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The germline DNA damage response actively truncates *C. elegans* lifespan

DISSERTATION

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by

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DEDICATION

To

my wife Crystal
for putting up with me
being a grad student
for seven years

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- Gerontology
- Limb development

SKILL LIST

- General molecular biology techniques
- Cloning
- Genetic engineering (including CRISPR)
- Immunohistochemistry
- Confocal microscopy
- Micro-dissection
- qPCR
- IR spectroscopy
- HPLC
- General wet chemistry procedures

ABBREVIATIONS

DDR	DNA damage response
DR	Dietary restriction
WNPRC	Wisconsin National Primate Research Center
NIA	National Institute on Aging
TOR	Target of rapamycin
IIS	Insulin/IGF-1 signaling
IGFIR	Insulin-like growth factor I receptor
ROS	Reactive oxygen species
SOD	Superoxide dismutase
DSB	Double-stranded break
IR	Ionizing radiation
HR	Homologous recombination
NHEJ	Non-homologous end joining
CPD	Cyclobutane pyrimadine dimers
NER	Nucleotide excision repair
BER	Base excision repair
MMR	Mismatch repair
ICL	Interstrand crosslink
PRR	Post-replication repair
MZ	Mitotic zone
PZ	Pachytene zone

ABSTRACT OF THE DISSERTATION

The DNA damage response in germ cells actively curtails *C. elegans* lifespan

by

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Lifespan varies dramatically between species, from weeks in the case of the nematode *C. elegans* to thousands of years in the case of the the conifer *Pinus longaeva* (bristlecone pine)[1]. What causes some organisms to live so briefly while others live for so long? The search for the answer to this question has resulted in the discovery of many experimental interventions that extend lifespan. One such intervention is the ablation of germ cells in the reproductive system. Lifespan control by the reproductive system has been demonstrated in *C. elegans*, in the fruit fly *D. melanogaster,* in the mouse species *Mus musculus*, and has even been suggested to apply to humans[2-6]. This indicates that the effect of the reproductive system on lifespan may be conserved between invertebrates and mammals.

The various experimental interventions known to increase lifespan have been used to evaluate a range of theories on aging. These theories attempt to explain the cause of aging from either a molecular or an evolutionary angle. Lifespan control by the reproductive system may be particularly useful in evaluation of the evolutionary theories of aging. However, current knowledge of lifespan control by the reproductive system is incomplete. While a number of molecular intermediaries in this control have been identified, upstream signals are largely unknown[2,7]. The result is a fundamental lack of understanding of why the reproductive system controls lifespan.

In this thesis, I identify the germline DNA damage response (DDR) as a possible driving force in the control of lifespan by the reproductive system. I find that levels of DDR activation rapidly increases with age in the germline of *C. elegans*. Increased germline DDR activation, effected by either targeted irradiation or genetic manipulation, leads to decreased lifespan. Conversely, the suppression of germline DDR through genetic means leads to increased lifespan. The checkpoint proteins ATM and ATR as well as insulin signaling play a central role in these lifespan effects.

The lifespan increase caused by reduced germline DDR does not come at the cost of decreased reproductive activity. This suggests that the disposable soma theory is insufficient to explain this lifespan effect. I propose a model in which germline DDR actively decreases lifespan as part of a mechanism to limit post-reproductive lifespan. Such a mechanism is consistent with the kin-selection theory of aging.

In addition to further elucidating the influence of the reproductive system on lifespan, I also demonstrate radiation hormesis in *C. elegans* through somatic cell

irradiation. Previously reports have presented conflicting results when testing for radiation hormesis in worms, perhaps due to the obscuring effects of germline irradiation[8,9]. My results demonstrate a reproducible way to increase lifespan by irradiation, opening the possibility of further investigation of the mechanism behind radiation hormesis using *C. elegans* as a model system.

CHAPTER 1: INTRODUCTION

The purpose of this introduction is familiarize the reader with the scientific background behind aging and lifespan control. I go over four well-established interventions known to extend lifespan. I then go over several major molecular and evolutionary theories of aging, and how these can be used to explain the discussed experimental interventions that extend lifespan. Germline ablation is an especially important intervention because it may provide a way to differentiate between the evolutionary theories of aging. I outline a potential molecular mechanism by which kin-selection driven senescence might be controlled. This mechanism is centered around the control of lifespan by the germline DNA damage response (DDR). I therefore review the molecular mechanisms of DDR, with particular emphasis on the central factors ATM and ATR. Finally, I review the known connections between DDR and lifespan.

1.1. Lifespan and the rate of aging

Aging is a phenomenon that occurs in a wide range of organisms, from plants to yeast to mammals. It is characterized by three trends: 1) a decline in organismal functions, such as immune response and cognitive function, 2) a decline in reproductive output, and 3) an increase in the rate of mortality[10-13]. This third characteristic means that the rate of aging in an individual organisms is, on average, inversely proportional to its lifespan.

1.1.1. The importance of aging in biology and medicine

Aging is an important problem in biology. As mentioned, organisms experience decreases in reproduction and survival with age. Aging should therefore lead to reduced fitness due to fewer progeny being produced, compared to a hypothetical state in which aging does not occur. The potential for aging to negatively impact fitness seems at odds with its prevalence in so many species. This problem is specifically addressed by Charles Darwin in The Origin of Species[14]. A criticism he received was that the ubiquity of aging is contrary to the idea that natural selection weeds out deleterious traits. Darwin's answer was that longevity is related to "the standard of each species" - the way that an organism develops and lives determines how long it will live. So why has aging not been selected against, to the point that all species are biologically immortal? One potential answer to this question is that most organisms in the wild die long before they are subjected to the negative effects of aging. Such early extrinsic mortality prevents selective forces from having any influence on the rate of aging.

The idea that aging does not occur in wild populations implies that there is no purpose in studying aging from an ecological point of view. However, aging has been documented in many different species in the wild. A recent review found that aging in the wild has been demonstrated in over a hundred different species, including both vertebrates and invertebrates[15]. This suggests that some organisms in the wild do experience the effects of aging. Focusing on a single species, a study of a wild population of *Sula nebouxii*, the blue-footed booby, found that about one third of males showed signs of aging[16]. These older males had higher levels of DNA damage in their

sperm and decreased levels of colorful foot pigmentation[16]. Reduced foot pigmentation decreases the chances of a blue-footed booby attracting a mate, and studies in humans have shown that increased DNA damage in sperm is associated with decreased fertilization efficiency[16-18]. Both characteristics displayed by the aged individuals would therefore be expected to decrease fitness[16]. The fact that a third of the population displayed these characteristics suggests that aging has a potentially large impact on fitness in individuals of this species. It is therefore insufficient to say that aging is a result of a lack of selective pressure.

Aging is also particularly relevant to the field of medicine. One characteristic of aging is a decline in organismal function. This decrease in function leads to an age-associated increase in the risk of cardiovascular disease, cancer, and certain infectious diseases[19]. Despite age being a major risk factor in so many diseases, current medical interventions do not target the cause of aging. Instead, the symptoms of aging are targeted. A preventative strategy towards aging may be a more efficient strategy of maintaining the health of patients. Such a strategy will require that we first understand the fundamental cause of aging.

1.1.2. Variation in lifespan and the rate of aging across species

One way to begin investigating the cause of aging is to compare the rate of aging and lifespan between different species. Lifespan varies dramatically between species. The nematode *C. elegans* lives about two weeks on average. In contrast, bowhead whales are estimated to live over 100 years, making them the longest-lived mammals known[20]. Such a difference in lifespan is not surprising, considering the different physiology and ecology of these species. However, even fairly similar species can display dramatically different lifespans. The common lab mouse *Mus musculus* typically lives between two and three years while the similarly sized naked mole rat *Heterocephalus glaber* can live for well over 25 years[21]. This vast differences in lifespans between species are important because they suggest that evolutionary forces have somehow resulted in different rates of aging between these species. Selection for different rates of aging may be direct due to selection for a particular lifespan or indirectly due to selection for traits which influence lifespan as a side-effect.

Most organisms that have been studied show signs of aging. However, there are some organisms that show negligible signs of aging. This does not mean that these organisms are immortal, but rather that they display no detectable increase in mortality or organismal function and no decrease in reproductive output with chronological age. Such species represent a diverse array of lifeforms, including angiosperms, molluscs, echinoderms, and turtles[22-25]. It may even be possible for mammals to display negligible signs of aging. Naked mole rats display no increase in mortality rate or decrease in reproduction with age, although they do show some of the superficial signs of aging[26]. The existence of species that do not display signs of aging raises the possibility that aging is not inevitable, given the right conditions.

1.2. Interventions that increase lifespan

The importance of aging has led to a vast amount of scientific literature on the subject. Many experimental interventions are known to either increase or decrease lifespan. Interventions that increase lifespan are especially important, as they may hint at the factors which ultimately limits lifespan. In this section, I will focus on several general categories of interventions that increase lifespan. These are particularly important due to both their efficacy across multiple species and the extent to which the underlying molecular mechanisms have been described. A graphical summary of these interventions is given in Figure 1.

1.2.1. Lifespan extension by dietary restriction

One of the oldest and best described interventions that increases lifespan is dietary restriction (DR). The first scientific report on this subject, published over 80 years ago, showed that feeding rats a reduced calorie diet from birth results in dramaticly increased lifespan[27]. Lifespan extension by DR has since been demonstrated in a wide range of species. Nematodes grown in dilute bacterial cultures live longer than those grown in more dense cultures[28]. Yeast grown in media containing lower glucose or amino acid concentrations live longer than those grown in standard media[29]. DR has recently been shown to extend lifespan in rhesus monkeys, according to a study conducted by the Wisconsin National Primate Research Center (WNPRC)[30]. However, the findings of this report conflict with another study carried out by the National Institute on Aging (NIA), which reported that DR does not significantly affect mortality[31]. The difference between the conclusions of these two studies may be due to the different feeding regimes used for the controls in each. The WNPRC study fed control monkeys a diet composed of almost 30% sucrose, whereas the NIA study fed controls a diet composed of 4% sucrose[30,31]. In addition, the WNPRC study provided control monkeys with an amount of food equivalent to ad libitum conditions, whereas the NIA study provided control monkeys with what is considered a healthy amount of food[30,31]. These two differences suggest that the WNPRC study may have detected a significant difference in mortality in part because animals subjected to DR were compared to animals on an unhealthy diet. Regardless, both studies observed that DR led to a decrease in age-related disease, suggesting a decrease in the rate of aging[30,31]. These primate studies are particularly exciting as they suggest that the effects of DR may be shared between simple model organisms and primates.

It is tempting to think that DR extends lifespan due to a general reduction in calorie use. However, there is compelling evidence that lifespan extension after DR is actually due to the activation of genetic programs which alter protein homeostasis and increase respiration. The complex molecular pathway behind DR has been worked out in some detail, although considerable unknowns remain.

One of the central players in DR-induced lifespan extension in worms is *skn-1*, homolog of the human gene Nrf2. The mutation of *skn-1* abolishes DR-induced lifespan extension in worms[32]. Rescuing expression of *skn-1* in the two ASI neurons rescues the effect of DR on lifespan[32]. This suggests that neurons may play a central role in the effect of DR on lifespan. Along the same lines, DR-induced lifespan extension is also

dependent on several genes involved in neuronal signaling. Knockdown of *nlp-7*, a neuropeptide which is regulated by *skn-1*, abolishes DR-induced lifespan extension while leaving other forms of lifespan extension unaffected[33]. In addition, knockdown of *ckr-1* or *ckr-2*, two neuropeptide receptors, reduces the effect of DR on lifespan[33].

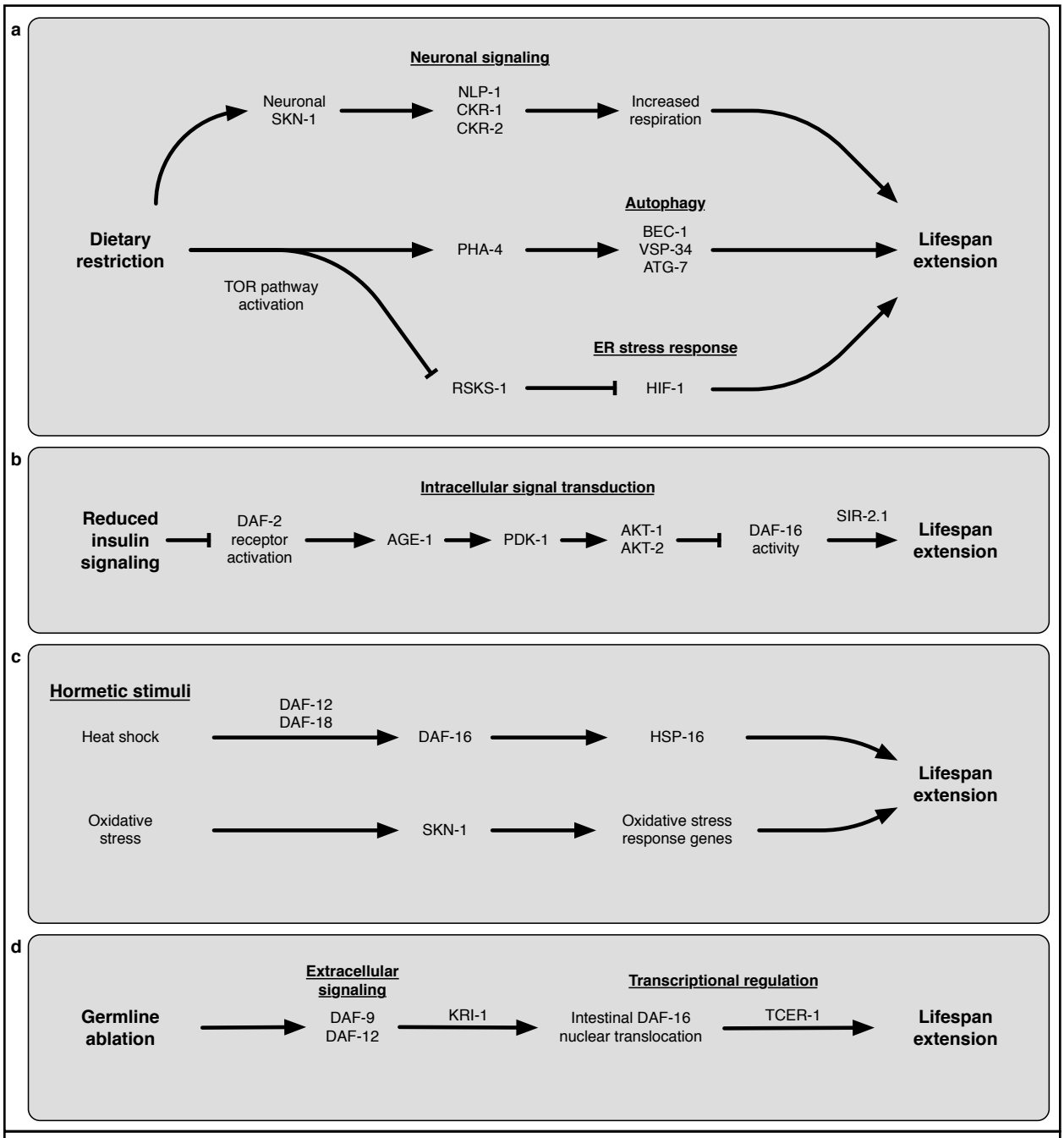

Figure 1: Experimental interventions that extend lifespan.
Summary of the basic mechanism behind lifespan extension after **a)** dietary restriction, **b)** reduced insulin signaling, **c)** hormesis, and **d)** germline ablation.

These reports support the idea that DR modulates lifespan through signaling from neurons. The downstream result of this signaling is an increase in respiration[32]. This increased respiration may counterintuitively increase lifespan, as administration of antioxidants abolishes the lifespan effect of certain types of DR[34].

Another important mechanism behind DR-induced lifespan extension is the target of rapamycin (TOR) pathway. The TOR pathway affects lifespan in yeast, worms, and flies[35-37]. TOR repression plays a role in DR-induced lifespan extension in worms through two mechanisms. First, DR activates the FOXA transcription factor PHA-4, leading to an increase in autophagy[38,39]. Mutation of the autophagy genes *bec-1*, *vsp-34*, or *atg-7* abolish the lifespan effect of DR[38,39]. Second, DR results in a decrease in *rsks-1*, the worm homolog of the ribosomal protein S6K, leading to reduced protein translation[36,40]. This decrease in *rsks-1* also activates *hif-1*, the worm homolog of the mammalian hypoxia-inducible factor HIF-1, which decreases ER stress[41]. Mutation of *hif-1* blocks DR-induced lifespan extension[41]. These results suggest that the lifespan effect of DR is due to an increase in autophagy, a decrease in protein translation, and an increase in ER stress. All three of these mechanisms provide a connection between protein homeostasis and DR-mediated lifespan extension

1.2.2. Lifespan extension by reduction of insulin signaling

Another well established method of increasing lifespan is by the reduction of insulin/IGF-1 signaling (IIS). The first single-gene mutation that was found to increase lifespan in a metazoan was in the *C. elegans* gene *age-1*, a worm homolog of mammalian PI3-kinase which is involved in IIS transduction[42,43]. In *Drosophila*, mutation of the insulin signaling components *chico*, *InR*, or *dFOXO* result in increased lifespan[44-46]. Genes involved in the insulin signaling pathway have also been implicated in the control of aging in humans. Certain variants of the human gene FOXO3a, a homolog of *dFOXO*, were found to associate with increased lifespan in two independent GWAS studies[47,48]. Another study found that variants in IGFIR (insulin-like growth factor I receptor) associate with both decreased IGFIR activity and increased lifespan[49]. These studies suggest that the role of insulin signaling in lifespan control is conserved across many species.

The molecular details of the IIS pathway have been extensively studied in *C. elegans*. An early step in IIS activation is binding of extracellular insulin-like peptides to the receptor protein DAF-2[50]. *daf-2* hypomorphic mutants live about twice as long as wild-type worms, illustrating how dramatically the IIS pathway can affect lifespan[51]. Activated DAF-2 in turn activates the PI3K ortholog AGE-1, which as mentioned plays a role in lifespan control[42]. Activated AGE-1 then activates the serine/threonine kinase PDK-1, which in turn activates the kinases AKT-1 and AKT-2[52,53]. AKT-1 and AKT-2 phosphorylate DAF-16, preventing its nuclear import[54]. *daf-16*, a transcription factor homologous to human FOXO3a, regulates the expression of a very large number of genes, many of which function in stress resistance and metabolism[55]. *daf-16* is required for the lifespan effects of a number of different interventions, including germline ablation and *daf-2* mutation[2,51]. In addition, *daf-16* interacts with other nuclear factors that are important for lifespan control, such as the sirtuin *sir-2.1*[56].

1.2.3. Lifespan extension by hormesis

A third way in which lifespan can be extended is through exposure to low levels of a toxic agent. This is referred to as hormesis - the concept that something that is toxic at high doses can be beneficial at low doses. Hormetic lifespan extension has been demonstrated in several model organisms using multiple types of stresses, such as heat, oxidative conditions, and radiation. Radiation hormesis will be discussed in more detail in a later section.

Hormetic lifespan extension has been observed in response to short periods of elevated temperature. *Drosophila*, which are normally maintained at 25°C, display increased lifespan in response to multiple 36°C heat shocks[57]. Similarly, *C. elegans*, which are normally maintained at 25°C, display increased lifespan in response to one or multiple 33°C heat shocks[58]. Mutation of *daf-12*, *daf-16*, or *daf-18* block the lifespan extension caused by heat shock in worms, suggesting that insulin signaling may play a role in this type of hormesis[59]. One possible way that insulin signaling mediates the effect of heat stress is through the regulation of heat shock proteins. Heat shock proteins function to ensure the proper folding of other proteins. *age-1* mutants, in which *daf-16* activity is increased, display increased expression of the heat shock protein *hsp-16*[60]. *hsp-16* expression is also increased in worms by heat shock[58]. Similarly, hsp70 expression is increased by heat shock in *Drosophila*[57]. *hsp-16* overexpression alone increases lifespan in worms, independently of heat shock itself[61]. These reports together support a model in which exposure to elevated temperature reduces insulin signaling, leading to increased heat shock protein expression. These proteins lead to improved proteostasis in an organism, an effect which may far outlast any damage from the initial heat shock.

Hormetic lifespan extension has also been observed after subjection to oxidative stress. Exposure of worms to 100% atmospheric oxygen for 8 hours increases lifespan[9]. Low levels of paraquat or arsenite, two chemicals which induces ROS formation in cells and are very toxic at high doses, also increase lifespan in worms[62,63]. The lifespan increase observed after arsenite exposure is abolished by the administration of antioxidants, suggesting that the effect is dependent on elevated ROS formation[63]. Arsenite exposure upregulates the expression of *skn-1*, an important transcription factor involved in DR-induced lifespan extension, and the mutation of *skn-1* abolishes the lifespan extension seen after arsenite exposure[63]. *skn-1* mutants are also hypersensitive to oxidative stress, suggesting that one of the functions of the gene is to regulate the oxidative stress response[64]. The hormetic effect of oxidative stress may be due to an overcompensation of this oxidative stress response, the effects of which may last much longer than the immediate damage caused by exposure to oxidative conditions.

1.2.4. Lifespan extension by germ cell ablation

A fourth way in which lifespan can be extended is by germ cell ablation. The physical ablation of all germ cells, through the use of laser targeting early in life, dramatically extends lifespan in *C. elegans*[2]. Similarly, lifespan is extended when the

germline is ablated genetically through the mutation *glp-1*, a gene required for the maintenance of dividing germ cells[65]. A similar effect has been shown in *drosophila*. The ablation of the germline through overexpression of the *bam* gene in germ cells, leading to their premature differentiation, extends lifespan[3]. There is also evidence that lifespan is affected by germ cells in mammals, including humans. Two studies found that transplanting young ovaries into older mice increaseslifespan[4,66]. Spaying and neutering correlate with increased lifespan in domestic dogs[67]. One human study looked at historical data on eunuchs and intact males in the Korean royal court[5]. The authors found that castrated males lived longer than similarly ranked in-tact males, on average[5]. Another study in mental patients found that castrated males lived significantly longer than intact males housed in the same institution[6]. These reports suggest that the reproductive system negatively affects lifespan throughout the animal kingdom.

The link between lifespan and reproduction may even extend to the plant kingdom. Soy plants produce fruiting pods in order to reproduce. The formation of these pods is accompanied by yellowing of the surrounding leaves of the plant[68]. This localized deterioration is not due to nutrient loss from the surrounding leaves to the fruiting pod, as yellowing still occurs when the phloem, which is responsible for nutrient transport, is destroyed between the pod and leaf[68]. Nitrogen content does not decrease in these yellowing leaves, further supporting the idea that nutrient loss is not responsible for this localized deterioration[68]. In contrast, depodding dramatically increases the lifespan of soybean plants[69]. These reports suggest that the reproductive system negatively affects lifespan in plants as well.

It is not known if the genetic mechanism behind this control of lifespan by the reproductive system is conserved between such distantly related species as worms, humans, and soybeans. However, the selective pressures that link reproduction and lifespan may be similar. This may lead to commonalities in the way that lifespan is controlled by the reproductive system. The general trend that emerges is that the removal of certain reproductive tissues increases lifespan. However, this link is not as simple as it first appears.

There are two main requirements which must be met in order for germ cell ablation to increase lifespan. I will focus here on *C. elegans*, as it is the model in which the link between the reproductive system and lifespan has been the most thoroughly studied. The first requirement is that the somatic structures of the reproductive system are retained. While germ cell ablation dramatically increases lifespan, ablation of the entire reproductive system does not increase lifespan compared to intact worms[2]. The current dominant model is that germ cell ablation increases lifespan by modifying signaling from the somatic structures of the reproductive system to the rest of the soma.

A second requirement for germ cell ablation to increase lifespan is IIS pathway function[65]. Germ cell ablation does not increase lifespan when certain components of the insulin signaling pathway, such as *daf-9, daf-12, kri-1*, or the downstream transcription factor *daf-16,* are mutated[2,7,65,70]. Several studies suggest that IIS activity in the intestine mediates the effect of germ cell ablation. First, germ cell ablation does not increase lifespan in *daf-16* mutant worms[71]. However, the lifespan effect of germ cell ablation is rescued in these worms when *daf-16* is expressed specifically in the intestine[71]. Second, germline ablation leads to increased nuclear translocation of DAF-16::GFP in the intestine of transgenic worms[54]. Third, *kri-1*, which is involved in

daf-16 nuclear translocation in response to germ cell ablation, is expressed primarily in the intestine[7]. Fourth, the transcription elongation factor *tcer-1*, which interacts with *daf-16*, is required for lifespan extension after germline ablation[72]. Expression of TCER-1::GFP protein increases in intestinal nuclei after germline ablation in transgenic worms[72]. Together, these data suggest that signaling from the somatic reproductive system ultimately affects lifespan through a change in IIS activity in the intestine.

It is important to note that the influence of the reproductive system on lifespan is not dependent on reproduction itself. The chemical sterilization of worms using FuDR does not lead to increased lifespan[73]. In addition, the genetic ablation of germ cells increases fly lifespan even in sterile individuals[3]. However, reproduction does have some effect on lifespan. For instance, the mating of flies, which is required for reproduction, dramatically decreases lifespan[74]. These data suggest that the reproductive system is controlling lifespan in a way that is related to reproduction, but not simply based on reproductive output itself.

1.3. Molecular theories of aging

The search for the causative factor of aging has led to a slew of theories, each with supporting and detracting evidence. I have divided them into two categories for this introduction: 1) molecular theories, and 2) evolutionary theories. Molecular theories attempt to explain the physical mechanisms that cause aging. Evolutionary theories attempt to explain the selective forces which give rise to different rates of aging across species. These theories are not mutually exclusive.

Each theory attempts to explain some of the observations that have been made about aging. These include the decrease in function with age, the variation in the rate of aging between species, and the various experimental interventions known to increase lifespan. While I will not cover the entire list of aging theories exhaustively, I will explain each major theory in this section.

1.3.1. Telomere theory of aging

One influential theory of aging is that it is caused by telomere shortening[75]. At the end of each linear chromosome are telomeres, which are composed of repeats of a specific DNA sequence bound by protein. Telomeres serve the dual purpose of preventing the loss of coding regions of the genome and preventing chromosomal abnormalities due to end-to-end fusions[76]. Each round of cell division results in a slight erosion of telomere length. Cells become senescent once telomere length is sufficiently short. The telomere theory of aging posits that as an organism ages telomere shortening causes senescent cells to accumulate, exhausting stem cell pools. This ultimately leads to aging due to a decline in organismal function, as damaged cells cannot be replaced.

There is some evidence in support of the telomere theory of aging. Telomere length decreases in a wide range of human tissues with age[77]. Telomere length is predictive of lifespan, based on comparisons between human twins[78]. Premature telomere shortening is observed in certain progeroid syndromes, such as Fanconi's

anemia and Werner's syndrome[79,80]. These observations strongly support an inverse correlation between telomere length and remaining lifespan.

In addition to correlation, there is some evidence that the manipulation of genes controlling telomere length affects lifespan. Mice in which mTERT, an important component of telomerase, is expressed throughout the body via an injected transgene are longer lived than controls[81]. Overexpression of *hrp-1* over successive generations in *C. elegans*, which results in abnormally long telomeres, also increases lifespan[82]. In contrast, mutation of *tert*, a homolog of mTERT, severely truncates lifespan in zebrafish[83]. Such observations give some credence to the idea that telomere length may actually play a role in determining lifespan.

While the telomere theory of aging has some support, it is inconsistent with certain empirical observations. The largest problem with this theory is that many organisms age independently of any change in telomere length. Yeast have a limited replicative lifespan and display many molecular signs of aging, yet do not display telomere shortening with age[84]. This is also the case in *C. elegans*, where telomere length does not decrease with age[85]. This is not surprising, as the somatic cells of adult *C. elegans* do not cycle. In addition, telomere length does not correlate with lifespan when comparing individual *C. elegans*, based on a study which took advantage of the natural variation in telomere length that occurs in wild-type worms[85]. This raises the possibility that the overexpression of *hrp-1* in C. elegans, which as mentioned increases lifespan, may affect lifespan for reasons independent of telomere length[82,85]. In mice, the knockout of mTERT, which causes telomere shortening, does not lead to a decrease in lifespan unless the mutation is maintained for several generations[86]. Together these reports suggest that telomere shortening is not the ultimate cause of normal aging in yeast, worms, or mice.

The telomere theory of aging also fails to explain many of the experimental interventions known to increase lifespan. Why would exposure to a stress early in life slow the erosion of telomeres? Why would the ablation of germ cells affect telomere length in the soma? There is no clear reason why these experimental interventions would extend lifespan if telomere length is the limiting factor which determines lifespan.

1.3.2. Free radical theory of aging

Another theory of aging is that it is caused by damage induced by reactive oxygen species (ROS)[87]. ATP is constantly being produced through oxidative phosphorylation in mitochondria. This process is not perfectly efficient and produces small amounts of superoxide and peroxide. These ROS react with lipids, proteins, and DNA within the cell. The free radical theory of aging posits that these damaged molecules accumulate, leading to a decline in cellular function with age.

There are numerous pieces of evidence supporting the free radical theory of aging. Levels of oxidized lipids increase with age in several human tissues[88]. Levels of oxidatively damaged protein increase with age in human erythrocytes, fibroblasts, and neural tissue based on protein carbonyl content[89,90]. Levels of oxidative DNA damage increase with age in a wide range of rat tissue, based on 8-oxoguanine levels[91,92]. These reports suggest that oxidative damage generally increase with age.

Another line of evidence supporting the free radical theory of aging is that ROS production and oxidative damage both correlate with lifespan, in certain cases. Longer-lived individuals within a population of houseflies were shown to have higher superoxide dismutase activity and lower peroxide content compared to shorter-lived individuals[93]. DR, which increases lifespan, also leads to a decrease in oxidatively damaged proteins and peroxide levels in mice, as well as a decrease in protein and lipid oxidative damage in rat lymphocytes[94,95]. These reports suggest that increased levels of ROS and oxidative damage correlate with decreased lifespan.

In addition to correlation, there are reports suggesting that oxidative damage determines lifespan. The overexpression of superoxide dismutase (SOD) both increases lifespan and decreases oxidative protein damage in *Drosophila melanogaster*[96]. Conversely, the mutation of certain SOD genes leads to reduced lifespan in *Drosophila*[97]. The most straightforward interpretation of these reports is that lifespan is determined by the ability of an organism to neutralize ROS.

The interpretation that SOD gene expression affects lifespan through an effect on ROS levels may be an oversimplification. The overexpression of SOD in *Drosophila* leads to increased lifespan[96]. However, a later report showed that the overexpression of a human SOD gene specifically in the motor neurons of *Drosophila* also leads to increased lifespan[98]. This suggests that lifespan extension may not necessarily be due to a decrease in oxidative damage throughout the fly. Several other studies have cast doubt on the observation that SOD affects lifespan through its influence on oxidative damage. Overexpression of *sod-3*, one of the *C. elegans* SOD genes, leads to an increase in lifespan due to an induction of the ER stress response[99]. Another study found that the simultaneous mutation of all five *C. elegans* SOD genes did not lead to a decrease in the lifespan of worms raised under standard lab conditions[100]. In addition, a study of 18 different mouse mutants with reduced expression of endogenous antioxidant enzymes showed that lifespan was reduced in only one hypomorph[101]. These reports suggest that ROS levels are not the driving force behind aging.

The ROS theory of aging is called into further question by reports that, in certain cases, oxidative damage and ROS production are not inversely correlated to lifespan. For instance, a comparison between many species of either birds or mammals found no significant correlation between several markers of oxidative damage and lifespan in either group, when correcting for body mass[102]. There is even evidence that increased ROS levels can increase lifespan, under certain conditions. Feeding *C. elegans* 2-deoxy-D-glucose results in an inability to carry out glycolysis and an extension in lifespan, but also an increase in both oxygen consumption and the formation of ROS[34]. The lifespan increase is abolished by treatment with N-acetylcysteine, an ROS scavenger, suggesting that it is actually a hormetic response to increased oxidative stress[34]. Another study found that two different mutant worm strains that produce abnormally high amounts of peroxide live longer than wild-type, and that this lifespan increase is abolished in the presence of antioxidants[103]. Another study found that exposure of worms to low levels of paraquat, a chemical which induces superoxide formation in mitochondria, hormetically increases lifespan[62]. These experimental observations suggest that, contrary to the free radical theory of aging, ROS may be important in determining lifespan due to the cellular response that they induce, rather than through oxidative damage itself.

1.3.3. Rate of living theory of aging

The free radical theory of aging shares many similarities with the rate of living theory of aging. The general idea behind the rate of living theory, first proposed by Max Rubner in 1908 and later refined by Raymond Pearl, is that an organism's rate of aging is determined by two qualities: 1) its metabolic rate, and 2) some inherent vital quality that is gradually exhausted by metabolism[104,105]. The rate of living theory gained popularity due to its straightforward explanation of why DR and reduced temperature increase lifespan in various organisms[28,106,107]. Both treatments were thought to slow the 'rate of living', either through decreased metabolism due to DR or through a decrease in the rate of chemical reactions due to low temperature. In addition, the rate of living theory explains the general correlation between metabolic rate and lifespan, when no other characteristics are considered.

More recent studies have suggested that the rate of living theory is overly simplistic. Comparisons between species have revealed a number of trends which are not consistent with the rate of living theory. For instance, birds generally have a higher body temperature and metabolic rate than size-matched mammals, yet generally have several-fold longer lifespans[108]. This particular observation may be attributed to differences between classes of animals. However, two other studies comparing metabolic rate and lifespan found no correlation when correcting for body mass[102,109]. One study compared almost 250 mammalian species, while the other separately compared over 200 species of mammals and almost 50 species of birds[102,109]. These studies suggests that metabolic rate and lifespan appear related only because they inversely and directly correlate with body mass, respectively.

The rate of living theory is also called into question by the mechanistic details of DR-induced lifespan extension. DR was previously held up as evidence in support of the rate of living hypothesis[28]. However, it has since been shown that many aspects of metabolism do not slow down during DR. DR does not lead to a change in oxygen consumption or heat production in *Drosophila*[110]. DR actually increases both oxygen consumption and heat production in *C. elegans*, suggesting that certain aspects of metabolism are actually increased by DR[111,112]. These observations suggest that simply considering the "rate of living" is too simplistic to explain the cause of aging.

1.3.4. DNA damage theory of aging

The DNA damage theory of aging posits that aging occurs because of the accumulation of DNA damage and mutations in the genome of somatic cells[113]. Damage may be caused by endogenous factors, such as free radicals generated during metabolic processes. Such oxidative damage connects the DNA damage theory of aging with the free radical theory of aging. In addition, mutations may arise during cell cycling due to errors during DNA replication. Such genomic deterioration leads to the declining function of individual cells, which has the cumulative effect of reducing organismal function.

There are several lines of evidence supporting the DNA damage theory of aging. The first is that mutation frequency and levels of DNA damage, which can ultimately

lead to mutations, both increase with age. Mutation frequency exponentially increases in human kidney cells with age[114]. As mentioned previously, oxidative DNA damage increases with age in various human tissues[91,92]. Gross chromosomal abnormalities increase with age in the livers of mice[115]. Cells from older mice contain higher numbers of γ-H2AX foci, which localize to sites of DNA damage[116]. These reports are consistent with a correlation between age and the frequency of both mutations and potentially mutagenic DNA damage.

The second line of evidence supporting the DNA damage theory of aging is that DNA damage accumulates with age more slowly in longer-lived species compared to shorter-lived species. Two different studies have found that the repair rate of UV-induced DNA lesions, based on labeled nucleotide incorporation in the cultured cells, positively correlates with maximum species lifespan[117,118]. Another study found that the rate of double-stranded DNA break recognition, based on DNA end binding by proteins in gamma irradiated cultured cells, positively correlates with maximum species lifespan[119]. These studies suggest a connection between DNA repair efficiency and lifespan. However, this does not mean that DNA damage is a limiting factor in the determination of lifespan under normal conditions. More active DNA damage repair systems may have been selected for in longer-lived species because they have more time in which to accumulate mutations.

The third line of evidence supporting the DNA damage theory of aging is that multiple human progeroid syndromes are associated with mutations in DNA damage repair genes. Werner syndrome, caused by the mutation of a gene involved in double-stranded DNA break repair, leads to both premature aging and an increased rate of mutation[120]. Xeroderma pigmentosum, caused by mutations in the nucleotide excision repair pathway, leads to both premature aging and an inability to repair UV-induced DNA damage[121]. These are just two of many progeroid syndromes caused by DNA damage repair gene mutation[122]. The connection between premature aging and DNA repair defects suggests that DNA damage can limit lifespan in certain cases.

The main weakness with the DNA damage theory of aging is that its support is based on either correlation or on interventions which decrease lifespan. This brings into question whether the accumulation of DNA damage is really a causative factor in aging under normal circumstances.

There are some reports that contradict the DNA damage theory of aging. The theory is contradicted by mutant organisms that display mutation accumulation phenotypes, yet live a normal lifespan. Mutation of eight different thiol peroxidases in yeast mutants dramatically increases mutation rate without affecting lifespan[123]. Mutation of *nth-1* in worms increases mutation rate by 7-fold without affecting lifespan[124]. These reports suggest that mutation rate is not the driving force behind aging.

1.3.5. Proteostasis theory of aging

The molecular theory I will discuss is the proteostasis theory of aging. The idea behind this theory is that the proteosome of an organism becomes increasingly chaotic with age[125]. This chaos takes the form of increasing levels of improperly folded, damaged, and aggregated proteins, as well as generally misregulated expression

levels. This results in a decrease in tissue function, to the ultimate point that an organism cannot sustain its own life.

The proteostasis theory of aging is supported by the increase in damage to and misregulation of the proteosome that occurs with age. As mentioned previously, levels of oxidatively damaged proteins increase with age in a number of different human tissues[89,90]. There is increasing heterogeneity in gene expression both between cells in the same individual with age, based on a study of rats and humans[126]. There is also increased variance in gene expression between individuals with age, based on a study of worms[127]. These reports suggest a link between age and proteosome damage and misregulation. However, this alone does not reveal whether problems with the proteosome are the cause of aging.

The second line of evidence in support of the proteostasis theory of aging is that lifespan can be altered by modifying various genes related to protein folding, repair, and recycling. Overexpression of the heat shock factor *hsf-1*, a protein chaperone, in *C. elegans* increases lifespan[128]. Conversely, the knockdown of the heat shock genes *hsf-1*, *hsp-16,* or *sip-1* in worms decreases lifespan[128,129]. In *Drosophila*, overexpression of the protein repair enzyme PCMT was increases lifespan[130]. Increased protein recycling, which may improve proteostasis, is also associated with increased lifespan. As mentioned previously, DR-induced lifespan extension in worms is dependent on several genes involved in autophagy[38,39]. Increased levels of autophagy in the neurons of *Drosophila*, accomplished by Atg8a overexpression, leads to increased lifespan[131]. Overexpression of the autophagy gene Atg5 increased lifespan in mice[132]. However, overexpression also decreased obesity, which may explain the increased lifespan of these transgenic mice[132]. Together, these reports suggest that decreasing levels of damaged protein increase lifespan.

A variation in the proteostasis theory of aging is that aging is specifically caused by the accumulation of protein aggregates, rather than general levels of protein damage or expression. Protein aggregates become more prevalent with age in a variety of organisms. Protein aggregation increases with age in *C. elegans*, in the muscles of *Drosophila*, and in the cytoplasm with successive divisions in aging yeast[133-135]. In humans, some age-related diseases, such as Alzheimer's disease, are characterized by the formation of insoluble protein aggregates[136]. These reports suggest a connection between protein aggregates and aging.

The proteostasis theory is supported by its ability to explain many of the experimental interventions that are known to increase lifespan. As discussed above, DR-induced lifespan extension is associated with increased autophagy[38]. Decreased IIS activity results in increased expression of multiple heat shock proteins, which ensure proper protein folding[55]. The hormetic response to heat shock also results in increased heat shock protein expression[57,58]. The ability to explain various interventions that increase lifespan makes the proteostasis theory of aging very attractive.

1.4. Evolutionary theories of aging

There are many theories attempting to explain the cause of aging at the molecular level. However, such molecular explanations of lifespan determination raise the question: why do these lifespan-determining characteristics vary between species?

If one assumes that longer-lived species have a more robust system to maintain proteostasis with age, this would not answer the question of why these longer-lived species have more robust systems to begin with. In order to answer this more basic question, researchers have turned to evolutionary theories of aging. Multiple evolutionary theories have been proposed to explain the variation in lifespan between species. As with the molecular theories, I will explain only the major evolutionary theories in this introduction. For each theory, I will emphasize its compatibility with the known effects of the reproductive system on lifespan. This is because reproduction plays a central role in each evolutionary theory of aging. For this reason, experimental investigation of the link between the reproductive system and lifespan may provide a means to distinguish the different evolutionary theories of aging.

1.4.1. Mutation accumulation theory

The simplest evolutionary theory of aging is the mutation accumulation theory, first proposed by Medawar[137]. This theory posits that selective pressure, which is maximal until the beginning of reproduction, decreases as an organism ages due to an inherently higher chance of having died with time. This reduced selective pressure with age means that there is less selection against deleterious mutations whose effects only occur later in life. The "accumulation" of mutations in this theory does not refer to the frequency of mutations increasing with age, but rather that the genomes of any given species accumulates mutations that are not selected against because the phenotypic effect of these mutations occur too late in life. A classic example that fits with the mutation accumulation theory is Huntington's disease, in which a mutation in the HTT gene causes no obvious phenotype early in life but leads to fatal health problems that emerge later in reproductive life[138].

There is some experimental support for the mutation accumulation theory of aging. One prediction of the theory is that species with a high rate of extrinsic mortality - death by age-independent causes - should have shorter lifespans than those with low rates of extrinsic mortality. This is because species with high rates of extrinsic mortality are unlikely to live long enough to actually be affected by aging. There is no selective pressure against late-acting deleterious mutations in such short-lived species. This prediction has held true experimentally. One study compared multiple species of birds and found a direct correlation between extrinsic mortality in the wild and the rate of aging in captivity[139]. Another study in *C. elegans* showed that experimentally increasing extrinsic mortality leads to a decrease in average lifespan after just 12 generations[140]. These studies suggest that high rates of extrinsic mortality not only correlate with by also cause shorter lifespan, confirming one prediction of the mutation accumulation theory

A second prediction of the mutation accumulation theory is an accelerated force of mortality with age[137]. This is because the selective force against deleterious mutations become increasingly weak with age. This prediction is supported by the survival curves of familiar species, such as worms, flies, mice, and humans, which show increasing forces of mortality with age[141]. However, it is in complete contradiction to data from many other species, some of which show decreasing forces of mortality with age[141]. Such species include diverse forms of life such as *Gopherus agassizii* (desert

tortoise), the bird *Ficedula albicollis* (collared flycatcher), and the tree *Quercus rugosa* (netleaf oak)[141]. This suggests that the mutation accumulation theory alone is insufficient to explain the determination of lifespan in all species.

One serious limitation to the mutation accumulation theory that it does not clearly address why certain experimental interventions increase lifespan. As previously discussed, the status of the germline has a large effect on lifespan independent of any genetic change[2]. It is not obvious why the late-acting deleterious mutations would take longer to manifest as aging phenotypes in animals without a germline. This does not mean there is no merit to the mutation accumulation theory, but does suggest that other factors are at play in the evolutionary determination of lifespan.

1.4.2. Disposable soma theory

Another explanation for the evolution of lifespan is the disposable soma theory[142]. This theory posits that there is a finite level of resources that must be distributed between the soma, where they will be used for maintenance, and the reproductive system, where they will be used for progeny production. The lifespan of a species is a result of a trade-off between reproductive output and longevity.

The main piece of evidence supporting the disposable soma theory is that germ cell ablation extends lifespan[2,3,65]. Conversely, reproduction decreases lifespan[74]. These observations are explained by the disposable soma theory as due to an increase in resource availability to the soma after germ cell ablation, and a decrease in resource availability when reproduction occurs. The identity of the limiting resources has not been identified, making the confirmation of this explanation difficult.

Despite its use to explain the effect of germline ablation on lifespan, the disposable soma theory fails to explain the main requirements for this control. Germ cell ablation extends lifespan only in animals with an intact somatic gonad[2]. When germ cells are absent, resource use by the reproductive system is zero and therefore a longer lifespan would be expected regardless of the presence of somatic reproductive structures. One possible explanation is that germline-ablated worms behave as if they must devote resource to reproduction due to the alteration of signaling from the reproductive system. However, this is not consistent with the timing requirements of the IIS pathway for lifespan extension. The knockdown of *daf-16* specifically during adulthood abolishes the lifespan increase caused by germline ablation[65]. Presumably, resource use by the soma is altered not only by the presence of germ cells in adulthood but also by the initial development of the germline, which requires the generation of two-thirds of the cells of an adult animal. This suggests that the effect of the reproductive system on lifespan cannot be attributed completely to resource use.

The idea that resource use by the reproductive system limits lifespan is also inconsistent with the two mouse ovary transplant studies mentioned previously[4,66]. One of these studies observed that young ovaries continue to cycle after transplantation, while the original ovaries of the transplant recipients would have since ceased cycling[4]. In addition, investment in DNA damage repair, another potential resource sink, appears to be higher in the ovaries of younger mice compared to older mice[143]. These results suggest that the transplantation of younger ovaries into older mice may actually

increase resource use while simultaneously increasing lifespan. This is not consistent with the disposable soma theory.

1.4.3. Antagonistic pleiotropy theory

A third evolutionary theory of aging is the antagonistic pleiotropy theory. This theory posits that processes which increase fitness, such as early reproduction and fast development, are inherently linked to an increased rate of aging later in life. The reasoning behind this is that the positive fitness effect of early reproduction far outweighs the negative fitness effect of premature death later in life.

The antagonistic pleiotropy theory is supported by the observation that long-lived mutants often display trade-offs in reproduction or other characteristics that ultimately lower their fitness. *daf-2* mutant worms, which are long-lived, are quickly outcompeted by wild-type worms due to a slightly lower early reproductive output[144]. Long-lived *age-1* mutants, which are competitive with wild-type worms under standard laboratory conditions, are outcompeted by wild-type worms under conditions of periodic starvation[145]. In addition, a study of long-lived yeast mutants showed that many had reduced fitness compared to wild-type yeast[146]. These studies clearly demonstrate that some genetic alterations that extend lifespan have pleiotropic effects which reduce fitness. The disadvantage of these studies is that they rely on single gene mutations. Genes generally play roles in multiple processes. For instance, the *daf* genes in *C. elegans* play roles in the dauer stage of the life-cycle. Therefore, hypomorphs naturally have phenotypes that are not directly related to aging and which may affect fitness.

As with the disposable soma theory, the antagonistic pleiotropy theory has limitations in explaining the effect of the reproductive system on lifespan. The ablation of germ cells increases lifespan, even in genetically identical individuals[2]. The antagonistic pleiotropy theory would predict that this occurs because certain processes involved in germ cell production are linked to shortened lifespan. Exactly why this linkage should occur is not explained by the theory. Similarly, the antagonistic pleiotropy theory does not explain why young ovary transplantation increases lifespan[4,66]. Therefore, the antagonistic pleiotropy is currently insufficient to explain all aspects of lifespan control by the reproductive system.

1.4.4. Kin-selection theory of aging

A final evolutionary theory is the kin-selection theory of aging. The roots of this theory go back over 100 years to August Weismann[147]. Simply put, he proposed that animals die in order to make room for the next generation. This idea soon fell out of favor, and even Weismann himself abandoned it[148]. However, phenoptosis, the purposeful ending of life by an organism in order to benefit successive generations, is well accepted in particular cases. Certain conditions are thought to induce phenoptosis in salmon, yeast, and worms. Salmon die in spawning grounds shortly after reproduction. This die-off occurs both in species that make long upstream migrations to spawn and also in land-locked species, suggesting it is not simply due to exhaustion[149]. Death can be prevented by infection with pearl muscle larvae[150,151]. Infected salmon display considerably extended lifespan and are capable of repeated spawning[151]. The

fact that the death can be prevented while preserving reproduction suggests that this phenomenon is not simply a matter of exhaustion or resource use, but may actually be an adaptation to increase resource availability for the next generation of salmon[152]. An analogous phenomenon has been observed in yeast, in which a pathway similar to programmed cell death is activated at later ages and leads to increased nutrient release for successive generations[153]. Similarly, gravid *C. elegans* retain hatching eggs in response to starvation conditions, resulting in the worm being consumed by its progeny[154]. In all three cases noted, phenoptosis is likely a strategy to increase resource availability to the next generation. Such cases are often dismissed as being exceptions to the rule that only evolve under very specific conditions[155]. This raises the question: what are the conditions that give rise to phenoptosis?

Two recent reports have shown that the evolution of phenoptosis is theoretically favored given certain general conditions[156,157]. In both reports, the authors performed computer simulations of individual organisms competing for space. Each individual had either a normal allele or an aging allele. Individuals with the normal allele had a constant risk of death at any given age. Individuals with the aging allele had the same initial death rate as individuals with the normal allele, but were additionally subjected to an increasing risk of death with age. No other characteristic was conferred by either allele. Both reports found that the allele which accelerated the risk of death outcompeted the normal allele when two conditions were met: 1) reproductive lifespan must be sufficiently shorter than total lifespan - this ensures that post-reproductive adults compete for space with their offspring without any fitness benefit, and 2) population viscosity must be high - this means that individuals do not migrate too far away from their parent, and ensures that there is competition between generations for resources[156,157].

Not all species meet these conditions in nature. Some species, such as *Hydra magnipapillata* or the desert tortoise *Gopherus agassizii*, appear to have no end to their reproductive lifespan[141]. Interestingly, these two species do not display an increasing force of mortality with age[141]. However, *C. elegans* is consistent with both of the above conditions. The first condition is met for *C. elegans* since reproductive lifespan is about one week while total lifespan is about two weeks[12,158]. In addition, developmental time is only three days, leading to overlap of multiple generations in the same culture[159]. The second condition may be met for *C. elegans* due to their natural ecology. Wild *C. elegans* reproduce on isolated food sources such as rotting fruit[160]. This may lead to localized populations of genetically related individuals, ensuring intergenerational competition. Their consistency with the two above conditions make *C. elegans* an excellent candidate for development of phenoptosis.

Besides being candidates for phenoptosis, *C. elegans* are also favorable candidates for the evolution of altruistic characteristics in general. The selective advantage of altruistic behavior is determined by genetic relatedness between individuals[161]. Wild isolates of *C. elegans* show relatively little genetic variation with one another compared to other species[162]. The amount of genetic variation that a wild population of worms encounters while expanding on a food source may be particularly low. This is because the wild *C. elegans* life cycle is characterized by primarily hermaphroditic reproduction and repeated bottlenecks due to the colonization of new

food sources by individual worms[163]. The high level of genetic relatedness between worms in the wild increases the favorability of altruistic behavior.

1.4.5. Experimentally distinguishing between evolutionary theories of aging

Evolutionary theories of aging can be evaluated based on their ability to explain experimental interventions that increase lifespan. Reproduction plays a central role in each evolutionary theory of aging discussed in this section. This suggests that lifespan extension by germline ablation may be particularly useful in differentiating between the different theories. The mutation accumulation theory does not offer an obvious explanation for the influence of the reproductive system on lifespan, so it will be ignored in this section.

A fundamental difference between the remaining evolutionary theories of aging is the type of trade-off each uses to explain the influence of the reproductive system on lifespan. The disposable soma theory posits that aging is a result of a trade-off in resource use between the reproductive system and soma of an individual organism. Antagonistic pleiotropy theory posits that aging is the result of a trade-off between early reproduction and longevity in an individual organism. Both theories predict that it is impossible to increase lifespan through manipulation of the reproductive system without decreasing or delaying reproduction. In stark contrast, the kin-selection theory of aging posits that programmed aging is a result of a trade-off between individual- and population-level fitness. Therefore, this theory allows for the possibility that manipulation of the reproductive system may increase both lifespan and reproduction simultaneously.

1.5. How is phenoptosis related to lifespan control by the reproductive system?

C. elegans meets the theoretical conditions that favor the evolution of phenoptosis. However, the general concept of phenoptosis does not explain why the status of the reproductive system influences lifespan. The reports discussed above, which demonstrate the theoretical feasibility of phenoptosis, relied on relatively simple simulations based on alleles that increase the risk of death with age[156,157]. Feedback between the reproductive system and lifespan was not considered. However, it makes logical sense that such feedback would play a role in phenoptosis, as the selective benefit of phenoptosis is gained by limiting post-reproductive lifespan. If feedback were introduced into a mechanism of phenoptosis, then an organism could display an increased chance of death specifically once it reaches the end of its reproductive life. Such a system would ameliorate the problem of premature aging before the end of reproduction. This might be especially important in *C. elegans*, which shows variation in reproductive lifespan even amongst genetically identical individuals raised in identical environments[164].

A system that provides feedback between reproductive status and lifespan may improve the fitness benefit of phenoptosis. For such a system to exist, there would have to be some way for an organism to determine when it has reached post-reproductive life. The simplest strategy would be for an organism to detect when it has stopped reproducing. However, this is clearly not the case, as germ cell ablation, starvation, and entry into dauer all halt reproductive output and would therefore be expected to reduce

lifespan. In reality, all three of these conditions increase lifespan dramatically[2,165,166]. In addition, halting reproduction without the removal of germ cells does not increase lifespan in worms[73]. What is being used to determine when post-reproductive life has been reached? Germ cell ablation is particularly important to consider when answering this question. Whatever is being used as a readout of reproductive status, it is presumably detected as low in individuals lacking a germline.

It is also important to consider that an organism may benefit from determining not only when it is post-reproductive, but also when offspring quality begins to precipitously decrease. Many organisms show a decline in offspring quality with age. For example, the offspring of older worms produce fewer progeny than the offspring of younger worms[167]. In *Arabidopsis*, increased parental age correlates with an increase in the incidence of certain types of somatic mutations[168]. In humans, the incidence rates of a number of disorders increase with parental age. For example, the incidence of Down syndrome and Apert syndrome increases with maternal and paternal age, respectively[169,170]. It would not benefit an organism to continue producing progeny if the health of those progeny drops below a certain level. Producing unhealthy progeny might actually be detrimental, as they may compete with healthy progeny produced earlier in life. For this reason, an ideal readout of the state of the reproductive system would also give an indication of the quality of progeny being produced, as well as remaining reproductive potential.

What characteristic of germ cells might be used as a readout for both reproductive potential and progeny quality? Up until this point, most experimental manipulations performed to investigate how the reproductive system controls lifespan have been limited to either ablating germ cells or halting germline stem cell cycling - an intervention which ultimately decreases germ cell number. Very few studies have manipulated the characteristics of germ cells and measured the effect on lifespan. Notable exceptions are the two studies, discussed previously, in which ovaries were transplanted between mice of different ages[4,66]. Both studies reported that transplanting young ovaries into older mice leads to increased lifespan[4,66]. These results suggest that the age of the reproductive system has some role in lifespan control. To put this another way, some characteristic of the reproductive system is increasing with age, and it is this characteristic that decreases lifespan. According to this explanation, the removal of germ cells increases lifespan due to the absence of this age-dependent characteristic.

One characteristic that increases with age in germ cells is the level of DNA damage. Age correlates with increased DNA damage in the sperm of rodents[171]. Age also correlates with increased DNA fragmentation in human sperm[172]. Not only does DNA damage increase with age, but this increase may be partially responsible for the decline in male fertility with age[173]. DNA damage may also lead to mutations, which are the cause of certain disorders whose incidence rates increase with parental age[169,170]. This makes DNA damage a particularly attractive candidate to use as a readout of reproductive potential. Perhaps the detection of DNA damage in germ cells leads to the activation of an aging program in individual organisms.

1.6. Structure of the DDR pathway in *C. elegans*

Knowledge from previous reports has led to the hypothesis that the detection of DNA damage in germ cells leads to the truncation of lifespan. In order to experimentally test this, it is necessary to understand the structure of the DDR pathway. The DDR pathway is responsible for the detection, repair, and downstream response to DNA lesions. This pathway is very complex, due in part to the variety of damage types that DNA can incur. Separate branches of the pathway are responsible for the repair of different types of lesions, although some redundancy exists between certain branches. In this section I will give a brief introduction to the major branches of the DDR pathway, focusing on those branches that are most relevant to this thesis. When possible, I will focus on the structure of the DDR pathway in *C. elegans*.

1.6.1. Homologous recombination repair

Double-stranded DNA breaks (DSB) can be induced by exogenous factors such as ionizing radiation (IR). In addition, DSB can be formed through intrinsic events such as replication over an unresolved single-stranded nick, replication fork collapse, or fragile site breakage[174]. DSB are particularly harmful to cells because their presence during cell division can result in the loss of genetic material. This is because any genetic material not bound to a centromere may fail to segregate to either daughter cell.

There are multiple pathways that are capable of repairing DSBs. One such pathway is Homologous Recombination (HR)[175]. HR is initiated when PARP1 and the MRN complex localize to a DSB and resect the surrounding DNA[175]. *pme-1* is the worm homolog of mammalian PARP1[176]. In response to DNA damage, this enzyme converts NAD+ molecules into ADP-ribose polymers[177]. These polymers interact with other components of the DDR[177]. The MRN complex in mammals is composed of Mre11, Rad50, and Nbs1[178]. The respective worm homologs are *mre-11* and *rad-50*, although the homolog of Nbs1 has yet to be identified[179,180]. The MRN complex binds the free ends of DSBs and anchors additional damage response proteins to these sites, resulting in DNA resection[178]. The MRN complex is also involved in the phosphorylation of human ATM, a protein which will be discussed at length in a later section[181]. The homolog of ATM in worms is *atm-1*[182]. DNA resection is followed by the recruitment of RPA, the homolog of worm *rpa-1*, to the resulting single-stranded DNA[183]. RPA is replaced by RAD51 and a Holliday junction forms between the damaged DNA strand and a matching DNA sequence, usually belonging to the sister chromatid[175,184]. The homolog of RAD51 in worms is *rad-51*[185]. Resolution of the Holliday junction results in the incorporation of sections of the sister chromatid into the repaired DNA sequence, which is why HR is referred to as homologous recombination.

The reliance by HR on Holliday junction formation has advantages and disadvantages. The advantage is that damaged nucleotides will be replaced by synthesis using a homologous template. The result is that HR repair is very accurate. Accurate DNA repair is very important in germ cells, as any errors that arise during repair may become mutations in the next generation. The disadvantage of the use of Holliday junction formation is that it requires the presence of a sister chromatid. This restricts the use of HR to G2- and S- phase cells. Germ cells in the MZ of *C. elegans*

use HR for the repair of DSBs[186]. The importance of HR repair in the germ cells of *C. elegans* is illustrated by a number of germline phenotypes associated with the mutation or knockdown of HR genes. The mutation of *mre-11* or the knockdown of *rad-51* or *rad-50* results in increased apoptosis in the germline in response to ionizing radiation, presumably due to reduced DNA repair efficiency[187-189].

1.6.2. Non-homologous end joining repair

An alternative to the HR pathway for the repair of DSB is non-homologous end joining (NHEJ) repair. NHEJ repair in *C. elegans* is initiated by the binding of Ku70 and Ku80 to the broken ends of DNA[190]. *cku-70* and *cku-80* are the worm homologs of mammalian Ku70 and Ku80[186]. Ku protein binding is followed by the recruitment of several other repair factors. The identity of several of these factors has not yet been elucidated in worms[175]. In humans, one of these factors is the DNA-protein kinase encoded by the PRKDC gene, which activates several other proteins through phosphorylation in order to prepare the broken DNA ends for repair[191]. Another recruited factor is Xrcc4[192]. Xrcc4 recruits Ligase IV, the homolog of the *C. elegans lig-4*[193,194]. Ligase IV, as its name implies, ligates the two broken DNA ends, resulting in resolution of the DSB[195].

The NHEJ is primarily used in the somatic cells of *C. elegans*. Late-stage NHEJ mutant worm embryos display higher rates of lethality in response to gamma irradiation compared to wild-type worms[186]. In contrast, irradiated late-stage HR mutant worm embryos do not display increased lethality compared to wild-type[186]. This suggests that the somatic cells of worms rely primarily on NHEJ to repair DSBs. While HR mutants display decreased progeny survival in response to adult irradiation compared to wild-type, NHEJ mutant worms do not display such an increase[186]. This suggests that germ cells rely primarily on HR, as opposed to NHEJ, for DSB repair. However, there is some evidence that *C. elegans* germ cells maintain the ability to activate NHEJ repair. NHEJ is active in the germ cells of inter-strand crosslink repair mutants[196]. In addition, inactivation of NHEJ by *cku-80* knockdown leads to increased reliance on HR pathway during genome modification by CRISPR[197]. This suggests that NHEJ can be used by the germ cells of *C. elegans* under certain circumstances, such as when HR repair is unavailable.

1.6.3. Alt-NHEJ repair

In addition to HR and NHEJ, a third DSB repair pathway, atl-NHEJ, exists in many organisms. atl-NHEJ is used in some organisms when repair by the canonical NHEJ pathway is not possible. As with HR repair, alt-NHEJ starts with PARP1 and the MRN complex localizing to DSB sites[198,199]. The endonuclease CtIP is then recruited, leading to processing of the DNA around the DSB in preparation for repair[200]. The homolog of CtIP in worms is *com-1*[201]. DNA ligase III the joints the two free DNA ends, resolving the DSB[202]. It is not clear that this pathway exists in *C. elegans* as ligase III has no known homolog in worms[203].

1.6.4. Nucleotide excision repair

Another type of DNA damage that cells encounter is the chemical modifications of nucleotides, which can lead either stalled replication forks or mutations due to misreading by the DNA replication machinery. There are many types of chemical modifications that can occur. Covalent links between adjacent nucleotides can be induced by UV light exposure, which generates cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts[204]. Chemical mutagens can also alter DNA, leading to individual modified nucleotides such as cyclopurines[205]. These types of lesions can be repaired by the Nucleotide Excision Repair (NER) pathway.

NER is initiated when helix-distorting lesions are recognized by the XPC complex, which in worms includes RAD-23 and the DNA-binding protein XPC-1[206-208]. The DNA surrounding the lesion is then unwound and the resulting ssDNA is bound by XPA-1 and RPA-1[207,208]. This allows for excision of the damaged strand and synthesis of new nucleotides based on the undamaged strand.

The NER pathway is active in both the soma and germline of worms. The importance of NER in *C. elegans* germ cells is illustrated by several studies. *xpa-1* mutants display an increase in spontaneous mutation rate over multiple generations[209]. Knockdown of multiple NER genes, including *xpa-1* and *xpc-1*, leads to decreased rates of germ cell apoptosis after UV-C exposure, suggesting decreased detection of damage[208]. The somatic activity of the NER pathway is illustrated by the decreased survival rate of *xpa-1* mutant worms after UV-C exposure, compared to wild-type worms[210].

1.6.5. Base excision repair

Chemically modified nucleotides that are not helix-distorting are preferentially repaired by the base excision repair (BER) pathway[206,211]. This is in contrast to NER, which is preferentially utilized to repair helix-distorting chemical modifications of nucleotides[206]. Non-helix-distorting lesions can be generated by oxidative damage, which can be induced by exposure to mutagens such as IR or to reactive oxygen species generated during normal respiration[212]. Oxidatively damaged nucleotides can cause incorrect nucleotide incorporation by the DNA replication machinery, if such lesions are present during S-phase[212]. For instance, guanine can be oxidized to 8-oxoguanine, which pairs with adenine[212]. This can lead to to G->T transversion during DNA replication[212]. This type of oxidative damage can be repaired by the BER pathway, alhtough NER also plays a role in repairing oxidative DNA damage[213].

The molecular mechanism behind BER varies depending on the type of chemical modification being repaired. These mechanisms can be divided into short- and long-patch BER, which refers to the length of the nucleotide sequence which is replaced during repair[214]. Both pathways are initiated by the glycosylation of the damaged nucleotide, which can be carried out by a number of different enzymes depending on the identity of the lesion[215]. This is followed by the removal of nucleotides from the damaged strand. This removal involves XRCC1 or FEN for short- or long-patch BER,

respectively[216,217]. Replacement of the excised sequence is then carried out by DNA polymerase and either ligase III or ligase I for short- or long-patch BER, respectively[218].

Many of the genes known to be involved in BER in other systems have no known homologs in *C. elegans*[219]. Two BER enzymes with known homologs in worms are *nth-1*, homolog of the human glycosylase hNTH, and *crn-1*, homolog of FEN1[220,221]. BER activity occurs in both somatic and germline cells in *C. elegans*. Mutation of the BER gene *nth-1* results in an increased rate of spontaneous mutation between generations, suggesting that this pathway plays a role in germ cells[209]. Knockdown of *nth-1* results in increased oxidative stress in somatic cells, suggesting that the pathway also plays a role in somatic cells[124].

1.6.6. Mismatch repair

Mutations can be generated by the misincorporation of nucleotides due to errors during DNA replication or repair. This can take the form of either mismatched nucleotides within a single base pair or short deletions or insertions forming small helix-distorting bulges[222]. These mutagenic sites are repaired through the Mismatch Repair (MMR) pathway.

The MMR can proceed through a number of different mechanisms, depending the nature of the mismatch being repaired[223]. However, all mechanisms involve the recognition of mismatched nucleotides by MSH2 and MSH6[223,224]. This is followed by the recruitment of MLH1 and PMS1[225]. The MLH1-PMS1 complex activates the endonuclease EXO1, which excises the mismatched nucleotides[226]. The excised nucleotides are then replaced by polymerase delta[227].

MMR is known to be active in the germline and soma of *C. elegans*. The homologs of MSH2, MSH6, and MLH1 in worms are *msh-2*, *msh-6*, and *mlh-1* respectively[223,228,229]. The mutation of *msh-2*, *msh-6, or mlh-1* leads to an increased rate of spontaneous mutations between generations, suggesting a role for MMR in the germline[209,229]. The knockdown of *msh-2*, *msh-6*, or *msh-1* leads to an increased rate of somatic mutation, based on a study utilizing a transgenic mutation reporter strain[229].

1.6.7. Interstrand cross-link repair

Another form of DNA damage is the covalent cross-linking of nucleotides on complementary strands of DNA. This is especially problematic because it prevents the separation of complementary DNA strands, which can lead to stalled replication forks during S-phase or broken chromosomes during M-phase. Interstrand cross-links can form due to exposure to chemicals that are both naturally occurring, such as psoralens or nitrous acid, or artificial, such as nitrogen mustard[230]. They are repaired through the Interstrand Cross-link (ICL) repair pathway, which is defective in Fanconi anemia patients[230].

The ICL pathway begins with the detection of cross-link and the recruitment of the FA core complex, a process which involves ATR activation[231]. DNA on both sides of the crosslinked nucleotides are cleaved by the endonuclease complexes XPF-ERCC1 and MUS81-EME1[232,233]. DNA repair then proceeds through resection, strand-invasion, and holiday junction resolution, similar to HR repair[231].

The ICL pathway is active in the germ cells of C. *elegans*. Exposure to psoralen, a compound that induces interstrand crosslinks, leads to foci formation by FCD-2, the worm homolog of FANCD2, and RAD-51 on the DNA of mitotic region nuclei[62]. In addition, mutation of the FANCJ homolog *dog-1* causes a mutator phenotype in worms[234,235].

1.6.8. Stalled replication fork resolution

Many types of DNA lesions, such as inter-strand cross links, can lead to replication fork stalling[236]. In addition, replication forks can stall at certain repetitive sequences or at complex DNA secondary structures[236]. While replication forks themselves are not a form of damage, unresolved replication forks are particularly harmful to cells as they can result in incomplete chromosome replication and failure to properly segregate DNA during mitosis. The DDR pathway includes a mechanism for stabilizing stalled replication forks, activating the checkpoint response to prevent cell progression, and resolving the replication fork.

Normal replication forks have the DNA binding protein RPA bound to their exposed ssDNA. When replication fork stalling occurs, there is an accumulation of RPA, due to the continued unwinding of DNA while new strand synthesis is stalled. The RPA homolog in worms is *rpa-1*[237]. The kinase ATR is then recruited to the stalled replication fork[238]. The ATR homolog in worms is *atl-1*[239]. ATR phosphorylates rad17, causing it to load the 9-1-1 protein complex onto DNA near the replication fork[240]. The worm homolog of the clamp loader rad17 is *hpr-17*[241]. The 9-1-1 complex is a ring composed RAD9, RAD1, and HUS1, homologs of the worm genes *hpr-9*, *mrt-2*, and *hus-1*, respectively[242]. This process leads to checkpoint activation by ATR, repair of the original damage, and ultimately resolution of the replication fork.

Stalled replication fork repair plays an important role in the germline of *C. elegans*. Mutation of *mrt-2* or *hus-1* results in an increased rate of mutations between generations[243]. In addition, *hus-1* is primarily expressed in the germline of adult worms, based on a transgenic reporter strain[244]. Stalled replication fork repair is not utilized in the somatic cells of adult *C. elegans*. Adult somatic cells are post-mitotic, so replication forks are not formed.

1.6.9. Translesion synthesis

An alternative mechanism to overcome stalled replication forks is translesion synthesis. Translesion synthesis has the advantage of not activating a checkpoint response. However, it does not actually repair the original lesion which caused replication fork stalling. Instead, the lesion is bypassed using a polymerase which tolerates damage to the template strand[245].

During development, the somatic cells of *C. elegans* utilize translesion synthesis rather than replication fork repair[246]. This may be in order to prevent delays in cell division, which could potentially disturb developmental timing[246]. Replication fork repair involves the activation of an ATR-dependent checkpoint response, while translesion synthesis does not. The mutation of genes involved in translesion synthesis severely interferes with the ability of developing *C. elegans* to cope with DNA damage[247].

Knockdown of *polh-1*, an ortholog of the polymerase POLH using in translesion synthesis, results in increased embryonic lethality in response to UV exposure[247].

1.7. The checkpoint proteins ATM and ATR

There are many different branches of the DDR pathway, many of which are active in *C. elegans* germ cells. Despite the complexity of the DDR pathway, most

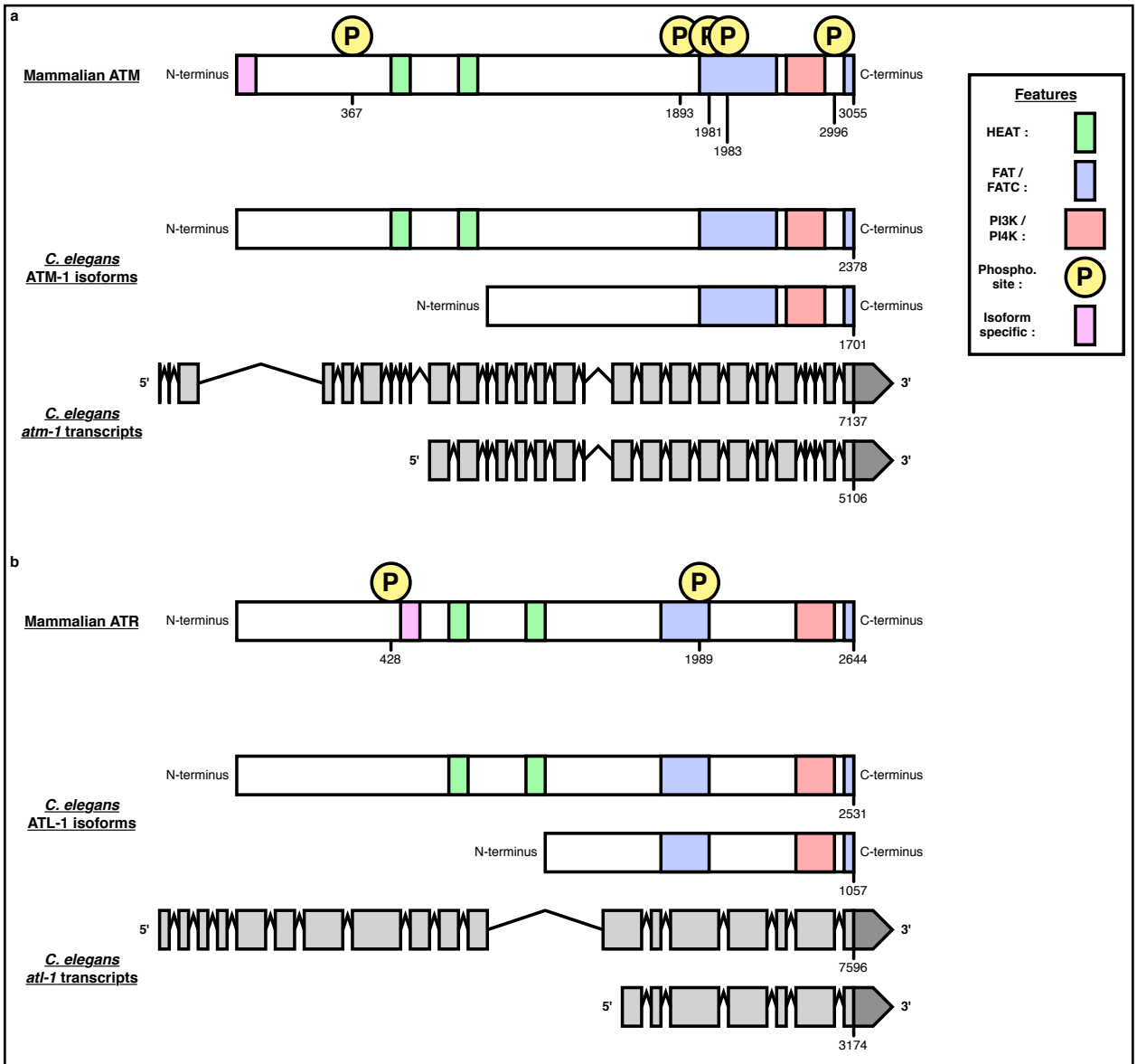

Figure 2: Structure of ATM and ATR.
The overall structure of **a)** ATM and **b)** ATR. For each, experimentally verified domains and phosphorylation sites for the mammalian protein are included. Peptide positions are given based on the human protein. The locations of domains on the *C. elegans* proteins are based on amino acid identity to the human proteins. The isoform-specific regions of the mammalian proteins are based on pig ATM and humans ATR.

branches lead to the activation of the downstream checkpoint proteins ATM (Ataxia Telangiectasia Mutated) and ATR (Ataxia Telangiectasia and Rad3-related), homologs of the C. elegans genes *atm-1* and *atl-1*[248]. ATM and ATR are both serine/threonine protein kinases which are themselves phosphorylated during DDR activation[249,250]. They in turn phosphorylate a number of downstream proteins, providing a link between upstream damage detection and repair and downstream cellular responses[248].

1.7.1. Cellular level responses to checkpoint activation

One important cellular-level change induced by ATM and ATR activation is the upregulation of certain genes involved in the DDR pathway itself[251]. ATM can directly activate the transcription factor p53[252,253]. Activation of ATM and ATR leads to the phosphorylation of a large number of proteins involved in the control of gene expression[254]. This feedback presumably results in more efficient repair of future DNA damage due to the increased expression of repair genes.

Another response to DDR activation is cell cycle arrest. Some DNA lesions can create devastating problems to the genome if present during DNA replication or cell division, as discussed previously. It is therefore advantageous for the cell to stop cycling upon the detection of DNA damage. Checkpoint activation is accomplished by the phosphorylation of Chk1 and Chk2 by ATM and ATR, respectively[248]. The worm homologs of Chk1 and Chk2 are *chk-1* and *chk-2*[255,256].

A final response to DDR activation is apoptosis. Apoptosis can be triggered by the phosphorylation of p53 by ATM and ATR[248]. Apoptosis may be preferable to repair in some situations, as incorrectly repaired DNA damage can lead to mutations and cancer in many organisms. DDR induced apoptosis does not occur in *C. elegans* somatic cells, even in response to high levels of exogenous DNA damage[257]. However, apoptosis is a very common fate for *C. elegans* germ cells[258]. It is estimated that 50% of germ cells ultimately undergo apoptosis under normal conditions[258,259]. This rate can be further increased by exogenous DNA damage[260]. Apoptosis in the C. elegans germline is limited to cells in the pachytene zone (PZ)[261].

1.7.2. Interaction between ATM and ATR

ATM and ATR are activated by different branches of the DDR pathway[248]. During replication fork stalling, the 9-1-1 complex binds to stalled replication forks. This binding is dependent on ATR and eventually leads to its activation[262]. Conversely, when DSB are induced by ionizing radiation, the MRN complex is activated at the site of damage, resulting in the activation of ATM[263]. In addition, ATM and ATR have unique downstream targets. For example, ATM and ATR phosphorylate Chk1 and Chk2, respectively[248].

While each have independent functions, ATM and ATR also interact with one another. The activation of ATM is dependent on ATR in some contexts[264,265]. In other contexts, activation of ATR is dependent on ATM[266,267].

1.7.3. Structure of ATM and ATR

ATM and ATR have similar overall protein structures[268]. A diagram of the structure of each protein in mammals and *C. elegans*, as well as the worm transcripts can be found in Figure 2. The N-terminal halves of both proteins contain several HEAT domains. HEAT domains are involved in protein-protein interactions[269]. The HEAT domain of Mec1, the yeast homolog of ATR, interacts with Lcd1, the homolog of ATRIP[270]. ATRIP is important for the G2/M phase checkpoint response[271,272]. The HEAT domain of ATM interacts with the MRN complex during DSB repair[273]. This suggests that the N-terminal halves of both ATM and ATR may play an important role in both DNA damage repair and the checkpoint response.

Both ATM and ATR contain a FAT domain and a related C-terminal FATC domain. The FAT domain contains an important phosphorylation site, which will be discussed below. The FATC domain is important for the regulation of kinase activity[274]. In addition, mutation of the FATC domain in Tel1, the yeast homolog of ATM, prevented the recruitment of the protein to DNA[275]. The kinase activity of ATM and ATR is carried out by conserved PI3K domains[276].

Both ATM and ATR contain several phosphorylation sites that are important for their activation. The phosphorylation of ATR on threonine-1981, which lies within the FAT domain, is particularly important for its activation in response to DNA damage[250]. Checkpoint activation fails to occur when this phosphorylation site is mutated[277]. DNA damage also induces the phosphorylation of serine-428 on ATR[278]. However, this residue is dispensable for checkpoint activation[277]. The phosphorylation of ATM on serine-1981 is induced by DNA damage[279]. Phosphorylation also occurs on serine-367 and -1893 in response to DNA damage[280]. Both of these phosphorylation sites are required for efficient DNA damage repair after irradiation[280].

1.7.4. Tissue specificity of ATM and ATR isoforms

There is evidence that multiple isoforms of ATR exist. One study looked at cDNA from various human tissues found two isoforms of ATR[281]. Some tissues contained a truncated isoform of ATR, which lacked one of the 5' exons[281]. This truncation removed 65 amino acids that lie towards the N-terminus of the protein[281]. However, no functional significance for this alternative splicing was determined[281].

There is also evidence that multiple isoforms of ATM exist. Multiple alternatively-spliced transcripts of ATM are present in pigs, based on studies of cDNA libraries[282]. Some of these alternative transcripts lack the canonical ATM start site, resulting in a truncated protein[282]. The resulting truncated isoforms lacked a key protein-binding domain involved in the activation of p53 and BRCA1[282]. Expression of the different isoforms varied between tissues, although the functional significance of this was not determined[282]. Alternative splicing of ATM transcripts also occurs in humans[283]. However, the described splice variants only affect the 5' untranslated region[283]. Different isoforms of the ATM protein have not been described in humans.

1.8. Known effects of DNA damage and DDR genes on lifespan

The central hypothesis of this thesis is that the level of DNA damage detected in germ cells controls lifespan. There are two obvious experimental strategies to test such a hypothesis: 1) decrease expression of DDR genes, in order to decrease the detection of damage, and 2) induce exogenous DNA damage, in order to increase the amount of damage that can be detected. In this section, I will review data which is directly relevant to both of these experimental strategies.

1.8.1. Loss of DDR genes

Disturbance of the DDR pathway has been shown to limit lifespan in a number of species. As discussed previously, many human progeroid syndromes are characterized by mutations in DNA damage repair genes[284]. This suggests that removing components of the DDR pathway may decrease lifespan. The mutation of a number of DNA damage repair genes also leads to reduced lifespan in worms. The loss of *xpa-1*, the homolog of XPA whose mutation is responsible for some forms of Xeroderma pigmentosum syndrome in humans, leads to decreased lifespan in *C. elegans*[124]. Similarly, the loss of *wrn-1*, homolog of WRN whose mutation is responsible for Werner Syndrome in humans, also leads to decreased lifespan in worms[285]. The fact that these mutations decrease lifespan is interesting because the somatic cells of adult worms do not undergo apoptosis and are post-mitotic. This suggests that DDR gene mutation can reduce lifespan independent of changes in somatic cell cycling rate, cell death, or exhaustion of stem cell pools. The simplest explanation for the lifespan effect of the DDR gene mutation is that it is a direct effect of decreased DNA damage repair efficiency.

It is not surprising that the mutation of DDR pathway genes often decreases lifespan. However, certain genetic manipulations of the DDR pathway actually increase lifespan in *C. elegans*. The lifespan of worms is increased by a reduction-in-function mutation of *hus-1*[286]. The lifespan of *daf-2* mutant worms is increased by knocking down *cku-70*, although the same effect is not seen in a wild-type background[287]. *atl-1* mutants live longer than wild-type worms[288]. These reports suggest that in some contexts, decreased expression of certain components of the DDR pathway actually increases lifespan.

These seemingly contradictory data lead to the question: why does the abrogation of some DDR components increase lifespan, while for other components this decreases lifespan? It is particularly interesting that *atl-1* mutation increases worm lifespan[288]. Mutation of the homologous gene in mice, ATR, leads to premature symptoms of aging and a decrease in lifespan[289]. One possible explanation for this difference is that *atl-1* and ATR have slightly different functions in worms and mice, respectively.

One reason that *atl-1* and ATR may have different functions is that the somatic cells of adult worms are post-mitotic, while mitosis continues in the soma of mice throughout life. For this reason the checkpoint function of *atl-1* may be dispensable in the somatic cells of worms, while this same function is required in the somatic cells of mice. There is some evidence that the checkpoint activation exists in the germline but

not the soma of adult worms. The activity of ATL-1 and ATM-1, which are both serine/threonine kinase, can be measured by antibody staining against phosphorylated S/TQ amino acid motifs. pS/TQ staining is dramatically increased in the germ cells but not the somatic cells of worms after irradiation[244]. There are some caveats in interpreting these data, which will be discussed in the results section of this thesis. Nevertheless, these data suggest that checkpoint function does not occur in the somatic cells of worms. The mutation of *atl-1* may increase worm lifespan due to its primary effect being the removal of checkpoint activity in germ cells. The mutation of ATR, on the other hand, may decrease mouse lifespan due to the loss of somatic checkpoint activity, which is harmful for cycling cells. This raises the possibility that the inactivation of genes in the soma and germline may have opposing effects on lifespan.

1.8.2. Exogenous DNA damage and radiation hormesis

The DNA damage theory of aging predicts that an increase in DNA damage will result in a decrease in lifespan. As discussed previously, many progeroid syndromes are accompanied by a decreased efficiency of DNA damage repair[284]. Exogenous DNA damage can also be induced by radiation. Mice exposed to moderately-high doses of gamma radiation have decreased lifespans[290]. This supports the idea that increased DNA damage leads to decreased lifespan. However, other studies that have shown that irradiation can lead to increased lifespan. This phenomenon is referred to as radiation hormesis. The general concept of hormesis is that exposure to a low dose of a normally toxic stress can have beneficial health effects. The hormetic response to heat and oxidative stress were discussed in a previous section. In this section I will discus the hormetic response to radiation. Radiation hormesis is important in the context of this thesis because of the connection between radiation and DNA damage.

There are several reports supporting the existence of radiation hormesis in model organisms. Gamma irradiation of the Caribbean fruit fly *A. suspensa* leads to a significant increase in lifespan[291]. Interestingly, this effect is sex dependent - only female flies display a hormetic lifespan increase. Radiation hormesis has also been observed in *Drosophila melanogaster* in response to gamma irradiation[292,293]. There is also evidence that radiation hormesis can occur in mammals. Low-doses of gamma rays have been observed to significantly increase lifespan in female mice[294].

A few studies support the existence of radiation hormesis in humans. One study of survivors of the atomic bombing of Nagasaki found that individuals who were a sufficient distance from the epicenter of the blast had lifespans that were significantly longer than the general population that was not exposed to the blast[295]. Another study showed that nuclear shipyard workers, who are exposed to about three times the level of ionizing radiation as the general population, have reduced mortality compared to other shipyard workers[296]. In addition, the prevalence of certain types of cancer have been shown to be lower in individuals subjected to low doses of radiation[297]. This suggests that the phenomenon of radiation hormesis may exist in mammals as well as invertebrates.

What little is known about the genetics of radiation hormesis comes from studies in *Drosophila*. One study looked at lifespan after low-dose gamma irradiation in several different mutant backgrounds[292]. Lifespan extension in response to radiation was absent

in *foxo* mutants, implicating the IIS pathway in this phenomenon[292]. Another study looked at gene expression at several time-points following irradiation[293]. The heat-shock protein *hsp70* was upregulated for at least three days following irradiation[293]. These findings are interesting when compared to data from heat shock induced hormesis. As mentioned previously, heat shock induced hormesis in worms is dependent on *daf-16*, a homolog of *foxo*[59]. Heat shock also leads to upregulation of *hsp70* in flies[57]. These two results hint that there may be some commonalities in the genetics behind the hormetic responses to heat and radiation.

C. elegans would be a very useful system in which to tease apart the genetic mechanisms behind radiation hormesis. Unfortunately, evidence supporting the existence of radiation hormesis in *C. elegans* remains very weak. Gamma irradiation was reported to increase the lifespan of worms in one study[8]. However, the effect was inconsistent and only occasionally significant, as acknowledged by the authors[8]. Another study reported no increase in worm lifespan after either gamma or UV irradiation[9]. It is surprising that radiation hormesis has been consistently observed in flies but not in worms, especially considering that heat shock induced hormesis has been observed in both species[57,58]. Why is radiation hormesis observed in flies and mice, but not in worms?

One way in which *C. elegans* are different from flies and mice is that the reproductive system comprises the majority of cells in worms. An adult *C. elegans* contains around 3,000 cells, two thirds of which are germ cells[298,299]. It is not currently known whether radiation hormesis occurs through an effect on germ cells or somatic cells. This means that, all other things being equal, the relative dose of radiation absorbed by the germline compared to the soma in worms is much higher than that of mice or flies. I discussed in a previous section the idea that worms may actively truncate their lifespan in response to DNA damage in the germline. The negative lifespan effect of germ cell irradiation may cover up any hormetic lifespan extension.

If the irradiation of germ cells decreases lifespan, this implies that any lifespan increase observed after irradiation would have to be due to an effect on somatic cells. This leads me to propose that the lifespan effect of irradiation is determined by two contradictory effects: 1) DNA damage induced in germ cells leads to decreased lifespan, due to signaling from the germline to the soma, and 2) DNA damage induced in somatic cells leads to increased lifespan, due to the induction of a stress response. The mechanism behind either of these effects is currently unknown. However, the demonstration of radiation hormesis in *C. elegans* would offer researchers a new tool in which to investigate these mechanisms.

1.9. Central hypothesis and outline of this thesis

In this thesis I will attempt to explain the effect of the reproductive system on aging. For reasons explained above, germline DDR is chosen as a possible factor that determines the effect of germ cells on lifespan. I will also attempt to clarify the effect of DNA damage on lifespan. DNA damage is typically thought of as detrimental to lifespan. However, the phenomenon of radiation hormesis seems to conflict with this idea. I will resolve this conflict using a model which differentiates between the lifespan effects of DNA damage in the germline and soma. The central hypotheses of this thesis are: 1)

germline DDR decreases lifespan, 2) this lifespan affect is not due to an effect on reproductive output, and 3) somatic DNA damage can hormetically increase lifespan.

I will use the model organism *C. elegans*. There are several characteristics of this model that make it ideally suited for this thesis. The first is that many of the mechanisms behind lifespan determination are conserved between *C. elegans* and higher organisms. The second is that their short lifespan and ease of handling make large-scale lifespan experiments practical. The third is that the wide range of genetic tools available allow for many of the experiments that will be presented in this thesis.

The general results of this thesis are presented in the following order: 1) I begin by showing that DNA damage and DDR signaling increase in germ cells with age, 2) I show that increasing DNA damage in germ cells through physical means decreases lifespan, 3) I show that irradiation of somatic cells leads to a hormetic increase in lifespan, 4) I show that decreasing DDR in germ cells using genetic means increases lifespan, 5) I show that this lifespan increase is not due to a change in reproductive output, 6) I show that decreasing DDR in somatic cells using genetic means decreases lifespan, and 7) I elaborate on the molecular mechanism behind my proposed model for the regulation of lifespan by germ cells.

CHAPTER 2: Materials and methods

ATM-1 and ATL-1 domain prediction

Approximate domains locations for were predicted for ATM-1 and ATL-1 by aligning each known domain from human and mouse ATM and ATR to the full length *C. elegans* homolog. Protein alignment was carried out using EMBOSS Needle through the EMBL-EBI website.

Nematode maintenance and lifespan assays

Worms were maintained as described using *E. coli* strain HB101 as a food source, and kept at 20°C unless otherwise specified[300]. Details of the worm strains used are provided in Table 1. Lifespan was scored as described, censoring worms that bagged, “exploded”, or crawled off their plate from the day at which the corresponding event occurred[51]. Curves for a single experiment are displayed for all figures. Data for all survival experiments are provided in Tables 3-9.

Immunohistochemistry

Immunohistochemistry was performed modified versions of previously described procedures[301]. Extrusion and fixation with 1.6% PFA was carried out for staining of RPA-1::YFP, PH3, and pS/TQ. For RPA-1::YFP, goat anti-GFP antibody (Abcam, catalog number ab5450) was used at 1:1,000 dilution. For M-phase cell number assays, mouse anti-PH3 antibody (Cell Signaling, catalog number 9706) was used overnight at 4°C, at 1:200 dilution. pS/TQ staining was used to detect phosphorylation of a number of ATM/ATR targets[302]. For pS/TQ staining, extruded germlines were freeze-cracked and stained with rabbit anti-pS/TQ (Cell Signaling, catalog number 2851) overnight at 4°C, at 1:200 dilution[301].

Extrusion and collagenase digestion were carried out for the staining of pATM. The antibody used for pATM staining detects phosphorylation on serine-1981[279]. Extruded germlines were freeze-cracked, washed for 1 min in 100% and then in 50% ethanol, washed 3 times with PBST (PBS supplemented with 0.4% Triton-X100), digested for approximately 10 min in 10µL of 100U/mL collagenase (Worthington, catalog number LS4004174), washed briefly in PBST ten times using Coplin jars, incubated in PBSBT (PBS supplemented with 0.5% BSA; Sigma, catalog number A7906), washed in 0.1% Triton-X100 for one hour, incubated with mouse anti-pATM (Rockland, catalog number 200-301-500) at 1:200 dilution overnight at 4°C, and finally washed 3 times in PBSBT before mounting. Alternatively, whole-worm peroxide fixation was carried out for some pATM staining experiments, following previously described procedures[303,304]. Specificity of pATM staining was verified by dramatically increased staining after gamma irradiation. Whole-worm staining and analysis was carried out by Amanda Cinquin.

In all cases DNA was stained by including 1µg/mL DAPI in the secondary antibody solution to stain for DNA. When possible, the cytoskeleton was visualized by staining F-actin with 0.16µM Alexa 594-conjugated Phalloidin (Life Technologies,

catalog number A12381) added to the secondary antibody solution. Imaging was performed with a Zeiss 780 confocal microscope using a 63x objective for most experiments. Verification of TUNEL staining foci was performed with a Leica SP8 confocal microscope using a 100x objective.

DAF-16::GFP imaging

DAF-16::GFP transgenic worms were immobilized on 10% a agarose pad between a coverslip and slide. Fluorescent images were captured using a Zeiss Axiovert 200M inverted microscope.

TUNEL staining

TUNEL staining was used to detect single- and double- stranded DNA breaks[305]. TUNEL staining was carried out in a similar fashion as pATM staining using collagenase digestion. Digest for TUNEL staining was performed with 10µL of either 100U/mL collagenase (Worthington, catalog number LS4004174) or 0.3U/mL protease K (New England Bioscience, catalog number P8107S) in M9. After digestion, slides were washed briefly in PBST ten times using Coplin jars, incubated in PBST for one hour, incubated in 20µL of TUNEL reaction solution (2µL enzyme solution, 18µL label solution) derived from In Situ Cell Death Detection Kit (Roche, catalog number 11-684-795-910), washed three times with PBST, incubated in PBSBT for one hour, and incubated overnight at 4°C with rabbit anti-fluorescein (Invitrogen, catalog number A-889) diluted 1:200 in PBSBT. TUNEL staining was validated by dramatically reduced staining when TdT enzyme was omitted, increased staining after DNA break induction using 1U of RQ1 DNase I (Promega, Catalog Number 25308622) for 10 minutes at 37C or 20 krad gamma irradiation (Figure 5a), and by localization of foci to DNA (Figure 5b).

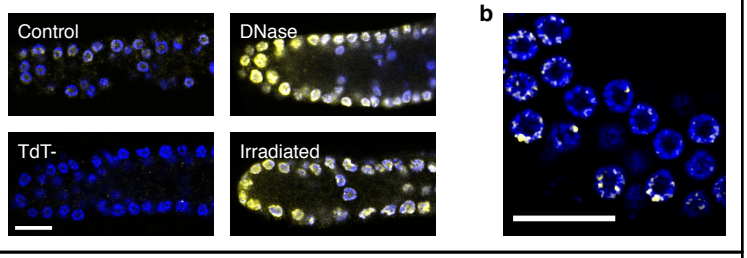

Figure 3: TUNEL validation.
a) TUENL staining (yellow) in MZ cells is increased by treatment with DNase or by irradiation. Staining is decreased when TdT enzyme is excluded from the TUNEL procedure. **b)** TUNEL staining localizes to DNA stained with DAPI (blue). Scale bars represents 10µm.

Image quantification

Germline images were stitched together when necessary[306]. Segmented masks for individual cells were generated using active contours run using the Parismi software pipeline for ImageJ[307]. Cell position along the distal-proximal axis was computed by this software; cells were manually annotated based on nuclear morphology as being in M-phase, and as being part of the mitotic zone, transition zone, or pachytene region. Fluorescence signals were subjected to median filtering and manually thresholded. For TUNEL staining, a DNA mask was created by thresholding of the DNA signal. In order to minimize the impact of fluorescence attenuation along the z-axis of the images, only

"top layer" cells were used (cells whose projection to the top of the stack was at least 70% free of overlap with nuclei closer to the top of the stack). Fluorescence from “top layer” cells is minimally attenuated since there is a minimal amount of tissue for photons to traverse. Signals from TUNEL, RPA-1::YFP, pS/TQ, and pATM staining were summed on a cell by cell basis, and averaged across germlines.

Targeted irradiation

Targeted irradiation was carried out on individual immobilized worms in conjunction with Veronica Gomez-Godinez. This was accomplished using a setup based on a near-infrared femtosecond laser, designed to induce finely-targeted damage in human and rat-kangaroo chromosomes[308,309]. Hermaphrodites were staged at L4 on the day of irradiation and immobilized on “slides” consisting of polystyrene microspheres(Polysciences Inc, catalog number 00876) and a 10% agarose pad sandwiched between two coverslips (Fisher Scientific, catalog number 12-548-A)[310]. Between 4 and 7 worms were interspersed on the slide. Germ cells were identified using transmitted phase contrast light microscopy with a Zeiss 63X 1.4NA objective. A line starting at the distal end of the targeted gonadal arm and covering ~15 cell rows was drawn using the Robolase graphical user interface; the system then performed targeted irradiation along that line using a near-infrared 780 nm 200 femtosecond laser operating at 76 MHZ[311]. The line was drawn by a series of 10 msec exposures as described previously[309]. An irradiance of 3.84×10^{11} W/cm^2 in the laser focal spot was identified as being optimal; this irradiance caused recruitment of RPA-1::YFP to irradiated regions (Figure 4a-d), while higher irradiances caused a high frequency of cavitation bubbles (identified in the live transmitted light image). Both gonadal arms were targeted in each worm; worms were killed by targeting the intestine at high enough doses to cause large cavitation bubbles if they had a gonadal arm that could not be properly targeted, e.g. because it was obscured by the intestine, or if a cavitation bubble occurred during gonadal arm targeting. Each irradiated slide was paired with a non-irradiated control slide that was prepared and treated in an identical way, and was kept near the microscope during the irradiation procedure — so that it was exposed to the same small-amplitude temperature fluctuations as the matched slide with treated worms. Exposure to temperature fluctuations was minimized by keeping the time for irradiation of each slide under 30 minutes; recorded temperature fluctuations did not exceed 2ºC. Worms were

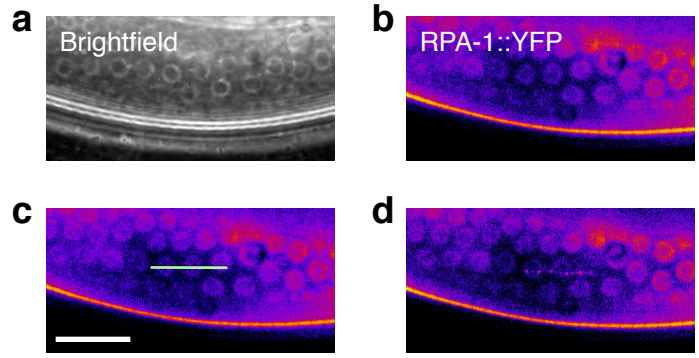

Figure 4: Targeted laser irradiation induces RPA-1::YFP foci formation.
Germ cells of immobilized RPA-1::YFP transgenic worms before irradiation under **a)** brightfield and **b)** fluorescent imaging. **c)** Laser irradiation was targeted to the overlaid green line. **d)** Multiple fluorescent foci along the targeted line are visible five minutes after irradiation. Scale bar represents 10μm.

recovered by opening the “sandwich” and washing both sides with 50µL of M9 onlto an *E. coli* seeded agar plate. Lifespan was then assayed as described above.

Whole-worm irradiation

Staged worms were subjected to 70 krad of gamma irradiation at a dose rate of 349 rad/min using a Cs^{137} source. This dose was chosen after initial tests with a variety of doses revealed it to be the most effective at extending the life of *kri-1* mutant worms (3.5, 7, 21 krad were also tested). Worms on agar plates with bacteria were placed in the irradiator. After irradiation worms were immediately transferred to new plates. Unirradiated controls were treated in an identical way, except that their plates were placed right next to the irradiator instead of inside the irradiator. Lifespan was then assayed as described above.

RNA interference

RNA interference was performed by feeding, as previously described[312]. RNAi plates were prepared by pouring standard NGM plates with the addition of 1mM IPTG, carbenicillin, and tetracycline. RNAi plates were seeded with HT115 bacteria expression dsRNA with sequences matching the ORF of the target gene. HT115 liquid cultures were grown to an optical density of 0.4. Cultures were then spun down for 3 minutes at 3,000 rpm to concentrate the bacterial culture. Pellets were immediately resuspended in LB containing IPTG, carbenicillin, and tetracycline. The resuspended bacteria was then seeded onto RNAi plates that were no more than two months old. RNAi plates were used within one week of seeding.

Worms were prepared for knockdown in the following manner. Eggs laid during the first two days of adulthood were subjected to alkaline bleaching, as previously described, and allowed to hatch in S-medium overnight[313]. Synchronized L1s were transferred to the appropriate RNAi plates within 16 hours of bleaching. After approximately 48 hours, L4 worms were staged based on vulval morphology. Double gene knockdown was accomplished using RNAi vectors containing two ORFs on a single construct, in order to optimize efficiency[314]. For lifespan assays after knockdown, worms were transferred to fresh RNAi plates every day during adulthood.

RNAi constructs were either purchased (Dharmacon, catalog number RCE1182) or generated in lab. The generation of RNAi constructs was carried out by PCR amplification of the target ORF from cDNA and ligation or recombination of this sequence into the L4440 plasmid. In the case of targets with very large ORFs, approximately 3kb of the ORF was cloned into the L4440 plasmid. The identity of each RNAi construct was confirmed by sequencing the junctions between the L4440 plasmid and the inserted ORF. Knockdown was confirmed by qPCR using primers whose binding sites do not overlap with the sequence of the RNAi construct. qPCR data for knockdown confirmation can be found in Table 2. This was generally accomplished using primers binding to the 3‘UTR of the target transcript.

Germline-sensitive RNAi strain

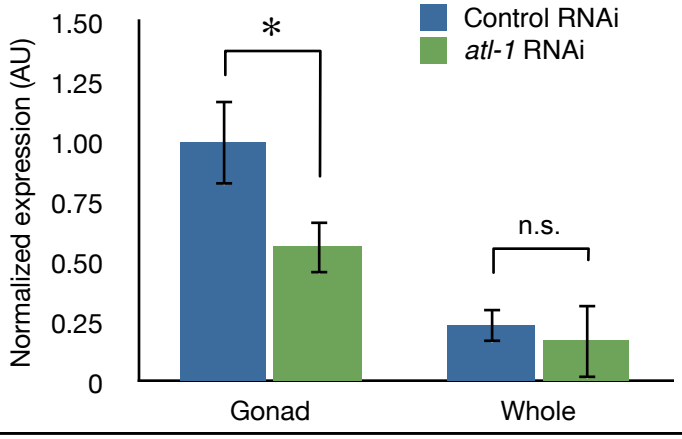

Figure 5: CNQ64 validation.
RNAi against *atl-1* in CNQ64 worms resulted in 44% decreased expression in isolated MZ, and 27% decreased expression throughout the entire worm. * $p < 0.05$.

Generation of transgenic worms sensitive to RNAi specifically in the germline was accomplished by rescuing *rde-1* expression in mutant worms using a germline-specific promoter. The starting strain was WM27, which has a mutant in *rde-1*, a gene required for sensitivity to RNAi[315]. A plasmid was generated which contained several kb of the promoter for *glh-1*, a germline-specific gene, followed by the *rde-1* coding region and the 3'UTR of the *glh-1* gene[316]. The 3' UTR is important because of the general role of such sequences in the regulation of germline expression[317]. Cloning fragments were generated by PCR using Phusion high-fidelity polymerase (NEB, catalog number M0530). Ligation was carried out using T4 DNA ligase (NEB, catalog number M0202). Genomic insertion of the transgene into a site on chromosome II was carried out using the Mos1-mediated single-copy insertion (MosSCI) technique described previously[318]. Injections were carried out by Amanda Cinquin.

Insertion was verified by PCR amplification across both insertion junctions using Taq polymerase (NEB, catalog number M0320). The correct overall structure of the insert was confirmed by amplification across the entire insertion using LongAmp Taq DNA polymerase (NEB, catalog number M0323) followed by separate restriction digests with XhoI, HindIII, and EcoRI. The sequence of the insert was also verified by Sanger sequencing. The resulting banding patterns were consistent with a single-copy insertion of the transgene. Specificity of RNAi sensitivity was confirmed by qPCR. Worms raised on bacteria expressing *atl-1* dsRNA had significantly lower *atl-1* expression in the MZ of the germline ($p < 0.03$), but showed no significant change in expression in whole worms ($p > 0.5$; Figure 3).

Progeny production assay

Singled worms were passaged daily, starting from L4 larval stage. Live progeny and dead eggs were counted from each plate one day after passaging, in order to allow all live eggs to hatch. Plates were censored upon disappearance or death of the parent by any cause.

The progeny production of mated worms was determined in a similar fashion. Mating was accomplished by placing a single L4 stage hermaphrodite on a small spot of bacteria with three young adult males overnight. Males were removed the following day. Successful mating was confirmed by the production of male progeny by the tracked hermaphrodite.

Germline-specific *atl-1* expression strains

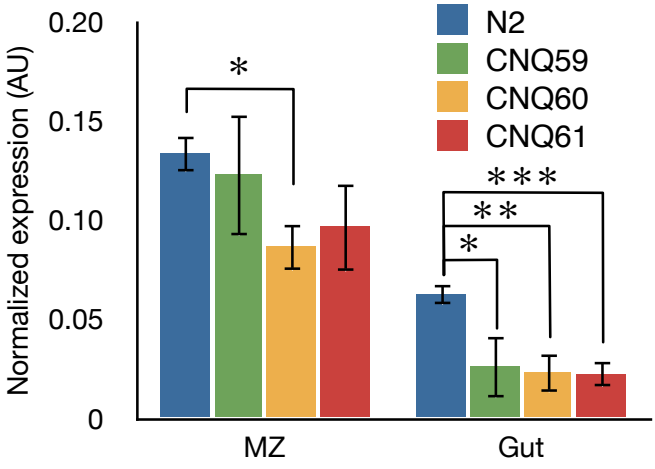

Figure 6: *atl-1* expression in CNQ59, CNQ60, and CNQ61.
Expression was measured by qPCR using primers binding near the 5' end of the *atl-1* transcript. Expression in the MZ was not significantly decreased in two of the transgenic lines analyzed. Expression in the gut was significantly decreased in all three transgenic lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Germline- and soma-specific expression of *atl-1* was accomplished by inserting the *glh-1* or sur-5 promoter in front of the endogenous *atl-1* start site. Constructs were generated which contained the *glh-1* or *sur-5* promoter flanked by the genomic regions immediately upstream and downstream of the *atl-1* start site. A stop sequence was inserted just after the *atl-1* start site, in order to prevent protein generation by the endogenous promoter. The construct was primarily assembled using the NEBuilder DNA HiFi kit (NEB, catalog number E2621) and amplicons produced using Q5 polymerase (NEB, catalog number M0491). The stop sequence was inserted using T4 DNA ligase (NEB, catalog number M0202).

Insertion of the stop region and *glh-1* or *sur-5* promoter into the endogenous *atl-1* locus was accomplished by a CRISPR-Cas9 based method outlined previously[319]. Commercially available Cas9-NLS protein was used (PNA Bio, catalog number CP02). The DNA repair template was obtained by digestion of repair constructs with EcoRI followed by gel purification. Repair template was injected at a concentration of around 5,000ng/uL. Injections were carried out by Amanda Cinquin.

Three independent lines were obtained for the *Pglh-1::atl-1* insertion - CNQ59, CNQ60, and CNQ61. Insertion was confirmed by sequencing of the endogenous *atl-1* promoter region. Potential off-target sites for the *atl-1* and co-CRISPR *dpy-10* sgRNA were predicted using an online resource (http://crispr.mit.edu/). Potential off-target sites with a score of 0.5 and above were checked in each line by sequencing. N2 genomic sequence at these sites was confirmed for all strains generated. Tissue-specific expression of the long *atl-1* transcript was confirmed by qPCR using primers that that specifically detect the long transcript but not the short transcript. CNQ59, CNQ60, and CNQ61 had reduced expression of the long *atl-1* transcript in the gut compared to wild-type ($p < 0.05$, 0.01, and 0.001; Figure 6). Only CNQ60 transgenic lines had significantly reduced expression of the long *atl-1* transcript in the MZ compared to wild-type ($p < 0.01$; Figure 6).

Gene expression

Gene expression was assayed by qPCR analysis of cDNA by Pete Taylor. Whole worms, isolated MZs, bodies, or intestines were mechanically isolated and put into a small volume of PBS buffer on dry ice. RNA was isolated using an RNeasy Mini kit (Quiagen, catalog number 74104). cDNA was generated using an ProtoScript First Strand cDNA Synthesis kit (NEB, catalog number E6300S). qPCR was carried out using iTaq Universal SYBR Green Supermix (Bio-Rad, catalog number 1725120) and a CFX96 RT-PCR Detection System (Bio-Rad, catalog number 1855195).

Telomere assay

Average telomere length was determined using a modified version of previously described procedures[320]. DNA was isolated from either whole worms or isolated germlines using a DNeasy Blood and Tissue kit (Quiagen, catalog number 69581). The primer sequences “CGC TTT GTT TGG CTT TGG CTT TGG CTT TGG CTT TGG CTT” and “GGG TTG CCT TAG CCT TAG CCT TAG CCT TAG CCT TAG CCT” were used, due to differences between mice and nematode telomere repeat sequences[321]. Samples were analyzed using primers binding to either the telomeres or to a single copy sequence in the genome. qPCR was carried out as it was for gene expression analysis. qPCR conditions were as follows: 1x 95°C for 3 minutes; 40x 95°C for 15 seconds, 52°C for 20 seconds, 72°C for 30 seconds. Specificity of this assay for telomeres was confirmed by measuring telomere length after the digestion of gDNA with exonuclease. Digest resulted in a rapid decrease in measured telomere length.

Statistics

Median lifespans were compared using the log-rank test[322]. Progeny production rates, cell numbers, mitotic index, telomere length, and staining intensity were compared using the Wilcoxon rank-sum test[323]. Gene expression data was compared using Welch’s t-test[324]. Hochberg’s step-up procedure was used where noted to account for multiple hypothesis testing[325]. Computations were performed using R[326]. All error bars shown represent 95% confidence intervals.

CHAPTER 3: Control of lifespan by the germ cell DNA damage response

3.1. DNA damage and DDR increase with age in the germline

Levels of DNA damage increase with age in the germ cells of several different organisms[171-173]. This age-related increase could potentially serve as proxy by which an organism assays its own reproductive potential. However, it is not known whether such an age-related increase in DNA damage occurs in *C. elegans* germ cells. I therefore determined whether this is the case by performing immunohistochemistry against markers of both DNA damage and DDR activation.

I focused on the Mitotic Zone (MZ) that contains germline stem cells and ensures self-renewal throughout reproductive life. This is because more differentiated germ cells can be replaced when damaged, which potentially means that reproductive potential can still be high even if DNA damage is prevalent in these cells. In contrast, MZ cells can only be replaced by other MZ cells. This means that high levels of DNA damage in the MZ are likely to indicate permanently decreased reproductive potential.

I first compared germ cell DNA damage in worms at the onset of reproductive activity (day one of adulthood), when reproduction has ceased (day seven), and at an intermediate time (day four). I utilized TUNEL staining to label single- and double-stranded breaks, which can result from primary damage or from intermediary states

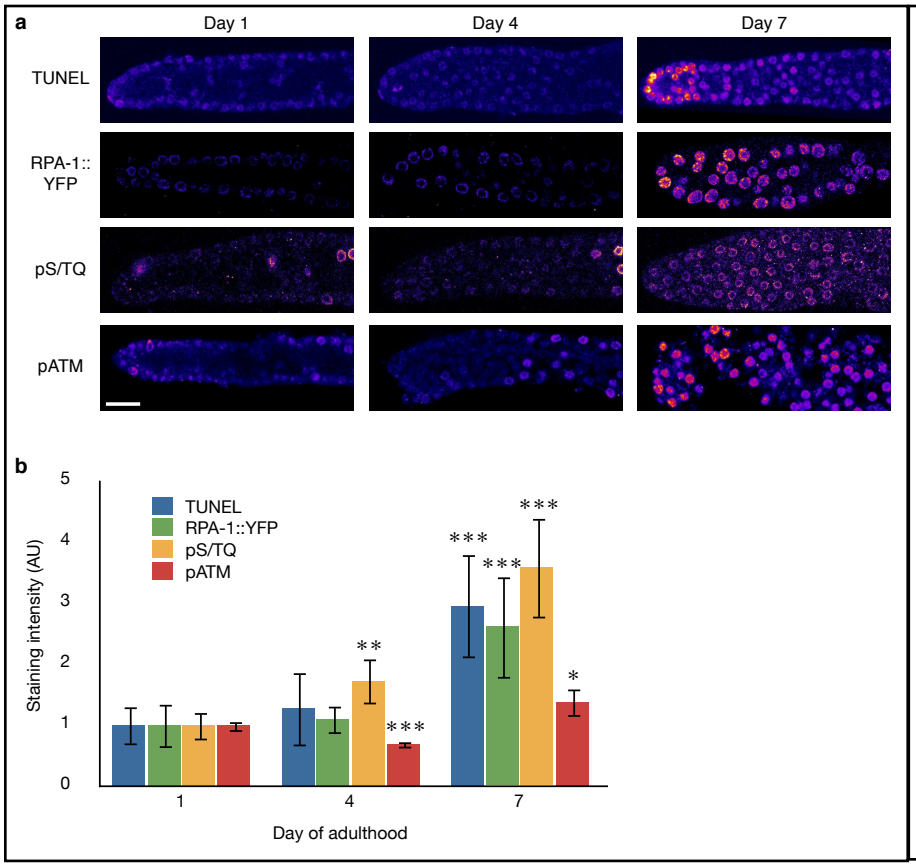

Figure 7: DNA damage and DDR increase in MZ cells with age.
a) Representative images of MZ at days one, four, or seven of adulthood stained using TUNEL or immunohistochemistry against RPA-1::YFP, pS/TQ, or pATM. Warmer colors correspond to higher staining intensity for all images. Scale bar represents 10μm. **b)** Quantification of staining for each condition. Staining significantly increases between days one and seven of adulthood. Significance is given in comparison to day one. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

during repair of other forms of damage[305]. TUNEL staining was performed in such a way that it detected individual DNA breaks, rather than the massive fragmentation that occurs during apoptosis. I found that TUNEL staining increases 2.5- and 3-fold in the MZ between days one and seven of adulthood, based on two independent experiments ($p < 0.001$ and < 0.0001; Figure 7a, b). This demonstrates that older MZs have elevated DNA damage, as expected.

TUNEL staining did not significantly increase between days one and four of adulthood ($p > 0.05$; Figure 7a, b). Instead, the age-dependent increase was almost entirely due to an increase in TUNEL staining between days four and seven. This is interesting because reproductive potential quickly decreases between days four and seven of adulthood. This indicates that a dramatic increase in levels of DNA damage in germ cells roughly coincides with a period of rapidly declining reproductive potential in worms, which is consistent with germ cell DNA damage being a suitable readout of reproductive potential.

I next asked whether increased DNA damage in older MZs resulted in increased DDR activation. I first looked at the ssDNA binding Replication Protein A (RPA-1 in worms), because of its role in multiple forms of DNA damage repair. I tested whether there are increased levels of RPA-1 in the nuclei of older germ cells by staining for RPA-1::YFP in transgenic worms. I found that RPA-1::YFP nuclear signal increases by 162% in the MZ between days one and seven of adulthood ($p < 0.001$; Figure 7a, b). This increase is primarily due to a 137% increase between days four and seven ($p < 0.01$; Figure 7a, b). This increase indicates that more damage is being detected in the germ cells of older worms.

I next looked at ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR), because of their central role in transducing DNA damage signals[327]. As readouts of ATM and ATR I used an antibody raised against pS/TQ peptides that detects activation of a broad range of ATM/ATR targets, as well as a more specific antibody detecting ATM auto-phosphorylation that occurs as part of its activation[279]. Staining with these antibodies increases by 259% and by 38%, respectively, between days one and seven of adulthood ($p < 0.001$ and $P < 0.015$; Figures 7a, b). As with TUNEL and RPA-1::YFP, this is primarily due to an increase in staining between days four and seven of adulthood. These data show that DDR activation increases in aging MZs around the end of reproductive lifespan.

The observation that DDR activation increases so dramatically over the span of several days raises the question: what is responsible for this increase? One possible reason for the increase in DDR activation with age is the erosion of telomeres in germ cells after multiple cell divisions, as DDR is known to be activated by critically shortened telomeres[328,329]. I asked whether the increase in DDR activation observed in older germlines is due to a decrease in germ cell telomere length with age. Average telomere length has previously been reported to remain constant with age in *C. elegans*, based on the analysis of whole worms[85]. However, such analysis may be unable to detect changes in telomere length in MZ cells due to the inclusion of both somatic cells and proximal germ cells. I therefore measured telomere length by collecting genomic DNA from the distal half of germlines on days one, four, seven, and ten of adulthood. Telomere length was quantified using qPCR by comparing amplification of the telomeric repeat sequence to amplification of a reference sequence which appears only once in

the genome, based on procedures previously developed for use in mice[320]. Average telomere length did not significantly decrease between days one and ten of adulthood ($p > 0.1$; Figure 8a). This suggests that decreasing telomere length is not the source of the increased DDR activation observed in germ cells with age.

Another possible reason for the increase in DNA damage with age is that cell cycling leads to the accumulations of lesions. This may be due to increased metabolic activity leading to the generation of free radicals, which can cause oxidative damage to DNA. Alternatively, it may be due to an accumulation of damaged cellular components, similarl to what has been observed in dividing fission yeast mother cells[330]. I therefore asked if increased cell cycling leads to increased DNA damage in MZ cells. Worms were mated for 24 hours starting at the L4 larval stage, a process which increases germ cell cycling, and their germlines were subjected to TUNEL staining at day seven of adulthood. TUENL staining in mated worms was not significantly higher at day seven compared to unmated worms ($p > 0.8$; Figure 8b). This suggests that cell cycling is not the cause of increased DNA damage with age in germ cells.

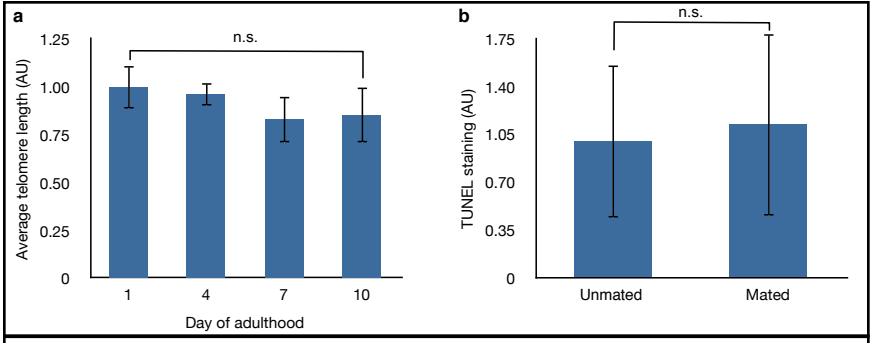

Figure 8: The age-related increase in damage is not due to telomere shortening or cell cycling.
a) Average telomere length in the distal germline on days one, four, seven, and ten of adulthood. No significant decrease is detected. **b)** TUNEL staining in the MZ on day 7 of adulthood is not significantly different between unmated worms and those mated early in life.

3.2. Increased DNA damage in germ cells decreases lifespan

I next asked whether experimental manipulation of germline DDR affects lifespan. In a first set of experiments, I induced localized DNA damage using targeted multiphoton irradiation in collaboration with Veronica Gomez-Godinez. Carefully dosed irradiation activated DDR in the germline, as shown by recruitment of RPA-1::YFP to DNA (Figure 4a-d), without compromising the structural integrity of cells. Irradiation targeted to MZ nuclei decreased lifespan by 11% and 10% in two independent experiments ($p < 0.015$ and < 0.05; Figure 9a; Table 3). In contrast, irradiation targeted to the nuclei of differentiated germ cells in the pachytene region did not significantly affect lifespan (-2%, $P > 0.6$; Figure 9b; Table 3). This suggests that the targeting procedure by itself is not decreasing lifespan. Instead, targeting must be directed specifically to MZ nuclei to decrease lifespan. My results suggest that activation of the DDR pathway in MZ cells decreases lifespan.

I next wanted to check that targeted irradiation was not decreasing lifespan due to collateral somatic cell irradiation - for instance, somatic cells which are not in the focal plane of the laser hypothetically may still incur DNA damage. I therefore asked what effect the irradiation of somatic cells has on lifespan. The set-up used for targeted germ

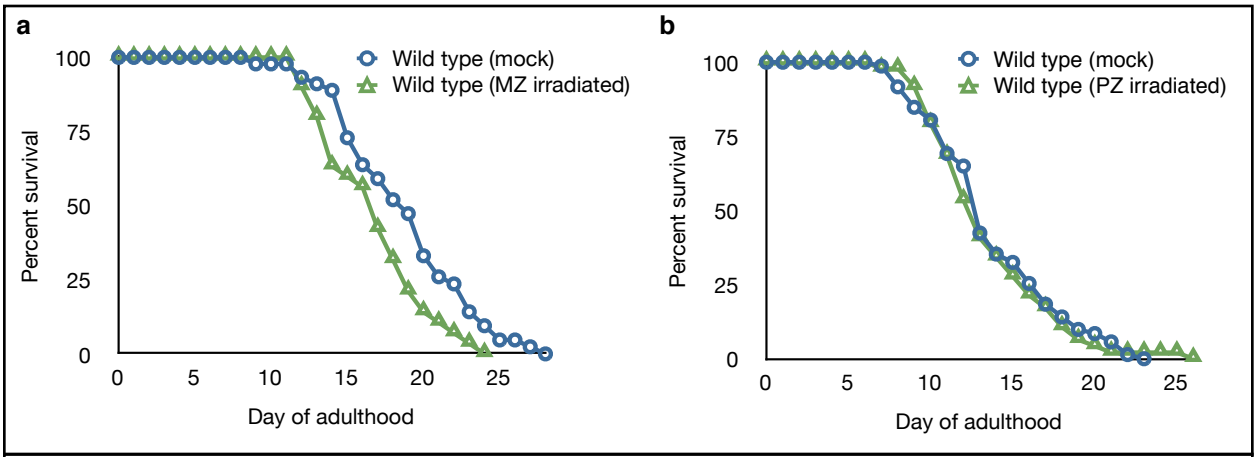

Figure 9: MZ cell irradiation decreases lifespan.
a) Targeted irradiation of MZ cells decreased lifespan by 10% and 11% in two independent experiments ($p < 0.05$ for both). **b)** Targeted irradiation of pachytene germ cells did not significantly affect lifespan ($p > 0.6$).

cell irradiation was not practical to use to target somatic cells for two reason. The first reason is that the somatic nuclei of *C. elegans* are not aligned in a regular pattern and do not lie on the same plane on the Z-axis, as germ cells do. This increases the time required to target a large number of cells in the soma by orders of magnitude. The amount of time that a worm is kept immobilized during the procedure must be kept to a minimum to avoid induction of a starvation response. The second reason is that many somatic cells contain autofluorescent foci. Contact of these foci by the laser induces cavitation bubbles. These bubbles are often tens of microns in diameter, which is larger than may of the cells of the worm. These practical constraints led me to try a different method to achieve somatic cell irradiation.

I chose to use whole-worm irradiation in order to determine the lifespan effect of somatic cell irradiation. In order to separate the effect of somatic and germ cell irradiation, I used a *kri-1(ok1241)* mutant strain, in which control of lifespan by the reproductive system is abolished[7]. By removing the influence of the germline on lifespan, the effect of irradiation should be solely due to somatic cell exposure. Surprisingly, I found that *kri-1* mutants displayed a significant lifespan increase upon gamma irradiation (+11%, +19%, and +25%, $p < 10^{-4}$, < 0.001, and < 0.01 in 3 independent experiments; Figure 10a; Table 4). This suggests that the irradiation of somatic cells actually increases lifespan. One implication of this is that targeted irradiation of MZ cell nuclei is not decreasing lifespan due to collateral absorption by somatic cells.

In addition to *kri-1* mutants, I also tested the lifespan effect of irradiation on wild-type worms. Unlike *kri-1* mutants, wild-type worms did not display an increase in lifespan after exposure to the same dose of gamma irradiation (-9%, +2%, and +5%, $p > 0.05$, > 0.9, and > 0.3 in 3 independent experiments; Figure 10a; Table 4). The simplest way to interpret both the targeted and whole-worm irradiation results is that irradiation of MZ cells decreases lifespan, while irradiation of somatic cells increases lifespan. This suggests that radiation hormesis occurs in worms, but is masked in wild type because the damage incurred by germ cells reduces lifespan in a way that cancels out the beneficial effects of somatic irradiation.

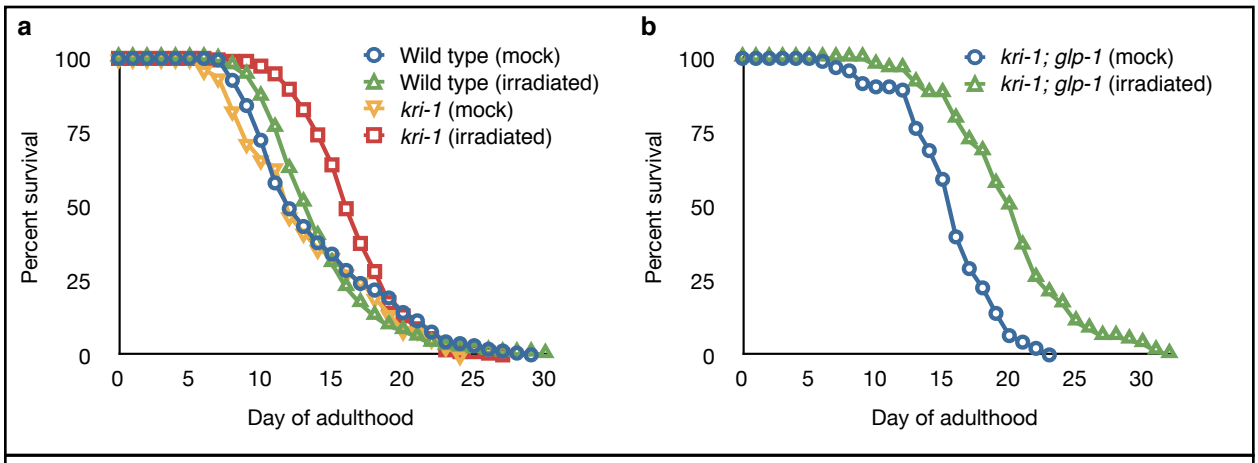

Figure 10: Whole worm irradiation increases lifespan in *kri-1* mutants.
a) Whole-worm gamma irradiation increased lifespan in *kri-1* mutants by 11%, 19%, and 25% in three independent experiments ($p < 10^{-4}$, 0.001, and 0.01). The same procedure did not significantly affect the lifespan of wild-type worms in three independent experiments ($p > 0.05$ in all cases). **b)** Whole-worm gamma irradiation increased the lifespan of sterile *kri-1; glp-1* mutants by 28% and 29% in two independent experiments ($p < 10^{-6}$ and 10^{-12}).

The radiation hormesis observed in *kri-1* mutants could conceivably be due to an indirect effect on the soma through germ cell irradiation. Cell non-autonomous effects of irradiation have been reported previously[331-333]. I therefore asked whether the lifespan extension observed after irradiation was present after the ablation of germ cells. I tested this by subjecting *glp-1;kri-1* double mutants, which lack germ cells, to gamma irradiation. Unlike *glp-1* single mutants, which are long-lived, *glp-1; kri-1* double mutants have a roughly wild-type lifespan[7]. Irradiation increased *glp-1;kri-1* mutant lifespan by 28% and 29% in two independent experiments ($p < 10^{-6}$ and $< 10^{-12}$; Figure 10b; Table 4). This strongly suggests that radiation hormesis works through a direct effect of irradiation on somatic cells.

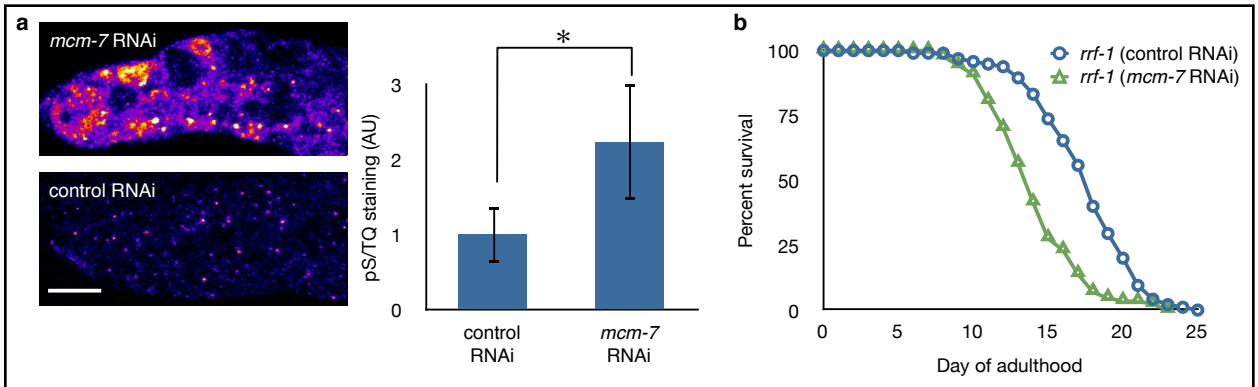

Figure 11: *mcm-7* knockdown leads to increased DDR activation and decreased lifespan.
a) Representative images and quantification of pS/TQ staining in the MZ of *rrf-1* mutants after *mcm-7* and control knockdown. Knockdown significantly increased pS/TQ staining. Warmer colors correspond to higher staining intensity. Scale bar represents 10µm. **b)** *mcm-7* knockdown reduces the lifespan of *rrf-1* mutants by 19% ($p < 10^{-9}$). * $p < 0.05$.

I next wanted to confirm that increased DDR in the germline leads to decreased lifespan by a method independent of irradiation. I therefore knocked down *mcm-7* in *rrf-1* mutant worms. *mcm-7* is a component of the MCM replicative helicase complex whose knockdown results in sterility in worms due to an inability to cope with replicative stress[334]. Mutation of *rrf-1* causes somatic insensitivity to RNAi specifically while maintaining sensitivity in germ cells[335]. Knockdown of *mcm-7* resulted in severely deformed MZ cell nuclei and a dramatic increase in pS/TQ staining in germ cells ($p < 0.01$; Figure 11a). Knockdown of *mcm-7* also reduced lifespan by 19% ($p < 10^{-10}$; Figure 11b; Table 5). These results support the idea that increased DDR in germ cells leads to decrease lifespan.

3.3. Reduced DDR in germ cells increases lifespan

Increased DNA damage in germ cells leads to decreased lifespan, which may be due to increased activation of DDR. If this is true, it would be expected that decreased DDR in germ cells leads to increased lifespan. I tested this in a second set of experiments by using genetic manipulation of components of the DDR pathway. I first performed simultaneous RNAi knockdown of *atm-1* and *atl-1*, the worm homologs of ATM and ATR, due to their central role in DDR activation. I initially knocked down both genes simultaneously because of partial redundancy between ATM and ATR in other systems[336,337]. Knockdown increased *rrf-1* mutant lifespan by 12%,14%, 15%, and 25% in four independent experiments ($p < 0.05$ and < 0.05, < 0.01, and $< 10^{-6}$; Figure 12; Table 5). This supports the idea that reduced DDR in germ cells increases lifespan.

I next asked whether increasing knockdown strength would further increase lifespan. I tested this by performing simultaneous knockdown of *atm-1* and *atl-1* in an *rrf-1; rrf-3* double mutant background. The *rrf-3(pk1426)* mutant allele results in hypersensitivity to RNAi[338]. Knockdown in *rrf-1; rrf-3* mutants increased lifespan by 21% and 31% in two independent experiments($p < 0.01$ and < 0.001; Figure 12; Table 5). The lifespan increase observed in *rrf-1; rrf-3* double mutants was generally stronger than those observed in *rrf-1* single mutants. This suggests that the effect of germline DDR reduction on lifespan is dose dependent.

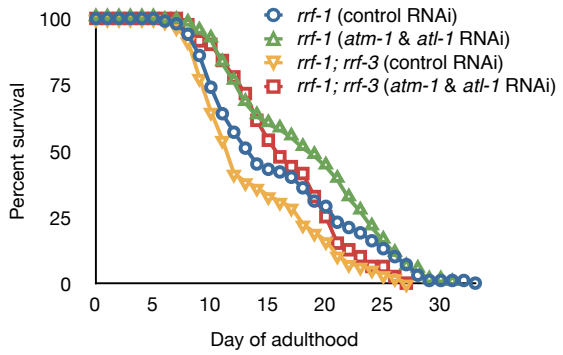

Figure 12: Knockdown of *atm-1* and *atl-1* in germ cells increases lifespan.
Simultaneous knockdown of *atm-1* and *atl-1* increases the lifespan of *rrf-1* mutants by 12%, 14%, 15%, and 25% in four independent experiments ($p < 0.05$, < 0.05, 0.01, and $< 10^{-6}$). The same knockdown increases the lifespan of *rrf-1; rrf-3* double mutants by 21% and 31% in two independent experiments(p 0.01 and 0.001).

I next asked whether both *atm-1* and *atl-1* play a role in lifespan control. I tested this by knocking down each gene individually. The knockdown of *atl-1* in *rrf-1* mutants led to a 12% increase in lifespan ($p < 0.05$; Table 5). The individual knockdowns of *atm-1* and *atl-1* in *rrf-1;rrf-3* mutants led to 11% and 24% increases in lifespan ($p < 0.05$ and 0.001, respectively; Table 5). The decreased

lifespan effect of *atm-1* knockdown compared to that of *atl-1* may be due to decreased efficiency of *atm-1* knockdown. Knockdown of *atm-1* in *rrf-1;rrf-3* mutant worms resulted in a 31% decrease in expression, while knockdown of *atl-1* in this strain resulted in a 41% decrease in expression ($p < 0.05$ and 0.001, respectively; Table 2). These results suggest that both *atm-1* and *atl-1* play a role in lifespan determination, although the relative importance of each gene cannot be determined from these data.

rrf-1 mutants have been shown to retain some somatic RNAi sensitivity, particularly in the intestine[339]. This raises the possibility that the lifespan increases observed after knockdown in *rrf-1* mutant worms may be due to a direct effect on gene expression in the intestine. I tested this by simultaneously knocking down *atm-1* and *atl-1* in the strain VP303, in which *rde-1*, a gene required for RNAi sensitivity, is expressed under the control of an intestinal specific promoter[315]. Knockdown significantly decreased lifespan by 11% in VP303 worms ($p < 0.001$; Table 6). This suggests that the lifespan increase observed in *rrf-1* mutants after *atm-1* and *atl-1* knockdown is not due intestinal RNAi sensitivity.

Another caveat introduced by the use of *rrf-1* mutants is the potential connection between the RNA interference and DDR pathways. The knockdown of Dicer, a component of the RNAi machinery, results in increased DDR activation in human cells[340]. This raises the possibility that lifespan extension after knockdown is an artifact of *rrf-1* mutation. I therefore confirmed the lifespan effect of germline DDR gene knockdown using a method independent of the *rrf-1* mutation. I generated a strain of worms in which expression of *rde-1* is under the control of the promoter and 3‘UTR of *glh-1*, a gene that is highly specific to the germline[341]. This strain will be referred to as CNQ64. The simultaneous knockdown of *atm-1* and *atl-1* in CNQ64 worms increased lifespan by 20% ($p < 10^{-5}$; Table 5). This confirms that the results observed in *rrf-1* mutant worms are due to knockdown in the germline, and that these results are not due to an artifact of *rrf-1* mutation.

I next wanted to confirm that the reduction of germline DDR increases lifespan using a method that is completely independent of knockdown. I first tested this by assaying the lifespan of *atl-1(tm853)* mutants. I found that lifespan was 37% higher in *atl-1* mutants compared to wild-type worms ($p < 10^{-20}$; Figure 13a; Table 7). This is consistent with previously published results[288]. Unfortunately, these data does not distinguish whether thais lifespan increase is due to the loss of ATL-1 in the soma or germline.

I next attempted to test the effect of limiting expression of *atl-1* to the soma. I did this by using a CRISPR-Cas9 based strategy to drive *atl-1* expression using the soma-specific *sur-5* promoter[319]. However, transgenic *Psur-5::atl-1* worms could not be recovered, even after two separate attempts to generate the strain. Instead, a large number of dead eggs were produced by the procedure. One explanation for the presence of these dead eggs is that the *Psur-5::atl-1* transgene resulted in a dominant embryonic lethal phenotype, due to somatic expression of *atl-1*.

I next tested the effect of decreased expression of *atl-1* in the soma. I again used a CRISPR-Cas9 based strategy to drive *atl-1* expression using the germline-specific *glh-1* promoter[316]. Three independent lines were generated - CNQ59, CNQ60, and CNQ61. These worms displayed reduced expression of *atl-1* in the soma but not the germline (Figure 6). The lifespan of CNQ59, CNQ60, and CNQ61 worms were not

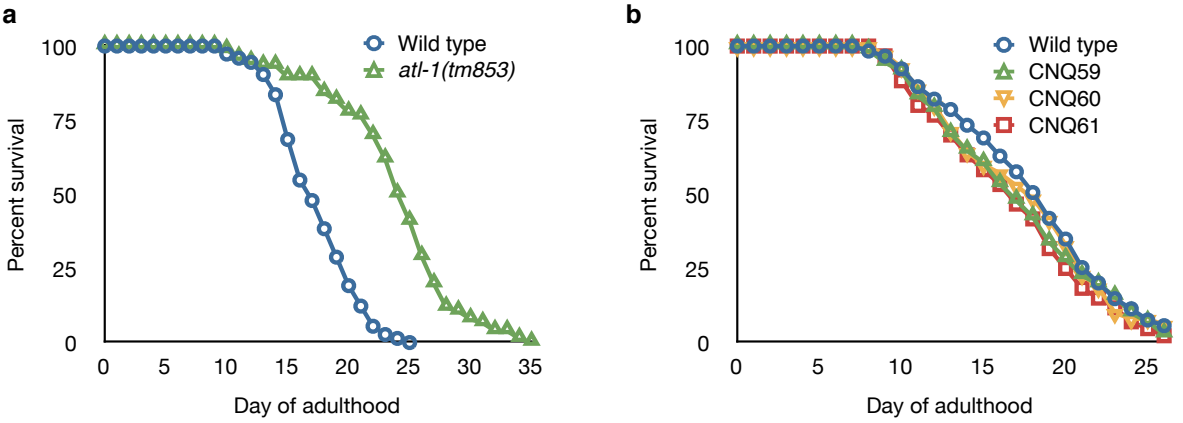

Figure 13: *atl-1* mutation increases lifespan due to an effect on the germline.
a) Mutation of *atl-1* leads to a 37% increase in lifespan relative to wild type ($p < 10^{-20}$). **b)** Decreased somatic expression of *atl-1* does not significantly affect lifespan based on experiments using three independent transgenic lines ($p > 0.4$ in all cases).

significantly different from wild-type ($p > 0.4$ in all cases; Figure 13b; Table 7). This suggests that decreased somatic expression of *atl-1* does not increase lifespan. This suggests that the increased lifespan observed in *atl-1(tm853)* worms is specifically due to an effect on germline *atl-1* expression. These results support the idea that the reduction of DDR in germ cells increases lifespan.

3.4. Lifespan extension by reduced germline DDR is not due to decreased reproductive activity

I next asked if the increased lifespan observed after germ cell DDR gene knockdown could be a secondary effect of altered reproductive activity. One concern is that DDR gene knockdown may decrease reproduction and thereby allow more resources to be allocated to somatic maintenance, following a trade-off posited by the disposal soma theory[142]. I first tested this idea by simultaneously knocking down *atm-1* and *atl-1* in either *rrf-1* mutants or in CNQ64 worms and measuring the resulting mitotic index in the MZ on days one, four, and seven of adulthood. Knockdown did not significantly decrease the mitotic index of either strain on any of the days measured (Figure 14a). Instead, mitotic index was increased by knockdown in four day old CNQ64 worms and in seven day old *rrf-1* mutant worms, compared to controls ($p < 0.01$ and < 0.05; Figure 14a). This result is surprising given that it has previously be suggested that germ cell cycling causes decreased lifespan[65]. The increased mitotic indices after knockdown suggest that the lifespan increase caused by germline DDR knockdown is not a byproduct of reduced germ cell cycling.

In addition to mitotic index, I also counted the number of MZ cells in CNQ64 worms and *rrf-1* mutants after knockdown. While knockdown did not affect MZ cell number on the first day of adulthood, knockdown increased MZ cell number in both strains on later days. Knockdown increased MZ cell number by 44% on day seven of adulthood in *rrf-1* mutant worms ($p < 0.05$; Figure 14b) and by 21% and 43% on days four and seven of adulthood in CNQ64 worms ($p < 0.05$ and < 0.01; Figure 14b). In

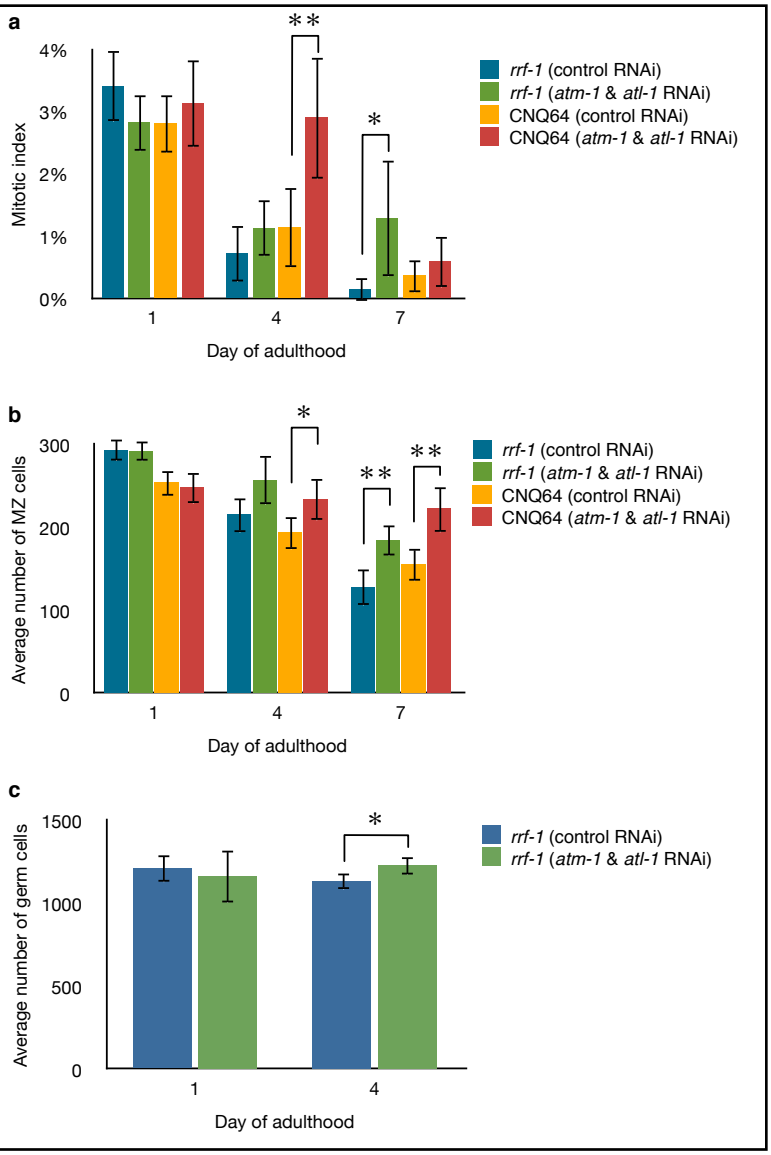

Figure 14: Knockdown of *atm-1* and *atl-1* increases germ cell production.
a) Simultaneous *atm-1* and *atl-1* knockdown increases mitotic index in CNQ64 worms on day four and in *rrf-1* mutants on day seven of adulthood. **b)** The same treatment significantly increases MZ cell number in CNQ64 worms on days four and seven and in *rrf-1* mutants on day seven of adulthood. **c)** The same treatment increases total germ cell number in *rrf-1* mutants on day four of adulthood. * $p < 0.05$, ** $p < 0.01$. Other differences are not significant.

addition, I counted the total number of germ cells present in *rrf-1* mutant worms on days one and four of adulthood. Knockdown did not affect total cell number on day one of adulthood, and increased total cell number by 9% on day four of adulthood ($p > 0.9$ and < 0.05; Figure 14b). These results show that the germline knockdown of *atm-1* and *atl-1* increase the production of germ cells, an idea consistent with the elevated mitotic indices observed after knockdown. This suggests that the lifespan increase observed after knockdown is not due to a decrease in germ cell number.

I next asked whether the knockdown of *atm-1* and *atl-1* affected reproductive output. I tested this by counting the number of progeny laid after knockdown in mated and unmated worms. Knockdown did not significantly affect progeny production in mated worms (Figure 15a, b). However, knockdown significantly increase total progeny production in unmated worms by 14% ($p < 0.001$; Figure 15a, b). This is consistent with a previous report which showed that unmated *atl-1* mutant worms lay more eggs than

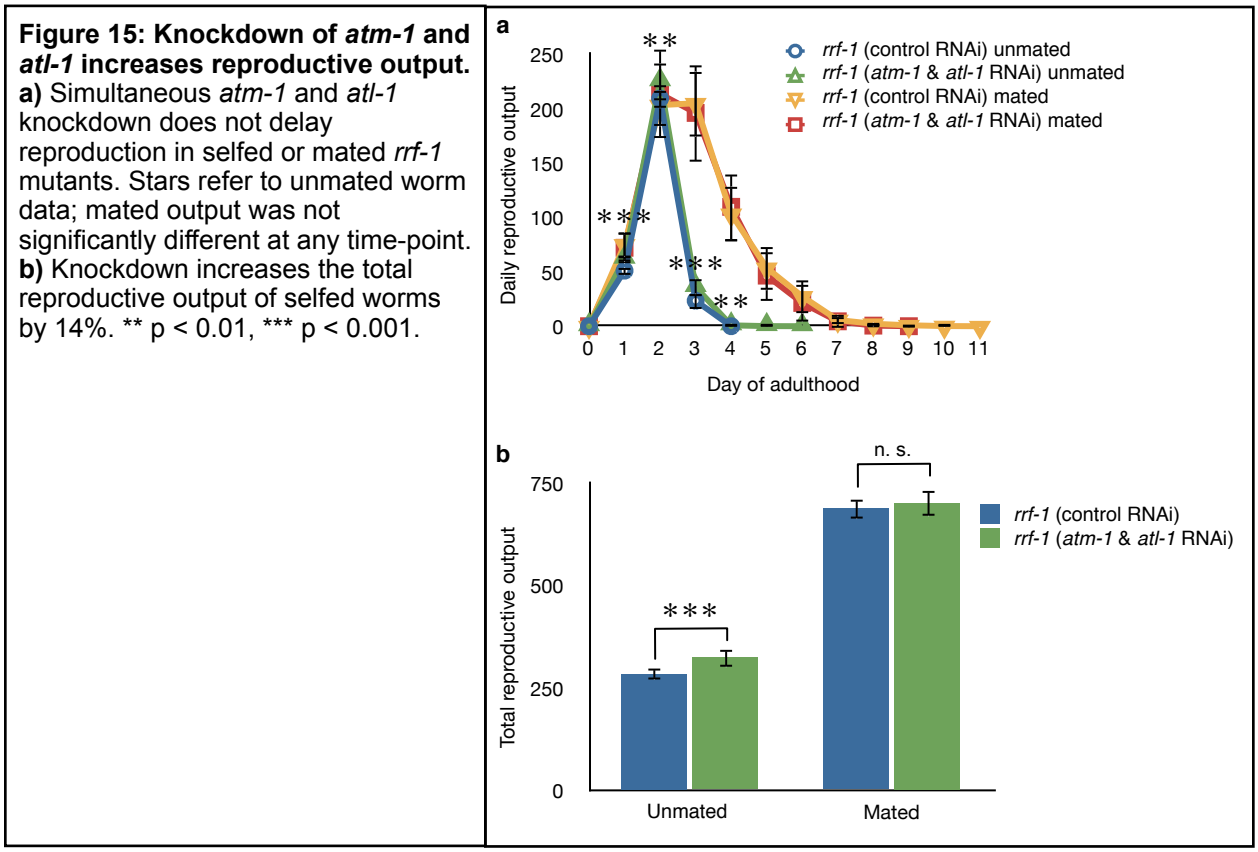

Figure 15: Knockdown of *atm-1* and *atl-1* increases reproductive output. **a)** Simultaneous *atm-1* and *atl-1* knockdown does not delay reproduction in selfed or mated *rrf-1* mutants. Stars refer to unmated worm data; mated output was not significantly different at any time-point. **b)** Knockdown increases the total reproductive output of selfed worms by 14%. ** p < 0.01, *** p < 0.001.

wild-type worms[342]. These results show that the increase in lifespan observed after knockdown is not due to a decrease in reproductive output.

I next asked whether the knockdown of *atm-1* and *atl-1* affects lifespan indirectly due to an effect on apoptosis. This was a concern because one of the possible outcomes of DDR activation is apoptosis[343]. I tested this possibility by knocking down *atm-1* and *atl-1* in an *rrf-1; ced-3* mutant background, in which apoptosis is abolished[344]. Knockdown strongly increased the lifespan of *rrf-1; ced-3* mutants (Table 8). This indicates that knockdown is not increasing lifespan due to an effect on germline apoptosis.

A final possibility I addressed is whether knockdown affects lifespan through a change in the rate of early development. This is a concern because some long-lived mutants display delayed development[345]. I tested this by knocking down *atm-1* and *atl-1* beginning on the first or third day of adulthood, rather than at the first larval stage as in other experiments. Knockdown at day one or three of adulthood resulted in lifespan increases of 12% and 10%, respectively ($p < 0.05$ for both; Table 5). This shows that the increase in lifespan caused by knockdown is not due to an effect on larval development.

3.5. Multiple branches of the DDR pathway affect lifespan

Up to this point I have focused on *atm-1* and *atl-1*, due to their central role in the DDR activation. I next asked about the role of individual DDR pathway genes in controlling worm lifespan. I performed germline knockdown of a broad set of genes

belonging to the HR, NHEJ, NER, ICL, MMR, and stalled replication fork repair pathways, as well as of *cep-1*, the worm homolog of p53, and *hus-1*, a member of the 9-1-1 complex. Knockdown of seven of eleven genes tested led to significant lifespan increases (Figure 16a; Table 5). This list includes *mre-11*, a member of the MRN complex which activates ATM[181], and *hus-1*, a member of the 9-1-1 complex which activates ATR[346]. This supports the idea that both *atm-1* and *atl-1* play a role in lifespan control. Only the knockdown of *rpa-1* led to decreased lifespan in *rrf-1* mutant worms. Knockdown of *rpa-1* also led to a 132% increase in pS/TQ staining in germ cells, compared to controls ($p < 0.05$). This is consistent with germline DDR activation being inversely correlated with lifespan.

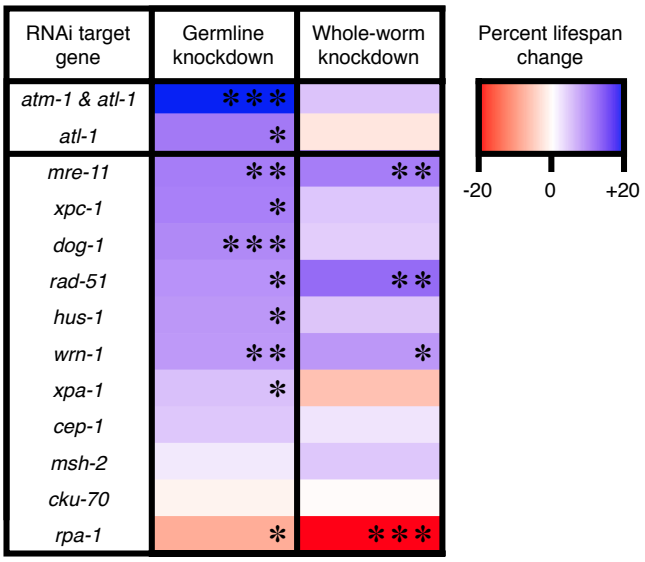

Figure 16: DDR gene knockdown in the germline and whole worm.
Knockdowns of a variety of genes involved in the DDR pathway were performed in the germline and whole worm, using *rrf-1* mutants and wild-type worms, respectively. Of the eleven genes tested (aside from *atm-1* and *atl-1*), the knockdown of seven in the germline significantly increased lifespan. In contrast, only three whole-worm knockdowns significantly increased lifespan. Percent lifespan change is relative to control knockdowns performed simultaneously. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Significance adjusted for multiple hypothesis testing.

The list of genes whose germline knockdown extends lifespan includes members of the NER, ICL, HR, and PRR pathways. This raises the possibility that multiple types of DNA damage in the germline play a role in the regulation of lifespan. This is not surprising, as multiple damage repair pathways are known to activate ATM and ATR. The knockdown of *cep-1* did not result in increased lifespan. Its homolog p53 is phosphorylated by ATM during DNA damage response activation in other systems[252,253]. This result suggests that lifespan control may be determined specifically by the activity of ATM-1 and ATL-1, as opposed to further downstream by the activation of CEP-1.

I next asked whether the mutation of other genes involved in the DDR pathway had similar lifespan effects as the mutation of *atm-1* or *atl-1*. I tested this by measuring the lifespan of *wrn-1*, *mre-11*, *rad-51*, and *hus-1* mutants. All four mutants had reduced lifespan compared to wild-type worms (Figure 17a; Table 7). This was interesting given that the knockdown of three of these four genes resulted in increased lifespan in wild-type worms. It was also surprising that the putative null mutant for *hus-1* was short lived, given that a *hus-1* hypomorphic mutant was previously reported to be slightly longer lived than wild-type[286].

One way to explain the decrease in lifespan after the mutation of certain DDR genes is that such interventions might paradoxically increase downstream activation of DDR, similar to the increased DDR activation observed after *rpa-1* knockdown. This may be due to decreased efficiency of DNA damage repair, resulting in higher levels of

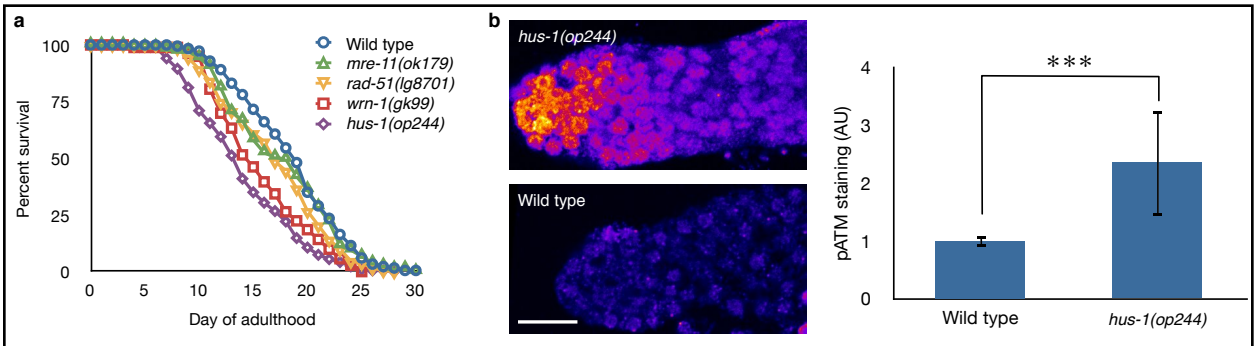

Figure 17: Decreased lifespan in some DDR mutants.
a) Lifespan is decreased by mutation of *mre-11* (-10%, $p < 0.5$), *rad-51* (-8%, $p < 0.05$), *wrn-1* (-20%, $p < 10^{-7}$), and *hus-1* (-10% and -33%, $p < 0.05$ and 10^{-8}). **b)** Representative images of pATM staining in the MZ of *hus-1(op244)* mutants and wild-type worms on day four of adulthood. *hus-1* mutants stain more intensely for pATM. Scale bar represents 10µm. *** $p < 0.001$.

unrepaired damage which is detected through alternative branches of the DDR pathway. I tested this by staining for pATM in wild-type and *hus-1* mutant germlines. Staining was dramatically higher in the MZs of *hus-1* mutants on the fourth day of adulthood compared to wild-type worms (Figure 17b). This suggests that severe *hus-1* mutation may decrease lifespan due to increased activation of DDR in germ cells. However, I cannot rule out the possibility that the lifespan effect is due to a somatic role of *hus-1*.

3.6. Reduced DDR in somatic cells decreases lifespan

The observation that decreased germline DDR extends lifespan was surprising since hypomorphic mutations in the DNA repair machinery, including ATM and ATR, are generally associated with accelerated aging[347]. This raised the possibility that DDR in the soma and germ line have opposing effects on lifespan. A reduction in germline DDR may increase lifespan, while a reduction in somatic DDR may decrease lifespan. I tested this idea by returning to the genes that I had already knocked down in the germ line, and comparing the effects of knockdown in the germline with knockdown in the whole worm using wild-type worms. I found that only three of the eleven knockdowns tested increased lifespan in wild-type worms (Figure 16; Table 9). The weaker lifespan extensions caused by knockdown in wild-type worms compared to *rrf-1* mutants may be due to the added effect of somatic knockdown. This is consistent with the idea that the germline knockdown of DDR genes increases lifespan while somatic knockdown of DDR genes decreases lifespan. This suggests that somatic DDR extends lifespan.

I next asked what the effect of knocking down DDR genes specifically in somatic cells would be. I tested this using *ppw-1* mutant worms, which display reduced sensitivity to RNAi in germ cells but efficient RNAi sensitivity in somatic cells[348]. Knockdown of *atm-1* and *atl-1* in *ppw-1* mutants resulted in lifespan decreases of 7% and 6% in two independent experiments, although only one result was significant ($p < 0.05$ and < 0.6; Table 6). This supports the idea that somatic DDR has a positive effect on lifespan.

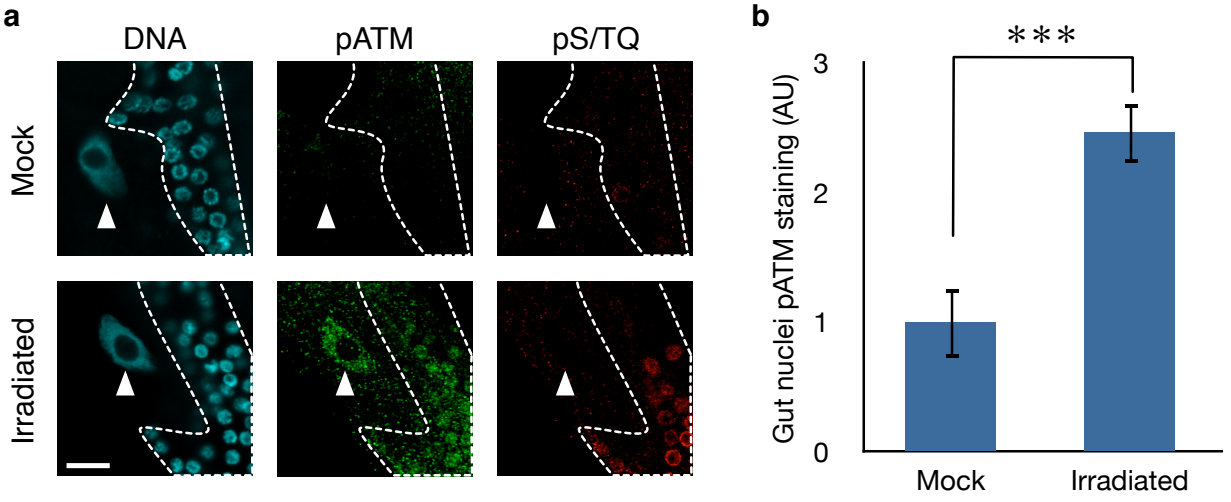

Figure 18: Irradiation causes ATM phosphorylation in somatic cells
a) Representative images of whole-mount staining using DAPI and antibodies against pATM and pS/TQ in irradiated and non-irradiated worms. The germline is outlined by a dashed line. Intestinal nuclei are indicated by white arrowheads. The dark region in the center of the nucleus is the nucleolus. pS/TQ staining only increases in germ cells after irradiation, while pATM staining increases in germ cells and intestinal nuclei. Scale bar represents 10μm. **b)** Irradiation increases pATM staining in intestinal nuclei by 146%. *** $p < 0.001$.

This result was surprising in light of a previous report suggesting that the DDR, and particularly *atm-1* and *atl-1*, do not play a role in the somatic cells of adult *C. elegans*[244]. This conclusion was based on the observation that pS/TQ, a common readout of ATM and ATR activity, was not induced in somatic cells by irradiation based on antibody staining[244]. However, pS/TQ is a relatively general phosphorylation motif, and staining may not be as sensitive to changes in DDR activation compared to staining for a more specific motif. I therefore asked whether ATM-1 activation can occur in the somatic cells of *C. elegans*. I worked with Amanda Cinquin to test this by gamma irradiating worms and staining them for phosphorylated ATM. Staining with pATM antibody increased by 147% in intestinal nuclei after irradiation ($p < 10^{-7}$; Figure 18a, b). This suggests that somatic cells are capable of activating DDR, although possibly to a lesser extent than germ cells.

3.7. Germline DDR controls lifespan through insulin signaling

I next asked what extracellular signaling pathways link germline ATM/ATR activity to somatic lifespan. Insulin signaling is a prime candidate given its requirement for lifespan extension in response to germline ablation[2]. I tested its role in the link between germline DDR and lifespan by knocking down *atm-1* and *atl-1* in a double mutant for *rrf-1* and *daf-16*, a FOXO homolog acting in the insulin signaling pathway. While the knockdown of *atm-1* and *atl-1* significantly increased lifespan in an *rrf-1* background, it did not significantly increase lifespan in *rrf-1 daf-16* mutants (-19%, $P < 10^{-6}$; Figure 19a; Table 8). In addition, targeted germ cell irradiation did not decrease lifespan in *daf-16* mutants, instead increasing it slightly (by 6%; $P < 0.03$; Figure 19b; Table 3). This

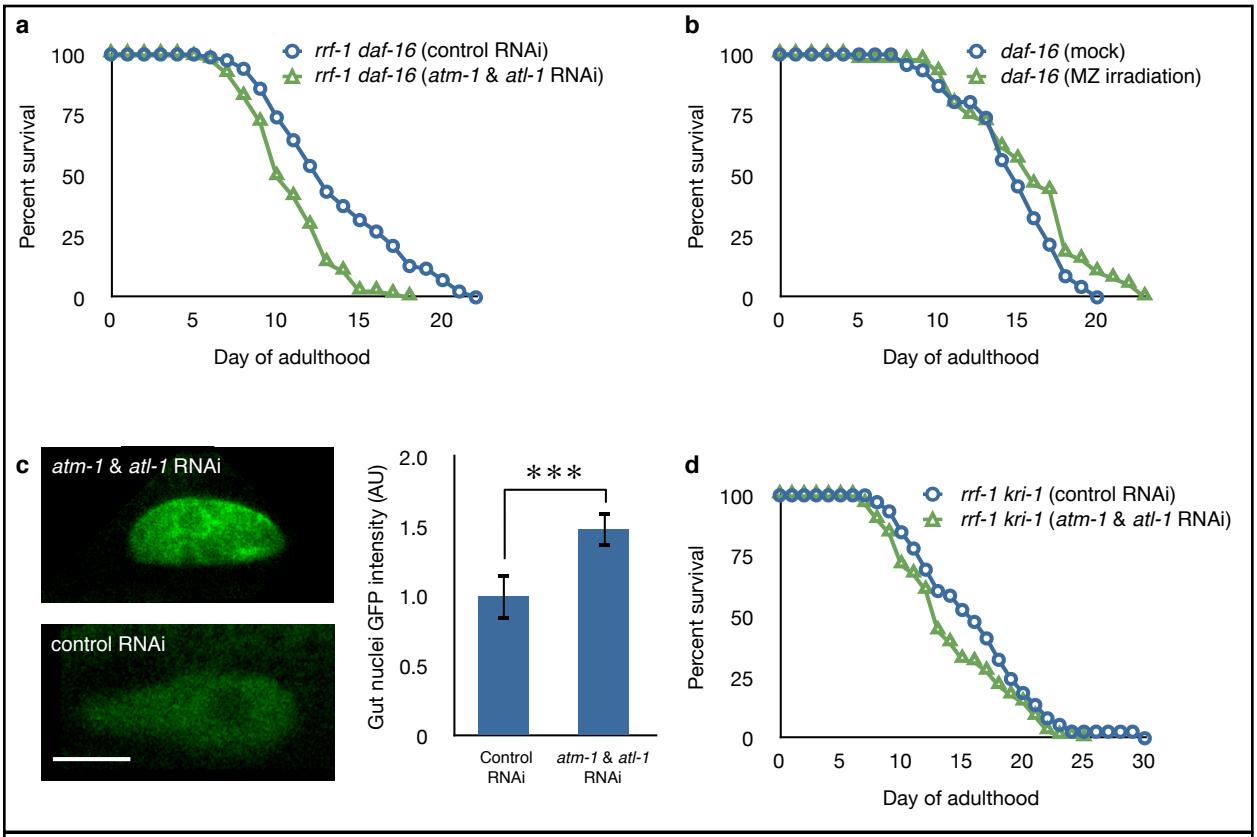

Figure 19: Germline DDR controls lifespan through insulin signaling.
a) The lifespan increase caused by *atm-1* and *atl-1* knockdown is abolished in *rrf-1 daf-16* double mutants (-20%, $p < 10^{-6}$). **b)** Targeted laser irradiation of MZ cells increases lifespan by 6% in *daf-16* mutants ($p < 0.05$). **c)** Representative images of DAF-16::GFP in the intestinal nuclei of *rrf-1* mutants in controls and after knockdown of *atm-1* and *atl-1*. GFP intensity was 48% higher after knockdown. Scale bar represents 10μm. **d)** The lifespan increase caused by *atm-1* and *atl-1* knockdown is abolished in *rrf-1 kri-1* double mutants (-11%, $p < 0.05$). *** $p < 0.001$.

suggests that germline DDR is controlling lifespan at least in part through the insulin signaling pathway.

I further asked whether *daf-16* activity is affected by germline DDR, rather than simply being a limiting factor in lifespan control. I worked with Amanda to test this by measuring DAF-16::GFP nuclear localization in the intestine after knockdown of *atm-1* and *atl-1* in *rrf-1* mutants. DAF-16::GFP was more abundant in intestinal nuclei after knockdown (Figure 19c). This suggests that decreased germline DDR leads to increased *daf-16* activity in the intestine, something which also occurs after germ cell ablation[54].

daf-16, while required for the lifespan effect of germline ablation, is also important in other lifespan extending manipulations. The *kri-1* gene is specifically required for lifespan extension after germline ablation, while not affecting other manipulations[7]. I therefore asked whether the lifespan effect of germline DDR gene knockdown is dependent on *kri-1*. I tested this by knocking down *atm-1* and *atl-1* in a *rrf-1 kri-1* double mutant background. Knockdown did not significantly increase lifespan

in this background (Figure 19d; Table 8). This suggests that germline DDR, like germline ablation, affects lifespan through *kri-1*.

The data presented so far leads me to propose the model shown in Figure 20. DNA damage accumulates in germ cells with age, leading to DDR pathway activation. This leads to a decrease in lifespan through decreased activity of *kri-1* and *daf-16*. In contrast, DDR in somatic cells increases lifespan independently of *kri-1*. This model summarizes the insights that this thesis provides both on the control of lifespan by the germline and on radiation hormesis.

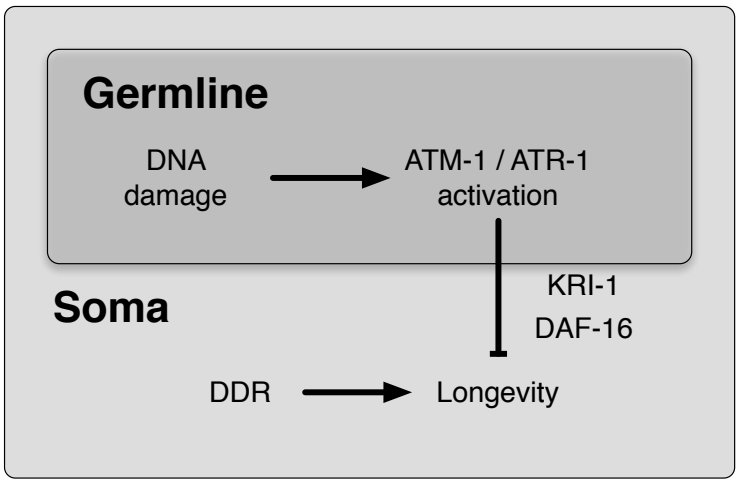

Figure 20: Summary of the proposed model
DNA damage leads to increased DDR in the germline, leading to decreased lifespan through an effect on insulin signaling. In contrast to the germline, DDR in the soma increases lifespan.

CHAPTER 4: Conclusions and future directions

The results presented in this thesis reveal a novel connection between germline DDR and lifespan control. The results also demonstrate the reproducible induction of radiation hormesis in *C. elegans*, which had not been previously reported. In this conclusion, I will briefly restate the major findings of this thesis and explain their broader implications for the mechanism behind control of lifespan by the reproductive system, for the evolutionary theories of aging, and for radiation hormesis. For each of these three topics I will propose future avenues of research.

4.1. The connection between germline DDR and lifespan

4.1.1. Germline DDR decreases lifespan

The results presented in this thesis demonstrate control of lifespan by the germline DDR. A connection between germline DDR and lifespan has not been previously reported. This finding is particularly interesting because so few previous studies have attempted to determine what aspects of germ cells affect lifespan. Instead, reports have focused on the downstream factors involved in the control of lifespan by the reproductive system. The result is that little is known about what is upstream in this control.

One previous report attributed the lifespan increased caused by germ cell ablation to a lack of germline stem cell cycling[65]. However, this conclusion was based on a genetic modification which results in the arrest and differentiation of germline stem cells. The result of this treatment is a lack of MZ cells, a halt to new pachytene cell generation, and a decrease in reproductive output. It is therefore impossible to say that germline stem cell cycling itself is responsible for lifespan control. The findings of this thesis shed new light on this report. Simultaneous knockdown of *atm-1* and *atl-1* in the germline leads to an increase in germline stem cell cycling and an increase in lifespan. This suggests that germline stem cell cycling does not directly lead to decreased lifespan. Instead, germline stem cell cycling may indirectly decrease lifespan by increasing the rate at which DNA damage accumulates with age.

4.1.2. Can germline DDR explain the effect of germline ablation on lifespan?

An exciting possibility is that control of lifespan by germline DDR might explain lifespan extension by germline ablation. The lifespan extensions caused by decreased germline DDR and by germline ablation are both dependent on *kri-1* and *daf-16*. Both decreased germline DDR and germline ablation cause increased DAF-16::GFP levels in intestinal nuclei. Why do worms respond similarly these two experimental manipulations?

One possible answer is that germline DDR activation causes germ cells to release signals which control lifespan. The reduction of DDR results in lower levels of signals being emitted. The ablation of germ cells abolishes these signals, as there are no cells left to release such signals. If this is the case, then completely blocking germline DDR activation may increase lifespan to a similar extent as germline ablation.

My results give some support to this possibility. The simultaneous knockdown of *atm-1* and *atl-1* in *rrf-1* mutants results in lifespan increases of between 12% and 21%. This falls short of the 50-60% lifespan increases observed after germ cell ablation[2,65]. However, knockdown only decreases expression of *atm-1* and *atl-1* on the fourth day of adulthood by 49% and 67%, respectively. Knockdown of these two genes in *rrf-1; rrf-3* double mutants resulted in a more dramatic increase in lifespan of 21% and 31%. The most obvious phenotype of *rrf-3* mutation is an increase in sensitivity to RNAi, including in germ cells[338]. This suggests that stronger knockdown of *atm-1* and *atl-1* leads to a greater increase in lifespan. A further increase in lifespan may be seen if germline expression of *atm-1* and *atl-1* can be completely removed. The *(atl-1)tm853* allele, which results in a frameshift mutation in *atl-1*, causes a 37% increase in lifespan. In contrast, the knockdown of *atl-1* alone in the germline results in a lifespan increase of 12% - about a third as much as mutation. This data suggests that the complete knockout of *atl-1* and *atm-1* specifically in germ cells may lead to a lifespan increase approaching the 60% observed after complete germline ablation.

4.1.3. What ATM and ATR targets are involved in lifespan control?

The results presented in this thesis implicate the germline activity of ATM-1 and ATL-1 in lifespan control. However, the molecular mechanism behind this control is still largely unknown. ATM-1 and ATL-1 are serine/threonine kinases with many known phosphorylation targets. Presumably, they affect lifespan through the phosphorylation of at least one target protein in the germline. The identity of these targets can be experimentally determined through three steps: 1) identify every target of ATM-1 and ATL-1 in the *C. elegans* germline, 2) determine which of these proteins are increasingly phosphorylated at later ages, and 3) test each target's effect on lifespan through knockdown in the germline.

The first step is to identify every target of ATM-1 and ATL-1 in the *C. elegans* germline. This can be carried out following the procedures outlined in previous reports. One report determined proteome-wide phosphorylation targets of ATM and ATR in cultured human cells by co-IP of proteins using pS/TQ antibody after irradiation[254]. One disadvantage of this procedure is that it does not distinguish targets of ATM and ATR from other PIK3/4 targets. This problem was avoided in a report that determined proteome-wide phosphorylation by mass spectrometry in yeast cells[349]. This report utilized yeast with mutations in *mec1* and *tel1*, the homologs of ATR and ATM[349]. The phosphorylation in these cells were compared to wild-type in order to determine which proteins are targets of *mec1* and *tel1*, although direct and indirect targets cannot be distinguished in this way[349].

The second step is to determine which of these proteins are increasingly phosphorylated at later ages. This can be carried out by analyzing the proteome-wide phosphorylation pattern of younger and older germlines. Proteins which display increasing levels of phosphorylation with age are ideal candidates for further analysis. Not all of these candidates would necessarily be involved in lifespan control. Instead, they could be involved in other age-related changes such as decreased cell cycling. However, this second step should narrow down the number of candidate genes for further investigation.

The third step is to test each target's effect on lifespan through knockdown in the germline. The previously discussed report that identified *mec1* and *tel1* targets in yeast found 55 different phosphorylation sites[349]. It is possible that the number of candidate genes in worms will be similarly large. Any attempt to assay the lifespan effect of this many genes should utilize high-throughput techniques, discussed previously[350]. This experimental approach should lead to the identification of the genes through which germline ATM and ATR affect lifespan.

4.2. Evolutionary implications of the control of lifespan by germline DDR

4.2.1. Lifespan control by germline DDR is inconsistent with the disposable soma theory

The results of this thesis show that knockdown of *atm-1* and *atl-1* in the germline leads to increased lifespan. This same experimental manipulation also leads to an increase in MZ cell cycling rate, germ cell number, and progeny production, all of which require that additional resources be committed to the reproductive system. These results parallel the previously published observation that the transplantation of younger ovaries into older mice increases lifespan, despite continued cycling by the transplanted ovaries[66]. These results are inconsistent with the disposable soma theory of aging, which predicts that increased resource use for reproduction leads to decreased lifespan.

There is the possibility that the knockdown of *atm-1* and *atl-1* actually does decrease resource use by the germline, due to decreased DDR activation. This would require that the resources saved by decreased repair efficiency is larger than the resources expended by increased cell cycling and progeny production. This is an inherently difficult statement to disprove, as the identities of the relevant resources are unknown. One way to convincingly show that germline DDR is not affecting lifespan through resource use would be if lifespan can be extended to the same degree as by germline ablation through the modification of DDR gene expression, while at the same time preserving reproductive function. An experimental strategy to accomplish this was discussed in a previous section.

4.2.2. Can kin-selection driven senescence explain the effect of germline DDR on lifespan?

One way to explain the evolution of the control of lifespan by germline DDR is through kin-selection driven senescence. Germline DDR may be used as a readout by an organism to determine when post-reproductive life has been reached. This may then activate an aging program in order to benefit their progeny by the truncation of post-reproductive lifespan. It may be possible to empirically test this explanation. As discussed in the introduction to this thesis, certain conditions can theoretically drive the evolution of phenoptosis. One of these conditions is high population viscosity. If the control of lifespan by germline DDR really is due to phenoptosis, then low population viscosity will strengthen the connection between germline DDR and lifespan, while high population viscosity will weaken this connection.

Many of the procedures for experimental evolutionary studies in *C. elegans* have been previously outlined[351-353]. Three important considerations are that the starting

population should be genetically heterogeneous, around 50 generations should be allowed to pass, and multiple independent lines should be maintained. The proposed experiment will require that *C. elegans* are passaged under conditions that mimic either high or low population viscosity. Low population viscosity is easily achieved by passaging groups of freely-mixing worms on large plates. The advantage of kin-selection under these conditions should be close to zero, as worms are competing against all neighbors equally, regardless of genetic relatedness. High population viscosity can be achieved by passaging individual worms onto small plates, allowing them to form a population, and then mixing all of the worms from multiple small plates together on larger plates before passaging another group of individual worms. The advantage of kin-selection under these conditions should be high. Alleles are most likely to survive passaging if they contribute towards the expansion of the whole population on the small plates, as this increases that population's representation upon transfer to the larger plates. After about 50 generations, the lifespan effect of germline *atm-1* and *atl-1* knockdown can be compared between lines which evolved under high and low population viscosities. If conditions of higher population viscosity leads to an increased effect of germline DDR gene knockdown on lifespan, then it supports the idea that kin-selection is responsible for the evolution of a connection between germline DDR and lifespan.

4.2.3. How might phenoptosis be beneficial?

The decrease in lifespan in response to germline DDR may be an example of phenoptosis. The selective advantage of phenoptosis is dependent on a trade-off between limitation of an organism's lifespan, which potentially truncates reproduction, and the benefit obtained by that organism's progeny. It is not advantageous for an organism to prematurely age if no benefit will be obtained. What is the benefit obtained by phenoptosis in the case of lifespan control by the germline DDR?

The commonality between many of the described instances of phenoptosis is that the goal is provide resources to successive generations[149,153,154]. Similarly, the goal of lifespan control by the germline DDR may be to limit post-reproductive lifespan in order to conserve resources for successive generations. Phenoptosis is therefore only beneficial if successive generations are present. Perhaps worms are more likely to activate a program of phenoptosis under these conditions.

Conditions that might favor the activation of phenoptosis may include the following: 1) the presence of a high density of neighboring worms, 2) the presence of reproductively active neighbors, or 3) the presence of genetically related neighbors. It is possible that worms are able to detect the presence and status of neighbors and modulate their rate of aging accordingly. The lifespan effect of all three of these conditions can be tested in a similar fashion. First, a population of worms whose lifespan would be assayed must be age-synchronized. These will be referred to as "tracked" worms. Second, plates containing the right density, age, and identity of neighboring worms would be prepared. Worms with a easily identifiable GFP expression will be used as neighbors, in order to distinguish them from tracked worms. Tracked worms would be transferred to new neighboring worm plates each day. The lifespan of the tracked worms would then be compared to control worms, passaged normally on

blank plates. This experimental setup would allow for the determination of the influence of neighboring worms on lifespan.

The first condition that may favor phenoptosis is the presence of a high density of neighboring worms. This can be done by using a non-synchronized population of worms as the neighbors in the above experiment. The second condition that may favor phenoptosis is the presence of reproductively active neighbors. This can be tested by comparing the lifespan of tracked worms in the presence of two different types of neighboring worms: reproductively active young worms, and reproductively exhausted old worms. The third condition that may favor phenoptosis is the presence of genetically related neighbors. This can be tested by comparing the lifespan of tracked worms in the presence of three different types of neighboring worms: genetically identical neighbors (for instance, N2 worms if the tracked worms are derived from this strain), wild-isolates of *C. elegans*, and other species of nematode (for instance, *C. brenneri*). If the presence of reproductively active, genetically related neighbors leads to a decrease in the lifespan of tracked worms, this would suggest that phenoptosis may be specifically activated under conditions that maximize the benefit derived from it.

4.3. Somatic DDR and radiation hormesis

4.3.1. The opposing effects of somatic and germline DDR on lifespan

In this thesis I showed that the somatic knockdown of several different genes involved in DNA damage repair led to decreased lifespan. This is consistent with previously published reports testing the effect of DNA damage repair gene mutation in worms. The mutation of *wrn-1* or *xpa-1* leads to a decrease in worm lifespan[124,285]. This is also consistent with the effect of DDR gene disruption in other systems. Human progeroid syndromes are associated with mutations in genes involved in DNA damage repair[79,80]. The link between somatic DDR and lifespan is presumably due to the importance of the DDR pathway in repairing DNA damage and preventing mutations.

The effect of the somatic DDR on lifespan is in stark contrast to the effect of germline DDR. The knockdown of DDR genes in the germline increases lifespan, while experimentally increasing DDR in the germline decreases lifespan. The opposing lifespan effects of germline and somatic DDR may explain several previously published results. Certain DDR gene mutants have been reported as having increased lifespan[286-288]. Such a lifespan increase may be due to the effect that these mutations have on somatic DDR.

4.3.2. Radiation hormesis

Previously published reports have failed to consistently demonstrate lifespan increases after irradiation in *C. elegans*[8,9,354]. In this thesis I demonstrate how radiation hormesis can be consistently induced. This was accomplished by using *kri-1* mutant worms, in which the connection between the reproductive system and lifespan has been abolished[7]. The simplest explanation for the consistent induction of radiation hormesis in *kri-1* mutants is that the irradiation of germ cells has a negative effect on lifespan while the irradiation of somatic cells has a positive effect on lifespan. This is further

supported by the observation that radiation hormesis is induced in sterile *kri-1; glp-1* mutant worms. The demonstration of a simple procedure to induce radiation hormesis in *C. elegans* provides a very useful tool for the study of this phenomenon, due to the technical benefits of using worms to study lifespan. Several experimental questions can be asked using this system: 1) what type of molecular damage is responsible for radiation hormesis, 2) what somatic tissues are responsible for radiation hormesis, and 3) what is the mechanism behind radiation hormesis?

The first question is what type of molecular damage is responsible for radiation hormesis. The simplest answer is that radiation-induced DNA damage is responsible for the hormetic lifespan increase. However, gamma irradiation in bacteria also leads to the oxidative damage of proteins[355]. It is therefore possible that radiation hormesis in worms is due to the induction of protein damage. Differentiating between DNA and protein damage as the cause of radiation hormesis can be accomplished by using more specific sources of damage. One way to do this is by utilizing the components of the CRISPR system. CRISPR-based genome editing can be carried out in worms through feeding of sgRNA to transgenic worms which ubiquitously express Cas9 protein[356]. This transgenic strain could be modified to express Cas9 in the soma using the *sur-5* promoter and 3‘UTR[316]. Additionally, an extrachromosomal array containing a repetitive DNA sequence capable of being targeted by Cas9 would be added. Exogenous DNA damage in somatic cells could be induced in such a strain by feeding it bacteria expressing sgRNA targeting the extrachromosomal array. If this system can be used to induce increased lifespan, it would suggest that DNA damage can indeed have a hormetic effect.

The second question is which somatic tissues play a role in radiation hormesis. It is technically impractical to irradiate individual tissues in *C. elegans*. An alternative is to use the system described in the previous paragraph. Rather than driving Cas9 expression using a pan-somatic promoter, expression can be driven using tissue specific promoters. Promoters that have previously been used for tissue-specific gene expression can be used for this purpose[357-360]. This will allow for the induction of DNA damage in a tissue-specific manner. Such a strategy should reveal which tissues are important in radiation hormesis.

The third question is what is the mechanism behind radiation hormesis. One potential answer is that DNA damage early in life results in a long-lasting increase in DNA damage repair activity. This explanation is consistent with the previously discussed report which found that the overexpression of certain DNA damage repair proteins increased lifespan in *Drosophila*[361]. If the increased expression of repair proteins is responsible for radiation hormesis, than preventing this expression increase should abolish the lifespan increase observed after irradiation. This can be tested by knocking down DNA damage repair genes after irradiation. Knockdown should bring the lifespan of irradiated and non-irradiated worms to approximately the same length. This would suggest that radiation hormesis is due to overcompensation by the DNA damage repair machinery.

4.3.3. Closing remarks

The ultimate goal of the field of aging research is to determine the fundamental causes of aging, both in terms of molecular and evolutionary mechanisms. My results support the idea that one of the evolutionary drivers of aging is the reduction of post-reproductive lifespan. However, a prerequisite of the selection for programmed aging is that reproductive aging occurs. If somatic aging is driven by reproductive aging, then we must then ask the question: what are the fundamental causes of reproductive aging? In order to fully understand aging, it will be necessary to understand the causes of reproductive aging.

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Appendix A: Strains and data tables

Table 1: Worm strains used in this thesis.

Strain name	Genotype	Relevant phenotype(s)
Previously existing strains		
N2	wild type	Wild type
NL2098	*rrf-1(pk1417) I*	Germline specific RNAi sensitivity
VP303	*rde-1(ne219) V; kbIs7[Pnhx-2::rde-1 rol-6(su1006)]*	Intestine specific RNAi sensitivity
WS4581	*unc-119(ed3) III; opIs263[Prpa-1::rpa-1::yfp unc-119(+)]*	Expression of RPA-1::YFP fusion transgene
WS2265	*hus-1(op244) I / hT2[bli-4(e937)let-?(q782)qIs48] (I;III)*	*hus-1* null mutant
CA538	*rad-51(lg8701) / mIs11[Pmyo-2::gfp Ppes-10::gfp PF22B7.9::gfp] IV*	*rad-51* mutant
VC174	*wrn-1(gk99) II*	*wrn-1* mutant
AV112	*mre-11(ok179IV / nT1[unc-?(n754) let-?] (IV;V)*	*mre-11* mutant
CF2052	*kri-1(ok1251) I*	Germline control of lifespan is abolished
CF2065	*kri-1(ok1251) I; glp-1(e2141) III*	Germline control of lifespan is abolished; temperature-sensitive loss of germ cells
NL2250	*ppw-1(pk2505) I*	Soma-specific RNAi sensitivity
CF1038	*daf-16(mu86) I*	Defective insulin signaling
MAH97	*rrf-1(pk1417) I; muIs109[Pdaf-16::GFP::DAF-16 cDNA + Podr-1::RFP]*	Germline-specific RNAi sensitivity; expression of GFP::DAF-16 fusion transgene
Derived for this thesis		
CNQ23	*rrf-1(pk1417) I; ced-3(n717) IV*	Germline specific RNAi sensitivity; apoptosis defective
CNQ27	*rrf-1(pk1417) daf-16(mu86) I*	Germline specific RNAi sensitivity; defective insulin signaling
CNQ36	*rrf-1(pk1417) I; rrf-3(pk1426) II*	Germline specific RNAi sensitivity; RNAi hypersensitive
CNQ37	*rrf-3(pk1426) II; rde-1(ne219) V; cinEx1[Psur-5::rde-1 Pmyo-3::mcherry]*	Somatic specific RNAi sensitivity; RNAi hypersensitive
CNQ55	*rrf-1(pk1417) kri-1(ok1251) I*	Germline-specific RNAi sensitivity; germline control of lifespan is abolished
CNQ59, CNQ60, and CNQ61	*atl-1(cin1[Pglh-1::atl-1]) V*	Germline-specific expression of *atl-1*; lines #1, 2, and 3
CNQ64	*cinIs4[Pglh-1::rde-1 ORF::glh-1 3'UTR] II; rde-1(ne219) V*	Germline specific RNAi sensitivity

Table 2: qPCR data for gene expression.

"Percent change" and "p-value" are relative to control RNAi in the case of knockdowns and N2 in the case of mutants or transgenics. All data are based on at least three biological replicates.

Worm strain	Collected tissue	RNAi target	qPCR target	Percent change	Welch's t-test p-value
rrf-1	MZ	*cku-70*	*cku-70*	-74%	9.65×10^{-6}
rrf-1	MZ	*xpa-1*	*xpa-1*	-80%	0.000487
rrf-1	*MZ*	*msh-2*	*msh-2*	-83%	3.58×10^{-5}
rrf-1	MZ	*cep-1*	*cep-1*	-49%	0.00103
rrf-1	MZ	*hus-1*	*hus-1*	-51%	0.00439
rrf-1	MZ	*atm-1 & atl-1*	*atm-1*	-49%	0.0356
rrf-1	MZ	*atm-1 & atl-1*	*atl-1*	-67%	0.00183
rrf-1	MZ	*atl-1*	*atl-1*	-29%	0.0460
CNQ64	MZ	*atm-1 & atl-1*	*atl-1*	-44%	0.0224
CNQ64	Whole worm	*atm-1 & atl-1*	*atl-1*	-27%	0.534
N2	MZ	*atm-1 & atl-1*	*atl-1*	-55%	0.0439
N2	Whole worm	*atm-1 & atl-1*	*atl-1*	-64%	0.0237
N2	MZ	*cku-70*	*cku-70*	-47%	0.0304
N2	Whole worm	*cku-70*	*cku-70*	-82%	0.00266
rrf-1; rrf-3	MZ	*atm-1 & atl-1*	*atl-1*	-38%	0.00284
rrf-1; rrf-3	MZ	*atm-1 & atl-1*	*atm-1*	-29%	0.0277
rrf-1; rrf-3	MZ	*atl-1*	*atl-1*	-42%	0.000422
rrf-1; rrf-3	MZ	*atl-1*	*atm-1*	-21%	0.125
rrf-1; rrf-3	MZ	*atm-1*	*atl-1*	+1%	0.747
rrf-1; rrf-3	MZ	*atm-1*	*atm-1*	-31%	0.0132
CNQ59	MZ	-	*atl-1*	-8%	0.564
CNQ59	Gut	-	*atl-1*	-60%	0.0307
CNQ60	MZ	-	*atl-1*	-35%	0.00472
CNQ60	Gut	-	*atl-1*	-64%	0.00626
CNQ61	MZ	-	*atl-1*	-28%	0.0674
CNQ61	Gut	-	*atl-1*	-65%	0.000738

Table 3: Lifespan data for targeted irradiation experiments.
'Percent change' and 'Log rank p-value' are relative to mock treatment of the same worm strain performed simultaneously.

Experiment number	Genetic background	Irradiation target	n	Mean lifespan	Percent change	Log rank p-value
1	wild-type	(mock)	43	18.7		
1	wild-type	MZ	29	16.7	-10.7%	0.0144
2	wild-type	(mock)	148	18.1		
2	wild-type	MZ	63	16.3	-9.5%	0.0408
3	wild-type	(mock)	71	13.8		
3	wild-type	PZ	47	13.6	-1.6%	0.607
4	*daf-16*	(mock)	46	14.8		
4	*daf-16*	MZ	39	15.7	+6.0%	0.029

Table 4: Lifespan data for whole-worm irradiation experiments.
'Percent change' and 'Log rank p-value' are relative to mock treatment of the same worm strain performed simultaneously.

Experiment number	Genetic background	Irradiated / mock	Fertile / sterile	n	Mean lifespan	Percent change	Log rank p-value
1	wild-type	Mock	Fertile	34	16.9		
1	wild-type	Irradiated	Fertile	36	17.7	+5.2%	0.352
1	*kri-1*	Mock	Fertile	41	15.6		
1	*kri-1*	Irradiated	Fertile	37	18.6	+19.1%	0.000248
2	wild-type	Mock	Fertile	90	18.8		
2	wild-type	Irradiated	Fertile	86	17.1	-9.0%	0.0573
2	*kri-1*	Mock	Fertile	82	17.9		
2	*kri-1*	Irradiated	Fertile	93	19.8	+10.7%	1.73×10^{-5}
3	wild-type	Mock	Fertile	184	14.0		
3	wild-type	Irradiated	Fertile	187	14.3	+1.8%	0.998
3	*kri-1*	Mock	Fertile	37	13.3		
3	*kri-1*	Irradiated	Fertile	187	16.6	+25.0%	0.00105
4	*kri-1; glp-1*	Mock	Sterile	93	11.7		
4	*kri-1; glp-1*	Irradiated	Sterile	85	15.0	+28.3%	4.34×10^{-7}
5	*kri-1; glp-1*	Mock	Sterile	93	15.8		
5	*kri-1; glp-1*	Irradiated	Sterile	82	20.3	+28.6%	1.73×10^{-13}

Table 5: Lifespan data for germline knockdown experiments.
'Percent change' and 'Log rank p-value' are relative to control knockdown performed simultaneously.

Experiment number	Genetic background / strain	RNAi target gene	RNAi started at	n	Mean lifespan	Percent change	Log rank p-value
1	*rrf-1*	control	L1	95	17.5		
1	*rrf-1*	*dog-1*	L1	94	19.4	+10.6%	0.000347
1	*rrf-1*	*rad-51*	L1	95	19.2	+9.7%	7.44×10^{-5}
1	*rrf-1*	*wrn-1*	L1	97	19.1	+9.1%	0.0066
1	*rrf-1*	*xpa-1*	L1	78	18.5	+5.7%	0.0137
1	*rrf-1*	*cep-1*	L1	83	18.4	+5.0%	0.102
1	*rrf-1*	*msh-2*	L1	89	17.9	+2.1%	0.529
2	*rrf-1*	control	L1	89	14.2		
2	*rrf-1*	*mre-11*	L1	90	15.8	+11.7%	0.00722
2	*rrf-1*	*cku-70*	L1	93	14.0	-1.0%	0.522
2	*rrf-1*	*rpa-1*	L1	92	12.9	-8.8%	0.0327
4	*rrf-1*	control	L1	155	15.9		
4	*rrf-1*	*atm-1 & atl-1*	L1	165	18.1	+14.0%	0.0482
5	*rrf-1*	control	L1	91	14.9		
5	*rrf-1*	*hus-1*	L1	91	16.9	+13.4%	0.00475
6	*rrf-1*	control	L1	87	16.0		
6	*rrf-1*	*atl-1*	L1	80	17.9	+12.1%	0.0113
6	*rrf-1*	*atm-1 & atl-1*	L1	77	18.4	+15.2%	0.0011
6	*rrf-1*	*atm-1 & atl-1*	L4+12hrs	65	17.9	+12.3%	0.0182
6	*rrf-1*	*atm-1 & atl-1*	L4+72hrs	81	17.6	+10.4%	0.0133
7	*rrf-1*	control	L1	87	14.5		
7	*rrf-1*	*atm-1 & atl-1*	L1	75	16.2	+11.8%	0.0118
7	*rrf-1*	*rpa-1*	L1	87	14.5	-15.3%	5.07×10^{-5}
8	*rrf-1*	control	L1	90	15.0		
8	*rrf-1*	*gfp*	L1	65	15.3	+2.3%	0.398
8	*rrf-1*	*hus-1*	L1	90	16.3	+9.3%	0.0343
8	*rrf-1*	*atm-1 & atl-1*	L1	90	18.7	+25.3%	1.87×10^{-7}

Experiment number	Genetic background / strain	RNAi target gene	RNAi started at	n	Mean lifespan	Percent change	Log rank p-value
9	*rrf-1; rrf-3*	control	L1	94	13.9		
9	*rrf-1; rrf-3*	*atm-1 & atl-1*	L1	80	16.7	+20.5%	0.00502
10	*rrf-1; rrf-3*	control	L1	67	14.2		
10	*rrf-1; rrf-3*	*atm-1*	L1	54	15.7	+11%	0.0153
10	*rrf-1; rrf-3*	*atl-1*	L1	69	17.6	+24%	0.000883
10	*rrf-1; rrf-3*	*atm-1 & atl-1*	L1	61	18.5	+31%	0.000322
11	CNQ64	control	L1	89	14.5		
11	CNQ64	*atm-1 & atl-1*	L1	72	17.3	+19.5%	3.96×10^{-6}

Table 6: Lifespan data for somatic knockdown experiments.

'Percent change' and 'Log rank p-value' are relative to control knockdown performed simultaneously.

Experiment number	Genetic background	RNAi sensitive tissue	RNAi target gene	n	Mean lifespan	Percent change	Log rank p-value
1	*ppw-1*	Soma	control	55	17.8		
1	*ppw-1*	Soma	*atm-1 & atl-1*	54	16.7	-6.1%	0.571
2	*ppw-1*	Soma	control	51	20.9		
2	*ppw-1*	Soma	*atm-1 & atl-1*	58	19.5	-7.1%	0.0101
3	VP303	Intestine	*control*	67	15.3		
3	VP303	Intestine	*atm-1 & atl-1*	74	13.6	-11.1%	0.000817

Table 7: Lifespan data for DDR mutant experiments.

'Percent change' and 'Log rank p-value' are relative to wild-type worms assayed simultaneously.

Experiment number	Genetic background / strain	n	Mean lifespan	Percent change	Log rank p-value
1	wild-type	116	19.4		
1	*wrn-1*	75	15.6	-19.9%	$-9.86E \times 10^{-8}$
1	*mre-11*	104	17.6	-9.7%	0.0267
1	*rad-51*	103	17.9	-7.9%	0.0171
2	wild-type	32	22.7		
2	*hus-1*	34	15.2	-33.2%	7.63×10^{-9}
3	wild-type	67	17.4		
3	*hus-1*	56	15.7	-9.7%	0.0495
4	wild-type	73	17.4		
4	*atl-1*	76	23.8	36.8%	$< 10^{-20}$
5	N2	104	17.2		
5	CNQ59	69	16.97	-1.3%	0.661
5	CNQ60	65	16.9	-1.9%	0.789
5	CNQ61	57	16.4	-4.4%	0.471

Table 8: Lifespan data for apoptosis and IIS mutant experiments.
'Percent change' and 'Log rank p-value' are relative to control knockdown performed simultaneously.

Experiment number	Genetic background	RNAi target gene	n	Mean lifespan	Percent change	Log rank p-value
1	*rrf-1; ced-3*	control	81	15.1		
1	*rrf-1; ced-3*	*atm-1 & atl-1*	73	17.9	+18.1%	4.57×10^{-7}
2	*rrf-1 daf-16*	control	85	13.7		
2	*rrf-1 daf-16*	*atm-1 & atl-1*	85	10.9	-19.9%	3.09×10^{-7}
3	*rrf-1 kri-1*	control	103	15.8		
3	*rrf-1 kri-1*	*atm-1 & atl-1*	104	14.0	-11.4%	0.0236

Table 9: Lifespan data for whole-worm knockdown experiments.
'Percent change' and 'Log rank p-value' are relative to control knockdown performed simultaneously.

Experiment number	Genetic background	RNAi target gene	n	Mean lifespan	Percent change	Log rank p-value
1	wild-type	control	41	18.8		
1	wild-type	*rad-51*	46	21.3	+13.3%	0.00114
1	wild-type	*mre-11*	71	21	+11.7%	0.00137
1	wild-type	*wrn-1*	47	20.6	+9.3%	0.0104
1	wild-type	*atm-1 & atl-1*	83	19.8	+5.3%	0.117
1	wild-type	*msh-2*	85	19.8	+5.0%	0.285
1	wild-type	*dog-1*	58	19.7	+4.5%	0.299
1	wild-type	*cep-1*	57	19.3	+2.5%	0.622
1	wild-type	*cku-70*	89	18.8	-0.1%	0.474
1	wild-type	*xpa-1*	21	17.6	-6.4%	0.586
2	wild-type	control	90	18.2		
2	wild-type	*rpa-1*	93	13.5	-25.9%	2.46×10^{-13}
3	wild-type	control	88	18.0		
3	wild-type	*hus-1*	81	18.9	+5.2%	0.492