The Role of Mitochondrial Plasticity for Intermittent Fasting Mediated Longevity

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The Role of Mitochondrial Plasticity for Intermittent Fasting Mediated Longevity

A dissertation presented by

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to The Committee on Higher Degrees in Biological Sciences in Public Health.

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Abstract

Aging is the single greatest risk factor for most chronic diseases that plague modern society. Research in the past two decades has demonstrated that dietary interventions can be utilized to modulate the rate of biological aging and these pathways can be targeted to promote health and longevity. Dietary restriction (DR) is the most well-studied and conserved method of increasing lifespan in many organisms, but it has associated negative side-effects and low rates of compliance in humans. Intermittent Fasting (IF), the modulation of the timing of eating with prolonged periods of fasting, is an alternative intervention shown to promote healthy aging, independent of reduced overall caloric intake. IF leads to lifespan extension in multiple model organisms and evidence is beginning to suggest that these mechanisms may be partially distinct from those of traditional chronic dietary restriction (DR). In my dissertation research, I show that mitochondrial dynamics are integral to IF-mediated longevity, and interestingly these requirements are different than those for chronic DR. Specifically, the ability of mitochondrial networks to remodel in response to feeding and fasting, what I define as mitochondrial plasticity, is necessary for IF-longevity. IF-longevity is abolished in *C. elegans* that have adynamic mitochondrial networks, but astonishingly mitochondrial plasticity is dispensable for DR-mediated longevity. Using an inducible system of mitochondrial fusion and fission expression, I show that synthetic manipulation of mitochondrial plasticity mimics IF at the level of mitochondrial morphology, and this is sufficient to extend lifespan in *C. elegans*. Finally, by comparing the effects of fasting on transcription that only occur specifically in wild type animals and not *drp-1;fzo-1* double mutant animals that lack mitochondrial plasticity, I show that rewiring of metabolism is an important and potentially causal mechanism coupling IF to longevity. Together my data suggest that mitochondrial plasticity and metabolic flexibility are key mechanisms of longevity mediated by intermittent fasting.

Key words: Intermittent Fasting, Mitochondrial Dynamics, Mitochondrial Plasticity, Metabolic Flexibility, *C. elegans*, Longevity, Aging

Contents

List of Figures

List of Tables

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1 Introduction

1.1 Aging as a risk factor for disease

To outsiders, the field of aging sciences may seem like an elusive realm in futile pursuit of an elixir to immortal life and youthful beauty. The maintenance of youthfulness holds an utmost value in modern society, with shocking global trends in plastic surgery and a multibillion dollar cosmetics and beauty industry targeted at anti-aging. The significance of aging sciences however penetrates far deeper than superficial beauty. Age is in fact the single greatest risk factor for most mortalities and co-morbidities – infectious disease, cancer, cardiovascular disease, stroke, and neurodegenerative disorders (Burkewitz et al., 2016; Escoubas et al., 2018). As advances in medical sciences and public health measures have improved, human life expectancy has achieved the remarkable feat of nearly doubling since the turn of the 20th century. Globally, the population aged 65 and over is the fastest growing age group, projected to make up more than 20% of the world population by 2100 (Escoubas et al., 2018). In 2018, for the first time in history, persons aged 65 or above outnumbered children under five years of age globally (United Nations Department of Economic and Social Affairs, 2022).

These statistics may gleam with positivity, but the shadowed reality is that these added years of life are often spent in a vulnerable, decrepit, and disease-ridden state. Age-associated diseases often occur as comorbidities, drastically reducing the quality of life in the older population

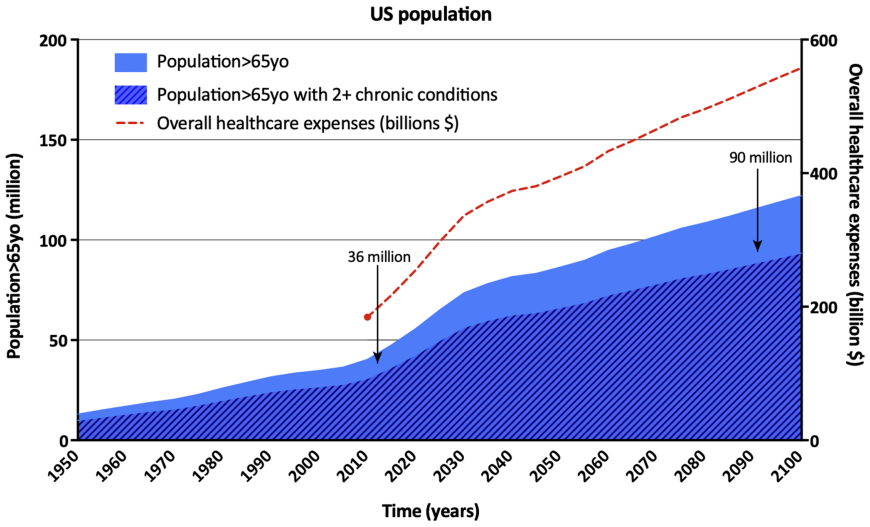


Figure 1.1: **Increased human life expectancy has resulted in an aged population suffering from multiple chronic diseases and increased challenges on healthcare.**
Life expectancy has been increasing since the last century and the number of persons aged over 65 years is predicted to represent up to 22% of the world population by 2050, with a proportional increase in people >65 years of age suffering from two or more chronic diseases. This comorbidity is expected to come with increased healthcare expenses. .
Figure adapted from Escoubas et al., 2018 with permission

(Burkewitz et al., 2016). Moreover, for those of the younger and still vibrant generation, the increase of older persons with multiple comorbidities poses a hefty burden on the health care system (Chang et al., 2019; Christensen et al., 2009; Figure 1.1). Most recently, we have seen during the COVID-19 pandemic that those of old age and pre-existing health concerns were of the most vulnerable to the devastating effects of the virus (Flaherty et al., 2020). However, the consequences spread beyond the elderly as a burdened health care system translated also to less treatment for non-covid related ailments (Kaye et al., 2021). Minimizing the risk to the older generation and saturation of the health care system therefore meant that children and healthy persons were forced to home confinement, and a slow-down of economic development having lasting effects as the world enters into a recession today (Sarkodie and Owusu, 2021). It is therefore imperative to find effective strategies that reduce the overall risk of disease and improve liveliness in old age.

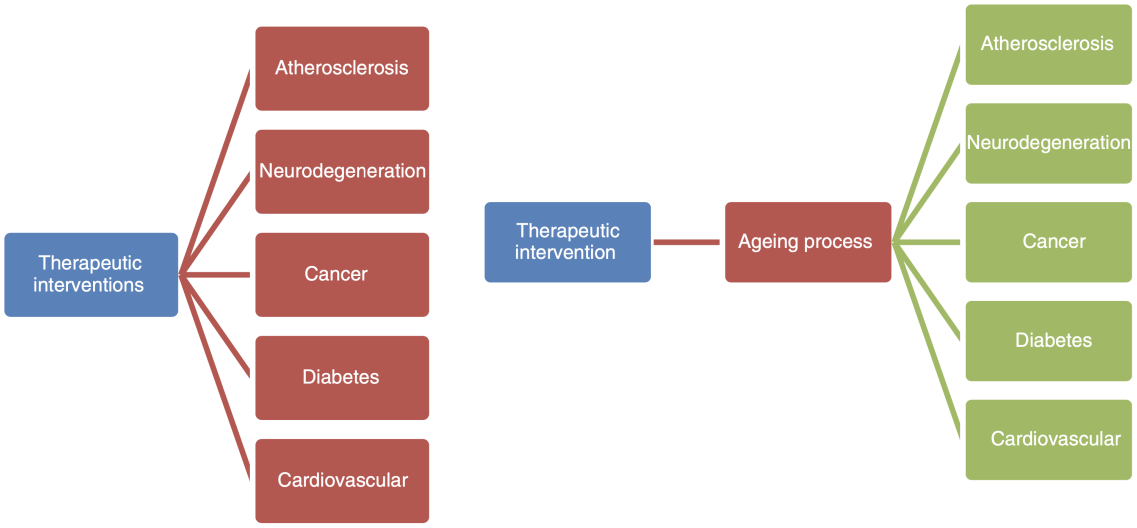


Figure 1.2: **Strategies to treat diseases that occur in old age.**
Current therapies target individual diseases in isolation, but therapies targeted to the aging process itself may be more efficient at treating multiple diseases simultaneously.
Figure adapted from Niccoli and Partridge, 2012 with permission

1.2 Anti-aging interventions as a strategy to target multiple age-related comorbidities

A flaw in the current medical strategy is a single disease centric approach to try and resolve the pathogenic mechanisms of individual diseases that incur with age. The downfall to this approach is that in an aged individual who likely has more than one non-communicable condition, i.e. diabetes, neurodegeneration and cancer, the treatment of diabetes alone does little to improve overall health when the patient is still left with two other debilitating diseases (Burkewitz et al., 2016). The goal of the study of aging is to target the unifying risk factor that underlies most diseases: patient age itself. By understanding the biological mechanisms that modulate the process of aging, we can theoretically target pathways that ameliorate all age-associated corbidities at once (Figure 1.2).

1.3 Dietary Restriction (DR) is a robust and conserved intervention to extend lifespan

Research over the last two decades has led to the concept that chronological age can be dissociated from biological age. Biological age is malleable, and the mechanisms that modulate it can be targeted to promote health and longevity (Fontana and Partridge, 2015). Much of this work has been centered around an intervention called dietary restriction (DR), which is defined as a chronic reduction in caloric intake without malnutrition (Longo and Anderson, 2022). From the first DR studies published in rat in 1935 to the now thousands of results that exist on PubMed, the study of DR in multiple organisms has uncovered how conserved nutrient sensing pathways can be manipulated to increases longevity and disease resistance (McCay et al., 1935). Broadly speaking, metabolic processes that are activated during conditions of low nutrient availability have been shown to slow the rate of biological aging and burden of disease in old age (Lopez-Otin et al., 2016; Fontana and Partridge, 2015). In two iconic 40-year longitudinal studies of DR conducted at the National Institute of Aging and at the Wisconsin National Primate Research Center, DR significantly reduced neoplasia, cardiovascular disease and metabolic syndrome in Rhesus monkeys (Colman et al., 2009; Mattison et al., 2012). In humans, DR has also been shown to improve health outcomes in patients with cancers (Brandhorst and Longo, 2016), neurodegenerative (Fontana and Partridge, 2015), and metabolic diseases (Kraus et al., 2019; Most et al., 2017; Larson-Meyer et al., 2006).

The unfortunate side of this research is that the positive effects of DR on longevity and health, are also accompanied by several negative associated physiological and psychological side-effects (Dirks and Leeuwenburgh, 2006). These include stunted growth, reproductive capacity, reduced thermoregulation, wound healing and bone density, and altogether severely hinder the therapeutic potential of DR as an anti-aging intervention in humans (Dirks and Leeuwenburgh, 2006; Redman and Ravussin, 2011). Moreover, it has been shown that the positive effects of DR are limited only to the period in which the organism is maintained on the restricted diet (Mair et al.,

2003). Unfortunately, switching back to an *ad libitum* diet immediately reverses any benefit that DR had on mortality rate, as has been shown in *Drosophila* (Mair et al., 2003). In human trials of DR, reports show that those on the restricted diet also have lower cognitive function (Redman and Ravussin, 2011). The specific dilution of calorie shortages also has a significant impact on the robustness to which the intervention has beneficial or deleterious outcomes on longevity (Mair et al., 2009). There is therefore an ongoing need to find more translatable therapies from this research.

Uncovering the cellular and signaling pathways that mediate both the positive and negative outcomes of DR enables the possibility of uncoupling the longevity benefits from the growth and reproductive failures. While these mechanisms remain mostly elusive, studies have shown that pathways that lie within the signaling network of key anabolic and catabolic nodes of metabolism are prime candidates. AMPK (AMP Activated Protein Kinase) and mTOR (Mechanistic Target of Rapamycin) are two important reciprocal energy sensors that modulate cellular responses to energy availability. AMPK is activated when nutrient availability is low. Constitutive activation of AMPK promotes lifespan extension and is required for DR-mediated lifespan extension, but also results in small body size (Mair et al., 2011). Within this pathway, CRTC-1 (CREB-regulated transcriptional co-activator-1) is a direct downstream target of AMPK, and has been shown to mediate the longevity-specific effects of AMPK activation (Burkewitz et al., 2015). By blocking CRTC-1 phosphorylation by AMPK in neurons, the longevity effects could be effectively uncoupled from growth retardation (Burkewitz et al., 2015). On the counter side of the metabolic scale, mTORC1 (Mechanistic Target of Rapamycin Complex 1) is a central anabolic regulator and activated by excess nutrients and growth signals (Saxton and Sabatini, 2017). High mTORC1 activity leads to accelerated aging and cancer (Zou et al., 2020; Weichhart, 2018). Reduced mTORC1 signaling leads to lifespan extension in worms, flies and mice, but also small body size (Bjedov et al., 2010; Harrison and Archer, 1987; Schreiber et al., 2010). New studies have also shown that by reducing mTORC1 signaling in only neurons of worms, and leaving somatic mTORC1 activity normal,

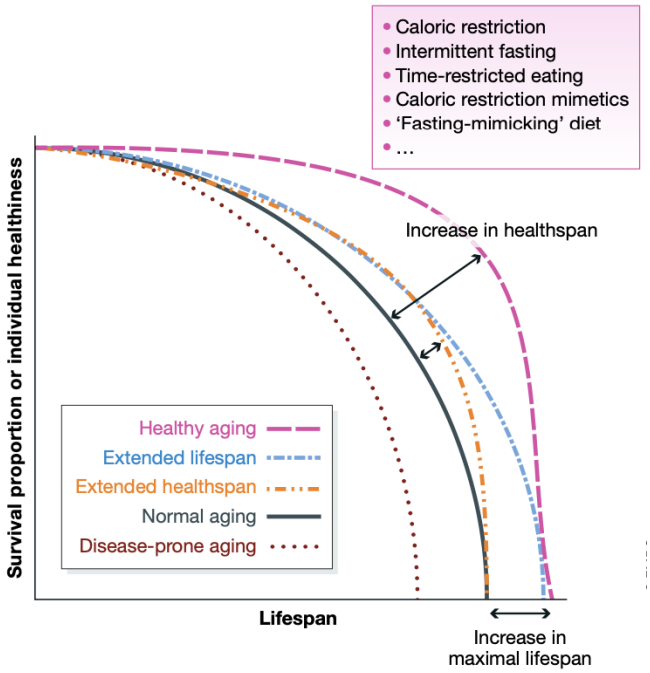


Figure 1.3: **Concepts underlying dietary interventions that influence healthy aging.** Courses of aging can be modulated by dietary interventions such as CR and IF to increase lifespan and healthspan. Y-axis shows survival proportions of a population; X-axis shows the relative lifespan. *Figure adapted from Hofer et al., 2022 with permission.*

lifespan is extended without the defect of small body size (H. J. Smith et al., 2021). Together these studies begin to delineate the specific pathways and signaling tissues that are required to uncouple the positive longevity outcomes from the negative growth outcomes.

1.4 *C. elegans* is a powerful model organism to study the genetic and cell biology mechanisms of aging

Major advances in the molecular mechanisms of aging have been made possible through studies in model organisms. The first long-lived genetic mutant was discovered in *Caenorhabditis elegans* (*C. elegans*) (Kenyon, 2010) and has set the precedence for some of the most pioneering work in the field of aging biology. These 1mm long free-living nematodes were first used to study important biological processes in the lab in 1963 by Sydney Brenner, work for which he was

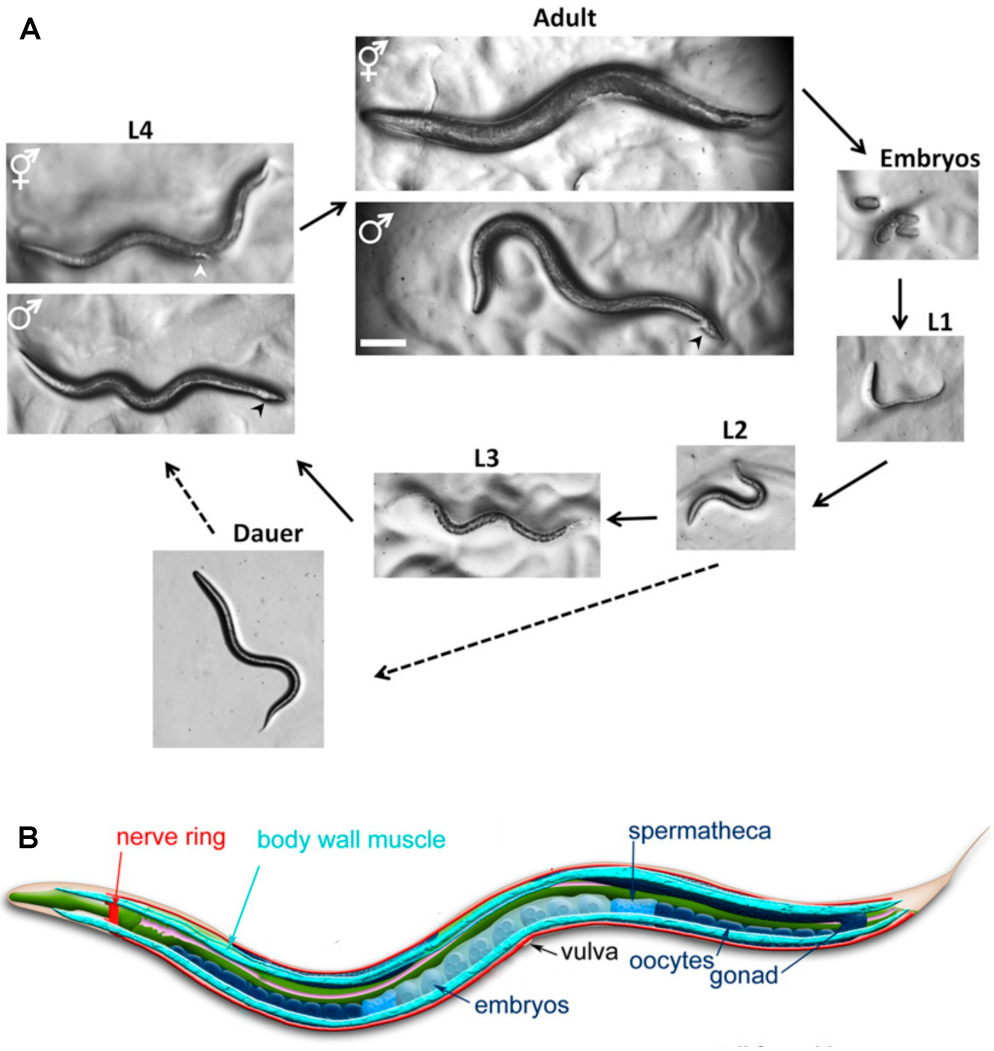


Figure 1.4: **Life cycle and anatomy of *C. elegans*.**
A. *C. elegans* develop through four larval stages (L1-L4), approximately 72 hours, before reaching adulthood. Larvae at the pre-L2 stage can undergo an alternative development pathway in response to stress, to become stress-resistant dauer larvae, characterized by their skinnier appearance than all other larval stages. Dauer larvae are capable of surviving up to months until conditions become favorable to resume growth and development into reproductive adults. In adults, the two sexes can be distinguished by the wider girth and tapered tail of the hermaphrodite and slimmer girth and fan-shaped tail (black arrowhead) of the male. Oocytes can be fertilized by sperm from the hermaphrodite or sperm obtained from males through mating. (Bar, 0.1 mm.)
B. Major anatomical features of a hermaphrodite.
Figure adapted from Corsi et al., 2015 with permission

awarded the 2002 Nobel Prize in Physiology and Medicine (Brenner, 1974; Tissenbaum, 2015). Several characteristics of the nematode make it a powerful and versatile genetic system to probe conserved genes and pathways that modulate organismal aging (Figure 1.4). First of all, the *C. elegans* genome has been fully sequenced, made up of 20.5 thousand genes with 38-70% homology to human orthologues (Shaye and Greenwald, 2011). They are incredibly genetically tractable and can be time-efficiently and cost-effectively used to modulate genes involved in a variety of aging-related pathways. With the modern onset and availability of CRISPR/Cas9 gene-editing technology, endogenous genes can be edited with precision at specific loci to generate point mutations, full genetic deletions, and insertion of fluorescence markers for microscopy or other tags for biochemistry (Paix et al., 2015). Large-scale EMS mutagenesis screens have been performed in *C. elegans* to generate a comprehensive library of mutants which are publicly available (Corsi et al., 2015). Two RNAi libraries, the Ahringer (Ahringer, 2006) and the Vidal library (Rual et al., 2004), are also available to knock down almost any gene in worms. Furthermore, their anatomy has been completely documented: the adult *C. elegans* hermaphrodite has 959 somatic cells, a neuronal network of 302 neurons has been completely mapped, and robust tissue-specific promoters have been characterized (Okkema and Krause, 2005). The naturally short lifespan of wildtype *C. elegans* (roughly 2-3 weeks) makes it possible to dissect the mechanisms within an aging pathway on a short timescale. *C. elegans* are hermaphrodites and each adult can lay 250-300 eggs, meaning that large numbers of isogenic populations can be cultured in a very short amount of time. Moreover, male progeny can be induced for use in making genetic crosses and tracing of Mendelian genetics straightforward. Finally, the cuticle of *C. elegans* is transparent, meaning that fast-growing technologies for high-resolution optical microscopy can be applied to visualize subcellular structures in a free-living animal *in vivo*. This combined with the genetically manipulatable traits of the worm set the mark for *C. elegans* as an optimal model organism for the study the genetics and cell biology of aging.

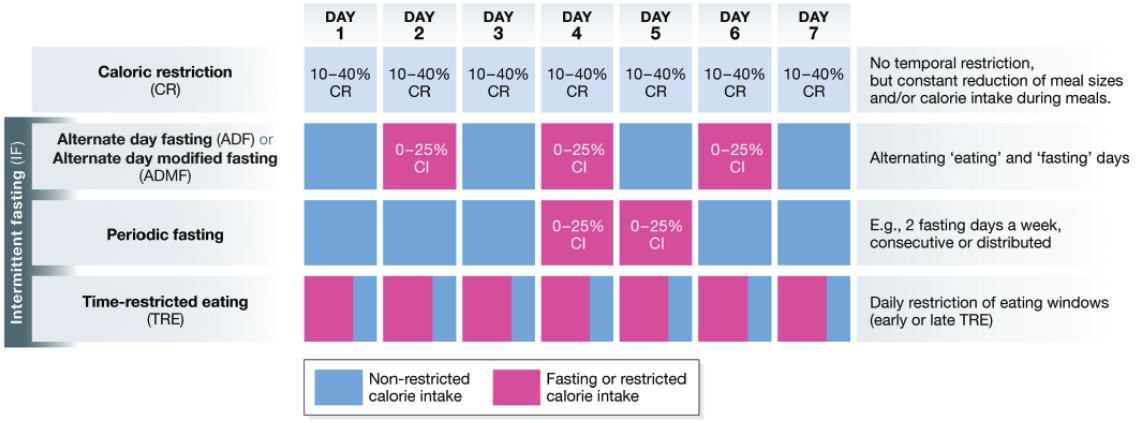


Figure 1.5: **Regimens of caloric restriction and intermittent fasting.**
(Blue = non-restricted calorie intake, magenta = fasting or restricted calorie intake, CI = calorie intake.)

Figure adapted from Hofer et al., 2022 with permission.

1.5 Methods of Dietary Restriction and nutritional interventions that promote longevity in *C. elegans*

Dietary restriction was first demonstrated to extend lifespan in *C. elegans* by Michael Klass in 1977 using bacterial dietary restriction (bDR), a method that relied on growing worms in liquid culture containing different dilutions of *E. coli* food source available (Klass, 1977). Since then, multiple methods of DR have been developed and utilized for longevity studies in the worm.

A second method of dietary restriction using liquid bacterial cultures, called liquid DR (lDR), was developed to culture worms on standard agar plates seeded with the bacterial food source throughout larval development then transferred to liquid medium with bacteria controlled at different densities (Bishop and Guarente, 2007). Methods of bacterial dilution were also adapted for use on solid agar plates (Greer et al., 2007), which permitted culturing of *C. elegans* on solid matter to mimic their naturally occuring growing conditions and to avoid confounding physiological changes that occur when worms are grown in liquid media (Lev et al., 2019). This method was called solid plate DR (sDR) whereby killed or arrested bacteria were diluted and seeded onto plates such that less food was available to DR worms. Genetic models of DR have

also been widely used when it was discovered that *eat-2* animals with mutation in the non-α-nicotinic acetylcholine receptor subunit had reduced pharyngeal pumping, reduced food intake, and correspondingly increased lifespan (Lakowski and Hekimi, 1998). Other methods of DR include bacterial deprivation (BD), the complete removal of food in adulthood (T. L. Kaeberlein et al., 2006), the use of axenic or chemically defined liquid medias (CDLM) that induce DR-like phenotypes (Houthoofd et al., 2002; Szewczyk et al., 2006), and dilution of peptone in agarose plates, which reduces the growth of bacteria (DP) (Hosono et al., 1989).

Notably, these variations in methodology have elucidated both independent and overlapping mechanisms by which different methods of DR extend lifespan in *C. elegans* Greer and Brunet, 2009). sDR and DP were shown to require AMPK/*aak-2* and the downstream phosphorylation of the Forkhead transcription factor FoxO/*daf-16* (Greer et al., 2007). In contrast, neither AMPK nor FoxO were necessary for the longevity induced by bDR or *eat-2* (Lakowski and Hekimi, 1998; Curtis et al., 2006). The stress transcription factor Nrf1/skn-1 was shown to be necessary for lDR, but not for sDR. mTOR was shown to be required in some DR longevity studies (Kapahi et al., 2004; M. Kaeberlein et al., 2005; Hansen et al., 2007), but not others (Henderson et al., 2006), and these observations may be due to differences in DR regimes. The transcription factor FoxA/*pha-4* downstream of mTOR was necessary for bDR and *eat-2* longevity, but dispensible for sDR. Some DR methods have also been shown to elicit partially overlapping mechanisms, such as *eat-2* and sDR, which both required the gene encoding a mitochondrial protein involved in ubiquinone synthesis, *clk-1*, but ultimately induced longevity via primarily independent pathways (Greer and Brunet, 2009). In addition to *clk-1*, sDR also required AMPK/*aak-2* and FoxO/*daf-16*, whereas *eat-2* required FoxA/*pha-4* but not AMPK/*aak-2* nor FoxO/*daf-16*. When sDR and *eat-2* were both performed in the same worm to test their combined effect on lifespan, it was shown that these two DR regimens were additive, therefore suggesting that *eat-2* and sDR evoke mostly independent, with some overlapping, mechanisms to extend lifespan (Greer and Brunet, 2009). Overall, these studies highlight that different dietary regimes can elicit distinct regulatory networks in the organism to promote longevity. Understanding how different dietary

interventions induce lifespan extension will be pivotal for identifying multiple components of the gene network that orchestrate maximal longevity extension in response to nutrient deprivation.

1.6 Intermittent Fasting and alternative methods of nutrient restriction that promote healthy aging

In recent years, there has been rising interest in various methods of nutrient restriction beyond the reduction of caloric intake alone. The benefits garnered from DR were initially attributed to the chronic reduction in caloric intake. However, evidence now suggests that the explanation for DR's beneficial gains may be more nuanced (Longo and Mattson, 2014; Mair et al., 2005). Modulating the nutrient composition of the diet, timing of food intake, duration of fasting, circadian rhythms, age of intervention onset, and sex are all variables that influence the efficiacy of DR on healthy aging. These findings have led to branching fields of work in alternative dietary interventions that may promote healthy aging without such stringent measures as chronic calorie deprivation. Restriction of single amino acids, such as methionine, increases lifespan in rodents, worms (Cabreiro et al., 2013) and flies (B. C. Lee et al., 2014). A growing body of compelling literature also indicates that dietary regimens modulating the timing of food intake to increase periods of fasting promotes longevity and disease resistance in multiple organisms (Di Francesco et al., 2018). Fasting extends lifespan in mice (Goodrick et al., 1990), yeast (Wei et al., 2009) and worms (T. L. Kaeberlein et al., 2006). Fasting has also been practiced historically in human populations, especially amongst religious groups, and emerging evidence from clinical trials indicate that IF may delay age-related pathologies and optimize health in humans (de Cabo and Mattson, 2019; Di Francesco et al., 2018).

Furthermore, in most chronic DR studies, prolonged fasting periods represent a consistent and frequently overlooked variable in energy homeostasis. Under DR, animals often self-impose chronic cycles of 1-hour feeding and 23-hour fasting (Acosta-Rodriguez et al., 2017). When fed

a restricted portion of food once per day, this meal is consumed rapidly within the first hour, with the remaining 23 hours between meals spent in an extended period of fasting. Their *ad libitum* (AL)-fed counterparts are given continuous access to food throughout the day. The possibility that benefits of restricting the timing of food intake can be separable from those associated with overt caloric intake reduction was demonstrated in a study by Mitchell et al. 2018. The authors investigated the effect of eating patterns on morbidity and mortality in mice fed a 30% calorie-restricted diet (DR) or fed a daily single-meal (MF) isocaloric to that of *ad libitum* (AL)-fed mice. A consequence of the MF paradigm was that mice consumed their meals within 12 to 15 hours and fasted for the remaining 9 to 12 hours. This prolonged period of fasting alone was sufficient to significantly increase mean survival 11-14% compared to AL even in the absence of reduced calorie intake (Mitchell et al., 2019). The importance of fasting duration was also supported in a study where 30% calorie restriction was implemented by dietary dilution (Solon-Biet et al., 2014). When mice compensated for the low energy density of the diet by eating throughout the entire day, the intervention produced no extension of lifespan. Therefore, the beneficial effects of dietary regimens may not be due to calories alone, but also the length of fasting periods, acting in independent or interacting mechanisms. Another recent study in mice showed that 30% calorie restriction extends lifespan by 10% (Acosta-Rodriguez et al., 2022). CR combined with daily fasting interval and circadian alignment of feeding however extended lifespan by 35%, and CR combined with Fasting and misaligned timing extended lifespan by 20% (Acosta-Rodriguez et al., 2022). Omics profiling in recent years have revealed that many characteristics of the transcriptomic and metabolomic reprogramming that occurs in CR are also induced by prolonged fasting (Green et al., 2019; Pak et al., 2021). These results altogether suggest that the effects of CR, Fasting, and Circadian timing may be additive and therefore act via some non-overlapping mechanisms.

Intermittent Fasting (IF) is the term used to describe the series of dietary interventions that incorporate prolonged periods spent in a state of fasting. There have been several methods of IF

utilized for studies in the lab and amongst human populations. Alternate day fasting (ADF) is performed by intermittent switching between one day of normal *ad libitum* feeding, followed by one day of fasting. Periodic fasting (PF) describes patterns of feeding:fasting that range from 5 days of feeding:2 days of fasting per 7-day week, up to a few weeks of fasting every few months. Time-restricted feeding is a feeding pattern tuned to a 24-h day clock that resticts food intake within 4-10 hours during the active phase, and the remaining 14+ hours spent in a state of fasting (Figure 1.5). Studies have shown that short-term periods of adopting an IF diet could also have lasting effects on physiology even after switching back to normal *ad libitum* feeding (Catterson et al., 2018). This could have major implications for clinical applicability, since IF may be a more viable intervention than DR on a psychological level, and result in greater compliance. Furthermore, IF improves many clinical parameters beyond longer lifespan, including protection against obesity, hypertension, diabetes, neurodegenerative diseases, and cancer (Mattson et al., 2014; C. Lee et al., 2012; Xie et al., 2017). Although there is promising evidence to support that IF improves multiple risk factors for disease, the specific mechanisms are not well understood. Additionally, studies of its effect on long-term organismal health are limited and a critical next step is to elucidate the molecular mechanisms underlying IF and longevity.

1.7 Molecular mechanisms of Intermittent Fasting mediated longevity

The molecular pathways underlying IF are relatively understudied compared to traditional chronic DR, but there is evidence to suggest that the mechanisms by which prolonged periods of fasting promote healthy aging can be distinguished from those of chronic reduced calorie intake. In *C. elegans*, few studies exist describing signaling pathways required for IF-longevity. The first study of IF in worms showed a small GTPase RHEB-1 to be an essential signaling node for IF-longevity (Honjoh et al., 2009). This RHEB-1 axis was suggested to mediate IF-longevity partially through interaction with the IIS pathway, by downregulation of an insulin-like peptide INS-7, and activation of transcription factor FoxO/DAF-16 (Honjoh et al., 2009). Important to

note however, is that while FoxO/DAF-16 is also reported to mediate the effects of DR-longevity, its activity seems to be regulated by a distinct mechanism. AMPK is required as an upstream activator of FoxO/DAF-16 for DR-longevity, whereas AMPK is dispensable for IF-longevity, a result I have also replicated in the lab (Honjoh et al., 2009; Supplemental Figure A.1). The same group later published that KGB-1, one of the *C. elegans* c-JUN N-terminal kinases (JNKs), directly activates transcription factor complex AP-1 in a second signaling axis important for IF-longevity (Uno et al., 2013). Fasting-induced transcriptional changes that were dependent on AP-1 and DAF-16 most notably encoded Skp1-related protein components of the Skp1-Cullin-F-box (SCF) E3 ligase complex, suggesting enhanced ubiquitin-proteasome activity and proteostasis to be an output of IF-longevity. Interestingly, another study in which our lab collaborated with, used a systems approach to map gene clusters and networks important for DR and IF (Hou et al., 2016). This identified two genes linked to IF that have been implicated in lifespan regulation, but not in the context of DR. These were *gas-1*, encoding a mitochondria complex I component, and *ogt-1*, encoding an orthologue of O-linked N-acetylglucosamine (O-GlcNAc) transferase (Hou et al., 2016). These findings suggest that studying the mechanisms of IF will likely lead to discovery of cellular pathways to target for longevity independent of those for DR. The molecular mechanisms of IF are currently not well-defined and few studies have been conducted to make direct mechanistic comparisons between chronic DR and IF longevity. Whether IF increases longevity through independent and/or overlapping cellular pathways remains a mostly untouched area of investigation.

In my thesis work, I therefore aimed to investigate outstanding questions in the mechanisms of IF longevity and whether these mechanisms could be capitalized upon to elicit health benefits independently from reducing calorie intake. Using the model organism *C. elegans*, I asked whether fasting promotes longevity through independent and/or overlapping pathways as traditional chronic sDR. We previously identified that several proteins involved in modulating mitochondrial shape are required for sDR longevity in *C. elegans* (Weir et al., 2017). The specific functions of mitochondria required for sDR longevity however are not fully defined. Furthermore, we observed

that mitochondrial shape is remodelled upon fasting, but what these structural changes mean for IF longevity is not known.

1.8 Mitochondrial dynamics: the relationship between form and function

The shape and ultrastructural hallmarks of mitchondria were first revealed by seminal electron microscopy work in the 1950s that led to the canonical textbook diagram of mitochondria as fascinating bean-shaped organelles with double lipid membranes and convoluted inner membrane folds termed cristae (Sjostrand, 1956; Palade, 1952). Since then, our understanding of mitochondria has evolved to appreciate the diverse shapes and sizes that mitochondria can take on, ranging from small spheres to long tubules (Detmer and Chan, 2007). The need for mitochondria in healthy tissues is ubiquitous, but the morphology of mitochondria can vary drastically across cell types and tissues (Sharma et al., 2019). Moreover, mitochondria within the cell are rarely found in isolation. Mitochondria are motile and interact with each other to form complex web-like networks that undergo constant remodeling. The collective processes that govern mitochondrial movement and remodeling are termed 'mitochondrial dynamics.' This dynamic restructuring of mitochondrial networks is coordinated by fusion and fission of the outer and inner membranes. Increasing fusion leads to elongated mitochondria and interconnected networks, whereas increasing fission leads to fragmented mitochondria and disconnected networks (Detmer and Chan, 2007; Tilokani et al., 2018).

Why do mitochondria come in such diverse shapes and sizes, engage in such complex network interactions, and undergoing constant remodeling? The answer lies in the relationship between form and function (Friedman and Nunnari, 2014). Mitochondria are multifaceted organelles that lie at the nexus of an intricate cellular signaling network that governs metabolism, cell fitness, and fate (Spinelli and Haigis, 2018; Mishra and Chan, 2016). They receive and integrate molecular

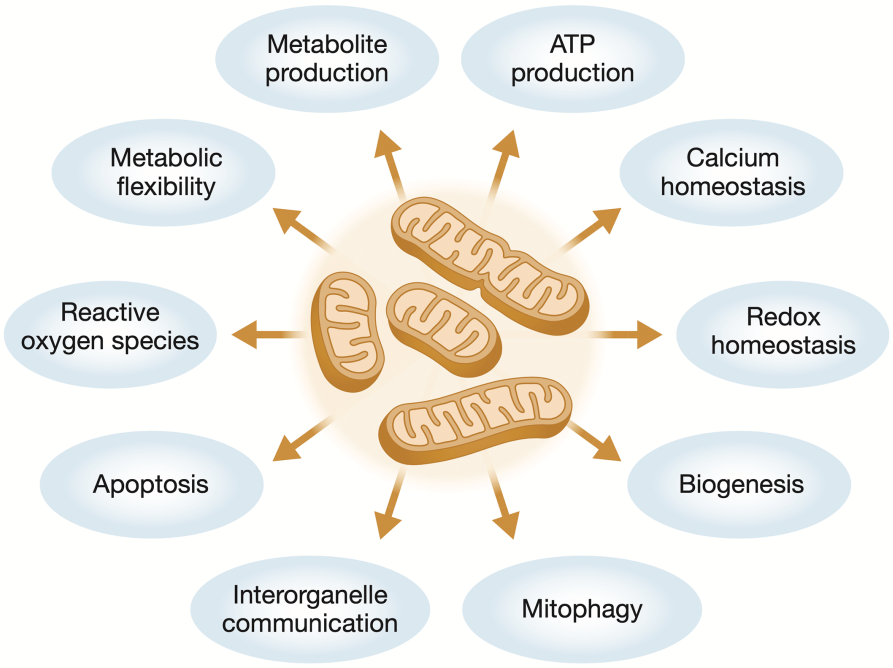


Figure 1.6: **Mitochondria perform a variety of functions that are essential for cellular health.** Mitochondria perform many functions including ATP generation, production of intermediate metabolites, inter-organelle signaling, and Ca2+ and ROS homeostasis. These functions are dependent upon the integrity of mitochondrial dynamics.
Figure adapted from Sharma et al., 2019 with permission.

cues about nutrient availability and cellular stress, and adapt their functions accordingly to meet bioenergetic demands. In addition to generating ATP, mitochondria produce biosynthetic precursors for macromolecules (i.e. lipids proteins, DNA, RNA), compartmentalize metabolites for redox homeostasis, and function as hubs for metabolic waste management (Spinelli and Haigis, 2018). An ever-expanding body of research has demonstrated that the myriad of functions performed by mitochondria are optimized by modulating their shape (Figure 1.6).

Mitochondrial dynamics modulates metabolism and health

Mitochondria play a central role in celullar metabolism as the site for oxidative phosphorylation (OXPHOS), the tricarboxylic acid (TCA) cycle, β-oxidation of fatty acids, redox homeostasis and calcium handling. Evidence shows that specific mitochondrial network morphologies

are associated with changes in the energetic state of the cell. Mitochondrial networks under starvation conditions appear preferentially more fused and have increased ATP synthesis capacity (Gomes et al., 2011). The fusion process is well-known to be important for OXPHOS activity. In yeast, nonfermentable culture conditions require increased OXPHOS activity, and this was accompanied by elongation of the mitochondrial network (Egner et al., 2002; Jakobs et al., 2003). In human cells, mitochondria elongate during growth in galactose media, which forces cells to rely more heavily on OXPHOS for ATP production (Rossignol et al., 2004). Mitochondrial elongation has also been observed during acute oxidative stresses associated with a need for increased ATP production (Tondera et al., 2009). In contrast, mitochondrial networks in nutrient-rich environments have been shown to be preferentially more fragmented (Molina et al., 2009). In response to nutrient excess, mechanisms are recruited to utilize nutrients by storage and heat generation. Studies show that mitochondrial fission is stimulated and fusion is inhibited in depolarized and uncoupled mitochondria. Uncoupled respiration is, for example, important for thermogenesis in brown adipose tissue, and involves activation of Drp1 and mitochondrial network fragmentation (Wikstrom et al., 2014). Uncoupled respiration also relieves ROS toxicity under conditions of nutrient excess by uncoupling the proton gradient. Increasing mitochondrial fission is also essential for programmed cell death via stress-induced hyperfission (Mao et al., 2013; Fannjiang et al., 2004; Thomenius et al., 2011). Mitochondrial morphology is sensitive to cellular calcium buffering signals with the ER (Pfluger et al., 2015), and controls its interaction with other cellular organelles (See Chapter 1.12).

Mitochondrial dynamics modulates mtDNA maintenance, quality control and life cycle

Beyong the role of modulating mitochondrial dynamics to meet the needs of the cell, mitochondrial dynamics also plays an integral role in its own maintenance (Friedman and Nunnari, 2014). As organelles of protobacterial origin, mitochondria have their own genome encoding core constituents of the mitochondrial respiratory complexes. Maintenance of mitonchondrial DNA (mtDNA) relies on both fusion and fission events (R. Yu et al., 2020). Attenuation of fusion

or fission causes mtDNA loss during cell division with associated severe defects in oxidative phosphorylation (H. Chen et al., 2005; Hanekamp et al., 2002). In human cells, a subset of ER-mitochondria contacts spatially couple mtDNA replication with downstream mitochondrial division (Lewis et al., 2016). ER-associated mitochondrial division also links the distribution of mitochondria and mitochondrial DNA in yeast (Murley et al., 2013). Disruption of mitochondrial fusion leads to mtDNA instability (H. Chen et al., 2010) and is important in mtDNA replication (Silva Ramos et al., 2019). Fusion enables mixing of matrix contents to exchange proteins, dilute accumulation of mtDNA mutations and to buffer transient defects that arise in mitochondria (Rolland et al., 2013; H. Chen et al., 2005). Fission is also critical for mitophagy, the recycling of damaged mitochondria. In the case of starvation, hyperfusion has been proposed to protect mitochondria from autophagic degradation or mitophagy through steric hinderance (Rambold et al., 2011). These studies altogether highlight that carefully orchestrated processes of mitochondrial fusion and fission are critical for maintaining a healthy population of mitochondria.

1.9 The molecular mediators of mitochondrial dynamics and their role in aging

Partial excerpts adapted from Review Causal roles of mitochondrial dynamics in longevity and healthy aging. Sharma et al., 2019 with permission.

Aberrant mitochondrial structures have long been observed in aging and age-related diseases, indicating that mitochondrial dynamics are compromised as cells age (Brandt et al., 2017; Short et al., 2005; Kuka et al., 2013). All of the processes mentioned in (Figure 1.6) have also been implicated in neurodegeneration, obesity, diabetes, cardiovascular disease, and cancer, with associated disruptions in mitochondrial dynamics (Liesa et al., 2009). In addition to pathological states, mitochondrial function actively modulates normal aging, and modulating mitochondrial form or function can directly affect organismal longevity (Sharma et al., 2019).

Mitochondrial fission

Mitochondrial fission is essential for mtDNA replication, removal of defective mitochondria via mitophagy, mitochondrial transport and programmed cell death via stress-induced hyperfission (Mao et al., 2013; Fannjiang et al., 2004; Thomenius et al., 2011). The central protein which orchestrates mitochondrial fission is the cytosolic dynamin-related protein 1 (DRP-1). DRP-1 is primarily localized in the cytosol, but assembles on mitochondrial networks in response to phosphorylation at Ser 616 to mediate membrane constriction and scission (R. Liu and Chan, 2015). Imaging and protein-protein interaction studies show several mitochondrial outer membrane proteins recruit DRP-1 to initiate scission, including mitochondrial fission factor (MFF), fission protein-1 (FIS1), mitochondrial dynamics protein-49 (MiD49) and mitochondrial dynamics protein-51 (Mid51) (J. E. Lee et al., 2016; Loson et al., 2013). MTP18 faces the intermembrane space to assist in inner mitochondrial membrane fission (Tondera et al., 2005). Several proteins involved in mitochondrial fission are known to be dysregulated with age in various animal models. Aged mice demonstrate reduced DRP1 activity and altered mitochondrial morphology in several tissues including neurons, skeletal muscle, and oocytes (Udagawa et al., 2014; Kageyama et al., 2012). Aged skeletal muscle in mice shows increased mitofusin 2-to-DRP1 ratio and longer intermyofibrillar mitochondria (Leduc-Gaudet et al., 2015). Aged human endothelial cells in vitro show a similar downregulation of both DRP1 and FIS1 expression as well as elongated mitochondrial networks (Mai et al., 2010). Induction of Drp1p expression in *Drosophila* midlife prolongs both lifespan and health span via improved mitochondrial respiration and autophagy (Rana et al., 2017). In both worms and flies, induction of mitochondrial fragmentation specifically in the intestine is known to increase lifespan, suggesting that maintaining mitochondrial fission might have pro-longevity effects (Weir et al., 2017). Paradoxically however, in yeast, mitochondrial fragmentation is observed with age, and maintaining fusion via deletion of the DRP-1 ortholog Dmn1p delays aging without impairing growth rate or fertility (Scheckhuber et al., 2007). Findings from mouse skeletal muscle have also shown that increased mitochondrial fission is correlated with impaired insulin signaling and mitochondrial dysfunction (Jheng et al.,

2012). Taken together, these data indicate that while it is clear that the processes regulating mitochondrial fission alter with age, promoting mitochondrial fission can increase or decrease mitochondrial fitness and function depending on the context, tissue or organism.

Mitochondrial fusion

Mitochondrial fusion is achieved by membrane-bound dynamin-related proteins. Mitofusin 1 (MFN1) and MFN2 mediate the fusion of the outer membrane, and optic atrophy 1 (OPA1) mediates the fusion of the inner membrane. Loss of either outer mitochondrial membrane (OMM) or inner mitochondrial membrane (IMM) fusion proteins results in a hyperfragmented mitochondrial network (Sharma et al., 2019; Hall et al., 2014). Metabolically active cells often have interconnected mitochondrial networks and is known to increase mitochondrial production of ATP (Rafelski, 2013). Fused mitochondrial networks allow efficient exchange of metabolites, transmission of membrane potential from mitochondria in one part of the cell to the other, increased ATP production, and decreased ROS production (Rafelski, 2013; Yao et al., 2019). Additionally, fusion reduces endoplasmic reticulum (ER)-mitochondrial calcium transfer to prevent calcium-induced cell death (H. Chen et al., 2005). Mitochondrial fusion also is known to help mitigate mitochondrial stress by fusing the contents of partially damaged mitochondria with healthy mitochondria as a form of complementation (Bereiter-Hahn, 2014). Dietary restriction (DR) increases mitochondrial fusion by increasing cyclic AMP (cAMP) levels, activating protein kinase A (PKA), and inhibiting DRP1 via PKA-mediated phosphorylation (Jheng et al., 2012). MFN2 expression is directly proportional to skeletal muscle insulin sensitivity and decreases with age, indicating decreased fusion is consistent with age-related dysfunction in mitochondrial dynamics and metabolic function (Bach et al., 2005).

Mitochondrial fusion in *C. elegans* increases and facilitates lifespan extension in various known pro-longevity interventions such as insulin/ IGF-1-like signaling (IIS) inactivation, DR, germline depletion, electron transport chain (ETC) dysfunction, mechanistic target of rapamycin (mTOR)

inactivation, sirtuin overexpression, AMP-activated protein kinase (AMPK) over-expression, impaired glycolysis, and exercise (Chaudhari and Kipreos, 2017). Lifespan extension for these longevity pathways was significantly reduced by *eat-3* RNAi, the *C. elegans* homolog for mammalian fusion protein OPA1. *eat-3* RNAi by itself did not alter the lifespan of wildtype animals. Additionally, RNAi of *drp-1* or overexpression of the mitofusin ortholog FZO-1, both of which increase mitochondrial fusion, does not increase lifespan in *C. elegans* (Weir et al., 2017). Converse to findings in *C. elegans* however, reduction in mitofusin levels in aging flies extends both mean lifespan and maximum lifespan (Rana et al., 2017). Collectively these studies again suggest that although mitochondrial fusion is a conserved mechanism to increase metabolic efficiency and is required for multiple pathways of lifespan extension in *C. elegans*, the effects on longevity may be context-dependent and differ depending on the tissue and organism.

Interestingly, mitochondrial dynamics have furthermore been shown to follow a diurnal pattern controlled by the circadian regulator brain and muscle arnt-like protein-1 (Bmal1), which regulates mitochondrial fusion by modulating MFN1 and MFN2 expression to impact mitochondrial metabolism during feeding-to-fasting transitions. *C. elegans* Bmal1 ortholog, *aha-1*, also modulates oxidative metabolism and promotes longevity via fusion of mitochondrial networks (Jacobi et al., 2015). The specific mechanisms by which aging affects mitochondrial dynamics and whether these changes are causally or casually associated with cellular and organismal aging remain open questions to be investigated.

1.10 Context-specific requirement of mitochondrial dynamics

While many studies have attempted to decipher the relative importance of mitochondrial fusion and fission in aging by perturbing these processes or by testing for their requirement in various lifespan-extending interventions, the varied results of these studies have highlighted the fact that neither process is universally pro- or anti-aging. The requirements for mitochondrial fusion and fission vary by cell type, the technique used, and the organism in which it is applied.

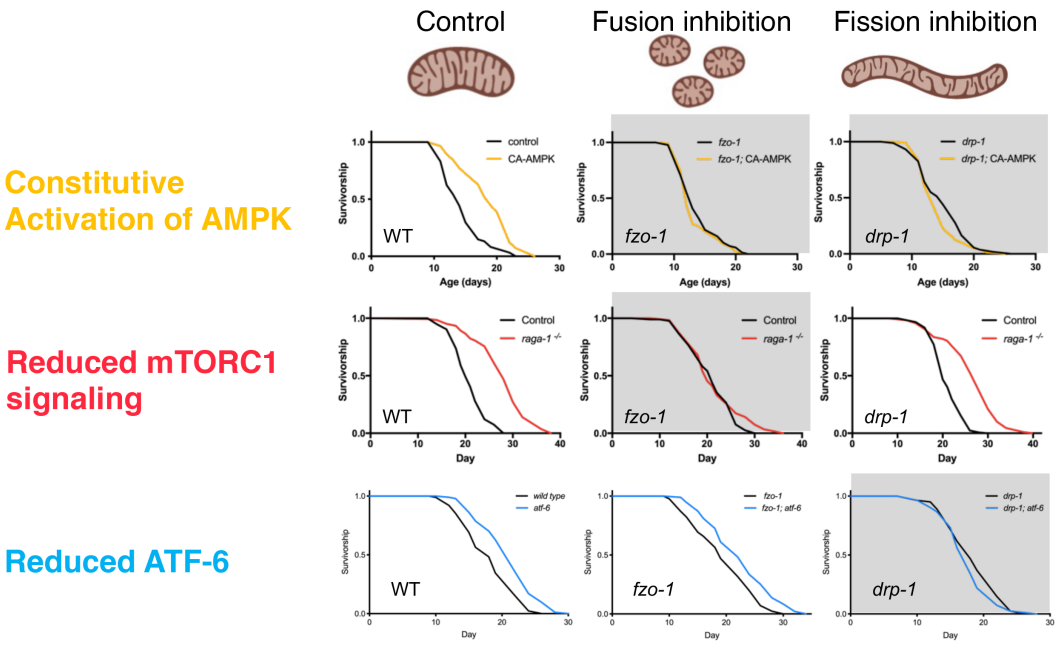


Figure 1.7: **Mitochondrial fusion and fission are differentially required for pathways affecting aging and longevity in *C. elegans*.**
Mitochondrial fusion (*fzo-1*) and fission (*drp-1*) are both required for longevity mediated by constitutive activation of AMPK (CA-AMPK) (Row 1, yellow). Inhibition of *fzo-1* abolishes lifespan extension by CA-AMPK; inhibition of *drp-1* also abolishes lifespan extension by CA-AMPK. Mitochondrial fusion is required for longevity mediated by reduced TORC1 signaling (Row 2, red). Inhibition of *fzo-1* abolishes lifespan extension by reduced TORC1; inhibition of *drp-1* has no effect on lifespan extension by reduced TORC1. Mitochondrial fission is required for longevity mediated by reduced ATF-6 (Row 3, blue). Inhibition of *drp-1* abolishes lifespan extension by reduced TORC1; inhibition of *fzo-1* has no effect on lifespan extension by reduced ATF-6.
Figure adapted from data in Weir et al., 2017; Burkewitz et al., 2020; Y. Zhang et al., 2019 with permission

This view can also be extended to the timing in which different mitochondrial functions are more or less necessary throughout an organism's lifetime. A study in *C. elegans* showed that loss of fission decreased neuronal function and performance across several behavioral tests primarily during development (L4 stage) and in aged animals (day 7 and day 11 adults), but not during early adulthood (day 3 adults). Loss of fusion produced more consistent and progressive neurological and behavioral defects all across development and adulthood (L4, day 3, day 7, and day 11 adults) (Byrne et al., 2019). In *Drosophila*, induction of mitochondrial fission in

midlife, but not in early adulthood, extended lifespan (Rana et al., 2017). Midlife upregulation of dMFN shortened lifespan (Rana et al., 2017). Another study showed in mice and *C. elegans* that increasing mitochondrial fission and mitophagy in middle-aged animals correlates with lifespan extension, whereby depletion of PUM2, an RNA-binding protein that inhibits Mff RNA translation, in old mice or the *C. elegans* homolog PUF-8 in middle-aged worms improved mitochondrial homeostasis, mitochondrial fission, and promoted mitophagy (D'Amico et al., 2019). Given that a major role of mitochondrial fission is to facilitate clearance of defective mitochondria via mitophagy, the fluctuating importance of fission during development and midlife may be a reflection of changes in the cellular demand for mitophagy.

Three studies from our lab also showed distinct requirements of mitochondrial fusion and/or fission in three conserved longevity pathways (Figure 1.7). In three separate genetic pathways that increase lifespan in *C. elegans*– constitutive activation of AMPK, reduction of mTORC1 signaling, and downregulation of the ATF-6 branch of the UPR_{ER}– we observed differential requirements of mitochondrial fusion and/or fission to mediate longevity. In the AMPK pathway, both fusion and fission was required for lifespan extension (Weir et al., 2017). In the mTORC1 pathway, only fusion was required for lifespan extension (Y. Zhang et al., 2019). In the ATF-6 pathway, only fission was required for lifespan extension (Burkewitz et al., 2020). These observations further support that there are distinct functional consequences of different mitochondrial network organizations, and they each contribute to the unique cellular milieu required during these longevity interventions in specific mechanisms.

1.11 Balanced fusion and fission dynamics versus mitochondrial plasticity

Another theme emerging from these studies is that the balance between fusion and fission may be more important than the modulation of either process in isolation. Despite the requirement

of fusion for lifespan-extending interventions, most studies show that driving fusion or fission alone is not sufficient to increase lifespan and sometimes negatively affects health and longevity (Chaudhari and Kipreos, 2017; Weir et al., 2017). Pathologies that are associated with deletion of *drp-1*, *fzo-1*, or *eat-3* in *C. elegans* can often be restored by inhibiting the other (Byrne et al., 2019). Yeast cells deficient for fission and fusion (*dnm1;mgm1* double knockout) have healthy-looking mitochondria with a wild-type morphology, although these yeast do have a shortened lifespan (Bernhardt et al., 2015). In *C. elegans*, double *drp-1;fzo-1* knockouts also have healthy-looking mitochondria, but their simultaneous inhibition can prolong lifespan (Weir et al., 2017). The mechanisms underlying this longevity effect require further investigation, including how it effects other mitochondrial stress responses and interactions between mitochondria and other organelles.

This requirement for mitochondrial network homeostasis may also be conserved in mammals. Inhibiting fission or fusion in mouse hearts by deletion of Drp1 or Mfn2, respectively, leads to mitochondrial disruption, cardiac pathology, and rapid lethality (Song et al., 2017). On the other hand, promoting fission or fusion by overexpressing the corresponding gene leads to changes in mitochondrial morphology but does not lead to cardiac pathology, suggesting that cardiac pathology did not entirely result from the fragmented or fused mitochondrial networks, but from the disruption of the fission/fusion balance. In support of this concept, triple Drp1, Mfn1, and Mfn2 knockout in adult mouse hearts showed slower progression of cardiac pathology and an extended lifespan compared with either the Drp1 KO or the Mfn1/Mfn2 KO (Song et al., 2017). Similarly in another study, Mff/Mfn1 double mutants mice were alive and healthy after one year, whereas single Mff mutants died prematurely due to cardiomyopathy (H. Chen et al., 2015).

Aging is often described as a loss of homeostasis, assuming that age is a deviation from a fixed equilibrium. In reality, cells at their prime are in constant dynamism, and it can be argued that the readiness to adapt to fluctuating conditions or stresses in the external or internal milieu is the truest form of a youthful cell. While studies in worms and yeast may have conflicting results

regarding the longevity effects of an adynamic mitochondrial network, they both highlight the importance of mitochondrial plasticity. In both organisms, although the morphology of the mitochondria of the double mutants resembles that of wild-type mitochondria, they respond very differently to stress and changes in their environment. In worms, intermittent fasting (IF) extends lifespan and remodels the mitochondrial network as they are switched between fasted and fed states. The *drp-1;fzo-1* double mutant worms are unable to remodel their mitochondrial networks in response to the feeding and fasting phases (Weir et al., 2017). In yeast, the double mutant responds poorly to various stress tests (Bernhardt et al., 2015). These results indicate that eliminating mitochondrial dynamics through simultaneous disruption of fusion and fission can result in normalized mitochondrial morphology and possibly increased lifespan, but it also results in an inability to respond to stress and changes in nutrient availability. While this static mitochondrial network may be better equipped to maintain metabolic homeostasis throughout an organism's life, it may be less able to respond to stress and changes in nutrient availability, and again dependent on the cellular context and metabolic demands.

1.12 Mitochondrial dynamics regulates inter-organelle communication

Furthermore, it is becoming increasingly appreciated that mitochondria engage in physical interactions, transient contacts, vesicular transport, molecular exchange and close proximal localization with not only other mitochondria, but also various other cellular organelles (Petkovic et al., 2021). These connections with other organelles can directly influence the function and dynamics of mitochondria.

ER-mitochondria interactions

Perhaps the best studied organelle connection so far is between ER and mitochondria. The ER forms close physical contacts with the mitochondrial network and directly modulates mi-

tochondrial dynamics via its role in membrane fission (Elgass et al., 2015). ER tubules wrap around mitochondria, marking sites for DRP1 recruitment and constriction, as well as mediating DRP1-independent constriction of mitochondria (Friedman et al., 2011; Chakrabarti et al., 2018). A subpopulation of the tail-anchored proteins MFF and FIS1 which are receptors for DRP1 recruitment on mitochondria also localize to the ER, where they can assemble DRP-1 oligomers that can be transferred to mitochondria or peroxisomes (Ji et al., 2017). A subpopulation of the fusion receptor MFN2 also localizes to ER and regulates organelle contacts via homotypic interactions with MFN2 or heterotypic interactions with MFN1 (de Brito and Scorrano, 2008). Reduced mitochondria-ER contacts have been reported in aged cells and is a likely contributor of age-associated imbalance in mitochondrial dynamics. Dysregulation in ER-mitochondria contacts has also been implicated for a variety of other diseases of aging, including obesity, diabetes, and neurodegeneration. In addition to the ER's role in mediating mitochondrial fission, contact site disruption also affects processes that ER and mitochondria regulate cooperatively, such as cellular calcium buffering and mitophagy. Aged cells show decreased mitochondrial Ca^{2+} uptake from the ER, resulting in excess ER-derived Ca^{2+} in the cytoplasm (Fernandez-Sanz et al., 2014). Cytoplasmic Ca^{2+} can regulate the activity of dynamin and promote mitochondrial translocation of DRP1 and enhanced mitochondrial fission observed with aging in many models (Cribbs and Strack, 2007). A recent study from the Mair lab by Burkewitz et al. show that modulating subcellular calcium compartmentalization and ER-mitochondria calcium signaling is indeed a mechanism of both aging and longevity (Burkewitz et al., 2020). The loss of ATF-6, a conserved mediator of the unfolded protein response, increases lifespan in *C. elegans* due to disruptions in ER calcium retention. ER calcium release via the inositol triphosphate receptor (IP3R/*itr-1*) was required for ATF-6 longevity, and IP3R/*itr-1* gain of function was sufficient to extend lifespan. The inhibition of IP3R had ripple effects on mitochondrial bioenergetics and caused mitochondrial network hyperfusion. The mitochondrial calcium import channel *mcu-1* was also required for *atf-6* longevity. Together these results highlight the importance of ER-mitochondria communication in mediating the effects of a longevity pathway (Burkewitz

et al., 2020). Inter-organelle communication modulation of aging is a relatively new area of research, but these studies indicate the importance of pathways that regulate signaling between organelles in the aging process and the need to further investigate these mechanisms so that they can be targeted as anti-aging therapeutics.

Peroxisome-Mitochondria interactions

Another exciting area of inter-organelle communication in the regulation of aging is the interaction between mitochondria and peroxisomes, which has been highly relevant to my thesis studies (See Chapter 2). Peroxisomes are ubiquitous, multifunctional, and dynamic organelles that are indispensable for organismal health and viability. Like mitochondria, peroxisomes modulate their morphology, abundance, and function to meet fluctuating bioenergetic needs (Schrader et al., 2016). The mechanism of peroxisome membrane fission largely parallels that of mitochondrial fission and requires DRP1 (Sesaki et al., 2014). FIS1 and MFF are also found on peroxisome membranes and interact with DRP1 and PEX11 to regulate peroxisome fission (Kobayashi et al., 2007; Kobayashi et al., 2007; Joshi et al., 2012). PEX11β, a member of the highly conserved PEX11 protein family known to regulate peroxisome size and number, accumulates at the constriction sites, where it functions in the early fission phase. It has been reported that PEX11β itself is also able to generate sites of constriction along these protrusions, and contributes to the recruitment and activity of DRP1 (Yoshida et al., 2015). Although PEX11β does not have intrinsic membrane scission activity, loss of Pex11β results in enlarged and elongated peroxisomes (Williams et al., 2015). A study in *Saccharomyces cerevisiae* also suggested that PEX11 interacts with the ERMES complex component MDM34 to establish contact sites between peroxisomes and mitochondria (Mattiazzi Usaj et al., 2015). Manipulation of *Drosophila* Pex11 and *C. elegans* (*prx-11*) was linked to lifespan regulation in a study where knockdown of several Pex genes involved in peroxisome biogenesis led to reduced levels of endogenous ROS and increased lifespan (Zhou et al., 2012).

Mitochondrial shape and function can be influenced by peroxisomal ROS and metabolites.

Localized oxidative damage to peroxisomes and increased intraperoxisomal ROS production causes mitochondrial fragmentation (B. Wang et al., 2013). Early observations reported the peroxisomal ROS- detoxifying enzyme, catalase, to decrease in old rodent livers and human cells (Ivashchenko et al., 2011; Xia et al., 1995), and loss of catalase caused accelerated aging in *C. elegans* (Petriv and Rachubinski, 2004). Several recent studies have continued to elucidate how peroxisomes play a critical role in the aging process, through independent or interacting functions with mitochondria. In work I contributed to in the beginning of my PhD, interplay between mitochondrial dynamics and peroxisome metabolism was shown to be essential to longevity mediated by DR and constitutively activated AMPK (CA-AMPK)in *C. elegans* (Weir et al., 2017). In this study, direct manipulation of mitochondrial dynamics by simultaneous inhibition of fusion and fission induced longevity and revealed that peroxisome dynamics and very-long-chain fatty acid metabolism were part of this mechanism. This seems to hold true in yeast as well, where the supply of acetyl-CoA by peroxisomal β-oxidation helped prevent mitochondrial network fragmentation and contributed to DR-induced extension of chronological lifespan. In *C. elegans*, peroxisome matrix protein import was necessary for DR and CA-AMPK mediated longevity. RNAi knockdown of *prx-5*/PEX5 (involved in recognition and matrix import of proteins containing the C-terminal peroxisome targeting sequence) abolished AMPK-longevity. I showed that *prx-5* RNAi also severely blunted *eat-2* longevity, a genetic model of DR (Supplemental Figure A.8). I performed experiments that showed aging resulted in decline of PRX-5-mediated peroxisomal matrix protein import. This age-associated decline in PRX-5 function was delayed by DR and activation of AMPK (Supplemental Figure A.8). RNA-seq analysis furthermore revealed that DR increased global peroxisome gene expression (Weir et al., 2017). This is interesting because with normal aging, it was reported that peroxisome proteins are among the most significantly down-regulated in the *C. elegans* proteome, suggesting that these longevity-promoting interventions may work in part by reversing age-associated decline of peroxisome function (Narayan et al., 2016). Even more promising is the likely conserved role of peroxisomes in aging. A study of calorie restriction (CR) in Rhesus monkeys similarly found that even short-term CR increases

peroxisome gene and protein expression in the liver (Rhoads et al., 2018).

1.13 Open questions in the mechanisms of IF-mediated longevity

It is clear that aging as a process is malleable and some of the most robust and conserved methods of slowing aging are via modulation of cellular metabolism and growth pathways. While dietary restriction is well-accepted to increase lifespan, the nuances of this intervention are only just gaining appreciation. Specifically, the health-promoting effects of DR are not due to reduced calories alone, but that macronutrient composition, timing of food intake to circadian rhythms, and prolonged periods of fasting are factors that are integral to DR longevity. Many of these studies open avenues for more clinically realistic approaches to promoting healthy aging in humans. Intermittent fasting is one of these interventions that has gained considerable traction. The specific mechanisms underlying IF are understudied, but evidence suggests that some of these mechanisms may be distinct from those of DR.

We previously identified mitochondrial dynamics to be required for sDR longevity in *C. elegans*. Blocking mitochondrial fusion or fission via genetic deletion of *fzo-1* or *drp-1*, respecitvely, abolished lifespan extension by sDR. The downstream functions of mitochondria resulting from inhibition of *fzo-1* or *drp-1* and how they regulate DR longevity are not fully understood. IF also extends lifespan in *C. elegans*, and changes in mitochondrial morphology have been observed upon fasting (Weir et al., 2017). The role of mitochondrial dynamics in IF longevity have not been well studied. The work presented in my thesis aims to address some of the key outstanding questions in this field:

1. What are the molecular mechanisms mediating IF longevity in *C. elegans*? 2. Are the mechanisms by which IF promote longevity independent or overlapping to those of chronic DR?
3. What are the mechanisms underlying the differential requirement of mitochondrial dynamics for IF and DR longevity?

4. Is mitochondrial plasticity required for IF-longevity?

5. What are the functional outputs of mitochondrial dynamics that couple to IF-longevity?

To summarize the work I have done, my results suggest that there is an interesting differential requirement of mitochondrial fusion, fission and plasticity for DR and IF longevity. Co-inhibition of fusion and fission does not affect global mitochondrial architecture, but blocks mitochondrial plasticity. Mitochondrial plasticity is specifically required for lifespan extension by IF, but not DR. In addition to multiple new tools to study mitchondrial structure and function in *C. elegans*, I also generated an inducible system to synthetically manipulate mitochondrial plasticity. I showed that this was sufficient to mimic intermittent fasting and extend lifespan in *C. elegans*. Finally, I used a transcriptomic profiling approach to identify genes and pathways that specifically explain loss of IF longevity when mitochondrial networks are made adynamic. While further mechanisms and follow up studies are necessary to define their causality for IF longevity, my findings show important new knowledge that perturbation of metabolic networks are a central mechanism coupling mitochondrial plasticity to IF longevity.

2 Generating improved microscopy tools for visualizing mitochondrial structure, function and inter-organelle interactions during aging in *C. elegans*

2.1 Introduction

Mitochondrial dysfunction has long been established as a classical hallmark of aging and contributes to many age-related pathologies such as metabolic disorder, cancer, and neurodegeneration (Sharma et al., 2019; Y. J. Liu, McIntyre, Janssens, and Houtkooper, 2020). Alterations in mitochondrial morphology have been reported in multiple organisms from yeast, worms, flies and mice during aging and in diseased states. Studies are beginning to elucidate that these changes in mitochondrial morphology during aging and disease also lead to associated mitochondrial dysfunction. Aged animals often present with more heterogeneous, fragmented, and large, swollen mitochondria that also have impaired mitophagy. Moreover, conserved pathways that mediate longevity in organisms require the mitochondrial membrane fusion and fission proteins that modulate mitochondrial morphology (Weir et al., 2017). Interestingly, our lab previously showed that the organization of mitochondrial networks are distinct in multiple longevity pathways. While fused networks are required for reduced mTOR longevity, fragmented networks are required for the ATF-6 longevity pathway in *C. elegans* (Y. Zhang et al., 2019; Burkewitz et al.,

2020). These studies suggest that different morphologies of the mitochondrial network play distinct roles in specific longevity pathways.

While many studies now show the correlation between mitochondrial form, aging, and longevity, evidence in multicellular organisms that mitochondrial dynamics causally regulate lifespan remain rather limited. Moreover, little is known about the specific functional consequences of modulating mitochondrial dynamics during aging. Cell biology work in cell models or unicellular organisms (yeast) have begun to characterize how modulating mitochondrial form has direct consequence on its function, but few studies have yet to be translated into multicellular organisms in the context of organismal aging.

A study from our lab that I contributed to at the start of my PhD (Weir et al., 2017) was one of the first to show that modulation of mitochondrial shape can directly influence organismal longevity(Weir et al., 2017; Rana et al., 2017). Maintaining balanced mitochondrial dynamics in aged *C. elegans* via genetic co-inhibition of *drp-1* and *fzo-1* increased maximal lifespan, supporting that mitochondrial dynamics causally regulate aging. Subsequent imaging and metabolomic studies revealed that co-inhibition of *drp-1;fzo-1* also caused changes to peroxisomal morphology and very long-chain fatty acid metabolism, suggesting that the longevity benefits were due to functions of both mitochondria and peroxisomes. My work in this study showed that peroxisome matrix protein import was necessary for DR and CA-AMPK mediated longevity; RNAi knockdown of *prx-5*/PEX5 (involved in recognition and matrix import of proteins containing the C-terminal peroxisome targeting sequence) abolished AMPK-longevity. I showed that *prx-5* RNAi also severely blunted *eat-2* longevity, a genetic model of DR (Supplemental Figure A.8). Aging resulted in decline of PRX-5-mediated peroxisomal matrix protein import, and this age-associated functional decline was delayed by DR and activation of AMPK (Supplemental Figure A.8). Other studies in recent years have also begun to elucidate the critical influence that peroxisome morphology and function have on aging. Many peroxisomal proteins are significantly decreased in old animals (Narayan et al., 2016), and DR was shown to increase peroxisomal gene expression in

monkeys (Rhoads et al., 2018) and worms (Weir et al., 2017).

Direct crossover between mitochondrial dynamics and peroxisomes is also highlighted by their shared membrane fission machinery. DRP1 is recruited to peroxisomes and mediates the final step of peroxisome fission (Sesaki et al., 2014). FIS1 and MFF are found on peroxisome membranes and interact with DRP1 and PEX11 to regulate peroxisome fission (Kobayashi et al., 2007; Kobayashi et al., 2007; Joshi et al., 2012). Manipulation of *Drosophila* Pex11 and *C. elegans* (*prx-11*) has been linked to lifespan regulation, where knockdown of several Pex genes involved in peroxisome biogenesis led to reduced levels of endogenous ROS and increased lifespan (Zhou et al., 2012). Therefore anti-aging interventions that require mitochondrial fission may in part be due to peroxisome fission, and most studies have failed to consider the consequences that altering DRP1, FIS1 or MFF could have on peroxisome dynamics and function. Few methods of specifically perturbing peroxisome dynamics without affecting mitochondrial dynamics (and vice versa) have been developed as a result.

These many unanswered questions on mitochondrial dynamics– (1) its causal role in multicellular organismal aging, (2) its direct consequence on mitochondrial function, (3) its temporal requirement for longevity, and (4) its effects on inter-organelle communication– have largely been hindered by a lack of appropriate tools. Therefore, I aimed to develop a series of improved *in vivo* tools for quantitative microscopy and spatiotemporal control of mitochondrial form and function in the multicellular model organism *C. elegans*. In this chapter of my thesis, I show new single-copy, integrated fluorescence markers of the other mitochondrial membrane translocase TOMM-20, an endogenous TOMM-70 marker, and a functional reporter of mitochondrial fatty acid β-oxidation. I also show a new single-copy, integrated peroxisome matrix import reporter and a new genetic mutant of *prx-11* that affected peroxisome morphology. These improved tools enabled better quantitative microscopy to be done, and have been used in my subsequent studies and the work of other labs interested in the role of mitochondrial dynamics and peroxisomes in a freely-living and aging *C. elegans*.

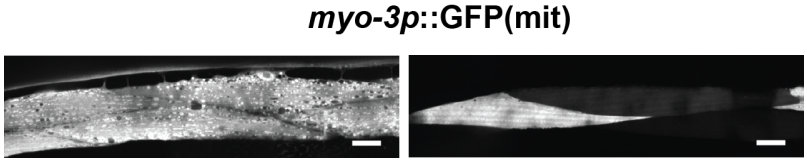


Figure 2.1: **Current tools to study mitochondrial structure in *C. elegans* are outdated and show variability in labeling of mitochondria.**
Representative images of muscle mitochondria shows that at Day 1, the existing *myo-3p::GFP(mit)* strain had inconsistent targeting of GFP to the mitochondria. Left panel shows muscle cells in which GFP adequately labeled the mitochondrial matrix; Right panel shows different muscle cells in the same strain in which GFP appeared mostly cytosolic. Scale bar 10 μm.

Results

2.2 Generating improved mitochondrial markers for quantitative fluorescence microscopy in *C. elegans*

Work on the critical role that mitochondrial dynamics play in multiple scenarios of aging and pathways of longevity is rising to the forefront of current cutting edge aging research, however the tools used to probe mitochondrial morphology remain rather archaic. The predominant reporter of mitochondrial morphology in most publications was generated in 2006, a GFP-targeted to mitochondria via a mitochondrial targeting sequence (Benedetti et al., 2006). In the lab, I have found that this tool presents several caveats that hinder its usefulness in aging cell biology studies. First, as a mito-targeted fluorophore that is non-integrated into the membrane or constitutively residing in the lumen, visualization of mitochondrial networks relies on the intact ability of mitochondrial import function. Mitochondrial protein import of GFP is inconsistent even in young day 1 worms, as shown by inadequate GFP labeling of the mitochondrial network and remaining mostly cytosolic (Figure 2.1). This would likely be exacerbated with age. Second, this model was generated using extrachromosomal plasmid arrays and integrated in the genome via gamma irradiation in unknown copy number and at random loci. This variability in baseline expression levels makes quantitative microscopy nearly impossible within the same strain, and especially across multiple conditions. With advances in targeted gene editing technologies like

CRISPR, it is necessary to upgrade our tools to probe mitochondrial morphology with age.

To address these concerns in morphometric studies, I generated several new fluorescence reporters to visualize mitochondrial morphology that had the following improvements: (1) single-copy integration into the genome to allow quantitative measurements without potential toxicicity associated with introducing high-copy number of fluorophores; (2) CRISPR-assisted integration at specific intergenic loci known to give stable expression without affecting other genes that may cause confounding biological changes; (3) fluorescent labeling of endogenous mitochondrial proteins to show relative endogenous abundance of mitochondria across different tissues.

To generate new outer mitochondrial membrane (OMM) fluroescence markers, I utilized a single-copy knock-in loci for defined gene expression (SKILODGE) system previously developed in the lab, which enables insertion of any DNA by CRISPR/Cas9 at defined safe harbors in the *C. elegans* (Silva-Garcia et al., 2019). The lab previously showed that this system is a fast, economical and effective method to introduce single-copy ectopic transgenes in *C. elegans*, and released a suite of tool-building strains regulated by multiple tissue-specific promoters. I created a new construct carrying the 1-49aa intermembrane fragment of the outer mitochondrial membrane translocase TOMM-20 (TOMM-20) fusion to GFP or mCherry, driven by the *eft-3p* for ubiquitous tissue expession (Figure 2.2 A). Using CRISPR/Cas9 editing, this construct was integrated in single-copy at a defined intergenic region on Chromosome V, previously characterized to give stable expression with no silencing (Silva-Garcia et al., 2019). I showed that this strain offered several improvements upon the mito-GFP strain, showing more consistent expression and accurate labeling of mitochondrial networks in young (Day 1) and old (Day 8) animals (Figure 2.2 B-C). In contrast, the mito-GFP strain shows variability in labelling of mitochondrial networks, even in young day 1 adults (Figure 2.1). Furthermore, by crossing these strains into *drp-1* or *fzo-1* deletions, I was able to show perturbations of the mitochondrial network towards hyperfragmentation or hyperfusion (Figure 2.2 C). Aged day 8 adult worms also presented with age-related mitochondrial fragmentation, indicating that this mitochondrial label responds in

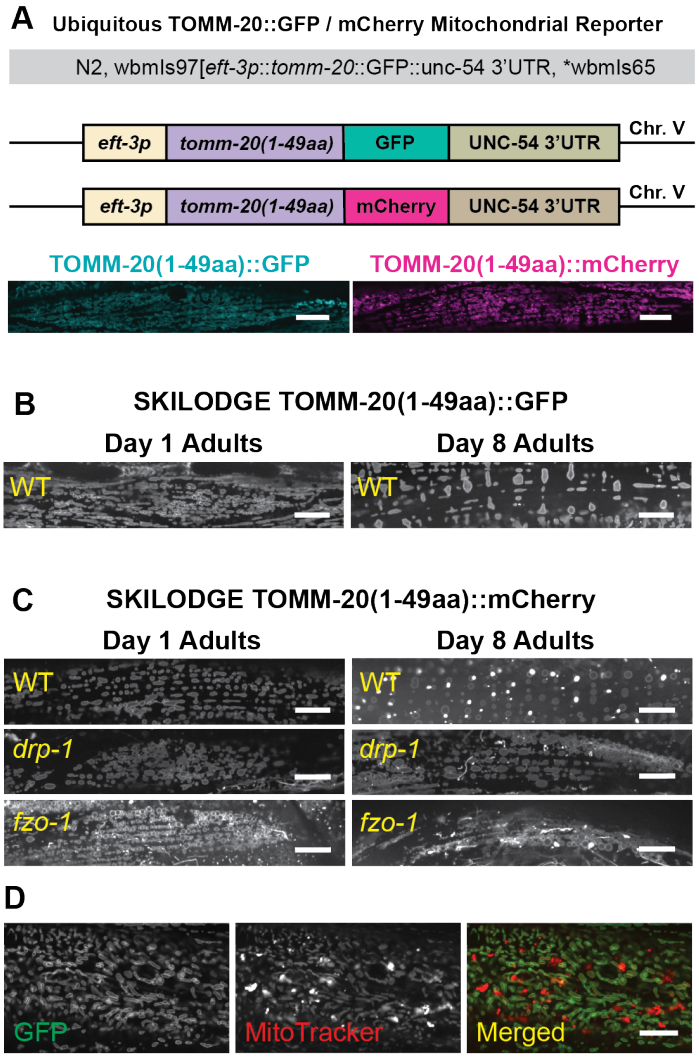


Figure 2.2: **Generation of new SKILODGE ubiquitous mitochondrial outer membrane TOMM-20(1-49aa) fluorescence reporters in *C. elegans* for quantitative microscopy.**
A. Schematic of mitochondrial TOMM-20(1-49aa) reporter with ubiquitous expression generated using the SKILODGE system. Representative confocal image of muscle mitochondria at taken at 100x. Scale bar 10 μm.
B. Representative images of ubiquitous mitochondrial outer membrane TOMM-20(1-49aa)::GFP generated using the SKILODGE method at Day 1 and Day 8. Day 8 mitochondria appear more fragmented compared to Day 1. Scale bar 10 μm.
C. Representatitve images of ubiquitous mitochondrial outer membrane TOMM-20(1-49aa)::mCherry generated using the SKILODGE method in WT, *drp-1* and *fzo-1* at Day 1 and Day 8. At Day 1, mitochondria in *drp-1* null are hyperfused compared to WT, whereas *fzo-1* null are hyperfragmented compared to WT. Age related fragmentation of mitochondria is seen at Day 8 in WT, but also in *drp-1* and *fzo-1* null. Scale bar 10 μm.
D. Representative images of SKILODGE *eft-3p::TOMM-20(1-49aa)::GFP* signal co-localizes with MitoTracker Red CMSRos in WT. Scale bar 10 μm.

ways previously reported in literature (Figure 2.2 B-C). To verify that the new *eft-3p::TOMM-20(1-49aa)::GFP* strain expressed GFP labeling specifically to mitochondria, I assessed co-localization of GFP with MitoTracker Red CMXRos, a cell permeable dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential. Mitochondria in the intestine showed GFP signal along the OMM and MitoTracker Red signal in the matrix, indicating that *eft-3p::TOMM-20(1-49aa)::GFP* was indeed labeling mitochondria (Figure 2.2 D).

To show that the new SKILODGE ubiquitous TOMM-20(1-49aa) reporter did not present overt phenotypic differences due to fluorophore or off-target toxicity, I measured their growth, development and fertility. The mito-GFP strain has been reported to have slight developmental delay and reduced brood size, likely due to both overexpression of multiple extrachromosomal copies of GFP(mit), as well as potential off-target gene effects from gamma irradiation as a method of integrating GFP(mit) randomly in the genome. The SKILODGE method I used involved precision gene editing with CRISPR at known intergenic regions to introduce only one copy of the *TOMM-20(1-49aa)::GFP* construct. It should therefore reduce these deleterious side-effects for development and fertility. Indeed SKILODGE *eft-3p::TOMM-20(1-49aa)::GFP/mCherry* strains showed no significant differences in developmental rates, brood size, and adult body size compared to wild type N2, whereas the mito-GFP strain showed significantly delayed development, reduced brood size and reduced body size (Figure 2.3 A-C).

Since the expression of TOMM-20(1-49aa)::GFP or mCherry in the SKILODGE strain is driven by a ubiquitous promoter, it does not show relative endogenous abundance of mitochondria in the worm. To generate a new tool that was able to show the endogenous expression levels of mitochondria, I used CRISPR to generate a strain expressing GFP on the C-terminus of endogenous outer membrane translocase TOMM-70 (Figure 2.4) (Paix et al., 2015). This strain showed relative levels of mitochondrial expression across different tissues, which was not possible in previous extrachromosomal strains or the SKILODGE strain.

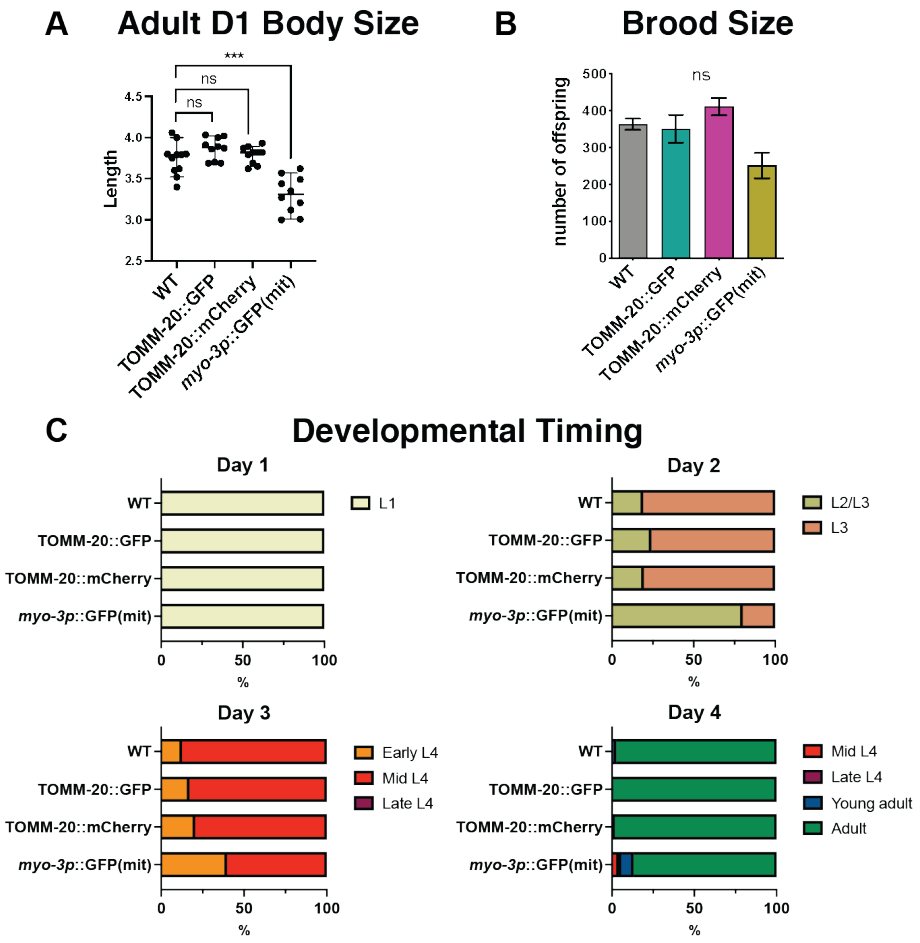


Figure 2.3: **SKILODGE mitochondrial fluorescence reporters do not show stunted growth, development and fertility as did pre-existing tools.**
A. Adult day 1 body size measurements in wild type and SKILODGE mitochondrial reporter strains (TOMM-20::GFP/mCherry) are not significantly different, but the pre-existing extrachromosomal mito-targeted GFP strain (*myo-3p*::GFP(mit)) is significantly smaller. (*** p = 0.0001 by Student's t-test, n = 2 biological replicates)
B. Brood size measurements in wild type, SKILODGE mitochondrial reporter strains, and pre-existing extrachromosomal mito-targeted GFP strain. (ns $p > 0.05$ by Student's t test, n = 1 biological replicate)
C. Developmental timing measurements in wild type, SKILODGE mitochondrial reporter strains, and pre-existing extrachromosomal mito-targeted GFP strain. WT and SKILODGE worms develop through L1-L4 larval stages to gravid adults at similar rates, but the *myo-3p*::GFP(mit) strain shows delayed development (n = 1 biological replicate).

Figure 2.4: **Generation of a new endogenous mitochondrial outer membrane TOMM-70 fluorescence reporter in *C. elegans* for quantitative microscopy.**
Representative images of endogenous mitochondrial outer membrane TOMM-70::GFP expression in various tissues, with highest expression in the gonad.

2.3 Validation of mitochondrial fluorescence reporters using two-photon microscopy to visualize mitochondrial architecture in *C. elegans*

To validate that the SKILODGE mitochondrial morphology reporters do not have artifacts due to insertion of a fluorescence tag, I used multiphoton imaging to visualize mitochondrial morphology in the absence of any exogenous labeling. This method relies on endogenous two-photon excited fluorescence (TPEF) and fluorescence lifetime (FLIM) of cellular NADH (nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide), which are naturally bioluminescent molecules (Liu et al., 2015). I captured TPEF and FLIM in young and old wildtype, *drp-1*, *fzo-1* and *drp-1;fzo-1* double mutants (Figure 2.5). Confocal images of wildtype, *drp-1* and *fzo-1* worms expressing the single copy insertion *eft-3p::TOMM-20(1-49aa)::mCherry* were also taken at Day 1 for morphometric comparison to NADH signal. Mitochondrial morphology of the TOMM20 labeled strain in each condition (wild type, hyperfragmented, hyperfused or adynamic) were comparable to that detected using purely endogenous mitochondrial fluorescence. These results support the advances of the new tool and show that the SKILODGE method of fluorescently labeling mitochondria does not disrupt organelle morphology at baseline. These pilot studies were done in collaboration with Irene Georgakoudi at the Tufts School of Biomedical Engineering. The combination of these optical measurements with other targeted approaches and metabolomics could potentially be used to detect both functional and structural information related to mitochondrial morphology and metabolic flexibility.

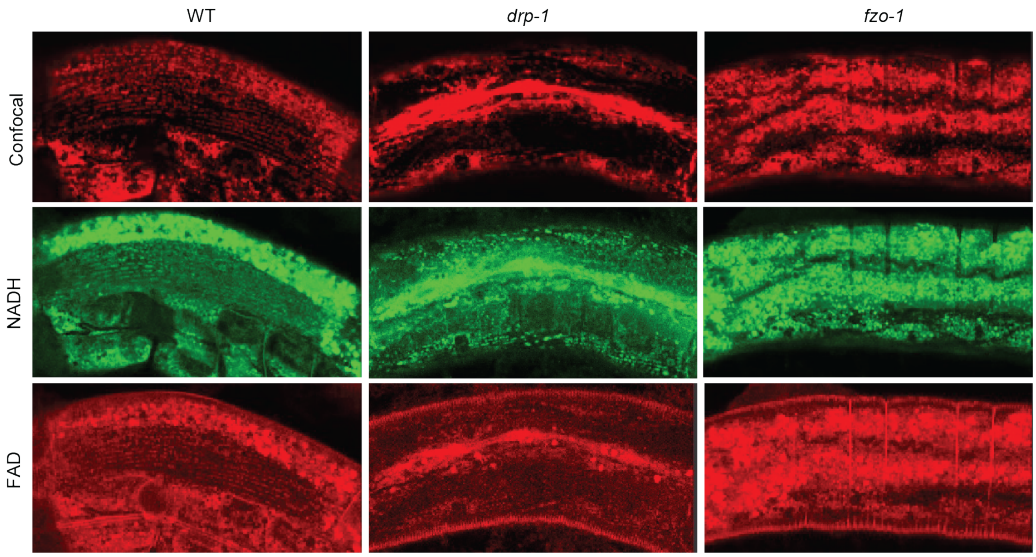


Figure 2.5: **Representative images of two-photon excited fluorescence signal from endogenous NADH and FAD signal, primarily arising from mitochondria.**
Top row shows confocal microscopy of mCherry-tagged outer membrane TOMM-20(1-49aa) fluorescence in WT, *drp-1* and *fzo-1* mutant animals. Mitochondrial network architecture is detectable using this non-labelled method and exhibits gross morphology comparable to that of fluorophore tagging methods.

2.4 Generating an *in vivo* functional reporter of mitochondrial fatty acid β-oxidation in *C. elegans*

We have previously observed that unique mitochondrial network architectures are required for different longevity pathways. How network morphologies affect the various functions of mitochondria in these longevity pathways is still not well understood. Since many longevity pathways in the worm are signaling networks involved in metabolic sensing, I first wanted to generate reporters that would give real-time readouts of changes in mitochondrial metabolism *in vivo* during aging. Many studies in *C. elegans* indicate that lipid metabolism lies at a central metabolic node of multiple longevity mechanisms. To generate an *in vivo* reporter of mitochondrial fatty acid oxidation, I targeted the mitochondrial enzyme acyl-coA synthetase-2 (ACS-2) in *C. elegans*. ACS-2 belongs to a family of acyl coenzyme A (CoA) synthetase (ACS) enzymes that catalyze the activation of free fatty acids (FAs) to CoA esters by a two-step thioesterification

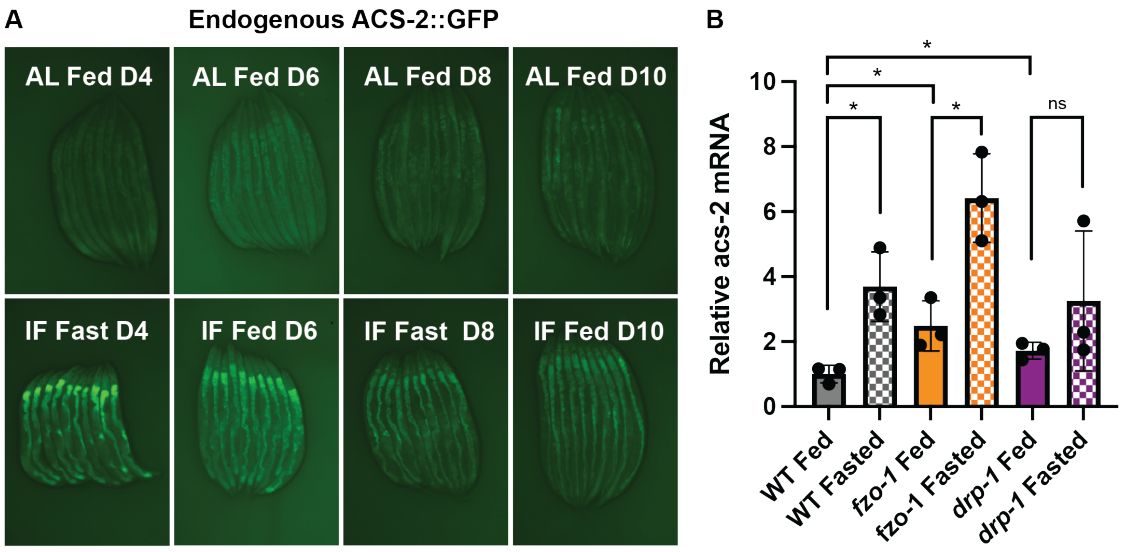


Figure 2.6: **An *in vivo* functional reporter of mitochondrial fatty acid oxidation shows induction of endogenous acs-2 expression upon fasting.**
A. ACS-2::GFP expression shows robust induction upon 48 hours of fasting at day 4. ACS-2::GFP in day 6 IF worms was reduced following 48 hours of feeding. IF worms maintain higher levels of ACS-2::GFP until day 10. Representative images are shown. (n=2 biological replicates)
B. qPCR showing relative *acs-2* mRNA following a 6h fast. *Fzo-1* animals showed higher basal and fasting-induced *acs-2* expression compared to WT and *drp-1* animals. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test; n=2 biological replicates)

reaction. Activated FAs participate in a variety of anabolic and catabolic lipid metabolic pathways, including *de novo* complex lipid biosynthesis, FA β-oxidation, and lipid membrane remodeling. Mitochondrial ACS-2 functions to activate fatty acids for transport into the mitochondrial matrix via carnitine palmitoyltransferase I (CPT-I) shuttle system for subsequent β-oxidation. Studies have shown that ACS-2 expression robustly increased upon fasting-induced fatty acid β-oxidation in *C. elegans* (Van Gilst et al., 2005; Ashrafi et al., 2003), suggesting that this would be a good enzyme to target as a reporter of increased mitochondrial FAO. I therefore used CRISPR to insert a C-terminal GFP at the endogenous *acs-2* locus. I showed that this reporter responded to regulation by fasting and refeeding (Figure 2.6). Through this work, I hope to show that *C. elegans* are a useful model to study changes in mitochondrial structure and function in an aging organism, and present the new tools developed to better study mitochondrial dynamics and organelle biology *in vivo*.

2.5 Peroxisome reporters and uncoupling mitochondrial and peroxisomal fission

Since regulation of mitochondrial and peroxisome dynamics are intimately coupled through their overlapping mechanisms of membrane fission, it is important to consider that manipulation of these factors will affect the morphology, and likely function, of both organelles. Studies that implicate a role for mitochondrial fission in aging by targeting DRP1 could, in fact, be due to peroxisome fission. Limited work has been done to delineate the contribution of mitochondrial and peroxisome fission to aging. In yeast *S. cerevisiae*, inhibiting fission via deletion of *Fis1* (present on both mitochondria and peroxisomes) increases chronological lifespan, and there is evidence to suggest that inhibition of peroxisome fission is a major contributor to these longevity effects. In yeast containing a *Fis1* deletion background, the authors reintroduced a *Fis1-Pex15* fusion construct that was directed specifically to peroxisomes. This effectively rescued peroxisome fission, but not mitochondrial fission, yet was sufficient to abolish longevity of the *Fis1* deletion mutant (Lefevre et al., 2015).

In previous publications, we observed that animals with null mutations in *drp-1* and *fzo-1* displayed altered mitochondrial and peroxisome morphology, where in both cases the organelles formed more elongated and tubular networks due to defective fission (Weir et al., 2017; Figure 2.7 A-B). The objective of these following experiments was to uncouple the regulation of peroxisomal fission from mitochondrial fission. While the majority of their fission machinery are shared, a few components are exclusive to each organelle. PEX11 is a peroxisome-specific membrane protein required for the initial phase of peroxisome fission. Previous studies in yeast and mammals showed that knockdown of PEX11 resulted in reduced number and larger peroxisomes. In mammals, there are three isoforms of PEX11 (α, β, γ); the single *C. elegans* homologue is PRX-11. I validated that *prx-11* RNAi impaired peroxisome fission in *C. elegans*, and resulted in larger peroxisomes that trend towards the morphologies observed on *drp-1* RNAi (Figure 2.7 C). Peroxisomes were visualized with a strain that expresses intestinal-specific GFP fused to a C-terminal Peroxi-

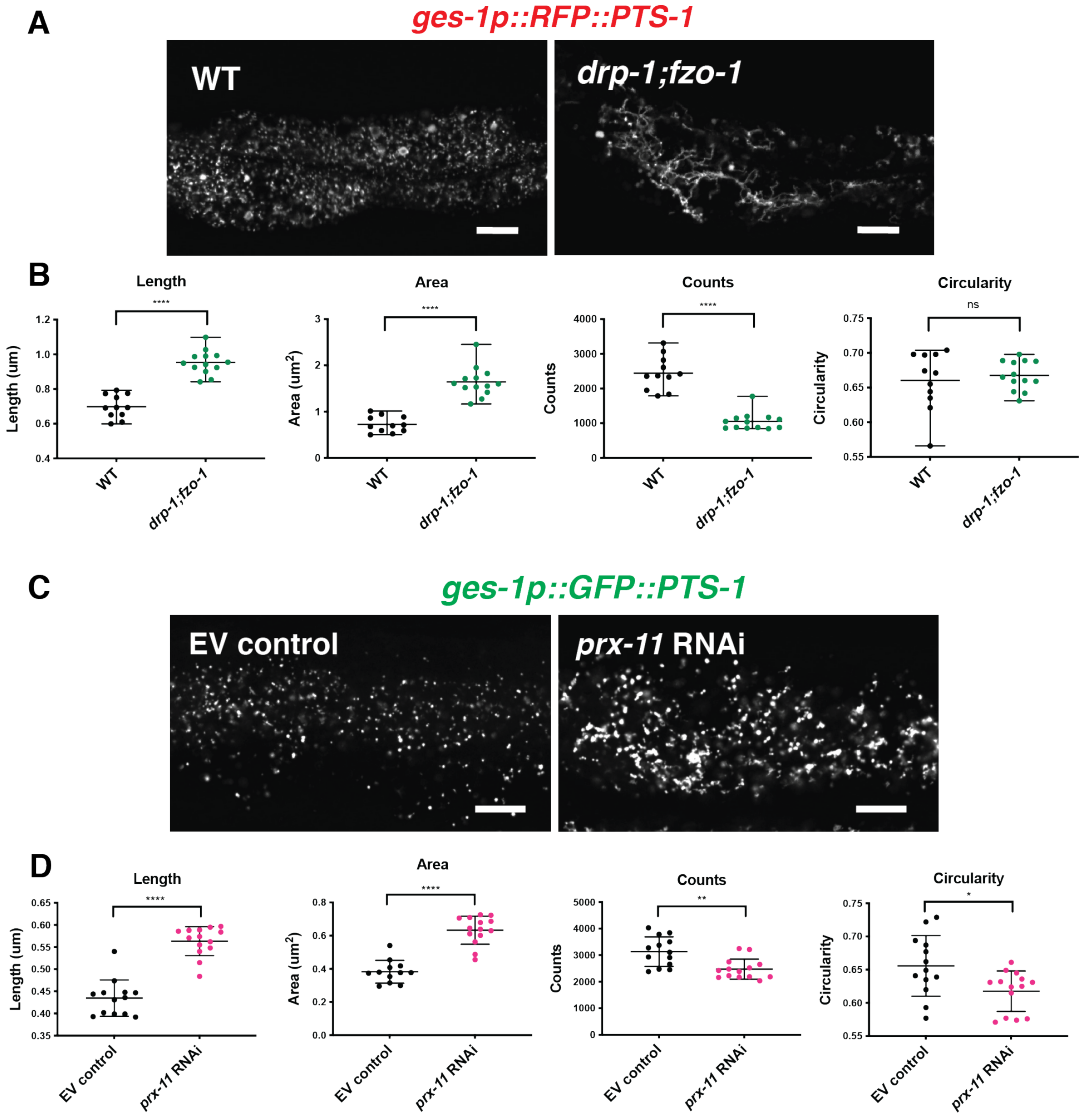


Figure 2.7: **Peroxisome morphology is elongated in *drp-1;fzo-1* mutants and *prx-11* knockdown.**

A. Representative images of peroxisomes in *drp-1;fzo-1* double mutant animals shows larger and hypertubular morphology compared to wild type. Scale bar 10μm.

B. Quantification of peroxisome length, size, counts, and circularity. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test with Welch's correction; n = 2 biological replicates)

C. Representative images show that knockdown of *prx-11* with RNAi resulted in enlarged and more tubular peroxisomes than those on control RNAi. Scale bar 10μm.

D. Quantification of peroxisome length, size, counts, and circularity. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test with Welch's correction; n = 2 biological replicates)

some Targeting Sequence (PTS). PTS1 is a three amino acid Serine-Lysine-Leucine signature that is recognized by the PEX5 membrane complex importing peroxisome-targeted proteins into the organelle matrix. Extrachromosomal arrays of intestinal *ges-1p::GFP::PTS1* were integrated into the genome using the Mos1-mediated single copy insertion (MosCI) system (Frokjaer-Jensen et al., 2008). Peroxisome morphology was quantified using a modified version of MitoMAPR, a macro tool we developed for ImageJ to quantify the mitochondrial network architecture (Y. Zhang et al., 2019). MitoMAPR was built using pre-existing ImageJ plugins, compiled in a single macro code that measures and collates data on the morphology of mitochondrial networks in *C. elegans*. For quantification of peroxisomes, the object masking radius was adjusted to identify particles within the 0.1-1 μm peroxisome size range. Using this program, I extracted quantitative attributes on peroxisomes, including area, length, count, and circularity (Figure 2.7 D).

Importantly, there was no significant effect of *prx-11* RNAi on mitochondrial morphology, whereas *drp-1* RNAi caused mitochondria to become hypertubular (Figure 2.8 A-B). Based on the promising result that knockdown of *prx-11* specifically affects peroxisome fission, I went on to generate a *prx-11* null mutant using CRISPR to make a clean deletion of the entire gene from start to stop codon and verified that like *prx-11* RNAi, the CRISPR *prx-11* null mutant displayed enlarged peroxisomes (Figure 2.8 C). I also generated a new peroxisome targeted GFP strain using the SKILODGE method, which provides the benefit of known single copy GFP insertion at a defined genomic intergenic loci, not previously possible using the MoSCI system.

For temporal and tissue-specific degradation of peroxisome import function, I utilized an Auxin-inducible degradation (AID) system in *C. elegans* (L. Zhang et al., 2015). I used CRISPR/Cas9 to introduce a 44aa degron tag to the N-terminus of the endogenous worm peroxisomal translocase PRX-5, a protein involved in the recognition and matrix import of peroxisome targeting sequence (PTS)-1 containing proteins. These animals were crossed into strains expressing the plant-derived components TIR1 (F-box protein of an E3 ligase complex from Arabidopsis). Upon exposure to the plant hormone Auxin, degron-tagged proteins are targeted for rapid proteasomal

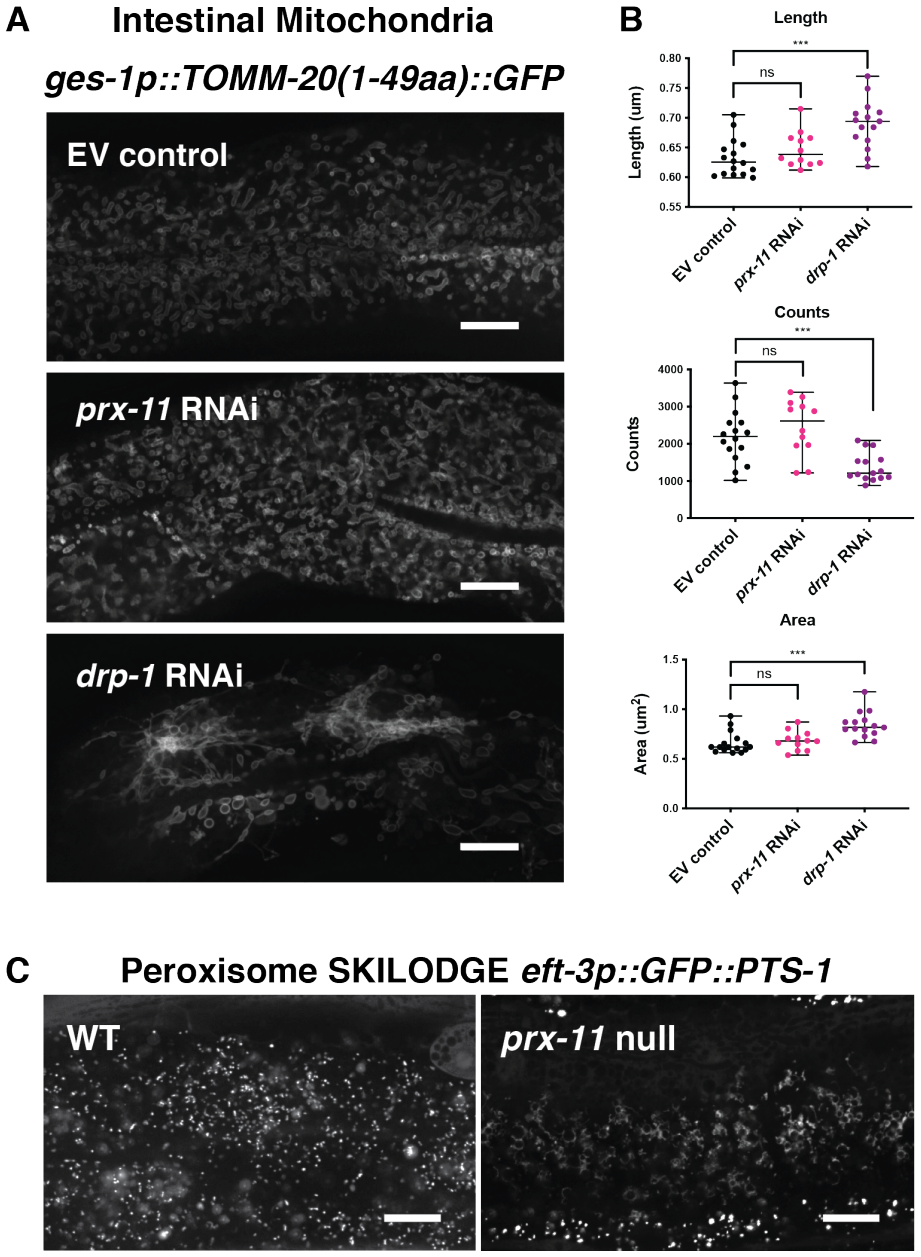


Figure 2.8: **Mitochondrial morphology is not affected by *prx-11* knockdown.**
A. Representative images show *prx-11* RNAi did not alter the shape of mitochondria, whereas *drp-1* RNAi significantly results in hypertubular mitochondria. Scale bar 10μm.
B. Quantification of mitochondrial length, size, and counts. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test with Welch's correction; n = 3 biological replicates)
C. CRISPR generated full *prx-11* null showed increased peroxisome size compared to WT. Representative images are shown using the newly generated SKILODGE *eft-3p::GFP::PTS-1*. Scale bar 10μm. (n=1 biological replicate)

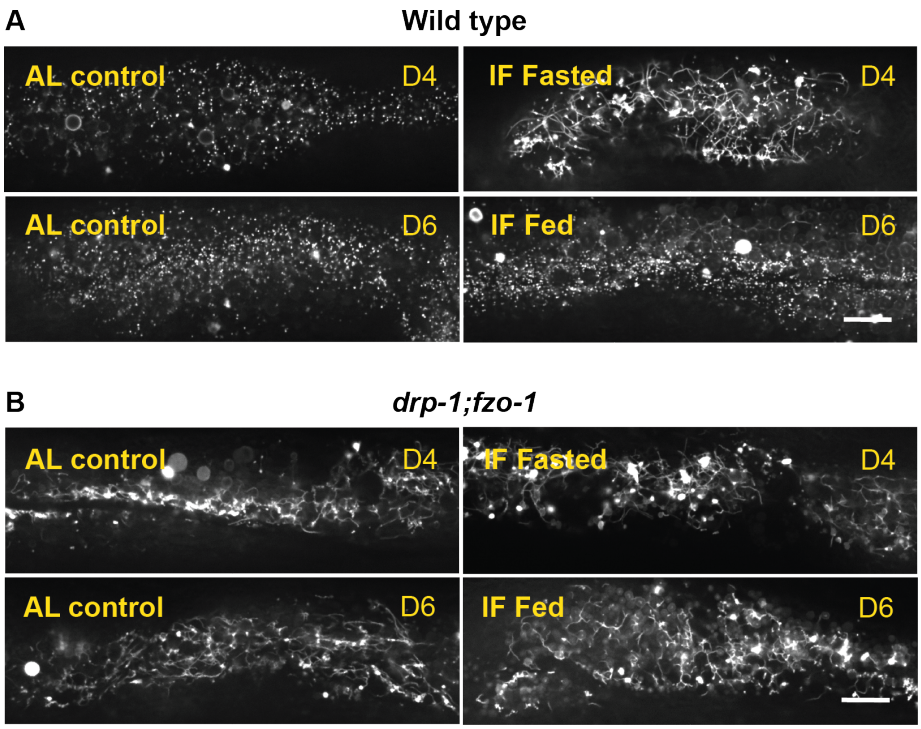


Figure 2.9: **Peroxisome morphology is dynamically remodelled by feeding and fasting.**
A. Peroxisomes in wild type animals under *ad libitum* fed conditions appear punctate in morphology. Upon 48 hours of fasting peroxisome morphology was remodelled to form hypertubular and elongated networks. Following 48 hours of refeeding, peroxisomes appeared punctate again, indicating that peroxisomal networks show dynamic remodelling in response to IF. Scale bar 10 μm.
B. Peroxisomes in *drp-1;fzo-1* double mutant animals appeared hypertubular as previously reported (Weir et al., 2017). Upon fasting, there was no further alteration in morphology. Scale bar 10 μm.

degradation. Importantly, proteasomal degradation is reversed within hours of auxin removal, enabling temporal control of degron-tagged protein expression. Tissue-specific control of PRX-5 can be achieved by crossing the *AID::prx-5* strain into strains expressing TIR1 under tissue-specific promoters. The strains we have available in the lab are neuronal (*rab-3p*), intestinal (*ges-1p*), germline (*pie-1p*), and muscle (*myo-3p*).

Very interestingly, I observed that peroxisomes were dynamically remodelled in response to Intermittent Fasting (Figure 2.9). Given my interest in understanding the mechanisms of

IF-longevity, I subsequently tested if peroxisomes were required for IF-longevity using a *prx-5(ku517)* mutant (R. Wang et al., 2013). *prx-5(ku517)* animals showed mildly blunted lifespan extension by IF compared to wild type control animals (Supplementary Figure A.4). The *prx-5(ku517)* mutation is a C>T point mutation in the C-terminal coding region of *prx-5*. While these observations hint that peroxisomes may be involved in IF-longevity, further studies are necessary. The new *AID::prx-5* strain will be particularly useful for robust degradation of PRX-5 to elucidate its potential role in IF longevity. These studies are beyond the scope of my thesis work, but are active research pursuits in the lab.

2.6 Mitochondrial dynamics are affected by antibiotics

In developing tools and methods to better dissect the role of organelle dynamics in aging pathways in the model organism *C. elegans*, one important factor to consider is the use of antibiotics. *C. elegans* are bacterivore nematodes that interact with a variety of bacterial species in their natural environment (Frezal and Felix, 2015). In the laboratory setting, *C. elegans* are raised on agar petri plates seeded with bacterial monocultures. The standard bacterial food source is the *Escherichia coli* B strain OP50; its use was advocated by Sydney Brenner as it grows in thin lawns and allows easier visualization of worms for experimental purposes. Maintenance of *C. elegans* in the laboratory standardly includes the removal of spontaneously occurring microbial contaminants to limit experimental variations, since diet, including the presence of contaminants, has been shown to exert potent influences over animal physiology, such as development, reproduction, healthspan, and longevity. This is routinely achieved by use of a single or combination of antibiotics. Bacterial food sources have also been engineered to carry specific resistance for selection, and this is particularly relevant for RNA interference studies in *C. elegans*, where worms are fed RNAi-containing bacteria. Carbenicillin is always present in these studies for the purpose of ensuring that only RNAi or control bacteria carrying carbenicillin resistance are present.

However, many antibiotics widely used in research have profounds effect on mitochondrial function and dynamics, which ultimately affect cell viability, metabolism, and organismal physiology. Considering the proteobacterial origin of the eukaryotic mitochondria (Margulis, 1975), and the similarity of ribosomal machinery between bacteria and mitochondria, it is not surprising that antibiotics that inhibit bacterial protein synthesis also have powerful inhibitory effects on mitochondrial translation. Several studies have demonstrated that tetracyclines interrupt mitochondrial proteostasis in round worms, fruit flies, mice and human cell lines. Tetracyclines are potent inhibitors of mitochondrial translation in rat heart and liver (McKee et al., 2006). Another study showed that Linezolid and other a ribosomal-targeting antibiotics perturbed mitochondrial translation in differentiating T-cells (Almeida et al., 2021). Doxycycline was also shown to disturb mitochondrial proteostasis and induce mito-nuclear protein imbalance in mouse and human cells, causing major changes in mitochondrial function (oxygen consumption rate) and mitochondrial dynamics (induced fragmented mitochondria) (Moullan et al., 2015). In *C. elegans* and *D. melanogaster*, doxycycline not only impaired mitochondrial function, but also impaired development (Moullan et al., 2015). In *C. elegans*, chloramphenicol induced mitonuclear imbalance, activated UPR(mt) and reduced mitochondrial respiration (Houtkooper et al., 2013). In addition to antibiotics that directly target the mitochondrial ribosome, a number of antibiotics including b-lactams (i.e. ampicillin) and aminoglycosides (i.e. kanamycin), induced oxidative stress via depletion of NADH in bacterial and mammalian cells (Kalghatgi et al., 2013).

Considering the evidence presented in these studies, I made an important re-evaluation of the protocol we were using in the laboratory for intermittent fasting in *C. elegans*. Previously, we had performed IF in *C. elegans* by transferring worms to plates containing (0.1 mg/ml) carbenicillin with no food every 48 hours during the Fasting interval of IF (Weir et al., 2017). Worms fed ad libitum were not exposed to carbenicillin. It was observed that the combination of Fasting + carbenicillin induced greater mitochondrial network fragmentation, compared to Fed controls. I replicated this result using the SKILODGE TOMM-20(1-49aa)::GFP reporter strain (Figure 2.10

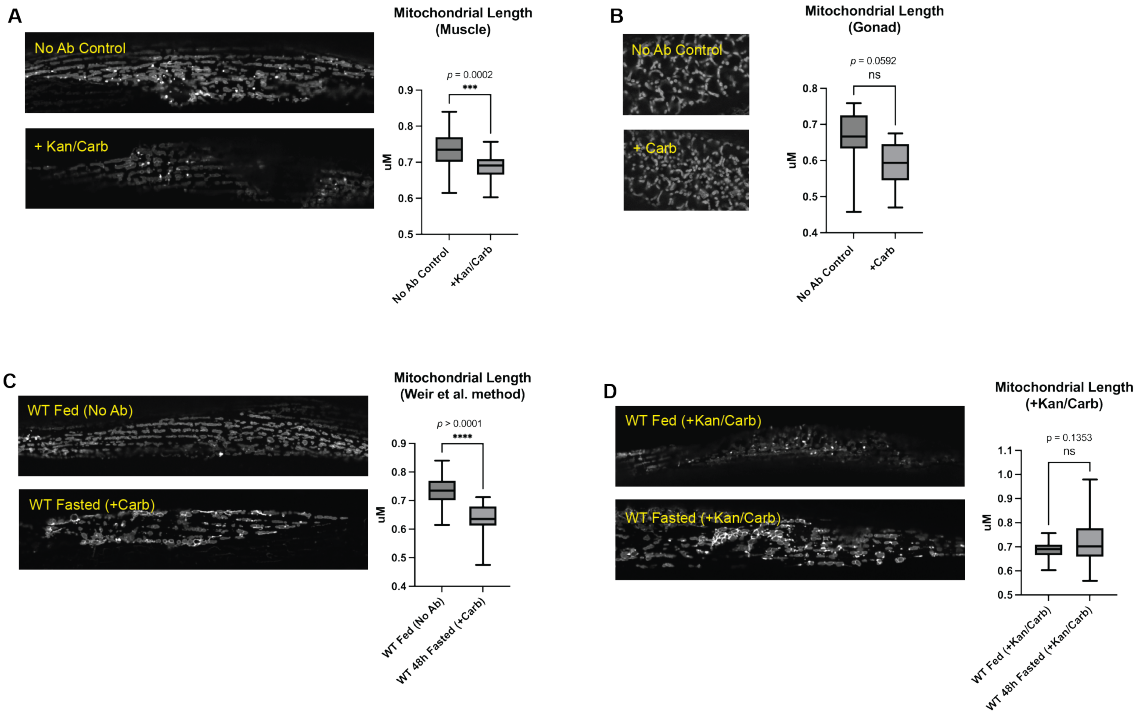


Figure 2.10: **Antibiotics affect mitochondrial morphology in muscle and gonadal cells of *C. elegans*.**
A. Mitochondria in muscle cells of animals exposed to a combination of the antibiotics kanamycin (0.05 mg/ml) and carbenicillin (0.1 mg/ml) for 48 hours were significantly more swollen or fragmented compared to control animals that were not exposed to antibiotics. Representative images are shown for minimum n = 15 images across 20-30 animals. (***p = 0.0002 by Unpaired t test with Welch's correction). B. Mitochondria in the gonad of animals exposed to carbenicillin (0.1 mg/ml) for 48 hours appeared, with trending towards significance, more swollen or fragmented compared to control animals that were not exposed to antibiotics. Representative images are shown for minimum n = 15 images across 20-30 animals. (*p < 0.01 by Unpaired t test with Welch's correction). C. Mitochondrial morphology of 48h-Fasted worms were significantly more fragmented (measured in length) than mitochondria in Fed worms when only the Fasted group was exposed to Carbenicillin. Wild type worms that were 48h-Fasted (and exposed to 0.1 mg/ml carbenicillin) had significantly more fragmented muscle mitochondria compared to worms that were Fed (without exposure to antibiotics). This data replicates the imaging findings observed during IF in Weir et al., 2017. This method of IF was also used for lifespan analyses in Weir et al., 2017. Representative images are shown for minimum n = 15 images across 20-30 animals. (****p < 0.0001 by Unpaired t test with Welch's correction). D. Mitochondrial morphology of 48h-Fasted worms were not significantly different from Fed worms when both groups were exposed to Kanamycin and Carbenicillin. Wild type worms that were 48h-Fasted (and exposed to 0.05 mg/ml kanamycin and 0.1 mg/ml carbenicillin) showed no significant change in mitochondrial morphology compared to worms that were Fed (and exposed to 0.05 mg/ml kanamycin and 0.1 mg/ml carbenicillin). Representative images are shown for minimum n = 15 images across 20-30 animals. (ns, p = 0.1353 by Unpaired t test with Welch's correction)

C). To test whether the presence of carbenicillin itself induced mitochondrial fragmentation, I evaluated mitochondrial morphology in the gonad using a TOMM-70::GFP reporter. There was a trend towards mitochondria with shorter length, indicating greater fragmentation, with a *p*-value of 0.0592 (Figure 2.10 B). A combination of kanamycin (0.05 mg/ml) and carbenicillin (0.1 mg/ml) is routinely used for solid plate dietary restriction experiments in *C. elegans* to arrest bacterial growth. I examined mitochondrial morphology in the presence of kanamycin and carbenicillin in muscle using the SKILODGE TOMM-20(1-49aa)::mCherry reporter and observed that mitochondria exposed to kanamycin and carbenicillin were significantly more fragmented compared to the control with no antibiotics (Figure 2.10 A). I also examined mitochondrial morphology in Fed versus 48h-Fasted animals exposed to kanamycin and carbenicillin across both groups, and observed no significant difference in mitochondrial network morphologies (Figure 2.10 D). Together, these data suggest that kanamycin and carbenicillin induce mitochondrial fragmentation in *C. elegans*. There is a clear need to define cleaner research tools that do not affect the function of mitochondria, as well as greater awareness of the undesired confounders caused by using antibiotics. The use of antibiotics should be carefully considered in research on mitochondrial biology and longevity in *C. elegans*.

2.7 Discussion

There is immense room for development of improved tools to study organelle biology and inter-organelle communication within the field of aging. Here I presented several improved fluorescence markers of mitochondrial membranes using targeted gene-editing strategies that enable single copy insertion of fluorophores at defined loci. This strategy is a significant improvement from extrachromosal methods because it allows stable and uniform expression within an isogenic population of worms, which is important for quantitative assessment of optical microscopy. It also improves upon gamma irradiation or MosCI methods of integrating edits into the *C. elegans* genome as it has less off-target effects, and bypasses the need for co-selection

with other phenotypic markers such as the *unc-119* mutation. I also generated a mitochondrial marker by using CRISPR to tag endogenous *tomm-70* in *C. elegans*. This strain reliably shows levels of endogenous TOMM-70 expression across various tissues in the worm. Although fluorescence levels are significantly lower than the SKILODGE reporters I generated, when used in combination with confocal or other super-resolution microscopy techniques, gives useful quantitative information on mitochondrial morphology. It is particularly useful for examining mitochondrial structure in the gonad of the worm, which contains 60-70% of the total mitochondria in *C. elegans*. As one method to validate that these new fluorescent reporters accurately label mitochondrial membranes and that insertion of the fluorophore does not disrupt mitochondrial morphology, I performed two-photon microscopy of endogenous NADH and FAD fluorescence in *C. elegans* muscle mitochondria. This was able to capture attributes of mitochondrial morphology in a live animal without the use of any exogenous fluorophore tags. These images showed comparable mitochondrial organization in muscle cells compared to the SKILODGE tools I generated, providing insurance that the SKILODGE tools accurately reflect mitochondrial networks in the worm. These studies also open the possibilities for application of two-photon microscopy for morphometric studies of mitochondria *in vivo* in the worm, as well as acquisition of redox balance and metabolism in *C. elegans* mitochondria. In-depth analysis of two-photon miscropy in *C. elegans* is out of the scope of my thesis, but is an area that shows promise for future development. Further validation of these tools would be made stronger by additional experiments showing co-localization of SKILODGE *eft-3p::TOMM-20(1-49aa)::GFP* or *mCherry* signal with other known mitochondrial localized proteins or mtDNA.

In addition to improved tools to investigate structural and functional questions related to mitochondrial dynamics and aging, there is now growing evidence for the contribution of peroxisomes to these mechanisms. Previous work I contributed to at the beginning of my PhD showed that peroxisomes are involved in AMPK and DR longevity. To ask whether peroxisomes are required for DR and IF longevity, it was necessary to develop better fluorescence reporters of peroxisome morphology in *C. elegans*, as well as methods to target peroxisome dynamics, independently

from mitochondrial dynamics. I generated a new peroxisome reporter using the SKILODGE system– GFP-targeted to peroxisomes, integrated in single copy under the ubiquitous *eft-3p*. Although previous versions of such a tool in *C. elegans* exist– an intestinal promoter-driven peroxisome targeted GFP generated using the MosCI system– the new tool I generated is integrated in single copy and driven by a ubiquitous promoter, which allows visualization of peroxisomes in intestine and other tissues. My new peroxisome reporter was used by a collaborator to define the role of peroxisome-lipid droplet signaling (Brunet et al., 2021), and its application will be widely useful for other future studies. I then targeted the peroxisome-specific fission protein PRX-11, and showed that RNAi knockdown of *prx-11* dampened peroxisome fission mechanisms and resulted in larger and fewer peroxisomes. Importantly, RNAi knockdown of *prx-11* did not significantly perturb mitochondrial morphology, suggesting that targeting *prx-11* is a valid approach to manipulate peroxisome dynamics without affecting mitochondrial dynamics. I then generated a new null mutant of *prx-11* as a model of inhibited peroxisome fission. Although application of this strain for its functional role in IF, DR or other longevity pathways is out of the scope of my thesis, this strain is actively being used by others in the lab for follow up research on the contribution of peroxisome dynamics to aging. This chapter summarizes the new tools I developed for the field of cell biology and aging. I have also used these tools to aid in the research questions to follow in Chapter 3.

2.8 Methods

RNA interference (RNAi) in *C. elegans*

There are three ways to carry out RNAi in *C. elegans*: injection (Fire et al., 1998), soaking (Tabara et al., 1998), and feeding (Timmons and Fire, 1998). In this study, RNAi knockdown of genes was performed in *C. elegans* via feeding with bacteria engineered to express double-stranded RNA (dsRNA) targeted to degrade mRNAs of specific genes of interest (Sijen et al., 2001; Timmons and

Fire, 1998). This method uses the *E. Coli* strain HT115 strain which lacks RNaseIII, an enzyme that degrades most dsRNAs. Two genome-scale RNAi libraries currently available, generated by Julie Ahringer lab (Ahringer, 2006) and Marc Vidal lab (Rual et al., 2004). The Ahringer library was made from genomic DNA; the Vidal library was constructed from the worm ORFome.

All RNAi used in this study were obtained from the Ahringer and Vidal library. The appropriate bacteria were streaked out and sequence-verified to contain fragments of target genes before use. RNAi experiments were done using *E. coli* HT115 bacteria on standard NGM plates containing 100 μg/ml Carbenicillin. HT115 bacteria expressing RNAi constructs were grown overnight in LB supplemented with 12.5ug/ml Tetracycline and 100 μg/ml Carbenicillin. Plates were seeded with 100 μl of the bacterial culture 48 hours before use. At least 1-2 hours before introducing worms to the plate, 100 μl IPTG (100mM) solution was added to the bacterial lawn to induce dsRNA expression. RNAi was induced from hatch or at Day 1 of adulthood as specified. Worms grown on empty vector HT115 RNAi bacteria are represented as control.

Genotyping

Worms were individually lysed in 5 μl single worm lysis buffer (30 mM Tris pH 8, 8 mM EDTA, 100 mM NaCl, 0.7% NP40, 0.7% Tween-20), with Proteinase K added to 100 μg/ml just before use. Incubate worm in lysis buffer for 60 minutes at 60 °C then at 15 minutes at 95 °C to inactivate proteinase K. 1 μl lysate was used as template for PCR reactions with the respective genotyping primers that detect bands of different sizes for distinguishing wild type and mutant alleles. PCR products were visualized on 1-2% agarose gels, run at 90-110V, depending on the expected band sizes.

Microinjection

Microinjection technique was used to introduce foreign DNA/RNA/protein to modify the *C. elegans* genome. Transgenic animals were generated by introduction of extrachromosomal

arrays or by CRISPR- Cas9 genome editing. Glass capillaries were heated and pulled to generate a pointed tip using a flaming/brown micropipette puller (Sutter Instrument, P-1000). Miniprepped DNA or CRISPR mixes were made and centrifuged to separate any needle-clogging particle or debris from the DNA solution. 0.5 μl-1 μl of DNA solution was loaded to each needle. Worms at the young adult to gravid day 1 adult stage with distinct gonads were selected for microinjection. Worms were mounted on a 2% agarose pad with a drop of halocarbon oil. The mix was injected into the gonads using FemtoJet Express (Eppendorf) following standard procedures (Evans, 2006). Following injection, the animals were recovered in M9 and each individual animal that is injected is isolated into a new plate to monitor transgenic progeny.

CRISPR-Cas9 genome editing

All CRISPR edits were performed using the CRISPR protocol developed by (Paix et al., 2015). Homology repair templates were amplified by PCR using primers that introduced a minimum stretch of 35 bp homology arms flanking the site of insertion at both ends. The CRISPR injection mix contained 2.5 μl tracrRNA (4 μg/μl), 0.6 μl dpy-10 crRNA (2.6 μg/μl), 0.5 μl target gene crRNA (2.6 μg/μl), 0.25 μl dpy-10 ssODN (500 ng/μl), homology repair template (200 ng/μl final in the mix), 0.375 μl Hepes pH 7.4 (200mM), 0.25 μl KCl (1M) and RNase free water to make up the volume to 8 μl. 2 μl purified Cas9 (12 μg/μl) was added at the end, mixed by pipetting, spun for 2 min at 13000 rpm and incubated at 37 °C for 10 minutes. *dpy-5* was used as a co-injection marker instead of *dpy-10* in case the edits were made on Chromosome II. Mixes were microinjected into the germ line of day 1 adult hermaphrodites using standard protocol. Screening worms and genotyping was performed as described earlier (Silva-Garcia et al., 2019). Worms generated using CRISPR were outcrossed at least six times before being used for experiments to remove the co-injection marker phenotype and other off-target edits.

Table 2.1: **crRNA constructs used to generate new strains**

crRNA name	**Target gene**	**Sequence 5' > 3'**
dpy-10 crRNA	*dpy-10*	gctaccataggcaccacgag
dpy-5 crRNA	*dpy-5*	ccaggaatgccaggaccacg
acs-2 crRNA	*acs-2*	agctgtcgacaattaaaatc
fzo-1 N-term crRNA	*fzo-1*	ttaagttttcaggtaatgtc
drp-1 N-term crRNA	*drp-1*	aaaattttaaattttacaga
prx-5 N-term crRNA 2	*prx-5*	ggacagcagaatgctcttgt
prx-11 N-term crRNA	*prx-11*	atgaagagagtactgtagtg
prx-11 C-term rcrRNA	*prx-11*	cgaagccaacaatgaaaacg
tomm-70 C-term crRNA	*tomm-70*	aaaaaaaacggggaaatgag

Body size measurements

Worms were synchronized by a 1-hour egg lay on OP50-1 seeded NGM plates. 3 days later, Day 1 adult worms picked onto a fresh NGM plate and anesthetized with sodium azide. Sodium azide anesthetizes worms immediately and causes body to become rigid, allowing better measurements of body length. Worms were imaged on a Zeiss Discovery V8 dissection microscope with an Axiocam camera. Length of worms was measured in FIJI from tip of nose to tail.

Developmental timing measurements

To measure developmental timing, animals were synchronized at the L1 larval stage. Synchronizing at the L1 stage allows for better controlled experiments than measuring from eggs, since ex utero egg development in C. elegans can take up to 9 hours until hatching. To obtain synchronized L1s, animals were bleached and embryos were incubated overnight at 20 °C in 1X M9 buffer and gentle rocking to allow hatching. After overnight incubation, OP50-1 plates were seeded with L1 larvae. Developmental stages were evaluated every 24 hours under a dissecting scope and scored for frequency of each developmental stage present.

Confocal microscopy

Worms were anesthetized in 0.1 mg/ml tetramisole in 1X M9 buffer on empty NGM plates

and mounted on thin 2% agarose pads on glass slides with 0.05 mm Polybead microspheres (Polysciences) for immobilization. A No. 1.5 cover glass was gently placed on top of worms and sealed with clear nail polish. Images were performed on a Yokogawa CSU-X1 spinning disk confocal system (Andor Technology, South Windsor, CT) with a Nikon Ti-E inverted microscope (Nikon Instruments, Melville, NY), using a Plan-Apochromat 100x/1.45 objective lens. Images were acquired using a Zyla cMOS camera and NIS elements software was used for acquisition parameters, shutters, filter positions and focus control. Image analysis was performed using Fiji software.

Two-photon microscopy

Confocal or two photon excited fluorescence (TPEF) were acquired on a Leica (Wetzlar, Germany) DMIRE2 microscope with a TCS SP2 scanner. TPEF images (512 x 512 pixels; 238 x 238 mm) were acquired with 400Hz scanning frequency, a 63x/1.2NA or 40x (1.57x zoom)/1.1NA water immersion objective at 755 and 860nm excitation and with emission bands centered at 460 ± 20 and 525 ± 25 nm for NAD(P)H and FAD signal acquisition, respectively. Samples were prepared on glass microscope slides with a 1.5 cover glass at room temperature. TPEF fluorescence intensity images were normalized for photomultiplier (PMT) gain and laser power. Image processing was performed with the Leica Confocal Software and Matlab.

RNA isolation and cDNA synthesis

Total RNA was extracted using Qiazol (QIAGEN), column purified by RNeasy mini kit (QIAGEN) according to manufacturer's instructions. cDNA was synthesized using SuperScript®VILO Master mix (Invitrogen).

Quantitative RT-PCR

StepOne Plus instrument from Applied Biosystems was used to perform real-time qPCR exper-

iments following the manufacturer's instructions. For each qPCR reaction, 5ng of cDNA was used. Relative expression differences were calculated with the comparative $2\Delta\Delta$Ct method using Y45F10D.4 (Ce02467253_g1) as the endogenous control. For each gene in each strain, fold-change relative to the average of wild type control group was calculated and statistical significance evaluated using Welch's t test. GraphPad Prism 9 was used for all statistical analysis and graph plotting.

3 Mitochondrial plasticity is required for Intermittent Fasting mediated longevity

3.1 Introduction

Mitochondria are organized in dynamic and interconnected networks within the cell to support metabolic health. These network structures are regulated by trafficking, membrane fusion and fission, collectively termed 'mitochondrial dynamics'. Many studies have demonstrated the importance of both mitochondrial fusion and fission in aging. Importantly, these studies have highlighted that the relative requirement of fusion and fission for longevity vary by cell type, the technique used, the organism in which it is applied (Bernhardt et al., 2015; Weir et al., 2017), and the timing in which different mitochondrial functions are more or less necessary throughout an organism's lifetime (Rana et al., 2017).

Our lab previously showed that mitochondrial dynamics are required for different longevity pathways in a context-specific manner. In three separate genetic pathways that increase lifespan in *C. elegans*– constitutive activation of AMPK, reduction of mTORC1 signaling, and downregulation of the ATF-6 branch of the UPR– mitochondrial fusion and/or fission were differentially required to mediate longevity. In the AMPK pathway, both fusion and fission are required for lifespan

extension (Weir et al., 2017). In the mTORC1 pathway, only fusion is required for lifespan extension (Y. Zhang et al., 2019). In the ATF-6 pathway, only fission is required for lifespan extension (Burkewitz et al., 2020). This interesting observation suggests that there are distinct functional consequences of different mitochondrial network organizations, and they each contribute to the unique cellular milieu required during these longevity interventions in specific mechanisms. In *Drosophila*, induction of mitochondrial fission in midlife, but not in early adulthood, extended lifespan (Rana et al., 2017). Midlife upregulation of dMFN shortened lifespan (Rana et al., 2017). Moreover, most studies have shown that driving fusion or fission alone is not sufficient to increase lifespan and sometimes negatively affects health and longevity (Chaudhari and Kipreos, 2017; Weir et al., 2017). Pathologies that are associated with deletion of *drp-1*, *fzo-1*, or *eat-3* in *C. elegans* can often be restored by inhibiting the other (Byrne et al., 2019). Yeast cells deficient for fission and fusion (*dnm1;mgm1* double knockout) have healthy-looking mitochondria with a wild-type morphology, although these yeast do have a shortened lifespan (Bernhardt et al., 2015). In *C. elegans*, double *drp-1;fzo-1* knockouts also have healthy-looking mitochondria, but their simultaneous inhibition can prolong lifespan (Weir et al., 2017). In mice, independently inhibiting fission (Drp1 KO) or fusion (Mfn2 KO) in cardiac tissue led to mitochondrial disruption, cardiac pathology, and rapid lethality (Song et al., 2017). On the other hand, simultaneous inhibtion of fission and fusion (triple Drp1, Mfn1, and Mfn2 KO) in adult mouse hearts showed slower progression of cardiac pathology and an extended lifespan compared with either the Drp1 KO or the Mfn1/Mfn2 KO (Song et al., 2017). Similarly in another study, Mff/Mfn1 double mutants mice were alive and healthy after one year, whereas single Mff mutants died prematurely due to cardiomyopathy (H. Chen et al., 2015). The mechanisms and downstream functions underlying why unique mitochondrial states are required for different longevity pathways is a question in need of further investigation. Furthermore, why simultaneous inhibition of both fusion and fission improves longevity under some circumstances, but may be of detriment to stress resistance is not clear.

In my work, I observed there is contextual requirement of mitochondrial dynamics for two dietary interventions that extend lifespan in *C. elegans*: Dietary Restriction (DR) and Intermittent Fasting (IF). Dietary restriction, the chronic reduction in caloric intake without malnutrition, is the most well-studied method of promoting longevity in many species, including the nematode worm. Intermittent fasting, on the other hand, describes patterns of food intake that increase periods in which the organism is spent in a state of fasting, without overall deficit in caloric intake. The mechanisms of IF that promote longer lifespan and better healthspan are vastly understudied compared to chronic DR. There is some evidence to suggest that IF modulates longevity in mechanisms that are distinct from those of chronic DR, but many mechanisms remain to be elucidated. A study by Mitchell et al., 2019 directly tested the possibility that benefits of restricting the timing of food intake can be separable from those associated with overt caloric intake reduction or macronutrient composition. Compared to mice given *ad libitum* (AL) access to food at all hours of the day, mice that were given only one daily meal (MF) isocaloric to that of AL mice lived longer. The eating pattern of these two groups of mice differed only in their timing, where single-meal fed mice showed increased time spent in a fasted state (9 to 12 hours), and this was sufficient to significantly increase mean survival 11-14% compared to AL. The molecular mechanisms by which increased fasting promotes longevity and whether these pathways are independent or overlapping to chronic DR have not been described.

My results show that DR and IF longevity require mitochondrial dynamics in independent ways, providing new evidence that DR and IF increase lifespan in *C. elegans* via some non-overlapping pathways. It was previously shown that single inhibition of *drp-1* or *fzo-1* blocked sDR longevity (Weir et al., 2017). In this study, I showed that, in contrast to sDR, single inhibition of *drp-1* or *fzo-1* had no effect on IF longevity. Remarkably, co-inhibition of *drp-1;fzo-1* completely abolished IF longevity, whereas it rescued sDR longevity. Imaging of mitochondria during IF revealed that mitochondrial networks are remodelled in response to feeding and fasting. These changes did not occur in double *drp-1;fzo-1* mutants, suggesting that mitochondrial plasticity

was inhibited. I tested the hypothesis that plasticity at the level of mitochondrial networks was causal to lifespan extension by IF. Using an inducible system of mitochondrial fusion and fission protein degradation, I intermittently induced mitochondrial fission in 48-hour intervals to mimic mitochondrial network changes observed during IF. Intermittent induction of mitochondrial fission was specifically sufficient to extend lifespan, whereas no effect was seen on lifespan by intermittent induction of fusion, nor chronic midlife induction of fission. Transcriptomic profiling and downstream functional analyses indicated that co-inhibition of *drp-1;fzo-1* altered important metabolic adaptations to fasting, which may explain the loss of IF-longevity.

Results

3.2 Establishing a robust and reliable method of intermittent fasting in *C. elegans*

The mechanisms of IF-longevity have not been well studied and methodologies for performing IF in *C. elegans* with minimal confounding variables are lacking. I therefore began this project by optimizing a strategy of intermittent fasting in *C. elegans* that produced robust and reproducible lifespan extension. To perform IF, animals were synchronized and fed on standard OP50-1 *E. coli* throughout development. On Day 1 of adulthood, animals were exposed to 5-Fluoro-2'-deoxyuridine (FUDR), a uridine analogue routinely used, and necessary, to prevent larval development of *C. elegans* progeny under conditions of stress, such as fasting/ starvation. On Day 3 of adulthood, animals were subjected to 48-hour intervals of feeding and fasting until Day 13 (IF condition), or maintained on an *ad libitum* OP50-1 diet (AL condition). In wild type N2 animals, intermittent fasting significantly increased median and maximum lifespan compared to *ad libitum* controls (Figure 3.1 A). However, I noticed there were peculiar interactions between specific genotypes and FUDR. FUDR significantly extended lifespan in *a drp-1* null mutant (Figure 3.1 C). As a result, I sought to remove the variable of FUDR from the IF protocol, first by beginning IF at midlife (Day 6 of adulthood) following the reproductive period. I found that

the longevity effects of IF were not induced late-onset (Figure 3.1 B). I next turned to the auxin-inducible degradation (AID) system to induce sterility in *C. elegans*. This method was previously published: a 44 a.a. degron tag was introduced to *spe-44*, a gene expressed in germline at the onset of spermatogenesis (L. Zhang et al., 2015). Degradation of SPE-44 results in sterility upon simultaneous expression of germline-specific (*pie-1p*) TIR1, an F-box protein component of an E3 ligase complex derived from Arabidopsis that upon exposure to the plant hormone Auxin, targets degron-tagged proteins for rapid proteasomal degradation. I exposed animals to 0.15 mM auxin from hatch throughout development and showed that this concentration had no effect on wildtype *C. elegans* lifespan and retained robust lifespan extension by IF (Figure 3.1 D). In the auxin-inducible sterile strain that expresses *spe-44::degron* and germline TIR1, intermittent fasting also robustly extends lifespan (Figure 3.1 F). Considering my interest in understanding the role of mitochondrial dynamics in aging pathways, I chose to continue with the AID-sterility method (IF Method 3 in Figure 3.1 E) for subsequent studies.

Figure 3.1: **Methods of Intermittent Fasting in *C. elegans*.**

A. Intermittent fasting starting in early life (Day 3 of adulthood) extends lifespan in *C. elegans*. Method 1 of Intermittent fasting uses 5-fluoro-2'-deoxyuridine (FUDR), a chemical cell cycle inhibitor that prevents embryos from developing. FUDR is necessary during intermittent fasting to prevent death due to bagging in worms under fasting and/or starvation conditions. (****p < 0.0001 by log-rank (Mantel-Cox) test; n = 4 biological replicates)

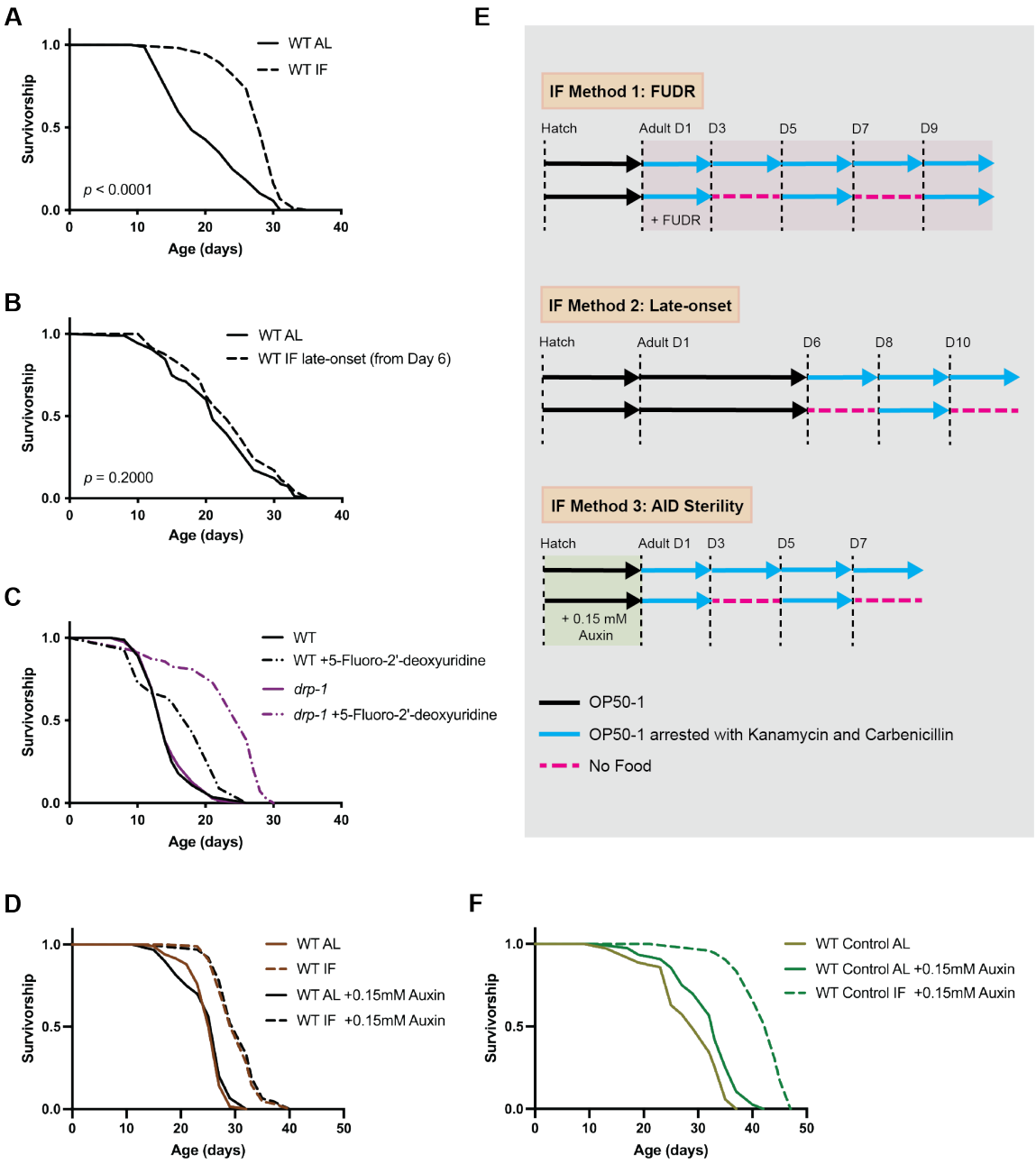
B. Intermittent fasting starting in midlife (Day 6 of adulthood) does not extend lifespan in *C. elegans*. (ns, p = 0.2000 by log-rank (Mantel-Cox) test; n = 2 biological replicates)

C. FUDR affects lifespan in different genetic backgrounds. Here I show that FUDR significantly extends the lifespan of *drp-1* null animals on a standard OP50-1 *E. coli ad libitum* diet. (****p < 0.0001 between *drp-1* +/- FUDR by log-rank (Mantel-Cox) test; n = 2 biological replicates)

D. Auxin at a concentration of 0.15 mM does not affect lifespan in wild type N2 animals and does not affect their ability to respond to the longevity effects of intermittent fasting. (ns, no significance between N2 +/- Auxin by log-rank (Mantel-Cox) test; n = 4 biological replicates)

E. Schematic diagram of three different methods of intermittent fasting in *C. elegans*, using FUDR, late-onset initiation of IF, and auxin inducible sterility.

F. Intermittent fasting starting in early life (Day 3 of adulthood) extends lifespan in auxin-inducible sterile animals, without the use of FUDR. 0.15mM Auxin treatment itself does not affect lifespan. (****p < 0.0001 between AL and IF, ns, no significance between +/- Auxin by log-rank (Mantel-Cox) test; n = 4 biological replicates)


A
WT AL
WT IF
Survivorship
p < 0.0001
Age (days)
B
WT AL
WT IF late-onset (from Day 6)
Survivorship
p = 0.2000
Age (days)
C
WT
WT +5-Fluoro-2'-deoxyuridine
drp-1
drp-1 +5-Fluoro-2'-deoxyuridine
Survivorship
Age (days)
D
WT AL
WT IF
WT AL +0.15mM Auxin
WT IF +0.15mM Auxin
Survivorship
Age (days)
E
IF Method 1: FUDR
Hatch
Adult D1
D3
D5
D7
D9
+ FUDR
IF Method 2: Late-onset
Hatch
Adult D1
D6
D8
D10
IF Method 3: AID Sterility
Hatch
Adult D1
D3
D5
D7
+ 0.15 mM Auxin
OP50-1
OP50-1 arrested with Kanamycin and Carbenicillin
No Food
F
WT Control AL
WT Control AL +0.15mM Auxin
WT Control IF +0.15mM Auxin
Survivorship
Age (days)

3.3 Mitochondrial dynamics are differentially required for longevity mediated by Intermittent Fasting and Dietary Restriction

Mitochondrial dynamics are required for many aging pathways and a body of evidence supports that membrane fusion, fission, and mitochondrial trafficking vary depending on the cell type and cellular context (See Chapter 1.10). The different shapes that mitochondria take on are intimately related to optimizing specific functions required in these various cellular states. **Here, I present some interesting new data to suggest that the mechanisms of DR and IF differ in their requirements for mitochondrial dynamics**. We know from past work that mitochondrial fusion and fission are both essential for DR mediated longevity in *C. elegans* (Weir et al., 2017). When DR was implemented using the solid plate method, it extended median and maximum lifespan robustly in wild type worms (Figure 3.2 A). Inhibition of either mitochondrial fission *drp-1* (Figure 3.2 B) or fusion *fzo-1* (Figure 3.2 C) alone abolished the lifespan extending effects of DR. Surprisingly, mitochondrial dynamics appeared to be dispensible for IF longevity. When IF was implemented using 48-hour cycles of alternate day fasting, it extended median and maximum lifespan of wild type worms (Figure 3.2 E). Inhibition of either *drp-1* (Figure 3.2 F) or *fzo-1* (Figure 3.2 G) had no effect on the robust lifespan extending properties of IF. These results support the prevailing notion that DR and IF modulate organismal aging in some nonoverlapping mechanisms (Chapter 1.6).

However, this outlook on the requirement of mitochondrial dynamics for DR and IF may be an oversimplified view. Previous studies in mice have shown that while single inhibition of both fusion and fission alone results in premature death and cardiac pathology, simultaneous fusion and fission co-inhibition can counteract these negative effects likely by re-establishing a balance in mitochondrial dynamics. **Intriguingly, when I simultaneously inhibited both fusion and fission, IF longevity was completely abolished** (Figure 3.3 B), **while remarkably, simultaneous inhibition of both fusion and fission had the completely opposite outcome for chronic DR. Simultaneous inhibition of *drp-1* and *fzo-1* completely restored DR longevity** (Figure 3.3 A).

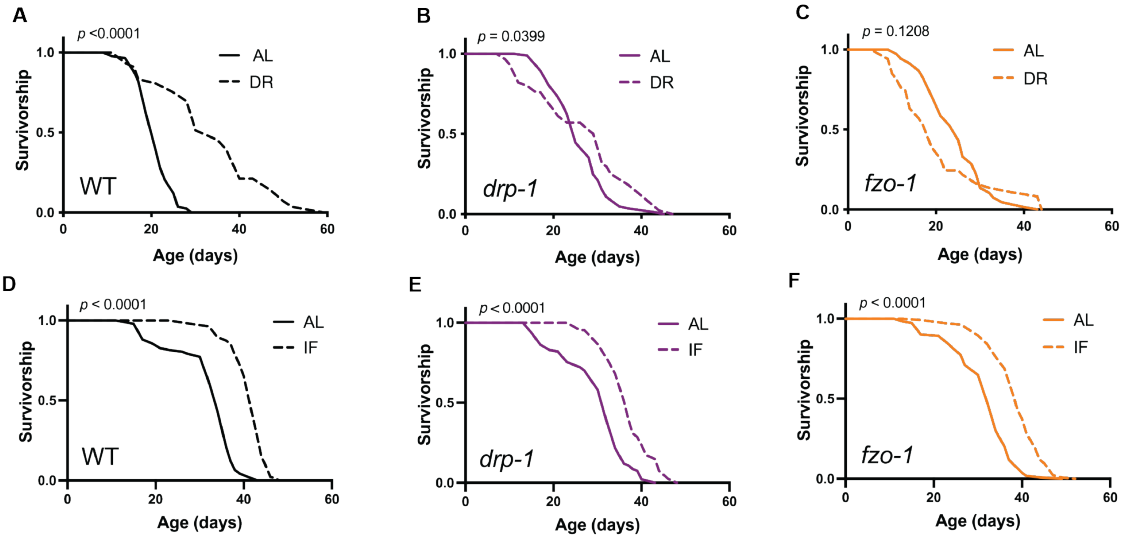


Figure 3.2: **Differential requirement of mitochondrial dynamics for longevity pathways mediated by Dietary Restriction and Intermittent Fasting.**
A. DR extends lifespan in WT animals. (****p < 0.0001 by log-rank (Mantel-Cox) test; n = 3 biological replicates)
B. Perturbing mitochondrial networks towards hyperfusion by genetic deletion of *drp-1* abolishes DR-longevity. (ns p > 0.05 by log-rank (Mantel-Cox) test; n = 3 biological replicates)
C. Perturbing mitochondrial networks towards hyperfragmentation by genetic deletion of *fzo-1* abolishes DR-longevity. (ns p > 0.05 by log-rank (Mantel-Cox) test; n = 3 biological replicates)
D. IF extends lifespan in WT animals. (****p < 0.0001 by log-rank (Mantel-Cox) test; n = 4 biological replicates)
E. *drp-1* is dispensible for IF-longevity. Perturbing mitochondrial networks towards hyperfusion by genetic deletion of *drp-1* has no significant effect on lifespan extension mediated by IF. (****p < 0.0001 by log-rank (Mantel-Cox) test; n = 4 biological replicates)
F. *fzo-1* is dispensable for IF longevity. Perturbing mitochondrial networks towards hyperfragmentation by genetic deletion of *fzo-1* has no significant effect on lifespan extension mediated by IF. (****p < 0.0001 by log-rank (Mantel-Cox) test; n = 2 biological replicates)

These results indicate that mitochondrial dynamics are indeed important for both DR and IF longevity, however the differences may lie in requirements for balanced mitochondrial network organization versus mitochondrial plasticity.

My hypothesis is that co-inhibition of fusion and fission restores the balance in mitochondrial network organization, however it leads to loss of mitochondrial plasticity. Mitochondrial

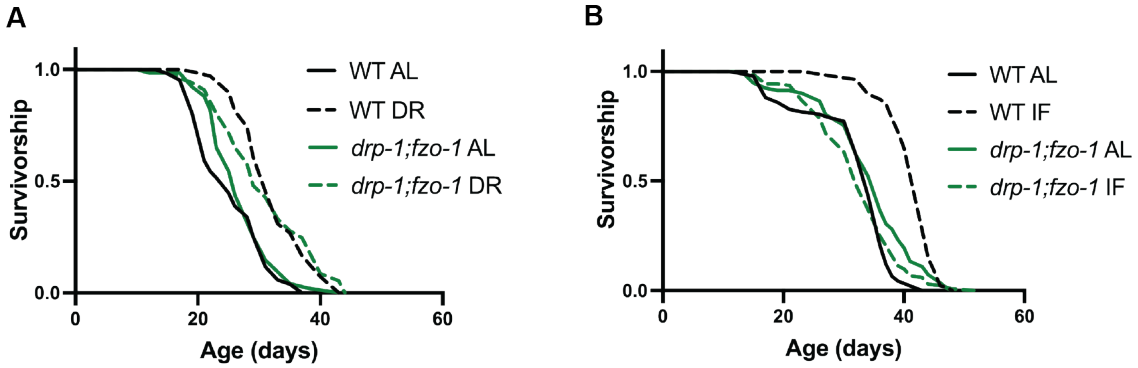


Figure 3.3: **Co-inhibition of mitochondrial fusion and fission specifically abolishes IF-longevity.**
A. Co-inhibition of both fusion (*fzo-1*) and fission (*drp-1*) remarkably de-represses the lifespan abolishing effects of each of the single mutants. Double *drp-1;fzo-1* animals respond to the full lifespan extending effects of dietary restriction. (****p < 0.0001 by log-rank (Mantel-Cox) test; n = 2 biological replicates)
B. Co-inhibition of both fusion (*fzo-1*) and fission (*drp-1*) completely abolishes IF-mediated longevity. (****p < 0.0001 by log-rank (Mantel-Cox) test; n = 2 biological replicates)

plasticity is specifically required for metabolic adaptations to fasting during IF longevity. Animals on IF are presented with acute energetic challenges between high-nutrient availability during feeding and complete nutrient deprivation during fasting. Plasticity in the mitochondrial network may be critical to adapt to fluctuating energetic states. Conversely, there are no acute changes to nutrient supply and demand during chronic DR, and maintenance of a balanced mitochondrial network may be preferred.

3.4 Mitochondrial networks are remodeled in response to cycles of feeding and fasting

To test the hypothesis that mitochondrial plasticity is an important mechanism of IF, I proceeded to observe how mitochondrial networks respond to cycles of feeding and fasting *in vivo*. I used the SKILODGE ubiquitous TOMM20(1-49aa)::GFP reporter to assess mitochondrial morphology periodically on days 4, 6, 8 and 10 of intermittent fasting. In the previous chapter, I showed evidence that mitochondrial dynamics are sensitive to antibiotics (Figure 2.10). I therefore modified

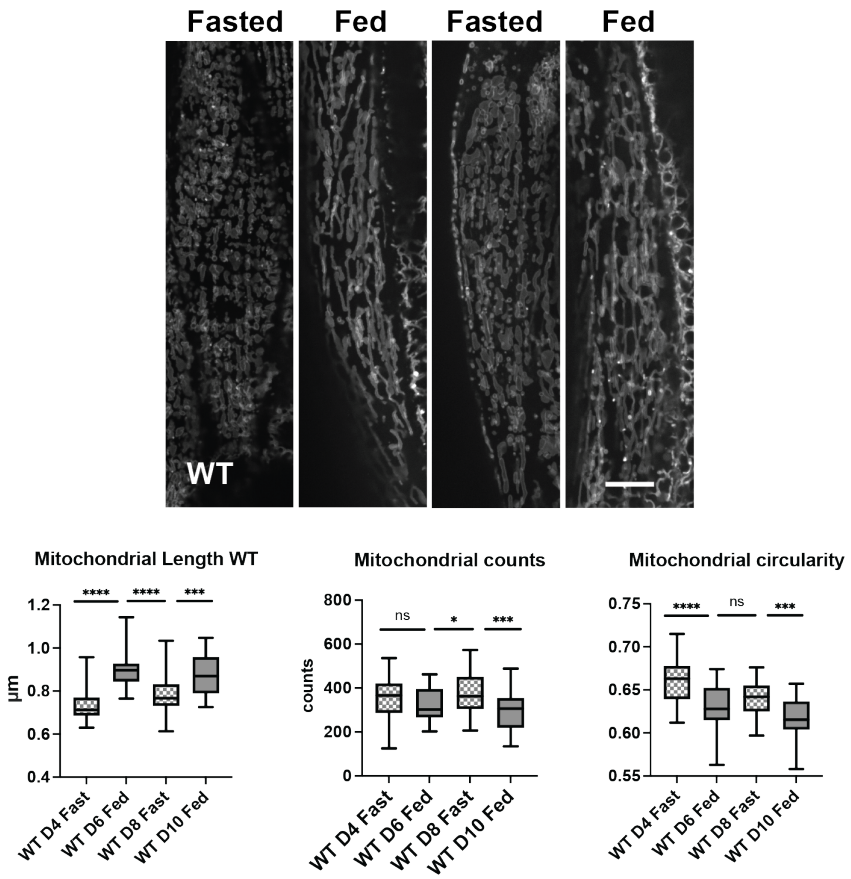


Figure 3.4: **Mitochondrial networks are remodeled in response to feeding and fasting.**
A. Representative confocal images of muscle mitochondrial morphology during 48h intervals of intermittent fasting. The SKILODGE *eft-3p::tomm-20(1-49aa)::GFP* strain was used. Scale bar 10 μm.
B–D. Quantification of mitochondrial morphology measured in mitochondrial length, circularity and number during 48h intervals of intermittent fasting. (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.001$, *$p < 0.05$, ns $p > 0.05$ by unpaired t test with Welch's correction)

the methods previously used in Weir et al. for imaging of mitochondria during IF, which used carbenicillin to inhibit bacterial growth on fasting days, but not on fed days. I performed IF and assessed mitochondrial morphology without the use of antibiotics, and the results suggest that mitochondria dynamically remodel their network morphology in response to 48-hour cycles of feeding and fasting (Figure 3.4 A). This was quantified by changes in length, circularity and counts (number of mitochondria per cell) (Figure 3.4 B-D). The length of mitochondria can

be interpreted as a readout of the network connectivity. Longer length indicates a more fused mitochondrial network, whereas shorter length indicates a more fragmented or disconnected mitochondrial network. On days following a 48 period of fasting (Day 4 and Day 6) mitochondrial length was decreased, indicating that mitochondria were more fragmented and the network was more disconnected, as compared to fed conditions. On days following 48 hours of refeeding (Day 8 and Day 10), mitochondrial length was increased, indicating that mitochondrial networks were more fused. These data indicate that 48 hours of fasting in *C. elegans* induced mitochondrial network remodeling towards increased fragmentation, whereas refeeding promoted mitochondrial network remodeling towards increased fusion. These observations suggest that fission of mitochondrial networks is an important adaptation to fasting in the worm. Quantitative PCR of worms with hyperfragmented mitochondria show upregulated expression of acetyl-coA synthetase-2 (*acs-2*), an enzyme required for priming fatty acids for beta-oxidation, hinting that mitochondrial fission increases mitochondrial beta-oxidation. Ongoing work that the lab is collaborating on with Nika Danial's lab has also indicated that mammalian cancer cells that are reliant on beta-oxidation have more fragmented mitochondria, and that the greater curvature on fragmented mitochondria is linked to carnitine palmitoyltransferase I (CPT1) activity (Choi et al., unpublished).

3.5 Simultaneous inhibition of mitochondrial fusion and fission by genetic deletion of DRP-1 and FZO-1 leads to loss of mitochondrial plasticity

Upon observation that IF induced both mitochondrial remodeling and increased longevity in wild type worms, I next wanted to examine if mitochondrial remodeling was responsible for the longevity benefits. If mitochondrial remodeling was required for IF longevity, I would expect to see that single *drp-1* and *fzo-1* mutants that respond to IF-longevity would also show mitochondrial remodeling by IF, whereas double *drp-1;fzo-1* mutants that are unable to respond to IF-longevity would also be unable to remodel their mitochondria. Therefore, I next imaged mitochondrial morphology during IF in each of the single and double *drp-1* and *fzo-1* mutants. Single deletion *drp-1* and *fzo-1* mutants showed remodeling of their mitochondrial networks in response to 48 hours of feeding and fasting, in a pattern similar to wild type worms (Figure 3.5 A-B). Conversely, double *drp-1;fzo-1* mutants showed complete lack of remodeling of mitochondrial networks in response to cycles of feeding and fasting (Figure 3.5 C). Quantifications show measurements of average mitochondrial length. Since neither *drp-1* nor *fzo-1* were individually required for IF-longevity, but the double *drp-1;fzo-1* was required for IF-longevity, these results suggest that inhibition of mitochondrial plasticity in the double *drp-1;fzo-1* null specifically is also coupled to the inhibition of lifespan extension by IF.

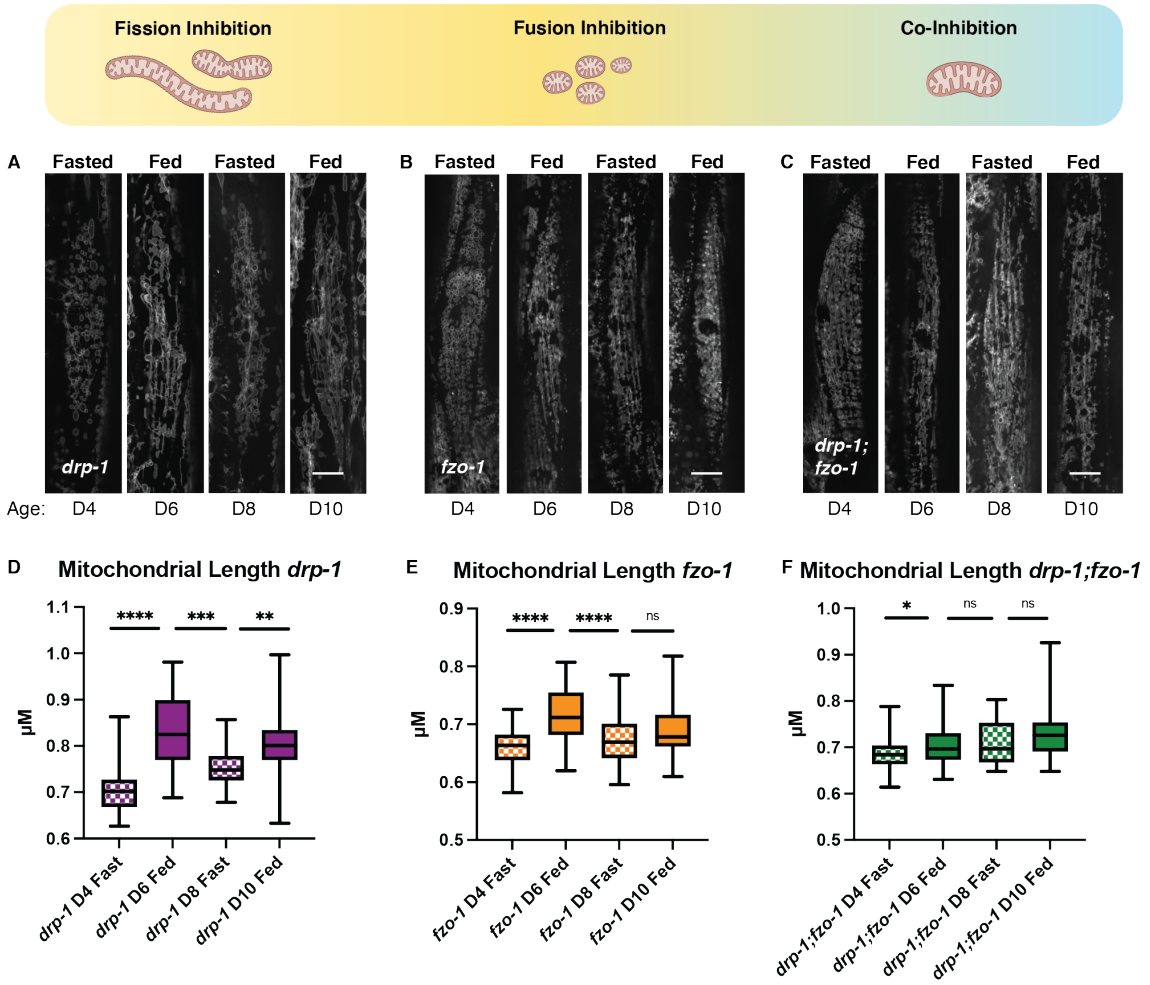


Figure 3.5: **Simultaneous inhibition of DRP-1 and FZO-1 abolishes mitochondrial remodeling in response to IF.**

A, D. Mitochondrial length in *fzo-1* worms show dynamic remodeling in response to 48h intervals of intermittent fasting. Greater mitochondrial length indicates mitochondria are more fused on Fed days (D6 and D10). Reduced mitochondrial length indicates mitochondria are more fragmented on Fasted days (D4 and D10). (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test) Scale bar 10 μm.

B, E. Mitochondrial length in *drp-1* worms show dynamic remodeling in response to 48h intervals of intermittent fasting. Greater mitochondrial length indicates mitochondria are more fused on Fed days (D6 and D10). Reduced mitochondrial length indicates mitochondria are more fragmented on Fasted days (D4 and D10). (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test) Scale bar 10 μm.

C, F. Mitochondrial length in *drp-1;fzo-1* worms on the contrary, do not show dynamic remodeling in response to 48h intervals of intermittent fasting. Mitochondria are statistically unchanged in length during Fed and Fasted days, indicating that networks are static and refractive to morphological changes induced by Feeding and Fasting. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test) Scale bar 10 μm.

3.6 Mitochondrial ultrastructure is intact in animals lacking both fusion and fission

Double *drp-1;fzo-1* mutants specifically abolished IF longevity, yet retained full responsiveness to DR longevity, suggesting that co-inhibition of fusion and fission were not detrimental to overall mitochondrial health and instead inhibited specific mitochondrial functions that were important for IF. This idea is supported by previous findings that double *drp-1;fzo-1* worms have normal (or even slightly elevated) basal respiration compared to wild type (Weir et al., 2017). Maximal uncoupled respiration was also slightly elevated in double Drp1;Mfn1 KO mammalian Hek293 cells (Weir et al., 2017; Arpit Sharma, unpublished). To further examine the consequences of inhibiting mitochondrial fusion and fission on mitochondrial integrity, I used electron microscopy to capture mitochondrial ultrastructure in wildtype, *drp-1*, *fzo-1*, and double *drp-1;fzo-1* mutant *C. elegans*. While fluorescence microscopy can detect overall organization of mitochondrial networks, electron microscopy enables detection of finer changes to mitochondrial membranes and cristae organization. Consistent with fluorescence microscopy results, electron microscopy showed that inhibition of *drp-1* resulted in larger and hyperfused mitochondria, whereas inhibition of *fzo-1* resulted in smaller and more circular mitochondria compared to their wild type controls (Figure 3.6 A-C). *Fzo-1* null animals also appeared to have more disrupted mitochondria cristae. Double inhibition of *drp-1;fzo-1* resulted in greater variability in mitochondrial shape. Hyperfragmented and hyperfused morphologies were found more frequently than in wild type worms. However, compared to their single mutant counterparts, *drp-1;fzo-1* worms showed overall more tubular mitochondrial networks (Figure 3.6 D). Overall mitochondrial ultrastructure and cristae were preserved in the double *drp-1;fzo-1* mutant, giving confidence that double inhibition of *drp-1;fzo-1* did not ablate global mitochondrial inner and outer membrane organization.

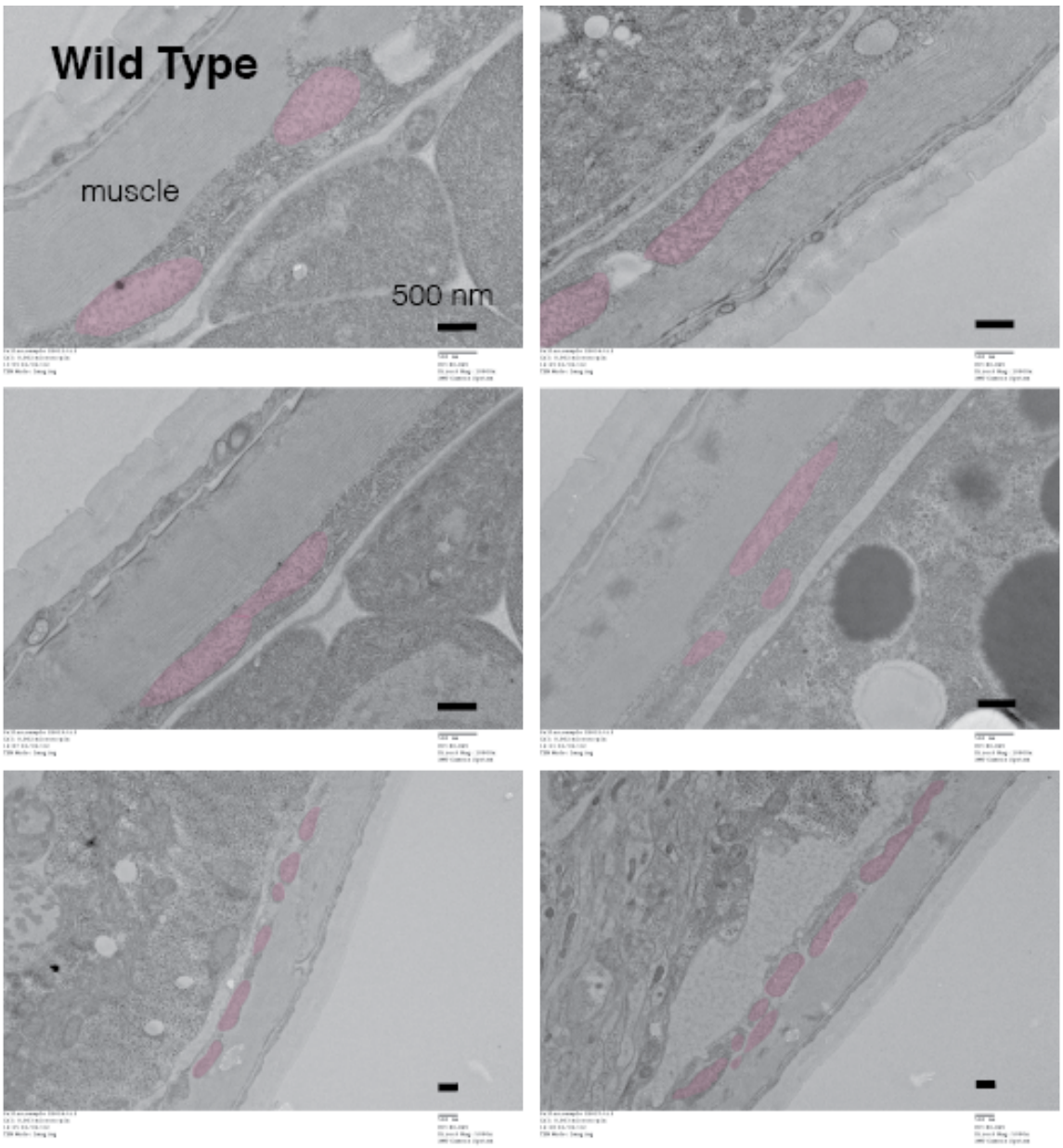


Figure 3.6: **Representative electron microscopy images of mitochondrial morphology and ultrastructure in *C. elegans* body wall muscle.**
A. Representative electron microscopy images of mitochondrial morphology and ultrastructure in wild type worms. Mitochondria are highlighted in pink. (Representative images are shown, Scale bars represent 500nm.)

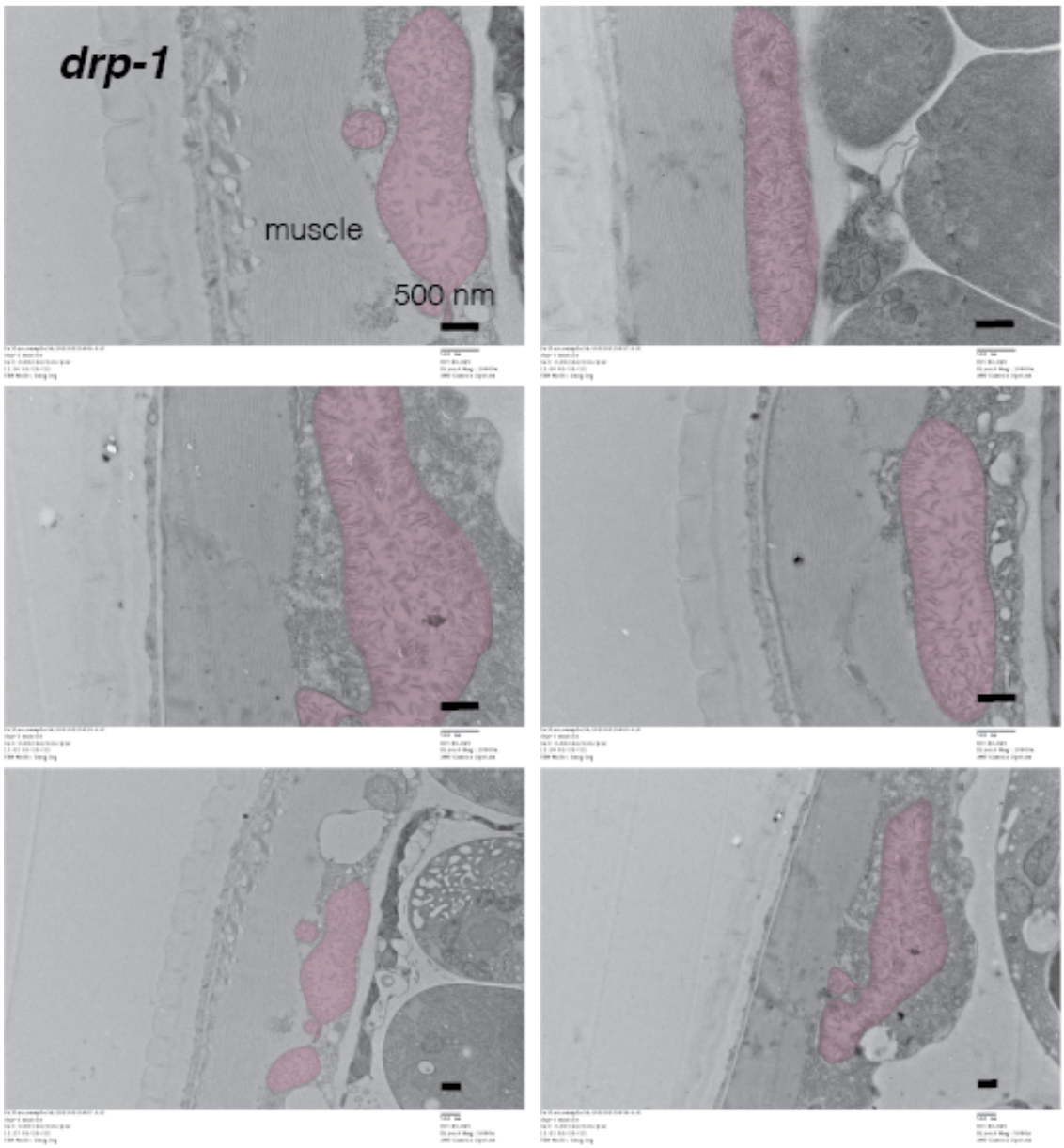


Figure 3.6: *Continued.* **Representative electron microscopy images of mitochondrial morphology and ultrastructure in *C. elegans* body wall muscle.**
B. Mitochondria in *drp-1* mutants are larger and hyperfused. (Representative images are shown, Scale bars represent 500nm.)

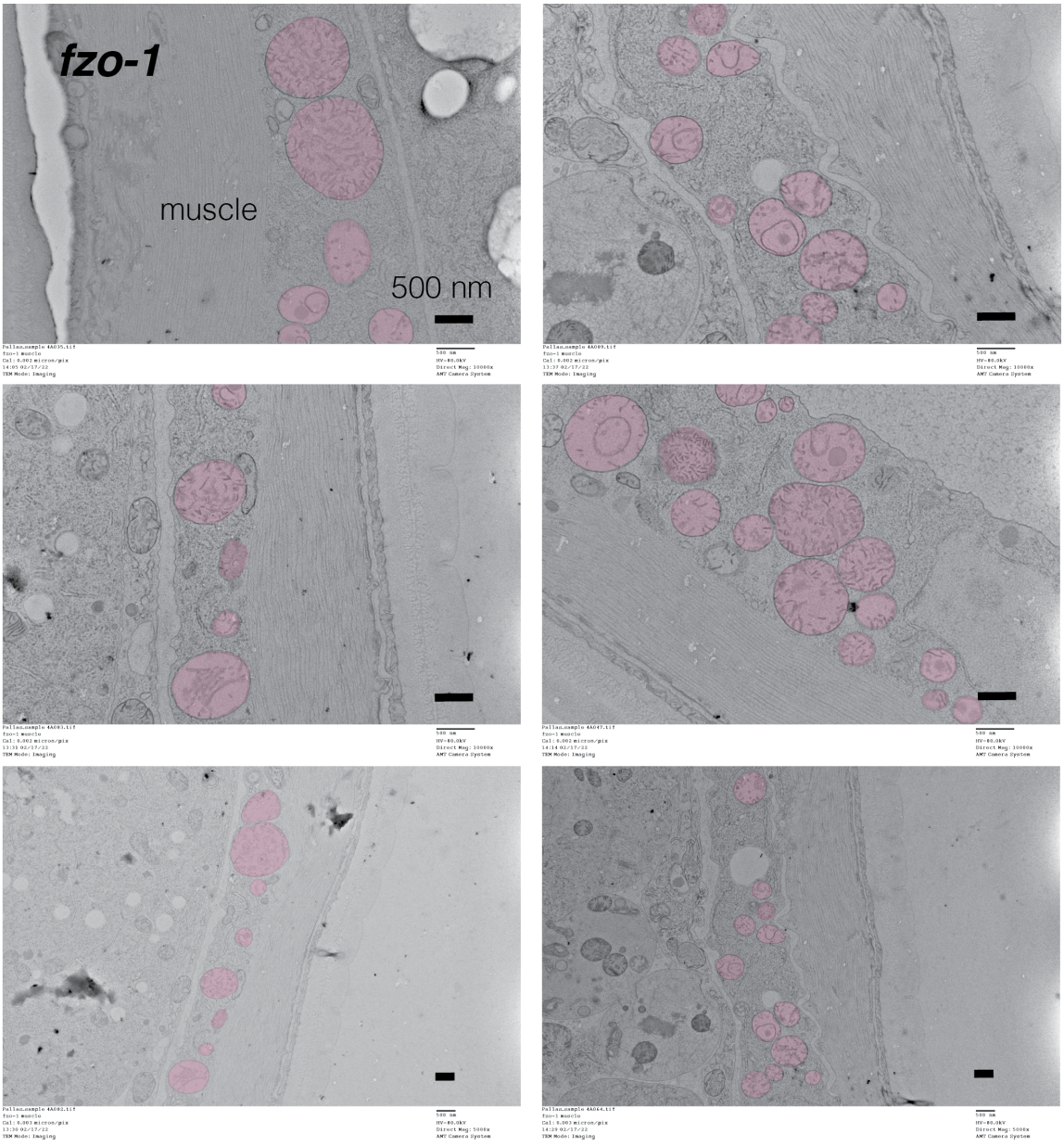


Figure 3.6: *Continued.* **Representative electron microscopy images of mitochondrial morphology and ultrastructure in *C. elegans* body wall muscle.**
C. Mitochondria in *fzo-1* mutants are hyperfragmented and have a high degree of circularity. *Fzo-1* mutant mitochondria also appear to have disrupted cristae. (Representative images are shown, Scale bars represent 500nm.)

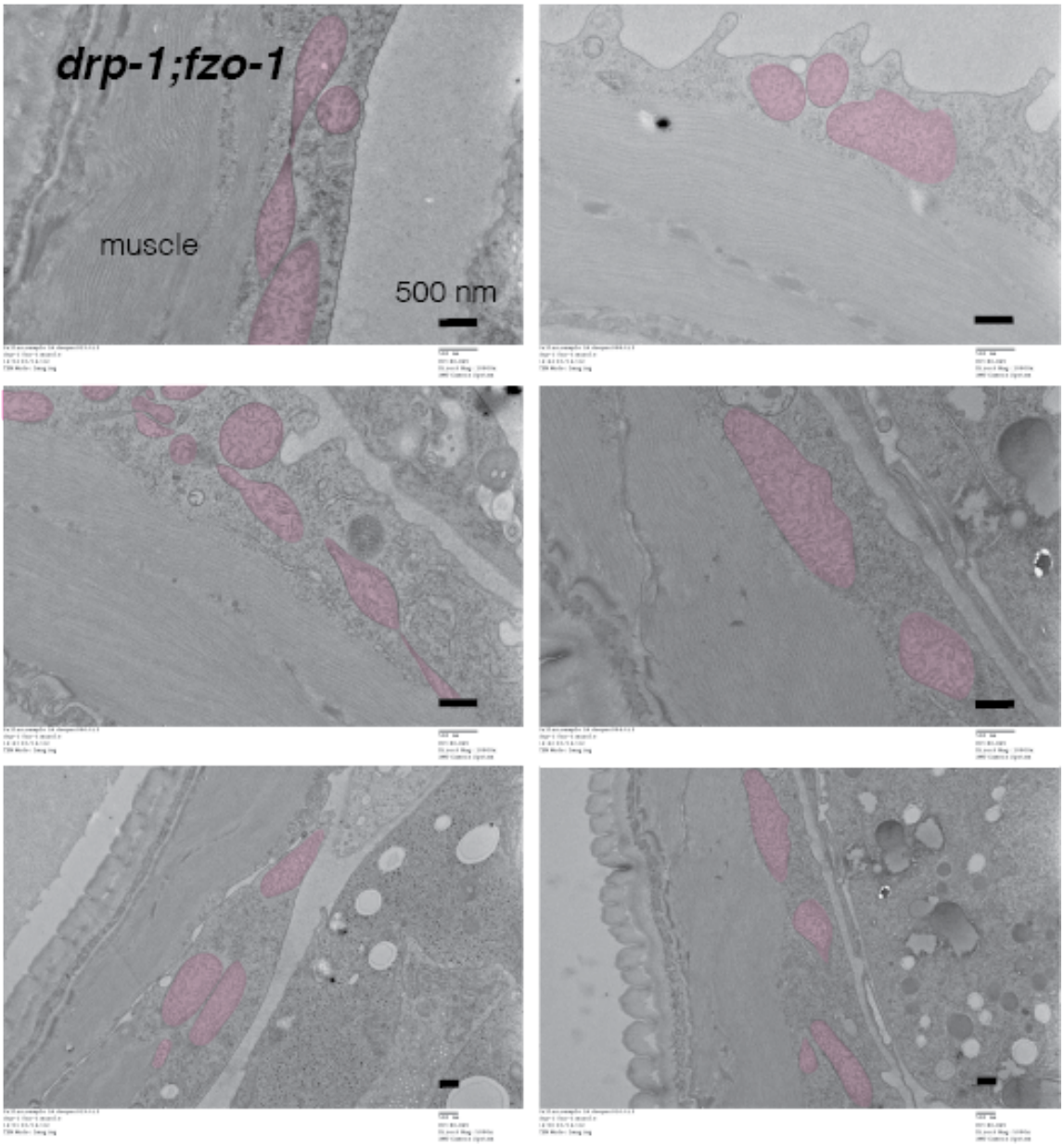


Figure 3.6: *Continued.* **Representative electron microscopy images of mitochondrial morphology and ultrastructure in *C. elegans* body wall muscle.**
D. Mitochondria in the double *drp-1;fzo-1* mutants show greater variability in shape of mitochondria – hyperfragmented and hyperfused morphologies are found more frequently in *drp-1;fzo-1* than in wild type worms, however *drp-1;fzo-1* worms also contain more tubular mitochondrial networks than their single mutant counterparts. Overall mitochondrial ultrastructure is preserved in the double *drp-1;fzo-1* mutant. (Representative images are shown, Scale bars represent 500nm.)

3.7 Simultaneous ablation of fusion and fission using the Auxin Inducible Degradation system abrogates lifespan extension by Intermittent Fasting.

In animals constitutively null for *drp-1* and *fzo-1*, I saw that double mutant *drp-1;fzo-1* specifically blocked IF-longevity (Figure 3.3 B). To support these findings with different *drp-1* and *fzo-1* mutant alleles, I generated conditional *drp-1* and *fzo-1* deletions using the Auxin-Inducible Degradation system in *C. elegans* (L. Zhang et al., 2015). I integrated the 44 a.a. AID sequence to the N-terminus of endogenous *drp-1* and *fzo-1* using CRISPR. These lines were then crossed into the background of ubiquitously expressed TIR1 driven by the *eft-3p*. First, to validate that this system indeed perturbs mitochondrial morphology, I crossed these strains into the background of the SKILODGE TOMM20::GFP reporter. I confirmed that exposure of these strains to the 0.15mM Auxin resulted in degradation of DRP-1 and FZO-1, and perturbed mitochondrial network morphology at Day 1 to hyperfusion and hyperfragmentation, respectively (Figure 3.7 A-D). Double *drp-1;fzo-1* AID deletion animals were resilient to age-related fragmentation of mitochondrial networks, as seen previously in the constitutive genetic nulls (Figure 3.7 E-F).

Upon establishing that the AID system sufficiently induced degradation of DRP-1 and FZO-1 and perturbed mitochondrial networks as expected, I next assessed lifespan and response to IF-longevity in animals with AID-mediated depletion of DRP-1 and FZO-1. For these lifespan assays, I exposed animals to auxin from hatch, and throughout life. Auxin-inducible degradation of *drp-1;fzo-1* significantly reduced lifespan extension by IF (Figure 3.8 B). In the absence of auxin (Control) these animals responded to IF-longevity, showing that our inducible deletion system indeed replicated previous findings in the genetic null models (Figure 3.8 A).

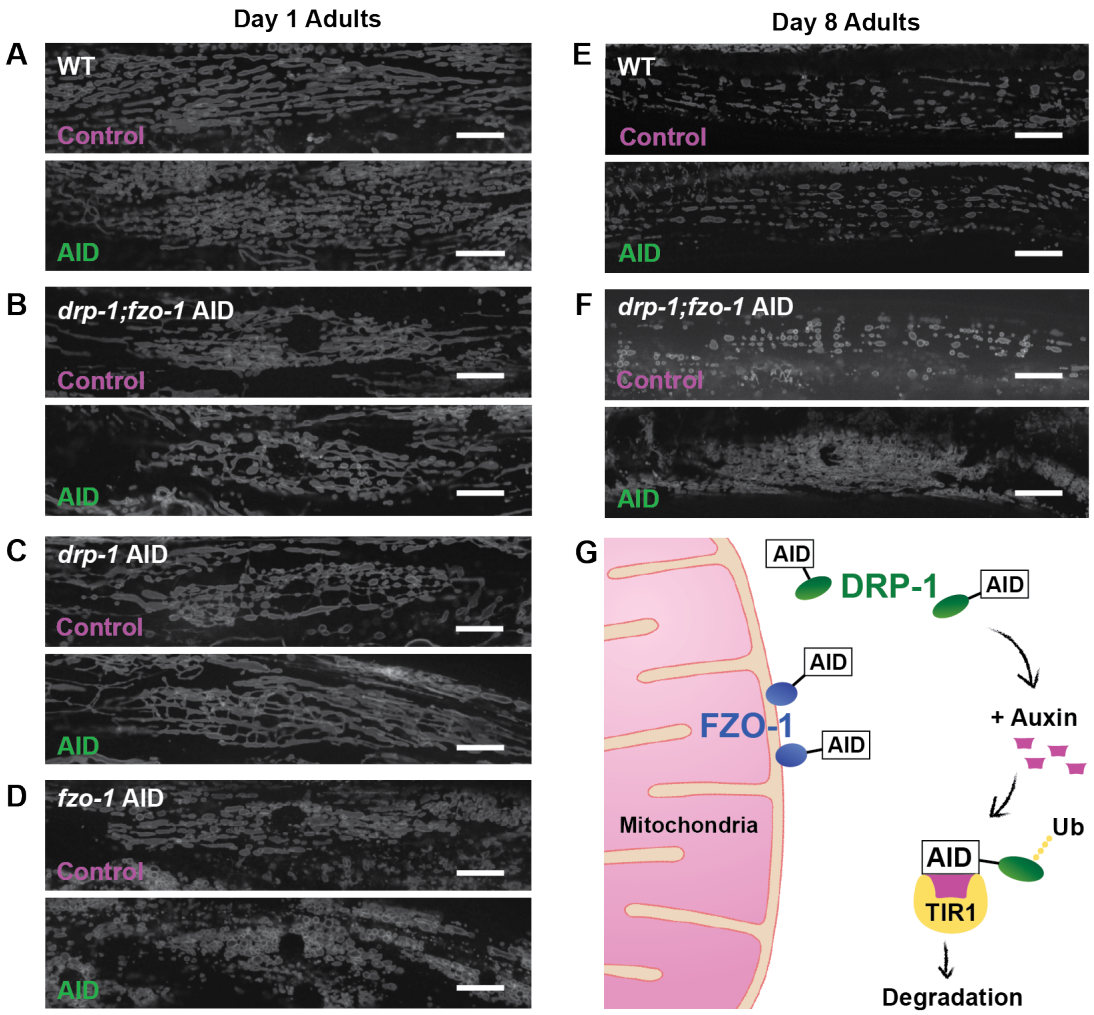


Figure 3.7: **AID-mediated degradation of mitochondrial fusion and fission results in changes in mitochondrial morphology similar to genetic nulls.**
A. Representative images of mitochondrial morphology in wild type animals on Day 1 of adulthood in Control and presence of 0.15 mM Auxin from hatch. All images in following panels A-F from n = 2 biological replicates. Scale bar 10 μm
B. Representative images of mitochondrial morphology in double *drp-1;fzo-1* animals on Day 1 of adulthood in Control and presence of 0.15 mM Auxin from hatch. Scale bar 10 μm
C. Representative images of mitochondrial morphology in *drp-1* animals on Day 1 of adulthood in Control and presence of 0.15 mM Auxin from hatch. Scale bar 10 μm
D. Representative images of mitochondrial morphology in *fzo-1* animals on Day 1 of adulthood in Control and presence of 0.15 mM Auxin from hatch. Scale bar 10 μm
E. Representative images of mitochondrial morphology in wild type animals on Day 8 of adulthood in Control and presence of 0.15 mM Auxin from hatch to Day 8. Scale bar 10 μm
F. Representative images of mitochondrial morphology in double *drp-1;fzo-1* animals on Day 8 of adulthood in Control and presence of 0.15 mM Auxin from hatch to Day 8. Scale bar 10 μm
G. Schematic diagram of AID system for inducible deletion of mitochondrial fusion and fission.

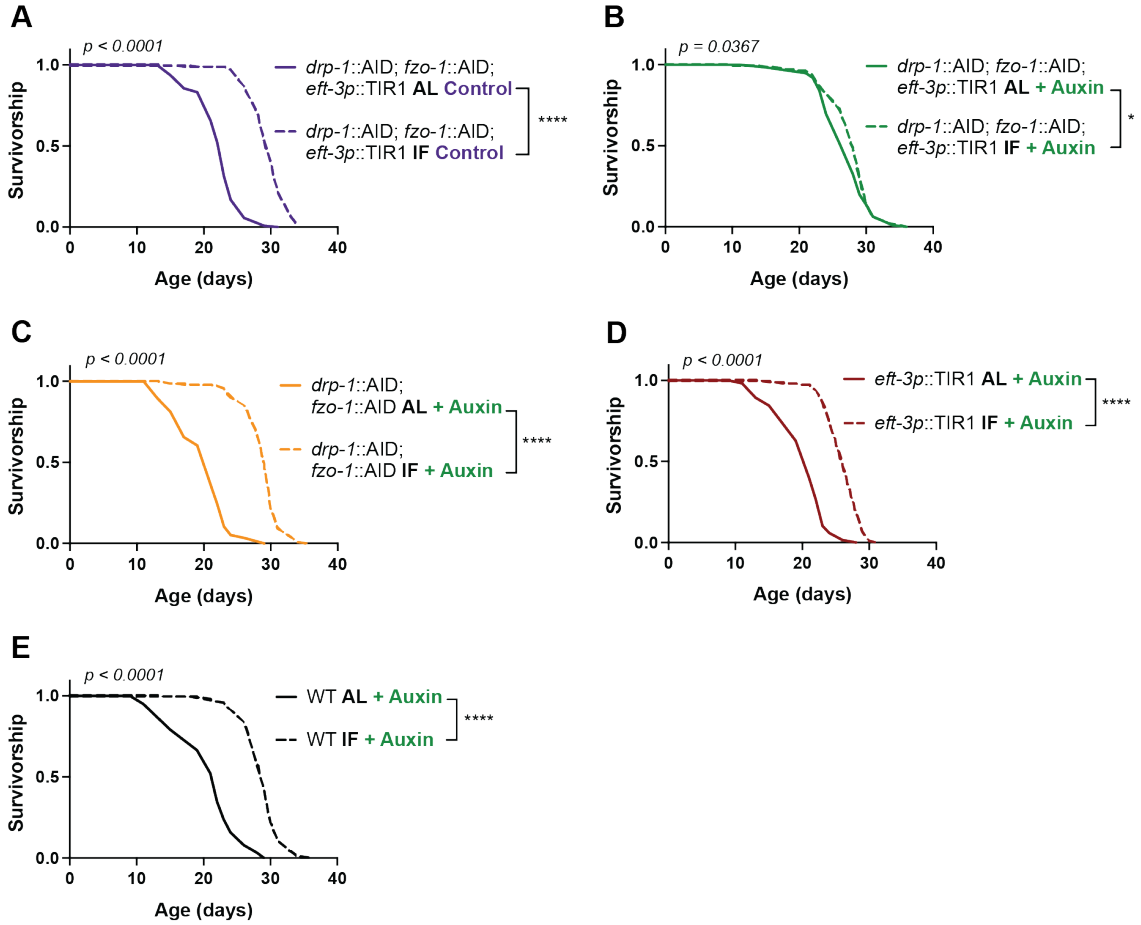


Figure 3.8: **Simultaneous ablation of DRP-1 and FZO-1 using the Auxin-Inducible Degradation system abrogates lifespan extension by Intermittent Fasting.**
A. In animals that express *drp-1*::AID, *fzo-1*::AID and ubiquitous TIR1, intermittent fasting extends lifespan in control conditions with no auxin. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by log-rank test; n = 2 biological replicates)
B. In animals that express *drp-1*::AID, *fzo-1*::AID and ubiquitous TIR1, intermittent fasting is abrogated in animals that were exposed to 0.15 mM Auxin. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by log-rank test; n = 2 biological replicates)
C–E. Intermittent fasting extends lifespan in AID control strains and wild type N2s exposed to 0.15 mM Auxin. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by log-rank test; n = 2 biological replicates).

3.8 Functional outputs of inhibiting mitochondrial network plasticity during Intermittent Fasting

Confirming that co-inhibition of mitochondrial fusion and fission at both the gene and protein level abolished IF-longevity, I next asked whether there were specific fasting-responsive pathways that were perturbed by *drp-1;fzo-1* inhibition and could explain loss of IF lifespan extension. To do so, I took an unbiased transcriptomic profiling approach. RNA was extracted from whole worm lysates and sent for RNA sequencing from each of the four conditions: wild type fed, wild type 48-h fasted, double *drp-1;fzo-1* mutant fed, and double *drp-1;fzo-1* mutant 48-h fasted. *drp-1;fzo-1* mutants were confirmed to have reduced expression levels of both *drp-1* and *fzo-1* (Figure 3.9 A). I probed for differentially expressed genes among the four conditions (WT Fed, WT Fasted, *drp-1;fzo-1* Fed, *drp-1;fzo-1* Fasted) using likelihood ratio test (LRT), pairwise analysis, and an interaction term of strain and condition to identify genes that responded differently to the condition Fed versus Fasted in the wild type and *drp-1;fzo-1* strains (Figure 3.9 B). These genes were categorized into groups based on the pattern of expression between genotype and feeding condition.

LRT identified that 48 hours of fasting induced changes in approximately 10,000 differentially expressed (DE) genes at an adjusted p value (padj) of 0.0001 in both wild type and *drp-1;fzo-1* animals. I next examined the expression patterns of the DE genes identified in the LRT analysis. Pattern analysis by pairwise comparison showed that the top 3000 genes ranked by padj showed similar patterns, both increasing or deceasing when fasted in both strains. These analyses indicated that co-inhibition of *drp-1;fzo-1* retained global fasting-induced transcriptomic remodeling. My hypothesis however was that there were a subset of DE genes that differed in response to fed or fasted between wild type and *drp-1;fzo-1* strains that could be mapped to specific cellular pathways causally coupled to loss of IF-longevity upon co-inhibition of *drp-1;fzo-1*.

To filter the specific DE genes that differed in response to fed or fasted between wild type and

drp-1;fzo-1 strains, I therefore used an interaction term of strain:condition at an FDR cutoff of 0.01. These genes were subsequently categorized into eight groups based on the pattern of expression between genotype and feeding condition. The most interesting clusters of genes were those in groups 1, 4, 6, 7, and 8, in which the transcription in response to Fasting were either blunted (groups 1 and 8), downregulated (group 7), or had opposite transcriptional responses (groups 4 and 6). I performed downstream functional analyses for Gene Ontology (GO) terms and KEGG pathways in each group of genes. These first analyses suggested that there were interesting metabolic changes in the aforementioned gene groups of interest, suggesting that specific metabolic remodeling upon co-inhibition of *drp-1;fzo-1* could likely explain the loss of IF-longevity.

In Group 8, there were 17 genes upregulated by fasting in WT and specifically showed no change in *drp-1;fzo-1*. WT and Mutants showed comparable expression levels in the Fed state, however Fasting upregulated expression in WT and showed no inductioin in Mutant. The functional analyses of this cluster of few genes did not identify significantly enriched GO terms or KEGG pathways. Manually curating the gene list, I saw that several genes were implicated in mitochondrial function and metabolism. Interestingly, *mrps-23*, a 28S mitochondrial ribosomal protein S23, was identified in Group 8. It could be speculated that mitochondrial translation is affected by structural changes in the mitochondrial membrane, considering that mitochondrial ribosomes are assembled on the inner mitochondrial membrane. Mitochondrial oxidative phosphorylation (OXPHOS) enzymes are made up of dual genetic origin from both nuclear and mitochondrial genomes. OXPHOS activity in cells has been linked to mitochondrial morphology and it is possible that changes in mitochondrial translation are critical to this process. Whether mitochondrial translation mediates important metabolic adaptations during feeding/feeding transitions and ultimately contributes to longer lifespan in IF animals is a fascinating question to explore in follow-up mechanistic studies. *cyp-33D3*, a protein of the cytochrome P450 family was also identified in this cluster of genes. Based on sequence anlysis, *cyp-33D3* is predicted to

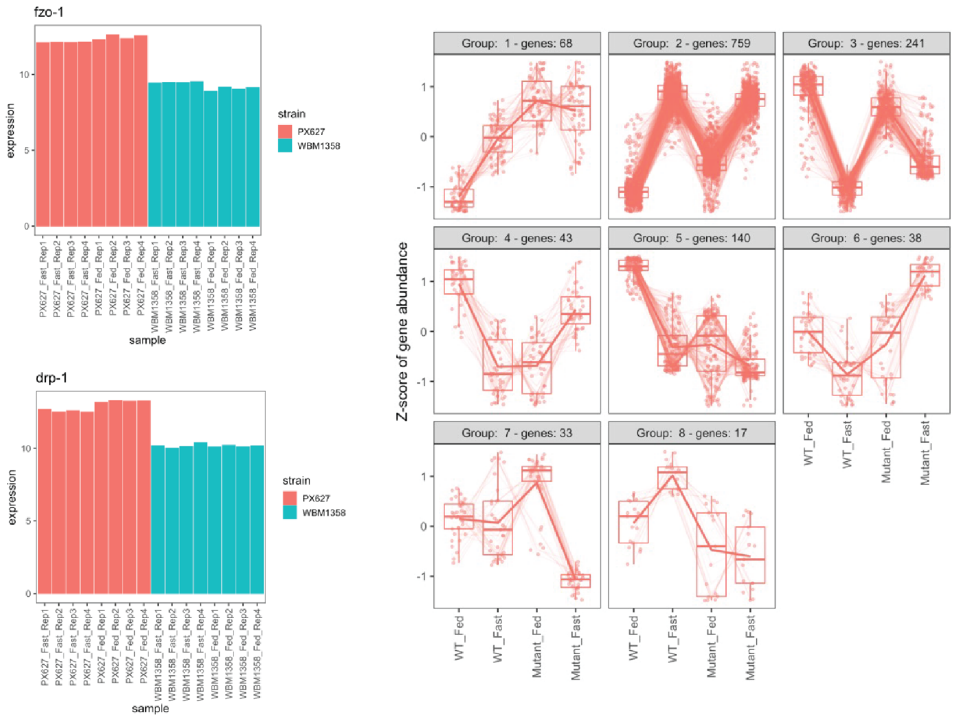
have oxidorectuctase activity, flavoprotein reducing activity, and be involved in organic acid and xenobiotic metabolic processes. It contains an ATFS-1 (activating transcription factor associated with stress-1) binding site, a transcription factor known to sense mitochondrial stress and communicates with the nucleus during the mitochondrial unfolded protein response (UPR(mt)) in *C. elegans*. ATFS-1 contains both an N-terminal mitochondrial targeting sequence and a nuclear localization sequence, and studies have demonstrated that during mitochondrial stress, ATFS-1 accumulates in the cytosol and is trafficked to the nucleus to coordinate the level of mitochondrial dysfunction with a protective transcriptional response (Nargund et al., 2012). Furthermore, ATFS-1 activity is responsive to manipulations in mTORC1 signaling in a manner that correlates with protein synthesis (Shpilka et al.,2021).

Group 1 also contained genes that showed increased expression induced by fasting in wildtype and no change in *drp-1;fzo-1*. KEGG pathway enrichments in Group 1 genes included terms tyrosine metabolism, retinol metabolism, pyruvate metabolism, sphingolipid metabolism and glycolysis. Several proteins of the cytochrome P450 family were found in this cluster of genes, including *cyp-33C8* and *cyp-14A1* also containing an ATFS-1 binding site. Nuclear hormone receptor family transcription factors (*nhr-123, nhr-115*), lipase related genes (*lips-12*), alcohol dehydrogenase (*sodh-2*), ceramide glucosyltransferase 1 (*cgt-1*), and UDP-glucuronosyltransferases (*ugt-47, ugt-32*), which catalyze conjunction of UDP-activated sugar donors to small lipophilic chemicals, were also identfied. Interestingly, the mammalian orthologues of *nhr-123*, RORs/NR1F, have been reported to regulated circadian rhythms (Akashi and Takumi, 2005; André et al., 1998; Sato et al., 2004).

In Group 7, 33 genes were specifically downregulated by fasting in *drp-1;fzo-1* animals, and included *dhs-14* and *dhs-20* (short chain dehydrogenases), *mppb-1* (mitochondrial processing peptidase beta), *acdh-1* (acyl CoA dehydrogenase), *cyp-33E2* (cytochrome P450 family), *ugt-62* (UDP-glucuronosyltransferase). The genes idenfied within these groups represent prime candidates for further mechanistic experiments investigating their causal role in IF-longevity.

The most significantly enriched KEGG pathway of Group 3 was Glycolysis, in which both WT and Mutants showed the same pattern of downregulation by fasting. Since glucose availability as a fuel source is decreased upon fasting, this suggests that some canonical metabolic adaptations were retained in *drp-1;fzo-1* and supports the idea that other specific metabolic pathways are responsible for loss of longevity in *drp-1;fzo-1*.

In summary, these data show that although adynamic mitochondria fully block the effects of IF on lifespan, mitochondria dynamics is not required for much of the transcriptional remodeling that is seen in response to fasting. Comparing the effects of fasting on transcription that only occur specifically in WT animals and not *drp-1;fzo-1* double mutants allows us to filter the transcriptomic changes coupled to when IF slows the aging process. Although this filtering does not define causality, it is a powerful approach to begin to focus in on what those causal changes may be. Next steps will be further mechanistic probing of specific genes and processes enriched in groups 1, 4, 6, 7, and 8, that represent the transcriptional response to fasting differentially regulated between *drp-1;fzo-1* and wild type and thus coupled to longevity. These initial analyses suggest that specific perturbations occur in fasting-induced metabolic networks that are require functional fusion and fission and these changes are blocked when mitochondria are adynamic. It will be pertinent to follow up on specific genes enriched in these clusters, and to determine their functional role in the mechanisms coupling mitochondrial morphology to lifespan extension during IF. This transcriptomic data set will also be of great value for ongoing investigations of mitochondrial dynamics and aging in the lab.



Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | Group 6 | Group 7 | Group 8

Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	geneID	Count
Tyrosine metabolism	1/3	24/2484	0.029	0.069	0.032	179628	1
Retinol metabolism	1/3	24/2484	0.029	0.069	0.032	179628	1
Pyruvate metabolism	1/3	33/2484	0.039	0.069	0.032	179628	1
Sphingolipid metabolism	1/3	39/2484	0.046	0.069	0.032	188169	1
Glycolysis / Gluconeogenesis	1/3	42/2484	0.050	0.069	0.032	179628	1
Metabolism of xenobiotics by cytochrome P450	1/3	47/2484	0.056	0.069	0.032	179628	1
Fatty acid degradation	1/3	52/2484	0.062	0.069	0.032	179628	1
Drug metabolism - cytochrome P450	1/3	52/2484	0.062	0.069	0.032	179628	1
Phagosome	1/3	63/2484	0.074	0.074	0.035	179680	1

Figure 3.9: **Metabolic pathways are perturbed by loss of *drp-1;fzo-1* in response to fasting.**
A. Expression levels of *drp-1* and *fzo-1* in wild type (PX627) or *drp-1;fzo-1* (WBM1358) (n = 4 biological replicates).
B. Interaction term of strain and condition to identify the genes that respond differently to Fasting in wild type and *drp-1;fzo-1*. $p < 0.05$ by BH adjusted Wald test (corrected for multiple comparisons; FDR) (n = 4 biological replicates).
C. KEGG Pathway enrichment analysis of genes in Group 1, that are upregulated by fasting in wild type, but show no change in *drp-1;fzo-1* (n = 4 biological replicates).

3.9 Synthetic manipulation of mitochondrial network plasticity mimics Intermittent Fasting

Thus far, I showed that (1) mitochondrial networks were remodeled between fused and fragmented states during IF; (2) co-inhibition of *drp-1* and *fzo-1* resulted in loss of mitochondrial remodeling, which could be interpreted as inhibition of mitochondrial plasticity; (3) inhibition of mitochondrial plasticity specifically abolished IF-longevity; and (4) functional analyses downstream of inhibiting mitochondrial plasticity indicate altered mitochondrial function and metabolism. Whether mitochondrial plasticity plays a causal role in IF longevity that can be targeted directly to increase lifespan is not known. To determine if mitochondrial plasticity is both necessary and sufficient for IF lifespan extension, I next asked if promoting mitochondrial plasticity itself would be sufficient to mimic IF and extend lifespan. By manipulating mitochondrial shape, can we by-pass upstream cues from nutrient sensing pathways, and directly affect downstream functions coupled to alterations in shape? I have reason to think this is possible, based on the previously reported differences in *acs-2* induction in WT, *fzo-1*, *drp-1* animals with perturbed mitochondrial networks (Figure 2.6), suggesting that FAO activity was altered by different mitochondrial network morphologies.

I set out to determine if intermittent cycles of fusion and fission could promote lifespan extension without associated feeding and fasting cycles. To synthetically manipulate mitochondrial plasticity, I utilized the AID system I generated in *C. elegans* to gain temporal control of DRP-1 and FZO-1 protein levels. In animals engineered to express DRP-1::AID and/or FZO-1::AID, and ubiquitous TIR1, I showed that auxin exposure in adulthood induced mitochondrial fission and fusion by proteasomal degradation of FZO-1 and DRP-1, respectively (Figure 3.10 A-D). The advantage of the AID system is the reversibility of these effects upon removal of auxin. Upon removal of auxin, FZO-1 and DRP-1 levels can be recovered and mitochondrial morphology restored to baseline. This system therefore allowed me to synthetically manipulate mitochondrial network morphology simply by the addition or absence of Auxin. To mimic a model of mitochondrial plasticity, I

subjected FZO-1::AID animals to intermittent treatment of Auxin on Day 1 of adulthood, such that animals were exposed to Auxin for 48 hours, followed by 48h off auxin, in repeating cycles until Day 13. As a result, mitochondrial fragmentation was induced every alternate 48h cycle when auxin was present (Figure 3.10 E-G). Mitochondrial fusion was restored on days with no auxin resulting in increased tubular mitochondria. Intermittent auxin treatment resulted in modulation of mitochondrial network shape between fused and fragmented states every 48h, effectively mimicking those changes seen on IF. I also performed intermittent auxin treatment in alternate 24h cycles beginning on day 1 of adulthood, and the pattern of mitochondrial networks was consistent with those seen during 48h cycles. (Figure 3.10 A-D). Overall, these experiments suggest that at the level of mitochondrial morphology, it was possible to synthetically induce mitochondrial plasticity to mimic IF.

3.10 Synthetic manipulation of mitochondrial plasticity is sufficient to extend lifespan

Establishing that mitochondrial networks can be synthetically induced to undergo remodeling reminiscent of IF, I proceeded to examine the outcome that synthetic manipulation of mitochondrial plasticity had on *C. elegans* lifespan. Remarkably, synthetic induction of mitochondrial plasticity resulted in significant lifespan extension compared to controls (Figure 3.11 A). It has been previously reported in *Drosophila* that chronic mid-life induction of mitochondrial fission, either via induction of *Drp1* or deletion of *dMfn*, is also able to extend lifespan by facilitating late-life mitophagy (Rana et al., 2017. To test whether the lifespan extending effects of intermittent AID in worms was due to late-life fission, I performed chronic midlife degradation of FZO-1 from Day 6 of Adulthood. Chronic midlife degradation of FZO-1 did not show significant differences in lifespan from Control animals not exposed to Auxin (Figure 3.11 B), indicating that late-life fission did not account for any longevity benefits in worms. Chronic midlife degradation of FZO-1 from Day 1 of Adulthood also did not significantly affect lifespan compared to Control animals (Supplemental Figure A.6). Together these results indicate that induction of

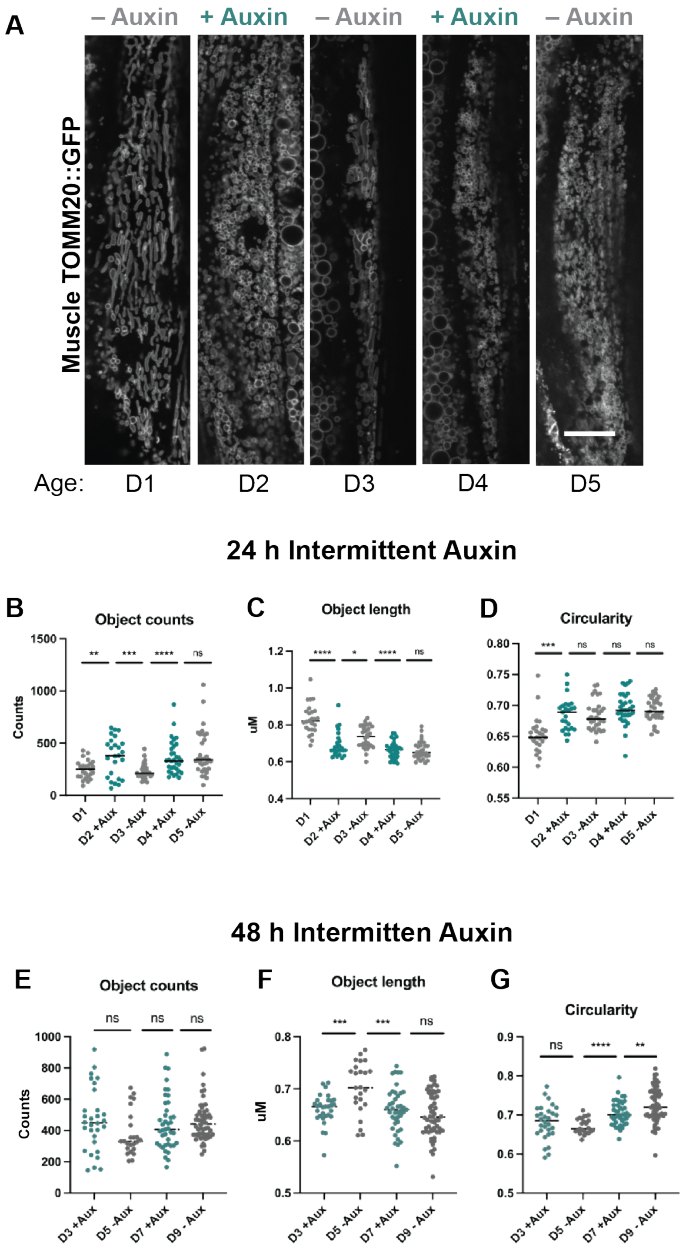


Figure 3.10: **Synthetic manipulation of mitochondrial plasticity mimics intermittent fasting.**
A. Representative confocal images of muscle mitochondria upon 24h cycles of intermittent auxin treatment show that mitochondrial morphology can be manipulated to mimic morphology changes induced by cycles of feeding and fasting (Scale bar 10 μm).
B-G. Quantification of mitochondrial number, length and circularity during 24h and 48h cycles of intermittent exposure to auxin. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test with Welch's correction; n = 2 biological replicates)

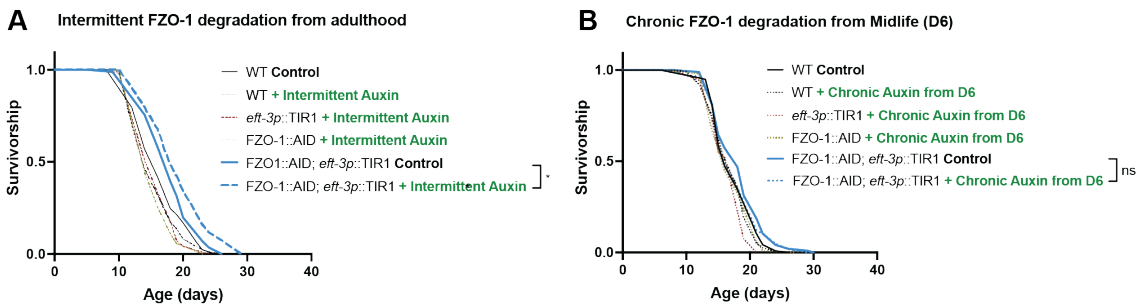


Figure 3.11: **Synthetic manipulation of mitochondrial plasticity is sufficient to extend lifespan.** A. Intermittent auxin treatment to mimic mitochondrial remodeling during IF was sufficient to increase lifespan compared to controls. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by log-rank test; n = 2 biological replicates)
B. Chronic midlife degradation of FZO-1 from Day 6 of adulthood did not significantly affect lifespan compared to controls. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by log-rank test; n = 2 biological replicates)

mitochondrial plasticity via intermittent auxin treatment throughout early life was specifically coupled to increased longevity. Mitochondrial plasticity is therefore likely to play a causal role in IF longevity in worms.

3.11 Discussion

The results I presented in this chapter show a fascinating, yet differential requirement of mitochondrial fusion, fission and plasticity for DR and IF (Figures 3.2 and 3.3). Previous studies I contributed to showed that inhibition of either mitochondrial fusion or fission alone abolished DR-longevity (Weir et al., 2017 and Figure 3.2), suggesting that disruption of the balance between fusion and fission was detrimental. IF is a related dietary intervention that has been gaining interest in recent years due to its protective effects against age-related pathologies, longevity inducing qualities, and more clinically relevant application than chronically reduced caloric intake (See Chapter 1.6). Studies are beginning to elucidate that IF modulates organismal aging in some mechanisms that are nonoverlapping to those that mediate DR (Mitchell et al., 2019; Uno et al., 2013). In this study, I showed that DR and IF indeed had differential requirements for mitochondrial fusion and fission for longevity, supporting the notion that DR and IF have

some distinct mechanisms. In contrast to DR, genetic ablation of either *fzo-1* or *drp-1* alone during IF had no effect on its lifespan extending properties (Figure 3.2). **Strinkingly, simultaneous inhibition of both fusion and fission completely abolished IF-longevity**, which was recapitulated at both the gene (Figure 3.3) and protein (Figure 3.11) level. The significance of these findings became even more intriguing upon co-deletion of *fzo-1* and *drp-1* during DR. **In stark contrast to IF, simultaneous inhibition of both fusion and fission completely restored DR-longevity** (Figure 3.3). These results led to my hypothesis that co-inhibition of fusion and fission restored the balance in mitochondrial network organization, however it led to loss of mitochondrial plasticity. Mitochondrial plasticity was required for specific rewiring of metabolic networks directly and causally mediating IF-longevity.

3.11.1 Balanced mitochondrial network morphology does not equate to mitochondrial plasticity

These findings are significant because it indicates the nuances in how specific mitochondrial network configurations are required for their various functional outputs, which may differ depending on the cellular context (i.e. the relationship between form and function; see Chapter 1.8). Fusion:fission balance versus plasticity may be associated to different functions of the mitochondria, and the importance of each may be more or less important under different physiological conditions. In the context of DR-longevity, maintaining balanced mitochondrial networks was beneficial and preferred, at the dispense of mitochondrial plasticity. In contrast, in the context of IF-longevity, abolishing mitochondrial plasticity was entirely detrimental. Whereas perturbing mitochondrial network organization towards states of hyperfusion or hyperfragmentation was detrimental for DR-longevity, it was dispensible for IF-longevity.

This idea has also been supported by other studies. In mouse heart, disrupting the balance of mitochondrial fusion and fission led to cardiac pathology, but restoring the balance through

co-inhibition of both fusion and fission led to slowed progression of cardiac pathology (Song et al., 2017). The importance of balance versus plasticity may also differ from organism to organism, or between tissues. In *C. elegans*, co-inhibition of fusion and fission did little to affect baseline survival, and even led to mild increased lifespan (Weir et al., 2017). In yeast however, it led to shortened lifespan (Bernhardt et al., 2015) as well as poor performance in various stress tests (Bernhardt et al., 2015). These studies and mine suggest that inhibition of mitochondrial plasticity may have deleterious outcomes when it is necessary to adapt to energetic challenges. Going forward, it will be important to assess the epistasis of DR and IF longevity. A combination intervention of DR and IF to determine whether the longevity benefits are additive would help to delineate whether their pathways are independent or overlapping.

3.11.2 Changes in mitochondrial shape are associated with unique bioenergetic functions

I showed that IF induced mitochondrial remodeling, and that cycling between periods of feeding and fasting caused switching between fused and fragmented mitochondrial states (Figure 3.4). Simultaneous inhibition of *drp-1* and *fzo-1* specifically abolished the ability of mitochondrial networks to remodel in response to feeding and fasting (Figure 3.5). During DR, mitochondrial networks remain relatively constant throught life and the advantage of co-deleting fusion and fission were attributed to the maintenance of a balanced mitochondrial network that counteracted age-related deterioration of tubular mitochondrial morphology (Weir et al., 2017). Since DR works via a chronic reduction in caloric intake, there are no acute changes to nutrient supply and demand. Therefore, it may not be necessary for acute adaptations in mitochondrial shape and maintenance of a balanced network architecture is optimal for the mitochondrial functions necessary to mediate DR-longevity. In Weir et al., 2017, it was suggested that balanced mitochondrial morphology was important for its interaction with peroxisomes to induce pro-longevity metabolic signatures. Conversely, animals on IF are presented with fluctuating energetic chal-

lenges through alternating cycles of high-nutrient influx during feeding and complete nutrient deprivation during fasting. The results I presented in this chapter suggest that the oscillation between these two extreme nutrient states requires acute metabolic switching and rewiring, and is directly linked to remodeling of mitochondria network plasticity. Hindering mitochondrial and metabolic remodling via co-inhibition of fusion and fission causally led to loss of IF-longevity.

Since different mitochondrial morphologies have been shown be associated with its various bioenergetic capabilities, I hypothesized that inhibiting mitochondrial plasticity also inhibits the ability of mitochondria to optimize their function during feeding and fasting. For example, fusion is known to promote higher OXPHOS potential when there is a need for increased ATP production during overall nutrient deprivation, or deprviation of specific substrates. Yeast cultured in nonfermentable conditions require increased OXPHOS activity, and this was accompanied by elongation of the mitochondrial network (Egner et al., 2002; Jakobs et al., 2003). In human cells, mitochondria elongate during growth in galactose media, which forces cells to rely more heavily on OXPHOS for ATP production (Rossignol et al., 2004). Under conditions of starvation, transition to an elongated mitochondrial network state might represent a shift from glycolysis toward mitochondrial respiration, as respiration can produce more ATP per molecule of glucose. Elongation of the mitochondrial network can also prevent starvation-induced autophagic clearance of mitochondria. Fission on the other hand, although commonly seen during states of nutrient excess, also promotes mitophagy, which can be beneficial during fasting. Fission is also necessary for mtDNA replication and transmission, which is important for mitochondrial biogenesis and increasing mitochondrial turnover together with mitophagy.

In *C. elegans*, I observed that mitochondrial networks were remodeled during IF, but appeared relatively more fragmented following 48h of fasting and relatively more fused following 48h of refeeding. While these observations may contradict some of the pre-existing knowledge of mitochondrial restructuring during starvation and nutrient excess, most of the studies mentioned were done in cell culture models, isolated from the environment of the whole organism. My

studies were done in an *in vivo* context, and could account for some differences. It is also possible that some organism variation exists between nematodes and mammals or yeast. Another possibility is that preferential utilization of different substrates may account for the variation. There is little known about how changes in substrate utilization are associated with changes in mitochondrial morphology. I observed that 48h fasting increases ACS-2 levels using a reporter I generated using CRISPR to introduce a GFP molecule at the C-terminus of endogenous worm *acs-2*. In qPCR data of wildtype, *fzo-1* and *drp-1* worms, *fzo-1* mutants that have highest degree of mitochondrial network fragmentation also showed highest expression of *acs-2*. Unpublished work from collaborators in the Nika Danial Lab at Dana Farber Cancer Institute reported that different subtypes of cancer cells have different mitochondrial morphologies depending on their fuel preference – cancer cells reliant on FAO consistently have more fragmented mitochondrial networks, whereas glycolytic cancer cells consistently showed more fused mitochondrial networks. The relationship between fatty acid oxidation and mitochondrial fission has also been indicated in some studies of brown adipose tissue during thermogenesis (Wikstrom et al., 2014). Drp1 phosphorylation at S616 and mitochondrial fission was induced upon activation of uncoupled respiration in brown adipocytes. Activated brown fat preferentially oxidized fatty acids in uncoupled mitochondria to increase heat production, as opposed to ATP synthesis. Through unclear mechanisms, mitochondrial fission promoted enhanced uncoupling and sensitivity to fatty acids during heat generation. Although current knowledge is limited as to the ways in which mitochondrial morphology may facilitate choice of substrate utilization, these studies and my findings highlight that further work in this area will greatly ameliorate our understanding of mitochondrial morphology and metabolic flexibility (see Chapter 4.2).

3.11.3 Functional outputs of mitochondrial plasticity for IF-longevity

The specific functions coupled to different mitochondrial network states are not well-understood in the context of aging. The results I presented in this chapter suggested that mechanisms

affecting specific metabolic rewiring downstream of mitochondrial network remodeling were directly linked to lifespan extension mediated by IF. To understand the functional consequences on the organismal level, I probed transcriptomic changes in wild type animals and in animals with adynamic mitochondria during Fed and Fasted conditions. The goal of this experiment and analyses were to extract information on fasting-induced gene changes that occured under wild type conditions, but were differentially regulated upon co-inhibition of *drp-1;fzo-1*. Using DE and interaction analyses followed by GO term and KEGG pathway analyses, I was able to detect a subset of genes and cellular pathways that indicated specific changes to ABC transporters and cellular metabolism (Figure 3.9), notably tyrosine metabolism, retinol metabolism, pyruvate metabolis and sphingolipid metabolism. These data provide the first evidence for a direct link between mitochondrial plasticity, rewiring of specific metabolic pathways, and IF longevity.

Among the genes that were specifically upregulated by fasting in WT but failed to show induction in Mutants was mrps-23, a 28S mitochondrial ribosomal protein. Mechanistic studies investigating how mitochondrial translation could be perturbed and contribute to inhibition of IF longevity by *drp-1;fzo-1* have not been done, but it can be speculated that mitonuclear translation imbalances resulting from altered mitochondrial translation could impact OXPHOS or other mitochondrial functions that hinder metabolic adaptations to IF. Validation of *mrps-23* expression changes using qPCR will be necessary to corroborate RNA-seq results. It would be interesting to measure changes in mitochondrial translation through western blots assessing levels of nuclear-encoded versus mitochondrial-encoded OXPHOS proteins, such as ATP5A (ATP synthase F1 subunit alpha) versus MTCO1 (mitochondrially encoded cytochrome c oxidase I), respectively. We can monitor mitochondrial translation using metabolic labeling after inhibition of cytosolic translation by cycloheximide. I have also worked on methods to directly measure mitochondrial translation in *C. elegans*, by generating a worm that expresses 3xFlag-tagged *mrpl-23*, a mitochondrial ribosome subunit that shows homology to yeast mitoribosome subunit MrpS17, previously used for successful mitoribosome immunoprecipitation and profiling (Couvillion et al., 2016). Preliminary testing suggested we can specifically pull-down *C. elegans*

mitoribosomes, but the technique has not been optimized and the use of this tool is out of the scope of my thesis work.

The list of genes and pathways also identified *cyp-33D3* and several other cytochrome P450 family proteins with ATFS-1 binding sites, *lips-12* lipase related gene, alcohol dehydrogenase *sodh-2*, and cermaide glucosyltransferase *cgt-1* as prime candidates for validation and downstream mechanistic studies. Since results from transcriptomics revealed that the most significant DE genes clusters lie in metabolic pathways, it will be important to directly follow up on these findings through metabolomics, lipidomics, and metabolic tracing, to determine sytems level changes to organismal metabolism resulting from inhibition of *drp-1* and *fzo-1* during fasting. Baseline measurements of mitochondrial respiration (Seahorse) in *drp-1;fzo-1* were performed previously and showed slight elevated basal respiration. Assessing *drp-1;fzo-1* respiration upon fasting challenge should be done next. Furthermore, it is imperative to examine specific changes to mitochondrial metabolism. In the lab, we have been developing a method for rapid mitochondrial immunoprecipitation in worms. We HA-tagged the mitochondrial scaffolding protein-1 (*mics-1*), which has homology to the human outer mitochondrial membrane protein 25 (OMP25) and has been shown to enable rapid mito-pulldown for matrix metabolite profiling in cell culture (W. W. Chen et al., 2017). Once this tool is optimized will be useful for investigating changes to mitochondrial specific proteome or metabolome.

To understand pathway components that could be involved in IF longevity, I performed targeted epistasis studies and found that mitochondrial sirtuins *sir-2.2; sir-2.3* are seemingly not involved in IF-longevity (Supplemental Figure A.2). I tested if peroxisome matrix import was involved in IF-longevity using a partial loss of function mutant in *prx-5* (Supplemental Figure A.4), but further studies should be done using AID-mediated degradation of endogenous PRX-5 using the strain I generated (Chapter 2). Interestingly, I observed that the *C. elegans* orthologues of CREB transcription factor, *crh-1* and *crh-2*, blocked IF-longevity (Supplemental Figure A.3). There is no current evidence linking mitochondrial plasticity and CREB-regulated transcription, but should

be investigated in the context of IF.

While a specific causal mechanism has not been delineated at this stage of my work, the data I collected are among the first evidence for a direct connection between mitchondrial dynamics, downstream metabolic reprogramming that requires mitochondrial plasticity during IF, and organismal longevity. Direct next steps would be to investigate specific gene candidates identified from my RNA-seq, such as the mitochondrial ribosome protein *mrpl-23*, the several cytochrome P450 family proteins with ATFS-1 binding sites, the lipase *lips-12*, the alcohol dehydrogenase *sodh-2*, and the cermaide glucosyltransferase *cgt-1*. Metabolomics would be a useful systems approach to corroborate transcriptomic changes indicative of specific fasting-induced metabolic reprogramming impaired by *drp-1;fzo-1*. For these future systems approaches, in addition to WT Fed, WT Fast, *drp-1;fzo-1* Fed, and *drp-1;fzo-1* Fasted, I would also include conditions for WT Refed and *drp-1;fzo-1* Refed to assist in filtering out acute fasting changes versus long-term systemic changes that are more likely to be coupled to the longevity phenotype. Overall, this work has provided novel evidence for a direct link between mitochondrial dynamics, metabolic reprogramming and IF longevity. I have elucidated multiple gene candidates to follow up on with direct mechanistic experiments to establish the causality of specific metabolic rewiring during IF longevity.

3.11.4 A causal role of mitochondrial plasticity for aging and longevity

Finally, in this chapter, I utilized a new inducible model for temporal and tissue-specific control of DRP-1 and FZO-1 in *C. elegans*. The novelty of this system was that it enabled me to temporally degrade FZO-1 and intermittently induce mitochondrial network fission. Using this technique, I was remarkable able to show that intermittent mitochondrial fission to mimic those changes observed during natural cycles of intermittent fasting was sufficient to extend lifespan in *C. elegans* (Figures 3.10 and 3.11). This suggests a potential causal link between mitochondrial plasticity

and IF-longevity, and it is critical going forward to elucidate the specific functional mechanisms by which mitochondrial remodeling mediates lifespan extensions. The first functional studies I have performed indicate that rewiring of specific metabolic pathways are core mechanisms coupling mitochondrial plasticity to IF longevity. To determine the causality and sufficiency of mitochondrial plasticity for lifespan extension, it will be important to assess whether synthetic induction of intermittent mitochondrial fission is able to recapitulate any pro-longevity signatures of IF on the level of the organism transcriptome or metabolome, and mitochondrial specifc functions.

Overall, my results indicate that there DR and IF promote longevity in *C. elegans* via some independent pathways that require different states of the mitochondrial network. These results would be strengthened by additional epistasis analyses to test whether the lifespan extending effects are additive. If lifespan extension is further increased by IF and DR than each intervention alone would sugest that they function in distinct mechanisms. I also showed that intermittent induction of mitochondrial fission was sufficient to increase longevity using the AID system. In addition to the imaging results showing perturbed mitochondrial network morphology upon treatment of Auxin, more robust control experiments showing direct degradation of DRP-1 and FZO-1 at the protein level should be made using Western blots. Transcriptomic profling and functional analyses support the hypothesis that metabolic adaptations are an important mechanism for IF-longevity. However, since DRP-1 is a large GTPase of the dynamin superfamily, alternative hypothesis that should be tested is whether depletion of mitochondrial fusion and fission proteins increases longevity via perturbation of organismal GTP levels that either directly or indirectly affect other known longevity pathways, such as mTORC1, that rely on GTPase signaling. Control experiments making direct measurements of GTP concentration and supplementation of GTP in *drp-1;fzo-1* animals should be done to eliminate this alternative explaination for results.

3.12 Methods

Source for *C. elegans* strains

Wild type N2 strain and transgenic strains, unless otherwise noted, were directly obtained from Caenorhabditis Genetics Center (CGC) or made in the lab. The *C. elegans* strains used for this work are listed below.

Table 3.1: ***C. elegans* strains made and/or used in this study, genotype and source**

Strain Name	Genotype	Notes	Source
N2 Bristol	Wild type	-	CGC
CU6372	*drp-1 (tm1108) IV*	-	CGC
WBM1273	*drp-1(tm1108) IV*	outcrossed 6x to lab maintained N2	Mair lab
CU5991	*fzo-1(tm1133) II*	-	CGC
WBM1420	*fzo-1(tm1133) II*	outcrossed 6x to lab maintained N2	Mair lab
WBM653	*drp-1(tm1108) IV;fzo-1(tm1133) II*	-	Mair lab
PX627	*fxIs1[pie-1p::TIR1::mRuby,I:2851009] I;spe-44(fx110[spe-44::degron]) IV*	Outcrossed 5x (Kasimatis et al., 2018)	CGC
WBM1219	*drp-1(tm1108) IV; fxIs1[pie-1p::TIR1::mRuby] I; spe-44(fx110[spe-44::degron]) IV*	*drp-1(tm1108)* mutant crossed into auxin-inducible sterile background	Mair lab
WBM1220	*fzo-1(tm1133) II; fxIs1[pie-1p::TIR1::mRuby] I; spe-44(fx110[spe-44::degron]) IV*	*fzo-1(tm1133)* mutant crossed into auxin-inducible sterile background	Mair lab
WBM1540	*drp-1(tm1108) IV; fzo-1(tm1133) II; fxIs1[pie-1p::TIR1::mRuby] I; spe-44(fx110[spe-44::degron]) IV*	*drp-1(tm1108);fzo-1(tm1133)* double mutant crossed into auxin-inducible sterile background	Mair lab

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Table 3.1 – *Continued from previous page*

Strain Name	Genotype	Notes	Source
WBM1480	*drp-1(wbm88) [degron::drp-1] IV; fzo-1(wbm84) [degron::fzo-1]; ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II; wbmIs97[eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]*	*drp-1::AID; fzo-1::AID; eft-3p::TIR1; eft-3p::tomm-20(1-49aa)::GFP* used for imaging	Mair lab
WBM1541	*drp-1(wbm88) [degron::drp-1] IV; fzo-1(wbm84) [degron::fzo-1]; ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II*	*drp-1::AID; fzo-1::AID; eft-3p::TIR1* used for lifespan	Mair lab
WBM1479	*drp-1(wbm88) [degron::drp-1] IV; ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II; wbmIs97[eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]*	*drp-1::AID; eft-3p::TIR1; eft-3p::tomm-20(1-49aa)::GFP* used for imaging	Mair lab
WBM1459	*drp-1(wbm88) [degron::drp-1] IV; ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II*	*drp-1::AID; eft-3p::TIR1* used for lifespan	Mair lab
WBM1455	*drp-1(wbm88) [degron::drp-1] IV*	5x Outcrossed	Mair lab
WBM1424	*fzo-1(wbm84) [degron::fzo-1] II; wbmIs97 [eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]; ieSi57 [eft-3p::TIR1::mRuby::unc-54 3' UTR +Cbr-unc-119(+)] II*	*fzo-1::AID; eftp3p::TIR1; eft-3p::TOMM20::GFP* used for imaging	Mair lab
WBM1467	*fzo-1(wbm84) II [degron::fzo-1]; ieSi57 [eft-3p::TIR1::mRuby::unc-54 3' UTR +Cbr-unc-119(+)] II*	*fzo-1::AID; eft-3p::TIR1* used for lifespan	Mair lab
WBM1393	*fzo-1(wbm84) [degron::fzo-1] II; wbmIs97 [eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]*	*fzo-1::AID; eft-3p::tomm-20(1-49aa)::GFP*	Mair lab
WBM1466	*fzo-1(wbm84) II [degron::fzo-1]*	6x Outcrossed	Mair lab
WBM1443	*fzo-1(wbm84) [degron::fzo-1] II ; wbmIs83 [rab-3p::3xflag::TIR1::rab-3 3'UTR *wbmIs66] IV*	*fzo-1::AID* with neuronal TIR1	Mair lab
WBM1255	N2, *wbmIs102[eft-3p::crh-1 cDNA::unc-54 3' UTR, *wbmIs65]*	-	Mair lab

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Table 3.1 – *Continued from previous page*

Strain Name	Genotype	Notes	Source
WBM1226	*crh-1(n3315) III; fxIs1I; spe-44(fz110[spe-44::degron]) IV*	*crh-1* in auxin-inducible sterile background	Mair lab
crh-1 (n3315)	*crh-1(n3315) III*	-	-
VS10	*hjIs37 [Pvha-6::mRFP-PTS1 + C. briggsae unc-119(+)]*	2x Outcrossed	CGC
VS15	*hjIs8 [ges-1p::GFP-PTS1]*	5x Outcrossed	CGC
SJ4103	*zcIs14[myo-3p::GFP(mit)]*	3x Outcrossed	CGC
WBM1231	N2, *wbmIS97[eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]*	6x Outcrossed	Mair lab
WBM1232	N2, *wbmIS98[eft-3p::tomm-20(1-49aa)::mCherry::unc-54 3'UTR, *wbmIs65]*	-	Mair lab
WBM1233	*drp-1 (tm1108) IV; wbmIS97[eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]*	-	Mair lab
WBM1234	*drp-1 (tm1108) IV; wbmIS98[eft-3p::tomm-20(1-49aa)::mCherry::unc-54 3'UTR, *wbmIs65]*	-	Mair lab
WBM1235	*fzo-1(tm1133) II; wbmIS97[eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]*	-	Mair lab
WBM1236	*fzo-1(tm1133) II; wbmIS98[eft-3p::tomm-20(1-49aa)::mCherry::unc-54 3'UTR, *wbmIs65]*	-	Mair lab
WBM1237	*prx-11(wbm49) I; wbmIS97[eft-3p::tomm-20(1-49aa)::GFP::unc-54 3'UTR, *wbmIs65]*	-	Mair lab
WBM1444	*tomm-70(wbm81) III*	Endogenous TOMM70::GFP marker 6x Outcrossed	Mair lab
WBM1177	N2, *wbmIs81[eft-3p::3xFlag::GFP::PTS1::unc54 3'UTR, *wbmIs65]*	Ubiquitous peroxisome targeted GFP	Mair lab

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Table 3.1 – *Continued from previous page*

Strain Name	Genotype	Notes	Source
WBM1221	*prx-11(wbm49) I; wbmIs81[eft-3p::3xFlag::GFP::PTS1::unc54 3'UTR, *wbmIs65]*	3x Outcrossed	Mair lab
WBM1222	*prx-11(wbm49)*	6x Outcrossed	Mair lab
WBM1446	*prx-5(wbm87) II [degron::prx-5]*	5x Outcrossed	Mair lab
MH5239	*prx-5(ku517) II*	-	CGC
WBM938	*acs-2(wbm9) V*	-	Mair lab
WBM1303	*fzo-1(tm1133) II; acs-2(wbm9) V*	-	Mair lab
WBM1304	*drp-1(tm1108) IV; fzo-1(tm1133) II; acs-2(wbm9) V*	-	Mair lab

C. elegans husbandry

C. elegans were cultured and maintained on standard sterilized nematode growth media (NGM) plates seeded with *Escherichia coli* (OP50-1). *E. coli* bacteria were cultured overnight in LB at 37 °C, after which 100 μl of liquid culture was seeded on plates to grow for 2 days at room temperature.

Preparation of Nematode Growth Medium (NGM) plates

C. elegans were maintained on 60 mm plates where sterilized Nematode Growth Medium (NGM) containing 12 g NaCl, 10 g Bacto-peptone, 80 g Agar-agar, 4000 ml of MilliQ water, 100 ml 1 M Potassium phosphate buffer pH 6, 4 ml 1M $CaCl_2$, 4 ml 1M $MgSO_4$, 4 ml 5 mg/ml cholesterol (dissolved in ethanol), 4 ml 100 mg/ml carbenicillin (only if preparing NG Carb plates for RNAi). MEDIACLAVE 10 (INTEGRA) was used for the preparation of NGM plates for worm culturing.

Note: Plates used for experiments should be at room temperature. If using plates stored at 4 °C, remove plates from 4 °C 24-48 hours before use and store on bench surface.

Preparation of *C. elegans* bacterial food source

C. elegans were maintained on standard streptomycin-resistant *E. Coli* strain OP50-1 from CGC. To prepare *E. coli* for *C. elegans* feeding, OP50-1 frozen glycerol stocks were streaked on LB plates containing 100 μg/ml streptomycin and incubated overnight at 37 °C to allow colonies to grow. Single colonies were selected to inoculate LB broth and grown overnight at 37 C with constant agitation. 100 μl OP50-1 cultures were seeded to the center of 60 mm NGM plates. Plates seeded with OP50-1 were incubated at room temperature for 2 days to allow bacterial lawn to grow to adequate density to attract worms to the center of the plate. For 10 cm NGM plates, 500 μl OP50-1 cultures was used and spread to cover the entire surface of the plate. For all experiments, plates were prepared with fresh 2-day seeded OP50-1 and used immediately. Plates with bacteria can be stored at 4 °C for up to 1 month and used for routine maintenance of *C. elegans* stocks and for crosses.

Maintaining *C. elegans* stocks for experiments

C. elegans stocks were maintained at 20 °C. Stocks were fed for at least 2 generations before experiments. Larvae can survive complete starvation for up to a few months in the dauer state, but starvation can significantly affect physiology and lifespan due to epigenetic changes that can persist for multiple generations (Rechavi et al., 2014). To maintain *C. elegans* stocks, a few worms of different stages were transferred to new NGM plates with OP50-1 every 3 to 4 days using a platinum worm pick. The tip of the worm pick is sterilized on a Bunsen burner after every transfer to prevent contaminants from growing on plate surfaces and more importantly, to prevent the cross contamination of worm strains of different genotypes. Fresh thaw of frozen worm stocks were requested after worms have been in culture for more than 3 months to avoid genetic drift.

Freezing *C. elegans*

C. elegans can be frozen in media containing glycerol and stored for years in -80 °C freezer or liquid nitrogen. All strains used in the lab were frozen in two types of freezing media:

Liquid freezing media was used for frozen stocks of *C. elegans* designated for long-term storage, as it gives better viability. To make 1L, combine 5.8 g sodium chloride, 50 ml 1M potassium phosphate buffer pH 6, 300 ml glycerol and water, autoclave to sterilize.

Soft agar freezing media was used for frozen stocks of *C. elegans* designated for working stocks, as it allows for multiple thaws from one vial. To make 500 ml, combine 2.9 g sodium chloride, 3.4 g monobasic potassium phosphate (KH_2PO_4), 4.4 g dibasic potassium phosphate (K_2HPO_4), 150 ml glycerol and water; adjust pH to 6.0; aliquot to 50 ml bottles and autoclave to sterilize, then add 0.2 g agar to each bottle.

All strains received from external sources or new transgenic strains generated were frozen immediately to prevent genetic drifting. Fresh thaws of strains were obtained from these frozen stocks every three months to prevent genetically drifted strains in our working cultures and improve reproducibility between experiments.

Thawing *C. elegans* frozen stocks

For thawing worm strains from a soft agar stock, use a sterile 1000 μl pipette tip to scoop around the edge of the vial and remove 1/4 of the frozen solution onto an OP50-1 seeded NGM plate. Return the vial immediately to its storage box in the -80 °C freezer. Once the soft agar liquid thaws and dries on the plate, check for sufficient larvae in the thaw. Allow the worms to recover by putting the plate in 20 °C. Stocks with liquid freezing media can be thawed at room temperature and the entire contents poured onto a 60 mm NGM plate with bacteria.

Bleaching *C. elegans*

Bleaching is a procedure routinely used to clean up *C. elegans* cultures contaminated with

unwanted fungi or bacteria, to shift worm cultures from one bacterial food source to another, or to age-synchronize a population. Bleach dissolves the cuticle of worms and other contaminants, but does not destroy eggs due to their protective eggshell. A population of many gravid adults is the best stage for bleaching.

To bleach C. elegans:

Gravid adult worms were washed from NG plates with M9 buffer. Worm pellets were centrifuged at 2500 rpm for one minute at room temperature. Pellets were washed with 15 ml M9 buffer to remove residual bacteria by inverting the tube several times. Bleach solution was added to worm pellets and shaken or vortexed rigorously for 2-5 minutes at RT with constant monitoring for release of eggs. Once all the worms dissolved, released eggs were centrifuged at 2500 rpm for one minute at RT to pellet. Egg pellets were washed three times with 15 ml M9 buffer to get rid of remaining bleach solution. Bleached egges were pipetted to new NGM plates with desired food source.

Genetic Crosses

C. elegans sex is determined by the number of X chromosomes: hermaphrodites have two and males have one X chromosome (Nigon and Dougherty, 1949). *C. elegans* strains are mostly maintained as self-fertilizing hermaphrodite cultures. Males are particularly useful for crosses and can be made by heat shocking to promote chromosomal nondisjunction.

To make crosses between genotypes:

L4 stage hermaphrodites were picked to a plate with a small amount of food, along with 8-10 males at the young adult–day 1 adult stage. Worms were allowed to mate and reproduce for 3-4 days, then F1 generation progeny that were hermaphrodites at the L4 stage were picked onto individual plates and allow to self-fertilize. Another 3 days later, F2 generation progeny that are hermaphrodites at the L4 stage were singled out onto individual plates. F2s are allowed to self-fertilize for 2-3 days before being lysed for genotyping.

To make male worms:

L4 larvae were heat-shocked at 30 °C for 4-6 hours. 1% population of males is expected in the F1 progeny. Males were crossed with N2 hermaphrodites to maintain a 50% N2 male population.

Lifespan assays

Lifespan assays were performed on standard NGM plates at 20 °C. Animals were fed with OP50-1 *E. coli* except in the case of RNAi experiments, which employed dsRNA-expressing HT115 bacteria. All worms were kept fed for at least two generations after thawing to minimize transgenerational effects of starvation on lifespan (Rechavi et al., 2014). To obtain a synchronized population of worms for lifespan assays, gravid adult worms were bleached. Approximately three days later, 10 gravid day 1 adult worms were picked onto each plate for a short, timed egg lay, after which the adult worms were removed. When working with genotypes that were developmentally delayed, bleaches and egg lays were staggered so that all worms reached Day 1 adult stage for beginning of lifespan. Day 1 of lifespan is defined as the day worms start to lay eggs. When progeny from the egg lay reached Day 1 of adulthood, 100 worms were transferred to 5-10 fresh plates at 20-25 worms per plate and this was considered time = 0. Worms were transferred to fresh bacterial lawns every 1-2 days to separate from progeny until day 10-13. Survival of populations were scored every 1-2 days as “Alive” or “Dead” or “Censored”.

Animals were scored as “Alive” if they moved spontaneously or moved in response to gentle prodding of the head or tail.

Animals were scored as “Dead” if they displayed no spontaneous movement of any part of their body and were unresponsive gentle taps of the head and tail three times.

Animals were censored from statistical analyses if progeny hatched internally, if worms left the agar media and desiccated on the walls of petri dishes, or if worms “exploded” due to loss of vulval integrity and could be seen with significant portions of internal tissues hanging outside of the cuticle.

Intermittent fasting lifespans using FUDR:

A population of 100-160 worms per condition were synchronized using bleaching and egg laying. Worms were bleached/egg layed onto NGM plates seeded with standard OP50-1. For the lifespans presented in Chapter 1.1, worms were transferred to plates containing the drug 5-fluoro-2'-deoxyuridine (FUDR) on Day 1 adulthood to prevent internal egg hatching. To prepare FUDR plates, 60 mm NGM plates were seeded with OP50-1 bacteria 2.5 days prior to use, plates were swirled to increase the surface area that bacterial lawn covered the plate, and incubated at RT for 48 hours to grow. After 48 hours, 100 μL of antibiotic solution (5 mg/mL Kanamycin and 10 mg/mL Carbenicillin in sterile milliQ water) was applied directly on top of the bacterial lawn to arrest bacteria growth (Total antibiotic concentration in plate 0.05 mg/mL Kan, 0.1 mg/mL Carb). Once antibiotic solution was completely dry, 1 mg/mL FUDR was diluted 50X in M9 solution, filter sterilized through a 0.2 μm Supor®Membrane filter, and 100 μL was seeded on top of the bacteria lawn of each plate. FUDR was allowed to diffuse into the plates overnight, covered with aluminum foil because FUDR is light-sensitive. Plates were used for lifespan transfers the following morning. On Day 3, worms were transferred to fasted plates containing kanamycin (0.05 mg/mL), carbenicillin (0.1 mg/mL), FUDR (0.05 mg/mL), prepared in the same manner above, directly on top of plates, one day before use. On Day 5, worms were transferred to fed plates containing kanamycin (0.05 mg/mL), carbenicillin (0.1 mg/mL), FUDR (0.05 mg/mL). Continue 48h intervals of alternate day feeding/fasting until Day 13. On Day 13, worms were transferred to fed plates containing kanamycin (0.05 mg/mL), carbenicillin (0.1 mg/mL), without FUDR. Worms were thereafter scored every 1-2 days for survival. After all worms were marked dead, survival data were analyzed with GraphPad Prism 9 and statistical significance was determined with Log-rank (Mantel-Cox) test.

Intermittent fasting lifespans using Auxin-inducible sterility system:

A population of 100-160 worms per condition were synchronized using bleaching and egg laying. Worms were bleached/egg layed onto NGM plates containing 0.15 mM Auxin seeded

with standard OP50-1. On Day 1 adulthood, worms were transferred off Auxin to standard NGM plates seeded with OP50-1 bacteria arrested with kanamycin (0.05 mg/mL) and carbenicillin (0.1 mg/mL). On Day 3 adulthood, worms were transferred to fasted NGM plates without food containing kanamycin (0.05 mg/mL) and carbenicillin (0.1 mg/mL). This 48h interval of alternate day feeding/fasting continued until Day 13. On Day 13, worms were transferred to fed OP50-1 plates containing kanamycin (0.05 mg/mL), carbenicillin (0.1 mg/mL). Worms were thereafter scored every 1-2 days for survival. After all worms were marked dead, survival data were analyzed with GraphPad Prism 9 and statistical significance was determined with Log-rank (Mantel-Cox) test.

For other lifespans using the AID system, animals were exposed to 0.15 mM Auxin from hatch or from Day 1 adulthood, as mentioned. Since auxin is dissolved in ethanol, Control plates for AID experiments contained an equal concentration of ethanol, without auxin.

RNA isolation and cDNA synthesis

Total RNA was extracted using Qiazol (QIAGEN), column purified by RNeasy mini kit (QIAGEN) according to manufacturer's instructions. cDNA was synthesized using SuperScript®VILO Master mix (Invitrogen).

Quantitative RT-PCR

StepOne Plus instrument from Applied Biosystems was used to perform real-time qPCR experiments following the manufacturer's instructions. For each qPCR reaction, 5ng of cDNA was used. Relative expression differences were calculated with the comparative $2\Delta\Delta$Ct method using Y45F10D.4 (Ce02467253_g1) as the endogenous control. For each gene in each strain, fold-change relative to the average of wild type control group was calculated and statistical significance evaluated using Welch's t test. GraphPad Prism 9 was used for all statistical analysis and graph plotting.

RNA-Sequencing

More than 1000 worms were used for each sample. Four biological replicate samples were collected for each genotype. Worms were collected in M9 buffer and washed three times in M9 buffer to remove bacteria. Liquids were removed after last centrifugation, and QIAzol lysis reagent (Qiagen, 79306). Samples were snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. To break the worm cuticle and improve RNA yield, all samples underwent five freeze-thaw cycles. In each cycle, samples were thawed at 37 °C and then snap-frozen in liquid nitrogen. RNA extraction was performed using QIAGEN RNeasy Mini Kit (QIAGEN, 74104) following manufacturer's instructions. Libraries were prepared using mRNA HyperPrep, poly-A selection (Roche) sample preparation kits according to the manufacturer's protocol. Library quality was confirmed using TapeStation system (Agilent Technologies). Uniquely indexed libraries were pooled at an equimolar ratio and sequenced on an Illumina NovaSeq SP Flow Cell using paired-end 50bp read length by the Harvard University Bauer Sequencing Core.

Differential Gene Expression Analysis

The sequencing data were processed using the bcbio-nextgen RNA-Seq pipeline (Chapman et al., 2020). The sequencing reads were merged for each sample and the quality was examined using FastQC (Andrews, 2010). STAR (Dobin et al., 2013) was used to align the reads to the *C.elegans* reference genome (WBcel235, Ensembl release 104). The alignment metrics were checked using QualiMap (Okonechnikov et al., 2016) and Samtools (Li et al., 2009) and summarized using MultiQC (Ewels et al., 2016). Gene expression was obtained by estimating transcript abundance using Salmon (Patro et al., 2017) and aggregating to gene-level counts using tximport (Soneson et al., 2015). DESeq2 (Love et al., 2014) was used for identifying differentially expressed genes and ClusterProflier (G. Yu et al., 2012) was used for functional analysis.

Confocal microscopy

Worms were anesthetized in 0.1 mg/ml tetramisole in 1X M9 buffer on empty NGM plates and mounted on thin 2% agarose pads on glass slides with 0.05 mm Polybead microspheres (Polysciences) for immobilization. A No. 1.5 cover glass was gently placed on top of worms and sealed with clear nail polish. Images were performed on a Yokogawa CSU-X1 spinning disk confocal system (Andor Technology, South Windsor, CT) with a Nikon Ti-E inverted microscope (Nikon Instruments, Melville, NY), using a Plan-Apochromat 100x/1.45 objective lens. Images were acquired using a Zyla cMOS camera and NIS elements software was used for acquisition parameters, shutters, filter positions and focus control. Image analysis was performed using Fiji software.

Electron microscopy

High Pressure Freezing in C. elegans:

Worms were washed off the fed plate with M9 buffer and swirled gently to suspend in liquid. Worms were washed twice with M9 at 2500 rpm for 60 seconds to get rid of bacteria. A small droplet containing 6-10 worms suspended in M9 were pipetted onto a 0.4 um, 100 μm deep side of type A 6 mm Cu/Au carriers (Leica), sandwiched with the flat side of type B 6 mm Cu/Au carriers (Leica). A 13% polyvinylpyrrolidone (PVP) cryoprotectant solution was added to the carrier such that the entire disk was covered with a liquid or filler. This was frozen in a high-pressure freezer (EM ICE, Leica) and followed by freeze substitution protocol.

Freeze substitution:

Freeze substitution protocol followed as described in Kristopher Burkewitz lab, Vanderbilt University.

Cocktail 1: 0.5% glutaraldehyde, 0.1% tannic acid in anhydrous acetone

Cocktail 2: 2% osmium tetroxide in anhydrous acetone

Program:

Cocktail	Temperature (start)	Temperature (end)	Time
Cocktail 1	-120 °C	-90 °C	3 h
Cocktail 1	-90 °C	-90 °C	114 h
Acetone wash (6x)	-90 °C	-90 °C	2 h
Cocktail 2	-90 °C	-20 °C	12 h
Cocktail 2	-20 °C	-20 °C	12 h
Cocktail 2	-20 °C	0 °C	4 h
Acetone wash (6x)	0 °C	0 °C	2 h

Samples were infiltrated in 1:1 TAAB Epon (https://taab.co.uk) as follows:
3:1 Acetone:TAAB Epon - 3hrs at RT 1:1 Acetone:TAAB Epon – ON at 4 °C 1:3 Acetone:TAAB Epon - 4hrs at RT 100% Acetone:TAAB Epon – ON at 4 °C

Samples were embedded in fresh TAAB Epon between two sheets of Aclar plastic (in order to create a thin layer of resin) and polymerized at 60 degrees °C for 48 hours.

After polymerization, the Aclar plastic was peeled off and individual animals were cut out and glued onto a block of resin. Ultrathin sections (approximately 80nm) were cut on a Reichert Ultracut-S microtome, picked up on to formvar/carbon coated copper grids and stained with lead citrate. The sections were examined in a JEOL 1200EX Transmission electron microscope and images were recorded with an AMT 2k CCD camera.

4 Discussion, Significance, and Future Directions

4.1 Variable efficacy of dietary interventions that promote healthy aging

As our scientific knowledge of the basic molecular mechanisms by which DR and IF promote longevity in multiple species expands, it is becoming increasingly evident that we must pivot the future of aging research towards emphasis on individual variability and personalized medicine. In multiple instances, the interaction of diet with sex, genotype, age, metabolic state, or physiological condition, can lead to beneficial, ineffective, or worse, harmful outcomes on health and longevity. The specific dilution of DR has been shown to have different effects on lifespan in different genotypes (Lucanic et al., 2017; Mitchell et al., 2016; Wilkie et al., 2020). In some mice, DR does not provide longevity benefits or is minimally effective (Harper et al., 2006; Turturro et al., 1999; Colman et al., 2009), and in other mice strains, DR can be deleterious (Barrows and Roeder, 1965; Fernandes et al., 1976; Forster et al., 2003; Harrison and Archer, 1987; Liao et al., 2010).

Similar response variability has been observed for IF. One of the earliest IF studies in rats reported up to 80% lifespan extension by IF (Kendrick, 1973; Carlson and Hoelzel, 1946. While numerous subsequent studies since then have backed these findings, often the magnitude of lifespan

extension was less dramatic. A study in male C57BL/6 mice however showed that IF delayed the onset of lethal neoplastic disorders, but without changes in most other parameters of aging, such as neurobehavioral functions, platelet counts, optical lens density, plasma immunoglobulin (Xie et al., 2017). Some parameters such as thickness of cortical bone and responsiveness to auditory function were even decreased in the IF group. Detrimental effects on longevity have even been observed in some cases. In a study using different mouse genetic backgrounds (C57BL/6J, A/J, or a hybrid of the two), when alternate day feeding was initiated in early life at 1.5 or 6 months of age, mean and maximum lifespan were increased in all three strains. However, when IF was initiated in late life (at 10 months), C57BL/6J and hybrid mice showed no significant increase in mean lifespan. In A/J mice, mean and maximum lifespan were reduced 15% by late-life IF (Goodrick et al., 1990). In another study, old male C57BL/6J mice fed a different variation of IF, a time-restricted high fat diet (with 16-hour daily fasting), showed protection against obesity, hyperinsulinemia, hepatic steatosis and inflammation with improved motor coordination. In rodents, IF enhances cognitive performance, improves insulin sensitivity and reduces blood pressure. Many studies in humans also support that IF improves many parameters of aging: protection against obesity and diabetes, cardiovascular incidents, improved cognitive function. However other studies indicate that the duration of fasting can have negative outcomes in some groups. A study in humans showed that the risk of gallstone disease nearly doubles between women who fast for 8 hours per day and those who fast for over 14 hours per day (Longo et al., 2021). Meta-analysis studies have also shown that skipping breakfast, the most common method of TRF used to reach a daily 14- to 18-hour daily fasting period, is associated with an increased risk of mortality from cardiovascular and all-cause mortality in the US population (Sun et al., 2022).

Furthermore, the time window of fasting to match natural circadian cycles may affect its efficacy. Studies have shown that aligning feeding periods to the active phase (nighttime for rodents and daytime for humans) prevents weight gain and metabolic disorders (Woodie et al., 2018), while

mismatching feeding periods to inactive phases could have the opposite effect (de Goede et al., 2019; Opperhuizen et al., 2016; de Goede et al., 2022). Aligned with the benefits of time-restricted feeding (TRF) on metabolic regulation if feeding is tuned to circadian rhythms, a recent study show that this could be due to improved rhythmicity in mitochondrial respiration. Specifically, strong rhythmicity was found for maximal OXPHOS capacity with combined NADH-substrates and succinate and uncoupled respiration if feeding was tuned to the active phase (de Goede et al., 2022). These enhancements were dampened or reversed if animals were fed "at the wrong time." TRF during the inactive phase reduced uncoupled respiration, had overall lower 24h respiration rate, and dampened the rhythmicity in expression of the core clock gene Bmal1, uncoupling protein 3 (Ucp3) and fatty acid synthase (Fasn) (de Goede et al., 2022). The expression of genes involved in mitochondrial biogenesis and the fission/fusion machinery were also altered in these animals, with a concomittant alteration in metabolite and lipid fluctuations (de Goede et al., 2022).

These studies taken together suggest that IF can have positive, negative or neutral effects on longevity depending on species, genotype, age, and sex. Fasting may be beneficial and protective against aging in young and middle-aged animals, but may have some detrimental effects in old animals after they begin to lose weight or become frail. Very limited studies have examined micronutrient intake and potential deficiencies that could be induced by IF that may have harmful effects in old individuals. One study reported mild reductions in sodium intake and adequate calcium intake for both CR and IF during the intervention period (Conley et al., 2018). More studies conducting direct comparisons between IF at different biological ages (young, middle, and old age) and disease states must be performed before IF becomes a mainstream and accepted therapeutical approach.

I have observed effects in my studies of intermittent fasting in worms that parallel these ideas. I observed immense interactions between genotypes and germline function mediating longevity responses to IF. Specifically, there was a peculiar interaction between *drp-1* null animals and

5-Fluoro-2'-deoxyuridine (FUDR), a uridine analogue routinely used, and necessary, to prevent larval development in *C. elegans* under conditions of stress, such as fasting/ starvation. FUDR significantly extended lifespan of *C. elegans* in a *drp-1* null mutant. Existing methods of performing IF in worms has been done using FUDR to inhibit larval development and death due to bagging. Using this method of IF, I and others observed that *drp-1* + FUDR extended lifespan to a degree mimicking IF-longevity. As a result, I sought to remove the variable of FUDR from the IF protocol, first by beginning IF later in life, at Day 6 following the reproductive period. However, I found that the longevity effects of IF were not induced late-onset. I next turned to the auxin-inducible degradation (AID) system to induce sterility in *C. elegans*. This method was previously published and utilizes a 44 a.a. degron tag on *spe-44*, a gene expressed in germline at the onset of spermatogenesis. Degradation of SPE-44 results in sterility upon simultaneous expression of germline-specific (pie-1p) TIR1, an F-box protein component of an E3 ligase complex derived from Arabidopsis that upon exposure to the plant hormone Auxin, targets degron-tagged proteins for rapid proteasomal degradation. I exposed animals to 0.15mM auxin from hatch throughout development and showed that this concentration had no effect on wildtype N2 *C. elegans* lifespan and retained robust lifespan extension by IF. The AID-sterility background also had no significant interaction when crossed into the *drp-1* null background at baseline (no exposure to auxin). *Drp-1;spe-44::degron;pie-1p::TIR1* worms had lifespans that were the same as their wildtype controls, suggesting that there is no confounding interaction between AID-sterility and *drp-1* genotypes. Exposure to auxin throughout the 3-day *C. elegans* developmental period, induced sterility in adult worms. I showed that IF extends lifespan in wildtype AID-sterility worms, as well as *drp-1* AID sterility worms. This response to IF is contrary to previous results with FUDR, and suggests that response to IF in different genotypes can vary depending on the sterility method. Since many interventions that extend lifespan (including DR, CA-AMPK, reduced mTOR, ETC deficiency) are also known to cause trade-offs in growth and reproduction, it is possible that the interactions between germline function and genotypes have significance in predicting responses to these types of longevity interventions. In this case, these

confounding interactions make the longevity-response to IF difficult to interpret and it is worth investing future research efforts to understand the mechanisms that link germline function and IF-longevity. Worms are hermaphrodites, but these interactions with the reproductive system highlight the need to conduct IF studies in both sexes to determine sex-specific efficacy in both males and females. Multiple studies in mammals have already shown sex-specific responses to DR, DR mimetics and IF, some of which are described above. Much of the evidence of longevity benefits of IF has been extrapolated from these animal studies, although early data in humans are also encouraging. No studies of any fasting regimes have been performed in children, pregnant or lactating women, the very old or underweight individuals, and it is possible that IF could show different, and potentially harmful results in these groups.

Considering that humans are genetically heterogenous, further understanding of these confounding interactions with IF must be determined to accurately predict an individual's response to the dietary intervention as an anti-aging therapy. A high degree of genetic or epigenetic variation also exists within an isogenic population, and it has been seen in laboratory grown wildtype *C. elegans*, or inbred *Drosophila* and mouse strains that are for the most part genotypically controlled, high variance exists both for lifespan itself and response to IF, DR, or DR mimetics. For interventions such as single amino acid methionine restriction, the median lifespan of mice and rats is substantially increased, but there is a subpopulation that die sooner than their non-restricted counterparts (Miller et al., 2005). Despite extensive research, the type, quantity, and combination of nutrients that optimize healthy longevity remain controversial due to the oftentimes variable results seen in different groups. Understanding the mechanisms driving differences in response to IF within diverse genetic, epigenetic, and physiological backgrounds will be important to prescribe a personalized approach to optimize health and longevity.

4.2 Metabolic flexibility during aging

In this study, I showed that interfering with mitochondrial dynamics, and specifically mitochondrial plasticity, was detrimental for the longevity effects of intermittent fasting. By ablating key membrane fusion and fission factors, FZO-1 and DRP-1 respectively, at both the gene and protein level, I was able to force mitochondrial networks into a static or adynamic state. Doing so abolished the natural rhythmic response of mitochondria to remodel in response to periods of feeding and fasting. Mitochondrial machinery are responsible for carbon combustion switches between alternative fuel sources, and these periodic transitions in fuel choice, i.e. gluocose or fat oxidation, depending on the physiological circumstances are hallmarks of normal energy metabolism. My results suggest that these metabolic switches occur not only at the biochemical level, but may be facilitated, or even orchestrated at the level of physical organelle remodeling. It is well-known that many diseases of aging stem from dysregulated activity of fatty acid and glucose metabolism pathways (e.g. elevated glucose leading to insulin resistance and diabetes, and favorable environments for cancer cells with high reliance on glucose utilization). The paradigm of intermittent fasting and the specific requirement of mitochondrial plasticity for its longevity effects highlight that mechanisms regulating the metabolic switch itself and the flexibility to switch freely between alternative forms of carbon energy may play dominant roles in aging.

Isolated skeletal muscle mitochondria from rats fed high-sugar or high-fat diets showed reduced metabolic flexibility, indicating that substrate switching begins at biochemical network interactions within the mitochondria (Jorgensen et al., 2017). The term metabolic flexibility was first coined by Kelley et al. in a study reporting that lean individuals readily adapted fuel preference to fasting or insulin infusions (Kelley et al., 1999). Obese patients did not increase fatty acid oxidation after fasting, or reduce FAO after insulin infusion, and this apparent stiffness in mitochondrial substrate selection was deemed to be a metabolically inflexible state (Kelley et al., 1999; Kelley and Mandarino, 2000). Metabolic flexibility is orchestrated by local feedback regulation

mechanisms within the mitochondrial matrix, and extend to complex cell and systemic signaling networks. After feeding, glucose and insulin are high, glucose uptake, glycolysis, and pyruvate oxidation are favored and FAO is suppressed. This results in an increase in concentration of malonyl-CoA, which allosterically inhibits carnitine palmitoyltransferase-1 (CPT-1), lowering transport of fatty acids into the mitochondria for beta-oxidation. During fasting, AMPK is activated and phosphorylates acetyl-CoA carboxylase (ACC), leading to its inhibition. Inhibition of ACC lowers malonyl-CoA concentrations, resulting in increased activity of CPT-1 and amplified transport of fatty acids into the mitochondria for beta-oxidation. Increases in acetyl-coA, NADH and ATP from upregulated FAO then allosterically inhibit pyruvate dehydrogenase, the mitochondrial enzyme coupling glycolysis to glucose oxidation (R. L. Smith et al., 2018). These glucose-fatty acid feedback and feedforward loops, first described by Randle et al. initiate the biochemical transition between feeding and fasting (Randle et al., 1963; Randle et al., 1998). On top of these local mechanisms, metabolic flexibility is also regulated by transcriptional reprogramming, endocrine mediators of cross-tissue metabolism, and epigenetic modifications. (R. L. Smith et al., 2018). These mechanisms assist metabolic adaptation to acute bioenergetic challenges, but emerging evidence also suggests that long-term benefits for health and longevity can accrue from intermittent metabolic switching. Metabolic flexibility is negatively correlated with aging (Stull et al., 2010). During prolonged fasting the metabolic switch from glycolysis to ketosis (keto-adaptation) is necessary for survival as fat-derived ketone bodies become the predominant energy source (McSwiney et al., 2018). Aged animals take longer than young to produce high levels of circulating ketone bodies, and time-restricted feeding reversed markers of insulin-related metabolic deficits and accelerated metabolic switching in aged animals (Hernandez et al., 2020). In *Drosophila*, just one month of an IF regime at the beginning of adulthood was sufficient to extend lifespan (Catterson et al., 2018). This was not due to reduced fecundity. IF led to higher lipid content in old flies, showed a significant reduction in age-related pathologies and improved gut barrier function. These mechanisms were found to be independent of the TOR pathway. Furthermore, many studies are emerging that the metabolic switch between fed

and fasted states is among the variables that drive DR's improvement in health and survival (Mitchell et al., 2019; Green et al., 2019; Pak et al., 2021). A recent study measured a series of physiological and molecular markers across DR and fasted mice, showing that prolonged fasting was responsible for many of the metabolic and geroprotective benefits of DR. Fasting was required for DR-induced changes in insulin sensitivity and fuel selection. Metabolic chambers were used to measure respiratory exchange ratio (RER) and determine substrate utilization. Both DR and Fasted animals showed increased fatty acid oxidation, but not animals fed a diluted AL diet. Additionally, fasting alone without reduced energy intake was sufficient to recapitulate the transcriptomic and metabolomic signature of a CR diet. Fasting was also required for DR-induced improvements in glucose metabolism, frailty and lifespan in C57BL/6J male mice (Pak et al., 2021).

The core concept of metabolic flexibility is linked to the mitochondria and the functional capacity of mitochondria to switch fuel preferences in response to bioenergetic changes (Muoio, 2014). Many studies support the idea that mitochondrial dysfunction underlies the onset of metabolic inflexibility (Muoio, 2014), although a causal link between the two still remains to be fully established. Here, I establish a link between mitochondrial morphology, mitochondrial plasticity and longevity mediated by intermittent fasting. Furthermore, I showed that inducing mitochondrial plasticity at the level of organelle remodeling was sufficient to promote longevity in animals, even in the absence of any dietary intervention. These results suggest that therapeutics targeted at direct manipulation of mitochondrial dynamics have potential to improve health and survival in animals without the need for dietary restriction of intermittent fasting regimens that are difficult to implement and have low compliance long-term. Directly targeting mitochondrial dynamics to affect downstream functional outcomes is potentially able to bypass the associated negative side effects of DR and IF on growth retardation, fecundity and mental health. The link between mitochondrial plasticity and metabolic flexibility remains unclear, but are immediate next questions of deep interest. In the lab, we have begun to generate tools for mitochondrial

pull-downs to assess mitochondrial metabolites. Metabolomics in fed versus fasted states in animals with wild type or adynamic mitochondria should be performed to assess alterations in specific metabolic pathways affected by loss of mitochondrial plasticity. These experiments that establish a causal link between mitochondrial plasticity and metabolic flexibility are open research questions of profound importance.

4.3 Homeodynamics: a new perspective on the process of aging

The process of aging has been historically described as a progressive loss of cellular homeostasis. The work I presented here challenges this perspective and proposes that as a field, we should deviate from the notion that aging is a mere loss of homeostasis around a fixed equilibrium of youth. Living systems are dynamic in nature, and nonlinearities and coupling between processes are inherent to most biological networks. Organisms cannot be considered in isolation from its environment, as the exchange of matter and energy with their surroundings, the coordination of fluxes between the internal and external milieu, and the tuning of biological rhythms with the periodic or oscillatory patterns of the natural environment (i.e. circadian) all suggest that biological systems exists in a dynamic equilibrium. Therefore, the ability to coordinate cellular 'homeodynamics', rather than 'homeostasis', may be a more accurate measure of healthy aging.

Therapeutic solutions invested in restoring the inherent dynamism of biological systems therefore represent important approaches to promote healthy aging. One great example of this is the field of circadian biology and how interventions that enhance circadian rhythms also protect against age-related pathologies and metabolic dysregulation (Longo and Panda, 2016; Manoogian and Panda, 2017; Zarrinpar et al., 2016). Modulating the activity of the major circadian regulators BMAL1 (also known as ARNTL; aryl hydrocarbon receptor nuclear translocator-like) and CLOCK (clock circadian regulator) results in premature aging. Importantly, studies of time-restricted feeding (TRF), one of the variations of intermittent fasting, have demonstrated that synchroniz-

ing feeding and fasting times to the circadian nature of many metabolic processes is a viable method to ameliorate healthy aging. In mice, TRF can counteract the detrimental effects of high-fat diets on metabolism, including slowed body fat accumulation, improved glucose tolerance, and improved dyslipidemia. In humans, TRF can also lower blood pressure and improve glycemic control in obese and overweight individuals. In *Drosophila*, it was recently shown that TRF increases lifespan by 18% in females and 13% in males, and protects against declines in muscular, neuronal and intestinal function with age (Ulgherait et al., 2021). Moreover, if the TRF schedule was misaligned to the active and inactive phases, TRF produced no benefit on longevity through mechanisms linked to enhanced clock-driven autophagy at night. Longevity was also maximized when initiated at day 10 of adulthood and terminated at day 40. Beyond day 40, there was no clear lifespan benefit of TRF, and even possible shortening of lifespan when TRF was continued at old age. These studies teach us important lessons about how enhancing biological rhythmicity can counteract the onsought of metabolic inflexibility during aging.

4.4 Final Summary and future directions

My PhD work demonstrates that mitochondrial dynamics are differentially required for Dietary Restriction and Intermittent Fasting mediated longevity. In particular, this work highlights the context-dependent role of balanced mitochondrial dynamics versus mitochondrial plasticity in these two longevity paradigms. I establish a causal link between mitochondrial plasticity and IF-longevity by showing that direct synthetic manipulation of mitochondrial plasticity to mimic IF conditions are sufficient to extend lifespan without the need for dietary alterations. To accomplish this work, I generated a series of new imaging tools for studying mitochondrial biology, and its interactions with other metabolic organelles such as the peroxisome. These tools will be widely applicable for research in the field of mitochondrial dynamics and aging in *C. elegans*.

Some of the immediate next steps derived from my work will be to establish the specific mechanisms coupling mitochondrial plasticity, metabolic flexibility, and IF-longevity. Moreover, a causal link between mitochondrial plasticity and IF-longevity should be determined if these mechanisms are conserved upon induction of mitochondrial plasticity directly at the level of organelles without upstream changes to dietary nutrient signaling. Revealing the specific tissue and temporal requirement of mitochondrial plasticity will also further our understanding of the mechanisms of IF-longevity. I have generated tools to ask these follow-up questions, which are already being implemented by others in the lab to address these outstanding knowledge gaps.

Finally, to gain a fuller understanding of the specific functional outputs associated with balanced mitochondrial dynamics versus mitochondrial plasticity, it will be important to investigate why co-inhibition of *drp-1* and f*zo-1* is able to restore DR-longevity but completely abolish IF-longevity. I performed RNA-seq in *drp-1;fzo-1* animals for IF, but a comparison of the differences in organismal level changes to transcriptome, metabolome, lipidome, or proteome in *drp-1;fzo-1* animals on DR need to be done. The results I presented in this thesis open doors to a myriad of follow up experiments required to understand the specific mechanisms by which balanced mitochondrial dynamics promotes DR longevity and mitochondrial plasticity promotes IF longevity. These are fascinating objectives for future research and ultimately pivots the direction of aging research towards inclusion of cellular homeodynamics and metabolic flexibility as hallmarks of healthy aging.

A Appendix

A.1 Supplemental data figures

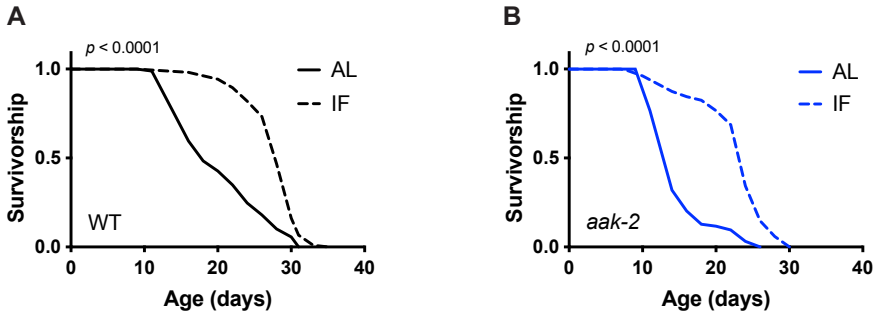

Figure A.1: **Deletion of *aak-2* does not affect IF-longevity in *C. elegans*.**

A. IF extends lifespan in wild type animals. (****p < 0.0001 by log-rank test, n=2 biological replicates)

B. IF extends lifespan in *aak-2(ok524)* animals that lack the α subunit of AMPK, suggesting *aak-2* is not involved in the mechanism of IF-longevity in *C. elegans*. (****p < 0.0001 by log-rank test, n=2 biological replicates)

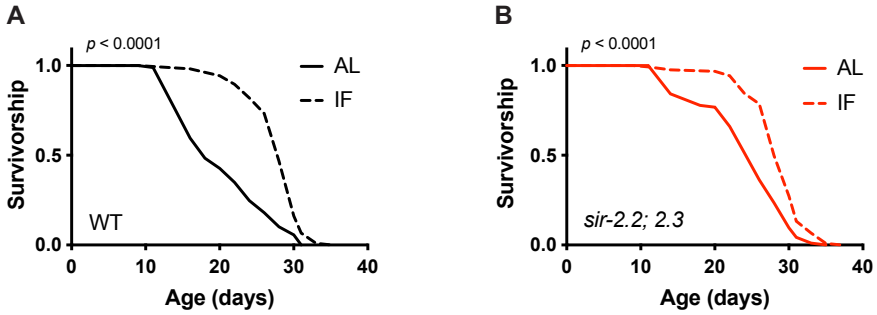

Figure A.2: **Double knockout of mitochondrial *sir-2.2; sir-2.3* does not affect IF-longevity.**

A. IF extends lifespan in wild type animals. (****p < 0.0001 by log-rank test, n=1 biological replicates)

B. IF extends lifespan in *sir-2.2; sir-2.3(wbm38)* animals that lack the C. elegans homologue of the mammalian mitochondrial SIRT4. *sir-2.2* and *sir-2.3*, suggesting they are not involved in the mechanism of IF-longevity in *C. elegans*. (****p < 0.0001 by log-rank test, n=1 biological replicates)

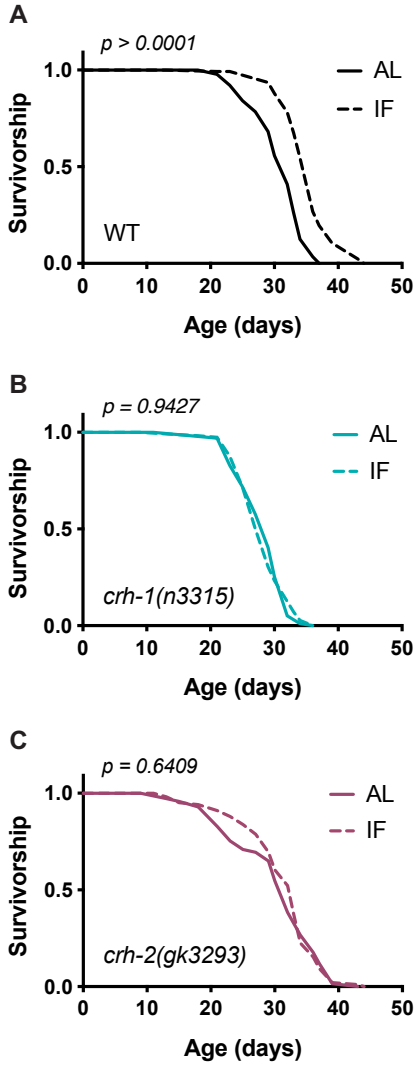

Figure A.3: **Deletion of *crh-1* and *crh-2* abolished IF-longevity in *C. elegans.***

A. Wild type animals respond to IF-longevity. (****p < 0.0001 by log-rank test, n = 3 biological replicates)

B. *crh-1(n3315)* animals that lack the worm orthologue of the CREB transcription factor do not respond to IF-longevity. This suggests that *crh-1* may be involved in the mechanism of IF-longevity in *C. elegans.* (ns p = 0.9427 by log-rank test; n = 3 biological replicates)

C. *crh-2(gk3293)* animals that lack the worm orthologue of the CREB2L transcription factor do not respond to IF-longevity. This suggests that *crh-2* may be involved in the mechanism of IF-longevity in *C. elegans.* (ns p = 0.9427 by log-rank test; n = 2 biological replicates)

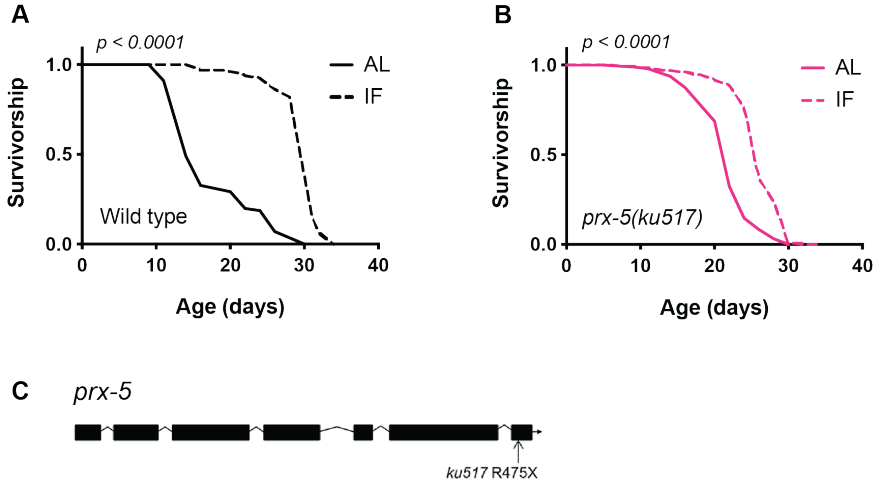

Figure A.4: **Mutants in peroxisomal matrix import showed mildly blunted response to IF-longevity**

A. Wild type animals respond to IF-longevity. (****p < 0.0001 by log-rank test, n = 1 biological replicates)

B. *prx-5(ku517)* animals that have a point mutation in C-terminal portion of the worm orthologue of PEX5 had mildly blunted response to IF-longevity. (****p < 0.0001 by log-rank test, n = 1 biological replicates)

C. Diagram showing position of mutation in *prx-5(ku517)* allele. The *ku517* allele contains a C-T substitution that changes CGA coding for Arginine 475 to a TGA stop codon in the *prx-5* coding sequence. *Figure adapted from R. Wang et al., 2013 with permission.*

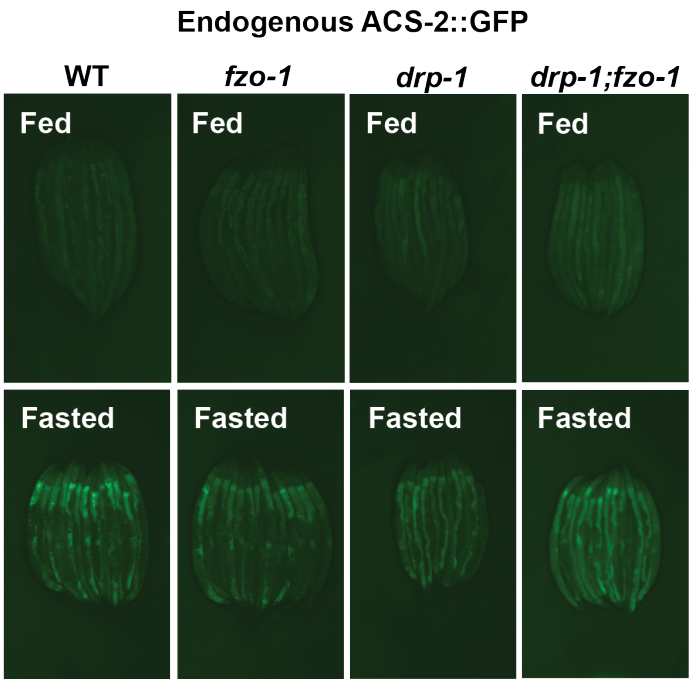

Figure A.5: **Endogenous ACS-2 induction upon 48 hours fasting in *C. elegans* with perturbed mitochondrial dynamics.**
Wild type, *fzo-1*, *drp-1* and *drp-1;fzo-1* animals showed induction of *acs-2* expression upon 48 hours of fasting. *Acs-2* induction was assessed using a reporter expressing a C-terminal GFP fusion to endogenous *acs-2*. Images were taken at day 4 of adulthood on a Zeiss Discovery V8 microscope equipped with an Axiocam camera at 1300 ms exposure. Worms were anaesthetized using tetramisole were placed on NGM plates.

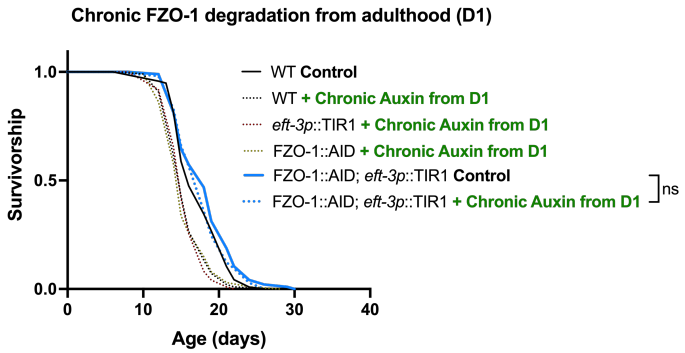

Figure A.6: **Chronic FZO-1 degradation from day 1 adulthood did not affect lifespan.** Chronic FZO-1 degradation upon exposure to 0.15 mM auxin from day 1 of adulthood resulted in no significant difference in lifespan compared to no auxin controls (ns p = 0.3530 by log-rank test; n = 2 biological replicates).

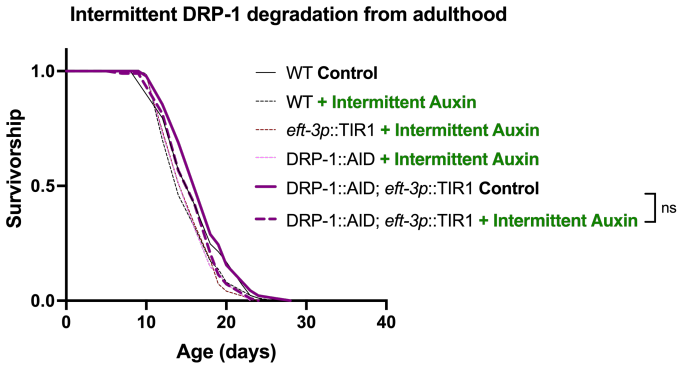

Figure A.7: **Intermittent DRP-1 degradation from day 1 adulthood did not affect lifespan.** Intermittent DRP-1 degradation upon exposure to 0.15 mM auxin from day 1 of adulthood resulted in no significant difference in lifespan compared to no auxin controls (ns p = 0.0899 by log-rank test; n = 2 biological replicates).

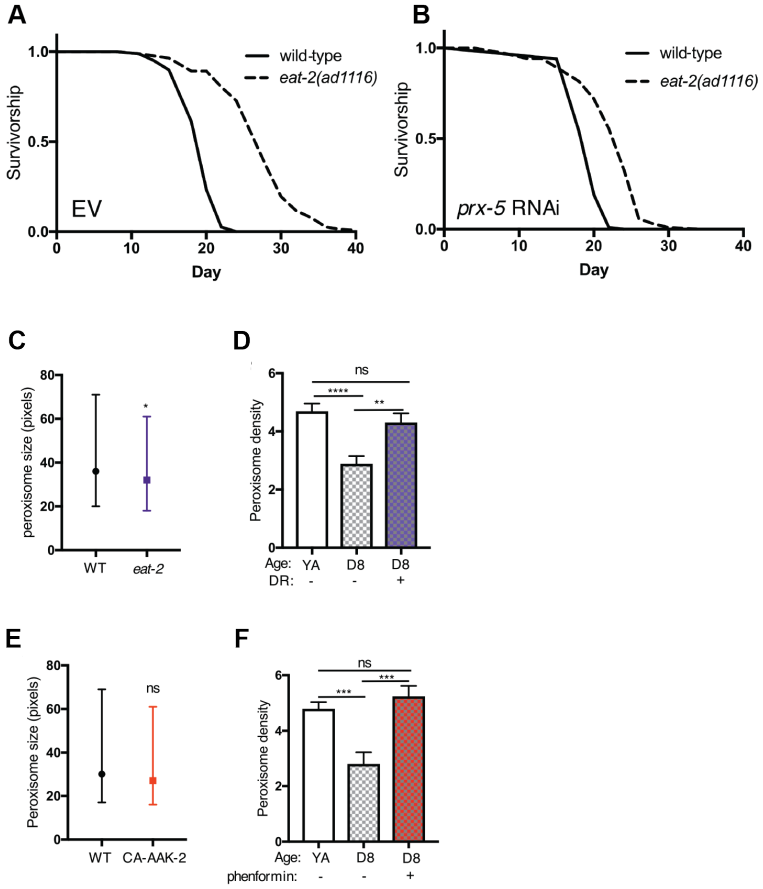

Figure A.8: **DR and AMPK promote longevity through delaying of age-related dysfunction of peroxisomes.**
A-B. Knockdown of *prx-5* severely blunted DR-longevity.
C-D. Peroxisome size and density were measured in *ad libitum* or DR animals, at young adults and day 8. sDR and *eat-2* protected against age-associated decline in peroxisome density. (****p < 0.0001 by log-rank test, n = 3 biological replicates)
E-F. Peroxisome size and density were measured in wild type control animals or animals with activated AMPK at young adults and day 8. Pharmacological activation of AMPK with phenform protected against age-associated decline in peroxisome density. (****p < 0.0001, ***p < 0.001, **p < 0.001, *p < 0.05, ns p > 0.05 by unpaired t test with Welch's correction; n = 3 biological replicates)

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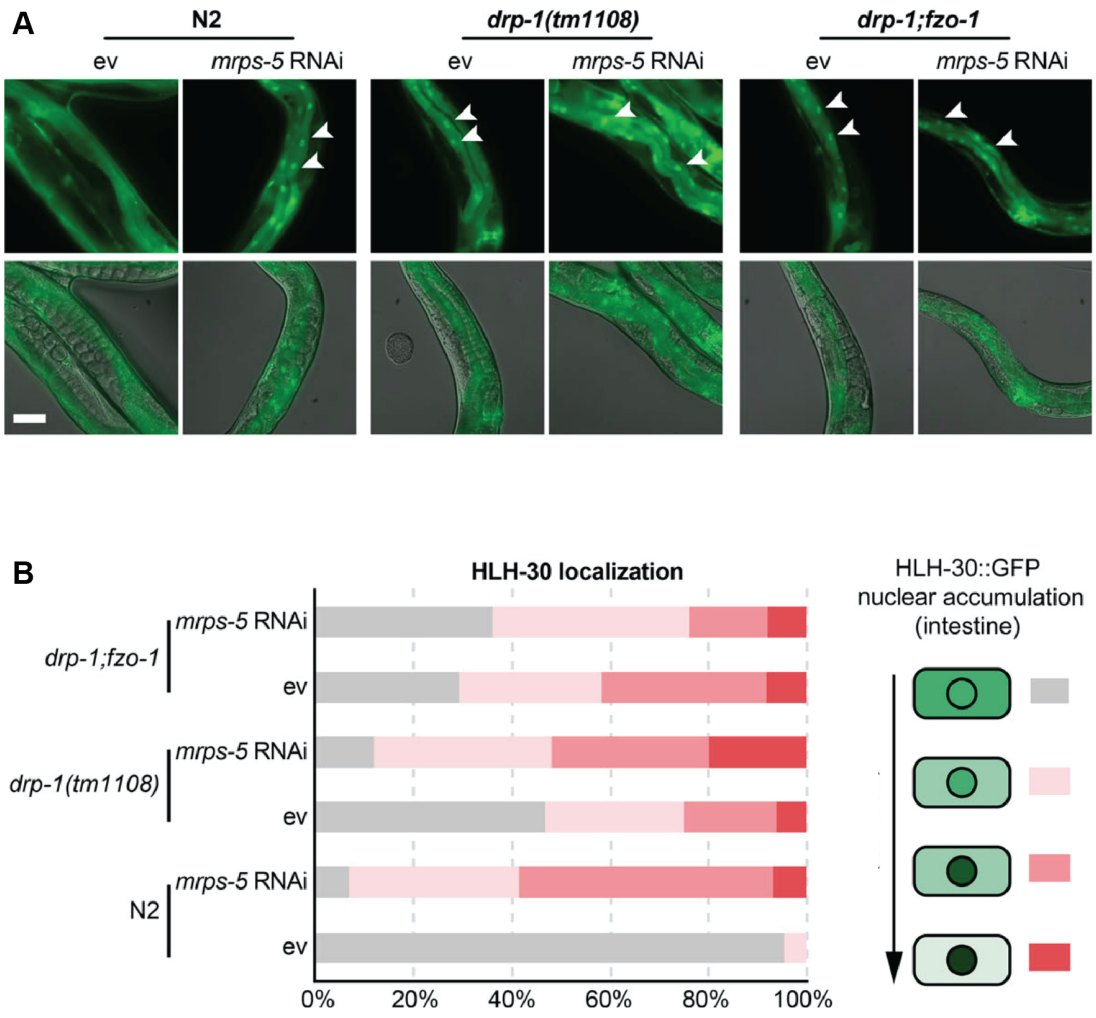

Figure A.9: **HLH-30/TFEB drives longevity from combined inhibition of mitochondrial translation and dynamics.**
A. Representative images of HLH- 30::GFP nuclear localization in intestinal cells of wild-type, *drp-1(tm1108)* mutant, and *drp-1;fzo-1* double mutant worms at day 2 of adulthood. HLH-30 is mostly cytosolic in WT ev and *mrps-5* RNAi induces nuclear translocation of HLH-30. Baseline nuclear localization of HLH-30 is increased in *drp-1* mutants, and even more so in *drp-fzo-1* double mutants. Scale bar 100 μm. White arrowheads indicate intestinal nuclei.
B. Quantification of HLH-30 nuclear enrichment. n = 21–32 different animals per RNAi treatment, pooled from two independent experiments.χ^2 tests were performed to assess the significance of differences in the levels of HLH-30::GFP nuclear enrichment in N2, drp-1(tm1108) mutant, and drp-1;fzo-1 mutant upon mrps-5 RNAi. WT ev v. *mrps-5* RNAi (p= 0), *drp-1* ev v. *mrps-5* RNAi (p = 0.0314), *drp-1;fzo-1* ev v. *mrps-5* RNAi (p = 0.5533).

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A.2 List of abbreviations

AID	Aucin-inducible degradation
AMPK	AMP-activated Protein Kinase
DR	Dietary Restriction
C. elegans	Caenorhabditis elegans
bDR	Bacterial Liquid Dietary Restriction
sDR	Solid Plate Dietary Restriction
BD	Bacterial Deprivation
Bmal1	Brain and muscle arnt-like protein-1
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DRP-1	Dynamin-related protein 1
ER	Endoplasmic reticulum
ERMES	ER-mitochondria encounter structures
FIS1	Mitochondrial fission 1 protein
FOXO	Forkhead box O
FZO-1	Fuzzy onioins
IF	Intermittent Fasting
IIS	Insulin/IGF-1 like signaling
IMM	Inner mitochondrial membrane
IP3R	Inositol 1,4,5-triphosphate receptor
MFF	Mitochondrial fission factor
MFNs	Mitofusins
MiD49/51	Mitochondrial dynamics protein 49/51
mtDNA	Mitochondria DNA
MTFP1	Mitochondrial fission process protein 1
NAD	Nicotinamid adenine dibucleotide
NIA	National Institute of Aging
OMM	Outer mitochondrial membrane
OPA1	Optic Atrophy 1
PUM2	Pumilio2
SCFA	Short-chain fatty acid
SERCA	Sarco/endoplasmic reticulum Ca2+ ATPase
SKILODGE	Single-copy knock-in loci for define gene expression
S6K	Ribosomal Protein S6 Kinase
TFEB	Transcription Factor EB
TOR	Target of Rapamycin
TORC1	Target of Rapamycin Complex 1
UPR	Unfolded protein response
UPRmt	Mitchondrial unfolded protein response

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