Coupling Protein Catabolism to Lifespan and Reproduction in *Caenorhabditis elegans*

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirement for the Degree of
Doctor of Philosophy in the Department of Biology
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By

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ABSTRACT

There is an undisputable link between aging and reproduction and it has both puzzled and fascinated biologists for decades. Evolutionary biologists suggest that the rate of aging depends on the complex tug-of-war between maintenance of the soma and maintenance of the germ cells. The former is essential for longevity while the latter is essential for transmitting genetic information from parents to progeny. Any perturbation in the fertility and fecundity of *C. elegans* influences lifespan and vice-versa. Interestingly, germline deficient animals have increased resistance to environmental and proteotoxic stress.

All eukaryotic cells reproduce for a finite amount of time before irreversibly ceasing reproduction, a phenomenon called reproductive senescence. Recent research has been aimed at trying to elucidate the genetic factors that regulate reproductive and post-reproductive lifespan. As part of this ongoing process, the initial part of my work aims to characterize the increased reproductive lifespan of a *C. elegans* mutant that is deficient for the gene *rer-1*. I further demonstrate that *rer-1* mutants show a higher level of autophagy which is responsible for the enhanced reproductive lifespan of these mutants.

Aging is thought to be a stochastic process, and cessation of reproduction is one of the biological hallmarks of aging. Although both reproduction and aging are well studied processes, there is very little mechanistic understanding of how these processes are connected and coordinated. The latter part of this study aims to answer some of these questions about the cross-talk between the germ cells and somatic tissue in *C. elegans* using a panel of sterile hermaphrodites impaired for specific stages of reproduction. My findings show that cessation of fertilization triggers a signalling cascade from the germ cells to the soma and that this signalling is brought about by steroid hormones, presumably synthesized by the somatic gonad. In actively reproducing worms, the forkhead transcription factor DAF-16 drives expression of *vha* genes which encode a multi-subunit proton pump that is responsible for maintaining lysosomal acidity. Nuclear exclusion of DAF-16 in post-reproductive worms co-ordinately reduces the expression of *vha* genes resulting in lysosomal alkalinization that culminates in acute loss of fitness and contributes to organismal senescence. My data shows a plausible mechanistic pathway by which lysosomal acidity is regulated via gonad to soma signalling in young (reproducing) animals.

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LIST OF ABBREVIATIONS USED IN THIS STUDY

AIR	Aurora/Ipl-1 related kinase
ALP	Autophagy lysosome pathway
APC	Anaphase promoting complex
ATF	cAMP-dependent transcription factor
ATM	Ataxia telangiectasia
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
C. elegans	*Caenorhabditis elegans*
cDCFDA	5-(and-6)-carboxy-2',7-dicholorofluorescein diacetate (carboxy-DCFDA)
cDNA	Complimentary DNA
CGC	Caenorhabditis genetics center
ChIP	Chromatin immunoprecipitation
COPI	Coat protein complex I
COPII	Coat protein complex II
DA	Dafachronic Acid
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid, a molecule that encodes genetic information
DR	Dietary restriction
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMB	Abnormal embryogenesis
ER	Endoplasmic reticulum
ERAD	Endoplasmic Reticulum-associated protein degradation
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box protein O
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLH	Helix loop helix

IGF-1	Insulin-like growth factor 1
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRE	Inositol requiring protein kinase
KDa	Kilodaltons
LIPL	Lipase like
LMP	Lysosome-associated membrane protein
NBRP	National Bioresource Project
NGM	Nematode growth media
NHR	Nuclear hormone receptor
PAGE	Polyacrylamide gel electrophoresis
PBS-T	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
qPCR	Quantitative polymerase chain reaction
RER	Retention in Endoplasmic Reticulum
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RT-PCR	Real-time polymerase chain reaction
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TFEB	Transcription Factor EB
TM	Tunicamycin
TMP	Trimethyl psoralen
TOR	Target of Rapamycin
UPP	Ubiquitin proteasome pathway
UPR	Unfolded protein response
UV	Ultraviolet

v-ATPase	Vacuolar-adenosine triphosphatase
VHA	Vacuolar H^+ ATPase
VMA	Vacuolar membrane ATPase

1 INTRODUCTION

NOTE: This is a general introduction section which gives a brief overview about current theories of aging. Each chapter will also have a specific introduction section focused on the current status of research for that particular chapter.

1.1 Aging

Aging can be defined as the gradual functional decline of complex organisms that eventually results in death. Aging is arguably the biggest risk factor for countless human diseases; therefore, understanding the aging process may facilitate the discoveries of novel treatments for age-associated diseases. It is estimated that at least 80% of health care costs arise after the age of 45 (Alemayehu et al., 2004), suggesting that aging is also an enormous economic challenge. Hence, it is imperative that strategies to ensure healthy aging be designed to allow some of the growing healthcare costs to be alleviated.

One of the popular model systems to study the cellular basis of human aging is the *in vitro* culturing of human cells (de Magalhaes et al., 2008). However, *in vitro* studies of cellular processes might not accurately reflect the *in vivo* physiology of a multicellular system such as the human body (Mondello et al., 1999). Moreover, a major concern for all cellular models of aging is that such studies rely on measuring cellular replicative senescence as a marker for aging, which is not necessarily indicative of cellular vitality (Cristofalo, 2001), and hence do not provide a direct readout of aging. To address these shortcomings of cellular studies on human aging, several human genetic studies have been carried out; these studies have focused primarily on longevity (total lifespan) rather than aging (cell, tissue and organ deterioration) (T. Perls et al., 2002; T. T. Perls, 2006; Puca et al., 2001). Even so, longevity studies only provide an indirect assessment of aging without allowing us to understand its underlying physiological mechanisms, thereby questioning the relevance of such studies. Moreover, human aging takes decades to progress and is much harder to study *in vivo*.

Much of our current knowledge and understanding about human aging derives from eukaryotic model organisms such as yeast, nematodes and fruit flies. The advantages to using such model organisms are numerous: a) inexpensive growth and maintenance requirements b) abundant availability of protocols and reagents c) shorter organismal lifespan and d) possibility of conducting high-throughput drug and genetic screens (Hamilton et al., 2005; Petrascheck et al., 2007). While some scientists have questioned the correlation between mechanisms of aging in these model

organisms with human aging, it is becoming increasingly clear that the pathways and processes that influence aging in each of these species are similar (Longo, 1999; Longo et al., 2002).

1.2 Theories of aging

Over the past century, various theories have been postulated to explain aging. Some of these theories argue that aging is a stochastic process, while others propose that aging is genetically programmed. Each theory has its own merits, and currently no single theory provides a complete explanation and understanding of the aging process. Some of the more well-known theories of aging are discussed below.

1.2.1 Cellular senescence (telomere) theory of aging

During the 1930s and 1940s, Hermann Muller and Barbara McClintock postulated that eukaryotic chromosomes possess structures (referred to as "telomeres" by Muller) prevented end-to-end chromosome fusions (McClintock, 1941; Muller, 1938). This was a time when not much was known about the structure of the DNA molecule, and absolutely nothing was known about the process of DNA replication. As more and more discoveries elucidated the structure of the DNA double helix and later on the mechanism of DNA replication (which occurs simultaneously at the leading and lagging strand), it became clear that during DNA replication of linear chromosomes (such as most eukaryotic chromosomes), lagging strand DNA synthesis would stall at the last RNA primer and then stop, eventually leading to loss of the downstream DNA sequence from the chromosome. Therefore, each round of DNA replication would lead to loss of 50 to 200 bp of DNA at the 3' end; this was termed as the "end replication problem" (Watson, 1972). Alexander Olovnikov theorized that this "end replication problem" would lead to shortening of telomeres during each round of DNA replication (Olovnikov, 1971, 1973). Telomeres were therefore thought to protect the genetic information encoded by the chromosomes at the cost of telomere length. Telomere shortening was seen as a plausible explanation of why primary culture cells divided a limited number of times (called the Hayflick limit) and then irreversibly exit mitosis (Hayflick, 1965; Hayflick et al., 1961). It was hypothesized that the Hayflick limit had a direct correlation with telomere length, and that cells stopped dividing once telomeres were shortened beyond a critical threshold. In 1970s and 1980s, Carol W. Greider and Elizabeth Blackburn discovered the enzyme telomerase, a reverse transcriptase that adds TTGGGG tandem repeats to cell-free extracts

of *Tetrahymena* (Blackburn et al., 1978; Greider et al., 1985), thereby replacing the lost bit of DNA. Cancerous cells bypass the Hayflick limit by promiscuously activating telomerase activity, thereby allowing cells to divide indefinitely (Hanahan et al., 2011). This is precisely why telomerase is only expressed in certain adult stem cells and activated lymphocytes, besides male germ cells (Cong et al., 2002).

Initially, telomeres were seen as a biomarker of aging since telomere shortening was thought to be responsible for replicative senescence. Indeed, aberrations in telomere length in *S. cerevisiae* arising due to telomerase mutations was shown to lead to cellular senescence (Lowell et al., 1998; Lundblad et al., 1989). However, in other model organisms such as *C. elegans*, telomerase defects do not appear to influence aging (Raices et al., 2005). In *Drosophila*, not only are telomere sequences dramatically different from other eukaryotes, but also the entire process of telomere maintenance relies on retrotransposons instead of a canonical telomerase (Mason et al., 2008). Telomerase-deficient mice display a normal aging phenotype up to four generations following which they show some symptoms of accelerated aging (Blasco et al., 1997; Rudolph et al., 1999). Interestingly, overexpression of telomerase in mice does not alter their aging rate (Artandi et al., 2002). In humans, telomerase dysfunction causes a disease termed dyskeratosis congenita, which is somewhat reminiscent of fifth or sixth generation telomerase-deficient mice (Blasco, 2007). In conclusion, different model organisms differ vastly in their chromosomal physiology, so it is almost impossible to determine the role that telomeres play in human aging by drawing parallels with the role of telomeres in model organisms.

1.2.2 Rate of living hypothesis

More than a century ago, Max Rubner made an observation for six animal species that larger animals had a slower metabolic rate and longer lifespan compared with smaller animals (Rubner, 1908). Kleiber and others later expanded this finding to several more species (Kleiber, 1932). This led to numerous hypotheses suggesting that aging and longevity are regulated by an organism's metabolic rate. Raymond Pearl went on to suggest that animal tissues could only undergo finite chemical reactions, and exhaustion of these chemical reactions would lead to mortality (Pearl, 1928). Ultimately, organisms with higher metabolic rates would age and die sooner. This theory was termed as the rate of living hypothesis. At a time when scientists were just starting to comprehend the biology of free radicals (Gerschman et al., 1954) and how they might influence

aging, Denham Harman put forth the idea that reactive oxygen species (ROS) produced by metabolism might perhaps link metabolic rate to aging and lifespan by causing cumulative damage resulting in aging followed by death (Harman, 1956).

In order to determine whether reactive oxygen species production has a direct influence on lifespan, experiments were carried out in which mice were fed antioxidants and their average lifespan was compared to a control group of mice. A few of these studies showed that these antioxidants did decrease oxidative damage and increase average lifespan, (Heidrick et al., 1984; Holloszy, 1998; Quick et al., 2008; Saito et al., 1998) but other studies concluded that feeding mice antioxidants had no discernible effect on their lifespan (Lipman et al., 1998). Experiments that knockout or overexpress genes encoding antioxidants in mice have shown that in most cases, longevity is not affected when compared to control mice, leading to the conclusion that although oxidative damage increases during aging, there is no clear evidence to suggest that oxidative stress contributes to aging (Ho et al., 1997; Lapointe et al., 2010; Schriner et al., 2000; Sohal et al., 2002). Later on, experiments performed with mice heterozygous for the superoxide dismutase gene (encoding a protein that converts toxic superoxide (O_2^-) radicals, a byproduct of the mitochondrial electron transport chain, into hydrogen peroxide and diatomic oxygen) SOD2 or mice overexpressing SOD1 failed to show any changes in longevity, albeit the mice heterozygous for SOD2 did show increased oxidative damage (T. T. Huang et al., 2000; Van Remmen et al., 2003). Moreover, even though certain animals are longer lived than others (e.g. the naked mole-rat lives longer than mice), they do not seem to have higher levels of antioxidants compared to mice (Andziak et al., 2005). In summary, even though certain antioxidants might increase longevity, there are some antioxidants that have no effect on lifespan at all, thereby questioning the efficacy of antioxidant treatment in increasing lifespan. The enormous variation in data from different genetic and physiological studies undertaken to study the effect of antioxidants on aging suggests that the physiology of the aging process is unaltered by the production or removal of reactive oxygen species (ROS) (Sohal et al., 1990).

1.2.3 DNA damage theory

Owing to its nature as the repository for all genetic information, DNA was one of the earliest molecules to be implicated in aging. Failla put forth the earliest proposal implicating DNA damage accumulation as a cause of aging in 1958, followed by a similar proposal by Leo Szilard (Failla,

1957, 1958; Szilard, 1959). As more and more types of DNA damage and mutations were discovered, the DNA damage theory of aging changed over the years (Hoeijmakers, 2009; Vijg et al., 2002).

Experiments in mice and humans suggested that DNA modifications and chromosomal abnormalities increase with age (Lu et al., 2004; Vijg et al., 2002) and that longevity might be directly correlated with DNA repair abilities (Lu et al., 2004). One of the biggest conundrums of the DNA damage theory of aging is that it is virtually impossible to tell whether these DNA modifications are actually the effects or causes of aging. Hypothetically, even a miniscule increase in DNA repair rate over a large timespan and thousands of cell divisions will have huge consequences and could influence rate of aging. On the flip side, it could be argued that such correlations may be coincidental, because larger animal species with considerably more DNA content (and therefore a higher risk of genomic mutations) live longer than smaller animal species (Promislow, 1994).

One of the leading arguments that seeks to demonstrate a connection between DNA damage and aging is the study of certain accelerated aging (progeria) disorders in humans and mice. Most dramatic progeroid syndromes such as Werner's, Hutchinson-Gilford's, and Cockayne syndrome are the results of alternation in genes responsible for DNA repair/metabolism (Martin et al., 2000). Werner's syndrome, for example, is caused by a recessive mutation in the WRN helicase gene that also possesses an exonuclease activity, which suggests that it is involved in DNA repair (Gray et al., 1997; S. Huang et al., 1998). In fact, cells isolated from Werner's syndrome patients have increased genomic instability (Fukuchi et al., 1989). Similar to humans, various accelerated aging syndromes in mice are caused by alterations in DNA repair genes such as mouse homologues of ataxia telangiectasia mutated or ATM, *xeroderma pigmentosum* and p53 (Cao et al., 2003; de Boer et al., 2002; Wong et al., 2003).

On the other hand, mice deficient in the DNA repair protein Pms2 had higher than normal mutation levels across multiple tissues and yet seemed to show no signs of accelerated aging compared to control mice (Narayanan et al., 1997). Similarly, another study showed that mice and fly embryos bombarded with x-rays (which presumably generates thousands of double stranded DNA breaks resulting in DNA mutations) do not age faster than control animals (Cosgrove et al., 1993). Another elegant study that contradicted the DNA damage theory of aging showed that while haploid wasps that undergo DNA damage live shorter than diploid wasps, in a normal physiological

environment (without any DNA damage) haploid and diploid wasps both show similar lifespan (Clark et al., 1961), which definitely should not be the case if DNA damage causes accelerated aging.

A more plausible explanation for these conflicting opinions on the DNA damage theory of aging could be that only specific types of DNA damage might influence aging, which would explain why mutations in some genes affect aging while others do not.

1.3 Pathways influencing aging

In the past few decades, research has dramatically improved our understanding of the biochemical mechanisms that regulate the pace of aging and facilitated extraordinary breakthroughs into designing therapies that may increase both quality as well as quantity of lifespan.

1.3.1 mTOR (TOR) pathway

Mechanistic target of rapamycin, also sometimes referred to as mammalian target of rapamycin (mTOR in humans; TOR in yeast), is a protein encoded by the *mtor* gene in humans (Brown et al., 1994). MTOR is a serine/threonine protein kinase (belonging to the PI3K protein family) that has been shown to be a master regulator of various processes such as cell growth, motility, proliferation, survival and protein synthesis (Hay et al., 2004). MTOR is one of the first human targets of the antifungal agent Rapamycin (produced by *Streptomyces hygroscopicus*) that was discovered in a soil sample isolated from the island of Rapa Nui (Easter Island) in the mid-1970s (Vezina et al., 1975).

Research in the last decade has shown that reduced TOR activity leads to an increase in lifespan in yeast, nematodes and flies (Harrison et al., 2009; Jia et al., 2004; Kaeberlein et al., 2005; Kapahi et al., 2004). Subsequent research showed that inhibition of mTOR activity also increases lifespan across vertebrates (Lamming et al., 2012) (Harrison et al., 2009). It has been suggested that caloric restriction and methionine restriction increase lifespan via reduction of mTOR activity (Anderson et al., 2012; Kaeberlein et al., 2005; R. W. Powers, 3rd et al., 2006). Rapamycin has also been shown to alleviate the effects of progeroid diseases such as Hutchinson-Gilford progeria and Cockayne syndrome as well as extending lifespan in mice (Neff et al., 2013).

1.3.2 Sirtuins

Another discovery supporting the use of model organisms in aging research was the finding that overexpression of the Sir2 (silent mating-type information regulation 2) gene in yeast, flies and nematodes leads to increase in lifespan (Kaeberlein et al., 1999; Rogina et al., 2004; Tissenbaum et al., 2001). The Sir2 gene in yeast encodes a nicotinamide–adenine dinucleotide (NAD)-dependent histone deacetylase that is involved in gene silencing by forming a more closed regional chromatic structure that renders the DNA within it inaccessible (Bi et al., 1997). This led to the discovery of a small molecule called resveratrol which was shown to activate Sir2 as well as its mammalian homolog SIRT1. Resveratrol also extends the lifespan of mice fed a high-fat diet, besides providing beneficial effects in nonhuman primates that were fed a high-sugar, high-fat diet (Baur et al., 2006; Jimenez-Gomez et al., 2013). Resveratrol is now being replaced by artificially synthesized SIRT1 activating compounds (SRT1720 and SRT2104) with better specificity and efficacy and these compounds have been shown to extend lifespan in mice fed a standard diet (Mercken et al., 2014; Mitchell et al., 2014). These studies have led to the introduction of several human clinical trials using SIRT1 activators intending to find the ones with higher impact on aging and lifespan.

1.3.3 Insulin signalling pathways & Dietary Restriction (DR)

In the 1930s, Mary Crowell and Clive McCay at Cornell University found that mice fed with a low calorie diet lived up to twice as long as mice fed a normal diet (McCay et al., 1989), providing the first insight into what would be later referred to as Dietary Restriction (DR) or Caloric Restriction (CR). Decades later, Richard Weindruch showed that restricting caloric intake of mice proportionally increased their lifespan compared to a group of mice with a normal diet. These calorie-restricted mice also seemed more youthful and active and showed a delay in age-related diseases (Weindruch, 1985; Weindruch et al., 1986). At around the same time, Michael Klass performed a genetic screen in *C. elegans* to isolate mutants that had increased lifespan. Klass attributed the longevity of these *C. elegans* mutants to Dietary Restriction (DR), owing to the fact that they were feeding defective (Klass, 1983). Later on, another genetic screen in Cynthia Kenyon's lab identified *daf-2(e1370)* mutants as being long-lived, with lifespan up to twice as long as the wild type (Kenyon et al., 1993).

The *daf-2(e1370)* strain was also unique in that when grown at high temperature, worms entered a state of diapause, similar to a bacterial spore. This state, termed "dauer" state, is a type of growth arrest checkpoint under low nutrition or crowding conditions. Dauer stage worms are thin, small, juveniles (with arrested growth) that have a special morphology. Dauers do not eat or reproduce, and are extremely long lived. Evolutionarily, the dauer stage probably allowed the juvenile worm to live out harsh environmental conditions until such a time when favourable conditions arose again. When optimum nutritional conditions are restored, the dauers resume normal development and grow on to become fertile adults. Interestingly, only young larvae can become dauers; once worms are fully grown adults, they cannot transition to the dauer state. In fact, some of the long-lived mutants isolated by Michael Klass in his genetic screen were dauer-constitutive mutants which he mistook for being feeding-defective.

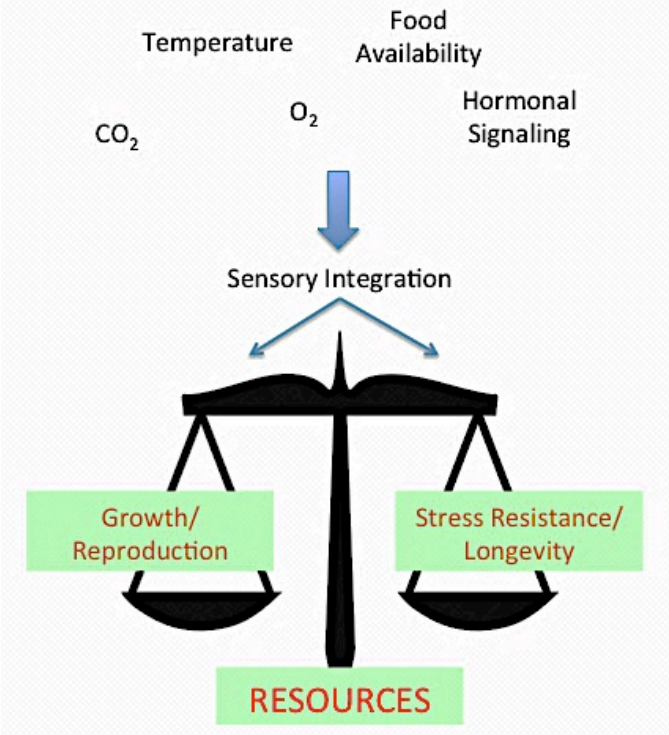


Figure 1.1: Resource allocation for growth/reproduction vs. stress resistance/longevity
Living organisms are evolutionarily programmed to undergo greatly enhanced rates of growth and reproduction when they encounter suitable environmental conditions such as food and oxygen availability as well as optimum temperatures. Any variation in one or more of these conditions leads to activation of genetic and biochemical pathways that result in increased stress resistance and increased lifespan.

Later on, research in the Kenyon lab showed that the protein DAF-16 is required for dauer formation of *daf-2* mutants at high temperature, and also for their extended lifespan phenotype (Kenyon et al., 1993). Evidence from genetic assays demonstrate that DAF-2 prevents DAF-16

from a) promiscuously activating the dauer state during worm development and b) extending lifespan of the *daf-2* mutants. Data from multiple experiments performed in various labs support the hypothesis that when environmental and growth conditions are favorable, DAF-2 inhibits the activity of DAF-16 and allows worms to grow to be adults. Under unfavourable environmental conditions, DAF-2 activity levels get reduced dramatically, thereby allowing DAF-16 to become active and promote dauer formation. Reduction in DAF-2 activity during adulthood allows DAF-16 to promote lifespan extension. Curiously, dietary restriction during middle age also extends lifespan in *C. elegans* and it has been shown that DAF-16 is required for this lifespan extension (Greer et al., 2007). Thus, *C. elegans* utilizes its sensory abilities to determine environmental cues such as temperature and food availability and based on these factors, a programmed genetic switch is activated which commits the animal to a phase of growth/reproduction (during optimal environment conditions) or the dauer phase (that promotes longevity and stress resistance during environmental stress). This has been diagrammatically illustrated in Figure 1.1 above.

While searching for more *C. elegans* mutants with increased lifespan, the Ruvkun lab (and others) showed that mutations in the *daf-23* gene (later shown to be *age-1*) also resulted in increased lifespan (Larsen et al., 1995; Malone et al., 1996; Morris et al., 1996). In August 1997, the Ruvkun lab reported that *daf-2* encoded a homolog of the human insulin and IGF1 receptors, which basically implicated that aging and lifespan are controlled by hormones (Kimura et al., 1997). The notion that inhibiting the very hormonal pathways that lead to the onset of diabetes when they become dysfunctional could prolong lifespan was a novel concept. Later that same year, the Ruvkun and Kenyon lab together published papers showing that DAF-16 is a FOXO transcription factor that can dramatically increase the lifespan of *C. elegans* worms (Lin et al., 1997; Ogg et al., 1997). At this point, it is important to highlight the difference between lifespan and healthspan in longevity studies: while lifespan is the obvious readout for establishing the rate of aging, it is not always correlated with healthy aging or "healthspan". While lifespan measurements only assess longevity, healthspan assesses tissue integrity assessment and functionality as well as resistance to a variety of stresses.

The entire body of literature regarding the insulin signalling pathway and how it regulates growth, development and longevity in *C. elegans* (and possibly other model organisms) is a topic of considerable depth, hence information about some of its aspects will be shared in the chapters below as needed.

2 MATERIALS AND METHODS

2.1 Techniques and *C. elegans* husbandry

2.1.1 Lifespan analysis for temperature sensitive (ts) sterile *C. elegans* mutants

Lifespan analysis of temperature sensitive sterile *C. elegans* mutants (such as *glp-1, fem-2* and *spe-26)* was carried in the following manner: gravid *C. elegans* adults were recovered into 15ml falcon tubes using sterile M9 buffer and pelleted by centrifugation for 1 minute at 2000 RPM. The worms were then suspended in 20% alkaline hypochlorite solution for ~3 minutes and then washed thrice with sterile M9 buffer. This procedure kills all the worms while leaving eggs intact, since the egg shell protects the embryo within from the 20% alkaline hypochlorite solution. The eggs were placed onto an NGM plate without food and larvae were allowed to grow overnight upto L1 stage. The next morning, larvae were transferred to NGM plates with OP50 food and grown in these plates until late L4 stage at 25°C before being singled onto NGM plates containing OP50 food and were shifted to the normal physiological temperature of 20°C. N2 worms were put through a similar temperature shift regimen for these experiments.

2.1.2 Analysis of *C. elegans* lifespan (RNAi)

RNAi experiments used wild type (N2) animals unless otherwise stated. Embryos were synchronized by bleaching and larvae subsequently allowed to develop upto late L4 stage at either 25°C for temperature sensitive strains (24hr post de-arrest of L1 diapause on food) or 20°C for wild type before being singled onto RNAi plates containing HT1115 control RNAi food or the appropriate RNAi food. Animals were left at 20ºC after larval development for the rest of the experiment and viability was determined by scoring every two days for movement after repeated poking of the head and tail with a platinum wire. In the lifecycle of *C. elegans*, day 1 of adulthood, when animals acquired reproductive competence after vulval development completes, is standardized at 24-30hrs post the L3/L4 molt at 20ºC. This timetable for synchronizing adult life has been used throughout the experiments in this thesis. RNAi clones were obtained from the *C. elegans* v1.1 Orfeome RNAi library (Source BioScience). All RNAi experiments were performed on NGM plates supplemented with 100μg/ml Ampicillin without IPTG at 20ºC.

2.1.3 Statistical analysis

Statistical analysis of all worm lifespan data was carried out using the OASIS lifespan analysis suite available online at http://sbi.postech.ac.kr/oasis. All other statistical analysis was performed using the R statistical software.

2.1.4 Quantification of *C. elegans* progeny outputs

Synchronized hermaphrodites were singled onto NGM-OP50 plates and incubated at 20ºC. Upon the start of progeny generation (day 1), animals were transferred to fresh NGM-OP50 plates daily. Plates were scored for viable larvae 24 hrs after the progenitor had been transferred.

2.1.5 Mating protocol

Wild type worms were synchronized by bleaching with 20% alkaline hypochlorite solution (as described in 2.2.1) and allowed to develop on NGM plates to L4 stage at 20ºC when they were transferred to mating plates (with a small OP50 lawn). A 1:1 male to female ratio was used. After 14 hours, hermaphrodites were removed and singled on individual NGM plates. Presence of F1 L4 males in the progeny 48 hrs after singling was used to exclude progenies generated by selfing.

2.1.6 cDCFDA and Nile Red staining of intestinal lysosomes

Synchronized worms were grown overnight in plates seeded with OP50 supplemented with 10mM cDCFDA (Molecular Probes) in DMSO. Plates were protected from light during this incubation period. For co-staining experiments, Nile Red (Sigma Aldrich) was added to OP50 lawns at a 15µg/ml concentration. After an ON incubation, animals were placed in fresh plates without bacteria to clear out excess dye for a few minutes and then anesthetized using 10mM Azide and imaged live on mounted agarose pads using a Zeiss LSM 510 confocal microscope.

2.1.7 Immunostaining of intestinal cells

Worms were dissected on glass cover slips in M9 Buffer and then freeze-cracked on an aluminum block on dry ice. Dissected intestines were fixed in cold methanol for 30 seconds followed by a 20-minute fixation in 4% formaldehyde solution (1X PBS-T, 80mM HEPES pH7.4, 0.8mM EDTA, 37% formaldehyde and 1.6mM MgSO4) at room temperature. Worms were washed 2x in PBS-T, then blocked for 1 hour in 0.5% BSA. Samples were then incubated in 1:100 dilution of

primary antibodies (polyclonal rabbit anti-DAF-16 (Santa Cruz Biotech) or polyclonal rabbit anti-RFP (Rockland)) and incubated overnight at 4°C. Immunostaining of a null *daf-16* mutant with the commercial anti-DAF-16 antibody used in this study does not reveal any signal in the intestine (Figure 2.1). Samples were washed twice with PBS-T the subsequent day followed by incubation with 1:500 FITC-conjugated goat anti-rabbit secondary antibody (Abcam) for 2 hours at room temp in a humid chamber. Slides were then washed 3x with PBST and DAPI stained for 10 mins followed by another wash for 1 hour in PBST prior to imaging. For VHA-17 and LMP-1 co-staining experiments, dissected intestines were fixed in -20ºC methanol for 30 min. Primary antibody dilutions were 1:100 while secondary antibody dilutions were 1:250.

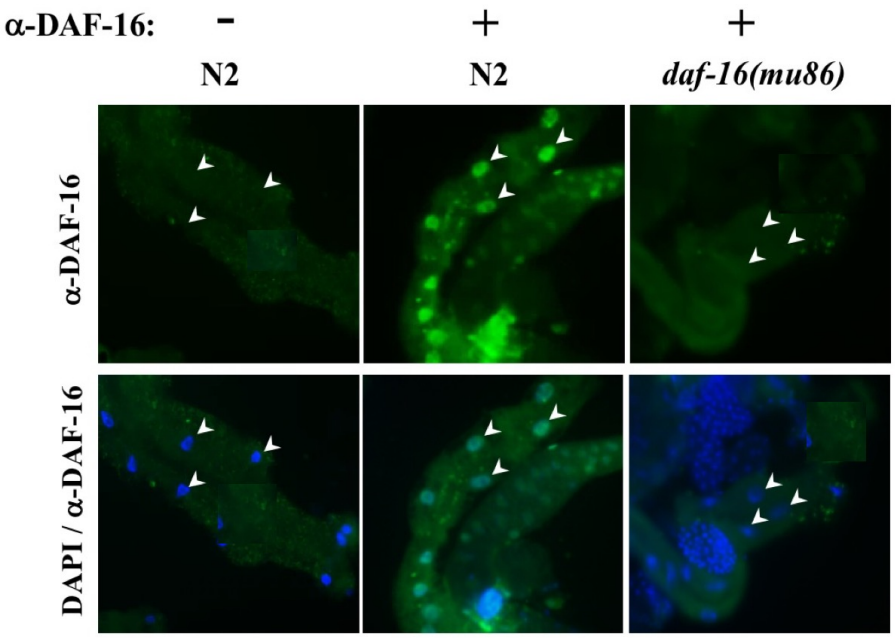


Figure 2.1: Immunostaining control tests for the anti-DAF-16 antibody
In a no-primary control or in a *daf-16(mu86)* mutant, there was no evidence of green fluorescence, whereas in wild type worms stained with the anti-DAF-16 antibody, there is clear green fluorescence seen in nuclei and cytoplasm in the intestine.

2.1.8 Continuous DA feeding protocol

Wild type worms were synchronized by bleaching and allowed to grow on NGM plates seeded with OP50 at 20° C until L4, following which they were transferred to either a) NGM plates with OP50 supplemented with freshly made 1µM, 10µM or 100µM Δ^4-Dafachronic Acid or b) NGM plates with OP50 supplemented with ethanol (control). For cDCFDA analysis following DA feeding, worms were transferred every day to fresh NGM plates supplemented with ethanol or Δ^4-Dafachronic Acid as appropriate. 100µM cDCFDA was added to NGM plates containing OP50

(with or without Δ^4-Dafachronic Acid). Worms were grown overnight on these plates for at least 14 to 16 hours, washed off excess bacteria and then used for microscopy.

2.1.9 Microscopy

Animals were anesthetized in 35µl of 10mM Sodium Azide and mounted on 2% agarose pads spotted prior to imaging. For imaging of intestinal lysosomes, a Zeiss LSM510 confocal laser scanning microscope was used. Z-stacks were acquired under identical imaging parameters and processed using the Zeiss LSM software package. cDCFDA spectrometric measurements consisted of a series of 12 excitation scans (480–600 nm in 10 nm steps). Alternatively, a Delta Vision Elite® microscope (GE) was used. Image processing was performed using Softworxs® software (G.E./Applied Precision). DIC images of post-mated *fem-2* worms were recorded with a Nikon SMZ1500 stereomicroscope. The LSM510 microscope was always used with a pinhole setting of 300. The detector gain of the microscope was determined at the start of every microscopy session using 2 day old N2 worms (stained with cDCFDA) and was kept constant for the entire duration of the microscopy session.

2.1.10 Generation of the pHTomato line

pHTomato is a pH sensitive fluorophore that does not show any fluorescence at acidic pH (pH 4 and below), but increases linearly in fluorescence intensity as the pH increases (Y. Li et al., 2012). The LMP-1 protein is a lysosomal membrane protein with its N terminus protruding into the lysosomal lumen (Kostich et al., 2000), hence it was selected for generating an LMP-1::pHTomato reporter fusion. The fragment encoding the gene encoding the pH sensitive fluorophore pHtomato was PCR amplified and fused downstream of a 1.5 kb of *lmp-1* promoter fragment through overlap extension PCR. The resultant cassette was then fused in frame to genomic *lmp-1* (without the start codon), thereby creating a *lmp-1p::pHTomato::lmp-1* fragment used for microinjections. CEC146 was generated by injecting wild type hermaphrodites with 20ng/µl of gel purified *lmp-1p::pHTomato::lmp-1* fragment in a mix containing 50ng/µl of pRF4 (*rol-6(su1006))* as a co-injection marker. The *rol-6(su1006)* is a marker that makes worms roll and is often used as a co-injection marker.

2.1.11 Quantification of αsynuclein::GFP foci

Z-stacked images of age-matched worms were obtained by confocal microscopy using standardized exposure settings and image dimensions. Single projections were scored for number of GFP foci and the averages calculated manually. Twenty worms were scored per genotype.

2.1.12 RT-PCR and qPCR analyses

Ten animals were collected from NGM-OP50 plates and RNA was extracted using Trizol (Invitrogen). After a 30-minute DNase I treatment (Fermentas), cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad) (after inactivating DNase). 10 ng of cDNA (quantified by nanodrop) was used in real-time PCR amplification using the iQ SYBR Green Supermix (Bio-Rad) and the iCycler system (Bio-Rad). The relative expression level of each gene was determined using the Comparative CT Method and normalized to 28S rRNA internal control in the same sample. Measurements were performed in triplicates for 2 biological samples for each condition.

2.1.13 Western blot analysis

For SDS-PAGE analysis, approximately 300 animals were collected, washed in M9 buffer, and resuspended in lysis buffer (20 mM Tris pH 7.4, 2% Triton X-100, 1 mM EDTA, 1 mM DTT, and protease inhibitor cocktail (Roche Diagnostics). Worms were lysed by 2x 20 second glass bead beating. Urea was added to a final 8M in order to solubilize α-synuclein and polyQ inclusions. Following a 30-min incubation on an end to end rotor at 4°C, samples were microfuged at 5000g for 10 minutes and heated in a 65°C water bath for 5 minutes prior to loading. Total protein lysates were resolved on a 10% SDS-PAGE and probed by immunoblotting. A rabbit polyclonal DAF-16 antibody (Santa Cruz) was used at 1/2000. β actin and GFP antibodies (AbCam) were used at 1/5000. The chemiluminescent blots were imaged with a ChemiDoc MP imager (Bio-Rad). ImageLab software version 4.1 (Bio-Rad) was used for image acquisition and densitometric analysis.

2.1.14 Quantification of nuclear::cytoplasmic DAF-16 IF

Images were acquired on a Delta Vision Elite® microscope (GE) and image processing was performed using Softworxs® software (G.E./Applied Precision). Digitized images were used to quantify nuclear::cytoplasmic DAF-16 immunofluorescence signal ratios using ImageJ software.

2.1.15 Native ChIP

Approximately 200 worms were lysed in ChIP buffer (20mM Tris-HCl pH 8, 80mM KCl, 0.1% Triton X-100, 1mM DTT, protease inhibitors) by glass bead beating for 2x20 seconds. The slurry was sonicated on ice and spun at 5000g for 10 minutes. DAF-16 complexes were immunoprecipitated from supernatant using a rabbit polyclonal DAF-16 antibody (Santa Cruz), washed extensively in lysis buffer, and eluted in Glycine 0.1 M (pH 1.3) at RT for 20 minutes. Eluates were neutralized with 1.5M Tris-HCl (pH 8.8) and incubated with 2µl of 0.5M DNase-free RNase A (Fermentas) at 37°C for 30 minutes. Recovered protein-DNA complexes were deproteinated by treating with Proteinase K (Fermentas) at 42°C for 30 minutes, phenol chloroform extracted, supplemented with 1µl glycogen as carrier, and ethanol precipitated overnight at -20°C. Pellets were washed in 70% Ethanol and resuspended in nuclease free water. This DNA was then used as a template for PCR using primers for the respective genes.

2.1.16 Worm motility analysis

Three age-matched worms of the same treatment were randomly picked and placed on a fresh NGM plate and imaged for 1 minute using a Basler acA2500 camera mounted on a Nikon 60mm f/2.8D micro lens. Videos were subsequently processed and each worm tracked for movement (µm/minute) using WormLab v4.05 (MBF Biosciences).

2.1.17 Generation of anti-RER-1 antibody

Mouse polyclonal antibody against C. elegans RER-1 was raised at the College of Medicine, University of Saskatchewan with the kind assistance of Dr. Barry Ziola by immunizing mice with a synthetic 14 amino acid peptide (GenScript) VGKPRMAGKEDTGK corresponding to amino acids 174 – 187 at the C-terminus of *C. elegans* RER-1. The peptide was conjugated to BSA at the N terminus via a cysteine residue.

2.1.18 Monitoring autophagy in *C. elegans*

Expression of LGG-1 was monitored at 20°C in adult transgenic worms carrying an integrated *Pnhx-2*::*mCherry*::*lgg-1* transgene. Worms were synchronized (age-matched) by bleaching and these age-matched wild type and *rer-1(tm5219)* animals were grown and imaged in parallel.

Table 2.1 LIST OF STRAINS USED IN THIS STUDY

Strain	Genotype	Source
CEC16	*rer-1(tm5219)*	NBRP
SJ4005	*zcIs4[hsp-4::GFP] V.*	CGC
VK1093	*vkEx1093[nhx-2p::mCherry::lgg-1]*	CGC
UA49	*[baInl2;Punc-54::a-syn::gfp, rol-6 (su1006)]*	Caldwell Lab
UA50	*[baInl13;Punc-54::a-syn::gfp,Punc-54::tor-2, rol-6 (su1006)]*	Caldwell Lab
DH245	*fem-2(b245) III.*	CGC
CF1903	*glp-1(e2144) III.*	CGC
BA821	*spe-26(hc138) IV.*	CGC
AA292	*daf-36(k114) V.*	CGC
AE501	*nhr-8(ok186) IV.*	CGC
EU630	*air-2(or207) I.*	CGC
DG627	*emb-30(tn377) III.*	CGC
CB1393	*daf-8(e1393) I.*	CGC
JT191	*daf-28(sa191) V.*	CGC
CB1386	*daf-5(e1386) II.*	CGC
RB711	*pqm-1(ok485) II.*	CGC
STE68	*nhr-49(nr2041) I.*	CGC
KQ1564	*sgk-1(ft15) X.*	CGC
RG1228	*daf-9(rh50) X.*	CGC

Table 2.2 LIST OF OLIGONUCLEOTIDES USED IN THIS STUDY (5' to 3')

Oligonucleotides for ChIP

vha-2 **forward**	CCCTTATTCTTGGAGCTACATA
vha-2 **reverse**	CCGAAGATGCTCTATAACATT
vha-8 **forward**	CTTCAAACAGGAAAACCCACC
vha-8 **reverse**	CCCACTGGAGGCCAATAATCAA
vha-13 **forward**	CTGGAGTCGTAGGTCTATCC
vha-13 **reverse**	CTTGTGATTTACTTTTGTAATG
vha-17 **forward**	TCACCTCGATGGAACCTTTC
vha-17 **reverse**	AAAATGTCGGCTTTCTGGTG
sod-2 **forward**	AATTCGGCTCAAAAAGCAAC
sod-2 **reverse**	TCGCAGAAACATATCAAGCTG

Oligonucleotides for RT-PCR

vha-2 **forward**	GATTTGGAAACCGCCGAAAG
vha-2 **reverse**	CGTCTCCGACAATTCCGATAG
vha-8 **forward**	GCTGAGGAGATCGATGCTAAAG
vha-8 **reverse**	TCCGGAAATACGGGAGAGAT
vha-9 **forward**	GCTGCGAAGGGTAAGATTTTG
vha-9 **reverse**	CCTCTGGGTTGAACAATCCAC
vha-10 **forward**	GCTGAGGTGGAGAAGTACAAG
vha-10 **reverse**	CGTGAAGTTTCTTCTGGAGAGT
vha-17 **forward**	GCAGATTAGCCTTTAACACCTAG
vha-17 **reverse**	GTTGAGTCAAGGGTGACCAAG
18s rRNA forward	TGGAGCTTGCGGCTTAAT
18s rRNA reverse	TTACCGATACCTTTCGGCATAG
daf-16 **forward**	CGAACCTCTCGATTCCTGGATC
daf-16 **reverse**	TCCAATTGTGCCAAGCACTAACTTCAA

3 RER-1: THE CONNECTING LINK BETWEEN ER HOMEOSTASIS AND REPRODUCTIVE SENESCENCE

Note: Data in this chapter have been published in ((Ghavidel et al., 2015))
All the *C. elegans* data in this study has been generated by me (Figure 3.7 and beyond). All yeast data (Figure 3.1 to Figure 3.6) has been generated by Dr. Ghavidel.

3.1 Background

3.1.1 Genetic screens for isolating lifespan mutants

Eukaryotic cells undergo a finite number of cell divisions, both *in vitro* and *in vivo*, before irreversibly exiting mitosis (Hayflick, 1965; Smith et al., 1996). Mutations in even a single gene can dramatically extend or reduce the number of cell divisions, indicating that mitotic lifespan can be genetically regulated (Jazwinski et al., 1989; Kaeberlein et al., 2005; Lesur et al., 2004). There is growing evidence that mitotic senescence is not a default outcome of stochastic cell deterioration, but is instead governed via intricate pathways that in concert limit the number of cell divisions and the onset of complex phenotypes associated with mitotic exit. Elucidating mechanisms that limit the number of cell divisions has been the subject of intensive research in part because failure to exit mitosis is tightly associated with cell immortalization, a hallmark of neoplastic growth (Longo et al., 2008). Owing to the broad conservation of mitotic longevity pathways, the budding yeast *S. cerevisiae* has proven remarkably well suited to unraveling molecular mechanisms that govern longevity in eukaryotic cells (Jazwinski et al., 1989; Longo et al., 2012). Large-scale screens of yeast mutants designed to map the underlying longevity networks have already been reported (Kaeberlein et al., 2005; Sutphin et al., 2012).

In 1977, Klass proposed that the nematode *C. elegans* could be used for aging research due to its short lifespan as well as the introduction of consistent protocols for measuring lifespan (Klass, 1977). In addition, *C. elegans* lifespan has little variation, thereby allowing isolation of mutants with a confidence of 10-15% change in average lifespan. Moreover, the entire *C. elegans* genome has been sequenced and annotated, with over 80% of the genes in the genome available as feeding RNAi libraries (Kamath et al., 2003). A large number of genes identified in *S. cerevisiae* genetic screens possess functional counterparts in *C. elegans*, thereby allowing the characterization of these genes in both unicellular and multicellular model systems.

3.1.2 ER-Golgi trafficking

In most eukaryotic cells, the endoplasmic reticulum (ER) is the site for protein synthesis and folding as well as glycosylation of a substantial portion of the cellular pool of proteins (Harding et al., 1999). Most proteins synthesized in the ER are transported to the Golgi inside cargo vesicles constituted of secretory proteins, and upon reaching the Golgi they are then modified, processed, sorted and secreted outside the cell, or transported to various other organelles within the cell (Palade, 1975) (Pfeffer et al., 1987). There exists a bi-directional membrane trafficking system between the ER and Golgi facilitated by distinct cellular proteins. Most of our current knowledge about eukaryotic membrane trafficking across the ER-Golgi network derives from studies in *S. cerevisiae*, plant and mammalian systems. Membrane trafficking between the ER and Golgi consists of: a) ER to Golgi transport (anterograde transport) and b) Golgi to ER transport (retrograde transport). The coat protein complex II (COPII) is a part of the anterograde pathway that transports newly synthesized proteins from the ER to the Golgi whereas the coat protein complex I (COPI) is a part of the retrograde pathway that transports proteins from the Golgi back to the ER (Szul et al., 2011). It is essential that newly synthesized proteins be correctly delivered to the Golgi while simultaneously ensuring that protein components of the secretory pathway be retrieved back to the ER (Castro-Fernandez et al., 2005).

A large-scale genetic screen designed to isolate yeast mutants with altered mitotic lifespan in the Harkness lab (University of Saskatchewan) (Figure 3.1 below) identified, among others, a candidate gene *RER1* (**r**etention in **e**ndoplasmic **r**eticulum) (Ghavidel et al., 2015). *RER1* is highly conserved in both sequence and function across eukaryotes (as illustrated in Figure 3.2), and this is exemplified by a study showing that human RER1 can functionally complement the yeast mutant (Fullekrug et al., 1997). In *S. cerevisiae*, retrieval of protein components of the secretory pathway occurs either by a) COPI mediated recognition and binding of KDEL/HDEL or KKXX (dilysine) motifs found at the C-terminal of these proteins or b) via retrieval by RER1 (Sato et al., 2003). *In vitro* and *in vivo* studies have shown that RER1 recognizes transmembrane domains (TMDs) present on various secretory proteins, and this recognition is critical for RER1-mediated retrieval of these secretory proteins back to the ER (Sato et al., 2003).

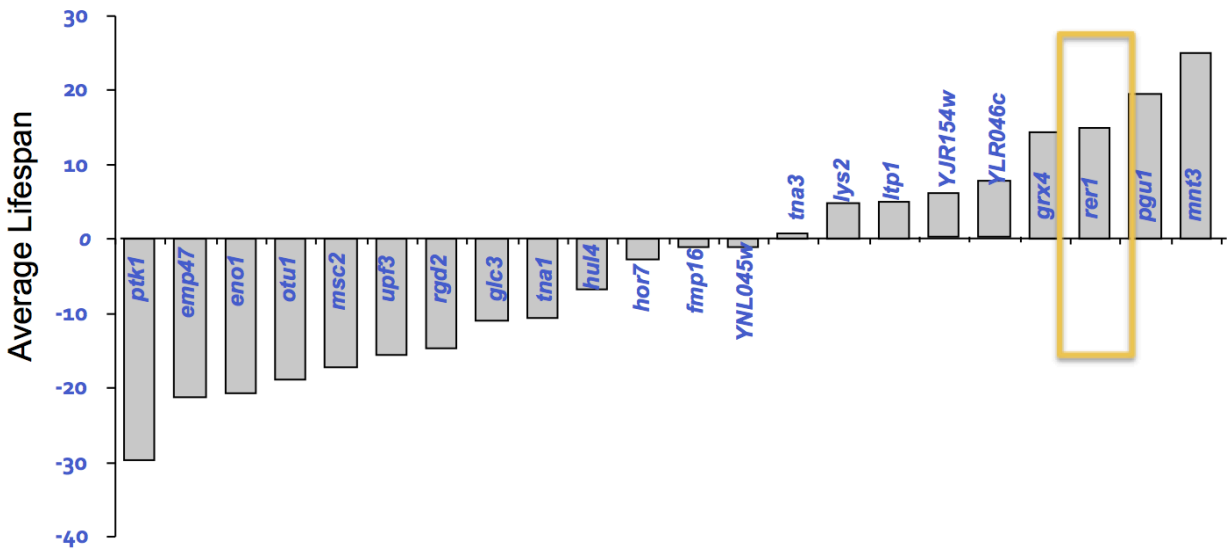


Figure 3.1: A high throughput genome scale genetic screen in *S. cerevisiae* revealed genes with altered lifespan.
A list of various genes isolated from this genetic screen; these genes are involved in processes as diverse as transcription, histone modification and ER-Golgi transport (Ghavidel et. al, unpublished data).

```
human          MSEGDSVGESVHGKPS-VVYRFFTRLGQIYQSWLDKSTPYTAVRWVVTLGLSFVYMIRVY 59
worm           MDDG------LRDRPG-VTSRFFHSLEVKYQYYLDRLTPHTAFRWVIALISLVFFASRII 53
yeast          MDYDSS--DTMNGGSSNPLITKMNTMKLLYQHYLDKVTPHAKERWAVLGGLLCLFMVRIT 58
               *. .      :.. ..      :  :   ** :**: **::  **.:      .:  *:

human          LLQGWYIVTYALGIYHLNLFIAFLSPKVDPSL-MEDSDDGPSLPTKQNEEFRPFIRRLPE 118
worm           LLQGFYIVAYAVGIYYLNLFLLFLTPSIDPALEFEDEDDGPVLPSKTNDEFRPFMRRLPE 113
yeast          MAEGWYVICYGLGLFLLNQFLAFLTPKFDMSL-QQDEENNELEAGEKSEEFRPFIRRLPE 117
               : :*:*:: *.:*:: ** *: **:*..* :*  :*.::.   . : .:*****:*****

human          FKFWHAATKGILVAMVCTFFDAFNVPVFWPILVMYFIMLFCITMKRQIKHMIKYRYIPFT 178
worm           FKFWHSFMKATLIAITCTFFEFFDVPVFWPILVMYFFILTFLTLKRQIMHMIKYRYIPFT 173
yeast          FKFWYNSIRATVISLLLSLFSIFDIPVFWPILLMYFILLFFLTMRRQIQHMIKYRYIPLD 177
               ****:   :. ::::  ::*. *::********:***::*  :*::*** *********:

human          HGKRRYRGKEDAGKAFAS 196
worm           VGKPRMAGKEDTGKVVVG 191
yeast          IGKKKYSHSSN------- 188
                ** :   ..:
```

Figure 3.2: RER-1 is highly conserved across the eukaryotes.
C. elegans RER-1 has 58% identity to human RER1 and 41% identity to yeast Rer1.

3.1.3 Membrane trafficking quality control and UPR

The membrane trafficking system that transports cargo back and forth across the ER-Golgi network is quite sophisticated, and there exist multiple quality control mechanisms that ensure efficiency and high fidelity (Ellgaard et al., 1999). For a protein to be exported from the ER, it has to be in the correct conformation and oligomerization (if required) (Ellgaard et al., 1999). Misfolded and non-oligomerized proteins are sensed by molecular chaperones and retained in the ER until they are folded and/or oligomerized properly (Ellgaard et al., 1999). Misfolded and aggregated proteins that cannot be repaired within the ER are targeted for degradation via either the ubiquitin-proteasome pathway (UPP) or the autophagy-lysosomal pathway (ALP) (discussed in more detail below) (Feng et al., 2014; Glickman et al., 2002). Accumulation of unfolded/aggregated proteins in the ER in spite of these quality control mechanisms culminates in the induction of the **u**nfolded **p**rotein **r**esponse (UPR) (Ma et al., 2001). UPR is a targeted cellular response that serves to: a) attenuate global protein synthesis b) upregulate autophagy and c) upregulate chaperones that enhance the protein folding capacity of the ER (Hotamisligil, 2010).

In *C. elegans,* autophagy plays a critical role in several processes including survival during nutrient deprivation, dauer and reproductive development, lifespan extension and programmed cell death (Melendez et al., 2009). Autophagy has been proposed to have a direct role in *C. elegans* dauer development and lifespan (Melendez et al., 2003). Autophagy is indispensable for the increased lifespan of feeding deficient *C. elegans* mutants such as *eat-2* as well as worms that have been subjected to dietary restriction (Hansen et al., 2008). The process of autophagy and its physiological importance has been elucidated in further detail in chapter 4 of this thesis.

The process of autophagy can be further divided into three separate components: 1) chaperone mediated autophagy 2) microautophagy and 3) macroautophagy. Of these, macroautophagy is the only form of autophagy capable of degrading large protein aggregates and cellular organelles, and is the primary focus of this study. Macroautophagy will herewith be referred to as autophagy throughout this study.

Even though the cellular events of autophagy in *C. elegans* are not known in great detail, it is presumed that the primary steps involved are conserved from yeast to mammalian cells. Autophagy is initiated by the formation of a phagophore (isolation membrane) via the synchronized activity of the autophagy core proteins at the site of phagophore assembly (**p**hagophore **a**ssembly **s**ite or PAS). The phagophore isolation membrane is extended to form an autophagosome which is

capable of engulfing bulk cytoplasmic proteins and organelles (non-specifically) or via specific targeting of certain cargo proteins. The outer membrane of the autophagosome then fuses with an endosome (to form an amphisome that then fuses with the lysosome) or directly fuses with a lysosome to form an autophagolysosome. Finally, the material inside the autophagolysosome is degraded by the action of lysosomal hydrolytic enzymes and then the degraded material is recycled. A summary of the process of autophagy is illustrated diagrammatically below in Figure 3.3.

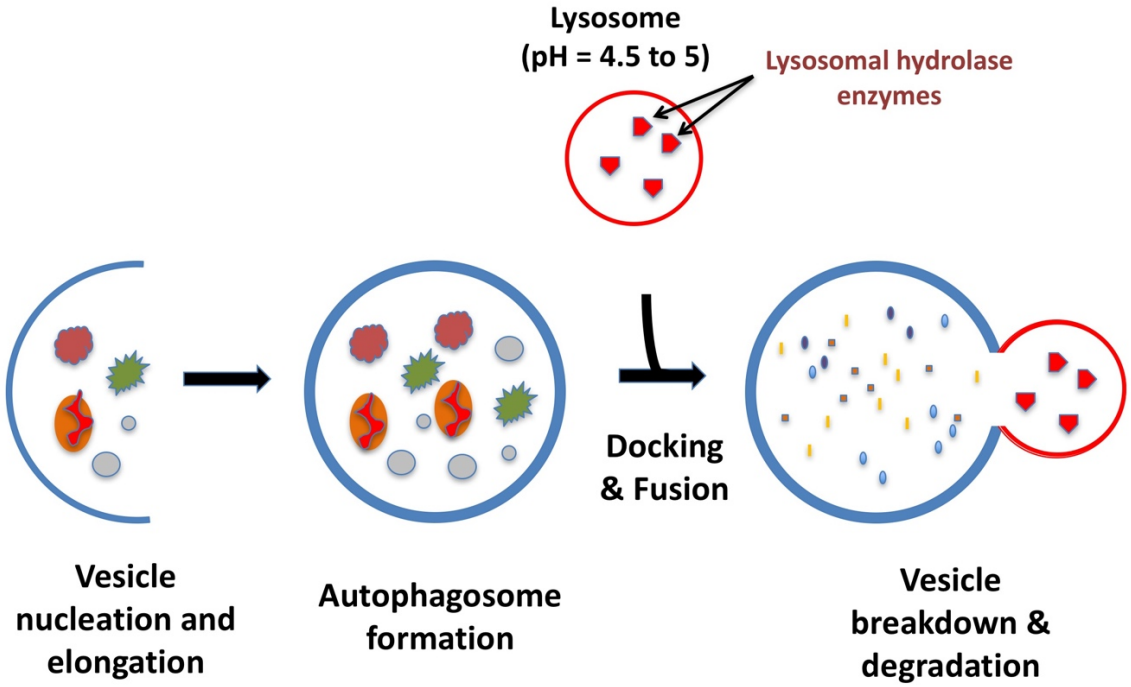


Figure 3.3: Diagrammatic illustration of autophagy
Autophagy is initiated by formation of an isolation membrane around proteins and organelles that are destined for proteolysis. The isolation membrane expands to form an autophagosome that then docks and fuses with the low pH lysosome (containing lysosomal proteases and hydrolases) that then break down the components of the autophagosome (adapted from wormbook.org).

During conditions of ER stress, three transmembrane signal transducers are activated to initiate a response cascade. Two of these are mediated by protein kinases: a) **I**nositol **RE**quiring kinase **1** (IRE1) and b) dsRNA-activated **P**rotein kinase-like **ER** **K**inase (PERK) while the third is a transcription factor called c) **A**ctivating **T**ranscription **F**actor **6** (ATF6) (Harding et al., 2000; Sidrauski et al., 1997; Yoshida et al., 2001). All three signal transducers serve to activate specialized transcriptional regimens mediated by distinct transducers – ATF4 (activated by PERK), cleaved ATF6 (activated by ATF6), and spliced XBP1 (sXBP1) (activated by IRE1). Briefly, PERK enables the dedicated translation of transcripts with an alternate open reading frame

including the key transducer ATF4, while inhibiting general protein translation. ATF4 has also been implicated in activation of cell death programs under extreme stress conditions (Hetz, 2012; Rutkowski et al., 2010). IRE1, on the other hand, functions as an RNAse that mediates activation via splicing of the XBP-1 transcription factor which leads to expression of ER-associated degradation (ERAD) components and chaperones (Maurel et al., 2014). Finally, ATF6 migrates to the Golgi after sensing ER stress where it is cleaved to produce an active form of a transcription factor that moves to the nucleus and regulates genes such as grp78 and CHOP (M. Wang et al., 2010). IRE1 was the first UPR component to be identified and is conserved in all eukaryotic cells. Higher eukaryotes possess PERK and ATF6 as additional sensors of ER stress besides IRE1 (Malhotra et al., 2007).

3.2 Rationale and specific aims

Most animals undergo an initial reproductive phase early in their life following which they enter a period of reproductive senescence that can last a variable time before death. While most longevity studies focus on organismal lifespan, other studies focus specifically on the reproductive or post-reproductive phase of eukaryotic lifespan. *C. elegans* nematodes, similar to humans, reproduce for the initial 1/3rd phase of their adult lifespan followed by a period of reproductive decline. It is well known that genetic ablation of the *C. elegans* reproductive system significantly increases lifespan; similarly, castration increases lifespan in men (Hsin et al., 1999). Interestingly, in *C. elegans*, this increase in lifespan is abrogated when the somatic gonad is removed (Arantes-Oliveira et al., 2002; Hsin et al., 1999; Min et al., 2012). Given the complex interplay between reproduction and lifespan, it is not surprising that a majority of genes implicated in enhancing *C. elegans* longevity do so by increasing post-reproductive lifespan (S. E. Hughes et al., 2007). Using elaborate genetic screens in yeast and *C. elegans,* researchers are trying to understand if either the reproductive lifespan or the post-reproductive lifespan can be increased without affecting the other, although this has proved challenging since genes that affect reproductive lifespan also affect longevity (Luo et al., 2011; M. C. Wang et al., 2014). Besides reproduction, there are a variety of factors, both internal and external, that influence lifespan in *C. elegans*. For example, studies have shown that ER stress (and subsequent UPR induction) also leads to increased lifespan, although there is considerable speculation about the molecular mechanism(s) for such a lifespan extension (Labunskyy et al., 2014). How would ER stress influence lifespan? What is the molecular

mechanism behind such a lifespan extension? To answer some of these questions linking ER-stress induced UPR to organismal and reproductive lifespan, the following specific aims were investigated in this part of my study:

a) Validation of tunicamycin induced ER stress (and subsequent UPR) in *C. elegans*
b) Impact of increased ER stress on reproductive and organismal lifespan in *C. elegans*
c) Role of *rer-1* inactivation in *C. elegans* on UPR activation and reproductive lifespan
d) Test whether increased autophagy in *rer-1* mutants contributes to increased reproductive lifespan.

S. cerevisiae cells treated with the glycosylation inhibitor tunicamycin accumulate unfolded proteins in the ER lumen, thereby leading to ER stress and upregulating UPR (as measured by a *lacZ* inducible UPR reporter system) (Ghavidel et al., 2015). Interestingly, these tunicamycin treated cells also undergo an increased number of mitotic cell divisions. This "reproductive lifespan" of yeast can be functionally compared to the reproductive lifespan of *C. elegans* since both are reflections (direct and indirect) of progressive loss of proliferative capacity in progenitor cells (Figure 3.4). Tunicamycin treatment leads to redistribution of GFP-Rer1 from the ER to the Golgi in yeast (Figure 3.5). Given the role of Rer1 in maintaining ER compartmentalization by retrieving the components of the vesicles that transport cargo from ER to Golgi, it is plausible that this redistribution is due to a previously unknown component of UPR evoked to reduce ER load by increasing trafficking of misfolded proteins from the ER to Golgi where these misfolded proteins are then packaged into autophagosomes and delivered to the lysosomes for destruction (van der Vaart et al., 2010). Moreover, *S. cerevisiae* Δ*rer1* mutants, similar to tunicamycin treated cells, also undergo increased mitotic cell divisions (Figure 3.6) while upregulating UPR (data not shown).

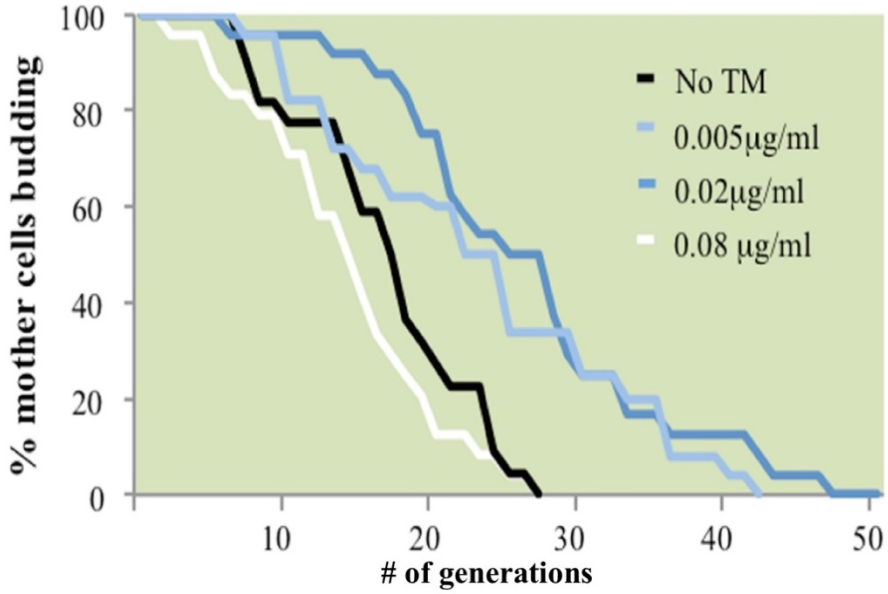


Figure 3.4: Tunicamycin induced ER stress leads to increased replicative lifespan in *S. cerevisiae*
Replicative lifespan of wild type yeast grown on YPD supplemented with the indicated concentrations of TM. Mean replicative lifespans for DMSO-treated control and cells treated with TM at 0.005, 0.02 and 0.08 µg/ml are plotted (p values: (a) control vs TM 0.0005 ug/ml $p < 0.0001$ (b) control vs TM 0.02 ug/ml $p < 0.0001$ (c) control vs TM 0.08 ug/ml $p < 0.0001$). (data from *(Ghavidel et al., 2015))*

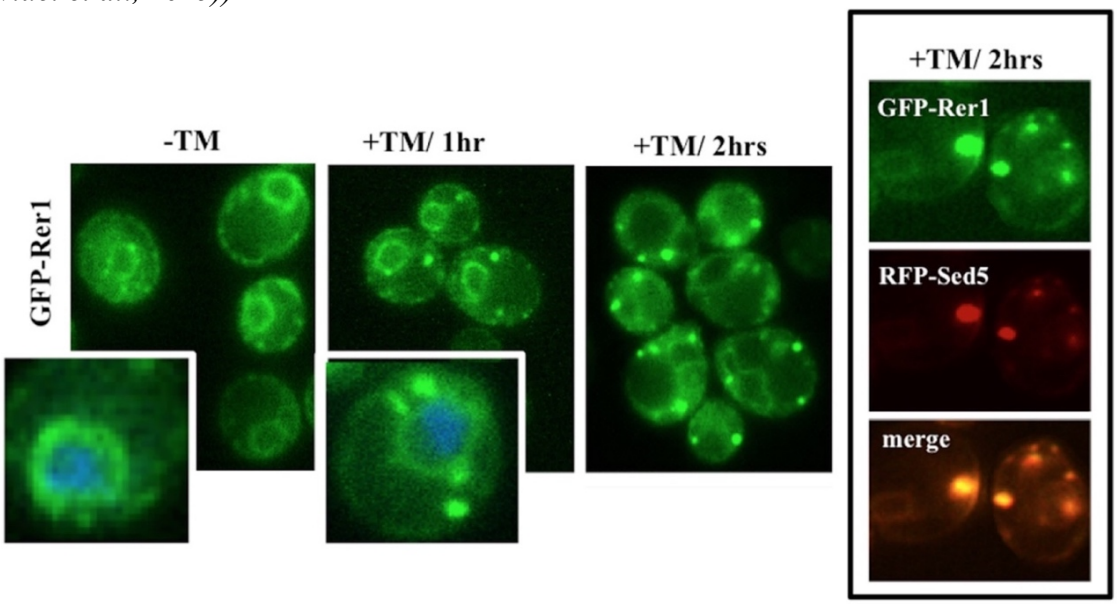


Figure 3.5: Tunicamycin-induced ER stress leads to Golgi localization of Rer1 in *S. cerevisiae*
Live cell fluorescence microscopy of GFP-Rer1 expressed from a plasmid in yeast *rer1Δ* mutants treated with DMSO (control) or after exposure of 0.2 µg/ml TM. GFP-Rer1 localizes to perinuclear ER in untreated cells and redistributes to Golgi in cells treated with TM. Insets show cells counterstained with DAPI. Boxed images denote GFP-Rer1 co-localization with Golgi resident RFP-Sed5. (data from *(Ghavidel et al., 2015))*.

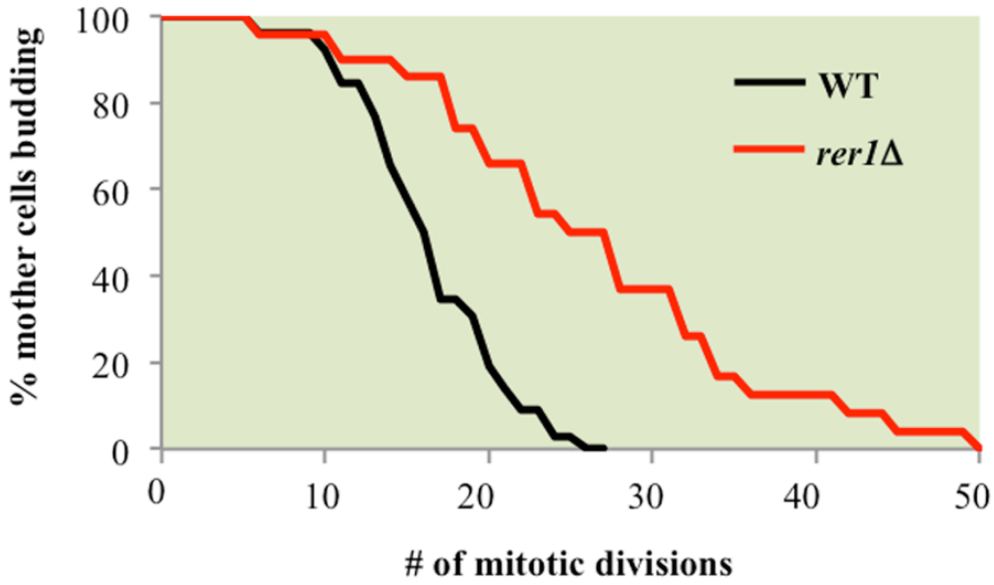


Figure 3.6: *S. cerevisiae rer1Δ* mutants show increased mitotic lifespan
Replicative lifespan of WT and isogenic *rer1*Δ mutants grown on YPD at 30°C (p value < 0.0001, nonparametric Mann-Whitney U test). (data from *(Ghavidel et al., 2015))*

Given the sequence conservation of Rer1 proteins across eukaryotes, it is likely that deleting *rer-1* would extend reproductive lifespan in *C. elegans*, similar to *S. cerevisiae.* Therefore, I asked: a) whether **inactivation of *rer-1* in *C. elegans* leads to increased UPR and b) whether triggering UPR influences reproductive lifespan.** For this purpose, I utilized a *C. elegans rer-1(tm5219)* mutant harbouring a 167 bp deletion in exon 2, which was obtained from the NBRP (National Bioresource Project) in Japan. This deletion removes almost the entire exon 2 of *rer-1* and generates a premature stop codon resulting in a 32-amino acid protein, making *tm5219* a putative null mutant (Figure 3.7). This *rer-1(tm5219)* mutant was outcrossed 4 times into the wild type strain to remove secondary mutations.

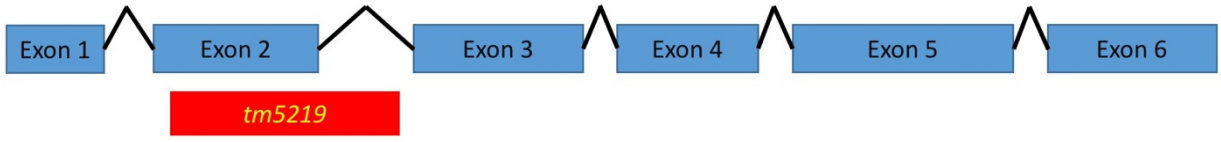


Figure 3.7: Genomic layout for *C. elegans rer-1*
Representative layout of the *C. elegans rer-1* introns and exons with the *tm5219* deletion marked (not drawn to scale); (data from www.wormbase.org).

3.3 Results

3.3.1 RER-1 is localized to the perinuclear region of intestine and germ cell nuclei

In order to probe the cellular localization of RER-1 in worms, I performed immunofluorescence microscopy on adult worms using a mouse anti-RER-1 polyclonal antibody and detected with a FITC conjugated rabbit anti-mouse secondary antibody. As shown in Figure 3.8a and 3.8b, RER-1 was found to be localized to the perinuclear region of both intestinal and germ cell nuclei, which agrees with the localization of Rer1 in *S. cerevisiae*.

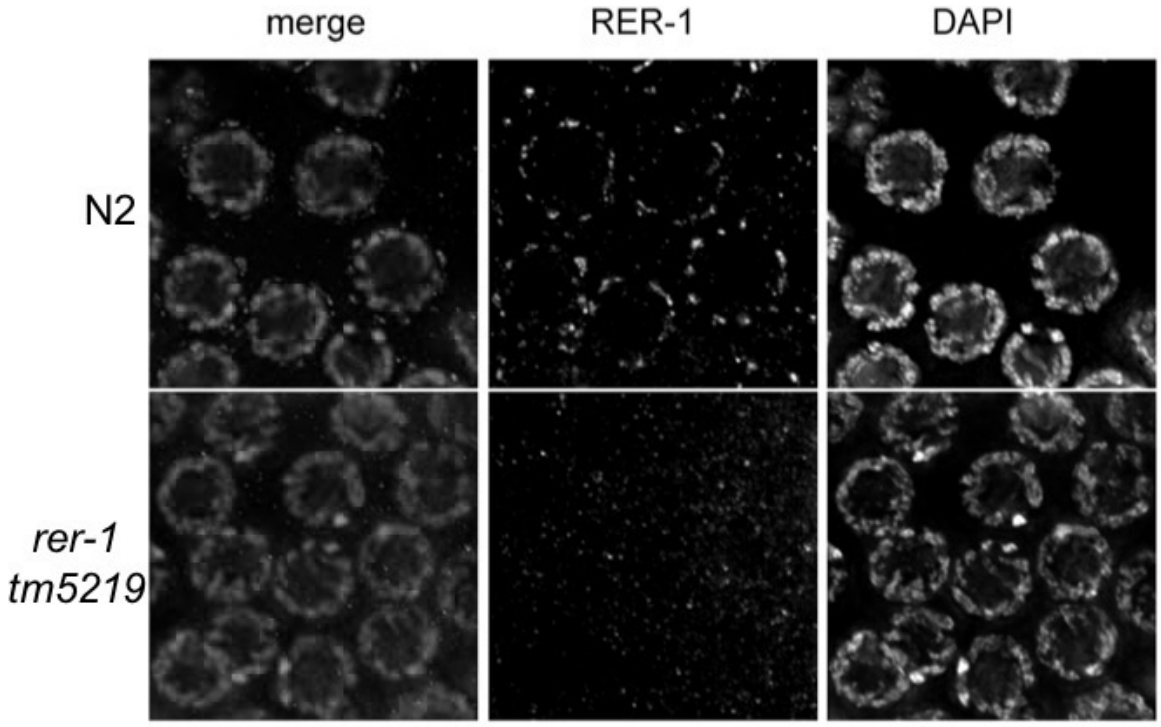


Figure 3.8(a): RER-1 localizes to the perinuclear region in gonadal nuclei.
Immunostaining of *C. elegans* gonads (WT and *rer-1* mutants) using mouse polyclonal anti-RER-1 antibody. Blue represents DAPI stained nuclei whereas green represents RER-1.

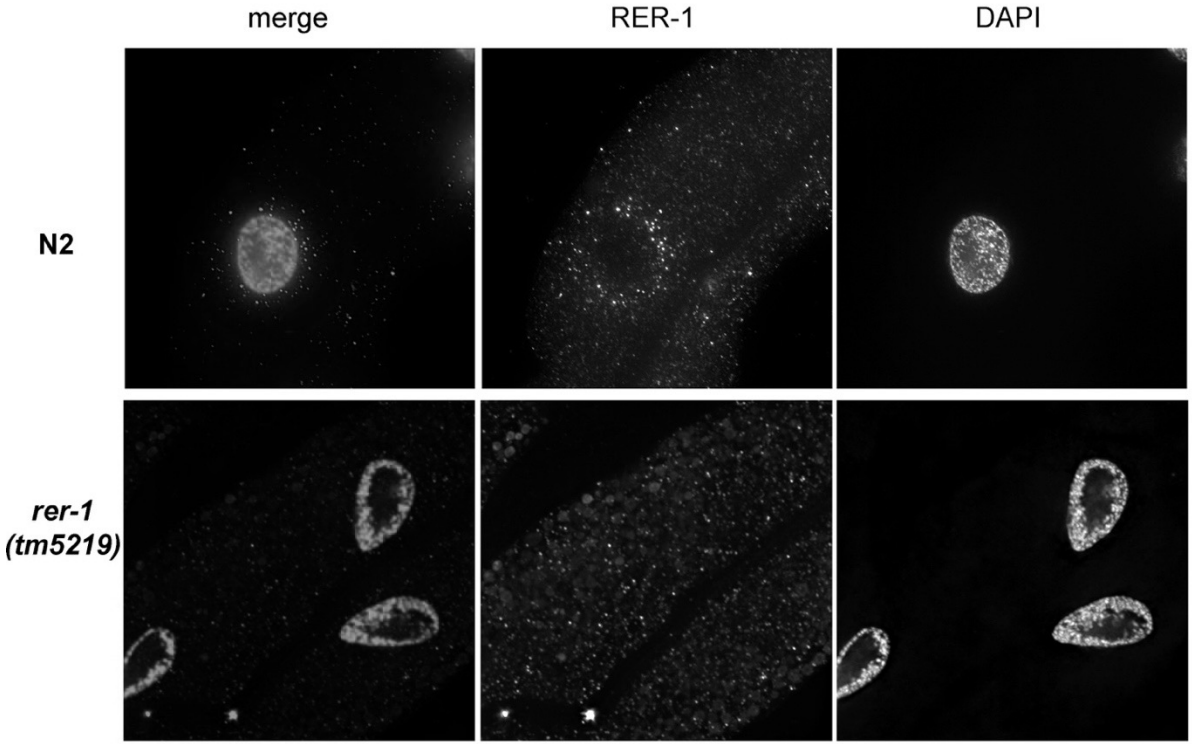


Figure 3.8(b): RER-1 localizes to the perinuclear region in intestinal nuclei.
Immunostaining of *C. elegans* intestines (WT and *rer-1* negative control) using mouse polyclonal anti-RER-1 antibody. Blue represents DAPI stained nuclei whereas green represents RER-1. (The variation in the shape of the nuclei is due to slight variations in fixing worms and is inconsequential for the scope of this experiment.)

3.3.2 Validation of tunicamycin as an ER stressor in *C. elegans*

In order to determine the influence of tunicamycin on *C. elegans* physiology, worms expressing a fluorescent UPR reporter (*Phsp-4*::*GFP*) were treated with tunicamycin and the level of GFP expression was compared with untreated worms (Calfon et al., 2002). As shown in Figure 3.9 below, worms treated with tunicamycin showed much higher GFP expression compared to untreated worms, thereby suggesting a higher level of UPR in these worms.

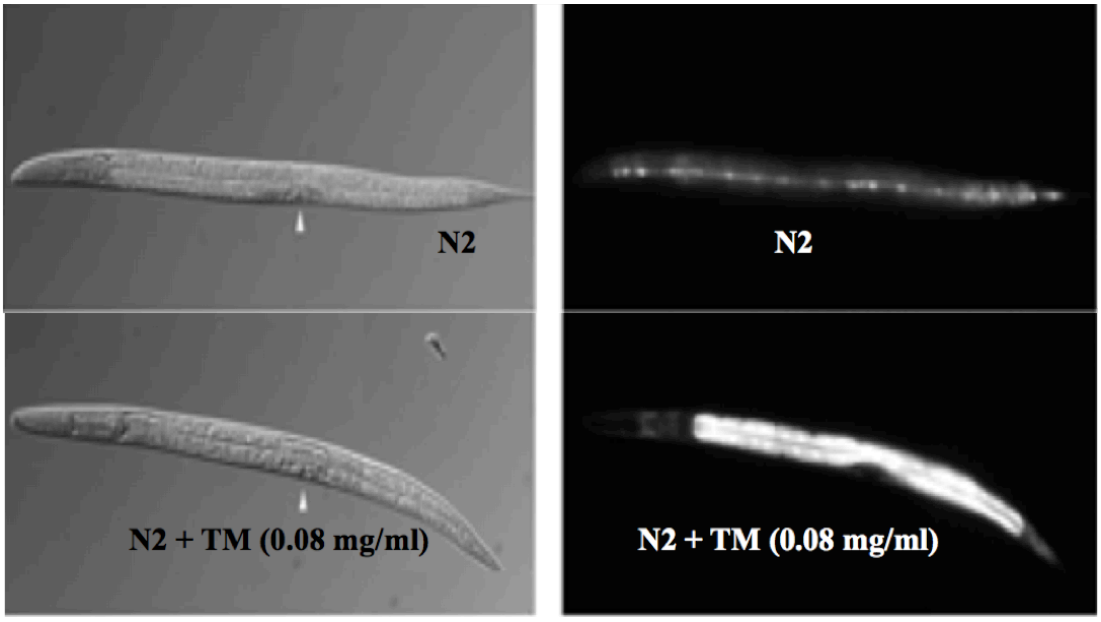


Figure 3.9: Tunicamycin induces ER stress in *C. elegans*.
Age matched N2 wild type worms were grown on NGM plates that were supplemented with either DMSO (control) or tunicamycin (0.08 mg/ml). Arrow heads indicate the vulva region.

3.3.3 Tunicamycin extends *C. elegans* organismal and reproductive lifespan

To monitor changes in organismal and reproductive lifespan following tunicamycin treatment, worms were fed OP50 food supplemented with tunicamycin (0.02 µg/ml or 0.1 µg/ml) and their organismal and reproductive lifespan was compared with worms that were fed control OP50 food. As shown in Figure 3.10(a) and 3.10(b), worms fed OP50 supplemented with tunicamycin showed increased organismal and reproductive lifespan.

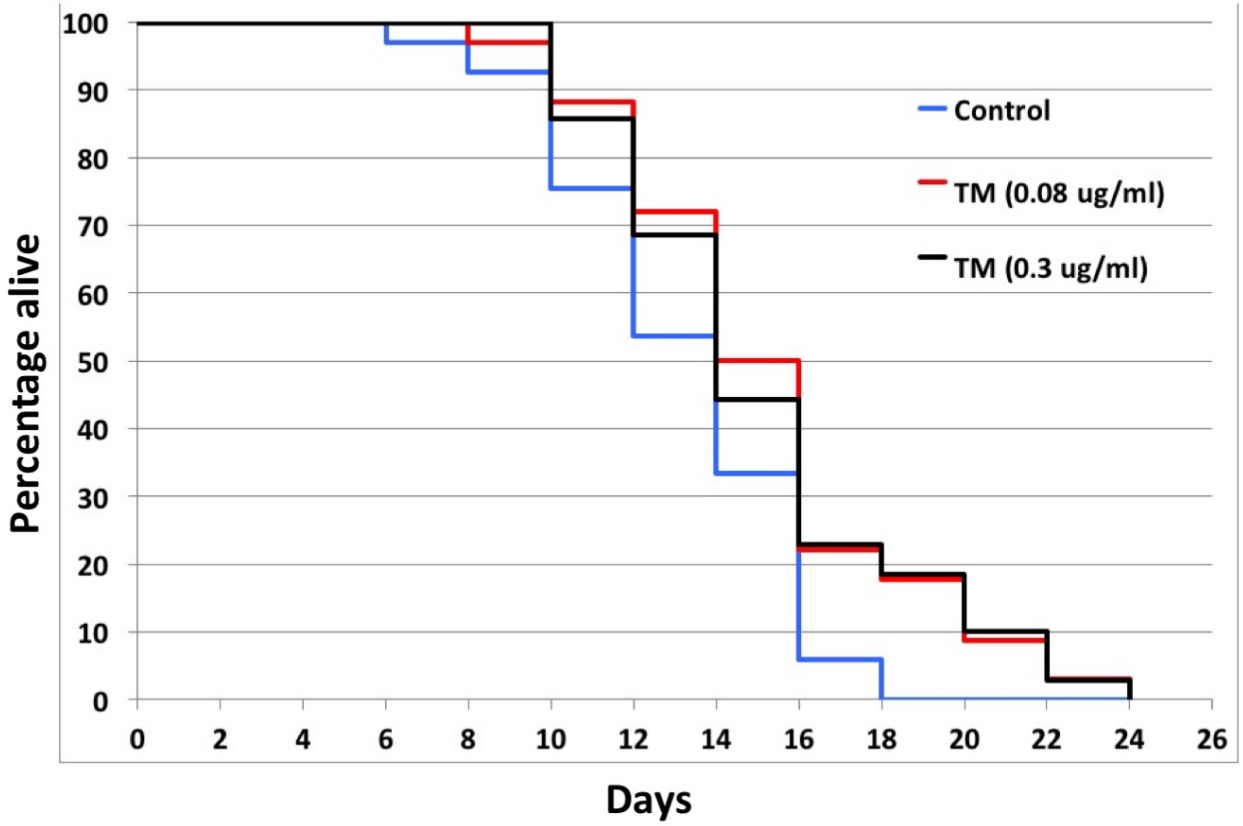


Figure 3.10(a): Tunicamycin increases *C. elegans* organismal lifespan (n=70).
Wild type (N2) worms were grown on NGM plates that were supplemented with either DMSO (control) or tunicamycin (0.1 ug/ml or 0.02 ug/ml) (p values: (a) control vs TM 0.08 ug/ml p = 0.000032 (b) control vs TM 0.3 ug/ml p = 0.0004 (c) TM 0.08 ug/ml vs TM 0.3 ug/ml p = 0.7097). All p values were calculated using Mantel-Cox test.

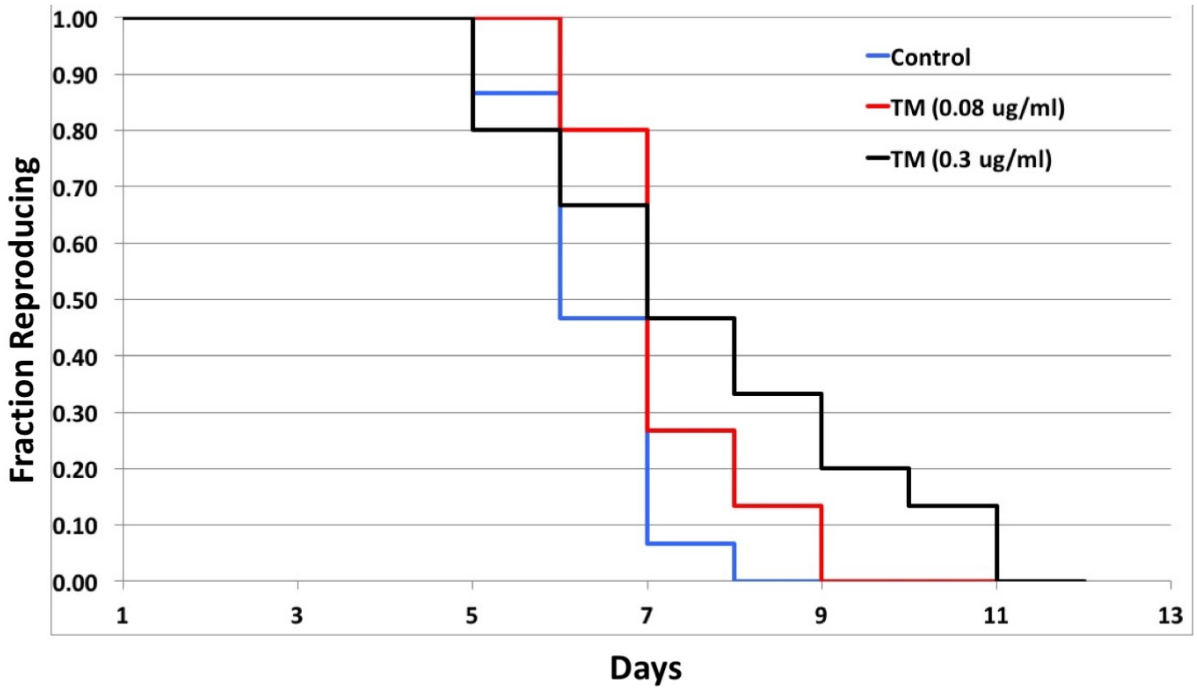


Figure 3.10(b): Tunicamycin treatment shifts the dynamics of *C. elegans* reproduction (n=15).
Wild type (N2) worms were grown on NGM plates that were supplemented with either DMSO (control) or tunicamycin (0.08 ug/ml or 0.3 ug/ml) (p values: (a) control vs TM 0.08 ug/ml $p < 0.0001$ (b) control vs TM 0.3 ug/ml $p < 0.0001$ (c) TM 0.08 ug/ml vs TM 0.3 ug/ml $p = 0.009$). All p values were calculated using Mantel-Cox test.

3.3.4 *rer-1* mutants undergo increased ER stress

In order to assess the level of ER stress in *rer-1* worms, I constructed *rer-1* worms expressing a UPR inducible reporter gene fusion (*Phsp-4*::GFP) (Calfon et al., 2002). As shown in Figure 3.11 below, *rer-1* worms expressing this reporter have a much higher GFP expression than control (*Phsp-4*::GFP) worms, indicating that *rer-1* mutant are in a constant state of low grade stress.

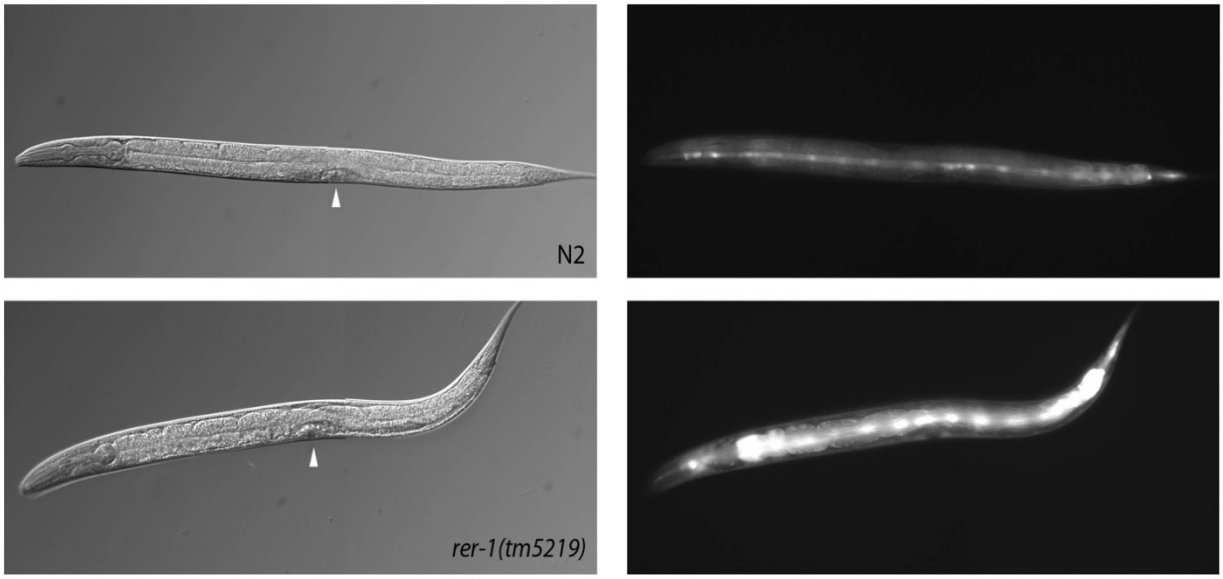


Figure 3.11: UPR induction in *rer-1* mutants.
*Phsp-4::*GFP expression in wild type and *rer-1* mutants. Arrow heads indicate the vulva region.

3.3.5 *rer-1* worms display extended reproductive lifespan

In view of the extended replicative lifespan in *S. cerevisiae rer1*Δ mutants (Figure 3.10b) and given the extensive sequence and functional conservation of RER1 proteins across eukaryotes (Fullekrug et al., 1997), I examined whether loss of *rer-1* impacts reproductive lifespan in *C. elegans* by monitoring the number of worms producing viable progeny as a function of time. Wild-type *C. elegans* worms exhibit a progressive decline in germ cell proliferation and oocyte fertilization capacity over time that contributes to reproductive cessation. This effect has been shown to be independent of sperm repository, brood size, and alterations in reproductive schedule. It is suggested that this is due to a physiological deterioration in germ cell quality and/or maintenance that normally occurs as animals age (Luo et al., 2010). In *rer-1* mutants, reproductive decline was slower compared to wild type worms such that during later reproductive life (day 6 post L4), 51% of *rer-1* hermaphrodites generated viable progeny whereas only 19% of wild type animals generated viable progeny (Figure 3.12, $p< 0.0001$). This prolonged reproductive lifespan in *rer-1* worms was eventually hampered by a sharp increase in cases of matricide, a process where eggs hatch inside the uterus of the hermaphrodite and devour the insides of the mother, eventually bursting out of the cuticle (Figure 3.12, $p<0.0001$). A comparatively milder RNAi knockdown of *rer-1* resulted in no detectable matricide and led to delayed reproductive senescence in wild type hermaphrodites from day 4 to day 7 of adulthood while increasing the median reproductive lifespan

by 66% (Figure 3.13, $p < 0.0001$). This experiment was undertaken using an *rrf-3* mutant strain at 16°C because this strain is sterile at 20°C or 25°C due to meiotic progression defects that affect progeny numbers. Even though this experiment was conducted at 16°C where this strain is viable, the reproductive lifespan curves for control and *rer-1* RNAi in Figure 3.13 are significantly shorter than the one presented in Figure 3.12. Taken together, these results suggest that inactivating RER-1 thus extends the window for reproduction.

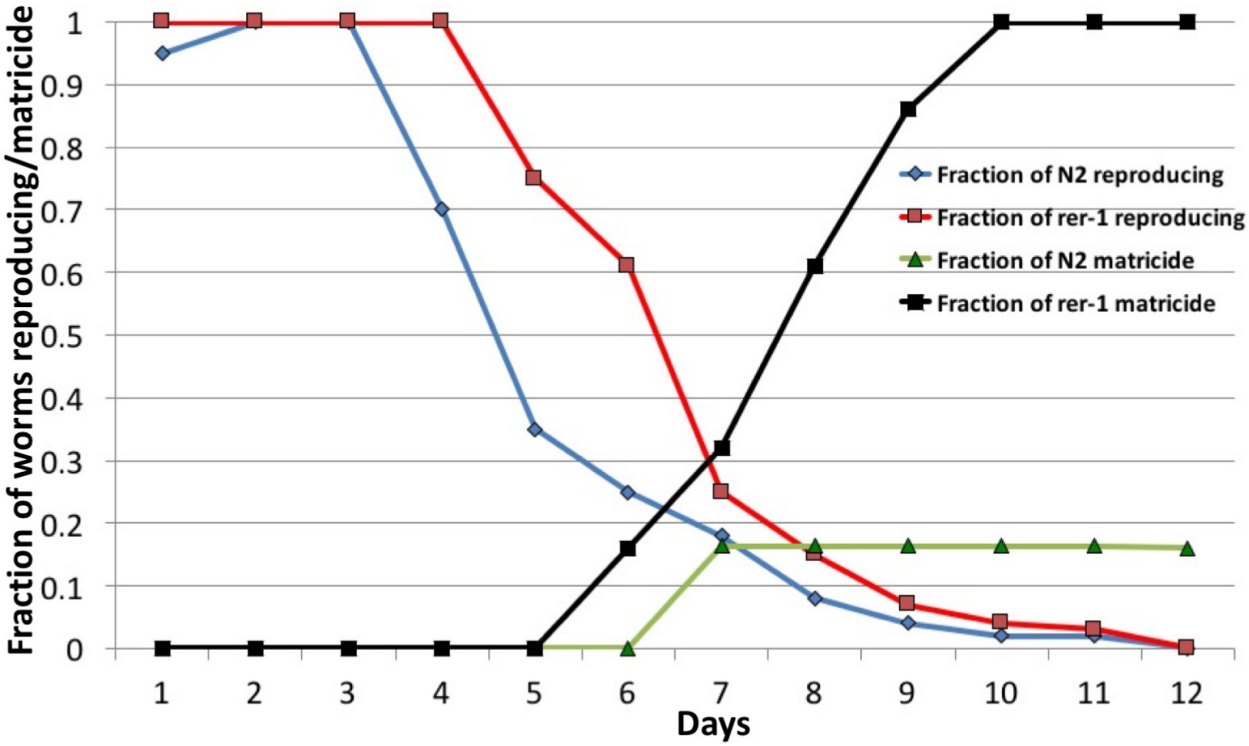


Figure 3.12: *rer-1* inactivation extends reproductive lifespan but increases matricide. Wild type (N2) and *rer-1* worms were grown on NGM plates seeded with OP50 and transferred every day to a fresh NGM plate. Worms were scored for the absence or presence of progeny after 24 hrs. Worms that did not generate any viable progeny for 2 consecutive days were deemed to have ceased reproducing (p values: (a) Fraction of N2 reproducing vs fraction of *rer-1* reproducing $p < 0.0001$ (b) Fraction of N2 matricide vs fraction of *rer-1* matricide $p < 0.0001$). All p values were calculated using Mantel-Cox test.

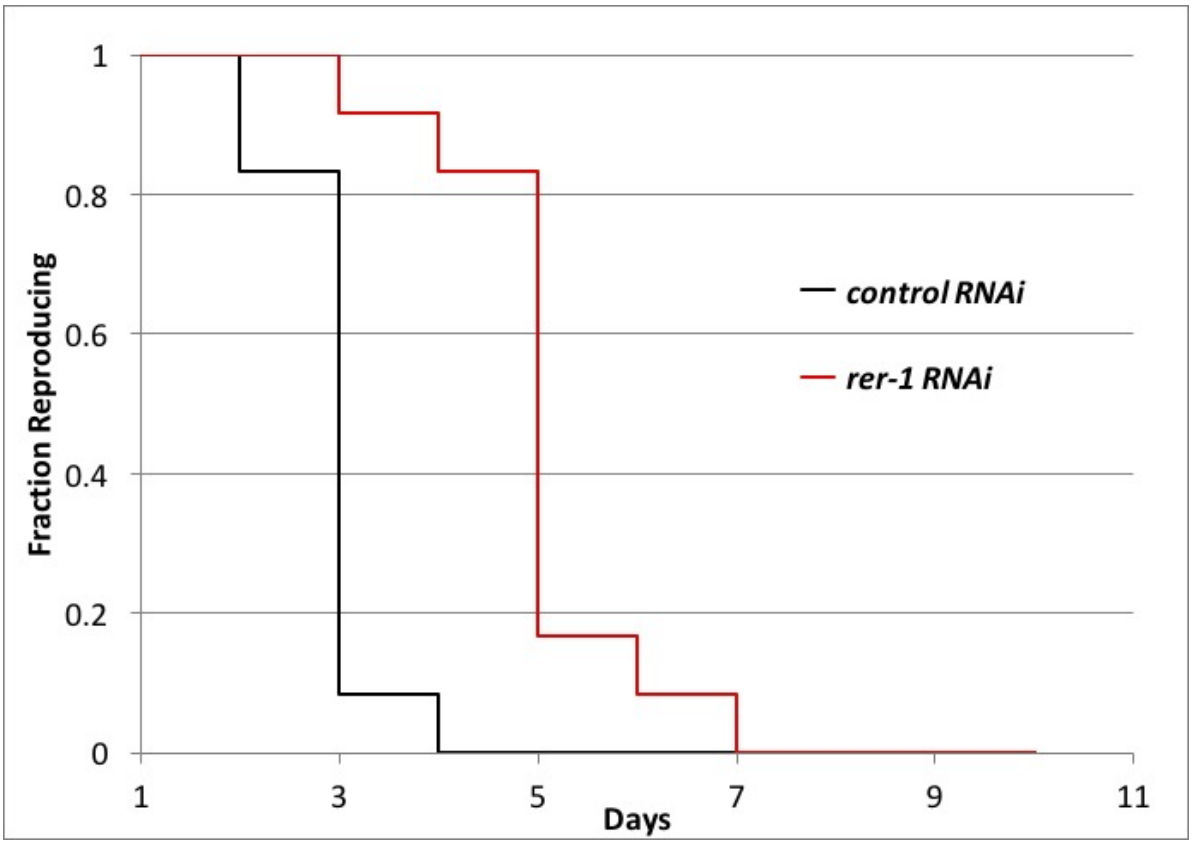


Figure 3.13: A mild *rer-1* RNAi knockdown extends reproductive lifespan (n=12). RNAi-hypersensitive *rrf-3* worms were grown on RNAi plates seeded with either HT1115 control food (empty pL4440 vector) or *rer-1* RNAi food and transferred every day to a fresh plate. Worms were scored for the absence or presence of progeny after 24 hrs. Worms that did not generate viable progeny for 2 consecutive days were deemed to have ceased reproducing ($p < 0.0001$; Mantel-Cox test)

3.3.6 *rer-1* mutants shift the dynamics of reproduction to later life stages.

To determine if the late cessation of reproduction in *rer-1* mutants produced overall more progeny, I monitored the production of progeny per day in both wild type (N2) and *rer-1* worms. I observed that while *rer-1* worms did not differ much in terms of total progeny production (Figure 3.14), they produced fewer progeny during day 1 and 2 but significantly higher progeny production during day 4 and 5 (Figure 3.15 below). The prolonged reproductive lifespan in *rer-1* worms was eventually tampered by a sharp increase in cases of matricide.

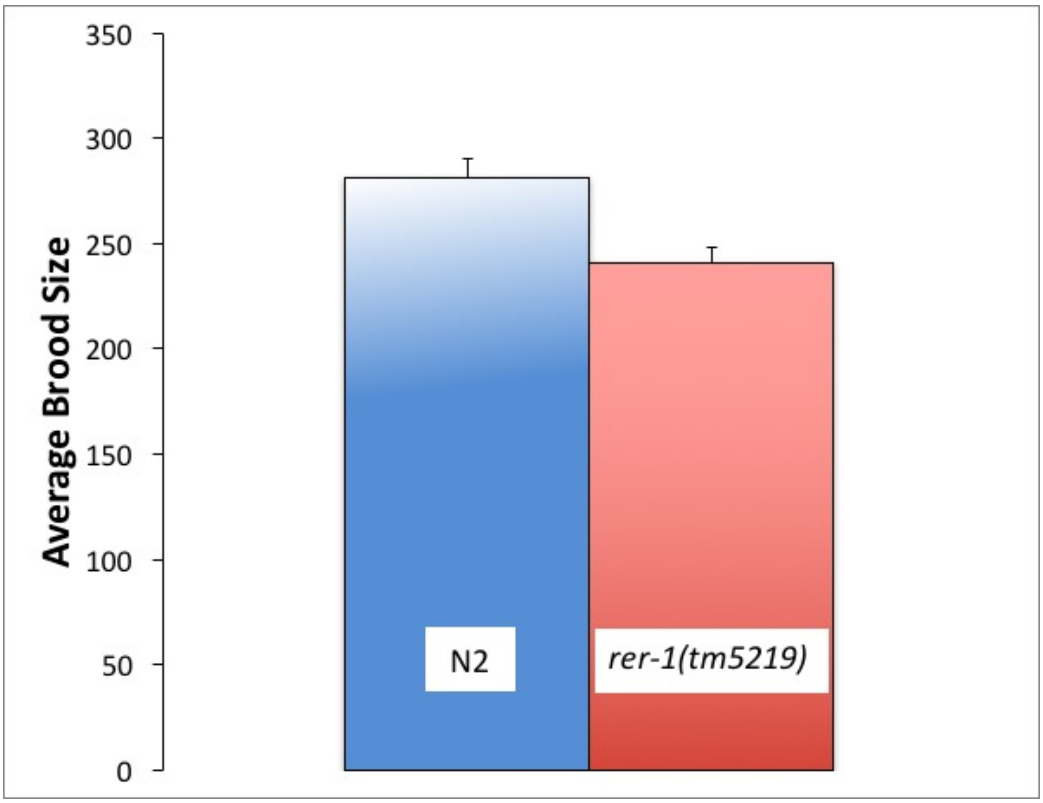


Figure 3.14: *rer-1* mutants generate slightly lower brood size compared to wild type (n=22). Wild type (N2) and *rer-1* worms were grown on NGM plates seeded with OP50 and transferred every day to a fresh NGM plate. The number of viable progeny in each plate was scored after 48 hrs ($p = 0.011$; unpaired t test).

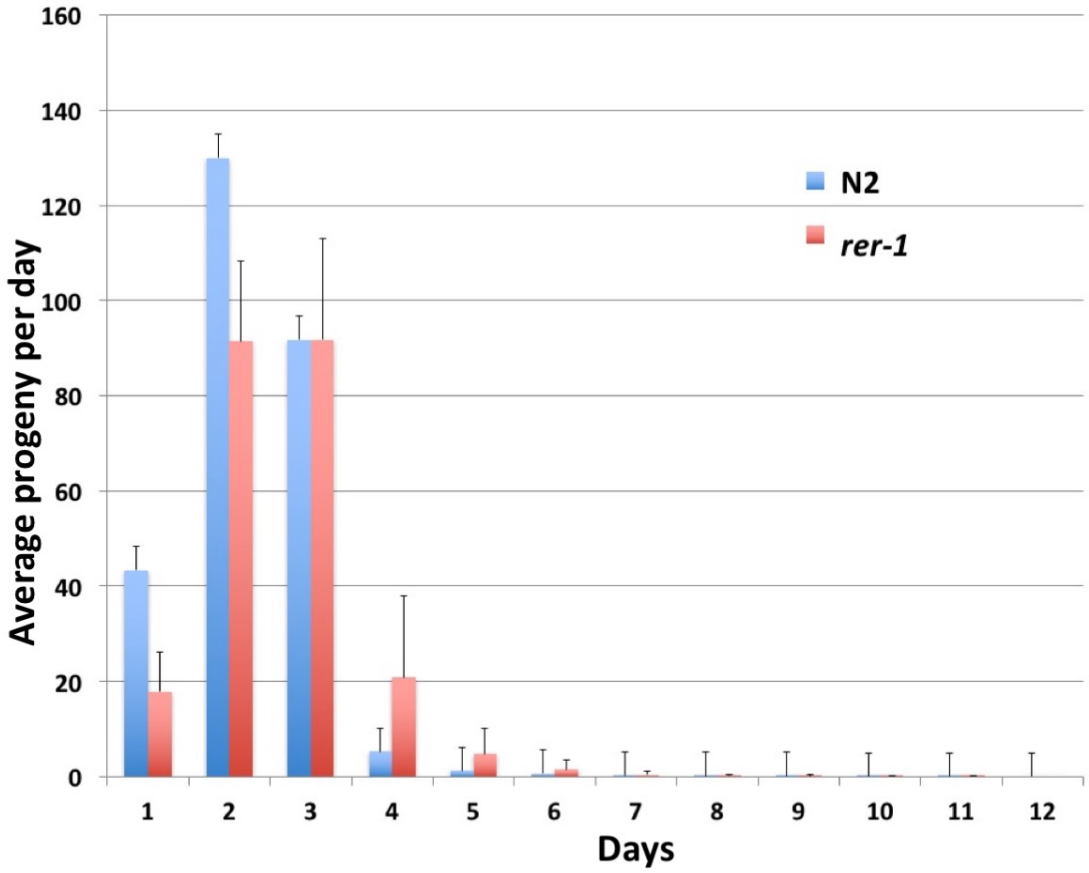


Figure 3.15: *rer-1* mutants shift the dynamics of reproduction (n = 22).
Wild type (N2) and *rer-1* worms were grown on NGM plates seeded with OP50 and transferred every day to a fresh NGM plate. The number of viable progeny in each plate was scored after 48 hrs (p value of average progeny for each day p = 0.0478; unpaired t test).

3.3.7 *rer-1* worms resist age-dependent decline in reproduction capacity

In *C. elegans*, the pre-meiotic tip (PMT) of the gonad is the only region that contains actively dividing (mitotic) germ cells. These stem cells divide throughout the adult lifespan of the worm to replenish the germ cell population entering meiosis, thereby providing a continuous supply of oocytes during adulthood. As worms reach the end of reproductive life, the proliferative index of this cell population drops, a fact that is thought to contribute to the reduced pace of oogenesis later in life and the decline in reproductive capacity (Luo et at., 2010). Proliferation in the distal cell population can be assayed by assessing levels of histone H3 phosphorylation, a hallmark of chromatin condensation during mitosis (Rogers et al., 2002). One plausible explanation for the extended reproductive lifespan in *rer-1* worms could involve a delay in the loss of mitotic activity in the pre-meiotic tip of the gonad as worms progress to later stages of reproduction. In order to

test this hypothesis, I measured the histone H3 phosphorylation of nuclei in the pre-meiotic tip region of the gonads of young and old *rer-1* mutants and compared them to wild type. As shown in Figure 3.16 and Figure 3.17, the mitotic index of wild type and *rer-1* mutants was comparable in young worms (day 2). By day 9 the mitotic activity in wild type worms had declined from 4.29 foci/gonad to 0.81 foci/gonad, whereas the *rer-1* mutants retained a significantly higher amount of mitotic activity (from 5.16 to 2.53 foci/gonad). This is the first report where removal of an ER-Golgi protein leads to an age-dependent decline in mitotic activity in the pre-meiotic tip of the *C. elegans* gonad.

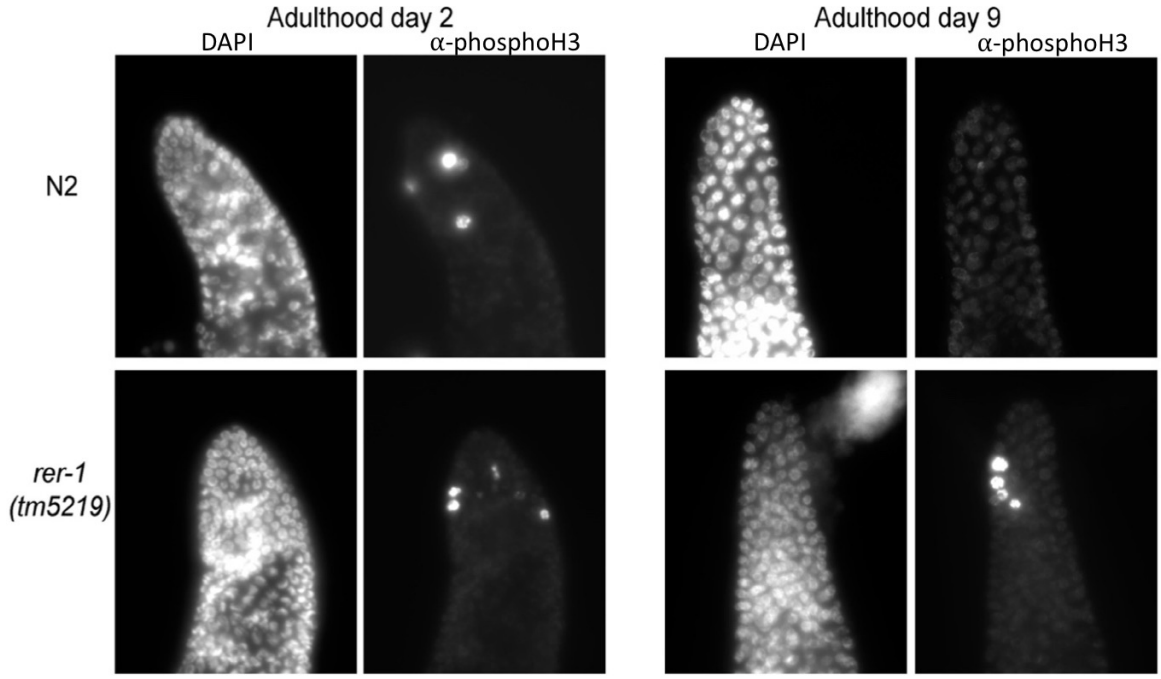


Figure 3.16: *rer-1* worms show higher mitotic activity in gonads compared to wild type. Representative immunofluorescence images of germ cells at the (pre-meiotic) distal tip region (farthest from the vulva). Worms were dissected and stained via freeze-cracking using anti-phosphoH3 antibody combined with DAPI staining.

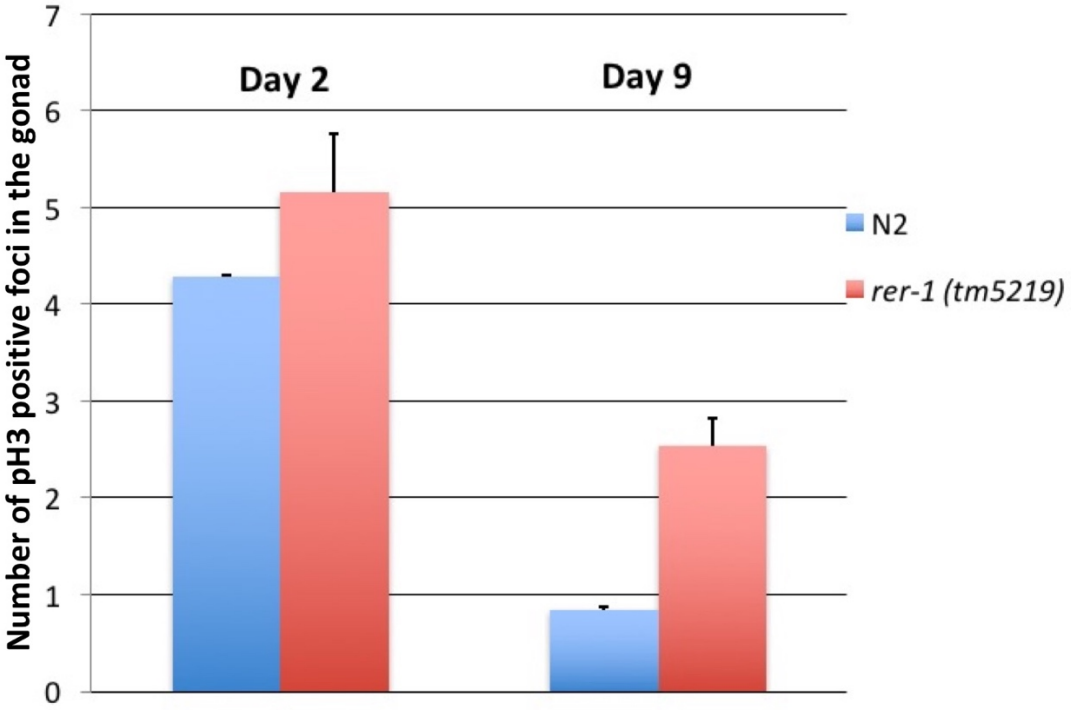


Figure 3.17: *rer-1* worms show higher mitotic activity in gonads compared to wild type (n=30).
Average number of histone H3 phosphorylated nuclei in the distal tip (pre-meiotic) region in day 2 and day 9 old wild type and *rer-1* mutants (p values: (a) p value for N2 day 2 vs *rer-1* day 2 p=0.1789 (b) p value for N2 day 9 vs *rer-1* day 9 p=0.0148 (c) p value for N2 day 2 vs N2 day 9 p<0.0001 (d) p value for *rer-1* day 2 vs *rer-1* day 9 p=0.03007). All p values were calculated using unpaired t test.

3.3.8 *rer-1* mutants undergo enhanced autophagic flux and increased proteolysis

Of all the downstream effects of UPR, perhaps the best studied is the process of autophagy. Autophagy is one of the most important processes of *C. elegans* physiology and is essential for dauer development and lifespan extension (Melendez et al., 2003). Studies have shown that certain lifespan extending paradigms such as caloric restriction are known to rely on autophagy (Hansen et al., 2008; Jia et al., 2007). It is therefore quite likely that the increased reproductive lifespan seen in *rer-1* mutants is a consequence of increased autophagy.

In tandem with inducing UPR, ER stress signals the activation of autophagy in yeast and human cells (Ogata et al., 2006; Yorimitsu et al., 2006). Autophagy, as explained earlier, is a conserved housekeeping mechanism whereby bulk cytoplasm including proteins and organelles are sequestered into membrane-bound vesicles (autophagosomes) which subsequently fuse with lysosomes where their content is degraded by a complement of lysosomal proteases (Cuervo et al.,

2005). While mechanistically distinct, UPR and autophagy synergistically mitigate ER protein load.

In order to monitor autophagic flux in the intestine of worms, I utilized a strain expressing mCherry fused to LGG-1 (*Pnhx-2::*mCherry::LGG-1), the *C. elegans* ortholog of ATG8 (Gosai et al., 2010). In wild type worms, mCherry::LGG-1 exhibited a diffuse intestinal signal with sporadic punctate foci formation. In *rer-1* worms, however, the number of foci was significantly increased, suggesting a higher abundance of autophagosomes (Figure 3.18 below). Elevated ER stress in *rer-1* mutants therefore results in robust formation of autophagosomes in *C. elegans*, suggesting enhanced autophagic flux.

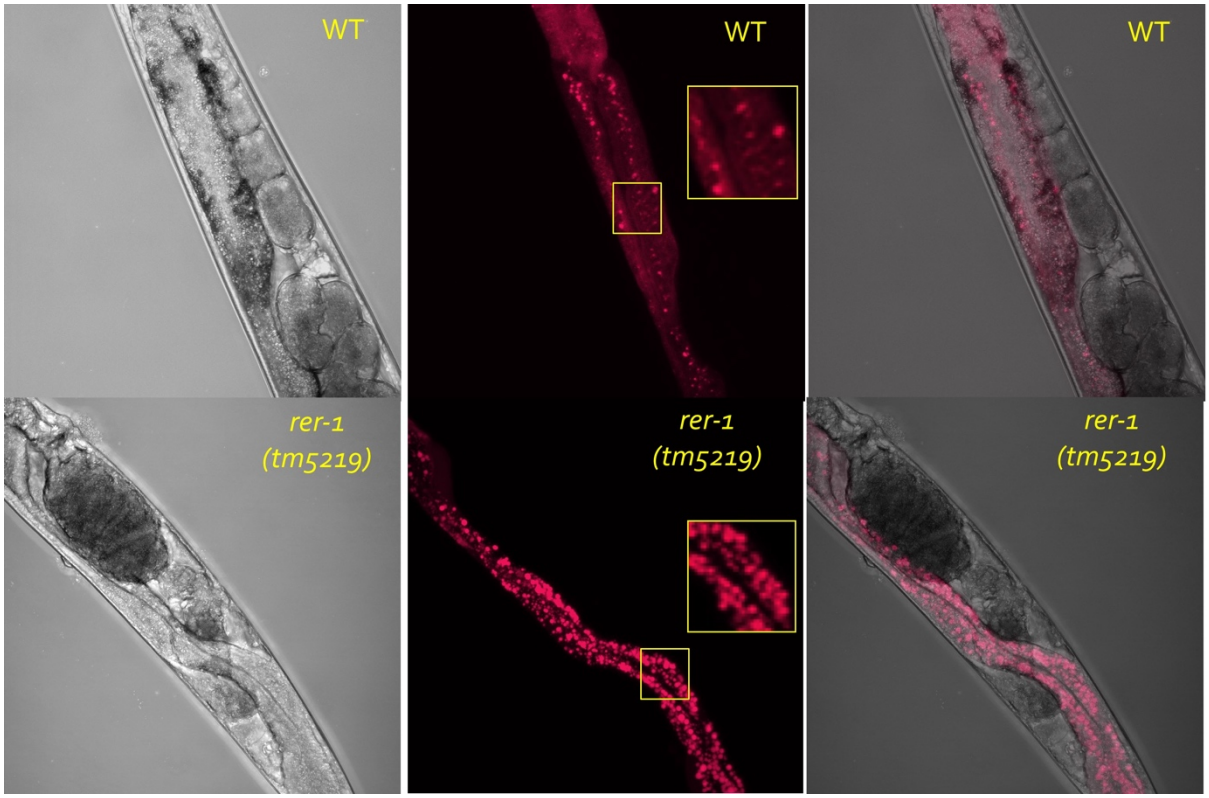


Figure 3.18: Induction of autophagy in *C. elegans rer-1* mutants. Intestinal expression of mCherry::LGG-1 from an intestine-specific promoter (*nhx-2*) in day 2 wild type and *rer-1* worms. Insets represent magnification of the boxed sections.

Protein aggregation is a hallmark of aging cells in which autophagy is overloaded and protein turnover impaired. In order to know whether the higher autophagic flux in *rer-1* mutants functionally translates into improved protein clearance, I monitored the age-dependent accumulation of α-syn::GFP in body wall muscles of a *C. elegans* strain expressing human α-

synuclein fused to GFP (*Punc-54::α-syn*::GFP) (Hamamichi et al., 2008). In young animals, GFP signal is observed diffused throughout the muscle cells, indicating a rapid GFP protein turnover under regular autophagy levels. As worms age, reduced autophagic flux leads to accumulation of GFP into non-soluble foci. Consistent with their high basal autophagic flux, *rer-1* worms (as well a control strain co-expressing the heat shock chaperone protein torsinA (*Punc-54::α-syn*::GFP; *Punc-54::tor-2*)) displayed a marked reduction in the number of GFP foci compared to wild type worms expressing the same *Punc-54::α-syn*::GFP construct (Figure 3.19 below). This suggests that the expansion in the pool of autophagosomes in *rer-1* mutants is accompanied with a quantitative reduction in cytoplasmic protein inclusions and increased protein homeostasis.

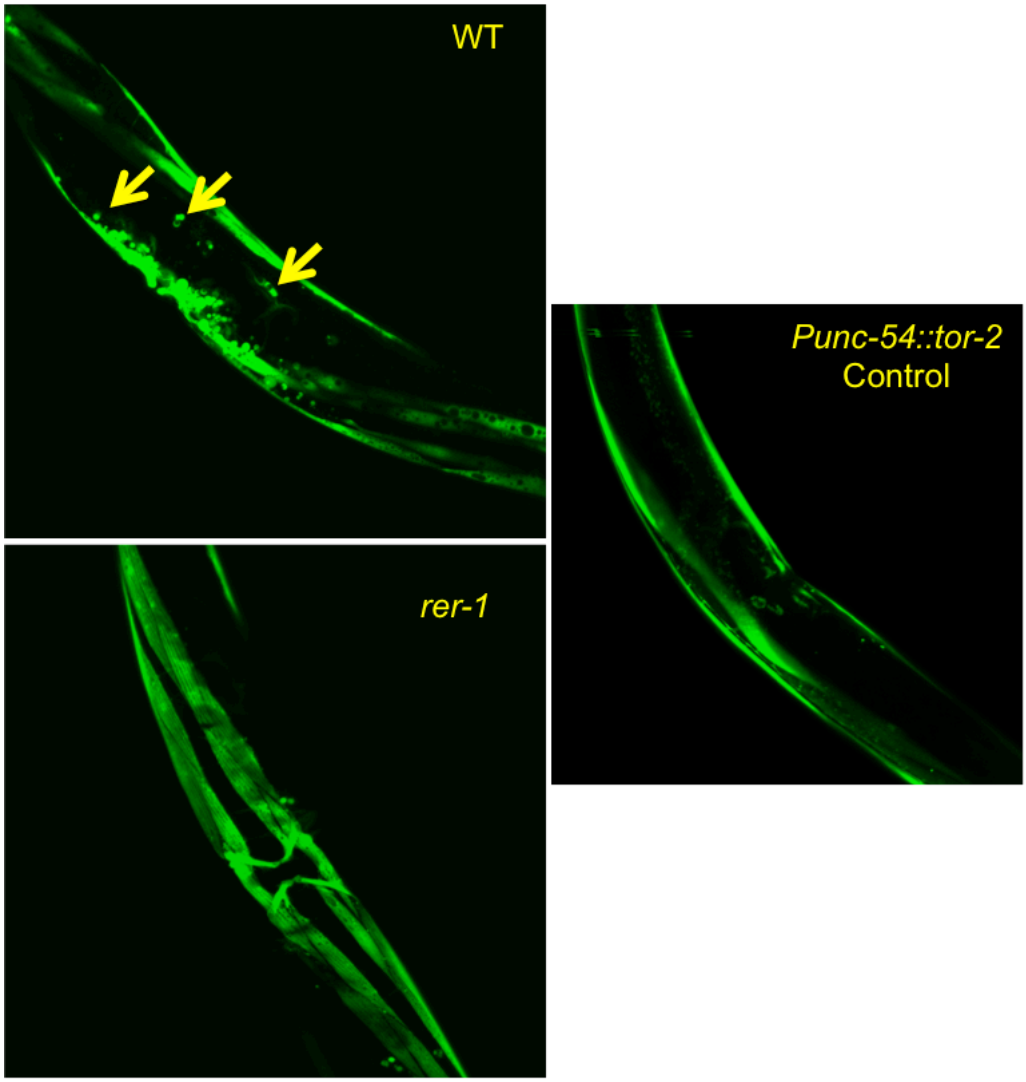


Figure 3.19: Enhanced clearance of protein aggregates in *rer-1* mutants.
Representative fluorescent images of *C. elegans* worms expressing human α-syn::GFP under a body wall muscle promoter (*Punc-54::α-syn*::GFP). Note the soluble GFP profile highlighting the longitudinal muscle fibers as well as the crossing vulval muscles. Arrows denote a subset of the insoluble α-syn-GFP foci. Worms over expressing heat shock protein torsinA (*tor-2*) in the same cells (*Punc-54::tor-2*) have increased chaperone function that can overcome the protein overload in these cells (Hamamichi et al., 2008).

3.3.9 Autophagy is required for extended reproductive lifespan in *rer-1* mutants

To investigate whether the increased autophagic flux in *rer-1* mutants is necessary and sufficient to explain the extended reproductive lifespan observed in these mutants, I used *lgg-1* RNAi knockdown to reduce autophagy. In order to first verify the *lgg-1* RNAi construct, I tested its effect on a strain expressing (*Pnhx-2::mCherry::lgg-1*). As seen in Figure 3.20 below, worms

fed *lgg-1* RNAi showed a dramatic reduction in mCherry::LGG-1 fluorescence in the intestine compared to control (empty vector) RNAi, thereby validating the use of this clone for *lgg-1* transcript knockdown .

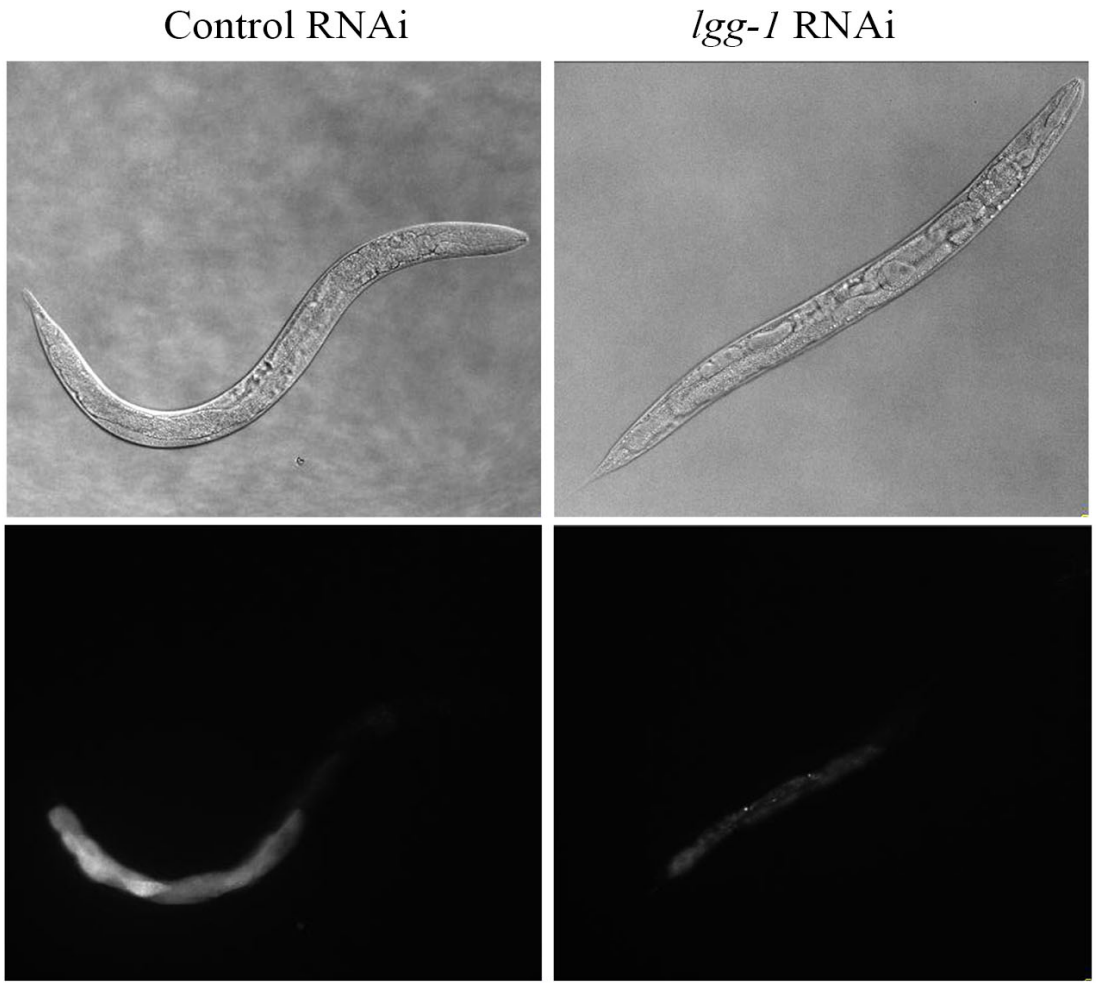


Figure 3.20: Verification of *lgg-1* RNAi knockdown.
Fluorescent images of *C. elegans* worms expressing mCherry::LGG-1 fed either control RNAi or *lgg-1* RNAi.

To verify that the diminished mCherry signal in mCherry::LGG-1 worms fed with *lgg-1* RNAi corresponded with reduced autophagic flux, I assayed the age-dependent accumulation of α-syn::GFP in *rer-1* worms that were fed *lgg-1* RNAi. As seen in Figure 3.21(a) and (b) below, *lgg-1* RNAi restored the aggregation of α-syn::GFP in *rer-1* worms, highlighting a requirement for autophagy in removal of these aggregates in worms and suggesting that *lgg-1* RNAi was indeed reducing autophagosome formation in *rer-1* worms.

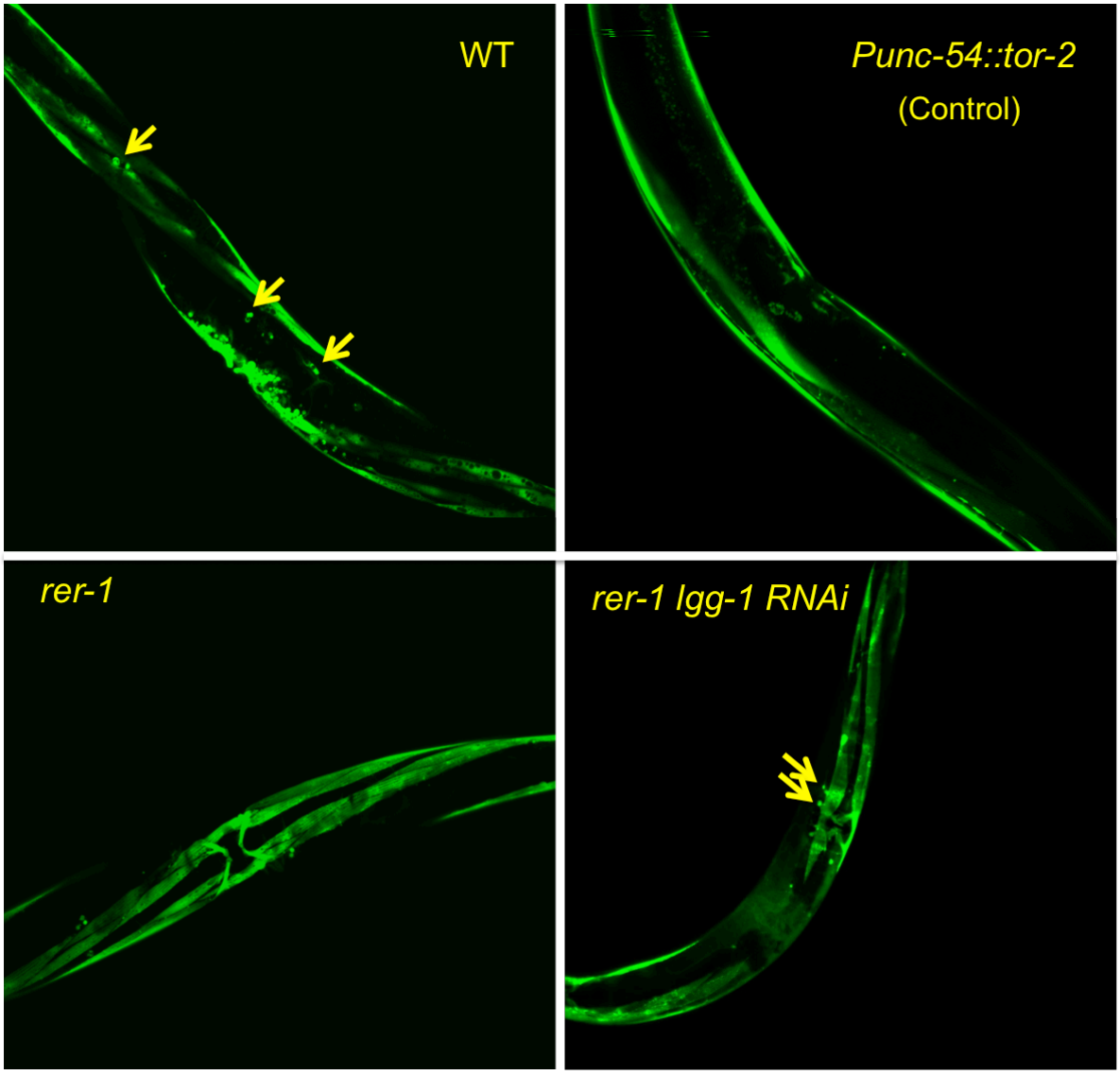


Figure 3.21(a): Autophagy is required for clearance of protein aggregates in *rer-1* mutants. *C. elegans* worms expressing human α-syn::GFP from a body wall muscle promoter (*Punc-54::α-syn*::GFP). Arrows denote a subset of the α-syn-GFP foci. Worms co-expressing heat shock protein torsinA (*tor-2*) in the same cells (*Punc-54::tor-2*) served as a positive experimental control.

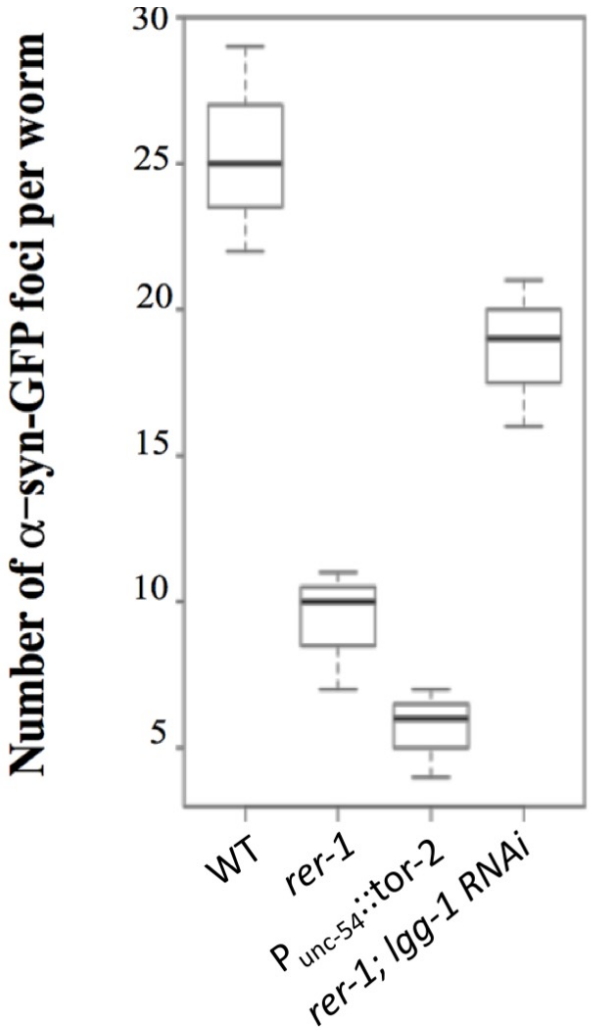


Figure 3.21(b): Autophagy is required for clearance of protein aggregates in *rer-1* mutants (n=10).
Average number of α-syn::GFP foci per worm scored from flattened Z-stack images such as the ones in Figure 3.21(a) above. (p values: WT vs *rer-1* p<0.0001; WT vs *Punc-54::tor-2* p<0.0001; WT vs *rer-1 lgg-1* RNAi p < 0.0001; *rer-1* vs *Punc-54::tor-2* p=0.038; *rer-1* vs *rer-1 lgg-1* RNAi p < 0.0001). All p values were calculated using unpaired t test.

Finally, to test whether the extended reproductive lifespan in *rer-1* mutants is due to increased autophagic flux, I performed RNAi-mediated *lgg-1* knockdown in *rer-1* and wild type worms and scored for reproducing worms throughout adulthood. As seen in Figure 3.22 below, *lgg-1* RNAi knockdown in *rer-1* worms brought the extended reproductive lifespan of *rer-1* worms back to wild type levels.

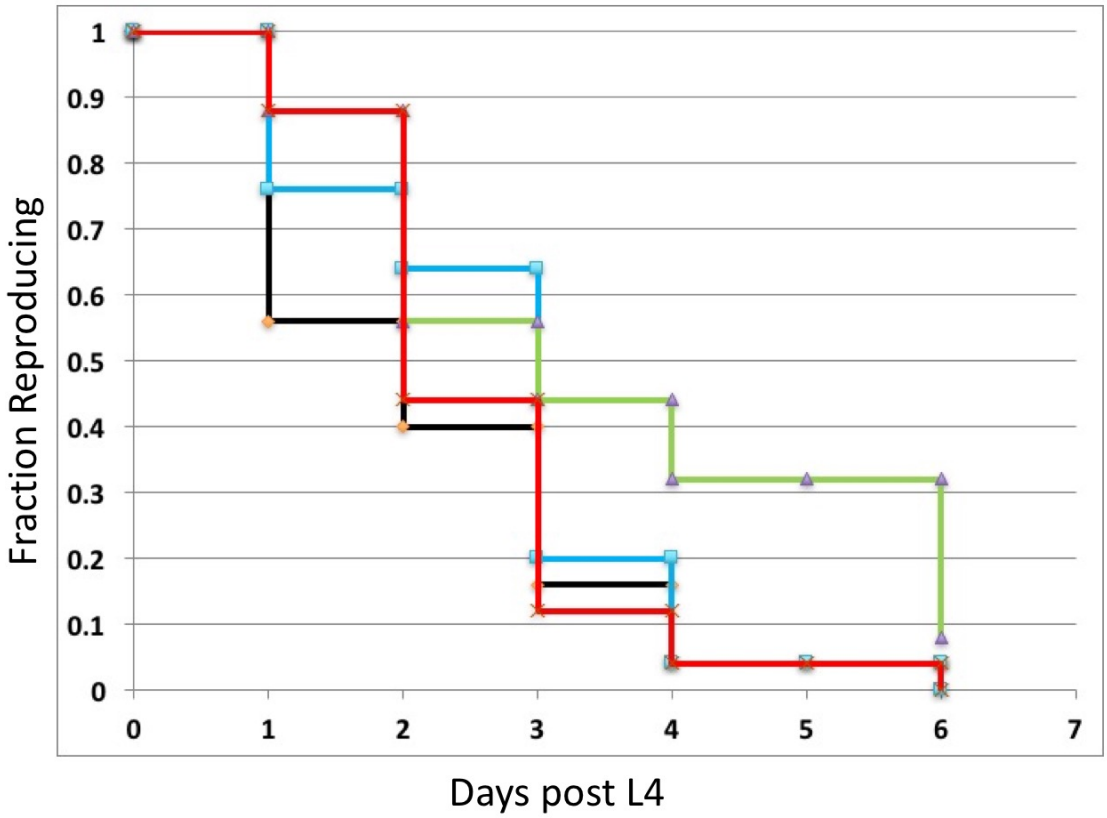


Figure 3.22: Reproductive lifespan extension in *rer-1* mutants requires autophagy-mediated cellular catabolism.

Reproductive lifespan in N2 and *rer-1* worms fed either control or *lgg-1* RNAi food. (p values: (a) N2 control RNAi vs N2 *lgg-1* RNAi p=0.0185 (b) N2 control RNAi vs *rer-1* control RNAi p<0.0001 (c) N2 *lgg-1* RNAi vs *rer-1 lgg-1* RNAi p=0.1 (d) *rer-1* control RNAi vs *rer-1 lgg-1* RNAi p<0.0001). All p values were calculated using unpaired t test.

3.4 Discussion

A genetic screen for identification of yeast mutants that undergo a higher than wild type number of cell divisions before senescing revealed a gene cluster that functions in ER to Golgi protein transport. In this chapter, I used *C. elegans* to validate the relevance of these findings in a multicellular eukaryote by focusing on one such gene named *rer-1*. This work led to insights on the integration of ER functions and reproductive longevity and exposed reproductive lifespan extension as a likely consequence of constitutive ER-induced stress. Data from *S. cerevisiae* studies showed that Rer1 is localized to perinuclear ER during unperturbed growth and is re-distributed to Golgi during ER stress (data not shown). I show that *C. elegans rer-1(tm5219)* mutants (169 bp deletion in exon 1) are under constitutive ER stress and UPR induction that culminates in extended reproductive lifespan, albeit this extended reproductive lifespan is eventually tampered by matricide, revealing a disconnect between somatic and reproductive aging in these mutants. Similar to *rer-1* mutants, wild-type worms treated with sub-lethal concentrations of ER stressors such as tunicamycin undergo increased ER stress and UPR induction that ultimately leads to a small but significant increase in reproductive lifespan. These observations suggest that RER-1 inactivation leads to induction of ER stress that likely contributes to ER homeostasis by removing misfolded proteins from the ER lumen to Golgi for their subsequent transport to lysosomes by autophagosomes. I have also provided evidence to show that the elevated autophagic flux in *rer-1* mutants is necessary and sufficient to extend reproductive lifespan. The induction of autophagy thus appears to be a broad adaptive response evoked to augment UPR in cells with impaired protein trafficking (*rer-1*) across the ER-Golgi network. The induction of autophagy in *rer-1* mutants is accompanied by a marked clearance of α-syn::GFP cytoplasmic inclusions, an observation consistent with the role of autophagy as the principle pathway for removing high molecular weight protein aggregates. Protein aggregates form prominent cytological features that segregate with and contribute to senescence in post mitotic models including *C. elegans* and mice (David et al., 2010; Lee et al., 2010). My data suggest that similar to post-mitotic aging in yeast, formation of protein aggregates also serves as a determinant of reproductive senescence in worms. Taken together, these results indicate that treatment of worms with tunicamycin or inactivation of RER-1 function leads to ER stress that signals UPR induction and increased autophagic flux. This increase in autophagic flux leads to and overall enhanced clearance of protein aggregates in the worm via the autophagy-lysosome pathway. At the level of

the germline, improved proteolysis may be important to maintain a robust proliferative level in older animals while likely also affecting other aspects of oocyte maturation downstream. These changes in the proteolytic capacity of germ cells, I speculate, ultimately delay the loss of oocyte quality, improve fertilizability and allow older animals to produce more viable (Figure 3.23 below).

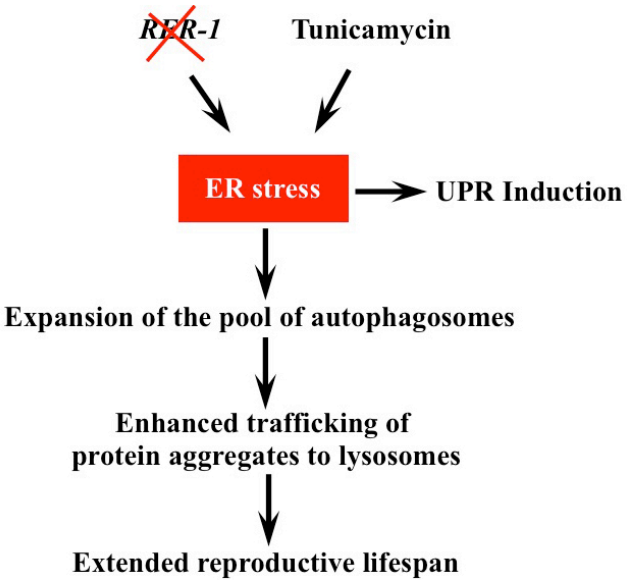


Figure 3.23: Relationship between ER stress, autophagy and lifespan.
Autophagy, induced in response to elevated ER stress in *rer-1* worms or following exposure to the ER stressor tunicamycin, traffics cytoplasmic aggregates to the lysosomes and in doing so delays mitotic senescence (Ghavidel et al., 2015)

4 COUPLING LYSOSOMAL FUNCTION TO LIFESPAN AND REPRODUCTIVE STATUS IN *C. elegans*

4.1 Background

4.1.1 Protein catabolism and aging in *C. elegans*

Aging in eukaryotic cells is accompanied by large, insoluble protein aggregates (David et al., 2010; Erjavec et al., 2007; Madeo et al., 2009; Rubinsztein, 2006). These protein aggregates are often deleterious, and one of the best examples of such protein aggregates are those involved in human neuropathies such as Alzheimer's and Parkinson's disease (Bence et al., 2001; Rubinsztein, 2006). It is also widely accepted that ectopic expression of aggregation-prone proteins leads to early onset of age-related phenotypes (Cohen et al., 2006; R. W. Powers, 3rd et al., 2006). Progressive formation of such large protein aggregates has been suggested to be one of the primary driving factors of cellular senescence.

Proteostasis in *C. elegans* is maintained by various quality control mechanisms that are involved in surveillance of protein synthesis, protein folding as well as protein degradation (E. T. Powers et al., 2009). The two main pathways of protein degradation are the autophagy lysosome pathway (ALP) and the ubiquitin proteasome system (UPS). While UPS is a selective process of protein degradation that relies on ubiquitination of proteins (Schmidt et al., 2014), ALP is a bulk degradation process that depends on sequestering proteins targeted for degradation and delivering these proteins to the lysosomes, which are the terminal end-point of autophagy (Vilchez et al., 2014).

4.1.2 Reproduction and aging

When the *C. elegans* germline (but not the somatic gonad) is removed either by genetic means or by laser ablation, worms live more than twice as long as wild type and are more resistant to environmental and nutritional stresses (Arantes-Oliveira et al., 2002; Hsin et al., 1999). One prevalent hypothesis was that the extended lifespan in this context arises as the consequence of extra resources becoming available in animals where germ cells are not produced. This hypothesis was dispelled when it was shown that removing the entire reproductive system had no effect on lifespan (Hsin et al., 1999). It was later postulated that signals from the somatic gonad are responsible for directly regulating *C. elegans* lifespan (M. C. Wang et al., 2008).

In worms, mutations in germ cell proliferation genes such as *glp-1* results in ablation of the germline without affecting the formation of the gonad. GLP-1 is expressed in the germline stem cells and promotes mitotic proliferation (Austin et al., 1987); mutations in *glp-1* result in premature entry into the meiotic program in detriment self-renewal which leads to long-lived adults with very few germ cells (Arantes-Oliveira et al., 2002). Removal of the *C. elegans* germline promotes increased lifespan by inducing a signalling cascade invoking the activation (and nuclear localization) of the (FOXO) forkhead transcription factor DAF-16 (Panowski et al., 2009). Besides DAF-16, germline-less mutants also require the proteins DAF-12 and DAF-9 for lifespan extension (Gerisch et al., 2001). While *daf-12* encodes a steroid hormone receptor, *daf-9* encodes a cytochrome P450 that is involved in the production of a lipophilic steroid ligand for DAF-12. The DAF-9/DAF-12 axis synergizes with DAF-2/Insulin signalling in interpreting metabolic cues to converge downstream in the regulation of DAF-16 (see below).

DAF-16 is the master transcription regulator that acts downstream of the *daf-2* insulin/insulin-like growth factor (IGF) signalling (IIS) pathway. Under conditions of nutritional abundance, the insulin/IGF-1 receptor activates a conserved signalling cascade leading to DAF-16 phosphorylation, thereby inactivating it and excluding it from the nucleus. During nutrition deprivation (as well as in *glp-1* mutants), IGF signalling is reduced and this leads to DAF-16 activation and translocation to the nucleus. This activates genes that are involved in lifespan extension and enhanced stress resistance (Figure 4.1) (Honda et al., 1999; Kenyon et al., 1993; Murakami et al., 1996). Even though both germline ablation and reduced IGF signalling act via the FOXO transcription factor DAF-16, the longevity conferred by germline ablation and reduced IGF signalling are synergistic to one another; loss of germline therefore increases the already-extended lifespan of IGF mutants such as *daf-2*. Reduction in IIS signalling has also been shown to delay accumulation of proteotoxic aggregates, suggesting that the IIS signalling pathway can regulate the protein homeostasis network (Morley et al., 2002).

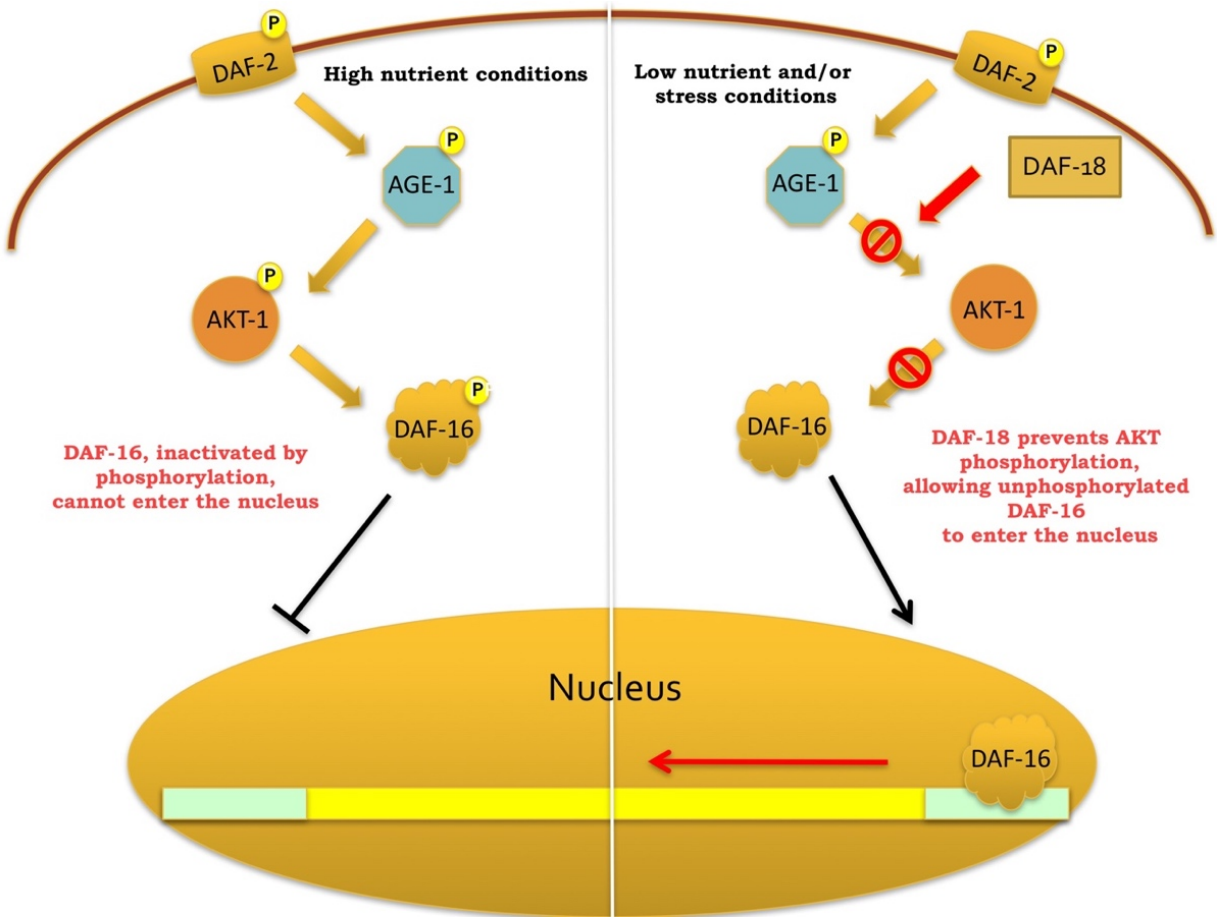


Figure 4.1: Diagrammatic illustration of the insulin/IGF-1 pathway.
Under conditions of adequate nutrition, DAF-16 is inactivated by a cascade of phosphorylation events and hence cannot enter the nucleus. However, under low nutrient or stressful conditions, DAF-16 is de-phosphorylated and enters the nucleus to transcribe genes that are involved in stress resistance. Some of these genes are also known to enhance lifespan.

4.1.3 Reproduction and protein catabolism

Recent data suggests that *C. elegans* worms lacking germline are more resistant to protein aggregation as well as thermal stress, and mutations that cause germline arrest delay the decline in proteostasis of somatic cells (Shemesh et al., 2013). Some of the genes involved in regulating somatic proteostasis in germline-less animals are also involved in their extended lifespan, and these include genes such as *daf-9, daf-12, nhr-80, pha-4* as well as *daf-16* (Shemesh et al., 2013; Vilchez et al., 2012). Such an enhanced proteostasis machinery in somatic tissues of germline-less animals could be explained by increased activity of the autophagy lysosome pathway as well as the ubiquitin proteasome degradation pathway. Not surprisingly, *C. elegans* worms lacking a germline have increased autophagy and enhanced *lipl-4* mediated lipolysis (Lapierre et al., 2011), thereby

promoting longevity via the nuclear hormone receptors NHR-80 and NHR-49 (Folick et al., 2015). The exact mechanisms by which NHR-49 and NHR-80 modulate lifespan, however, remains unclear. Besides LIPL-4, the transcription factor HLH-30 also influences lifespan in germline-less animals via regulation of autophagy, but evidence seems to suggest that HLH-30 might also regulate lifespan in other mutants with intact germline (Lapierre et al., 2013).

It is interesting to note that there is negligible evidence of the cross-talk between the autophagy lysosomal pathway and the reproductive system in *C. elegans* and most of the work done in this area has utilized sterile mutants that do not provide proper framework for explaining the connection between reproduction and autophagy in wild type worms.

4.1.4 Lysosomes

Lysosomes (referred to as vacuoles in yeast) are acidic (pH 4.5 to 5) organelles found within eukaryotic cells that are the terminal end-point of protein catabolism via the autophagy lysosome pathway. Besides their role in autophagy, lysosomes are also involved in endocytosis of yolk proteins in the intestine, although this role of lysosomes is outside the scope of this study (Sato et al., 2014). During autophagy, proteins and other macromolecules are encompassed within a membrane bilayer called the autophagosome and delivered to the lysosomes for degradation (Figure 4.). The acidic milieu of the lysosomes is maintained at a low pH by evolutionarily conserved ATP-dependent proton pumps (encoded by the *vma*/*vha* group of genes) that continuously transport H^+ (protons) across the lysosomal membrane (Figure 4.). These pumps are composed by 13+ different subunits that fall within one of two separate domains, namely the V_1 (cytosolic) domain (650 kDa) that generates energy via ATP hydrolysis for pumping protons and the V_0 domain (260 kDa) that is embedded in the lysosomal membrane and is involved in transporting protons across the lipid bilayer (Forgac, 2007; Jefferies et al., 2008). Lysosomal acidification is essential for various physiological functions such as activation of hydrolases and vesicle trafficking. Loss of function *vha* genes or RNAi mediated knockdown of any *vha* transcripts leads to embryonic lethality, presumably due to impairment of receptor mediated endocytosis that prevented yolk protein from being transported to the embryo (Choi et al., 2003; Oka et al., 2000).

Defects in lysosomal function leads to over 40 neurodegenerative and neurodevelopmental diseases in humans, and has been implicated in the pathogenesis of various disorders such as Alzheimer's and Parkinson's disease (Menzies et al., 2015; Rubinsztein et al., 2005). Mutations

that inactivate even a single lysosomal protein can lead to congenital or age-associated neurodegenerative disease, with the brain being the most prominent organ affected by lysosomal dysfunction (Cuervo et al., 2000; Rubinsztein et al., 2011). Until the last few years, most research focused on lysosomal proteases and their disorders. Recently though, more efforts are being made to understand the importance of lysosomal acidification as a readout of lysosomal competence. Lysosomes have recently been implicated as biosensors that sense nutrition levels and general cellular stress (S. Wang et al., 2015). The existence of lysosome-to-nucleus signalling has recently been shown to regulate aging in *C. elegans* by regulating the nuclear localization of various nuclear hormone receptors (Folick et al., 2015). The low pH is critical for modifying cargoes delivered to the lysosomes such as dissociation of lipids from proteins, dissociation of receptors from ligands and dissociation of metal ions from proteins (Asano et al., 2011; Singh et al., 2009; Yamashiro et al., 1984). Lysosomes have an internal feedback and quality control mechanism that serves to regulate autophagy induction by sensing the release of amino acids following effective substrate cleavage and degradation (Zoncu et al., 2011). Indeed, acidification of the yeast lysosomal apparatus has been shown to enhance mitochondrial stability and lifespan in *S. cerevisiae* (A. L. Hughes et al., 2012). Lysosomal acidification and autophagic induction are also crucial in mediating *S. cerevisiae* lifespan increase after caloric and methionine restriction, and overexpression of the v-ATPase components have been shown to increase lifespan (A. L. Hughes et al., 2012; Molin et al., 2014; Ruckenstuhl et al., 2014).

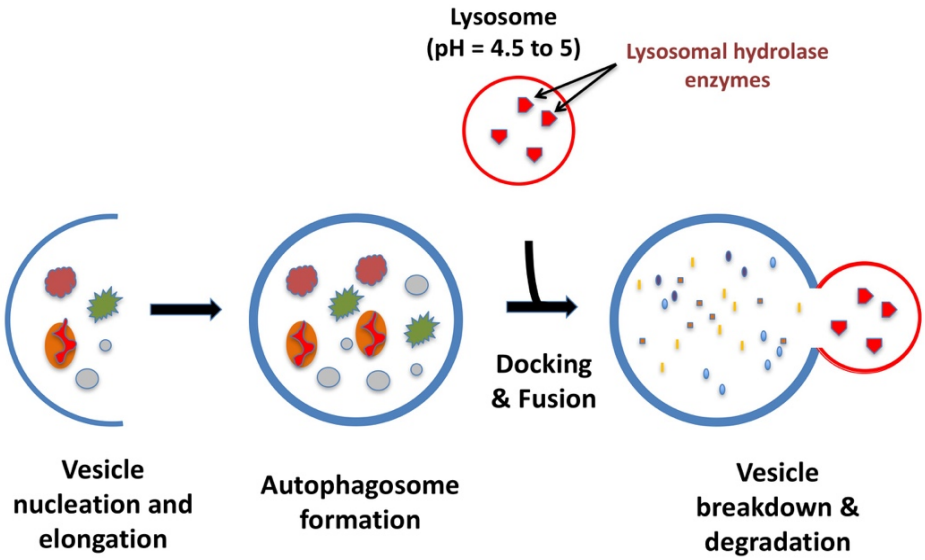


Figure 4.2: Diagrammatic illustration of autophagy.
Autophagy is initiated by formation of an isolation membrane around proteins and organelles that are destined for proteolysis. The isolation membrane expands to form an autophagosome that then docks and fuses with the low pH lysosome (containing lysosomal proteases and hydrolases) that then break down the components of the autophagosome (adapted from WormBook).

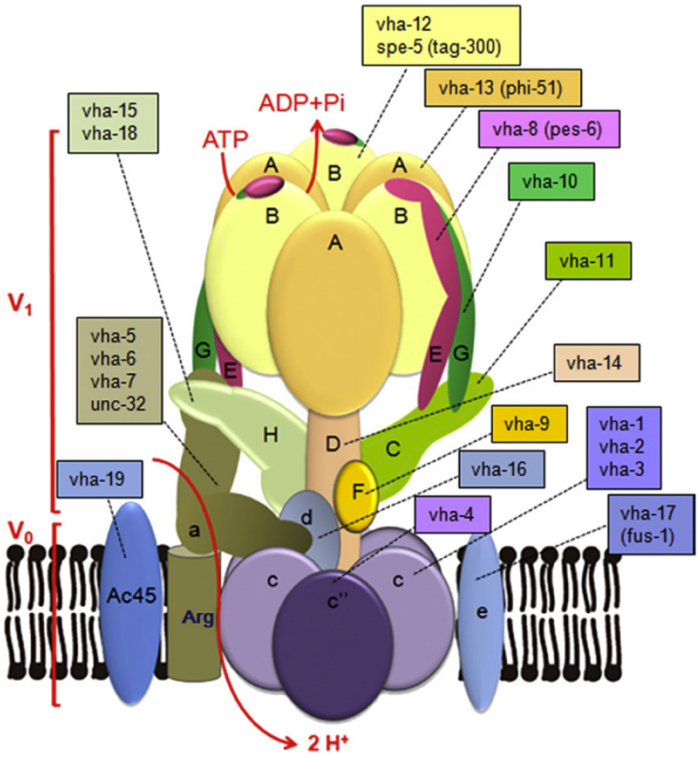


Figure 4.3: Diagrammatic representation of *C. elegans* vacuolar ATPase.
The *C. elegans* lysosomal membrane is embedded with numerous such membrane-spanning multi-subunit ATP-dependent proton pumps – each v-ATPase proton pump comprises more than 20 proteins that serve to pump protons into the lysosomal lumen, thereby acidifying it (diagram from (Choi et al., 2003)

4.2 Rationale and specific aims

In the previous chapter, I showed that accumulation of misfolded proteins coincides with reproductive senescence and that upregulation of autophagy delays reproductive senescence in yeast and *C. elegans*. It has been previously shown that autophagic flux declines in an age dependent manner in eukaryotic cells (Cuervo, 2008). One plausible explanation for this reduction in autophagy could be to allow the lysosomes to cope with accumulated misfolded/damaged proteins in the cytosol. Recently, the focus of scientific interest has shifted from autophagy to the proteolytic capacity of lysosomes, with more findings suggesting a link between lysosomal competence and aging (Carmona-Gutierrez et al., 2016; Folick et al., 2015; Han et al., 2015). There are numerous age-related changes that occur in lysosomal enzymes (mainly proteases and hydrolases) in eukaryotic cells, but the changes seem to vary based on the type of enzyme assayed and the type of tissue used (Cuervo et al., 1998). Moreover, there is strong evidence that the activities of lysosomal enzymes are not the rate-limiting component of lysosomal competence. Conversely, changes in the intralysosomal pH that license lysosomal enzymatic function appear to be more relevant in the prolonged function of these organelles (Cuervo et al., 2000).

In *C. elegans,* the intestine comprises one third of the somatic tissue and it is the site for food digestion, storage of macromolecules, and regulation of metabolism. As the major metabolic hub in the organism, it is not surprising that the *C. elegans* intestine is a critical node in the cross-tissue signalling pathways that regulate lifespan. The majority of *C. elegans* lysosomes are also localized in cells of the intestine. To date, there are few studies, if any, that have looked at the influence of reproduction status on *C. elegans* lysosomes. Instead, studies in *C. elegans* have mostly focused on the interplay between autophagy and lifespan in ***sterile*** mutants, but there is very little data showing the correlation between reproduction status and lysosomal health in wild type animals. Thus, there is a need for a more thorough evaluation at the physiological levels on the connection between somatic proteostasis, reproductive capacity and lifespan. The initial goals of this study were to:

a) **Design an *in vivo* assay to assess intestinal lysosomal pH over time**
b) **Compare the changes in lysosomal pH between actively reproducing and sterile mutants**
c) **Assess the impact of changes in lysosomal pH in organismal lifespan**

4.3 Results

4.3.1 cDCFDA – a novel lysosomal pH indicator for *C. elegans*

cDCFDA (5-(6)-carboxy-2',7'-dichlorofluorescein diacetate) is a cell membrane permeable probe that acts as a fluorescent pH indicator. With a pKa of 4.8, cDCFDA can be used to mark acidic environments in an otherwise neutral cytosol. Free cDCFDA can cross cell membranes passively and reach all cell compartments and organelles. In normally neutral environments, the dye does not fluoresce. In the lysosome, however, cDCFDA is hydrolyzed by esterases to carboxy-dichlorofluorescein, a fluorescent compound. It has been used previously in yeast where it provides an *in vivo* readout of acidic (and hence functional) vacuoles (Zheng et al., 1998). In order to determine the feasibility of cDCFDA as a pH sensitive marker for intestinal lysosomes in *C. elegans*, I supplemented OP50 food with 100µM cDCFDA + 60 µg/ml Nile Red, a vital dye that has been shown to mark intestinal lysosomes (also known as gut granules or lysosomes-related organelles) in worms (O'Rourke et al., 2009). Animals fed overnight on this dye mixture were imaged the next day via confocal microscopy. My results showed that cDCFDA stained organelles (green) co-stained with Nile Red (Figure 4.4). Importantly, cDCFDA reported emission spectra generated a single prominent peak at 520nm, ruling out possible interference with the notoriously autofluorescent signals of gut granules (Figure 4.5). Also notable is the fact that no cDCFDA signal was ever detected outside the intestine, suggesting that the dye, while imported from the intestinal lumen, cannot cross the basal membrane of the intestine.

Having established that cDCFDA localizes to intestinal lysosomes, I then sought to determine whether cDCFDA is able to report changes in lysosomal pH *in vivo*. In order to do so, worms were fed OP50 supplemented with 200mM of the v-ATPase inhibitor chloroquine (reviewed in (Pivtoraiko et al., 2009) until day 2 post-L4, following which worms were transferred to OP50 food supplemented with chloroquine and cDCFDA and imaged via confocal microscopy the day after. Confocal microscopy results showed that chloroquine-induced inhibition of v-ATPase dramatically reduced cDCFDA signals in the intestine. This is consistent with a corresponding increase in lysosomal pH in these cells validating the use of cDCFDA as a live pH indicator (Figure 4.6). Worms grown on OP50 food supplemented with cDCFDA for 3 days showed no overt impairment on reproduction or motility and were phenotypically indistinguishable from untreated worms, suggesting that prolonged exposure to cDCFDA does not lead to any

deleterious effect (data not shown). Taken together, these results indicate that not only does cDCFDA stain lysosomes, but it can detect dynamics changes (alkalinization) in pH in the lumen of intestinal lysosomes in *C. elegans*.

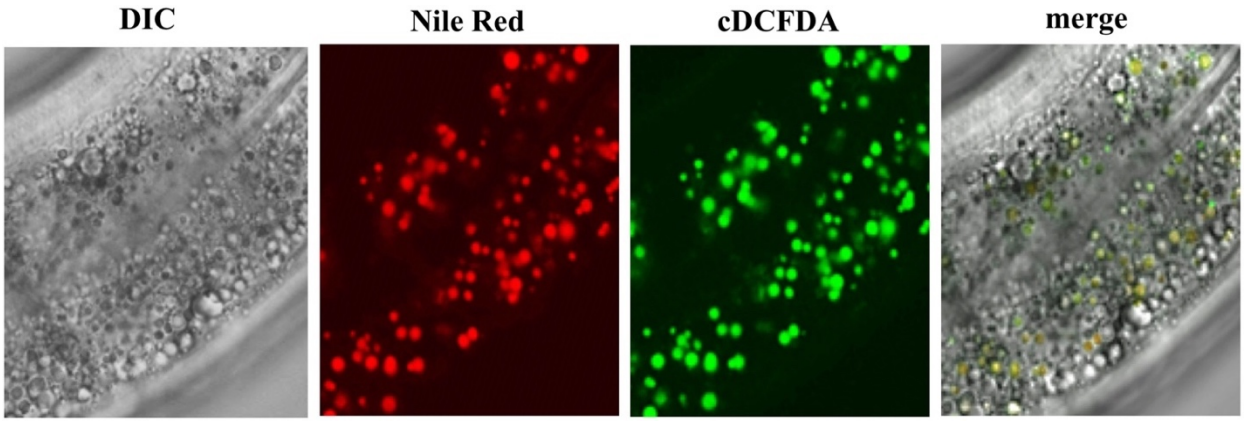


Figure 4.4: cDCFDA co-localizes with lysosomal marker Nile Red.
2-day post L4 *C. elegans* worms were fed OP50 food supplemented with Nile Red and cDCFDA overnight. Worms were imaged the next day using confocal microscopy.

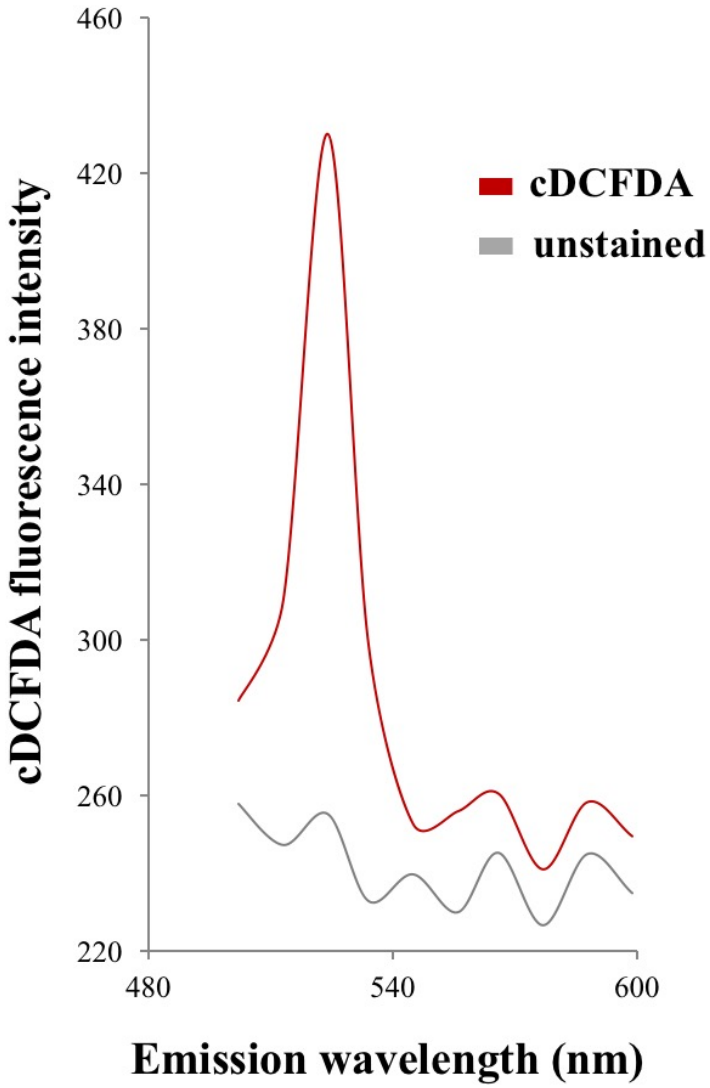


Figure 4.5: Emission spectra of cDCFDA.
2-day post L4 *C. elegans* worms were fed OP50 food supplemented with cDCFDA overnight. Worms were imaged the next day using confocal microscopy.

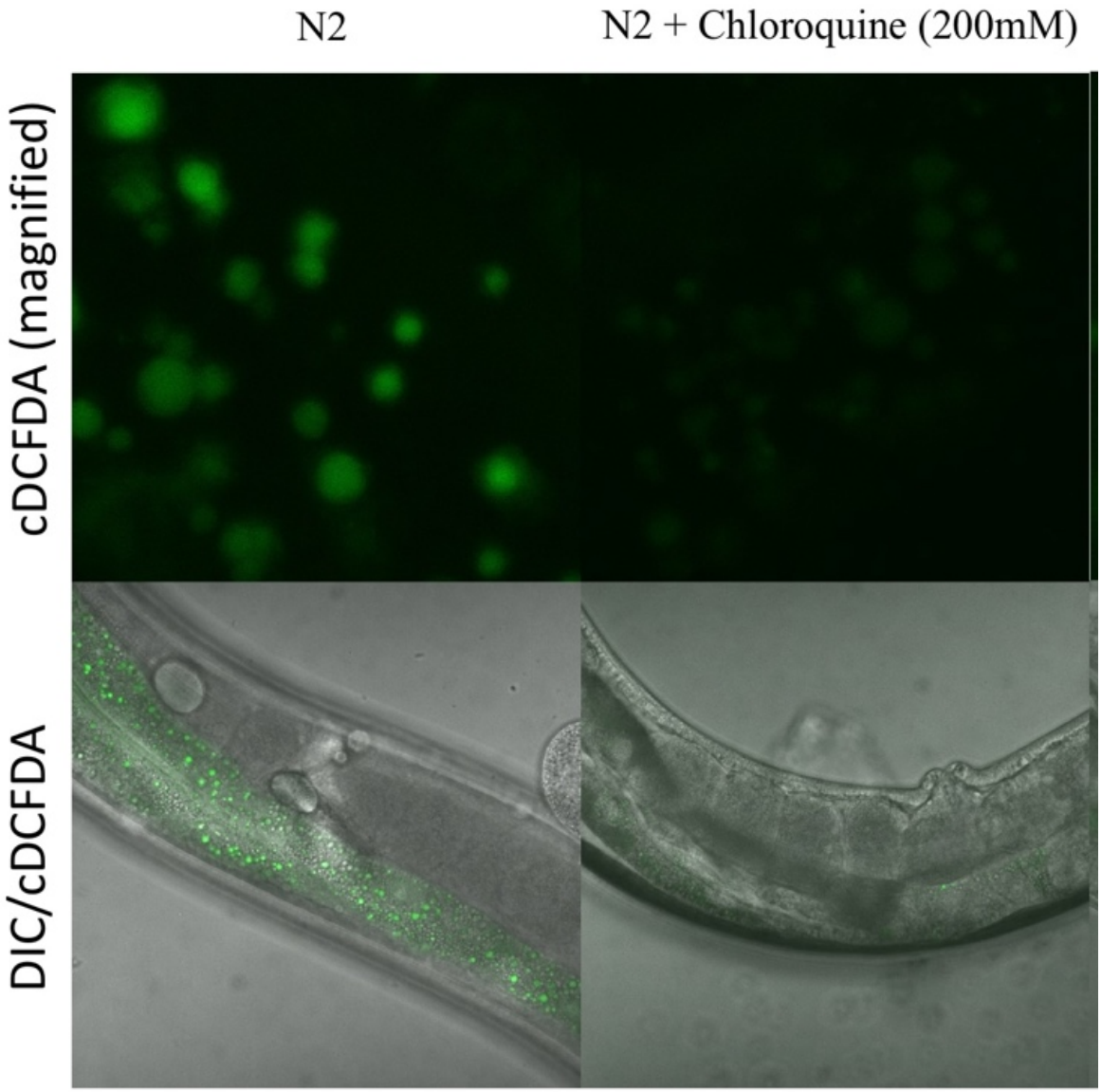
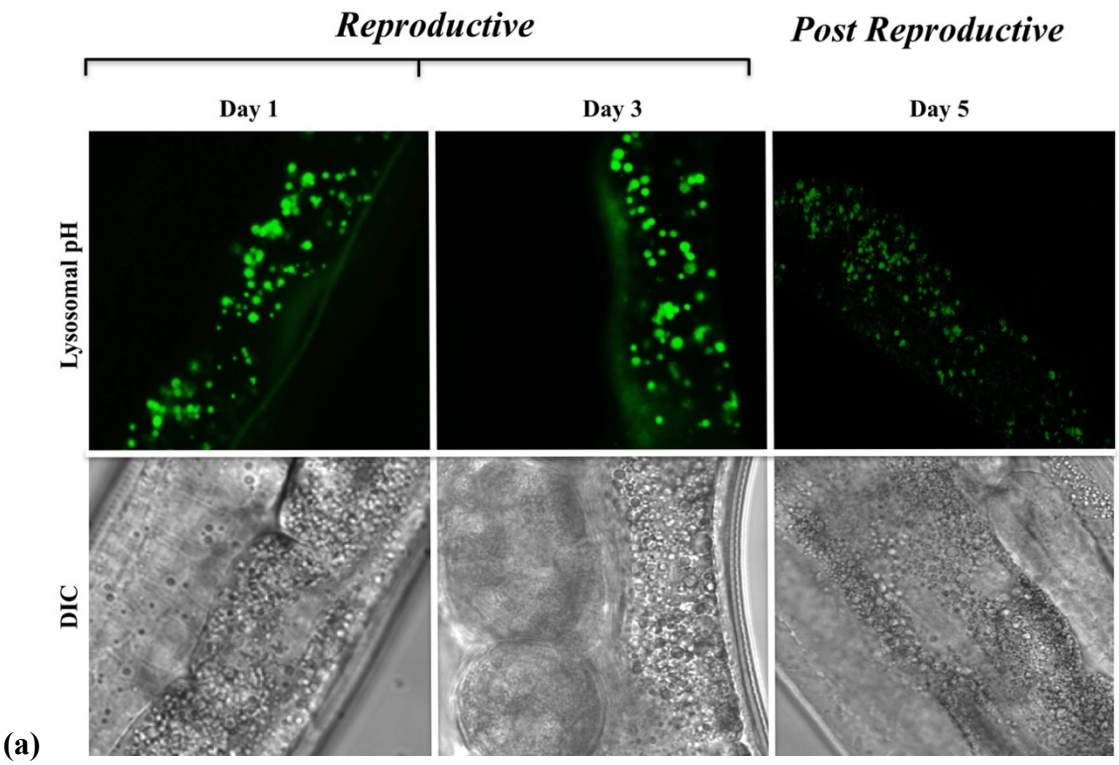


Figure 4.6: Chloroquine induces premature lysosomal alkalinization
Wild type worms were fed OP50 supplemented with chloroquine (200 mM) for 2 days post L4 and imaged via confocal microscopy.

4.3.2 Lysosomal pH homeostasis is linked to reproduction status

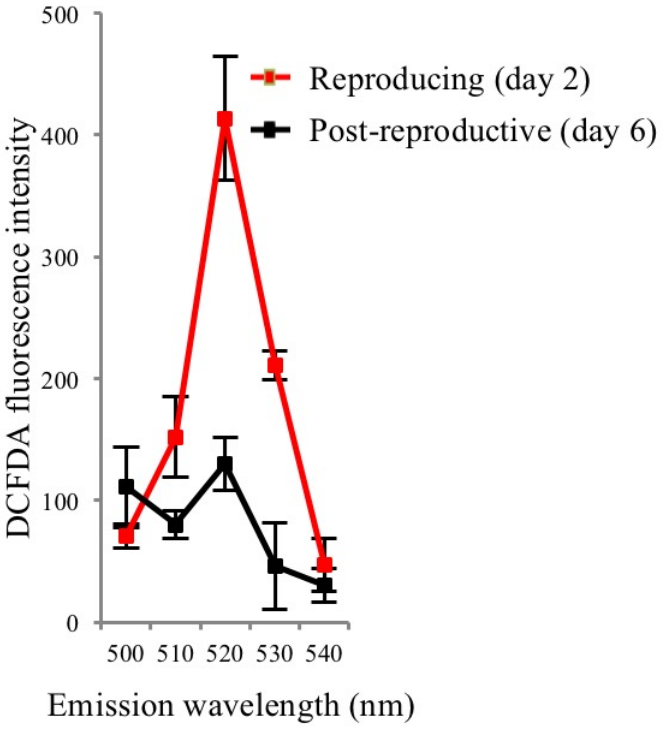
In wild type (unmated) *C. elegans* hermaphrodites, the bulk of progeny production occurs during days 1 to 3 of adulthood with reproduction ceasing at around days 4-5. In order to measure the dynamics of lysosomal pH during and after the reproductive period, I stained *C. elegans* lysosomes using cDCFDA at days 1, 3 and 5 of adulthood. During the initial 3 days, intestinal lysosomes appeared as discrete organelles of high cDCFDA fluorescence signal. In contrast, at day 5 (and beyond – data not shown), lysosomes were greatly enlarged, tightly packed, and displayed a marked reduction in cDCFDA fluorescence, indicating reduced lysosomal pH (Figure 4.7(a) and Figure 4.7(b)). This age-dependent transition of lysosomal pH was abrupt and often complete within 48 hours following the cessation of egg-laying. The apparent morphology of lysosomes in post-reproductive hermaphrodites was strikingly similar to that of young (day 2) worms after chloroquine treatment, suggesting that impaired acidification of lysosomes also implicates in other aspects of lysosomal function.

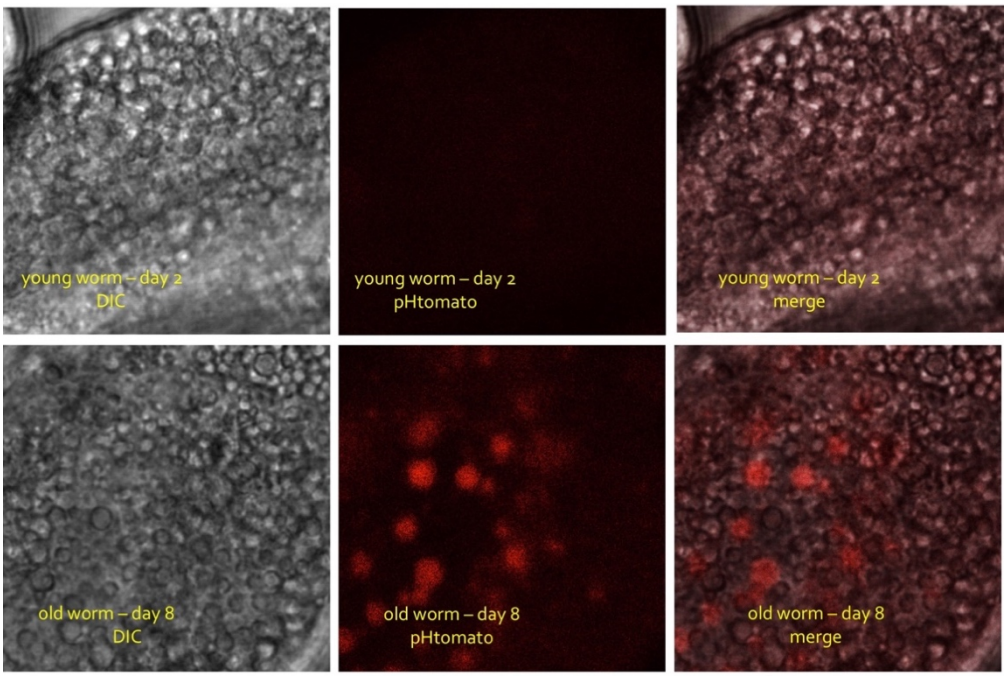
The loss of cDCFDA signal in post-reproductive worms indirectly points to the alkalinization of lysosomes. To obtain direct evidence of a rise in pH in these cells, I generated a line expressing pHTomato, a fluorescent pH sensor that fluoresces only when in neutral or alkaline environments (Y. Li et al., 2012). pHTomato was targeted to the lysosomal membrane facing the lumen by fusion to the amino terminus of the integral lysosome membrane glycoprotein LMP-1 (*Plmp-1::pHTomato::lmp-1*). *pHTomato* fluorescence sharply increases in pH 5 and above in reverse dynamics of that observed with cDCFDA fluorescence. As anticipated, while cDCFDA signal weakens at day 8 hermaphrodites, intestinal pHTomato fluorescence intensity was greatly elevated. Thus, lysosome alkalinization in the intestine is a physiological process taking place in post-reproductive worms (Figure 4.7 (c) and (d)).


Reproductive
Post Reproductive
Day 1
Day 3
Day 5
Lysosomal pH
DIC

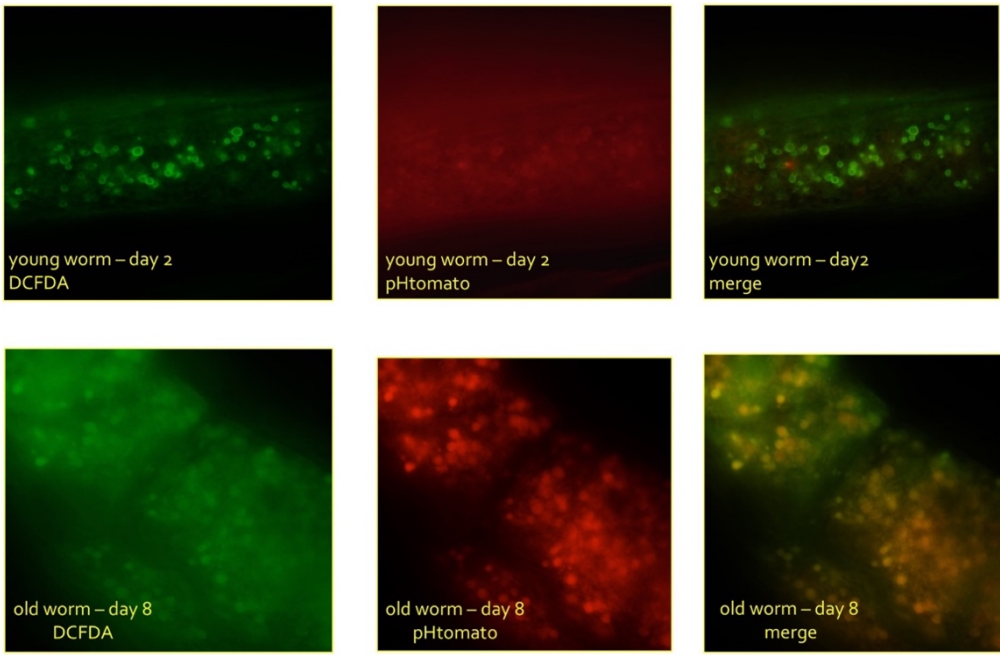
(a)

(b)


500
400
300
200
100
0
DCFDA fluorescence intensity
Reproducing (day 2)
Post-reproductive (day 6)
500 510 520 530 540
Emission wavelength (nm)



(c)



(d)

Figure 4.7: Validation of lysosomal pH increase following cessation of reproduction.
(a) Time course of cDCFDA stained lysosomes during (day 1 and day 3) and after cessation of reproduction (day 5). (b) Day 2 and day 6 worms were fed cDCFDA overnight (approximately 16 hours) and imaged the next day using a Zeiss LSM 510 confocal microscope. Relative cDCFDA fluorescence intensity (arbitrary units) from at least 5 worms was measured and plotted ($p < 0.0001$). (c) Confocal microscopy images of young (day 2) and old (day 8) worms expressing *Plmp-1::pHTomato::LMP-1*. (d) Fluorescence microscopy images of young (day 2) and old (day 8) worms expressing Plmp-1::pHTomato::LMP-1 co-stained with cDCFDA and imaged after ~16 hours.

4.3.3 *vha* genes are essential for lysosomal pH homeostasis and lifespan.

In order to provide a secondary source of validation for cDCFDA as a lysosomal pH indicator as well as to study the effects of v-ATPase knockdown on lysosomal pH, I fed worms with *vha-2* and *vha-8* RNAi food and then assessed lysosomal pH using cDCFDA. Knockdown of either *vha-8* (coding to a cytoplasmic ATPase subunit) (Figure 4.8) or *vha-2* (coding to a membrane-spanning component) (data not shown) led to a dramatic reduction in cDCFDA fluorescence. The tightly packed, enlarged lysosomes with reduced cDCFDA fluorescence seen after *vha* RNAi resemble lysosomes seen in post-reproductive worms after cDCFDA staining. Consistent with a role of v-ATPases in stabilizing autophagic flux in the intestine, subtle RNAi knockdown of *vha-8* (Figure 4.9) as well as *vha-2* (data not shown) dramatically reduced lifespan of wild type worms, revealing the importance of lysosome acidification in regulating organismal lifespan (Figure 4.9). In all, the results above validate the use of cDCFDA as an *in vivo* probe to investigate pH changes in intestinal lysosomes and provide evidence that mild disturbance of the regulatory mechanism behind this process can have severe systemic implications that ultimately impact lifespan.

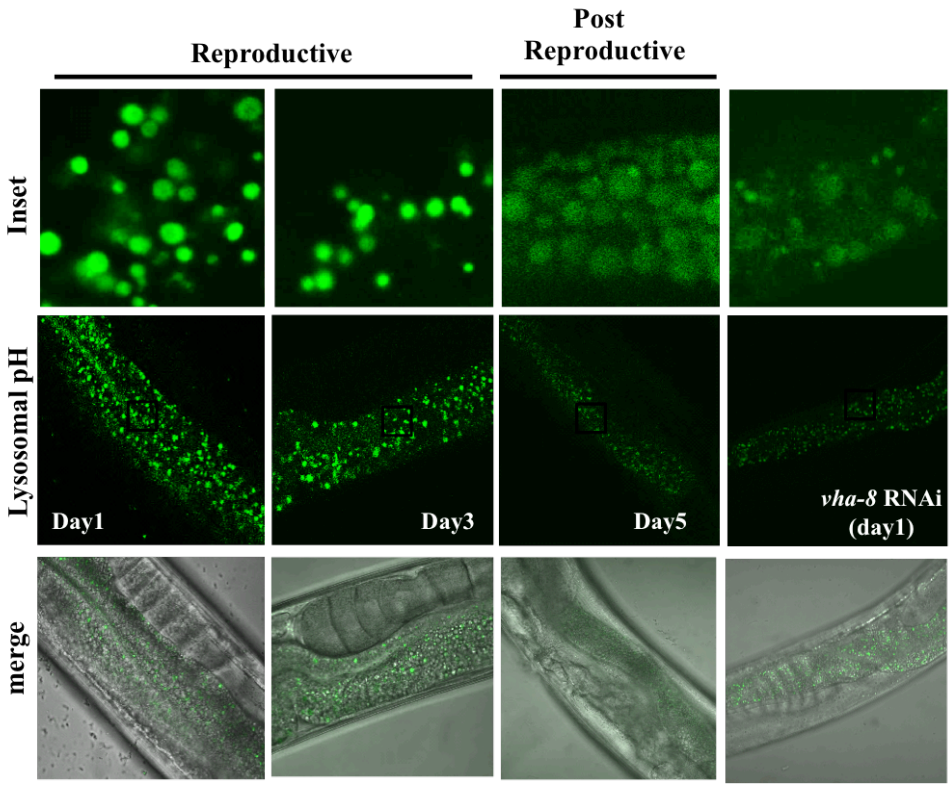


Figure 4.8: RNAi against *vha* subunits leads to premature lysosomal alkalinisation.
cDCFDA stained lysosomes during and after cessation of reproduction along with a young day 1 worm stained with cDCFDA following *vha-8* RNAi.

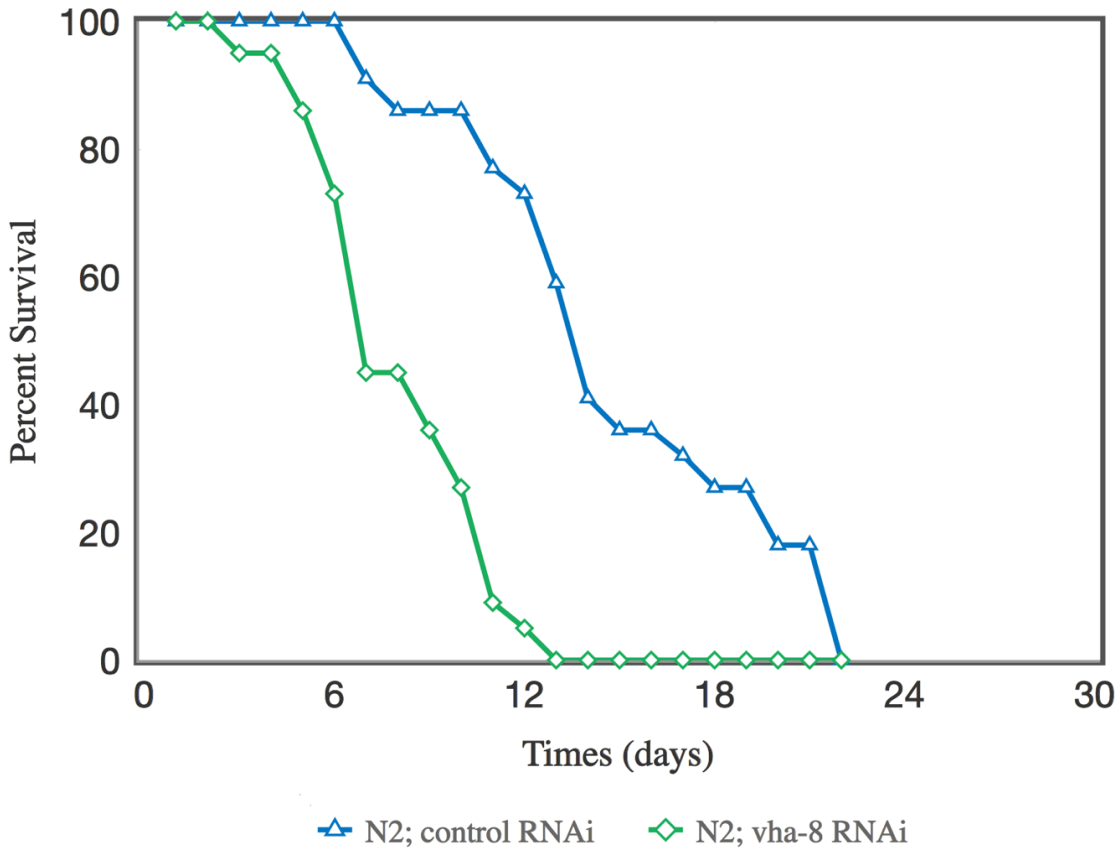


Figure 4.9: *vha-8* RNAi leads to loss of viability.
Worms fed with *vha-8* RNAi show greatly reduced lifespan compared to worms fed control RNAi (HT1115) ($p<0.0001$; Mantel Cox test).

4.3.4 *vha* genes are downregulated following cessation of reproduction

What is the mechanism behind lysosomal alkalinization that is triggered upon cessation of reproduction? Lysosomes in post-reproductive worms or after *vha-2* or *vha-8* knockdown in reproducing worms displayed similar morphology both in lysosome enlargement and loss of cDCFDA fluorescence levels. This suggested a dosage reduction of *vha* gene transcription could underlie the process in old worms. Indeed, analysis of *vha-2* and *vha-8* gene expression via qRT-PCR revealed a coordinately reduction during the latter days of reproduction (Figure 4.10). The drop in transcript abundance was abrupt and largely complete by day 4, coincident with the final days of progeny production and, importantly, preceding the loss of lysosome acidity as detected by cDCFDA staining (Figure 4.7a). Thus, a controlled downregulation of *vha* genes expression upon cessation of reproduction provides a plausible explanation for the lysosomal alkalinization observed in post-reproductive worms and suggests the existence of a dynamic transcriptionally-based regulatory system that interprets the reproductive status in the germline to optimize lysosomal function in the soma.

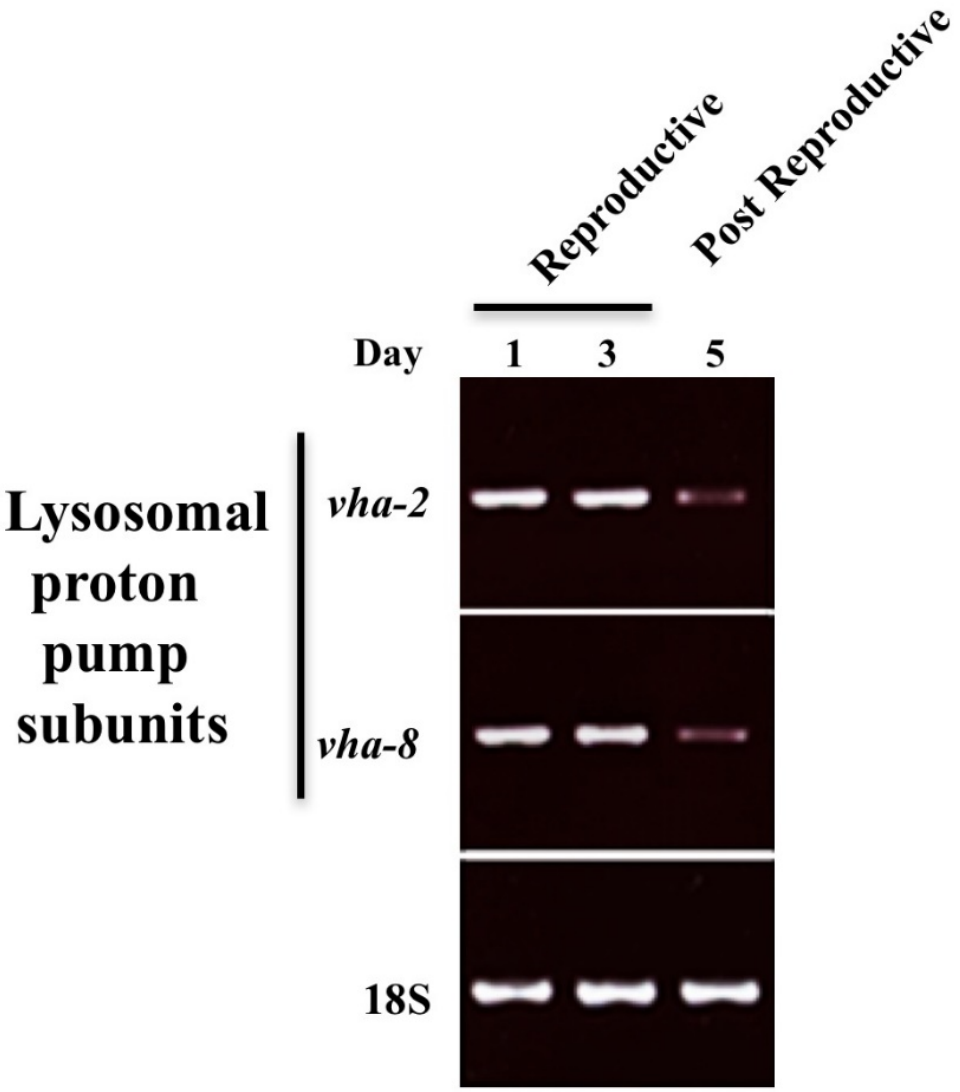


Figure 4.10: Transcriptional decline of *vha* gene transcription following cessation of reproduction.
Agarose gel electrophoresis for semi-quantitative PCR of *vha* genes during and after cessation of reproduction (18S rRNA as control).

4.3.5 *fem-2* worms maintain acidic lysosomal pH longer than wild type

The results above support a working model in which reproduction somehow regulates somatic senescence in the context of a normally aging animal. To investigate the link between reproduction in the gonad and lysosomal pH changes in the intestine, I utilized a temperature sensitive sterile mutant (*fem-2(b245))*. FEM-2 is a protein phosphatase that is required to promoter the male fate in the germ and soma during a short window in larval development. *fem-2* X0 and XX worms failed to make sperm and develop otherwise as viable females that undergo normal oogenesis (Pilgrim et al., 1995). Females are only fertile if mated, since they cannot self-fertilize. To determine the role of reproduction in lysosomal pH decline, I grew synchronized *fem-2(b245)* worms at 25^{o} C until L4 and then moved them to 20^{o} C. Based on my results (Figure 4.11), feminized *fem-2(b245)* worms (henceforth referred to only as *fem-2* worms) delayed lysosomal alkalinization in the intestine for an extended period of time in adult life. While wild type worms showed progressive lysosomal alkalinization at day 5 and beyond, cDCFDA signal intensity in *fem-2* intestine were similar past day 9 of adulthood. These results support the interpretation that preventing reproduction delays the deterioration of lysosomal function in aging *C. elegans*.

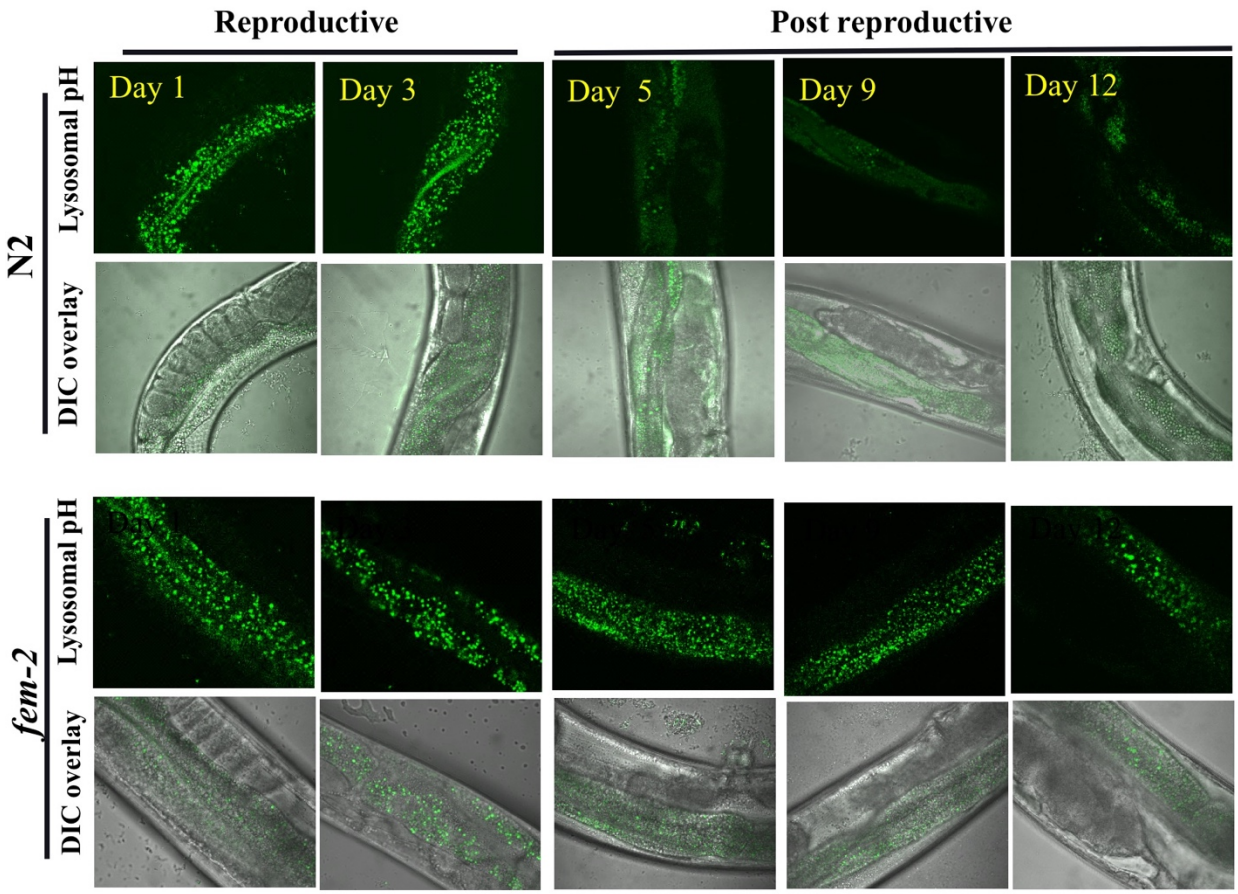


Figure 4.11: Sterile *fem-2* worms maintain acidic lysosomes for longer than wild type. Confocal microscopy images of wild type and *fem-2* worms fed cDCFDA at various time points.

4.3.6 *fem-2* worms delay downregulation of *vha* gene expression

In an effort to elucidate the molecular mechanism behind extended acidic pH in lysosomes of *fem-2* worms, the expression of *vha* genes in these animals was measured using semi-quantitative PCR. During day 5 and beyond, when wild type worms are normally ceasing to generate progeny and show a concomitant reduction in *vha* gene expression, *fem-2* worms displayed levels of *vha* transcripts similar to younger stages (Figure 4.12). Sustaining high levels of *vha* gene expression is therefore one plausible explanation for why *fem-2* worms extend lysosomal acidity well past day 5, whereas wild type worms do not.

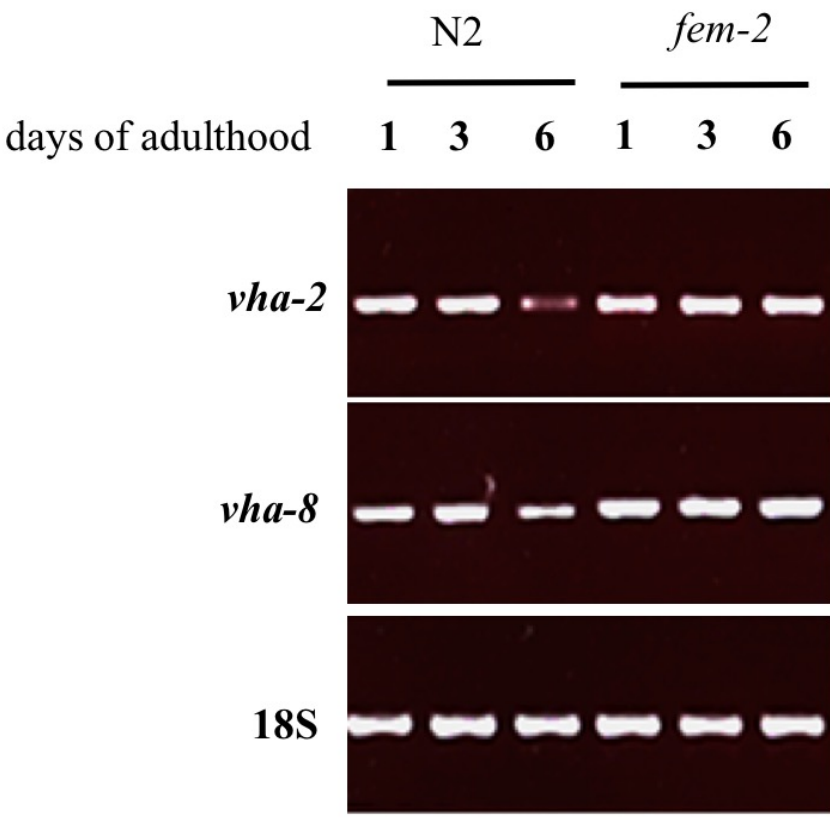


Figure 4.12: *fem-2* worms show sustained *vha* gene expression unlike N2 worms
Semi-quantitative PCR of *vha* gene expression in *fem-2* vs wild type worms. Day 1 and 3 correspond to the time frame during which wild type worms are actively reproducing, whereas day 6 represents a post-reproduction time point.

4.3.7 VHA-17 levels in older *fem-2* worms do not drop as compared to wild type

The molecular evidence collected above reveals that the decline in *vha* gene expression underlies lysosomal pH increase in post-reproductive worms. It is possible, however, that this change in gene expression does not necessarily impact VHA protein levels or if it does, the stoichiometry of v-ATPase assembly on the lysosome membrane may still remain unaffected despite these changes. Alternatively, the drop in mRNA levels for *vha* genes may directly determine a loss of v-ATPase complexes on lysosomes. To account for these possibilities, I looked at relative changes in protein levels of VHA-17, a V_0 subunit of the protein pump by IF analysis. Dissected intestines of young (day 2) and old (day 8) worms were co-immunostained using a polyclonal anti-VHA-17 (FUS-1) antibody as well as an anti-LMP-1 antibody (that marks lysosomal membranes). In agreement with previous data, VHA-17 protein levels on lysosomal membranes are seemingly reduced in post-reproductive worms but not *fem-2* worms at corresponding age. No obvious difference in the number of lysosomes is observed between the samples. These results confirm that the lysosome alkalinization program is triggered in post-reproductive worms at the transcriptional level by downregulation of *vha* genes expression that ultimately leads to a reduction in v-ATPase pumps on the lysosomal membrane and the consequent rise in intra-lumen pH. Sterile animals bypass this sensitive time point in adulthood and retain *vha* expression levels consistent with those observed in reproductive stages of wild type animals. (Figure 4.13 & Figure 4.14).

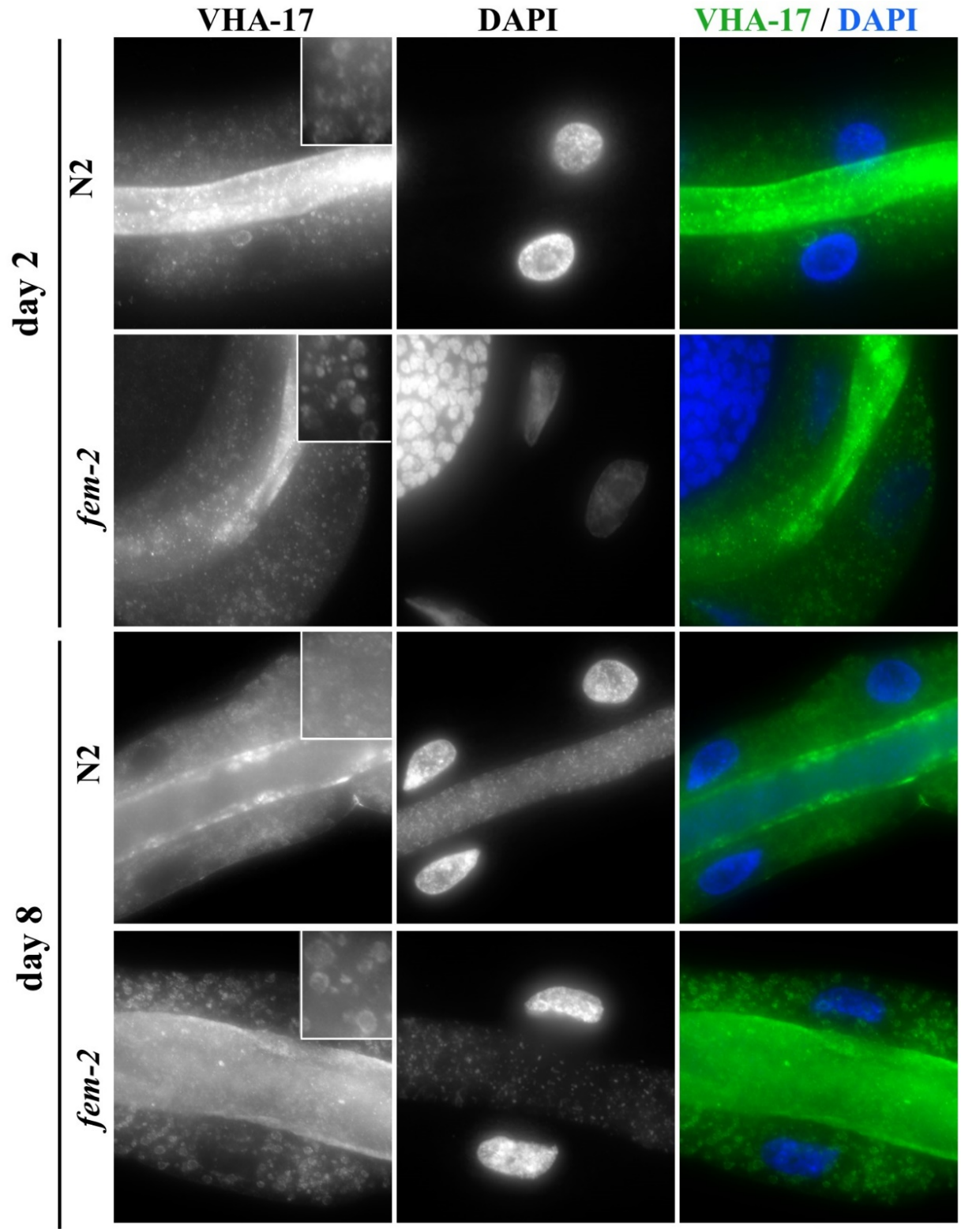


Figure 4.13: 8-day old *fem-2* mutants retain more VHA-17 on lysosomal membrane than wild type.
Young (day 2) and old (day 8) N2 and *fem-2* worms were dissected and immunostained with anti-VHA-17 antibody and subsequently analyzed by fluorescence microscopy.

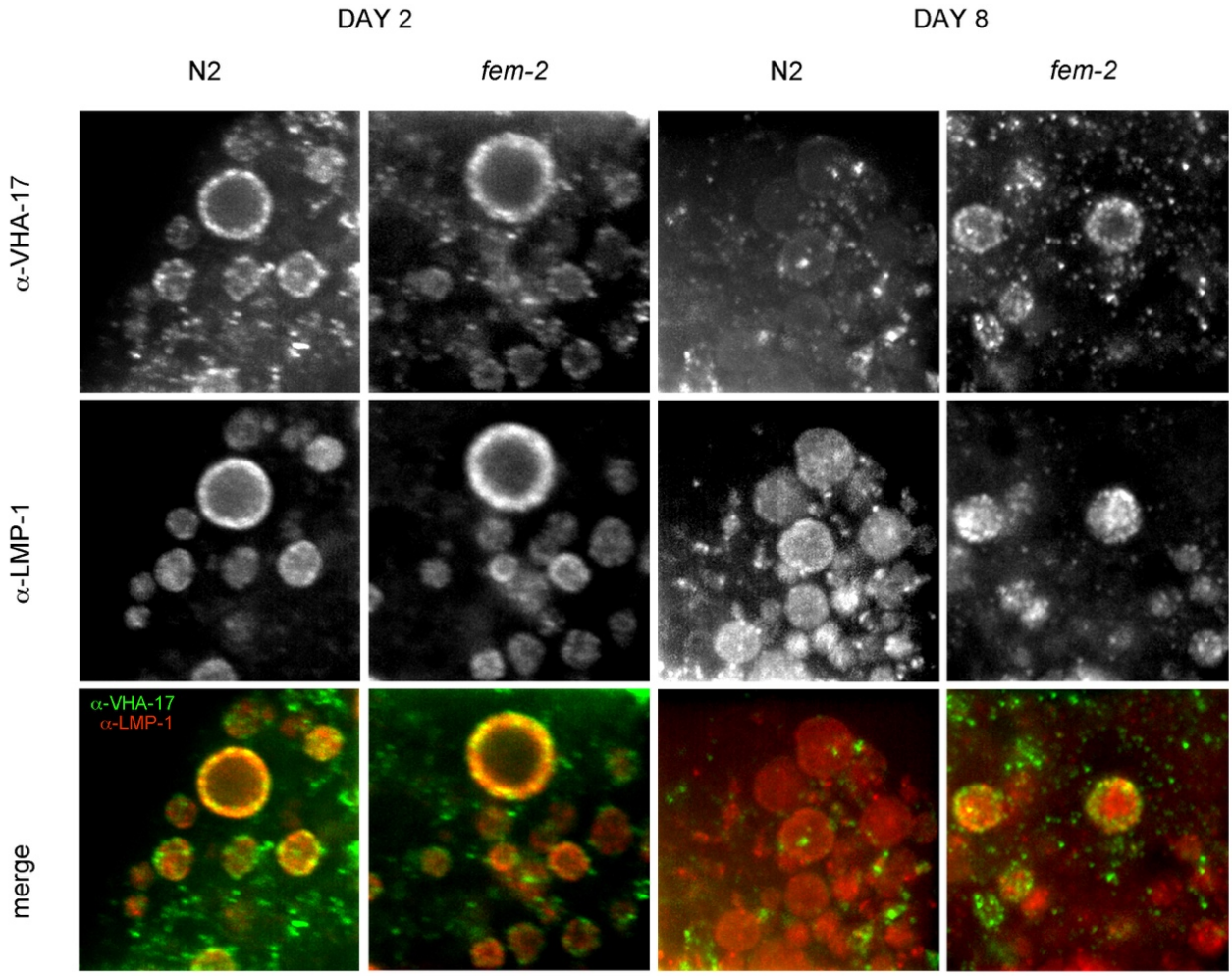


Figure 4.14: 8-day old *fem-2* mutants retain more VHA-17 on lysosomal membrane than wild type.
Young (day 2) and old (day 8) N2 and *fem-2* worms were dissected and co-immunostained with anti-VHA-17 and anti-LMP-1 antibody and subsequently analyzed by fluorescence microscopy.

4.3.8 Sterile worms display increased lysosomal pH homeostasis and extended lifespan

One interpretation of the *fem-2* results (above) is that in reproducing animals, the cessation of reproduction triggers lysosomal alkalinization in wild type worms. If so, disrupting progeny production at different levels may impair the signalling originating from the gonad and prolonged lysosomal acidity in the intestine. To test this hypothesis, I examined the dynamics of pH change in intestinal lysosomes in a panel of sterile hermaphrodite with defects in specific stages of reproduction; inactivating *glp-1(e2141)* abolishes germ cell proliferation, denoted by the absence of oocytes and an empty spermatheca in adult *glp-1* hermaphrodites (Priess et al., 1987). In contrast *spe-26(hc140ts)* mutants, similar to *fem-2* animals, produce a full complement of fertilizable oocytes, but defective sperm that fails to fertilize (Varkey et al., 1995). Unlike wild type hermaphrodites, and irrespective of the presence of germ cells in the gonads, age-matched sterile worms maintained a robust lysosomal pH past day 6 of adulthood (Figure 4.15). Even though the metabolic requirement for oogenesis is among the most resource intensive developmental undertaking in adult hermaphrodites, sterile *fem-2* and *spe-26* worms, in spite of producing a full complement of oocytes, still displayed a lysosomal pH profile indistinguishable from *glp-1* worms that lack germ cells entirely. Thus, the maintenance of lysosomal acidity in sterile worms cannot be fully explained as a consequence of resource re-allocation from a gamete producing germline to the soma and instead likely reflects a discrete failure to trigger a senescence program that senses and actively responds to the progressive loss of reproductive capacity in normally reproducing animals. This time-sensitive process can be understood as a *reproductive timer* started at the time of reproductive maturity and winding down around days 5 and 6 of adulthood. Importantly, and similar to germline-less mutant such as *glp-1*, sterile mutants with normal germ cell differentiation but impaired in progeny production displayed extended lifespan (Figure 4.16 a). Moreover, *fem-2* worms also showed increased motility compared to age-matched N2 worms, indicating enhanced fitness (Figure 4.16 b). Taken together with the *fem-2* data, these results seem to suggest that enhanced proteolysis in post-reproductive time points is achieved when animals fail to fertilize, irrespective of germ cell production.

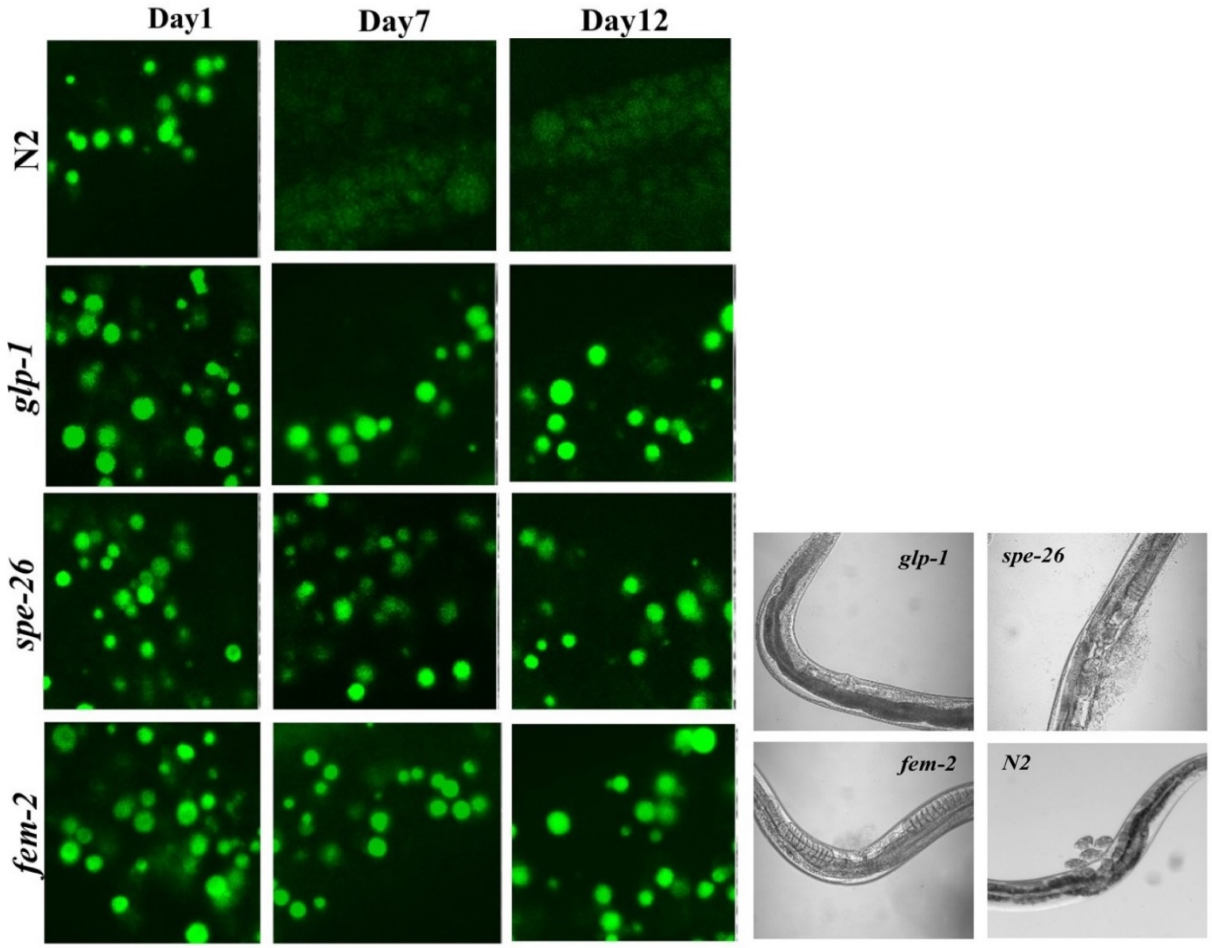
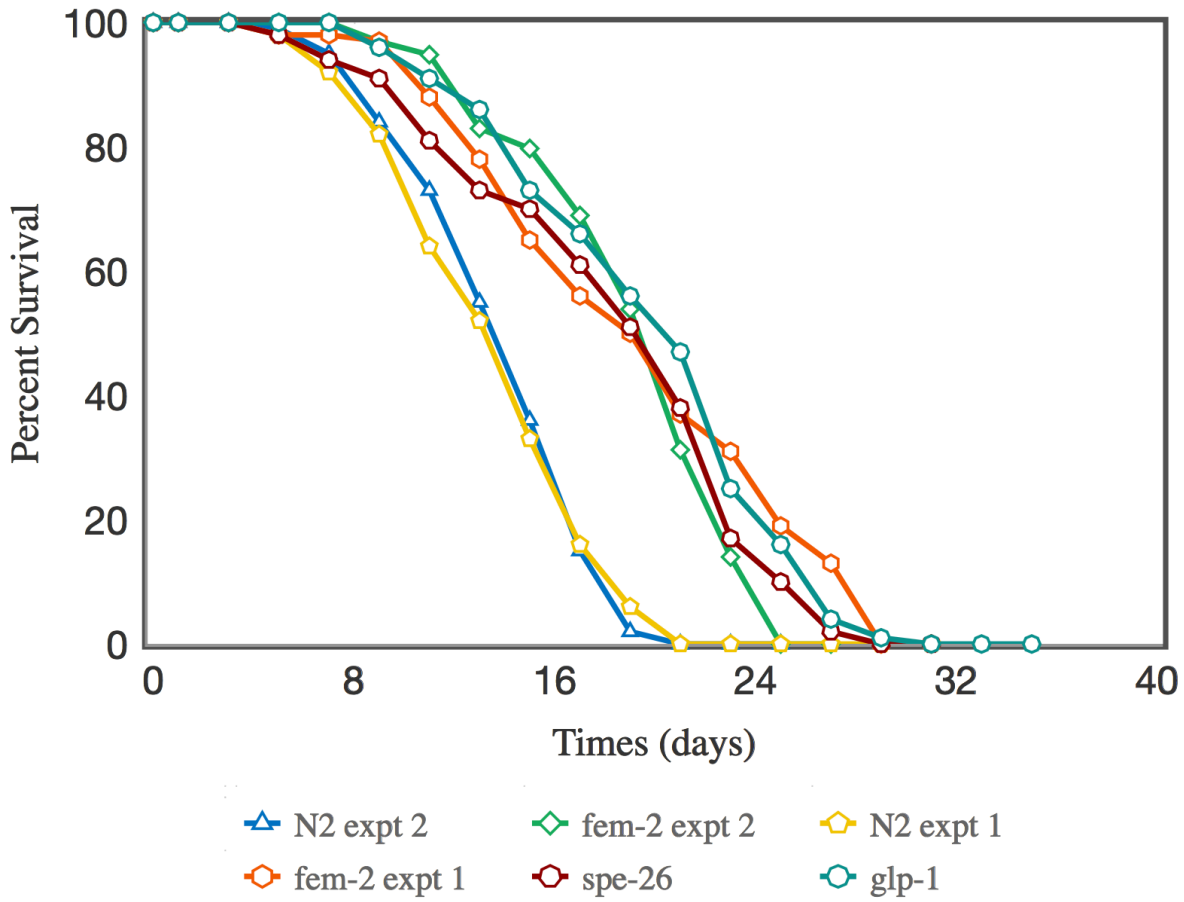


Figure 4.15: Sterile *C. elegans* worms maintain acidic lysosomal pH in the intestine for longer than wild type.
Sterile *C. elegans* mutants (*fem-2, glp-1* and *spe-26*) were analyzed for loss of lysosomal pH compared to wild type by staining with cDCFDA followed by confocal microscopy after ~16 hours.


100
80
60
40
20
0
Percent Survival
0
8
16
24
32
40
Times (days)
N2 expt 2
fem-2 expt 2
N2 expt 1
fem-2 expt 1
spe-26
glp-1

(a)

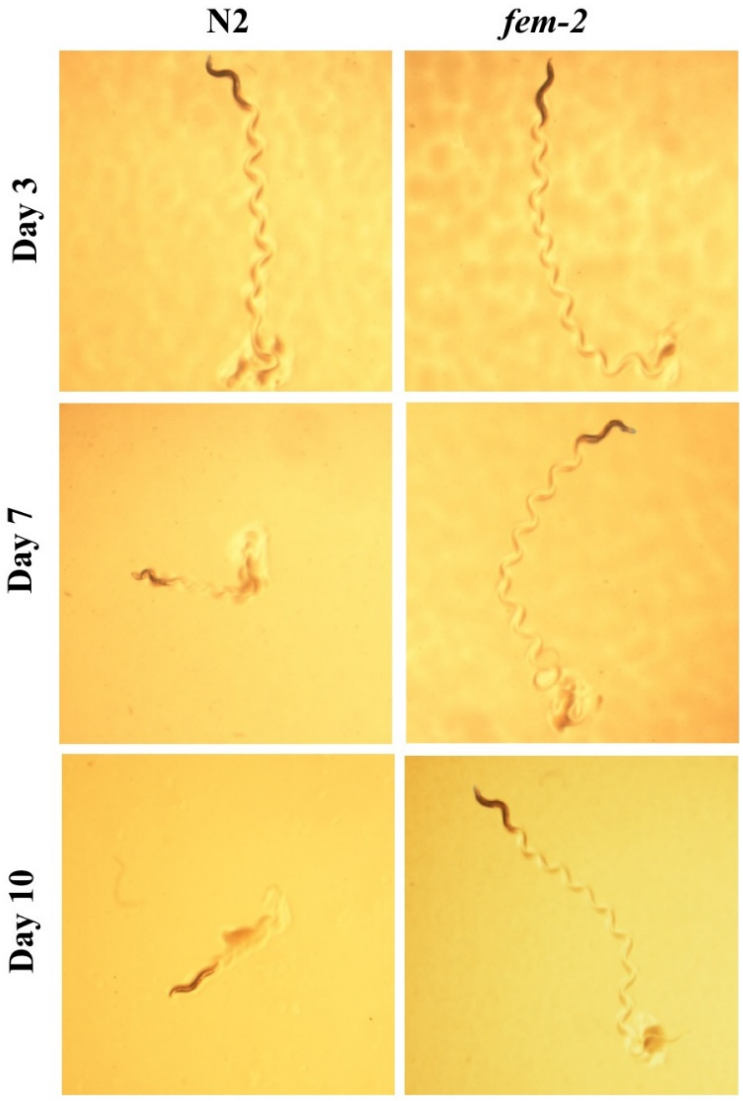


Figure 4.16: Sterile mutants show increased lifespan and fitness compared to wild type.
(a) Lifespan plot for sterile mutants (*fem-2, glp-1* and *spe-26)* compared with wild type. Worms were grown at 25^{o}C until L4 and then shifted to 20^{o}C post L4. (p values: (a) N2 expt 1 N2 expt 2 p=0.9516 (b) *fem-2* expt 1 vs *fem-2* expt 2 p=0.0449 (c) N2 expt 1 vs *fem-2* p<0.0001 (d) N2 expt 1 vs *spe-26* p <0.0001 (e) N2 expt 1 vs *glp-1* p<0.0001 (f) *fem-2* expt 1 vs *spe-26* p=0.08 (g) *fem-2* expt 1 vs *glp-1* p=0.8052). All p values calculated using Mantel Cox test.
(b) The overall fitness of N2 and *fem-2* worms was assessed by motility analysis at day 3, day 7 and day 10 of lifespan.

4.3.9 Re-establishing reproduction in sterile worms leads to lysosomal pH collapse

Though reversing defects in germ cell proliferation post-development is not feasible, one can easily reconstitute reproduction at normal levels in feminized mutants by simply mating adult females with wild type males. This replenishes the sperm stock in XX animals and immediately triggers ovulation and fertilization at wild type rates. If, therefore, the lack of fertilization alone in sterile mutants prevents the onset of the somatic senescence program that converges on lysosomal pH regulation, it should be possible to reconstitute it by initiating reproduction upon mating. I therefore asked whether supplying sterile *fem-2* worms with exogenous sperm would impact the dynamics of lysosomal alkalinization in these mutants. To that effect, I mated *fem-2* adult worms with N2 males to restore progeny production. Mating reduces the average lifespan of feminized worms independent of receipt or storage of sperm, brood size, or structural damage during copulation (Gems et al., 1996). Indeed, restoration of progeny production in *fem-2* worms was sufficient to accelerate loss of lysosome acidity with a profile similar to age-matched wild type worms (Figure 4.17). Moreover, mating *fem-2* worms also abrogated the prolonged expression of *vha* genes otherwise seen in sterile *fem-2* worms (Figure 4.18(a) and (b)). These observations collectively highlight that intestinal lysosomal pH in wild type worms is coupled to the reproductive state in the gonad via a process that relies on successful sperm entry in the oocyte.

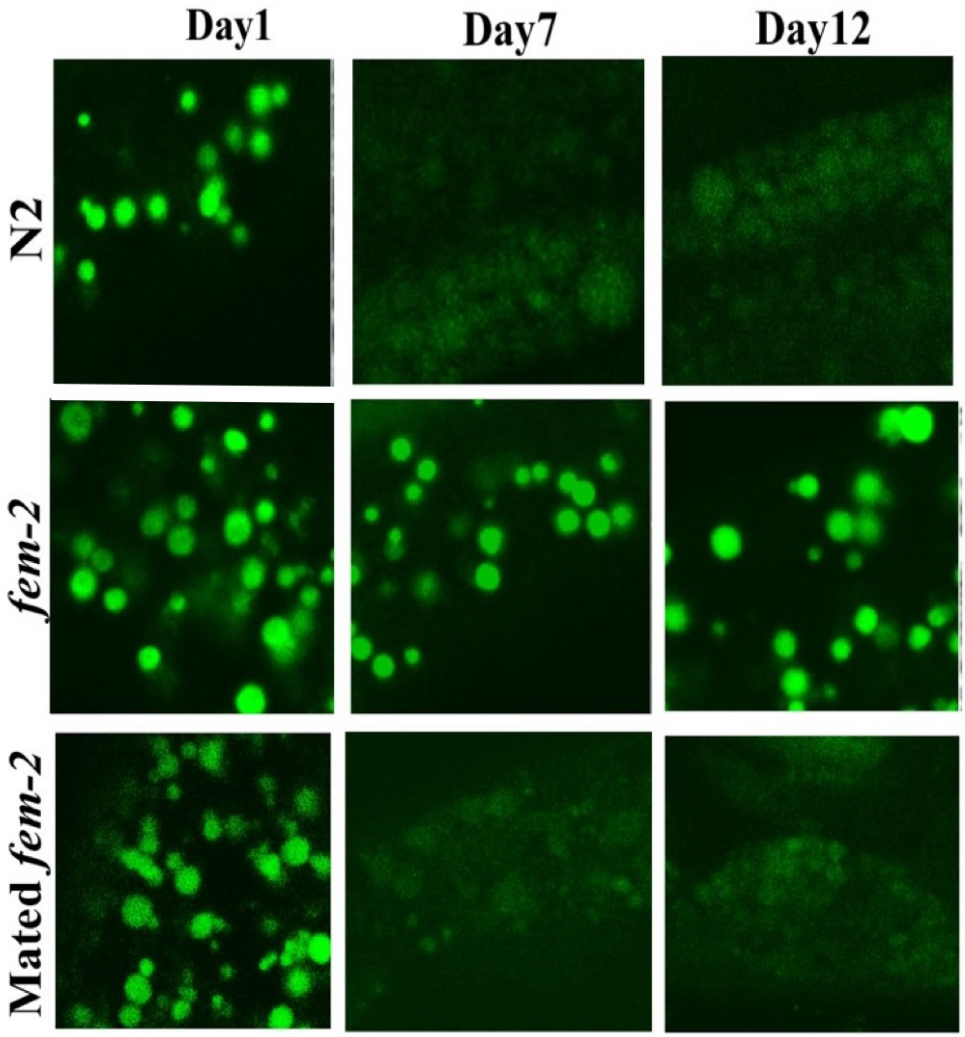
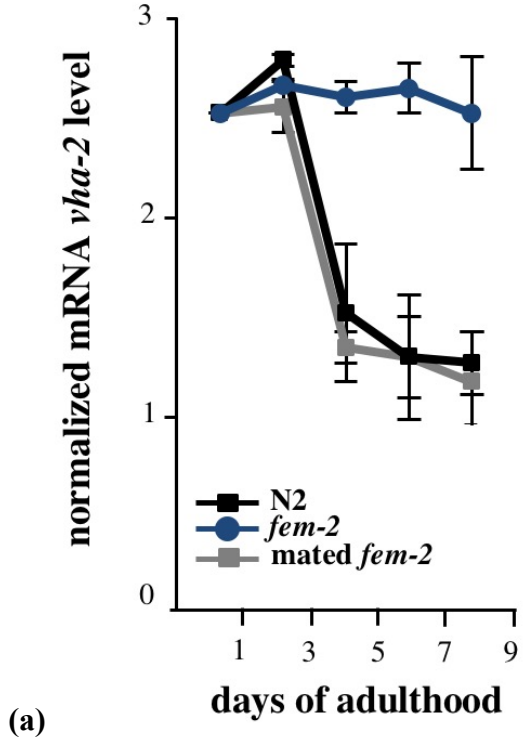
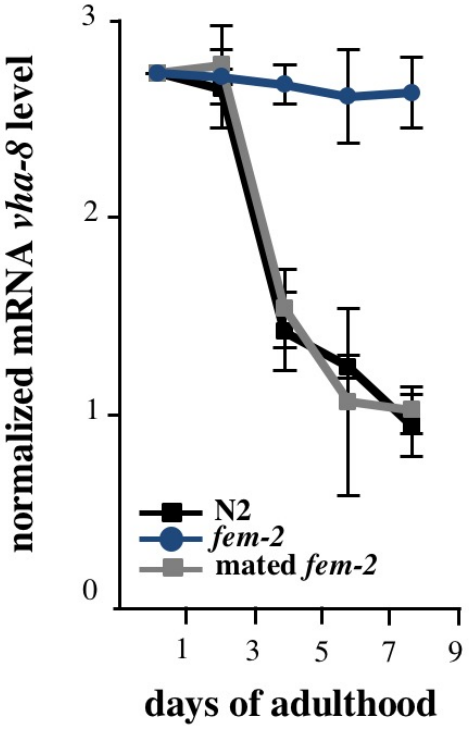


Figure 4.17: Mating reverses *fem-2* prolonged lysosomal acidification.
Mated and non-mated (sterile) *fem-2 C. elegans* worms were analyzed for loss of lysosomal pH compared to wild type by staining with cDCFDA followed by confocal microscopy after ~16 hours as per time points indicated above.



(a)



(b)

Figure 4.18: *vha* expression is downregulated post reproduction in wild-type and mated *fem-2* but not feminized *fem-2* worms.
RT-PCR analysis to monitor (a) *vha-2* and (b) *vha-8* gene expression in *fem-2,* mated *fem-2* and wild-type worms. Days 1 and 3 represent the reproductive phase in wild type worms, whereas days 5 and beyond represent the post-reproductive phase.

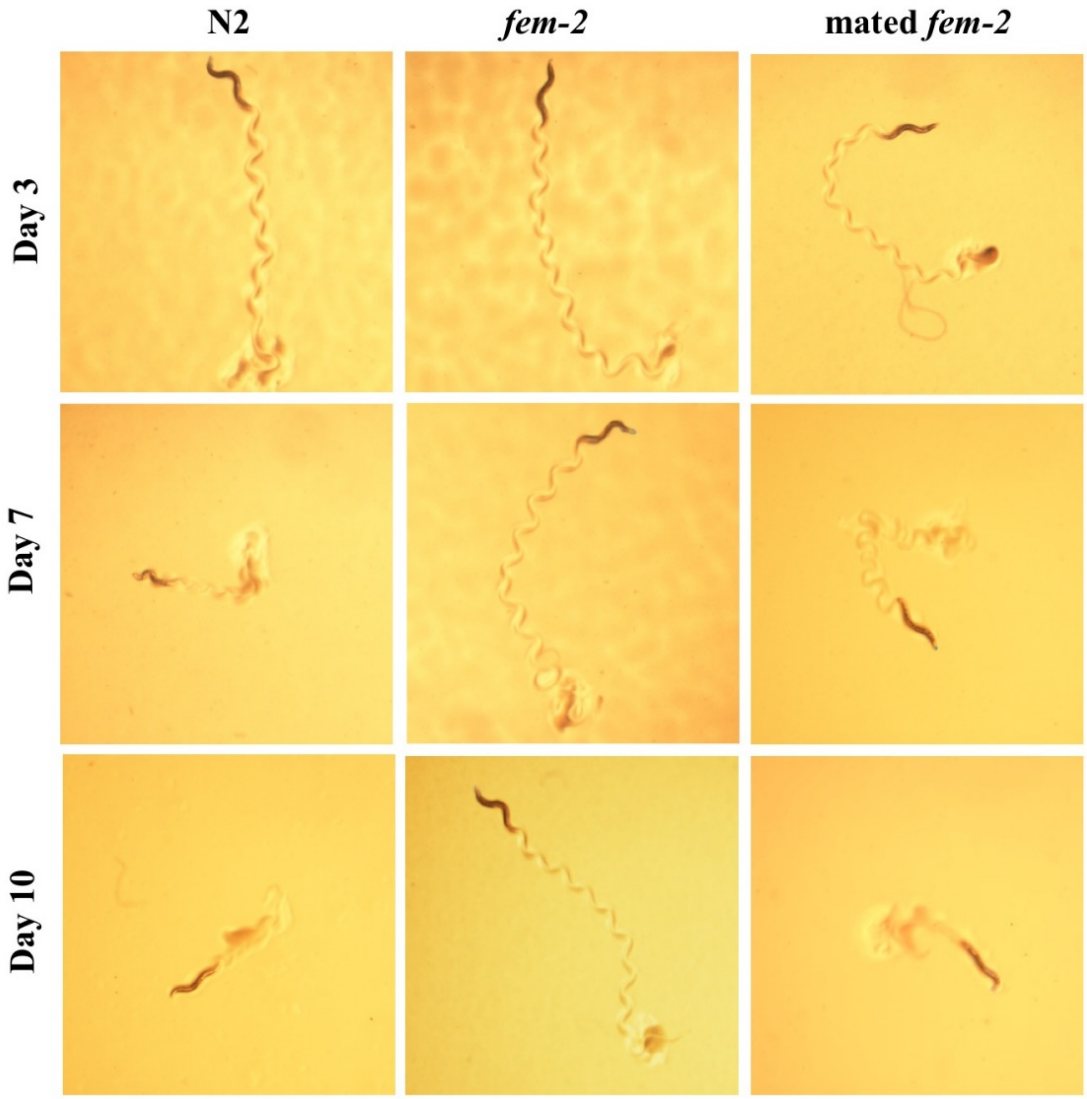
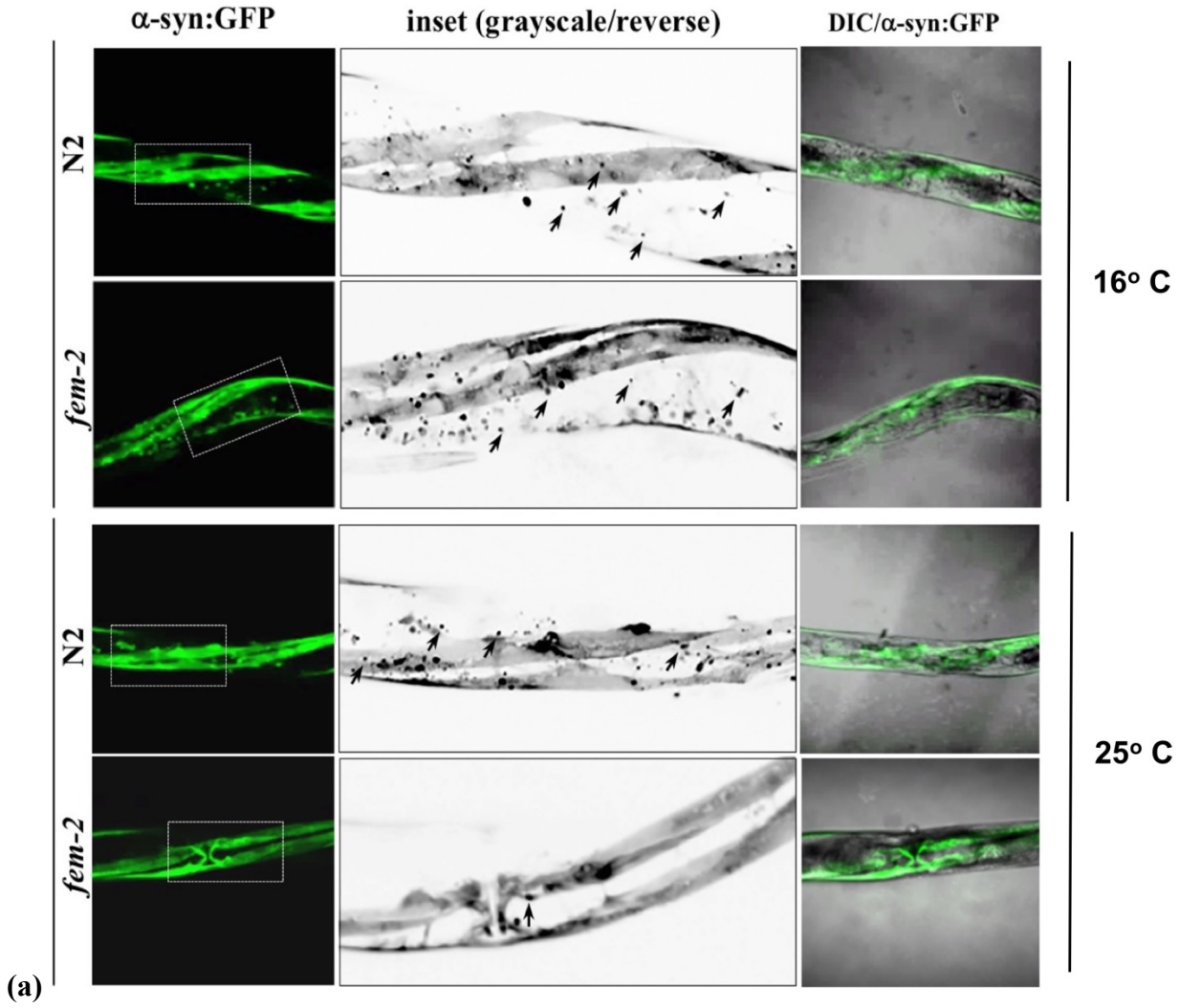


Figure 4.19: Sterile *fem-2* mutants remain more mobile than wild type and mated *fem-2*
Image showing relative motility of mated *fem-2* and fertile *fem-2* as well as wild type worms. Worms were placed on a plate containing OP50 food and allowed to move for 1 minute, and the distance travelled by each worm was measured at the end of 1 minute. (At least 5 worms per genotype were analyzed.)

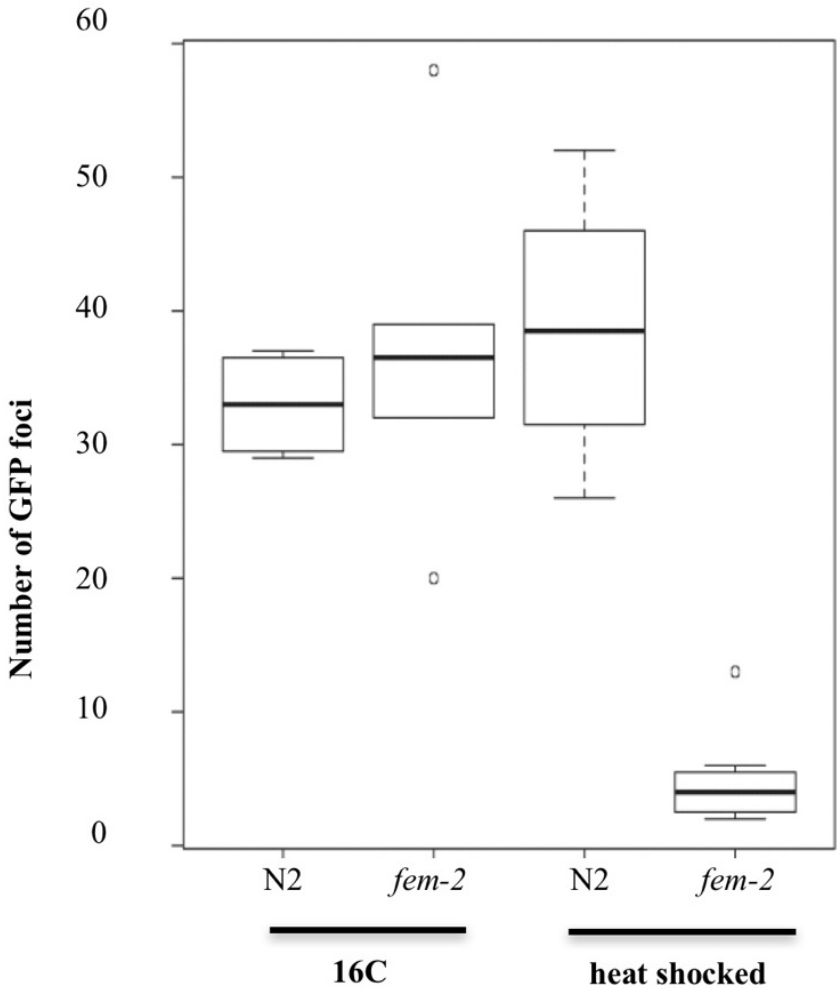
4.3.10 Sterile *fem-2* worms show enhanced proteostasis

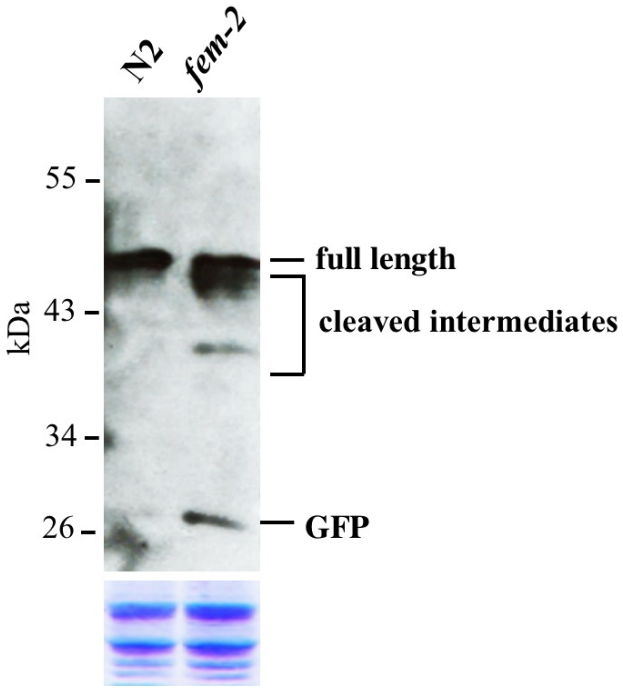
To probe whether the extended lysosomal acidity in intestine translates to a global increase in enhanced proteolysis across the tissues of *C. elegans*, I examined the formation of α-synuclein aggregates in muscle cells of older worms. Ectopic expression of a GFP fusion of human α-synuclein in body wall muscle cells (P_{unc-54}::α-syn::GFP (Harrington et al., 2011)) gives rise to prominent foci in older worms that can be detected and quantified by confocal microscopy. These aggregates (foci) provide a direct readout of the proteolytic capability of the organism. In post-reproductive (day 8) wild type worms (Figure 4.20(a)), muscle cells accumulated a high number of α-syn::GFP aggregates in the cytoplasm. In contrast, age-matched feminized *fem-2* worms (grown at 25ºC then shifted to 20^{o}C), but not self-fertile *fem-2* worms (grown at 16ºC throughout) displayed a significantly reduced number of *in situ* GFP foci (quantified in Figure 4.20(b)). In line with their robustly acidic lysosomes, the *in-situ* clearance of protein aggregates in *fem-2* worms was indeed accompanied with their enhanced intralysosomal proteolysis (Figure 4.20(c)). This enhanced clearance of protein aggregates, besides being conserved amongst all sterile worms (data not shown), was also independent of changes in the autophagic flux, as observed by similar expression levels of *lgg-1* and loading of LGG-1::mCherry foci in the intestine of age-matched N2 and *fem-2* worms (Figure 4.21(a) and Figure 4.21(b)). The results above, however must be reproducible in both muscle and intestinal cells before it can be interpreted as representing a systemic regulation of autophagy. Formation of protein aggregates within cells is one of the principle drivers of reproductive senescence and functional decline of somatic tissue (Ghavidel et al., 2015), hence I asked if a subtle increase in lysosomal pH would influence lifespan in *fem-2* mutants. In agreement with my hypothesis, a mild RNAi knockdown of *vha-2* abrogated the extended lifespan observed in feminized *fem-2* mutants (Figure 4.22).

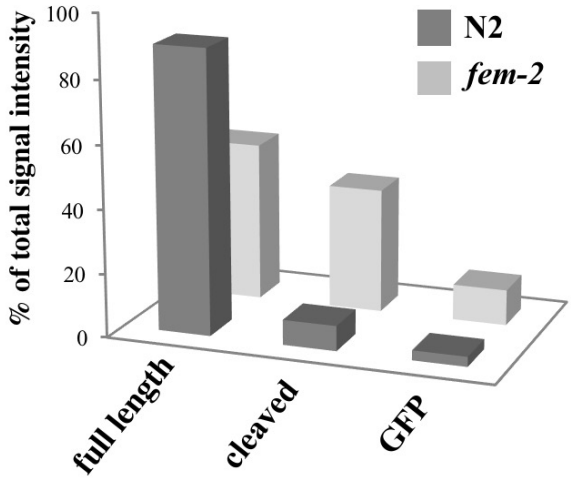

α-syn:GFP
inset (grayscale/reverse)
DIC/α-syn:GFP
N2
fem-2
16° C
N2
fem-2
25° C

(a)

(b)


60
50
40
30
20
10
0
Number of GFP foci
N2
fem-2
N2
fem-2
16C
heat shocked





(c)

Figure 4.20 (a), (b) and (c): Enhanced proteostasis of human αsyn::GFP in *fem-2* worms.
(a) Fluorescent micrographs of sterile *fem-2* (grown at 25° C) as well as self-fertile *fem-2* (grown at 16° C) worms. (b) Plot showing average number of αsyn::GFP foci in self-fertile and sterile *fem-2* mutants. (c) GFP immunoblot of total lysates prepared from post-reproductive (day 8) wild type and age-matched feminized *fem-2* worms expressing a GFP fusion of human α-syn in muscle cells. A coomassie blue stained gel shows loading input (bottom panel). The relative abundance of full length protein, the intermediate cleaved products, and the cleaved GFP moiety as a percentage of the total signal in each lane was determined by densitometry of the chemifluorescence blot.
(p values for Figure 4.20b: (a) N2 – 16°C vs *fem-2* – 16°C p=0.5551 (b) N2 – 25°C vs *fem-2* 25°C p<0.0001). All p values calculated using unpaired t test.

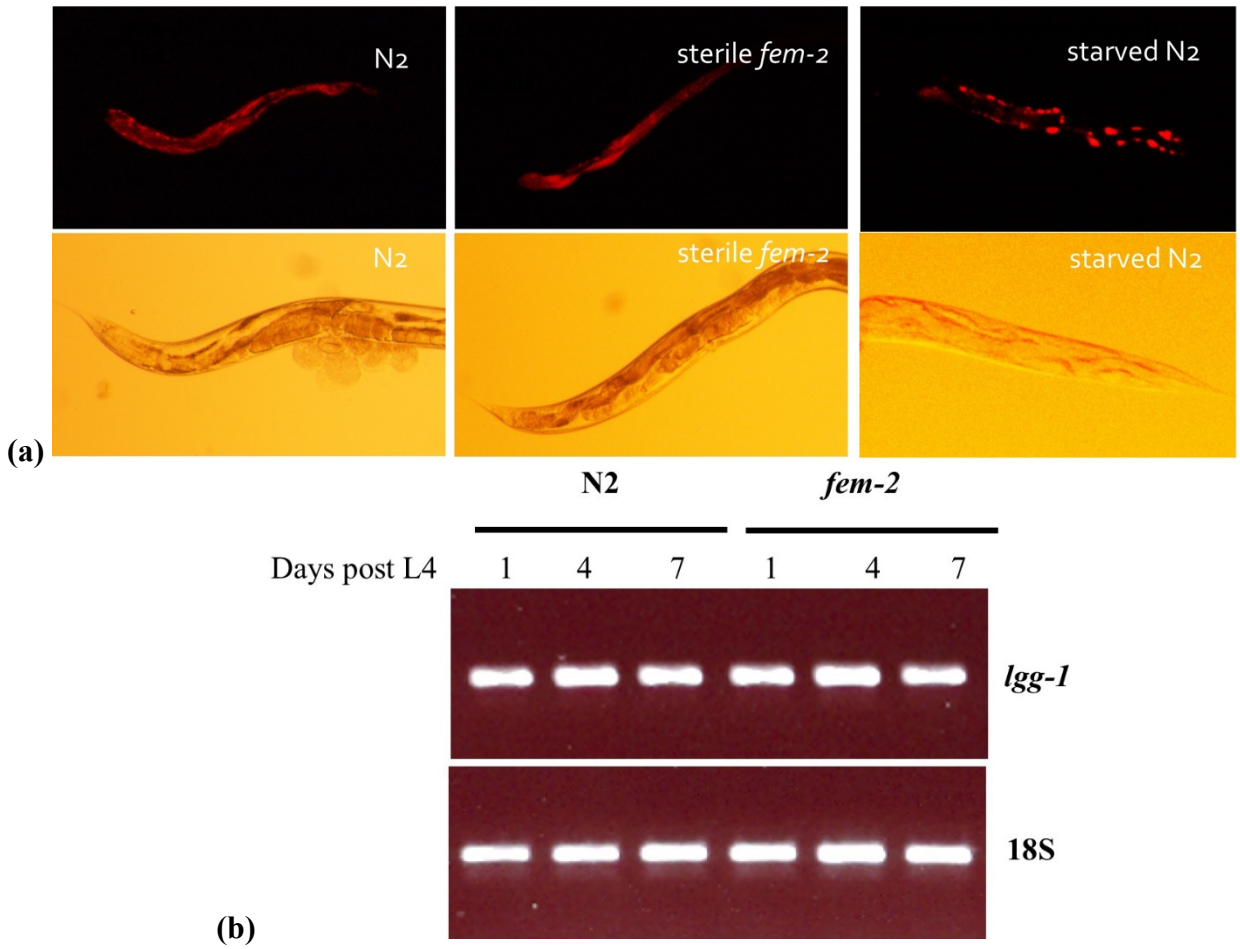


Figure 4.21: Autophagic flux remains unchanged in sterile *fem-2* worms.
(a) Fluorescence microscopy images of 2-day old wild type and sterile *fem-2* worms expressing the autophagy reporter fusion LGG-1::mCherry. Starved N2 worms were used as a positive control since starvation has been known to induce autophagy. (b) Semi-quantitative PCR showing *lgg-1* expression in wild type and *fem-2* worms during days 1, 4 and 7 of adulthood.

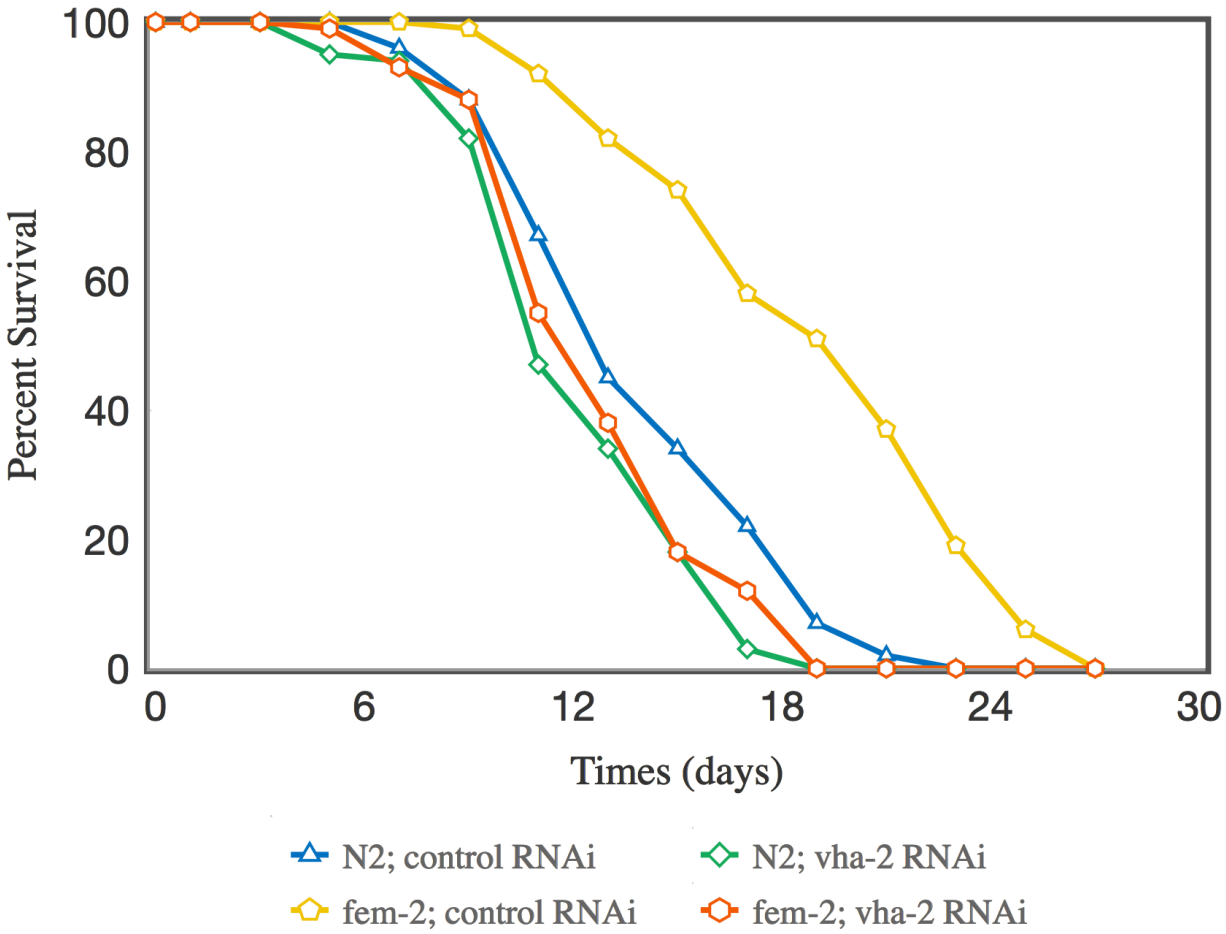


Figure 4.22: *vha-2* RNAi knockdown abrogates extended *fem-2* lifespan (n=100).
In order to achieve a subtle *vha-2* knockdown, worms were fed with *vha-2* RNAi food without IPTG supplementation. Worms were transferred every day to fresh *vha-2* or control RNAi food and the number of viable worms were counted at the time of transfer. Worms that did not move after repeated touches with the worm pick were deemed as dead. (p values: (a) N2 control RNAi vs N2 *vha-2* RNAi – p=0.0002 (b) N2 control RNAi vs *fem-2* control RNAi p<0.0001 (c) N2 *vha-2* RNAi vs *fem-2 vha-2* RNAi p=0.145 (d) *fem-2* control RNAi vs *fem-2 vha-2* RNAi p<0.0001). All p values were calculated using Mantel Cox test.

4.3.11 DAF-16 is the master regulator of *vha* genes

Having determined the role of *vha* gene expression if the control of lysosome function via reproductive inputs, I next focused on upstream components of this pathway, particularly in identifying transactivators of *vha* regulatory regions. It had been previously reported that *vha-3* promoter sequences co-IP with the FOXO transcription factor DAF-16 (Oh et al., 2006). To ascertain whether DAF-16 targets other *vha* genes for expression, an *in-silico* analysis of the 5'-upstream regions of v-ATPase genes in *C. elegans* was performed. This analysis yielded conserved DAF-16 consensus binding sites in nine out of twenty *vha* genes (Murphy, 2006) (Figure 4.23). A native (non-crosslinking) ChIP assay was then carried out to test whether these putative interactions were real. As is seen in Figure 4.24, *vha-2, vha-8, and vha-17* as well as the bona fide DAF-16 target *sod-2* were pulled down with the anti-DAF-16 antibodies. Taken together, both the *in situ* and the ChIP data suggested that DAF-16 may play a role in regulation of *vha* gene expression *in vivo*. In order to test this hypothesis, I performed a mild RNAi knockdown of *daf-16* (corresponding to a 40% reduction in *daf-16* transcript levels – Figure 4.25). This RNAi knockdown resulted in a coordinated downregulation of *vha* mRNAs (Figure 4.25) and a premature rise in lysosomal pH n the intestine of actively reproducing worms (Figure 4.26). A subtle knockdown of *daf-16* also abrogated the extended lifespan seen in *fem-2* mutants. These results indicate that DAF-16 is the master regulator that intermediates regulation of lysosomal pH in the soma in response to cues from the gonad. Moreover, DAF-16-dependent lysosomal acidification is one of the driving forces leading to extended lifespan in *fem-2* mutants (Figure 4.27).

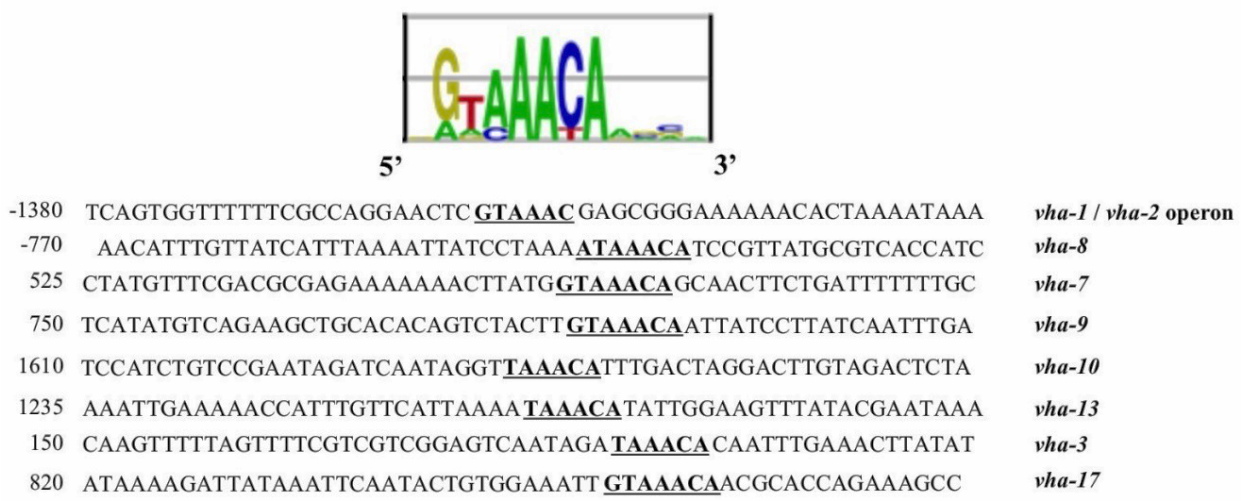


Figure 4.23: *In silico* analysis of putative DAF-16 binding sites in *vha* gene promoters.
In order to identify putative DAF-16 binding sites in *vha* genes, 2000 bp of *vha* promoter sequences were curated from WormBase and analyzed for DAF-16 consensus motifs (underlined).

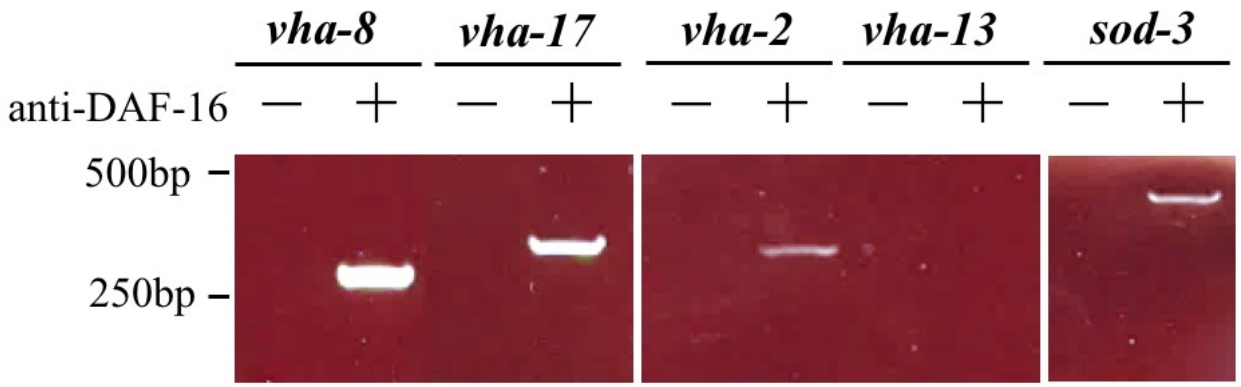


Figure 4.24: Native ChIP analysis to identify putative *vha* genes regulated by DAF-16.
Binding of DAF-16 to *vha* promoters in day 2 wild type worms assayed by ChIP. All queried genes contained a conserved binding motif (Figure 4.23). Failure to recover *vha-13* likely reflects a previous observation where the presence of binding motifs alone does not ensure DAF-16 binding (Oh et al., 2006)

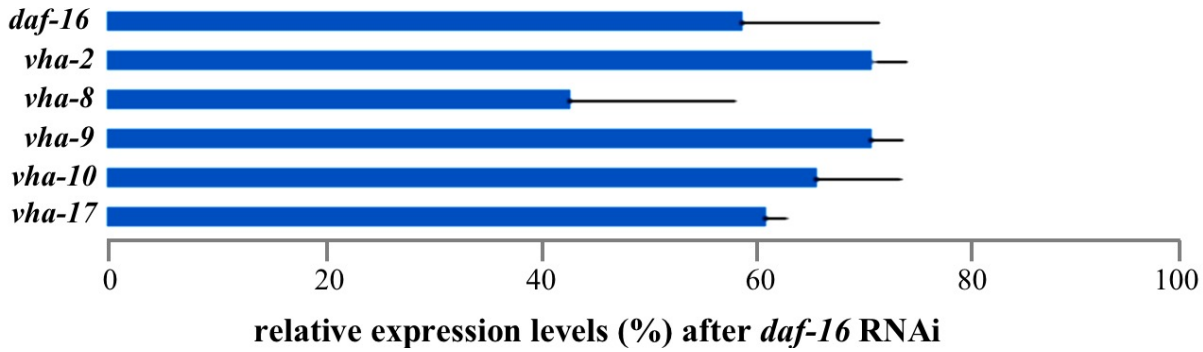


Figure 4.25: *daf-16* RNAi leads to a reduction in *vha* transcript levels.
Relative transcript levels of *vha* genes after a mild RNAi knockdown (40% depletion in mRNA level) of *daf-16*. Two samples were analyzed in triplicates. (All p values between control and *daf-16* RNAi were calculated to be $p < 0.05$; unpaired t test).

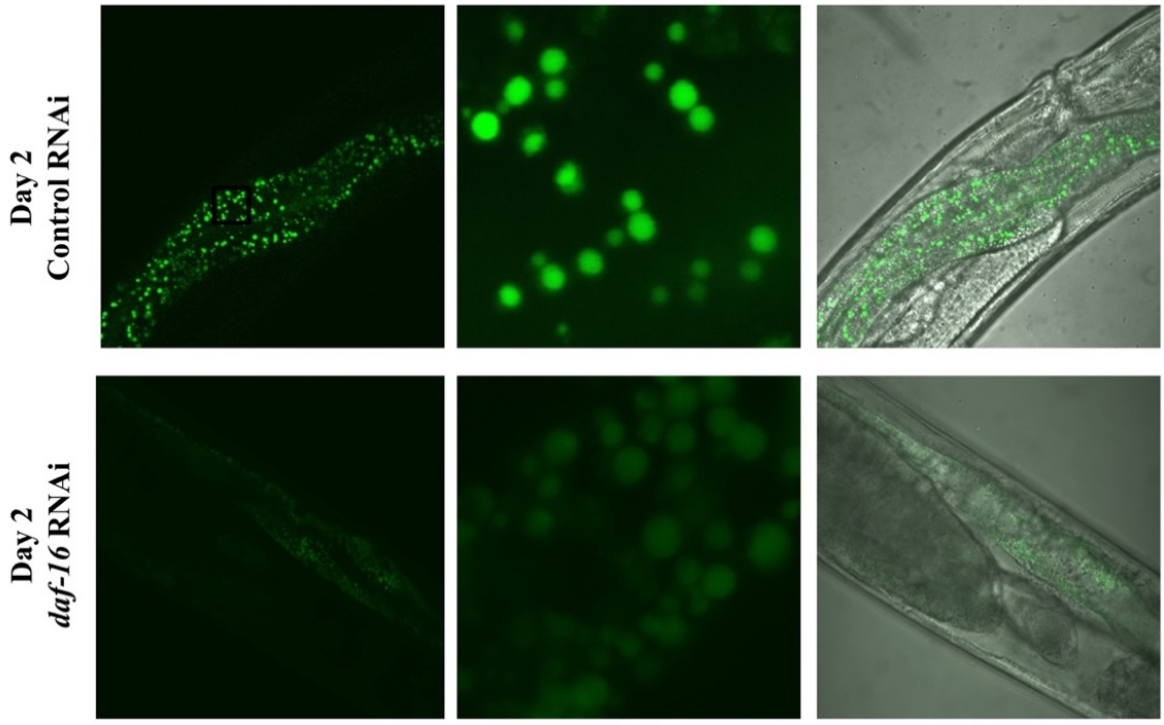


Figure 4.26: *daf-16* RNAi knockdown leads to impaired lysosomal acidification.
In order to perform a subtle *daf-16* RNAi knockdown, wild type day 1 adult worms were fed *daf-16* RNAi food without IPTG supplementation for 48 hours and were imaged the next day (day 3) using cDCFDA.

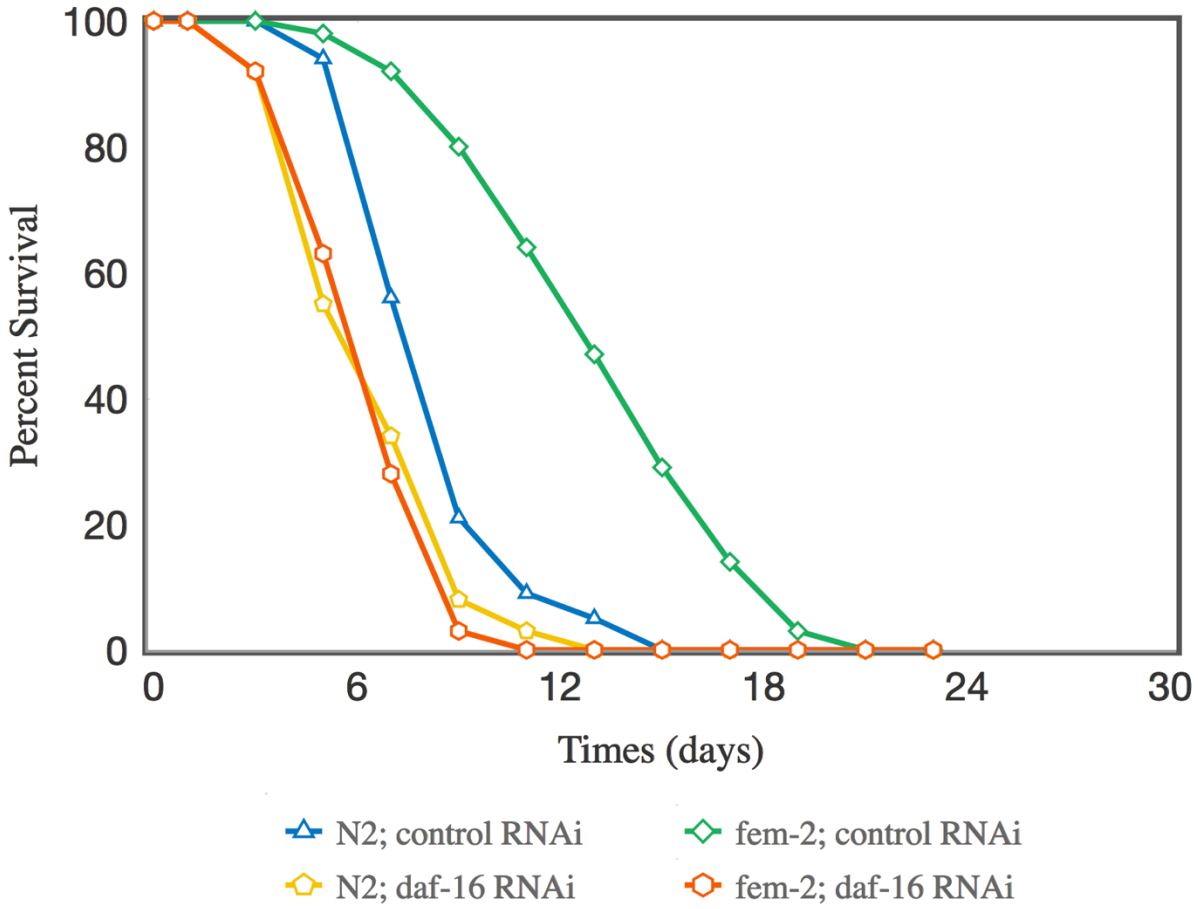


Figure 4.27: *daf-16* RNAi knockdown abrogates extended *fem-2* lifespan (n=100).
In order to achieve a subtle *daf-16* knockdown, worms were fed with *daf-16* RNAi food without IPTG supplementation. Worms were transferred every day to fresh *daf-16* or control RNAi food and the number of viable worms were counted at the time of transfer. Worms that did not move after repeated touches with the worm pick were deemed as dead. (p values: (a) N2 control RNAi vs N2 *daf-16* RNAi p <0.0000017 (b) *fem-2* control RNAi vs *fem-2 daf-16* RNAi p<0.0001 (c) N2 *daf-16* RNAi vs *fem-2 daf-16* RNAi p=0.432). All p values were calculated using Mantel Cox test.

4.3.12 DAF-16 subcellular localization regulates *vha* gene expression

One plausible mechanism explaining the decline in *vha* gene expression mediated by DAF-16 considers the downregulation of DAF-16 expression itself in post-reproductive animals. To test this idea, DAF-16 transcript levels were measured via semi-quantitative PCR in both young and old (post reproductive) worms. As seen in Figure 4.28 (a), DAF-16 transcript levels as well as protein loads (b) remained relatively unchanged during and after reproductive cessation. Nor was any difference found in the relative amounts of DAF-16 in *fem-2* versus wild type animals that could explain the increase DAF-16-mediate transcription in the latter (Figure 4.28. b). This is not surprising, as the primary mechanisms by which DAF-16 regulates expression in *C. elegans* appears to rely on post-translational modifications that either potentiate or impede nuclear translocation (Lin et al., 2001; Yen et al., 2011). When DAF-16 is activated, it translocates to the nucleus and transcribes genes that are responsible for stress response and longevity. When DAF-16 becomes inactivated it relocates to the cytoplasm and ceases transcription of genes involved in longevity and stress resistance (Henderson et al., 2001). Loss of DAF-16 nuclear localization during post-reproductive periods was therefore a strong possibility to explain the downregulation of *vha* gene expression in these animals. Figures 4.29 and 4.30 clearly show that DAF-16 displays a strong nuclear signal in the intestinal cells of young wild type and *fem-2* worms, but while *fem-2* worms retain nuclear DAF-16 well into day 10 of adulthood, wild type worms show a discrete loss of nuclear signal at these stages. Migration of DAF-16 back to the cytoplasm following reproductive decline thus provides a plausible molecular mechanism for the loss of *vha* gene expression following cessation of reproduction. Somehow, *fem-2* (and possibly other sterile worms) that never initiated the reproductive cycle bypass this inhibitory effect on DAF-16 localization, thus avoiding a decline in *vha* gene expression. Remarkably, a short heat shock at 30^{o}C (~1 hr) or overnight nutritional deprivation restored DAF-16 nuclear localization in (day 10) post-reproductive animals (Figure 4.31). The *hsf-1*-mediated heat stress response (Hsu et al., 2003), or the IGF1-induced nutritional stress pathway (Lin et al., 2001) exert their effects in part by signalling DAF-16 nuclear localization. Induction of either pathway thus overrides the effect of the germline signalling on intestinal DAF-16. This observation suggests that nuclear localization of DAF-16 and its subsequent cytoplasmic translocation via different genetic pathways can be overridden under certain stresses when DAF-16 nuclear activity is again required. Overall, these findings indicate a level of functional plasticity of DAF-16, which converges signals for several

different pathways, in the intestine and provides evidence that its nuclear recruitment via converging pathways can be hierarchical.

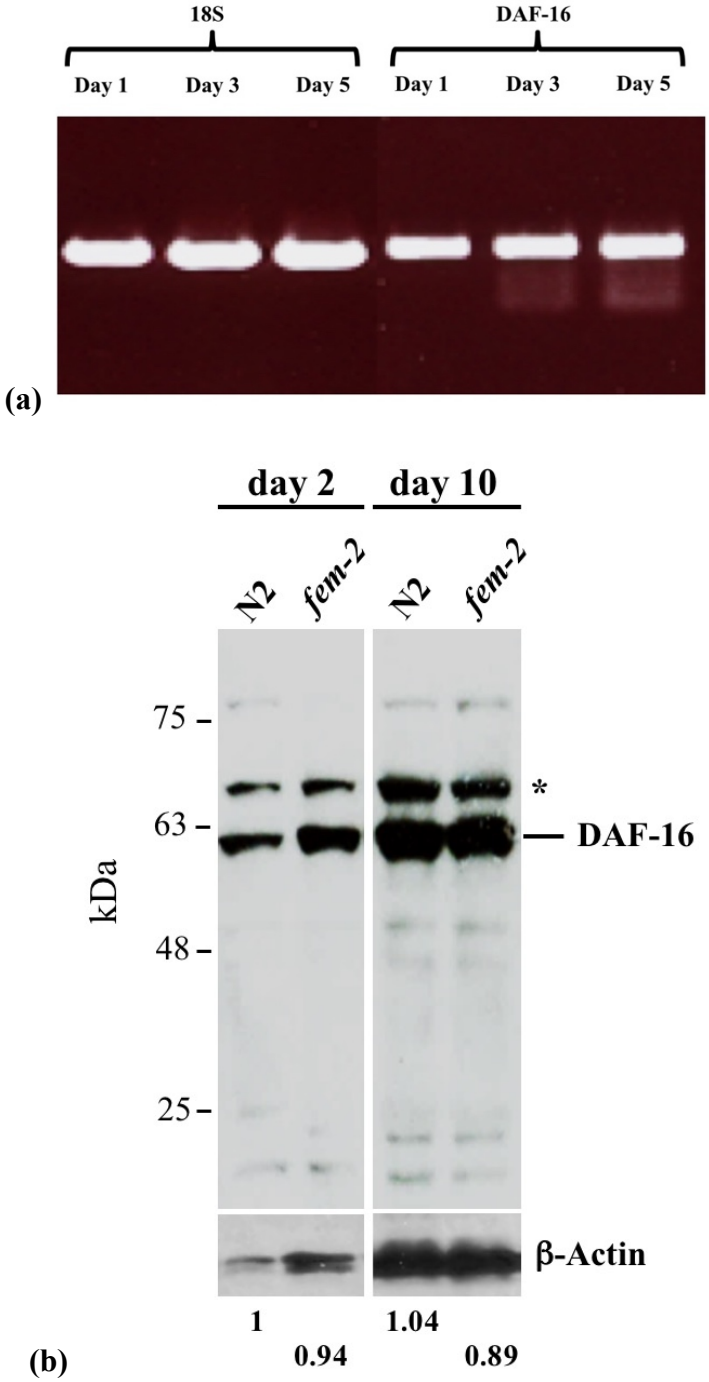
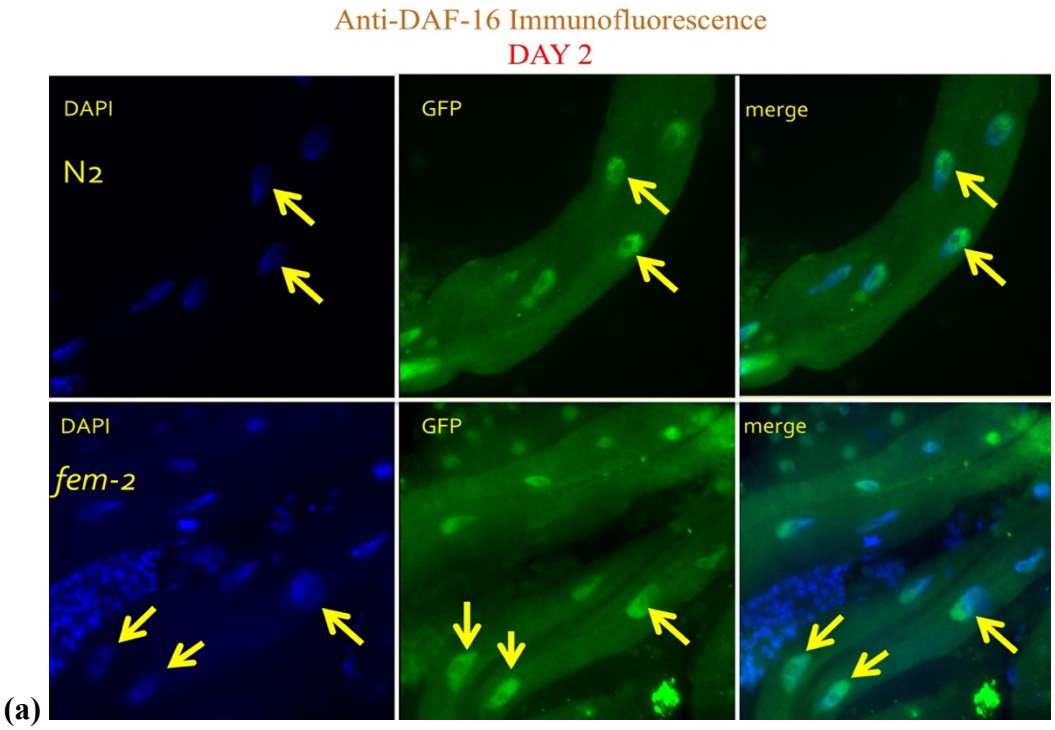
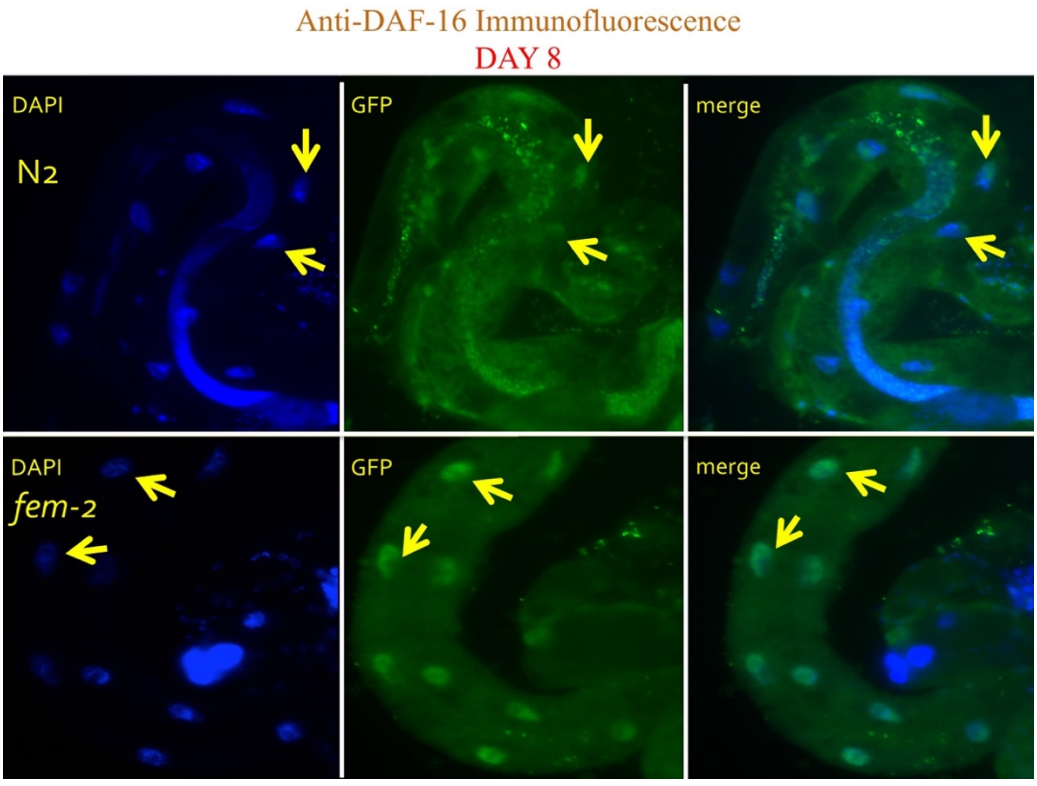


Figure 4.28: DAF-16 levels remain unchanged during and after cessation of reproduction. (a) Agarose gel for determining the relative levels of DAF-16 via semi-quantitative PCR during and after cessation of reproduction in wild type worms. (b) DAF-16 immunoblot using total lysate prepared from reproductive (day 2) and post-reproductive (day 10) wild type and *fem-2(b245)* worms. DAF-16 abundance, determined by densitometric analysis of the chemifluorescence blot and normalized to ß-actin loading control, is shown at the bottom.



(a)



(b)

Figure 4.29: DAF-16 remains nuclear in post-reproductive *fem-2* mutants.
Immunofluorescence images of dissected worms during and after cessation of reproduction. Worms were freeze-fractured and probed with an anti-DAF-16 antibody raised against the last 300 amino acids of the C terminus of DAF-16.

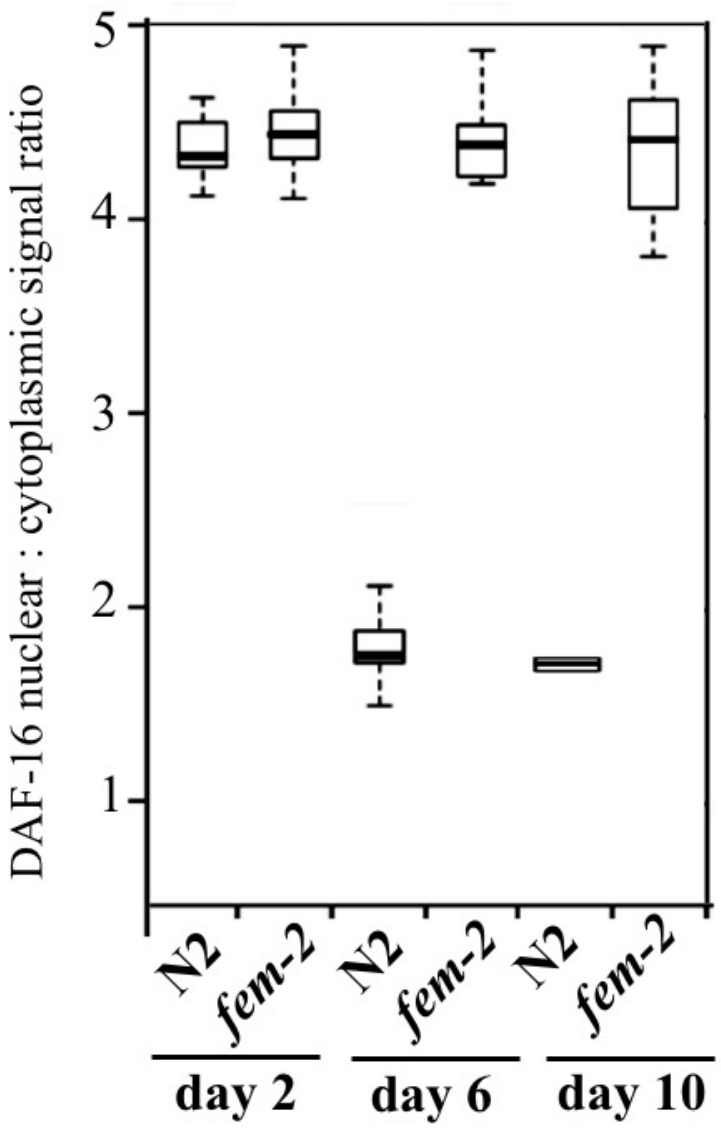


Figure 4.30: DAF-16 is predominantly nuclear in older *fem-2* but not wild type worms. Nuclear:cytoplasmic DAF-16 fluorescence signal ratios in wild-type and *fem-2* worms were plotted for day 2, day 6 and day 10 of lifespan. Images were obtained as a Z-stack and were first collapsed into a single image and then analyzed by ImageJ.

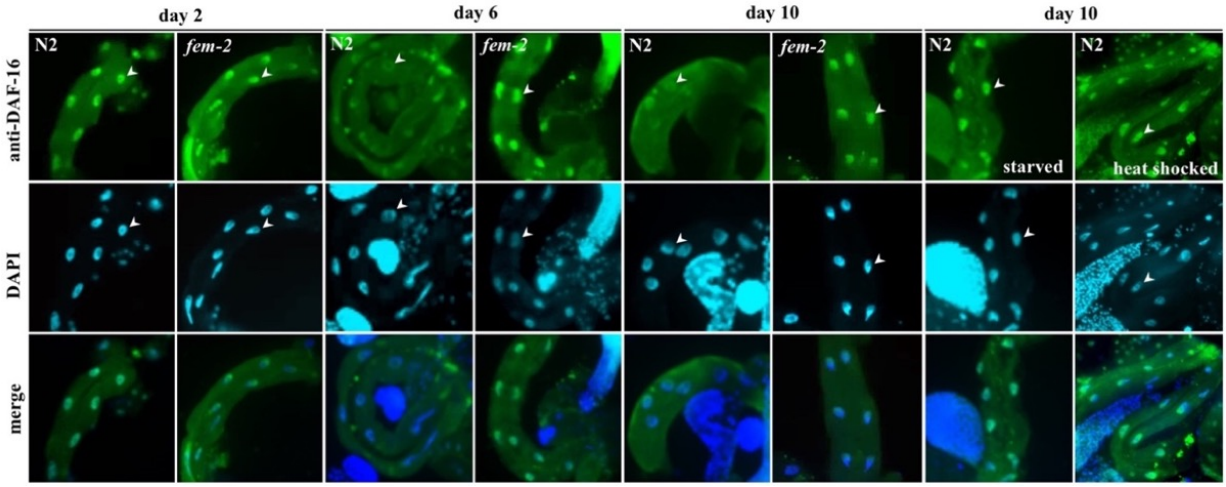


Figure 4.31: Starvation or heat shock forces DAF-16 to migrate back to the nucleus in post-reproductive wild type animals.

Fluorescence microscopy images showing DAF-16 localization for day 1, day 6 and day 10 wild type and *fem-2* worms. The last two panels show day 10 wild type worms that have been either starved overnight or heat shocked for 1 hour at 30^{o} C.

4.3.13 DAF-12/DAF-9 hormone signalling is required for lysosomal pH homeostasis

In animals without a germline, DAF-9 catalyzes the synthesis of lipophilic steroid hormones such as Δ^4-dafachronic acid (DA) that activate the nuclear hormone receptor (NHR) DAF-12 to upregulate the expression of genes whose activities extend lifespan by promoting nuclear localization of DAF-16 (Antebi et al., 2000; Berman et al., 2006; Gerisch et al., 2001; Shen et al., 2012). This increase in lifespan triggered by loss of the germ cells requires intact sterol hormone signalling as mutations in *daf-12* or *daf-9* prevents the extension of lifespan in animals without germ cells (Gerisch et al., 2001; Hsin et al., 1999; Yamawaki et al., 2010). It has been shown that supplementing Δ^4-dafachronic acid with OP50 food is sufficient to restore the expression of DAF-12 target genes and the longevity in animals lacking the entire gonad (Gerisch et al., 2007; Yamawaki et al., 2010). Thus, the DAF-9/DAF-12 hormonal signalling axis responds to germ cell proliferation to regulate DAF-16 recruitment. In mutants without a germline, constitutive activation of this pathway underlines the extended longevity observed in these animals.

I first asked whether lysosome acidification in the intestine of *fem-2* mutants responds to DAF-9/DAF-12 signalling. Similar to *daf-16* RNAi, a knockdown of *daf-12* leads to premature lysosome alkalinization in actively reproducing worms (Figure 4.32) and suppression of the extended lifespan in *fem-2* mutants (Figure 4.33).

Consistent with its established role in transducing signals from the germline to the soma, a *daf-9* mutant (*rh50*) showed premature lysosomal alkalinization (Figure 4.34). Not surprisingly, the *daf-9(rh50)* mutation also led to premature lysosomal alkalinization in *fem-2* worms (Figure 4.35 (a) and (b)) mirrored by an increase in the nuclear exclusion of DAF-16 in the intestine in these animals (Figure 4.36 (a) and (b)). Thus, acidic lysosomal pH in the intestine requires the activation of DAF-9/DAF-12/DAF-16 signalling axis.

A transcriptomic analysis of the gonad throughout adulthood provides further evidence of the close association of DAF-9 with the reproductive status of the animal (Figure 4.37). Coincident with the end of reproductive life (day 5), *daf-9* transcript levels decrease sharply. This drop in *daf-9* mRNA levels is not recapitulated later on, nor is it the product of a general decline in germline gene expression, since other gene families highly expressed in this tissue such as the piwi-related gene responsible for maintaining germ-cell proliferation continue to be highly expressed past the reproductive span (Lund et al., 2002; Reinke et al., 2000) (Figure 4.37).

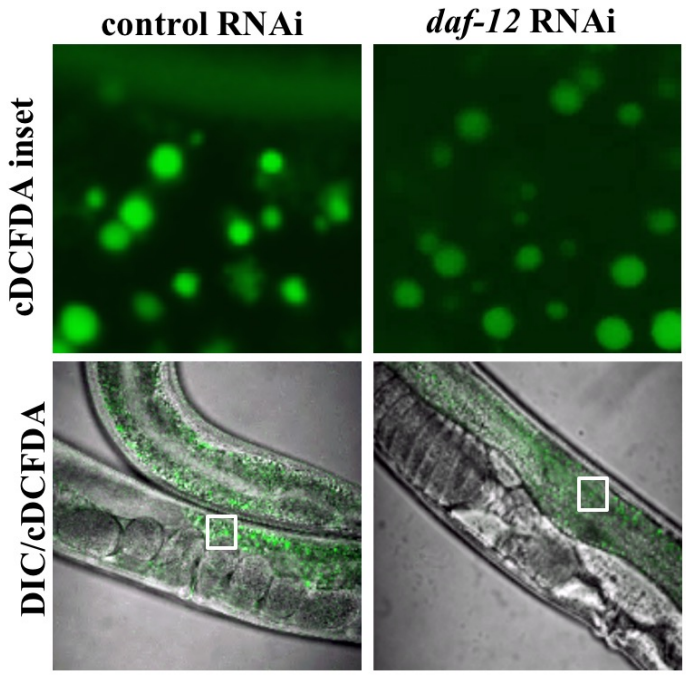


Figure 4.32: *daf-12* RNAi knockdown leads to premature lysosomal alkalinization.
A mild *daf-12* RNAi knockdown (no IPTG supplementation) leads to premature lysosomal alkalinization of 2-day old wild type worms.

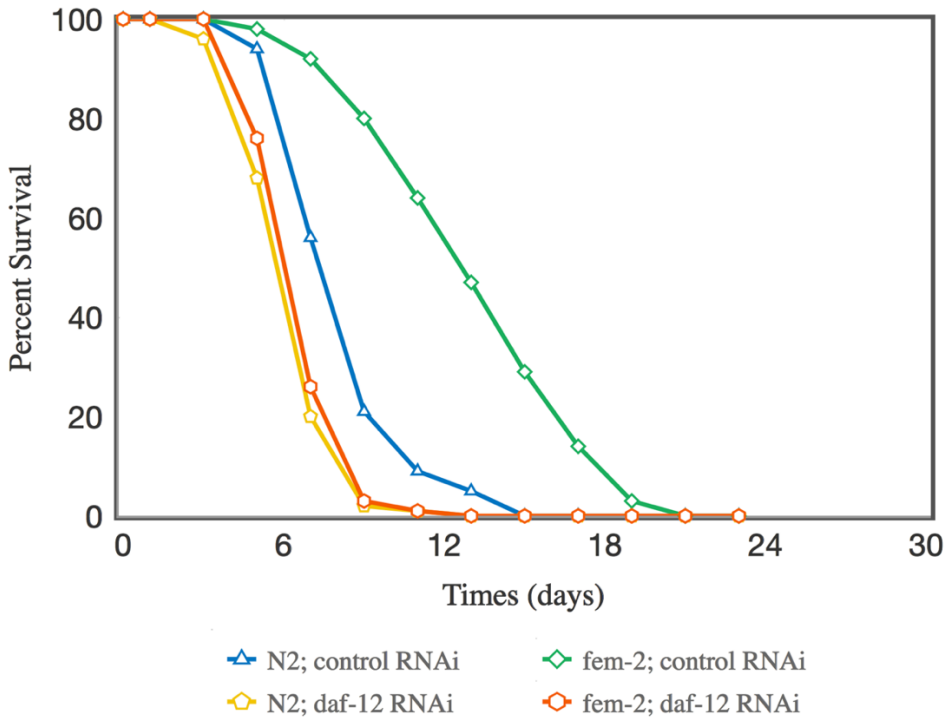


Figure 4.33: *daf-12* RNAi knockdown abrogates extended *fem-2* lifespan (n-100).
Lifespan plot of *fem-2* vs wild type worms that have been fed either control or *daf-12* RNAi food. (p values: (a) N2 control RNAi vs N2 *daf-12* RNAi $p<0.0001$ (b) *fem-2* control RNAi vs *fem-2 daf-12* RNAi $p<0.0001$ (c) N2 *daf-12* RNAi vs *fem-2 daf-12* RNAi $p=0.1629$). All p values calculated using Mantel Cox test.

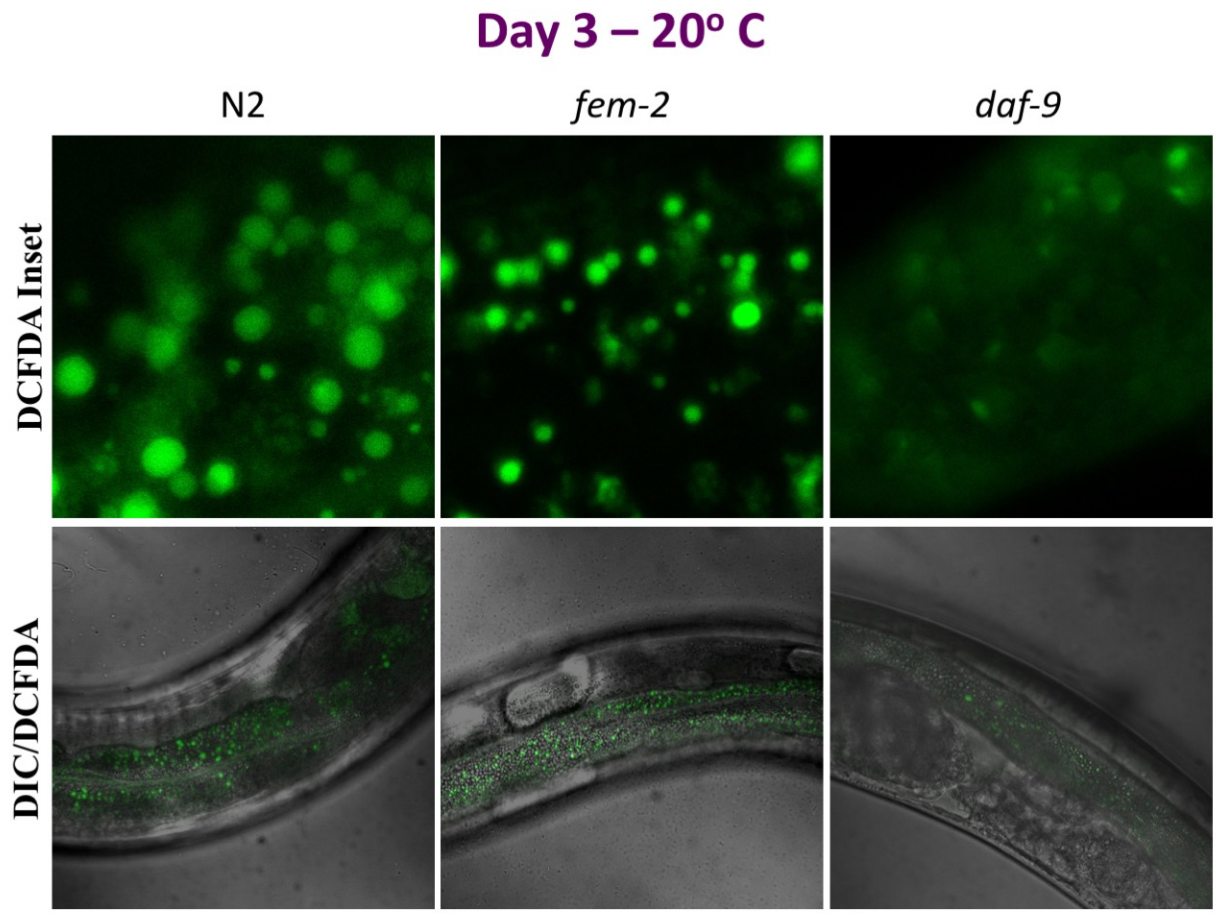


Figure 4.34: *daf-9* mutants show premature lysosomal alkalinization.
Wild type, *fem-2* and *daf-9* worms were grown at 25°C until adulthood and then allowed to grow at 20°C until day 3. Worms were then added to OP50 food supplemented with 100µg/ml of cDCFDA and imaged via confocal microscopy the day after.

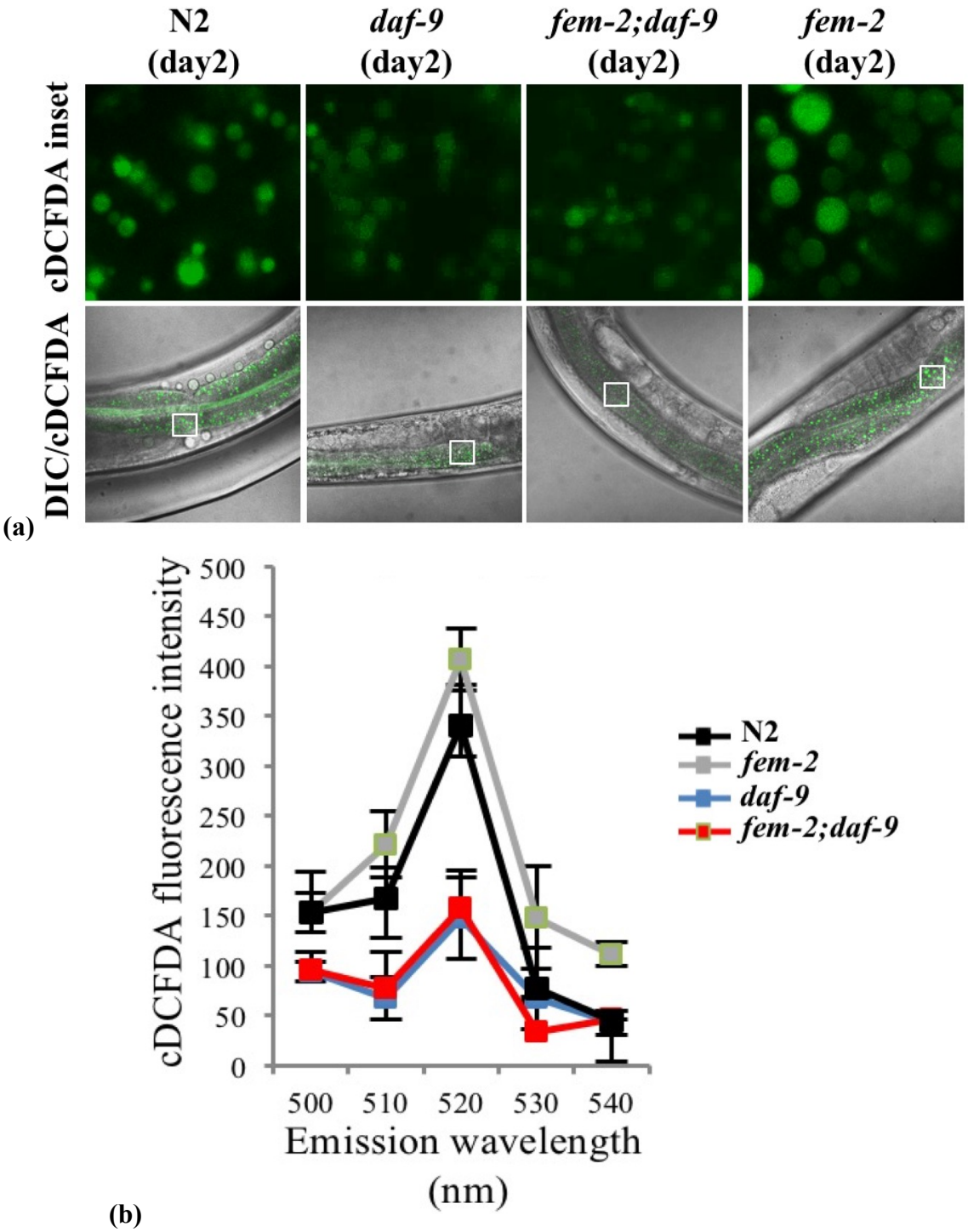


Figure 4.35: Inactivation of *daf-9* leads to premature lysosomal alkalinization in *fem-2* mutants.

Wild type, *fem-2, fem-2;daf-9* and *daf-9* worms were grown at 25°C until adulthood and then allowed to grow at 20°C until day 2. Worms were then added to OP50 food supplemented with 100μM of cDCFDA and imaged via confocal microscopy after ~16 hours. (a) Confocal microscopy of 2-day old wild type, *fem-2, daf-9* and *fem-2;daf-9* worms after cDCFDA staining. (b) Relative fluorescence intensity plot of wild type, *fem-2, daf-9* and *fem-2;daf-9* worms. Relative fluorescence intensities were calculated using at least 5 different lysosomes for 5 different worms.

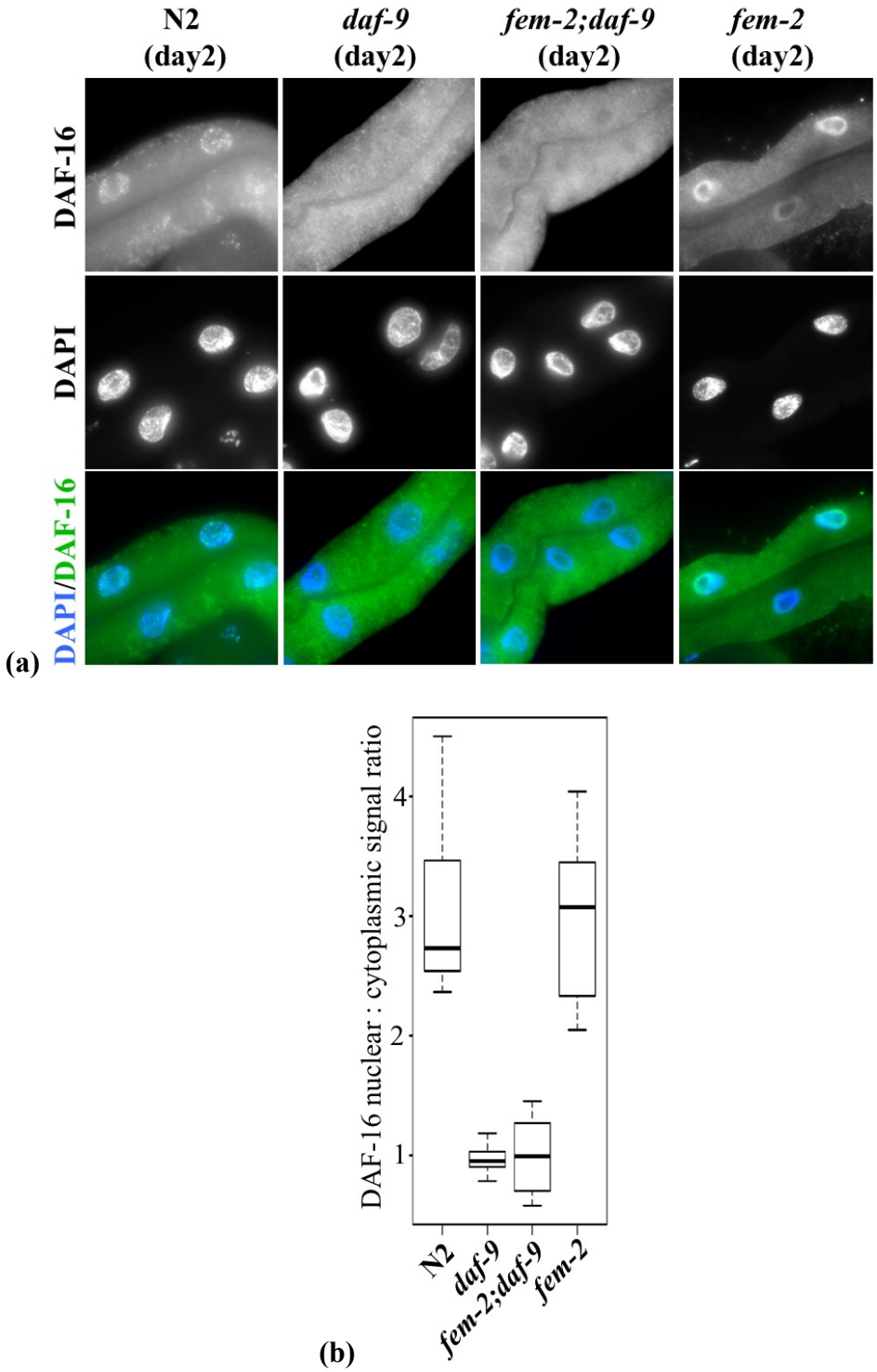


Figure 4.36: DAF-16 is predominantly cytoplasmic in *daf-9* and *fem-2;daf-9* mutants.
Wild type, *daf-9, fem-2* and *fem-2;daf-9* worms were grown at 25°C on OP50 food until adulthood and then switch to 20°C until day 2. Worms were then dissected and immunostained with anti-DAF-16 antibody and imaged via fluorescence microscopy. (a) Fluorescence microscopy images of day 2 old wild type, *fem-2, daf-9* and *fem-2;daf-9* worms (b) Quantification of nuclear:cytoplasmic DAF-16 ratios calculated from fluorescence microscopy images. All calculations were done using ImageJ from a total of at least 15 nuclei per genotype.

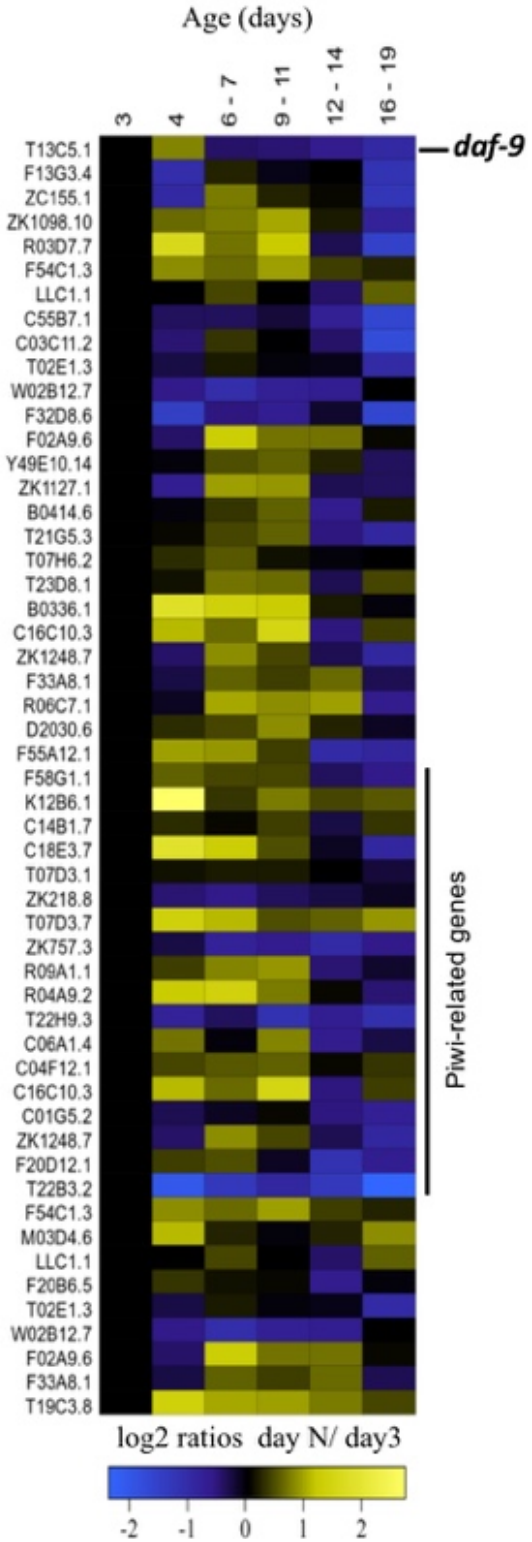


Figure 4.37: DAF-9 levels are reduced following cessation of reproduction.
RNA expression data was curated from (Lund et al., 2002) and projected as the $\log_2$ ratios of day N/day 3. DAF-9 is predominantly expressed in the somatic gonad. The piwi-related genes are annotated to germ cells and function in proliferation or maintenance of sperm and oocytes (Reinke et al., 2000).

4.3.14 Δ_4-DA supplementation is sufficient to restore lysosomal acidity in DAF-9 deficient worms

The involvement of DAF-9 in promoting lysosomal acidification predicts that supplementation with Δ_4-DA, a product of DAF-9/p450 activity and the ligand for DAF-12 should bypass the defect in the cytochrome. Indeed, supplementing *daf-9* mutants with 100 μM Δ_4-DA restore lysosomal acidification to wild type levels (Figure 4.38 (a) and (b)). Consistent with the reactivation of the nuclear hormonal pathway in Δ_4-DA-fed animals, DAF-16 can be detected again in the nuclei of intestinal cells (Figure 4.39 (a) and (b)). In these animals, even a 10 μM Δ_4-DA supplementation delayed age-associated fitness decline in wild type worms by restoring motility in 7 day old worms (Figure 4.40.), presumably by enhancing proteostasis in later life stages.

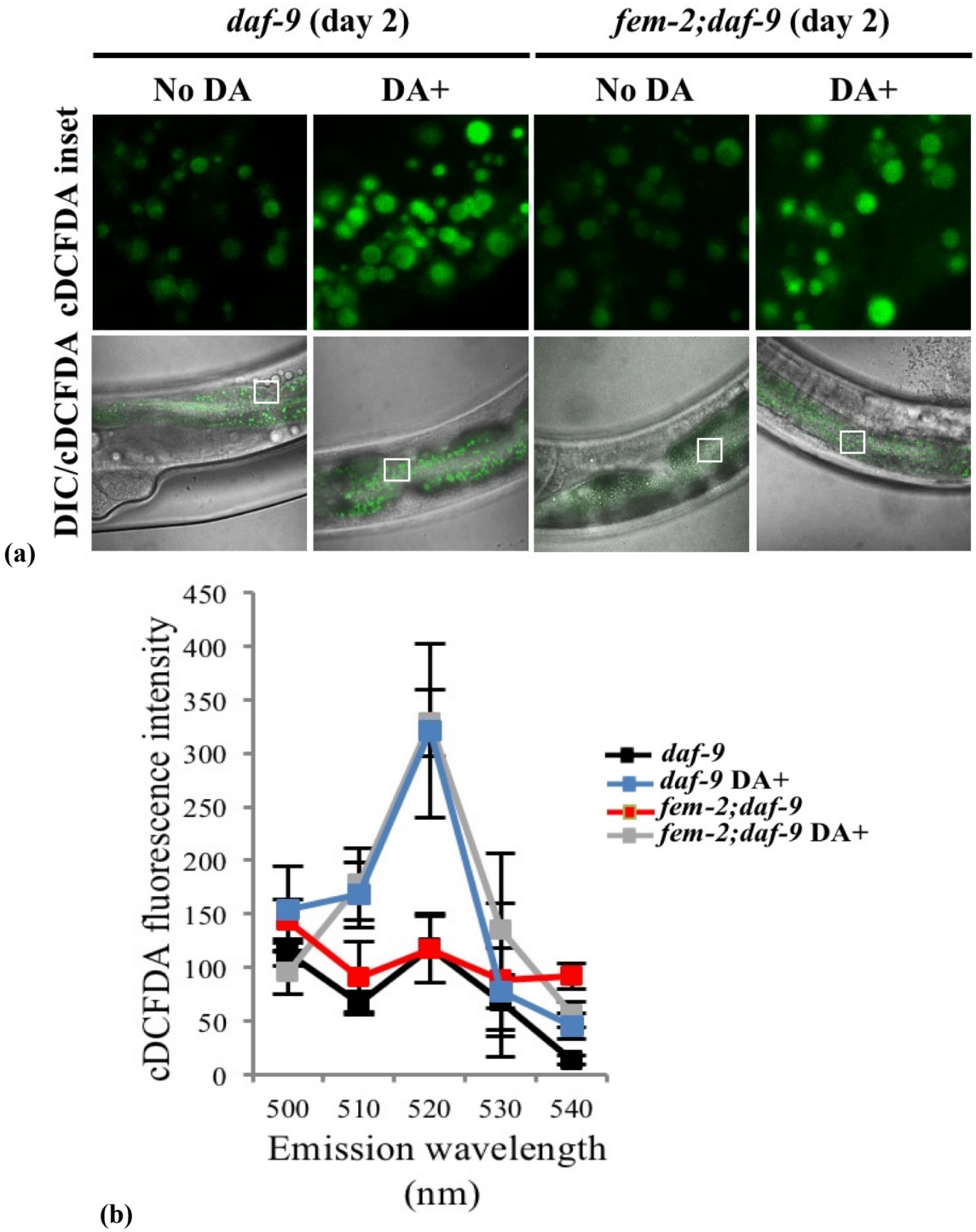


Figure 4.38: Δ_4-DA restores lysosomal pH of *daf-9* and *fem-2;daf-9* back to wild type levels. *fem-2;daf-9* and *daf-9* worms were grown at 25°C on OP50 food supplemented with 100μM Δ_4-DA until adulthood and then grown at 20°C on OP50 food supplemented with 100μM Δ_4-DA at 20°C until day 2. At day 2, worms were transferred to fresh OP50 containing 100μM cDCFDA + 100μM Δ_4-DA and imaged via confocal microscopy after ~16 hours. (a) Confocal microscopy of 2-day old *fem-2* and *fem-2;daf-9* worms after cDCFDA staining. (b) Relative fluorescence intensity plot of *fem-2* and *fem-2;daf-9* worms. Relative fluorescence intensities were calculated from fluorescence intensity values of at least 5 different lysosomes from 5 different worms.

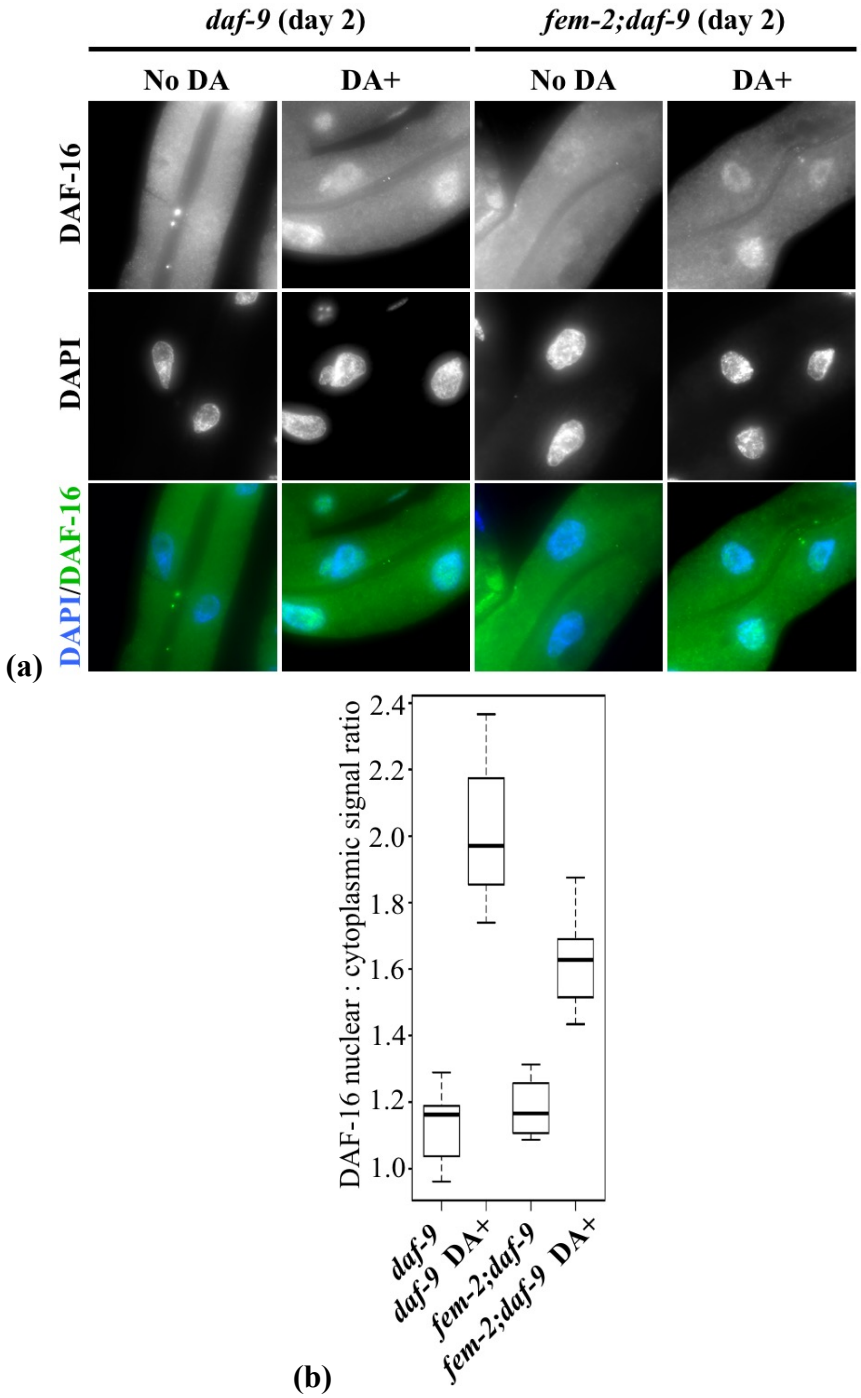


Figure 4.39: Δ_4-DA supplementation leads to nuclear translocation of DAF-16 in *daf-9* and *fem-2;daf-9* mutants.

daf-9 and *fem-2;daf-9* worms were grown at 25°C on either OP50 + ethanol (control) food or OP50 + 100μM Δ4-DA food until adulthood and then transferred to 20°C until day 2. Worms were then immunostained with anti-DAF-16 antibody and imaged via fluorescence microscopy. (a) Fluorescence microscopy images of 2-day old *daf-9* and *fem-2;daf-9* worms that were fed either control or Δ4-DA supplemented food (b) Plot showing nuclear:cytoplasmic DAF-16 ratios calculated from fluorescence microscopy images. All calculations were done using ImageJ from a total of at least 15 nuclei per genotype.

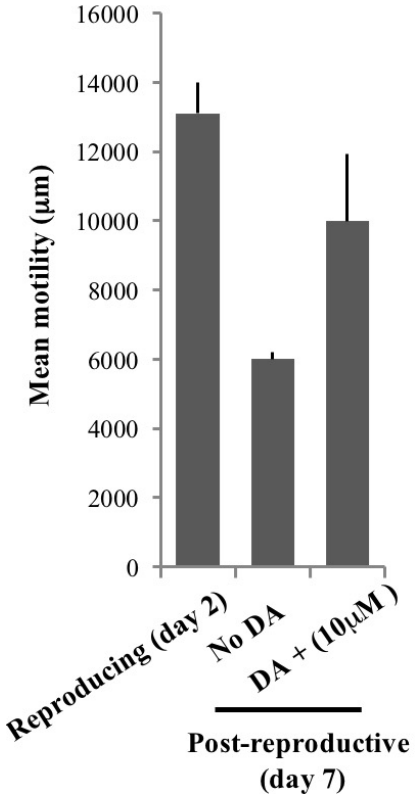


Figure 4.40: Δ_4-DA supplementation delay age-associated fitness decline in old worms. Wild type worms were grown on OP50 food supplemented with 10 µM Δ_4-DA from day 1 of adulthood. (a) Fluorescence microscopy images of day 7 worms with or without 10 µM Δ_4-DA supplementation. (b) Relative motility of old (day 7) worms with or without 10 µM Δ_4-DA supplementation was measured using WormLab software (MBF Bioscience).

4.3.15 Extending the reproduction window delays lysosomal alkalinization

If acidification of lysosomes is potentiated during the time of progeny generation, then extending the window of reproduction may result in delayed alkalinization induced by a prolonged DAF-9/DAF-12 signalling period. In order to test this hypothesis, I mated wild type worms on day 4 post L4 (when the XX sperm repository has been almost exhausted) and then assessed lysosomal pH and DAF-16 localization in the mated worms on day 7. Mating extends by a few days the reproductive period by supplying hermaphrodites with an exogenous supply of fresh sperm, the limiting factor in the number of progeny generated by selfing. As anticipated, 7 day old mated worms showed delayed lysosomal alkalinization and nuclear DAF-16 localization (Figure 4.41 and 4.42). Thus, extending the reproductive lifespan can maintain high levels of DAF-9/DAF-12 signalling and delay aging.

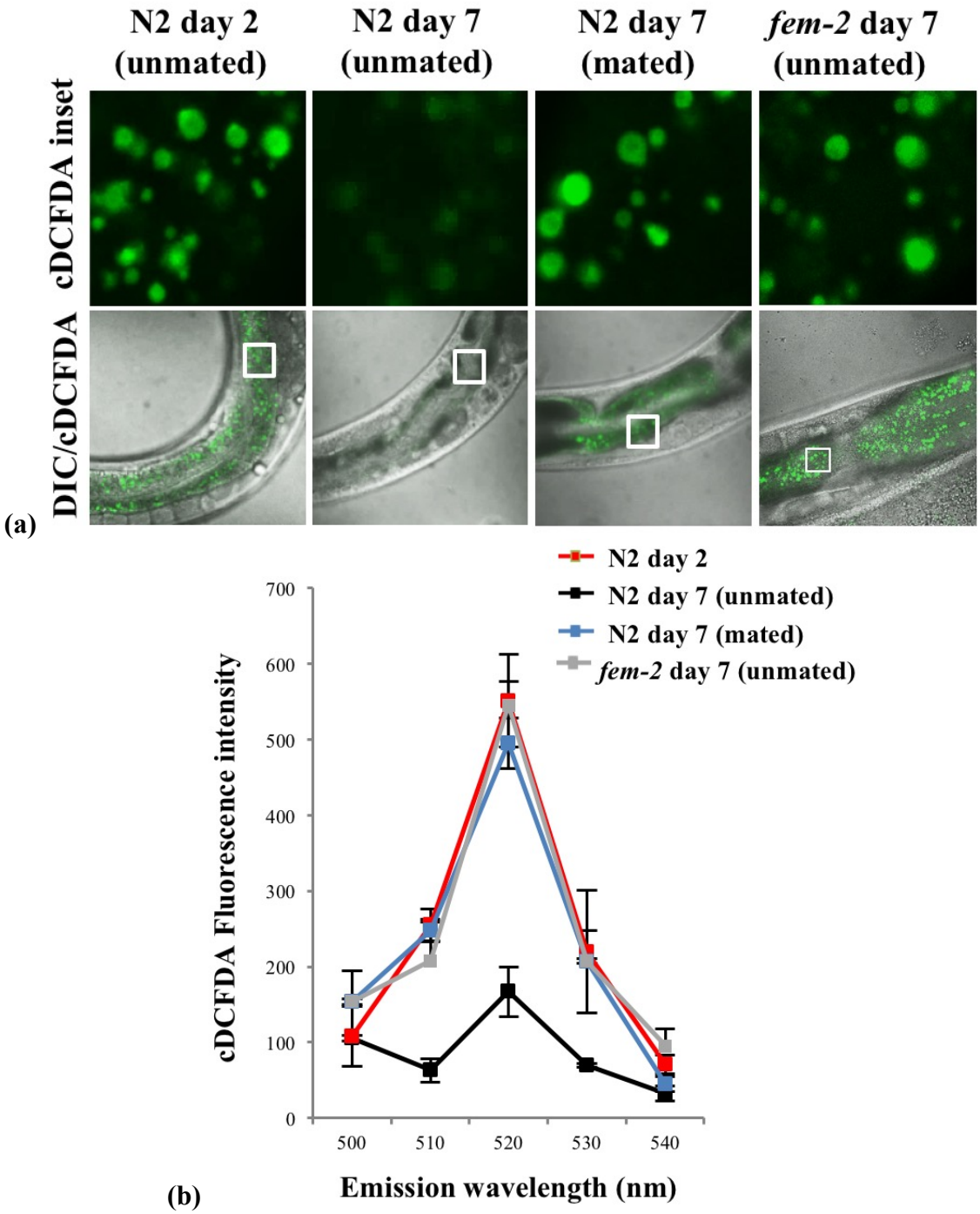


Figure 4.41: Delaying reproductive cessation prolongs lysosomal homeostasis.
4-day old (post L4) wild type hermaphrodites were mated overnight and singled out on OP50 plates the next day. After 48 hours, worms that generated male progeny were deemed to have mated successfully and these mated hermaphrodites were transferred to OP50 food supplemented with 100μM cDCFDA (around day 7 post L4). (a) Confocal microscopy of 7-day old mated and unmated wild type worms (with 2-day old wild type and 7-day old unmated *fem-2* worms as control). (b) Relative fluorescence intensity plot of mated and unmated wild type worms. Relative fluorescence intensities were calculated from fluorescence intensity values of at least 5 different lysosomes from 5 different worms.

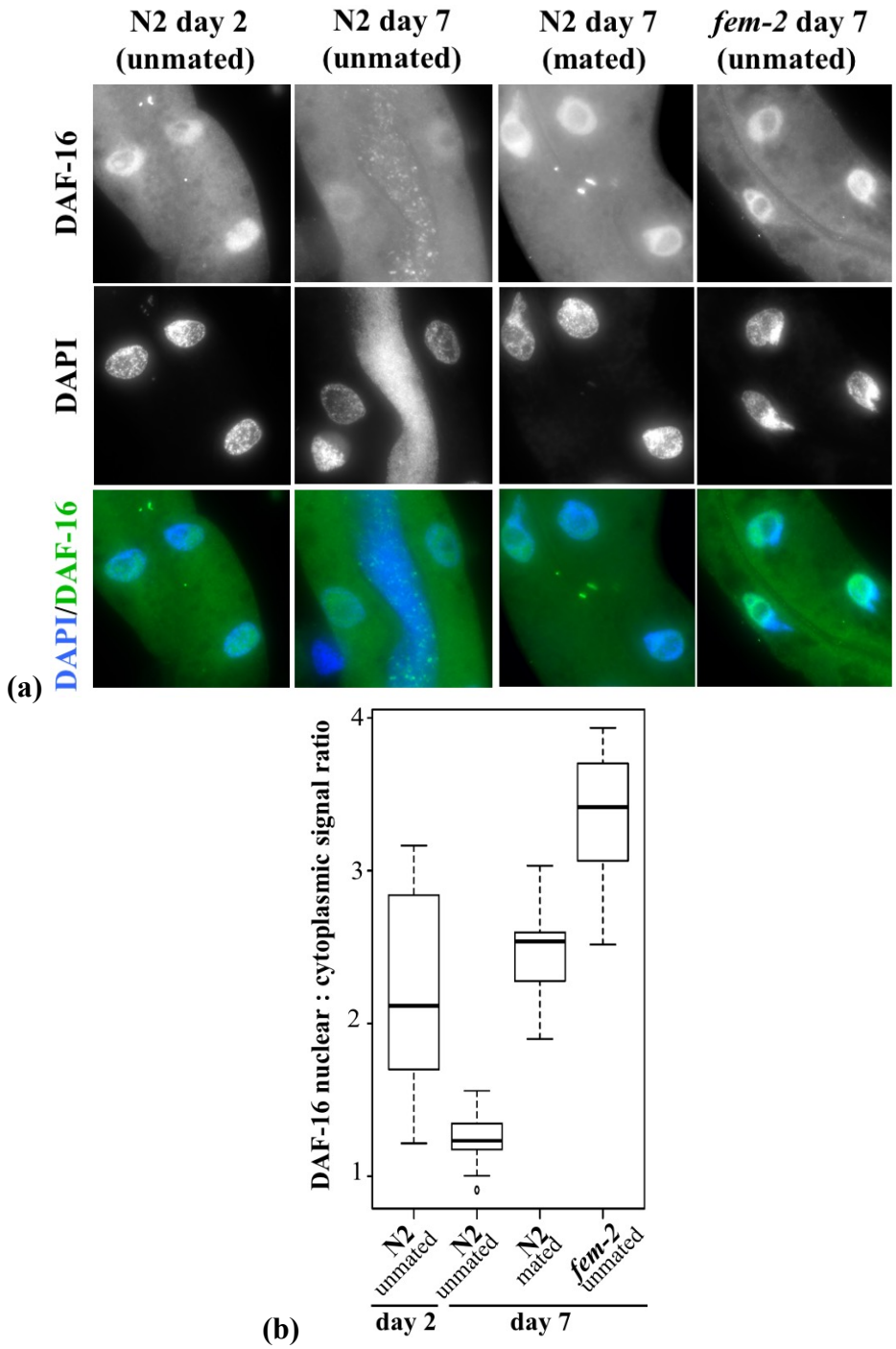


Figure 4.42: Mating induces nuclear localization of DAF-16.
4-day old (post L4) wild type hermaphrodites were mated overnight and singled out on OP50 plates the next day. After 72 hours, worms that generated male progeny were deemed to have mated successfully and these mated hermaphrodites were immunostained with anti-DAF-16 antibody and imaged via fluorescence microscopy. (a) Fluorescence microscopy images of 7-day old mated and unmated wild type worms along with 7-day old *fem-2* and 2-day old wild type worms as control. (b) Plot showing nuclear:cytoplasmic DAF-16 ratios calculated from fluorescence microscopy images. All calculations were done using ImageJ from a total of at least 15 nuclei per genotype.

4.3.16 Δ_4-DA supplementation in post-reproductive worms is sufficient to restore lysosomal acidity and nuclear DAF-16 localization

Extending reproduction appears to delay loss of lysosome acidity, presumably by maintaining active DAF-9/DAF-12-mediated signalling for longer periods (see above). This raises the question of whether this signalling could be re-activated altogether after animals have stopped reproducing and lysosome alkalinization in the intestine started. To investigate this possibility, post-reproductive worms (day 5) were supplemented with Δ_4-DA for 48 hours and cDCFDA signal intensity was assessed a day after. Surprisingly, these worms were able to respond to Δ_4-DA supplementation regimen and showed cDCFDA signals in the intestine consistent with acidified lysosomes at concentrations of 10 and 100 μM Δ_4-DA (but not 1 μM Δ_4-DA) (Figure 4.43). To investigate whether a continuous feeding of Δ_4-DA would similarly alleviate lysosomal alkalinization, N2 worms were fed Δ_4-DA until day 7 of adulthood following which cDCFDA signal and DAF-16 localization was assessed a day after (Figure 4.44 and 4.45). Not surprisingly, worms that were fed continuously with Δ_4-DA until day 7 of adulthood did not show the same dramatic decline in lysosomal pH as control worms and they retained a higher amount of DAF-16 in the nucleus compared to control worms. The results above indicate that the regulatory program driving lysosomal acidification can be maintained (or even reactivated) when the original endogenous signal is depleted.

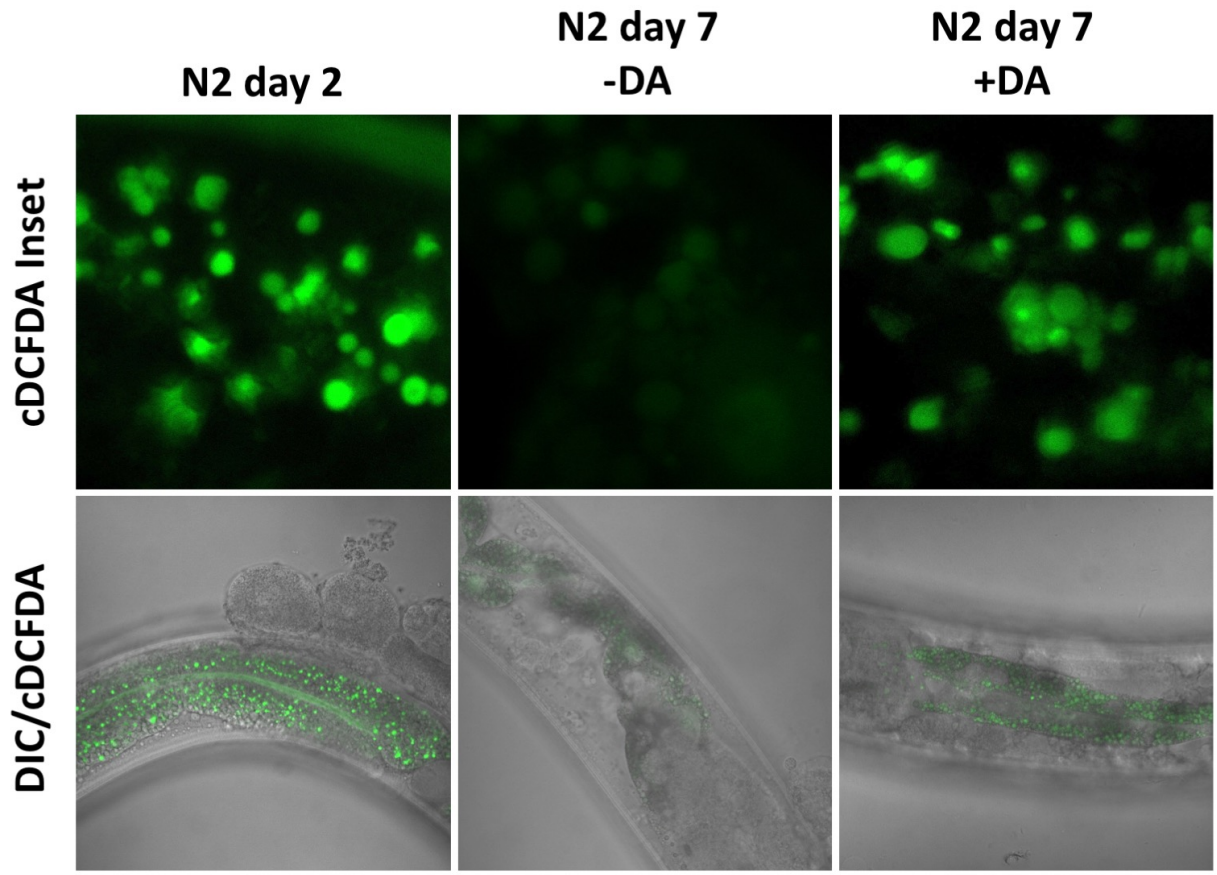


Figure 4.43: Δ_4-DA supplementation in post-reproductive worms recapitulates lysosomal pH homeostasis.

Wild type worms were grown on OP50 food until cessation of reproduction (day 5) following which they were transferred to OP50 food supplemented with ethanol (control) or 1, 10 or 100 μM Δ_4-DA for 48 hours. Thereafter, worms were fed cDCFDA supplemented OP50 food containing either ethanol (control) or varying concentration of Δ_4-DA and analyzed by confocal microscopy.

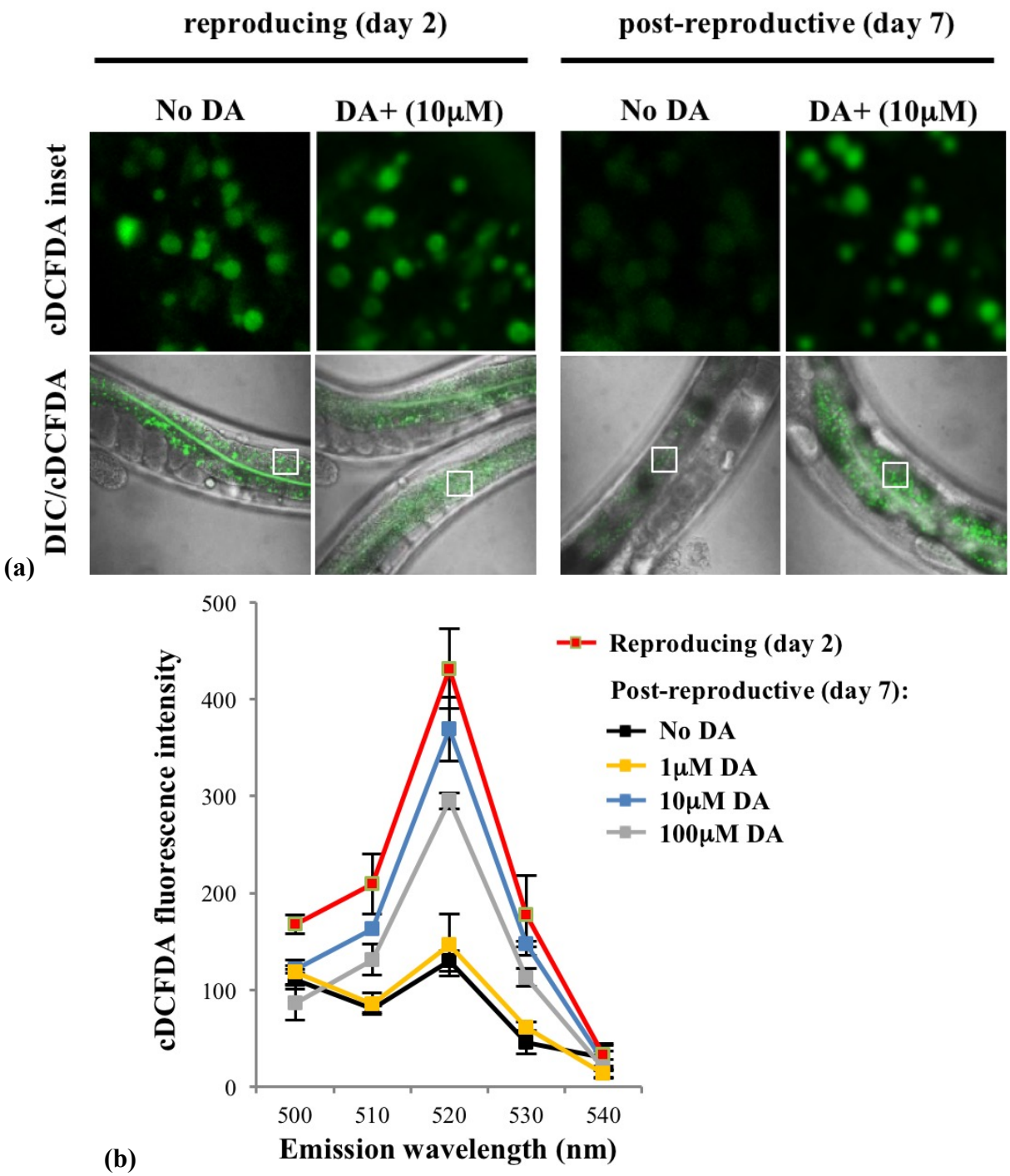


Figure 4.44: Δ_4-DA supplementation maintains lysosomal pH homeostasis.
Wild type worms were grown on OP50 food supplemented with ethanol (control) or 1, 10 or 100 µM Δ_4-DA until day 7. Thereafter, worms were imaged via confocal microscopy. (a) Confocal microscopy of Δ_4-DA (or control) fed worms after cDCFDA staining. (b) Relative fluorescence intensity plot of Δ_4-DA fed and control fed worms. Relative fluorescence intensities were calculated from fluorescence intensity values of at least 5 lysosomes from 5 different worms.

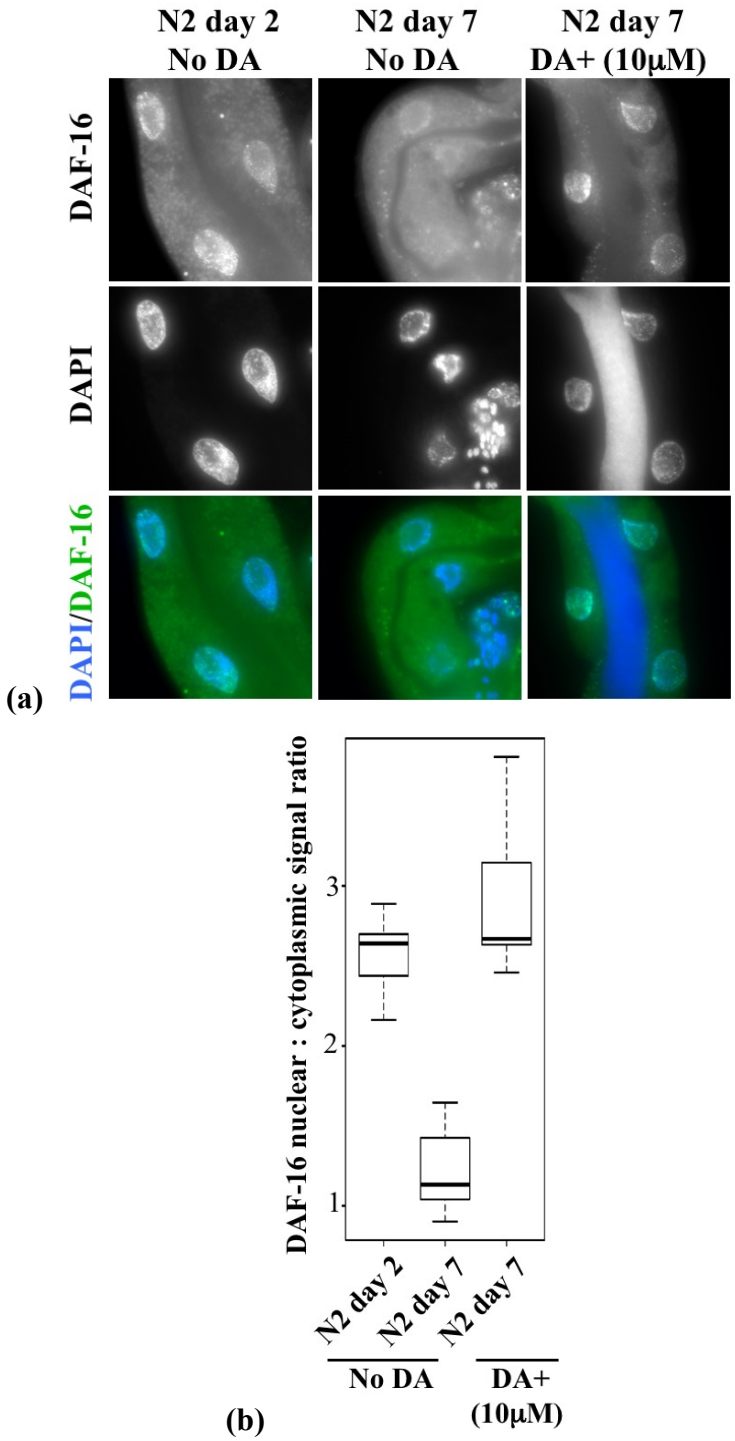


Figure 4.45: Δ_4-DA supplementation in post-reproductive worms results in nuclear localization of DAF-16.

Wild type worms were grown on OP50 food supplemented with ethanol (control) or 10 μM Δ_4-DA for 48 hours. Thereafter, worms were dissected and immunostained with anti-DAF-16 antibody and analyzed via fluorescence microscopy. (a) Fluorescence microscopy images of 7-day old wild type worms fed either OP50 + ethanol or OP50 + 10 μM Δ_4-DA stained with cDCFDA. (b) Plot showing nuclear:cytoplasmic DAF-16 ratios calculated from fluorescence microscopy images. All calculations were done using ImageJ from a total of at least 15 nuclei per genotype.

4.4 Discussion

An important question in biology is the interplay between reproduction and aging and how the production of progeny affects longevity. So far, various studies have looked into elucidating the pathways for longevity in sterile mutants, but this study is one of the few that attempts to provide a connection between progeny production and somatic aging in actively reproducing (wild type) *C. elegans*. My results show that lysosomal alkalinization via downregulation of *vha* genes may serve as a novel biomarker of aging in worms and offer a quantifiable measure of somatic fitness. The results presented in this chapter suggest that the loss of acidity in lysosomes is not a stochastic event, but rather coupled to the reproductive cycle of *C. elegans*. Genetic or direct interventions that alter the length of reproduction (by using sterile alleles or mating, for example) can modulate this signal and impact lifespan. In this context, reproduction can be perceived as another biological timer that signals the decline of somatic proteostasis once progeny generation is no longer possible. By using sterile *fem-2* worms, I show that this reproductive timer can be bypassed to prolong somatic proteostasis, indicating that reproduction (as a timer) has to start in order for its end to be sensed (Figure 4.46). Mating *fem-2* worms restores sperm supply, re-initiates fertilization and reconstitutes the reproductive timer. As such, mated *fem-2* worms can again signal the end of reproduction and lysosomes are alkalinized as in wild type. On the other hand, extending slightly the window of reproduction by providing additional sperm to reproducing hermaphrodites re-sets the reproductive timer and delays senescence. Taken together, these results support the hypothesis that reproduction and somatic proteostasis evolved a complex molecular cross talk that aims at ensuring that fitness of the soma is not compromised during time of progeny generation.

This study implicates DAF-16 as the downstream regulator of *vha* gene expression that responds to cues from the germline. DAF-9 and DAF-12 mediate this connection upstream of DAF-16 (diagrammatic illustration in Figure 4.47). There are still several questions about this proposed pathway that remain unanswered. For instance, do *daf-9* larvae have alkalinized lysosomes or does lysosomal alkalinization in these mutants occur after reaching adulthood (post L4)? Furthermore, what is the molecular mechanism that senses reproductive decline in the gonad? Further work is needed to understand what signals the end of reproduction and how this signal is transmitted across the gonad to the intestinal cells.

Besides the *vha* genes, DAF-16 has been shown to regulate the expression of numerous genes directly or indirectly involved in mediating longevity and stress resistance in worms. These

include genes that function in epigenetic regulation of gene expression (Jin et al., 2011), mitochondrial respiration (Felkai et al., 1999), lipid metabolism (Lapierre et al., 2011), antioxidant and stress response (Murphy, 2006), protein folding and degradation (Oh et al., 2006), and innate immunity (Murphy et al., 2003). The nuclear exclusion of DAF-16 following reproductive decline may therefore influence other processes in the soma that could either directly or indirectly contribute to aging in post-reproductive worms.

One of the other overarching questions in this study that is yet to be answered is how exactly lysosomal alkalinization promotes aging. One possible answer could be the progressive accumulation of unprocessed "age-pigments" such as lipofuscin within the lysosomes that interfere with normal hydrolysis. This would eventually result in the functional collapse of these organelles and ultimately cell death (Gerland et al., 2004; Yin, 1996)

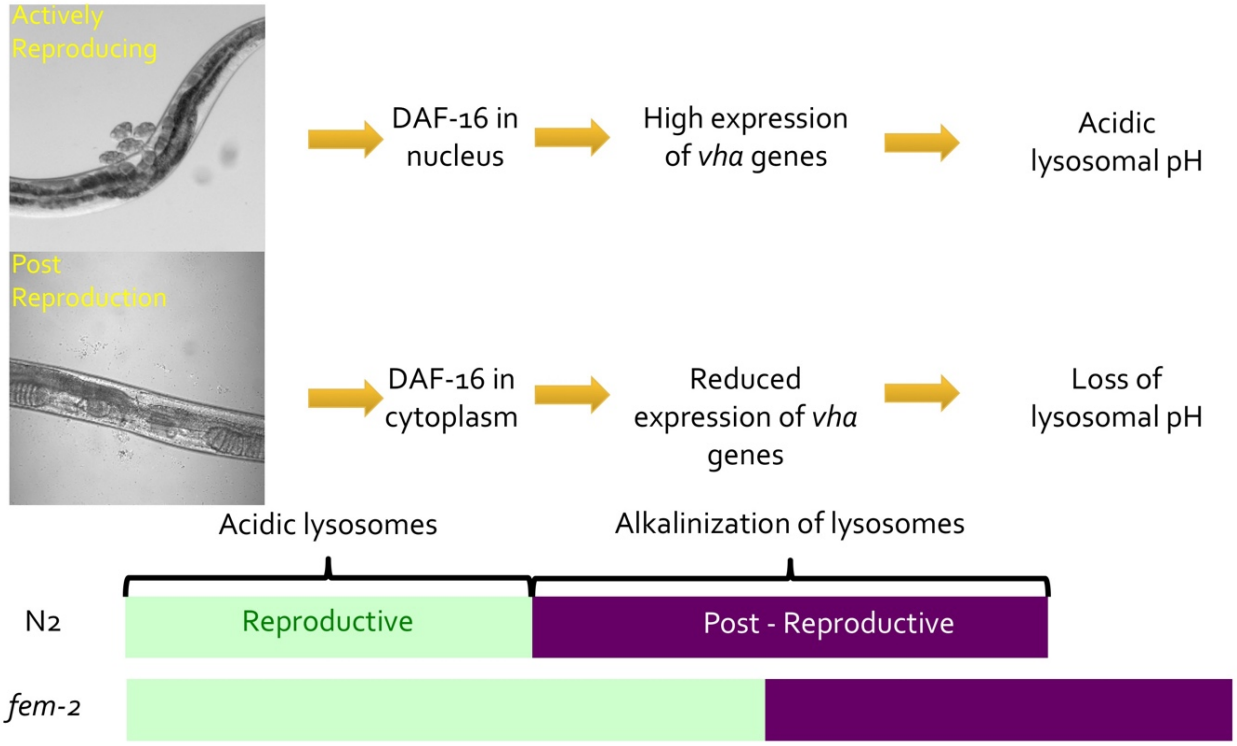


Figure 4.46: Model showing the connection between reproduction and lysosomal homeostasis.

In actively reproducing worms, DAF-16 translocates to the nucleus and drives expression of *vha* genes thereby maintaining acidic lysosomal pH. Once worms cease reproducing, nuclear exclusion of DAF-16 leads to a global reduction of *vha* gene expression and the resulting inability to actively maintain a low pH in the lumen of lysosomes.

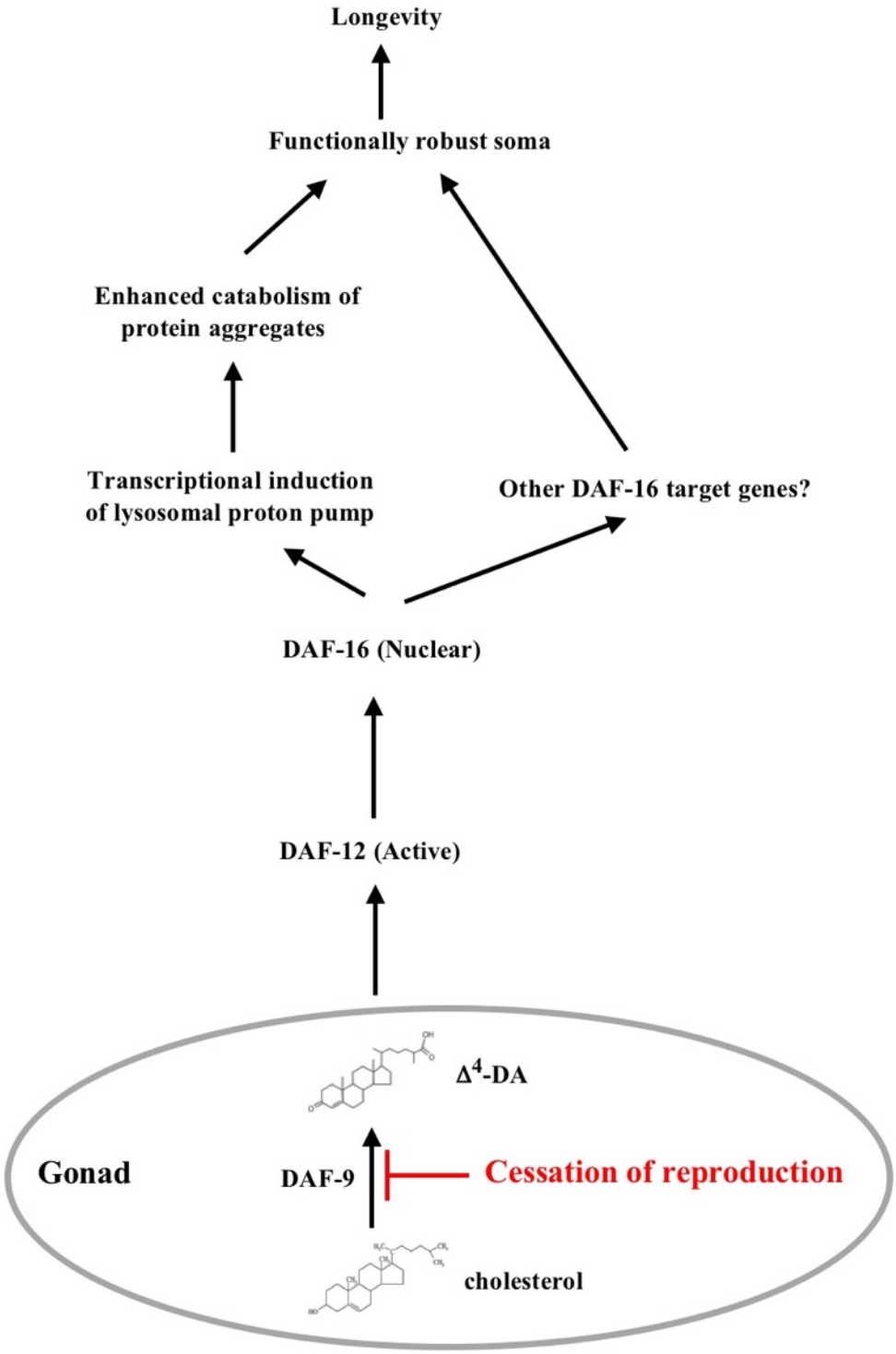


Figure 4.47: Diagrammatic illustration of the hormonal signalling pathway that links reproduction to lifespan and proteostasis.
In actively reproducing wild type worms, DAF-9 levels are adequately maintained, thereby leading to production of Δ_4-DA that in turn activates DAF-16 via a DAF-12 dependent pathway and leads to transcription activation of *vha* gene subunits. Once reproduction ceases (or in *daf-9* worms), production of Δ_4-DA is impaired, leading to inactivation of DAF-16 which leads to progressive downregulation of *vha* gene subunits, thereby leading to lysosomal alkalinization.

5 A TARGETED GENETIC SCREEN FOR PROBING GENES INVOLVED IN LYSOSOMAL pH HOMEOSTASIS

5.1 Background

In the previous chapter, I defined the relationship between progeny production and somatic aging in normally reproducing (wild type) *C. elegans*. I identified the collapse of lysosomal acidity as a novel biomarker of aging in *C. elegans* and provided evidence to show that lysosomal pH is actively maintained by a signalling cascade that has been previously implicated in mediating the cross talk between germline and soma. Changes in lysosomal pH depend on the levels of *vha* gene expression which in turn respond to the levels of the *C. elegans* FOXO transcription factor DAF-16 present in the nucleus. This body of evidence supports the notion that the functional collapse of lysosomes is not an *ad hoc* consequence of aging and is instead genetically programmed and timed with the cessation of reproduction. A possible molecular pathway to regulate v-ATPase loading on intestinal lysosomes emerged from these findings and is corroborated partially from published literature: DAF-9 in the cells of the somatic gonad is activated to produce Δ_4-DA by an unknown signal released upon fertilization, likely from the sperm. Δ_4-DA, a small lipophilic ligand, is in turn released into the pseudocoelom. Δ_4-DA could then easily reach the intestine since it is in direct contact with the gonad in *C. elegans*. Inside intestinal cells, Δ_4-DA presumably binds and activates its receptor DAF-12. Activated DAF-12 in the nucleus would then act upon genes that ultimately promote DAF-16 nuclear translocation where *vha* gene expression would be finally upregulated. As the upstream reproductive signal dwindles with the exhaustion of sperm supply, DAF-9 activity is reduced in the somatic gonad and the pathway is shut off, leading to alkalinization of lysosomes in the intestine.

This multi-tissue signalling must have several other nodes still uncharacterized. Likewise, it is possible that other pathways regulate lysosomal pH independently of DAF-9/DAF-12/DAF-16, and that these too are aligned with the reproductive outputs somehow (or not). To identify genes encoding proteins playing a role in the regulation of lysosomal pH, I have taken a targeted genetic screen approach. To do so, I undertook a comprehensive literature analysis to select candidate genes that when inactivated would:

a) Disrupt the signalling from the germline to the soma by interfering with cholesterol metabolism.

b) Disrupt one or more metabolic processes that impact lifespan/reproduction.

c) Impair the ability of the germline to correctly sense reproduction.

I grouped these candidate genes into the following 7 test groups:

1) Genes that impact the hormonal signalling pathway (*daf-36*)
2) Genes that influence cholesterol homeostasis (*nhr-8*)
3) Genes that might be involved in germline to soma signalling
4) Genes involved in lipid metabolism
5) Genes involved in the dauer pathway
6) Genes that are responsible for nutrition sensing/metabolism
7) Genes that may be involved in modulation of DAF-16 targets

Using either hypomorphs or RNAi treatment, I tested each of the genes that I had selected for either premature or delayed loss of lysosomal pH. Section 5.2 provides a brief functional overview of the gene tested, along with a rationale of why it was selected for the genetic screen.

5.2 Rationale and results

1) Hormonal signalling (*daf-36*)

In germline-less *C. elegans*, steroid hormone signalling controls lifespan, a process that depends on the somatic gonad, the nuclear hormone receptor DAF-12, the FOXO transcription factor DAF-16 as well as the DA biosynthetic pathway components DAF-9 and DAF-36 (Gerisch et al., 2007; Hsin et al., 1999; Rottiers et al., 2006). Germline-less *C. elegans* worms have been shown to live longer but this longevity is abrogated when both the germline and somatic gonad are abrogated via genetic or surgical means (Hsin et al., 1999). Worms lacking both the somatic gonad and germline live longer if they are supplemented with exogenous Δ_4-dafachronic acid, and this longevity requires both DAF-12 and DAF-16 (Yamawaki et al., 2010). These results suggest that dafachronic acids (DAs) are produced in response to germline ablation, either in the somatic gonad or in other tissues via signals mediated by the somatic gonad. DAF-36 is an enzyme required for the first step in cholesterol metabolism that feeds into the Δ_7-DA biosynthetic pathway. As such, *daf-36* likely interferes with the lysosomal acidification pathway at the level of Δ_4–DA production. I analyzed a *daf-36* mutant (*daf-36(k114)* (Yoshiyama-Yanagawa et al., 2011)) for premature loss of lysosomal pH in this study.

Confocal microscopy showed that *daf-36* mutants, like *daf-9*, *daf-12* RNAi and *daf-16* mutants (this study), display premature loss of lysosomal acidity early on at day 2 of adulthood. These worms show a cDCFDA staining pattern that is reminiscent of day 8 wild type worms (Figure 5.1). It is interesting to note that lysosomes in *daf-36* mutants do not actually display the enlargement that was seen in day 8 wild type or day 2 *vha* RNAi worms.

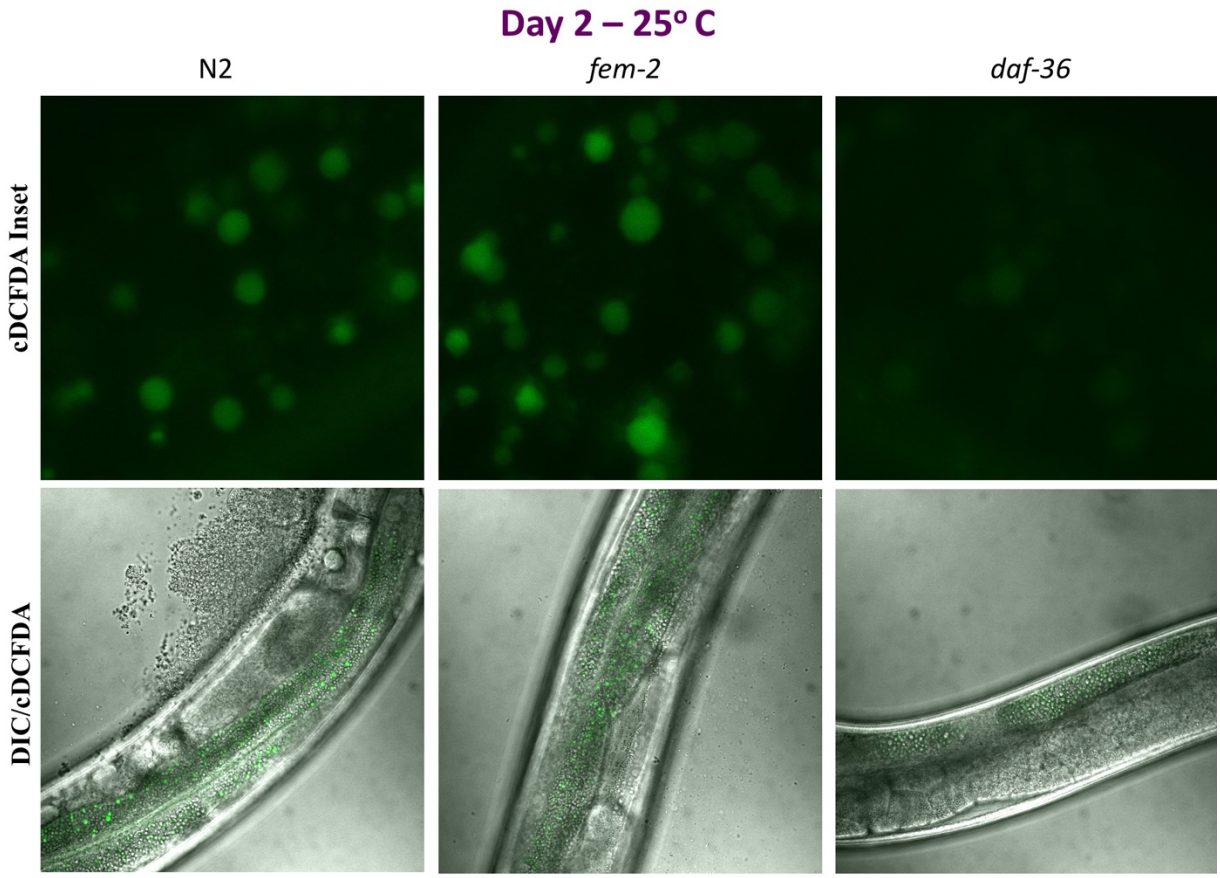


Figure 5.1: Lysosomes in *daf-36* mutants show premature alkalinization.
daf-36 mutants were grown at 25° C until day 2 post L4 and then transferred to OP50 food supplemented with cDCFDA overnight. Worms were imaged the next day via confocal microscopy.

2) Cholesterol homeostasis (*nhr-8*)

The *C. elegans* NHR-8 nuclear hormone receptor has been shown to regulate cholesterol balance and bile acid metabolism. Loss of *nhr-8* results in a deficiency of DA synthesis and leads to reduced lifespan. These phenotypes have been shown to be alleviated following cholesterol supplementation (Magner et al., 2013). Given its role in cholesterol homeostasis and biosynthesis of dafachronic acids, an *nhr-8* mutant was tested in this study to assay for perturbations in lysosomal pH. Figure 5.2 shows that similar to a *daf-36* or *daf-9* mutant, the *nhr-8* mutant shows premature lysosomal alkalinization, suggesting that inhibiting DA biosynthesis by limiting the availability cholesterol presumably disrupts the germline to soma signalling.

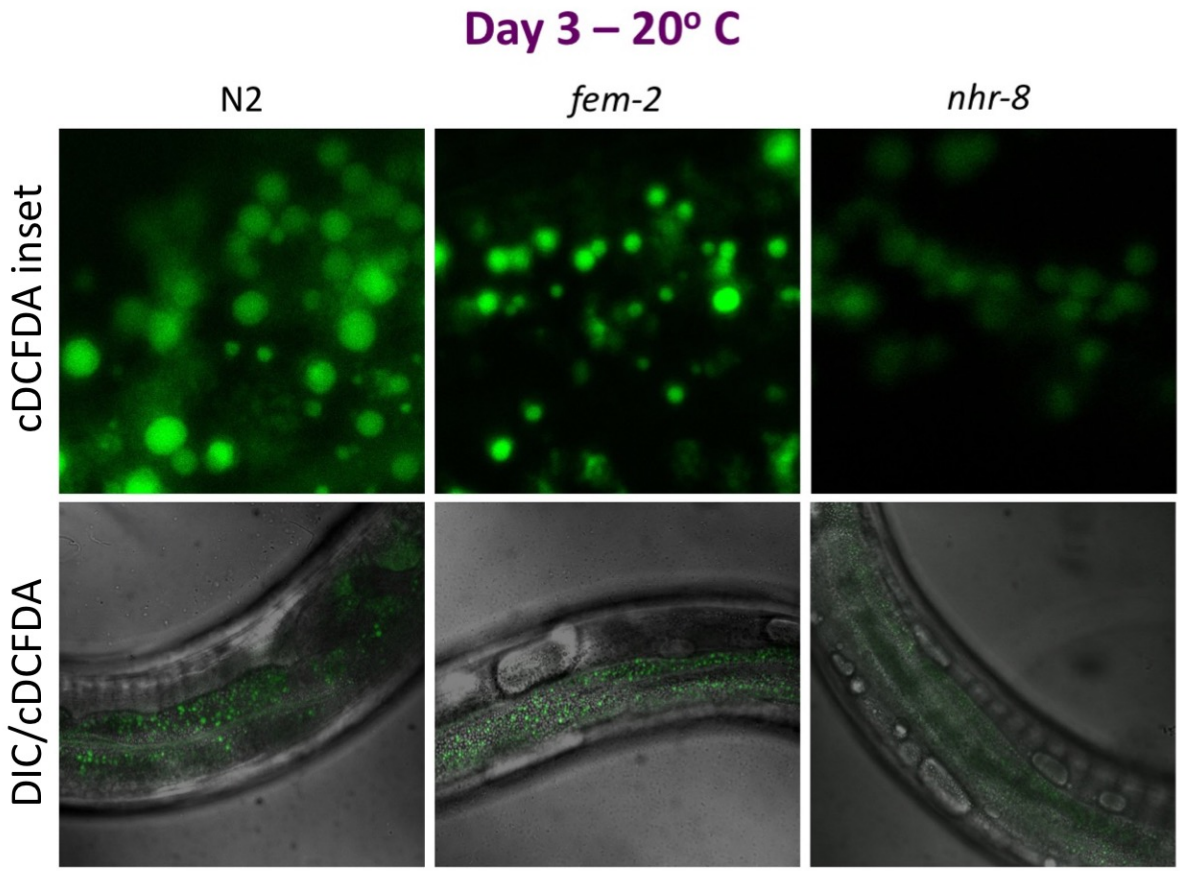


Figure 5.2: *nhr-8* mutants show premature lysosomal alkalinization.
nhr-8 mutants were grown at 20° C and transferred to OP50 supplemented with cDCFDA on the days indicated (above) and imaged via confocal microscopy the next day.

3) Germline signalling (*air-2* and *emb-30*)

Results in chapter 2 revealed that progeny production is necessary for timely lysosomal alkalinization to ensue in wild type intestinal cells after the end of reproduction. The presence of germ cells (oocytes or sperm) alone does not seem to be sufficient for this change, which suggested a requirement for fertilization in the germline. Fertilization is a complex process that involves sperm fusion leading to several physiological alterations that ultimately leads to egg activation and pronuclei fusion. In theory, any of these early zygotic events prior to the formation of the egg shell happening while the embryo is still inside the uterus could be the source of the secreted signal molecule. I therefore sought to determine the window of time in which embryos must advance to trigger the lysosomal alkalinization program in the intestine (presumably because a signal was successfully made and exported) to narrow down the possible process required. As a first attempt, I chose to arrest development in two critical points; early on in metaphase I of meiosis (Anaphase-promoting complex (APC)/*emb-30*) and the other after pronuclear fusion during the first mitotic metaphase (AuroraB/*air-2*). I tested two temperature-sensitive mutants (*emb-30(tn377ts)* and *air-2(or207ts))* that once grown at the restrictive temperature produced non-viable embryos. Importantly, neither prevents sperm entry or fertilization. *emb-30* is required for metaphase to anaphase transition during meiosis and its depletion causes failure to extrude polar bodies, thereby leading the 1-cell embryo to arrest soon after fertilization but prior to pronuclear fusion (Furuta et al., 2000). *air-2,* on the other hand, is primarily involved in chromosome segregation. The *or207* allele of *air-2* specifically disrupts chromosome segregation in mitosis, while disjunction in meiosis I and II are not affected. Thus, I used *or207* to arrest embryos later in the metaphase of the first mitotic division in the embryo (Severson et al., 2000). Synchronized worms were shifted to 25ºC at L4 and exposed to cDCFDA feeding protocol as young adults. As seen in Figure 5.3, the dynamics of lysosomal acidification in reproducing worms was not affected in these mutants, consistent with the interpretation that the ensuing arrests occurred after the release of the putative signal molecule in the gonad. Thus, the germline signal regulating intestinal lysosome pH is produced/released after sperm entry and before pronuclei fusion.

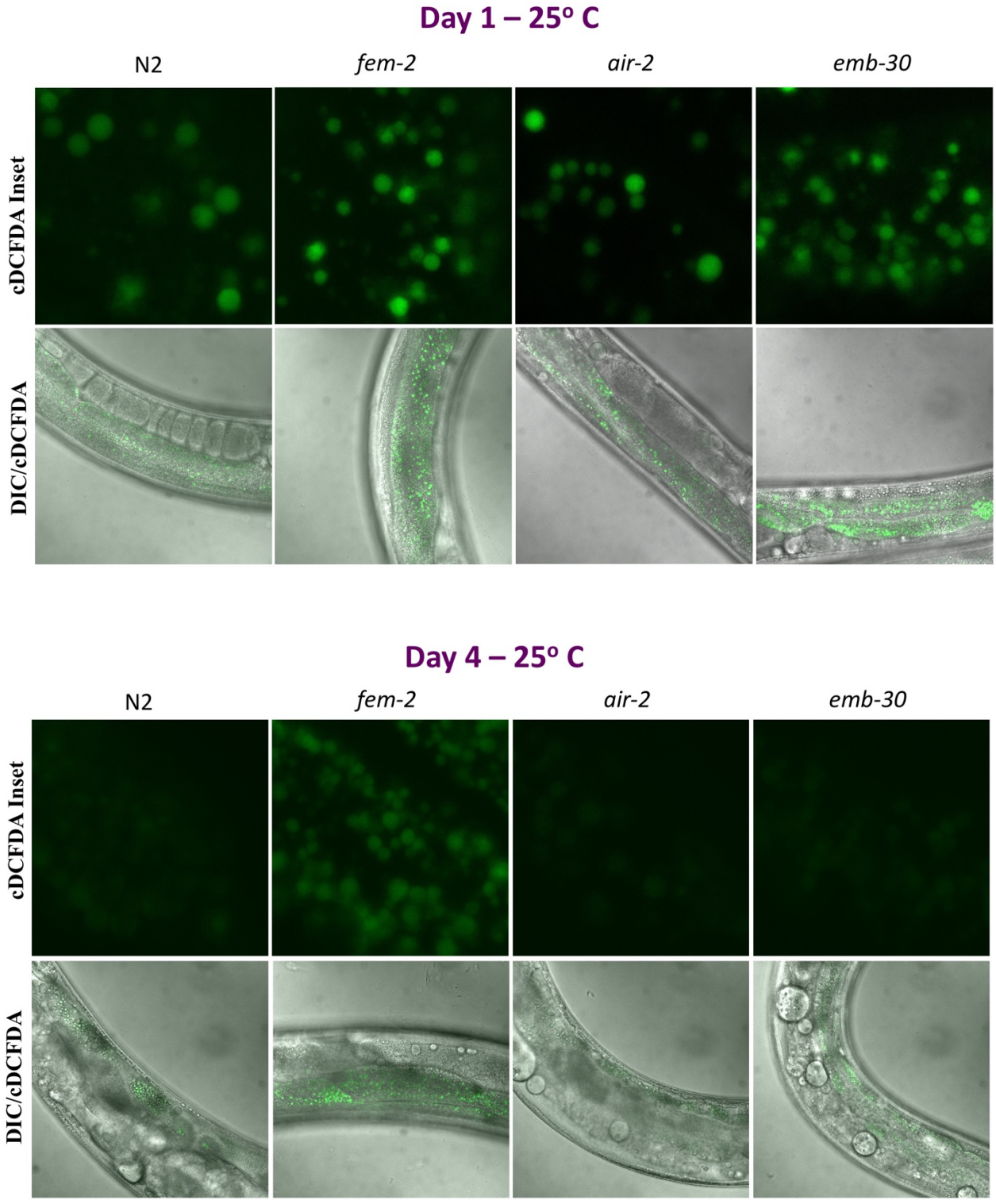


Figure 5.3: *air-2* and *emb-30* mimic wild type lysosomal pH homeostasis.
Wild type, *fem-2, air-2* and *emb-30* mutants were grown at 25° C and transferred to cDCFDA on the days indicated (above) and imaged via confocal microscopy the next day.

4) Lipid metabolism (*hlh-30, lipl-4* and *nhr-80*)

A major factor in determining lifespan in several long-lived *C. elegans* mutants is the process of intracellular recycling mediated by autophagy (Lapierre & Hansen, 2012). This is a multistep process that sequesters and transports material to be degraded into a compartment called an autophagosome, which then fuses to a lysosome that ultimately breaks down all the cargo by means of lysosomal proteases and lipases (Gelino et al., 2012; He et al., 2009). One important aspect of autophagy that has recently been shown to impinge upon longevity is the process of lipophagy, where lipids are broken down via the autophagy lysosome pathway (Lapierre et al., 2011; Lapierre, Melendez, et al., 2012). Given the importance of prolonged lysosomal pH homeostasis in *fem-2* worms and its role in longevity, I sought to ascertain whether perturbations in lipid metabolism would alter the lysosomal pH profile in *fem-2* worms. To this end, I selected three genes that have been implicated in lipid metabolism in *C. elegans* and used an RNAi based approach to test whether knockdown of these three genes would change the dynamics of lysosomal pH in young *fem-2* worms (i.e. prematurely alkylate lysosomes).

hlh-30 is the *C. elegans* homolog of the mammalian transcription factor EB (TFEB) that has been implicated in regulating autophagy in mammals (Settembre et al., 2011). HLH-30/TFEB has been shown to regulate genes that are responsible for lipid metabolism and autophagy, thereby suggesting that under nutrient stress, survival of the animal relies on the rigorous coordination between lipid metabolism and autophagy pathways. (O'Rourke et al., 2013; Settembre et al., 2013). In mammalian systems TFEB has been shown to translocate to the nucleus in response to starvation, partly via means of a TOR-dependent mechanism, to induce the expression of various genes involved in autophagy and lysosome function (Roczniak-Ferguson et al., 2012; Settembre et al., 2012). In *C. elegans, hlh-30* was recently shown to be required for the enhanced lifespan of germline-less and TOR-mediated longevity, while its mammalian counterpart TFEB was found to be activated in dietary restricted mice, a known longevity regimen (Lapierre et al., 2013; Settembre et al., 2013).

The *lipl-4* gene is highly conserved between nematodes to humans and encodes a triglyceride lipase. Depletion of *lipl-4* in germline-less worms abolishes their extended lifespan while overexpression of *lipl-4* is sufficient to increase lifespan of wild type worms (Lapierre et al., 2011; M. C. Wang et al., 2008).

It was recently shown that signals from the lysosome activate nuclear hormone receptor NHR-80 via a LIPL-4-dependent mechanism, thereby promoting longevity (Folick et al., 2015). This implicates a direct role of lysosomes in regulating transcription with obvious important consequences. In *C. elegans, nhr-80* is one of multiple homologs of HNF4-like nuclear hormone receptors. In mammals, the HNF4-like nuclear hormone receptor regulates fat metabolism and glucose homeostasis (Brock et al., 2006). NHR-80 is activated in germline-less *C. elegans* and is one of the regulators of longevity of such animals. Overexpression of *nhr-80* leads to extended lifespan, and this increase in lifespan requires the nuclear receptor DAF-12 but not the FOXO transcription factor DAF-16 (Goudeau et al., 2011). Loss of NHR-80 does not impact lifespan in wild type nematodes, or long-lived mutants with reduced insulin/IGF-1 signalling nor the lifespan extension in dietary restricted animals (Goudeau et al., 2011).

In order to determine if knockdown of *hlh-30, lipl-4* or *nhr-80* would affect lysosomal pH of young *fem-2* worms, I fed *fem-2* worms with RNAi food and assessed changes in lysosomal pH after day 4 of adulthood. As shown in Figure 5.4 below, none of the RNAi treatments resulted in premature lysosomal alkalinization in *fem-2* worms.

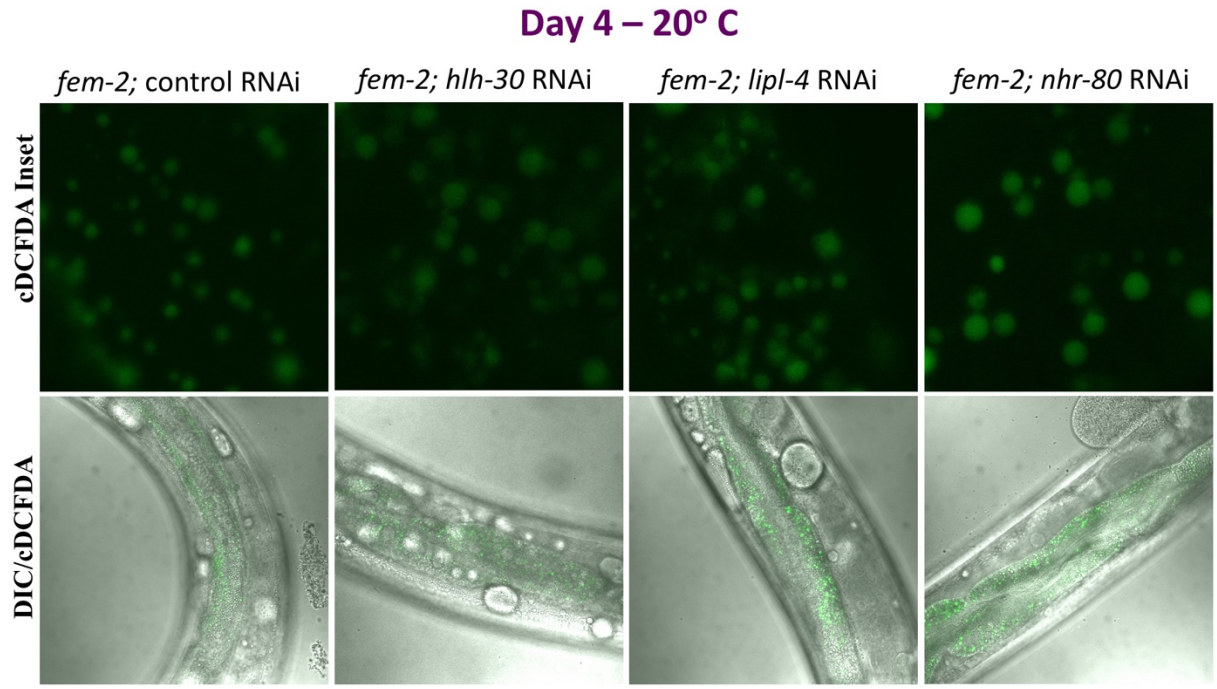


Figure 5.4: *hlh-30, lipl-4* and *nhr-80* do not affect lysosomal pH homeostasis in *fem-2* worms.

fem-2 mutants were grown at 25° C until L4 and then transferred to RNAi food on day 1 of adulthood. Worms were grown at 20° C until day 4 of adulthood after which they were fed RNAi food supplemented with cDCFDA and imaged via confocal microscopy the next day.

5) Dauer pathway (*daf-8, daf-28* and *daf-5*)

In *C. elegans*, the dauer stage is a developmentally arrested larval stage that is highly resistant to nutritional starvation as well as thermal stress. The genetic programs controlling dauer development versus reproductive development act antagonistically. This dichotomy in fate decisions influences lifespan in opposite ways and could play a role in the regulation of lysosomal function in the adult. For example, mutations in genes that prolong the reproductive phase or delay the onset of reproduction would be expected to force lysosomal pH to remain acidic for a much longer duration. On the other hand, mutations that shorten the reproductive phase or accelerate the cessation of reproduction would lead to a much faster lysosomal alkalinization.

The decision to enter dauer stage is triggered by signals from sensory neurons that analyze and respond to environmental cues, including a dauer pheromone (Golden et al., 1982, 1984). Although food availability and temperature are both factors affecting dauer formation, the dauer pheromone is the most potent regulator of dauer fate decision, and can override the sensory input of low temperature and high food concentration, both of which trigger reproductive development and repress dauer entry (W. Li et al., 2003). The DAF-2/insulin signalling pathway and the DAF-7/TGF-ß signalling pathway mediate signals that regulate dauer formation. *daf-28* encodes an insulin-like protein that when inactivated results in dauer arrest and downregulation of DAF-2 signalling. The role of *daf-28* is therefore to promote reproductive growth. The allele used in this study *daf-28(sa191)* was isolated in a genetic screen for mutants that arrest at the dauer stage (although this particular allele is unique in that the dauers recover within 2 hours) and shows 10-13% increased lifespan, (Malone et al., 1994). The *daf-28(sa191)* allele also causes a slight but statistically significant increase in lifespan (Malone et al., 1996).

The *daf-5* and *daf-8* genes have opposite roles in *C. elegans* development and lifespan. While DAF-5 promotes dauer formation (via interaction with DAF-3), DAF-8 represses it. The *daf-8* and *daf-5* alleles used in this study have opposite effects on *C. elegans* lifespan – while *daf-8(e1393)* mutants have slightly longer lifespan than wild type, *daf-5(e1386)* have slightly shorter lifespan than wild type. DAF-7 promotes continuous, non-dauer development via interaction with the DAF-1 and DAF-4 receptors which then transduce the signal via DAF-8 and DAF-14. When DAF-8 and DAF-14 are activated, they (redundantly) inhibit DAF-3 and DAF-5 transcription factors, both of which promote dauer development. Both the *daf-8(e1393)* and *daf-5(e1386)* (temperature sensitive) alleles used in this study were chosen because of their weak daf-c (dauer-

constitutive) and daf-d (dauer defective) phenotypes respectively at 20^{o} C, hence were ideal for the purpose of this study.

As shown in Figure 5.5, none of the *daf* genes tested showed any variation in lysosomal pH homeostasis, and all of them behaved similar to wild type.

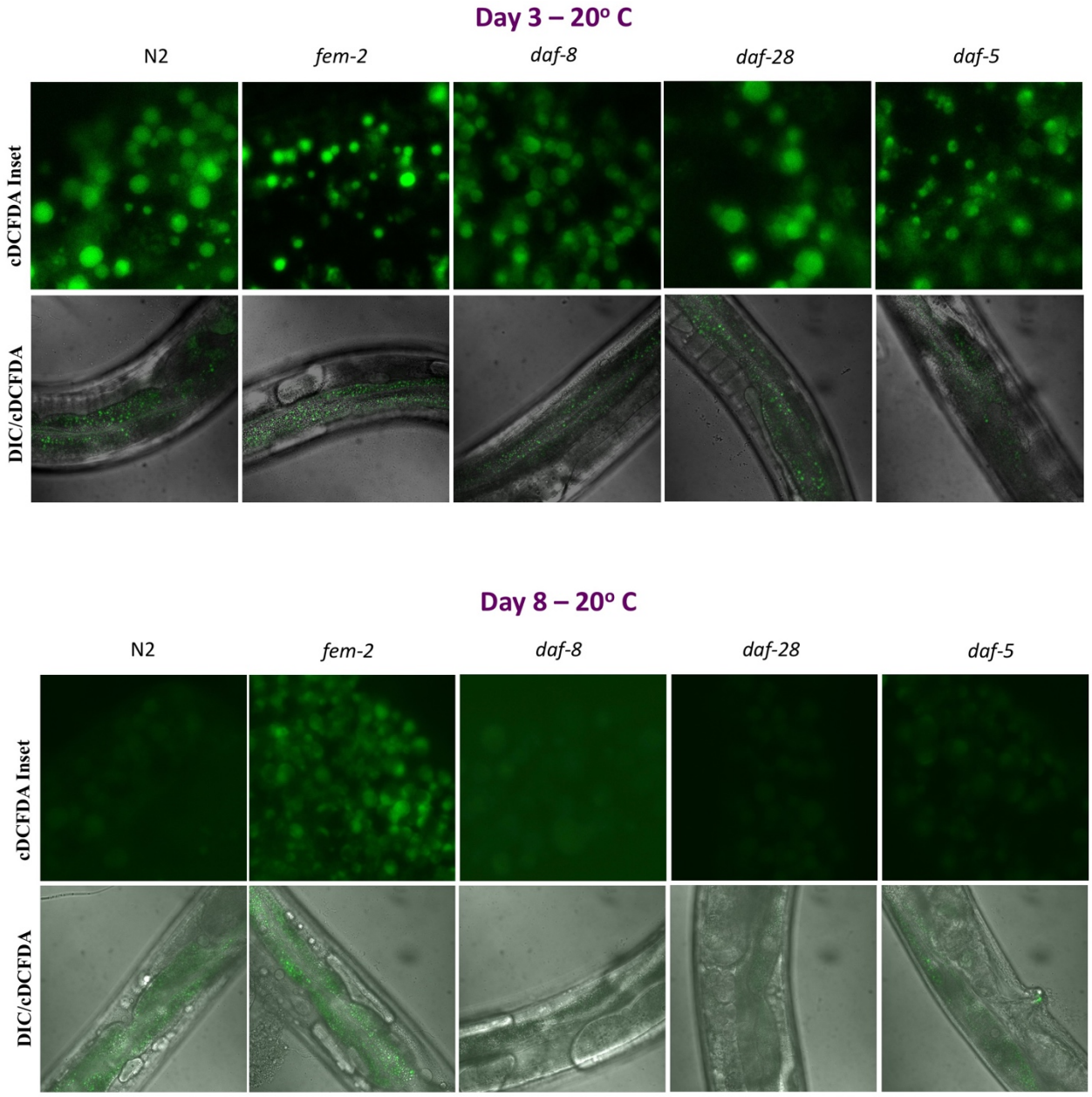


Figure 5.5: Lysosomal pH homeostasis is not affected by mutations in the *daf* genes.
daf-8, daf-28 and *daf-5* mutants were grown at 20° C until L4 and transferred to OP50 supplemented with cDCFDA on the days indicated (above) and imaged via confocal microscopy the next day.

6) Nutrition sensing pathways (*nhr-49 and pqm-1*)

One of the factors that presumably plays a major role in *C. elegans* development and reproduction is the availability of nutrition and the physiological process of sensing nutrition levels in the environment. The two gene candidates that I selected in this genetic screen, *nhr-49* and *pqm-1*, have been implicated in signalling pathways downstream of nutrition sensing and ultimately regulate global metabolism and stress response genes (Burkewitz et al., 2015).

In germline-less *C. elegans,* the nuclear hormone receptor NHR-49 plays a role in lifespan extension by upregulating genes involved in fatty-acid desaturation and mitochondrial ß-oxidation. The coordinated upregulation of these two processes is crucial for worms lacking germline to sustain lipid synthesis as well as maintaining lipid reserves (Ratnappan et al., 2014). The expression of NHR-49 is upregulated in somatic cells of germline-less animals by DAF-16 and TCER-1, although overexpression of NHR-49 in fertile *C. elegans* only leads to a very modest increase in lifespan (Ratnappan et al., 2016). The *nhr-49(nr2041)* allele used in this study harbors a deletion in part of the NHR-49 DNA binding domain (DBD) as well as more than half of its ligand binding domain (LBD), thereby making this allele a putative null mutant (Liu et al., 1999). *nhr-49(nr2041)* worms have a shorter lifespan at 23^{o} C although they do not show any delayed development or fertility phenotypes (Van Gilst et al., 2005). These results suggest that NHR-49 is presumably not required for development or fertility but is essential for maintaining normal lifespan. From these phenotypic assessments, it is unclear if the shortened life span of *nhr-49(nr2041)* is due to accelerated aging or due to other physiological defects that somehow lead to a shorter lifespan.

PQM-1, as DAF-16, is a transcription factor that modulates various aspects of stress response and development via inputs from the insulin/IGF-1 signalling (IIS) pathway. The nuclear localization of DAF-16 and PQM-1 is mutually antagonistic and is controlled in opposite ways. Loss of PQM-1 abolishes *daf-2* longevity and also slows down development (Tepper et al., 2013). The *pqm-1(ok485)* strain used in this study was generated by the *C. elegans* Gene Knockout Project at the Oklahoma Medical Research Foundation using UV/TMP mutagenesis.

Since both *pqm-1* and *nhr-49* mutants were available as hypomorphs from the Caenorhabditis Genetics Center (CGC), I decided to test these mutants directly instead of via RNAi knockdown. Hence, these two genes have been placed in a separate section rather than grouped with the RNAi treatment in a *fem-2* background. As shown in Figure 5.6, neither *pqm-1* nor *nhr-49* influenced the pH dynamics of intestinal lysosomes.

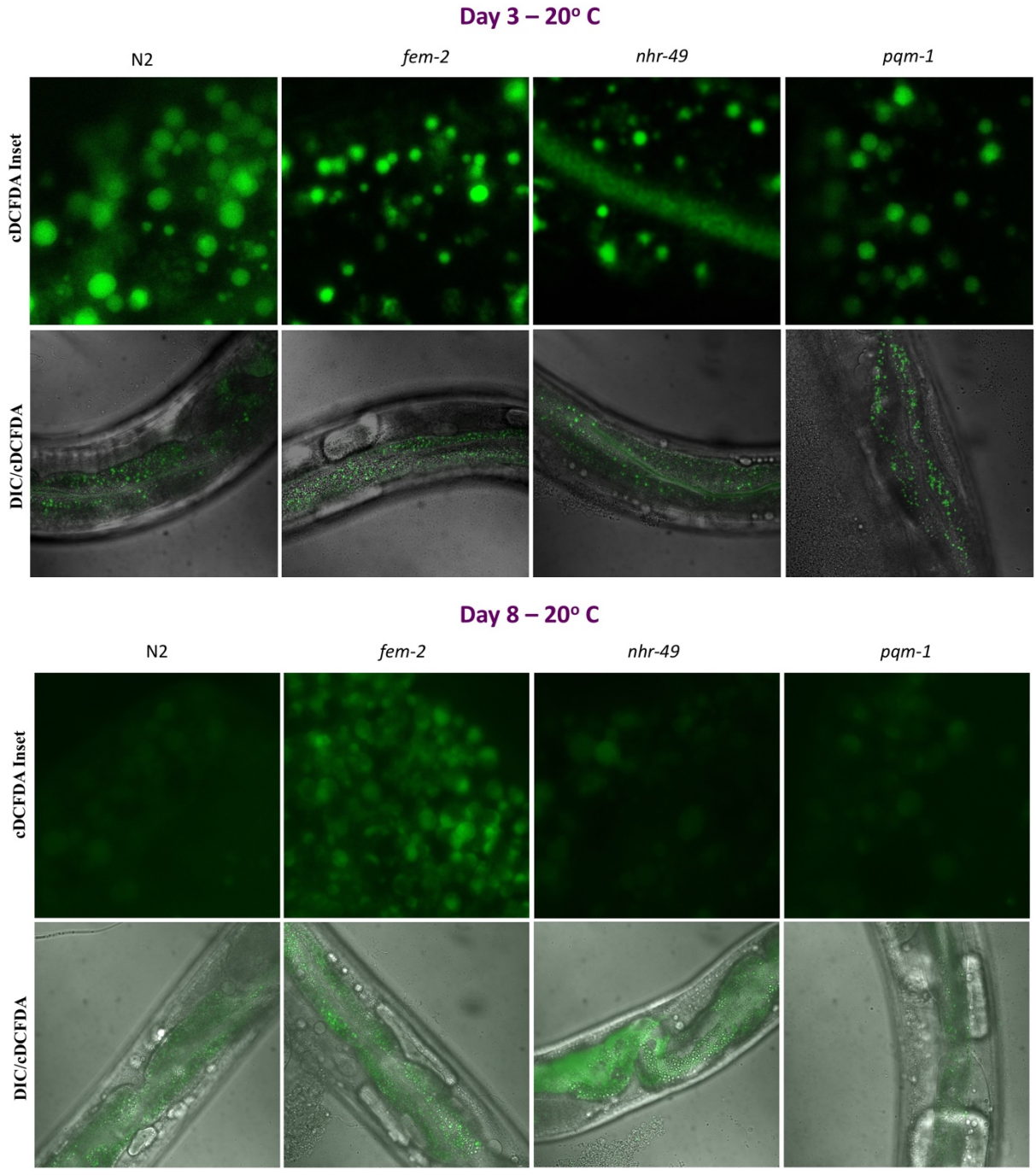


Figure 5.6: Lysosomal homeostasis is independent of sperm sensing, stress response or fat metabolism.
nhr-49 and *pqm-1* mutants were grown at 20° C until L4 and transferred to OP50 supplemented with cDCFDA on the days indicated (above) and imaged via confocal microscopy the next day.

7) Modulator of DAF-16 targets (*sgk-1*)

The *s*erum- and *g*lucocorticoid-inducible *k*inase (SGK-1) in *C. elegans* acts downstream of DAF-2 and in parallel to AKT-1/AKT-2 to mediate DAF-16 activity. SGK-1 has been shown to be able to phosphorylate DAF-16 *in vitro* while *sgk-1* RNAi leads to nuclear translocation of DAF-16, thereby extending lifespan in a DAF-16 dependent manner (Hertweck et al., 2004). However, published studies have shown that contrary to expectations, *sgk-1* null mutants have a shorter lifespan than wild type (Alam et al., 2010; Kwon et al., 2010; Soukas et al., 2009). In fact, *sgk-1* null mutants as well as gain of function mutants do not influence DAF-16 localization, but rather impact lifespan by indirectly regulating DAF-16 targets (Chen et al., 2013). In this study, I utilized a gain-of-function mutation in *sgk-1,* known as *sgk-1(ft15),* in order to determine the influence of this essential kinase on lysosomal pH in reproductive and post-reproductive worms.

As shown in Figure 5.7, *sgk-1(ft15)* failed to elicit any change in lysosomal pH dynamics with the cDCFDA staining profile was similar to wild type.

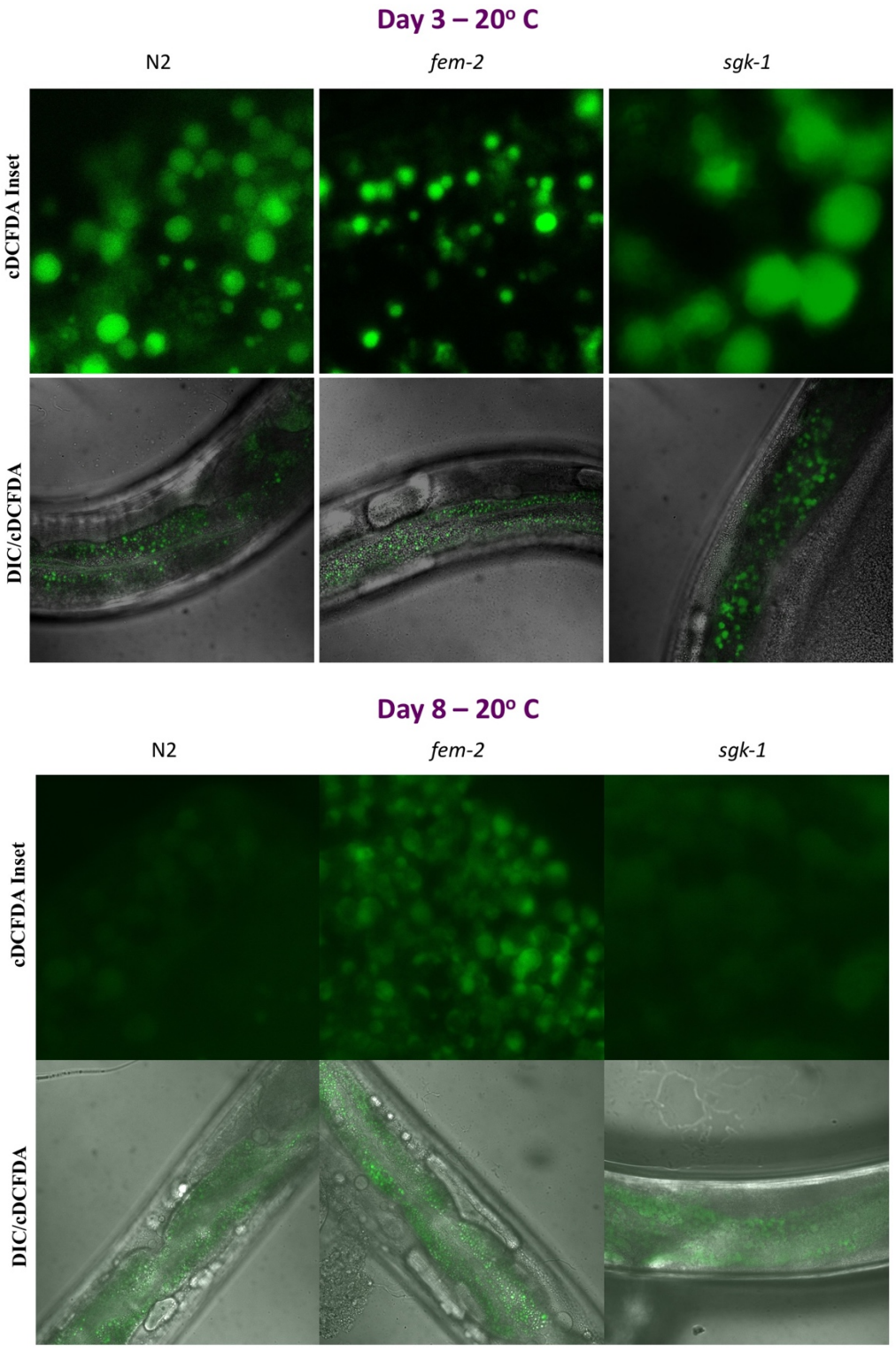


Figure 5.7: Inactivation of *sgk-1* does not affect lysosomal pH dynamics.
sgk-1 mutants were grown at 20° C until L4 and transferred to OP50 supplemented with cDCFDA on the days indicated (above) and imaged via confocal microscopy the next day.

5.3 Discussion

In this chapter, I tested different genes that are known to be involved in dauer formation, lifespan extension, cholesterol metabolism and reproduction. Consistent with its role in catalyzing the first step of cholesterol conversion, a *daf-36* mutant showed low cDCFDA staining, implying premature lysosomal alkalinization (Figure 5.1). This result is both interesting as well as intriguing, since DAF-36 has been shown to catalyze the conversion of cholesterol into 7-dehydrocholesterol, which is the first step towards biosynthesis of Δ_7-DA, **but not Δ_4-DA** (Rottiers et al., 2006; Wollam et al., 2011; Yoshiyama-Yanagawa et al., 2011). In fact, available evidence suggests that the 3-hydroxysteroid dehydrogenase HSD-1 and not DAF-36 likely catalyzes the first step in the biosynthetic pathway that converts cholesterol into Δ_4-DA. There is growing evidence that Δ_7-DA is more biologically relevant for reproduction and dauer formation than Δ_4-DA (Sharma et al., 2009). On another hand, Δ_4-DA can rescue *daf-36* mutant phenotypes, indicating that at least *in vivo,* Δ_4-DA can compensate for a reduction in Δ_7-DA levels (Gerisch et al., 2007; Rottiers et al., 2006). In studies using a metabolomics approach, Δ_4-DA could not be detected in wild type *C. elegans,* suggesting that either Δ_4-DA is produced transiently or in very little amounts, or maybe acts as a fallback mechanism when Δ_7-DA levels are perturbed (Mahanti et al., 2014). From data shown previously in this study (Chapter 4), there is no doubt that Δ_4-DA alleviates lysosomal pH decline in post-reproductive *daf-9* and wild type worms. I have not validated if Δ_7-DA supplementation would rescue impaired lysosomal pH homeostasis in *daf-36* mutants, although given the role of DAF-36 in Δ_7-DA biogenesis, it is certainly a plausible prediction.

NHR-8 is homologous to the vertebrate Vitamin D receptor group of proteins and is primarily expressed in the intestine where it controls production of DAs through regulation of cholesterol metabolism via the DAF-36/Rieske oxygenase. Consistent with this information, young *nhr-8* mutants showed quite a dramatic reduction in cDCFDA staining intensity in my genetic screen (Figure 5.2), indicating premature lysosomal alkalinization. *nhr-8* mutants display lower levels of DAF-36 and show lower levels of the cholesterol metabolite, 7-dehydrocholesterol (Magner et al., 2013). The importance of NHR-8 in regulating DA synthesis via DAF-36 transcription is highlighted by the role of DA in *C. elegans* metabolism – DA binding to DAF-12 has been shown to promote reproductive development whereas unliganded DAF-12 leads to dauer arrest (Motola et al., 2006). Although NHR-8 has been shown to regulate *daf-36* expression, the ligand that binds to it to modulate its activity has yet to be determined.

In order to zoom in on the reproductive event that is critical to sustain lysosome acidification, I tested *air-2* and *emb-30* mutants in my genetic screen. Both of these genes are required downstream of fertilization (Furuta et al., 2000; Severson et al., 2000) and both showed lysosomal pH profile similar to wild type during their lifespan (Figure 5.3). This data combined with the observation from Chapter 4 that extending reproduction by mating sustains acidified lysosomes indicates that cessation of reproduction is perceived when fertilization is no longer possible. If so, then *C. elegans* worms that display reduced brood size or early cessation of reproduction should show premature lysosome alkalinization. There in fact exist several mutations that reduce brood size, so such a prediction is testable.

I had previously shown in Chapter 4 (Figure 4.21) that *fem-2* mutants do not show increased autophagic flux compared to wild type worms. However, further tests on the contribution of *hlh-30* in maintaining acidic lysosomes in *fem-2* worms must be investigated. HLH-30 may transactivate genes in *fem-2* that could impact autophagy independently of DAF-16. For example, long-lived *glp-1(e2141)* mutants showed increased *vha-15, vha-16* and *vha-17* expression compared to wild type worms, and this increased expression of *vha* genes was downregulated in *glp-1(e2141); hlh-30(tm1978)* worms. Knockdown of *vha-16* via RNAi reduces the lifespan of long-lived *glp-1(e2141)* mutants to wild type levels (Lapierre et al., 2013). This suggests that HLH-30 might be involved in modulating lifespan in germline-less animals via other targets that could act upstream of autophagy, such as lysosome biogenesis or autophagosome-lysosome fusion. However, *hlh-30* RNAi did not disrupt proper acidification of lysosomes in young *fem-2* worms (Figure 5.4) indicating that *hlh-30* does not function in regulating lysosome pH.

In order to determine if lysosomal pH in *fem-2* mutants are subject to feedback regulation via signals originating from the lysosome itself, *fem-2* worms were fed *lipl-4* RNAi and assayed for premature lysosomal alkalinization. LIPL-4 has been known to promote longevity via nuclear translocation of the lysosomal lipid chaperone LBP-8 that activates the transcription factors NHR-49 and NHR-80 (Folick et al., 2015). Germline removal leads to upregulation of *lipl-4* expression via DAF-16/FOXO while overexpression of *lipl-4* leads to extended lifespan in animals with intact gonads (M. C. Wang et al., 2008). My results from the *lipl-4* RNAi experiment showed no change in lysosomal pH in young *fem-2* worms that were fed *lipl-4* RNAi (Figure 5.4), suggesting that LIPL-4 mediated transcriptional feedback does not impact lysosomal pH maintenance in *fem-2* worms. It is entirely possible that LIPL-4 activates other lysosomal proteases or lipases that might

enhance lysosomal protein degradation, but such a change would not be detected via cDCFDA staining.

The nuclear hormone receptor NHR-80 is one of the lesser studied nuclear hormone receptors in *C. elegans,* and not much is known about it except that it is involved in fatty acid desaturation and needed for the extended lifespan of germline-less *C. elegans* (Goudeau et al., 2011). Deletion of *nhr-80* suppresses the longevity of *glp-1* mutants. Evidence provided in this thesis (Figure 5.4) show that NHR-80 likely does not impact lysosomal pH in *fem-2* worms. A more plausible explanation is that NHR-80 either activates other unrelated genes involved in lifespan extension, or is involved in the production of steroid hormone signals (assuming these signals are steroid in nature) that communicate the reproductive status of the germline to the soma.

In order to ascertain the role of the TGF-ß dauer signalling pathway in lysosomal pH homeostasis, I used various hypomorphs in the TGF-ß/DAF-7 signalling pathway and as shown in Figure 5.5, none of the mutants tested (*daf-28, daf-8* and *daf*-5) resulted in premature alkalinization of lysosomes, nor did these mutants lead to prolonged lysosomal acidity. The TGF-ß pathway in *C. elegans* has discrete roles during larval stage in regulating entry into dauer and in reproductive aging. While the TGF-ß dauer pathway is involved in embryonic viability and oocyte quality (Luo et al., 2010). A parallel TGF-ß pathway in worms, named TGF-ß Sma/Mab pathway impacts reproductive lifespan. The scope of this study only covers the TGF-ß dauer pathway, as it plays an important roles in the assessment of metabolic conditions and driving developmental programs that control germline and somatic fate (Luo et al., 2009). It is also plausible that the *daf* mutants tested here might have redundant (overlapping) functions with other genes, and hence these single mutants might not show altered lysosomal pH profile compared to wild type. It is also possible that these *daf* mutants might retain partial activity, thereby confounding the cDCFDA analysis.

Since PQM-1 has been shown to complement DAF-16 as a regulator of certain genes associated with longevity and development and given that PQM-1 and DAF-16 mutually antagonize each other via nuclear/cytoplasmic localization (Tepper et al., 2013), I tested whether a loss of function *pqm-1* mutant displayed defects in lysosomal acidification. This does not appear to be the case, since cDCFDA staining in these animals is comparable to wild type. PQM-1 regulates expression of class II insulin signalling genes involved in development, but not the expression of class I insulin signalling genes involved in stress response, perhaps indicating a lesser role in proteolysis control of this gene. Alternately, if PQM-1 redundantly activates transcription

of *vha* genes in the absence of DAF-16, perhaps a double RNAi of *daf-16* and *pqm-1* would reduce *vha* transcription levels to even more than a *daf-16* RNAi treatment. This is something that could be tested in the future.

The NHR-49 transcription factor has been implicated in fatty acid desaturation, and was thus investigated for a possible role in maintenance of lysosomal pH in wild type worms, mainly as a downstream target of LIPL-4 as well as its role in maintaining fatty acid reserves following germline ablation. Similar to *lipl-4* RNAi or *nhr-80* mutants, the *nhr-49* mutant failed to elicit any change in lysosomal pH homeostasis during and after cessation of reproduction (Figure 5.6).

SGK-1 was tested primarily because of its involvement in promoting longevity in a DAF-16 dependent manner, but it does not control the subcellular localization of DAF-16. Instead, it may rely on interaction with other proteins to influence expression of DAF-16 target genes (Chen et al., 2013). The *sgk-1(ft15)* mutant used in this study is a gain of function mutant, and as such was used because of its small but statistically significant lifespan that relies on DAF-16 activity. From Figure 5.7, it is clear that although *sgk-1* might influence longevity via DAF-16 dependent manner, it does not influence *vha* gene expression during or after cessation of reproduction.

There are likely many other genes that modulate lysosomal pH by influencing either reproduction or hormonal signalling, but these would need to be fished out using a more generalized genetic screen (presumably employing RNAi) using a worm strain expressing a pH sensitive fluorophore such as pHTomato. Undoubtedly, some of the genes involved in this process would affect the duration of reproduction or delay/accelerate the onset of reproductive maturation, but these genes can be refined further once the initial hits from the genetic screen have been curated.

6 CONCLUSION AND FUTURE DIRECTIONS

Overall, the goal of this study was to identify genetic pathways that influence aging, reproduction and healthspan in *C. elegans*. The first part of this study sought to elucidate the role of ER stress in autophagy and how it relates to organismal and reproductive aging. This study validated previous data from other labs that that a small increase in ER stress levels (via tunicamycin treatment, etc.) leads to an increase in both reproductive and organismal lifespans. Such an increase in lifespan was also observed in *C. elegans rer-1* mutants due to increased autophagic flux mediated via increased basal levels of ER stress and UPR. Interestingly, *rer-1* mutants were able to alleviate human αsyn::GFP aggregation compared to wild type worms, presumably due to increased proteolytic ability via the autophagy lysosome pathway. Moreover, *rer-1* mutants were also able to shift the dynamics of reproduction, thereby able to reproduce for longer than wild type worms. An RNAi knockdown of LGG-1 reduced autophagy levels back to wild type and abolished the enhanced proteolytic phenotype of *rer-1* worms, as well as reducing reproductive lifespan back to wild type levels. Even though it seems that the extended window of reproduction may be advantageous for *rer-1* mutants, such mutations are probably not favorable from an evolution point of view, since these worms need to utilize whatever nutrition source is available and generate as many progenies as possible before passing. This might be why mutations in genes such as *rer-1* do not arise during evolution, and even if they do, they are quickly selected against. While this study answers some of the questions about the connection between ER stress and reproductive lifespan, it raises others. For example, the exact mechanism by which a slight increase in autophagic flux leads to an increase in organismal and reproductive lifespan could be a source of further studies. Perhaps the increased autophagic flux leads to availability of extra nutrients that are channeled for reproduction. It would also be interesting to see if moderate amounts of stress can lead to increased fitness and lifespan in humans.

One observation from yeast as well as *C. elegans* data is that as organisms age, autophagic flux increases while global transcription levels decline (Chapin et al., 2015). This induction of autophagic flux seems to be a compensation mechanism for higher than normal levels of protein aggregation in older cells. Despite such measures, these organisms accumulate a high amount of protein aggregates as they age, implying a systemic deficiency in destruction of protein aggregates (Outeiro et al., 2003). The second part of this study was therefore aimed at assessing the functional competence of lysosomes as worms age using lysosomal pH as a readout. Initially, this study

established the usefulness of cDCFDA as a lysosomal marker, following which I explored its use as a pH sensitive fluorophore. I found that lysosomal pH increased sharply as soon as worms ceased reproduction, but sterile mutants such as *fem-2* showed a much longer period of lysosomal acidification. I showed that the vATPase subunits (encoded by the *vha* genes) that are embedded in the lysosomal membrane and which acidify lysosomes are transcriptionally downregulated in wild type but not sterile (*fem-2*) worms following cessation of reproduction. Re-establishing progeny production by mating sterile *fem-2* worms abolished the prolonged lysosomal acidification. I demonstrated that lysosomal pH regulation was achieved *in situ* by DAF-16, the FOXO transcription factor that controls longevity and stress response. In young reproductive worms, DAF-16 is nuclear where it drives *vha* expression and upon cessation of reproduction DAF-16 becomes progressively cytoplasmic, thereby leading to a drop in *vha* transcript levels. A vast amount of scientific data points to a role of the cytochrome P450 DAF-9 in the production of a steroid hormone that conveys the reproductive status of the germline to the somatic tissue. In view of this, I tested lysosomal pH dynamics in a *daf-9* mutant and I found that these worms showed reduced lysosomal acidification. Supplementing *daf-9* worms with Δ_4-DA alleviated this effect. Moreover, I showed that lysosomal pH collapse following cessation of reproduction can be delayed by extending the window of reproduction in wild type worms. Taken together, reproduction seems to act as a biological timer that regulates lysosomal proteostasis by regulating lysosomal pH by controlling the *vha* gene transcription. It is still unclear how the germline conveys reproductive status to other somatic tissues and the exact biological event that triggers this signalling. Also, it is unclear how lysosomal collapse eventually leads to cell death, and how lysosomes cope with increased autophagic flux. I observed lysosomal size and morphology to change as worms age such that young worms have smaller lysosomes that are spaced apart. As worms age, lysosomes become enlarged and are clustered together. It is unclear whether this represents lysosomal fragmentation or if this is a normal physiological response to cope with increased proteolytic requirements. The answers to these questions would allow us to better understand and improve the human body's abilities to counter proteotoxic stress.

The final part of this study was aimed at identifying the various genes involved in maintaining lysosomal pH homeostasis. Even though only a handful of candidate genes were tested as a proof of concept, much remains to be uncovered. Using this candidate based approach, I identified NHR-8 as one of the proteins that influences lysosomal pH, presumably due to its role in cholesterol

biogenesis. Even though none of the other genes tested in this study showed any influence on lysosomal pH, it is likely that an RNAi based screen using a pH sensor (fluorophore) would provide a better platform for identifying other genes involved in regulating lysosomal pH.

C. elegans is an excellent model organism to study aging and reproduction, and many of the genes examined in this study have known human homologues. Some of the genes and processes in this study have also been implicated in various neurodegenerative disorders such as Alzheimer's and Parkinson's. I am confident that gaining further insight about *C. elegans* aging will allow us to understand the aging process in humans and the various physiological changes that take place as humans age. It would also allow us to develop strategies for promoting health aging, thereby reducing healthcare cost.

7. REFERENCES

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