.
12. Keithley J, Swanson B. Glucomannan and obesity: a critical review. Altern Ther Health Med. 2005;11(6):30-4.
13. Tester RF, Al-Ghazzewi FH. Beneficial health characteristics of native and hydrolysed konjac (Amorphophallus konjac) glucomannan. J Sci Food Agric. 2016;96(10):3283-91.
14. Al-Ghazzewi FH, Khanna S, Tester RF, Piggott J. The potential use of hydrolysed konjac glucomannan as a prebiotic. Journal of the Science of Food and Agriculture. 2007;87(9):1758-66.
15. Connolly ML, Lovegrove JA, Tuohy KM. Konjac glucomannan hydrolysate beneficially modulates bacterial composition and activity within the faecal microbiota. Journal of Functional Foods. 2010;2(3):219-24.
16. Ramberg JE, Nelson ED, Sinnott RA. Immunomodulatory dietary polysaccharides: a systematic review of the literature. Nutr J. 2010;9:54.

17. Al-Ghazzewi F, Elamir A, Tester R, Elzagoze A. Effect of depolymerised konjac glucomannan on wound healing. Bioactive Carbohydrates and Dietary Fibre. 2015;5(2):125-8.
18. Suwannaporn P, Tester RF, Al-Ghazzewi FH, Artitdit P. Effect of short term administration of konjac glucomannan hydrolysates on adult blood lipid parameters and glucose concentrations. Nutrition & Food Science. 2015;45(4):616-24.
19. Jasper H. Exploring the physiology and pathology of aging in the intestine of Drosophila melanogaster. Invertebr Reprod Dev. 2015;59(sup1):51-8.
20. Bonfini A, Liu X, Buchon N. From pathogens to microbiota: How Drosophila intestinal stem cells react to gut microbes. Dev Comp Immunol. 2016;64:22-38.
21. Guo L, Karpac J, Tran SL, Jasper H. PGRP-SC2 promotes gut immune homeostasis to limit commensal dysbiosis and extend lifespan. Cell. 2014;156(1-2):109-22.
22. DeGruttola AK, Low D, Mizoguchi A, Mizoguchi E. Current Understanding of Dysbiosis in Disease in Human and Animal Models. Inflamm Bowel Dis. 2016;22(5):1137-50.
23. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. Cell. 2012;148(6):1258-70.
24. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu Rev Immunol. 2010;28:573-621.
25. Uronis JM, Muhlbauer M, Herfarth HH, Rubinas TC, Jones GS, Jobin C. Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. PLoS One. 2009;4(6):e6026.
26. Biagi E, Candela M, Turroni S, Garagnani P, Franceschi C, Brigidi P. Ageing and gut microbes: perspectives for health maintenance and longevity. Pharmacol Res. 2013;69(1):11-20.
27. Ottaviani E, Ventura N, Mandrioli M, Candela M, Franchini A, Franceschi C. Gut microbiota as a candidate for lifespan extension: an ecological/evolutionary perspective targeted on living organisms as metaorganisms. Biogerontology. 2011;12(6):599-609.
28. Micchelli CA, Perrimon N. Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature. 2006;439(7075):475-9.
29. Ohlstein B, Spradling A. The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature. 2006;439(7075):470-4.
30. Biteau B, Karpac J, Supoyo S, Degennaro M, Lehmann R, Jasper H. Lifespan extension by preserving proliferative homeostasis in Drosophila. PLoS Genet. 2010;6(10):e1001159.
31. Buchon N, Broderick NA, Chakrabarti S, Lemaitre B. Invasive and indigenous microbiota impact intestinal stem cell activity through multiple pathways in Drosophila. Genes Dev. 2009;23(19):2333-44.
32. Rera M, Clark RI, Walker DW. Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in Drosophila. Proc Natl Acad Sci U S A. 2012;109(52):21528-33.
33. Rera M, Bahadorani S, Cho J, Koehler CL, Ulgherait M, Hur JH, et al. Modulation of longevity and tissue homeostasis by the Drosophila PGC-1 homolog. Cell Metab. 2011;14(5):623-34.
34. Jiang H, Patel PH, Kohlmaier A, Grenley MO, McEwen DG, Edgar BA.

Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell. 2009;137(7):1343-55.
35. Jiang H, Edgar BA. EGFR signaling regulates the proliferation of Drosophila adult midgut progenitors. Development. 2009;136(3):483-93.
36. Park JS, Kim YS, Yoo MA. The role of p38b MAPK in age-related modulation of intestinal stem cell proliferation and differentiation in Drosophila. Aging (Albany NY). 2009;1(7):637-51.
37. Yang JS, Nam HJ, Seo M, Han SK, Choi Y, Nam HG, et al. OASIS: online application for the survival analysis of lifespan assays performed in aging research. PLoS One. 2011;6(8):e23525.
38. Deshpande SA, Carvalho GB, Amador A, Phillips AM, Hoxha S, Lizotte KJ, et al. Quantifying Drosophila food intake: comparative analysis of current methodology. Nat Methods. 2014;11(5):535-40.
39. Sambrook J, Russell DW. Purification of nucleic acids by extraction with phenol:chloroform. CSH Protoc. 2006;2006(1).
40. Callahan BJ, Sankaran K, Fukuyama JA, McMurdie PJ, Holmes SP. Bioconductor Workflow for Microbiome Data Analysis: from raw reads to community analyses. F1000Res. 2016;5:1492.
41. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.
42. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8(4):e61217.
43. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-40.
44. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590-6.
45. McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol. 2014;10(4):e1003531.
46. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
47. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.
48. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.
49. Malick LE, Kidwell JF. The effect of mating status, sex and genotype on longevity in Drosophila melanogaster. Genetics. 1966;54(1):203-9.
50. Partridge L, Piper MD, Mair W. Dietary restriction in Drosophila. Mech Ageing Dev. 2005;126(9):938-50.
51. Pan XD, Chen FQ, Wu TX, Tang HG, Zhao ZY. Prebiotic oligosaccharides change the concentrations of short-chain fatty acids and the microbial population of mouse bowel. J Zhejiang Univ Sci B. 2009;10(4):258-63.
52. Elamir AA, Blades M, Tester RF, Al-Ghazzewi FH, Kaal HY, Ghalbon AA, et al.

Effects of konjac glucomannan hydrolysates on the gut microflora of mice. Nutrition & Food Science. 2008;38(5):422-9.
53. Wong CN, Ng P, Douglas AE. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ Microbiol. 2011;13(7):1889-900.
54. Broderick NA, Lemaitre B. Gut-associated microbes of Drosophila melanogaster. Gut Microbes. 2012;3(4):307-21.
55. Li H, Qi Y, Jasper H. Preventing Age-Related Decline of Gut Compartmentalization Limits Microbiota Dysbiosis and Extends Lifespan. Cell Host Microbe. 2016;19(2):240-53.
56. Libert S, Pletcher SD. Modulation of longevity by environmental sensing. Cell. 2007;131(7):1231-4.
57. He Y, Jasper H. Studying aging in Drosophila. Methods. 2014;68(1):129-33.
58. Aleksandrov ID, Aleksandrova MV, Goriacheva, II, Roshchina NV, Shaikevich EV, Zakharov IA. [Removing endosymbiotic Wolbachia specifically decreases lifespan of females and competitiveness in a laboratory strain of Drosophila melanogaster]. Genetika. 2007;43(10):1372-8.
59. Chow CY. Bringing genetic background into focus. Nat Rev Genet. 2016;17(2):63-4.
60. Ayyaz A, Jasper H. Intestinal inflammation and stem cell homeostasis in aging Drosophila melanogaster. Front Cell Infect Microbiol. 2013;3:98.
61. Buchon N, Broderick NA, Lemaitre B. Gut homeostasis in a microbial world: insights from Drosophila melanogaster. Nat Rev Microbiol. 2013;11(9):615-26.
62. Ren C, Webster P, Finkel SE, Tower J. Increased internal and external bacterial load during Drosophila aging without life-span trade-off. Cell Metab. 2007;6(2):144-52.
63. Biteau B, Hochmuth CE, Jasper H. Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. Cell Stem Cell. 2011;9(5):402-11.
64. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B. Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe. 2009;5(2):200-11.
65. Kim T, Kim YJ. Overview of innate immunity in Drosophila. J Biochem Mol Biol. 2005;38(2):121-7.
66. Schley PD, Field CJ. The immune-enhancing effects of dietary fibres and prebiotics. Br J Nutr. 2002;87 Suppl 2:S221-30.
67. Al-Ghazzewi FH, Tester RF. Inhibition of the adhesion of Escherichia coli to human epithelial cells by carbohydrates. Bioactive Carbohydrates and Dietary Fibre. 2014;4(1):1-5.

Chapter 3 – Oligofructose supplementation extends lifespan in *Drosophila melanogaster*

3.1 Introduction

Inulin is a natural polysaccharide that can be found in many plants including onion, garlic, asparagus, and chicory (1, 2). Inulin is a heterogeneous blend of fructose monomers with a degree of polymerization of two to 60+, while oligofructose is the lower-molecular-weight form of inulin with a degree of polymerization of three to eight (3). The fructose monomers are linked by β-2-1 bonds, thus making both inulin and oligofructose indigestible in the upper gastrointestinal tract (4). It is well established that inulin and oligofructose has the ability to promote the growth of *Bifidobacteria* in the colon, which is a strain that has health-promoting functions such as inhibiting the growth of pathogenic bacteria in the intestine, aiding the absorption and the synthesis of B vitamins, and stimulating the immune system (3, 5). Therefore, inulin and oligofructose have been considered as prebiotics, which can exert beneficial effects by stimulating the growth of benign gut bacteria. Additionally, studies in human subjects have also suggested that administration of inulin or oligofructose has beneficial effects on the modulation of lipid metabolism, bone mineralization and a reduction in colon cancer risk (6-8). However, to the best of our knowledge, only one paper has investigated the lifelong supplementation effect of inulin on the survival rate of rats (9). They reported that the survival rate at 24 months of age is about 30% greater in the inulin-supplemented group than the control group, together with the observation of reduced body weight and lower cholesterol level (9). No studies have investigated the impact of oligofructose supplementation on the lifespan and the potential underlying

mechanism. To examine whether oligofructose can extend the lifespan, we chose *Drosophila melanogaster* as the model organism. Unlike rats having a lifespan of two-four years, *D. melanogaster* is ideal for aging studies due to its relatively short lifespan (two to three months), and ease of environmental and genetic manipulations (10).

As the frontline in contact with dietary supplementation and gut microbiome, the intestine is not only responsible for physiological functions like nutrient absorption, but also acting as the defense barrier to control both commensal and pathogenic bacteria in both human and *D. melanogaster* (10). The intestine is a high-turnover tissue, and intestinal stem cells (ISCs) proliferation is critical for flies to maintain its homeostasis (11). However, hyper-proliferation of ISCs in aged flies may result in dysregulation of gut proliferative homeostasis and even premature death (12). Therefore, preservation of gut homeostasis by dietary supplementation may be a feasible approach to managing healthy lifespan. The relationship between mitochondrial function and lifespan is another popular topic in the field of aging studies. Mitochondria are involved in various physiological processes including metabolism, apoptosis, and signaling (13, 14). In particular, the major function of mitochondria is to produce ATP as the cellular energy through the electron transport chain (ETC), which is essential for all organisms. However, it is interesting that mild inhibition of mitochondrial respiration is able to extend the lifespan of various species (15-17), including *D. melanogaster* (18). It has been shown that the lifespan is extended by RNAi knockdown of genes encoding components in the ETC complex I, III, IV or V (18). In this article, we will present data for the lifespan-extending effect of oligofructose. With the aid of biochemical assays and transcriptome analysis, we will show that both ISC proliferation and mitochondrial

respiration are altered after oligofructose supplementation, suggesting the potential underlying mechanisms.

3.2 Materials and methods

Fly Stocks and Husbandry

As this study was carried out as a concurrent study of GMH supplementation project, all the control data are shared between these two projects. All flies were reared under standard laboratory conditions with a 12-h light/dark cycle at 25°C in vials containing agar–dextrose–yeast medium. Nine different strains of wild-type flies were used in this study. Besides Canton-S and Oregon-R, we also included one strain (B18) from the Global Diversity Panel and six strains (DGRP-21, DGRP-38, DGRP-40, DGRP-85, DGRP-105, DGRP-136) from Drosophila Genetic Reference Panel (DGRP). To examine whether *Wolbachia* infection status modulates the lifespan-extending effect, we chose three *Wolbachia* positive strains and three negative strains from the DGRP.

Oligofructose Supplementation and Survival Assay

The oligofructose supplemented medium was prepared by adding the oligofructose (Sigma) powder into the control agar-dextrose-yeast medium at a concentration of 0.25% w/v. Flies were collected right after eclosion and housed at a density of 20-30 flies per vial. At least 6 replicates with a minimum of 120 flies were tested for each condition. Flies were transferred to fresh medium every two days for mated females, and every three to five days for virgin females and males. During each transfer, dead flies in each vial were counted. Flies escaped or died accidentally were recorded as missing. All data were analyzed with Log-Rank test using the Online Application for

the Survival Analysis (OASIS) of lifespan assays (19).

Long-term Feeding Assay

Both control medium and oligofructose supplemented medium were prepared with the addition of a blue indigestible dye, FD&C blue No.1, at a concentration of 1% w/v (20). Dyed growth medium was poured into the cap of an aerated 50 ml Falcon centrifuge tube. Mated females were transferred into Falcon tubes with the medium-containing cap on. Falcon tubes were placed upside down (cap side down) in incubators for two days. Flies were then removed from the tube, and two ml of PBS was added into the falcon tube to wash all the colored-feces off the side of tubes. The total amount of food that flies consumed across two days was measured as a function of the amount of blue in the feces. This calculated from the optical density of a serial dilution of FD&C blue No.1 solution.

Gut Microbiome Sequencing and Analysis

Guts of flies were dissected in PBS after 10, 20, 30 and 40 days of treatments. DNA was extracted from a pool of 20 guts for each sample using phenol-chloroform extraction method (21). According to Illumina 16s metagenome library preparation guide, 16s ribosomal RNA gene amplicons were prepared for the Illumina MiSeq System. Briefly, two rounds of PCR reactions were carried out to first amplify the hypervariable regions (V3 and V4) of the bacterial 16s rRNA genes, and then adaptors and barcodes were added for Illumina sequencing.

Microbiome sequencing reads were analyzed following a Bioconductor workflow based on dada2, phyloseq, and EdgeR (22-25). Low-quality reads were filtered: those

containing N, with the base quality score less than or equal to two, or with more than two expected errors. Additionally, the first ten bases and all bases after position 230 were trimmed. De-replication was performed to combine identical reads while keeping track of their abundance. The core dada2 ribosomal sequence variants (RSVs) inference algorithm was applied to the de-replicated data to infer sample sequences exactly and resolve differences of as little as one nucleotide by modeling and correcting sample-specific sequencing errors (23). We then merged the inferred forward and reverse sequences, removing paired sequences that do not overlap perfectly. Chimera sequences were further removed. The taxonomy of RSVs was assigned based on the SILVA database (version 128) (26). Sequences assigned to the genus of *Wolbachia* were removed. Principal coordinates analysis was performed on log-transformed abundance data using Bray-Curtis dissimilarity. Constrained correspondence analysis was performed to evaluate the contribution of diet and treatment length to the variation in microbiome composition. Alpha diversity was measured with the Shannon index and the Simpson index. Differential abundance across control and supplementation groups was tested with edgeR (25, 27).

Gut RNA-Seq and Data Analysis

After 10, 30 and 50 days of oligofructose treatment, 50 guts were dissected and pooled for each replicate. Total RNA was extracted using Trizol (Invitrogen) and RNeasy mini plus kit (Qiagen). mRNA was isolated using magnetic mRNA isolation kit (NEB). KAPA stranded RNA-Seq library preparation kit was used to construct libraries for Illumina sequencing.

Raw sequencing reads were firstly processed with Trimmomatic (Version 0.33) (28) to trim adaptor sequences and low-quality bases and then mapped to the reference genome of *Drosophila melanogaster* (FlyBase Dmel Release 6.09) with STAR (Version 2.5.1b) (29). Differentially expressed genes were identified with edgeR (25). Only genes expressed (defined as count per million > 1) in at least two out of four samples were included in the analysis. Significant differentially expressed genes were defined with FDR < 0.05 and $\log_2$(fold change) >= or <= 0.5. Pathway enrichment of differentially expressed genes were evaluated with DAVID 6.8 (30).

PH3 Immunostaining and Gut Length Measurement

Guts were dissected in PBS and fixed for 30 min in PBS with 0.1% Tween 20 (PBT) and 4% paraformaldehyde. They were subsequently rinsed with PBT, and incubated with primary antibodies (1/1000 anti-PH3 (Upstate/Millipore) or 1/1000 anti-GFP, Roche) in PBT 1% BSA. Staining was revealed after a second incubation with Alexa488- or Alexa594-coupled anti-mouse antibodies (Invitrogen), and nuclei were stained with DAPI (Sigma).

3.3 Results

3.3.1 Oligofructose extends the lifespan in specific strains of *Drosophila melanogaster*

We first tested whether oligofructose supplementation affects the lifespan of *Drosophila melanogaster*. In strain B18, survival tests in both mated male and female flies showed significant lifespan extension (Figure 3.1 A, B). The mean lifespan in control groups was 57.39 days for male flies and 58.05 for female flies. In the oligofructose treatment groups, an increase of 12.96% for the mean lifespan was observed in males (p = 2.1e-

08), and an increase of 10.44% for the mean lifespan was observed in females (p = 0.0008). As mating status is known to markedly affect lifespan (31), we then examined whether oligofructose can also extend the lifespan in virgin female flies of strain B18. No significant results were observed in virgin females (Figure 3.1 C) so that we focused on mated females for the rest of studies. In addition to gender and mating status, we want to evaluate if the genetic background is able to affect the lifespan-extending effect of oligofructose. We repeated the survival test in another two wild-type strains (Oregon-R and Canton-S) and six strains from DGRP. Oligofructose supplementation increased the mean lifespan by 5.6% in Canton-S (Figure 3.1 D, p = 0.046) and 4.48% for DGRP-136 (Figure 3.2 A, p = 0.016), but had no effects on Oregon-R and four out of six DGRP strains (21, 38, 40, 85, Figure 3.2 B-E). Surprisingly, in the strain of DGRP-105, oligofructose supplementation resulted in a significant reduction in the mean lifespan by 11.68% (Figure 3.2 F, p = 0.021). These results suggest that oligofructose supplementation can prolong the lifespan in some strains. Both mating status and genetic backgrounds may moderate the effect of oligofructose supplementation on lifespan.

3.3.2 Oligofructose has little effect on food intake and gut microbiome composition

Dietary supplementation might change the amount of food consumption and calorie restriction is an effective way to extend lifespan (32). Therefore, before we start to investigate the potential underlying mechanisms, we firstly evaluated the impact of oligofructose supplementation on feeding behavior using a long-term feeding assay (Figure 3.3 A). No significant difference in the amount of food intake was discovered

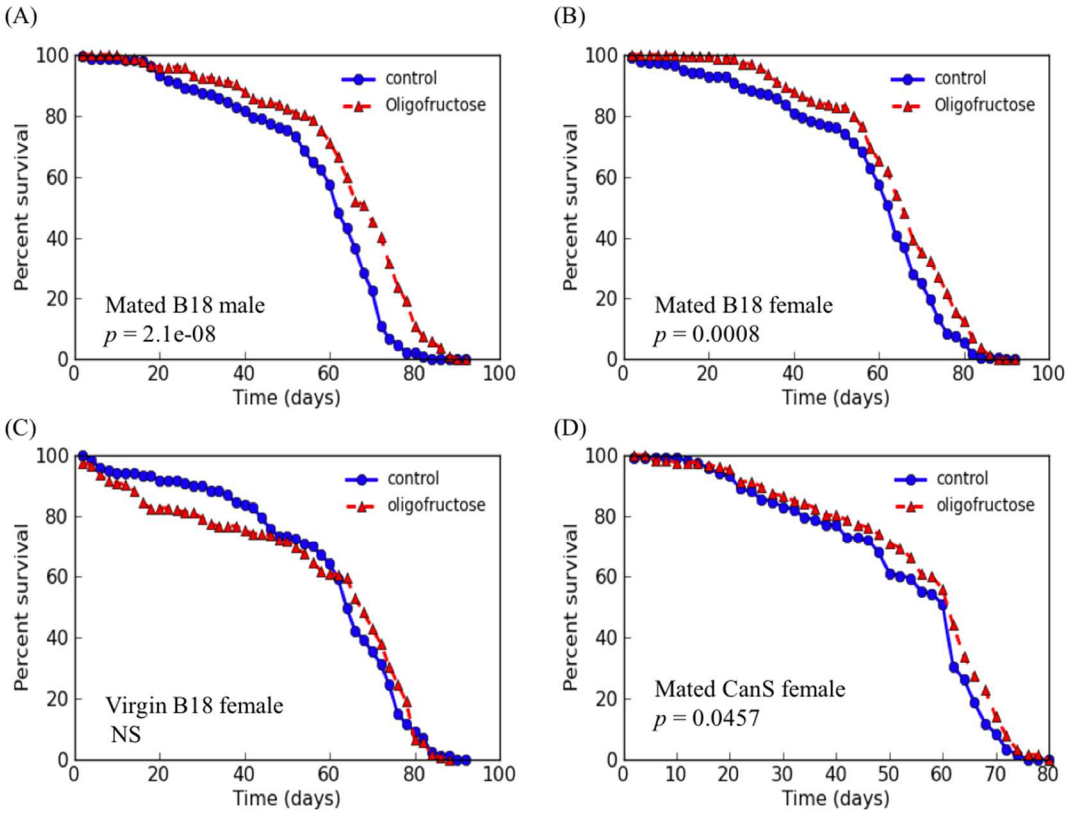

Figure 3. 1 Oligofructose extends lifespan in mated flies of strain B18 and Canton-S Oligofructose supplementation increase the mean lifespan of (A) mated male flies of strain B18; (B) mated female flies of strain B18. Oligofructose has no effect on (C) virgin female flies of strain B18; and slight effect on (D) mated female flies of strain Canton-S. For all of the survival tests, N>120.

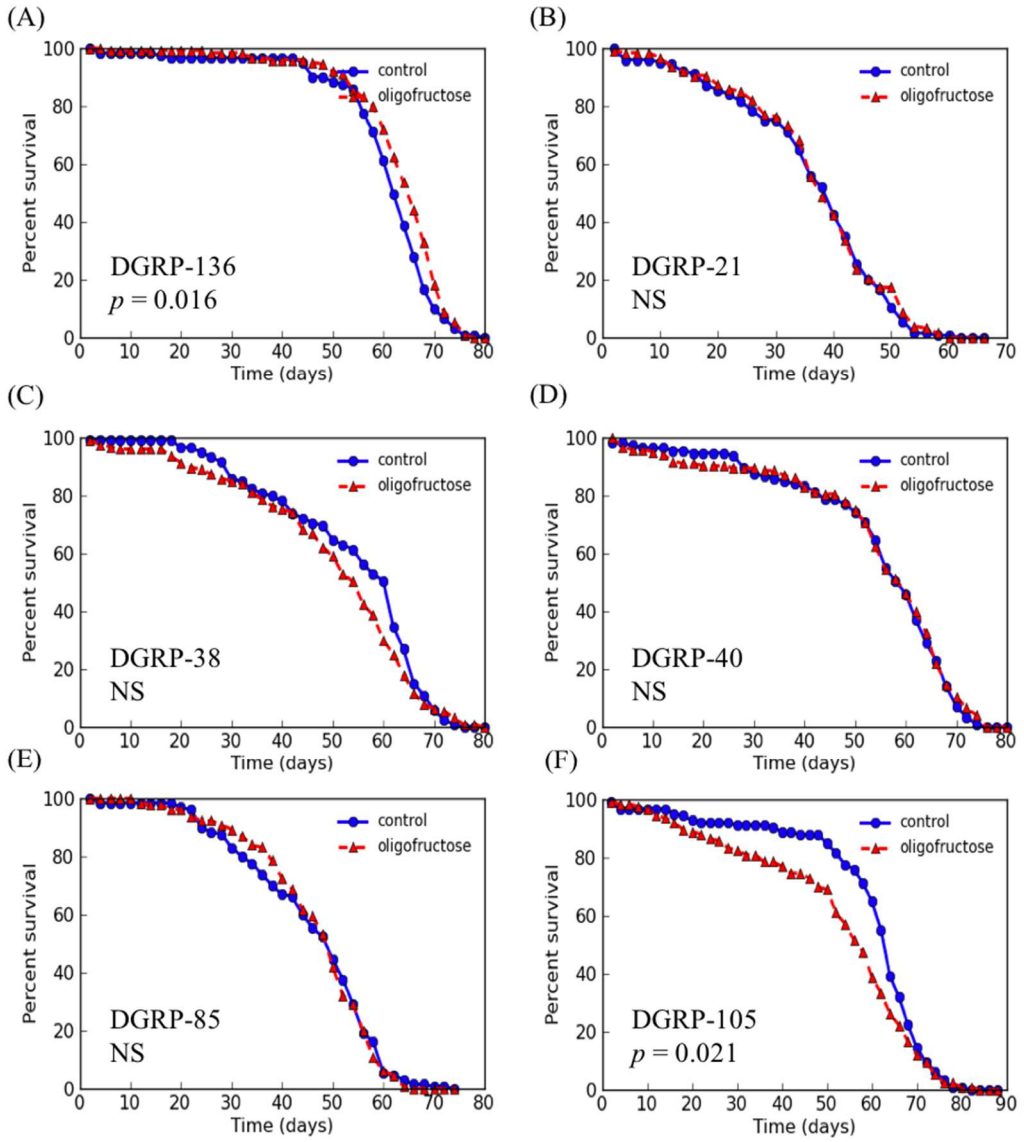

Figure 3. 2 The effect of oligofructose on lifespan in DGRP strains
Oligofructose has significant effect on lifespan in (A) DGRP-136, but has no effect on lifespan in (B) DGRP-21, (C) DGRP-38, (D) DGRP-40, and (E) DGRP-85. The lifespan is significantly decreased in (F) DGRP-105 after oligofructose supplementation. For all of the survival test, N>120.

between control and oligofructose treatment groups, ruling out the involvement of calorie restriction in the observation of the lifespan-extending effect of oligofructose supplementation. It has been well established that oligofructose has prebiotic effect in human and is able to promote the growth of beneficial bacteria in the intestine. Therefore, we hypothesized that oligofructose may extend the lifespan through its impact on the gut microbiome. To test this hypothesis, we performed gut microbiome sequencing to quantify the relative abundance changes of the commensal bacteria in flies along the process of aging. According to the result of the principal coordinates analysis (PCoA), there is a clear pattern that samples were clustered based on the sampling times, but not on the diet groups (Figure 3.3 B). Constrained correspondence analysis further confirmed that the sampling time explains 49.80% ($p < 0.001$) of the variance in microbiome composition while the type of diet explains only 5.55%. When analyzing the differential abundance at the genus level, only one genus of *Stenotrophomonas* showed a significantly lower abundance in oligofructose group on day 10. Additionally, we observed a shift toward the high relative abundance of *Acetobacter* in old flies in both control and oligofructose groups (Figure 3.3 C), which is in accordance with previous findings (33). Altogether, our results suggest that oligofructose has little effect on food intake and gut microbiome composition, indicating the lifespan-extending effect of oligofructose is not mediated by its prebiotic effect.

3.3.3 The effect of oligofructose on gut proliferative homeostasis

As the gut is a tissue with a high turnover rate, the proliferative activity is critical in maintaining the gut integrity (34). It has also been proved that preserving the intestinal

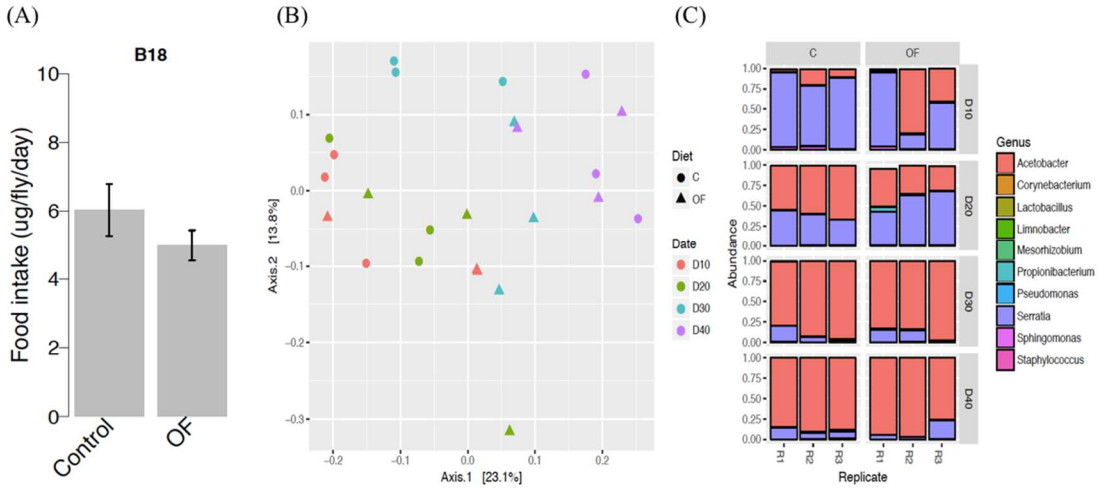

Figure 3. 3 Oligofructose has little effect on both food intake and gut microbiome composition

(A) There is no significant difference in long-term food intake between control and oligofructose group, n = 3 biological replicates with 20 individual flies per replicate; (B) Principal coordinate analysis of gut microbiome compositions in control and oligofructose groups at different time points; (C) Each bar represents average relative abundance of each bacterial taxon (top 10 taxa) within a group at genus level.

proliferative homeostasis is sufficient to extend the lifespan of *D. melanogaster* (34). Therefore, we used anti-phosphohistone H3 (anti-PH3) antibody as the marker to measure the gut stem cell proliferation rate in the gut of flies fed on control and oligofructose food at different time points. Consistent with former studies (35, 36), low levels of homeostatic proliferation were observed in young and healthy guts on day 10, and higher levels on day 50 in both control and oligofructose groups. However, we found that the number of PH3 positive cells was significantly higher in the oligofructose group (Figure 3.4 A) than that of the control group on both day 30 ($p = 0.021$) and day 40 ($p = 0.016$). Although no significant difference was observed on day 50 between control and oligofructose group, there was an obvious trend that the number of PH3 positive cells was lower in oligofructose group on day 50. We also measured gut length as another parameter of the physiological status of the gut. In accordance with a previous study (37), we observed significant gut shortening along the process of aging in both groups (Figure 3.4 B). However, on day 10, guts from the oligofructose group were significantly shorter than the control group ($p = 0.000791$), and no differences were observed at other time points. Taken together, oligofructose supplementation shortens the gut at an early life stage and promotes gut stem cell proliferation at middle life stages.

3.3.4 Oligofructose affects the regulation of stress signaling pathways and mitochondrial respiration processes

To explore the potential underlying mechanisms of the lifespan-extending effect of oligofructose supplementation, we carried out mRNA-sequencing experiment to identify differentially expressed genes between control and supplementation groups.

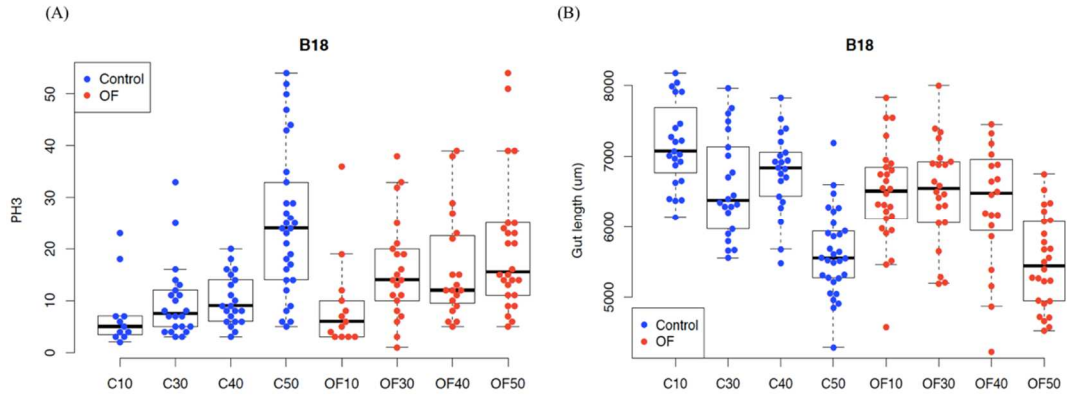

Figure 3. 4 The effect of oligofructose on intestinal stem cells proliferation

(A) Quantification of PH3 positive cells in mid-guts from flies fed on either control or oligofructose supplemented food at different time points. (B) Measurement of the mid-gut length from flies fed on either control or oligofructose supplemented food at different time points.

The guts of flies were dissected after 10, 30 and 50 days of treatment, and the total RNA was isolated from these guts to construct the mRNA-sequencing library. Overall, 95 differentially expressed genes were found in guts collected on day 10, 355 genes on day 30, and 285 genes on day 50 (Figure 3.5). Only six genes were shared among all three time-points, 16 genes were shared between day 10 and day 30, 64 genes were shared between day 30 and day 50, and 24 genes were shared between day 10 and day 50. To interpret the data at the biological pathway level, we annotated genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) to perform the pathway enrichment analysis. We found that on day 10, only one pathway of ribosome biogenesis is upregulated in the oligofructose group. On day 30, the EGFR/MAPK and Toll/Imd signaling pathways are significantly up-regulated, both of which are involved in the intestinal immune responses. At the same time, oxidative phosphorylation (OXPHOS) and citrate cycle (TCA cycle) together with pathways related to carbon metabolism and fatty acid degradation are all down-regulated in the oligofructose group. The OXPHOS activity was kept down-regulated on day 50, with additional pathways involved in cytochrome P450 metabolism. Since the ISC proliferation is regulated by these stress-signaling pathways and mitochondrial OXPHOS and TCA cycle are tightly involved in the energy metabolism, we next mainly focused on genes related to these pathways. As early as day 10, we found that several differentially expressed genes in the oligofructose group were involved in the EGFR/MAPK and Toll pathways (Figure 3.6 A). However, inconsistent with the KEGG pathway enrichment analysis on day 30, most of the genes are down-regulated on day 10, except one antimicrobial peptide, Drosomycin (*Drs*). Meanwhile, although we did not observe significant results at the pathway level on day

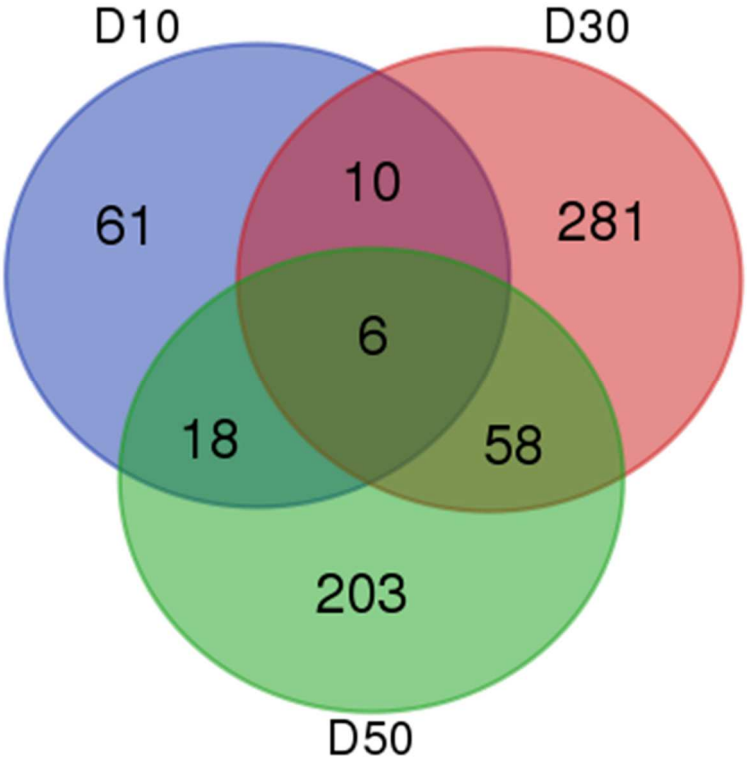

Figure 3. 5 Venn diagram of the differentially expression genes at three time points There are 95 differentially expressed genes between control and oligofructose groups on day 10, 355 genes on day 30, and 285 genes on day 50. 6 genes are shared among all three time points, 16 genes shared between day 10 and day 30, 64 genes shared between day 30 and day 50, and 24 genes shared between day 10 and day 50.

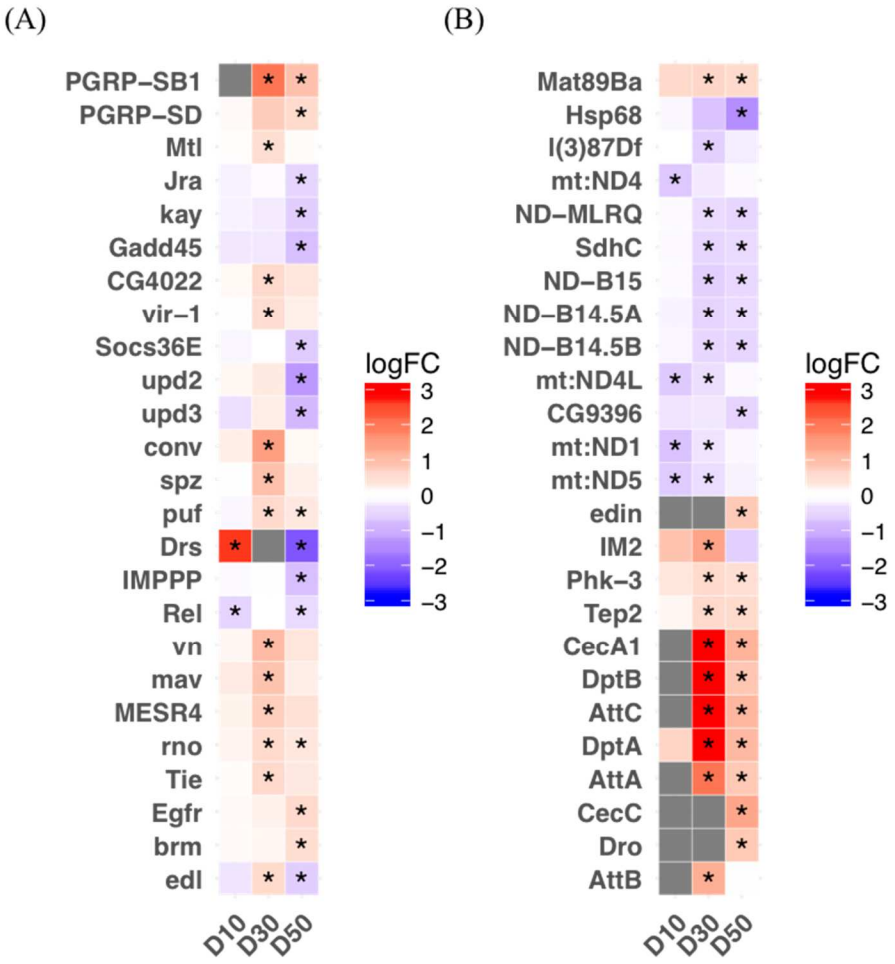

Figure 3. 6 Heatmap of selected differentially expressed genes between control and oligofructose groups

Heat map shows expression levels of selected genes of interest across three time points, * indicates FDR < 0.05. Genes with low expression levels in half of the samples or more were not included in the differential expression analysis, and indicated as grey here

10, four genes encoding proteins in the mitochondrial electron transport chain (ETC) were all significantly down-regulated in oligofructose group (Figure 3.6 B). For day 30, an increasing number of genes in the stress signaling pathways were found to be differentially expressed (Figure 3.6 A). One of the major ligands for the EGFR pathway, Vein (*vn*), was significantly up-regulated in the oligofructose group, together with another two regulators of the EGFR pathway, *Edl* and *Rno*. Multiple genes in the Toll/Imd, JAK/STAT, and JNK pathways were also up-regulated after 30 days of oligofructose supplementation. On the other hand, the expression of genes encoding NADH dehydrogenase and succinate dehydrogenase were significantly down-regulated in the oligofructose group (Figure 3.6 B), which is in accordance with the pathway enrichment analysis.

When analyzing the transcriptome data collected on day 50, we found dramatic changes in the stress-signaling pathways. Except for two genes in the EGFR pathway still up-regulated in the oligofructose group, all other genes were down-regulated in the stress-signaling pathways, including the two main ligands of the JAK/STAT pathway, unpaired 2 (*Upd2*) and unpaired 3 (*Upd3*) (Figure 3.6 A). We still observed the same pattern on genes involved in mitochondrial respiration: genes encoding NADH dehydrogenase and pyruvate dehydrogenase were kept down-regulated on day 50 (Figure 3.6 B). In addition to the above-mentioned genes, there are numerous genes encoding antimicrobial peptides (AMP) or involving in antibacterial responses were up-regulated in oligofructose group on both day 30 and day 50 (Figure 3.6 B). It is also noteworthy that two genes functioning in responses to starvation were up-regulated in flies fed on oligofructose diet, one on day 30 and the other on day 50. To summarize, the

differentially expressed genes are enriched in gut stress-signaling pathways and mitochondrial respiration processes. The stress-signaling pathways tended to be inhibited on both day 10 and day 50, but were significantly promoted on day 30. For the mitochondrial respiration process, it was inhibited in the oligofructose treatment group at all three time-points.

3.4 Discussion

In this study, we use *Drosophila melanogaster* as the model organism to investigate the effect of life-long oligofructose supplementation on the lifespan. We found that in the genetic background of B18, oligofructose can significantly extend the lifespan in mated male and female flies, but not in virgin female flies, suggesting the effect of oligofructose in B18 is affected by the mating status but not gender. When repeating the experiment using flies with different genetic backgrounds, oligofructose supplementation extends the average lifespan of two strains out of eight wild-type strains we examined. In one of the DGRP strains, oligofructose even has detrimental effects on the average lifespan. Therefore, we found that there is a strong genetic influence on the lifespan-extending effect of oligofructose supplementation. Future studies are needed to explain the interaction between the oligofructose treatment and genetic factors in the process of aging. As oligofructose is known to promote the growth of beneficial bacteria in the gut (1), we then examined if we can observe the same effect in *D. melanogaster*. The results of 16s rRNA sequencing indicated that dietary supplementation of oligofructose cannot alter the gut microbiome composition in flies, suggesting gut microbiome may not play a role in increasing the lifespan after

oligofructose treatment.

Combining the results of PH3 immunostaining experiment and gut transcriptome sequencing, we discovered that the ISC proliferation rate and mitochondrial respiration process may be the two key factors mediating the lifespan-extending effect of oligofructose. First, ISC proliferation is essential for the gut to maintain the high-turnover rate for the epithelial recoveries after damage or infection (38, 39). However, at the later life stage, the hyperproliferation of ISCs can lead to the disruption and perturbation of normal intestinal organization and function, which may ultimately lead to the death of the animal (12). Therefore, it is critical for the animal to maintain the proliferative gut homeostasis. The over-activation of several growth and stress signaling pathways is known to be responsible for the hyper-proliferation of ISCs, such as MAPK/EGFR, JAK/STAT, Toll/Imd, and JNK pathways (38, 40, 41). Therefore, we have observed a consistent pattern on day 30 that oligofructose group has both elevated amounts of PH3 positive cells and promoted expression of MAPK/EGFR and Toll/Imd pathways, indicating oligofructose may stimulate ISC proliferation via the stress signaling pathways on day 30. It is known that the hyper-proliferation in the aging intestine is detrimental for flies. However, it is still uncertain whether it is also harmful to the middle-aged flies. On day 50, the transcriptome analysis showed that stress signaling pathways like JAK/STAT and JNK are inhibited under oligofructose supplementation. However, the general effect of aging may dominate the outcome so that we have only observed the trend of reduction in ISC proliferation in oligofructose group on day 50, but not significant. In comparison, in the examination of another compound named glucomannan hydrolysate (GMH), we have observed a more potent

reduction in ISC proliferation in aging flies together with a longer lifespan extension. It is very likely that the oligofructose supplementation is not as efficient as GMH, especially on the ISC proliferation.

The second key factor in the potential underlying mechanism of oligofructose supplementation is the mitochondrial respiration. Evidence from RNA-seq demonstrated that mitochondrial respiration is inhibited after oligofructose treatment. The expression of genes encoding NADH dehydrogenase and succinate dehydrogenase is down-regulated. These two enzymes are required for the oxidation of NADH and succinate to provide the energy for the first step in ETC. Therefore, the ETC capacity is significantly suppressed under oligofructose supplementation across the process of aging. According to the literature, the extension of lifespan by mild inhibition of mitochondrial respiration has been demonstrated from yeast to mice (15-17). It has also been proved that RNAi knockdown of components in ETC complexes extends lifespan in *D. melanogaster* (18). However, the molecular mechanism of this phenotype is still under debate, with several theories including reduced metabolic rate, retrograde signaling from mitochondria to the nucleus, and elevated ROS level (14). To elucidate the connection between oligofructose supplementation and mitochondrial respiration, further studies are needed to explore the molecular mechanism of this association.

Overall, we have demonstrated that oligofructose can extend the lifespan of *D. melanogaster* in a genetics-linked manner. Unlike the established prebiotic effect in human, oligofructose has little impact on gut microbiome growth in flies. However, it showed a consistent impact on gut homeostasis as revealed by the biochemical

experiment and the transcriptome analysis that oligofructose is able to promote the ISCs proliferation at middle age of flies, but inhibit the hyper-proliferation of ISCs in the aging intestine. Moreover, mitochondrial respiration capacity is also inhibited by oligofructose supplementation. Taken together, the involvement of both ISC proliferation and mitochondrial respiration may contribute to the lifespan-extending effect of oligofructose supplementation. Meanwhile, future studies are required to further investigate the molecular mechanisms of the interaction between oligofructose supplementation, gut homeostasis, and mitochondrial metabolism.

3.5 Acknowledgement

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3.6 References

1. Roberfroid MB. Introducing inulin-type fructans. Br J Nutr. 2005;93 Suppl 1:S13-25.
2. Roberfroid MB. Concepts in functional foods: the case of inulin and oligofructose. J Nutr. 1999;129(7 Suppl):1398S-401S.
3. Niness KR. Inulin and oligofructose: what are they? J Nutr. 1999;129(7 Suppl):1402S-6S.
4. Coudray C, Bellanger J, Castiglia-Delavaud C, Remesy C, Vermorel M, Rayssignuier Y. Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men. Eur J Clin Nutr. 1997;51(6):375-80.
5. Slavin J. Fiber and prebiotics: mechanisms and health benefits. Nutrients. 2013;5(4):1417-35.
6. Delzenne NM, Daubioul C, Neyrinck A, Lasa M, Taper HS. Inulin and oligofructose modulate lipid metabolism in animals: review of biochemical events and future prospects. Br J Nutr. 2002;87 Suppl 2:S255-9.
7. Watzl B, Girrbach S, Roller M. Inulin, oligofructose and immunomodulation. Br J Nutr. 2005;93 Suppl 1:S49-55.
8. Rafter J, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, et al. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr. 2007;85(2):488-96.
9. Rozan P, Nejdi A, Hidalgo S, Bisson JF, Desor D, Messaoudi M. Effects of lifelong intervention with an oligofructose-enriched inulin in rats on general health and lifespan. Br J Nutr. 2008;100(6):1192-9.
10. Jasper H. Exploring the physiology and pathology of aging in the intestine of Drosophila melanogaster. Invertebr Reprod Dev. 2015;59(sup1):51-8.
11. Buchon N, Broderick NA, Lemaitre B. Gut homeostasis in a microbial world: insights from Drosophila melanogaster. Nat Rev Microbiol. 2013;11(9):615-26.
12. Ayyaz A, Jasper H. Intestinal inflammation and stem cell homeostasis in aging Drosophila melanogaster. Front Cell Infect Microbiol. 2013;3:98.
13. Osellame LD, Blacker TS, Duchen MR. Cellular and molecular mechanisms of mitochondrial function. Best Pract Res Clin Endocrinol Metab. 2012;26(6):711-23.
14. Hwang AB, Jeong DE, Lee SJ. Mitochondria and organismal longevity. Curr Genomics. 2012;13(7):519-32.
15. Feng J, Bussiere F, Hekimi S. Mitochondrial electron transport is a key determinant of life span in Caenorhabditis elegans. Dev Cell. 2001;1(5):633-44.
16. Dillin A, Hsu AL, Arantes-Oliveira N, Lehrer-Graiwer J, Hsin H, Fraser AG, et al. Rates of behavior and aging specified by mitochondrial function during development. Science. 2002;298(5602):2398-401.
17. Dell'agnello C, Leo S, Agostino A, Szabadkai G, Tiveron C, Zulian A, et al. Increased longevity and refractoriness to Ca(2+)-dependent neurodegeneration in Surf1 knockout mice. Hum Mol Genet. 2007;16(4):431-44.
18. Copeland JM, Cho J, Lo T, Jr., Hur JH, Bahadorani S, Arabyan T, et al. Extension of Drosophila life span by RNAi of the mitochondrial respiratory chain. Curr Biol.

2009;19(19):1591-8.
19. Yang JS, Nam HJ, Seo M, Han SK, Choi Y, Nam HG, et al. OASIS: online application for the survival analysis of lifespan assays performed in aging research. PLoS One. 2011;6(8):e23525.
20. Deshpande SA, Carvalho GB, Amador A, Phillips AM, Hoxha S, Lizotte KJ, et al. Quantifying Drosophila food intake: comparative analysis of current methodology. Nat Methods. 2014;11(5):535-40.
21. Sambrook J, Russell DW. Purification of nucleic acids by extraction with phenol:chloroform. CSH Protoc. 2006;2006(1).
22. Callahan BJ, Sankaran K, Fukuyama JA, McMurdie PJ, Holmes SP. Bioconductor Workflow for Microbiome Data Analysis: from raw reads to community analyses. F1000Res. 2016;5:1492.
23. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581-3.
24. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One. 2013;8(4):e61217.
25. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-40.
26. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41(Database issue):D590-6.
27. McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol. 2014;10(4):e1003531.
28. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
29. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.
30. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4(1):44-57.
31. Malick LE, Kidwell JF. The effect of mating status, sex and genotype on longevity in Drosophila melanogaster. Genetics. 1966;54(1):203-9.
32. Partridge L, Piper MD, Mair W. Dietary restriction in Drosophila. Mech Ageing Dev. 2005;126(9):938-50.
33. Wong CN, Ng P, Douglas AE. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ Microbiol. 2011;13(7):1889-900.
34. Biteau B, Karpac J, Supoyo S, Degennaro M, Lehmann R, Jasper H. Lifespan extension by preserving proliferative homeostasis in Drosophila. PLoS Genet. 2010;6(10):e1001159.
35. Micchelli CA, Perrimon N. Evidence that stem cells reside in the adult Drosophila midgut epithelium. Nature. 2006;439(7075):475-9.
36. Ohlstein B, Spradling A. The adult Drosophila posterior midgut is maintained by pluripotent stem cells. Nature. 2006;439(7075):470-4.
37. Li H, Qi Y, Jasper H. Preventing Age-Related Decline of Gut

Compartmentalization Limits Microbiota Dysbiosis and Extends Lifespan. Cell Host Microbe. 2016;19(2):240-53.
38. Biteau B, Hochmuth CE, Jasper H. Maintaining tissue homeostasis: dynamic control of somatic stem cell activity. Cell Stem Cell. 2011;9(5):402-11.
39. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B. Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe. 2009;5(2):200-11.
40. Jiang H, Grenley MO, Bravo MJ, Blumhagen RZ, Edgar BA. EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in Drosophila. Cell Stem Cell. 2011;8(1):84-95.
41. Jiang H, Patel PH, Kohlmaier A, Grenley MO, McEwen DG, Edgar BA. Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the Drosophila midgut. Cell. 2009;137(7):1343-55.

Chapter 4 – The association of mitochondrial DNA content and the survival rate under various conditions in *Drosophila melanogaster*

4.1 Introduction

Mitochondria are ubiquitous organelles in eukaryotic organisms which play a key role in multiple cell functions, including producing ATP via respiration (1), apoptosis (2), and Ca2+ signaling (3). Mitochondria are also unique as they are the only organelles that host their own genome, mitochondrial DNA (mtDNA). Unlike nuclear genome, mtDNA does not follow the Mendelian inheritance system and shows a maternal inheritance pattern. More importantly, multiple copies of mtDNA exist within each cell, ranging from hundreds to thousands of copies per cell. Altered mtDNA copy number regulation may lead to changes in the expression of mtDNA encoded genes and result in mitochondrial dysfunction, which may further damage the nuclear genome (4, 5).

Aging is a complex biological process characterized by systemic declining of physiological functions. The causes and mechanisms of aging are still not well understood. Different theories have been proposed, including telomere shortening, genome instability, and mitochondrial dysfunction. Among them, the mitochondrial dysfunction theory has received increasing attention with the support of numerous studies. As mtDNA copy number is believed to be a good indicator of mitochondrial function (6, 7), various studies have been carried out to examine the association between mtDNA copy number and aging or aging-associated diseases. The aging-associated decline in mtDNA copy number and respiration chain function have been observed in different organisms including humans (8-10). Surprisingly, one study on the

nonagenarians and centenarians in China revealed that these long-lived people have a significantly higher mtDNA copy number than the subjects aged between 50 and 70 years (11). More interestingly, in another study carried out by the same group demonstrated that the offspring of the centenarians also have a significantly higher mtDNA copy number than their spouses (12). Consistently, in a Danish study, they found that higher mtDNA copy number in blood is associated with better health and survival among the elderly (13). As the main function of mitochondria is to supply energy, aging-associated decline in mitochondrial function may have more tremendous impact on energy-demanding tissues like the brain. It has been shown that mtDNA content is reduced in cerebrospinal fluid of subjects with Alzheimer's disease (14), which is considered as an aging-associated disease.

In most cells and tissues, there are hundreds to thousands of copies of mtDNA, which seem to be more than what is needed to maintain basic functions of mitochondria. From a perspective of evolution, some selection pressures might exist to sustain a high cellular mtDNA copy number, perhaps related to immune functions (15). Ever-growing evidence in literature links mitochondria to the immune system, most notably to innate immune responses to cellular damage, stress and pathogen infections (16). The bacterial origin of mitochondria results in a mtDNA methylation pattern distinctly from that of nuclear DNA. The mtDNA methylation pattern appears to be more like that of "foreign" than "self" DNA (17). Recent works in circulating cell-free mtDNA have suggested that upon tissue damage, the release of mtDNA into plasma activates the innate immune responses, which may further lead to chronic inflammation (18, 19). However, mtDNA stress can also prime the antiviral innate immune response. Researchers found that in a

TFAM (transcription factor A, mitochondrial) deficient mouse model, aberrant mtDNA packaging promotes the escape of mtDNA into the cytosol to enhance the expression of antiviral signaling pathways (20). Furthermore, the same mechanism was observed upon herpesviruses infection that mtDNA stress acts as a cell-intrinsic trigger of antiviral immune responses (20). It has also been shown that mtDNA is a potent inducer of neutrophil extracellular traps, which are critical for the anti-bacterial activity of the innate immune system (21). Therefore, mtDNA may contribute to chronic inflammation diseases, but at the same time, it is also a central participant in innate immunity.

It has been demonstrated that mtDNA copy number can be altered under various pathologic conditions and is associated with multiple diseases. However, in apparently healthy individuals, the mtDNA copy number still varies over a 2-10 fold range (22). Although it is considered clinically normal when the mtDNA content is between 40 and 150 percent of the average (23), we are interested in the potential consequences of such dramatic variation among healthy populations, especially when facing environmental stresses. In this article, we use *Drosophila melanogaster* as the model organism to study the mtDNA copy number variation in healthy individuals. By implementing both survival test and transcriptome analysis, we found a possible connection between mtDNA copy number variation and immune responses in male flies.

4.2 Materials and methods

Fly husbandry and different diet treatments

All flies were reared under standard laboratory conditions with a 12-h light/dark cycle at 25°C in vials containing agar–dextrose–yeast medium. We used one strain (B18) from

the Global Diversity Panel as the wild-type strain. For normal diet groups, flies were fed with the food containing 12 g agar, 100 g glucose, 100 g yeast and 10 ml propionic acid every one-liter medium. In high sucrose diet group, based on the same food recipe, glucose was substituted into sucrose at a concentration of 34% (w/v), which was proved to shorten the lifespan of fruit flies (24). For starvation treatment, flies were fed with 1% agar plate with the addition of sucrose at a concentration of 2.5%.

DNA extraction

Classic phenol-chloroform method was implemented when purifying DNA from a single fly. For wing DNA purification, a modified protocol was used based on the method developed by Gil B. Carvalho *et al.* (25). Briefly, wings were cut after eclosion when wings were spread but still soft, then frozen in liquid nitrogen and stored at -80 °C if not proceeding to DNA extraction. When purifying DNA from the wing sample, add 100 μL lysis buffer (10 mM Tris-Cl at pH 8.2, 1 mM EDTA and 1% SDS) with protease K at a concentration of 200 μg/mL. Optimal results were obtained when the wing sample was entirely submerged in the buffer. The tubes were incubated at 55 °C for 1 h and then phenol-chloroform were added to inactivate protease. Afterwards, DNA was extracted the same way as the single fly DNA extraction.

Droplet digital PCR measurement

To measure mitochondrial copy number using ddPCR, two specific sets of primers and TaqMan probes were designed and purchased from VWR, one set measuring nuclear copies (Forward:5'-AATTGGGTTGCTACCAGGTC, Reverse:5'-GACTAGAGGCG GATTGAAGG, Probe: 5'-FAM- CCAGGCACTTGTGCCCACGT) and one set

measuring mitochondrial copies (Forward: 5'-AAGGAACACCCGCTATTCTTATAC, Reverse: 5'-AATGTCCAGCAATTATATTAGCAGTT, Probe: 5'-HEX-TCGACCTG GAACATTAGCTGTTCGA). Depending on the concentration of genomic DNA, one to eight μL of purified genomic DNA was added to 10 μL of ddPCR 2× PCR master mix (Bio-Rad), together with nuclear and mitochondrial primers and probes at a final concentration of 900 nM for primers and 250nM for probes. In total, 20 μL of the master mix, genomic DNA and primers and probes mixture was loaded into the cartridges together with 70 μL of generator oil, and then placed in the automated droplet generator. 40 μL of picoliter droplet emulsion was transferred to a PCR plate by an autopipette to reduce the risk of disrupting the droplets. The PCR plate was sealed by the foil (Bio-Rad) and placed in the thermocycler with the following program: 95 °C for 10 min; 40 cycles of 94 °C for 30 s, 60 °C for 1 min; and 98 °C for 10 min. The plates were then placed into the ddPCR reader to read the fluorescent signal in each droplet. The number of molecules was then estimated by the Quantalife ddPCR using an internal Poisson algorithm. As nuclear and mitochondrial probes are labeled with different fluorescent, we can measure the absolute number of both nuclear and mitochondrial molecules at the same time as long as they both fall in the range of 50-5000 copies/ μL, otherwise, a separate reaction for mitochondrial copies measurement is required.

RNA-seq and data analysis

Individual flies were placed in 1.5 ml tubes and then placed into liquid nitrogen and stored at -80°C if not proceeding to RNA extraction. Total RNA was extracted using Allprep DNA/RNA mini kit (Qiagen), and the DNA was also extracted at the same time. After ddPCR measurement, three samples with the lowest mitochondrial copy numbers

and three with the highest were selected for each gender. mRNA was isolated using magnetic mRNA isolation kit (NEB) for selected samples. KAPA stranded RNA-Seq library preparation kit was used to construct libraries for Illumina sequencing.

Bacterial infection experiment

The oral bacterial infection experiment is performed according to the published protocol (26). Experiment flies were flipped into empty vials and starved for 2 h under 29 °C. A 1:1 mixture of 5% sucrose and concentrated bacteria (OD 200) solution was prepared as the infection mix. An absorbent pad was cut into the diameter of the fly vials, placed into a tube, and pushed down until contact with the diet. The pad should completely cover the diet. 150 μL of the infection mix were added immediately to the pad for infection treatment. Starved flies were then flipped into the infection vials and incubated at 29°C. Flies were flipped into new vials with the fresh bacterial solution every other day, and death was recorded every day.

4.3 Results

4.3.1 A large variation of mitochondrial DNA copy number in healthy flies

Similar to observations from previous studies, we found that there is a six-fold variation of the mtDNA copy number among the healthy newly-eclosed individual flies (Figure 4.1 A), with the lowest mtDNA copy number of 38.28 and the highest mtDNA copy number of 253.83. The mean mtDNA copy number for male individuals is 115.56, and 106.27 for female individuals. There is no significant difference in the distribution of mtDNA copy number between the male and female groups. In order to measure mtDNA

copy number without killing the flies, we modified the method developed by Carvalho *et al.* to extract DNA from the wings of flies (25). Similarly, a variation of mtDNA copy number was observed in wing samples as well (Figure 4.1 B). To examine whether wing mtDNA copy number is representative for the whole-body mtDNA content, we performed the association analysis between the wing and whole-body mtDNA copy number. A borderline significant association ($p = 0.055$, R = 0.21) was observed for the wing and whole-body mtDNA association in the combined data of male and female (Figure 4.1 C). When separating the data by gender, there is a significant association in the female group (Figure 4.1 D, $p = 0.00066$, R = 0.47), but not significant in the male group. Therefore, we observed a wide distribution of mtDNA copy number in both whole-body and wing samples, in male and female flies, but the association between the wing and whole-body mtDNA copy number is only significant in female flies.

4.3.2 mtDNA content is associated with multiple stress responses in male flies

To further investigate the consequence of mtDNA copy number variation in healthy individuals, we performed survival test to examine if it is associated with lifespan variation of *D. melanogaster*. A negative correlation between mtDNA copy number and lifespan was observed in both male and female individuals. Although the overall analysis showed that it is not statistically significant (Figure 4.2 A), data from the male flies have a marginal *p*-value of 0.056 (R = -0.64) (Figure 4.2 C). To explore whether mtDNA copy number variation is related to the ability to withstand extrinsic stress, we implemented different stress assays including high-sucrose, bacterial infection, and

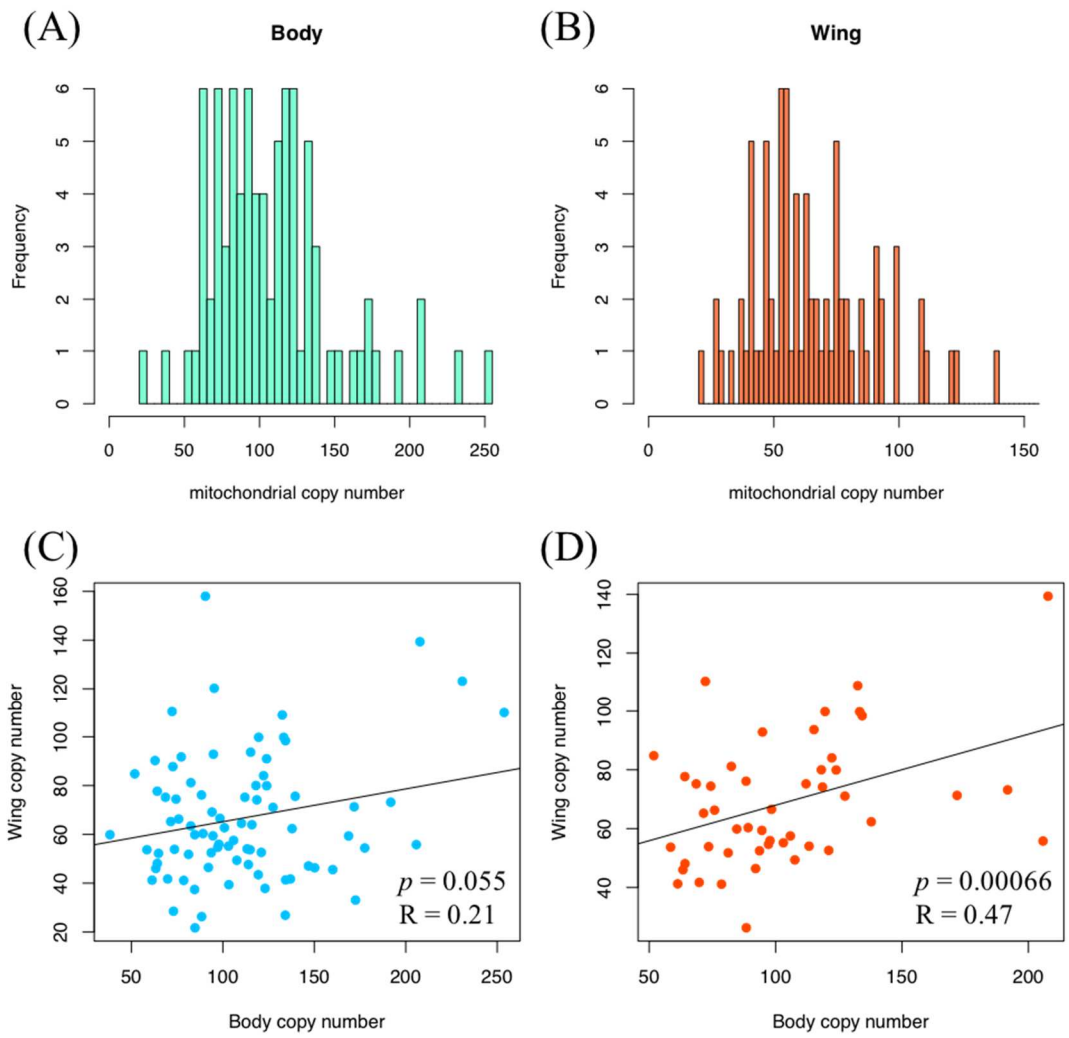

Figure 4. 1 A large variation of mtDNA copy number and mtDNA copy number correlation between wing and body samples

There is a large variation of the mtDNA copy number in both (A) whole flies body samples, and (B) flies wing samples; There is (C) a borderline significant correlation of mtDNA content between wing and body samples, and (D) a very significant correlation of mtDNA content between wing and body samples in female flies.

starvation assays. In high-sucrose feeding experiment, we discovered a similar pattern as that in the normal survival test: there is a significant negative correlation between mtDNA copy number and lifespan in male flies ($p = 0.044$, R =-0.63), but not in female flies (Figure 4.2 D-F). Then we carried out bacterial infection assay to investigate if the response to oral bacterial infection is associated with mtDNA content. Interestingly, we still found a significant association between mtDNA copy number and the survival time after oral infection in male flies again ($p = 0.022$, R = 0.51), and not in female flies (Figure 4.3 A-C). Different from the negative correlation observed in both the normal survival assay and the high-sucrose survival assay, a positive correlation was observed in the bacterial infection study. Due to the special design of oral bacterial infection assay, flies are exposed to a combination of stresses from bacterial infection and partial starvation. Therefore, we also performed a starvation survival assay to evaluate its impact on the oral infection experiment. We did not find any significant results in the starvation survival test in either male or female groups (Figure 4.3 D-F), suggesting it is unlikely that starvation plays a role in the correlation between mtDNA content and the survival time after bacterial infection.

4.3.3 mtDNA content is associated with gene expression changes in multiple pathways

To explore the potential molecular mechanism underlying the association between mtDNA copy number and stress responses, we selected individual flies with extreme mtDNA copy number to identify differentially expressed genes between flies with low and high mtDNA copy number in both male and female individuals. Both DNA and RNA were extracted at the same time for individual flies. After mtDNA copy number

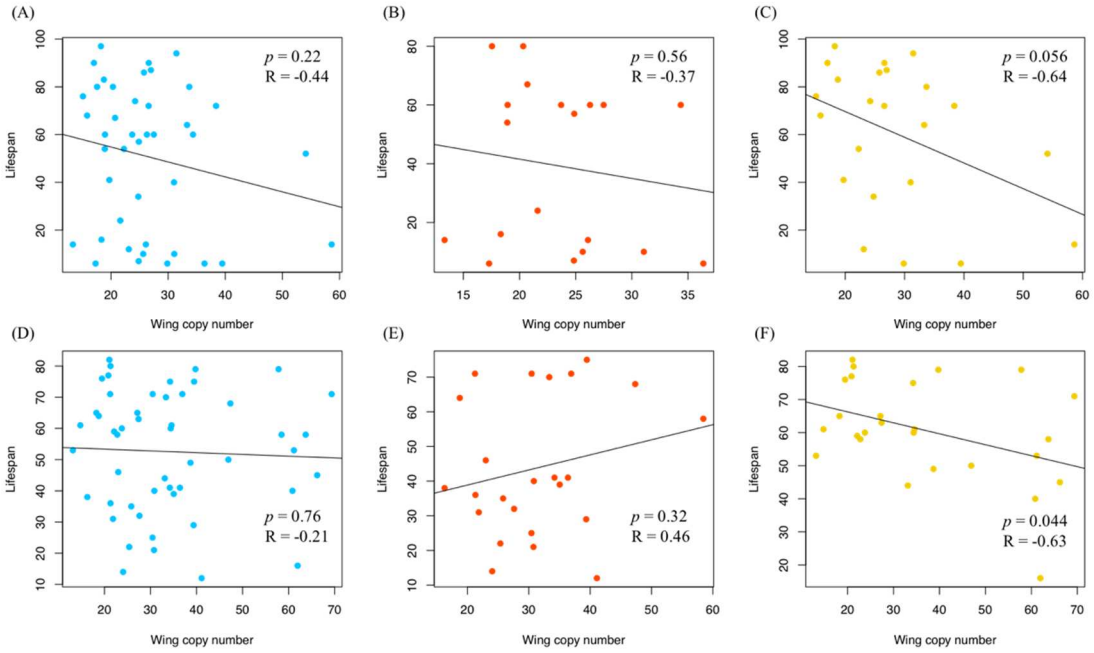

Figure 4. 2 Correlation between mtDNA content and survival rate of flies fed on normal and high-sucrose diet

When flies are fed on the normal diet, there is no correlation between mtDNA content and survival rate when analyzing the data of (A) combination of male and female flies, and (B) female flies only, and there is a borderline significant correlation when analyzing the data of (C) male flies only; When flies are fed on the high-sucrose diet, there is no significant correlation results in (D) combination of male and female flies, and (E) female flies only, but significant result for (F) male flies.

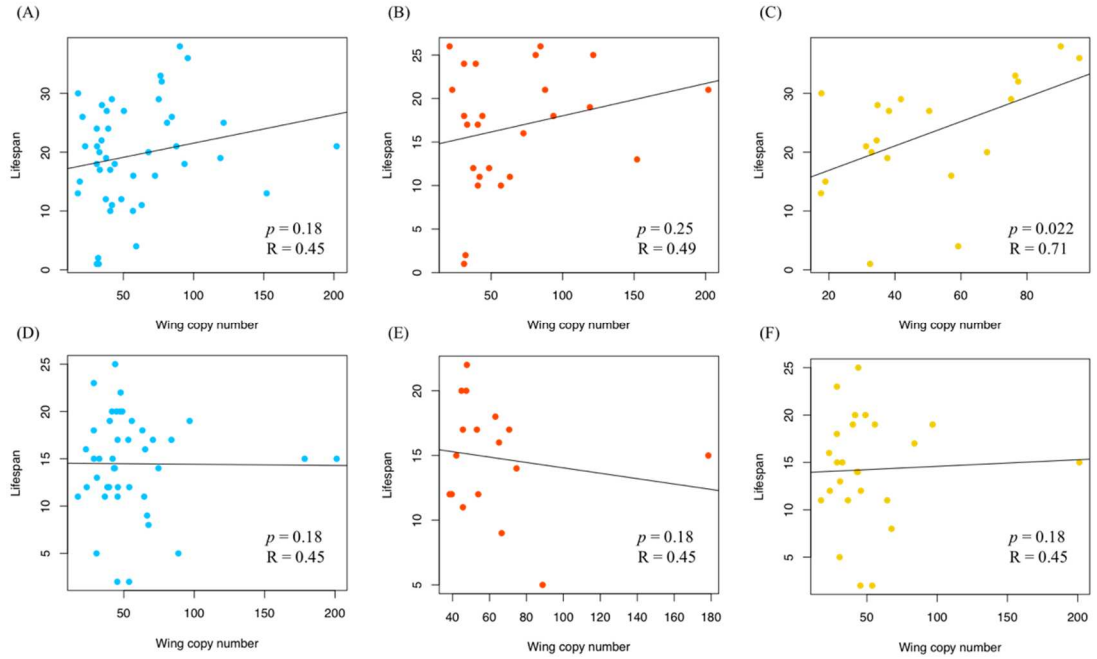

Figure 4. 3 Correlation between mtDNA content and survival rate of flies under bacterial infection and starvation

When flies are under oral bacterial infection, there is no correlation between mtDNA content and survival rate when analyzing the data of (A) combination of male and female flies, and (B) female flies only, and there is a significant correlation when analyzing the data of (C) male flies only ($p = 0.022$); When flies are under starvation, there is no significant correlation results in (D) combination of male and female flies, (E) female flies only, and (F) male flies only.

is determined by ddPCR, we chose 3 individuals with extreme copy number from both ends of the distribution to perform mRNA-sequencing in both genders. Overall, we found four genes differentially expressed in male flies, and 105 genes in female flies between the low and high mtDNA copy number groups. All of the four genes were down-regulated in male flies with high mtDNA copy number compared with the ones with low mtDNA copy number. Among the 105 differentially expressed genes in the female flies, 66 genes were up-regulated and 39 were down-regulated in the high copy number group. When further examined the function of the four differentially expressed genes in male, we found that two of them have unknown functions, while the other two are related with sperm production, including *Dnah3* and *Mst36Fb*. *Dnah3* is involved in the function of sperm competition, and *Mst36Fb* plays an important role in spermatogenesis. Consistently, gene sets enrichment analysis revealed that most of the down-regulated gene sets are enriched in the processes related to spermatogenesis and sperm motility (Figure 4.4 A). Moreover, processes of directing and transporting proteins into mitochondria also tend to be down-regulated in the high copy number male flies. Multiple metabolic processes are up-regulated in males with high copy number, such as amino acids, glucose and cellular carbohydrate metabolic processes (Figure 4.4 B). For the female flies, as more genes are found to be differentially expressed between low and high mtDNA copy number groups, we started by examining the significantly regulated pathways or gene sets. A large number of cell-cycle-related gene sets were down-regulated in the high mtDNA copy number group (Figure 4.5 A). Although no up-regulating pathways survived multiple-testing correction, most of the top ones are related to anti-microbial responses (Figure 4.5 B). Interestingly, in addition to genes

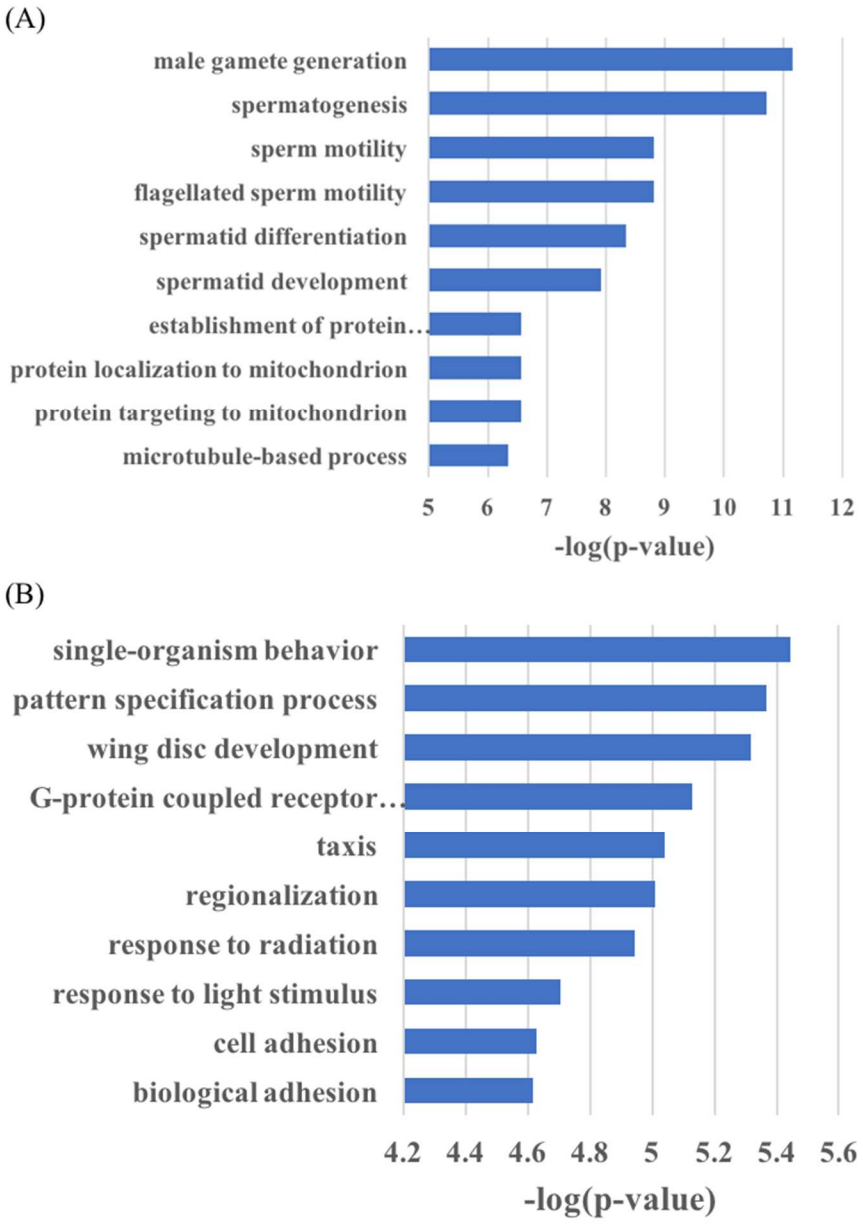

Figure 4. 4 Top GO enrichment pathways in male flies
(A)Down-regulated pathways in the high copy number group, and (B) up-regulated pathways in the high copy number group

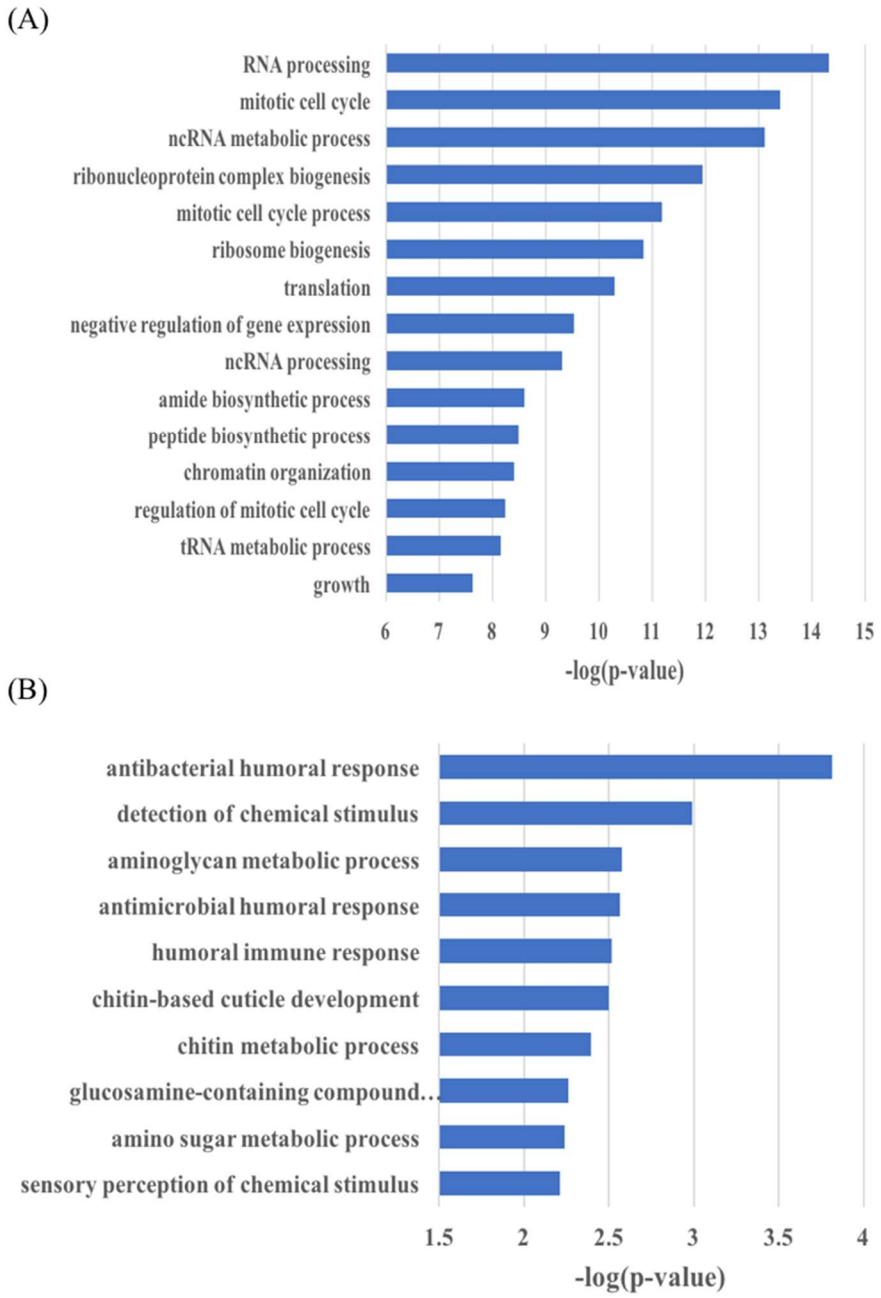

Figure 4. 5 Top GO enrichment pathways in female flies
(A)Down-regulated pathways in the high copy number group, and (B)up-regulated pathways in the high copy number group

involved in the process of cell-cycle and immune responses, we also discovered several mitochondria-related genes including *spargel*, which is the *Drosophila* PGC-1 homolog. Altogether, there is a clear pattern in male flies that the male fertility tends to be inhibited in flies having a higher mtDNA content comparing with the ones having a lower mtDNA content. However, it is more complicated for female flies, with the possible involvement of genes regulating cell-cycle and immune responses.

4.4 Discussion

Revealed by the survival assays together with the transcriptome analysis, there is a gender-specific effect regarding the impact of mtDNA content variation in healthy individuals. In male flies, a higher mtDNA copy number is associated with inhibited sperm production and motility, and with promoted amino acids and carbohydrate metabolism. From previous studies, we learned that mitochondrial fusion and fission are required for the process of spermatogenesis (27), and mtDNA copy number could be affected by the changes of mitochondrial dynamics (28). Thus, it is very likely that the observation of association between mtDNA content and male fertility is related to mitochondrial dynamic changes. In terms of carbohydrate metabolism, it is much clearer in the literature that the reduction of mtDNA copy number is associated with the failure of glucose metabolism in the diabetic patient (29). Therefore, comparing with the flies having low mtDNA copy number, the ability to metabolize glucose and carbohydrate is improved in high mtDNA copy number group. In female flies, flies with higher mtDNA content have down-regulated cell-cycle and growth-related pathways. Although it is not clear if and how mtDNA replication is linked to the cell cycle, several studies have

reported a correlation between mitochondrial biogenesis and progression of the cell cycle (30, 31). In particular, it has been demonstrated that the stimulation of growth factors can also induce mtDNA replication (32). However, in the high mtDNA copy number group, we observed inhibited cell-cycle and growth pathways. Further assays are needed to demonstrate the potential relationship between mtDNA copy number and cell cycle.

Even though these differentially regulated pathways have some underlying connection with mitochondrial DNA, the reason of having such a huge difference between male and female flies is still unknown. Given the exclusive maternal inheritance of mitochondria, there may be fundamental sex differences in regulating mitochondrial metabolism to cope with different stressful conditions between male and female individuals (33). Similarly, in the normal and stress survival assays, only male flies have shown the significant correlation between mtDNA copy number and responses to high-sucrose diet and oral bacterial infection, indicating mtDNA copy number variation may have a stronger influence on male flies, especially when facing stressful conditions. From a perspective of evolution, the maternal inheritance implies that selection might only be effective on mitochondria carried by females, thus making mitochondrial DNA more compatible with female nuclear DNA (33). Hence, it is very likely that female has a better buffering capacity for mtDNA mutations and copy number variations. Therefore, male flies harboring high mtDNA copy number may have a different metabolic capacity comparing with the flies have lower mtDNA copy number, which leads to a shorter lifespan when feeding on both control and high-sucrose diet, but a better survival rate under bacterial infection. Specifically, the results of the high-sucrose

survival assay and transcriptome analysis have suggested carbohydrate metabolism may be the mediator of the mtDNA copy number variations in males. For the positive correlation between mtDNA copy number and lifespan under bacterial infection, flies with high copy number have a better survival rate. Evidence from other studies showed that the release of mtDNA may prime the innate immune system (20). Therefore, it is possible that individuals harboring higher mtDNA copy number may have a higher chance of releasing mtDNA to stimulate the immune responses.

As an exploratory research, there are also some limitations and caveats in this study. The main obstacle is to find a non-invasive method to obtain the information of mtDNA copy number in an early life stage when using *D. melanogaster* as the model organism. We chose to cut the wings off flies for mtDNA measurement so that flies are still alive for further survival assays. Although most of the flies can survive the wing cutting procedure, there is a small portion of them died afterward. When comparing the mtDNA content between the flies survived and died after the wing-cutting procedure, we discovered that the flies with relatively lower mtDNA copy number tend to have a lower survival rate of wing-cutting. Therefore, the procedure of wing-cutting may introduce possible bias for further survival assays by eliminating the flies with lower mtDNA copy number. However, in *D. melanogaster*, it is very challenging to develop a non-invasive method to collect the data of mtDNA copy number without affecting the performance of flies. In future studies, using larger experimental organisms will be a more promising approach to investigate the potential consequence of mtDNA copy number variation. For mtDNA copy number variation studies, another inevitable issue is to address the tissue-specific manner of mtDNA copy number (34). Due to the limitation of using *D.*

melanogaster as the experimental organism, wing mtDNA copy number has been chosen as a biomarker for this study, which only has a significant correlation with the whole-body mtDNA copy number in male flies. Even though it is unclear whether wing mtDNA copy number is representative for other tissues in the body, our results suggest that mtDNA content in wing samples does have an association with the lifespan of male flies under high-sucrose and oral bacterial infection treatments.

In summary, we found that there is a broad distribution of mtDNA copy number even in *D. melanogaster* with an isogenic background. To explore the potential consequence of such a huge variation of mtDNA content in healthy individuals, we discovered that in male flies, higher mtDNA content tends to associate with a shorter lifespan in normal and high-sucrose diet, but a better survival rate when facing bacterial stresses. Future studies are needed to confirm the generalizability of this relationship in other organisms and to elucidate its underlying molecular mechanisms.

4.5 Acknowledgement

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4.6 References

1. Attardi G, Schatz G. Biogenesis of mitochondria. Annu Rev Cell Biol. 1988;4:289-

333.
2. Wallace DC. Mitochondria as chi. Genetics. 2008;179(2):727-35.
3. Nicholls DG. Mitochondria and calcium signaling. Cell Calcium. 2005;38(3-4):311-7.
4. Corral M, Paris B, Baffet G, Tichonicky L, Guguen-Guillouzo C, Kruh J, et al. Increased level of the mitochondrial ND5 transcript in chemically induced rat hepatomas. Exp Cell Res. 1989;184(1):158-66.
5. Delsite RL, Rasmussen LJ, Rasmussen AK, Kalen A, Goswami PC, Singh KK. Mitochondrial impairment is accompanied by impaired oxidative DNA repair in the nucleus. Mutagenesis. 2003;18(6):497-503.
6. Jeng JY, Yeh TS, Lee JW, Lin SH, Fong TH, Hsieh RH. Maintenance of mitochondrial DNA copy number and expression are essential for preservation of mitochondrial function and cell growth. J Cell Biochem. 2008;103(2):347-57.
7. Malik AN, Czajka A. Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction? Mitochondrion. 2013;13(5):481-92.
8. Cree LM, Patel SK, Pyle A, Lynn S, Turnbull DM, Chinnery PF, et al. Age-related decline in mitochondrial DNA copy number in isolated human pancreatic islets. Diabetologia. 2008;51(8):1440-3.
9. Hartmann N, Reichwald K, Wittig I, Drose S, Schmeisser S, Luck C, et al. Mitochondrial DNA copy number and function decrease with age in the short-lived fish Nothobranchius furzeri. Aging Cell. 2011;10(5):824-31.
10. Lee JW, Park KD, Im JA, Kim MY, Lee DC. Mitochondrial DNA copy number in peripheral blood is associated with cognitive function in apparently healthy elderly women. Clin Chim Acta. 2010;411(7-8):592-6.
11. He YH, Lu X, Wu H, Cai WW, Yang LQ, Xu LY, et al. Mitochondrial DNA content contributes to healthy aging in Chinese: a study from nonagenarians and centenarians. Neurobiol Aging. 2014;35(7):1779 e1-4.
12. He YH, Chen XQ, Yan DJ, Xiao FH, Lin R, Liao XP, et al. Familial longevity study reveals a significant association of mitochondrial DNA copy number between centenarians and their offspring. Neurobiol Aging. 2016;47:218 e11- e18.
13. Mengel-From J, Thinggaard M, Dalgard C, Kyvik KO, Christensen K, Christiansen L. Mitochondrial DNA copy number in peripheral blood cells declines with age and is associated with general health among elderly. Hum Genet. 2014;133(9):1149-59.
14. Podlesniy P, Figueiro-Silva J, Llado A, Antonell A, Sanchez-Valle R, Alcolea D, et al. Low cerebrospinal fluid concentration of mitochondrial DNA in preclinical Alzheimer disease. Ann Neurol. 2013;74(5):655-68.
15. West AP, Shadel GS. Mitochondrial DNA in innate immune responses and inflammatory pathology. Nat Rev Immunol. 2017;17(6):363-75.
16. West AP, Shadel GS, Ghosh S. Mitochondria in innate immune responses. Nat Rev Immunol. 2011;11(6):389-402.
17. Hong EE, Okitsu CY, Smith AD, Hsieh CL. Regionally specific and genome-wide analyses conclusively demonstrate the absence of CpG methylation in human mitochondrial DNA. Mol Cell Biol. 2013;33(14):2683-90.
18. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature.

2010;464(7285):104-7.
19. Fang C, Wei X, Wei Y. Mitochondrial DNA in the regulation of innate immune responses. Protein Cell. 2016;7(1):11-6.
20. West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, Lang SM, et al. Mitochondrial DNA stress primes the antiviral innate immune response. Nature. 2015;520(7548):553-7.
21. Itagaki K, Kaczmarek E, Lee YT, Tang IT, Isal B, Adibnia Y, et al. Mitochondrial DNA released by trauma induces neutrophil extracellular traps. PLoS One. 2015;10(3):e0120549.
22. Stringer HA, Sohi GK, Maguire JA, Cote HC. Decreased skeletal muscle mitochondrial DNA in patients with statin-induced myopathy. J Neurol Sci. 2013;325(1-2):142-7.
23. Nakano Y, Murayama K, Tsuruoka T, Aizawa M, Nagasaka H, Horie H, et al. Fatal case of mitochondrial DNA depletion with severe asphyxia in a newborn. Pediatr Int. 2011;53(2):240-2.
24. Na J, Musselman LP, Pendse J, Baranski TJ, Bodmer R, Ocorr K, et al. A Drosophila model of high sugar diet-induced cardiomyopathy. PLoS Genet. 2013;9(1):e1003175.
25. Carvalho GB, Ja WW, Benzer S. Non-lethal PCR genotyping of single Drosophila. Biotechniques. 2009;46(4):312-4.
26. Buchon N, Broderick NA, Poidevin M, Pradervand S, Lemaitre B. Drosophila intestinal response to bacterial infection: activation of host defense and stem cell proliferation. Cell Host Microbe. 2009;5(2):200-11.
27. Fabian L, Brill JA. Drosophila spermiogenesis: Big things come from little packages. Spermatogenesis. 2012;2(3):197-212.
28. Hori A, Yoshida M, Ling F. Mitochondrial fusion increases the mitochondrial DNA copy number in budding yeast. Genes Cells. 2011;16(5):527-44.
29. Park KS, Nam KJ, Kim JW, Lee YB, Han CY, Jeong JK, et al. Depletion of mitochondrial DNA alters glucose metabolism in SK-Hep1 cells. Am J Physiol Endocrinol Metab. 2001;280(6):E1007-14.
30. Van den Bogert C, Muus P, Haanen C, Pennings A, Melis TE, Kroon AM. Mitochondrial biogenesis and mitochondrial activity during the progression of the cell cycle of human leukemic cells. Exp Cell Res. 1988;178(1):143-53.
31. Lee HC, Wei YH. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. Int J Biochem Cell Biol. 2005;37(4):822-34.
32. Trinei M, Berniakovich I, Pelicci PG, Giorgio M. Mitochondrial DNA copy number is regulated by cellular proliferation: a role for Ras and p66(Shc). Biochim Biophys Acta. 2006;1757(5-6):624-30.
33. Demarest TG, McCarthy MM. Sex differences in mitochondrial (dys)function: Implications for neuroprotection. J Bioenerg Biomembr. 2015;47(1-2):173-88.
34. Kelly RD, Mahmud A, McKenzie M, Trounce IA, St John JC. Mitochondrial DNA copy number is regulated in a tissue specific manner by DNA methylation of the nuclear-encoded DNA polymerase gamma A. Nucleic Acids Res. 2012;40(20):10124-38

Chapter 5 – Summary and future directions

In summary, I have reviewed the current knowledge of mitochondrial DNA genetics and the involvement of mitochondrial dysfunction in human diseases, emphasizing the importance of investigating the role of mitochondria in the progression of complex diseases. By discussing the interaction between mitochondria and different nutrients or dietary patterns, I suggested that identifying nutritional interventions to restore mitochondrial function may offer a new approach to managing complex diseases. I have also presented data of two compounds that have the ability to extend the lifespan of *Drosophila melanogaster*, konjac glucomannan hydrolysate (GMH) and oligofructose. I found that GMH can extend the lifespan in different genetic backgrounds and in both virgin and mated flies, possibly through regulating the gut microbiome and intestinal proliferative homeostasis. For oligofructose, it can also extend the lifespan of *Drosophila melanogaster*, but in a genetics-linked manner. I have identified three possible underlying mechanisms, including mild starvation, regulation of stress signaling pathways, and slight inhibition of mitochondrial respiration. Moreover, in an exploratory project, I have investigated the potential association between mitochondrial copy number and the survival rate under various conditions. I found significant associations between wing mtDNA copy number and survival time under both high-sucrose and oral bacterial infection conditions in male flies only.

My studies have generated valuable insights and suggestions for future studies. My GMH supplementation study was the first to show that it can extend the lifespan of *Drosophila melanogaster*. Future studies are needed to examine if the same effect and

molecular mechanisms are generalizable to other model organisms, such as mice. Considering the lifespan-extending effect of oligofructose is in a genetics-linked manner, using flies from the DGRP to perform the survival assay at the population level may help us discover the genetic factors that are linked with the effect of oligofructose supplementation. As for the exploratory project studying the potential association of wing mtDNA copy number and lifespan, there are still some issues that need to be addressed in the future. With the limitation of the body size, I chose to extract DNA and measure the copy number from the wing samples of flies. However, the wing mtDNA copy number is not associated with body mtDNA copy number in male flies. Further studies are needed to first calibrate the measurement of wing mtDNA copy number and also examine whether wing mtDNA copy number is representative of other tissues. Due to the fact that flies with lower mtDNA copy number tend to die after the wing-cutting procedure, it brings us a dilemma that we could never obtain the information of flies mtDNA copy number without affecting their performance. Therefore, I propose that using mice as the experimental organism in the future may be a more feasible approach to investigate the impact of mitochondrial copy number in early life stage.