The role of *daf-16* in *C. elegans* radiation resistance

By

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A Thesis Submitted to The W.A. Franke Honors College

In Partial Fulfillment of the Bachelor's degree

With Honors in

Molecular and Cellular Biology

THE UNIVERSITY OF ARIZONA

December 2024

Approved by:

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Abstract

Medical research is usually focused on diseases and their unique characteristics that could be targeted for therapy. Age is the primary risk factor for most chronic diseases in the modern era. There is a more direct focus on the biology underlying the aging process and the cellular stress individuals are put through during this period. Oxidative stress, DNA damage, and protein misfolding are just a few of many common causes for activating cellular stress pathways. Eukaryotic cells have developed various molecular pathways for dealing with the deleterious effects of different stresses. As the aging process continues, dysregulation of these stress pathways increases the risk for many age-associated diseases. By slowing this process, we can work towards maintaining health even in old age. My work with *Caenorhabditis elegans* delves into the effects of radiation on aging and how manipulation of specific molecular genotypes affects the lifespan of these nematodes. The DAF-16/FOXO pathway regulates genes involved in aging, stress, and metabolism, enhancing antioxidant defenses and metabolic shifts under stress conditions like starvation. My focus on this pathway and its regulation of stress response and longevity demonstrates how its activation influence survival and stress resistance under exposure to radiation. My thesis will discuss the effects of radiation and induced stress on *C. elegans*. It will outline the results of my experiments and how age-related deterioration is affected by specific changes in genotype.

Introduction

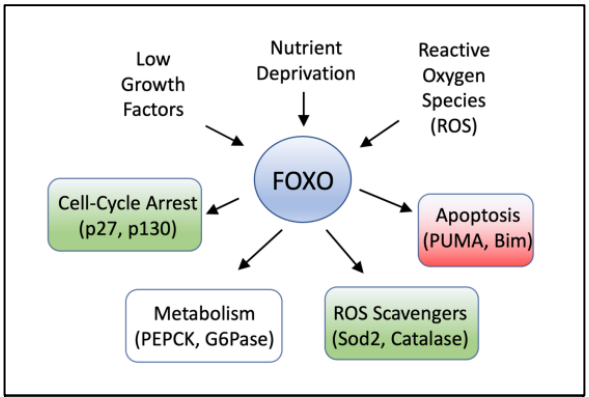

Figure 1: FOXO transcription factors control a range of cellular processes, most notably inhibition of the cell cycle, regulation of cell death, protection from oxidative stress, and regulation of cellular metabolism.

The insulin/insulin-like signaling pathway is one of the most important cellular processes linked to aging, regulating a wide range of critical functions, including cell cycle inhibition, apoptosis, and protection against oxidative stress (Murtaza et al., 2017). The accumulation of reactive oxygen species (ROS), protein misfolding, and DNA damage are associated with aging and play a role in the development of various age-related diseases. Genetic evidence suggests a prominent role for FOXO in lifespan regulation in several animal models such as *Caenorhabditis elegans*, *Drosophila*, and mice (Santos et al., 2023). Notably, FOXO3 is the second most replicated gene associated with extreme human longevity in genome-wide association studies, suggesting that targeting FOXO pharmacologically could be a promising step towards improving long-term health (Santos et al., 2023).

Preliminary research shows a relationship between radiation and DAF-16, the FOXO ortholog in *Caenorhabditis elegans.* DAF-16 is the only FOXO homolog in *C. elegans*, showing integration of signals from upstream pathways to elicit transcriptional changes in many genes involved in aging, stress, and metabolism (Zečić & Braeckman, 2020). During stress conditions such as starvation, *daf-16* upregulates the transcription of genes that improve antioxidant defenses and shift metabolism, allowing the worms to resist various forms of cellular stress (Kenyon et al., 1993). SOD-3p::GFP is a *daf-16* reporter which can be used to measure physiological age, as its expression declines with age (Sánchez-Blanco & Kim, 2011). Higher *sod-3* expression from worm to worm is correlative with longer individual lifespan. A major regulator of the transcription of this gene is the insulin signaling pathway (IGF-1). When this pathway is reduced, past research leads to an increase in lifespan in worm models, flies, mice, and humans. We suggest that starvation may play a role in radiation sensitivity in *C. elegans,* and the *daf-16* gene plays a protective role in radiation resistance in these nematodes.

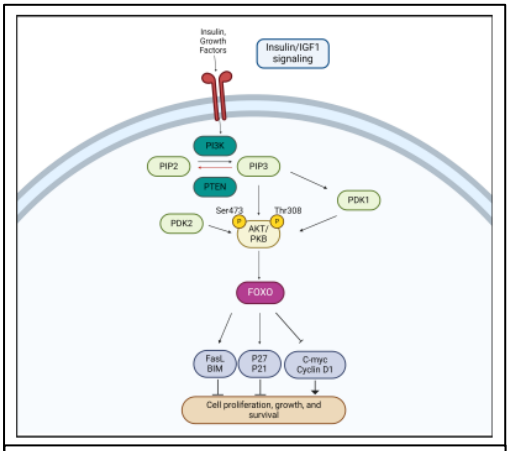

Figure 2: Diagram of the insulin signaling pathway

Materials and Methods

Strains

The following strains were obtained from the *Caenorhabditis* Genetic Center (CGC) at the College of Biological Sciences at the University of Minnesota: *daf-16*(mu86) I (CF1038); *sod-3p*::*GFP* (muls84) I (CF1553); DAF-16::GFP (TJ356). Wild type (N2) worms were originally obtained from Dr. Matt Kaeberlein (University of Washington, Seattle, WA, USA).

C. elegans culture and maintenance

We kept the worms on 60 mm plates containing Nematode Growth Media (NGM) spotted with OP50 *Escherichia coli* (Sutphin & Kaeberlein, 2009) unless described differently. The worms were maintained at 20°C and transferred to new plates at least twice per week to prevent starvation. For RNAi experiments, OP50 was replaced with the HT115 *E. coli* strain containing the indicated RNAi plasmid. Finally, 50 µL of carbenicillin and 100 µL of IPTG per 100 mL of NGM were added to RNAi plates (Sutphin & Kaeberlein, 2009).

Age synchronization

The bleaching technique was used for age synchronization of *C. elegans*. To do this, we washed the desired plate with sterile water to loosen the eggs and worms that were stuck to the plate. Then, we transferred this liquid into a sterile centrifuge tube. We prepared a mixture of 10M NaOH, water, and bleach. Next, we placed the tube in a centrifuge for 60 seconds at 1700 RPM. We added 5 mL of the bleach solution and placed it on a nutator for no more than 3 minutes. We then aspirated the supernatant and repeat the previous step one more time. Then we added 10 mL of sterile water and placed it in the centrifuge for 60 seconds at 1700 RPM. We removed the excess liquid and placed the desired number of eggs on the selected plate (Porta-de-la-Riva et al., 2012).

Body size measurements

Worms were bleached following the previously described protocol and grown on NGM plates. At the L4 larval stage, all worms were transferred to NGM plates supplemented with FUdR to prevent reproduction. Once they reached the L4 stage, worms were moved to either control plates or plates which were then irradiated. Images were captured at L4 immediately after radiation (D0) and on day 1 (D1) of adulthood, and body size was assessed using the measurement tool in arivis Vision4D software (version 4.1.1, https://www.arivis.com/products/pro). The settings were maintained consistently across all images.

ROS measurements

2',7'-dichlorofluorescein diacetate (H_2DCFDA) is used to detect the presence of ROS in *C. elegans.* It penetrates the cellular membrane and then is deacetylated by esterases which turns it into H_2DCF, a non-fluorescent compound. This compound is rapidly oxidized to DCF, which is highly fluorescent (Yoon et al., 2018). After we treated our worms (approximately 50), we washed the worms from the NGM plate with M9 buffer and transferred them to a centrifuge tube. We then washed them three times with M9 buffer. These worms were transferred to an OP50 agar plate for 5- 10 minutes, so they were able to crawl from the liquid. Approximately 15 animals were transferred onto an agarose pad with a drop of levamisole. Pictures were taken with a Leica microscope to measure the fluorescence signal.

Results

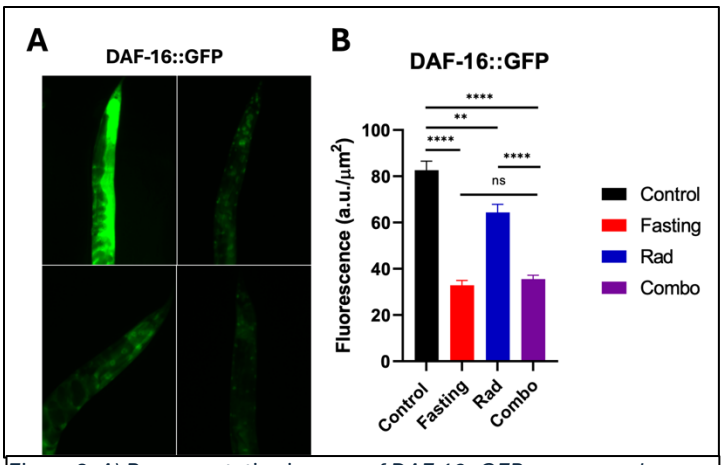

Figure 3: A) Representative images of DAF-16::GFP worms under respective conditions. B) Fluorescence (a measure of expression) is reduced in fasting and irradiated models. Expression is localized to the nucleus as seen in the images taken. Combination of fasting and radiation does not present a significant difference from fasting models.

Radiation increases DAF-16 expression in *C. elegans*

Previous research in our lab demonstrated a direct relationship between radiation-induced stress and *daf-16* expression in *C. elegans* (Figure 3). DAF-16, an ortholog of FOXO (Senchuk et al., 2018), is recognized for its essential role in managing stress responses. Building on this foundation, we designed an age-synchronization experiment to investigate the relationship further by comparing wild type and *daf-16* mutant strains. Figure 4 shows that *daf-16* knockout worms exhibit a notable decrease in lifespan, highlighting the gene's critical function in stress resilience and longevity.

The relationship between radiation stress and *daf-16* expression highlights an essential survival mechanism linked to the DAF-16/FOXO pathway. Activation of DAF-16 initiates the transcription of genes involved in antioxidant defense, DNA repair, and cell cycle regulation—

key processes for countering radiation-induced cellular damage. In wild type *C. elegans*, exposure to radiation markedly upregulates *daf-16*, enhancing their resilience by promoting these protective pathways. The reduced lifespan observed in *daf-16* knockout strains suggests that, without *daf-16*, the worms lack upregulation of genes that would otherwise mitigate radiation-induced reactive oxygen species (ROS) damage. Consequently, these worms experience accelerated cellular damage and aging, highlighting the protective function of *daf-16* in extending lifespan under stress. These findings provide strong evidence that the DAF-16/FOXO pathway is essential for radiation resistance in *C. elegans* and suggest that similar mechanisms may operate in other organisms, where DAF-16/FOXO could play a pivotal role in managing cellular stress and promoting repair pathways.

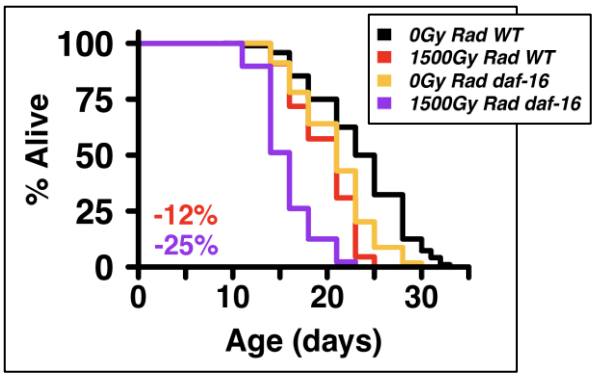

Figure 4: daf-16 mutants are more sensitive to X-ray irradiation compared to WT, suggesting a possible role for daf-16 in radiation resistance.

Short Term Starvation (STS) has no significant effect in radiation sensitivity in day 1 adults

We examined the impact of Short-Term Starvation (STS) on the survival of day 1 adult *C. elegans* following exposure to 1500 Gy of radiation and decided to use fasting to activate DAF-16 since the presence of insulin acts as a negative regulator in the insulin/insulin-like signaling pathway. As depicted in Figure 5, there was no significant difference in survival between wild type worms subjected to STS and those on an ad libitum diet. Similarly, *daf-16* mutants under STS and ad libitum conditions exhibited comparable survival profiles to the wild type groups. These findings support STS did not alter the worms' sensitivity to radiation under these conditions.

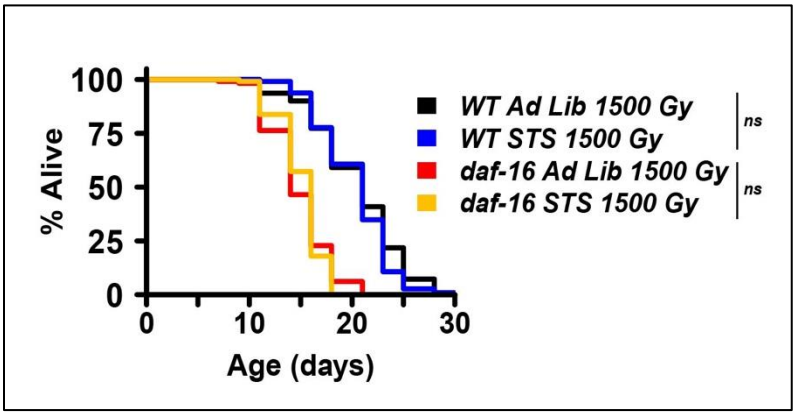

Figure 5: There is no significant difference between STS (short-term starvation) and Ad Lib (ad libitum) daf-16 mutants as compared to WT, suggesting a larger starvation period may be required.

The lack of impact from STS on radiation sensitivity in day 1 adult *C. elegans* highlights the specificity of the DAF-16/FOXO pathway in addressing certain types of cellular stress. DAF-16's activation is typically responsive to direct stress signals, like those from reactive oxygen species (ROS) generated by radiation exposure.

When *C. elegans* face oxidative stress due to radiation, DAF-16

translocates to the nucleus and activates a suite of protective genes, including antioxidant genes like *sod-3*, which combats ROS and mitigates cellular damage. This pathway not only helps to repair radiation-induced DNA damage but also enhances cellular resilience. By contrast, STS triggers a metabolic response, primarily aimed at energy conservation, which does not appear to stimulate DAF-16 activity in the same way.
This specificity indicates that the DAF-16/FOXO pathway is not a generalized stress response but a targeted mechanism that responds more robustly to environmental stressors directly threatening cellular integrity, such as oxidative damage from radiation.

FOXO/DAF-16 mediates a *sod-3* dependent response to radiation

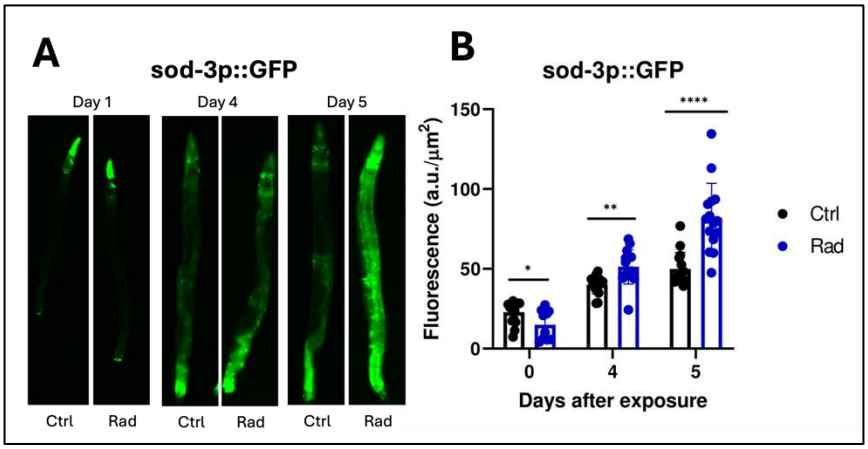

Figure 6: A) Representative images of sod-3p::GFP worms at day 1, 4, and 5 following radiation exposure vs. control. B) L4 worms tagged with sod3p::GFP on OP50 were treated with 500 Gy ionizing radiation or control. Gene expression was measured following radiation exposure.

As seen in Figure 6, exposure to radiation elevates the expression of *sod-3*, a gene linked to oxidative stress management, as evidenced by increased fluorescence in sod-3p::GFP worms. SOD-3 serves as a transcriptional reporter for DAF-16 activity, which is activated by radiation exposure. This response appears useful for monitoring DAF-16 activation under stress conditions. The protective mechanism mediated by FOXO/DAF-16 likely involves a broad range of downstream stress response pathways rather than relying solely on SOD-3 activation. The relationship between DAF-16 and SOD-3 expression is straightforward—DAF-16, as a transcription factor, directly drives SOD-3 transcription upon activation. This simple and well-established connection supports the use of SOD-3 as a DAF-16 reporter. Future directions could include investigating other downstream targets of DAF-16 and their roles in stress resistance and radiation response. Additionally, these insights could be applied to mammalian systems, including human cancer models, to explore their potential relevance.

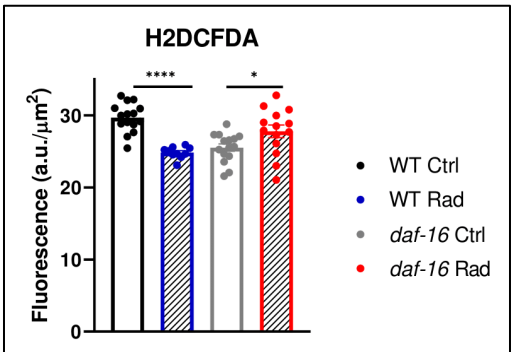

Figure 7: WT and daf-16 mutant C. elegans were treated with 1500 Gy ionizing radiation and resulting oxidative stress was measured using H2DCFDA.

Figure 7 further explores this relationship using a H2DFCDA fluorescent probe. Wild type organisms showed lower ROS levels immediately following radiation, reflecting DAF-16's

influence on oxidative stress. In contrast, DAF-16 mutants had higher ROS immediately after radiation compared to wild type. These findings emphasize DAF-16's importance in ROS regulation and potential alternative pathways in its absence.

Radiation stunts growth in *daf-16* mutants

Body size serves as an important measure of radiation resistance, as it reflects *C. elegans'* ability to maintain normal growth and development under stress conditions. Figure 8 highlights the relationship between body size and various strains of *daf-16*. In *daf-16* mutants, a stunted body size is observed following radiation exposure, highlighting their diminished capacity to cope with stress compared to wild type. Wild type models exposed to radiation maintain a significantly larger body size due to stress resistance mechanisms mediated by the DAF-16/FOXO pathway. In contrast, the stunted growth in *daf-16* mutants highlights their vulnerability, as the loss of *daf-16* disrupts the activation of protective responses, such as oxidative stress mitigation and DNA repair, which are crucial for sustaining normal development.

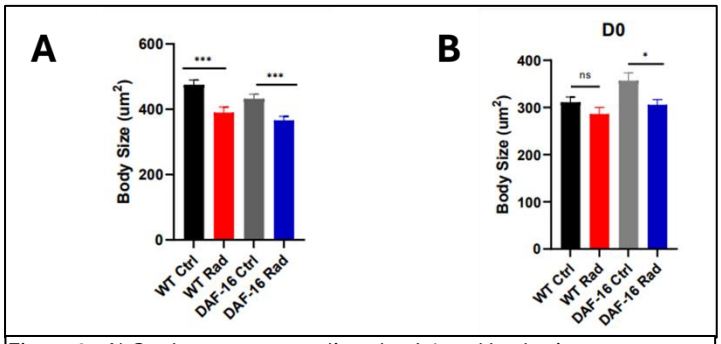

Figure 6: A) C. elegans were radiated at L4 and body size was measured at D1 to determine its response to radiation. B) L4 worms were measured immediately following radiation treatment and subsequent body size was measured.

As expected, our body size experiment showed a decrease in size for both wild type and *daf-16* mutants. However, the figure shows that D1 control and irradiated *daf-16* mutants were lower in body size as compared to wild type models. We analyzed the body size of L4 animals immediately following radiation exposure as well to determine whether this change was due to shrinkage in body size or stunted development. This data leads us to believe that radiation leads to a stunted growth in *daf-16* mutants.

Discussion and Future Directions

The relationship between age and stress resistance in *C. elegans* presents a complex interaction that is not fully understood. Future studies will be designed to examine how sensitivity to radiation and oxidative stress varies across different developmental stages, identifying key time points at which resistance mechanisms are most robust or vulnerable. By conducting experiments on *C. elegans* at various life stages, we hope to map out age-dependent changes in stress response pathways, which may reveal optimal intervention periods for promoting resistance to radiation and other forms of cellular stress.

Future research will delve into the specific mechanistic and biological roles of downstream targets in the FOXO pathway, focusing on how these genes contribute to cellular resilience and lifespan extension. By identifying and analyzing the individual and collective impacts of genes regulated by DAF-16, we hope to determine the molecular processes that protect cells from oxidative damage, DNA instability, and other stressors. This line of investigation could clarify how alterations in the FOXO pathway influence aging and disease resistance, potentially paving the way for targeted therapies that enhance longevity and stress resilience in *C. elegans* and eventually human models.

The impact of starvation on stress resistance will be further explored by extending the fasting period from 24 to 48 hours in future experiments. Research outlined in this thesis has suggested that STS may not significantly alter radiation sensitivity in adult *C. elegans*, but longer starvation periods could induce more pronounced adaptive changes. By extending the starvation duration, we can investigate whether prolonged nutrient restriction amplifies FOXO/DAF-16 pathway activation, potentially enhancing radiation resistance and providing deeper insights into the connections between metabolic stress and lifespan extension.

To bridge insights gained from *C. elegans* research with potential applications in human health, studies will translate findings on ROS and radiation resistance to human cancer cell lines. This transition is crucial for assessing how FOXO/DAF-16-mediated pathways perform in complex mammalian cells, which share conserved mechanisms but possess additional layers of regulation. We hope to better understand how manipulating ROS and stress response pathways could improve resistance to radiation therapy, potentially offering new avenues for enhancing cancer treatment efficacy.

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