Influences of nutrition and pathogenicity from a microbial diet on immunity and longevity in *Caenorhabditis elegans*

by

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Abstract

Interactions with the environment play a critical role in animal physiology and evolution. Animals alter their cellular functions and organismal behavior to maximize survival in a given setting, or make the decision to seek out a new environment that is more compatible with life. Responses to external conditions can be carried out via sensory perception of environmental cues, or as a result to decreased cellular energy or tissue damage. Both modes of modification present complex biological questions as to how animals recognize the need to adapt, make the decision to adapt, and relay that decision into a physical outcome.

This thesis focuses on how we can answer some of these questions through study of the model organism, *Caenorhabditis elegans*, and its interactions with its microbial environment, which serves as both a nutrient source as well as a potential pathogenic threat. In Chapter One, I provide an overview of aging and infection in *C. elegans*. Many of the pathways involved in regulating longevity and immunity in *C. elegans* are conserved in mammals, and work in this system has revealed a surprising amount of intersection of these two seemingly separate matters. Chapter Two focuses on how a TGFβ neuroendocrine signaling pathway contributes to lifespan extension as a result of reduced nutrient availability in adulthood, commonly known as dietary restriction. Chapter Three explores how a bZIP transcription factor works to regulate the response to pathogenic bacteria downstream of p38 MAP Kinase signaling. In Chapter Four, I present ideas for exploring the future directions of these two projects that focus on untangling how *C. elegans* respond to a changing microbial environment.

Thesis supervisor: Dennis H. Kim
Title: Professor of Biology

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Chapter One

Introduction

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Overview

The ability to detect and process information about the environment and respond by adjusting physiological processes accordingly is critical to survival for all species. From bacteria to mammals, there are countless examples of conserved stress responses, all with the common goal of keeping organisms alive in fluctuating environmental conditions. Animals alter their behavior to seek out the most beneficial living conditions, and plants have evolved methods to reposition themselves throughout daylight hours to maximize exposure to the moving sun. With external conditions being a major factor influencing living and growth, all species must adapt to their environments to achieve maximal fitness. In the simplest of terms, this requires obtaining adequate nutrition to support growth and reproduction, while evading demise from dangers such as toxic microbes, chemicals, or otherwise inhospitable habitats.

For the bacteriovore *Caenorhabditis elegans,* the requirement to properly respond to surroundings is no less critical. Of particular challenge is the need to seek out bacterial species that are rich food sources, while simultaneously avoiding or initiating immune defense responses against harmful pathogens. As such, microbial species have a sizable influence on the nutritional state and infection status of *C. elegans*, and these states in turn have a considerable effect on the aging process and survival rate of these animals (Figure 1). The influence of microbial species on health and lifespan has been well documented, if not completely understood, in *C. elegans*, as well as in humans and other mammals (Kim 2013; Clemente et al. 2012; Cabreiro and Gems 2013). This chapter will give an overview of the interplay between nutrition and aging, as well as a review of host defense mechanisms in *C. elegans*.

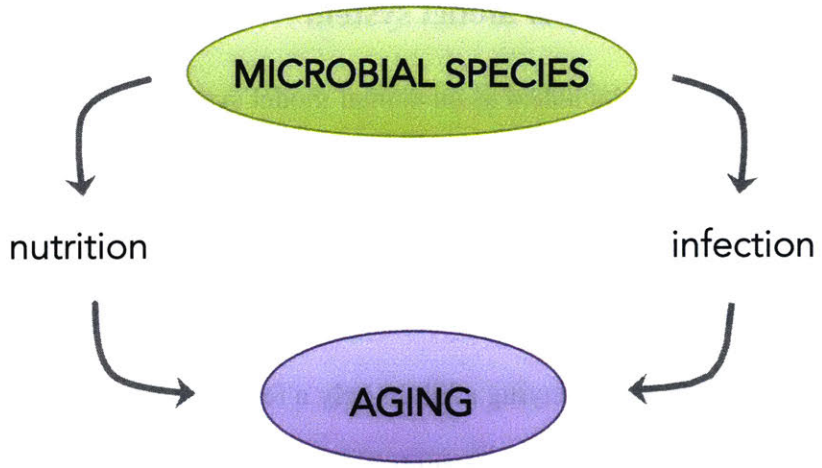

Figure 1: Microbial species serve as both sources of nutrition and possible infection in *C. elegans*, which in turn influence aging and survival.

An introduction to the *C. elegans* model system

C. elegans was initially established as an animal model system due to its relative simplicity and genetic tractability (Brenner 1974). An adult hermaphrodite has just under 1000 somatic cells that rise from an invariant developmental lineage which compose of several organs including an intestine and a nervous system (Sulston and Horvitz 1977). These small nematodes are fast living, going from egg to egg-laying adult in only a few days, and living for only a few weeks in total, making them an ideal model to study everything from development to behavior to aging (Corsi, Wightman, and Chalfie 2015).

The genetic tractability of *C. elegans* has led to many key discoveries over the last few decades, including identification of genes and pathways that are conserved in all animals such as genes regulating apoptosis, development, longevity, and microRNAs (Hedgecock, Sulston, and Thomson 1983; Ellis and Horvitz 1986; Ambros and Horvitz 1984; Kimura et al. 1997; Kenyon et al. 1993; R. C. Lee, Feinbaum, and Ambros 1993). Due to these and other advantages, many have turned to *C. elegans* to study conserved elements of the biology of aging and host-pathogen interactions. In doing so, we must consider how interfaces with these animals' bacterial food source influences these efforts.

Living and aging in a microbial environment

The natural habitat of *C. elegans* lies in microbe-rich decomposing vegetative matter, requiring that these animals evolve mechanisms by which to seek out nutritionally beneficial microbial species and to avoid detrimental ones (Schulenburg and Félix 2017). In the lab setting, *C. elegans* are raised almost exclusively on a diet of the benign *E. coli* strain, OP50, and the presence of this food source influences many aspects of worm physiology such as pharyngeal

pumping, chemotaxis behavior, reproductive egg laying, and locomotion (Brenner 1974; Avery and Horvitz 1990; Sawin, Ranganathan, and Horvitz 2000; Colbert and Bargmann 1997; Horvitz et al. 1982). In many of these cases, the exact neuronal circuits and signaling pathways involved have been elucidated, contributing to our overall understanding of how food cues are detected to modulate animal behavior. However, while all of these studies have relied on *E. coli* OP50 as a food source, it is well documented that not all bacterial species are of equal nutritional value, and that *C. elegans* exhibit dietary choice behaviors (Shtonda 2006).

In addition to showing preference based on food quality, *C. elegans* exhibit avoidance behavior of pathogenic bacterial species, similar to the innate and learned avoidance behaviors across animal species, including humans (Zhang, Lu, and Bargmann 2005; Pradel et al. 2007; Meisel and Kim 2014; Stensmyr et al. 2012; V. A. Curtis 2014). Further, deviations from the standard live *E. coli* OP50 diet have been shown to influence many aspects of *C. elegans* physiology, including growth rate and longevity (Garigan et al. 2002; Garsin et al. 2003; Gems and Riddle 2000; Gusarov et al. 2013). These observations are perhaps not surprising, given that dietary microbes have also been linked to influencing development, immunity, and aging in mammals (Kau et al. 2011; Nicholson et al. 2012; Clemente et al. 2012).

A brief overview of aging in *C. elegans*

The utility of *C. elegans* as a model in which to investigate the basic biology of aging was identified early on, and the influences of environmental factors such as temperature, food availability, and parental age were described in detail (Klass 1977). From this study, it was clear that, similar to humans and other animals, the living environment contributes to the aging and longevity of *C. elegans*. When the first aging gene, *age-1*, was identified a few years later, it was

established that these animals would also be a suitable model in which to examine genetic contributions to lifespan (Friedman and Johnson 1988). Subsequent studies placed the *age-1* gene in the conserved insulin/insulin-like growth factor-1 signaling (IIS) pathway (Malone, Inoue, and Thomas 1996; Morris, Tissenbaum, and Ruvkun 1996; Kimura et al. 1997). Since then, several other evolutionarily conserved pathways have been linked to aging in *C. elegans* such as TOR signaling, sirtuins, mitochondrial and oxidative stress response genes, and proteostasis maintenance pathways (Vellai et al. 2003; Tissenbaum and Guarente 2001; Dillin et al. 2002; S. S. Lee et al. 2003; Lakowski and Hekimi 1996; An and Blackwell 2003; Garigan et al. 2002; Hsu, Murphy, and Kenyon 2003). Intriguingly, there have been several studies that emphasize the importance of some of these pathways in specific tissues, particularly in the nervous system, which can affect longevity in a cell-nonautonomous manner.

Hermaphrodite *C. elegans* animals have exactly 302 neurons, and the anatomical positions and synaptic connectivity of these neurons have been carefully mapped (White et al. 1986). Eleven bilaterally symmetric neuron pairs make up the amphid chemosensory neurons, which extend cilia directly into the environment through openings created by glial cells (Bargmann 2006). Sensation of environmental cues was initially linked to longevity in *C. elegans* by study of mutants defective in sensory perception (Apfeld and Kenyon 1999). Later, ablation studies found that specific gustatory, olfactory and thermosensory neurons contribute to lifespan (Alcedo and Kenyon 2004; S.-J. Lee and Kenyon 2009). These works provided the first evidence that the ability to faithfully sense the environment influences the aging process in *C. elegans*.

Subsequent studies revealed that neurons could have dramatic, cell-nonautonomous effects on longevity. Neuron-specific overexpression of stress response mediators AAK-2 (AMP

activated kinase) or HIF-1 (hypoxia-inducible factor) is sufficient to extend lifespan (Burkewitz et al. 2015; Leiser et al. 2015). Pan-neuronal overexpression of the spliced form of unfolded protein response (UPR) mediator XBP-1 increases longevity, and overexpression of HSF-1 (heat shock factor) improves thermotolerance as well as lifespan (Taylor and Dillin 2013; Douglas et al. 2015). Together, these and many other studies highlight the importance that external signals and chemosensation have on regulating aging and longevity.

Chemosensory neurons are particularly tuned to detect changes in food availability, and in turn modulate animal behavior through neurotransmitter secretion to relay those cues to the rest of the animal. For example, serotonin and dopamine have been shown to promote behaviors that occur in the presence of food, while octopamine mimics food-absence and therefore opposes these behaviors (Horvitz et al. 1982; Sawin, Ranganathan, and Horvitz 2000). Food availability not only influences animal behaviors during development and the reproductive period, but also impacts aging and lifespan.

Dietary restriction: a method to extend longevity in diverse species

Nutrient sensing and consumption have been implicated in the longevity process in many studies using various model systems (Fontana and Partridge 2015). Dietary restriction (DR), defined as a chronic decrease in caloric intake without malnutrition, was first shown to be capable of extending lifespan in rats in 1935 (McCay, Crowell, and Maynard 1989). Since then, several studies have confirmed these results in rats and mice, and found that DR further improves many aspects of health throughout the aging process, including increasing the proportion of animals that die absent any pathological signs of disease (Maeda et al. 1985; Ikeno et al. 2006; Shimokawa et al. 1993). Shorter-term food restriction studies in humans have found results

similar to some of the physiological changes exhibited by animals on DR, such as lessened age-associated myocardial stiffness, lower core body temperatures, and decreased IIS and inflammation in skeletal muscle (Mercken et al. 2013; Cava and Fontana 2013).

However, widespread application of human DR through the practice of food restriction is unrealistic, largely because it is simply difficult to sustain properly for the average person. Among other things, reducing food intake without ensuring adequate nutritional content can lead to increased risk of anemia, cardiac arrhythmia, and reduced bone density (Fairburn and Harrison 2003). As such, pharmacological interventions that target the key cellular mediators of DR benefits could lead to healthy aging in humans, without the potentially disastrous consequences of DR practice. Of course, to develop a DR mimetic, the mechanisms of DR-mediated lifespan extension must first be elucidated.

In addition to promoting healthy aging in mammals, DR increases the lifespan of many shorter-lived model organisms, including *C. elegans* (Fontana, Partridge, and Longo 2010). Thanks to studies in these simple models, many of the molecular regulators that respond to DR have been identified, even if exact mechanism of DR-mediated longevity has not been fully explained (Koubova and Guarente 2003). In *C. elegans,* DR can be induced in a variety of ways, but all methods generally reduce the ingestion of bacterial food. While there is some controversy over which particular form of DR is best to use in studying this complicated process, the key players that have been identified through various methods of DR are those that also play conserved longevity roles in evolutionarily diverse species (summarized in Figure 2) (Greer and Brunet 2009; Mair and Dillin 2008). Specifically, there is evidence that nutrient and energy sensing pathways, such as IIS, TOR, sirtuins, and AMPK are critical mediators of longevity as a result of DR (Greer et al. 2007; Hansen et al. 2007; Panowski et al. 2007). Additionally, stress

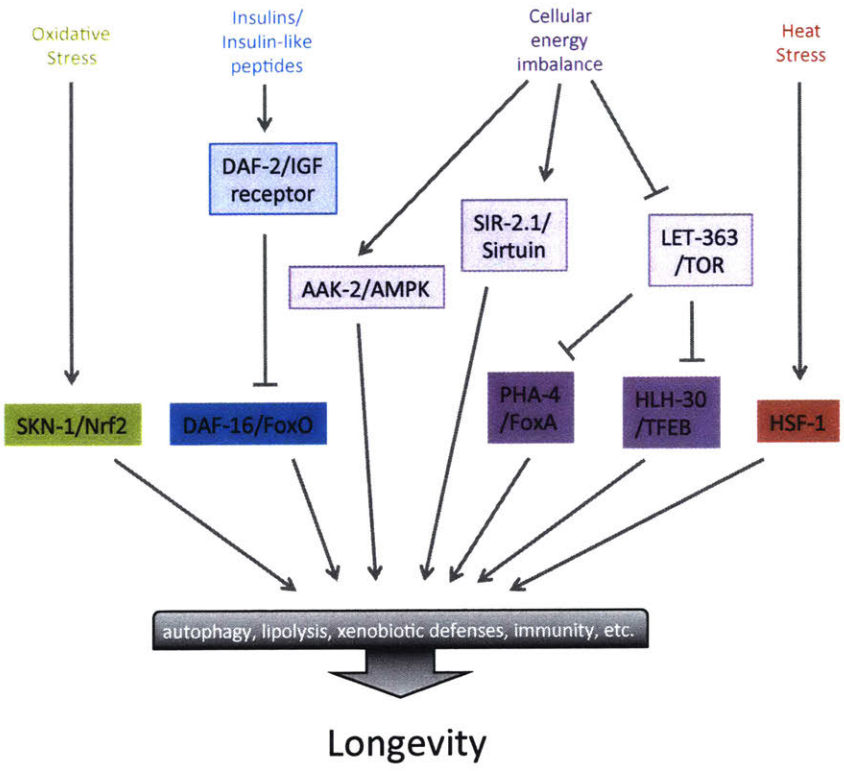

Figure 2: An incomplete representation of genes regulating DR-induced longevity in *C. elegans*.

A simplified overview of genes that implicated in regulating both longevity and lifespan extension as a result of dietary restriction. Not diagrammed is the immense amount of crosstalk that has been documented between these major aging pathways. For example, DAF-16 interacts with AAK-2, SKN-1 and HSF-1 to co-regulate transcription, and DAF-16 is required for lifespan extension as a result of SKN-1 stabilization. DAF-16 and SKN-1 are also required for longevity resulting from decreased TOR signaling (Summarized in (Lapierre and Hansen 2012)). Additionally, HLH-30 acts to extend lifespan downstream of reduced IIS (as well as inhibited TOR activity), and lifespan extension by sirtuins requires DAF-16 and AMPK activity (Lapierre et al. 2013; Tissenbaum and Guarente 2001; R. Curtis, O'Connor, and DiStefano 2006).

response pathways such as the oxidative stress response (under the control of SKN-1) and heat shock response (regulated by HSF-1) also appear to be involved in the DR response (An et al. 2005; Bishop and Guarente 2007; Greer et al. 2007; Steinkraus et al. 2008). Many studies further indicate crosstalk between these and other pathways, both in normal aging and in aging under DR conditions (Tullet et al. 2008; Greer and Brunet 2009; Apfeld et al. 2004; Chen et al. 2013; Tissenbaum and Guarente 2001; Berdichevsky et al. 2006; Wang and Tissenbaum 2006; Greer et al. 2007; Robida-Stubbs et al. 2012; Uno and Nishida 2016)

In addition to the complex cell-autonomous components of dietary restriction, there is also evidence that sensory perception and endocrine signaling are key to mediating lifespan extension as a result of DR. Studies in *C. elegans* and *Drosophila* have found that the efficacy of DR treatment is reduced by olfactory sensation of food odors (Libert et al. 2007; Smith et al. 2008). Similar to the influence of altered chemosensory perception on lifespan in *C. elegans* described above, *Drosophila* longevity is extended by reduced olfactory function alone (Libert et al. 2007). Further, the ASI neurons, a gustatory neuron pair in the *C. elegans* amphid, are required for lifespan extension in response to dietary restriction, emphasizing the importance of sensory perception in this aging intervention (Bishop and Guarente 2007).

Chapter Two of this thesis will focus on the DAF-7/TGFβ neuroendocrine signaling peptide, which is produced in the ASI neuron pair. This conserved signaling pathway has a long history in mediating development and behavioral responses to environmental food, pathogen, and pheromone (Ren et al. 1996; Schackwitz, Inoue, and Thomas 1996; Meisel et al. 2014; Entchev et al. 2015; Hilbert and Kim 2017). DAF-7/TGFβ is also implicated in longevity. Animals carrying loss-of-function *daf-7* alleles are long-lived in an IIS-dependent manner (Shaw et al. 2007). Microarray analysis has revealed there is low correlation between the influence of

DAF-7 on transcriptional outputs in aging adults compared to outputs regulating larval developmental, suggesting that DAF-7-regulated gene expression in response to environmental stimuli in aging animals may be distinct from those early in life and suggests a role for this neuroendocrine pathway in aging and age-related decline (Liu, Zimmerman, and Patterson 2004; Shaw et al. 2007). In Chapter Two, I will describe a role for DAF-7/TGFβ neuroendocrine signaling in mediating lifespan extension in response to DR treatment. These findings add to a growing body of literature that sensory perception is a key component of longevity and of lifespan extension by DR treatment.

Infection: the flip side of a microbial diet

While the lab diet of *E. coli* OP50 is a relatively innocuous food source, it is also a potential source of infection for *C. elegans*. Lifespan assays conducted on OP50 that has been arrested through UV irradiation or antibiotic application results in increased *C. elegans* survival relative to a standard live OP50 culture (Gems and Riddle 2000; Garigan et al. 2002). Feeding animals *Bacillus subtilis*, which is thought to be less pathogenic to *C. elegans* than *E. coli*, increases longevity without altering developmental rates or brood size, suggesting that infection during aging is responsible for the relatively shorter lifespan on *E. coli* OP50 (Garsin et al. 2003; Gusarov et al. 2013).

Strong evidence for bacterial infection contributing to aging in *C. elegans* can be found in studies of intestinal accumulation of *E. coli* OP50 in aging adult animals. As *C. elegans* age, their intestine becomes filled with bacteria at both the anterior and posterior ends, often resulting in distension (Garigan et al. 2002). This bacterial packing is similar to intestinal accumulation in younger animals that are exposed to highly pathogenic bacterial species that quickly kill *C.*

elegans, indicating that *E. coli* infection contributes to the mortality of these animals (Irazoqui et al. 2010; Tan, Mahajan-Miklos, and Ausubel 1999). Interestingly, the pro-longevity benefits of a *B. subtilis* diet requires gut colonization and biofilm formation, suggesting there are some gut microbial species that can be beneficial for *C. elegans* (Donato et al. 2017).

In addition to the lifespan-shortening impact of chronic infection by harmful bacterial species, there are several microbial pathogens that infect in a more acute manner, causing death in only a few days (Darby 2005). A particularly well-studied pathogen is the Gram-negative bacterium, *Pseudomonas aeruginosa*, which is commonly found in soil and water and is capable of infecting a wide range of hosts, including plants and animals. While it is fairly innocuous to healthy human individuals, *P. aeruginosa* can be quite dangerous for humans who are immune-compromised in any manner, and is a leading culprit in hospital-acquired infections (Lyczak, Cannon, and Pier 2000). Studies of *P. aeruginosa* in *C. elegans* have helped identify virulence factors of this bacterium, as well as conserved innate immune response factors in *C. elegans* (discussed below) (Tan et al. 1999). *P. aeruginosa* can kill *C. elegans* by two mechanisms. Toxin-mediated killing is a rapid process that kills in only a few hours and does not require bacterial growth (Mahajan-Miklos et al. 1999). Alternatively, *P. aeruginosa* can act via a "slow killing" mechanism in which the bacteria establish an infection through colonization of the *C. elegans* gut (Tan, Mahajan-Miklos, and Ausubel 1999). The slow killing model has been more commonly used to study host-pathogen interactions in *C. elegans*, as this process takes days rather than hours and more closely models *P. aeruginosa* infections in mammals (Tan et al. 1999).

Innate immunity in *C. elegans*

While *C. elegans* lack the cellular arm of the immune response that jawed vertebrates possess, they not completely defenseless against invading microbes. In addition to the behavioral avoidance mentioned earlier in this chapter, *C. elegans* also mount an innate immune response upon infection by a pathogenic species. Since *C. elegans* do not have dedicated immune cells to fight infection, their innate immune defenses comprise primarily of secreted antimicrobial peptides, as well as activation of general stress response pathways.

Over the last few decades, work from several groups has begun to characterize innate immunity in *C. elegans*. Toll-like receptor (TLR) signaling plays a major role in triggering innate immune responses in *Drosophila* and vertebrate animals (Medzhitov 2001). The *C. elegans* genome contains only one TLR homolog, *tol-1*, which is not required for resistance to pathogenic bacteria (Pujol et al. 2001). However, *tol-1* is required for avoidance of the pathogen *Serratia marcescens*, and several studies suggest that *tol-1* functions in the neurons to promote pathogen avoidance behaviors (Pujol et al. 2001; Pradel et al. 2007; Tenor and Aballay 2008; Brandt and Ringstad 2015).

The p38/PMK-1 mitogen-activated protein kinase (MAPK) signaling cascade is a conserved, central regulator of *C. elegans* immunity and is activated upon encounter with many pathogenic microbial species, including *P. aeruginosa* (Figure 3) (Kim et al. 2002). This signaling cassette contains the core NSY-1(MAPKKK)-SEK-1(MAPKK)-PMK-1(MAPK), which is homologous to the mammalian ASK1-MKK3-p38 MAPK pathway. Interestingly, the Toll/IL-1 resistance domain containing protein, TIR-1 (an orthoglog to mammalian SARM), acts upstream of the p38/PMK-1 signaling pathway in *C. elegans* to promote an immune response upon infection (Couillault et al. 2004; Liberati et al. 2004). p38/PMK-1 activation results in

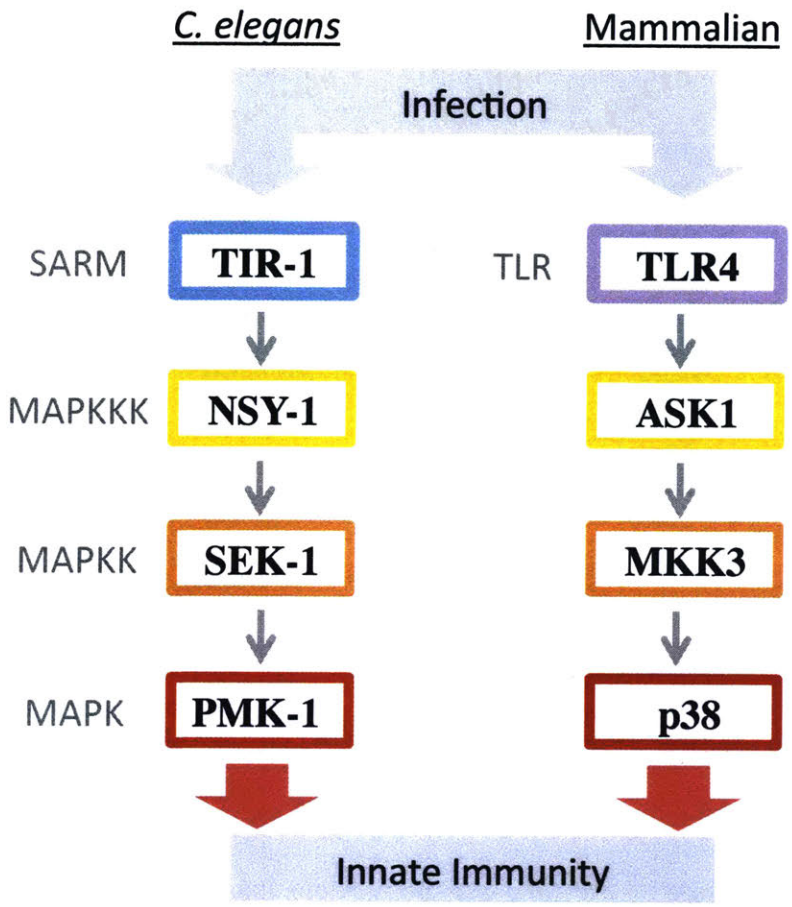

Figure 3: p38/PMK-1 signaling.

C. elegans gene names are listed on the left, mammalian counterparts on the right. Grey text refers to the protein family.

broad transcriptional changes, including the induction of many genes that are predicted to have antimicrobial activity such as genes containing c-type lectins, lysozymes, and ShK-like toxins (Troemel et al. 2006). These secreted, antimicrobial immune effector molecules presumably act in a cell-autonomous manner at the site of infection, the intestine of the animal. However, TIR-1-NSY-1-SEK-1 also contributes to neuronal phenotypes, including serotonin-mediated avoidance of *P. aeruginosa*, and PMK-1 has been implicated in the response to other, abiotic stressors (discussed below) (Shivers et al. 2009; Pagano, Kingston, and Kim 2015). This diversification of an ancient immunity pathway underscores the importance that interactions with microbes have had on shaping the evolution of response pathways in *C. elegans*.

Longevity and immunity

Long-lived animals harboring mutations that promote longevity through canonical longevity pathways show resistance to infection. Animals with reduced insulin signaling by genetic mutation of the insulin-like receptor gene, *daf-2*, are resistant to killing by *P. aeruginosa* and exhibit reduced intestinal bacteria packing with age (Garigan et al. 2002; Garsin et al. 2003). This decreased sensitivity to infection requires activity of the DAF-16/FOXO transcription factor, which is negatively regulated by IIS. Further, transcriptional profiling studies have revealed that DAF-16 promotes expression of genes annotated to be part of the innate immune response (McElwee, Bubb, and Thomas 2003; Murphy et al. 2003). While loss-of function-mutations of *pmk-1* do not appear to influence longevity on *E. coli* OP50, PMK-1 pathway mutations do reduce the longevity of *daf-2* mutant animals, further suggesting that the longevity conferred by reduced IIS is, in part, a result of improved pathogen resistance (Troemel et al. 2006). Infection-independent induction of immunity genes by FOXO has also been documented

in studies of *Drosophila* and human cell lines, indicating that regulation of innate immunity by IIS is evolutionarily conserved (Becker et al. 2010).

Additional interactions between aging and immunity pathways have been found. The transcription factor SKN-1/Nrf2 contributes to lifespan extension as a result of oxidative stress or decreased *daf-2* activity (An and Blackwell 2003; Tullet et al. 2008; Oliveira et al. 2009; Schmeisser et al. 2013). SKN-1 is also activated by PMK-1 in response to oxidative stress and infection, suggesting yet another intersection of longevity and immunity regulators (H. Inoue et al. 2005; Hoeven et al. 2011). Similarly, mutation in the heat shock factor, HSF-1, results in many phenotypes, including reduced lifespan and decreased resistance to external stressors such as thermal heat and immune challenge (Singh and Aballay 2006). PMK-1 also regulates the hypoxic response pathway through the prolyl hydroxylase, EGL-9, which promotes degradation of HIF-1 in an oxygen dependent manner (Park and Rongo 2016).

PMK-1/p38 MAPK activity itself also exhibits age-related decline. As animals age, they show reduced levels of activated PMK-1, and this decay in PMK-1 activity is associated with an age-related decline in pathogen resistance, or immunosenescence (Laws et al. 2004; Youngman, Rogers, and Kim 2011). This immunosenescence could partially account for the accumulation of intestinal bacteria with age, and may also explain why mutants in the PMK-1 pathway do not show reduced lifespan relative to wild-type animals on OP50. That is, older wild-type animals are essentially *pmk-1* mutants in that they lack basal levels of PMK-1 activity and are therefore comparably susceptible to infection at later life stages.

p38/PMK-1 signaling converges on ATF-7

To identify how PMK-1 regulates gene expression and in the context of infection, our lab used forward genetic screens to identify the conserved basic-region leucine zipper (bZIP) transcription factor, ATF-7, as a direct target of PMK-1 kinase activity (Shivers et al. 2010). This work suggested a model by which ATF-7 may be switched from a transcriptional repressor to an activator as a result of phosphorylation by active PMK-1. In Chapter Three, I investigate how ATF-7 functions to regulate gene expression upon exposure to pathogenic *P. aeruginosa*. I will present evidence that supports the model suggested by Shivers et al., as well as some tantalizing data that may further cement a role PMK-1 and ATF-7 as a more central regulator of *C. elegans* physiology, separate from immune challenge.

Concluding remarks

Interaction with environmental factors is an essential part of life for all organisms, and properly responding to those factors is crucial for survival. In *C. elegans*, microbial encounters are exceptionally important for life, as bacteria serve as a source of nutritional content but can also pose the fatal threat of infection. This thesis will focus on two projects that both relate to the question of how animals respond to their environments to modulate their physiology accordingly. Chapter Two will explore contributions of the DAF-7/TGFβ neuroendocrine signaling pathway in responding to nutrient poor conditions in adulthood to drive the DR response. Chapter Three will explore how the bZIP transcription factor, ATF-7, contributes to the innate immune response upon exposure to pathogenic bacteria. Together, this work adds to increasing evidence that interaction with microbial species can define animal health and longevity.

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Chapter Two

Age-Dependent Neuroendocrine Signaling from Sensory Neurons Modulates the Effect of Dietary Restriction on Longevity of *Caenorhabditis elegans*

Marissa Fletcher and Dennis H. Kim

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Abstract

Dietary restriction extends lifespan in evolutionarily diverse animals. A role for the sensory nervous system in dietary restriction has been established in *Drosophila* and *Caenorhabditis elegans*, but little is known about how neuroendocrine signals influence the effects of dietary restriction on longevity. Here, we show that DAF-7/TGFβ, which is secreted from the *C. elegans* amphid, promotes lifespan extension in response to dietary restriction in *C. elegans*. DAF-7 produced by the ASI pair of sensory neurons acts on DAF-1/TGFβ receptors expressed on interneurons to inhibit the co-SMAD DAF-3. We find that increased activity of DAF-3 in the presence of diminished or deleted DAF-7 activity abrogates lifespan extension conferred by dietary restriction. We also observe that DAF-7 expression is dynamic during the lifespan of *C. elegans*, with a marked decrease in DAF-7 levels as animals age during adulthood. We show that this age-dependent diminished expression contributes to the reduced sensitivity of aging animals to the effects of dietary restriction. DAF-7 signaling is a pivotal regulator of metabolism and food-dependent behavior, and our studies establish a molecular link between the neuroendocrine physiology of *C. elegans* and the process by which dietary restriction can extend lifespan.

Author Summary

Reductions in food intake have long been observed to improve longevity, extending lifespan in many evolutionarily divergent organisms. While great progress has been made in identifying the mechanisms by which nutritional interventions act to delay the aging process, much remains unclear. Particularly, while work in multiple species has found evidence that the sensation of food availability by the nervous system contributes to lifespan extension in response to reduced food levels, little is known about how these contributions are executed. Here, we have

characterized how a specific neuroendocrine peptide, expressed in a set of sensory neurons, responds to changes in food conditions to modulate lifespan effects of dietary restriction at the organismal level. We further find that age-related changes in expression of this neuroendocrine signal contribute to the declining efficacy of nutritional interventions as animals get older. This work highlights the importance of neuroendocrine regulation in both the aging process and in treatments aimed at increasing longevity.

Introduction

Adult reduction in caloric intake and restriction of feeding periods have been shown to substantially increase lifespan across evolutionarily diverse organisms (Mair and Dillin 2008; Longo and Panda 2016). Collectively, such treatments have been referred to as dietary restriction (DR). DR has been shown to be effective even when initiated in later phases of adult life, although the efficacy of the treatment has been observed to diminish with advancing age in *Caenorhabditis elegans* (G. D. Lee et al. 2006; Kaeberlein et al. 2006). Genetic studies in *C. elegans* have defined roles for mediators of stress response pathways, such as DAF-16/FoxO, PHA-4/FoxA and SKN-1/Nrf2, as well as the intracellular energy sensors TOR and AMPK in mediating the effects of DR on longevity (Panowski et al. 2007; Bishop and Guarente 2007; Hansen et al. 2007; E. L. Greer et al. 2007). Other studies have suggested that external cues are also critical in eliciting a DR response that extends lifespan in *C. elegans* (Lucanic et al. 2016). In both *C. elegans* and *Drosophila,* the efficacy of DR treatment can be abrogated by the addition of food odors, and longevity in *Drosophila* can be extended by reduction of olfactory function (Libert et al. 2007; Smith et al. 2008). Similarly, studies in *C. elegans* have shown that mutation of genes implicated in sensory systems or ablation of chemosensory neurons results in extended lifespan (Apfeld and Kenyon 1999; Alcedo and Kenyon 2004; Maier et al. 2010). Specifically, a pair of gustatory neurons in *C. elegans*, the ASI neuron pair, have been shown to be required for lifespan extension in response to dietary restriction (Bishop and Guarente 2007).

In the present study, we sought to explore the signaling mechanisms by which perceptions in the nervous system of food availability contribute to the DR response in peripheral tissues. We have focused our attention on the gene *daf-7*, which encodes a TGFβ ligand that is secreted from the ASI neurons to control diverse behaviors of *C. elegans* (Ren et al. 1996;

Schackwitz, Inoue, and Thomas 1996; E. R. Greer et al. 2008; Meisel et al. 2014). DAF-7 has previously been implicated in longevity and food sensing; *daf-7* mutant animals are reported to be long-lived in a manner that is dependent on food levels and also exhibit defects in adjusting feeding behaviors in response to periods of starvation (Shaw et al. 2007; You et al. 2008; Entchev et al. 2015). However, the role of DAF-7 in lifespan extension in response to DR has not been fully investigated.

Here, we have focused on expanding understanding of the role that the DAF-7 signaling pathway has in lifespan extension in response to limited nutrient availability. We have determined that DAF-7 is a key neuroendocrine signal required in the ASI neurons for response to dietary restriction. Moreover, we find that age-related changes in *daf-7* expression contribute to the reduced sensitivity that older animals have to DR treatment, suggesting that the efficacy of DR interventions that delay aging can be modulated by neuroendocrine signaling.

Results

Neuronal DAF-7/TGFβ signaling promotes lifespan extension in response to dietary restriction

We investigated the role of the DAF-7/TGFβ pathway in lifespan extension in response to dietary restriction using the bacterial deprivation (BD) method, where animals are moved to solid media completely lacking a bacterial food source during adulthood (Kaeberlein et al. 2006; G. D. Lee et al. 2006). Using this protocol (see Methods for details) at 25°C and initiating BD treatment at day 3 of adulthood, we observed an average 19.5% extension of mean lifespan in wild-type animals, comparable to what has been reported previously when taking into account changes in experimental temperature (Figure 1B,E; Table S2). Using multiple loss-of-function

alleles, we observed that mutations in the *daf-7* gene, encoding a TGFβ family ligand, or in the *daf-1* gene, encoding the Type I TGFβ receptor, abrogated the lifespan extension conferred by BD (Figure 1C,E; Table S2). This is consistent with a prior report, which found that *daf-7* mutant animals are resistant to longevity fluctuations due to altered food levels (Entchev et al. 2015). We observed that the strong dependence of lifespan extension conferred by BD on DAF-7 was temperature dependent, as *daf-7* mutant animals retained lifespan extension, albeit reduced relative to wild type, when propagated 20°C (Table S2), as reported previously (Thondamal et al. 2014).

Different regimens of dietary restriction have been found to extend lifespan in *C. elegans* through separate genetic pathways (E. L. Greer and Brunet 2009). To ensure the effects we observed were not an outcome specific to the BD method of DR, we also tested *daf-7* pathway mutants in a second, distinct protocol for dietary restriction, referred to as solid dietary restriction (sDR), in which adult animals are exposed to a diluted bacterial food source that is refreshed every other day (E. L. Greer et al. 2007). Using the sDR method, we observed results consistent with our BD data, where mutants in either *daf-7* or *daf-1* have diminished lifespan extension in response to sDR (Figure S1; Table S3). DAF-7 signaling through DAF-1 has been shown to act through inhibition of the co-SMAD DAF-3 (Figure 1A) (Thomas, Birnby, and Vowels 1993; Patterson et al. 1997). We found that *daf-3* mutation could suppress the loss of sensitivity to dietary restriction observed in *daf-7* and *daf-1* mutants (Figure 1D-E, Figure S1; Tables S2, S3).

Mutations in *daf-7* have previously been observed to result in phenotypes such as diminished pumping, increased dauer entry, and increased fat storage (E. R. Greer et al. 2008). Genetic analysis of the individual phenotypes of *daf-7* mutant animals has identified distinct downstream genetic pathways that act to mediate each of these DAF-7-dependent phenotypes

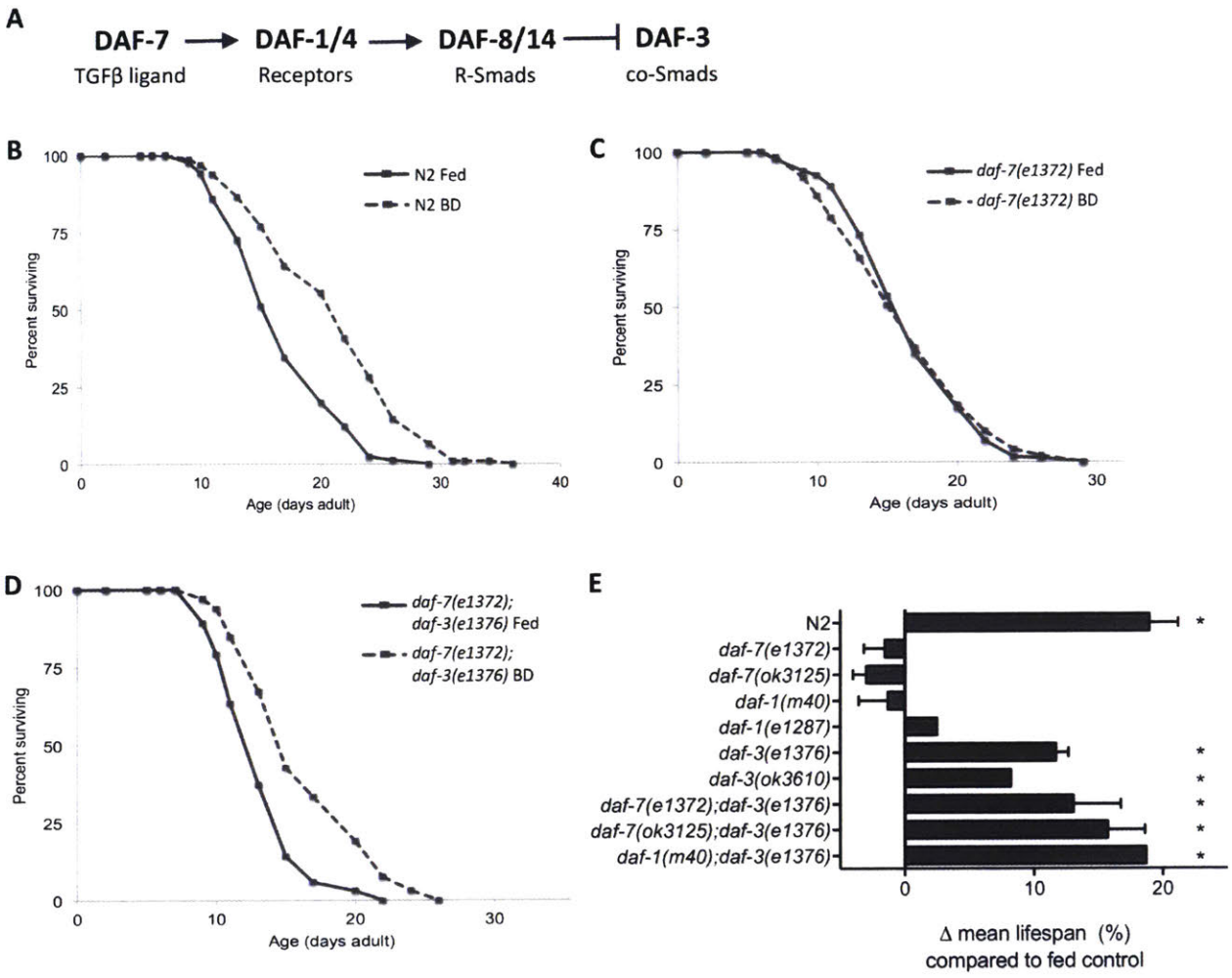

Figure 1: The DAF-7 signaling pathway is required for lifespan extension in response to dietary restriction.

(**A**) Summary of the DAF-7/TGFβ pathway (**B-D**) Representative lifespan curves of N2 (B), *daf-7(e1372)* (C), and *daf-7(e1372);daf-3(e1376)* (D) animals subjected to control (fed, solid lines) or bacterial deprivation (BD, dashed lines) diets. (**E**) Summary of all alleles tested for BD response. * indicates BD lifespan was significantly different ($p \leq 0.001$) than fed control group in all experiments, error bars reflect SEM. See Supplemental Table 2 for individual experiment details.

(E. R. Greer et al. 2008), enabling us to determine if any of these pleiotropies might be associated with the diminished ability of DAF-7 pathway mutants to respond to DR. The pumping defect of *daf-7* mutants is small in magnitude compared to the decrease in pharyngeal pumping observed in feeding-defective *eat* mutants that are used as genetic models of DR (Avery 1993; Lakowski and Hekimi 1998). Nonetheless, to test this possibility, we determined the effects of combining a *daf-1* mutation with mutations in *tbh-1* and *tdc-1*, which have been shown to suppress the feeding rate changes in *daf-1* and *daf-7* mutants (E. R. Greer et al. 2008). To determine if signaling through pathways promoting dauer formation might be involved in the DR phenotype, we examined a *daf-1;daf-12* double mutant. To determine if fat storage might be contributing to the DR defects we observed, we constructed *daf-1 mgl-3;mgl-1* mutants, in which fat storage increases arising from diminished DAF-7 signaling are specifically suppressed (E. R. Greer et al. 2008). None of these secondary mutations were able to suppress the BD defect of *daf-1* mutant animals, decoupling these three phenotypes from the DR response that is dependent on DAF-7 signaling (Figure S2; Table S2).

Prior studies established that *daf-7* is expressed principally in the ASI neuron pair, but also in additional sensory neurons when *C. elegans* is propagated on *E. coli* bacterial food, and that *daf-7* expression is induced in the ASJ neuron pair upon exposure to metabolites of *Pseudomonas aeruginosa* (Schackwitz, Inoue, and Thomas 1996; Ren et al. 1996; Meisel et al. 2014). We found that reintroducing wild-type *daf-7* into *daf-7(ok3125)* mutants rescued the BD defect of these animals (Figure 2A,B). Additionally, *daf-7(+)* driven by ASI or ASJ specific promoters was also sufficient to rescue the BD defect of *daf-7* mutant animals, consistent with the secretory nature of the DAF-7 ligand (Figure 2C). Unlike the expression of the DAF-7 ligand, the DAF-1 receptor is broadly expressed in the *C. elegans* nervous system (Patterson et al. 1997; Gunther,

Georgi, and Riddle 2000). To determine the functional targets receiving DAF-7 signal, we examined the ability of *daf-1(m40)* animals to respond to DR when a wild-type *daf-1* transgene had been expressed in different subsets of cell types under heterologous promoters (E. R. Greer et al. 2008). *daf-1* expression in the nervous system was sufficient to restore lifespan extension in response to BD. Furthermore, as has been demonstrated for other *daf-7* regulated phenotypes (E. R. Greer et al. 2008), we observed that the RIM/RIC interneurons are the specific sites of action for the *daf-1* receptor for lifespan extension in response to BD treatment (Figure 2D-F).

Bacterial deprivation acutely induces expression of *daf-7* in the ASI neurons of adult animals

Given the results of our genetic analysis of the DAF-7 signaling pathway in dietary restriction, we sought to examine how *daf-7* expression might change in response to DR intervention. We were unable to detect a change in expression using quantification of the transcriptional reporter, *ksIs2[daf-7p::GFP],* in fed versus BD treated animals (Figure 3A). We have previously observed that fluorescent in situ hybridization (FISH) provides more precise kinetic resolution of the dynamics of *daf-7* transcription than does the *ksIs2* GFP reporter (Meisel et al. 2014). By performing FISH on animals subjected to BD, we were able to detect a slight but consistent upregulation of *daf-7* mRNA transcription. Worms exhibited an increase in *daf*-7 mRNA in ASI neurons in animals fixed 24 hours after BD treatment was initiated, but no detectable difference was found after a period of 5 days had passed (Figure 3B). Of note, we observed that aging adult animals began to exhibit low-level expression in the ASJ neurons, but we did not observe any changes in *daf-7* mRNA in the ASJ neurons in response to BD (Figure 3A,C). These data suggest

Figure 2

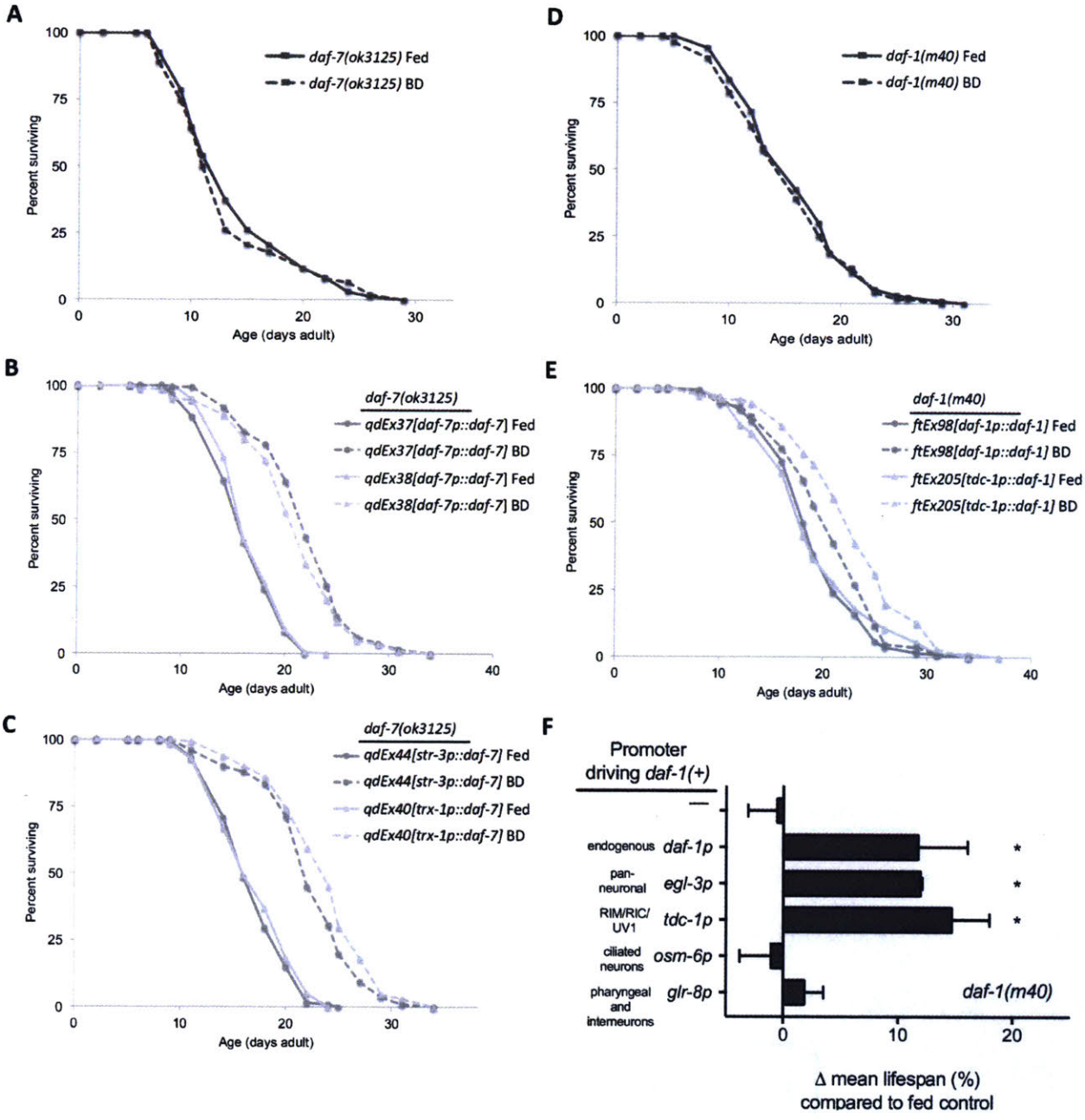
A
daf-7(ok3125) Fed
daf-7(ok3125) BD
Percent surviving
Age (days adult)
D
daf-1(m40) Fed
daf-1(m40) BD
B
daf-7(ok3125)
qdEx37[daf-7p::daf-7] Fed
qdEx37[daf-7p::daf-7] BD
qdEx38[daf-7p::daf-7] Fed
qdEx38[daf-7p::daf-7] BD
E
daf-1(m40)
ftEx98[daf-1p::daf-1] Fed
ftEx98[daf-1p::daf-1] BD
ftEx205[tdc-1p::daf-1] Fed
ftEx205[tdc-1p::daf-1] BD
C
daf-7(ok3125)
qdEx44[str-3p::daf-7] Fed
qdEx44[str-3p::daf-7] BD
qdEx40[trx-1p::daf-7] Fed
qdEx40[trx-1p::daf-7] BD
F
Promoter driving daf-1(+)
endogenous daf-1p
pan-neuronal egl-3p
RIM/RIC/UV1 tdc-1p
ciliated neurons osm-6p
pharyngeal and interneurons glr-8p
daf-1(m40)
Δ mean lifespan (%) compared to fed control

Figure 2: DAF-7 originating from chemosensory neurons acts on RIM/RIC interneurons to promote lifespan extension in response to DR.

(**A-C**) Lifespan curves displaying rescue of BD defect of *daf-7(ok3125)* animals (A) by reintroducing wild-type *daf-7* under the endogenous promoter (B), the ASI specific *str-3* promoter, or the ASJ specific *trx-1* promoter (C). (**D-E**) Representative curves displaying rescue of BD defect of *daf-1(m40)* animals (D) by expressing wild-type *daf-1* under its own promoter or the RIM/RIC/UV1 specific promoter, *tdc-1* (E). (**F**) Summary of all *daf-1(m40)* rescue experiments performed. * indicates BD lifespan was significantly different ($p \leq 0.05$) than fed control group in all experiments, error bars reflect SEM. See Supplemental Table 2 for individual experiment details.

Figure 3

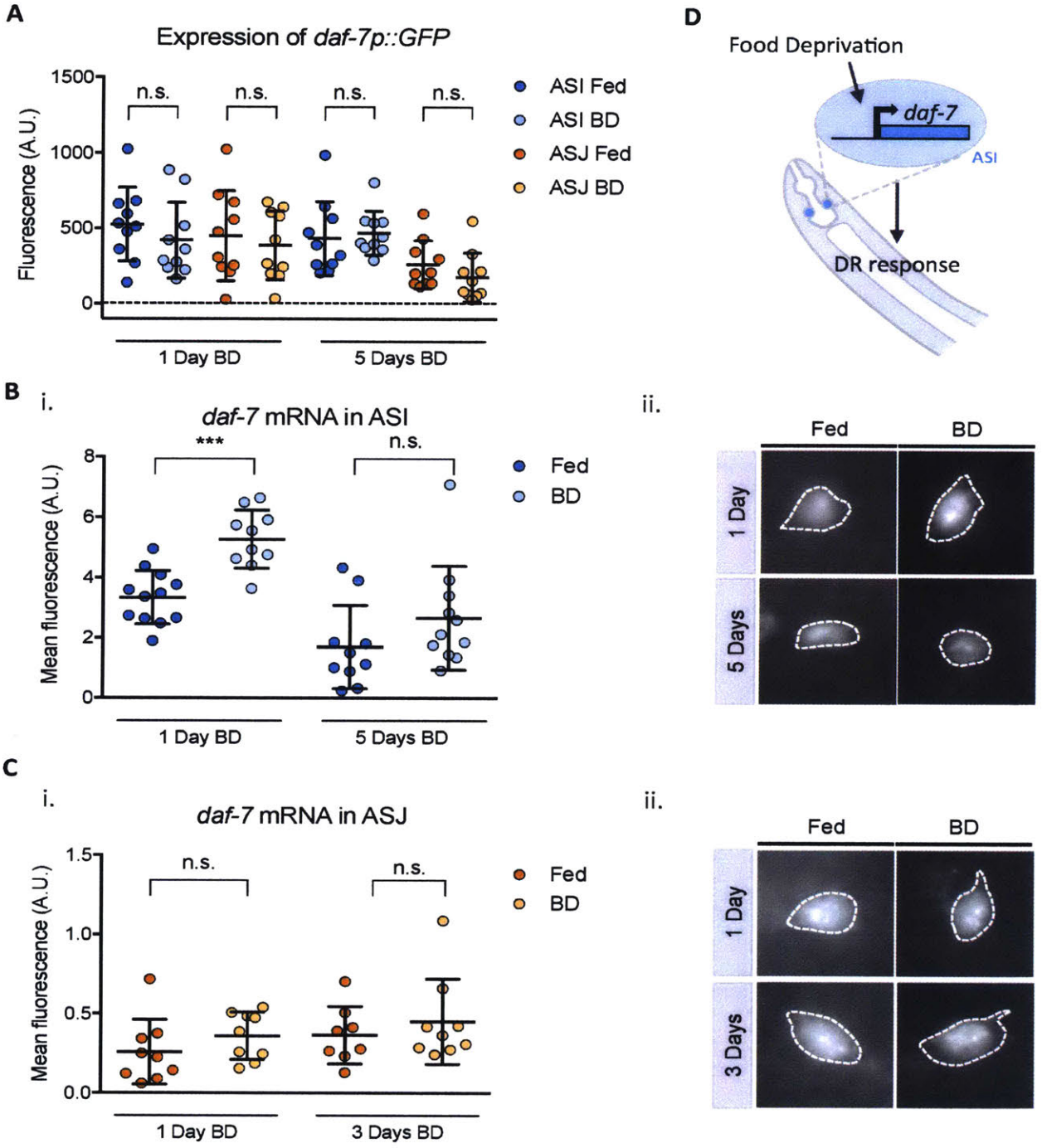
A
Expression of daf-7p::GFP
Fluorescence (A.U.)
1500
1000
500
0
n.s.
n.s.
n.s.
n.s.
1 Day BD
5 Days BD
ASI Fed
ASI BD
ASJ Fed
ASJ BD
D
Food Deprivation
daf-7
ASI
DR response
B
i.
daf-7 mRNA in ASI
Mean fluorescence (A.U.)
8
6
4
2
0
***
n.s.
1 Day BD
5 Days BD
Fed
BD
ii.
Fed
BD
1 Day
5 Days
C
i.
daf-7 mRNA in ASJ
Mean fluorescence (A.U.)
1.5
1.0
0.5
0.0
n.s.
n.s.
1 Day BD
3 Days BD
Fed
BD
ii.
Fed
BD
1 Day
3 Days

Figure 3: Dietary restriction acutely increases *daf-7* expression in adult animals.

(**A**) Quantification of GFP expression driven by *ksIs2[daf-7p::GFP]*, normalized to exposure time in fed and BD conditions. Representative of 3 replicates performed with n>10 animals per condition. B,C) (i.) Quantification of fluorescence of FISH probes designed against *daf-7* in ASI (identified by co-localization with *str-3p::GFP*) (**B**) and ASJ (identified by co-localization with *trx-1p::GFP*) (**C**) under fed and BD conditions. *** represents $p < 0.001$ by unpaired t-test. (ii.) Representative images of ASI or ASJ neurons corresponding to the quantifications presented in (i). All images taken with the same exposure time. (**D**) Model of *daf-7* expression change in response to dietary restriction.

that in response to food deprivation, *daf-7* transcription is acutely activated in the ASI neuron pair, which promotes lifespan extension mediated by DR (Figure 3D).

DAF-7 is required for intestinal DAF-16/FoxO translocation in response to food deprivation

In response to food cues, neuroendocrine signals originating from chemosensory neurons can influence the activity of DAF-16/FoxO in the intestine (Artan et al. 2016; Lin et al. 2001). To determine if DAF-7 signaling contributes to the DR response via DAF-16/FoxO activation, we monitored the localization of the *zIs356[daf-16p::daf-16::GFP]* transgene in wild-type and *daf-7* mutant backgrounds. In response to food deprivation, wild-type animals shift from mostly cytosolic to nuclear localized DAF-16::GFP (Artan et al. 2016). A *daf-7* loss-of-function mutation abrogated this intestinal DAF-16::GFP translocation in BD conditions compared to wild-type animals (Figure 4). These data were surprising particularly considering that DAF-16 activation has been implicated in the setting of *daf-7* loss-of-function (R. Y. Lee, Hench, and Ruvkun 2001). However, we note that consistent with reports by others (Shaw et al. 2007), we did observe an increase in nuclear DAF-16::GFP in the *daf-7(e1372)* background in other tissues such as the muscle and hypodermis in both fed and BD conditions (Figure S3). This observation suggests that specifically in response to BD, an increase in *daf-7* expression stimulates activation of DAF-16 in the intestine, which helps to promote longevity. This model is fitting with prior reports that have implicated a role for DAF-16/FoxO in mediating lifespan extension in response to various forms of DR (E. L. Greer et al. 2007; E. L. Greer and Brunet 2009) and in food sensing mutants (Apfeld and Kenyon 1999).

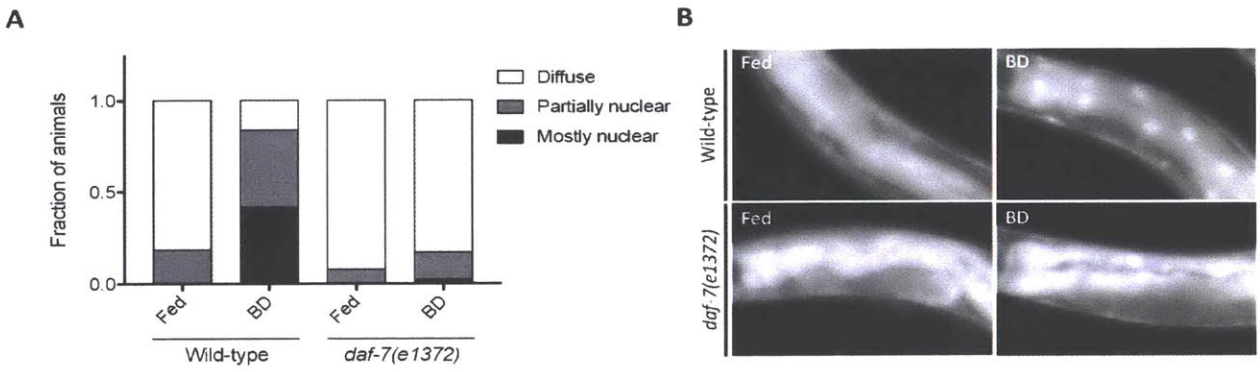

Figure 4: *daf-7* is required for DAF-16/FoxO nuclear translocation in response to BD.

(**A**) State of DAF-16a/b::GFP localization pattern in fed versus BD conditions. Representative of 2 replicates with total n=116-154 animals per condition. (**B**) Representative images of the *zIs356[daf-16p::daf-16a/b::GFP]* reporter in the intestine of wild-type or *daf-7(e1372)* animals in fed and BD conditions.

Decline of *daf-7* expression in aging animals reduces organismal sensitivity to dietary restriction

We measured *daf-7* expression as animals aged during adulthood using the *ksIs2[daf-7p::GFP]* reporter strain. We observed that *daf-7* expression is maintained throughout the life of adult animals in the ASI neurons. As noted above, we also observed *daf-7* expression in the ASJ neuron pair as animals age, with all animals exhibiting ASJ expression by day 3 of adulthood (Figure 5A). In contrast to the marked induction of *daf-7* expression in both ASI and ASJ neurons in response to *P. aerugionsa* (Meisel et al. 2014), in aging animals, *daf-7* expression in ASJ remained relatively low (Figure 5B). Moreover, we observed that *daf-7* expression in the ASI neuron pair significantly decreased with age (Figure 5B). We confirmed these findings by FISH using probes targeted to endogenous *daf-7* mRNA to eliminate the possibility that these observations were an artifact of using a transgenic reporter. Our FISH results support our observations of the *ksIs2* GFP reporter strain. ASI neurons from aged animals show decreased *daf-7* expression; and while there is no detectable *daf-7* mRNA in ASJ neurons of young animals, we were able to observe *daf-7* mRNA in older adults (Figure S4).

We sought to corroborate these changes in *daf-7* expression in these sensory neurons with a measure of how much functional DAF-7 was secreted, so we utilized the *cuIs5[C183::GFP]* reporter of DAF-3 activity. DAF-3 negatively regulates *C183* enhancer activity *in vivo*, resulting in low GFP fluorescence when DAF-3 is active (Thatcher, Haun, and Okkema 1999). The transgenic *cuIs5[C183::GFP]* reporter provides a measure of DAF-7 signal production by examining the downstream effects on DAF-3 in a neighboring tissue. We found that GFP fluorescence was diminished in an age-related, DAF-7-dependent manner, consistent with less overall DAF-7 signaling in aging worms (Figure 5C).

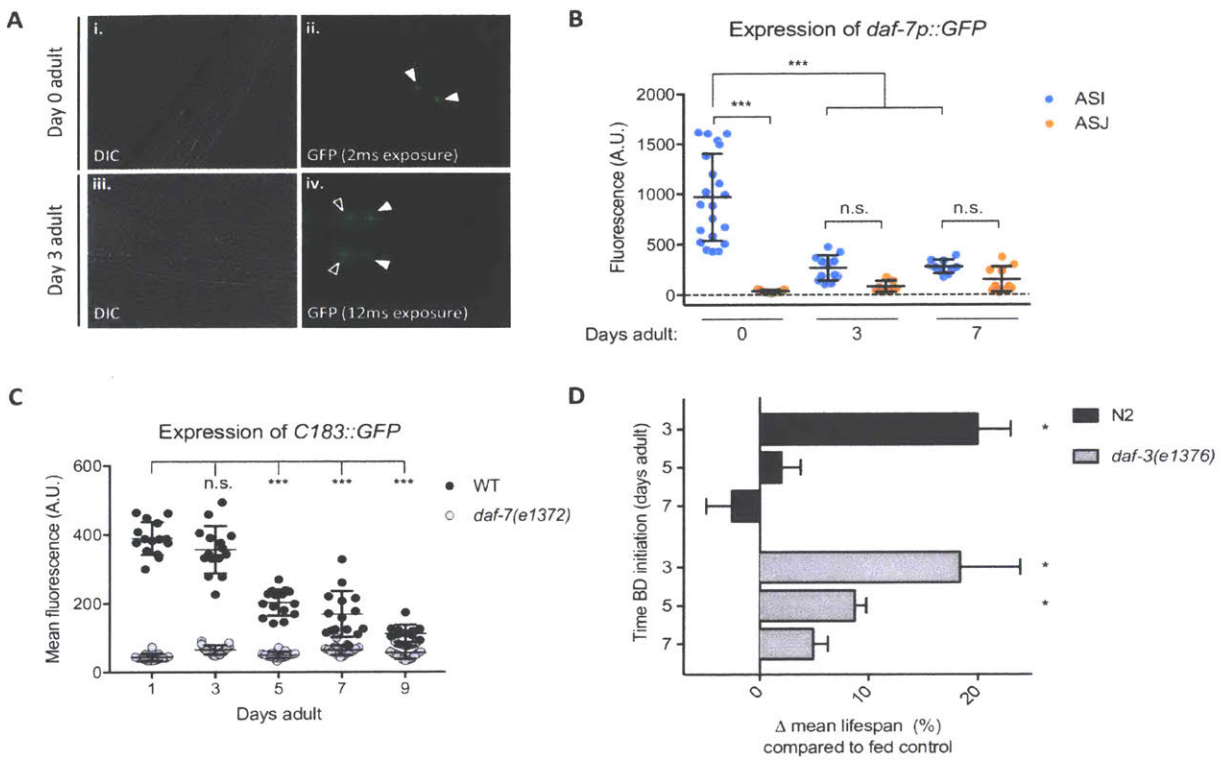

Figure 5: *daf-7* expression declines in aging animals.

(**A**) Expression pattern of *ksIs2[daf-7p::GFP]* reporter in young (i,ii) and aged (iii,iv) animals. Solid triangles indicate ASI neurons, open triangles indicate ASJ neurons. (**B**) Quantification of GFP expression driven by *ksIs2[daf-7p::GFP]*, normalized to exposure time. Representative of 4 replicates with n>10 animals per day. *** represents $p < 0.001$ by one-way ANOVA (**C**) Quantification of GFP expression driven by *cuIs5[C183::myo-2p::GFP]* in aging animals, normalized to exposure time. WT statistics indicated on graph, *daf-7(e1372)* statistics- Day 3 p=0.0002; Day 5 p=0.5225; Day 7 p=0.0005; Day 9 p=0.0445; all differences in *daf-7(e1372)* are a result of increased GFP fluorescence later in life. Representative of 4 replicates with n>10 animals per genotype per day. *** represents $p < 0.001$ by one-way ANOVA (**D**) Summary of lifespan experiments initiating BD at various times in adulthood. * indicates BD lifespan was significantly different ($p < 0.005$) than fed control group in all experiments. See Supplemental Table 4 for individual experiment details.

In addition to experiencing declines in healthspan indicators such as feeding rate and mobility, aging worms also become diminished in their ability to respond to dietary restriction treatment to extend lifespan (G. D. Lee et al. 2006; Kenyon et al. 1993). We wondered if part of the insensitivity older animals have to dietary restriction treatment could be attributed to diminished levels of DAF-7 that cause an increased amount of DAF-3 activity that blocks responses leading to lifespan extension in response to DR in aging animals. To test this hypothesis, we conducted BD experiments where BD treatment was initiated at multiple time points, beginning on days 1, 3, 5 or 7 of adulthood, in wild-type or *daf-3* mutant animals. We found that wild-type animals experience a robust lifespan extension when BD was began on days 1 or 3, but were unable to respond when BD was started on days 5 or 7 (Figure 5D, Table S4), consistent with prior studies (G. D. Lee et al. 2006). By contrast, *daf-3* mutant animals were able to maintain the ability to respond to BD on day 5 (Figure 5D), suggesting that age related decline in the ability to respond to dietary restriction can be attributed, in part, to increased DAF-3 activation as a result in diminished *daf-7* expression. Additionally, animals overexpressing *daf-7* retain the ability to respond to BD and extend lifespan late in life at a time when wild type animals no longer exhibit lifespan extension in response to BD (Figure S5).

Discussion

DAF-7 is at the nexus of feeding behaviors and fat metabolism (E. R. Greer et al. 2008; You et al. 2008), suggestive of neuroendocrine links between the nervous system and secondary tissues. We have described how neuroendocrine signaling through the DAF-7/TGFβ pathway is required for lifespan extension in response to DR in *C. elegans*. Whereas canonical energy sensing pathways, such as AMPK and TOR, have been shown to be involved in lifespan

extension in response to DR, the role of neural regulation by sensory systems of the DR response is less understood (Libert et al. 2007; Smith et al. 2008; Mair and Dillin 2008). Prior studies have established the ASI neuron pair as a cell non-autonomous regulator of the DR response, identifying the insulin-like peptide INS-6 and the SKN-1/Nrf2 transcription factor as relevant agents in initiating communication to downstream cells and tissues (Bishop and Guarente 2007; Artan et al. 2016). We have shown that in response to DR, the ASI pair also secretes the neuroendocrine ligand, DAF-7, which signals to the RIM/RIC interneurons to suppress the co-SMAD DAF-3. In the absence of negative regulation by DAF-7, increased DAF-3 activity blocks the lifespan extension caused by DR (Figure 6).

In the developing animal, the DAF-7 ligand is produced in favorable conditions that promote entry into reproductive development, specifically in the presence rather than the absence of bacterial food (Ren et al. 1996; Schackwitz, Inoue, and Thomas 1996). Our data are suggestive of an acute increase in *daf-7* expression in the ASI neuron pair in response to the withdrawal of bacterial food, indicating that the dynamic expression of *daf-7* of developing larvae may differ from that of adult animals in response to changing environmental conditions such as DR treatments. Indeed, while bacterial deprivation extends the lifespan of adult animals, the introduction of DR-like treatments in young larvae either prompts entry into the dauer state or has detrimental effects on developing animals that have already surpassed the dauer decision checkpoint (Cassada and Russell 1975; Klass 1977).

Whereas a recent study showed that adult animals exposed to diminishing amounts of bacterial food exhibit decreased *daf-7* expression in the ASI neurons after a period of four days (Entchev et al. 2015), our data, recording levels of *daf-7* mRNA using FISH-based detection at multiple time points after the complete withdrawal of food, reveal a complex relationship pattern

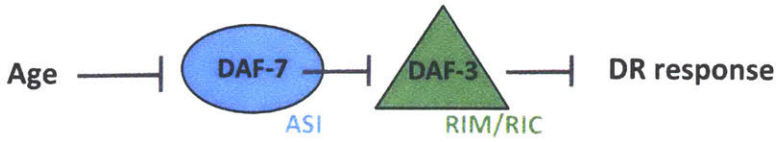

Figure 6: A decline in neuronal *daf-7* expression with advancing age alters sensitivity to effects of DR on lifespan.

Declines in *daf-7* expression in ASI chemosensory neurons with age inhibit activity of *daf-3* in RIM/RIC interneurons, the active form of which is able to interfere with the DR response in other tissues.

of dynamic *daf-7* expression in the ASI neurons of adult animals in response to the withdrawal of bacterial food. We observe an initial increase in *daf-7* expression in animals subjected to BD conditions, consistent with our genetic data implicating a requirement for DAF-3 inhibition for lifespan extension in response to BD. We observe that at later times following the withdrawal of bacterial food, *daf-7* expression is maintained relative to initial levels of expression, in marked contrast to what has been observed when developing larvae are subjected to starvation conditions (Ren et al. 1996).

Our study builds upon previous observations that have linked the *daf-7* gene with aging and the influence changing food levels has on longevity (Entchev et al. 2015; Shaw et al. 2007). Together, our genetic findings and expression analyses support a model where active DAF-3 is sufficient to disrupt the animals' sensory abilities and prevent lifespan extension in response to DR (Figure 6). Because *daf-3* mutant worms are capable of responding to DR, the DAF-7 signaling pathway does not seem to have a direct role in altering metabolism in other tissues to extend lifespan in response to limited food levels. Rather, DAF-7 secreted by the chemosensory neurons seems to be a key neuroendocrine signal that allows animals to properly sense reductions in nutrient availability, which eventually results in activation of DAF-16/FoxO in the intestine under food deprivation. Moreover, our data suggest that an age-dependent decline in neuronal *daf-7* expression also underlies the diminished sensitivity of aging animals to the lifespan effects of DR, linking a decline in neuroendocrine function to the loss of DR efficacy with advancing age.

In human aging, decline in olfactory function is one of the largest predictors of mortality- a stronger independent risk factor for death than causes such as cancer or heart failure (Pinto et al. 2014). Our study suggests that the modulation of a specific neuroendocrine signaling pathway

active in chemosensory neurons involved in the sensation of bacterial food may alter the sensitivity of *C. elegans* to the effects of DR. We speculate that therapeutic strategies targeting analogous neuroendocrine pathways in mammals may be able to function in concert with dietary modifications to promote longevity.

Materials and Methods

C. elegans Strains

C. elegans were maintained at 16°C on *E. coli* OP50 as previously described (Brenner 1974). For a list of all strains used in this study, see Table S1.

Lifespan assays

Due to the egg-laying defect of *daf-7* pathway mutant animals, synchronized populations were prepared by egg-prep of gravid adult worms in bleach followed by L1 arrest overnight in M9 buffer. L1s were placed on OP50 seeded Nematode Growth Media (NGM) plates and raised to the L4 larval stage at 16°C. Upon reaching L4, worms were transferred onto NGM plates containing 12 μM FUDR (to prevent matricidal effects of *daf-7* pathway mutants as well as progeny production) and 0.01 mg ampicillin seeded with 10X concentrated OP50 from an overnight culture and moved to 25°C (to avoid AVID (Leiser et al. 2016) as well as enhance *daf-7* mutant phenotypes (Ren et al. 1996; Riddle, Swanson, and Albert 1981)). Unless otherwise noted, on day 3 of adulthood (where day 0 is defined as L4 stage), worms were transferred to either fed or DR conditions on NGM plates made without peptone to prevent bacterial growth and rimmed with 150 μL of 10 mg/mL palmitic acid to prevent worms from crawling off the plates. For BD experiments, fed plates were seeded with 200 μL of 10X concentrated OP50 from an overnight culture and BD plates were unseeded. For sDR experiments, fed plates were seeded with 200 μL of OP50 at a concentration of $2x10^{10}$ bacteria/mL and sDR plates with 200 μL of OP50 at $5x10^{8}$ bacteria/mL. At least 2 plates per condition were used in all experiments. Worms were scored for death (defined as failure to respond to prodding with a platinum wire) every 1-3 days beginning around day 4 of adulthood. Animals exhibiting vulval rupture were censored.

Worms that crawled off the plate were never considered. Representative experiments are presented here. For lifespan statistics of individual experiments, see Tables S2-S4.

GFP expression experiments

Synchronized populations were prepared as above and treated in the same manner as worms subjected to lifespan analysis (raised to L4 16°C, then shifted to ampicillin/FUDR plates and placed at 25°C). Animals were examined for GFP fluorescence on the indicated days. All images were acquired with an Axioimager Z1 microscope using animals mounted on glass slides, anesthetized by 100mM sodium azide. Quantification of *daf-7p::GFP* was performed by taking the maximum intensity by FIJI software (Schindelin et al. 2012) within the ASI or ASJ neuron at 40X magnification. Quantification of *C183::GFP* was done by taking the average intensity by FIJI software (Schindelin et al. 2012) within the entire pharynx at 20X magnification. All quantifications were normalized by exposure time and background fluorescence (measured individually for each image). Day 3 adult *zIs356[daf-16p::daf-16a/b::GFP]* strains were examined on a fluorescent dissecting microscope after 4 hours of bacterial deprivation. Representative images were taken at 20X magnification. Two to four replicates were performed for all experiments presented.

Fluorescent In Situ Hybridization

Synchronized populations were established as above. FISH was performed as previously described (Raj et al. 2008). At the indicated times and treatments, animals were washed twice with M9 buffer before fixation with 4% formaldehyde at room temperature, followed by PBS washes and suspension in 70% RNase free ethanol and stored at 4°C. To image, all samples from

an individual experiment were incubated overnight with FISH probes designed against *daf-7* mRNA (coupled to Cy5 dye) (Meisel et al. 2014) in hybridization solution at 30°C. The next day, animals were imaged with a Nikon Eclipse Ti Inverted Microscope outfitted with a Princeton Instruments PIXIS 1024 camera. A GFP marker was used to focus on the neuron of interest and obtain a single image using a Cy5 filter. This method of image acquisition does not allow resolution of single mRNA molecules, thus quantification of *daf-7* was done using FIJI software (Schindelin et al. 2012) to outline either ASI or ASJ and obtaining the mean intensity and subtracting background fluorescence (measured by obtaining the mean intensity of a small space immediately adjacent to the neuron being quantified). A minimum of 2 replicates was performed for all experiments presented.

Statistical analysis

The log-rank statistical test was used to determine *p*-values for lifespans. Using Graphpad Prism, an unpaired t-test, one-sample t-test, or one-way ANOVA was used to determine significance in quantification of expression experiments.

Author Contributions

M.F. performed all experiments. M.F. and D.H.K. analyzed data, interpreted results, and wrote the paper.

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Supporting Information

Figure S1

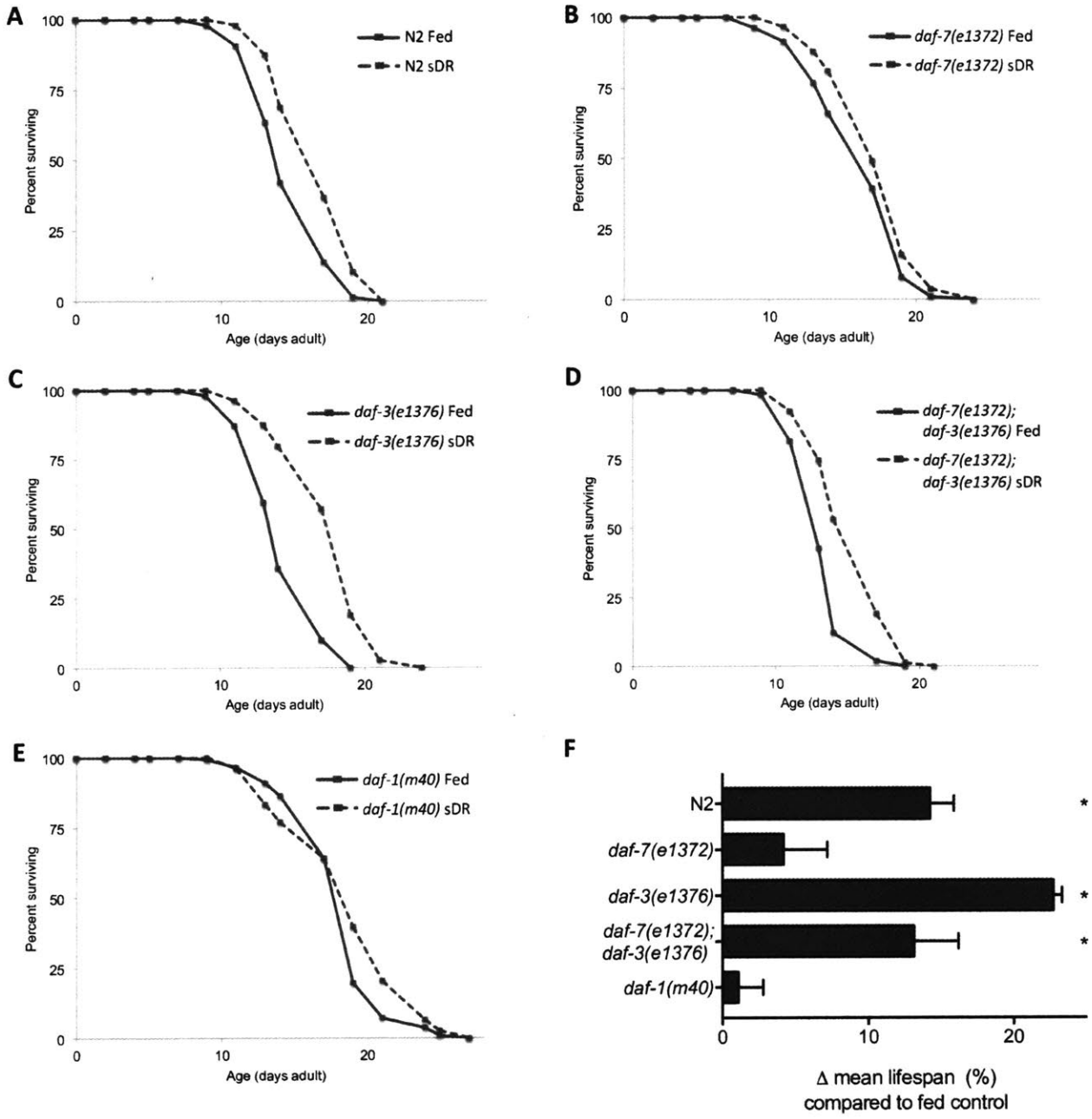

Figure S1: *daf-7* signaling is required for additional forms of dietary restriction.

(A-E) Representative curves of animals subjected to control fed (solid lines) or sDR (dashed lines) dietary regimens. **(F)** Summary of all sDR experiments. * indicates BD lifespan was significantly different ($p \leq 0.001$) than fed control group in all experiments, error bars reflect SEM. See Supplemental Table 3 for individual experiment details.

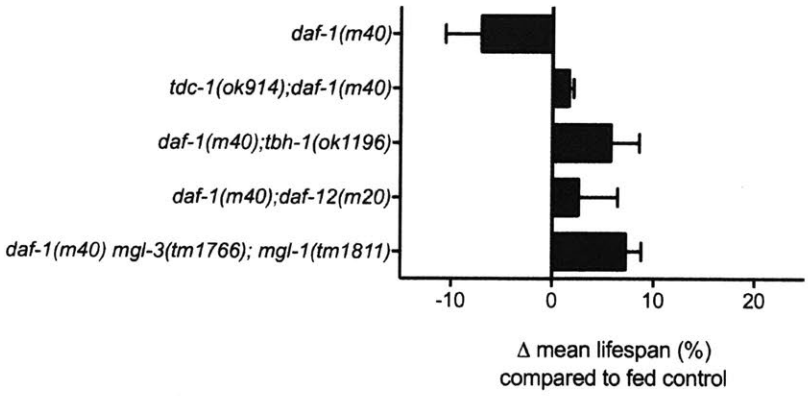

Figure S2: Feeding behavior, dauer formation, and fat storage phenotypes of *daf-7* pathway mutants can be uncoupled from the lack of lifespan extension in response to BD. Summary of lifespan experiments suppressing specific phenotypes caused by increased *daf-3* activity. Introducing suppressor mutations in genes controlling changes to feeding rate (*tdc-1* and *tbh-1*), dauer formation (*daf-12*), and fat storage (*mgl-3;mgl-1*) was insufficient to restore lifespan extension by BD in *daf-1(m40)* mutant animals. * indicates BD lifespan was significantly different ($p \leq 0.001$) than fed control group in all experiments, error bars reflect SEM. See Supplemental Table 2 for individual experiment details.

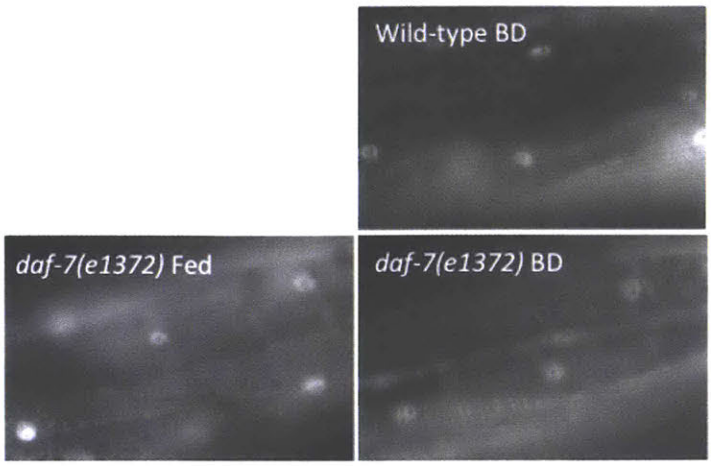

Figure S3: *daf-7(e1372)* mutants have increased basal nuclear DAF-16::GFP.

Representative images of the *zIs356[daf-16p::daf-16a/b::GFP]* reporter in muscle cells of fed wild-type or *daf-7(e1372)* animals in fed and BD conditions.

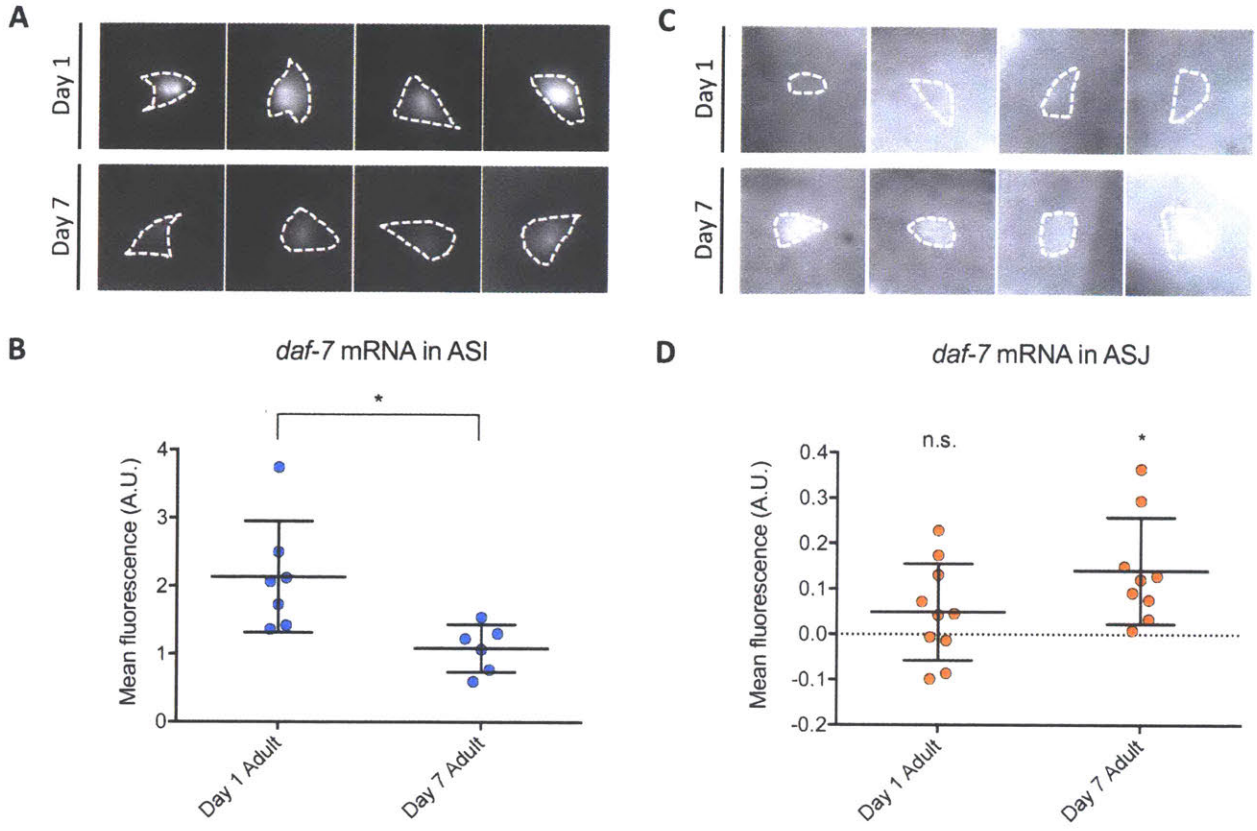

Figure S4: FISH of *daf-7* mRNA.

Images and quantification of fluorescence of FISH probes designed against *daf-7* in ASI (identified by co-localization with *str-3p::GFP*) (A,B) and ASJ (identified by co-localization with *trx-1p::GFP*) (C,D) in young and aged animals.

(B) * indicates $p < 0.05$ by unpaired t-test. (D) * indicates $p < 0.05$ significantly different from zero by one-sample t-test.

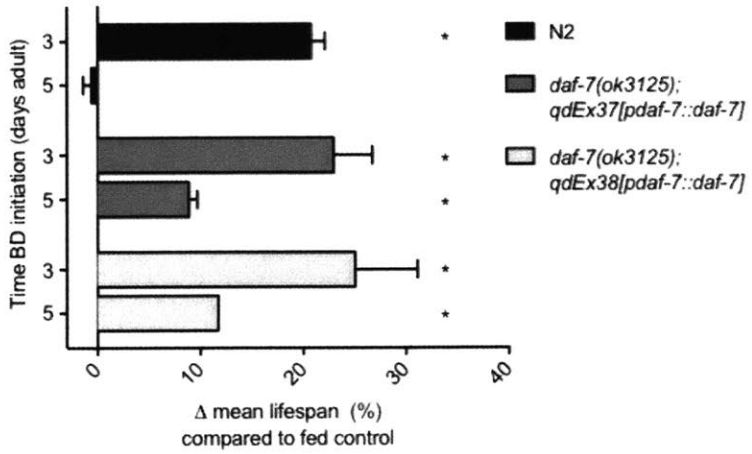

Figure S5: *daf-7* overexpression extends the DR response window.

Summary of lifespan experiments in two rescue lines overexpressing *daf-7*. * indicates BD lifespan was significantly different ($p \leq 0.001$) than fed control group in all experiments, error bars reflect SEM. See Supplemental Table 4 for individual experiment details.

Table S1: Strain list

Strain	Genotype
N2	Wild-type Bristol isolate
DR40	*daf-1(m40)*
CB1287	*daf-1(e1287)*
CB1372	*daf-7(e1372)*
RB2302	*daf-7(ok3125)*
CB1376	*daf-3(e1376)*
RB2589	*daf-3(ok3610)*
DR114	*daf-7(e1372);daf-3(e1376)*
ZD907	*daf-7(ok3125);daf-3(e1376)*
ZD1641	*daf-1(m40);daf-3(e1376)*
KQ363	*tdc-1 (ok914); daf-1(m40)*
KQ364	*daf-1 (m40); tbh-1 (ok1196)*
DR213	*daf-1(m40); daf-12(m20)*
ZD1424	*daf-1(m40) mgl-3(tm1766); mgl-1(tm1811)*
ZD729	*daf-7(ok3125);qdEx37[daf-7p::daf-7 + ges-1p::GFP]*
ZD730	*daf-7(ok3125);qdEx38[daf-7p::daf-7 + ges-1p::GFP]*
ZD736	*daf-7(ok3125);qdEx44[str-3p::daf-7 + ges-1::GFP]*
ZD732	*daf-7(ok3125);qdEx40[trx-1p::daf-7 + ges-1p::GFP]*
KQ251	*daf-1(m40); ftEx69[egl-3p::daf-1::GFP + odr-1::dsRED]*
KQ256	*daf-1(m40); ftEx74[glr-8p::daf-1::GFP + odr-1::dsRED]*
KQ265	*daf-1(m40); ftEx83[osm-6p::daf-1::GFP + odr-1::dsRED]*
KQ280	*daf-1(m40); ftEx98[daf-1p::daf-1::GFP + odr-1::dsRED]*
KQ380	*daf-1(m40); ftEx205[tdc-1p::daf-1:gfp + odr-1:dsRED]*
FK181	*ksIs2[daf-7p::GFP + rol-6(su1006)]*
TY3856	*cuIs5[myo-2 C183::GFP + pRF4(rol-6(su1006)]*
TY3862	*daf-7(e1372); cuIs5[myo-2 C183::GFP + pRF4(rol-6(su1006)]*
OE3010	*lin-15B(n765); ofEx4[BLH98p::lin-15(+) + trx-1::GFP]*
CX3596	*kyIs128[lin-15(+) + str-3::GFP]; lin-15B(n765)*
TJ356	*zIs356[daf-16p::daf-16a/b::GFP + rol-6(su1006)]*
ZD1839	*zIs356[daf-16p::daf-16a/b::GFP + rol-6(su1006)]; daf-7(e1372)*

Table S2: BD lifespan data.

Results for individual lifespan assays with BD treatment.

Experiments performed at 25° C

Genotype	Experiment #	Condition	Mean LS	N	Mean % change	p-value	Responds?	Note
N2	1	Fed	16.8	166				Graph shown in Fig. 1B
	1	BD	21.1	125	25.2	<0.001		
	2	Fed	15.9	170				
	2	BD	19.9	143	25.8	<0.001		
	3	Fed	16.5	148				
	3	BD	21.4	112	29.4	<0.001		
	4	Fed	17.1	157				
	4	BD	18.9	135	11.1	<0.001		
	5	Fed	16.9	124				
	5	BD	18.7	126	11	0.012		
	6	Fed	16.8	171				
	6	BD	19.4	125	15.4	<0.001		
	9	Fed	13.9	146				
	9	BD	16.2	124	16.4	<0.001		
	10	Fed	17	136				
	10	BD	10.7	86	21.4	<0.001		
daf-1(m40)	1	Fed	23	142				
	1	BD	22.7	136	6.5	0.134		
	3	Fed	17.2	168				
	3	BD	17.1	162	-0.6	0.822		
	5	Fed	18.1	131				
	5	BD	16.6	139	-7.9	0.01		
	6	Fed	17.7	135				
	6	BD	18	147	2	0.503		
	8	Fed	16	134				Graph shown in Fig. 2D
	8	BD	15.5	121	-3.1	0.414		
	9	Fed	18.5	145				
	9	BD	16.1	113	-12.7	0.001		
	10	Fed	19.2	135				
	10	BD	20	125	4.1	0.313		
daf-1(e1287)	1	Fed	12.7	16				
	1	BD	13	17	2.5	0.815		
daf-7(e1372)	1	Fed	16.7	172				Graph shown in Fig. 1C
	1	BD	16.4	169	-1.8	0.451		
	4	Fed	14	155				
	4	BD	14.1	118	0.8	0.636		
	6	Fed	14.3	109				
	6	BD	13.4	112	-6	0.199		
daf-7(ok3125)	2	Fed	13.7	161				Graph shown in Fig. 2A
	2	BD	13.2	153	-4	0.225		
	4	Fed	13.9	123				
	4	BD	13.6	56	-1.9	0.428		
daf-3(e1376)	1	Fed	15.4	84				
	1	BD	17.2	169	11.9	0.001		
	4	Fed	15.8	156				
	4	BD	17.9	135	13.8	<0.001		
	6	Fed	16.7	150				
	6	BD	18.7	109	11.8	<0.001		
	9	Fed	17	130				
	9	BD	19.2	77	13	<0.001		
daf-3(ok3610)	4	Fed	15.7	155				
	4	BD	17	178	8.2	<0.001		
daf-1(m40);daf-3(e1376)	10	Fed	18.5	130				
	10	BD	22	95	18.7	<0.001		
daf-7(e1372);daf-3(e1376)	1	Fed	13.2	175				Graph shown in Fig. 1D
	1	BD	16.4	162	24.1	<0.001		
	4	Fed	15.6	172				
	4	BD	17.2	141	10	<0.001		
	6	Fed	15.6	144				
	6	BD	17.2	117	10	0.004		
daf-7(ok3125);daf-3(e1376)	4	Fed	15.6	165				
	4	BD	18.4	137	18.6	<0.001		
	6	Fed	16.5	137				
	6	BD	18.7	98	12.9	<0.001		
daf-7(ok3125);qdEx37[pdaf-7::daf-7]	2	Fed	16.3	177				daf-7 rescue line 1, Graph shown in Fig. 2B
	2	BD	21.9	131	34.3	<0.001		
daf-7(ok3125);qdEx38[pdaf-7::daf-7]	2	Fed	16.9	175				daf-7 rescue line 2, Graph shown in Fig. 2B
	2	BD	20.9	125	23.6	<0.001		
daf-7(ok3215);qdEx44[pstr-3::daf-7]	2	Fed	17.1	116				daf-7 rescue ASI only, Graph shown in Fig. 2C
	2	BD	22.4	138	31.1	<0.001		
daf-7(ok3125);qdEx40[ptrx-1::daf-7]	2	Fed	17.3	163				daf-7 rescue ASJ only, Graph shown in Fig. 2C
	2	BD	23.6	128	36.9	<0.001		

daf-1(m40);ftEx98[pdaf-1::daf-1::GFP]	6	Fed	20.9	159				daf-1 rescue, endogenous promoter
	6	BD	22.5	108	7.5	0.013		
	7	Fed	17.4	125				
	7	BD	21	79	20.4	<0.001		
	8	Fed	19.2	139				Graph shown in Fig. 2E
	8	BD	20.6	85	7.3	0.014		
daf-1(m40);ftEx69[pegl-3::daf-1::GFP]	6	Fed	21.6	128				daf-1 rescue, >80 neurons
	6	BD	24.2	106	11.8	0.004		
	8	Fed	18.9	122				
	8	BD	21.2	97	12.1	0.003		
daf-1(m40);ftEx83[posm-6::daf-1::GFP]	7	Fed	16	100				daf-1 rescue, 56 ciliated neurons
	7	BD	15.4	106	-3.8	0.194		
	8	Fed	17.6	91				
	8	BD	17.9	90	1.7	0.62		
daf-1(m40);ftEx74[pglr-8::daf-1::GFP]	7	Fed	19.6	93				daf-1 rescue, 25 pharyngeal & interneurons
	7	BD	19.6	103	0.2	0.654		
	8	Fed	17.4	89				
	8	BD	18	97	3.5	0.416		
daf-1(m40);ftEx205[ptdc-1::daf-1::GFP]	7	Fed	17.3	119				daf-1 rescue, RIM/RIC/UV1
	7	BD	19.3	87	11.4	0.007		
	8	Fed	19.3	95				Graph shown in Fig. 2E
	8	BD	22.8	71	18	<0.001		
tdc-1(ok914);daf-1(m40)	3	Fed	11.7	157				
	3	BD	12	154	2.1	0.117		
	9	Fed	12.1	147				
	9	BD	12.2	139	1.2	0.712		
tbh-1(ok1196);daf-1(m40)	3	Fed	14	158				
	3	BD	14.4	152	3	0.434		
	9	Fed	15.4	154				
	9	BD	16.7	142	8.6	0.002		
daf-12(m20);daf-1(m40)	3	Fed	14.9	159				
	3	BD	15.9	137	6.5	0.146		
	9	Fed	16.1	125				
	9	BD	15.8	121	-1.3	0.692		
daf-1(m40) mgl-3(tm1766); mgl-1(tm1811)	5	Fed	15	131				
	5	BD	16.3	135	8.8	0.068		
	9	Fed	14.3	108				
	9	BD	15.1	141	5.8	0.162		

Experiments performed at 20° C

Genotype	**Experiment #**	**Condition**	**Mean LS**	**N**	**Mean % change**	**p-value**	**Responds?**	**Note**
N2	18	Fed	28.9	132				
	18	BD	37.3	38	29	<0.001		
daf-7(e1372)	18	Fed	28.3	171				
	18	BD	33.4	88	17.8	<0.001		

KEY	
	Positive extension, p <0.001 (unless transgenic, then p<0.05)
	Postitive extension, 0.01>p>0.001
	Negative or no extension (p>0.01)

Table S3: sDR lifespan data.

Results for individual lifespan assays with sDR treatment.

Genotype	Experiment #	Condition	Mean LS	N	Mean % change	p-value	Responds?
N2	11	AL	15	95			
	11	sDR	16.8	97	12.6	<0.001	
	12	AL	12.3	61			
	12	sDR	13.2	86	15.9	<0.001	
daf-1(m40)	11	AL	18.4	111			
	11	sDR	18.9	78	2.8	0.221	
	12	AL	16.3	92			
	12	sDR	16.2	72	-0.6	0.914	
daf-7(e1372)	11	AL	16.5	102			
	11	sDR	17.6	57	7.2	0.049	
	12	AL	13.1	87			
	12	sDR	13.3	86	1.2	0.669	
daf-3(e1376)	11	AL	14.6	101			
	11	sDR	17.8	79	22.1	<0.001	
	12	AL	12.7	88			
	12	sDR	15.7	80	23.3	<0.001	
daf-7(e1372);daf-3(e1376)	11	AL	13.4	108			
	11	sDR	15.6	90	16.2	<0.001	
	12	AL	12	87			
	12	sDR	13.2	85	10.1	<0.001	

KEY	
	Positive extension, p <0.001
	Postitive extension, 0.01>p>0.001
	Negative or no extension (p>0.01)

Supplemental Figure 1 graphs results of Experiment #11

Table S4: Late BD lifespan data.

Results for individual lifespan assays where BD treatment was initiated at multiple ages.

Genotype	Experiment #	Condition	Mean LS	N	Mean % change	p-value	Responds?
N2	13	Fed Day 1	18	157			
	13	BD Day 1	23.8	78	32.3	<0.001	
	13	Fed Day 3	17	134			
	13	BD Day 3	19.1	98	12.3	<0.001	
	13	Fed Day 5	16.9	129			
	13	BD Day 5	16.7	100	-1.3	0.425	
	13	Fed Day 7	17	132			
	13	BD Day 7	17.1	87	0.8	0.942	
	14	Fed Day 1	17	148			
	14	BD Day 1	19.6	104	15.3	<0.001	
	14	Fed Day 3	15.8	128			
	14	BD Day 3	18.3	87	15.9	<0.001	
	14	Fed Day 5	14.2	129			
	14	BD Day 5	15.2	105	6.8	0.06	
	14	Fed Day 7	13.9	135			
	14	BD Day 7	12.9	107	-7.1	0.008	
	15	Fed Day 1	16.3	143			
	15	BD Day 1	21.2	44	29.9	<0.001	
	15	Fed Day 3	15	142			
	15	BD Day 3	19.5	101	30.2	<0.001	
	15	Fed Day 5	13.1	103			
	15	BD Day 5	13.8	98	5.6	0.181	
	15	Fed Day 7	13.8	133			
	15	BD Day 7	13.6	110	-1.4	0.378	
	16	Fed Day 3	15.2	125			
	16	BD Day 3	18.2	75	19.4	<0.001	
	16	Fed Day 5	13.7	123			
	16	BD Day 5	13.5	127	-1.4	0.545	
	17	Fed Day 3	13.2	83			
	17	BD Day 3	16.1	67	22.1	<0.001	
	17	Fed Day 5	13.8	69			
	17	BD Day 5	13.8	62	0.2	0.95	
daf-3(e1376)	13	Fed Day 1	16.5	174			
	13	BD Day 1	20.3	71	23.6	<0.001	
	13	Fed Day 3	16.4	133			
	13	BD Day 3	18.9	92	15.1	<0.001	
	13	Fed Day 5	16.3	139			
	13	BD Day 5	18	97	10.5	<0.001	
	13	Fed Day 7	17.1	123			
	13	BD Day 7	17.8	125	3.8	0.012	
	14	Fed Day 1	16.5	156			
	14	BD Day 1	18.7	93	13	<0.001	
	14	Fed Day 3	16.7	132			
	14	BD Day 3	18.5	96	10.9	<0.001	
	14	Fed Day 5	16	133			
	14	BD Day 5	17	123	6.8	0.004	
	14	Fed Day 7	15.8	131			
	14	BD Day 7	17	108	7.6	0.002	
	15	Fed Day 1	16.1	99			
	15	BD Day 1	18.9	49	17.8	<0.001	
	15	Fed Day 3	15.7	119			
	15	BD Day 3	20.3	93	29.1	<0.001	
	15	Fed Day 5	14	121			
	15	BD Day 5	15.2	108	8.9	<0.001	
	15	Fed Day 7	13.9	115			
	15	BD Day 7	14.4	112	3.4	0.165	

daf-7(ok3125);qdEx37	16 Fed Day 3	16.8	118			
	16 BD Day 3	20	91	19.2	<0.001	
	16 Fed Day 5	13.8	128			
	16 BD Day 5	15.1	107	9.7	0.001	
	17 Fed Day 3	13.9	64			
	17 BD Day 3	17.6	61	26.7	<0.001	
	17 Fed Day 5	14.5	73			
	17 BD Day 5	15.6	75	8.1	0.001	
daf-7(ok3125);qdEx38	16 Fed Day 3	16.4	102			
	16 BD Day 3	19.5	57	19	<0.001	
	16 Fed Day 5	13.9	123			
	16 BD Day 5	15.4	110	11.3	0.001	
	17 Fed Day 3	13.5	35			
	17 BD Day 3	17.8	32	31.1	<0.001	
	17 Fed Day 5	13.9	18			
	17 BD Day 5	15.6	17	12.2	0.038	

KEY	
	Positive extension, p <0.001
	Postitive extension, 0.01>p>0.001
	Negative or no extension (p>0.01)

Chapter Three

Global regulation of innate immunity through a single p38 MAPK – ATF transcription factor pathway in *C. elegans*

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This chapter is currently in preparation for publication.

Abstract

The nematode *Caenorhabditis elegans* has emerged as a simple animal host in which to study evolutionarily conserved mechanisms of innate immune signaling. We previously showed that the PMK-1 p38 mitogen-activated protein kinase (MAPK) pathway regulates innate immunity of *C. elegans* through phosphorylation of the CREB/ATF bZIP transcription factor, ATF-7. Here, we have undertaken a global genetic and genomic analysis of the regulation of the transcriptional response of *C. elegans* to infection by *Pseudomonas aeruginosa*, combining genome-wide expression analysis by RNA-seq with ATF-7 chromatin immunoprecipitation followed by sequencing (ChIP-Seq). We observe that PMK-1-ATF-7 activity regulates more than half of all genes induced by pathogen infection, with ATF-7 occupancy in regulatory regions of many of these pathogen-induced genes in a PMK-1-dependent manner. Moreover, functional analysis of a subset of these ATF-7-regulated pathogen-induced target genes, including genes encoding C-type lectins, support a direct role for this transcriptional response in host defense. The observed striking degree of genomic, organism-wide regulation through PMK-1-ATF-7 reveals the global organismal control over the innate immune response by a single transcriptional regulator in the *C. elegans* host.

Introduction

Convergent genetic studies of host defense of *Drosophila melanogaster* and mammalian innate immune signaling revealed a commonality in signaling pathways of innate immunity that has helped motivate the study of pathogen resistance mechanisms in genetically tractable host organisms such as *Caenorhabditis elegans* (Schulenburg, Kurz, and Ewbank 2004). The simple *C. elegans* host lacks specialized immune cells, but this simplicity has in particular, facilitated the dissection of integrative stress physiology orchestrating immune responses of *C. elegans*. Genetic analysis of resistance of *C. elegans* to infection by pathogenic *Pseudomonas aeruginosa* has defined an essential role for a conserved p38 mitogen-activated protein kinase pathway that acts on a CREB/ATF family bZIP transcription factor, ATF-7, in immune responses (Kim et al. 2002; Shivers et al. 2010). A complementary approach to characterizing the host response has been organismal transcriptomic characterization of genes induced upon infection by a number of different bacterial pathogens. Putative effector genes encoding lysozymes and C-type lectin domain (CTLD)-containing proteins have been identified that have also served as useful markers of immune induction, facilitating the dissection of integrative stress physiology orchestrating immune responses of *C. elegans* (Schulenburg et al. 2008). However, these genetic and gene-expression based approaches have not provided information about whether transcriptional regulators identified from genetic studies directly regulate the genes induced by infection. Here, we report the genome-level characterization of the *C. elegans* response to *P. aeruginosa*, combining RNA-seq analysis of pathogen-induced gene expression with ChIP-seq analysis of ATF-7 binding, which suggest a dramatic degree of global regulation of the immune response of *C. elegans* through a single MAPK- transcription factor pathway.

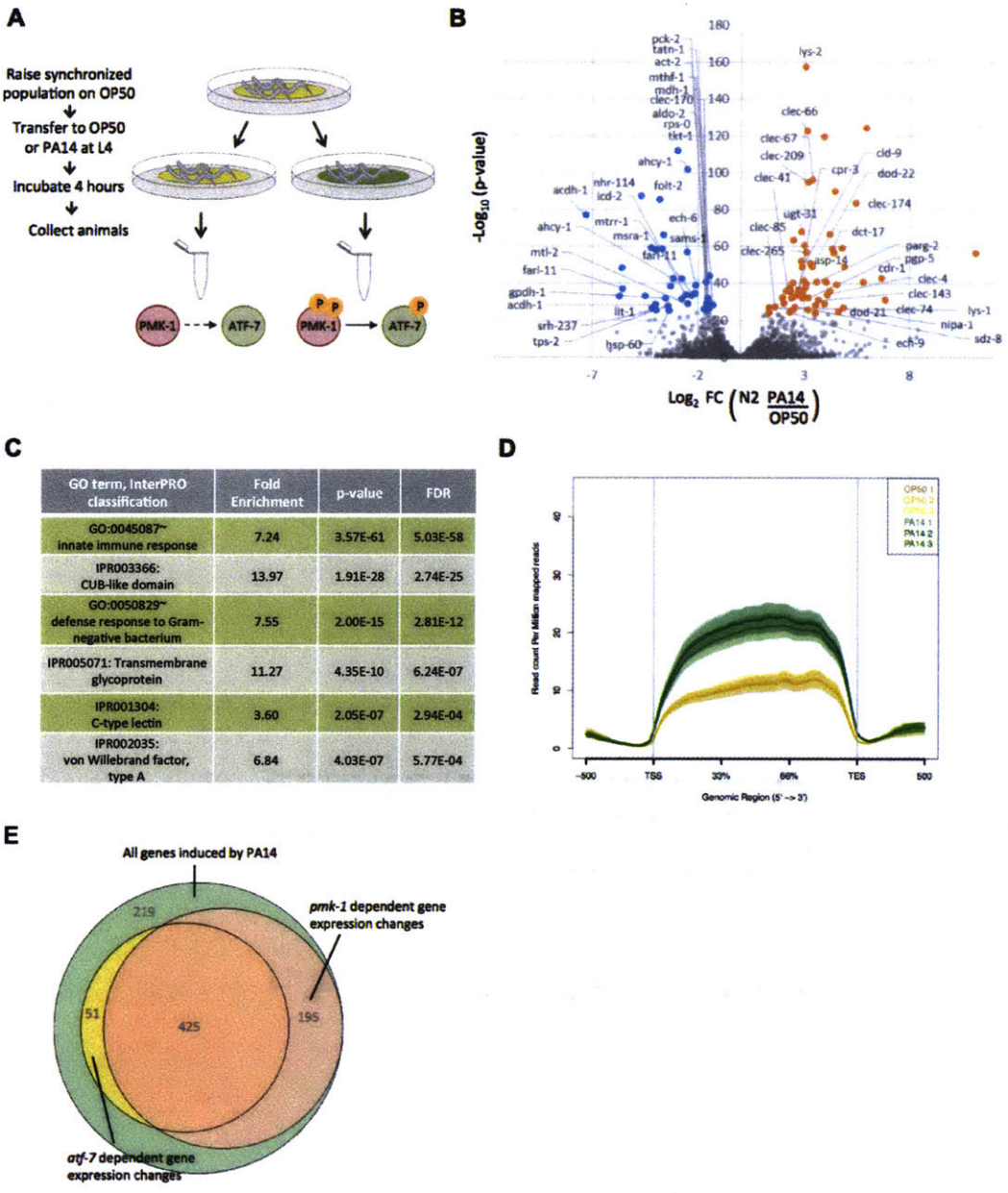
Results and Discussion

We performed RNA-seq on wild-type (N2), *pmk-1* mutant, or *atf-7* mutant animals exposed to *E. coli* OP50 or *P. aeruginosa* PA14 to identify genes that are differentially regulated upon infection that also require *pmk-1* or *atf-7* for induction (Figure 1A). We found that in wild-type animals, 890 genes were significantly two-fold upregulated and 803 genes were two-fold downregulated by *P. aeruginosa* exposure, compared to animals exposed in parallel to *E. coli* (Figure 1B; Table S1). Many of these upregulated genes have been previously implicated in the *C. elegans* immune response and included genes encoding C-type lectin domain (CTLD)-containing genes and lysozymes, which corroborates prior microarray-based gene expression studies (Figure 1C) (Mallo et al. 2002; Huffman et al. 2004; Troemel et al. 2006; O'Rourke et al. 2006; Wong et al. 2007; Engelmann et al. 2011). In contrast, genes that are decreased in expression upon *P. aeruginosa* exposure are largely associated with growth, development and reproduction, (Figure S1A). Of note, many of the genes upregulated in response to *P. aeruginosa* exposure exhibit relatively low expression when animals are propagated on *E. coli*, whereas genes that are decreased in expression upon *P. aeruginosa* exposure are expressed at a relatively high basal level during normal growth conditions on *E. coli* OP50 (Figure 1D, S1B). In parallel, we analyzed PA14 induction in *pmk-1* and *atf-7* mutants to identify the proportion of genes induced by *P. aeruginosa* exposure that required *pmk-1* and/or *atf-7* for induction (Figure S2). We observed that 70% of genes induced 2-fold or greater by *P. aeruginosa* exposure required *pmk-1*, and that 53% of these genes required *atf-7* (Figure 1E, Table S1). We also found that 41% of genes reduced two-fold or more by PA14 required *pmk-1*, and 50% required *atf-7* (Figure S2B, Table S1). These data implicate a pivotal role for PMK-1-ATF-7 signaling in the transcriptional response to infection by *P. aeruginosa*.

To evaluate the role of ATF-7 in the direct regulation of genes induced by *P. aeruginosa* infection, we performed chromatin immunoprecipitation followed by sequencing (ChIP-seq) on animals carrying a GFP-tag fused to the C-terminal end of the endogenous *atf-7* locus. Using a GFP polyclonal antibody for immunoprecipitation, we generated ChIP binding profiles for animals in either the wild-type background (*atf-7(qd328[atf-7::2xTY1::GFP])*) or the *pmk-1* mutant background (*pmk-1(km25);atf-7(qd328[atf-7::2xTY1::GFP])*) after a four hour exposure to either *E. coli* OP50 or *P. aeruginosa* PA14, for a total of four conditions, similar to the treatment described in Figure 1A. In all conditions analyzed, ATF-7 exhibits abundant binding throughout the genome, with around 9,000 total peaks called, corresponding to 23% of genes and 25% of transcription start sites (TSSs) (Table S2). Of these binding sites, 43% contained the Jun D bZIP motif GACgTCA (Figure 2A).

Analysis of the ATF-7 binding profile across all genes, as well as just those altered in expression by *P. aeruginosa* in wild-type animals according to our RNA-seq data, revealed that ATF-7 preferentially binds promoter regions of genes that are increased in expression by PA14, and that this enhancement is lessened by *pmk-1* loss (Figure 2B, Figure S3A). We assessed the enhanced ATF-7 binding profile at *P. aeruginoasa*-induced genes using a Gene Set Enrichment Analysis (GSEA), which tests whether a set of genes (genes bound by ATF-7) shows biased enrichment among genes associated with one state (PA14-induced genes) versus another (PA14-decreased genes). GSEA of ATF-7 binding across all expressed genes, ranked by induction in response to PA14 as measured by RNA-seq, showed that ChIP peaks were significantly enriched for association with transcripts that are positively changed upon pathogen exposure in both OP50 and PA14 ChIP conditions (Figure 2C, Figure S3B). Strikingly, this association is dependent on *pmk-1* (Figure2D, Figure S3C). Moreover, we also evaluated ATF-7 binding at individual gene

Figure 1



GO term, InterPRO classification	Fold Enrichment	p-value	FDR
GO:0045087~ innate immune response	7.24	3.57E-61	5.03E-58
IPR003366: CUB-like domain	13.97	1.91E-28	2.74E-25
GO:0050829~ defense response to Gram-negative bacterium	7.55	2.00E-15	2.81E-12
IPR005071: Transmembrane glycoprotein	11.27	4.35E-10	6.24E-07
IPR001304: C-type lectin	3.60	2.05E-07	2.94E-04
IPR002035: von Willebrand factor, type A	6.84	4.03E-07	5.77E-04

Figure 1: Exposure to *P. aeruginosa* prompts gene expression changes.

(A) Schematic of experimental design. Yellow bacterial lawn indicates *E. coli* OP50, green bacterial lawn indicates *P. aeruginosa* PA14. PMK-1 and ATF-7 activation states are indicated below each condition. **(B)** Volcano plot of transcripts corresponding to differentially expressed protein-coding genes by exposure to *P. aeruginosa* PA14 versus *E. coli* OP50 in N2 animals. Orange and blue colored dots denote the top 100 outliers that are increased or decreased, respectively, and are annotated with their gene names. **(C)** Top GO terms and InterPRO classifications of transcripts that are significantly upregulated (adjusted p-value < 0.05) in N2 animals exposed to PA14 versus OP50. **(D)** Average expression (RPM) across the gene body of genes that are two-fold upregulated by exposure to pathogenic PA14. **(E)** Venn diagram of induced genes that are dependent upon *pmk-1* or *atf-7* for complete upregulation.

Figure 2

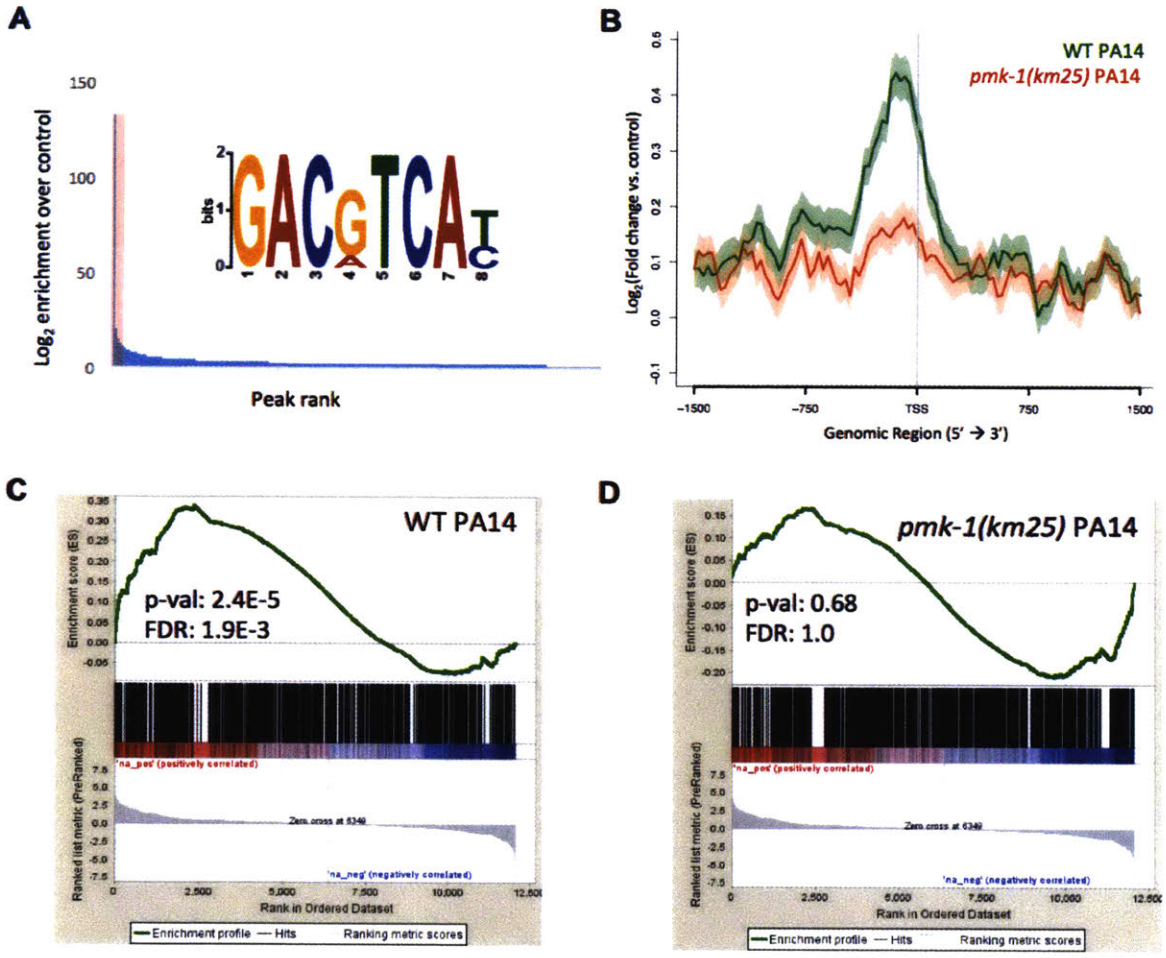
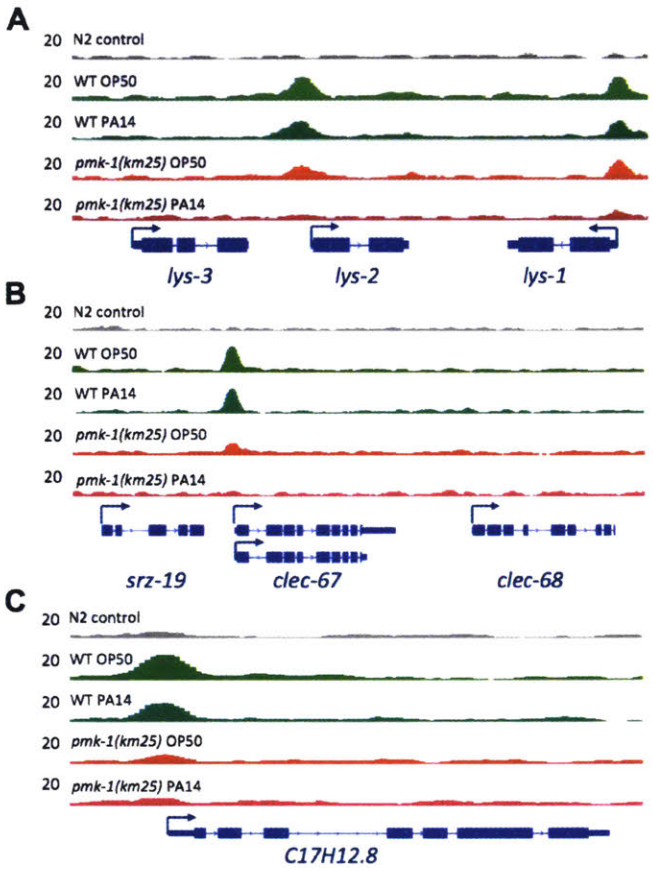

A
Log2 enrichment over control
Peak rank
bits
B
WT PA14
pmk-1(km25) PA14
Log2(Fold change vs. control)
Genomic Region (5' → 3')
TSS
C
WT PA14
p-val: 2.4E-5
FDR: 1.9E-3
Enrichment score (ES)
Ranked list metric (PreRanked)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
D
pmk-1(km25) PA14
p-val: 0.68
FDR: 1.0

Figure 2: ATF-7 associates with genes that are differentially expressed upon exposure to pathogenic *P. aeruginosa*.

(A) Motif analysis of ATF-7::GFP ChIP peaks. The top 400 peaks (indicated by red shading) were considered for motif analysis. **(B)** Metagene analysis of ATF-7::GFP binding profile in WT or *pmk-1(km25)* mutant animals exposed to PA14 across genes that are two-fold upregulated (by RNA-seq) upon exposure to PA14. **(C,D)** Gene Set Enrichment Analysis (GSEA) of transcripts detected by RNA-seq (ranked from most upregulated to most downregulated upon PA14 exposure in N2 animals) for association with ATF-7::GFP peaks in WT (C) or *pmk-1(km25)* mutant (D) animals exposed to PA14.

loci induced by *P. aeruginosa* infection that were dependent on ATF-7 for full upregulation. Examinations of distinct genetic loci further support the conclusions drawn from the metagene analyses described above (Figure 3). These observations suggest a direct transcriptional role for ATF-7 in the induction of broad transcriptional changes upon immune challenge involving activation of p38/PMK-1 MAPK signaling in response to *P. aeruginosa* infection.

For functional validation of putative ATF-7-regulated immune response target genes, we focused on genes that were upregulated at least two-fold by *P. aeruginosa* exposure in an *atf-7*-dependent manner and that were also bound by ATF-7 in any of our four ChIP-seq conditions. Included among these putative ATF-7 targets were genes encoding antimicrobial effector molecules, such as CTLD-containing proteins and lysozymes (Table S3). We determined whether RNAi-mediated knockdown of these genes resulted in enhanced susceptibility to killing by *P. aeruginosa* and observed that 13 of 43 genes exhibited a consistently significant enhanced sensitivity to killing by *P. aeruginosa*, without affecting development and survival on relatively non-pathogenic *E. coli* (Table S3, Figure S4). Our data suggest that ATF-7 is a direct regulator of immune effector genes, and that activation of ATF-7 by PMK-1/MAPK induces transcriptional changes of ATF-7 target sequences. We previously proposed a model in which PMK-1 phosphorylates ATF-7 in response to pathogen infection, switching the activity of ATF-7 from that of a transcriptional repressor to that of an activator, allowing the induction of immune response genes (Shivers et al. 2010). Our data here are consistent with this model, showing a strong dependence of pathogen-induced gene induction on PMK-1 and ATF-7, and a high degree of occupancy of regulatory regions of pathogen-induced genes by ATF-7 under basal and pathogen-induced conditions, with ATF-7 occupancy of pathogen-induced genes being strongly dependent on PMK-1.



Average TPMs: (OP50 / PA14)	lys-3	lys-2	lys-1
N2	2 / 6	210 / 2032	322 / 1032
pmk-1(km25)	1 / 29	12 / 62	135 / 222
atf-7(qd22 qd130)	3 / 16	40 / 242	254 / 440

Average TPMs: (OP50 / PA14)	srz-19	clec-67	clec-68
N2	0 / 0	15 / 189	0 / 3
pmk-1(km25)	0 / 0	0 / 0	0 / 0
atf-7(qd22 qd130)	0 / 0	1 / 1	0 / 0

Average TPMs: (OP50 / PA14)	C17H12.8
N2	148 / 489
pmk-1(km25)	0 / 0
atf-7(qd22 qd130)	83 / 35

Figure 3: ATF-7 binding at PA14-induced genes.

Examples of ATF-7::GFP read pileup at individual loci in all four ChIP conditions. Expression in transcripts per kilobase million (TPM) in all RNA-seq conditions are displayed to the right of each locus.

PMK-1 signaling has also been implicated in a number of non-infection contexts in *C. elegans* (Inoue et al. 2005; Richardson, Kooistra, and Kim 2010; Chikka et al. 2016). Interestingly, we observed that ATF-7 binds quite strongly to several key regulators of stress response pathways. We found that ATF-7 exhibits binding affinity to regulators of autophagy (*lgg-1*), the Unfolded Protein Response (*xbp-1*), and the oxidative stress response (*skn-1*), as well as several immunity regulators (*hlh-30*, *zip-2*, and interestingly, *atf-7*) (Figure 4). These observations suggest that initiation of other stress responses may be integrated with the immune response. For example, we have previously shown that immune response activation in developing larval is lethal without compensatory XBP-1 activity, establishing an essential role for XBP-1 during activation of innate immunity during infection of *C. elegans* (Richardson, Kooistra, and Kim 2010). We speculate that ATF-7 may function to activate anticipatory stress responses that can be activated in concert with innate immunity to promote host survival during microbial infection in a context-dependent manner.

Our genomic and genetic findings in the simple, genetically tractable *C. elegans* host reveal a striking degree of global regulation of the organismal response to pathogenic bacteria through a single p38 MAPK-regulated transcriptional regulator. Our data support the idea that ancient innate immune signaling pathways, even on a global, genome-wide and organism-wide level, are under the control of a limited number of stress-activated signaling pathways that converge on pivotal transcriptional regulators.

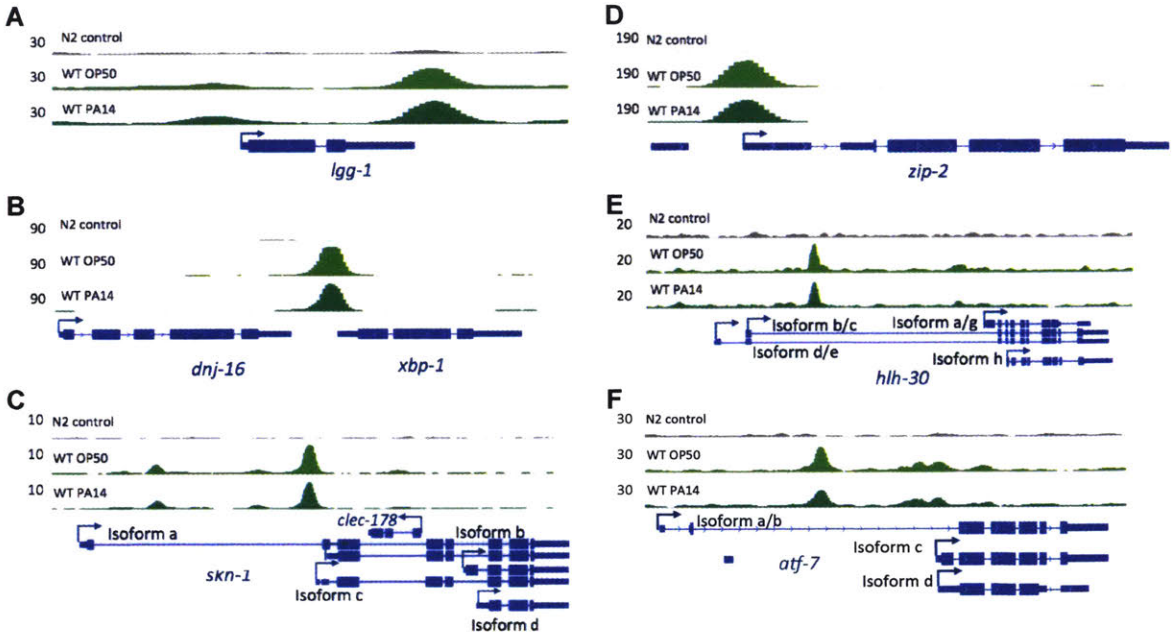


Figure 4: ATF-7 binds key regulators of animal physiology.

Examples of ATF-7::GFP read pileups stress response (A-C) and immune (D-F) regulators.

Materials and Methods

C. elegans Strains

Strains used: N2, ZD386 (*atf-7(qd22 qd130)*), KU25 (*pmk-1(km25)*), ZD1807 (*atf-7(qd328[atf-7::2xTY1::GFP])*), ZD1976 (*atf-7(qd328[atf-7::2xTY1::GFP]);pmk-1(km25)*). *C. elegans* were maintained at 16°C on *E. coli* OP50 as described by Brenner 1974. The *atf-7(qd328)* allele was generated by the CRISPR-Cas9 system (as described by Arribere et al. 2014) and verified by Sanger sequencing. GFP expression in ZD1807 (*atf-7(qd328)*) was verified by immunobloting, and pull-down was assessed by IP-IB. The *atf-7(qd238)* allele was confirmed to function as wild-type, as assayed by susceptibility to *P. aeruginosa* strain PA14 in a slow kill assay, and then crossed into the *pmk-1(km25)* mutant background.

Preparation of Animals for Sequencing Experiments

SKA plates were prepared as previously described (Tan, Mahajan-Miklos, and Ausubel 1999). *P. aeruginosa* strain PA14 or *E. coli* OP50 was grown overnight in Luria Broth (LB), seeded onto SKA media and then grown overnight at 37°C, followed by an additional day at room temperature as described by Meisel et al. 2014. Large populations of animals were synchronized by egg-preparation of gravid adult worms in bleach, followed by L1 arrest overnight in M9 buffer. L1 animals were dropped onto concentrated OP50 lawns seeded onto Nematode Growth Media (NGM) and raised to the L4 larval stage at 20°C (about 40 hours). Upon reaching L4, worms were washed off growth plates with M9 and placed on SKA plates prepared as described above, seeded with either PA14 or OP50 and incubated at 25°C for four hours. At this time, worms were harvested by washing for downstream applications.

Chromatin Immunoprecipitation Followed by Sequencing

After three washes in M9 buffer, animal pellets were resuspended in an equal volume of PBS + complete ULTRA protease inhibitor tablets (Roche), flash frozen in liquid nitrogen, and stored at -80°C until chromatin immunoprecipitation (ChIP). ChIP was preformed as described (Ercan et al. 2007; Gerstein et al. 2010) using Ab290, a ChIP-grade polyclonal GFP antibody (Abcam). Libraries were prepared using the SPRIworks Fragment Library System (Beckman Coulter) and single-end sequenced on an Illumina HiSeq2000 sequencer. Three biological replicates of at least 15,000 animals were prepared and sequenced for each condition, with the exception of only two replicates for *atf-7(qd328)* on PA14, as one of the samples failed to pass quality control.

ChIP-seq reads were aligned against the *C. elegans* WBPS9 assembly using bwa v. 0.7.12-r1039 (H. Li and Durbin 2009) and the resulting bam files were sorted and indexed using samtools v. 1.3 (H. Li et al. 2009). Sorted bam files were pooled by strain and microbial treatment, and peaks were called using MACS2 (v. 2.1.1.20160309), as follows: callpeak on specific strain bam file ("-t" flag) against the N2_PA14 control sample bam file ("-c" flag) callpeak -c N2_PA14_control.sorted.bam -g ce --keep-dup all --call-summits --extsize 150 -p 1e-3 --nomodel -B. Peak locations were intersected with regions +/-0.5kb around annotated TSS based on the WBPS9/WS258 annotation using bedtools intersect (v2.26.0) (Quinlan and Hall 2010), and in cases of multiple peaks associated with a given TSS, peaks with maximal enrichment over N2 control were retained. For the purpose of motif identification, peaks were ranked by fold-enrichment over N2 control in descending order and the top 400 peaks were retained, regions +/- 200 bps around the summit were retrieved and sequences were obtained with bedtools getfasta. MEME-ChIP v. 4.12.0 (Machanick and Bailey 2011) was used to call

motifs using the following parameters: meme-chip -oc . -time 300 -order 1 -db db/JASPAR/JASPAR2018_CORE_nematodes_non-redundant.meme -meme-mod anr -meme-minw 5 -meme-maxw 30 -meme-nmotifs 8 -dreme-e 0.05 -centrimo-local -centrimo-score 5.0 -centrimo-ethresh 10.0 . Presence of the top motifs under each peak called by macs2 was assessed using Mast v.5.0.1 (Bailey and Gribskov 1998) on the same +/- 200bp region around the summit of each peak. The number of peaks with one or more occurrences of the motif was tallied using a 200-peak window, and plotted across all peaks ranked either by log-fold enrichment over N2 or –log-transformed p-values. Inflection points in the motif density function were used to narrow down the number of peaks retained for downstream analyses.

RNA Sequencing

After three washes in M9 buffer, TRIzol™ Reagent (Invitrogen) was added to worm pellets and flash frozen in liquid nitrogen. Following an additional round of freeze-thaw, RNA was isolated using the Direct-zol™ RNA MiniPrep kit (Zymo Research). Libraries were prepared using the Kapa mRNA Hyperprep kit and paired end reads were sequenced on the Illumina NextSeq500 sequencer. Three biological replicates of at least 1,000 animals were prepared and sequenced for each condition, with the exception of only two replicates for *atf-7(qd22 qd130)* on PA14, as one of the samples failed to pass quality control.

Reads were aligned against the *C. elegans* WBPS9 assembly/ WS258 annotation using STAR v. 2.5.3a (Dobin et al. 2013) with the following flags: -runThreadN 16 --runMode alignReads --outFilterType BySJout --outFilterMultimapNmax 20 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --outFilterMismatchNmax 999 --alignIntronMin 10 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --outSAMtype BAM SortedByCoordinate --quantMode

TranscriptomeSAM . with --genomeDir pointing to a low-memory footprint, 75nt-junction WBPS9/WS258 STAR suffix array. Gene expression was quantitated using RSEM v. 1.3.0 (B. Li and Dewey 2011) with the following flags for all libraries: rsem-calculate-expression --calc-pme --alignments -p 8 against an annotation matching the STAR SA reference. Posterior mean estimates (pme) of counts and estimated "transcript-TPMs" were retrieved for genes and isoforms. Subsequently, counts of isoforms sharing a transcription start site (TSS) were summed, and differential-expression analysis was carried out using DESeq2 (Love, Huber, and Anders 2014) in the R v3.4.0 statistical environment, building pairwise models of conditions to be compared (microbial exposures within each genotype). Sequencing library size factors were estimated for each library to account for differences in sequencing depth and complexity among libraries, as well as gene-specific count dispersion parameters (reflecting the relationship between the variance in a given gene's counts and that gene's mean expression across samples).

Differences in gene expression between conditions (expressed as log2-transformed fold-changes in expression levels) were estimated under a general linear model (GLM) framework fitted on the read counts. In this model, read counts of each gene in each sample were modeled under a negative binomial distribution, based on the fitted mean of the counts and aforementioned dispersion parameters. Differential expression significance was assessed using a Wald test on the fitted count data (all these steps were performed using the DESeq() function in DESeq2) (Love, Huber, and Anders 2014). P-values were adjusted for multiple-comparison testing using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995).

Data availability

The data discussed in this chapter have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO SuperSeries accession number GSE119294, which contains SubSeries GSE119292 (RNA-seq data) and SubSeries GSE119293 (ChIP-seq data).

Evaluation of ATF-7 binding and modulation of gene expression

Metagene analyses of gene expression and ATF-7 binding enrichment were generated by ngs.plot as described in Shen et al. 2014. Correlations between ATF-7 binding and regulation of gene expression were interrogated using the gene set enrichment analysis (GSEA) framework (Subramanian et al. 2005). Briefly, all transcription start sites (TSSs) associated with a protein-coding transcript were ranked based on differential expression results from DESeq2 (log2 fold-changes), which is a measure of the correlation between their expression and the host response to infectious agents. Biases in expression of ATF-7-bound TSSs were assessed using a walk down the list tallying a running-sum statistic, which increases each time a TSS is part of the list and decreases otherwise. The maximum of this metrics (i.e. where the distribution if furthest away from the background) is called the enrichment score (ES). Significance is estimated using random permutations of the TSSs to generate p-values gauging how often the observed ES can be seen in randomized gene sets, for each direction of the expression biases independently. Multiple-testing correction is addressed using a false-discovery rate calculation on permuted datasets.

Gene Ontology analysis

Genes with adjusted p-values <0.05 were considered for Gene Ontology enrichment analysis using the DAVID online webtool, considering as a background the union of all genes with a non-zero baseMean value across any of the DE comparison, based on unique WormBase IDs.

Killing Assays and Bacterial Strains

PA14 plates were prepared as described as above. N2 animals were grown on NGM, supplemented with 25 ug/mL carbenicillin and 2mM isopropyl b-D-1 thiogalactopyranoside (IPTG), that was seeded with either the *E. coli* HT115 expressing plasmids targeting the gene of interest or the empty L4440 vector backbone for two generations prior to each experiment. Animal populations were synchronized by egg lay. At the L4 larval stage, approximately 30 worms were transferred to prepared SKA plates and incubated at 25°C. Animals were scored for killing twice daily until the majority of animals had died. Within each experiment, three plates were prepared and scored per RNAi treatment. All clones were obtained from the Ahringer or Vidal RNAi libraries (Kamath et al. 2003; Rual et al. 2004) and were verified by sequencing. For a list of all RNAi clones used, see Table S4.

Author Contributions

M.F. and E.J.T. performed all experiments. V.B. and S.S.L. performed bioinformatics analysis of RNA-seq and ChIP-seq datasets. M.F. and D.H.K analyzed data, interpreted results, and wrote the paper with input from E.J.T.

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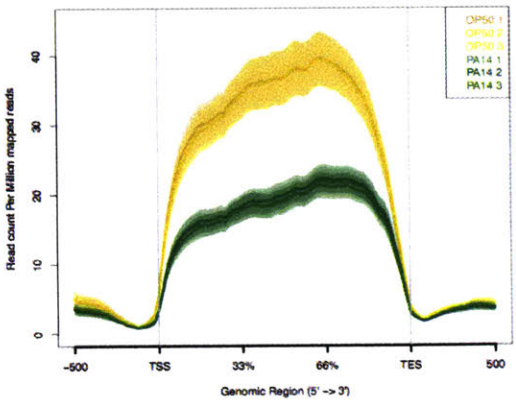
Supporting Information

Supplemental Figure 1

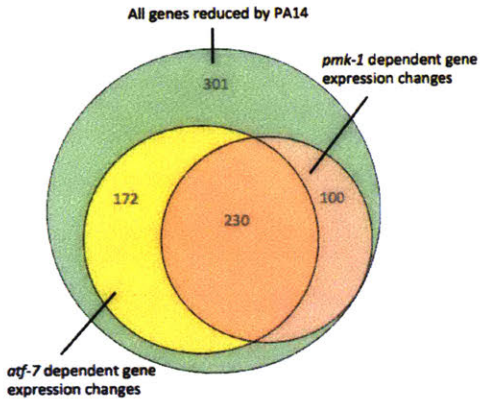
A

GO term, InterPRO classification	Fold Enrichment	p-value	FDR
GO:0000003~ reproduction	1.90	4.14E-17	6.13E-14
GO:0006412~ translation	4.34	2.11E-16	3.33E-13
GO:0002119~ nematode larval development	1.89	5.90E-15	8.72E-12
GO:0009792~ embryo development ending in birth or egg hatching	1.59	5.47E-12	8.11E-09
GO:0008340~ determination of adult lifespan	2.04	1.35E-09	2.00E-06
GO:0055114~ oxidation-reduction process	2.51	1.40E-08	2.08E-05
GO:0006915~ apoptotic process	2.49	8.73E-08	1.29E-04
GO:0006096~ glycolytic process	8.97	8.04E-07	1.19E-03

B

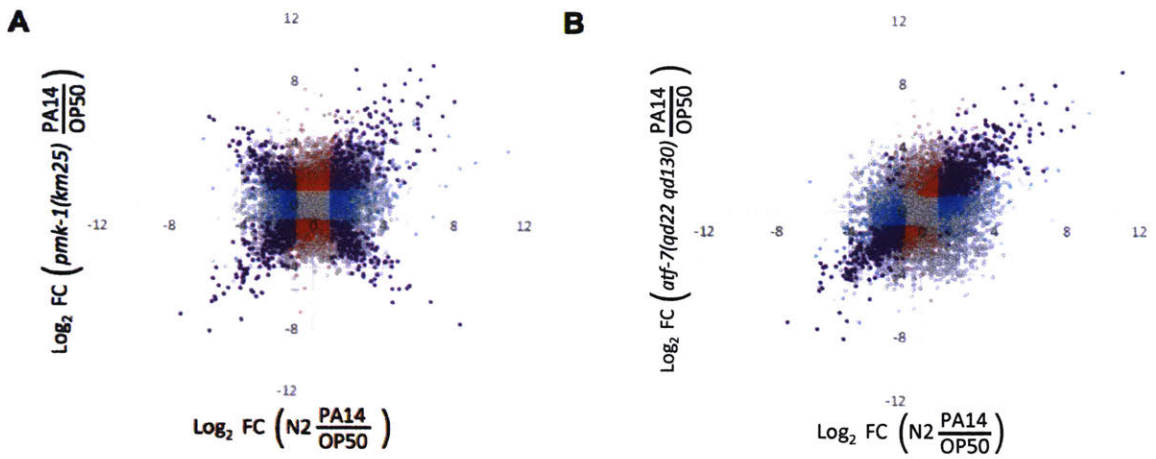


C



Supplemental Figure 1: Expression of genes decreased by PA14 exposure.

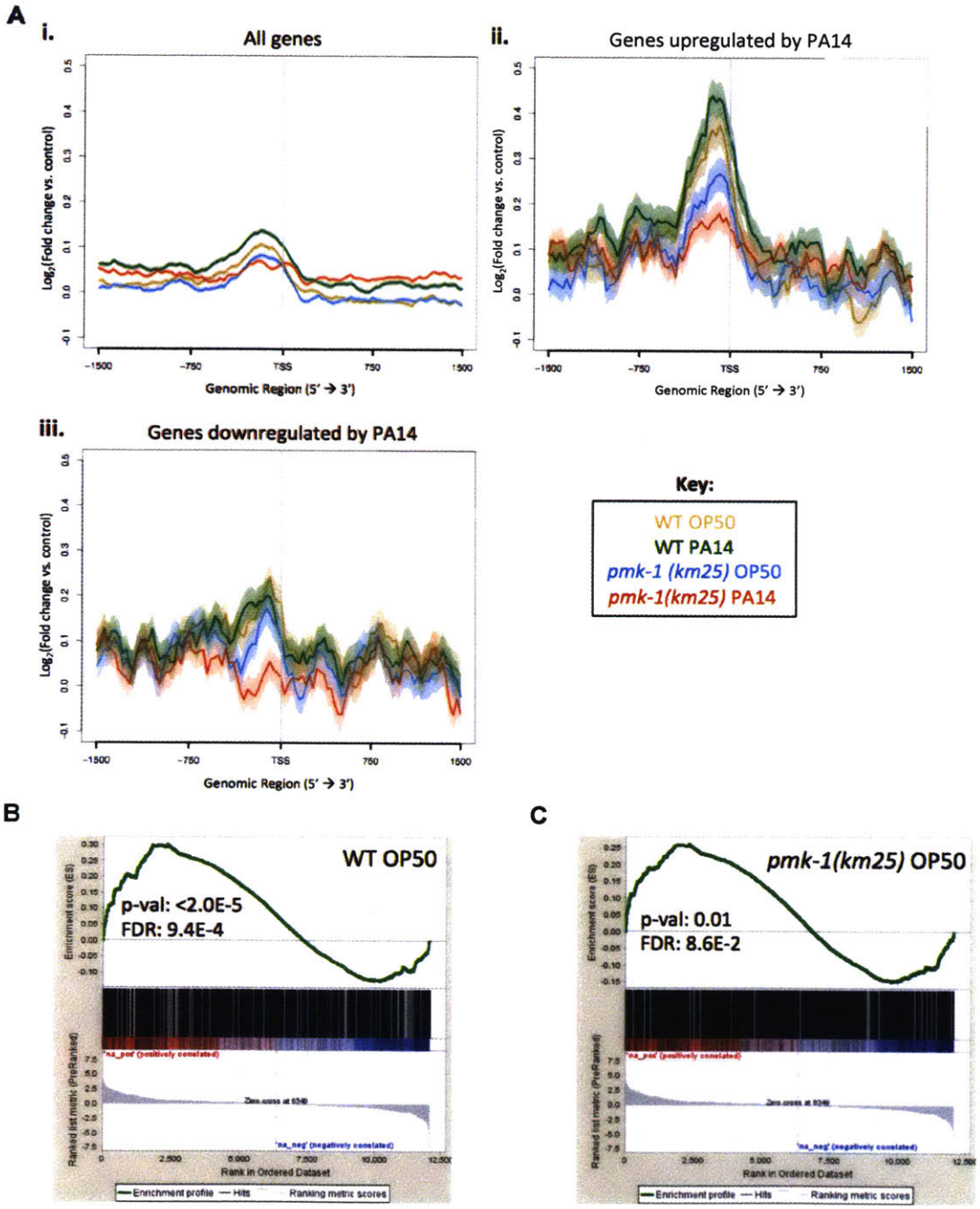
(**A**) Top GO terms and InterPRO classifications of transcripts that are significantly downregulated (adjusted p-value < 0.05) in N2 animals exposed to PA14 versus OP50. (**B**) Average expression (RPM) across the gene body of genes that are two-fold downregulated by exposure to pathogenic PA14. (**C**) Venn diagram of decreased genes that are dependent upon *pmk-1* or *atf-7* for complete downregulation.



Supplemental Figure 2: Differential PA14-induced gene expression in *pmk-1* and *atf-7* mutant animals.

2x2 comparison of genes differentially expressed by exposure to PA14 in N2 animals (x- axis) or upon loss of *pmk-1* (A) or *atf-7* (B) (y-axis). Transcripts highlighted in purple correspond to genes that were significantly different compared to OP50 in both genotypes (adjusted p-value of <0.05). Blue dots indicate genes that are significantly different in the N2 PA14/OP50 comparison only. Red dots represent genes that reach significance in only the mutant condition. Grey dots indicate genes with detected transcripts in at least one condition being compared, but that failed to reach significance cutoffs in either data set.

Supplemental Figure 3



Supplemental Figure 3: Evaluation of ATF-7::GFP peaks.

(A) Metagene analysis of ATF-7::GFP binding in all four ChIP conditions across all genes (i), genes that are upregulated (ii) or downregulated (iii) by RNA-seq upon exposure to PA14 in a wild-type (N2) background. **(B,C)** Gene Set Enrichment Analysis (GSEA) of transcripts detected by RNA-seq (ranked from most upregulated to most downregulated upon PA14 exposure in N2 animals) for association with ATF-7::GFP peaks in WT (B) or *pmk-1(km25)* mutant (C) animals exposed to OP50.

Supplemental Figure 4

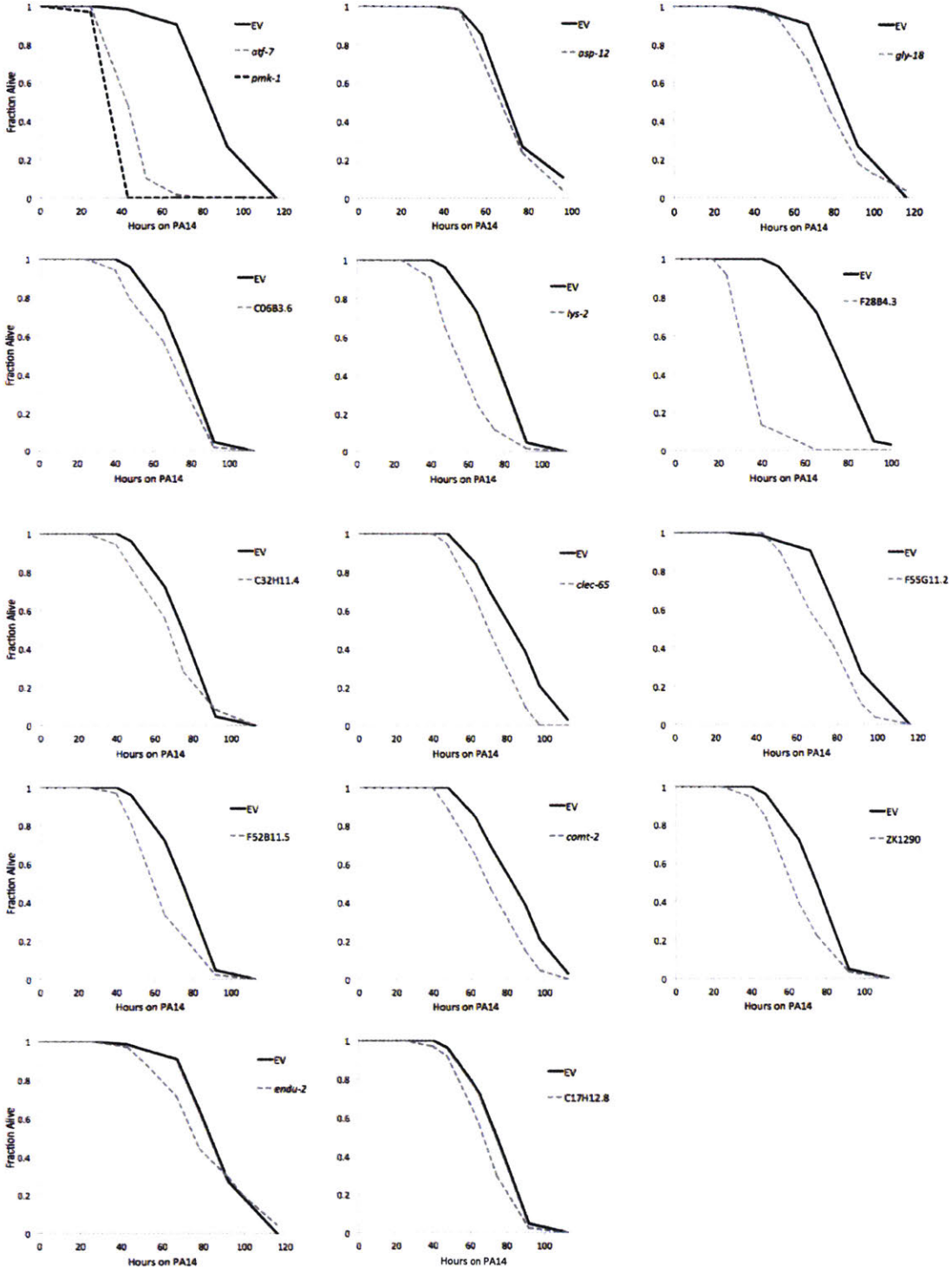


Figure S4: ATF-7 target genes that effect survival on *P. aeruginosa* PA14.

Representative survival curves of animals treated with RNAi against indicated genes that resulted in a significant (p-value < 0.05 by log-rank test) reduction in survival on PA14 compared to EV controls in 2/2 experiments. Animals were treated with RNAi for two generations prior to exposure to PA14. EV refers to HT115 carrying the Empty Vector control plasmid, L4440.

Table S1: RNA-seq summary

Please see separate electronic (.xlsx) file that accompanies this thesis.

Table S2: ATF-7::GFP peaks from ChIP-seq.

Please see separate electronic (.xlsx) file that accompanies this thesis.

Gene	Conserved protein domains	esp phenotype upon knockdown?
asp-12	aspartyl protease	yes
F28B4.3	c-type lectin	yes
clec-65	c-type lectin	yes
clec-41	c-type lectin	no
clec-67	c-type lectin	no
clec-186	c-type lectin	no
cnc-4	caenacin	no
cav-2	caveolin	no
C32H11.4	CUB like domain	yes
C17H12.8	CUB like domain	yes
F55G11.2	CUB like domain	yes
dod-22	CUB like domain	no
K10D11.2	CUB like domain	no
F08G5.6	CUB like domain	no
H20E11.1	CUB like domain	no
cld-9	CUB like domain	no
K08D8.6	CUB like domain	no
dod-21	CUB like domain	no
endu-2	endoribonuclease XendoU	yes
fbxa-105	F-box domain	no
Y9C9A.8	F-box domain	no
Y17G7B.8	F-box domain	no
gly-18	glycosyl transferase	yes
fut-6	glycosyl transferase	no
hrg-1	heme transporter	no
lys-2	lysozyme	yes
lys-1	lysozyme	no
oac-5	o-acyltransferase	no
oac-6	o-acyltransferase	no
oac-3	o-acyltransferase	no
comt-2	o-methyltransferase	yes
twk-28	potassium channel	no
C16D9.4	Protein of unknown function D	no
R03G8.3	Protein of unknown function D	no
ZK1290.10	Shk domain-like	yes
F01D5.1	Shk domain-like	no
F49H6.13	tight junction protein	no
C08F11.13	transmembrane protein	no
C51E3.10	transmembrane protein	no
B0310.3	transmembrane protein	no
ugt-7	UDP-glucosyl transferase	no
C06B3.6	Uncharacterized protein	yes
F52B11.5	Uncharacterized protein	yes

Table S3: Genes tested for *esp* phenotype by RNAi knockdown.

Protein domains classified using the David 6.8 Functional Annotation Tool. "Yes," indicates a significant (p-value < 0.05 by log-rank test) reduction in survival on PA14 compared to Empty Vector control in 2/2 experiments.

Table S4: RNAi clones used in this study.

Target gene	Library	Plate	Well
asp-12	Ahringer- Main	144	G7
atf-7	Vidal	11007	F4
B0310.3	Vidal	11076	B1
C06B3.6	Ahringer- Main	157	G4
C08F11.13	Ahringer- Main	117	B2
C16D9.4	Ahringer- Main	144	F11
C17H12.8	Ahringer- Main	101	D10
C32H11.4	Ahringer- Main	115	E1
C33D9.5	Ahringer- Main	106	A4
C51E3.10	Ahringer- Supplemental	S38	C8
C53D6.11	Ahringer- Supplemental	S29	E9
cav-2	Ahringer- Main	156	G10
cld-9	Ahringer- Main	157	C4
clec-186	Ahringer- Main	115	D1
clec-65	Ahringer- Main	61	A11
clec-67	Vidal	11050	A8
cnc-4	Ahringer- Main	127	G9
comt-2	Ahringer- Main	165	C3
dod-19	Ahringer- Main	125	F2
dod-22	Ahringer- Main	115	F3
endu-2	Ahringer- Main	188	H8
F01D5.1	Ahringer- Main	62	H12
F08G5.6	Ahringer- Main	114	E8
F28B4.3	Ahringer- Main	180	F7
F47D12.6	Ahringer- Main	75	D11
F52B11.5	Ahringer- Main	117	G5
F55G11.2	Ahringer- Main	115	E12
fbxa-105	Ahringer- Main	142	D9
fbxa-51	Ahringer- Main	67	D8
fbxa-88	Ahringer- Main	164	A12
fip-4	Ahringer- Main	145	G2
fut-6	Ahringer- Supplemental	S11	E3
gly-18	Ahringer- Main	11	A6
H20E11.1	Ahringer- Main	101	E5
hrg-1	Vidal	11080	E3
irg-7	Ahringer- Main	180	G1
K09C4.5	Ahringer- Main	180	G7
K10D11.2	Ahringer- Supplemental	S27	F12
lmp-2	Ahringer- Main	177	B10
lys-1	Ahringer- Main	149	F3
lys-2	Ahringer- Main	149	F4
nhr-54	Ahringer- Main	165	C7
oac-3	Ahringer- Main	157	F12
oac-5	Ahringer- Main	164	F1
oac-6	Ahringer- Main	164	F5
pmk-1	Vidal	10003	A2
R03G8.3	Ahringer- Main	196	F9
sri-36	Ahringer- Main	37	E11
sru-44	Ahringer- Supplemental	S39	E9
T15B7.17	Ahringer- Main	141	A12
twk-28	Ahringer- Main	182	D12
ugt-7	Ahringer- Main	137	B3
Y17D7C.2	Ahringer- Main	169	H5
Y17G7B.8	Ahringer- Main	59	E7
Y47H10A.4	Ahringer- Main	21	B10
Y9C9A.8	Ahringer- Supplemental	S25	C2
ZK1290.10	Ahringer- Main	49	G11

Chapter Four

Future Directions

Marissa Fletcher

Responding to environmental conditions is critical to survival and longevity. Work presented in this thesis has focused on understanding responses to environmental challenges in two distinct contexts: in the aging intervention of dietary restriction and in the immune challenge of infection. While I presented progress towards comprehension in these two specific areas, much of the biology of how DAF-7/ TGFβ signaling contributes to the DR response and how p38/PMK-1 signaling contributes to immunity remains elusive. Thinking about my findings in the context of prior studies, I propose a few follow up inquiries for each of these two areas of study.

Continuing our understanding DAF-7/TGFβ and dietary restriction

Dynamic expression of *daf-7* has been linked to changes in food availability, pathogen exposure, and developmental status (Ren et al. 1996; Schackwitz, Inoue, and Thomas 1996; Meisel et al. 2014; Hilbert and Kim 2017). As such, signaling through DAF-7 is thought to help *C. elegans* decode its external environment. However, the mechanistic details of how this signaling pathway is activated and how that activation leads to changes in physiology are yet to be elucidated. While our lab has described the role of DAF-7 in the detection of pathogenic *P. aeruginosa* in more detail, defining aspects upstream and downstream of DAF-7 in dietary restriction presents its own challenges. Unlike the dramatic expression changes our lab has observed in *P. aeruginosa* exposure and male development, DAF-7 expression is much more subtly altered under DR conditions. Additionally, as with many age-related studies, forward-genetic screens of aging phenotypes are both labor intensive and technically difficult. Nonetheless, I think there are a few genetic strategies that would help illuminate how DAF-7/TGFβ signaling contributes to lifespan extension via dietary restriction.

Most studies of DAF-7/TGFβ signaling in *C. elegans* focus on *daf-7* (TGFβ ligand) and *daf-1* (receptor) gene expression. It is clear from the work I presented in Chapter Two that DAF-3 (Smad) activity is also a critical component of this neuroendocrine signaling pathway. Yet, relatively little is known about how DAF-3 works to regulate gene expression in response to regulation by DAF-7. TGFβ signaling pathways regulate central processes in a manner that is conserved among *C. elegans*, *Drosophila*, and mammals (Patterson and Padgett 2000). Interestingly, *C. elegans* DAF-3 regulation seems to be distinct from other species in that it is inhibited rather than activated by DAF-7 signaling (Figure 1) (Patterson et al. 1997). While this feature is useful for genetic studies of DAF-7/TGFβ dependent processes, it leaves questions as to how DAF-3 regulates gene expression.

To address these and other questions, it would be useful to identify direct targets of DAF-3 regulation through a combination of ChIP-seq and RNA-seq studies, similar to those performed in Chapter Three. In this way, we could determine what types of genes change in expression as a result of DAF-3 activation (such as in a *daf-7* loss-of-function mutant animals) or repression (such as by overexpression of *daf-7*). Preforming ChIP-seq using an endogenously-tagged allele of *daf-3* (generated by use of the CRISPR-Cas9 endonuclease system) could identify DAF-3 binding sites and motifs. Paired with expression data in a variety of contexts (such as animals exposed to DR conditions, animals fed *ad libitem,* or *daf-7* mutant animals on DR), we may be able to provide insight as to which genes promote the DR response downstream of DAF-7/ TGFβ signaling, and which genes disrupt the DR response as a consequence of overactive DAF-3 activity. With a tagged version of DAF-3 in hand, it would also be interesting to perform ChIP followed by mass spectrometry (ChIP-MS) to identify additional factors that DAF-3 may interact with under both low and high activation states.

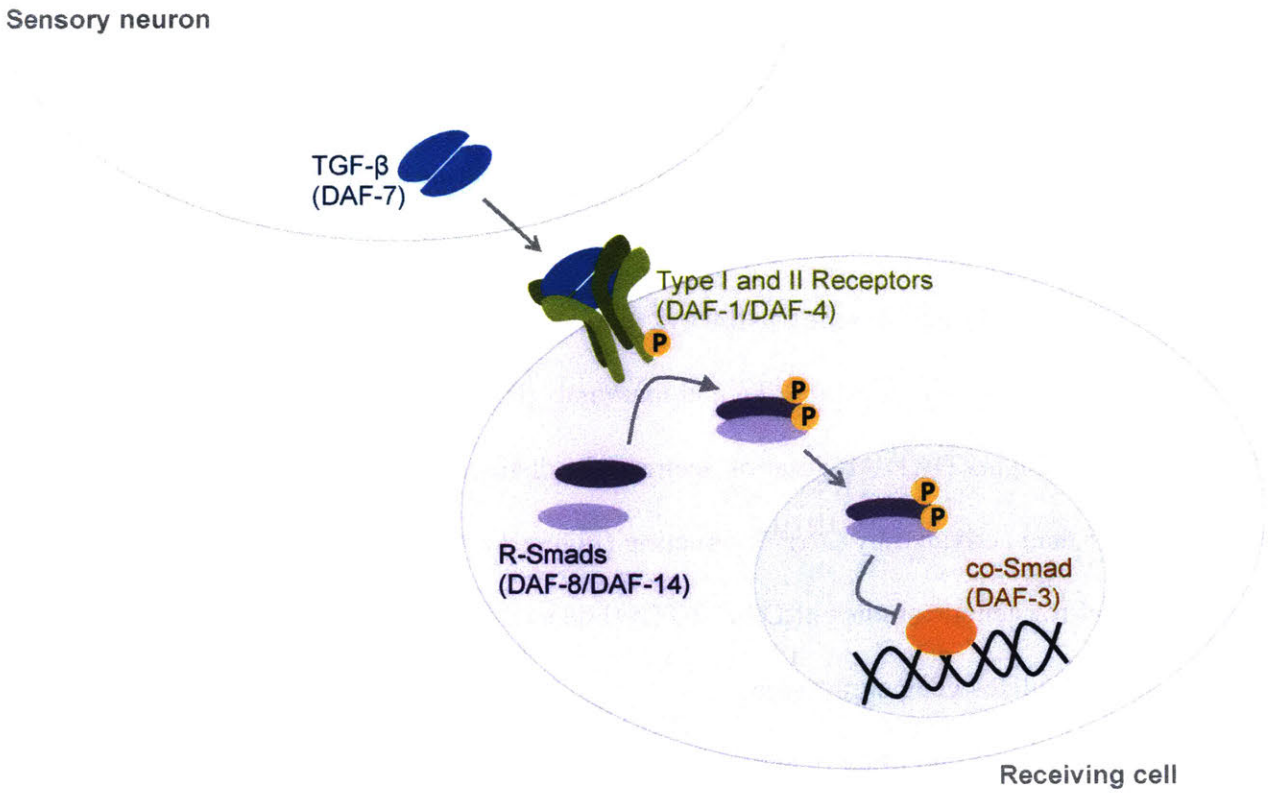


Figure 1: DAF-7/TGFβ signaling in *C. elegans*.

DAF-7, secreted from sensory cells, binds and activates the type I and type II serine-threonine kinase receptors (DAF-1 and DAF-4 respectively). This leads to phosphorylation of the R-Smads (DAF-8 and DAF-14), enabling interaction with the co-Smad (DAF-3), which (presumably) inhibits transcriptional activity.

In addition to discovering how gene expression changes downstream of DAF-3 regulation function in longevity extension in response to DR, characterizing how the DAF-7/ TGFβ signaling pathway is involved in sensing reductions in nutrient availability could lend insight into how food sensation drives response to DR conditions. In Chapter Two, I described an experiment that used GFP expression downstream of a DAF-3 target sequence as a proxy for DAF-3 activation status. I found that as animals age, GFP expression declines, corresponding to an increase in DAF-3 activity. This phenotype may be one that is amenable to forward genetic screening, since it can be easily observed on a dissecting microscope and the differences I saw in GFP fluorescence were readily apparent by eye. Mutagenizing animals carrying this construct and screening for animals that stay bright with age may contain mutations that promote expression of *daf-7*, or transmission of DAF-7 signal to receiving cells. These mutations would perhaps be in genes involved in chemosensation upstream of *daf-7* transcription, and animals carrying these mutations would be predicted to have an extended window of DR efficacy, similar to *daf-3* mutant animals as shown in Chapter Two. However, these criteria may only fit gain-of-function mutations in genes that are specific to the pathway of interest and therefore this screening strategy may not yield many worthwhile results. Therefore, we could also use this construct to screen for animals that exhibit low GFP fluorescence at a young age, producing mutants that would be predicted to be defective at promoting DAF-7 signaling and, consequently, DR non-responsive. One major caveat to this approach is the high chances of selecting animals that carry mutations that are either nonspecific to our DR phenotype or are in previously identified components of DAF-7/ TGFβ signaling. As such, these genes would need to be ruled out by complementation prior to additional characterization of selected mutants.

Another unanswered question from the work presented in Chapter Two is how does DAF-3 regulation in the RIM and RIC interneurons result in lifespan extension on the organism level as a result of DR? I showed that *daf-7* is necessary for DR-induced DAF-16::GFP nuclear localization. This presents us with a tool with which we may be able to answer this question. Screening for animals that fail to re-localize DAF-16 in response to DR in the intestine (that is, GFP signal stays diffuse) should identify genes that are important for relaying information received by RIM and RIC to the rest of the animal. This would, however, be a rather low throughput screen, as it would need to be a clonal screen for a few reasons. First, the DR methods I used in Chapter Two were applied to animals that were largely in the post-reproductive period. It is possible that with some optimization, this timeline could be moved up, but this phenotype is best assayed at the population level, as there is a decent amount of variability from animal to animal. However, identification of factors that connect neuronal, environmental perceptions to intestinal, physiological responses would be well worth the effort. Genes identified in either of these screens may also encode important elements in initiating and transmitting DAF-7 signal in additional contexts.

Additional characterization of ATF-7-mediated responses

In Chapter Three, I showed that ATF-7 exhibits widespread binding throughout the genome. Yet, expression of many of these genes is unaffected by the loss of *atf-7*. Additionally, our ChIP-seq experiments did not detect any major changes in ATF-7 occupancy by *P. aeruginosa* exposure or by *pmk-1* loss. These results suggest that 1) ATF-7 acts redundantly to regulate expression of many of its target genes and 2) phosphorylation by PMK-1 does not change the DNA binding capacity of ATF-7.

The fact that ATF-7 binds so many loci is particularly intriguing and hints at a central role for ATF-7 in mediating physiological responses to environmental stimuli. We were able to link ATF-7 binding to genes that change upon pathogen exposure, so it would be interesting to repeat this analysis for gene expression changes in other contexts, such as oxidative stress and ER stress, since ATF-7 binds strongly to mediators of these stress responses to which p38/PMK-1 signaling has been previously linked (Figure 2) (H. Inoue et al. 2005; Richardson, Kooistra, and Kim 2010).

Because occupation status of ATF-7 was largely unaffected by a null mutation in *pmk-1*, phosphorylation downstream of the p38/PMK-1 signaling cascade likely changes ATF-7 interactions with additional, unknown cofactors. DNA binding and transcription activation by bZIP transcription factors is dependent on formation of homo- and heterodimeric complexes with other bZIP proteins, and these interactions are in part facilitated by MAPK signaling pathways in mammals (van Dam and Castellazzi 2001). The *C. elegans* genome contains 22 genes encoding bZIP domain containing proteins that could potentially be binding partners for ATF-7, but physical interactions with any of these genes have not yet been described for ATF-7. However, given that the esp phenotype has been screened extensively by our lab, it is more likely that ATF-7 functions as a homodimer. This suggests that phosphorylation by PMK-1 modifies interaction of ATF-7 with other co-activators or co-repressors, rather than interactions with other bZIP transcription factors. To identify these ATF-7 interactors, we could use the strain generated in Chapter Three, which contains a GFP-tagged allele of *atf-7*, to perform ChIP-MS. Performing such an experiment under conditions with active PMK-1 (such as during pathogen exposure) or inactive PMK-1 (such as in a *pmk-1* loss-of-function mutant) could reveal how phosphorylation status changes ATF-7 binding partners and, consequently, target gene expression.

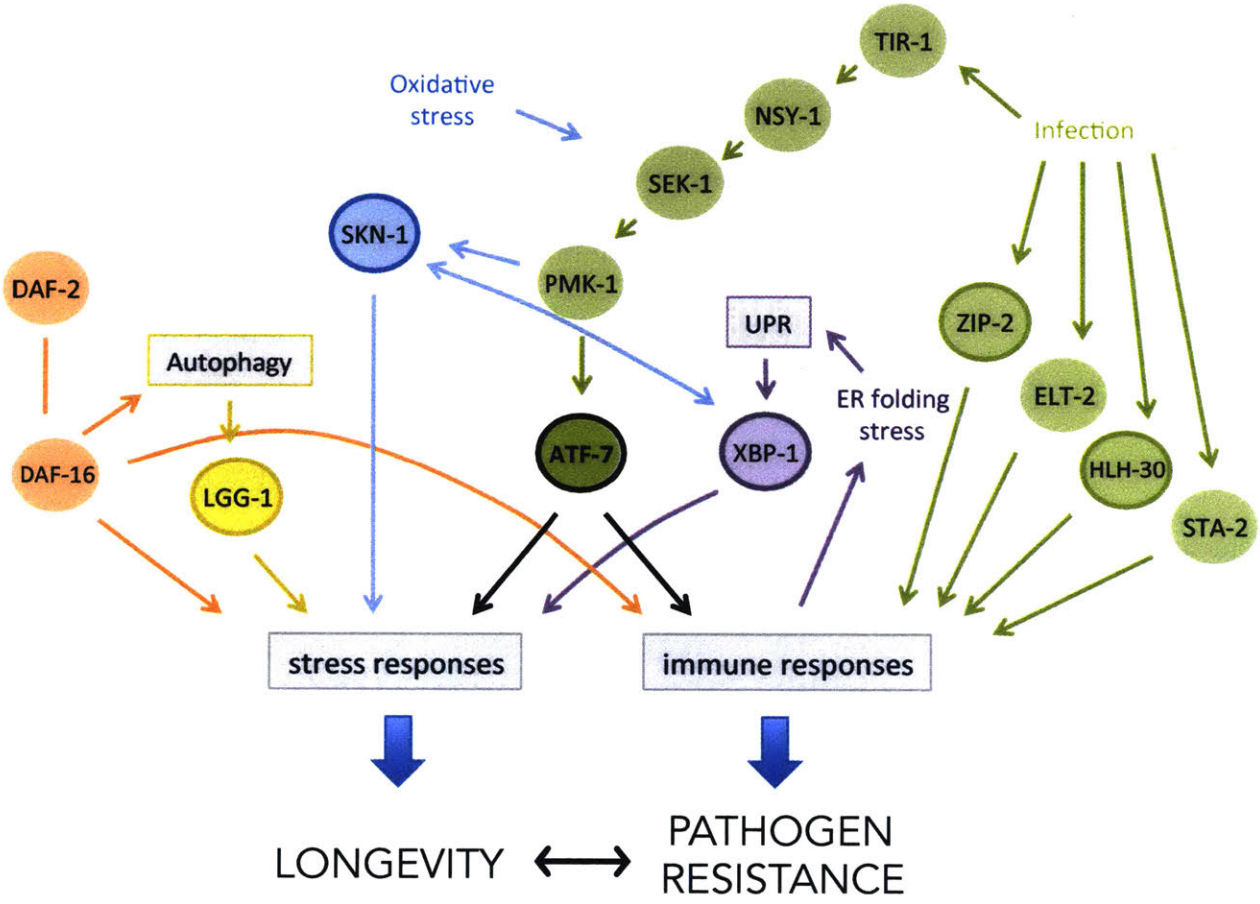


Figure 2: ATF-7 as a central regulator of *C. elegans* physiology.

Several stress and immune response pathways contribute to pathogen resistance and longevity. p38 MAPK signaling has been implicated in many of these pathways, and ATF-7 directly binds mediators of these responses (ATF-7 binding to genetic loci are indicated by the outlined factors in the above diagram).

Finally, functional classification of antimicrobial effectors has been challenging in *C. elegans*, presumably due to redundancy. For example, the *C. elegans* genome contains nearly 300 c-type lectin domain- (CTLD-) containing genes, many of which are induced upon pathogen exposure (Pees et al. 2016). CTLD-containing genes can function in pathogen recognition or elimination, yet evidence for either of these activities has yet to be identified in *C. elegans* (Dierking, Yang, and Schulenburg 2016). The identification of the CTLD-containing gene F28B4.3 as an immune effector molecule presents a good candidate for future characterization. Other genes identified in Chapter Three as contributing to pathogen resistance also warrant additional studies; especially those containing domains associated with antimicrobial activities such as CUB-like, aspartyl protease, and lysozyme domains. More detailed characterization of some of these candidates could provide experimental evidence that solidify our understanding of the innate immune response in *C. elegans* and other animal species.

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